



Universidade de Aveiro

2015-2016

Departamento de Química

**Leonor Sofia Oliveira Castro** Purificação de anticorpos utilizando sistemas micelares de duas fases aquosas

**Antibodies purification using aqueous micellar two-phase systems**





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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Biotecnologia Molecular, realizada sob a orientação científica da Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora do Departamento de Química, CICECO, da Universidade de Aveiro, e coorientação da Doutora Ana Paula Moura Tavares, Estagiária de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.



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**palavras-chave**

Imunoglobulina Y, purificação, sistemas micelares de duas fases aquosas, Triton X-114, líquidos iônicos

**resumo**

Nos últimos anos, terapias à base de anticorpos mono e policlonais têm atraído muita atenção por parte da indústria farmacêutica levando a um aumento da investigação neste campo. A imunoglobulina G (IgG), um dos anticorpos já aprovados pela *Food and Drug Administration* (FDA) é uma das imunoglobulinas mais abundantes nos mamíferos, cuja obtenção requer o uso de técnicas invasivas. Como alternativa, e devido às semelhanças estruturais com a IgG, surge o uso da imunoglobulina Y (IgY) que se encontra presente na gema de ovo de galinha. Além disso, as quantidades de IgY presente na gema do ovo são bastante altas (100-150 mg/ovo) quando comparadas com a quantidade de IgG no soro (200mg/40mL de sangue). Contudo, o isolamento da IgY a partir da gema de ovo é bastante dispendioso e moroso, uma vez que requer o uso de processos com várias etapas. Tendo em conta estas desvantagens torna-se indispensável o desenvolvimento de um método mais económico e mais biocompatível. Os sistemas micelares de duas fases aquosas, um tipo específico de sistemas de duas fases aquosas que recorrem ao uso de surfactantes, surge como alternativa para a extração, purificação e/ou concentração de (bio)moléculas. Adicionalmente, o uso de líquidos iônicos (LIs) como co-surfactantes pode modificar as propriedades do surfactante e dessa forma conduzir a mudanças na extração das (bio)moléculas. Neste sentido, o objetivo deste trabalho é o desenvolvimento de um novo método para a extração, purificação e concentração da IgY através do uso de sistemas micelares de duas fases aquosas convencionais e mistos com LIs pertencentes à família dos imidazólios e fosfónios a atuar como co-surfactantes. Para tal, foram otimizados parâmetros como a concentração de surfactante e da fração de proteínas solúveis em água, a ausência e presença de (LIs) bem como o efeito da sua concentração e estrutura química. Após toda a otimização, os melhores resultados foram obtidos com o sistema convencional e sistema misto com [C<sub>16</sub>mim]Cl, onde se obtiveram purificações de 51.2 % e 64.5% e rendimentos de 100% e 74.7% respetivamente.



**Keywords**

Immunoglobulin Y, purification, aqueous micellar two-phase systems, Triton X-114, ionic liquids

**Abstract**

In the past few years, therapies based on mono- and polyclonal antibodies have attracted attention from pharmaceutical industries leading to a drastic research increment in this field. Immunoglobulin G (IgG), one of the antibodies already approved by Food and Drug Administration (FDA), is the most abundant mammalian immunoglobulin, whose acquisition requires the use of invasive techniques. As an alternative to the use of mammalian antibodies, Immunoglobulin Y (IgY) from hens' egg yolk emerges due to its structural similarity to IgG. Furthermore, the IgY amount present in egg's yolk can be quite high (100-150 mg/egg) when compared with the IgG amount in the blood serum (200 mg/40 mL of blood). However, IgY isolation from the egg's yolk is quite expensive and time consuming, since it requires multistep processes. Taking these disadvantages into account, the development of a cheaper and more biocompatible technique for IgY extraction and purification becomes mandatory. Aqueous micellar two-phase systems (AMTPS) are a special type of aqueous two-phase systems that comprise micellar solutions of surfactants for (bio)molecules extraction, purification and/or concentration. Additionally, the use of ionic liquids (ILs) as co-surfactants can modify the surfactant properties, leading to changes in the phase separation as well as in the (bio)molecules fractionation. In this sense, this work aims at the development of a new extraction, purification and concentration technique for IgY from egg's yolk using both conventional and mixed AMTPS with tensioactive ILs belonging to two distinct families, namely imidazolium and phosphonium, acting as co-surfactants. Thus, parameters like the surfactant and the water-soluble proteins fraction (WSPF) concentration, the presence/absence of IL as well as the effect of its concentration and structural features were optimized. After the optimization procedure, the best results were obtained with the conventional system and mixed AMTPS with [C<sub>16</sub>mim]Cl, were a purification of 51.2 % and 64.5 % and yields of 100% and 74.7%, respectively, were obtained.



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## List of Abbreviations

[C<sub>10</sub>mim]Cl – 1-decyl-3-methylimidazolium chloride

[C<sub>12</sub>mim]Cl – 1-dodecyl-3-methylimidazolium chloride

[C<sub>14</sub>mim]Cl – 1-methyl-3-tetradecylimidazolium chloride

[C<sub>16</sub>mim]Cl – 1-Hexadecyl-3-methylimidazolium Chloride

[C<sub>18</sub>mim]Cl – 1-Octadecyl-3-methylimidazolium Chloride

[P<sub>6,6,6,14</sub>]Br – trihexyltetradecylphosphonium bromide

[P<sub>6,6,6,14</sub>]Cl – trihexyltetradecylphosphonium chloride

[P<sub>6,6,6,14</sub>]Dec – trihexyltetradecylphosphonium decanoate

[P<sub>6,6,6,14</sub>]TMPP – trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl)phosphinate

AMTPS – Aqueous micellar two-phase systems

ATPS – Aqueous two-phase systems

C<sub>6</sub>H<sub>8</sub>O<sub>7</sub> – Citric acid

CMC – Critical micellar concentration

DAD – Diode array detector

DTT – Dithiothreitol

FDA – Food and Drug and Administration

FT-IR – Fourier transform infrared spectroscopy

IgG – Immunoglobulin G

IgY – Immunoglobulin Y

IL – Ionic liquid

Na<sub>2</sub>HPO<sub>4</sub> – Disodium phosphate

NaH<sub>2</sub>HPO<sub>4</sub> – Sodium dihydrogenphosphate

PEG – Polyethylene glycol

POEAES – Poly(oxyethylene) alkyl ether

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SE-HPLC – Size exclusion - high performance chromatography

TBS – Tris-buffered saline

TCA – Trichloroacetic acid

WSPF – Water soluble protein fraction

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IgY in AMTPS with 1 wt% of Triton X-114; **(e)** Pure IgY in 20 wt% of Triton X-114; **(f)** Pure IgY in AMTPS with 0.3 wt% of [C<sub>14</sub>mim]Cl; **(g)** Pure IgY in AMTPS with 0.3 wt% of [P<sub>8,8,8</sub>]Br.



# **1. General Introduction**



# **1. General Introduction**

## **1.1. Scope and Objectives**

In the past few years, therapies based on mono- and polyclonal antibodies have attracted much attention from pharmaceutical industries leading to a drastic research increment in this field. However, any new compound within the medical field must undergo several steps and clinical trials in order to avoid human casualties, and it starts with the Food and Drug Administration (FDA) approval. So far, several antibody-based therapies have been developed in order to treat different disorders and/or diseases, such as cancer [3,4], transplant rejection [5], auto-immune disorders [6], asthma [7] and infectious diseases [8]. Immunoglobulin G (IgG), one of the antibodies already approved by FDA [9], is the most abundant mammalian immunoglobulin and it is responsible for the transition between innate and cell mediated immune responses [2]. However, to acquire IgG from mammal serum, the use of invasive techniques such as animal bleeding is required, leading sometimes to the animal's death. As an alternative, monoclonal IgG antibody can also be obtained through mammalian culture cells; however, this approach involves high production costs since it needs the acquisition of transfected cells, culture media and laborious procedures [10]. As a substitute for the use of mammalian antibodies, immunoglobulin Y (IgY) from hens' egg yolk plays a promising role. The IgY molecule has a similar structure to IgG and does not require animals suffering, since it is obtained from the egg yolk. Furthermore, the quantities present in the egg yolk can be quite high (100-150 mg/egg) when compared with the amount of IgG in blood serum (200 mg/40 mL of blood) [1]. IgY also displays phylogenetic distance from mammals, and thus, allowing the production of antibodies against highly conserved mammalian proteins. Moreover, the use of hens as the host for IgY production has lower costs when compared to the use of mammals. The recommendations of European Center for Validation of Alternative Methods specifies that, considering animal welfare reasons, IgY acquired from egg's yolk should be used instead of mammalian IgG [11].

Currently, the IgY isolation from egg's yolk is quite expensive and time consuming since it requires multistep processes [12], usually carried out through liquid-liquid extraction using organic solvents, which are hazardous to the environment and non-biocompatible when dealing with proteins [13,14]. Other methods such as precipitation [15], chromatography with the use of affinity ligands [16] or filtration [17] have also been attempted, but are difficult to be used in an industrial scale. In this sense, it is mandatory

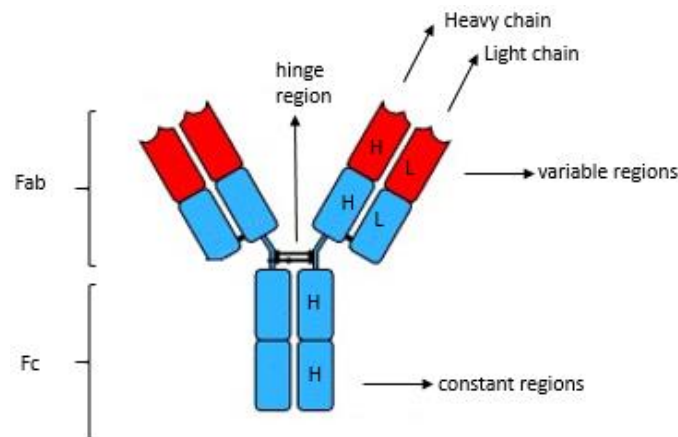
the development of cheaper and more biocompatible techniques for IgY extraction and purification. For this purpose, the use of aqueous two-phase systems (ATPS), a particular type of liquid-liquid extraction, is a promising alternative. ATPS have already been applied in the extraction of several (bio)molecules, such as proteins [18,19], DNA plasmids [20], antibiotics [21], dyes [22], hormones [23] and cells [24] without the significant denaturation, degradation or loss of biological activity. Aqueous micellar two-phase systems (AMTPS) are a special type of ATPS that comprise micellar solutions of surfactants for (bio)molecules extraction, purification and/or concentration [25–28]. Furthermore, surfactant micellar solutions allow the extraction of (bio)molecules with distinct polarities since they offer, simultaneously, both hydrophobic and hydrophilic environments [26]. Changes on surfactant type, temperature and pH can additionally improve the (bio)molecules separation and purification [29–33]. Additionally, the use of ionic liquids (ILs) as co-surfactants can modify the polymer or surfactant properties, which leads to changes in phase separation and (bio)molecules extraction (yields and selectivity) [34,35]. In this sense, this work aims at the development of a new extraction, purification and concentration technique for IgY using both conventional and novel AMTPS. This study will be accomplished by using the non-ionic surfactant Triton X-114 and tensioactive ILs as co-surfactants, those belonging to two distinct families, the imidazolium- and phosphonium-based ones.



## 1.2 State of the art

### 1.2.1. Immunoglobulins structure

Antibodies, also named immunoglobulins (Ig), are glycoproteins secreted by specialized plasma cells, namely the B lymphocytes. Structurally, immunoglobulins are composed of four polypeptides: two heavy chains (H) and two light chains (L), as presented in **Figure 1**. Those chains have two main regions: the N-terminal region also known as the variable (V) region and a C-terminal or constant (C) region, connected by disulfide bonds. Immunoglobulins can suffer proteolytic digestion and originate two separated fragments, the Fab and the Fc, connected by a hinge region rich in proline, threonine, and serine. The Fc fragment is responsible for the effector properties, such as natural killer cells activation, classical complement pathway activation and phagocytosis. The Fab fragment is the one containing the antigen-binding domains [2].



**Figure 1:** General immunoglobulin structure. Adapted from [2].

Antibody functions are dependent on the interactions between antibodies and antigens, though they are conditioned by the complexity and number of antigen's epitopes present [2]. One antigen can be recognized by a large number of lymphocytes T through antigen-presenting cells, such as macrophages, that will consequently activate different plasma cells, thus resulting in a polyclonal response by polyclonal antibodies [2]. If only one B lymphocyte is activated and proliferates, the response will be through monoclonal antibodies with a single specificity. Rabbits, sheep and goats are usually used for the polyclonal antibodies production due to their size. From this group, the most used animal is the rabbit since their maintenance is less expensive. On the other hand, for monoclonal antibodies, mice are the most used species owing to their small size [36]. The mammalian immune system has several clonal lymphocytes populations that exhibit antigen-receptor

specificity. Due to this lymphocyte diversity, the immune system will respond to numerous antigens, corresponding to foreign proteins, carbohydrates, peptides, bacterial and viral components [11]. In this sense, depending on the antigen, different types of immunoglobulins will be produced and will circulate in the blood stream. Mammals produce five different types of immunoglobulins: IgG, IgM, IgA, IgD, and IgE; whereas for avian species only three types of immunoglobulins are found: IgY, IgM, and IgA. The last two immunoglobulins are identical to the mammalian correspondent ones and the IgY is similar to the mammalian IgG; nonetheless, with some differences which are presented in **Table 1** [36]. When considering the host for antibodies production, it is also important to take into account the main goal of the European Center for Validation and Alternative Methods, which requires the reduction or replacement of laboratory animals. In the case of IgG, non-mammalian species can be used for antibodies production without the use of invasive techniques, namely by the use of cells cultures. As aforementioned, the use of hens as an antibody host not only eliminates the use of invasive techniques but also offers a phylogenetic divergence [11]. Due to this phylogenetic distance between birds and mammals, IgY leads to an enhanced immune response in mammals when compared with IgG.

