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Use of Ionic Liquids as Cosurfactants in Mixed Aqueous Micellar Two-Phase Systems to Improve the Simultaneous Separation of Immunoglobulin G and Human Serum Albumin from Expired Human Plasma

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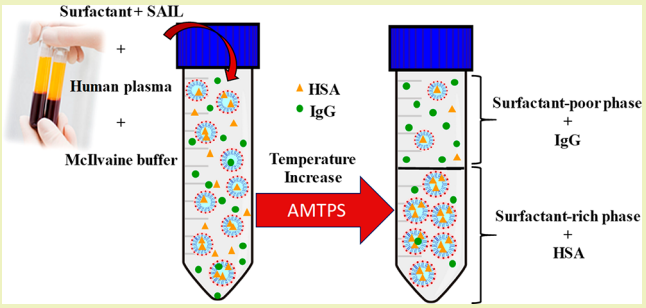
Supporting Information

ABSTRACT: A large fraction of unused clinical transfusion plasma is nowadays discarded. Nevertheless, some proteins, including serum albumin, immunoglobulins, and clot factors, are still viable for use in diagnosis or research. However, lasma is a complex matrix, requiring multiple steps and time-consuming methods for the isolation of proteins. In this work, aqueous micellar two-phase systems (AMTPS) composed of nonionic surfactants (Triton X-114 or Tergitol 15-S-7) were investigated to simultaneously separate immunoglobulin G (IgG) and human serum albumin (HSA) from human expired plasma. A factorial planning was applied to the nonionic surfactant and plasma concentrations to enhance the systems separation performance. After identifying the best conditions with AMTPS formed by nonionic surfactants, mixed AMTPS formed by Tergitol 15-S-7 and various surface-active ionic liquids (SAILs) acting as cosurfactants were additionally investigated to tailor the proteins partition between the two phases. The mixed AMTPS composed of Tergitol 15-S-7 as the nonionic surfactant and tributyltetradecylphosphonium chloride as the cosurfactant at pH 8.0 improved the simultaneous separation of both proteins to the opposite phases. IgG purification of 1.14-fold in the surfactant-poor phase and HSA purification of 1.36-fold in the surfactant-rich phase were obtained. Method reproducibility was investigated using additional samples of human serum and plasma and serum mixtures. Although improvements in the purification factor of each protein are still a future goal, mixed AMTPS comprising nonionic surfactants and SAILs as cosurfactants may be considered as novel platforms to be used in the simultaneous separation of value-added compounds from complex and natural matrices.



KEYWORDS: *Immunoglobulin G, Human serum albumin, Downstream process, Aqueous micellar two-phase systems, Ionic liquid, Cosurfactant*

INTRODUCTION

According to the World Health Organization (WHO), plasma-derived medicinal products have application in a daily basis.[1] These products, comprising albumin, clot factors, and immunoglobulins, are recurrently applied in trauma incidents and surgeries, as well as in the treatment of several diseases such as hemophilia, autoimmune pathologies, systemic inflammatory, and/or bleeding disorders and congenital deficiencies.[1−3] The availability of plasma and plasma-derived products is however mainly dependent on the goodwill of donors. Therefore, limited plasma availability is a critical issue, contributing to the expensive cost of plasma-derived products. On the other hand, the recovery and purification of these products require numerous steps, additionally contributing to the overall cost of plasma-derived therapeutics.[4,5]

Clinical transfusion plasma is obtained after platelets removal or by apheresis in clinical blood collection centers. Depending on its treatment, plasma can be categorized as fresh frozen plasma (frozen within 8 h after collection) or frozen plasma (frozen from 8 to 24 h after collection). Both types can be thawed and kept for 5 days at temperatures ranging from 1 to 6 °C and fractionated into distinct products.[6] Included in plasma-derived products are immunoglobulins, which represent the major plasma product in the blood and plasma global market.[5] Not all hospitals and health care centers have plasma manufacturers in their vicinities, and most of the unused

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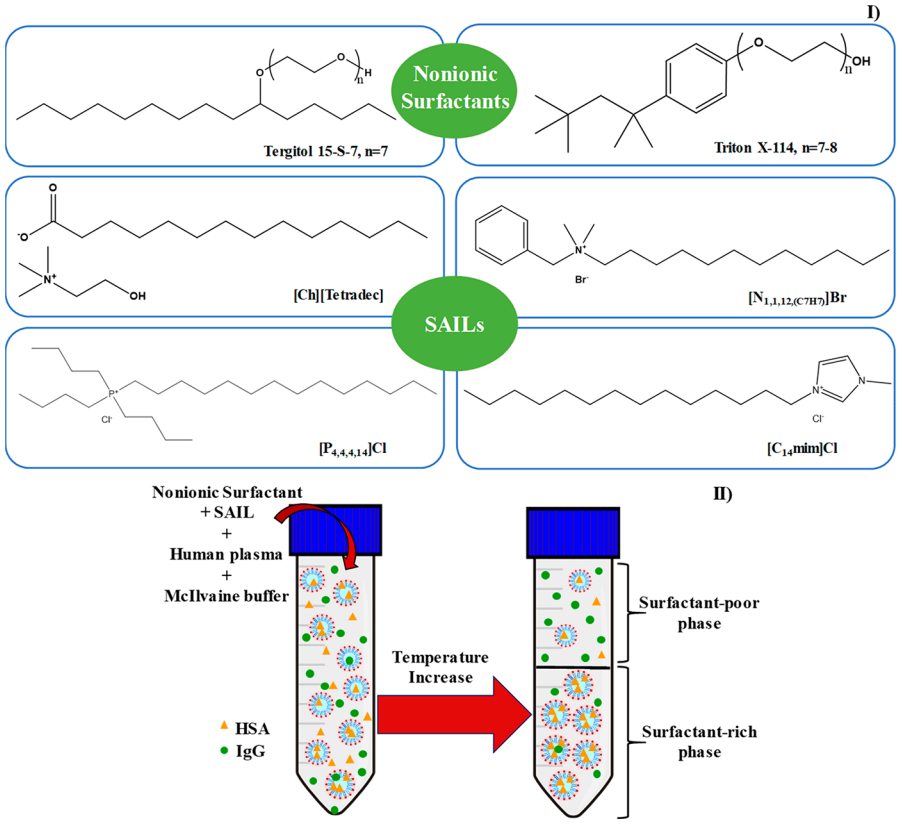

Figure 1. (I) Chemical structures and abbreviations of the investigated nonionic surfactants and SAILs. (II) Schematic representation of the simultaneous separation process of IgG and HSA from human plasma using mixed AMTPS.

clinical transfusion plasma is discarded. However, the present proteins, including serum albumin, immunoglobulins, and clot factors may be still viable for use in diagnosis or research.[7]

Immunoglobulins or antibodies represent a large market sector with an estimated global production of USD 13.28 billion by 2021.[8] In this sector, recombinant monoclonal antibodies are an attractive option as specific therapeutics since they recognize a single antigen with high specificity.[9] These antibodies are currently considered high-cost products, mainly due to the complexity of the original medium in which they are produced.[5,10,11] In addition, immunoglobulins obtained from human blood are polyclonal antibodies derived from different B cells, being able to recognize multiple epitopes on the same antigen. Immunoglobulins from human plasma, namely, immunoglobulin G (IgG), are obtained by the traditional or adapted Cohn method,[12] comprising several steps of precipitation (induced by ethanol, caprylate, or polyethylene glycol), filtration, and distinct types of chromatography.[5,11,13,14] Blood and derived fractions are also rich in human serum albumin (HSA), the most abundant protein in blood. HSA is a plasma derivative widely used to treat shock as a protein/volume replacement or as an adjunct in renal dialysis or cardiac bypass surgeries. Although it is not included in the essential medicines list, the average HSA demand per country accounts for the need of 59 000 L of plasma.[7]

Given the current high cost and potential use of immunoglobulins as biopharmaceuticals,[15] significant consideration has been given to the development of alternative purification platforms for IgG, the most abundant antibody in blood accounting for 75% of all immunoglobulins.[16] Among these platforms, large attention has been given to aqueous two-phase systems (ATPS).[10,11,17−19] Most of these studies were however carried out with monoclonal antibodies, leading to recovery yields ranging between 76 and 99% and purity levels ranging from 76 to 100%. To the best of our knowledge, IgG purification from human plasma using ATPS has only been attempted twice, as part of a multistep approach with a final chromatographic step resulting in high-purity IgG.[13,14] In addition to ATPS, aqueous micellar two-phase systems (AMTPS) are biphasic systems formed merely by water and a surfactant. In these systems and for a given mixture composition, the phase separation is triggered by changes in temperature. At the cloud point temperature, there is the system coacervation and the formation of two distinct environments, namely, a hydrophobic surfactant-rich phase and a hydrophilic surfactant-poor phase.[20,21] AMTPS have been studied in the purification of several biomolecules, namely, antibiotics,[22] single-chain antibody fragments,[23] and proteins such as cytochrome c and bromelain.[24,25] Nevertheless, when dealing with the purification of labile products, such as proteins or antibodies, it is of high relevance to work with AMTPS that phase-separate with low concentrations of surfactant and at low temperatures. The process separation performance can be improved by the manipulation of the surfactant concentration, temperature, size, and charge at the micelles surface. More recently, the creation of mixed AMTPS incorporating other surface-active compounds acting as cosurfactants, such as surface-active ionic liquids (SAILs), have been explored.[24] SAILs display most of the properties of ionic liquids, in addition to their amphiphilic character, for which there is the possibility of designing the surfactant accordingly to the final application.[20,24,26] Furthermore, if

