Universidade de Aveiro Departamento de Química
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Purificação de anticorpos utilizando sistemas aquosos bifásicos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo de Métodos Biomoleculares, realizada sob a orientação científica do Professor Doutor João Manuel da Costa Araújo Pereira Coutinho, Professor Associado com agregação do Departamento de Química da Universidade de Aveiro e co-orientação da Doutora Mara Guadalupe Freire Martins, Investigadora Auxiliar do Departamento de Química, CICECO, da Universidade de Aveiro.

Aos meus pais e aos meus irmãos…

o júri

presidente

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agradecimentos

Antes de mais, gostaria de agradecer ao meu orientador, o Prof. João Coutinho, pela oportunidade de poder trabalhar no excelente grupo que é o Path. Um especialíssimo obrigado à minha co-orientadora, a Dr^{a}. Mara, por me ter acompanhado de forma extraordinária e incansável, e por toda a sua boa disposição e otimismo que anima qualquer dia menos bom. A todos os membros do Path e mini-Path, obrigada por tudo, trabalhar em equipa é fundamental. Em especial, deixo um muito obrigado à Helena e ao Matheus por toda a paciência que gastaram comigo e por toda a ajuda. Sempre que precisei vocês estavam lá.

Às minhas amigas, Carla, John, Luísa, Mónica, Ritinha e Sara. Agradeço-vos por todos os momentos bons e menos bons, por todos os convívios, desabafos e noitadas. Este trabalho tem um bocadinho de cada uma de vocês. Obrigada ainda a todos os que conheci nesta jornada.

Pai e Mãe, muito obrigada por me permitirem chegar até aqui. Sem vocês, sem os vossos conselhos e sem o vosso apoio incondicional, nada disto seria possível. Obrigada por serem os melhores pais do Mundo. João e Francisco, obrigada também a vocês por serem os manos mais tolinhos e chatinhos do Universo e por todas as discussões que só nos deixam mais unidos. Dé, Avô Rodrigues e Avó Teresa, obrigada por acreditarem tanto em mim. Obrigada a toda a minha família.

Samuel, obrigada por compreenderes e apoiares as minhas decisões. Obrigada por todas as gargalhadas que me fazes dar e por nunca me deixares desanimar. Tu és o melhor de mim…

palavras-chave

Extração líquido-líquido, sistemas aquosos bifásicos, líquidos iónicos, biofármacos, imunoglobulina Y.

resumo

Com o aparecimento de microrganismos resistentes a antibióticos, de doenças que não respondem a terapias comuns recorrendo a fármacos e de indivíduos que são incapazes de responder ao método de vacinação tradicional, hoje em dia, é de grande preocupação para a sociedade humana, o desenvolvimento de anticorpos específicos com aplicação em imunoterapia passiva. Apesar de os anticorpos de mamíferos serem mais específicos, os anticorpos obtidos a partir de gema de ovo de galinhas imunizadas, imunoglobulina Y (IgY), são uma alternativa possível onde a quantidade de anticorpos obtida é maior e recorrendo ainda a métodos não invasivos. Esta grande quantidade disponível de anticorpos permite explorar um novo tipo de biofármacos menos dispendiosos que os convencionais. No entanto, o custo da produção de IgY com elevado grau de pureza continua a ser mais elevado do que o de outras terapias convencionais devido à falta de uma técnica de purificação eficiente. Assim, neste trabalho, estudaram-se três tipos de sistemas aquosos bifásicos (SAB) constituídos, nomeadamente, por polímeros e Na_2SO_4, por polímeros, Na_2SO_4 e líquidos iónicos (LIs) como adjuvantes, e por Na_2SO_4 e LIs, como uma técnica de extração e purificação alternativa para o IgY a partir da gema de ovo. De acordo com os resultados obtidos pode-se constatar que a purificação do IgY não foi conseguida num único passo de extração (sendo a β-livetina o maior contaminante encontrado). No entanto, e dos resultados conseguidos, mostrou-se que os SAB utilizando LIs como adjuvantes permitem uma extração seletiva da β-livetina para uma das fases e será possível purificar a imunoglobulina ao aplicar extrações sucessivas. De entre os vários SAB avaliados, os que permitiram melhores resultados em termos de extração seletiva e posterior purificação do IgY são constituídos por polietilenoglicol (PEG) com uma baixa massa molecular e por LIs com o anião dicianamida.

keywords

Liquid-liquid extraction, aqueous two-phase systems, ionic liquids, biopharmaceuticals, immunoglobulin Y.

abstract

With the emergence of antibiotic-resistant microorganisms, diseases that are unresponsive to drug therapy, and the appearance of individuals that are unable to respond to conventional vaccines, the development of antigen-specific antibodies for use in passive immunotherapy is, nowadays, a major concern in human society. Despite the most focused mammal antibodies, antibodies obtained from egg yolk of immunized hens, immunoglobulin Y (IgY), are an alternative option that can be obtained in higher titres by non-stressful and non-invasive methods. This large amount of available antibodies opens the door for a new kind of cheaper biopharmaceuticals. Nevertheless, the production cost of high-quality and/or high-purity IgY still remains higher than other drug therapies due to the lack of an efficient purification method. Therefore, in this work, three types of aqueous two-phase systems (ATPS) were studied constituted by polymers and Na_2SO_4, polymers, Na_2SO_4 and ionic liquids (ILs) as adjuvants and by Na_2SO_4 and ILs, as an alternative technique for the selective extraction, and thus purification, of IgY from egg yolk. According to the obtained results, the complete purification of IgY was not achieved in a single step procedure (with β-livetin being the major contaminant found). However, the results obtained reveal that ATPS using ILs as adjuvants allow the selective extraction of β-livetin for one phase and it is possible to purify the immunoglobulin if successive extractions are applied. Amongst the various ATPS evaluated, the improved results on the selective extraction and subsequent purification of IgY were obtained with ATPS consisting of polyethylene glycol (PEG) of low molecular mass and ILs with the anion dicyanamide.

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List of Symbols

Abs – absorbance (dimensionless);

K_{IL} – partition coefficient of ionic liquid (dimensionless);

$K_{Protein}$ – partition coefficient of protein (dimensionless);

Mw – molecular weight ($g \cdot mol^{-1}$);

R^2 – correlation coefficient (dimensionless);

wt % – weight percentage (%);

λ – wavelength (nm);

ρ – density ($g.cm^{-3}$);

σ – standard deviation;

$[IL]_{PEG}$ – concentration of ionic liquid in the PEG-rich phase (wt %);

$[IL]_{salt}$ – concentration of ionic liquid in the salt-rich phase (wt %);

$[Na_2SO_4]$ – concentration of Na_2SO_4 (wt % or $mol \cdot kg^{-1}$);

[PEG 300] – concentration of PEG 300 (wt % or $mol \cdot kg^{-1}$);

[PEG 400] – concentration of PEG 400 (wt % or $mol \cdot kg^{-1}$);

[PEG] – concentration of PEG (wt % or $mol \cdot kg^{-1}$);

$[PEG]_M$ – concentration of PEG in the initial mixture (wt %);

$[PEG]_{PEG}$ – concentration of PEG in the PEG-rich phase (wt %);

$[PEG]_{salt}$ – concentration of PEG in the salt-rich phase (wt %);

$[Protein]_{PEG}$ – concentration of protein in the PEG-rich phase (wt %);

$[Protein]_{salt}$ – concentration of protein in the salt-rich phase (wt %);

[salt] – concentration of salt (wt % or $mol \cdot kg^{-1}$);

$[salt]_M$ – concentration of salt in the initial mixture (wt %);

$[salt]_{PEG}$ – concentration of salt in the PEG-rich phase (wt %);

$[salt]s_{alt}$ – concentration of salt in the salt-rich phase (wt %);

List of Abbreviations

Asn – asparagine;

ATPS – aqueous two-phase systems;

b **–** bottom;

C – constant region of antibody;

CF – cystic fibrosis;

C_H – constant domain of heavy chain of antibodies;

CL – constant domain of antibodies;

DNA – deoxyribonucleic acid;

DTT – dithiothreitol;

Fc – fragment crystallizable region of antibodies;

FCA – Freund's complete adjuvant;

FIA – Freund's incomplete adjuvant;

H. pylori – Helicobacter *pylori*;

HBC – hiperimmune bovine colostrum;

HIF-1 – hypoxia-inducible factor 1;

Ig – immunoglobulin;

IL – ionic liquid;

NMR – nuclear magnetic resonance;

PBS – phosphate buffer saline solution;

PEG – polyethylene glycol;

PEG 200 – polyethylene glycol with a molecular weight of 200 $g \cdot mol^{-1}$;

PEG 300 – polyethylene glycol with a molecular weight of 300 $g \cdot mol^{-1}$;

PEG 400 – polyethylene glycol with a molecular weight of 400 $g \cdot mol^{-1}$;

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

SDS – sodium dodecylsulfate;

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis;

TL – tie-line;

TLL – tie-line length;

TNF – tumor necrosis factor;

Tris – tris(hydroxymethyl)aminomethane;

UV – ultraviolet;

V – variable region of antibody;

V_H – variable domain of the heavy chain of antibodies;

VL – variable domain of antibodies;

WSPF – water soluble proteins fraction;

$[C_2mim][N(CN)_2]$ – 1-ethyl-3-methylimidazolium dicyanamide;

$[C_4mim][C(CN)_3]$ – 1-butyl-3-methylimidazolium tricyanomethane;

$[C_4mim][CH_3CO_2]$ – 1-butyl-3-methylimidazolium acetate;

$[C_4mim][N(CN)_2]$ – 1-butyl-3-methylimidazolium dicyanamide;

$[C_4mim][SCN]$ – 1-butyl-3-methylimidazolium thiocyanate;

$[C_4mim][TOS]$ – 1-butyl-3-methylimidazolium tosylate;

$[C_4mpy][N(CN)_2]$ – 1-butyl-3-methylpyridinium dicyanamide;

$[C_6mim][N(CN)_2]$ – 1-hexyl-3-methylimidazolium dicyanamide;

$[THTDP][N(CN)_2]$ – trihexyltetradecylphosphonium dicyanamide.

1. General introduction

1.1. Scopes and objectives

The emergence of drug-resistant microorganisms, diseases that are unresponsive to drug therapy and the appearance of individuals with impaired immune systems who are unable to respond to conventional vaccines make the search of effective alternative therapeutics a crucial goal to be achieved within the next few years [1]. Biopharmaceuticals have greatly improved the treatment of many diseases and sometimes are the only approved therapies available for a particular disease. These biological-based products, including recombinant therapeutic proteins, antibody-based products for *in vivo* medical purposes and nucleic-acid-based products have shown application in several medicinal areas such as vaccination, immunization and oncology, and autoimmune, cardiovascular, inflammatory and neurological diseases [2].

In the past 10 years an increase of the commercial potential of antibodies as major therapeutic drugs has been observed. The ability of antibodies to bind an antigen with a high degree of affinity and specificity has led to their ubiquitous use in a variety of scientific and medicinal disciplines [3]. However, the production cost of high-quality antibodies, and in particular of immunoglobulin Y (IgY, hen antibody), still remains higher than other drug therapies due to the absence of a cost-effective purification method. Despite the advantages of IgY over mammal antibodies, only less than 2 % of the total polyclonal antibodies produced worldwide are IgY. This low percentage is due to the difficulties in isolating antibodies from egg yolk, which is fundamentally composed of lipoproteins [4].

The main objective of this work is the development of an alternative purification platform for the selective extraction, and thus purification, of IgY from egg yolk using aqueous two-phase systems (ATPS). The use of ATPS for the selective extraction of mammal antibodies was previously addressed using conventional polymer-polymer or polymer-salt combinations [5-8]. Typical polymer-based ATPS display two hydrophobic phases, or a hydrophobic polymer-rich phase and a highly hydrophilic and ionic salt-rich phase. Therefore, there is always a restricted polarity difference between the two phases which has limited their success in the selective extraction and purification of monoclonal antibodies from complex matrices [7]. On the other hand, for the purification of IgY, a much less studied class of antibodies, only one work using micelle-based ATPS composed of a phosphate salt and Triton X-100 was found [9]. Previous literature results have

confirmed the enhanced ability of IL-based ATPS to separate and purify target biomolecules from complex matrices [10-17]. The major reason behind such improved capability relays on the possibility of tailoring the phases' polarities and affinities by a proper choice of the IL which constitutes a given ATPS. Indeed, there are *circa* one million of different ILs that can be synthesized [18]. Therefore, in this work, several ATPS were studied to infer on their potentiality to purify IgY from egg yolk, and composed of polyethylene glycol and Na_2SO_4; polyethylene glycol, Na_2SO_4 and ionic liquids (ILs) as adjuvants; and Na_2SO_4 and ILs.

This work starts with an introduction to IgY and their physicochemical properties followed by the description of the methods commonly applied for the separation of antibodies from related contaminants.

As a first approach several experimental studies were carried out using literature methodologies whereas the feedstock composition was followed/confirmed by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) applied to egg yolk samples and pure IgY commercially acquired. After the proper evaluation of the feedstock, the studies on the selective partitioning and purification of IgY were initiated using ATPS. Several ATPS composed of polyethylene glycols (PEGs) of different molecular weight and Na_2SO_4 were initially investigated. After the selection of the most adequate polymer, one polymer-salt ATPS was used combined with different ILs and at different concentrations aiming at allowing the selective partitioning of IgY for a specific phase (whereas the impurities must be retained in the other phase). Finally, the ATPS composed of Na_2SO_4 and one of the improved ILs was also evaluated. To evaluate the potential systems capable of selectively extracting IgY from egg yolk, the total protein partition coefficients in each ATPS and the respective proteins profile were also determined. Novel phase diagrams composed of PEG + Na_2SO_4 + water and PEG + Na_2SO_4 + IL + water were also determined to ascertain on the biphasic region and phases' compositions.

1.2. Antibodies

Antibodies are proteins found in plasma and extracellular fluids of all vertebrate species and are members of a particular family of molecules, the immunoglobulins (Ig), which constitute the humoral branch of the immune system and form approximately 20 %

of the plasma proteins in humans [3, 19, 20]. Different populations of immunoglobulins are found on the surface of lymphocytes, in exocrine secretions and in extravascular fluids [19]. Antibodies are host proteins produced in response to bacteria, viruses, foreign molecules or other agents in the body [19, 20]. This response is a key mechanism used by a host organism to protect itself against the action of foreign molecules or organisms [19]. Each animal can produce millions of different antibodies, and each antibody is able to bind specifically to a particular foreign substance known as an antigen [3]. B-lymphocytes carrying specific receptors recognize and bind the antigenic determinants of the antigen and this motivates a process of division and differentiation, transforming the B-lymphocytes into plasma cells. There are these lymphoid or plasma cells that predominantly synthesize antibodies [19].

1.2.1. Antibody structure and function

Antibodies are glycoproteins composed of four polypeptides. They contain a common structural domain found in many proteins and hence they are also referred to as immunoglobulins [3]. All antibodies have a common Y-shape based on the four polypeptide chain [20]. A pair of identical heavy chains, each of them carrying covalently attached oligosaccharide groups, is linked to another identical pair of non-glycosylated light chains by disulfide bonds resulting in a molecule of ~150 kDa [19, 20] (Figure 1).

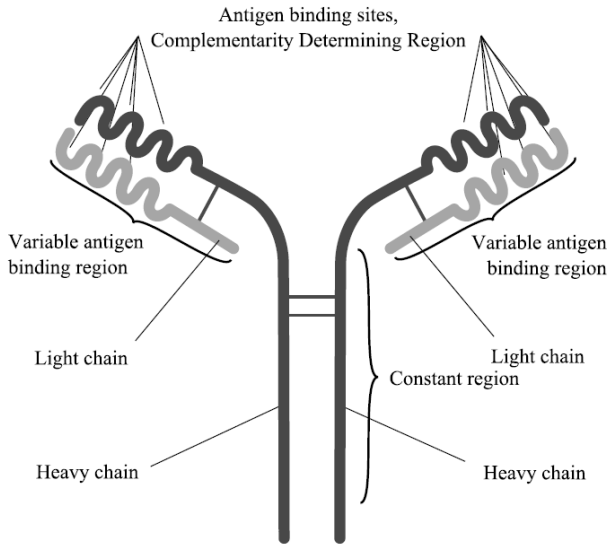

Figure 1 - Schematic representation of an antibody [20].

In antibodies, the heavy chains are joined to each other by disulfide bonds located in a flexible region of the heavy chain known as the *hinge*, a region of approximately twelve amino acids that is exposed to enzymatic or chemical cleavage. Each globular region formed by the folding of the polypeptide chains as a result of the disulfide bonding is termed a *domain*. All polypeptide chains contain constant (C) and variable (V) regions, found at the carboxyl and the amino terminal portions, respectively [19]. Heavy and light chains have a single V region, while light chains possess a single C region. Heavy chains contain three C regions. The V regions of both heavy and light chains combine to form two identical antigen binding sites [19]. The heavy chains are responsible to give the characteristic of the immune system. Still, the light chains are the responsible to define the antigen-binding specificity [20]. The effect or functions of antibodies, such as placental transport or antigen-dependent cellular toxicity, are mediated by structural determinants within the fragment crystallizable (Fc) region of the immunoglobulin [19]. Depending on the Ig class that is determined by their heavy chain components, five structural molecules may be combined to form an antibody [3]. In mammals, there are five classes of Ig (IgG, IgM, IgA, IgD and IgE) whereas in hens there are three classes (IgY, IgM and IgA) [3]. Ig classes are distinguishable in the structure and in the temporal nature of the immune response [3, 21].

The interaction of an antigen is crucial to the antibody's natural biological functions [3]. The specificity of the antibody response is mediated by T and/or B cells through membrane-associated receptors that bind a target antigen of a single specificity [3]. After binding to an appropriate antigen and receipt of various other activating signals, the B-lymphocytes divide, which produce memory B cells as well as antibody secreting plasma cells clones [3, 19]. The memory of B-lymphocytes remains latent until they are subsequently activated by their specific antigen [3]. Lymphocytes provide the cellular basis memory and the resulting escalation in antibody response when re-exposed to a specific antigen [3]. Because most antigens are highly complex, they present numerous *epitopes* (antibody binding site) that are recognized by a large number of lymphocytes [3].

1.2.2. Polyclonal and monoclonal antibodies

Monoclonal and polyclonal antibodies are different in nature and the differences between those antibodies define the limitations of their use [20]. Polyclonal antibodies are most frequently produced in animals and recognize independent *epitopes* on the antigen and are able to bind to different substances [19, 20]. Each specific polyclonal antibody is produced by a clone of plasma cells because B-lymphocytes are activated to proliferate and differentiate into plasma cells [19, 20]. Serum is a very good source of polyclonal antibodies [19]. These antibodies are commonly used in immunochemical techniques to detect denatured proteins, using crude serum as the main source [19, 20]. In contrast, monoclonal antibodies are only produced by a single B-lymphocyte clone; therefore, all of them are identical [3, 20]. The high specificity of a monoclonal antibody, since it can only interact with a specific and unique substance, is a significant advantage, particularly in therapeutic applications [19, 20]. Monoclonal antibodies are frequently used in the form of tissue culture supernatants harvested from the hybridoma culture, or as crude extracts produced from hybridoma cells grown as tumors in syngenic mice. Production of monoclonal antibodies using hybridoma technology has been successful in the production of mouse monoclonal antibodies, but this has meant that therapeutic applications have always been associated with the risk of immunogenic reactions [19]. The main difference between them is that monoclonal antibodies are more specific than polyclonal antibodies. Due to their neat binding to the antigen, monoclonal antibodies are extremely efficient in the purification of mixtures of molecules. Unfortunately, their homogeneity also makes them less tolerant to denaturation (induced by pH or temperature variations) and degradation when compared to polyclonal antibodies. If denaturation occurs, the monoclonal antibodies become useless for applications, while the denatured polyclonal antibodies can still be used in some applications [20]. An example of this application is, for instance, immunoblotting from denaturing gels such as SDS-PAGE.

1.3. Hen antibodies

Hen egg is the largest biological cell known resulting from one cell division [21]. It is a storehouse of variable nutrients, namely proteins, lipids, carbohydrates and other

substances with biological functions that extend beyond basic nutrition [21, 22]. Hence, since the mid-1970s, many studies on the characterization on the biophysiological functions of egg components and the seeking of novel biologically active substances in hen eggs have been conducted [23]. Egg yolk, particularly, is considered as a major source of active principles because they also include a large quantity of antibodies which may be used in the medicinal, pharmaceutical, cosmetic and biotechnological areas [1, 22, 24]. In chicken there are three classes of antibodies: IgY, IgA and IgM [1]. IgA and IgM, similar to mammalian IgA and IgM, are secreted in the ripening egg follicle together with other proteins and are deposited into the egg white [1, 4, 21]. IgY is the functional equivalent of mammal IgG 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 developing of chicks [1, 4]. In the egg yolk, IgY is the predominant and most important fraction of γ-livetin [22]. Livetins are a fraction of water-soluble proteins that have three main components including α-, β-, and γ-livetin [22]. Egg white contains IgA and IgM at concentrations of around 0.15 a 0.7 $mg \cdot mL^{-1}$, respectively, whereas the yolk may contain from 5 to 25 $mg \cdot mL^{-1}$ of IgY [4, 24]. Mammalian equivalents of IgE and IgD have not been identified in chickens [4].

1.4. Molecular properties of IgY

In 1969, Leslie and Clem reported the existence of an IgG-like molecule in chickens [4]. For many years, in older literature and even in commercial product catalogues, IgY is often mislabeled as IgG, due to its functional similarity to mammalian IgG. However, this older nomenclature is outdated, since the molecular properties of IgY and mammalian IgG are substantially different.

1.4.1. Structure

IgY contains two heavy and to light chains which are linked by a disulfide bridge, and has a molecular mass of ~180 kDa (larger than mammalian IgG (~159 kDa)) [1, 4, 25].

IgY possesses a larger molecular weight heavy chain (68 kDa) as compared to that from mammals (50 kDa). The light chain of IgY consists of one variable (VL), and one constant domain (CL), like mammalian IgG [1, 25]. The intra-chain disulfide linkage between the VL region and the CL region of the light chain that exists in IgG is absent in the IgY light chain, and thus, intra-molecular forces of IgY are weaker than those of mammalian IgG [25]. The heavy chain of IgY has one variable domain (V_H) and four constant domains (C_H1; C_H2; C_H3 and C_H4), in contrast to mammalian IgG which has three constant domains (C_H1; C_H2 and C_H3) [1]. In the heavy chain of IgG, the C_H1 and the C_H2 domains are separated by a *hinge* region, which gives considerable flexibility to the Fab fragment (the portion which contains the antigen-binding activity) [25]. In contrast to IgG, IgY has a shorter and less flexible *hinge* region [1]. Comparisons of C-domain sequences in IgG and IgY have shown that the C_H2 and C_H3 domains of IgG are the equivalents of the C_H3 and C_H4 domains of IgY, respectively, and the equivalent of C_H2 domain of IgY is absent in the heavy chain of IgG [25]. In addition, the content of β-sheet structure in C domains of IgY is lower than that of mammalian IgG suggesting that the conformation of IgY domains is more disordered and less stable in comparison to mammalian IgG [1, 25]. As for IgG, the Fc part of IgY is the site with the most biological effector functions and contains two carbohydrate side-chains, in contrast to only one verified in IgG [25]. Figure 2 shows the differences between IgY and IgG. Both IgG and IgY are known to contain Asn-linked oligosaccharides. However, the structure of oligosaccharides in IgY differs from those of any mammalian IgG containing unusual monoglucosylated oligomannose type oligosaccharides with $Glc_1Man_{7-9}GlcNAc_2$ structure [1, 4].

