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Development and Comparison of Alternative Methods to Purify Adalimumab Directly from the Harvested Cell Culture Fluid

Running title: Adalimumab Purification from Harvested Cell Culture Fluid

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SUMMARY

Research background. Protein A affinity chromatography is a well-established method currently used in the pharmaceutical industry. However, the high costs usually associated with Protein A chromatographic separation and its difficulties in continuous operation make the investigation of alternative purification methods of great importance.

Experimental approach. In this study, the development and optimization of extraction-back extraction and precipitation-dissolution methods were performed, and they were compared with Protein A and cation exchange chromatographic separations in terms of mAb yield and concentration of residual impurities, such as DNA, host cell proteins, and mAb aggregates. To perform a comprehensive comparison of the different methods, experiments were carried out from the same cell-free fermentation broth containing adalimumab.

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Results and conclusions. Protein A and cation exchange chromatographic separations resulted in high adalimumab yield and purity. The precipitation-based process resulted in high yield, but with lower purity. The extraction-based purification resulted in low yield and purity. Thus, the precipitation-based method was found to be more promising for direct purification of adalimumab from harvested cell culture fluid compared to the extraction-based method.

Novelty and scientific contribution. Although alternative purification methods could offer the advantages of simplicity and low-cost operation, further significant improvements are still required to compete with the performance of the chromatographic separations of adalimumab from real fermentation broth.

Keywords: monoclonal antibody; purification technology; Protein A chromatography; precipitation; extraction

INTRODUCTION

The United States Food and Drug Administration (US FDA) approved the first monoclonal antibody (mAb) in 1986. Since then, the market for therapeutic antibody drugs has experienced explosive growth as new drugs have been approved for treating various human diseases, including cancer, autoimmune, metabolic, and infectious diseases (*1*). The global therapeutic mAb market was valued at approximately US$ 115.2 billion in 2018 and is expected to generate revenue of US$ 300 billion by 2025 (*2*). The widespread application of mAbs in clinical practice requires the development of cost-effective production routes. In general, a significant percentage (50-80 %) of the total manufacturing cost of mAb is incurred during the downstream processing. Critical challenges in producing new antibody therapeutics include improving process economics and efficiency, furthermore, fulfilling the increasingly demanding quality criteria for FDA approval (*3*). Although a wide range of technology is available for the downstream processing of proteins, most mAb purification systems are based on the use of Protein A affinity chromatography (*3*). Protein A affinity chromatography is highly selective for antibodies, allowing the removal of more than 95 % of the impurities from a complex fermentation broth culture in one step (*4*). However, using Protein A has some disadvantages, which are essentially related to the high cost and sensitivity of Protein A resins and possible leakage of the ligand from the resin matrix (*5*). In most industrial processes, two further chromatography steps, typically a cation and an anion exchange step, are inserted to remove remaining host cell proteins, DNA, leached Protein A ligands, and mAb aggregates from the eluate of the Protein A chromatography step (*6*). Furthermore, the process also includes virus inactivation steps

by using low pH and virus filtration steps to ensure an acceptable level of virus clearance (*4,7,8*). Since Protein A chromatography is considered the most critical and costly step in the general purification process of mAbs, intensive research efforts have been made in the past years to develop alternatives, including selective extraction or precipitation methods. Partitioning in aqueous two-phase systems (ATPSs) has proved to be a valuable tool for separating and purifying mixtures of biomolecules by extraction. ATPS is an ideal technology where protein clarification, concentration, and partial purification can be integrated into one step. This purification technique can be highly selective and can be suited for continuous operation on a large scale, thus allowing wider biotechnological applications. Both polymer-polymer (e.g., polyethylene glycol (PEG)-dextran, PEG-starch) and polymer-salt (e.g., PEG-phosphate, PEG-citrate) ATPSs have been developed and studied to purify different immunoglobulin proteins (*9*). The ATPS is a good solution to provide a moderate environment for antibodies, as both phases (polymer and salt) have high water content (80-90 %). In addition, phase-forming polymers can stabilize the tertiary structure of the target protein (*10*). Sequential precipitation of impurities and mAb in a selective manner is another promising strategy to substitute Protein A chromatographic separation. The precipitation method offers a fast process for protein separation with high yield. It is easy to scale up and operates with low costs (*11*). However, to achieve sufficient purity of the recovered mAb fraction, a combination of different precipitating agents (e.g., CaCl2 and PEG) in sequential precipitation steps would be required. The addition of CaCl2 to precipitate high molecular weight impurities (HMWI) such as dsDNA, host cell proteins (HCPs), and protein aggregates prior to the selective precipitation of mAbs by using PEG as a precipitating agent was found to be an interesting strategy for efficient, cost-effective purification of mAbs (*12*). Precipitation of mAbs by addition of PEG can be combined with other precipitating agents, such as caprylic (octanoic) acid, to enhance the final purity of the recovered antibody (*13*).

Although promising results were achieved in the field of mAb purification by using extraction and precipitation methods, the yield and purity of mAbs should be significantly enhanced for industrial implementation. Optimization of the different process variables affecting the purification of mAbs by extraction and precipitation is a prerequisite to make these methods competitive with the Protein A chromatographic step. The purification of mAbs by using ATPSs is strongly affected by the characteristics and concentration of the phase-forming agents, ionic strength, and pH, among other factors (*13,14*). The efficiency and selectivity of PEG precipitation of mAbs are highly dependent on the molecular mass and concentration of the PEG molecule, and the pH applied. Several studies have been dedicated recently to investigate the effects of different factors on the performance of mAb purification by extraction (*15*) and precipitation methods (*13,16*), and optimizing these strategies

(*10,14,17*). However, major part of the available studies focused on the investigation of model systems, and in many cases, the target immunoglobulin proteins were not specified (*10,14,17*). Thus, limited data are available in the literature regarding the purification of certain monoclonal antibodies from real fermentation broth (*16*). In addition, most of the studies focused on investigating and developing one alternative recovery method of the selected protein, making the exact comparison of different purification methods extremely hard and uncertain.

Thus, the aim of this work was to investigate the effects of different process variables on the recovery and purification of adalimumab from cell-free fermentation broth by using chromatographic, extractive, and precipitation-dissolution methods. Adalimumab recovery by alternative purification methods was optimized, and these methods (extraction and precipitation-dissolution) were compared to each other and with the conventional chromatographic method. The comparison was based on the adalimumab yield obtained during purifications, the amount of remaining dsDNA and HCP, and the presence of aggregates.

MATERIALS AND METHODS

Harvested Cell Culture Fluid

Fermentation broth containing adalimumab was derived from fed-batch cultures of recombinant Chinese hamster ovary (CHO) cells. The fermentation process was performed following the method detailed by Júlia Domján *et al.* (*18*). The fermentation broth was centrifuged at 4000 rpm for 40 minutes (Rotanta 460R, Hettich, Germany) at 20 °C and filtered through a bottle top vacuum filtration PES membrane (VWR International, Hungary) having a pore size of 0.2 µm to remove cells, cell debris and other insoluble contaminants. The obtained supernatant was stored in a refrigerator (-20 °C) until further use.

