Evaluation of downstream methods for monoclonal antibodies produced from CHO cell lines

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UNIVERSITY OF ZAGREB
FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

GRADUATE THESIS

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EVALUATION OF DOWNSTREAM METHODS FOR MONOCLONAL ANTIBODIES PRODUCED FROM CHO CELL LINES

This study was carried out in the BioIndustrial Pilot Plant and at the Department of Downstream Processing University of Natural Resources and Life Sciences (BOKU), Vienna, under supervision of Bernhard Sissolak, PhD and assistance of Gabriele Recanati, MSc.

I would like to thank Dr. Bernhard Sissolak, for accepting my application for the student exchange program and for giving me valuable instructions throughout my research project. Furthermore, I would like to thank Gabriele Recanati for the patience, help, and much needed instructive input for my experiment. Lastly, I would like to thank my entire research group at BOKU, Vienna, for all the help, advice, and memorable moments that I will never forget.

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EVALUATION OF DOWNSTREAM METHODS FOR MONOCLONAL ANTIBODIES PRODUCED FROM CHO CELL LINES

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Abstract: CHO culture is a well-charaterized cell line that has been used for years in the field of biomanufacturing to produce various valuable pharmaceutical components, such as monoclonal antibodies that are used for the treatment of numerous diseases. Novel, more cost-effective downstream techniques and methods are continuously being developed by large companies. This thesis focused on the characterization of downstream equipment, mainly three components: depth filters (used for filtration), hollow fibers (used for concentration), and ion exchange membrane (used for anionic exchange, or AEX). For the specific process of trastuzumab purification, ErtelAlsop B4E7 filters were proven to be effective in the DNA removal step. In the critical flux experiment, the Microza hollow fibers (0.2 μm pores) could sustain the highest transmembrane flux before significant fouling occurred. Lastly, AEX was effective in removing host cell proteins/DNA.

Keywords: *monoclonal antibodies, downstream processing, filters, hollow fibers, ion exchange*
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EVALUACIJA METODA ZA PROČIŠĆAVANJE MONOKLONALNIH PROTUTIJELA PROIZVEDENIH IZ CHO STANIČNE LINIJE

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Sažetak: CHO stanice dobro su definirana kultura koje se koriste u proizvodnji mnogih farmaceutskih komponenata, poput monoklonalnih protutijela koja se koriste za prevenciju mnogih bolesti. U svrhu smanjenja troškova, mnoga velika poduzeća kontinuirano razvijaju nove metode pročišćavanja ovih vrijednih spojeva. Tijekom ovog rada ispitana je oprema za pročišćavanje, uz naglasak na tri glavna tipa: dubinski filteri (korišteni za filtraciju), membrane (korištene za koncentraciju) i membranski adsorberi (korištene za anionsku izmjenu). Za specifičan proces pročišćavanja Trastuzumab antitijela, ErtelAlsop B4E7 filteri su dokazano efektivni. Nadalje, ispitivane Microza membrane (pore veličina 0.2 μm) su izdržale najveći protok bez pojave začepljenja. Na kraju, anionska izmjena pokazala se najefektivnijom pri uklanjanju neželjenih staničnih proteina i DNK.

Ključne riječi: *monoklonalna protutijela, procesi izdvajanja, filteri, membrane, ionska izmjena*
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1. INTRODUCTION

The global monoclonal antibody market is expected to hit around 524 billion dollars by 2030 (Precedence Research). These high value compounds are being produced by biotechnological companies all over the world in order to prevent diseases and medical conditions such as cancer, inflammatory disorders, allergies, osteoporosis etc., with the majority being used in cancer treatment. Over the last decade, biotechnological manufacturing of various antibodies has seen significant changes and improvements, whether in the form of better cell lines, advances in bioreactor processes, novel purification methods or single-use technologies (Chon and Zarbis-Papastoitsis, 2011).

Purification of high value compounds produced from various bioprocesses (such as monoclonal antibodies) has always been a significant challenge for biotechnological companies. New downstream processes are continuously being developed to achieve optimal product quality as well as purity, but also to cut down on enormous costs in the downstream steps. Developing these novel methods are enabling the companies to stay one step ahead of their competitors and to remain competitive on the ever-growing pharmaceutical market. A completely generic downstream process, however, is impractical due to significant biochemical differences between biological products (Shukla et al., 2006). This master thesis focuses on constructing and evaluating a complete downstream process for the purification of antibodies, as well as comparing and testing the efficiency of several different purification methods (filtration, concentration, ion exchange).

The goal of the master thesis was to evaluate the performance of filtration equipment used in the downstream process of monoclonal antibodies. The equipment mainly consisted of three categories:

1. hollow fibers (used for the mAb concentration step)
2. depth filters (used for filtration to clean the impurities and lower turbidity)
3. anion exchange membrane (used to purify the final solution of any residual HCPs and DNA impurities)

Various downstream processing equipment is available on the market: the aim is to find the best possible according to the requirements of the process (operational cost, effectiveness, desired purity etc.). Moreover, the same CHO cell culture supernatant solution was used throughout the

entire downstream process, and while screening the filters/membranes, so that the same conditions were fulfilled. The target protein was trastuzumab and the process was optimized to achieve the highest yield of trastuzumab possible, as well as getting rid of as many impurities as possible. When it comes to depth filters, the best performing ones are the ones where the pressure is kept constant throughout the process, and where no clogging occurs, which means that the equipment can be used for longer periods of time without cleaning/exchanging. The same is true for the hollow fibers, which is why the critical flux experiment was performed. Determining the behavior of the membranes when applying certain flow rates and seeing if any issues with transmembrane pressure occur was crucial. By screening multiple membranes, the ones who show reliability and robustness throughout the test would be used in the experiment. Lastly, the anion exchange membrane was applied in the "polishing step", after most of the purifying steps were performed, to dispose of any final (negatively charged) impurities. The goal was to test the membrane adsorbers for their longevity and to see at which point during the process membrane saturation would occur. Since membrane adsorbers are commonly used in the industry in the polishing step, for more extensive purification of the remaining impurities, they were applied at the end of the process for the same purpose as well.

2. THEORY

2.1. Animal cell cultivation

With recent advances in cell culture technology over the last few decades, animal cell lines have now become a reliable way to produce high-quality biopharmaceuticals. The biopharmaceuticals produced by various cell lines can be used for both commercial purposes and clinical studies, but the robust implementation of this technology requires optimization of several variables such as operating cost, culture conditions, appropriate on-line and off-line sensors to enhance process control, and good understanding of culture performance at different scales to ensure smooth scale-up (Li et al., 2010). One of the very valuable biopharmaceutical components for which there is a consistent need on the market are monoclonal antibodies, which can be produced by using productive mammalian cell lines and optimize the conditions and process parameters to enhance protein expression. Since antibody therapies may require large doses over a longer period, the manufacturing capacity of those antibodies can become an issue – in response to the strong demand, many companies have built large scale manufacturing plants containing multiple 10.000L or larger cell culture bioreactors (Li et al., 2010). When it comes to cultivating the cells with the desired media, a lot of bioprocessing issues have been present in the industry over the years, such as undefined nature of biological constituents and increased risk of contamination, which consequently led many research groups to develop serum-free, chemically defined culture media through analysis of cellular composition, serum composition, and identification of key constituents needed to support growth (Ritacco et al., 2018).

2.1.1. CHO cell culture

One of the cell cultures used for the production of biopharmaceuticals is the Chinese Hamster Ovary Line (CHO). CHO cells have become the standard mammalian host cells used in the production of recombinant proteins, the biggest advantage being that there are well-characterized platform technologies that allow for transfection, amplification, and selection of high-producer clones (Butler, 2005). Moreover, it's important to note that CHO cells can grow in chemically defined and serum-free media enabling standardization of the production process and Good Manufacturing Practices compliance (Pan et al., 2017). Great characteristics of CHO cells, like good adaptability to genetic manipulations and changing culture conditions have rendered them extremely useful in the pharmaceutical industry, although their high propensity for genomic

rearrangements leads to cell line instability during the bioproduction process (Tihanyi and Nyitray, 2021). Nevertheless, advances in host cell engineering have led to the development of CHO cell lines with extremely high volumetric productivities up to 3 – 7 g/L, and it is unlikely that the scientific and the industrial community will abandon this expression system in the near future (Kelley et al., 2018; Tihanyi and Nyitray, 2021). As is the case with the cultivation of any cell line, the components of the media need to be clearly defined and present in just the right amount to achieve the desired result. The components present in the media need to support cell growth while simultaneously providing optimal conditions to achieve the desired product. The key components of media used to cultivate CHO cells are water (highly purified to prevent contamination), energy sources, amino acids (which can't be synthesized by the cells), lipids, vitamins, trace elements, salts, growth factors, polyamines and non-nutritional components (Ritacco et al., 2005). Some examples of them and their respective roles on the cell line are listed in Table 1.

Table 1. Key components of CHO cell culture media (Ritacco et al., 2005)

MEDIA COMPONENT	EXAMPLES	ROLE
Water	Highly purified, endotoxin-free water	Needed to formulate the liquid media
Energy sources	Carbohydrates (mainly glucose), glutamine, glutamate	Generating energy through glycolysis/pyruvate oxidation
Amino acids	Essential amino acids, Glycine, L-Histidine, L-Asparagine, L- Arginine etc.	Enhance cell density and titer, protective effects, eliminating osmolality
Lipids	Linoleic Acid	Membrane components, energy sources, signaling molecules
Vitamins	Biotin, Folic Acid, Riboflavin, Thiamine etc.	Coenzymes, prosthetic groups, cofactors in signal cascade, enzyme activity
Trace elements	Ferrous Sulfate, Sodium Selenite, Zinc Sulfate etc.	Regulation of metabolic pathways, enzyme activity

Salts	Calcium/Sodium Chloride, Magnesium Sulfate, Sodium Phosphate etc.	Osmolality, buffering, maintenance of membrane potential
Growth factors	i-Inositol	Cell growth, recovery, proliferation, differentiation
Polyamines	Putrescine 2HCl	Transcription, ribosome function, cell signaling
Non-nutritional components	Additives	Cell productivity

Cell culture media are nowadays completely chemically defined, and in some cases protein-free. This evolution of medium design was the result of optimizing the medium for maximum performance and consistency in biomanufacturing (Ritacco et al., 2018).

2.1.2. Perfusion cell cultivation

Mammalian cells can be cultivated in many ways, using different methods, bioreactors etc. Fed-batch culture is still the most common industrial process for CHO cell culture (Pan et al., 2017), but since the main focus of this thesis was obtaining antibodies produced by CHO cells cultivated through the perfusion process, the perfusion cell cultivation needs to be closely examined and explained. There are many different methods of retaining the cells in the bioreactor – one of these methods is retention by applying hollow fiber membranes, which are commonly used in ATF/TFF processes (methods described later in this chapter, with ATF being "state of the art"). Therefore, the two main components of a system designed for perfusion cell cultivation are the reactor (where the cells are grown) and the hollow fiber (where the product is harvested through the permeate line, while the cells recirculate back into the reactor through the retentate line). In this way, the cells are retained in the system and their concentration is maintained constant by bleeding the excess of dead cells from the system, while the medium is continuously fed to the bioreactor at a certain rate, enabling the growth of new cells. The schematic representation of a perfusion cell cultivation process is shown in Figure 1.

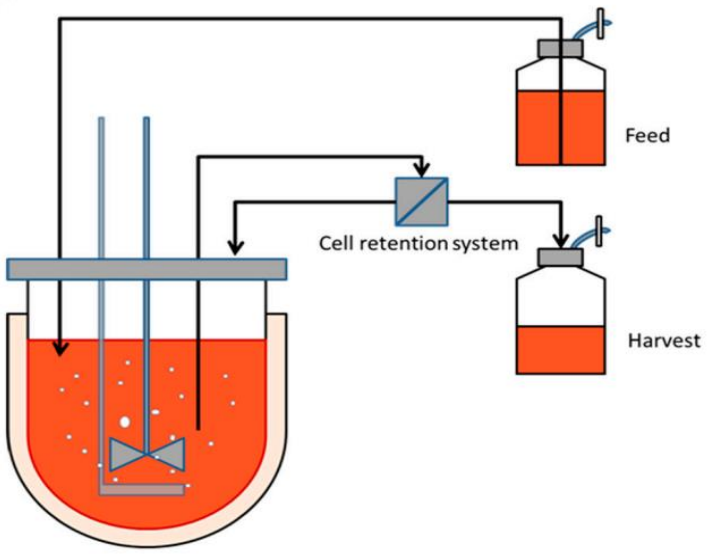


Figure 1. Schematic representation of a perfusion cell culture setup in a stirred tank reactor in an ATF (alternate flow filtration) configuration (Cole et al., 2015)

The adoption of perfusion cell lines was influenced by two important factors: the need to reduce bioreactor residence time for unstable molecules (such as blood factors and enzymes) and the need to increase protein productivity (Wong et al., 2021). The major advantages of cultivating cells in perfusion as opposed to other methods of cultivation are high cell numbers and high total production in a relatively small size bioreactor, with perfusion being optimal when the product of interest is unstable or if the product yield is low (Clincke et al., 2011). The rise of modern perfusion processes is supported by three key technology advancements: cell line engineering (which focused on enabling cell stability), cell retention technology development (improving scalability, efficiency, and robustness), and process intensification (enhancement of economic attractiveness when compared to other processes) (Wong et al., 2021). Although numerous cell retention technologies are available on the market, the main one consists of applying external hollow fibers in two main methods: either TFF (tangential flow filtration) or ATF (alternate flow filtration), with the main difference being the flow direction of the medium (which stays the same during TFF and alternates in ATF). In one study that compared the TFF and ATF cell separating techniques and their influence on the total mAb production/cell density, it was found that both higher cell densities (200 x 10^6 cells/mL) and higher retentions of mAb were observed using the TFF system, with the ATF system being more showing itself more favorable for production at stable cell density

maintained by cell bleeds (30% of mAb removed in cell bleeds in TFF compared to 19% in ATF) (Clincke et al., 2011). Furthermore, membrane-based techniques such as alternating tangential flow filtration (ATF) and tangential flow filtration (TFF) have proven to be suitable for integration with perfusion reactors coupled with continuous protein A chromatography (Somasundaram et al., 2018). To successfully carry out a perfusion cultivation process, a continuous insight into the metabolic activity of the cells is crucial. Key parameters such as pH and CO_2/O_2 levels can be monitored via various direct/indirect instruments to provide a general overview of the bioprocess.

2.2. Monoclonal antibodies

Monoclonal antibodies (mAbs) are immunoglobulins designed to target a specific epitope on an antigen (Posner et al., 2019). Antibodies are produced by B lymphocytes (plasma cells), as a body's response to external antigens to fight off diseases. Additionally, the word "monoclonal" describes their shape and, consequentially, their specific function. These molecules consist of four polypeptide chains: two identical heavy ones (~50 kDa each) and two light chain units (~25 kDa each) (Posner et al., 2019). Their very specific and recognizable Y-shaped structure is shown in Figure 2.

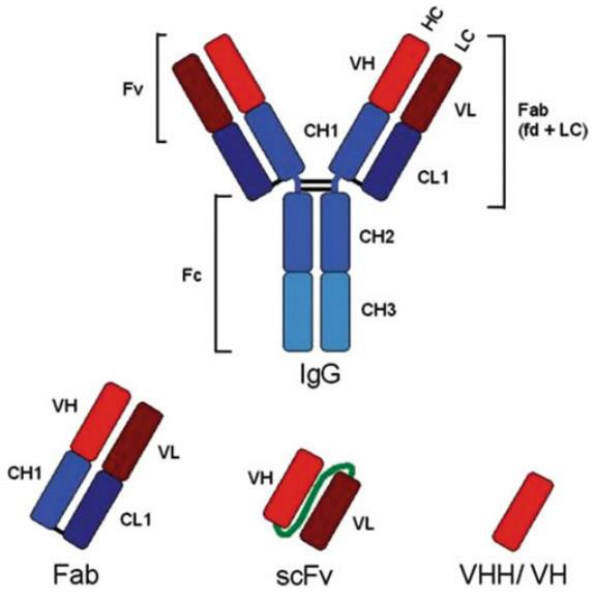


Figure 2. Representation of an IgG (immunoglobulin)/therapeutic mAbs structural unit. Each chain consists of constant (C_H and C_L) and variable (V_H and V_L) domains. The Fc region can interact with various cellular receptors. Fv represents the antigen-binding region. (Posner et al., 2019)

Structure of proteins used in pharmaceutical applications dictates not only their function, but the complexity of their production. Molecules that are extremely structurally complex are difficult to produce and develop as therapeutic agents. Because of this, the majority of marketed mAb therapeutics are developed from three out of four IgG subclasses: IgG1, IgG2, and IgG4, with IgG3 being structurally too complex to develop into therapeutic agents because of a much longer hinge region that renders it difficult to optimize for target binding and drug-ability characteristics (Vidarsson et al., 2014; Ryman and Meibohm, 2017). The structure of the four IgG subclasses is shown in Figure 3.