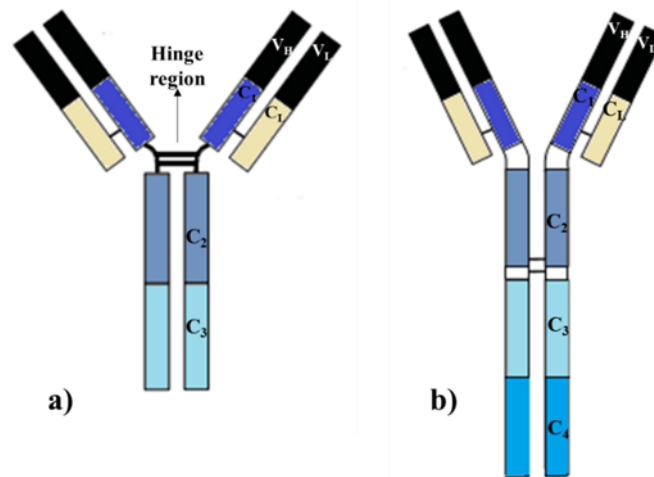
**Table 1:** Differences between the mammalian IgG and IgY. Adapted from [1].

	<i>Rabbits (IgG)</i>	<i>Hens (IgY)</i>
<i>Antibody source</i>	Blood serum	Egg's yolk
<i>Type of antibody</i>	Polyclonal	Polyclonal
<i>Quantities</i>	200 mg/40 mL blood	100-150 mg/egg
<i>Protein A/G binding</i>	Yes	No
<i>Interaction with mammalian Ig G</i>	Yes	No
<i>Activation of complement</i>	Yes	No
<i>Interaction with rheumatoid factor</i>	Yes	No

### 1.2.2. Immunoglobulin Y

The hen immune system is different from the mammalian one; in mammals, the transference of the antibodies occurs after birth [2], whilst in the avian immune system, antibodies are transferred to the egg in order to offer the chick an immune protection [1]. The egg is composed of the shell, albumen and yolk, and the yolk constituents are synthesized in the hens liver and then transferred to the blood, where they are carried to be later transferred to the egg [1]. Amongst distinct avian antibodies, IgY is the most prominent one, presenting concentrations ranging from 5 to 15 mg/mL, while IgA and IgM concentrations are 0.3-0.5 mg/mL and 1-3 mg/mL, respectively [37]. Although egg yolk is a highly complex matrix, it can be separated by high-speed centrifugation into granules and plasma. The granules are composed of 70% of  $\alpha$ - and  $\beta$ - lipovitellins, 60% phosphovitin and 12% of low-density lipoproteins. On the other hand, the plasma is composed of 33% of low-density lipoprotein fraction and 5% of water soluble protein fraction (WSPF) [1].

IgY and IgG, **Figure 2**, are similar, only with some differences in their structure: IgG presents 3 C regions in its H chain, whereas IgY holds 4 C regions, which is then translated in a larger molecular weight (180 kDa) when compared with IgG (150 kDa). The addition of an extra C region also leads to the loss of flexibility in IgY since it loses the hinge region [38]. In relation to pH stability, IgY activity suffers a decline at pH 3.5, however, at pH 3, there is a complete loss of the IgY activity [39]. On the other hand, IgG does not suffer any changes in its activity for values above pH 2 [39]. Furthermore, in relation to temperature, IgY suffers a loss of activity at 70°C and IgG at 75-80°C [39].



**Figure 2:** Structure of IgG (a) and IgY (b) representation of H and L chains, hinge region and C and V domains. Adapted from [1].

Despite the clear advantages of IgY, one of the biggest drawbacks of the IgY large-scale application is its problematic isolation from the complex egg yolk matrix. Usually, the methods employed are divided into two distinct steps: the first one involves the recovery of the WSPF, which is rich in immunoglobulins, among other water-soluble proteins [12]. In this sense, precipitation, dilution and freeze and thaw cycles are usually applied to precipitate the yolk's granules and to acquire the WSPF as the supernatant [12]. Once the WSPF is attained, it is necessary to eliminate the other contaminant proteins in order to collect the pure IgY. Throughout the years, different methods have been investigated to this end [15–17,40,41]. Hatta and co-workers [40] applied natural gums for the precipitation of lipoproteins from egg yolk. For this, a solution with 0.15% of  $\lambda$ -carrageenan was added to the diluted egg yolk and then centrifuged; afterwards, the supernatant was filtered and submitted to three chromatographic cycles with sodium sulphate obtaining IgY at 98% of purity and with a yield of 73%. Kim and Nakai [17] implemented ultrafiltration for IgY purification using diverse membranes with different cut-off values and achieved purification values between 74-99% and yields from 72 to 85%. Moreover, Verodilva and co-workers [16] tested a chromatography column with an affinity ligand for IgY purification and attained a purity around 90% and an yield of 10 mg of IgY/mL of resin. The IgY purification was also pursued using water dilution and salt induced precipitation steps [42]. Zhen and co-workers [42] diluted the egg yolk in water followed by the proteins precipitation using ammonium sulphate and sodium sulphate, followed by an ultrafiltration step using a 100 kDa cut-off membrane. The level

of purity accessed was around 86% after several purification steps [42]. Later on, in 2010, Deignan and co-workers [15] compared different purification methods. For the lipidic fraction removal from the egg yolk, the following approaches were investigated: (i) freeze and thaw cycles at neutral pH, followed by precipitation with 3.5% of polyethylene glycol (PEG) of the diluted egg yolk in a phosphate buffer; (ii) precipitation with dextran sulphate and calcium chloride, and also (iii) precipitation with phosphotungstic acid and magnesium chloride of the diluted egg yolk in a Tris-buffered saline (TBS) solution. All these methods were followed by centrifugation. After the lipids removal, the IgY precipitation was carried out by precipitation with sodium sulphate at pH 6, at two different concentrations, and at 30°C, followed by centrifugation and dialysis against TBS for the removal of sodium sulphate. The same procedure was also attempted with ammonium sulphate. The precipitation of IgY was also carried out with a solution of 12% of PEG with a molecular weight of 8000 followed by centrifugation and dialysis. The purification results for IgY after the lipids removal were around 60% [15]. More recently, Priyanka and co-workers [41] used a PEG 1500/potassium phosphate ATPS and a three-phase partitioning system with *t*-butanol and ammonium sulphate for the extraction and purification of IgY. The results obtained showed higher yields for the ATPS (9.0 mg/mL) than for the three-phase partitioning process (6.0 mg/mL). Considering that these methods usually involve multistep approaches and the use of organic solvents (hazardous for the environment and proteins), it becomes urgent the development of an effective, cheaper and more biocompatible method for the IgY purification.

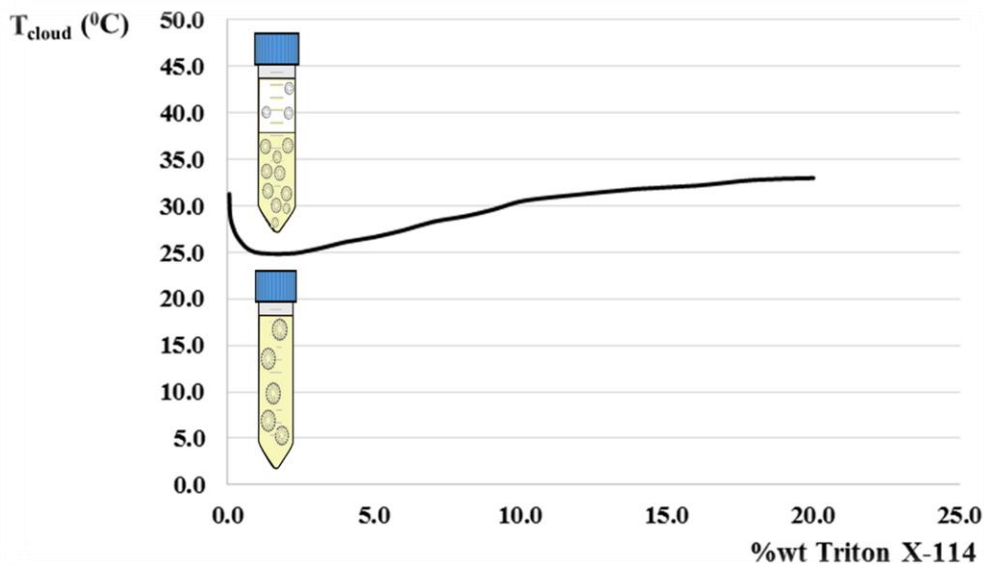
### **1.2.3. Extraction and purification processes using aqueous (micellar) two-phase systems**

Advances in biotechnology have created a number of opportunities for (bio)molecules large-scale production and promoted the development of more efficient, cheaper and sustainable techniques. The use of liquid-liquid extraction systems offers the possibility of combining several steps in only one process, namely the compound's extraction and purification, and involves the transference of components from one phase to the other when immiscible or partially soluble liquid phases are in equilibrium [43]. ATPS comprise liquid-liquid extraction principles and are formed when two different aqueous solutions are mixed above a certain concentration of different components, namely polymer-polymer [44], polymer-salt [19,20,45], IL-salt [46,47], and more recently, polymer-surfactant [48] combinations. These different types of ATPS have already been applied for the recovery and purification of several (bio)molecules, such as proteins [18,19,45,49–51], plasmid DNA [20], antibiotics [21], dyes [22], hormones [23] and cells [24], without the denaturation, degradation or loss of biological activity of the compounds. Human antibodies are another type of biomolecules already extracted using ATPS by combining PEG 3350/dextran [44] and PEG 6000/NaCl [45] pairs. However, to increase the purity levels, additional steps requiring the use of affinity ligands are usually employed [44]. ATPS have other advantages, namely their potential for scale-up and capacity to purify and concentrate various (bio)molecules in a single-step [45]. The (bio)molecules interaction with the system components can be promoted through hydrogen bonding, ionic and hydrophobic interactions, and thus the net effect is different in both phases. This normally allows the preferential migration/partition of the target (bio)molecules to one of the phases [52]. Moreover, the (bio)molecules migration is also influenced by their intrinsic properties, for instance, size, electrochemical properties, surface hydrophobicity/hydrophilicity, conformational characteristics, and extrinsic properties such as the ionic strength, molecular weight, concentration of the phase forming components, pH, and temperature [45,50,53]. Nonetheless, conventional ATPS present some limitations since several of the polymers applied have high viscosities, a limited range of polarities and some can also be quite expensive making it difficult to be employed at an industrial level [54]. In order to overcome these difficulties, Rogers and co-workers [55] reported the possibility of using ILs in the ATPS formation, thus revolutionizing the process for (bio)molecules extraction [23,47,56–61].

ILs are salts composed of a large organic cation and a small organic or inorganic anion, conferring them an asymmetrical ionic structure and higher temperatures of crystallization. As salts, ILs present a negligible vapor pressure and non-flammability, being also characterized by their high chemical and thermal stabilities. However, one of the most interesting properties of ILs is the possibility to combine several anions and cations, which gives them the so called “*designer solvents*” character [62]. Depending on their cation, ILs belonging to phosphonium, ammonium and cholinium families are usually more compatible with (bio)molecules, allowing them to be more adequate for their extraction [47,63]. Compared to imidazolium-based ILs, the phosphonium-based fluids have a greater ability to form ATPS in the presence of the same anions [47,60]. This behavior can be related to the different affinities of both classes of ILs for water [47,60]. (Bio)molecules extraction using ILs as phase-forming components of ATPS is also dependent on the use of different salts, temperatures and pH values [56]. In some cases, lower pH values improve the purification results since the proteins partitioning can be manipulated by changes in their isoelectric points [61]. When applied in ATPS, ILs allow the broadening of polarities and affinities [62]. In addition to the use of ILs to form IL-salt and IL-polymer ATPS, the use of ILs as co-adjuvants (in low quantities) alongside other polymers can improve the (bio)molecules separation, since it causes alterations in the aqueous phase polarities allowing the manipulation of the (bio)molecules extraction [35,59]. Studies have shown that the addition of 5 wt% of  $[C_n\text{mim}]\text{Cl}$ -based ILs increase the two-phase region and the (bio)molecules extraction results following the increment of their hydrophobic nature ( $[C_2\text{mim}]\text{Cl} > [C_4\text{mim}]\text{Cl} > [C_6\text{mim}]\text{Cl}$ ) [35]. However, even with the incorporation of ILs into ATPS, these systems still present some drawbacks, for instance the increased economic impact derived from the use of ILs (*e.g.*, in the previous mentioned study [35] 5 wt% of IL are required in each ATPS). In order to overcome this problem, a step of back extraction is normally added to recover the IL [47]. Furthermore, in some cases the (bio)molecule of interest is extracted in the IL rich-phase, which could lead to biocompatibility issues since the IL can interact negatively with the (bio)molecule. In this sense, AMTPS, a specific type of ATPS, appear as an attractive alternative to these systems since they are only composed of surfactants and water (or a buffer if the control of the system pH is required) as the system components [25–28,64], thus reducing the amount of solvents required for the system preparation. Even when combined with ILs as co-surfactants, significantly lower amounts are employed, ranging from 0.3 to 0.5 wt% [34,65]. Besides, AMTPS are capable of extracting, purifying and even concentrate

several biomolecules, specially proteins, without losses on their activity and native conformation, which is commonly justified by the lack of interactions between the system components and the proteins [66]. In particular, the (bio)molecules extraction, separation and purification is mainly dependent on the surfactants micellar aggregation capacity [66]. Surfactants are amphiphilic molecules formed by a hydrophilic (polar) moiety, known as the head, and a hydrophobic (nonpolar) moiety established as the tail. When dissolved in water above its critical micellar concentration (CMC), surfactants aggregate in a noncovalent way and form micelles in order to minimize the water contact with the hydrophobic moiety [67,68]. Moreover, these systems are dependent of specific temperatures and surfactant concentrations. Usually, the experimental procedure applied to determine the binodal curves is based on the method described by Huang [69]. In this method, a solution with a certain concentration of surfactant is set up in a controlled temperature bath and slowly heated until the solution becomes turbid and suffers phase separation. This specific temperature known as the cloud point or  $T_{\text{cloud}}$ , when plotted with the different surfactant concentrations under study, describe the binodal curve obtained for this specific system, similar to that presented in **Figure 3**. Under an appropriated temperature and surfactant concentration, the system displays one micelle-poor phase and one micelle-rich phase that usually corresponds to the top and bottom phases, respectively. In the particular case of AMTPS of non-ionic surfactants from the poly(oxyethylene) alkyl ether (POEAEs) family, the temperature at which the AMTPS suffers separation depends on the balance between the alkyl chain length and the length of the poly(oxyethylene) chain of the surfactant. For POEAEs with the same alkyl length, the  $T_{\text{cloud}}$  increases with the poly(oxyethylene) alkyl chain length increment [30].