Purification methods

Protein A affinity chromatography

HiTrap® MabSelect SuReLX resin (4.24 mL, GE Healthcare, Chicago, US) was filled into a glass column (10 cm × 5.5 cm) for the preparative purification of adalimumab from the fermentation broth by using Äkta pure fast protein liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden) at room temperature (25 °C). The column was equilibrated with 4 column volumes (CVs) of equilibration buffer (20 mM sodium phosphate,15 mM sodium chloride, pH=7.4). at a flow rate of 1 mL/min and the sample (120 mL) was loaded at 1 mL/min. Thereafter, the column was washed with 6 CVs of washing buffer (10 mM EDTA, 1.5 M sodium chloride, 40 mM sodium phosphate, pH=7.4)

at 1 mL/min flow rate. Elution of adalimumab was performed with 10 CVs elution buffer (100 mM sodium citrate, pH=3.3) at 1 mL/min flow rate. The eluate was collected, and its pH was adjusted to 5.00 with 1 M NaOH solution. Until further analysis, the eluate was stored in a refrigerator (-20 °C).

Quality parameters of the column, such as the asymmetry factor and the height equivalent to the theoretical plate (HETP), were examined before adalimumab purifications by following the protocol of the producer (*19*). Measurements were performed in down-flow modes at three different flow rates (1.5 mL/min, 2.0 mL/min, and 2.5 mL/min). Measurement of dynamic binding capacity (DBC) was performed using purified adalimumab solution by following the method detailed by the producer of the resin (*20*), and the influence of residence times (3.6 min, 2.05 min, and 1.44 min) on DBC was analyzed. The loading of purified adalimumab solution was stopped when the concentration of mAb in the column effluent (detected at UV 280 nm) was 10 % relative to the mAb concentration of the feed. This loading is defined as 10 % breakthrough, and DBC was determined at this point.

Cation exchange chromatography

The Protein A eluate was loaded onto a 5.9 mL packed POROSTM 50 HS column (Thermo Fischer Scientific, Waltham, USA) with a flow rate of 1 mL/min at room temperature (25 °C). The column was equilibrated with 4 CVs of equilibration buffer (20 mM sodium citrate, pH=4.5) and the Protein A eluate (10 mL), then loaded onto the column at 1.0 mL/min. The column was washed with 2 CVs of washing buffer (20 mM sodium citrate, pH=5.0) at a flow rate of 1 mL/min. Before the actual elution phase, the ratio of the different elution solutions was set to the appropriate value within 2-5 CVs. Elution of the adalimumab was performed by gradient elution, varying the NaCl concentration (salt gradient elution) or the pH and NaCl concentration simultaneously (salt-mediated pH gradient elution) at 1 mL/min flow rate. During the salt gradient elution, NaCl concentration was changed within 5 CVs from 25 mM to 200 mM or from 25 mM to 100 mM, while the pH was kept constant at 8.1 or 8.6, respectively. The elution solutions were based on Tris (20 mM) buffer. During the salt-mediated pH gradient elution, the pH was changed from 7 to 9.1 at 1 mL/min flow rate, while the NaCl concentration was varied in different ranges within different CVs: 50-100 mM NaCl within 10 CVs, 62.5-75 mM NaCl within 10 CVs, and 25-50 mM within 15 CVs. The elution solutions were based on sodium-citrate (20 mM) and Tris (20 mM) buffers. The eluates were collected and stored in a refrigerator (-20 °C) until further analysis.

One-step, PEG-Buffer aqueous two-phase extraction

PEG-buffer (PEG-phosphate and PEG-citrate) aqueous two-phase extractions were performed as a one-step, batch extraction process to recover adalimumab in the PEG phase from the cell-free fermentation broth. The PEG-buffer extraction systems were prepared by mixing the appropriate amount of PEG3350 stock solution, phosphate or citrate buffer stock solution, fermentation broth, NaCl, and water. PEG3350 stock solution contained 50 *w/w* % PEG having an average molar mass of 3350 Da (PEG3350). The concentration of the phosphate and citrate buffer stock solutions was 40 *w/w* %, and they were prepared by using an appropriate amount of dipotassium hydrogen phosphate and sodium dihydrogen phosphate, or citric acid and tri-sodium citrate, respectively, according to the required pH. Phosphate buffer stock solutions with pH=6 and 8 and citrate buffer stock solutions with pH=5 were used in this study. The pH of the system was assumed to be equal to the pH of the stock solution applied. The extraction system contained 7 *w/w* % PEG3350 and 25 *w/w* % fermentation broth in all the cases, while the amount of NaCl, phosphate, or citrate and the pH of the system was varied (Table 1). The extraction systems were supplemented with an appropriate amount of water to reach the final total weight of 10 g in all cases. The extractions were performed in 15 mL centrifuge tubes in triplicates. After adding all the components of the extraction system, it was properly mixed by vortex, three times each for 20 seconds. The mixed system was kept at 25 °C for 5 minutes and then centrifuged (Rotanta 460R, Hettich, Germany) at 5000 g for 10 minutes to separate the PEG and buffer phases. The PEG and buffer phases were analyzed for adalimumab content.

Table 1

Two-step, PEG-dextran, and PEG-buffer aqueous two-phase extractions

The two-step extraction process consisted of a first step referred to as the extraction step and a second step referred to as the back-extraction step, performed in PEG-dextran and PEG-phosphate extraction systems, respectively. After the extraction step, the major part of the adalimumab was obtained in the PEG phase, so the PEG phase was separated and subjected to the back-extraction step (Fig. S1).

The PEG-Dextran ATPSs were prepared by weighting the appropriate amount of stock solutions of 16 *w/w* % PEG3350 (Sigma-Aldrich; USA) and 25 *w/w* % 500 kDa dextran (Pharmacosmos; Denmark), 10 *w/w* % phosphate buffer, 25 *w/w* % fermentation broth, NaCl and ultrapure water. Phosphate buffers with different pH values (5; 8) were obtained by using 1 M monopotassium phosphate (KH_2PO_4; Merck; Germany) and 1 M disodium hydrogen phosphate

(Na_2HPO_4; Sigma-Aldrich; Germany) in the appropriate ratio. The extraction (first extractive step of the two-step extraction process) was conducted at room temperature (25 °C) with 10 g total weight in 15 mL centrifuge tubes. All the components of the system were thoroughly mixed in a vortex for 20 seconds three times. The mixed system was kept at 25 °C for 5 minutes and then centrifuged (Rotanta 460R, Hettich, Germany) at 5000 g for 10 minutes. The bottom (dextran-phase) and top (PEG-phase) phases were separated, and the volumes and weight of the phases were measured. The top phase was subjected to the back-extraction process step and/or analysed to determine the adalimumab concentration. Designed experiments were performed to optimize selected process variables of the extraction step, such as pH (5,8), PEG to dextran ratio (6 %:10 %; 8 %:5 %), and concentration of NaCl (0 %; 1 %).

Back extraction was performed in a PEG-buffer system with a total weight of 0.7 g in 2 mL Eppendorf tubes at room temperature (25 °C). The back-extraction system was composed of 0.5 g of PEG-phase derived from the extraction step and 0.2 g, 0.35 g, or 0.5 g of buffer phase (40 *w/w* % phosphate buffer with pH=8). The system was mixed in a vortex for 20 seconds three times and then centrifuged at 5000 g for 10 minutes. The bottom and top phases were separated, and their volumes and weights were determined. The bottom phase (buffer phase) was analyzed for adalimumab concentration. Extraction and back-extraction process steps were performed in triplicates.