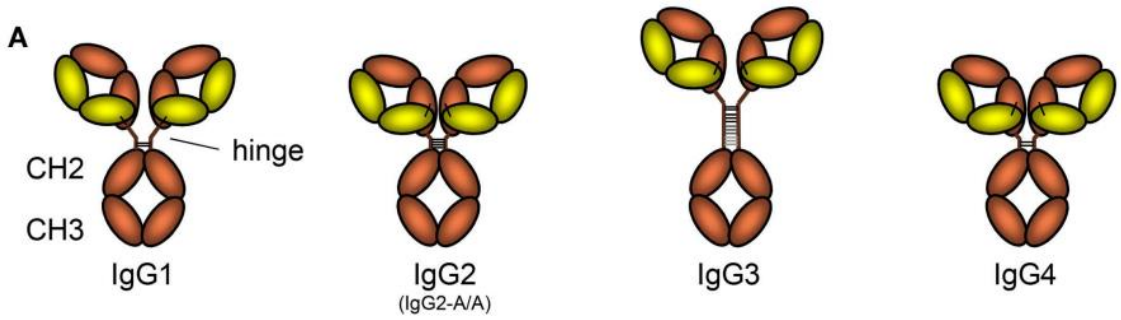


Figure 3. Human IgG subclasses and their structure. (Vidarsson et al., 2014)

The number of new mAbs on the market is increasing almost every year – from 2016 to 2018, 27 new antibodies were approved for marketing by the FDA, with over 500 different ones being in early-stage clinical development (Posner et al., 2019).

2.2.1. Trastuzumab (brand name *Herceptin*)

Trastuzumab (branded as *Herceptin*) is a humanized monoclonal antibody developed to target the HER2 receptor which is overexpressed by some cancer cells (25 to 30% of breast cancer) (McKeage and Perry, 2002). Its mechanisms render it extremely useful in battling cancer, which is why it is commonly used as medicine to prevent the proliferation of tumor cells. It's generally well received by most patients, with serious events such as anaphylaxis and death occurring in only 0.25% of patients (McKeage and Perry, 2002).

The biopharmaceutical industry needs to continuously fulfill the market demand for all kinds of biological products. Increasing the productivity of antibody-producing cells is the best way to meet these growing demands, which is still a real challenge (Li et al., 2006). The CHO cell line (explained in detail in **Chapter 2.1.1.**) was shown to be a good strategy to produce these

monoclonal antibodies, with a lot of research projects still being conducted to improve process titre, product quality, cell productivity etc. In one study conducted by Zhang et al. (2019), an auxotrophic CHO-K1 cell line to produce recombinant monoclonal antibodies was developed. The clones with the highest productivity have successfully sustained their productive capacity for at least 3 months, while expressing trastuzumab at a relatively high level. The study is just one out of numerous studies which shows that specific clones of a CHO host cell line are a promising approach for recombinant protein manufacturing. The biggest concern, however, is the possible instability of expression of the protein of interest (Zhang et al., 2019).

2.3. Downstream processing of monoclonal antibodies

Downstream methods and separation techniques are an enormous part of the final cost of any bioprocess where a high product purity is required. Therefore, lowering the costs of these methods and technologies will significantly decrease the overall cost and improve economic feasibility of the entire bioprocess. When it comes to producing monoclonal antibodies, high product purity is required due to strict requirements of the pharmaceutical industry. Other considerations of the downstream process are yield and process throughput, as well as robustness, reliability, and scalability (Shukla et al., 2007). From the standpoint of product safety, purity is also an extremely important consideration: while yield and throughput may be necessary from an economic perspective, no product is even possible without meeting the purity requirements of the pharmaceutical industry (Fahrner et al., 2013). According to Fahrner et al., there are six main purity considerations for the recovery of pharmaceutical antibodies.

1. Host cell proteins (HCPs). Present in high amounts in the harvested cell culture fluid, typically reduced to a total reduction of at least 10^5.
2. DNA. Also present in high amounts. <10 ng/dose allowed by The World Health Organization.
3. Aggregate. Mostly dimers of antibody, need to be removed because of the possible immunogenicity of the aggregate. Total content in the harvested cell culture fluid is about 5-15%.
4. Small molecules. These components are created during the process by the CHO cells.
5. Leached protein A. This protein is present in the antibody pool because it leaches from the chromatography column used in the purification step.

6. Virus. Biological pharmaceuticals are allowed to have 1 theoretical virus per 10^6 doses, which is an extremely strict requirement. In general, protein A affinity chromatography step provides 10^7 of virus clearance. Additional process steps such as viral filtration may be required after this step to ensure exceptionally high product quality.

The main step of the downstream processing is the Protein A affinity chromatography method, which is proven to be highly selective for monoclonal antibodies. Other modes of chromatography have also been combined with Protein A chromatography to achieve pharmaceutically acceptable purity levels (Shukla et al., 2007). In one study, an anion-exchange chromatography as a second chromatographic step for DNA and endotoxin clearance was applied, after which aggregates and degradation products were removed by employing a size-exclusion chromatography step (Gottschalk, 2005). Since no single chromatography step can achieve the necessary antibody purity, three chromatography steps have also been employed in the industry: protein A affinity chromatography, followed by cation exchange chromatography (both in bind-and-elute modes), followed by anion exchange chromatography (run in flow-through mode), which produces a high-yield process capable of meeting the purity requirements (Fahrner et al., 2013). The three step purification method (Protein A chromatography – flow-through anion exchange – mixed-mode cation exchange) of monoclonal antibodies was also successfully carried out in continuous operation, which resulted in higher productivity and reduced resin volume required for Protein A and mixed mode chromatography. The employed method is the standard, "state of the art" procedure of purifying biopharmaceuticals. The experiment proved that significant efficiency and quality advantages, as well as reductions in both capital and operating expenditure, can be realized by connecting multiple unit operations together and moving from batch to continuous operation (Gjoka et al., 2017). This type of process intensification paves the way for new drug products to be released very quickly and will ultimately lower the cost of that drug to the patient, as well as reduce the need for large inventories of that drug product (Gjoka et al., 2017). Continuous biomanufacturing technologies and their advantages are described in more detail in **Chapter 2.4**.

Future challenges in mAb production depend on the technological advancement of the downstream methods since they are the limiting factor in the bioprocess. Chromatographic operations become limited in terms of the throughput they can provide – on the other hand, non-chromatographic purification techniques such as selective precipitation or liquid – liquid separations employing highly selective ligands are likely to emerge (Shukla et al., 2007). A figure representing a downstream process of mAbs is shown in Figure 4.

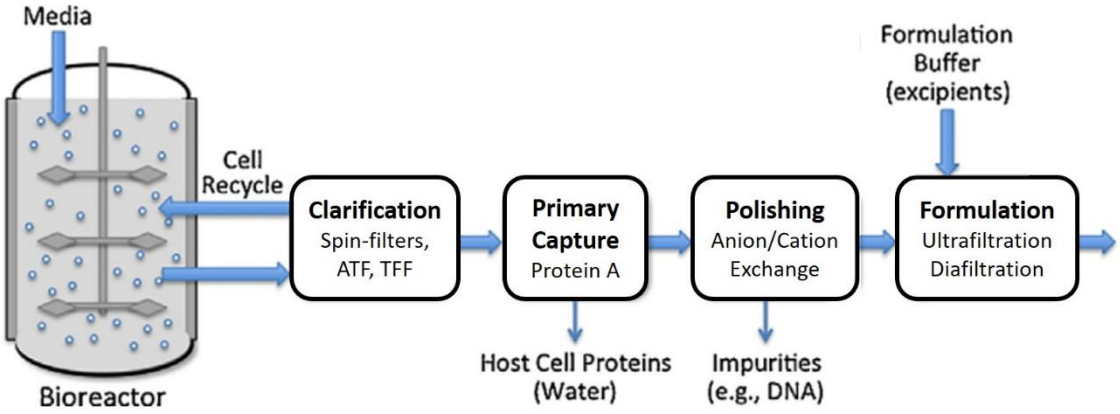


Figure 4. An industry example of a generic continuous downstream operation as part of perfusion bioprocess for mAb production (Adapted from Zydney, 2015)

2.3.1. Analytical methods

Naturally, the monoclonal antibody is not the only component that is produced by the mammalian cell line during the bioprocess – a lot of impurities are produced along with the protein of interest. To know which methods should be applied to purify the product, these contaminants need to be qualified and quantified, depending on the nature of the component. Although mammalian cells generate a lot of various components during the production of antibodies, in the following section, quantification methods that were used in the experimental part of the master thesis (quantification of HCPs, DNA, and antibodies), will be explained in detail.

Quantification of HCPs using the ELISA method

Because different cells have different genomic profiles, the profile of HCPs generated during the bioprocess depends on the type of cell line (plant cells, mammalian cells, insect cells etc.). Most of the HCPs present in the supernatant of CHO cell lines come from lysis or breakage of cells rather than secretion of protein material from within the cell (Tait et al., 2012). These proteins are

key quality attributes – in theory, residual HCP could pose a risk to patient safety by potentially causing an unwanted immunological response, which is why its clearance from biologic products is employed as a benchmark to demonstrate the robustness of a bioprocess (Zhu-Shimoni et al., 2014). To analyze HCP content of CHO cell line supernatant, an enzyme-linked immunosorbent assay (ELISA) is often the preferred method. Advantages of this method include high-throughput, sensitivity, and selectivity, although the method shows some limitations such as detection issues due to low/high concentration of antibodies and poor immunoreactivity of some antigens (Tait et al., 2012). Despite these limitations, HCP ELISA is still widely utilized in the biopharmaceutical industry for the control of HCP.

Quantification of dsDNA using the Picogreen assay kit

Along with HCPs, a lot of double-stranded (ds) DNA impurities are present in the cell line supernatant. Detecting and quantitating small amounts of DNA is extremely important in a wide variety of biological applications such as subcloning, cDNA library production, various diagnostic techniques etc. (Singer et al., 1997). The way this DNA is quantified is by applying the PicoGreen dsDNA quantitation reagent, which binds to nucleic acids in the solution and gives off an intense fluorescent response which can then be measured by a specific spectrofluorometric device. The absorbance for DNA analysis is measured at 260 nm. Major disadvantages of this method include the large relative contribution of single-stranded DNA to the total signal, the interfering effect of contaminants, and the relative insensitivity of the assay (Singer et al., 1997). To face these issues, significantly more sensitive reagents, like PicoGreen reagent, have been developed in the past, paving a way to new analytical methods which are successfully being applied in the biopharmaceutical industry for the detection of low amounts of dsDNA in a given solution. The PicoGreen dye-based assay is readily suited to rapid, high-throughput automation since it is a single-step procedure with only a brief incubation period and is spectrally compatible with mercury-arc lamp or other broad-spectrum excitation sources as well as with commercially available lasers (Singer et al., 1997).

Quantification of trastuzumab with Protein A chromatography

Protein A chromatography is an affinity chromatography method used for the purification of antibodies which specifically bind to the ligands originating from the bacterial species

Staphylococcus. However, protein A columns can also be used in the quantification of antibodies like trastuzumab, in configuration with HPLC.

2.3.2. Process equipment

Depth filters

Depth filtration has a long history in bioprocessing, with the earliest records of water purification dating back 4000 years (Nejatishahidein and Zydney, 2021). The renewed interest in depth filtration has been driven by both the push towards single-use manufacturing technologies and improvements in cell density and product titer, which gave way to increased turbidity (Nejatishahidein and Zydney, 2021). There are many different configurations of depth filters depending on the material, pore size, volumetric loading, capacity, type of impurities that need to be filtered etc. The first step of the downstream process of cell culture supernatant includes getting rid of initial impurities (clarification of cell suspension), where single-use depth filters are useful. Depth filtration can also be applied for primary separation of cells and product during these initial steps of the process. Some other isolation techniques of cell debris like centrifugation and microfiltration are available, however, they are significantly more expensive, which is also an advantage of depth filters (Hadpe et al., 2020). Approaches that combine multiple techniques to improve process performance are also possible, with centrifugation or flocculation pretreatment being applied before depth filtration to further remove cells and cell debris. These pretreatment alternatives can play a significant role in relieving some of the challenges by removing the impurities that add further pressure on the downstream processing (Hadpe et al., 2020). Depth filters can be applied at various steps of the process to reduce the turbidity of the solution at any point (which was the case during the master thesis as well) and to minimize the amount of impurities before moving on to the next purification step. Turbidity (measured in NTU: nephelometric turbidity unit) is an indication of cloudiness or haziness of a fluid caused by individual particles (suspended solids), and its removal indicates solution clarity

Hollow fibers

Hollow fiber membranes are used in bioprocessing methods such as diafiltration, ultrafiltration, pervaporation, air separation, reverse osmosis etc. The perfusion processes (explained in **Chapter 2.1.2.**) also depend on the quality and performance of hollow fiber membranes used for cell retention, with the major drawbacks being fouling and product retention (Nadar et al., 2021).

Several criteria need to be carefully examined when selecting a membrane device for a perfusion bioreactor, such as process duration, operation cost, maintenance, desired product recovery, fouling, scalability, cell viability etc. (Voisard et al., 2002).

In recent years, the applicability of membrane-based unit operations in the downstream process of monoclonal antibody production has also been explored (Nadar et al., 2021). Due to advances in polymer chemistry and membrane support matrices over the past decades, modern membranes have been engineered with selectivity to achieve separation based on molecular weight, surface charge and hydrophobicity, combining high throughput advantages of membranes with separation properties of resin-based chromatography (Ma et al., 2009). This has broadened the applicability of membranes for the removal of the process- and product-related variants, which is driven by improvements in several processes such as:

- Clarification of proteins (to reduce soluble and insoluble impurities coming as a result of increased levels of cell biomass)
- Capture, intermediate, and polishing purification (membrane adsorbers with enhanced binding capacity and module design)
- Pre-Formulation of proteins (to concentrate/exchange the protein into a stable buffer system) (Nadar et al., 2021).

Ionic exchangers

Ion exchange chromatography (IEX) is a widely used technique for the purification of many protein products in the biopharmaceutical industry. This method relies on the use of specifically charged resins to purify the sample by removing the charged molecules which adsorb to the resin due to their charge. IEX is based on differential adsorption of charged substances at oppositely charged surfaces of porous chromatographic media (Harinarayan et al., 2006). A key parameter that is thoroughly investigated is the dynamic binding capacity of a protein (DBC). To put it simply, this parameter indicates the maximum amount of protein that can be loaded onto the column without causing unnecessary loss (Cytiva, 2018). In general, the capacity of the resin decreases with increasing buffer conductivity and decreasing protein charge (Brooks and Cramer, 1992). When it comes to ion exchange of monoclonal antibodies, two main mechanisms were defined: exclusion and equilibrium (Harinarayan et al., 2006). The two applications of ionic

exchange chromatography are cationic exchange (CEX) and anionic exchange (AEX). CEX columns bind cationic species such as protonated bases through ionic interactions with negatively charged acidic functional groups, while AEX columns bind anionic species with positively charged basic groups, such as carboxylic acids (Fekete et al., 2015). To date, IEX has been successfully employed for the characterization of charge variants on the peptide chains of numerous therapeutic proteins, and it will certainly, despite its disadvantages, remain one of the gold standards for the analysis of biopharmaceuticals (Fekete et al., 2015).

Target proteins produced in mammalian cell cultures can also be captured by using specific membrane adsorbers. These adsorbers show high binding efficiency, scalability and flexibility, as well as easy incorporation into a process for the removal of various impurities such as HCPs, viral vectors, coagulation factors etc. (Pall, 2023). Coupled with subsequent membrane-based intermediate purification and polishing steps, such high-capacity non-affinity based adsorbers may lead to more integrated processes from capture to final product without the use of an expensive protein A-based capture step (Nadar et al., 2021). Membrane chromatography differs from column chromatography and is a relatively new approach of separation technologies, showing many advantages compared to traditional column chromatography. These advantages include reduced mass transfer resistance (which results in a fast binding behavior), reduced buffer usage due to low void volume, lowered pressure drops, high scalability etc. However, it doesn't come with its disadvantages, the main one being flow distribution due to non-uniform membrane porosity and thickness, which has shown to be a concern for resin (column) chromatography as well (Orr et al., 2013). The specific membrane adsorber used for the purpose of this experiment, the 3M™ Polisher ST, contains two complementary AEX-functional media: a quaternary ammonium functional nonwoven (which provides reduction of turbidity, DNA, and endotoxin), and a guanidinium functional membrane (which provides high HCP and virus reduction), and is designed to improve mAb development and manufacturing processes (3M™ Polisher ST Data Sheet).

2.4. Continuous biomanufacturing

Continuous processing is necessary when talking about novel and effective methods in the field of biomanufacturing. In some cases, the transition from traditional batch processes to continuous processing in the biopharmaceutical industry in general was highly disruptive – some companies that were unable (or unwilling) to adapt to novel techniques were ultimately outcompeted by others

(Zydney, 2015). Although the details of each industry are clearly unique, the benefits of moving to continuous operations are similar and include:

- significant reductions in capital equipment costs and facility size,
- significant increases in productivity (largely because all equipment is in use at all times),
- greater flexibility (because of more efficient utilization of the facility),
- improved product quality (because of greater uniformity in process time) (Zydney, 2015).

