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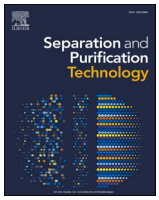

Using three-phase partitioning for the purification and recovery of antibodies from biological media

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ABSTRACT

Antibodies, in particular immunoglobulin G (IgG), are one of the biopharmaceutical industry highflyers, with relevance for the treatment of several diseases. However, the recovery of antibodies from complex biological media with high quality and purity is difficult and requires multi-step and expensive approaches. Herein, we propose a cost-effective approach using three-phase partitioning (TPP) systems based on polyethylene glycol (PEG)-salt aqueous biphasic systems (ABS) with ionic liquids (ILs) as adjuvants for the purification and recovery of IgG antibodies from three biological media, i.e., human serum, and serum-containing and serum-free Chinese hamster ovary (CHO) cell culture supernatants. The economic analysis of the developed process was carried out. The results obtained using PEG-salt ABS without ILs and human serum show that IgG could be recovered either at the interphase of the TPP or in the top phase, depending on the molecular weight of the PEG. The system composed of PEG with a molecular weight of 1000 g/mol is the PEG-salt system enabling the highest purity of human polyclonal IgG at the interphase (80.7 %, with a recovery yield of 65.8 %). Still, by adding 1 wt% of the ILs tetra(*n*-butyl)ammonium bromide ($[N_{4444}]Br$) and 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$), it is possible to compete or even outperform the PEG 1000-salt system with no IL regarding purity/recovery performance and production costs under given operation conditions. The best systems were then applied for the purification and recovery of monoclonal antibodies from serum-containing and serum-free culture supernatants. Improved recovery of monoclonal antibodies from serum-free Chinese hamster ovary (CHO) cell culture supernatants with a reduction of the host cell proteins (HCPs) content are obtained by introducing ILs as adjuvants in PEG-salt systems, with $[N_{4444}]Br$ providing the lowest production costs. Overall, TPP systems were shown to be not only cost-effective, but also robust and flexible routes to purify and recover IgG from complex biological matrices as shown here with human serum, serum-containing and serum-free cell culture supernatants.

1. Introduction

Relevant advances have been accomplished in the last years regarding the development of effective alternative therapies, with biopharmaceuticals being among the most favourable options [1]. They have showed the potential to widen the available options in the treatment of certain diseases, including also rare conditions [2]. Amongst biopharmaceuticals, immunoglobulin G (IgG) antibodies are widely applied for therapeutic purposes, especially monoclonal antibodies (mAbs), which have shown remarkable contributions in clinical practice for the treatment of oncologic and autoimmune diseases [3]. In turn, polyclonal antibodies (pAbs) derived from human serum are usually applied for the prevention and treatment of infections in immunodeficient patients, and also for the treatment of autoimmune and

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inflammatory diseases [4].

In the production of therapeutic antibodies, bioprocessing technologies must provide high recovery yields and purities of the final product, obeying with the rigorous quality and safety criteria imposed by the regulatory agencies [5]. Even though their upstream processing is well-established, the high cost of the currently used downstream technologies represents a stumbling block to the generalized use of antibodies as recurrent therapies [6]. The purification schemes usually followed by most (if not all) manufacturers rely on a complex multi-step platform comprising high resolution technologies, such as chromatographic steps which include affinity chromatography (with biological ligands – protein A) [7]. Due to the technological specificities of the downstream processing, it usually contributes to up to 80 % of the antibodies total production costs [8]. Therefore, there is a critical need to develop simpler and cost-effective purification platforms for antibodies.

To overcome the described concerns, the extraction and purification of antibodies using aqueous biphasic systems (ABS) represents a promising alternative [9]. ABS are liquid–liquid ternary systems that are formed by dissolving polymer–polymer or polymer-salt pairs in aqueous media above given concentrations [10]. More recently, the types of existing systems have been diversified by using other phase-forming components as well as by adding adjuvants (i.e., a fourth component added to tailor the properties and affinities of the ABS phases). Examples of alternative phase-forming components/adjuvants include ionic liquids (ILs), surfactants, amino acids and carbohydrates [11–18]. In ABS, each phase is enriched in each of the phase-forming components and has a high-water content, providing gentle conditions for biomolecules. Due to this advantage, ABS have been successfully used for the recovery of several types of proteins, ranging from transport and hormonal proteins to enzymes and antibodies [19–26].

Three-phase partitioning (TPP) is a type of extraction technique that usually uses a water-miscible aliphatic alcohol (usually *t*-butanol) and an aqueous solution of ammonium sulphate ($(NH_4)_2SO_4$) [27]. In TPP, the purification and recovery of target products, mostly proteins, is achieved through the formation of an enriched precipitate at the interphase [28–30]. In this way, a streamlined recovery of the final product directly from its raw source is granted, simplifying, or even dismissing additional polishing steps and facilitating the recovery/reuse of the TPP components. However, the application of traditional TPP is mainly constrained by a limited range of phase-forming components, which is strongly reliant on the use of *t*-butanol. Seeking for alternatives to *t*-butanol, the use of hydrophobic deep eutectic solvents (DES) or common phase-forming components of ABS has been brought to light [31–34]. Promising performance has been obtained using hydrophobic DES-based TPP in the recovery of polysaccharides, enzymes, and lipids from natural sources [31–33]. Furthermore, Belchior *et al.* [34] recently investigated the performance of ABS-TPP composed of polyethylene glycol (PEG) of different molecular weights and potassium phosphate buffer at pH 7 for the fractionation of three main proteins from egg white, by their differential partition between the three phases of the system. The association of ABS and TPP technologies (ABS-TPP) allows the development of more versatile bioseparation techniques since both phases are water-rich and the range of phase-forming components is amplified, thus allowing to better tailor selectivity and yield of the target compound.

The use of ionic liquids (ILs) has shown potential to enhance the extraction efficiency and selectivity of ABS and/or TPP due to their high chemical diversity and ability to be tailored [35]. Ionic-liquid-based ABS-TPP (IL-ABS-TPP) systems were firstly proposed by Alvarez-Guerra *et al.* [36,37] for the recovery of food proteins (e.g., lactoferrin). More recently, Castro *et al.* [38] used IL-ABS-TPP composed of PEG and poly(propylene) glycol (PPG) with ILs as adjuvants (at 5 wt%) for the purification of recombinant proteins (interferon alpha-2b) from *Escherichia coli* BL21 (DE3) inclusion bodies. The authors [38] showed that interferon alpha-2b purification was possible at the PEG-rich phase with the simultaneous precipitation of the remaining proteins at the ABS interphase.

The precipitation of antibodies in ABS has been reported in the literature, but often seen as leading to low yields [39–42]. Yet, IgG precipitation can be used as a way of recovering them by an alternative process. The association of ABS and TPP for the purification of antibodies remains still underexplored, especially considering mAbs [43–45]. This work aims to investigate ABS-TPP as alternative platforms for the purification of antibodies from three biological sources, i.e., human serum, and serum-containing and serum-free Chinese hamster ovary (CHO) cell culture supernatants. Systems formed by polymer-salt combinations, namely PEG with different molecular weights (600, 1000, 1500 and 2000 g/mol) and citrate buffer ($K_3C_6H_5O_7/C_6H_8O_7$) at pH 7, were investigated to selectively precipitate IgG antibodies from human serum at the interphase of the ABS. Distinct ILs were further added as adjuvants to tune the selectivity of the polymer-salt ABS-TPP towards IgG, and their chemical structure and concentration were optimized. Finally, the most promising ABS-TPP were applied to the remaining biological matrices to evaluate the robustness and flexibility of the developed process, for which an economic analysis was finally performed.

2. Experimental section

2.1. Materials

The ABS-TPP studied in this work were prepared using PEGs of different molecular weights, namely 600, 1000, 1500 and 2000 g/mol (hereafter abbreviated as PEG 600, PEG 1000, PEG 1500, and PEG 2000, respectively). PEG 600 and PEG 1000 were purchased from Alfa Aesar, while PEG 1500 and PEG 2000 were supplied by Acros Organics and Sigma Aldrich, respectively. Potassium citrate tribasic monohydrate ($K_3C_6H_5O_7{\cdot}H_2O$, $\geq$ 99 wt% purity) was acquired from Acros Organics, and citric acid ($C_6H_8O_7$, $\geq$ 99.5 wt% purity) was supplied by Panreac. Phosphate buffered saline solution (PBS, pH $\approx$ 7.4) pellets were acquired from Sigma-Aldrich, and prepared according to the indications of the supplier (by solubilizing each pellet in 200 mL of distilled water).

The ILs studied in this work were the following: 1-butyl-3-methylimidazolium bromide ([C_4mim]Br, 98 % purity), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl, 99 % purity), tetra(*n*-butyl)ammonium bromide ([N_{4444}]Br, 98 % purity), tetra(*n*-butyl)ammonium chloride ([N_{4444}]Cl, 97 % purity), tetra(*n*-butyl)phosphonium bromide ([P_{4444}]Br, 95 % purity), and tetra(*n*-butyl)phosphonium chloride ([P_{4444}]Cl, 95 % purity). All ILs were acquired from Iolitec, with the exception of [N_{4444}]Br and [N_{4444}]Cl that were supplied from Fluka and Sigma Aldrich, respectively. The chemical structures of the studied ILs are depicted in Fig. 1.