**Figure 3:** Binodal curve for Triton X-114. At temperatures below the  $T_{cloud}$ , the AMTPS only has one homogeneous phase and above the  $T_{cloud}$ , the homogeneous mixture is separated into two phases: one micelle-poor phase and one micelle-rich phase. Adapted from [34].

The  $T_{cloud}$  can be modified by the addition of inorganic salts: if the salt has the ability to promote the *salting-out*, such as chloride- or sulphate-based salts, the system  $T_{cloud}$  is lower; on the other hand, if the salt induces the *salting-in*, e.g. nitrate- or thiocyanate-based salts, the system  $T_{cloud}$  is higher [70,71]. The salt effect on the system  $T_{cloud}$  is related with the hydration of the surfactant poly(oxyethylene) chains. In this sense, *salting-in* inducing salts have weaker electric fields and a smaller hydration shell, which leads to an increment on the concentration of free water molecules that form hydrogen bonds with the poly(oxyethylene) chains. On the other hand, *salting-out* salts possess high electric fields and bind strongly with the water molecules, thus competing with the poly(oxyethylene) chains for the water molecules, which leads to a dehydration of the chains and consequent  $T_{cloud}$  reduction [71]. Moreover, the (bio)molecules extraction is dependent on their properties, so hydrophobic and hydrophilic proteins should migrate to the micelle-rich and micelle-poor phases, respectively, as firstly demonstrated by Bordier [26] and more recently by Vicente *et al.* [34]. The (bio)molecules separation/purification is also dependent on repulsive and steric interactions as well as on the molecules size. If the (bio)molecule molecular weight is higher than the micelle size, then the molecule partitions preferably towards the micelle-poor phase due to the micelles exclusion volume effect [25,27,66]. The exact mechanism for this separation phenomenon is not completely understood but it is possibly associated with competitions between entropic and enthalpic

factors [29]. It should be noted that the phase separation is reversible and can be associated with temperature variations, since below the  $T_{\text{cloud}}$ , the surfactant micelles are solubilized in water due to hydrogen bonds promoted between the solvent and the poly(oxyethylene) chains. With the temperature raising, the hydrogen bonds are reduced, and the aggregation phenomenon starts [30].

In proteins separation/purification, the most common surfactant reported is Triton X-114 [72] due to its relatively low  $T_{\text{cloud}}$ , around 22°C [28,71]. However, other surfactants could also be applied in the proteins purification, namely alkylglucosides [31], zwitterionic surfactants [32], triblock copolymer surfactants [29,73], and mixtures of cationic and anionic surfactants [33]. The mixture of different surfactants allows an adequate manipulation of different factors, namely variations on CMC, micelle shape [29,74], (bio)molecules solvation and extraction abilities [33], among others. Nonetheless, when ionic surfactants are employed, it should be taken into account that, in order to avoid protein denaturation, the amount of ionic surfactant in the system should only be high enough to induce changes in the protein partitioning behavior, but without compromise the proteins conformational structure of activity [29]. Changes in pH also change the (bio)molecules extraction, and those alterations could be related with modifications on the electrical charge of the (bio)molecule, which can affect the repulsive and/or attractive interactions between the (bio)molecules and the surfactant [75], consequently promoting a distinct migration behavior.

Some ILs were found to exhibit surface active properties similar to surfactants, having the ability to form micelles as demonstrated by several studies of small angle neutron scattering analysis (SANS), surface tension, conductivity [76], and steady-state fluorescence measurements [77]. The ILs CMC is dependent on the number of methylene (-CH<sub>2</sub>-) groups present in the aliphatic moiety, and for which ILs with more methylene groups require a lower concentration in order to form micelles [78]. Different anions also influence the ILs' CMC even if the surface-active feature arises from the IL cation: the larger the anion, the lower the CMC is, since ions that are less hydrated will be more easily adsorbed and suffer lesser electrostatic repulsions, leading to an easier aggregation [79]. Furthermore, temperature variations lead to changes in CMC values of ILs in aqueous solutions since it also reduces the hydration of the IL anion, favoring the IL aggregation [80]. Considering the IL ability to self-aggregate, the addition of IL to other surfactants can originate mixed micelles [81–83] with different diameters, CMC, and surface charge [84]. The pioneer work of Vicente *et al.* [34] demonstrated the possibility

of using Triton X-114 and ILs as co-surfactants for the selective extraction of target (bio)molecules, namely cytochrome c and rhodamine 6G. It was demonstrated that the use of different ILs influences the  $T_{\text{cloud}}$  of the AMTPS: imidazolium-based ILs require superior temperatures to create the AMTPS due to their higher hydrophilic nature; on the other hand, phosphonium- and ammonium-based ILs need lower temperatures to promote the same phase separation owing to its more hydrophobic character [34]. Variations on the ILs concentration can also induce changes in the system  $T_{\text{cloud}}$  by pronouncing the previous effects [34]. Additionally, these systems were proven to enhance the extraction performance and to be very selective with a selectivity improvement from 925.25 of conventional AMTPS to 3418.89 for a mixed AMTPS with ILs acting as co-surfactants [34]. The enhanced extraction was also explained by an increment in the surfactant concentration, which increases the system entropy, thus leading to micelles fusion that results in micelles with larger diameters and better separation results [66]. This selectivity can be further explained by hydrophobic, electrostatic and excluded-volume phenomenon's with hydrophilic proteins presenting an higher affinity to the micelle-poor phase but also with smaller (bio)molecules migrating to the micelle-rich phase [34]. Taking into account the nature and characteristics of AMTPS employing ILs as co-surfactants, it seems that they may be an attractive alternative for IgY purification.



## **2.Experimental Section**



## 2. Experimental Section

### 2.1. Materials and reagents

The nonionic surfactant Triton X-114 used in the AMTPS preparation was supplied by Sigma Aldrich/Acros Organics and the McIlvaine buffer components, particularly citric acid ( $C_6H_8O_7$ , purity = 99.5%) and disodium phosphate ( $Na_2HPO_4$ , purity = 99%) were acquired from Panreac and Merck, respectively. For the mixed AMTPS preparation using ILs as co-surfactants, the ILs used, namely 1-decyl-3-methylimidazolium chloride [ $C_{10}mim$ ]Cl (purity > 98 wt%), 1-dodecyl-3-methylimidazolium chloride [ $C_{12}mim$ ]Cl (purity > 98 wt%) and 1-methyl-3-tetradecylimidazolium chloride [ $C_{14}mim$ ]Cl (purity > 98 wt%) were acquired from Iolitec (Ionic Liquid Technologies, Heilbronn, Germany). All the phosphonium-based ILs, namely trihexyltetradecylphosphonium chloride, [ $P_{6,6,6,14}$ ]Cl (purity > 93 wt%); trihexyltetradecylphosphonium bromide, [ $P_{6,6,6,14}$ ]Br (purity > 96.0 wt%); trihexyltetradecylphosphonium decanoate, [ $P_{6,6,6,14}$ ]Dec (purity > 97 wt%); and trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl)phosphinate [ $P_{6,6,6,14}$ ]TMPP (purity > 93.0 wt%) were kindly offered by Cytec. The components of the phosphate buffer used as the mobile phase for Size Exclusion High Performance Liquid Chromatography (SE-HPLC) analysis, namely  $Na_2HPO_4 \cdot 7H_2O$ ,  $NaH_2HPO_4$  and NaCl were attained at Panreac with purities above 98%. The staining solution Coomssie Blue (G-250) was purchase from AMRESCO, whereas the destaining solution components, methanol and acetic acid, were supplied by Fisherchem. The components of the loading and running buffer for the electrophoresis, bromophenol blue, were provided by Merck, Tris base by Pronolab, glycine with 99% of purity by Acros, glycerol by Sigma-Aldrich and dithiothreitol (DTT) by Acros Organics. The gel of electrophoresis used in the SDS-PAGE analysis was purchased from Amersham.

### 2.2. Methods

#### 2.2.1. Preparation of the Water Soluble Protein Fraction

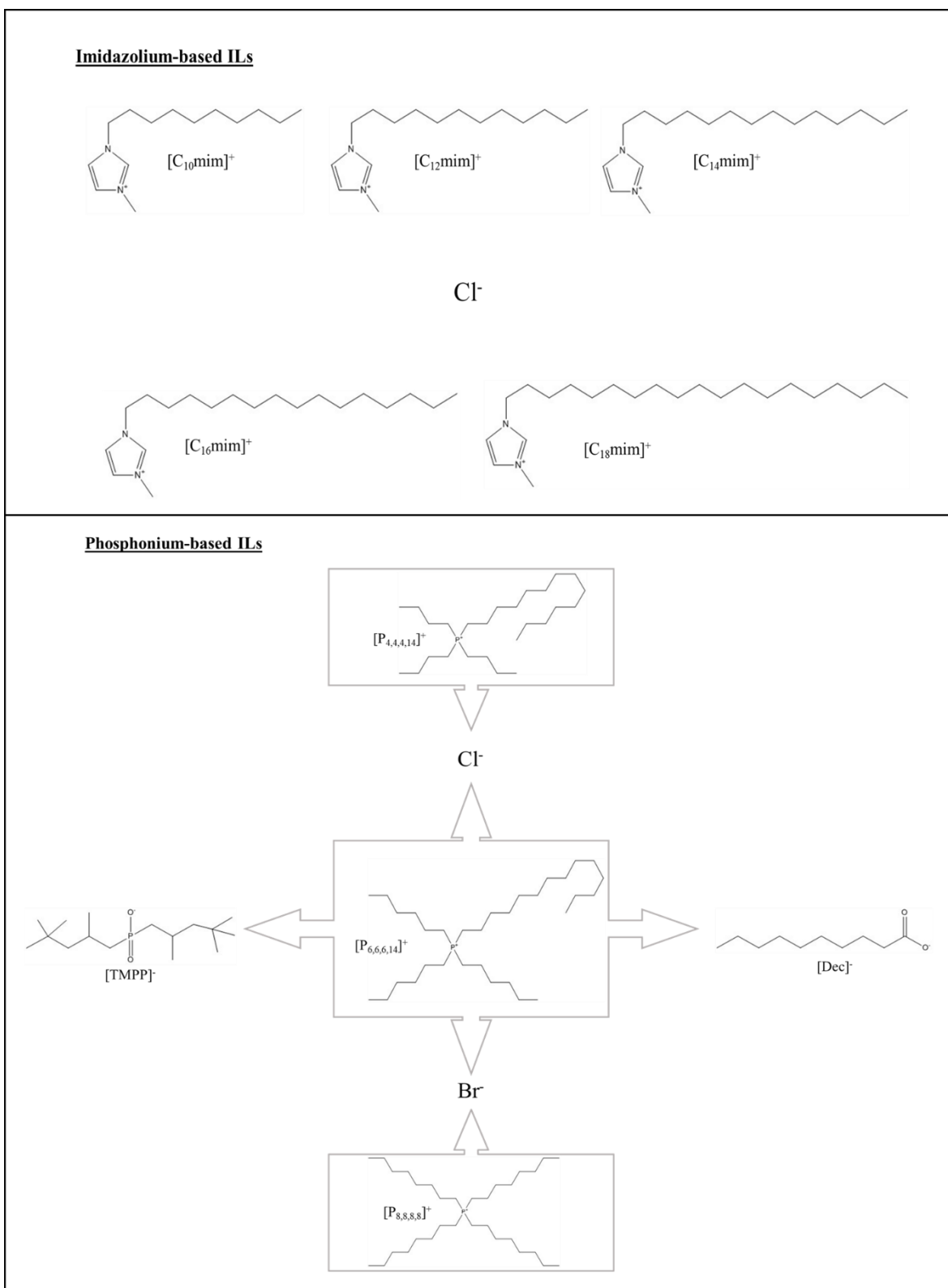
In order to acquire the IgY-rich WSPF from chicken's egg yolk, it was followed the standard experimental protocol reported by Liu *et al.* [85].

#### 2.2.2. Preparation of the AMTPS for the IgY extraction and purification

For the optimization studies of the surfactant and WSPF concentrations, several conventional AMTPS were prepared. Firstly, the surfactant concentration study was

carried out by weighting all the systems components into a falcon tube: 1, 5, 10, 15 or 20 wt % of Triton X-114 + 10 wt% of WSPF + McIlvaine buffer pH 6.0 (184.25 mL of  $C_6H_8O_7 \cdot H_2O$  at 0.1M + 315.75 mL of  $Na_2HPO_4$  at 0.2M) to complete a final volume of 10 mL. Then, the AMTPS were homogenized at 4°C in a freezer for at least 2 hours, using a rotor apparatus Stuart SB3 at 35 rpm. Afterwards, the systems were left at 35°C in a Venticell incubator overnight in order to reach the thermodynamic equilibrium and to guarantee the complete phase separation. The result was a micelle-rich phase (bottom phase) and a micelle-poor phase (top phase). These were carefully separated and their volumes and weights collected. After the proper optimization of the surfactant concentration, this procedure was repeated for the WSPF concentration study using 20 wt % of Triton X-114 + 10, 17.5 or 25 wt % of WSPF + McIlvaine buffer (pH 6.0) to complete the final volume of 10 mL. Finally, the complete optimized system was carried out to study the effect of the IL addition as co-surfactant (**Figure 4**) to the AMTPS: 20 wt % of Triton X-114 + 0.3 or 0.5 wt % of IL + 25 wt % of WSPF + McIlvaine buffer pH 6.0 to complete the final volume of 10 mL. It should be stressed that all studies were performed in triplicate and the respective average and standard deviations were determined. Moreover, when working with mixed AMTPS, an interval of temperatures (35 - 50°C) was studied due to the  $T_{cloud}$  variations imposed by the ILs addition. For ILs belonging to the imidazolium family, the phase separation occurs at 37°C and higher temperatures due to its higher hydrophobicity. Regarding the phosphonium-based ILs, the tests of purification of IgY were carried at 35°C and 37°C.





**Figure 4:** Chemical structure of the anions and cations that composed the imidazolium-based and phosphonium-based ILs applied to the AMTPS.