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properly designed, then ionic liquids are improved candidates to stabilize labile molecules, such as antibodies[27] and other proteins,[28] as well as RNA[29] and DNA.[30]

In this work, AMTPS formed by nonionic surfactants, namely, Triton X-114 or Tergitol 15-S-7, and mixed AMTPS composed of the nonionic surfactant Tergitol 15-S-7 and several SAILs as cosurfactants, were investigated for the separation of IgG and HSA from unused clinical transfusion plasma (not in quarantine). Samples of human serum and mixtures of human serum and plasma were used to address the reproducibility of the systems' separation performance. According to the WHO, 9.3 million liters of recovered plasma from donation are wasted every year.[7] If still viable, then this plasma volume would allow the recovery of 1.4 billion international units (IU) of FVIII, 2.3 billion IU of FIX, 3.2 billion IU of prothrombin complex concentrate, 37 tons of IgG, and 230 tons of HSA.[7] Thus, the development of sustainable strategies to recover plasma-derived medicinal products, particularly from expired plasma, significantly contributes to the Sustainable Development Goals of Agenda 2030.[31]

EXPERIMENTAL SECTION

Materials. Nonionic surfactants, namely, Triton X-114 (technical grade) and Tergitol 15-S-7 (purity ≥ 99%), were acquired at Acros Organic and Sigma-Aldrich, respectively. SAILs used as cosurfactants to create mixed AMTPS with Tergitol 15-S-7 were 1-methyl-3-tetradecylimidazolium chloride ([C_{14}mim]Cl, purity > 98 wt %) purchased from Iolitec (Ionic Liquid Technologies, Heilbronn, Germany); tributyltetradecylphosphonium chloride ([$P_{4,4,4,14}$]Cl, purity = 95%) provided by Iolitec; benzyldodecyldimethylammonium bromide ([$N_{1,1,12,(C_7H_7)}$]Br, purity > 99%) obtained from Sigma-Aldrich; and cholinium tetradecanoate ([Ch][Tetradec], purity = 99.7%) synthesized by us according to established protocols.[32] The chemical structures of the investigated compounds are shown in Figure 1I). The McIlvaine buffer was prepared by mixing sodium phosphate dibasic anhydrous (Na_2HPO_4, purity ≥ 99%) and citric acid anhydrous ($C_6H_8O_7$, purity ≥ 99.9%), acquired from EMSURE ACS, Reag. PhEur, and VWR Chemicals BDH Prolabo, respectively. The mobile phase used in HPLC analysis is composed of sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$, purity = 98−102%), sodium phosphate monobasic (NaH_2PO_4, purity = 99−100.5%), and sodium chloride (NaCl, purity = 99.5%), all acquired from Panreac AppliChem. Human plasma was obtained from whole blood donated by healthy individuals, drawn according to well-established protocols[4] and by authorized personnel. These plasma samples were not in quarantine and were about to be discarded. Pure IgG (purity ≥ 95%) and HSA (purity = 96%) were acquired at Sigma-Aldrich and Alfa Aesar, respectively. Human serum and bicinchoninic acid protein assay (BCA) kit were acquired from Sigma-Aldrich.

Methods. *Determination of the AMTPS and Mixed AMTPS Binodal Curves.* Binodal curves for the two AMTPS composed of nonionic surfactants and mixed AMTPS with ionic liquids used as cosurfactants were determined according to the cloud point method.[33] The cloud point corresponds to the temperature at which each surfactant solution becomes turbid upon heating due to the system's coacervation and therefore separates into two macroscopic phases: a surfactant-poor phase and a surfactant-rich phase. Two to three cloud point temperature measurements were visually performed for each nonionic surfactant composition, with the average temperature used in the representation of the solubility curves. AMTPS and mixed AMTPS composed of 4−21 wt % each nonionic surfactant in absence and presence of 0.3 wt % each SAIL, respectively, were considered, with each mixture being completed with the McIlvaine buffer (pH 6.0, at 0.16 M) up to 10 mL of final volume. To determine the cloud points, 10 °C was used as the lowest and starting temperature to guarantee that each cloud point temperature is correctly determined. Each mixture composition was heated up to 55 °C in a temperature-controlled water bath with a precision of ±0.01 °C (ME-18 V Visco-Thermostat, Julabo).

Characterization of Plasma and Serum Samples. Pure IgG and HSA, as well as plasma samples from two donors (1 and 2), were analyzed through size-exclusion high-performance liquid chromatography (SE-HPLC) equipped with a diode array detector (DAD) and a size-exclusion column Shodex Protein KW-802.5 (8 mm × 300 mm). Each protein solution and biological sample was diluted (1:10, v/v) in phosphate buffer (pH 7, 100 mM) and NaCl at 0.3 M aqueous solutions, which correspond to the mobile phase, injected into the HPLC apparatus and run isocratically with a flow rate of 0.5 mL·min^{-1} at 40 °C. The injection volume was 25 μL, and the wavelength was set at 280 nm. IgG and HSA quantifications were carried out by an external standard calibration method in the range from 1 to 14 mg·mL^{-1} and from 5 to 50 mg·mL^{-1}, respectively.

The total protein content in both human serum and plasma samples was determined by applying the BCA method and using bovine serum albumin as standard. Both samples were incubated at 37 °C for 30 min, and the absorbance was determined at 562 nm using a Molecular Device Spectramax 384 Plus UV−Vis Microplate Reader.

The dry matter content of human serum and plasma samples was determined up to a constant weight at 70 °C in an air oven (VWR INCU-Line).

Separation of IgG and HSA Using AMTPS and Mixed AMTPS. Each mixture, composed of nonionic surfactant, SAIL (when applicable), biological sample, and McIlvaine buffer, was gravimetrically prepared, homogenized for ∼2 h using a tube rotator apparatus model 270 from Fanem at 40 rpm, and left overnight at 40.0 ± 0.1 °C. At this temperature, the complete separation of the phases was guaranteed after reaching the thermodynamic equilibrium as well as the stability of both IgG and HSA since they are known to maintain their stability up to 70 °C[34] and 60 °C[35] at a pH ranging from 5[34] and 5.4[35] to 10,[34,35] respectively. A schematic representation of the separation process is displayed in Figure 1II. The phases were carefully separated, and their volumes and weights were determined and registered before the proteins quantification. The mixture compositions were chosen taking into account the binodal curves determined for each system.

Although the surfactant-poor phase was directly diluted in the respective buffer and injected in the HPLC, to analyze the surfactant-rich phase, a protein precipitation step was previously performed to avoid damaging the HPLC column with the surfactants. To this end, 4 mL of cold acetone was added to 1 mL of each surfactant-rich phase sample and left for 4 h at −20 °C. Each sample was centrifuged for 30 min at 4 °C and 5500 rpm, and the supernatant was discarded. The protein-rich pellet was resuspended in 1 mL of McIlvaine aqueous buffer (pH 6.0, 0.16 M).

The first set of studies to identify the best nonionic surfactant and its concentration was performed using plasma from donor 1, whereas the remaining optimization experiments were carried out using plasma from donor 2. The reproducibility of the separation methods was addressed using serum samples, which contain IgG and HSA but no clot factors, and mixtures of human serum and plasma from donor 2.

The methods previously described were repeated at pH 7.0 (using 0.18 M McIlvaine aqueous buffer) and 8.0 (using 0.20 M McIlvaine aqueous buffer) to evaluate the pH effect on the separation performance. The same protocol was used to perform the screening of SAILs in the formation of mixed AMTPS (using the nonionic surfactant Tergitol 15-S-7 and SAILs as cosurfactants) for IgG and HSA separation.

The protein separation and the systems' purification performance was evaluated through the purity factor (PF, eq 1) of each protein in each phase. The IgG purity and yield were also determined, according to eqs 2 and 3, respectively.

$$PF_{IgG} = \frac{\text{IgG purity}_{phase}(\%)}{\text{IgG purity}_{initial}(\%)} \quad (1)$$

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$$\text{IgG purity (\%)} = \frac{\text{IgG area}}{(\text{Other Proteins} + \text{IgG})\text{area}} \times 100 \quad (2)$$

$$\text{IgG yield (\%)} = \frac{\text{IgG weight}_{\text{phase}}}{\text{IgG weight}_{\text{initial}}} \times 100 \quad (3)$$

where IgG purity$_{phase}$ and IgG purity$_{initial}$ correspond to the antibody purity in each phase and in plasma, respectively. IgG weight$_{phase}$ represents the IgG weight in each phase, known by the determination of the IgG concentration and volume of the respective phase, and IgG weight$_{initial}$ corresponds to the IgG weight in plasma, by knowing the IgG concentration, total volume of each sample, and volume of plasma added. The same parameters were determined for HSA using eqs 1−3, where HSA is considered instead of IgG.

A response surface methodology was carried out to optimize the separation of IgG and HSA for opposite phases from expired human plasma samples. A 2^2 factorial planning was used to allow the simultaneous optimization of the nonionic surfactant and plasma concentrations. These two parameters are the independent variables while the IgG and HSA purification factors are the response variables. Detailed data are given in Table S1, including 4 factorial points, 4 axial points, and 3 replicates of the central point, with additional replicas of each condition tested to guarantee the accuracy of the data. The respective coded and decoded matrices are given in Tables S1 and S2. The results obtained were statistically analyzed considering a confidence level of 95%. The software Statsoft Statistica 10.0 was used in the statistical analysis and preparation of the response surface plots.

