Universidade de Aveiro Departamento de Química
2018

SANDRA CRISTINA DA SILVA BERNARDO

ESTRATÉGIAS DE PURIFICAÇÃO DE ANTICORPOS POLICLONAIS A PARTIR DA GEMA DO OVO

PURIFICATION STRATEGIES FOR POLYCLONAL ANTIBODIES FROM EGG YOLK

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica do Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora do Departamento de Química, CICECO, da Universidade de Aveiro, e coorientação do Professor Doutor João Manuel da Costa e Araújo Pereira Coutinho, Professor Catedrático do Departamento de Química, CICECO, da Universidade de Aveiro.

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“Imperfection is beauty, madness is genius and it’s better to be absolutely ridiculous than absolutely boring.”

o júri

presidente

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palavras-chave

Anticorpos, Imunoglobulina Y, gema do ovo, técnicas de purificação, sistemas aquosos bifásicos, líquidos iónicos, partição trifásica

resumo

Esta tese de doutoramento prende-se com o desenvolvimento de plataformas de purificação mais eficazes para imunoglobulina Y (IgY), anticorpo presente na gema de ovo, visando a sua possível utilização como biofármaco alternativo. Apesar do anticorpo imunoglobulina G (IgG) ser o biofármaco mais utilizado, este apresenta algumas desvantagens, nomeadamente baixos rendimentos de produção e reatividade cruzada com anticorpos humanos ou outras proteínas. Nas últimas décadas, têm sido realizados esforços de modo a utilizar IgY como uma alternativa terapêutica, uma vez que estes anticorpos podem ser obtidos em maiores quantidades e por métodos menos invasivos. No entanto, o custo de produção de IgY com elevada qualidade, e particularmente para aplicações em larga escala, é extremamente elevado quando comparado com outros agentes terapêuticos, o que se deve principalmente à falta de uma estratégia de purificação rentável e eficaz. Assim, o objetivo principal deste trabalho é superar esta grande limitação associada à purificação de IgY.

Neste trabalho foi inicialmente realizada uma análise crítica dos métodos de purificação de IgY descritos na literatura, levando à conclusão que são necessários pelo menos dois passos para purificar este anticorpo da gema de ovo. O primeiro passo envolve a remoção de lipídios e lipoproteínas e recuperação da fração de proteínas solúveis em água (FPSA) que contém o anticorpo alvo, seguido da purificação de IgY a partir da FPSA, num segundo ou mais passos. Com base nesta informação, foram posteriormente avaliados dois tipos de métodos de purificação, nomeadamente sistemas aquosos bifásicos (SAB) e sistemas de partição trifásica (SPT). No primeiro tipo de sistemas, onde o objetivo consiste em extrair seletivamente IgY para uma das fases coexistentes, foram estudados sistemas formados por polímeros e sais e por polímeros e líquidos iónicos. O primeiro conjunto de resultados demonstrou que os SAB apresentam baixa seletividade para IgY, especialmente quando considerando a partição preferencial do anticorpo para uma das fases em detrimento das restantes proteínas da FPSA. Por outro lado, a abordagem de partição trifásica, estudada com SAB formados por dois polímeros e líquidos iónicos como eletrólitos, levou a melhores resultados em termos de pureza de IgY, alcançados pela precipitação seletiva do anticorpo alvo na interface do SAB. Sob condições otimizadas, estes sistemas permitiram recuperar IgY com uma pureza superior a 90%. Finalmente, com base no potencial de mercado de IgY como um anticorpo alternativo é também apresentado um plano de negócios preliminar.

keywords

Antibodies, Immunoglobulin Y, egg yolk, purification techniques, aqueous biphasic systems, ionic liquids, three-phase partitioning.

abstract

This PhD work is focused on the development of effective purification platforms for immunoglobulin Y (IgY), antibodies present in high quantities in egg yolk, while foreseeing their widespread use as alternative bio-based therapeutics. Although immunoglobulin G (IgG) antibodies are the most used biopharmaceuticals, they display some disadvantages, namely their low production yields and cross reactivity with human antibodies or other human proteins. In the last decades, some efforts have been placed on the use of IgY as a promising alternative therapeutic option since these can be obtained in higher titters by less stressful and invasive methods. Nevertheless, the production cost of high-quality IgY, particularly for large-scale applications, remains higher than other drug therapies mainly due to the lack of a cost-effective purification strategy. Thus, the main objective of this work is to surpass this major limitation associated to IgY.

In this work, a comprehensive and critical analysis of the IgY purification methods described in the literature was first performed. At least two steps are required to purify IgY from egg yolk, the first to remove lipids and lipoproteins and to recover the water soluble-protein fraction (WSPF) that contains the target antibody, followed by the purification of IgY from the WSPF in a second or further steps. Based on this information, two types of purification methods were then investigated, namely aqueous biphasic systems (ABS) and three-phase partitioning (TPP) systems. In the first type of systems, where the goal is to selectively extract IgY for one of the coexisting phases, ABS formed by polymers and salts and by polymers and ionic liquids were investigated. The first set of results demonstrated that ABS have low selectivity for IgY, particularly regarding the preferential migration of IgY for one of the phases over the remaining proteins in the WSPF. On the other hand, the TPP approach, studied with ABS formed by two polymers and ionic liquids as electrolytes, led to better results in terms of IgY purity, achieved by inducing the selective precipitation of the target antibody at the ABS interphase. Under optimized conditions, these systems allow to recover IgY with a purity higher that 90%. Based on the market potential of purified IgY, a preliminary business plan is finally presented.

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NOTATION

List of symbols

wt%	Weight fraction percentage
A_i	Absorbance of component i
[i]	Concentration of component i
t	Time
K_i	Partition coefficient of component i
$EE_i\%$	Extraction efficiency percentage of component i
$Y_i\%$	Recovery yield of component i
K_{OW}	Octanol-water partition coefficient
R^2	Correlation coefficient

List of ionic liquids cations

$[Ch]^+$	Cholinium

List of ionic liquids anions

Cl^-	Chloride
$[Ac]^-$	Acetate
$[DHcit]^-$	Dihydrogencitrate
$[DHP]^-$	Dihydrogenphosphate
$[Bit]^-$	Bitartrate

List of common salts

NaCl	Sodium chloride
$Na_3C_6H_5O_7$	Sodium citrate
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Sodium phosphate
Na_2SO_4	Sodium sulphate
NaH_2PO_4	Sodium dihydrogenphosphate
$NaNO_3$	Sodium nitrate
NH_4SO_4	Ammonium sulphate

List of abbreviations

ABS	Aqueous biphasic systems
Ab	Antibody
BP	Bottom phase of ABS
CD	Circular Dichroism
DSC	Differential scanning calorimetry
FT	Freeze and thaw method
FTIR	Fourier transform infrared spectroscopy
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
IL	Ionic liquid
IP	Interphase of ABS
pI	Isoelectric point
LDL	Low density lipoproteins
LLE	Liquid–liquid extraction
MW	Molecular weight
NaPA	Sodium polyacrylate
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Poly(ethylene)glycol
PPG	Polypropylene glycol
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size Exclusion Chromatography
TL	Tie-line
TLL	Tie-line length
TP	Top phase of ABS
TPP	Three phase partitioning
UV-Vis	Ultraviolet-Visible
WSPF	Water soluble protein fraction

1. Avian Immunoglobulins:
An Introduction

1.1. Immunoglobulins: Structure and Function

In 1890, von Behring and Kitasato [1] showed that the transfer of serum from immunized animals against diphtheria to infected animals could treat them; the immunity process was described as a result of substances in the blood that von Behring then named antitoxins or antibodies [2,3]. In 1900, Ehrlich defined the main function and way of action of antibodies, proposing the "side-chain theory" [4], in which the antibody, also described as "specific receptors", was branched and consisted of multiple binding sites for the foreign material, the antigen [5]. Ehrlich [4] additionally proposed that these receptors are either associated with cells or distributed in the blood stream in response to antigens, and after the antibody-antigen interaction there is the activation of the complement pathway [6,7], explaining why the immune response occurs in reaction to infections.

Nowadays, it is known that immunoglobulins (Igs) or antibodies are host proteins produced by jawed vertebrates in response to bacteria, viruses, or other foreign molecules or agents [8]. Immunoglobulins are glycoproteins that constitute the humoral branch of the immune system and represent approximately 20% of the plasma proteins in humans [8,9], being this response a key mechanism used by the host organism for protection (immunity). Each animal can produce millions of different specific antibodies, and each antibody specifically binds to a particular substance, known as an antigen [9].

Immunoglobulins are glycoproteins that belong to the immunoglobulin super-family, which includes cell surface antigen receptors, co-receptors and other molecules of the immune system, all containing a structural domain known as an Ig domain (tightly packed anti-parallel β-sheets) [10,11]. In 1959, Porter [12] published the first model for the molecular structure of antibodies. The author observed that when digested by papain, Ig results in three fragments (corresponding to at least 90% of the original molecule): "fragments I and II", which are extremely similar in chemical and biological properties, and the "fragment III" that differs very extensively [12]. This initial study on the application of enzymes to fragment IgG allowed to obtain the first insights about its structure. Porter latter concluded that papain digests IgG into a single (Fc) fragment and two identical fragments, named Fab fragments, in which each is able to bind to an antigen (Figure 1) [9,13,14].

The first atomic resolution structure of an antibody fragment (Fab) was published in 1973 by Poljak [15]. Later, Huber et al. [16] performed crystallographic structure studies of IgG molecules and Fc fragments enabling the description of the structure of an antibody molecule. Antibodies comprise Y-shaped monomeric subunits with a basic H_2L_2 structure, composed of four polypeptide

chains, consisting of two identical heavy chains (H_2, each with *circa* 50–75 kDa) paired with two identical light chains (L_2, each with *circa* 25 kDa), held together by disulfide and noncovalent bonds [9,17]. Within this basic H_2L_2 structure, Igs have two major regions, the Fc ("fragment, crystallizable") and the two Fabs ("fragment, antigen binding") (Figure 1) [18]. The Fab fragments are part of the two arms of the Y shape, and are formed by the packing of the variable domains of both heavy and light chains (V_L and V_H), forming the antigen-binding region responsible for target recognition [17,19]. The tail section of the Y, i.e. the fragment crystallizable (Fc), is composed of the unpaired sections of the heavy chains, and is linked to the arms by a flexible linker called "hinge" (Figure 1). This Fc region is common to all antibodies of the same type and provides the signal for effector functions [17,18]. For several years it has been assumed that antibodies possessed extraordinary flexibility, in which the Fc had the ability to twitch back and forth, and the Fab arms could wave, bend and also rotate on the hinge [20–22]. It was then proved that the hinge region provides the antibody with the ability to adopt several structural forms in solution with apparent constant motion and flexing [23,24].

At the top of each Fab arms, the complementarity determining regions (CDRs) are present, which comprise the antigen binding site, also called antigen combining site or paratope, defined by a specific set of amino acid residues contacting with the specific epitope of an antigen (Figure 1) [18]. The sequence of CDRs varies between distinct antibodies and is primarily responsible for determining the specificity of a given antibody [17].

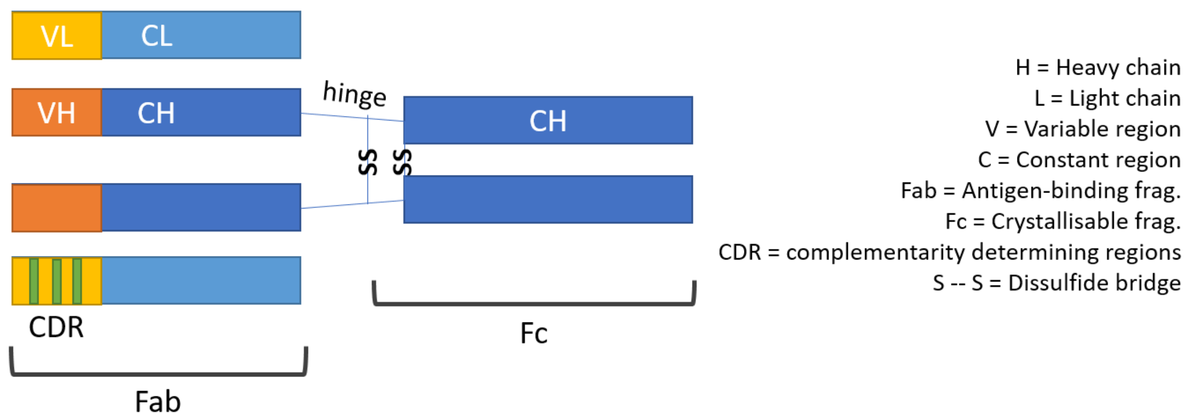

Figure 1. Structure of IgG: schematic diagram showing the antibody structure. The H and L chains at the top deconstruct the antibody at a nucleotide level. The chains at the bottom deconstruct the protein sequence [14].

1.1.1. Immunoglobulins diversity

Immunoglobulins or antibodies are expressed only in jawed vertebrates, including fish, amphibians, reptiles, birds, and mammals [25]. It is estimated that the diversity in antibodies specificity is close to 1011, which is enough to cover the antigens repertoire [26]. Depending on the Ig class, the structures of the corresponding immunoglobulins are different, namely in the number of constant domains within the heavy chains [27]. The types of mammal Igs are defined according to the heavy chain they contain: α, δ, ε, γ or μ, corresponding to IgA, IgD, IgE, IgG and IgM, respectively [9,28,29]. Furthermore, in certain mammals, such as humans, IgG and IgA are further subdivided into sub-classes, referred to as isotypes, due to polymorphism in the conserved regions of the heavy chain [9]. The isotype is defined by the amino acid sequence differentiation, created by the combination of a particular heavy chain class or subclass of Ig with one of the light chain genes κ or λ [28].

While in mammals there are five classes of Ig, in avian there are only three classes: IgY, IgM, and IgA [9]. As the most conserved Ig class, the IgM in birds has a similar structure to the mammalian IgM [25]. Despite being orthologous to mammalian IgA, avian IgA differs structurally from its mammalian counterpart, since it has four constant domains and no hinge region, whereas the same structural difference is observed for IgY and the functional analogue IgG [25]. The avian IgY comprises four constant domains, such as the mammalian IgE and IgM, while the mammalian IgG contains only three [27,30]. This fact is most likely due to the reducing of one constant domain in the case of mammals to a short segment that comprises the hinge region typically present in the mammalian IgG [27,31]. Avian immunoglobulins will be described in more detail in Chapter 1.2.

The diversity in Igs classes occurs in the sequence and number of constant domains, hinge structure and in the valency of the antibody, i.e. in the number of "arms" available to bind to the antigen [32]. Hence, these classes exist in different molecular forms: monomers (IgG, IgA, IgD, IgE and IgY), dimers (IgA) or pentamers (IgM). Each antibody class has different functional properties since each isotype is in a specific location of the body, having a specific role to play in natural defence/immunity against extracellular pathogens and their toxic products [29].

IgG is a monomeric protein with a molecular weight of about 150 kDa, being the major antibody class of the secondary response of the immune system and the major immunoglobulin class present in normal serum, accounting for 70-75% of the total immunoglobulins population [33]. Furthermore, IgG can be found in the blood and extracellular fluids, being responsible to effectively neutralize toxins, viruses and bacteria, and efficiently opsonize them for phagocytosis, activating the complement [29,33]. In addition, in mammals, maternal IgG is extremely relevant since it can

be transported across the placenta to provide protection to the fetus [29].

Although IgG corresponds to the most abundant Ig present in mammal serum, IgA is actually the most abundant Ig class present in mammals, in which approximately 60% of all immunoglobulins produced belong to the IgA isotype [34]. However, IgA is more abundant in the mucosal tissues since more than half of the IgA produced is selectively transported into external secretions [29,35]. IgA exists in two molecular forms, the monomeric (with a molecular weight of about 160 kDa) that is mostly present in serum (although in small concentrations of about 3-5 mg/mL), and dimeric forms that are present in the mucosa, mostly in a secreted form (sIgA) comprising both a joining chain (J-chain) and the secretory component chain, with a size of *ca.* 400 kDa [29,35]. The main function of IgA is to neutralize toxins and viruses at mucosal surfaces and to block the entrance of bacteria across the intestinal epithelium, acting as the first line of humoral defence [29,34].

IgM is a highly conserved and ancient Ig that can be found in the blood of all jawed vertebrates, from sharks to mammals [35]. The IgM molecule normally assumes a star-shaped pentameric form of five of the general four-chain immunoglobulin unit, with a total size of 900 kDa [36]. It can also be found in a hexameric form in a lower amount (5% of the circulating IgM) [35]. Like IgA, IgM also contains a J-chain in its pentameric form, conferring the ability to bind the polymeric Ig receptor (pIgR) and allowing the transition from the vasculature to mucosal surfaces [35]. IgM is the first antibody produced by activated B cells in the primary antibody response [29,36]. In fact, IgM corresponds to the largest fraction of what is termed “natural antibodies” (non-immune antibodies), which have low-affinity, are polyreactive, and are produced in the absence of antigen stimulation [35,37]. Still, the multivalency of IgM makes of it a potent binder, and as such, it can efficiently agglutinate and eliminate large particles such as microorganisms [29,36].

IgE is the less abundant immunoglobulin in the mammalian body (about 0.002% of the total Ig pool, with a molecular weight of about 190 kDa), and half of it can be found in the intravascular space with an exceedingly low quantity present in the serum (17-420 ng/mL) [29]. IgE is monomeric and mainly exists bound to receptors on the surface of basophils and mast cells [29,38]. In the case of allergic conditions or parasitic infections, the normal amount of circulating IgE is high. Allergy is thus caused by an overproduction of IgE in response to external agents, like pollen, house dust mites, animal danders, food and insect venoms, leading to disorders like asthma, allergic rhinitis, atopic dermatitis and, in the worst case, to anaphylactic shock [29,38]. In spite of this, IgE acts through a network of cells and receptors, turning it very effective both as part of the first-line defence mechanism and as a mediator of harmful allergic reactions [29,38]. The binding of IgE to antigen results in several cellular responses, including inflammation, itching, coughing,

bronchoconstriction and mucus secretion, with subsequent elimination of the invading organisms from the body [29,38].

IgD does not occur in all species, but it is present in most mammalian and in some fish and xenopus [39,40]. It is found as the major membrane-bound receptor on B lymphocytes, where it is co-expressed with IgM [40]. IgM is secreted upon antigen stimulation, while the expression of IgD is turned off, being thus much less abundant than IgM (IgD corresponds to *circa* 0.25% of all the Ig population) [29]. IgD has been suggested to play a role in B-cell triggering, development of tolerance and in the process of immunological memory, though the precise function of IgD still remains to be revealed and it has been associated to certain autoimmune diseases [29,39].

1.1.2. Immunity terminology

The immune system is present in the body in order for the host to defend itself against a certain pathogen [41]. This defence system can be divided in the innate (native) and the acquired (adaptive) immune system. The acquired immune system is also known as the specific part of the response and it is divided into the cellular branch (T and B lymphocytes) and the humoral branch [42].

The innate immune system is the first line of defence against all pathogens; this is a general mechanism, or nonspecific, and do not require previous exposure to the offending pathogen. There are always certain molecules, cells, and receptors present that can protect the host against these pathogens, for instance in the skin and mucosal surfaces [32]. This type of immunity is inherent to the host organism, being always present. In contrast to innate immunity, the acquired immunity occurs due to an antigen-specific response that generates protective resistance against an infectious agent after a previous contact with the specific pathogen. Acquired immunity can occurs as a consequence of a clinical or subclinical infection, for instance, or by deliberate immunization with the target agent [32]. B and T lymphocytes are involved in this antigen-specific response that comprises the clonal selection of these lymphocytes and the development of an immunological “memory” [32].

Acquired immunity can be developed by natural ways, in which the protection is provided by the primary exposure of the host to a pathogenic or antigenically related organism [32]. Furthermore, artificially acquired immunity develops as a result of an immunization process, which can be then a passive (temporary) or active (long-term) immunization process. Acquired active immunization, also known as vaccination, refers to the process of exposing the individual to a target antigen, either with attenuated/killed organisms or toxoid/subunit components, to generate an adaptive immune response and long-term duration immunity [30,43]. On the other hand, acquired

passive immunization involves the use of antigen-specific antibodies from a different source, such as from an immune individual or animal, followed by the administration of specific antibodies to give temporary protection (passive immunity) [43]. It is quite important to distinguish between natural passive immunity and artificially (acquired) one. In the first type, there is the transference of antibodies between individuals by a natural process, i.e. they are passively transferred across the placenta or by breast milk from the mother to the child or by the ingestion of colostrum in some mammals, while in the latter the immunity is conferred artificially, i.e. by the administration of antibodies taken from a different source through vaccination [30].

The artificial passive immunization, formerly referred as "serum therapy", was developed in the 19th century by von Behring to protect humans against diphtheria and tetanus toxins [1,3]. This was the first developed effective therapy against infectious disease agents, occurring before the “antibiotic era” and common vaccines (active immunization). Passive immunization is characterized by the transfer of a specific antibody or of sensitized lymphoid cells from an immune to a previously nonimmune recipient host, conferring resistance against a specific pathogen from an hyperimmune individual to a susceptible recipient [30,44,45]. Contrarily to active immunization (vaccines) that may concede long duration protection [45], passive immunization provides an immediate but short-lived protection, lasting several weeks to three or four months at most, in which no immunological memory is established [30,44,45]. The source of passively administered antibodies may be human blood donors, immunized humans or other animals, or hybridoma cell lines [45]. However, human sera are always preferred instead of other mammals to avoid side effects induced by foreign serum proteins [44]. Since the protection provided by transferred antibodies are of a transient nature, the repeated or continuous antibody administration is necessary, thus requiring large amounts of these antigen-specific antibodies [30]. These aspects make of passive immunization a highly expensive and extremely difficult process to achieve as an effective widespread therapy.

1.2. Avian Immunoglobulin Y (IgY)

Leslie and Clem [46] reported the existence of immunoglobulin Y (IgY) in 1969 as an immunoglobulin (Ig)G-like molecule present in chickens, although with some differences in the heavy (H) chains that were larger than in mammalian IgG [46]. Only few years later, it was realized the wide distribution of IgY in non-mammalian vertebrates, such as other birds, reptiles and amphibia [47]. After these evidences, it was finally concluded that although IgY and IgG display similar functions, there are several differences between both types of antibodies [48,49]. Additional reports by molecular cloning techniques led to the suggestion that avian IgY is the evolutionary

ancestor of mammalian IgG and IgE [18,50]. This fact is not completely unforeseen, as birds and mammals evolved from a common reptilian ancestor more than 200 million years ago [51]. Such common features, yet with significant differences between their immune systems, make of avian antibodies an interesting class of antibodies to study.

Contrarily to mammals, chickens and other birds express only three classes of Ig chains, namely IgM, IgA, and IgY. Chicken IgA and IgM are similar to mammalian counterparts in terms of functional properties, molecular weights, morphology and immunoelectorophoretic mobility, being the IgM highly conserved between species. On the other hand, IgY can be found in chickens and oviparous (egg laying) animals, being known as the functional equivalent of immunoglobulin G (IgG) found in mammals [48,49]. In terms of activity, IgY combines the two functions of mammalian IgG and IgE; IgY has the ability to mediate anaphylactic reactions, such as IgE, and like IgG is the major serum antibody and the main defence mechanism against systemic infections in hens and other oviparous [31]. Although both chickens and ducks express the intact form of IgY, a truncated IgY form designated as IgY(ΔFc) and lacking the last two CH domains is also expressed in ducks. Structurally, IgY(ΔFc) has antigen-binding activity but lacks the effector function due to the absence of the Fc region. Thus, this truncated IgY will not be addressed in this thesis, since neither is present in chicken nor can be studied as a regular antibody common to chicken, other birds (e.g. quails and ostriches), reptiles and amphibia [25].

IgY is present in the blood of chickens, like IgG is in mammals' serum. During egg formation, IgY is transferred and concentrated in the yolk to protect the development of chick embryo, in a natural passive immunization process, as previously described for mammals [27,30,49]. IgA and IgM are later secreted and deposited into the egg white in the oviduct (Figure 2) [27,30]. In Figure 2, it is represented the anatomy of the oviduct, and the composition of a chicken egg.

Eggs consist of about 9.5% of shell, 63% of albumen, and 27.5% of yolk [49], where the total dry matter of freshly laid egg yolks is *circa* 52% and slightly increases with the age of the laying chicken [52]. The avian eggshell is made of an organic matrix (3.5%), comprising the eggshell membrane and some constituents embedded in the layer of calcium carbonate (95%) in the form of calcite [52]. Inside the shell and the membrane, it is the albumen, the well-known egg white. The egg white is mainly composed of water and proteins, in which proteins correspond to about 9.7% to 10.6% (w/w) [53]. The albumen main role is to provide water and proteins for the growing chick and to support the yolk through the chalazae, two fibrous proteins that connect the yolk to the inner membrane. Egg white corresponds to an aqueous solution enriched in proteins, such as ovalbumin, lysozyme, ovotrasferrin, ovomucoid, ovomucin, and globulins (IgA and IgM) [52,53]. The vitelline membrane

separates the egg white from the yolk (Figure 2) and corresponds to a permeable membrane, allowing the diffusion of water and small molecules from the egg white to the yolk, during the storage of eggs, that leads to the small decrease of the dry mass of the yolk [52,53].

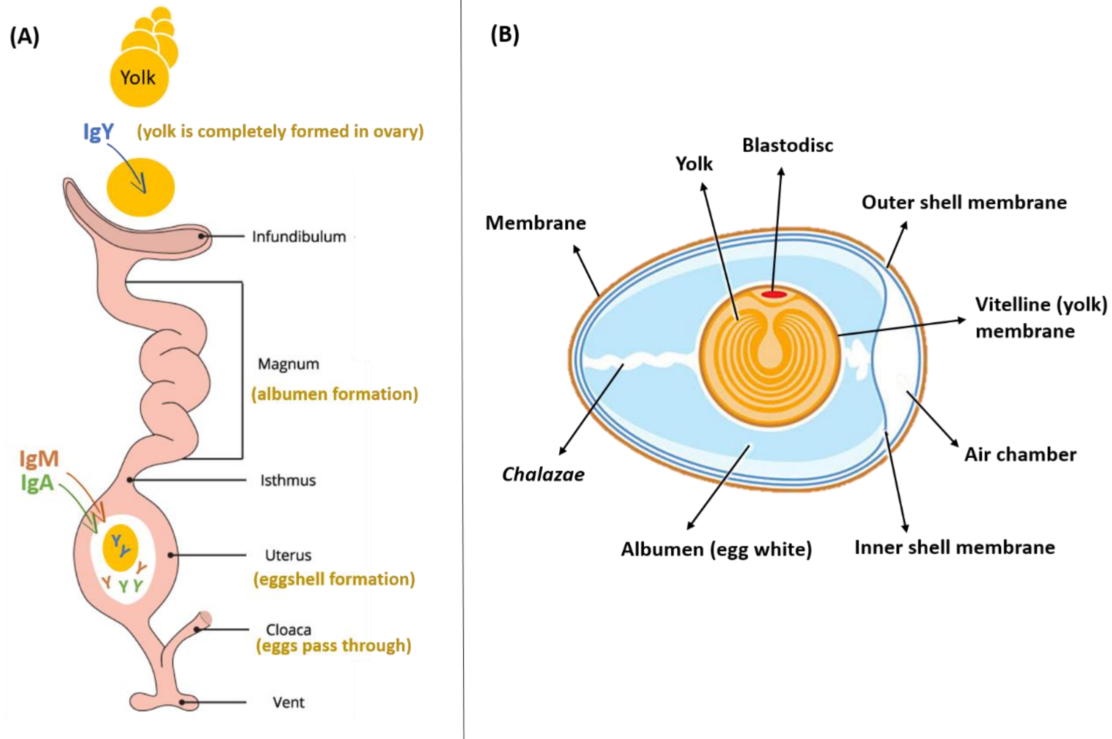

Figure 2. Schematic representation of the oviduct anatomy **(A)** and egg composition **(B)**.

Egg yolk provides vital nutrients (proteins, lipids, vitamins, and minerals) to the chicken embryo [52], being a very complex matrix composed of two main fractions, the plasma and granules [54]. Egg yolk is mainly constituted by lipids, proteins, carbohydrates and minerals [52]. The major constituents of the solid matter of yolk are lipids and proteins, in a ratio of 2 to 1 [49,52,55]. Considering the lipids (about 65% of the dry matter), they are also present in the form of lipoproteins [55]. Five groups of lipoproteins are present in egg yolk: 68% of low-density lipoproteins (LDL), 16% of high-density lipoproteins (HDL), 10% of globular proteins (livetins), 4% of phosphoproteins (phosvitin), and 2% of other proteins [52]. The proteins present as free proteins correspond to antibodies and apolipoproteins. The detailed composition of an egg yolk is reported in Table 1.

Table 1. Average composition of a fresh egg yolk (information taken from [53]).

Component	Content (in w/w%)	
Water		48
Lipids	Triglycerides	22.6
	Phospholipids	9.6
	Cholesterol	1.8
	Caratenoids	<1
Proteins	Lipovitellin (HDLs)	5.8
	Livetins (including the IgY)	5
	Lipovitellinin (LDLs)	3.5
	Phosvitin	1.8
Carbohydrates	Glucose	<1
Vitamins	A, D, E, K, B1…	<1
Minerals	P, Ca, Na, K, Cl, S, Mg, Fe	1

Livetins are globular proteins which are not bound to lipids, corresponding to water-soluble proteins, and having three main components: α-, β-, and γ-livetins [53,56]. The α-livetin corresponds to albumin, whereas α-2-glycoprotein is the main component of β-livetin, and IgY corresponds to γ-livetin [49]. The relative proportion of the three livetins in the yolk is 2:5:3, respectively [57]. The IgY concentration in the chicken blood is 5-6 mg/mL, while in egg yolk it is 10-25 mg/mL [27,58]. Furthermore, the total IgY concentration is estimated to be 100-200 mg *per* egg (about 1.5% of total weight of an egg yolk) [52]. Based on these values, it is reasonable to assume that egg yolk is a potential source of antibodies. In fact, during the past 20 years, the use of chickens instead of mammals for antibodies production has increased. This fact is complemented by the advantage of painless harvesting of chicken eggs over the collecting of mammals serum [59].

1.2.1. Biochemical and Immunological Properties

The general structure of IgY is similar to the mammalian IgG, presenting two heavy (H) chains and two light (L) chains, in a Y shape with a valency for two epitopes as well [26,31,52,59,60]. The

H chains have a molecular weight of 67-70 KDa each, and typically possess one variable (V)- and four constant (C)- region domains [31]. The main structural difference between IgG and IgY is on the number of these regions, in which IgG has three C regions (Cγ1-Cγ3) and IgY has four (Cυ1-Cυ4) - Figure 3 [59]. Like IgG, the L chain of IgY also contains one V and one C domain with a molecular weight of about 25 KDa of each chain [26,31,59]. Due to these differences, IgY has a higher molecular weight (180 kDa) when compared to IgG (150 KDa) [59].

Contrarily to IgG, the heavy chain of IgY does not present a hinge region (Figure 3) [61], and its absence may result in a reduced flexibility of the Fab moiety and could be responsible for some differences when considering their antigen recognition ability [31,52,59]. This hinge absence results in a steric hindrance which affects the cross-linking of the formed antigen-antibody complex [27], also required for a commonly applied immunochemical method for identification and quantification of IgG (immunoprecipitation reaction) [26]. IgY also contains two carbohydrate side chains, contrarily to the single one present in IgG (Figure 3) [52]. Both Igs contain Asn-linked oligosaccharides in the C_H2 regions; nevertheless, IgY contains an additional and unique high-mannose-type oligosaccharide [30]. Therefore, the major structural difference between IgG and IgY is located in the Fc region, where the most biological effector functions of immunoglobulins take place [59].

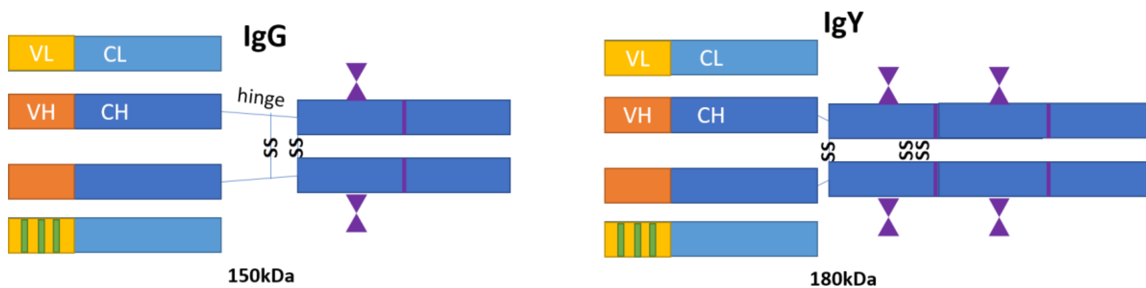

Figure 3. Schematic representation of IgG and IgY structures. H: Heavy chain; L: Light chain; V: Variable region; C: Constant region; S-S: Dissulfide bridge. Carbohydrates chains are signed with purple triangles. Adapted from [31,62].

In agreement with the lipid-rich environment of the yolk, IgY is also more hydrophobic than IgG and presents a lower isoelectric point [30,42], ranging between 5.7 and 7.6, whereas that of IgG is in the physiologic range, between 6.1 and 8.5 [42,52,59,60]. In addition, the β-sheet content of IgY has been reported to be lower than that of IgG, suggesting that the conformation of IgY is more disordered [62]. Thus, IgY tends to be less stable when compared to IgG, yet it is a reasonably stable protein that can be stored at 2-4 °C preserving its structure and antibody activity [26]. Due to

relatively high core body temperature of chickens (41 °C) it is not surprising that IgY could retain their activity for about 6 months at room temperature (20 °C) or for a month at 37 °C [59,63]. In summary, IgY is fairly heat stable; nevertheless, the activity of IgY has been shown to decrease with increasing temperature and time [30]. Minimal loss of activity was observed when heating between 60-65 °C, and decreased remarkably by heating for 15 min at 70 °C or higher temperatures [30,62]. Concerning the pH range stability, IgY is relatively stable between pH 4 and pH 11, but displays a rapid decrease in activity above pH 12 and below pH 3.5, being almost completely lost at pH 3 [30,62]. As with other proteins, also the chicken antibodies are susceptible to proteolytic digestion [64]. The IgY activity is completely lost by pepsin digestion below pH 4.5 [30]. Though, in a trypsin and chymotrypsin digestion, IgY retained 39% and 41% of its activity, respectively, after 8 h of digestion [65].

1.2.2. Advantages of IgY *versus* IgG

Polyclonal antibodies have been conventionally isolated from the sera of several animals hyperimmunized with a target antigen, such as rabbits, goats and sheep [66]. Nevertheless, it should be highlighted that IgG monoclonal antibodies produced in culture cells lines have been the most investigated, mainly because these correspond to monoclonal antibodies and target-specific antibodies with a more effective response [67]. However, the use of chickens for the production of specific polyclonal antibodies affords several advantages, particularly if compared with the IgG counterpart obtained from mammals serum [49].

IgY is recovered by a non-invasive method which does not cause pain to animals or leads to their dead; although the immunization is required, it is based on the collection of eggs [8,68]. Moreover, few eggs *per* week can provide the same amount of immunoglobulins obtained from repeated bleeding of immunized mammals - more than 100 mg of IgY can be isolated *per* egg [69].

Several works published comparative studies on the production of antibodies by immunized hens and mammals [68,70,71]. Jensenius et al. [71] estimated that the total antibody activity of the eggs laid by a hen in a month is equivalent to that produced in a half litre of serum of an immunized rabbit. Additionally, Gottstein and Hemmeler [70] reported that the quantity of IgY obtained from eggs laid by an immunized hen is 18 times higher than that of IgG isolated from rabbit serum. Kim et al. [68] 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, exceeding by far the productivity of IgG.

Compared to its mammalian IgG counterpart and due to the differences in their structure, IgY

antibodies have several immunoreactivity advantages. They recognize more epitopes on a mammalian protein than the corresponding IgG antibodies do, thus displaying superior immunogenicity (high avidity) against highly conserved mammalian proteins [59,72]. IgY lacks in cross-reactivity (high specificity and low background), important to reduce unwanted reactions in many assays using anti-IgG antibodies [73]. Due to the phylogenetic distance, there is also a better immune response of IgY to mammalian antigens [73,74]. As it was stated before the typical main differences in IgY molecule is relating with the Fc domain; therefore, their effector functions are essentially different [59]. For instance, IgY does not react with mammalian antibodies [75], and thus IgY does not react with human anti-murine antibodies (HAMA) nor bind to the rheumatoid factors (RF), which are autoantibody reacting with the Fc portion of IgG found in many autoimmune diseases [30,63,73]. IgY does not activate mammalian complement factors nor bind to mammalian and bacterial cell surface Fc receptors, such as staphylococcal protein A or streptococcal protein G, indicating definitely an immunological difference of the Fc region from that found in IgG [30,73,76]. Table 2 provides a summary on the advantages of IgY over IgG and their main characteristics.

By analysing all the information discussed above and presented in Table 2, it can be inferred that there are several advantages by choosing chickens rather than rabbits or goats to produce polyclonal antibodies. Not only it is the most ethical method to produce polyclonal antibodies since no bleeding is required, but also high-affinity antibodies can be produced, particularly relevant to the conserved proteins in mammals. Compared to rabbits or other mammals, IgY is also less subjected to variability between batches. Additionally, with small quantities of administered antigens, a single chicken can produce up to 2 g of IgY *per* month, 10 times higher than antibodies obtained from the serum of a rabbit. It is also cheaper to feed and house chickens than rabbits or other larger mammals.

Table 2. Differences and advantages/disadvantages between mammalian IgG and avian IgY (adapted from Kovacs-Nolan and Mine [49] and Schade et al. [61]).

Features of comparison	IgG	IgY
Animal	Mammals	Birds (+ reptiles and amphibian)
Source of antibody (Ab)	Blood serum	Egg yolk
Ab type	Polyclonal	Polyclonal
Molecular Weight (kDa)	150	180
Number of C domains	3	4
Number of carbohydrate side chains	2 – less sites for labelling	4 – more labelling sites, leads to improved signals
pI range	6.1 - 8.5	5.7 - 7.6
Hinge region	Present	Absent
Ab sampling	Invasive - bleeding	Non-invasive – collecting eggs
Ab yield	200 mg / bleed (400 mL blood)	100-150 mg / egg (5-7 eggs per week)
Ab yield per month	200 mg	~ 1500 mg
Protein A/G binding	Yes - easily purified by affinity chromatography	No
Interaction with rheumatoid factor	Yes	No
Activation of mammalian complement	Yes	No
Interaction with the antigen (conserved mammalian proteins)	Weak interaction - low avidity	Strong interaction - high avidity (due to the high phylogenetic distance)
Interaction with other mammalian proteins (e.g. IgG)	High reactivity - cross reactivity and false results	Low reactivity - less background and high specificity

1.3. Applications of IgY

As discussed above, polyclonal antibodies from chicken egg yolk offer many advantages for diverse applications compared to polyclonal IgG. Therefore, during the last decades it was observed a significant increase in the research on IgY, ranging from its application in immunodiagnostic techniques and in immunotherapy [77].

1.3.1. IgY as a research tool in immunoassays

There are some reports in the literature showing the feasibility of IgY application in immunoaffinity chromatography [27,49,78]. Immunoaffinity chromatography is a powerful technique with high specificity that comprises the isolation and selective purification of target molecules from a complex mixture, using immobilized ligands, being in this case antibodies with specificity to the target molecule [49,79]. A major drawback on the implementation of this technique at a large scale is its current high cost, derived from the costs associated to the production of antibodies and their low efficiency in immobilization [80]. Due to the high yield that can be obtained with IgY recovered from egg yolk, IgY can be considered an ideal replacement in immunoaffinity chromatography, thus capable of lowering the considerable the costs of this type of chromatography [49]. Since IgY has high avidity, showing better affinity for the highly conserved mammal proteins, this polyclonal antibody can perform as good as a monoclonal, which are also very expensive to produce [9,26,49]. Based on these advantages, immobilized IgY has been used for the isolation of added-value proteins from dairy products, such as lactoferrin [81], and in the separation of IgG subclasses from colostrum, milk, and cheese whey samples [82]. Others studies using IgY immunoaffinity columns have been described in the literature for the purification of biological molecules from human serum [49], including the purification of tetrachlorodibenzo-p-dioxin [78], prekallikrein [83] and human alpha-2 antiplasmin [84].

An additional application of IgY in research comprises it applicability in rocket electrophoresis to quantify immunoglobulins of mammalian sera [85]. The principle underlying rocket electrophoresis is that the antigen should migrate rapidly in an electric field in a gel and produce a rocket-shaped precipitate by interacting with antibodies. In the present context, the target antigen is the immunoglobulin to be tested in the serum and the antibody is an anti-immunoglobulin serum, being regularly used mammalian IgG from a different source (e.g. rabbit). However, human IgG migrates thorough a gel containing mammalian antiglobulin antibodies which have a similar electrophoretic mobility, representing thus a major drawback in this technique. By using IgY instead, and as the average pI of avian antibodies is about 1 pH unit lower than mammalian IgG, proper rockets can be formed and Igs from serum can be properly quantified [27,85].

There are several reports showing the applicability of IgY in other different research assays. For instance, Camenisch et al. [86] described the use of a specific HIF-1a antibody obtained from chicken egg yolks to detect the HIF-1a protein by Western blot, electrophoretic mobility shift assays (EMSAs), immunoprecipitation, and immunofluorescence experiments using hypoxic cells from different species; in contrast, the application of mouse IgG monoclonal antibodies for the same

purpose was found to be more restricted [86,87].

Chicken antibodies can also be used instead of IgG to improve immunological assays, enhancing their sensitivity and reducing the false positive/negative results in clinical applications employing enzyme-linked immunosorbent assays (ELISA) [49,59,63,77]. For instance, human serum samples might contain human anti-mouse IgG antibodies (HAMA), which can be found naturally in the serum or specially in individuals treated with mouse antibodies for therapeutic purposes. Thus, these specific antibodies will bind to any mouse antibodies if these are used in immunoassays, being one of the well-known cause for false positive results [49,63,77]. Additionally, the interferences between the human rheumatoid factor (RF) with IgG from different mammalian species could also pose a problem in some tests [63]. RF is usually found in serum samples from patients with rheumatoid arthritis, but can also be found in patients with other diseases and even in 3-5% of healthy individuals [63,77]. Unlike IgG, chicken antibodies (IgY) do not react with RF or HAMA and can be used to avoid these interference problems [49,77]. Other interferences in these assays are usually caused by mammalian complement activation that can be triggered after adding a new an undiluted human serum sample to an assay. By using IgY, this type of interference is eliminated [49,77,88]. A common problem also found in immunoassays is the cross-reactivity that leads to false positive results, either by the interaction between IgG from different mammalian species in a multi-antibody assay or by the reaction between the Fc region of mammalian antibodies and staphylococcal protein A or streptococcal protein G [49,59,71]. Hence, whenever there is a risk of false positive results due to cross-reactions, chicken antibodies can be used as a tool to avoid this drawback - IgY antibodies do not bind to bacterial Fc receptors or with mammalian IgG [49,63,77]. If a mammalian protein is used, IgY recognizes more epitopes. Therefore, this feature can result in the amplification of the signal, representing an advantage of using IgY over IgG in immunoassays [63,77].