### 2.2.3. AMTPS preparation for the consecutive IgY purification cycles

After the optimization of the best ILs for the purification of IgY, new consecutive extraction cycles were applied to the resulting top-phase of the mixed AMTPS. In the case of the AMTPS with 0.3 wt% of [C<sub>14</sub>mim]Cl, the system top-phase was applied in consecutive extraction cycles. The new AMTPS composition was constituted by 25 wt% of the AMTPS top-phase, 20 wt% of Triton X-114, 0.3 wt% of IL and the appropriated amount of McIlvaine buffer at pH 6.0 in order to obtain the same concentration of the original AMTPS components, from where the top-phase was attained. A new consecutive cycle was also applied to the micelle-rich phase in an attempt to investigate the possibility of its reuse for a continuous IgY purification using a new WSPF. In this case, an appropriated amount (20.3 wt%) of the micelle-rich phase was added to a new WSPF in order to obtain the second AMTPS components in the same concentration prepared for the first one.

### 2.2.4. Protein quantification of the micelle-poor phase by SE-HPLC analysis

Proteins from both the WSPF and the micelle-poor phase of each AMTPS were quantified through size exclusion high performance liquid chromatography (SE-HPLC) with a size exclusion column Shodex Protein KW-802.5 (8 mm x 300 mm). Initially, the micelle-poor phase was diluted (1:10) in 100 mM of phosphate buffer + NaCl at 0.3 M and pH 7.0 (mobile phase), injected into the HPLC and run isocratically with a flow rate of 0.5 mL.min<sup>-1</sup> at 25°C. The injection volume was 25µL and the wavelength was set at 280 nm using a diode array detector (DAD). The IgY quantification was carried out by an external standard calibration method in the range from 0.1 to 1.0 g/L. The chromatograms acquired from the HPLC were used for the determination of the IgY purity and recovery, which were estimated by Eqs 1 and 2, respectively:

$$\text{IgY purity (\%)} = \frac{\text{IgY area}}{(\text{Contaminat Proteins} + \text{IgY}) \text{ area}} \times 100 \quad (\text{Eq. 1})$$

$$\text{IgY yield (\%)} = \frac{\text{IgY weight}_{\text{Top phase}}}{\text{IgY weight}_{\text{initial}}} \times 100 \quad (\text{Eq. 2})$$

where IgY weight<sub>Top phase</sub> represents: [IgY] in top phase × volume of top phase, and added IgY weight<sub>initial</sub> corresponds to: [IgY] in WSPF × volume of WSPF added. Lastly, it should be noted that in the SE-HPLC analysis, due to the column sensitivity, it was not

always possible to use the same column for every system prepared. Therefore, and in order to minimize the effect of using a new column, a new calibration curve (depicted in **Figure 1** of supporting information) was prepared for each column used. Furthermore, with the column usage the protein separation can be affected so, each set of results were prepared and analyzed at the same time in order to overcome those effects.

### **2.2.5. SDS-PAGE analysis of both micelle-poor and micelle-rich phases**

Considering the impossibility to analyze the AMTPS micelle-rich phase through SE-HPLC due to the surfactant and IL interference with the column and in order to analyze the micelle-rich phase, it was necessary to proceed to the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Thus, the SDS-PAGE analysis allowed the demonstration of the contaminants proteins presence in the micelle-rich phase through the determination of the proteins profile of both phases. In this context, three different methods were evaluated to precipitate the proteins and to allow the removal of all the surfactant and IL used in the AMTPS. The first methodology consisted in adding 500  $\mu\text{L}$  of acetone and 100  $\mu\text{L}$  of a sample from each phase [86]. The second method comprised the addition of 200  $\mu\text{L}$  of 20 % (w/v) of trichloroacetic acid (TCA) in acetone to 100  $\mu\text{L}$  of a sample from each phase [87]. The third method involved 1000  $\mu\text{L}$  of acetone/methanol (8:1) in 100  $\mu\text{L}$  of a sample from each phase [86]. After the precipitation and the discard of the supernatant, the pellet was dissolved in 100  $\mu\text{L}$  of the McIlvaine buffer. All samples were diluted in water so that the amount of protein in each lane was around 0.5  $\mu\text{g}/\text{mL}$ . The samples were then diluted in a dissociation buffer consisting of 2.5 mL of 0.5 M Tris-HCl at pH 6.8, 4.0 mL of 10 % (w/v) of a SDS solution, 2.0 mL of glycerol, 2.0 mg of bromophenol blue and 310 mg of dithiothreitol (DTT), followed by a 5 minutes incubation at 95°C. This step allowed the protein denaturation and the disulfide linkage reduction, thus allowing the breakup of the protein quaternary structure when present in an unfolded state. The electrophoresis was carried out with a polyacrylamide gel (stacking and resolving gels at 4 and 20 %, respectively) from Amersham ECL Gel Box alongside with the running buffer (25 mM Tris, 192 mM glycine and 0.1% of SDS, pH 8.3), which was submitted to a pre-run during 12 minutes at 160 V. Afterwards, the samples and the full-range Amersham rainbow marker (12 to 225 kDa) were loaded into the gel and submitted to a run during 1.30 h at 135 V. Then, the gel was incubated overnight in an IKA KS 4000 ic control orbital shaker with 0.1 % (w/v), 50 % (v/v), 7 % (v/v) and 42.9 % (v/v) of Coomassie Brilliant Blue G-250, methanol, acetic

acid and water, respectively, in order to stain the proteins. The final step was the gel destaining with 7 % (v/v), 20 % (v/v) and 73 % (v/v) of acetic acid, methanol and water, respectively, in the same orbital shaker at 80 rpm during 3-6 hours at room temperature.

#### **2.2.6. IgY stability studies**

In order to study the effect of the surfactant as well as the IL (as co-surfactant) in the IgY stability, the best (conventional AMTPS with 20 wt % of Triton X-114 and the mixed AMTPS with 0.3 wt % of [C<sub>14</sub>mim]Cl) and worst (conventional AMTPS with 1 wt% of Triton X-114 and the mixed AMTPS with 0.3 wt% of [P<sub>8,8,8,8</sub>]Br) AMTPS towards the IgY purification, were analyzed by attenuated total reflection Fourier transform infrared spectroscopy (ATR- FT-IR) and SDS-PAGE. It should be stressed that in this step pure IgY was used, which was previously purified from an egg's yolk by a Pierce Chicken IgY Purification Kit from Thermo Scientific. In both SDS-PAGE and FT-IR analysis only the top-phases were analyzed since the IgY, due to its hydrophilic nature, migrates preferentially towards the micelle-poor (top) layer. Owing to the interferences that protein concentration can display in a SDS-PAGE analysis and considering that AMTPS have the ability to concentrate the IgY, the control system in this case was prepared in the lower concentration that the antibody can be present in the top-phase. The FT-IR analysis were performed in a Perkin Elmer Spectrum Bx spectrophotometer and scanned between [2000-1000] cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> and 64 scans. These conditions were chosen since this is the most important interval when studying proteins owing to the localization of the amide I and II regions for the protein secondary structure determination [88]. Background (water vapour), McIlvaine buffer and each AMTPS blank spectra were also subtracted from all samples prior to data analysis in order to eliminate their interference from the samples containing pure IgY.

## **2. Results and Discussion**

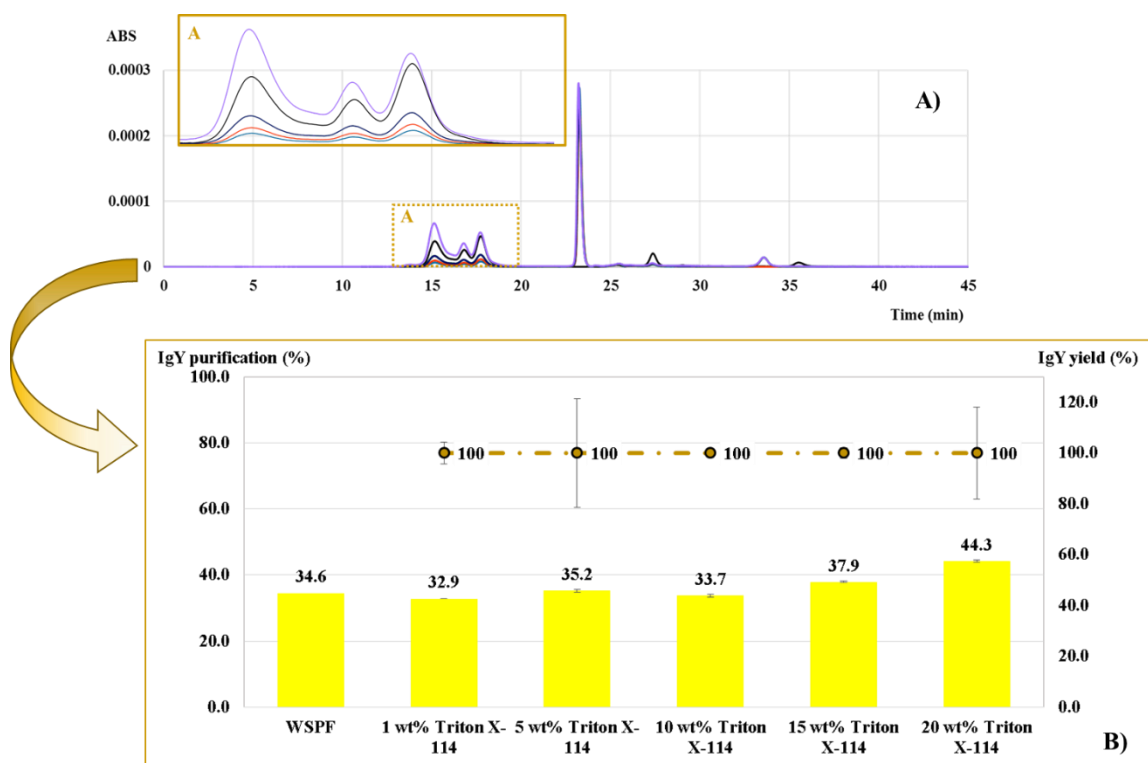


### 3. Results and Discussion

#### 3.1. IgY purification using the conventional and mixed AMTPS with ILs acting as co-surfactants

Considering the aspects mentioned before, the main goal of this work is the development of a new IgY purification method using conventional and mixed AMTPS with ILs acting as co-surfactants. In this context, and considering that the biomolecules separation in these systems is dependent on surfactant concentration and temperature [30,70,71], the first step was the optimization of Triton X-114 and WSPF concentration on the IgY purification. Therefore, several conventional and mixed AMTPS in concentrations of 0.3 and 0.5% of IL were prepared, since it has been reported that the addition of an IL into the AMTPS improved significantly the (bio)molecules selectivity [34]. Furthermore, different ILs families were carefully chosen in order to study the effect of tensioactive ILs' structure, namely the imidazolium-based ILs that possess a more hydrophilic nature and the phosphonium-based ILs with a more hydrophobic character.

As far as the surfactant concentration effect is concerned, the optimization study was evaluated using distinct conventional AMTPS composed of 1, 5, 10, 15 and 20 wt% of Triton X-114 for the IgY extraction and purification, being the results from the micelle-poor phase displayed in **Figures 5.A) and B)**.



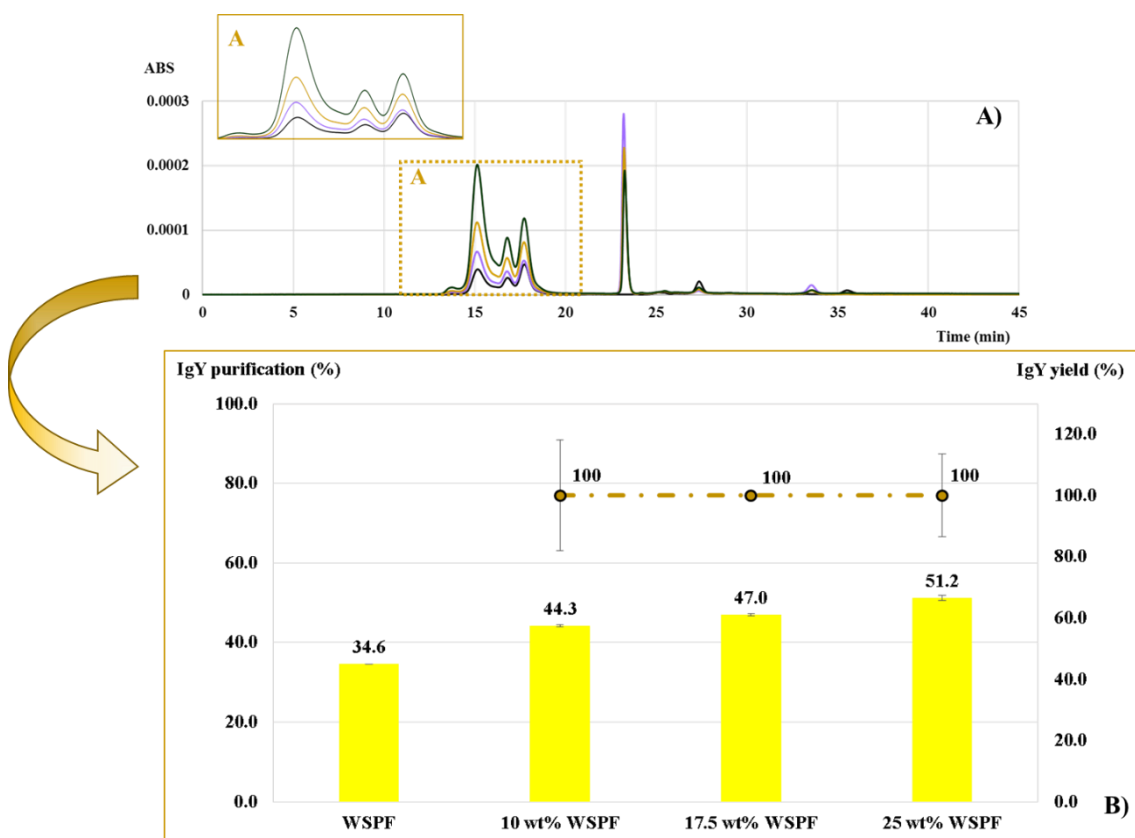
**Figure 5:** A) Chromatograms for the AMTPS top phase at different surfactant concentrations upon phase separation at 35°C: —, WSPF; —, 1 wt% Triton X-114; —, 5 wt% Triton X-114; —, 10 wt% Triton X-114; —, 15 wt% Triton X-114; and —, 20 wt% Triton X-114 and B) IgY purification (bars) and yield (line) in percentage (%), obtained for each AMTPS with different surfactant concentrations, (wt%) of Triton X-114, upon phase separation at 35°C.