Integrated and continuous biomanufacturing (ICB) is one modern approach to the production of recombinant protein therapeutics from mammalian cell lines, which links the bioreactors and the process of cell cultivation with a continuous downstream process. To carry out a process successfully, consistency of upstream/downstream flow rates is crucial (Coffman et al., 2021). One example for the need for high productivity ICB would be the impact of the manufacturing of SARS-CoV-2 antibodies, which has caused a sudden shortage of large-scale capacity (Coffman et al., 2020). A desirable continuous manufacturing process must have all process unit operations fully integrated with appropriate PAT tools and process model-based "soft" sensors to enable real-time decisions to be undertaken autonomously based on process deviations or disturbances (Chopda et al., 2021). An overview of a continuous mAb production is shown in Figure 5.

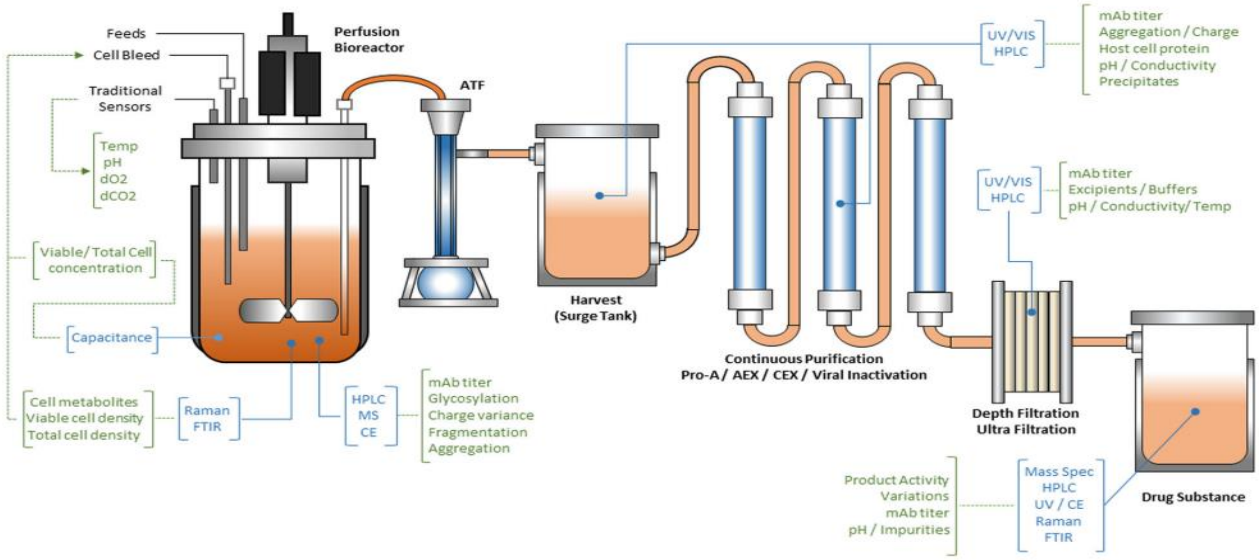


Figure 5. Overview of a continuous mAb production process, along with specific PAT tools required to monitor the process. Blue denots the specific analytical techniques, while green denotes specific critical quality attributes (CQAs) that are measured during each step (Chopda et al., 2021)

3. EXPERIMENTAL PART

3.1. MATERIALS

3.1.1. Samples

The sample solution for the hollow fiber critical flux experiment, called the "bleed", was taken from the downstream unit of the process, after the trastuzumab precipitation step. After the sample was taken, it was stored in the cold room at 4°C. In order to obtain enough volume for the entire experiment, certain volumes of samples taken during different days were combined, resulting with a sample of 5 g of precipitated trastuzumab/L.

All the other samples that were analyzed for DNA, HCP, and trastuzumab content were taken during various points of the purification process and stored in the cold room at 4°C until they were analyzed.

3.1.2. Chemicals

0.5 M NaOH

20% ethanol solution

200 mM phosphate buffer (pH 8.0)

50 mM potassium phosphate (pH 2.5)

50% PEG in 100 mM MOPS (pH 7.0)

18% PEG in 100 mM MOPS (pH 7.0)

1M $CaCl_2$ in 100 mM MOPS

1M phosphoric acid

3.1.3. Equipment

2L Bioreactor, Labfors, Switzerland

Masterflex pump, Germany

Magnetic pump, Levitronix, Switzerland

Silicone tubings (various diameters)

Flexboy bag (10L), Sartorius, Germany

Single use pressure sensors, PendoTECH, USA

PendoTECH PressureMAT Sensor Monitor, USA

Agilent HPLC System, USA

Magnetic stirrer

2100Q Portable Turbidimeter, HACH, USA

Watson Marlow Pump 120U, United Kingdom

Cable ties

Luer locks

Pyrex bottle (500 mL)

Glass beakers (100 mL, 600 mL, 2L, 5L)

Plastic beakers

Micropipette, Mettler Toledo, United States

0.22 µm microfilters, Sartorius, Germany

Microza UJP-1047R, Asahi Kasei, Japan

Hollow fiber screening:

- Microza UJP-0047R, Asahi Kasei, Japan
- Microza UMP-0047R, Asahi Kasei, Japan
- Cytiva CFP-2-E-3MA, USA
- Repligen, USA

Depth filtration screening:

- ErtelAlsop P2E4, USA
- ErtelAlsop B1E5, USA
- ErtelAlsop B4E7, USA

- Millipore Millistak, Merck, Germany

DNA/HCP analysis

Orbital shaker, Thermo Fisher Scientific, USA

96-well plates

Black 96-well incubation plates

Multichannel pipette, Thermo Fisher Scientific, USA

TMB substrate (Tetramethylbenzidine)

Anti-CHO:HRP conjugate

Stop solution (0.5 M H_2SO_4)

dsDNA PicoGreen Assay Kit, Invitrogen

Polishing experiment:

ÄKTA protein purification system, GE Healthcare, USA

CHO-K1 Mammalian Cell Line

For the purposes of trastuzumab antibody production, a mammalian cell line CHO-K1 was used. CHO-K1 (Chinese Hamster Ovary) cell lines are used for the production of most new recombinant protein therapeutics due to their various advantages (Kelley et al., 2018).

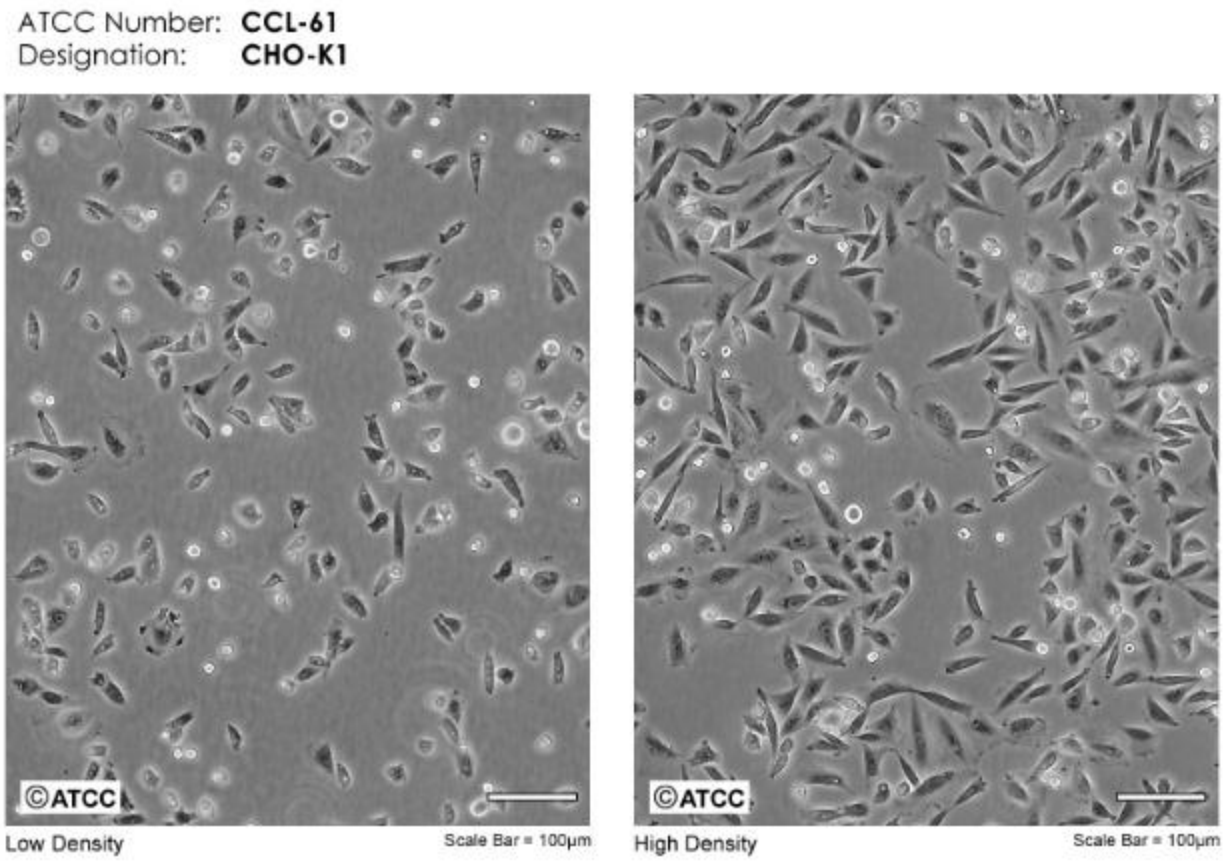


Figure 6. CHO-K1 mammalian cell line (ATCC, 2022)

3.2. METHODS

3.2.1. Cell cultivation

Cells were cultivated in a perfusion bioreactor setup with a Labfors bioreactor, Levitronix pump and a Microza UMP-1047R membrane (0.2 μm pore size, 0.09 m^2 effective membrane area) used as a cell retention device. The cell concentration in the bioreactor was kept at a constant of ~60 x 10^6 cells/mL. The "harvest" of the process (captured through the permeate line of the Microza membrane) was pumped into a 10L Flexboy bag with a Masterflex pump, and then later used in the antibody purification process. Key parameters of the bioprocess, such as cell concentration, O_2 concentration, pH values and stirrer speed were measured using both on-line (pH, O_2, biomass probes) and off-line methods (Vi-Cell XR Cell Viability Analyzer, CEDEX Bio-Analyzer).

3.2.2. Hollow fiber critical flux experiment

Redissolution and neutralization step of trastuzumab

The precipitated trastuzumab solution that was used for both the critical flux experiment (with membranes) and depth filter screening experiments was collected from the capture unit, a separate downstream process unit used in a different research project, and it contained 5 g/L of PEG-precipitated trastuzumab. To prepare the media for the experiments, redissolution and neutralization steps were applied.

The media was redissolved with a 50 mM phosphate buffer (pH 2.5) in a ratio of 1:5 (100 mL of media was mixed with 500 mL of buffer), after which the antibodies became soluble again. The solution was incubated for 1 hour at low pH. After the redissolution step, neutralization was performed with a 200 mM phosphate buffer (pH 8.0); 150 mL of neutralization buffer was added to the bottle. The final pH after neutralization was 6.96.

Critical flux experiment

The system for the critical flux experiment was the same for all the hollow fibers tested. It consisted of a 250 mL Pyrex bottle with feed, tubings, Masterflex pump (connected to the feed line), Watson Marlow pump (used for the permeate line), three PendoTECH pressure sensors (one for the feed line, one for the retentate line and one for the permeate line) and a hollow fiber of choice. The Masterflex pump was used for the feed line since it can operate at high flow rates, and the retentate

flow rate is much higher than the permeate one for all four membranes, which meant a stronger pump needed to be used. A scheme of the setup is shown in Figure 7.

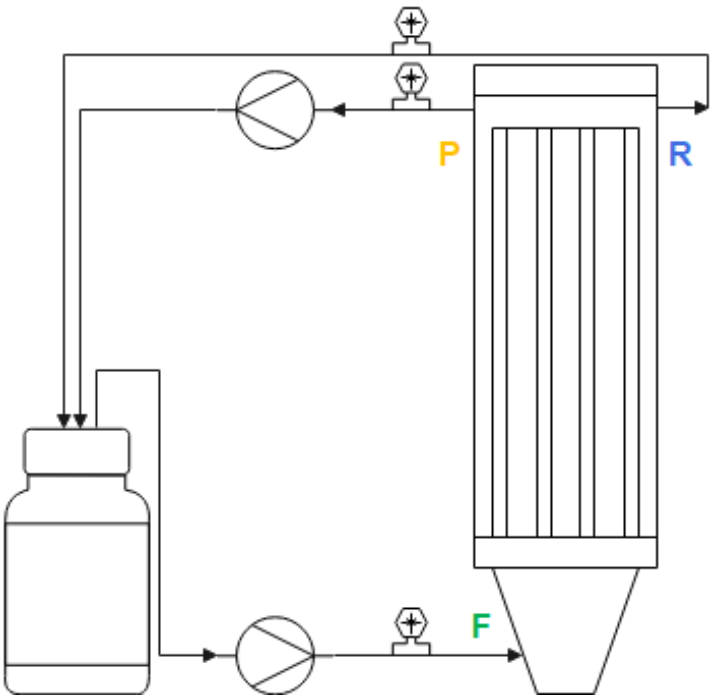


Figure 7. Critical flux experiment system setup (F – feed line, R – retentate line, P – permeate line)

Since the four membranes differ in terms of geometry (area size, pore size, fiber number etc.), the shear rate in the retentate side of the hollow fibers was maintained constant by modifying the inlet (feed) flow rate. The shear rate can be calculated for each membrane, and is a good indication of the operating parameters applied in the system:

$$\text{Shear rate} = \frac{4 \cdot Q}{r^3 \, x \, n \, x \, \pi} \qquad [1]$$

In which:

Q = volumetric flow rate (mL/min)

R = radius of the hollow fiber (cm^3)

n = number of fibers in the cartridge.

Depending on the parameters of the membrane r^3, recirculation and permeate flow rate were adjusted to keep the shear rate at around 1600 s^{-1}. Table 2 displays the values of applied flow rates for the critical flux experiment according to the geometry of the membrane.

Table 2. Applied flow rates for each membrane based on membrane geometry

	Microza 0.65 µm	Microza 0.2 µm	Cytiva	Repligen
Membrane area (cm^2)	0.01	0.02	0.01	0.01
Area size (cm^2)	0.02	0.02	0.011	0.014
Fiber ID (mm)	1.1	1.4	1	0.5
Pore size (µm)	0.65	0.2	0.2	0.2
Fiber number	27	21	13	45
Recirculation flow rate (mL/min)	**338**	**542**	**122**	**53**

The critical flux test is performed in the following way: retentate/feed flow rates are kept constant throughout the entire experiment, while the permeate flow is adjusted every 30 minutes (each 30-minute sequence is called a "step"). After the initial step, the permeate flow is increased for another 30 minutes (second step). The permeate flow is then decreased to the value from the previous step. During the first few steps, no sudden/abrupt changes in the system (when it comes to transmembrane pressure) are expected, because the permeate flow rates are still low. After this first 90-minute stage, the process continues by increasing the flow rates even higher than step 2. After every increase of the permeate flow, it's adjusted back to what it was in the previous step to determine whether irreversible fouling has occurred. When the TMP increases sharply, and, what's more, does not drop with a decrease in flow rate, it's safe to say that irreversible fouling occurred, and the process for that membrane can be stopped. This is also the difference between reversible and irreversible fouling – after reversible fouling occurs, the TMP values can still drop back down with a decrease in flow rate because the fiber pores are not irreversibly clogged. The steps are graphically shown in Figure 8.

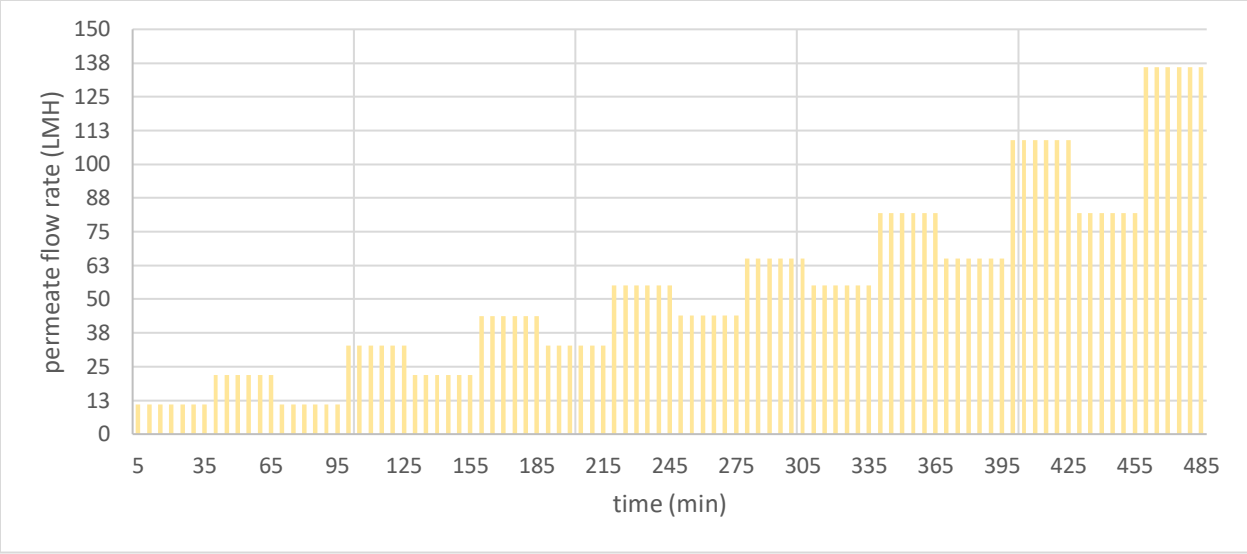


Figure 8. Critical flux steps (permeate flow values)

The transmembrane pressure at each measuring point was manually calculated using the following equation:

$$TMP\ (bar/psi) = \frac{Pf + Pc}{2} - Pp \qquad [2]$$

In which:

Pf = feed pressure [bar/psi]

Pc = recirculation pressure [bar/psi]

Pp = permeate pressure

After the critical flux experiment was performed, the hollow fibers were cleaned by recirculating warm water, followed by recirculating a 0.5 M NaOH solution. Later, hollow fibers were stored in 20% ethanol to keep the membrane clean until its next usage.