Several studies have shown promising results by using IgY in immunodiagnostics [77], for instance in immunoassays tests able to detect the circulating antigen of *Schistosoma japonicum* [89], which remains as the most prevalent parasitic infection in the world. The respective circulating antibodies tend to remain high for a considerable time after treatment, making it difficult to distinguish between past and current infections. The authors developed a new ELISA method capable to detect this antigen with better results than previous ones using the commonly applied IgG [89]. IgY antibodies can be also used in diagnosis in veterinary. Rangel et al. [90] successfully developed an assay using IgY capable of detecting proteins of *Pythium insidiosum* in affected horses in Brazil. By using a host with significant phylogenetic divergence like chickens, IgY demonstrated to act as a valuable tool in the detection of antigens of these pathogens [90].

Summing up, IgG has been the antibody of choice for immunoassays in diagnosis in humans and mammals [88]; however, the evolutionary distance between chicken and mammalian immunoglobulins and their structural differences in the Fc domain make of IgY a better alternative over IgG in this type of assays. Furthermore, chickens produce higher amounts of antibodies and it is not required the invasive step of blood collection [26,77].

1.3.2. IgY as prophylactic and therapeutic agents

Nowadays, one of the most serious threats to both mankind and the ecosystem is the uprising of antibiotic-resistant bacteria at alarming rates [91,92]. This is mostly due to the fact that antibiotics are ubiquitously found in our environment since they are extensively used in livestock farming and also in aquaculture to both prevent diseases and promote growth [30,92]. Although it has been forbidden to use them as "growth factors" in livestock production in Europe (since 2006) and also in some states of USA, they are still widely used in other countries [92]. Therefore, it is highly relevant to find effective alternatives to antibiotics [91]. As such, oral immunotherapy with specific antibodies, namely IgY, has been considered in the past years, and used as a feed additive aiming the elimination of specific pathogens and to improve animal growth [30,91]. Feed grade antibodies from egg yolks of immunized chickens have the advantage of being produced in high contents, and where eggs represent a low-cost source of polyclonal antibodies [93].

Oral administration of spray dried whole eggs or yolks has been used in the treatment of enteric diseases in veterinary medicine [30,94]. For instance, IgY has been produced against the porcine enterotoxigenic Escherichia coli (ETEC) in the attempt of protecting the pathogenic diarrhoea in piglets [30,91,95]. A passive protective effect of anti-ETEC IgY in cattle has also been successfully applied, whereas neonatal calves that had transient diarrhoea were fed with milk containing IgY, and an improved body weight gain was observed with 100% of survival [30]. Other uses of IgY in cattle include the prevention of respiratory diseases and mastitis. Dahlen et al. [96] found that an IgY preparation reduced morbidity and mortality in calves due to several bovine pathogens, such as mastitis-causing *Escherichia coli* and *Staphylococcus aureus* [30,96], and *Campylobacter* and *Clostridium* [95] [96]. Moreover, specific IgY for *Salmonella Enteritidis* (SE) and *Salmonella Typhimurium* (ST) has also been suggested in the passive immunization of several animals, such as calves, mice and also in poultry [30,95]. By feeding chickens with egg powder containing the SE-specific IgY it was found a decrease on the faecal shedding, faecal colonization, and in the number of Salmonella-contaminated eggs [30,97]. New-born calves where also protected from diarrhoea

induced by bovine rotavirus (BRV) by orally administering anti-BRV IgY [30,91].

Passive immunization has been applied in aquaculture, where infectious diseases represent significant economic losses [30]. Still, the number of reports regarding the application of IgY in aquaculture is more limited when compared to calves, goats, poultry, cats, dogs and humans [98]. Even so, IgY has shown to be effective against diseases like white spot disease (WSD) in shrimps and crayfishes, *Vibrio anguillarum* and *Yersinia ruckeri* in rainbow trout, *Aeromonas hydrophila* in polyploid gibel carps (*Carassius auratus gibelio*), and *Edwardsiellosis* in Japanese eel (*Anguilla japonica*) [98].

IgY has also been suggested for the treatment of *E. coli* infections in humans [30]. Like in other mammals, enterotoxic *Escherichia coli* (ETEC) is a frequent cause of diarrhoea in children, especially in developing countries, accounting for *circa* one million deaths *per* year [92]. Oral administration of anti-ETEC IgY allowed the successful treatment of gastrointestinal infections in animals [92], indicating that this strategy will be of value also for humans. Furthermore, IgY against adherence-associated proteins of ETEC reduced in vitro bacterial adherence in cultured cells, suggesting that they may have the same potential in human hosts [30]. Another potential application of IgY in human nutrition is for the prevention of *Helicobacter pylori* infection, being the common cause of gastritis and gastric ulcers [30]. Due the concurrent infection by this and the potential development of new antibiotic-resistant strains, the investigation into alternative treatment methods has driven the study of IgY against *H. pylori* that showed, together with lanzoprazol in human volunteers, good results [92].

In summary, in times of increasing antibiotic resistance, the oral administration of specific antibodies is an attractive approach against bacterial infections of the digestive system not only in animals, but in humans as well [99]. This approach is especially advantageous for the treatment of gastrointestinal or airways-related infections that are difficult to treat with traditional antibiotics [91]. IgY can be introduced in several daily products to improve human health in order to prevent or cure diseases, such as nose sprays, cosmetics, body lotions, functional foods like yogurts, nutraceutics or food supplements [99].

There are studies showing that antibodies are absorbed in the intestine of young piglets and calves (<48 h old), but only low levels of IgY can be detected in the circulation of those that are treated orally with IgY during the first 24-48 h *post natum* [100,101]. Like any other protein, orally administered antibodies are subjected to denaturation by the acidic pH of the stomach and degradation by proteases, such as pepsin, trypsin, chymotrypsin, carboxypeptidase and elastase [30,62,91]. Still, several studies have shown that part of the antibodies remain intact after full

digestion, and besides being subjected to a considerable cleavage into Fab, Fab'2 and Fc fragments, the fragments Fab'2 and Fab have yet the capability to bind to the antigen and exhibit neutralizing activity in the various segments of the gastrointestinal tract [91].

IgY can be also useful as a novel therapy against dental caries and periodontitis by preventing oral infections caused by *Streptococcus mutans* (caries' cause) or *Prevotella intermedia*, *Fusobacterium nucleatum* and *Porphyromonas gingivali* (gingivitis and halitosis' causing bacteria) [92]. It was shown that the mouth rinse containing IgY against *S. mutans* reduces the establishment of these bacteria in dental plaques of humans [92]. In Japan, chewing gums with anti-*S. mutans* IgY are commercially sold to prevent caries formation [92].

During the past decade, IgY has been reported as a feasible immunotherapeutic agent against pathogens that are difficult to treat with traditional antibiotics [102]. These biologic-based therapeutics are not only relevant for dealing with multiresistant bacteria, but are also of high value in passive immunization of immunocompromised individuals and for combating rare or emerging diseases [103], such as cystic fibrosis, Ebola, or pandemic influenza [17]. In fact, passive immunization has been used for long to prevent or to treat several infectious diseases, such as rabies, diphtheria, tetanus, hepatitis B, cytomegalovirus (CMV), respiratory syncytial virus (RSV) and botulism [17,103]. IgY has also been reported as an anti-venom for the treatment of stings and snakebites [17].

Throughout times, the best defence in humans, mammals and other vertebrates against all kind of infectious agents has been the immune system [92], and in the last decades, antibodies have become one of the leading classes of biotechnology-derived drugs in passive immunization [17]. Although hyperimmune serum is still the primarily used form of antibody therapeutics for prophylaxis and treatment of several infections, this approach has several disadvantages. There are significant side effects due to the inherent immune response against animal-derived antibodies. Furthermore, since these correspond to polyclonal antibodies mixtures containing undefined concentrations of multiple specific and non-specific antibodies, it is extremely difficult to standardize serum quality and to ensure the efficacy of these therapeutic serum products [17]. Therefore, a new form of antibody therapy using intravenous immunoglobulin (IVIG) has also been introduced. Contrarily to hyperimmune sera, IVIG is a preparation of human polyclonal antibodies pooled from a large number of healthy donors, being not enriched in pathogen-specific antibodies [17]. The described drawbacks also led researchers to explore the possibility of replacing polyclonal antibodies by monoclonal antibodies (mAbs) [103]. After the development of the hybridoma technology in 1975 (by the immortalization of murine B lymphocytes through fusion with a

myeloma cell) [104] it was possible to produce high-affinity specific mAbs [17]. After these findings, a high development on finding new methods for obtaining human mAbs and on the increase of their production has been observed. mAbs are being studied as alternative therapeutics for cancer, autoimmune diseases and other chronic and/or rare diseases [17,103]. Unfortunately, mAbs are relatively expensive to manufacture, require systemic administration and are only specific to a particular pathogen, being thus highly specific for passive immunization therapies [17]. Contrarily, polyclonal antibodies recognize more epitopes than mAbs, which makes it difficult for bacteria to avoid the less specific antibodies by mutations [91]. Hence, IgY can be seen as the ideal choice for passive immunization purposes. Still, clinical applications of IgY in humans are scarce [92], being predominantly used in oral administration for the treatment of localized infections [30]. Unlike the therapies for enteric diseases, therapeutic antibodies need to be administered systemically. Although systemic administration ideally requires antibodies of the same species in order to avoid anti-isotype immune reaction against the therapeutic agent, by using IgY this secondary effect is not expected to occur, being a relevant candidate to fight the increasing risk of novel antibiotic-resistant strains [105,106].

The increase in antibiotic-resistant bacteria, such as the MRSA (multiresistant *Staphylococcus aureus*), the ESBL (extended-spectrum β-lactamase)-producing bacteria and the VRE (vancomycin resistant enterococci) [92], has prompted the research into the application of IgY for the prevention and treatment of infections caused by these highly pathogenic bacteria as a potential alternative over antibiotics [49]. For instance, IgY against ESBL has been produced from immunized chickens with bacteria carrying ESBL genes and found to have specific binding capacity for those [92]. Nevertheless, despite the demand on finding an effective treatment for ESBL, MRSA and VRE infections, there is a lack of studies for these bacteria with IgY, and clinical studies on this subject are urgently needed [92]. Even so, IgY has found to be effective against a number of human pathogens and diseases, both in vitro and in laboratory animal studies and clinical settings [60,92]. Furthermore, one of the most successful clinical applications of IgY has been against infectious diseases in patients with cystic fibrosis (CF), namely to reduce the chronic infection by *Pseudomonas aeruginosa* (PA) [91,107]. Once a chronic infection has been established it is very difficult to eliminate it even by using antibiotics [92]. In order to prevent PA colonization and to reduce the need for antibiotics in CF patients, Carlander et al. [91] studied the benefits of IgY as a prophylactic tool against this infectious disease [92]. In 2008, an orphan drug designation was granted for IgY antibody against PA for the treatment of CF in humans by the European Medicines Agency, in which a mouth rinse containing purified anti-PA IgY is given on a continuous basis to reduce and prevent

PA colonization [91,92,100].

Another promising clinical application of IgY in humans is for the prevention of influenza virus, which still remains a major threat to global health due to its ability to undergo changes through antigenic drift and antigenic shift [92]. In a mouse model, IgY anti-H5N1 protected mice with an efficacy of 100% when administered by an intranasal way 1h prior to infection. Based on these reported preliminary results, it is plausible to admit that in the near future IgY-Influenza can be used in the fight against this pandemic problem [92].

IgY can also be used in Crohn's disease or ulcerative colitis, two chronic inflammatory bowel diseases that increase the burden of hospitals in terms of medication costs [95,108]. The use of antibiotics in these diseases is limited by side effects, immunosuppression, and incomplete efficacy; therefore, alternative therapies have been investigated [95]. Immunotherapy using monoclonal mouse antibodies directed against tumour necrosis factor (TNF) has been approved for use. However, it can be costly and adverse side effects have been reported [95,109]. More recently, anti-TNF antibodies produced in chickens were able to effectively treat acute and chronic phases of colitis in rats [110], and were also found to neutralize the human TNF in vitro, indicating its possible use in human inflammatory bowel disease in the near future [95].

In summary, due to the availability and widespread use of nonspecific antibacterial therapies with broad spectrum, namely antibiotics, the multiresistant bacteria phenomenon is nowadays a serious concern [111]. Nonspecific antibacterial and antifungal therapies also damage the human microbial flora, which is essential for the development of the immune system and to protect hosts by niche-denial to more pathogenic microbes [111]. Hence, the development of novel therapies with pathogen-specificity and less adverse effects, like antibodies, is mandatory. In this field, egg yolk antibodies represent a viable alternative.

1.4. Production of IgY

The production of IgY antibodies was named as “IgY Technology” in 1995 [31], being nowadays the internationally accepted term for describing the production and use of this type of antibodies [59]. The European Centre for the Validation of Alternative Methods (ECVAM) strongly recommends that avian antibodies should be used as an alternative to mammalian Abs, specially due to the animal welfare reasons [59,61]. Avian immunoglobulins will be soon accepted as a viable alternative over IgG, particularly taking into account the previously described applications and advantages [27]. Even though, it is still required to prompt the IgY technology and to convince the scientific and medical community of these antibodies advantages [42]. Once accepted and widely used, the IgY

technology will offer alternatives and solutions to science, medicine and society. However, from a realistic point of view, it must be disclosed that IgY Abs will not be able to completely replace IgG in the near future [42]. Their widespread use is also limited from difficulties on their purification from the complex egg yolk matrix.

The use of specific pathogen free (SPF) chickens are mandatory when the produced antibodies are to be used for therapeutic purposes [61]. Different variables could influence the immune response of the immunized chickens, namely the antigen (dose and MW), the type of adjuvant used, the route of immunization, and the chicken itself (age, breed, egg laying capacity, etc.) [42]. Different immunization protocols have been studied in order to improve IgY production [59]. Usually, 10-100 µg of antigen in a volume of 1 mL are administered intramuscularly in the breast muscle at two or three injection sites of a 7 to 8 week-old chicken [59]. Nevertheless, different immunization protocols for each antigen and for each animal have to be tested to find out which method induces the highest antibody titer [59]. Oil-based adjuvants, such as Freund's incomplete adjuvant (FIA), could be used to increase the immune response and also to avoid an eventual local tissue reaction, while the vaccination frequency and interval depend on the immunogenic potential of the antigen itself and on the adjuvant used [59,112]. It is recommended to immunize chickens before they begin to produce eggs since the stress induced by immunization could decrease their laying performance [61]. The number of immunizations is also variable; for chickens it is common at least 2 injections, where the presence of antibodies should be checked two weeks after the second immunization. When the antibody titer decreases, booster immunizations can be given during the whole laying period [59]. For additional protocols and guidelines there is a detailed report with a wide-broad recommendations from the workshop organized by ECVAM on "The Production of Avian Antibodies" in 1996 [61].

One of the advantages of using chickens instead of other mammals is related with the antibody productivity and the long-lasting high titers. An hen lays five to six eggs *per* week, with an average volume of 15 mL of egg yolk containing 50-150 mg of IgY, of which 2 to 10% are specific antibodies [49,59]. When a chicken is immunized against a microbe, only around 5%-10% of the IgY will react to this microbe [92]. Even so, Gottstein and Hemmeler [70] demonstrated that over a period of slightly less than 6 weeks, 298 mg of a specific IgY against *Echinococcus granulosus* was obtained from eggs, compared to only 16.6 mg from the rabbit's blood [49,70]. Moreover, chickens usually lay about 280 eggs *per* year, corresponding to 14-42 g (30 g in average) of IgY *per* year *per* chicken [49,61,92]. At least 10 rabbits (or a larger mammal) are needed to obtain the same amount of antibodies *per* year [49,61,92].

Although chicken eggs represent an ideal alternative antibody source, the production cost of high-quality IgY for large-scale applications still remains higher than that of other drug therapies, such as common antibiotics [30,113]. This is mainly due to difficulties in the extraction and isolation of IgY from a complex matrix such as the egg yolk. The separation of IgY at least involves two steps, one where the yolk lipoproteins are removed allowing the recovery of the water-soluble protein fraction (WSPF) containing IgY, followed by the use of this fraction in a subsequent or multiple steps to purify the target antibody [114]. One of the major challenges in IgY purification is to efficiently separate the WSPF from the yolk lipoproteins in the first step [30,115]. Furthermore, the usual employed methods for the isolation of IgY from the WSPF are highly expensive, in which chromatography and ultrafiltration are commonly applied. The purification of IgY is not only required to use this antibody in significant doses in a regular basis by patients, but also due to the prevalence of individuals with allergies to egg proteins [49]. A critical overview and validation of the methods reported for the purification of IgY is described in Chapter 2. In summary, the production of low-cost IgY biopharmaceuticals is a mandatory challenge to allow their use in passive immunization therapies [30].

1.5. Scope and objectives

Antibodies are amongst the top selling biopharmaceuticals and are of high relevance in health care. Although IgG-based products are still the most focused antibodies, they display some disadvantages, namely low production yields and cross reactivity with human antibodies or proteins. In the last decades, some efforts have been placed on demonstrating the relevance of IgY, as a promising alternative therapeutic option. IgY can be obtained in higher titters by less stressful and less invasive methods when compared to mammals' polyclonal antibodies, while also avoiding cross reactivity effects in humans. Although several approaches have been formerly developed to purify IgY from egg yolk, the developed purification processes are still of high cost and difficult to be scaled-up. In this context, this PhD thesis focuses on the development of new and cost-effective strategies for the purification of IgY from egg yolk using two types of approaches: liquid-liquid extractions using aqueous biphasic systems (ABS) and three-phase partitioning systems (TPP). Accordingly, this thesis comprises the following topics:

(i) critical evaluation of the purification methodologies reported up to date in order to address which factors and conditions give rise to high purification levels and yields of IgY;

(ii) investigation of liquid-liquid strategies based on ABS aiming the selective migration of IgY for one of the phases;

(iii) investigation of TPP systems as alternative strategies to purify IgY by selectively inducing its precipitation;

(iv) evaluation of the commercial potential of IgY and preparation of a preliminary business plan.

A schematic representation of the current thesis is given in Figure 4.

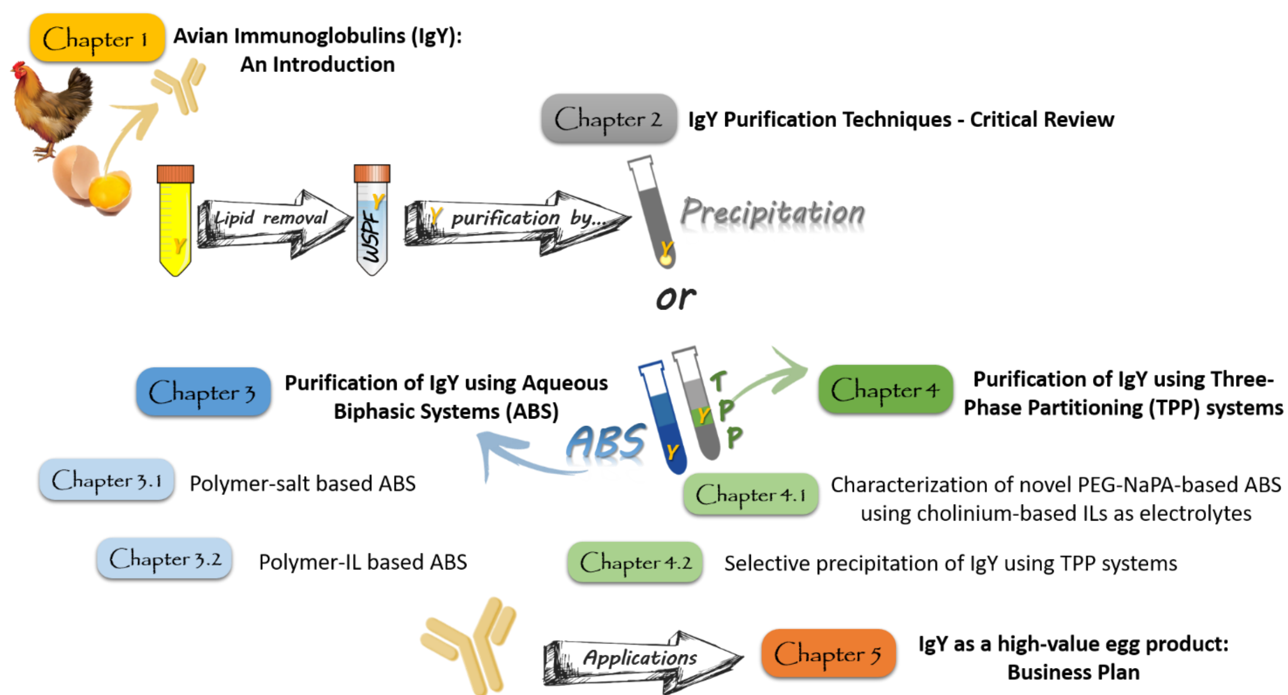

Figure 4. Schematic illustration of the thesis layout.

Chapter 1 includes a brief introduction and description of the main characteristics of the protein of interest, immunoglobulin Y (IgY), such as molecular properties, stability, comparison with the mammalian IgG, main applications, and current difficulties in purification. In Chapter 2, it is provided a comprehensive and critical analysis of the reported purification processes for IgY, in which most of the reported methods were tested by us to appraise on their performance using the same raw materials. In general, the purification of IgY inherently requires (at least up to date and given the results obtained) the use of at least a two-step approach: (i) preparation of a water-soluble protein fraction (WSPF) containing IgY and other water-soluble proteins by the removal of lipoproteins from egg yolk; and (ii) purification of IgY from the WSPF. Afterwards, two types of purification platforms have been investigated: liquid-liquid extractions using aqueous biphasic systems (ABS) – Chapter 3, and IgY selective purification by induced precipitation using three-phase partitioning (TPP) systems – Chapter 4. Concerning the ABS-based purification platform, we have first studied several polymer-

salt ABS – Chapter 3.1 – using more benign organic salts, such as sodium citrate, instead of the commonly used polymer-phosphate-based ABS. In Chapter 3.2, cholinium-based ionic liquids (ILs) were investigated as more biocompatible phase-forming compounds for the purification of IgY from the prepared WSPF solution, and the results were compared with the previously studied polymer-salt based ABS. Instead of using the conventional polymer-polymer (mainly polyethylene glycol(PEG)-dextran) ABS, which are highly viscous and present a limited polarity range and poor selectivity, we investigated a different class of polymer-based systems, composed of PEG and sodium polyacrylate (NaPA) – Chapter 4. PEG-NaPA ABS commonly require the addition of electrolytes for phase splitting, and in this work, we tested the ability of cholinium-based ILs for such a purpose and have studied the underlying mechanisms of ABS formation – Chapter 4.1. Additionally, we studied the potential of these PEG-NaPA ABS using cholinium-based ILs as electrolytes for the purification of IgY by means of TPP, where the IgY was purified by inducing its selective purification at the interphase of these systems – Chapter 4.2. The best results were obtained with TPP systems, in which IgY with a purity higher than 90% was obtained from the egg yolk WSPF. Finally, a preliminary business plan regarding the commercialization of IgY was prepared, providing information on the market, product competitors and a value-proposition – Chapter 5.

2. IgY Purification techniques: Critical Overview

This chapter is based on:

Sandra C. Bernardo, Mafalda R. Almeida, Pedro Domingues, João A. P. Coutinho and Mara G. Freire

"Critical analysis on the available techniques for the purification of Immunoglobulin Y (IgY)", manuscript under preparation[1]*.*

2.1. Abstract

Immunoglobulins from egg yolk (immunoglobulin Y, IgY) have been proposed as viable alternatives to mammal antibodies, namely immunoglobulin G (IgG), within passive immunization. IgY can be obtained in higher titers and by less stressful and less invasive methods than that applied to polyclonal IgG, having therefore an outstanding potential to reach a central place in the biopharmaceuticals market. Nevertheless, the cost of producing high-quality/high-purity IgY, particularly for large-scale applications, still remains higher than other drug therapies due to the lack of current efficient purification processes. In this context, efforts have been carried out in order to purify IgY from egg yolk. Several procedures have been reported, yet corresponding to isolated works, resulting in the lack of a critical analysis on their comparative performance. In this work, the reported procedures for the purification of IgY were experimentally addressed, and the IgY was identified and quantified by size exclusion high performance liquid chromatography (SE-HPLC) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The performance of two commercial kits to purify IgY was also addressed. This experimental part allowed us to provide a critical analysis on the reported methods in terms of purification levels and yields of IgY. An overview on the several approaches and their feasibility, helpful for defining the correct pathway for future developments in IgY purification, is presented. Two steps are usually applied, the first to remove lipids and lipoproteins from egg yolk to obtain the water-soluble protein fraction (WSPF) containing IgY, followed by a second step in which IgY is purified from this fraction. The best identified protocol to recover the WSPF corresponds to the dilution of egg yolk (8-fold), followed by freezing/thawing and centrifugation. Concerning the IgY purification from the WSPF, the method using sodium chloride to induce the selective precipitation of IgY was identified as the most efficient in terms of purity and recovery yield.

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[1]**Contributions:** M.G.F. conceived and directed this work. S.C.B. performed the most part of the experimental work. The manuscript was mainly written by S.C.B., with significant contributions from the remaining authors.

2.2. Introduction

Immunoglobulins (Igs) or antibodies are glycoproteins found in plasma and extracellular fluids of all vertebrate species, being a key response mechanism used by the host organism for its protection against pathogens or foreign molecules or microorganisms [8,9]. In mammals, there are five classes of Igs (IgG, IgM, IgA, IgD and IgE), whereas in hens there are three classes (IgY, IgM and IgA) [9,30]. The general features of the major avian antibody (IgY) and its functional equivalent found in mammals (IgG) are described in more detail in the introduction of this thesis (sub-chapter 1.2).

Polyclonal antibodies are usually isolated from the serum of several animals, such as rabbits, goats and sheep, ideally super immunized with an aimed antigen [49]. However, the use of chickens for the production of specific polyclonal antibodies affords several advantages (see Chapter 1.2.2) [49]. As IgY can be recovered by a less invasive method, such as collecting eggs, the pain caused to animals is minimized [8,69]. The use of chickens is also a more straightforward method for polyclonal antibodies production due to the availability of a large number of chicken farms and automation of egg breaking and processing [69]. Therefore, eggs can be considered as a less expensive and more ethical source for the large-scale production of polyclonal antibodies [56]. Additionally, few eggs *per* week can provide the same amount of immunoglobulins obtained by repeated bleeding of immunized mammals. More than 100 mg of IgY can be isolated *per* egg [69]. Jensenius et al. [71] estimated that the total antibody activity obtained from eggs laid by a hen in a month is equivalent to that produced in a half litre of serum from an immunized rabbit. Gottstein and Hemmeler [70] reported that the quantity of IgY obtained from eggs laid by an immunized hen is 18 times higher than that of IgG isolated from rabbit serum. Kim et al. [69] 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, demonstrating that the productivity of IgY exceeded by far the productivity of IgG [69].

Antibodies as a class of biopharmaceuticals, such as IgG or IgY, can be used in passive immunotherapy, in immunoassays, and other applications (see Chapter 1.3). Still, the major shortcoming on the large-scale application of IgY and its adoption by biopharmaceutical companies results from the lack of current cost-effective techniques able to purify IgY from the complex egg yolk matrix [30,113]. Several purification methods for IgY were described in the last two decades; nevertheless, most of them are isolated works from different authors and there are few comparative studies. Moreover, most of the authors did not provide quantification-related parameters (such as yield and purity of IgY) needed to perform an adequate comparison. Most of the published works

are based on a qualitative analysis carried out by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Herein, an intensive comparison on the performance of the most relevant purification methods reported for IgY was carried out. These methods were reproduced and tested by us and the identification and quantification of IgY was carried out by SEC-HPLC (size exclusion high-performance liquid chromatography) and SDS-PAGE, allowing to determine the yield and purification level of IgY. The overall results allowed us to provide a critical analysis on the reported purification methods and to provide insights on novel techniques to be developed in the future for an adequate purification of IgY from egg yolk by cost-effective strategies.

2.3. IgY Purification techniques

The difficulties in IgY purification arise from the fact that egg yolk is a very complex matrix. Egg yolk is mainly composed of lipids, proteins, carbohydrates and minerals, whereas the major constituents of its solid matter are lipids and proteins, in a ratio of 2 to 1, respectively [49,52,55]. All methods reported up to date are based on at least two steps (Figure 5), where in the first step the lipids and lipoproteins are removed, followed by the isolation of IgY from the recovered water-soluble proteins fraction (WSPF). The current main problems in isolating IgY from egg yolk arise from both the first step comprising the separation/removal of lipids/lipoproteins from egg yolk and on the absence of selective methods to separate IgY from the remaining proteins in the WSPF [49].

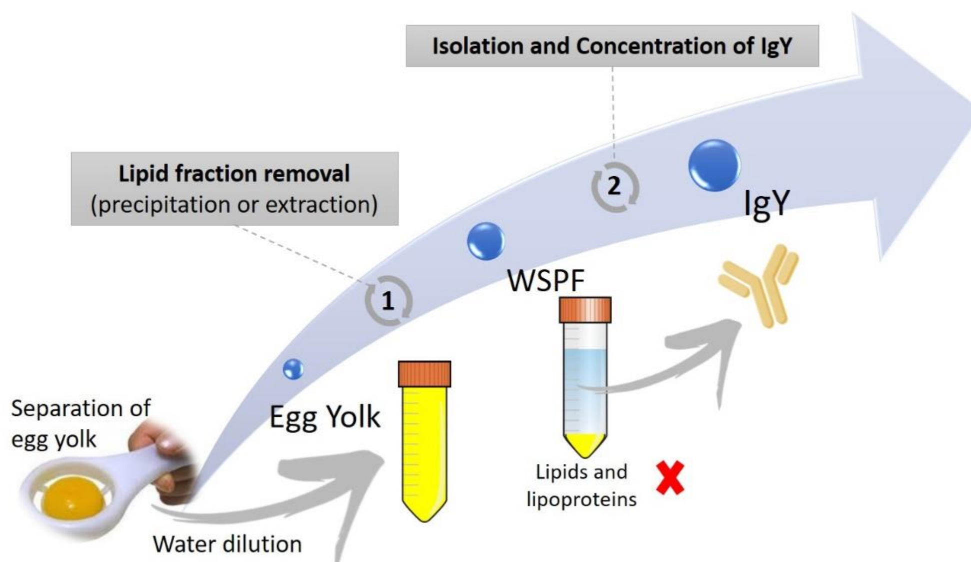

Figure 5. Schematic representation of the two-step process required to purify IgY purification from egg yolk. These methods are in general divided into two major steps: (1) removal of lipids and lipoproteins from egg yolk to obtain the water-soluble proteins fraction (WSPF) containing IgY; and (2) isolation of immunoglobulins from the remaining water-soluble proteins present in the WSPF.

The methods reported in the literature for the purification of IgY from egg yolk can be divided into three main groups, namely: (i) precipitation methods, involving simple water dilution, and the addition of salts [116], organic solvents [117] or polymers, such as polyethylene glycol (PEG) [118] or natural gums [66,119]; (ii) chromatographic methods, namely ion exchange [120–122], gel-filtration [120], hydrophobic interaction [123,124] and affinity chromatography [117]; and (iii) ultrafiltration [120,125,126]. In this work, only the performance of the precipitation methods was addressed since these correspond to the majority of the published works (from 70% to 80% of the total published methods), and also correspond to the most cost-effective processes reported. A technique reporting on the use of chloroform [117] for the removal of lipids and lipoproteins by means of extraction and creation of three phase systems (TPP) is also included for comparison purposes. In order to remove the lipids fraction from the WSPF (Step 1) and to further purify IgY from the WSPF (Step 2), several approaches have been proposed in the literature. These methods, which are described below, were experimentally reproduced and analysed by us, allowing to provide a critical analysis on the IgY purity and yield, and thus on these purification techniques performance.

2.3.1. Precipitation (and extraction) of lipids and lipoproteins from egg yolk to recover the WSPF

The best methods described in the literature to achieve an effective removal of lipids and lipoproteins and to recover the WSPF containing the target antibody are water dilution, followed or not by freezing/thawing, addition of PEG and natural gums, and chloroform extraction [42,117,118,120,127–132]. These methods are described below. In this section a total of 10 manuscripts was analysed.

2.3.1.1. Water dilution (WD) and freezing/thawing (FT)

A simple and frequently used procedure to remove lipids and lipoproteins from egg yolk consists on successive water dilution (WD) steps, as proposed by Akita and Nakai in 1992 [120]. The water dilution of egg yolk leads to the aggregation of lipoproteins since this phenomenon is promoted at a low ionic strength [49]. Afterwards, the lipids fraction is removed by low speed centrifugation. For these methods, the optimization of the operating conditions, such as pH, temperature, dilution factor, etc., was previously reported [120,127]. Nevertheless, different authors accomplished different results and have different perspectives. While Akita and Nakai [120] reported that the best results are obtained using a ten-fold water dilution at pH 5.0, Luo et al. [132] reported that the enhanced results are obtained with a 15-fold water dilution step. Furthermore, Ahn and co-workers

[127] concluded that increasing the dilution factor more than 10 times facilitates the precipitation of the lipids; yet, higher dilutions also turn difficult the additional purification steps. Freezing and thawing (FT) of the diluted yolk results in the formation of lipid aggregates as well [131]. It was latter demonstrated that by applying FT it is possible to use only 6- to 8-fold dilutions of the egg yolk in water instead of the commonly used 10-fold [131]. Based on the exposed information, and only for the water dilution methods, it is clear that there is some controversy in the literature in what concerns the best strategy to remove lipids and lipoproteins from egg yolk in order to obtain the WSPF that contains the target antibody. Furthermore, these methods should be analysed considering both the IgY yield and purity achieved in this first step.

2.3.1.2. **Precipitation with polyethylene glycol (PEG) and natural gums**

Polson and co-workers [118] proposed, in 1985, the precipitation of lipids and lipoproteins by the addition of polymers, namely PEG. This approach has been described as the most effective and is the most used protocol by the research community working with IgY since it is of low-cost, fast, and easy to perform [42]. The authors demonstrated that a concentration of 3.5 wt% of the polymer with a molecular weight of 6000 $g.mol^{-1}$ (PEG 6000) allows the separation of the lipids from the livetin fraction [118]. This method was also previously compared with the WD-induced precipitation method by other authors [128,130]. Akita and Nakai [128] found that the WD method is superior based on the yield and purity of the IgY obtained. In the same line, Deignan et al. [130] compared the PEG precipitation method with the FT method, in which they demonstrated that the former has a higher performance in terms of IgY purity. Besides a comprehensive comparison between the different methods, the analysis of the effect of the molecular weight of PEG used in the precipitation step is still missing and was additionally addressed in this work.

Hatta and co-workers [66] investigated the effect of several natural gums on the precipitation of yolk lipoproteins. The authors [66] concluded that the addition of λ-carrageenan leads to a complete removal of the lipids from the WSPF. Similar results were obtained with the addition of xanthan gum and some other carrageenan, while furcellaran, arabic gum and sodium alginate were shown to be less effective [66]. The authors concluded that this method is cost-effective and leads to a high purity (~80 %) of chicken IgY from egg yolk after additional precipitations steps [66]. Later, similar results were achieved by Tan et al. [72], which described a method comprising a mixture of natural gums containing 0.06% of κ-carrageenan and 0.18% of low-methoxyl pectin in presence of $CaCl_2$.

2.3.1.3. **Chloroform extraction**

The removal of the lipids fraction from egg yolk is also possible using organic solvents, such as propan-2-ol, acetone and chloroform [117,133]. Bade and Stegemann [133] described a method where the yolk proteins were first precipitated by cold propane-2-ol, and then the lipoproteins and the yolk lipids were removed by repeated washing with propane-2-ol and acetone. The resulting solution contains ca. 10 proteins, meaning that this method is not efficient and further purification is still needed [133]. One of the first works where chloroform was employed was reported by Ntakarutimana et al. [117], in which three phases were obtained: an orange organic phase at the bottom, a yellow semi-solid phase at the interface, and an aqueous phase at the top. The organic phase contains the lipids, while the aqueous phase contains the WSPF. The middle semi-solid phase is a mixture of the two phases that can be diluted and centrifuged once more to achieve higher separation efficiencies of the lipids [117]. Bizhanov and Vyshniauskis [129] compared the chloroform method with the PEG 6000 precipitation approach described by Polson and co-workers [118]. They found that by using the chloroform approach, the supernatant obtained (WSPF) contains more proteins, including IgY. However, the purity of IgY was lower than that obtained by the PEG approach [129]. In general, the main proteins identified in the recovered WSPF correspond to α-livetin, i.e. chicken serum albumin (65–70 kDa) [134], β-livetin also known as α-2-glycoprotein (45 kDa) [135], and γ-livetin that corresponds to IgY [49]. A less purified WSPF still presents low-density lipoproteins (LDLs), which have higher molecular weights than the previously identified ones [54,136].

2.3.2. Purification of IgY from the WSPF

The purity of IgY in the WSPF samples after step 1 is still low (ranging between 19.5% [66] and 32% [128] according to several authors); hence, when envisaging the use of IgY as a target biopharmaceutical, a further purification step is still required. The purification of IgY from the WSPF in subsequent steps has been carried out by precipitation with several salts and PEG [116,128,131], as described below. The attempt has always been to induce the selective precipitation of IgY instead of inducing the precipitation of the remaining water-soluble proteins.

2.3.2.1. **Salts precipitation**

Akita and Nakai [128] proposed the selective precipitation and recovery of IgY from the WSPF (obtained with the WD method) using sodium sulphate. The authors were able to recover 83% of the IgY present in egg yolk with a purity of 82% [128]. In 2004, Bizhanov et al. [116] described a

modification to this protocol, in which they used other salts instead of sodium sulphate, namely ammonium sulphate, sodium citrate and lithium sulphate. Using sodium citrate and lithium sulphate, higher purification factors were achieved with more than 90% of IgY purity and about 80% of recovery yield being reported [116]. In all situations, concentrations of salts ranging from 14 to 35 wt% were employed, and studies were conducted at room temperature. Recently, Hodek et al. [131] reported a new method, in which after the first precipitation step carried out with the FT dilution method, the authors used sodium chloride (NaCl) with a concentration of 8.8% (w/v) to precipitate IgY, at pH 4 [131]. Using this procedure, highly purified IgY antibodies (97%) with 89% of recovery yield were obtained as described by the authors. The authors [131] optimized both the pH and concentration of salt for the IgY precipitation. However, the range of NaCl concentration studied was narrow, only up to 10 % (w/v), and it is not clear if higher concentrations of this salt could lead to better results.

2.3.2.2. **PEG precipitation**

Akita and Nakai [128] were the first authors comparing the efficacy of the IgY precipitation from the WSPF using PEG or a salt (sodium sulphate). Their results suggest that PEG is more efficient to induce the selective precipitation of IgY [128]. However, the salt used is not amongst the best ones as compared to other salts discussed above [116,131]. More recently, Bizhanov et al. [116] compared the performance of sodium citrate with the PEG 6000 precipitation method and found that the IgY purification by PEG results in a significantly lower yield of total proteins, as well as of IgY. Furthermore, the yield of IgY by the PEG precipitation method (4.4 mg/mL egg yolk) is amongst the lowest ones, by at least 50% when compared with other precipitation agents [116]. Nevertheless, these authors also reported that using PEG as a precipitation agent, an IgY precipitate with 95.6 % of purity is obtained [116], which could be beneficial since eggs correspond to low-cost materials. Additionally, the effectiveness of the IgY precipitation using PEG as reference was compared with the sodium chloride method developed by Hodek et al. [131], in which the new protocol (using NaCl) results in at least a 24% higher IgY yield. In summary, the lower recovery of IgY precipitated using PEG seems to be one of the major disadvantages of this approach. Still, there is a lack on the study of a wide range of salts and their concentrations. These comparisons on the precipitating agents' performance also should be carried out using the same samples and with similar analytical techniques to address the IgY purity and yield.

2.4. Experimental procedure

Materials. Fresh eggs were obtained from a local market and were stored at 4 °C for 2 to 4 weeks. Chicken IgY isotopic control was purchased from Gallus Immunotech Inc. (Cary, United States of America). The chemicals investigated as precipitation agents were chloroform, polyethylene glycol (PEG) with the molecular weights of 600, 2000, 6000 and 10000 $g \cdot mol^{-1}$ (PEG 600, PEG 2000, PEG 6000 and PEG 10000), sodium chloride (99.5% pure) and sodium citrate (99% pure), all purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, pH 7.4) was used for IgY dilution procedures, and was acquired from Sigma-Aldrich, in which one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4.

Isolation of Egg Yolk. The eggshell was carefully broken, and the egg yolk was separated from the egg white, using a "yolk spoon" in order to remove as much egg white as possible. The yolk was then carefully transferred to a filter paper and rolled to remove the remaining adhering egg white. The egg yolk was positioned near the edge of one side of the paper towel and the yolk skin was cut with a lancet and transferred into a graduated cylinder in order to register the yolk's volume. The methods used to recover the WSPF (step 1) and to isolate IgY from this fraction (step 2) are described below.

Water dilution method (according to Ahn et al. [127]***).*** Egg yolk was diluted 10 times (v/v) with distilled water and thoroughly mixed. Solutions of HCl and NaOH (0.5 M) were used in order to adjust the pH of the mixture, at 3 pH values (5, 6 and 7). The mixture was incubated overnight at 4 °C and centrifuged at 4500 rpm for 30 min. The pellet (lipids fraction) was discarded and the supernatant was filtered through a cotton wool plug placed in a funnel. The supernatant that corresponds to the WSPF and contains IgY was used in the analysis of the IgY yield and purity. This fraction can be stored at -8 °C for a long period or at 4 °C for 2 to 3 weeks, as reported in the literature [117].

Freezing & Thawing method (according to Hodek et al. [131]***).*** Egg yolk was diluted 7 times (v/v) with distilled water and thoroughly mixed. As described before, solutions of HCl and NaOH (0.5 M) were used in order to adjust the pH of the mixture, at 3 pH values (5, 6 and 7). The mixture was frozen overnight at -8 °C and after thawing, the aggregated egg yolk granules were obtained as an isolated precipitate by centrifugation at 4500 rpm for 45 min. The pellet (lipids fraction) was discarded and the supernatant was filtered through a cotton wool plug. This supernatant corresponds to the WSPF and was used analysed and used in further purification steps.

