T.C.
MANİSA CELAL BAYAR UNIVERSITY
GRADUATE SCHOOL OF NATURAL and APPLIED SCIENCE INSTITUTE

MASTER THESIS
BIOENGINEERING DEPARTMENT
BIOENGINEERING DISCIPLINE

ANION EXCHANGE CHROMATOGRAPHY METHOD OPTIMIZATION TO mAb PROTEIN PURIFICATION

Fatih ÖZTÜRK

Assist. Prof. Dr. Mehmet Emin USLU

MANİSA–2024

FATİH ÖZTÜRK	**ANION EXCHANGE CHROMATOGRAPHY METHOD OPTIMIZATION TO mAb PROTEIN PURIFICATION**	**2024**

COMMITMENT

I declare that this thesis was written in Manisa Celal Bayar University, Faculty of Engineering, Department of Bioengineering in accordance with academic and ethical rules, and all the literature information used is included in the thesis with reference.

FATİH ÖZTÜRK

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LIST OF SYMBOLS AND ABBREVATIONS

AEX: Anion Exchange Chromatography

BHK: Baby Hamster Kidney

C_H: Constant Heavy Chain

CHO: Chinese Hamster Ovary

C_L: Constant Light Chain

Conc: Concentration

Cond: Conductivity

CV: Column Volume

CQA: Critical Quality Attribute

Fab: Antigen Binding Fragment

Fc: Crystallizable Fragment

FDA: Food and Drug Administration

HCP: Host Cell Protein

HEK: Human Embryonic Kidney

HPLC: High Performance Liquid Chromatography

IEC: Ion Exchange Chromatography

kDa: Kilodalton

M: Molar

mAb: Monoclonal Antibody

mM: Milimolar

PCR: Polymerase Chain Reaction

pI: Isoelectronic Point

pKa: Acid Ionization Constant

Rec: Recovery

RT: Residence Time

SEC: Size Exclusion Chromatography

V_H: Variable Heavy Chain

V_L: Variable Light Chain

WFI: Water for Injection

µm: Micrometer

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ACKNOWLEDGEMENT

First of all, I would like to express my deepest gratitute to Ali ÖZÜER, vice president of AbdiBio department of Abdi İbrahim Pharmaceuticals company, who enabled me to use all the opportunities to carry out the work on my thesis. Then, thank to all my AbdiBio R&D department colleagues for their endless support in carrying out all the research and experiments. I am deeply greatfull to my valuable supervisor Assist. Prof. Dr. Mehmet Emin USLU and co-supervisor Assoc. Prof. Dr. Irem DENIZ CAN, who shared all their assistance and guidence throughout this master thesis. Finally, I would like to thank my dear wife Ecem DOKUMACI ÖZTÜRK, who is my biggest moral supporter and endure my grumpiness.

Fatih Öztürk
Manisa, 2024

ABSTRACT

M.Sc. Thesis

Fatih ÖZTÜRK
Manisa Celal Bayar University
Graduate School of Education
Department of Bioengineering

Supervisor: Assist. Prof. Dr. Mehmet Emin USLU

The effects of resins with different ligands and different pH values on human IgG4 protein were investigated by anion exchange chromatography In the first step, a resin screening study with 5 different positively charged ligands from 4 different brands was applied to the largely purified monoclonal antibodies protein using the Protein A capture step. Following this study, the effect of different pH (6-7-8.1) values on an IgG4 protein with an isoelectric point of 6.9 was investigated on a single resin. During the resin screening process, all protein quality analyzes were examined aiming to reach the resin with the most compatible ligand. According to the studies on the effect of pH values on monoclonal antibody protein, when the pH value was higher than 6.9 different fragments of the protein were removed, and it had a direct effect on the protein charge variant. When the protein pH value was studied equal or below to the isoelectric point value with the anion exchange chromatography flow through method was determined that protein recovery was at the maximum value of %92-%98.

Keywords: Anion Exchange Chromatography, Purification, Monoclonal antibody, IgG, Protein, Resin Screening, Downstream

2024, 37 pages

ÖZET

Yüksek Lisans Tezi

Fatih ÖZTÜRK
Manisa Celal Bayar Üniversitesi
Lisansüstü Eğitim Enstitüsü
Biyomühendislik Anabilim Dalı

Danışman: Dr. Öğr. Üyesi Mehmet Emin USLU

Farklı ligandlara ve farklı pH değerlerine sahip reçinelerin insan IgG4 proteini üzerindeki etkileri anyon değişim kromatografisi ile araştırıldı. İlk adımda, büyük ölçüde saflaştırılmış monoklonal antikor proteinine, Protein A yakalama adımı kullanılarak 4 farklı markaya ait 5 farklı pozitif yüklü ligandla reçine tarama çalışması uygulandı. Bu çalışmanın ardından tek bir reçine üzerinde farklı pH (6-7-8.1) değerlerinin izoelektrik noktası 6.9 olan bir IgG4 proteini üzerindeki etkisi araştırıldı. Reçine tarama sürecinde tüm protein kalite analizleri incelenerek en uyumlu ligandlı reçineye ulaşılması hedeflendi. pH değerlerinin antikor proteini üzerindeki etkisi üzerine yapılan çalışmalara göre, pH değeri 6,9'dan yüksek olduğunda proteinin farklı fragmanları uzaklaştırılmış ve protein yük varyantı üzerinde doğrudan etki oluşmuştur. anyon değişim kromatografisi flow through yöntemi ile protein pH değeri izoelektrik noktası değerine eşit veya altında incelendiğinde protein geri kazanımının %92-%98 maksimum değerde olduğu belirlendi.

Anahtar Kelimeler: Anyon Değişim Kromatografisi, Saflaştırma, MonoKlonal antikor, IgG, Protein, Rezin Tarama, Alt Akım

2024, 37 sayfa

1. INTRODUCTION

The biopharmaceutical industry has developed significantly since the first approval of a recombinant protein, recombinant insulin, in the early 1980s by the Food and Drug Administration (FDA). More than half of the approved recombinant proteins are produced from mammalian cell lines, such as Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and mouse myeloma cells (NS0 and SP2/0) and human cell lines (HEK293) (Kunert & Reinhart, 2016). Mammalian cell lines are preferred because of their innate protein processing machinery, which resembles the post-translational modifications in humans. CHO cells are mostly preferred in the pharmaceutical industry for their ability to i) express complex proteins with desired quality; ii) serve as a safe host for the synthesis of proteins since human pathogenic viruses can not replicate in CHO cells; and iii) secrete proteins into the cell culture, which ease the downstream process (World Health Organization, 2021) (Li, Fan, Lin, & Wang, 2021).

Monoclonal antibodies (mAb) are by far the largest class of biopharmaceutical proteins. It is also of great importance in the growth of the field of biopharmaceuticals. The most basic feature that distinguishes monoclonal antibodies from polyclonal antibodies is that it is the only antibody that recognizes a single epitope region and produces a single class of antibodies. With these features, monoclonal antibodies have been used frequently in the diagnosis and treatment of various diseases. As the structure and function of antibodies are investigated, antibodies have become the most effective components of humoral immunity (Lu, ve diğerleri, 2020) (World Health Organization, 2022).

Antibodies, also known as an immunoglobulins (Ig), are heavy proteins with approximately 150 kDa and 10 nm in size. An antibody has an Y-shaped structure that consist of two identical heavy chains and two identical light chains (as shown in Figure 1.1). Each chains have their own domains with constant and variable domains. Light chains contain one variable (V_L) and one constant domain (C_L). On the other hand, heavy chains consists of one variable (V_H) and three to four constant domains ($C_{H\,1,2,3,4}$). Antibodies can be divided into two fragments, depend on their function, antigen-binding region (Fab) and crystallizable region (Fc). That unique structure allows antibody molecules to fulfil their dual functions, as the antigen binding and biological activity. The Fab fragment contains the antigen-binding site at the amino terminal end of the monomer. The Fc region interacts with cell surface receptors and/or complement system proteins which activate the immune system. Hinge region is placed between two fragments which confers flexibility to antibodies and helps antibodies to bind to their distant epitopes (Janeway, Travers, Walport, & Shlomchik, 2001).

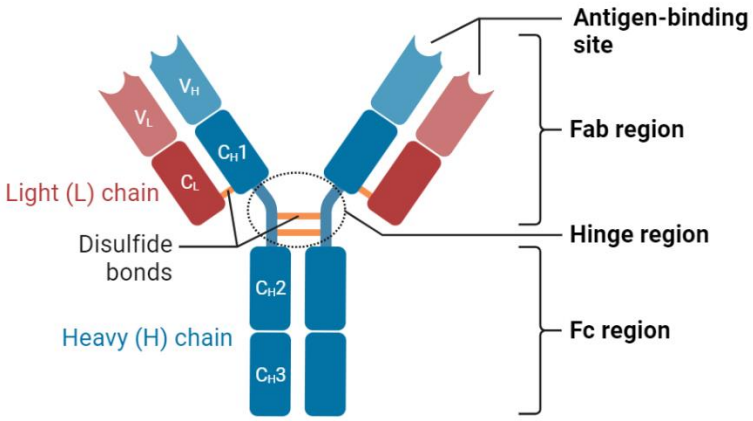
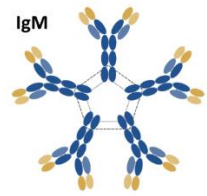
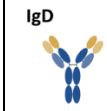
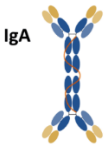


Figure 1.1. An example for antibody structure (created with BioRender.com).

There are five types of antibodies in humans; IgM (μ), IgD (δ), IgG (γ), IgA (α) and IgE (ε) (Table 1.1). IgM is the first antibody to be expressed on the surface of a B cell during early stage of development. IgM contains five or six subunits which consists of two heavy chains (μ-chains) and two light chains, which are bound by disulfide bonds. It involves in primary immune response and enhances the phagocytosis. As the B cells leave the bone marrow and migrate to secondary lymphoid organ, IgD is started to be express on the B cell surface. IgD can be also produced in secreted form which is found at low level in the serum. It plays a role in antibody production. They are highly abundant in mucosal surfaces and in secretions, including the saliva and breast milk. IgA confers protection against bacteria and viruses by neutralization or by prevention of binding to the mucosal surface, such as the intestines, nose and lungs. IgE antibodies are monomers which only found in mammals which are produced by plasma cells. They bind to mast cells, eosinophils and basophils, which are further participated in the immune response. Finally, IgG antibodies are large tetrameric proteins that contain two identical γ heavy chains and two identical light chains. It allows long term protection against bacteria and viruses. It neutralizes bacterial toxins, activates the complement proteins systems and binds to antigens in order to increase the phagocytosis (Schroeder & Cavacini, Structure and function of immunoglobulins, 2010).

