FTB Food Technology & Biotechnology

original scientific paper
ISSN 1330-9862
https://doi.org/10.17113/ftb.61.03.23.8094

Development and Comparison of Alternative Methods for the Purification of Adalimumab Directly from Harvested Cell Culture Fluid

Dorottya Vaskó[1], Júlia Domján[1], Bence Szűcs[1], László Bakk[1], Péter Hajdinák[2], György Marosi[1], Zsombor K. Nagy[1], Edit Hirsch[1]* and Csaba Fehér[2]*

[1]Department of Organic Chemistry and Technology, Faculty of Chemical Technology and Biotechnology, Budapest University of Technology and Economics, Műegyetem rkp. 3, 1111 Budapest, Hungary
[2]Department of Applied Biotechnology and Food Science, Faculty of Chemical Technology and Biotechnology, Budapest University of Technology and Economics, Műegyetem rkp. 3, 1111 Budapest, Hungary

Received: 23 January 2023
Accepted: 11 July 2023

*Corresponding author:
E-mail: hirsch.edit@vbk.bme.hu
feher.csaba@vbk.bme.hu

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 chromatographic separation of protein A and the difficulties in continuous operation make the investigation of alternative purification methods very important.

Experimental approach. In this study, extraction/back-extraction and precipitation/dissolution methods were developed and optimised. They were compared with protein A and cation exchange chromatography separations in terms of yield of monoclonal antibody (mAb) and amount of residual impurities, such as DNA and host cell proteins, and amount of mAb aggregates. For a comprehensive comparison of the different methods, experiments were carried out with the same cell-free fermentation broth containing adalimumab.

Results and conclusions. Protein A and cation exchange chromatographic separations resulted in high yield and purity of adalimumab. 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 proved to be more promising than the extraction-based method for direct purification of adalimumab from harvested cell culture fluid.

Novelty and scientific contribution. Although alternative purification methods may offer the advantages of simplicity and low-cost operation, further significant improvements are required to compete with the performance of chromatographic separations of adalimumab from true 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 (*1*). Since then, the market for therapeutic antibody drugs has experienced rapid growth as new drugs have been approved for the treatment of various human diseases, including cancer, autoimmune, metabolic and infectious diseases. The global market for therapeutic mAb was estimated at approx. US$ 115.2 billion in 2018 and is expected to reach US$ 300 billion by 2025 (*2*). The widespread use of mAbs in clinical practice requires the development of cost-effective production routes. In general, downstream processing accounts for a significant percentage (50–80 %) of the total manufacturing cost of mAb. Key challenges in the production of new antibody therapeutics include improving process economics and efficiency, as well as meeting the increasingly demanding quality criteria for FDA approval (*3*). Although a wide range of technologies are available for 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 and allows the removal of more than 95 % of the impurities from a complex fermentation broth culture in one step (*4*). However, the use of protein A has some disadvantages, mainly related to the high cost and sensitivity of

protein A resins and the possible leakage of the ligand from the resin matrix (*5*). In most industrial processes, two further chromatography steps, usually a cation and an anion exchange step, are added to remove residual host cell proteins, DNA, leached protein A ligands and mAb aggregates from the eluate of the protein A chromatography step (*6*). In addition, the process also includes virus inactivation steps 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 recent years to develop alternatives, including selective extraction or precipitation methods. Partitioning in aqueous two-phase systems (ATPS) has proven to be a valuable tool for the separation and purification of mixtures of biomolecules by extraction. ATPS is an ideal technology where clarification, concentration and partial purification of proteins can be integrated into one step. This purification technique can be highly selective and is suitable for a large-scale continuous operation, enabling broader biotechnological applications. Both polymer-polymer (*e.g.* polyethylene glycol (PEG)-dextran, PEG-starch) and polymer-salt (*e.g.* PEG-phosphate, PEG-citrate) ATPS have been developed and studied to purify various immunoglobulin proteins (*9*). The ATPS is a good solution to create a moderate environment for antibodies because both phases (polymer and salt) have a high water content (80–90 %). In addition, the phase-forming polymers can stabilise the tertiary structure of the target protein (*10*). Sequential precipitation of impurities and mAb in a selective manner is another promising strategy to replace chromatographic separation of protein A. The precipitation method offers a rapid process for protein separation with high yield. It is easy to scale up and operates at low cost (*11*). However, to achieve sufficient purity of the recovered mAb fraction, a combination of different precipitating agents (*e.g.* $CaCl_2$ and PEG) would be required in sequential precipitation steps. The addition of $CaCl_2$ to precipitate high-molecular-mass impurities (HMMI) such as dsDNA, host cell proteins (HCPs) and protein aggregates prior to the selective precipitation of mAbs using PEG as a precipitating agent proved to be an interesting strategy for efficient, cost-effective purification of mAbs (*12*). Precipitation of mAbs by the addition of PEG can be combined with other precipitating agents, such as caprylic (octanoic) acid to increase the final purity of the recovered antibody (*13*).

Although promising results have been achieved in the field of mAb purification using extraction and precipitation methods, the yield and purity of mAbs for industrial application should be significantly improved. The optimisation of various process variables affecting the purification of mAbs by extraction and precipitation is a prerequisite for these methods to compete with the protein A chromatography step. The purification of mAbs by ATPS is strongly affected by the properties 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 and the pH used. Several studies have recently focused on investigating the effects of various factors on the performance of mAb purification by extraction (*15*) and precipitation methods (*13*,*16*), and optimising these strategies (*10*,*14*,*17*). However, most of the available studies have focused on the investigation of model systems and in many cases the target immunoglobulin proteins have not been specified (*10*,*14*,*17*). Therefore, few data are available in the literature on the purification of specific monoclonal antibodies from fermentation broth (*16*). Furthermore, most studies have focused on the investigation and development of an alternative method of recovery of the selected protein, which makes the accurate comparison of different purification methods extremely difficult and uncertain.