3.2.2. Depth filter screening

Buffer preparation

Two buffers were prepared for this experiment: one for the redissolution step (50 mM K-phosphate, pH 2.5) and one for the neutralization step (200 mM phosphate, pH 8.0). 1.5L of K-phosphate buffer (pH 2.5) was prepared by dissolving 10.2 g of KH_2PO_4 in HQ water. The solution was titrated with 1M phosphoric acid until the pH dropped to the desired value of 2.5. 600 mL of

phosphate buffer (pH 8.0) was prepared by dissolving 20.9 g of K_2HPO_4 in HQ water. The solution was titrated with 200 mM KH_2PO_4 until the pH dropped to the desired value of 8.0. The details of the step are explained in the following section.

Redissolution and neutralization step of trastuzumab

The PEG-precipitated antibody (5 g/L of trastuzumab) was redissolved with 50 mM sodium phosphate buffer, pH 2.5 (1:5 ratio of feed to buffer). Low pH incubation lasted for one hour. After the redissolution step, neutralization with 200 mM phosphate (pH 8.0) was performed. (1:1.25 ratio of feed to buffer). After stirring for 15 minutes, the solution was ready to be used for the screening of depth filters.

Depth filter screening experiment

To maintain the same conditions for all filters, the tests were performed with the same flow rate (1.0 mL/min) and the same volumetric loading (150 L/m^2) across all depth filters. Since all the filters have the same volume, the same volume of ~340 mL of redissolved/neutralized trastuzumab solution was applied to all filters as well.

After performing the depth screening at a flow rate of 1 mL/min, the same filters were backflushed with clean water and used again in the same screening, only this time applying a flow rate of 5 mL/min. Since the same volumetric loading was applied (meaning the same volume was ran through the filter), the process was five times shorter (340 minutes at 1 mL/min, 70 minutes at 5 mL/min).

3.2.3. Sequential antibody purification process

Schematic representation of the process for antibody purification is shown in Figure 9. The applied procedure is called “sequential” because it’s performed sequentially (in four steps) rather than continuously. Samples were taken during each performed step of the process, as well as before every process, to determine the total loss of antibodies and, consequently, the final antibody yield.

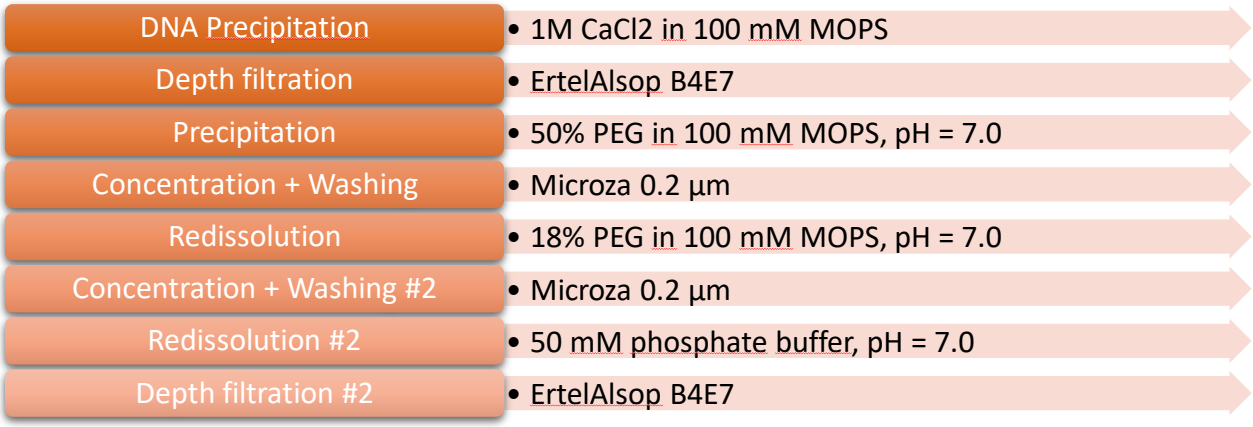


Figure 9. Steps of the sequential mAb downstream process

The low pH incubation step, as well as the neutralization step that was applied during depth filtration screening, were skipped in this procedure. The redissolution was performed with a phosphate buffer at pH 7.0. Instead of low pH incubation step, other methods of viral inactivation could be performed here (like the use of detergents when operating with pH-sensitive proteins). Since low pH incubation was not applied, the neutralization step with another buffer was not necessary.

Step 1: Depth filtration/DNA precipitation

To get rid of initial impurities and to precipitate DNA, the first step of the purification process was the depth filtration of the harvest media (collected from the perfusion run). To successfully precipitate the DNA, 2520 mL of media harvested from the perfusion process was mixed with 280 mL of 1M $CaCl_2$ in 100 mM MOPS solution in a 5L glass beaker and stirred for 30 minutes. The mechanism of $CaCl_2$ precipitation can be described by the formation of an insoluble calcium phosphate salt, consisting of Ca^{2+} ions from the salt, phosphate from DNA backbone as well as free phosphate (Sommer et al., 2015).

The depth filter used for this process was ErtelAlsop B4E7, which was determined to be a good choice for the desired process after the screening of multiple depth filters. The flow rate for the depth filtration was kept at a constant 5 mL/min using a Watson Marlow pump. Inlet pressure was constantly monitored via PendoTECH pressure sensors. Since the pressure increased significantly over the first 5 hours, the filter was exchanged with a new one after about 5 hours and 20 minutes into the process. To determine if the filter maintains the same performance, the turbidity was

measured every 30 minutes by taking the samples in a vial and using the HACH device. The device gives out a turbidity value measured in NTUs, which is a good indication of product clarity. After measurements were performed, the sample volumes were returned to the total pool. The collected pool was stored in the cold room at 4°C.

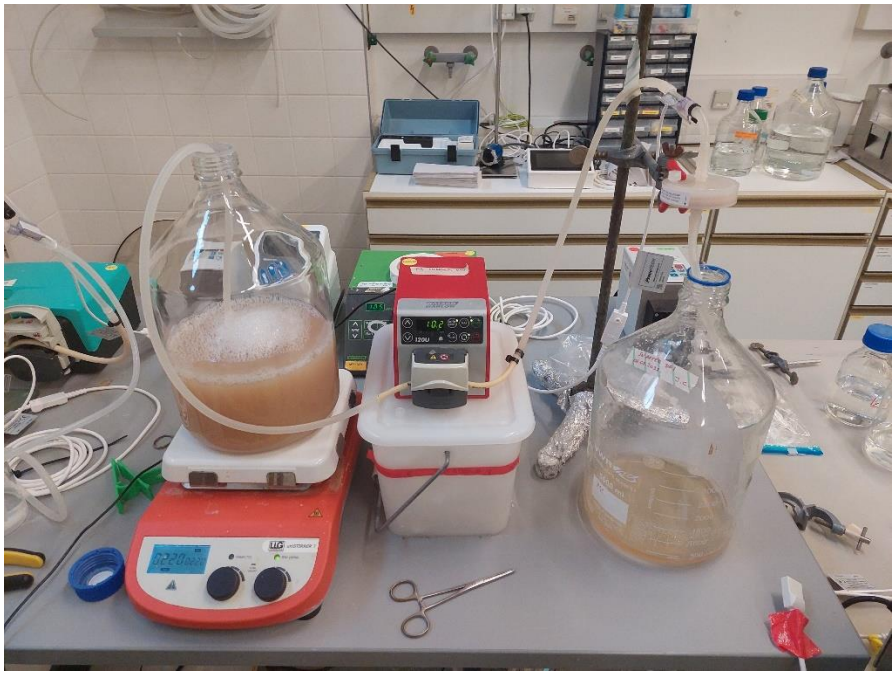


Figure 10. Depth filtration system of cell culture supernatant

Step 2: Concentration + Washing + Redissolution

To precipitate the antibody, 2680 mL of the depth filtrated pool from the first step was mixed with 1512 mL of 50% PEG in 100 mM MOPS buffer in a 5L glass beaker. The solution was left stirring for 30 minutes. The phenomenon of protein precipitation with PEG could be explained through a theory of excluded volume, which states that the accessibility of a certain volume to a molecule in the system is reduced because of the presence of another molecule. Therefore, the accessibility of water molecules to proteins (i.e., protein solubility) is reduced because of the presence of PEG which dissolves in the water. Washing the precipitate with fresh buffer with added PEG was reported to remove the soluble HCPs from the volume (Kuczewski et al., 2011). Combining the $CaCl_2$ precipitation from the previous step and PEG precipitation in this step is expected to result in a cost-effective method of antibody purification – $CaCl_2$ precipitation separates the dsDNA and

aggregates, while PEG precipitation separates the antibody of interest from low molecular weight impurities (LMWI), mainly HCPs (Sommer et al., 2015).

Concentration and recovery of the precipitated antibody was performed by applying a tangential flow filtration process (Microza hollow fiber, 0.2 μm). Figure 11 represents a simple TFF system built for this purpose, with the only pump used as a recirculation feed pump. Both feed and permeate flow rates were constant throughout the process. The recirculation flow rate was kept at a constant 350 mL/min, and the permeate line was left unrestricted to collect the filtered water in a graduated cylinder. The process was stopped when the volume of clean water reached 3990 mL (meaning that the antibody was concentrated twenty times). Three PendoTech pressure sensors were connected to the feed, retentate, and permeate lines to track the transmembrane pressure in case of a sudden spike, which was done via PendoTECH PressureMAT. This was an important parameter to consider – as PEG, a very viscous solution, is being used for antibody precipitation, lower transmembrane fluxes and, hence, higher pressures, are to be expected. Samples of the permeate were taken to determine if there are antibodies that were lost during the concentration. After the procedure ended, the membrane was washed twice with 40 mL of 18% PEG in 100 mM MOPS (stored in 50 mL Falcon tubes) to collect any residual antibodies that were left in the system after the run, as well as to determine the quantity of antibodies that weren't collected during the run. The concentrated antibody solution was stored in the cold room at 4°C.

Figure 11. Concentration of mAb with hollow fiber membranes

Step 3: Concentration + Washing + Redissolution

The procedure for step 3 was carried out exactly like the one for the previous step, only using 18% PEG in 100 mM MOPS instead of 50% PEG in 100 mM MOPS. A concentrated solution of antibodies from step 2 was redissolved in 18% PEG in 100 mM MOPS – the amount of solution added corresponded to the amount of water that was removed during concentration (3990 mL). Washing was performed exactly like step 2 as well. Just like the previous step, the concentrated antibody solution was stored in the cold room at 4°C.

Step 4: Depth filtration

The final 170 mL of supernatant after step 3 was dissolved in 850 mL of 50 mM phosphate buffer (pH 7.0) and stirred for 30 minutes. This results in a final PEG concentration of 3% (an 18% solution of PEG from the previous step is diluted 6 times). This concentration value of 3% has been reported as the limit beyond which there might be a significant reduction in antibody yield due to low solubility (Hammerschmidt et al., 2016). Depth filtration was performed exactly step

1, by using the same filter and the same flow rates. The final pool was once again stored in the cold room at 4°C.

Sample analysis

All the samples taken during various steps of the process were stored in the cold room at a temperature of 4°C, in 2 mL Eppendorf tubes. They were analyzed for the concentration of:

1. Antibodies (protein A chromatography)
2. DNA (Picogreen method).
3. Host cell protein (ELISA method)

DNA Analysis (Picogreen assay)

DNA content was analyzed by applying the method using a PicoGreen reagent. 20x stock TE buffer, DNA standard and PicoGreen reagent contained in the PicoGreen kit from Invitrogen were used for this method, as well as a 96-well plate. 1x TE buffer was prepared in a 50 mL Falcon tube (1:20 dilution of 20x TE buffer). 150 µL of 1x TE buffer was pipetted to each well. The buffer/samples/standards were transferred to the first row in the plate in such a way that the first two columns contained 150 µL of the standard, the third column contained 150 µL of the blank (buffer) and columns 4 to 12 contained 150 µL of the sample. Each well in the first row contained 300 µL of liquid, while the other wells contained 150 µL of 1x TE buffer. The samples were then sequentially diluted by pipetting 150 µL of samples from the first row and continuously diluting them towards the bottom row. Since each time 150 µL of components are pipetted to 150 µL of 1x TE buffer, samples in each row are twice as diluted as samples from the previous row. After The procedure is carried out in such a way to accurately assess the DNA concentration across multiple dilutions, since it can only be correctly evaluated when it is inside a certain range. After preparing the dilutions, 100 µL of samples from each well were added to a black incubation plate. Just before the analysis, 11 mL of reagent solution was prepared by adding 55 µL of the Picogreen reagent (from the Invitrogen kit) to 10.945 µL of HQ water. 100 µL of the prepared reagent solution was added to each well in the incubation plate and, to prevent the influence of light on the reagent, quickly evaluated using the dedicated software.

HCP Analysis (ELISA method)

The ELISA method is a great way to determine the presence of host cell protein impurities. To quantify the HCPs, a kit from Cygnus Technologies was used, which contained all the necessary chemicals to conduct the assay protocol, which goes as follows: 100 µL of anti-CHO:HRP substrate are pipetted into each well. 50 µL of standards, controls and samples were pipetted into wells indicated on the work list. The plate was covered and incubated on an orbital shaker for 2 hours at room temperature, after which the contents of the wells were dumped into the waste and the wells were washed with diluted wash solution (4 times in total). Absorbent paper was gently and carefully tapped over the plate (to not cause dissociation of antibody bound material) to remove any residual liquid. 100 µL of TMB substrate was pipetted into each well and the wells were incubated for another 30 minutes at room temperature, this time without shaking as it may lead to worse precision. 100 µL of Stop Solution was pipetted into each well. Finally, the absorbance was read at 450/650 nm.

3.2.4. Polishing experiment

The polishing experiment is the last step of the antibody purification process performed for the purpose of this thesis. Typically, this process is more extensive and itself consists of several parts, such as anionic exchange chromatography, cationic exchange chromatography, hydrophobic chromatography etc. (with their order depending on the process). These downstream purifications steps are described in more detail in **Chapter 2.3**. However, due to lack of time and equipment, a reduced version the polishing step was carried out, with the only step being anion exchange (AEX). This step is performed using a ÄKTA Pure Protein Purification System from GE Healthcare (shown in Figure 12), a product used in biopharmaceutical processing for purifying various pharmaceutical compounds such as drugs and vaccines (more on this purification device in **Chapter 2.3.2.**). Depending on the characteristics of the target compound and the impurities from the solution, numerous different methods can be applied. Since the antibody of interest is trastuzumab, which is positively charged, the membrane used for the purification process is a single-use anion exchange product (3M Polisher ST), which is intended to remove the negatively charged contaminants such as viruses, HCPs, DNA etc.

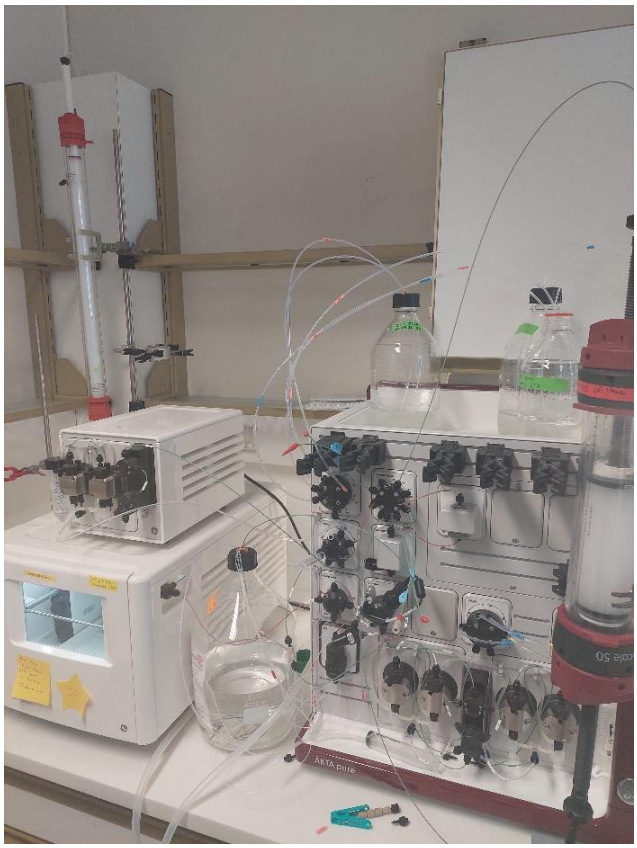


Figure 12. ÄKTA Pure Protein Purification System

Before purifying the final pool obtained from the sequential process, two things needed to be performed. First, the redissolved mAb was depth filtered once again (with the same filter as in the sequential process described in **Chapter 3.2.3.**) because the turbidity was relatively high. Second, the pH and conductivity of the feed media needed to be adjusted since the binding capacity of the contaminants (interaction strength) is limited by these operating conditions and higher binding capacity is observed when purifying a feed with lower conductivity values. This was also reported in the 3M support file (data displayed on Figure 13). Therefore, 940 mL of feed obtained from the sequential process was mixed with 5.5 mL of 1% HCl (for pH adjustment) and 440 mL of HQ water (for conductivity adjustment).