Precipitation-dissolution method

The investigation and development of a precipitation and dissolution method for adalimumab purification from fermentation broth were based on the work of Sommer *et al.* (*17*). In this study, adalimumab recovery from cell-free CHO fermentation broth by a precipitation-dissolution method was developed. The process was optimized by changing one factor at a time. The effects of $CaCl_2$ concentration (1.25, 2.5, 3.75 mM), PEG molecular weight (3350; 4000; 8000 Da), PEG4000 concentration (10; 12; 14; 16; 18 *w/w* %), and system pH (5.5; 6; 6.5; 7; 7.5; 8.5; 8.7; 8.9; and 9.1) were investigated. All the experiments were performed in triplicates. Experiments of $CaCl_2$ precipitations were carried out at room temperature (25 °C) with a 5 mL reaction volume. Samples were mixed with phosphate buffer (0.5 M, pH=8.0) to reach 5 mM phosphate concentration (4.95 mL cell culture supernatant with 0.05 mL phosphate buffer). Then 0.125 mL from the supernatant was replaced by 0.125 mL of $CaCl_2$ solution (50 mM, 100 mM, or 150 mM). The mixture was mixed in an end-over-end rotator for 60 min at 5 rpm. The obtained precipitate was separated by centrifugation at 4000 g for 15 min. Then, 3.5 mL from the supernatant and 1.5 mL of PEG solution (with the appropriate concentration to reach the desired PEG concentration in the system) were mixed and

incubated for 60 min at 5 rpm. The obtained mAb precipitate was separated by centrifugation at 4000 g for 15 min. The supernatant was discarded, and the precipitate was dissolved in 0.75 mL histidine buffer (20 mM Histidine; 100 mM NaCl) (Fig. S1). Liquid samples were analyzed to determine adalimumab, HCP, and dsDNA concentrations. Precipitation experiments were performed in triplicates.

Analytical methods

Adalimumab quantification

Determination of monomer adalimumab and small aggregates was performed by analytical size-exclusion chromatography (SEC). It was performed using a TSK-GEL G3000SWXL column obtained from Tosoh Bioscience (PA, USA) with a Shimadzu HPLC system (Ontario, Canada). The column was equilibrated at a flow rate of 1.0 mL/min with a phosphate solution containing 20 mM sodium phosphate (pH=6.8**)** and 100 mM sodium chloride. Before analysis, samples were filtered through a 0.22 µm PES membrane (Millipore Millex®-GP, Merck, Germany). Isocratic elution was performed at 25 °C column temperature by using the phosphate solution at a flow rate of 1.0 mL/min. Qualitative and quantitative analyses of adalimumab were performed by monitoring the elution at a wavelength of 280 nm by a UV detector (Shimadzu SPD-10A). For qualitative analysis of adalimumab monomers, a calibration curve was obtained by using a standard solution. The amount of adalimumab aggregates was determined as percentage of the amount of monomer adalimumab by calculating it from the ratios of the corresponding peak areas.

Determination of Host Cell Proteins (HCP)

HCP concentrations were determined in adalimumab solutions purified by various methods using the commercially available ELISA kit from Alpha Diagnostic Intl (San Antonio, USA) (*21*). The concentration of HCPs present in samples was calculated based on a calibration curve.

Determination of dsDNA

DsDNA concentrations were determined in adalimumab solutions purified by various methods using the Quant-iT™ PicoGreen® dsDNA reagent kit (Thermo Fisher Scientific, Waltham, USA) (*22*). The samples were excited at 480 nm, and the fluorescence emission intensity was measured at 520 nm with a spectrofluorometer (Thermo Scientific, Waltham, USA).

RESULTS AND DISCUSSION

Purification of adalimumab by chromatography

Before affinity chromatography purification of the fermentation broths on the Protein A column, quality parameters of the column were examined to verify the suitability of the filled resin for the chromatographic separation and to determine the appropriate flow rates for adalimumab purification. In general, an asymmetry factor between 0.8 and 1.8 is considered acceptable in this case (*23*). During the measurements by using the Protein A column, asymmetry factors of 1.16, 1.15, and 1.16 were obtained at flow rates of 1.5, 2, and 2.5 mL/min, respectively. The theoretical plate height is not limited in numbers, but the lower the HETP value, the better the column is. The theoretical plate height was around 0.05 cm (0.47; 0.05; 0.05) at all three flow rates, so the filling of the column was considered adequate. The DBC value specified by the manufacturer for the Protein A resin at 10 % breakthrough is 39 mg/mL at 2.4 minutes residence time by using a mAb solution (*24*). In our measurements using purified adalimumab solution (3 g/L), DBC values of 59.3, 42.3, and 32.5 mg/mL were obtained at residence times of 4.2, 2.4 and 1.7 minutes, respectively. Thus, the residence time of 4.2 minute was chosen for the following purifications of adalimumab, which corresponded to the flow rate of 1 mL/min. The flow rate of 1 mL/min was also selected for the washing and elution steps, resulting in good separation of adalimumab from other proteins based on the chromatogram detected at 280 nm (data not shown). Adalimumab yield of a certain purification step is calculated from the amount of adalimumab obtained after the purification step and expressed as a percentage of the initial amount of adalimumab. Protein A chromatography resulted in an adalimumab yield of 91 %. The amount of adalimumab aggregates after Protein A chromatography was 0.65 % of the monomer adalimumab content.

Cation exchange chromatographic purification of the eluate derived from the affinity chromatography step was performed with the main aims of reducing the amount of small adalimumab aggregates and separating charge variant fractions of monomer adalimumab. To accomplish adequate separation of the main fraction from the acidic and basic charge variants of adalimumab, salt gradient elution and salt-mediated pH gradient elution methods were investigated. The salt gradient elution was tested at pH=8.1 and pH=8.6, varying the NaCl concentration between 25 mM and 200 mM. However, salt gradient elution under the examined conditions did not result in the separation of the charge variants and main fraction of adalimumab. Thus, a salt-mediated pH gradient elution method was tested. The pH during the elution phase varied from 7 to 9.1 in all cases. The salt concentrations were changed between 25 mM and 100 mM with different ranges and gradients. Satisfactory separation of the charge variants from the main fraction of adalimumab was achieved when pH and NaCl concentration changed from 7 to 9.1 with a gradient of 0.14 pH unit/CV and from

0 mM to 50 mM with a gradient of 1.67 mM NaCl/CV, respectively (Fig. S2). The peak of the main fraction was eluted at around pH=8.3 (adalimumab pI=8.2) and the resolutions from the peaks of acidic and basic charge variants were 1.14 and 1.96, respectively. The adalimumab yield (in the main fraction of monomer adalimumab) of the cation exchange chromatography was 90 % and the amount of aggregates were 0.36 % of monomer adalimumab. Thus, affinity and cation exchange chromatography resulted in an overall adalimumab yield of 82 %.

