Integrating Upstream and Downstream Process Development Strategies for Mammalian Cell Derived Therapeutic Antibodies

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for the degree of Doctor of Philosophy (PhD)

by

Louisa Jane Wilson

Department of Biochemical Engineering,

University College London,

Bernard Katz Building,

Gordon Street,

London WC1H 0AH

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DECLARATION

I, Louisa Jane Wilson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

ABSTRACT

Recent improvements in volumetric antibody productivity (often in excess of 5 g/L) have been achieved by advances in cell lines and upstream processing, but often lead to harvest material becoming more difficult to recover. These intensified upstream operations require a renewed prioritisation of the integration of upstream and downstream process development to ensure product purification issues are taken into consideration, to avoid extensive and expensive clearance strategies downstream.

Here, it was demonstrated that changes to upstream process parameters at the bioreactor stage of monoclonal antibody production affect product quantity and quality. Culture pH, temperature and seed density setpoints leading to high titre are commonly also linked to higher post-protein A HCP levels, reduced monomer percentages and increased percentages of undesirable glycan structures.

To predict post-protein A product quality, several potential indicators that can be measured in harvest material (prior to using expensive purification resources) were explored, including culture viability and osmolality, revealing unexpectedly that culture viability could not be used for such a purpose, but that osmolality has the potential to be used as a product quality indicator.

The impact of culture duration on product quality was also investigated and it was shown that as cultivation progressed and antibody titre increased, product quality declined, in one case due to post-protein A HCP levels increasing by 75% from day 14 to day 17 of culture. HCP identification by mass spectrometry was applied to this system to provide insights into cellular behaviour and HCP carryover during protein A purification. It showed increases in several classes of post-protein A HCPs (e.g. stress response proteins) as the culture progressed, particularly on days 15 and 17 of culture which were associated with significant increases in total HCP levels. This provides a new level of insight into

HCPs that are retained during mAb purification which may be used to aide process development strategies.

IMPACT STATEMENT

There are several possible and promising impacts deriving from this thesis project, e.g. a) on commercial drug manufacture and patient safety as well as b) in an academic context as university research projects.

a) Commercial drug manufacture and patient safety

First and foremost, the discoveries presented in this thesis demonstrate how upstream and downstream processing of biopharmaceuticals are interconnected. The holistic approach implemented during the studies discussed here has a high potential to be used as a tool for future process development in order to gain significant insight into biopharmaceutical production and ultimately accelerate drug manufacture. The methodology of this integrated upstream / downstream approach serves to optimise industrial processes by streamlining the entire process development rather than having segregated units of operations. This will not only improve production timelines and resource management due to (amongst other parameters) a “fail early, fail cheap” mentality, but also will result in being more cost efficient. Another important aspect with regards to manufacturing costs would be that this holistic approach enables the companies’ employees to interact more efficiently with colleagues from different stages of the production process leading to an optimisation of the workflow.

Further to its use in future biopharmaceutical process development, this approach can also help to ensure that regulatory requirements for patient safety are met.

All of these benefits will ultimately offer significant advantages regarding a company’s competitiveness in the international market.

b) Academic research

Implementation of the knowledge acquired throughout this work also offers possible applications in the field of academic research. Specifically, further research projects can be based upon my work in the form of M.Sc. student projects, PhD or EngD studies or

postdoctoral work, in order to broaden the understanding for the (bio-) chemical engineering community, both within university and industry. Example projects that would be particularly relevant and which would offer great potential for ground-breaking new discoveries are those involving host cell protein profile characterisation of material at different stages of the drug development process, the results of which will enable a deeper understanding of the host cells being used during therapeutic antibody production and the cells' behaviour under varying process parameters.

As this topic is currently receiving much international attention, there is scope for extensive collaborations between universities and/or research institutes all over the world. This will consequently lead to many novel ground-breaking discoveries that are suitable for publication.

Similarly, collaborations between universities and industrial companies might occur, which will prove mutually beneficial due to the availability of state-of-the-art laboratory equipment and cost-efficient researchers.

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ABBREVIATIONS OF COMMONLY USED TERMS

CHO - Chinese hamster ovary

ELISA - Enzyme linked immunosorbent assay

ER - Endoplasmic reticulum

HCCF - Harvested cell culture fluid

HCP - Host cell protein

ICH - International Council for Harmonisation

IgG - Immunoglobulin G

LC - Light chain (of an antibody)

mAb - Monoclonal antibody

PLBL2 - Phospholipase B-Like 2

ppm - parts per million

SEC - Size exclusion chromatography

1 INTRODUCTION

1.1 The industry

Recombinant therapeutic antibodies are a major part of today's pharmaceutical industry. From January 2014 to July 2018, 129 distinct biopharmaceutical active ingredients have been approved in the EU and US, and monoclonal antibodies (mAbs) generated sales of ca. $ 100 billion in 2017 (Walsh, 2018). In order to remain competitive in this market, biopharmaceutical companies should explore strategies for intensifying manufacturing processes to maximise productivity whilst following best practice to meet regulatory expectations regarding product quality. To this end, much research is carried out to investigate innovative technologies such as single use bioreactors or new separation techniques. In addition, more sensitive assays and analytical tools are always of interest in order to enable better characterisation of ever improving product quality.

However, biopharmaceutical manufacture comprises a series of segregated units of operation and development activity is focused on each individual unit. Characterising interactions between unit operations is a key challenge. Over the past decade, significant improvements made to upstream process parameters have led to increasingly higher productivity. Antibody titres of 5 g/L are routinely produced but can be as high as 20 g/L (Grönemeyer et al., 2014). These optimisations made upstream can result in unfavourable conditions for downstream purification and stability profiles as well as potentially cause increased production costs. Higher levels of titre will soon represent a serious concern for downstream processing as the purification equipment was not intended for these amounts. Capacity limits will be reached and consequently, processing time, material consumption and downstream processing costs will increase. If these costs outweigh the decreased cost per gram gained from higher titres, then this may even raise the overall manufacturing costs (Strube et al., 2012). It may therefore be more beneficial to take a holistic approach

to biomanufacturing and aim for total process optimisation, rather than focusing the optimisation on individual unit operations.

To date, limited research has been published about investigating the interface between upstream and downstream processing and defining which upstream parameters are critical for not only high product titre but also for efficient downstream purification and high product purity.

An example of published research in this field include Goey et al. (2017) who studied one particular upstream parameter (i.e. a temperature downshift to mild hypothermia on day 5 of fed-batch CHO cell bioreactors) and the effect this had on antibody titre, HCP concentration and HCP species. They concluded that CHO cell cultures grown at standard physiological temperature followed by a shift to mild hypothermia on day 5 of culture resulted in material with similar antibody titre and HCP concentration, but with noticeably different HCP composition. They also noted a lower cell growth rate but higher percentages of healthier cells and a less apparent onset of apoptosis, leading to less varied HCPs, particularly intracellular ones or those localised to the cell membrane. While this research is very interesting, it focussed only on one upstream parameter (i.e. culture temperature) whereas the aim of this thesis is to take a much more holistic approach (as will be discussed more extensively later).

Another published paper of interest is by Agarabi et al. (2017) which has highlighted the importance of linking upstream and downstream studies during process development. They investigated how changes to upstream process parameters can influence capture chromatography performance and discovered that higher dissolved oxygen levels (DO%) and higher sparge rates were associated with fewer HCPs, increased antibody monomer levels and improved protein stability with properly folded native structures. The authors suggest this is possibly because well-aerated and oxygenated cultures might be healthier and have intact machinery for protein assembly and processing which should result in

fewer misfolded HCPs. This research is more in line with the approach that is presented in this thesis, although Agarabi et al. (2017) focussed on different upstream parameters compared to those discussed here. Other literature of interest will be discussed throughout this thesis.

Recent advances in technology allow for a thorough investigation at the interface between upstream and downstream processing: The ambr15 and ambr250 scale-down bioreactor systems from TAP Biosystems enable efficient cell culture experiments using the statistical Design-of-Experiments (DoE) approach where a large number of screening experiments can be done with only small amounts of material. Several factors can be changed within one set of experiments which decreases the number of required experiments and allows the influence of several parameters to be determined and significant ones to be identified (Zhang et al., 2013; Grönemeyer et al., 2014). Moreover, the use of scale down high-throughput downstream processing equipment like the liquid handling robots from TECAN allow rapid purification of the large number of harvested cell culture fluid (HCCF) samples supplied by the upstream experiments. The ambr250 scale down bioreactors and the small scale, high-throughput TECAN robot in particular enable extensive yet time-efficient research into the interactions between upstream and downstream processing as various different upstream process parameters can be tested for their impact on downstream performance with regards to fast and cost-effective separation and high product purity. Highly sensitive instrumentation such as mass spectrometry and automatable assays like the enzyme-linked immunosorbent assay (ELISA) for the quantification of host cell protein impurities enable precise analytical support and fast analysis of a large amount of data. Due to these advances and the limited knowledge about the impact of upstream operating conditions on downstream performance, it is highly advisable to investigate the interface between upstream and downstream processing and define critical process parameters (CPPs) associated with

upstream HCCF production. Once these are defined, the optimal operating ranges of the CPPs can be investigated and a traffic light system can be used to highlight which operating conditions have an advantageous (red) or disadvantageous (green) effect on the most important critical quality attributes (CQAs). This would efficiently demonstrate whether a robust process with high productivity and purity is achievable for a certain set of upstream operating conditions.

1.2 Mammalian biopharmaceutical processing and optimisation

Monoclonal antibodies represent an increasingly higher share of biological products on the market. As these molecules require post-translational modifications such as glycosylation for efficient activity, mammalian host expression systems that are capable of making these modifications such as Chinese hamster ovary (CHO) cells are progressively preferred for the production of therapeutic antibodies (Walsh, 2014). Microbial expression systems are unable to fold the proteins correctly and to carry out the necessary post-translational modifications in ways that are favourable for use in humans. Currently, biopharmaceutical manufacturing processes are developed and delivered by several segregated units of operation and of particular interest here are two groups: one is the upstream processing group which selects cell clones for the production of therapeutic antibodies and defines operating conditions under which cells grow well and produce high amounts of antibody. The second group of interest is the downstream processing group which subsequently purifies the produced antibody and separates out process- and product related impurities.

1.2.1 Upstream processing (USP)

The upstream processing group grows different cell lines that are transfected with a desired gene and evaluates the cell line stability in various media processes. The best performing cell lines in terms of high productivity are then scaled up in bioreactors and

grown under certain process parameters until the cells are harvested, usually between days 12 and 18. Optimisation in upstream processing is usually focused around cell line development, media and feed development, scale up, process control and cell harvesting (Li et al., 2010; Zhu, 2012). The main focus of this group is on making sure cells show high viability and healthy cell growth in order to produce high amounts of antibody titre, whereas the ease of purification can be a rather negligible concern at this stage (Bhoskar et al., 2013).

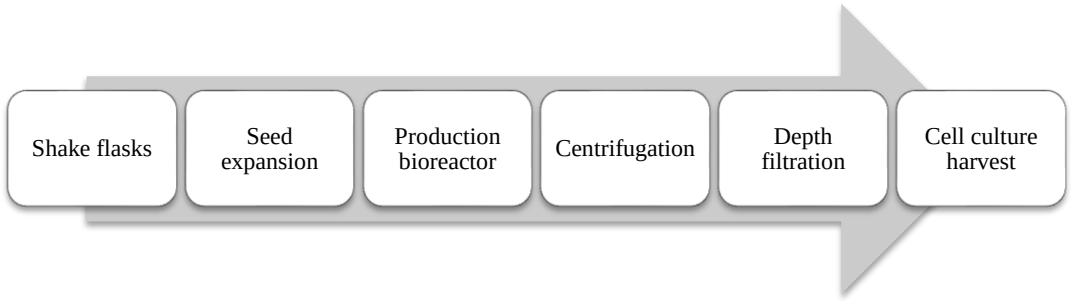


Figure 1. Generic upstream process flow diagram for a monoclonal antibody.

1.2.2 Downstream processing (DSP)

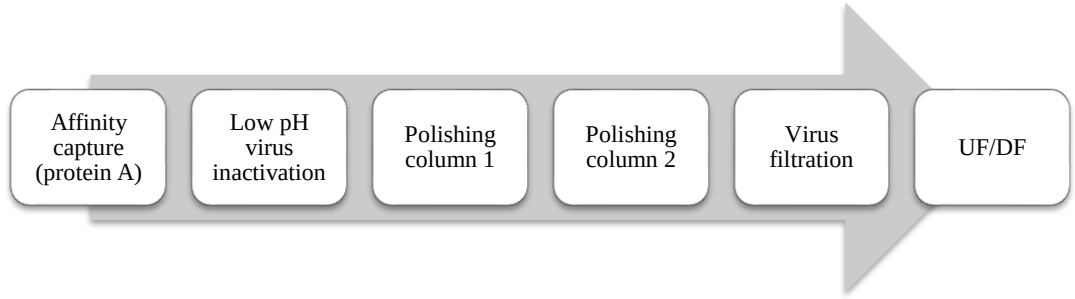


Figure 2. Generic downstream process flow diagram for a monoclonal antibody.

The filtered harvested cell culture fluid (HCCF) is passed onto the downstream processing group for protein purification where the desired pharmaceutical protein is separated from most process- and product-related impurities. A typical downstream process for the purification of therapeutic mAbs is done in two or three column steps depending on the molecule expressed by the selected cell lines. The initial affinity chromatography

commonly uses protein A to bind the Fc region in immunoglobulins (Igs) while most cell impurities flow through the column without binding and are thus separated from the therapeutic product. This very powerful step is considered a gold standard as the initial purification step due to its very high affinity specifically for the Fc region in Igs and therefore its capability of removing most impurities including the majority of host cell proteins (HCPs). Protein A affinity capture is commonly followed by low pH viral inactivation which inactivates or denatures potential (enveloped) viruses by altering their surface chemistry. Further separation techniques can include anion and cation exchange chromatography. The former can be used to separate out negatively charged impurities such as DNA, endotoxins and HCPs, while the latter can be used to improve monomer purity if required. Cation exchange chromatography removes product aggregates by binding the highly positively charged aggregates more tightly than the monomers and this causes the aggregates to elute after the monomers. The eluate is collected in fractions and those fractions containing the product monomers are pooled.

Optimisation in downstream processing is focused mainly around improving individual chromatography steps such as the implementation of superior resins with new matrix or ligand chemistry, or the use of intermediate washing steps to remove remaining impurities. In addition, a large amount of research has been carried out to find alternatives to protein A resin as it is the most expensive chromatography step (Grönemeyer et al., 2014).

1.2.3 Critical parameters in USP and DSP

Due to the segregated approach to antibody production and process development, process optimisations are carried out within each individual group rather than for the whole production process and this lack of alignment can cause disadvantages to the overall manufacturing process. For instance, certain cell lines or bioreactor processing parameters will increase product titre but may increase the amount of impurities as well.

These impurities will need to be removed again during downstream processing which may affect workload and process cost. To prevent increasingly higher downstream processing costs with further increasing product titre, critical parameters in process development need to be thoroughly investigated.

Critical quality attributes (CQA) are defined by the Food and Drug Administration (FDA) (2009) as being "physical, chemical, biological, or microbiological [properties] or characteristic[s] that should be within an appropriate limit, range, or distribution to ensure the desired product quality", for example aggregates, fragments and host cell proteins which can affect monomer purity, product stability and efficacy. As such they need to be closely monitored. Parameters of the manufacturing process that can affect these CQAs are considered critical process parameters (CPPs). Potential upstream process parameters that can affect product CQAs are generally at the bioreactor stage (Figure 3). Examples include the seed density, the temperature and pH under which the cells are grown and the process duration (Nagashima et al., 2013). Seed density and temperature impact the growth profile and viability of the culture, while the amount of time for which the cells are grown has an effect on the culture viability at harvest. As such, cultures which are grown for an extended duration may start showing declining viability and may be associated with higher amounts of host cell proteins. Sparge rate and agitation rate are also potential CPPs. It is possible that changes to these parameters have a big impact on the product quality, and as such they may be considered critical (Agarabi et al., 2017). However, the parameters mentioned previously are generally easily regulated with current bioreactor controls, so as soon as an optimal operating range for these parameters is determined and they are maintained within this design space, they can be considered less critical (Haigney, 2013). On the other hand, parameters such as dissolved oxygen and carbon dioxide concentration as well as the concentration of critical nutrients are harder to control and may therefore need to be regarded as highly critical. The concentration of

nutrients reportedly affects the molecule's glycosylation pattern and thereby the efficacy of the drug (Hossler et al., 2009). Reducing the concentration of CO_2 but avoiding interference with the pH is a fine balance that can influence the drug quality (Zhu et al., 2005; Darja et al., 2016).

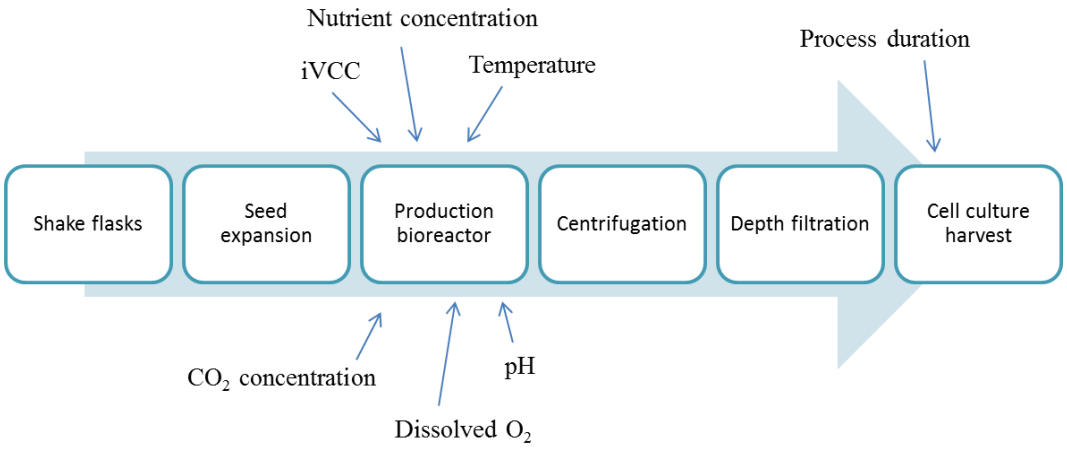


Figure 3. Potential critical process parameters (CPP) in a generic upstream process. Note, iVCC stands for initial viable cell density, or seed density.

In terms of the downstream purification process, potential CPPs include the protein load and flow rate during each chromatography step, as well as the pH and conductivity during the wash and elution phases (Figure 4). The elution gradient during chromatography may also affect product quality. All of the above determine how well the protein of interest is separated from the product- and process-related impurities and therefore how good the quality of the final drug substrate will be (Rathore & Winkle, 2009). Similarly, the pH, hold time and temperature during the viral inactivation step as well as the membrane characteristics, filtration volumes and pressure during viral filtration all have an impact on the drug CQAs (Jin et al., 2019; Dizon-Maspat et al., 2011; Haigney, 2013).

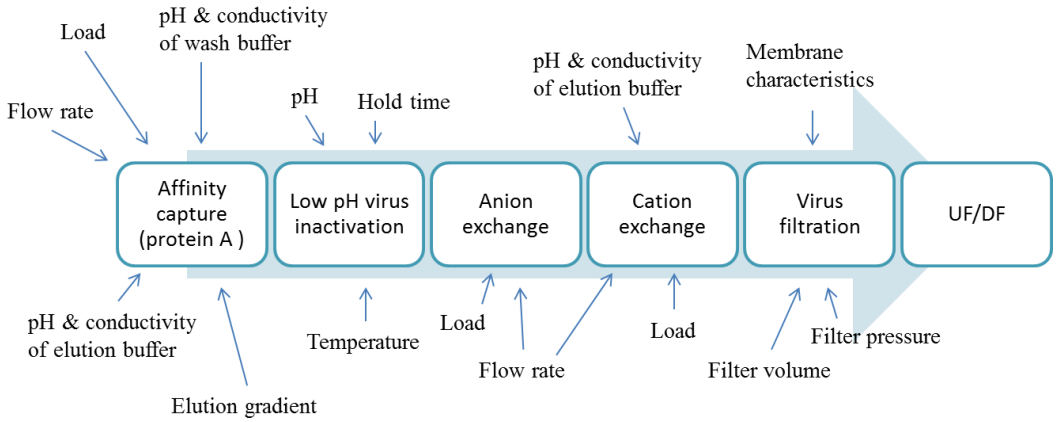


Figure 4. Potential critical process parameters (CPP) in an example downstream process.

1.3 Impurities in biopharmaceutical manufacturing

Biopharmaceutical manufacturing is associated with several types of impurities, such as product related impurities (e.g. aggregates) and process related components (e.g. HCPs, DNA, residual media components, protein A leachables). All impurities need to be monitored and if necessary, removed during product purification in order to achieve drug quality specifications. These are set in accordance with guidelines published by the ICH and are harmonised between the regulatory authorities and the pharmaceutical industries in Europe, Japan and the United States of America (Oshinbolu et al., 2017).

1.3.1 Aggregation

Aggregation is the self-association of proteins with each other and can vary in structure, bonding, reversibility and solubility. Particularly during cellular stress and/or when high amounts of proteins are expressed and ER chaperones do not function sufficiently, proteins can unfold/misfold resulting in the exposure of hydrophobic patches of the peptide structures. This can cause accumulation of numerous unfolded proteins into aggregates. Such conditions can occur during the expression of recombinant antibodies, where aggregation might occur both intracellularly during the actual synthesis of antibodies as well as in the culture medium after the proteins are secreted (Bhoskar et al., 2013). Aggregates can also form when therapeutic proteins are exposed to chemical (e.g.

pH), physical (e.g. aeration or agitation), and/or biological damage (e.g. proteases) which can cause conformational changes or might even form on the chromatography column during protein purification (Farys et al., 2018). As these aggregates have different activity and efficacy compared to the product monomers and can pose a safety concern due to adverse immune responses in patients (Moussa et al., 2016), they need to be removed during downstream purification.

As mentioned, for the production of recombinant proteins, cells are expressing and folding a higher amount of proteins than normal and this can cause stress, particularly if chaperones in the endoplasmic reticulum (ER) such as BiP (binding immunoglobulin protein) are unable to fold the nascent proteins in a timely and precise manner and these unfolded or incorrectly folded proteins are prone to aggregation (Bhoskar et al., 2013; Schröder et al., 2002). It has been found that heterotetrameric antibodies with two heavy chains and two light chains (H_2L_2) assemble in a manner that requires correctly folded and functional light chain (LC) domains to be present in the ER. In the absence of light chains, the ER chaperone BiP binds tightly to one particular heavy chain domain (CH1) and prevents the folding of this domain and thus the assembly of a complete antibody. If functional light chains are present, BiP and CH1 dissociate from each other and the latter is able to fold and subsequently assemble with all subunits to create an immunoglobulin (Bhoskar et al., 2013; Lee et al., 1999). Free LC domains are easily transported from the ER and the cell, so a high amount of light chains in the culture media is thought to be indicative of their concentration within the cell. Based on this knowledge, Bhoskar et al. (2013) have published that a high amount of antibody light chains in the culture media is linked with high antibody productivity, high cell viability and low aggregation. Of particular interest here is the correlation between high levels of free light chains (i.e. LC in the culture media) and low product aggregation. This research suggests that the concentration of antibody LC domains in the culture media might be used as an indicator

of issues pertaining to product aggregation: low levels of free light chains imply a lack of intracellular LC domains, which means CH1 is unable to fold and assemble with the other subunits into a full immunoglobulin. If unfolded proteins are not degraded by the cells in a timely manner but instead accumulate, aggregation will occur. These cells with low LC domains would therefore be associated with high aggregation levels. Bhoskar et al. (2013) have found that such cells also have low viability which may be due to the resulting toxicity. As such, functional light chain domains are a pre-requisite for immunoglobulin assembly and they minimise the risk of aggregation of unfolded heavy chains, which might classify free LC in culture media as a critical parameter of upstream processing. However, this research was only carried out with an IgG1-type mAb, so further research is required to verify these finding with other types of recombinant antibodies (Bhoskar et al., 2013). Also, it would need to be determined at which point the light chain concentration can be considered too low to produce high quality drugs and becomes critical, although this may very well vary between different antibodies.

1.3.2 Glycosylation

During protein synthesis in eukaryotic cells, one significant post-translational modification that is carried out is the attachment of oligosaccharides to specific amino acids of the protein, known as glycosylation. The most common type of glycosylation seen in antibodies is N-linked glycosylation where glycan structures are attached to asparagine at the following consensus site: Asn-Xaa-Ser/Thr where Xaa is not a proline amino acid. This post-translational modification is very complex and a cause for great heterogeneity in mAbs. There are three different types of N-glycans found on IgGs – complex, high mannose and hybrid (Figure 5), of which the complex and hybrid types occur either with or without a core fucose residue attached to the innermost N-acetylglucosamine (GlcNAc) residue. Usually, mAbs are associated with high amounts of complex bi-antennary glycans with core fucosylation (Higel et al., 2016).

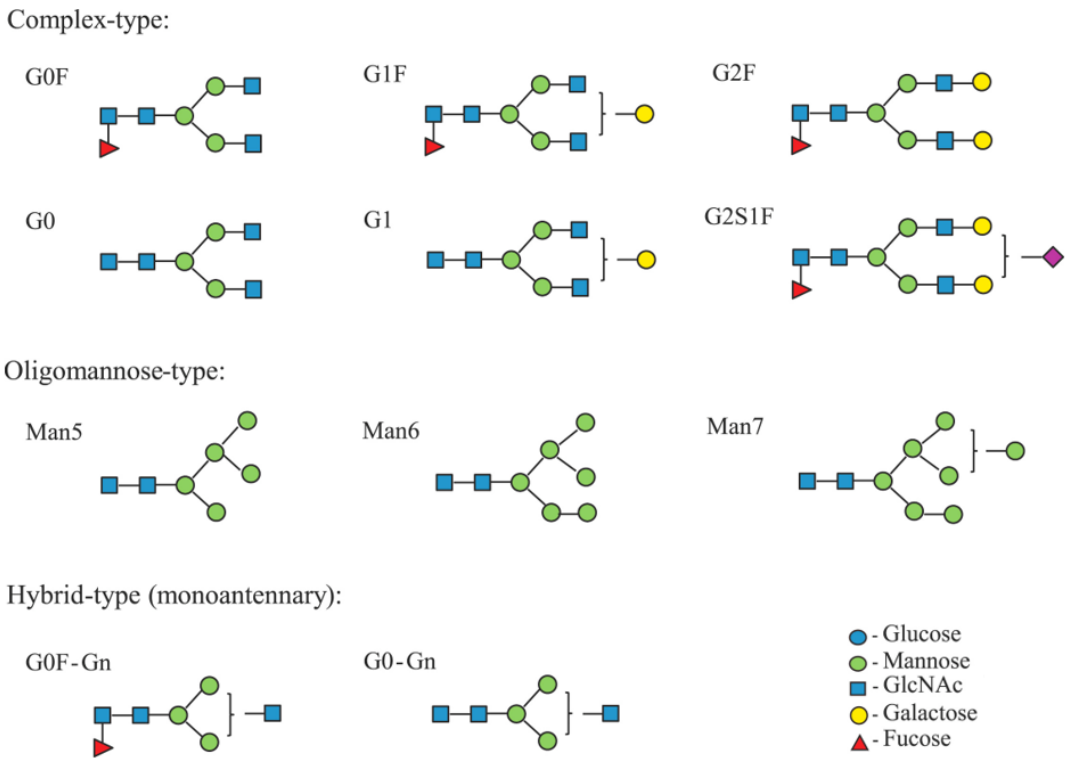


Figure 5. Diagrams of glycan structures commonly found on immunoglobulins. Taken from Zhou & Qiu, (2019, figure 2).

Upstream processing conditions directly influence correct glycosylation formation since the expression system and growth conditions have an impact on the glycosylation pathways (Hossler et al., 2009).

Glycosylation itself greatly affects product solubility and stability and influences the mechanisms by which therapeutic mAbs work. A lack of glycan residues makes mAbs more susceptible to unfolding and aggregation (Zheng et al., 2011). Furthermore, certain types of glycan species have the undesirable effect of clearing the product from the bloodstream too quickly. Mannose receptors, which bind to mannose and N-acetylglucosamine residues of N-glycans and which are most noticeably expressed on immune cells (Allavena et al., 2004), are responsible for selective clearance of glycoproteins, thereby reducing the half-life of affected mAbs in patients. Similarly, mAbs with N-glycans lacking galactosylation (and therefore having a terminal GlcNAc residue) have a higher rate of clearance from circulation according to Huang et al. (2006).

Glycan species also influence the way by which therapeutic mAbs can act. For mAbs that rely on Fc-mediated effector function, changes in Fc glycans can affect the mechanism of action, e.g. IgGs with afucosylated Fc glycans increase antibody-dependent cell-mediated cytotoxicity (ADCC) (Reusch & Tejada, 2015). Therefore, products with an incorrect glycan profile will be restricted in their functionality, which makes correct glycosylation a crucial quality attribute.

There has been a lot of development to measure and monitor glycosylation patterns, which has become particularly relevant for the manufacture and approval of biosimilars. The first therapeutic mAb has lost patent protection in the US in 2016 and there has since been a rise in the production of biosimilars. Given the effect that incorrect glycosylation can have on drug efficacy, it is important to monitor glycan species in biosimilars to ensure that they have the same pharmacokinetics and immunogenicity as the original mAbs (Kang et al., 2018).

As mentioned, it is important to control the production of only specific glycan structures. This can be achieved in several ways, e.g. by

a) controlling culture pH, temperature, media and nutrients, which are parameters that affect high mannose and terminal galactosylation levels (Zupke et al., 2015), or by

b) creating knockout cell lines with silenced genes for Fucosyltransferase 8 or GDP-Mannose 4,6-dehydratase (Yamane-Ohnuki et al., 2004; Kanda et al., 2007) which results in reduction of fucosylation levels. The absence of core fucose is responsible for enhanced ADCC and increased drug efficacy, which proved especially beneficial for oncology products (Higel et al., 2016). On the other hand, in diseases where ADCC is not the desired effector function, the absence of core fucose is unfavourable.

1.3.3 Host cell proteins

One product quality attribute in particular has proven to be quite challenging to monitor and to remove from the final drug product and has thus come under increasing scrutiny

over the last few years – host cell proteins. The host cells that are implemented for the expression of recombinant therapeutics (e.g. monoclonal antibodies) produce not only the desired antibody but also co-express indigenous proteins that enable the cells to survive, known as host cell proteins (HCPs). They are present in the harvested cell culture fluid (HCCF) and are process related impurities requiring separation from the drug product during downstream processing.

HCPs are a complex mixture of a huge variety of proteins with significantly diverse physicochemical properties (e.g. isoelectric point, molecular weight and degree of hydrophobicity) (Jin et al., 2010; Nogal et al., 2012), which makes it necessary to use multiple techniques for efficient clearance. Shukla et al. (2008) have investigated two strategies to demonstrate robust HCP clearance during downstream purification steps. The reason why HCP removal is so essential is not only due to the possibility that they might influence the efficacy of the drug product, but also because they can cause adverse immune reactions in patients, including cross-reactivity and autoimmunity, and as such are a major safety concern (Bracewell et al, 2015; FDA, 2014; Gao et al., 2011; Nogal et al., 2012; Singh, 2011). One notable case was published by Genentech about phase III clinical trials for the asthma drug Lebrikizumab. Material for this study was found to contain high amounts of the HCP species PLBL2 which caused a notable immunogenic response in ~90% of subjects, although no adverse safety effects were observed (Fischer et al., 2017). For these reasons, the Food and Drug Administration recommends reducing HCPs to acceptably low levels (<100ppm) (Chon and Zarbis-Papastoitsis, 2011), although in reality HCP limits are case-by-case dependent and are defined from (pre-) clinical studies and manufacturing consistency lots (ICH, 1999; Oshinbolu et al., 2017). The recommended limit is a guideline only and aims to reduce the impurities as much as possible as our limited understanding about the exact types of HCP species that are being retained in the final drug product means it is unclear how dangerous their presence may

be to the patient. Low levels of HCPs overall reduce the possibility that harmful types of HCP species are still present in the final drug substrate and pose a risk to patients.

1.3.3.1 Select HCPs co-purify with mAbs

When it was first discovered that significant amounts of host cell proteins were not being efficiently separated during the antibody purification process but were in fact retained during protein A affinity chromatography, it was unclear whether the host cell proteins co-eluted with antibodies by non-specifically binding to the resin or by associating with the bound antibodies. Shukla & Hinckley (2008) have demonstrated that the latter was the case and that column wash buffers that disrupt mAb – HCP interactions (rather than resin – HCP interactions) need to be developed and applied after loading material onto columns in order to maximise HCP clearance. Subsequently, Nogal et al. (2012) have also shown that HCPs co-elute with bound mAbs by performing two protein A chromatography runs: in the first case, mAb free HCP material was loaded onto clean protein A resin, whereas in the second case, the mAb free HCP material was loaded onto a protein A column that already had antibodies bound to it. The design of this experiment effectively allowed a conclusion to be drawn on whether host cell proteins co-purify during protein A affinity chromatography by binding to the resin or the bound antibodies: if the eluate from the first experiment contained high HCP levels, the impurities must have bound to the chromatography resin because there were no antibodies present yet to which they could have bound; if the eluate from the second experiment contained high HCP levels, the impurities bound to the antibodies that were already bound to the resin. It was found that upon elution, the HCP levels were significantly higher in the second run, demonstrating that most HCP species co-purify during protein A affinity chromatography due to their interactions with the bound antibodies.

Nogal et al. (2012) also demonstrated that only select subpopulations of host cell proteins co-elute with the antibody during protein A affinity chromatography. They loaded harvested cell culture fluid containing antibody and HCPs onto protein A resin, spiked the flow-through with purified antibody again and recycled this material for a total of five cycles. This experimental design allowed a conclusion to be drawn on whether all or only select subpopulations of host cell proteins co-elute with the antibody during protein A affinity chromatography: if only specific subpopulations of HCPs co-elute with the antibody, then these populations should be depleting from the CHO culture and the eluate samples should contain decreasing amounts of HCPs over the course of the five cycles. However, should all HCPs co-elute with the antibody similarly, any depletion from the large supply that is present in the CHO culture is unlikely. In this case, the HCP levels in the five eluate samples should remain similar. And indeed, Nogal et al. (2012) noted that HCP levels in the flow-through samples remained fairly consistent while eluate samples from the five cycles contained rapidly decreasing levels of HCPs. They concluded that only specific subpopulations of HCPs co-elute with the antibody, which were gradually depleted from the load material. Other research groups have confirmed these findings (Aboulaich et al., 2014; Bailey-Kellogg et al., 2014; Hogwood et al., 2014; Levy et al., 2014; Sisodiya et al., 2012), and have furthermore focussed research on identifying specific HCP species as discussed in the next sections and throughout this thesis.

1.3.3.2 Identification of co-eluting HCP species

Considerable research has been done recently to identify the specific HCP species that are being retained during protein A affinity chromatography with certain antibodies expressed in CHO cells. HCPs reported to be present in high amounts include those that are involved in essential cell survival processes such as in translation (e.g. elongation factor 2), in protein folding (e.g. heat-shock proteins Hsp70 and Hsp90 and clusterin), and in glucose or lipid metabolism (e.g. Glyceraldehyde 3-phosphate dehydrogenase;

pyruvate kinase, lactate dehydrogenase; PLBL2) (Albrecht et al., 2018; Farrell et al., 2015; Tait et al., 2012; Zhang et al., 2014). In addition, proteases such as cathepsins and serine protease HTRA1 have been identified (Albrecht et al., 2018; Farrell et al., 2015; Tait et al., 2012; Zhang et al., 2014), particularly during late stages of the culture process when they are suggested to cause fragmentation of HCPs (Bee et al., 2015; Farrell et al., 2015). Levy et al. (2016) have also identified difficult-to-remove HCP species from non-affinity chromatographic polishing resins commonly used in polishing steps for mAb purification (i.e. ion-exchange, hydrophobic interaction, and multimodal).

Identified post-protein A HCPs	**Source**
78 kDa glucose-regulated protein	Farrell (2015), Zhang (2014), Zhang (2016)
Actin cytoplasmic 1	Farrell (2015), Zhang (2014), Zhang (2016)
Clusterin	Farrell (2015), Zhang (2014), Zhang (2016)
Elongation factor 1-alpha 1	Zhang (2014), Zhang (2016)
Elongation factor 2	Albrecht (2018), Tait (2012), Zhang (2014), Zhang (2016)
Glutathione S-transferase P	Albrecht (2018), Zhang (2016)
Glyceraldehyde-3-phosphate dehydrogenase	Albrecht (2018), Farrell (2015), Zhang (2016)
Heat shock cognate 71kDa protein	Albrecht (2018), Zhang (2016)
Peptidyl-prolyl cis-trans isomerase	Albrecht (2018), Tait (2012), Zhang (2016)
Peroxiredoxin-1	Albrecht (2018), Farrell (2015), Zhang (2016)
Phosphoglycerate kinase 1	Zhang (2016)
Pyruvate kinase	Tait (2012), Zhang (2014), Zhang (2016)
Serine protease HTRA1	Farrell (2015), Zhang (2016)

Table 1. Selection of host cell protein species that have been identified by different research groups to be co-eluting with monoclonal antibodies.

1.3.3.3 Effects of upstream process parameters on HCP composition

In addition to carrying out HCP research in the downstream space, work has also been done to investigate whether certain upstream process conditions affect the composition of HCPs present in harvested cell culture fluid. For instance, Jin et al. (2010) researched how media, temperature, feeding strategy, agitation speed, process duration and cell viability impact the levels of total HCPs. They found low cell viability to have the most significant effect on HCP composition. Not only did they measure higher levels of HCPs at day 15 – when viability was only 11% – but they also discovered that low molecular weight species were more abundant at this time in the culture process, suggesting the release of proteases and the associated degradation of proteins at low viability. This suggests that viability needs to be closely monitored, although it should be noted that the viability of 11% observed in this research is not a realistic manufacturing process viability. Low viability cultures are likely to be avoided for the production of therapeutic antibodies anyway but even well behaving cell cultures need to be monitored to prevent such cultures from producing high amounts of HCP at the end of the culture process when viability might be decreasing and extra care must be taken to harvest cells at high viability and not extend the process duration too far. Tait et al. (2012) have also reported that the changes in environment, metabolism and declining viability at the end of the culture period result in different compositions of HCPs at the end of the process compared to earlier days. Both the work from Jin et al. (2010) and Tait et al. (2012) suggest that the time of harvest is a crucial parameter with regards to HCP composition and that cell culture duration and cell viability should be controlled for process consistency. Further research is required to investigate if an earlier harvest might be worthwhile to obtain HCCF with easier separable HCP species.

However, there has been little research to link these HCP discoveries to upstream process conditions. Experiments are often focussed either on downstream HCP analysis without

consideration of the upstream process conditions under which the material was produced, or they are focussed on upstream process conditions and their effect on HCP composition in the harvested cell culture fluid, but not on the HCP characteristics in processed material. One paper of interest that does link upstream and downstream studies is by Agarabi et al. (2017) who have investigated how changes to upstream process parameters can influence capture chromatography performance.

1.4 Analytical tools for the measurement of CQAs

1.4.1 Analytics for aggregation quantification

There are several techniques which can measure the percentage of differently sized particles in a solution. Size exclusion chromatography (SEC) separates the molecules of a sample by their respective sizes, using resin beads with different pore sizes. During the chromatography run, large molecules (such as product aggregates) are unable to enter any of the pores or only diffuse into a few of the larger pores and are thus eluted from the chromatography column first. Small molecules like product fragments on the other hand will diffuse into many if not all the pores and are thus retained in the column for the longest period and elute last. Medium sized proteins like product monomers can diffuse into some but not all the pores so they are eluted in between the large and small sized molecules. As the proteins are eluted, UV (ultraviolet light) absorbance is measured and the relative percentage of each group of molecules can be calculated to determine how high monomer purity is. However, since separation depends on the pore dimensions, it is crucial to select the appropriate range of pores for the respective sizes of the proteins that are to be separated. SEC is the most common technique to quantify mAb aggregates and ensure that the amount meets regulatory guidelines. It is considered a gold standard in industry to monitor mAb monomer purity and support process development / manufacture.

Dynamic light scattering (DLS) is another orthogonal tool to measure particle size distribution. It measures the scattered light from the molecules that are moving in solution, which is dependent on the rate of diffusion and the hydrodynamic radius of each molecule (Wyatt Technologies). Accurate measurement depends on sufficient protein concentration.

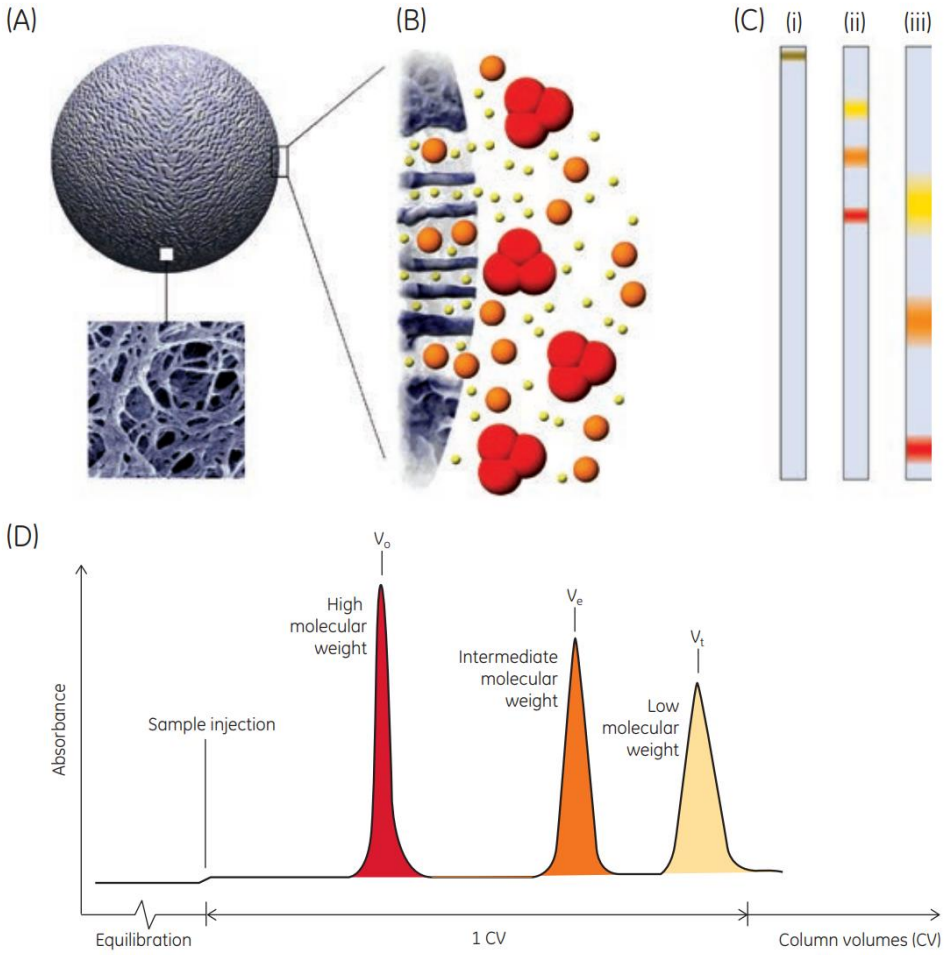


Figure 6. Process of size exclusion chromatography. (A) Schematic picture of a particle with an electron microscopic enlargement. (B) Schematic drawing of sample molecules diffusing into the pores of the particle. (C) Graphical description of separation: (i) sample is applied to the column; (ii) the smallest molecule (yellow) is more delayed than the largest molecule (red); (iii) the largest molecule is eluted first from the column. Band broadening causes significant dilution of the protein zones during chromatography. (D) Schematic chromatogram. Figure and legend taken from GE Healthcare (2018).

1.4.2 Analytics for glycosylation quantification and identification

Characterising the glycosylation of a therapeutic protein is critical. According to the ICH Q6B guideline, "for glycoproteins, the carbohydrate content should be determined. In addition, the structure of the carbohydrate chains, the oligosaccharide pattern and glycosylation sites of the polypeptide chain should be analysed to the extent possible" (ICH, 1999). And the European Medicines Agency states that "glycan structure should be characterised, and particular attention should be paid to their degree of mannosylation, galactosylation, fuscosylation and sialylation. The distribution of main glycan structure present (often G0, G1 and G2) should be determined" (EMA, 2008).

Glycan profiling can be achieved by means of two strategies, the first of which requires cleavage of the protein peptide chain, e.g. using trypsin, while the second removes the glycan structures from the protein peptide chain (either by chemical means or by enzymatic cleavage). In order to detect and quantify the released glycopeptides or oligosaccharides respectively, several analytical techniques can be used. There are commonly two groups of analytical techniques for the analysis of mAb glycans, one being mass spectrometric techniques and the other being separation-based techniques (del Val et al., 2010).

An example of a MS technique is MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight), while an example of the separation based techniques is HILIC-HPLC (Hydrophilic Interaction High Performance Liquid Chromatography) or HILIC-UPLC (Hydrophilic Interaction Ultra Performance Liquid Chromatography) (Figure 7). In HILIC, separation of compounds occurs based on their differential capacity of producing hydrogen bonds with the polar media of the stationary phase. UPLC systems as opposed to HPLC systems have improved resolution, are more sensitive and are capable of quantifying mAb glycans within 30 minutes (Clarke et al., 2009).

Mass spectrometry is considered the gold standard for qualitative characterisation of glycans. Tandem MS is a possible approach to obtain glycan identification and sequencing and can provide purification and analysis in one system (Wuhrer et al., 2007). However, for quantification of oligosaccharides, techniques such as liquid chromatography which render direct results due to fluorescence or UV absorbance detectors are more commonly used compared to mass spectrometry which only quantifies results relative to other glycan structures in a sample.

High sensitivity of an analytical technique used to quantify glycans is very important in order to allow detection of glycans present in low concentrations as well as to enable analysis of low volume samples, as is commonly the case during small scale process development (del Val et al., 2010).

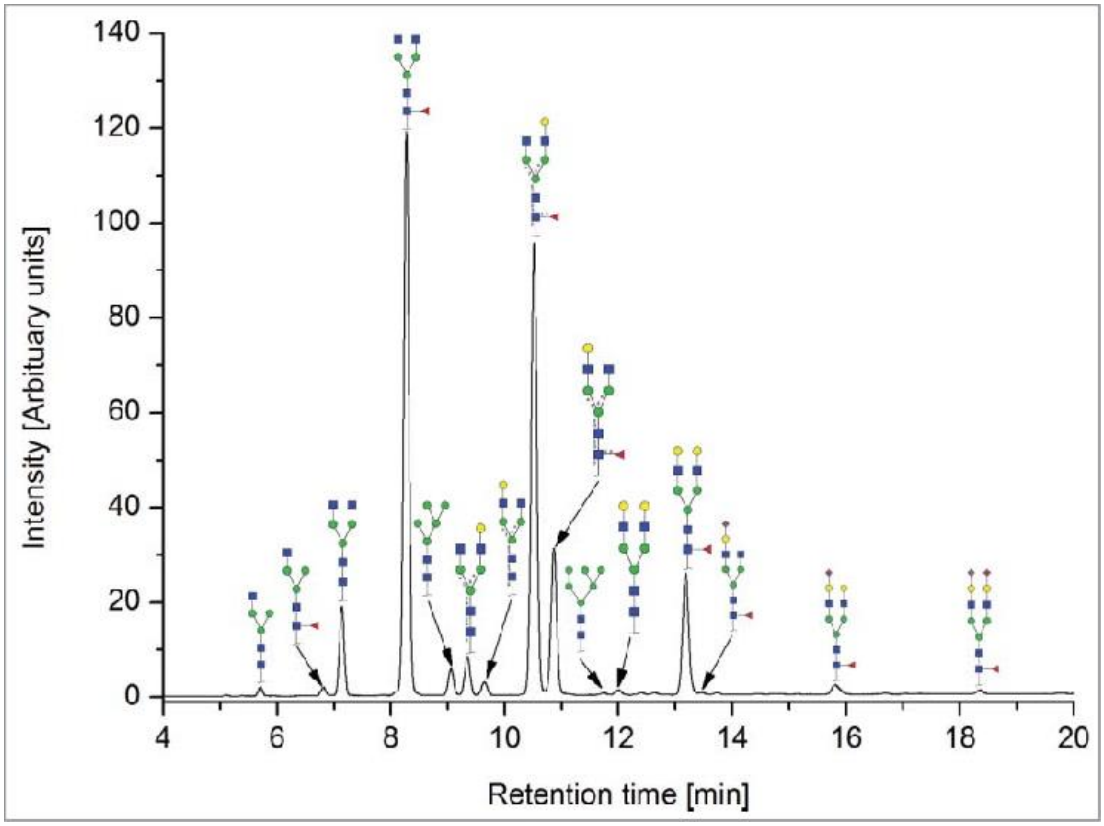


Figure 7. Example of HILIC-UPLC for N-glycan identification, taken from Reusch et al. (2015, figure 2).

1.4.3 Analytics for HCP quantification and identification

Enzyme-linked immunosorbent assays (ELISAs) are considered a gold standard for the quantification of HCPs and are most commonly used to ensure the drug specification limits of HCP impurities are met. The antibodies used in such assays are produced by immunised animals such as goats or rabbits. Therefore, only HCPs with immunogenicity in the host animals will be accurately measured. For HCPs which do not cause an immune reaction in the immunized animals, there will be no specific antibody produced and so they will not be detected in the corresponding ELISA. As an ELISA is unlikely to detect 100% of HCPs, its quantification is limited and does not provide absolute results about the total mass of HCPs present in a sample (Champion, 2005).

Moreover, this type of ELISA does not provide any information about the identity of the HCP species that are present.

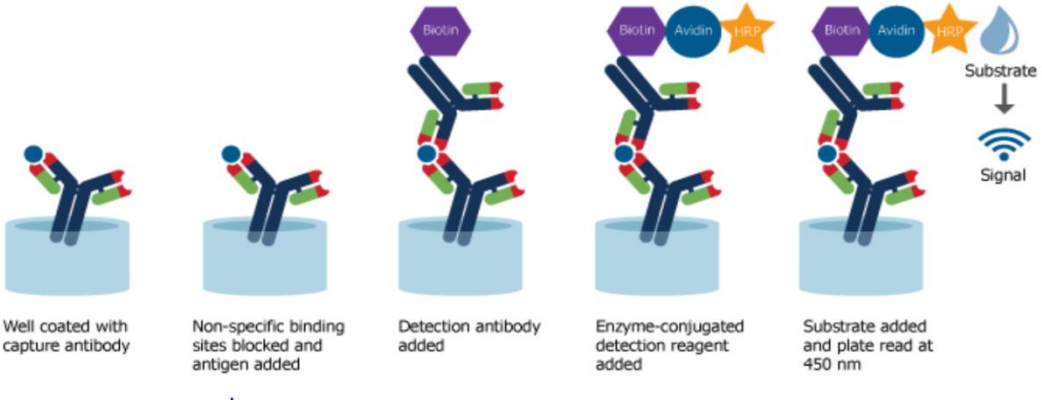


Figure 8. Schematic of a sandwich ELISA, taken from Rockland Immunochemicals Inc. (2019).

The identity of specific HCP species can be investigated by 2D-PAGE (two-dimensional polyacrylamide gel electrophoresis) and LC-MS/MS (liquid chromatography with tandem mass spectrometry). Electrophoresis with sensitive protein staining enables the visualization of the proteins and 2D electrophoresis supplies information about the distribution of HCP species according to molecular weight and isoelectric point. Coupled with LC-MS/MS, these species can be identified and quantified. This approach allows for

HCP species with low immunogenicity to be recognized as well. On the other hand, low abundant proteins are masked by high abundant ones and are less likely to be identified (Hogwood et al., 2014; Lu et al., 2015). This is a concern when trying to quantify and identify low abundance HCPs in the presence of highly abundant antibody product. To address this issue, recent advances in mass spectrometry have enabled improvements in the specificity and dynamic range using targeted mass spectrometry techniques, e.g. using a Q-Exactive mass spectrometer which contains a quadrupole mass filter and orbitrap mass analyser for high resolution and accurate mass (HR/AM) measurements (Picotti & Aebersold, 2012; Gallien et al., 2012; Gillet et al., 2012). Recent research has demonstrated that it is possible to quantify HCPs down to single-digit ppm range within 1 hour for real time bioprocess development support (Walker et al., 2017).

An example of a workflow used to identify HCP species by mass spectrometry is presented in Figure 9, which shows that samples of interest are digested overnight with trypsin with the purpose of cleaving HCP proteins into peptides. The reducing agent DTT (dithiothreitol) is added the following day for a duration of 30 minutes, after which the digestion is stopped, samples are dried, and then analysed on a nano-LC Orbitrap mass spectrometer. The data is then processed using software for identifying peptides and proteins by correlating the detected peptides against a database which contains a list of previously identified CHO HCPs.

Common contaminants as well as HCPs that are identified on the basis of only one detected peptide are filtered out, and manual data validation is performed based on the MS/MS data quality and the isotope plot data, which removes false or uncertain results and increases confidence in the accuracy of the identified host cell proteins.

However, it should be noted that MS-based HCP detection has its limitations, one of which might be the software (if this is not programmed to search for certain species, then these will not be detected), another limitation can be the database used to correlate the

detected peptides against (if the database is incomplete, then the HCPs can't be identified). A third possible factor that might prove to be an issue for getting good quality data, is the sensitivity of the instrument used for detection, as instruments which have a low resolution won't be able to detect proteins that are present in very low abundance.

If a certain HCP species of interest needs to be identified, ELISAs using antibodies raised against a specific problematic HCP species can be carried out. Alternatively, western blots can be performed – again, either using a polyclonal mix of anti-HCP antibodies to verify the presence of HCPs in general, or using antibodies raised against a specific HCP species to identify the particular HCP species in question (Hogwood et al., 2014). However, this approach is very time consuming and it can be expensive to raise antibodies against several specific types of HCPs. Additionally, only HCP species that are immunogenic and against which antibodies can be raised in the first place, will be able to be identified using this technique (Hogwood et al., 2014). Table 2 summarizes the advantages and disadvantages of each analytical assay, and often it may be necessary to use several orthogonal assays to ensure confident HCP detection and characterisation.

Technique	**Advantages**	**Disadvantages**
ELISA	Highly sensitive & fast measurements of HCP amounts	Only semi-quantitative and unable to identify HCP species
2D-PAGE or 2D-DIGE	Visualizes HCPs and provides information about molecular weight and isoelectric point	Abundant proteins mask less abundant ones and make detection of the latter impossible
LS-MS/MS (coupled with 2D electrophoresis)	Identifies HCP species of interest	Takes a lot of time and work
Western blotting	Confirms presence of (specific) HCP species	Only works on immunogenic HCPs for which antibodies are available; can be expensive

Table 2. Advantages and disadvantages of several analytical tools for the measurement of HCPs.

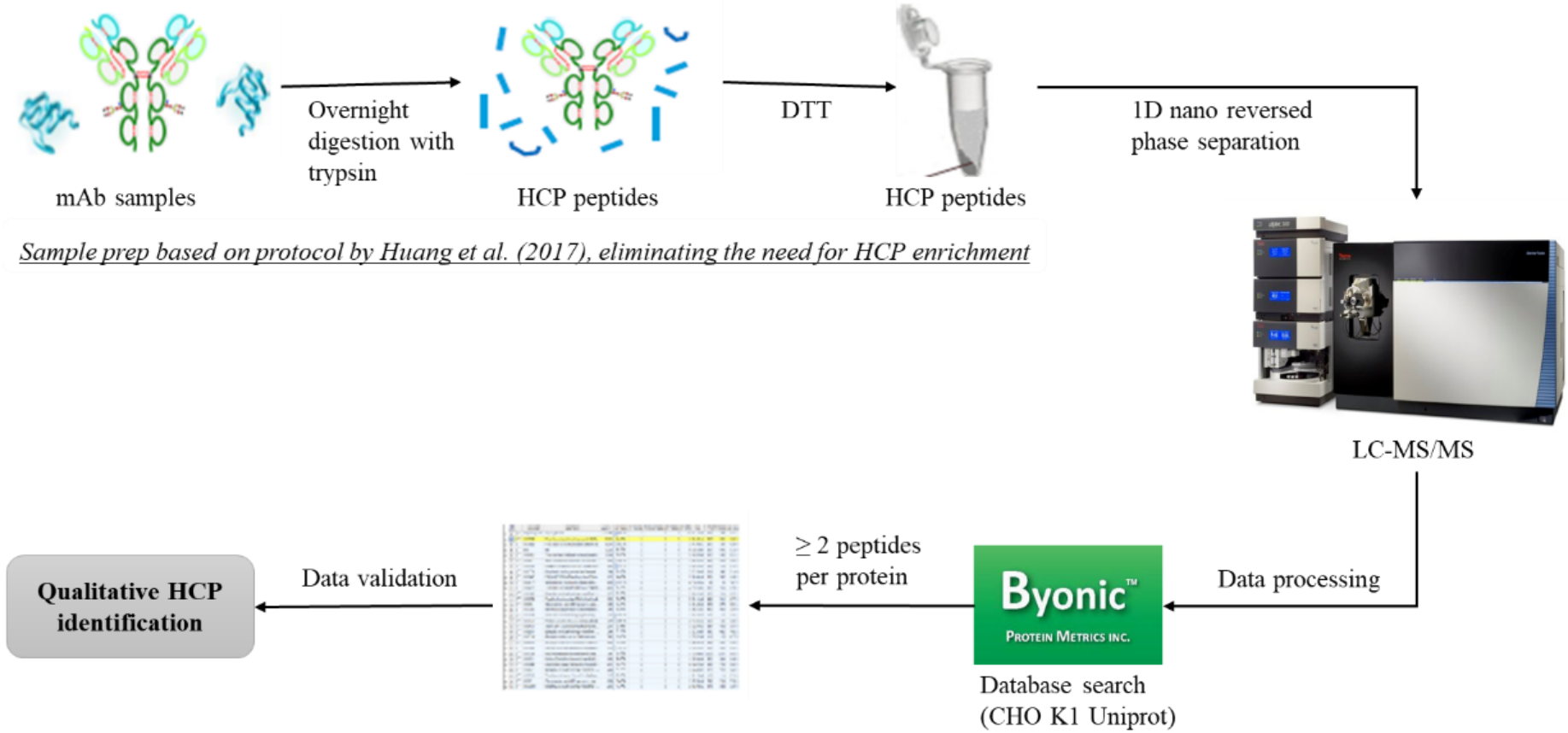


Figure 9. Example workflow for HCP identification by mass spectrometry. Figure adapted from Mitul Patel (GSK) and Huang et al. (2017).