In the chromatogram depicted in **Figure 5.A)**, it can be seen the presence of several peaks that correspond to IgY (15 minutes), main contaminant proteins (16, 17 minutes) and other small contaminants (27 and 37 minutes). At 23 minutes it is represented a peak associated with the residual presence of surfactant micelles. The analysis of both **Figures 5.A)** and **B)** demonstrates that with the surfactant concentration increase, the IgY purification from the WSPF was improved from 34.6 to 44.3%, a profile accompanied by the complete recovery of the IgY into the micelle-poor (top) phase. This effect could be related with the fact that an increment in the surfactant concentration seems to increase the size of the micelles (*i.e.* with larger diameters and different shapes [68]), which resulted from the micelles fusion. Consequently, with the increase of the micelles diameter, the system has a greater capacity to concentrate a larger amount of contaminant proteins inside the micelles, which is more pronounced in the bottom phase. On the other hand, and due to the higher molecular weight of IgY, as well as its hydrophilicity, its entrance into the micelles is physically impossible, leading to their separation from the



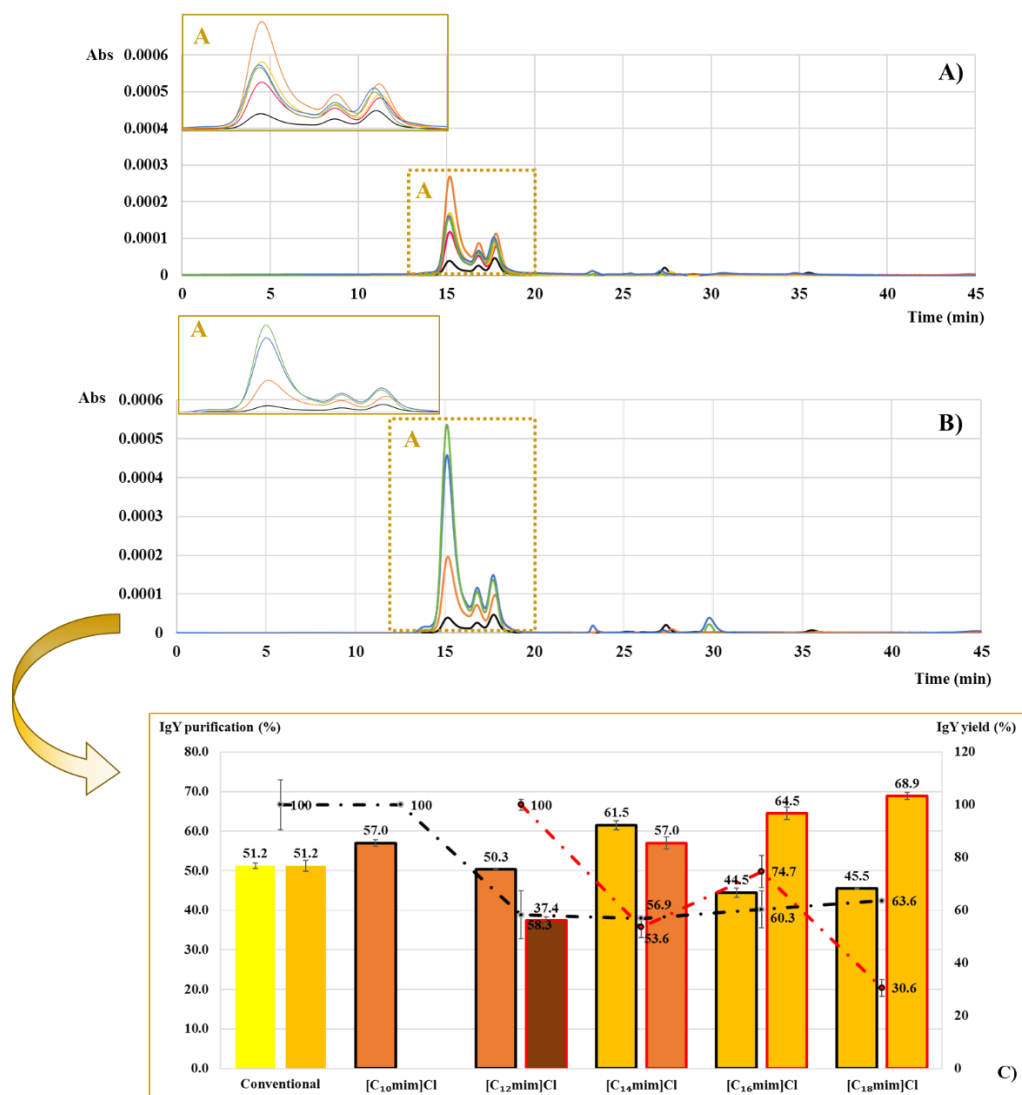
main contaminant proteins. Both phenomenon, micelles size and hydrophilicity of IgY, are thus contributing to a higher IgY purification in the micelle-poor phase. Thereby, molecules with bigger sizes than the size of the micelles formed, will be located into the micelle-poor phase independently of the biomolecules hydrophobicity [25,27,66].

As a second step of optimization, the WSPF concentration was studied on the IgY purification. In this context, several AMTPS composed of 10, 17.5 and 25 wt % of WSPF were performed. The chromatograms and the IgY purification and recovery are presented in **Figures 6.A)** and **B)**, respectively. In this study, it was possible to verify that the increment in the WSPF concentration leads to an increase in the IgY purification since the amount of proteins added to the systems is also higher. These results can also suggest the possibly that the micelle-rich phase has not achieved saturation when a lower WSPF concentration was used. Additionally, it was possible to confirm that the systems do not present any IgY losses, as it can be seen in **Figure 6.B)**.



**Figure 6:** A) Chromatograms for the top phase of the AMTPS at different wt% of WSPF at pH 6 at 35°C for —, WSPF; —, 10 wt% WSPF; —, 17.5 wt% WSPF; and —, 25 wt% WSPF and B) IgY purification (%) (bars) and yield (%) (lines), obtained for each system with different amounts of WSPF (wt%), and 20 wt% of Triton X-114, upon phase separation at 35°C.

Once the optimization study was finished, and aiming at designing a more efficient AMTPS for the purification of IgY, the mixed AMTPS were tested, namely those using ILs from the phosphonium and imidazolium families as co-surfactants, in order to study their potential in the enhancement of the biomolecules selectivity [34]. In this context, several modifications at the level of the alkyl side chain, the anion moiety and the cation core as well as the ILs' concentration were studied. The alkyl side chain effect was evaluated through the imidazolium family,  $[C_n\text{mim}]\text{Cl}$ , by varying  $n$  between 10 and 18 as presented by the chromatograms in **Figure 7.A)** and **B)**, where it can be clearly seen a decrease in the protein contaminant peaks (16 and 17 minutes). This decrease is clearly demonstrated by the improvements of the IgY purification and yield as represented in **Figure 7.C)**. Moreover, the anion influence was studied using the phosphonium family, through the cation  $[P_{6,6,6,14}]^+$  while using  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{Dec}^-$  and  $\text{TMPP}^-$  as the anions conjugated. This family was also used to investigate the cations' symmetry and the alkyl side chain by comparing  $[P_{6,6,6,14}]\text{Br}$  with  $[P_{8,8,8,8}]\text{Br}$  and  $[P_{6,6,6,14}]\text{Cl}$  with  $[P_{4,4,4,14}]\text{Cl}$ , respectively, since variations on the cation can be related to changes in viscosity, density and atom charge [89]. Thus, changes on the cation in the phosphonium family could be translated in different purification results as presented in **Figures 8.A) and B)** and **Figure 9.A) and B)**, respectively. It should be stressed that some of the chromatograms obtained in this study are displayed in **Figure 2** of Supporting Information. Finally, the ILs concentration effect is visible over **Figures 7, 8 and 9**.



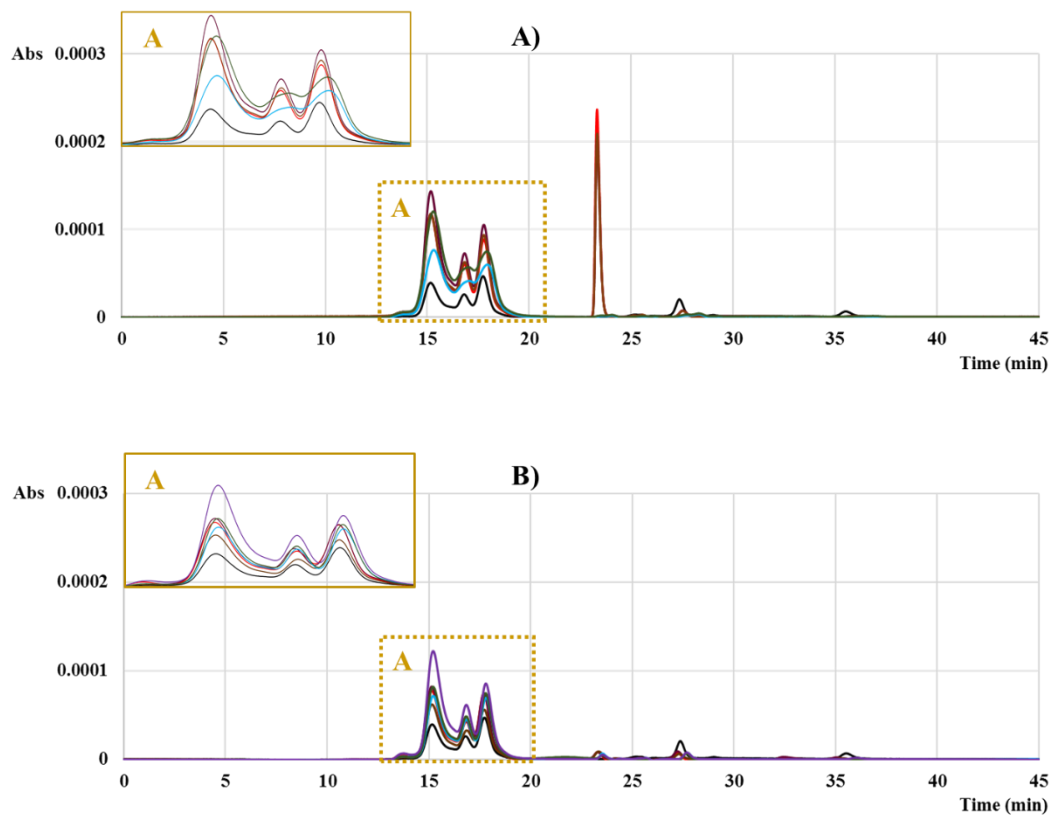
**Figure 7:** A) Chromatograms for the top-phase of the AMTPS with [C<sub>n</sub>mim]Cl-based ILs at A) 0.3 wt% and B) 0.5 wt% at pH 6 for: —, WSPF; —, [C<sub>10</sub>mim]Cl; —, [C<sub>12</sub>mim]Cl; —, [C<sub>14</sub>mim]Cl; —, [C<sub>16</sub>mim]Cl; —, [C<sub>18</sub>mim]Cl. C) IgY purification (%) (bars) and yield (%) (line), obtained for each system with: —, 0.3 or —, 0.5 wt% of the imidazolium-based ILs at the optimized system conditions (20 wt% of Triton X-114 and 25 wt% of WSPF) at: ■, 35°C; ■, 37°C; ■, 40°C; and ■, 50°C.

Considering the results evidenced in **Figures 7.A)** and **B)**, it is possible to verify that the best and worst imidazolium-based AMTPS for the IgY purification are the ones that use [C<sub>18</sub>mim]Cl and [C<sub>12</sub>mim]Cl, respectively, being both developed with 0.5 wt% of IL. The achieved results can be explained by the formation of mixed micelles, since the addition of IL as co-surfactants seems to lead to the incorporation of some IL monomers alongside the Triton X-114 monomers, thus driving the system to exhibit mixed micelles with different diameters and shapes as well as superficial charges [81–84]. However, it is important to notice that the results for the imidazolium-based ILs were not studied at the