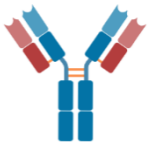
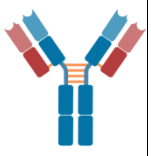
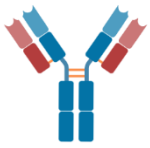
Table 1.1. Types of antibodies in humans.

Isotype					
Subtypes	None	None	4	2	None
H chain	μ	δ	γ	α	ε
H chain molecular weight (kDa)	70	60	50-60	55	70
L chain molecular weight (kDa)	23	23	23	23	23
Total molecular weight (kDa)	970	180	150-170	165-350	190
Abundance	5-10%	<1%	70-85%	5-15%	0.002%
Serum half-life	5 days	3 days	23 days	6 days	2.5 days

Among different antibody isotypes, IgG is the predominant antibody in blood and extracellular fluid. The Fc region of the IgG contains conserved N-glycosylation site locating at Asparagine 297 in the heavy chain. IgGs are further divided into four different subclasses; IgG1, IgG2, IgG3 and IgG4, named depend on the relative abundance (as shown in Table 1.2) (Sadeghalvad & Rezaei, 2021). They have approximately 90% identical amino acid structures but differ in antigen binding affinity, complement activation level, their half-life, etc.

IgG1 antibodies are the most abundant IgG types and they give response to soluble antigens, which is mainly mediated by Fc region. IgG1s are mainly produced under anaphylactic or allergic, in combination with IgE. On the other hand, IgG2s are primarily associated with the bacterial capsular polysaccharide antigens. IgG3 antibodies are usually found in relation with the effector response to foreign antigen as well as parasite infection. Viral infections lead to production of IgG1 and IgG3 subclasses, while IgG3 subclass is the first appearing antigen during the infection. The final IgG antibody subclass IgG4 is mainly correlated with chronic antigen-exposure, some autoimmune disease such as asthma and several allergies. During the immunotherapy, symptoms are relived upon the IgG4 production (Vidarsson, Dekkers, & Rispens, 2014).

Table 1.2. Types of IgG antibodies.

Isotype				
Molecular weight (kDa)	150	150	170	150
Amino acids in hinge region	15	12	62	12
Inter-H chain disulfide bonds	2	4	11	2
Half life (days)	14-21	14-21	7	14-21
Relative abundance (%)	60	32	4	4

Recovery and purification of antibodies produced with the selected clone with minimal loss from the cell culture medium are one of the most important steps in the antibody production process (Zhu, Mollet, Hubert, Kyung, & Zhang, 2017) Impurities from the cell line (i.e host cell proteins (HCP), DNA, endotoxin and media additives) and impurities from the product (i.e high molecular weight and low molecular weight species) must be removed from the target protein during the purification. In addition, viruses that may be present in the system must be removed to obtain fully purify the product (World Health Organization, 2022) (Han, Hewig, & Vedantham, 2011).

The occurrence of variety of variants with difference in charge and molecular weight is a common in mAb production. These charge variants are acidic or basic compared to the main mAb species. It reflects the heterogeneity of mAb and the analytical analysis ensures product consistency with no changes to the protein. The acidic and basic variants of a given mAb show altered antibody structure, stability and biological functions, which might be resulted in unwanted immunogenic response (Du, ve diğerleri, 2012).

One of the most important quality aspect of mAb is the monomeric purity and aggregate formation, which can be influenced by multiple factors of the bioprocess, such as extreme pH, temperature and shear stress. Aggregates are generally large clusters of denatured mAb molecules that are formed either during upstream cell culture, downstream protein purification or storage of drug product. Aggregates may expose normally unexposed epitopes which cause unintended immunogenicity (Zhang, Chen, & Li, 2019). Downstream process should be designed accordingly to obtain mAb molecule with low aggregate levels (0.5-2%). Generally, anion exchange chromatography is performed in downstream processes with a flow-through mode in order to remove DNA and some viruses that are negatively charged at neutral pH (Joshi, Yadav, & Rathore, 2013)

Host cell proteins are endogenous proteins produced by the host cells used in upstream bioprocess, therefore they are considered as major process-related impurities. Even low levels of HCP can be potentially immunogenic and can affect the safety and efficacy of biopharmaceuticals. Due to these potential effects, the amount of residual HCP in drug product is considered as critical quality attribute (CQA) and it is a regulatory requirement to monitor the amount of HCP during process development. HCPs are required to be eliminated during the downstream process to a certain level (Li Y. , 2017).

The purification of antibodies is accomplished by several downstream process steps. These purification processes usually start with the capture step (Gomis-Fons, Andersson, & Nilsson, 2020) (Matte, 2020). In the capture step, chromatographic purification is aimed by using a resin-containing ligand that specifically binds to the target protein. Protein A chromatography is the most preferred method and provides the removal of impurities in a single step (Matte, 2020) (Ramos-de-la-Peña, González-Valdez, & Aguilar, 2019) (Cataldo, Burgstaller, Hribar, Jungbauer, & Satzer, 2020). The other chromatography step is the polisher. More than one type of chromatography can be used in polisher step. In this step the goal is to obtain the target protein with maximum impurity. Ion exchange chromatography (cation exchange and anion exchange chromatography), hydrophobic interaction and multi-model chromatography are the most used chromatography types (Nadar, ve diğerleri, 2022) (Müller-Späth, ve diğerleri, 2010). At the last step, the protein purification is completed with the virus filtration step, and the final concentration was adjusted, following the initiation of formulation process. Furthermore, the virus inactivation process is often performed after the capture step, in order to inactivate the enveloped viruses (Gomis-Fons, Andersson, & Nilsson, 2020). The production of mAb and purification process flow summary can be seen in Figure 1.2.

Anion exchange chromatography (AEX) is one of the most widely used purification steps in the polisher step because it is fast, reliable and inexpensive method.

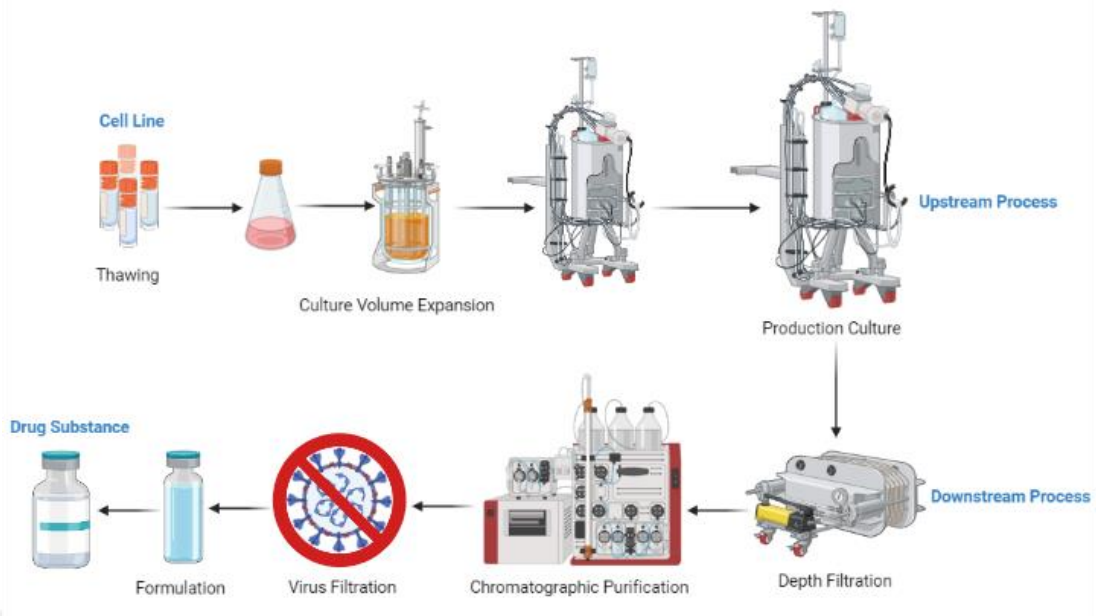


Figure 1.2. Monoclonal antibody production and purification process flow (created with BioRender.com).

1.1. Anion Exchange Chromatography

AEX chromatography is one of the chromatography method using resins containing positively charged ligands that generally have an affinity for negative surface charge molecules, as illustrated in Figure 1.3 (Kang, ve diğerleri, 2015). Molecules differ in their total charge based on differences in charge densities and surface charge distributions. In this case, the molecules exhibit different degrees of interaction with the charged chromatography resin. Charged groups of a molecule that contribute to the net surface charge have different acid ionization constants (pKa) values depending on their structure and chemical microenvironment (Ichihara, Ito, & Gillespie, 2018). Net surface charges are generally pH dependent. The net surface charge of proteins changes depend on the pH of the resin. Each protein has a pH versus its specific net charge. At a pH above pKa, that protein becomes negatively charged, termed as an anion, at a pH below pKa, that protein becomes positively charged and termed as a cation (Sanchayita, Mi, Jia, John, & Steven, 2017).

AEX chromatography is a commonly used polishing step for monoclonal antibodies because most human antibodies have high pl. For the polishing step, pH condition is generally set lower than 7-8 to prevent the antibody from binding to AEX resins. The pH conditions higher than 7-8 may increase the resin binding capacity of the antibody, but high pH may also induce deamidation and proteolysis (Chen, ve diğerleri, 2022). To prevent this situation, high pH is generally avoided. Apart from these, impurities such as DNA, HCP and endotoxins are generally negatively charged and thus under the same conditions, the impurities bind strongly while human monoclonal antibodies do not bind to AEX resins. It has become a common method to perform flow-through AEX chromatography at alkaline pH conditions and low conductivity values, as it is time and cost-advantageous to collect the target protein without binding compared to binding the protein to the resin and eluate it. Flow-through AEX chromatography also provides effective virus removal and has been proven to be a successful step for viral safety. After the Protein A chromatography is applied in the capture step, the contaminants are significantly reduced (but the amount of the contaminant must be reduced to a very small extent by the AEX method to be able to administer to human body), which gives the AEX resin the advantage to obtain higher concentration of target protein and higher capacity of sample loading. Antibodies can bind to AEX resins when the distributions of their surface charges are adjusted to suitable conditions. Thus, AEX can be used in flow-through mode as well as in bind-eluate mode. This method is mostly used to separate two antibodies with different pKa values, rather than removing impurities. However, it is not recommended widely as it is necessary to reach high pH values (Jackobek, ve diğerleri, 2020). With all this information provided, the main purpose of this research article was to optimize the method in the polisher step by applying AEX chromatography to an IgG4 antibody produced by a CHO cell culture.