RESULTS AND DISCUSSION

AMTPS and Mixed AMTPS Binodal Curves. The cloud points (temperatures of phase transition) of the systems composed of Triton X-114 or Tergitol 15-S-7 in McIlvaine aqueous buffer (pH 6.0, 0.16 M) are given in Figure 2. These were previously determined as a function of the nonionic surfactant composition,[20,36] with our results being in close agreement with the literature. The two nonionic surfactants have identical molecular weights and hydrophilic−lipophilic balances (HLB, 12.4 and 12.1 for Triton X-114 and Tergitol 15-S-7, respectively),[37] yet there is a difference of ca. 10 °C between their cloud points at similar compositions. Given the nonionic nature of the studied surfactants, these differences can be attributed to hydrogen bonding and van der Waals forces. The clouding phenomenon in nonionic surfactants occurs when surfactant−surfactant interactions are enhanced and surfactant−water interactions are weakened, thus leading to phase separation.[38] Furthermore, for the investigated nonionic surfactants, it has been suggested that conformational changes of the polar poly(oxyethylene) chains on heating induce phase separation.[39] The conformation of the C−C bonds becomes less polar as temperature increases, resulting in stronger surfactant−surfactant interactions than surfactant−water interactions and phase separation.[39] As previously mentioned, the two studied nonionic surfactants have identical molecular weight and HLB. However, Tergitol is a linear surfactant, whereas Triton presents ramified tails (Figure 1), with a clear impact on the surfactant−water and surfactant−surfactant interactions. Accordingly, the cloud points obtained indicate that Tergitol requires a higher energy input to weaken the water−surfactant interactions and to establish stronger surfactant−surfactant interactions, being a direct result of its linear chemical structure.

Novel binodal curves in the presence of cosurfactants, namely, SAILs, have been determined in this work and are depicted in Figure 2. Regarding the mixed systems, i.e., when

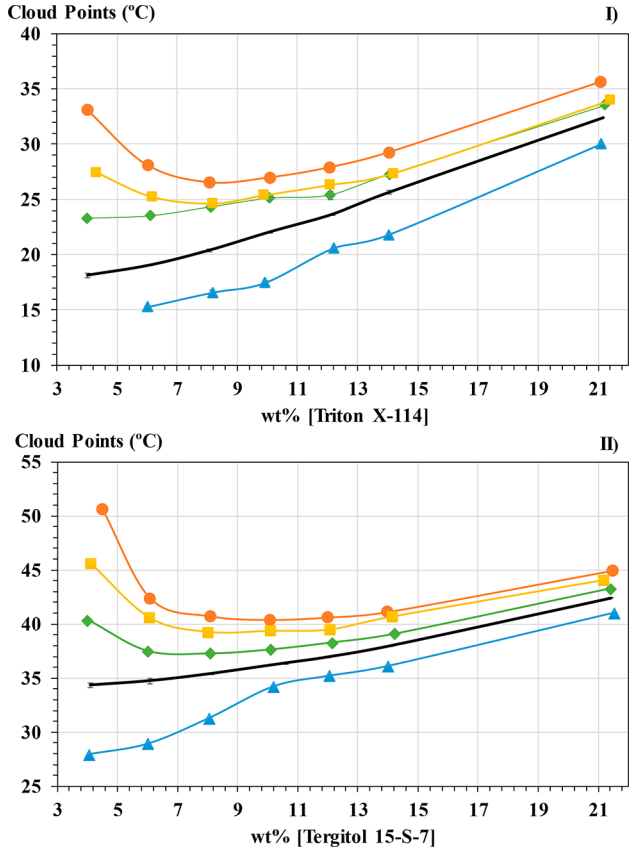

Figure 2. Binodal curves of AMTPS and mixed AMTPS composed of nonionic surfactants Triton X-114 (I) or Tergitol 15-S-7 (II) in the absence (line without symbols) and presence of SAILs as cosurfactants (symbols and line): ●, [C_{14}mim]Cl, ■, [$N_{1,1,12,(C_7H_7)}$]Br, ♦, [$P_{4,4,4,14}$]Cl, and ▲, [Ch][Tetradec]. All systems contain McIlvaine buffer (pH 6.0).

SAILs are used as cosurfactants at a concentration of 0.3 wt %, four SAILs were selected. These compounds not only allow evaluation of the effect of different ionic liquid families but also the study of both cationic and anionic-based SAILs. Figure 2 shows two different behaviors, namely, the increase or decrease of the cloud point temperature, when compared with the simple AMTPS (no cosurfactant present). The cloud point temperature increases when using cationic SAILs ([C_{14}mim]Cl, [$N_{1,1,12,(C_7H_7)}$]Br, and [$P_{4,4,4,14}$]Cl). These SAILs display a more hydrophilic nature and a critical packing parameter (CPP) lower than 0.5, thus promoting the formation of mixed and smaller micelles that require higher temperatures to undergo coacervation and phase separation.[20] Furthermore, a higher difference between the different ionic liquid cations is seen at lower surfactant concentrations due to the higher SAIL/nonionic surfactant ratio. The influence of the SAIL cation upon the cloud point temperature increase follows the rank phosphonium < ammonium < imidazolium. This trend clearly evidences the formation of micelles with distinct characteristics and different degrees of repulsion between neighbor micelles. The bulkier phosphonium cation with long alkyl side chains displays a more screened charge, resulting in a lower micelles repulsion and thus lower cloud point temperatures. In contrast, the imidazolium charge is less protected leading to a higher degree of repulsion between neighbor micelles, ending in higher cloud point temperatures.[40] In

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contrast, there is the cloud point decrease induced by the anionic SAIL, namely, [Ch][Tetradec]. This behavior may be attributed to the fact that both the ionic liquid cation (cholinium) and anion (tetradecanoate) are organic and large ions. This trend was also observed for other nonionic surfactants belonging to the Tergitol family.[20] These larger ions lead to the formation of larger micelles and to their coacervation at lower temperatures.[20] Overall, the AMTPS under study present a wide biphasic region, reaching at least 22 wt % nonionic surfactant with mild temperatures inducing phase separation. This is a crucial parameter to take into account when dealing with labile biomolecules, such as IgG and HSA proteins.

Characterization of Biological Samples. The plasma samples used in this work were initially characterized, allowing the identification of each protein and representative SE-HPLC chromatograms. The respective results are given in Figure 3,

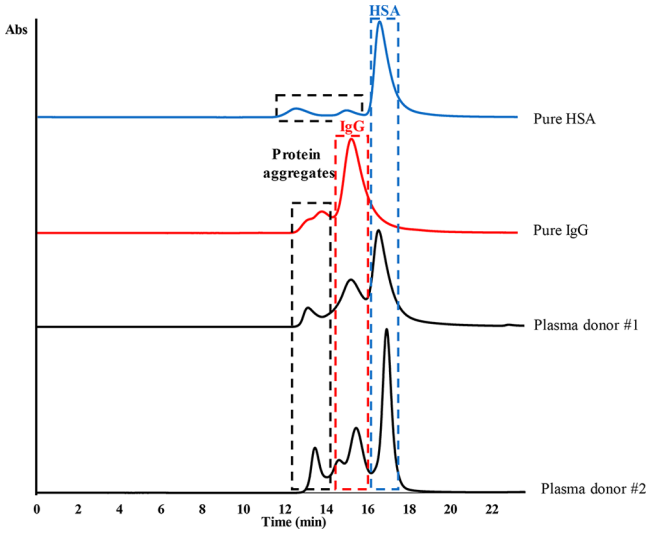

Figure 3. SE-HPLC chromatograms at 280 nm of pure aqueous solutions of HSA (blue) and IgG (red) and of the two expired human plasma samples. Protein aggregates (black rectangles) are visible in both pure commercial protein samples and human plasma samples.

where the peak corresponding to IgG appears at around 15 min and that of HSA appears at ca. 17 min. Chromatograms of both pure proteins show some higher molecular weight peaks, corresponding to protein aggregates, appearing at lower retention times when compared to those corresponding to IgG and HSA. There are 3 peaks in plasma samples from donor 1 and an additional peak in plasma from donor 2. In both samples, the peaks corresponding to IgG and HSA appear at similar retention times when compared to those of the pure proteins. It should be remarked that the partition coefficients, recovery yields and purification factors discussed below were determined for the nonaggregated forms of IgG and HSA.

In order to address the reproducibility of the technique applied on the separation of IgG and HSA, human serum was also used (chromatogram given in Figure S1).

Further characterization of both plasma and serum samples was carried out by the determination of the total proteins and dry matter contents. The used human serum sample has a dry matter content of 6 ± 1% and a total protein concentration of 76.6 ± 0.1 mg·mL^{-1}, while plasma samples have 10 ± 1% dry matter content and 81 ± 2 mg·mL^{-1} total protein concentration.

Separation of IgG and HSA Using AMTPS and Mixed AMTPS. After the determination of the cloud points of each AMTPS composed of nonionic surfactants and mixed AMTPS with SAILs used as cosurfactants, these were investigated for the simultaneous separation of IgG and HSA from expired human plasma. The major goal is to simultaneously separate both proteins by inducing the preferential migration of each protein to opposite phases, as schematized in Figure 1II. One of the advantages of these systems is that upon the AMTPS formation there is the establishment of two different environments, i.e., a hydrophobic surfactant-rich phase and a hydrophilic surfactant-poor phase. Accordingly, the separation of biomolecules in AMTPS may be governed by the phases' hydrophobicity/hydrophilicity and specific interactions established between each protein and the surfactant and/or water. However, in AMTPS comprising micelles, the protein molecular weight also plays a role, since a hydrophobic protein might be retrieved in the surfactant-poor phase if it displays a high molecular weight and size. These trends and phenomena are well-established in the literature.[41,42] In this sense, AMTPS seem an appropriate approach to simultaneously separate IgG from HSA due to different molecular weights, namely, 150 and 66.5 kDa.