1.5 Disadvantages of segregated bioprocessing and optimisation strategies

Since biopharmaceutical manufacturing processes are developed and delivered by segregated units of operation, all development activity is focused on the specific function of each individual group, such as increased antibody production in the upstream processing group, whereas HCP clearance is one of the aims of the downstream processing group and is not commonly monitored during upstream processing. There are certain disadvantages to this segregated approach: The upstream selection criteria for the best cell lines and culture conditions are based mainly on titre considerations, but adaptations/optimisations to the upstream process conditions with the intent to increase titre will also have an effect on the amount and composition of host cell proteins (Jin et al., 2010). It is therefore possible that certain process conditions cause the HCCF to contain high amounts of HCPs or species that bind particularly tightly to the therapeutic antibody, causing their retention during the purification process. The removal of these HCPs, but also the removal of other process related impurities like DNA and lipids presents a developmental challenge for the downstream group. If the clearance strategy necessitates the implementation of additional purification steps and each step is associated with a loss of product, then the high titre gained upstream may ultimately be negated again. As such, optimisations to the antibody production process in one segregated unit of operation may not necessarily lead to total process optimisation.

From an economic point of view, another disadvantage to this isolated approach is an increase in cost: As it currently stands, the increasingly higher titres produced by upstream processing groups over the last decade have caused the majority of the process costs to shift from upstream to downstream, resulting in the fact that downstream processing now contributes the majority of the cost for the manufacture of biopharmaceuticals (Guiochon & Beaver, 2011; Levy et al., 2014). There are several reasons for this cost shift. For one, the overall cost per gram decreases with increasing

titre, resulting in other costs becoming more pronounced such as the cost of protein A resin. The other effect of higher titres is that the protein A resin binding capacity may reach its limits which will either call for larger amounts of the expensive resin or longer processing times. The latter causes fewer batches per year which increases the COG/g. Lastly, since cultures which produce more titre may also be associated with higher levels of impurities that require a more extensive clearance strategy, the cost for downstream processing further increases due to higher buffer consumption during the purification process, either due to an extended number of purification steps or due to longer processing times. As such, higher titres produced upstream cause longer downstream processing times, more material consumption and overall higher costs to the manufacturing process when using the current downstream processing equipment and industry standard processes. Decisions made during upstream processing can therefore have a big impact on downstream operations.

To address this issue, a more holistic approach to process development is advisable, which considers the impacts on downstream purification during the development of upstream processing. For this, it is crucial to identify how exactly upstream and downstream processes affect each other and determine which operating conditions are critical not just for the best performance of one group but for an optimal entire process. The goal is to aim for total process optimisation rather than just optimisation of each segregated unit of operation. For example, rather than focusing on process conditions that yield the highest titre, process conditions that result in a trade-off between product quantity and purity should be used.

1.6 Conclusion and project aims

Over the past decade, significant improvements have been made to upstream processing and the optimisation of process parameters have led to increasingly higher antibody titres. However, antibody purification issues are not taken into consideration during upstream

development and one side effect of this is that downstream processing has become the most expensive part in the production of therapeutic drugs.

This is partly due to the challenges of carrying out a whole-bioprocess analysis type of research: Processes must be designed and evaluated for every new therapeutic protein candidate, which historically involved large-scale studies that consume resources (i.e. capital, workforce and time) (Titchener-Hooker et al., 2008). The amount of time such studies take may also affect product release and the duration for which the product has market exclusivity which in turn impacts sales proceeds. While it is important to gain process design information as early as possible to address any potential problems, these studies need to be cost-effective enough that it would not be a huge loss should the product fail during any subsequent trials. Capital, workforce and time can be saved by implementing small-scale biochemical engineering methods, which can provide early information about how large-scale downstream processes are likely to perform. (Titchener-Hooker et al., 2008). However, this requires very particular equipment (e.g. small-scale bioreactors, small scale columns, liquid handling robots, automated assays etc.) and if such equipment is not readily available, early process development research is severely limited.

However, another reason that antibody purification issues are not taken into consideration during upstream development is due to the segregated approach to bioprocessing. In order to deal with this aspect, several changes to the antibody production process will need to be implemented: While optimisation of current separation processes will continue, another approach to deal with the new challenges is to consider the manufacturing process more holistically and think about the ease of downstream protein purification during the selection of upstream operating conditions, and essentially integrate upstream and downstream processing. A pre-requisite for this approach is to determine which upstream process parameters are critical not only for high productivity and antibody titre but also

for the ease of purification and good product quality in terms of low aggregation, low fragmentation and low host cell protein impurities.

The impact of upstream operating conditions on downstream performance is still not very well understood, and it is thus highly advisable to investigate the interface between these two groups and define critical process parameters (CPPs) associated with upstream HCCF production. Once these are defined, the optimal operating ranges of the CPPs can be investigated and a traffic light system can be used to highlight which operating conditions have a bad (red) or good (green) effect on the most important critical quality attributes (CQAs). This would efficiently demonstrate whether a robust process with high productivity and purity is achievable for a certain set of upstream operating conditions. The robust operating conditions are likely to vary between molecules, but identification of important CPPs and CQAs should enable a more concise approach to finding a robust window of operation. Recent advances in technology allow for a thorough investigation at this interface of upstream and downstream biopharmaceutical processing. 250 mL or even 15 mL scale-down bioreactor systems enable efficient cell culture experiments using the statistical Design-of-Experiments (DoE) approach where a large number of screening experiments can be done with only small amounts of material. Several factors can be changed within one set of experiments which decreases the number of required experiments and allows the influence of several parameters to be determined and significant ones to be identified (Zhang et al., 2013). Moreover, the use of scale down high-throughput downstream processing equipment like liquid handling robots allow rapid purification of the large number of harvested cell culture fluid (HCCF) samples supplied by the upstream experiments. The 250 mL or 15 mL scale down bioreactors and the small scale, high-throughput robot in particular enable extensive yet time-efficient research into the interactions between upstream and downstream processing as various different upstream process parameters can be tested for their impact on downstream

performance with regards to fast and cost-effective separation and high product purity. Highly sensitive instrumentation such as mass spectrometry and high-throughput assays like the ELISA enable precise analytical support and fast analysis of a large amount of data.

The project described herein focuses on a systematic investigation of the interface between upstream and downstream manufacturing activities. Specifically, this project aims to combine off-line with online orthogonal analytics to generate multi-factorial signatures associated with upstream HCCF and thereafter understand how such outputs impact downstream events (including product recovery, purity and impurity). The following aims were set out at the beginning of the research and will be addressed in the following chapters:

a) Scope upstream processing parameters for several model IgG1 biopharmaceuticals using a range of different upstream operating conditions, and use online and off-line analytics, to identify critical upstream process parameters (CPPs) that impact HCCF quality and downstream product purification – do cell culture pH & temperature, seed density, culture duration have a critical impact on (i.e. significantly change) product amount, viability, post-protein A HCP quantity or product monomer purity? [1]

b) Scope upstream processing parameters for several model IgG1 biopharmaceuticals using a range of different upstream operating conditions to identify HCCF-associated critical quality signatures (indicators for the prediction of poor product quality) that impact HCCF quality and downstream product purification.

[1] Please note, investigated upstream parameters were chosen based on literature reviews (cf. Figure 3) and availability of GSK's resources.

c) Model a design space summarising the optimal process conditions for the entire antibody production process, resulting ideally in HCCF with high titre and low CQA-associated impurities.

d) Scope downstream processing parameters for several model IgG1 biopharmaceuticals using both fixed and variable upstream conditions to identify the robustness of the downstream processing space in terms of improving product quality – do wash buffer, column scale, polishing chromatography have a critical impact on monomer, post-protein A HCP quantity?

In summary, this thesis reflects the work that was done to investigate the interface between upstream and downstream processing and takes a holistic approach to mAb production to identify process parameters that significantly affect both upstream and downstream responses. As harvest material is becoming progressively more difficult to recover with intensified upstream operations, using an integrated approach to upstream and downstream process development strategies enables efficient whole process optimisation, as well as the design of a mutually beneficial operating space for biopharmaceutical production. The applications of the knowledge gained throughout this work will support future biopharmaceutical process development and -robustness strategies.

2 MATERIALS AND METHODS

2.1 Upstream experimental designs

2.1.1 A note on confidentiality

Upstream operating conditions and titre results are confidential and have therefore been redacted throughout this thesis. Operating setpoints have been described in a relative manner: e.g. in the first experiment the pH ranges have been described as –0.15 to + 0.15 in relation to the pH centre point of 0. Temperature and seed density ranges were described in a similar manner. In order to compare experimental setpoints between studies, subsequent experimental ranges were described relative to the first DoE study.

While not being able to disclose actual values might limit the analysis of certain observations or any potential future work involving the replication of the specific operating ranges used here, it does not restrict any conclusions for this thesis since the aim of this thesis is not to provide specific optimal values for upstream operating parameters as these vary from antibody to antibody and process to process anyway.

2.1.2 mAb 1 ambr250 DoE

A Design-of-Experiment (DoE) with a bespoke definitive screening design had been carried out on an ambr250 system (Sartorius, Göttingen, Germany) using a Chinese hamster ovary (CHO) cell line expressing an IgG1 monoclonal antibody (mAb 1) to explore the effects of cell culture seed density, pH, temperature and batch versus fed-batch mode on titre and product quality and to investigate if starting cell cultures with higher seed densities could circumvent the initial few days of cell growth in order to produce desired levels of antibody titre quicker. For this study, small-scale bioreactors were inoculated to the DoE seed density setpoints and maintained at a defined DO and the DoE temperature and pH setpoints. A pH range from -0.15 to +0.15, a temperature range from -3 to +3, and a seed density range from -6.3 to +5 were explored (redacted

seed density values represent ranges in units of 10^6 viable cells/mL and are relative to the seed density centre point) (full experimental design details in Figure 10 and Table 3). Samples were harvested on various days by centrifugation in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 5 mins at 4 °C. Antibody titre was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany), and culture viability was determined by the trypan blue exclusion method using a benchtop Vi-Cell XR (Beckman Coulter, Indianapolis, IN). Samples were then frozen at -80 °C and subsequently thawed prior to further analysis: free antibody light chain levels were measured as described in 2.3.2, samples were affinity purified as described in 2.2.1 and monomer and HCP levels were quantified as described in 2.4.1 and 2.4.2 respectively using GSK's in-house ELISA reagents.

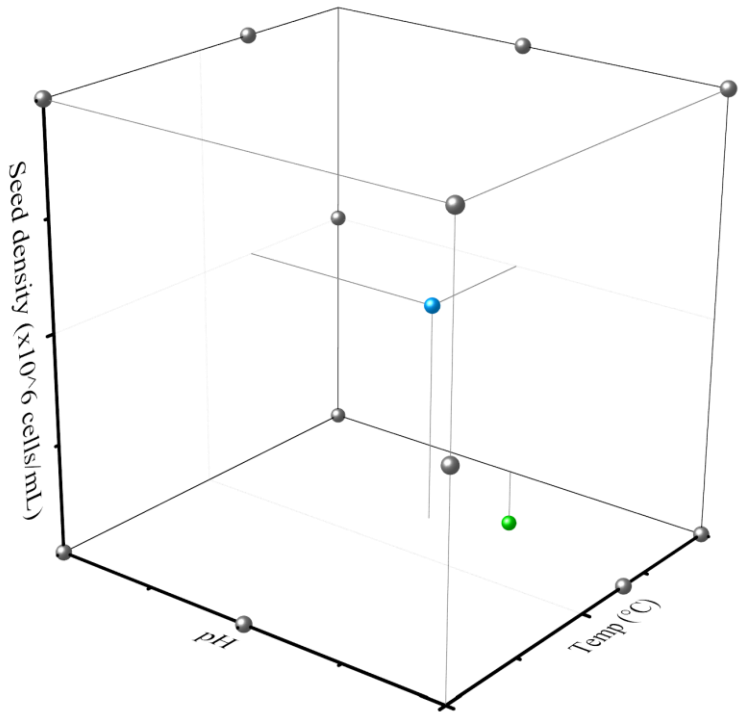


Figure 10. Representation of the experimental space explored in the mAb 1 ambr250 DoE. Setpoints are shown in grey, the 6 centre points (batch and fed-batch) in blue, and the green dot outside of the main frame represents the 2 replicates run at the operating conditions usually used for mAb 1 production.

Culture	Temp	pH	Seed density	Mode
1	+ 3	0	+ 5	Fed-Batch
2	- 3	0	- 5	Batch
3	- 3	+ 0.15	0	Fed-Batch
4	+ 3	- 0.15	0	Batch
5	+ 1	+ 0.15	- 5	Batch
6	+ 1	- 0.15	+ 5	Fed-Batch
7	- 3	+ 0.15	+ 5	Fed-Batch
8	+ 3	- 0.15	- 5	Batch
9	+ 3	+ 0.15	+ 5	Batch
10	- 3	- 0.15	- 5	Fed-Batch
11	+ 3	+ 0.15	- 5	Fed-Batch
12	- 3	- 0.15	+ 5	Batch
13	+ 1	0	0	Batch
14	+ 1	0	0	Batch
15	+ 1	0	0	Batch
16	+ 1	0	0	Fed-Batch
17	+ 1	0	0	Fed-Batch
18	+ 1	0	0	Fed-Batch
19	+ 3	0	- 6.3	Batch
20	+ 3	0	- 6.3	Batch

Table 3. Upstream process parameters of the mAb 1 ambr250 DoE. 0 = middle point of each parameter range, numbers indicate relative values based on 0. Redacted seed density values represent ranges in units of 10^6 viable cells/mL. Cultures were harvested when less than 150 mg/L of antibody was produced over 2 days, although cultures that never reached this rate were harvested discretionally to gather data for comparison. Harvest days for each culture are listed in the appendix. Cultures 13-18 are centre point repeats and 19-20 are replicates of the operating conditions usually used for mAb 1 production.

2.1.3 mAb 1 ambr15 DoE

A further Design-of-Experiment (DoE) – based on the previous design but adjusting for the fact that only two temperatures could be explored – was carried out on the smaller ambr15 system (Sartorius, Göttingen, Germany) using a Chinese hamster ovary (CHO)

cell line expressing the same IgG1 monoclonal antibody (mAb 1) to explore the effects of cell culture seed density, pH and temperature on titre and product quality at a refined range of parameters and to compare these findings between the two different upstream bioreactor scales. As before, small-scale bioreactors were inoculated to the DoE seed density setpoints and maintained at a defined DO and the DoE temperature and pH setpoints. A pH range from -0.15 to +0.25, a seed density range from -6.3 to 0 (relative to the previous DoE) and two different temperatures (-1 and +3; relative to the previous DoE) were explored (full experimental design details in Figure 11 and Table 4).

Samples were harvested on day 17 by centrifugation in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 5 mins at 4 °C. Antibody titre was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany), culture viability was determined by the trypan blue exclusion method using a benchtop Vi-Cell XR (Beckman Coulter, Indianapolis, IN). Samples were then affinity purified as described in 2.2.1 before HCP levels were quantified as described in 2.4.2 using GSK's in-house ELISA reagents.

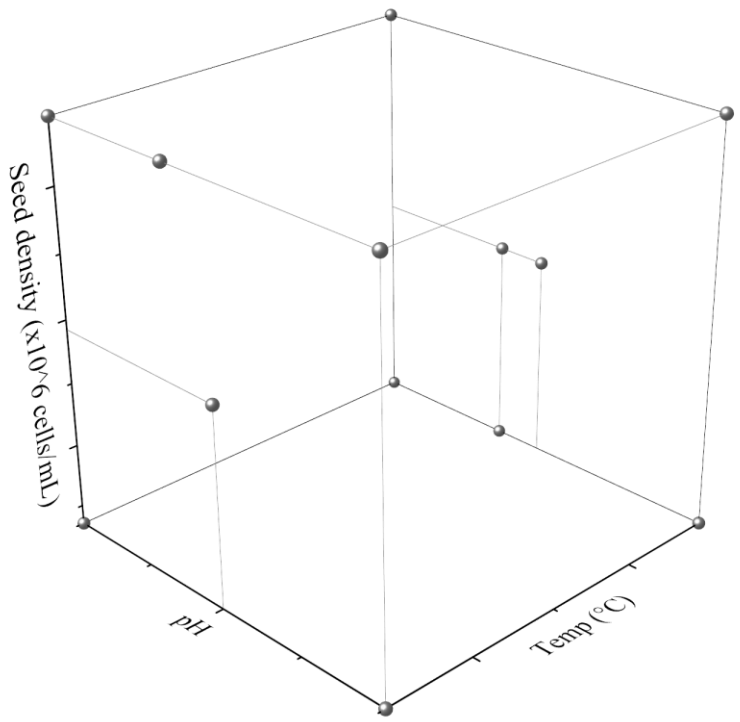


Figure 11. Representation of the experimental space explored in the mAb 1 ambr15 DoE.

Culture	Temp	pH	Seed density	Mode
1	- 1	+ 0.25	- 6.3	Fed-Batch
2	- 1	- 0.15	0	Fed-Batch
3	- 1	- 0.15	- 6.3	Fed-Batch
4	- 1	+ 0.05	- 3.15	Fed-Batch
5	- 1	- 0.15	- 6.3	Fed-Batch
6	- 1	+ 0.25	- 6.3	Fed-Batch
7	- 1	+ 0.25	0	Fed-Batch
8	- 1	+ 0.25	0	Fed-Batch
9	- 1	+ 0.05	- 3.15	Fed-Batch
10	- 1	- 0.15	0	Fed-Batch
11	- 1	0	0	Fed-Batch
12	- 1	0	0	Fed-Batch
13	+ 3	- 0.15	- 6.3	Fed-Batch
14	+ 3	+ 0.25	0	Fed-Batch
15	+ 3	- 0.15	- 6.3	Fed-Batch
16	+ 3	+ 0.25	- 6.3	Fed-Batch
17	+ 3	- 0.15	0	Fed-Batch
18	+ 3	+ 0.25	- 6.3	Fed-Batch
19	+ 3	+ 0.05	- 3.15	Fed-Batch
20	+ 3	+ 0.05	- 3.15	Fed-Batch
21	+ 3	- 0.15	0	Fed-Batch
22	+ 3	+ 0.25	0	Fed-Batch
23	+ 3	0	- 6.3	Fed-Batch
24	+ 3	0	- 3.15	Fed-Batch

Table 4. Upstream process parameters of the mAb 1 ambr15 DoE. 24 fed-batch cultures were grown in 15 mL small scale bioreactor vessels (TAP ambr250) under three different seed densities, 3 different pHs and 2 different temperatures. Numbers indicate relative values based on the process parameters of the previous experiment (mAb 1 ambr250 DoE). Redacted seed density values represent ranges in units of 10^6 viable cells/mL. All cultures were harvested on day 17. Centre points are highlighted in blue and platform conditions in green.

2.1.4 mAb 2 ambr250 DoE

Another Design-of-Experiment (DoE) with a central composite design and with 6 centre point replicates was carried out on the ambr250 system (Sartorius, Göttingen, Germany) using a Chinese hamster ovary (CHO) cell line expressing an IgG1 monoclonal antibody (mAb 2) to explore the effects of cell culture pH and temperature on titre and HCP levels. For this fed-batch process, all vessels were inoculated to the same defined seed density, performed with defined DO and temperature setpoints, and maintained at the same defined pH for the first three days, before the pH was reduced to the various DoE setpoints. A pH range from -0.15 to +0.15 and a temperature range from -2 to +4 (relative to the first DoE) were explored (pH and temperature setpoints are illustrated in Figure 12 and Table 5).

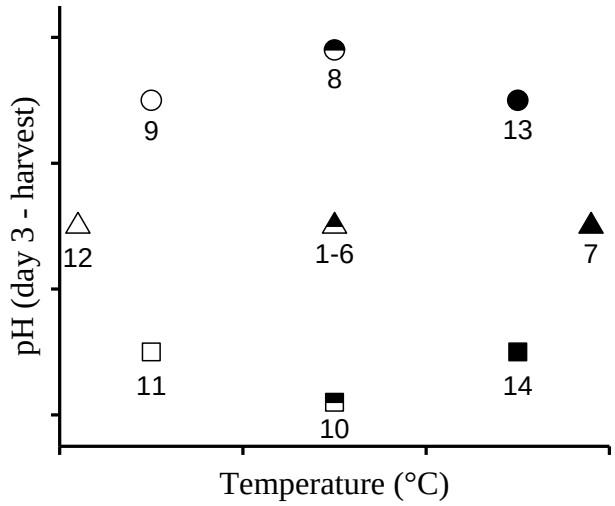


Figure 12. Representation of the experimental space explored in the mAb 2 ambr250 DoE. 14 fed-batch cultures were grown in 250 mL small scale bioreactor vessels (TAP ambr250) under a range of different pHs and temperatures. The empty, half-filled and filled symbols represent low, medium and high temperatures respectively, whereas the squares, triangles and circles represent low, medium and high pH points. All cultures were harvested on day 16.

Samples were harvested on day 16 by centrifugation in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 5 mins at 4 °C. Antibody titre was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany), while culture

viability was determined by the trypan blue exclusion method using a benchtop Vi-Cell XR (Beckman Coulter, Indianapolis, IN). Samples were then frozen at -80 °C and subsequently thawed prior to further analysis: samples were affinity purified as described in 2.2.1 and HCP quantification was carried out on the purified samples as described in 2.4.2 using Cygnus reagents.

Culture	Temp	pH	Seed density	Mode	Harvest day
1	+ 1	0	- 6	Fed-Batch	16
2	+ 1	0	- 6	Fed-Batch	16
3	+ 1	0	- 6	Fed-Batch	16
4	+ 1	0	- 6	Fed-Batch	16
5	+ 1	0	- 6	Fed-Batch	16
6	+ 1	0	- 6	Fed-Batch	16
7	+ 3.8	0	- 6	Fed-Batch	16
8	+ 1	+ 0.14	- 6	Fed-Batch	16
9	- 1	+ 0.1	- 6	Fed-Batch	16
10	+ 1	- 0.14	- 6	Fed-Batch	16
11	- 1	- 0.1	- 6	Fed-Batch	16
12	- 1.8	0	- 6	Fed-Batch	16
13	+ 3	+ 0.1	- 6	Fed-Batch	16
14	+ 3	- 0.1	- 6	Fed-Batch	16

Table 5. Upstream process parameters of the mAb 2 ambr250 DoE. 14 fed-batch cultures were grown in 250 mL small scale bioreactor vessels (TAP ambr250) under a range of different pHs and temperatures. Numbers indicate relative values based on the process parameters of the first experiment (mAb 1 ambr250 DoE). Redacted seed density values represent ranges in units of 10^6 viable cells/mL. All cultures were harvested on day 16. Cultures 1-6 are centre point replicates.

2.1.5 mAb 3 time-course study

This experiment was performed with a Chinese hamster ovary (CHO) cell line expressing an IgG1 monoclonal antibody (mAb 3) to investigate how much impact the culture duration has on the amount of host cell proteins present in post-protein A material, and

whether there is a point during the culture when product titre is already quite high but impurity levels of HCPs are still relatively low. Cells were grown in a 50 L Single Use Bioreactor (Sartorius, Göttingen, Germany) using chemically defined media under defined pH, temperature and DO setpoints. On days 1, 3, 7, 10, 14 and 17 samples were taken and centrifuged in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 5 mins at 4 °C. Antibody titre was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany), while culture viability was determined by the trypan blue exclusion method using a benchtop Vi-Cell XR (Beckman Coulter, Indianapolis, IN). 6 mL per sample were then affinity purified as described in 2.2.1 and HCP quantification was carried out on purified samples as described in 2.4.2 using GSK's in-house ELISA reagents.

2.1.6 mAb 1 shear study I

CHO-expressed IgG1 monoclonal antibody (mAb 1) was cultivated in shake flasks under fixed culture parameters (batch process with defined temperature, DO and pH setpoints, and with glucose addition on day 7) using chemically defined media. Samples from the shake flasks were taken and pooled at four time points (days 10, 17, 20 and 24) to generate samples with a wide range of viability percentages. To mimic harsh mechanical conditions that might cause cell breakage during cultivation (e.g. bioreactor impeller type, impeller speed or sparge rate) and/or harvesting (e.g. improper use of a centrifuge), a rotating cell shearing device (with a stainless-steel chamber of 50 mm diameter and 10 mm height, and a rotating disc of 40 mm diameter and 1 mm thickness) was used. This ultra-scale-down (USD) centrifugation technology was developed at UCL and has previously been described in Hutchinson et al. (2006) and Tait et al. (2009).

Half of the material from each time point was sheared at 12,000 rpm for 20 seconds which is equivalent to 0.53 x 10^6 W/kg. These settings have previously been determined by Hutchinson et al. (2006) and Rayat et al. (2016) to be sufficient time for full cellular

break-down which might occur during harsh treatment. The shear device chamber was carefully filled with 20 mL aliquots of cell culture material using a 50 mL syringe – excess material was used to flush out any air bubbles trapped in the chamber. The selected shear was then applied to the material for 20 seconds, after which the material was removed from the chamber with a second syringe. This process was repeated with fresh cell culture material until enough material for protein A purification was obtained. The other half of the cell culture material was not exposed to this particular shearing technique. Both sheared and non-sheared material was subsequently centrifuged in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 20 mins at 4 °C and filtered using 0.2 µm syringe filters (Mini Kleenpak™ 25 mm syringe filters with 0.2 µm Supor® EKV membrane, Pall Corporation, Portsmouth, UK). Antibody titre was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany), while culture viability was determined by the trypan blue exclusion method using a benchtop Vi-Cell XR (Beckman Coulter, Indianapolis, IN). Samples were affinity purified as described in 2.2.2 using 4.7 mL HiScreen columns pre-packed with protein A resin, as well as polished by anion and cation exchange chromatography (2.2.4 and 2.2.5 respectively). HCP quantification was carried out on the purified samples as described in 2.4.2 using GSK's in-house ELISA reagents.

2.1.7 mAb 1 shear study II

A second shear study was carried out to further examine the shear sensitivity of cells by analysing culture time points that hadn't been investigated in the first shear study. As such, this study was performed with the same mAb 1 that was also used in the first shear study, and as before, the antibody was cultivated in shake flasks under fixed culture parameters (batch process with defined temperature, DO and pH setpoints, and with glucose addition on day 7) using chemically defined media. Additionally, mAb 1 was also cultivated in 2 L bioreactors under the same operating conditions. Samples were taken on

days 8, 10, 13, 15 and 17 (and 21 in the case of shake flask cultures). Once again, a rotating shear device was used to apply mechanical stress to the cells. In addition to shearing cells at 12,000 rpm as in the first study, material was also sheared at 6,000 rpm for 20 seconds (equivalent to 0.06 x 10^6 W/kg which cells are likely to experience in the feed zone of a hydro-hermetic disk stack centrifuge (Chatel et al., 2014)). All material was subsequently centrifuged in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 20 mins at 4 °C and filtered using 0.2 µm syringe filters (Mini Kleenpak™ 25 mm syringe filters with 0.2 µm Supor® EKV membrane, Pall Corporation, Portsmouth, UK). The samples' turbidity was measured before and after filtration to determine clarification using the LaMotte 2020we/wi turbidity meter (LaMotte Company, Maryland, US), while antibody titre and culture viability were determined as described previously. Samples were then affinity purified as described in 2.2.2. using 1 mL HiTrap columns pre-packed with protein A resin. Monomer and HCP quantification was carried out on the purified samples as described in 2.4.1 and 2.4.2 using GSK's in-house ELISA reagents.

2.1.8 mAb 4 case study

A comprehensive DoE with a central composite design including 3x centre point replicates was performed on the ambr250 system (Sartorius, Göttingen, Germany) using a Chinese hamster ovary (CHO) cell line expressing an IgG1 monoclonal antibody (mAb 4). The DoE was carried out twice with two different upstream processes – fed-batch version A (FBvA) and fed-batch version B (FBvB). The differences between these two versions are summarised in Table 6.

Within each process version, the following factors and ranges were explored: pH -0.15 to +0.15, temperature 0 to +4, and seed density -6.5 to -5 (relative to the mAb 1 ambr250 DoE setpoints) (see experimental design details in Figure 13).

Fed-batch version A	Fed-batch version B
DO is x %	DO is y %
Initial pH is +0.25 ± 0.15	Initial pH is +0.05 ± 0.15
Final pH* is 0 ± 0.05	Final pH* is 0 ± 0.10
Day 3 supplement	No day 3 supplement
Different production medium and feeding strategy	Production medium contains more glucose and less salt; Nutrient feed is slightly richer; Feeding strategy and volume is slightly different to avoid weekend feeding
Culture duration is 17 days	Culture duration is 15 days

*Table 6. Differences between the two fed-batch processes. *pH shift is on day 3 prior to feeding.*

Note that during FBvA, two additional cultures were grown at the upstream industry standard conditions for mAb 4 (i.e. at the default upstream operating conditions used in the production of this molecule based on an extensive legacy of biopharmaceutical manufacture; marked in green) but using an alternative cell line, while during FBvB, two additional cultures were grown at the upstream industry standard conditions (marked in green) but using the FBvA process, coming to a total of 24 cultures.

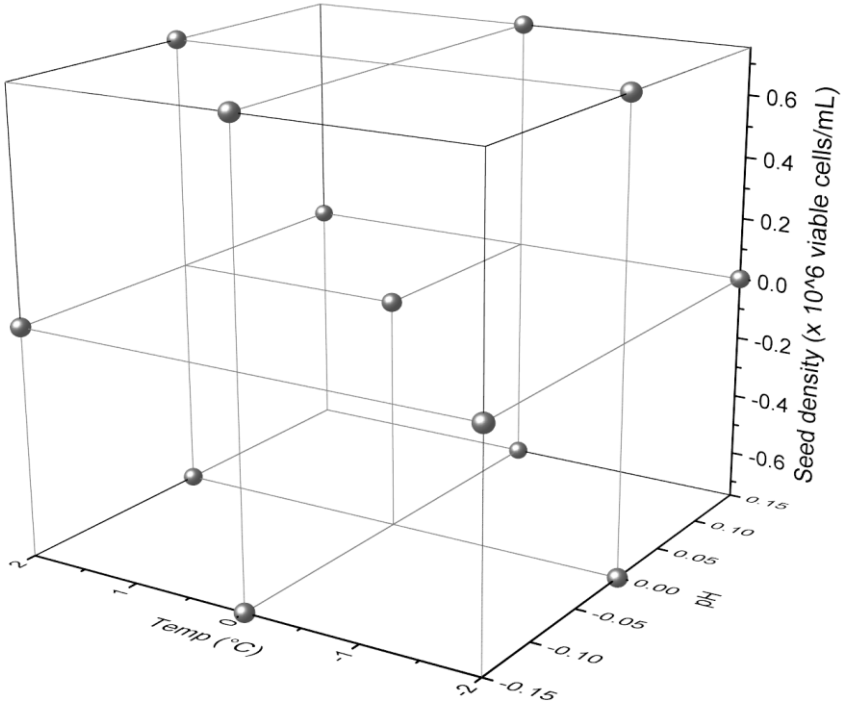
Samples were harvested by centrifugation in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 3300 g for 10 mins at 4 °C. Antibody titre was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany), before samples were affinity purified as described in 2.2.1. Monomer and HCP levels were quantified as described in 2.4.1 and 2.4.2 respectively using GSK's in-house ELISA reagents.

In addition to this, time-dependent effects on product quality were studied by taking samples on days 8, 10, 13 and 15 during cultivation of the mAb 4 culture grown using

FBvB and the upstream industry standard process conditions. These samples were processed in the same way as the DoE samples.

A few select samples (including the mAb 4 culture grown using the upstream industry standard process conditions) from FBvA were also chosen for investigations into alternative protein A wash buffers. These samples were protein A purified on 1 mL HiTrap columns as described in 2.2.2 with four different wash buffers (equilibration buffer, downstream industry standard buffer, alternative wash buffer A, alternative wash buffer B) before they were analysed as described in 2.4.1 and 2.4.2. These same samples were also protein A purified on 4.7 mL HiScreen columns as described in 2.2.2 in order to produce enough material for an investigation into polishing chromatography which was carried out as described in 2.2.4. After both the protein A purification and the AEX step, material was analysed as described in 2.4.1 and 2.4.2. Given the complexity of this integrated case study, the methods described herein have been summarised in Figure 14 and Figure 15.

(A)



(B)

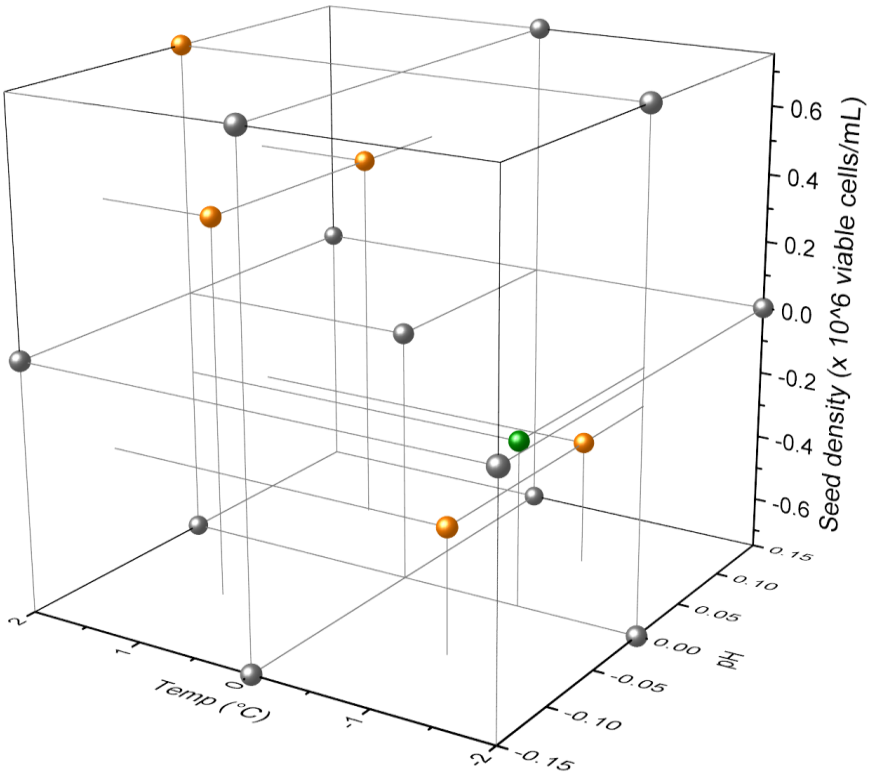


Figure 13. Representation of the experimental space explored in the mAb 4 ambr250 DoE. (A) 15 DoE points in grey (3x centre point replicates). (B) Additional runs to validate the model (orange) and duplicate mAb 4 industry standard conditions (green).

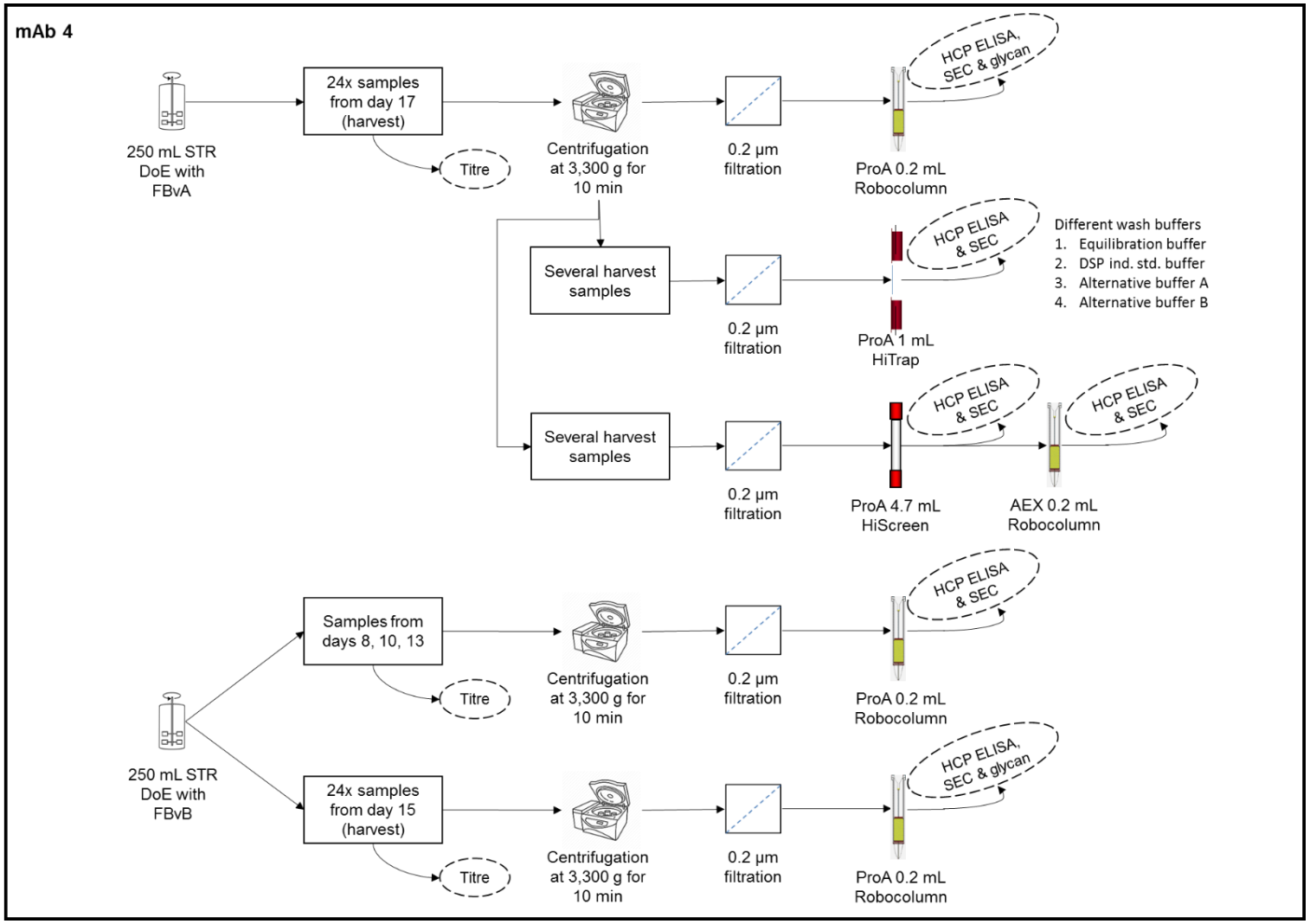


Figure 14. Summary of mAb 4 case study. DoEs, time-course studies and downstream robustness studies.

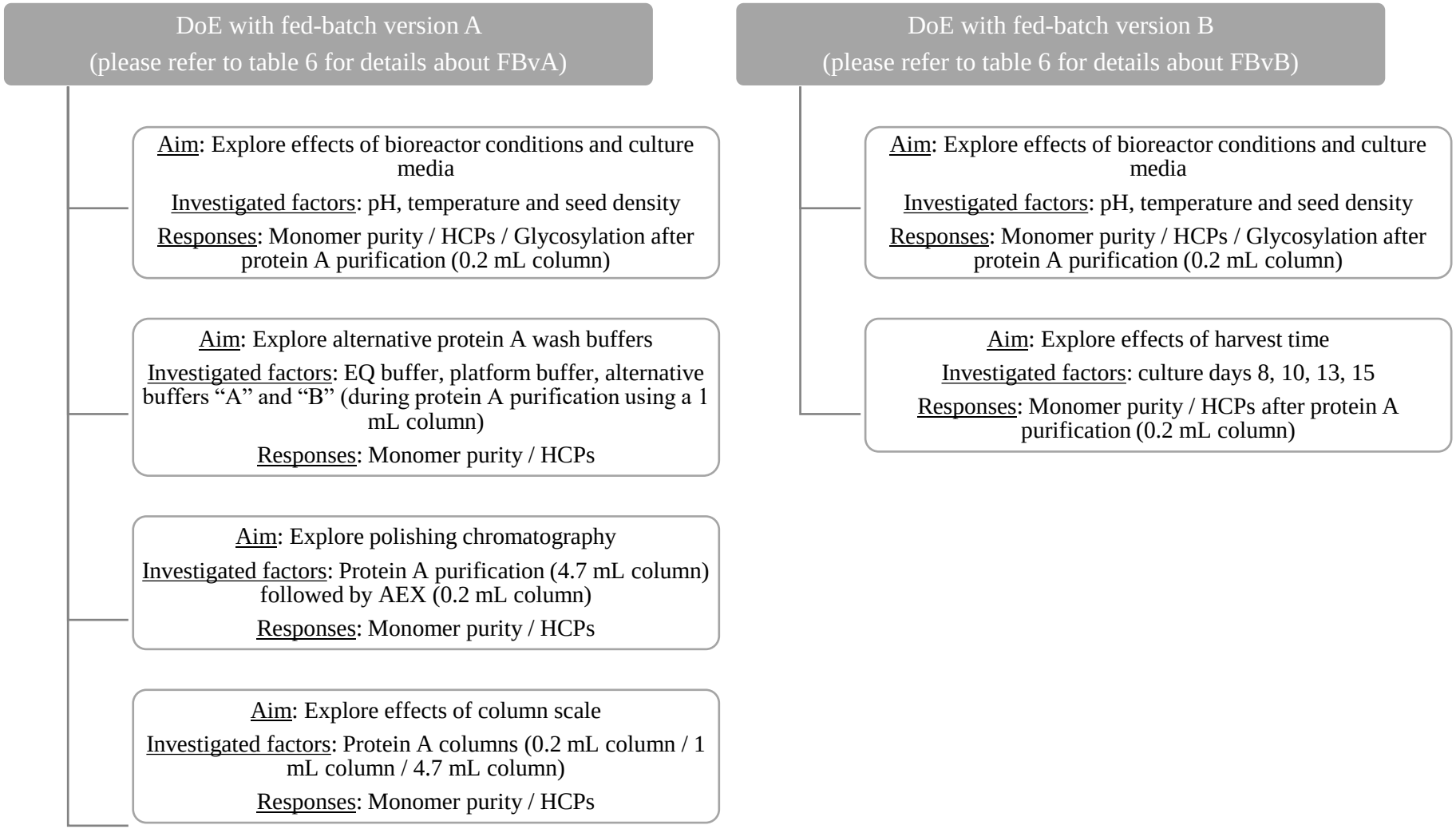


Figure 15. Diagram to visualise experimental aims as well as inputs and outputs of each study performed as part of this integrated case study.

2.2 Downstream purification

2.2.1 Protein A purification on the TECAN liquid handling robot

Supernatant samples were 0.2 µm filtered and affinity purified on prepacked 0.2 mL MabSelect SuRe™ (GE Healthcare, Uppsala, Sweden) RoboColumns (Atoll GmbH, Weingarten, Germany) using a liquid handling robot (Freedom Evo 200 Robot with EVOware standard software; Tecan, Mannedorf, Switzerland). The purification protocol was a scale-down mimic of the typical downstream processing method for mAbs. Columns were equilibrated with a Tris acetate buffer (pH 7.5) before loading 35 mg product / mL resin (unless otherwise specified) at a flowrate of 0.2 mL/min. This was then followed by a column wash step with a Tris acetate buffer containing caprylate (pH 7.5) and a re-equilibration step before product elution using a sodium acetate buffer (pH 3.6). The eluate from each column was collected across 10 fractions using a 96 well collection plate, lined with 10 µL of 1 M tris base to neutralise the eluate. The UV at 280 nm was read by a Magellan Infinite 200 plate reader (Tecan, Mannedorf, Switzerland) and fractions containing high levels of protein were pooled. Pooled protein concentrations were checked on a NanoDrop 1000.

2.2.2 Protein A purification on the AKTA Avant systems

Supernatant samples were 0.2 µm filtered to remove all cell debris and prepare samples for affinity purification. A 1 mL MabSelect SuRe protein A HiTrap column (GE Healthcare, Uppsala, Sweden) or a 4.7 mL MabSelect SuRe protein A HiScreen column (GE Healthcare, Uppsala, Sweden) (as specified) was used for the purification, using the same steps as previously described for the RoboColumns, and loading to 85% of the manufacturer's suggested dynamic binding capacity. Where specified, protein A eluate samples were then further processed by anion exchange chromatography as specified in 2.2.4.

2.2.3 *Low pH viral inactivation*

Low pH viral inactivation was done by titrating protein A eluates to pH 3.5 using 30 mM Acetic Acid / 100 mM HCl and holding samples at this low pH for 30 minutes before adding 1 M Tris base to raise the pH to 7.5 again. Samples were then 0.2 µm filtered to remove any precipitation caused by the low pH hold.

2.2.4 *AEX on the TECAN liquid handling robot*

Where specified, protein A eluate samples were further processed by anion exchange chromatography in flow-through mode using 0.2 mL pre-packed ATOLL RoboColumns. The flow-through material was collected and, where specified, polished with a final cation exchange chromatography step as described in 2.2.5.

2.2.5 *CEX on the AKTA Avant system*

Where specified, anion exchange flow-through material was polished with a final cation exchange chromatography step, using 1mL MiniChrom columns (Repligen, Waltham, MA).

2.3 Analytical assays on harvested cell culture fluid

2.3.1 *Lactate dehydrogenase*

LDH was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany).

2.3.2 *Osmolality*

Culture osmolality was measured using a OsmoPRO Multi-Sample Micro-Osmometer (Advanced Instruments, Horsham, UK).

2.3.3 *Free antibody light chains*

To measure the amount of free antibody light chains present in harvested cell culture fluid, samples were run on a ThermoScientific MabPac RP 4 µm 50 x 2.1 mm column

(Agilent HP1290) by loading duplicates of each sample onto the column with the following method conditions:

Load volume	5 µL
Mobile Phase A	Water + 0.1 % TFA (v/v)
Mobile Phase B	Acetonitrile + 0.1 % TFA (v/v)
Flow Rate	0.9 mL / min
Column Temperature	80 °C
Sample Temperature	5 °C
Wavelength	280 nm & 214 nm
Gradient	0 – 100 %

Table 7. HPLC method conditions for the measurement of free antibody light chains.

The elution peak sizes were compared against two markers, one for the whole mAb analysed and another for the mAb light chain, using Empower 3 software. Reported results are averages of the duplicate injections of each sample.

2.3.4 Cholesterol

Since cholesterol is a lipid that is present in cell membranes, it was explored if cholesterol content as a measure of cell lysis could be used as an indicator for post-protein A HCP levels. For this, a commercial cholesterol assay kit (E2CH-100, BioAssay Systems, California, USA) was used to determine the cholesterol content in various samples. Based on previous literature (Senczuk et al., 2016), the detectable levels were expected to be low, so the fluorometric procedure of the manufacturer's protocol was followed, for which the linear detection range is 0.2 to 10 mg/dL. A single working reagent is used in the EnzyChromTM cholesterol assay which combines cholesterol ester hydrolysis, oxidation and colour reaction in one step. The fluorescence intensity of the reaction product at λem/ex = 585/530 nm is directly proportional to total cholesterol concentration

in the sample, and is calculated using the following equation, where "F_{Blank}" is the reading obtained for the sample containing only assay buffer:

$$[\text{Cholesterol}] = \frac{F_{Sample} - F_{Blank}}{\text{Slope}} (\text{mg/dL})$$

2.4 Analytical assays on processed material

2.4.1 Size exclusion chromatography

Product monomer, aggregation and fragmentation percentages in processed samples were determined by size exclusion chromatography (HPLC-SEC) using an Agilent HPLC system (Agilent 1100 series) and a 7.8 mm x 300 mm TSKgel G3000SWXI column (Tosoh Bioscience) with a running buffer containing sodium phosphate (monobasic) and sodium chloride (pH 6.7). The flow rate was 1 mL/min and protein was detected using UV detectors at 214 nm and 280 nm. The SEC data was analysed on ChromView for ChemStation version 2.4.2 and has an accuracy of ±0.5% as previously established by GSK's analytical team.

2.4.2 HCP-ELISA

ELISAs were used to quantify total immunoreactive HCP content in processed samples using GSK's in-house anti-CHO HCP antibodies raised against media from null CHO cells with GSK's antigen standard (except for samples from the mAb 2 ambr250 DoE where commercial Cygnus anti-CHO HCP antibodies (3G-0016-AF and 3G-0016-AFB, Cygnus Technologies, Southport, NC) with antigen standard (F018H; Cygnus Technologies, Southport, NC) was used as the Cygnus antibodies were raised against CHO cell lysate which should detect more HCPs in lower viability cell cultures). The polyclonal mix of antibodies was immobilized on 96-well plates and diluted samples were

added to the wells. Serial dilution was performed for the samples to ensure results were within the quantification range of the standard curve. After sample incubation at 24 °C and 400 rpm for 1 hour, goat anti-HCP biotinylated antibody was incubated under the same conditions, followed by a final incubation with streptavidin–horseradish peroxidase (HRP). The HRP enzymatic activities were quantified using substrate and stop solution by SeraCare Life Sciences (Milford, MA, United States), and measuring UV absorbance with the SpectraMax190 Microplate Reader (Molecular Devices, San Jose, CA, United States). Results were analysed using SoftMax Pro version 5.4.4 and were only accepted if calibration curves had an R^2 value of at least 0.95 with concentrations of the calibration curves accurately back calculated to 100±20% of the expected concentrations with %CV's below 20%. The same criteria was applied to the high and low controls.

2.4.3 Mass spectrometry for HCP identification

HCP species present in various samples were identified by mass spectrometry. Samples were prepared by adding 40 µg of protein A purified mAb to 45 µL of 50 mM ammonium bicarbonate and then adding 1 µg/µL trypsin in a 20:1 mAb/trypsin ratio. Samples were incubated overnight at 37 °C, and the next day 5 µL of 100 mM DTT were added prior to a further incubation period of 30 minutes at 37 °C. The digestion was stopped by adding 1 µL neat formic acid and then drying the samples by speed-vacuum. Samples were re-dissolved in 40 µL 0.1% formic acid and then analysed on a nano-LC Orbitrap mass spectrometer. Common contaminants as well as HCPs with only one or two peptides (as specified) have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data. Biological process information was obtained for all identified HCP species by searching the UniProt database using protein accession numbers.

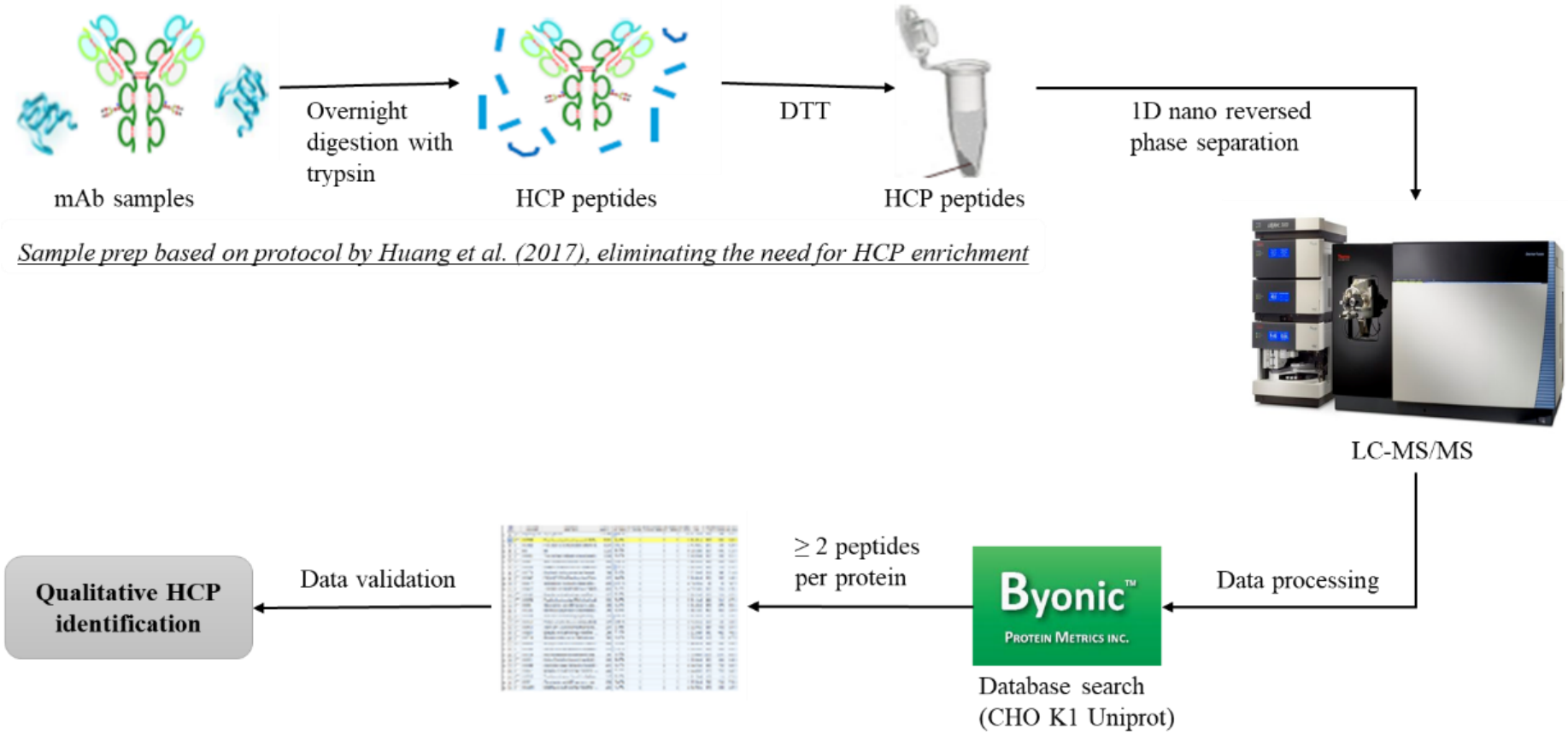


Figure 16. Workflow for HCP identification by mass spectrometry. Figure adapted from Mitul Patel (GSK) and Huang et al. (2017).

2.4.4 *Glycan analysis*

Glycan analysis was carried out by GSK's analytical group using Waters RapifluorMS UPLC to determine the relative % areas of observed N-glycans. Samples were denatured, N-glycans were released and labelled with RapifluorMS before subsequently being loaded onto a HILIC SPE cartridge. Samples were processed using MassLynx v4.1 software and a Waters RapifluorMS Performance Standard was used to check the system suitability.

2.5 Data analysis

DoE results were analysed and plotted with JMP 13.2.1 (SAS Institute, Inc., Cary, NC). All other graphs were created in OriginPro 2016 software (OriginLab Corporation, Northampton, MA).

The heat maps were created in JMP by displaying three variables (two operating factors and one response) in a two-dimensional view where the third variable is represented by contour curves of equal value. Contour values were specified so that minimum values, maximum values and increments were the same for all heat maps that were to be compared with each other.

Standard deviation was calculated using the following equation,

$$\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

where x is the sample mean average and n is the sample size.

Correlation coefficients were calculated using the following equation,

$$Correl(X,Y)=\frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\bar{x})^2\sum(y-\bar{y})^2}}$$

where $\bar{x}$ and $\bar{y}$ are the sample means average(array1) and average(array2).

3 EFFECTS OF UPSTREAM CULTURE CONDITIONS ON MAB-ASSOCIATED POST-PROTEIN A HCP IMPURITIES

3.1 Introduction

Traditionally, biopharmaceutical manufacturing processes are developed and delivered by several segregated units of operation. Isolated decisions made during upstream processing can result in unfavourable conditions for downstream product purification and stability profiles as well as cause increased production costs. For instance, certain bioreactor process parameters will increase product titre but may also negatively affect the product quality in terms of its monomer purity and HCP profile. All impurities need to be removed during downstream processing so if upstream conditions result in material with more impurities, these will need to be cleared during downstream processing which affects the workload and process cost. Due to the segregated approach to bioprocessing, antibody purification issues are not taken into consideration during upstream development and this can place increasing demands on downstream processing.

The aim of this chapter is to explore the impact of upstream operating conditions on downstream performance and evaluate potential trade-offs between titre and product quality, using a Design-of-Experiment (DoE) approach which is a common industry standard to optimise process parameters during process development. An example of the DoE approach is described extensively in “A-Mab: A Case Study in Bioprocess Development” (CMC Biotech Working Group, 2009).

The first DoE discussed in this chapter was carried out on an ambr250 system (Sartorius, Göttingen, Germany) using a Chinese hamster ovary (CHO) cell line expressing an IgG1 monoclonal antibody (mAb 1) to explore the effects of cell culture seed density, pH, temperature and batch versus fed-batch mode on titre and product quality and to investigate if starting cell cultures with higher seed densities could circumvent the initial

few days of cell growth in order to produce desired levels of antibody titre more quickly. For this study, small-scale bioreactors were inoculated to the DoE seed density setpoints and maintained at a defined DO and the DoE temperature and pH setpoints. A pH range from -0.15 to +0.15, a temperature range from -3 to +3, and a seed density range from -6.3 to +5 was explored (full experimental design details in Figure 10 and Table 3). While the upstream aspect of this experiment had been designed and carried out by GSK's upstream processing group prior to the start of this thesis work, it was deemed to be an excellent starting point to investigate the effects of various upstream process parameters on upstream and downstream responses.

Further to the first DoE, a second DoE with the same molecule was eventually carried out at an even smaller bioreactor scale (15 mL bioreactors compared to 250 mL bioreactors in the first DoE) and using slightly different upstream process parameter ranges, in order to expand the dataset and compare outputs between the two bioreactor scales.

A third DoE – which had also previously been designed and carried out by GSK's upstream processing group – was subsequently investigated in order to include a further model IgG1 mAb in the research. The third DoE was comparable in scale to the first DoE as both were carried out on 250 mL small-scale bioreactors, and both studies explored similar pH and temperature ranges, although seed density had not been included as a factor in the third DoE. A summary of all three experiments [2] is illustrated in Figure 17. Using these three experiments, this chapter explores effects of upstream culture conditions on harvested cell culture fluid (HCCF) quality – specifically antibody titre and culture viability – as well as on mAb product quality. It also investigates correlations amongst these outputs [3].

[2] The first and third DoE had been carried out by GSK's upstream processing group prior to the start of this research project, while the second DoE as well as the downstream processing, analytical assays and data analysis of all three DoEs was carried out by me.

[3] The window of operation shown in figure 33 (along with results discussed in chapter 5) has been published in Wilson et al., 2019.