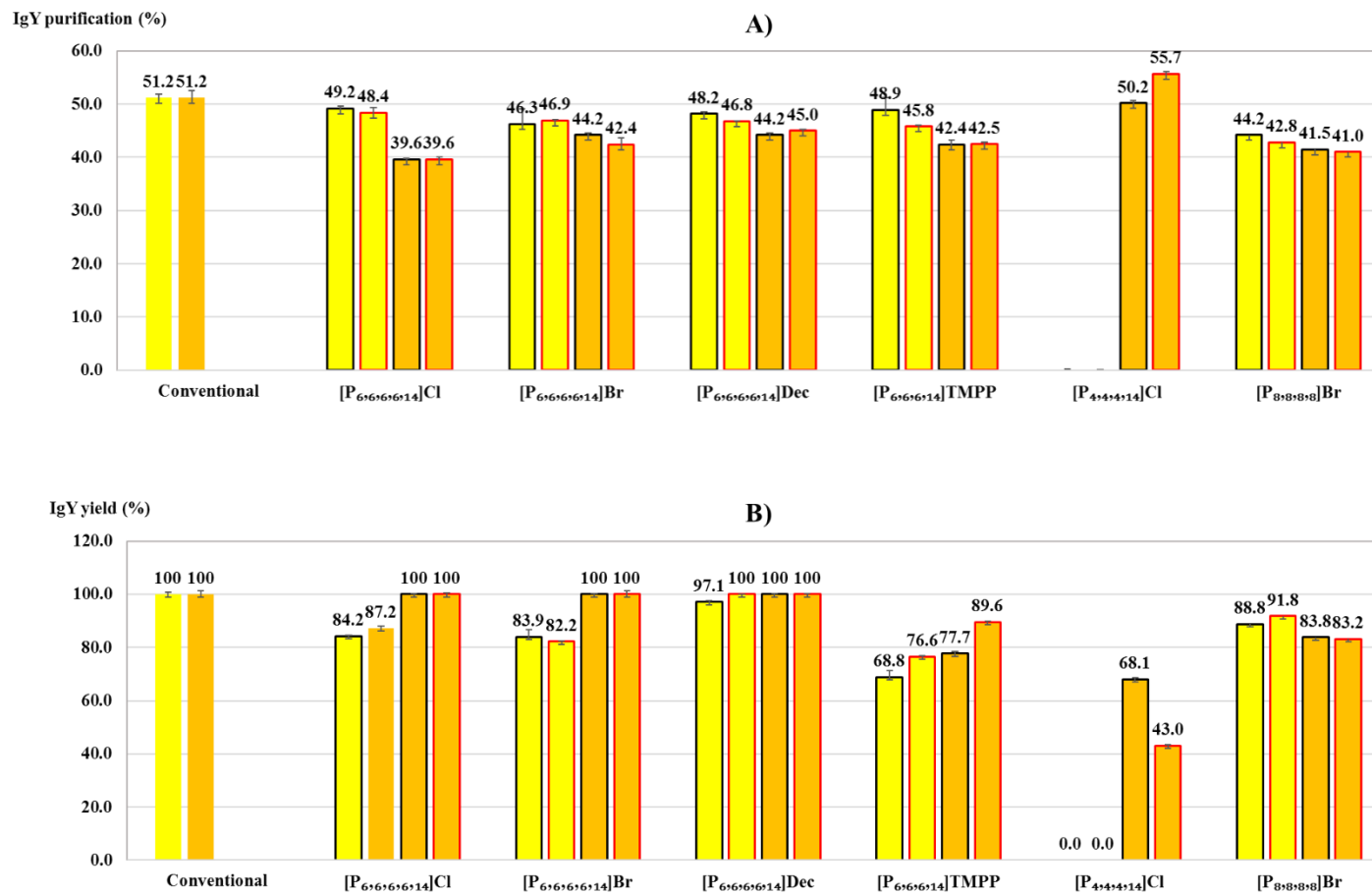
same temperature due to their  $T_{\text{cloud}}$  variation, *i.e.* when the alkyl side chain of the IL increases, so does the system hydrophobicity and thus, lowering the temperature required to promote the phase separation [34]. Therefore, when comparing these results, it was mandatory to have this fact into attention since the AMTPS phase separation is temperature dependent [67]. In this sense, it was impossible to exhibit the data for 0.5 wt% of [C<sub>10</sub>mim]Cl owing to the absence of a phase separation until 50°C. It is also important to note that alterations on temperature can also lead to changes in the CMC since as the temperature increases, the hydration shell of the imidazolium heads decreases, leading to an increased hydrophobicity [79,80]. This hydrophobicity increment can affect the phase separation, phase volume (data not shown) as well as the purification results. In this case, it seems that a temperature increment leads to a decrease in the IgY purification (**Figure 7**). However, the IgY loss should not be associated to the temperature increase since this antibody is stable until 70°C [39]. Furthermore, and according to the results from the IgY yield, it can be seen that the best results are attained for the highest temperatures tested, as 100% of IgY yield was obtained with [C<sub>10</sub>mim]Cl.

Additionally, and taking into consideration the results for [C<sub>16</sub>mim]Cl and [C<sub>18</sub>mim]Cl obtained in the same temperature (**Figure 7**), it was possible to conclude that when the IL's alkyl side chain increased, a better extraction of the contaminants proteins was obtained for the opposite phase of IgY, resulting in the higher antibody purity that is present in the micelle-poor phase. This effect is even more pronounced when the IL concentration increases from 0.3 to 0.5 wt%. Moreover, the increment of the IL's alkyl side chain can lead to an increase in the IL hydrophobicity, which in turn tends to create problems in its dissolution in the surfactant and buffer and also contributes to a decrease in the CMC values [78]. In summary, it is possible to state that the addition of the imidazolium-based ILs as co-surfactant to the AMTPS can improve the IgY purification from 51.2 % to 68.9 %.

Afterwards and as aforementioned, several studies were carried out incorporating the phosphonium based-ILs in the AMTPS for the extraction and purification of IgY and its results can be seen over **Figures 8.A and B**) and **9.A) and B**).



**Figure 8:** Chromatograms for the AMTPS top-phase at (A) 35°C and (B) 37°C with phosphonium-based ILs at 0.3 wt% and pH 6 for: —, WSPF; —, [P<sub>6,6,6,14</sub>]Cl; —, [P<sub>6,6,6,14</sub>]TMMP; —, [P<sub>6,6,6,14</sub>]Br; —, [P<sub>6,6,6,14</sub>]Dec; —, [P<sub>8,8,8,8</sub>]Br; —, [P<sub>4,4,4,14</sub>]Cl.

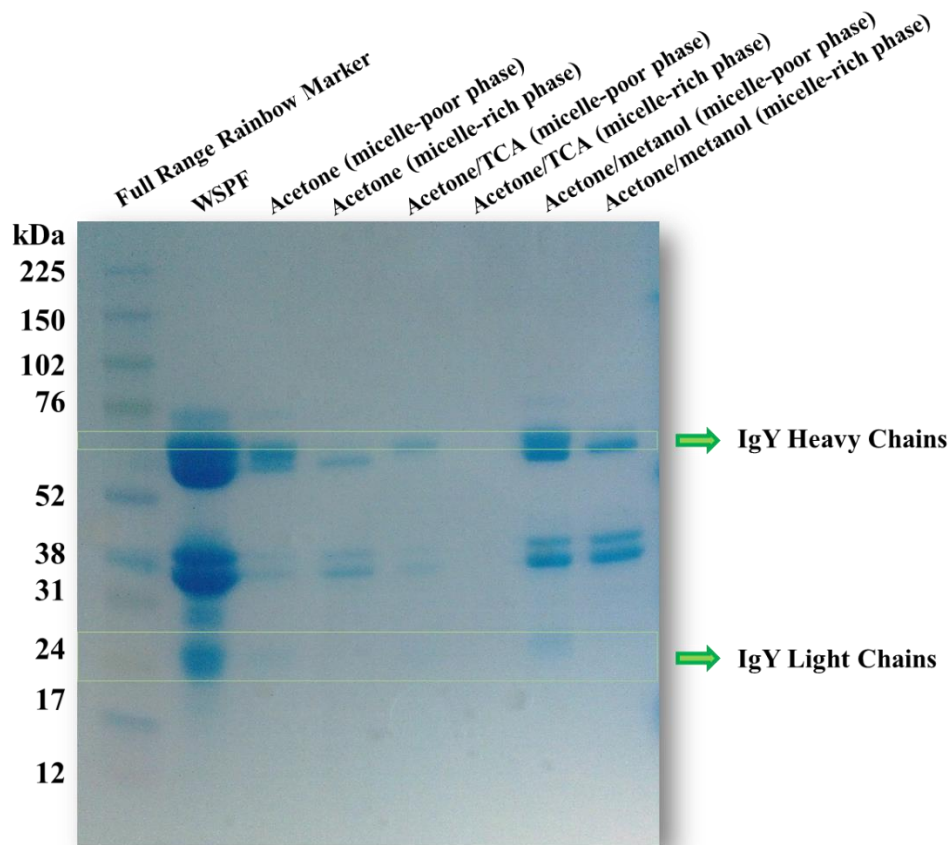


**Figure 9:** IgY purification (bars) and yield (line) in percentage (%) obtained for each system with —, 0.3 and —, 0.5 wt% of phosphonium-based IL at ■, 35°C and ■, 37°C for the same system with 20 wt% of Triton X-114 and 25 wt% of WSPF.

According to **Figure 9**, the addition of phosphonium-based ILs to the AMTPS reduces the IgY purification (results depicted as bars) in comparison with the results obtained for the conventional system. This effect could be related with the more hydrophobic nature of the IL, which can impose difficulties to the extraction of the hydrophilic contaminant proteins, even though these ILs have shown a better phase separation [90] (data not showed). Furthermore, through the results attained for the longer phosphonium-based ILs,  $[P_{6,6,6,14}]^+$ , at both concentrations (0.3 and 0.5 wt%), it was possible to verify that the anion effect does not seem to be significant for the purification of IgY and neither it is for the IL concentration. When the cation effect was concerned, the achieved results at 37°C showed that the IgY purification followed the increasing tendency:  $[P_{4,4,4,14}]^+ > [P_{6,6,6,14}]^+ > [P_{8,8,8,8}]^+$ , whereas the IgY yield presented a decreasing trend:  $[P_{6,6,6,14}]^+ < [P_{8,8,8,8}]^+ < [P_{4,4,4,14}]^+$ . Through the phosphonium family, it was proven once again that the temperature increase leads to a decrease in the IgY purification as well as a variation of the phase separation and phase volume (data not showed) as it was predicted from literature [67]. Considering these results, it was possible to conclude that the phosphonium-based ILs are less effective for the IgY extraction and purification when compared with imidazolium-based ILs, possibly due to hydrophilic/hydrophobicity differences.

### ***3.1.1. SDS-PAGE analysis of micelle-poor and -rich phases for conventional and mixed AMTPS***

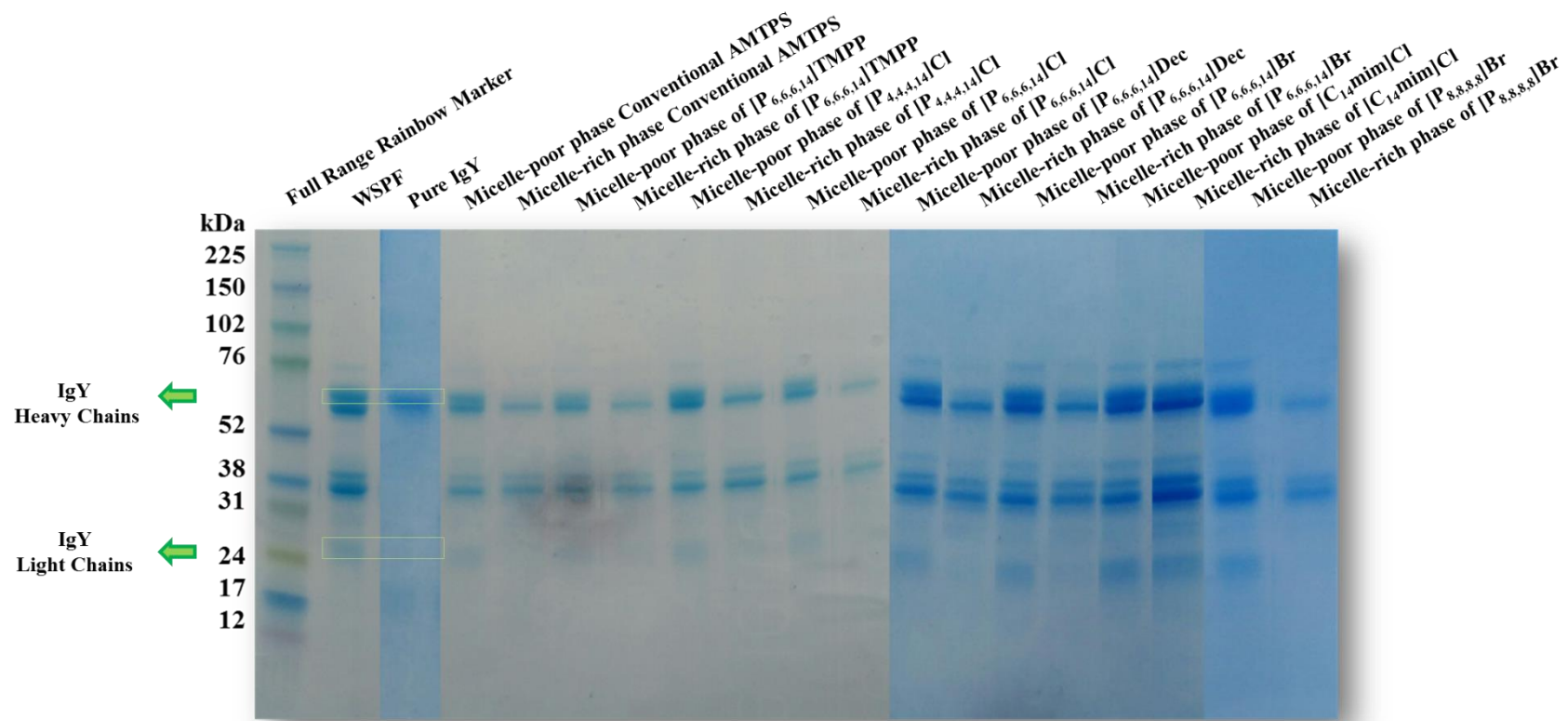
In view of the impossibility to analyze the AMTPS micelle-rich phase through SE-HPLC and in order to investigate the AMTPS capacity to extract the contaminant proteins in the micelle-rich phase, it was necessary to proceed to the use of SDS-PAGE analysis. However, since the surfactant (Triton X-114) and co-surfactant (IL) can interfere with the gel running, it was indispensable the proteins precipitation in both AMTPS phases. For this purpose, three different precipitations methods were tested, acetone, acetone/TCA and acetone/methanol being the results presented in **Figure 10**.



**Figure 10:** SDS-PAGE gel of the supernatant after different protein precipitation methods used in both micelle-poor and micelle-rich phases for the conventional AMTPS with 20 wt% of Triton X-114.

From the gel analysis, it is easily seen that the method showing better results was the one using acetone/methanol (8:1), exhibiting more defined bands for both IgY heavy (65-68 kDa) and IgY light (25 kDa) chains, as well as the contaminants. Furthermore, in the micelle-poor phase, it is not possible to distinguish the bands between the IgY heavy chain band and the contaminant proteins. Bearing in mind these results, the acetone/methanol precipitation method was also applied to the systems with IL as an approach to verify the IgY and the contaminant proteins migration during the purification tests (**Figure 11**).