Optimization of the Operational Conditions in AMTPS. The cloud point extraction was performed at a temperature of 40 °C to guarantee that the biphasic region is reached in the range of surfactant concentrations studied. To optimize the separation of both proteins to opposite phases with a maximum purity, a 2^2 factorial planning was applied to each nonionic surfactant. Two parameters, namely, the nonionic surfactant and plasma concentrations, were investigated. These factorial designs were analyzed using plasma from donor 1. The coded and decoded matrices are provided in Table S1 and are identical for both nonionic surfactants, whereas the experimental and theoretical data for the IgG and HSA purity factor (PFs) in both phases and the respective residues are displayed in Tables S3−S10. In general, these results show a negligible difference between the predicted and experimental data. The regression coefficients, standard deviation, Student's *t*, and *p*-values were also calculated and reported in Tables S11−S18. Partition coefficients of both IgG and HSA are given in Table S19. The response surface and contour plots of PF_{IgG} and PF_{HSA} as a function of the nonionic surfactant and plasma concentrations for the AMTPS composed of Triton X-114 or Tergitol 15-S-7 are displayed in Figures 4 and 5, respectively.

For a successful simultaneous separation of two proteins in AMTPS, each protein should preferentially partition toward opposite phases. The PF of each protein in each phase was used to evaluate the AMTPS separation performance. This parameter was used since it is a relative value, thus allowing us to suppress different purity levels in the original plasma samples (eq 1). This is particularly relevant when dealing with biological samples with significant differences in their composition, although not compromising the systems' separation performance reproducibility as shown below. Figures 4 and 5 simultaneously show the PF_{IgG} in the surfactant-poor phase and the PF_{HSA} in the surfactant-rich phase, with the results for PF_{IgG} and PF_{HSA} in the opposite phases displayed in Figures S2 and S3. These results show that IgG preferentially migrates toward the surfactant-poor phase, whereas HSA is more prone to be retrieved inside the micelles

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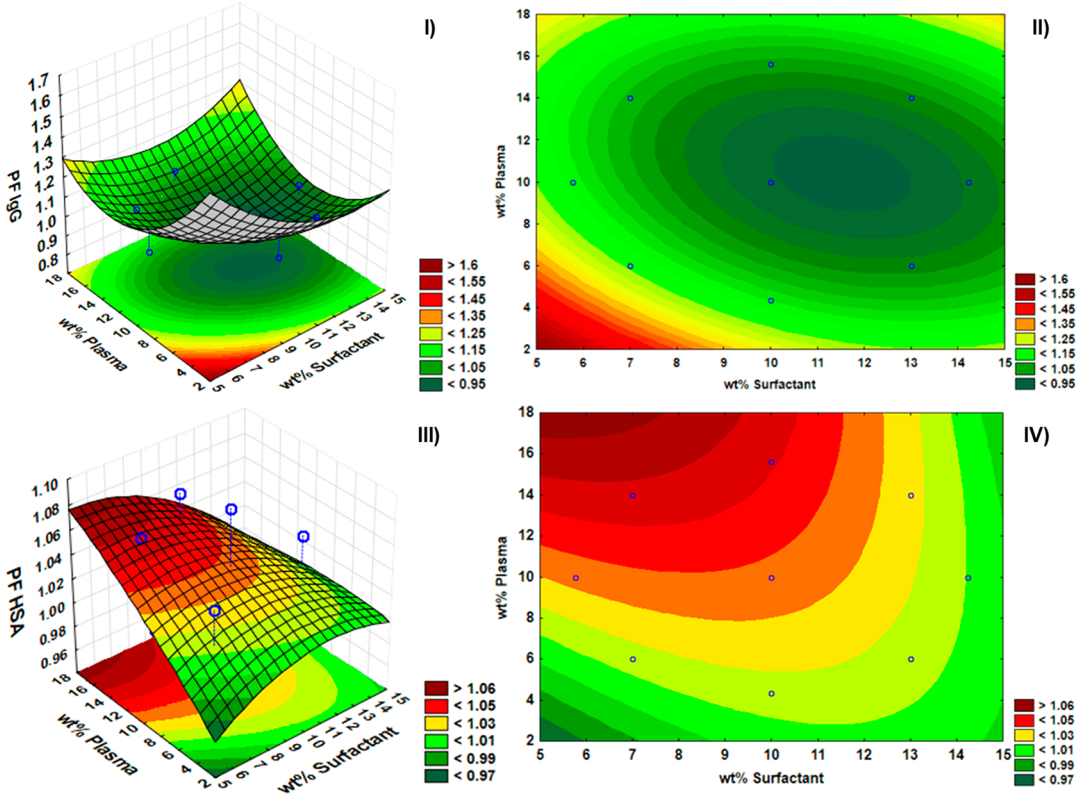

Figure 4. Response surface plots (left) and contour plots (right) of the PF_{IgG} in the surfactant-poor phase (I and II) and the PF_{HSA} in the surfactant-rich phase (III and IV) in the Triton X-114-based AMTPS. Data collected using plasma from donor 1.

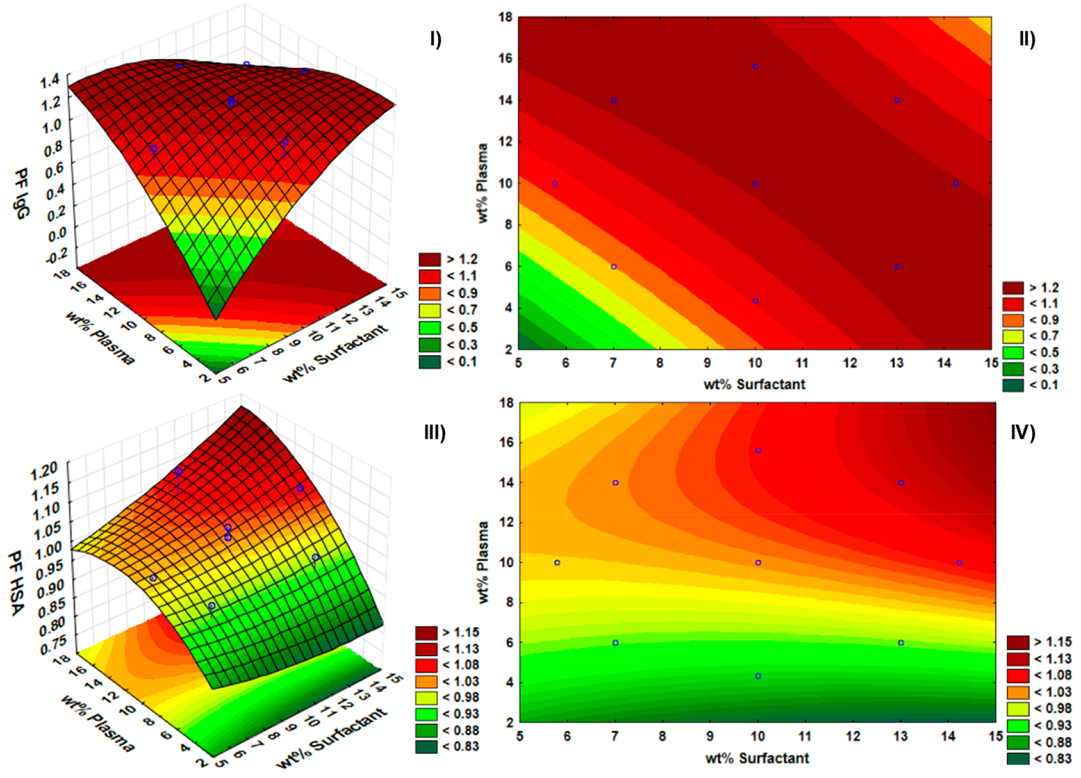

Figure 5. Response surface plots (left) and contour plots (right) of the PF_{IgG} in the surfactant-poor phase (I and II) and the PF_{HSA} in the surfactant-rich phase (III and IV) in the Tergitol 15-S-7-based AMTPS. Data collected using plasma from donor 1.

of the surfactant-rich phase. Taking into account that IgG is the most hydrophobic protein studied,[43,44] and yet preferentially enriches in the surfactant-poor phase, it is clear that the proteins molecular weight (150 kDa for IgG) is a relevant

