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Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography

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ABSTRACT

In this paper we explore an alternative process for the purification of human antibodies from a Chinese hamster ovary (CHO) cell supernatant comprising a ligand-enhanced extraction capture step and cation exchange chromatography (CEX). The extraction of human antibodies was performed in an aqueous two-phase system (ATPS) composed of dextran and polyethylene glycol (PEG), in which the terminal hydroxyl groups of the PEG molecule were modified with an amino acid mimetic ligand in order to enhance the partition of the antibodies to the PEG-rich phase. This capture step was optimized using a design of experiments and a central composite design allowed the determination of the conditions that favor the partition of the antibodies to the phase containing the PEG diglutaric acid (PEG-GA) polymer, in terms of system composition. Accordingly, higher recovery yields were obtained for higher concentrations of PEG-GA and lower concentrations of dextran. The highest yield experimentally obtained was observed for an ATPS composed of 5.17% (w/w) dextran and 8% (w/w) PEG-GA. Higher purities were however predicted for higher concentrations of both polymers. A compromise between yield and purity was achieved using 5% dextran and 10% PEG-GA, which allowed the recovery of 82% of the antibodies with a protein purity of 96% and a total purity of 63%, determined by size-exclusion chromatography. ATPS top phases were further purified by cation exchange chromatography and it was observed that the most adequate cation exchange ligand was carboxymethyl, as the sulfopropyl ligand induced the formation of multi-aggregates or denatured forms. This column allowed the elution of 89% of the antibodies present in the top phase, with a protein purity of 100% and a total purity of 91%. The overall process containing a ligand-enhanced extraction step and a cation exchange chromatography step had an overall yield of 73%.
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1. Introduction

Biopharmaceutical products, including proteins, nucleic acids and other bioproducts, have application in several focus areas, including vaccination, immunization, oncology, autoimmune, cardiovascular, inflammatory and neurological diseases. The market of biopharmaceutical products is one of the fastest growing segments of the pharmaceutical industry, and as the efficacy of traditional drugs is decreasing and population is aging, the demand for new therapies, that will allow the treatment of the *21st century diseases*, is increasing. Industry is thus focusing on developing new biotechnology-derived products. The annual sales of several biopharmaceuticals have surpassed the billion dollar mark [1]. In 2006, the global market for biopharmaceuticals was estimated to be worth around $50 billion [2] and is expected to overpass the $90 billion by 2010 [3]. With the completion of the primary DNA mapping of the human genome and the progress made in high-throughput technology for drug discovery, a remarkable growth in the development of therapeutic products was observed, with more than 500 products in active clinical trials [2].

Nevertheless, the demand for large quantities of therapeutic proteins for the treatment of diseases requiring high doses and/or chronic administration has brought several concerns about a potential shortage of the manufacturing capacity, which, in turn, intensified the pressure to improve cell culture productivity [4]. Advances in molecular biology have allowed the increase of cell line productivity, by optimizing media formulations and feed delivery strategies. Process control, bioreactor design, and host cell engineering have also been significant factors in improving upstream processes [4]. Antibody titers, for example, have increased from few milligrams a decade ago to multigrams per liter today, with reports of titers reaching 15 g/l [5]. Such increase has been attributed to an increase in both cell density and duration of cell viability [6].

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Nevertheless, while upstream productivity is increasing in line with demand, improvements in downstream processes have been neglected, as biopharmaceutical companies are reluctant to change long established processes for new ones. This is resulting in a production bottleneck that is shifting the costs of production downstream [7]. In fact, downstream processes of biopharmaceuticals can account for up to 80% of total manufacturing cost [8] and has been a long-standing market barrier. As a result, the need to improve process economics and efficiency, to reduce costs and to meet the increasingly demand of quality for market approval is forcing the development of more efficient and cost-effective separation and purification methods in order to keep up with the upstream gains.

The platform approach for the downstream processing of antibodies usually encompasses three chromatographic steps, a Protein A affinity capture step and two chromatographic polishing steps to remove host cell proteins, high molecular weight aggregates, low molecular weight clipped species, DNA and leached Protein A that remain after the capture step [9]. Protein A chromatography has become the traditional choice for antibody capture not only due to its specificity and purity levels obtained but also due to its ability to handle unconditioned feeds straight from the bioreactor. Nevertheless, Protein A chromatography has several inherent limitations because of its high cost and leachability.

Non-chromatographic methods could be a valuable alternative to some of the chromatographic steps as long as the final purification is not compromised. Several promising alternatives have been described in the literature, including, affinity precipitation [10,11], liquid–liquid extraction [12,13], high performance tangential flow filtration [14], membrane chromatography [15,16], high gradient magnetic fishing [17] and crystallization [18]. These alternative technologies, and in particular, aqueous two-phase partitioning, aim at high throughput and seek to avoid problems associated with cost, capacity and diffusional limitations encountered with most chromatographic supports [19].

Aqueous two-phase systems (ATPSs) composed of dextran and polyethylene glycol (PEG) have been shown suitable for the capture of human antibodies from a CHO cell supernatant, as long as the affinity of the antibody for the PEG-rich phase is enhanced by the addition of a ligand [20–22]. The modification of polyethylene glycol terminal hydroxyl groups with an amino acid mimetic ligand (glutaric acid) enhanced the partitioning of antibodies to the PEG-rich phase from 23% in the unfunctionalized system to more than 90% in the glutaric acid functionalized system [22]. In this paper, we have used a design of experiments to optimize the ATPS extraction step, in terms of system composition, by response surface analysis. This approach allows studying the effect of polyethylene glycol diglutaric acid (PEG-GA) and dextran concentration and their possible interaction without varying one condition at the time, and consequently reducing the number of required experiments. We also explore the possibility of integrating the ATPS extraction step with a cation exchange chromatography (CEX) step without any pre-conditioning step. This would not only simplify the process but would also reduce the overall costs, especially at large scale.

2. Materials and methods

2.1. Materials

Human IgG for therapeutic administration (product name: Gammanorm) was purchased from Octapharma (Lachen, Switzerland), as a 165 mg/ml solution containing 95% of IgG. The cell supernatant was produced and delivered by ExcellGene (Monthey, Switzerland) and contains a human IgG1, directed against a human surface antigen. An ExcellGene proprietary serum-free medium was used for production. Phenol red has been added to the medium as a pH indicator. According to Excellgene the antibody titer was 110 mg/l as determined by ELISA. Due to the low concentration of IgG, the supernatant was spiked with 1 g/l IgG from Gammanorm.