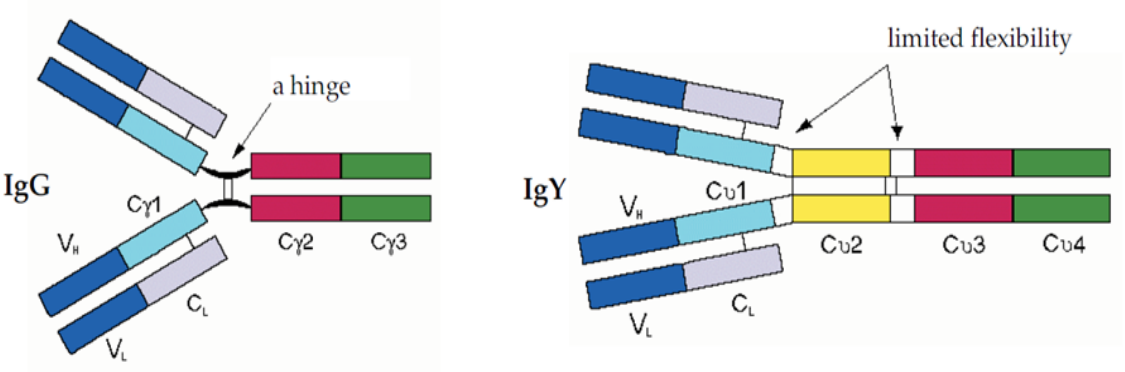

Figure 2 - Structure of IgG and IgY [26].

1.4.2. Physicochemical properties

The isoelectric point of IgY is lower than that of IgG [25]. It is in the range of 5.7 to 7.6, whereas that of IgG lies between 6.1 and 8.5 [1, 4, 25]. Since the Fc fragment (the most hydrophobic moiety of the antibody molecule) of the IgY is bigger than that of the IgG, the IgY molecule is more hydrophobic than the IgG molecule [25]. However, it has also been suggested that IgY can be more hydrophobic because of the lipid-rich fraction of egg yolk [1].

1.4.3. Stability

The stability of IgY to acid and alkali media has been studied under various conditions. It was found that the activity of IgY was decreased at pH 3.5 or lower and almost completely lost with irreversible change at pH 3 [25, 26]. The rapid decrease of the IgY activity at low pH values indicated conformational changes. Nevertheless, the polypeptide is not broken down as observed by SDS-PAGE [25, 26]. Under alkaline conditions, the activity of IgY did not changed until a pH of 11 [25, 26]. However, it was markedly diminished at pH 12 or higher [1, 25]. Finally, the pH stability of IgY may be improved by the addition of stabilizers, such as sugars, complex carbohydrates or polyols [1].

1.4.4. Proteolysis stability

IgY is relatively resistant to trypsin or chymotrypsin digestion; yet, it is fairly sensitive to pepsin digestion [25]. Hatta et al. demonstrated that almost all of the IgY activity was lost by the digestion with pepsin, but 39 % and 41 % of the activity remained even after 8 h of incubation with trypsin or chymotrypsin, respectively [27]. The sensitivity of IgY to pepsin is highly dependent on the pH and on the enzyme/substrate ratio [1, 25]. At pH 5 or higher, IgY was fairly resistant to pepsin and retained its antigen-binding and cell-agglutinating activities [25]. However, at pH 4.5 or below, the antibody activity was completely lost at an enzyme to substrate ratio of 1:20 [1]. The results of Hatta et al., who

also observed the IgY behavior with pepsin under different incubation times and pH, confirmed the susceptibility of IgY to pepsin at low pH [27]. Digestion of IgY with pepsin at pH 2 resulted in the complete hydrolysis of the antibody molecule, leaving only small peptides when pepsin was used at an enzyme/substrate ratio of 1:200. Even though IgY was digested by pepsin at pH 4 it retained 91 % and 63 % of its activity after 1 h and 4 h of incubation time with the same enzyme/substrate ratio [27]. Following a tryptic digestion, IgY retained its antigen binding and cell-agglutinating activities in spite of a definite breakdown of the polypeptides. Unlike the trypsin digestion, no definite cleavage of the IgY chains was observed for chymotryptic digestion and the activities of IgY remained high for these digests [25].

1.4.5. Temperature and pressure stability

The binding activity of IgY with antigen decreases with increasing temperature and heating time [1, 25]. Minimal loss of activity was observed at temperatures ranging between 60 °C and 65 °C but it decreased markedly by heating for 15 min at 70 °C [1, 25, 26]. At temperatures higher than 75°C, IgY almost complete denatures [25]. However, addition of carbohydrates has been found to improve its resistance [1].

IgY fractions have been stored in 0.9 % NaCl and 0.02 % NaN_3 at 4 °C for over 10 years without any significant loss of antibody titer [26]. The purified antibodies also retained their antigen binding capacity after 6 months at 20 °C or 1 month at 37 °C [26]. An egg yolk can be stored at 4 °C, with just a small loss of IgY activity, for at least six months [26].

IgY is relatively stable to pressure since no detectable inactivation of IgY was observed with pressures up to bar [25].

1.4.6. Freeze and spray-drying stability

Freezing and freeze-drying are processes that are usually considered to be less destructive and that do not affect the activity of IgY (unless repeated several times) [25]. However, Sunwoo et al. showed that frozen or freeze-dried IgY resulted in some loss of

antigen-binding activity and a substantial drop in the solubility under the conditions of high salt and protein concentrations [28]. Fu et al. studied the stability of IgY at various temperatures between 25 and 90 °C for a 15 min treatment, before and after a freeze-drying process [29]. The results provided by the authors reveal that freeze-dried IgY has a good thermal stability with no significant reduction in reactive activity, except at 90 °C [29]. This was also observed for the non-freeze-dried IgY [29]. Yokoyama et al. analyzed some properties of IgY powders obtained by spray or freeze-drying the water-soluble fraction of egg yolks from *Escherichia coli* immunized hens [30]. As compared to the freeze-dried powder, the spray-dried powder did not show a relevant alteration in antibody titres and yields, even with temperatures ranging between 140 to 170 °C [30]. Likewise, it has been shown that the addition of disaccharides or complex carbohydrates to IgY prior to freeze-drying can improve the IgY stability during storage [1].

1.5. Extraction/ purification of IgY

Although the chicken eggs are a convenient and economical source of antibodies, there are many difficulties on the extraction of IgY from egg yolk and, for this reason, the separation and purification of IgY from egg yolk has been considerably studied [4, 31]. Egg yolk is a fluid emulsion with a continuous phase of lipoprotein particles [4]. The major problem in isolating IgY from egg yolk relays on the separation of lipoproteins from egg yolk before the purification of IgY [4]. Based on this strategy, many purification methods of IgY have been reported, and are summarized and compared in Table 1.

Many methods have been described using, for instance, simple water dilution, followed by centrifugation or ultrafiltration, to isolate the lipoproteins of the water-soluble proteins fraction (WSPF) [31-33]. This method is based on the aggregation of yolk lipoproteins at low ionic strengths [4]. For these dilution methods, optimization of the operational conditions (pH, temperature, dilution factor, etc.) has allowed the increase on the IgY recovery up to 90 % [32]. However, different authors have accomplished different results and have thus different perspectives. Nakai et al. reported that the best results were obtained using a six-fold water dilution, at pH 5.0 [34]. Yao Luo et al. showed that the enhanced results were obtained with a fifteen-fold water dilution [35]. Marcet et al. advised that the egg yolk should suffer a 1.5 water dilution, at pH 7 [22].

According to literature the main components of WSPF are IgY, α-livetin, β-livetin, low density lipoproteins and albumin [33]. Therefore, IgY must be purified from WSPF after a first step involving the lipoproteins precipitation [4, 33]. The recovery of IgY has been carried out with the addition of ammonium sulfate [34-36], sodium sulfate [31, 34], sodium alginate [23], cadmium sulfate [37], zinc sulfate [37] and dextran sulfate [34].

Beyond the water dilution methods, other approaches were developed to separate IgY from the lipoproteins and other impurities including deslipidation by organic solvents (isopropanol [38] and chloroform [39]) and by a mixture with κ-carrageenan, low-methoxyl pectin and $CaCl_2$ [40], and lipoprotein precipitation using PEG [22, 34, 39, 41], dextran blue [42], natural gums (such as xanthan gum) [34] or caprylic acid [38].

Ion exchange chromatography has also been used as a final step in the IgY purification, as well as hydrophobic interaction chromatography [4]. Because of the failure of IgY to bind to proteins A and G, and its sensitivity to traditional affinity purification conditions, several other methods of affinity chromatography have been examined for the purification of IgY, including immobilized metal ion affinity chromatography, thiophilic interaction chromatography, affinity chromatography using alkaline conditions and synthetic peptide ligands specifically designed for immobilizing antibodies [4, 39].

The use of supercritical fluids has also been employed as a separation method. Nevertheless, high costs have deemed this process as non-viable [43].

Stimulated by an increasing demand for IgY-based technologies, several commercial suppliers have developed IgY purification kits. Two of the most prominent (Thermo Pierce's Eggcellent™ and Promega'sEGGstract®) are based on a delipidation step followed by the IgY precipitation/purification [40]. Although both kits are particularly user friendly and do not involve the use of organic solvents they are particularly expensive given the yield of IgY produced (up to $67 USD *per* egg). Furthermore, none of these commercially available options is available in a scalable commercial level [40].

Table 1 - Comparison of the literature methods for purifying IgY.

	Purification method	IgY recovery (%)	Total protein (mg)	IgY ($mg \cdot ml^{-1}$)	Purity of IgY (%)	Reference
	Water dilution	91	773	(----)	31	[34]
		(----)	(----)	(----)	85	[44]
	Ultrafiltration	87	(----)	(----)	> 93	[31]
		(----)	(----)	9.8	94	[34]
		91	(----)	(----)	85	[32]
Precipitation and extraction	Polyethylene glycol (PEG)	64.0	348	(----)	15.8	[39]
		(----)	(----)	4.9	89	[45]
		47	501.6	(----)	25	[34]
	Dextran sulfate	71	(----)	(----)	(----)	[34]
		(----)	(----)	7.5	87	[46]
	Sodium sulfate	(----)	(----)	9.8	94	[34]
	Ammonium sulfate	(----)	6.1	3.8	62.1	[37]
		(----)	(----)	60	~80	[40]
	Cadmium sulfate	(----)	6.2	1.9	31.8	[37]
	Dextran blue	79.2	ND	(----)	16.7	[39]
	Caprylic acid	97/90**		160/120	4.0/4.3 *	[38]
	Zinc sulfate	(----)	5.8	2.4	41.2	[37]
	Isopropanol	69/58**		160/120	1.0/1.1 *	[38]
	Chloroform	65.1	275	(----)	14.0	[39]
Natural gums	Xanthan gum	72	601.0	(----)	32	[34]
		(----)	(----)	7.3	89	[47]
Chromatography	Ion exchange chromatography	(----)	(----)	(----)	(----)	(----)
	Affinity chromatography	(----)	(----)	65	> 90	[39]
	Hydrophobic interaction chromatography	(----)	(----)	(----)	(----)	(----)
	Commercial kit	(----)	(----)	(----)	70-90	[40]

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

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

(----)– Non-determined.

All methods described before have in common the recovery and purification of IgY from the native egg yolk. Hence, IgY is the only compound recovered and many egg yolk compounds are lost [8]. Although several methods for the purification of IgY have been described in literature, all are time-consuming, labor intensive, low yielding, high cost and cannot easily be scaled-up for industrial applications [31, 39, 40]. In addition, in some of these protocols the solvents used are not innocuous and can lead to the appearance of solvent residuals in the final products and alter the IgY functionality.

In most of the studies reported in literature, SDS-PAGE was carried out as the main chromatographic technique to confirm the presence of IgY at the different steps of the isolation process [5, 22, 23, 31, 32, 40, 44].

1.6. Application of IgY

The polyclonal antibodies produced by chickens can be shaped against a large number of antigens [48]. They can be applied in many different methods for various purposes as academic research, diagnostic, therapeutic reagents, as a tool for purification or detection of antigens and as a protective agent in passive immunization. IgY are excellent alternatives for their mammalian counterparts in what concerns the passive immunization ability [48, 49]. In Table 2 some of the antigens produced and their applications are presented. IgY demonstrated to act in almost all the tested immunological methods that were traditionally developed for mammalian IgG (immunofluorescence, immnoenzyme techniques, immunoelectrophoresis and Western blotting, immunohistochemistry and many others) [48].

IgY is successfully used in immunochemistry to detect antigens of viral bacteria, plants and animal origins, and the contamination of foods with toxins and drugs [6].

Due to its distinctness from IgG, IgY has also been found to be advantageous in several immunodiagnostic techniques, as well as in immunoaffinity purification, in many cases replacing IgG, which is reflected in a great reduction of background and false results [4, 48].

Table 2 - Antigens used to produce specific polyclonal IgY and their applications [48].

Antigen	**Application**
Human IL-6	RE
Human manose 6-phosphate/insulin-like growth factor-II receptor	RE
Humantransferin	RE
α-subunit of hypoxia-inducible factor 1 (HIF-1)	RE
Helyobacterpylori	RE, TE
Choleratoxin B	RE
Rabies virus	RE
Sendaivirus	RE
Rabbitmuscleactin	RE
Humanrotavirus	RE, DE
Bovine growth hormone and prolactin	RE, DE
Lactoferin	RE, DE
Activin A	RE, DE
Mucin-likeglycoprotein A	DE
α-subunit of insulin receptor	DE
Toxoplasmagondii	DE
Rat liver cytosolic casein kinase II	DE
Chlamidiae	DI
Campylobacterfetus	DI
Cartilage gp-39	DI
Hepatitis B surfaceantigen	DI
Humanbloodantigens	DI, RE
Influenza virus	DI, RE
AsialoGM1 (T-antigen in human colorectal adenocarcinom)	DI, RE
Calf thymus RNA polymerase II	DI, RE, TE
Plasmid (naked) DNA	DI, TE
E7 oncogenic protein of human papillomavirus type 16	DI, TE
P110 protein (antigen of human stomach cancer)	DI, TE
Ig and blood components of several mammalian species	DI, PU
Human -2 plasmin (its carboxy-terminal peptide)	PU
Porcineendothelialcells	TE
E. coli	PI
Animal venoms (snake, viper, rattlesnake, scorpion)	PI
*Streptococcus mutans*glucan binding protein B	PI

RE, research reagent; **PU**, purification of antigens; **DE**, detection of antigens; **DI**, diagnostic reagent; **TE**, therapeutic reagent; **PI**, used in passive immunization

During the past decade, IgY was reported to be used as immunotherapeutic agents against pathogens that are difficult to treat with traditional antibiotics [48]. Shin et al. were able to identify the immunodominant proteins of *H. pylori* [50]. IgY antibodies with specificity against these proteins were effective and demonstrated a curative effect of the anti-*H. pylori* antibodies. In most cases no complete *H. pylori* eradication could be achieved, but in view of the increasing bacterial resistance, the use of specific IgY antibodies can minimizes the use of antibiotics [50]. The use of IgY for passive and protective immunization against gastrointestinal pathogens in humans and animals have also been studied extensively [1, 48].

Passive immunity refers to the process of providing preformed antibodies to protect against infection, and it provides immediate but short-lived protection lasting several weeks to three or four months at most. Passive immunity can be classified as natural, when maternal antibodies are transferred to the offspring or acquired, when high levels of antibodies specific for a pathogen or a toxin are recovered from immunized individuals or from patient recovering from the infection and administered to non-immune individuals [1, 25]. The transfer of antibodies may be carried out via systemic, intravenous or oral routes [25]. IgY has been found to be effective against a number of human pathogens and diseases, both in vitro and in laboratory animal studies and clinical settings [25]. Casswall, Carlander et al., and Sarker et al. investigated the action of hyperimmune bovine colostrum (HBC) and the oral administration of IgY antibodies with significant protective effect [51-53]. Worledge et al. demonstrated significant protective effects after oral application of specific IgY against tumor necrosis factor (TNF) in an experimental rat model for colitis [54]. TNF is implicated in the pathogenesis of inflammatory bowel disease [49]. Carlander et al. studied the benefits of IgY as a prophylactic tool against infectious diseases in patients with cystic fibrosis (CF), the most common fatal genetic disease of the Caucasian population in Europe and in the USA [55]. These oral IgY treatments were successful in reducing chronic *P. aeruginosa* infections in CF patients, and thus resulted in a decrease in antibiotic prescriptions [49].

A new and an interesting field of the use of the IgY-technology is the proteomic analysis. A major problem in the separation of complex protein mixtures by 2D-electrophoresis is the predominance of high-abundant proteins like albumin which disturb the monitoring of low-abundant proteins. Low-abundant proteins can be of high

importance for identification and monitoring several human (and animal) diseases. Recently, it has been shown that IgY antibodies directed against these high-abundant proteins are in fact useful tools for their removal. In addition, these antibodies work more specific than matrices with affinity to albumin like for example blue sepharose [49].

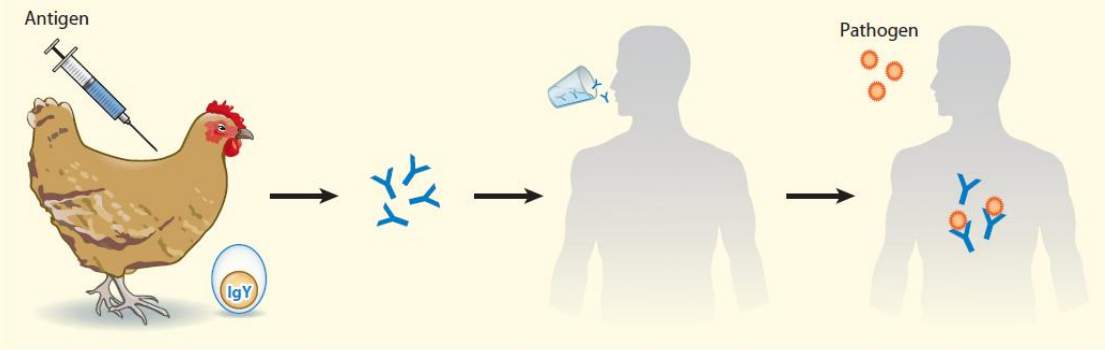

Figure 3 - Passive immunization process [1].

1.7. Production of IgY

The production of large amounts of IgY in a cost-effective manner is the key for a successful passive immunization. The development and production of specific IgY can be achieved by immunizing laying-hens with the target antigen [25]. The immune response of immunized hens cannot be very predictable due to five factors that influence the immune response and which are: the antigen dose and molecular weight, the type of adjuvant used the route of application, the immunization frequency, and the interval between immunizations [25].

1.7.1. Antigen

The dose of antigen influences the immune response and the antibody titre [25]. Too much or too little antigen dosages may induce suppression, sensitization, tolerance or other unwanted immunomodulation [25]. Behn et al. achieved better results when injecting hens with 0.1 mg of mouse IgG, instead of 1.0 mg. Schwarzkopf et al. found that the injection of

antigen concentrations ranging between 10 µg and 1 mg elicited good antibodies responses [56]. Therefore, for each antigen, various concentrations have to be tested since the type of antigen should be also considered [25]. Antigens can be present in the immune system as complex multiantigens (e.g., bacteria) or as single antigens (*e.g.*, proteins). Proteins are recognized to be the most efficient immunogens because of the polymorphism of their structure and the differences existing between species and individuals. As a general rule, only 10-100 µg of protein antigen is enough [25]. Peptides (with a molecular weight below 10 kDa) can also be used as antigens, but they should be coupled to carriers (e.g., bovine serum albumin). Polysaccharides antigens are efficient too. However, lipid and nucleic acids are not potent immunogens unless they are coupled to proteins or polysaccharides [25].

1.7.2. Adjuvant

The induction of high egg yolk antibody titres can be also achieved by the use of adjuvants [25]. There are more than 100 known adjuvants, which differ in their chemical characteristics, their efficacy in stimulating the immune system, and their secondary side-effects [25]. Oil-based adjuvants, such as Freund's complete adjuvant (FCA), remain the most effective for antibodies production in laboratory animals [1, 25]. However, FCA has been associated with potentially severe injection site reactions [1]. To avoid an eventual local tissue reaction, the Freund's incomplete adjuvant (FIA), which is the most effective substitute found to date, becomes now the most commonly used adjuvant to produce egg yolk antibody. Since FIA is less efficient than FCA, some investigators preferred the use of a combination of the two adjuvants: FCA for the first immunization and FIA for the following immunizations [25].

The use of DNA vaccines has also been reported for IgY production. In this process the animals are immunized with plasmid DNA encoding the antigen of interest and eliminating the potentially tedious and costly process of purifying antigens [1].

1.7.3. Route of application

The most common injection route in hens for IgY production is the intramuscular route. Intramuscular immunization results in higher levels of specific IgY when compared to antigen injected subcutaneously [1]. Injection is usually performed in the breast muscle [25]. Chicken can also be injected subcutaneously in the neck. With very young animals, it may be preferable to inject intramuscularly into the breast muscle, because subcutaneous injection is more difficult to perform and can therefore cause more distress [25].

1.7.4. Immunization frequency an interval between immunizations

The total number of immunizations necessary for the production of IgY depends on the type and dosage of the antigen as well as the adjuvant employed. At least two immunizations have to be given. Yolk antibody titres should be checked fourteen days after the last immunization. If antibodies titres begin to decrease, following immunizations can be given during the laying period to maintain production of high levels of specific antibodies up to one year [25]. The success of an immunization protocol depends also on the interval between the first and second and subsequent immunizations. This interval is between two to four weeks [25].

1.8. Advantages of IgY

Polyclonal antibodies have been conventionally isolated from sera of animals such as rabbits, goats and sheep superimmunized with an aimed antigen [2]. However, the use of chickens for the production of polyclonal antibodies provides several advantages [4, 19].

IgY is produced by a non-invasive method which does not cause pain to animals or lead to their dead since it is based on a simple act of collecting eggs, and so, the production of IgY from eggs is easier than that of IgG from serum [21, 22]. The preparations methods of IgG and IgY are shown in Figure 4. Moreover, few eggs *per* week can provide the same amount of immunoglobulin as repeated bleeding of an immunized mammalian. Usually more than 100 mg of IgY can be isolated *per* egg [19, 21]. Several works published

comparative studies on the production of antibodies by immunized hens and rabbits sera [21, 46, 57]. Jensenius et al. estimated that the total antibody activity of the eggs laid by a hen in a month is equivalent to that produced in a half liter of serum from an immunized rabbit [46]. Gottstein and Hemmler reported that the quantity of IgY obtained from eggs laid by an immunized hen was 18 times higher than that of IgY isolated from the serum of an immunized rabbit [57]. Mujo Kim et al. also compared the productivity of IgY from eggs laid by a hen over a year with that of IgG from the entire serum of a rabbit in which both animals were immunized with the same antigens and the productivity of IgY exceeded the productivity of IgG [21].

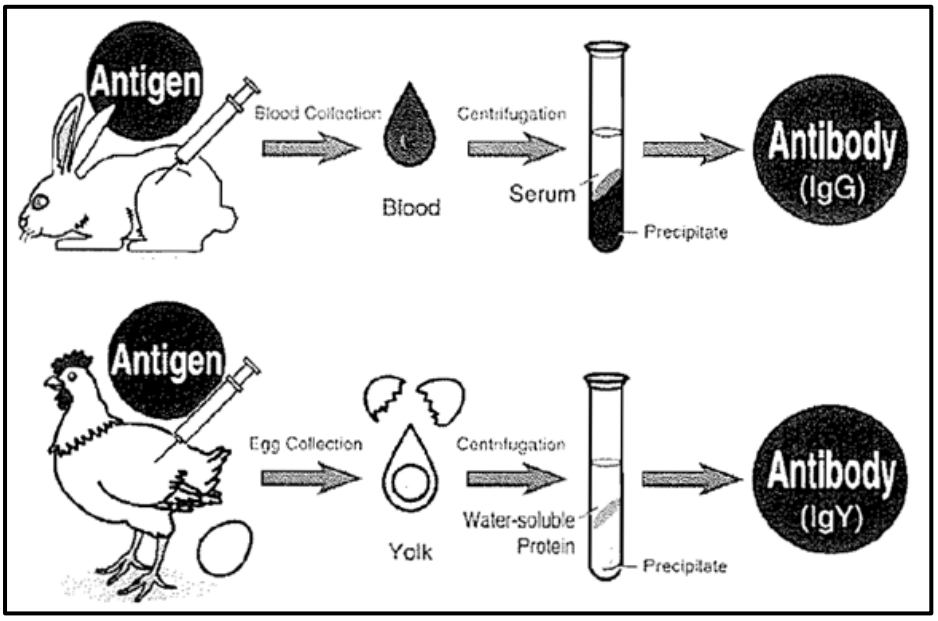

Figure 4 - Preparation of specific antibodies [21].