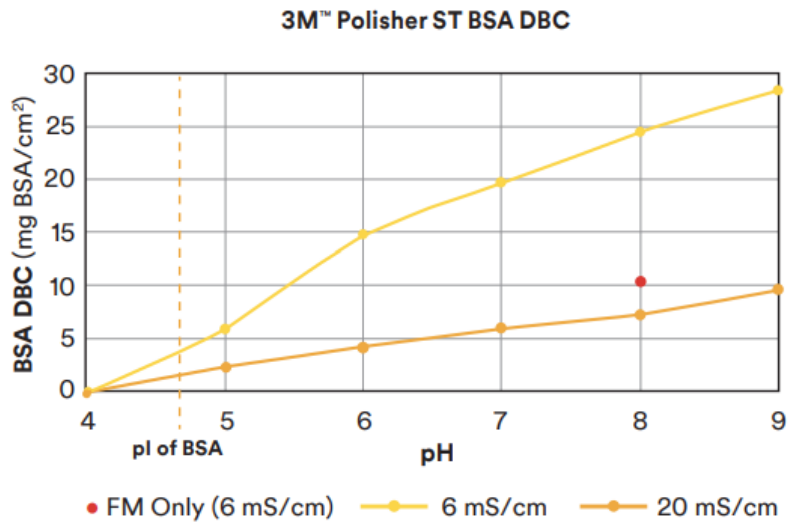


Figure 13. Dynamic binding capacity for BSA (bovine serum albumin) – effects of pH and conductivity. High salt (20 mS/cm) reduces the capacity of the membrane (3M™ Polisher ST Data Sheet)

The final volume of the feed (purified antibody solution) before starting the polishing step was 1385 mL. The feed media was kept in a 4°C room to prevent the growth of potential contaminants. The final pH and conductivity values were 5 mS/cm and 8, respectively. During the process, 24 fractions of the purified media were taken from the system to be analyzed for HCPs and DNAs (using the methods described in **Chapter 3.2.3).** The volume of each fraction was 2.5 mL.

4. RESULTS AND DISCUSSION

4.1. Hollow fiber screening (critical flux experiment)

Hollow fiber membranes were used as both 1) a cell retention device during the perfusion process and 2) a device for concentrating the precipitated antibody. The first step of the project was to screen the hollow fiber membranes for the critical flux by adjusting the permeate flow (while keeping the retentate flow constant) and observing at which flow rate irreversible fouling (clogging) occurs, i. e. when the transmembrane pressure rises sharply. Four membranes were tested: Cytiva, Repligen, and two Microza membranes (the number next to the name corresponds to the pore size in μm). Since the same shear rate was applied to each membrane, similar results are expected as well (detailed discussion in **Chapter 3.2.2.**). The results are shown in Figure 14.

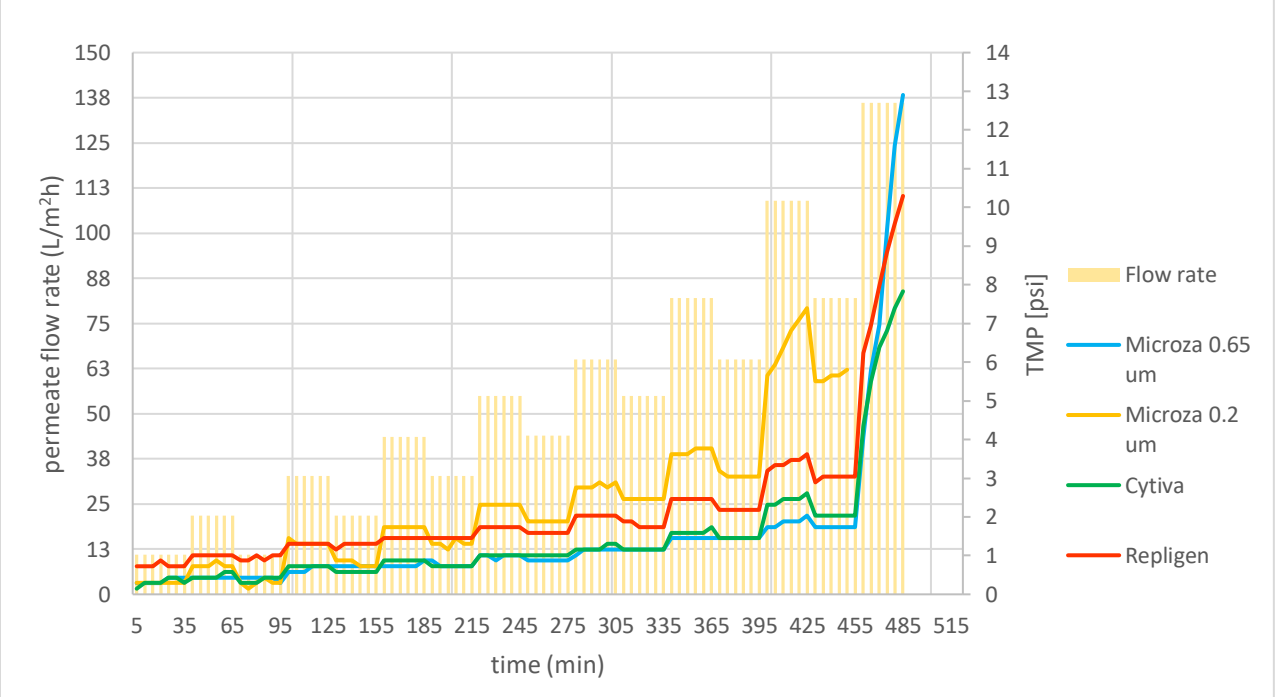


Figure 14. Critical permeate flow rates at which fouling (transmembrane pressure spike) occurred, shown for all four membranes

It is clearly visible that all the hollow fibers (Microza 0.65 μm, Microza 0.2 μm, Cytiva, and Repligen) have shown similar characteristics when it comes to changes in transmembrane pressure – all four TMP spikes occurred when applying the permeate pressure of around 109 LMH. A small spike was seen on the step before that as well, but it was low enough to keep the process going, especially because the membranes are tolerant of pressure increases up to 30 psi. However, a

significantly sharper spike was seen on the next step. At this point the process was stopped to avoid any potential damage to the system. The threshold for stopping the process is arbitrary and represents a sharp spike equivalent to about 60 Pa/min, or around 90 kPa/day. If the TMP continued to increase linearly at the same rate (which is not true, but for the purpose of the experiment it was taken into consideration in such a way), that would mean it would rise to about 90 kPa after a day of operating at those conditions. The hollow fiber TMP limit was around 100 kPa (taken from the hollow fiber catalogs), which is why that value was chosen as an arbitrary threshold which represents the point at which the experiment should be stopped. Finally, the entire experiment was safely conducted because it lasted only about 10 hours for each membrane, so it's safe to say that the risk of going over the pressure limit was non-existent.

The pressure spike for the Microza 0.2 μm membrane at 109 LMH is a bit sharper, indicating that fouling can occur at an earlier point compared to the other hollow fibers. This could be because the geometric parameters are way different: Microza 0.2 μm is the membrane with the highest fiber ID (internal diameter) of 1.4 mm, and the only membrane with the membrane area of 200 cm^2. As a consequence, applying the same shear rate of 1600 s^{-1} means significantly increasing the retentate flow rate compared to other membranes (~550 mL/min, compared to other flow rates which are all below 350 mL/min). This may lead to formation of bubbles, an undesirable occurrence in the system.

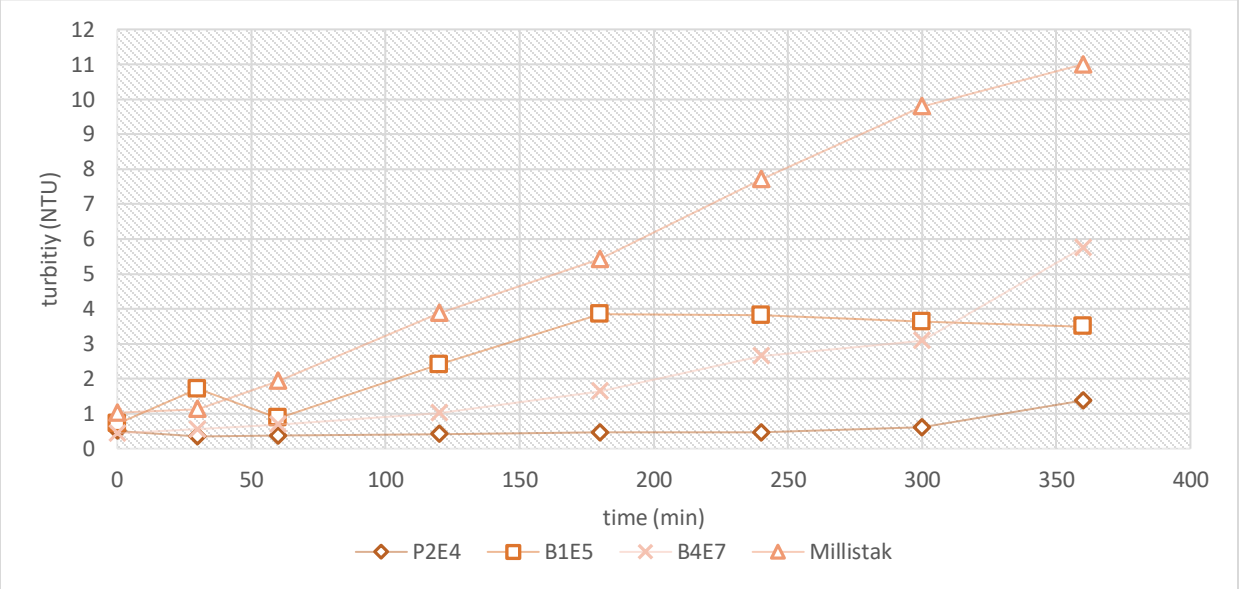
The values for all the membranes are relatively the same. What needs to be considered are the actual operating parameters – again, the applied shear rate was the same when calculated for the specific hollow fiber geometry, so similar critical flux values are expected. The downstream equipment needs to be screened and tested for the desired operating parameters before its utilization the process – the correct prediction of the behavior of the processing equipment is not possible simply based on smaller scale experiments, before testing the equipment for the desired operating parameters (such as flow rate, pressure, operating time etc.). A preliminary experiment should always be conducted beforehand.

4.2. Depth filter screening

Depth filters were screened in a similar way to membranes, with the only difference being that the feed flow rate was kept constant throughout the process, and no changes were applied. The same volumetric loading was applied to all four depth filters, which means the process lasted for 360

minutes (at 1 mL/min) and 70 minutes (at 5 mL/min). During each process, turbidity and inlet pressure values were measured at regular intervals: every 70 minutes and every 10 minutes, respectively.

a)



b)

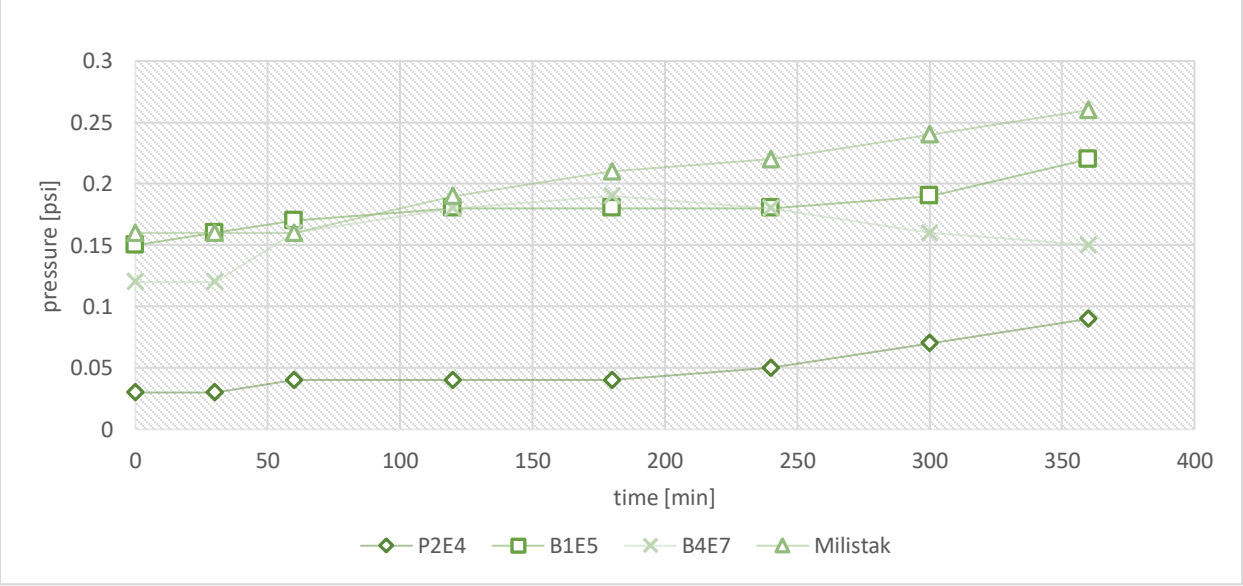
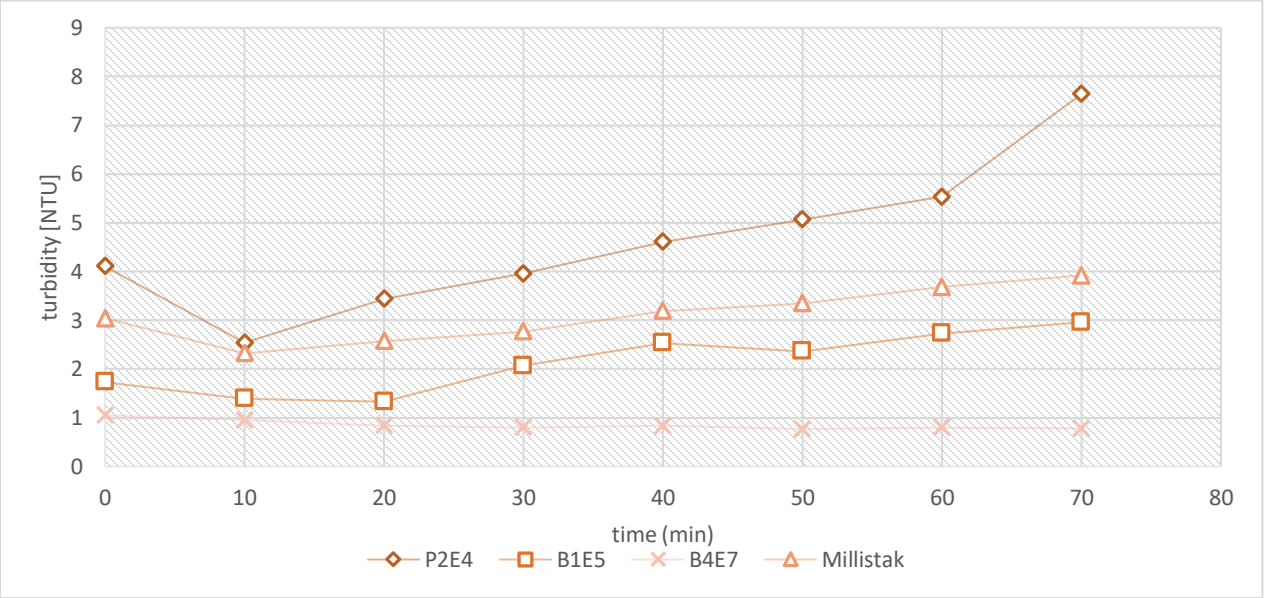


Figure 15. a) Turbidity and b) inlet pressure changes for each depth filter at a flow rate of 1 mL/min

The depth filter that can be deemed the most suitable for the process that was carried out is the one which shows consistent and reliable results (in this case, steady low pressure and turbidity values), without any abrupt changes occurring in the system results. In this case, inlet pressure and turbidity were continuously measured. The quality of the filter is determined by whether these values are kept at a relatively low level throughout the process. From the data shown in Figure 15, it is clear that the P2E4 filter was performing better at lower inlet flow rates (1 mL/min) than the other ones. Additionally, ErtelAlsop filters were more efficient in keeping the turbidity low than the Milistak filter, where the turbidity of the outlet was steadily increasing, although not significantly. From Figure 15. a) it is also clearly visible that the Milistak filter performs similarly to the other ones, but that ErtelAlsop depth filters are superior when it comes to longevity of the process, since the outlet turbidity measured later in the process is way lower for those depth filters.

