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Desenvolvimento de sistemas micelares de duas fases aquosas para a extração seletiva e purificação de IgG de matrizes reais

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Dr[a]. Mara Guadalupe Freire Martins, Investigadora Coordenadora do Departamento de Química, CICECO, da Universidade de Aveiro, e coorientação da Dr[a]. Sónia Patrícia Marques Ventura, Investigadora Auxiliar do Departamento de Química, CICECO, da Universidade de Aveiro

Dedico esta dissertação aos meus pais, à minha irmã e aos meus amigos por todo o apoio que me deram durante todo o percurso académico.

o júri

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palavras-chave

sistemas micelares de duas fases aquosas, purificação, Triton X-114, líquidos iónicos, anticorpos policlonais, IgG, soro de coelho.

resumo

Os anticorpos, mono e policlonais, têm sido alvo de estudo intenso pelas indústrias farmacêuticas e biotecnológicas, resultando num extraordinário crescimento do mercado de produção de anticorpos. Este crescente interesse deve-se principalmente às vantagens que estas biomoléculas compreendem, tais como a sua aplicação terapêutica e a sua incorporação numa vasta variedade de técnicas de quantificação. A imunoglobulina G (IgG) é um dos anticorpos já aprovados pela *Food and Drug Administration* (FDA) que oferece uma grande variedade de aplicações em diversas áreas e, portanto, reconhecida como uma proteína de valor acrescentado. Apesar da vasta oferta de técnicas de purificação de anticorpos, ainda existem grandes desvantagens associadas à sua purificação. As técnicas atualmente utilizadas centram-se em técnicas cromatográficas, as quais envolvem materiais dispendiosos e longos períodos de operação, sendo responsáveis por 50-80% dos custos totais do produto. Copulativamente, estas técnicas apresentam grandes limitações relativamente à escalabilidade do processo. Como alternativa, este trabalho foca-se na aplicação de sistemas micelares de duas fases aquosas convencionais e mistos, utilizando líquidos iónicos com carácter tensioativo, como uma técnica de purificação não cromatográfica para a extração e purificação de IgG a partir de soro de coelho. Foram otimizados alguns parâmetros associados ao processo de purificação, tais como a concentração de surfactante, a presença/ ausência de líquidos iónicos como co-surfactantes, o efeito da sua concentração e as suas características estruturais. Após o estudo de otimização, os sistemas convencionais foram confinados a uma percentagem de 10% de Triton X-114 a pH 7, alcançando purezas de IgG de 24,6%. Com a aplicação de sistemas micelares mistos de duas fases aquosas compostos por líquidos iónicos obtiveram-se ainda melhores fatores de purificação. Os sistemas que conduziram a melhores purezas foram os sistemas com adição de $[C_{16}mim]Cl$ a 0,7 wt%, $[P_{4,4,4,14}]Cl$ a 1,0 wt% e $[P_{6,6,6,14}]Br$ a 0,7wt%, obtendo-se purezas de IgG de 26,3%, 28,1% e 29,2%, respectivamente. Estes resultados de pureza são superiores aos reportados na literatura relativamente à purificação de IgG a partir do soro do coelho utilizando sistemas de duas fases aquosas, confirmando o potencial desta técnica na extração e purificação de anticorpos ou outros produtos de valor acrescentado de matrizes complexas.

keywords

aqueous micellar two-phase systems, purification, Triton X-114, ionic liquids, polyclonal antibodies, IgG, rabbit serum

abstract

Both mono and polyclonal antibodies have attracted ample consideration from pharmaceutical and biotechnological industries, leading to a remarkable global growth in the antibody production market. This interest is due to their numerous advantages, such as the wide therapeutic window they offer, as well as their applicability in a variety of quantification. Immunoglobulin G (IgG) is one of the antibodies already approved by the Food and Drug Administration (FDA) for a wide range of applications and, therefore, considered a high value protein. Despite the vast offer of purification techniques for antibodies, there are still major drawbacks associated to their purification. The current purification techniques involve chromatographic processes, which comprise expensive materials and time consuming processes, accounting for 50-80% of the total product cost. In addition, these techniques present major scalability limitations. As an alternative, this work focuses on the application of both conventional and mixed aqueous micellar two-phase systems (AMTPS) with tensioactive ionic liquids (ILs) as a non-chromatographic method to purify IgG from rabbit's serum. Parameters such as the surfactant concentration, the presence/absence of IL as co-surfactants, as well as their concentration and structural features, were optimized. After optimization, conventional AMTPS composed of 10 wt% of Triton X-114 at pH 7 are able to lead to an IgG purity of 24.6%. Moreover, regarding the application of mixed AMTPS composed of ILs it was possible to increase the IgG purity. The best systems identified are constituted by $[C_{16}mim]Cl$ at 0.7 wt%, which led to a purification yield of 26.3%, and by $[P_{4,4,4,14}]Cl$ at 1.0 wt% and $[P_{6,6,6,14}]Br$ at 0.7 wt%, with purification yields of 28.1% and 29.2%, respectively. The results obtained exceed the purity levels of IgG from rabbit serum reported in the literature using aqueous two-phase systems, demonstrating the potential of AMTPS as a purification method for IgG and other value-added compounds from complex matrices.

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LIST OF ABBREVIATIONS

AMTPS - aqueous micellar two-phase systems;

ATPS - aqueous two-phase systems;

C - constant region

CMC - critical micelle concentration;

[C_{10}mim]Cl - 1-decyl-3-methylimidazolium chloride;

[C_{12}mim]Cl - 1-dodecyl-3-methylimidazolium chloride;

[C_{14}mim]Cl - 1-methyl-3-tetradecylimidazolium chloride;

[C_{16}mim]Cl - 1-hexadecyl-3-methylimidazolium chloride;

DAD - diode array detector;

CD - circular dichroism

ELISA - enzyme linked immunosorbent assay;

Fab - fragment antigen binding;

Fc - fragment crystallization;

FDA - Food and Drug Administration;

FTIR - Fourier transform infrared;

H - heavy chain;

Ig - immunoglobulin;

IgG- immunoglobulin G;

IL - ionic liquid;

pI - Isoelectric point;

L - light chain;

LLE - liquid-liquid extraction;

MW – molecular weight;

[$P_{6,6,6,14}$]Br - trihexyltetradecylphosphonium bromide;

[$P_{6,6,6,14}$]Cl - trihexyltetradecylphosphonium chloride;

$[P_{6,6,6,14}]$[Dec] - trihexyltetradecylphosphonium decanoate;

$[P_{6,6,6,14}]$[TMPP] - trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl) phosphinate;

$[P_{4,4,4,14}]$Cl - tributyltetradecylphosphonium chloride;

$[P_{8,8,8,8}]$Br - tetraoctylphosphonium bromide;

SE-HPLC - size exclusion performance liquid chromatography;

T_{cloud} - cloud-point;

V – variable region.

1. GENERAL INTRODUCTION

1.1 STATE-OF-THE-ART

1.1.1 Antibodies

Antibodies or immunoglobulins (Ig) are host glycoproteins produced by the immune system of all vertebrate species in response to the exposure to foreign molecules, known as antigens. These foreign substances possess one or multiple small regions called epitopes that can be independently recognized by a small region present in an antibody. In this sense, the function of an antibody is to recognize and bind specifically to a certain epitope, then triggering the neutralization and/or elimination of the foreign material. As a result, an effective immune response takes place, often involving the production of a vast array of antibodies that are structurally similar, yet unique, thus enabling the multiple epitope binding onto an antigen. Primarily, the immune response is elicited by the recognition of the foreign molecule by specialized plasma cells, specifically the B-lymphocytes. These B-cells circulate throughout the blood and lymph streams in search for the antigens. At the recognition instant, these multiple and distinct B-cells are activated and the production of antibodies is initiated [1–3]. The generated heterogeneous population of antibodies, classified as polyclonal, comprise different paratope specificities and affinities allowing the recognition and binding of different epitopes [4,5]. Ultimately, this molecular recognition enables the neutralization and/or elimination of the antigen, permitting the host organism to protect itself against the action of microorganisms, such as bacteria, viruses, fungus or even cancer cells [1,6]. Due to this target specificity and affinity, antibodies are renowned as “magic bullets” and are applied in a vast array of biotechnological and medical applications [5–7].

Independently of their specificity, all immunoglobulins are heterodimer proteins composed of four polypeptides chains: two identical heavy chains (H) and two similar light chains (L). The terms *heavy* and *light* relate to the molecular weight of the chains, being the heavy chain approximately the double of the light one. To be more precise, each H chain has a molecular weight around 50 kDa and the L chain weights around 25 kDa, resulting in an immunoglobulin monomer with a molecular weight of approximately 150 kDa. Moreover, all four polypeptide chains contain variable regions (V), which show considerable variations in their amino-acid composition at the amino terminal region, and constant regions (C) located at the carboxyl terminal region. In fact, each L chain contains one variable domain, VL, and one constant domain, CL. On the other hand, the H chains

include one variable domain, VH, and three constant domains, CH1, CH2 and CH3. The V domain binds to the antigens while the C domain specifies the effector functions [1]. Disulfide bonds are the type of bond that hold the H and L chains together, while also linking the H chains. Structurally, the specific connections between H and L chains establish the hinge region that offers stability and flexibility to the antibody. As a result, the antigen molecule holds a bilateral structure that is often represented by a schematic Y-shaped molecule [1–3], as represented in **Figure 1 (A)**. Furthermore, the molecule can suffer proteolytic digestion originating biologically active antibody fragments that can be used as specific reagents. Depending on the enzyme applied for the proteolytic digestion, different fragments can be originated. In particular, the use of papain produces two fragment antigen binding (Fab) and one fragment crystallization (Fc), as represented in **Figure 1 (B).** On the other hand, the use of pepsin produces one large fragment called $F(ab')_2$ [8,9]. The Fab regions reside at the antibody arms and comprise the antigen-binding domains, which confer the unique binding specificity of an antibody. These domains are encompassed by the V region of both H and L chains, resulting in two identical antigen binding sites. Subsequently, Ig molecules are claimed as bivalent. In the case of the Fc region, it resides in the immunoglobulin tail and owns certain effector functions, including the activation of the complement systems (leading to enhanced phagocytosis), the binding to a wide range of cell-associated receptors (*e.g.* macrophages and monocytes) and placental transfer [1–3].