Chloroform Extraction method (according to Ntakarutimana et al. [117]***).*** Egg yolk was diluted in PBS aqueous solutions: 15 mL of yolk + 40 mL of aqueous solution of PBS. This mixture was homogenized for 10 min, and 40 mL of chloroform were added under continuous stirring. Afterwards, the mixture was incubated at 4 °C for 24 h. By centrifugation at 4000 rpm for 30 rpm at 4 °C, three phases of similar volumes are obtained: an orange bottom organic phase, a semi-solid middle phase, and an aqueous top phase, with the last one named as supernatant A. The organic phase that contains the lipids and lipoproteins was discarded, the supernatant A stored, and the semi-solid phase was diluted in 40 mL of PBS to extract all residual proteins. After an additional centrifugation step at 4000 rpm for 30 min at 4 °C, the obtained supernatant (supernatant B) was mixed with supernatant A. This mixture of supernatants (supernatants A+B) corresponds to the WSPF analysed and studied in this work.

PEG precipitation method (according to Polson et al. [118]***).*** Egg yolk was diluted 2 times (v/v) with aqueous solutions of PBS and mixed thoroughly. 3.5% (w/v) of PEG 6000 was added, and the mixture was vortexed for 10 min. Then, the mixture was centrifuged at 4500 rpm for 45 min at 4 °C, leading to the separation of the "yolk solids and fatty substances" (original quotation of Polson et al. [137]) from the water-rich phase containing IgY and other water-soluble proteins. The supernatant was poured through a cotton wool plug placed in a funnel and transferred to a new vial. This supernatant corresponds to the WSPF obtained through this method and was analysed in further purification steps of IgY.

Natural Gums Precipitation method (according to Hatta et al. [66]***).*** Egg yolk was diluted 5 times (v/v) with distilled water and vigorously mixed. 60 mg of natural gums (arabic gum or xanthan gum) *per* 10 mL of original yolk were added and mixed until each gum was completely dissolved. The mixture was incubated at room temperature for 30 min and centrifuged at 5000 rpm for 25 min at room temperature. The pellet was discarded, and the supernatant was filtered through a cotton wool filter. The supernatant obtained corresponds to the WSPF used.

Induced precipitation of IgY from the WSPF using the PEG precipitation method (according to Polson et al. [118]***).*** The WSPF was subjected to a PEG induced precipitation step with 8.5% (w/v) of PEG 6000, and vortexed for 10 min to induce the selective precipitation of IgY. The mixture was centrifuged at 4500 rpm for 45 min at 4 °C. The supernatant was discarded, and the pellet was carefully dissolved in 1 mL of a PBS aqueous solution. PBS was then added to a final volume of 10 mL and the solution was then mixed thoroughly with 12% (w/v) of PEG 6000 and vortexed for 10 min. The mixture was centrifuged at 4500 rpm for 45 min at 4 °C and the supernatant was discarded. The pellet obtained is enriched in IgY, and was dissolved in PBS up to 5 mL and used in the analysis

of IgY yield and purity. In this work, we additionally studied the effect of the molecular weight of PEG, namely with PEG 600, 2000 and 10000, in which a similar protocol was applied.

Induced precipitation of IgY from the WSPF using ammonium sulphate (according to Luo et al. [132]***).*** 35 % (w/v) of ammonium sulphate [$(NH_4)_2SO_4$] was added to the previously prepared WSPF and the mixture was stirred and incubated for 2 h at 4 °C. After settling for 30 min, the solution was centrifuged at 3000 rpm for 30 min at 4 °C, and the supernatant was discarded. The pellet is enriched in IgY, which can be dissolved in PBS (5 mL) and stored at 4 °C for further analysis.

Induced precipitation of IgY from the WSPF using Sodium Citrate (according to Bizhanov et al. [116]***).*** To the previously prepared WSPF, 34% (w/v) of sodium citrate was added and mixed thoroughly. The mixture was incubated at 4 °C for 2h. Afterwards, the mixture was centrifuged at 5000 rpm for 30 min at 10 °C and the supernatant was discarded. The pellet recovered contains IgY and was dissolved in PBS (5 mL) and stored at 4 °C for further analysis. In order to study the effect of the concentration of the salt, a wider range of concentrations compared to those published was investigated by us (the additional concentrations of 20, 30, 40 and 50% (w/v) of sodium citrate were investigated by us). In order to study the effect of the pH, we also studied a vaster range of pH values. To this end, the WSPF was adjusted to pH 4, 5, 6, 7 and 8 with HCl aqueous solutions (0.5 M).

Induced precipitation of IgY from the WSPF using Sodium Chloride (according to Hodek et al. [131]***).*** 8.8 % (w/v) of sodium chloride was added to the WSPF, and the pH was adjusted to 4 using HCl aqueous solutions (0.5 M). After mixing, the mixture was incubated at room temperature for 2h under moderate agitation. Afterwards, the mixture was centrifuged at 5000 rpm for 30 min at 10 °C and the supernatant was discarded. The obtained pellet was dissolved in 5 mL of PBS aqueous solutions and stored at 4 °C. Aiming at better understanding the salt concentration effect, we also studied the addition of 10, 15, 20, 25, 30 and 35% (w/v) of NaCl to the WSPF, following a similar procedure to that described for 8.8% (w/v) of NaCl.

pH measurements. All the pH values (± 0.02) were measured at 25 °C (± 1 °C) using a SevenMultiTM (Mettler Toledo Instruments). The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The protein profile of the samples obtained in steps 1 and 2 described above was appraised by SDS-PAGE using an Amersham ECLTM Gel from GE Healthcare Life Sciences. The samples were directly mixed with the Laemmli buffer (1:1, v:v) in presence or absence of the reducing agent, dithiothreitol (DTT), and then heated at 90 °C for 5 min for a complete denaturation of the proteins, and then subjected to

SDS-PAGE in 20 % polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue G-250 for 2-3 h, and then distained with a mixture of methanol, acetic acid and distilled water (20:7:73, (v:v:v)) at room temperature. The molecular weight marker used was the commercial Amersham ECLTM Full-Range Rainbow Molecular Weight Marker, with a size range from 12 to 225 kDa.

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). The identification and quantification of IgY and remaining proteins in the samples obtained by the best studied methods was performed by SE-HPLC. A Chromaster HPLC (VWR, Hitachi) coupled with a diode array detector was employed. SE-HPLC was performed on an analytical column (25 cm × 2 mm i.d., 25 µm), Shodex Protein KW-802.5. Before injection, each sample solution was diluted 10 or 5 times (v:v) in the mobile phase buffer (100 mM phosphate, 150 mM NaCl, at pH 7). The mobile phase was run isocratically with a flow rate of 0.5 mL/min. The column oven and autosampler were kept at constant temperatures of 25 and 10 °C, respectively. The injection volume was 25 µL and each sample was injected at least 2 times. At least two replicates for each series of experiments were performed. A range of wavelengths (200-400 nm) was analysed and the results were integrated at the wavelength of maximum absorption (280 nm). The retention time of IgY was found to be 15.3 min within an analysis time of 45 min. The quantification of the IgY was carried out by an external standard calibration method in the range from 0.1 to 1 g/L. Pure and commercial IgY was used to establish the calibration curve. The treatment of the experimental data was performed by the Origin Software, where the baselines of chromatograms were refined, and the areas of each peak were integrated. The content of IgY and total proteins (mg) in each sample and *per* mg of yolk was calculated. The purity of IgY (*Purity, %*) was determined by the ratio between the amount of IgY ($mass_{IgY}$) and the total amount of proteins ($mass_{protein}$) in the sample, defined according to Equation 1,

$$Purity\ (\%) = \frac{mass_{IgY}\ (mg)}{mass_{protein}\ (mg)} \times 100 \qquad \text{(Equation 1)}$$

2.5. Results and discussion

2.5.1. Comprehensive analysis on the removal of lipids and lipoproteins and recovery of the WSPF (step 1)

The results reported in the literature in terms of IgY purity and the best conditions found are given in Table 3. Although some purity values of IgY have been given by some authors, most of

these studies only addressed the determination of the purity level after the second step (or further steps) of purification. Below we provide and discuss the results obtained by us by applying the several methods described in the literature, and by exploring additional experimental conditions.

Table 3. Summary of the methods and best identified conditions reported in the literature regarding the lipids and lipoproteins removal and WSPF recovery.

Method	Best Conditions		Purity	Reference
Water dilution	15-fold	No pH adjustment	<20%	Luo et al., 2010
	10-fold	pH 5	18%	Akita and Nakai, 1992
Freezing and Thawing	6- to 8-fold	pH 5	-	Hodek et al., 2013
Polyethylene glycol (PEG) precipitation	3.5 wt % of PEG 6000 g·mol^{-1}		-	Polson et al., 1985
Natural gums precipitation	0.1% of λ-carrageenan		19.4%	Hatta et al., 1990
	0.06 % of κ-carrageenan + 0.18 % of low-methoxyl pectin in presence of $CaCl_2$		-	Tan et al., 2012
Chloroform extraction	2.5-fold dilution with PBS and 2.5-fold chloroform addition		<50%	Ntakarutimana et al., 1992

2.5.1.1. Water dilution (WD) and freezing and thawing (FT)

We first evaluated the WD method proposed by Akita and Nakai [120] and the FT method proposed by Hodek et al. [131] to remove lipids and lipoproteins to recover the WSPF from egg yolk. We carried out additional experiments to appraise the pH effect and the dilution factor on the recovery yield and purity of IgY. Figure 6 depicts the SDS-PAGE results, in which the proteins present in the WSPF samples are separated according to their molecular weight. In general, when the FT method (lanes 2-4) is employed, the intensity of the bands corresponding to IgY (bands B and D which correspond to the heavy and light chains, respectively) is higher than when the simpler WD method is used, meaning that the concentration of IgY present in those samples (FT method, lanes 2-4) is higher, corresponding thus to higher recovery yields. Additionally, with the FT method less contaminant proteins are identified in the WSPF, meaning that the purity of IgY is also superior. When the pH of the diluted yolk is changed to 5 (lanes 2 and 5), the recovery of IgY in the WSPF

sample is also higher and with less contaminant proteins present; the IgY corresponding bands (bands B and D) are more intense and no low-density lipoproteins (LDLs) are observed (lane 2, band A). It should be highlighted that, in this situation, the obtained aqueous solution is much clearer without yellowish residues, being an additional proof that lipoproteins were effectively removed. These changes on the removal of the lipids fraction according to the pH of the aqueous solution can be explained by the isoelectric points (pI) of the different proteins, including the IgY pI (≈ 6.3 [30,42]). At lower pH values the water molecules tend to bind more effectively to positively charged proteins and the lipids are thus more easily removed [127]. It can be concluded that it is extremely important to change the pH of the diluted egg yolk down to pH 5 for a better removal of the lipids fraction (as Akita and Nakai [120] described), and to apply freezing and thawing (as Hodek and co-workers [131] proposed) to achieve better results in terms of IgY purity and recovery from the egg yolk WSPF.

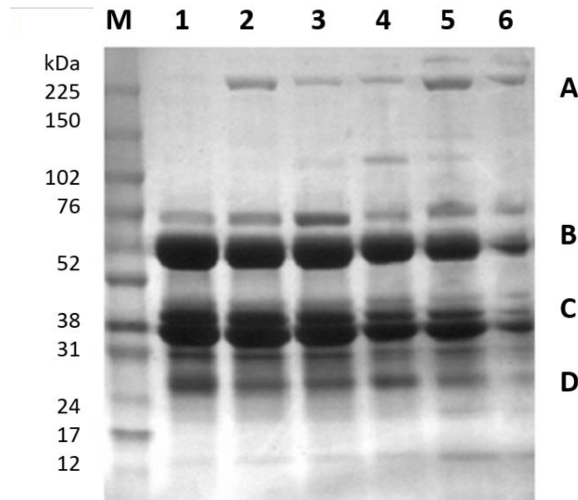

Figure 6. Coomassie stained SDS-PAGE gel of the WSPF samples obtained by WD and FT methods. Lane M: Molecular Weight Marker; Lanes 1-3: samples obtained with the FT method at pH 5, 6 and 7, respectively; Lanes 4-6: samples obtained with the simple WD method at pH 5, 6 and 7, respectively. Bands A: low-density lipoproteins; B: IgY heavy chain; C: β-livetin; D: IgY light chain. Electrophoresis was performed using reducing (DTT) conditions.

2.5.1.2. **Precipitation with polyethylene glycol (PEG) and natural gums**

Regarding the precipitation method with PEG to recover the WSPF, PEG with a molecular weight of 6000 g·mol^{-1} was reported as the most appropriate by Polson et al. [118]. To address this issue, PEGs of different molecular weight were also tested in this work, namely PEG 600, 2000 and 10000. It is here shown that the PEGs molecular weight can indeed influence the removal efficiency

of the lipids fraction - results reported in Figure 7(A). In all situations, a concentration of PEG of 3.5 wt% was used. For lower molecular weights of PEG (PEG 600 and 2000), the removal of lipids and lipoproteins is not effective, as seen by the presence of more contaminant proteins, namely LDLs, in the electrophoresis gel (Figure 7(A), lanes 1 and 2). On the other hand, PEGs with higher molecular weight, namely PEG 6000 and PEG 10000, lead to an increase on the recovery of IgY, where the lipoproteins were completely removed, particularly by applying PEG 6000 (Figure 7(A), lanes 3 and 4). Moreover, it is possible to observe that when PEG 6000 is used (Figure 7(A), lane 3), less contaminant proteins are present in the WSPF. Therefore, our results are in full agreement with those previously reported by previous works [118,130], indicating that PEG 6000 is one of the best polymers to induce the precipitation of lipoproteins and lipids from egg yolk and to recover IgY in the WSPF.

The efficacy of natural gums on the removal of lipoproteins from egg yolk was also investigated by us; we particularly carried out a comparison between arabic and xanthan gums. The first authors to propose the use of natural gums described concentrations of 0.15 % (w/v) as the improved conditions [66]. In Figure 7(B), it is possible to observe that the xanthan gum (lane 1) leads to better results than the arabic gum (lane 2) to remove lipids and lipoproteins from the WSPF. It is also possible to conclude that both gums are not significantly effective for the removal of lipoproteins (LDLs are still present). Hatta et al. [66] described that the moderate effectiveness of natural gums may be related with its acidic nature, assuming that the main forces which lead to the lipoproteins precipitation are electrostatic interactions established between the negative charge of the gums and the net positive charge of the lipoproteins. Our results are in good agreement with the findings of Hatta et al. [66].

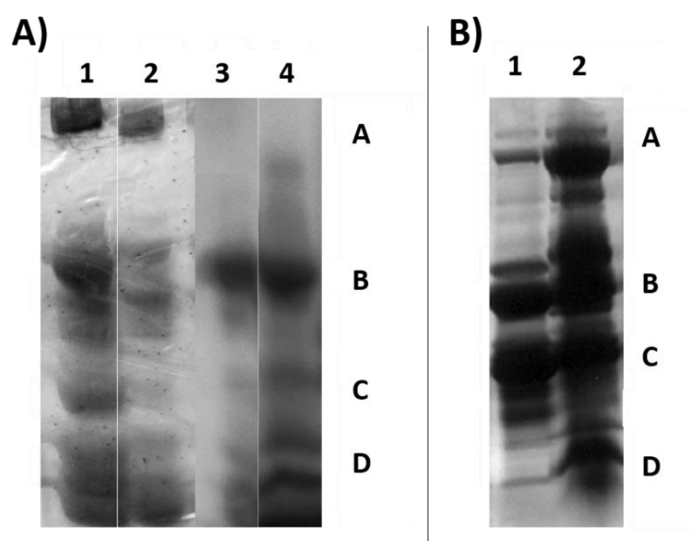

Figure 7. Coomassie stained SDS-PAGE gels of the WSPF samples obtained by: **A)** PEG precipitation method, using polymers of different molecular weight. Polymer 1: PEG 600; 2: PEG 2000; 3: PEG 6000; 4: PEG 10000. **B)** precipitation method using natural gums. Lanes 1: xanthan gum; 2: arabic gum. Bands A: low-density lipoproteins; B: IgY's heavy chain; C: livetin; D: IgY's light chain. Electrophoresis was performed using reducing (DTT) conditions.

2.5.1.3. Overall comparison and analysis on the precipitation methods to recover the WSPF

As previously stated, one of the major lacunas in the literature that we intend to overcome with this study, is that most of the times a proper quantification is not performed, or even when performed [131], the authors do not simultaneously compare all methods and with the same egg yolk samples. In addition to the discussion presented above and based on SDS-PAGE results, in this work, we additionally carried out the quantification of IgY by SEC-HPLC in each WSPF obtained by the three best methods previously described (FT, PEG precipitation and Chloroform). For the methods using natural gums this quantification analysis was not performed since a high lipoproteins content in the prepared WSPF samples was identified, meaning that this method is less effective to purify and recover IgY in a first step. The results obtained are reported in Figure 8, in which it is depicted the purification level of IgY, the recovery yield of IgY (mg IgY/g yolk), and the total weight of all proteins (mg) in the WSPF *per* g of yolk.

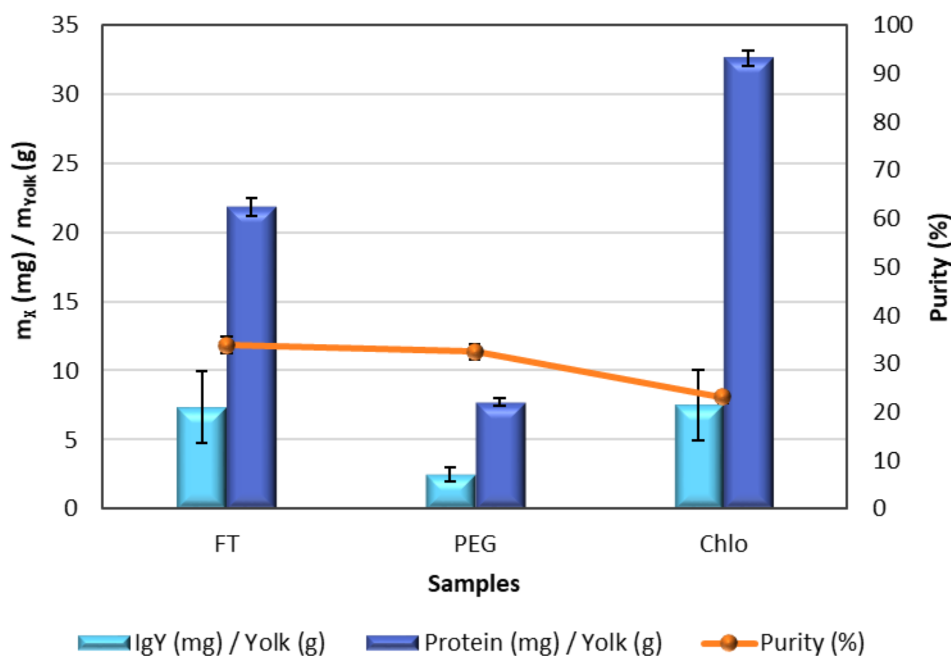

Figure 8. Amount of IgY and other proteins in the WSPF after the freezing and thawing (FT), precipitation with PEG 6000 (PEG), and chloroform extraction (Chlo) methods.

By the results shown in Figure 8, the FT and the chloroform extraction methods are the best methods in terms of IgY yield, in which 7.4 mg IgY/g yolk can be obtained; nevertheless, the chloroform extraction method lacks in selectivity for IgY recovering – the purity level of IgY with this method is *ca.* 23% in the WSPF. The PEG precipitation method, although displaying a high selectivity and leading to a high purity of IgY (32%) in the WSPF, leads to significant losses of IgY. The best method in terms of effectiveness (yield and purity of IgY) and applicability (simplicity and cost) is thus the water dilution method, followed by freezing and thawing. This simple method is less time-consuming, does not require the use of harmful solvents or more expensive compounds, and provides high recovery yields (7.4 mg IgY/g yolk) and purification (≈35%) of IgY.

2.5.2. Comprehensive analysis on the methods used to purify IgY from the WSPF

As described before, the isolation and purification of IgY from the obtained WSPF in step 1 has been carried out mostly by precipitation with several salts and PEG [116,128,131]. The best results in terms of purity and yield of IgY in the WSPF correspond to the water dilution method, followed by freezing and thawing. All the methods described below aim the selective precipitation of IgY from the WSPF pool, which can be easily collected as a pellet. As shown in the SDS-PAGE results and according to the literature [49], the main proteins present in the WSPF are α-livetin,

corresponding to chicken serum albumin (65–70 kDa) [134], β-livetin, also known as α-2-glycoprotein (45 kDa) [135], and IgY corresponding to the γ-livetin [49]. The relative proportion of the three livetins in the yolk is 2:5:3, respectively [57].

2.5.2.1. Induced precipitation of IgY from the WSPF using salts

Regarding the salts precipitation methods, several salts were investigated by us according to previously reported studies [116,128,131] and to extend the range of salts used and their concentrations. As reported in the literature [116,131] we also have found better results using sodium citrate or sodium chloride (Figure 9, lanes 2 and 3) instead of ammonium sulphate (Figure 9, lane 1). Therefore, sodium citrate and sodium chloride have a higher selectivity towards the salting-out of IgY, promoting the selective precipitation of this protein. It should be remarked that all the following figures comprising SDS-PAGE results correspond to a PBS (phosphate buffered saline, 0.01 M, pH 7.4) solution in which the pellet (precipitate enriched in IgY) was re-suspended.

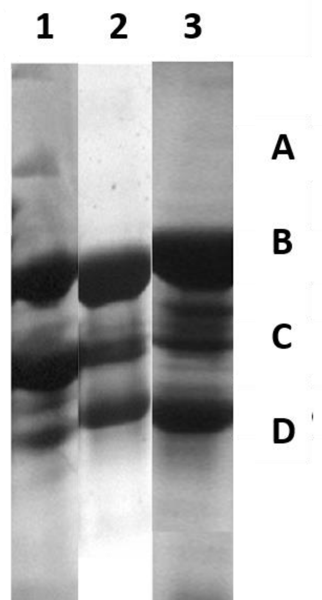

Figure 9. Coomassie stained SDS-PAGE gel of the samples obtained by the precipitation of IgY using salts induced precipitation. Lanes 1: ammonium sulphate; 2: sodium citrate; 3: samples sodium chloride. Bands A: low-density lipoproteins; B: IgY heavy chain; C: livetin; D: IgY light chain. Electrophoresis was performed using reducing (DTT) conditions.

One important factor that can influence the efficacy on the IgY selective precipitation is the concentration of salt used. Based on the results shown in Figure 9, sodium chloride and sodium

citrate were selected and these salts concentration upon the selective precipitation of IgY was evaluated. The obtained results are shown in Figure 10(A). As the salt concentration decreases from 35% (w/v) to 10% (w/v) (lanes 1 to 6), the amount of IgY that precipitates decrease as well. Hence, higher concentrations of sodium chloride lead to stronger salting-out effects, being 35% (w/v) the optimal concentration of this salt found by us. No higher concentrations of NaCl were investigated due to its saturation/solubility in water. Hodek et al. [131] reported a complete study of the effect of pH in the induced precipitation of IgY by salts addition, where the pH was adjusted with 0.5 M HCl from 4.0 to 7.2, identifying pH 4 as the best condition.

Regarding the sodium citrate salt, two effects were studied: concentration of salt and pH. The respective SDS-PAGE results are shown in Figure 10. As observed with NaCl, the effectiveness of IgY precipitation increases with the concentration of the salt, with 40% (w/v) as the optimum concentration identified for sodium citrate, being also this value the higher limit of this salt concentration according to its solubility in water. When analysing the results corresponding to the several pH values evaluated, the optimum pH for the selective precipitation of IgY is pH 5 (Figure 10). With sodium citrate at pH 5, a higher purification level of IgY is perceived since there is a lower concentration of contaminant proteins (lane p2) when compared with those identified in the processes carried out at other pH values (lanes p1, p3, p4 and p5); moreover, in the supernatant corresponding to this precipitation, only impurities are present (lane s2).

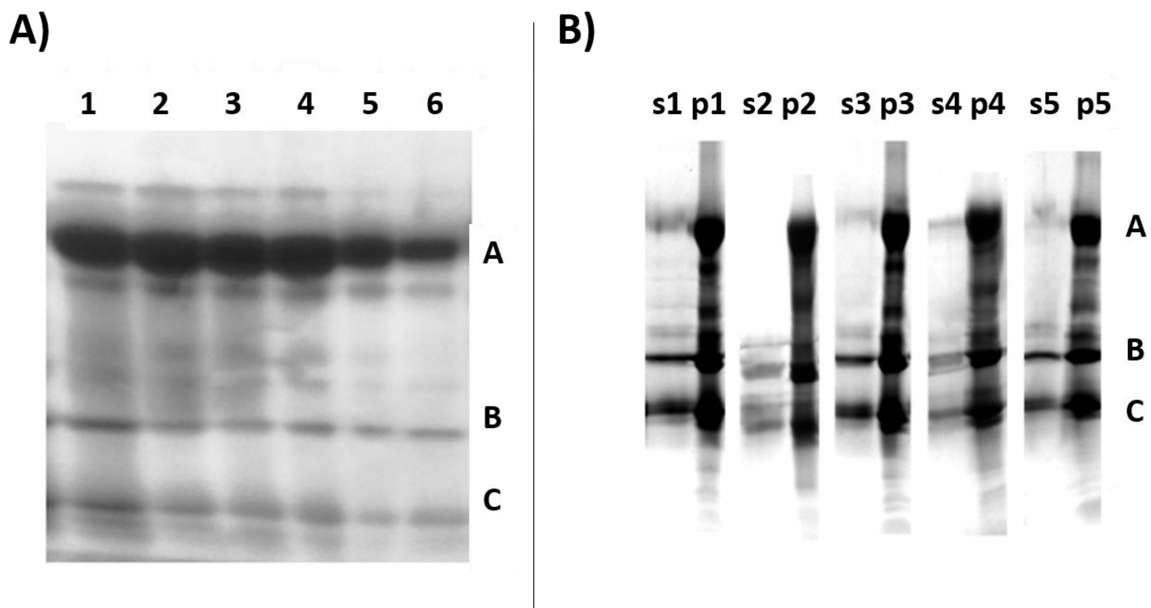

Figure 10. Coomassie stained SDS-PAGE gel of the samples obtained for: **A)** sodium chloride precipitation method using different concentrations of salt – lanes 1: 35 %, 2: 30 %, 3: 25 %, 4: 20 %, 5: 15 % and 6: 10 % (w/v); **B)** sodium citrate precipitation method performed at different pH values - lanes s1/p1: pH 4; s2/p2: pH

5; s3/p3: pH 6; s4/p4: pH 7; s5/p5: pH 8. “s” correspond to the supernatant and “p” signed lanes correspond to resuspended pellets. Electrophoresis was performed under non-reducing conditions (without DTT).

2.5.2.2. Overall comparison and analysis on the precipitation methods to recover IgY from the WSPF

After the studies regarding the selective precipitation of IgY attained by salts or polymers, a complete evaluation on the optimized methods for both steps (recovery of the WSPF followed by purification) was performed. The WSPF samples obtained by the methods described before were used for the subsequent purification step. At this stage, the quantification of IgY was performed by SEC-HPLC in each sample obtained by the best methods described above. The results obtained are depicted in Figure 11. In all situations, the purity of IgY always increases after the second purification step. However, this second purification step from the WSPF always leads to the loss of IgY, as confirmed by the decrease in the respective recovery yield.

Comparing the three best identified purification methods, reported by Bizhanov et al. (WD + NaCit) [116], Hodek et al. (FT + NaCl) [131] and Polson et al. (PEG) [118], it is possible to conclude that the Hodek’s method (FT + NaCl) is the one that leads to a higher IgY purity, despite the slightly inferior values obtained for the IgY recovery when compared to Bizhanov et al. (WD + NaCit) [116]. Regarding the PEG precipitation method reported by Polson et al. [118], it can be disclosed that besides the attainment of purity values of IgY higher than 90%, there is a significant compromise in the recovery yield. It should be however remarked that most of the IgY is lost in the first step of the overall process, as shown in Figure 8.

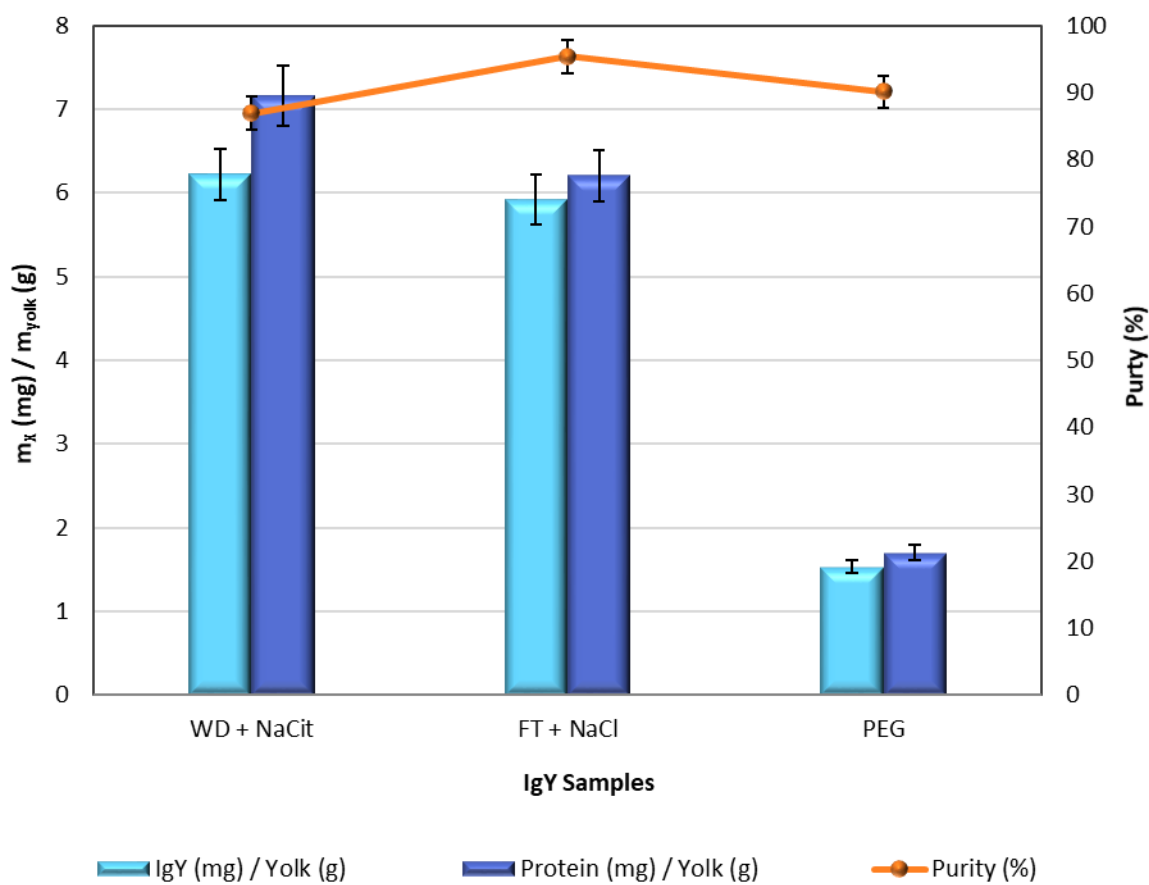

Figure 11. Recovery parameters, namely purity (orange line) and yield (light blue columns) of IgY obtained by each method evaluated. The dark blue columns correspond to the yield of total proteins present. The yield is represented as weight of IgY obtained in each sample *per* mg of initial egg yolk.

2.5.3. Performance of commercial kits to purify IgY from egg yolk

Two commercial kits (1: Thermo Pierce's Eggcellent™ and 2: AGRO-BIO's EggsPure™) are currently available in the market for IgY purification. These were commercially acquired and used to evaluate the purity and yield of IgY obtained from egg yolk, allowing to compare the performance of these two kits with the purification methods previously described and reported in the literature. It should be highlighted that these kits are based on several steps (usually 3), where IgY is finally obtained as a pellet. Therefore, both commercial kits are also based on precipitation induced methods. The results obtained with these kits were compared with the three best reported methods identified before, namely those reported by Polson et al. (PEG), Bizhanov et al. (WD + NaCit) and Hodek et al. (FT + NaCl). Again, the purity and recovery yield of IgY was determined by SEC-HPLC and the results obtained are depicted in Figure 12.

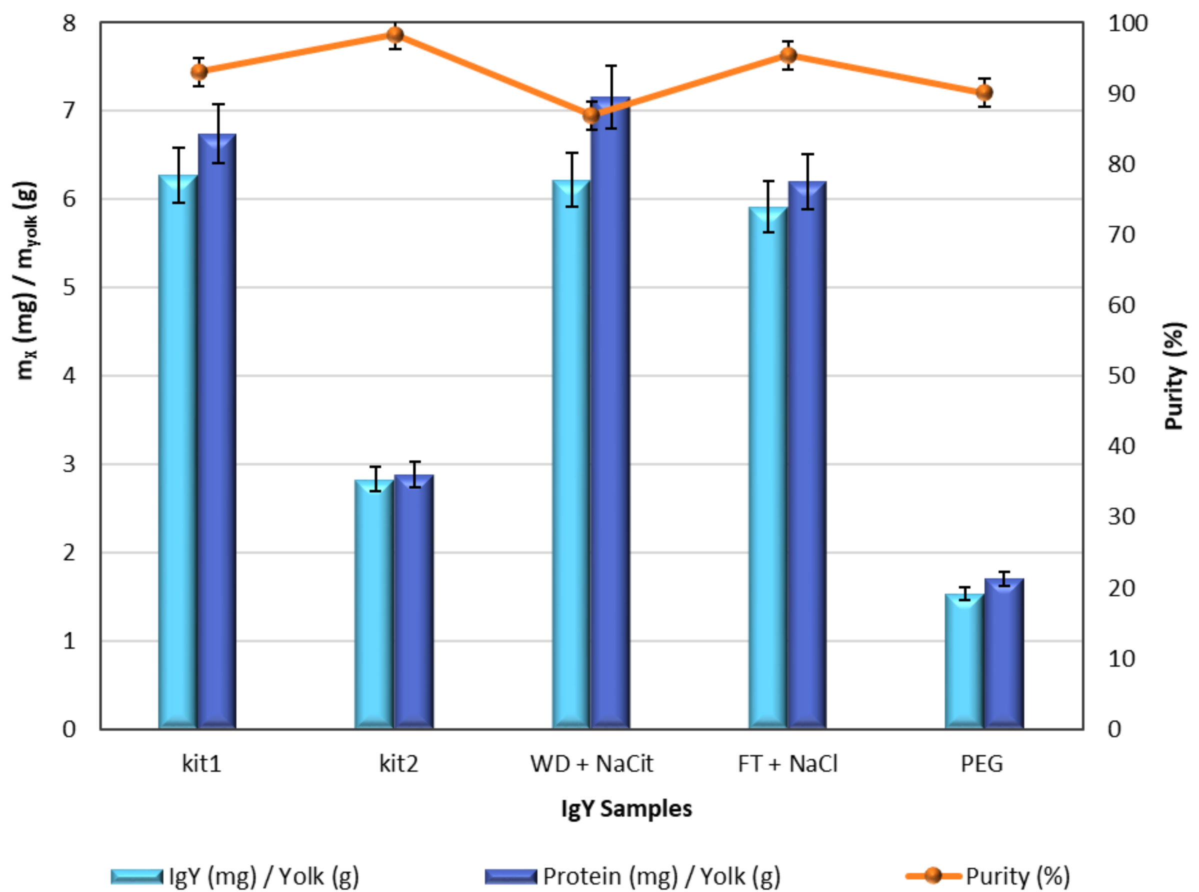

Figure 12. Recovery parameters, namely purity (orange line) and yield (light blue columns) of IgY obtained by the two commercial kits and each method evaluated. The dark blue columns correspond to the yield of total proteins present. The yield is represented as weight of IgY obtained in each sample *per* mg of initial egg yolk.

Both commercial kits are based on a first depletion step, followed by one (kit 1) or two (kit 2) precipitation steps to purify IgY. In general, both commercial kits provide high purification levels of IgY (> 95%), although the best one in terms of purity (kit 2: EggsPure™, 98 % of purity) leads to a higher loss of IgY (28% of IgY recovery assuming a total amount of IgY of 125 mg *per* yolk). Kit 2 (higher purity and lower yield) requires three precipitation steps, while kit 1 requires two, explaining the differences in terms of IgY purity and yield. This fact was commonly reported by almost all authors [127–129,131] dealing with the development of purification methods for IgY. In fact, to increase the purity of a protein we are increasing the number the purification steps, which ultimately leads to a decrease in the respective yield. This evidence was also verified in our experiments by using the different reported methods, in which that the Bizhanov's reported method (WD + NaCit) based on 2 steps (dilution with water followed by sodium citrate

precipitation) allows a higher IgY recovery, when compared for instance with the PEG precipitation method described by Polson et al. [118], in which 3 steps of precipitation are required.

Regarding the sample obtained by the Hodek et al. [131] reported method (FT + NaCl), it is possible to conclude that in terms of IgY purity it is comparable with the sample purified using kit 1 (93 % against 95 %). Nevertheless, the commercial kit is better in terms of IgY yield (59 % against 63 %). Although both kits are particularly user friendly and do not involve the use of organic solvents (like chloroform in the method proposed by Ntakarutimana at al. [117]) they are particularly expensive given the yield of IgY produced (ca. 40 € *per* egg).

2.6. General discussion and conclusions

Immunoglobulins from egg yolk (IgY) have been described as a viable alternative to mammal antibodies, namely IgG, for use in passive immunization. Contrarily to IgG, IgY can be recovered by a non-invasive method which does not cause pain to animals by blood collection since it is based on a simple act of collecting eggs. Moreover, higher titers of IgY can be attained; a dozen of eggs *per* week can provide the same amount of immunoglobulins as repeated bleeding of immunized mammalians. Nevertheless, the major shortcoming on their large-scale application and adoption by the biopharmaceutical companies results from the lack of current cost-effective techniques able to purify IgY from egg yolk. Several purification methods were described in the last two decades; still, most of them are isolated works from different authors, which lack on quantification studies by several techniques and no comparative studies on all these methods were performed by the same group of researchers and using the same egg yolk samples. Most of the published works are based on qualitative/quantitative analysis carried out by SDS-PAGE. In 2010, Deignan et al. [130] compared the performance of five methods for the lipids fraction removal from egg yolk (freezing and thawing at neutral pH, PEG precipitation, dextran sulphate and calcium chloride precipitation, phosphotungstic acid and magnesium chloride induced precipitation, and propan-2-ol and acetone extraction), and afterwards tested three methods of IgY precipitation on the obtained WSPF by each of the five methods previously described (PEG, sodium sulphate and ammonium sulphate). The authors addressed the purity of IgY by SDS-PAGE, which can be used as a quantitative method based on the optical density of the bands, but that could lead to under-/over-estimated results.

In this work, we carried out a comprehensive analysis and performed additional experimental studies on the reported purification methods for IgY, in which the IgY was quantified by SE-HPLC. This study allowed us to conclude that amongst the methods available for the lipids and lipoproteins

removal from egg yolk, the best choice is the dilution of egg yolk (8-fold dilution), followed by freezing/thawing and centrifugation. A clear WSPF can be obtained with a loss of only 25 % of IgY during this step (75% of IgY recovery yield). The high performance of this method was previously demonstrated by Hodek et al. [131]; however, the authors only investigated the optimal conditions to achieve higher purities and did not provide a comparative study using other methods. We investigated these methods thoroughly, allowing us to conclude that besides the large number of available reports based on the PEG precipitation method for the lipids removal, there are significant losses of IgY (with only 25 % of IgY present in the WSPF). Therefore, the best compromise in terms of effectiveness (yield and purity) and applicability (simplicity and costs) of all methods studied for the recovery of the WSPF corresponds to the one proposed by Hodek et al. [131], corresponding to the freezing and thawing precipitation method. Additionally, this method is simple, takes less than 1 h of labour work, and uses no armful solvents or expensive compounds, making of this method the most suitable and sustainable to be applied at research laboratories.

Concerning the IgY purification from the WSPF using sodium citrate and sodium chloride as precipitating agents, proposed respectively by Bizhanov et al. [116] and Hodek et al. [131], NaCl was identified as the best salt to induce the selective precipitation of IgY, without compromising the respective yield as verified in the PEG precipitation method described by Polson et al. [118]. By using the reported method by Hodek et al. [131], where a freezing and thawing precipitation method for the removal of the insoluble lipid fraction followed by a salt precipitation of the IgY from the WSPF using NaCl was performed, IgY with a final recovery yield of 60% and purity of 95% can be obtained.

Finally, the performance of two commercial kits for purifying IgY was also analysed, the Thermo Pierce's Eggcellent™ (kit 1) and the AGRO-BIO's EggsPure™ (kit 2). Although both kits are particularly user friendly they are particularly expensive given the yield of IgY produced (about 40 € *per* egg). From our data, it was possible to observe that kit 1 is better to achieve high yields, while with kit 2 higher purification factors are achieved (kit 1: 63 % of recovery and 93 % of purity; kit 2: 28 % of recovery and 98 % of purity). Besides the good performance of the commercial kits our results allowed us to conclude that the use of the commercial kits does not bring significant advantages comparing with the three best tested methods reported in the literature, especially the best reported method described by Hodek et al. [131].

3. Purification of IgY using Aqueous Biphasic Systems

3.1. Polymer-salt-based ABS

This chapter is based on:

Sandra C. Bernardo, João A. P. Coutinho and Mara G. Freire

"Purification of immunoglobulin Y (antibodies) using aqueous biphasic systems formed by polymers and salts", ongoing work and manuscript under preparation

[2].

3.1.1. Abstract

A large interest has been placed in egg yolk antibodies (immunoglobulin Y, IgY) as viable alternatives over mammal antibodies (IgG) for therapeutic purposes, such as in passive immunotherapy. However, due to their current high cost mainly derived from the lack of a cost-efficient purification technique, the use of IgY antibodies is still limited. Therefore, the search on effective purification platforms is a crucial demand, in which liquid-liquid extraction by means of aqueous biphasic systems (ABS) can be considered. In this work, ABS formed by sodium citrate and polyethylene glycol (PEG) or polypropylene glycol (PPG) to purify IgY antibodies from the egg yolk water-soluble protein fraction (WSPF) were investigated. With PEG-based ABS, no IgY is detected in both phases, with a significant precipitation of proteins that create an interphase in the studied ABS. On the other hand, with PPG-based ABS no precipitation of proteins was verified; however, all proteins tend to migrate to the bottom salt-rich phase, indicating no selectivity for IgY. The addition of NaCl to the ABS formed by PEG 2000 and sodium citrate was then investigated to tailor the coexisting phases properties, and thus the IgY and remaining proteins partitioning. By this last approach it is possible to increase the IgY purity in 35%. Based on the overall results, a possible strategy to recover IgY is by the use of the top phase from an aqueous system composed of 20 wt% of PEG 2000, 8 wt% of sodium citrate, 10 wt% of NaCl and 10 wt% of WSPF in the creation of a new ABS with no NaCl added, expected to induce the selective precipitation of IgY.