Furthermore, an industrial scale production of IgY is plausible because of the availability of a large number of chicken farms and automation of egg breaking and processing [21]. Therefore, chicken eggs are considered as a potential source of large-scale production of IgY [22].

On the other hand, compared to its mammalian IgG counterpart, IgY neither binds to the mammalian or bacterial Fc receptor, it does not activate the complement system or interact with the rheumatoid factor [40]. Not surprisingly, IgY antibodies also show far superior immunogenicity against highly conserved mammalian proteins making the

production of IgY orders of magnitude more efficient than current IgG production using rabbits, donkeys and mice [40].

Table 3 shows the advantages associated with the production of antibodies in chicken and compares the characteristics of mammalian IgG and avian IgY.

Table 3 - Comparison between the mammalian IgG and avian IgY [4, 58].

	Mammalian IgG	**Avian IgY**
Source of antibody	Blood serum	Egg yolk
Kind of antibody	Polyclonal	Polyclonal
Antibody sampling	Invasive - bleeding	Non-invasive – collecting eggs
Antibody yield	200 mg/ bleed (400 mL blood)	100-150 mg/ egg (5-7 eggs *per* week)
Antibody yield per month	200 mg	~ 1500 mg
Specific antibody yield	~ 5 %	2-10 %
Protein A/G binding	Yes	No
Interaction with rheumatoid factor	Yes	No
Activation of mammalian complement	Yes	No

1.9. Extraction of IgY using ATPS

1.9.1. Extraction of biomolecules using ATPS

There are two main processes commonly used to extract biomolecules from a liquid phase to another: liquid-liquid extraction using water immiscible organic solvents and aqueous two-phase systems (ATPS). This last extraction method uses two incompatible solutes dissolved in aqueous media and are known since 1896 when Beijerinck discovered agar and gelatin form two macroscopic liquid aqueous phases [2, 6, 59]. Nevertheless, ATPS as extractive/separation techniques were only introduced by Albertson in 1955 [2, 8, 60]. Albertson demonstrated that a concentrated solution of an organic polymer, such as PEG, and an inorganic salt, such as dibasic sodium or potassium

phosphate (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 [60]. ATPS are thus formed when two mutually incompatible hydrophilic solutes are dissolved in water above certain concentrations [6, 8]. These solutes can either be two polymers, a polymer and a salt, or two salts [2, 6].

ATPS are a powerful, non-chromatographic process for the separation and/or purification of biomolecules, and which have been successfully applied in the purification of different biological materials, such as cells, viruses, organelles, nucleic acids, lipids, amino acids, proteins, antibodies and enzymes without significant denaturing effects [6, 8, 60]. Moreover, this technique is relatively simple and inexpensive, is easily operated and scaled-up, has a high resolution capacity, and allows the purification and concentration to be integrated in a single step procedure [6, 8].

The partition of a target product in ATPS depends on a number of parameters related to the system properties [2, 5]. The extrinsic properties concern the type, molecular weight and concentration of phase forming components, ionic strength, pH, temperature and others [5]. The intrinsic properties include size, electrochemical properties, surface hydrophobicity and hydrophilicity and conformal characteristics of both the phases and of the solute to be extracted [5]. The complexity of the chemical and physical interactions involved in the partitioning process can make these systems very powerful in contrast to other established separations techniques [2].

Conventional ATPS are typically formed by polymer-polymer or by polymer-inorganic salt mixtures. These ATPS are usually chosen for the extraction/purification of biomolecules, such as proteins, due to their biocompatibility since both phases consist mainly of water (80-90 wt %) and most of the polymers have a stabilizing effect on the protein tertiary structure [5, 6, 8].

PEG is common used as one of the phase forming polymers in ATPS because it presents a high biodegradability, low toxicity, low volatility, low melting temperature, large water miscibility and low cost [12, 59]. PEG is as polyether diol that is commercially available in a variety of molecular weights [5]. Salt-polymer-type ATPS provide advantages over ATPS formed by polymer-polymer combinations, such as a low interfacial tension, fast and high phase separation rates and low cost, which makes them more viable for downstream processing [12]. Despite all these advantages, the hydrophilic nature of

PEG limits the applicability of this technique when the goal is to extract hydrophobic biomolecules [12]. To overcome this limitation, recent works have introduced ionic liquids (ILs) to tune the properties of PEG through the modification of its chemical structure and thus increase the extraction yield [61, 62]. Moreover, an additional work proved that the addition of small quantities of ILs to conventional polymer-salt-ATPS leads to different partitioning behaviors that are dependent on the IL employed as adjuvant (at 5 wt %) [12]. Therefore, the use of ILs in conventional systems seems to provide tailored and optimized extractions by a proper choice of the IL used as phase-forming component.

ILs are salts that are liquid below a conventional temperature of 100 ºC and they are usually composed of a large asymmetric organic cation and either an organic or inorganic anion. The most studied ILs are the nitrogen-based and their general cation structures are present Figure 5.

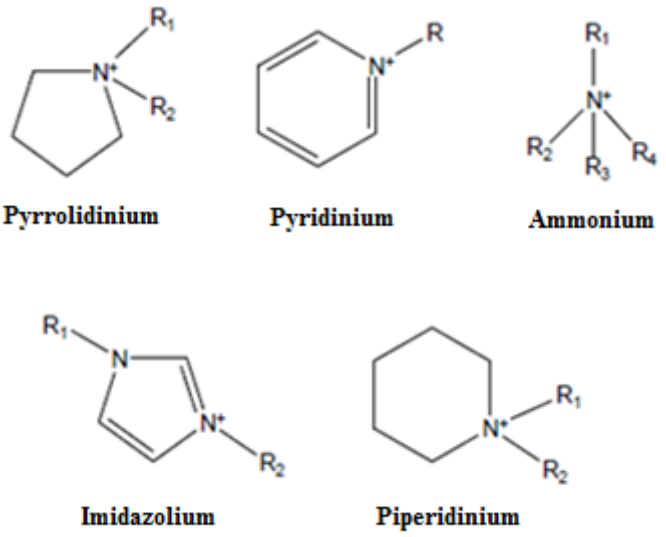

Figure 5 - Cation structures of nitrogen-based ILs.

Due to their ionic character, these fluids present outstanding properties such as a negligible vapor pressure, null flammability, high ionic conductivity, as well as high thermal and electrochemical stabilities. Besides the ILs negligible volatility and non-flammability, which have contributed to their being labeled as "green solvents", one of the main advantages of the use of ILs in ATPS is the possibility of tailoring their phases' polarities and affinities by an adequate manipulation of the cation/anion combinations -

"designer solvents". Unlike most traditional molecular solvents, ILs can be regarded as nano-segregated media, composed of a polar network permeated by non-polar domains [63]. These domains allow the ILs to interact selectively with different types of solute/solvent and improved properties and applications can always be foreseen. In this context, ILs cover a much wider hydrophilic-lipophilic range allowing for more extensive and selective separations. In fact, ATPS with ILs have been intensively investigated for the extraction of the most diverse (bio)molecules [10, 12, 13, 15] where results up to complete extraction were achieved.

1.9.1.1. Phase diagrams and respective properties

ATPS have a unique phase diagram under a particular set of conditions such as pH and temperature [59]. Phase diagrams provide information about the concentration of the phase forming components required to form a two-phase system, the concentration of the phase components in the top and bottom phases, and the ratio of phase volume or weight [59]. The binodal curve of a phase diagram divides two regions: the monophasic region (bellow the curve), where at these concentrations the system is monophasic, and the biphasic region (above the curve), where the concentration of the components will form two immiscible aqueous phases. The binodal data are thus initially required for the design of proper ATPS extraction processes and to the development of models to predict the partitioning of biomolecules [59]. Figure 6 shows an example of a binodal curve, TCB, and three mixture compositions at 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 will present the same top phase equilibrium composition (T_{PEG}, T_{Salt}) and the same bottom phase composition (B_{PEG}, B_{Salt}) [59].

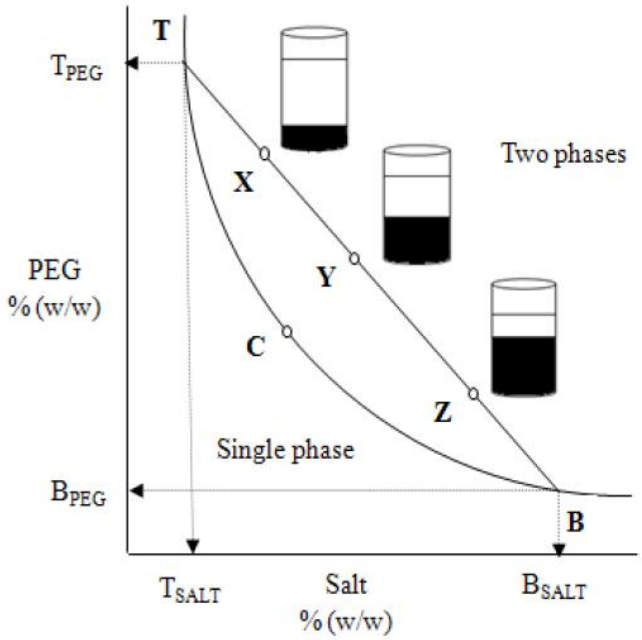

Figure 6 - Binodal curve: TCB = binodal curve; C = critical point; TB = tie-line; T = composition of the top phase; B = composition of the bottom phase; X, Y and Z = mixture compositions at the biphasic region [59].

The units of tie-line length (TLL) are given in the same units used for representing the components concentration [59]. The length of each tie-line is related to the weight of the phases and defines the difference in compositions among the coexisting phases [59].

2. Experimental section

2.1. PEG + Na_2SO_4 + H_2O ternary systems

2.1.1. Chemicals

The ATPS studied in this work were established by using an aqueous solution of sodium sulfate anhydrous, Na_2SO_4 (100 % pure from Prolabo), and different aqueous solutions of PEGs. The PEGs studied were of molecular weight 200 $g \cdot mol^{-1}$, 300 $g \cdot mol^{-1}$, 400 $g \cdot mol^{-1}$ and 600 $g \cdot mol^{-1}$ and abbreviated as PEG 200, PEG 300, PEG 400, and PEG 600. All the PEGs were from Fluka with the exception of PEG 300 that was from Sigma-Aldrich.

The water employed was double distilled, passed across a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

A saline buffered aqueous solution using commercial Phosphate Buffer Saline tablets (PBS), from Sigma-Aldrich, was also prepared and used as the aqueous phase to maintain the pH of the ATPS at 7.4.

2.1.2. Experimental procedure

2.1.2.1. Phase diagrams

The binodal curve of each phase diagram was determined through the cloud point titration method at 298 K (± 1 K) and atmospheric pressure [6]. This procedure was initially validated with the phase diagram of PEG 600 + Na_2SO_4 + water already reported in literature [12] (*cf.* Appendix A - Figure A 1). Aqueous solutions of Na_2SO_4 at 17 wt % and aqueous solutions of the different PEGs at 70 wt % were prepared and used for the phase diagrams determination. Repetitive drop-wise addition of the aqueous inorganic salt solution to each PEG solution was carried out until the detection of a cloudy biphasic solution, followed by the drop-wise addition of water until the detection of a monophasic region (clear and limpid solution). Drop wise additions were carried out under constant stirring. The ternary systems compositions were determined by the weight quantification of all components added within an uncertainty of ± 10^{-4} g.

2.1.2.2. Determination of TLs

The tie-lines (TLs) were determined by a gravimetric method originally described by Merchuck et al. [64]. A mixture at the biphasic region was gravimetrically prepared with PEG + salt + water, vigorously stirred, and allowed to reach the equilibrium by the separation of both phases for at least 12 h at 298 K (± 1 K). After the separation step, both top and bottom phases were weighted. Finally, each individual TL was determined by the application of the lever-arm rule to the relationship between the top phase weight and the overall system composition. The experimental binodal curves were fitted using Equation 1 [64]:

$$[\mathrm{PEG}] = A\exp[(B[\mathrm{salt}]^{0.5}) - (C[\mathrm{salt}]^3)] \quad (1)$$

where [PEG] and [salt] are, respectively, the PEG and salt weight percentages and A, B and C are constants obtained by the regression.

For the determination of TLs it was solved the following system of four equations (Equations 2 to 5) and four unknown values ($[\mathrm{PEG}]_{\mathrm{PEG}}$, $[\mathrm{PEG}]_{\mathrm{salt}}$, $[\mathrm{salt}]_{\mathrm{PEG}}$ and $[\mathrm{salt}]_{\mathrm{salt}}$):

$$[\mathrm{PEG}]_{\mathrm{PEG}} = A\exp\left[\left(B[\mathrm{salt}]_{\mathrm{PEG}}^{0.5}\right) - \left(C[\mathrm{salt}]_{\mathrm{PEG}}^{3}\right)\right] \quad (2)$$

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

$$[\mathrm{PEG}]_{\mathrm{PEG}} = \frac{[\mathrm{PEG}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{PEG}]_{\mathrm{salt}} \quad (4)$$

$$[\mathrm{salt}]_{\mathrm{PEG}} = \frac{[\mathrm{salt}]_{\mathrm{M}}}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[\mathrm{salt}]_{\mathrm{salt}} \quad (5)$$

The subscripts PEG, salt and M represent the top, bottom and the mixture phases, respectively. The parameter α is the ratio between the top weight and the total weight of the mixture. The solution of the referred system gives the concentration of the IL and salt in the top and bottom phases. For the calculation of the tie-line lengths (TLLs) it was applied Equation 6.

$$\mathrm{TLL} = \sqrt{([\mathrm{salt}]_{\mathrm{PEG}} - [\mathrm{salt}]_{\mathrm{salt}})^2 + ([\mathrm{PEG}]_{\mathrm{PEG}} - [\mathrm{PEG}]_{\mathrm{salt}})^2} \quad (6)$$

2.2. PEG + Na_2SO_4 + H_2O + IL quaternary systems

2.2.1. Chemicals

The ATPS studied in this work were established by using aqueous solutions of sodium sulfate anhydrous, Na_2SO_4 (100 % pure from Prolabo), PEG, from Sigma Aldrich, and several ILs of different chemical structures. The ILs studied were: 1-butyl-3-methylimidazolium thiocyanate, [C_4mim][SCN] (> 98 wt % pure); 1-butyl-3-methylimidazolium tosylate, [C_4mim][TOS] (98 wt % pure); 1-butyl-3-methylimidazolium dicyanamide, [C_4mim][$N(CN)_2$] (> 98 wt % pure); 1-butyl-3-methylpyridinium dicyanamide [C_4mpy][$N(CN)_2$] (> 98 wt % pure); 1-ethyl-3-methylimidazolium dicyanamide, [C_2mim][$N(CN)_2$] (98 wt % pure) and 1-butyl-3-methylimidazolium acetate, [C_4mim][CH_3CO_2] (98 wt % pure). All the ILs were supplied by Iolitec and their chemical structures are shown in Figure 7.

To reduce the water and volatile compounds content to negligible values, ILs individual samples were dried under constant agitation at vacuum and moderate temperature (≈ 323 K) for a minimum of 24 h. After this process, the purity of each IL was further checked by ^{1}H and ^{13}C NMR spectra and found to be in accordance with the purity given by the suppliers.

The water employed was double distilled, passed across a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

A saline buffered aqueous solution using the commercial Phosphate Buffer Saline tablets (PBS), from Sigma-Aldrich, was also prepared and used to prepare the phase diagrams and liquid-liquid extractive systems.

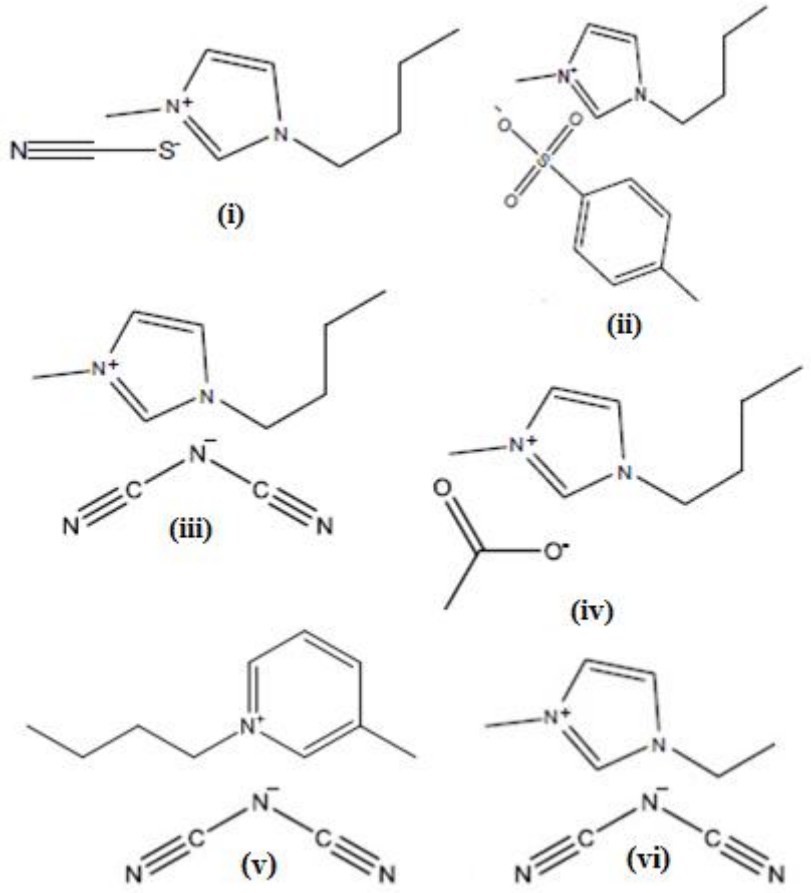

Figure 7 - Chemical structures of the studied ILs: (i) [C_4mim][SCN]; (ii) [C_4mim][TOS]; (iii) [C_4mim][$N(CN)_2$]; (iv) [C_4mim][CH_3CO_2]; (v) [C_4mpy][$N(CN)_2$]; (vi) [C_2mim][$N(CN)_2$].

2.2.2. Experimental procedure

2.2.2.1. Phase diagrams

The binodal curves of the quaternary phase diagrams were determined through the cloud point titration method at 298 K (± 1 K) and atmospheric pressure [6]. This procedure was initially validated with the phase diagram obtained for PEG 600 + Na_2SO_4 + [C_4mim]Cl + water quaternary system against literature data [12] (*cf.* Appendix A- Figure A 2). Aqueous solutions of Na_2SO_4 at 17 wt % and aqueous solutions of PEG 300 at 70 wt % were prepared and used for the phase diagrams determination. All the aqueous solutions used in the determination of the phase diagrams contained the same concentration of IL (5 and 10 wt %). Repetitive drop-wise addition of the aqueous IL-inorganic salt solution to

the IL-PEG solution was carried out until the detection of a cloudy and further biphasic solution, followed by the drop-wise addition of the IL-water mixture until the detection of a monophasic region. All this procedure was carried out under constant stirring. The quaternary systems compositions were determined by the weight quantification of all components added within an uncertainty of ± 10^{-4} g.

2.2.2.2. Determination of TLs

The tie-lines (TLs) were determined by a gravimetric method originally described by Merchuck et al. [64]. A mixture at the biphasic region was gravimetrically prepared with PEG + salt + water + IL, vigorously stirred, and allowed to reach the equilibrium by the separation of both phases for at least 12 h at 298 K (± 1 K). After the separation of the coexisting phases they were further weighted. Finally, each individual TL was determined by the application of the lever-arm rule to the relationship between the top phase weight and the overall system composition. The fitting of the experimental binodal curves, the determination of TLs and TLLs was determined according to Equations 1 to 6 described before.

2.3. Purification of IgY using ATPS

2.3.1. Chemicals

Fresh eggs were purchased in supermarkets of the Aveiro region. The aqueous solutions of egg yolk were prepared in saline buffered solutions using the commercial Phosphate Buffer Saline tablets (PBS) from Sigma-Aldrich to maintain the pH of the systems *circa* to 7.4. Sodium sulfate (Na_2SO_4), 100 % pure from Prolabo, PEGs of different molecular weight and several ILs were used for the formation of ATPS to be used as purification systems. The PEGs studied were: PEG 200, PEG 300, PEG 400, and PEG 600. All the polymers were acquired from Fluka with the exception of PEG 300 that was from Sigma-Aldrich. The ILs used were: 1-butyl-3-methylimidazolium thiocyanate, [C_4mim][SCN] (> 98 wt %); 1-butyl-3-methylimidazolium tosylate, [C_4mim][TOS] (98 wt

%);1-butyl-3-methylimidazolium acetate, [C_4mim][CH_3CO_2] (98 wt % pure); 1-butyl-3-methylimidazolium dicyanamide, [C_4mim][$N(CN)_2$] (> 98 wt % pure); 1-ethyl-3-methylimidazolium dicyanamide, [C_2mim][$N(CN)_2$] (98 wt % pure); trihexyltetradecylphosphonium dicyanamide, [THTDP][$N(CN)_2$] (95.7 wt % pure); 1-butyl-3-methylpyridinium dicyanamide, [C_4mpy][$N(CN)_2$] (> 98 wt % pure); 1-butyl-3-methylimidazolium tricyanomethane, [C_4mim][$C(CN)_3$] (98 wt % pure); 1-hexyl-3-methylimidazolium dicyanamide, [C_6mim][$N(CN)_2$] (99 wt % pure). The ILs molecular structures are shown in Figure 8. All the ILs were supplied by Iolitec with the exception of [THTDP][$N(CN)_2$] that was kindly supplied by Cytec Industries Inc. and [C_4mim][$C(CN)_3$] that was from Merck. To reduce the water and volatile compounds content to negligible values, the ILs were dried at vacuum and moderate temperature (≈ 350 K) for a minimum of 24 h under constant agitation. After this process, the purity of each IL was checked by ^{1}H and ^{13}C NMR spectra and found to be in accordance with the purity given by the suppliers.

The water employed was double distilled, passed through a reverse osmosis system and treated with a Milli-Q plus 185 water purification apparatus.

The material necessary to the SDS-PAGE analysis include: tris(hydroxymethyl)aminomethane, PA from Pronalab; sodium dodecyl sulfate, SDS (> 98.5 wt % pure), from Sigma-Aldrich; 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.