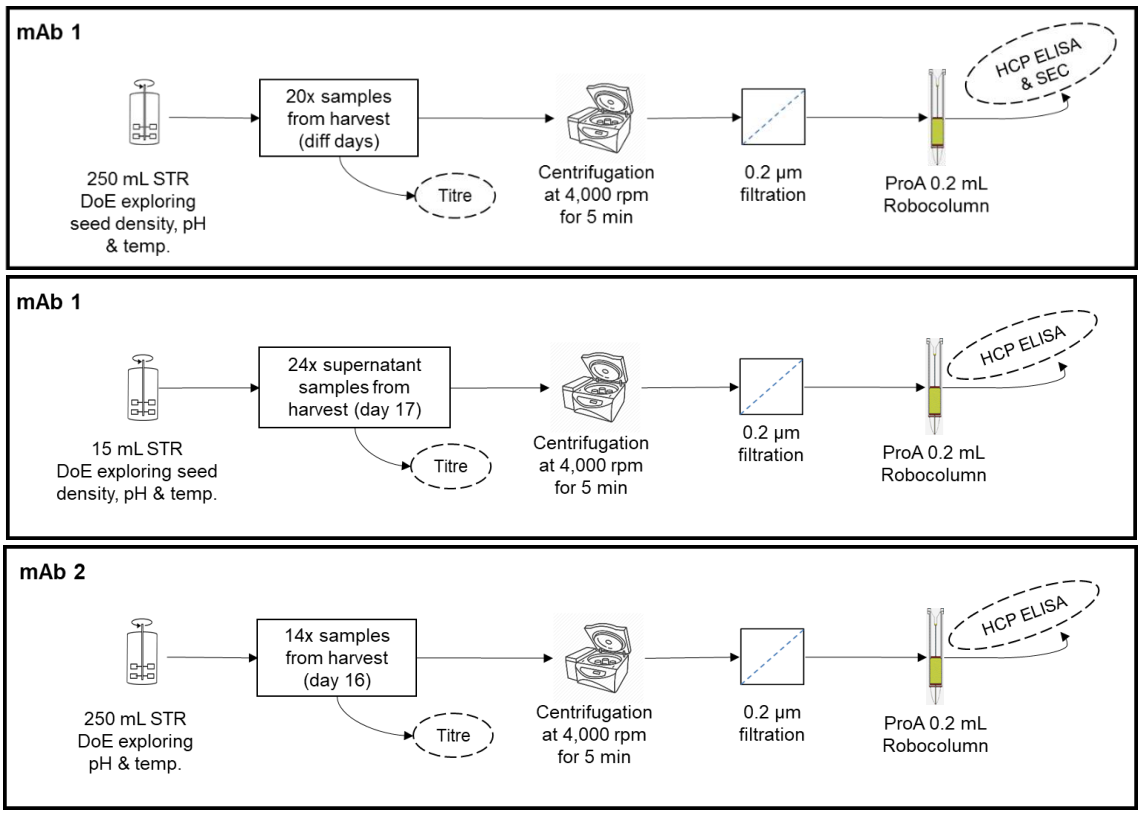


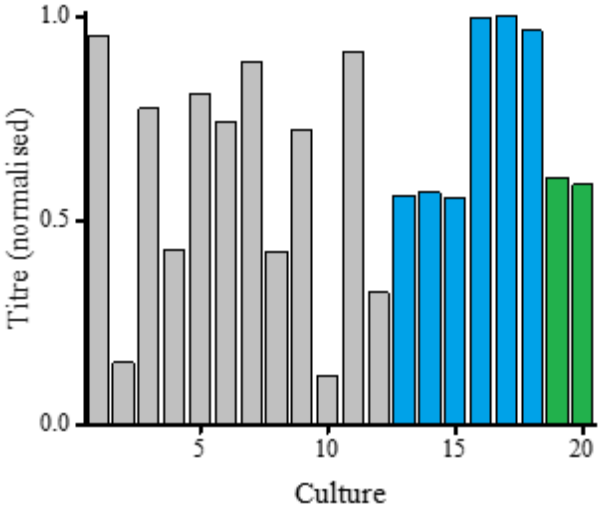
Figure 17. Summary of DoE experiments discussed in chapter 3 (mAb 1 ambr250 DoE, mAb 1 ambr15 DoE, mAb 2 ambr250 DoE).

3.2 Effects of upstream culture conditions on HCCF quality

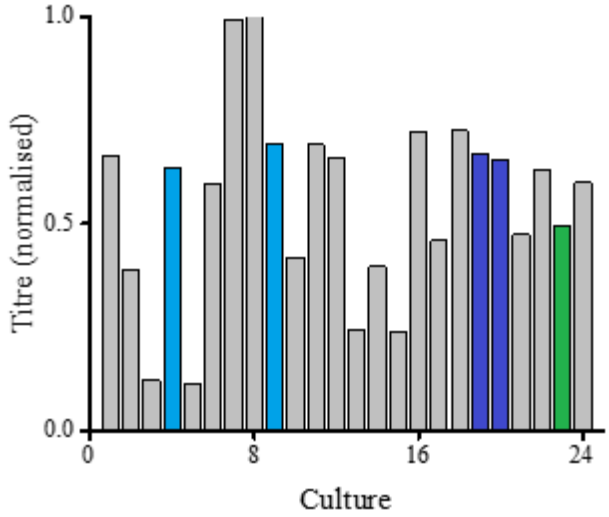
3.2.1 Titre

During upstream processing of biopharmaceuticals, the primary response of interest is antibody titre. As such, upstream process parameters are optimised with the goal of creating ideal culture conditions and increase titre as much as possible. The figure below shows the titre results for three different experiments – the 250 mL bioreactor DoE using mAb 1 (A), the 15 mL bioreactor DoE using mAb 1 (B), and the 250 mL bioreactor DoE using mAb 2 (C). As Figure 18 A-C illustrates, the titre produced by cultures grown under different bioreactor conditions varies quite significantly (e.g. by more than 150% in the first DoE). Figure 18 A also highlights how changes in operating conditions can improve or reduce titre (compared to the control in green) and demonstrates that similar titres are achieved by replicate operating conditions (vessels 13-15; 16-18; 19-20).

(A)



(B)



(C)

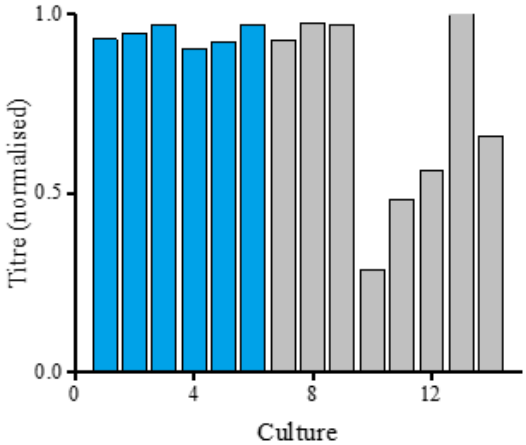


Figure 18. Titre results for the three DoEs. Due to confidentiality, results are normalised to show titre as a comparison to other samples of the same DoE, rather than as actual measurements. (A) mAb 1 ambr250 DoE, (B) mAb 1 ambr15 DoE, and (C) mAb 2 ambr250 DoE. Samples have been colour coded with blue samples representing the centre point conditions of each DoE and green samples representing the platform conditions (i.e. the control) where applicable. Note in (B) centre points are differentiated for low temperature samples (light blue) and high temperature samples (dark blue).

Since bioreactor operating conditions have such a big impact on the amount of antibody that is produced by cultures, their optimal ranges need to be identified. Each of the three DoE studies discussed in this chapter has been analysed with the statistical software JMP 13.2.1 (SAS Institute, Inc., Cary, NC). The prediction and interaction profilers below were generated by fitting least squares regression models for the titre results. For the mAb 1 ambr250 DoE (predicted RMSE = 211.07, R^2 = 0.95, p value < 0.0001), it can be seen that more titre is produced when cells are inoculated with a higher seed density and grown at higher pH in fed-batch mode. An increase in temperature is initially beneficial for higher titre, although it seems that a temperature above +1 / +2 results in lower titre for these mAb 1-producing CHO cells using this particular process (Figure 19 A). However, the prediction profiler is based on all data points from this experiment and does not distinguish between different factors. While the confidence intervals give an indication of the variance between results by showing a broader range at high temperature in this instance, it is important to look at the interaction profiler to determine how factors affect each other. In Figure 19 B, we can see that the impact of temperature on titre is linked with the seed density. An increase in temperature to +3 is productive for cultures seeded at -5 but less so at a seed density of +5. This is most likely because a temperature of +3 is promoting cell growth which is favourable for low density cultures to enhance productivity. In contrast, operating high density cultures at a temperature that promotes more cell growth will likely be detrimental and result in increased cell death due to limited nutrient supply. A slight interaction is also seen between temperature and feed mode with the benefits of fed-batch mode being enhanced at higher temperature perhaps due to cells being more metabolically active at higher temperature and thus consuming media components more readily. The effects of seed density, pH and temperature are summarised as heat maps in Figure 19 C-E with high titres shown in green and low titres in red.

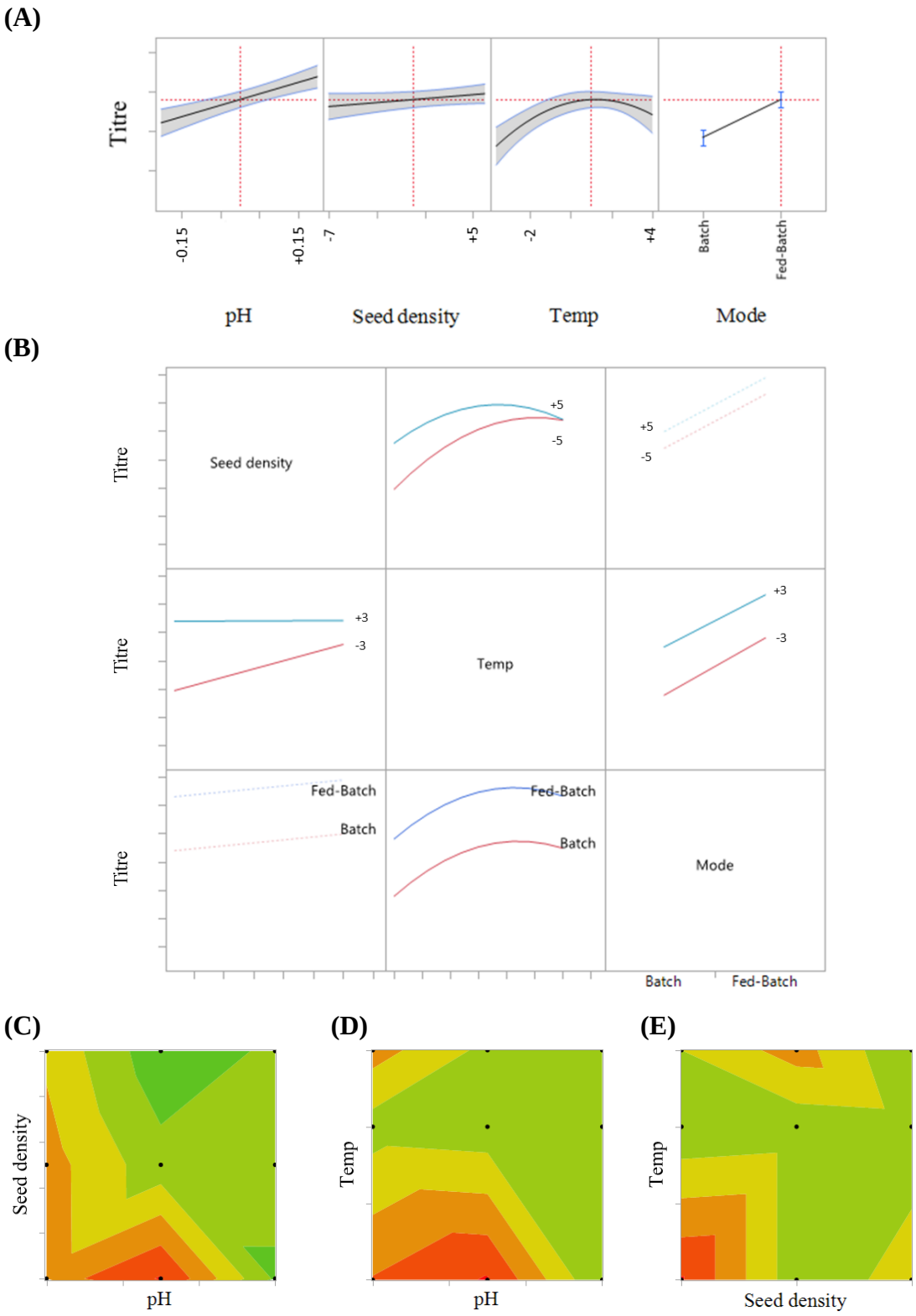


Figure 19. Effects of culture conditions on titre for the mAb 1 ambr250 DoE (R^2= 0.95) as prediction profiler (A), interaction profiler (B), and heat maps (C-E). In the heat maps, high titre levels are indicated in green and low titre levels in red. Contour values were specified so that minimum values, maximum values and increments were the same for all three heat maps with 0.5 g/L increments. Black dots represent the experimental data points.

For the mAb 1 ambr15 DoE (low temperature: predicted RMSE = 68.43, R^2 = 0.99, p value < 0.0001; high temperature: predicted RMSE = 162.5, R^2 = 0.91, p value = 0.004), the results are separated by low and high temperature. Since the culture temperature for the ambr system is controlled by culture stations, and only two of these were available for this study, only two temperature setpoints could be included in the experimental design (-1 and +3) and as such they were analysed as a categorical factor rather than a continuous one. Nonetheless, results show similar trends as in the first DoE: a higher seed density and culturing at higher pH leads to increased titre production, especially at a low temperature of -1 (Figure 20 A-C). This case study illustrates the importance of analysing factor interactions in order to explain the great variance seen in one of the prediction profilers (Figure 20 D). At a high temperature of +3, an increase in titre due to operating at higher pH is only effective in cultures that were inoculated to a low seed density (Figure 20 E-F). For high seed density cultures, an increase in pH only marginally increases the titre, presumably because both higher temperature as well as higher pH promote cell growth (Trummer et al., 2006) and operating high seed density cultures under these conditions will therefore likely result in decreased culture viability and be detrimental to an increase in antibody production. These observations correlate with the previous DoE, demonstrating that the investigated culture conditions have the same effect on antibody production at 15 mL as well as 250 mL bioreactor scale.

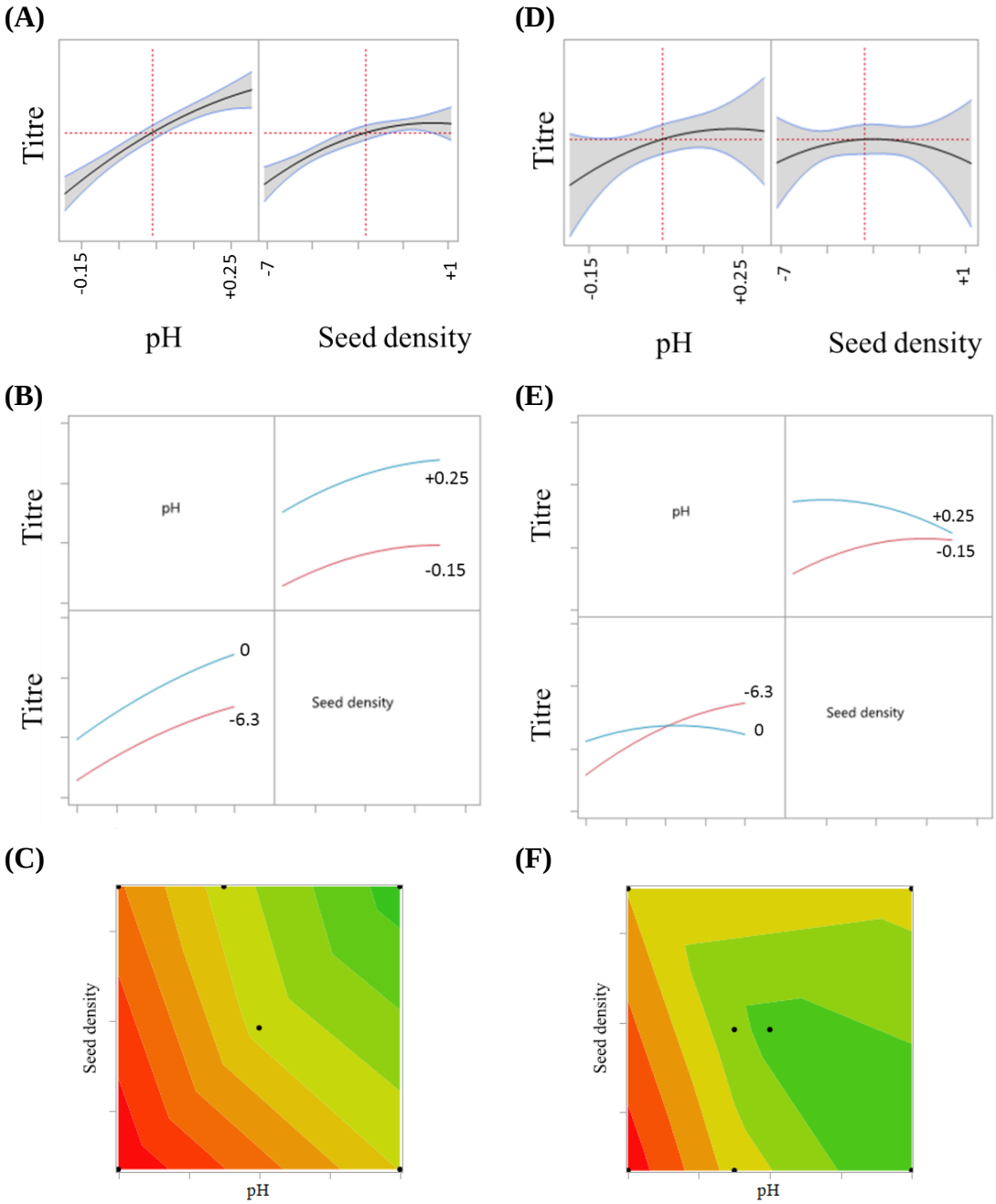


Figure 20. Effects of culture conditions on titre for the mAb 1 ambr15 DoE at low and high temperature. (A-C) Prediction profiler, interaction profiler and heat map showing effects of pH and seed density at low temperature on titre (R^2 = 0.99). (D-F) Prediction profiler, interaction profiler and heat map showing effects of pH and seed density at high temperature on titre (R^2 = 0.91). In the heat maps, high titre is indicated in green and low titre in red. Contour values were specified so that minimum values, maximum values and increments were the same for both heat maps with 0.25 g/L increments. Black dots represent the experimental data points.

As mentioned, the third DoE was comparable in scale to the first DoE (250 mL bioreactors), but was carried out with a different antibody (mAb 2) and all cultures were inoculated at the same seed density of -6 which is comparable to the seed densities referred to as "low" in the previous two studies and as such does not include a contrasting high seed density setpoint. In this third DoE (predicted RMSE = 208.88, R^2 = 0.95, p value < 0.0001), increasing the pH results in cultures producing more antibody titre, and increasing the temperature also leads to higher titre; however increasing temperature and pH together in order to produce more antibody only works to a certain point before titre declines again as already seen in the two previous DoEs. As such, these three studies demonstrate that the observed effects of culture conditions on antibody titre hold true for both investigated mAbs.

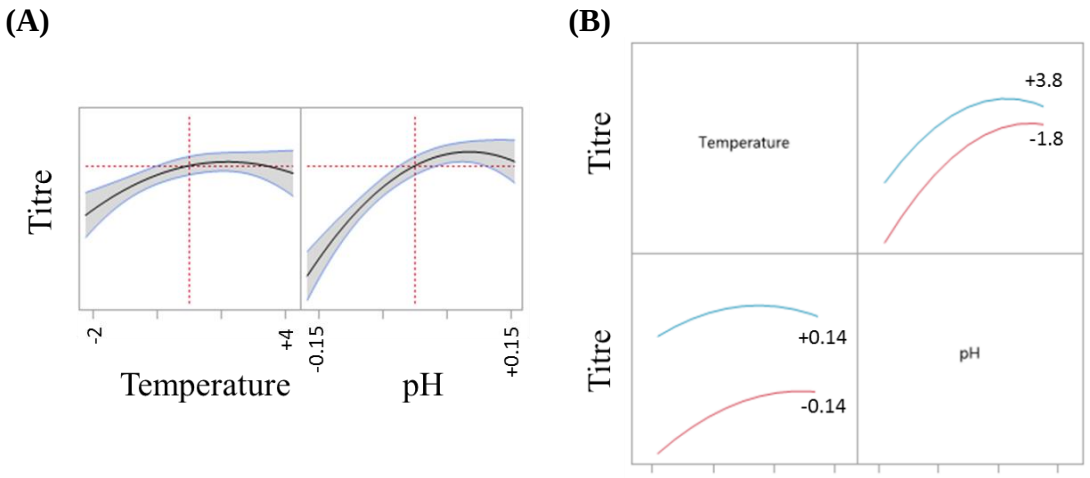


Figure 21. Effects of culture conditions on titre for the mAb 2 ambr250 DoE (R^2 = 0.95).

These results largely reflect what has previously been published in the literature, although while it is generally accepted that process parameters can influence product quality attributes (Goey et al., 2017; Schneider et al., 2019), this impact has also been reported to be protein dependent and therefore requiring investigation on an individual case basis (Hennicke et al., 2019).

With regards to culture temperature, growing mammalian cells at sub-physiological temperatures has been shown to result in slowed or arrested growth and reduced metabolism (Trummer et al., 2006), cell-cycle arrest in the G1 phase as well as increased viability for prolonged periods of time (Fogolin et al., 2004), and reduced transcription / translation (Mason et al., 2014). It has also been reported to result in increased specific productivity rates (Yoon et al., 2003) and maintained or even improved product quality and titre (Fogolin et al., 2004; Trummer et al., 2006; Schneider et al., 2019). Recent studies have suggested that improved protein titres at sub-physiological temperatures are due to an increase in mRNA half-life as well as extended stationary phase (Masterton et al., 2010; Mason et al., 2014).

However, there are also reports where decreased culture temperature had little or no effect on protein expression (Yoon et al., 2003). Mason et al. (2014) have further reported that reduced culture temperature had a differential effect on protein and mRNA expression of closely related antibody mutants from stable cell lines, and they therefore propose that the effect of reduced culture temperature on titre is protein-dependent, with the accuracy of protein folding and assembly being improved at lower temperatures, and therefore enhancing the expression of proteins that have a propensity to misfold.

Another effect of decreased temperature is the reduced consumption of glucose and therefore the associated production rate of lactate, which is a major by-product of the glycolytic pathway during incomplete oxidation of glucose. As accumulated lactate in the

culture medium has an inhibitory effect on cell growth and protein production, decreased temperature may prevent this (Eibl et al., 2009).

Culture pH is another parameter known to influence cell growth, cell metabolism, recombinant protein production and product quality (Trummer et al., 2006).

Trummer et al. (2006) used a recombinant CHO cell line expressing the fusion protein Epo-Fc to examine (among others) the effects of pH and temperature on cell growth and productivity. They reported that pH and temperature both had a strong effect on the performance of cells with a reduction in either parameter resulting in a significantly decreased specific growth rate (or even growth arrest at 30 °C). However, the specific productivity rate was enhanced at lower culture temperature (30 and 33 °C) and was maintained throughout the entirety of the batch cultivation, as opposed to a steady decrease in specific production rate during cultivation at 37 °C. With regards to a low culture pH, Trummer et al. (2006) observed an increase in product yield, but rather than being caused by a higher specific productivity rate (as was noted for low culture temperature), the improved product yield at pH levels lower than 7.1 was the result of a higher integral of viable cells (which in turn was due to high cell viability and a consequently prolonged cultivation time).

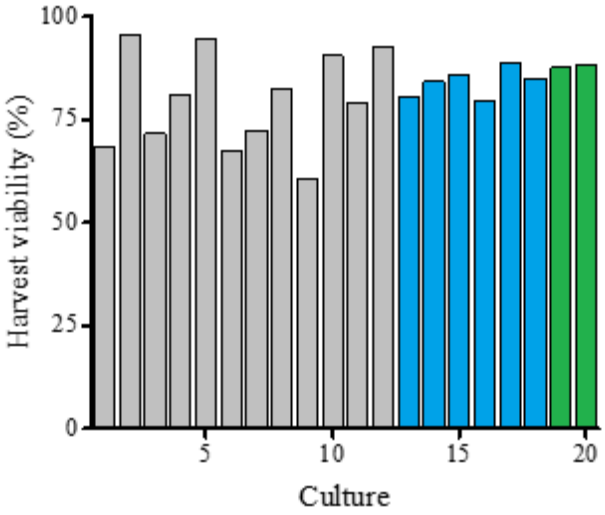
Hennicke et al. (2019) studied how culture pH and temperature affected IgM product quantity and quality by using a central composite design with five temperature levels (38.5 °C, 37 °C, 33.5 °C, 30 °C and 28.5 °C) and three pH levels (7.05, 6.9, 6.75) for a total of 12 batch fermentations. The initial growth phase was performed at 37 °C and pH 7.0 before the process parameters were subsequently switched to the various temperatures and pH values at production phase. Unlike previous literature, they noted no impact on cell growth or antibody production by changing the culture pH, and no significant effects on IgM product quality. Temperature also did not significantly affect product quality, but lower culture temperature did in fact substantially decrease IgM titres. Furthermore, at

sub-physiological temperatures, cell growth was decreased, possibly caused by cell cycle arrest in the G0/G1 phase and decreased metabolism. Lower volumetric and cell-specific productivity was also observed, which is contradictory to some literature that has reported optimised specific productivity following a shift to lower temperatures (Trummer et al., 2006). Hennicke et al. (2019) suggest that a temperature shift is not always beneficial for improved production of therapeutic proteins (as is commonly reported in the literature) and is strongly dependent on the cell line and the expressed product.

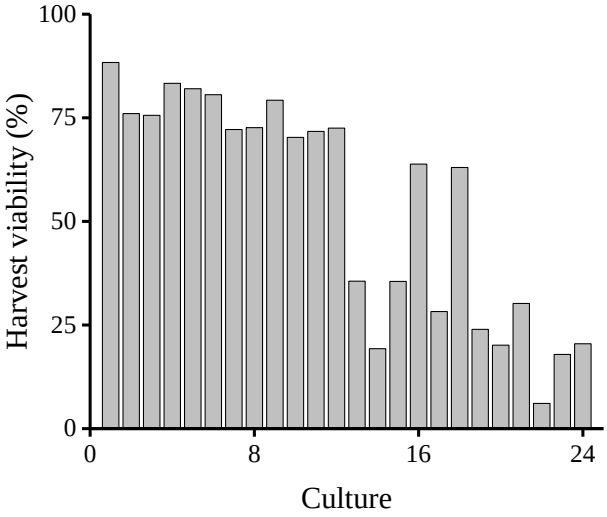
Schneider et al. (2019) reported that during the production of their product (an IL-2 variant immunocytokine) in CHO cells, fragmented product (and therefore reduced cytokine activity) was observed. To control product fragmentation, different production process conditions were investigated, and it was determined that decreasing the temperature or pH during their process led to a decrease in fragmentation. However, this was also accompanied by low product titre. Subsequently, they carried out a DoE to determine optimal values for temperature, pH, seed density, and harvest day in order to minimize product fragmentation while maximizing titre. The best results were achieved when inoculating with a higher cell density, shifting the temperature to a lower temperature and the pH value to a higher pH on day 8 of cultivation, and harvesting the product on day 14. This improved process reduced fragmentation to 4% (compared with 12% in the standard process) while keeping a titre of approximately 2 g/L (comparable with the standard process on day 14).

3.2.2 Correlation between titre and harvest viability

(A)



(B)



(C)

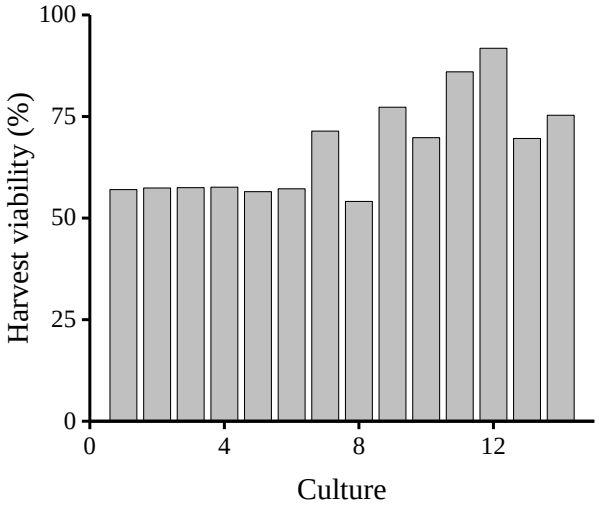
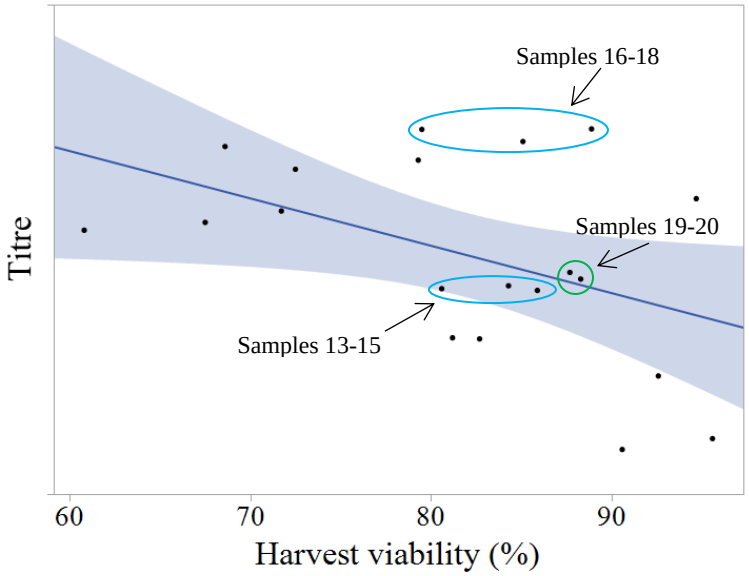


Figure 22. Harvest culture viability results for the three DoEs. (A) mAb 1 ambr250 DoE, (B) mAb 1 ambr15 DoE, and (C) mAb 2 ambr250 DoE. Samples in (A) have been colour coded according to Figure 10 and Table 3 with blue samples representing the centre point conditions of this DoE and green samples representing the platform conditions (i.e. the control).

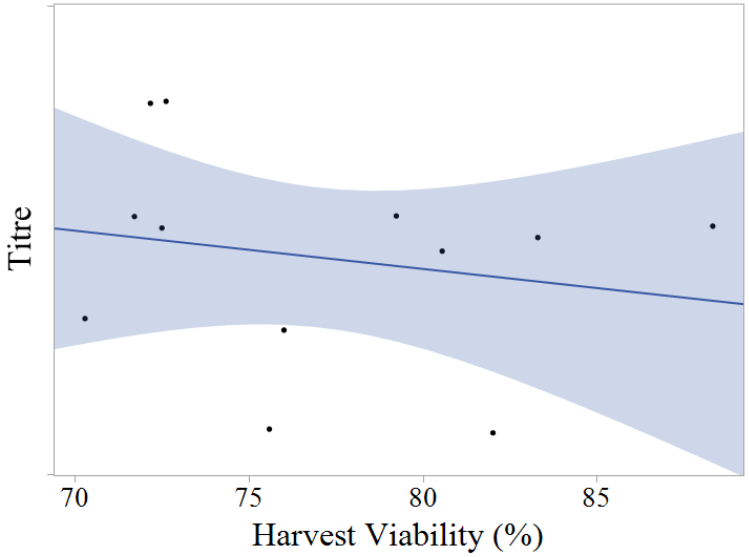
One of the most commonly monitored upstream responses is the viability of cultures during the production process. This is done to ensure that cells remain healthy and produce as much antibody as possible. If cells are experiencing suboptimal growth conditions, their energy will go towards supporting cell survival processes rather than antibody synthesis. Figure 22 A-C shows culture viability results for the same three experiments discussed previously – the 250 mL bioreactor DoE using mAb 1 (A), the 15 mL bioreactor DoE using mAb 1 (B), and the 250 mL bioreactor DoE using mAb 2 (C). As can be seen, the viability of cultures grown under different bioreactor conditions undeniably varies quite significantly, e.g. the variation in culture viability for the first DoE ranges from 55% to 96%.

However, titre and culture viabilities from the three DoE studies have been plotted against each other in Figure 23 which shows that there is a clear lack of linear correlation between high culture viability at the point of harvest and high titres. On the one hand, this is unexpected as one would assume that high viability cultures produce more titre than low viability ones, based on the assumption that low viability cultures focus more energy on cell survival processes than on the production of the therapeutic antibody. On the other hand, cultures starting with higher seed densities presumably produce antibody more quickly at the start of the culture as the higher cell density circumvents the need for the initial growth period, and then rapidly become less viable compared to low seed density cultures. Therefore, product titre and culture viability at the point of harvest need not necessarily correlate with each other in the investigated DoEs.

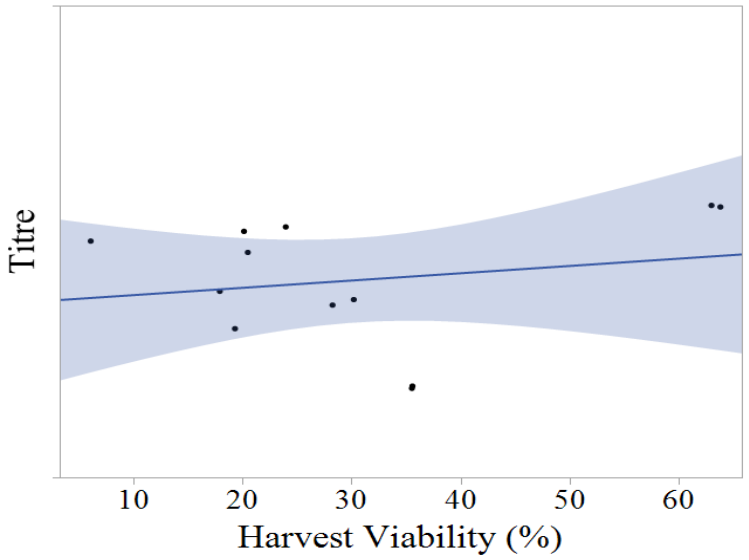
(A)


Samples 16-18
Samples 19-20
Samples 13-15
Titre
60
70
80
90
Harvest viability (%)

(B)


Titre
70
75
80
85
Harvest Viability (%)

(C)


Titre
10
20
30
40
50
60
Harvest Viability (%)

(D)

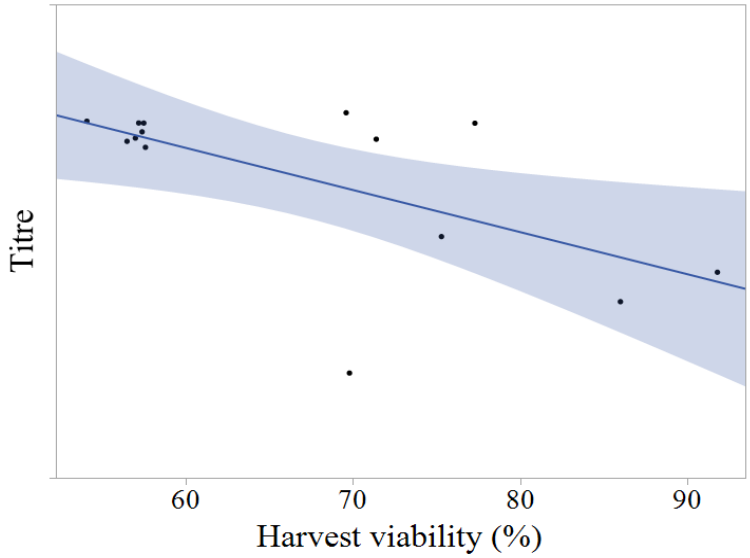


Figure 23. Correlations between harvest culture viability and titre in the three DoEs (A) mAb 1 ambr250 DoE (R^2 = 0.21), (B) mAb 1 ambr15 DoE at low temperature (R^2 = 0.04); (C) mAb 1 ambr15 DoE at high temperature (R^2 = 0.04), and (D) mAb 2 ambr250 DoE (R^2 = 0.38) showing that high harvest viability is not an indicator for high titre. Titre has been redacted due to confidentiality. Samples in (A) have been colour coded according to Figure 10 and Table 3 with blue samples representing the centre point conditions of this DoE and green samples representing the platform conditions (i.e. the control).

In addition, titre is determined by two factors – the integral viable cell density (IVCD) and the specific protein productivity rate (SPR or q_p) – and the lack of correlation between titre and harvest culture viability could be due to differential impacts on these two factors whereby there could be cultures with low viability (and therefore less viable cells) that might still produce high amounts of titre due to a higher q_p or alternatively, there might be cultures with high viability (which one might expect to be producing high amounts of antibody titre) that may have a low q_p.

The IVCD and SPR data for the samples from the first DoE are therefore illustrated as an example in Figure 24. It can be seen that titre is primarily affected by IVCD as an increase in IVCD is linked to a fairly steady transition from red cultures (i.e. low titres) to green cultures (i.e. high titres).

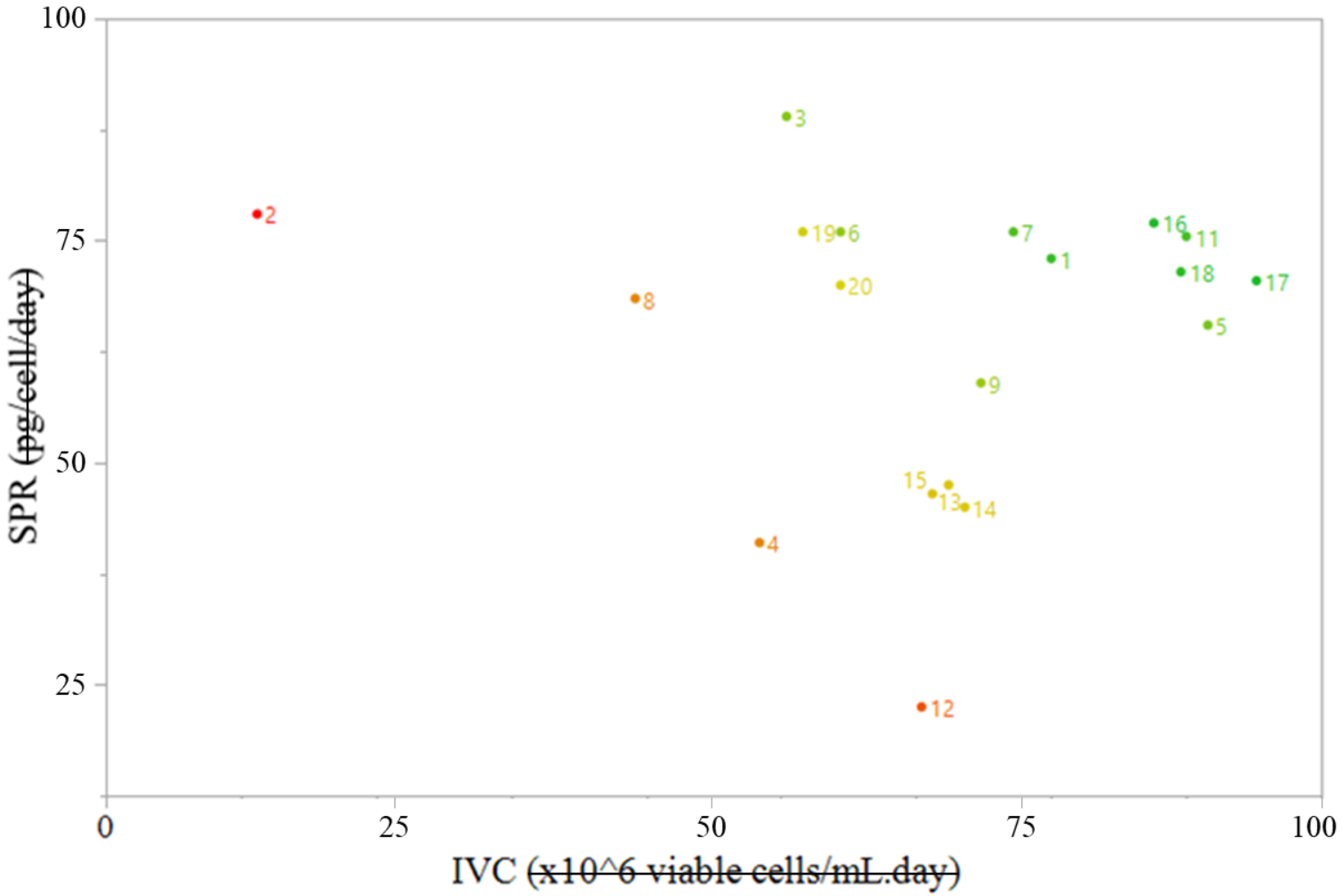


Figure 24. Specific protein productivity rate (SPR or q_p) versus integral viable cell density for all samples of the mAb 1 ambr250 DoE, overlaid with titre results (high values shown in green, low values marked in red). Please note that both SPR and IVC have been redacted due to confidentiality and that axes values are normalised to 100%.

The q_p seems to have a lesser impact, as demonstrated by the fact that cultures with a q_p of approximately 15 pg/cell/day produce a wide variety of titres, e.g. samples 16-18 have a similar q_p compared to the control group (samples 19 & 20) but a higher titre (cf. Figure 23 A) due to having a higher IVCD. Furthermore, culture 2 with a q_p of ~ 15 pg/cell/day has a much lower titre than cultures 9 or 13-15 which all have a lower q_p.

In general, none of the changes to process parameters have led to a higher specific protein productivity rate (compared to the control), apart from culture 3 which had the highest q_p but by no means the highest titre measured from all samples in this DoE. As such, the specific protein productivity rate has a lesser impact here than the integral viable cell density and the focus will therefore remain on cell counts rather than specific productivity rate.

In summary, high culture viability at harvest is thus not a suitable indicator for high titre. Nonetheless harvest culture viability may serve as an indicator for other responses such as product quality attributes and was therefore analysed in JMP in the same manner as was done for titre to examine which factors affected cell viability.

The first DoE (predicted RMSE = 4.85, R^2 = 0.84, p value = 0.0011) shows that harvest culture viability seems to be greatly influenced by the seed density with high seed densities resulting in lower cell viability at harvest. Viability is also decreased when operating in fed-batch mode or growing cells at higher pH (Figure 25 A-E) – all conditions that promoted higher titres which confirms once more that high culture viability does not necessarily correlate with high titres.

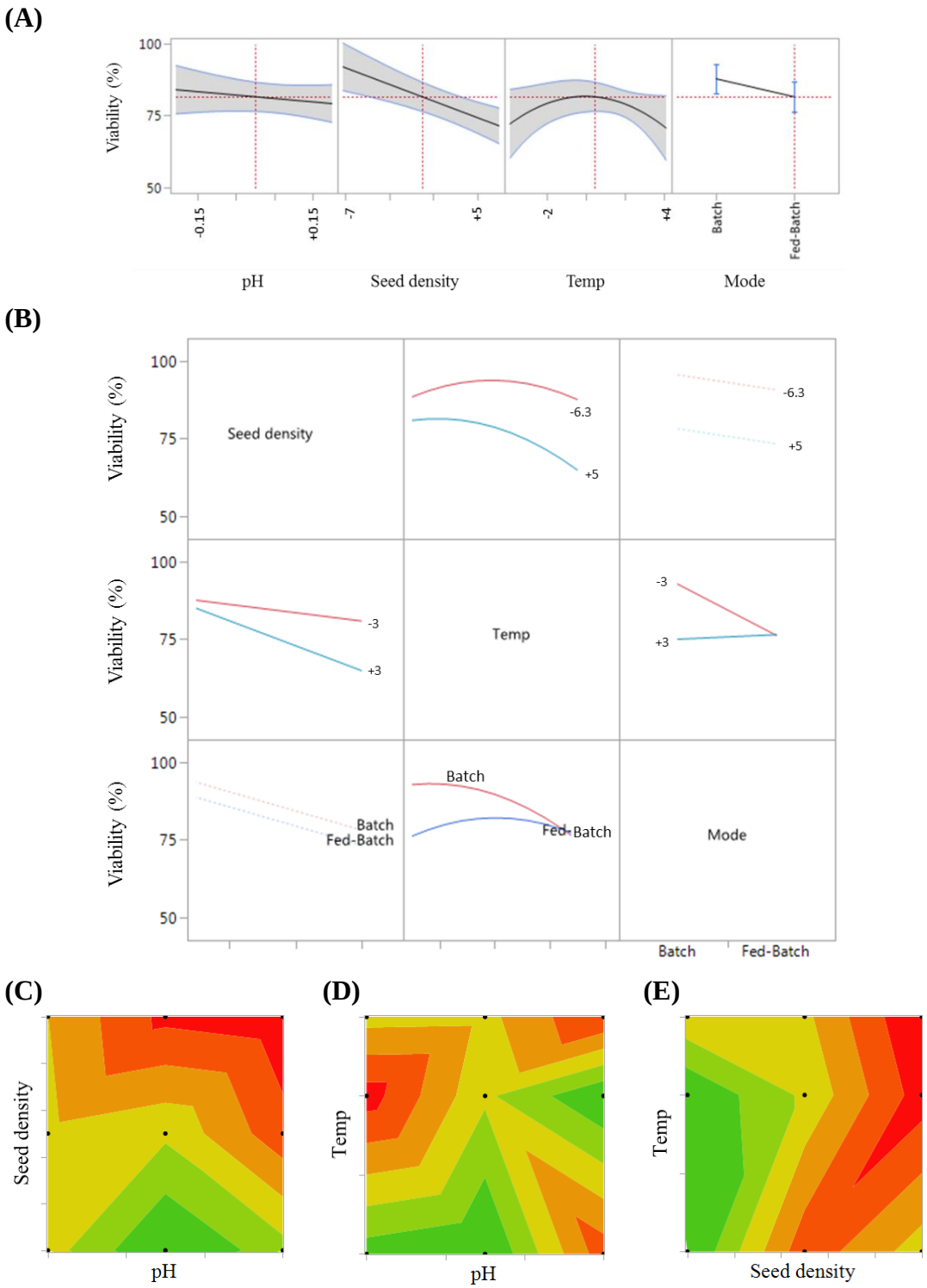


Figure 25. Effects of culture conditions on harvest culture viability for the mAb 1 ambr250 DoE (R^2 = 0.83) as prediction profiler (A), interaction profiler (B), and heat maps (C-E). In the heat maps, high viability is indicated in green and low viability in red. Contour values were specified so that minimum values, maximum values and increments were the same for all three heat maps with 5% increments. Black dots represent the experimental data points.

In addition, temperature has a strong effect in batch mode with cultures grown at low temperature (-3) being of higher viability compared to those grown at higher temperature (+3) (Figure 25 B). Interestingly, temperature does not seem to affect culture viability greatly when operating in fed-batch mode, although culture viability is worse when combining high temperature conditions with high pH or high seed density conditions. This supports the previously stated hypothesis that cultures grown under growth promoting conditions (high pH and high temperature) and inoculated with a high seed density likely exhaust their nutrient supply more rapidly which leads to increased cell death and overall less titre compared to those cultures grown at lower pH and/or temperature or those inoculated with fewer cells.

Figure 26 displays the effects of culture conditions on harvest culture viability for the second DoE (low temperature: predicted RMSE = 3.56, R^2 = 0.78, p value = 0.05; high temperature: predicted RMSE = 4.12, R^2 = 0.97, p value = 0.0002). As explained previously, the results for this DoE are separated by low and high temperature. At lower temperature, culture viability is not significantly affected by a different pH or seed density as all cultures remained fairly viable (above 70% of the total cell count), although harvest culture viability is slightly decreased in cultures that were inoculated with higher seed densities, and low seed density cultures are slightly more viable when grown at higher pH (Figure 26 A-C). In comparison, high temperature cultures were associated with much more varied culture viabilities. Much higher viabilities were observed in cultures that were grown at low seed density, especially when combined with high pH which confirms once more that high seed density, high pH and high temperature together causes increased cell death.

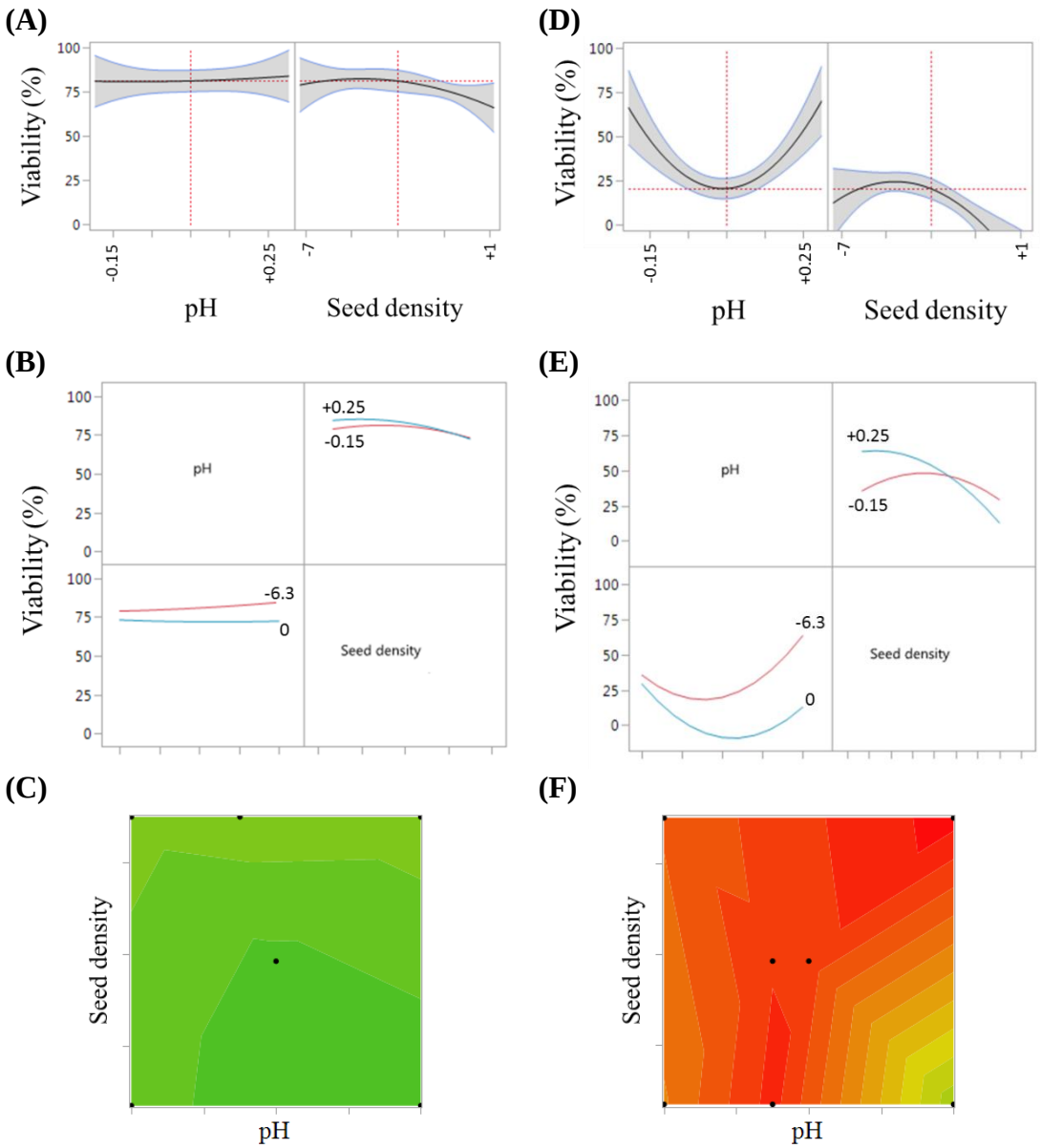


Figure 26. Effects of culture conditions on harvest culture viability for the mAb 1 ambr15 DoE at low and high temperature. (A-C) Prediction profiler, interaction profiler and heat map showing effects of pH and seed density at low temperature on harvest viability (R^2 = 0.78). In the heat maps, high levels are indicated in green and low levels in red. Contour values were specified so that minimum values, maximum values and increments were the same for both heat maps with 5% increments. Black dots represent the experimental data points. (D-F) Prediction profiler, interaction profiler and heat map showing effects of pH and seed density at high temperature on harvest viability (R^2 = 0.97).

Similar results were observed in the third DoE (predicted RMSE = 4.86, R^2 = 0.94, p value = 0.048) with higher pH and higher temperature causing decreased harvest culture viability, although some curvature effects were visible particularly when analysing the impact of temperature, with a temperature of approximately 0 to +2 resulting in even

worse culture viability than +3. This could potentially be explained by two different forces affecting culture viability – at low temperature, cells might be quite dormant and not very active in terms of antibody production and cell growth. In this latent state, cells might remain viable due to inactivity. At higher temperature however, cells become more active but if conditions are not yet ideal, cells may be more susceptible to cell death. A temperature of +3 is more ideal and growth promoting which might raise culture viability again compared to a slightly lower temperature, even though viability may not be as high as at the lowest temperature investigated here (-1). This correlates with the previous DoE study which showed that cultures grown at -1 had consistently higher culture viabilities while cultures grown at +3 were only associated with high harvest culture viabilities if they were grown at high pH and low seed densities.

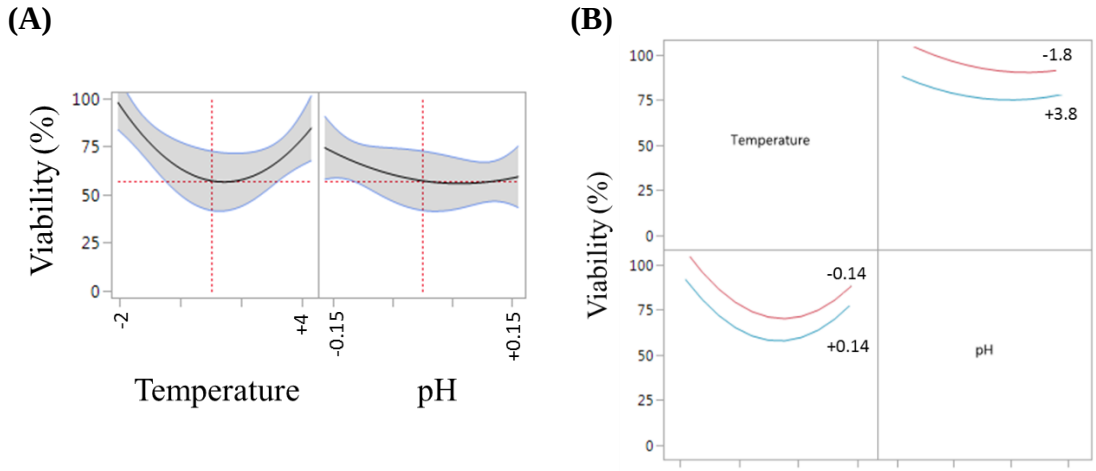


Figure 27. Effects of culture conditions on harvest culture viability for the mAb 2 ambr250 DoE (R^2 = 0.94).

In conclusion, while culture viability at harvest may not necessarily correlate with product titre and is therefore not a suitable indicator for high titre, it is nonetheless generally considered desirable to have healthy cells and culture viability may well impact upon the quality of the produced therapeutic antibody, if not the quantity. This will be investigated in section 3.4.2.

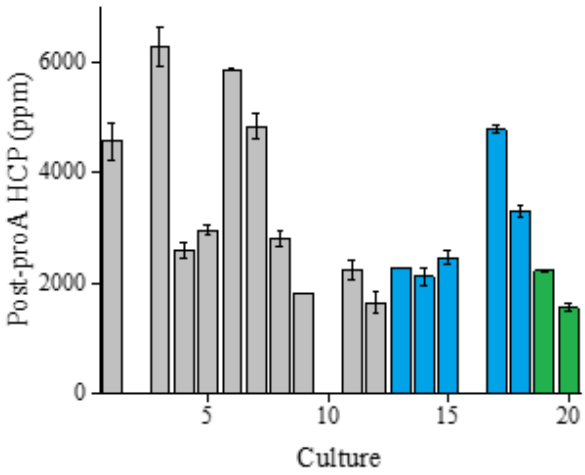
3.3 Effects of upstream culture conditions on post-protein A HCPs

3.3.1 Post-protein A HCP quantity

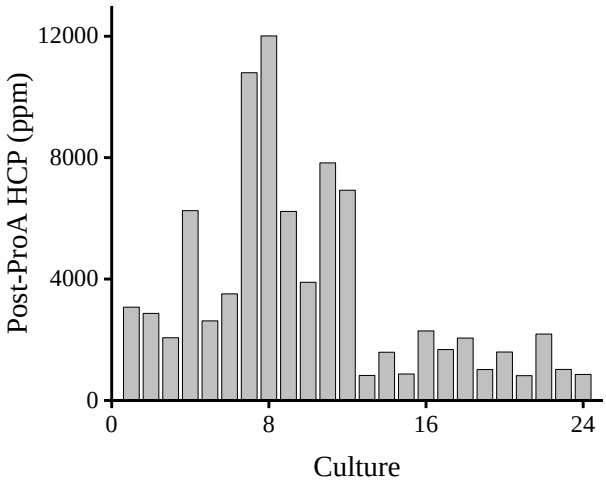
As discussed in great length in the introduction chapter, host cell proteins are process related impurities that need to be removed from the therapeutic protein during product purification. Initially, it was attempted to measure HCP quantities in harvested cell culture fluid (HCCF) as one of the product quality attributes. However, the quantification of HCPs in HCCF by use of an ELISA proved to be unreliable as the vast quantities of HCPs that were present seemed to saturate the detection antibodies used in this type of assay which meant that impurity levels could not confidently be determined (data not shown). However, this was not a major drawback as this thesis' focus is on the interface between upstream and downstream processing and as such, HCP impurities that are present in the HCCF are of less interest here than those that are retained during downstream purification, as it is the HCPs that are retained during drug purification that can influence final drug efficacy and pose potential safety risks to patients. The by far most effective purification step for the clearance of HCPs is protein A chromatography which utilities the strong affinity between protein A and the Fc region in antibodies to bind the product of interest and clear away the majority of impurities, making the saturation of ELISA detection antibodies less likely. As mentioned, of interest for this thesis are the HCP levels following protein A purification since too many HCPs remaining associated with the antibody require a more extensive HCP clearance strategy and might prove challenging for the downstream group to reduce.

Using the previously discussed three DoE studies, the post-protein A HCP levels for all samples were determined and are displayed in Figure 28. As can be seen, there is variation in HCP levels amongst the samples, showing that upstream conditions and process parameters affect the levels of impurities associated with a drug product.

(A)



(B)



(C)

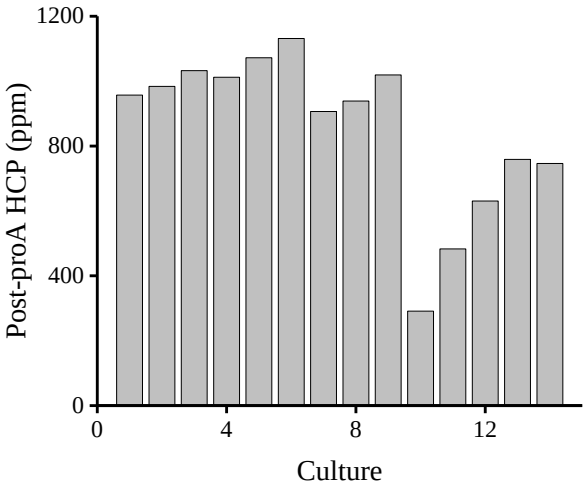


Figure 28. Post-protein A HCP results for the three DoEs (A) mAb 1 ambr250 DoE, (B) mAb 1 ambr15 DoE, and (C) mAb 2 ambr250 DoE. Samples in (A) have been colour coded according to Figure 10 and Table 3 with blue samples representing the centre point conditions of this DoE and green samples representing the platform conditions (i.e. the control).

