Universidade de Aveiro Departamento de Química

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

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Palavras-chave Biofármacos, Anticorpos, Imunoglobulina Y, Purificação, Sistemas Aquosos Bifásicos, Cromatografia de Partição de Força Centrífuga

Resumo A dificuldade em desenvolver antibióticos mais eficientes, numa altura em que a resistência microbiana tem vindo a aumentar, torna essencial o desenvolvimento de terapias alternativas, económicas e eficazes. Os anticorpos obtidos a partir da gema de ovo de galinha, imunoglobulina Y (IgY), têm-se destacado não só pela sua produção mais simples e em maior quantidade em relação aos anticorpos policlonais de mamífero, mas também devido às inúmeras vantagens em termos de aplicações. No entanto, atualmente não existe uma plataforma de purificação de IgY que seja económica, eficaz e passível de aplicação a nível industrial - uma lacuna que este trabalho se propõe a resolver. Assim, neste trabalho, estudou-se a possibilidade da utilização de sistemas aquosos bifásicos (SAB) compostos por PEG 1000 e tampão fosfato (K_2HPO_4/KH_2PO_4) ou K_2HPO_4, seguidos de um passo de ultrafiltração, ou acoplados à tecnologia de cromatografia de partição de força centrífuga (CPC), para a purificação de IgY. Foram avaliados os efeitos de pH (5,5; 6,0; 6,5; 7,5; e 8,0) e composição de PEG e sal na extração de IgY, bem como as condições utilizadas na CPC (fluxo da fase móvel, rotação e modo de operação). Foi estudada a estabilidade do anticorpo em soluções aquosas dos componentes utilizados nos SAB utilizando dicroísmo circular, assim como a atividade/estabilidade do anticorpo após o processo de purificação por ELISA, salientando assim o efeito do PEG 1000 na estrutura secundária e na atividade do IgY. O ATPS constituído por 18 % PEG 1000 + 13 % tampão fosfato a pH 6,0 conduz aos melhores resultados em termos de purificação, obtendo-se num único passo de extração uma pureza de IgY de 39 %. Após a aplicação de CPR, obteve-se IgY com um grau de pureza de 51 %, e com ultrafiltração, IgY com um grau de pureza de 47 %. Face aos resultados obtidos, destaca-se a CPR como a técnica mais adequada dado que permite obter IgY com um maior grau de pureza e ser passível de aplicação à escala industrial.

Keywords Biopharmaceuticals, Antibodies, Immunoglobulin Y, Purification, Aqueous Biphasic Systems, Centrifugal Partition Chromatography

Abstract The difficulty in developing more effective antibiotics, at a time where the microorganism's resistance to them has been increasing, turns essential the development of cheaper and effective alternative therapeutics. Antibodies obtained from the chicken's egg yolk, immunoglobulin Y (IgY), have stood out because of their production simplicity and production in higher quantity when compared to mammal polyclonal antibodies, and also because of their advantages in terms of applicability. Nonetheless, there is still no low-cost, effective and scalable platform for the IgY purification - a vacuity that this work aims to solve. Therefore, in this work, the possibility of using aqueous biphasic systems (ABS) composed of PEG 1000 and phosphate buffer (K_2HPO_4/KH_2PO_4) or K_2HPO_4, followed by an ultrafiltration step, or coupled with centrifugal partition chromatography (CPC), was studied. The effect of pH (5.5; 6.0; 6.5; 7.5 and 8.0) and the PEG + phosphate buffer composition in the extraction of IgY was investigated, as well as the conditions to be used in CPC (mobile phase flow rate, rotation, and the operation mode). The stability of the antibody in aqueous solutions of the components used in the ABS formation was studied using circular dichroism, as well as the activity/stability of the antibody after the purification process, by ELISA, primarily outlining the effect of PEG 1000 in the secondary structure and activity of IgY. The ABS composed of 18 wt % PEG 1000 + 13 wt % phosphate buffer at pH 6.0 yielded the best results in terms of purification, achieving a 39 % IgY purity in a single extraction process. After the application of CPC, an IgY purity of 51 % was obtained, and with the ultrafiltration technique a purity of 47 % was obtained. According to these results, CPC appears as the most adequate purification technique due to the higher purity of IgY obtained and possibility of being applied at an industrial level.

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Abbreviations

ABS	*Aqueous Biphasic Systems*
AEC	*Anion Exchange Chromatography*
CCD	*Counter Current Distribution*
CD	*Circular Dichroism*
CEC	*Cation Exchange Chromatography*
CPC	*Centrifugal Partition Chromatography*
Cr	*Constant Region*
ELISA	*Enzyme-Linked Immunosorbent Assay*
EU	*European Union*
Fab	*Antigen-binding Fragment*
Fc	*Crystallizable Fragment*
FCPC	*Fast Centrifugal Partition Chromatography*
Hc	*Heavy Chain*
HIV	*Human Immunodeficiency Virus*
IgG	*Immunoglobulin G*
IgY	*Immunoglobulin Y*
Lc	*Light Chain*
$\mathbf{K_{cont}}$	*Partition Coefficient of the contaminants*
mAbs	*Monoclonal Antibodies*
pAbs	*Polyclonal Antibodies*
PEG	*Poly Ethylene Glycol*
pI	*Isoelectric Point*
RPM	*Rotations Per Minute*
Sf	*Stationary phase retention*
TL	*Tie-line*
TLL	*Tie-line Length*
Vc	*FCPC column volume*
Vs	*FCPC stationary phase volume*
WSPF	*Water-Soluble Protein Fraction*

1. General introduction

1.1. Context and Objectives

While the use of antibiotics has been increasing, the antibacterial pipeline is scarce [1,2], giving rise to significant antimicrobial-resistant infections as a result of the microbe's potential to mutate and adapt to treatment [3,4]. Thus, there is an enormous need for alternative therapies, which include other pharmaceuticals, such as biopharmaceuticals, in addition to antibiotics. The classic definition of biopharmaceuticals refers to compounds of biological nature with biological/therapeutic activity, manufactured using biological sources and processes [5]. These compounds have been seen as promising alternatives to conventional pharmaceuticals, from the historical example of insulin for the treatment of diabetes [6], the use of coagulation factors, such as Factor VIII and IX, for the treatment of both A and B types of hemophilia [7–9], modified growth hormones against acromegaly [10], and the use of retroviruses against serious infectious diseases, like the Human Immunodeficiency Virus (HIV) [11]. In 2015, nearly 250 biopharmaceuticals have been approved for sale in the United States of America (USA) and in the European Union (EU), while the sales of therapeutic proteins cumulatively reached 140 billion US$ by 2013, with antibodies representing a large fraction of the overall biopharmaceutical approvals (27 %, 75.7 billion US$) [12].

Polyclonal antibodies, such as immunoglobulin G (IgG), can be obtained from mammal's blood, such as horses, sheep, and rabbits [13,14], while monoclonal antibodies are typically obtained by the use of cell cultures [15]. Nevertheless, viable alternatives from hen egg yolk have been suggested, namely the avian antibody immunoglobulin Y (IgY), as a functionally equivalent antibody of IgG [16]. The collection of IgY minimizes the ethical concerns with the standard practices of antibody production from mammal's blood [17]. Concerning the applicability of IgY, there are also advantages to be found by exploring the evolutionary distance between mammals and birds, since this fact allows the production of antibodies against highly conserved mammalian proteins, providing a more efficient immune response, and bypassing the activation of interference factors like the complement system or by human anti-mouse antibodies, unlike mammalian antibodies [18]. Despite these advantages, the common use of IgY is still restricted by difficulties in its isolation and purification from the egg yolk complex matrix, requiring improvements in the current purification techniques and/or development of new ones [19].

The downstream processing of biopharmaceuticals is a critical part of their production, accounting, for instance, with 50 % to 80 % of the total manufacturing costs in the case of therapeutic antibodies [20]. The need of optimizing this part of the process is a crucial demand to allow a viable upscale production of antibodies and to decrease their current cost. The antibodies separation and purification are usually performed by solid-liquid chromatography [21]; however, Aqueous Biphasic

Systems (ABS) have been studied as a possible alternative [22]. ABS are a liquid-liquid extraction technique, being a primary stage unit used in the downstream processing of many biological products, like cells, viruses, proteins, and antibodies [23]. These systems are prepared by the mixing of two phase-forming components in water, that above-given concentrations undergo phase separation while providing a suitable water-rich environment for maintaining the biological activity of many biomolecules [24,25]. Since the introduction of ABS by Albertsson [26], multistage extractions using ABS have been an ambition aiming at increasing the purification factors of target products. In 1964, the introduction of centrifugal partition chromatography (CPC) [27] turned this ambition into a reality, and, in recent years, there have been some examples of successful use of CPC for the separation of biochemical products [28–30]. CPC is a chromatographic separation technique, based on the principles of liquid-liquid extraction, that features a series of chambers mounted on a disk, connected from the top of one to the bottom of another, that rotates on an axis, and creating a centrifugal force field [27]. The stationary phase is loaded while the other phase is pumped through, creating a waterfall-like mechanism mixing the mobile phase and the stationary phase in each chamber [27]. This technique has several advantages, detailed in further chapters, but the easy scale-up and industrial adaptation of the process, as well as other economic and environmental factors [27,31], make it especially relevant to the goal of this work.

The main objective of this work conveys on the study of the fast centrifugal partition chromatography (FCPC) technique with ABS for the purification of IgY from egg yolk, envisaging the purification technique scale-up and a decrease of these biopharmaceuticals costs. The target antibody selected is IgY, not only due to the relative advantages in terms of applicability, but also because of the efficiency of its production [32]. PEG/salt-based ABS have been chosen since these systems have phase-forming components of relatively low cost, provide a stable and fast phase separation, display an adequate density difference between the two phases, and are well documented and characterized in terms of phase diagrams [33]. The use of the FCPC technology coupled with the bio-suitable environment provided by ABS may provide an important step into obtaining cheaper and widely available biopharmaceuticals, as envisioned and demonstrated with this work.

1.2. Production and applications of antibodies

Antibodies, found in plasma and extracellular fluids, are involved in the defense of an organism by connecting to foreign and adverse substances, further activating a cascade of reactions that ultimately lead to the neutralization and destruction of these. The compounds that antibodies

connect to are referred to as antigens and are usually proteins, glycoproteins or polysaccharides [34], but also encompasse a wide range of chemicals, like metals, toxins, among others [35].

The immune system branches out into two types of responses, innate and adaptive; the first occurs fast but is non-specific, while the second, albeit slower, is specific and can lead to long-term protection [36]. The innate response starts in the immediate moment of infection and, on a first stage, lasts for up to four hours. It is based on the deployment of preformed solutions in the blood, extracellular fluids, and epithelial secretion, to either kill the pathogen outright or weaken its effect. These solutions include several antimicrobial substances: enzymes, like lysozyme, to digest the bacterial walls; peptides, like defensins, that lyse the bacterial cell membranes directly; and the plasma proteins of the complement system responsible for the targeting of pathogens for lysis and phagocytosis [37]. After the first four hours, and until the activation of the adaptive response, the cells responsible for innate immunity begin recognizing molecules typical to microbes and foreign to the host – pathogen-associated molecular patterns – and start to activate effector cells and inducing inflammation, in an attempt to halt the pathogens progress [37,38].

There are two general types of antibodies, monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs), and the traditional production of both begin in the same manner - by inoculation with an antigen or a mixture of antigens and, if the antigens used are poorly immunogenic, with an adjuvant to increase the immune response [42]. The choice of the animal used plays a significant part in the production process, as factors like the amount of antibody needed and its intended use, the easiness of handling the animals, and the phylogenic distance between the antigen and the animal species, have to be taken into account [42]. After this first step, and after the time required for the immune response to occurs, the process differs between the two types of antibodies. Antigen inoculation for mAbs production trigger the activation of specific B-cells that then have to be harvested, usually from the animal's spleen, and fused with a type of cancerous immune cell that allows for the indefinite proliferation of the fused B-cell, a myeloma [43]. In the case of pAbs, the usual process relies on their extraction and purification from the serum of immunized mammals [44]. The production of antibodies from cell cultures is also an important alternative methodology since these cultures provide higher amounts of consistent products from a single cell clone [45]. This technique is based on the introduction of an expression vector plasmid, designed to contain the desired antibody's Lc and Hc genes and a special selector gene, to a cell culture that contains a selector agent [46]. The selector gene in the added plasmid, along with the selector agent in the culture, ensure that only the cells transformed by the plasmid survive after some time [46]. The surviving cells are then collected and allowed to proliferate in a second cultivation vessel, after which individual clones are tested for

production efficacy of the target antibody, with the most efficient clones being chosen for scale up and long-term expression [45].

The applications of pAbs and mAbs are diverse, with both types of antibodies presenting their advantages and disadvantages, not only in terms of production but also in applicability. Concerning the production of antibodies, there is a notable difference between these two classes. For instance, pAbs can be faster generated and at a lower expense, usually being ready some months after the first immunization [47]. In contrast, the process of generating hybridomas can add a considerable time expense to the production of mAbs, requiring up to a year in some cases and presenting significant costs [47]. The process for achieving effective antibody production with cell cultures is also significantly laborious, due to the requirement of analyzing and selecting efficient clones for production [15]. In spite of time-consuming and expensive production requirements, mAbs have considerable advantages in terms of homogeneity and consistency, since they are highly specific and thus useful in many therapeutic scenarios [48]. mAbs are also better suited for evaluating small changes in molecular conformation [49], protein-protein interactions [50], and phosphorylation state [51]. There are, however, situations where the advantages of mAbs are reduced, particularly regarding their specificity and homogeneity that can prevent them from adequately recognizing an epitope when small changes occur to it, be it by genetic polymorphism, glycosylation or even by small changes in the structure brought about by denaturation. Since they recognize several epitopes of an antigen, pAbs can be consistently used against highly mutagenic targets, like viruses, in analysis assays such as immunoprecipitation and enzyme-linked immunoassay (ELISA), considering not only their multiple epitope binding capabilities but also their usefulness as secondary anti-species antibody conjugates and binding effectiveness, even when covalently linked to a fluorochrome [42].

When it comes to diagnosis and research methods, mAbs are an essential piece in many established techniques, and generally more used than pAbs, due to their more specific nature and standardization of mAbs produced from hybridomas or cell cultures [52]. Since the development of a competitive binding assay using a radioisotope, by Yalow et al. [53], and after the design of the first ELISA quantitative assay by Engvall et al. [54], antibodies have proven to be a major scientific tool. The assays reliant on antibodies mostly function by detecting specific targets, which can be scientifically exploited for many uses. Immunocytochemical and immunohistochemical techniques are clinically vastly used to detect diseases biomarkers in cells or tissues [55,56], while techniques like Western blot are more suited towards the detection and classification of proteins in a mixture [57]. Besides the techniques focused on detection, some antibody-based methodologies also provide adequate tools for quantification, such as ELISA or radioimmunoassay [58,59], by specifically binding

to target antigens and then emitting a quantifiable signal, based on the concentration of the target. In addition to quantitative and detection applications, antibodies have an enormous potential for separation and purification, used in techniques like immunoprecipitation and immunoaffinity chromatography [60,61]. There are also some antibody-based techniques that are more flexible, making their use possible for many applications. Some notable examples include immuno-polymerase chain reaction methodologies, that can provide both sensitive real-time detection and quantification of proteins [62]. Another very flexible technique is flow cytometry, where antibodies serve as labels for a high number of cells that can then be processed by flow cytometry, capable of recording quantitative and qualitative information, thus enabling cell counting and sorting procedures [63].

1.3. Immunoglobulin Y (IgY): an avian antibody

The term IgY emerged in 1969 when Leslie and Clem identified the egg-laying avian homolog of the IgG mammalian antibodies [64]. The general structure of IgY is the same as IgG: composed of four polypeptides, with two identical heavy chains (Hc) presenting a molecular mass ranging from 67 to 70 kDa each in IgY, in contrast to the 55 kDa of IgG [65], and light chains (Lc) with a molecular mass of 25 kDa each [13], that are held together by disulfide and covalent bonds in a Y shape. Each arm of the "Y", the antigen-binding fragment (Fab), contains a binding site responsible for the antigen binding, and the tail of the "Y", the crystallizable fragment (Fc), imparts the antibody with its effector functions, such as pathway activation [47]. An important difference between IgY and IgG antibodies however exists. The number of constant regions (Cr) at the Hc in IgG is 3, while in IgY it is 4, explaining the molecular mass difference between IgG and IgY, 180 and 150 kDa, respectively [32]. IgY also lacks a hinge region, rich in proline, threonine, and serine, that is normally present in IgG, responsible for connecting the Fc and the Fab region of the antibody and providing it with a lateral and rotational movement for an improved antigen binding [47]. The IgY molecule may compensate its lack of a hinge region by the expression of proline and glycine residues near the boundaries of the Cr, spatially similar to where the hinge region appears in IgG [13]. These referred structural differences are shown in Figure 1.

Concerning their physicochemical parameters, IgY is more hydrophobic that IgG and its isoelectric point (pI) is 5.5, lower than that of IgG that fits within 6.1-6.5 [66 67]. Another important parameter to take into account, especially concerning the oral administration of antibodies, is their pH and temperature stability. In this regard, IgY is more sensitive than IgG to acid denaturation, rapidly losing its activity at pH values between 3 and 4 [68]. However, Lee et al. [68] found that the addition of sorbitol can almost completely suppress the acid-induced deactivation, even at pH 3. On

the other extreme of the pH scale, IgY has been found to stay active under severe alkaline conditions, up to pH 11, having nevertheless its activity severely diminished at pH 12 and higher values [69]. Finally, and in terms of temperature stability, IgY is stable in aqueous solutions at temperatures up to 60 ºC with the addition of sucrose [70].

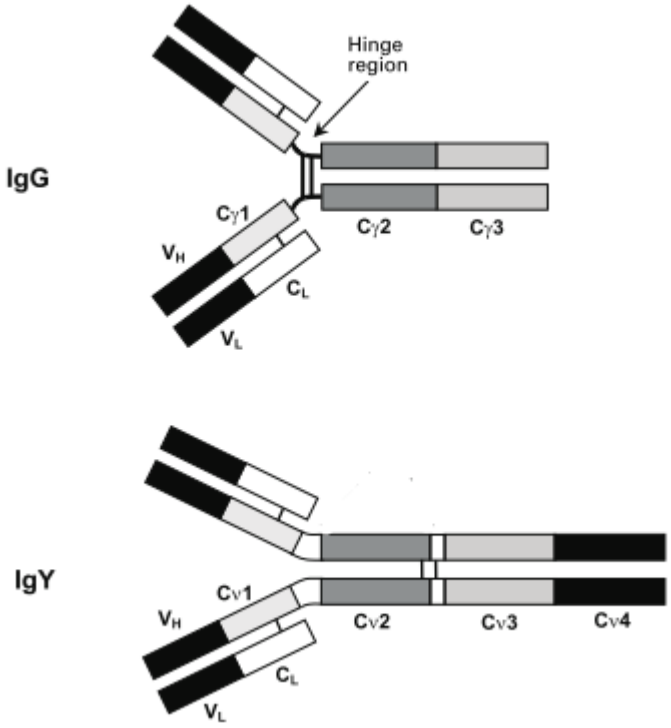

Figure 1 - Structural differences between IgY and IgG molecules [71].

Being one of the three classes of antibodies that birds produce (IgA, IgM, and IgY), IgY is by far the most relevant in terms of concentration, making up about 75 % of the total serum antibody population of chickens [72]. IgY antibodies also are transferred from hen to chick in the latent stage of egg development by receptor-mediated processes [73]. This fact makes the chicken egg a great source of antibodies, not only compared to the chicken's serum [72], but also compared to others mammals serum, showing a per weight productivity 18 times higher than rabbit serum, for instance [74]. Besides their higher production, there are other significant advantages connected to IgY, such as the antibodies production methodologies. The production of IgY requires only the collection of eggs, providing thus a more ethical and healthy treatment of animals than the traditional methodology used to obtain IgG where a significant amount of blood is necessary (obtained by repeated bleeding or heart puncture, possibly resulting in animal death) [17]. Furthermore, chickens not only tolerate the immunization process better than rabbits [17], but are also easier to house [75], whereas the process for their immunization and collection of eggs is already well established on an industrial scale.

In terms of functionality, the use of IgY has significant advantages that make it very useful, especially regarding their use in immunoassays, where the phylogenetic difference between birds and mammals plays an important role, making of avian antibodies better suited for detection and binding with very conserved proteins in mammals [76]. One specific case is the detection of proteins associated with human pregnancy, in which immunological cross-reactivity could not be achieved with rabbit or goat anti-serum, whereas it is possible with avian antibodies [77]. This phylogenetic difference can be further exploited by looking at the fact that avian antibodies do not show cross-reactivity with rheumatoid factors, enabling the monitoring of acute phase proteins expression as a response to inflammatory processes, like in rheumatoid patients, that cannot be achieved with mammalian antibodies because of cross-reactivity [78]. Further examples of the application of IgY, due to the inherent phylogenetic difference, lie in its lack of cross-reactivity with human antibodies [79]. Another large contributor to interferences in immunoassays is the activation of the complement system [80], that can bind to mammalian antibodies and block the antigen-binding site [81], something that does not occur when avian antibodies are used [80]. In spite of these advantages it is important to refer that antibodies can be humanized [82], normally using recombinant technology [83]; yet, these processes require more time, manpower and monetary expense that could, in some cases, be avoided by using avian antibodies. For the reasons referred above, the use of IgY in immunoassays and immunodiagnostics has been reported and reviewed thoroughly [84]. In spite of its lack of popularity, IgY is suited for practically all common immunologic methods [85], mainly because IgY can form conjugates with typical molecules used in these methods, like horseradish peroxidase or biotin [86,87]. Some notable cases include its use in a fluorescence polarization immunoassay [88], rocket electrophoresis [89] and antigen-capture ELISA [90].