Therefore, the aim of this work is to investigate the effects of different process variables on the recovery and purification of adalimumab from cell-free fermentation broth using chromatography, extraction and precipitation/dissolution methods. Adalimumab recovery by alternative purification methods was optimised and these methods (extraction and precipitation/dissolution) were compared with 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

The fermentation broth containing adalimumab was obtained from recombinant Chinese hamster ovary (CHO) cells in fed-batch cultures. Fermentation was performed according to Domján *et al.* (*18*) in a reactor with a working volume of 2 L. Dissolved oxygen (40 %), pH (7.1), temperature (37 °C), glucose (~10 mM) and glutamate (~3–10 M) values were regulated during cultivation in the bioreactor. The fermentation broth was centrifuged at 4000×*g* for 40 min (Rotanta 460R; Hettich, Darmstadt, Germany) at 20 °C and filtered through a bottle-top vacuum filtration PES membrane (VWR International, Budapest, Hungary) with 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, IL, USA) was loaded into a glass column (10 cm×5.5 cm) for the preparative purification of adalimumab from the fermentation broth using the 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 of equilibration buffer (20 mM sodium phosphate and 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. Then the column was washed with 6 column volumes of wash buffer (10 mM EDTA, 1.5 M sodium chloride and 40 mM sodium phosphate, pH=7.4) at a flow rate of 1 mL/min. Adalimumab was eluted with 10 column volumes of elution buffer (100 mM sodium citrate, pH=3.3) at a flow rate of 1 mL/min. The eluate was collected and its pH was adjusted to 5.0 with 1 M NaOH solution. It was stored in a refrigerator (–20 °C) until further analysis.

The quality parameters of the column, such as the asymmetry factor and the height equivalent to the theoretical plate (HETP), were examined before adalimumab purification according to the manufacturer's protocol (Merck KgaA, Darmstadt, Germany). Measurements were performed in down-flow mode at three different flow rates (1.5, 2.0 and 2.5 mL/min). Dynamic binding capacity (DBC) was measured with purified adalimumab solution and according to the method described by the manufacturer of the resin (*19*) and the influence of the residence times (3.6, 2.05 and 1.44 min) on DBC was analysed. Loading of purified adalimumab solution was stopped when the mAb amount in the column effluent (detected at UV λ=280 nm) was 10 % relative to the mAb amount 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, MA, USA) at a flow rate of 1 mL/min and room temperature (25 °C). The column was equilibrated with 4 column volumes of equilibration buffer (20 mM sodium citrate, pH=4.5) and the protein A eluate (10 mL) and then loaded onto the column at 1.0 mL/min. The column was washed with 2 column volumes 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 adjusted to the appropriate value within 2–5 column volumes. The adalimumab was eluted with gradient elution, varying the NaCl concentration (salt gradient elution) or the pH and NaCl concentration simultaneously (salt-mediated pH gradient elution) at a flow rate of 1 mL/min. During the salt gradient elution, NaCl concentration changed from 25 to 200 mM or from 25 to 100 mM within 5 column volumes, while the pH was kept constant at 8.1 or 8.6, respectively. The elution solutions were based on Tris buffer (20 mM). During the salt-mediated pH gradient elution, the pH was changed from 7 to 9.1 at a flow rate of 1 mL/min, while the NaCl concentration varied in different ranges within different column volumes: 50–100 mM NaCl within 10 column volumes, 62.5–75 mM NaCl within 10 column volumes and 25–50 mM within 15 column volumes. The elution solutions were based on buffers sodium citrate (20 mM) and Tris (20 mM). 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 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 (Millipore Sigma, St. Louis, MO, USA), phosphate or citrate buffer stock solution, fermentation broth, NaCl and water. The PEG3350 stock solution contained mass fraction of 50 % PEG with an average molar mass of 3350 Da. The mass fractions of phosphate and citrate buffer stock solutions were 40 % and they were prepared with an appropriate amount of dipotassium hydrogen phosphate and sodium dihydrogen phosphate or citric acid and trisodium citrate, respectively, depending on the required pH. In this study, phosphate buffer stock solutions with pH=6 and 8 and citrate buffer stock solutions with pH=5 were used. The pH of the system was assumed to be equal to the pH of the stock solution used. In all cases, the extraction system contained mass fractions of 7 % PEG3350 and 25 % fermentation broth, while the amount of NaCl, phosphate or citrate and the pH of the system varied (**Table 1**). The extraction systems were supplemented with an appropriate amount of water to reach the final total mass of 10 g in all cases. Extractions were performed in 15-mL centrifuge tubes in triplicate. After adding all the components of the extraction system, it was properly mixed by vortex, three times for 20 s each. The mixed system was kept at 25 °C for 5 min and then centrifuged (Rotanta 460R; Hettich) at 5000×*g* for 10 min to separate the PEG and buffer phases. The adalimumab content in PEG and buffer phases was determined.