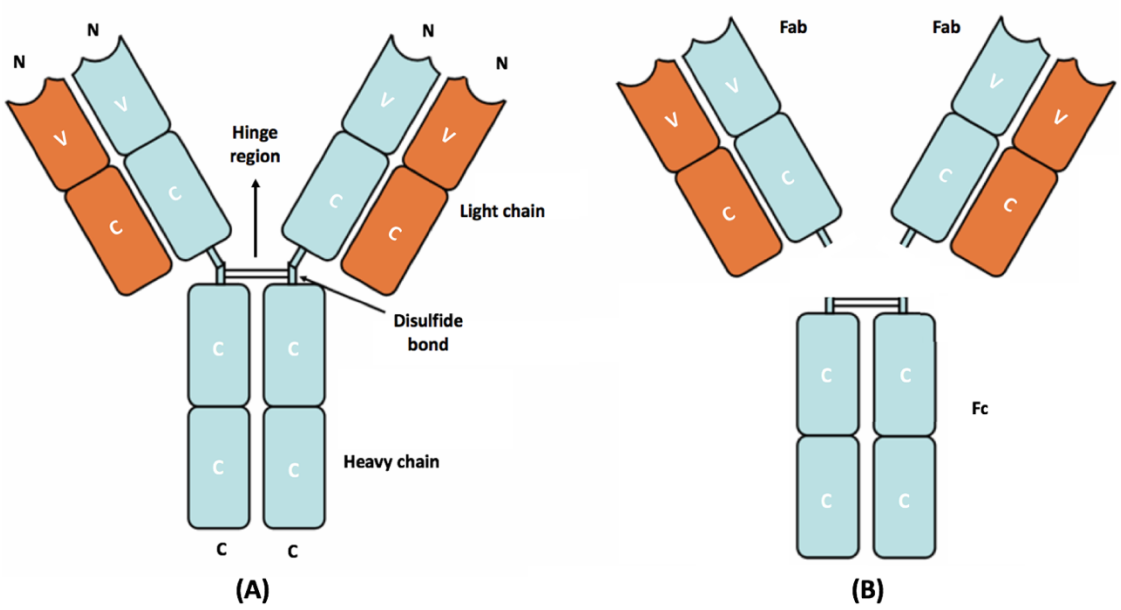


Figure 1 (A): Basic structure of an antibody molecule, consisting in a "Y"-shaped structure composed of two-identical heavy (blue) and light (orange) chains. Each of these chains contains multiple constant (C) and one variable (V) regions linked by disulfide bonds. The antigen-binding domains reside at the tip of the arms on the N-terminal region whereas their effector domains reside in the tail on the C-terminal region. **(B)**: Fab and Fc domains that can be separated from each other by proteolytic digestion through the cleavage of the heavy chain disulfide bonds. Adapted from [10].

In humans and in other mammals, there are two types of light chains (κ and λ) and five types of heavy chains (α, δ, ε, γ, and μ). According to the heavy chain constant domains, immunoglobulins are grouped into five classes: α (IgA), δ (IgD), ε (IgE), γ (IgG), and μ (IgM) as represented in **Figure 2** [8]. Besides, in certain mammals, IgG and IgA are further subdivided into subclasses, referred as isotypes. IgG can be split into 4 subclasses, IgG1, IgG2, IgG3, and IgG4, each with its own biologic properties, and IgA can similarly be split into IgA1 and IgA2 [11]. The most prevalent class of antibodies in humans is IgG and its majority is present in the blood stream. This immunoglobulin comprises approximately 80% of all the immunoglobulins and 15 % of the total proteins in serum, achieving a concentration of 10 to 16 mg/mL [12,13]. This class of antibodies has an isoelectric point (pI) of 6.6, a molecular weight (MW) of approximately 150 kDa and, when intact, has a valence of 2 in the blood (pH 7.35-7.45). Moreover, it is composed of two identical γ heavy chains, with a MW of 53 kDa each, and two light chains, either κ or λ. Furthermore, the four polypeptide chains are covalently held together by disulfide bonds. Due to its relative abundance and excellent specificity toward antigens, IgG is the main antibody applied in immunological research and clinical diagnostics [12,14].

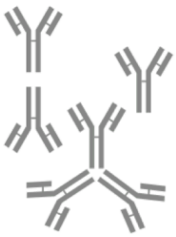
	IgG	IgM	IgA	IgE	IgD
Serum concentration (mg/mL)	12-14	1-2	3-3.5	Trace amounts	Trace amounts
Serum half life (days)	23	5	6	2	3
Heavy chain	γ	μ	α	ε	δ
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Y structure					

Figure 2. Characteristics and structures of mammal's immunoglobulins, adapted from [15].

As mentioned before, antibodies can be characterized as mono or polyclonal depending on the nature of the associated B-cell population. In the case of antibodies present in blood or serum they are classified as polyclonal, since the immunologic response elicits the production of distinct B-cells, and consequently, antibodies that differ in affinity and specificity are produced [4,7]. The mammals serum is considered a valuable source of this class of antibodies, and is currently the preferred and often the only therapeutic choice for selected acute medical emergencies to eliminate complex and poorly characterized mixtures of target antigens [16]. Contrarily, monoclonal antibodies are derived from a single cell line, and consequently all of them are identical. To obtain this class of antibodies, the isolation of myeloma cells is often required to achieve replication and cloning of a single B-lymphocyte cell. This technique allows the generation of identical antibodies with unique structure, affinity and specificity. Overall, the importance of differentiating these two categories is to define the limitations of their use in order to maximize their application. If on one hand, monoclonal antibodies can only interact with a specific and unique substance, thus being extremely efficient for a target antigen, on the other hand, polyclonal antibodies can bind to different substances, and have thus a wider range of applicability as well as lower probability to suffer denaturation. Furthermore, this last class has the ability to increase the signal and provides less sensitiveness to

antigen changes. Polyclonal antibodies are also extremely valuable at circumstances where the nature of the antigen is unknown. Polyclonal antibodies are normally recovered from animal serum, which has been considered a simple, efficient and inexpensive source, although highly invasive [16]. Rabbits are frequently the mammal's choice due to their size and their easy maintenance. Regarding IgG purity in this source, it is important to recognize that serum contains other proteins besides antibodies, like albumin. Consequently, in order to achieve a high quality product for different applications, a purification step is crucial to withdrawn these contaminants. Considering the application of polyclonal antibodies, those obtained from serum are routinely used as ligands for the preparation of immunoaffinity columns and as coating or labeling reagents for qualitative and quantitative determination of molecules [17]. Antibody reagents support traditional immunodetection tools, such as immunoblotting, immunohistochemical analysis, immunoprecipitation, flow cytometry, enzyme-linked immunosorbent assay (ELISA), as well as more advanced proteomic assay platforms, such as, planar or bead-based antibody multiplexing microarrays and antibody-oriented mass-spectrometry technologies [18,19], as summarized in **Table 1**.

Table 1. Application of polyclonal antibodies in the biotechnological and biological fields [18,19].

Application	***Detail***
Western Blot	Single-cell Western Probed isoelectric focusing
Immunohistochemistry	Fixation
Immunocytochemistry	Proximity ligation assay In-cell Western
Flow cytometry	Cell sorting
Immunoprecipitation	Chromatin immunoprecipitation Chromatin immunoprecipitation sequencing RNA immunoprecipitation Cross-linking immunoprecipitation
Immunoassay	ELISA Radioimmunoassay Enzyme immunoassay Immune-polymerase chain reaction
Functional assay	Activation Blocking/ neutralization
Electrophoretic mobility shift assay	
Mass spectrometry	Stable isotope standards and capture by antigen peptide antibodies Immuno-multiple reaction monitoring Immuno-matrix-assisted laser desorption/ionization Mass spectrometric immunoassay

1.1.2 Antibodies' purification: market opportunity and limitations

Antibodies or immunoglobulins represent a large market sector, which is undergoing an impressive global growth in the biopharmaceutics and biotechnology fields, demonstrating their high relevance. This achievement considers the numerous advantages that antibodies comprise, such as their wide therapeutic window (treatment of cancer, autoimmune, multisclerosis and rheumatoid arthritis diseases, and neural disorders) and the high efficacy to identify, localize, quantify and separate intracellular and extracellular proteins. Antibodies are currently one of the fastest growing classes of therapeutic molecules with an estimated global production market of USD 13.28 Billion by 2021 [20]. This estimation is based on the improved approval rate of therapeutic antibodies by regulatory authorities, the increased adoption of targeted immunotherapy and the boosted R&D supported by pharmaceutical and biotechnology companies, diagnostic laboratories and research institutes. Moreover, the upsurge prevalence of infectious and chronic

diseases is also responsible for the escalation demand on protein therapeutics like antibodies [20].

The general process for poly- and monoclonal antibody recovery, such as IgG, involves numerous steps that comprise harvest, clarification, concentration, purification, clearance and validation and quality control [21]. In the past decade, the biotechnology industry has improved substantially their upstream processes and, as an attempt to reduce the manufacturing costs, both poly and monoclonal antibodies have been obtained from animal serum, milk and eggs' yolk, and transgenic plants [7,22]. The downstream processing segment, on the other hand, exhibits the major challenges, since there is not a single process that on its own meets the regulatory standards for the antibody purification in an economical and scalable pathway. The commercially available antibodies are highly expensive, mainly due to the lack of cost-effective purifications techniques, inhibiting thus their widespread application. Indeed, the isolation of the final product requires numerous changeling steps with high energy and material consumption, contributing up to 50–80% of the total product costs [23–25]. The antibody purification can be achieved by a range of methodologies based on the specific physical and chemical properties of antibodies, such as size, solubility, hydrophobicity, charge and binding affinity, as showed in **Table 2**. Currently, numerous techniques and methodologies comprising electrophoretic separations, precipitations, filtrations, liquid-liquid extractions (LLE), ion-exchange and affinity chromatography can and are being applied for the antibody purification [23,26]. These processes can be divided into high resolution techniques, such as the chromatographic methods, and low resolution techniques, such as precipitation and LLE.

Table 2. Purification techniques currently applied in the purification of antibodies based on their physical and chemical properties [27].

Protein property	***Technique***
Charge	Ion exchange
Size	Gel filtration
Hydrophobicity	Hydrophobic interaction, Reversed phase, LLE
Biorecognition (ligand specificity)	Affinity chromatography
Charge, ligand specificity or hydrophobicity	Expended bed adsorption that follows the principles of affinity chromatograph, ion exchange or hydrophobic interactions

Despite the vast offer of techniques, chromatography-based purifications continue to be the most efficient and widely employed technique for the purification of antibodies, as it can result in high quality IgG. The chromatographic methodologies can incorporate different separation techniques, according to the target compound, which include affinity-tag binding, ion-exchange, size-exclusion or immunoaffinity chromatography [28,29]. Over the last few decades, protein A and G affinity chromatography have been the predominant approaches for IgG purification as they offer high selectivity and accurate resolution. These techniques are currently being applied to purify poly and monoclonal IgG antibodies, as well as IgG's subclasses from cell culture supernatants, serum, and ascites fluids [30]. However, and despite their high resolution and accuracy, these methodologies lack in economic viability and scalability, mainly due to the high cost of the columns, the high time consuming cycles, batch processing and pressure drops [24]. As a result, a lot of attention has been devoted to develop alternative methodologies to chromatography that can maximize product concentration and purification, foster the economic and scalable feasibility, while still guaranteeing the biomolecule's activity, structure and purity [14,26]. Consequently, non-chromatographic techniques have been revisited as potential alternative and strategic approaches to overcome the inadequacies referred above. Included in these non-chromatographic methods are the affinity precipitation [31–33], membrane filtration [34], crystallization [35,36], magnetic separation [37,38], reversed micelles [39] and LLE (*e.g.* aqueous two-phase systems - ATPS [12,40–45]).