The figure also shows that post-protein A HCPs levels vary between different mAbs - Figure 28 A and B both show results for mAb 1 studies and cultures from both studies were associated with levels of roughly between 2000 ppm and 6000 ppm, apart from two outliers in the second DoE (i.e. cultures 7 and 8 which were also associated with high levels of titre and were grown at conditions that were identified in section 3.2.1 as being particularly beneficial for protein production). Figure 28 C on the other hand shows results for the DoE using a different molecule – mAb 2, and cultures from this study were associated with much lower post-protein A HCP levels. This highlights the fact that HCP levels are not just determined by upstream culture conditions but are also dependent on the antibody being expressed.

DoE analysis using JMP software was performed as before to investigate which upstream factors affect post-protein A HCP levels. Figure 29 displays the effects of culture conditions on post-protein A HCP levels for the first DoE (predicted RMSE =715.15, R^2 = 0.88, p value = 0.0046), which shows that cells express higher levels of HCPs when grown in fed-batch mode as opposed to batch mode. The prediction profiler suggests that factors pH and seed density only contribute slightly to post-protein A HCP quantities with a slightly inverse correlation, whereas temperature appears to have a stronger negative correlation – however confidence intervals are fairly wide towards the extreme ends of the tested temperatures. Looking at the interaction profiler, it becomes apparent that there is a factor interaction between temperature and pH as well as temperature and seed density; at low temperature, the seed density has quite a strong impact on the level of HCP impurities with a low seed density resulting in fewer HCPs than high seed densities. At high temperature however, inoculating cultures with higher seed densities results in the production of more HCPs. Furthermore, pH has a much bigger impact on HCP production at high temperature in comparison to a lower temperature, with an increase in pH at high temperature correlating with a decreased number of HCPs. In summary, the

data from this first DoE suggest that cells produce fewer HCPs when they are inoculated with a low seed density and grown at high temperature and high pH.

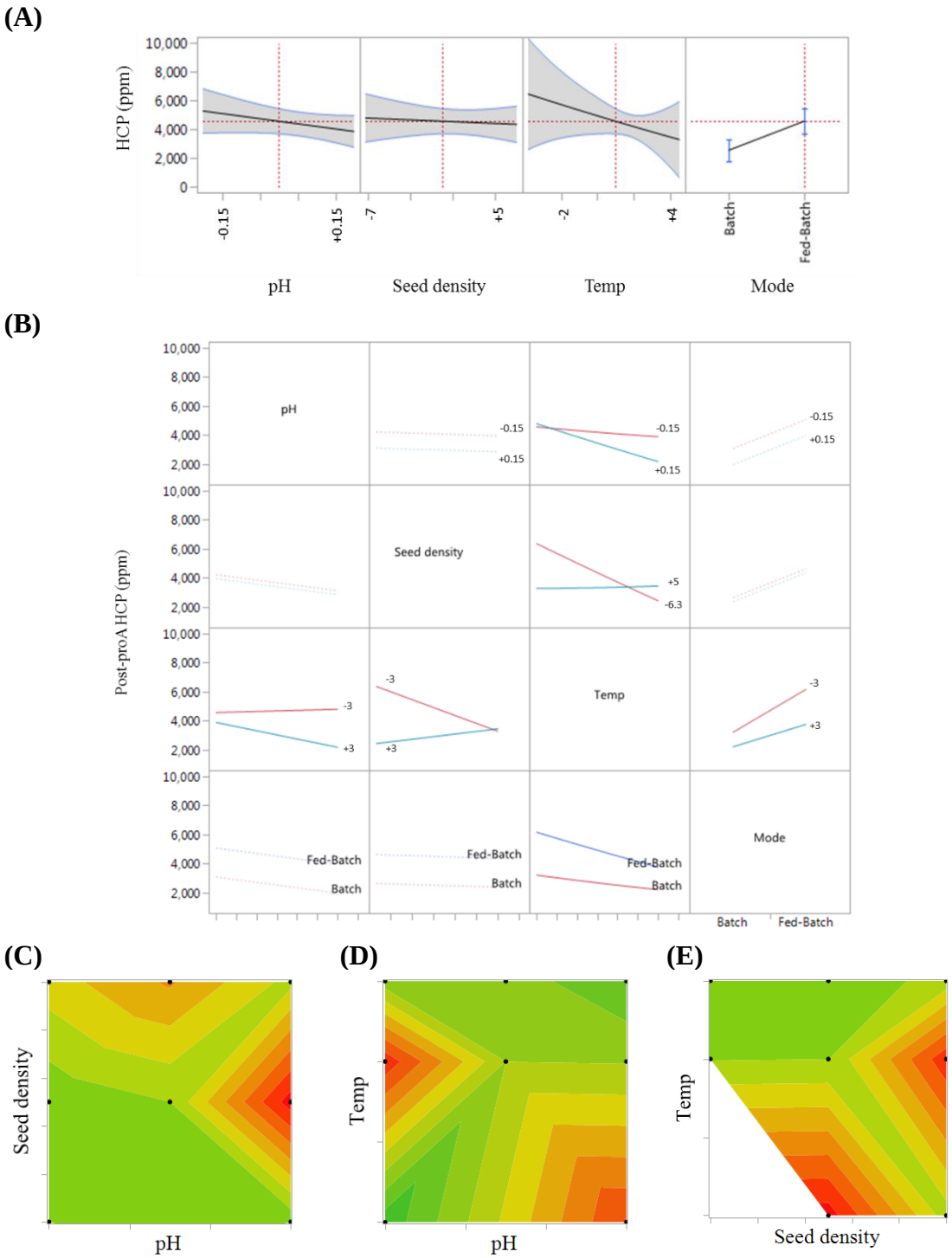


Figure 29. Effects of culture conditions on post-protein A HCP levels for the mAb 1 ambr250 DoE (R^2 = 0.88) as prediction profiler (A), interaction profiler (B), and heat maps (C-E). In the heat maps, low levels of HCP impurities are indicated in green and high levels in red. Contour values were specified so that minimum values, maximum values and increments were the same for all three heat maps with 500 ppm increments. Black dots represent the experimental data points.

The results for the second DoE (low temperature: predicted RMSE = 565.29, R^2 = 0.89, p value <0.0001; high temperature: predicted RMSE = 371.03, R^2 = 0.77, p value = 0.0629) can be seen in Figure 30. Cells grown at low temperature, low pH and low seed density appear to be mostly inactive (as discussed previously) and therefore do not produce many HCPs. Increasing the pH or the seed density individually causes the production of slightly more HCPs, and increasing both conditions together (i.e. inoculating cultures with high seed densities and growing cells at high pH – while maintaining the low temperature of -1) significantly increases the levels of HCPs that are associated with the material after protein A purification. In comparison, cells that are grown at a higher temperature (+3) behave slightly differently. A low pH and a low seed density still leads to minimal host cell protein production and increasing the seed density (while maintaining a low pH) still only results in slightly more HCPs being produced. But growing a low seed density culture at a higher pH (and the higher temperature) produces material with more than double the amount of HCP impurities. Of further interest is that seed density does not have as strong of an impact on post-protein A HCP levels as it did when operating at low temperature. At a temperature of +3 and a pH of +0.25, high seed density cultures seem to produce less HCPs than those inoculated with a low seed density. One possibility as to why the results of the first DoE do not correlate with the results from this second DoE could be because cultures from the first DoE were harvested on varying days. As mentioned, that particular DoE had previously been carried out by GSK's upstream processing group and as such was designed with a different aim in mind than to investigate the interface between upstream and downstream processing as is the aim of this thesis. Cultures were harvested as soon as a plateau of titre production had been reached which occurred between days 12 and 21. This additional factor likely had an influence on the varying HCP results seen in the first DoE as compared to the second DoE

in which all cultures were harvested on the same day. The removal of this variable factor provides more confidence in the data results from the second DoE.

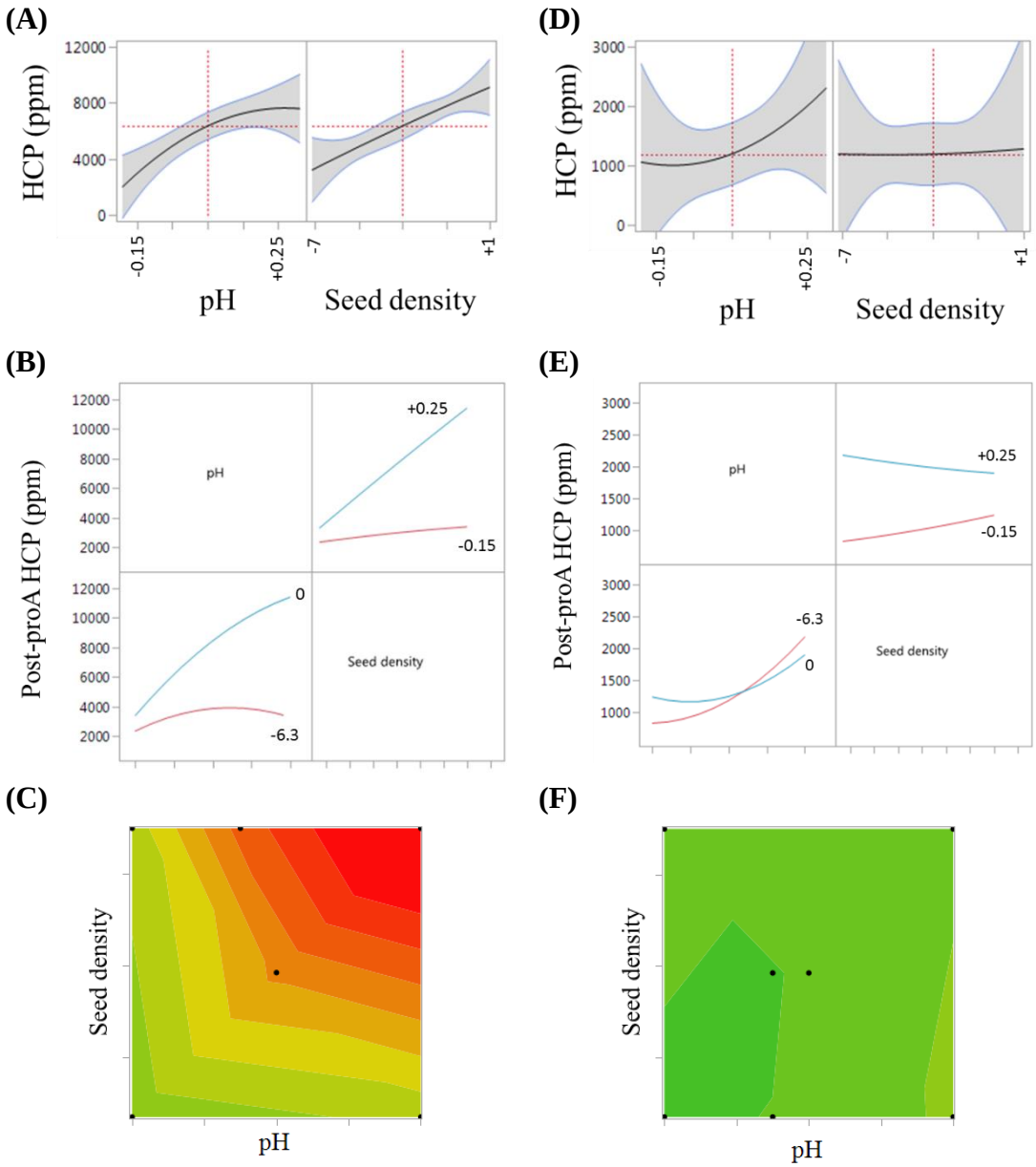


Figure 30. Effects of culture conditions on post-protein A HCP levels for the mAb 1 ambr15 DoE at low and high temperature. (A-C) Prediction profiler, interaction profiler and heat map showing effects of pH and seed density at low temperature on post-protein A HCP levels (R^2 = 0.98). In the heat maps, low levels of HCP impurities are indicated in green and high levels in red. Contour values were specified so that minimum values, maximum values and increments were the same for both heat maps with 1000 ppm increments. Black dots represent the experimental data points. (D-F) Prediction profiler, interaction profiler and heat map showing effects of pH and seed density at high temperature on post-protein A HCP levels (R^2 = 0.77).

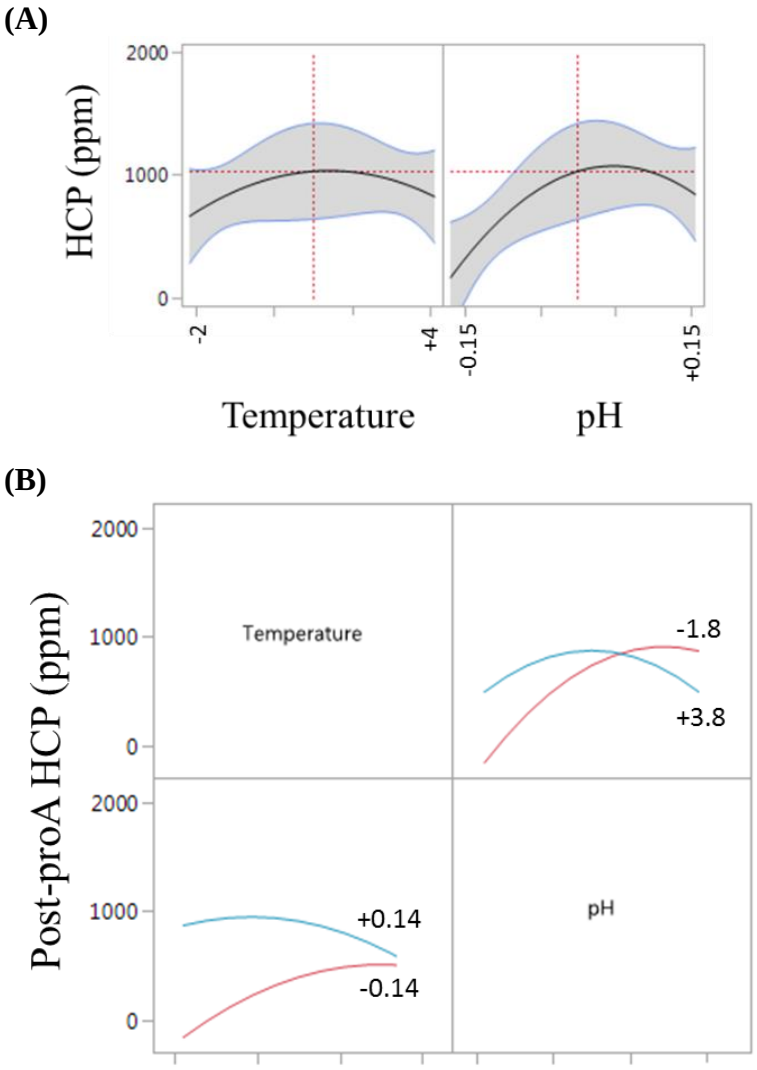


Figure 31. Effects of culture conditions on post-protein A HCP levels for the mAb 2 ambr250 DoE (R^2 = 0.91).

The third DoE (predicted RMSE = 122.77, R^2 = 0.91, p value = 0.0831) shows a factor interaction between pH and temperature (Figure 31). Cultures grown at low pH and low temperature appear to be mostly inactive (as discussed previously) and therefore do not produce many HCPs. If a low temperature is maintained but the pH is increased, cells become more active and produce more HCPs. Likewise, if the low pH is maintained but the temperature is increased, HCP levels also rise. If cells are grown at both high pH as well as high temperature, cultures are interestingly associated with fewer HCPs again. This same trend was also observed when the antibody titre for this DoE was analysed, suggesting that the production of antibody and the production of HCPs is affected by

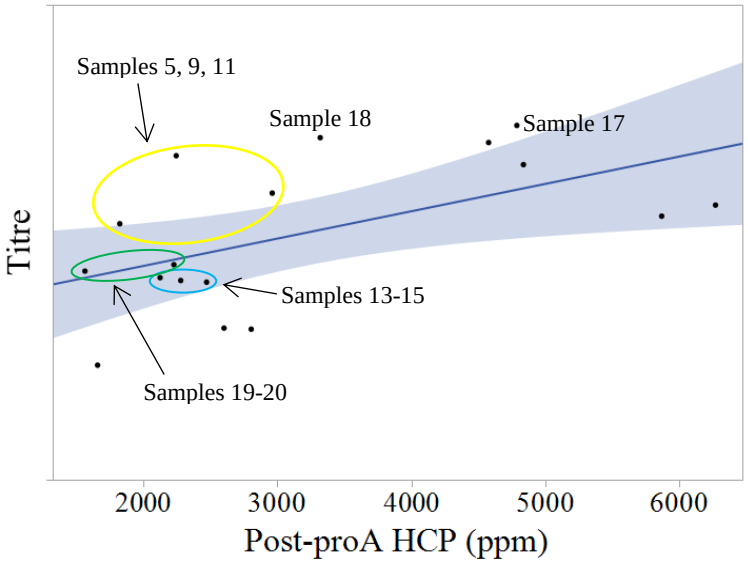
similar operating conditions, an observation that will be discussed further in the next section.

3.4 Correlations between HCCF quality and mAb product quality

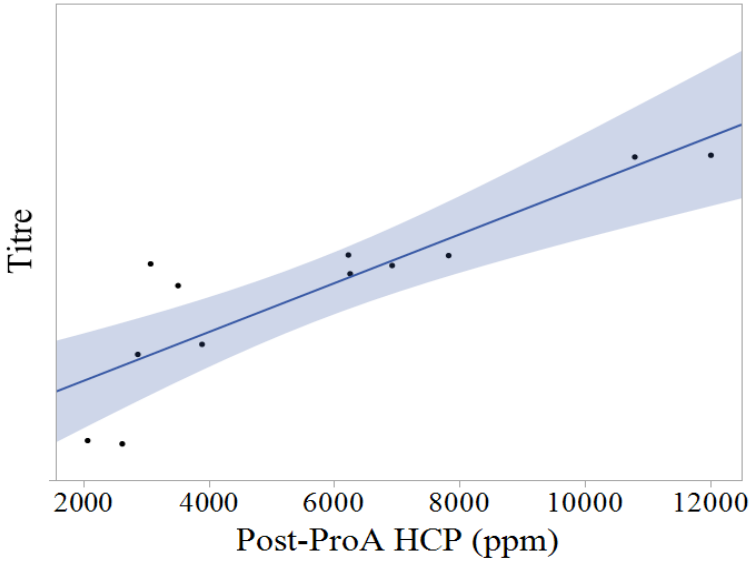
3.4.1 Titre

Of great interest for this research was to see if there is a correlation between antibody titre (the main output of interest to upstream processing) and post-protein A HCP impurities (one of the outputs of interest to downstream processing). The analysis of factors that contribute to high titre and high HCP levels already gave an indication that these two responses may be linked.

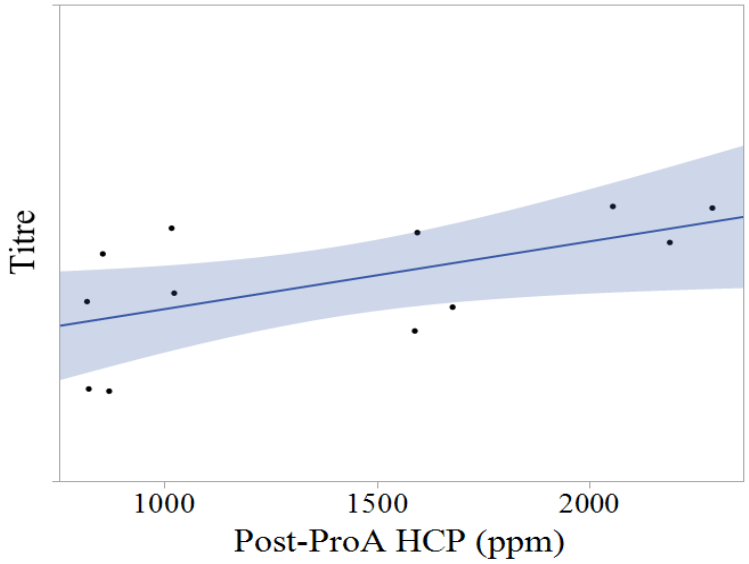
(A)



(B)



(C)



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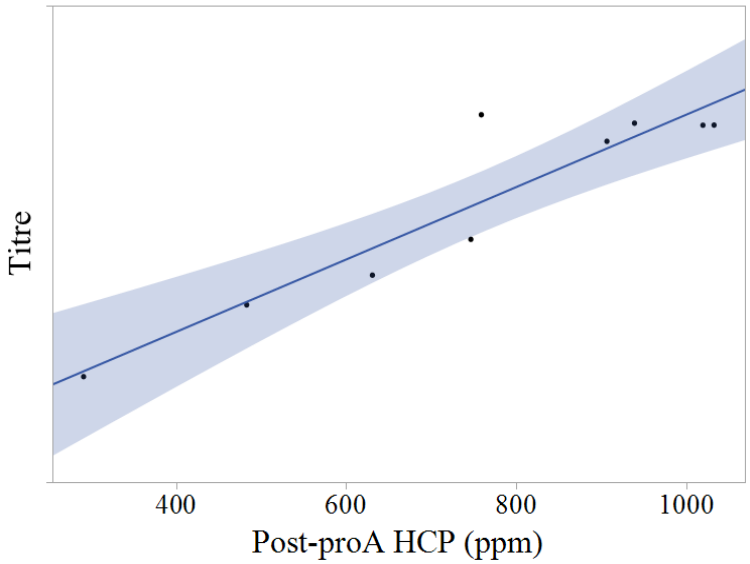
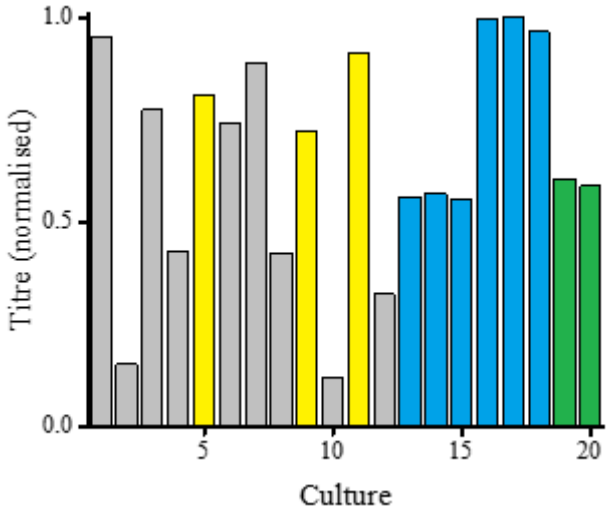


Figure 32. Correlations between titre and post-protein A HCPs in the three DoEs. (A) mAb 1 ambr250 DoE (R^2 = 0.3), (B) mAb 1 ambr15 DoE at low temperature (R^2 = 0.75), (C) mAb 1 ambr15 DoE at high temperature (R^2 = 0.35), and (D) mAb 2 ambr250 DoE (R^2 = 0.86) showing that higher titres are commonly associated with higher levels of post-protein A HCPs, although there are exceptions of high titre cultures that are associated with less HCPs (e.g. those highlighted in yellow in the first graph). Titres are redacted due to confidentiality.

Figure 32 shows the correlations between titre and post-protein A HCPs for each of the three DoEs. It can be seen that higher titres are commonly associated with higher levels of post-protein A HCPs, either due to cells grown under these conditions having a higher specific productivity rate and thus producing not only more antibody but also more host cell proteins, or due to a higher number of cells being present which together produce

more titre but more cells also imply an increase in cell death and the concomitant release of intracellular HCPs. It should be noted though that there are exceptions of high titre cultures that are associated with fewer HCPs, e.g. in the first DoE, cultures 5, 9 and 11 (illustrated in yellow in Figure 33) produced more titre than cultures 19 and 20 (the control group) but produced similar or fewer levels of post-protein A HCP levels:

(A)



(B)

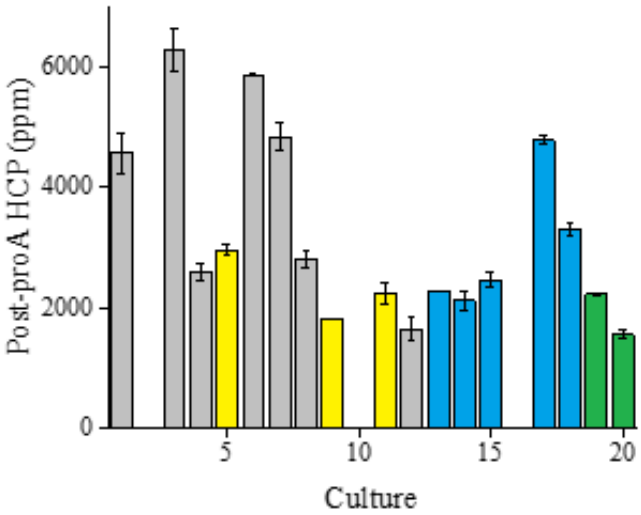


Figure 33.(A) Titre and (B) post-protein A HCP results from the mAb 1 ambr 250 DoE, taken from Figure 18 (A) and Figure 28 (A), but highlighting in yellow cultures 5, 9, and 11 as possible optimal conditions in terms of improved titre and product purity. Centre point conditions of this DoE are shown in blue and platform conditions (i.e. the control) are shown in green.

Given this observation, upstream operating conditions were compared in terms of titre and post-protein A HCPs. For the first DoE, Figure 34 compares the heat maps for titre and post-protein A HCPs that were already shown previously in Figure 19 and Figure 29.

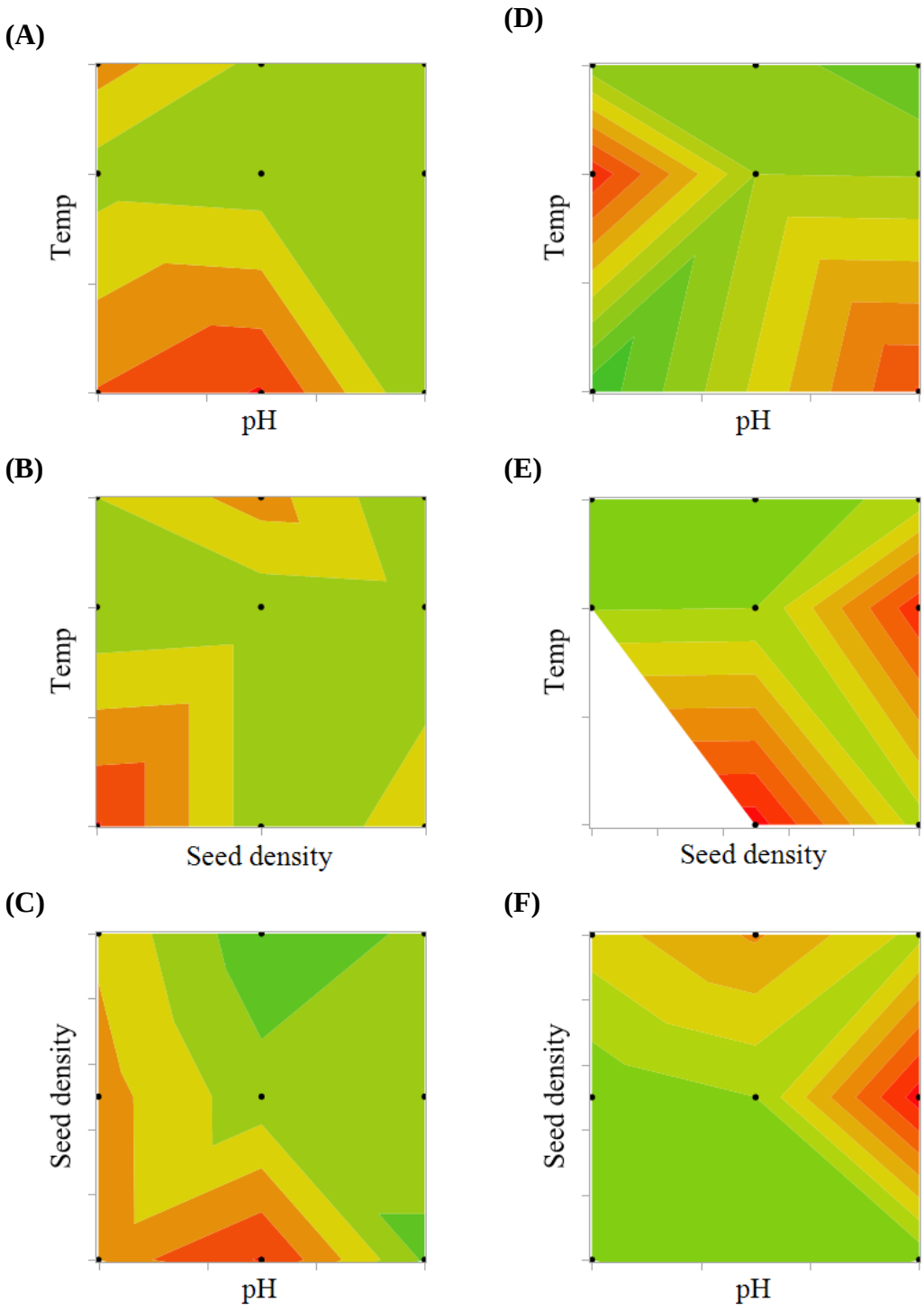


Figure 34. Heat map contour plots for titre (A-C) vs. post-protein A HCP levels (D-F) for the mAb 1 ambr250 DoE. Desirable results (high titre / low HCP) are indicated in green and undesirable results (low titre / high HCP) are shown in red. Conditions that result in high titre also result in high levels of post-protein A HCPs suggesting that operating conditions for titre and product quality contradict and that a trade-off between these responses may be necessary.

By looking at them next to each other, it is clear that conditions that result in high titre also result in high levels of post-protein A HCPs suggesting that operating conditions for titre and product quality contradict and that a trade-off between these responses may be necessary.

For the second and third DoE, operating windows were generated in JMP by fitting least squares regression models to overlay desirable responses for both upstream and downstream processing and to consider if there is an operating space that would be beneficial to product quantity and quality. Such windows of operation are commonly used to illustrate the operating conditions that are feasible for one or multiple desired responses (Woodley and Titchener-Hooker, 1996).

The contour plots for the mAb 1 ambr15 DoE (separated by temperature) were generated by fitting least squares regression models for titre (R^2 = 0.99 and 0.91), yield (R^2 = 0.75 and 0.62) and HCP (R^2 = 0.98 and 0.77) results, and illustrate how pH and seed density affect titre, post-protein A yield and post-protein A HCP levels at a temperature of -1 (Figure 35 A) and +3 (Figure 35 B). The boundary conditions for figures A and B have been set to slightly different values (in the case of yield) or vastly different values (in the case of post-protein A HCPs) in order to visualise the operating window most effectively here. Within the JMP software, these contour profilers are interactive but given the 2D representation here, the goal was to highlight that while the feasible operating windows (white area) look slightly different at low vs. high temperature, both plots show that a feasible operating window for maximum titre is constrained by the downstream responses (protein A yield and post-protein A HCP levels), confirming the results of the first DoE that a trade-off between these responses may be necessary. If the boundary conditions had been set to the same values in both figures, the constraint might not have been displayed on the profiler at all (e.g. if the same maximum HCP limit from figure A had been applied to figure B as well) or would have completely dominated the profiler with

no feasible / white area remaining (e.g. if the same maximum HCP limit from figure B had been applied to figure A).

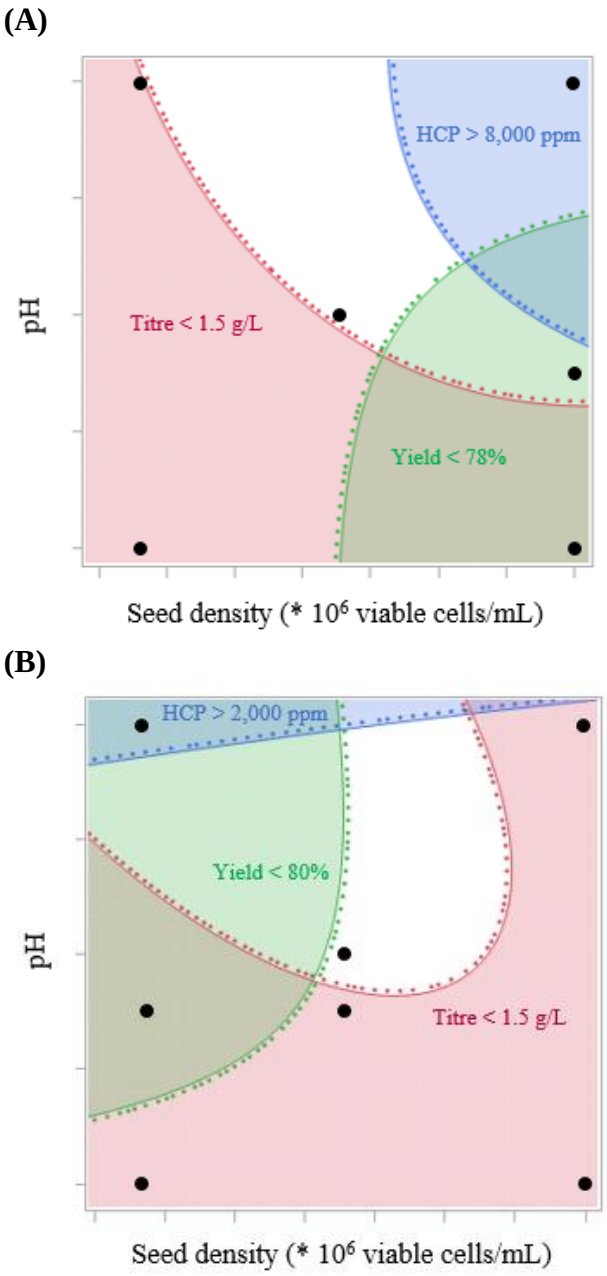


Figure 35. Window of operation for the mAb 1 ambr15 DoE. Using JMP software, contour plots were created to illustrate how pH and seed density affect titre, protein A yield and post-protein A HCP levels at a temperature of -1 and +3. The contour plots for low temperature (A) and high temperature (B) were generated by fitting least squares regression models for titre (R^2 = 0.99 and 0.91), yield (R^2 = 0.75 and 0.62) and HCP (R^2 = 0.98 and 0.77) results and are based on the black experimental data points. While the feasible operating windows (white area) look slightly different at low vs. high temperature, both plots show that a feasible operating window for maximum titre is constrained by the downstream responses (protein A yield and post-protein A HCP levels), confirming the results of the first DoE that a trade-off between these responses may be necessary.

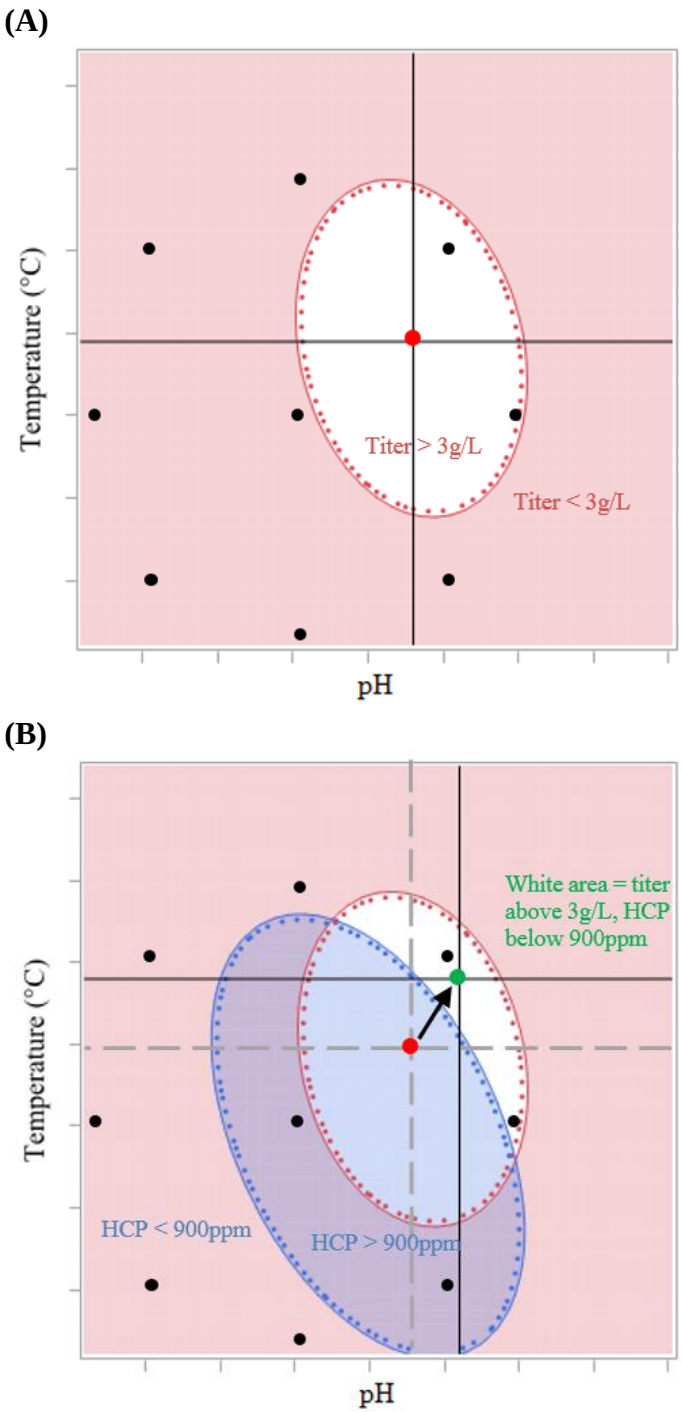


Figure 36. Window of operation for the mAb 2 ambr250 DoE. Using JMP software, a contour plot was created to illustrate how pH and temperature affect the titre and post-protein A HCP results, and how the feasible operating window (white area) gets smaller when considering not just the upstream requirement of high titre (A), but also the downstream requirement of low HCP levels (B). The contour plot was generated by fitting least squares regression models for the titre (R^2 = 0.95) and HCP (R^2 = 0.92) results and are based on the black experimental data points. These results correspond with the results from the first two DoEs that operating conditions optimised for upstream processing lead to lower product quality.

The contour plot for the mAb 2 ambr250 DoE was generated by fitting least squares regression models for the titre ($R^2 = 0.95$) and HCP ($R^2 = 0.92$) data to show the range of pH and temperature that would give rise to a window of operation, trading off both upstream and downstream responses in terms of high titre and low HCP levels respectively. Figure 36 A shows such a feasible operating window based purely on an upstream requirement (chosen here to be a titre of at least 3 g/L) which suggests operating at the midpoint of the feasible window to be optimal. However, Figure 36 B displays a smaller feasible operating window generated based on both an upstream and a downstream requirement (a titre of at least 3 g/L, as well as no more than 900 ppm post-protein A HCPs, which was deemed to be a suitable early development target based on industry trends). This second plot shows that the operating range needs to be shifted to a new optimum based on a trade-off between product titre and product quality (shift from red point to green point) resulting in a slight loss of mAb titre but increased product purity in terms of fewer post-protein A HCPs. In this instance, such a shift can be accomplished by increasing culture temperature by 1 °C and carefully maintaining culture pH at "+ 0.1" (cf. Table 5) ± 0.05, but the exact optimal culture conditions as well as the shape of the feasible window of operation will of course vary amongst different processes and molecules. Nonetheless, the contour plots discussed here highlight the issue that operating windows for optimised upstream responses can be constrained by downstream targets, and that trade-off decisions will likely be necessary.

It is noteworthy that the feasible window of operation in Figure 36 is based on HCP clearance after the critical protein A purification. The feasible operating space may widen when the whole downstream process is considered and HCP clearance during subsequent chromatography polishing steps is demonstrated. Alternatively, the operating space may become smaller when further desired responses are added to the contour plot, such as high product monomer percentage, but in this case study, all cultures were associated with low

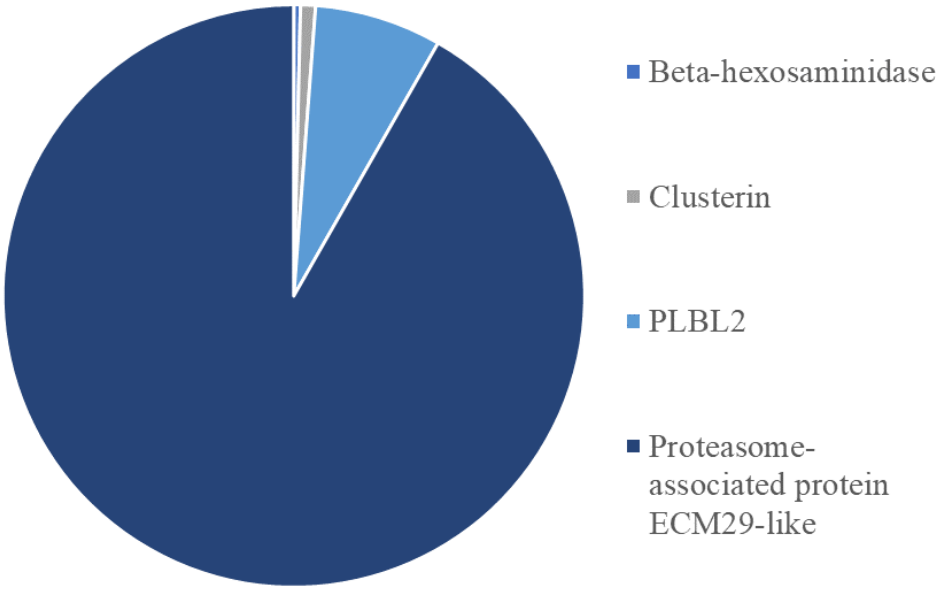
levels of aggregates and fragments post-protein A purification (data not shown) and therefore did not impact the window of operation.

Furthermore, the operating window in Figure 36 was produced under the assumption that lower HCP level are better for downstream clearance, without taking the species information into account which may show that at the "optimal operating window" more problematic or "sticky" HCP species are being produced.

To address this, mass spectrometry analysis (using the nano-LC Orbitrap) was carried out to evaluate potential differences in HCP profiles between a culture grown at optimal upstream conditions and a culture grown at the "compromise" conditions. The aim was to compare the red point and the green point from Figure 36, however none of the cultures from the third DoE were grown using conditions similar to the red point. Instead, the DoE centre point was chosen for this comparison. The green point is represented by the culture grown using very similar setpoints as the green point (cf. Figure 36).

Figure 37 shows the HCP species that have been identified by mass spectrometry to be co-eluting during protein A purification of the specified material. Slightly more clusterin and proteasome-associated protein ECM29-like were identified in cultures grown under the green setpoint conditions, while beta-hexosaminidase and slightly more PLBL2 were identified at the DoE centre point, however these differences are fairly minute. In this instance, the results therefore show that the HCP species which were detected in cultures grown using the "red" and "green" setpoint conditions were in fact very similar and thus it would indeed be beneficial from a combined upstream and downstream perspective to operate at the green culture conditions due to reasons explained previously. Had the HCP profile been different between these two conditions, with HCP species known to be problematic being produced at the green setpoints, it would have to be evaluated if continuing to operate at the red point might not be better after all.

(A)



(B)

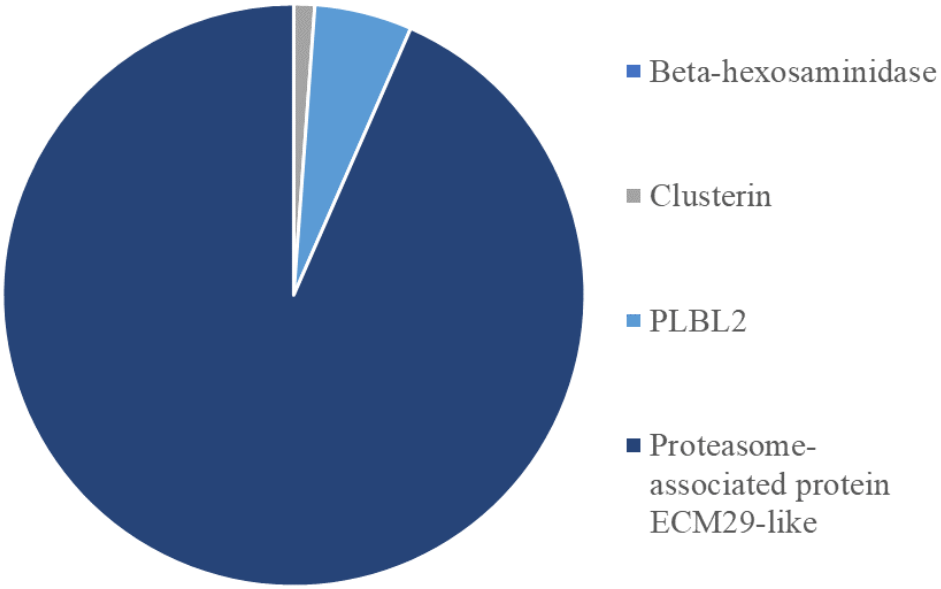


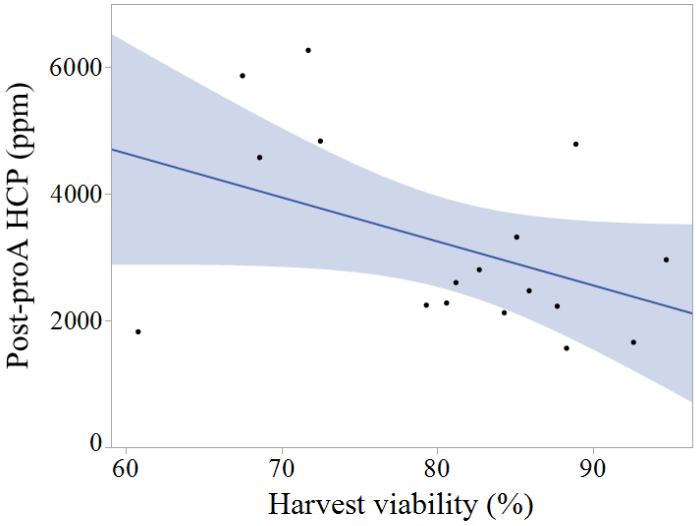
Figure 37. Comparison of identified HCP species at (A) the DoE centre point (1032 ppm post-protein A HPC levels quantified by ELISA) and (B) the green point (759 ppm post-protein A HPC levels quantified by ELISA). HCP species were identified by using nano-LC Orbitrap mass spectrometry. Common contaminants as well as HCPs with only one peptide have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data.

3.4.2 *Culture viability*

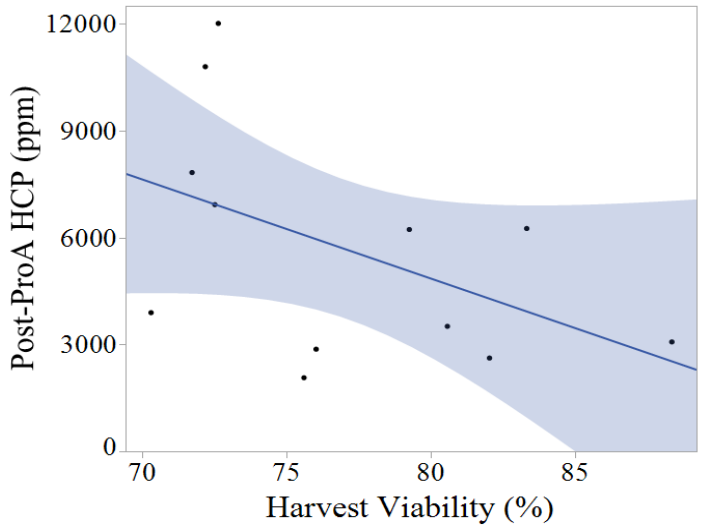
Assuming that decreasing product purity is caused by stressed or dying cells, culture viability may be an indicator that can predict poor product quality since low viability cultures are likely under a lot of cellular stress and may be producing more HCPs involved in the stress response that might be less efficiently cleared during affinity chromatography. This hypothesis is complemented by published literature (Jin et al., 2010; Goey et al., 2017). In this section, a correlation between culture viability at harvest and HCP levels in protein A purified material was therefore explored.

Surprisingly, there does not seem to be a strong inverse correlation between culture viability and post-protein A HCP levels i.e. high viability cultures being associated with fewer HCPs than low viability cultures (Figure 38). However, as previously discussed, culture viability at the point of harvest is unlikely to be an ideal indicator given the many variables involved in these DoEs such as varying seed densities and different harvest days. So, while culture viability at harvest will remain an important factor to monitor during production of biopharmaceuticals to ensure cells are healthy, in this research it was found that harvest day culture viability by itself cannot be used as a reliable indicator to predict product quantity or post-protein A HCP quality.

(A)



(B)



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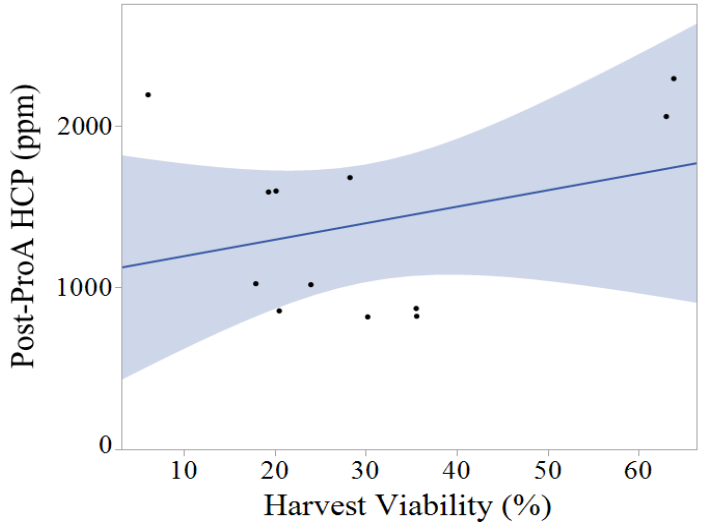


(D)

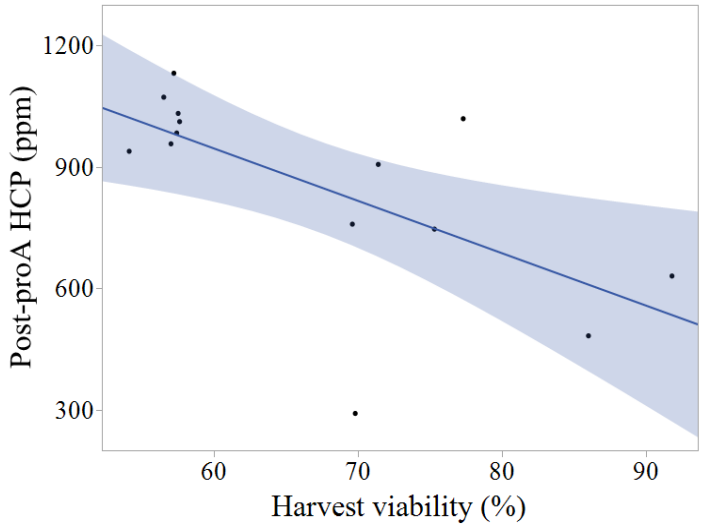


Figure 38. Correlations between harvest culture viability and post-protein A HCPs in the three DoEs. (A) mAb 1 ambr250 DoE (R^2 = 0.20), (B) mAb 1 ambr15 DoE at low temperature (R^2 = 0.23), (C) mAb 1 ambr15 DoE at high temperature (R^2 = 0.10), and (D) mAb 2 ambr250 DoE (R^2 = 0.42) showing that high harvest viability is not a reliable indicator for low HCP values.

3.5 Summary

In this chapter, the impact of upstream operating conditions on product titre and post-protein A HCP levels was explored and trade-offs between titre and product quality were evaluated, using three Design-of-Experiment (DoE) studies. Upstream process parameters that were studied were culture pH, temperature and seed density using two different mAbs and two different bioreactor scales (15mL and 250mL). Outputs of interest in this chapter were antibody titre, culture viability at harvest, and an important downstream response – post-protein A HCP levels. These upstream parameters have all been examined previously and their impacts on antibody titre in particular have been widely covered in the literature (Yoon et al., 2003; Trummer et al., 2006; Mason et al., 2014; Hennicke et al., 2019; Schneider et al., 2019). Effects on post-protein A HCP levels on the other hand are scarcer: Examples of published literature in this field specifically include Goey et al. (2017) who studied what effects a temperature downshift to mild hypothermia had on antibody titre, HCP concentration and HCP species. They concluded that CHO cell cultures grown at standard physiological temperature followed by a shift to mild hypothermia on day 5 of culture resulted in material with similar antibody titre and HCP concentration, but with lower cell growth rate, higher percentages of healthier cells and a less apparent onset of apoptosis, leading to a noticeably different HCP composition, particularly fewer intracellular HCPs or those localised to the cell membrane. And while this research is very interesting, it focussed only on one upstream parameter (i.e. culture temperature).

As such, what has been novel in this chapter is the holistic approach that was taken by combining upstream DoE studies with examinations into the impact on downstream responses, and determining that pH, temperature and seed density affect product quantity and quality in ways that necessitate trade-off decisions between upstream and downstream responses. This was shown in contour plots that illustrate feasible operating

windows which are of mutual benefit to both upstream and downstream processing and is something that has not even been published by Schneider et al. (2019) who carried out a DoE to determine optimal values for temperature, pH, seed density, and harvest day in order to minimize product fragmentation while maximizing titre.

This novel display of overlapping windows of operation for contradictory upstream and downstream targets has been published by us in Wilson et al. (2019).

Also in this chapter, and based on the realisation that upstream decisions affect downstream performance, a correlation between culture viability and post-protein A HCP levels was investigated in the hope of being able to predict the amount of HCP impurities that might be present after affinity chromatography. Being able to predict this, before carrying out the expensive protein A chromatography step would save costs and make process development more efficient. However, this chapter has shown that culture viability at harvest cannot be used as such an omnipotent prediction tool. The next chapter will therefore focus on exploring other potential indicators that can be measured in HCCF to predict post-protein A HCP levels.

4 IDENTIFICATION OF HCCF-ASSOCIATED QUALITY SIGNATURES

4.1 Introduction

Since harvest culture viability proved not to be a reliable indicator for the prediction of poor product quality, other options are investigated in this chapter. As we have learned in the previous chapter, upstream operating conditions that are optimised for high antibody titre can often also be the cause for higher levels of post-protein A HCP levels which necessitates the implementation of additional HCP clearance steps. Being able to predict post-protein A product quality prior to carrying out this expensive purification step would be immensely beneficial during the process development of a therapeutic protein and could save a lot of time and money, both in terms of preventing a waste of expensive resources as well as with regards to applying a "fail early, fail cheap" methodology.

The main focus of this chapter is therefore to investigate potential product quality indicators that can be measured in HCCF, but which correlate to and predict post-protein A product quality. Ideally such "signatures" could then be used to label upstream material as either good or bad – or green versus red, similar to a traffic light system which either approves upstream material being passed onto the next step (aka product purification) or stops any further processing of the material.

The potential HCCF-associated quality signatures that are presented in this chapter are cholesterol, ammonium, osmolality and antibody light chains. Various assays were used to measure each of these in the HCCF samples of the same DoE studies that were discussed in the previous chapter (Figure 39). Each of these potential signatures were then correlated to post-protein A product quality attributes (HCP and monomer purity).

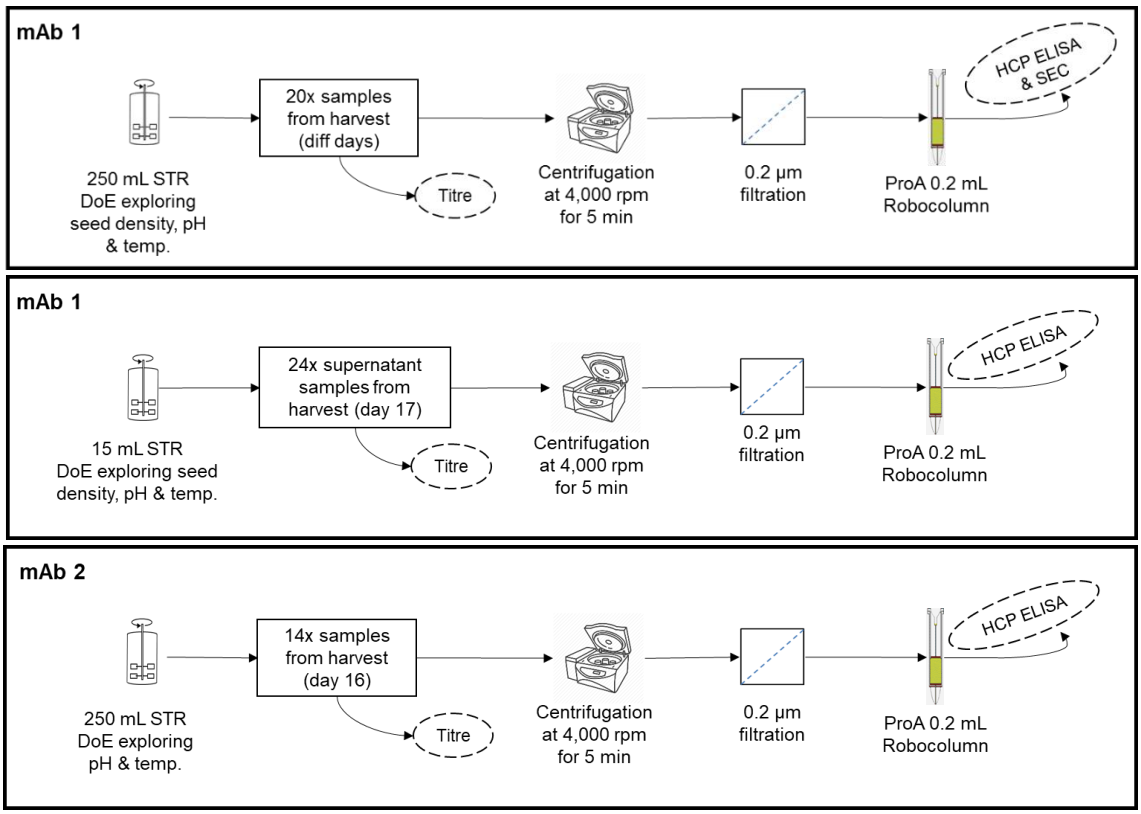


Figure 39. Summary of the DoE experiments discussed in chapter 4 (mAb 1 ambr250 DoE, mAb 1 ambr15 DoE, mAb 2 ambr250 DoE).