Polyethylene glycol with an average molecular weight of 3350 Da, sodium phosphate monobasic, sodium acetate and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). Dextran form *Leuconococcus*, with an average molecular weight of 500,000, and acetic acid were obtained from Fluka (Buchs, Switzerland). Chemicals used in the synthesis of PEG-GA were purchased from Acros Organics (Geel, Belgium).

2.1.1. Synthesis of polyethylene glycol diglutaric acid

PEG-GA was synthesized (Fig. 1) according to the procedure described in [21]. Briefly, PEG 3350 (60 g, 17.9 mmol) and glutaric anhydride (18 g, 157 mmol) were dissolved in pyridine (60 ml) and dichloroethane (900 ml). The reaction mixture was stirred at reflux temperature for 16 h. After cooling to room temperature, the reaction mixture was evaporated until dryness. The crude product was taken up in 0.5 M HCl (400 ml) and extracted with dichloromethane (2×500 ml). The organic layers were combined, dried over sodium sulfate and evaporated until dryness. The crude solid material was washed with hot diethyl ether (5×1 l). The last traces of solvent were removed at the rotary evaporator and PEG-GA (54.4 g, 87%) was obtained as a white solid. ^{1}H NMR ($CDCl_3$): δ 4.2 (m, 4H); 3.6 (large s, PEG); 2.4 (m, 8H), 1.9 (t, 4H).

2.2. Aqueous two-phase extraction

PEG-GA/dextran ATPS were prepared by mixing appropriate amounts of stock solutions of PEG-GA (50%, w/w), dextran (25%, w/w) and phosphate buffer at pH 7 (1 M) with the CHO cell supernatant in 15 ml graduated test tubes. The final weight was adjusted to 5 g by addition of water. All systems were prepared with a 35% loading of CHO cell supernatant. The components were added in the following order: water, phosphate buffer, CHO cell supernatant, dextran and finally PEG-GA. All components were thoroughly mixed before and after the addition of PEG-GA in a Vortex mixer (Ika, Staufen, Germany), equilibrated at 25 °C in a water bath and centrifuged for 5 min in a fixed angle rotor bench centrifuge (Eppendorf, Hamburg, Germany) at $1400 \times g$ to ensure total phase separation. The volumes of the phases were determined, and the samples from both top and bottom phases were carefully isolated and stored at 4 °C until further analysis. All systems were analyzed against blank systems, containing the same phase composition but with water instead of the CHO cell supernatant. The extraction yield of IgG was defined by the ratio between the mass of IgG in the top phase and the mass of IgG initially added to the system.

2.3. Cation exchange chromatography

Chromatographic runs on three different commercial prepacked cation exchange HiTrap columns ($0.7\,cm \times 2.5\,cm$) from GE Healthcare (Uppsala, Sweden) were preformed on an Äkta Purifier system from GE Healthcare. The CEX columns tested included a sulfopropyl Shepharose fast flow (SP FF), a sulfopropyl Shepharose XL (SP XL) and a carboxymethyl Shepharose fast flow (CM FF). The columns were equilibrated with 5 column volumes (CV) of 20 mM sodium acetate buffer at pH 5, prior to injection at 1 ml/min. ATPS top phases containing IgG (pH around 4.5) were injected without any pre-conditioning step at 0.5 ml/min using a 2 ml sample loop. After washing unbound compounds with 5 CV of the equilibration buffer, elution of bound components was triggered by increasing

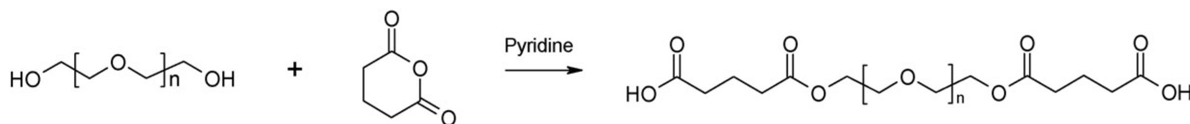

Fig. 1. Functionalization of PEG molecules with glutaric acid.

salt concentration from 0 to 1 M NaCl at 1 ml/min. Preliminary column screening studies were performed with a step gradient elution. However, the final CEX purification step was performed using a 10 CV gradient elution. Column flowthrough and eluate were continuously collected as 1 and 0.5 ml fractions, respectively, in a FRAC 950 fraction collector from GE Healthcare. Absorbance at 280 nm and conductivity was continuously monitored. The recovery yield of IgG was defined by the ratio between the mass of IgG in the eluted fractions and the mass of IgG initially loaded in the column.

2.4. Analytical methods

2.4.1. Quantification of IgG

The concentration of IgG was determined by affinity chromatography using an analytical POROS Protein A Affinity Column from Applied Biosystems (Foster City, CA, USA). Samples containing IgG were diluted 10 times in a sample buffer, containing 0.05% (w/v) Tween 80, 150 mM NaCl in 10 mM sodium phosphate buffer at pH 8.5. Adsorption of IgG to the column was performed in 10 mM sodium phosphate buffer at pH 8.5 containing 150 mM NaCl and elution was triggered by decreasing the pH to 2–3, using 12 mM HCl with 150 mM NaCl, according to a procedure previously described by [23]. Absorbance was monitored at 280 nm.

2.4.2. Determination of total purity by HPLC

The total purity of IgG sample was evaluated by size-exclusion chromatography. Samples from top and bottom phases and CEX fractions were diluted in PBS (phosphate buffer saline) and run in isocratic mode in a TSK-Gel Super SW3000 column coupled with a TSK-GEL Super SW guard column, both from Tosoh Bioscience (Stuttgart, Germany) at 0.35 ml/min for 25 min. Total purity was defined by the ratio between the area of the IgG peak and the total area of the chromatogram. All samples collected in the ATPS step were analyzed against a blank system with the same composition but with water instead of CHO cell supernatant. All chromatograms shown were plotted using the blank chromatogram as baseline.