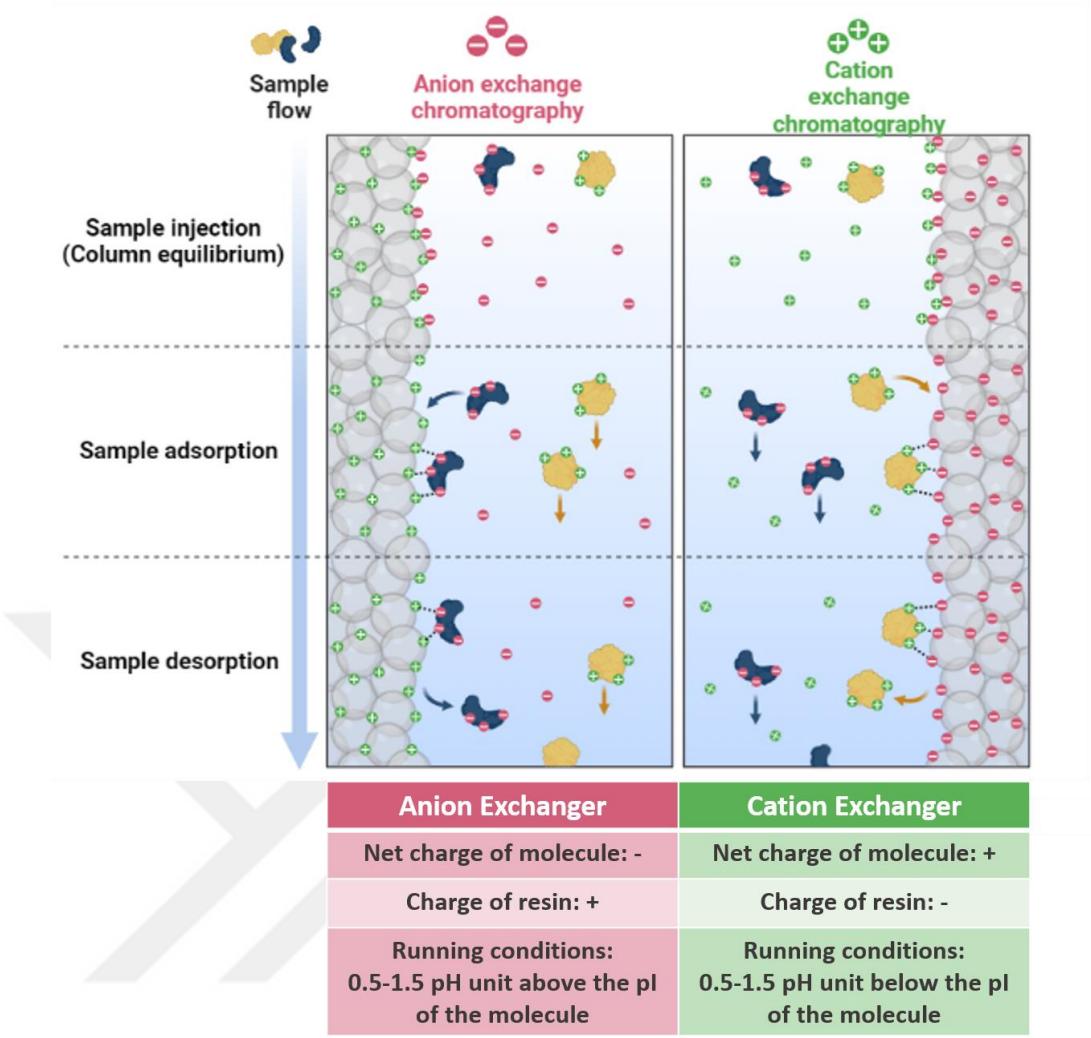


Figure 1.3. Schematic representation of Cation and Anion Exchange Chromatography (created with BioRender.com)

For many years, the biopharmaceutical industry has focused on increasing specific productivity and reducing costs (Butler & Meneses-Acosta, 2012). Recently, the focus of downstream bioprocesses began to change from productivity to the quality attributes of therapeutic proteins. These quality attributes include efficacy, function and safety characteristics; they mainly depend on the protein structures. To optimize the quality and production of mAb, various process parameters are extensively studied.

In this master's thesis, the aim is to optimize the AEX chromatography polishing step to obtain IgG with lower HCP, lower acidic variant with higher monomeric purity. To achieve this goal, the research project was divided into three sections. First step is the resin screening for AEX chromatography. The next step of process development is to determine best pH value and conductivity. A brief description of each part is given hereafter.

2. MATERIALS AND METHODS

All experiments and studies were carried out with the materials and equipment available in the Abdi İbrahim pharmaceutical company. After the production of target mAb produced by CHO cell cultured in a bioreactor in the Upstream Laboratory, the purification of mAb via Protein A chromatography, optimization and development of the polishing step AEX chromatography were performed in the Downstream Laboratory. Finally, the protein samples, which were obtained from optimization process, were analyzed in the Analytical Laboratory.

2.1. Monoclonal Antibody Production

The mAbs used for the experiment were humanized IgG4 proteins obtained after 14 days of production in recombinant Chinese Hamster Ovary (CHO) cells in a 10L BioFlo 320 (Eppendorf) bioreactor. Clarification of IgG4 proteins in the harvesting process after bioreactor production was performed with a 0.08 m2 Sartoclear depth filter (Sartorius). With this process, large cell fragments were removed from the mAbs on the basis of exclusion to ease the downstream chromatographic purification. The mAb protein that was used in this research has 150 kDa size and 6.9 isoelectric point (pI).

2.2. Protein A Chromatography Purification

The clarified cell culture fluid was purified with bind-eluate method by the Protein A chromatography, which is the first of the downstream chromatographic purification steps. MabSelect PrismA (Cytiva) resin which is driven from Alkaline-stabilized protein A (*Escherichia coli*) and ÄKTA avant 150 (Cytiva) device were used in Protein A chromatography. The MabSelect PrismA resin was packed into the HiScale 50/40 column (Cytiva) containing 294 mL of resin at 15 cm column height. The Protein A purification method includes column equilibration, three steps of column wash, elution and column sanitization. The mobile phases with their respective pH and volume are given in Table 2.1.

Table 2.1. Mobile phases of Protein A chromatography.

Protein A Chromatography	Mobile Phase	pH	Volume (CV*)
Equilibration	50 mM Tris-HCI 150 mM NaCI	7.4	5 CV
Wash 1	50 mM Tris-HCI 150 mM NaCI	7.4	5 CV
Wash 2	50 mM Tris-HCI 1 M NaCI	7.4	5 CV
Wash 3	50 mM Sodium Acetate	5.5	5 CV
Elution	50 mM Sodium Acetate	3.3	6 CV
Sanitization	0.1 M NaOH	-	5 CV

*CV is column volume of HiScale 50/40 column, which was 294 mL

2.3. Virus Inactivation Step

The pH of the eluate obtained from Protein A purification was first reduced to pH 3.5 with 2 M acetic acid. After 1-hour incubation at room temperature (20°C) with 100 rpm, the pH was adjusted to 8.1 with 2 M Tris base (Sigma-Aldrich).

2.4. Resin Screening in AEX Chromatography

As a first step of AEX polisher step, resin screening was performed. Five different resins were used for resin screening. Table 2.2 shows five different resins that were used for resin screening and their respective strong anion exchanger ligand and pore size information. Each resin has prepacked in 1 mL column supplied by the manufacturer. Each resin containing columns were attached to the ÄKTA avant 150 device at five different column attachment positions. After the virus inactivation process, some of sample portioned to resin screening experiment and it was adjusted to 3 mS/m by adding water for injection (WFI).

Table 2.2. Five different resins used for resin screening for AEX polishing step.

Resin Name (Brand)	Ligand	Particle Size
Capto Q (Cytiva)	Quaternary amine	~90 µm
Poros HQ (Thermo Scientific)	Quaternized Polyethyleneimine	~50 µm
Nuvia Q (Bio-Rad)	Trimetilamin	~85µm
Eshmuno Q (Merck)	Polyvinyl Ether	~85µm
Pros XQ (Thermo Scientific)	Proprietary quaternary amine	~50 µm

The AEX chromatography method includes column equilibration, column wash, strip and column sanitization steps. The mobile phases with their respective pH and volume are given in Table 2.3. The dynamic binding capacity of the working column was over 150 g/L, 15 mL of protein sample with 10.35 g/L concentration was loaded to the columns. As the residence time (RT) of the protein in the column was desired as 3 RT, the flow rate was determined as 0.33 mL/min.

Table 2.3. Mobile phases of AEX chromatography.

AEX Chromatography Steps	Mobile Phase	pH	Volume (CV*)
Equilibration	50 mM Tris	8.1	5 CV
Column Wash	50 mM Tris	8.1	5 CV
Strip	50 mM Tris, 2M NaCl	7.7	5 CV
Sanitization	0.5 M NaOH	-	5 CV

*CV is volume of prepacked column, which was 1 mL

2.5. pH Optimization of Protein in AEX Chromatography

After the AEX resin screening process, three independent protein samples were taken from the same Protein A chromatography and virus inactivation processes. The pH of the two samples was adjusted to pH 7 and pH 6 with 2 M acetic acid (Table 2.4 amd 2.5, respectively) while other sample was kept constant at pH 8 (Table 2.6). For the protein sample at pH 7, 50 mM Tris with pH 7 was used as equilibration and column washing mobile phases. For the protein sample at pH 6, 50 mM Tris with pH 6 was used as equilibration and column washing mobile phases. For the protein sample at pH 8, 50 mM Tris with pH 8 was used as equilibration and column wash mobile phases, which were same mobile phases used in resin screening.

The study was carried out with 1 mL prepacked column from Nuvia Q column (Bio-Rad). The dynamic binding capacity of the working column was over 150 g/L. The column loading volume of samples were 17.6 mL for protein sample at pH 6, 17.16 mL for protein sample at pH 7 and 16.93 mL for protein sample at pH 8 with the concentration of 8.18 g/L, 8.32 g/L and 8.28 g/L, respectively. Since the residence time (RT) of the protein in the column was desired to be 3 RT, the flow rate was worked as 0.33 mL/min.

Table 2.4. Mobile phases of AEX chromatography with pH experiment 1.

AEX Chromatography Steps	Mobile Phase	pH	Volume (CV*)
Equilibration	50 mM Tris	6	5 CV
Column Wash	50 mM Tris	6	5 CV
Strip	50 mM Tris, 2M NaCl	7.7	5 CV
Sanitization	0.5 M NaOH	-	5 CV

* CV is volume of prepacked column, which was 1 mL.