3.1.2. Introduction

Polyclonal antibodies, such as immunoglobulins from egg yolk (IgY), can be used as alternative biopharmaceuticals in passive immunotherapy, which is particularly relevant given the increasing

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[2] **Contributions:** M.G.F. and J.A.P.C. conceived and directed this work. S.C.B. performed all the experimental work and interpreted the experimental data. The manuscript was mainly written by S.C.B., with significant contributions from the remaining authors.

prevalence of antimicrobial resistance and the emergence of newly-recognized infectious diseases [80,94]. Nevertheless, the widespread use of IgY is still conditioned due to the current purification costs, associated to multi-stage and time-consuming techniques, such as precipitation followed by ultrafiltration or chromatographic processes [30,42,128]. Hence, the search of alternative and cost-effective purification platforms for IgY is a crucial requirement. In this context, liquid-liquid extraction by means of aqueous biphasic systems (ABS) could be a viable option [138]. ABS have been extensively exploited for the last two decades in order to recover, purify and/or concentrate a plethora of biocompounds, including IgG antibodies [67], enzymes [139], antibiotics [140], etc. Usually, ABS are formed by the combination of two water-soluble compounds, such as two polymers, a polymer and a salt, or two salts in aqueous media, and where above a given concentration there is the two-phase formation or creation of liquid-liquid systems [141]. ABS correspond to ternary mixtures with a high-water content, and where the only volatile component corresponds to water, thus supporting their more biocompatible nature when compared to organic-solvents-water binary systems. When defining a new ABS, the determination of the respective phase diagram is of significant importance in order to be able to choose the adequate solutes and their compositions to be applied as separation techniques [142]. ABS have a unique phase diagram under a particular set of conditions, such as pH, temperature, and other added components [143–145]. Figure 13 presents the phase diagram of an ABS, in an orthogonal representation where the content of water is omitted. Each phase diagram has a characteristic binodal curve (Figure 13), which divides the monophasic from the biphasic regions [145]. Above the binodal curve there is a clear interfacial boundary separating two liquid aqueous phases, in which one phase is enriched in one of the solutes and water [138]. The phase diagrams also comprise tie-lines (TLs), which give information on the composition of the top and bottom phases for an initial mixture point (Figure 13). By moving along a given tie-line, different total compositions and volume ratios occur, but presenting the same phase composition at the coexisting phases [138,145]. The most common methods to determine the binodal curves of ABS are: (1) cloud point method [146]; (2) turbidimetric titration method [147]; (3) node determination method [148].

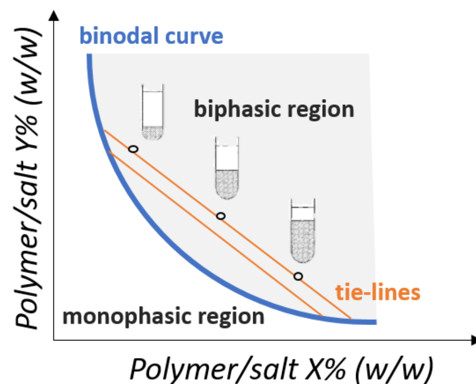

Figure 13. Illustration of an ABS phase diagram, where the binodal curve and tie-lines are represented in blue and orange, respectively.

Different types of ABS are formed by the following combinations of phase-forming components: (1) polymer-polymer (e.g., PEG/dextran) [146]; (2) polymer-salt (e.g., PEG/Na_2SO_4) [148]; (3) ionic-liquid-salt (e.g., [C_4mim]Br/$K_3C_6H_5O_7$) [149]. ABS offers many advantages over conventional separation techniques, namely they are easily scaled-up, they are mainly composed of water, and they can handle complex feeds at high target titers and large fermentation feeds [142,144]. The major drawbacks of ABS are related with the separation of the target product from the phase in which it is enriched after the separation step, and with the disposal and environmental impact of the phase-forming components if no recovery or recycling strategies are designed. Additionally, each type of system has its own advantages and disadvantages regarding the extraction and separation of different class of molecules [142]. In particular, ABS composed of polymers and inorganic salts have been reported as improved separation systems with some advantages: low interfacial tension, low viscosity, high phase separation rates and formed by low cost phase-forming components, particularly when compared with the common PEG/Dextran ABS [140,150]. Nevertheless, these systems also present major issues associated to the presence of high amounts of inorganic salts, which may compromise wastewater streams by the discharge of the commonly used phosphate-based salts [151,152]. One solution to overcome this drawback might be by using less toxic and biodegradable salts, such as citrate-based ones [152–154]. Citrate-based salts have been reported as biodegradable and non-toxic [151], and have been used in the formation of aqueous two-phase systems with both polyethylene glycol (PEG) and polypropylene glycol (PPG) polymers [151,155–157], and in some works applied to the extraction of biological materials [153]. Although their potential as alternative salts, a limited amount of research works have reported on the use of ABS constituted by citrate-based salts. In particular, these systems have

been used for the recovery of IgG antibodies, where the addition of NaCl was shown to be a critical factor to improve their selectivity for antibodies [152]. No similar works have however been found for the purification of IgY antibodies.

In this work, sodium citrate + citric acid at pH 7 was used as a phase-forming component with both polyethylene glycol (PEG) and polypropylene glycol (PPG) to create ABS, aiming at purifying chicken antibodies (IgY) from egg yolk. Initial studies using pure IgY were performed in order to select the best performing systems in terms of partition coefficient and extraction efficiency. Afterwards, the best selected polymer-salt systems were explored to purify IgY from the water-soluble proteins fraction (WSPF) obtained from egg yolk.

3.1.3. Experimental procedure

Materials. Poly(ethylene glycol) with molecular weights of 400, 600 and 2000 $g{\cdot}mol^{-1}$ (PEG 400, PEG 600 and PEG 2000, respectively) and poly(propylene glycol) with a molecular weight of 400 $g{\cdot}mol^{-1}$ (PPG 400) were purchased from Sigma-Aldrich. Trisodium citrate dihydrate was acquired from Merck (99% purity). Sodium chloride was purchased from Panreac (99.5% purity). Phosphate buffered saline (PBS, pH 7.4) was used for IgY dilution procedures, and was acquired from Sigma-Aldrich, in which one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. All other chemicals were of reagent grade. Fresh eggs were obtained from non-hyperimmune hens, provided by "HenBiotech" from Biocant. The egg yolk was carefully separated from the egg white as previously described [114], and used in the following described IgY purification methods.

Preparation of the egg yolk water-soluble protein fraction (WSPF) and pure IgY solution. Since egg yolk is a complex matrix, a first step was applied to remove lipids and lipoproteins, and to recover the water-soluble protein fraction (WSPF) from egg yolk that contains the target antibody (IgY). A water dilution method followed by freezing and thawing was applied, according to the procedure described by Hodek et al. [131]. The prepared WSPF was then used in the formulation of ABS to appraise their performance as purification strategies of IgY. A scheme of the protocol used is shown in Figure 14. A pure IgY aqueous solution at 2.5 g/L was used in ABS extraction procedures. This IgY was obtained using the sodium chloride precipitation method described by Hodek et al. [131], and previously described in Chapter 2.

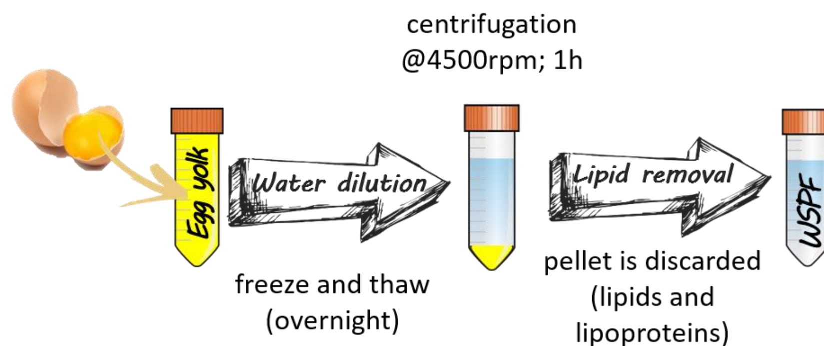

Figure 14. Scheme showing the process of preparation of the egg yolk water-soluble protein fraction (WSPF).

Determination of the ABS liquid–liquid phase diagrams. Binodal curves were determined by the cloud point titration method for each ABS at (27 ± 1) °C and atmospheric pressure. Briefly, the salt (sodium citrate + citric acid, pH 7) aqueous solution was added drop-wise to the polymers (PEG or PPG) aqueous solutions, under constant mixing, until the resulting mixture became cloudy (biphasic region); afterwards, water was added drop-wise to the mixture until a clear solution (monophasic region) was identified. Each mixture composition was determined by the weight quantification of all components added within $\pm 10^{-4}$ g (Mettler Toledo Excellence XS205 Dual Range). Tie-lines (TLs), which give the composition of each phase for an overall mixture composition, were gravimetrically determined at 27 °C, according to the original method reported by Merchuk et al. [158]. Briefly, several points were selected in the biphasic region, in which the phase-forming components were weighted, vigorously mixed and left to equilibrate overnight at 27 °C. These mixtures were centrifuged at 1500 rpm for 10 min, and the phases were carefully separated and weighted. TLs were then determined by the application of the lever-arm rule to the relationship between the weight of the top and bottom phases and the overall system composition, as described in the literature [158].

Extraction and purification of IgY using aqueous biphasic systems (ABS). The aqueous biphasic systems were prepared at different mixtures points by weighting the appropriate amounts of each component: PEG/PPG (18-26 wt%), sodium citrate (8-14 wt%), NaCl (0 or 10 wt%), pure IgY solution (at 2.5 g/L) or WSPF containing IgY (10 wt%), and water for a total mass of 1.5 or 3 g. The pH was then adjusted with citric acid until pH 7. Each mixture was stirred, left to equilibrate for at least 3 hours at 27 °C to allow the protein partitioning between the coexisting phases, and then centrifuged at 1000 rpm for 10 min for a complete phase separation. The top and bottom phases were separated and taken for analysis. In the cases were an interphase of precipitated protein was

created, it was resuspended in 500 µL of PBS aqueous solution and analysed in terms of IgY quantification and purity.

Ultraviolet–visible (UV-Vis) spectroscopy. For an initial screening on the ABS performance to extract IgY, pure IgY was used and was quantified in each phase by UV-spectroscopy, using a UV-Vis spectrophotometry (Infinite 200 PRO plate reader from Tecan), at a wavelength of 280 nm. A calibration curve using pure IgY was previously established, with an extinction coefficient (ε280) at 280 nm of 1.27 $mL.cm^{-1}g^{-1}$. Blank control samples of the ABS were also analysed to ascertain on possible interferences of the phase-forming components with the quantification method. The partition coefficients of IgY (K_{IgY}) were determined as the ratio between the concentration of IgY in the top phase and that in the bottom phase, while the percentage extraction efficiency of the studied systems for IgY (EE_{IgY}%) correspond to the ratio between the amount of IgY in the phase in which it is enriched and the total amount of IgY in both phases.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins profile of the analysed ABS phases was investigated by SDS-PAGE, using ClearPAGE™ Precast Gels from Expedeon. The samples were mixed with the Laemmli buffer (1:1, v:v), heated at 90 °C for 5 min for a complete denaturation of the proteins, and subjected to SDS-PAGE running in 4-20 % polyacrylamide gels with TEO-Tricine buffer for 2 h at 90 V. The proteins were stained with 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 50 rpm for 8 h, and then distained with a mixture solution of acetic acid at 7% (v/v), methanol at 20% (v/v), and water at 73% (v/v), 2-3 times for 2h at room temperature. The molecular weight marker used was the GRS Protein Marker Multicolour from GRiSP, which is composed of 12 bands in the range of 10-250 kDa.

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). The quantification of IgY and its purity in each ABS phase was addressed by SE-HPLC. A Chromaster HPLC (VWR, Hitachi) coupled with a binary pump, column oven, temperature controlled auto-sampler, and a diode array detector was employed. SE-HPLC was performed in an analytical column Protein KW-802.5 (8 mm × 300 mm, 5 µm) from Shodex. Before injection, each sample solution was diluted 10 times (v:v) in the mobile phase buffer (100 mM phosphate, 150 mM NaCl at pH 7). The mobile phase was run isocratically with a flow rate of 0.5 mL/min for 45 min. The column oven and autosampler were kept at constant temperatures of 40 and 10 °C, respectively. The injection volume was 25 µL. A range of wavelengths (200-400 nm) was analysed and the results were integrated at the wavelength of maximum absorption (280 nm). The retention time of IgY was found to be about 15 min. A calibration curve using pure IgY was established and used for the calculation of the IgY

concentration at the ABS phases. All chromatograms were analysed by the Origin 8.5 software in order to calculate the concentration of IgY and other proteins present in each sample. SE-HPLC was also applied to the ABS in which the WSPF was employed. In these, the percentage extraction efficiency of the studied systems for IgY (EE_{IgY}%) was determined as the ratio between the total amount of IgY in the phase in which it is enriched and the total amount of IgY in both phases. The IgY recovery yield (Y_{IgY}%) was determined by the ratio between the amount of IgY in the phase in which it is enriched and the amount of IgY present in the WSPF loaded in each ABS. The percentage of purity of IgY (Purity, %) was determined by the ratio between the amount of IgY and the total amount of proteins present in the samples or ABS phases.

3.1.4. Results and discussion

Several polymer-citrate-based ABS were investigated as alternative purification strategies of IgY from the egg yolk WSPF. The ABS phase diagrams were determined, and initial studies of partitioning with pure IgY were performed in order to identify the best systems. Finally, these systems were applied to purify IgY from the WSPF obtained from egg yolk that was obtained according to the method described by Hodek et al. [131].

3.1.4.1. ABS phase diagrams

In order to address the compositions required to form two-phase systems, and the phases compositions for a given mixture composition (TLs), the ABS ternary phase diagrams were firstly determined at 27° C. The phase diagrams were determined for ABS constituted by PEGs with different molecular weights (400, 600, 2000 g.mol^{-1}) or PPG (400 g.mol^{-1}) and sodium citrate + citric acid at pH = 7.

The solubility curves of each ABS for each polymer and sodium citrate + citric acid at pH 7 are shown in Figure 15. With mixture compositions above each binodal curve, a two-phase system can be formed, while mixture compositions below this curve result in a homogeneous and completely miscible solution. Moreover, the closer the solubility curve is to the axes, the higher the ability of a specific solute to undergo two-phase separation, meaning that a lower amount of phase-forming components is required to form ABS and a higher amount of water is present. The high amount of water can be advantageous when considering the use of ABS to extract and purify labile molecules such as antibodies.

The influence of the molecular weight of PEG (400, 600, 2000 g.mol^{-1}) on the ability to form ABS is depicted in Figure 15(A). By increasing the molecular weight of PEG the biphasic region also increases, being in agreement with the literature [152,159,160]. This tendency also confirms the

salting-out ability of the salt over the polymer, since the more hydrophobic the polymer is, lower amounts of salt are required to form two-phase systems. In this line, lower amounts of phase-forming components are required by using PEG 2000 with sodium citrate to create ABS, and thus a higher water content is present while increasing the biocompatible character of ABS to act as separation strategies for IgY.

The influence of the polymer nature (PEG vs. PPG) to form ABS with the salt was also evaluated (Figure 15(B)). PPG is a more hydrophobic polymer, and therefore there is an increase on the biphasic region area, particularly when comparing with the PEG with the same molecular weight (400 g.mol^{-1}). As highlighted before, ABS with PPG present a higher amount of water for a given mixture composition, which can be advantageous for the extraction of products with biological activity. Furthermore, PPG has been reported as a good alternative to PEG in several works to extract biomolecules, including other antibodies [161–163]. Phosphate-based salts have been used as the preferred salts in the formation of ABS, being however a strong salting-out species that could also lead to the aggregation/precipitation and consequent degradation/denaturation of the target protein [152,153,164], a problem that may be overcome using citrate-based salts.

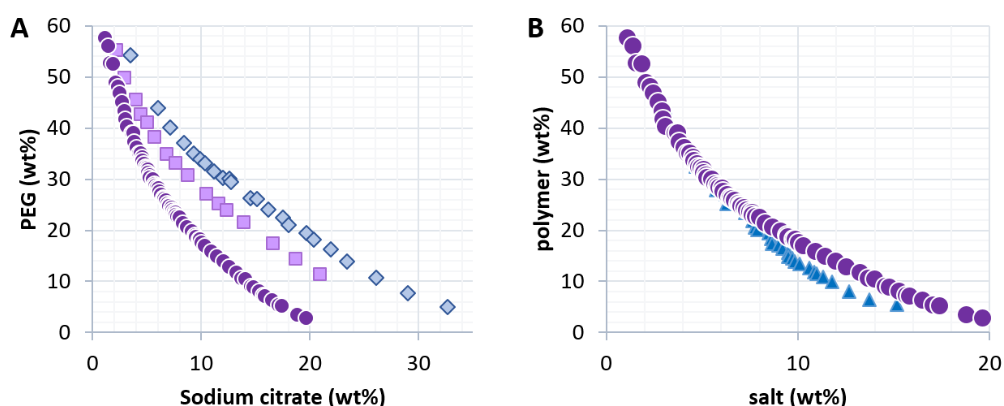

Figure 15. Phase diagrams at 27 °C for ABS composed of: **A)** PEG 400 (◆)/PEG 600 (■)/PEG 2000 (●) + sodium citrate/citric acid at pH 7 + H_2O. **B)** PPG 400 (▲)/PEG 2000 (●) + sodium citrate/citric acid at pH 7 + H_2O.

3.1.4.2. Partitioning of pure IgY

Given the phase diagrams determined before, the potential of the ABS formed by PEG 2000 and PPG 400 (plus sodium citrate/citric acid at pH 7) for the extraction of IgY was initially appraised using pure IgY. Only the systems formed by these two polymers were investigated in this part since these correspond to the ones with a higher water content for a given mixture composition.

Different mixture compositions were chosen at the biphasic region, and for each the respective tie-lines (TLs) and tie-line lengths (TLLs) were determined, being reported in Annexes, Table A.1. In all systems and mixtures evaluated, the top phase corresponds to the polymer-rich phase, whereas the bottom phase is mainly enriched in salt and water.

The extraction performance of these systems for IgY partitioning (at the mixture compositions given in Annexes, Table A.1 (TL: 1, 2 and 3)) was evaluated by the determination of the respective partition coefficients (K_{IgY}), which correspond to the ratio between the concentration of IgY at the top phase (polymer-rich) and the concentration of IgY at the bottom (salt-rich) phase. The experimental K_{IgY} and the percentage extraction efficiencies ($\%EE_{IgY}$) of the studied systems for IgY are shown in Figure 16. The partition coefficients are shown in the respective logarithmic value since it is easier to appraise the IgY specific migration for each of the ABS phases. Surprisingly, in the systems composed of PPG, the IgY partition mainly occurs towards the bottom phase (salt-rich phase, $\log(K_{IgY}) < 0$), while for the PEG-salt systems the partition of IgY occurs preferentially to the top phase (polymer-rich phase, $\log(K_{IgY}) > 0$). Therefore, a change in the polymer nature completely modifies the preferential partitioning of IgY amongst the two phases. This may be a direct result of the water content at the polymer-rich phase. Although a higher content of water in the PEG-based ABS is present in the overall mixture, the opposite occurs when analysing the polymer-rich phase. PPG, being more hydrophobic, displays a lower water content at the polymer-rich phase (as shown in the TL data given in Annexes, Table A.1.

At the working pH (pH 7), IgY is mostly negatively charged (pI around 6 [126]); therefore, electrostatic interactions, if playing a role, only seem relevant in ABS formed by PPG, where the target antibody migrates to the salt-rich phases. These results are in agreement with other published works, where other proteins (such as BSA and IgG antibodies) mainly partition to the salt-rich phases in ABS formed by PPG 400 [161–163]. In addition to the higher water content at the polymer-rich phase, when using ABS formed by PEG 2000, a salting-out effect of the citrate salt over IgY seems to be also a more relevant phenomenon, leading to its migration to the polymer-rich phase. In the case of ABS formed by PPG this phenomenon does not play a significant role in the IgY migration between the two phases. It seems thus that IgY tends to preferentially partition to moderately hydrophilic phases, i.e. with a higher water content. Furthermore, in the systems formed by PEG 2000, it was observed the precipitation of IgY at the interphase, which increases with the salt content at the mixture composition or TLL increase, which in turns corresponds to a lower water content at the PEG-rich phase (see TL data in Annexes, Table A.1). Comparing the ABS formed by PEG with those formed by PPG, it can be concluded that PPG-salt ABS seem to lead to a

better performance regarding the IgY recovery at the ABS phases since there is a lower precipitation or losses of IgY.

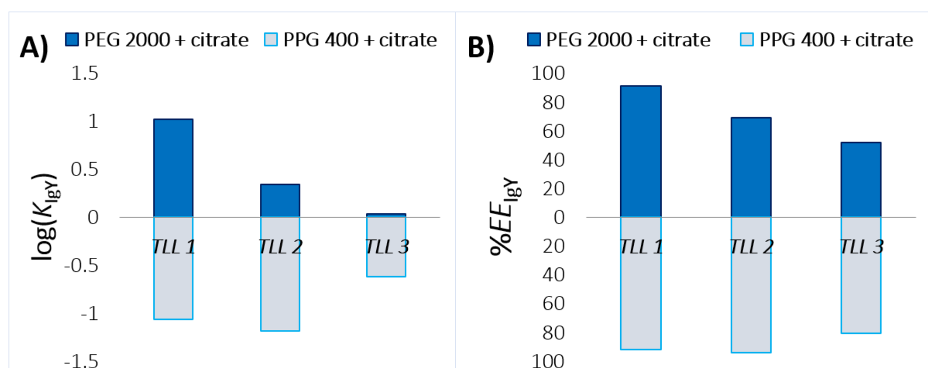

Figure 16. Partition coefficients (A) and extraction efficiencies (B) of pure IgY in ABS composed of PEG 2000/PPG 400 + sodium citrate/citric acid + water at pH 7 and 27°C. Mixture and phases' compositions are given in Annexes, Table A.1.

3.1.4.3. **Purification of IgY from the egg yolk WSPF**

After addressing the potential of the studied ABS to extract IgY, these systems were then investigated for the antibody purification from the WSPF recovered from egg yolk. The WSPF was obtained according to the protocol described in the literature [131]. The same mixture compositions, given in Table 4, were applied for both polymer-based ABS. IgY was quantified by SE-HPLC and some examples of the obtained chromatograms are depicted in Figure 17. Under the chromatographic conditions used, the retention time of IgY was found to be ≈ 15 min, within an analysis time of 45 min, and two other major peaks were found at longer retention times corresponding to α-livetin (65–70 kDa) [134] and β-livetin (45 kDa) [135], respectively. The coexisting phases used in the extraction of IgY from the WSPF of egg yolk were also analysed by SDS-PAGE.

When using PEG-based ABS to purify IgY from the WSPF, no IgY was detected in both phases (Figure 17). In fact, in all systems formed by PEG 2000, a significant precipitation of proteins was observed at the interphase. Comparing with the set of results with pure IgY, it is thus clear that the precipitation of proteins and particularly of IgY is more prone to occur with the WSPF and in presence of a "pool" of proteins, being in agreement with the "protein crowding" concept [165,166]. With ABS formed by PEG 2000, there is the preferential precipitation of antibodies, while the other proteins in the WSPF (2 main contaminants according to the chromatograms shown in Figure 17) are only partially precipitated, also partitioning between the ABS phases. Thus, these

systems are not promising for IgY purification when considering its preferential partition for a given phase using the ABS concept, especially due to the high precipitation phenomenon observed and lack of selectivity. By the Figure 17, it can be noted for the system A (showed as example), the peak corresponding to IgY (≈15 min) is absent on the profiles of the chromatograms corresponding to top and bottom phases of these PEG-citrate systems, since it is only present on the precipitate found on the interphase of those systems. Moreover, as it is also present the other two proteins in this precipitate (the chromatogram named "PP A" of Figure 17), the lack of selectivity upon precipitation also diminish the potential of the using these particular systems for IgY purification.

Table 4. Mixture compositions of the studied ABS for the purification of IgY from the WSPF, and visual observations.

System ID	wt% polymer	wt% citrate	Polymer	Polymer MW	Observations
A	22	11	PEG	2000	Precipitation
B	24	12			Precipitation
C	26	14			Precipitation
D	22	11	PPG	400	No visible precipitation
E	24	12			Partial precipitation
F	25	14			Precipitation

Contrarily to PEG-based systems, in which the precipitation of proteins, and mainly of IgY, in the interphase was significant, for almost all the studied PPG-based systems no significant precipitation was detected, being however more relevant in mixtures with higher TLL. In all PPG-based ABS, the antibody preferentially migrates to the more hydrophilic salt-rich phase, the bottom phase, in accordance with the results obtained with pure IgY. According to the SE-HPLC chromatograms shown in Figure 17, no IgY was detected at the PPG-400-rich phase (top phase). However, all proteins present in the WSPF migrate to the salt-rich phase, rendering these systems with no selectivity for IgY.

Aiming at increasing the selectivity of both PEG- and PPG-based ABS for IgY, additional ABS were prepared by adding sodium chloride salt that is a "neutral" salt within the Hofmeister series [167], and in accordance with previous studies by Azevedo et al. [168] to purify IgG. The respective phase diagrams for systems involving PEG, with 10 wt% of NaCl, were previously reported [168]. By adding NaCl there is the shift in the binodal curves towards the origin, meaning that it is possible to increase the water content and decrease the amount of phase-forming components (polymer and sodium citrate/citric acid) to create ABS. In this line, new mixture compositions were used, now with lower contents of polymer and citrate-based salt. The respective compositions of the ABS

investigated are given in Table 5. While in the PEG-citrate systems the addition of NaCl leads to the complete absence of precipitation of proteins in the interphase, in the PPG-based ABS the opposite effect occurs, not being suitable for proteins recovery by means of ABS.

The recovery yield, extraction efficiencies and purity of IgY with the PEG-based systems using NaCl are given in Figure 18. No losses were obtained in these studies by precipitation in the interphase; additionally, in the ABS composed of PEG 2000, sodium citrate + citric acid + NaCl, IgY partitioning occurs exclusively towards the top (polymer-rich) phase. In summary, increases in the IgY purity of 30%, 35% and 12% when compared with its purity in the WSPF were achieved with the mixtures 1, 2 and 3, respectively, identified in Table 5.

The enhanced purity of IgY in the PEG-rich phase is a result of the migration of the major contaminant proteins (livetins) to the salt-rich phase, as confirmed in the SDS-PAGE results shown in Figure 19. The main contaminant proteins present in the WSPF (with 65 and 45 kDa), contrarily to IgY, have more affinity towards the bottom phase. In particular, the protein with 65 kDa, which according to the literature corresponds to α-livetin, is completely enriched in the ABS bottom phase for almost all the studied systems, allowing to enhance the purity of IgY up to 35 %.

Table 5. Mixture compositions of the studied ABS in presence of NaCl for the purification of IgY from WSPF, and visual observations.

<table>
<tr><th>System ID</th><th>wt% polymer</th><th>wt% sodium citrate</th><th>Wt % NaCl</th><th>Polymer type</th><th>polymer MW</th><th>Observations</th></tr>
<tr><td>1</td><td>18</td><td>8</td><td rowspan="6">10</td><td rowspan="4">PEG</td><td rowspan="4">2000</td><td>No visible precipitation</td></tr>
<tr><td>2</td><td>20</td><td>8</td><td>No visible precipitation</td></tr>
<tr><td>3</td><td>21</td><td>12</td><td>No visible precipitation</td></tr>
<tr><td>4</td><td>24</td><td>12</td><td>Partial precipitation</td></tr>
<tr><td>5</td><td>22</td><td>10</td><td rowspan="2">PPG</td><td rowspan="2">400</td><td>Extensive precipitation</td></tr>
<tr><td>6</td><td>24</td><td>12</td><td>Extensive precipitation</td></tr>
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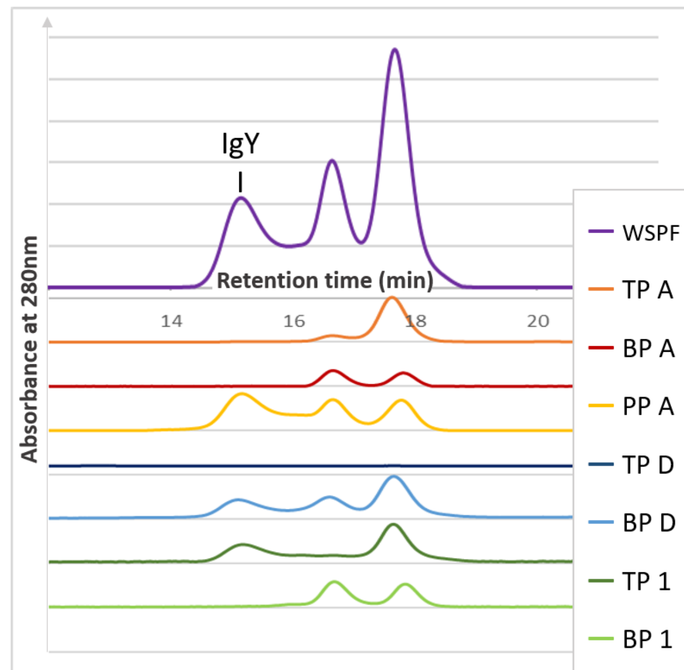

Figure 17. Size-exclusion chromatograms of the water-soluble protein fraction (WSPF) from egg yolk in phosphate buffer aqueous solution and of the top and bottom phases of ABS after the extraction of IgY from the WSPF. The IgY peak is characterized by a retention time at 15 min. Chromatograms correspond to samples of top phases (TP), bottom phases (BP) and precipitation pellet of interphase (PP) of the systems A, D and 1, presented in Tables 4 and 5, respectively, which was recovered and resuspended in a PBS aqueous solution.

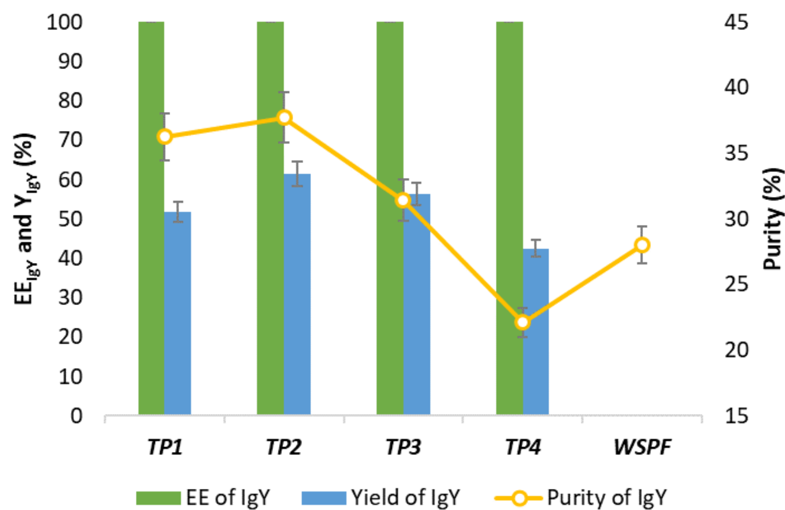

Figure 18. Extraction efficiency (EE_{IgY}%), recovery yield (Y_{IgY}%) and purity (%) of IgY extracted from the WSPF using ABS composed of PEG 2000 + sodium citrate/citric acid + 10 wt% of NaCl + 10 wt% of WSPF, where mixture points are shown in Table 5; pH ≈ 7. TP corresponds to the top phase.

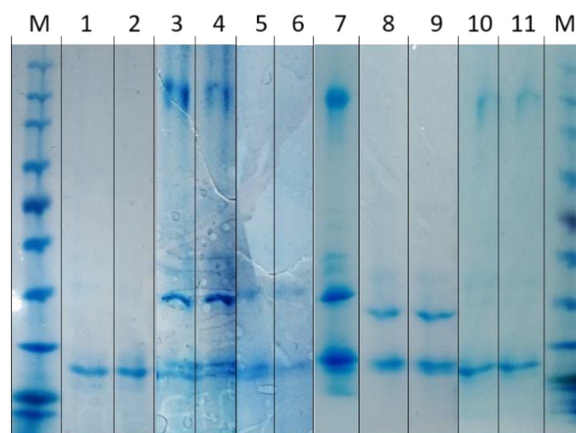

Figure 19. SDS-PAGE of a gel loaded with 0.02 mL of sample protein *per* well, stained with Coomassie blue. Lane (M): standard molecular weights. Lanes (1) and (2): top phases of the ABS constituted by PEG 2000 + sodium citrate without NaCl, namely systems A and B, respectively. Lanes (3) and (4): pellet of the interphases of the systems A and B, respectively. Lanes (5) and (6): bottom phases of the systems A and B, respectively. Lane (7): water-soluble protein fraction from egg yolk. Lanes (8) and (9): bottom phases of the ABS constituted by PEG 2000 + sodium citrate with NaCl, namely systems 2 and 1, respectively. Lanes (10) and (11): top phases of the systems 2 and 1, respectively.

As the presence of NaCl is the driving force for the selective partitioning of IgY to the PEG-rich phase, a possible strategy to recover IgY from the polymer is to create a new ABS composed of 20 wt% of PEG 2000 and 8 wt% of sodium citrate/citric acid, where no NaCl is added. By adding a fresh citrate-based buffer solution to the top phase of the extraction step, it will be "ideally" possible to obtain all the IgY in the interphase with higher purity, since in the first step of extraction the α-

livetin is completely removed from the top (increasing the IgY purity), and then all the IgY can be precipitated and recovered in the interphase in the following step (yield of 100%). This hypothesis will be explored in the near future and corresponds to projected future work. An additional hypothesis to improve the purity of IgY is to use the systems composed of PEG 2000, sodium citrate and NaCl, where no precipitation of proteins occurs and selectivity for IgY was identified in centrifugal partition chromatography (CPC), being also a target of future investigations.

3.1.5. Conclusions

In this work, ABS formed by polymers (PEG and PPG) and sodium citrate/citric acid (pH 7) were investigated as alternative processes to purify IgY from the WSPF from egg yolk. The respective ternary phase diagrams were determined, and their extraction performance for pure IgY was initially addressed. In these studies, IgY preferentially partitions to the polymer-rich phase in PEG-based ABS, and to the salt-rich phase in PPG-based systems. The best extraction efficiency for IgY (91%) was obtained with the system composed of 22 wt% PEG2000 + 10 wt% sodium citrate/citric acid (pH 7).

When using PEG-based ABS to purify IgY from the WSPF, no IgY was detected in both phases, especially due to high precipitation phenomena that is more favourable in presence of a pool of proteins, rendering these systems are not promising for IgY purification using the ABS concept. On the other hand, with the PPG-based ABS used to purify IgY from the WSPF, all proteins migrated to the salt-rich phase, rendering these systems with no selectivity for IgY. Additionally, NaCl was used in order to change the properties of the coexisting phases and to change the partitioning trend of the WSPF proteins, aiming the improvement in selectivity of these systems for IgY. It was found that the best ABS (composed of 20 wt% of PEG 2000, 8 wt% of sodium citrate/citric acid and 10 wt% of NaCl) leads to an increase of 35% in the IgY purity (against its purity in the original WSPF sample), although with a small compromise in the recovery yield (60%). Based on the overall results, it is expectable to be possible to increase the purity of IgY in a two step-approach, where the IgY is first partially purified in the PEG-rich phase using NaCl, and afterwards this phase is used with sodium citrate in order to create a new ABS (no NaCl added) to precipitate the IgY in the interphase. Being this method relatively easy to perform, fast and cheap, this approach will be carried out in the near future. Furthermore, given the selectivity for IgY observed with PEG-2000-based ABS when using NaCl, with no precipitation of proteins observed, these systems will be also investigated in centrifugal partition chromatography (CPC).

3.2. Polymer-IL-based ABS

This chapter is based on:

Sandra C. Bernardo, João A. P. Coutinho and Mara G. Freire

"Purification of immunoglobulin Y using aqueous biphasic systems formed by polymers and ionic liquids", ongoing work [3].

3.2.1. Abstract

Immunoglobulin Y (IgY) is an antibody present in egg yolk that has been studied as an alternative to immunoglobulin G (IgG) as a biological-based therapeutic agent. However, cost-efficient purification methods for IgY are still required since most of the reported methods are time consuming, of high cost and difficult to scale-up. In this work, aqueous biphasic systems (ABS) composed of polyethylene glycol (PEG) and cholinium-based ionic liquids (ILs) were investigated as alternative strategies to purify IgY. First, egg yolk was directly applied in ABS aiming the integration of the lipids fraction removal and the purification of IgY from the water-soluble protein fraction (WSPF) in one step. This approach was proven to be inefficient when compared to the reported methods. However, based on the gathered results, an aqueous homogeneous solution constituted by polymer and IL was used to induce the precipitation of the lipids and lipoproteins present in the yolk and to recover the WSPF. The obtained WSPF is very similar in terms of protein profile comparing with the commonly obtained ones applying reported methods. The prepared WSPF was then used in the preparation of ABS aiming the purification of IgY. Pure IgY aqueous solutions were also prepared in order to study the antibody partitioning between the ABS coexisting phases. However, when the WSPF was applied in ABS in order to purify IgY, an extensive precipitation of proteins was observed, creating a middle interphase enriched in proteins in the studied systems.

3.2.2. Introduction

Aqueous biphasic systems (ABS) have been reported as alternative and biocompatible liquid-liquid extraction techniques for the recovery and purification of several value-added biological products [138,141,169], such as antibodies [168,170]. ABS are mainly composed of water, in which

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[3] **Contributions:** M.G.F. and J.A.P.C. conceived and directed this work. S.C.B. performed all the experimental work and interpreted the experimental data. This chapter was mainly written by S.C.B., with significant contributions from the remaining authors.

two phases are formed upon mixing two structurally different components in aqueous media above given concentrations [141]. Conventional ABS are formed by two polymers, two salts, or one polymer and one salt in aqueous media [171]. Nevertheless, after the pioneering work reported in 2003 by Rogers and co-workers [172], a novel class of ABS composed of ionic liquids (ILs) + water + organic/inorganic salts, amino acids, polymers or carbohydrates [173] has been extensively studied and used to extract a wide variety of biomolecules [139,174–179]. ILs have also been used as adjuvants in conventional polymer-salt ABS to tailor the phase diagrams and biomolecules partitioning between the coexisting phases [180–182].

ILs are low-temperature molten organic salts [183], and due to their ionic nature ILs present several outstanding properties, such as negligible volatility at atmospheric conditions, non-flammability, high thermal and chemical stabilities, and an improved ability for the dissolution of several biomaterials [138,184]. Since it is possible to choose diverse combinations of cations and anions, ILs also have tuneable properties, meaning that the polarities and affinities of the phases of IL-based ABS can be tailored by a proper manipulation of the cation/anion design and their combinations [138,183,185]. This feature could overcome one of the most significant drawbacks in more conventional systems composed of polymer-polymer or polymer-salt combinations, i.e. the limited difference in polarities between the coexisting phases [138,186]. Nevertheless, the large-scale application of IL-based ABS is still limited by the ILs usual high price and by the hazardous features of some ILs [138,162,186]. More recently, natural-derived ILs, such as cholinium- and amino-acid-based ILs, have emerged as a new alternative to common ILs [186]. Cholinium-based ILs, which are derived from natural sources, were already synthesized and successfully used as phase-forming components of ABS [161,187–189]. ABS composed of cholinium-based ILs and polymers, such as polyethylene glycol (PEG), are a good option for the purification of value-added biomolecules [161,190]. Furthermore, it has been proven that by using cholinium-based ILs as phase-forming components of ABS, better extraction results involving proteins could be attained, and that proteins maintain their structure and function [161].

Immunoglobulin Y (IgY) is an antibody found in high concentrations in egg yolk of hens and other birds. This antibody has been studied as an alternative to immunoglobulin G (IgG) in passive immunization therapies and other important bio-based applications [49,59,61]. Given their potential, in the last decade, several methodologies have been investigated for the purification of IgY from egg yolk, including precipitation methods using polymers or salts, ultrafiltration, chromatography or extraction using organic solvents, like chloroform [130,191]. However, most of the reported methods are time consuming, are unable to provide a product with high purity level

at low cost and high yielding, and are difficult to be scaled-up. Aiming at finding alternative purification methods for IgY, ABS formed by cholinium-based ILs and PEG were here investigated. As a first attempt, the use of ABS for the simultaneous removal of lipoproteins (by precipitation) and purification of IgY directly from the complex egg yolk matrix was investigated. As a second approach, lipoproteins were initially precipitated using aqueous solutions constituted by polymers and ILs, and only the water-soluble protein fraction (WSPF) of egg yolk, which contains the target antibody, was used in the ABS formulation.

3.2.3. Experimental Procedure

Materials. Poly(ethylene glycol) with molecular weights of 600 and 2000 g·mol^{-1} (PEG 600 and PEG 2000) were purchased from Sigma-Aldrich. The following cholinium-based compounds were acquired from Sigma-Aldrich: cholinium chloride, [Ch]Cl (with > 98 % of purity) and cholinium dihydrogencitrate, [Ch][DHcit] (with 99% of purity). The cholinium acetate, [Ch][Ac] (with > 99% of purity) and cholinium dihydrogenphosphate, [Ch][DHP] (with > 98 % of purity), were obtained from Iolitec. Phosphate buffered saline (PBS, pH 7.4) was used for IgY dilution procedures, and was acquired from Sigma-Aldrich, in which one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. All other chemicals were of reagent grade. Fresh eggs were obtained from non-hyperimmune hens, provided by “HenBiotech” from Biocant. The egg yolk was carefully separated from the egg white as described in the literature [114], and used in the following described IgY purification methods.

Preparation of the egg yolk water-soluble protein fraction (WSPF) and pure IgY solution. In order to recover the water-soluble protein fraction (WSPF), that contains the target antibody (IgY), from the egg yolk, a water dilution method followed by freezing and thawing was applied, according to the procedure described by Hodek et al. [131]. The prepared WSPF was then used in the formulation of ABS to investigate their performance as purification strategies of IgY. A pure IgY aqueous solution at 2.5 g/L was used in ABS extraction procedures. This IgY was obtained using the sodium chloride precipitation method described by Hodek et al. [131], and previously described in Chapter 2.

Extraction and purification of IgY using aqueous biphasic systems (ABS). The aqueous biphasic systems were prepared at different mixtures points by weighting the appropriate amounts of each component: PEG + cholinium-based ILs + water + pure egg yolk or WSPF (10 wt%) for a total mass of 3 g. Each mixture was stirred and left to equilibrate for at least 3 h at 27 °C to allow the proteins partitioning between the coexisting phases. The top and bottom phases were separated and taken

for analysis. In the cases where an interphase of precipitated proteins was created, it was resuspended in 500 µL of PBS aqueous solution and analysed in terms of IgY purity.