For the preparation of the HPLC buffer, di-sodium hydrogen phosphate 7-hydrate ($Na_2HPO_4{\cdot}H_2O$) and di-sodium hydrogen phosphate anhydrous ($NaHPO_4$) were purchased from Panreac, while sodium chloride (NaCl) was obtained by Fisher Scientific. The water employed was double distilled, passed across a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus. The membrane filters (Whatman, 0.45 μm, diameter 47 mm) used for the mobile phase filtration were made of regenerated cellulose R55 and were provided by GE Healthcare Life Science. The disposable syringes (5 mL) used for the separation of the phases were purchased from Injekt.

The model proteins used as standards were: immunoglobulin G (IgG) for therapeutic administration (trade name: Gammanorm®), obtained from Octapharma (Lachen, Switzerland), as a 165 mg/mL solution containing 95 % of IgG (of which 59 % IgG1, 36 % IgG2, 4.9 % IgG3 and 0.5 % IgG4), human serum albumin (HSA, > 97 % purity) acquired from Sigma-Aldrich, and bovine serum albumin (BSA) (2 mg/mL) purchased from Thermo Scientific Pierce.

The human serum used in this study was from human male AB plasma, USA origin, sterile-filtered, obtained from Sigma Aldrich (H4522 Sigma), with a total protein content ranging between 40 and 90

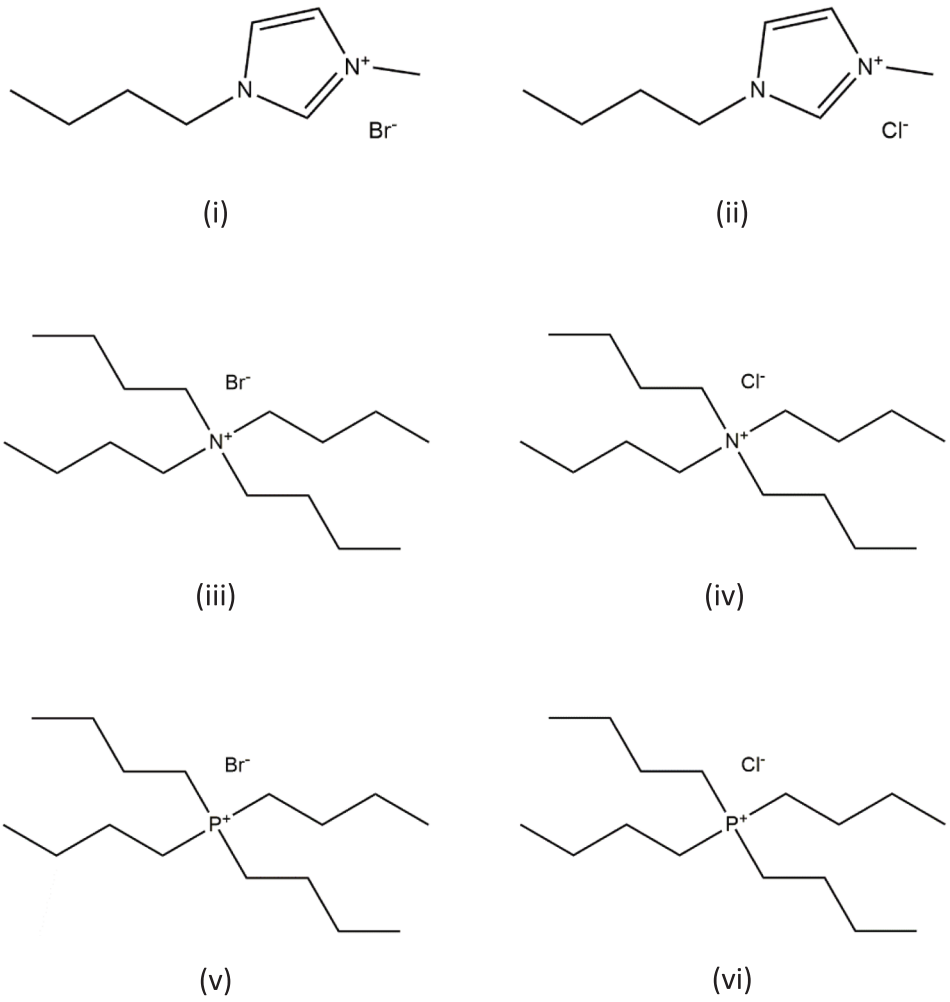

Fig. 1. Chemical structures of the investigated ILs: (i) [C_4mim]Br; (ii) [C_4mim]Cl; (iii) [N_{4444}]Br; (iv) [N_{4444}]Cl; (v) [P_{4444}]Br; (vi) [P_{4444}]Cl.

mg/mL. This product was stored at −20 °C up to use. Human serum was previously diluted to its use on each assay using PBS solution. Serum-free Chinese hamster ovary (CHO) cell culture supernatants were produced and delivered by Icosagen SA (Tartumaa, Estonia). These supernatants contain a humanized monoclonal antibody, from IgG1 class, derived from mouse anti-hepatitis C virus subtype 1b NS5B (nonstructural protein 5B) monoclonal antibody 9A2 expressed in mouse hybridoma culture. cDNA of antibody variable regions was isolated and cloned into the human IgG1 constant region-containing antibody expression vector. CHO cells were grown in a mix of two serum-free growth media, the CD CHO Medium (Gibco®, Carlsbad, CA) and the 293 SFM II Medium (Gibco®). The final concentration of IgG was around 100 mg/L. Serum-containing CHO cell culture supernatants containing anti-human interleukin-8 (anti-IL-8) monoclonal antibodies were produced in-house by a CHO DP-12 clone#1934 (ATCC CRL-12445) using DHFR minus/methotrexate selection system, obtained from the American Type Culture Collection (LGC Standards, Middlesex, UK). CHO DP-12 cells were grown in a mixture of 75 % (v/v) of serum-free media formulated with 0.1 % Pluronic® F-68 and without L-glutamine, phenol red, hypoxanthine, or thymidine (ProCHO™5, Lonza Group ltd, Belgium), and 25 % (v/v) of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % (v/v) of ultra-low IgG fetal bovine serum (FBS). ProCHO™5 formulation contains 4 mmol/L L-glutamine (Gibco®, Carlsbad, CA), 2.1 g/L $NaHCO_3$ (Sigma–Aldrich), 10 mg/L recombinant human insulin (Lonza), 0.07 % (v/v) lipids (Lonza), 1 % (v/v) antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Gibco®) and 200 nmol/L methotrexate (Sigma). DMEM was formulated to contain 4 mmol/L of L-glutamine, 4.5 g/L of D-glucose, 1 mmol/L of sodium pyruvate, 1.5 g/L of $NaHCO_3$, 2 mg/L of recombinant human insulin, 35 mg/L of L-proline (all acquired at Sigma), 0.1 % (v/v) of a trace element A, 0.1 % (v/v) of a trace element B (both from Cellgro®, Manassas, VA, USA), and 1 % (v/v) of antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin from Gibco®). The composition of trace element A includes 1.60 mg/L of $CuSO_4{\cdot}5H_2O$, 863.00 mg/L of $ZnSO_4{\cdot}7H_2O$, 17.30 mg/L of selenite·2Na, and 1155.10 mg/L of ferric citrate, while the trace element B is composed of 0.17 mg/L of $MnSO_4{\cdot}H_2O$, 140.00 mg/L of $Na_2SiO_3{\cdot}9H_2O$, 1.24 mg/L of molybdic acid, ammonium salt, 0.65 mg/L of NH_4VO_3, 0.13 mg/L of $NiSO_4{\cdot}6H_2O$, and 0.12 mg/L of $SnCl_2$. Cultures were carried out in T-75 flasks (BD Falcon, Franklin Lakes, NJ) at 37 (±1) °C and 5 % CO_2 with an initial cell density of 2.1 × 10^6 cells/mL. Cell passages were performed every 4 days in a laminar flow chamber. Cell supernatants were centrifuged in BD Falcon™ tubes at 175 × g for 7 min, collected and stored at −20 °C. This culture was maintained for several months, with the mAbs concentration varying between 40.5 and 99.4 mg/L. The produced anti-IL-8 mAb has an isoelectric point (pI) of 9.3 [46].

2.2. Methods

2.2.1. ABS-TPP and IL-ABS-TPP preparation

In this work, ABS composed of PEG and $K_3C_6H_5O_7/C_6H_8O_7$ at pH 7 were considered, in which the top phase corresponds to the PEG-rich

aqueous phase while the bottom phase is mainly composed of salt and water. The ternary mixture composition for the IgG purification/recovery was chosen based on the phase diagrams reported in the literature [25]: 20 wt% PEG + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% human serum/cell culture supernatant + 15 wt% H_2O/IL. All extraction/recovery studies involving the quaternary systems (i.e., ABS-TPP comprising ILs as adjuvants) were performed in the same mixture composition, where the ILs were introduced as adjuvants in substitution of the corresponding portion of water added in each system without IL. The purification/recovery performance of IgG with polymer-salt ABS-TPP and IL-ABS-TPP was investigated using human serum 20-fold diluted in PBS (phosphate buffered saline at 10 mmol/L, pH ≈ 7.4, at 25 °C) and CHO cell culture supernatants (serum-free or serum-containing). In each system, the biological sample was loaded at 40 wt % to reach a total weight of the mixture of 2.0 g of each ABS-TPP system. Each system was mixed in a vortex mixer (Ika, Staufen, Germany), centrifuged for 15 min in a fixed angle rotor bench centrifuge (Eppendorf, Hamburg, Germany) at 1372 × *g*. To further ensure total phase separation and interphase formation, all systems were then left in rest for 30 min. Then, both phases were carefully separated using syringes to extract the top and bottom phases. Both aqueous phases were analysed regarding their volumes as well as their pH values (at (25 ± 1) °C using a Mettler Toledo U402-M3-S7/200 micro electrode), showing that a pH of 7.0 ± 0.2 was maintained in all systems. In the cases in which an interphase precipitate was observed, i.e., for the ABS-TPP, the precipitate was isolated from the remaining phases by being centrifugated again for a short time and high rotation (for 2 min at 12000 × *g*), and then resuspended in 1.0 mL of PBS. Blank systems without biological sample were prepared to address the interference of the phase-forming compounds in the analytical methods.