Table 2.5. Mobile phases of AEX chromatography with pH experiment 2.

AEX Chromatography Steps	Mobile Phase	pH	Volume (CV*)
Equilibration	50 mM Tris	7	5 CV
Column Wash	50 mM Tris	7	5 CV
Strip	50 mM Tris, 2M NaCl	7.7	5 CV
Sanitization	0.5 M NaOH	-	5 CV

* CV is volume of prepacked column, which was 1 mL.

Table 2.6. Mobile phases of AEX chromatography pH experiment 3.

AEX Chromatography Steps	Mobile Phase	pH	Volume (CV*)
Equilibration	50 mM Tris	8.1	5 CV
Column Wash	50 mM Tris	8.1	5 CV
Strip	50 mM Tris, 2M NaCl	7.7	5 CV
Sanitization	0.5 M NaOH	-	5 CV

* CV is volume of prepacked column, which was 1 mL.

2.6. Conductivity Optimization of Protein in AEX Chromatography

With the completion of the pH study, the conductivity experiment, which is the final optimization study, was started. The sample obtained after ProteinA and virus inactivation and whose conductivity was not adjusted with WFI was divided into 4 equal parts and used it to conductivity optimization experiment. Before the study, the samples were adjusted to 3.6, 5, 6, 9.6 mS/cm conductivity values by adding WFI and were made suitable for the study. The buffer sets used in the experiment were used as shown in tables 7, 8, 9, 10.

The study was carried out with 1 mL prepacked column from Nuvia Q column (Bio-Rad). The dynamic binding capacity of the working column was over 150 g/L. The column loading volume of samples were 60 mL for protein sample at conductivity 3.6 mS/cm, 61 mL for protein sample at conductivity 5 mS/cm, 62 mL for protein sample at conductivity 6 mS/cm and 73 mL for protein sample at conductivity 9.6 mS/cm with the concentration of 2.51 g/L, 2.46 g/L, 2.43 and 2.06 g/L, respectively. The mobile phases for the conductivity experiment are given in Table 2.7. Since the residence time (RT) of the protein in the column was desired to be 3 RT, the flow rate was worked as 0.33 mL/min.

Table 2.7. Mobile phases of AEX Chromatography with conductivity experiment.

AEX Chromatography Steps	Mobile Phase	pH	Volume
Equilibration	50 mM Tris	8.1	5 CV
Column Wash	50 mM Tris	8.1	5 CV
Strip	50 mM Tris, 2M NaCl	7.7	5 CV
Sanitization	0.5 M NaOH	-	5 CV

* CV is volume of prepacked column, which was 1 mL.

2.7. Concentration and Recovery Analysis via Protein A High Performance Liquid Chromatography

Concentration analysis was performed in the analytical laboratory by connecting the MAbPac Protein A column (Thermo Scientific) to the Prominence i-Plus (Shimadzu) High Performance Liquid Chromatography (HPLC) device. At the first stage, a calibration curve was drawn in the range of 5-0.1 g/l with the original mAb protein at different concentrations by serial dilution. Then, since the concentration of the product after AEX chromatography would be above this value range, it was diluted 10 times before analysis. Finally, the column was equilibrated with 7.5 pH 150 mM NaCl-50 mM Sodium phosphate buffer before processing and the product was injected into the column for analysis and eluated with 2.5 pH 150 mM NaCl-50 mM Sodium phosphate mobile phase.

2.8. Monomeric Purity Analysis via Size Exclusion Chromatography HPLC

Monomeric purity analysis was performed by Size Exclusion Chromatography (SEC) in the Analytical Laboratory by connecting the TSKgel SW column (Tosoh) to the Prominence i-Plus HPLC device (Shimadzu). The concentrations of the original product and post-AEX mAb protein were diluted to 2 g/L before injecting into the column, then the column was equilibrated with 6.8 pH 0.2 M sodium phosphate buffer. The original product was first injected into the column and accepted as reference. Finally, flow-through AEX proteins were injected into the column and their separation time from the column was analyzed.

2.9. Charge Variant Analysis via Ion Exchange Chromatography HPLC

Charge variant analysis was performed by Ion Exchange Chromatography (IEC) in the Analytical Laboratory by connecting the ProPac WCX-10 HPLC column (Thermo Scientific) to the Prominence i-Plus HPLC device (Shimadzu). Prior to the study, the concentration of the original product and the protein after AEX chromatography was adjusted to 5 g/L. The column was equilibrated with 20 mM mass mobile phase before the products were injected. First, the column was calibrated with three injections of the original product, and then all samples were injected into the column. The proteins bound to the column were eluted in a linear gradient with 20 mM mass-1M NaCl mobile phase.

2.10. Residual DNA Analysis with Quantitative Polymerase Chain Reaction

Residual DNA analysis was performed in the Analytical Laboratory with resDNASEQ Quantitative CHO DNA Kits in a QuantStudio 5 (Thermo Scientific) PCR device. First, different dilutions were prepared in the concentration range of 300-0.003 pg/uL to create a reference curve. Flow-through AEX protein was diluted 10-fold to be fit into prepared reference range. As the first step, a lysis plate was created for DNA isolation, for this, Proteinase K buffer was added to the proteins, followed by the lysis mixture composition. This process was applied to both reference products and sample proteins. Finally, the prepared mixtures were placed in a 96-well PCR plate and analyzed. The process was carried out in the PCR device for 40 cycles and denaturation at 95 °C for 15 seconds and annealing at 60 °C for 1 minute in each cycle.

3. RESULTS AND DISCUSSION

3.1. Optimization of AEX Chromatography Polishing Step

When the AEX chromatogram images of five different resin screening trials were evaluated, similar chromatogram peaks were observed, as shown in Figure 3.1, 3.2, 3.4 and 3.5. The only difference is that in the study with Poros HQ resin, an extra peak was seen in the column wash step. The reason may be the resin ligand can hold even weak negative charges because a mobile phase with a different pH or conductivity value was not used in the column wash step and the impurity was removed from the column without any force. Apart from this, as expected in the chromatogram images, positively charged mAb proteins were collected without binding to the column, as seen in the sample application part and peak in the strip step where the negatively charged impurities attached to the resin were removed from the column with high salt concentration.

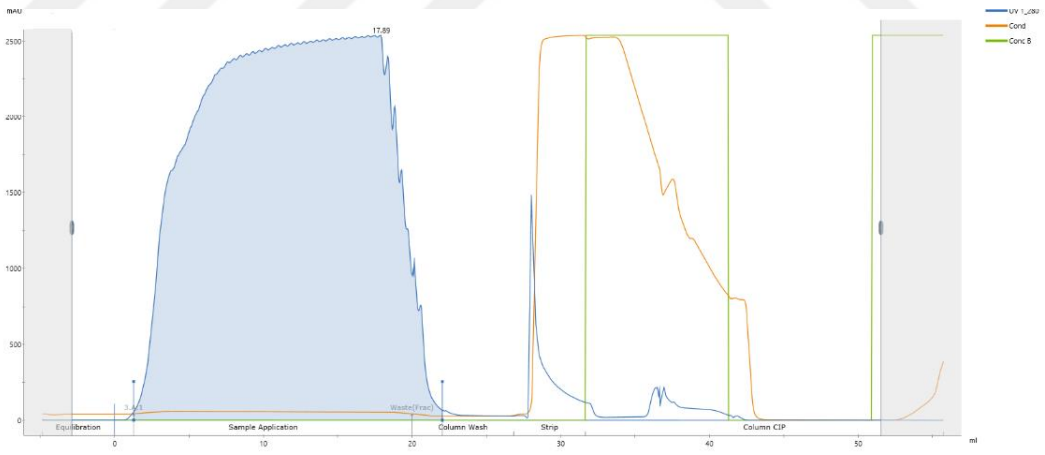


Figure 3.1. Flow-through AEX chromatogram results of Capto Q resin.

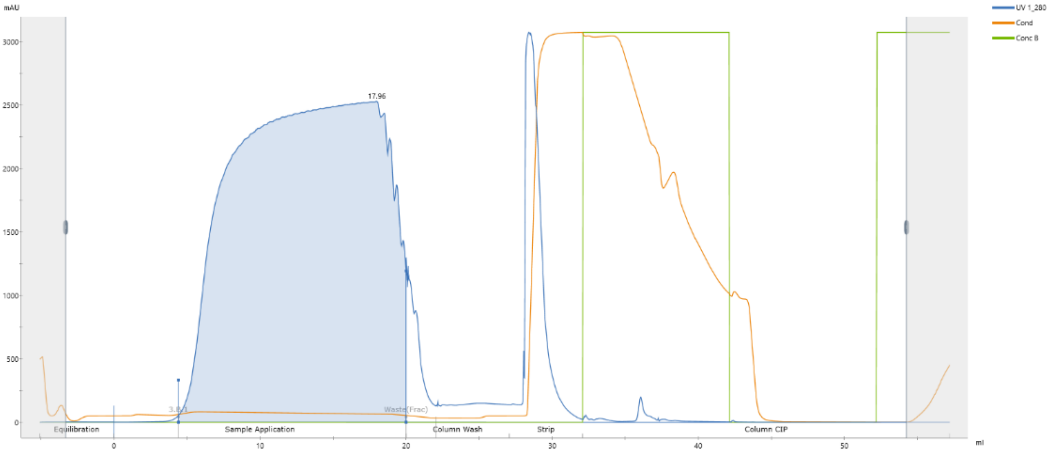


Figure 3.2. Flow-through AEX chromatogram results of Eshmuno Q resin.

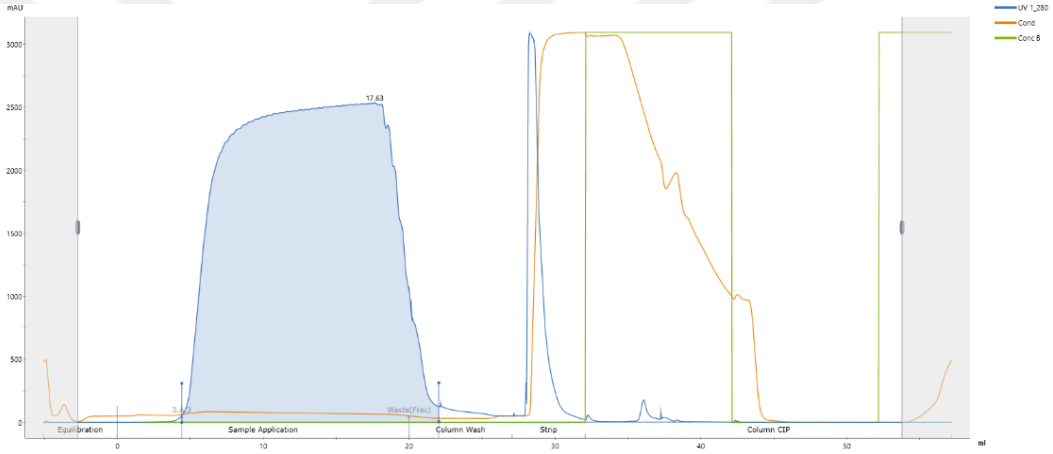


Figure 3.3. Flow-through AEX chromatogram results of Nuvia Q resin.