a)



b)

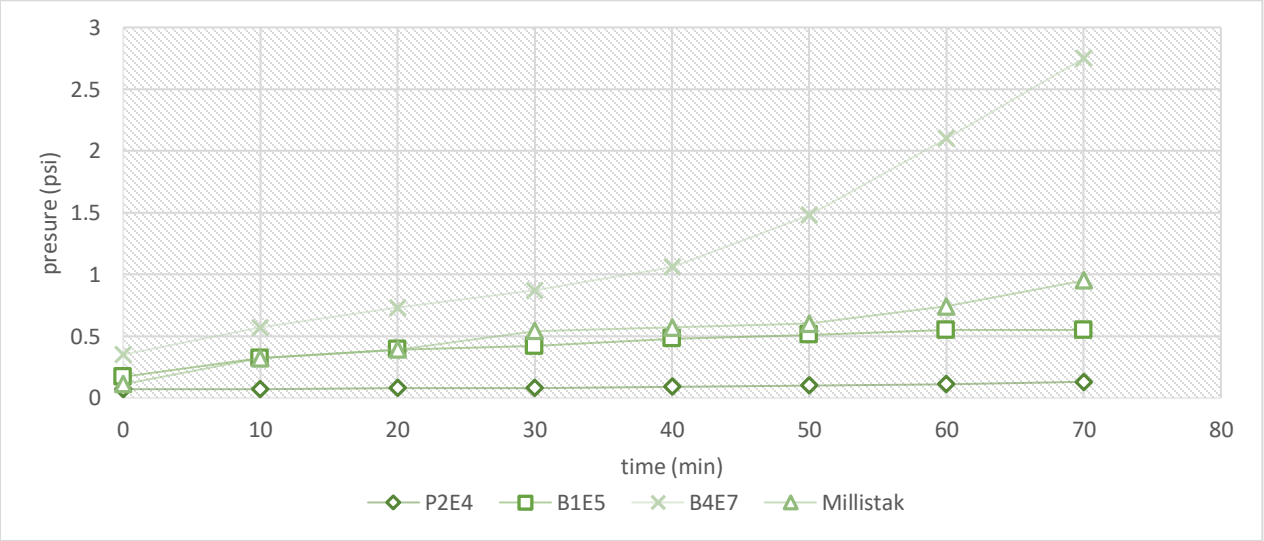


Figure 16. a) Turbidity and b) inlet pressure changes for each depth filter at a flow rate of 5 mL/min

To further confirm the results (and, additionally, to provide information on a possible scale-up experiment), the same experiment was conducted with a higher flow rate (5 mL/min). Here, ErtelAlsop B4E7 stood out as the filter that showed different characteristics than the other filters. When it comes to the turbidity measurements, it is apparent that B4E7 reduces the turbidity of the

solution in the best way. The higher pressure values are therefore to be expected – with more impurities caught by the filter, the inlet pressure will increase. Since this filter can sustain pressure values up to 36 psi, this was no issue. The potential issues could come up from either using the filter for too long or applying a significantly higher inlet flow rate. All the other filters showed a better product longevity (they could be used for a longer time), but the turbidity was several times higher at the end of the process (Figure 16. a)). Therefore, ErtelAlsop B4E7 was used in the next step: the sequential downstream process.

4.3. Sequential downstream process

The efficiency of the downstream process was evaluated by measuring the HCP/DNA concentration at each important step, as well as tracking the concentration of trastuzumab throughout the entire process to see where majority of the losses occurred. The moments where DNA and HCP content was measured include before the beginning of the sequential process (initial sample) and after the sequential process was finished. Naturally, the quantity of the antibody was adjusted for the total volume of supernatant used in the process, giving the mass of the antibody as the result. The overall yield was then determined by simply dividing the final mass with the initial mass of the antibody. The results for HCP and DNA content are shown in Figure 17.

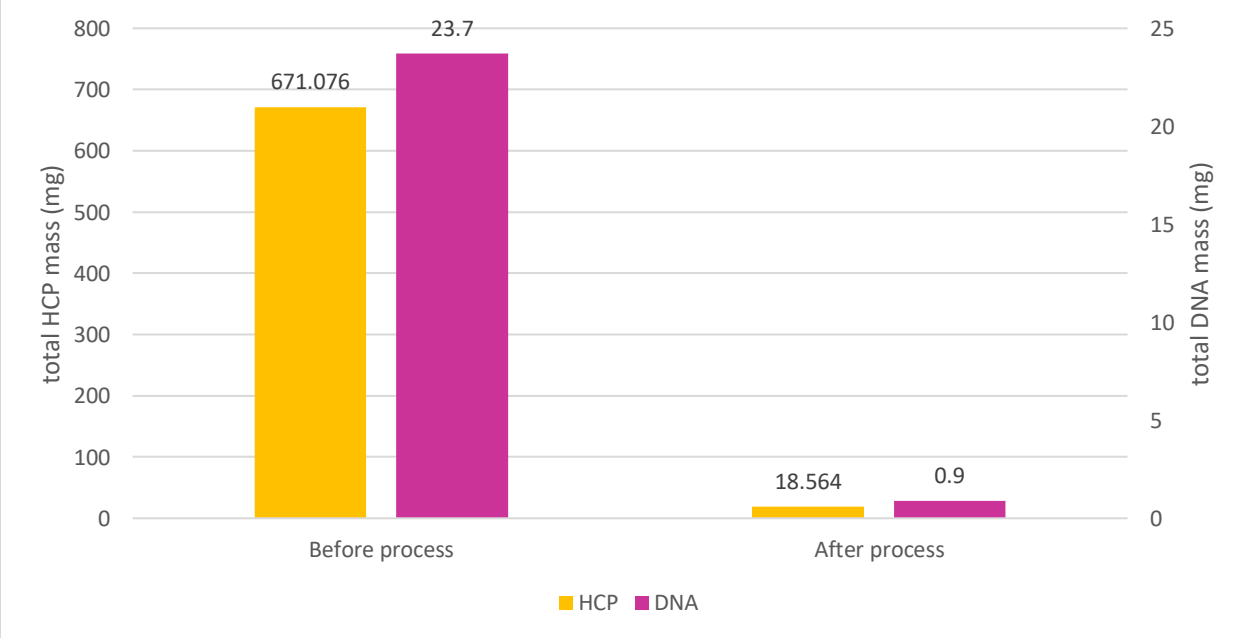


Figure 17. HCP and DNA content in the sequential downstream process

The graphs for both HCP and DNA content show similar results. It is clear that most of these impurities were removed at the final step, after the concentration and redissolution step. It is safe to say that the membrane concentration/redissolution of the antibodies was a success, with PEG predominantly precipitating antibodies, while HCPs and other LMWI were filtered through the membrane. Some HCPs and DNA were lost between the initial step and the final depth filtration, which can also be attributed to the denaturation of some of these molecules. However, most of the DNA and HCPs molecules were removed from the system - after the entire sequential downstream process was concluded, 97.72% of HCP and 96.20% of DNA were successfully removed. The small amount of DNA and HCPs that remained in the system was thought to be co-precipitated along with the antibody of interest.

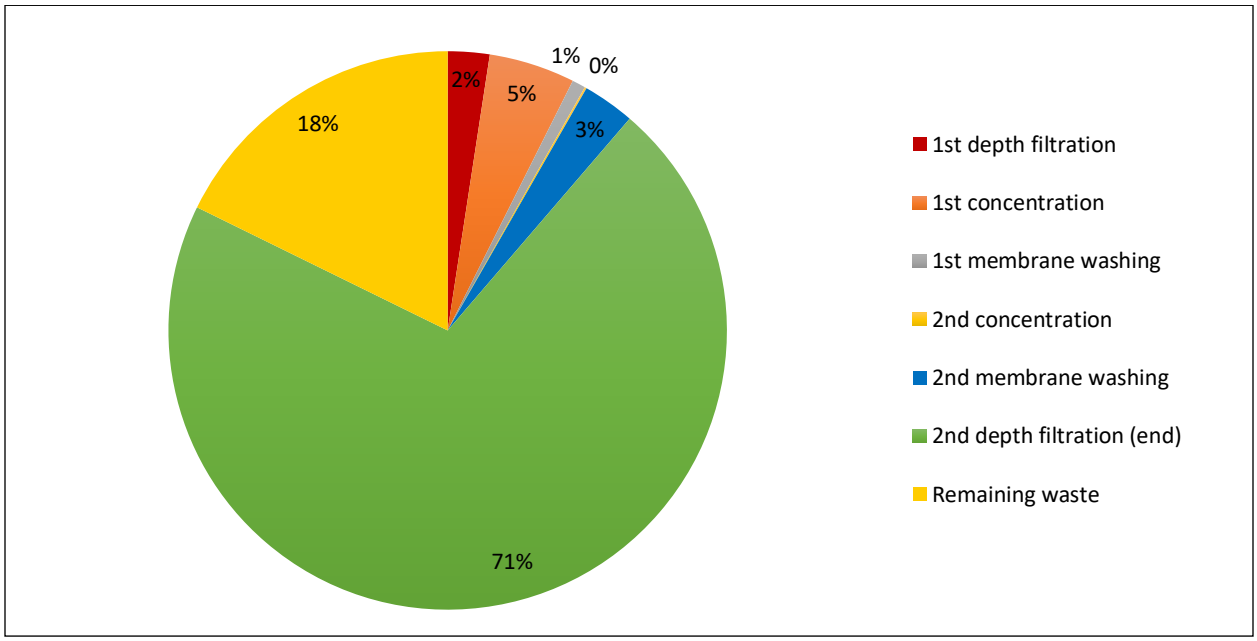


Figure 18. Trastuzumab losses during the sequential downstream process

The final yield of the sequential downstream process was 71%, meaning 71% of the initial antibody mass was successfully obtained in the final volume. At each step of the process, small amounts of the antibody were lost, however, that is not where most of the antibodies were lost. When the antibody masses from all steps were summed up and compared to the initial mass of 1.547 g, 18% of antibodies were still "missing". This amount is labeled as "remaining waste" in Figure 18. This means that, after all the calculations and measurements, a significant portion of the proteins were lost either inside the purification equipment (hollow fibers/filters/tubes) or they passed through the

membrane filter. A significant loss due to washing of the precipitated mAb was also reported by Li et al. (2019), where the mAb yield decreased from 98% in the precipitation step to 82% in the washing. This could be attributed to the resolubilization of some of the precipitated protein after adding the fresh buffer (Li et al., 2019). The buffer that was used in the study, however, did not contain PEG. It is unlikely that many aggregates formed during this process. Kuczewski et al. (2011) reported no formation of aggregates when precipitating antibodies with PEG and applying microfiltrational TFF for the washing of the antibody. Finally, a significant loss of mAbs due to a high concentration of PEG used for precipitation was also reported in the study by Hammerschmidt et al. (2016), which may have played a role during the sequential process of this experiment as well. Nevertheless, a yield of 71% was somewhat expected for a first-time experiment, especially because large amounts of volumes were used (where the likelihood of errors is higher). The yield is high enough for the experiment to be considered a good starting point for other research projects when it comes to novel downstream techniques and methods for monoclonal antibodies. A reasonable mass of antibodies (about 6% in total) was lost in the two concentration steps, one after the first depth filtration, and one after the second. This was determined by measuring the antibody concentration of the fresh buffer which was used to flush the membranes after the concentration process. Therefore, it can be concluded that a lot of proteins remain in the membrane even after it's emptied out – here, the application of lower recirculation flow rates and the use of different hollow fibers might have resulted in obtaining more antibodies. The poor influence of bubbles on antibody concentration was observed, which is the result of applying higher recirculation flow rates (~350 mL/min). Lower recirculation flow rates might have led to a more favorable hydrodynamic environment in the system with less bubbles. Factors like light exposure and agitation stress due to high flow recirculation flow rates could also be the reason that some of the antibodies were degraded.

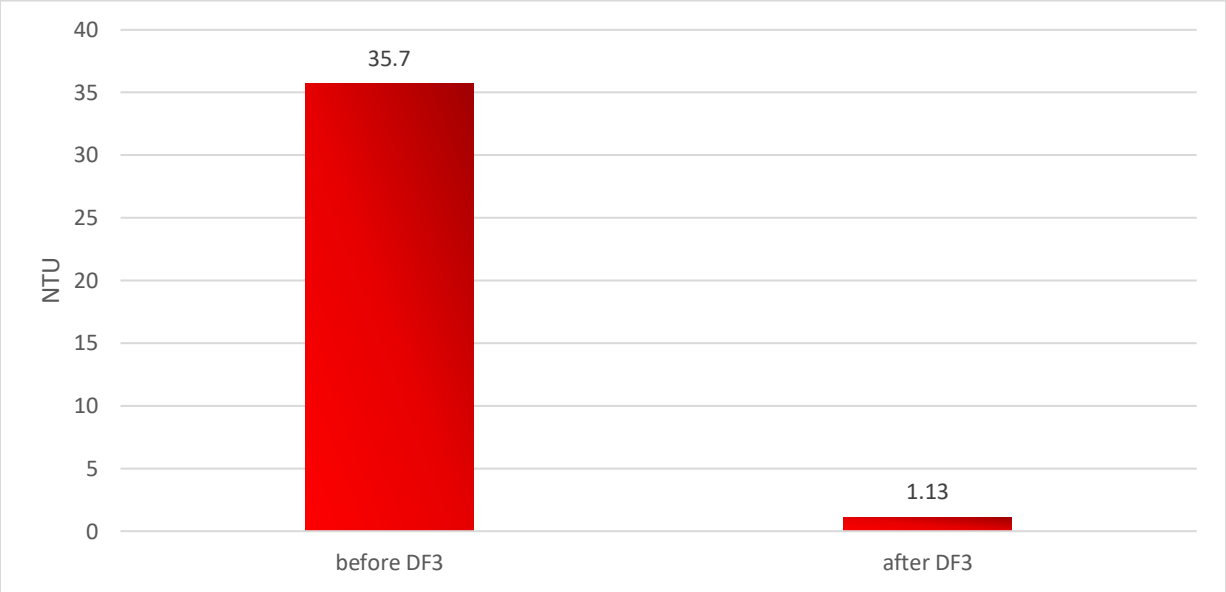
Different approaches to the redissolution phase could also be considered: especially the redissolution pH. Li et al. performed a similar continuous precipitation technique, using $ZnCl_2$ and PEG for the precipitation of impurities and mAb, respectively (2019). In this study, the precipitated protein was resolubilized at low pH (3.2), which disrupted the crosslinking by the Zn^{2+}. This resulted in nearly complete dissolution of the precipitated protein. Compared to this study, where a phosphate buffer of pH 7.0 was applied before the last depth filtration step, this might provide valuable information on how to achieve a higher mAb yield than 71% in the sequential process.

The study also included a depth filtration after the redissolution step to remove any insoluble material, meaning that any undissolved mAb before the depth filtration will be removed as well.

4.4. Polishing process

As discussed in **Chapter 3.2.4.**, the depth filtration (again with the B4E7 filter) was performed in order to reduce the turbidity of the final volume (about 1L of antibody solution) before the anionic exchange step. The final depth filtration of the sequential process (previous step) was carried out at a higher flux of 5 mL/min, so a breakthrough of impurities might have occurred at some point during the filtration step. Figure 19 shows the total amount of turbidity reduced, as well as the reduction of HCP/DNA content.

a)



b)

Figure 19. Turbidity (a), HCP and DNA (b) measurements before and after the final depth filtration

Reducing turbidity and bringing the concentration of impurities down to a minimum is necessary before the ionic exchange (polishing) step. These impurities are not desirable in the solution as they can interfere with the ionic interactions of other negatively charged impurities we want to remove from the solution, such as HCPs and DNA, and the ionic exchange membrane. This step was needed again because the previous depth filtration might have resulted in some errors (a breakthrough of components might have occurred). The performed filtration did not differ from the previous one (the same flow rate was applied, and the same depth filters were used), but it was still efficient in removing a significant amount of DNA content, as well a small number of residual HCPs. Two things are observed here:

1. HCPs are purified in a different way than DNA/high molecular weight impurities (HMWI), and the same conditions can not be applied for the simultaneous removal of both components. For significant HCP reduction, a method suitable for LMWI reduction needs to be applied (reported by Sommer et al., 2015).
2. A ~10-fold decrease in DNA content during the 3rd depth filtration indicates that there was a breakthrough of components at the end of the sequential process during the 2nd depth filtration step, and that not all DNA content was filtered during this step. Supplementary information to support this breakthrough is the high turbidity of ~35 NTU at the end of the sequential process, which is highly probable to be caused by the residual PEG, DNA, or other impurities that broke through the filter. The importance of a robust and reliable filtration method for the purification of monoclonal antibodies is crucial, as each further step increases the capital cost, operation time, and system complexity, which could result in a lower overall antibody yield.

The antibody concentration for each fraction is shown in Figure 20. As it was described in **Chapter 3.2.4.**, these fractions of 2 mL were taken during the ion exchange process (around every two minutes). The total volume of antibody solution treated with the ion exchange method was 680 mL. After the method, 550 mL of clear solution was obtained. In total, 25 samples were obtained (100 mL total volume).