2.2.2. Purification and recovery of IgG antibodies using ABS-TPP and IL-ABS-TPP

IgG and protein impurities, in particular HSA, were quantified in all feeds and in each ABS-TPP phase by size-exclusion high-performance liquid chromatography (SE-HPLC). Samples were diluted at a 1:2 (v/v) ratio in an aqueous potassium phosphate buffer solution (50 mmol/L, pH 7.0, with NaCl 0.3 mol/L), also used as the mobile phase. The equipment used was a Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven (operating at 40 °C), temperature controlled auto-sampler (operating at 10 °C), DAD detector and a Shodex Protein KW-802.5 (8 mm × 300 mm) analytical column. The mobile phase was run isocratically for 40 min, with a flow rate of 0.5 mL/min, the injection volume was 25 μL and the analyses were done at a wavelength of 280 nm.

A calibration curve was established with commercial human IgG, ranging from 5 to 200 mg/L. The performance of each ABS-TPP was evaluated by the determination of the recovery yields and purity levels of IgG. For each sample, the peak areas were estimated using PeakFit® software, and the remaining data was treated on Excel. The recovery yields (%$Yield_{IgG}$) in the ABS top and bottom phases were determined according to Equation (1):

$$\%\mathrm{Yield}_{\mathrm{IgG}} = \frac{[\mathrm{IgG}]_{\mathrm{TOP/BOT}} \times V_{\mathrm{TOP/BOT}}}{[\mathrm{IgG}]_{\mathrm{initial}} \times V_{\mathrm{initial}}} \times 100 \quad (1)$$

where $[IgG]_{TOP/BOT}$ and $[IgG]_{initial}$ represent the IgG concentration in the top or bottom phases of the ABS and in the initial biological matrix (human serum or CHO cell culture supernatants), respectively, and $V_{TOP/BOT}$ and $V_{initial}$ correspond to the volumes of the top or bottom phases and biological matrix (human serum or CHO cell culture supernatants) loaded in the system, respectively.

In the cases where a precipitate of proteins at the ABS interphase was formed, corresponding to the ABS-TPP approach, the %$Yield_{IgG}$ in the precipitate was determined according to Equation (2):

$$\%\mathrm{Yield}_{\mathrm{IgG}} = \frac{[\mathrm{IgG}]_{\mathrm{PP}} \times V_{\mathrm{final}}}{[\mathrm{IgG}]_{\mathrm{initial}} \times V_{\mathrm{initial}}} \times 100 \quad (2)$$

where $[IgG]_{PP}$ and V_{final} represent the IgG concentration and the final volume of the solution after resuspension, respectively.

According to Equation (3), the percentage purity level of IgG (%$Purity_{IgG}$) was calculated by dividing the SE-HPLC peak area of IgG (A_{IgG}) by the total area of the peaks corresponding to all proteins present in the respective sample (A_{Total}):

$$\%\mathrm{Purity}_{\mathrm{IgG}} = \frac{A_{\mathrm{IgG}}}{A_{\mathrm{Total}}} \times 100 \quad (3)$$

Since HSA is the main impurity of IgG in human serum, a calibration curve was also established for HSA with the commercially acquired protein, ranging from 50 to 1800 mg/L. The same performance parameters as for IgG were determined for HSA (%$Yield_{HSA}$ and %$Purity_{HSA}$) whenever indicated; for their determination, Equations 1–3 were applied using the concentration of HSA instead of IgG. At least two individual experiments were performed to determine the average of all described parameters, as well as the respective standard deviations.

An analysis of variance (*one way*-ANOVA) was performed to compare the yield and purity level of IgG enabled by distinct systems, followed by the Tukey *posthoc* test (a minimum significance level of 95 % — $p < 0.05$) using the JAMOVI software (1.6.6).

2.2.3. Circular dichroism

Circular dichroism (CD) assays were performed to infer the stability of the secondary structure of the purified samples, using a Jasco J-1500 CD spectrophotometer. CD spectra of aqueous solutions containing 0.25 g/L of commercial IgG, HSA and BSA in PBS (pH ≈ 7.4), human serum 400-fold diluted, cell culture supernatants and precipitates from selected ABS-TPP were acquired at 25 °C using a scanning speed of 100 nm/min, with a response time of 4 s over wavelengths ranging from 190 nm to 260 nm. The CD spectra of PBS (pH ≈ 7.4) was firstly taken as a blank control. The recording bandwidth was of 1 nm with a step size of 0.5 nm using a quartz cell with an optical path length of 1 mm. Three scans were averaged *per* spectrum to improve the signal-to-noise ratio. Measurements were performed under a constant nitrogen flow, which was used to purge the ozone generated by the light source of the instrument. The experiments were carried out in triplicate to ascertain the average values and associated standard deviations.

2.2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) assays were performed to assess the proteins profile and infer the IgG stability and integrity. The precipitates resulting from the TPP systems were analysed after pre-conditioning in PBS. All samples were diluted 1:1 in Laemmli loading buffer (4 % (w/v) of SDS, 20 % (w/v) of glycerol, 120 mM of Tris-HCl pH 6.8, 0.02 % (w/v) bromophenol blue), under reducing conditions with 200 mmol/L of dithiothreitol (DTT), and denatured by incubation at 95 °C for 5 min. The samples were then loaded in the polyacrylamide gel (Precast Gel SDS-PAGE 4 – 12 % from Expedeon) and ran at 90 mV. Protein staining was achieved by incubation with BlueSafe (from NZYTech) under mild stirring for 1 h. The gels were stored in distilled water at room temperature, until digital images of the gels were acquired.

2.2.5. Host cell proteins quantification

Host cell proteins (HCP) were quantified in the precipitates (IgG-rich samples) of the ABS-TPP applied for the processing of serum-free CHO cell culture supernatants, using a CHO Host Cell Proteins 3rd Generation ELISA kit from Cygnus Technologies (Southport, NC, USA). Briefly, standards (0 – 75 ng/mL), controls and samples (50 μL) were added to the wells of the ELISA microplate, as well as 200 μL of anti-CHO:alkaline phosphatase, and then covered and left to incubate at 400 – 600 rpm for

120 min at room temperature. Following, the content of each well was discarded, and each well was cleaned with diluted wash solution. Then, 200 µL of PNPP substrate was added to each well, and the microplate was covered again and left to incubate for 90 min at room temperature. The absorbance was finally measured at 405 and 492 nm in a BioTek SYNERGY|HT microplate reader.

The percentage of HCP removal (%HCP removal) was calculated using Equation (4),

$$\%\text{HCP removal} = \frac{m_{\text{HCP supernatant}} - m_{\text{HCP TOP/BOT/PP}}}{m_{\text{HCP supernatant}}} \times 100 \quad (4)$$

Where $m_{\text{HCP supernatant}}$ is the total amount of HCP present in the initial cell culture supernatant and $m_{\text{HCP TOP/BOT/PP}}$ is the amount of HCP quantified in a given phase, i.e. top (TOP), bottom (BOT) or precipitate (PP).

2.2.6. Economic evaluation

A critical aspect of new processes development is to determine production costs. As an initial approach, production cost was calculated only considering materials, as previously reported [47,48]. This allows a contrast between different conditions that will rely on the same type of equipment and operating conditions as the different ABS-TPP developed in this work. Equations (5) to (8) were used to obtain the production cost:

$$\frac{Cost\ of\ Materials}{Batch} = \sum_{i=1}^{n} \frac{Use\ of\ Material_i}{Batch} \times \frac{Cost\ of\ Material_i}{Unit\ of\ Material_i} \quad (5)$$

$$Product\ Obtained(IgG)[mg] = Product\ Concentration\left[\frac{mg}{mL}\right] \times Product\ Inlet[mL] \times Recovery\ Yield\ [\%] \quad (6)$$

$$\frac{Cost\ of\ Materials}{mg} = \frac{Cost\ of\ Materials/Batch}{Product\ Obtained} \quad (7)$$

$$\frac{Penalized\ Cost\ of\ Materials}{mg} = \frac{Cost\ of\ Materials/mg}{Purity[\%]} \quad (8)$$

To capture purity into production costs, a ratio of production cost and purity percentage was calculated. This allowed for a penalization of the cost if it does not achieve a 100 % purification. This in turn will proportionally increase the cost to less purified products. It is important to note that this strategy does not reflect an actual cost increase because of required subsequent unit operations, but rather a penalization that will help to select better cost scenarios and options. All calculations were performed for all ABS-TPP mixtures developed in this work. Economic data used for model construction is included in the Supplementary Material (Table S1).