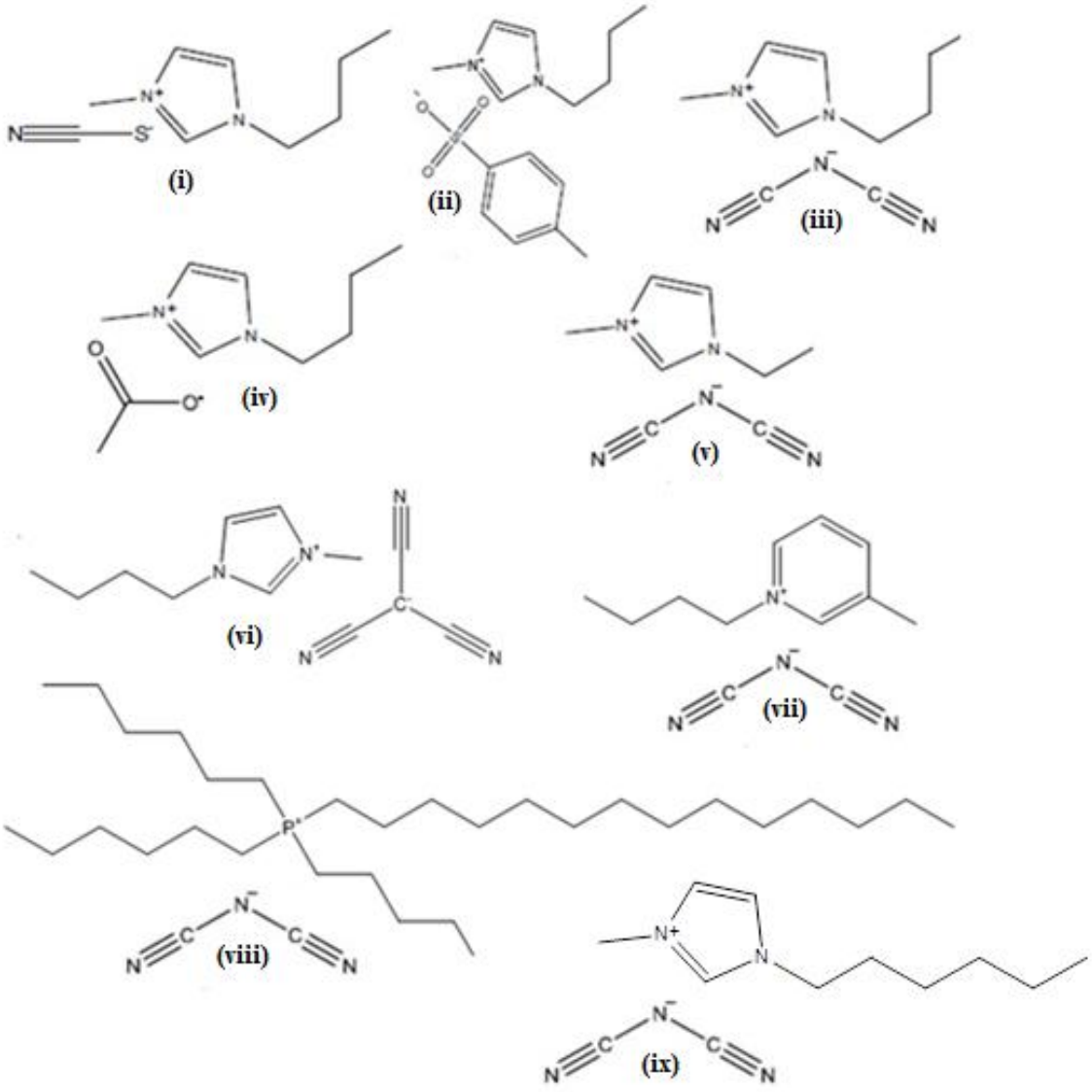

Figure 8 – Chemical structure of the ILs studied: (i) [C_4mim][SCN]; (ii) [C_4mim][TOS]; (iii) [C_4mim][$N(CN)_2$]; (iv) [C_4mim][CH_3CO_2]; (v) [C_2mim][$N(CN)_2$]; (vi) [C_4mim][$C(CN)_3$]; (vii) [C_4mpy][$N(CN)_2$]; (viii) [THTDP][$N(CN)_2$]; [C_6mim][$N(CN)_2$].

2.3.2. Experimental procedure

2.3.2.1. ATPS composed of PEG + Na_2SO_4

To obtain the initial aqueous solution of egg yolk from fresh eggs to be applied in the subsequent purification step the procedure described by Liu et al. was here used [31]. The water-phase was then recovered and used in the formation of each ATPS. The ternary

mixtures compositions were chosen based on the phase diagrams determined before for each PEG-Na_2SO_4 ATPS (without IL).

Each mixture was prepared gravimetrically within ± 10^{-4} g and vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments) and at 298 K, to achieve the complete partitioning of IgY and other contaminant proteins between the two phases.

In all the ternary mixtures evaluated, and at the compositions used, the PEG-rich aqueous phase is the top layer while the salt-rich aqueous phase corresponds to the bottom layer.

After the separation of the phases, using small ampoules designed for the purpose, the amount of total protein was quantified in each phase and the partition coefficients and the respective standard deviations were further determined. The total proteins content was quantified through UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 280 nm. A previous calibration curve was established using BSA (*cf.* Annex B - Figure B 1). The partition coefficients of the total protein, K_{Protein}, were determined according to Equation 7,

$$K_{\text{Protein}} = \frac{[\text{Protein}]_{\text{PEG}}}{[\text{Protein}]_{\text{salt}}} \qquad (7)$$

where $[\text{Protein}]_{\text{PEG}}$ and $[\text{Protein}]_{\text{salt}}$ are the concentration of total protein in the PEG and in the salt aqueous phases, respectively.

Possible interferences of Na_2SO_4 and PEGs with the analytical method were investigated and found to be not significant at the working conditions. As IgY is a protein and the aqueous solution of egg yolk is a complex matrix because it contains other proteins, it is not possible to calculate the specific partition coefficient of the IgY using of the UV-Vis data. It is possible thus, beforehand, to calculate the partition coefficient of the total proteins present in solution. To quantify the IgY and to determine the related parameters other quantification and identification techniques must be employed.

After quantification, the protein profile of the coexisting aqueous phases was investigated by SDS-PAGE.

2.3.2.1.1. pH measurement

The pH of both the PEG-rich and salt-rich aqueous phases were measured at 298 K (± 1 K) using a METTLER TOLEDO SevenMulti pH meter within an uncertainty of ± 0.02.

2.3.2.2. ATPS composed of PEG + Na_2SO_4 and PEG + Na_2SO_4 + IL

The buffered aqueous solution of egg yolk was prepared according the combined protocols described by Liu et al. and Pauly et al. [31, 65]. The ternary and quaternary mixtures compositions were chosen based on the phase diagrams determined before for each ATPS constituted by PEG-Na_2SO_4 and PEG-Na_2SO_4 with different ILs combinations. It should be remarked that the results obtained for the systems without IL are also presented in this section since the water soluble fraction obtained from egg yolk was obtained in a similar experimental step and are valuable for comparison purposes with the systems containing ILs as adjuvants.

Each mixture was vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments) and at 298 K, to achieve the complete partitioning of the proteins between the two phases. Again, the PEG-rich phase is the top layer while the salt-rich phase corresponds to the bottom layer.

After a careful separation the two phases, the protein content and respective partition coefficient (Equation 7) was determined by UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 280 nm. A previous calibration curve was established using of BSA (*cf.* Annex B - Figure B 1) as previously described. The protein profile of each aqueous phase was investigated by SDS-PAGE described below. Since the quaternary systems are also constituted by IL it the partitioning of the IL itself between the coexisting phases must be studied. The quantification of $[C_4mim][N(CN)_2]$ was carried out by UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 211 nm. Possible interferences of Na_2SO_4 and PEGs with the analytical method were investigated and found to be not significant at the dilutions performed for the quantification of the IL. The partition coefficient of IL, K_{IL}, was determined according to Equation 8,

$$K_{\mathrm{IL}} = \frac{[\mathrm{IL}]_{\mathrm{PEG}}}{[\mathrm{IL}]_{\mathrm{salt}}} \quad (8)$$

where $[\mathrm{IL}]_{\mathrm{PEG}}$ and $[\mathrm{IL}]_{\mathrm{salt}}$ are the concentration of IL in the PEG and in the salt aqueous phases, respectively.

2.3.2.3. ATPS composed of IL + Na_2SO_4

The preparation of the water-soluble fraction of egg yolk followed the same protocol described in section 2.3.2.2., and the final filtered aqueous solution was further employed in the composition of each ATPS.

The ternary mixtures compositions were chosen based on the phase diagram already available in literature [66].

Each mixture was vigorously stirred and left to equilibrate at the same conditions described before in section 2.3.2.2. In this type of systems, and at the compositions used, the top phase corresponds to the IL-rich aqueous phase whereas the salt-rich phase represents the bottom layer.

After a careful separation of the two phases, the protein profiles in each aqueous phase were investigated by SDS-PAGE.

2.3.2.4. Simulation of a countercurrent chromatography process

The preparation of the water-soluble fraction of egg yolk followed the same protocol described in section 2.3.2.2., and the final aqueous solution was further employed in the composition of each ATPS.

The mixture was vigorously stirred and left to equilibrate at the same conditions described before in section 2.3.2.2. After a careful separation of the two phases, the TL of this ATPS was determined according to the section 2.2.2.2. Then, the phases were carefully separated and the bottom phase was recovered and reused to form a new ATPS adding the amount of PEG, PBS and $[C_4mim][N(CN)_2]$ necessary to form a new biphasic

solution and with the same point of initial biphasic mixture. This procedure was repeated once again obtaining thus 3 cycles of partitioning.

The protein profiles of all ATPS-based samples were investigated by SDS-PAGE.

2.3.2.5. SDS-PAGE

The protein profile of the obtained aqueous rich phases was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All samples were diluted so that the amount of protein in each gel lane was about 0.5 µg. This amount was predicted taking into account the UV quantification of the total amount of proteins in each phase. Electrophoresis was run on polyacrylamide gels (stacking: 4 % and resolving: 20 %) with a running buffer consisting in 250 mM Tris HCl, 1.92 M glycine, 1 % SDS. The proteins were stained with the usual staining procedure (Coomassie Brilliant Blue G-250 0.1 % (w/v), methanol 50 % (v/v), acetic acid 7 % (v/v) and water 42.9 % (v/v)) in an orbital shaker at a moderate speed during 2-3 hours at room temperature. The gels were distained in a solution containing acetic acid 7 % (v/v), methanol 20 % (v/v) and water 73 % (v/v) in an orbital shaker at a moderate speed during 3-4 h at room temperature. SDS-PAGE Molecular Weight Standards, Marker molecular weight full-range (VWR), were used as protein standards. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis tool.

3. Results and discussion

3.1. PEG + Na_2SO_4 + H_2O ternary systems

Novel ternary phase diagrams were determined for several PEGs (PEG 200, 300, 400 and 600) + water + sodium sulfate, at 298 K and at atmospheric pressure. The respective ternary phase diagrams are illustrated in Figure 9. The experimental weight fraction data of each phase diagram are given at Appendix A (Table A 1 to Table A 2).

The system composed of PEG 600, sodium sulfate and water has already been reported in literature and the results obtained in this work are in close agreement with literature data [12] (*cf.* Appendix A- Figure A 1).

In the studied ATPS, the top phase corresponds to PEG-rich aqueous phase while the bottom phase is mainly composed of the Na_2SO_4 salt and water.

Figure 9 depicts the effect of the molecular weight of PEG in the formation of ATPS. The solubility curves are presented in molality units for a better understanding of the impact of the PEGs molecular weight on the phase diagrams behavior. In the presented phase diagrams the biphasic region is localized above the solubility curve. The larger this region the higher is the ability of PEG to undergo liquid-liquid demixing in the presence of Na_2SO_4 in aqueous media.

As expected, the influence of the length of the PEG chains on the phase diagram is notorious. For polymers of lower molecular weight the phase separation occurs at higher PEG and sodium sulfate concentrations. In general, the ability of PEG to form ATPS in the presence of the inorganic salt decreases in the following order: PEG 600 > PEG 400 > PEG 300 > PEG 200. Similar trends have been observed in other ATPS composed of polymer/salt or PEG/IL combinations [6, 67]. This behavior is a main consequence of the higher hydrophobicity displayed by PEGs of higher molecular weight, *i.e.*, they present a lower affinity for water, and are more easily salted-out by Na_2SO_4 [67].

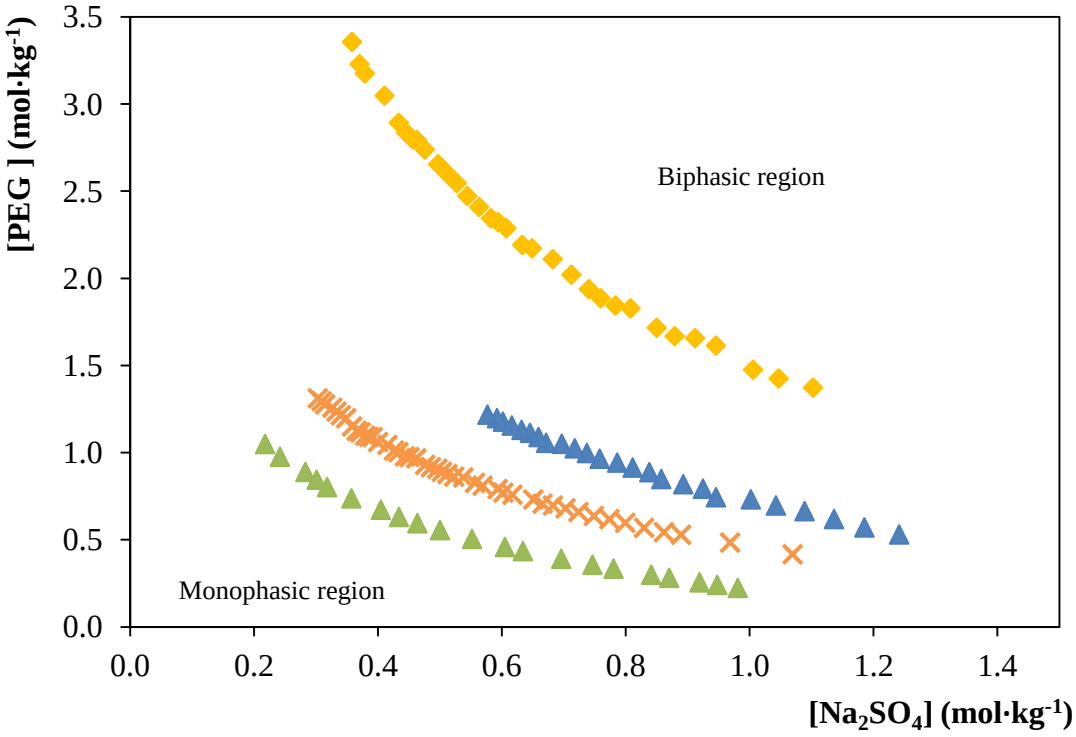

Figure 9 - Evaluation of the molecular weight of PEG in ternary phase diagrams composed of PEG + Na_2SO_4 + H_2O: PEG 600 (▲); PEG 400 (x), PEG 300 (▲); PEG 200 (◆).

For the studied systems, the experimental binodal data were further fitted by the empirical relationship described by Equation 1. The regression parameters were estimated by the least-squares regression method, and their values and the corresponding standard deviations (σ) are provided in Table 4. In general, good correlation coefficients were obtained for all systems, 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 TLs, along with their respective length (TLLs), are reported in Table 5. An example of the TLs obtained is depicted in Figure 10. In general, the TLs are closely parallel to each other.

Table 4 - Correlation parameters used to describe the experimental binodal data by Equation 1.

PEG	$A \pm \sigma$	$B \pm \sigma$	$10^5 (C \pm \sigma)$	R^2
PEG + Na_2SO_4 + water systems				
600	81.8 ± 0.6	-0.471 ± 0.004	20.0 ± 0.60	0.99964
400	85.6 ± 1.4	-0.449 ± 0.007	6.10 ± 0.77	0.99891
300	87.0 ± 1.2	-0.417 ± 0.006	6.12 ± 0.42	0.99916
200	91.5 ± 1.7	-0,376 ± 0,008	2.74 ± 0.57	0.99896

Table 5 - Experimental data of TLs and TLLs of PEG + Na_2SO_4 ATPS.

PEG	Weight fraction composition (wt %)						TLL
	$[PEG]_{PEG}$	$[salt]_{PEG}$	$[PEG]_M$	$[salt]_M$	$[PEG]_{salt}$	$[salt]_{salt}$	
600	40.36	2.23	23.87	10.09	1.62	20.72	42.93
400	40.06	2.86	30.05	9.96	1.31	30.35	47.51
	33.67	4.29	25.25	9.96	3.86	24.37	35.94
	36.87	3.51	26.99	10.02	3.07	25.81	40.49
300	32.59	5.44	19.27	13.94	6.13	22.33	31.87
	41.89	3.06	33.13	9.98	0.43	35.82	52.90
200	40.81	4.59	33.17	9.92	6.54	28.50	41.79

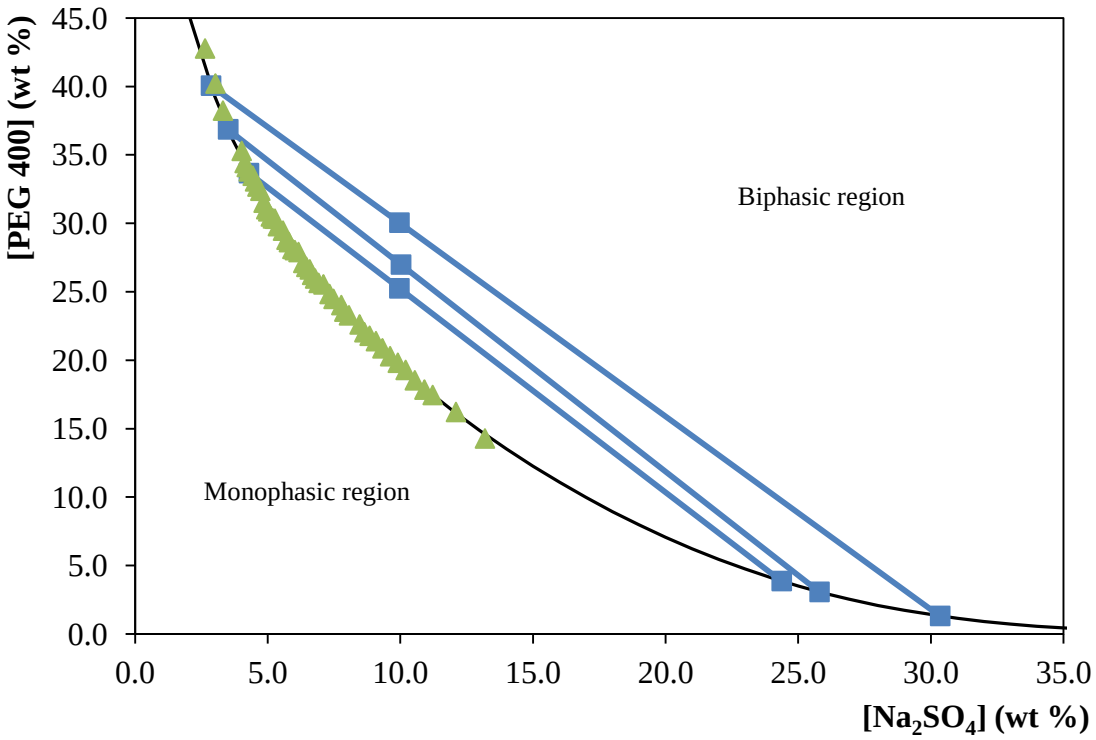

Figure 10 - Phase diagram for the ternary system composed of PEG 400 + Na_2SO_4 + H_2O: binodal curve data (▲); TL data (■); adjusted binodal data through Equation 1 (-).

3.2. PEG + Na_2SO_4 + H_2O + IL quaternary systems

Figure 11 and Figure 12 present the experimental phase diagrams at 298 K and atmospheric pressure for each PEG + Na_2SO_4 + H_2O + IL system. The experimental weight fraction data of each phase diagram are given at Appendix A (Table A 3 to Table A 5). It should be pointed out that the IL concentration was kept constant in all the phase diagrams determination. The binodal curves are reported in molality units for an enhanced understanding on the impact of distinct ILs in the formation of ATPS.

Figure 11 shows the influence of [C_4mim]-based ILs, and thus of the IL anion nature, on the phase diagrams behavior, where results for [C_4mim][TOS], [C_4mim][SCN], [C_4mim][CH_3CO_2] and [C_4mim][$N(CN)_2$] are depicted. In Figure 11 the phase diagram for the control system without IL is further represented for comparison purposes. In all the studied examples, the presence of IL facilitates the phase separation since all the biphasic regions are larger in the presence of IL when compared with the control system. The IL

anion ability to form ATPS is as follows: $[CH_3CO_2]^-$ < $[TOS]^-$ < $[N(CN)_2]^-$ ≈ $[SCN]^-$. There is a decrease on the ATPS formation ability with the increase in the IL anion affinity for water, following the trend of the anion hydrogen bond basicity [12]. Thus, the hydrophobicity of the IL anion or its ability to hydrogen-bond with water controls the ATPS formation [12].

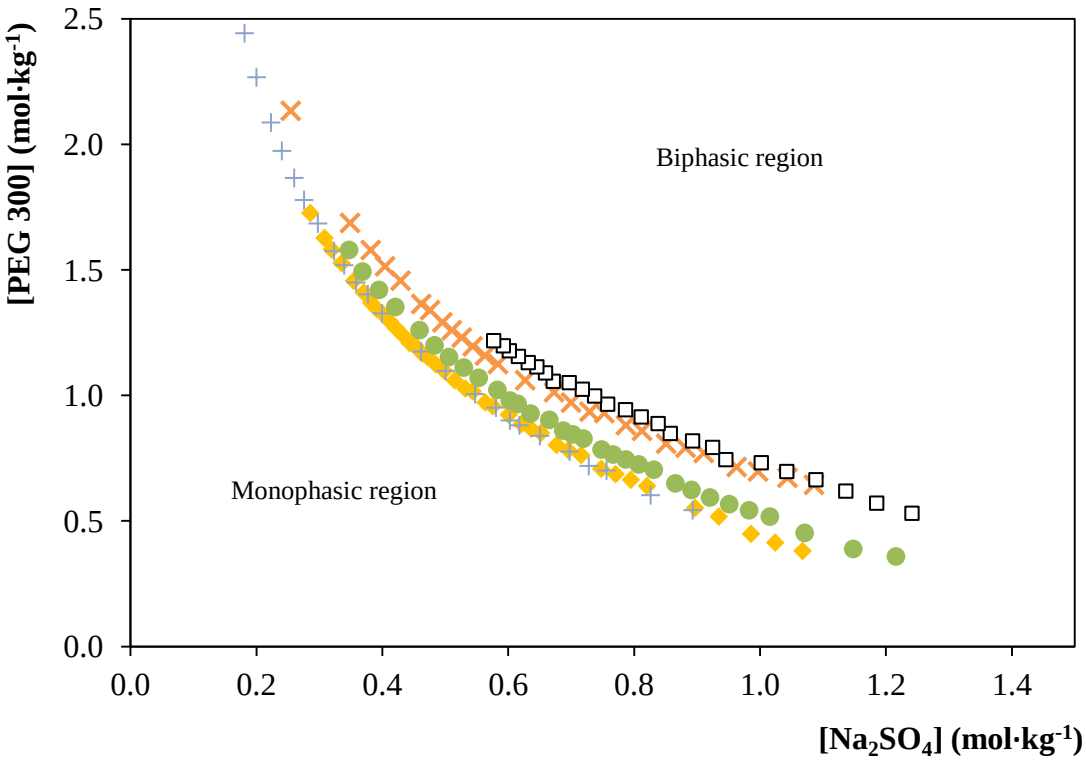

Figure 11 - Phase diagrams for the quaternary systems composed of PEG + Na_2SO_4 + H_2O + IL at 298 K: no IL (□); [C_4mim][CH_3CO_2] (×); [C_4mim][TOS] (●); [C_4mim][$N(CN)_2$] (◆); [C_4mim][SCN] (+).

In order to evaluate the effect of the IL concentration on the phase diagrams behavior, the binodal curves of the systems composed of PEG + Na_2SO_4 + H_2O + two concentrations of [C_4mim][$N(CN)_2$] were determined. Figure 12 shows the respective phase diagrams together with the control system without IL. In general, the higher the amount of IL the higher is the ability for two-phases formation. In summary, higher amounts of IL in the quaternary system leads to TLs or phases compositions with a higher water content which can be favorable for the extraction of proteins, and in this particular work, of antibodies.