In 1958, Albertson introduced the concept of (bio)molecules separation by their partition between two liquid aqueous-rich phases, that is a low cost, gentler and biocompatible alternative to chromatographic purification techniques [46]. Overall, ATPS have emerged as an alternative to more traditional LLE techniques able to offer a more biocompatible environment to (bio)molecules [47,48]. Traditional LLE makes use of volatile organic compounds which are chosen due to their immiscibility with the aqueous media, however with major drawbacks associated (high volatility and toxicity, high propensity to denature biomolecules such as proteins, etc.) [49]. These systems are known as ATPS since they consist of two immiscible aqueous-rich phases formed by polymer-polymer [50], polymer-salt [51], salt-salt [52] or surfactant [53,54] mixtures. Above a critical concentration of these components, a spontaneous phase separation takes place and the extraction/separation/purification of high-added value (bio)molecules can be achieved by the manipulation of their affinity to each of the aqueous-rich phases [47,55]. In the last

two decades, these systems, mainly the polymer-polymer- and polymer-inorganic-salt-based ATPS, have been intensively explored and used to separate, concentrate, recover and purify distinct (bio)molecules [44,47,56,57]. However, this type of systems presents several economic and efficiency drawbacks, such as the use of inorganic salts that can lead to changes in the biomolecules structure and bioactivity [58,59], or the use of polymers that lead to ATPS with a low polarity range, high viscosity and high cost [60,61]. The influence of inorganic salts on proteins solubility and stability was firstly report by Hofmeister [62]. Depending on the salt, two phenomena were described in protein-water mixtures, namely a *salting-out* effect, which promotes aggregation and stabilization of proteins, or a *salting-in* phenomenon that results in the destabilization of the proteins conformation [63,64].

Apart from these conventional systems, in 2003, it was reported that mixing ionic liquids (ILs) and inorganic salts in aqueous environments could also lead to ATPS formation. ILs are molten salts with unique properties, and most of them are water-soluble solvents. These systems have shown improved advantages when compared to polymer-based ATPS, such as low viscosity and quick phase separation, and great potential in the design of novel and target-specific separation [65,66] and concentration processes [67,68]. Moreover, these compounds are only constituted by ionic species, usually a large organic cation and an organic or inorganic anion, allowing them to display lower melting temperatures, by general definition below 100 ºC [69]. Based on their cation structure there are several classes of ILs, such as ammonium, cholinium, phosphonium, imidazolium, pyridinium, pyrrolidinium and piperidium. Their ionic character allow them to exhibit unique properties, such as a negligible vapor pressure, large liquid temperature range, high thermal and chemical stabilities, high ionic conductivity and an improved capacity to solvate a wide array of compounds [70,71]. ILs are also considered task-specific solvents with adjustable solubility in water and controllable biocompatibility since their physicochemical properties can be tailored by a proper combination of the anion or cation that composes them [72]. In fact, ILs have received an extensive attention as "*designer solvents*" due to this remarkable aptitude [73], which is highly relevant issue for their application as alternative solvents in different downstream and analytical technologies [67,68,74].

Regarding the reported literature, there have been a lot of efforts to investigate the application of ATPS, aiming at the reduction or even the complete elimination of chromatographic methodologies. Optimistic results have been reported by applying

ATPS prior the application of protein-A and size-exclusion chromatography for the IgG purification [75]. In this case, the system, on its own, allowed to reach a purity of 70%, which accounts for a big share of the total 99.5% purity obtained at the end of the process [75]. As a single purification methodology, there are also numerous reports concerning ATPS, which were able to achieve recovery yields ranging from 88 to 97% and an overall purity of 70%, addressing these systems as considerable valuable alternatives to chromatography [42,76]. Behind the significant purities and recovery yields attained by the application of ATPS, their use is also advantageous since these systems can comprise clarification, concentration and purification in just one step, while combining an easy and reliable scale-up process in a continuous operation mode. It was stated that ATPS-based extractions were shown to be considerably advantageous in terms of process economics for processing high titer cell culture supernatants, allowing a continuous purification and, most importantly, a reduction of the annual operating cost from 14.4 to 8.5 million (US$/Kg) [77]. Considering complex matrixes as sera, recently ATPS composed of bio-based ionic liquids (ILs) and biocompatible polymers were tested, with an IgG recovery yield of 85% of yield and a purity level of 30.4% [12]. Moreover, the application of ATPS composed of polymer and salts using ILs as adjuvants was also reported, leading to IgG purities of 26 % and recovery yields of 46% [45].

1.1.3 Liquid-liquid extraction and purification of biomolecules applying aqueous micellar two phase systems

Aqueous micellar two-phase systems (AMTPS) are a particular type of ATPS of major potential as a bioseparation technique due to their unusual physicochemical properties as a result of the surfactant monomers aggregation [78–80]. Surfactants, also known as surface-active agents, are amphiphilic molecules with both hydrophobic and hydrophilic moieties, namely a polar and/or ionic head connected to a long hydrocarbon tail [81]. At concentrations above the critical micelle concentration (CMC), they form aggregates known as micelles, where in aqueous media the hydrophobic tails flock to the interior, while the hydrophilic heads remain on the outer surface [82–84]. The micelle formation reflects a delicate balance of several intermolecular forces, including van der Waals, electrostatic, and hydrogen-bonding forces [82,83]. Their shape and size can be controlled by varying some processing conditions, such as the surfactant concentration,

temperature, salt type and concentration, and pH [82,85]. In fact, micelles can adopt a variety of shapes, ranging from roughly spherical to ellipsoidal, depending on these factors [81]. In addition, the micelles present in aqueous solution do not necessarily possess an uniform size but, instead, may exhibit a broad size distribution that can also be controlled by varying the solution conditions mentioned above [86]. This aggregation provides a medium, which is entirely different from the surrounding environment, creating the basis for suitable and useful separation, concentration and purification phenomena of several biomolecules [87,88]. Such micellar systems have different technological applications, namely as solubilizing and emulsifying agents [89], flow field regulators [90], nanoreactors for enzymatic reactions [91], and in the separation, concentration and purification of proteins [92,93]. The combination of surfactants and ATPS gives rise to AMTPS. In fact, a wide array of biocompounds, such as viruses [94], DNA [95], proteins [96–98] and antibiotics [12,45] have already been separated, concentrated and/or purified addressing this class of ATPS, due to the remarkable ability that AMTPS own to keep the native conformation and biological activities of target molecules [99].

The whole process of AMTPS resembles the traditional LLE except that, the ‘‘organic’’ phase is generated within the aqueous phase, converting a previously homogeneous solution into a heterogeneous one by simply changing temperature and pressure [87,100]. The phase separation provides a unique environment for effective bioseparations [87] since both phases created, one micelle-rich and one micelle-poor phases, present important physicochemical differences between them [99,101]. The types of surfactants employed can be either ionic (cationic and anionic) or non-ionic [84,102]. Actually, an aqueous solution of the non-ionic surfactant octylphenol ethoxylate (Triton X-114), for example, undergoes macroscopic phase separation upon increasing the temperature, known as the cloud-point of the surfactant (T_{cloud}), resulting in a bottom micelle-rich phase, and in a top micelle-poor phase [101,103,104]. Several studies have shown that such phase separation results from the competition between entropy and enthalpy phenomena, favoring the micelles miscibility and separation, respectively [105]. Moreover, their interplay is modulated by temperature and also by the type of surfactant [106,107]. The system turbidity and phase-separation procedure is reversible and the re-establishment of the initial solution conditions drives the micelles to merge with the aqueous phase, reproducing a homogeneous system [108]. A schematic representation of an AMTPS is shown in **Figure 3**.

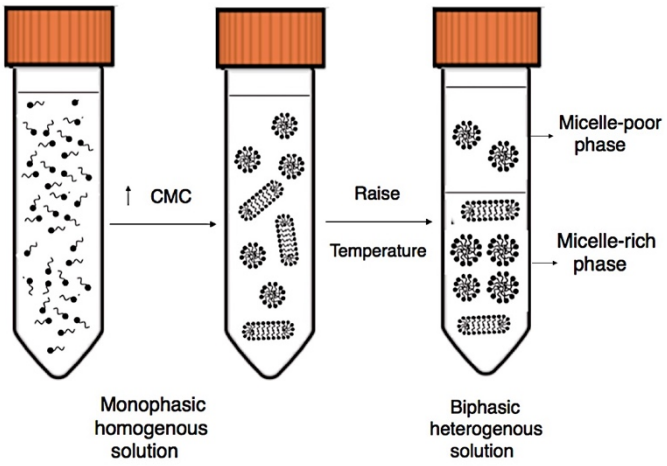


Figure 3. Schematic illustration of a particular AMTPS composed of Triton X-114, in which the concentration of surfactant is higher than the CMC to allow the two phase generation upon the temperature raise.

The phase separation phenomenon induced by raising temperature can be evaluated by establishing the respective binodal curves, *i.e.* by plotting the temperature of separation *versus* the surfactant concentration. **Figure 4** shows a concave-upperward curve typical of a lower critical point AMPTS, composed of a non-ionic surfactant (Triton X-114) and the McILvaine buffer at pH 7. In **Figure 4** it is represented the coexistence curve, *i.e.* the boundary curve separating the temperature and surfactant concentration conditions at which a homogeneous micellar solution (below the curve) separates into two macroscopic phases (above the curve). The minimum of the binodal curve is referred to as a lower critical point, characterized by a critical temperature, T_{cloud}, and a critical surfactant concentration [109]. It is noteworthy to mention that some micellar solutions undergo phase separation upon decreasing temperature. In this case, the binodal curve is concave-downward, and the system is characterized by an upper critical point. The non-ionic surfactant-based AMTPS have shown a major economic potential due to their reasonable low T_{cloud} values [110], contrarily to those obtained for ionic surfactants which present higher T_{cloud} values as a consequence of the high micellar repulsion conferred by the charged heads of the surfactant [111].

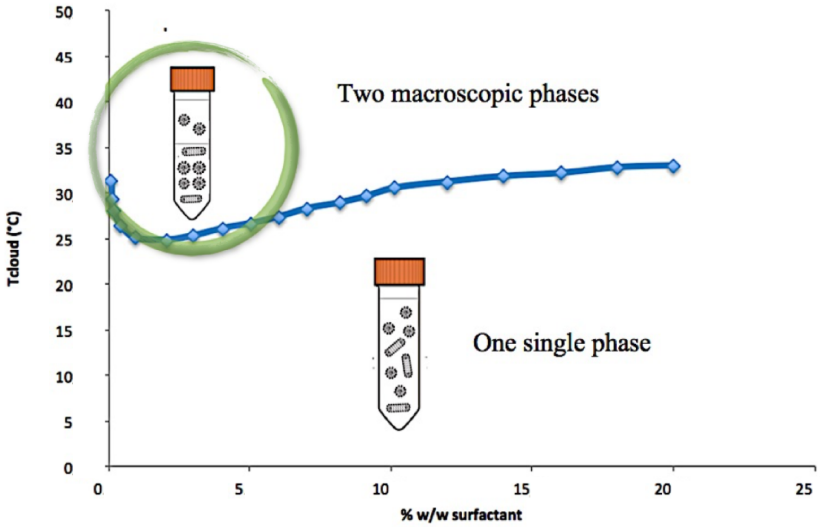


Figure 4. Binodal curve of Triton X-114 + McILvaine buffer at pH 7 adapted from the literature [109]. Above the binodal curve (*e.g.* the area inside the green circle) the system displays two macroscopic phases, while below/ the binodal curve only a single phase is generated.