3. Results and discussion

3.1. Characterization of biological media

Qualitative and quantitative characterizations of the feedstocks used in this work (human serum 20-fold diluted, serum-containing and serum-free CHO cell culture supernatants) were initially performed. These aimed to appraise the complexity and composition of the media from which IgG antibodies will be purified. The SE-HPLC chromatographic profiles of IgG, BSA and the several biological media are provided in Fig. 2.

Under the chromatographic conditions used, pure IgG samples present two chromatographic peaks: one corresponding to the IgG monomer and other to IgG aggregates, with a retention time of *ca.* 15.0 min and 13.8 min, respectively, being in good agreement with previous reports [43]. In human serum, the major protein impurity is human serum albumin (HSA), with a retention time of *ca.* 16.8 min. The IgG content in the human serum samples was ascertained before each assay, found to be 760 ± 80 mg/L, with a purity of 45.2 ± 0.9 %. For HSA, the average concentration in human serum was 2724 ± 211 mg/L, with a purity of 54.8 ± 0.9 %.

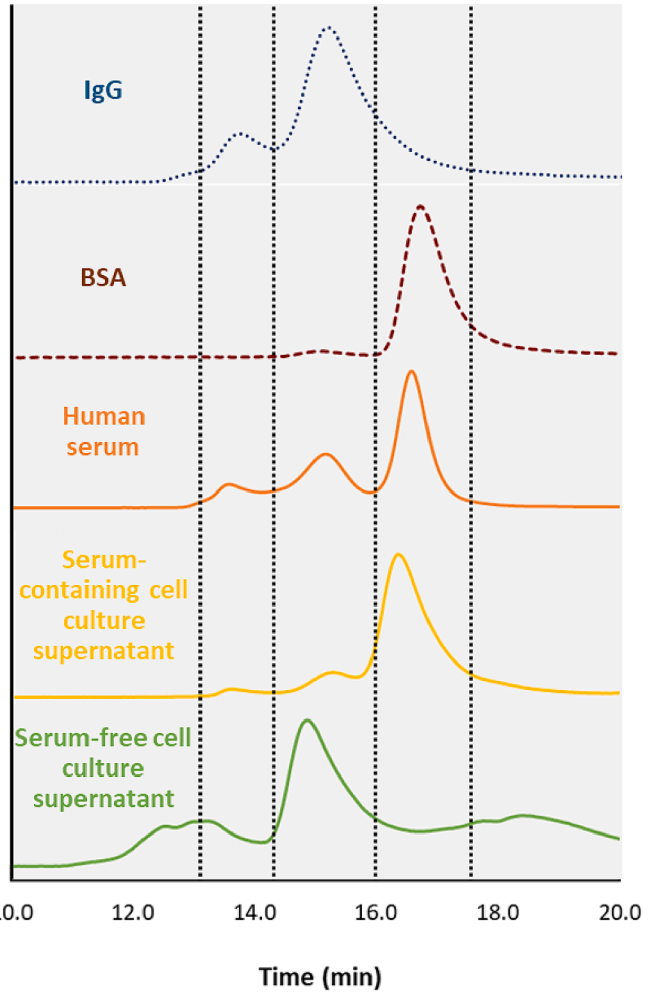

Fig. 2. Characterization of the cell culture supernatants: SE-HPLC chromatograms of pure IgG solution 100 mg/L (dotted blue line), pure BSA solution 200 mg/L (dashed brown line), human serum (solid orange line), serum-containing cell culture supernatant (solid yellow line), and serum-free cell culture supernatant (solid green line).

The serum-containing cell culture supernatant presents a similar chromatographic profile to human serum. Two peaks of IgG (corresponding to mAbs) are found, one corresponding to the IgG monomer and the other to IgG aggregates. However, these peaks present a smaller area than the respective peaks in human serum chromatograms, being related with the lower concentration of IgG in the cell culture supernatant. The major protein impurity in the feed is bovine serum albumin (BSA), a close analogue of HSA, also presenting a similar retention time. The mAb content in the serum-containing supernatant was 86 ± 1 mg/L, with a purity of 21 ± 1 %.

Serum-free cell culture supernatant presents a different chromatographic profile. In this matrix, IgG, in its monomeric form, is the main protein present, and, as expected, no peak corresponding to BSA is found since the supernatant is serum-free, and thus free of albumin. In the chromatographic profile are observed high molecular weight (HMW) protein impurities, with retention times below 15.0 min. Also, some low molecular weight (LMW) protein impurities with retention times above 17.0 min were found. The mAb content in serum-free supernatants was 275 ± 2 mg/L, with a purity of 36.5 ± 0.1 %.

3.2. Purification and recovery of antibodies from human serum samples

The potential of ABS-TPP as purification techniques for antibodies was initially addressed using human serum and conducting optimization studies of three parameters: (i) PEG molecular weight, (ii) addition of ILs as adjuvants, and (iii) ILs concentration. To have the minimum concentration of phase-forming compounds and the maximum concentration of water with the goal of developing a more economic and biocompatible process, but simultaneously being located in the biphasic region of all liquid–liquid phase diagrams [25], the PEG-salt composition of the systems was fixed throughout the optimization studies. PEG molecular weight is optimized as it is an essential parameter in the development of ABS-TPP for protein recovery, as supported by literature data [34]. Since the application of ILs as adjuvants in ABS allows adjusting the phases' polarities and affinities to target molecules [25], the main focus of this work is to address the impact of both IL structure and concentration to tailor the selectivity of IgG purification and recovery through precipitation by fixing the best performing PEG-salt ABS-TPP. For sake of clarity, the yields and purity levels of IgG referring to the interphase (as the final objective of an ABS-TPP is to precipitate target molecules at the interphase) will be considered. Whenever relevant, yield and purity level at the top or bottom phases will be mentioned. Additionally, the partitioning of HSA (major protein impurity of IgG in human serum) in the systems was evaluated to provide information on the ABS-TPP selectivity.

3.2.1. Effect of polymer molecular weight

The effect of the molecular weight of the phase-forming polymer was evaluated using four PEGs, namely PEG 600, PEG 1000, PEG 1500, and PEG 2000. Based on the relative position of the respective binodal curves taken from the literature [25], a common mixture point was chosen in the biphasic region of all systems: 20 wt% PEG + 25 wt% $K_3C_6H_5O_7$/$C_6H_8O_7$ + 40 wt% human serum 20-fold diluted + 15 wt% H_2O. The performance of the ABS-TPP to purify and recover IgG was investigated in terms of recovery yield (%$Yield_{IgG}$) and purity level (%$Purity_{IgG}$), whose results are shown in Fig. 3. The detailed data on the recovery yields and purity levels are given in the Supplementary Material (Table S2).

The results obtained for the IgG recovery indicate that, except for the ABS-TPP with PEG 600, there is a preferential precipitation of IgG at the interphase, with yields ranging from 65.8 % to 85.1 %. PEGs with higher molecular weight are more hydrophobic, promoting a higher precipitation of IgG at the interphase and following a similar molecular-level phenomenon as induced by *t*-butanol in conventional TPP. In turn, PEG 600 is not a proper candidate to develop ABS-TPP for the purification and recovery of IgG from serum since a preferential extraction of IgG to the top phase with a yield of 85.2 % was obtained, while only 7.9 % of IgG precipitated at the interphase. According to the literature [49], the partition of proteins in ABS is dependent on several interactions occurring between the ABS constituents and the protein under appraisal, including hydrophobic effects, electrostatic interactions, hydrogen-bonding and size-dependent phenomena. The relative contribution of each interaction further governs the extent of the partition. While ABS comprising polymers and salts have been investigated for the purification of IgG, the majority of TPP systems studied so far are formed by *t*-butanol and ammonium sulfate [45,50]. Overall, better precipitation results are reported in the literature with higher salt concentrations or strong salting-out salts, as the $K_3C_6H_5O_7$/$C_6H_8O_7$ here used, being this a common trend [34,40,42,51]. Additionally, more hydrophobic polymers, i.e., of higher molecular weight, lead to a lower solubility of IgG in the PEG-rich phase, thus inducing its precipitation [34,41,42]. According to our results, there is a trade-off between the solubility/saturation at the PEG-rich phase and a "salting-out" effect caused by the $K_3C_6H_5O_7$/$C_6H_8O_7$ to induce protein–protein interactions and consequent IgG precipitation.

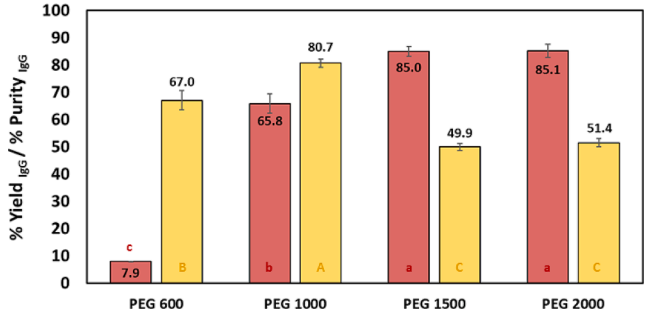

Fig. 3. Recovery yields (%$Yield_{IgG}$ – red bars) and purity levels (%$Purity_{IgG}$ – yellow bars) of human IgG at the interphase of ABS-TPP composed of 20 wt% PEG + 25 wt% $K_3C_6H_5O_7$/$C_6H_8O_7$ + 40 wt% human serum 20-fold diluted + 15 wt% H_2O. Different letters correspond to a significant difference in the IgG yield or purity between systems (*one way*-ANOVA: $p < 0.05$): lowercase letters refer to yield; uppercase letters refer to purity.