2.4.3. Determination of protein purity

Protein purity was determined by the ratio between the concentration of IgG, determined according to Section 2.4.1, and total protein. The total concentration of protein in the ATPS phases and CEX fractions was determined by the Bradford method, as this method is compatible with up to 0.5 g/l phenol red, present in the CHO cell supernatant as a pH indicator. The assays were set up in 96-well polystyrene microplates and 200 μl of Coomassie reagent (Pierce, Rockford, IL, USA) were added to 50 μl samples. Pure IgG from Gammanorm was used as protein standard. Absorbance was monitored at 595 nm in a microplate reader from Molecular Devices (Sunnyvale, CA, USA). To avoid interference from PEG and phosphate, all samples from top and bottom phases were analyzed against blanks systems containing the same phase composition.

2.4.4. Protein electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the purity of the ATPS and CEX fractions. Samples were diluted in a sample buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue and 10% glycerol and denatured under non-reducing conditions at 100 °C for 5 min. Samples were applied in a 4–15% gradient precast gel from Bio-Rad (Hercules, CA, USA), and run at 90 mV in a TGS buffer containing 25 mM Tris, 250 mM glycine and 0.1% (w/v) SDS. The gel was stained using a silver nitrate staining kit from Bio-Rad.

3. Results and discussion

3.1. Aqueous two-phase extraction optimization

The selective capture of human antibodies from a CHO cell culture supernatant by aqueous two-phase extraction using a polymer functionalized with an amino acid mimetic ligand (polyethylene glycol glutaric acid, PEG-GA) was optimized by response surface methodology (RSM), which comprises three general steps: the experimental design; modeling and statistical validation; and optimization [24–26].

3.1.1. Experimental design

A Box–Wilson central composite design (CCD) was selected for the optimization of the purification process in terms of system composition. In particular, three response variables, including extraction yield (y_1), total purity (y_2) and protein purity (y_3), have been experimentally determined and optimized as a function of two independent variables, the dextran concentration (x_1) and the PEG-GA concentration (x_2). Both independent variables were then studied at five levels, whose values were selected according to preliminary experiments carried out and taking into consideration the required experimental conditions to obtain phase separation, as illustrated in Fig. 2. According to these preliminary results, it was observed that a small variation in the concentration of one of the polymers could have a prominent effect on yield. For example, a reduction in the dextran concentration from 8 to 5%, for a PEG-GA concentration of 8%, allowed a 25% increase in the extraction yield. Fig. 3 depicts the SDS-PAGE which was run with samples from both top and bottom phases of these two systems. This gel clearly shows

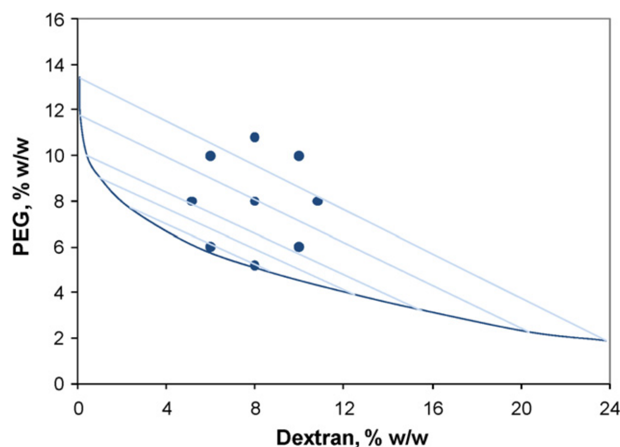

Fig. 2. Phase diagram of PEG/dextran system containing the factorial, axial and central points of the CCD.

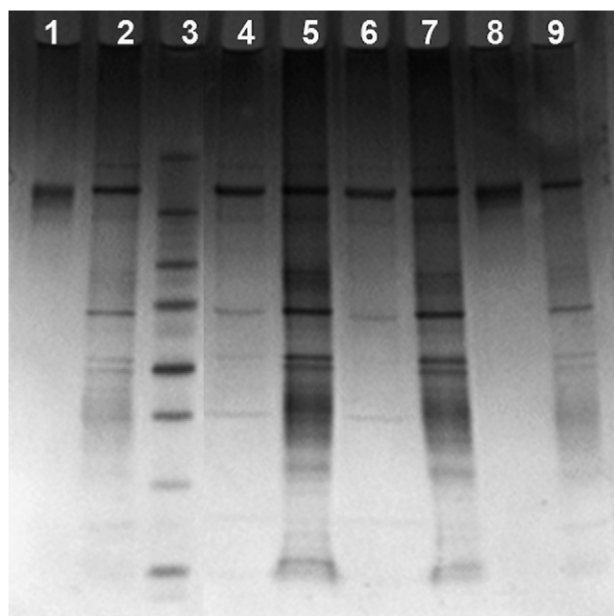

Fig. 3. Protein profile of the different top and bottom phases analyzed in a 4–15% gradient precast SDS-PAGE run in non-reduced conditions and stained with silver nitrate. Lanes 1 and 8: pure IgG from Gammanorm; lanes 2 and 9: CHO cell supernatant diluted 20×; lane 3: molecular weight markers (from top to bottom—250, 150, 100, 75, 50, 37, 25, 20, 15 kDa); lane 4: top phase of the system composed by 5% dextran, 8% PEG-GA; lane 5: bottom phase of the system composed by 5% dextran, 8% PEG-GA; lane 6: top phase of the system composed by 8% dextran, 8% PEG-GA; lane 7: bottom phase of the system composed by 8% dextran, 8% PEG-GA. All ATPS were prepared with 2% phosphate buffer pH 7.

that a higher protein clearance is observed in the top phases (lanes 4 and 6), confirming the selective extraction of IgG using this ligand.

The CCD consisted of 14 experimental points, including an imbedded 4 points 2^2 factorial matrix, with 6 center points and augmented with 4 "star" (axial) points. In order to obtain a rotable design, star points were located at an axial distance (α) of 1.414 from the center of the experimental domain [24,25]. Fig. 2 depicts the experimental domain within the PEG/dextran phase diagram. The design matrix, including both independent and response variables, is presented in Table 1.