Adalimumab purification by extraction and back-extraction

Extraction in PEG-buffer aqueous two-phase system

In the first step of investigating PEG-buffer systems in a one-step extraction of adalimumab from the cell-free fermentation broth, buffers of three different pH values were tested: pH=5 citrate buffer; pH=6 and 8 phosphate buffers (Table 1, Exp. 1-3). The highest adalimumab yield in the PEG phase (60 %) was obtained with a pH=5 citrate buffer. However, the results showed high standard deviations, which might be caused by partial precipitation of adalimumab during these extractions. Precipitation probably occurred because of the high ionic strength in the system, which could be caused by the high proportion of NaCl together with the high concentration of buffer components. Thus, the experiment was performed with pH=5 citrate buffer containing a reduced amount of NaCl (12.5 and 10 *w/w* % instead of 15 *w/w* % (Table 1, Exp. 4 and 5)). However, precipitation was still observed in these cases and the adalimumab yield significantly decreased in the case of the 10 *w/w* % NaCl system (45 %) compared to the previous measurements. As the presence of NaCl in an appropriate amount is considered to be essential for the efficient extraction of adalimumab to the PEG phase (*25*), ionic strength was reduced in the next step by decreasing the concentration of buffer components (8 *w/w* %), while the amount of NaCl (5, 10, and 15 *w/w* %) was varied (Table 1, Exp. 6-8). However, the achieved adalimumab yields were even lower (< 35 %) than in the previous experiments, and precipitation of adalimumab was still observed. Precipitation and partition of proteins in a polymer-buffer ATPSs might be strongly influenced not only by the ionic strength of the given phase but the type of the present ions, among many other factors (*26,27*). In our system, none of the investigated settings provided suitable conditions for efficient extraction of adalimumab into the PEG phase without considerable precipitation. Based on these results, this PEG-buffer system seems to be inefficient for adalimumab purification from our fermentation broth. In contrast, other investigations by using PEG-buffer (phosphate and citrate buffer) systems for mAb purification resulted in high (88-100 %) yields (Azevedo *et al.*, 2007, 2008; Azevedo, Gomes, *et al.*, 2009; Rosa *et al.*, 2007). In these studies, IgG protein, an artificial protein solution and CHO cells supernatant

(from Excellgene, Switzerland), and undefined antibodies were purified by PEG-buffer systems. In conclusion, although PEG-buffer systems work well in the case of different mAb solutions, it was not suitable with our fermentation broth containing adalimumab.

Extraction in a two-step process containing PEG-dextran and PEG-buffer aqueous two-phase systems.

The use of a polymer-polymer system could be preferred over a polymer-buffer system due to the lower ionic strength and the protein stabilizing effect of the phase-forming polymers. Thus, the PEG-dextran system was selected to investigate the extraction of adalimumab from cell-free CHO fermentation broth. Sequential extraction and back-extraction steps were investigated during the purification process. The extraction step was performed in the PEG-dextran system, and the back-extraction step was performed in the PEG-phosphate system. During the extraction step, the effects of pH (5, 8), NaCl concentration (0 %; 1 %), and PEG-dextran weight ratio (6 %:10 %; 8 %:5 %) on the adalimumab yield in the PEG phase were studied. The experiments were performed according to 2^3 experimental design. Based on the statistical evaluation, all factors (pH, NaCl concentration, PEG:dextran weight ratio) and their interactions significantly affect adalimumab yield (Fig. S3). The presence of NaCl (1 %) significantly improved the adalimumab yield and the PEG: dextran ratio of 8 %:5 % resulted in higher adalimumab yields compared to the ratio of 6 %:10 %. Furthermore, extractions performed at pH=8 were more favorable compared to pH=5 in terms of adalimumab yield (data not shown). Maximum adalimumab yield (75 %) was achieved with 1 % NaCl, pH=8, and 8 %:5 % PEG: dextran ratio. In the next step of our investigation, the effect of increasing the NaCl concentration (1 %, 2.5 %, and 5 %) on the adalimumab yield in the PEG phase was examined during the extraction step. By increasing the NaCl concentration, the adalimumab yield continuously increased in the PEG phase and decreased in the dextran phase. The adalimumab yield in the PEG phase was increased from 75 % to 95 % by increasing the NaCl concentration from 1 % to 5 % (Fig. 1). Adalimumab was enriched in the PEG phase during the extraction step, then fresh phosphate buffer (pH=8) was added to the separated PEG phase during a back-extraction step.

Fig. 1

The effect of NaCl concentration applied in the extraction step was also examined on the adalimumab yield obtained during the subsequent back-extraction step in the buffer phase. Moreover, the effect of different weight ratios of the PEG and buffer phases (1:0.4, 1:0.7, and 1:1) of the back-extraction step on the adalimumab yield in the buffer phase was also examined. Regarding the effect

of NaCl concentration, an opposite trend was experienced during the back-extraction compared to the extraction step: with increasing the NaCl concentration (in the extraction step), the adalimumab yield in the buffer phase decreased during the subsequent back-extraction step (Fig. 2).

Fig. 2

Moreover, precipitation was observed in the case of 5 % NaCl. Regarding the weight ratios of the PEG and buffer phases applied, increasing adalimumab yield was observed by decreasing the amount of the buffer phase (Fig. 2). Increased amounts of the added buffer phase (1:0.7 and 1:1 PEG: buffer ratios) caused adalimumab precipitation during the back-extraction steps, which explains that the lowest amount of buffer phase (1:0.4 PEG: buffer ratio), where no precipitation was observed, resulted in higher adalimumab yield. The highest adalimumab yield (91 %) during back-extraction was achieved by using a PEG: buffer weight ratio of 1:0.4 and 1 % NaCl in the extraction step. Although the highest yield of adalimumab was obtained with 5 % NaCl concentration during the extraction step, the 1 % NaCl extraction system was the best, with 91 % yield at the back-extraction step. The overall adalimumab yield of the extraction process, including the extraction and back-extraction steps, was calculated for all the cases, and the highest overall adalimumab yield (68 %) was achieved by using 1 % NaCl in the extraction step and a PEG: buffer ratio of 1:0.4 in the back extraction step. Thus, a two-step extraction process containing sequential extraction steps of PEG-dextran and PEG-buffer ATPSs was developed in our study, and it was found as promising strategy for the recovery of adalimumab from the fermentation broth produced in our laboratory. Comparable results have been presented for one-step extractions of IgG by using ATPSs based on PEG and dextran. Azevedo *et al.*;(*28*) achieved a 95 % yield of IgG in the upper (PEG) phase with an ATPS containing 5 % dextran, 8 % PEG with triethylene glycol diglutaric acid, and 10 mM phosphate buffer (pH=7). Rosa *et al.* (*29*) achieved 96 % of IgG in the PEG-rich phase in a single-stage extraction step, where the system was composed of 7 *w/w* % PEG (PEG3350), 5 *w/w* % dextrans, and 1.3 *w/w* % triethylene glycol diglutaric acid. In contrast to the one-step purifications, extraction systems containing two sequential steps have been barely studied so far, and they mainly focused on PEG-buffer systems in both steps (*10,30*). Azevedo et al (*30*) investigated a two-step extraction system (extraction: 8 *w/w* % PEG3350, 8 *w/w* % citrate, 15 *w/w* % NaCl (pH=6), back extraction: recovered PEG phase, 15 *w/w* % citrate, 5 *w/w* % NaCl) to purify IgG from a hybridoma cell culture supernatant. The process resulted in an overall IgG yield of 99 %. Rose *at el.* (*10*) obtained 76 % overall IgG yield from mixture of protein during a two-step extraction process based on PEG-buffer systems (extraction: 8 *w/w* % PEG3350, 10 *w/w* % phosphate buffer, 15 *w/w* % NaCl (pH=6), back-extraction: recovered PEG phase, 10 *w/w* % phosphate buffer (pH=6)).