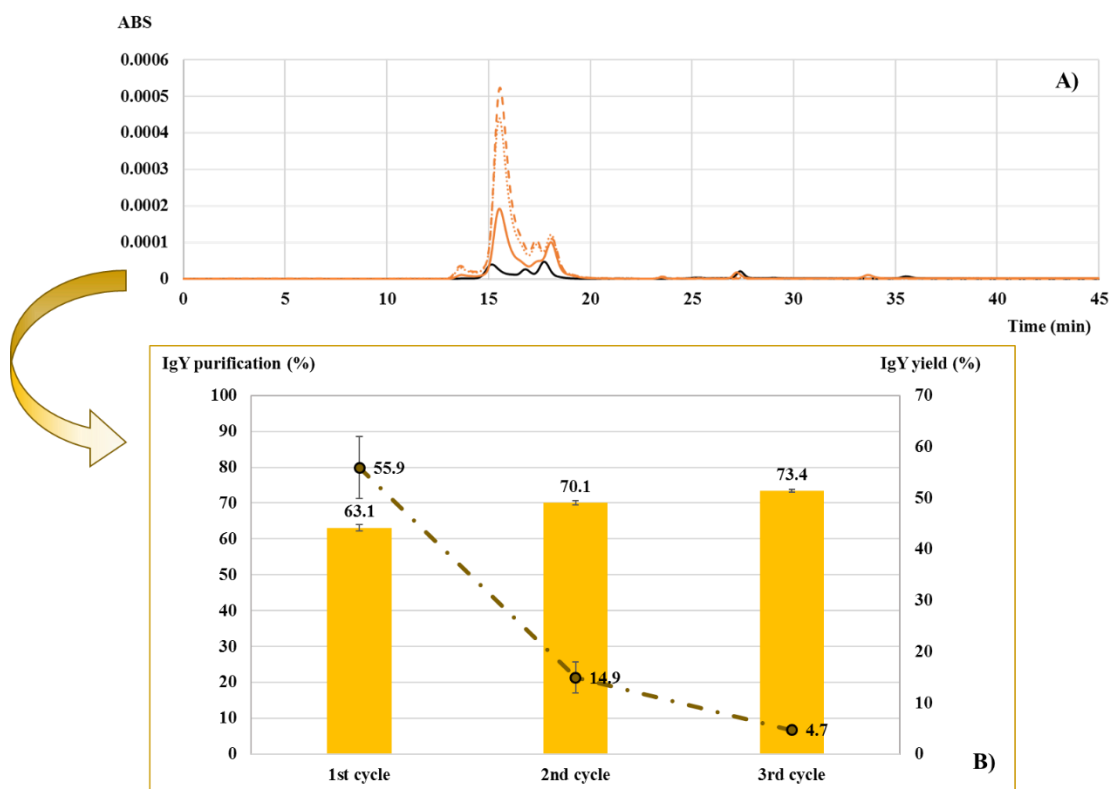


**Figure 11:** SDS-PAGE gel of the resulting from the protein precipitations both micelle-poor and micelle-rich phase of conventional AMTPS with 20 wt% of Triton X-114 and mixed AMTPS with 0.3 wt% IL acting as co-surfactant.

Considering the polyacrylamide gel presented in **Figure 11**, it is verified that for the conventional AMTPS there is some contaminant proteins in the micelle-rich phase, but this extraction was not complete since, in the micelle-poor phase, there are still some bands corresponding to contaminant proteins. From the observation of the lane corresponding to the micelle-poor phase of the conventional AMTPS, it can also be noted the presence of the IgY heavy chain band (65-68 kDa) and the IgY light chain band (25 kDa), however, these bands are not visible in the micelle-rich phase of the conventional AMTPS. Therefore, it can be stated that IgY was completely extracted for the micelle-poor phase as it was expected according to the yield results of 100% as previously shown. The same behavior can be observed for the mixed AMTPS with 0.3 wt% of IL at 37°C: [P<sub>6,6,6,14</sub>]TMPP, [P<sub>6,6,6,14</sub>]Cl, [P<sub>6,6,6,14</sub>]Dec, [P<sub>6,6,6,14</sub>]Br and [P<sub>8,8,8,8</sub>]Br. Focusing on the particular cases of [P<sub>4,4,4,14</sub>]Cl and [C<sub>14</sub>mim]Cl, it can be recognized that these systems can indeed extract a higher number of contaminant proteins. Furthermore, it was also possible to confirm that [P<sub>4,4,4,14</sub>]Cl is one of the best phosphonium-based ILs for the purification of IgY and that [C<sub>14</sub>mim]Cl is the overall system that presents a better IgY purification.

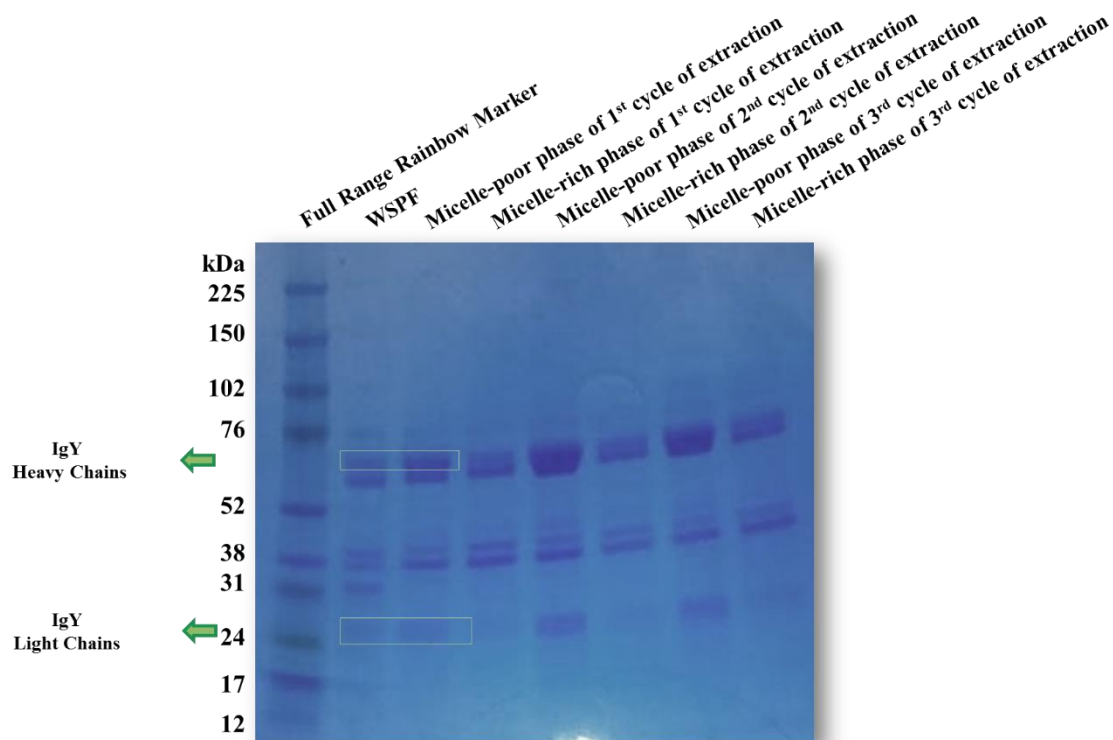
### **3.2. Consecutive extraction cycles using mixed AMTPS for the IgY extraction and purification**

After considering the results achieved in the extraction and purification of IgY using mixed AMTPS, extraction cycles were applied to the top-phases from the mixed AMTPS that exhibited better purification and yield results aiming to improve the purification of IgY as already reported to other compounds [91]. The AMTPS chosen was the one presenting better results during the ILs screening, corresponding to 0.3 wt% of [C<sub>14</sub>mim]Cl. This choice was made taking into account both the IgY purification and yield in order to attain the maximum purity and yield of IgY, being these results presented in **Figures 12.A) and B)**.



**Figure 12:** A) Chromatograms for the top-phase form the extraction cycles with 0.3 wt% of  $[C_{14}mim]Cl$  at pH 6 and  $37^{\circ}C$  for: —, WSPF; - - 1<sup>st</sup> extraction cycle; \*, 2<sup>nd</sup> extraction cycle; - · - ·, 3<sup>rd</sup> extraction cycle and B) IgY purification (%) (bars) and yield (%) (line) obtained for each extraction cycle with 0.3 wt% of  $[C_{14}mim]Cl$ , upon phase separation at  $37^{\circ}C$ .

When analyzing **Figure 12**, it was possible to observe that the addition of new extraction cycles improved the purification of IgY. However, these improvements come with significant IgY losses. Moreover, by comparing the second and third cycles, the second one seems to exhibit a higher efficiency since, in this case, the IgY is more purified than the one obtained for the third cycle besides the fact that IgY losses are much lower. When proceeding to the use of extraction cycles towards the purification of IgY, the same problem regarding the analysis of the micelle-rich phase was faced. Therefore, it became necessary once again the use of SDS-PAGE for its analysis. The results are shown in **Figure 13**.

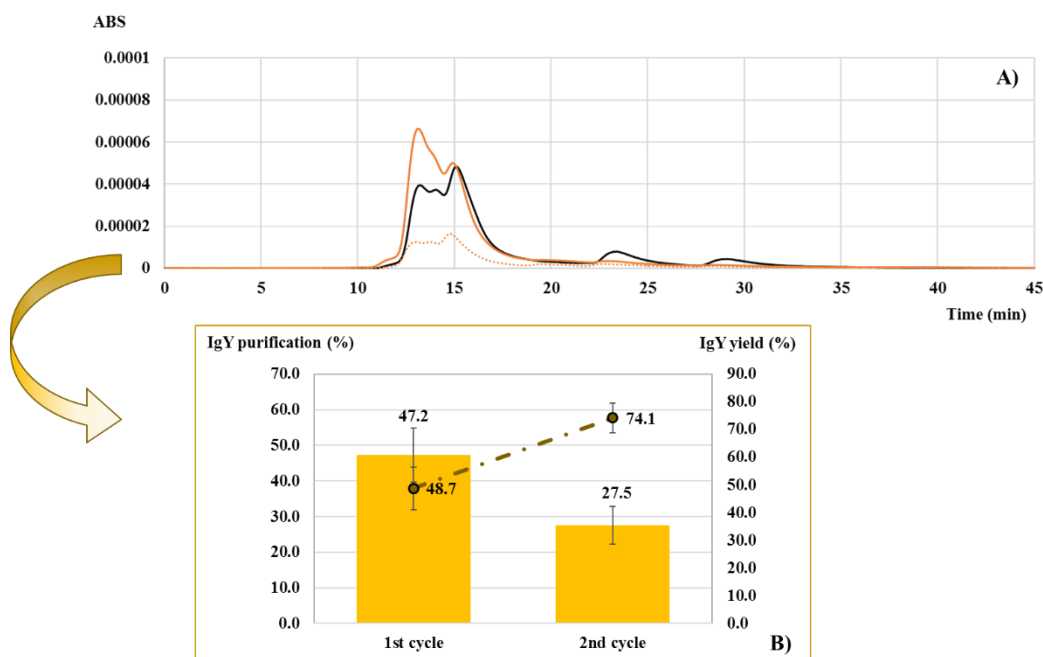


**Figure 13:** SDS-PAGE gel of the resulting from the protein precipitation of the micelle-poor and micelle-rich phase of the different extraction cycles using 0.3 wt% of  $[C_{14}mim]Cl$ .

According to the SDS-PAGE depicted in **Figure 13**, it is concluded that the new extraction cycles are able to extract the contaminant proteins from the WSPF. However, the presence of IgY bands was also observed in the micelle-rich phase of each cycle, which was in accordance with the decreasing values of IgY yield in each new AMTPS. In conclusion, this analysis confirms that the addition of new extraction cycles has the capacity of purify and concentrate the IgY, though the total extraction of contaminants is not achieved.

### 3.3. Consecutive extraction cycles for reuse of micelle-rich phase

Afterwards, the micelle-rich phase was studied considering its potential saturation, and in this sense, the bottom phase was reused for a second cycle of extraction with a new WSPF (**Figure 14**).

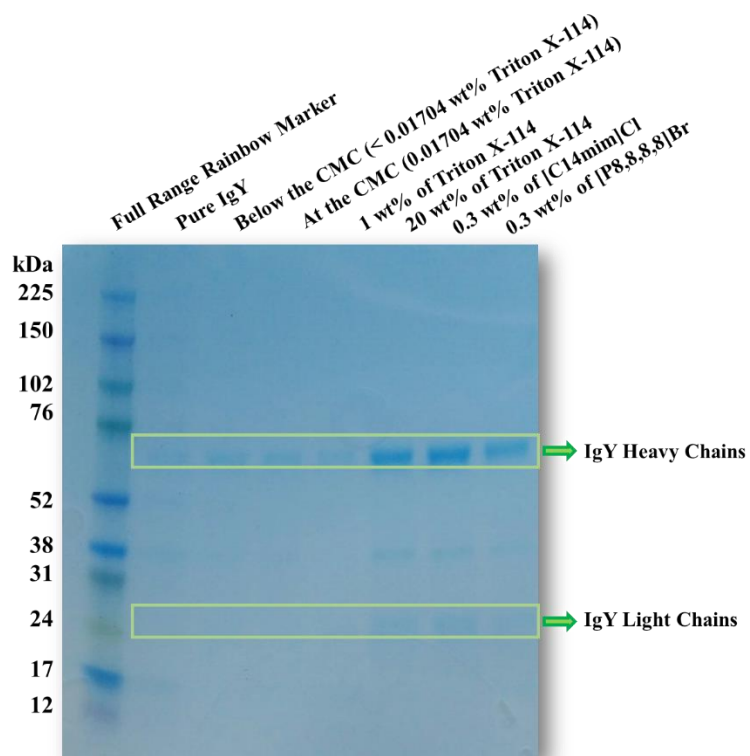


**Figure 14:** A) Chromatograms for the top-phase form the extraction cycles with 20 wt% of Triton X-114 and 0.3 wt% of  $[C_{14}mim]Cl$  at pH 6 and  $37^{\circ}C$  for: —, WSPF; —, 1<sup>st</sup> extraction cycle and \*, 2<sup>nd</sup> extraction cycle and B) IgY purification (%) (bars) and yield (%) (line) obtained for the new extraction cycle with 0.3 wt% of  $[C_{14}mim]Cl$  with micelle-rich phase reutilization, upon phase separation at  $37^{\circ}C$ .