3.1.2. Modeling and statistical validation

A second-order polynomial model was used to express the different response variables as a function of the independent variables. The model coefficients were estimated using least square fitting and the significance of each model factor was evaluated using a Student's t-test. Factors that were not statistically valid with 95% confidence were removed and pooled into the error term. The equations that describe the reduced models obtained for each response variables (in un-coded values) are the following:

$$\text{Yield}(\%) = 9.92 + 1.09 \times [\text{dextran}] + 13.78 \times [\text{PEG}] - 0.43 \times [\text{PEG}]^2 - 0.54 \times [\text{dextran}] \times [\text{PEG}] \quad (1)$$

$$\text{Total purity}(\%) = -74.30 + 19.50 \times [\text{dextran}] + 9.56 \times [\text{PEG}] - 0.75 \times [\text{dextran}]^2 - 0.73 \times [\text{dextran}] \times [\text{PEG}] \quad (2)$$

$$\text{Protein purity}(\%) = -71.52 + 17.23 \times [\text{dextran}] + 16.65 \times [\text{PEG}] - 0.70 \times [\text{dextran}]^2 - 0.56 \times [\text{PEG}]^2 - 0.74 \times [\text{dextran}] \times [\text{PEG}] \quad (3)$$

It was observed that the factor that had the greatest effect in the recovery yield was the dextran concentration. In addition, PEG-GA concentration had also a significant effect. Regarding the purity of the top phases, the factor that had the greatest effect was the PEG-GA concentration. For a more detailed analysis of the t-tests performed, the reader is referred to the supplement of this manuscript available on-line, specifically to Figure 1S that represents the Pareto charts of the standardized effect of the model factors.

The statistic validation of the reduced models, described by Eqs. (1)–(3), was conducted by means of an analysis of variance (ANOVA), using the Statistica 5.1 software (StatSoft, Tulsa, OK, USA). The outcome of this ANOVA is summarized in Table 2 for the three response variables and accordingly all predictive models are statistically significant with 95% confidence level ($p\text{-value}|_{model} < 0.05$) and do not exhibit any significant lack-of-fit ($p\text{-value}|_{LOF} > 0.05$) [26]. For more details regarding the significance of each parameter present in the ANOVA table, the reader is referred to the supplementary file available on-line.

3.1.3. Optimization

The predictive models obtained in the previous sections were plotted as a function of both polymers concentration. Figs. 4–6 show the response surfaces plots obtained for the recovery yield, total

Table 1
CCD matrix including the independent variables (x_1 and x_2) and the response variables experimentally determined (y_1, y_2 and y_3). CHO cell supernatant: [IgG] = 1.084 g/l; [Protein] = 4.104 g/l; total purity = 37%.

Run ID	Dextran% (x_1) (w/w)	PEG-GA% (x_2) (w/w)	Yield% (y_1)	Total purity% (y_2)	Protein purity% (y_3)
1	6.0	6.0	61.3	42.1	55.6
2	6.0	10.0	76.0	60.9	72.0
3	10.0	6.0	56.0	59.0	64.7
4	10.0	10.0	62.0	65.2	69.1
5	5.2	8.0	80.0	58.6	68.7
6	10.8	8.0	57.0	63.3	66.0
7	8.0	5.2	57.0	55.5	65.8
8	8.0	10.8	71.0	80.4	71.0
9 (C)	8.0	8.0	65.0	63.0	74.4
10 (C)	8.0	8.0	68.0	59.6	73.3
11 (C)	8.0	8.0	65.0	64.9	73.3
12 (C)	8.0	8.0	65.0	64.0	70.1
13 (C)	8.0	8.0	68.0	65.0	69.6
14 (C)	8.0	8.0	66.0	62.3	69.2

All systems contained 10 mM phosphate buffer. The pH values were around 4.5.

Table 2
ANOVA outcome for the three reduced models, showing for each response variable, the three main sources of variation (including a discrimination of the residual error variability into lack-of-fit and pure error).

Variable	Source	SS	DF	MS	*F*-value	*p*-value
Yield	Model	580.64	4	145.16	62.21	0.0002
	Error	46.64	9	5.18		
	Lack-of-fit	35.81	4	8.69	3.72	0.0909
	Pure error	10.83	5	2.33		
	Total	628.51	13			
Total purity	Model	394.40	4	98.60	23.99	0.0018
	Error	96.52	9	10.72		
	Lack-of-fit	75.97	4	18.99	4.62	0.0621
	Pure error	20.55	5	4.11		
	Total	490.93	13			
Protein purity	Model	229.87	5	45.97	8.82	0.0161
	Error	73.60	8	9.20		
	Lack-of-fit	47.53	3	15.84	3.04	0.1313
	Pure error	26.07	5	5.21		
	Total	296.91	13			

purity and protein purity, respectively, and were drawn using the Statistica software.

High recovery yields can be obtained using high concentrations of PEG-GA, as expected since the glutaric acid functionalities attached to the PEG molecule are the driving force for the IgG partitioning to the top phase. In addition, low concentrations of dextran also favored the partitioning of IgG to the top phase. If the phase diagram, depicted in Fig. 2, is taken into consideration, then the region that favors the extraction of IgG is characterized by high volume ratios and, consequently, higher top phase volumes, which are typically known to favor the extraction of molecules to the top phase. The highest recovery yield obtained experimentally was obtained in an ATPS composed of 5.15% dextran and 8% PEG-GA. According to Fig. 4, the optimum system composition appears to be 5% dextran and 10% PEG-GA.

Regarding the total purity, higher purities are observed for higher concentrations of both polymers (Fig. 5). However, it is also possible to observe a maximum of the purity with respect to the dextran concentration at high PEG-GA concentrations. The optimal conditions, according to Fig. 5, are obtained for PEG-GA concentrations higher than 9.5% and dextran concentrations ranging between 6.5 and 9%. The response surface plot obtained for the protein purity follows a similar trend, and the optimum range is composed by concentrations of dextran and PEG-GA between 6 and 9% and 8.5 and 10%, respectively. According to Figs. 5 and 6, for very low concentrations of both polymers there is a pronounced decrease in both purities. This happens because as the system approaches the critical composition (composition at which both phases have the same composition), the composition of both top and bottom phases becomes more similar and the partitioning of all components becomes less one-sided. This explains not only the decrease observed in purity but also in the recovery yield. On the other hand, as the concentration of the polymers increases, the composition of both phases becomes more distinct and the partition of the different components becomes more one-sided, with IgG favoring to the top phase due to the presence of the glutaric acid ligand, and remaining proteins partitioning mostly to the bottom, more hydrophilic phase.