All ABS-TPP provide satisfactory IgG purity results, ranging from 49.9 % to 80.7 %. Among all polymers studied, systems comprising PEG 1500 and PEG 2000 lead to the best recovery yields (85.0 % and 85.1 %, respectively) while leading to the lowest IgG purities (49.9 % and 51.4 %, respectively). This trend suggests that by increasing the molecular weight of PEG, not only more IgG but also other proteins are precipitated due to a reduced solubility in the PEG-rich phase. As a result, the systems selectivity towards the IgG precipitation is reduced. On the opposite, the application of the lower molecular weight PEG 600 is limited by low yields of IgG at the interphase (only 7.9 %), although an IgG purity of 67.0 % is obtained. Among all PEGs studied, PEG 1000 is a polymer of intermediary molecular weight and provides the best balance between IgG purification and recovery in the precipitate (yield of 65.8 % with 80.7 % purity), with HSA being mainly retained in the liquid phases (HSA yield of 70.0 % in the top phase and 17.7 % in the bottom phase) - *cf.* Table S2 in Supplementary Material. This result represents an improvement of 78.5 % in the IgG purity compared to the initial human serum sample. Thus, the ABS-TPP composed of PEG 1000 was used in the following studies.

3.2.2. Effect of ILs as adjuvants

Six structurally different ILs were added as adjuvants at 5 wt%, namely [C_4mim]Br, [C_4mim]Cl, [N_{4444}]Br, [N_{4444}]Cl, [P_{4444}]Br and [P_{4444}]Cl, as an attempt to improve the performance of polymer-salt ABS-TPP to purify human IgG. The following mixture composition was studied: 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7$/$C_6H_8O_7$ + 40 wt% human serum 20-fold diluted + 10 wt% H_2O + 5 wt% IL. These ILs were chosen to evaluate different cation structures combined with anions at extremes of hydrogen-bond basicity [52], thus covering a large hydrophobic/hydrophilic range and different types of interactions that may occur with proteins (e.g., dispersive forces, van der Waals, electrostatic and π··· π interactions).

The performance of all IL-ABS-TPP was investigated considering the IgG recovery yield (%$Yield_{IgG}$) and purity level (%$Purity_{IgG}$) at the interphase. The results are shown in Fig. 4, while detailed data are given in the Supplementary Material (Table S3). Although the addition of [P_{4444}]Br to the system resulted in the formation of an ABS-TPP with a third solid layer in its macroscopic appearance, no proteins could be detected in the SE-HPLC chromatogram, thus preventing the determination of the systems performance parameters. This event suggests a negative impact of this IL on the proteins structure, being not adequate to design ABS-TPP for the purification of human IgG (and/or other serum components, such as HSA).

Except for [P_{4444}]Br and [C_4mim]Cl, all the remaining ILs added to the ABS-TPP systems induced the IgG precipitation at the interphase, with yields ranging from 56.9 % to 100 % achieved in one-step. If compared to the ABS-TPP formed by polymer-salt, the introduction of properly designed ILs allows to maximize the precipitation yield of IgG.

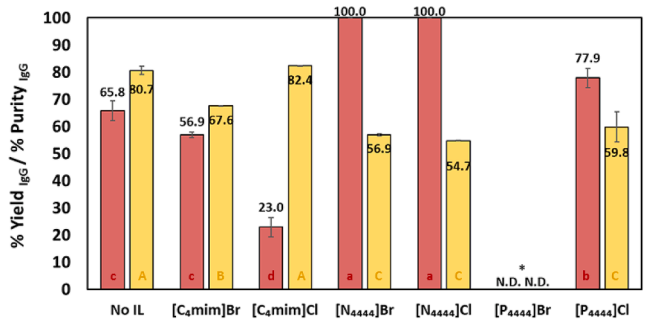

Fig. 4. Recovery yields (%$Yield_{IgG}$ – red bars) and purity levels (%$Purity_{IgG}$ – yellow bars) of human IgG at the interphase of IL-ABS-TPP composed of 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% human serum 20-fold diluted + 10 wt% H_2O + 5 wt% IL. N.D. means not determined. Different letters correspond to a significant difference in the IgG yield or purity between systems (*one way*-ANOVA: $p < 0.05$): lowercase letters refer to yield, while uppercase letters refer to purity. * identifies systems that have not been included in the statistical analysis.

Remarkably, in the presence of 5 wt% of the quaternary ammonium-based ILs, namely $[N_{4444}]Br$ and $[N_{4444}]Cl$, the IgG complete recovery was achieved. Considering the ILs tested, it is possible to improve the purity level of IgG to 82.4 % with $[C_4mim]Cl$, but compromising the recovery yield (only 23.0 %). Overall, $[C_4mim]Br$ was the identified IL providing the best balance between the yield and purity of IgG (recovery yield of 56.9 % and purity of 67.6 %). However, these performance parameters are lower than those obtained with the system without IL. Also, as with the system without IL, 68.3 % of HSA is retained in the top phase of the system composed of $[C_4mim]Br$, with 80.4 % of purity (*cf.* Table S3 in Supplementary Material). This result unveils the possibility to simultaneously purify and recover two different value-added proteins in a single step, i.e., IgG at the interphase and HSA at the top phase. This result is in line with the literature, where $[C_4mim]Br$ allowed remarkable selectivity in the extraction of proteins both using ABS and ABS-TPP [25,43].

As with higher molecular weight PEGs, the impact of the IL in the precipitation of IgG correlates well with the IL hydrophobicity. In general, the more hydrophobic the IL, the more extensive the precipitation. $[N_{4444}]Br$, $[N_{4444}]Cl$ and $[P_{4444}]Cl$ possess larger cations, with four butyl chains attached to the central heteroatom, than the aromatic ILs $[C_4mim]Br$ and $[C_4mim]Cl$. The former ILs are thus more hydrophobic, enhancing protein–protein interactions and, consequently, inducing precipitation at the interphase. Also, $[C_4mim]Br$ allows a higher precipitation than $[C_4mim]Cl$, in agreement with the hydrophobicity of these two IL anions. The Br^- anion presents a lower hydrogen-bond basicity (β) than the Cl^- anion ($\beta\ (Br^-) = 0.87 < \beta\ (Cl^-) = 0.95$), thus resulting in a more hydrophobic IL [52]. These results show that a molecular-level phenomenon similar to conventional TPP occurs, in which a highly hydrophobic phase is required to induce the precipitation of proteins. Ferreira et al. [25] previously reported that ILs preferentially migrate to the PEG-rich phase in ABS composed of PEG and $K_3C_6H_5O_7/C_6H_8O_7$. This IL partition justifies the increase of the PEG-rich phase hydrophobicity, and the subsequent more extensive protein precipitation in the IL-ABS-TPP herein studied. Nevertheless, a careful balance is required since high selectivity is also needed. As such, the ILs that lead to a higher precipitation are not the best ones to purify IgG since other proteins are co-precipitated. Based on the aforementioned results and discussion, $[C_4mim]Br$, $[C_4mim]Cl$ and $[N_{4444}]Br$ were considered in further optimization studies.

To appraise the influence of the IL concentration, $[C_4mim]Br$, $[C_4mim]Cl$ and $[N_{4444}]Br$ were added as adjuvants at 1, 2, 5, 10 and 15 wt% in systems with the following composition: 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% human serum 20-fold diluted + 15 wt % H_2O/IL. The IgG recovery yield (%$Yield_{IgG}$) and purity level (% $Purity_{IgG}$) at the interphase are shown in Fig. 5, whose detailed results are provided in the Supplementary Material (Table S4).

By changing the concentration of $[C_4mim]Br$ (Fig. 5A) from 1 wt% to 10 wt%, recovery yields ranging from 56.9 % to 77.7 % and purity levels ranging between 67.6 % and 70.0 % are obtained at the interphase of the respective IL-ABS-TPP systems. On the other hand, at higher $[C_4mim]Br$ concentrations (i.e., 15 wt%), a negative effect over antibodies is observed, since no peaks are found in the SE-HPLC chromatograms. For this reason, the determination of the respective performance parameters was not possible. The obtained results at the interphase suggest that the most efficient concentration of $[C_4mim]Br$ is the lowest under investigation (i.e., 1 wt%), yielding 77.7 % of IgG with 69.1 % of purity. Under these conditions, it is possible to simultaneously recover HSA, the major protein impurity of IgG in human serum and also an added-value protein, in the top phase of the ABS-TPP, with 49.7 % yield and 89.1 % HSA purity (*cf.* Table S4 in the Supplementary Material). The system with 1 wt% of $[C_4mim]Br$ thus outperformed the system without IL in terms of HSA purity (81.9 % in the ABS-TPP top phase).