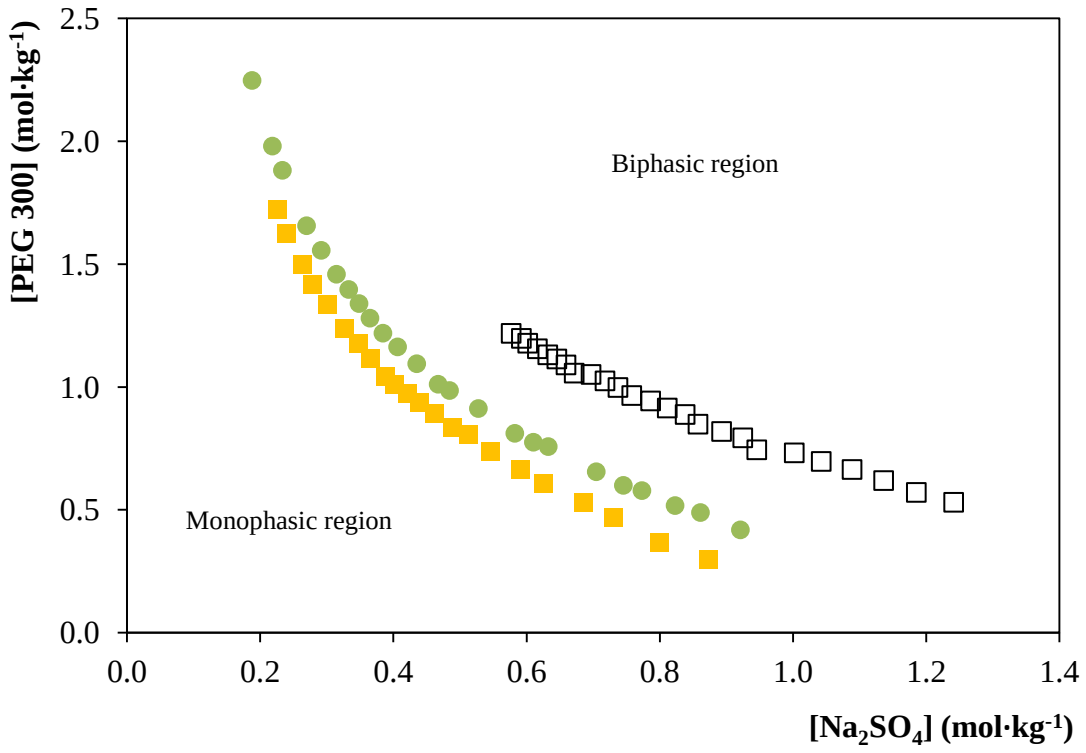

Figure 12 - Phase diagrams for the systems composed of PEG + Na_2SO_4 + H_2O + IL at 298 K: no IL (□); lower amount of [C_4mim][$N(CN)_2$] (●); higher amount of [C_4mim][$N(CN)_2$] (■).

Since a higher amount of IL improves the phase separation, the effect of the IL cation core and alkyl side chain length was further investigated fixing this amount of IL in the quaternary system. The respective phase diagrams are depicted in Figure 13 with results for [C_4mim][$N(CN)_2$], [C_2mim][$N(CN)_2$] and [C_4mpy][$N(CN)_2$]. The IL cation ability to form ATPS is as follows: $[C_2mim]^+ < [C_4mim]^+ \approx [C_4mpy]^+$. This trend reflects the capacity of the IL cation to be solvated by water (since the dicyanamide anion is the counterion common to all ILs), and which is regulated by steric and entropic contributions [68-70]. The trend obtained here is in agreement with the results previously reported for IL-based ATPS composed of different salts [69].

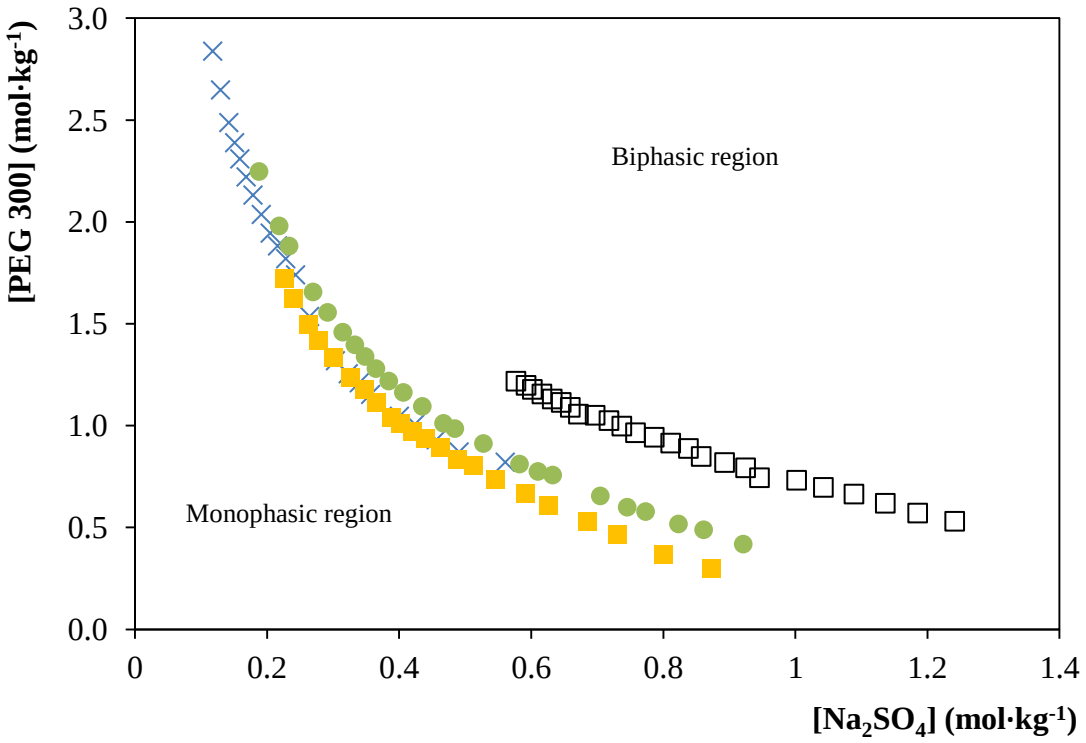

Figure 13 - Phase diagrams for the dicyanamide-based quaternary systems composed of PEG + Na_2SO_4 + H_2O + IL at 298 K: no IL (□); $[C_4mim][N(CN)_2]$ (■); $[C_2mim][N(CN)_2]$ (●); $[C_4mpy][N(CN)_2]$ (×).

For all the studied systems, the experimental binodal data were also fitted by the empirical relationship described by Equation 1. The regression parameters were estimated by the least-squares regression method, and their values and corresponding standard deviations (σ) are provided in Table 6. In general, highly satisfactory correlation coefficients were obtained for all systems.

The experimental TLs, along with their respective length (TLLs), are reported in Table 7. An example of the TLs obtained is depicted in Figure 14. As observed with the systems without IL, the TLs are closely parallel to each other.

Table 6 - Correlation parameters used to describe the experimental binodal data by Equation 1.

IL	$A \pm \sigma$	$B \pm \sigma$	$10^5 (C \pm \sigma)$	R^2
PEG + Na_2SO_4 + water + IL system				
$[C_4mim][TOS]$	80.4 ± 1.7	-0.420 ± 0.009	15.70 ± 0.66	0.99927
$[C_4mim][SCN]$	92.5 ± 1.0	-0.497 ± 0.006	14.66 ± 0.99	0.99966
$[C_4mim][CH_3CO_2]$	91.6 ± 1.4	-0.461 ± 0.008	2.09 ± 0.56	0.99946
$[C_4mim][N(CN)_2]$	81.8 ± 1.4	-0.442 ± 0.008	19.39 ± 0.80	0.99925
PEG + Na_2SO_4 + water + IL system				
$[C_4mim][N(CN)_2]$	93.3 ± 1.4	-0.5688 ± 0.014	35.65 ± 2.84	0.99864
$[C_2mim][N(CN)_2]$	100.1 ± 1.5	-0.569 ± 0.008	14.17 ± 1.22	0.99948
$[C_4mpy][N(CN)_2]$	102.1 ± 3.7	-0.615 ± 0.024	6.28 ± 11.19	0.99520

Table 7 - Experimental data of TLs and TLLs of PEG 300 + Na_2SO_4 + 5/10 wt % IL ATPS.

Weight fraction composition (wt %)							
IL	**$[PEG]_{PEG}$**	**$[salt]_{PEG}$**	**$[PEG]_M$**	**$[salt]_M$**	**$[PEG]_{salt}$**	**$[salt]_{salt}$**	**TLL**
$[C_4mim][TOS]$	34.16	4.06	16.94	15.46	0.53	26.31	40.32
$[C_4mim][SCN]$	39.05	2.98	30.01	9.991	0.02	33.23	49.38
	38.70	3.04	17.11	15.00	1.12	23.85	42.96
$[C_4mim][N(CN)_2]$	44.64	2.08	32.97	10.05	0.01	32.54	54.04
	43.23	2.30	30.94	10.19	0.01	30.02	51.31
	38.00	2.97	17.16	15.00	0.51	24.61	43.29
	39.86	2.62	10.00	20.15	0.31	25.84	45.87
IL	**$[PEG]_{PEG}$**	**$[salt]_{PEG}$**	**$[PEG]_M$**	**$[salt]_M$**	**$[PEG]_{salt}$**	**$[salt]_{salt}$**	**TLL**
$[C_4mim][N(CN)_2]$	29.98	3.84	19.78	10.12	0.11	22.23	35.08
	31.83	3.48	10.00	20.13	0.00	27.76	40.04
$[C_4mpy][N(CN)_2]$	35.23	2.99	24.99	9.99	1.44	26.11	40.95
$[C_2mim][N(CN)_2]$	38.00	2.88	34.95	5.04	0.11	29.66	46.39
	27.9	4.91	19.95	10.02	1.64	21.75	31.22

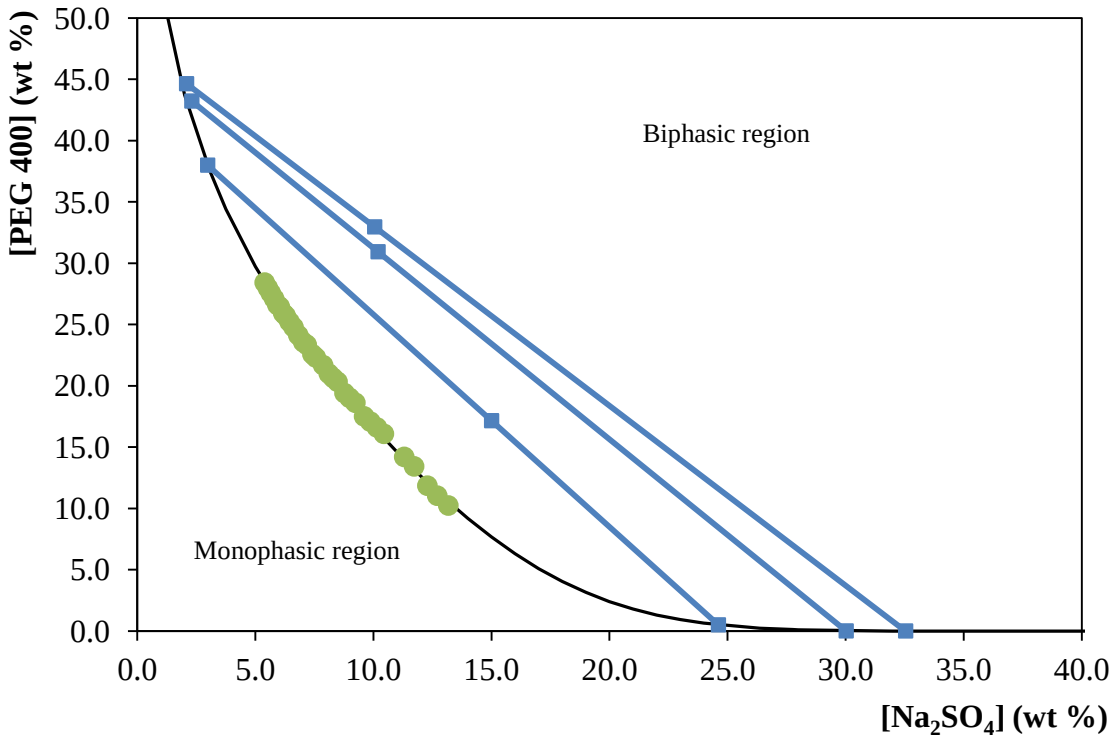

Figure 14 - Phase diagram for the quaternary system composed of PEG + Na_2SO_4 + H_2O + $[C_4mim][N(CN)_2]$: binodal curve data (•); TL data (■); adjusted binodal data through Equation 1 (-).

All the phase diagrams presented before were determined with aqueous solutions using ultra-pure water as the main solvent. However, and according the optimized protocol of purification of IgY described in section 2.3.2.2., the aqueous solution of egg yolk was initially prepared with PBS. Thus, novel ternary and quaternary phase diagrams were determined with aqueous solutions of PBS instead of pure water, at 298 K and at atmospheric pressure. These novel phase diagrams were determined to infer on the influence of PBS through the phase diagrams behavior. The experimental weight fraction data of each phase diagram are given at Appendix A (Table A 6 to Table A 7). The respective ternary phase diagrams are illustrated in Figures 15 and 16.

Figure 15 compare the PEG + Na_2SO_4 + H_2O ternary diagram with the PEG + Na_2SO_4 + water + PBS ternary diagram. As shown both phase diagrams are overlapping which means that the presence of the saline solution doesn't significantly influence the phase diagram and consequently the ATPS used for the IgY partitioning studies.

Figure 16 compares the PEG + Na_2SO_4 + H_2O + $[C_4mim][N(CN)_2]$ quaternary diagram with the PEG + Na_2SO_4 + water + PBS + $[C_4mim][N(CN)_2]$ diagram. As observed before, the presence of the "extra" saline solution doesn't influence the phase diagram behavior and consequently the phases compositions used in the partitioning investigations.

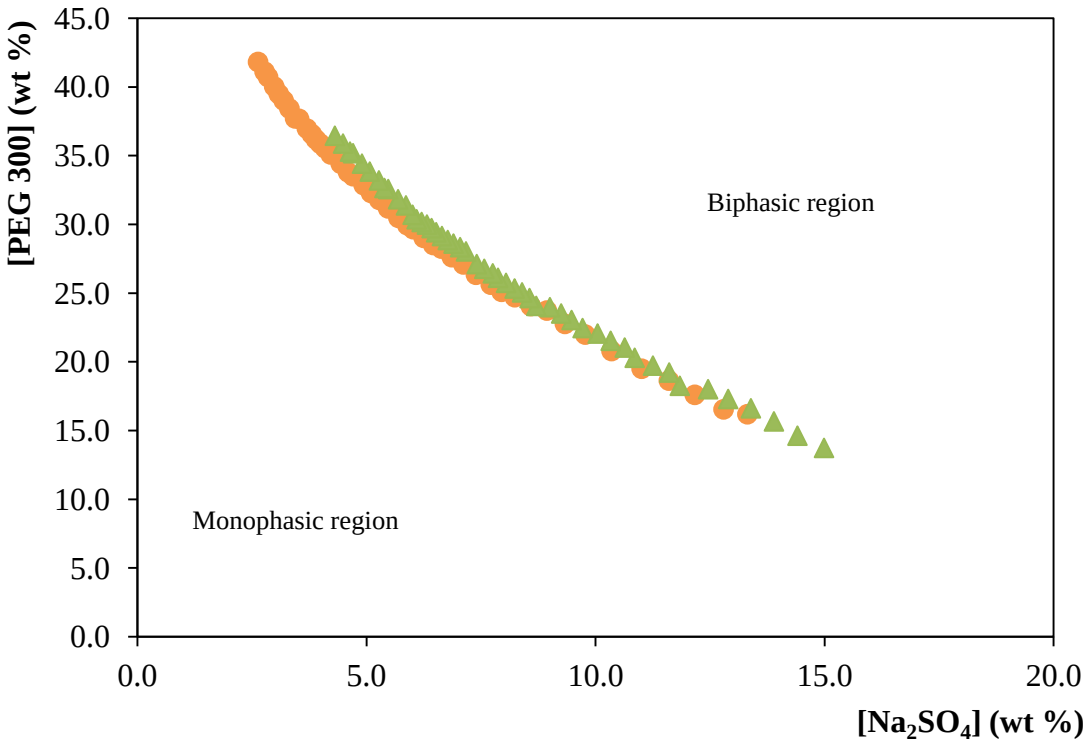

Figure 15 - Evaluation of the presence of PBS in phase diagrams for the ternary systems composed of PEG + Na_2SO_4 + H_2O (▲) or PBS (●).

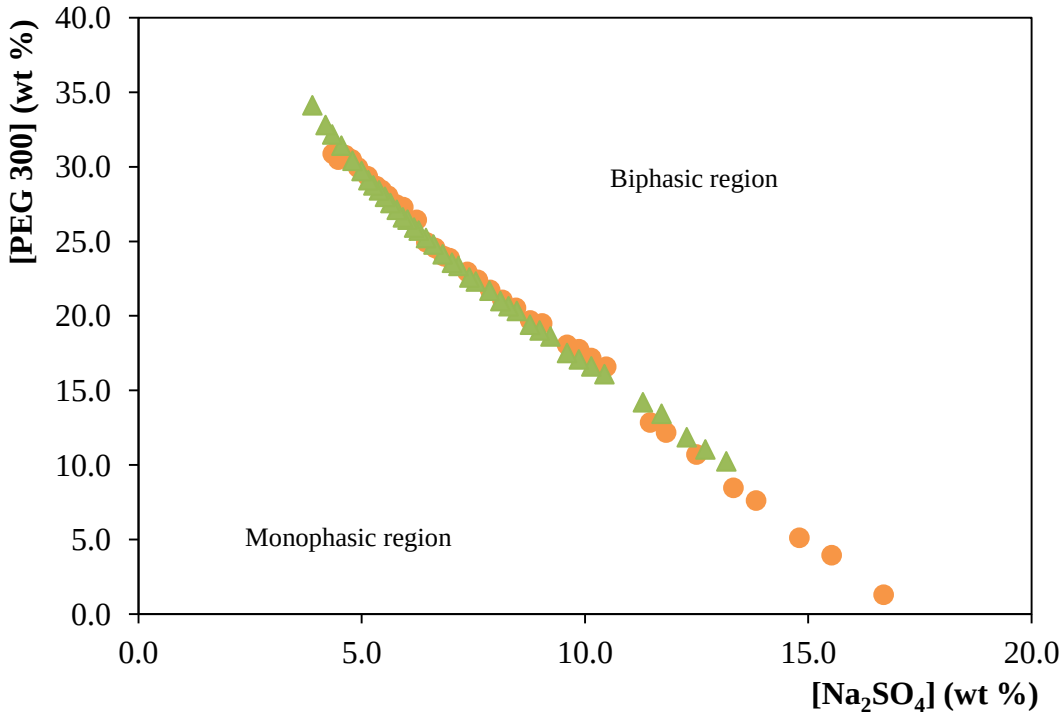

Figure 16 - Evaluation of the presence of PBS in phase diagrams for the quaternary systems composed of PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$ + H_2O (▲) or PBS (●).

3.3. Purification of IgY using ATPS

3.3.1. ATPS composed of PEG + Na_2SO_4

In Figure 17 it is presented the macroscopic appearance of the ternary mixtures with the compositions described in Table 8. It is clear that these mixtures and the preparation procedure are not appropriate for the purification of IgY since there is always an intermediate solid-like phase. The generation of this intermediate phase suggests that some proteins present in the aqueous solution of the water-soluble fraction of egg yolk suffer denaturation. In these preliminary studies the pH was slightly acidic (pH of the phases is *circa* 3.5 for ATPS with PEG 600, 4.0 for ATPS with PEG 400 and 6.0 for ATPS with PEG 300 according to pH measurements carried out in the separated phases) due to the absence of a buffered solution. All of these pH values are below the isoeletric point of some proteins in the medium (IgY, γ-livetin, has a pI between 5.7 and 7.6 and β-livetin has

a pI around 5) [25]. Moreover, it is notorious that PEGs of higher molecular weight lead to a larger amount of denaturated proteins. Besides the higher molecular weight of PEG that turns it into a more hydrophobic substance, this fact also seems to be related with the pH of each PEG solution. In other words, higher molecular weight PEGs result in aqueous solutions of lower pH values which lead to the proteins denaturation.

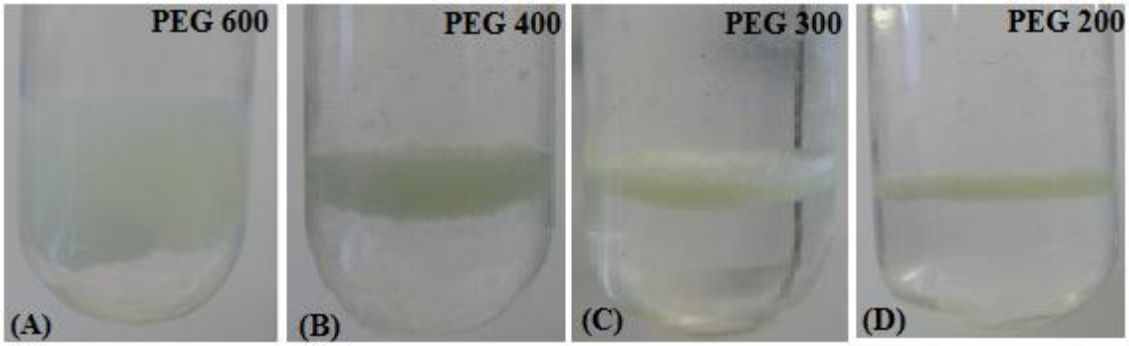

Figure 17 - ATPS formed by PEG + Na_2SO_4 + aqueous solution of egg yolk.

Table 8 - Mixture points of ATPS composed of PEGs of different molecular weight for the purification of IgY using the aqueous solution of egg yolk containing the water-soluble proteins.

ATPS Identification	***Mw* of PEG** **($g \cdot mol^{-1}$)**	**[PEG]** **(wt %)**	**[Na_2SO_4]** **(wt %)**	**[Aqueous Solution]** **(wt %)**
1	200	33	10	57
2	300	33	10	57
3	400	27	10	63
4	600	24	10	66

Despite the fact that a solid phase was always present in the investigated systems, the aqueous phases were separated to ascertain on the amount of total proteins present in each phase. The results obtained for the partition coefficients of the total proteins in the preliminary ATPS with an uncontrolled pH are shown in Figure 18. The partition coefficients of the systems formed by 33 wt % PEG 200 + 10 wt % Na_2SO_4 is not presented since when separating the coexisting phases (top and bottom) they immediately crystallized. The total amount of proteins in the systems composed of PEG 300, PEG 400 and PEG 600, is 0.013 g, 0.016 g and 0.002 g, respectively. Indeed, these values closely correlate with the macroscopic appearance of the ATPS shown in

Figure 17. The ATPS with the PEGs of higher molecular weight lead to a more intense precipitation of proteins, and thus, the total amount of proteins in solution is lower.

The partition coefficients of the total proteins range between 27.2 and 0.4 and are highly dependent on the molecular weight of the PEG used. In general, an increase in the molecular weight of PEG leads to a lower partitioning of the proteins for the polymer-rich phase. Water-soluble proteins prefer more hydrated phases, and thus, the increase of the molecular weight of PEG leads to more hydrophobic phases which are not "preferred" by the water-soluble proteins present in egg yolk [6]. PEGs of lower molecular weight favor the migration of proteins for the polymer-rich phase.

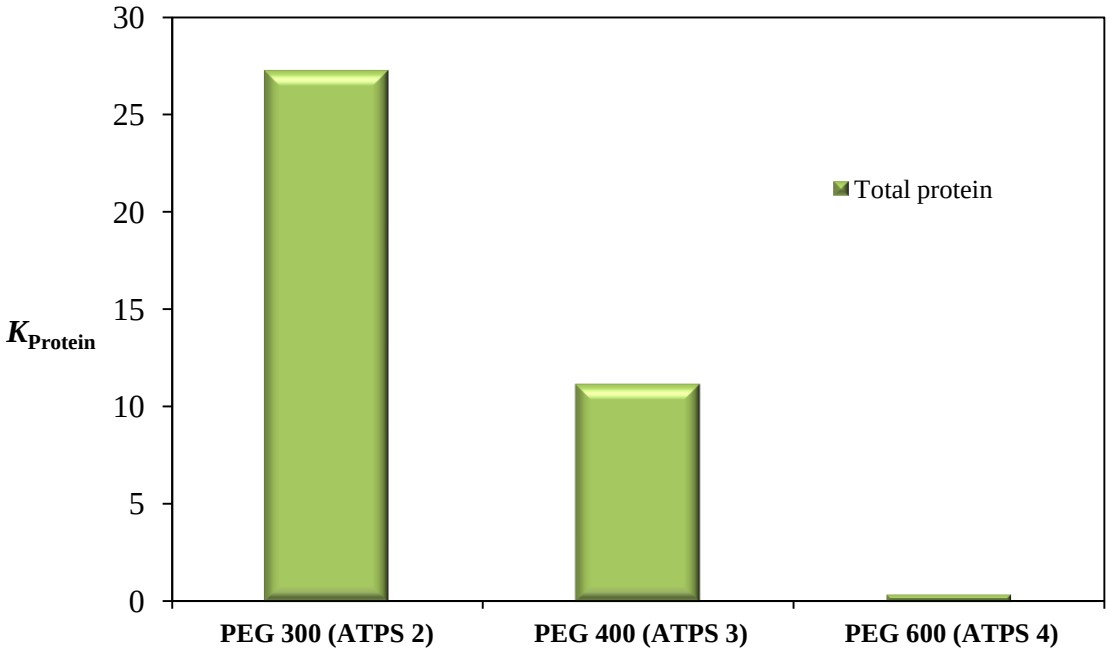

Figure 18 - Partition coefficients of total protein in ATPS formed by PEG + Na_2SO_4 + H_2O at 298 K.