Ultraviolet–visible (UV-Vis) spectroscopy. For an initial screening on the ABS performance to extract IgY, pure IgY was used and was quantified in each phase by UV-spectroscopy, using a UV-Vis spectrophotometry (Infinite 200 PRO plate reader from Tecan), at a wavelength of 280 nm. A calibration curve using pure IgY was previously established, with an extinction coefficient (ε280) at 280 nm of 1.27 $mL.cm^{-1}g^{-1}$. Blank control samples of the ABS were also analysed to ascertain on possible interferences of the phase-forming components with the quantification method. The partition coefficients of IgY (K_{IgY}) were determined as the ratio between the concentration of IgY in the top phase and that in the bottom phase, while the percentage extraction efficiency of the studied systems for IgY (EE_{IgY}%) correspond to the ratio between the amount of IgY in the phase in which it is enriched and the total amount of IgY in both phases.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins profile of the analysed ABS phases was investigated by SDS-PAGE, using ClearPAGE™ Precast Gels from Expedeon. Samples were mixed with the Laemmli buffer (1:1, v:v), heated at 90 °C for 5 min for a complete denaturation of the proteins, and subjected to SDS-PAGE running in 4-20 % polyacrylamide gels with TEO-Tricine buffer for 2 h at 90 V. The proteins were stained with 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 50 rpm for 8 h, and then distained with a mixture solution of acetic acid at 7% (v/v), methanol at 20% (v/v), and water at 73% (v/v), 2-3 times for 2h at room temperature. The molecular weight marker used was the GRS Protein Marker Multicolour from GRiSP, composed of 12 bands in the range of 10-250 kDa.

3.2.4. Results and discussion

The methods described in the literature involving the IgY purification are mainly divided into two steps. First, the fraction of egg yolk containing the water-soluble proteins fraction (WSPF), which contains IgY, is isolated from lipids and lipoproteins (Step 1). Then, the isolation of IgY from the WSPF is carried out (Step 2) [129,130,191]. Herein, we first attempt to integrate both steps using ABS composed of polyethylene glycol (PEG) and cholinium-based ILs. Then, we explored the use of the WSPF obtained from egg yolk in the formulation of ABS to decrease the complexity of the matrix, while envisioning higher IgY purification factors.

PEG has been reported as a precipitation agent in some methods to remove the lipids fraction from the WSPF [114,118]. In this sense, we first addressed the use of this polymer as a phase-forming component of ABS in order to induce the selective precipitation of the lipids fraction from egg yolk, while envisaging the selective and simultaneous extraction of IgY for one of the ABS phases. The mixture compositions were chosen based on the ABS phase diagrams reported by Pereira et al. [192]. ABS formed by PEG with a molecular weight of 600 $g.mol^{-1}$ and the ILs [Ch]Cl, [Ch][Ac], [Ch][DHP] and [Ch][DHcit] were investigated. Mixture compositions (given in Table 6) close to the binodal curves were chosen to have a higher amount of water, beneficial to maintain the stability of IgY while avoiding its precipitation. After mixing all phase-forming components, pure egg yolk was added (10 wt% of yolk to a total system of 5 g). The obtained results are shown in Figure 20.

Table 6. Mixture compositions of the studied ABS for the lipids fraction removal from egg yolk.

System ID	wt% PEG	wt% IL	IL anion	PEG MW	Observations
1	55	30	Cl^-	600	Extensive precipitation; no ABS formation.
2	35	40	$[Ac]^-$		
3	30	25	$[DHP]^-$		ABS formation; inefficient removal of the lipids fraction
4	50	35	$[DHcit]^-$		No ABS formation.

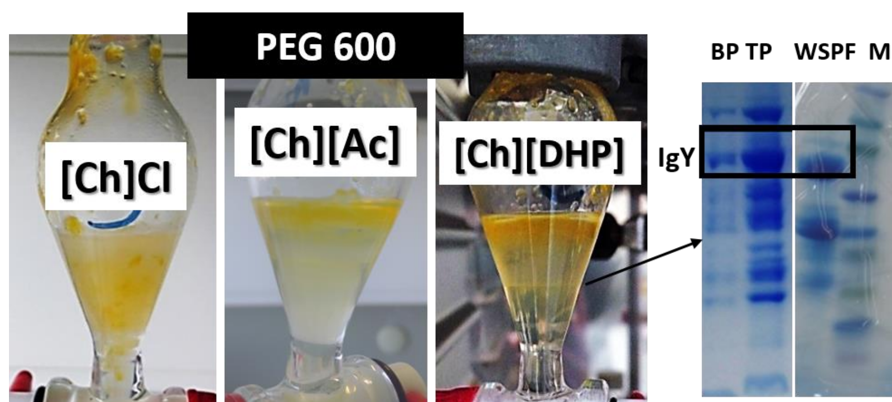

Figure 20. Visual aspect of the extractions carried out using ABS formed by PEG 600 + ILs + egg yolk. SDS-PAGE results demonstrating the inefficient removal of the contaminant proteins of egg yolk, where the top (TP) and bottom (BP) phases of the ABS composed of 30 wt% PEG 600 + 25 wt% [Ch][DHP] are compared with the WSPF obtained by reported methods.

Most of the studied systems lead to unsuccessful results since the precipitation of the whole proteins was verified or because the two-phase formation did not occur. The only exception was

verified with the IL [Ch][DHP], in which it is clear the formation of the ABS. Still, this system also presents a top small layer corresponding to the insoluble water fraction of egg yolk, and both phases are visibly contaminated with some lipids and lipoproteins (yellow colour). SDS-PAGE analysis (Figure 20) was performed for this system, showing that the top phase presents a large amount of contaminant proteins when compared with the usual obtained WSPF by the method described by Hodek et al. [131], whereas the bottom phase, although appearing as more selective towards IgY, reveals also a significant loss of the target antibody (that is majorly enriched in the top polymer-rich phase).

Besides the difficulty upon two-phase formation when combining [Ch][DHcit], PEG 600, water and egg yolk, Pereira et al. [192] reported that this IL induces an acidic pH in the coexisting phases (ca. 5), which can be beneficial to induce the complete precipitation of the lipids fraction from egg yolk – according to the data shown in Chapter 2. Hodek et al. [131] were able to show that the decrease of the pH value to 5 promotes the precipitation of lipids and lipoproteins from egg yolk. Based on this information, a new strategy was explored, in which no preparation of ABS was made to attempt the removal of the lipids, but instead an aqueous mixture solution composed of 30 wt% PEG 600 + 30 wt% [Ch][DHcit] + 30 wt% water (monophasic solution) was prepared, and the egg yolk (10 wt%) was added to this solution. Two phases were formed, in which the upper phase is mainly composed of lipids and lipoproteins from egg yolk, and the lower phase is an aqueous solution containing PEG 600, [Ch][DHcit], IgY and also other proteins (Figure 21). Nevertheless, our approach leads to a decrease in the IgY yield when compared to WSPF obtained by well reported methods. Therefore, the WSPF was obtained according to the protocol described by Hodek et al. [131] and further used in the ABS formation aiming the selective extraction of IgY for one of the phases, as shown and discussed below.

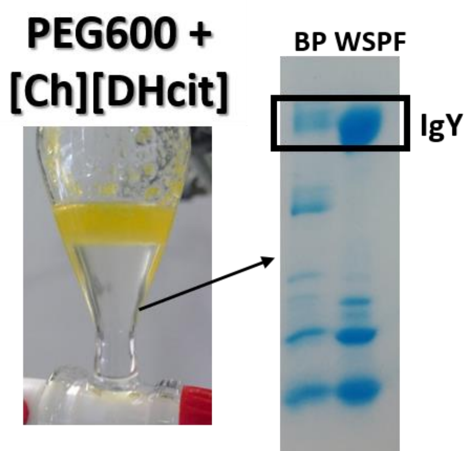

Figure 21. Precipitation of the lipids fraction from egg yolk using 30 wt% PEG 600 + 30 wt% [Ch][DHcit] + water. SDS-PAGE results for the bottom phase (BP) of the ABS formed by PEG 600 + [Ch][DHcit], and for the WSPF obtained by reported methods.

Initial studies using pure IgY, followed by the application of the WSPF obtained by the method reported by Hodek et al. [131], were carried out. The partitioning of pure IgY was addressed in ABS formed by PEG 2000 and [Ch][DHP] at different mixture compositions, as described in Table 7. The results shown in Figure 22(A) are given in the logarithm of partition coefficients (log(K_{IgY})) and extraction efficiencies of IgY (%EE_{IgY}) at 25°C. An inversion on the partition behaviour of pure IgY is observed at different mixture compositions. For mixture points closer to the binodal curve the antibody preferentially partitions to the bottom (IL-rich phase; log(K_{IgY}) < 0), while for mixture points far from the binodal curve (longer TLL) IgY preferentially migrates to the top phase (PEG-rich phase; log(K_{IgY}) > 0). With the last composition (25 wt% PEG 2000 + 24 wt% [Ch][DHP]), a better extraction efficiency of IgY at the polymer-rich phase is achieved (> 80%). Therefore, this system was further investigated to purify IgY from the egg yolk WSPF. However, when the WSPF containing IgY was used, an extensive precipitation of the proteins was observed, and an interphase was formed. Furthermore, it is shown an inversion on the partition of IgY when using the pure antibody *versus* the WSPF. The proteins pellet was resuspended in PBS (phosphate buffered saline, 0.01 M, pH 7.4) aqueous solutions and also analysed by SDS-PAGE. The proteins profile of the bottom and top phases, as well as of the ABS interphase, is shown in Figure 22(B), where all proteins are shown to partially precipitate and migrate to the bottom phase in lower amounts (Figure 22(B)), rendering this system with no selectivity for IgY. Nevertheless, in both the ABS bottom-phase and precipitate it seems that IgY presents a higher purity than that in the original WSPF

Table 7. Mixture compositions of the studied ABS for the partition studies of pure IgY.

System ID	w% PEG	w% [Ch][DHP]⁻	PEG (g·mol⁻¹)	Observations
1	25	20	2000	Extensive precipitation of proteins at the interphase.
2	25	22		
3	25	24		Moderate precipitation of proteins at the interphase.

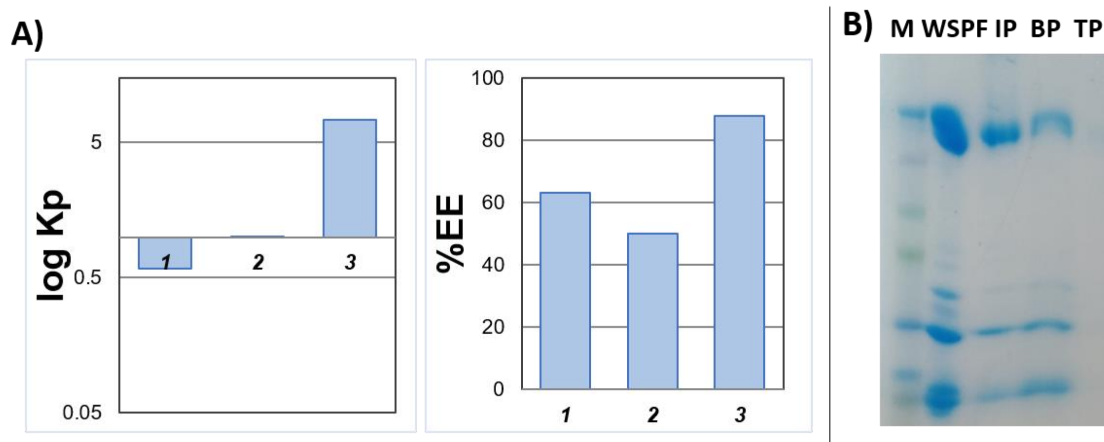

Figure 22. Partition and purification studies for IgY using ABS formed by PEG 2000 + [Ch][DHP]. A) Partition coefficients and extraction efficiencies of pure IgY in ABS composed of PEG 2000 + [Ch][DHP] + water at 27°C. B) A SDS-PAGE results for the top (TP) and bottom (BP) phases, and resuspended pellet of the precipitated proteins at the interphase (IP), compared with the WSPF obtained by reported methods.

3.2.5. Conclusions

A novel purification strategy was attempted herein, in which ABS composed of cholinium-based ILs and polymers, namely PEG, were investigated for the purification of IgY. First, the egg yolk was used directly in these systems aiming at developing an integrated strategy to remove the lipids fraction and simultaneously purify IgY. This step was proven to be inefficient given the complexity of egg yolk and presence of lipoproteins at the ABS coexisting phases. Furthermore, it was demonstrated that an aqueous solution composed of PEG 600 and [Ch][DHcit] allows the removal of lipoproteins and the recovery of a WSPF containing IgY. This trend was due to the presence of PEG and pH 5 induced by [Ch][DHcit]. Pure IgY partitioning studies, and the use of the WSPF obtained by this method and by reported ones in ABS formulation were then investigated. An inversion on the preferential partitioning of pure IgY amongst the phases was observed with an increase in the polymer concentration. Regarding the studies carried out with the WSPF, it can be

concluded that some of the contaminant proteins are removed by their migration towards the bottom phase. Therefore, a small increase in the purity of IgY was obtained in the recovered pellet.

Although no high purity levels of IgY were achieved, in this work we proposed a different methodology for the first step of IgY purification (the separation between the lipids fraction and the water-soluble proteins fraction), where no centrifugation or freezing is needed, which can lead to fast industrial processes. For future work, and based on the results discussed here and in Chapter 3.1, it will be important to explore the three-phase partitioning approach that is used in the next chapter of this thesis aiming the selective precipitation of IgY from the WSPF. To this end, several operating conditions, such as the molecular weight of the polymer and the IL nature, as well as their concentration, should be optimized. Finally, the stability and activity of the recovered IgY need to be addressed after the recovery step.

4. Purification of IgY using Three-Phase Partitioning Systems

4.1. Characterization of novel PEG-NaPA-based ABS using ionic liquids as electrolytes

This chapter is based on:

Sandra C. Bernardo, Jorge F. B. Pereira, Sónia P. M. Ventura, Mara G. Freire and João A. P. Coutinho

"Opposite effects induced by Cholinium-based Ionic Liquid Electrolytes in the Formation of Polyethylene glycol/Sodium Polyacrylate Aqueous Biphasic Systems", communication submitted to Physical Chemistry Chemical Physics [4].

4.1.1. Abstract

Cholinium-based ionic liquids ($[Ch]^+$-ILs) were investigated as electrolytes in the formation of aqueous biphasic systems (ABS) composed of polyethylene glycol (PEG) and sodium polyacrylate (NaPA) polymers. Both the enhancement and decrease of the liquid-liquid demixing ability induced by electrolytes in PEG/NaPA aqueous systems were observed for the first time. It is shown that the ILs that most extensively partition to the PEG-rich phase tend to act as inorganic salts enhancing the two-phase formation ability, while those that display a more significant partition to the NaPA-rich phase decrease the ABS formation capacity. The obtained results allow us to confirm the tailoring ability of ILs. The distribution of the electrolyte ions between the coexisting phases and the polyelectrolyte ions compartmentalization are key factors behind the formation of PEG/NaPA-based ABS.

4.1.2. Introduction

Liquid-liquid extractions by means of aqueous biphasic systems (ABS) have been extensively explored in the last decades to recover, purify and/or concentrate a large number of biocompounds [169,182]. ABS are formed by the combination in water of, at least, two water-soluble compounds, (polymers, salts, ionic liquids, carbohydrates, etc.) which act as phase-forming components [138,193]. Among these, polymer/polymer ABS have been the most studied since they have a high water content, and thus display a more biocompatible character, although being restricted to few combinations of polymers [164,194–198].

[4] **Contributions:** M.G.F. and J.A.P.C. conceived and directed this work. S.C.B. performed all the experimental work and interpreted the experimental data. The manuscript was mainly written by S.C.B., with significant contributions from the remaining authors.

A large number of water-soluble non-ionic polymers has been investigated as phase-forming agents of ABS; however, some of these polymers combinations do not undergo liquid-liquid demixing in aqueous media at convenient concentrations for separation purposes. The most commonly used polymer/polymer pair for ABS formation is polyethylene glycol (PEG)/dextran [196,199], although dextran is quite expensive in comparison with the polymers herein used, and leads to highly viscous aqueous solutions, imposing some limitations when the large-scale application of ABS is envisaged [144,154,200]. To overcome these shortcomings, polyelectrolytes have been investigated as phase-forming agents of ABS [201–203]. Due to the entropically driven dissociation of their counterions in aqueous solutions, and consequent large contribution of the entropy of mixing, these charged polymers are highly water soluble [194]. In addition, polyelectrolyte aqueous solutions usually exhibit lower viscosity than non-ionic polymer solutions [197,203].

Although PEG and the polyelectrolyte sodium polyacrylate (NaPA) with adequate molecular weights can form ABS, they may not be able to create liquid-liquid systems at suitable polymers concentrations [194]. In order to overcome this drawback, the addition of inorganic salts (1-5 wt%) as electrolytes in PEG/NaPA ABS is commonly used as a successful approach [201,203,204]. The addition of salts decreases the entropic penalty of the polyelectrolyte counterions compartmentalization, favouring phase separation [194]. Moreover, the decrease of the polymers content, while increasing the water amount, further decreases the overall system cost and the phases viscosities [198,203,204].

The effect of salts or electrolytes in the PEG/NaPA/water phase diagrams has been investigated by several authors, with some of these works focusing in a more fundamental perspective trying to better understand the molecular-level phenomena ruling the phase separation. Gupta et al. [203] correlated the capability of ions as water structure-breakers or water structure-makers (kosmotropic vs. chaotropic salt ions) and their ability to induce ABS. Perrau et al. [198] and Johansson et al. [204] proposed that the salt addition decreases the entropy penalty of the polyelectrolyte counterions compartmentalization by charge effects. All these studies employed high melting inorganic salts as additives/electrolytes, but when the application of these systems is foreseen for the separation for labile biomolecules separation, inorganic salts may raise some biocompatibility concerns [205,206].

Considering the relevance of salts or electrolytes in the formation of polymer/polyelectrolyte ABS, ionic liquids (ILs) have been investigated as potential alternatives to the widely studied high melting inorganic salts [207,208]. The use of ILs as additives, due to their tuneable characteristics,

was shown to effectively control the formation of PEG/NaPA ABS. By adding tensioactive [208] and non-tensioactive ILs [207], it was possible to design ABS with different phases' polarities and phase's separation ability. However, the ILs used as electrolytes in previous works [207,208] may still raise some environmental and biocompatibility issues [209–211].

Aiming at developing liquid-liquid systems with enhanced environmentally-friendly and biocompatible characteristics, in this work, we investigated PEG/NaPA-based ABS using cholinium-based ILs ($[Ch]^+$-ILs) as electrolytes. The effects of the PEG molecular weight (MW), electrolyte type and concentration on the formation ability of ABS were investigated at 27 °C.

4.1.3. Experimental Procedure

Materials. Poly(ethylene glycol) of molecular weights of 600, 2000, 4000 and 6000 $g.mol^{-1}$ and the aqueous solution of sodium poly(acrylate) of 8000 $g.mol^{-1}$ (45 wt%) were purchased from Sigma-Aldrich. Cholinium chloride, [Ch]Cl, and cholinium dihydrogencitrate, [Ch][DHcit], were acquired from Sigma-Aldrich; cholinium acetate, [Ch][Ac], and cholinium dihydrogenphosphate, [Ch][DHP], were obtained from Iolitec; cholinium bitartrate, [Ch][Bit], was purchased from Acros Organics. All ILs have a purity level higher than 98 wt%. All other chemicals were of reagent grade.

Determination of phase diagrams. Aqueous solutions of each phase-forming compound (PEG or NaPA) were prepared with a known concentration of each [IL and used to determine the respective binodal curves. The water samples used also contained each IL at the desired concentration to keep it constant along the determination of the phase diagrams. The phase diagrams were determined by the cloud point titration method at 27 °C and atmospheric pressure, according to previously described procedures [138,212]. The ternary system compositions were calculated by the weight quantification of all components added within ± 10^{-7} kg. The detailed experimental weight fraction data are reported in Tables A.2 to A.16 of Annexes.

Quantification of the IL electrolyte in the ABS coexisting phases. A mixture point within the ABS biphasic region was selected and an ABS of 3 g of total mass was prepared by weighting the appropriate amounts of each compound: 25 wt% of PEG 600 + 7.5 wt% of NaPA 8000 + 10 wt% of each IL + water. After mixing all components, the systems were allowed to equilibrate for 10 h, followed by centrifugation at 1500 rpm for 10 min. The phases were carefully separated and collected for the determination of their volumes, weight, and further quantification of each IL at the coexisting phase by proton Nuclear Magnetic Resonance (^1H NMR) spectroscopy. The amount of the cholinium cation present in each phase was quantified using a Bruker Avance 300 at 300.13 MHz. A NMR tube containing 500 µL of solvent plus the internal standard (dimethyl sulfoxide, DMSO, + 10% v/v benzene) and 100 µL of the ABS phase within a neat tube, to avoid precipitation

of the NaPA present in the samples by DMSO, was prepared. By quantifying the cholinium cation present in each phase using benzene as internal standard, the partition coefficient (K_{elect}) of $[Ch]^+$-ILs was determined, defined as the ratio between the concentration of each $[Ch]^+$-based IL present in the PEG-rich phase (top) in respect to that in the NaPA-rich phase (bottom), as described by the following equation:

$$K_{elect} = \frac{[\mathrm{Ch^+{-}IL}]_{\mathrm{PEG}}}{[\mathrm{Ch^+{-}IL}]_{\mathrm{NaPA}}} \qquad \text{(Equation 2)}$$

where $[\mathrm{Ch^+} - \mathrm{IL}]_{\mathrm{PEG}}$ and $[\mathrm{Ch^+} - \mathrm{IL}]_{\mathrm{NaPA}}$ are the concentration of each $[Ch]^+$-based IL in the PEG- and NaPA-rich phases, respectively.

4.1.4. Results and discussion

To evaluate the PEG MW influence on the formation of PEG/NaPA + $[Ch]^+$-ILs ABS, the phase diagrams composed of PEG polymers with different MW (600, 2000, 4000, 6000 and 8000 $g.mol^{-1}$), NaPA with a MW of 8000 $g.mol^{-1}$, and 5 wt% of each $[Ch]^+$-IL (cholinium chloride, [Ch]Cl, cholinium acetate, [Ch][Ac], cholinium dihydrogenphosphate, [Ch][DHP], cholinium dihydrogencitrate, [Ch][DHcit] and cholinium bitartrate, [Ch][Bit]) were determined at 27 °C by the cloud point titration method.

Figure 23 depicts the phase diagrams of the systems composed of PEG/NaPA + 5 wt% of [Ch]Cl. The remaining phase diagrams for systems containing the different $[Ch]^+$-ILs combined with PEGs of different MW are given in Annexes, Fig. A.1. The detailed weight fraction experimental data are provided in the Annexes, Tables A.2 to A.6. Compositions of PEG and NaPA above each solubility curve, while keeping the concentration of $[Ch]^+$-ILs at 5 wt%, lead to the creation of two-phase systems, whereas compositions below the respective binodal curve result in a homogenous solution.

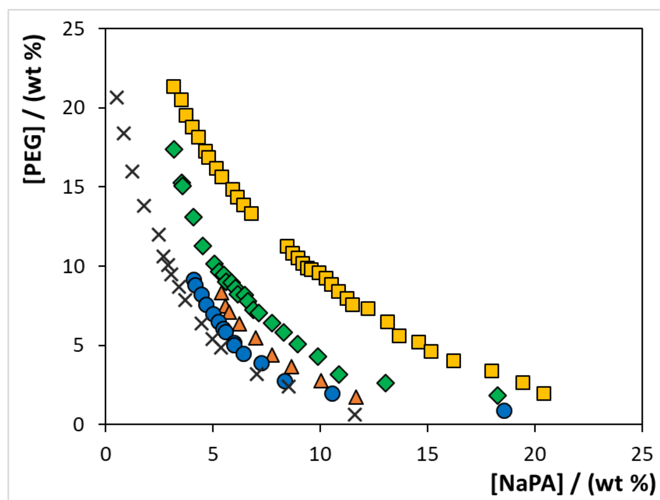

Figure 23. Solubility/binodal curves for ABS composed of PEG (different MW), NaPA 8000 + 5 wt% of [Ch]Cl, at 27 °C: (□) PEG 600; (◆) PEG 2000; (▲) PEG 4000; (●) PEG 6000; and (✕) PEG 8000. Data corresponding to the latter system were taken from the literature [207].

Figure 23 shows an increase in the biphasic region, i.e. of the ability to undergo liquid-liquid demixing, with the PEG MW increase, being this phenomenon independent of the $[Ch]^+$-IL used as electrolyte. The same behaviour is obtained with other ILs or when representing the respective phase diagrams in molality units – see Annexes, Figs. A.1 and A.2. In summary, lower MW PEGs reduce the PEG/NaPA ABS biphasic region. Our results using $[Ch]^+$-ILs agree well with those previously reported using inorganic salts as electrolytes [164,204,213]. For all PEG/NaPA systems investigated up to date, the biphasic region increases with the MW of PEG, meaning that lower amounts of phase-forming components are required to undergo liquid-liquid demixing when employing higher MW PEGs. The same trend has been reported for other ABS composed of PEG/polymer, PEG/salt and PEG/IL [214–216].

The influence of different $[Ch]^+$-ILs as electrolytes in the phase diagrams behaviour was investigated in the ABS composed of PEG 600, NaPA 8000 and water, at 27 °C. The ILs [Ch]Cl, [Ch][Ac], [Ch][DHP], [Ch][DHcit] and [Ch][Bit] were used as electrolytes at concentrations ranging from 0 to 10 wt%. The respective phase diagrams with PEG 600 are depicted in Figure 24. The detailed experimental weight fraction compositions are given in Annexes, Tables A.7 to A.15.

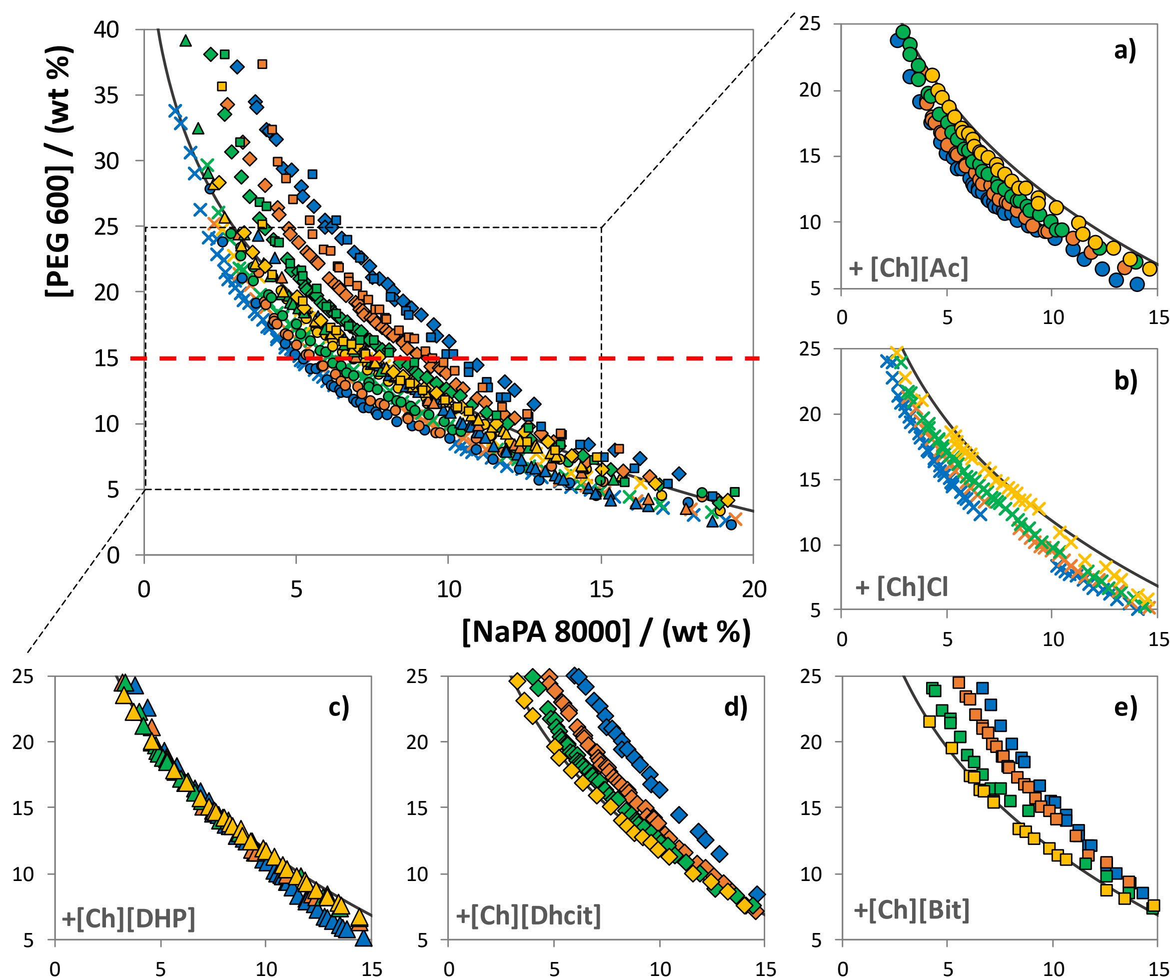

Figure 24. Experimental solubility data at 27 °C for ABS composed of PEG 600, NaPA 8000, and each $[Ch]^+$-based IL used as electrolyte: a) [Ch][Ac] (●); b) [Ch]Cl (✕); c) [Ch][DHP] (▲); d) [Ch][DHcit] (◆); and e) [Ch][Bit] (■). Distinct colours represent different concentrations, namely 10 wt% (**blue**), 5 wt% (**orange**), 2.5 wt% (**green**), 1 wt% (**yellow**). The ABS formed by PEG 600 and NaPA 8000 without electrolyte is represented by (—).

From the data shown in Figure 24, three different effects are identified when $[Ch]^+$-ILs are used as electrolytes in ABS formed by NaPA 8000 and PEG 600: *i)* increase of the biphasic region when increasing the concentration of [Ch]Cl and [Ch][Ac], acting as PEG/NaPA ABS formation enhancers; *ii)* decrease of the biphasic region with the [Ch][DHcit] and [Ch][Bit] concentration increase, acting as ABS formation depressants; and *iii)* no significant influence on the solubility/binodal curve of the PEG/NaPA ABS with [Ch][DHP]. It should be remarked that with the IL [Ch][DHP] a slight inversion in the trends behaviour seems to occur according to the polymer concentration (changing from a type *i)* to a type *ii)* effect as the systems moves from the NaPA-rich to the PEG-rich region). All previous reports suggest that the addition of electrolytes leads to an increase of the two-phase region of PEG/NaPA ABS [164,204,213], as also confirmed in this work using NaCl as electrolyte (data provided in Annexes, Fig. A.4 and Table A.16). These differences in the PEG/NaPA ABS phase

behaviour according to the electrolyte nature are here shown for the first time, while supporting the IL tailoring ability and their more complex nature in terms of chemical nature and possibility of interactions when compared to high melting temperature salts.

The changes in the ABS formation ability are also dependent on the polymers concentration, with the electrolyte induced changes in the binodal curves being more evident at the PEG-rich region. It seems thus that a saturation effect of the added electrolyte occurs at the PEG-rich phase. As previously reported for inorganic salts [203,204], the IL (electrolyte) concentration used to promote the ABS formation is a key parameter.

To fully understand the opposite effects promoted by $[Ch]^+$-ILs, it is important to look into the mechanisms behind the formation of non-ionic/ionic polymer-based ABS. When using combinations of non-ionic and ionic polymers to create ABS, still without considering the addition of an electrolyte, a phase containing a high concentration of the polyelectrolyte and the corresponding counterions, and another enriched in the uncharged polymer will be created, being both aqueous. In the absence of electrolytes or additives, the influence of the entropy of mixing of the polyelectrolyte counterions dominates the phase's separation [194]. However, when an electrolyte is introduced, the molecular-level mechanisms which rule the phase equilibria are modified, where "the entropy of mixing of the counterions no longer dominates over the entropy of the polyions", as discussed by Piculell and Lindman [194]. Previous works [198,203,204] provided two different explanations for the electrolytes influence on the PEG/NaPA phase equilibria, but all reported positive deviations or the enlargement of the biphasic region by the addition of salts. However, based on the opposite trends observed herein with the ILs evaluated as electrolytes in the PEG 600/NaPA 8000 ABS, additional interpretations on the molecular-level mechanisms ruling the two-phase formation of systems composed of non-ionic and ionic polymers are required. To better address these phenomena, the distribution of the IL electrolyte between the coexisting phases was experimentally determined. For all the $[Ch]^+$-ILs-based ABS, a mixture point within the biphasic region was selected and the ABS prepared (3 g of total weight; 25 wt% of PEG 600 + 7.5 wt% of NaPA 8000 + 10 wt% of the $[Ch]^+$-ILs + 57.5% water). After equilibrium, the phases were separated and the partition of the $[Ch]^+$-IL determined by proton nuclear magnetic resonance (^1H NMR). The electrolyte partition coefficients (K_{elect}), defined as the ratio of the concentration of electrolyte in the PEG-rich phase to that in the NaPA-rich phase, are depicted in Figure 25 (full experimental data are provided in Annexes, Table A.23).

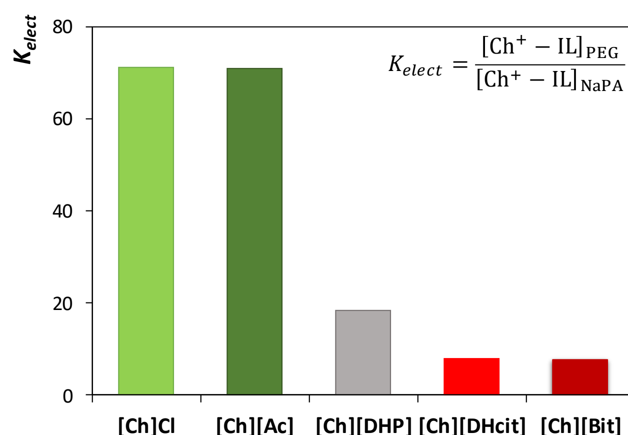

Figure 25. Electrolyte partition coefficients (K_{elect}) between the PEG-rich (top) phase and NaPA-rich (bottom) phase, determined for the $[Ch]^+$-based ILs used as electrolytes in the formation of ABS formed by PEG 600 + NaPA 8000 + water + 10 wt% of $[Ch]^+$-IL.

Figure 25 shows that the ILs partition to the PEG-rich phase follows the trend: [Ch]Cl and [Ch][Ac] (K_{elect} ≈ 71) >> [Ch][DHP] (K_{elect} ≈ 18) > [Ch][DHcit] and [Ch][Bit] (K_{elect} ≈ 8). Although all the $[Ch]^+$-ILs "prefer" more hydrophilic phases (octanol-water partition coefficients lower than 1 [192]), they have significant different solubilities in water. Pereira et al. [192] showed that the solubility of $[Ch]^+$-ILs in water ($mol.kg^{-1}$) at 298.15 K follows the trend: [Ch][DHcit] (3.2) < [Ch][Bic] (4.8) < [Ch][DHP] (13.5) < [Ch][Ac] (20.9) < [Ch]Cl (21.1). Based on these results, the most water soluble ILs are those that more extensively partition to the PEG-rich phase. This trend agrees with literature data on the partition of inorganic salts in PEG/NaPA ABS [204], in which more hydrophilic anions in terms of solvation free energy more extensively partition to the PEG-rich phase, as for example SO_4^{2-} with a K_{elect} = 1.0 *vs.* Cl^- with a K_{elect} = 1.14 [204]. Overall it could be said that electrolytes that extensively partition to the PEG-rich phase act as ABS enhancers, whereas those that are less enriched in the PEG-rich phase act as ABS depressants.

As expected, the ABS coexisting phases maintain their electroneutrality; yet, the addition of electrolytes can induce a dramatic effect on the phase equilibria, particularly in the compartmentalization mechanism of the NaPA 8000 counterions [198]. For instance, if a specific ion moves from the PEG-rich phase to the NaPA-rich phase, a counter-ion must be transferred in the opposite direction in order to maintain the electroneutrality in both phases. Therefore, when the IL electrolyte is only partially concentrated in the PEG-rich phase with a significant part of it in the NaPA-rich phase, as happens with [Ch][DHcit] and [Ch][Bit], there is the partition of Na^+ into the PEG-rich phase. On the opposite, when an electrolyte extensively partitions into the PEG-rich phase, as [Ch]Cl and [Ch][Ac], there is no significant Na^+ migration to the opposite phase. In summary, the

electrolytes that have a lower affinity to the PEG-rich phase, namely [Ch][DHcit] and [Ch][Bit], tend to increase the entropy of mixing of the polyelectrolyte counterions and consequently decrease the ABS liquid-liquid demixing ability. This negative influence over the phase equilibria was here observed with organic electrolytes, as $[Ch]^+$-ILs, being more evident when electrolytes with a lower affinity to the PEG-rich phase are used.

4.1.5. Conclusions

Opposite trends in the PEG/NaPA ABS formation ability were observed for the first time when using ILs as electrolytes. ILs that extensively partition to the PEG-rich phase, act as inorganic salts, enhancing the two-phase formation ability, while those that have a lower partition to the PEG-rich phase, decrease the ABS formation capacity. The distribution of the electrolyte ions between the coexisting phases and the polyelectrolyte ions compartmentalization are the key factors ruling the formation of PEG/NaPA-based ABS. The use of organic salts/ILs as electrolytes allows a wider diversity of phase equilibria, as well as the manipulation of their potential to improve the selectivity and efficiency of separation processes.

4.2. Selective precipitation of IgY using three-phase partitioning systems

This chapter is based on:

Sandra C. Bernardo, João A. P. Coutinho and Mara G. Freire

"Antibodies (Immunoglobulin Y) purification using three-phase partitioning (TPP) strategies",

ongoing work and manuscript under preparation[5].

4.2.1. Abstract

In the last decades, polymer-polymer aqueous biphasic systems (ABS) have been described as an effective approach for the purification of biologically active molecules, such as antibodies, since they have a large amount of water in their composition, thus providing a biocompatible environment for labile biomolecules. Among these, ABS formed by polyethylene glycol (PEG) and sodium polyacrylate (NaPA) with the additional of inorganic salts as electrolytes have been studied in order to purify several biocompounds. Still, and within the ABS concept, the main goal with previously published works was to induce the selective migration of the target molecule for one of the ABS phases, thus leading to its purification. Herein, we propose a different strategy on the application of these systems, i.e. as three-phase partitioning (TPP) systems, to purify the high-value immunoglobulin Y (IgY) from the water-soluble proteins fraction from egg yolk. Accordingly, the main goal is to induce the selective precipitation of the antibody at the interphase of the ABS, allowing its purification and easy recovery. Instead of the conventional inorganic salts applied, cholinium-based ionic liquids (ILs) were used as electrolytes in PEG-NaPA ABS. The effect of the polymer's molecular weight and their concentration and the nature and concentration of the IL electrolyte on the selective precipitation of IgY were investigated. At appropriate compositions and using adequate phase-forming components and IL electrolytes, the selective precipitation of IgY at the interphase was accomplished. IgY with 90% purity was obtained in a single-step from the WSPF of egg yolk.

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[5] **Contributions:** M.G.F. and J.A.P.C. conceived and directed this work. S.C.B. performed all the experimental work and interpreted the experimental data. The manuscript was mainly written by S.C.B., with significant contributions from the remaining authors.

4.2.2. Introduction

Significant efforts have been carried out on the finding of new and cheaper sources of antibodies. Antibodies or Immunoglobulins can be used in diagnosis and in many therapeutic applications, being nowadays one of the most promising classes of biopharmaceuticals [9,27,66]. Although large interest has been devoted to passive immunotherapy by the use of mammal antibodies, namely immunoglobulin G (IgG) taken from mammals serum, immunoglobulins from egg yolk (IgY) have also been reported as viable alternatives [9,27,42,49,66,71,120,123]. Furthermore, more than 100 mg of IgY can be isolated *per* egg, corresponding to the same amount that is obtained from 200 mL of animal serum (e.g. rabbit serum) [26,42,49]. Additionally, IgY is obtained by less-invasive methods, contrarily to IgG that requires the repeated bleeding of immunized mammals [30,42,191]. Despite these advantages, the production cost of high-quality IgY still remains higher mainly due to the lack of an efficient purification strategy [72,191]. Typically, the first step on the purification of IgY from egg yolk consists on the precipitation of the lipids fraction to obtain a water-soluble protein fraction, which contains IgY as well as a large number of other proteins [72,120,127,131,191,217,218]. In order to obtain a highly pure IgY sample, additional techniques are then applied to the WSPF, increasing the final cost of the overall process and of the target antibody [122–124,126]. Hence, the search of new and more scalable fractionation platforms for the purification of IgY is a crucial requirement. In this context, liquid-liquid extractions using aqueous biphasic systems (ABS) could be seen as a viable option [67,138].

Aqueous biphasic systems (ABS) were introduced in the 50s by Albertsson [219], and in the past decades have been described as a biocompatible extraction technique for numerous labile biomolecules (e.g. antibodies [67], enzymes [139], etc.). ABS are formed when two water-soluble compounds, such as two polymers, two salts, or one polymer and one salt, are mixed in water, and above a given concentration two liquid phases are formed [141]. For several years, ABS composed of polymer and inorganic salts have been reported as improved systems since they present low interfacial tension, high phase separation rates, and use low cost phase-forming components. Nevertheless, these systems also present major drawbacks associated to the high consumption of both polymers and salts [140,150], i.e. high amounts of polymers and salts are required to form two-phase (liquid-liquid) systems. Although described as systems of higher viscosity and higher cost, polymer-polymer ABS, such as PEG-Dextran, may have additional advantages compared to polymer-salt systems, namely the requirement of lower amounts of the phase-forming components to create ABS. In the past decades, several studies have been carried out in order to find low-cost polymers to replace dextran [164,220]. In particular, polyethylene glycol (PEG)-sodium polyacrylate

(NaPA) ABS have been proposed and successfully explored as an alternative class of polymer-polymer systems, of low viscosity and using low cost polymers [150,164,204,220–222]. Nonetheless, NaPA is a negatively charged polyelectrolyte, and in order to decrease the amount of polymers required to form ABS with PEG, inorganic salts as electrolytes are usually applied. These electrolytes contribute with the addition of a counter ion to the system, allowing the compartmentalization of the polyelectrolyte in one of the phases, thus enhancing phase separation [204,221]. Sodium chloride or ammonium sulphate are the electrolytes of choice for these PEG-NaPA systems [204]; recently, ionic liquids (ILs) were proposed as alternative electrolytes. The use of ILs was shown to be particularly relevant to tailor the partitioning of target molecule, such as cytochrome c and chloranilic acid, amongst the two phases [207]. Santos et al. [207] used imidazolium- and ammonium-based ILs as novel electrolytes to induce the two phases formation of ABS composed of PEG and NaPA (8000 $g \cdot mol^{-1}$) and to evaluate these systems extraction performance. Nevertheless, most of these ILs are not suitable for biological applications given their moderate toxicity and low biodegradability [161]. Given the plethora of ILs available nowadays, cholinium-based ILs as electrolytes could be used instead, and since the cholinium cation is derived from natural sources these ILs contribute to an increase on the PEG-NaPA ABS biocompatibility [187–189].