Adalimumab purification by precipitations and dissolution

Recovery of adalimumab from cell-free CHO fermentation broth was also investigated by using the precipitation-dissolution method. During the optimization of the process parameters affecting the recovery and purity of adalimumab obtained after the sequential precipitations and dissolution, one factor was changed at a time. As shown in Fig. S1, the process contained two sequential precipitations and a dissolution step. The first precipitation step was accomplished by adding $CaCl_2$ to remove high molecular weight contaminants (e.g., dsDNA and aggregates) (*31,32*). This was followed by the precipitation of adalimumab with PEG solution. The main goal of this step was to separate mAbs from lower molecular weight impurities (e.g., HCP) (*17*). The last step was the dissolution of the precipitated adalimumab in histidine solution.

The effect of different CaCl2 concentrations (1.25, 2.5, 3.75 mM) was investigated in the first step. Following the precipitation with CaCl2, the obtained supernatant was analyzed for adalimumab and dsDNA concentrations. The adalimumab content of the supernatant did not decrease significantly, with yield higher than 98 % in all of the cases. However, the dsDNA concentration, relative to the mAb concentration, significantly decreased, but no significant difference was found in the dsDNA concentrations (1382 and 1408 ppm) when using 1.25 mM and 2.5 mM $CaCl_2$ solution. Thus, this treatment was selected as the first step to remove large molecular mass impurities such as dsDNA from the fermentation broth during all the following experiments.

In the next step, PEG-mediated precipitation of adalimumab was studied by varying the molecular mass and concentration of PEG and pH of the system. The obtained precipitates were separated and dissolved in 20mM histidine solution. Adalimumab yield was calculated based on the adalimumab content before the PEG-mediated precipitation and after the dissolution in histidine solution. The concentration of remaining dsDNA after the dissolution step was also determined. Evaluation of the different settings was based on the adalimumab yield and dsDNA concentration. The effect of the molecular weight of PEG was examined by using PEGs with an average molecular mass of 3350, 4000, and 8000 Da, using the same concentration (14 %) in all cases. As shown in Fig. 3, the highest adalimumab yield (84.3 %) and lowest dsDNA concentration (1027 ppm) were achieved by using PEG4000. Based on these results, PEG4000 was found to be suitable for adalimumab purification by the precipitation-dissolution method, so it was used in all of the following experiments.

Fig. 3

The effect of PEG content was investigated at 10 %, 12 %, 14 %, 16 %, and 18 % PEG concentrations. As shown in Fig. 4, by increasing the PEG concentration from 10 % to 14 %, the adalimumab yield sharply increased, reaching its maximum value (81.4 %) at 14 % PEG concentration. Further increase in the PEG content caused a slight decrease in the adalimumab yield, which might have occurred due to the formation of bigger aggregates in the presence of elevated PEG concentration.

Regarding the amount of dsDNA, PEG concentrations of 12 % and 14 % resulted in the lowest values corresponding to 1218 ppm and 1027 ppm, respectively. Considering the achievable adalimumab yield and dsDNA removal efficiency together, 14 % PEG concentration was selected as the most favorable setting, and it was applied in the following experiments.

Fig. 4

In the last step, the pH of the precipitation step was varied within the range of pH=5.5-9.1 (Fig. 5). Investigating the adalimumab yield in the function of the pH applied during the precipitation step, a trend with a maximum adalimumab yield (85 %) at pH=7.5 was observed. In the meantime, the remaining dsDNA concentration showed an opposite trend in the function of pH, having a minimum value (1027 ppm) at pH=7.5. Thus pH=7.5 was chosen as the most suitable pH for adalimumab precipitation using 14 % PEG4000.

In summary, the investigated process of adalimumab purification by selective precipitation-dissolution method contained two sequential precipitation steps and a dissolution step. The application of 2.5 mM $CaCl_2$ was chosen for the first precipitation step to remove a considerable part of dsDNA and probably other high molecular mass impurities. In the second step of selective adalimumab precipitation, the following parameters were selected as the most favorable condition in terms of adalimumab yield and removal of dsDNA: 14 % PEG with the average molecular mass of 4000 Da at pH=7.5. This process resulted in an adalimumab yield of 85 % and dsDNA concentration of 1027 ppm after adalimumab dissolution.

Sommer *et al.* (*17*) developed a mAb purification method, in which $CaCl_2$ and PEG precipitations were used. They tested the proposed method on five different CHO cell-free supernatant and achieved IgG yields of 80 %-95 %. These results are in line with our yield acquired during the purification of adalimumab from cell-free fermentation broth.

Fig. 5

Comparison of the purification methods

After investigation of the novel purification methods based on precipitation and extraction, the most favorable conditions in terms of adalimumab recovery were selected for each method, and the purifications were performed in a new batch of cell-free CHO supernatant. During these experiments, the different purification processes were compared and contrasted in terms of adalimumab yield and the amount of HCP, dsDNA, and adalimumab aggregates in the final solutions (Table 2).

Table 2

HCP, dsDNA, and adalimumab aggregates were also determined after chromatographic purifications and in the initial fermentation broth (Table 2) to be able to evaluate the efficiency of each purification process. Affinity chromatography resulted in high adalimumab yield (91 %) and low amounts of HCP (3082 ppm), dsDNA (2.93 ppm), and aggregates (0.65 %) in the effluent. The amount of HCP, dsDNA, and adalimumab aggregates could be further reduced by the subsequent cation exchange chromatography (Table 2) in addition to the separation of adalimumab charge variants. However, the overall adalimumab yield was decreased to 82 %. Comparable adalimumab yield (78 %) was achieved during the investigation of the precipitation-dissolution method. It was the most effective alternative purification method in removing HCP and dsDNA (Table 2), although it resulted in a higher amount of aggregates (2.27 %) in the final adalimumab solution. Thus, considering the purity of the final solution (containing 767341 ppm HCP and 933 ppm dsDNA), the precipitation-dissolution method was unsatisfactory as an individual purification step; however, it could be a good process for pre-purification prior to other purification steps. In the case of the extraction-based purification method, both steps of extraction and back-extraction were examined. Compared to the precipitation-dissolution method, the extraction step resulted in similar adalimumab yield, but higher amounts of HCP, dsDNA, and adalimumab aggregates in the PEG phase (Table 2). The subsequent back extraction step decreased the concentration of HCP and dsDNA relative to the mAb concentration, and almost completely eliminated protein aggregates (<0.1 %). However, the overall adalimumab yield was significantly decreased to 54 %. Interestingly, the relative HCP content after extraction-back extraction was even higher than the relative HCP content of the initial fermentation broth, indicating the necessity of further development of this process. On the other hand, considering the amount of small aggregates, extraction provided better results than chromatographic separation. The performance of the extractive separation method might be improved by combining it with other pre-purification steps. Both purification alternatives (precipitation and extraction) resulted in lower overall adalimumab yields during these experiments compared to the previous experiments aimed at their optimizations (Section 3.1, 3.2, 3.3). It might be because the fermentation broths derived from two different fermentation runs, thus broths with different adalimumab concentrations were used in

the optimization experiments and the experiments performed for the comparisons. In the latter, the fermentation broth contained less adalimumab (0.9 g/L instead of 1.3 g/L), which might negatively affect the achievable adalimumab yields during precipitations and extractions (*13*).

In summary, the precipitation-dissolution method and one-step extraction provided adalimumab yield comparable with the purification by affinity and cation exchange chromatography but with lower purity. Based on adalimumab yield and relative HCP and dsDNS contents, precipitation was found to be more promising compared to the extractive method for adalimumab purification from cell-free fermentation broth.