The SDS-PAGE of ATPS 2, 3 and 4 was also carried out to identify the major proteins in solutions. However, the gel didn't show any bands. Therefore, it is impossible to present any protein profile for these mixtures. The main problem could be the low concentration of proteins in the samples prepared. It should be noted that in these mixtures it was macroscopically observed the denaturation of some proteins. Therefore, since the precipitation of proteins was observed in this type of mixtures, no additional tests were conducted and the procedure of using buffered solutions was further adapted.

3.3.2. ATPS composed of PEG + Na_2SO_4 and PEG + Na_2SO_4 + IL

The macroscopic appearance of the ATPS formed, now using the PBS buffer and the preliminary precipitation step, is shown in Figure 19. By adopting these procedures the denaturation of proteins was decreased or even completely avoided as the solid-like phase is now not visible.

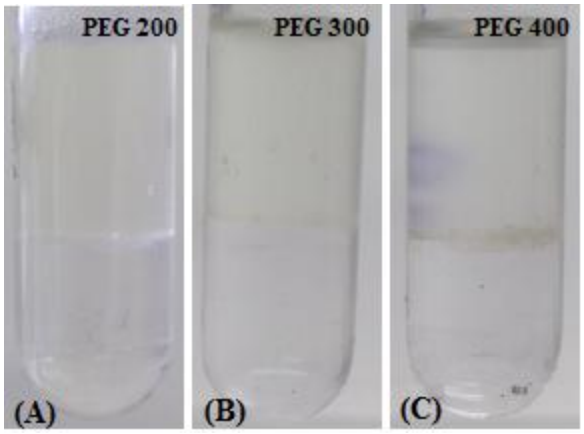

Figure 19 - ATPS formed by PEG + Na_2SO_4 + buffered aqueous solution of egg yolk.

Table 9 - Mixture points of PEG-based ATPS for the purification of IgY using the buffered aqueous solution of egg yolk containing the water-soluble proteins.

ATPS Identification	*Mw* of PEG ($g \cdot mol^{-1}$)	[PEG] (wt %)	[Na_2SO_4] (wt %)	[Aqueous Solution] (wt %)
5	200	22	14	64
6	300	19	14	67
7	300	17	15	68
8	300	10	20	70
9	400	16	14	70

The results obtained for the partition coefficients of total protein in the several ATPS with a controlled pH (~7) in the aqueous media are shown in Figure 20. The partition coefficients of total protein range between 3.2 and 18.5. As observed before with the ATPS where no control of the pH value was carried out, an increase in the molecular weight of PEG leads to lower partition coefficients. However, an exception is now observed with PEG 200 where the partition coefficient of total protein of ATPS 5 (22 wt % PEG 200 + 14

wt % Na_2SO_4 + 64 wt % buffered aqueous solution of egg yolk) is lower than in ATPS 6 (19 wt % PEG 300 + 14 wt % PEG 200 + 67 wt % buffered aqueous solution of egg yolk).

Comparing the results of the partition coefficients of total protein in ATPS using the buffered or non-buffered aqueous solutions of egg yolk it is visible that the buffered media lead to a decrease in the partition coefficients of ATPS composed of PEG 300 or PEG 400. In addition, the total amount of protein in each system is 0.080 g, 0.031 g and 0.047 g for the systems composed of PEG 200, PEG 300 and PEG 400, respectively. Therefore, these values confirm the precipitation of protein as observed before where no control of the medium pH was carried out. The high content of proteins is thus related with the absence of an intermediate phase with denaturated proteins and with the precipitation of the high molecular weight lipoproteins. The first reason allows the proteins to choose "better" the phase for which they have more affinity whereas the second reason decreases the total amount of contaminant proteins. Curiously, using the buffered medium it was not observed the crystallization of the coexisting phases of the system composed of PEG 200 as observed before.

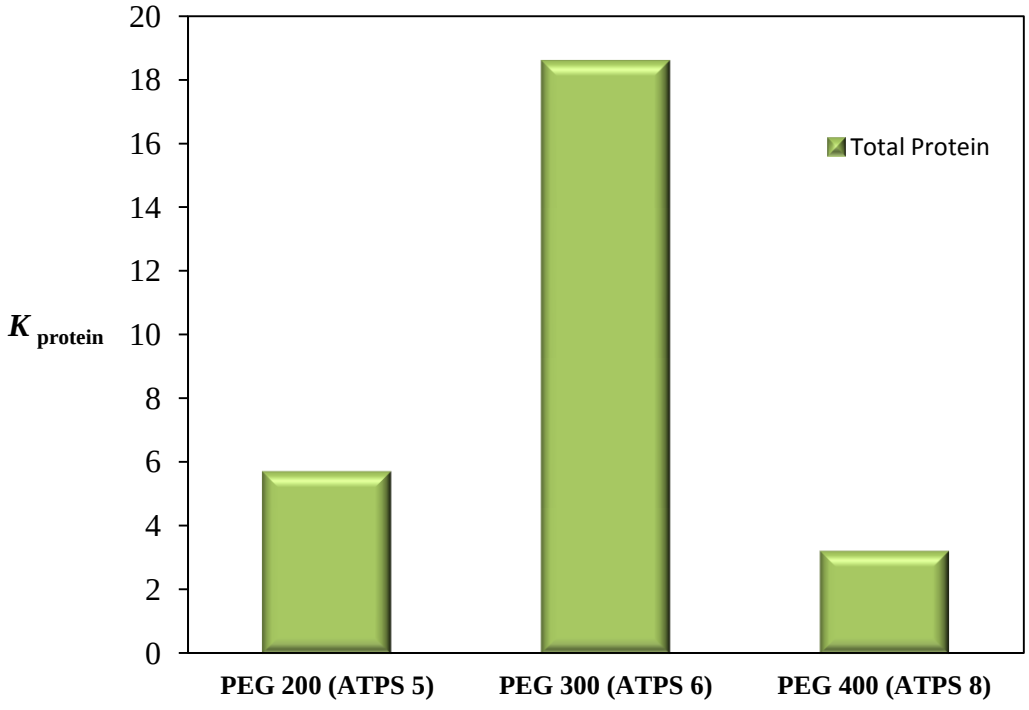

Figure 20 - Partition coefficients of total protein in ATPS formed by PEG + Na_2SO_4 + H_2O at 298 K**.**

The protein profiles obtained from SDS-PAGE of the egg yolk buffered aqueous solution, as well as of the coexisting phases of the ATPS composed of PEGs + Na_2SO_4 + buffered aqueous solution of egg yolk are shown in Figure 21. The commercial pure IgY, under reducing conditions, presents two major bands at 65 kDa and 25 kDa that correspond to two heavy chains and two light chains, respectively. In sample (A) these two bands are visible whereas in samples (B), (C), (D), (F), (G) and (H) only the heavy chain around 65 kDa is detectable. In all samples, with the exception of sample (E) where no proteins at all were detected possibly due to their low concentration, another significant band around 38 kDa, and that corresponds to β-livetin, a contaminant protein of the water-soluble proteins present in egg yolk, is always visible.

The absence of any band in sample (E) may be explained by a sample dilution higher than necessary or because all proteins in the aqueous solution are preferentially migrating to the top phase of the ATPS. This trend is plausible taking into account the high total protein partition coefficient values reported in Figure 20. Despite the bottom phase of the ATPS constituted by PEG 300, lane (E), seems to present no detectable amounts of proteins, this PEG is indeed the more efficient amongst the evaluated ones because in the top phase, lane (D), the band corresponding to β-livetin is not as evident as in the other top phases, lane (B) and lane (F), meaning that the main contaminant is being retained in the opposite phase.

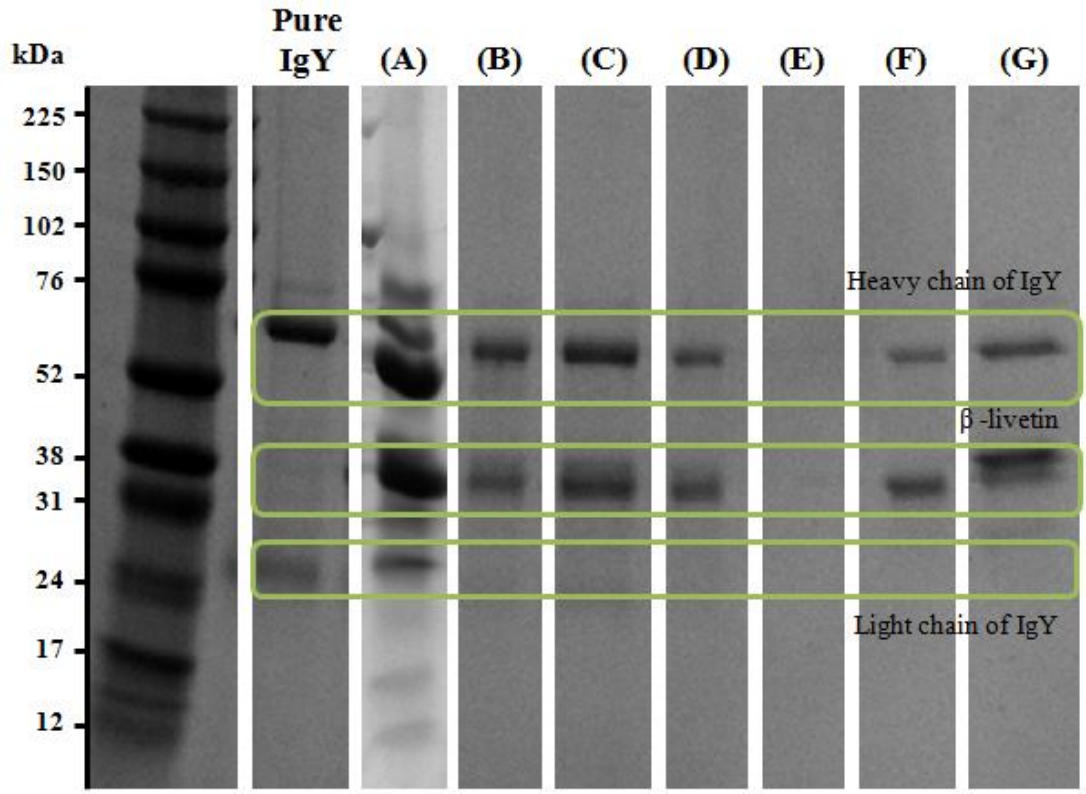

Figure 21 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A): Buffered egg yolk aqueous solution;

Lane 4 (B): and Lane 5 (C): Top and bottom phases, respectively, of the ATPS constituted by 22 wt % PEG 200 + 14 wt % Na_2SO_4;

Lane 6 (D) and Lane 7 (E): Top and bottom phases, respectively, of the ATPS constituted by 19 wt % PEG 300 + 14 wt % Na_2SO_4;

Lane 8 (F) and Lane 9 (G): Top and bottom phases, respectively, of the ATPS constituted by 16 wt % PEG 400 + 14 wt % Na_2SO_4.

As all the samples present contaminant proteins, from now on the partition coefficients of the total proteins will not be displayed because, at this stage, they do not provide any specific information regarding the selective migration of IgY.

Since the best results were obtained with the ATPS composed of PEG, PEG-based ATPS with ILs as adjuvants were further used to evaluate the tailoring ability of diverse ILs on the selective extraction of IgY. The IL was used at small concentrations. Table 10 presents the ATPS identification.

Table 10 – ATPS identification.

ATPS Identification	Polymer	IL Identification	[IL] (wt %)
10	PEG	$[C_4mim][TOS]$	5.0
11	PEG	$[C_4mim][SCN]$	5.0
12	PEG	$[C_4mim][CH_3CO_2]$	5.0
13	PEG	$[C_4mim][N(CN)_2]$	5.0
14	PEG	$[C_4mim][N(CN)_2]$	5.0
15	PEG	$[C_4mim][N(CN)_2]$	5.0
16	PEG	$[C_4mim][N(CN)_2]$	1.0
17	PEG	$[C_4mim][N(CN)_2]$	10.0
18	PEG	$[C_4mim][N(CN)_2]$	10.0
19	PEG	$[C_2mim][N(CN)_2]$	5.0
20	PEG	$[C_6mim][N(CN)_2]$	5.0
21	PEG	$[THTDP][N(CN)_2]$	5.0
22	PEG	$[C_4mpy][N(CN)_2]$	10.0
23	PEG	$[C_4mim][C(CN)_3]$	10.0
24	PEG	$[C_4mim][SCN]$	10.0

Figure 22 shows the macroscopic appearance of the ATPS containing the buffered egg yolk solution, PEG, Na_2SO_4 and each IL. For the ILs $[C_4mim][TOS]$ and $[C_4mim][N(CN)_2]$ it don't exist the formation of an intermediate phase as observed in the preliminary tests when using a non-buffered egg yolk aqueous solution. On the other hand, for the ILs $[C_4mim][SCN]$ and $[C_4mim][CH_3CO_2]$, there is the precipitation and/or denaturation of proteins. $[C_4mim][CH_3CO_2]$ is composed of a highly hydrophilic anion with a salting-out effect which could leads to the precipitation of proteins (according to the Hofmeister series) [71]. Therefore, the ATPS with $[C_4mim][CH_3CO_2]$ was immediately discarded and not used in the following experiments. Albeit some precipitation was also observed with the ATPS constituted by $[C_4mim][SCN]$, this was used in some following experiments to address on the effect through the IgY purification by the number of –CN groups at the anion.

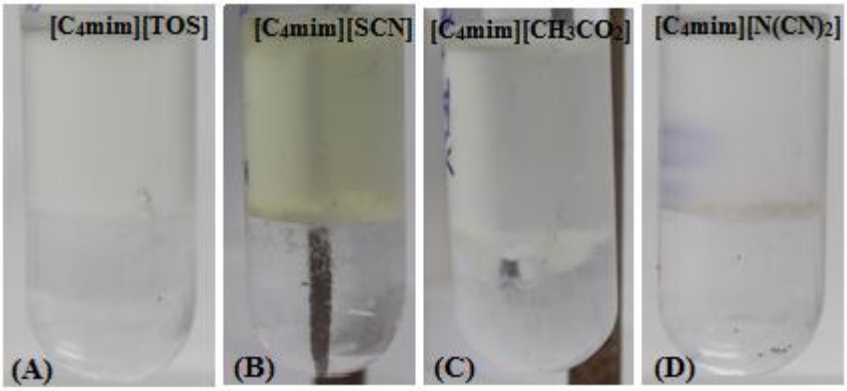

Figure 22 - ATPS formed by PEG + Na_2SO_4 + 5 wt % IL.

Figure 23 depicts the SDS-PAGE results or protein profile of samples from ATPS 10 and 14 formed by PEG + Na_2SO_4 + 5 wt % of $[C_4mim][TOS]$ or $[C_4mim][N(CN)_2]$, respectively.

In all ATPS-based samples the heavy chain of IgY is visible as well as the main impurity β-livetin. Particularly relevant, and seen in lanes (C) and (D) that belong to ATPS 13, that have in its composition $[C_4mim][N(CN)_2]$, is that IgY and β-livetin are displaying different affinities for the coexisting phases. The IgY has more affinity to the bottom phase (Na_2SO_4-rich aqueous phase) of the corresponding PEG-based ATPS and the β-livetin has enhanced affinity for the corresponding top phase (PEG-rich aqueous phase) as revealed by the intensity of the bands. This protein profile shows that the presence of $[C_4mim]N(CN)_2]$ leads to a preferential affinity of β-livetin to the top phase. As a result it seems that the IL is promoting the β-livetin migration for the top phase while leaving the γ-livetin (IgY) in the opposite phase.

Comparing the protein profile in the ATPS without IL (Figure 21) and with IL (Figure 23) it's clearly demonstrated that the presence of IL improves the selective separation of IgY and β-livetin for different phases.

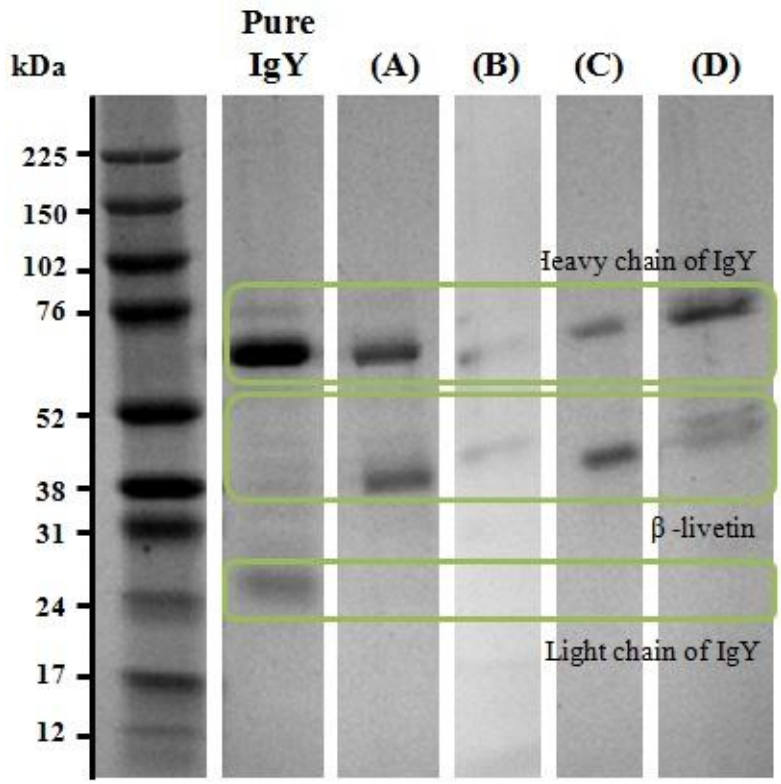

Figure 23 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A) and Lane 4 (B): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][TOS]$;

Lane 5 (C) and Lane 6 (D): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$.

Aiming at concentrating the major contaminant into the top phase, the amount of PEG was decreased while increasing the amount of Na_2SO_4, ATPS 14 and 15. The idea was to maintain the same phase compositions and to manipulate the concentration of β-livetin in the top phase. The last ATPS, with the higher concentration of the inorganic salt was impossible to create because the salt didn't solubilized completely and a solid phase was always present. Therefore, concentrations above 25 wt % were discarded and not used in the following experiments.

Figure 24 displays the protein profile in the ATPS 13 and ATPS 15 to compare the results obtained previously and the results increasing the amount of salt. In this figure it is

seen the prominent presence of β-livetin in band (C). These results are as expected and confirm the idea that β-livetin has more affinity to the top phase, the PEG-rich phase, and the amount of contaminant protein increased as revealed by the higher intensity of the band corresponding to β-livetin. The absence of any band in sample (B) may be explained by the increased amount of inorganic salt in the initial mixture that leads to a phase with more water. As desired and comparing the two protein profiles it can be seen that increasing the concentration of salt leads to the concentration of the major contaminant into the top phase.

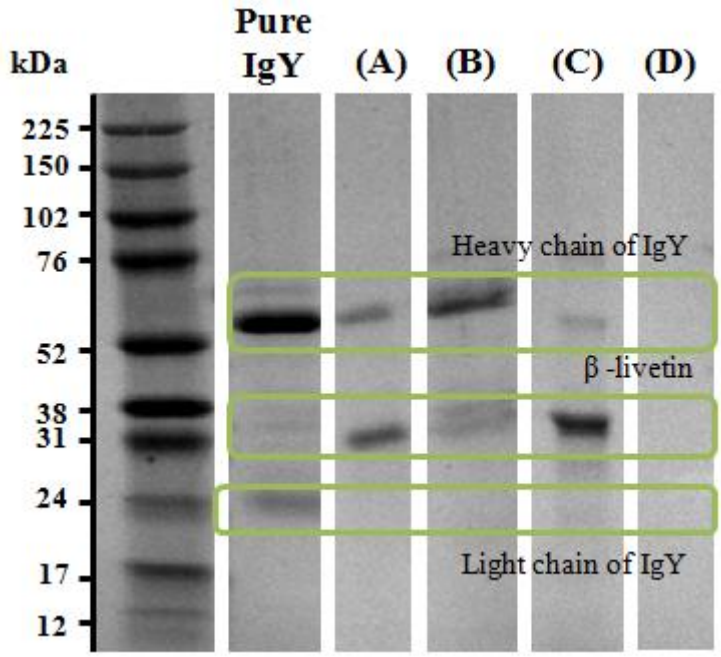

Figure 24 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A) and Lane 4 (B): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$;

Lane 5 (C) and Lane 6 (D): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$.

Because the presence of IL, and particularly of $[C_4mim][N(CN)_2]$, increases the affinity of β-livetin for the top phase, the weight percentage of IL added was studied with the ATPS 16 and 17. These ATPS have in their constitution fixed amounts of PEG and Na_2SO_4, because it was previously shown that with these values it was possible to properly

concentrate β-livetin, and different amount of ILs. Figure 25 shows the macroscopic appearance of the ATPS 15, 16 e 17 that contain different concentrations of $[C_4mim][N(CN)_2]$. For ATPS 15 and 16, there is no formation of an intermediate phase, while in ATPS 17 there is the precipitation and/or denaturation of a considerable amount of proteins. These results suggest that higher concentrations of IL favor the formation of an intermediate phase composed of denatured protein and are not favorable for the envisaged extraction and purification technique. Despite the significant amounts of proteins precipitated in ATPS 17, the two liquid phases were separated for all ATPS and examined by SDS-PAGE.

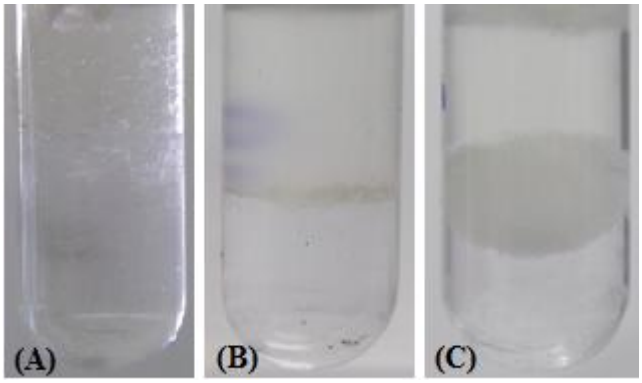

Figure 25 - ATPS formed by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$.

Figure 26 depicts the protein profile of the ATPS with variable concentrations of IL. In all the ATPS-based samples the impurity β-livetin remains visible whereas the heavy and light chains of IgY are only visible in some systems. IgY and β-livetin still have different affinities for the coexisting phases in the presence of different concentrations of $[C_4mim][N(CN)_2]$ as revealed by the intensity of the bands. In lane (A), that corresponds to the top phase with $[C_4mim][N(CN)_2]$, and as has been previosly shown, the band corresponding to β-livetin has higher intensity than the band corresponing to the heavy chain of IgY. In lane (C) that corresponds to the top phase of the ATPS with a lower amount of $[C_4mim][N(CN)_2]$, despite the existence of a large amount of β-livetin, the band of the heavy chain of IgY is still visible, meaning that the selective separation is not so effective at low concentrations of IL. In lane (E) that belongs to ATPS 17, only β-livetin is visible. The absence of any band of IgY in this sample means that increasing the concentration of IL favors the separation of the two proteins. It is thus clearly demonstrated

that an increased amount of IL favors the selective separation of IgY and β-livetin. However, it should be remarked that in the bottom phases of all ATPS (ATPS 14, 16 and 17) the band of IgY does not appear, unlike expected maybe due to the high concentration of Na_2SO_4 (20 wt %) used at this point that dilutes the respective phase.