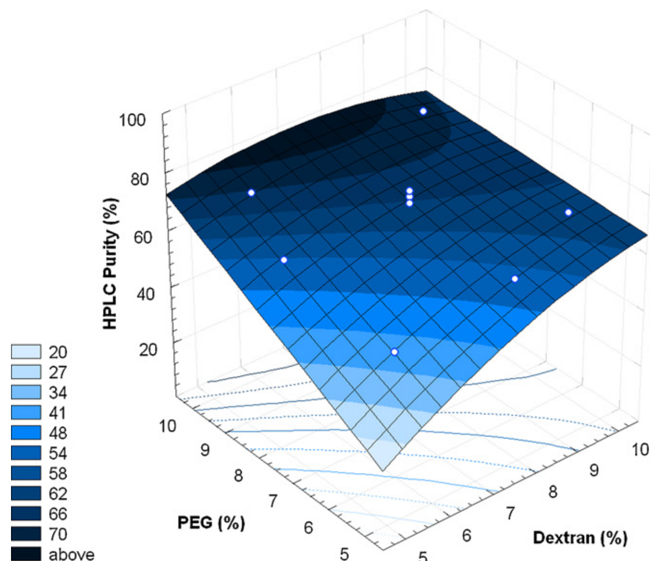

Fig. 5. Response surface plot obtained for the total purity determined by HPLC, as a function of dextran and PEG-GA concentration. $R^2 = 0.786$.

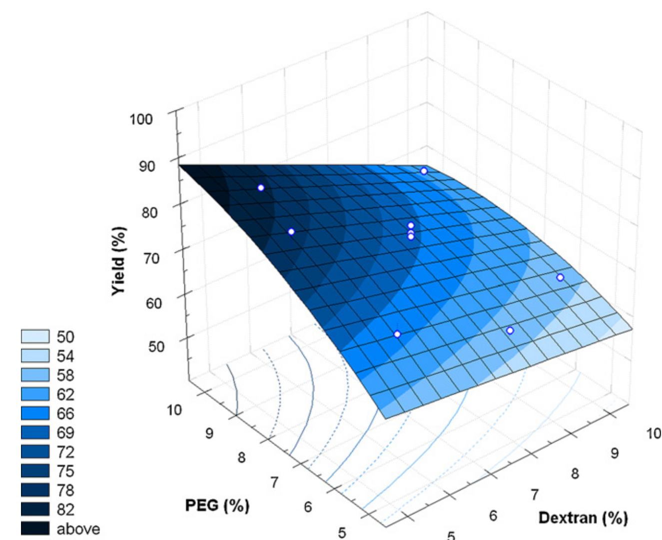

Fig. 4. Response surface plot obtained for the extraction yield, as a function of dextran and PEG-GA concentration. $R^2 = 0.926$.

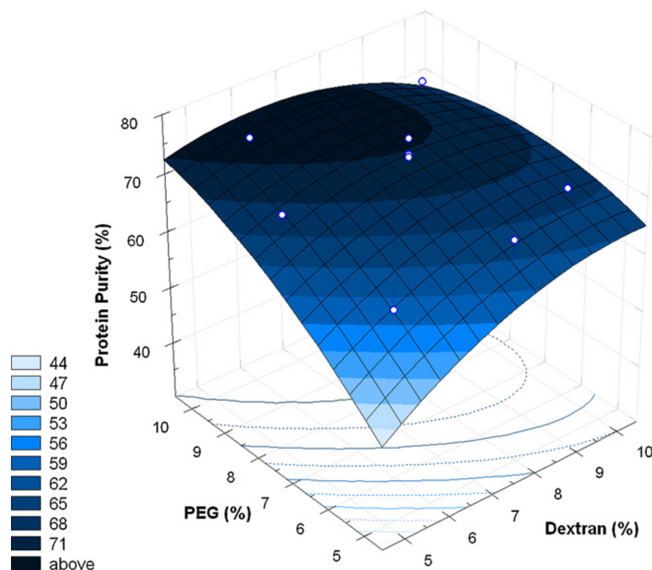

Fig. 6. Response surface plot obtained for the protein purity, as a function of dextran and PEG-GA concentration. $R^2 = 0.752$.

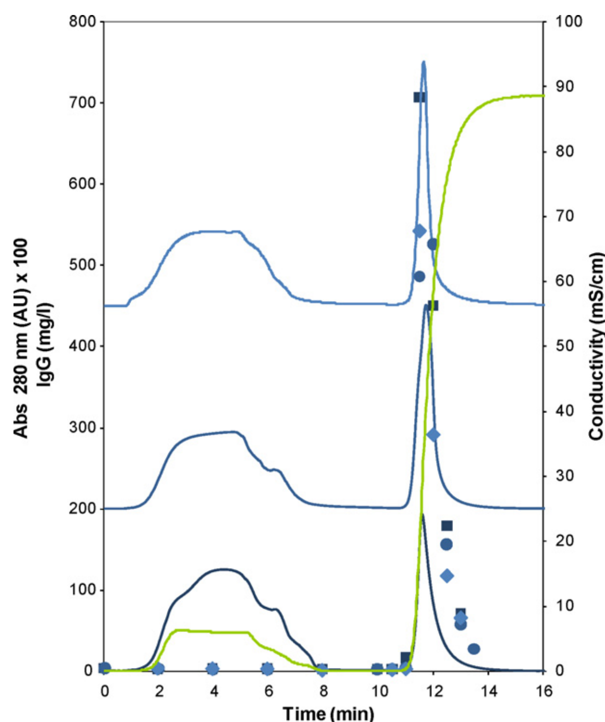

Fig. 7. Chromatography runs for the separation of IgG from the ATPS top phases, after loading 2 ml, on different cation exchange columns (from bottom to top): CM FF (▬), SP FF (▬) and SP XL (▬). Concentration of native IgG determined in the different fraction by Protein A affinity chromatography: CM FF (■), SP FF (●) and SP XL (♦). ATPS top phase: [IgG] = 420 mg/l, [Protein] = 620 mg/l and total purity = 56%. Overall composition of the ATPS: 5.17% dextran and 8% PEG-GA.

According to the most favorable compositions determined for each response variables and considering that this is the first step in the purification process, we have selected a composition of 5% dextran and 10% PEG-GA as the composition of the extraction system, since it allows the highest recovery of antibodies with a considerable increase in both purities, but specially of the protein purity.