CONCLUSIONS

In this study, two alternative methods based on extraction and precipitation were developed and investigated to purify adalimumab directly from harvested cell culture fluid. The adalimumab-containing fermentation broths were produced in our laboratory using CHO cell cultivations. The precipitation method contained two sequential steps of precipitation using $CaCl_2$ and PEG, followed by dissolution in histidine solution. The extractive process contained two sequential steps of extraction and back-extraction based on PEG-dextran and PEG-buffer ATPSs, respectively. Both purification methods were optimized in several steps, and the best parameters were selected in terms of adalimumab yield and purity in certain cases. Classic chromatographic purification containing affinity and cation exchange steps was also performed and enhanced. The three different strategies were compared in terms of (monomer) adalimumab yield and the relative concentration of impurities such as HCP, dsDNA, and adalimumab aggregates.

Subsequent affinity and cation exchange chromatography were found to be an efficient purification method for adalimumab resulting in high yield and excellent purity. However, there are several disadvantages of chromatographic purifications. One of the main drawbacks is the high price of affinity resins. A further disadvantage is that it is difficult to operate them in continuous mode, and the scale-up could also be challenging. To overcome these obstacles, alternative purification methods of precipitations-dissolution and extraction-back-extraction offer promising alternatives, and thus they were studied and compared with each other and with the chromatographic separation. The precipitation-based process resulted in good adalimumab yield however impurities with high concentrations were still present in the final solution. The extraction-based purification resulted in low adalimumab yield with low purity.

The precipitation-based method was found to be more promising for direct purification of adalimumab from harvested cell culture fluid compared to the extraction-based method. However,

further improvements are still required to compete with the performance of the chromatographic separations. However, considering the advantages of the alternatives (such as continuous operation, simplicity, and scalability), a cost-effective operation might be achieved without the same performance (e.g., mAb yield) that chromatographic purifications have. Also, the performance of these alternative mAb purifications might be significantly improved by combining them, which will be the goal of further studies.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

Design of the research work was performed by Cs.F. and E.H. Data collection and analysis was carried out by D.V., J.D., B.Sz., L.B. P.H. The article draft was written by D.V. Critical revision and final approval was performed by E.H. and Cs.F..

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Table 1. Composition of PEG-buffer extraction systems

Experiment	Buffer pH	Citrate/ Phosphate (*w/w* %)	NaCl (*w/w* %)	PEG3350 (*w/w* %)	Fermentation broth (*w/w* %)
1	5	14	15	7	25
2	6	14	15	7	25
3	8	14	15	7	25
4	5	14	12.5	7	25
5	5	14	10	7	25
6	5	8	5	7	25
7	5	8	10	7	25
8	5	8	15	7	25

Table 2. Comparison of different purification methods for adalimumab

Method	Adalimumab Yield (%)	Host cell protein [ppm HCP/ mAb]	dsDNA [ppm dsDNA/mAb]	Aggregates (% of the monomer adalimumab content)
Fermentation broth	-	5 433 032	522 000	15.02
Affinity chromatography	91	3 082	2.93	0.65
Affinity and Cation exchange chromatography	82	176	0.48	0.36
Precipitations-dissolution	78	767 341	933	2.27
Extraction	76	8 181 067	4270	4.52
Extraction and Back-extraction	54	6 133 057	3952	<0.10

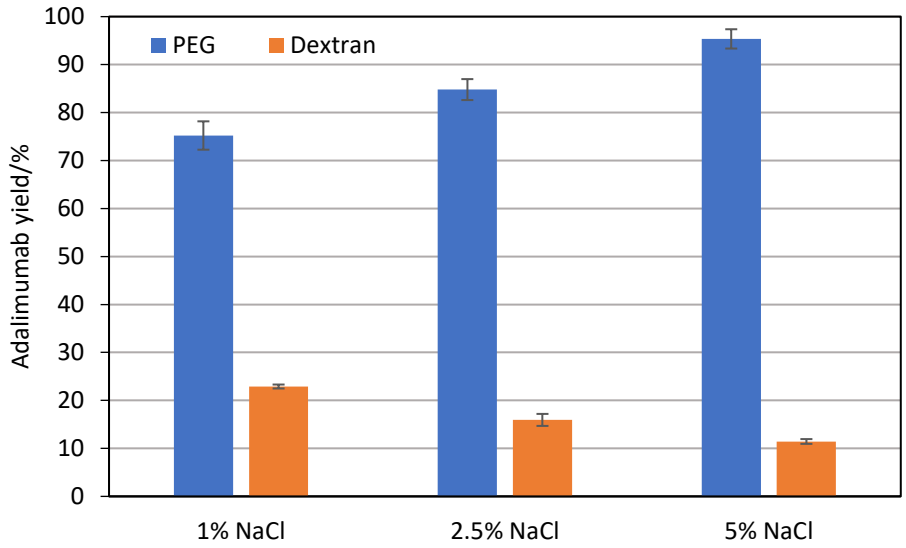

Fig. 1. Adalimumab yields in the PEG and dextran phases during the extraction step with different NaCl contents

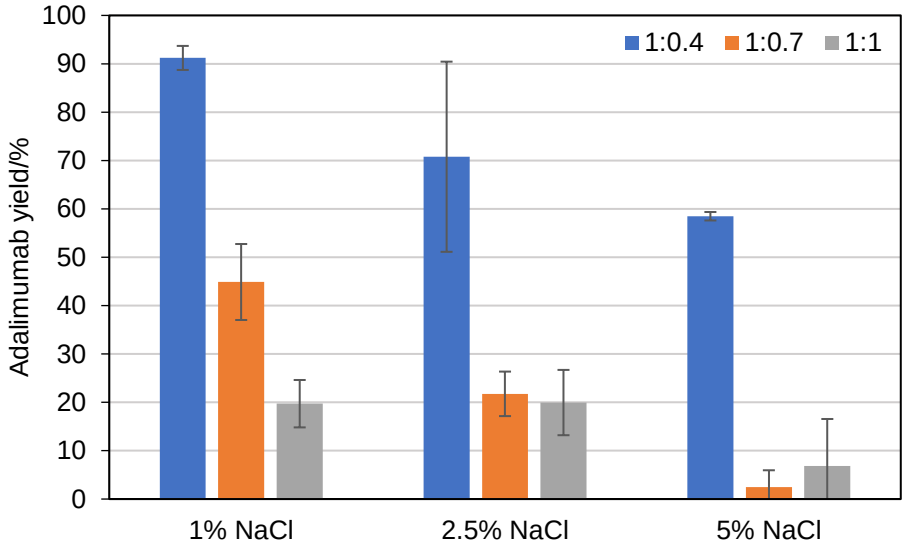

Fig. 2. Adalimumab yields in the buffer phase during the back-extraction experiments by using different NaCl concentrations in the extraction step (1, 2.5, and 5 w%) and the different weight ratio of PEG and buffer phases (1:0.4, 1:0.7, and 1:1 of PEG: buffer) during the back-extraction step