In addition to their notable advantages for immunoassays, avian antibodies also have amazing prophylactic capacities that can be tapped by exploiting the process of passive immunization. Passive immunity, described in detail in the previous chapter, refers to the process of providing preformed antibodies for immediate but short-lived protection, lasting several weeks to three or four months at most. Therefore, passive immunity requires repeated or continuous administration and large amounts of antibodies [91], making hen antibodies more adequate for this type of application because of the relative easiness of production and acquisition. Concerning veterinary applications, IgY has been examined as a feed additive for livestock to both target pathogens, especially enteric, and to improve growth and feed efficiency [92]. Some notable examples include the protection of pigs against E-coli infection by oral administration [93], the protection of newborn calves against Bovine rotavirus [94], and the reduction of immune-stimulating bacteria in the gut of animals by targeting molecules involved

in inflammation and animal growth, among other examples described in Table 1. When it comes to applications in human medicine, IgY has also proved its efficiency and viability, getting the designation of an orphan drug, an agent developed especially to treat a rare condition by the European Medicines Agency, namely on their use in the prevention of *Pseudomonas aeruginosa* in the airways of cystic fibrosis patients [95]. Other anti-bacterial effects of IgY include the suppression of *Heliobacter pylori* infection, a common cause of gastritis and gastric ulcers [96], and the reduction of the levels of *Porphyromonas gingivalis* [97] and *Streptococcus mutans* [98], responsible for plaque forming and tooth decay in humans.

Table 1 - Examples of IgY use in veterinary medicine (adapted from the literature [99]).

Pathogen/antigen	Target Species	Effects of IgY
Escherichia coli	Pigs	Protection against infection
	Cattle	Reduction of *E. coli* induced fecal shedding
	Chickens	Improvement of intestinal health and immune response following bacterial challenge
	Humans	Reduction of *in vitro* binding
Salmonella spp.	Chickens	Reduction of fecal shedding and cecal colonization
Bovine rotavirus (BRV)	Cattle	Protection of neonatal calves against BRV induced diarrhea
Infectious bursal disease virus (IBDV)	Chickens	Protection of chicks from IBDV infection
Eimeria spp.	Chickens	Protection of chicks against avian coccidiosis
Porcine epidemic diarrhea virus (PEDV)	Pigs	Protection from PEDV infection
Canine parvovirus	Dogs	Protection against CPV2-induced disease symptoms
Heliobacter pylori	Humans	Reduction of bacterial growth, urease activity and gastric mucosal injury in animal model
		Suppressed infection in humans when incorporated in yogurt

1.4. Conventional methods for the purification of antibodies

There are many techniques for the production of antibodies, developed and improved over the years, involving ascites fluid [100], plasma cells [101], cell cultures [15], bacteria and yeast [102], transgenic plants [103], and egg yolk. All of these production techniques result in many different end products that contain the target antibodies, as well as other impurities, like cellular components and debris, DNA, lipids or other proteins. For this reason, the purification of antibodies is a very important step in the development of a successful platform of antibodies production, commercialization, and application. Many techniques for the purification of antibodies have been used, and even though chromatographic methods are the most commonly used, there are many other techniques that should be discussed. The most common technique used for proteins purification is chromatography, and as such, it is mandatory to take into consideration affinity chromatography, one of the most applied techniques [104]. This technique is based on a reversible specific interaction of the target protein with a ligand, exploiting this specificity in order to separate the target compounds from a complex mixture. While there have been some efforts on the development of new types of ligands, protein-A is still the most

used [104]. Protein A, a cell wall component of *Staphylococcus aureus,* binds selectively to IgG, by the antibody's Fc regions, but binds weakly to human IgA or IgM [105], and the connection to IgY is almost negligible [106]. Despite its high binding capacity for IgG, and as such its main use is for IgG purification, there are some major disadvantages with the use of protein A, namely the high manufacturing and processing costs, difficulties in scaled-up applications, limited life-cycles and, since the production of these types of ligands is achieved through bacteria, there is a risk of contamination by viruses, pyrogens and DNA [104].

Another kind of chromatographic technique used for the purification of antibodies is ion-exchange chromatography, which includes anion exchange chromatography (AEC) and cation exchange chromatography (CEC). AEC has been a focal point of scientific innovation due to this technique importance for the removal of DNA, virus, endotoxin, leached protein-A and acidic host cell protein contaminants, being one of the most flexible tools in chromatography [107]. When it comes to chromatography it is necessary to distinguish between two main modes: bind elute, where the sample elements are bounded to the column, and flow through, where the column contents are flushed along with the solvent [108]. Bind elute AEC is useful when the sample contains impurities that bind less strongly to the used column, like in the case of murine IgG [109]. Flow through chromatography is more favorable when the antibodies bind weakly or not at all to the column, as is the case with human and chimeric IgG [109]. The flow-through mode is commonly conducted on membrane exchangers, since they are less expensive and provides a higher throughput than porous particles, without compromising the DNA or virus removal [110], with the host-cell protein removal usually being the limiting factor. In light of this fact, there have been studies detailing the use of displacement chromatography, where the antibody is loaded continuously in conditions where it binds weakly to the column, achieving more than 99 % of host cell protein removal, with a high ratio of antibody processed per liter of membrane [111]. On the other hand, with the introduction of new porous particle ion exchangers, there has been an interest in using CEC as a substitute for protein-A capture [112]. There are significant cost and cleaning advantages and high binding capacities [112]; however, the pH control has proved to be a challenge for the elution process for CEC [113]. When salt gradient elution is utilized, ions like sodium have a higher affinity for exchange groups than hydrogen ions, causing a displacement of hydrogen to the mobile phase, and consequently a pH drop [113].

When it comes to non-chromatographic methodologies, the discussion of filtration-based methods is of utmost importance. These techniques have maintained the interest of the scientific community, despite its industrial application being reliant on bulky and expensive hardware when some kinds of membrane filtration systems are used [114]. Although the high costs associated with this

technique, there are some examples of successful scale-up applications using cross-flow filtration for cell cultures [115]. Van de Reis et al. [116] developed a technique based on cross-flow filtration, yet using a charged ultrafiltration membrane that, via ion exclusion, repels positively charged proteins such as mAbs. As mAbs are small enough to pass through the pores, they are retained and concentrated, while alkaline, neutral and weakly acidic contaminants are released, and strongly acidic contaminants may be retained [116]. This technique can present some disadvantages since, at membrane conductivity values low enough to repeal antibodies, positively charged surfaces can bind DNA that, if present at a high enough concentration, can reduce the membrane's potential, thus significantly impairing the processes' effectiveness [117]. Utilizing this technique as a secondary step to other processes might solve this contamination problem, one example of which being co-precipitation. The use of positively charged polymers has led to the selective precipitation of acidic host proteins, DNA, and various other culture additives, and a multistep purification process using polyallylamine has led to results competitive with those obtained with protein-A based chromatographic processes [118].

1.4.1. IgY purification methods

To obtain pure IgY from egg yolk there are two crucial steps: (i) removal of the lipids and lipoproteins, obtaining a water-soluble protein fraction (WSPF); and (ii) purification of IgY from the WSPF which contains a large number of water-soluble proteins. One of the most used techniques for lipids removal from egg yolk, is water dilution, as proposed by Kwan et al. [119]. Further research by Akita et al. [120] led to an optimum recovery from 93 to 96 % of IgY by exploring a 6-fold water dilution at pH 5.0 and incubating the sample for 6 h at 4 ºC. Another methodology that is also simple consists on freezing and thawing the egg yolk samples, introduced by Jensenius et al. [121], who explored the aggregation of yolk lipids at neutral pH after a 9 volume water dilution and a freezing and thawing cycle, finally recovering the antibody by centrifugation [121]. Even though both of these processes have some disadvantages considering the increase of sample volume, and thus increasing the storage and handling requirements, the simplicity and cost can, in some cases, outweigh these drawbacks. Jensenius et al. [121] used a modified process for the separation of lipoproteins [122], achieving better results using dextran sulfate on a buffer-diluted yolk solution and then adding calcium chloride to precipitate the dextran in excess, followed by incubation at room temperature and centrifugation [121]. Other similar precipitation approaches include the use of phosphotungstic acid and magnesium chloride [123], and polyethylene glycol (PEG) [67]. The use of volatile organic solvents has also been explored, with some researchers utilizing propane-2-ol and acetone in a series of cycles of continuously-stirred mixing, followed by the collection of the resulting powder and its dissolution in

a buffer, finally recovering the proteins by centrifugation [124]. A comprehensive comparison of the lipids removal capacities of the referred processes revealed that the highest protein yields were obtained via methods based on the combination of polyanions and cations, the dextran sulphate and the phosphotungstic acid methodologies, achieving average IgY yields of 15.6 and 15.1 mg per mL of yolk, with purities of 64.3 and 69.8 %, respectively [125]. The simpler process of freezing and thawing at neutral pH led to IgY recoveries of 13.1 mg per mL of yolk, being this process the one that resulted in the highest purity level (71.1 %). The remaining two processes resulted in a low purity end product, capping their effective IgY yield at only 11.0 mg per ml of yolk, in the case of PEG precipitation, and 7.11 mg per ml of yolk for the propane-2-ol and acetone technique [125].

After the initial delipidation step, the obtained WSPF is then processed in order to be concentrated and further purified, normally using a combination of several techniques, which can be grouped into three types: precipitation, filtration and chromatographic techniques [19]. Akita and Nakai [120] showed the recovery and purity percentages of IgY after several stages of purification from the WSPF, starting with the precipitation with ammonium sulfate, followed by either ethanol precipitation at sub-zero temperatures or ultrafiltration, with a final chromatographic step using either AEC or size exclusion chromatography. These researchers found that while just the salt precipitation procedure was insufficient to obtain a pure sample, reaching only 30 % of purity, following it with the sub-zero temperature ethanol procedure or ultrafiltration could lead to high purity samples of over 93 % [120]. Following either of these techniques with Diethylaminoethyl–Sephacel, a kind of AEC, or gel filtration with Sephacryl S-200 led to even greater purities, over 99 % [120]. Deignan et al. [125] observed that the best precipitation method, when compared to ammonium sulfate and sodium sulfate salt precipitation, was the one developed by Polson et al. in 1985 [126], using 12 wt % of PEG 8000 followed by its removal with sub-zero ethanol. Kim and Nakai [127] demonstrated the possibility of scaling-up the IgY purification methods by utilizing several ultrafiltration systems on a WSPF delipidated using an octadecyl column after filtration with a cellulose powder column. They achieved a high IgY purity with all the systems tested, the best being the Amicon, reporting an IgY purity of 99 % and a recovery of 80 % [127]. An earlier publication by the same group [128], demonstrated purity levels over 90 % and a recovery of about 92 % of IgY, with the key methodologic difference being the use of the freeze and thaw method to obtain the WSPF, a cheaper and easier process when compared to the delipidation method used in their other work [127]. Lastly, but possibly the most used, are the chromatographic techniques used for IgY purification, even though they can be rather restricted if the sample is too complex [129], making it necessary to couple this technique with others. Fichtali et al. [130] achieved about 60 % of purity for IgY from a WSPF produced via the water dilution

method, using an HC-2 medium CEC. The process was then coupled with a salt precipitation process using 15 wt % of sodium sulfate, improving the sample's purity up to 90 %; yet, this technique leads to low recoveries (50 %) [130]. The purification techniques presented above all require some performance and quality compromises for monetary and simplicity advantages, or vice versa. Therefore, there is no end-all technique available for IgY purification that can be effective, cheap and able to be scaled-up. In this sense, there is a growing need to focus on unconventional and cost-effective techniques – a goal of this work, described in more detail in the following sections.

1.5. Aqueous Biphasic Systems (ABS)

The introduction of Aqueous Biphasic Systems (ABS) by Albertson [26] in the 50's revolutionized the field of liquid-liquid extractions [131,132] . ABS have several significant advantages over typical solvents used for liquid-liquid extraction, mainly because they provide a milder environment for biomolecules purification afforded by their high water content and low interfacial tension, which translates into lower losses of structure and biological activity of the target product [24,25]. The other key advantage of ABS is their tunability since the phase forming components can be altered not only in chemical nature but also in composition to achieve ideal separation conditions [133]. These aspects, coupled with the fact that this technique is relatively cheap and easy to be scaled-up [134], make of ABS a very valuable tool for the purification of antibodies (e.g. IgY).

The two-phase formation in ABS is achieved by mixing two polymers [135], a polymer and a salt [136], or two salts [137] in aqueous media above given concentrations. The choice of the phase forming components is a very delicate and critical step, as it dictates the distribution/partitioning of the target biomolecule between the coexisting phases [138]. This solute/biomolecule partition coefficient can be exploited by manipulating the phase forming components weight and concentration, the ionic strength of the medium, the system pH and through the use of adjuvants [133,139]. The composition of the ABS, i.e. the amount of phase forming components required to form two-phase systems, is usually taken from their phase diagrams – Figure 2 [23]. The so-called binodal curve of the phase diagram separates the monophasic and the biphasic regions. Another important information retained in the phase diagrams is the tie-line, that describe the composition of the two phases for a given mixture point [23].

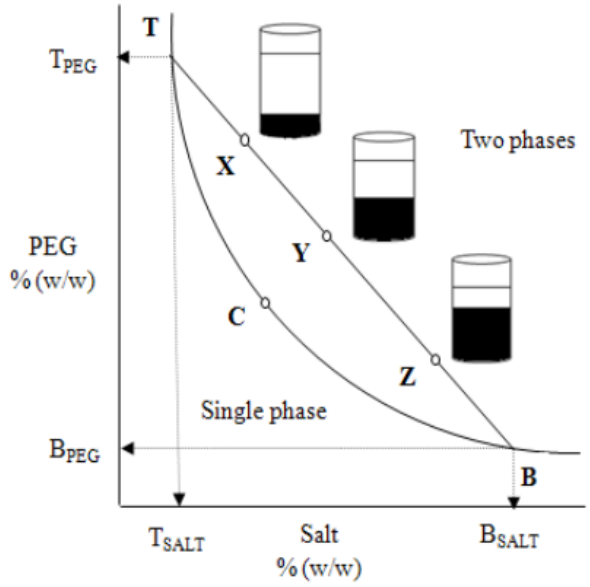

Figure 2- Example of a phase diagram for a polymer/salt system, showing the binodal curve, a tie-line and three ABS corresponding to different mixtures in the same tie-line, (X, Y and Z). C= critical point; T=composition of the top phase, B=composition of the bottom phase [23].

Concerning the use of ABS in biomolecules purification, there is an extensive number of examples including cells [140], viruses [141], and proteins [142], as a way to reduce the downstream processing time and costs associated. When it comes to antibodies, Eggersgluess et al. [143] demonstrated that a polymer/salt ABS, composed of 12 wt % of PEG 400 and 28 wt % of phosphate buffer, was effective as a single-step wash and extraction procedure for mAbs from a complex cell culture broth. Azevedo et al. [144] provided evidence towards the use of ABS for the initial purification step of IgG from hybridoma cell cultures and Chinese Hamster Ovary cell supernatants, recognizing an ABS composed of 12 wt % of PEG 6000, 10 wt % of NaH_2PO_4/K_2HPO_4 buffer and 15 wt % of NaCl as the most effective, giving rise to 90 % of recovery yields and purification factors of 4.0. The same group [138] also used polymer/polymer ABS, composed of PEG 3350 and Dextran 500000 modified with glutaric acid, to enhance the ABS purification yield by increasing the system's affinity to IgG, recovering 97% of IgG with a purity of 94 %. Yau et al. [22], in their review, described a series of applications of ABS, showing also the difference between the different kinds of phase forming components used.

1.6 Fast Centrifugal Partition Chromatography

Fast Centrifugal Partition Chromatography (FCPC) is a type of liquid-liquid chromatography requiring two immiscible liquid phases, one of which acts as the stationary phase and the other acts as the mobile phase [27]. The lack of a solid support distinguishes this technique from commonly used

chromatographic methods, like high-performance liquid chromatography, where a column with an adsorbent material is usually utilized [145]. While the basis of this technique is indeed common to other liquid-liquid chromatographic methods - partition of solutes between the two phases formed, the notable difference between them and FCPC is the use of a centrifugal force by the later, in order to hold the stationary liquid phase [27]. The concept of FCPC was introduced in 1982 by Murayama et al. [146], stemming from an already established technique developed in 1944 by Lyman Craig [147], the countercurrent distribution (CCD), with the first functional FCPC instrument being manufactured by Sanki Engineering [148]. A Sanki-type FCPC machine is composed of basic components, also encountered in most chromatographic systems, such as pumps for solvent delivery and valves to control them, a sample injector, a detector and a recorder, with the main difference between these and conventional systems being the rotor, the centerpiece of this equipment functionality [27]. The rotor is composed of several stacked discs engraved with a great number of cell channels, with each cell linked to the following one by a small duct, where the stationary phase is retained, stabilized by the centrifugal field generated by the spin of the rotors [27]. An example of this centerpiece can be found in Figure 3. The liquid nature of the applied phases and the mechanical construction of the FCPC apparatus allows for two modes of operation to be used, ascending and descending. The key difference in these modes is the density of the phase being used as the mobile phase: in the ascending mode the lightest phase is the mobile, flowing upwards through the heavier stationary phase, while the opposite occurs in the descending mode [149]. A representation of the ascending mode of operation can be found in Figure 3. The FCPC apparatus operation involves the pumping of the stationary phase into the channels, while the rotor is working at low speed, usually between 300 to 600 rpm, followed by the introduction of the mobile phase [27]. The FCPC system is usually cleaned with water and as such, in order to begin a new FCPC extraction, all of the water needs to be pumped out, something that is achieved by pumping an amount of stationary phase that can completely fill the FCPC cell channels, replacing the water that was inside. To make sure that the system is completely filled with the stationary phase it is necessary to collect a significant amount of the stationary phase, usually a volume corresponding to the total column volume. Only after this process can the pumping of the mobile phase be initiated and, after that, the sample can be introduced.

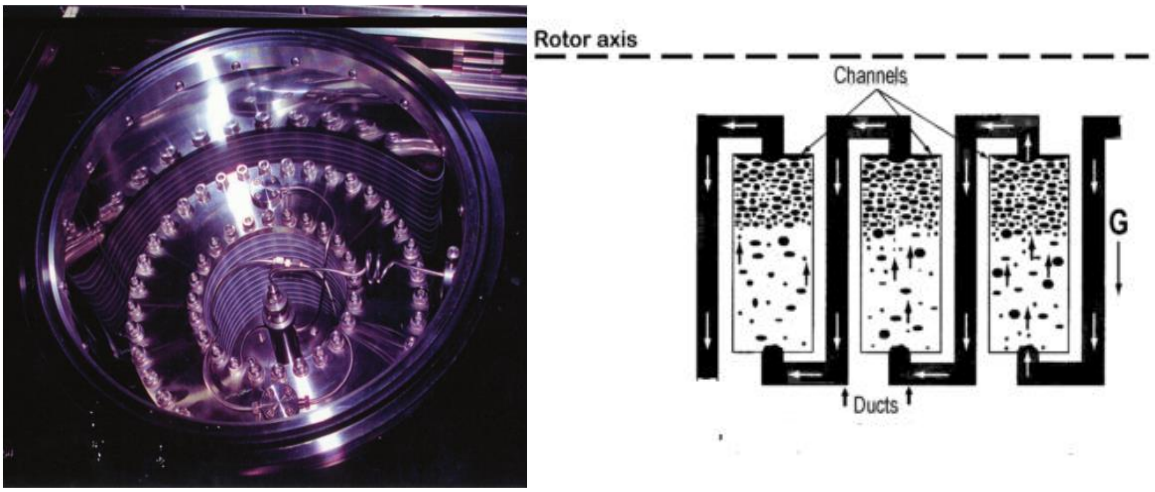

Figure 3 - Upper inside view of a pilot-scale Sanki FCPC [148] and a representation of the FCPC's ascending mode of operation, with the lighter mobile phased rising through the denser stationary phase [150].

FCPC works by the exploitation of the partition trend of different compounds between the two immiscible solvents/phases, as long as their densities are sufficiently different [27]. The main advantage of using FCPC comes from the liquid-liquid nature of this process, making it unnecessary to use solid chromatographic supports, guaranteeing therefore almost 100 % of the target compound recovery and an easy recyclability of the solvents, thus minimizing environmental problems [27], while also avoiding the need to buy, maintain and clean high-cost solid columns [149]. The high volume of the stationary phase that can be loaded into the FCPC is also a significant advantage, making this technique very suitable for industrial applications [150]. While this adaptability is seen as an advantage in terms of potential applications, it should be stressed the importance of a careful choice of the solvents/phases, which should be defined according to the following criteria: easiness of two-phase formation; capacity to be retained by the FCPC; and separation/purification effectiveness [150]. Even though the selection of a solvent system for use in FCPC can be in many ways similar to other chromatographic methods, when it comes to criteria such as polarity, charge state, and complexation, one also has to take into account the partition coefficients of the sample in that particular system [27]. While the tweaking of classical chloroform/methanol/water or n-hexane/ethyl acetate/methanol proportions can be a good starting point, allowing for some manipulation of the system's polarity in order to achieve the required sample distribution [27], there has been a major interest in utilizing ABS in FCPC. This increase is not only due to the ABS already outlined advantages in terms of tunability [144], a characteristic that is essential for the development of a good FCPC separation protocol, but also

because of the high biocompatible environment that ABS provide, especially when compared to common organic solvents that, in many cases, induce conformation changes in proteins [25,151].

While the separation capacity of the ABS is a really important parameter to acknowledge and tune, the first point of order for a successful FCPC protocol is the retention of the used system in the FCPC chamber. Without a proper retention and a minimal bleeding-effect, even if theoretically an ABS system is very successful in separating biomolecules, it will not lend itself to use in FCPC. In light of this concern, Schwienheer et al. [151] developed some guidelines to ensure a sufficient system retention, achieved by analyzing the phase's physicochemical properties, hydrodynamics and bleeding tendency of the phases of a PEG + NaH_2PO_4/K_2HPO_4 ABS [151]. Their main findings were that a low viscosity coupled with a high interfacial tension leads to the best stationary phase retention; however, an increase in density leads to an increase in the pressure drop [151]. While these were found to be the best conditions, if partition performance of the target molecule is not sufficient, i.e. the protein is not partitioning selectively to just one phase, some changes might be in order. To overcome this drawback, Schwienheer et al. [151] recommended the use of a polymer with higher molecular weight and a decrease of the content of the phase forming components. When it comes to picking the mode of FCPC operation, the mode that allows the lightest phase to be the mobile one should be the preferred choice, meaning, in the case of most PEG + salt systems, the descending mode. The choice of rotational speed should simply be the highest allowed by the system, in order to increase the mass transport between the phases while not allowing a significant pressure drop [151].