3.1.4. Validation

In order to test the accuracy of the models, two ATPSs were prepared and the experimental data obtained compared with the data predicted from the models. For the best predicted composition (5% dextran, 10% PEG-GA), a recovery yield of 84% was obtained experimentally, with a total purity of 63% and protein purity of 96%, against 83 ± 4, 63 ± 4, 71 ± 7% obtained, respectively, using Eqs. (1)–(3). For the best experimentally evaluated composition (5.17% dextran, 8% PEG-GA), a recovery yield of 78% was obtained experimentally, with a total purity of 58% and protein purity of 90%, against 76 ± 2, 53 ± 6, 65 ± 5% obtained, respectively, using Eqs. (1)–(3). Because this set of experiments was performed with a different CHO cell supernatant batch, containing a similar IgG concentration (1.08 g/l *vs.* 1.02 g/l) and total purity (35% *vs.* 28%) but a 50% higher protein purity (21% *vs.* 31%), higher protein purities were obtained in the second set of experiments. Curiously, this second batch had a slightly lower total purity but this decrease did not affect the purity of the top phases. Moreover, both yield and total purity obtained for the system containing 5.17% dextran and 8% PEG-GA are in accordance to the ones obtained in the CCD (run 5, Table 1).

3.2. Two-step extraction

In order to increase the recovery yield of IgG in the PEG-GA-rich phase, the IgG remaining in the bottom phase of an ATPS containing 5.17% dextran and 8% PEG-GA was further partitioned to a new top phase. To perform this second stage extraction, the top phase of the blank system was added to the first stage bottom phase, using the same phase volume ratio (top/bottom phase volume = 3). The recovery yield of IgG in the first stage was 78% and in the second stage was 83%. Hence, of the 19.7 mg of IgG loaded in this ATPS, 15.3 mg were extracted in the first stage, 3.7 mg were extracted in the second stage and 0.8 mg remained in the bottom phase, translating an overall yield of 96%.

Table 3
Recovery yield of IgG in the eluted fractions and total yield in all collected fraction (in parenthesis) on the three cation exchange columns using 0.5, 1 and 2 ml sample loops.

Column	0.5 ml loop	1 ml loop	2 ml loop
SP XL	43% (48%)	60% (63%)	68% (71%)
SP FF	–	69% (71%)	76% (77%)
CM FF	–	80% (83%)	85% (86%)

The volume injected in the loop was 2–3 times higher than the corresponding volume.

3.3. Cation exchange chromatography

Cation exchange chromatography was selected for the second purification step. Typically, feedstocks have to be pre-conditioned before loading on a CEX column since charged molecules are only able to bind to ion exchange media at low ionic strength, *e.g.* less than 5 mS/cm [27]. Since PEG/dextran systems are characterized by low ionic strengths, we have explored the feasibility of loading the ATPS top phases directly on the CEX columns.

Preliminary studies performed using pure IgG solutions showed that the pH profile obtained during the binding step, at pH 5, was more constant with acetate buffers than with phosphate solutions. Also during the elution step, the pH changed more linearly with the acetate buffer than with phosphate. Thus, 20 mM sodium acetate buffer at pH 5 was chosen for start buffer. Salt-gradient and pH-gradient elutions were then compared using the selected start buffer. The salt gradient was further selected as it originated narrower eluted peaks. Hence, 20 mM acetate buffer at pH 5 with 1 M NaCl was selected for the elution buffer.

3.3.1. Screening of columns

The screening for the best ion exchange ligand is an important factor in the optimization of a purification step. In this work, we choose to use the pre-packed HiTrap IEX selection kit commercialized by GE Healthcare. The media packed in the cation exchange columns are SP Sepharose Fast Flow, CM Sepharose Fast Flow and SP Sepharose XL. The first two media are based on a 6% highly cross-linked beaded agarose matrix, whereas SP Sepharose XL media have long chains of dextran coupled to the 6% highly cross-linked agarose matrix, which increase the exposure of the SP charged groups. SP columns have a total ionic capacity (TIC) of 0.18–0.25 mmol H^+/ml medium and CM columns have a TIC of 0.09–0.13 mmol H^+/ml medium.

Table 4
Characterization of the first IgG fraction eluted from the cation exchange columns, including total purity, and percentage of aggregates and multi-aggregates.

	CM FF	SP FF	SP XL
Elution yield (%)	85	76	68
IgG (mg/l)	707	486	542
Total purity (%)	93	79	75
Aggregates	3	6	9
Multi-aggregates (%)	0	13	18

Sample loop = 2 ml.

Table 5
Purification of IgG using an extraction step in an ATPS composed of 5% dextran and 10% PEG-GA and a cation exchange chromatographic step on a CM Sepharose FF column.

Purification step	[IgG] (mg/l)	Yield (%)	Total purity (%)	Protein purity (%)
CHO cell supernatant	1024	–	27	31
ATPS	386	84	63	97
CEX on CM FF	298	89	91	104

Top phases of an ATPS composed of 5.17% dextran and 8% PEG-GA were selected for this study. The system was scaled-up 10 times in order to have enough top phase volume for the different chromatographic runs. A recovery yield of 78%, with a 56% total purity and 67% protein purity was obtained and is consistent with the models predicted for this ATPS composition (the same CHO cell supernatant batch was used) showing that the scale-up of this unit operation is very reliable. The concentration of IgG in this top phase was 420 mg/l. Different volumes of this solution (0.5, 1 and 2 ml) were injected into the cation exchange columns and the bound IgG was eluted with a salt step gradient. The chromatograms obtained for the three columns were very similar (Fig. 7) but the mass of IgG recovered was very different, depending not only on the sample loop used but also on the column selected, as shown in Table 3. Since very low amounts of IgG were detected in the flowthrough, this decrease in the elution yield is related to a decrease in the overall yield probably due to partial fractionation, since samples from the elution peak tail with low absorbance were not collected, but can also be associated with denaturation.

The increase in the elution yield with the injection volume can be attributed to the fact that eluted samples, with less than 60 mAU, were not collected neither analyzed, and consequently, the mass of IgG rejected represents higher percentages of IgG lost, for the lower injection volumes. Nevertheless, for the same sample loop, fast flow (FF) columns exhibited higher yields, and among these, the carboxymethyl (CM) ligand performed better than the sulfopropyl (SP).