The aggregation properties of AMTPS can be modified, not only by changing the temperature and pressure but also, by the addition of some additives, namely co- and anti-solvents, co-surfactants, electrolytes, among others [78,112–115]. Recently, different types of ILs have been successfully employed to modulate the aggregation properties of surfactant systems, in which *Bowers et al.* [116] described the possibility of long alkyl chain ILs to self-aggregate and to confer different properties to conventional AMTPS. This ability to act as a co-surfactant can contribute to the modification of the physicochemical properties of micelles originated by the common surfactants, affecting not only their aggregation number but also their CMC values. This phenomenon results from the stronger electrostatic interactions between these salts and water molecules in comparison to the hydrogen bonds between the surfactant polar heads and the water molecules. Such behavior, known as the "*salting-out*" effect [117] is responsible for a decrease in the cloud point. A particular study has demonstrated the large implications of the ILs hydrophobicity on the impact of phase formation[109], in which two main tendencies were observed (**Figure 5**): imidazolium-based ILs produce an increase in the T_{cloud} whereas phosphonium- and quaternary ammonium-based ILs induce significant reductions in the T_{cloud}. This is a result of the IL hydrophobicity/hydrophilicity, *i.e.* the first group of compounds present a more hydrophilic character when compared to the phosphonium and ammonium families. Therefore, even though imidazolium-based ILs

are quite hydrophobic, they still confer some hydrophilicity to the medium due to their large affinity for water and ability to form hydration shells. As a result, higher energy is required to promote the phase separation [118,119]. Contrarily, the second group of ILs is much more hydrophobic owing to their long alkyl side chains and thus conferring a more pronounced hydrophobic environment, enhancing the ability to undergo phase separation at lower temperatures [84,120]. As a result, they are a more interesting group of co-surfactants from both operational and economic points of view for (bio)separation and concentration processes.

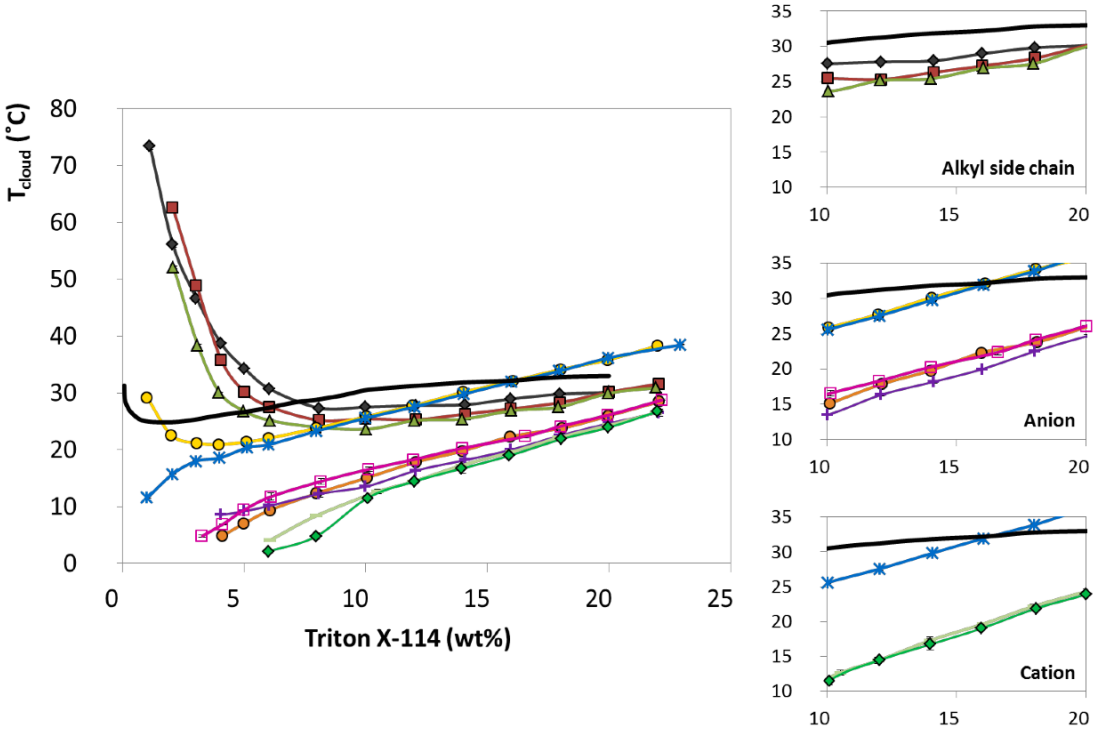


Figure 5. Binodal curves of AMTPS composed of Triton X-114, buffer McIlvaine pH 7 and 0.3 wt% of IL: —, without IL; ◆, [C_{10}mim]Cl; ■, [C_{12}mim]Cl; ▲, [C_{14}mim]Cl; ●, [$P_{6,6,6,14}$]Cl; ✳, [$P_{6,6,6,14}$]Br; ●, [$P_{6,6,6,14}$][Dec]; +, [$P_{6,6,6,14}$][$N(CN)_2$]; □, [$P_{6,6,6,14}$][TMPP]; —, [$P_{8,8,8,8}$]Br; ◆, [$N_{8,8,8,8}$]Br. The effect of the individual ILs' structural features is separately provided in the insets to facilitate the analysis [109].

The possibility of micelle formation with desirably modified physicochemical properties within aqueous IL solutions is exciting, useful, and economically convenient, due to the higher yields of purity and selectivity values that can be achieved [121–123]. Thus, the design of mixed AMTPS should be carefully conducted considering the chemical, physical and biological properties of the target molecule, as well as the nature of the surfactant and co-surfactant used [120]. Concerning the proteins' partitioning/migration, properties such as their isoelectric point, surface hydrophobicity, molecular weight,

medium variables like polymer molecular weight, pH, inorganic salt type and concentration, are crucial factors to tailor the process effectiveness [60,97,122]. By controlling these factors, namely through the addition of buffer solutions to stabilize the pH, as well as, to control the presence of charged/uncharged species of the molecules in the system, it can be expected the selective partition/migration of a target protein [124]. The different combination of phase forming agents allows the creation of tunable AMTPS, thus increasing the possibility to develop a more efficient separation system, considering their capacity to concentrate and purify the target molecule in just one step.

1.2 SCOPE AND OBJECTIVES

Regarding the potential of AMTPS as extraction/separation platforms for distinct (bio)molecules, as well as the downsides associated to the current techniques used for antibodies purification, this work intends to study the use of AMTPS as a more cost-effective and sustainable purification technique for IgG. The main goal of this study is to develop and design an integrated process for the purification of IgG from rabbit serum, aiming to achieve high yields and high purity levels with a simple and low cost process, while guaranteeing the IgG's activity and structure after the purification step. Considering this, to optimize the extraction and purification for the biomolecule of interest, several parameters were evaluated, namely the concentration of Triton X-114 as surfactant, the presence/ absence of ILs as co-surfactants (imidazolium- and phosphonium-based), as well as their concentration. Hence, as a first approach, conventional AMTPS composed of Triton X-114 + McIlvaine buffer at pH 7 were evaluated to purify IgG from rabbit's serum with the goal of identifying the best Triton X-114 concentration in the system. Furthermore, mixed AMTPS constituted of Triton X-114 + McIlvaine buffer at pH 7 + 10 ILs with tensioactive character were investigated. Through the development of these mixed AMTPS it was possible not only to study the effect of the IL and its structural features, such as the cation, anion and alkyl side chain length, but also the IL concentration in the system (0.3, 0.5, 0.7 and 1.0 wt%). The conditions such as the time and temperature for phase formation were maintained at 12 hours and 37º C, respectively, and the time of homogenization to 2 hours. In this work, rabbit serum was used, though the observation of promising purification results could lead to the application of the developed technique to purify antibodies from other mammals' matrices, such as human serum.

2. EXPERIMENTAL SECTION

2.1. MATERIALS

The nonionic surfactant Triton X-114 used in the AMTPS preparation was supplied by Acros Organics, with a CMC of 0.2-0.35 mM and a cloud point in water of 20-25ºC [125]. 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%), 1-methyl-3-tetradecylimidazolium chloride ([C_{14}mim]Cl, purity > 98 wt%) and 1-methyl-3-hexadecylimidazolium chloride ([C_{16}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%), trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate ([$P_{6,6,6,14}$][TMPP], purity > 93.0 wt%), tributyltetradecylphosphonium chloride ([$P_{4,4,4,14}$]Cl. purity > 97.1 wt%) and tetraoctylphosphonium bromide ([$P_{8,8,8,8}$]Br, purity > 95 wt%), were kindly offered by Cytec. The chemical structure of the anions and cations that compose the imidazolium- and phosphonium-based ILs applied in this work are presented in **Figure 6**. The rabbit's serum was purchased from Sigma-Aldrich (R9133 Sigma), with a total protein content between 40 and 70 $mg.mL^{-1}$ (determined by the Biuret method), pooled from a normal donor population. The components of the phosphate buffer used as the mobile phase for the Size Exclusion High Performance Liquid Chromatography (SE-HPLC) analysis, namely $Na_2HPO_4.7H_2O$, NaH_2HPO_4 and NaCl were acquired at Panreac with purities above 98%.