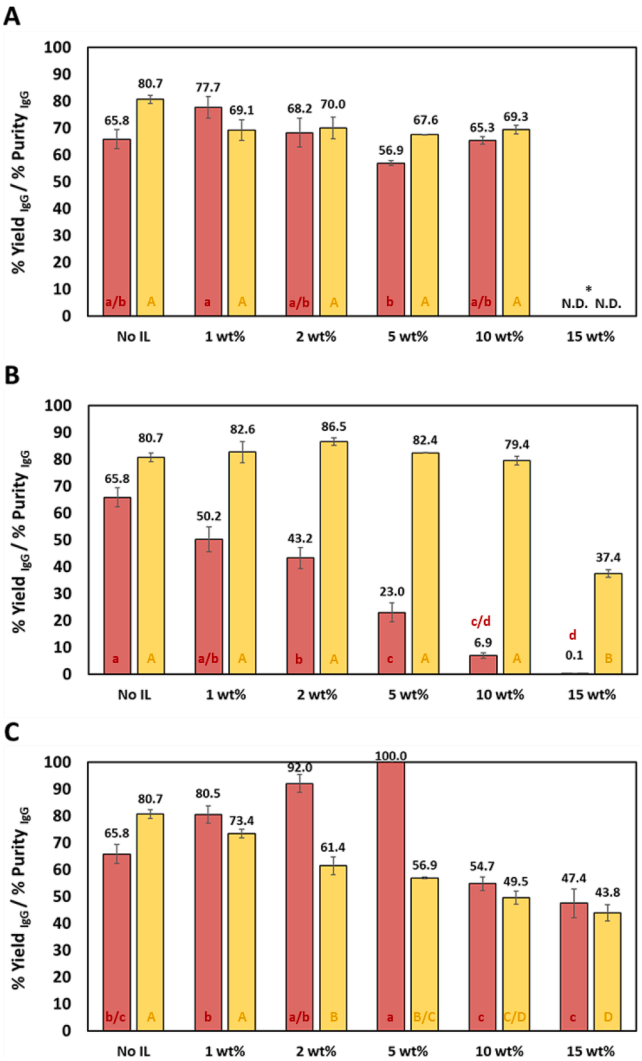

Fig. 5. Recovery yields (%$Yield_{IgG}$ – red bars) and purity levels (%$Purity_{IgG}$ – yellow bars) of human IgG at the interphase of ABS-TPP composed of 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% human serum 20-fold diluted + 15 wt% H_2O/IL: (A) $[C_4mim]Br$; (B) $[C_4mim]Cl$; and (C) $[N_{4444}]Br$. N.D. means not determined. Different letters correspond to a significant difference in the IgG yield or purity between systems (*one way*-ANOVA: $p < 0.05$): lowercase letters refer to yield, while uppercase letters refer to purity. * identifies systems that have not been included in the statistical analysis.

A decrease in the recovery yields of IgG following the [C_4mim]Cl concentration increase occurs, despite no significant variation in the purity levels are observed up to 10 wt% of IL – Fig. 5B. On the other hand, the recovery yield of IgG in the top phase of the ABS-TPP increases when using higher IL concentrations. This observation suggests that [C_4mim]Cl possibly improves protein solubility in the ABS top phase and/or establishes non-covalent interactions with the target protein, promoting its partition to the polymer-rich top phase (the phase where the IL is also enriched), and thus hampering its precipitation at the interphase. The further increase in the concentration of [C_4mim]Cl up to 15 wt% does not seem appropriate for the processing of human serum proteins due to the abrupt decrease in the performance of the system.

For the system containing [N_{4444}]Br (Fig. 5C), the maximum recovery performance is achieved using 5 wt% of IL. In what concerns the purification performance, it was found a continuous decrease in the purity level of IgG at the interphase, ranging between 43.8 % and 73.4 %. This set of results suggests that by increasing the amount of IL in the system, other proteins in addition to IgG also precipitate, thus decreasing the system selectivity. For this reason, the condition that allows a better compromise between the recovery yield and purity is with 1 wt% of [N_{4444}]Br, in which 80.5 % of IgG is recovered with 73.4 % of purity.

The described results show the role of ILs as adjuvants in polymer-salt ABS-TPP. By simply changing the IL content it is possible to maximize the recovery yield or purity of IgG. Despite no condition allowing to maximize both the recovery yield and purity level was found, some clues for the development of high-performance IL-ABS-TPP were provided. It was found that increasing the IL concentration has a negative impact on the recovery yield and purity of IgG at the interphase. The systems containing the highest amount of IL tested, i.e., 15 wt%, led to the complete loss of native IgG in the interphase or to a high decrease on its recovery yield.

For ILs to be efficient adjuvants for IgG purification, not only their chemical structure, but also their concentration in the ABS-TPP must be carefully optimized for any desired target compound. Nevertheless, lower amounts of IL are beneficial not only to the performance and selectivity of the system, but also to decrease the economic cost of the whole process. This concept has been reinforced by the calculation of production costs. [N_{4444}]Br and [C_4mim]Cl have demonstrated the best conditions for recovery yield and purification, and from an economic perspective they have also demonstrated to be cost-effective when compared with the system containing no IL. Considering the results from purity and recovery yields, regression equations were developed, correlating these values to the percentage of IL added (*cf.* Fig. S1 in the Supplementary Material). Using this information, it was possible to elucidate operational zones for IL concentration where ABS-TPP will always provide a better production cost (Fig. 6). Overall, IL concentrations below 1 wt% are recommended to maintain a lower production cost than the system with no IL, particularly when using [N_{4444}]Br that provides the lowest production cost at US$ 2.56 per mg.

Based on all the aforementioned information and discussed topics, the systems composed of 1 wt% of [C_4mim]Cl and 1 wt% of [N_{4444}]Br were selected as the best IL-ABS-TPP, allowing the best trade-off between yield and purity of IgG at the IgG-rich interphase. Therefore, these systems were further studied towards the purification and recovery of mAbs from cell cultures supernatants.

3.3. Purification and recovery of antibodies from cell culture supernatants

To evaluate their technological robustness and flexibility, the best identified IL-ABS-TPP were applied towards the purification and recovery of mAbs from cell culture supernatants and compared with polymer-salt ABS-TPP. For that purpose, two different biological matrices were considered: (i) a serum-containing CHO cell culture supernatant with anti-interleukin-8 (anti-IL-8) mAbs; and (ii) a serum-free CHO cell culture supernatant with anti-hepatitis C virus (anti-HCV) mAbs. The following mixture ABS-TPP or IL-ABS-TPP compositions were evaluated: (i) 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% (serum-containing or serum-free) CHO cell culture supernatant + 15 wt% H_2O; (ii) 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% (serum-containing or serum-free) CHO cell culture supernatant + 14 wt% H_2O + 1 wt% [C_4mim]Cl; and (iii) 20 wt% PEG 1000 + 25 wt % $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% (serum-containing or serum-free) CHO cell culture supernatant + 14 wt% H_2O + 1 wt% [N_{4444}]Br. All the detailed data obtained with serum-containing CHO cell culture supernatant with anti-interleukin-8 (anti-IL-8) mAbs is given in the Supplementary Material (Table S5).

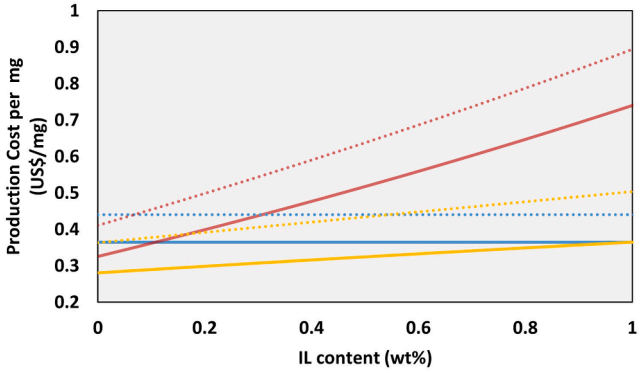

Fig. 6. Production cost based on materials for ABS-TPP with recovery yields and purity predicted by regression equations: No IL (solid blue line); [C_4mim]Cl (solid red line); [N_{4444}]Br (solid yellow line); No IL (penalized) (dotted blue line); [C_4mim]Cl (penalized) (dotted red line); [N_{4444}]Br (penalized) (dotted yellow line). Full graph until 5 wt% is included in the Supplementary Material (Fig. S2).

In what concerns TPP formation, deviations to the behavior observed with human serum, in which TPP was formed regardless of the IL structure and concentration, were found when dealing with cell culture supernatants. Although some small particles could be found at the interphase of the ABS without IL and with 1 wt% of [C_4mim]Cl when using serum-containing cell supernatants, the precipitation was too small to allow a proper recovery of a third solid interphase for analysis (*cf.* Fig. S3 in the Supplementary Material). A TPP could only be formed when using 1 wt% of [N_{4444}]Br, thus allowing to evaluate its performance. Using this ABS-TPP, 46.2 % of anti-IL-8 mAbs could be directly recovered in the precipitate layer, with a purity of 64.4 %. These results are lower than those obtained with the same system for human IgG from serum samples (yield of 80.5 % of IgG with 73.4 % of purity; improvement of 62.4 % in purity in comparison with the initial serum sample). Still, a large improvement, when comparing with the initial purity of the feedstock, was accomplished (purity of 64.4 % versus 21.0 % in the initial supernatant; an improvement of 200+% in purity in comparison with the initial supernatant). When processing human serum, the TPP composed of [N_{4444}]Br revealed a good aptitude to precipitate proteins (leading to the highest recovery yields). This is probably the reason why the system composed of 1 wt% of [N_{4444}]Br was the only capable to form a ABS-TPP with serum-containing cell supernatants. These results highlight the importance of adding ILs to the process, since it was only possible to create an ABS-TPP in the presence of an IL (and not in the system without IL).