Because the bottom phase might be very diluted, this phase was concentrated by decreasing the amount of Na_2SO_4 and increasing the content of PEG, yet maintaining the concentration of IL at 10 wt %). Although some precipitation of proteins was observed with 10 wt % of IL, the best results in terms of selective extraction were precisely obtained with this amount of IL, and thus, it was used in the following experiments.

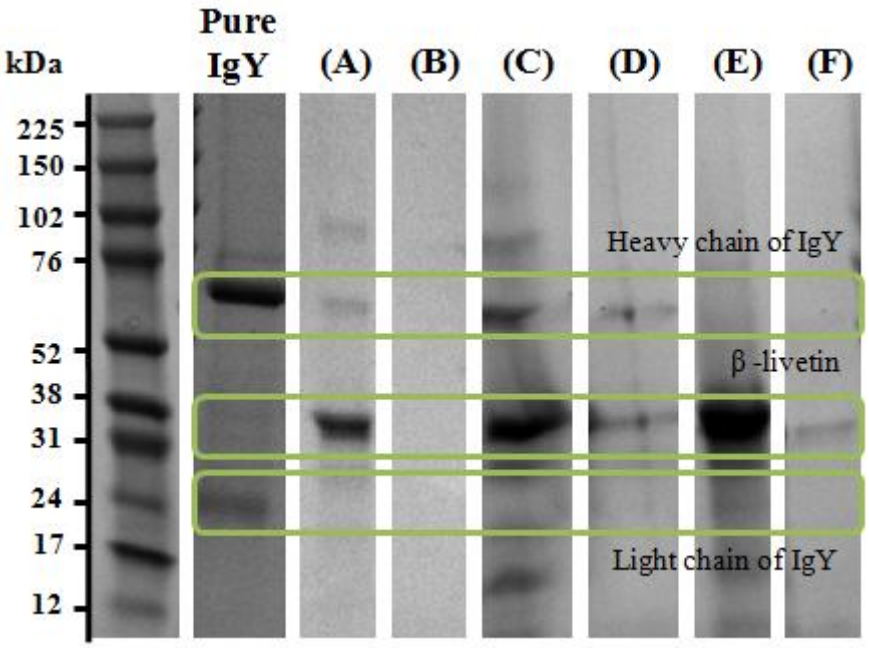

Figure 26 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A) and Lane 4 (B): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$;

Lane 5 (C) and Lane 6 (D): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$;

Lane 7 (E) and Lane 8 (F): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$.

Figure 27 exhibits the protein profile of ATPS 18. It can be seen that in lanes (A) and (B), top and bottom phases, IgY and β-livetin are present. Therefore, in this ATPS, the selective separation of proteins for each phase decreases compared with ATPS 17. As in this ATPS the concentration of salt was decreased aiming at concentrating the IgY in the bottom phase, but the concentration of IL was mantained, it means that the concentration of salt influences also the partition behavior of proteins. In lane (A), top phase, the concentration of both proteins are higher than in lane (B), bottom phase, as revealed by the intensity of the bands. This fact might happen because the IL is majorly concentrated in the PEG-rich phase (top phase) increasing therefore the affinity of both proteins for that layer since both proteins have similar characteristics (IgY is a livetin too).

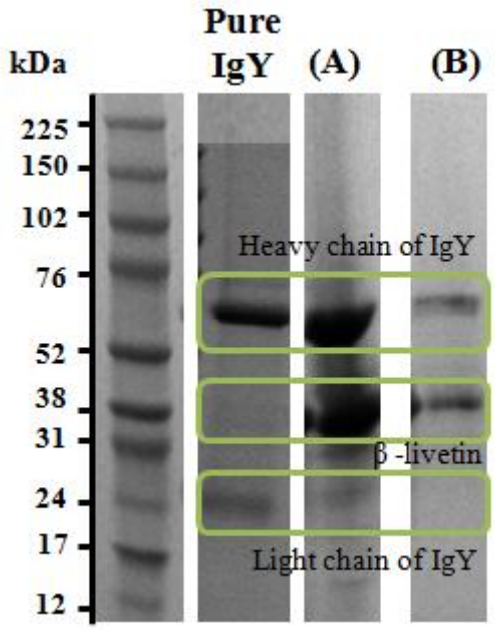

Figure 27 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A) and Lane 4 (B): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$.

Since decreasing the concentration of Na_2SO_4 didn't result in a better selective separation of IgY and of the major contaminant protein (β-livetin), and the best results so far were achieved with PEG, Na_2SO_4 and higher amounts of $[C_4mim][N(CN)_2]$, this point

of mixture was applied to form other ATPS, where the IL was changed to study the effects of the IL cation while maintaining the $[N(CN)_2]$ anion. For this purpose, four new ATPS were prepared with different ILs: $[C_2mim][N(CN)_2]$, $[C_6mim][N(CN)_2]$, $[THTDP][N(CN)_2]$ and $[C_4mpy][N(CN)_2]$ (ATPS 19, 20, 21 and 22). These ILs allow the evaluation of the IL cation core and alkyl side chain length influence on the selective separation of IgY from β-livetin. For these ATPS (ATPS 19, 20 and 21 which correspond to $[C_2mim][N(CN)_2]$, $[C_6mim][N(CN)_2]$ and $[THTDP][N(CN)_2]$, respectively), the concentration of IL was kept at a low concentration to avoid the precipitation and denaturation of proteins. For ATPS 22 that have in its composition $[C_4mpy][N(CN)_2]$ it was possible to maintain the IL at a higher concentration without observing proteins precipitation. Figure 28 exposes the macroscopical appearance of ATPS with $[C_2mim][N(CN)_2]$, $[C_6mim][N(CN)_2]$, $[THTDP][N(CN)_2]$ and $[C_4mpy][N(CN)_2]$. A significant amount of denatured proteins is present in the ATPS with $[THTDP][N(CN)_2]$ (Figure 28-(C)). Hence, this ATPS was discarded and not used in the following experiments. The main reason should be associated to the high hydrophobicity displayed by the quaternary phosphonium cation with relatively long aliphatic chains. The ATPS formed with $[C_2mim][N(CN)_2]$ and $[C_6mim][N(CN)_2]$ also show a thin intermediate phase of denatured protein and $[C_4mpy][N(CN)_2]$ didn't show any visible denaturated protein. Since $[THTDP][N(CN)_2]$ and $[C_6mim][N(CN)_2]$ are the most hydrophobic ILs investigated it seems that the best results are achieved with an IL with an intermediate hydrophobic character, such as $[C_2mim][N(CN)_2]$, $[C_4mim][N(CN)_2]$ or $[C_4mpy][N(CN)_2]$.

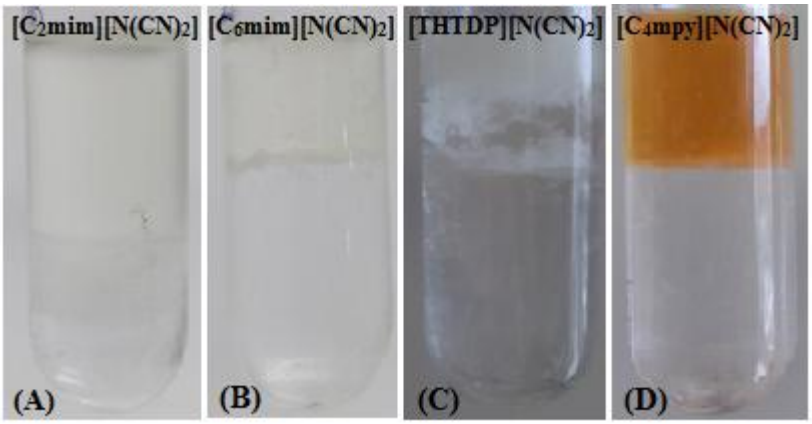

Figure 28 - ATPS formed by PEG + Na_2SO_4 + IL.

The protein profiles in the ATPS with $[C_2mim][N(CN)_2]$, $[C_6mim][N(CN)_2]$ and $[C_4mpy][N(CN)_2]$ are depicted in Figure 29. In general, in the three ATPS there is a similar protein profile. The β-livetin is in higher concentrations in the top phase, (lanes (A), (C) and (E)) as revealed by the intensity of the corresponding bands. However, no proteins were detected at the bottom phases because the samples were more diluted than necessary. However, major conclusions can be drawn without the need of repeating these partitioning experiments. Two differences can be seen in the protein profiles of lane (C) and lane (E). In lane (C), that correspond to the top phase of ATPS with $[C_2mim][N(CN)_2]$, there is a totally absence of IgY and, in lane (C), belonging to the ATPS with $[C_4mpy][N(CN)_2]$, it can be seen the band corresponding to IgY which means that this IL is not so effective in the selective separation of IgY from β-livetin. Thereby, $[C_2mim][N(CN)_2]$ was more efficient than $[C_4mpy][N(CN)_2]$ and $[C_4mim][N(CN)_2]$ (Figure 26) In summary, it is clear that ILs composed of the $[N(CN)_2]^-$ anion provide the best results combined with imidazolium-based cations afford the best separation performances.

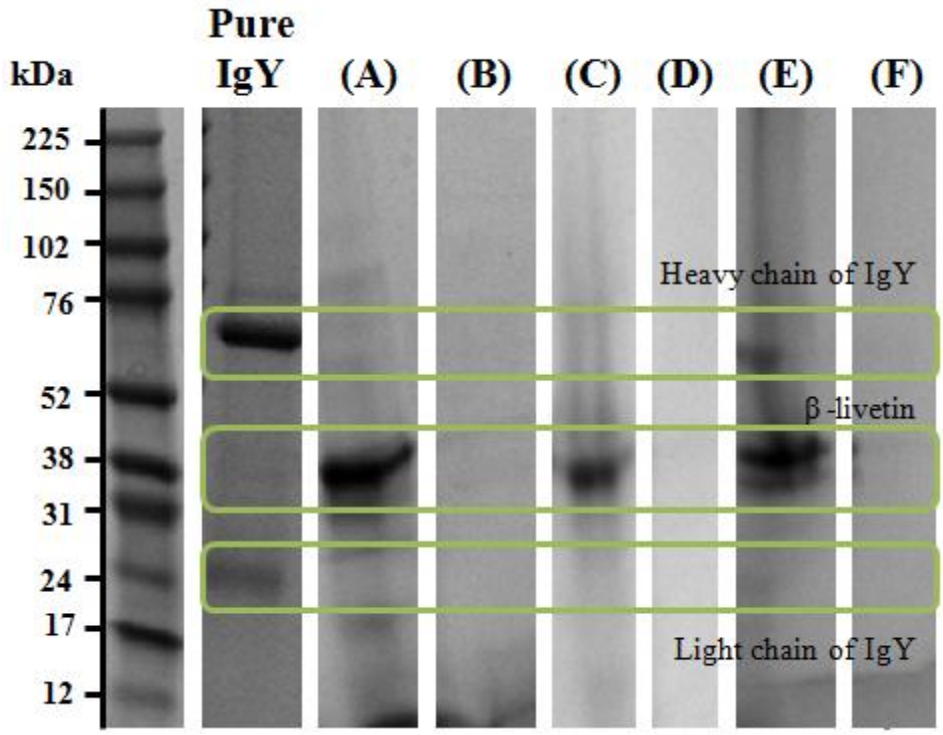

Figure 29 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A) and Lane 4 (B): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_2mim][N(CN)_2]$;

Lane 5 (C) and Lane 6 (D): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_6mim][N(CN)_2]$;

Lane 7 (E) and Lane 8 (F): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mpy][N(CN)_2]$.

According to the results shown in Figure 28 and Figure 29 the ATPS with an IL with an imidazolium cation and a -CN-based anion revealed the best results so far. Thus, novel ATPS composed of ILs with the common $[C_4mim]^+$ cation and several -CN-based ILs were prepared, namely ATPS 23 and 24. In ATPS 23 it was used a "new" IL, $[C_4mim][C(CN)_3]$, whereas in ATPS 24, it was used an IL applied before, $[C_4mim][SCN]$. Nevertheless, better results are now observed due to an adjustment of the mixture composition.

For a better comparison of all the ILs, the ATPS 17 with $[C_4mim][N(CN)_2]$ was prepared again and novel results were acquired to confirm the initial data.

The macroscopic appearances of the prepared ATPS are shown in Figure 30. As can be seen, increasing the number of -CN groups leads to a decreased formation of an intermediate phase (lower amounts of denaturated proteins). Although, based on Figure 31, these results didn't influence positively the separation of β-livetin and IgY. The lanes (A) and (B) corresponding to ATPS with [C_4mim][$C(CN)_3$] (IL with the higher number of -CN groups) don't show any band at all. This fact is indeed not expected because the mixture point is the same for all ATPS and the problem of dilution cannot be considered in this case. By now, the only explanation can be associated to the IL that does not allow, or interferes with the use of the SDS-PAGE. Comparing the two other ATPS, in Figure 31, the ATPS with [C_4mim][$N(CN)_2$], lanes (C) and (D) (top and bottom phases), leads to better results than the ATPS with [C_4mim][SCN], lanes (E) and (F) (top and bottom phase). The selective separation of IgY from its major contaminant is of better performance with [C_4mim][$N(CN)_2$] because, in the top phase, lane (A), there is a higher concentration of β-livetin, while, in the bottom phase, lane (B), there is a higher concentration of IgY. In the ATPS with [C_4mim][SCN] the selective separation is not so effective. In the top phase, lane (E), the band corresponding to β-livetin is intense, whereas, in the bottom phase, lane (F), the intensity of the band of IgY is equal or maybe lower than the band of β-livetin. Thus, despite the not very favorable macroscopic appearance of the ATPS shown in Figure 30, the ATPS with [C_4mim][$N(CN)_2$] as adjuvant is the most effective on the selective partition of IgY and the contaminant protein for opposite phases. However, and based on all ATPS studied throughout this work, [C_2mim][$N(CN)_2$] proved be the most effective IL on the partition of IgY and β-livetin.

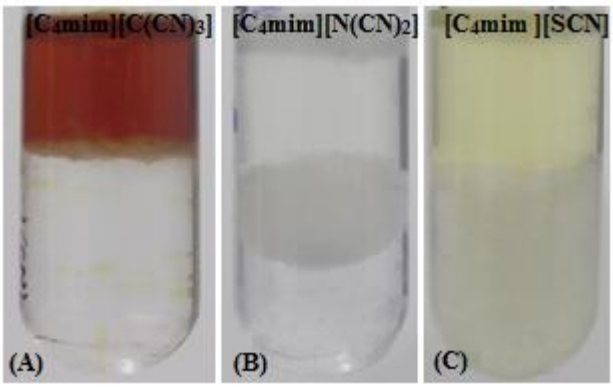

Figure 30 - ATPS formed by PEG + Na_2SO_4 + IL.

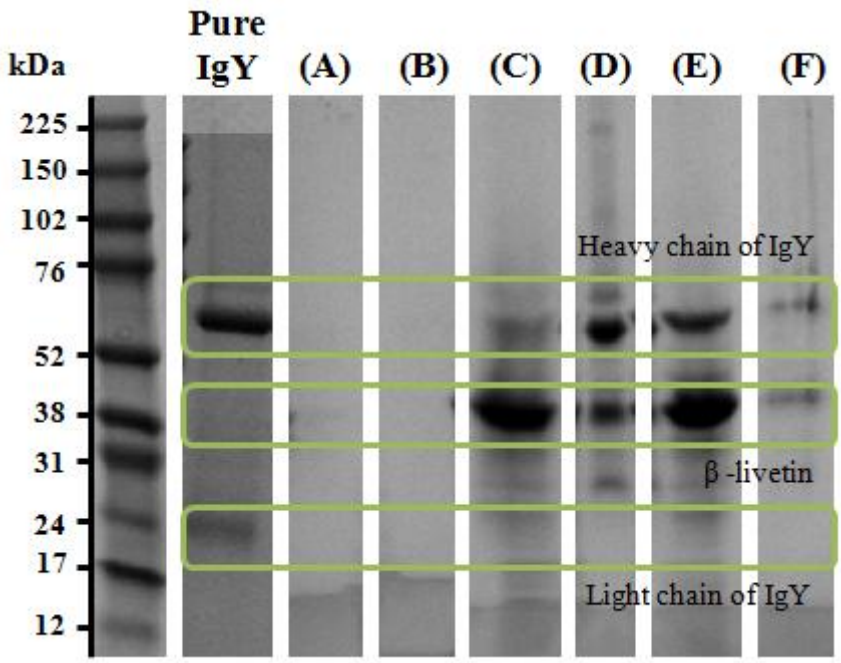

Figure 31 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A) and Lane 4 (B): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][C(CN)_3]$;

Lane 5 (C) and Lane 6 (D): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][N(CN)_2]$;

Lane 7 (E) and Lane 8 (F): Top and bottom phases, respectively, of the ATPS constituted by PEG + Na_2SO_4 + $[C_4mim][SCN]$.

3.3.3. ATPS composed of IL + Na_2SO_4

The results obtained so far with PEG-based ATPS suggest that the presence of some ILs as adjuvants increases the affinity of the major contaminant protein, β-livetin, for the top phase while retaining the IgY in the bottom phase. Since the best results were obtained with $[N(CN)_2]$-based ILs, an IL-based ATPS already reported in literature [66], without PEG and composed only of $[C_4mim][N(CN)_2]$ and Na_2SO_4 was tested. The phase diagrams for this type of IL-based ATPS are already published in literature and the biphasic regions were chosen according to these published data [66]. Three mixture compositions were

prepared, namely ATPS 25, 26 and 27 according to data previously published [66] – and increasing the amount of IL while decreasing the amount of salt.

The macroscopic appearance of three prepared ATPS is shown in Figure 32. In general, an increase in the IL content leads to a higher amount of denatured proteins.

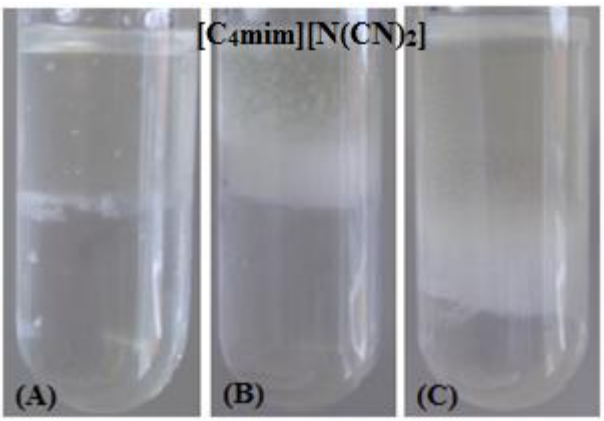

Figure 32 - ATPS formed by IL + Na_2SO_4 + aqueous solution of egg yolk buffered with progressively higher amounts of IL and lower amounts of salt.

Figure 33 exhibits the protein profile observed in each phase of the IL-based ATPS. Lanes (A) and (B) belong to ATPS 26. In these lanes it can be seen that β-livetin has more affinity to the top phase, lane (A), like previously observed in PEG-based ATPS where the same IL was used as adjuvant. On the other hand, the bottom phase must be too much diluted and no proteins are detected at all.

For ATPS 26, IgY also migrates to the top phase together with most part of β-livetin (lane C). Moreover, some β-livetin also starts to migrate to the bottom phase, lane (D), and meaning that, in general, the selective separation is becoming worst. With the subsequent increasing of IL, ATPS 27, the IgY still continues to show preferential affinity to top phase while half of the amount of β-livetin migrates to the top phase and the other half migrates to the bottom phase.

By the results depicted in Figure 33 it is clear that the affinity of both proteins changed with different concentrations of IL in the ATPS. With the increase of the IL content there is a reduction on the selective partitioning of IgY and β-livetin for opposite phases.

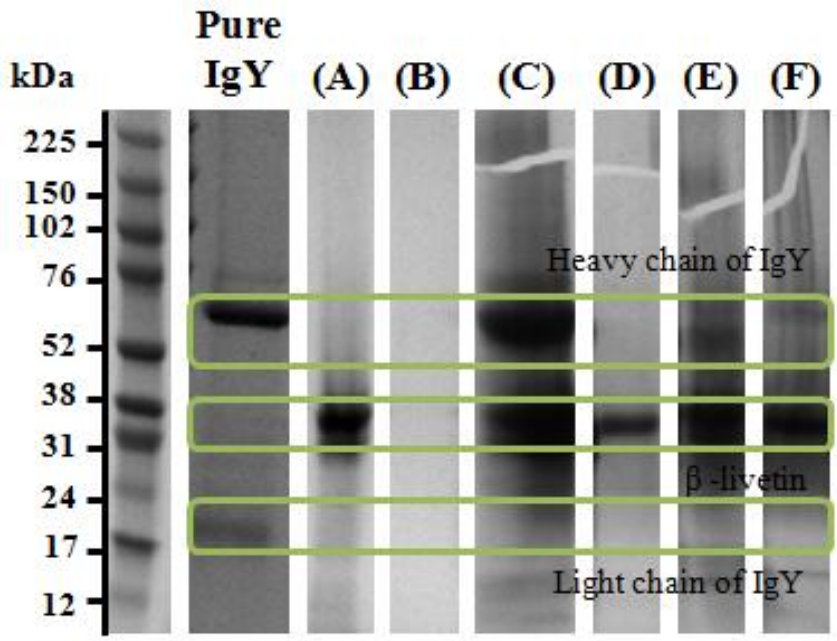

Figure 33 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A) and Lane 4 (B): Top and bottom phases, respectively, of the ATPS constituted by $[C_4mim][N(CN)_2]$ + Na_2SO_4;

Lane 5 (C) and Lane 6 (D): Top and bottom phases, respectively, of the ATPS constituted by $[C_4mim][N(CN)_2]$ + Na_2SO_4;

Lane 7 (E) and Lane 8 (E): Top and bottom phases, respectively, of the ATPS constituted by $[C_4mim][N(CN)_2]$ + Na_2SO_4.

3.3.4. Simulation of a countercurrent chromatography process

The results obtained so far suggest that β-livetin can be partially and selectively removed from the phase containing IgY. Therefore, and trying to simulate a "countercurrent chromatography" approach three successive extractions wherein the bottom phase, the salt-rich phase, was "recycled" and reused were carried out.

In the three cycles and after a proper separation of the phases it was investigated the protein profile in each ATPS. The results obtained are shown in Figure 35. In the first ATPS almost all β-livetin was removed because only in the top phase of the first ATPS, lane (A), is evident the band corresponding to β-livetin. In the second and third ATPS, β-

livetin was completely removed because in the respective top phases, lanes (B) and (C), no longer appears the band corresponding to β-livetin.

The absence of any band in sample (D), that corresponds to the bottom phase of the third ATPS, and where it was expected the single band of IgY may be explained again by a higher sample dilution than really necessary due to the point mixture chosen. However, the presence of IgY in this phase is assured in the top phase, lane (C), because although the presence of this protein is not detected it was not visually observed any denaturation behavior between the three cycles.

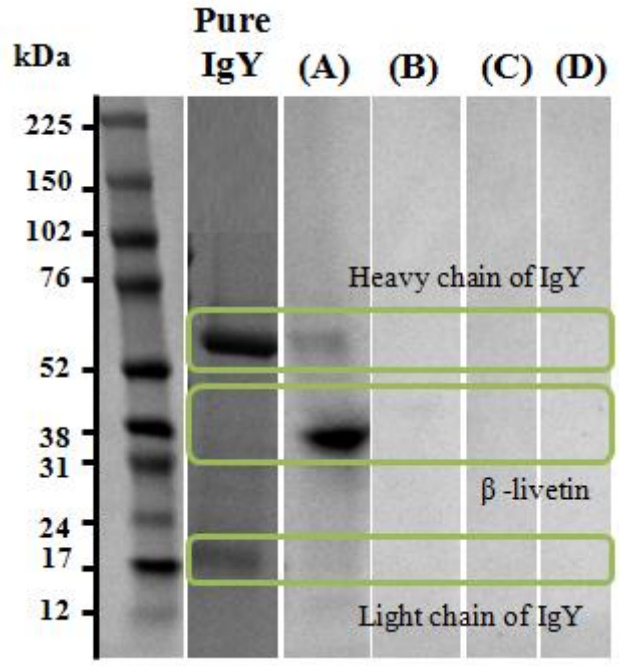

Figure 34 - SDS-PAGE of a gel loaded with 0.5 µg of protein/well, stained with Coomassie blue.