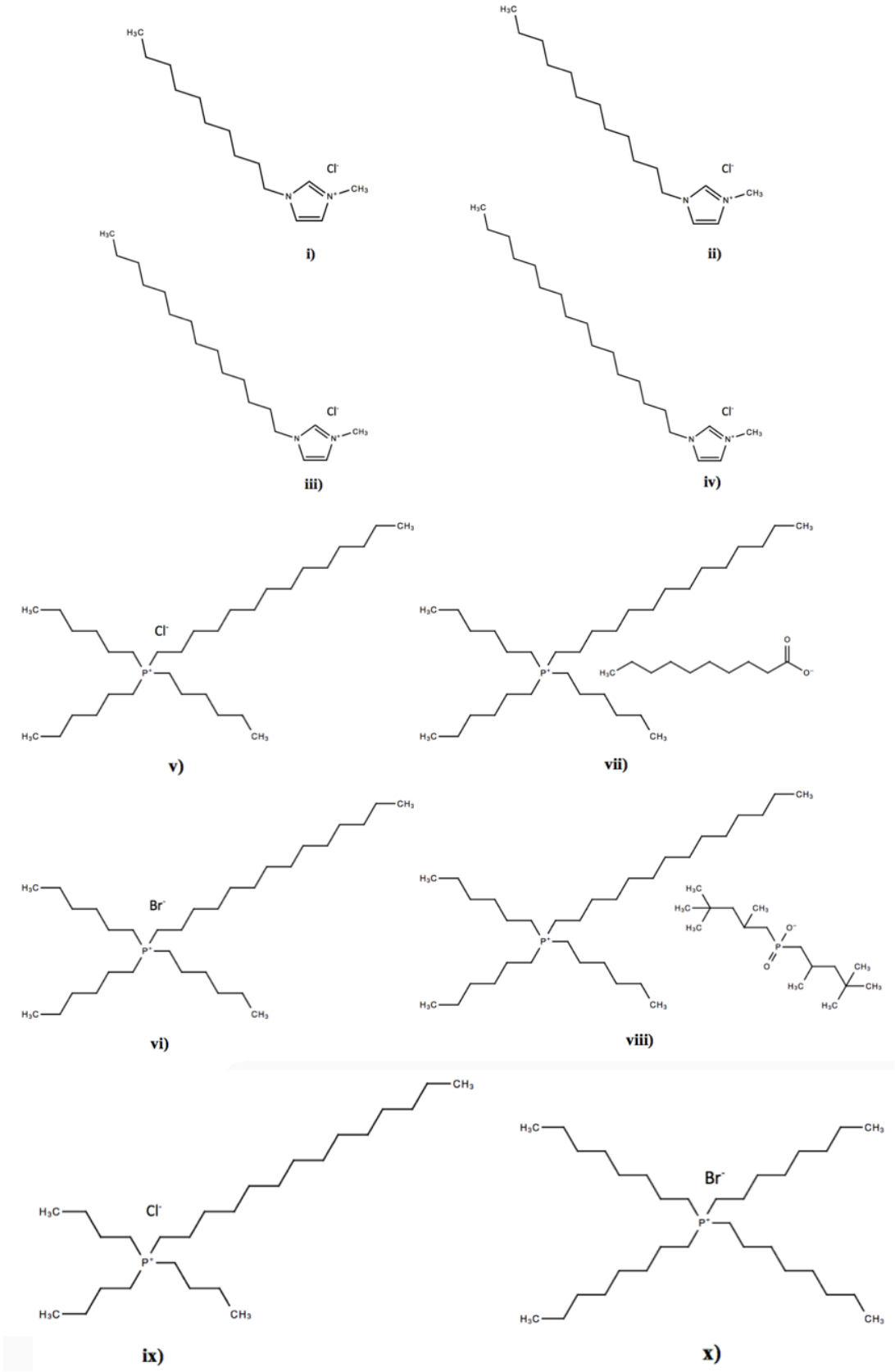


Figure 6. Chemical structure of the anions and cations that compose the imidazolium- and phosphonium-based ILs applied in the investigated AMTPS: i) $[C_{10}mim]Cl$. ii) $[C_{12}mim]Cl$, iii) $[C_{14}mim]Cl$ iv) $[C_{16}mim]Cl$, v) $[P_{6,6,6,14}]Cl$, vi) $[P_{6,6,6,14}]Br$, vii) $[P_{6,6,6,14}][Dec]$, viii) $[P_{6,6,6,14}][TMPP]$, ix) $[P_{4,4,4,14}]Cl$ and x) $[P_{8,8,8,8}]Br$.

2.2. METHODS

2.2.1 Partition studies of IgG from rabbit's serum applying aqueous micellar two-phase systems

To optimize the AMTPS for the IgG purification from the rabbit's serum, three main conditions were evaluated, specifically the surfactant concentration, the addition of ILs as co-surfactants as well as their concentration in the system. First, the surfactant concentration was evaluated by preparing conventional AMTPS at the following compositions (final volume of 2 mL): 10, 15, 17.5 and 20 wt % of Triton X-114 + 10 wt% of rabbit serum (diluted 1:10 (v:v)) + 80, 75, 72.5 and 70 wt% of McILvaine buffer (0.18 M) at pH 7.0 (3.53mL of $C_6H_8O_7.H_2O$ at 0.1M + 16.47 mL of Na_2HPO_4 at 0.2 M). Then, the AMTPS were homogenized at 40 rpm for at least 2 hours at 4ºC, using a rotor apparatus Stuart SB3. Afterwards, the systems were left at 37ºC in a Venticell incubator overnight in order to reach the thermodynamic equilibrium and to guarantee the complete phase separation. The result was the formation of a two-phase system composed of a micelle-rich phase (bottom phase) and a micelle-poor phase (top phase). The objective of this work is to recover the target IgG at the micelle-poor phase and to separate the remaining proteins present in serum by tuning their migration to the micelle-rich phase. These were carefully separated, their volumes and weights measured, and the quantification of IgG and other contaminant proteins addressed. After the optimization of the surfactant concentration, this procedure was repeated for the study of the ILs addition as co-surfactants using the following system: 10 wt % of Triton X-114 + 0.3% of imidazolium or phosphonium ILs + 89.7 % of McILvaine buffer (pH 7.0), for a final volume of 2 mL. Finally, the most promising systems were used for further studies, specifically the effect of the IL concentration as co-surfactant using AMTPS with the following compositions: 10 wt % of Triton X-114 + 0.3, 0.5, 0.7 and 1.0 wt % of IL + 89.7, 89.5, 89.3 and 89.0 % of McILvaine buffer at pH 7.0, to a final volume of 2 mL. All systems were prepared gravimetrically within ± 10^{-4} g. It should be stressed that all studies were performed at least in duplicate and the respective average and standard deviations were determined.

2.2.2. Proteins profile and their quantification in the micelle-poor phase by SE-HPLC

Proteins in both the original rabbit serum and in the micelle-poor phase of each AMTPS were analyzed and quantified by a Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven, temperature controlled auto-sampler and diode array detector (DAD). SE-HPLC was performed using an analytical column, Shodex Protein KW-802.5 (8 mm x 300 mm). Before the HPLC injection, 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 $mL.min^{-1}$ at 25ºC. The injection volume was of 25 μL and the wavelength was set at 280 nm. The chromatograms acquired from the HPLC were used for the determination of the IgG purification yield, which was estimated by **Eq 1**:

$$IgG\ purification\ yield\ (\%) = \frac{IgG\ peak\ area}{(Contaminant\ proteins + IgG)\ peak\ area} \times 100 \quad \text{(Eq. 1)}$$

where the *IgG peak area* represents the area of the peak with a retention time 15.7 min (peak 3, corresponding to IgG)) and *(Contaminant proteins + IgG) peak area* is the sum of the peak areas of all proteins present in the micelle-poor phase.

3. RESULTS & DISCUSSION

3.1 Protein profile of the rabbit serum samples

As a first approach, the rabbit serum, diluted at 1:10 (v:v) with distilled water, was injected into the SE-HPLC to address the complexity of the original matrix. The acquired chromatogram is depicted in **Figure 7**.

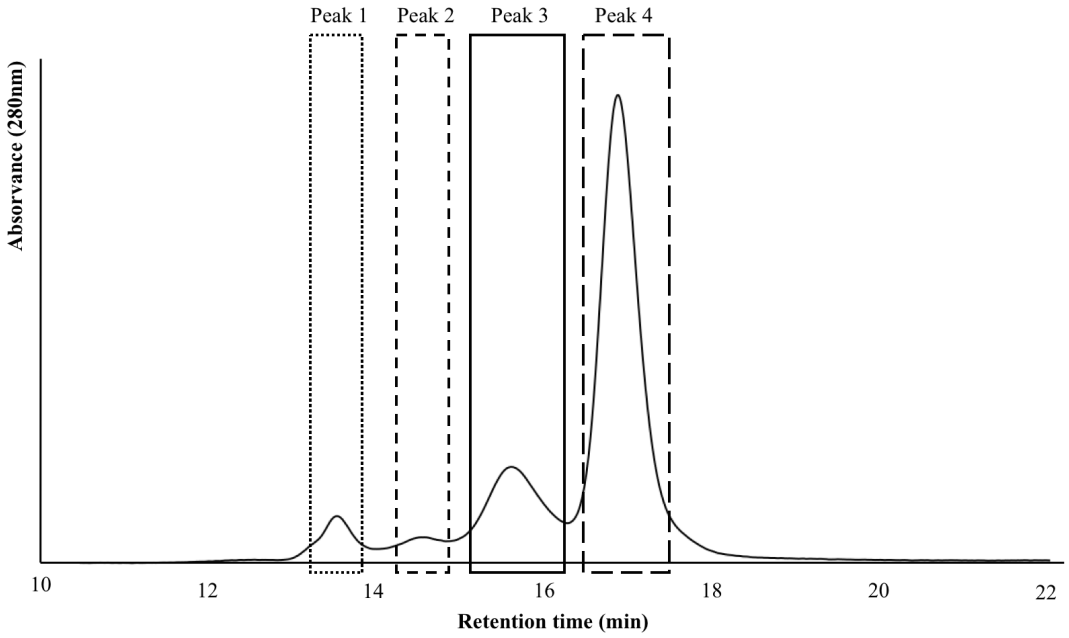


Figure 7. SE-HPLC chromatogram of the rabbit serum samples (dilution factor of 1:10 (v:v)).

From the obtained chromatogram four peaks are identified, specifically peak 1 at 13.5 min, peak 2 at 14.5 min, peak 3 at 15.7 min and peak 4 at 17 min. Through a simultaneous injection of a pure IgG solution into the SE-HPLC, it was possible to identify the peak of IgG, which discloses a retention time of 15.7 min and thus, accounting for peak 3 of the serum sample chromatogram. In this sense, the purification process will face the selective separation of three contaminant proteins from IgG, those represented by peaks 1, 2 and 4. In literature, two major proteins composing the rabbit's serum have been reported, specifically albumin and globulins (alpha 1, alpha 2, beta and gamma) [126]. Accordingly, the major contaminant is believed to be albumin with a retention time of 17 min (peak 4), due to its longer retention time and high concentration in the serum [126]. As a matter of fact, albumin has a MW of 69 kDa, which is lower than that of IgG (150 kDa), and thus, the appearance of its peak is observed after IgG. Moreover, since albumin has a higher serum's concentration, normally ranging from 3.5 to 5.0 g/L, the peak is more intense than the one for IgG. The other two peaks (1 and 2), on the other hand,

cannot be alpha or beta globulins, since these have a lower MW than IgG, approximately 93 kDa and, therefore, should appear after the IgG peak. Consequently, due to the MW of the first two contaminants it is assumed that these correspond to protein agglomerates or IgG dimers.

3.2 Purification of IgG from rabbit serum using aqueous micellar two-phase system

Considering the fact that the biomolecules separation using AMTPS depends on the surfactant concentration [109], the first approach here considered was the evaluation of conventional AMTPS composed of different Triton X-114 concentrations (10, 15, 17.5 and 20 wt%, concentrations all above the CMC). Upon phase separation at 37ºC, a micelle poor-phase and a micelle rich-phase are spontaneously generated occupying the top- and bottom-phases, respectively, as depicted in **Figure 8**. Each phase has its unique environment, so the different proteins present in the solution partition between the two phases depending on their physical properties.

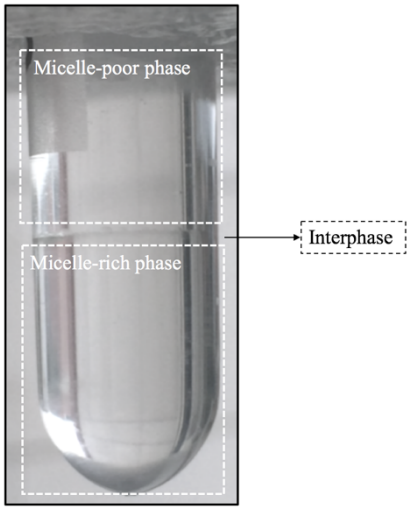


Figure 8. Conventional AMTPS composed of 10 wt% of Triton X-114 and rabbit's serum with a dilution of 1:10 (v:v) and 80 wt% of McILvaine buffer at pH 7.

Taking into consideration the concepts mentioned in the state-of-the-art, it is expected that the IgG will partition preferably towards the top phase. This anticipation is based on the high MW of IgG and on a possible size-exclusion effect at the micelle rich-phase. In

addition, its hydrophilic character favors its preferential migration to the micelle-poor phase.