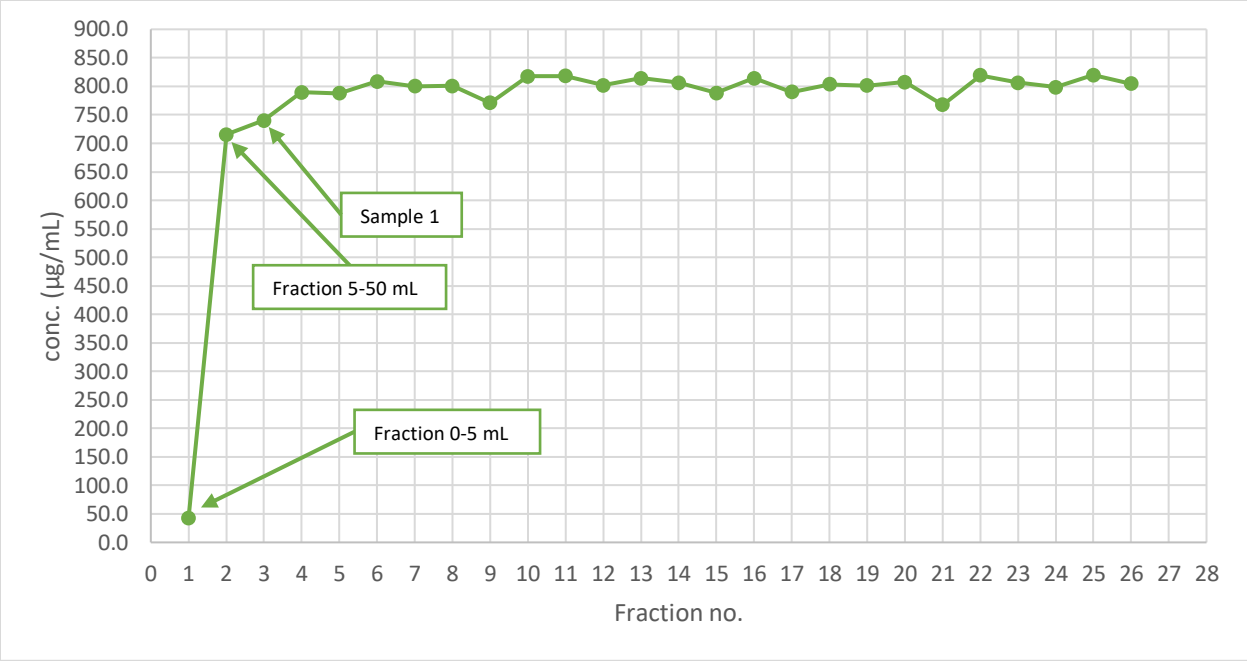


Figure 20. Trastuzumab concentration for each fraction

It is assumed that the lower concentration of antibodies in the initial fractions is because of the dead volume of the system remaining in the device before use (the device needs some time to flush the buffer out, after which the sample reaches the membrane adsorber). The antibody concentration stayed relatively constant throughout the entirety of the process – the small errors can be attributed to the differences in HPLC measurements and results. Since Sample 1 represents the first sample taken after the solution has already entered the system, this has been considered the initial sample for HCP/DNA analysis. The results for HCP/DNA analysis are shown in Figure 21.

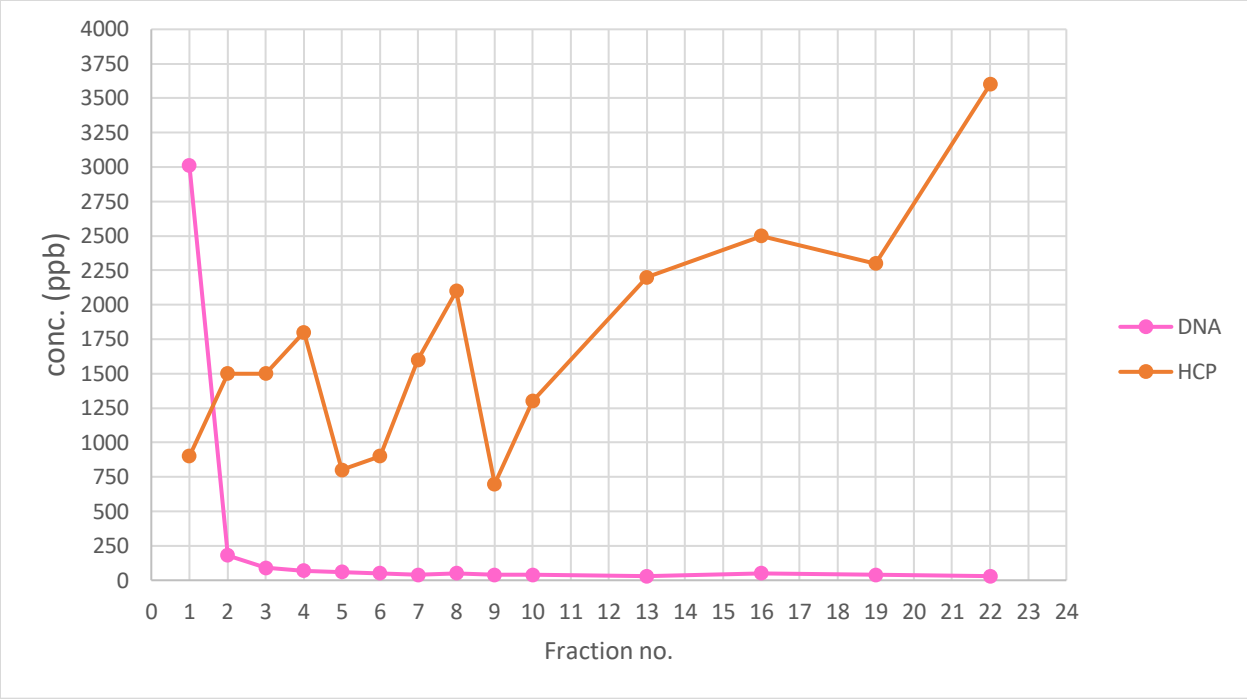


Figure 21. HCP/DNA concentration for each fraction

The two lines representing DNA and HCP concentration show quite different results – over time, the concentration of DNA in the collected samples (and, therefore, in the solution) stayed relatively low, which means the anionic exchange membrane successfully purified the sample solution from most of the DNA impurities. However, the measurements also indicate an inconsistency of HCP content, with it slowly increasing in the second half of the process. By comparing the results before and after the polishing step (displayed in Figure 22), it is shown that both the DNA and HCP content is reduced by about 7.7-fold, meaning the increased concentration of HCPs towards the end of the process does not mean that the membrane binds DNA preferentially to HCPs, since the HCP content is much higher in the initial sample (over 150 higher when comparing the mass amount). It does, however, indicate that the membrane is almost saturated, since less HCPs are binding to the membrane, and that the process should be stopped.

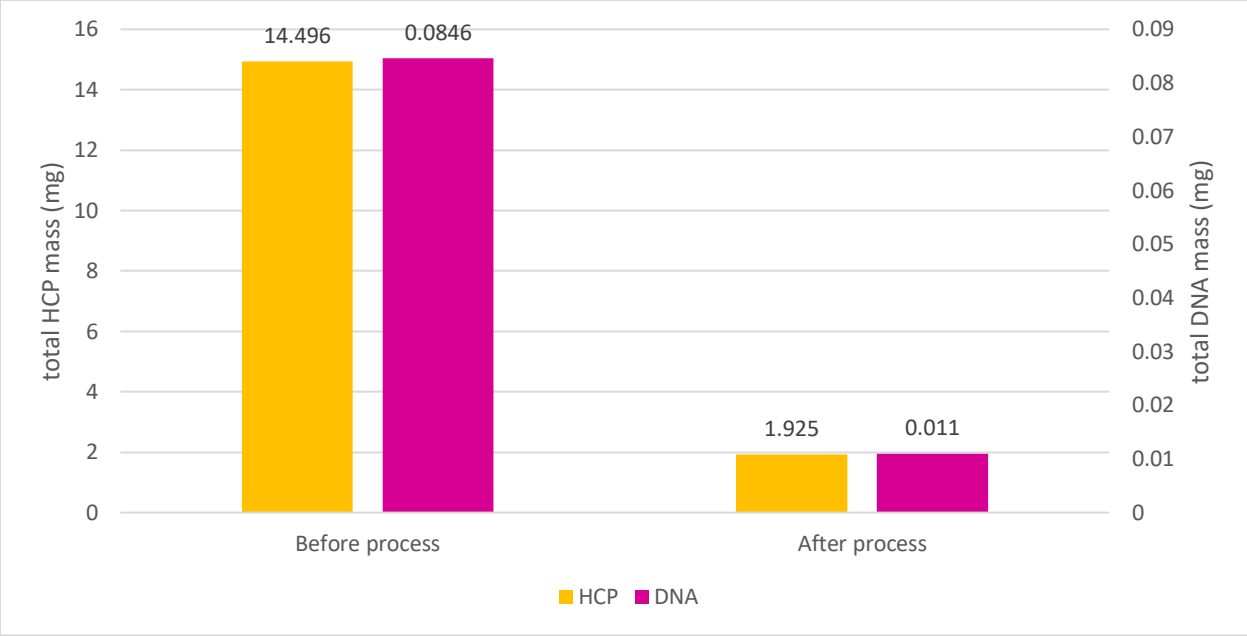


Figure 22. HCP and DNA measurements before and after the polishing step

At the end of the polishing step (and, for that matter, the entire monoclonal antibody purification experiment), 1.925 mg of HCPs and 0.011 mg of DNA were present in the purified antibody solution. Other methods could be used in this step to improve the selectivity of anion-exchange. Previously, PEG precipitation of antibodies was discussed - however, PEG has also been shown to alter the retention behavior of proteins during ion-exchange chromatography (Gagnon et al., 1996). This should only be a consideration, however, as the study also points out various flaws with the addition of PEG to alter selectivity, like elevated viscosity (which resulted in a reduced flow rate) and depressed dynamic binding capacity for smaller proteins (even though it appeared to be maintained/increased for larger ones).

4.5. Determining the final yield and purification efficiency

The final yield of the entire process (sequential purification + polishing step) can be determined by simply dividing the initial antibody mass from the cell culture supernatant with the antibody mass. Naturally, the same can be applied to calculate how much DNA/HCP content has been purified from the sample and to determine purification efficiency. Calculations for purification efficiency are shown in Table 3.

Table 3. Final purification efficiency

sample	DNA mass (mg)	HCP mass (mg)	Antibody mass (µg)
initial (cell culture supernatant)	**16.124**	**456.331**	**1547.08**
final (after polishing)	**0.011**	**1.925**	**1033.024**
yield (% mass removed/obtained)	**99.93 (removed)**	**99.58 (removed)**	**66.77 (obtained)**

It needs to be mentioned that some of the volume was not purified – the polishing step was stopped when the inlet pressure exceeded the recommended one (the data was available in the 3M brochure). From the starting feed volume of 1385 mL, only 550 mL of completely purified product was obtained, with 680 mL passing through the system in total (the remaining volume was contained in the ÄKTA fractions). Therefore, the yield calculation shown in Table 3 was adjusted based on the actual loaded volume of 680 mL, instead of the entire volume of antibody solution. It is to be expected that more of this feed volume could be purified for DNA: however, if the polishing step was continued further, there is a high probability that more host cell proteins (and potentially some other impurities which do not have a very high affinity for the ionic exchange membrane) could be found in the final product. If purification was to be continued further, a used anionic exchanger would have needed to be exchanged with a new one. In the end, about 66.77% of trastuzumab from the starting supernatant cell culture was successfully obtained after the polishing. Comparing it to the yield of around 71% after the sequential process, it can be concluded that about 4% of the antibodies were lost during this polishing experiment. Here, careful consideration of the bioprocess parameters when operating with the ÄKTA device is crucial to achieve as high a yield as possible – predominantly sample conductivity, which can be optimized depending on the process. The biggest improvement to the process can be done in the initial steps – since the experiment required the operation with large volumes, some methods (like centrifugation) were not possible for precipitation steps. What's more, during centrifugation, the precipitate flocs are compacted to a dense, paste-like mass, whose resolubilization is an extremely slow process (Hammerschmidt et al., 2016). As the resolubilization of the precipitate was crucial here because the precipitate is not being discarded (since it contains the product), centrifugation is

not a viable option here, which is why it was replaced by concentration of the precipitate with hollow fibers. Hammerschmidt et al. (2016) precipitated DNA and impurities at a low pH in an experiment similar to this one, where recombinant antibodies were precipitated using PEG, and TFF was used as an alternative precipitate capture step. A significant reduction in soluble DNA concentration was reported by pH reduction alone (most efficient at pH 4.0 - 4.5). This low-pH step could be introduced to the process to enhance DNA removal, which might lead to a higher antibody yield. As the isoelectric point of trastuzumab antibodies is much higher (above 8.0), a low-pH step would increase its solubility and precipitate the impurities in a more effective way. Additionally, operating parameters such as PEG buffer concentration, retentate flow rate during concentration step or depth filtration could be altered to achieve maximum product yield. Other hollow fibers could also be applied – differences in their structure are the most likely causes for different behaviors (Hammerschmidt et al., 2016). Nevertheless, hollow fibers with a 0.22 μm pore size are still recommended here, since it was also used in other experiments where the precipitated antibody was recovered by applying a membrane concentration process (Kuczewski et al., 2011; Li et al., 2019). When considering other hollow fibers, parameters like fiber number and filtration area could be altered. Lastly, ensuring maximum removal of HMWI before moving on to the antibody precipitation step is crucial. Large impurities usually co-precipitate with the target protein, as the PEG separation is size-based, which was already reported by Sim et al. (2011). It is also possible that the co-precipitation of large impurities and antibodies took part in this experiment, at least to some extent, resulting in a decreased yield.

Some impurities that may be present in the final volume of antibody solution were small aggregates (although unlikely, as explained previously) and viruses whose presence was not assessed. It nevertheless represents a crucial part of any purification experiment. When it comes to leached protein A molecules, it is safe to say that there aren't any in the final volume, since no protein A affinity column was used to purify the sample (a well-known protein purification step in the industry). A small protein A column was used only for the detection and quantification of antibodies during HPLC analysis. In fact, studies similar to this one, which test ways to purify monoclonal antibodies using alternative ways such as precipitation, are conducted exactly for the purpose of finding a decent substitute to the well-known protein A chromatography method. Larger chromatography columns are necessary due to increased cell culture titers that are

continuously being reported in recent years, and with it, new challenges arise – mainly cost constraints, large buffer volumes required and long processing times (Giese et al., 2013).

Since the entire purification experiment consisted of a lot of steps, it might be confusing go through it step by step, without having a general overview of the whole process. Therefore, an entire scheme of the process is represented in Figure 23, to sum up the entirety of work done for the purpose of this master thesis.

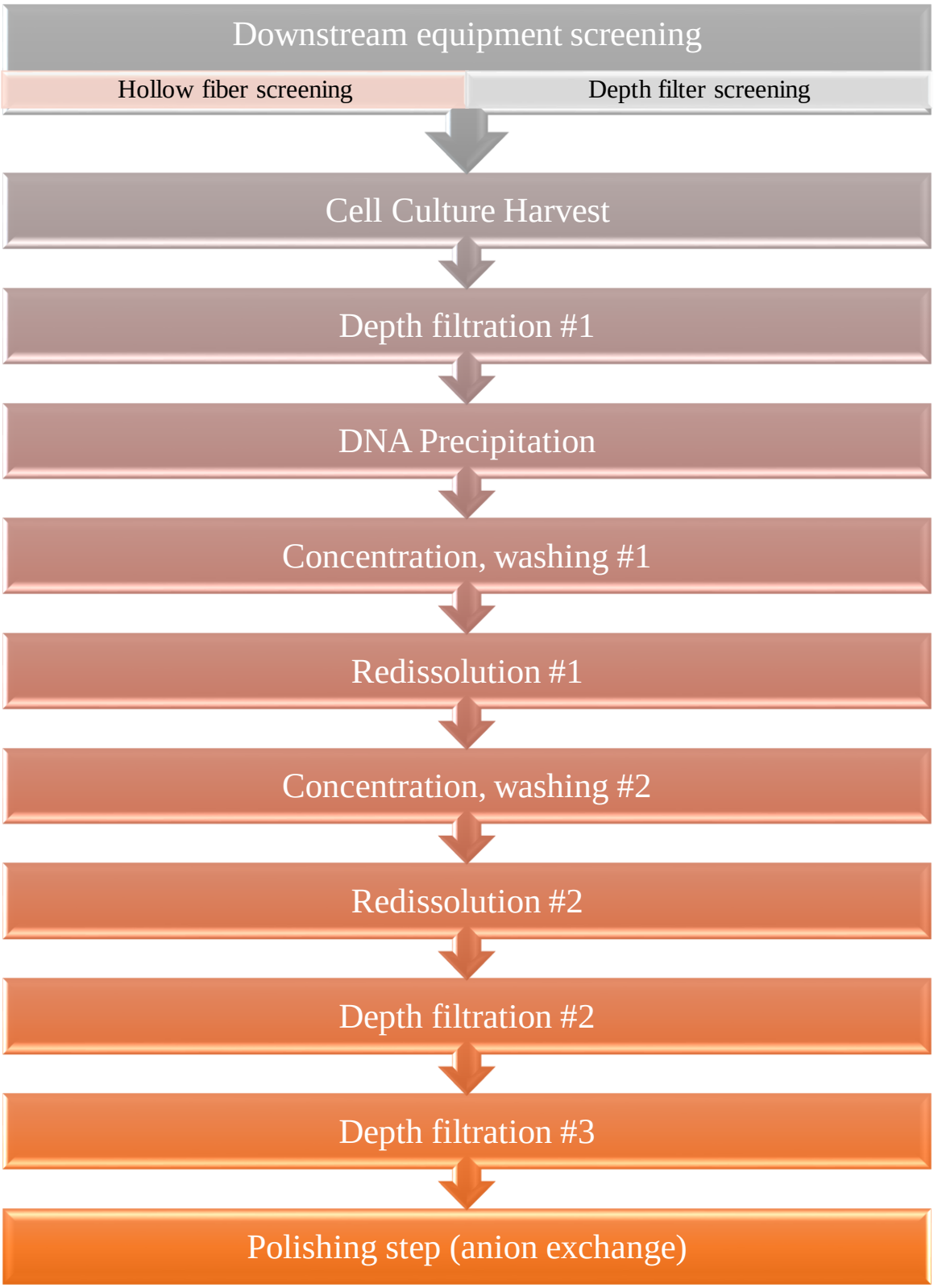


Figure 23. Flow diagram of the entire master thesis experiment

5. CONCLUSIONS

1. The geometry of hollow fiber membranes does not have an impact on the final critical flux achieved, as long as the hollow fiber operate under the same conditions (shear rate).