On the opposite, with serum-free CHO cell culture supernatants, all the systems under study were capable to form ABS-TPP, despite the completely different protein profile of both biological fluids (*cf.* Fig. S3 in Supplementary Material). It is interesting to notice that the serum-containing cell supernatant that presented a similar chromatographic profile to that of human serum was not able to form ABS-TPP in most of the selected conditions. These observations suggest that the biological matrix composition plays a key role in ABS-TPP formation. The influence that each protein exerts on the others in specific macromolecular

crowded pools is commonly observed, which in this case leads to ABS-TPP formation only with the less complex medium. However, a higher mAbs concentration is present in the serum-free cell supernatants (274.9 mg/L versus 85.8 mg/L in the serum-containing), suggesting that high concentrations of proteins are required to induce the formation of TPP. This evidence agrees with previous observations in the field of protein precipitation by salts and with the molecular-level mechanisms of TPP formation previously discussed [51].

Precipitate layers enriched in anti-HCV mAbs were obtained in all systems. The performance of these systems was also investigated in terms of IgG recovery yield (%$Yield_{IgG}$) and purity level (%$Purity_{IgG}$) at the interphase. All related results and detailed data are shown in Fig. 7 and in the Supplementary Material (Table S6), respectively. ABS-TPP provide purity levels higher than that of the initial cell supernatant (36.5 %), with mAbs recovery yields ranging from 55.3 % to 79.7 %, and purity levels between 82.8 % and 89.2 %.

The conventional ABS-TPP leads to the lowest IgG recovery performance, yielding 55.3 % of anti-HCV mAbs with 82.8 % of purity. The purified sample was also analysed by ELISA towards the host cell proteins (HCP) content, revealing HCP removal values of 94.7 % from the original feed. Remarkably, by using ILs as adjuvants, better recovery yields were obtained with an equivalent purity. Also, the system composed of 1 wt% of [C_4mim]Cl proved to be an efficient TPP to deplete HCPs (100 % HCP removal), since the results obtained were below the limit of detection of the method.

The SE-HPLC chromatograms of the precipitates are presented in the Supplementary Material (Fig. S4A–C), as well as the representative chromatograms of the coexisting phases of the best ABS-TPP containing 1 wt% of [C_4mim]Cl in the Supplementary Material (Fig. S4D). The analysis of the SE-HPLC chromatograms corroborates the high purities achieved with the ABS-TPP under study, since the main peak present corresponds to mAbs (retention time of *ca.* 15.0 min); only a small peak is noticed, corresponding to HMW impurities (or IgG aggregates). The remaining IgG that is not recovered in the precipitate fraction partition to the PEG-rich phase with the majority of the LMW protein impurities. On the other hand, HMW protein impurities are preferentially retained in the salt-rich phase.

From an economic perspective, current estimates indicate that the recovery of IgG from serum-free supernatant is low-cost alternative when applying IL-ABS-TPP (Fig. 8A). By adding 1 wt% of [N_{4444}]Br it is possible to obtain a production cost (considering only materials) of US$ 0.36 per mg of IgG. The cost reduction comes from the ability of the ABS-TPP to handle larger concentrations of IgG present in the sample and still recover it with a high yield and purity.

Works in the literature have shown that the purification of

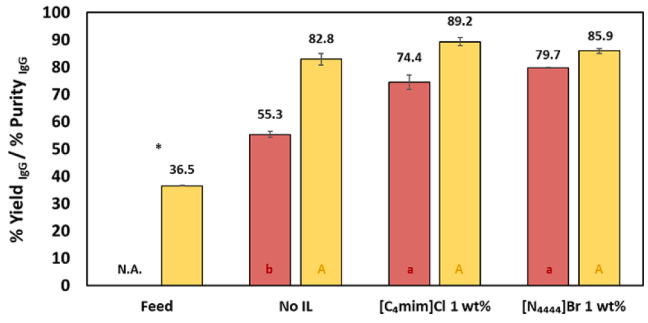

Fig. 7. Recovery yields (%$Yield_{IgG}$ – red bars) and purity levels (%$Purity_{IgG}$ – yellow bars) of IgG at the interphase of ABS-TPP composed of 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% serum-free CHO cell culture supernatant + 15 wt% H_2O/IL. For the feed, the %$Yield_{IgG}$ is not applicable (N.A.). Different letters correspond to a significant difference in the IgG yield or purity between systems (*one way*-ANOVA: $p < 0.05$): lowercase letters refer to yield, while uppercase letters refer to purity. * identifies systems that have not been included in the statistical analysis.

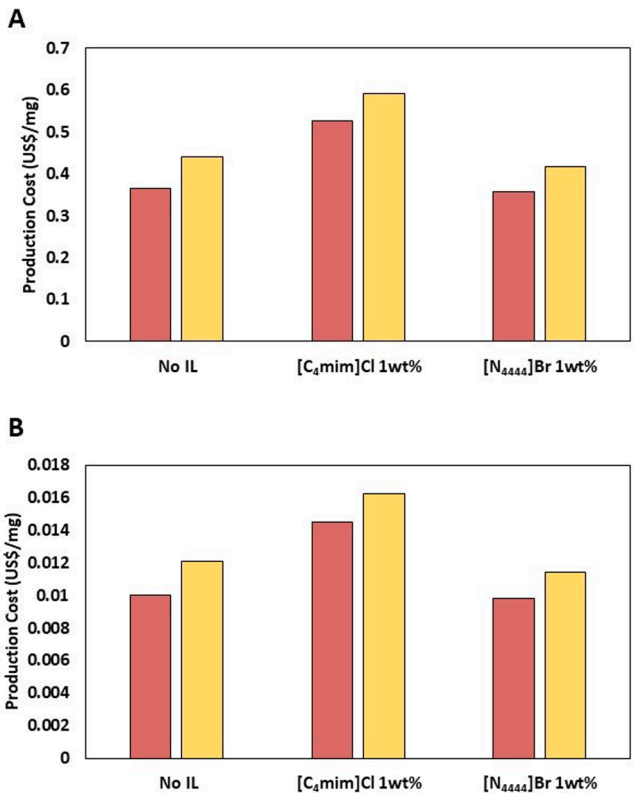

Fig. 8. Production costs (red bars) and its penalized version (yellow bars) for the inclusion of 1 wt% of [N_{4444}]Br and [C_4mim]Cl when recovering IgG from serum-free CHO cell culture supernatant. Figures show the results when using an inlet containing a concentration of 274.9 mg/L (A) as quantified in this work and a reported 10 g/L (B) for other potential antibodies.

monoclonal antibodies provided production costs ranging from US$ 31 to 45 per gram of antibodies [53]. Costs obtained here are considerably higher, but it is important to note that typical antibody titers are much higher (approximately 10 g/L) in the published work [53]. Considering an improved product inlet, production costs decrease to a range between US$ 11.45 to 16.25 per gram, depending on the IL used (Fig. 8B). The strategy developed here still needs to be polished and tested against higher production levels of antibodies and at larger scales. Still, current results show a very promising strategy to reduce the length and duration of bioprocesses, which in turn have demonstrated to be critical parameters for production costs in different markets.

Circular dichroism (CD) spectroscopy was used to examine the secondary structure of mAbs after purification and recovery using ABS-TPP. The spectrum of a standard solution of IgG 250 mg/L was also appraised and included for comparison purposes. For all systems, the CD spectra depicted in Fig. 9 clearly show that the secondary structure of mAbs after the recovery procedure resembles the one of the freshly prepared solutions of IgG. All spectra exhibit typical shape of a β-sheet protein as IgG, with a characteristic maximum of negative ellipticity at *ca.* 216 nm [26]. Thus, after the recovery of mAbs with the investigated ABS-TPP, their conformation is not affected, and the recovered proteins maintain their secondary structure.

In Fig. 10, the SDS-PAGE gel of all the recovered fractions is provided, allowing not only to confirm the mAbs integrity, but also to corroborate the discussed purification and recovery results.

3.4. Comparative assessment of antibody purification and recovery using IL-ABS-TPP and state-of-the-art techniques

During the bioprocessing of therapeutic antibodies, there are several requisites to be fulfilled. These entail product purity, safety and yield,

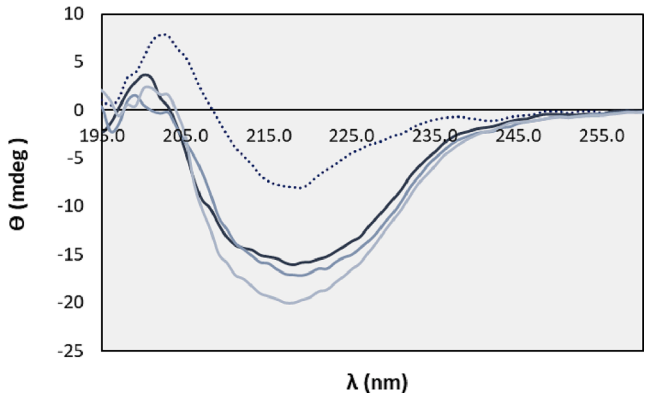

Fig. 9. Circular dichroism (CD) spectra (ellipticity, θ, in mdeg) for the recovered mAbs-rich precipitate fractions of the ABS-TPP composed of 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% serum-free CHO cell culture supernatant + 15 wt% H_2O/IL: pure IgG solution 250 mg/L in PBS (dotted dark blue line), TPP with no IL (solid dark blue line), TPP with 1 wt% $[N_{4444}]Br$ (solid light blue line), TPP with 1 wt% $[C_4mim]Cl$ (solid medium blue line).