Within liquid-liquid separation strategies, three phase partitioning (TPP) systems have been described as a novel bioseparation strategy, originally created by t-butanol and ammonium sulphate in aqueous media to precipitate proteins from crude matrices [223–225]. In these systems, the protein of interest is insoluble and precipitates at the interphase between the upper butanol-rich phase and the lower salt-rich phase, forming a third middle phase [223–225]. Alvarez-Guerra et al. [226,227] demonstrated that this concept can be applied to IL-based ABS. This type of IL-TPP, contrarily to conventional TPP systems, does not require the use of volatile organic solvents, such as t-butanol, while taking advantage of the tailoring nature of ILs. In summary, TPP can be considered a method of choice for large scale proteins purification, since it is simple, does not require the use of sophisticated equipment, is easily scalable, and a fast technique that enables the purification and concentration of the target protein in a single-step [227]. Moreover, TPP can be seen as a better method when comparing with ABS, since most of the times in the latter process the protein must be recovered from the phase in which it is enriched [223].

Herein, we investigate the application of ABS composed of PEG, NaPA and cholinium-based ILs as novel electrolytes in the purification of IgY from the water-soluble fraction of proteins (WSPF) obtained from egg yolk. As a first approach, the selective extractive performance of these systems

for IgY was ascertained, by determining some parameters such as the recovery yield and purity of IgY upon changing the composition of the ABS in terms of the polymer's molecular weight, their concentration, and added electrolyte and its concentration. After this initial screening, the TPP concept was applied to induce the selective precipitation of IgY at the ABS interphase.

4.2.3. Experimental Procedure

Materials. Poly(ethylene glycol) of molecular weights of 600, 1000, 4000 and 8000 $g{\cdot}mol^{-1}$ (PEG 600, 1000, 4000 and 800, respectively) and the aqueous solution of Na-poly(acrylate) of 1200 and 8000 $g{\cdot}mol^{-1}$ (45 wt%) were purchased from Sigma-Aldrich. Cholinium chloride, [Ch]Cl, and cholinium dihydrogencitrate, [Ch][DHcit], were acquired from Sigma-Aldrich. Cholinium acetate, [Ch][Ac], and cholinium dihydrogenphosphate, [Ch][DHP], were acquired from Iolitec. Cholinium bitartrate, [Ch][Bit], was purchased from Acros Organics. Phosphate buffered saline (PBS, pH 7.4) was used for IgY dilution procedures, and was acquired from Sigma-Aldrich, in which one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. All other chemicals were of reagent grade. Eggs were obtained from non-hyperimmune hens provided by "HenBiotech" from Biocant.

Preparation of water-soluble protein fraction (WSPF). In order to obtain the containing IgY water-soluble protein fraction (WSPF), the egg yolk was carefully separated from the egg white, followed by a water dilution method and freezing and thawing, as proposed by Hodek et al. [131]. This prepared WSPF was used in the formulation of ABS and TPP aiming the purification of IgY.

Preparation of aqueous biphasic systems (ABS) and three-phase partitioning (TPP) systems. The phase diagrams of the systems investigated here were previously determined, and are discussed in detail in Chapter 4.1. Each ABS was prepared by weighting the appropriate amounts of each component: PEG, NaPA, [Ch]-based ILs, WSPF and water, up to total mass of 3 g. Each mixture was stirred, and left to equilibrate for at least 30 min at (25 ± 1)°C, allowing the proteins partitioning between the coexisting phases, or their precipitation at the interphase, and then centrifuged at 1000 rpm for 10 min. Afterwards, the top and both phases were completely separated and taken for further analysis, as well as the precipitated pellet of proteins at the interphase, which was carefully recovered and resuspended in PBS aqueous solution for IgY quantification and to appraise the antibody purity.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins profile of the analysed samples was investigated by SDS-PAGE using an Amersham ECLTM Gel from GE Healthcare Life Sciences. Samples were directly mixed with the Laemmli buffer (1:1, v:v) in presence

or absence of the reducing agent, dithiothreitol (DTT), and then heated at 90 °C for 5 min for a complete denaturation of proteins, and subjected to SDS-PAGE in 20 % polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue G-250 for 2-3 h and then distained with a mixture of methanol, acetic acid and distilled water (50:37:413, (v:v:v)) at room temperature. All gels were analysed and processed using the ImageJ (NIH) analysis tool. The purity of IgY in each sample was quantify by densitometry of the bands in the gels. The molecular weight marker used was the commercial Amersham ECLTM Full-Range Rainbow Molecular Weight Marker, from 12 to 225 kDa.

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). The quantification of IgY and in the samples obtained by the best studied methods was additionally performed by SE-HPLC. A Chromaster HPLC (VWR, Hitachi) coupled with and diode array detector was employed. SE-HPLC was performed in an analytical column (25 cm × 2 mm i.d., 25 µm), Shodex Protein KW-802.5. Before injection, each sample solution was diluted 10 times (v:v) in the mobile phase buffer (100 mM phosphate, 150 mM NaCl at pH 7). The mobile phase was run isocratically with a flow rate of 0.5 mL/min. The column oven and autosampler were kept at constant temperatures of 40 and 10 °C, respectively. The injection volume was 25 µL and each sample was injected at least 2 times. A range of wavelengths (200-400 nm) was analysed and the results were integrated at the wavelength of maximum absorption (280 nm). The retention time of IgY was found to be 13.5 min within an analysis time of 40 min. Due to UV interferences of NaPA we could not analyse and quantify most of the bottom NaPA-rich phases. In these cases, a mass balance approach was applied.

4.2.4. Results and discussion

Herein we report the use of cholinium ([Ch])-based ILs as a new class of electrolytes in the formation of ABS composed of PEG and NaPA, and how these systems act as enhanced purification platforms of IgY from the WSPF of egg yolk. The experimental binodal curves of the studied ABS, required to infer the mixture compositions required to form two-phase systems to act as separation strategies, are described and discussed in Chapter 4.1. Several parameters that could influence the proteins partition of the WSPF were evaluated, such as the polymers molecular weight and their concentration, and the added electrolyte nature and concentration. ABS were first evaluated as extraction platforms, where the goal was to selectively extract IgY for one of the phases while the remaining proteins should partition to the opposite phase. After identifying the systems which allow the IgY precipitation, the TPP concept was explored, aiming the selective precipitation of IgY

and the creation of an interphase (ideally corresponding to pure IgY that could be easily recovered). In all systems investigated, the top phase corresponds to the PEG-rich phase while the bottom phase is mainly enriched in NaPA and water.

4.2.4.1. Feedstock (WSPF) characterization

The WSPF was recovered from egg yolk, after the removal of lipoproteins and lipids, as described by Hodek et al. [131]. The obtained WSPF was used in the formulation of ABS and TPP systems aiming the purification of the target antibody. IgY corresponds to about 30% of all proteins in the WSPF, as shown in Figure 26(A) (lane 1), which contains a total protein concentration of 5 g/L (as determined by us). Even after the removal of lipoproteins, the IgY purity in the WSPF is significantly low (*ca.* 30%), reinforcing the complexity of the matrix used and the current high cost of high-quality/high-purity IgY.

4.2.4.2. Extraction of IgY from the WSPF for one of the phases of ABS

An important factor that influences the protein partitioning in ABS is the molecular weight of the polymer [144]. Therefore, we initially studied the proteins partition profile in ABS formed by PEGs with a molecular weight ranging between 600 and 8000 $g{\cdot}mol^{-1}$. The mixture composition used corresponds to 20 wt% PEG (600, 1000, 4000 and 8000 $g.mol^{-1}$) + 8 wt% NaPA + 1 wt% [Ch][DHP] + 20 wt% WSPF. The IL electrolyte concentration was kept at 1 wt% in these studies. Figure 26 shows both SDS-PAGE and SE-HPLC results. Figure 26(A) depicts the SDS-PAGE results, showing that with PEG 600 no selective extraction of IgY was obtained. In this system, all the proteins of the WSPF migrate to the top (PEG-rich) phase (Figure 26(A), lane 2). This trend was also confirmed by the SE-HPLC chromatograms shown in Figure 26(B). Furthermore, all the studied ABS present a pH value higher than the IgY pI (6-7) [30,42], meaning that the target antibody is negatively charged at the working pH (7 to 8). Therefore, the IgY partitioning among the phases is not ruled by electrostatic interactions since the charged species used in the ABS formation corresponds to NaPA and IgY preferentially partitions to the PEG-rich phase.

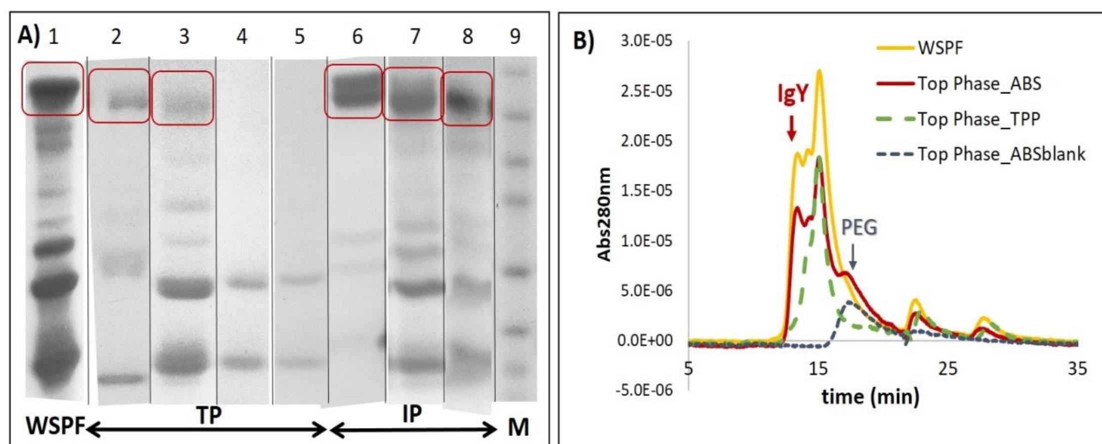

Figure 26. PEG molecular weight effect on the IgY partitioning and precipitation. **A)** SDS-PAGE: WSPF in lane 1; top phases of the systems in lanes 2 to 5; interphases of the systems in lanes 6 to 8; molecular weight marker in lane 9. ABS composed of 20 wt% PEG (X) + 8 wt% NaPA + 1 wt% [Ch][DHP] + 20 wt% WSPF, where X: PEG 600 (lane2), PEG 1000 (lanes 3 and 6), PEG 4000 (lanes 4 and 7) and PEG 8000 (lanes 5 and 8). The IgY band under non-reducing conditions (ca. 180 kDa) is identified with the red squares. **B)** SE-HPLC chromatograms of the WSPF feedstock (yellow), of the top phases of ABS extractions (red) and TPP systems (dashed green line), and the blue dashed line corresponding to a blank of the top PEG-rich phase, for the systems composed of 20 wt% PEG (600 and 1000) + 8 wt% NaPA + 1 wt% [Ch][DHP] + 20 wt% WSPF.

When PEG 1000 or a polymer with a higher molecular weight is used, a precipitation phenomenon occurs, creating an interphase of precipitated proteins in the ABS. After the proteins pellet recovery and resuspension in PBS (phosphate buffered saline, 0.01 M, pH 7.4) aqueous solutions, and its analysis by SDS-PAGE, it is possible to observe that the main protein present corresponds to the target IgY protein (Figure 26(A), lanes 6-8). Furthermore, in the SE-HPLC chromatogram of the corresponding top phases it is shown the disappearance of the peak corresponding to IgY at 13.5 min (Figure 26(B) – "WSPF" vs "Top Phase_TPP"), meaning that IgY it is not present in top phase. However, one of the main contaminant proteins is still present at these systems top phases, indicating that IgY is being selectively precipitated at the interphase. Additionally, with the increasing of the PEG molecular weight, the quantity of total proteins that precipitates also increases, leading to a decrease on the IgY purity (Figure 26(A), lanes 6-8).

In summary, regarding the PEG molecular weight effect, it is important to differentiate two phenomena: one occurring with lower molecular weight polymers (PEG < 1000 $g{\cdot}mol^{-1}$) where the proteins do not precipitate and are completely soluble in the PEG-rich phase, but no selectivity for IgY was identified, and the other occurring with higher molecular weights of PEG (PEG ≥ 1000 $g{\cdot}mol^{-1}$), where the proteins precipitation occurs leading to the creation of an interphase in the ABS, in

which the IgY is the main component and fitting within the TPP approach. In the last example, the selective precipitation of IgY over the remaining proteins favourably occurs with PEG 1000.

Since the target protein is enriched at the liquid-liquid interface of the ABS, we are purifying the IgY not by a common partitioning between the two phases of the ABS, but by the so called TPP method. The precipitation of proteins is particularly relevant when using ABS formed by polymers with higher molecular weight since in these ones there is a lower water content at the polymer-rich phases, thus decreasing the proteins solubility [141], and as shown and discussed in Chapters 3.1 and 3.2.

Additional studies were performed in order to ascertain if the NaPA molecular weight could affect the IgY purification. NaPA with a molecular weight of 1200 g·mol^{-1} was also used to create ABS with different compositions, while maintaining the PEG composition. PEG 600 was chosen since it does not lead to proteins precipitation according to the results discussed above. Contrarily to the effect of the PEG molecular weight, the molecular weight of NaPA does not affect the partition behaviour of IgY amongst the coexisting phases (Figure 27(A)), where all proteins present in the WSPF preferentially migrate to the top (PEG-rich) phase.

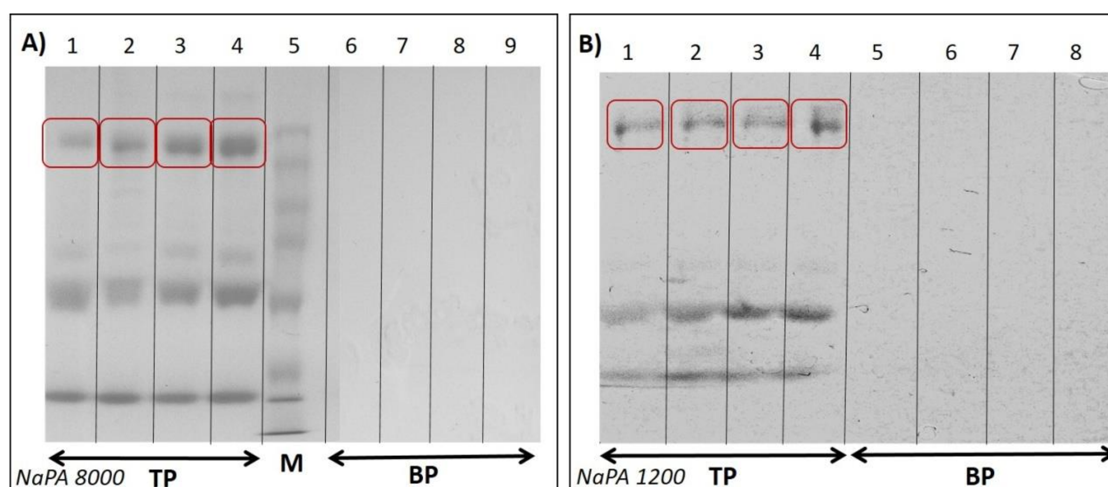

Figure 27. NaPA molecular weight effect on the selective IgY partitioning appraised by SDS-PAGE: ABS composed of PEG 600 and NaPA 8000 **(A)** or NaPA 1200 **(B)**. The first lanes correspond to the ABS top phases, whereas the last lanes correspond to the ABS bottom phases.

4.2.4.3. Purification of IgY from the WSPF using TPP systems

As described above, when PEG 600 is used no precipitation of proteins occurs in the ABS interphase. This result was obtained for all the studied conditions, i.e. by changing the amount of

phase-forming components of each phase (PEG and NaPA), the NaPA molecular weight, and the IL electrolyte nature. Therefore, the selective partitioning of IgY in the studied ABS is difficult to achieve since both IgY and the remaining proteins are enriched in these systems top phases. The most promising results were obtained with ABS formed by PEG of higher molecular weight, in which proteins precipitation occurs and a pellet is formed at the interphase. In this set of results, some selectivity for IgY was observed, leading us to the study these PEG-NaPA systems within the TPP approach.

We first tested the proteins precipitation by using isolated aqueous solutions of the phase-forming components to address if the formation of an ABS is a required step. By using NaPA or ILs aqueous solutions (without the presence of PEG) no precipitation of the proteins present in the WSPF was observed. On the other hand, when using aqueous solutions of PEG, well known for its capacity to precipitate proteins and as shown in Chapter 2, we observed the precipitation of all proteins from the WSPF, yet with no selectivity for IgY. Therefore, the use of all phase-forming components and creation of ABS is a required issue to induce the selective precipitation of IgY. Furthermore, there is a significant difference between the molecular-level mechanisms involved in proteins precipitation by TPP systems and the commonly used precipitation of proteins by agents such as PEG. In TPP, proteins are excluded from the two aqueous phases creating a third phase, in which this precipitate can be concentrated into a thin disk by low speed centrifugation [225]. Dennison and Lovrien described that there are essentially two mechanisms for protein precipitation, pulling and pushing, in which both involve macromolecule hydrate water [223]. Pushing is a thermodynamic consequence of an exclusion crowding mechanism accomplished by a large concentration of the precipitation agent, like PEG, resulting in lower water contents and changes on protein conformation. Pulling results in a protein conformation tightening, but for different reasons, much lower concentrations of salt are used and electrostatic interactions between the agent and the protein take place [223,225]. With the PEG induced precipitation, the protein starts to precipitate when there is not sufficient water [225]. Nevertheless, the latter requires high speed centrifugation, while in TPP, which is a hybrid mechanism where both pushing and pulling effects occur, only low speed centrifugation is required to concentrate the pellet into a thin phase between the two phases. Furthermore, we believe that the presence of the NaPA in TPP is also highly relevant since this polymer presents a highly hygroscopic nature, thus affecting the proteins structure by sequestering hydration water molecules to the respective phase. This will induce a salting-out-like phenomenon of the protein by exposing the hydrophobic parts at the surface, leading ultimately to the protein precipitation at the interphase.

Based on the possibility of using TPP systems formed by PEG, NaPA and ILs electrolytes to purify IgY by induced (selective) precipitation, the influence of the nature of the added electrolyte was first addressed, namely with [Ch]Cl, [Ch][Ac], [Ch][DHP], [Ch][DHcit] and [Ch][Bit]. In this study, the following mixture composition was used: 20 wt% PEG 1000 and 8 wt% NaPA 8000 with 1/5 wt% of [Ch]-IL.

The proteins profile appraised by SDS-PAGE is shown in Figure 28. ABS formed by ILs constituted by [DHP]-, [DHcit]- and [Bit]- anions are the ones that more effectively induce the IgY precipitation at the interphase (lanes 1, 4 and 5 of Figure 28 (A)), while the other two ABS composed of Cl- and [Ac]- anions are less prone for IgY precipitation (lanes 2 and 3 of Figure 28(A)). This effect is even more pronounced when 5 wt% of electrolytes is used (lanes 3 and 8 of Figure 28(B)). Therefore, both the IL nature and its concentration influence the intensity of proteins precipitation. Even that the increase of the IL concentration is favourable to induce the precipitation of IgY, it is also favourable to induce the precipitation of the remaining WSPF proteins, therefore decreasing the IgY purity (lanes 4 and 5 of Figure 28(B)).

PEG-NaPA systems are usually investigated in the presence of electrolytes, both to be able to create ABS as well as to decrease the amount of phase-forming components required for liquid-liquid demixing [164,204]. In fact, no electrolyte is required to form ABS with PEG with 600 and 1000, allowing us to study the PEG-NaPA ABS capacity (with no electrolyte added) to induce the IgY precipitation. Without the addition of the IL electrolyte there is also the precipitation of proteins at the interphase; nevertheless, the yield of the purification process is inferior compared to the values obtained when using ILs, as shown in Figure 28 (lane 12), in which the top phase also contains IgY. The IgY precipitation is thus favoured in presence of ILs electrolytes (Figure 28(A), lane 1-5 and 7-11), being in agreement with the results of Alvarez-Guerra et al. [226], where the authors found that higher recovery yields of the target protein at the interphase are obtained in presence of ILs. In summary, the type and nature of the added IL electrolyte has a significant influence on the precipitation phenomenon, in which [Ch][DHcit] and [Ch][DHP] are more prone to induce the selective precipitation of IgY.

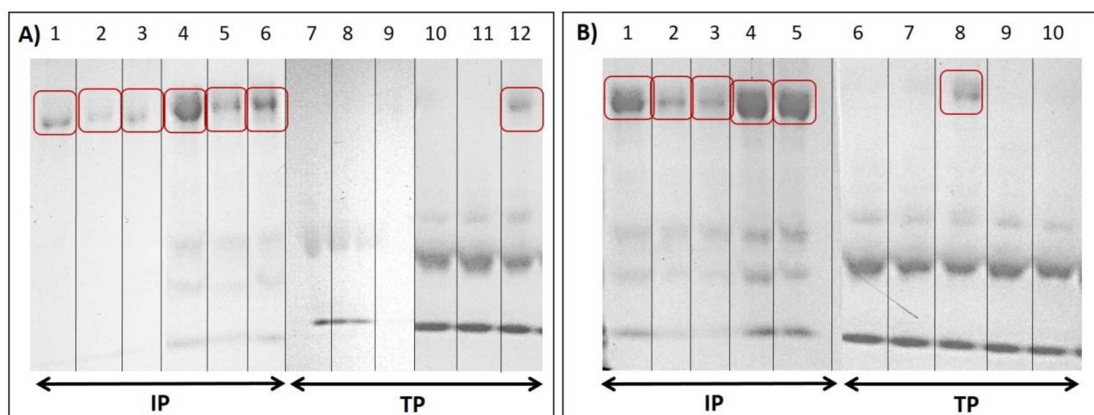

Figure 28. Effect of the type and concentration of the added [Ch]-IL on the IgY purification appraised by SDS-PAGE. ABS composed of 20 wt% PEG1000 and 8 wt% NaPA 8000 and [Ch]-IL. (A): systems constituted by 1 wt% of [Ch]-IL, except lanes 6 and 12 (no IL added). (B) constituted by 5 wt% of [Ch]-IL. ILs are according to the order, from left to right: [Ch][DHP], [Ch][Ac], [Ch]Cl, [Ch][DHcit] and [Ch][Bit]. IP: interphase; TP: top phase. The IgY band is marked in the red boxes.

To explore the influence of the molecular weight of the polymers and the composition of the phase-forming components in the IgY purification by TPP, the systems described in Table 8 were prepared and evaluated. According to the results shown in Figure 29, by using the ABS formed PEG 1000, it is possible to selectively induce the precipitation of IgY, which can be recovered with a purity higher than 70%. In fact, all systems that allow to obtain IgY with a purity higher than 70% are composed of PEG 1000 (as highlighted by the dashed line in Figure 29). Only one exception was observed with PEG 1000, in which the TPP does not occur and no interphase pellet was formed, when using lower concentrations of the phase-forming components which seem not enough to increase the hydrophobicity of the PEG-rich phase. Poorer results are observed with ABS composed of PEG with higher molecular weight (PEG 4000 and 8000). Also, with these two PEGs, when lower amounts of phase-forming components are used no precipitation occurs (Table 8). Moreover, an increase in the PEG or NaPA content leads to higher IgY purification values. By increasing the PEG content in the ABS from 16 wt% to 20 wt% (system 7) or the NaPA from 8 wt% to 12 wt% (system 6), IgY with a purity ca. 90% is obtained at the interphase.

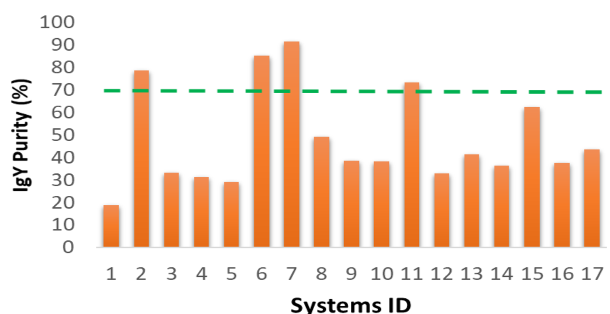

Figure 29. IgY purity (%) in the recovered pellet using the ABS and TPP approach using the systems described Table 8.

Table 8. Studied ABS within the TPP approach to purify IgY from the egg yolk WSPF: PEG concentration (16–20 wt%), NaPA concentration (8–12 wt%), and PEG molecular weight (1000–8000 g.mol^{-1}); and IgY purity (%) in the recovered pellet.

System	PEG / wt%	NaPA / wt%	PEG / (g·mol^{-1})	IgY Purity (%)
1	16	8	1000	18.82
2	20	12	1000	78.39
3	16	12	8000	33.14
4	20	8	8000	31.14
5	18	10	4000	28.94
6	16	12	1000	85.11
7	20	8	1000	91.37
8	16	8	8000	49.06
9	20	12	8000	38.54
10	18	10	4000	37.98
11	18	10	1000	73.25
12	18	10	8000	32.69
13	16	10	4000	41.13
14	20	10	4000	36.23
15	18	8	4000	62.15
16	18	12	4000	37.60
17	18	10	4000	43.50

4.2.5. Conclusions

ILs have been extensively used as phase-forming components of ABS or used as adjuvants in polymer-salt systems to extract biomolecules. Herein, we use ABS composed of PEG/NaPA and ILs as electrolytes to purify IgY from the egg yolk WSPF. A range of conditions, such as the molecular

weight of the polymers, the concentration of the phase-forming components, and IL anion nature, were investigated. When using PEG with a lower molecular weight, all the proteins present in the WSPF of egg yolk preferentially partition to the upper PEG-rich phase. Nevertheless, when higher molecular weight PEGs are used it is possible to obtain a precipitated pellet containing the target protein (IgY) at the interphase of the ABS, fitting within the TPP approach. By the optimization of additional parameters high purity and recovery levels of IgY were obtained, being the best results achieved with the system composed of 20 wt% PEG1000 + 8 wt% NaPA + 1wt% [Ch][DHP] + 20 wt% WSPF, in which IgY is obtained with a purity higher than 90%.

The application of the TPP concept represents a promising approach to purify IgY. Furthermore, TPP presents additional advantages over ABS in which the selective separation of a target protein to a given phase is envisioned: (i) the phase-forming components can be easily recovered and recycled; and (ii) there is no need to apply a subsequent step to recover the target protein from the ABS phase in which it is enriched. Nevertheless, future studies are still required, namely on the increase of the loading capacity of the WSPF feedstock, on the validation of the phase-forming components recyclability, and an economical evaluation of the proposed process.

5. IgY as a High-Value Egg Product: Business Plan

This chapter is based on a final report presented in the discipline "Entrepreneurship" of the PhD Program in Chemical Engineering of University of Aveiro (final classification of 19, in a 0-20 scale)

Sandra C. Bernardo

"AveIGs – A high-value egg product"

5.1. Summary

"AveIGs" is a putative biotechnology company in which its main goal is to produce pure Immunoglobulins from egg yolk (IgY) at significantly low production costs, expectable to produce 100 mg of highly pure IgY (with *circa* 90% purity) for about 10€ of production costs.

Strongly motivated by the fact that the technologies nowadays available to produce pure antibodies from egg yolk (IgY) are not cost effective, and that this antibody can be used as an alternative biopharmaceutical in passive immunotherapy, our proposal is to provide pure IgY using low-cost techniques to allow its widespread use in the near future. Moreover, there is a strong pressure to withdraw or limit antibiotic used in animal feed and to look for viable alternatives. Thus, IgY can be used as a food additive for livestock to target specific pathogens and also to improve the growth and feeding efficiency of animals. IgY has been found to be effective against a number of human pathogens and diseases; hence, antibodies, namely IgY, may be useful for passive immunization in humans when the immune system of those patients is compromised or when the foreign pathogens get resistance to conventional and cheaper antimicrobials. Alternatively, to the use of an antigen-specific antibody for passive immunization applications, these avian antibodies can be also used to improve the overall human health, by enhancing our immune system.

IgY can be obtained from egg yolk, allowing to generate large quantities of antibodies in a non-stressful and non-invasive way and at lower costs, instead of using animal blood as an antibody (IgG) source. Additionally, no toxic chemicals are used during the whole process. Therefore, the wasted products can be used afterwards for by-products valorisation.

We believe to be able to provide cheaper and high-quality antibodies derived from a natural source (egg yolk) that can be used as new bioactive ingredients on nutraceutical products, which ultimately will benefit the final consumer by improving its immune system, health in general and quality of life.

5.2. Introduction

5.2.1. What is Immunoglobulin Y (IgY) and its Relevance?

IgY is a type of antibody that is the functional equivalent of the mammal immunoglobulin G (IgG), produced during egg formation of some hens [49]. Immunoglobulins (Ig) or antibodies are glycoproteins found in plasma and extracellular fluids of all vertebrate species, which constitute the humoral branch of the immune system and approximately correspond to 20 % of the plasma proteins in humans [8,9]. Antibodies are produced in response to bacteria, viruses, foreign molecules or other agents [8,228]. The production of antibodies acts as a response or key mechanism used by the host organism to protect itself against foreign molecules or organisms [228]. Thus, antibodies such as IgY can be used in passive immunization applications [30]. The commercially available polyclonal antibodies have been mainly produced by mammals (mice, rats, rabbits, sheep, goats, and horses), and are obtained from the sera of these animals after their immunization [27]. Still, these polyclonal antibodies (IgG) cannot be prepared on a large commercial scale because of the difficulty in obtaining high quantities of sera. On the other hand, the method of producing antibodies in hens (IgY) is less invasive and stressful to animals, requiring the collection of eggs instead of blood collection [27,102]. Additional to the fact that the IgY production is more viable than IgG, avian antibodies also present immunogenicity advantages since IgY does not activate mammalian complement or interact with mammalian Fc receptors that could mediate an inflammatory response [27,102], meaning that the unwanted immune reactions to the therapeutic antibodies can be avoided.

5.2.2. Where is the "Pain"?

In egg yolk, IgY is the predominant and most important fraction of antibodies, belonging to the livetins (α-, β-, and γ-livetin) class of proteins that constitute the water-soluble protein fraction [56]. However, egg yolk is mainly composed of lipids, proteins, carbohydrates and minerals, being this a very complex matrix, which makes the extraction and purification of the target IgY extremely difficult. Thus, the current commercially available IgY is still highly expensive [229], and either higher quantities of antibodies are produced at low concentration or lower quantities of IgY at high concentration are commercialized. In summary, besides the advantages of using IgY instead of using IgG, the production cost of high-quality IgY for large-scale applications still remains higher than other drug therapies [30], thus raising the requirement of developing cost-effective purification techniques for this alternative class of antibodies.

5.2.3. Which Market Segments can be addressed?

Antibodies were one of the first agents used to fight specific infectious diseases [105]. The first successful case involving the use of antibodies to treat illness by passive immunization was reported in the late 19th century, to treat patients with diphtheria, a dangerous disease that obstructs the throat and airways [1,3]. Although their potential effect as therapeutics, their widespread use is conditioned by the requirement of high amounts of high-purity antibodies. On the other hand, with the appearance of low cost antibiotics, and as vaccines were developed, the use of passive immunization lagged behind [230]. Nevertheless, during the last years, antibiotics have been playing a major role in the development of multi-drug resistant bacteria, mostly due to their over-prescription and incorrect use [231]. Antimicrobials also have been largely used in the poultry and cattle industries for growth promotion, disease prevention, and treatment of infections for many years, leading to an enhancement of the bacteria resistance problem [92,231]. Accordingly, nowadays there is a strong pressure for these industries to withdraw or limit the antibiotic use in animal feed and to look for viable alternatives. Hence, IgY can be used as a food additive for livestock to target specific pathogens and also to improve the growth and feeding efficiency of the animals [49].

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, namely against some bacteria, such as *Pseudomonas aeruginosa* colonization in the airways of cystic fibrosis patients, and also against fungal infections, like the prevention of the growth, adherence, and biofilm formation of *Candida albicans* [30,59]. Antibodies such as IgY may be useful for passive immunization in humans when the immune system of those patients is compromised or when the foreign pathogens get resistance to the conventional and cheaper antimicrobials. Moreover, antibodies can be effective against virus, such as the pandemic influenza virus H5N1 or H1N1, and since this type of immunization is a fact process, rather than developing the vaccines (which can take several months in some cases) this immediate protection may be a preferable option [30]. Likewise, in a bioterrorism event this fast protection afforded by antigen-specific IgY has also been suggested as a major emergency response solution [30].

Alternatively to the use of antigen-specific antibodies for passive immunization applications, IgY antibodies can be used to improve the overall human health, by enhancing the immune system [99]. One of the benefits of egg consumption corresponds to the improvement of our natural defences against common diseases, which is accomplished by the presence of naturally present antibodies in egg yolk. However, egg yolk presents a high lipid content and low IgY content. As a

result, the antibodies that are normally ingested in our diet are not sufficient for an effective immune modulation [99]. IgY should be purified and recovered from egg yolk, and concentrated, allowing therefore its use as a primer ingredient on nutraceutical products. In addition to their therapeutic relevance, antibodies play a major role in biological and medical research, used in a variety of diagnostic tools [49].

5.3. Market Segmentation

Based on the exposed, there are 3 possible market segments for the developed pure IgY product: the nutraceutical industry (for both animal and human food supplements); the pharmaceutical industry (for passive immunization); and the industry of biochemical suppliers (for bioassays used in diagnostic tools). For each market segment, an evaluation of its size, value and readiness for our product, with values ranging from 1 to 3 (1= low and 3= high), is given in Table 9.

Table 9. Market Analysis: readiness, size and value. *Rating: 1- weak; 2- medium; 3 – strong.

Market Segment	Market Readiness*	Market Size*	Market Value*
Food Supplement (Animals)	③	③	①
Food Supplement (Human)	③	③	②
Pharmaceutical	①	②	③
Bio-products Suppliers (Bioassays)	②	②	②

The pharmaceutical segment is not an immediate viable choice for our IgY product, since at first plenty of clinical trials are required, meaning that more resources (time and money) are mandatory. We believe that the pharmaceutical market is not currently ready for this product to be used in passive immunization. Besides the high-value of this market segment, its size is not big enough for our type of product since there are also a lot of competitors that offer high quality/high quantity antibodies, namely the human poly- and mono-clonal antibodies that are produced in mammal cells culture. However, the major drawback of these products still is their high cost, a problem that IgY may overcome but requiring at least a decade of further studies/clinical trials and investment.

Concerning the industry of the biochemical suppliers for laboratorial purposes, like Sigma-Aldrich for instance, this market still needs to grow, since nowadays the most common antibodies that are used for bioassays are obtained from a mammal origin (IgG). We believe that the scientific community may change their approaches along time if IgY antibodies start to appear in the market, opening a window of opportunity for our business in the future. Moreover, if IgY antibodies (IgY) could be produced at lower costs, the costs of bioassays will be also lower.

Finally, the industry of food supplements, either for feedstock animals or for the nutrition supplementation in humans, is the segment with highest potential in the near future, particularly taking into account the developed IgY purification technologies. We believe that this market is ready to embrace our product, particularly because immunoglobulins are proven to be helpful on improving the immune system of both animals and humans [30], and because this segment does not require clinical trials. However, there are some evidences suggesting that the cost of eggs may still be an obstacle for the broader use of IgY in some commercial applications, particularly in animal feed supplements [30]. On the other hand, the market of human food supplements is vast, with a tendency to grow in the future (see the analysis below). Therefore, and although the biopharmaceutical market still is our main goal, in the near future, we will concentrate our efforts on commercializing IgY antibodies to human nutraceutical companies.

5.3.1. Nutraceutical Market Analysis

From the early 90s, there has been a considerable shift in the nutraceuticals use. Nowadays, consumers (especially from developed countries) are more conscious and aware about their health. Moreover, food supplements are not only being consumed to meet the recommended dietary income, but also as a mechanism of performance enhancement, by athletes or students for instance, and for diseases prevention, such as cancer, cardio-vascular and degenerative diseases. In addition, consumers' preference has now undertaken a paradigm shift from synthetic ingredients toward natural and organic foods, beverages and supplements [232]. Hence, the World nutraceutical market has been growing exponentially in the last years. From 1999 to 2002, the nutraceutical industry grew at an Annual Average Growth Rate (AAGR) of 7.3 percent, while from 2002 to 2010, the AAGR doubled to 14.7 percent [232]. According to a new market report published by Transparency Market Research "Nutraceuticals Product Market: Global Market Size, Segment and Country Analysis & Forecasts (2007-2017)", the Global Nutraceutical Product market reached $142.1 billion in 2011, and is expected to reach $204.8 billion by 2017, growing at a Compound Annual Growth Rate (CAGR) of 6.3% from 2012 to 2017 [233].

The nutraceuticals market in Europe is mainly driven on the basis of health claims [232]. Furthermore, the intake of nutraceuticals may be of special relevance for the elderly population. In Figure 30 it is possible to observe the countries in which these products have a higher impact, namely Germany, France and Italy, which are key markets, while UK and Spain have emerged as the key test markets, resulting in a high frequency of product launches. The nutraceutical global market in Europe in 2010 corresponded to US$ 35 billion, while the specific nutraceutical ingredients

market represented only US$ 4.1 billion. Additionally, in the Europe ingredients market, immunoglobulins correspond to 35% of this global portion (others), meaning that this type of ingredient represents only a small portion of this market (about 2-5%). Regarding the Portuguese nutraceutical market, a TGI study from Marktest stated that about 1 million of Portuguese habitants consumed feed supplements and related products in the year of 2013.

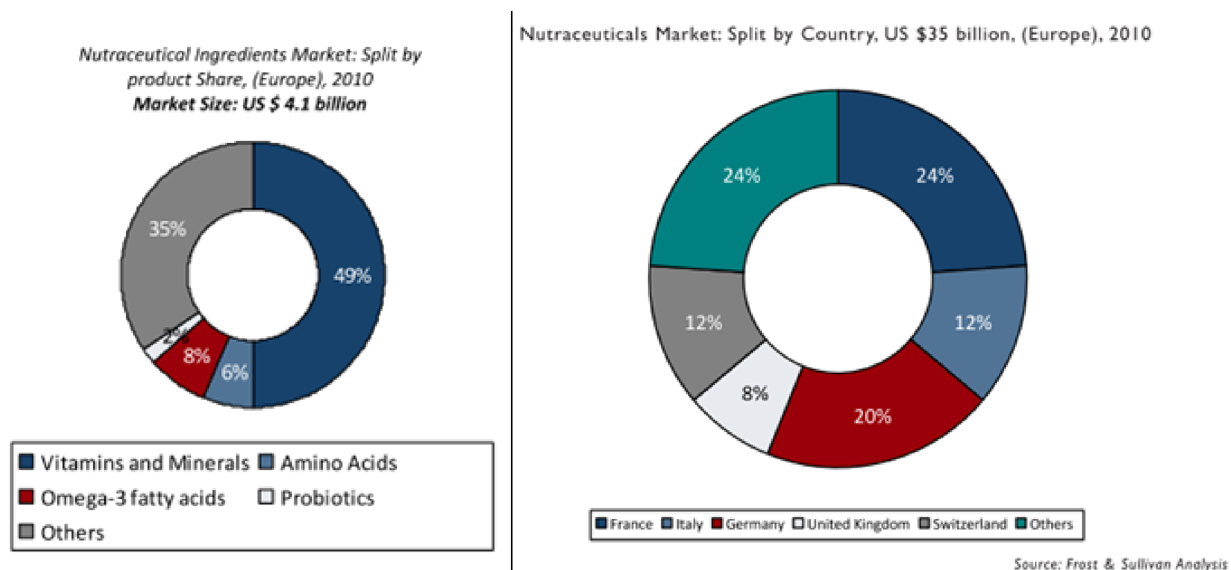

Figure 30. Graphical representation of the nutraceutical ingredients market in Europe and global market divided by countries.

5.4. Product Positioning in the Market

The current commercially available IgY is still expensive, which represents a huge challenge on using these antibodies in healthcare therapies. However, we were able to develop an easy, fast and relatively inexpensive method that leads to a high-quality purified IgY product (> 90% purity). By considering only the raw materials (eggs and chemicals) needed to produce 100 mg of IgY, less than 10€ is spent, while the same amount obtained by commercial kits corresponds to 80€. Moreover, in order to achieve this high purity by the common industrial purification methods, such as chromatography or ultrafiltration techniques, a large investment on sophisticated equipment is initially required, and more raw materials are needed to produce the same amount of antibodies (lower recovery yields).

There are few current companies that are able to deliver this type of high-quality antibody at such lower costs. We thus believe that for several years we could thrive in the market of the food supplements. As the pharmaceutical industry will embrace this new type of antibodies (IgY) as viable choice for immunization therapies, we could also expand our product to other segments and deal with future competitors.

5.5. Value Proposition

IgY with a purity higher than 90% can be obtained at low cost, with potential to enhance the content of human feed supplements, or in a later stage to be used in passive immunization therapies.

5.5.1. How can we create additional value?

There is a current need on finding substitutes to replace the intensive use of antibiotics in both livestock production and in human infection diseases (pain), and the polyclonal antibodies produced by hens (IgY) represents a good candidate (pain reliever). However, the production cost of antibodies for large-scale applications still remains higher than other drug therapies (pain). IgY can be recovered from egg yolk to generate large quantities of antibodies in a non-stressful and non-invasive way (gain creators) and at lower costs (pain reliever), instead of using the animal blood (IgG) as an antibody source. Additionally, no toxic chemicals are used during the whole process, thus the wasted products, which are the lipids, carotenoids (yellow pigments) and other proteins, can be used afterwards for by-products valorisation, fitting within the circular economy concept (gain creators).

We are able to provide cheaper and high-quality antibodies (product) derived from a natural source (egg yolk) that can be used as new bioactive ingredients in nutraceutical products (gains), which ultimately will benefit the final consumer by improving its immune system, overall health and quality of life (customer job).