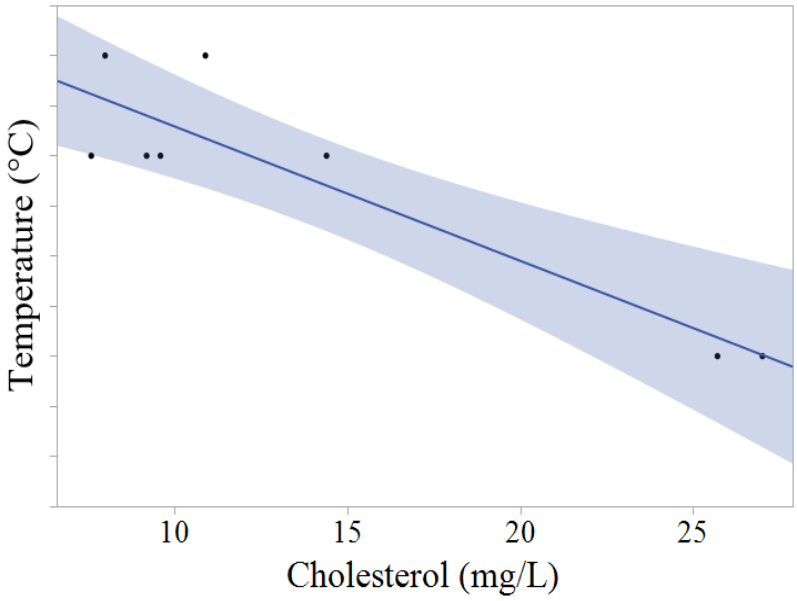
4.2 Cholesterol

Assuming that decreasing product purity is caused by stressed or dying cells and knowing that the Trypan blue exclusion method to measure culture viability is not an accurate way to define apoptotic and lysed cells, other options were explored. Assays that measure lactate dehydrogenase (LDH) or DNA could be used, since these are both localised intracellularly and would thus only be detectable upon lysis of the cell, with higher levels corresponding to a higher degree of lysed cells. Another possible assay, and the one that is discussed here, detects levels of cholesterol in HCCF. Since cholesterol is a lipid that is present in cell membranes and can thus serve as a measure of cell lysis, it was explored if cholesterol content in HCCF could be used as an indicator for post-protein A HCP levels (based on Senczuk et al., 2016). To test this hypothesis, a commercial cholesterol assay kit (E2CH-100, BioAssay Systems, California, USA) was used to measure cholesterol content in samples from the DoE studies discussed in the previous chapter.

Subsequently cholesterol values were correlated to the upstream operating conditions as well as to product quality measurements.

The first DoE study showed that cholesterol values in fed-batch samples correlate strongly with temperature – cultures that were grown at lower temperature were associated with more cholesterol (Figure 40 A), presumably due to cultures being grown at low temperature having a lower viable cell density (Figure 40 B).

(A)



(B)

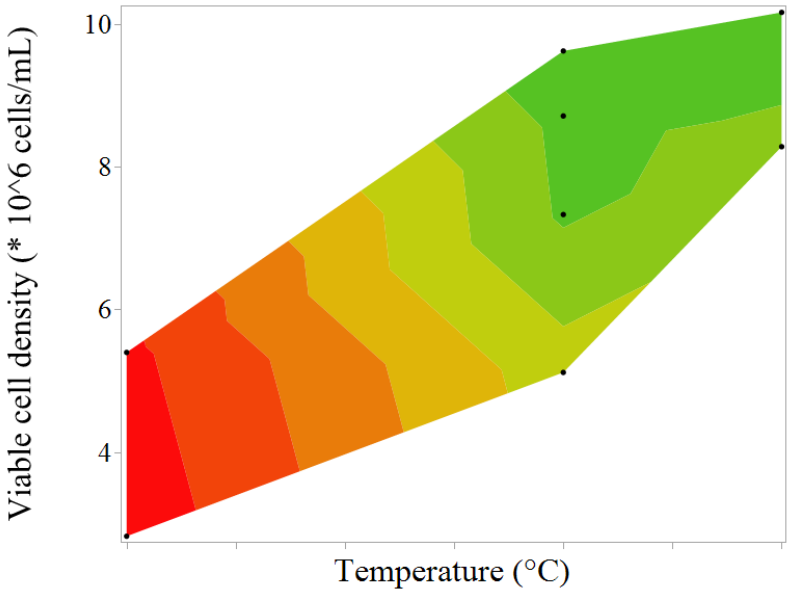


Figure 40. (A) Correlation between temperature and cholesterol in the first DoE (fed-batch cultures) (R^2 = 0.83). (B) Heat map of cholesterol levels as a function of temperature and viable cell density. Low cholesterol levels are shown in green (< 10 mg/L) while high cholesterol levels are shown in red (> 25 mg/L), with contour intervals of 3 mg/L. Cholesterol levels increase with decreasing temperature and viable cell density.

However, only a weak correlation to post-protein A HCP levels and no correlation to monomer purity could be observed (Figure 41), although the latter is not unusual, as cell lysis is not expected to have a strong impact on product monomer percentage.

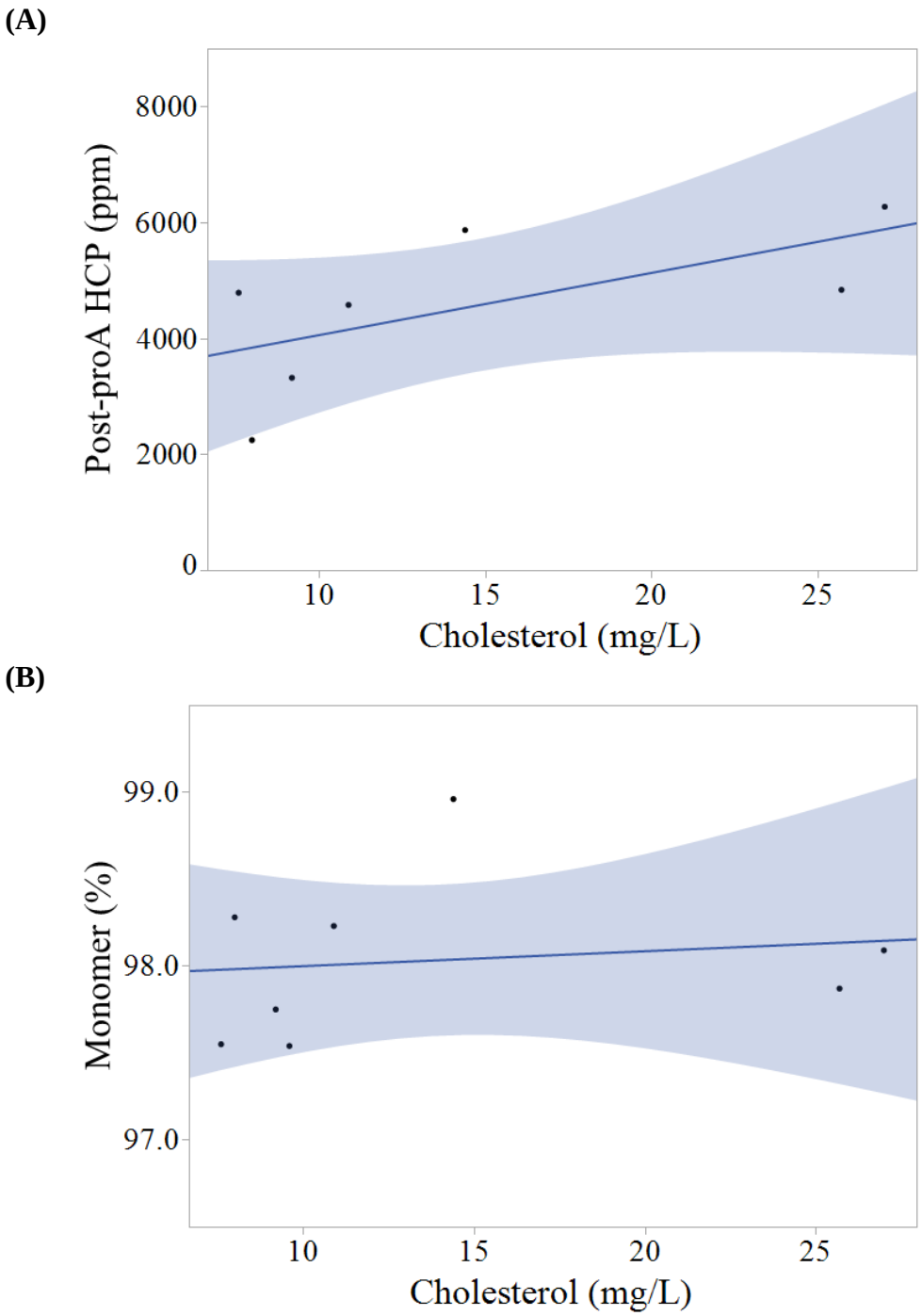


Figure 41. Correlation between cholesterol and (A) post-protein A HCPs (R^2 = 0.41) and (B) post-protein A mAb monomer (R^2 = 0.02) in the first DoE (fed-batch cultures). While upstream conditions seem to have an effect on cholesterol, cholesterol has only weak correlations to product quality attributes.

Similar correlations to temperature and post-protein A HCPs were seen in another DoE study (Figure 42).

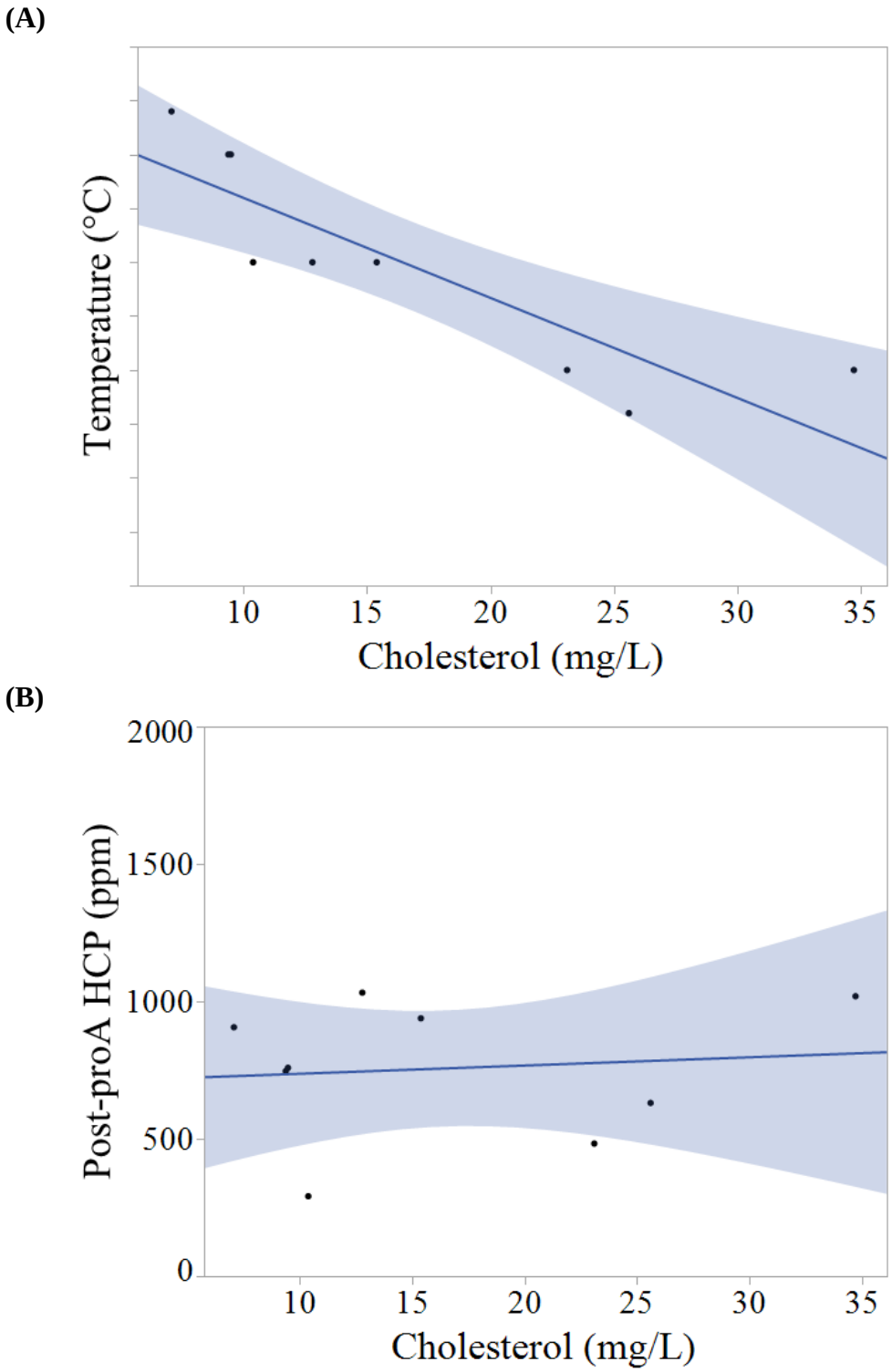


Figure 42. (A) Correlation between temperature and cholesterol (R^2 = 0.76) in the third DoE. Low temperature cultures are associated with high levels of cholesterol as in mAb 1 ambr250 DoE, however no correlation to HCP is seen (R^2 = 0.01) (B).

The ambr15 DoE study showed that growing cultures at low pH (whether at low or high temperature) results in quite poor titres (equal to or less than 1 g/L) as shown in Figure 43.

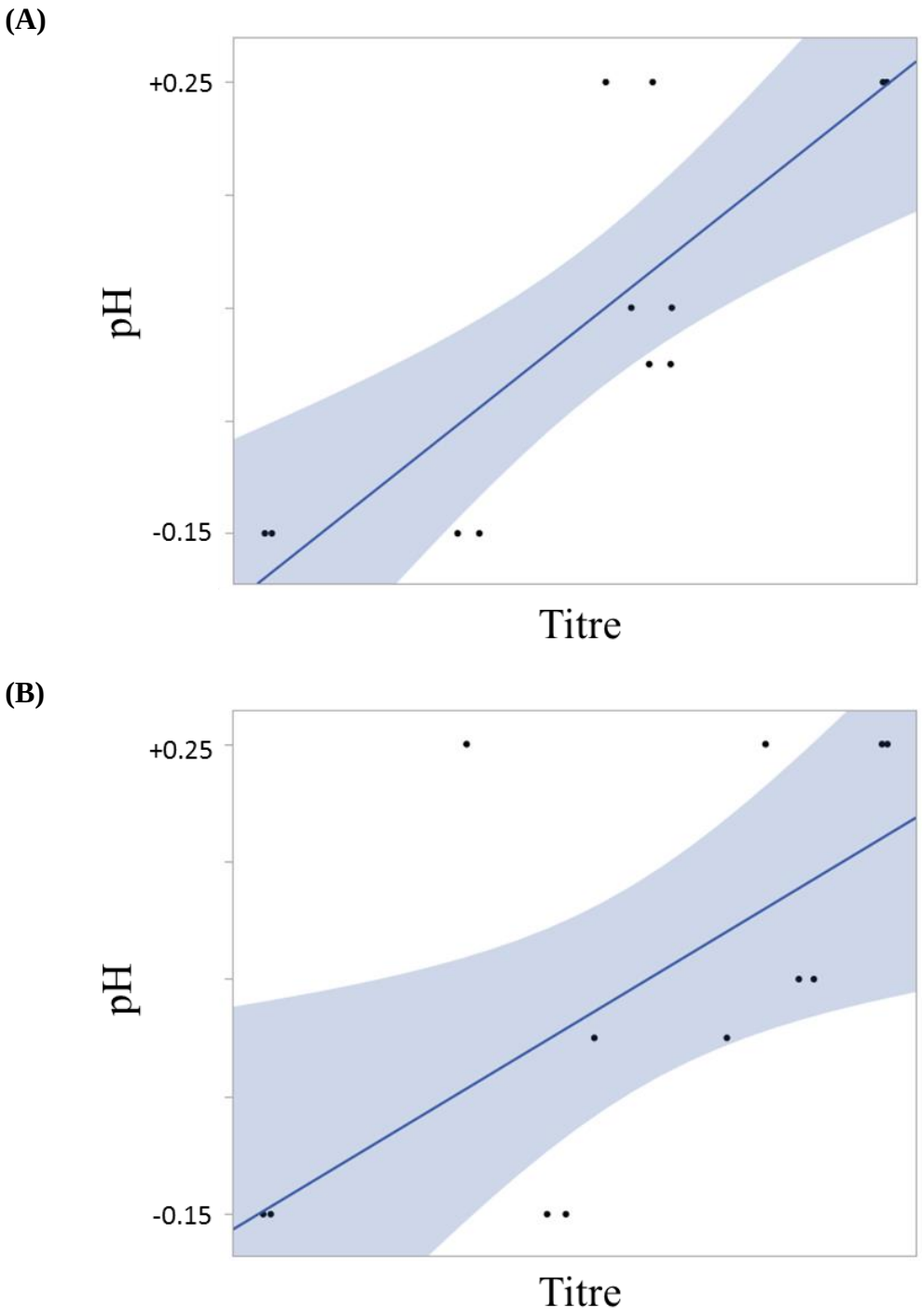


Figure 43. Correlation between pH and product titre at (A) low temperature (R^2 = 0.66) and (B) high temperature (R^2 = 0.42) in the second DoE.

As upstream engineers would not operate at these conditions, low pH samples have been excluded from Figure 44, which shows that samples containing less than 10 mg/L cholesterol (indicated by the orange line) are associated with fewer HCPs.

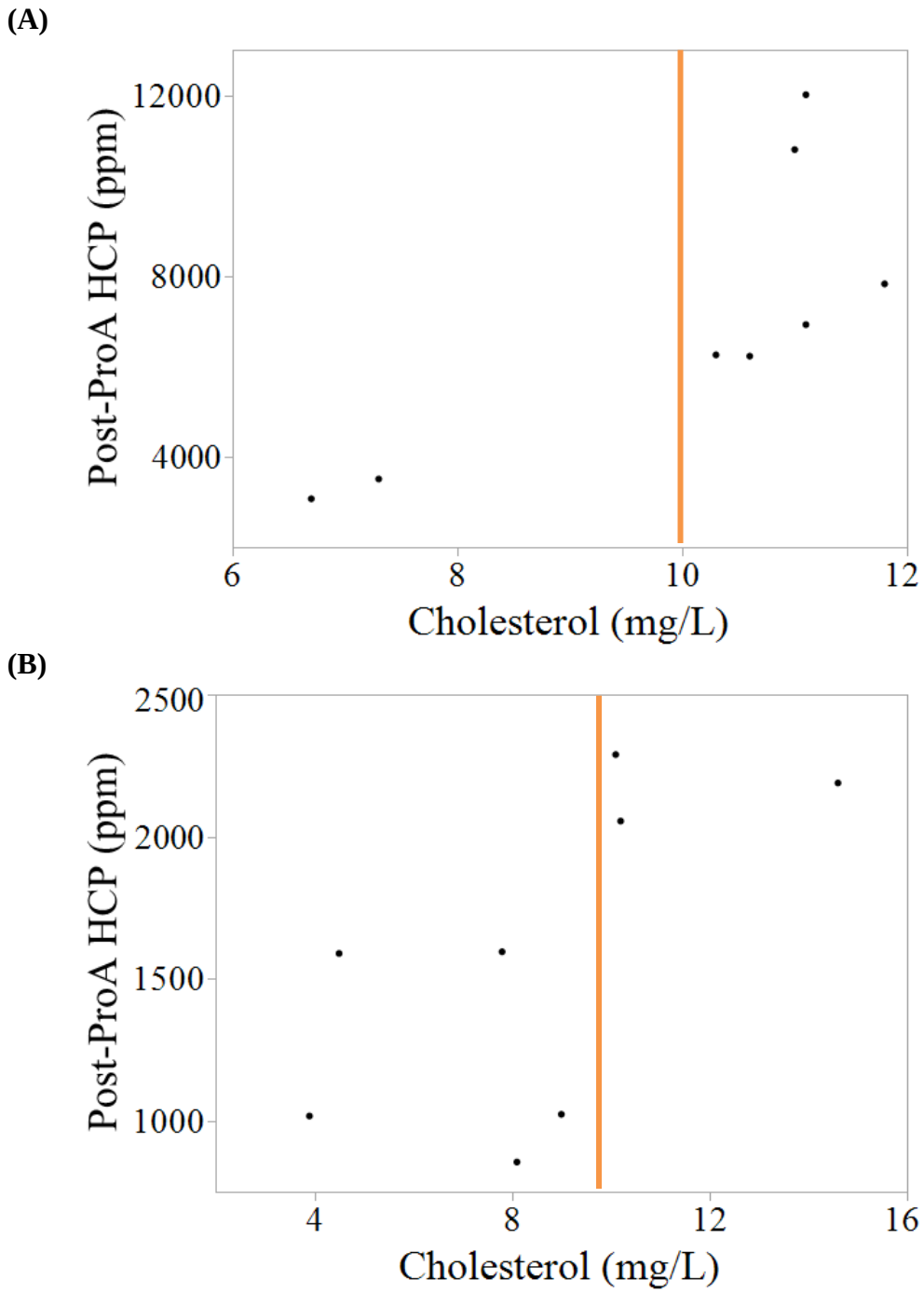


Figure 44. Correlation between cholesterol and post-protein A HCP levels at a temperature of -1 (A) and +3 (B) in the second DoE. Samples associated with less than 10 mg/L of cholesterol (indicated by the orange line) contain generally fewer HCPs.

These results have been summarised in Figure 45 which shows the temperature and pH operating conditions as a heat map and the results for cholesterol, titre and post-protein A HCPs as a traffic light system. Low temperature and pH are shown in blue, while high temperature and pH are illustrated in red. Cholesterol levels of 10 mg/L or less were deemed acceptable based on Figure 44, and are indicated by a green traffic light. Antibody titre of more than 1.5 g/L has also been marked by a green traffic light. In terms of HCPs, different constraints have been placed on the material, depending on whether cultures were grown at low or high temperature, since low temperature resulted in much higher HCPs. Therefore, HCP values up to 4,000 ppm for low temperature samples are indicated with a green traffic light, while the cap for high temperature samples was set at a maximum of 2,000 ppm. If the same limits were applied to all samples, the low temperature cultures would likely all have a red traffic light while high temperature cultures would all have a green traffic light. Setting individual conditions for the two groups of samples enables a more meaningful analysis.

At low temperature, samples with less than 10 mg/L cholesterol were associated with 3,000 – 3,500 ppm HCPs, and samples with more than 10 mg/L cholesterol are associated with HCP values between 6,000 – 12,000 ppm. At high temperature, samples with less than 10 mg/L cholesterol are associated with equal to or less than 1,600 ppm HCPs (Figure 44). In this study, cholesterol measurements can therefore predict post-protein A HCP levels, and together with acceptable titres can be used to choose upstream operating conditions that are favourable for both upstream and downstream processing.

While all these results together do show that a recommended cholesterol limit differs for each molecule or process, cholesterol as a potential product quality indicator will be explored further in chapter 6.

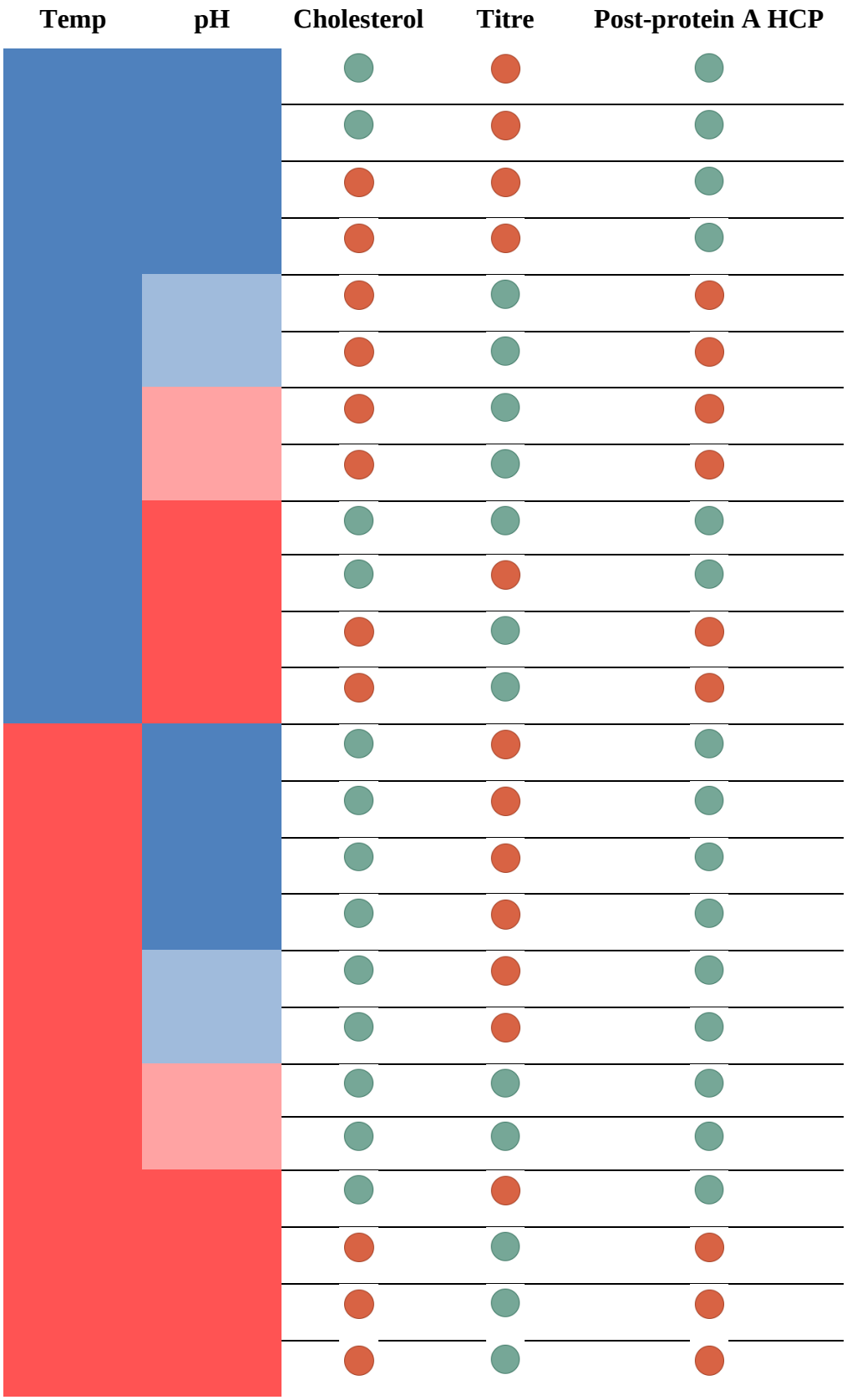


Figure 45. Heat map with traffic light system for cholesterol as a possible product quality indicator (second DoE). Low temperature and pH conditions are illustrated in blue, high levels in red. Symbols are green if the following conditions are met: less than 10 mg/L cholesterol, more than 1.5 g/L antibody titre and less than 4,000 ppm HCPs (for low temperature samples) or less than 2,000 ppm HCPs (for high temperature samples).

4.3 Ammonium

Ammonium is part of a cell's nitrogen metabolism along with glutamine and glutamate. It is known that culture overfeeding leads to an accumulation of ammonium which affects culture pH and is growth and productivity inhibitory (Chen & Harcum, 2005; Reinhart et al., 2015). Ammonium levels were therefore investigated in the mAb 1 and mAb 2 ambr250 DoEs. Cultures from these two studies were associated with wide ammonium ranges which were then correlated to product quality attributes (Figure 46).

As Figure 46 shows, ammonium correlates differently to product quality depending on cell line. For mAb 1, barely any of the linear correlations can be considered true as the confidence intervals are too wide for post-protein A HCP levels and monomer / aggregate percentages. The results for mAb 2 are more promising, showing that increasing levels of ammonium are associated with reduced antibody titre and post-protein A HCP levels (most probably caused by the inhibitory effect on growth and productivity) as well as with increased percentages of aggregation.

The contradictory results between the two studies could be due to the poor model fit for the mAb 1 DoE which in turn is likely the result of this particular bespoke DoE design. The model fit for the mAb 2 DoE is better and provides more confidence in the accuracy of the data. Another possibility for the varying responses could be the use of different cell lines, which would suggest that ammonium is not a reliable indicator for product quality. While it would be possible to assess the suitability of ammonium as an indicator for poor product quality by performing DoE studies and small-scale protein A purification runs for each therapeutic antibody or process, this defeats the purpose of identifying a suitable marker that can be measured in HCCF in order to avoid the costly affinity purification step on unsuitable material.

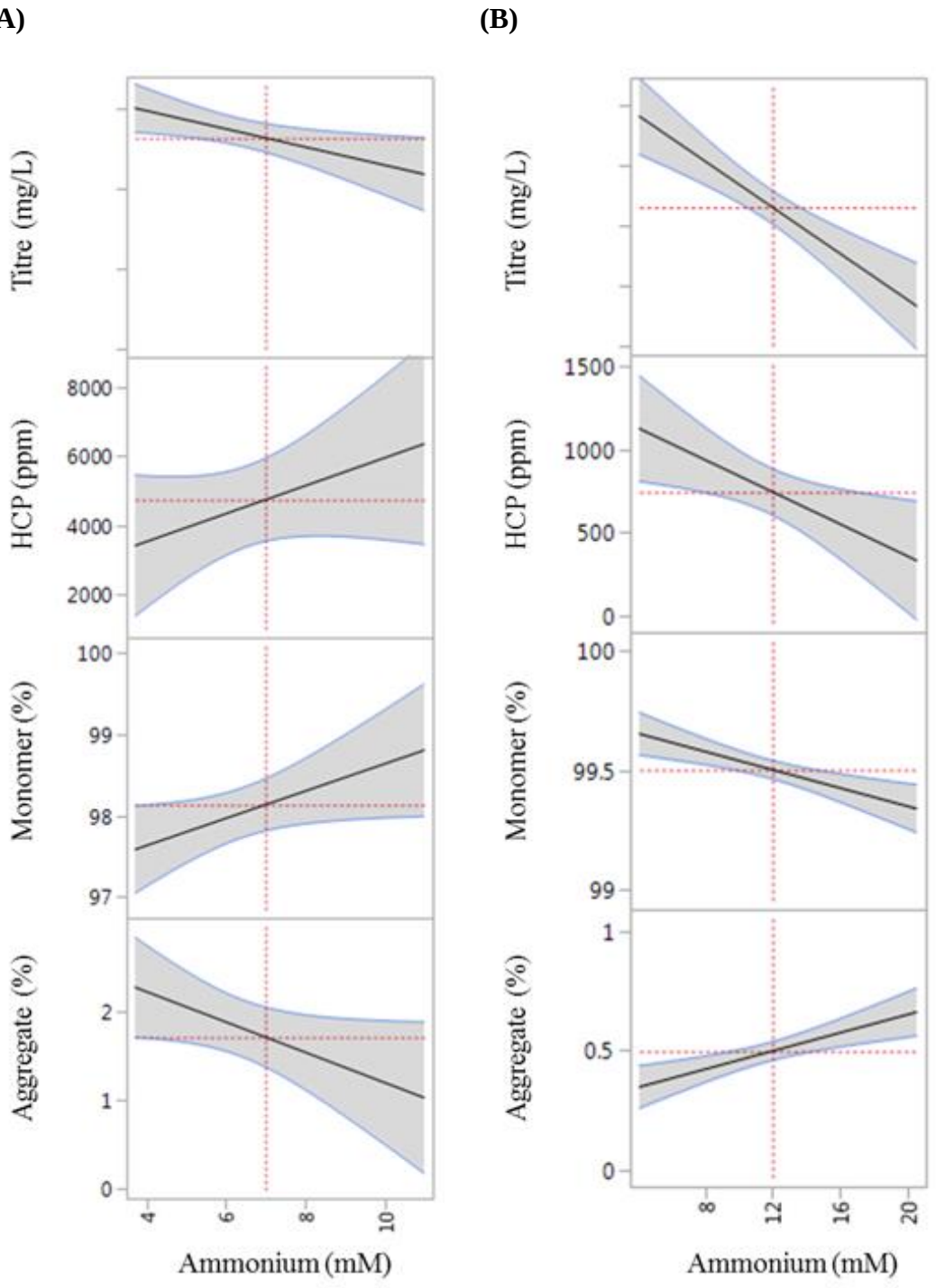


Figure 46. Correlations between ammonium and titre / product quality in (A) mAb 1 ambr250 DoE fed-batch cultures and (B) mAb 2 ambr250 DoE. R^2 values for the model fit in (A) are 0.6 for titre, 0.4 for post-protein A HCPs, 0.5 for monomer and 0.5 for aggregation. R^2 values for the model fit in (B) are 0.84 for titre, 0.57 for post-protein A HCPs, 0.72 for monomer and 0.72 for aggregation.

4.4 Osmolality

Osmolality measures the concentration of solutes (such as salt, amino acids, trace elements) in solution. Values below 300 mOsm/kg are generally considered beneficial for growth, whereas higher osmolality (>500 mOsm/kg) slows down cell growth (Kim et al. 2002). Increased osmolality can be caused by increased pCO_2 which in turn is caused by a build-up of carbon dioxide, a by-product of mammalian cell metabolism. Elevated pCO_2 levels have been indicated with inhibiting cell growth and/or recombinant protein production, although in bench-top bioreactors, increased CO_2 levels are usually not a problem (Kimura & Miller, 1996).

Hyperosmolality is also caused by an accumulated feed – which is why high osmolality is more commonly considered to be an indicator of overfeeding (Yu et al., 2011; Reinhart et al., 2015) – or can occur due to the addition of a base for optimal pH control (Han et al., 2010). This can lead to a reduction in cell growth, viability, and recombinant protein yields (Reinhart et al., 2015).

Osmolality was measured in the mAb 2 ambr250 DoE and plotted against post-protein A HCP levels. Figure 47 shows that there is a strong correlation between these two, with low osmolality being associated with high HCP values and vice versa. The figure also shows product titre for each sample (with green dots representing high titre and orange / red dots representing low titre). This highlights the fact that cultures with high osmolality values may be associated with low HCPs but they also produce less antibody, most likely due to the previously mentioned inhibitory effects of high osmolality on growth and productivity. This suggests that mid-range of the measured osmolality values would be a good compromise for titre and product purity.

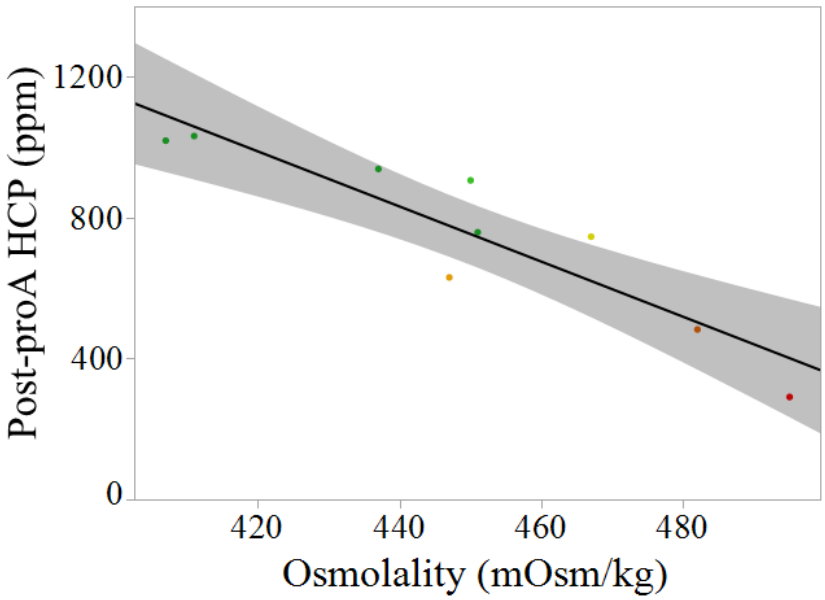


Figure 47. Correlation between osmolality and post-protein A HCPs (R^2 = 0.83), overlaid with titre results from the third DoE (high titres in green and low titres in red, where the lowest titre was 26.6% compared to the highest titre in this dataset).

In the literature, hyperosmolality has been shown to increase specific antibody productivity (q_{Ab}) in mammalian cells but decrease specific growth rate (Zhu et al., 2005; Han et al., 2009; Yu et al., 2011), unless an osmoprotective compound (e.g. glycine betaine) is used (Ryu et al., 2000). Increased specific productivity as a result of increased osmolality may be linked to improved nutrient transport into the cells, G1 cell cycle arrest or higher transcription / translation rates (Han et al., 2009). However, the exact impact on productivity and growth is cell line dependent and can result in the final antibody titre being either increased, decreased, or comparatively unaffected (Ho et al., 2006; Yu et al., 2011).

Therefore, the results discussed here are consistent with some previously published literature such as Ryu et al. (2000) who noted a 60% decrease in final mAb titre over an osmolality increase from 305 to 537 mOsm/kg, despite a 410% increase in q_{Ab} (most likely due to a more severe decrease in growth rate).

Han et al. (2010) have also demonstrated in their study that while q_{Ab} was increased with increasing osmolality, antibody titre was highest at 310 mOsm/kg (compared to 410

mOsm/kg, 510 mOsm/kg and 610 mOsm/kg) because of inhibited cell growth and cell death at higher osmolality.

However, opposite effects to those observed here have also been published, e.g. Han et al. (2009) have seen increased IFN-β concentrations at 470 mOsm/kg compared to 310 mOsm/kg despite a reduced cell growth, due to significantly increased specific productivity rates (17-fold higher q_p resulting in 4.8-fold higher titre).

Another example was published by Zhu et al. (2005) who reported that hyperosmolality (450 mOsm/kg compared to the control at 316 mOsm/kg) resulted in a 60% decreased cell growth rate, but final titre remained unaffected due to an increase in specific productivity rate.

While the correlation between osmolality, titre and post-protein A HCP levels shown here is promising for the implementation of osmolality as a HCCF-associated quality signature, this conclusion is based on only one dataset as osmolality was not recorded for the first two DoEs. This potential product quality indicator will therefore be discussed further in chapter 6.

4.5 Antibody light chain levels

Previous literature has suggested that the amount of antibody light chain is indicative of sufficient peptide synthesis, correct protein folding and antibody assembly, and thus high levels of extracellular antibody light chains are correlated to high antibody production and low aggregation (Bhoskar et al., 2013).

Free antibody light chain (LC) measurements have been compared both to HCP quantification data as well as to aggregation data from the first DoE. Regarding the comparison to HCP data, Figure 48 shows that cultures with high levels of free LC are associated with low levels of post-protein A HCPs. However, the data does not confirm the theory that cultures with low free LC are potentially stressed and produce higher

amounts of HCP species that are less efficiently cleared during affinity chromatography, as there are low-LC cultures that are associated with both high and low HCP levels.

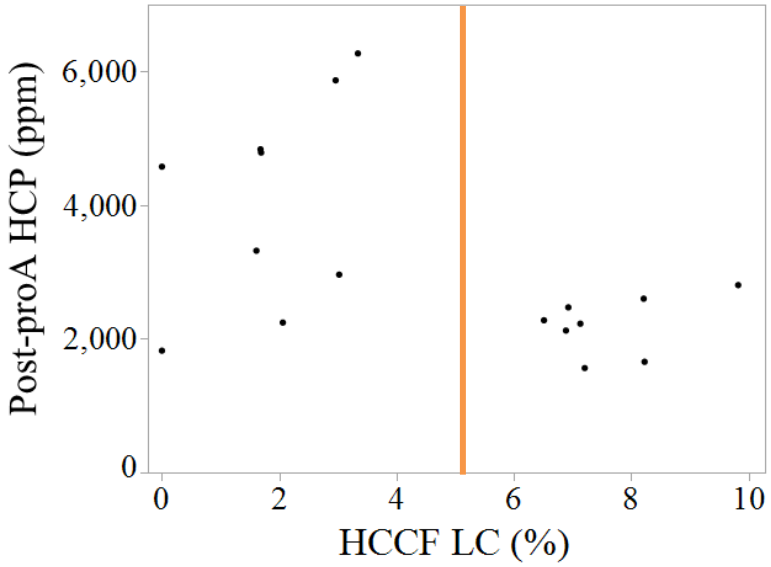


Figure 48. Correlation between free antibody light chain (HCCF LC) and post-protein A HCP levels in the first DoE. Cultures with low levels of free antibody light chains (to the left of the orange line) are illustrated with a red traffic light in Figure 50, while cultures with high levels of light chain (to the right of the orange line) are symbolised with a green traffic light.

Comparing free light chain levels with aggregation, Figure 49 shows that product aggregation did not vary significantly in cultures grown under different upstream operating conditions. There are only three samples that are associated with slightly more than 2% aggregation and none of the samples are therefore associated with problematic levels of aggregates. Due to the similarity of the aggregation results, there is no visible correlation between free antibody light chain and aggregation – the theory being that cultures associated with high levels of free LC are less likely to aggregate, whereas low levels of free LC might suggest that not enough light chain is produced for full Ig assembly, and so the remaining peptides could aggregate and cause (further) cell stress.

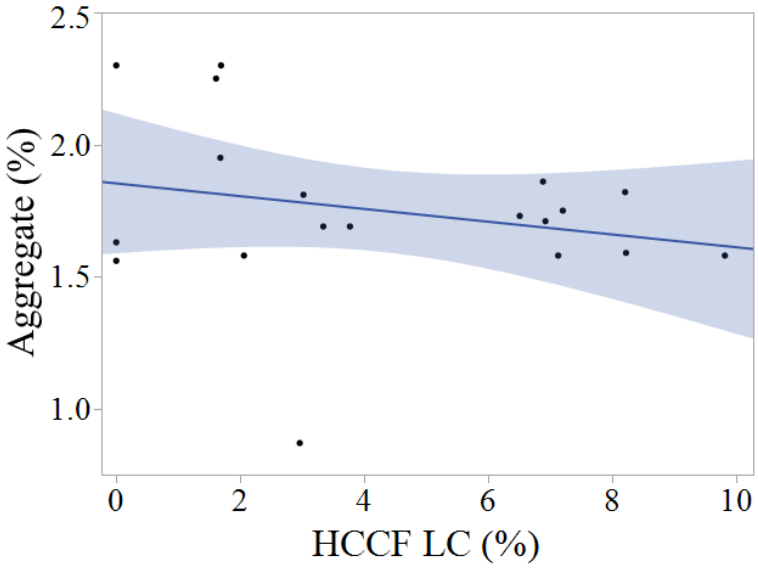


Figure 49. Correlation between free antibody light chain and aggregation in affinity purified samples in the first DoE (R^2 = 0.06). While cultures with more than 2% aggregation are associated with low levels of free antibody light chains, low levels of light chains are not indicative of high aggregation. As such, free mAb light chain quantity is not a suitable indicator for high aggregation.

Another observation made by Bhoskar et al. (2013) was that cultures with low free LC are associated with low productivity. This has also been contradicted in this instance as all low-LC cultures have produced relatively high antibody titre (cf. Figure 50), so antibody assembly does not seem to be an issue. It is possible that cultures which had a very high seed density and thus circumvented the initial required growth phase produced high amounts of antibody early on. The low levels of free light chain could in this case indicate that no new antibodies were being produced towards the end of the culture and would not reflect on the antibodies that had already been produced.

Another possible explanation for the contradictory results seen here compared to the data from Bhoskar et al. (2013) could of course be the different cell lines that were used. The relationship between the production of antibody and the levels of free antibody light chains is dependent on the design of the vector that was used to integrate the antibody's gene into the host genome – a different vector design, a different ratio of gene copy numbers for the heavy and light chains, or a different integration of the recombinant DNA

will all affect the observed relationship between free antibody light chains and titre productivity (Li et al., 2007; Davies et al., 2011; Gion et al., 2013). And in this instance, using mAb 1, antibody light chain measurements were found to be unsuitable as a global indicator for poor product quality.

Figure 50. Traffic light system for antibody light chain as a possible product quality indicator (first DoE). For mAb light chains, green symbols represent more than 5% while red symbols represent less than 5%. For Titre, more than 70% (relative to the highest producing culture) are indicated in green, between 50-70% are shown in yellow, and less than 50% are shown in red. For post-protein A HCPs, green symbols represent less than 2,500 ppm HCPs, while red symbols represent more than 2,500 ppm. For post-protein A aggregation, levels above 2% are shown in red.

To highlight this once again, Figure 50 summarises the data discussed here in the form of a traffic light system, showing that antibody light chain measurements are unsuitable as a global indicator for poor product quality. While cultures with a green traffic light for

mAb light chain levels are mostly associated with low HCP levels, they are also associated with low titre. Red traffic light cultures on the other hand would be ruled out with this system even though there are cultures associated with high titre, low HCP levels and low product aggregation. This figure also shows again that high titre is generally associated with high HCP levels and vice versa.

4.6 Summary

In this chapter, several potential HCCF-associated quality signatures (cholesterol, ammonium, osmolality and antibody light chains) were explored for their potential to be used as prediction tools in estimating post-protein A product quality, prior to carrying out the expensive protein A chromatography step, as this would make process development more efficient. Ideally such "signatures" could be used to label upstream material as either good or bad – or green versus red, similar to a traffic light system which either approves upstream material being passed onto the next step (aka product purification) or stops any further processing of the material.

It was determined that cultures which were grown at lower temperature were associated with more cholesterol, and cholesterol measurements were shown to be able to predict post-protein A HCP levels in the mAb 1 ambr15 DoE, demonstrating that together with acceptable titres, cholesterol could potentially be used to choose upstream operating conditions that are favourable for both upstream and downstream processing.

Ammonium was deemed to be an unreliable prediction tool due to varying responses with different cell lines. This would make it necessary to first perform small-scale studies to determine the suitability of ammonium for each potential drug product which is counter-productive to the purpose of identifying a suitable marker that can be measured in HCCF in order to avoid the costly affinity purification step on unsuitable material.

Osmolality was discovered to be a very promising prediction tool, although further datasets are required to validate the observations made in this chapter. This potential product quality indicator will therefore be discussed further in chapter 6.

Antibody light chain levels were also deemed unreliable as a prediction tool due to a lack of linear correlations to post-protein A HCP levels or monomer purity. A potential traffic light system for antibody light chains as a quality indicator was created to highlight how such a system would fail at identifying cultures that are associated with high titre, low HCP levels and low product aggregation.

While some of these results are promising and a good basis for further research, the challenge to find HCCF-associated quality signatures that can be used to create suitably reliable and robust traffic light systems for mAb processing meant that other options needed to be considered.

The next chapter will therefore explore the impacts of upstream processing on product quality from a different angle and investigate the potential benefits of harvesting upstream material earlier.

5 EFFECTS OF UPSTREAM HARVEST TIME ON MAB PRODUCT QUALITY

5.1 Introduction

As studied in the third chapter, upstream operating conditions that are optimised for higher levels of antibody can result in decreased product quality. One upstream operating condition that has not been investigated in the third chapter is the length of cultivation. CHO-expressed mAbs are commonly produced in fed-batch processes, during which time the CHO cells grow in number and produce increasing amounts of protein – both the desired antibody as well as the host cell proteins that are required for the host cells to survive.

Assuming that increased culture duration is associated with aging cells becoming less sufficient at transcribing, translating, folding, and/or secreting antibody or with dead cells releasing intracellular impurities – including proteases which are increasingly produced as part of the cell stress response during late stage culture (Jin et al, 2010; Tait et al, 2012) – product quality may get progressively worse and it may be feasible to harvest the mAb product earlier.

This chapter investigates the effects of upstream harvest time on mAb product quality as well as harvest time interactions with subsequent downstream processing steps, in order to determine how much impact the culture duration has on desirable upstream responses, i.e. antibody titre, as well as downstream responses such as product monomer levels and the amount of host cell proteins present in purified material [4]. The aim of this chapter is to explore whether there is an ideal harvest point during the culture when product titre is already quite high, but impurity levels are still relatively low [5].

[4] I was provided with upstream material for the first study discussed in this chapter. All other experimental work discussed in this chapter (whether upstream, downstream, or analytical) as well as data analysis was carried out by me. For the second shear study, I had help to operate the bioreactors and had guidance on how to use the mass spectrometer.

[5] The results discussed in sections 5.2 – 5.4 have been published in Wilson et al., 2019.

5.2 Harvest time vs. product quality

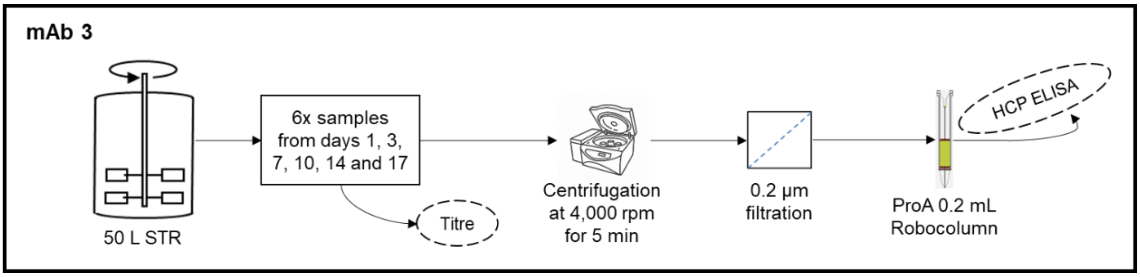


Figure 51. Summary of mAb 3 time-course study.

In the first study for this chapter, I was provided with samples that were taken throughout the production bioreactor run of mAb 3 (including on the harvest day when culture viability was at the lowest point of 93%) in order to investigate the effect that culture duration has on not only product titre but also post-protein A HCP levels. The purpose of this study was to investigate whether there is a trade-off between titre and HCP clearance with cell cultivation time, and whether it would ultimately save cost and work force to harvest the product earlier when it is purer and requires a less extensive downstream clearance strategy.

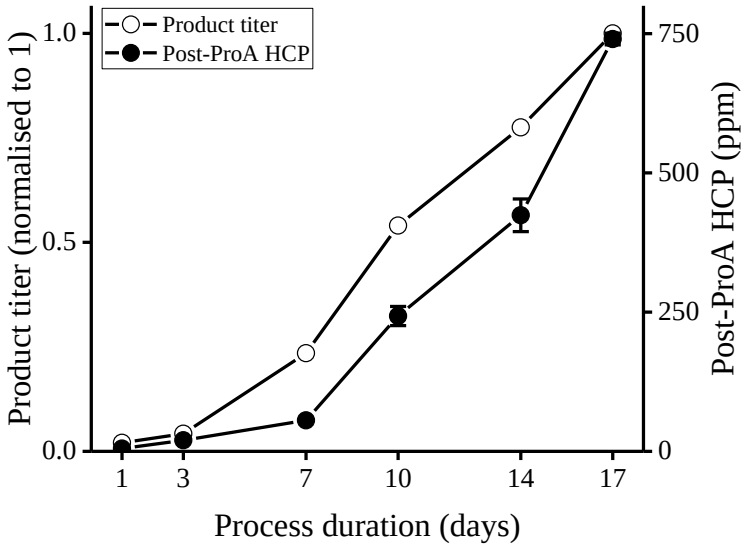
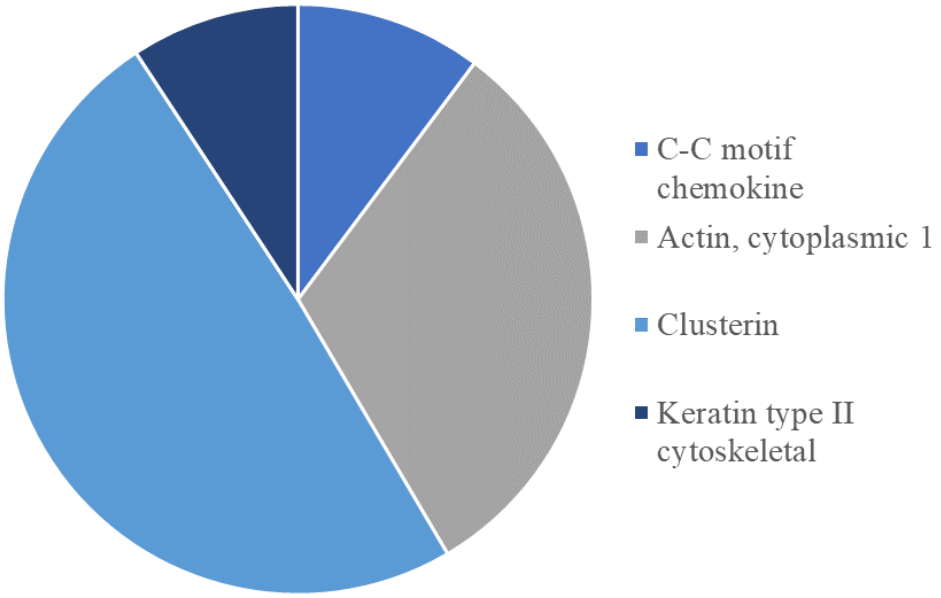


Figure 52. Effects of harvest time on product titre and HCP levels. Increasing product amount over the course of a culture is accompanied by decreasing product purity. Supernatant samples were taken throughout the 50 L STR production bioreactor run of mAb 3 (including on harvest day) and antibody concentration was measured and normalised to 1 due to confidentiality. Supernatant samples were filtered, protein A purified, and analysed for HCP quantities by ELISA. Note that the HCP data points for days 1, 3 and 7 are estimated on titre as HCP levels were below detection (< 2 ng/mL). Descriptive error bars are based on assay serial dilutions and show one standard deviation.

Figure 52 illustrates that as product titre increased throughout a typical mAb culture, so did the amount of post-protein A HCP impurities, meaning that product amount and purity are negatively correlated. The amount of product increased by 28% from day 14 to day 17, however post-protein A HCP levels increased by 75% during this time frame (Figure 52), demonstrating that material which is harvested later potentially requires a more extensive HCP clearance strategy. The HCP profiles at different stages of the cultivation (day 14 versus day 17) were compared by using mass spectrometry (nano-LC Orbitrap) and the identified species are shown in Figure 53. As can be seen, the HCP profiles at both days are very similar – the presence of keratin is likely indicative of contamination during sample handling. Of notable interest is the relative amount of each species: clusterin was present in higher levels on day 17 compared to day 14. Clusterin functions as an extracellular chaperone that maintains partially unfolded proteins in a state appropriate for subsequent refolding by other chaperones and prevents aggregation of non-native proteins. Its presence is therefore an indication that cells are struggling to maintain protein synthesis in late stage culture.

Previous literature (Jin et al., 2010; Tait et al., 2012) has suggested that the time of harvest is a crucial parameter with regards to the HCP composition in HCCF, and our data shows that harvest time further also affects the efficiency of HCP removal during protein A chromatography, with a later harvest time resulting in decreased post-protein A product purity. However, previous literature (Tait et al., 2009) also states that younger, more viable cells are more shear sensitive. An earlier harvest for material with fewer HCPs may therefore increase the risk of breaking cells and releasing any intracellular impurities during the harvest process, which is a challenge that will be addressed later in this chapter.

(A)



(B)

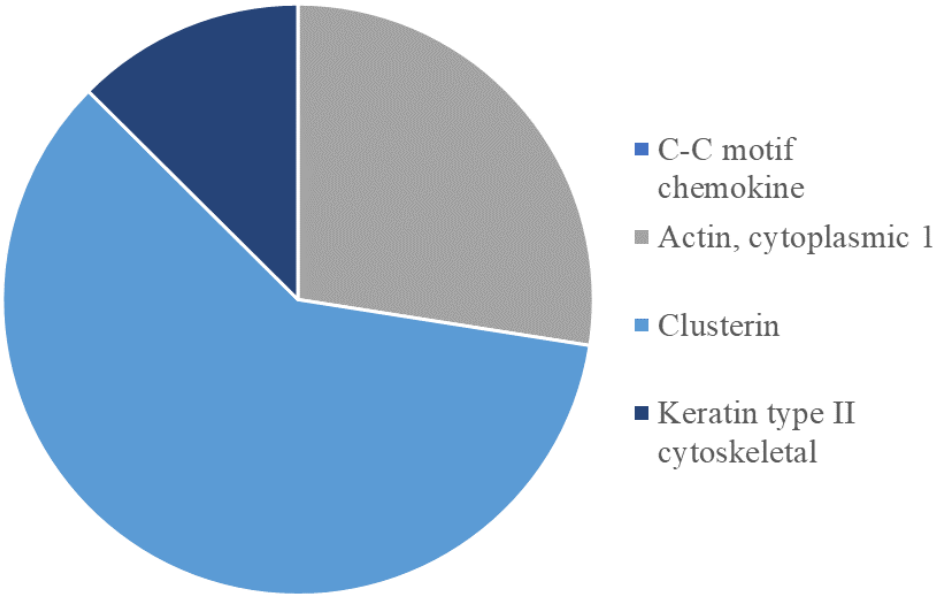


Figure 53. Comparison of identified post-protein A HCP species at (A) day 14 (424 ppm post-protein A HPC levels quantified by ELISA) and (B) day 17 (740 ppm post-protein A HPC levels quantified by ELISA) of the mAb 3 culture. HCP species were identified by using nano-LC Orbitrap mass spectrometry. Common contaminants as well as HCPs with only one peptide have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data.

5.3 Exploring the possibility of an earlier harvest

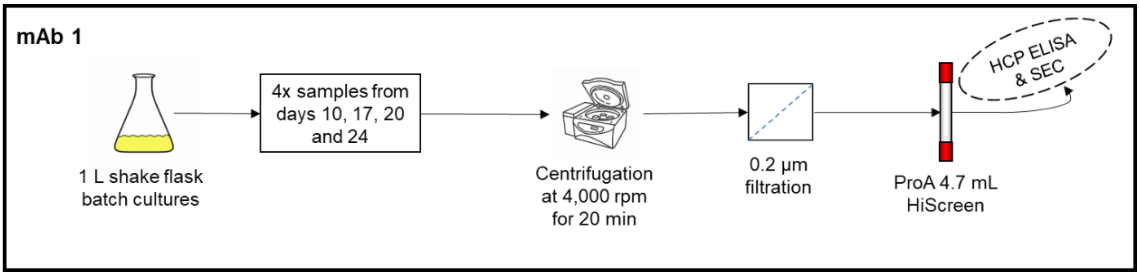


Figure 54. Summary of basic experiments in mAb 1 shear study I.

In the second study, another molecule (mAb 1) was used to explore the downstream process beyond protein A capture, and to consider how process development decisions might be affected by a mAb that is susceptible to fragmentation. This study also explored the effect of shear damage – representative of that which might occur in a feed zone of disc stack centrifugation upon scale-up (12,000 rpm for 20 seconds as determined by Hutchinson et al. (2006)) – at different harvest points. Cells were cultured in 29x 1 L shake flasks for over three weeks to enable the analysis of material that had been grown for an extended process duration and exhibited a wide range of viabilities. During the culture, all shake flasks were regularly monitored to ensure that cells were behaving similarly in terms of culture viability, production of antibody titre, and metabolite profiles. The results (see Figure 87 in the appendix) gave confidence that the material from 29x shake flasks could be pooled together in order to create enough material for all subsequent processing steps.