Table 1. Composition of PEG-buffer extraction systems

Experiment	Buffer pH	(*m*(citrate)/*m*(phosphate))/%	*w*(NaCl)/%	*w*(PEG3350)/%	*w*(fermentation broth)/%
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

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

The two-step extraction consisted of a first step referred to as the extraction step and a second step referred to as the back-extraction step, which was carried out in PEG-dextran and PEG-phosphate extraction systems, respectively. After the extraction step, most 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 aqueous two-phase system (ATPS) was prepared using mass fractions of 16 % PEG3350 stock solution (Millipore Sigma) and 25 % 500 kDa dextran (Pharmacosmos, Holbæk, Denmark), with the addition of 10 % phosphate buffer, 25 % fermentation broth, NaCl and ultrapure water. Phosphate buffers with different pH values (5 and 8) were prepared with 1 M monopotassium phosphate (Millipore Sigma) and 1 M disodium hydrogen phosphate (Millipore Sigma) in the appropriate ratio. Extraction (first extraction step of the two-step procedure) was carried out at room temperature (25 °C) with 10 g total mass in 15-mL centrifuge tubes. All the components of the system were thoroughly mixed three times for 20 s in a vortex at 10×*g*. The mixed system was kept at 25 °C for 5 min and then centrifuged (Rotanta 460R; Hettich) at 5000×*g* for 10 min. The lower (dextran) and upper (PEG) phases were separated and the volumes and mass of the phases were measured. The upper phase was subjected to the back-extraction step and/or analysed to determine the adalimumab concentration. Experiments were performed to optimise selected process variables of the extraction step, such as pH (5 and 8), PEG to dextran mass ratio (6:10 and 8:5) and the mass fraction of NaCl (0 and 1 %).

Back-extraction was performed in a PEG-buffer system with a total mass of 0.7 g in 2 mL Eppendorf tubes at room temperature (25 °C). The back-extraction system consisted of 0.5 g of the PEG-phase derived from the extraction step and 0.2, 0.35 or 0.5 g buffer phase (40 % phosphate buffer with pH=8). The system was mixed three times in a vortex at 10×*g* for 20 s and then centrifuged at 5000×*g* for 10 min. The lower and upper phases were separated, and their volumes and mass were determined. The adalimumab concentration was determined in the lower (buffer) phase. The extraction and back-extraction steps were performed in triplicate.

Precipitation/dissolution method

In this study, the recovery of adalimumab from cell-free CHO fermentation broth was developed using a precipitation/dissolution method based on the work of Sommer *et al.* (*17*). The process was optimised by changing one factor at a time. The effects of $CaCl_2$ concentration (1.25, 2.5 and 3.75 mM), PEG molecular mass (3350, 4000 and 8000 Da), PEG4000 mass fraction (10, 12, 14, 16 and 18 %) and pH of the system (5.5, 6, 6.5, 7, 7.5, 8.5, 8.7, 8.9 and 9.1) were investigated. All experiments were performed in triplicate. Experiments on precipitation of $CaCl_2$ were carried out at room temperature (25 °C) with a reaction volume of 5 mL. Samples were mixed with phosphate buffer (0.5 M, pH=8.0) to achieve a phosphate concentration of 5 mM (4.95 mL cell culture supernatant with 0.05 mL phosphate buffer). Then 0.125 mL of the supernatant was replaced with 0.125 mL of $CaCl_2$ solution (50, 100 or 150 mM). The mixture was mixed in an end-over-end rotator at 5×*g* for 60 min. The obtained precipitate was separated by centrifugation at 4000×*g* for 15 min. Then 3.5 mL of supernatant and 1.5 mL of PEG solution (with the appropriate mass fraction) were mixed and incubated at 5×*g* for 60 min. 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 and 100 mM NaCl) (**Fig. S1**). The liquid samples were analysed to determine adalimumab, HCP and dsDNA amounts. The precipitation experiments were performed in triplicate.

Analytical methods

Adalimumab quantification

Monomeric adalimumab and small aggregates were determined by analytical size-exclusion chromatography (SEC) using a TSK-GEL G3000SWXL column (Tosoh Bioscience, Philadelphia, PA, USA) with a Shimadzu HPLC system (SPD-10A VP, 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. Prior to analysis, samples were filtered through a 0.22-µm PES membrane (Millipore Millex®-GP; Merck). Isocratic elution was performed at 25 °C column temperature 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 using a UV detector (Shimadzu SPD-10A). For the qualitative analysis of adalimumab monomers, a calibration curve was prepared using a standard solution. The amount of adalimumab aggregates was determined as a percentage of the amount of monomeric adalimumab by calculating it from the ratios of the corresponding peak areas.

Determination of host cell proteins

Host cell protein (HCP) concentrations were determined in adalimumab solutions purified by various methods (protein A affinity chromatography, extraction/back-extraction, precipitation/dissolution) using the commercially available CHO HCP ELISA kit from Alpha Diagnostic Intl (San Antonio, TX, USA). The amount of HCP in the samples was calculated using a calibration curve and expressed in relation to the mAb amount.

Determination of dsDNA

The dsDNA quantities were determined in adalimumab solutions purified by various methods (protein A affinity

chromatography, extraction and back-extraction, precipitation and dissolution) using the Quant-iT™ PicoGreen® dsDNA reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were excited at 480 nm and the intensity of fluorescence emission was measured at 520 nm using a spectrofluorometer (Thermo Fisher Scientific). DNA quantities were expressed in relation to the mAb content.

RESULTS AND DISCUSSION

Purification of adalimumab by chromatography

Prior to purification of the fermentation broths on the protein A column by affinity chromatography, the quality parameters of the column were investigated to verify the suitability of the resin used for 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 (*20*). In measurements with 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. There is no numerical limit to the theoretical plate height, but the lower the HETP value, the better the column. The HEPT value was about 0.05 cm (0.47, 0.05 and 0.05 cm) at all three flow rates, so the filling of the column was considered sufficient. The DBC value specified by the manufacturer (GE Healthcare, Chicago, IL, USA) for protein A resin at 10 % breakthrough is 39 mg/mL at 2.4 min residence time using mAb solution. In our measurements with purified adalimumab solution (3 g/L), DBC values of 59.3, 42.3 and 32.5 mg/mL were obtained with residence times of 4.2, 2.4 and 1.7 min, respectively. Therefore, the residence time of 4.2 min was chosen for the subsequent purification of adalimumab, which corresponded to a flow rate of 1 mL/min. The flow rate of 1 mL/min was also chosen for the washing and elution steps, resulting in good separation of adalimumab from other proteins based on the chromatogram at 280 nm (data not shown). The adalimumab yield of a given 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 concentration.