After phase separation, the recovery of both phases was performed, succeeding by the injection of the micelle-poor phase (top phase) into the SE-HPLC. The decision of not injecting the micelle-rich phase was ground on the high Triton X-114 concentration and presence of micelles that can lead to column clogging and, hence, unsuitable for SE-HPLC analysis. Even so, by the analysis of the micelle-poor phase and considering the proteins profile of the original matrix (**Figure 7**) it is possible to infer on the proteins migration pattern and purification yields. The obtained chromatograms for the micelle poor-phases of the first 4 prepared systems are display in **Figure 9**.

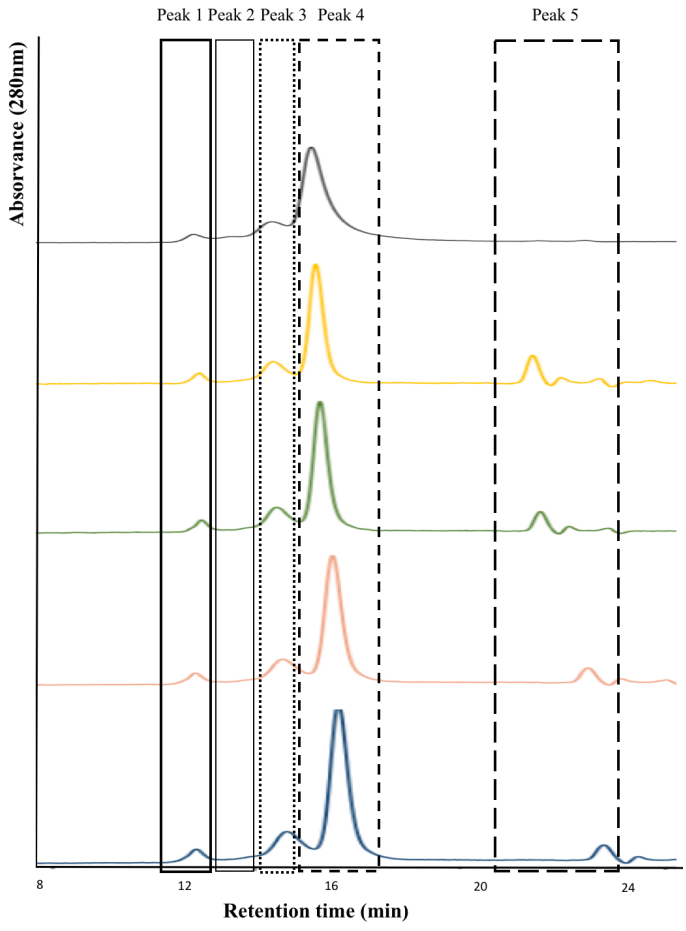


Figure 9. SE-HPLC chromatograms of the micelle-poor phase (top phase) of conventional AMTPS with different Triton-114 concentrations: 10 wt% (—), 15 wt% (—), 17.5 wt% (—), and 20 wt% (—), and buffer McILvaine at pH 7.0, upon phase separation at 37ºC. The top chromatogram (—) corresponds to the original rabbit serum sample (diluted 1:10 (v:v)).

In all chromatograms it can be seen several peaks corresponding to different proteins and/or aggregates. However, compared to the serum's chromatogram, it is observed an additional peak (5) and a depletion of peak 2 in the samples of the micelle-poor phases of the investigated AMTPS. The peak 2 at 14.5 min (contaminant 2) is no more visible in all micelle poor-phase's chromatograms, meaning that this aggregation phenomenon is avoided in the presence of surfactant-based systems or that this type of proteins aggregates migrate completely to the micelle-rich phase. In order to infer on the systems performance as purification platforms, the purification yield was determined and the respective results are shown in **Figure 10**.

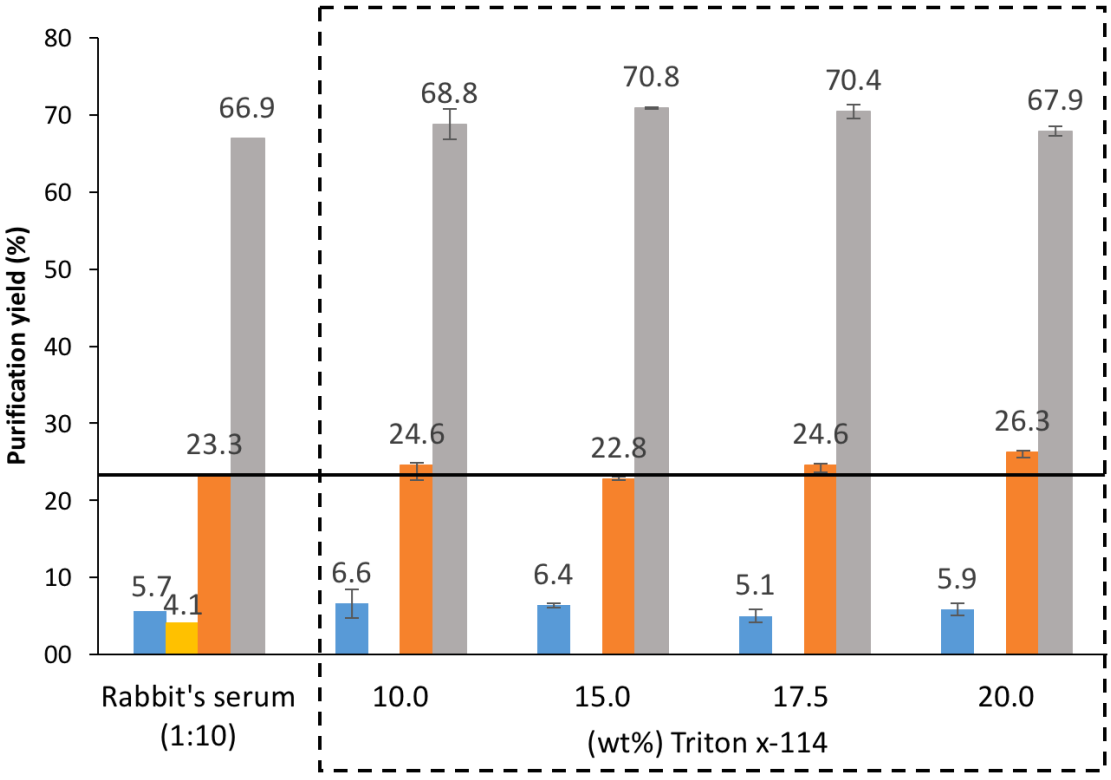


Figure 10. Purification yields (%) of IgG, illustrated by orange bars, obtained for each AMTPS with different surfactant concentrations (wt%) of Triton X- 114, upon phase separation at 37ºC. The purification of the three other contaminants, specifically contaminant 1 (retention time of 13.5 min), contaminant 2 (retention time of 14.5 min) and contaminant 3 (retention time of 17 min) are represented by the blue, yellow and grey bars, respectively.

The analysis of **Figure 10** demonstrates that the use of conventional AMTPS can slightly improve the purification yield of IgG from the rabbit's serum, which is dependent on the surfactant concentration applied and where the best purification levels were obtained with the higher concentrations of surfactant. However, the increase in the purification level observed is not significant, since when the surfactant concentration was doubled (10 wt% to 20 wt%), it was only detected an increase of *circa* 1.5% on the IgG purity. This increment suggests that the increase in the concentration of Triton X-114, which leads to the increase of the system's entropy and, consequently, to the fusion of micelles, does not influence the capture of the contaminants. However, it is interesting to notice the volume decrease of the micelle poor-phase as Triton X-114 concentration increases. This could be profitable to obtain the IgG more concentrated if the system can be simultaneously more selective towards this protein.

Due to the depletion of peak 2, with a retention time of 14.5 min in the serum's chromatogram (**Figure 7**), it was possible to increase the IgG purification yield in the top phase of all systems. It should be remarked the absence of any precipitation in the interphase, and thus it is believed that this aggregate migrates towards the micelle-rich phase or that the presence of a surfactant avoids the formation of these protein aggregates. For a reliable classification of each contaminant and to confirm the presence of the protein corresponding to peak 2 in the HPLC chromatogram, SDS-PAGE analysis should be carried out in the future.

Focusing on the additional peak observed (peak 5), the injection of a micelle poor-phase of a control system, more precisely a conventional system without rabbit's serum, was essential to understand its origin. The chromatogram of the AMTPS without serum showed the same peak, leading to the conclusion that this peak corresponds to the buffer Triton X-114 used in the AMTPS formation.

In general, the high MW as well as the hydrophilic character of IgG allows its preferential partition to the micelle-poor phase. This preference is based on the reduced size that the micelles might provide to foster larger biomolecules, such as IgG (~150 kDa), as well as the higher hydrophilic character that the micelle-poor phase offers. Relatively to the partition of albumin, it is also observed its fondness for the micelle-poor phase and an almost unapparent decrease as the concentration of surfactant increases. Regarding contaminant 1 (peak 1), the AMTPS with 17.5 wt% of Triton X-114 shows better results when compared to the remaining systems. Overall, considering the goal of developing a cost-effective process and since the increment of the IgG purity was only 1.7% when the

concentration of surfactant was doubled, the most suitable system considered to be used in the further studies was the AMTPS composed of 10 wt% of Triton X-114. This AMTPS was used as model system to evaluate other conditions, namely the use of ILs as co-surfactants and their concentration.

Considering the possibility of designing mixed AMTPS with controlled physicochemical properties [78,112–115], two distinct families of ILs, specifically imidazolium and phosphonium, were studied as co-surfactants. In general, the imidazolium family presents a more hydrophilic character when compared to the phosphonium family [109]. Four imidazolium-based ILs, namely [C_{10}mim]Cl, [C_{12}mim]Cl, [C_{14}mim]Cl and [C_{16}mim]Cl, and six phosphonium-based ILs, namely [$P_{6,6,6,14}$]Cl, [$P_{6,6,6,14}$]Br, [$P_{6,6,6,14}$][Dec], [$P_{6,6,6,14}$][TMPP], [$P_{4,4,4,14}$]Cl and [$P_{8,8,8,8}$]Br, were used. The micelle-poor phases of these mixed AMTPS were injected into the SE-HPLC and the obtained chromatograms are depicted in **Figure 11A** and **B**. Moreover, the purification yields were also determined from the peak areas of the chromatograms and are presented in **Figure 12.**