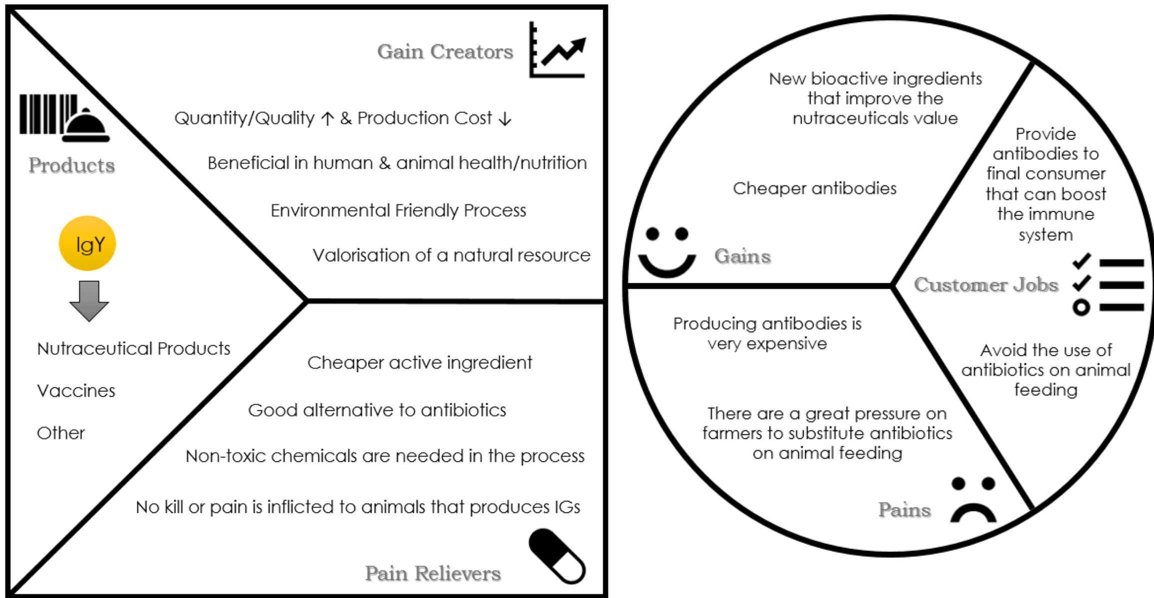

Figure 31. Schematic representation of our Value Proposition Canvas.

5.6. Network Positioning

The supply chain (Figure 32) of our business encompasses all the inbound logistics, the manufacturing operations, and the outbound logistics. We still need to know who the suppliers of the required raw-materials are, and who will buy our product (customers). We can identify as major stakeholders the suppliers, customers, employees, creditors or investors, and the owners, while the minor stakeholders correspond to the general public community, healthcare providers, government, pharmaceutical industries and a specific group of people (elders or sportsmen, for instance).

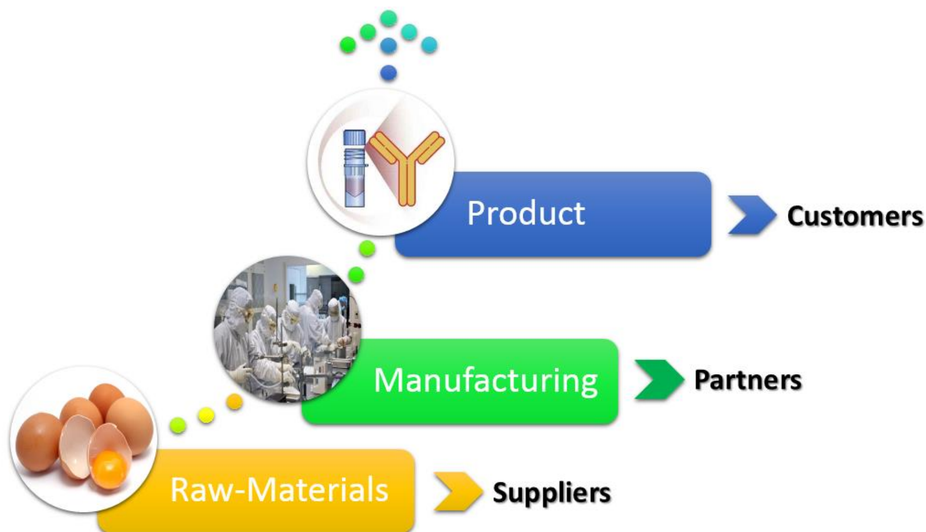

Figure 32. Schematic representation of our supply chain and its relationship with our major stakeholders.

5.6.1. Key Suppliers

Regarding the suppliers, our main concern is to ensure the necessary amount of eggs. Hen eggs are one of the most versatile foods, containing high-quality proteins and lipids, as well as valuable minerals, carbohydrates, and vitamins. We have the advantage of counting on the fact that the egg industry is wide and well established across Europe. In Portugal, the production exceeds its consumption (Figure 33) by more than 20000 tons of eggs; therefore, we believe that there is enough production of this essential raw-material for starting our business. One of the biggest distributers of eggs in Portugal is the "Companhia Avícola do Centro, S.A." (CAC), being this company our primer supplier of eggs. As the production of IgY will increase during the expansion of our business, we also expect a higher demand for eggs, meaning that we have to look for other producers in Europe, for instance in Spain or Germany. We have identified 3 major egg/egg products distributers in Europe as our secondary potential suppliers, namely the "Interovo Egg Group", "Ovobest Eiprodukte" and "OvoMarket".

An additional possibility will be to buy only the yolks instead of the entire eggs, which could be a more economical solution. However, these yolks might containing residues of egg whites, and since the process of the separation, cleaning and bursting of the yolk is a crucial step for the integrity of our final product, we prefer to carry out the entire process. We can however develop a plan to commercialize egg white or to develop other strategies to purify value-added proteins from egg white and from the discarded portion of egg yolk.

Concerning our ingredients suppliers, such as distilled water, polymers and ILs, we intent to contact common laboratorial suppliers, which we already have experience on working with, for instance Sigma-Aldrich.

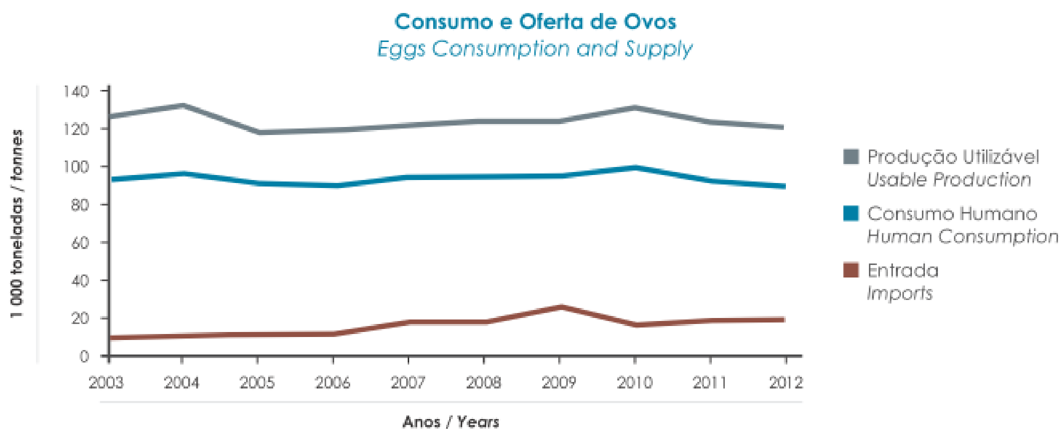

Figure 33. Graphic showing the relationship between the production of eggs in Portugal, their consumption and imports.

5.6.2. Key Costumers

At the beginning we intent to commercialize our high-value IgY product to Portuguese nutraceutical companies, and as our business expands, we intent to reach the European market. As primary customers we identified two major companies in the Portuguese nutraceutical market, namely the "EcoNutraceuticos" and the "KimiPharma". The first is more focused on sport supplements, being the GoldNutrition® products their major source of sales. The second company (KimiPharma) has been developing supplement products to improve both human and animal health. We expect to establish a partnership with both companies, selling our IgY as an essential component for improving either the sports supplements, offered by the first company, or the animal supplements, produced by the last.

Concerning our potential international customers, we have found two giant companies in Europe, one that produces nutraceutical products – "DSM", and also one big company that could be our primary customer if we could produce the highly pure and specific IgY product that could be used in passive immunization therapies – "Merck".

DSM is a full value-chain player, providing active ingredients, delivery systems, and nutritional and premix solutions, globally and at a local level. The focus of this company is on nutritional ingredients and additives segments of these markets. According with the DSM report of 2014, the Animal Nutrition & Health (ANH) business achieved sales of € 2,084 million in 2014, compared to € 1,935 million in 2013. The Merck company develops, among other pharmaceuticals, several of the vaccines used nowadays, improving health and well-being around the world. Additionally, they also have a broad portfolio of vaccines and pharmaceutical medicines to maintain and improve animal health (Merck Animal Health business). Likewise, as our business expands, new partnerships to selling our product across Europe have to be established.

5.6.3. Key Partners

In addition to our key suppliers and customers, we also need to establish other important partnerships to launch our business, namely with certain entities that could help us to find a place, license and finance our manufacturing process. The most obvious option for starting this business could be as a start-up company of the University of Aveiro (UA). There is a unity of technology transfer in UA (UATEC) that is responsible for helping young entrepreneurs on the academic community to develop their business, mainly on mentoring the business plan and on finding investors. Moreover, by using UA's network, it could be easier for us to get the attention of angel and venture capital investors.

One potential investor for our business could be the “Biocant Ventures”, for instance. This entity invests in "seed capital" life science projects, creating conditions to pass through the "valley of death" until its organization as a company, and also provides attractive laboratory facilities to the start-up of the companies.

Finally, we should attempt partnerships for the valorization of the remaining egg components that are useless to us, such as the egg shell, egg white and the lipid fraction recovered from the egg yolk. Egg shells are a source of natural calcium and proteins, which can be used to complement the animal feeding in small farms. Egg whites are rich in proteins, like lysozyme and ovalbumin, which have a high commercial value for biochemical supplier industries. Also, the egg white is used in bakery and alimentary industries, which represent also a partnership opportunity for us. Our wasted lipid fraction contains carotenoids (yellow pigments) that are valuable for alimentary industry, phospholipids that correspond to high value biocompounds and that can be sold for scientific purposes, and fatty acids that are also valuable in the food industry.

5.7. Operations

After the identification of the key stakeholders for our business, namely the suppliers and customers (Figure 34), it is also required to describe the key activities and resources.

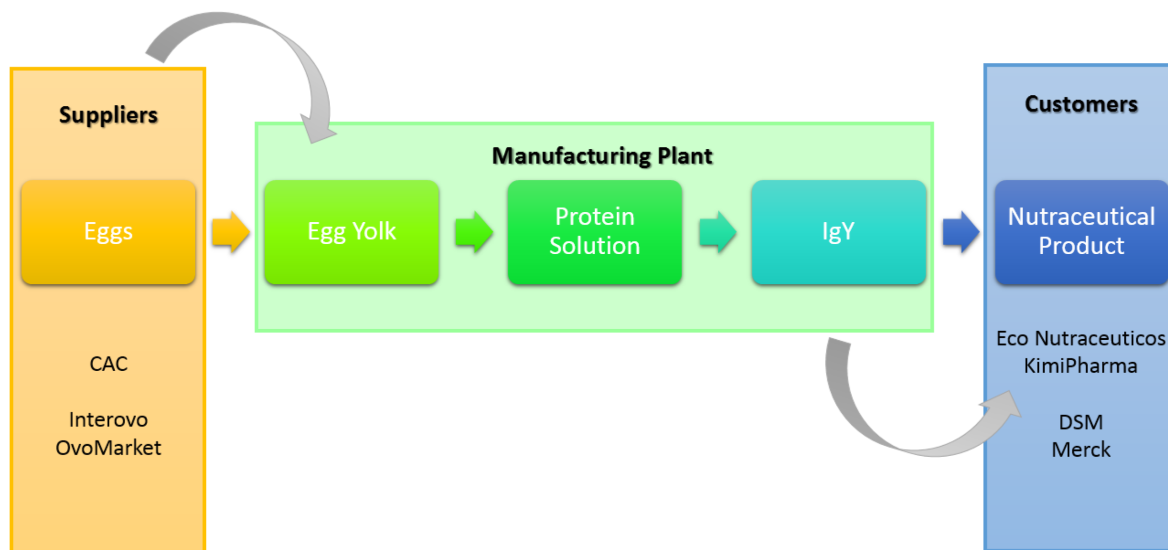

Figure 34. Scheme of our complete supply chain, where our possible major stakeholders are identified.

5.7.1. Location and Facilities

For this type of business, it is required a small factory installation, being the Biocant park a good choice for us, since “Biocant Venture” could be our partner in terms of capital investment and/or by leasing installations. Additionally, we also have investigated alternative locations, in which we

found an incubation centre in Aveiro (or other locations on the country), designed for young entrepreneurs by "ANJE – Associação Nacional de Jovens Empresários", which has industrial installations for renting at affordable values (250 m^2 for about 700 €/month). The required facilities have to include a warehouse to store raw-materials, a managing office, bathrooms, and a manufacturing plant, where the main operations to develop the target product occur.

5.7.2. Key resources

Besides the raw-materials that were previously discussed (eggs and others), additional resources are required, such as utilities (water, electricity, etc.), man power (engineers, technicians, accountants, administrators, managers and non-specialized workers), specific equipment (industrial egg separator, laboratorial freezer, industrial centrifuge, pH meter), specific tools (spatulas, egg spoon separators, tubes, etc.), packaging refrigerated boxes, administrator and managing equipment (computers, software, etc.), and also capital funds.

5.7.3. Manufacturing Process

We can divide our process in 3 stages according to the business key operations:

1. Inbound Logistics: Acquiring and storing the raw-materials (mainly eggs) – Warehousing;
2. Manufacturing Process: Processing of egg yolk up to the final product (IgY);
3. Outbound Logistics: Packaging and selling of the product on refrigerated conditions – Distribution Centre.

During the manufacturing process, we have some key operations, such as: cracking the egg and separation of the egg yolk from the white; cleaning and processing the yolk in a careful and specific manner; freezing and thawing operations, followed by a centrifugation step, where a protein solution is obtained; a specific mixture is performed, followed by new centrifugation steps until final product obtainment; finally, quality analysis of the batch production is performed by an out-side entity.

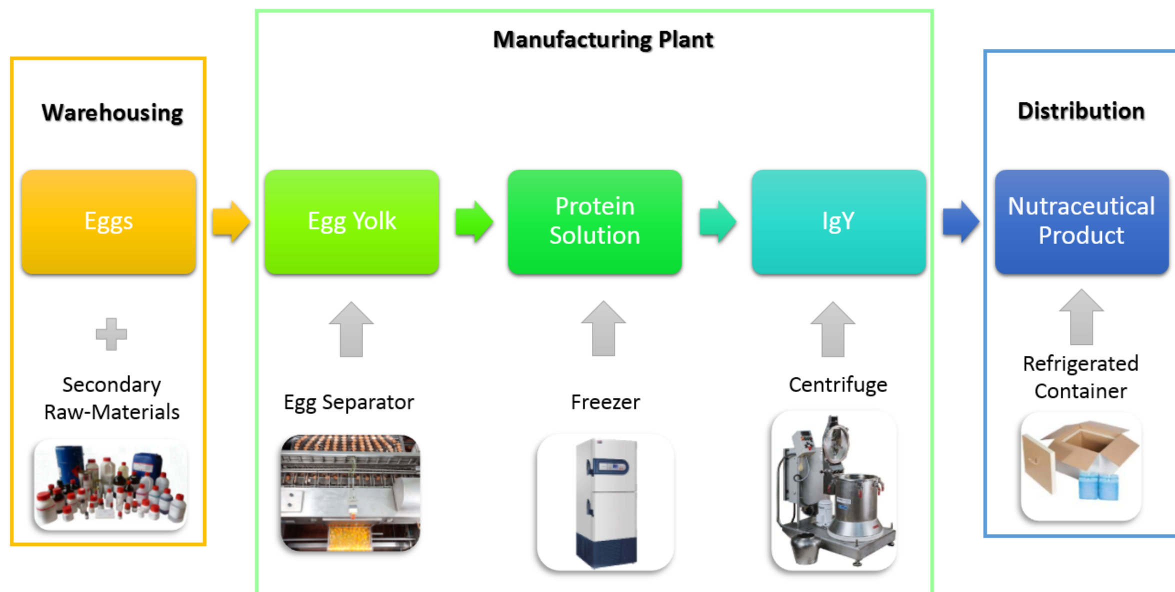

Figure 35. Scheme of our complete supply chain, where the key resources are identified.

5.7.4. Management Team

In order to implement and grow this business we have to develop a managing team with distinct characteristics. For instance, one person should have the scientific knowledge for managing all the manufacturing operations and to develop new processes along time to expand the company. We also need one person able to manage the inbound logistics and other responsible for the outbound logistics and to sell the product.

5.8. Financial Forecast

In order to provide a truthful financial forecast of our business, we believe that more studies based on our real market are needed. Therefore, additional contacts with our potential suppliers, customers and final consumers would be essential for a good revenue prediction.

As mentioned before, our initial target customers are nutraceutical companies that could sell our IgY product as a feed supplement to humans. In Portugal, there are about 1million consumers of these kind of products (numbers reviewed on market analysis). Since this product is very specific and somewhat expensive, we predict that, at the time of launching the business, we could reach 10% of this total market, meaning that it would be possible to sell 100000 units of 60 capsules *per* year plus the repeaters buyers.

Taking into account the financial and investment needs we considered a preliminary business financial balance, considering the data on investment needs, to pay the company workers, and all other operational costs for the first year of production. Based on these projections, we need *ca.*

about of 1M € of initial investment capital, being our Break Even Point occurrence between the years 2 and 3 (Figure 36).

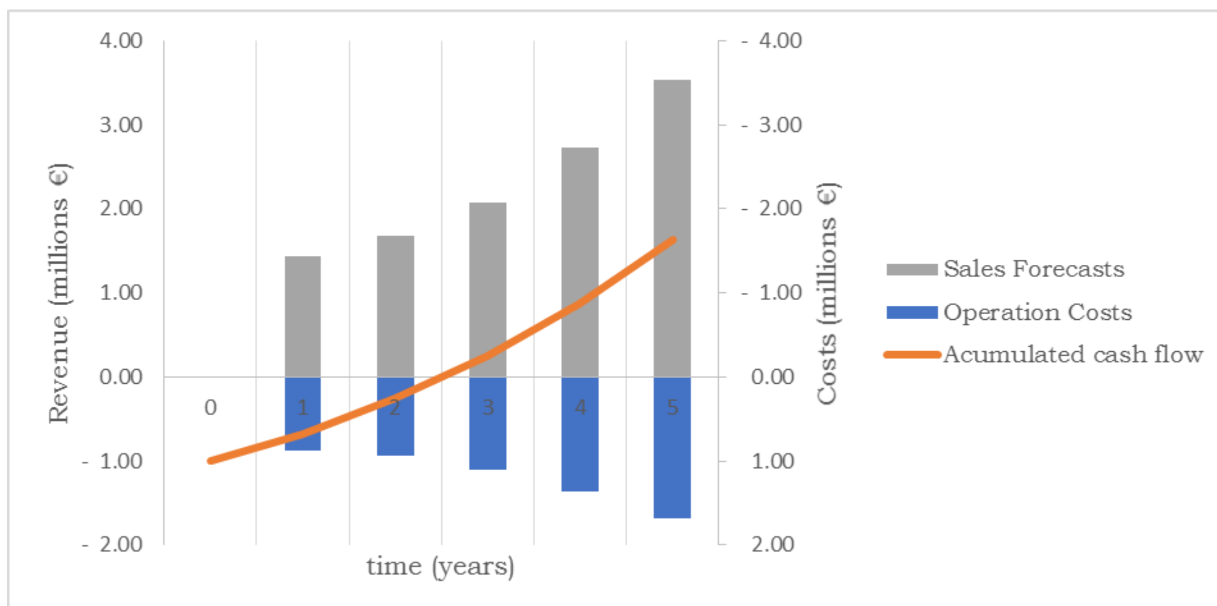

Figure 36. Projected sales and costs for the first 5 years after launching the business.

It should be remarked that a more detailed financial information will be required when presenting this information to investors and partners during the due diligence phase of the investment.

5.9. Risk Assessment

The appraisal of the risk assessment is of extreme importance, mainly because introducing a new brand and a new product in the market represents many risks. It should be however remarked that the study presented in this document does not include an optimized risk analysis.

5.9.1. What are our main uncertainties?

People may not accept our product, or the advertising campaign performed by our customers to the final consumer may turn out to be unsuccessful. Also, the actual sales can be inferior from the ones predicted and therefore will not be able to compensate costs. A complete risk analysis would allow our team to assess some of these possibilities' consequences and also investors would also use it as a way to assess a range of possible scenarios. It is therefore essential to perform a risk analysis before launching our product.

The main difficulties that we may encounter during the development of this project are mainly scaled-up related issues, since we found a method on a lab scale in which we can obtain 3 mg of a

90% pure IgY *per* gram of egg yolk. However, in a higher level or scaled-up process this might not be reproducible. Additionally, since we believe that this product can be a good candidate for passive immunization therapies, we also have to overcome the barriers of the approvals by the healthcare entities (FDA and EMA). An additional difficulty that we will find, common to other new companies that try to enter in the market, is to find investors, suppliers, partners and buyers.

In the following SWOT analysis, it is shown a representation of what we have and do not have, and what we need to succeed, representing also a guide for subsequent steps.

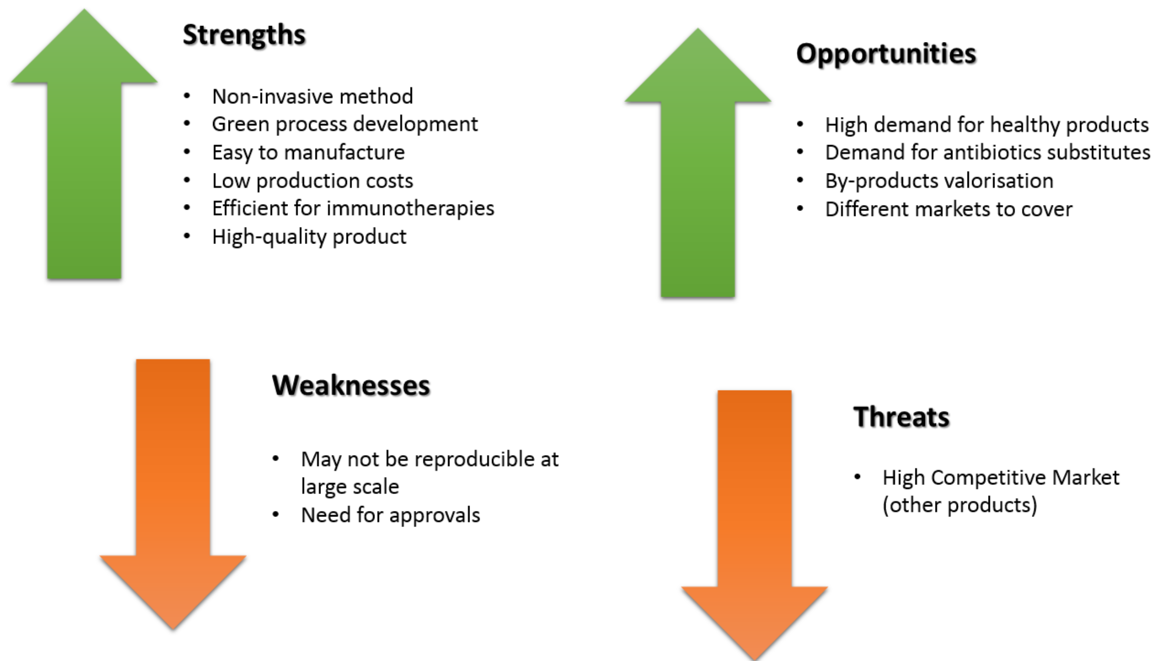

Figure 37. SWOT analysis for the development of a high-quality IgY product.

5.10. Competitors

We can divide our competitors in two different groups: the companies that purify and commercialize IgY or those that produce hyperimmune egg products. Nowadays, there are already a number of IgY or hyperimmune egg products for sale to treat specific diseases or for promoting the overall health in humans and livestock [30]. Moreover, current commercially available products are mainly hyperimmune feed supplements containing IgY, which consists on spray-drying the egg yolk and using this powder-containing the antibodies along with other nutritional products. However, besides IgY, these products have as well high concentrations of lipids that are present in the yolk. Thus, these types of products are mainly for nutritional purposes, such as the "OVOPRON IgY" from "Pharma Foods International Co. Ltd" that contains antibodies against *Helicobacter pylori* and has been incorporated into a number of finished products, like yogurts for instance. A similar product, called "IgY Recovery ProteinsTM", combines the egg yolk powder with other proteins to

obtain shorter recovery time, reducing muscle soreness, and improving overall performance in athletes. Additionally, “EW Nutrition” produces IgY supplements for both livestock and pets (Globigen®), as well as a full line of products for human health (Ovalgen®) containing IgY against a number of pathogens targeting oral, stomach, intestinal, and skin and mucosa care. Similarly, “Aova Technologies” sells a line of IgY products (BIGTM) for pigs, cows, poultry, and aquaculture industry that contain antibodies against a specific enzyme, which can significantly improve feed efficiency, growth rate, carcass yield, and general health in livestock [30]. It is important to notice that these companies are not able to provide a high-quality and purified IgY product at low production costs. Thus, they are not able to purify and concentrate the IgY from egg yolk; instead, they only dry out the egg yolk containing the IgY, meaning that they can release a relatively inexpensive product into the market. However, these products contain a large amount of lipids, which are not beneficial from the health perspective. On the other hand, the IgY purity in these products is far from the required for an effective immunotherapy.

There are some companies that commercialize IgY with high purity, but at small quantities and high cost – 50 µg for 270€ from Sigma-Aldrich®. Other companies provide a cheaper product at higher quantities, but with lower purity – 10 mg for 50€ from Gallus Immunotech Inc. The most relevant company in the field is the “IGY Life Sciences (ILS) & Technology Inc.”, which is a privately held Canadian biotechnology company specialized in the extraction, development and commercialization of avian antibodies (IgY) that can be used in over-the-counter (OTC) nutraceutical products or in human immunotherapies applications, such as the pathogen-specific target drugs. This company claims that they are able to produce a high-quality IgY product for a very low operation costs. Thus, ILS is our main competitor; however, we believe that the market is big enough for both companies and, besides, we also think that we can benefit from the fact that this company already started to modify the current immunotherapies market, making the penetration on this market easier for us. Even so, our company (AveIGs) fully distances itself in terms of presenting a high-quality product at low production costs when compared to most companies currently in the market.

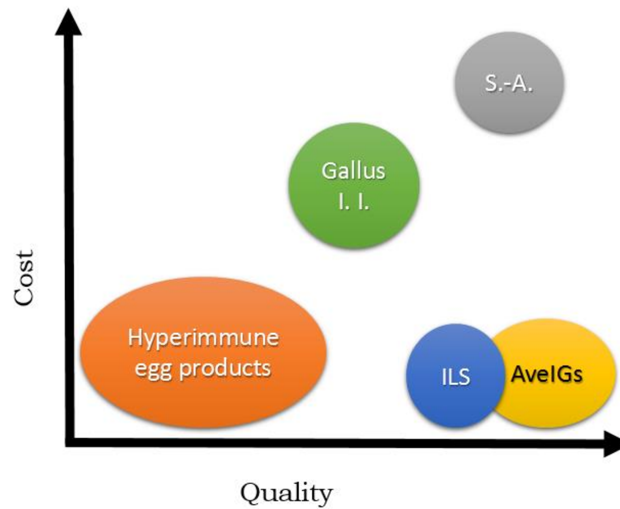

Figure 38. Product-Market Positioning Matrix, where the key competitors are compared in terms of cost and quality with our solution.

5.11. Conclusions and Future Perspectives

We believe that we are able to provide an attractive low-cost product to the market. Antibodies can be highly efficient against some diseases, and thus have a great potential for resolving emergent healthcare issues. On the other hand, they can be used in nutraceutical applications to improve human health and performance. IgY is particularly relevant since it can be obtained from a low-cost and renewable resource. None of the other IgY-producing companies is able to offer highly pure IgY at our low costs. Therefore, we believe that we have a new technology able to provide a distinct and valuable product that can benefit all stakeholders (costumers, suppliers, employees, partners and investors). Additionally, the market size of nutraceuticals is growing, and we could also consider other perspectives, such as producing for other markets and moving into international markets. We can also adapt our final product in terms of its quality and final price (lowering one and rising the other, and vice-versa) to be able to provide IgY for different markets.

6. FINAL REMARKS
AND FUTURE WORK

The finding of a cost-efficient purification method for IgY from egg yolk could be the key for developing a novel source of low cost antibodies for passive immunotherapy applications, with relevant social and economic impacts. Accordingly, the major goal of this thesis consisted on the development of cost-effective purification strategies for IgY from egg yolk.

The starting point of this work comprised a critical and extensive analysis of the commonly used and reported techniques and methods for the purification of IgY from egg yolk, specifically precipitation and extraction methods, which were revised in Chapter 2. These methods involve at least two steps. In the first step it is attempted the removal of the lipids fraction of the yolk from the WSPF, where the best method identified corresponds to the dilution of egg yolk with water in acidic conditions (pH 5), followed by the freezing and thawing of the mixture. In a second step, it is aimed the selective precipitation of IgY from the prepared WSPF, using aqueous solutions of salts or polymers. The best precipitation agent identified was NaCl.

Taking into account the results for the IgY precipitation methods reviewed in Chapter 2, two types of purification platforms for IgY from egg yolk have been investigated: liquid-liquid extractions using aqueous biphasic systems (ABS) – Chapter 3, and IgY induced precipitation using three-phase partitioning (TPP) systems – Chapter 4. Concerning the ABS-based purification platforms, we have first studied several polymer-salt ABS – Chapter 3.1 – using more benign organic salts, such as sodium citrate, instead of the commonly used polymer-phosphate-based ABS. In Chapter 3.2, ABS formed by polymers and cholinium-based ionic liquids (ILs) were investigated as alternative phase-forming compounds for the purification of IgY. These works comprised investigations on the partition behaviour of pure/commercial IgY, and on the separation of IgY from the WSPF and directly from egg yolk. Although not promising results have been obtained when attempting the IgY selective extraction for one of the phases using ABS, and thus the IgY purification, these studies allowed to conclude that IgY can be selectively precipitated (over the remaining WSPF proteins) at the interphase of ABS. This evidence was then investigated by means of TPP systems, shown and discussed in Chapter 4.

In Chapter 4, a novel class of polymer-based systems were studied, composed of PEG and sodium polyacrylate (NaPA) and using cholinium-based ILs as electrolytes. First, we investigated the ability of these phase-forming components to create ABS, particularly regarding the ILs electrolyte nature, and inferred the molecular-level mechanisms responsible for ABS formation – Chapter 4.1. We have found opposite trends in the PEG/NaPA ABS formation ability when using [Ch]-ILs as electrolytes, shown here for the first time. [Ch]-ILs that extensively partition to the PEG-rich phase, acting like inorganic salts, enhance the two-phase formation ability, while those that have a lower

partition to this phase also have a lower capacity of inducing ABS formation. Additionally, we studied the potential of these novel PEG-NaPA ABS using cholinium-based ILs as electrolytes for the purification of IgY by means of TPP. In this approach, IgY was purified by inducing its selective purification at the interphase of these systems – Chapter 4.2. Using this methodology, IgY with a purity higher than 90% was obtained from the egg yolk WSPF.

According to the obtained and discussed results, future investigations should be addressed in order to complete these studies. In some developed methods it is still required to confirm the purity of IgY by SE-HPLC. Furthermore, the TPP approach should be preferentially investigated as the most effective purification strategy in the systems shown in Chapter 3. Finally, the IgY stability and activity need to be evaluated, particularly for the samples obtained with the most promising purification processes. This work will lead to the development of more efficient purification platforms, which could have a major impact in the development of new therapies and antibody-based products, as discussed in Chapter 5.

Although not shown and discussed in this work due to confidentiality issues, we were able to develop a more efficient technique which leads to higher recovery yields and purity of IgY (> 95%). Stability and activity studies were performed for the recovered IgY, and more than 200 eggs were processed to confirm the reproducibility of the developed method. At this point, its large-scale viability needs to be addressed, as well as its potential to purify specific IgY from immunized egg samples.

List of Publications

List of publications in the current thesis:

1. Sandra C. Bernardo, Mafalda R. Almeida, Pedro Domingues, João A. P. Coutinho and Mara G. Freire, “Critical analysis on the available techniques for the purification of Immunoglobulin Y (IgY)”, manuscript under preparation.
2. Sandra C. Bernardo, João A. P. Coutinho and Mara G. Freire, “Purification of immunoglobulin Y (antibodies) using aqueous biphasic systems formed by polymers and salts”, manuscript under preparation.
3. Sandra C. Bernardo, Jorge F. B. Pereira, Sónia P. M. Ventura, Mara G. Freire and João A. P. Coutinho, “Opposite effects induced by Cholinium-based Ionic Liquid Electrolytes in the Formation of Polyethylene glycol/Sodium Polyacrylate Aqueous Biphasic Systems”, manuscript submitted to Physical Chemistry Chemical Physics.
4. Sandra C. Bernardo, João A. P. Coutinho and Mara G. Freire “Antibodies (Immunoglobulin Y) purification using three-phase partitioning (TPP) strategies”, manuscript under preparation.

List of other outputs:

1. Mara G. Freire, Sandra C. Bernardo and João A. P. Coutinho “Processo para purificar um anticorpo a partir da gema de ovo”, patent application requested to INPI at 20.09.2018.

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Annexes

3.1. Polymer-salt-based ABS

Determination of the tie-lines (TLs) and tie-lines lengths (TLLs)

The determination of the TLs was carried out by a gravimetric approach initially proposed by Merchuk et al. [158].

Table A.1. Mixture compositions, and respective TLs and TLLs, of ABS formed by PEG2000 or PPG400, sodium citrate/citric acid at pH 7 and water, used in the partitioning studies of pure IgY.

Phase Component		TL	top phase composition (wt%)		Initial mixture composition (wt%)		bottom phase composition (wt%)		TLL
Salt	Polymer		[Salt]	[Polymer]	[Salt]	[Polymer]	[Salt]	[Polymer]	
sodium citrate	PEG2000	*TL1*	5.978	29.639	10.009	21.981	20.137	2.736	30.401
		TL2	4.230	37.341	12.011	24.043	25.808	0.463	42.727
		TL3	3.227	43.248	14.082	25.971	30.359	0.064	51.000
	PPG400	*TL1*	1.161	66.297	10.298	20.033	12.618	8.286	59.132
		TL2	0.700	76.616	12.266	19.961	15.547	3.893	74.224
		TL3	0.328	89.744	14.211	20.229	17.887	1.825	89.655

4.1. Characterization of novel PEG-NaPA-based ABS using ionic liquids as electrolytes

Effect of the PEG molecular weight in the formation of ABS composed of PEG + NaPA 8000 + water + 5 wt% of [Ch]⁺-IL as electrolyte

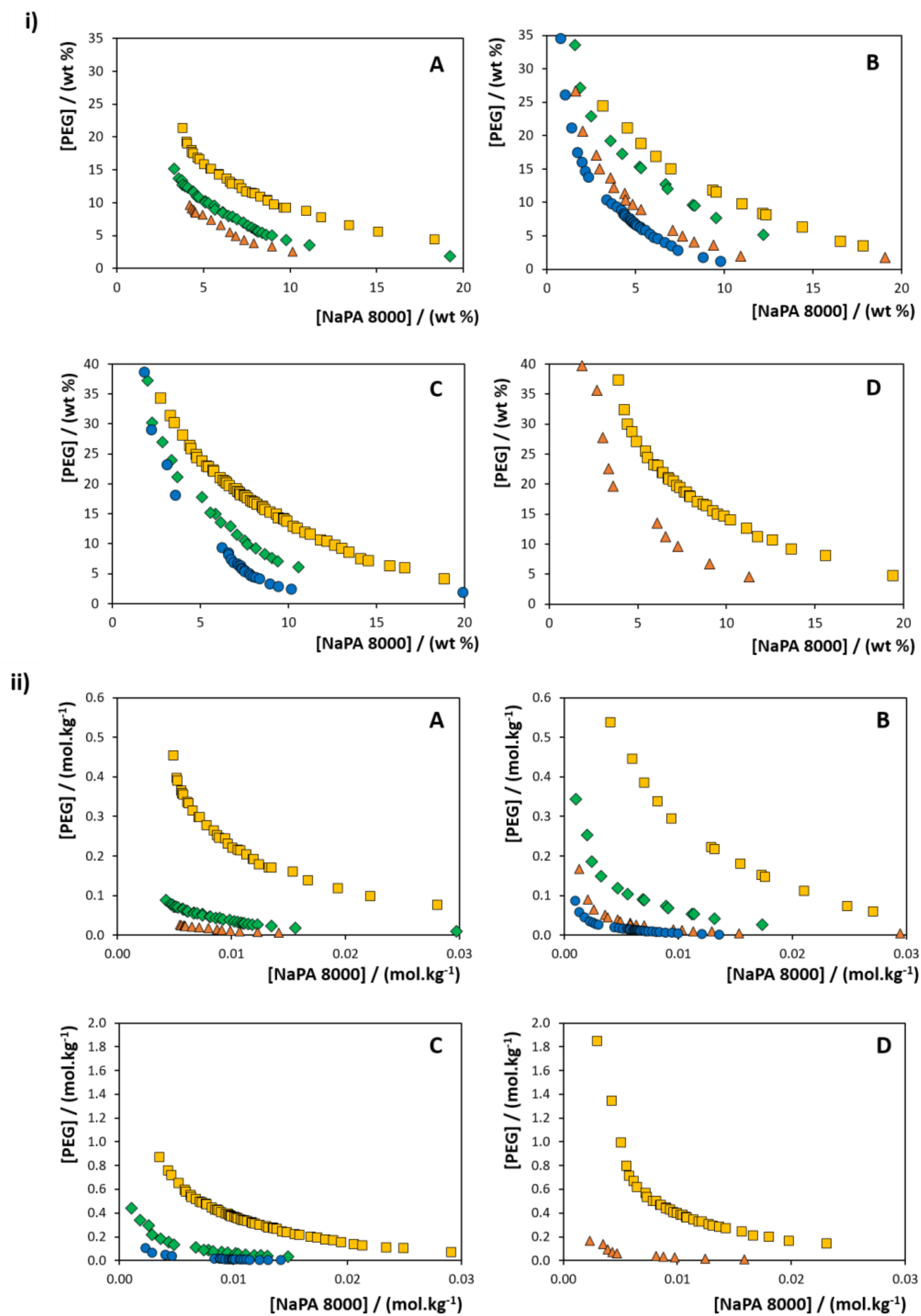

Fig. A.1. Binodal curves for the systems formed by PEG (different molecular weight) + NaPA 8000 + water + 5 wt% of [Ch]$^+$-IL as electrolyte: [Ch][Ac] (A), [Ch][DHP] (B), [Ch][DHcit] (C) and [Ch][Bit] (D). PEG molecular weight: PEG 600 (□); PEG 2000 (◇); PEG 4000 (△); PEG 6000 (○).

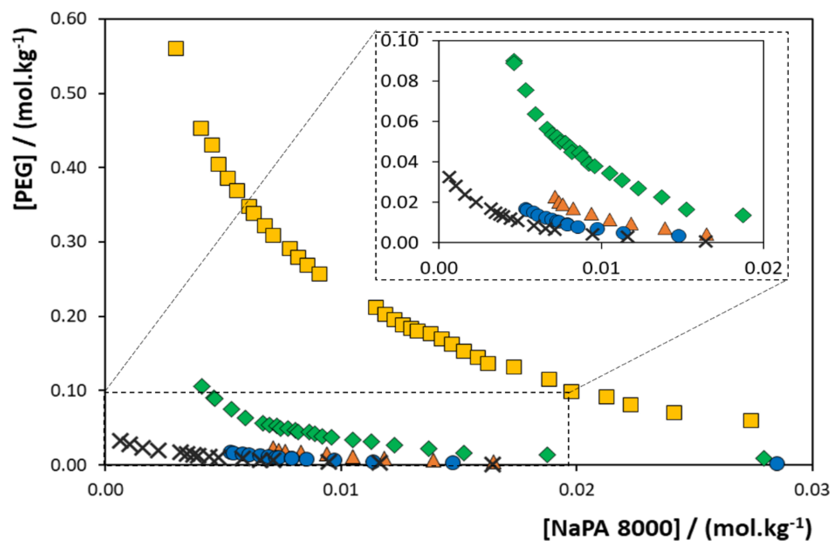

Fig. A.2. Binodal curves for the ABS formed by PEG (different molecular weight) + NaPA 8000 + water + 5 wt% of [Ch]Cl as electrolyte. PEG molecular weight: PEG 600 (■); PEG 2000 (◆); PEG 4000 (▲); PEG 6000 (●); PEG 8000 (✕) from Santos et al. [207].