The eluate from the affinity chromatography step was purified by cation exchange chromatography to reduce the amount of small adalimumab aggregates and to separate the charge variant fractions of adalimumab. For adequate separation of the main fraction from the acidic and basic charge variants of adalimumab, the 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, with NaCl concentration varying between 25 and 200 mM. However, salt gradient elution did not result in the separation of the charge variants and the main fraction of adalimumab under the tested conditions. Therefore, a salt-mediated pH gradient elution method was tested. The pH during the elution phase varied from 7 to 9.1 in all cases, and the salt concentrations varied between 25 and 100 mM with different ranges and gradients. Satisfactory separation of the charge variants from the main fraction of adalimumab was achieved when the pH and NaCl concentration were changed from 7 to 9.1 with a gradient of 0.14 pH unit/column volume and from 0 to 50 mM with a gradient of 1.67 mM NaCl/column volume, respectively (**Fig. S2**). The peak of the main fraction was eluted at about pH=8.3 (adalimumab pI=8.2), and the resolutions from the peaks of the acidic and basic charge variants were 1.14 and 1.96, respectively. The adalimumab yield (in the main fraction of monomeric adalimumab) of the cation-exchange chromatography was 90 % and the percentage of aggregates was 0.36 % of monomeric 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 the experiment, PEG-buffer systems in a one-step extraction of adalimumab from the cell-free fermentation broth, buffers with three different pH values were tested: pH=5 (citrate buffer), pH=6 and 8 (phosphate buffers) (**Table 1**, experiments 1–3). The highest adalimumab yield in the PEG phase (60 %) was obtained with a citrate buffer at pH=5. However, the results showed high standard deviations, which could be due to partial precipitation of adalimumab during these extractions. The precipitation probably occurred due to the high ionic strength in the system, which could be caused by the high mass fraction of NaCl (15 %) together with the high mass fraction of the buffer. Therefore, the experiment was performed with citrate buffer at pH=5, which contained lower mass fractions of NaCl, 12.5 and 10 % (**Table 1**, experiments 4 and 5). Since the presence of NaCl in sufficient quantity is considered essential for the efficient extraction of adalimumab into the PEG phase (*21*), the ionic strength was reduced in the next step by decreasing the mass fraction of the buffer components (8 %), while the mass fraction of NaCl (5, 10 and 15 %) was varied (**Table 1**, experiments 6–8). However, the obtained adalimumab yields were even lower than in the previous experiments, and precipitation of adalimumab was still observed. The precipitation and partition of proteins in a polymer-buffer ATPS can be strongly influenced not only by the ionic strength of the given phase, but also by the type of the present ions, among many other factors (*22*,*23*). In our system, none of the investigated settings provided suitable conditions for efficient extraction of adalimumab into the PEG phase without significant precipitation. Based on these results, this PEG-buffer system appears to be inefficient for the purification of adalimumab from fermentation broth in our experiments. In contrast, other studies using PEG-buffer systems (phosphate and citrate buffers) resulted in high (88–100 %) yields for mAb purification

(*7*,*14*,*24*–*26*). In these studies, IgG protein, an artificial protein solution and CHO cell supernatant (Excellgene, Monthey, Switzerland) as well as undefined antibodies were purified with PEG-buffer systems. In summary, although PEG-buffer systems worked well with different mAb solutions, they were not suitable for our fermentation broth with 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-stabilising effect of the phase-forming polymers. Therefore, the PEG-dextran system was selected to study the extraction of adalimumab from cell-free CHO fermentation broth. Sequential extraction and back-extraction steps were investigated during purification. The extraction step was performed in the PEG-dextran system, and the back-extraction step in the PEG-phosphate system. During the extraction step, the effects of pH=5 and 8, NaCl mass fractions 0 and 1 % and PEG-dextran mass ratio (6:10 and 8:5) on adalimumab yield in the PEG phase were investigated. The experiments were performed according to a 2^3 experimental design. Statistical analysis revealed that all factors (pH, NaCl mass fraction, PEG:dextran mass ratio) and their interactions significantly affected adalimumab yield (**Fig. S3**). The presence of NaCl (1 %) significantly improved adalimumab yield and the *m*(PEG):*m*(dextran)=8:5 resulted in higher adalimumab yields than the 6:10 ratio. In addition, extractions at pH=8 were more favourable for adalimumab yield than at pH=5. Maximum adalimumab yield (75 %) was obtained with 1 % NaCl, pH=8 and *m*(PEG):*m*(dextran)=8:5 (data not shown).

The next step of our study was to investigate the effect of increasing the NaCl mass fraction (1, 2.5 and 5 %) on the adalimumab yield in the PEG phase during the extraction step. By increasing the NaCl mass fraction, the adalimumab yield continuously increased in the PEG phase and decreased in the dextran phase. The adalimumab yield in the PEG phase increased from 75 to 95 % by increasing the NaCl mass fraction from 1 to 5 % (**Fig. 1**), then fresh phosphate buffer (pH=8) was added to the separated PEG phase during a back-extraction step.

The effect of NaCl mass fraction in the extraction step on adalimumab yield in the subsequent back-extraction step in the buffer phase was also investigated. In addition, the effect of different mass ratios of the PEG and buffer phases (1:0.4, 1:0.7 and 1:1) of the back-extraction step on adalimumab yield in the buffer phase was investigated. An opposite trend was observed in the effect of NaCl mass fraction during the back-extraction compared to the extraction step: with increasing NaCl mass fraction (in the extraction step), the adalimumab yield in the buffer phase decreased during the subsequent back-extraction step (**Fig. 2**).