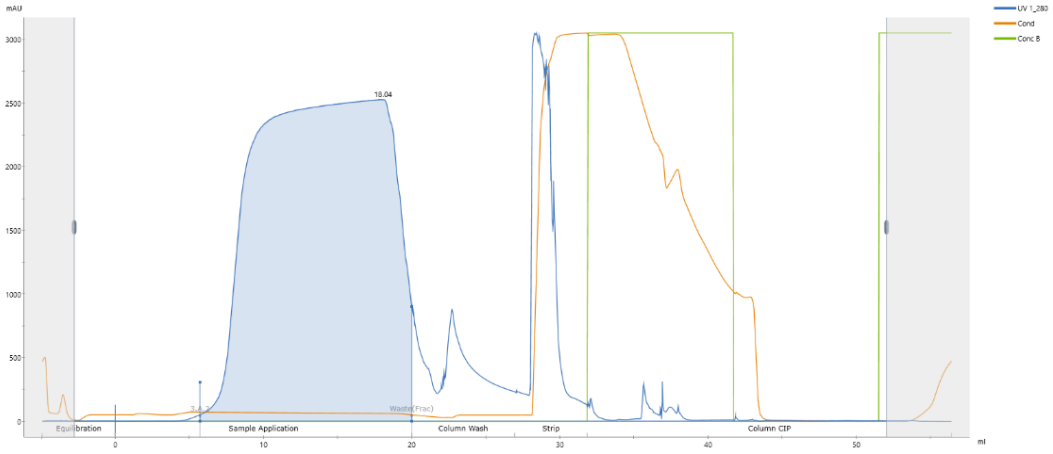


Figure 3.4. Flow-through AEX chromatogram results of Poros HQ resin.

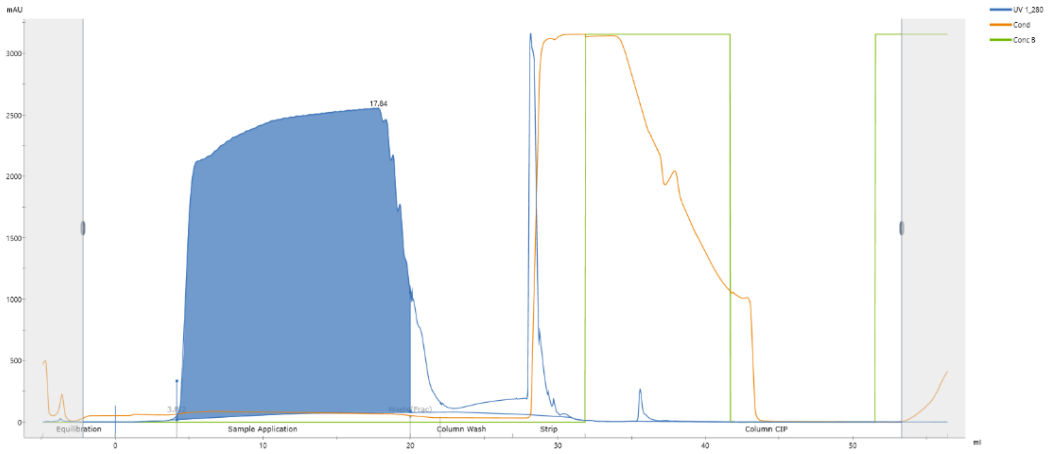


Figure 3.5. Flow-through AEX chromatogram results of Poros XQ resin.

In the second study, the effect of the pH value of the protein on the surface charge and the quality differences that would occur in the mAb protein were examined. The isoelectric point of the target mAb protein is 6.9, but as seen in Figure 3.8, the product at 8.1 pH value was collected in the sample application step without binding to the resin, which shows that the product is still in a positive charge. In some cases, even if the total charge becomes negative, the surface charges can still remain positive, and this might have happened in the sample with pH 8.1. In the chromatography images of the protein samples at three different pH, the protein sample at pH 6 showed no peaks in the column washing and strip step (Figure 3.6). This indicates the amount of impurity is very low and the entire mAb protein has a positive charge. The protein sample at pH 7 had small peaks in strip step whereas the protein sample at pH 8.1 showed a large strip peak (Figure 3.7 and 3.8, respectively). The reason for these peaks may be due to impurity or weak mAb proteins with different surface charges at different pH values. In other words, with AEX chromatography, it is possible to avoid impurities and increase the quality of the product.

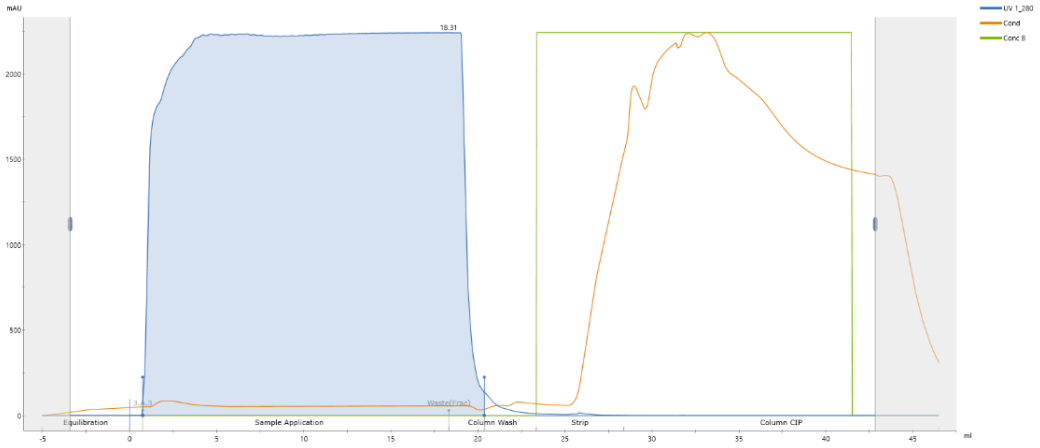


Figure 3.6. Flow-through AEX chromatogram results with pH 6.

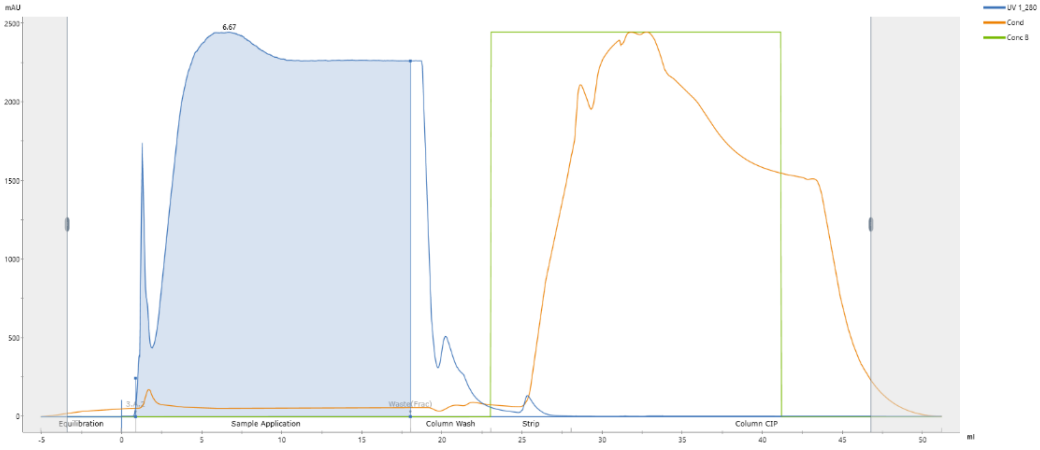


Figure 3.7. Flow-through AEX chromatogram results with pH 7.

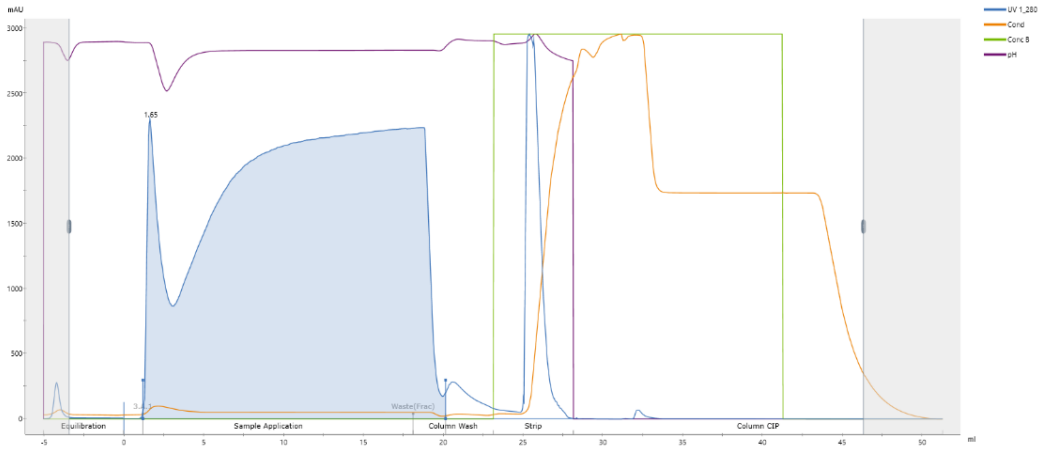


Figure 3.8. Flow-through AEX chromatogram results with pH 8.

In the last study, the effect of different conductivity values on AEX purification was examined. When looking at the chromatogram images, no difference is observed during sample loading in flow through mode (Figure 3.9, 3.10, 3.11 and 3.12) . The only difference in the images is seen in the extra impurity peaks that come from the washing step applied to collect the product remaining in the column in flow through mode after sample application. This difference is not seen at the low conductivity values of 3.6, 5 and 6 mS/cm, but only at the 9.6 mS/cm conductivity value. Considering the studies on this subject, it is known that low conductivity value is more effective in removing host cell DNA and host cell protein in AEX purification (Kurák & Polakovič, 2022). It is very likely that the difference in the impurity peak observed on the chromatogram images is due to the host cell DNA or host cell protein binding to the column during sample application, separating from the target protein, and then moving away from the column in the washing step.