To further evidence the advantages of using FCPC for the separation/purification of biomolecules, it is important to look at previous cases of its successful applications. In recent years, FCPC has been used to isolate and purify many natural products, from phenolic compounds (chlorogenic acids) [152], terpenes (geniposide) [153], alkaloids (sinomenium) [154], and antibiotics (aldecalmycin) [155]. Yoon et al.[156] provided a review on the use of FCPC for the purification of many compounds, including works from 1994 to 2009, and that can serve as a follow-up to the examples presented here. In the case of protein extraction, while not yet a common mainstay in the subject, FCPC has seen also used. Foucalt et al. [157] successfully used FCPC to separate two membrane protein fragments, dubbed C1 and C2, utilizing FCPC with a three solvent system consisting of CH_3COOH/HCOOH/$CHCl_3$ and water at 800 rpm, with a flow rate of 1 ml/min, making use of different concentrations of CH_3COOH and HCOOH to control the partitioning of the fragments from either 100 % on the bottom phase, in systems poor in HCOOH, or 100 % to the top phase, in systems rich in HCOOH. The separation of the two types of fragments was then achieved using reversed phase chromatography, for the HCOOH poor systems, or cation exchange chromatography for the HCOOH

rich systems, in which both methodologies the C2 fragments elute first. Another work on the separation of peptide fragments via FCPC was developed by Amarouche et al. [158], presenting two new solvent systems, composed of heptane/tetrahydrofuran/CH_3CN/dimethyl sulfoxide/water and heptane/methyl-tetrahydrofuran/N-methyl-2-pyrrolidone/water, to be used at a 8 mL/min flow rate and 1400 rpm. These systems allowed for a recovery of 85.7 % with 98.7 % purity, for the first system, and a recovery of 71 % with 92.1 % purity for the second system, as determined by HPLC [158]. This work demonstrated the FCPC's effectiveness at separating synthetic peptides used for the drugs Bivalirudin and Exenatide, whereas separation by HPLC had proven to be difficult [158]. For the extraction of proteins, like laccases from fermentation broth, Schwienheer et al. [31] used FCPC with a buffered PEG 3000/NaH_2PO_4 ABS, at 1000 rpm with a flow rate of 5.0 mL/min, achieving a separation of highly active laccases, demonstrating that the use of ABS systems with FCPC can provide conditions for gentle separation of enzymes. Other applications have also marked the significance of FCPC protocols, as shown by Bérot et al. [159], who achieved purification rates of above 95 % with much higher yields than other common methodologies for the preparative purification of pea albumin. These researchers used a *n*-butanol/water system at a 2.5 mL/min flow rate at both 500 and 1200 rpm, acknowledging that while the difference in rotor speed did not affect purification results, the lower speed generated less pressure in the system [159]. When it comes to antibodies, the focus of this work, Oelmeier et al. [160] presented FCPC as an alternative separation step that, when combined with other precipitation and resolubilization techniques, can purify monoclonal antibodies in an easily scalable manner, while providing a high concentration of target proteins with insignificant contamination. These researchers found that after removing the cells from a culture supernatant, either via regular means like centrifugation or by using liquid-liquid separation approached by ABS, and performing a FCPC run in dual-mode, an upper phase rich in antibodies could be attained while removing host cell proteins [160]. The solute system used in the FCPC run was PEG 400/Citrate, at 21.02 % and 18.99 wt % %, determined by an high-throughput development approach introduced by the same authors [29]. Testing several operational parameters, in terms of flow rate and rotation speed, they also found that the system's retention capacity diminished with an increase in flow rate, and while the rotation speed did not significantly affect this parameter, the column's back-pressure is heavily influenced by it [160]. The FCPC run was performed at a 5 mL/min flow rate and 1500 rpm, achieving a compromise between functional speed and effectiveness. This extraction step, done via FCPC, was then combined with a subsequent precipitation of the antibodies by addition of PEG 4000. After this step, the precipitate was washed with a 3.0 pH citrate buffer, that not only removes the polymer but also provides a virus inactivation effect, in a process similar to what is performed by

protein-A affinity chromatography [160,161]. In the end of this procedure, the clearance of host cell proteins surpassed 99 %, while still achieving an impressive 93 % recovery of antibodies [160]. Despite these remarkable results, no more literature reports were found on this topic.

2. Experimental section

2.1 Aqueous biphasic systems: PEG 1000 + phosphate salt + H_2O

2.1.1 Chemicals

The ABS studied in this work were prepared using an aqueous solution of PEG with a molecular weight of 1000 g/mol (PEG 1000), supplied by Fluka. The phosphate buffer used is composed of potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$, purity > 99%) and monopotassium phosphate (KH_2PO_4, purity > 99.5%), acquired from PanReac and Sigma-Aldrich, respectively. The phosphate buffer was prepared at different pH values using different molar ratios of the two phosphate-based salts: 5.5 (±0.22), 6.0 (±0.16), 6.5 (±0.16), 7.5 (±0.02) and 8.0 (±0.05). The unbuffered aqueous solution was prepared using only $K_2HPO_4 \cdot 3H_2O$ (pH value *ca*. 9.0). The water utilized was double distilled, treated with a Milli-Q plus 185 water purification apparatus.

2.1.2 Experimental procedure

2.1.2.1 Ternary phase diagrams

The binodal curve of each phase diagram was determined through the cloud point titration method at 298 K (± 1 K) and atmospheric pressure [162]. The phase diagrams were determined using a solution of PEG 1000 at 60 wt %, and aqueous salt solutions with different concentrations, detailed in Table 2. The repetitive drop-wise addition of each aqueous salt solution to the PEG 1000 solution was carried out until the detection of a cloudy biphasic solution, followed by the drop-wise addition of water until the detection of a monophasic region, limpid solution (direct method). To obtain a well-defined phase diagram, the inverse approach was performed for all systems, with two exceptions – unbuffered systems and the one composed of the buffered salt solution with a pH value of 5.5. To this end, the repetitive drop-wise addition of the PEG 1000 aqueous solution to each aqueous salt solution was carried out until the detection of a cloudy biphasic solution, followed by the drop-wise addition of water until the detection of a monophasic region. Dropwise additions were carried out under constant stirring. The ternary systems compositions were determined by the weight quantification of all components added within an uncertainty of ± 10^{-4} g.

Table 2- Concentration of the initial phosphate salt solutions used for the phase diagrams determination of the ABS composed of PEG 1000 + phosphate salt + H_2O.

Phosphate salt solution / pH	Concentration of salt (wt %)	
	Direct Method	Inverse Method
5.5	26	n.d.
6.0	35	35
6.5	33	50
7.5	32	60
8.0	31	60
Unbuffered	39	n.d.

* n.d.: non-determined

2.1.2.2. Determination of tie-lines and tie-line lengths

The tie-lines (TLs), representing the composition of each phase for a given initial mixture composition, was determined by a gravimetric method originally described by Merchuk et al. [163]. A previously selected mixture at the biphasic region was prepared, vigorously stirred, and allowed to reach the equilibrium by the separation of both phases for at least 4 h at (298 ± 1) K. Each phase was separated and the top and bottom phases were weighted. Finally, each individual TL was determined by the application of the lever-arm rule.

The experimental binodal curves were fitted using Equation 1 [163];

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

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

For the determination of the TLs the following system of four equations (Equations 2 to 5) was used to estimate the concentration of polymer and salt at each phase ($[PEG]_{PEG}$, $[PEG]_{salt}$, $[salt]_{salt}$, $[salt]_{PEG}$),

$$[PEG]_{PEG} = Aexp[(B[salt]_{salt}^{0.5}) - (C[salt]_{salt}^3)] \quad (2)$$

$$[PEG]_{salt} = Aexp[(B[salt]_{salt}^{0.5}) - (C[salt]_{salt}^3)] \quad (3)$$

$$[PEG]_{PEG} = \frac{[PEG]_M}{\propto} - \frac{1-\propto}{\propto}[PEG]_{salt} \quad (4)$$

$$[salt]_{PEG} = \frac{[salt]_M}{\propto} - \frac{1-\propto}{\propto}[salt]_{salt} \quad (5)$$

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

In order to calculate each tie-line length (TLL), Equation 6 was applied:

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

The correlation parameters of Equation 1 and the compositions of the top and bottom phases, were determined using the software Matlab R2015a.

2.2 Purification of IgY from egg yolk

2.2.1 Chemicals and materials

The water-soluble protein fraction (WSPF) of the egg yolk was obtained from fresh eggs, provided by Dr. Ricardo Pires from Biocant – Associação de Transferência de Tecnologia, Cantanhede, Portugal. The water-soluble protein fraction (WSPF) of the egg yolk was prepared following a protocol described by Liu et al. [164]. A commercial kit (ABIN410202) from EggsPure IgY was used to purify IgY in order to determine the calibration curve to calculate the concentration and purity of IgY in each phase of the ABS. The ultrafiltration filters used (Amicon Ultra-0.5 mL), with a molecular weight limit of 100 kDa, were provided by Merck. The water employed was double distilled, and treated with a Milli-Q plus 185 water purification apparatus. The polymer and salts used are described in section 2.1.1.

2.2.2 Experimental procedures

2.2.2.1 Preparation of ABS

The composition of the ABS used was chosen by the analysis of the phase diagrams. The mixture points selected are composed of 18 wt % of PEG 1000 + 15 wt % of phosphate-based salt + 67 wt % of WSPF and 18 wt % of PEG 1000 + 13 wt % of phosphate-based salt + 69 wt % of WSPF. All components of the ABS were stirred at a moderate speed in a VWR vortex mixer until the solution turned cloudy, and left to rest for 4 h at (298 ± 1) K, to achieve the complete partitioning of IgY and contaminant proteins between the two phases. In all the mixtures evaluated, and at the compositions

used, the PEG-rich aqueous phase was the top phase while the salt-rich aqueous phase was the bottom phase. The pH values of the phosphate buffer solutions were additionally determined at (298 ± 1) K, using a METTLER TOLEDO SevenMulti pH meter with an uncertainty of ± 0.02.

2.2.2.3. Quantification of IgY by Size-Exclusion HPLC (SE-HPLC)

After a careful separation of the ABS phases, SE-HPLC, using a Chromaster HPLC (VWR Hitachi), was used with the aim of quantifying IgY in each phase, as well as to determine its purity. A calibration curve was determined for this purpose, shown in Appendix A (Figure A 1), using pure IgY obtained from a commercial kit, EggsPure IgY. A phosphate buffer solution (1000 mL) was prepared using 47 mL of a Solution A (27.8 g NaH_2PO_4), 203 mL of a Solution B (53.65 g $Na_2HPO_4 \cdot 7H_2O$) and 35 g of NaCl. Each phase was diluted at a 1:9 (v:v) ratio in this phosphate buffer solution before injection. The SE-HPLC was performed on an analytical column Shodex Protein KW-802.5 (8 mm x 300 mm). A 100 mM phosphate buffer + NaCl 0.3 M solution was run isocratically with a flow rate of 0.5 $mL.min^{-1}$. The column oven and autosampler temperatures were kept at 298 K and at 283 K, respectively. The injection volume was 25 µL. The wavelength was set at 280 nm using a DAD detector. The obtained chromatograms were treated and analyzed using the PeakFit v4 software. The partition coefficients of contaminant proteins, K_{cont}, were calculated according to equation 7, where $[Contaminant]_{PEG}$ and $[Contaminant]_{salt}$ correspond to the concentration of each contaminant protein in the polymer- and salt-rich phase, respectively.

$$K_{Cont} = \frac{[Contaminant]_{PEG}}{[Contaminant]_{salt}} \quad (7)$$

The extraction yield of IgY, $Yield_{IgY}$ (%), was calculated according to equation 8, where m_{PEG} and m_{WSPF} are the mass of the top phase and the mass of WSPF added to the system, respectively, with $[IgY]_{PEG}$ and $[IgY]_{WSPF}$ being the concentration of IgY in the top phase and in the WSPF added to the system, respectively.

$$Yield_{IgY}\,(\%) = \frac{mPEG \times [IgY]_{PEG}}{mWSPF \times [IgY]_{WSPF}} \times 100 \quad (8)$$

The IgY purity, $Purity_{IgY}$ (%), in the top phase was calculated according to equation 9,

$$\text{Purity}_{\text{IgY}}\,(\%) = \frac{[IgY]_{PEG}}{[IgY]_{PEG} + [Contaminants]_{PEG}} \text{ x } 100 \quad (9)$$

2.2.2.4. Fast Centrifugal Partition Chromatography (FCPC)

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

The FCPC assays were carried out using the system composed of 18 wt % of PEG 1000 + 13 wt % of phosphate buffer at pH 6.0 and 67 wt % of water. This system was set to work in the ascending mode. The rotor was entirely filled with the bottom-rich phase at 600 rpm to achieve the homogeneous solvent re-equilibration on the rotor. Then, the rotation was set to the intended speed, needed for the appropriate stationary phase retention. After the working rotational speed was set up, the PEG 1000-rich (top) phase was pumped through the stationary phase to reach the equilibrium.. The stationary phase retention, S_f, was calculated by the ratio of the stationary phase volume (V_S) and the column volume (V_C), as described in equation 10,

$$S_f\,(\%) = \frac{\text{Vs}}{Vf} \text{ x } 100 \quad (10)$$

The sample loop was filled with 8 mL of WSPF and 2 mL of polymer-rich phase from the ABS composed of 18 wt % of PEG 1000 + 13 wt % of the phosphate buffer at pH 6.0 and 67 wt % water.

2.2.2.5. Ultrafiltration

Ultrafiltration was performed on the most promising ABS, composed of 18 wt % PEG 1000 + 13 wt % phosphate salt buffer at 6.0 pH + 69 % WSPF, as an alternative purification technique to FCPC. The top phase of the ABS was collected and placed in. The filters were then placed inside an appropriate Eppendorf and centrifuged at 14,000 g for the duration of 6 cycles, 15 min each. Between each centrifugal cycle, the filtrate found in the filter exterior was collected and analyzed by SE-HPLC, also adding 300 µL of phosphate buffer to the filter interior. The final concentrate was mixed with 200 µL of phosphate buffer and analyzed with SE-HPLC. Figure 4 describes the ultrafiltration process.

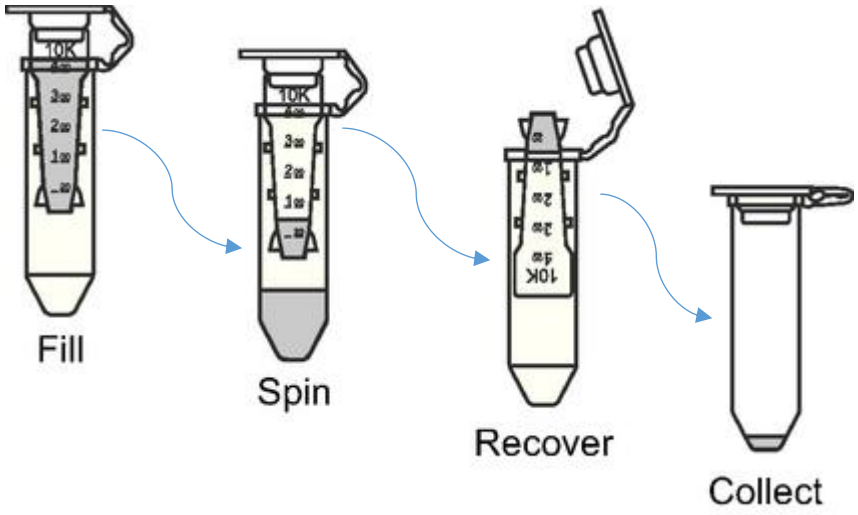

Figure 4- Schematization of the ultraperformance assay [165].

2.3. IgY Stability

2.3.1 Chemicals and materials

The IgY stability was evaluated in different aqueous solutions of phosphate-based salts and PEG 1000. The chemicals used were described in section 2.1.1. Pure IgY obtained from the commercial kit (ABIN410202), from EggsPure IgY, was used for comparison purposes. Circular dichroism spectra were recorded on a JASCO J-1500 spectropolarimeter (JASCO, Hiroshima, Japan). The ELISA assay was done using an IgY ELISA Kit obtained from Abnova Corporation, Taiwan, containing a dilution solution, wash solution, an anti-IgY-HRP conjugate, a chromogenic substrate, the stop solution, an IgY calibrator. The ELISA microplaque was analyzed in a BioTeck Synergy HT

microplate reader. The water utilized was double distilled, and treated with a Milli-Q plus 185 water purification apparatus.

2.3.2 Experimental Procedure

2.3.2.1 Circular Dichroism

The IgY secondary structure was evaluated in different aqueous solutions by Circular Dichroism (CD). IgY (3 mg/mL) was mixed in a 1:1 w:w proportion with all of the following aqueous solutions: phosphate buffer at pH values of 5.5, 6.0, 6.5, 7.5, 8.0 and K_2HPO_4 at 26-36 wt %; PEG 1000 at 10%, 20% and 30 wt%. IgY in the top phase of the following ABS was also analysed by CD for the following systems: 18 wt % of PEG 1000 + 15 wt % phosphate buffer at pH 6.0 + 67 wt % of the IgY aqueous solution; and 18 wt % of PEG 1000 + 13 wt % phosphate buffer at pH 6.0 + 69 wt % of the IgY aqueous solution. Standard samples of all the previously mentioned solutions were also prepared using water instead of the IgY solution. Circular dichroism spectra were recorded from 200 to 260 nm using quartz circular dichroism cuvettes (0.1 cm) at room temperature (*ca.* 298 K). Each circular dichroism spectrum is the result of four accumulations recorded in millidegrees. The following acquisition parameters were used: data pitch, 0.5 nm; bandwidth, 1.0 nm; response, 1 s; and scan speed, 50 nm min^{-1}.

2.3.2.2 ELISA

The top phase of the ABS composed of 18 wt % of PEG 1000 + 15 wt % phosphate buffer at pH 6.0; the concentrated rich fraction in IgY obtained by ultrafiltration; and the highest IgY purity fraction obtained from FCPC were analyzed by ELISA. The IgY standards were prepared according to the indication provided by the kit's manufacturer. Using the provided calibrator, standard solutions were prepared at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 0 ng/mL, using the ELISA dilution solution. All samples and standards were added to the microplaque wells in duplicate, forming IgY-Anti IgY complexes due to the Anti-IgY adsorbed in them. After 30 min of incubation, the wells were aspirated and washed with the provided washing solution in order to remove all proteins that did not form complexes. The anti IgY-HRP conjugate was then added to the wells, binding to the previously recognized IgY. After 30 min of incubation the wells were washed again and the chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB), was added, allowing the HRP enzyme to catalyze the chromophoric reaction. Following 10 min of incubation, the provided stop solution was added and the samples absorbances were measured at 450 nm - calibration curve given in Appendix E (Figure E 1).

3. Results and Discussion

3.1 Evaluation of the FCPC operational parameters and system composition

As described in section 1.6, there are three main parameters that should be analyzed for the selection of a proper solvent to be used in FCPC: the easiness of two-phase formation; the retention capacity in the FCPC cells; and the separation/purification effectiveness [150,151]. The first and most pressing characteristic for any prospective solvent system is its retention capacity in the FCPC cells. In order to move on with an adequate system composition ensuring a minimal bleeding out effect, a literature review was previously carried out, summarized in Table 3.

Table 3 - ATPS used in FCPC to separate proteins.

Components	Concentration (wt %)	Flow Rate / (mL min^{-1})	Rotation / (rpm)	Extracted Protein	Reference
PEG 1000	12.50	10.00	2000	Lysozyme and Myoglobin	Sutherland et al. [166]
K_2HPO_4	12.50				
PEG 400	21.02	5.00	1500	mAbs from cell culture	Oelmeier et al. [160]
$Na_3C_6H_5O_7$	18.99				
PEG 1000	19.20	0.45	1200	Ovalbumin	Foulcault et al. [167]
$(NH_4)_2SO_4$	14.50				
PEG 4000	13.00	5.00	2000	Cianovirin-N from transgenic tobacco cell culture	Grudzien et al. [168]
NaH_2PO_4	13.00				
PEG 3000	13.00	5.00	1000	Lacase from *Pleurotus Sapidus* culture	Schwienheer et al. [31]
K_2HPO_4	6.00				
NaCl	2.50				

Foucault et al. [167], in 1990, compared several polymer/salt and polymer/polymer ABS in FCPC assays, concluding that while all of the systems analyzed have an effective retention capacity, the major problem conveys in the mass transfer effect, requiring significant reductions in the apparatus' flow-rate to overcome this effect. Advances in FCPC technology [148] have permitted the effective use of higher flow rates with polymer/salt ABS, as demonstrated by Sutherland et al. [166], using ABS formed by 12.50 wt % of PEG 1000 + 12.50 wt % of K_2HPO_4, with a 10.0 mL/min flow-rate. This system was used in the first step of this work. This decision was based on the system's previously portrayed effectiveness when it comes to retention [166], but also because of the remarkable results provided by this system for the purification of enzymes and proteins [169,170], including antibodies [171,172], and the work already done on the characterization of antibodies' partition behavior

in this kind of ABS [173]. It was however decided to use a lower flow rate, 1.5 mL/min, in order to establish a functional starting point in the FCPC's operating parameters. This decrease in the flow rate was due to concerns of possible ineffective retention that could be exacerbated by running the assay at a higher flow-rate. While the compromise of a lower purification time in order to maintain adequate stationary phase retention was kept in mind, its limits were pushed by increasing the flow rate to 2.0 mL/min, with good results. Both the ascending and descending mode of FCPC operation were tested, maintaining the rotation value of 2000 rpm for the ascending mode used by Sutherland et al. [166], and then increasing it to 2500 rpm for the descending mode. In general, higher retention values seem to lead to a better system retention, as long as the apparatus' pressure limit is not reached [158].

The process consisted of filling the column with the phase that would serve as stationary, the phase rich in PEG 1000 for the descending mode, and the phase rich in K_2HPO_4 for the ascending mode, and then pump the mobile phase to achieve the equilibrium between the phases. The stationary phase retention, shown in Table 4, was calculated by equation 10, achieving S_f values of 47.30 % and 46.00 % for the ascending mode of operation at 2000 rpm, with 1.5 mL min^{-1} flow rate, and 48.52 % for the descending mode at 2000 rpm and with a 1.5 mL min^{-1} flow rate. An increase of the flow rate and rotation, to 2.0 mL min^{-1} and 2500 rpm, respectively, in the descending mode was also tested, but yielded the worst stationary phase retention, namely 44.90%. All the assays revealed to be well above the 20 % value that was deemed as the minimum adequate value for sufficient phase retention. This system composition has an effective retention, enabling its further use in this work, with slightly better results for lower flow rates and rotation speed, using the salt as a mobile phase in the descending mode.