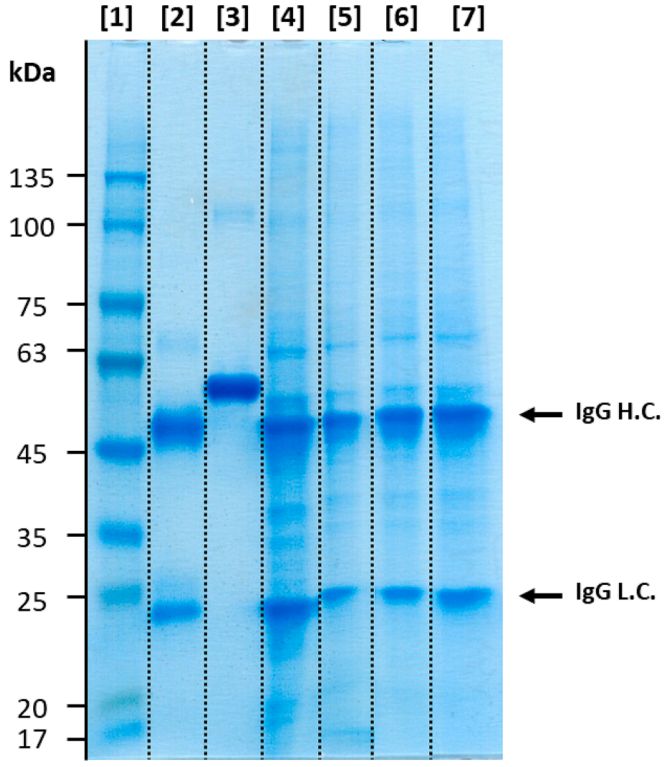

Fig. 10. SDS-PAGE of the recovered fractions using the ABS-TPP composed of 20 wt% PEG 1000 + 25 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 40 wt% serum-free CHO cell culture supernatant + 15 wt% H_2O/IL. Lane 1 – molecular weight marker (kDa); Lane 2 – pure IgG at 100 mg/L; Lane 3 – pure BSA 200 mg/L; Lane 4 – serum-free CHO cell culture supernatant; Lane 5 – precipitate of the ABS-TPP with no IL; Lane 6 – precipitate of the ABS-TPP with 1 wt% $[C_4mim]Cl$; Lane 7 – precipitate of the ABS-TPP with 1 wt% $[N_{4444}]Br$. The bands corresponding to IgG heavy chain (H.C.) and IgG light chain (L.C.) are also labelled.

short manufacturing time and process robustness [54]. By meeting most of these criteria, protein A chromatography remains the standard technique in the purification of antibodies. Either alone or combined with other chromatographic approaches, such as ion-exchange and size exclusion, protein A chromatography enables high purity levels (>95 % purity) that are of chief importance when envisioning therapeutic applications of IgG [54]. Despite the remarkable selectivity, issues related with proteolysis, column regeneration and cost pose technological barriers to reduce IgG biopharmaceuticals cost [54]. Alternatively, precipitation methods have been implemented as simpler and cost-effective technologies for the purification and recovery of antibodies. Cold ethanol and PEG are among the most widely adopted precipitating agents due to the high product yields usually enabled [55]. These alone, however, cannot compete with protein A chromatography in terms of selectivity and, consequently, product purity [55]. Due to the co-precipitation of impurities (e.g., HCPs, DNA), multiple precipitation steps using conventional or even expensive affinity-based precipitating agents may need to be implemented [56–58]. Thereby, to approach the purity levels of standard chromatographic techniques, the technological simplicity of precipitation is jeopardized.

Compared to the previously described methods, the proposed IL-ABS-TPP can combine, in a certain degree, the most advantageous features of both approaches: technological simplicity and cost-efficiency of precipitation and high selectivity of chromatographic techniques. Despite product purity can be further increased for therapeutic applications, the IL-ABS-TPP herein proposed successfully remove impurities (i.e., HSA from serum and HCPs from cell supernatants) in a single step approach.

Besides standard techniques, it is also important to understand how the results obtained in this work compare with those obtained with their parent TPP or ABS. Regarding conventional TPP formed by *t*-butanol and ammonium sulphate, Dennison [59] observed that these are not capable of purifying IgG. Despite promising, the available literature on ABS-TPP for the recovery and purification of antibodies is still scarce. In a previous work we proposed using ILs as main phase-forming agents instead of adjuvants in polymer-salt ABS, allowing to obtain a recovery of 41.0 % with 60.9 % purity [43]. On the other hand, as recently reviewed by us, large efforts have been carried out with traditional polymer-based ABS to purify antibodies [60]. Commonly, a better performance is achieved resorting to multi-stage approaches, adding electrolytes, using affinity ligands or these strategies combined. The described limitations are here overcome using common ABS constituents such as PEG, salts and ILs instead of *t*-butanol. Although the complete recovery of IgG was not achieved, IgG could be recovered with purity values up to 89.2 %. Furthermore, the developed TPP strategy is robust since different antibodies (pAbs, anti-IL-8 mAbs and anti-HCV mAbs) can be successfully purified and recovered from different matrices (human serum and serum-containing and serum-free CHO cell cultures supernatants). Overall, the ABS-TPP here proposed can be considered a valuable strategy for the one-step purification and recovery of antibodies, particularly mAbs. Finally, the results here obtained exceed those previously reported and bring additional advantages regarding the reduction of the costs of the process.

4. Conclusions

In this work, it is proposed a cost-effective approach for the purification and recovery of human antibodies from biological complex matrices. The proposed approach resorts to ABS-TPP composed of PEG and $K_3C_6H_5O_7/C_6H_8O_7$ at pH 7, either in the absence or presence of ILs as adjuvants. During process development and optimization using human serum, all process parameters evaluated, i.e., PEG molecular weight, IL structure and IL concentration, were shown to have an impact on the final IgG purity and yield. Most of the investigated systems allowed the formation of an IgG-rich precipitate layer, with the best performance being achieved with systems composed of the intermediate molecular weight PEG (PEG 1000) and by using low amounts (1 wt%) of the ILs $[C_4mim]Cl$ and $[N_{4444}]Br$. Under the optimum conditions, recovery yields of IgG from human serum correspond to 80.5 % and purity levels to 82.6 %, while decreasing the costs of the process.

To demonstrate the robustness and flexibility of the proposed technology, the best IL-ABS-TPP systems were investigated for the purification and recovery of mAbs from cell culture supernatants. It was

observed an influence of the molecular crowding effect of the matrices and of the effect of the chemical structure of the IL. For the serum-containing CHO cell culture supernatant with anti-IL-8 mAbs, only the system composed of 1 wt% of $[N_{4444}]$Br was able to create an ABS-TPP, yielding 46.2 % of mAbs with a purity of 64.4 %. For serum-free CHO cell culture supernatant with anti-HCV mAbs, presenting a higher IgG concentration, all the systems under study were able to create ABS-TPP. The use of ILs as adjuvants not only allowed the best mAb recovery and purification balance, with an exceptional ability to remove HCP, but also showed potential to reduce production costs. The stability and integrity of the recovered antibodies was proven by CD spectroscopy and SDS-PAGE.

Overall, it is here shown that the association of the ABS and TPP concepts leads to a promising technology to purify and recover proteins from complex biological matrices, particularly antibodies, in a single step. The addition of ILs to ABS-TPP also brings advantages to the process in terms of selectivity or recovery yield, and cost reduction, which is due to their designer solvent feature. Although there is still room for further improvement and a path to follow to fully explore the potential of ABS-TPP in the bioprocessing of biopharmaceuticals, the developed process proved to be robust, as shown with purification and recovery of different antibodies from multiple biological samples. In addition to achieving high recovery yields and purity levels, this technology allows an easy recovery of the protein of interest, which could be further resuspended in an appropriate buffered formulation for storage and further commercialization.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

Emanuel V. Capela: Investigation, Methodology, Data curation, Formal analysis, Validation, Visualization, Writing – original draft. **Ilaria Magnis:** Investigation, Data curation. **Ana F.C.S. Rufino:** Investigation, Methodology. **Mario A. Torres-Acosta:** Methodology, Formal analysis, Writing – review & editing. **M. Raquel Aires-Barros:** Resources, Supervision. **João A.P. Coutinho:** Supervision, Writing – review & editing. **Ana M. Azevedo:** Resources, Supervision, Writing – review & editing. **Francisca A. e Silva:** Supervision, Writing – review & editing, Funding acquisition, Project administration. **Mara G. Freire:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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