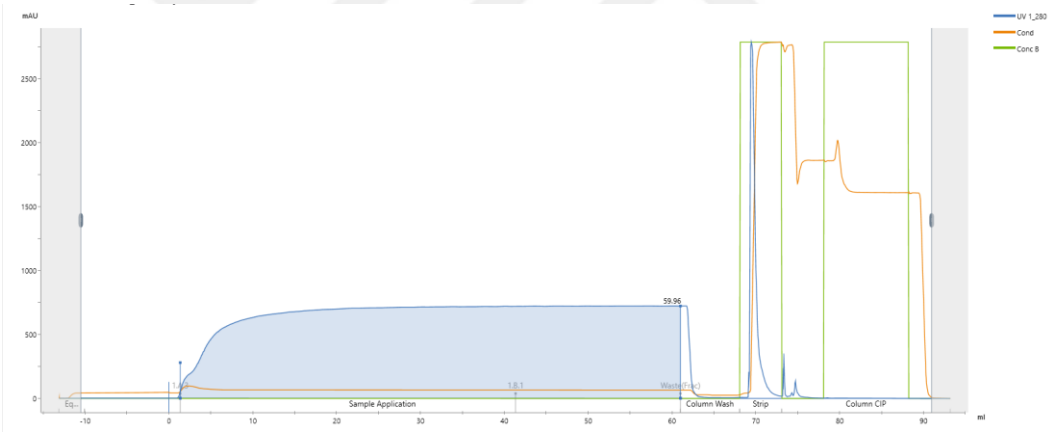


Figure 3.9. Flow-through AEX chromatogram results with conductivity 3.6 mS/cm.

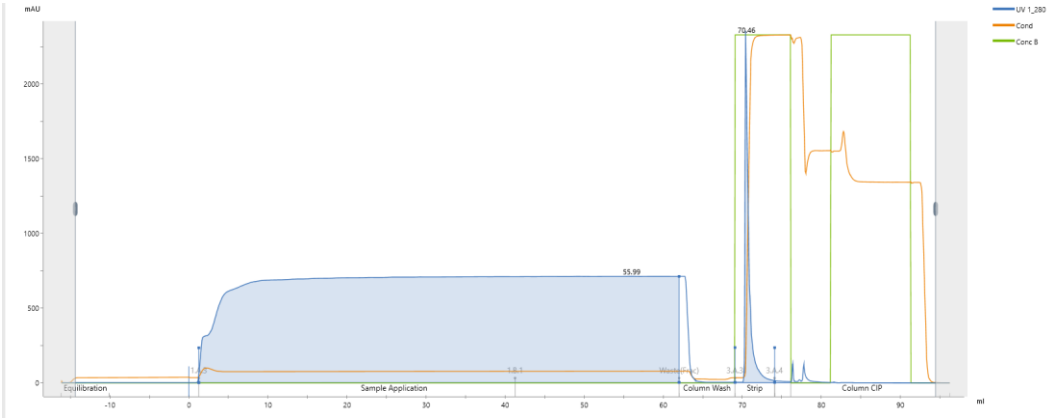


Figure 3.10. Flow-through AEX chromatogram with conductivity 5 mS/cm.

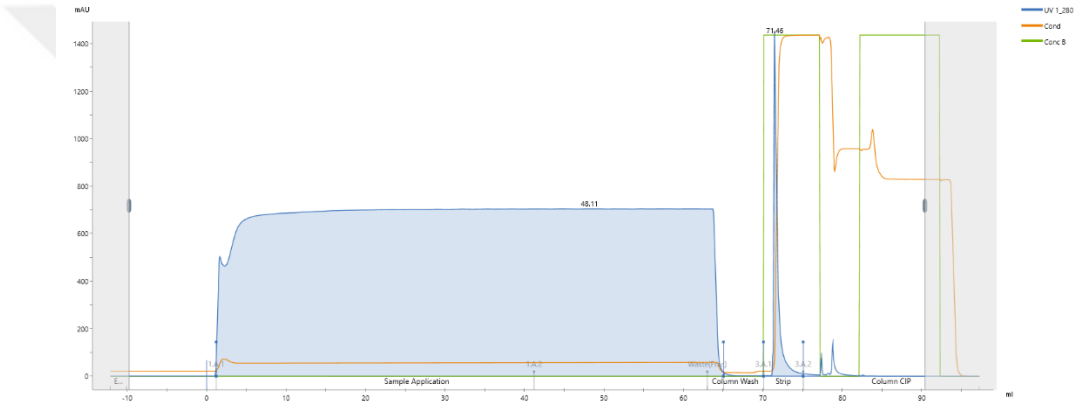


Figure 3.11 Flow-through AEX chromatogram results with conductivity 6 mS/cm.

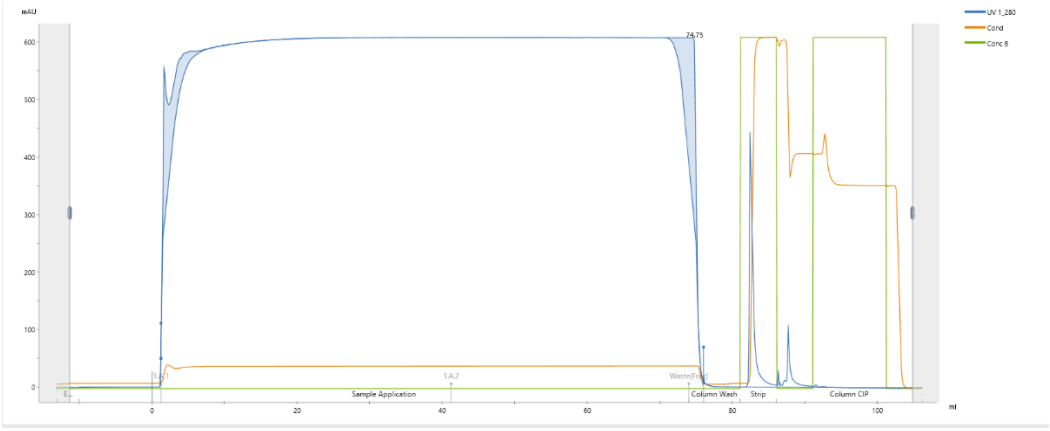


Figure 3.12. Flow-through AEX chromatogram results with conductivity 9.6 mS/cm.

3.2. Product Concentration Analysis and Recovery Measurement

The recovery rates of the target protein after processing were compared as the first step in the evaluation of the resin screening study with five different resins. Table 4 shows the protein amounts of the loaded protein samples before and after the AEX chromatography, and the percentage of recovery calculated by dividing the amount of protein leaving the column by the protein sample entering and multiplying by 100. As the recovery rates of different resins are compared, and the best recovery was found in the Poros XQ column (Thermo Scientific). It would be wrong to choose the best column according to the product recovery alone without evaluating the protein quality analysis. Therefore, protein quality was evaluated, and all data should be taken into consideration for a decision.

Since the mode of the AEX chromatography is flow-through, it was expected to show higher binding recovery compared to bind-elute methodology (Pergande & Cologna, 2017). The increase in pH above pI value of protein decreases the recovery of chromatographic method while increases the removal of weak variants of the protein sample, such as proteins with different post-translational modifications (Zhu, Mollet, Hubert, Kyung, & Zhang, 2017) (Becerra & Buyel, 2022). As the pH condition of resin screening experiment was higher than the pI value of protein, the recovery was around 70%. The reduction of pH to 6 and 7 have resulted in increase in higher recovery value, as indicated in Table 3.1. The recovery results of protein purification with different pH conditions in Nuvia Q column prove that some of the target proteins turn negative with values above the pI point and therefore bind to the column. This was expected due to the absence of a strip peak in the chromatogram images. The lowest recovery was found in experimental setup with Poros HQ resin. Unlikely, Poros HQ resin was found to be relatively stable in high pH conditions and provide high recovery rates (Gupte, Gavasane, Bhabal, & Cheulkar, 2021).

Table 3.1. IgG titer results and the calculated recovery values of five different resins, three different pH and conductivity value.

Resin (Brand)	Loaded protein sample volume (mL)	Loaded protein sample conc. (mg/mL)	Loaded protein sample amount (mg)	Flow through protein volume (mL)	Flow through protein conc. (mg/mL)	Flow through protein amount (mg)	Rec. (%)
Capto Q (Cytiva)	18.72	10.35	193.75	18.72	7.86	147.139	76%
Poros HQ (Thermo Scientific)	14.287	10.35	147.87	14.287	7.385	105.509	71%
Nuvia Q (Bio-Rad)	15.58	10.35	161.25	15.58	7.945	123.783	77%
Eshmuno Q (Merck)	15.58	10.35	161.25	15.58	7.545	117.551	73%
Pros XQ (Thermo Scientific)	15.825	10.35	163.79	15.825	8.335	131.901	81%
Nuvia Q (Bio-Rad) pH: 6	17.6	8.18	143.97	17.6	7.995	140.712	98%
Nuvia Q (Bio-Rad) pH: 7	17.16	8.32	142.77	17.16	7.6615	131.471	92%
Nuvia Q (Bio-Rad) pH: 8	16.93	8.28	140.18	16.93	6.5187	110.362	79%
Nuvia Q (Bio-Rad) Cond: 3.6 mS/cm	60	2.51	150.6	60	2.29	137.4	91%
Nuvia Q (Bio-Rad) Cond: 5 mS/cm	61	2.46	150.06	61	2.32	141.52	94%
Nuvia Q (Bio-Rad) Cond: 6 mS/cm	62	2.43	150.66	62	2.35	145.7	96%
Nuvia Q (Bio-Rad) Cond: 9.6 mS/cm	73	2.06	150.38	73	1.87	136.51	90%

3.3. Monomeric Purity Analysis

AEX chromatography is one of the most preferred monomeric impurity removal steps (Parra & Gebski, 2011). This is clearly seen in the test results. As shown in Table 3.2, the monomeric purity of the Protein A elution product increased in all studies except Capto Q resin after AEX purification. The aggregate amount was found below 1% in all resin studies. Purity levels of 99% indicate that the protein sample did not contain any aggregates and that there was no need for an extra purification step that may be required to remove away.

Capto Q resin is widely used in purification via AEX chromatography. In the current research, Capto Q resin was tested to remove monomeric impurity, but it showed a monomeric purity level of around 99.25%. This was the lowest monomeric purity level among the resins tested. However, successful results have been obtained with Capto Q resin in many studies. In a patent study conducted in 2014, the depth filtrate was purified using Capto Q resin to remove impurities and obtain a product of the desired purity, and very high purity rates were obtained (Parra & Gebski, 2011) (Yüce, ve diğerleri, 2021). It shows that specific proteins do not always produce the same successful results in the same resin.