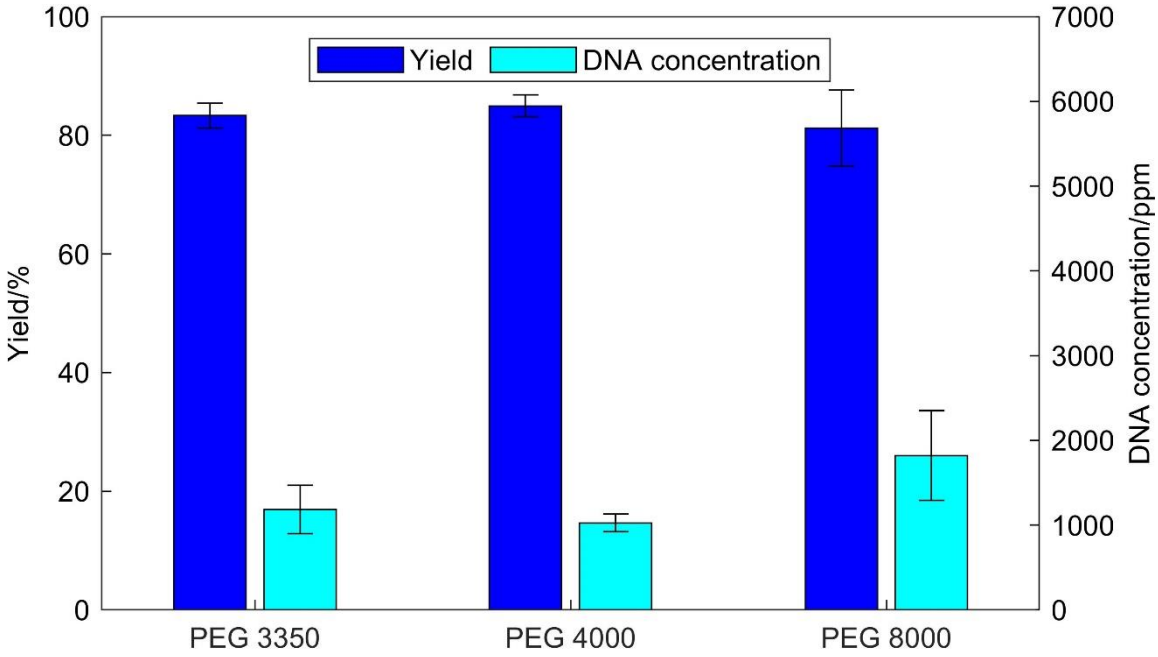

Fig. 3. Adalimumab yields and ds DNA concentration after precipitation and dissolution of adalimumab by using different molecular weights of PEG

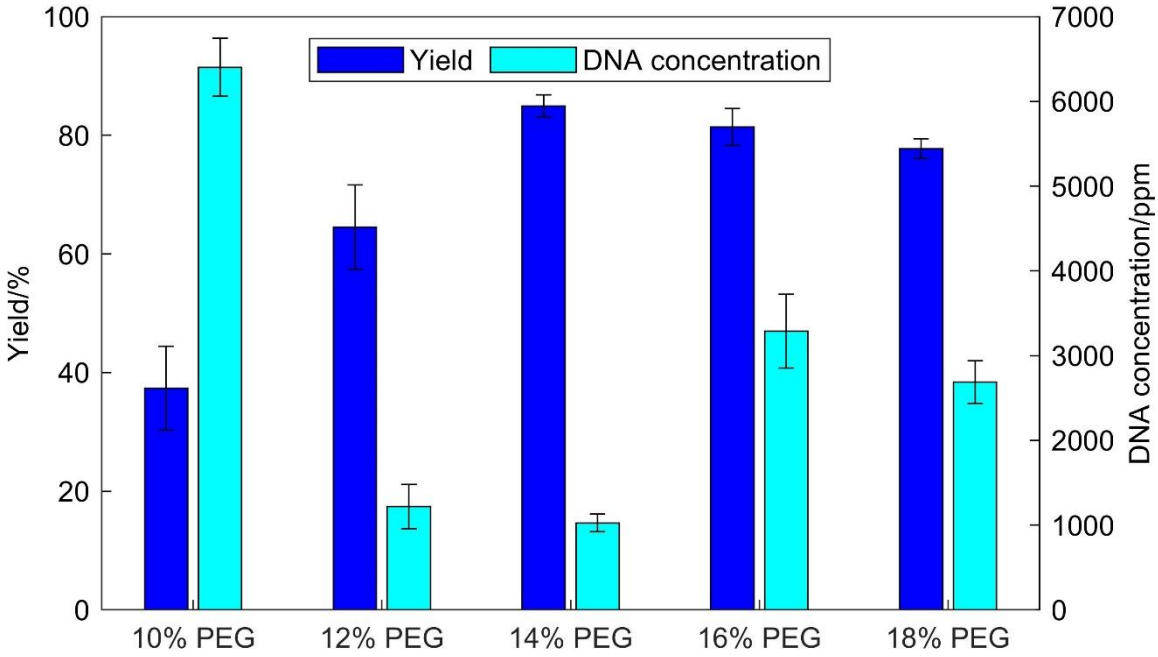

Fig. 4. Adalimumab yields and ds DNA concentration after precipitation and dissolution of adalimumab by using different concentrations of PEG4000

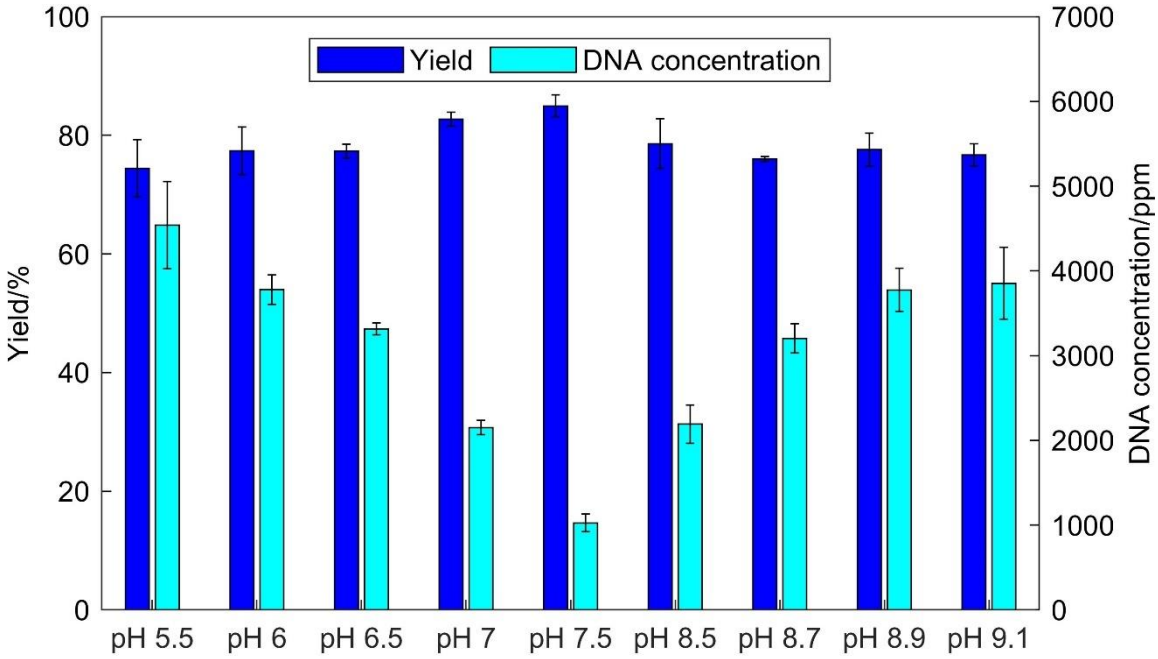

Fig. 5. Adalimumab yields and dsDNA concentration after precipitation and dissolution of adalimumab by using different pH-s during precipitation