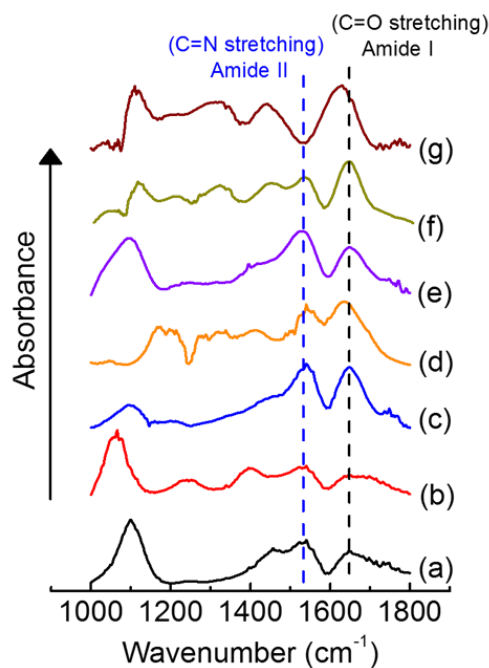
In **Figure 14.A)** and **B)**, it can be seen that the purification of IgY decreased when the micelle-rich phase was reused. Therefore, this study indicates that the micelle-rich phase seems to be saturated, which was translated in its inability to extract more contaminant proteins in presence of a new WSPF.

### 3.4. IgY stability studies

As a way to validate the results acquired for the extraction and purification of IgY, stability studies were conducted to determine if the AMTPS interfere with the molecular structure of IgY. Thus, new conventional AMTPS and mixed AMTPS were prepared with pure IgY in order to assure that any variations in the molecular structure were only associated with the antibody and not with the contaminants. The achieved micelle-poor phases were then analyzed by SDS-PAGE and FI-TR and the antibody stability was evaluated, being its results presented in **Figures 15** and **16**.



**Figure 15:** SDS-PAGE gel of some conventional and mixed AMTPS micelle-poor phases with pure IgY at distinct surfactant concentrations; stained with Coomassie G-250.



**Figure 16:** Infrared spectra of pure IgY in conventional and mixed AMTPS. (a) Pure IgY; (b) IgY in AMTPS below the CMC; (c) Pure IgY in AMTPS in the CMC; (d) Pure IgY in AMTPS with 1 wt% of Triton X-114; (e) Pure IgY in 20 wt% of Triton X-114; (f) Pure IgY in AMTPS with 0.3 wt% of [C<sub>14</sub>mim]Cl; (g) Pure IgY in AMTPS with 0.3 wt% of [P<sub>8,8,8,8</sub>]Br.

From the results presented in **Figure 15**, it is possible to detect that the band corresponding to the IgY heavy chain (65-68 kDa) only suffers a slight alteration for the AMTPS with 0.3 wt% of [P<sub>8,8,8</sub>]Br, which could indicate some change on the antibody molecular structure. There are also changes in the band intensity corresponding to concentration variations, which can be explained by the ability of these specific AMTPS to concentrate this antibody [66]. The next step was the analysis of the same top-phases through FT-IR, (**Figure 16**). From the FT-IR spectra of IgY, two different regions were identified as important for the structural integrity analysis of the secondary structure: *i*) the amide I, which corresponds to stretching vibration of the group C=O, and *ii*) the amide II that corresponds to stretching on C=N group. Variations on this region can be attributed to  $\beta$ -sheet structures ( $\approx 1635\text{ cm}^{-1}$ ), as well as  $\alpha$ -helices ( $\approx 1660\text{ cm}^{-1}$ ), random coil ( $\approx 1645\text{ cm}^{-1}$ ) and  $\beta$ -turn ( $\approx 1670\text{ cm}^{-1}$ ). These regions are usually used to identify several elements from secondary structures of proteins [88]. Considering these amide I and II regions, it can be seen some peak variations for the systems below the CMC (b), with 1 wt% of Triton X-114 (d), and with 20 wt% of Triton X-114 and 0.3 wt% of [P<sub>8,8,8</sub>]Br (g), when compared to the pure IgY in the McIlvaine buffer. These results could be translated in possible changes in the molecular structure of the IgY. When analyzing the AMTPS corresponding to very low amounts of surfactant, for instance the systems below the CMC (b) and at the CMC (c), some alterations on the amide region were also detected. These results could be associated with surfactant interferences in the IgY molecular structure since the systems do not present surfactant micelles formation for the case below the CMC. Overall, these results indicate that, the systems that present more alterations in the amide I and II regions, are the same that exhibit worst purification results: the conventional AMTPS with 1 wt% of surfactant and the mixed AMTPS with 0.3 wt% [P<sub>8,8,8</sub>]Br.

Lastly, it should be stressed that, even though SE-HPLC analysis is more widely used for quantification purposes, it can also be used to investigate the formation of aggregates as well as protein denaturation [92]. In this sense, taking into consideration the findings discussed throughout this work and the absence of any new peaks, it can be corroborated that these systems do not seem to cause the biomolecules denaturation and/or aggregation.

### **3.5. Conventional vs novel methods for antibodies extraction and purification**

Over the years, a considerable number of methods have been applied in the purification of IgY as well as IgG, as presented in **Table 2**. These methods are usually coupled with other techniques in order to improve the purification of the antibody. However, in this work, it is presented for the first time the use of conventional and mixed AMTPS for the purification of IgY from WSPF originated from hens' egg yolk in a single step purification. Nevertheless, in this study, the achieved purification levels are dependent on the AMTPS applied and the number of extraction cycles. The purity values ranged between 51.2 % with a yield of 100% for conventional AMTPS and 64.5% with 74.7% of yield when mixed AMTPS are used. Moreover, the purification results can be improved when consecutive extraction cycles are imposed, reaching purity values of 73.4%. Overall, the application of AMTPS for IgY purification seems to be more advantageous when compared with literature (**Table 2**) because, even though the results can be slightly inferior to the ones reported, the use of AMTPS allows the purification of IgY without requiring the addition of another technique, specially an expensive one. Additionally, AMTPS avoids the use of organic solvents usually applied in conventional liquid-liquid extraction methods, making this method more environmental friendly and biocompatible.



**Table 2:** Comparison of different methods for antibody extraction and purification.

<u>Antibody</u>	<u>Method</u>	<u>Purification</u>	<u>Yield</u>
<b>IgG</b>	From colostrum whey with reverse micelles [93]	90%	90%
	With water solution, salt precipitation and gel chromatography [94]	99%	-
	Membrane based two-stage ultrafiltration process [85]	93%	87%
	Natural gums ( $\lambda$ -carrageenan) and isolation of IgY by chromatography [40]	98%	86%
	Single step chromatography with affinity columns with TG19318 ligand [16]	90%	-
<b>IgY</b>	Lipids removal, followed by different precipitation methods [15]	The best methods had purifications of 60%	-
	Antibody partitioning using PEG 1500/potassium phosphate [41]	With a IgY yield of 9mg/ml of egg yolk	
	Ultrafiltration with several membranes with different cut-offs [17]	74-99%	72-85%
	Preparative electrophoresis [95]	-	80%
	Conventional AMTPS	51.2%	100%
	Mixed AMTPS with ILs as co-surfactant	68.9%	30.6%
	Consecutive extraction cycles with mixed AMTPS	73.4%	4.7%



## **4. Final Remarks**



## **4. Final remarks**

### **4.1. General conclusions and future work**

In this work, the use of AMPTS is reported for the first time regarding the purification of IgY from the WSPF from hens' egg yolk. The application of these systems allowed an improvement of the IgY purification from 34.6% to 51.2%. in the case of conventional AMTPS. Furthermore, the use of mixed AMTPS with imidazolium-based IL as co-surfactants showed an enhanced capacity to increase even more the purification results up to 68.9% with [C<sub>18</sub>mim]Cl. Moreover, these systems seem to be a biocompatible alternative since the IgY molecular stability does not suffer any significant alterations upon phase separation.

In summary, AMTPS seem to be a viable method to IgY purification; however, further research should be made in order to evaluate the antibody activity as well as its recovery from the top-phase. In this sense, dialysis could be a good method to recover IgY in phosphate saline buffer (PBS) instead of the top-phase that could still exhibit some micelles even though in a very low concentration. Furthermore, considering the amounts of surfactant used here and as a way to avoid any environmental impact, the recovery and reuse of the surfactant and IL should be attempted, so the costs of IgY purification could be minimized. In this case, micellar-enhanced ultrafiltration (MEUF) [96] or a foam separation process [97] could be used in order to remove surfactant micelles.



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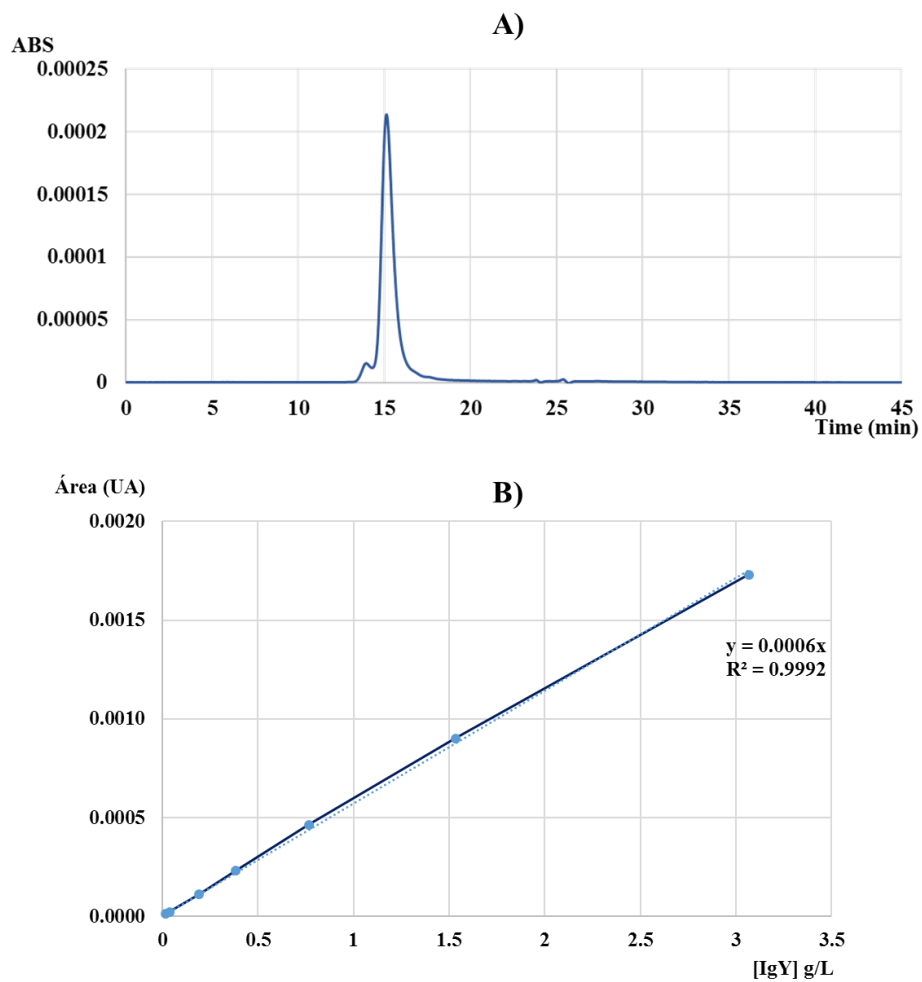
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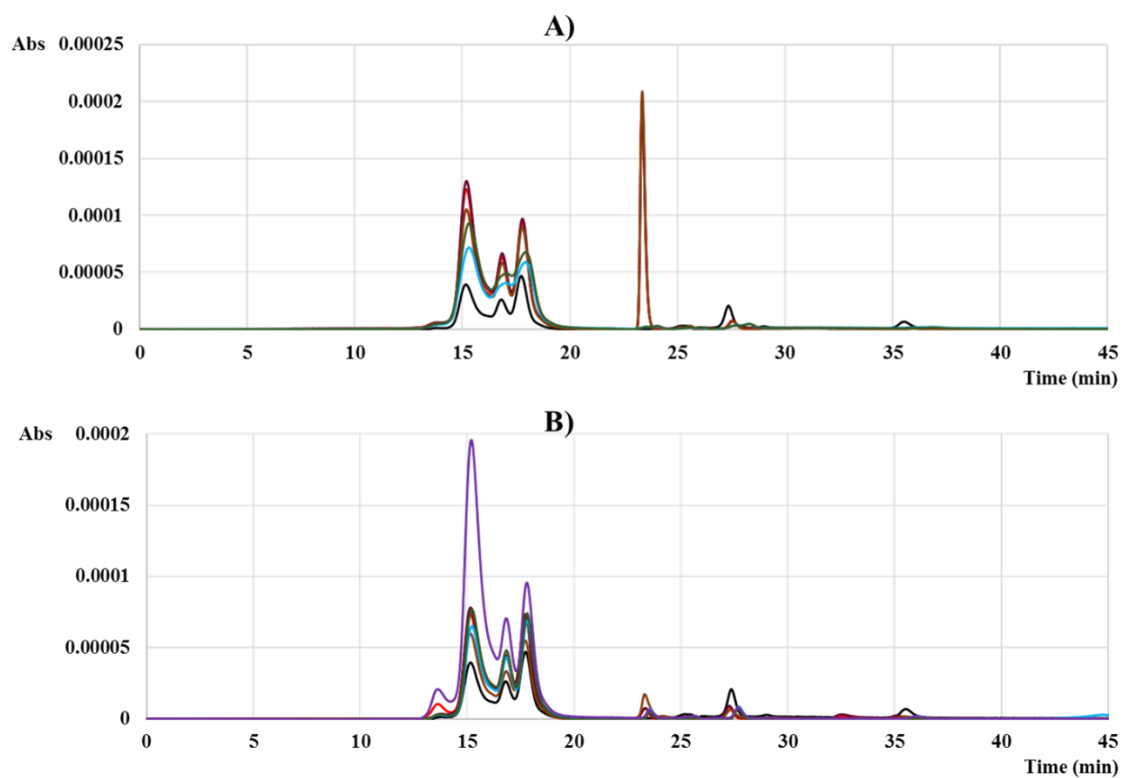


## **6. Supporting Information**





**Figure 1:** SE-HPLC pure IgY control **A)** and calibration curve **B)** for the total IgY concentration at  $\lambda=280\text{nm}$ .



**Figure 2:** Chromatograms for the top-phase of the AMTPS at **a)** 35°C and **b)** 37°C with phosphonium-based ILs at 0.5 wt% and pH 6 for: —, WSPF; —, [P<sub>6,6,6,14</sub>]Cl; —, [P<sub>6,6,6,14</sub>]TMMP; —, [P<sub>6,6,6,14</sub>]Br; —, [P<sub>6,6,6,14</sub>]Dec; —, [P<sub>8,8,8,8</sub>]Br; —, [P<sub>4,4,4,14</sub>]Cl.