Cell broth samples for subsequent processing were taken on days 10, 17, 20 and 24 when culture viability was 97, 76, 68 and 43% respectively, and at each time point half of the collected material was exposed to shear to see if the process duration and/or culture viability impacted upon cellular behaviour during disc stack centrifugation, which will be discussed in more detail later on.

In the first instance, monomer purity and HCP levels were measured following protein A purification of HCCF which had not undergone any shear treatment (as illustrated in the

experimental work flow in Figure 54). Figure 55 shows that throughout the culture the product quality decreased due to increasing levels of post-protein A HCPs (as already observed in the first study with mAb 3) as well as increasing levels of product aggregates and fragments measured after affinity purification. Aggregate levels on days 10 and 17 are close to the typical industry threshold of 2%, while aggregation on days 20 and 24 exceeds the maximum limit. Fragment levels increase from day 10 to day 24 and are very close to the 2% threshold in late stage culture. Taking the 0.5% assay variability into account, fragmentation on day 24 could either be of negligible concern or might in fact exceed the threshold.

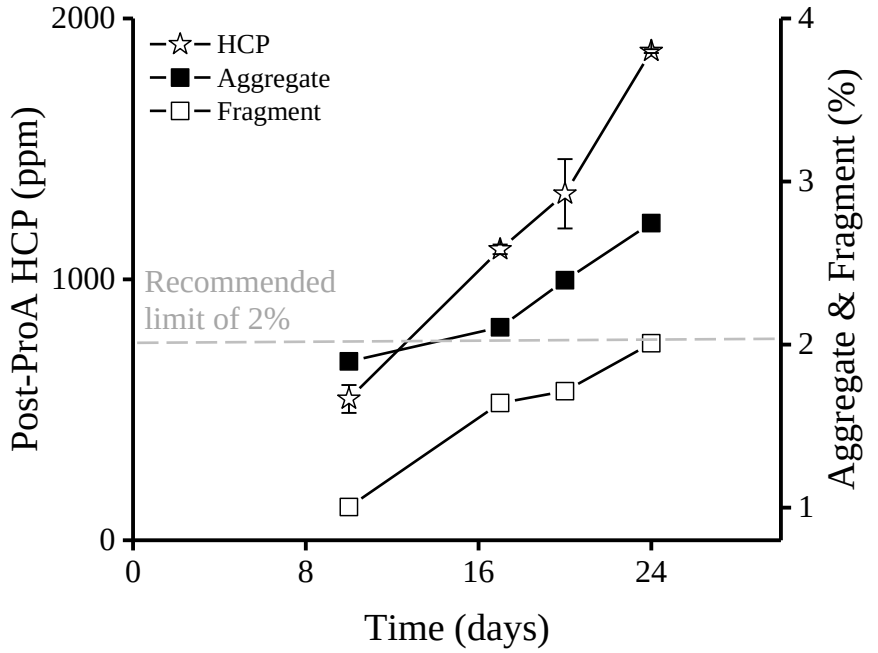


Figure 55. Effects of harvest time on product quality in the unmodified process, i.e. non-sheared samples only (mAb 1 shear study I). Aggregate and fragment percentages as well as HCP levels were measured after affinity purification and were determined by analytical SEC or CHO-HCP ELISA respectively. Descriptive error bars are based on HCP assay serial dilutions and show one standard deviation. A typical industry threshold for aggregation and fragmentation has been indicated by the grey line.

Interesting to note is that in the previous case study, protein A columns were loaded according to volume rather than total amount of protein, and the observed HCP increase could be explained by the increasing load levels of antibody molecules to which the HCPs

could bind. However, in this case study, the same mass of antibody was loaded to each column and shows the increase in post-protein A HCP levels with progressing culture duration as well. Regarding the increase in aggregates, previous research has linked aggregation to the low pH conditions required for protein A elution (Shukla et al., 2007) as well as to a low pH hold following protein A purification as commonly used for viral inactivation (Mazzer et al., 2015), and while material in this case study was eluted from the protein A resin with a pH 3.6 buffer, all samples were treated in the same way and should be affected in a similar manner if the observed aggregation were purely pH dependent. Increasing product aggregation due to a concentration effect is also unlikely given that the same mass was loaded onto the column for all samples. A more likely cause for the increase in aggregates and fragments could be the presence of proteases – Jin et al. (2010) and Tait et al. (2012) have previously reported that proteases are produced during late stage cell culture and can cause product fragmentation. Cathepsin D in particular is known to co-purify and cleave the antibody product (Robert et al., 2009; Bee et al., 2015; Lim et al., 2018). Accumulation of fragments could also lead to the formation of aggregates.

5.4 Complications of an earlier harvest

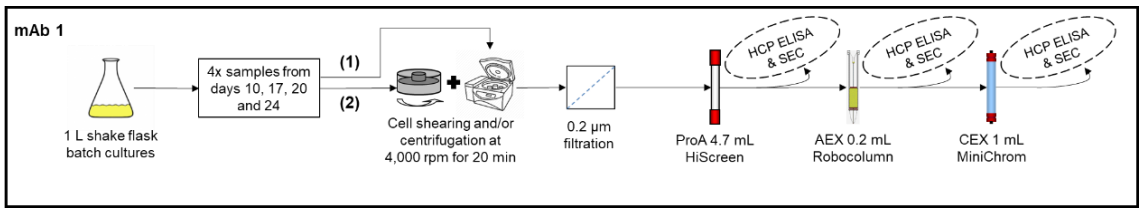


Figure 56. Summary of entire mAb 1 shear study I.

Further to the initial basic investigation, it was then considered how mechanical damage to cells during culture and harvest (mimicked here using an ultra-scale down shear device) affected post-protein A HCP levels. Interestingly, it was determined that shearing did not cause a statistically significant increase in HCPs in the investigated samples (results not

shown). However, shearing did have a significant effect on cell breakage on samples taken on day 10 of culture. While cell viability remained unaffected, total cell number was reduced by 60% (determined through the Vi-Cell cell counter; images displayed in Figure 57 A-D), at which point the levels of total HCPs produced by the cells is expected to be low (cf. Figure 52 and Figure 55), whereas the samples from day 17 onward were more shear-resistant and did not suffer significant morphological damage.

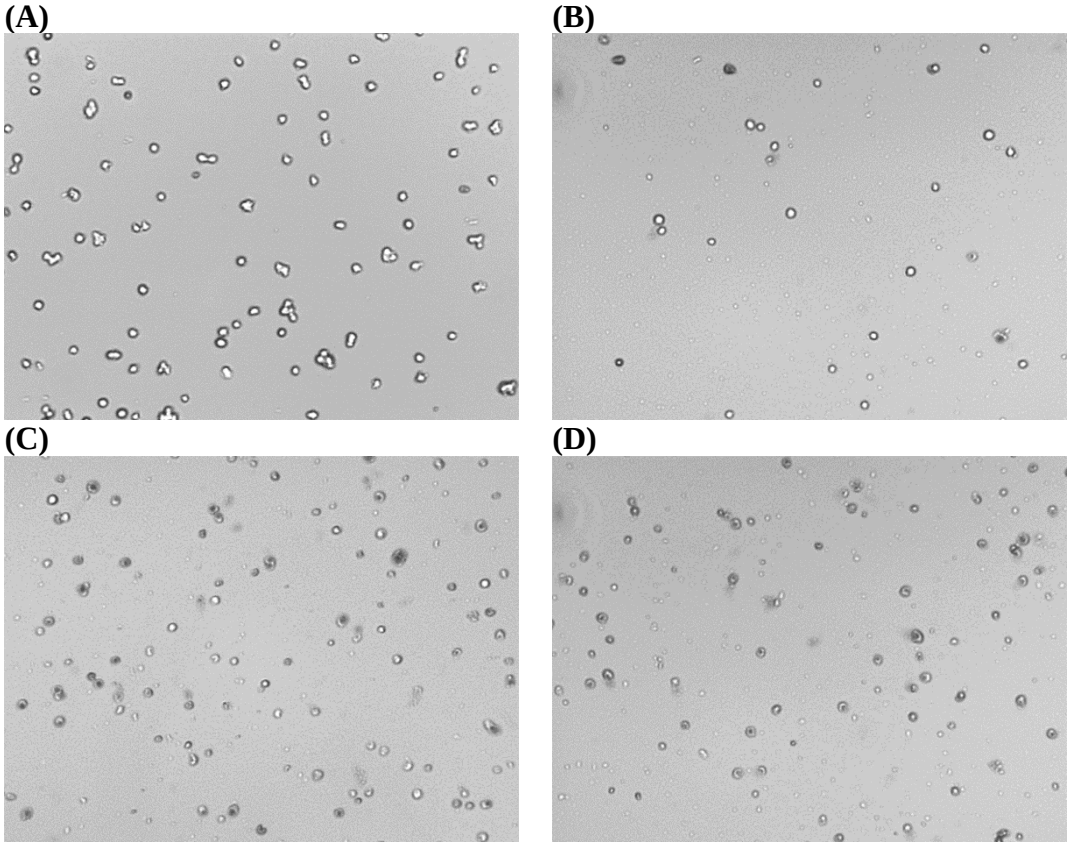


Figure 57. Changes to cell morphology upon shear exposure (12,000 rpm for 20 seconds) (mAb 1 shear study I). Vi-Cell cell count images. Material from day 10 prior to shearing (A) and afterwards (B), in comparison to material from day 17 prior to shearing (C) and afterwards (D). Whereas the total cell count has been dramatically reduced by 60% in day 10 material, no obvious breakage has occurred in day 17 and subsequent days (images from days 20 and 24 not shown).

This is consistent with previous literature such as Tait et al. (2009) who found that the more permeable outer membrane structure of apoptotic and non-viable cells can potentially absorb the impacts of stress better and thus these cells are less susceptible to it.

In addition to HCP levels, the extent of shear on product aggregation and fragmentation was also studied. While some slight increases in the percentage of aggregated and fragmented antibodies were detected after shear exposure (not shown), these changes were within assay variability and were therefore not considered to be significant. Further examination is required in order to conclusively establish a relationship between shear impact and product aggregation and will be addressed later in this chapter.

Lastly, monomer purity and HCP clearance were explored during intermediate and polishing chromatography steps. Cation exchange chromatography (CEX) was found to dramatically reduce HCP levels to below the guidance limit of 100 ppm (Figure 58 A). Any HCP species still remaining in the drug product after CEX imply a potential safety risk to patients by having the potential to cause adverse immune reactions. Tools like CHOPPI (Bailey-Kellogg et al., 2014) can be used to estimate the immunogenicity of any residual HCPs in order to determine how safe the therapeutic drug is.

Removing HCP species is crucial in drug manufacture, and in this study CEX was a required step. However, this step also resulted in increased product fragmentation in the case of this particular mAb 1 (Figure 58 B).

Figure 55 B shows three interesting observations: Firstly, fragmentation increases up to 4-fold from day 10 to day 24 – even prior to CEX – which could be caused by the release of proteases. Secondly, fragmentation is increased even further by the cation exchange chromatography step, suggesting either that a component of the CEX process itself causes the unstable mAb 1 to fragment, or that protease-dependent fragmentation is activated on the CEX column. Thirdly, on day 10 the post-CEX fragmentation in non-sheared material is similarly low to fragment levels prior to CEX (0.5-0.8%) whereas the fragment level in the sheared material has increased by 1%. Comparing this observation to day 24, the fragmentation levels post-CEX are similarly high (3-3.2%) in non-sheared and sheared material.

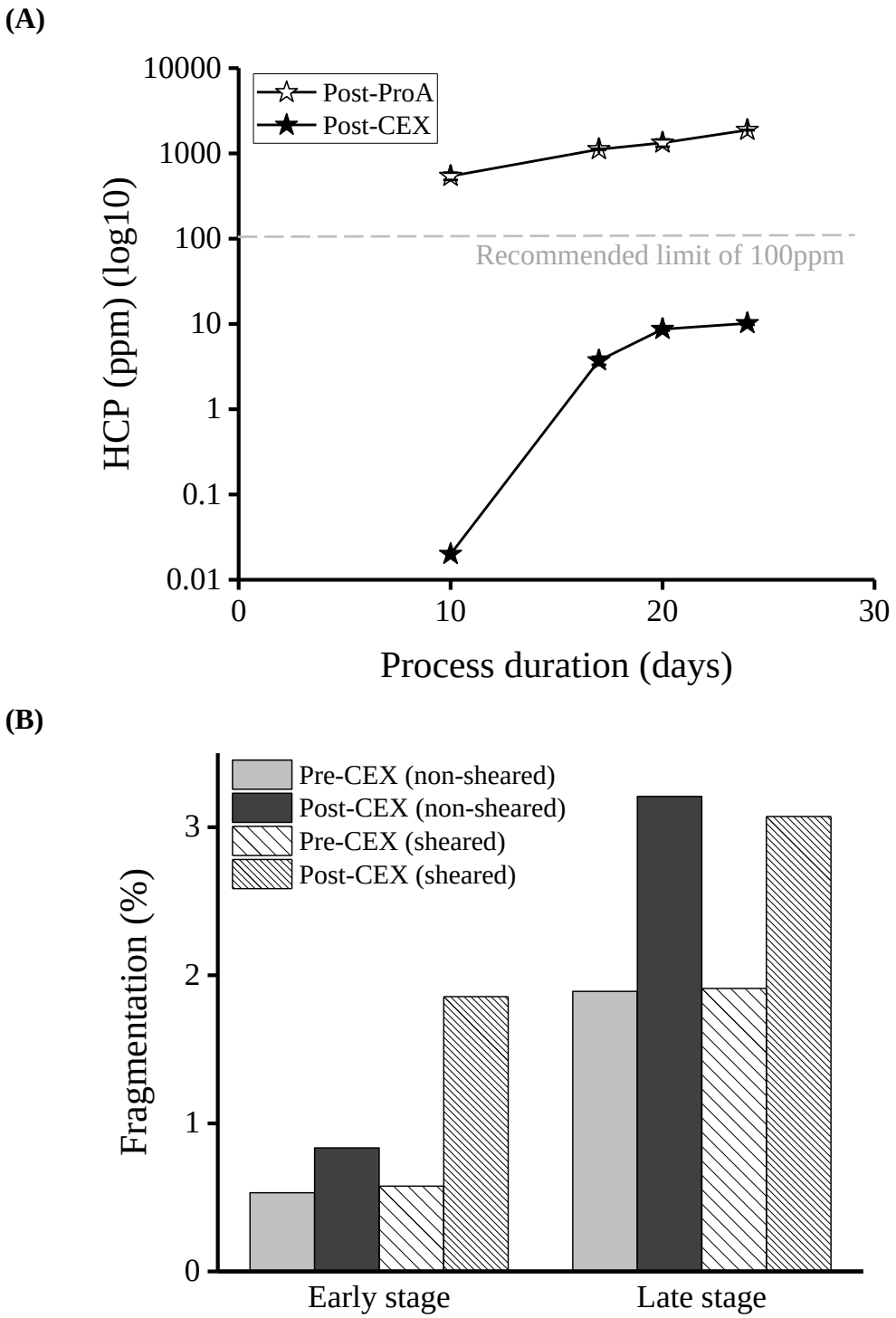


Figure 58. Effects of a polishing chromatography step on product quality (mAb 1 shear study I). (A) HCP levels in non-sheared samples before and after cation exchange chromatography, as determined by ELISA. HCP data point for day 10 after CEX is approximated based on titre as HCP levels were below detection (< 2 ng/mL). Descriptive error bars are based on HCP assay serial dilutions and show one standard deviation. (B) Fragmentation percentages in non-sheared and sheared samples before and after cation exchange chromatography during early stage (day 10) and late stage (day 24) culture, as determined by analytical SEC.

This supports the theory that fragmentation is caused by proteases which on day 10 are only released into the cell culture fluid upon shear-induced cell breakage whereas on day 24 they are thought to be present in the HCCF regardless of shear exposure.

While CEX is essential for HCP clearance, the fact that it can cause fragmentation of unstable mAb product further increases the challenges for downstream purification. Previous literature has also shown that the use of the CEX polishing step can result in lower product quality due to on-column aggregation of mAbs (Farys et al., 2018; Guo & Carta, 2015). If the upstream process were amended to create material with fewer HCPs, the CEX polishing step might not be required, which would avoid a trade-off decision between two downstream responses (HCP clearance vs high monomer purity). This could be done e.g. by optimising culture conditions such as pH, temperature and seed density, by decreasing the process duration and harvesting the antibody product sooner, or even by genetically modifying the CHO cells to prevent the expression of problematic HCP species (i.e. those that are present in particularly high levels or immunogenic species causing adverse reactions in patients). Alternatively, if CEX is definitely required, genetic modification could also be used to prevent expression of the HCP species that cause product fragmentation during CEX as shown here.

In summary, the results presented thus far raise interesting contradictory arguments to the question of whether product should be harvested earlier to avoid the presence of high HCP levels, or later to ensure cells are more shear-resistant and able to withstand potentially harsh harvest conditions. A decision for when to harvest needs to weigh not only the advantage of purer product versus the disadvantage of less product, but also consider the additional risk of harvesting shear-sensitive cells that might break and release intracellular impurities. In order to make an informed decision, the window between days 10 and 17 that was determined to be of interest during this case study, needs to be explored

further to determine if there is an optimal harvest point. Financial analysis could also help determine optimality (Farid 2007).

5.5 Investigating shear resistance

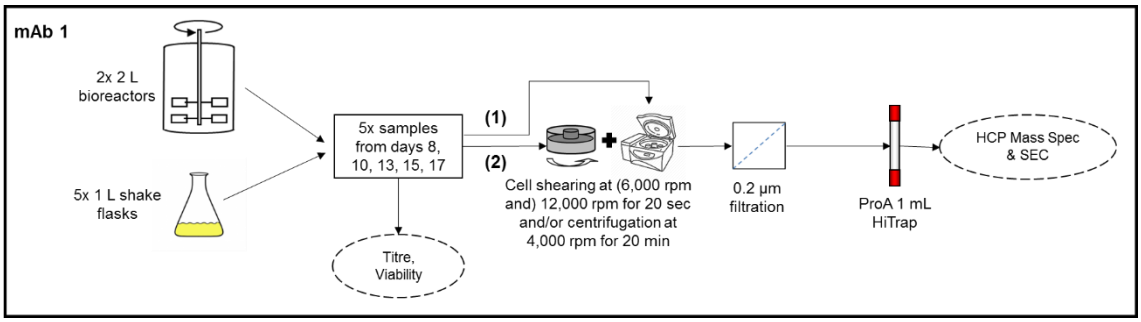


Figure 59. Summary of mAb 1 shear study II.

The findings from the previous two studies suggested that purer product could be obtained by harvesting the material earlier. However, the shear study showed that the shear sensitivity of cells during early stage culture could pose a risk during an earlier harvest. To address this issue, a second shear study was carried out with the aim of exploring at what timepoint cells become more shear-resistant and thus less susceptible to morphological damage during harvest. It was expected that the new and improved shear study would provide an optimal harvest point between days 10 and 17. In addition to replicating the operating conditions of the previous shear study with extra sample points, material was also grown in 2 L bioreactors in order to compare material from shake flasks and stir-tank reactors. Furthermore, material was not only separated into non-sheared and sheared at 12,000 rpm (representative of harsh disc stack centrifuge conditions) but also sheared at 6,000 rpm (representative of normal disc stack centrifugation).

Cultures grown in both shake flasks and bioreactors behaved according to expected growth profiles in terms of antibody production, culture viability and viable cell counts (see Figure 88 in the appendix for the bioreactor data).

However, contrary to expectation, the impact of shear on cell breakage did not decline linearly with increasing process duration – based on the data points of the first shear study,

which suggested that cells were sensitive to shear during early stage culture, and became more robust against shear during late stage culture, it was expected that the impact of shear on cell breakage would decline as a liner trend. Instead, the amount of cell breakage (determined as the difference between viable cell counts before and after shearing) increased initially up to day 13 of culture, and only then did shear stress have less of an impact on cell morphology (Figure 60).

There are two further notes of interest here: Firstly, material grown in shake flasks was not, as originally intended, harvested on day 17 but instead was maintained until day 21 to add an extra sample point to the analysis, which fits in with the data from the previous culture days and supports the observation that the impact of shear stress on cells decreases from day 13 onwards. Secondly, the material grown in the two bioreactors was pooled together during most of the study (once it had been confirmed that cultures from both bioreactors were behaving similarly), except on day 17, since the culture grown in bioreactor #2 ran out of glucose on day 16 or 17, whereas the culture in bioreactor #1 still had small amounts of glucose left (most likely due to a slight variation in glucose addition on day 7 between bioreactor #1 and #2). Consequently, on day 17 the culture viabilities between the two bioreactors varied between 89% and 66%, and it was thus decided not to pool the material together, but to analyse it individually instead, and as Figure 60 B and C demonstrates the two cultures reacted to shear exposure quite differently with the viable cell count from bioreactor #2 decreasing much less than those from bioreactor #1, despite both cultures being sampled on the same day.

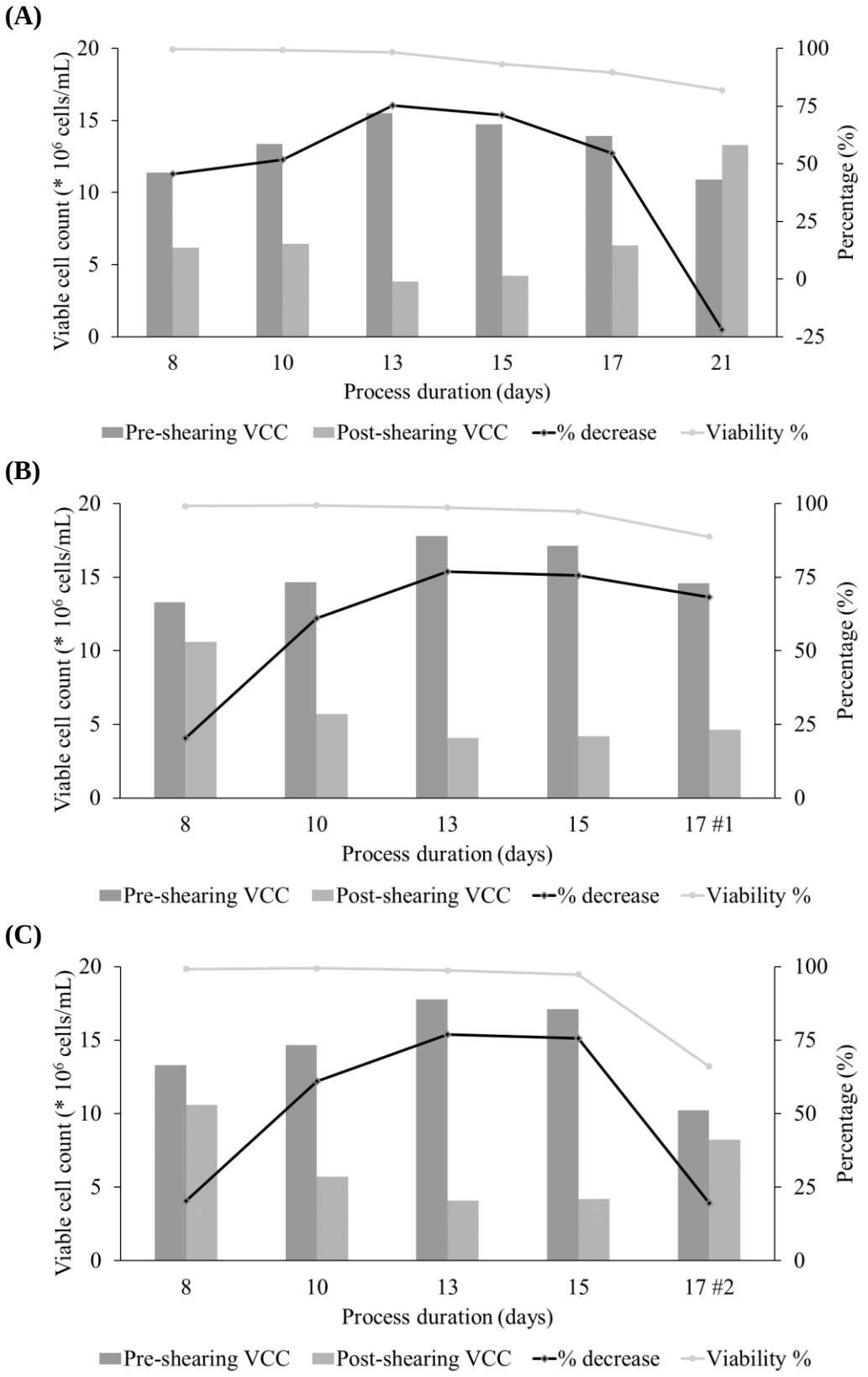


Figure 60. Culture viability and effect of shear on viable cell counts in material grown in shake flasks (A) and bioreactors #1 (B) and #2 (C). Shear impact is defined as the difference in viable cell counts before and after shearing and is represented by the percentage difference between non-sheared and sheared cells.

Taking all these observations into account, process duration by itself does not seem to cause the extent to which cells are damaged by shear. Instead, the culture viability looks to correlate to shear impact much better than process duration, as indicated not only by the fact that the day 17 cultures from the two bioreactors (with vastly different culture viabilities) behaved differently to shear stress, but also by the fact that the results from the second shear study differ compared to the first shear study: while the first study showed a shear-dependant reduction of 60% in viable cell counts on day 10 of culture and no significant shear-induced damage to cells on day 17 of culture, the second study showed that cells grown in shake flasks suffered a 52% reduction in viable cell counts upon exposure to shear on day 10 but also a similarly significant 55% decrease on day 17, whereas cells on day 21 of culture were not significantly damaged by the application of shear. Note that the culture viability on day 21 of shear study II was much more comparable to the culture viability of day 17 of shear study I.

In addition to determining the impact of shear damage by measuring viable cell counts using the trypan blue exclusion method on the Vi-Cell, the turbidity of samples was also determined by using a nephelometer to measure the concentration of suspended particulates in cell culture fluid. Sample turbidity was determined after centrifugation to remove crude particulates like cell debris. Figure 61 displays the amount of turbidity that was measured in each bioreactor sample before and after the application of shear, and illustrates three main observations: firstly, the level of particulates in harvested cell culture fluid increases as a function of process duration and/or viability even before any application of shear stress, so the longer a culture is maintained and the more culture viability decreases, the more turbid the upstream material becomes which can affect primary recovery. Secondly, HCCF becomes significantly more turbid after the material is exposed to strong shear. This observation corresponds with the previously discussed shear-induced cell breakdown that was measured using the Vi-Cell. Lastly, the graph

shows that the impact of shear on turbidity initially increases from day 8 to day 13 and then lessens with decreasing culture viability. This trend fits with the observed impact of shear on cellular breakdown measured on the Vi-Cell.

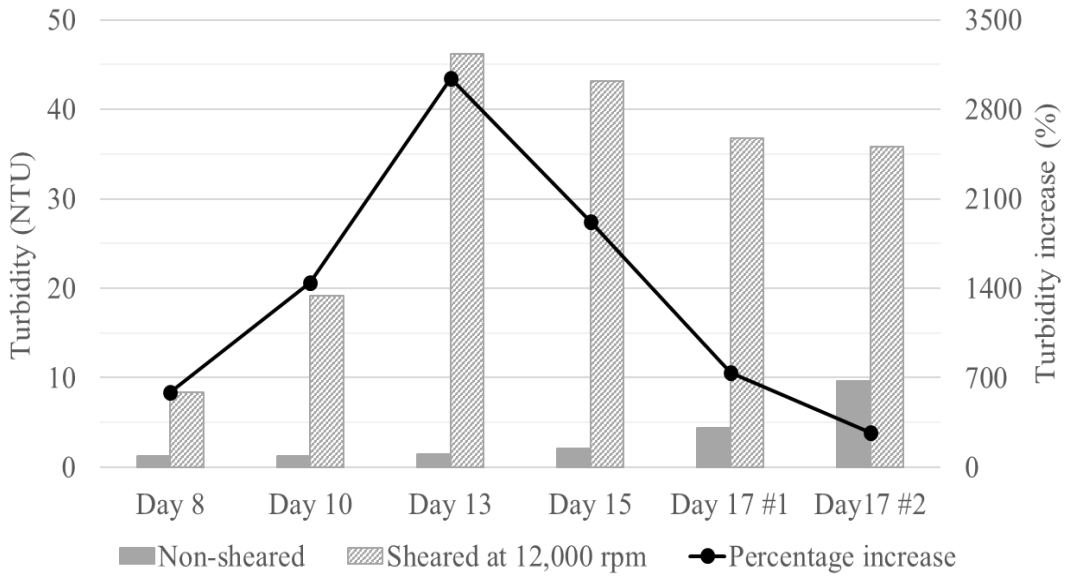


Figure 61. Turbidity in non-sheared (dark grey) and sheared (light grey) material prior to filtration, overlaid with a line diagram displaying the percentage increase in turbidity after shearing at 12,000 rpm.

If culture viability were the only factor to correlate with the extent of shear damage, the exposure to shear should have the same strong effect on cell damage during early culture as viability is consistently high during this time. Instead, the extent of shear damage is becoming stronger from day 8 to day 13 meaning that an increase in shear breakdown can be observed alongside an increase in cell count typical of the log phase of cell growth. Once cell counts plateau and subsequently decrease again during the death phase, along with the gradual decrease in culture viability, shear-induced cell breakdown decreases concomitantly. One hypothesis that could explain the unexpected observation that shear has a reduced impact on cells during early culture is based on the total volume that was injected into the shear device each time. Since the sample chamber of the shear device is designed to hold 20 mL, material was injected according to total sample volume rather

than cell count. For example, while 310 million viable cells (per 20 mL aliquot) were sheared on day 13 of the bioreactor culture, only 228 million viable cells were loaded into the sample chamber of the shear device on day 8 of the same culture. This means it is possible that fewer cells became exposed to the shear rotor compared to material taken while the culture was at peak cell counts. This could of course also contribute to the observation of decreasing shear impact in late stage culture since viable cell counts decrease again to similar numbers as on days 8 and 10 of culture. It could also be that an increase in cellular debris (caused by declining culture viability) has a buffering effect in the sense that the debris might shield the remaining viable cells from the maximum impact of the shear rotor. Another theory discussed in previous literature is that increased shear resistance may be an effect of cell age since apoptotic and non-viable cells suffer a gradual breakdown of cells' lipid bi-layer resulting in increased porosity of the membrane and a loss of membrane integrity (Tait et al., 2009).

5.6 Mass spec analysis of bioreactor time-course samples

To confirm the theory from Tait et al. (2009) about cell age affecting shear resistance, and to gain a deeper understanding of cellular behaviour, protein A purified time-course samples of the cultures grown in the 2 L bioreactors were analysed by mass spectrometry and post-protein A HCP profiles for the cultures were produced. The MS instrument used was a highly sensitive nano-LC Orbitrap system which can quantify HCPs down to single-digit ppm range. The amount of HCP peptides that were identified in each sample of this study are illustrated in Figure 62 and the specific HCP species are listed in Table 8. All biological process information from the identified proteins has been obtained from the UniProt database (UniProt Consortium, 2019).

Figure 62 confirms once again that post-protein A HCP levels increase as culture duration progresses.

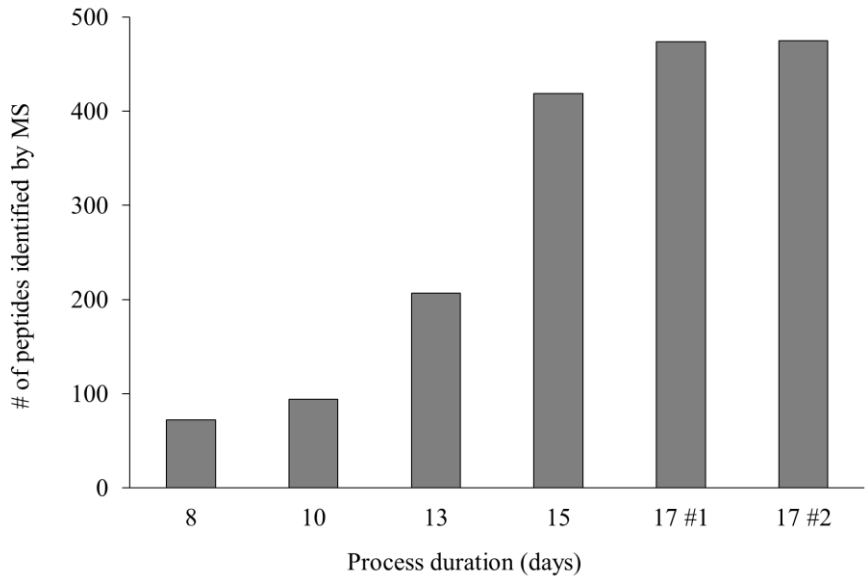


Figure 62. Amount of post-protein A HCP peptides identified by nano-LC OrbiTrap in mAb 1 2 L bioreactor cultures on days 8 – 17. Note that on days 8 – 15 material from both bioreactors was pooled after confirmation of similar growth and metabolite profiles, whereas on day 17 the cultures from bioreactor #1 and #2 were analysed separately due to varying viability levels (89% and 66% respectively). During MS data validation, common contaminants as well as HCPs with only two peptides have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data.

As can be seen in Table 8, HCPs present in high amounts include those that are involved in essential cell survival processes such as in crucial glucose or lipid metabolism pathways (e.g. Glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, alpha-enolase, lipoprotein lipase, phospholipid transfer protein) (UniProt Consortium, 2019). These proteins were expected to be highly abundant and were indeed mostly present throughout the entire duration of the culture.

Additionally, further carbohydrate metabolism proteins were detected during later stages of the culture (from days 13 and 15), e.g. lysosomal alpha-glucosidase, which is an enzyme usually located in the lysosome rather than the cytosol and could thus be an indicator of cell membrane breakdown (UniProt Consortium, 2019).

Further HCPs that were identified are those involved in the crucial cell process of translation. Of these, the most abundant protein and one which also was present from day 8 until harvest, was elongation factor 1-alpha. Further elongation factor proteins were

measured during later stages: elongation factor 1-gamma and elongation factor 2 (from day 13); elongation factor 1-delta (day 17 / harvest).

Similarly, ribosomal proteins as well as enzymes necessary for aminoacylation of tRNA were detected during later stages of the process, namely 40S ribosomal protein SA (from day 13), 40S ribosomal protein S15a and 60S acidic ribosomal protein P0 (both from day 15), 40S ribosomal protein S16 (from day 17); glycyl-tRNA synthetase (from day 17 in both bioreactors) and serine-tRNA ligase as well as valyl-tRNA synthetase (both from day 17, only bioreactor #2 which had extremely low culture viability) (UniProt Consortium, 2019).

The fact that these proteins can be measured in HCCF towards the end of the process suggests that cells need to produce higher amounts of such proteins during later stages of the culture. Given that more and more antibody titre is being produced as time progresses, cells might be struggling to "keep up" with protein synthesis, which of course also includes the limited availability of proteins that are involved in the process of translation. Consequently, cells need to produce more of those proteins, which might explain why they were only measured towards the end of the culture. Alternatively, these findings might be an indicator of significant cell lysis, with intracellular proteins being more prevalent in the HCCF at late-stage culture. This could be confirmed by carrying out assays that detect the degree of cell lysis, such as those measuring DNA, LDH or cholesterol, which are all localised either intracellularly or within the cell membrane and would thus only be detectable upon lysis of the cell (as discussed in chapter 4).

Perhaps most interesting is the detection of HCPs that are commonly produced as a response to stress. While proteins like clusterin and cathepsin L1 were identified in all samples, regardless of process duration, other stress-response proteins weren't detected until later. For example, endoplasmic reticulum chaperone BiP, heat shock protein HSP 90-alpha, heat shock cognate 71 kDa protein, hypoxia up-regulated protein 1 (all from

day 13 onwards); heat shock protein HSP 90-beta, heat shock-related 70 kDa protein 2, endoplasmin, calreticulin, T-complex protein 1 subunit alpha / delta / theta (all from day 15 onwards); and lastly T-complex protein 1 subunit beta and zeta (both only on day 17) (UniProt Consortium, 2019; Albrecht et al., 2018; Farrell et al., 2015).

Additionally, ubiquitin activating enzyme E1 – a protein involved in proteasomal degradation – was only detected in the second bioreactor on the last day of the process.

The presence of HCPs such as BiP, endoplasmin, heat shock protein HSP 90-alpha, heat shock cognate 71 kDa protein and hypoxia up-regulated protein 1 are strong indicators of stress within the endoplasmic reticulum (ER), possibly caused by glucose starvation, lack of protein glycosylation, or oxygen deprivation which all lead to the accumulation of unfolded proteins in the ER and in turn to the activation of the unfolded protein response pathway (Schröder & Kaufman, 2005). The oxidative stress response is further confirmed by the presence of HCPs like peroxiredoxin-1, glutathione S-transferase P, protein disulfide-isomerase A3, and peroxidasin-like.

In summary, the fact that these proteins – which are all involved in chaperoning unfolded proteins, in telomere maintenance, or in proteasomal degradation (UniProt Consortium, 2019; Albrecht et al., 2018) – are accumulating considerably at a later stage in the culture is a strong indication of several types of stress, induced by factors such as a lack of nutrients and cell age. Particularly the presence of proteins involved in telomere maintenance strongly suggests an age-related impact.

Reiterating the previously mentioned theory that increased shear resistance may be an effect of cell age since apoptotic and non-viable cells suffer a gradual breakdown of cells' lipid bi-layer resulting in increased porosity of the membrane and a loss of membrane integrity (Tait et al., 2009), it can therefore be concluded that the MS data presented here supports this hypothesis as several HCP species were detected which are indicators of cell age and cellular membrane breakdown.

Protein name	Biological process (*)	Day 8	Day 10	Day 13	Day 15	Day 17-1	Day 17-2
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	x	x	x	x	x	x
Pyruvate kinase	Glycolysis	x	x	x	x	x	x
Alpha-enolase	Glycolytic process		x	x	x	x	x
Phosphoglycerate kinase	Glycolysis				x	x	x
Transketolase	Glyceraldehyde-3-phosphate biosynthesis, glycolysis				x	x	
Lipoprotein lipase	Lipid metabolism	x	x	x	x	x	x
Phospholipid transfer protein	Lipid transport	x	x	x	x	x	x
Lysosomal alpha-glucosidase	Carbohydrate metabolism			x	x	x	x
6-phosphogluconate dehydrogenase, decarboxylating	Carbohydrate metabolism, pentose phosphate pathway			x	x	x	x
Neutral alpha-glucosidase AB	Carbohydrate metabolism				x	x	x
UDP-glucose 6-dehydrogenase	Carbohydrate metabolism, glycosaminoglycan biosynthesis				x	x	x
Malate dehydrogenase	Carbohydrate metabolism, TCA				x	x	x
Tissue alpha-L-fucosidase	Carbohydrate metabolism				x	x	
Sialidase-1	Carbohydrate metabolism				x		
Elongation factor 1-alpha	Translation, protein biosynthesis	x	x	x	x	x	x
Elongation factor 1-gamma	Translation, protein biosynthesis			x	x	x	x
Elongation factor 2	Translation, protein biosynthesis			x	x	x	x

Table 8. HCP species identified by nano-LC OrbiTrap in mAb 1 2 L bioreactor cultures on days 8 – 17. Note that on days 8 – 15 material from both bioreactors was pooled after confirmation of similar growth and metabolite profiles, whereas on day 17 the cultures from bioreactor #1 and #2 were analysed separately due to varying viability levels. During MS data validation, common contaminants as well as HCPs with only two peptides have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data. (*) Biological process information was obtained from the UniProt database (UniProt Consortium, 2019). [Table continues on the next page.]

Protein name	Biological process (*)	Day 8	Day 10	Day 13	Day 15	Day 17-1	Day 17-2
40S ribosomal protein SA	Translation, ribosome constituent			x	x	x	x
60S acidic ribosomal protein P0	Translation, ribosome biogenesis				x	x	x
40S ribosomal protein S15a	Translation, ribosome constituent				x	x	x
D-3-phosphoglycerate dihydrogenase	L-serine biosynthesis				x	x	x
40S ribosomal protein S16	Translation, ribosome constituent					x	x
Elongation factor 1-delta	Translation, protein biosynthesis					x	x
C-1-tetrahydrofolate synthase, cytoplasmic-like protein	Amino acid biosynthesis, one-carbon metabolism, tetrahydrofolate interconversion					x	x
Glycyl-tRNA synthetase	Translation, aminoacylation of tRNA					x	x
Serine--tRNA ligase, cytoplasmic	Translation, aminoacylation of tRNA						x
Valyl-tRNA synthetase	Translation, aminoacylation of tRNA						x
Clusterin	Chaperone, protein folding	x	x	x	x	x	x
Cathepsin L1	Proteolysis	x	x	x	x	x	x
Serine protease HTRA1	Proteolysis		x	x	x	x	x
Endoplasmic reticulum chaperone BiP	Chaperone, unfolded protein response			x	x	x	x
Heat shock protein HSP 90-alpha	Stress response (cytosolic), protein folding			x	x	x	x
Heat shock cognate 71 kDa protein	Stress response (ER), protein folding			x	x	x	x
Hypoxia up-regulated protein 1	Stress response (ER), cellular response to hypoxia			x	x	x	x
Peroxiredoxin-1	Stress response to oxidation, cell redox homeostasis			x	x	x	x

Table 8. HCP species identified by nano-LC OrbiTrap in mAb 1 2 L bioreactor cultures on days 8 – 17. Note that on days 8 – 15 material from both bioreactors was pooled after confirmation of similar growth and metabolite profiles, whereas on day 17 the cultures from bioreactor #1 and #2 were analysed separately due to varying viability levels. During MS data validation, common contaminants as well as HCPs with only two peptides have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data. (*) Biological process information was obtained from the UniProt database (UniProt Consortium, 2019). *[Table continues on the next page.]*

Protein name	Biological process (*)	Day 8	Day 10	Day 13	Day 15	Day 17-1	Day 17-2
T-complex protein 1 subunit gamma	Chaperone, protein folding, telomere maintenance			x	x	x	x
Glucosylceramidase	Stress response to starvation, lipid glycosylation			x	x	x	
Heat shock protein HSP 90-beta	Stress response (cytosolic), protein folding				x	x	x
Heat shock-related 70 kDa protein 2	Stress response, protein folding				x	x	x
Endoplasmin	Stress response (ER), protein folding				x	x	x
Calreticulin	Chaperone, cellular senescence				x	x	x
T-complex protein 1 subunit alpha	Chaperone, protein folding, telomere maintenance				x	x	x
T-complex protein 1 subunit delta	Chaperone, protein folding, telomere maintenance				x	x	x
T-complex protein 1 subunit theta	Chaperone, protein folding, telomere maintenance				x	x	x
Glutathione S-transferase P	Stress response, detoxification				x	x	x
Protein disulfide-isomerase A3	Cell redox homeostasis				x	x	x
Peroxidasin-like	Stress response to oxidation				x		
Metalloendopeptidase	Proteolysis				x		
Peptidyl-prolyl cis-trans isomerase	Protein folding acceleration, cell cycle				x		x
T-complex protein 1 subunit beta	Chaperone, protein folding, telomere maintenance					x	x
T-complex protein 1 subunit zeta	Chaperone, protein folding, telomere maintenance					x	x
T-complex protein 1 subunit eta-like protein	Chaperone, protein folding, telomere maintenance					x	
Peroxiredoxin-2	Stress response to oxidation, cell redox homeostasis					x	
Ubiquitin activating enzyme E1	Ubiquitin activation, proteasome degradation						x

Table 8. HCP species identified by nano-LC OrbiTrap in mAb 1 2 L bioreactor cultures on days 8 – 17. Note that on days 8 – 15 material from both bioreactors was pooled after confirmation of similar growth and metabolite profiles, whereas on day 17 the cultures from bioreactor #1 and #2 were analysed separately due to varying viability levels. During MS data validation, common contaminants as well as HCPs with only two peptides have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data. (*) Biological process information was obtained from the UniProt database (UniProt Consortium, 2019). *[Table continues on the next page.]*

Protein name	Biological process (*)	Day 8	Day 10	Day 13	Day 15	Day 17-1	Day 17-2
Actin, cytoplasmic	Cytoskeleton, cell motility	x	x	x	x	x	x
Procollagen C-endopeptidase enhancer	Collagen binding		x	x	x	x	x
Tubulin alpha chain	Cytoskeleton, microtubule			x	x	x	x
Tubulin beta chain	Cytoskeleton, microtubule			x	x	x	x
Torsin-1B	ER organisation			x			
Nidogen-1	Extracellular matrix structural constituent				x		
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Collagen fibril organisation, response to hypoxia				x		
Dihydropyrimidinase-related protein 2	Cytoskeleton organisation, axon guidance				x	x	x
Cofilin-1	Cytoskeleton organisation					x	x
Myosin-9	Cytoskeleton reorganisation, cytokinesis					x	x
Guanine nucleotide-binding protein subunit beta-2-like 1	Cell growth inhibition, translational repression, apoptosis			x	x	x	x
GTP-binding nuclear protein Ran	Protein transport			x	x	x	x
Tubulointerstitial nephritis antigen-like	Polysaccharide/laminine binding			x	x	x	
Proliferation-associated protein 2G4	Cell differentiation				x	x	x
Tubulin--tyrosine ligase-like protein 12	Unknown				x	x	x
Chondroitin sulfate proteoglycan 4	Cell proliferation, migration				x		
14-3-3 protein eta	Signalling protein binding					x	
Aldose reductase-related protein 2	Oxidoreductase activity					x	

Table 8. HCP species identified by nano-LC OrbiTrap in mAb 1 2 L bioreactor cultures on days 8 – 17. Note that on days 8 – 15 material from both bioreactors was pooled after confirmation of similar growth and metabolite profiles, whereas on day 17 the cultures from bioreactor #1 and #2 were analysed separately due to varying viability levels. During MS data validation, common contaminants as well as HCPs with only two peptides have been filtered out and a MS/MS score of 150 was applied to accept the MS/MS data quality. The remaining data was manually evaluated based on the isotope plot data. (*) Biological process information was obtained from the UniProt database (UniProt Consortium, 2019).

Some of the proteins listed in Table 8 have also been identified in previous literature (Albrecht et al., 2018; Farrell et al., 2015; Zhang et al., 2014; Zhang et al., 2016) although HCP identification data has not commonly been presented in relation to culture duration and biological processes.

Literature	Samples	Related to culture duration?	Related to biological process?
Albrecht et al., 2018	HCCF	No	Yes
Farrell et al., 2015	Post-protein A	Yes (days 5 & 7)	No
Zhang et al., 2014	HCCF Post-protein A Post-viral inactivation Post-ion exchange	No	No
Zhang et al., 2016	Post-protein A	No	No

Table 9. Literature containing HCP identification data.

Albrecht et al. (2018) have carried out mass spectrometry to study HCP profile changes during cell stress and cell death using apoptosis and necrosis models. They identified 23 HCP species that were present in the apoptotic or necrotic model (or in both models) and listed the biological processes that these proteins were involved in. These HCPs were measured in HCCF rather than in protein A purified material, but several of the species identified by Albrecht et al. (2018) in apoptotic and/or necrotic models have also been detected here in late stage culture material following protein A purification, i.e. they have been carried over during protein A purification, e.g. heat shock cognate 71 kDa protein and heat shock protein HSP 90-alpha (detected from day 13 onward), endoplasmin, glutathione S-transferase P, and heat shock protein HSP 90-beta (detected from day 15 onward), and cofilin-1 (detected on day 17).

Farrell et al. (2015) have used mass spectrometry to determine post-protein A HCP profiles as a function of culture harvest time – although only comparing day 5 (the start of the stationary phase) and day 7 (the end of the stationary phase). They found that product which is harvested at the later stage of cell culture contained higher

concentrations of HCPs. Furthermore, the HCPs identified on day 5 were mainly secreted proteins (such as clusterin and procollagen C-endopeptidase enhancer), whereas most HCPs (>70%) identified on day 7 were intracellular proteins (e.g. 78 kDa glucose-regulated protein, calreticulin, glyceraldehyde-3-phosphate dehydrogenase, histone H2AX, and serine protease HTRA1). They theorised this was likely due to cell lysis rather than increased secretion of proteins. The results from Farrell et al. (2015) are congruent with the data presented in this chapter; however, the culture duration investigated here is far more extensive than the one studied by Farrell et al. (2015).

Zhang et al. (2014) have used mass spectrometry to track HCP species from HCCF (where they were able to identify approximately 500 HCPs) through the downstream purification steps: protein A purification, viral inactivation, and polishing chromatography, at which point they were unable to confidently identify any HCPs. They used nine mAbs for the study and further demonstrated that clusterin and actin were highly abundant in most of the protein A purified mAbs, suggesting that these two HCP species are difficult to remove during purification. In the case of the first HCP species, this could be due to clusterin binding to both Fc and Fab regions of mAbs (Wilson & Easterbrook-Smith, 1992). The second HCP species, actin, is known to engage in numerous protein-protein interactions (Dominguez & Holmes, 2011).

In another publication (Zhang et al., 2016), this research group has further compared post-protein A HCP profiles among 15 different mAbs, and found that on average only 10% of post-protein A HCPs were specific for each individual mAb (except for one mAb which was associated with over 65% specific HCPs), while the remaining post-protein A HCPs were common to all mAbs. HCPs that were common to all investigated mAbs were e.g. clusterin, actin, elongation factor 1 alpha 1, heat shock cognate 71 kDa protein, 78 kDa glucose regulated protein, glyceraldehyde-3-phosphate dehydrogenase, glutathione S transferase P and serine protease HTRA1.

Zhang et al. (2016) have also studied how these common HCPs bind to mAbs and discovered that instead of using specific binding sites, HCPs seemed to be using multiple non-specific interaction sites in both Fab and Fc regions of the antibodies. They conclude that several of these common HCPs are likely to be present in other CHO-produced mAbs as well, and indeed also in CHO-produced Fc fusion antibodies.

Zhang et al. (2014) have published approximately 40 HCP species, many of which have also been detected here and are presented in Table 8. Unfortunately, neither Zhang et al. (2014) nor Zhang et al. (2016) specify how long their cultures were maintained for and on which days material was harvested, so it is impossible to link the published HCP species to a particular harvest timepoint, and fully compare the data to the results presented in this chapter, where an extensive culture duration context is provided.

Additionally, the work by Zhang et al. (2016) focused on identifying common HCP species and how they interact with mAbs in order to provide knowledge on how HCPs co-elute during product purification and how their retention could be prevented. The HCPs identified by Zhang et al. (2016) have therefore not been assigned to the biological processes that they are involved in, which would provide insight into cells' behaviour during mAb production.

In conclusion, information about the specific HCPs that co-purify with mAbs during protein A chromatography is progressively increasing with each new published dataset. Understanding the mechanism by which HCPs are retained during protein A purification is crucial to enable the development of a targeted HCP clearance strategy. The HCP species identification presented here provides a new level of insight into HCPs that are retained during mAb purification which can be applied to increase our understanding of cellular behaviour during production of therapeutic antibodies as well as to design targeted HCP clearance strategies during protein A purification, both of which may be used to aide process development strategies.

5.7 Effects on filter efficiency

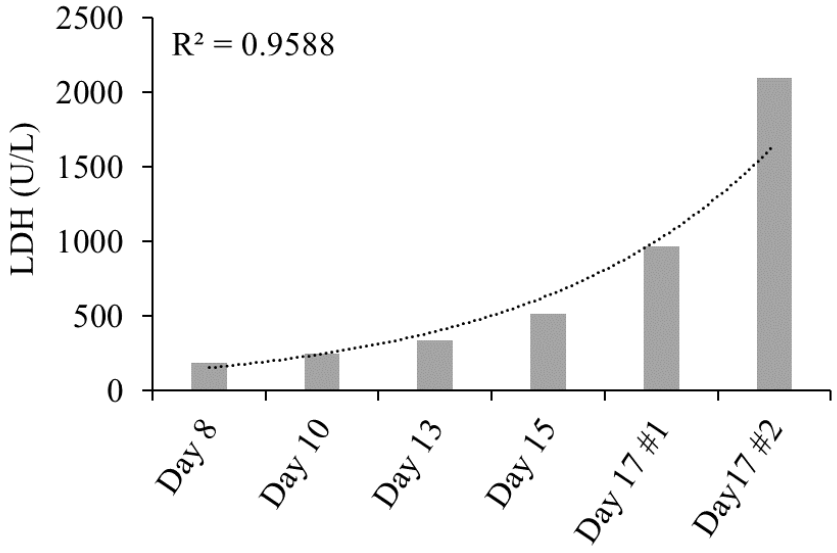
Initially, the intention of this study was to identify an optimal harvest point when product quality had not decreased yet and cells had developed some shear-resistance so as not to risk cell breakage during an earlier harvest. However, it appears that the development of shear resistance has caveats of its own. Both a loss of membrane integrity as well as a potential shielding effect from more cellular debris is undesirable for the process: an associated release of intracellular impurities would result in less pure product while larger amounts of particulates / cell debris negatively affect the efficiency of the subsequent filtration step prior to downstream purification. Unfortunately, due to the small volumes of material used in this study, the pressure during filtration could not be measured, but as a rough approximation Table 10 compares how many 0.2 µm syringe filters (Mini Kleenpak™ 25 mm syringe filters with 0.2 µm Supor® EKV membrane, Pall Corporation, Portsmouth, UK) had to be implemented to filter the non-sheared versus sheared material from each sampling day which shows that sheared material is blocking the filter membranes very quickly and is significantly more difficult to clarify, especially towards the end of cultivation.

	Viable cell counts (x 10^6 viable cells/mL)	**Non-sheared material**	**Sheared material**
Day 8	13.29	1x	3x
Day 10	14.64	1x	3x
Day 13	17.78	1x	4x
Day 15	17.12	2x	4x
Day 17 (#1)	14.57	2x	5x
Day 17 (#2)	10.21	3x	6x

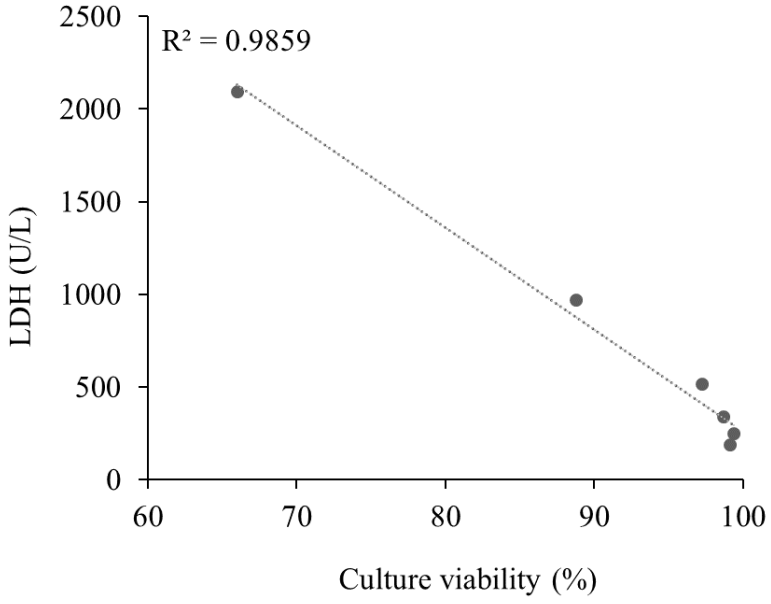
Table 10. Number of 0.2 µm syringe filters (Mini Kleenpak™ 25 mm syringe filters with 0.2 µm Supor® EKV membrane, Pall Corporation, Portsmouth, UK) required to filter non-sheared and sheared (12,000 rpm) material on each sampling day as a rough measurement of filter efficiency.

An indicator for both culture viability as well as the turbidity of centrifuged material was found to be lactate dehydrogenase (LDH). Known for being an indicator of cell lysis due to its intracellular localization (Albrecht et al., 2018), LDH is increasing exponentially during the cultivation (Figure 63 A) and was shown to correlate linearly with culture viability and sample turbidity (Figure 63 B and C respectively). As such, LDH – which is measured in harvested cell culture fluid prior to filtration – may very well be useful as an indicator to predict the filter efficiency of upstream material.

(A)



(B)



(C)

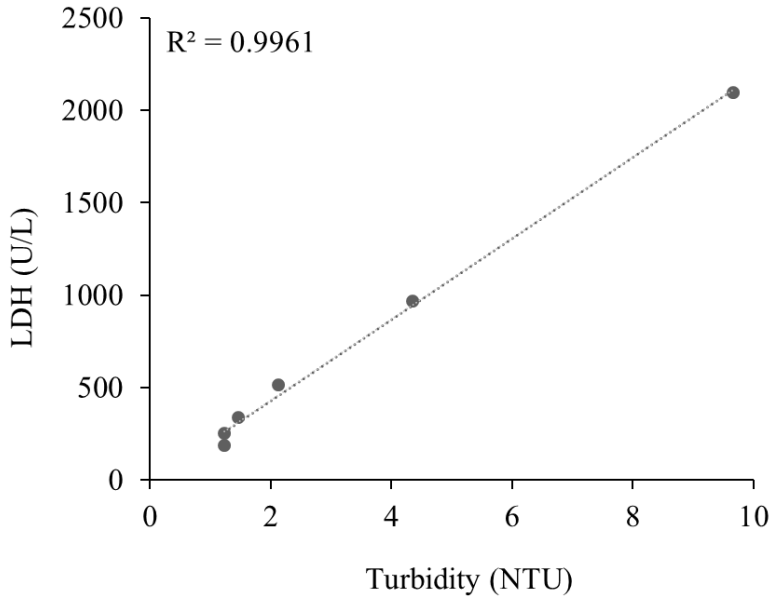


Figure 63. (A) Lactate dehydrogenase increase during cultivation. (B) Correlation between LDH and culture viability. (C) Correlation between LDH and turbidity.

5.8 Conclusions for downstream processing

Analytical analysis of the purified material generally confirmed previous observations that product quality decreases in a time-dependant manner (cf. Figure 62; Figure 64).