In addition, precipitation was observed at 5 % NaCl. Regarding the mass ratio of the PEG and the buffer phases used, an increasing adalimumab yield was observed by decreasing

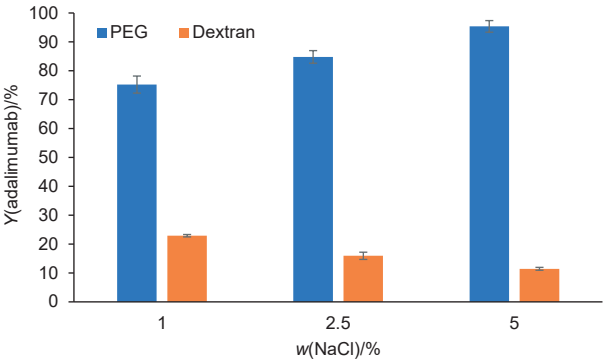

Fig. 1. Adalimumab yields in the PEG and dextran phases during the extraction step with different NaCl mass fractions

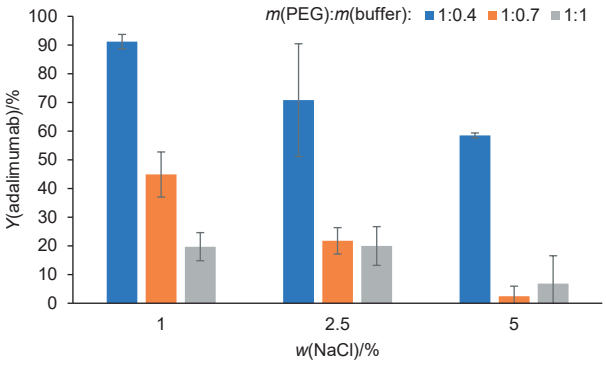

Fig. 2. Adalimumab yield in the buffer phase during back-extraction experiments using different NaCl mass fractions in the extraction step (1, 2.5 and 5 %) and the different *m*(PEG):*m*(buffer)=1:0.4, 1:0.7 and 1:1 during the back-extraction step

the amount of buffer phase (**Fig. 2**). Higher amounts of the added buffer phase (*m*(PEG):*m*(buffer)=1:0.7 and 1:1) resulted in the precipitation of adalimumab during the back-extraction steps, which explains why the lowest amount of buffer phase (*m*(PEG):*m*(buffer)=1:0.4), where no precipitation was observed, resulted in higher adalimumab yield. The highest adalimumab yield (91 %) during back-extraction was obtained with an *m*(PEG):*m*(buffer)=1:0.4 and 1 % NaCl in the extraction step. Although the highest yield of adalimumab was obtained with a 5 % NaCl during the extraction step, the extraction system with 1 % NaCl was the best with 91 % yield during the back-extraction step. The total adalimumab extraction yield, including the extraction and back-extraction steps, was calculated for all cases, and the highest total adalimumab yield (68 %) was obtained with 1 % NaCl in the extraction step and a PEG:buffer mass ratio of 1:0.4 in the back--extraction step. Thus, in our study, a two-step extraction with sequential extraction steps of PEG-dextran and PEG-buffer ATPS was developed and it proved to be a promising strategy for the recovery of adalimumab from the fermentation broth prepared in our laboratory. Comparable results were presented for the one-step extraction of IgG with ATPS based on PEG and dextran. Azevedo *et al.* (*26*) obtained 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.* (*25*) achieved 96 % of IgG in the PEG-rich phase in a one-step extraction, where the system consisted of 7 % PEG (PEG3350), 5 % dextran and 1.3 % triethylene glycol diglutaric acid. In contrast to the one--step purifications, extraction systems with two consecutive steps have hardly been investigated so far, and they mainly focused on PEG-buffer systems in both steps (*10*,*26*). Azevedo *et al.* (*26*) investigated a two-step extraction system (extraction: 8 % PEG3350, 8 % citrate and 15 % NaCl (pH=6), back extraction: recovered PEG phase, 15 % citrate and 5 % NaCl) to purify IgG from a hybridoma cell culture supernatant. The procedure resulted in a total IgG yield of 99 %. Rosa *et al.* (*10*) obtained a total IgG yield of 76 % from a protein mixture during a two-step extraction based on PEG-buffer systems (extraction: 8 % PEG3350, 10 % phosphate buffer and 15 % NaCl (pH=6), back-extraction: recovered PEG phase and 10 % phosphate buffer (pH=6)).

Adalimumab purification by precipitation and dissolution

The recovery of adalimumab from the cell-free CHO fermentation broth was also investigated using the precipitation/dissolution method. One factor was changed at a time during the optimisation of the process parameters affecting the recovery and purity of adalimumab obtained after the sequential precipitations and dissolution. As shown in **Fig. S1**, the process consisted of two sequential precipitations and one dissolution step. The first precipitation step was performed by adding $CaCl_2$ to remove high-molecular-mass contaminants (*e.g.* dsDNA and aggregates) (*27*,*28*). This was followed by the precipitation of adalimumab with PEG solution to separate the mAbs from low-molecular-mass impurities (*e.g.* HCP) (*17*). The final step was to dissolve the precipitated adalimumab in histidine solution.

The effect of different $CaCl_2$ concentrations (1.25, 2.5 and 3.75 mM) was investigated in the first step. After precipitation with $CaCl_2$, the amounts of adalimumab and dsDNA in the obtained supernatant were analysed. The adalimumab content of the supernatant did not decrease significantly, with the yield exceeding 98 % in all of the cases. However, the dsDNA amount decreased significantly in relation to the mAb amount, but no significant difference in the dsDNA amounts (1382 and 1408 ppm) was observed using 1.25 and 2.5 mM $CaCl_2$ solution. Therefore, this treatment was chosen as the first step to remove large-molecular-mass impurities such as dsDNA from the fermentation broth in all subsequent experiments.