Lane 1 (Std): Standard molecular weights;

Lane 2 (Pure IgY): Commercial and pure IgY from chicken egg yolk from by Sigma-Aldrich®;

Lane 3 (A): Top phase of the 1st ATPS.

Lane 4 (B): Top phase of the 2nd ATPS.

Lane 5 (C): Top phase of the 3rd ATPS.

Lane 6 (D): Bottom phase of the 3rd ATPS.

3.4. Conclusions

In this work novel phase diagrams for ATPS composed of PEG 200, PEG 300, PEG 400 and PEG 600, sodium sulfate and water, as well as constituted by PEG , sodium sulfate, water and different ILs were determined. Moreover, the corresponding TLs and TLLs were also ascertained. The diversity of PEGs and ILs investigated leads to different abilities in the liquid-liquid demixing behavior. In general, PEGs of higher hydrophobicity or higher molecular weight lead to an improved ability for phase separation. On the other hand, with a common PEG and with different ILs at two concentrations, the phase separation is favored by ILs of higher hydrophobicity, or less affinity to hydrogen-bond with water, or with the increased amount of IL in the medium. After the proper characterization of these ATPS they were further used in the purification of IgY from egg yolk.

For the first time, it was studied the use of ATPS to purify IgY from aqueous solutions of raw egg yolk. Amongst the several systems, the most promising results were obtained with ILs constituted by the anion $[N(CN)_2]^-$. In the ATPS formed by IL + Na_2SO_4 it was verified that lower concentrations of IL provide enhanced selective separations.

Huge advances were obtained in this work allowing to conclude that the use of ILs as adjuvants in conventional PEG-salt systems provide an improvement for the purification of IgY from egg yolk in what concerns the development of a more benign and cost-effective process. Although the complete purification was not achieved in a single-step, it was observed a preferential partitioning of β-livetin for the top phase whereas the IgY was mainly retained in the bottom phase. In this context, the complete purification of the Ig seems plausible by applying consecutive separation steps to completely remove β-livetin from the phase containing IgY. To this end, it was used a three-cycle process to simulate a "countercurrent chromatography" approach reveling that the major contaminant was completely removed from the phase containing the immunoglobulin. The use of countercurrent chromatography is thus a viable and effective option that should be explored as a future strategy.

4. Final remarks

4.1. General conclusions and future work

In this work, it was studied a new method to purify IgY from raw egg yolk. Several ATPS were studied, either composed of polymers and salts, polymers, salts and ILs as adjuvants or of ILs and salts. Since the purification of IgY was not achieved in a single-step procedure further investigations are still required.

Based on the results gathered in this work the next step should involve the application of the improved system in countercurrent chromatography to completely purify IgY. Moreover, and after the complete purification of IgY, the recuperation step from the salt-rich phase should be further addressed, as well as the recycling nature of the ATPS used. Additional investigations on the protein stability and activity should also be conducted to ascertain on the effects of the phase-forming components through IgY. Given the remarkable results obtained, the use of the investigated ATPS is also highly recommended in the purification of other biopharmaceuticals, such as antibodies from mammal sources, nucleic acids and recombinant proteins.

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Appendix B: *calibration curves*

B. 1. Calibration curve for the quantification of the total amount of proteins

Figure B 1 depicts the calibration curve (absorbance *vs.* concentration of total amount of protein). The calibration curve was made with BSA.

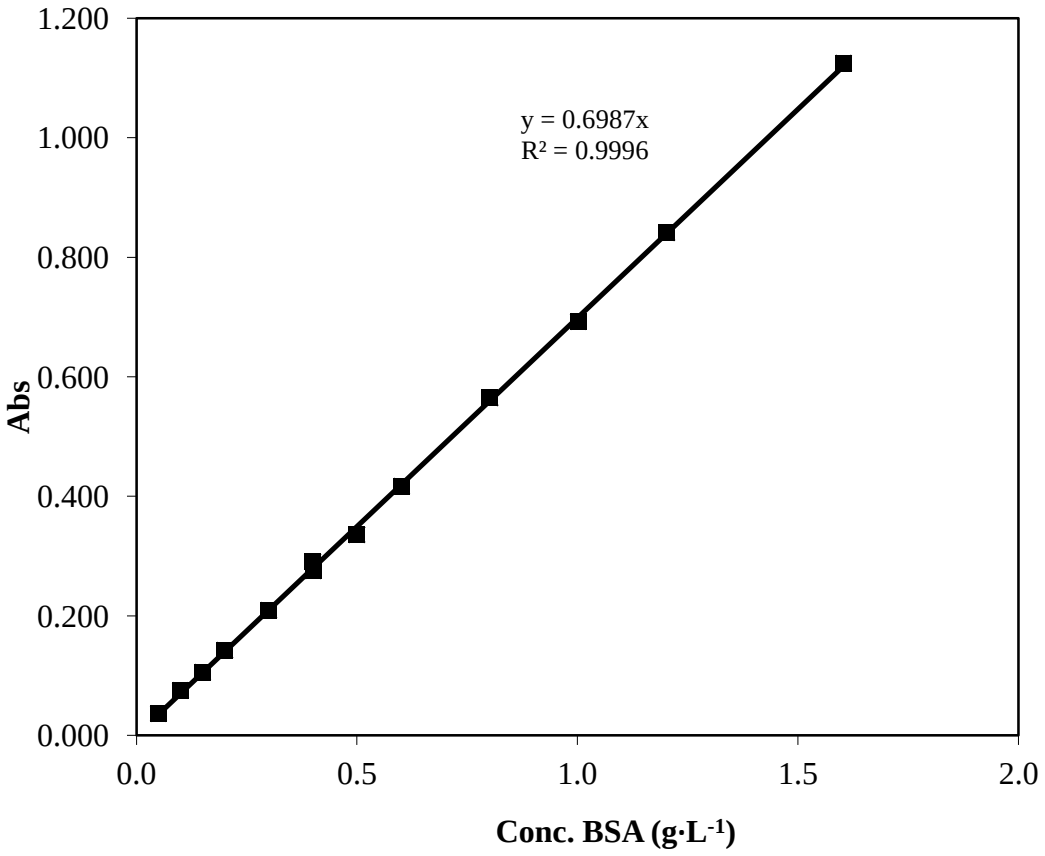

Figure B 1 - Calibration curve for total amount of protein at λ = 280 nm.

Appendix A: *experimental binodal curves*

A.1. Experimental binodal data for systems composed of PEG 600 + Na_2SO_4 + H_2O and PEG 600 + Na_2SO_4 + $[C_4mim]Cl$ + H_2O

The phase diagrams of the ternary systems composed of PEG 600 + Na_2SO_4 + H_2O obtained in this wok and the one reported in literature are shown in Figure A 1.

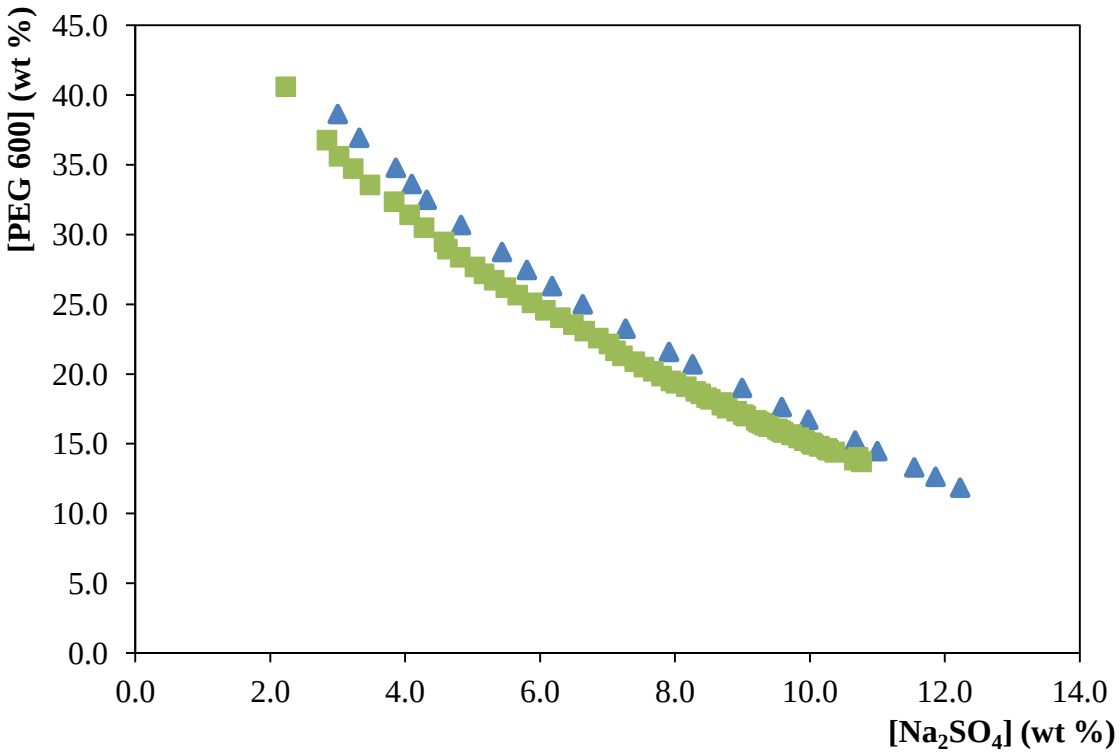

Figure A 1 - Phase diagrams for the ternary systems composed of PEG 600 + Na_2SO_4 + H_2O at 298 K: this work (▲); literature data (■) [12].

The phase diagrams of the ternary systems composed of PEG 600 + Na_2SO_4 + $[C_4mim]Cl$ + H_2O obtained in this work and reported in literature are shown in Figure A 2.

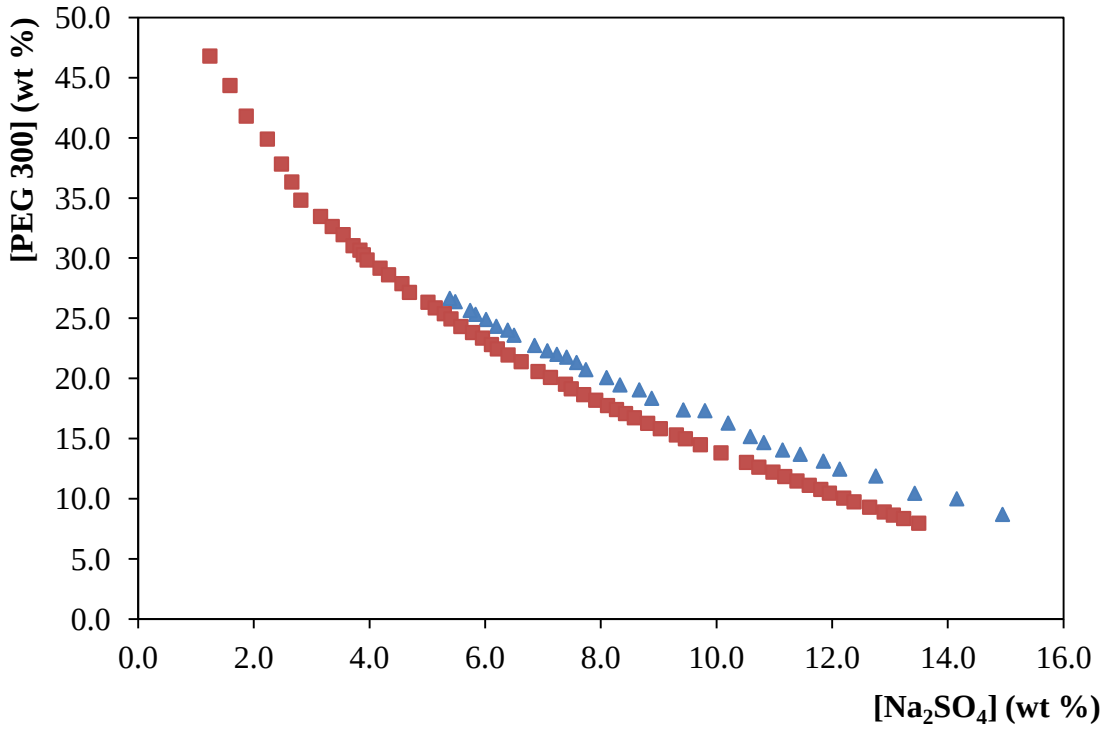

Figure A 2 - Phase diagrams for the quaternary systems composed of PEG 600 + Na_2SO_4 + $[C_4mim]Cl$ + H_2O at 298 K: this work (▲); literature data (■) [12].

The experimental weight fraction data for the phase diagrams of the systems composed of PEG + Na_2SO_4 + H_2O and PEG + Na_2SO_4 + IL + H_2O are presented in Table A 1 to Table A 5.

Table A 1 - Experimental weight fraction data for the systems composed of PEG (1) + Na_2SO_4 (2) + H_2O (3) at 298 K and atmospheric pressure.

***Mw* of PEG 600 g·mol^{-1}**		***Mw* of PEG 400 g·mol^{-1}**			
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
38.638	2.999	14.277	13.183	26.818	6.449
36.938	3.318	16.214	12.090	27.107	6.334
34.781	3.860	17.454	11.213	27.900	6.154
33.625	4.096	17.850	10.902	28.038	6.017
32.490	4.318	18.529	10.540	28.132	5.924
30.678	4.826	19.287	10.194	28.624	5.793
28.745	5.434	19.811	9.898	28.801	5.701
27.459	5.802	20.288	9.608	29.462	5.570
26.298	6.176	20.865	9.319	29.792	5.378
24.996	6.631	21.390	9.074	30.334	5.266
23.249	7.266	21.784	8.835	30.358	5.190
21.583	7.910	22.042	8.637	30.499	5.112
20.689	8.260	22.611	8.459	30.876	5.014
18.997	8.993	23.274	8.057	31.029	4.937
17.622	9.581	23.548	7.881	31.507	4.839
16.718	9.975	24026	7.768	32.379	4.716
15.223	10.670	24.480	7.475	32.686	4.606
14.468	10.998	24.837	7.326	33.066	4.517
13.299	11.546	25.536	7.094	33.475	4.429
12.624	11.861	25.647	6.913	33.794	4.278
11.846	12.226	25.968	6.780	34.114	4.203
		26.236	6.669	34.406	4.123
		26.632	6.579		

Table A 2 - Experimental weight fraction data for the systems composed of PEG (1) + Na_2SO_4 (2) + H_2O (3) at 298 K and atmospheric pressure.

***Mw* of PEG 300 g·mol^{-1}**				***Mw* of PEG 200 g·mol^{-1}**	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
13.730	14.987	29.723	6.420	21.541	13.536
14.620	14.407	29.982	6.318	22.179	12.942
15.659	13.893	30.169	6.203	22.783	12.496
16.612	13.391	30.367	6.093	24.400	11.840
17.290	12.895	30.696	6.005	24.885	11.466
18.002	12.456	31.375	5.860	25.017	11.097
18.251	11.839	31.823	5.689	25.554	10.770
19.215	11.606	32.565	5.474	26.769	10.289
19.719	11.252	32.642	5.391	26.940	10.012
20.290	10.854	33.194	5.270	27.383	9.735
21.033	10.635	33.836	5.071	27.928	9.514
21.524	10.329	34.413	4.900	28.782	9.186
22.051	10.044	35.170	4.706	29.674	8.832
22.452	9.717	35.288	4.640	30.283	8.434
23.036	9.476	35.873	4.487	30.474	8.246
23.512	9.249	36.443	4.308	31.387	7.939
23.970	9.003			31.718	7.778
24.063	8.705			31.918	7.640
24.635	8.558			32.516	7.407
25.045	8.395			33.083	7.173
25.330	8.232			33.738	6.966
25.740	8.045			34.197	6.782
26.111	7.871			34.680	6.592
26.433	7.756			35.392	6.326
26.762	7.570			35.842	6.168
27.106	7.406			35.866	6.089
28.028	7.171			36.180	5.952
28.337	7.043			36.651	5.800
28.597	6.898			37.873	5.509
28.857	6.773			38.840	5.106
29.148	6.646			39.238	4.994
29.424	6.520			40.164	4.839

Table A 3 - Experimental weight fraction data for the systems composed of PEG (1) + Na_2SO_4 (2) + H_2O (3) + 5 wt % IL at 298 K and atmospheric pressure.

[C_4mim]Cl		**[C_4mim][TOS]**		**[C_4mim][SCN]**	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
8.703	14.944	9.707	14.725	14.005	11.253
10.001	14.153	10.435	14.017	15.307	10.500
10.461	13.425	11.945	13.199	17.370	9.696
11.895	12.753	13.431	12.604	17.744	9.368
12.470	12.129	13.995	12.244	18.887	9.012
13.133	11.846	14.531	11.899	20.107	8.453
13.700	11.446	15.111	11.559	20.898	8.064
14.058	11.141	15.758	11.233	21.263	7.883
14.676	10.817	16.308	10.944	22.207	7.614
15.177	10.583	17.437	10.558	23.171	7.209
16.296	10.200	17.864	10.283	24.778	6.636
17.327	9.798	18.266	10.048	26.060	6.151
17.389	9.425	18.655	9.817	28.468	5.367
18.354	8.878	19.049	9.604	29.614	5.079
19.056	8.663	19.896	9.269	30.312	4.841
19.467	8.329	20.219	9.070	31.300	4.595
20.076	8.098	20.502	8.893	32.084	4.388
20.737	7.740	21.311	8.631	33.574	4.052
21.332	7.579	21.769	8.275	34.790	3.764
21.778	7.407	22.473	8.030	35.894	3.558
22.012	7.238	22.715	7.880	37.195	3.299
22.307	7.073	23.464	7.642	38.506	3.069
22.752	6.853	24.302	7.281	40.485	2.760
23.599	6.498	24.996	6.991	42.293	2.504
24.023	6.388	25.699	6.701		
24.343	6.191	26.464	6.416		
24.909	6.014	27.430	6.119		
25.336	5.832	28.861	5.634		
25.658	5.739	29.878	5.306		
26.390	5.482	30.940	4.970		
26.661	5.387	32.153	4.696		

Table A 4 - Experimental weight fraction data for the systems composed of PEG (1) + Na_2SO_4 (2) + H_2O (3) + IL at 298 K and atmospheric pressure.

[C_4mim][CH_3CO_2]		**[C_4mim][$N(CN)_2$]**			
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
16.184	13.358	10.241	13.164	27.117	5.785
16.780	12.907	11.040	12.696	27.570	5.642
17.293	12.400	11.857	12.278	27.987	5.520
17.661	12.027	13.432	11.715	28.420	5.387
18.778	11.450	14.204	11.297	28.753	5.260
19.190	11.125	16.087	10.435	29.101	5.148
19.504	10.779	16.608	10.141	29.716	4.996
20.490	10.343	17.080	9.862	30.405	4.792
20.917	10.051	17.509	9.598	31.417	4.545
21.802	9.654	18.621	9.227	32.169	4.339
21.907	9.383	19.021	8.984	32.801	4.190
22.569	9.038	19.408	8.767	34.124	3.894
23.302	8.720	20.330	8.473		
24.136	8.174	20.649	8.286		
25.232	7.653	20.991	8.104		
25.796	7.402	21.689	7.859		
26.398	7.164	22.305	7.555		
26.960	6.950	22.574	7.409		
27.434	6.757	23.352	7.166		
27.918	6.571	23.563	7.020		
28.671	6.328	24.130	6.812		
29.043	6.153	24.787	6.606		
30.419	5.740	25.222	6.439		
31.239	5.428	25.720	6.276		
32.146	5.137	25.923	6.173		
33.605	4.716	26.454	6.025		
39.029	3.486	26.608	5.922		

Table A 5 - Experimental weight fraction data for the systems composed of PEG (1) + Na_2SO_4 (2) + H_2O (3) + IL at 298 K and atmospheric pressure.

[C_4mim][N(CN)$_2$]		**[C_4mpy][N(CN)$_2$]**		**[C_2mim][N(CN)$_2$]**	
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
9.895	10.200	19.754	7.373	11.151	11.567
12.298	9.397	20.712	6.514	12.791	10.896
13.668	8.871	21.827	6.083	13.433	10.464
15.440	8.159	23.197	5.674	14.781	9.893
16.637	7.738	23.883	5.377	15.242	9.570
18.103	7.197	25.703	4.820	16.421	9.095
19.455	6.788	26.643	4.599	18.511	8.241
19.994	6.481	27.348	4.383	18.864	7.973
21.084	6.158	28.345	4.129	19.573	7.636
21.916	5.873	31.542	3.616	21.486	6.968
22.577	5.637	34.297	3.335	22.817	6.433
23.276	5.392	35.309	3.136	23.271	6.220
23.812	5.230	36.088	2.971	24.718	5.817
25.045	4.943	36.848	2.821	25.865	5.453
26.117	4.696	37.924	2.641	26.773	5.172
27.060	4.425	39.005	2.471	27.744	4.923
28.598	4.100	39.985	2.330	28.662	4.712
29.813	3.802	40.927	2.201	29.528	4.512
31.010	3.603	41.748	2.095	30.438	4.273
32.766	3.296	42.734	1.973	31.822	3.974
34.068	3.104	44.269	1.802	33.192	3.685
35.453	2.852	45.987	1.639	36.081	3.204
37.653	2.582			37.267	3.004
				40.271	2.594

The experimental weight fraction data for the phase diagrams of the systems composed of PEG + Na_2SO_4 + PBS and PEG + Na_2SO_4 + IL + PBS are presented in Table A 6 to Table A 7.

Table A 6 - Experimental weight fraction data for the systems composed of PEG (1) + Na_2SO_4 (2) + PBS (3) at 298 K and atmospheric pressure.

100 w_1	**100 w_2**	**100 w_1**	**100 w_2**
16.181	13.314	31.159	5.471
16.536	12.794	31.778	5.278
17.593	12.166	32.280	5.098
18.628	11.600	32.845	4.939
19.492	11.006	33.501	4.705
20.779	10.350	33.783	4.597
21.978	9.774	34.404	4.438
22.755	9.331	35.084	4.218
23.725	8.937	35.535	4.095
24.050	8.585	35.866	3.990
24.695	8.236	36.180	3.895
25.089	7.948	36.554	3.806
25.610	7.713	36.967	3.699
26.329	7.389	37.672	3.527
27.081	7.114	37.706	3.441
27.609	6.862	38.422	3.317
28.234	6.634	39.012	3.190
28.496	6.456	39.482	3.085
29.014	6.248	40.021	2.985
29.646	6.018	40.716	2.850
29.932	5.890	41.105	2.776
30.469	5.697	41.811	2.632

Table A 7 - Experimental weight fraction data for the systems composed of PEG (1) + Na_2SO_4 (2) + PBS (3) + IL at 298 K and atmospheric pressure.

$[C_4mim][N(CN)_2]$			
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**
1.292	16.688	22.948	7.364
3.94	15.523	23.870	6.970
5.113	14.800	24.002	6.839
7.612	13.831	24.552	6.645
8.463	13.324	24.934	6.446
10.697	12.496	26.436	6.231
12.173	11.816	27.293	5.930
12.847	11.459	27.470	5.771
16.586	10.475	28.057	5.589
17.171	10.139	28.439	5.445
17.761	9.866	28.699	5.329
18.054	9.599	29.361	5.134
19.490	9.040	29.949	4.917
19.690	8.773	30.496	4.774
20.535	8.453	30.776	4.633
21.071	8.150	30.481	4.474
21.739	7.876	30.872	4.351
22.414	7.600		