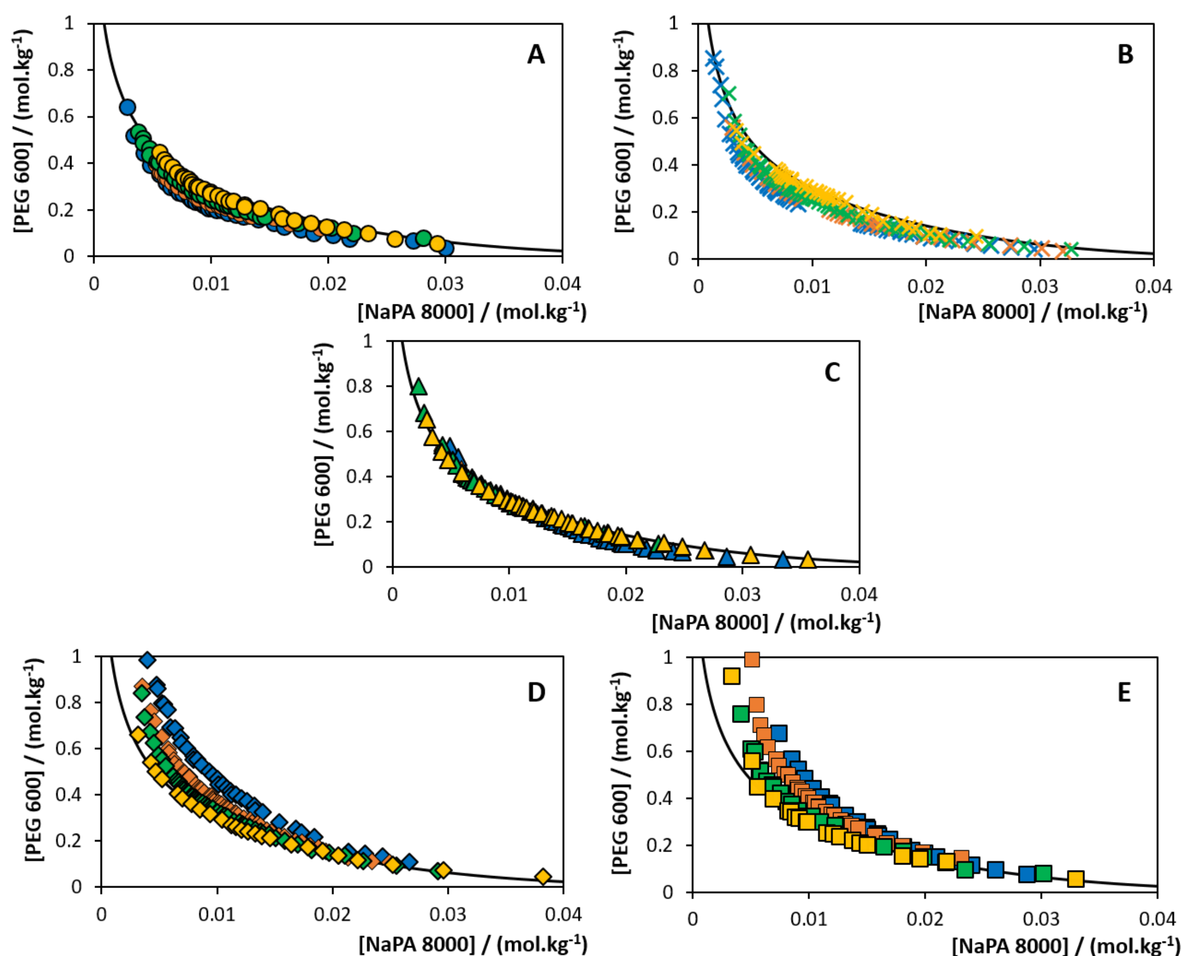

Fig. A.3. Binodal curves, at 27 °C, for ABS composed of PEG 600, NaPA 8000, and each $[Ch]^+$-based IL used as electrolyte: **a)** [Ch][Ac] (●); **b)** [Ch]Cl (✕); **c)** [Ch][DHP] (▲); **d)** [Ch][DHcit] (◆); and **e)** [Ch][Bit] (■). Distinct colours represent different concentrations, namely 10 wt% (**blue**), 5 wt% (**orange**), 2.5 wt% (**green**), 1 wt% (**yellow**). The ABS formed by PEG 600 and NaPA 8000 without electrolyte is represented by (—).

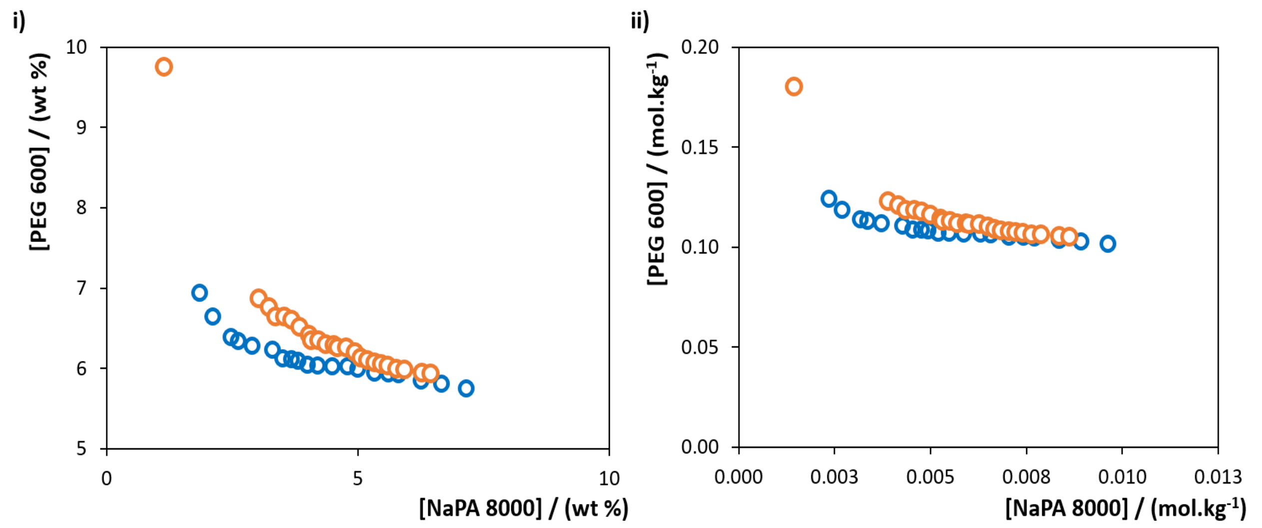

Fig. A.4. Binodal curves, at 27 °C, for ABS composed of PEG 600, NaPA 8000, and NaCl used as electrolyte. Distinct colours represent different concentrations, namely 10 wt% (**blue**), 5 wt% (**orange**).

Experimental weight fraction data corresponding to the solubility curves

Table A.2. Experimental binodal weight fraction data for the systems composed of PEG (1) + NaPA 8000 (2) + 5 wt% [Ch]Cl + water, determined at 27 °C.

PEG 600		PEG 2000		PEG 4000		PEG 6000	
100 $w1$	**100 $w2$**	**100 $w1$**	**100 $w2$**	**100 $w1$**	**100 $w2$**	**100 $w1$**	**100 $w2$**
25.1491	2.3364	17.3944	3.1726	8.3313	5.3989	9.1430	4.0836
21.3523	3.1556	15.2522	3.5561	7.4996	5.5688	8.8037	4.1666
20.5117	3.5010	15.0750	3.5767	7.0830	5.7551	8.2269	4.4431
19.5203	3.7108	13.0980	4.1009	6.3609	6.2144	7.5748	4.6539
18.7926	4.0016	11.2728	4.5327	5.4744	6.9948	6.9902	5.0067
18.1432	4.2878	10.1529	5.0678	4.4121	7.7595	6.5283	5.2529
17.2884	4.6484	9.6631	5.2854	3.6436	8.6418	6.0622	5.4773
16.8672	4.7913	9.4591	5.5026	2.7929	10.0172	5.8532	5.5693
16.1764	5.1497	9.0366	5.6252	1.7434	11.6509	5.1807	5.9555
15.6390	5.4029	8.9884	5.8491			5.0350	5.9559
14.8483	5.8827	8.6352	6.0549			4.4882	6.4069
14.3791	6.1344	8.2402	6.1377			3.9017	7.2251
13.8735	6.4168	8.1707	6.4707			2.7645	8.3239
13.3383	6.7710	7.7865	6.6300			2.0052	10.5569
11.2692	8.4250	7.2584	6.8632			0.9007	18.5574
10.8306	8.6766	7.0430	7.1218				
10.5197	8.9421	6.4155	7.7440				
10.1831	9.1599	5.8331	8.2823				
9.9022	9.3963	5.1084	8.9478				
9.7855	9.5786	4.2865	9.8825				
9.5854	9.9409	3.1842	10.8559				
9.2636	10.2435	2.6150	13.0353				
8.8711	10.5180	1.8522	18.2685				
8.4300	10.8399						
7.9695	11.2180						
7.5918	11.4997						
7.3320	12.1887						
6.4983	13.1027						
5.6207	13.6698						
5.2406	14.5508						
4.6566	15.1248						
4.0526	16.1773						
3.4361	17.9787						
2.6852	19.4121						
1.9918	20.3835						

Table A.3. Experimental binodal weight fraction data for the systems composed of PEG (1) + NaPA 8000 (2) + 5 wt% [Ch][Ac] + water, determined at 27 °C.

PEG 600		PEG 2000		PEG 4000	
100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***
21.4017	3.7864	15.1460	3.3006	9.6174	4.2166
19.2553	3.9999	13.6992	3.5856	9.1064	4.2968
18.9732	4.0378	13.4232	3.7430	8.9808	4.4429
17.9705	4.3047	12.6516	3.8075	8.6367	4.5057
17.7180	4.3110	12.4560	3.9420	8.4873	4.5405
17.5451	4.4039	12.2705	4.0911	8.1821	4.9635
16.7975	4.6627	11.6826	4.3879	7.3844	5.4372
16.6444	4.7474	11.5988	4.4120	6.5967	6.0116
15.8893	5.0285	10.8861	4.6215	5.5113	6.5327
15.1910	5.3814	10.8032	4.7175	4.9028	6.8400
15.1512	5.4554	10.1876	5.0888	4.3457	7.3458
14.3308	5.8885	10.0487	5.0911	3.8404	7.8922
14.3043	5.8977	9.9626	5.3045	3.2958	8.9568
13.7125	6.3169	9.5534	5.6218	2.5007	10.1638
13.1869	6.5189	8.9641	5.6489		
12.8489	6.6510	8.5555	6.0932		
12.7660	7.0384	8.4145	6.1125		
12.1765	7.1943	7.9354	6.4491		
11.7017	7.4732	7.8962	6.6856		
11.5019	7.7788	7.4211	6.9153		
11.3975	7.9332	7.0224	7.2999		
10.9419	8.2892	6.4482	7.5787		
10.3918	8.6923	6.1606	7.7734		
10.3231	8.7050	5.8875	7.9909		
9.7496	9.0447	5.6539	8.1069		
9.2713	9.6181	5.5980	8.1839		
9.2632	9.7591	5.3625	8.4008		
8.7851	10.9494	5.1170	8.6253		
7.7252	11.8046	4.9913	8.9642		
6.6225	13.3984	4.3138	9.7724		
5.5533	15.0832	3.5596	11.1107		
4.3823	18.3445	1.8910	19.2213		

Table A.4. Experimental binodal weight fraction data for the systems composed of PEG (1) + NaPA 8000 (2) + 5 wt% [Ch][DHP] + water, determined at 27 °C.

PEG 600		PEG 2000		PEG 4000		PEG 6000			
100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***
24.4297	3.1373	40.6956	0.8139	40.1585	1.0337	34.4945	0.7568	6.9796	4.8275
21.0999	4.5286	33.5871	1.5930	26.6726	1.6020	26.0814	1.0361	6.9490	4.8923
18.8096	5.3104	27.1566	1.8711	20.6659	2.0199	21.1803	1.4024	6.7227	4.9847
16.8888	6.1427	22.9208	2.4979	17.1142	2.7721	17.4782	1.7365	6.7185	5.0277
15.0411	6.9715	19.1875	3.5885	15.0704	2.9723	16.0258	1.9903	6.5236	5.0809
11.7951	9.3382	17.2279	4.2429	13.6400	3.5964	14.6184	2.1740	6.3523	5.2549
11.5536	9.5282	15.3528	5.2193	12.2534	3.7621	13.7749	2.3465	6.2266	5.2721
9.8168	10.9834	15.0861	5.3178	11.3541	4.4049	10.3681	3.3771	5.9715	5.3706
8.3572	12.1637	12.7565	6.6735	10.3885	4.4326	9.8122	3.6758	5.7921	5.5544
8.1333	12.3493	12.0768	6.7858	9.7035	4.8143	9.2789	3.9486	5.2872	5.7989
6.2687	14.3867	9.6191	8.2111	8.9501	5.3091	8.8003	4.1911	4.7896	6.0468
4.2163	16.5466	9.5248	8.3384	5.8503	7.0902	8.2964	4.3033	4.5827	6.2645
3.4545	17.8155	7.6681	9.5471	4.9581	7.6451	8.2094	4.3717	4.0318	6.6381
		5.1469	12.1886	4.0733	8.2967	7.9321	4.4122	3.5199	7.0101
				3.6442	9.3823	7.9146	4.4346	2.8077	7.3688
				1.9809	10.9239	7.6513	4.5819	1.7734	8.8212
				1.7982	19.0826	7.5988	4.6636	1.1364	9.7894
						7.3190	4.7252	1.1345	20.1040
						7.2579	4.7600		

Table A.5. Experimental binodal weight fraction data for the systems composed of PEG (1) + NaPA 8000 (2) + 5 wt% [Ch][DHcit] + water, determined at 27 °C.

PEG 600				PEG 2000		PEG 6000			
100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***
57.6120	0.7478	17.2403	7.8416	46.9251	0.8473	38.6150	1.8036	5.3881	7.5172
34.3054	2.7416	17.0602	7.9706	40.6456	1.4423	29.0086	2.2189	5.0224	7.7864
31.3698	3.2824	16.8770	8.0700	37.1967	2.0046	23.1217	3.1035	4.7198	7.8390
30.1640	3.4983	16.6071	8.2083	30.2088	2.2366	18.1094	3.5725	4.4872	7.9944
28.0897	3.9969	16.2660	8.4998	26.9246	2.8231	9.3457	6.2399	4.3237	8.1565
26.4449	4.3812	16.0804	8.5396	23.9236	3.3458	8.5274	6.5801	4.1221	8.3500
25.9101	4.4693	15.6927	8.6625	21.1660	3.6994	8.1735	6.6038	3.2682	8.9434
24.8802	4.7702	15.2476	8.9554	17.8206	5.0826	7.4045	6.7464	2.8488	9.4386
24.3640	4.8088	14.9280	9.3237	14.9694	5.8651	6.8973	6.8790	2.4648	10.1561
23.7758	5.0868	14.3502	9.4292	12.8949	6.7083	6.6356	7.1129	1.8362	19.9296
22.9845	5.3585	14.2403	9.7241	11.4689	7.0586	6.3505	7.1843		
22.8497	5.4682	14.0816	9.7553	10.5672	7.5269	6.2807	7.2981		
22.2941	5.7055	13.7589	9.9232	9.9017	7.6790	5.8581	7.3242		
22.1434	5.7341	12.9219	10.2577	9.2061	8.1421	5.7508	7.3802		
21.0172	6.0988	12.6329	10.4988	8.3119	8.6505	5.5907	7.4979		
20.5719	6.3000	11.8999	10.9395	7.5862	9.0439				
20.3958	6.4215	11.5913	11.2237	7.0916	9.4050				
20.1778	6.4756	10.6640	11.8196	6.0671	10.5600				
19.7313	6.6378	10.4120	12.1759	13.5170	6.1651				
19.1847	6.9432	9.7228	12.6220	15.1955	5.5796				
19.1301	7.0480	9.2715	13.0348						
18.7089	7.1195	8.5963	13.4540						
18.5329	7.2034	7.5510	14.1189						
18.2212	7.2600	7.1311	14.5535						
18.0815	7.4733	6.3375	15.7635						
17.9678	7.5607	5.9758	16.6236						
17.6495	7.6487	4.1325	18.8669						

Table A.6. Experimental binodal weight fraction data for the systems composed of PEG (1) + NaPA 8000 (2) + 5 wt% [Ch][Bit] + water, determined at 27 °C.

PEG 600				PEG 4000	
100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***
62.2136	1.7814	19.4539	7.3749	39.6989	1.8437
52.5733	2.3021	18.7232	7.6140	35.6251	2.6937
44.6771	3.2771	18.6921	7.7185	27.7177	3.0314
37.3126	3.8869	18.0215	7.9331	22.5812	3.3373
32.3674	4.2323	17.9061	7.9695	19.6730	3.6096
29.9647	4.4224	17.1094	8.3706	13.4993	6.0994
28.6764	4.6788	16.5921	8.6832	11.2195	6.5543
27.0796	4.9042	16.4422	8.8648	9.6106	7.2659
25.4448	5.4349	15.5315	9.2420	6.7214	9.0401
24.3991	5.5574	14.9597	9.4808	4.5749	11.2690
23.2431	5.9013	14.6365	9.8653	1.7670	20.0187
23.1088	6.1244	14.0050	10.2305		
21.9647	6.3742	12.6883	11.1395		
21.9000	6.4050	11.2239	11.7630		
21.0747	6.7205	10.6607	12.5969		
20.8167	6.7377	9.2180	13.6766		
20.4978	6.9869	8.0577	15.5889		
19.7306	7.1758	4.8035	19.3919		

Table A.7. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 1 wt% of [Ch]X, X = Cl and [Ac], determined at 27 °C.

[Ch]Cl				[Ch][Ac]			
100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***
24.7644	2.5866	8.7921	11.4887	21.1471	4.2748	8.0249	12.8621
22.8099	2.9850	8.2995	12.4953	20.0032	4.5362	7.2308	13.6659
21.1635	3.7729	7.6290	12.8927	19.4416	4.7412	6.4584	14.5580
18.6200	5.1626	7.3259	13.2123	18.7358	5.0813	5.6921	15.7508
18.3432	5.3492	6.2233	14.0118	17.9019	5.3799	4.4936	17.0376
17.9359	5.4424	5.8839	14.5126	17.1447	5.6854	3.2654	18.9647
17.4975	5.5353	5.4269	16.3113	16.8365	5.7414		
17.3001	5.7004			16.6972	5.9994		
17.1200	5.8521			16.2704	6.2119		
16.9294	6.1327			15.7760	6.2494		
16.6101	6.1703			15.3494	6.5435		
15.8423	6.7780			15.1687	6.6279		
15.5939	7.0285			14.8980	6.9343		
14.9019	7.3140			14.3730	7.3603		
14.7492	7.6450			13.9868	7.3612		
14.6913	7.6576			13.6478	7.7530		
14.3648	7.8848			13.1188	8.0730		
14.1696	8.0844			12.6152	8.3937		
13.9223	8.3110			12.5757	8.6880		
13.4772	8.3933			11.8770	9.2930		
13.4633	8.6620			11.4258	9.3214		
13.1352	8.9489			11.0650	10.1871		
12.7467	9.3719			9.8967	11.2003		
10.9479	10.3489			9.0564	11.3919		
10.2497	10.8720			8.4841	12.0351		

Table A.8. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 1 wt% of [Ch]X, X = [DHP], [DHcit] and [Bit], determined at 27 °C.

[Ch][DHP]				[Ch][DHcit]		[Ch][Bit]	
100 *w1*	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***
28.2076	2.2987	8.7364	12.3446	28.3673	2.4709	35.6599	2.5653
25.6730	2.6584	8.3535	12.8696	24.5402	3.2896	25.1579	3.8820
23.5209	3.2325	8.1968	12.8998	23.0655	3.5776	21.3774	4.2111
22.1828	3.6726	7.7622	13.3778	21.9428	4.0033	19.3431	5.2412
20.0503	4.5662	7.5309	13.5488	19.6006	5.0370	17.3022	6.1283
17.8177	5.6243	6.7364	14.3643	18.7389	5.2753	17.1258	6.3010
16.8300	6.1749	6.0453	15.6966	17.8160	5.8428	16.2485	6.5636
15.7126	6.8737	5.2533	16.5986	16.8660	6.3675	16.0653	6.8077
15.0580	7.2959	4.2589	17.6372	15.9030	7.0111	15.3889	7.2372
14.6884	7.5361	3.1925	19.7201	15.0135	7.7197	15.2781	7.2661
14.6338	7.5885	2.0471	22.1697	13.9400	8.2194	13.2115	8.4185
14.2320	7.9715			13.5506	8.5153	13.0044	8.7555
13.7331	8.2703			13.0627	8.8457	12.5520	9.1417
13.5107	8.4096			12.6685	9.1976	11.7446	9.8744
13.3123	8.7727			12.2728	9.6172	11.2502	10.3094
12.8512	8.8393			11.7206	9.9579	10.9512	10.6760
12.4411	9.2440			11.2720	10.4491	8.6116	12.5882
11.8839	9.8384			9.9805	11.5979	7.9057	13.4808
11.6682	9.9531			9.3224	12.4804	7.3917	14.8568
11.2657	10.3691			8.5829	13.2832	3.3296	20.8293
10.5741	10.7631			7.5314	14.0628		
10.3213	10.9652			6.4754	15.0422		
9.7359	11.4273			5.4520	16.7978		
9.4573	11.8174			4.1225	19.1531		
9.2159	11.8894			2.6295	23.4147		

Table A.9. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 2.5 wt% of [Ch]X, X = Cl and [Ac], determined at 27 °C.

[Ch]Cl				[Ch][Ac]			
100 *w1*	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***
29.6776	2.1156	13.6448	7.0901	24.3756	2.9152	10.0362	9.8804
26.0777	2.4486	13.5190	7.2218	23.3797	3.2038	9.3952	10.1686
23.9821	2.8264	13.3243	7.3614	22.7240	3.2194	9.3624	10.4365
21.7580	3.1643	13.1955	7.4897	21.8338	3.6184	9.0611	11.4447
21.6817	3.2976	12.7675	7.7748	20.8342	3.6407	8.0281	12.2205
21.0214	3.4262	12.4112	8.1036	19.7260	4.1170	7.0118	13.9448
19.7696	3.8520	11.9521	8.3230	19.5468	4.2379	5.6270	15.0265
19.2867	4.1178	11.5887	8.5021	18.1224	4.6052	4.6331	18.3442
18.9950	4.1551	11.3073	8.8798	17.5007	5.0399		
18.3678	4.3825	10.7977	9.1568	16.7868	5.1870		
18.1599	4.5712	10.2048	9.4998	16.2257	5.4972		
18.0802	4.5834	9.7665	9.9999	15.4808	5.8008		
17.5848	4.7993	9.5280	10.3049	15.4076	6.0075		
17.4243	4.8166	8.0102	11.6228	14.5772	6.2251		
17.1615	4.9825	7.5713	11.9171	14.3455	6.5046		
16.8446	5.1393	7.2065	12.2980	13.7975	6.6872		
16.7013	5.3058	6.7393	12.6590	13.6026	6.9376		
16.6000	5.3555	6.3636	13.2113	13.0951	7.4091		
16.2403	5.4926	5.9752	13.8277	12.7339	7.4263		
15.7187	5.7442	5.3474	14.3589	12.4609	7.6928		
15.2978	5.9354	4.7927	14.9810	11.9406	8.0407		
15.1719	6.1026	4.4077	15.9317	11.6639	8.3294		
14.9314	6.2885	3.8118	16.9360	11.5966	8.4569		
14.6389	6.4750	3.2162	18.6362	11.2212	8.7950		
14.3080	6.6787	2.4686	20.7526	10.9275	8.9281		
14.0932	6.8984			10.6003	9.3934		

Table A.10. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 2.5 wt% of [Ch]X, X = [DHP], [Bit] and [DHcit], determined at 27 °C.

[Ch][DHP]		[Ch][Bit]		[Ch][DHcit]			
100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***	100 ***w1***	100 ***w2***
48.4052	0.6514	38.0560	2.6711	38.1256	2.2127	16.9202	7.1553
39.1697	1.3816	31.4109	3.1864	33.4958	2.6858	16.3463	7.3697
32.4314	1.7746	26.8374	3.8153	30.6888	2.9078	16.3142	7.4397
29.0554	2.1187	26.5532	4.0584	28.7751	3.2251	15.9326	7.6402
24.4036	3.2898	23.9127	4.3209	27.2878	3.5105	15.6017	7.8093
22.2024	3.9447	23.7192	4.4333	25.6090	3.7999	15.5912	7.8427
21.1886	4.1691	22.2060	4.8198	24.8546	3.9990	15.5405	8.0414
19.8640	4.5427	21.6017	5.1743	24.0053	4.2628	14.6877	8.4119
19.7366	4.7663	21.2426	5.2137	22.4668	4.7181	14.4452	8.7066
19.2505	4.8147	20.2243	5.6645	21.6957	4.8771	14.0338	8.8727
19.0756	4.9146	18.8472	5.9919	21.5741	5.0818	13.9237	8.9325
18.7354	5.0977	18.3591	6.3342	21.0519	5.1199	13.6906	9.0280
18.6701	5.2802	17.4041	6.7259	20.7797	5.2891	13.4776	9.2883
18.4214	5.2825	16.3409	7.0843	20.3360	5.3060	13.0519	9.6308
17.6771	5.6816	16.2930	7.5520	20.1393	5.3853	12.6131	9.9355
17.1730	5.9750	15.3690	8.0543	19.8464	5.5274	12.3253	10.0561
16.9045	6.2674	14.6267	8.8768	19.7785	5.6375	12.1291	10.3837
16.0503	6.5848	10.6126	11.5955	19.7209	5.6752	11.3310	10.7489
15.4969	6.9046	9.6025	12.6109	19.1566	5.8076	10.7664	11.2389
14.7707	7.4838	8.3853	13.6789	19.0180	5.9136	9.9905	11.9503
14.0350	7.7969	7.2142	14.7947	18.9047	6.1008	8.7796	12.6744
13.9763	8.1935	5.4628	15.7811	18.5814	6.1067	8.2214	13.6262
13.0778	8.7481	4.7131	19.4162	18.4113	6.2271	7.5739	14.4080
12.4113	9.3075			18.3170	6.2912	6.4155	15.3259
10.3199	10.9585			18.1290	6.3572	5.2809	16.9510
9.7430	11.6136			17.9483	6.5183	3.9536	18.8896
8.1999	12.6479			17.6931	6.6947		
7.3703	13.4506			17.4005	6.8032		
6.6204	14.4006			17.3349	6.8522		
5.7693	15.4119			17.0689	6.9978		

Table A.11. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 5 wt% of [Ch]X, X = Cl and [Ac], determined at 27 °C.

[Ch]Cl				[Ch][Ac]			
100 *w1*	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***
25.1491	2.3364	5.6207	13.6698	21.4017	3.7864	7.7252	11.8046
21.3523	3.1556	5.2406	14.5508	19.2553	3.9999	6.6225	13.3984
20.5117	3.5010	4.6566	15.1248	18.9732	4.0378	5.5533	15.0832
19.5203	3.7108	4.0526	16.1773	17.9705	4.3047	4.3823	18.3445
18.7926	4.0016	3.4361	17.9787	17.7180	4.3110		
18.1432	4.2878	2.6852	19.4121	17.5451	4.4039		
17.2884	4.6484	1.9918	20.3835	16.7975	4.6627		
16.8672	4.7913			16.6444	4.7474		
16.1764	5.1497			15.8893	5.0285		
15.6390	5.4029			15.1910	5.3814		
14.8483	5.8827			15.1512	5.4554		
14.3791	6.1344			14.3308	5.8885		
13.8735	6.4168			14.3043	5.8977		
13.3383	6.7710			13.7125	6.3169		
11.2692	8.4250			13.1869	6.5189		
10.8306	8.6766			12.8489	6.6510		
10.5197	8.9421			12.7660	7.0384		
10.1831	9.1599			12.1765	7.1943		
9.9022	9.3963			11.7017	7.4732		
9.7855	9.5786			11.5019	7.7788		
9.5854	9.9409			11.3975	7.9332		
9.2636	10.2435			10.9419	8.2892		
8.8711	10.5180			10.3918	8.6923		
8.4300	10.8399			10.3231	8.7050		
7.9695	11.2180			9.7496	9.0447		
7.5918	11.4997			9.2713	9.6181		
7.3320	12.1887			9.2632	9.7591		
6.4983	13.1027			8.7851	10.9494		

Table A.12. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 5 wt% of [Ch]X, X = [DHP], [DHcit] and [Bit], determined at 27 °C.

[Ch][DHP]		[Ch][DHcit]				[Ch][Bit]			
100 *w1*	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***
24.4297	3.1373	57.6120	0.7478	17.2403	7.8416	62.2136	1.7814	14.9597	9.4808
21.0999	4.5286	34.3054	2.7416	17.0602	7.9706	52.5733	2.3021	14.6365	9.8653
18.8096	5.3104	31.3698	3.2824	16.8770	8.0700	44.6771	3.2771	14.0050	10.2305
16.8888	6.1427	30.1640	3.4983	16.6071	8.2083	37.3126	3.8869	12.6883	11.1395
15.0411	6.9715	28.0897	3.9969	16.2660	8.4998	32.3674	4.2323	11.2239	11.7630
11.7951	9.3382	26.4449	4.3812	16.0804	8.5396	29.9647	4.4224	10.6607	12.5969
11.5536	9.5282	25.9101	4.4693	15.6927	8.6625	28.6764	4.6788	9.2180	13.6766
9.8168	10.9834	24.8802	4.7702	15.2476	8.9554	27.0796	4.9042	8.0577	15.5889
8.3572	12.1637	24.3640	4.8088	14.9280	9.3237	25.4448	5.4349	4.8035	19.3919
8.1333	12.3493	23.7758	5.0868	14.3502	9.4292	24.3991	5.5574		
6.2687	14.3867	22.9845	5.3585	14.2403	9.7241	23.2431	5.9013		
4.2163	16.5466	22.8497	5.4682	14.0816	9.7553	23.1088	6.1244		
3.4545	17.8155	22.2941	5.7055	13.7589	9.9232	21.9647	6.3742		
		22.1434	5.7341	12.9219	10.2577	21.9000	6.4050		
		21.0172	6.0988	12.6329	10.4988	21.0747	6.7205		
		20.5719	6.3000	11.8999	10.9395	20.8167	6.7377		
		20.3958	6.4215	11.5913	11.2237	20.4978	6.9869		
		20.1778	6.4756	10.6640	11.8196	19.7306	7.1758		
		19.7313	6.6378	10.4120	12.1759	19.4539	7.3749		
		19.1847	6.9432	9.7228	12.6220	18.7232	7.6140		
		19.1301	7.0480	9.2715	13.0348	18.6921	7.7185		
		18.7089	7.1195	8.5963	13.4540	18.0215	7.9331		
		18.5329	7.2034	7.5510	14.1189	17.9061	7.9695		
		18.2212	7.2600	7.1311	14.5535	17.1094	8.3706		
		18.0815	7.4733	6.3375	15.7635	16.5921	8.6832		
		17.9678	7.5607	5.9758	16.6236	16.4422	8.8648		
		17.6495	7.6487	4.1325	18.8669	15.5315	9.2420		

Table A.13. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 10 wt% of [Ch]X, X = Cl and [Ac], determined at 27 °C.

[Ch]Cl				[Ch][Ac]			
100 *w1*	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***	**100 *w1***	**100 *w2***
33.8098	1.2398	8.1705	10.4166	27.8541	2.1921	11.1646	7.2578
29.0288	1.6864	8.0353	10.5816	23.7725	2.6356	11.1336	7.4373
24.1185	2.3956	7.8069	10.7905	21.0038	3.2577	10.8658	7.6617
22.4232	2.6884	7.6421	11.1562	19.0572	3.7062	10.6459	7.6953
20.9179	3.0110	7.3157	11.4379	17.4765	4.2566	10.6348	8.0266
19.8901	3.2123	7.0162	11.6977	16.0626	4.6955	10.1059	8.3503
19.1240	3.4469	6.7447	11.9426	15.1802	4.9763	9.7746	8.8310
18.5193	3.6591	6.5595	12.4101	14.8770	5.2821	9.3868	9.2572
17.8678	3.9131	6.2415	12.7316	14.0971	5.4846	8.7534	10.0790
17.3037	4.1315	5.9018	13.0505	14.0638	5.6902	7.9924	10.9372
16.6415	4.3996	5.5615	13.6539	13.2902	6.0645	7.1675	11.4964
16.1184	4.6542	5.0932	14.0409	12.8598	6.1815	6.4608	12.3581
15.6775	4.8248	4.8981	14.6600	12.7232	6.2690	5.6129	13.0196
15.0979	5.1190	4.3832	15.4369	12.5508	6.4575	5.3527	13.9997
14.7138	5.2535	3.8651	16.2147	12.3397	6.4896	4.4551	14.8685
14.0937	5.6117	3.4888	17.0616	12.2866	6.6947	3.9074	17.8842
13.5232	5.8942	2.9925	18.0287	11.7350	6.9114	2.1795	19.3297
12.9339	6.2679	2.5739	19.0805	11.5904	6.9924		
8.4247	10.2259			11.5075	7.1677		

Table A.14. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water + 10 wt% of [Ch]X, X = [DHP], [DHcit] and [Bit], determined at 27 °C.

[Ch][DHP]		[Ch][DHcit]				[Ch][Bit]	
100 *w1*	100 *w2*	100 *w1*	100 *w2*	100 *w1*	100 *w2*	100 *w1*	100 *w2*
24.2622	3.7772	57.7008	0.7470	18.2422	9.1211	53.9838	2.0020
22.5645	4.3317	43.6067	2.2085	17.5090	9.5403	29.0091	5.5580
19.2803	5.1759	41.8212	2.5107	16.6847	9.6117	25.4684	6.3242
18.1361	5.7163	37.1924	3.0831	16.2740	10.0302	23.9590	6.7033
16.2240	6.6013	34.4426	3.6504	14.4220	10.9499	22.6321	7.1083
14.4133	7.4698	34.0532	3.7162	13.1445	11.8696	21.0374	7.5796
13.0852	8.0834	32.4130	4.0329	12.4994	12.1696	19.6465	8.1072
12.8711	8.2794	32.2996	4.1263	11.4569	12.8258	18.6153	8.5936
11.5513	9.0994	31.5820	4.3774	8.3868	14.6167	18.2847	8.6814
10.1235	10.0553	29.3701	4.5725	7.9932	15.4458	16.5173	9.4150
9.9401	10.0842	29.2441	4.8510	7.3632	16.3044	16.5084	9.4426
8.8776	10.9433	28.0329	5.1974	6.1353	17.5624	15.3194	9.9462
8.0100	11.5083	27.3236	5.2199			15.2765	10.1471
7.6770	11.8974	26.4838	5.6045			14.3184	10.6612
7.1653	12.2866	25.5004	5.9582			13.9177	10.6845
6.6981	12.7102	24.9494	5.9719			13.1460	11.2538
6.3612	12.8534	24.8973	6.1904			12.6974	11.2884
5.8344	13.4980	24.0957	6.5038			11.9484	11.7203
5.7456	13.6158	23.0773	6.9327			11.9304	11.8820
4.6166	14.8434	22.6083	7.1602			10.5957	12.5898
4.1600	15.2644	21.9295	7.4383			10.4950	12.6188
3.7230	16.1346	21.0814	7.4995			9.8048	13.0426
2.5807	18.4114	21.0100	7.7438			9.1006	13.7079
		20.5494	7.9938			8.3796	14.3174
		20.0131	8.2012			7.4384	15.1124
		19.4158	8.2610			6.5307	16.0870
		19.3408	8.4990			5.4420	17.2158
		18.7788	8.8020			4.4598	18.6537

Table A.15. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) + water, determined at 27 °C.

0% electrolyte			
100 *w1*	100 *w2*	100 *w1*	100 *w2*
28.1862	2.3155	11.5343	10.3369
26.3684	2.4134	11.1387	10.5996
24.4414	3.1864	11.1088	10.8985
21.7966	3.8956	10.6981	10.9633
20.0505	4.7358	10.4649	11.4550
19.0319	4.9585	10.2736	11.5971
17.9428	5.5830	9.7014	12.3906
16.8543	6.1602	8.5098	13.0199
15.9753	6.8495	8.0010	13.4899
14.9248	7.5844	7.6666	14.0553
14.1999	8.1486	7.1280	14.6505
14.0782	8.1741	6.4816	15.2321
13.8352	8.4629	6.0404	15.8321
13.4004	8.8689	5.4144	16.6881
12.9887	9.0204	4.6406	17.5635
12.9746	9.3627	3.7826	18.3779
12.3537	9.7816	3.2772	19.8021
12.0381	9.8544	2.3425	22.2530
11.7856	10.2621	1.2223	26.8263

Table A.16. Experimental binodal weight fraction data for the systems composed of PEG 600 (1) + NaPA 8000 (2) +5/10 wt% NaCl + water, determined at 27 °C.

5% NaCl		10% NaCl	
100 *w1*	**100 *w2***	**100 *w1***	**100 *w2***
9.7524	1.1381	6.9431	1.8493
6.8700	3.0212	6.6506	2.1035
6.7609	3.2235	6.3935	2.4682
6.6485	3.3607	6.3454	2.6134
6.6475	3.5243	6.2873	2.8898
6.6038	3.6724	6.2315	3.2978
6.5173	3.8395	6.1299	3.5028
6.4152	4.0290	6.1156	3.6748
6.3559	4.0727	6.1001	3.8034
6.3523	4.2108	6.0451	3.9976
6.3005	4.3604	6.0377	4.1979
6.2874	4.5180	6.0259	4.4846
6.2630	4.5827	6.0238	4.7984
6.2612	4.7656	5.9965	4.9936
6.2071	4.9325	5.9468	5.3306
6.1371	5.0532	5.9416	5.6081
6.1083	5.1791	5.9248	5.8107
6.0770	5.3271	5.8545	6.2596
6.0606	5.4523	5.8133	6.6605
6.0320	5.5932	5.7491	7.1507
5.9968	5.7574		
5.9918	5.9227		
5.9521	6.2681		
5.9379	6.4509		

The experimental binodal curves of each ABS were correlated using the following equation:

$$Y = A\exp(BX^{0.5} - CX^{3}) \qquad \text{(Equation A.1)}$$

where A, B and C are correlation constants, and X and Y are the weight concentrations (wt%) of NaPA 8000 and PEG 600, respectively.

Table A.17. Correlation parameters to describe the binodal curve, where A, B and C are constants obtained by the regression of the experimental binodal data through the application of Eq. (1) (and respective standard deviations, σ, and correlation coefficients, R^2) for the system composed of PEG 600 + NaPA 8000.

0 wt % of [Ch]X	
R^2	0.9977
$A \pm \sigma$	54.17 ± 2.03
$B \pm \sigma$	-0.45 ± 0.02
$10^5C \pm \sigma$	9.65 ± 1.28

Table A.18. Correlation parameters to describe the binodal curves, where *A*, *B* and *C* are constants obtained by the regression of the experimental binodal data through the application of Eq. (1) (and respective standard deviations, σ, and correlation coefficients, R^2) for the systems composed of PEG 600 + NaPA 8000 + [Ch]Cl.

	1 wt % of [Ch]Cl	2.5 wt % of [Ch]Cl	5 wt % of [Ch]Cl	10 wt % of [Ch]Cl
R^2	0.9954	0.9938	0.9990	0.9971
$A \pm \sigma$	44.31 ± 1.35	62.76 ± 1.79	56.58 ± 0.87	68.84 ± 1.48
$B \pm \sigma$	-0.37 ± 0.01	-0.57 ± 0.01	-0.54 ± 0.01	-0.67 ± 0.01
$10^5C \pm \sigma$	18.14 ± 1.31	8.24 ± 1.30	9.58 ± 0.60	2.11 ± 1.16

Table A.19. Correlation parameters to describe the binodal curves, where *A*, *B* and *C* are constants obtained by the regression of the experimental binodal data through the application of Eq. (1) (and respective standard deviations, σ, and correlation coefficients, R^2) for the systems composed of PEG 600 + NaPA 8000 + [Ch][Ac].

	1 wt % of [Ch][Ac]	2.5 wt % of [Ch][Ac]	5 wt % of [Ch][Ac]	10 wt % of [Ch][Ac]
R^2	0.9972	0.9977	0.9920	0.9941
$A \pm \sigma$	62.07 ± 2.36	69.26 ± 1.41	71.75 ± 3.40	75.18 ± 2.33
$B \pm \sigma$	-0.53 ± 0.02	-0.62 ± 0.01	-0.66 ± 0.02	-0.70 ± 0.01
$10^5C \pm \sigma$	7.90 ± 0.99	0.83 ± 0.88	-1.42 ± 1.61	1.31 ± 1.42

Table A.20. Correlation parameters to describe the binodal curves, where *A*, *B* and *C* are constants obtained by the regression of the experimental binodal data through the application of Eq. (1) (and respective standard deviations, σ, and correlation coefficients, R^2) for the systems composed of PEG 600 + NaPA 8000 + [Ch][DHP].

	1 wt % of [Ch][DHP]	2.5 wt % of [Ch][DHP]	5 wt % of [Ch][DHP]	10 wt % of [Ch][DHP]
R^2	0.9986	0.9920	0.9988	0.9994
$A \pm \sigma$	55.87 ± 1.65	78.45 ± 1.91	59.03 ± 2.08	78.88 ± 1.94
$B \pm \sigma$	-0.47 ± 0.01	-0.63 ± 0.01	-0.49 ± 0.02	-0.60 ± 0.01
$10^5C \pm \sigma$	10.14 ± 1.12	0.00 ± 2.51	13.68 ± 1.22	1.56 ± 0.80

Table A.21. Correlation parameters to describe the binodal curves, where *A*, *B* and *C* are constants obtained by the regression of the experimental binodal data through the application of Eq. (1) (and respective standard deviations, σ, and correlation coefficients, R^2) for the systems composed of PEG 600 + NaPA 8000 + [Ch][DHcit].

	1 wt % of [Ch][DHcit]	2.5 wt% of [Ch][DHcit]	5 wt % of [Ch][DHcit]	10 wt % of [Ch][DHcit]
R^2	0.9985	0.9918	0.9980	0.9984
$A \pm \sigma$	61.65 ± 2.49	92.46 ± 2.63	95.45 ± 0.97	90.76 ± 0.96
$B \pm \sigma$	-0.51 ± 0.02	-0.64 ± 0.01	-0.60 ± 0.01	-0.51 ± 0.01
$10^5C \pm \sigma$	6.48 ± 1.35	2.33 ± 1.34	6.54 ± 0.68	11.69 ± 0.79

Table A.22. Correlation parameters to describe the binodal curves, where *A*, *B* and *C* are constants obtained by the regression of the experimental binodal data through the application of Eq. (1) (and respective standard deviations, σ, and correlation coefficients, R^2) for the systems composed of PEG 600 + NaPA 8000 + [Ch][Bit].

	1 wt % of [Ch][Bit]	2.5 wt % of [Ch][Bit]	5 wt % of [Ch][Bit]	10 wt % of [Ch][Bit]
R^2	0.9782	0.9865	0.9917	0.9996
$A \pm \sigma$	105.68 ± 8.30	122.12 ± 9.17	196.48 ± 6.83	138.99 ± 1.29
$B \pm \sigma$	-0.72 ± 0.04	-0.76 ± 0.04	-0.87 ± 0.02	-0.67 ± 0.00
$10^5C \pm \sigma$	-1.61 ± 2.78	-2.64 ± 2.69	-5.40 ± 2.14	9.74 ± 0.44

Quantification of the IL electrolyte in the ABS coexisting phases

By quantifying the cholinium cation present in each phase by means of the benzene internal standard by ^{1}H NMR spectroscopy, the partition coefficient (K_{elect}) of $[Ch]^{+}$-ILs was determined, defined as the ratio between the concentration of each $[Ch]^{+}$-based IL present in the PEG-rich phase (top) and NaPA-rich phase (bottom).

Table A.23. Number of moles of $[Ch]^{+}$ at each phase, volume of the phase, and K_{elect} values of $[Ch]^{+}$-based ILs.

	[Ch]Cl		**[Ch][Ac]**		**[Ch][DHP]**		**[Ch][DHcit]**		**[Ch][Bit]**	
Phase	**top**	**bot**	**top**	**bot**	**top**	**bot**	**top**	**bot**	**top**	**bot**
n $[Ch]^{+}$ (mol)	4.29E-03	1.47E-05	3.89E-03	1.37E-05	3.10E-03	7.62E-05	3.78E-03	1.32E-04	3.30E-03	1.18E-04
Volume (mL)	3.7	0.9	3.6	0.9	3.3	1.5	3.6	1.0	3.6	1.0
K_{elect}	71.14		71.00		18.47		7.99		7.81	