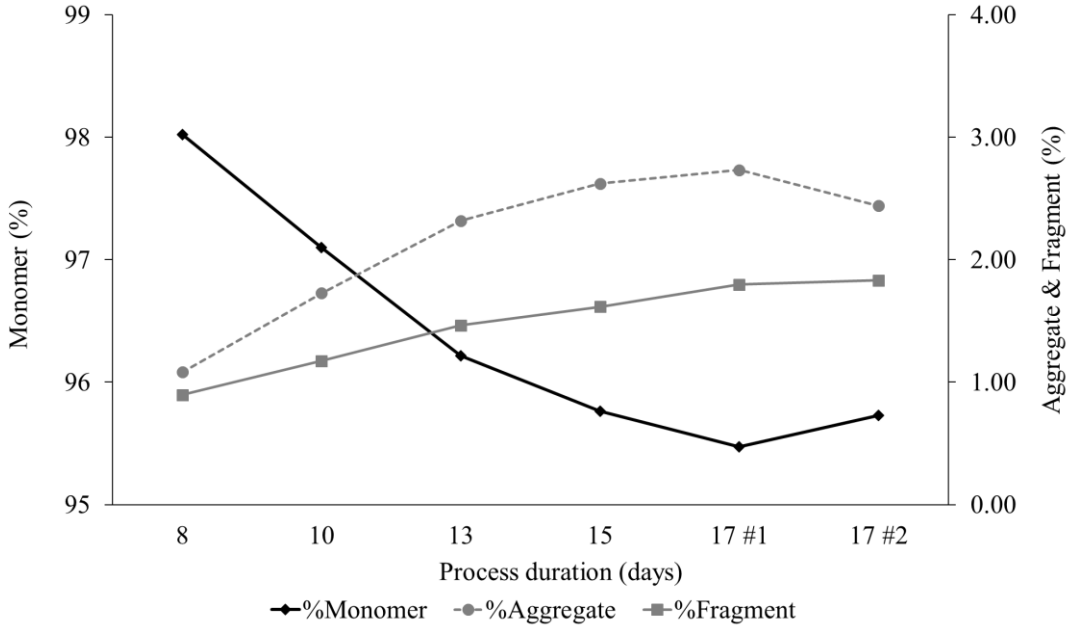


Figure 64. Effects of process duration on product monomer, aggregation and fragmentation in mAb 1 2 L bioreactor samples. Data was obtained by SEC following protein A purification.

Figure 64 also shows that product fragmentation doubles from 0.9% to 1.8% throughout the culture from days 8 to 17 for both bioreactors, whereas product aggregation steadily increases only in bioreactor #1 from 1% to 2.7% while in the material from the second bioreactor, product aggregation was measured to be slightly lower in sample 17 #2 compared to day 15. This observation, along with the HCP species identification information (Table 8) suggests that product fragmentation and aggregation initially increase as cells are struggling to continue protein biosynthesis, particularly protein folding. However, eventually, cellular stress response pathways are activated / accelerated leading to progressively more protein degradation.

In the context of product quality, process duration seems to be the primary factor influencing the levels of impurities, as opposed to culture viability being the defining factor as seems to be the case for shear-resistance of cells. This conclusion is supported by Figure 62 and Figure 64 as well as previously presented results (Figure 52 & Figure 55) which show that product quality readily decreases even during early stage culture when viability is still consistently high.

Interestingly, cells' exposure to shear was not seen to have a significant effect on product quality such as the release of intracellular HCP impurities or an increase in product aggregates (not shown). While this is similar to the previous shear study, earlier conclusions were based on very limited data points (mainly day 10 and day 17 of culture), especially since shearing on day 17 of the former shear study did not result in significant cell breakdown. As further data points were included in the present study, it was expected that shear would have an impact on product quality between days 10 and 17. Although, it might be the case that any potential increase in HCPs or aggregates was negated by the shear-induced increase of cell debris and particulates which affected primary recovery, such that some of these impurities may have been reduced alongside the cell debris when the material was centrifuged and filtered.

One important thing to note is that the observed impact of shear exposure is based on data from material that was sheared at 12,000 rpm for 20 seconds, i.e. representative of harsh disc stack centrifuge operating conditions. As a comparison, material grown in bioreactors was also sheared at 6,000 rpm for 20 seconds which is more characteristic of proper disc stack centrifugation. As demonstrated in Figure 65, cells that are exposed to this level of shear are not as negatively affected as those which experience stressful conditions.

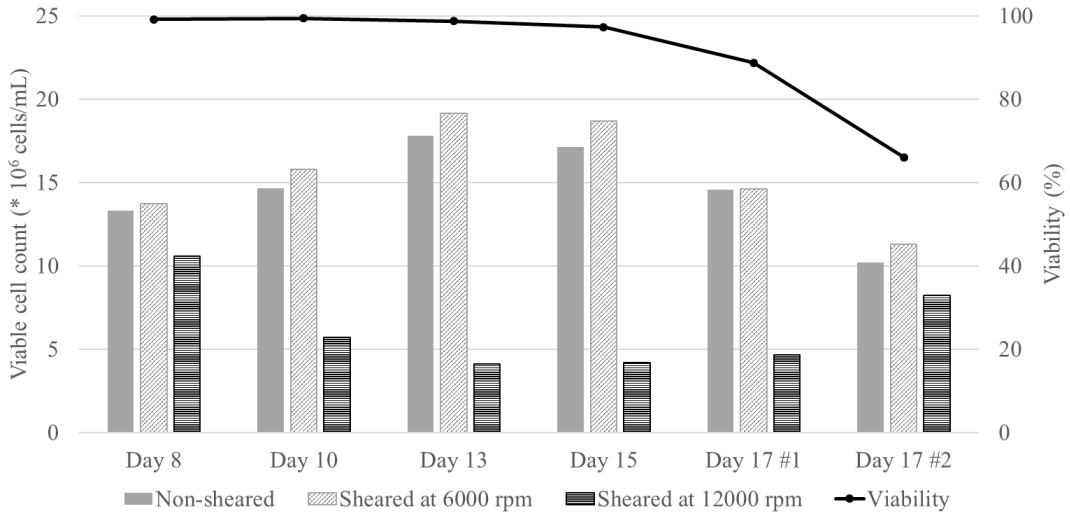


Figure 65. Effect of different levels of shear on viable cell counts.

So, while the data presented in this chapter helps establish worst case scenario outcomes which are crucial for designing a robust process, sensibly designed operating conditions and carefully executed processes may not be impacted in such harsh manners as shown here.

5.9 Summary

To investigate the impact of cell culture duration on critical quality attributes post protein A purification, a CHO-expressed IgG1 was cultivated in a 50 L bioreactor and samples were taken on days 1, 3, 7, 10, 14 and 17. Antibody titre was assessed prior to filtration and protein A purification via 0.2 mL columns on an automated liquid handling system.

Host cell protein (HCP) analysis was performed using a CHO-HCP-ELISA. Results showed that as cultivation progressed, and antibody titre increased the post protein A HCP impurities also increased indicating that harvest material is becoming progressively more difficult to recover using this purification scheme. It was then considered how product quality is impacted by shear damage at different harvest time points. For this, a CHO-expressed IgG1 was grown in shake flasks and cell broth samples were taken at four time points. Half of the collected material was sheared, to mimic disc stack centrifugation, and all material was then centrifuged, filtered and protein A purified on a 4.7 mL HiScreen column. HCP levels were measured by ELISA. Shearing caused a 60% decreased cell count of young cells (measured by a Vi-Cell cell counter) while older, less viable cells were quite shear-resistant. This is consistent with previous literature stating that younger, more viable cells are more shear sensitive. HCP levels were not significantly affected, confirming that young viable cells did not produce many HCPs yet. An earlier harvest for material with fewer HCPs may therefore increase the risk of breaking shear-sensitive cells and releasing any intracellular impurities during the harvest process.

However, this study had a limited amount of data points as samples were only taken and analysed on days 10, 17, 20 and 24. To address this issue, a second more extensive shear study was carried out with additional sampling days in order to explore at what timepoint cells become more shear-resistant and thus less susceptible to morphological damage during harvest. It was expected that the new and improved shear study would provide an optimal harvest point between days 10 and 17. But instead of identifying an ideal harvest day, it was found that increased shear resistance in later culture appears to be defined more by low culture viability than by the culture day itself. Furthermore, it was discovered that rather than being beneficial, shear-resistance is associated with increased sample turbidity and decreased filter efficiency, of which LDH levels seem to be a useful indicator. In addition, HCP species identification by mass spectrometry has confirmed a

previously published theory that increased shear resistance may be an effect of cell age since apoptotic and non-viable cells suffer a gradual breakdown of cells' lipid bi-layer resulting in increased porosity of the membrane and a loss of membrane integrity (Tait et al., 2009). The MS data presented in this chapter supports this hypothesis as several HCP species were detected which are indicators of cell age and cellular membrane breakdown. Lastly, with regards to downstream product quality, this study has shown that the impact of shear on downstream responses is negligible compared to the impact that culture duration has, as revealed by the additional data points between days 10 and 17, as well as those created by shearing the material at 6,000 rpm. In conclusion, the results presented in this chapter suggest that culture duration has a larger impact on the production of pure product than the shear sensitivity of cells.

The next chapter will apply the knowledge gained in the current and the previous two chapters, which is summarised in Table 11 below as a reminder:

Investigated factors	Conclusions
Upstream culture conditions	Bioreactor conditions that are optimised for maximum titre have been shown to commonly also result in higher levels of post-protein A HCPs. However, operating windows that are feasible for upstream and downstream targets are achievable.
Culture viability	Harvest culture viability is not a reliable indicator to predict product quantity or post-protein A HCP quality (in studies with many variables such as varying seed densities and different harvest days as was the case here).
Cholesterol	Cholesterol levels were able to predict post-protein A HCP levels in one study and can be used (together with acceptable titres) to choose upstream operating conditions that are favourable for both upstream and downstream processing. However, this conclusion only applied to one out of three studies!
Osmolality	Osmolality seems to be a promising prediction tool, although further work is required to validate this.
Free antibody light chains	Free antibody light chain levels are not a reliable indicator to predict titre, post-protein A HCPs or aggregation as results seem to be dependent on vector design / the ratio of gene copy numbers for the heavy and light chains / the integration of the recombinant DNA.

Culture duration	As product titre increases throughout a typical mAb culture, post-protein A HCP levels tend to increase as well, meaning that material which is harvested later likely requires a more extensive HCP clearance strategy (which can be a concern when polishing chromatography is required for HCP clearance of an unstable mAb, e.g. a mAb that fragments during CEX). HCP species involved in chaperoning unfolded proteins, telomere maintenance, and proteasomal degradation are accumulating at late stage culture which is a strong indication of ER stress and oxidative stress, induced by factors such as glucose starvation, lack of nutrients, lack of protein glycosylation, oxygen deprivation and cell age.
Cell age	Non-viable / apoptotic cells seem to be more shear-resistant than viable cells, with minimal cell breakage upon shear exposure, possibly due to a gradual breakdown of cells' lipid bi-layer resulting in increased porosity of the membrane and a loss of membrane integrity.
Lactate dehydrogenase	LDH seems to be a good indicator to predict filter efficiency of upstream material, as it was found to correlate linearly with culture viability and turbidity.

Table 11. Summary table of the knowledge that was gained during chapters 3, 4 & 5 (effects of upstream bioreactor conditions, product quality indicators, and harvest time).

6 IMPLEMENTATION OF AN INTEGRATED CASE STUDY

6.1 Introduction

Taking into consideration all the knowledge that was gained during the previous three chapters, an extensive DoE study was used to tie all investigations into upstream culture conditions, harvest time points and product quality indicators together, and to further explore the robustness of some aspects of the downstream process. For this, two DoEs were carried out by GSK's upstream group which explored the same pH, temperature and seed density ranges but used two different upstream processes – fed-batch version A (FBvA) and fed-batch version B (FBvB) (for full experimental details refer to section 2.1.8). A diagram of all the experiments carried out in this case study can be seen in Figure 66 [6].

Further to the DoE approach which investigated the effects of culture media and bioreactor conditions, time-dependent effects on product quality were studied by taking samples on several days during cultivation (on days 8, 10, 13 and 15 for FBvB). A few select samples were also chosen for investigations into the robustness of the downstream process such as the effectiveness of alternative protein A wash buffers, different purification column scales, and the application of polishing chromatography steps.

[6] The upstream experimental work for this study was carried out by GSK's upstream processing group, and the 0.2 mL protein A purification for the DoE using FBvA was carried out by GSK's downstream processing group. All other downstream experimental work (i.e. the 1 mL and 4.7 mL protein A purification and the AEX for the DoE using FBvA, as well as all downstream purification for the DoE using FBvB) was carried out by me. ELISAs and glycan analysis was carried out by GSK's analytical group, while all other assays as well as all data analysis for results presented in this chapter was carried out by me.

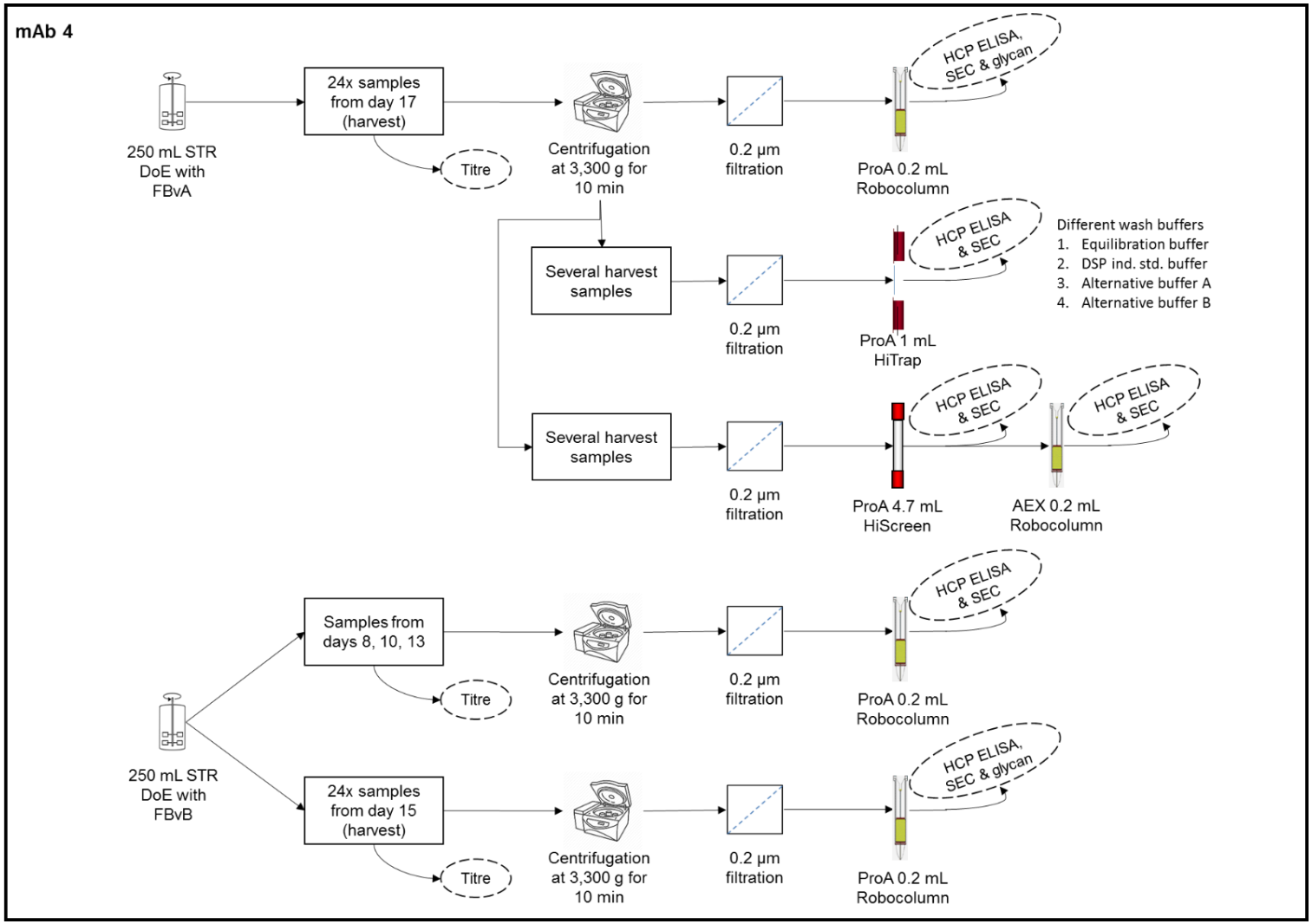


Figure 66. Summary of mAb 4 case study. DoEs, time-course studies and downstream robustness studies.

6.2 Impact of culture conditions on product quality

As mentioned, the first aspect to this case study was to apply the understanding gained in the third chapter by once more determining the effects of upstream bioreactor conditions on product quantity and quality in a DoE approach. By performing two DoEs (FBvA and FBvB), two different culture media / process configurations were compared in this case study, in addition to the factors pH, temperature and seed density that were already examined in the third chapter. Moreover, additional product quality attributes were measured in order to determine the impacts of upstream process parameters not just on HCP impurities, but also on monomer purity and glycosylation profiles.

As with all previous DoE studies, results have been analysed with the statistical software JMP 13.2.1 (SAS Institute, Inc., Cary, NC) and least squares regression models were used to generate prediction profilers for the outputs of interest. For the first DoE using fed-batch version A, it can be seen in Figure 67 that the effects of culture conditions on titre correlate with the findings discussed in chapter 3 – namely that a higher pH increases titre to a certain extent, that a temperature of +1 is optimal for titre production with a higher temperature resulting in decreased antibody production, and that a slightly higher seed density also results in increased titre (keeping in mind that the maximum seed density of -5 examined in this DoE is a lot lower than the high seed densities examined in some of the DoEs that were discussed in chapter 3).

Figure 67 also shows that for FBvA, the pH, temperature and seed density setpoints that are optimal for the production of mAb 4 can be undesirable from a product quality perspective. For instance, cultures that were inoculated with higher seed densities (one of the parameters that promotes increased titre) are associated with decreased monomer purity in comparison to those cultures that were inoculated with fewer viable cells. Perhaps the higher cell density resulted in the production of more HCP species that negatively affected antibody monomers such as proteases which cleaved the product into

fragmented antibodies. This is supported by the prediction profiler for post-protein A HCP levels which also shows an increased amount of HCPs in cultures that were inoculated with higher seed densities, and while the model for the FBvA HCP data is not a very good fit and is characterised by quite broad confidence intervals, the model fit is much better in FBvB (Figure 68) which supports the same theory.

For the second DoE using fed-batch version B (Figure 68), the previous findings about the effects of temperature and seed density on titre still hold true, however unlike in previous DoEs, a higher pH seems to result in less antibody production, suggesting that for this fed-batch version B (with a higher dissolved oxygen percentage and a media with more glucose and a richer nutrient feed), a pH around -0.05 and a temperature of +1 is optimal for titre production. In terms of product quality, we can see once more that operating conditions that are optimal for antibody production are also associated with decreased product purity due to an increase in post-protein A HCP levels as well as higher percentages of the undesirable glycan structures mannose 5 and G0F(-GlcNAc) (minus N-acetylglucosamine) (Figure 68). Mannose 5 is an undesirable glycan structure because high mannose glycan species like mannose 5 bind to mannose receptors in patients. These receptors are responsible for selective clearance of glycoproteins, and mAbs which are associated with high levels of mannose 5 are therefore removed from blood circulation much quicker than drug products without this type of oligosaccharide attachment (Allavena et al., 2004). This results in an undesirable reduction of the half-life of such affected mAbs.

With regards to G0F(-GlcNAc), this is an example of an undesirable glycan species containing a core fucose residue. As mentioned in the introduction, the absence of a core fucose residue is responsible for enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) and increased drug efficacy in certain types of drug products (Higel et al., 2016; Reusch & Tejada, 2015). As such, the presence of the fucose residue here is

disadvantageous. Having said that, most mAbs contain fucose (unless a knockout cell line with a silenced gene for Fucosyltransferase 8 is used). Therefore, levels of up to 10% are generally considered acceptable.

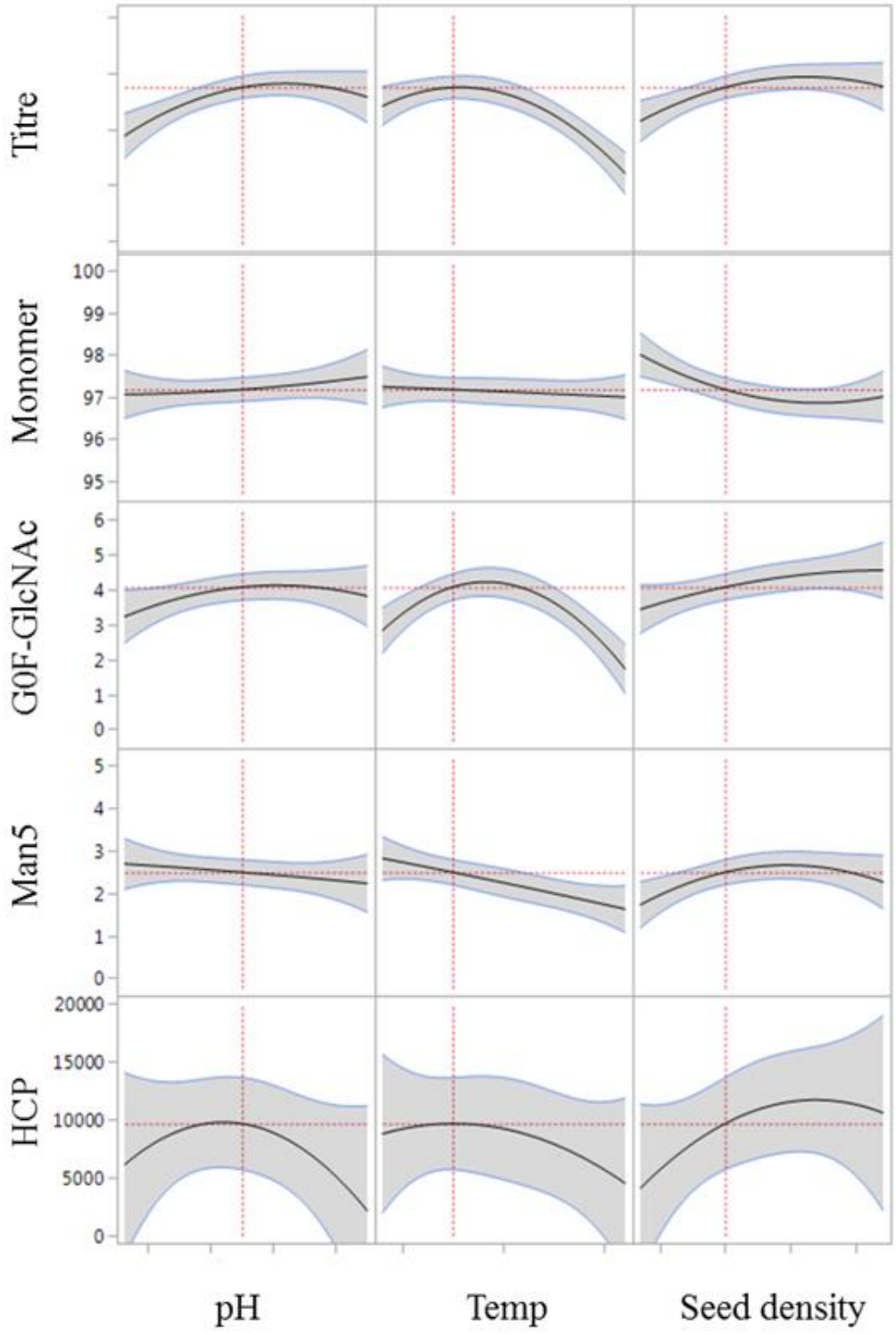


Figure 67. Effects of culture conditions on titre and product quality in the mAb 4 FBvA process. Model fit: R^2 values for parameters: Titre 0.93, Main isoform (%) 0.57, Monomer (%) 0.67, G0F-GlcNAc (%) 0.9, Man5 (%) 0.8, post-protein A HCP (ppm) 0.56 (mAb 4 FBvA). Increasing titre is accompanied by decreasing product quality.

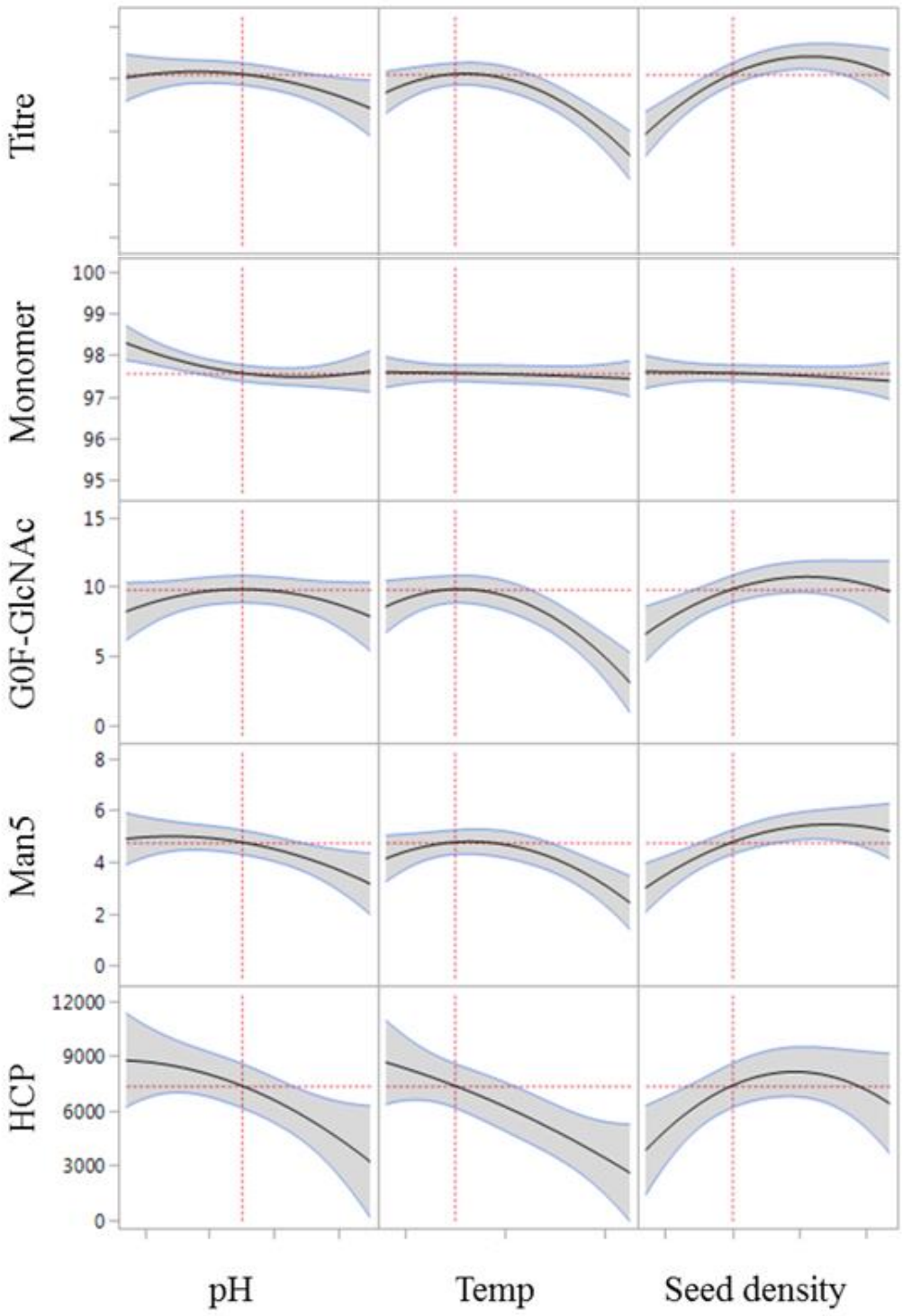


Figure 68. Effects of culture conditions on titre and product quality for the mAb 4 FBvB process. Model fit: R^2 values for parameters: Titre 0.91, Main isoform (%) 0.93, Monomer (%) 0.57, G0F-GlcNAc (%) 0.87, Man5 (%) 0.83, post-protein A HCP (ppm) 0.82 (mAb 4 FBvB). Increasing titre is accompanied by decreasing product quality.

Particularly regarding product glycosylation, another interesting observation is that cells grown using the fed-batch version B process seem to have added more undesirable glycan structures to the antibody of interest during post-translational modifications as the

percentage of mannose 5 and G0F(-GlcNac) residues measured is higher compared to FBvA (1.2 – 3% in FBvA versus 1.7 – 5.9% in FBvB for mannose 5 and 1.9 – 5.5% in FBvA versus 2.4 – 10.9% in FBvB for G0F(-GlcNac) as shown in Figure 69). Nonetheless, as mentioned, levels of up to 10% are generally still considered acceptable.

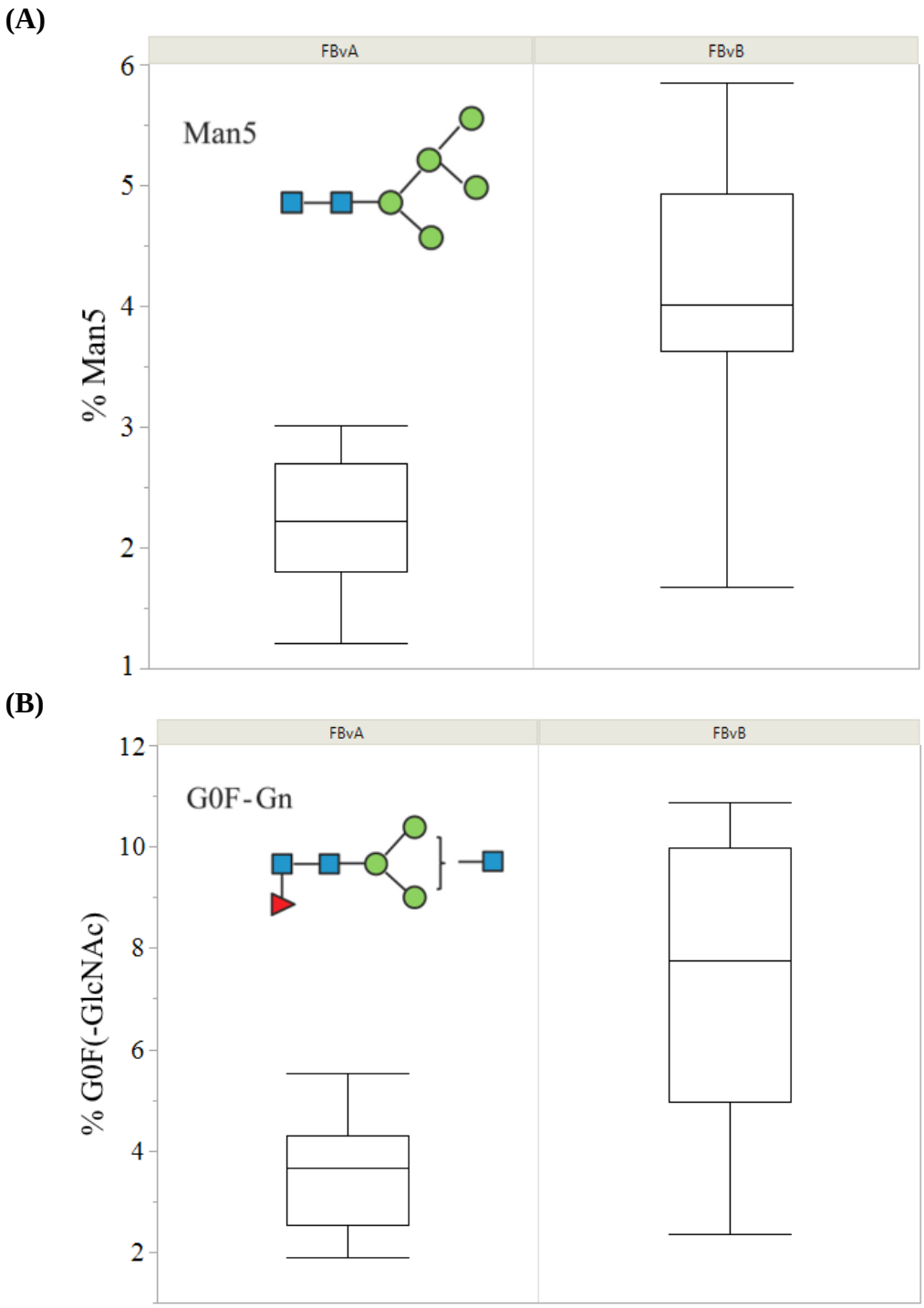


Figure 69. Box plots comparing (A) mannose 5 and (B) G0F(-GlcNac) percentages between FBvA and FBvB.

Since product glycosylation is generally considered to be unaffected by downstream purification and therefore cannot be altered, it is an important quality attribute that needs to be monitored during upstream process modifications. As this two-part DoE study has shown, a different type of media (such as a more glucose- and nutrient-rich media) and slight changes in upstream process configurations (e.g. higher percentages of dissolved oxygen) can completely change the percentages of certain product glycosylations.

Based on the discussed data, feasible operating windows for both fed-batch versions have been created by fitting least squares regression models and applying the constraints shown in Table 12. As can be seen in Figure 67, media and process configurations have a big impact on titre and product quality and influence the effects of culture conditions on product quality attributes. FBvB in this instance leads to increased production of mAb 4 compared to FBvA, as well as reduced levels of product fragmentation and post-protein A HCPs, which creates a larger feasible operating window based on those three responses. However, FBvB also resulted in higher levels of certain glycan structures – a quality attribute that isn't of concern when using FBvA. As was the case in previous chapters, trade-off decisions will need to be made.

	FBvA	**FBvB**
Titre	min. 2.5 g/L	min. 2.5 g/L
Post-protein A HCP	max. 15,000 ppm	max. 7,000 ppm
Product fragments	max. 2%	max. 2%
Mannose 5	max. 4.5%	max. 4.5%
G0F(-GlcNac)	max. 9%	max. 9%

Table 12. Constraints applied to the feasible operating windows for FBvA and FBvB.

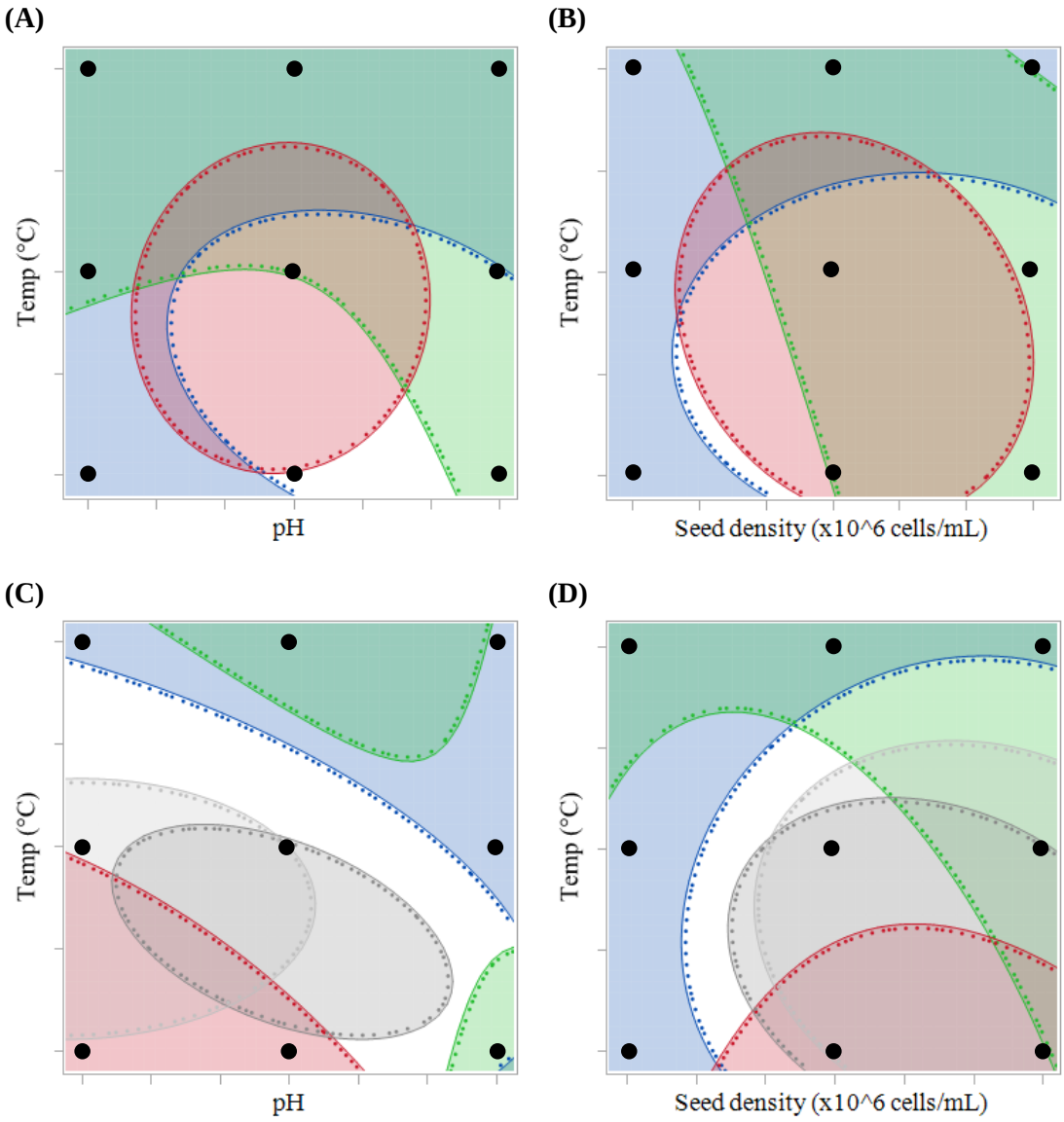


Figure 70. Window of operation for the mAb 4 FBvA and FBvB process. Using JMP software, contour plots were created to illustrate how temperature, pH and seed density affect titre, post-protein A HCP levels, product fragmentation and glycosylation in FBvA (A-B) compared to FBvB (C-D). The contour plots are based on the black experimental data points and were generated by fitting least squares regression models. As can be seen, the feasible operating window (white area) is slightly wider when using FBvB compared to FBvA. (A-B) Restraints: titre (blue) min. 2.5 g/L, HCP (red) max. 15,000 ppm, fragment (green) max. 2%, [man5 max. 4.5%, G0F(-GlcNac) max. 9% - did not impact on feasible window of operation due to measured values being lower than restraints]; Statistics of fit: R^2 for titre 0.93, HCP 0.67, fragment 0.89, man5 0.90, G0F(-GlcNac) 0.93. (C-D) Restraints: titre (blue) min. 2.5 g/L, HCP (red) max. 7,000 ppm, fragment (green) max. 2%, man5 (light grey) max. 4.5%, G0F(-GlcNac) (dark grey) max. 9%; Statistics of fit: R^2 for titre 0.95, HCP 0.86, fragment 0.89, man5 0.89, G0F(-GlcNac) 0.88.

The results discussed here highlight once more the importance of using an integrated approach as presented here during biopharmaceutical process development research in order to consider all impacts of upstream process modifications on upstream and downstream responses and thereby aid in the design of a robust process for every monoclonal antibody.

6.3 Impact of harvest time on product quality

The next aspect to this integrated case study was to combine the knowledge gained in the fifth chapter about earlier harvests with the DoE approach in order to create and compare feasible operating windows for different harvest days.

Prior to purifying and analysing time-course samples from multiple DoE cultures, one culture was chosen as a test sample – the culture grown under the upstream industry standard operating conditions for mAb 4 using FBvB. Small samples were taken from this culture on days 8, 10, 13 and 15, and the titre was determined before protein A purification was carried out on these four samples. Post-protein A HCP levels and the percentages of monomers in the eluate were then measured and plotted to illustrate the impact of culture duration on these responses (Figure 71).

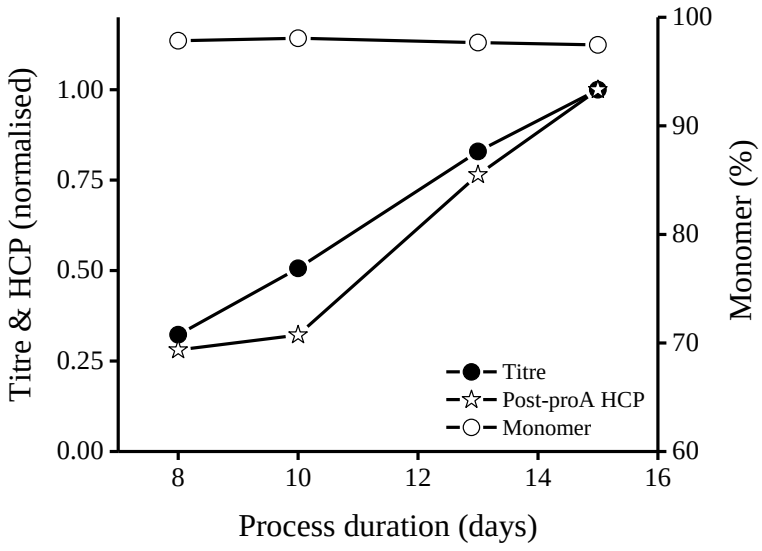


Figure 71. Impact of harvest time on product titre and product quality of mAb 4 using FBvB and the upstream industry standard culture condition.

A similar trend as the one presented in chapter 5 can be observed in terms of product titre and post-protein A HCP levels increasing concomitantly. Monomer purity in this instance did not decrease significantly by any means but remained between 98% and 97.5% during days 8 and 15.

The most interesting product quality attribute was therefore deemed to be post-protein A HCP levels. In order to see if a feasible operating window would be significantly wider when cultures are harvested on day 13 compared to day 15, the day 13 material from all 15x core DoE datapoints was processed in the same way as the day 15 material, and heat maps were created to highlight the difference in titre and post-protein A HCP levels on day 13 compared to day 15 (Figure 72 and Figure 73).

While on day 15, low post-protein A HCP levels are more easily achieved at culture conditions that unfortunately also result in low titre (i.e. high temperature and high pH), on day 13 post-protein A HCP values are much lower in general (500 - 1,800 ppm compared to 1,000 - 10,500 ppm on day 15). Furthermore, titre is only slightly lower (decreased by 3 – 10% for cultures with lowest and highest productivity respectively), suggesting that an earlier harvest results in purer product quality (in terms of HCP quantities) without too much loss of product titre.

Regarding a time-dependent comparison of monomer purity, the SEC data suggested that monomer levels were approximately 1% lower on day 13 compared to day 15 (not shown), but this is more likely due to the fact that material from day 13 was kept frozen until these samples were analysed in this manner, whereas material from day 15 (i.e. harvest day) was immediately analysed and did not undergo any freeze-thaw cycles. This assumption is supported by the data presented in Figure 71 which shows that monomer percentage did not change much for the duration of these cultures. Monomer purity should therefore not be detrimentally affected by an earlier harvest in this instance. Previous research has shown that product aggregation or fragmentation can be problematic and

should therefore always be considered a potentially critical quality attribute that requires investigating (c.f. chapter 5; Chung et al., 2017).

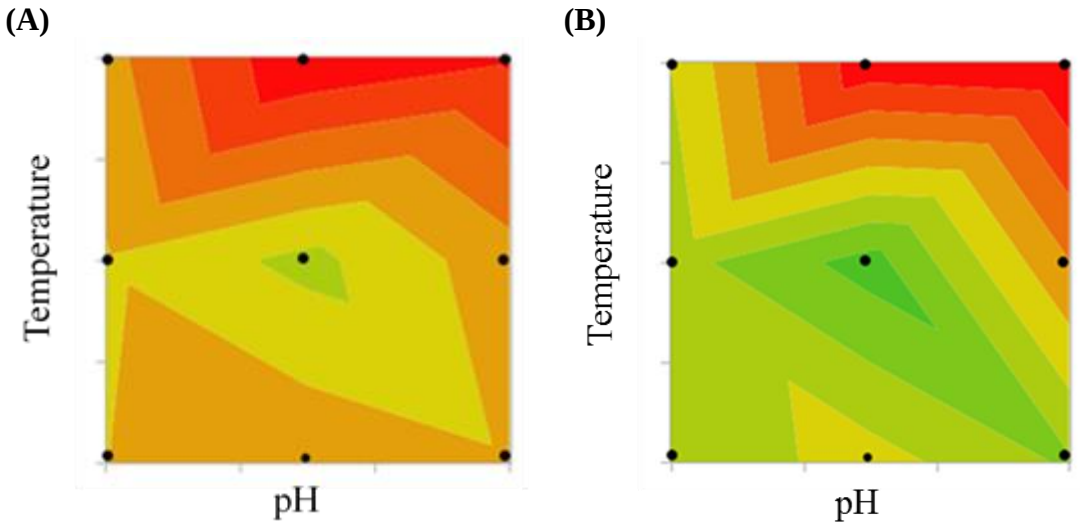


Figure 72. Harvest time dependent effect of pH and temperature on mAb 4 titre using FBvB. The contour plots compare product titre on (A) day 13 and (B) day 15 of culture and were generated based on the 15x DoE data points from FBvB (shown in black). Contour values were specified so that minimum values, maximum values and increments were the same for both heat maps.

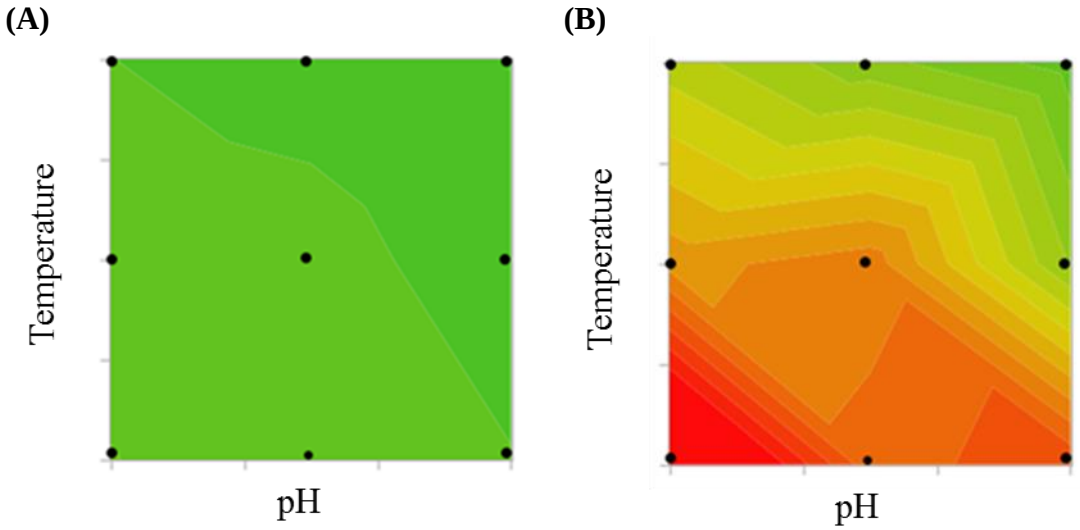


Figure 73. Harvest time dependent effect of pH and temperature on mAb 4 post-protein A HCP levels using FBvB. The contour plots compare HCP levels on (A) day 13 and (B) day 15 of culture and were generated based on the 15x DoE data points from FBvB (shown in black). Contour values were specified so that minimum values (500 ppm), maximum values (8000 ppm) and increments (500 ppm) were the same for both heat maps.

However, in this particular case product monomer purity was deemed not to be a critical criteria, so the SEC data was not included in the next figure, which presents the results as feasible operating windows on two potential harvest days (Figure 74).

The previous figures have shown that desirable levels of antibody titre can clash with desirable results for product quality attributes at harvest day 15 – an earlier harvest such as on day 13 in this case, can reduce this phenomenon.

Figure 71 demonstrates that an earlier harvest allows for wider operating ranges and purer product quality without too much loss of product titre.

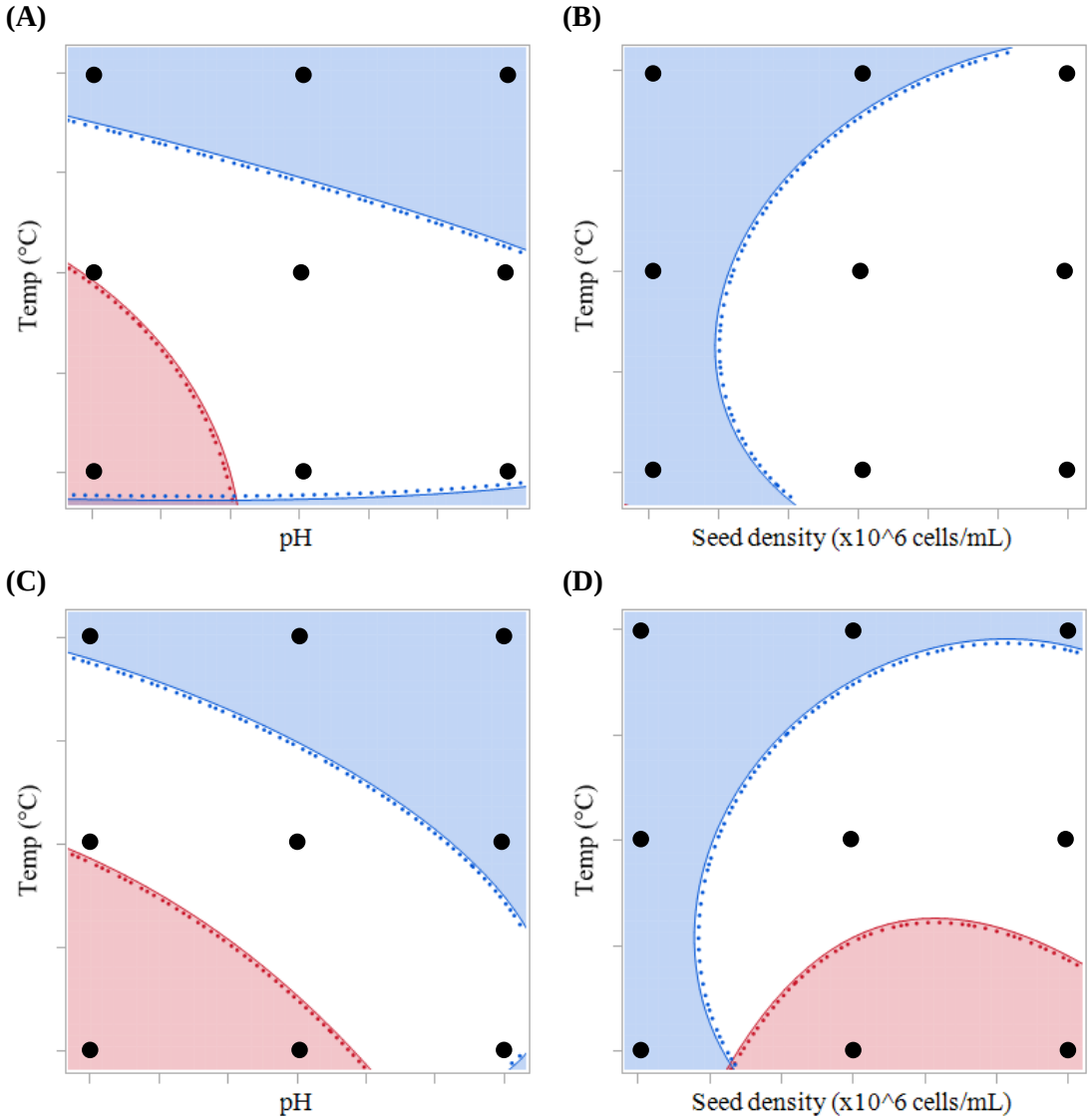


Figure 74. Comparison of feasible operating windows on day 13 (A-B) versus day 15 (C-D) of culture. Using JMP software, contour plots were created to illustrate how the effects of temperature, pH and seed density on titre and post-protein A HCP levels change from day 13 to 15. The contour plots are based on the black experimental data points and were generated by fitting least squares regression models. Titre is shown in blue while post-protein A HCP values are represented by the red contours. As can be seen, the feasible operating window (white area) is wider on day 13 of culture. (A-B) Restraints and statistics of fit: min. 2.2 g/L titre (R^2 = 0.94) and max. 1500 ppm HCPs (R^2 = 0.88). (C-D) Restraints and statistics of fit: min. 2.5 g/L titre (R^2 = 0.93) and max. 7000 ppm HCPs (R^2 = 0.86).

6.4 Indicators for poor product quality

In addition to confirming the findings discussed in the third and fifth chapter about the impacts of culture conditions and harvest time, this case study was also used to further explore the potential product quality indicators that seemed promising from the fourth chapter, one of which was cholesterol content measured in HCCF.

To recollect, in chapter 4 it was found that cultures which were grown at lower temperature were associated with more cholesterol, and cholesterol measurements were shown to be able to predict post-protein A HCP levels in the mAb 1 ambr15 DoE, demonstrating that together with acceptable titres, cholesterol could potentially be used to choose upstream operating conditions that are favourable for both upstream and downstream processing.

For this final DoE study, cholesterol content was measured in the HCCF of cultures grown using the FBvB process. It can be seen once more that cultures grown at higher temperature are associated with lower levels of cholesterol, although the linear correlation is not as strong as in the previous studies (Figure 75).

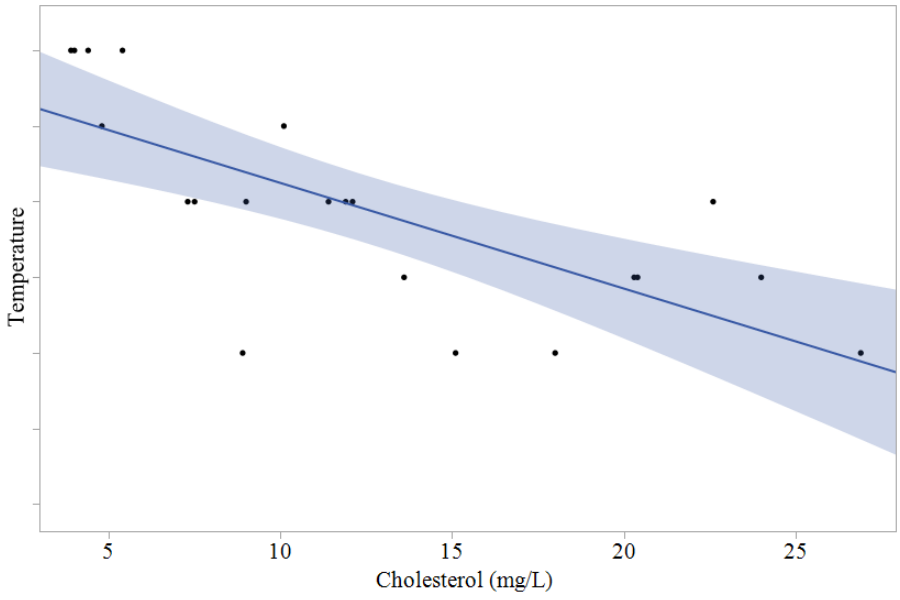
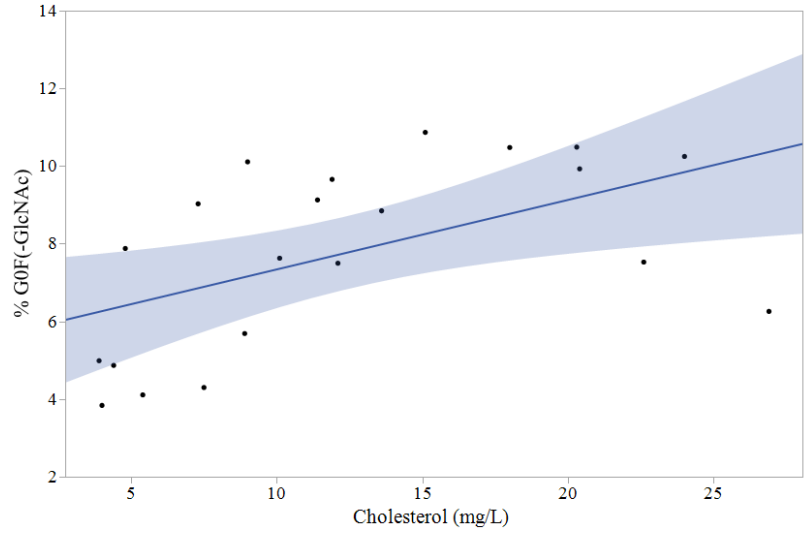
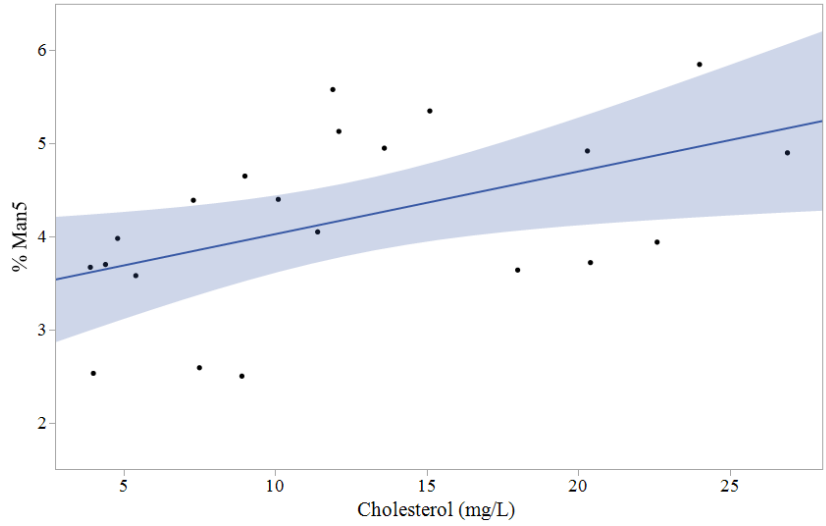


Figure 75. Correlation between cholesterol and temperature using the FBvB process (R^2 = 0.52).

(A)



(B)



(C)

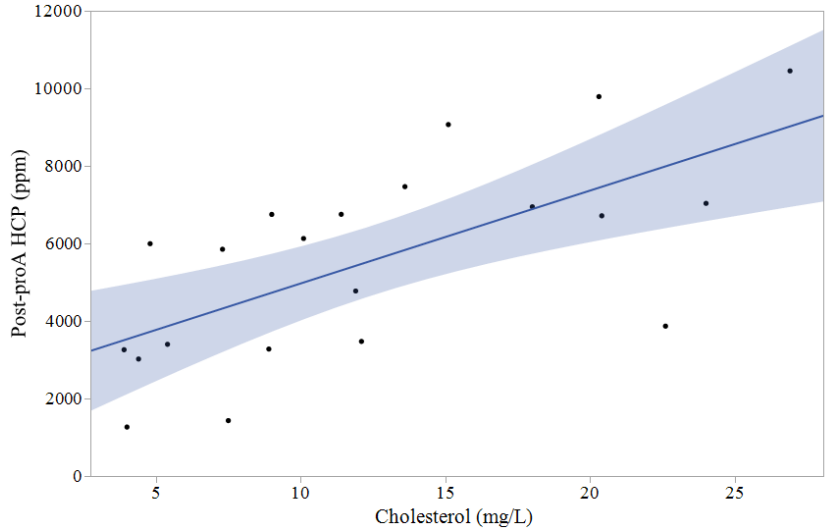


Figure 76. Correlations between cholesterol and mAb 4 product quality attributes using FBvB. Plots are based on linear regression models with confidence intervals for (A) G0F(-GlcNAc) (R^2 = 0.29), (B) mannose 5 (R^2 = 0.25) and (C) post-protein A HCP (R^2 = 0.44).