These assays provided a meaningful basis for further FCPC experiments, by testing the effective operational parameters and to determine a base ABS composition to be used in FCPC. An initial ABS was chosen from a literary review done on the subject, taking into account its retention in FCPC and its purification effectiveness. The 12.50 wt % of PEG 1000 + 12.50 wt % of K_2HPO_4 ABS was tested in terms of retention, by changing several FCPC operational parameters, namely rotation, flow rate and operation mode (ascending/descending). All the operational conditions evaluated led to an effective retention of the phases inside the FCPC apparatus, as demonstrated by their S_f values of ≈46-49%, well above the 20% deemed adequate. In this sense, PEG 1000 and K_2HPO_4 were chosen to be used as components of ABS, with the system composition possibly benefiting from further tweaking to maximize extraction performance, as presented in following sections. The operational parameters evaluated did not show a significant negative impact when it

comes to system retention, demonstrating that the determining factor for the choice of effective operational conditions is not the retention but, possibly, the extraction performance. The impact of the operation conditions on extraction performance was evaluated in the subsequent sections.

Table 4- Retention values achieved for the FCPC assays and their respective operational conditions.

S_f (%)	FCPC Operation Mode	Rotation / (rpm)	Flow Rate / (mL min^{-1})
48.52	Descending	2000	1.5
47.30	Ascending	2000	1.5
44.90	Descending	2500	2.0
46.00	Ascending	2000	1.5

3.2 Aqueous biphasic systems: PEG 1000 + phosphate-based salt + H_2O

After demonstrating that the selected system has a sufficient retention to be used in a FCPC assays, further optimizations of the ABS were carried out. To this end, phosphate buffer solutions at different pH values were prepared, namely 5.5, 6.0, 6.5, 7.5 and 8.0, in order to appraise the effect of the pH on the ABS extraction capacity. However, to address which compositions could be used, i.e. fitting within the biphasic region, the respective ternary ABS phase diagrams were determined at 298 K and atmospheric pressure. The respective ternary phase diagrams are illustrated in Figure 5. The experimental weight fraction data of each phase diagram are given in Appendix B (Tables B 1 and B 2). In the studied ABS, the top phase is the PEG-rich phase, while the bottom phase is primarily composed of salt and water.

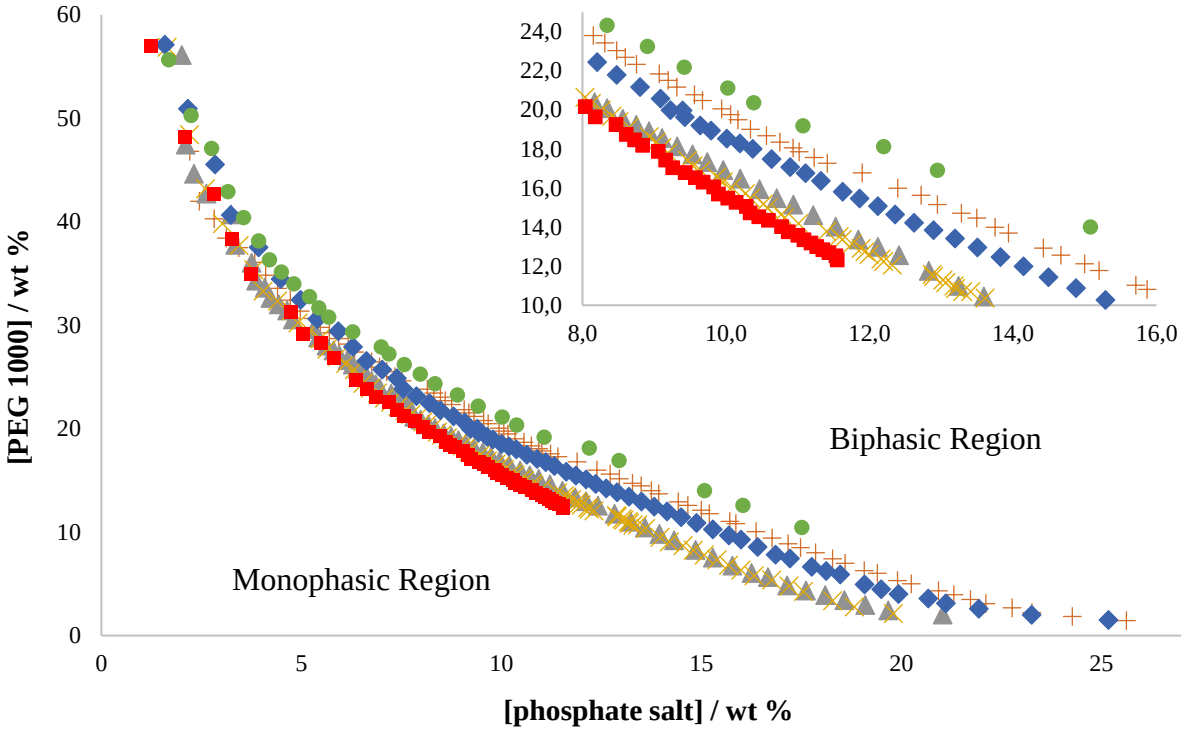

Figure 5- Phase diagrams of the ABS composed of PEG 1000 + phosphate-based salt + H_2O at different pH values: 5.5 (●); 6.0 (+); 6.5 (♦); 7.5 (▲); 8.0 (x); unbuffered (■).

In the phase diagrams presented in Figure 5, the biphasic region, which corresponds to the area which leads to ABS formation, i.e. two phases, is located above the solubility curve. In general, there is an increase in the biphasic region area with the increase in pH, consequently meaning that the system's capacity to form an ABS follows the trend: pH 5.5 < 6.0 < 6.5 < 7.5 ≈ 8.0 ≈ unbuffered. The separation of a polymer/salt aqueous solution into two phases is main a result of the salting-out effect of the salt over the polymer, in which ions that are more easily hydrated are more able to induce phase splitting [174]. The main physicochemical characteristic associated with the salting-out ability is the ions' free energy of hydration, usually described according to the Hofmeister's series [175]. The Hofmeister's series [176] rates ions according to their salting-out capacity, e.g. in the following well-established orders: $F^- > SO_4^{2-} > HPO_4^{2-} > C_2H_3O_2^- > Cl^-$ and $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+}$ (this is only a partial rank as many more ions were ranked). With the increase in the pH of the system there is a higher concentration of K_2HPO_4, and as such, higher concentrations of HPO_4^{2-}, being this ion a stronger salting-out agent than $H_2PO_4^-$. This trend on the ABS behavior along the pH has been observed previously by Glyk et al. [177] on ABS of similar composition.

The experimental binodal data were fitted according to the empirical relationship described by equation 1. The regression parameters *A*, *B* and *C*, which were estimated by the least-squares

regression method, are provided in Table 5, along with their corresponding standard deviations (σ). Overall, good correlation coefficients were obtained, indicating that these fittings can be used to predict data in any given region of the phase diagram, without the need to resort to experimental data.

Table 5- Correlation parameters used to describe the experimental binodal data by equation 1, and respective standard deviations (σ).

pH value	$A \pm \sigma$	$B \pm \sigma$	10^{-5} ($C \pm \sigma$)
5.5	99.2 ± 4.0	-0.49 ± 0.02	2.7 ± 2.0
6.0	93.9 ± 2.0	-0.48 ± 0.01	7.5 ± 0.9
6.5	97.9 ± 3.2	-0.49 ± 0.02	10.7 ± 1.2
7.5	98.3 ± 3.5	-0.52 ± 0.02	15.2 ± 1.7
8.0	98.4 ± 3.2	-0.53 ± 0.02	14.9 ± 1.5
Unbuffered	92.4 ± 4.4	-0.51 ± 0.03	18.9 ± 4.4

The experimental data for the TLs and their respective length (TLL) are reported in Table 6. In Appendix C (Figures C 1 – 5) are reported the TLs for each ABS combined with the respective phase diagram. In general, the total composition of the system does not usually have a significant effect on the slope of the TLs. This implies that the TLs are generally parallel to each other, as presented in the example shown in Figure 6, allowing the phase compositions for any given system to be estimated.

While the description of TLLs and the binodal data for the systems are important for further work using these systems, at the referred pH values, the main conclusion drawn from this section lies on the effect of pH on the binodal curve. In this sense, a clear correlation between the pH and the position of the binodal curve can be established, with higher pH values yielding systems with wider ranges of composition that present biphasic behavior.

Table 6- Weight fraction data for the TLs of the studied PEG 1000 + phosphate-based salt ABS.

pH Value	Weight Fraction Composition / (wt %)					
	$[PEG]_{PEG}$	$[salt]_{PEG}$	$[PEG]_{Mix}$	$[salt]_{Mix}$	$[PEG]_{salt}$	$[salt]_{PEG}$
5.5	3.935	37.812	15.011	17.913	18.861	9.200
	1.594	53.742	15.059	24.633	23.351	6.703
6.0	4.687	32.872	14.830	18.311	26.117	2.108
	2.765	42.115	14.904	24.859	32.021	0.524
6.5	4.415	34.376	14.961	17.854	25.470	1.391
	2.921	41.994	15.136	24.689	32.457	0.153
7.5	3.583	36.429	14.963	17.974	25.723	0.526
	2.398	43.797	14.979	25.048	31.761	0.040
8.0	3.268	37.816	14.855	18.317	25.375	0.612
	2.417	43.340	15.005	24.984	32.113	0.036
Unbuffered	2.779	39.549	15.034	17.978	25.034	0.376
	1.919	45.740	14.869	25.209	30.754	0.023

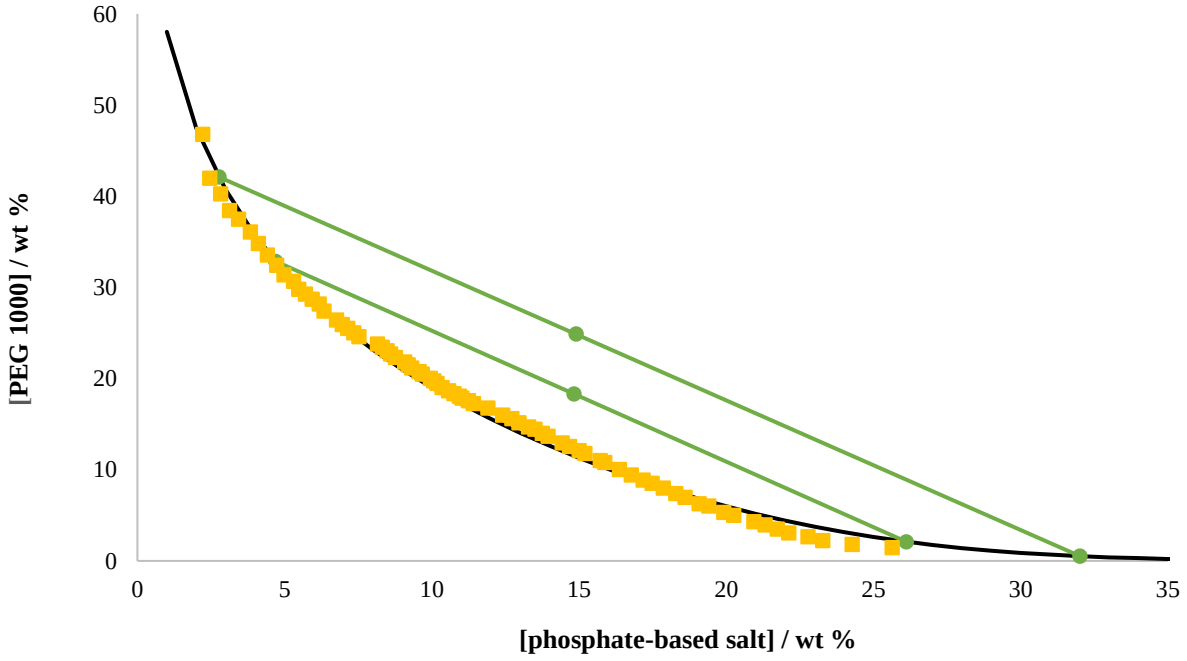

Figure 6- Phase diagram of the ABS composed of PEG 1000 + Phosphate buffer at 6.0 pH + H_2O: tie-line data (●); adjusted binodal data obtained through equation 1 (−); experimental binodal data (■).

3.3 Purification of IgY from egg yolk using ABS

3.3.1 Screening of the pH in ABS to purify IgY

After describing the PEG 1000 + phosphate-based salts ABS by their phase diagrams, the mixture point for the extraction was selected to be composed of 18 wt % PEG 1000 + 15 wt % of phosphate-based salt, and 67 wt % of the WSPF prepared from egg yolk. This mixture point was selected to fit within the biphasic area of the phase diagram, but as close as possible to the binodal curve to maximize the water content on the system. Figure 7 shows the macroscopic appearance of the systems prepared, at different pH values. The appearance of these systems suggests that proteins in the WSPF suffer denaturation and precipitate, with a noticeable increase in the amount of precipitate found in systems with higher pH, with the unbuffered system also presenting some precipitation. Another important macroscopic characteristic is the precipitation of the K_2HPO_4 salt present in the ATPS at pH 5.5, as can be seen in Figure 7.

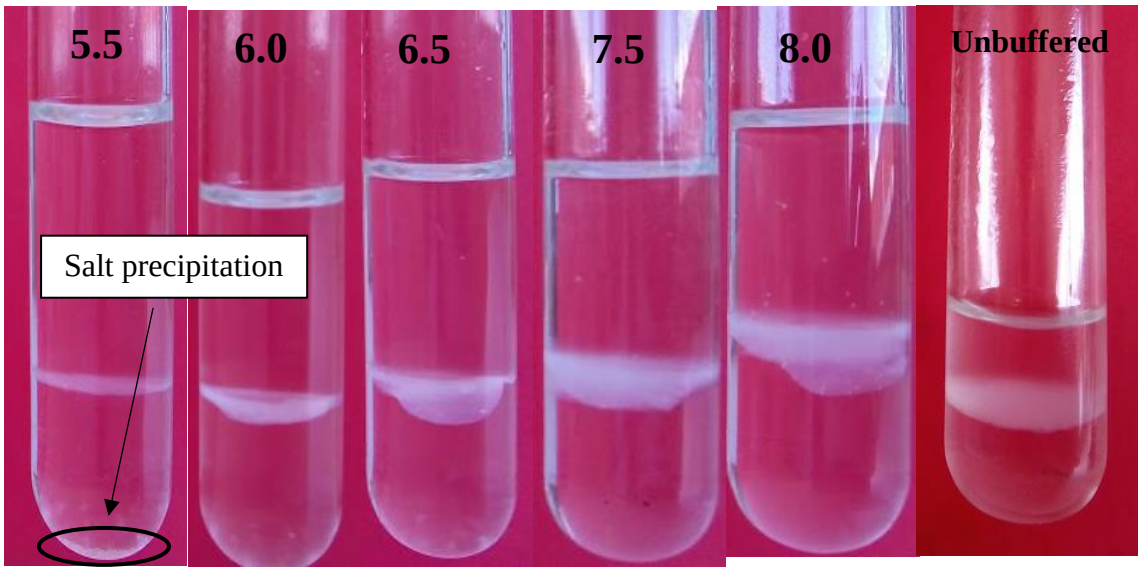

Figure 7- ABS composed of 18 wt % PEG 1000 + 15 wt % Salt Buffer and WSPF, at several pH values. From left to right: 5.5 pH; 6.0 pH; 6.66 pH; 7.5 pH; 8.0 pH; Unbuffered.

In spite of the precipitation of proteins, the purification effect of these systems cannot be ruled out, as the precipitated proteins can be contaminant proteins present in the WSPF. The occurrence of precipitation and denaturation of the proteins is due to the strong salting-out capacity of the salt used, and the amount of polymer added, as previously described in the literature [176,178] and better discussed in the next section – stability assays.

Both phases of the systems were collected and analyzed by SE-HPLC, without removing the precipitate in the interphase which was then discarded, in order to calculate the partition coefficients of the contaminant proteins, IgY recovery yield, and purity. The results obtained are shown in Table 7 and Figure 8. The partition coefficient of IgY was not determined because, as can be seen in the chromatogram presented in Figure 9, as an example, no IgY peak was found in the bottom phase. This indicates a complete partition (selectivity) of IgY to the top phase (polymer-rich phase), in agreement with previous studies on the partition of other antibodies (IgG) in similar systems [173]. In general, the values of the partition coefficients of the contaminant proteins to the polymer-rich phase tend to increase with the pH increase (Table 7), and thus, the selectivity of the system decreases with an increase in pH. The partition coefficients of the contaminant proteins are all above 1, meaning that the contaminant proteins are migrating mainly to the top phase, which in turn explains the relatively low purity of IgY obtained in a single-step with all systems. Overall, the pH value and/or the phosphate buffer used affects the purity of IgY, as extractions done at lower pH lead to the best results.

While the extractions with phosphate buffers at pH 5.5 and 6.0 result in similar purity levels, the ABS composed of phosphate buffer at 5.5 is harder to form, resulting in some precipitation of the K_2HPO_4 salt, as shown in Figure 7, making this system less reliable to work with. Therefore, the ABS buffered to pH 6.0 was deemed more adequate, showing comparable purity and yield values, and thus further evaluated in ultrafiltration and CPC approaches.

Table 7 - Partition coefficients of contaminant proteins ($K_{cont1\ and\ 2}$) present in the WSPF, and IgY purity and recovery yield using ABS.

pH Value	Purity ± σ (%)	Yield ± σ (%)	K_{cont1} ± σ	K_{cont2} ± σ
5.5	41.79 ± 0.12	89.10 ± 3.36	1.57 ± 0.04	1.44 ± 0.03
6.0	41.07 ± 0.46	79.80 ± 3.09	2.04± 0.04	1.65 ± 0.12
6.5	33.93 ± 3.58	79.88 ± 0.74	12.74 ± 8.83	2.52 ± 0.91
7.5	27.19 ± 3.97	74.89 ± 3.41	10.75 ± 3.08	3.02 ± 0.66
8.0	28.11 ± 1.60	79.93 ± 5.55	25.47 ± 12.35	3.54 ± 0.64
Unbuffered	38.00 ± 5.78	89.64 ± 0.19	4.93 ± 0.87	6.07 ± 4.54

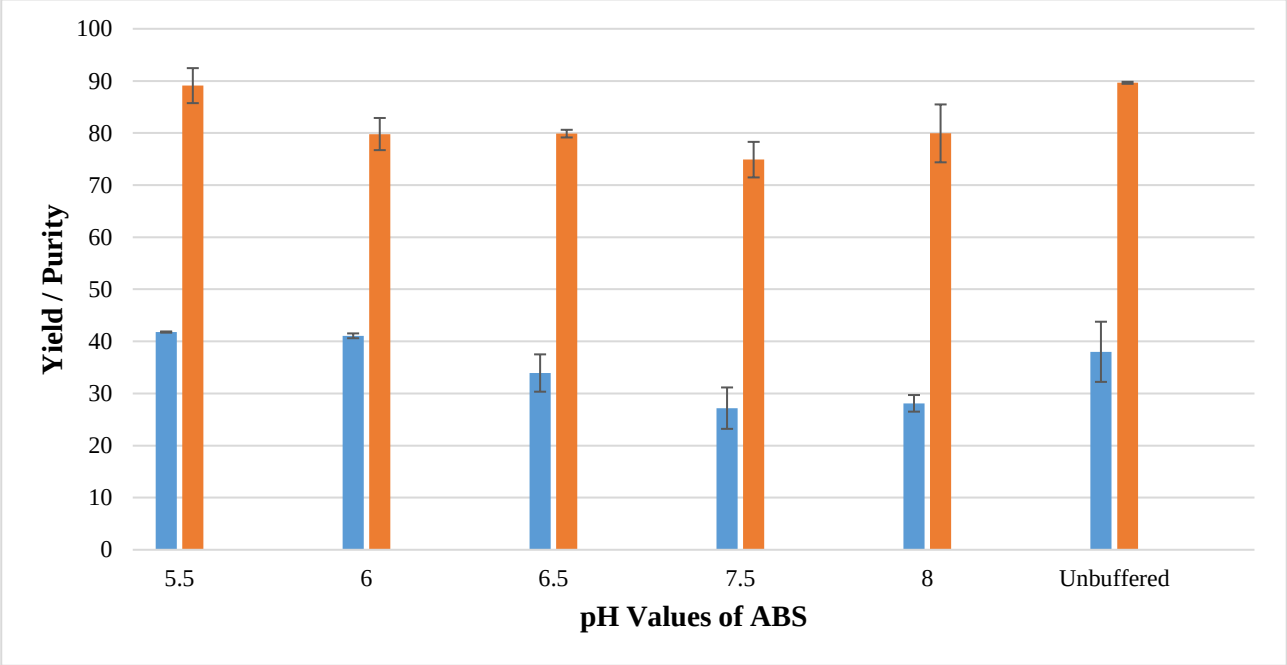

Figure 8 - IgY purity (blue) and recovery yield (orange) from the WSPF of egg yolk using ABS at different pH values.

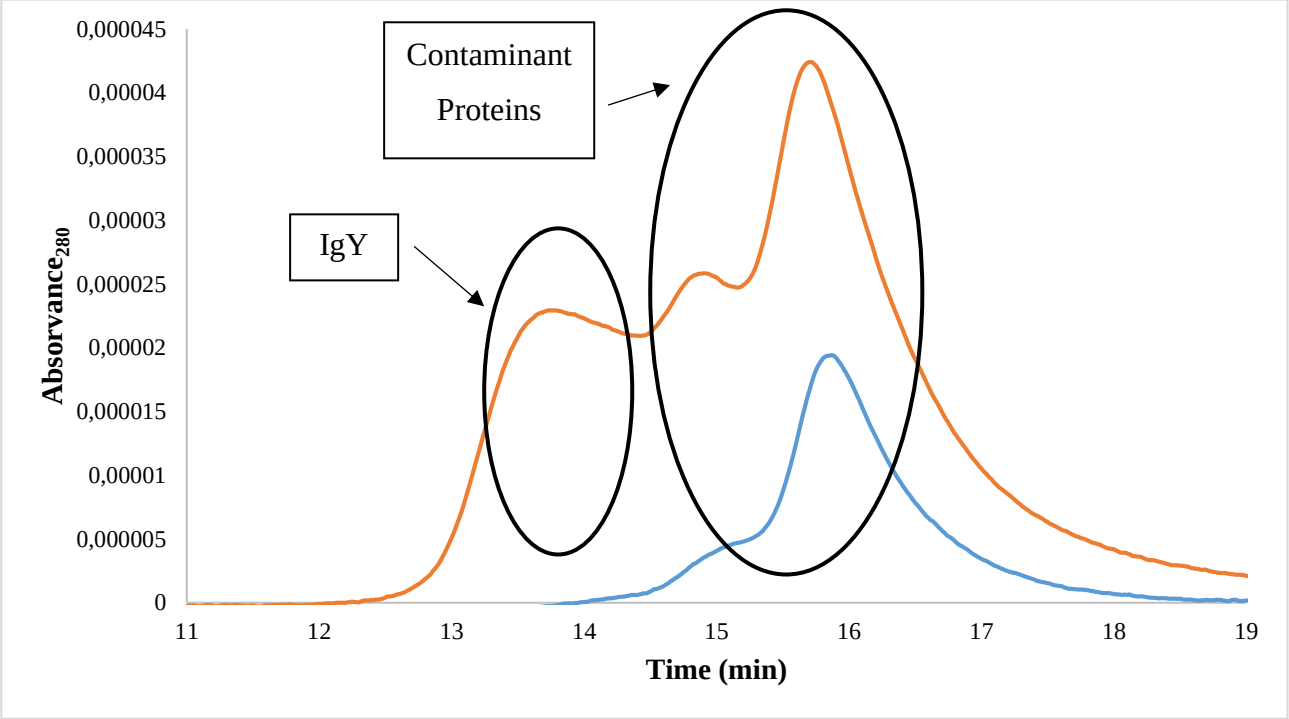

Figure 9 - SE-HPLC chromatograms of the top phase (orange) and bottom phase (blue) of the phosphate-based salt (pH 6.0) + PEG 1000 ABS.