In the next step, the PEG-mediated precipitation of adalimumab was investigated by varying the molecular mass and mass fraction of PEG and the pH of the system. The obtained precipitates were separated and dissolved in 20 mM histidine solution. The adalimumab yield was calculated from 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 amount. The effect of the molecular mass of PEG was examined using PEGs with average molecular masses of 3350, 4000 and 8000 Da using the same mass fraction (14 %) in all cases. As shown in **Fig. 3**, the highest adalimumab yield (84.3 %) and the lowest dsDNA amount (1027 ppm) were obtained with PEG4000. Based on these results, PEG4000 proved to be suitable for adalimumab purification by the precipitation/dissolution method, so it was used in all subsequent experiments.

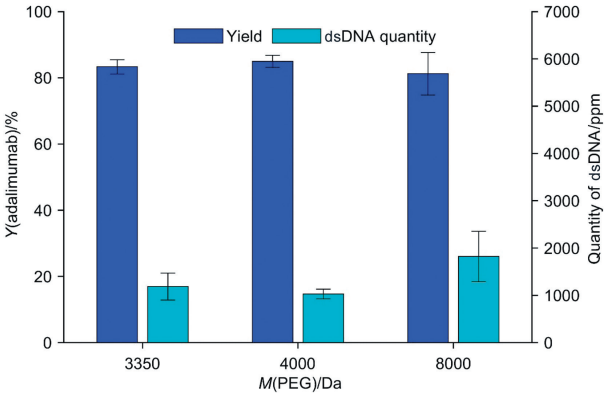

Fig. 3. Adalimumab yield and dsDNA quantity after precipitation and dissolution of adalimumab using different molecular masses of PEG

The effect of PEG content was studied at the mass fractions of PEG of 10, 12, 14, 16 and 18 %. As shown in **Fig. 4**, increasing the PEG mass fractions from 10 to 14 % sharply increased the adalimumab yield, reaching its maximum value (81.4 %) at 14 % PEG. Further increase in the PEG mass fraction resulted in a slight decrease in the adalimumab yield, which could be due to the formation of larger aggregates in the presence of an increased amount of PEG.

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

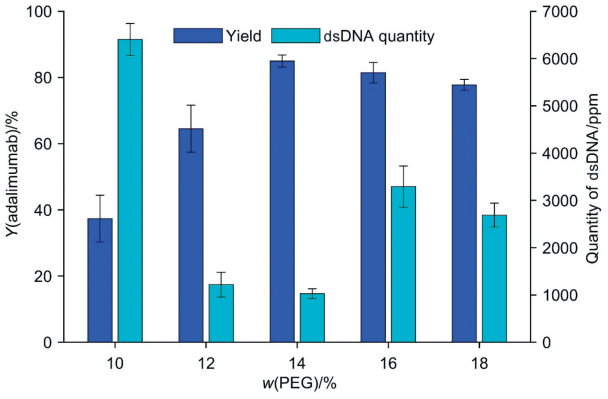

Fig. 4. Adalimumab yield and dsDNA quantity after precipitation and dissolution of adalimumab using different mass fractions of PEG4000

In the final step, the pH of the precipitation step was varied in the range of pH=5.5–9.1 (**Fig. 5**). When the adalimumab yield was examined as a function of the pH applied during the precipitation step, a trend was observed with a maximum adalimumab yield (85 %) at pH=7.5. Meanwhile, the remaining amount of dsDNA showed an opposite trend as the function of pH, having a minimum value (1027 ppm) at pH=7.5. Therefore, pH=7.5 was chosen as the most appropriate pH for adalimumab precipitation using 14 % PEG4000.

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

Sommer *et al.* (*17*) developed a mAb purification method using $CaCl_2$ and PEG precipitates. They tested the proposed method on five different CHO cell-free supernatants and achieved IgG yields of 80–95 %. These results are in line with the yield we obtained when purifying adalimumab from cell-free fermentation broth.

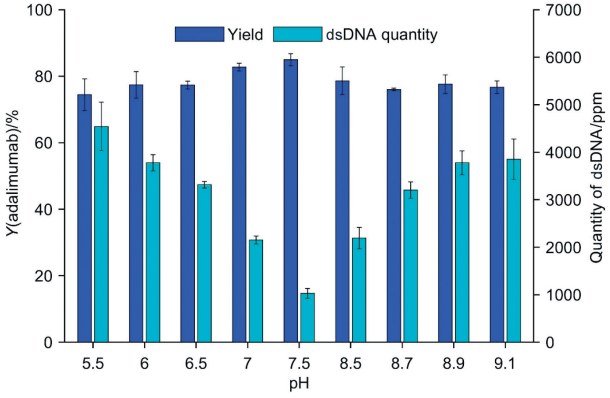

Fig. 5. Adalimumab yield and dsDNA quantity after precipitation and dissolution of adalimumab using different pH values during precipitation

Comparison of the purification methods

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

HCP, dsDNA and adalimumab aggregates were also determined after chromatographic purifications and in the initial fermentation broth (**Table 2**) to evaluate the efficiency of each purification procedure. Affinity chromatography resulted in a 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 can be further reduced by subsequent cation exchange chromatography (**Table 2**) in addition to separation of the charge variants of adalimumab. However, the overall adalimumab yield decreased to 82 %. A comparable adalimumab yield (78 %) was obtained when the precipitation/dissolution method was investigated. It was the most effective alternative purification method for the removal of 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 767 341 ppm HCP and 933 ppm dsDNA), the precipitation/dissolution method was not satisfactory as a single purification step; however, it could be a good method for purification before other purification steps. For the extraction-based purification method, both extraction and back-extraction steps were investigated. 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 reduced the amount of HCP and dsDNA relative to the amount of mAb and almost completely eliminated the protein aggregates (<0.1 %). However, the total adalimumab yield decreased significantly to 54 %. Interestingly, the relative HCP content after extraction/back-extraction was even higher than the relative HCP content of the initial fermentation broth, suggesting that this process needs further development. On the other hand, considering the amount of small aggregates, extraction gave better results than chromatographic