Fig. 8 compares the size-exclusion chromatograms of the fractions eluting form the three columns with the column feedstock (ATPS top phase). According to Fig. 8a, the CEX feedstock is characterized by an IgG peak with a retention time of 8.8 min; a higher molecular weight impurity, consistent with IgG aggregates, eluting one minute before IgG (7.7 min); and several low molecular weight components with residence times equal to 11.5, 12.4, 12.5, 13 and 14.8 min. In all fractions eluting from the three cation exchange columns there is a very high clearance of all low molecular weight components, however there is a completely different profile regarding the higher molecular weight compounds. While the fraction eluted from the CM FF column (Fig. 8b) contains just two peaks

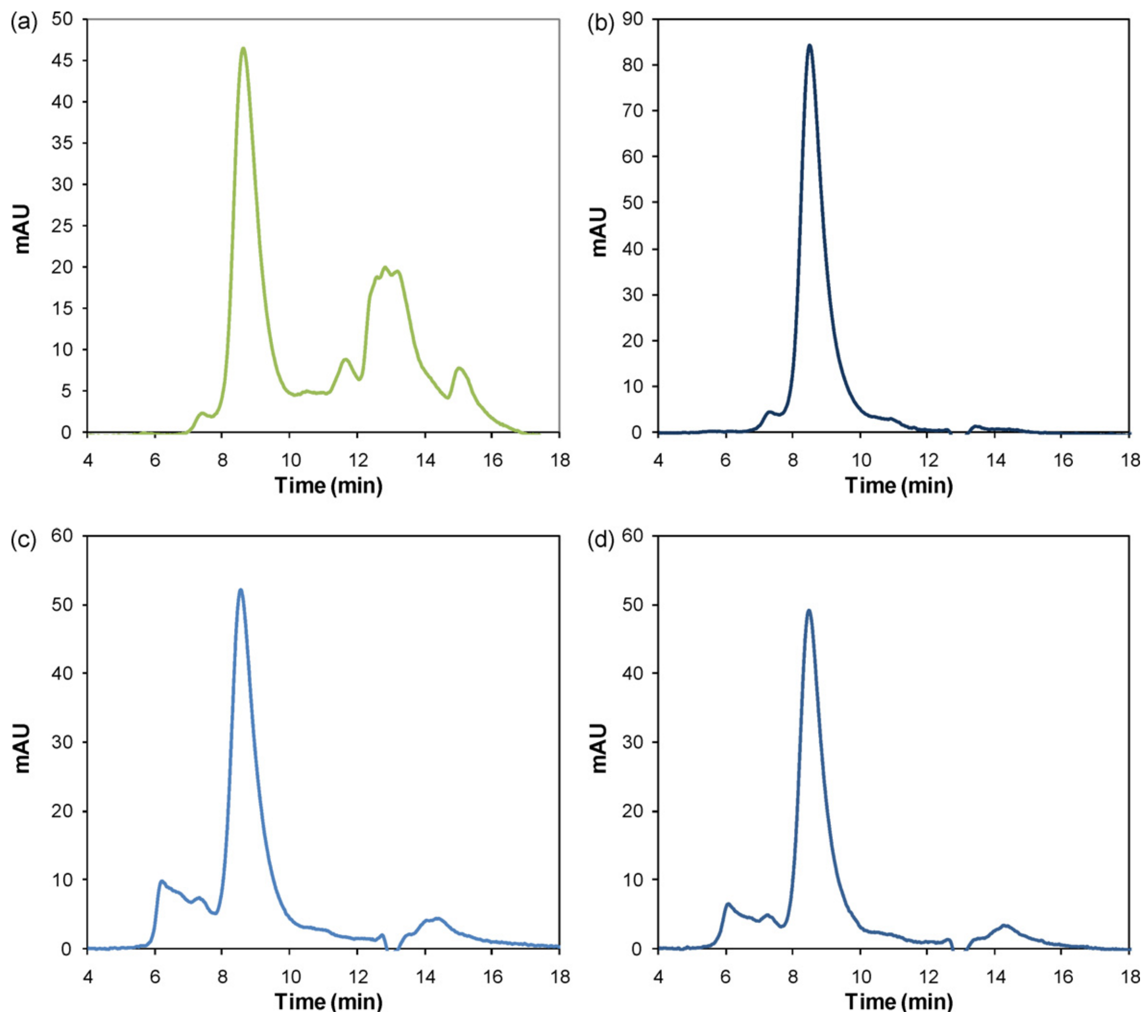

Fig. 8. Size-exclusion chromatograms showing the purity profile of the columns feedstock (a) and the fraction eluted from the columns CM FF (b), SP XL (c) and SP FF (d). ATPS top phase (column feedstock): [IgG] = 420 mg/l, [Protein] = 620 mg/l and total purity = 56%. Overall composition of the ATPS: 5.17% dextran and 8% PEG-GA.

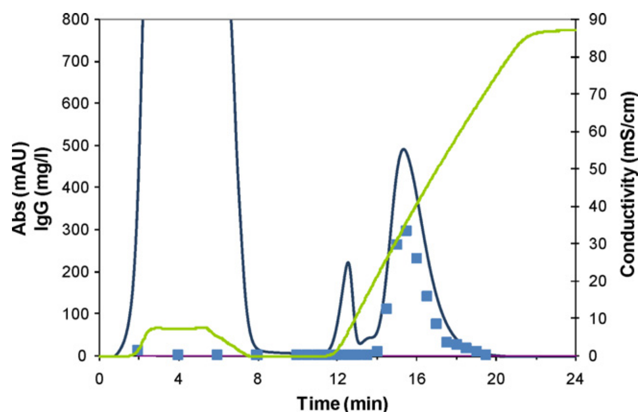

Fig. 9. Chromatography runs for the separation of IgG from the ATPS top phase, after loading 2 ml, on a CM FF cation exchange columns: (——) Absorbance at 280 nm; (——) conductivity; (■) native IgG concentration. Overall composition of the ATPS: 5% dextran and 10% PEG-GA. Sample volume: 2 ml; gradient length: 10 CV.

consistent with IgG and IgG aggregates, the fractions eluted from both SP columns (Fig. 8c and d) present not only a higher IgG aggregates content but a new peak consistent with multi-aggregates or unfolded IgG, which lack the ability to bind to protein A analytical column, and hence decrease the elution yield of native IgG (Table 4). An explanation for this induced denaturation can reside in the lower ligand density of the CM FF column in comparison with both SP columns. The carboxymethyl Sepharose fast flow was thus selected for the purification of IgG from ATPS top phases.