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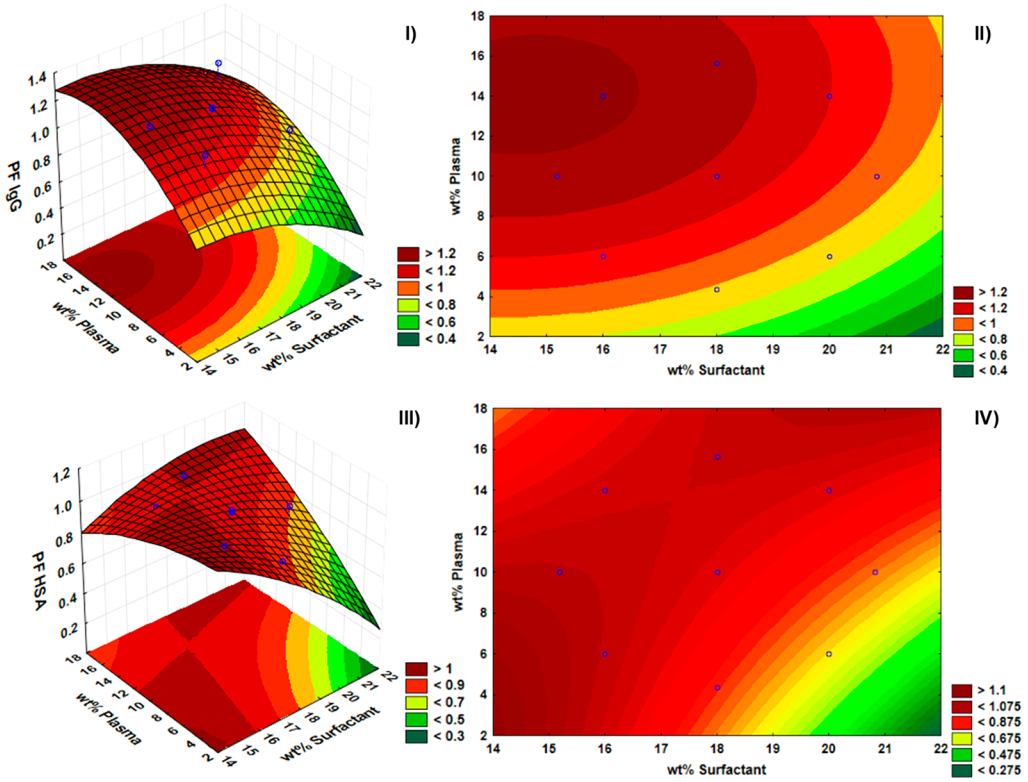

Figure 6. Response surface plots (left) and contour plots (right) of the PF_{IgG} in the surfactant-poor phase (I and II) and the PF_{HSA} in the surfactant-rich phase (III and IV) using a second 2^2 factorial planning for Tergitol 15-S-7. Data collected using plasma from donor 1.

parameter dictating the proteins partition behavior. HSA, being a smaller protein (66.5 kDa), is retrieved in the micelles core. This trend is in agreement with the literature, as shown by da Silva and Arruda[45] with a Triton X-114-based AMTPS.

Figure 4 shows that by using Triton X-114 and the investigated plasma concentrations, it is not possible to successfully separate IgG and HSA into distinct phases. While there are some experimental conditions that slightly improve the PF_{HSA} to the surfactant-rich phase, namely, a PF_{HSA} of 1.059 ± 0.002 with 14 wt % plasma and 7 wt % nonionic surfactant and a PF_{HSA} of 1.07 ± 0.02 with 15.6 wt % plasma and 10 wt % nonionic surfactant, none of these conditions allow a significant increase in the PF_{IgG} in the surfactant-poor phase. The only option to increase the IgG purity in this phase would be to reduce both plasma and nonionic surfactant concentrations. However, this is not economically viable since it would result in significantly lower amounts of IgG being available to purify. Additionally, at these experimental conditions, PF_{HSA} is far from its highest value, indicating a poor separation of both proteins. Herein, none of the independent variables are statistically significant as demonstrated by the Pareto charts (Figure S4). Therefore, Triton X-114-based AMTPS are not a good option to separate IgG and HSA from human plasma.

The replacement of Triton X-114 by Tergitol 15-S-7 leads, however, to promising results, as shown in Figure 5. Apart from the mixture point composed of 6 wt % plasma and 7 wt % nonionic surfactant (PF_{IgG} = 0.6 ± 0.2), all remaining experimental conditions result in improvements in the PF_{IgG} in the surfactant-poor phase, ranging between 1.1 (±0.2) and 1.25 (±0.01). Furthermore, most nonionic surfactant and plasma concentrations studied do not lead to high HSA purification factors. However, the contour plots obtained indicate that by further increasing the nonionic surfactant and plasma concentrations the PF_{HSA} could be increased. However, since the goal is to simultaneously separate both proteins yet keeping in mind that IgG is the most expensive and relevant protein to be recovered, a critical analysis of the conditions identified was carried out, leading us to the conclusion that the plasma concentration should be kept at intermediate values in order to maintain the PF_{IgG} at the highest values. Pareto charts (given in Figure S5) demonstrate that the plasma concentration is the only parameter significantly affecting the PF_{HSA} in the surfactant-rich phase. Concerning the PF_{IgG} and PF_{HSA} in the opposite phases, the results corroborate an enhanced separation in the mentioned phases as the PF_{IgG} in the surfactant-rich phase displays an average value of 0.58 ± 0.05 with a maximum of 0.65 ± 0.03, obtained with the mixture point of 6 wt % plasma and 7 wt % nonionic surfactant (the condition leading to the lowest IgG purification in the surfactant-poor phase). PF_{HSA} in the surfactant-poor phase also presents a low average value (0.88 ± 0.05) and a maximum of 1.03 ± 0.04 for the aforementioned concentrations, indicating that lower nonionic surfactant and plasma concentrations are definitely not appropriate for a successful separation process.

Aiming at improving the results obtained for the separation of HSA and IgG, a second 2^2 factorial planning was applied with increased nonionic surfactant concentrations and intermediate plasma concentrations, being the coded and decoded matrices provided in Table S2. Experimental and predicted PF_{IgG} and PF_{HSA} data in surfactant-poor and -rich phases, as well as their differences (residues), are presented in Tables S20−S23. These results show small differences between the model and experimental data. The regression coefficients, standard deviation, Student's *t*, and *p*-value were also

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calculated and are reported in Tables S24–S27. Partition coefficients of both IgG and HSA are shown in Table S28. The response surface and contour plots of the PF_{IgG} in the surfactant-poor phase and the PF_{HSA} in the surfactant-rich phase as a function of the nonionic surfactant and plasma concentrations are shown in Figure 6, with the results for the opposite phase depicted in Figure S6.

Figure 6 shows that by the proper choice of the experimental conditions it is possible to achieve a good separation of IgG and HSA from expired human plasma in a single step, particularly when using the mixture point composed of 10 wt % plasma and 15.2 wt % nonionic surfactant. At these concentrations, the PF_{IgG} in the surfactant-poor phase reaches its maximum (1.26 ± 0.01), whereas the PF_{HSA} in the surfactant-rich phase is 1.01 ± 0.02. Pareto charts (Figure S7) show that individual plasma and nonionic surfactant concentrations, as well as a combined effect, i.e., plasma concentration × nonionic surfactant concentration, display a significant influence upon the PF_{IgG} in the surfactant-rich phase. In contrast, only the plasma concentration and the combined effect of plasma and nonionic surfactant concentrations statistically influence the PF_{HSA} in the surfactant-poor phase. Hence, 10 wt % plasma and 15.2 wt % Tergitol 15-S-7 were selected as the most selective experimental conditions to be used in further optimization studies at different pH values to create mixed AMTPS involving SAILs as cosurfactants.

pH Influence in the IgG and HSA Separation Using AMTPS. While the previously results were obtained at pH 6.0, additional pH values of 7.0 and 8.0 were investigated to evaluate the influence of pH on the IgG and HSA simultaneous separation. HSA displays an isoelectric point (p*I*) of 4.7;[46,47] therefore, it has a negative charge in the studied pH range. IgG has a p*I* ranging between 6.6 and 7.2,[48] with a large fraction of the protein negatively charged at pH 8. This set of studies was done with plasma from donor 2. The pH effect on the PF_{IgG} in the surfactant-poor phase and on the PF_{HSA} in the surfactant-rich phase is shown in Table 1, being the data for the opposite phases presented in Table S29. It should be highlighted that the purification analysis was carried out considering PF since this parameter overcomes the different purity levels of each protein in the original plasma samples, thus allowing an accurate analysis of the AMTPS separation performance.

Table 1. PF_{IgG} and IgG Yield in the Surfactant-Poor Phase and PF_{HSA} and HSA Yield in the Surfactant-Rich Phase, with the Respective Standard Deviation (σ), using AMTPS at Several pH Values and Plasma from Donor 2

	$IgG_{surfactant\text{-}poor\text{-}phase}$		$HSA_{surfactant\text{-}rich\text{-}phase}$	
pH	PF + σ	(yield + σ)/%	PF + σ	(yield + σ)/%
6.0	1.03 ± 0.02	47 ± 2	1.27 ± 0.01	54 ± 2
7.0	1.03 ± 0.05	75 ± 4	1.42 ± 0.06	25 ± 1
8.0	1.02 ± 0.01	89 ± 4	1.6 ± 0.2	12 ± 2

The data given in Table 1 demonstrate a small influence of pH over the PF_{HSA} and no effect on the PF_{IgG}. Nonetheless, it plays an important role on the yield of both proteins: there is a decrease on the yield of HSA and an increase on the yield of IgG as the pH increases. The differences in the yield of HSA are more pronounced as a result of its lower p*I*, thus leading to a more significant pH contribution toward proteins aggregation and losses. Similar behaviors have been reported in the literature.[45] Although pH can be a significant parameter in the separation performance of other separation techniques, e.g., ionic chromatography, this does not apply in the current work. pH mainly affects the proteins yields but not the purification performance. Overall, it was found that pH is not the driving force on the partitioning of both proteins to different phases in AMTPS. Instead, the separation of the proteins in AMTPS is governed by specific interactions established between each protein and the phase-forming components and by the proteins molecular weight, as discussed above. Overall, considering the importance and cost associated with antibodies, pH 8.0 was selected for further optimizations since it leads to the highest IgG yield.