Table 2. Comparison of different purification methods for adalimumab

Method	*Y*(adalimumab)/%	Relative quantity		*w*(aggregate in the monomer adalimumab)/%
		HCP/mAb	dsDNA/mAb	
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
Precipitation/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

HCP=host cell protein, mAb=monoclonal antibody

separation. The performance of the extraction as separation method could be improved if combined with other pre-purification steps. Both purification alternatives (precipitation and extraction) resulted in a lower overall yield of adalimumab in these experiments than in the previous experiments for their optimisation. This could be due to the fact that both the optimisation experiments and the comparison experiments used fermentation broths from two different fermentation runs, *i.e.* broths with different adalimumab concentrations. In the latter, the fermentation broth contained a lower concentration of adalimumab (0.9 instead of 1.3 g/L), which could negatively affect the achievable adalimumab yields during precipitations and extractions (*13*).

In summary, the precipitation/dissolution method and one-step extraction provided an adalimumab yield comparable to purification by affinity and cation exchange chromatography but with lower purity. Based on adalimumab yield and relative HCP and dsDNS content, precipitation proved more promising than the extraction 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 fermentation broths containing adalimumab were prepared in our laboratory using CHO cell cultures. The precipitation method involved two consecutive steps of precipitation with $CaCl_2$ and PEG, followed by dissolution in histidine solution. The extraction involved two consecutive steps of extraction and back-extraction based on PEG-dextran and PEG-buffer ATPS, respectively. Both purification methods were optimised in several steps and in certain cases the best parameters in terms of adalimumab yield and purity were selected. The classical chromatographic purification with affinity and cation exchange steps was also performed and improved. The three different strategies were compared in terms of (monomeric) adalimumab yield and relative amounts of impurities such as HCP, dsDNA and adalimumab aggregates.

Subsequent affinity and cation exchange chromatography proved to be an efficient purification method for adalimumab, resulting in high yield and excellent purity. However, chromatographic purification has several disadvantages. One of the main disadvantages is the high price of the affinity resins. Another disadvantage is that it is difficult to run them in continuous mode, and scale-up could also be challenging. To overcome these obstacles, the alternative purification methods precipitation/dissolution and extraction/back-extraction offer promising alternatives and were therefore studied and compared with each other and with chromatographic separation. The precipitation-based process resulted in a good adalimumab yield, but impurities were still present in high amounts in the final solution. The extraction-based purification gave a low adalimumab yield with low purity.

The precipitation-based method proved to be more promising for direct purification of adalimumab from harvested cell culture fluid compared to the extraction-based method. However, further improvements are needed to compete with the performance of chromatographic separations. However, considering the advantages of the alternatives (such as continuous operation, simplicity and scalability), a cost-effective operation could be achieved without the same performance (*e.g.* mAb yield) that chromatographic purifications have. Furthermore, the performance of these alternative mAb purifications could be significantly improved by combining them, which will be the aim of further studies.

FUNDING

This research was funded by the National Research, Development and Innovation Fund of Hungary under grant K143039 and ÚNKP-21-4-II-BME-331 New National Excellence Program of the Ministry of Culture and Innovation. The research was carried out in the frame of RRF-2.3.1-21-2022-00015 project provided by the European Union.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

Cs. Fehér and E. Hirsch designed the research. D. Vaskó, J. Domján, B. Szabó, L. Bakk and P. Hajdinák collected data and carried out the analysis. D. Vaskó wrote the draft of the article. E. Hirsch and Cs. Fehér critically revised and approved the final version of the manuscript.

ORCID ID

D. Vaskó https://orcid.org/0000-0002-2502-0644
J. Domján https://orcid.org/0000-0001-5040-751X
P. Hajdinák https://orcid.org/0000-0002-7705-2074
Gy. Marosi https://orcid.org/0000-0002-4774-2023
Zs. K. Nagy https://orcid.org/0000-0003-2651-7756
E. Hirsch https://orcid.org/0000-0002-4672-9572
Cs. Fehér https://orcid.org/0000-0002-5659-5985

REFERENCES

1. Brekke OH, Sandlie I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. Nat Rev Drug Discov. 2003;2:52–62. https://doi.org/10.1038/nrd984
2. Lu RM, Hwang YC, Liu IJ, Lee CC, Tsai HZ, Li HJ, Wu HC. Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci. 2020;27(1):1. https://doi.org/10.1186/s12929-019-0592-z