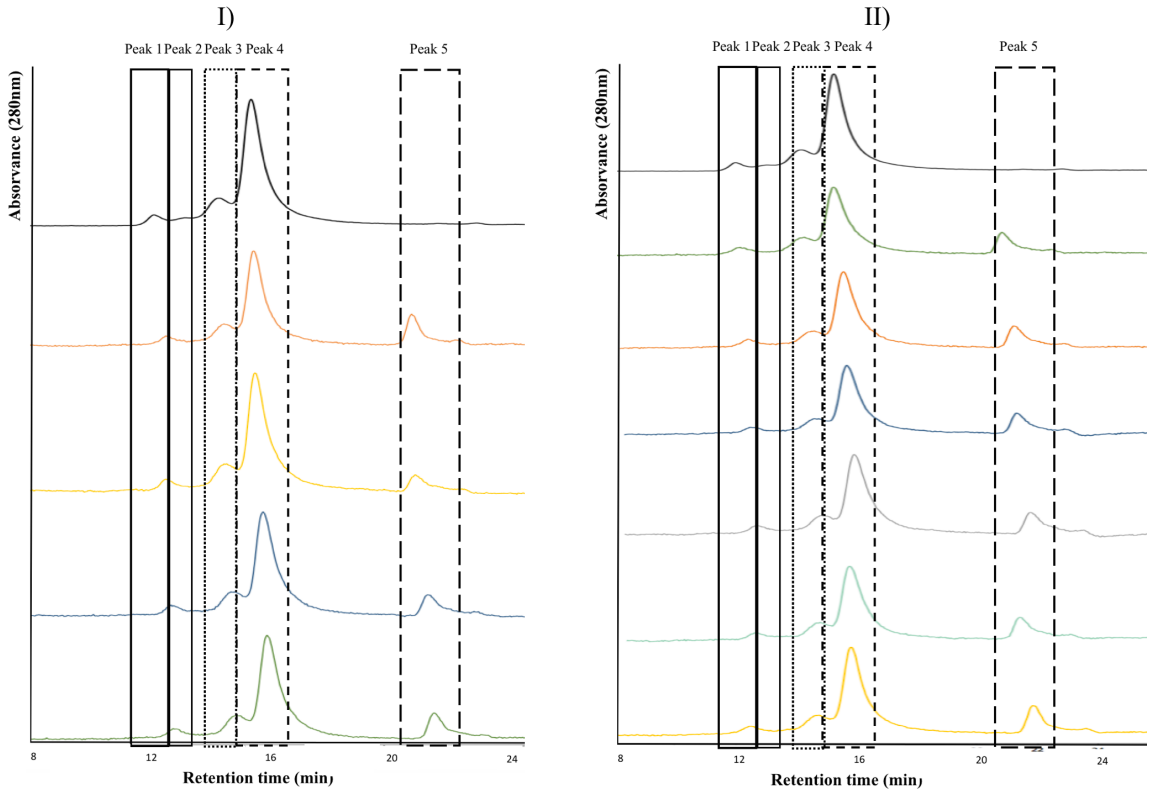


Figure 11. SE-HPLC chromatograms of the micelle-poor phase of mixed AMTPS composed of 10 wt% of Triton X-114, 0.3 wt% of IL and 89.7 wt% of McILvaine buffer (pH 7), upon phase separation at 37ºC. Figure I for imidazolium-based ILs: (—) [C_{10}mim]Cl; (—) [C_{12}mim]Cl; (—) [C_{14}mim]Cl and (—) [C_{16}mim]Cl; and Figure II for phosphonium-based ILs: (—) [$P_{6,6,6,14}$]Cl; (—) [$P_{6,6,6,14}$]Br; (—) [$P_{6,6,6,14}$][Dec]; (—) [$P_{6,6,6,14}$][TMPP]; (—) [$P_{8,8,8,8}$]Br; and (—) [$P_{4,4,4,14}$]Cl. The top chromatogram (—) corresponds to the original rabbit serum (diluted in 1:10 (v:v)).

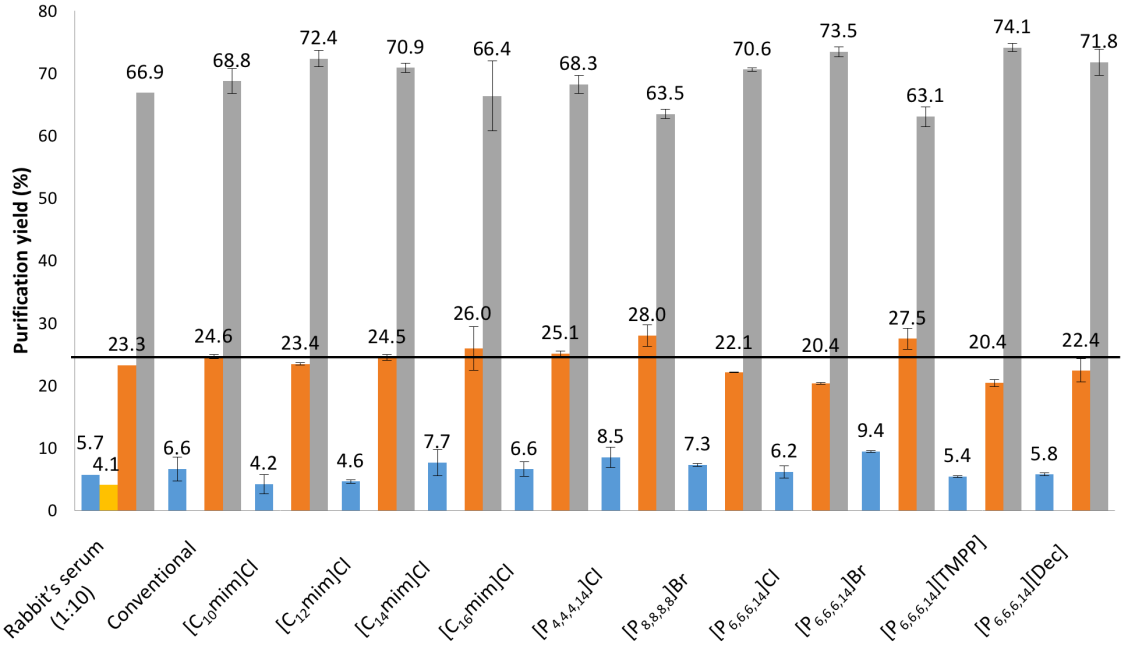


Figure 12. Purification yields (%) of IgG, illustrated by orange bars, obtained for each AMTPS with 10 wt% of Triton X-114 and a concentration of co-surfactants (ILs) at 0.3 wt%, upon phase separation at 37ºC. The purification of the three other protein contaminants, specifically contaminant 1 (retention time of 13.5 min), contaminant 2 (retention time of 14.5 min) and contaminant 3 (retention time of 17 min) are represented by the blue, yellow and grey bars, respectively.

By analyzing the chromatograms shown in **Figure 11,** the same peaks detected in the conventional AMTPS were observed, namely peak 1 (contaminant 1), peak 3 (IgG), peak 4 (contaminant 3) and peak 5 (buffer's salts and/or Triton X-114). In general, the addition of ILs as co-surfactants at 0.3 wt% does not allow the complete elimination of the contaminants in the micelle rich-phase, however it is noticeable an improvement of the IgG purification yield in some of the mixed AMTPS using phosphonium-based ILs. When the imidazolium-based AMTPS are considered, there is not a significant difference in the IgG purification yield if the standard deviations are taken into account. Some phosphonium-based AMTPS display better abilities to improve the IgG purification yield, and follow the sequence: $[P_{6,6,6,14}]Cl = [P_{6,6,6,14}][TMPP] < [P_{8,8,8,8}]Br \approx [P_{6,6,6,14}][Dec] < [P_{6,6,6,14}]Br < [P_{4,4,4,14}]Cl$. By comparing these mixed AMTPS with the conventional AMTPS, it is noticed a decrease of the IgG purification yield in the micelle poor-phase; however, the ILs $[P_{6,6,6,14}]Br$ and $[P_{4,4,4,14}]Cl$ stand out with superior purifications yields of 27.5 and 28.0 %, respectively. As a matter of fact, the best IL ($[P_{4,4,4,14}]Cl$) has shown an increase of almost 4% in the purification yield when compared to the conventional

AMTPS. Consequently, it seems that the proper choice of the phosphonium-based IL has an effect in the purification and recovery of IgG in the micelle poor-phase, and where it is important to highlight the contrast in behavior between the ILs that share the same anion Cl^-, more specifically $[P_{6,6,6,14}]Cl$ and $[P_{4,4,4,14}]Cl$. These ILs offer distinct purification yields, with almost 8% of purity difference, evidencing the important effect of the cation alkyl side chain length of the co-surfactants. Moreover, considering the anion effect, $[P_{6,6,6,14}]Cl$ and $[P_{6,6,6,14}]Br$ evidence a purification yield difference of approximately 7 %.

Finally, it is noteworthy to mention that the achieved 28.0% of purity with the $[P_{4,4,4,14}]Cl$ co-surfactant is an improved result regarding the purification of IgG from rabbit's serum by applying LLE. The most significant result obtained in this work in terms of IgG purification, from a similar source, is superior to that obtained with polymer/salt ATPS using ILs as adjuvants [45] and identical to that obtained with ATPS composed of polymer/bio-based ILs [12]. Consequently, these results highlight the potential and success of AMTPS as a promising purification technique to purify antibodies, as IgG, from complex matrixes.

Taking into account the results on the improvement of the IgG purification yield and the standard deviations, the effect of the IL concentration in the AMTPS formation was also ascertained, by applying the systems based in $[C_{16}mim]Cl$, $[P_{4,4,4,14}]Cl$ and $[P_{6,6,6,14}]Br$. To maintain their role as co-surfactants, the stipulated concentrations here studied are 0.3, 0.5, 0.7, and 1.0 wt%. The acquired chromatograms and purification yields are shown in **Figures 13** and **Figure 14**, respectively.