2. The choice of the optimal membrane for any given bioprocess mostly comes down to the capacity of the bioprocess (the volume that needs to be processed) and capital expenditures (how much money can be spent on cleaning or replacing used membranes).

3. Efficiency and longevity of depth filters in downstream processing depend on filter pore size and inlet flow rates.

4. B4E7 filters can successfully reduce the turbidity of antibody solution and hold most of the impurities over a longer period (a few hours), without any sudden pressure increases. They were effective in DNA purification, reducing the amount of DNA by more than 99.5% – however, three filters needed to be used to achieve maximum removal.

5. PEG precipitation combined with $CaCl_2$ was shown to be an effective way of removing most of the impurities from the cell culture supernatant, particularly DNA and HCPs.

6. Anionic exchange polishing step can successfully remove any residual DNA impurities from the antibody solution, as well as residual host cell proteins, which are mostly negatively charged. During this step (performed with a 3M™ Polisher ST membrane adsorber), the DNA and HCP content were both reduced by about 7.7-fold.

7. The trastuzumab yield of 66.77% reached after the polishing steps shows that the applied downstream procedure has the potential to be developed into a regular monoclonal antibody purification approach, but that there is much room for improvement as well, particularly in the precipitation step, to achieve higher antibody yields.

6. LITERATURE

ATCC (2022) ATCC: The Global Bioresource Center, https://www.atcc.org/products/ccl-61 Accessed on 10th of January 2022.

Bielser JM, Wolf M, Souquet J, Broly H, Morbidelli M (2018) Perfusion mammalian cell culture for recombinant protein manufacturing – A critical review. *Biotechnol Adv* **36**, 1328 – 1340. https://doi.org/10.1016/j.biotechadv.2018.04.011

Brooks CA, Cramer SM (1992) Steric mass-action ion exchange: Displacement profiles and induced salt gradients. *AIChE Journal* **38**, 1969 – 1978. https://doi.org/10.1002/aic.690381212

Butler M (2005) Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. *Appl Microbiol Biotechnol* **68**, 283–291. https://doi.org/10.1007/s00253-005-1980-8

Chaturvedi A (2022) Investigation of process conditions and chemical modulators on CHO-cell based trastuzumab production. https://doi.org/doi:10.7282/t3-r4fh-fd45

Chopda V, Gyorgypal A, Yang O, Singh R, Ramachandran R, Zhang H, i sur. (2021) Recent advances in integrated process analytical techniques, modeling, and control strategies to enable continuous biomanufacturing of monoclonal antibodies. *J Chem Technol Biotechnol* **97**, 2317 – 2335. https://doi.org/10.1002/jctb.6765

Clincke M - F, Mölleryd C, Zhang Y, Lindskog E, Walsh K, Chotteau V (2011) Study of a recombinant CHO cell line producing a monoclonal antibody by ATF or TFF external filter perfusion in a WAVE Bioreactor™. *BMC Proc* **5**. https://doi.org/10.1186/1753-6561-5-S8-P105

Clincke M, Mölleryd C, Zhang Y, Lindskog E, Walsh K, Chotteau V (2013) Very high density of CHO cells in perfusion by ATF or TFF in WAVE bioreactor™. Part I. Effect of the cell density on the process. *Biotechnol Prog* **29**, 754 – 767. https://doi.org/10.1186/1753-6561-5-s8-p105

Coffman J, Brower M, Connell-Crowley L, Deldari S, Farid SS, Horowski B, i sur. (2021) A common framework for integrated and continuous biomanufacturing. *Biotechnol Bioeng* **118**, 1735 – 1749. https://doi.org/10.1002/bit.27690

Cole H, Demont A, Marison I (2015) The Application of Dielectric Spectroscopy and Biocalorimetry for the Monitoring of Biomass in Immobilized Mammalian Cell Cultures. *Processes* **3**, 384 – 405. https://doi.org/10.3390/pr3020384

Cytiva Life Sciences. How to determine dynamic binding capacity of chromatography resins. https://www.cytivalifesciences.com/en/us/news-center/how-to-determine-dynamic-binding-capacity-10001 Accessed on 8th of January 2023.

Fahrner RL, Knudsen HL, Basey CD, Galan W, Feuerhelm D, Vanderlaan M, i sur. (2001) Industrial Purification of Pharmaceutical Antibodies: Development, Operation, and Validation of Chromatography Processes. *Biotechnol Genet Eng Rev* **18**, 301 – 327. https://doi.org/10.1080/02648725.2001.10648017

Fekete S, Beck A, Veuthey J - L, Guillarme D (2015) Ion-exchange chromatography for the characterization of biopharmaceuticals. *J Pharm Biomed* **113**, 43 – 55. https://doi.org/10.1016/j.jpba.2015.02.037

Gagnon ' P, Godfrey B, Ladd D (1996) Method for obtaining unique selectivities in ion-exchange chromatography by addition of organic polymers to the mobile phase a~. J *Chromatogr A* **743**, 51 – 55**.** https://doi.org/10.1016/0021-9673(96)00125-2

Giese G, Myrold A, Gorrell J, Persson J (2013) Purification of antibodies by precipitating impurities using Polyethylene Glycol to enable a two chromatography step process. *J Chromatogr B* **938**, 14 – 21. https://doi.org/10.1016/j.jchromb.2013.08.029

Gjoka X, Gantier R, Schofield M (2017) Transfer of a three step mAb chromatography process from batch to continuous: Optimizing productivity to minimize consumable requirements. *J Biotechnol* **242**, 11–18. https://doi.org/10.1016/j.jbiotec.2016.12.005

Gottschalk U (2005) Downstream processing of monoclonal antibodies: from high dilution to high purity. *Biopharm Int* **18**(**6**), 42 - 58.

Gunawan F, Nishihara J, Liu P, Sandoval W, Vanderlaan M, Zhang H, i sur. (2017) Comparison of platform host cell protein ELISA to process-specific host cell protein ELISA. *Biotechnol Bioeng* **115**, 382 – 389. https://doi.org/10.1002/bit.26466

Hadpe SR, Mohite V, Alva S, Rathore AS (2020) Pretreatments for enhancing clarification efficiency of depth filtration during production of monoclonal antibody therapeutics. *Biotechnology Progress* **36** https://doi.org/10.1002/btpr.2996

Hammerschmidt N, Hobiger S, Jungbauer A (2016) Continuous polyethylene glycol precipitation of recombinant antibodies: Sequential precipitation and resolubilization. *Process Biochemistry* **51**, 325 – 33. https://doi.org/10.1016/j.procbio.2015.11.032

Harinarayan C, Mueller J, Ljunglöf A, Fahrner R, Van Alstine J, van Reis R (2006) An exclusion mechanism in ion exchange chromatography. *Biotechnol Bioeng* **95**, 775 – 787. https://doi.org/10.1002/bit.21080

Hober S, Nord K, Linhult M (2007) Protein A chromatography for antibody purification. *J of Chromatogr B* **848**, 40–47. https://doi.org/10.1016/j.jchromb.2006.09.030

Kelley B, Kiss R, Laird M (2018) A Different Perspective: How Much Innovation Is Really Needed for Monoclonal Antibody Production Using Mammalian Cell Technology? U: Kiss B, Gottschalk U, Pohlscheidt M (ured.) New Bioprocessing Strategies: Development and Manufacturing of Recombinant Antibodies and Proteins. Advances in Biochemical Engineering/Biotechnology, vol 165, Springer, Cham 443 – 462. https://doi.org/10.1007/10_2018_59

Kuczewski M, Schirmer E, Lain B, Zarbis-Papastoitsis G (2010) A single-use purification process for the production of a monoclonal antibody produced in a PER.C6 human cell line. *Biotechnol J* **6**, 56 – 65. https://doi.org/10.1002/biot.201000292

Li F, Vijayasankaran N, Shen A (Yijuan), Kiss R, Amanullah A (2010) Cell culture processes for monoclonal antibody production. *mAbs 2*, 466 – 479. https://doi.org/10.4161/mabs.2.5.12720

Li F, X Zhou, J Yang X, Tressel T, Lee B (2006) Current Therapeutic Antibody Production and Process Optimization. *Bioprocess J* **5**, 16 – 25. https://doi.org/10.12665/J54.LiZhou

Li Z, Gu Q, Coffman JL, Przybycien T, Zydney AL (2019) Continuous precipitation for monoclonal antibody capture using countercurrent washing by microfiltration. *Biotechnol Prog* **35** https://doi.org/10.1016/j.jchromb.2009.09.019

Ma Z, Lan Z, Matsuura T, Ramakrishna S (2009) Electrospun polyethersulfone affinity membrane: Membrane preparation and performance evaluation. *J of Chromatogr B* **877** 3686 – 3694. https://doi.org/10.1016/j.jchromb.2009.09.019

Mahboudi R, Samavat S, Afrah A, Khorshidtalab M, Tehran AF, Motahari P, i sur. (2019) Implementation of Design of Experiments (DOE) for Optimization of Feeding Strategy and Glyco-Engineering of Trastuzumab Biosimilar. Cold Spring Harbor Laboratory. https://doi.org/10.1101/584144

Mckeage K, Perry CM, Seshadri R (2002) Trastuzumab A Review of its Use in the Treatment of Metastatic Breast Cancer Overexpressing HER2. *Drugs* **62**, 209 – 243. https://doi.org/10.2165/00003495-200262010-00008

Membrane chromatography. Single-use chromatography solution to purify viral vectors and remove impurities from therapeutic proteins. https://www.pall.com/en/biotech/technologies/chromatography/membrane-chromatography.html Accessed on 24th of January, 2023.

Nadar S, Shooter G, Somasundaram B, Shave E, Baker K, Lua LHL (2021) Intensified Downstream Processing of Monoclonal Antibodies Using Membrane Technology. *Biotechnol J* **16**. https://doi.org/10.1002/biot.202000309

Nejatishahidein N, Zydney AL (2021) Depth filtration in bioprocessing - new opportunities for an old technology. *Curr Opin Chem Eng* **34** https://doi.org/10.1016/j.coche.2021.100746

Orr V, Zhong L, Moo-Young M, Chou CP (2013) Recent advances in bioprocessing application of membrane chromatography. *Biotechnol Adv* **31**, 450 – 465. https://doi.org/10.1016/j.biotechadv.2013.01.007

Pan X, Streefland M, Dalm C, Wijffels RH, Martens DE (2016) Selection of chemically defined media for CHO cell fed-batch culture processes. *Cytotechnology* **69**, 39 – 56. https://doi.org/10.1007/s10616-016-0036-5

Plosker GL, Keam SJ (2006) Trastuzumab. *Drugs* **66**, 449–475. https://doi.org/10.2165/00003495-200666040-00005

Posner J, Barrington P, Brier T, Datta-Mannan A (2019) Monoclonal Antibodies: Past, Present and Future. U: Barrett J, Page C, Michel M (ured.) Concepts and Principles of Pharmacology, Springer, Cham, str. 81 – 141. https://doi.org/10.1007/164_2019_323

Puente-Massaguer E, Badiella L, Gutiérrez-Granados S, Cervera L, Gòdia F (2019) A statistical approach to improve compound screening in cell culture media. *Eng Life Sci* **19**, 315 – 327. https://doi.org/10.1002/elsc.201800168

Ritacco FV, Wu Y, Khetan A (2018) Cell culture media for recombinant protein expression in Chinese hamster ovary (CHO) cells: History, key components, and optimization strategies. *Biotechnol Prog* 34, 1407–1426. https://doi.org/10.1002/btpr.2706

Ryman JT, Meibohm B (2017) Pharmacokinetics of Monoclonal Antibodies. *CPT: Pharmacometrics & Systems Pharmacology* **6**, 576–588. https://doi.org/10.1002/psp4.12224

Shukla AA, Hubbard B, Tressel T, Guhan S, Low D (2007) Downstream processing of monoclonal antibodies - Application of platform approaches. *J of Chromatogr B* **848**, 28 – 39. https://doi.org/10.1016/j.jchromb.2006.09.026

Singer VL, Jones LJ, Yue ST, Haugland RP (1997) Characterization of PicoGreen Reagent and Development of a Fluorescence-Based Solution Assay for Double-Stranded DNA Quantitation. *Anal Biochem* **249**, 228 – 238. https://doi.org/10.1006/abio.1997.2177

Somasundaram B, Pleitt K, Shave E, Baker K, Lua LHL (2018) Progression of continuous downstream processing of monoclonal antibodies: Current trends and challenges. *Biotechnol Bioeng* **115**, 2893 – 2907. https://doi.org/10.1002/bit.26812

Sommer R, Satzer P, Tscheliessnig A, Schulz H, Helk B, Jungbauer A (2014) Combined polyethylene glycol and CaCl2 precipitation for the capture and purification of recombinant antibodies. *Process Biochem* **49**, 2001 – 2009. https://doi.org/10.1016/j.procbio.2014.07.012

Tait AS, Hogwood CEM, Smales CM, Bracewell DG (2011) Host cell protein dynamics in the supernatant of a mAb producing CHO cell line. *Biotechnol Bioeng* **109**, 971–982. https://doi.org/10.1002/bit.24383

Tihanyi B, Nyitray L (2020) Recent advances in CHO cell line development for recombinant protein production. *Drug Discovery Today: Technologies* **38**, 25 – 34. https://doi.org/10.1016/j.ddtec.2021.02.003

Turbidity < https://www.sciencedirect.com/topics/chemistry/turbidity > Accessed on 3rd of February 2023.

Vidarsson G, Dekkers G, Rispens T (2014) IgG Subclasses and Allotypes: From Structure to Effector Functions. *Frontiers in Immunology* **5**. https://doi.org/10.3389/fimmu.2014.00520

Voisard D, Meuwly F, Ruffieux P-A, Baer G, Kadouri A (2003) Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells. *Biotechnol Bioeng* **82**, 751–765. Voisard, D., Meuwly, F., Ruffieux, P. A., Baer, G., & Kadouri, A. (2003). https://doi.org/10.1002/bit.10629

Wong HE, Chen C, Le H, Goudar CT (2021) From chemostats to high-density perfusion: the progression of continuous mammalian cell cultivation. *J Chem Technol Biotechnol* **97**, 2297 – 2304. https://doi.org/10.1002/jctb.6841

Yigzaw Y, Piper R, Tran M, Shukla AA (2006) Exploitation of the Adsorptive Properties of Depth Filters for Host Cell Protein Removal during Monoclonal Antibody Purification. *Biotechnol Progress* **22**, 288 – 296. https://doi.org/10.1021/bp050274w

Zhang Q, Jiang B, Du Z, Chasin LA (2020) A doubly auxotrophic CHO-K1 cell line for the production of recombinant monoclonal antibodies. *Biotechnol Bioeng* **117**, 2401 – 2409. https://doi.org/10.1002/bit.27367

Zhu-Shimoni J, Yu C, Nishihara J, Wong RM, Gunawan F, Lin M, i sur. (2014) Host cell protein testing by ELISAs and the use of orthogonal methods. *Biotechnol Bioeng* **111**, 2367 – 2379. https://doi.org/10.1002/bit.25327

Zydney AL (2015) Continuous downstream processing for high value biological products: A Review. *Biotechnol Bioeng* **113**, 465 – 475. https://doi.org/10.1002/bit.25695

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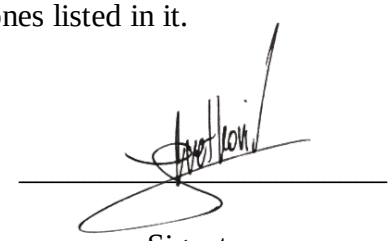
I, JAKOV CVETKOVIĆ, declare that this master's thesis is an original result of my own work and it has been generated by me using no other resources than the ones listed in it.



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