3.3.2 Screening of the mixture composition in ABS to purify IgY

After the initial screening on the pH effect towards the IgY purification, different ABS mixture compositions, by changing the concentration of PEG 1000 and phosphate salt at pH 6.0, were tested. The phase diagram, presented in Figure 10, depicts three new mixture compositions were tested: 18 wt % PEG 1000 + 13 wt % phosphate salt buffer; 21 wt % PEG 1000 + 16 wt % phosphate salt buffer; 21 wt % PEG 1000 + 14 wt % phosphate salt buffer. The extraction results, in terms of purity, yield and partition coefficient of contaminant proteins are described in Table 8.

Table 8 - Partition coefficients of contaminant proteins ($K_{cont1\ and\ 2}$) present in the WSPF, and IgY purity and recovery yield using three new mixture compositions of the ABS at pH 6.0.

System composition (PEG 1000 wt % / Salt Buffer wt %)	Purity ± σ (%)	Yield ± σ (%)	K_{cont1} ± σ	K_{cont2} ± σ
18 / 15	41.07 ± 0.46	79.80 ± 3.09	2.04± 0.04	1.65 ± 0.12
18 / 13	39.31 ± 0.97	103.83 ± 2.36	3.90 ± 0.05	1.80 ± 0.39
21 / 16	27.19 ± 0.09	53.81 ± 0.56	n.d.*	8.81 ± 2.67
21 / 14	22.81 ± 2.02	35.94 ± 3.30	n.d.*	n.d.*

* n.d.: non-determined

Comparing the three new mixture compositions with the initially studied ABS, a new mixture reveals a top contender to be used in further FCPC trials, with some advantages. In terms of purity, no mixture surpassed the first studied system, with only the 18 wt % PEG 1000 + 13 wt % phosphate salt buffer system achieving comparable results (39.31 ± 0.97% *versus* 41.07 ± 0.46%). The loss of some purification capacity on the first step, using only the ABS as a purification tool, is an adequate trade-off considering that the goal is the application of the ABS in FCPC. When it comes to yield, the 18 wt % PEG 1000 + 13 wt % phosphate salt buffer system clearly performs better. The yield value of the mentioned system is 103.83 ± 2.36 %, showing not only that this system is partitioning IgY exclusively to its top phase, as thoroughly justified in section 3.3.1 and Figure 9, but that there is also no loss of IgY. In comparison, the first studied system leads to an yield of 79.80 ± 3.09 %.

The last parameter that demonstrates the 18 wt % PEG 1000 + 13 wt % phosphate salt buffer system as a more suitable candidate for FCPC assays is the partition coefficient of the contaminant proteins. The K values relate to the exit time of its compound in a chromatographic assay, due to its affinity to the mobile and stationary phases. Therefore, a higher difference between K_{cont1} and K_{cont2} will allow an easier separation of the different contaminant proteins in FCPC. In fact, the K values of both contaminant proteins, 3.90 ± 0.05 and 1.80 ± 0.39, are more distinct than the ones reported for first system studied (2.04± 0.04 and 1.65 ± 0.12). It should be remarked that the remaining systems do not present peaks for both groups of contaminant proteins (in the case of the 21 wt % PEG 1000 + 14 wt % phosphate salt buffer system) or for one of them (in the case of the 21 wt % PEG 1000 + 16 wt % phosphate salt buffer system) in the bottom phase. This indicates that the separation of IgY from the contaminants is not being achieved, with the partitioning of IgY and contaminants occurring to the same phase.

In summary, the 18 wt % PEG 1000 + 13 wt % phosphate salt buffer ABS is the clear choice to pursue the FCPC trials, due to the greater distinction between the partition coefficients for the contaminants, and high extraction yield and purity.

3.3.4 Purification of IgY using ABS and ultrafiltration

Ultrafiltration was investigated as a subsequent method of ABS to improve the purification of IgY. Ultrafiltration was applied to the top phase of the ABS also chosen for FCPC assays, identified in the previous section, and composed of 18 wt % PEG 1000 + 13 wt % phosphate salt buffer at pH 6.0. The SE-HPLC chromatograms of the top phase (PEG-rich phase enriched in IgY) of the original ABS and after ultrafiltration are presented in Figure 10, along with the chromatogram of the WSPF used in the ABS composition. The analysis of the results obtained reveals that an ultrafiltration step after the IgY extraction improves the IgY purity (in 8 %, from 39 % to 47 %).

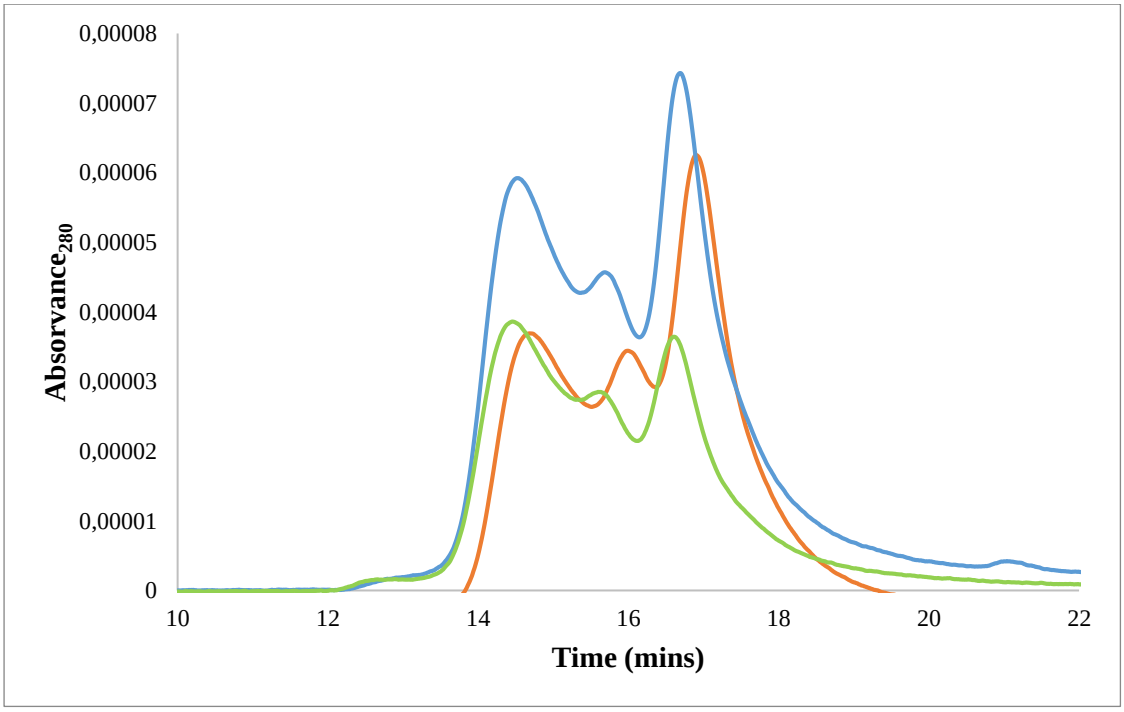

Figure 10- SE-HPLC spectra of the top phase of the 18 wt % PEG 1000 + 13 wt % phosphate buffer at pH 6.0: top-phase of the ABS (blue), top phase of the same ABS after six ultrafiltration cycles (green), and WSPF used in ABS formation (orange).

The fractions collected from each ultrafiltration cycle were also analyzed in SE-HPLC, in order to verify the possible effectiveness of extra ultrafiltration cycles. The chromatograms obtained are shown in Appendix D (Figures D 1 – 5), and a summary of the percentage of contaminants in

each fraction is given in Figure 11. The results from fraction 3 are not presented due to an anomaly found in the chromatogram, assumed to be due to a mechanical error in the SE-HPLC apparatus. From the gathered data, it is apparent that the first and last filtration steps are the most effective in removing impurities from the sample, with the first being composed of about 74 % impurities, and fraction 6 presenting about 75 % of contaminants. While the initial cycle is effective in removing contaminants, the trend for the subsequent cycles shows an increase in impurity percentage, revealing that more cycles can be incorporated on the ultrafiltration assay until a plateau is reached.

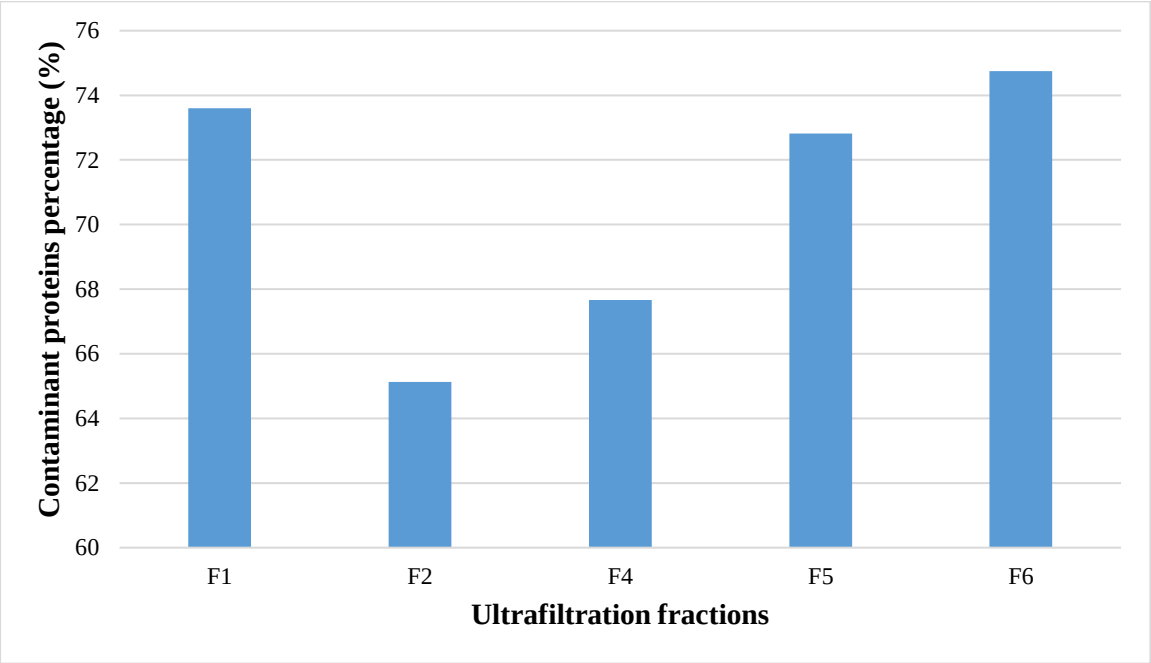

Figure 11 – Contaminant proteins percentage in the fractions obtained by ultrafiltration, calculated from the SE-HPLC spectra. F1-6 represent the fraction collected after each of the six ultrafiltration cycles performed.

Although it was not observed a significant improvement on the IgY purity after ultrafiltration, it should be highlighted that this approach allows the removal of PEG 1000. Most applications of IgY, clinical or laboratory ones, require it to be not only pure but also solubilized in a salt solution, while other applications, such as using IgY as a food additive, do not benefit from polymer removal. The chromatograms presented in Appendix D (Figures D 1-5) were additionally analyzed to infer the polymer presence, concluding that all of the PEG 1000 is removed after the forth ultrafiltration cycle. Figure 12 shows the chromatograms of fraction 4 and the top phase of the ABS, used for ultrafiltration.

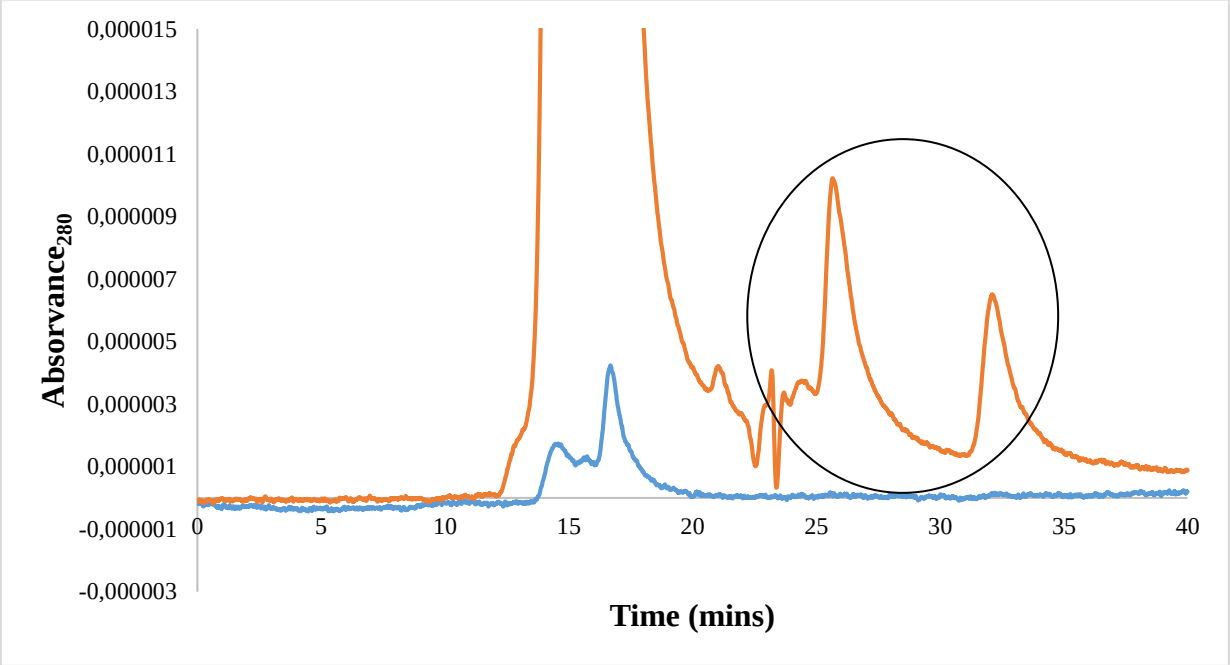

Figure 12 – SE-HPLC spectra of the top phase of the 18 wt % PEG 1000 + 13 wt % phosphate salt buffer at pH 6.0: ABS (orange) and the fraction obtained after the 4th ultrafiltration cycle (blue), with the peaks pertaining to PEG 1000 outlined.

The last important parameter to investigate in the performance of ultrafiltration for the purification of IgY is the loss of the target protein. In this sense, the mass of IgY present in the concentrated sample, after all the filtration cycles, and on the original top phase of the ABS used was calculated. Ultrafiltration leads to a loss of *ca*. 36 % of IgY, with the top phase of the ABS containing 73.2 µg, compared to the 46.7 µg found in the concentrated sample. This results reinforces a potential negative point, in concern to the overall yield of the process, and that might be exacerbated with more cycles.

The use of ultrafiltration leads to an increase in the IgY purity, when compared to the single-step extraction by ABS, while also providing a complete removal of the polymer. An important negative aspect, is however the loss of IgY. Ultrafiltration might thus be useful for laboratory purification assays due to its simplicity and overall low cost.

3.3.5 Purification of IgY in FCPC using ABS

With the previous screening on the ABS ability to purify IgY, and after confirming their feasibility for use in FCPC, ABS were finally tested for their capacity to extract and purify IgY in FCPC. While the 18 wt % of PEG 1000 + 13 wt % of phosphate salt buffer at pH 6.0 was considered

optimal, additional tests were carried out using the 18 wt % of PEG 1000 + 15 wt % of phosphate salt buffer ABS at pH 6.0.

Since the validation of the system, described in section 3.1, was carried out using an ABS composed of 12.5 wt % of PEG 1000 + 12.5 wt % of phosphate salt, it is imperative to confirm that the behavior is the same for the ABS constituted by 18 wt % of PEG 1000 + 15 wt % of phosphate salt buffer, when it comes to the stationary phase retention. A process similar to the one detailed in section 3.1. was carried out, mainly regarding the determination of the S_f value for the FCPC assay with this system composition. The ascending mode of operation was chosen since IgY was found to partition exclusively to the top phase, rich in PEG 1000. Therefore, it would be the first protein to be collected in the chromatographic assay. With 2000 rpm of rotation speed and 2.0 mL min^{-1} of flow rate, an S_f of 39.0 % was achieved, a lower value than the S_f values obtained in section 3.1, but well above the 20 % established minimum.

The first purification trial was performed with the injection of 5 g of both phases of an ABS composed of 18 wt % of PEG 1000 + 15 wt % of phosphate salt buffer at pH 6.0 + 67.0 wt % WSPF, using the following operational conditions: ascending mode, 2000 rpm at a 2.0 mL min^{-1} flow rate. In this trial, no IgY was detected in any collected fraction, and during the procedure a loss of some stationary phase inside the column was detected. A follow-up run was performed, this time injecting only the top phase of a 10 g ABS composed of 18 wt % PEG 1000 + 15 wt % of phosphate salt buffer at pH 6.0 + 67 wt % WSPF, reducing the flow rate to 1.5 mL min^{-1} and maintaining the rotation at 2000 rpm, in the ascending mode. Also with this system and conditions, IgY was not identified in the collected fractions. Furthermore, a severe pressure drop and loss of stationary phase in the column in a short period was observed, indicating an inefficient stationary phase retention. A different approach was then considered, using the same conditions, but instead of injecting the top phase of a PEG 1000 + phosphate salt buffer at pH 6.0 + WSPF, the same system was prepared with water instead of the WSPF. The top phase of the PEG 1000 + phosphate salt buffer at pH 6.0 + H_2O was then mixed with WSPF and injected, resulting in a stationary phase retention of 48.5 %. Despite the adequate S_f value obtained, IgY was not identified in the collected fractions.

In the previous trials, IgY was not identified, evidencing that the protein is being lost. Therefore, in an attempt to solve this problem, a different ABS was tested, namely the 18 wt % PEG 1000 + 13 wt % of phosphate salt buffer at pH 6.0 + 69 wt % WSPF ABS. The second set of FCPC assays were validated for its capacity to extract IgY from the WSPF in section 3.3.2. While this system led a lower IgY purity level, it was considered as more adequate for use in FCPC do to a

higher difference in the contaminant's partition coefficient. Similarly to the previous purification assays, the S_f value was calculated and taken into account.

Considering the loss of protein that occurred in previous FCPC assays, most likely due to denaturation, it was decided that the sample injected into the FCPC loop should not be the result of a one-step ABS extraction, as done previously with the phases from the PEG 1000 + phosphate salt buffer at pH 6.0 + WSPF. To lower the denaturation effect resulting from the one-step ABS extraction, the sample injected in the following assays was composed of 8g of WSPF + 2g of the top phase of a 18 wt % of PEG 1000 + 13 wt % of phosphate salt buffered at pH 6.0 ABS + 69% H_2O. An increase in the flow-rate was also deemed necessary, postulating that the contact time between the IgY and the phases was causing this issue. In that sense, the first assay was done in the ascending mode of operation, using a 2.5 mL/min flow-rate, also increasing the rotation to 2500 rpm, in an attempt to offset a possible hit to phase retention as a result of the increased flow-rate. While this assay presented a remarkably low S_f value, 16.7%, lower than the 20% required, some IgY purification was indeed achieved, as demonstrated in the chromatogram presented in Figure 13. As the purity of the end sample was only about 29.5%, possibly due to low retention of the stationary phase, further optimizations were performed.

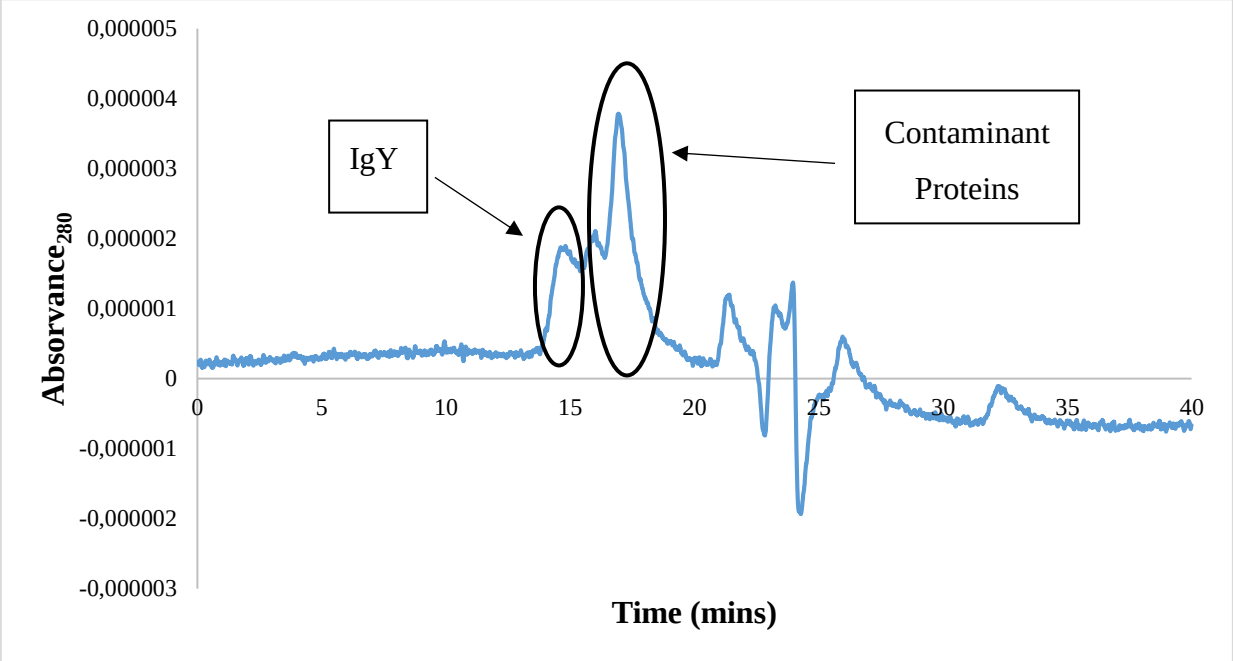

Figure 13 - SE-HPLC spectra of fraction 9 collected from the FCPC assay using the 18 wt % PEG 1000 + 13 wt % phosphate buffer at pH 6.0 ABS, performed with 2.5 mL/min flow rate and 2500 rpm in the ascending mode.