3.3.2. Gradient elution

The best ATPS composition has been determined to be 5% dextran and 10% PEG-GA (Section 3.1). In Fig. 9, the chromatogram of the purification of IgG from the corresponding top phase is shown. In this run, 2 ml of top phase were loaded on the CM FF column at 0.5 ml/min. IgG was then eluted using a 10 CV gradient elution at 1 ml/min, and contrary to what was observed in the previous section, during the step gradient elution, two peaks are now observed. According to HPLC analysis (Fig. 10), the first eluted peak contains low molecular weight components and the second peak contains the IgG, as depicted in Fig. 10c and d, respectively. The gradient elution enabled the separation of a small molecular weight impurity, but prevented the concentration of IgG in the pool of fractions. In this run, 89% of IgG was recovered in the elution pool of fractions, with a total purity of 91% and protein purity of 100% (Table 5).

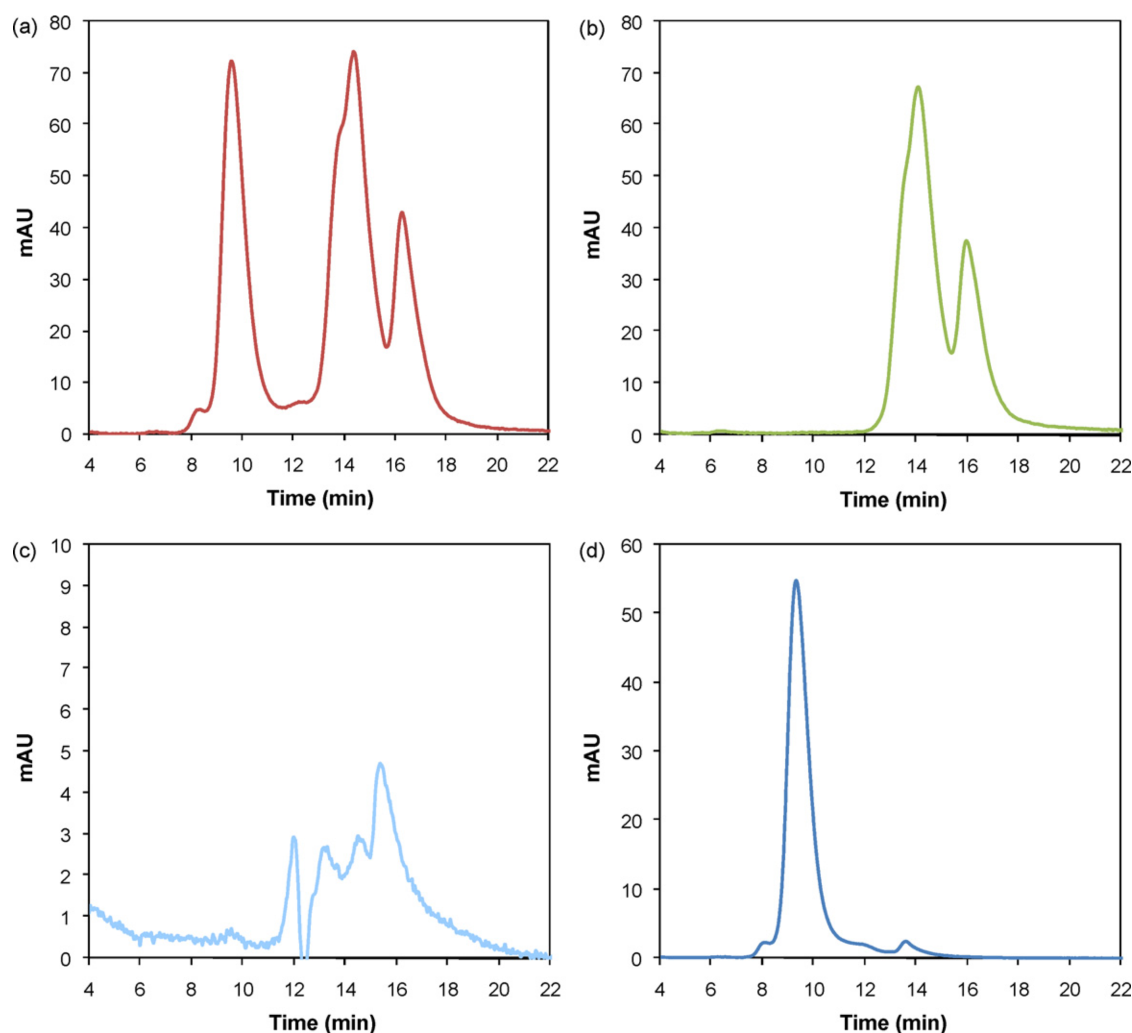

Fig. 10. Size-exclusion chromatograms showing the purity profile of the column feedstock (a) and the collected fractions from the columns flowthrough (b), first peak eluted (c) and second peak eluted (d). ATPS top phase (column feedstock): [IgG] = 370 mg/l, [Protein] = 380 mg/l and total purity = 63%. Overall composition of the ATPS: 5% dextran and 10% PEG-GA.

4. Conclusion

We have developed a process for the purification of human antibodies that allows the successful integration of affinity aqueous two-phase extraction with cation exchange chromatography. This process has an overall yield of 75% and allows the recovery of the antibodies with a total purity of 91% and about 100% protein purity. The capture of IgG from the CHO cell supernatant was successfully accomplished with a PEG/dextran system in which PEG hydroxyl groups were modified with an amino acid mimetic ligand (glutaric acid). Using just one step, the maximum recovery yield obtained was 82% but it can be increased to 96% using two extraction stages. ATPSs top phases can be directly loaded on a cation exchange column without any need for pre-conditioning, allowing a substantial simplification of the purification process. CM Sepharose FF columns were the most suitable for the purification of IgG, since SP columns induced the formation of unfolded variants.

The ATPS step allowed a considerable reduction on the amount of protein impurities, while the CEX step removed almost all low molecular weight compounds. In addition, this process also allows a significant reduction in the percentage of aggregates from 25% in the CHO cell supernatant, to 3% in the ATPS top phase to less than 2% in the eluted fraction from the CM FF column.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.jchromb.2008.11.014.

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