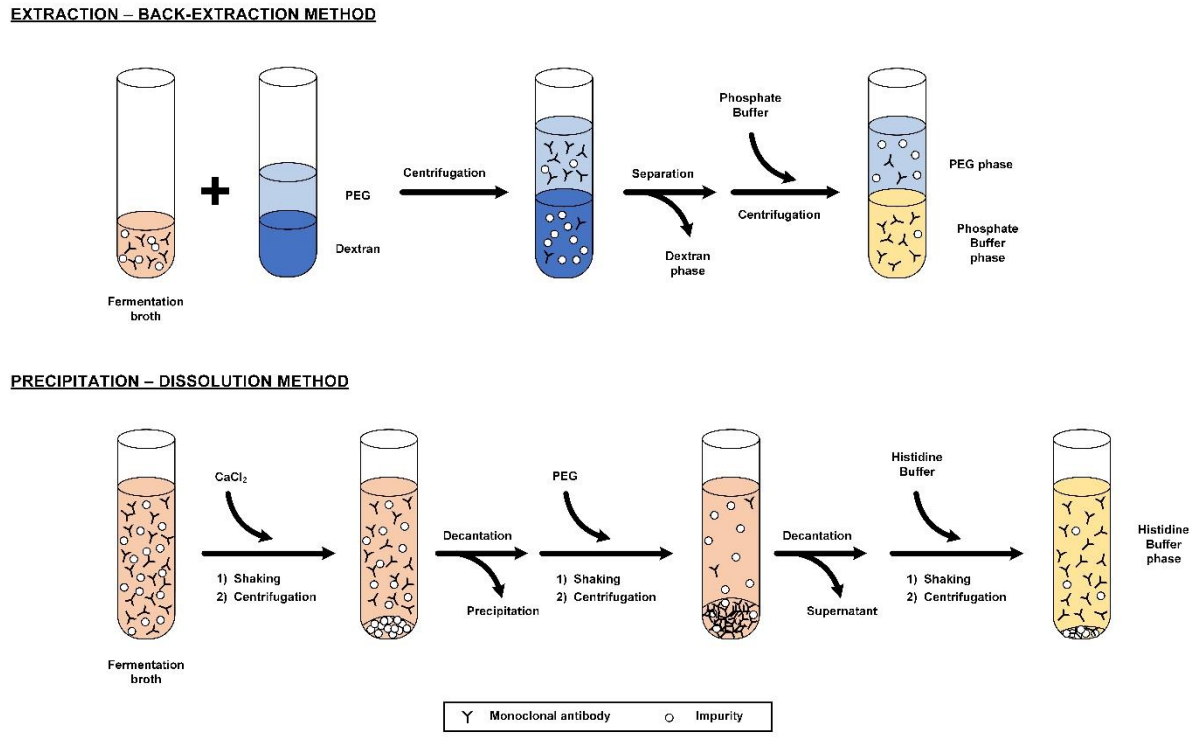

Fig. S1. Purification process of extraction-back extraction and precipitation-dissolution methods

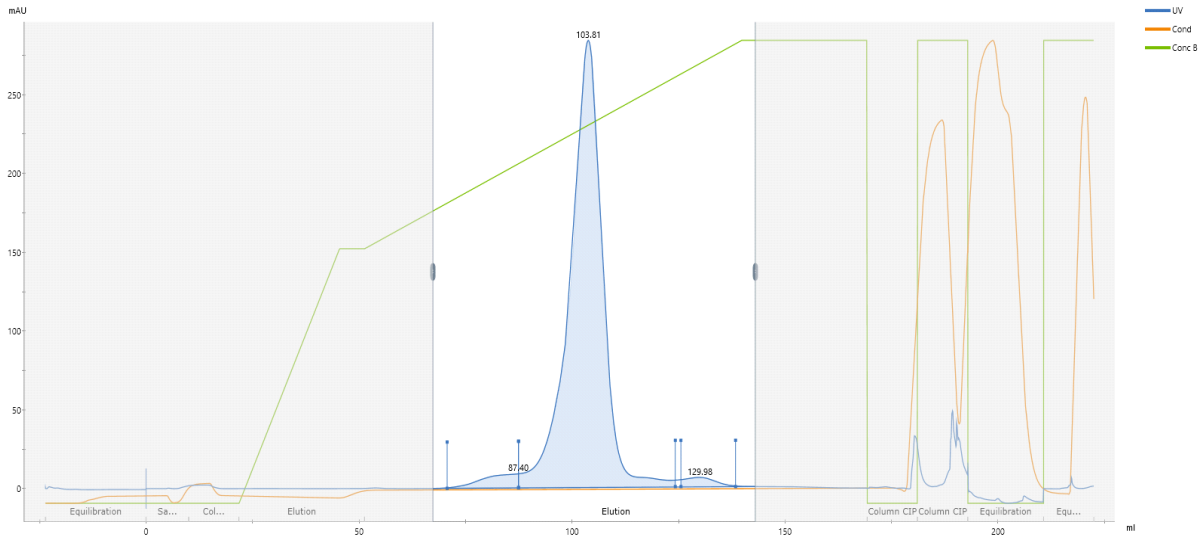

Fig. S2. Chromatogram, salt-mediated pH elution experiment pH=5.0-9.1 and 0-50mM NaCl

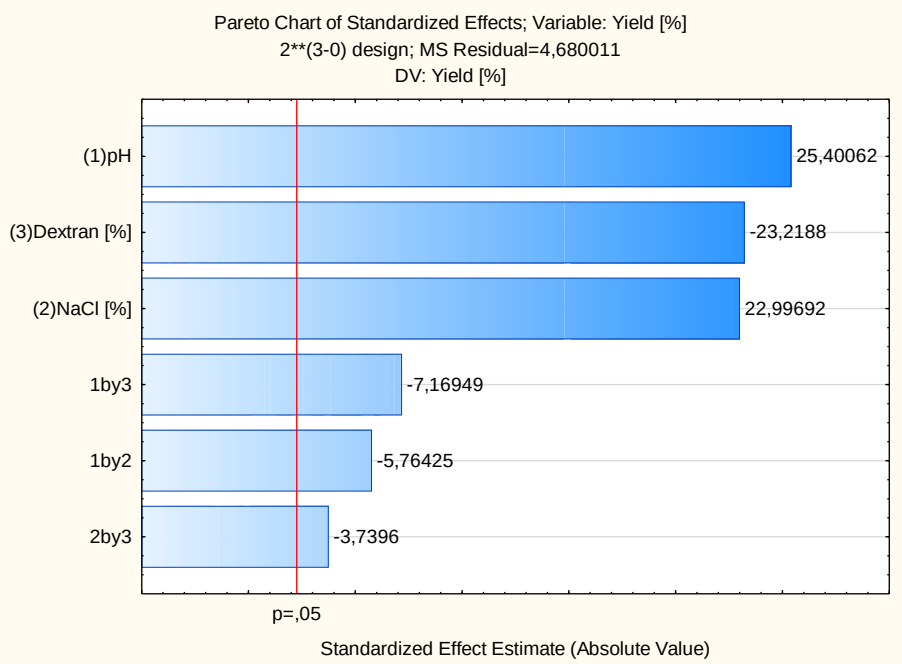

Fig. S2. Pareto chart of the effects of different factors on adalimumab yield during extraction in the PEG-dextran system