As an increase in the rotation speed does not lead to a sufficient S_f value, an additional assay was performed in the ascending mode using 2000 rpm and a 3.0 mL/min flow rate. The chromatogram of the seventh fraction collected from this assay is presented in Figure 14. This assay leads to more promising results, not only presenting a higher S_f than the previous one, but also a higher IgY purity (50.6 %). This purity effectiveness surpassed both the extraction assays using only the ABS and the combination of ABS extraction and ultrafiltration, disclosing the higher potential of FCPC for the purification of biopharmaceuticals.

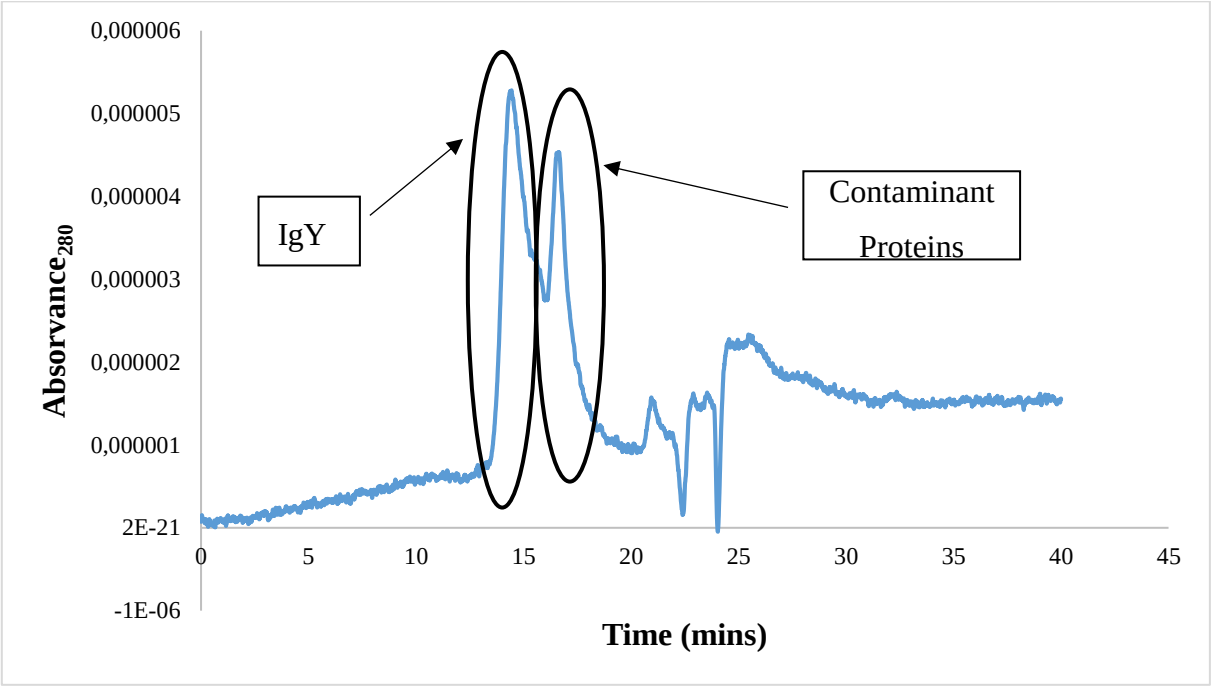

Figure 14 - SE-HPLC spectra of fraction 7 collected from the FCPC assay using the 18 wt % PEG 1000 + 13 wt % phosphate buffer at 6.0 pH ABS, performed with 3.0 mL/min flow rate and 2000 rpm in the ascending mode.

Overall, the first FCPC assays were performed using the 18 wt % of PEG 1000 + 15 wt % of phosphate salt buffer at pH 6.0 + 67 wt % WSPF, using the ascending mode of FCPC operation, at 2000 rpm. A flow rate of 2.0 mL/min was tested first and then lowered to 1.5 mL/min. The samples injected in the FCPC loop were the phases obtained after a single-step IgY extraction, using the ABS referred above. These first assays revealed a complete loss of IgY. As such, the following assays attempted to thwart the IgY loss by injecting a mixture of 8 g of WSPF + the top phase of a 18 wt % of PEG 1000 + 13 wt % of phosphate salt buffer at pH 6.0 + 69 wt % H_2O. This system was deemed more appropriate than the previously one. The flow rate was increased to 3.0 mL/min to reduce the

time IgY is present inside the FCPC apparatus and, as such, to decrease its loss. These optimization steps allowed to obtain IgY with a purity level of 51%.

3.4 Secondary structure and stability of IgY

Circular Dichroism (CD) can be defined as the unequal absorption of left-handed and right-handed circularly polarized light, in which the differential absorption effect of light by asymmetric molecules can be appraised [179]. CD is appropriate for determining the secondary structure of proteins due to the amides present in the polypeptide backbone, that are characteristic of proteins, and which have a chromophoric activity that effectively shifts or splits light into multiple transitions due to exciton interactions between electrons [180]. Because protein CD spectra are dependent on their conformation, this technique can be used to monitor and evaluate conformational changes due to binding interactions [179].

The evaluation of the IgY stability was carried out by the analysis of CD spectra, in comparison to a solution of pure IgY commercially acquired. This analysis was carried out according to the representative CD spectra of proteins shown by Greenfield [179], presented in Figure 15. A typical CD spectra of proteins rich in α-helixes show a negative band at 222 and 208 nm, while proteins composed mainly of β-sheets can be identified by a negative band at 218 nm. Disordered or otherwise very unstable proteins can be identified by relatively low, but not negative, CD values above 210 nm [179].

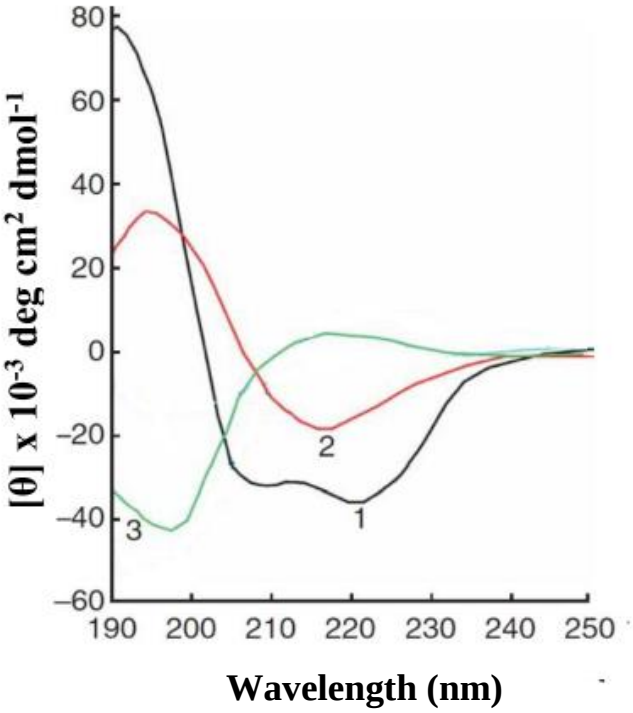

Figure 15 - Representative CD spectra for proteins, adapted from Greenfield [179]. 1: α-helix; 2: β-sheet; 3: disordered proteins.

The results for the IgY samples obtained according to the protocols described in section 2.3.2.1, will be shown separately in the following figures, with the IgY standard 8commercially acquired sample) shown in all of them for comparison. Figure 16 shows the CD results for the IgY in PEG 1000 solutions, at 10, 20 and 30 wt %. IgY in the 10 and 20 wt% of PEG 1000 solutions does not present a significant loss of secondary structure, as shown by their spectra resemblance with the IgY standard, with a negative band at 218 nm characteristic of proteins mainly comprising β-sheets [37,69]. However, for 30 wt% of PEG 1000, IgY displays a highly significant loss of its secondary structure, as shown by the spectras lack of a smoothly defined curve, and by presenting a less noticeable negative band at 218 nm. Another indication of the negative effect of the high concentration of PEG 1000 towards IgY is the signal intensity. A loss of the CD signal intensity indicates two phenomena: a loss of protein, due to precipitation, making these unable to be detected by CD; and an increased contribution of the positive band above 210 nm, characteristic of disordered proteins. This result is however expected, considering the strong capacity of polymers like PEG to precipitate proteins, more significant at higher concentrations [178].

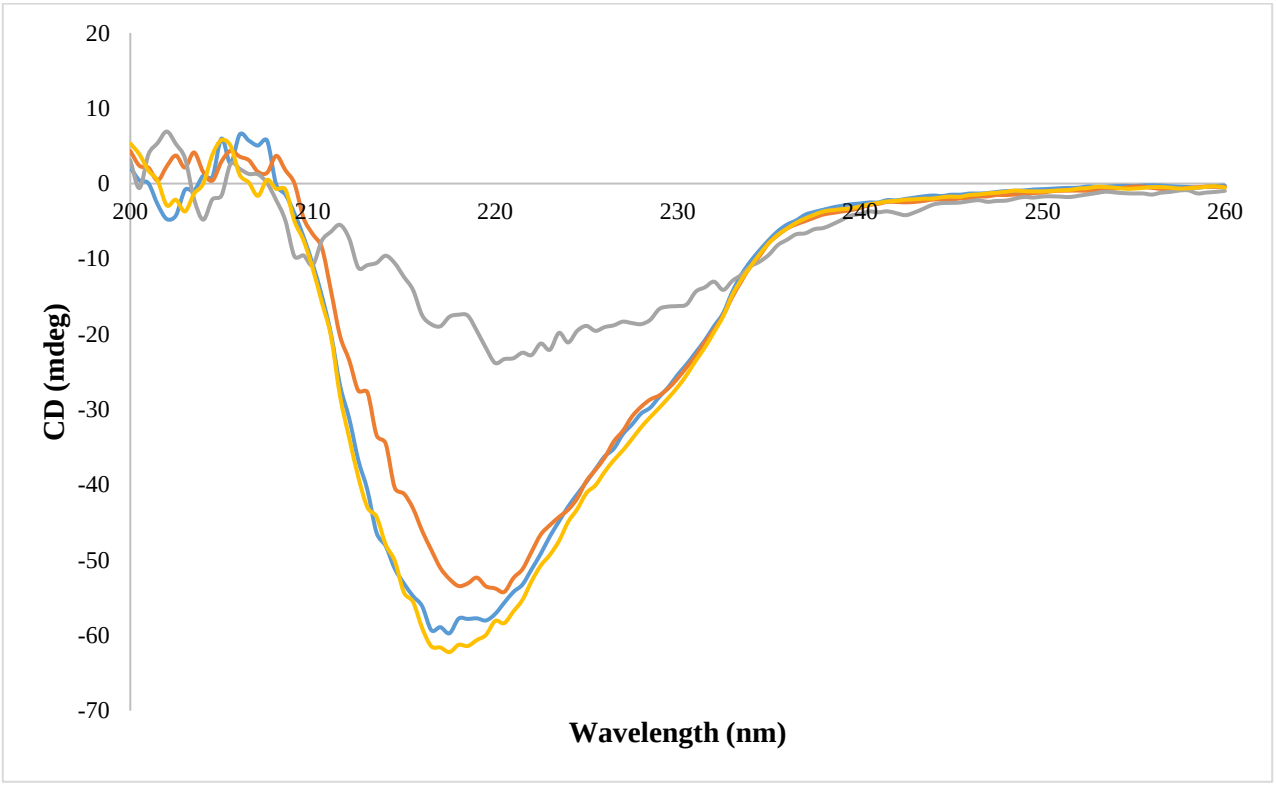

Figure 16 - CD spectra of the IgY standard in PBS at 1.5 mg/mL (yellow), IgY + PEG 1000 at 10 wt % (blue), IgY + PEG 1000 at 20 wt % (orange), and IgY + PEG 1000 at 30 wt % (gray).

Figure 17 presents the CD spectra for the IgY + phosphate salt solutions at 5.5, 6.0, 6.5, 7.5 and 8.0 pH values. Almost all of the samples composed of salt buffer + IgY revealed similar CD spectra, although with a rougher pattern than that observed in the IgY standard, but still presenting a noticeable negative band at the 218 nm. The sample at pH 8.0 shows the most distinct spectral profile, with a shift of its negative band from the expected 218 nm to 222 nm, indicating a significant loss of the protein structure. A reduction in the signal intensity is also noticeable in these spectra, with a more pronounced effect for samples prepared at higher pH values. These results show that the strong salting-out effect caused by the ions K^{2+} and HPO_4^{-} [176], when present in higher concentrations at alkaline medium, is relevant to induce the protein loss of stability.

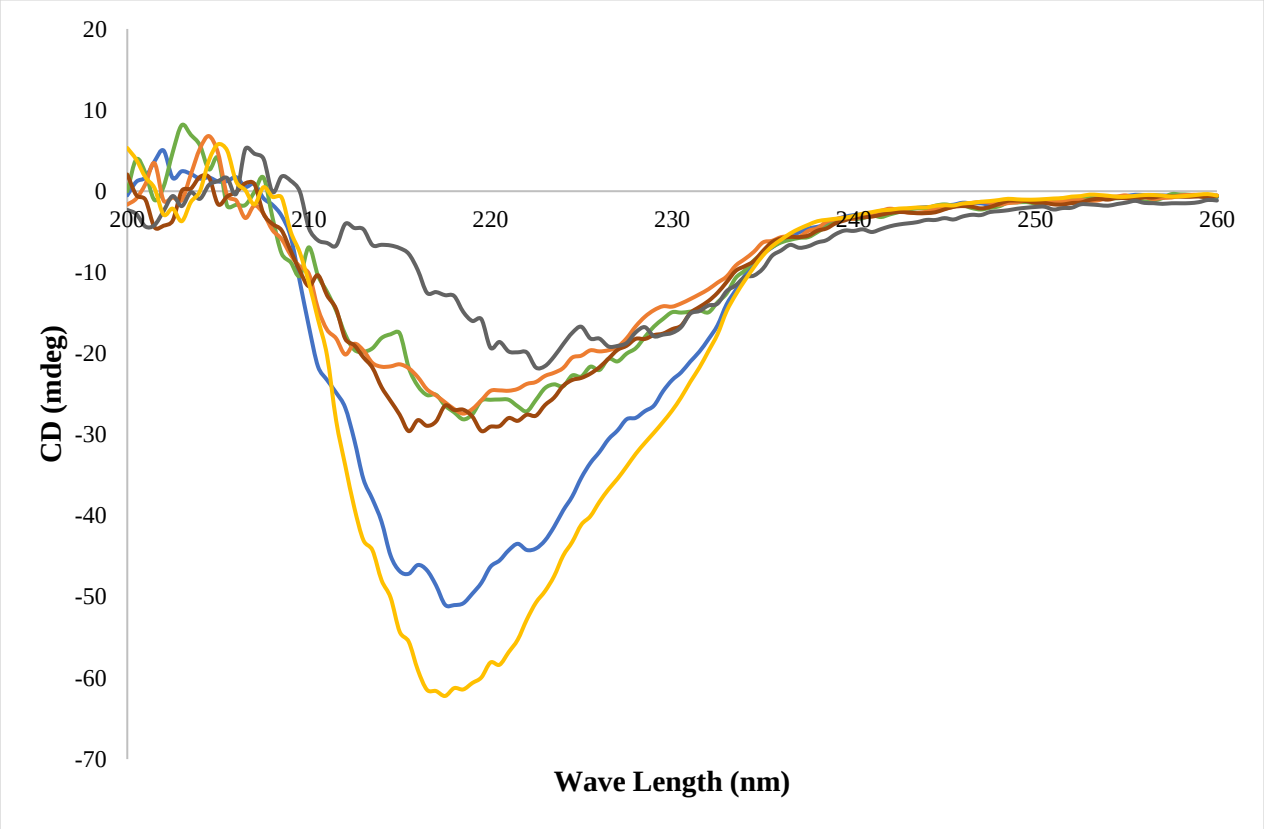

Figure 17 - CD spectra of the IgY standard in PBS at 1.5 mg/mL (yellow) and the IgY + phosphate salt buffers at 5.5 (blue), 6.0 (green), 6.5 (orange), 7.5 (brown) and 8.0 (gray) pH values.

Figure 18 shows the CD spectra for the top phase of the two ABS analyzed: 18 wt % of PEG 1000 + 15 wt % phosphate buffer at pH 6.0 + 67 wt % of the IgY aqueous solution (ABS 18/15/67) and 18 wt % of PEG 1000 + 13 wt % phosphate buffer at pH 6.0 + 69 wt % of the IgY aqueous solution (ABS 18/13/69). While both samples show similar spectra, they differ however in the signal intensity, indicative of loss of the protein secondary structure. The ABS 18/15/67 spectrum has a remarkably lower signal intensity when compared to both the IgY standard and ABS 18/13/69, indicating a more significant loss of secondary structure. The spectrum of the ABS 18/13/69 differs from the IgY standard, mainly in a shift from the typical depression at 218 nm band (characteristic of β-sheets) to 222 nm (characteristic of α-helix). While this fact could indicate a possible loss of secondary structure, it is important to acknowledge that, since the other parameters are highly comparable to the IgY standard, other factors could be at play, requiring deeper analyses. Considering that the shift occurs to bands characteristic of the α-helix secondary structure, it is possible that this sample may be contaminated with other proteins.

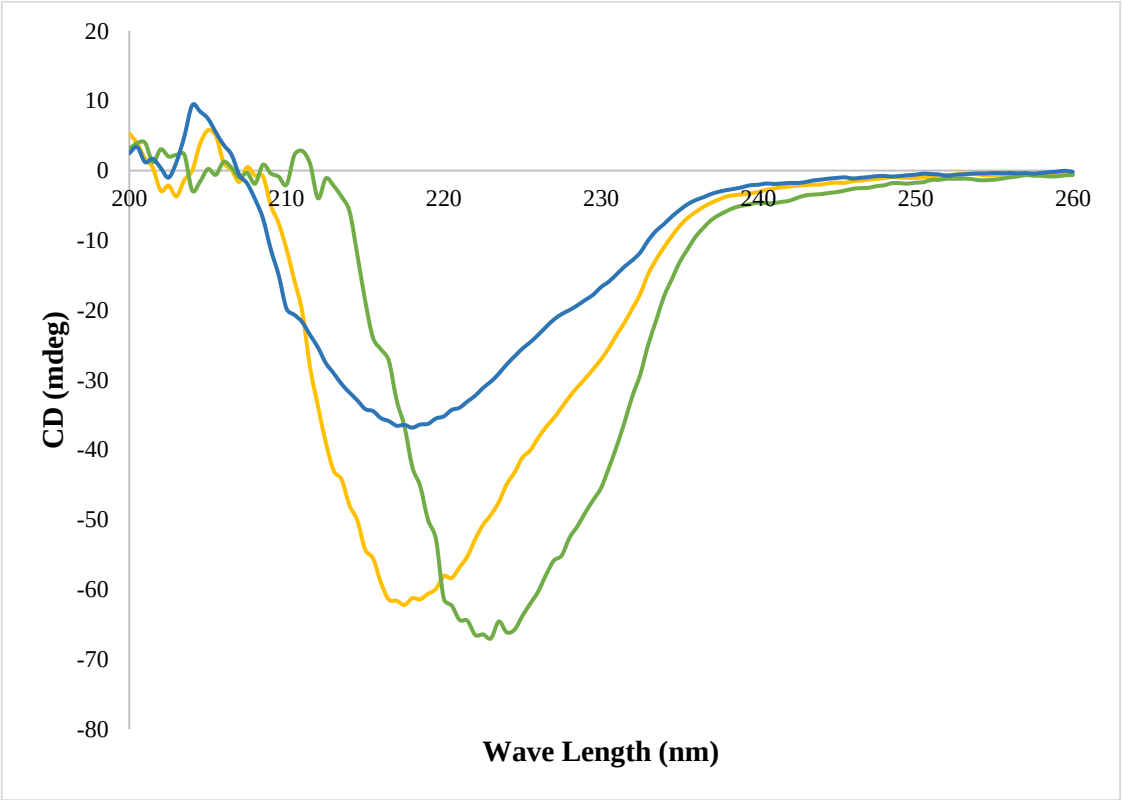

Figure 18- CD spectra of the IgY standard in PBS at 1.5 mg/mL (yellow), the IgY + TP of the 18 wt % PEG 1000 + 15 wt % phosphate-based salt buffers at 6.0 ph (blue) and the IgY + TP of the 18 wt % PEG 1000 + 13 wt % phosphate-based salt buffers at 6.0 ph (green).

Additionally to the CD analysis, stability and activity studies were carried out with ELISA for the IgY obtained from the 3 purification approaches presented in this work: one-step purification method with the 18 wt % of PEG 1000 + 13 wt % phosphate buffer at pH 6.0 + 69 wt % WSPF ABS; two-step purification method with ABS and ultrafiltration; and multiple-step purification approach using the same ABS in FCPC. Since these techniques result in samples with similar purity, it is expected that the concentration of active IgY determined by ELISA will change primarily due to the effect of each of these techniques on the IgY activity, and as such, IgY stability. The IgY concentration results are presented in Figure 19. The analysis of these results reveals a much higher concentration of biologically active IgY for the process using ultrafiltration, with 553.77 ng/mL, against the ABS extraction and the FCPC results, with 50.07 and 29.19 ng/mL of IgY, respectively. The ELISA results for active IgY after the ABS extraction followed by ultrafiltration should not be higher than the result for the IgY purified using just the one-step ABS extraction. This discrepancy can be attributed to the blocking effect of PEG 1000 in ELISA assays [181], and therefore, after the ultrafiltration step, the polymer has been removed and the antibody concentration/activity increases.