It should be stressed that although we only determined the binodal curves for pH 6.0 while also studying pH 7.0 and 8.0 in the separation step we are always working in the biphasic region as demonstrated elsewhere.[20] Binodal curves with these mixed systems involving SAILs at pH 8.0 were not previously reported; nonetheless, it was shown that there is not a significant difference in the binodal curves of the respective AMTPS at pH 7.0 and 8.0.[20]

SAILs Effect in the IgG and HSA Separation Using Mixed AMTPS. Mixed AMTPS have shown to considerably enhance the extraction performance of different compounds, including proteins,[24,49] and to increase their stability.[25] Therefore, we investigated the addition of several SAILs as cosurfactants at 0.3 wt % to the best identified AMTPS (10 wt % plasma and 15.2 wt % Tergitol 15-S-7). The studied SAILs comprise different cation core families, namely, imidazolium ($[C_{14}mim]$-Cl), phosphonium ($[P_{4,4,4,14}]Cl$), and tetraalkylamonium ([Ch][Tetradec] and $[N_{1,1,12,(C_7H_7)}]Br$), and different anions, namely, chloride, bromide, and tetradecanoate. The set of chosen SAILs allows the analysis of both cationic and anionic cosurfactants on the separation process performance. Both PF_{IgG} and PF_{HSA} in the surfactant-poor and -rich phases, respectively, and the correspondent recovery yields obtained with the mixed AMTPS are given in Table 2. The PF for both proteins in the opposite phase as well as their yields and partition coefficients are given in Table S30.

Table 2. PF_{IgG} and IgG Yield in the Surfactant-Poor Phase and PF_{HSA} and HAS Yield in the Surfactant-Rich Phase, with the Respective Standard Deviation (σ), Using Mixed AMTPS and Plasma from Donor 2

	$IgG_{surfactant\text{-}poor\ phase}$		$HSA_{surfactant\text{-}rich\ phase}$	
AMTPS	PF + σ	(yield + σ)/%	PF + σ	(yield + σ)/%
without SAIL	1.02 ± 0.01	89 ± 4	1.6 ± 0.2	12 ± 2
$[C_{14}mim]Cl$	1.08 ± 0.01	78 ± 1	1.24 ± 0.02	26 ± 2
$[P_{4,4,4,14}]Cl$	1.14 ± 0.07	86 ± 1	1.36 ± 0.05	15 ± 1
[Ch][Tetradec]	1.00 ± 0.03	94 ± 3	1.6 ± 0.1	8.068 ± 0.003
$[N_{1,1,12,(C_7H_7)}]Br$	1.083 ± 0.001	82 ± 2	1.669 ± 0.004	16.3 ± 0.3

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Table 3. PF_{IgG} and IgG Yield in the Surfactant-Poor Phase and PF_{HSA} and HSA Yield in the Surfactant-Rich Phase, with the Respective Standard Deviation (σ), Using Plasma, Serum, and Plasma/Serum Mixture Samples

	$IgG_{surfactant\text{-}poor\ phase}$		$HSA_{surfactant\text{-}rich\ phase}$	
AMTPS	PF + σ	(yield + σ)/%	PF + σ	(yield + σ)/%
plasma 2	1.14 ± 0.07	86 ± 1	1.36 ± 0.05	15 ± 1
serum	1.01 ± 0.01	88 ± 2	1.01 ± 0.01	25 ± 1
plasma/serum mixture (50:50, wt %/wt %)	1.02 ± 0.02	90 ± 7	1.021 ± 0.003	20 ± 1

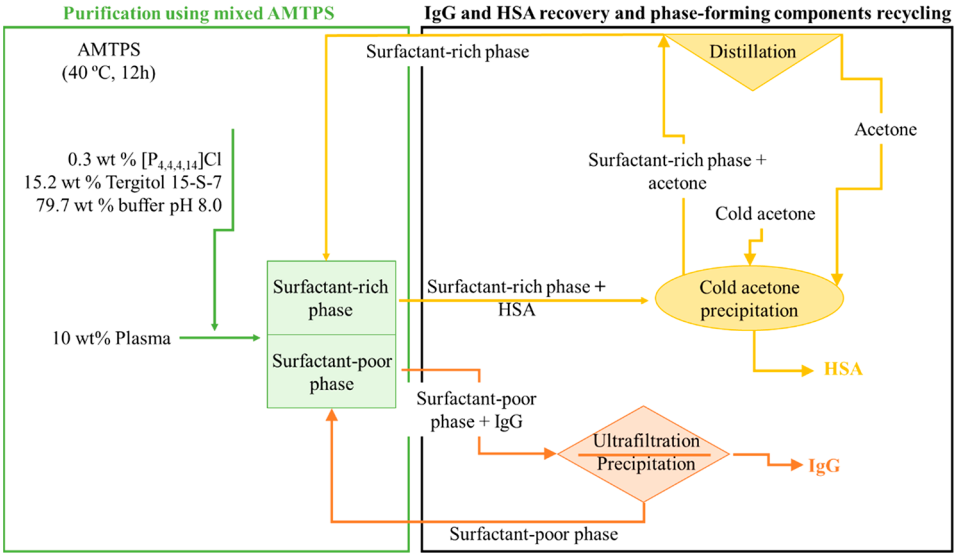

Figure 7. Diagram of the envisioned process for the simultaneous separation of IgG and HSA from expired human plasma.

By the proper choice of the SAIL in the mixed AMTPS, the simultaneous separation of IgG and HSA can be enhanced. PF_{HSA} increases in the following order: $[C_{14}mim]Cl < [P_{4,4,4,14}]Cl <$ no SAIL ≈ [Ch][Tetradec] < $[N_{1,1,12,(C_7H_7)}]Br$, whereas PF_{IgG} follows the increasing trend: [Ch][Tetradec] ≈ no SAIL < $[C_{14}mim]Cl$ ≈ $[N_{1,1,12,(C_7H_7)}]Br$ < $[P_{4,4,4,14}]Cl$. The $[P_{4,4,4,14}]Cl$-based AMTPS is able not only to increase (by 0.12-fold) the IgG purification in the surfactant-poor phase compared to the system without SAIL but also to increase (by 3%) the HSA recovery yield in the opposite phase. Although the recovery of HSA is still low, it should be remarked that it exists in higher quantities in blood and plasma and that antibodies are a main target due to their higher cost and use as potential therapeutics. Furthermore, IgG is present in the surfactant-poor phase, which is mainly composed of McIlvaine buffer and can be used for antibody preservation.

To evaluate the reproducibility of the proposed AMTPS, additional studies were carried out using a human serum sample and a 50:50 (wt %/wt %) mixture of human serum and plasma with the AMTPS composed of 15.2 wt % nonionic surfactant + 0.3 wt % $[P_{4,4,4,14}]Cl$ + 10 wt % serum or mixture of serum and plasma +74.5 wt % McIlvaine buffer at pH 8.0. The obtained results are given in Tables 3 and S31. The respective partition coefficients are also provided in Table S31. Although dealing with biological samples that inherently display different compositions, the obtained results demonstrate that the preferential enrichment of each protein in each phase is kept, even when applied to a different type of matrix such as serum and serum−plasma mixtures. The main difference between these samples is that human serum does not contain clotting factors in its composition and presents more water in comparison with human plasma. These results confirm thus the reproducibility of the proposed AMTPS process. Although the purification factors are not as high as desired, the main advantage of the current process is that it allows the enrichment of both proteins in opposite phases. This approach is not attempted with chromatographic methods, and no works in the literature proposed the simultaneous separation of both proteins from plasma samples in a single-step.

The results achieved for the antibody purity are lower than those obtained for the IgG purification from human plasma with a traditional polymer−salt ATPS.[13] Nevertheless, our extraction yield is 13% higher, probably due to the fact that IgG is never precipitated during the fractionation process as it occurs in the PEG-based systems in the work of Vargas and co-workers.[13] Additionally, both extraction yields correspond to more than double the yield achieved with the conventional Cohn method, which is ca. 20−50%.[13] Regarding the HSA separation and purification, our yields are lower than previously reported ones.[13] It should be stressed however that even though IgG purification is a hot topic[10,13,14,17−19,50−54] only in this work and in the studies developed by Vargas and co-workers[13,14] the use of human plasma was addressed. Furthermore, previous works[13,14] reported the use of a multistep approach, where the immunoglobulin-rich phase is run through different steps of purification, namely, caprylic acid precipitation, followed by filtration and dialysis and a final step of anionic exchange chromatography.[13] This process was latter improved, where the immunoglobulin-rich phase was first precipitated with caprylic acid and filtrated before being applied in a diafiltration unit, followed by anionic chromatography and a final diafiltration stage combined with ultrafiltration steps.[14]

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Authors were able to fractionate human plasma within the distinct IgG isoforms, clot factors, plasmin, and thrombin.[14] Despite these promising results, we focused on the development of a more sustainable downstream process to separate IgG and HSA from human plasma in a single step, while maintaining their structural integrity and stability, as proved by the absence of new peaks or increase of protein aggregates in the SE-HPLC chromatograms (cf. Figure S8). It is noteworthy to mention that the final purification levels achieved for both IgG and HSA are still below the purity levels achieved with chromatographic techniques, and additional work for optimization should be carried out. Still, the effect of SAILs to improve these systems separation performance was demonstrated, deserving attention by researchers working on the development of purification platforms for value-added biomolecules. For further purification of the proteins present in each phase, low-cost ultrafiltration or precipitation steps can be included to increase the purification factor.[55,56] An overview of the overall process and options to improve the separation performance are given in Figure 7. It is also proposed the possibility of recycling the system phase-forming components, contributing to the sustainability of the envisioned separation process.