Poros HQ resin has been shown to provide the best removal of monomeric impurities. Previously, Poros HQ resin was found to have high binding capacity against impurities, which improved cleanliness under different process conditions (Matos, Mohamed, & Queiroz, 2016). Poros HQ resin also gave successful results in achieving high monomeric purity after high recovery percentage.

According to the monomeric impurity results of the protein sample with different pH, the pH value did not show any significant change in monomeric purity. Therefore, it can be concluded that pH change does not trigger aggregate formation. However, serious peak size differences are clearly observed in the strip step in different pH experiments. It is seen in the SEC result that this is not due to monomeric impurity. A different impurity is removed in this step. When the studies were examined, it was known that lower monomeric purity and lower host cell DNA removal rate would be obtained due to the ionic charge that would occur at high conductivity values (Trnovec, Doles, Hribar, Furlan, & Podgornik, 2020).

In the study, a lower monomeric purity value was obtained at high conductivity value (9.6 mS/cm) than at low conductivity value (3 mS/cm). This is because the salt in the sample creates an ionic charge and causes the protein to bind to impurities (Trnovec, Doles, Hribar, Furlan, & Podgornik, 2020). Since this ionic force is lower at low conductivity value, lower binding and higher monomeric purity are obtained.

Table 3.2. Monomeric purity results of flow-through AEX chromatography studies.

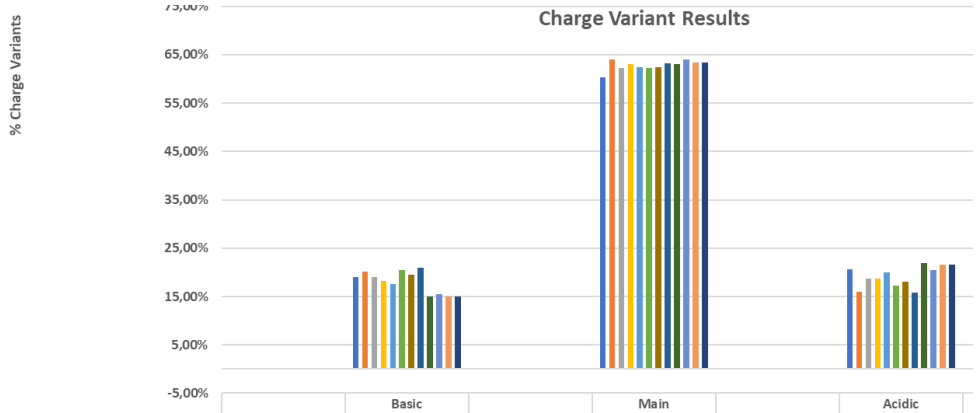
Resin (Brand)	**Monomeric Purity (%)**
Protein A Eluate	99,43
Capto Q (Cytiva)	99,25
Poros HQ (Thermo Scientific)	99,78
Nuvia Q (Bio-Rad)	99,55
Eshmuno Q (Merck)	99,46
Pros XQ (Thermo Scientific)	99,56
Nuvia Q (Bio-Rad) pH:6	99,69
Nuvia Q (Bio-Rad) pH:7	99,69
Nuvia Q (Bio-Rad) pH:8	99,7
Nuvia Q (Bio-Rad) Cond: 3.6 mS/cm	99,64
Nuvia Q (Bio-Rad) Cond: 5 mS/cm	99,14
Nuvia Q (Bio-Rad) Cond: 6 mS/cm	99,35
Nuvia Q (Bio-Rad) Cond: 9.6 mS/cm	98,89

3.4. Charge Variant Analysis

One of the most important protein quality parameters in mAb production is to obtain the charge variants of the product at the desired parameters. In particular, the acidic variant of the protein may increase during stability studies, so it is an important factor to decrease the level as close as possible to the lower limit. Considering the different studies performed, different variants of proteins with specific pI values may have different pH values (Matos, Mohamed, & Queiroz, 2016). In particular, the different pH conditions were expected to bring protein to the desired acidic variant level. The aim was to reduce the weak and acidic variant as much as possible.

As shown in Figure 3.13, the Poros HQ column in the resin screening study had the lowest acidic value and was in the conformity value range. For the pH conditions of protein sample was below the pI point or at neutral, the protein sample remained completely positive and there was no change in the acidic variant. According to the comprehensive studies examined, it was seen that the variants of the protein began to turn negative above the pI value. By using this change in the study, only acidic variants were removed from the protein before the mAb protein turned completely negative (Matos, Mohamed, & Queiroz, 2016). According to the results of the analysis, we can confirm the accuracy of this situation. At pH values 6 and 7, a value of 17% was obtained in the acidic variant, while at pH 8.1 above the pI value, a value of 15% of the acidic variant was obtained, resulting in a visible removal of the acidic variant.

Surface potentials are known to vary as a function of the ionic strength of the surrounding solution. Increasing the ionic strength screens the surface charge, which reduces the magnitude of the surface potential and the distance over which it affects penetrating ions (Green & Andersen, 1991). When Figure 3.13 is examined, protein charge variant changes at different conductivity values, which is the 3rd working condition, are seen. Since the protein could not change its surface tension when the conductivity charge of the medium was 3.5 and 6 mS/cm, there was no change in its net charge and the charge variant results were similar. However, at a conductivity value of 9.6 mS/cm, the ionic strength affected the surface charge of the protein and caused acidic variants to bind to the column.



	Basic	Main	Acidic
Capto Q (Cytiva)	19,03%	60,34%	20,64%
Poros HQ (Thermo Scientific)	20,08%	64,01%	15,92%
Nuvia Q (Bio-Rad)	19,05%	62,21%	18,75%
Eshmuno Q (Merck)	18,16%	63,12%	18,73%
Pros XQ (Thermo Scientific)	17,50%	62,48%	20,03%
Nuvia Q (Bio-Rad) ph:6	20,47%	62,29%	17,24%
Nuvia Q (Bio-Rad) ph:7	19,52%	62,49%	17,99%
Nuvia Q (Bio-Rad) ph:8	20,97%	63,16%	15,88%
Nuvia Q (Bio-Rad) Conductivity: 3.6 mS/cm	15,07%	63,03%	21,90%
Nuvia Q (Bio-Rad) Conductivity: 5 mS/cm	15,47%	64,02%	20,50%
Nuvia Q (Bio-Rad) Conductivity: 6 mS/cm	15,07%	63,38%	21,55%
Nuvia Q (Bio-Rad) Conductivity: 9.6 mS/cm	14,98%	63,42%	21,59%

Figure 3.13. Charge variants results of flow-through AEX chromatography studies.

3.5. Host Cell DNA and Protein Analysis

One of the most important data obtained after AEX purification was host cell DNA, as the main purpose of the polishing step is known to eliminate the host cell DNA from the protein sample (Stone, Borman, Ferreira, & Robbins, 2018).

As shown in Table 3.3, host cell DNA was mostly removed from the target protein in five different resin studies, 3 different pH and 4 different conductivity value studies, As expected from previous studies, there were no significant differences [30]. The biggest reason for this finding is the negative charge of DNA in all conditions, while AEX resin ligand is always positively charged. According to the pH value of the protein sample, it is possible to evaluate whether the product will bind to the column only. Although there was no significant difference, in previous examinations, Capto Q resin was found to be quite successful in removing host cell DNA (Stone, Borman, Ferreira, & Robbins, 2018), and as the result of the study is examined, a DNA result below 0 (ng/mg) is seen and this confirms these examinations.

When different studies with different pH values were examined, it was found that higher pH value was more effective in removing host cell DNA. However, the results of the study show that lower pH values for the protein used were more effective in removing host cell DNA. The reason for this difference is that at high pH values, the protein may also gain a surface charge due to the pI point difference, and the host cell DNA and protein may be bound to each other, which seems to be a common result (Stone, Borman, Ferreira, & Robbins, 2018).

When Table 3.3 is examined, it is seen that there is a more obvious separation of host cell DNA at different conductivity values. Studies on this subject have shown that better host cell DNA removal is achieved with low electrostatic attraction. The study yielded results that support these studies (Stone, Borman, Ferreira, & Robbins, 2018). A better host cell DNA separation was obtained at conductivity values of 3, 5 and 6 mS/cm compared to the conductivity value of 9.6 mS/cm. The reason for this is that the host cell DNA can bind to the protein instead of the column due to the electrostatic attraction that will occur at high conductivity values. This situation can be prevented with low conductivity.

Table 3.3. Residual DNA results of flow-through AEX chromatography studies.

Resin (Brand)	**Residual DNA (ng/mg)**
Capto Q (Cytiva)	<0.0000
Poros HQ (Thermo)	0,00001
Nuvia Q (Bio-Rad)	0,00544
Eshmuno Q (Merck)	0,00508
Pros XQ (Thermo)	0,00432
Nuvia Q (Bio-Rad) pH:6	0,00453
Nuvia Q (Bio-Rad) pH:7	0,00573
Nuvia Q (Bio-Rad) pH:8	0,00978
Nuvia Q (Bio-Rad) Cond: 3.6 mS/cm	0,009
Nuvia Q (Bio-Rad) Cond: 5 mS/cm	0,009
Nuvia Q (Bio-Rad) Cond: 6 mS/cm	0,009
Nuvia Q (Bio-Rad) Cond: 9.6 mS/cm	0,016

4. CONCULUSION

Polishing step is an important method for removing impurities from the cell line such as host cell DNA, monomeric impurities and cell culture media additives. Considering the removal of all these impurities, cost and time saving, AEX chromatography is one of the best mAb protein polishing methods. In this report, were investigated the effect on the protein recovery, monomeric purity, host cell DNA removal and charge variant of the mAb polishing purification step by working with different pH and conductivity values and resin screening using resins with different ligands.

Even though each AEX resin has a positive charge, it is not possible to get the same result in mAb proteins as each resin is positively charged with different ligands. Therefore, it was aimed to find the best results using resins with different ligands. Most analysis results observed to be better for Poros HQ resin, but considering cost and especially recovery, it was decided that Nuvia Q resin would be more suitable. In Nuvia Q resin, both the recovery result and the protein quality analysis results met the expectations. Specially, %77 recovery and %18.75 acidic variant values are most critical parameters to chosed it.