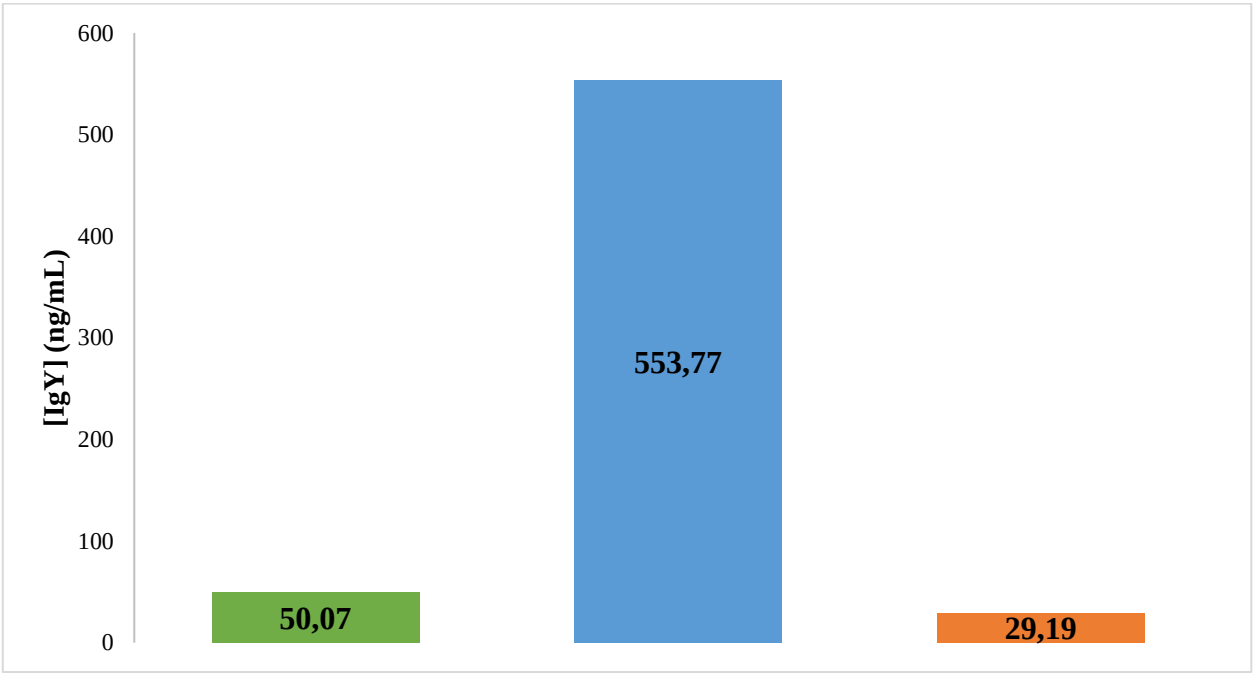

Figure 19 – Concentration of IgY determined by ELISA for the top phase of the 18 wt % of PEG 1000 + 13 wt % phosphate buffer at pH 6.0 + 69 wt % WSPF ABS (green), after ultrafiltration (blue), and after FCPC purification (orange).

In summary, the stability assays using CD allowed the evaluation of the effect of the ABS phase-forming components on the IgY stability. The most important set of data is the for IgY stability in PEG 1000 solutions at 30 wt%, mainly because the top phase of the studied ABS is composed of *ca.* 30 wt% of PEG, as determined using the systems TLLs and binodal data. The CD spectra reveal that at this PEG concentration there is significant loss of the target protein secondary structure, a result that coincides with the analysis done on the FCPC assays and loss of protein. The results for the CD spectra of IgY + phosphate buffer at different pH values are less important, particularly considering that the IgY partitions almost exclusively to the PEG-rich phase. Yet, they provide relevant information on the effect of the pH in the IgY secondary structure, which could be a limiting factor if a high pH buffer solution is used. While the ELISA assays did not permit a direct comparison of the several purification methodologies, it revealed an important blocking effect exerted by the polymer that should be taken into account when considering the aimed application of IgY.

4. Final Remarks

4.1 Conclusions and future work

This work aimed to introduce aqueous biphasic systems and the FCPC technology as a purification platform for IgY from egg yolk. A literature review was firstly carried out in order to find a starting point for a system that should have two essential characteristics: being able to effectively separate IgY from the contaminant proteins of egg yolk and be feasible to be used in FCPC. This research work showed that a PEG 1000 + K_2HPO_4 ABS has the potential for such an approach, and as such, the first step consisted on the validation of the system viability in FCPC. The system capacity to be retained inside the FCPC column was tested through the calculation of the stationary phase retention.

After the validation of the system applicability in FCPC, the next step comprised the description of these systems phase diagrams, by including the analysis of the effect of the pH in their capacity to form two phases, i.e. ABS. This step revealed that higher pH values are more favorable for the separation into two phases. With the binodal curves presented, for which a good correlation was achieved, the respective tie-lines were determined, allowing to conclude on the composition of each phase in equilibrium for the mixtures for which the partition studies were performed.

After the determination of the ABS phase diagrams, the mixture formed by18 wt % of PEG 1000 and 15 wt % of phosphate salt buffer was chosen, aiming at maximizing the water content of the system while still guaranteeing that it presents a biphasic behavior. In the extraction studies of IgY, some protein precipitation was evident at the interphase, more pronounced in ABS of higher pH values . By the analysis of both phases by SE-HPLC it was revealed that all the proteins in the WSPF have a higher affinity to the top-rich phase (polymer-rich phase), in which high yields were found, yet with low IgY purity levels (≈ 30-40 %). SE-HPLC also revealed that the system is quite specific for IgY, which partitions completely to the top phase, while the contaminants partition among both phases, being thus promising to be applied in FCPC. Further optimization of the system compositions was attempted, demonstrating that an ABS consituted by 18 wt % of PEG 1000 + 13 wt % phosphate salt buffer is more favorable for FCPC purification due to the IgY higher yield obtained.

The purification of Igy using FCPC was best achieved for ABS formed by 18 wt % PEG 1000 + 13 wt % phosphate salt buffer at pH 6.0, using the ascending mode, a 3.0 mL/min flow-rate and 2000 rpm of rotation speed. An adequate system retention was guaranteed by a high S_f value for the same system, and IgY with ≈ 51 % purity was obtained. An alternative purification method based on ultrafiltration using the same ABS was also attempted, achieving a lower purity, 47 %, but enabling the removal of the polymer which might be useful depending on the intended use of IgY.

The stability of the target protein was to be assessed by circular dichroism, concluding that the protein did not show significant signs of degradation of its secondary structure, rich in β-sheets, in PEG 1000 aqueous solutions of low concentration (up to 20 wt%). In phosphate salt buffer solutions some loss of stability of IgY was found, particularly for higher pH values. The ELISA assays revealed an important blocking effect for IgY, as detailed by the much higher concentration of active IgY after the ultrafiltration assays, while also demonstrating that FCPC purification further promotes the loss of biological activity, when compared to the single-step extraction using just the ABS.

Overall, the methods investigated in this work for IgY purification have some advantages and disadvantages. While FCPC is the best method in terms of possible industrial application, due to the scale-up possibilities and IgY purity obtained, it is more disruptive towards the protein stability than just the single-step extraction using ABS. The ultrafiltration and one-step ABS methodologies also lead to high purity levels of IgY, albeit more limited in terms of scale-up, and being more suitable for laboratory purifications.

As future work, and based on the work developed, other operational conditions for the FCPC should be tested, particularly in the finding of other phase-forming components and compositions which could allow the complete purification of IgY, and using higher flow-rates to turn the process faster and more profitable. The two-step purification method with ABS and ultrafiltration should also be refined by applying more filtration cycles in an attempt to increase the end product purity, while still considering the final mass of IgY obtained, that can be reduced with more cycles. Further evidence of the blocking effect of PEG 1000 should be obtained by ELISA analysis of IgY purified by FCPC, yet after the polymer removal.

5. References

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Appendix A: Calibration curve for the IgY quantification by SE-HPLC

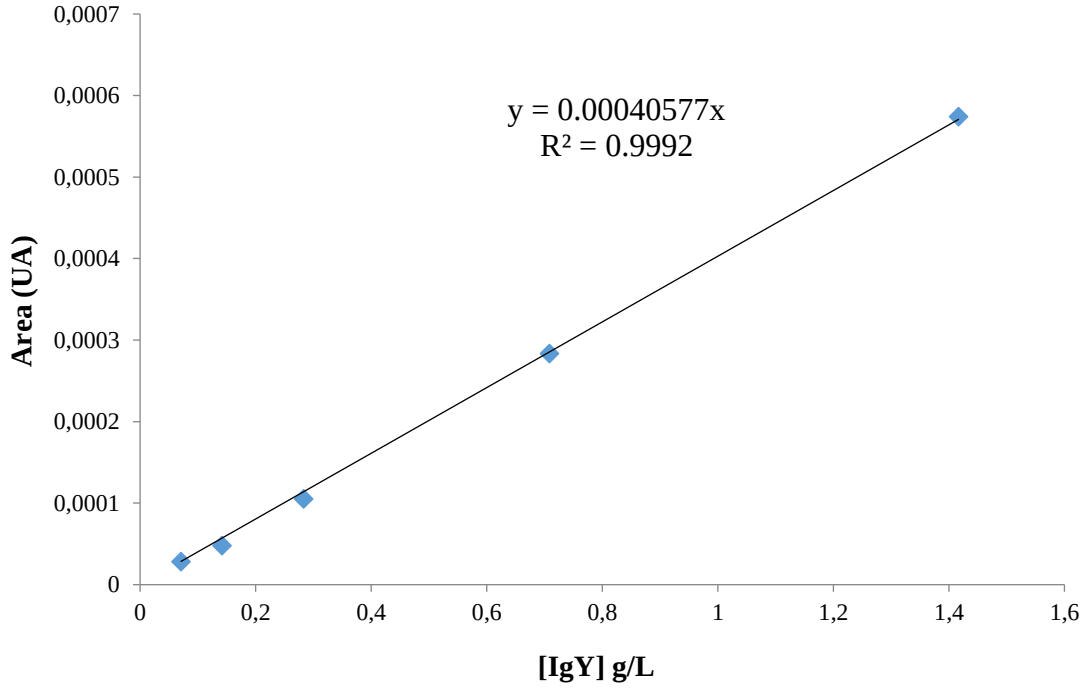

Figure A 1- Calibration curve (absorbance vs concentration of IgY) by SE-HPLC.

Appendix B: *Experimental binodal data for the PEG + phosphate-based salts + H_2O Ternary Systems*

Table B 1 - Experimental weight fraction data for the systems composed of PEG 1000 (1) + phosphate-based salt (2) + H_2O, at pH 5.5 and 6.0, at 298 K and atmospheric pressure.

pH 5.5		pH 6.0					
100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2
55.680	1.680	46.812	2.212	21.179	9.317	9.426	16.776
50.288	2.242	41.979	2.449	20.788	9.563	8.871	17.173
47.080	2.753	40.286	2.822	20.490	9.674	8.514	17.483
42.901	3.168	38.434	3.124	20.056	9.945	7.996	17.863
40.423	3.555	37.504	3.435	19.773	10.067	7.415	18.283
38.153	3.935	36.075	3.838	19.500	10.169	6.975	18.601
36.336	4.208	34.835	4.109	19.016	10.343	6.283	19.076
35.138	4.501	33.556	4.405	18.681	10.565	6.045	19.404
34.018	4.812	32.424	4.726	18.360	10.753	5.329	19.913
32.755	5.208	31.378	4.970	18.063	10.939	5.014	20.256
31.697	5.434	30.677	5.297	17.864	11.021	4.328	20.936
30.774	5.690	29.800	5.478	17.573	11.233	3.957	21.315
29.352	6.288	29.271	5.705	17.281	11.414	3.508	21.731
27.906	6.999	28.711	5.932	16.791	11.901	3.082	22.120
27.232	7.190	28.190	6.174	16.002	12.397	2.674	22.776
26.201	7.572	27.428	6.336	15.631	12.720	2.228	23.273
25.285	7.975	26.449	6.757	15.164	12.951	1.839	24.279
24.342	8.343	25.950	6.955	14.715	13.283	1.456	25.634
23.256	8.905	25.523	7.138	14.481	13.496		
22.195	9.422	25.041	7.340	14.007	13.754		
21.130	10.025	24.625	7.520	13.701	13.941		
20.372	10.386	23.816	8.152	12.941	14.426		
19.195	11.075	23.442	8.313	12.582	14.671		
18.137	12.201	23.051	8.478	12.127	15.004		
16.921	12.948	22.699	8.598	11.781	15.201		
14.017	15.082	22.333	8.757	11.035	15.714		
12.605	16.047	21.848	9.072	10.824	15.871		
10.459	17.516	21.523	9.195	10.041	16.368		

Table B 2 - Experimental weight fraction data for the systems composed of PEG 1000 (1) + phosphate-based salt (2) + H_2O, at pH 6.5 and 7.5, at 298 K and atmospheric pressure.

6.5 pH				7.5 pH					
100 w_1	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**	**100 w_1**	**100 w_2**
57.131	1.584	15.084	12.119	56.144	2.013	20.881	7.982	3.445	18.579
50.928	2.163	14.654	12.359	47.468	2.106	20.412	8.173	2.971	19.105
45.557	2.843	14.236	12.624	44.638	2.318	20.089	8.343	2.442	19.674
40.648	3.241	13.853	12.897	42.737	2.625	19.547	8.562	2.037	21.039
37.545	3.936	13.429	13.196	40.715	3.246	19.247	8.751		
34.461	4.486	12.970	13.510	37.780	3.349	18.907	8.932		
32.452	4.982	12.481	13.828	36.045	3.755	18.563	9.112		
30.585	5.396	12.004	14.150	34.303	3.870	18.141	9.322		
29.432	5.923	11.444	14.497	33.628	4.088	17.734	9.535		
27.905	6.292	10.879	14.882	32.658	4.232	17.337	9.742		
26.545	6.630	10.273	15.292	32.015	4.438	16.937	9.964		
25.703	7.023	9.669	15.689	31.438	4.644	16.491	10.202		
24.884	7.393	9.285	15.997	30.560	4.792	15.949	10.471		
23.865	7.552	8.571	16.408	29.646	5.309	15.499	10.711		
23.148	7.877	7.829	16.857	28.828	5.419	15.160	10.941		
22.451	8.205	7.452	17.223	28.017	5.633	14.619	11.220		
21.796	8.481	6.663	17.765	27.576	5.802	14.029	11.533		
21.180	8.807	6.257	18.126	27.122	5.950	13.377	11.849		
20.594	9.089	5.904	18.480	26.639	6.155	13.006	12.121		
20.000	9.230	4.948	19.089	26.193	6.290	12.571	12.414		
19.986	9.396	4.487	19.502	25.761	6.413	11.780	12.830		
19.630	9.430	4.027	19.935	25.364	6.544	11.001	13.246		
19.207	9.645	3.581	20.678	24.979	6.648	10.468	13.598		
18.954	9.794	3.126	21.117	24.613	6.755	9.845	13.955		
18.545	10.012	2.594	21.941	24.291	6.861	9.232	14.325		
18.291	10.196	2.008	23.265	23.888	6.969	8.283	14.861		
18.010	10.377	1.518	25.184	23.403	7.250	7.535	15.290		
17.492	10.639	0.867	33.334	23.091	7.356	6.771	15.779		
17.074	10.896			22.769	7.466	6.047	16.258		
16.769	11.111			22.460	7.560	5.648	16.665		
16.382	11.326			22.179	7.648	4.844	17.146		
15.825	11.629			21.880	7.738	4.357	17.618		
15.470	11.867			21.173	7.821	3.919	18.104		

Table B 3 - Experimental weight fraction data for the systems composed of PEG 1000 (1) + phosphate-based salt (2) + H_2O, at pH 8.0 and with no buffer, at 298 K and atmospheric pressure.

8.0 pH				No Buffer			
100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2	100 w_1	100 w_2
56.884	1.647	15.719	10.274	56.951	1.247	12.853	11.351
48.417	2.204	15.499	10.344	48.221	2.090	12.698	11.435
43.182	2.603	15.185	10.524	42.723	2.811	12.540	11.535
39.835	3.030	14.978	10.585	38.314	3.265	12.333	11.553
37.723	3.442	14.671	10.778	34.971	3.741		
35.357	3.815	14.479	10.816	31.268	4.742		
33.293	4.046	14.204	11.012	29.104	5.043		
32.349	4.402	13.788	11.406	28.296	5.503		
31.374	4.757	13.526	11.579	26.821	5.822		
30.216	4.917	13.370	11.626	24.687	6.360		
29.434	5.204	13.147	11.759	23.895	6.652		
28.705	5.474	12.917	11.888	23.055	6.877		
27.684	5.647	12.754	11.940	22.547	7.202		
26.991	5.907	12.614	11.998	21.847	7.383		
26.315	6.108	12.383	12.165	21.183	7.569		
25.713	6.330	12.252	12.201	20.759	7.836		
24.907	6.412	12.064	12.321	20.185	8.041		
24.373	6.545	11.596	12.880	19.648	8.182		
23.919	6.725	11.471	12.898	19.266	8.469		
23.464	6.901	11.288	13.024	18.758	8.612		
22.968	7.079	11.171	13.064	18.474	8.727		
22.565	7.229	10.998	13.180	18.194	8.841		
22.149	7.423	10.872	13.204	17.886	9.059		
21.745	7.588	10.713	13.301	17.443	9.162		
21.369	7.744	10.700	13.412	17.049	9.259		
21.026	7.899	10.374	13.613	16.804	9.433		
20.652	8.036	9.498	13.968	16.547	9.577		
20.347	8.125	9.051	14.206	16.315	9.684		
20.004	8.265	8.704	14.550	16.068	9.830		
19.691	8.411	8.267	14.800	15.703	9.898		
19.412	8.511	7.781	15.076	15.489	10.028		
19.106	8.653	7.322	15.392	15.273	10.145		
18.642	8.936	6.808	15.681	15.059	10.288		
18.361	9.029	6.333	16.000	14.744	10.340		
18.084	9.107	5.793	16.325	14.546	10.458		
17.816	9.193	5.395	16.768	14.366	10.596		
17.419	9.464	4.792	17.194	14.032	10.775		
17.152	9.546	4.279	17.554	13.763	10.870		
16.900	9.655	3.380	18.289	13.590	11.005		
16.572	9.870	2.772	18.826	13.353	11.088		
16.322	9.963	2.123	19.803	13.192	11.187		
16.051	10.080			13.015	11.266		

Appendix C: Phase Diagrams and TLs for PEG 1000 + phosphate-based salts + H_2O ternary systems

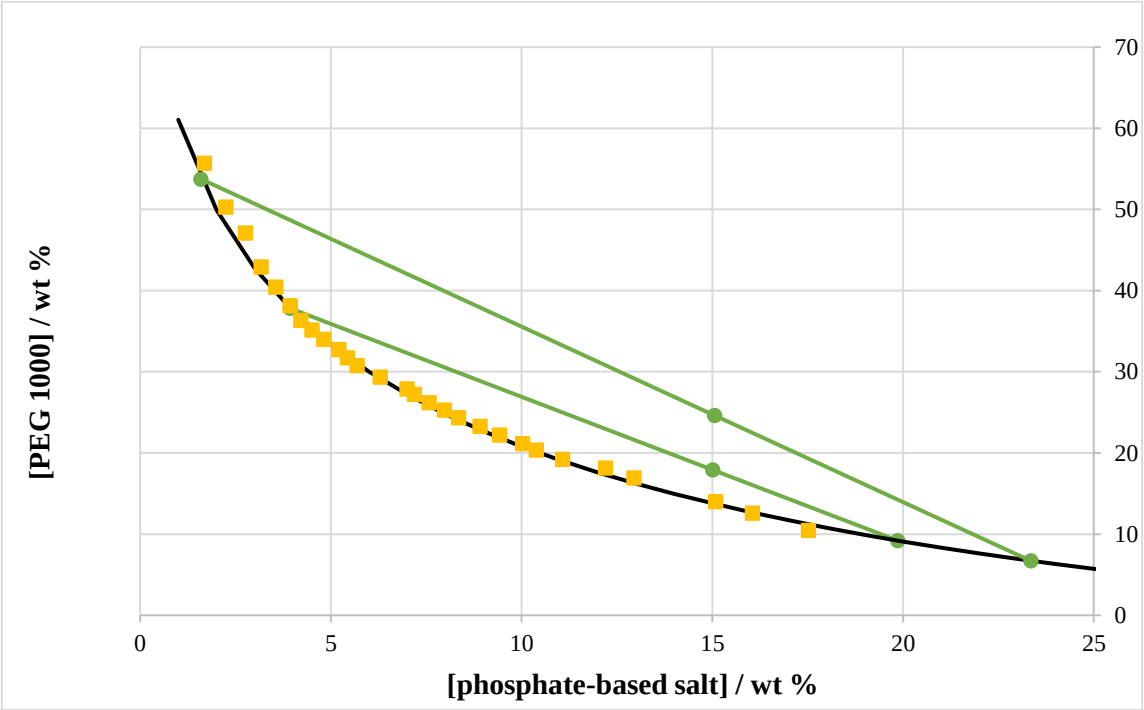

Figure C 1 - Phase diagram of the ABS composed of PEG 1000 + Phosphate buffer salt at pH 5.5 + H_2O: tie-line data (●); adjusted binodal data obtained through equation 1 (−); experimental binodal curve data (■).