CONCLUSIONS

Not all hospitals and health care centers have plasma manufacturers in their vicinities, and most of the unused clinical transfusion plasma is discarded. However, the present proteins, including serum albumin, immunoglobulins, and clotting factors, may be still viable to be used in diagnosis or research. Herein, AMTPS composed of nonionic surfactants, namely, Triton X-114 or Tergitol 15-S-7, and mixed AMTPS comprising Tergitol 15-S-7 and SAILs used as cosurfactants were characterized and applied toward the separation of IgG, the most abundant and valuable antibody present in human plasma, from HSA, the most abundant protein in plasma. In all studies, expired plasma samples were used, contributing to several items included in the Sustainable Development Goals of Agenda 2030. The separation process performance was optimized in terms of plasma and nonionic surfactant concentrations, pH, and SAIL type applied to create mixed AMTPS. The best results were achieved with the mixed AMTPS composed of Tergitol 15-S-7 and the SAIL [$P_{4,4,4,14}$] Cl at pH 8.0, resulting in a IgG purification factor of 1.14-fold in the surfactant-poor phase and a HSA purification factor of 1.36-fold in the surfactant-rich phase. The separation performance reproducibility was ascertained by applying the best identified mixed AMTPS to samples of human serum and mixtures of serum and plasma. Although the purification factors are not as high as desired, the best AMTPS allowed the enrichment of both proteins in opposite phases. This approach is not attempted with chromatographic methods, and no works in the literature proposed the simultaneous separation of both proteins from plasma samples in a single-step. Overall, mixed AMTPS comprising nonionic surfactants and SAILs as cosurfactants can be used to simultaneously separate IgG and HSA from expired plasma samples and should be a target of future attention to be used in the fractionation of other value-added compounds from complex and natural matrices.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.9b03841.

Experimental data used in the factorial planning, purification factors and yields experimentally obtained and respective fitted values, statistical analysis, partition coefficients, and SE-HPLC chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Grazzini, G.; Mannucci, P. M.; Oleari, F. Plasma-Derived Medicinal Products: Demand and Clinical Use. *Blood Transfus.* **2013**, *11*, s2−s5.

(2) Farrugia, A.; Cassar, J. Plasma-Derived Medicines: Access and Usage Issues. *Blood Transfus.* **2012**, *10* (3), 273−278.

(3) Liumbruno, G.; Bennardello, F.; Lattanzio, A.; Piccoli, P.; Rossettias, G. Recommendations for the Use of Albumin and Immunoglobulins. *Blood Transfus.* **2009**, *7* (3), 216−234.

(4) Celis, P.; Silvester, G. European Regulatory Guidance on Virus Safety of Recombinant Proteins, Monoclonal Antibodies and Plasma Derived Medicinal Products. *Dev. Biol. (Basel)* **2004**, *118*, 3−10.

(5) Buchacher, A.; Iberer, G. Purification of Intravenous Immunoglobulin G from Human Plasma − Aspects of Yield and Virus Safety. *Biotechnol. J.* **2006**, *1* (2), 148−163.

(6) Hess, J. R. Conventional Blood Banking and Blood Component Storage Regulation: Opportunities for Improvement. *Blood Transfus.* **2010**, *8*, s9−s15.

(7) *Improving Access to Safe Blood Products Through Local Production and Technology Transfer in Blood Establishments*; World Health Organization, 2017.

(8) *Antibody Production Market by Process (Upstream Processing, Downstream Processing, and Filtration), End-User (Pharmaceutical & Biotechnology Companies, Diagnostic Laboratories, and Research Institutes) - Global Forecast to 2021*; Markets and Markets, 2016.

(9) Lipman, N. S.; Jackson, L. R.; Weis-Garcia, F.; Trudel, L. J. Monoclonal *versus* Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR J.* **2005**, *46* (3), 258−268.

DOI: 10.1021/acssuschemeng.9b03841
ACS Sustainable Chem. Eng. 2019, 7, 15102−15113

(10) Rosa, P. A. J.; Azevedo, A. M.; Aires-Barros, M. R. Application of Central Composite Design to the Optimisation of Aqueous Two-Phase Extraction of Human Antibodies. *J. Chromatogr. A* **2007**, *1141* (1), 50−60.
(11) Low, D.; O'Leary, R.; Pujar, N. S. Future of Antibody Purification. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2007**, *848* (1), 48−63.
(12) Cohn, E. J.; Strong, L. E.; Hughes, W. L.; Mulford, D. J.; Ashworth, J. N.; Melin, M.; Taylor, H. L. Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids. *J. Am. Chem. Soc.* **1946**, *68* (3), 459−475.
(13) Vargas, M.; Segura, Á.; Herrera, M.; Villalta, M.; Angulo, Y.; Gutiérrez, J. M.; León, G.; Burnouf, T. Purification of IgG and Albumin from Human Plasma by Aqueous Two Phase System Fractionation. *Biotechnol. Prog.* **2012**, *28* (4), 1005−1011.
(14) Vargas, M.; Segura, Á; Wu, Y.-W.; Herrera, M.; Chou, M.-L.; Villalta, M.; León, G.; Burnouf, T. Human Plasma-Derived Immunoglobulin G Fractionated by an Aqueous Two-Phase System, Caprylic Acid Precipitation, and Membrane Chromatography Has a High Purity Level and Is Free of Detectable in Vitro Thrombogenic Activity. *Vox Sang.* **2015**, *108* (2), 169−177.
(15) Flickinger, M. C. *Downstream Industrial Biotechnology: Recovery and Purification*; John Wiley & Sons, 2013.
(16) Painter, R. H. IgG. In *Encyclopedia of Immunology*, 2nd ed.; Delves, P. J., Roitt, I. M., Eds.; Elsevier: Oxford, U.K., 1998; pp 1208−1211.
(17) Azevedo, A. M.; Gomes, A. G.; Rosa, P. A. J.; Ferreira, I. F.; Pisco, A. M. M. O.; Aires-Barros, M. R. Partitioning of Human Antibodies in Polyethylene Glycol−Sodium Citrate Aqueous Two-Phase Systems. *Sep. Purif. Technol.* **2009**, *65* (1), 14−21.
(18) Azevedo, A. M.; Rosa, P. A. J.; Ferreira, I. F.; Pisco, A. M. M. O.; de Vries, J.; Korporaal, R.; Visser, T. J.; Aires-Barros, M. R. Affinity-Enhanced Purification of Human Antibodies by Aqueous Two-Phase Extraction. *Sep. Purif. Technol.* **2009**, *65* (1), 31−39.
(19) Ferreira, I. F.; Azevedo, A. M.; Rosa, P. A. J.; Aires-Barros, M. R. Purification of Human Immunoglobulin G by Thermoseparating Aqueous Two-Phase Systems. *J. Chromatogr. A* **2008**, *1195* (1−2), 94−100.
(20) Vicente, F. A.; Cardoso, I. S.; Sintra, T. E.; Lemus, J.; Marques, E. F.; Ventura, S. P. M.; Coutinho, J. A. P. Impact of Surface Active Ionic Liquids on the Cloud Points of Nonionic Surfactants and the Formation of Aqueous Micellar Two-Phase Systems. *J. Phys. Chem. B* **2017**, *121* (37), 8742−8755.
(21) Leite, A. C.; Ferreira, A. M.; Morais, E. S.; Khan, I.; Freire, M. G.; Coutinho, J. A. P. Cloud Point Extraction of Chlorophylls from Spinach Leaves Using Aqueous Solutions of Nonionic Surfactants. *ACS Sustainable Chem. Eng.* **2018**, *6* (1), 590−599.
(22) Santos, V. C.; Hasmann, F. A.; Converti, A.; Pessoa, A. Liquid−Liquid Extraction by Mixed Micellar Systems: A New Approach for Clavulanic Acid Recovery from Fermented Broth. *Biochem. Eng. J.* **2011**, *56* (1−2), 75−83.
(23) Malpiedi, L. P.; Nerli, B. B.; Abdala, D. S. P.; Pessôa-Filho, P. de A.; Pessoa, A. Aqueous Micellar Systems Containing Triton X-114 and Pichia Pastoris Fermentation Supernatant: A Novel Alternative for Single Chain-Antibody Fragment Purification. *Sep. Purif. Technol.* **2014**, *132*, 295−301.
(24) Vicente, F. A.; Malpiedi, L. P.; e Silva, F. A.; Pessoa, A.; Coutinho, J. A.P.; Ventura, S. P.M. Design of Novel Aqueous Micellar Two-Phase Systems Using Ionic Liquids as Co-Surfactants for the Selective Extraction of (Bio)Molecules. *Sep. Purif. Technol.* **2014**, *135*, 259−267.
(25) Vicente, F. A.; Lario, L. D.; Pessoa, A., Jr.; Ventura, S. P. M. Recovery of Bromelain from Pineapple Stem Residues Using Aqueous Micellar Two-Phase Systems with Ionic Liquids as Co-Surfactants. *Process Biochem.* **2016**, *51* (4), 528−534.
(26) de Faria, E. L. P.; Shabudin, S. V.; Claúdio, A. F. M.; Válega, M.; Domingues, F. M. J.; Freire, C. S. R.; Silvestre, A. J. D.; Freire, M. G. Aqueous Solutions of Surface-Active Ionic Liquids: Remarkable Alternative Solvents To Improve the Solubility of Triterpenic Acids and Their Extraction from Biomass. *ACS Sustainable Chem. Eng.* **2017**, *5* (8), 7344−7351.
(27) Mazid, R. R.; Vijayaraghavan, R.; MacFarlane, D. R.; Cortez-Jugo, C.; Cheng, W. Inhibited Fragmentation of MAbs in Buffered Ionic Liquids. *Chem. Commun.* **2015**, *51* (38), 8089−8092.
(28) Kumar, A.; Bisht, M.; Venkatesu, P. Biocompatibility of Ionic Liquids towards Protein Stability: A Comprehensive Overview on the Current Understanding and Their Implications. *Int. J. Biol. Macromol.* **2017**, *96*, 611−651.
(29) Pedro, A. Q.; Pereira, P.; Quental, M. J.; Carvalho, A. P.; Santos, S. M.; Queiroz, J. A.; Sousa, F.; Freire, M. G. Cholinium-Based Good's Buffers Ionic Liquids as Remarkable Stabilizers and Recyclable Preservation Media for Recombinant Small RNAs. *ACS Sustainable Chem. Eng.* **2018**, *6* (12), 16645−16656.
(30) Singh, N.; Sharma, M.; Mondal, D.; Pereira, M. M.; Prasad, K. Very High Concentration Solubility and Long-Term Stability of DNA in an Ammonium-Based Ionic Liquid: A Suitable Medium for Nucleic Acid Packaging and Preservation. *ACS Sustainable Chem. Eng.* **2017**, *5* (2), 1998−2005.
(31) Transforming our world: the 2030 Agenda for Sustainable Development. *United Nations*. https://sustainabledevelopment.un.org/post2015/transformingourworld.
(32) Sintra, T. E.; Vilas, M.; Martins, M.; Ventura, S. P. M.; Lobo Ferreira, A. I. M. C.; Santos, L. M. N. B. F.; Gonçalves, F. J. M.; Tojo, E.; Coutinho, J. A. P. Synthesis and Characterization of Surface-Active Ionic Liquids Used in the Disruption of *Escherichia coli* Cells. *ChemPhysChem* **2019**, *20* (5), 727−735.
(33) Blankschtein, D.; Thurston, G. M.; Benedek, G. B. Phenomenological Theory of Equilibrium Thermodynamic Properties and Phase Separation of Micellar Solutions. *J. Chem. Phys.* **1986**, *85* (12), 7268−7288.
(34) Moggridge, J.; Biggar, K.; Dawson, N.; Storey, K. B. Sensitive Detection of Immunoglobulin G Stability Using in Real-Time Isothermal Differential Scanning Fluorimetry: Determinants of Protein Stability for Antibody-Based Therapeutics. *Technol. Cancer Res. Treat.* **2017**, *16* (6), 997−1005.
(35) Picó, G. A. Thermodynamic Features of the Thermal Unfolding of Human Serum Albumin. *Int. J. Biol. Macromol.* **1997**, *20* (1), 63−73.
(36) Spir, L. G.; Ataide, J. A.; De Lencastre Novaes, L. C.; De Borba Gurpilhares, D.; Moriel, P.; Silveira, E.; Pessoa, A.; Tambourgi, E. B.; Mazzola, P. G. Application of an Aqueous Two-phase Micellar System to Extract Bromelain from Pineapple (Ananas Comosus) Peel Waste and Analysis of Bromelain Stability in Cosmetic Formulations. *Biotechnol. Prog.* **2015**, *31* (4), 937−945.
(37) (a) Triton X-114. https://www.sigmaaldrich.com/catalog/product/sial/x114?lang=en&=US&gclid=Cj0KCQjwhdTqBRDNARIsABsOl9-MXb9EEl7_ZbODRRQDjmvJqqoJq6qeIOwIR-zKIcnXnrl2JPcVqoQaAthiEALw_wcB. (b) Tergitol 15-S-7. https://www.sigmaaldrich.com/catalog/product/sigma/15s7?lang=en&region=US.
(38) Naqvi, A. Z.; Kabir-ud-Din. Clouding Phenomenon in Amphiphilic Systems: A Review of Five Decades. *Colloids Surf., B* **2018**, *165*, 325−344.
(39) Lindman, B.; Medronho, B.; Karlström, G. Clouding of Nonionic Surfactants. *Curr. Opin. Colloid Interface Sci.* **2016**, *22*, 23−29.
(40) Vicente, F. A. A. Design and Application of Ionic Liquid-Based Thermo-Responsive Systems to Purify Therapeutic Proteins. University of Aveiro, 2019.
(41) Liu, C. L.; Nikas, Y. J.; Blankschtein, D. Novel Bioseparations Using Two-Phase Aqueous Micellar Systems. *Biotechnol. Bioeng.* **1996**, *52* (2), 185−192.
(42) Rangel-Yagui, C. O.; Pessoa-Jr, A.; Blankschtein, D. Two-Phase Aqueous Micellar Systems: An Alternative Method for Protein Purification. *Braz. J. Chem. Eng.* **2004**, *21* (4), 531−544.