Different pH values were examined on the mAb protein have pI value of 6.9, and the recovery analyzes were found similar under pH conditions equal or below the pI value. However, as the pI point was exceeded, weakly acidic variants of mAb proteins turned into negative charges was concluded. Under pH condition of 8.1, the recovery was found to be decreased while the acidic variant value decreased. Under normal conditions, as the pI value is exceeded, the protein turns negative. However, various studies have shown that protein surface charges can remain positive. When working with the mAb protein, it is important to think broadly and study different conditions. It is possible to bring the charge variant to the desired level with different polishing steps but achieving these results with such a low recovery loss shows the success of the study. For the protein we studied, it was found that it was more advantageous to work with a pH of 6 when high protein recovery value and high host cell DNA removal were targeted in the process, but if the acidic variant removal was targeted in the process, it was found to be more advantageous to work with a pH value

of 8.1.

When different conductivity values were examined, it was seen that lower monomeric purity and lower host cell DNA removal data were obtained due to the increase in electrostatic charge at higher conductivity values. At all conductivity values studied below 9.6 mS/cm, impurities were more easily bound to the column and removed from the protein due to the provision of ion balance.

REFERENCES

Becerra, A., & Buyel, J. (2022). Optimizing downstream purification of high-quality plasmid DNA with POROS™ Chromatography Resins. *Cell & Gene Therapy Insight, 8*(1), 87.

Butler, M., & Meneses-Acosta, A. (2012). Recent advances in technology supporting biopharmaceutical production from mammalian cells. *Appl Microbiol Biotechnol, 96*(4), 885-94. doi:10.1007/s00253-012-4451-z

Cataldo, A. L., Burgstaller, D., Hribar, G., Jungbauer, A., & Satzer, P. (2020). Economics and ecology: Modelling of continuous primary recovery and capture scenarios for recombinant antibody production. *J Biotechnol, 308*, 87-95.

Chen, S.-T., Xu, W., Cai, K., Ferreira, G., Wickramasinghe, R., & Qian, X. (2022). Factors affecting robustness of anion exchange chromatography: Selective retention of minute virus of mice using membrane media. *J Chromatogr B Analyt Technol Biomed Life Sci*.

Du, Y., Walsh, A., Ehrick, R., Xu, W., May, K., & Liu, H. (2012). Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies. *MAbs, 4*(5), 578-585.

Gomis-Fons, J., Andersson, N., & Nilsson, B. (2020). Optimization study on periodic counter-current chromatography integrated in a monoclonal antibody downstream process. *J Chromatogr A*. doi:10.1016/j.chroma.2020.461055

Goodrich, E. M., Bohonak, D. M., Genest, P. W., & Peterson, E. (2020). Recent advances in ultrafiltrationand virus filtration for production ofantibodies and relatedbiotherapeutics. *Approaches to the Purification, Analysis and Characterization of Antibody-Based Therapeutics* (s. 137-165). içinde Elsevier Limited.

Green, W., & Andersen, O. (1991). Surface Charges And Ion Channel Functıon. *Annual Review of Physiology, 53*, pp. 341-359.

Gupte, P., Gavasane, M., Bhabal, T., & Cheulkar, S. (2021). *Removing Oligomers of a Recombinant Human Therapeutic Hormone: Evaluation of Chromatographic Options for Effectiveness*. BioProcess International: https://bioprocessintl.com/downstream-processing/chromatography/removing-oligomers-of-a-recombinant-human-therapeutic-hormone-evaluation-of-chromatographic-options-for-effectiveness/ adresinden alındı

Han, X., Hewig, A., & Vedantham, G. (2011). Recovery and Purification of Antibody. *Antibody Expression and Production Cell Engineering*, 305-40.

Hober, S., Nord, K., & Linhult, M. (2007). Protein A chromatography for antibody purification. *Journal of Chromatography, 88*(1), 40-47.

Ichihara, T., Ito, T., & Gillespie, C. (2018). Polishing approach with fully connected

flow-through purification for therapeutic monoclonal antibody. *Eng Life Sci, 19*(1), 31-36.

Jackobek, R., Herrick-Wagman, S., Zhu, L., Francis, C., Solbrand, A., Eriksson, C., . . . D'Antona, A. M. (2020). Automated pH and conductivity conditioning using feedback control to support a two-step continuous purification process. *Journal of Chromatography A, 1630*.

Janeway, C., Travers, P., Walport, M., & Shlomchik, M. (2001). The structure of a typical antibody molecule. *Immunobiology: The Immune System in Health and Disease.* içinde New York: Garland Science.

Joshi, V., Yadav, N., & Rathore, A. (2013). Aggregation of Monoclonal Antibody Products: Formation and Removal. *BioPharm International, 26*(3).

Kang, Y. (., Ambat, R., Hall, T., Sauffer, M. D., Ng, S., Healy-Fried, M. L., . . . Balde, P. (2015). Development of an acidic/neutral antibody flow-through polishing step using salt tolerant anion exchange chromatography. *Pharmaceutical Bioprocessing, 3*(8), 477-487.

Kunert, R., & Reinhart, D. (2016). Advances in recombinant antibody manufacturing. *Appl Microbiol Biotechnol*, 3451-61. doi:10.1007/s00253-016-7388-9.

Kurák, T., & Polakovič, M. (2022). Adsorption Performance of a Multimodal Anion-Exchange Chromatography Membrane: Effect of Liquid Phase Composition and Separation Mode. *Membranes, 12*(21), 1173.

Li, W., Fan, Z., Lin, Y., & Wang, T.-Y. (2021). Serum-Free Medium for Recombinant Protein Expression in Chinese Hamster Ovary Cells. *Front Bioeng Biotechnol, 9*(64).

Li, Y. (2017). Effective strategies for host cell protein clearance in downstream processing of monoclonal antibodies and Fc-fusion proteins. *Protein Expr Purif, 134*, 96-103.

Lu, R.-M., Hwang, Y.-C., Liu, I.-J., Lee, C.-C., Tsai, H.-Z., Li, H.-J., & Wu, H.-C. (2020). Development of therapeutic antibodies for the treatment of diseases. *J Biomed Sci, 27*(1).

Matos, T., Mohamed, E., & Queiroz, J. (2016). Capto™ Resins for Chromatography of DNA: A Minor Difference in Ligand Composition Greatly Influences the Separation of Guanidyl-Containing Fragments. *Chromatographia, 79*(19-20), 1277-1282.

Matte, A. (2020). Recent Advances and Future Directions in Downstream Processing of Therapeutic Antibodies. *Int J Mol Sci, 23*(15).

Müller-Späth, T., Aumann, L., Ströhlein, G., Kornmann, H., Valax, P., Delegrange, L., . . . Morbidelli, M. (2010). Two step capture and purification of IgG2 using multicolumn countercurrent solvent gradient purification (MCSGP). *Biotechnol Bioeng, 107*(6), 974-984.

Nadar, S., Somasundaram, B., Charry, M., Billakanti, J., Shave, E., Baker, K., & Lua,

L. (2022). Design and optimization of membrane chromatography for monoclonal antibody charge variant separation. *Biotechnology Progress, 38*(6).

Parra, S. C., & Gebski, C. (2011). Benefits of a Revised Approach to Anion Exchange Flow-Through Polish Chromatography. *BioPharm International, 11*(3).

Pergande, M., & Cologna, S. (2017). Isoelectric Point Separations of Peptides and Proteins. *Proteomes, 5*(1), 4.

Ramos-de-la-Peña, A., González-Valdez, J., & Aguilar, O. (2019). Protein A chromatography: Challenges and progress in the purification of monoclonal antibodies. *J Sep Sc,, 42*(9), 1816-1827.

Reed, C., Koza, S. M., & Chen, W. (2018). *Protein A for Monoclonal Antibody Titer Measurements with ACQUITY Arc Bio.* Retrieved from Waters Technology Brief: https://www.waters.com/webassets/cms/library/docs/720006297en.pdf

Sadeghalvad, M., & Rezaei, N. (2021). Introduction on Monoclonal Antibodies. *IntechOpen*. doi:10.5772/intechopen.98378

Sanchayita, G., Mi, J., Jia, L., John, H., & Steven, L. (2017). Integrated polishing steps for monoclonal antibody purification. *Process scale purification of antibodies* (s. 145-167). içinde

Schroeder, H., & Cavacini, L. (2010). Structure and function of immunoglobulins. *J Allergy Clin Immunol, 125*(2), 41-52. doi:10.1016/j.jaci.2009.09.046

Schroeder, H., & Cavacini, L. (2010). Structure and Function of Immunoglobulins. *J Allergy Clin Immunol, 125*, 41-52.

Sodavarapu, S., Ghotra, G. S., Obad, N., Goyal, M., & Gill, A. (2020). IgG4-Related Diseases–Continues To Be a Cancer Mimicker. *Cureus, 12*(1).

Stone, M., Borman, J., Ferreira, G., & Robbins, D. (2018). Effects of pH, conductivity, host cell protein, and DNA size distribution on DNA clearance in anion exchange chromatography media. *Biotechnol Prog., 34*, pp. 141–149.

Trnovec, H., Doles, T., Hribar, G., Furlan, N., & Podgornik, A. (2020). Characterization of membrane adsorbers used for impurity removal during the continuous purification of monoclonal antibodies. *J Chromatogr A.*

Vidarsson, G., Dekkers, G., & Rispens, T. (2014). IgG subclasses and allotypes: from structure to effector functions. *Front. Immunol.*

World Health Organization. (2021, 10 12). *WHO Guideline for the safe production and quality control of monoclonal antibodies for use in humans.* World Health Organization: https://cdn.who.int/media/docs/default-source/biologicals/mabs-manufacture-guideline-draft-for-1st-public-comment.pdf adresinden alındı

World Health Organization. (2022, 04 22). *Guidelines for the production and quality control of monoclonal antibodies and related products intended for medicinal*

use. World Health Organization: https://cdn.who.int/media/docs/default-source/biologicals/final-who-guidelines-on-mab-production-and-quality-control-annex-4---7-jun-2022.pdf?sfvrsn=8c542f00_1&download=true adresinden alındı

Yüce, M., Sert, F., Torabfam, M., Parlar, A., Gürel, B., Çakır, N., . . . Çapan, Y. (2021). Fractionated charge variants of biosimilars: A review of separation methods, structural and functional analysis. *Anal Chim Acta*.

Zhang, X., Chen, T., & Li, Y. (2019). A parallel demonstration of different resins' antibody aggregate removing capability by a case study. *Protein Expression and Purification, 153*, 59-69.

Zhu, M. M., Mollet, M., Hubert, R. S., Kyung, Y. S., & Zhang, G. G. (2017). Industrial Production of Therapeutic Proteins: Cell Lines, Cell Culture, and Purification. *Handbook of Industrial Chemistry and Biotechnology.* (s. 1639-1669). içinde USA. doi:10.1007/978-3-319-52287-6_29

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