3. Roque ACA, Lowe CR, Taipa MÂ. Antibodies and genetically engineered related molecules: Production and purification. Biotechnol Prog. 2004;20(3):639–54. https://doi.org/10.1021/bp030070k
4. Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. Downstream processing of monoclonal antibodies – Application of platform approaches. J Chromatogr B. 2007;848(1):28–39. https://doi.org/10.1016/j.jchromb.2006.09.026
5. Roque ACA, Lowe CR. Advances and applications of *de novo* designed affinity ligands in proteomics. Biotechnol Adv. 2006;24(1):17–26. https://doi.org/10.1016/j.biotechadv.2005.05.001
6. Follman DK, Fahrner RL. Factorial screening of antibody purification processes using three chromatography steps without protein A. J Chromatogr A. 2004;1024(1–2):79–85. https://doi.org/10.1016/j.chroma.2003.10.060
7. Azevedo AM, Rosa PAJ, Ferreira IF, Aires-Barros MR. Integrated process for the purification of antibodies combining aqueous two-phase extraction, hydrophobic interaction chromatography and size-exclusion chromatography. J Chromatogr A. 2008;1213(2):154–61. https://doi.org/10.1016/j.chroma.2008.09.115
8. Low D, O'Leary R, Pujar NS. Future of antibody purification. J Chromatogr B. 2007;848(1):48–63. https://doi.org/10.1016/j.jchromb.2006.10.033
9. Iqbal M, Tao Y, Xie S, Zhu Y, Chen D, Wang X, *et al.* Aqueous two-phase system (ATPS): An overview and advances in its applications. Biol Proced Online. 2016;18(1):18. https://doi.org/10.1186/s12575-016-0048-8
10. Rosa PAJ, Azevedo AM, Aires-Barros MR. Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies. J Chromatogr A. 2007;1141(1):50–60. https://doi.org/10.1016/j.chroma.2006.11.075
11. Mahadevan H, Hall CK. Statistical-mechanical model of protein precipitation by nonionic polymer. AIChE J. 1990;36(10):1517–28. https://doi.org/10.1002/aic.690361007
12. Wilson SP, Liu F, Wilson RE, Housley PR. Optimization of calcium phosphate transfection for bovine chromaffin cells: Relationship to calcium phosphate precipitate formation. Anal Biochem. 1995;226(2):212–20. https://doi.org/10.1006/abio.1995.1216
13. Sommer R, Tscheliessnig A, Satzer P, Schulz H, Helk B, Jungbauer A. Capture and intermediate purification of recombinant antibodies with combined precipitation methods. Biochem Eng J. 2015;93:200–11. https://doi.org/10.1016/j.bej.2014.10.008
14. Azevedo AM, Rosa PAJ, Ferreira IF, Aires-Barros MR. Optimisation of aqueous two-phase extraction of human antibodies. J Biotechnol. 2007;132:209–17. https://doi.org/10.1016/j.jbiotec.2007.04.002
15. Rosa PAJ, Azevedo AM, Sommerfeld S, Mutter M, Aires-Barros MR, Bäcker W. Application of aqueous two-phase systems to antibody purification: A multi-stage approach. J Biotechnol. 2009;139:306–13. https://doi.org/10.1016/j.jbiotec.2009.01.001
16. Dutra G, Komuczki D, Jungbauer A, Satzer P. Continuous capture of recombinant antibodies by $ZnCl_2$ precipitation without polyethylene glycol. Eng Life Sci. 2020;20(7):265–74. https://doi.org/10.1002/elsc.201900160
17. Sommer R, Satzer P, Tscheliessnig A, Schulz H, Helk B, Jungbauer A. Combined polyethylene glycol and $CaCl_2$ precipitation for the capture and purification of recombinant antibodies. Process Biochem. 2014;49(11):2001–9. https://doi.org/10.1016/j.procbio.2014.07.012
18. Domján J, Fricska A, Madarász L, Gyürkés M, Köte Á, Farkas A, *et al.* Raman-based dynamic feeding strategies using real-time glucose concentration monitoring system during adalimumab producing CHO cell cultivation. Biotechnol Prog. 2020;36(6):e3052. https://doi.org/10.1002/BTPR.3052
19. GE Healthcare Life Sciences application note 28-9872-25 AA. Dynamic binding capacity study on MabSelect SuRe LX for capturing high-titer monoclonal antibodies. GE Healthcare, Little Chalfont, UK; 2020. Available from: https://cdn.cytivalifesciences.com/api/public/content/digi-15746-pdf.
20. Foley JP, Dorsey JG. Equations for calculation of chromatographic figures of merit for ideal and skewed peaks. Anal Chem. 1983;55(4):730–7. https://doi.org/10.1021/ac00255a033
21. Huddleston J, Veide A, Köhler K, Flanagan J, Enfors SO, Lyddiatt A. The molecular basis of partitioning in aqueous two-phase systems. Trends Biotechnol. 1991;9(1):381–8. https://doi.org/10.1016/0167-7799(91)90130-A
22. Kinugasa T, Kondo A, Mouri E, Ichikawa S, Nakagawa S, Nishii Y, *et al.* Effects of ion species in aqueous phase on protein extraction into reversed micellar solution. Sep Purif Technol. 2003;31(3):251–9. https://doi.org/10.1016/S1383-5866(02)00202-2
23. Yun HJ, Jeong SJ, Lee Y, Kim HR, Kim B, Lee S. Effects of various extraction factors on protein yield of *Haliotis discus hannai* (Abalone). Appl Sci. 2021;11(23):11239. https://doi.org/10.3390/app112311239
24. Azevedo AM, Rosa PAJ, Ferreira IF, Pisco AMMO, de Vries J, Korporaal R, *et al.* Affinity-enhanced purification of human antibodies by aqueous two-phase extraction. Sep Purif Technol. 2009;65(1):31–9. https://doi.org/10.1016/j.seppur.2008.03.006
25. Rosa PAJ, Azevedo AM, Ferreira IF, Sommerfeld S, Bäcker W, Aires-Barros MR. Downstream processing of antibodies:

Single-stage *versus* multi-stage aqueous two-phase extraction. J Chromatogr A. 2009;1216(50):8741–9. https://doi.org/10.1016/j.chroma.2009.02.024

26. Azevedo AM, Gomes AG, Rosa PAJ, Ferreira IF, Pisco AMMO, Aires-Barros MR. Partitioning of human antibodies in polyethylene glycol-sodium citrate aqueous two-phase systems. Sep Purif Technol. 2009;65(1):14–21. https://doi.org/10.1016/j.seppur.2007.12.010

27. Lis JT, Schleif R. Size fractionation of double-stranded DNA by precipitation with polyethylene glycol. Nucleic Acids Res. 1975;2(3):383–9. https://doi.org/10.1093/nar/2.3.383

28. Satzer P, Tscheließnigg A, Sommer R, Jungbauer A. Separation of recombinant antibodies from DNA using divalent cations. Eng Life Sci. 2014;14(5):477–84. https://doi.org/10.1002/elsc.201400080