DOI: 10.1021/acssuschemeng.9b03841
ACS Sustainable Chem. Eng. 2019, 7, 15102−15113

(43) Malmsten, M.; Lassen, B. Competitive Adsorption at Hydrophobic Surfaces from Binary Protein Systems. *J. Colloid Interface Sci.* **1994**, *166* (2), 490−498.
(44) Svendsen, I. E.; Santos, O.; Sotres, J.; Wennerberg, A.; Breding, K.; Arnebrant, T.; Lindh, L. Adsorption of HSA, IgG and Laminin-1 on Model Hydroxyapatite Surfaces − Effects of Surface Characteristics. *Biofouling* **2012**, *28* (1), 87−97.
(45) da Silva, M. A. O.; Arruda, M. A. Z. An Aqueous Two-Phase System as a Strategy for Serum Albumin Depletion. *Talanta* **2009**, *77* (3), 985−990.
(46) Vlasova, I. M.; Saletsky, A. M. Study of the Denaturation of Human Serum Albumin by Sodium Dodecyl Sulfate Using the Intrinsic Fluorescence of Albumin. *J. Appl. Spectrosc.* **2009**, *76* (4), 536−541.
(47) Fogh-Andersen, N.; Bjerrum, P. J.; Siggaard-Andersen, O. Ionic Binding, Net Charge, and Donnan Effect of Human Serum Albumin as a Function of pH. *Clin. Chem.* **1993**, *39* (1), 48−52.
(48) Molecular weight and isoelectric point of various animal immunoglobulins. *Agrisera*. https://www.agrisera.com/en/info/molecular-weight-and-isoelectric-point-of-various-immunoglobulins.html.
(49) Vicente, F. A.; Cardoso, I.; Martins, M.; Gonçalves, C.; Dias, A.; Domingues, P.; Coutinho, J. A. P.; Ventura, S. P. M. R-Phycoerythrin Extraction and Purification from Fresh Gracilaria Sp. Using Thermo-Responsive Systems. *Green Chem.* **2019**, *21*, 3816.
(50) Mondal, D.; Sharma, M.; Quental, M. V.; Tavares, A. P. M.; Prasad, K.; Freire, M. G. Suitability of Bio-Based Ionic Liquids for the Extraction and Purification of IgG Antibodies. *Green Chem.* **2016**, *18* (22), 6071−6081.
(51) Mao, L. N.; Rogers, J. K.; Westoby, M.; Conley, L.; Pieracci, J. Downstream Antibody Purification Using Aqueous Two-phase Extraction. *Biotechnol. Prog.* **2010**, *26* (6), 1662−1670.
(52) Azevedo, A. M.; Rosa, P. A. J.; Ferreira, I. F.; Aires-Barros, M. R. Optimisation of Aqueous Two-Phase Extraction of Human Antibodies. *J. Biotechnol.* **2007**, *132* (2), 209−217.
(53) Rosa, P. A. J.; Azevedo, A. M.; Sommerfeld, S.; Mutter, M.; Aires-Barros, M. R.; Bäcker, W. Application of Aqueous Two-Phase Systems to Antibody Purification: A Multi-Stage Approach. *J. Biotechnol.* **2009**, *139* (4), 306−313.
(54) Ferreira, A. M.; Faustino, V. F. M.; Mondal, D.; Coutinho, J. A. P.; Freire, M. G. Improving the Extraction and Purification of Immunoglobulin G by the Use of Ionic Liquids as Adjuvants in Aqueous Biphasic Systems. *J. Biotechnol.* **2016**, *236*, 166−175.
(55) dos Santos, N. V.; Martins, M.; Santos-Ebinuma, V. C.; Ventura, S. P. M.; Coutinho, J. A. P.; Valentini, S. R.; Pereira, J. F. B. Aqueous Biphasic Systems Composed of Cholinium Chloride and Polymers as Effective Platforms for the Purification of Recombinant Green Fluorescent Protein. *ACS Sustainable Chem. Eng.* **2018**, *6* (7), 9383−9393.
(56) Santos, J. H. P. M.; Trigo, J. P.; Maricato, É.; Nunes, C.; Coimbra, M. A.; Ventura, S. P. M. Fractionation of Isochrysis Galbana Proteins, Arabinans, and Glucans Using Ionic-Liquid-Based Aqueous Biphasic Systems. *ACS Sustainable Chem. Eng.* **2018**, *6* (11), 14042−14053.

DOI: 10.1021/acssuschemeng.9b03841
ACS Sustainable Chem. Eng. 2019, 7, 15102−15113