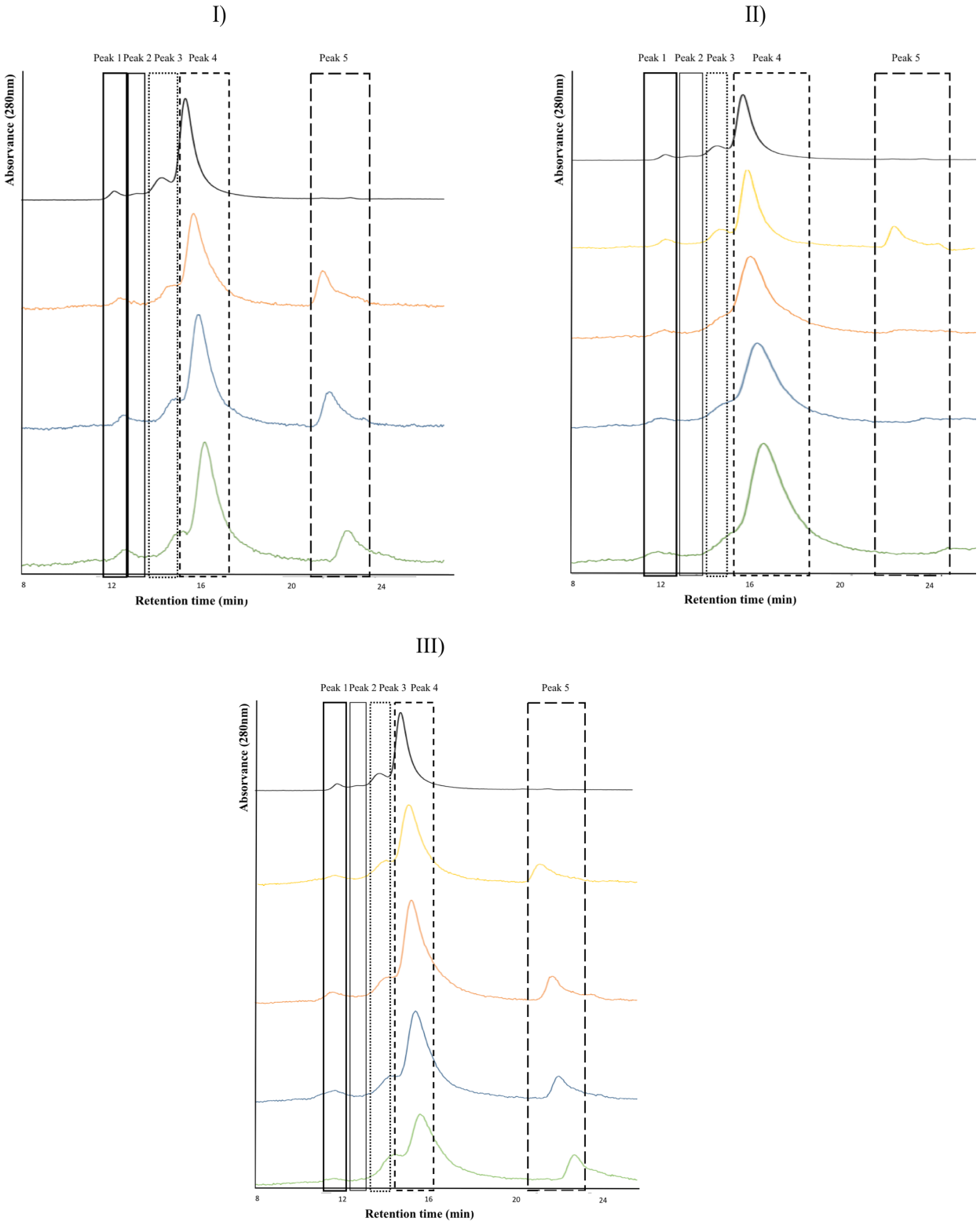


Figure 13. SE-HPLC chromatograms of the micelle-poor phase of mixed AMTPS composed of 10 wt% of Triton X-114, McIlvaine buffer (pH 7) and different IL concentrations of $[P_{6,6,6,14}]Br$ and $[P_{4,4,4,14}]$ upon phase separation at 37ºC. Figure I for $[C_{16}mim]Cl$: (—) 0.3% wt; (—) 0.5% wt and (—) 0.7% wt%; Figure II for $[P_{6,6,6,14}]Br$: (—) 0.3% wt; (—) 0.5% wt; (—) 0.7% wt% and (—) 1.0% wt%; and Figure III for $[P_{4,4,4,14}]$: (—) 0.3% wt; (—) 0.5% wt; (—) 0.7% wt% and (—) 1.0% wt%. The top chromatogram (—) corresponds to the original rabbit serum sample (diluted in 1:10 (v:v).

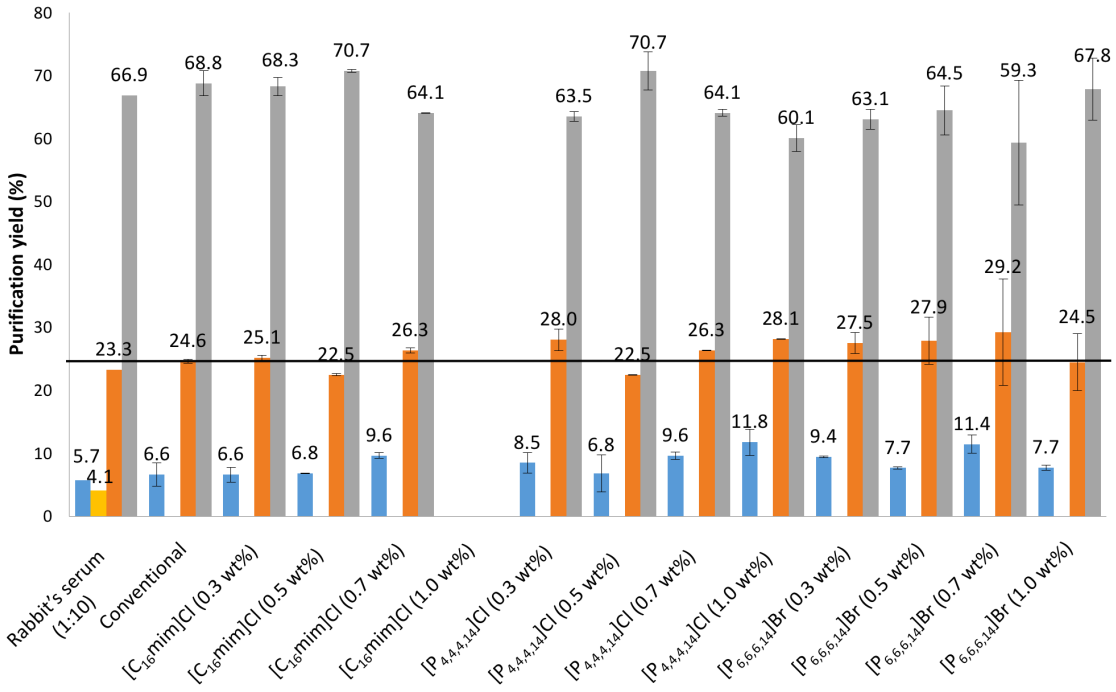


Figure 14. Purification yields (%) of IgG, illustrated by orange bars, obtained for each AMTPS with different co-surfactant concentrations (wt%) of three ILs ($[P_{4,4,4,14}]Cl$, $[P_{6,6,6,14}]Br$ and $[C_{16}mim]Cl$) upon phase separation at 37ºC. The purification of the three other contaminants, specifically contaminant 1 (retention time of 13.5 min), contaminant 2 (retention time of 14.5 min) and contaminant 3 (retention time of 17 min) are represented by the blue, yellow and grey bars, respectively.

First of all, it needs to be mentioned that the AMTPS with 1.0 wt% of $[C_{16}mim]Cl$ is not represented in both **Figures 13** and **14,** since the two-phase formation for this IL-based AMTPS was not achieved at 37ºC, which is probably justified by the capacity of ILs to modify, and in this case, to increase the T_{cloud} of AMTPS [120].

By the analysis of the overall results, the main conclusion is that the IL concentration does not follow a well-defined tendency, which can be justified by the fact that the (bio)molecule's migration tendencies are based on a delicate balance involving dispersion and electrostatic interactions and excluded-volume effects [120]. However, and even though the lack of any tendency, it seems that the IL concentration can slightly improve the purification of IgG. The best purity data achieved was with the systems formed by $[C_{16}mim]Cl$ at 0.7 wt%, $[P_{4,4,4,14}]Cl$ at 1.0 wt% and $[P_{6,6,6,14}]Br$ at 0.7 wt%, which allow purification yields of IgG of 26.3, 28.1 and 29.2%, respectively. As the IgG in the physiologic pH (pH ~7) has a valence of 2 but the surfactant Triton X-114 is a non-ionic species, the addition of ILs confers positive electric charges to the micelles that may

causing repulsive forces between the mixed micelles and the protein, improving IgG purification. Considering the contaminants, the contaminant 1 has a slight increase towards the micelle poor-phase. Contrarily, from **Figure 14**, it can be observed a slight decrease in the purification yield of contaminant 3, which partitions slightly more to the micelle-rich phase. As a result, the electrostatic charges seem to improve the partition of contaminant 1 to the micelle-poor phase and the partition of contaminant 3 (albumin) to the micelle-rich phase, although not in a significant way. Summing up, and after a careful analysis of all parameters and conditions, the best purification yield of IgG observed was obtained with the system composed of 0.7 wt% of $[P_{6,6,6,14}]$Br achieving a 29.2% purity for IgG. This value is higher to that obtained with other liquid-liquid extraction approaches reported in the literature, namely polymer/salt ATPS using ILs as adjuvants [45] or with ATPS composed of polymer/bio-based ILs [12]. The results obtained highlight the potential and success of AMTPS as a promising purification technique to purify antibodies, as IgG, from complex matrixes. However, future studies are still required, in particular regarding the system pH optimization aiming at tailoring the selective partition of the diverse proteins between the two phases.

4. CONCLUSIONS, FINAL REMARKS &FUTURE WORK

In this work, it was evaluated the application of AMTPS as a non-chromatographic method to purify IgG from a complex matrix, namely rabbit serum. It was possible to achieve a purity of 24.6% of IgG by applying conventional AMTPS at pH 7.0. Moreover, in order to obtain higher purification yields, the addition of co-surfactants was evaluated and an enhanced capacity was perceived by the application of mixed AMTPS composed of ILs. The best mixed systems reported in this study are based on $[C_{16}mim]Cl$ at 0.7 wt%, which led to a purification yield of 26.3%, and phosphonium ILs, namely $[P_{4,4,4,14}]Cl$ at 1.0 wt% and $[P_{6,6,6,14}]Br$ at 0.7 wt%, which led to 28.1% and 29.2% IgG purity yield, respectively. These results exceed the purity levels of IgG extracted from the same source using ATPS reported in the literature [12,45], demonstrating the potential of the studied micellar systems as a potential purification technique. Overall, considering the optimization studies performed and the economical flexibility stipulated in this work, it is concluded that for the purification of IgG from rabbit's serum, the AMTPS with 10 wt% of Triton X-114 and 1.0 wt% of $[P_{4,4,4,14}]Cl$ and 0.7 wt % of $[P_{6,6,6,14}]Br$ are the most suitable.

Considering the work here performed, there are still some parameters and conditions that should be improved and investigated, such as the concentration/dilution factor of the serum employed in the formation of each AMTPS, the pH of the system, and the time and temperature of phase separation. Furthermore, the quantification of IgG should be assessed in all studied AMTPS in order to obtain the IgG recovery yield in the micelle poor-phase. This is achievable using a calibration curve and a simultaneously injection into the SE-HPLC of the serum and of the micelle-poor phases of each AMTPS evaluated. Most importantly, the effect of the surfactant as well as of the IL on the IgG stability should be, undoubtedly, evaluated to validate the applicability of the technique under study to purify antibodies. Well established techniques such as Fourier transform infrared (FTIR) spectroscopy and Circular Dichroism (CD) are some of the techniques that can be used. Depending on the IgG stability obtained in the micelle-poor phase, the decision and the development of processes for the recovery of IgG should be carried out. Furthermore, considering the quantities of surfactant applied, the recovery and reuse of the surfactant and of the IL should be additionally attempted in order to minimize the costs and the environmental impact of the process. In this case, micellar-enhanced ultrafiltration (MEUF) [127] or a foam separation [128] processes could be used in order to remove the surfactant micelles.

Recently, it has been reported the application of novel ILs composed of the cholinium cation combined with anions derived from plants natural acids for the purification of IgG from rabbit's serum, achieving a ~85% of recovery yield and a purification of ~30% [12]. Taking this into account, the synthesis and the assessment of bio-based ILs with tensioactive character, such as with the cholinium cation, should be performed to evaluate their role as co-surfactants in AMTPS to purify IgG. If successful, the incorporation of bio-based ILs can improve the benign character of the designed AMTPS.

Despite all the considerations described before, the purification yields obtained in this work are far from the aspired results. Therefore, the development of a novel parallel and more integrated approach to achieve better purification yields has been already planned and considered. In the future, the application of ILs directly to serum, prior the applicability of conventional AMTPS, is going to be investigated. The basis of this approach comprises the use of ILs to induce first the selective precipitation of the contaminant proteins present in the serum, and further use of these aqueous solutions to form AMTPS and IgG purification.

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