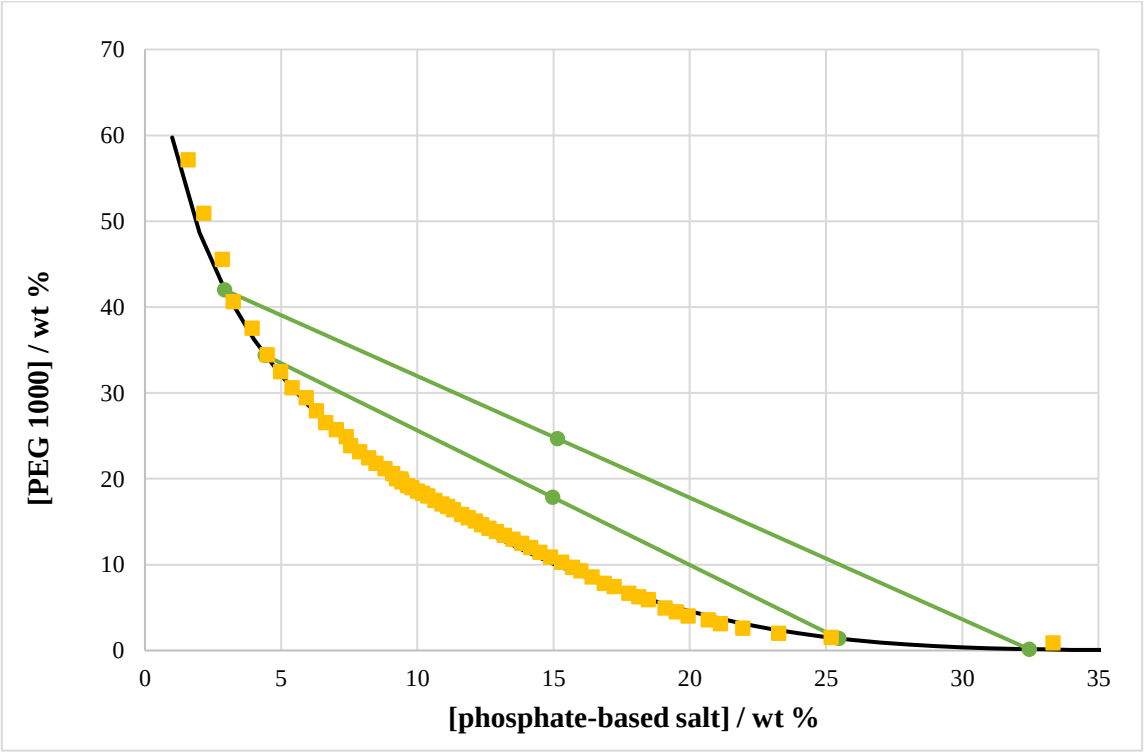

Figure C 2 Phase diagram of the ABS composed of PEG 1000 + Phosphate buffer salt at pH 6.5 + H_2O: tie-line data (●); adjusted binodal data obtained through equation 1 (−); experimental binodal curve data (■).

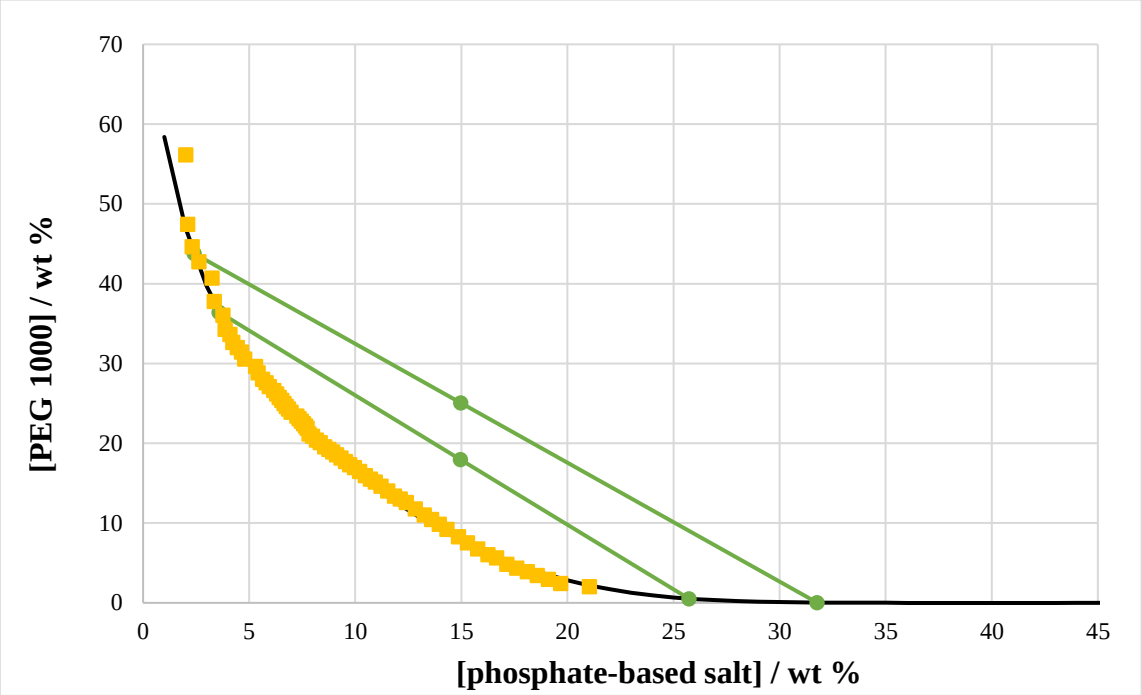

Figure C 3 - Phase diagram of the ABS composed of PEG 1000 + Phosphate buffer salts at pH 7.5 + H_2O: tie-line data (●); adjusted binodal data obtained through equation 1 (−); experimental binodal curve data (■).

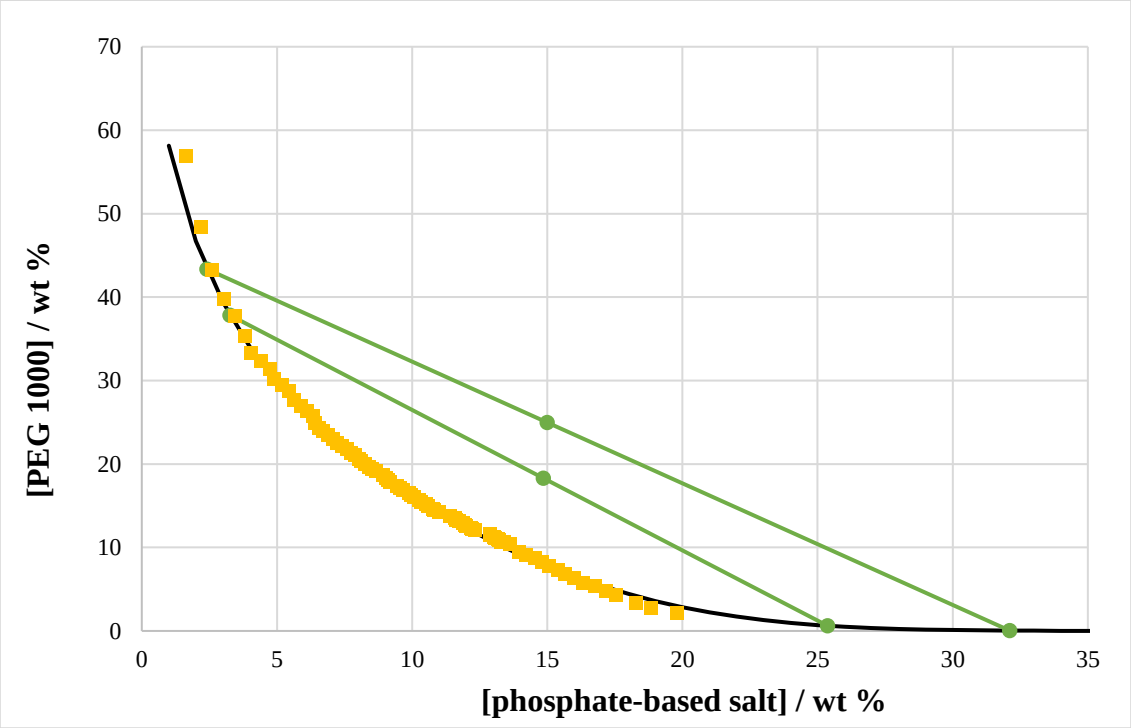

Figure C 4 - Phase diagram of the ABS composed of PEG 1000 + Phosphate buffer salts at pH 8.0 + H_2O: tie-line data (●); adjusted binodal data obtained through equation 1 (−); experimental binodal curve data (■).

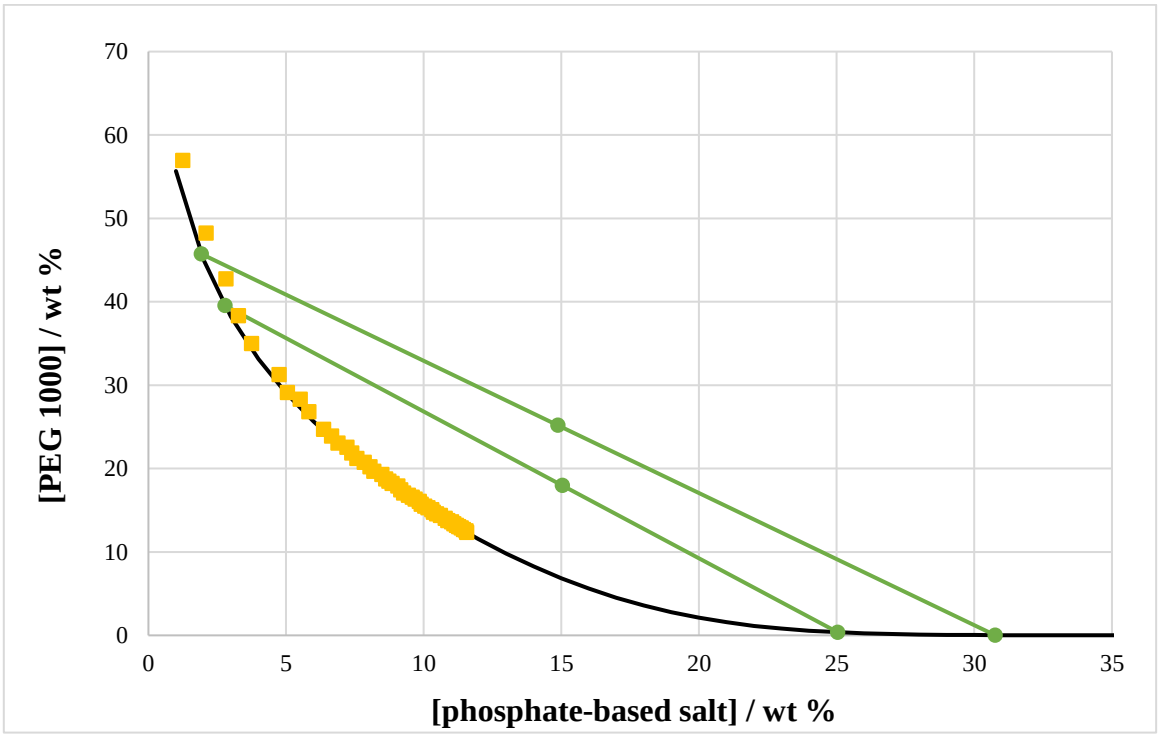

Figure C 5 - Phase diagram of the ABS composed of PEG 1000 + K_2HPO_4 salt without pH buffering + H_2O: tie-line data (●); adjusted binodal data obtained through equation 1 (−); experimental binodal curve data (■).

Appendix D: SE-HPLC chromatograms for the collected fractions of each ultrafiltration cycle

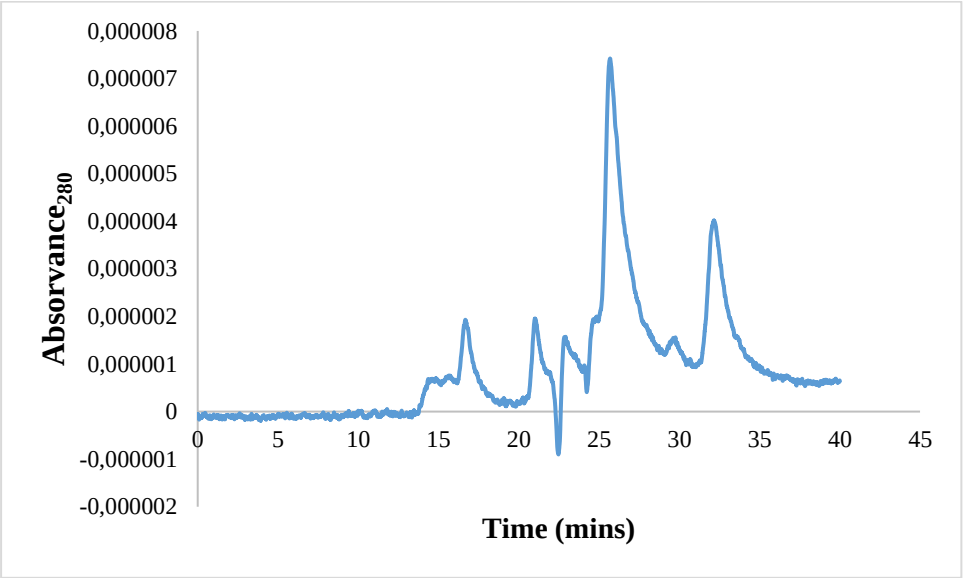

Figure D 1 - SE-HPLC chromatogram of fraction 1 from the ultrafiltration assay.

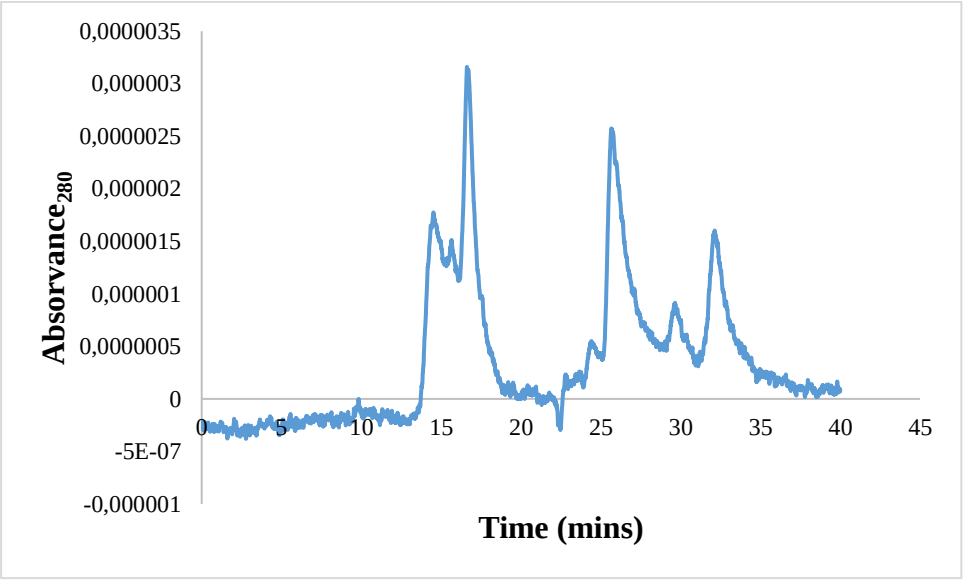

Figure D 2 - SE-HPLC chromatogram of fraction 2 from the ultrafiltration assay.

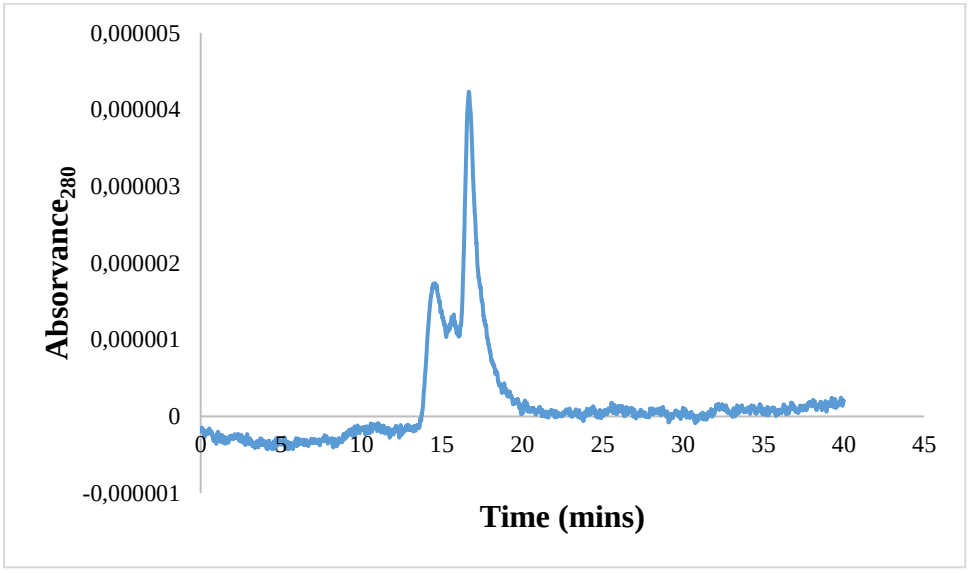

Figure D 3 - SE-HPLC chromatogram of fraction 4 from the ultrafiltration assay.

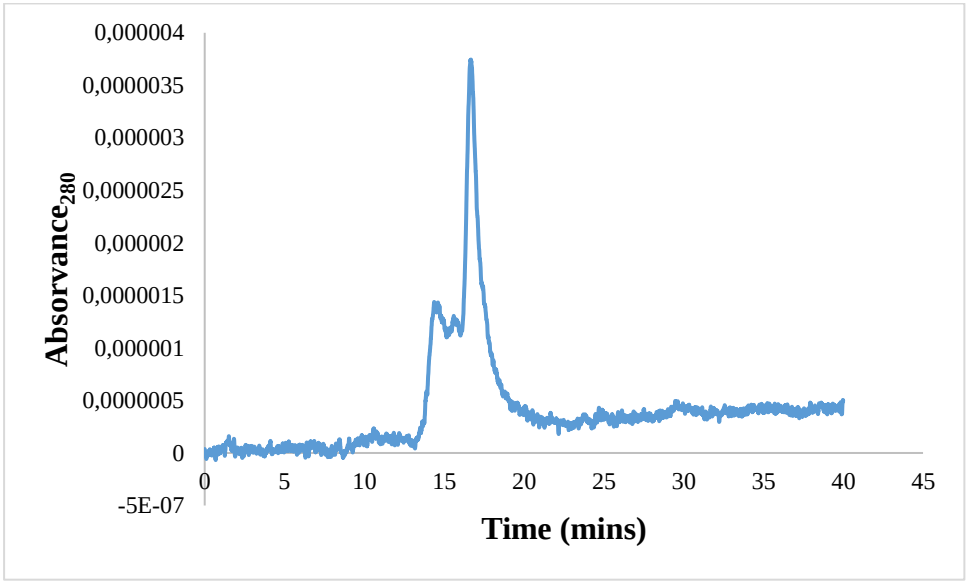

Figure D 4 - SE-HPLC chromatogram of fraction 5 from the ultrafiltration assay.

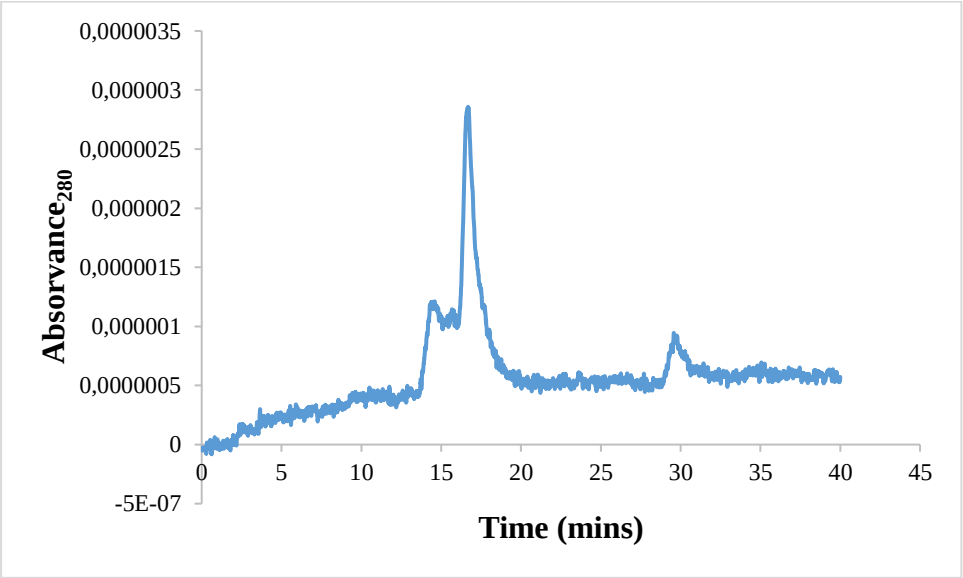

Figure D 5 - SE-HPLC chromatogram of fraction 6 from the ultrafiltration assay.

Appendix E: Calibration curve for ELISA

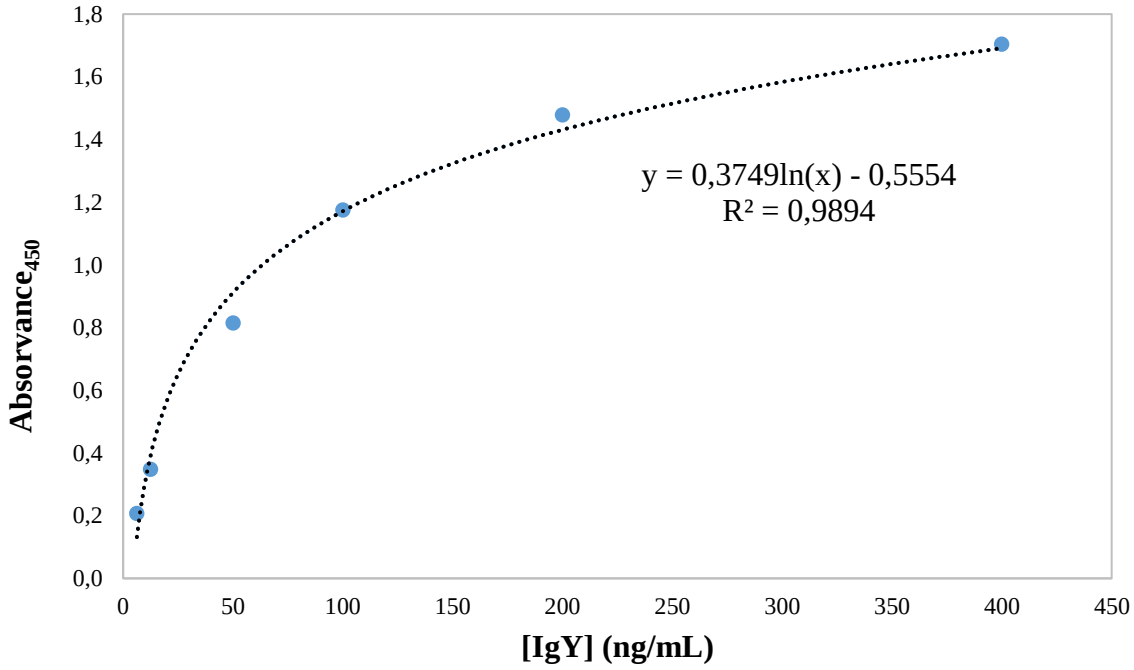

Figure E 1- Calibration curve (absorbance vs concentration of IgY) by UV-spectroscopy.