Furthermore, weak correlations between cholesterol and product quality attributes can be observed– material containing more cholesterol is generally linked to worse product quality with higher levels of post-protein A HCPs and higher levels of certain undesirable glycan species (Figure 76), although the model fit it not very strong. However, unlike in the mAb 1 ambr15 DoE, a maximum limit for cholesterol cannot be easily defined. The maximum cholesterol limit of 10 mg/L that was defined in chapter 4 for example, is here associated with material containing between 1,000 – 7,000 ppm HCPs (Figure 76 C). No linear correlations between cholesterol and titre or product monomer were detected.

While the cholesterol results from all investigated DoEs together seem to suggest that material containing less cholesterol is associated with better product quality, a recommended cholesterol limit is subjective and seems to differ for each molecule or process.

Given the varying results in each DoE study, cholesterol does not seem to be a reliable objective indicator for product quality. Any changes in pH and temperature affect the cells' metabolic state, thereby impacting upon the production of cholesterol which is of course dependent on protein synthesis like all other proteins which the cells produce. It is therefore likely that levels of cholesterol are different at each set of conditions making it difficult to have as a global indicator. While it would be possible to determine acceptable ranges of cholesterol levels for each therapeutic antibody or process by performing DoE studies and small-scale protein A purification runs, this defeats the purpose of identifying a suitable marker that can be measured in HCCF in order to avoid the costly affinity purification step on unsuitable material.

Nonetheless, while cholesterol may not seem to be a reliable indicator for product quality, previous literature (Senczuk et al., 2016) has linked cholesterol levels to filterability with material containing less cholesterol being easier to clarify and thus process. In chapter 4 and here, it was shown that temperature has a strong effect on cholesterol levels – cultures

that were grown at higher temperature were associated with less cholesterol – which based on the research by Senczuk et al. (2016) suggests that material produced at higher temperatures will be easier to clarify. Cholesterol may thus be of interest to establish manufacturability

Lactate dehydrogenase is a cytosolic enzyme which is generally considered to be a marker of broken-down cells if it is detected in HCCF. As such, it is a more suitable indicator of cell number than the Trypan Blue exclusion method (which does not detect lysed cells anymore) and is orthogonal to the measurement of cholesterol. Figure 77 shows how well cholesterol and LDH correlate in samples from the mAb 4 FBvB DoE. As expected, there is a rough correlation between these two indicators of cell lysis, with the exception of some outliers.

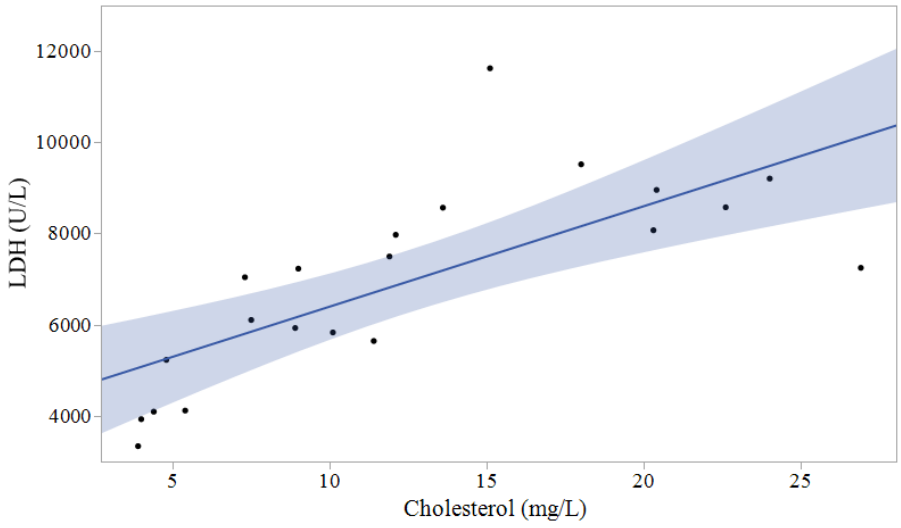


Figure 77. Correlation between cholesterol and LDH (R^2 = 0.54), both thought to be indicators for cell lysis, in the mAb 4 case study using the FBvB process.

LDH was subsequently plotted against several product quality attributes (Figure 78). For both FBvA and FBvB, the same general trends between LDH and product quality attributes can be observed, although the model fit is better for FBvB than for FBvA – with increasing levels of LDH (which is representative of a greater breakdown of cells), the product is associated with more post-protein A HCPs and more undesirable glycan species (mannose 5, G0F-GlcNac and G0-GlacNac). Commonly it is the case that LDH

levels increase towards the end of a culture when cells are dying more readily. Based on this, it is possible that during late stage culture, cells break open and release product which has not yet been fully synthesised, leading to an increase in glycan species without the GlcNac residue. Alternatively, it is also possible that cells run out of the required nutrients and are unable to attach the GlcNac residues during late culture.

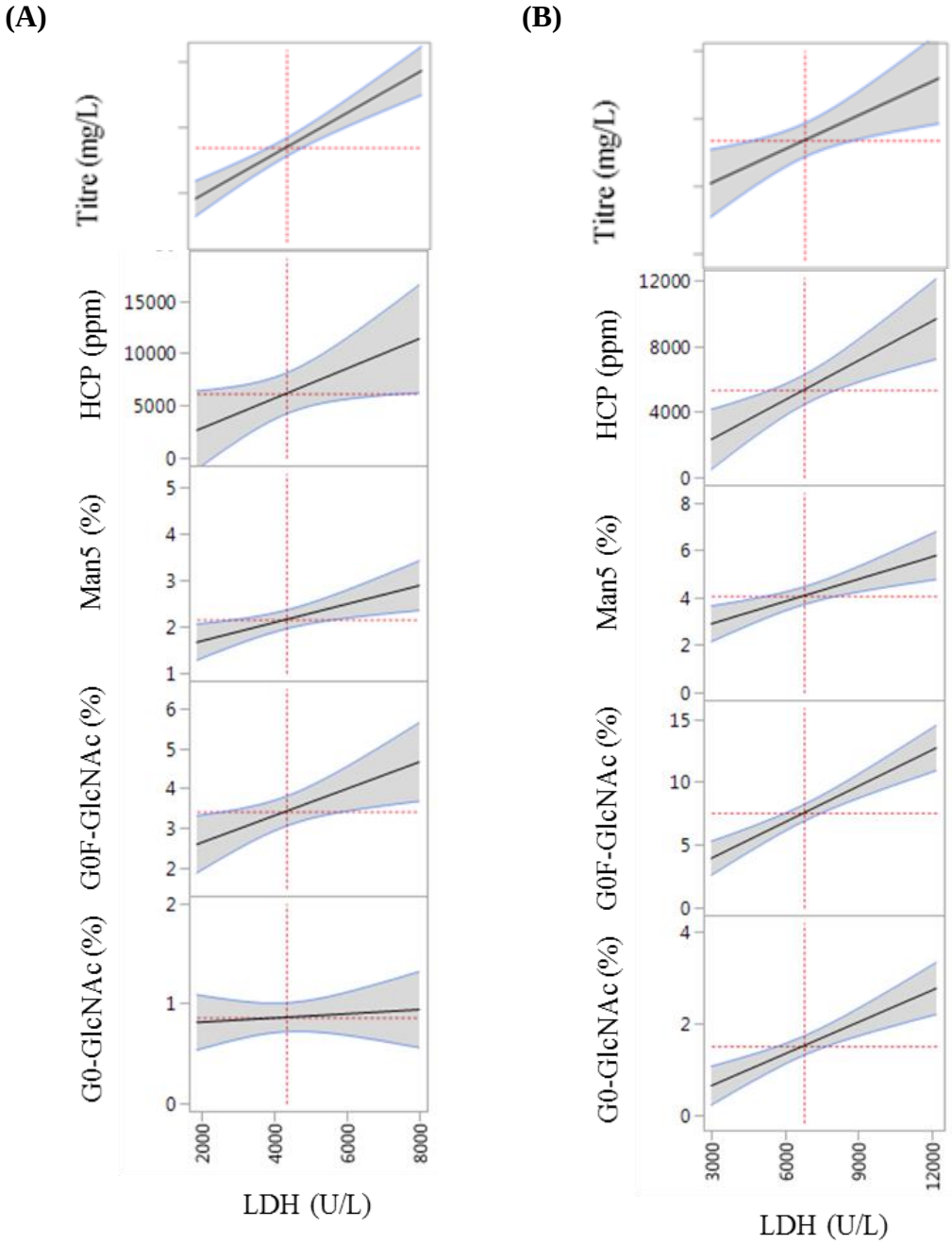


Figure 78. Correlations between LDH and titre / product quality attributes in (A) FBvA and (B) FBvB. Very poor model fit for FBvA (R^2 values: titre 0.74, HCP 0.23, Man5 0.35, G0F-GlcNAc 0.31, G0-GlcNAc 0.01). Better fit for FBvB (R^2 values: titre 0.32, HCP 0.44, Man5 0.41, G0F-GlcNAc 0.67, G0-GlcNAc 0.55).

With regards to post-protein A HCPs, Figure 79 further demonstrates that while there may be a broad trend of post-protein A HCP levels increasing with increasing LDH levels, defining a clear cut-off point for LDH is not easy since there is still a wide range of HCPs associated with a given LDH value, e.g. at 6000 U/L LDH, cultures may be associated with 1000 – 7000 ppm HCPs. While LDH on its own is not a reliable indicator, very low LDH levels do seem to be associated with better product quality – if an acceptable titre is therefore produced by cultures with low LDH, product quality is presumably better than from cultures associated with higher levels of LDH.

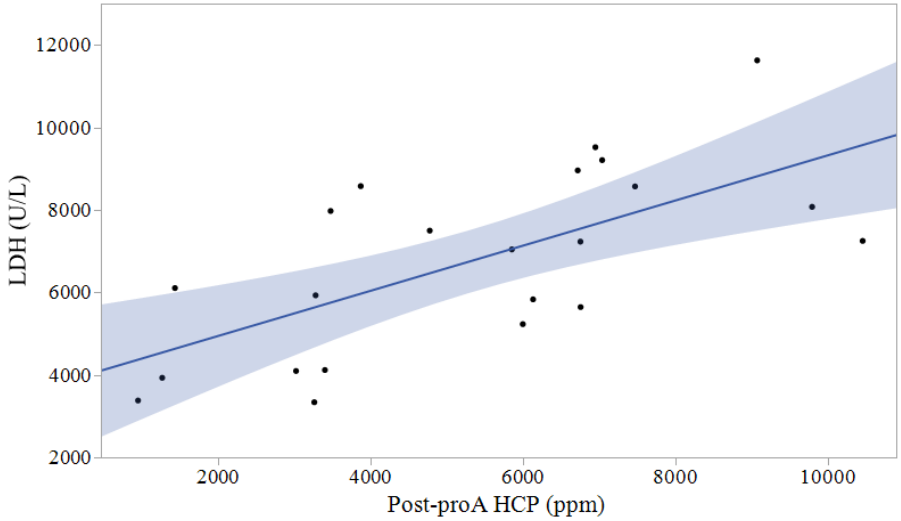


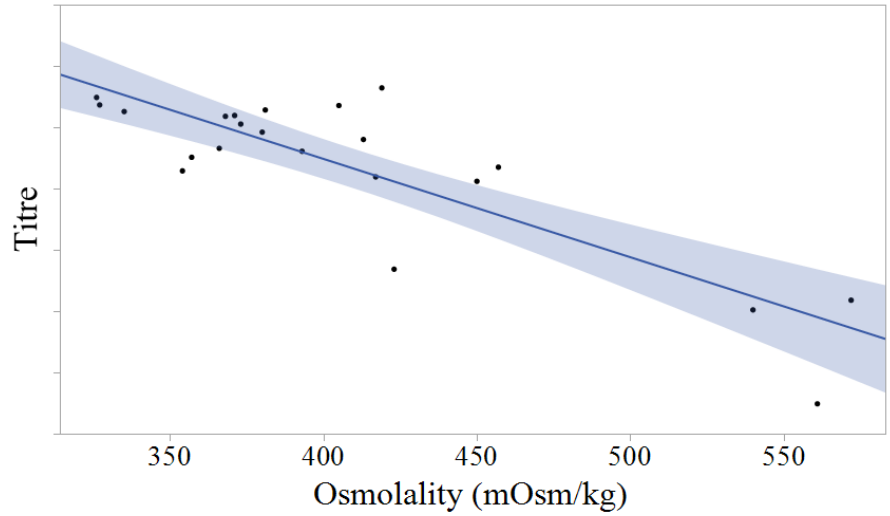
Figure 79. Correlation between LDH and post-protein A HCPs (R^2 = 0.44) using FBvB.

Another potential product quality indicator that was discussed in chapter 4 was osmolality. As was mentioned in chapter 4, osmolality measures the concentration of solutes (such as salt, amino acids, trace elements) in solution. Values below 300 mOsm/kg are generally considered beneficial for growth, whereas higher osmolality (>500 mOsm/kg) slows down cell growth (Kim et al. 2002). An accumulation of feed leads to higher osmolality, which is why high osmolality is commonly considered to be an indicator of overfeeding (Yu et al., 2011; Reinhart et al., 2015). But hyperosmolality can also occur due to the addition of a base for optimal pH control (Han et al., 2010). This

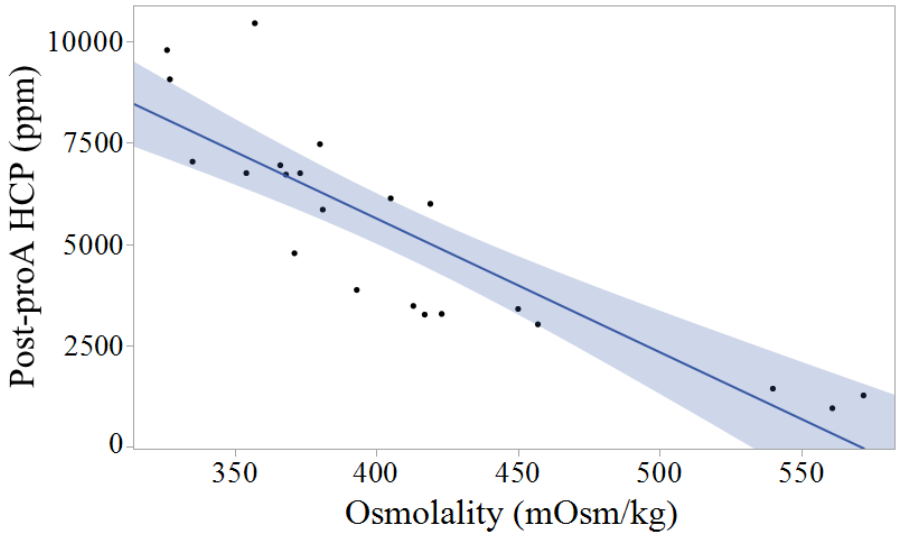
can lead to a reduction in cell growth, viability, and recombinant protein yields (Reinhart et al., 2015).

Osmolality values from the mAb 4 FBvB DoE were plotted against two important product quality attributes – post-protein A HCP levels and percentages of certain glycan species (Figure 80). The data shows that there is a solid correlation between osmolality and post-protein A HCPs, as well as between osmolality and glycosylation.

(A)



(B)



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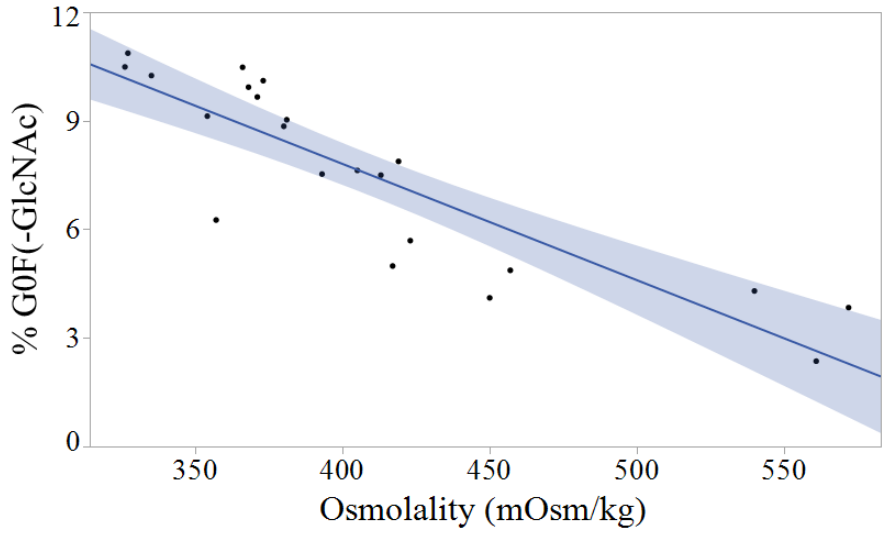


(D)

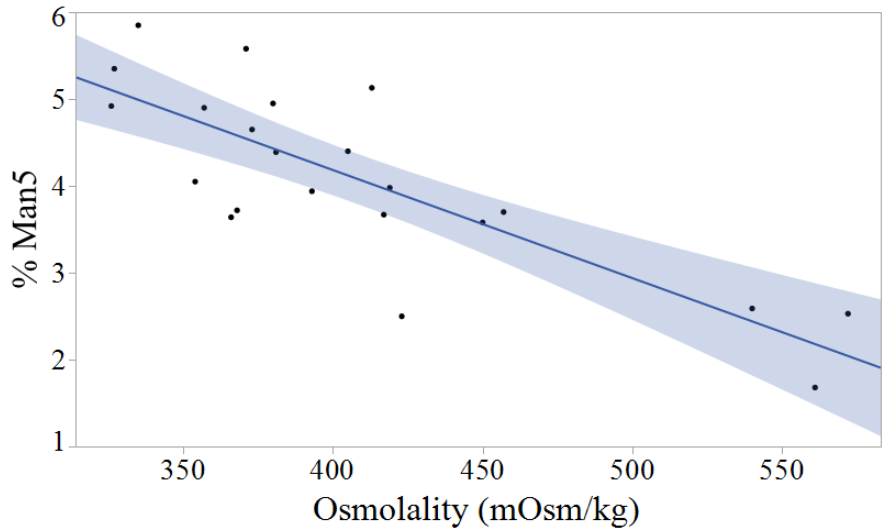


Figure 80. Correlations between osmolality and product quality using FBvB. Product quality improves with increased osmolality, however too high osmolality is associated with low titre presumably due to inhibition of cell growth and productivity. Osmolality between 400 – 500 mOsm/kg results in a trade-off between antibody titre and product quality. R^2 values: Titre 0.72, post-protein A HCP 0.75, G0F-GlcNAc 0.76, Man5 0.66.

There does not seem to be a strong linear correlation between osmolality and titre, as the data rather looks to be clustered in a group with three outliers towards the high osmolality / low titre range.

For the product quality attributes, however, the spread of data corresponds to a much better linear correlation, with product quality improving with increased osmolality. As mentioned, high osmolality is considered to be an indicator of accumulated feed; with regards to decreased percentages of undesirable glycan species, this is likely related to

the fact that more nutrients would be available per cell, allowing cells to fully synthesise correctly glycosylated product and create less undesirable glycan species such as those lacking the GlcNac residue, e.g. G0F-GlcNac and G0-GlacNac.

The decrease in post-protein A HCPs is most likely due to the previously mentioned inhibitory effects of high osmolality on growth and productivity which would inhibit not just the synthesis of host cell proteins but would also slow down cell count growth, meaning that the culture would consist of fewer cells to produce HCPs.

Figure 81 overlays the effects of osmolality on titre and post-protein A HCP results for the FBvB process, demonstrating once more that low osmolality is associated with high titre and high HCP values while high osmolality is associated with low titre and low HCP values. This suggests that osmolality values between 400 – 500 mOsm/kg would result in a trade-off between antibody titre and product quality.

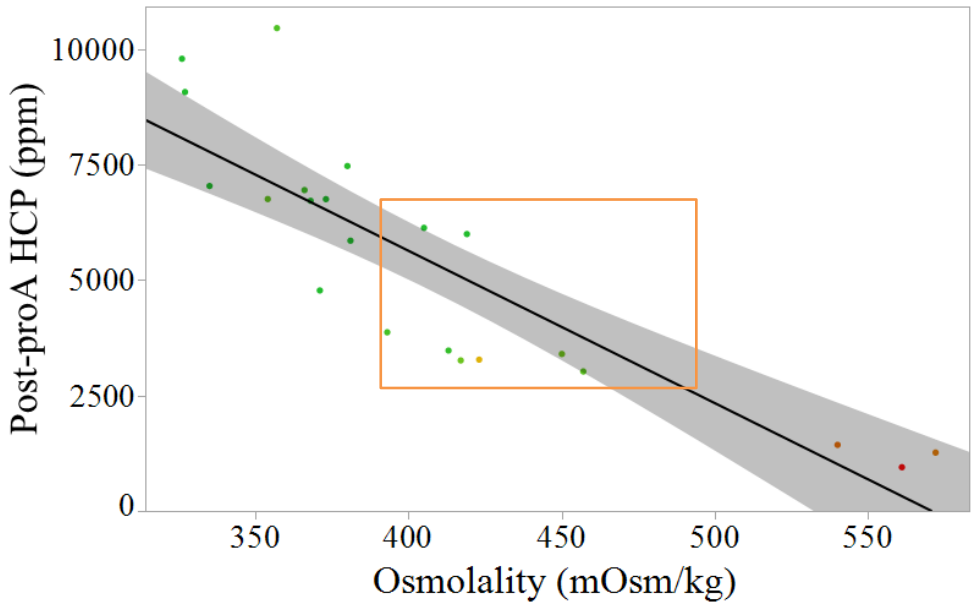


Figure 81. Correlation between osmolality and post-protein A HCP levels in FBvB (R^2 = 0.75), overlaid with titre results (high titres in green and low titres in red).

As discussed in chapter 4, hyperosmolality has been shown to increase specific antibody productivity (q_{Ab}) in mammalian cells but decrease specific growth rate (Zhu et al., 2005; Han et al., 2009; Yu et al., 2011). However, the exact impact on productivity and growth is likely cell line dependent and can result in the final antibody titre being either increased, decreased, or comparatively unaffected (Ho et al., 2006; Yu et al., 2011).

While some literature thus regards hyperosmolality positively due to its enhancing effect on specific productivity rate (Ho et al., 2006), other articles express concern that hyperosmolality is problematic due to stress-induced cell death (Han et al., 2010).

Despite contradictory data published in literature, it is promising that in this thesis, two different cell lines have both shown similar trends – mAb 2 which was used in the study discussed in the fourth chapter, as well as mAb 4 which was used in the study shown here. Furthermore, even if the use of osmolality as a global indicator might vary between cell lines, its applicability could be confirmed relatively simply; as osmolality can be easily measured in HCCF, correlations to titre can quickly be confirmed, and if a similar correlation between osmolality and titre is observed, a correlation to post-protein A HCP levels might exist as well. Of course, further studies are required to confirm this.

In conclusion, osmolality has been found to be one of the most promising product quality indicators that were investigated so far, and has a strong potential to be used as a HCCF-associated quality signature to predict desirable levels of certain glycan species as well as post-protein A HCPs.

6.5 Exploring downstream robustness with alternative protein A wash buffers

This study was also used to explore the robustness of some aspects of the downstream process, such as the implementation of alternative wash buffers during protein A chromatography. Figure 82 displays an overlay of chromatograms from four purification runs using the same upstream sample and four different wash buffers – a control wash

buffer (using only equilibration buffer), the downstream industry standard wash buffer, an alternative wash buffer “A”, and an alternative wash buffer “B”.

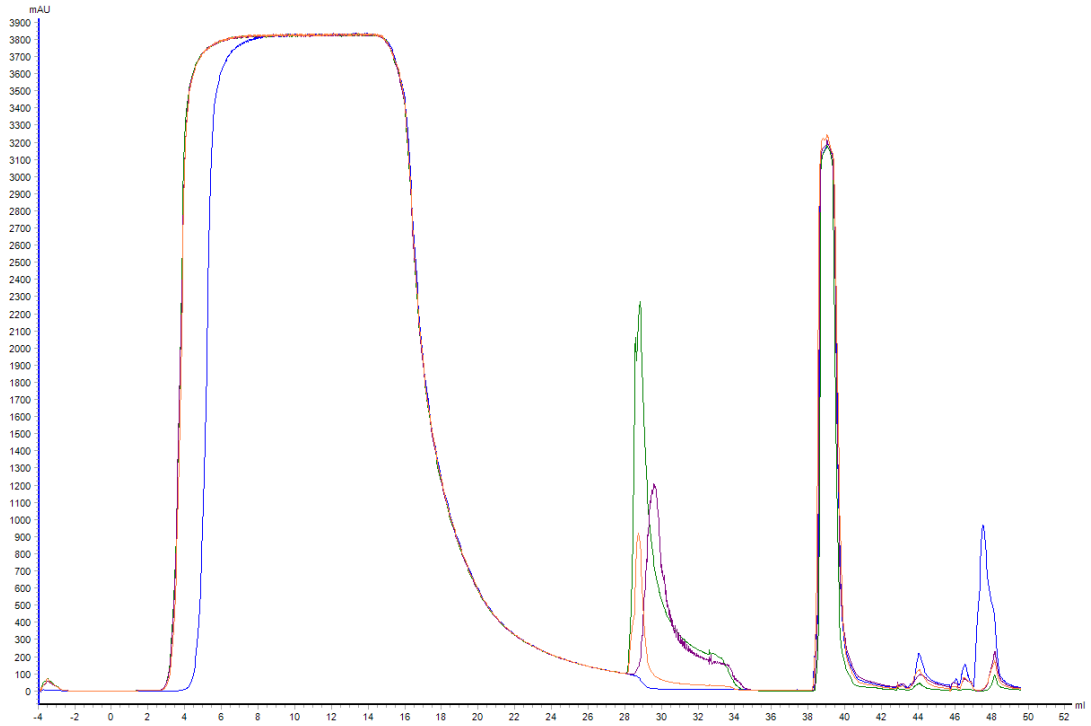
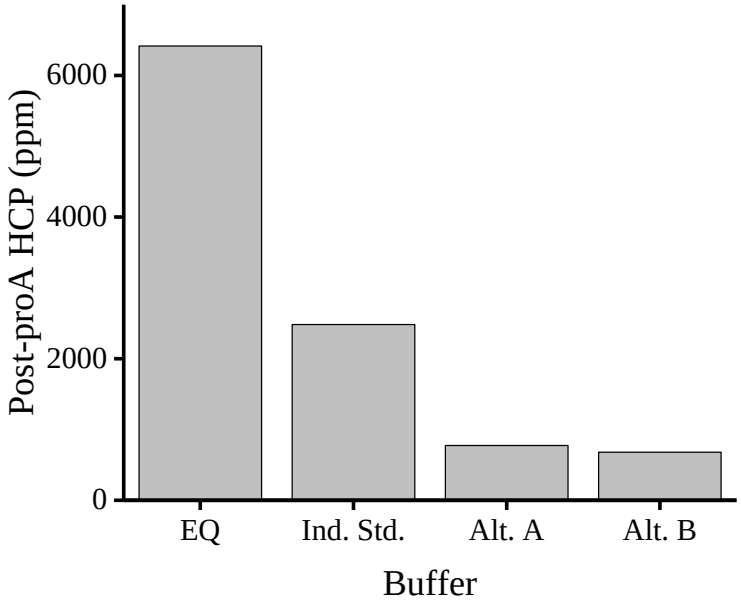


Figure 82. Overlay of chromatograms of protein A purification using four different wash buffers (for the culture grown under upstream industry standard condition using FBvA). EQ buffer (blue), downstream industry standard buffer (orange), wash buffer “A” (purple), wash buffer “B” (green).

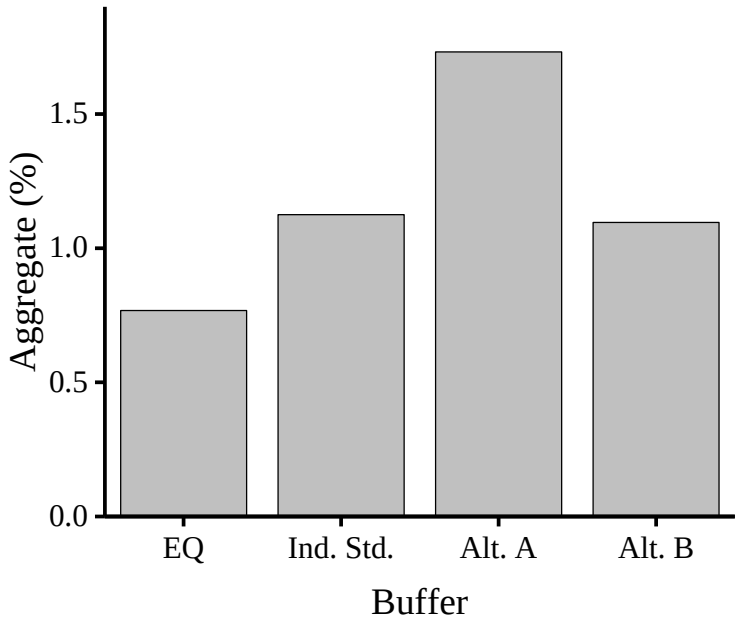
The chromatograms show that when using only equilibration buffer (blue) after loading the material, many loosely bound HCPs are not washed away and are therefore eluted with the product or during the subsequent column sanitisation step. The orange line in the chromatograms is from the run using the default downstream industry standard wash buffer, whereas the purple and green lines are from the runs with the alternative wash buffers “A” and “B” respectively, which shows that both alternative wash buffers are better than the industry standard wash buffer in removing loosely bound HCPs from the column prior to elution of the desired antibody.

To confirm these observations that were suggested by the chromatograms, the eluate samples from all four purification runs were analysed for post-protein A HCP levels using an ELISA. The eluate samples were further analysed to determine product recovery and aggregation. Results are summarised in Figure 83.

(A)



(B)



(C)

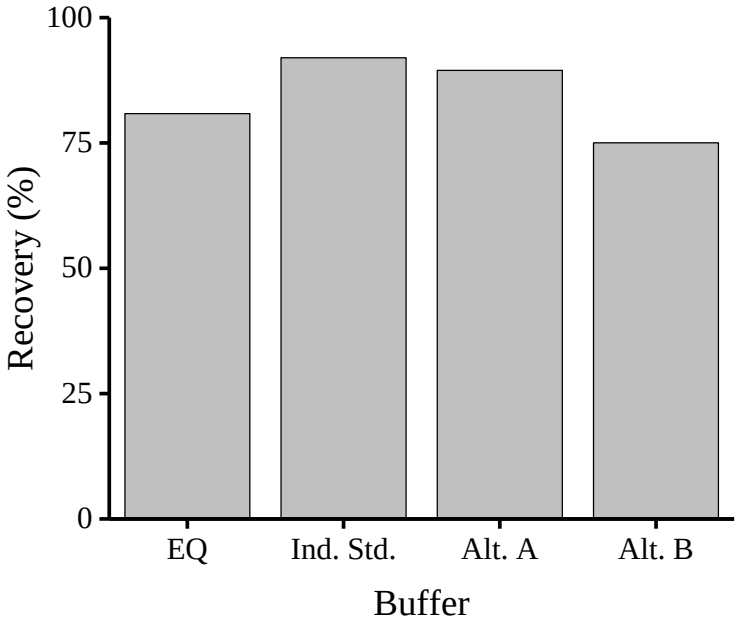


Figure 83. Impact of wash buffers on (A) HCP levels, (B) product aggregation and (C) product recovery. While both of the alternative wash buffers clear HCPs more than 3-fold compared to the downstream industry standard wash buffer, the alternative wash buffer A causes increased product aggregation while the alternative wash buffer B results in lower product recovery (mAb 4 FBvA).

As can be seen, post-protein A HCP levels are indeed highest when not using a specific wash buffer after sample loading. HCP levels are much lower when using the downstream industry standard wash buffer. However, when using one of the two alternative wash buffers, post-protein A HCP quantities are further decreased by more than two thirds. From an HCP clearance perspective, either one of the two alternative wash buffers would therefore be a feasible option to use during protein A purification.

More than one response needs to be considered during downstream purification though, such as product aggregation or product recovery during chromatography purification. The results show that while the two alternative wash buffers may be a suitable choice to separate the antibody from loosely associated HCPs, wash buffer “A” causes higher aggregation of the eluted drug product, and wash buffer “B” results in decreased product recovery suggesting that not just HCPs are washed away before elution but small amounts

of the protein of interest as well. The same trends could also be observed when using these four wash buffers during the purification of several other upstream samples (not shown). The effect of these different wash buffers on glycan composition were not examined since downstream purification has little to no effect on this quality attribute.

To summarise, three important downstream responses (product recovery, aggregation and HCP quantities) have been taken into consideration in this study, which has demonstrated that while choosing alternative wash buffers during protein A purification can positively contribute to the desired outcome of one or two quality attributes, it is very challenging to achieve optimal results for all three responses. Trade-off decisions will need to be made.

6.6 Exploring downstream robustness with larger column scales

In addition to exploring the robustness of the downstream space with alternative wash buffers, chromatography column scale was another factor that was studied. Due to the nature of this thesis research (large amounts of samples available in small volumes), most of the purification runs were carried out on small-scale 0.2 mL chromatography columns using a liquid handling robot. In order to represent column sizes more commonly used in biopharmaceutical process research environments (such as during the preparation of material for tox studies), the same upstream material was purified on several different chromatography column scales. Two samples were chosen for this mini-study – (A) the sample that was cultured under the upstream industry standard conditions for this particular mAb 4 and (B) the sample that was cultured under the same bioreactor operating conditions but using an alternative cell line. Both of these samples were protein A purified on 0.2 mL, 1 mL and 5 mL columns, and two quality attributes (post-protein A HCPs and product monomer percentage) were measured in order to compare these outputs amongst the different chromatography column scales.

While product monomer percentages remained unaffected by the column scale used to purify material (data not shown), Figure 84 shows that for post-protein A HCP levels, there is a significant discrepancy between large and small scale purification, with an inverse correlation between HCP values and column scale. This holds true for both tested samples, regardless of cell line.

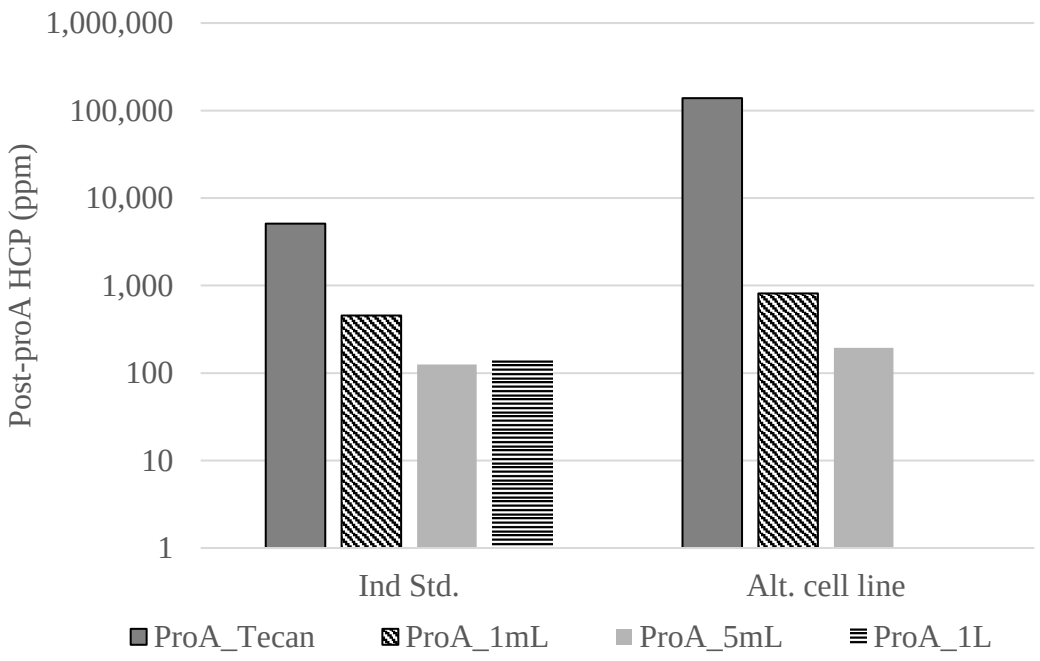


Figure 84. Impact of column scale on product quality in the culture grown under the upstream industry standard condition and an alternative cell line. While monomer purity is comparable between the scales (not shown), there is a significant discrepancy between HCP values for large and small scale purification (mAb 4 FBvA).

Due to different eluate volumes, material was treated slightly differently after it had eluted and before it was analysed by ELISA. While the small amount of volume that had been eluted from the 1 mL column had merely been spun down prior to an ELISA (to avoid cross-contamination of sample compontents with the assay reagents), the eluate from the 5 mL column was 0.2 µm filtered before HCP quantification by ELISA was carried out. It was therefore checked whether the discrepancy between HCP values was indeed due to scale differences or whether the treatment prior to the ELISA had an impact. For this,

more mAb 4 material was purified on a 5 mL column in the same manner as the previous material was purified. Half of the eluate was then 0.2 µm filtered while the remaining material was not. Both the non-filtered and filtered eluate was then analysed by carrying out an ELISA. As seen in Figure 85, while filtration does remove some of the HCPs (approximately 100 ppm in this instance), this step does not contribute to HCP clearance to the same extent as column scale seems to do.

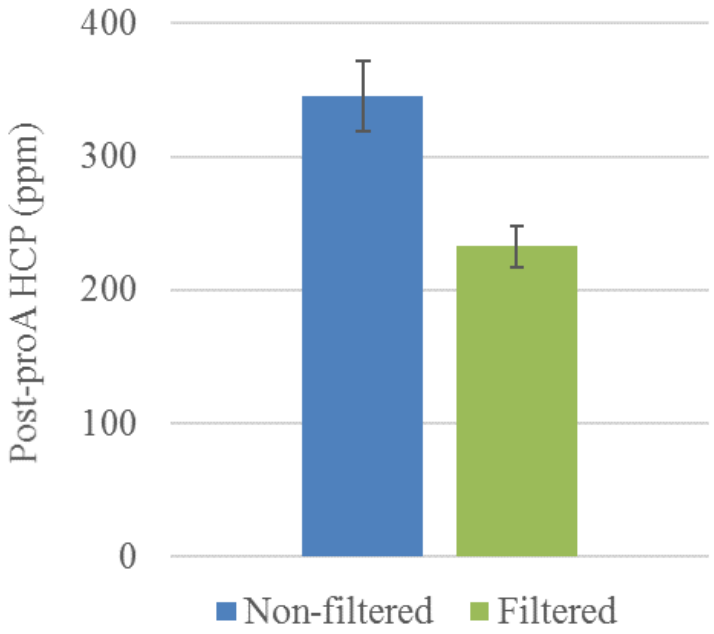


Figure 85. Impact of pre-ELISA treatment. Error bars are based on triplicate samples run at the same time and show one standard deviation.

In conclusion, material that has been purified on larger columns seems to be associated with fewer post-protein A HCP quantities. This is advantageous as purification column sizes are scaled up from research environments to manufacturing processes and means that the actual HCP values need not be of huge concern. Nonetheless, using small scale columns during process development research remains practical and efficient, as long as relative trends in HCP values amongst DoE samples stay the same, which has been shown to be the case by GSK's analytical group (confidential data).

6.7 Exploring downstream robustness with polishing chromatography

Further to the previous two variables, it was also considered how the inclusion of a polishing chromatography step might make downstream processing more robust and able to cope with increasing challenges on product purification. For this, the two samples which had been purified on 5 mL chromatography columns (as mentioned in the previous section) were further processed by carrying out the viral inactivation step and subsequently purifying the material on a 0.2 mL anion exchange chromatography column. As before, HCP quantities and product monomer percentage were determined. Improving HCP clearance by implementing an additional chromatography step is a possibility that has already been discussed in chapter 5. As was already demonstrated in section 5.4, here we also see that HCP reduction is achieved by further processing steps. Monomer purity was not significantly affected by the implementation of further polishing steps (data not shown).

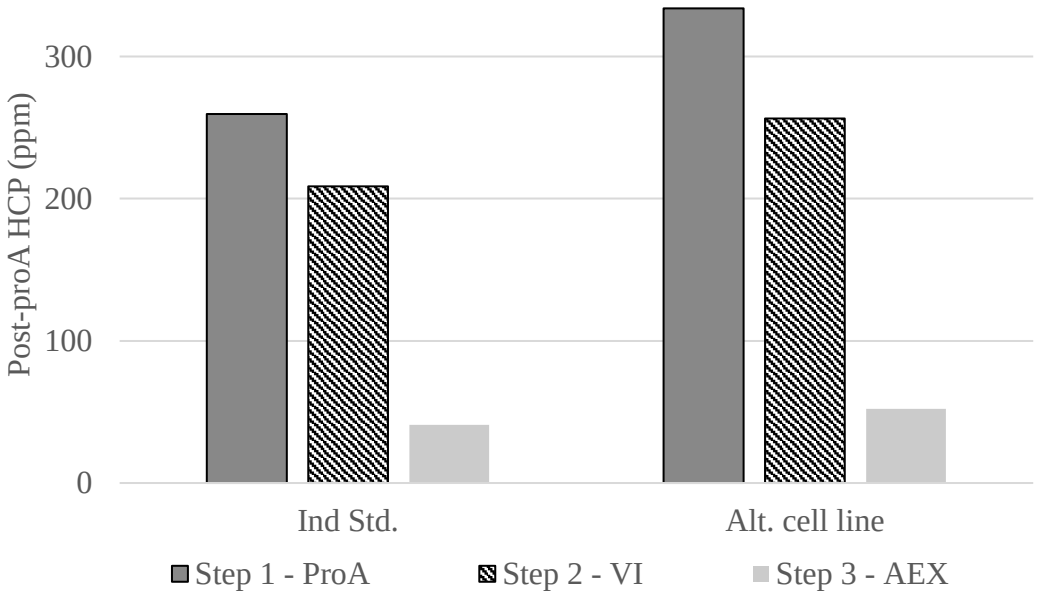


Figure 86. Impact of anion exchange polishing chromtography on post-proA HCP levels in the culture grown under the upstream industry standard condition and an alternative cell line (mAb 4 FBvA). AEX reduces HCP levels to below the recommended limit of 100 ppm in both cases.

While it is good that additional downstream processing steps are able to compensate for worse initial product quality and thus achieve the required product quality specifications in the final drug product, all extra steps in the process are of course associated with extra expenses as well as a potential loss of product yield at each step. The antibody purification process should therefore be kept to the fewest steps possible. Wherever possible, the drug production process should be developed for an optimal integrated process with an efficient number of steps. Nonetheless, for situations where this isn't possible, it is very beneficial to know which factors increase the robustness of the downstream space.

6.8 Summary

In this chapter, an extensive integrated DoE study was carried out to tie all investigations into upstream culture conditions, harvest time points and product quality indicators together, and to further explore the robustness of some aspects of the downstream process. Further to the DoE approach which investigated the effects of culture media and bioreactor conditions, time-dependent effects on product quality were studied by taking time-course samples and studying the variation in visible effects. Some samples were also chosen for further investigations into the robustness of the downstream process in terms of protein A wash buffers, purification column scales, and polishing chromatography steps.

It was confirmed that culture pH, temperature and seed density setpoints that result in high titre are commonly also linked to decreased product quality in terms of high post-protein A HCP levels, reduced monomer percentages and increased percentages of undesirable glycan structures. Furthermore, it was found that the type of media (such as a more glucose- and nutrient-rich media) and slight changes in upstream process configurations (e.g. higher percentages of dissolved oxygen) have a big impact on product quality. FBvB for instance resulted in more titre, reduced levels of product fragmentation and post-protein A HCPs but more undesirable glycan structures.

With regards to harvest time, contour plots have shown that ideal ranges for product quality attributes can clash with desirable levels of antibody titre on day 15. An earlier harvest such as on day 13 in this case, was shown to reduce this impact and allow for wider operating ranges and purer product quality, without too much loss of product titre. This integrated case study was also used to re-address some of the potential product quality indicators that seemed promising from the fourth chapter. While cholesterol initially seemed to be a promising predictor of product quality, it was ultimately deemed unreliable since defining a maximum cholesterol limit appeared to differ for each molecule or process. This would make it necessary to first perform small-scale studies to determine the appropriate cholesterol limits for each potential drug product which is counter-productive to the purpose of identifying a suitable marker that can be measured in HCCF in order to avoid the costly affinity purification step on unsuitable material. LDH as an orthogonal measurement to cholesterol was also investigated here and while LDH on its own may not be a reliable indicator, very low LDH levels do seem to be associated with better product quality, so if an acceptable titre is produced by cultures with low LDH, product quality is presumably better than from cultures associated with higher levels of LDH.

Another potential prediction tool that was explored here was osmolality. Osmolality has been found to be one of the most promising product quality indicators out of all the ones that were investigated and presented in this thesis, and has a strong potential to be used as a HCCF-associated quality signature to predict post-protein A HCPs as well as percentages of certain glycan species.

The research into the robustness of the downstream process that was carried out also generated some interesting findings. In terms of protein A wash buffers, it was discovered that while optimised wash buffers during protein A purification may very well be able to reduce HCP levels further than standard wash buffers, some alternative wash buffers can

cause higher product aggregation while others may result in lower product recovery. So, while the downstream space may be robust enough to adjust to higher quantities of HCP impurities, other downstream responses may be affected by such an adjustment. As such, it should still be considered a priority to produce upstream material with fewer HCPs, otherwise trade-off decisions between several downstream responses (HCP clearance, product aggregation and product recovery) may be necessary.

Another aspect of the downstream space that was studied was purification column scales and it was shown that post-protein A HCP levels decreased with increasing column scale, which is very advantageous. It suggests that when using small-scale columns during process research, the actual HCP values need not be of huge concern, but they do provide valuable information about the relative trends in HCP values amongst samples from various process conditions.

One final observation was related to polishing chromatography steps. As expected and previously demonstrated, HCP reduction is achieved by further processing steps, which is good for those scenarios where no other alternatives are available, and bad product quality needs to be compensated for in order to achieve the required product quality specifications for the final drug product. But ideally, the process should be amended further upstream if possible, since all extra steps in the purification process are of course associated with extra expenses and potential losses of product yield at each step.

On the other hand, cost savings due to fewer processing steps, fewer resources and less product loss at each processing step will of course have to be weighed against the costs of carrying out extensive small-scale high-throughput studies. If the necessary equipment (e.g. ambr bioreactors, small scale columns, liquid handling robots, automated assays etc.) is not readily available, then the cost of acquiring this equipment or the loss of time from waiting for this equipment to be accessible, quite possibly negates any savings.

To summarise, the table of conclusions listed at the end of the previous chapter has been updated with conclusions from this chapter in Table 13 below:

Investigated factors	Conclusions
Upstream culture conditions	Bioreactor conditions that are optimised for maximum titre have been shown to commonly also result in decreased product quality (HCP and monomer). Also, different media (e.g. a more glucose- and nutrient-rich media) and changes in upstream process configurations (e.g. higher percentages of dissolved oxygen) can change glycan species percentages.
Culture viability	Harvest culture viability is not a reliable indicator to predict product quantity or post-protein A HCP quality (in studies with many variables such as varying seed densities and different harvest days as was the case here).
Cholesterol	Cholesterol levels seem to be different at each set of culture conditions (due to the impact on cells' metabolic state and protein synthesis) making it difficult to have as a global indicator for product quality.
Osmolality	Osmolality has been found to be one of the most promising product quality indicators that were investigated so far, and has a strong potential to be used as a HCCF-associated quality signature to predict desirable levels of certain glycan species as well as post-protein A HCPs.
Free antibody light chains	Free antibody light chain levels are not a reliable indicator to predict titre, post-protein A HCPs or aggregation as results seem to be dependent on vector design / the ratio of gene copy numbers for the heavy and light chains / the integration of the recombinant DNA.
Culture duration	As product titre increases throughout a typical mAb culture, post-protein A HCP levels tend to increase as well, meaning that material which is harvested later likely requires a more extensive HCP clearance strategy (which can be a concern when polishing chromatography is required for HCP clearance of an unstable mAb, e.g. a mAb that fragments during CEX). HCP species involved in chaperoning unfolded proteins, telomere maintenance, and proteasomal degradation are accumulating at late stage culture which is a strong indication of ER stress and oxidative stress, induced by factors such as glucose starvation, lack of nutrients, lack of protein glycosylation, oxygen deprivation and cell age.
Cell age	Non-viable / apoptotic cells seem to be more shear-resistant than viable cells, with minimal cell breakage

	upon shear exposure, possibly due to a gradual breakdown of cells' lipid bi-layer resulting in increased porosity of the membrane and a loss of membrane integrity.
Lactate dehydrogenase	LDH seems to be a good indicator to predict filter efficiency of upstream material, as it was found to correlate linearly with culture viability and turbidity. In terms of predicting product quality, LDH on its own is not a reliable indicator but very low LDH levels do seem to be associated with better product quality – if an acceptable titre is therefore produced by cultures with low LDH, product quality is presumably better than from cultures associated with higher levels of LDH.
Alternative protein A wash buffers	Alternative wash buffers during protein A purification can positively contribute to the improved outcome of one or two quality attributes, but it is very challenging to achieve optimal results for all three of the investigated downstream responses (product recovery, aggregation and HCP quantities), meaning that trade-off decisions will need to be made.
Purification column scale	Material that is purified on larger columns seems to be associated with fewer post-protein A HCP levels.

Table 13. Summary table of the knowledge that was gained during this thesis – updated from previous chapter.

7 CONCLUSIONS

As an increasing number of biopharmaceuticals are being brought to market, creating an ever more competitive market environment, biopharmaceutical companies need to speed up their drug production timelines by streamlining process development and optimisation. An integral part of this is to improve the understanding between upstream and downstream process operations in order to describe how changes to process parameters in any one operation affect the efficiency of therapeutic drug manufacturing throughout the whole process.

As significant improvements to antibody productivity have been achieved within upstream processing, and harvest material is becoming progressively more difficult to recover with these intensified upstream operations, one important factor has remained largely unchanged – the segregation of upstream and downstream processing. With limited interaction between these two groups during the process development stage, any potential product purification issues are not taken into consideration during the optimisation of culture conditions, which could potentially make it necessary to use extensive and expensive clearance strategies.

In order to avoid the costly processing of material that may not even get past the development phase, it is necessary to take a holistic approach to process development and consider the ease of downstream protein purification when designing upstream operating conditions, and thereby essentially integrating upstream and downstream processing. This approach also supports the “fail early, fail cheap” mentality.

This thesis presents a methodology based on such an integrated process development approach, which can be used to determine the effects of upstream processing conditions on various major critical quality attributes – by combining upstream DoEs with downstream studies and high-throughput analytical assays, feasible upstream operating

conditions for any given process can be determined which are not just optimised for high antibody productivity but also low impurity profiles.

7.1 Effects of upstream culture conditions on mAb-associated post-protein A HCP impurities

The work discussed in the third chapter has demonstrated that even small changes to upstream process parameters at the bioreactor stage of monoclonal antibody production can greatly influence the amount of product being produced as well as the host cell protein impurities associated with each culture.

Using Design-of-Experiment (DoE) studies and contour plots, it was demonstrated that factors such as culture pH, temperature and seed density affect product quantity and quality in ways that necessitate trade-offs between upstream and downstream operations, in order to avoid producing high levels of product with poor product quality. It should of course be noted that neither the assessed upstream process parameters nor the upstream- and downstream-related quality attributes examined here form an exhaustive list as there are several alternative parameters and quality attributes that might be of interest to a bioengineer (such as aeration rate, impeller speed, or dissolved oxygen / carbon dioxide percentages). But the parameters and attributes examined here were of special interest and were considered to be very relevant based on other published literature.

Complementary work that has been carried out and published by others include research by Agarabi et al. (2017) who have investigated how changes to upstream process parameters can influence capture chromatography performance and who have concluded that DO% and sparge rate greatly affect downstream purification. Other work of interest resolves around culture temperature – operating cultures at 37 °C for several days to promote cell growth and then shifting temperatures to 28 – 35 °C extends culture viability (Trummer et al. 2006) and increases cell specific productivity (Yoon et al. 2003). Another effect of decreasing temperature is the reduced consumption of glucose and therefore the

associated production rate of lactate, which is a major by-product of the glycolytic pathway during incomplete oxidation of glucose. As accumulated lactate in the culture medium has an inhibitory effect on cell growth and protein production, decreased temperature may prevent this (Eibl et al., 2009).

This chapter has further shown that culture viability at harvest cannot be used alone as a tool to predict post-protein A HCP levels and so other potential indicators that can be measured in HCCF to predict post-protein A product quality were explored in the next chapter.

7.2 Identification of HCCF-associated quality signatures

In the fourth chapter, several potential product quality indicators were investigated in the hope that these could be measured in harvested cell culture fluid (prior to using expensive purification resources) and used to predict post-protein A product quality such as HCP levels. While cholesterol seemed promising, it was ultimately deemed unreliable since defining a maximum cholesterol limit appeared to differ for each molecule. This makes it necessary to first perform small-scale studies to determine the appropriate cholesterol limits for each potential drug product which is counter-productive to the purpose of identifying a suitable marker that can be measured in HCCF in order to avoid the costly affinity purification step on unsuitable material. However, cholesterol has previously been linked to filterability with material containing less cholesterol being easier to clarify and thus process. In this chapter, it was shown that temperature has a strong effect on cholesterol levels – cultures that were grown at higher temperature were associated with less cholesterol – which based on the research by Senczuk et al. (2016) suggests that material produced at higher temperatures will be easier to clarify. Cholesterol may thus be of interest to establish manufacturability.

Ammonium and antibody light chain levels were also deemed to be unreliable product quality predictors due to varying reasons, but osmolality was discovered to be a promising

quality indicator and has a strong potential as a predictor of post-protein A HCPs as well as percentages of certain glycan species.

Other potential quality indicators that would be interesting to explore in the future are certain HCP species that are associated with the cellular stress response such as proteases, cathepsins or heat shock proteins. As these proteins are produced in higher levels during stress, they could give an indication of suboptimal conditions for cells to grow and produce therapeutic antibody and might therefore be linked to poor product quality.

7.3 Effects of upstream harvest time on mAb product quality

In the fifth chapter, the impact of cell culture duration on critical quality attributes was investigated and it was shown that as cultivation progressed and antibody titre increased, the post protein A HCP impurities also increased indicating that harvest material is becoming progressively more difficult to recover using this purification scheme.

It was also demonstrated that cells become more robust against shear damage as cultivation progresses, and that an earlier harvest can therefore lead to greater cell breakage. Nonetheless, it was ultimately discovered that shear-induced cell breakage affects the product less than the decline in product quality caused by a longer culture duration. Furthermore, shear-resistance is associated with increased sample turbidity and decreased filter efficiency, of which LDH levels seem to be a useful indicator. Consequently, culture duration has a large impact on the production of pure product, more so than any shear sensitivity of cells.

HCP identification by mass spectrometry was performed on material from different timepoints to provide insights into cellular behaviour and HCP carryover during protein A purification. It showed increases in several classes of post-protein A HCPs (e.g. stress response proteins) as the culture progressed, particularly on days 15 and 17 of culture which were associated with significant increases in total HCP levels. This provides a new

level of insight into HCPs that are retained during mAb purification which may be used to aide process development strategies.

7.4 Implementation of an integrated case study

The sixth chapter can be regarded as a case study which applies the methodology or decision-making framework developed in this thesis. It tied together all investigations into upstream culture conditions, harvest time points and product quality indicators, and further explored the robustness of some aspects of the downstream process. This case study implemented commonly used high throughput scale-down mimics of unit operations which enabled a fast evaluation of the effects that process conditions have on upstream responses and downstream product quality attributes. This approach confirmed that upstream operating conditions which result in high titre are commonly also linked to decreased product quality in terms of high post-protein A HCP levels, reduced monomer percentages and increased percentages of undesirable glycan structures. It also highlighted that an earlier harvest (here on day 13) allows for wider operating ranges and purer product quality, without too much loss of product titre.

In terms of the robustness of the downstream space, this case study explored alternative protein A wash buffers, chromatography column scale and the inclusion of polishing chromatography steps, which all showed that protein A HCP levels could be reduced to acceptably low levels, but commonly by affecting other downstream factors (e.g. increased product aggregation, decreased product recovery, extra steps in the purification process which are associated with extra expenses and potential losses of product yield at each step).

As such, this integrated approach to upstream and downstream process development enables the selection of upstream operating conditions that are mutually beneficial for both upstream and downstream targets, and it is a methodology that may well need to be applied for every future molecule and process change. The scale-down approach

described in this thesis can be used in early process development to help select operating conditions that maximise product titre and quality.

7.5 Future work

As mentioned, only a select few upstream and downstream parameters were investigated in this thesis. There are many more factors at the interface of upstream and downstream processing that can be studied to continue building a complete picture. Upstream bioreactor conditions like temperature shifts, dissolved oxygen percentage, sparge rate or impeller speed can be included in future integrated DoE studies to link these upstream factors to downstream responses. Alternatively, factors affecting primary recovery and filterability can be explored, rather than focussing on post-protein A product quality. Cholesterol levels in HCCF seemed to be a promising indicator of filter efficiency and might be further examined as a potential critical attribute for robust manufacturability.

For the near future, I would personally recommend focussing on osmolality and establishing this upstream measurement as a HCCF-associated quality signature to predict critical product quality attributes like glycosylation and post-protein A HCP levels. As mentioned, high osmolality is considered to be an indicator of accumulated feed, i.e. more nutrients are available per cell, allowing cells to fully synthesise correctly glycosylated product and create less undesirable glycan species such as those lacking the GlcNac residue. High osmolality also has inhibitory effects on growth and productivity, limiting the levels of HCPs. Carrying out DoE studies with cultures exhibiting a range of different osmolality levels will establish a more conclusive relationship between osmolality and product quality in order to create a robust traffic light system as mentioned in the fourth chapter of this thesis.

For a longer project, such as maybe the next PhD project, I believe it would be incredibly valuable to focus on host cell protein identification by mass spectrometry and combine a process engineering approach with a strong biochemical analysis of the identified HCP

species present under various process conditions. This serves several purposes – not only will such research help identify process conditions resulting in product which is associated with HCP species that are known to be a safety risk to patients (e.g. PLBL2) or those that negatively affect product stability (e.g. lipoprotein lipase), and thus help avoid growing cells in such conditions, but it can also greatly enhance our understanding of the cells we use to synthesise therapeutic proteins. Identifying the proteins that host cells produce under different growth or stress conditions and at different times during culture allows us to use biochemical analysis to better understand cellular behaviour, e.g. which metabolic pathways are active; are cells overstrained and activating the unfolded protein response pathway or even stressed to the point that apoptotic pathways are being activated. This level of understanding will be greatly beneficial in attempts to influence cellular behaviour in order to create optimal conditions for high production of good quality therapeutic proteins.

One possible way this could be done is by understanding the factors that lead to apoptosis and the particlar apoptotic pathways that are activated and then by exploring ways to prevent or counter-act the activation / progression of the apoptotic pathways. Similar could be done with the identification of proteins involved in stress response pathways like chaperones involved in the unfolded protein response pathway which could be used as early indicators of cellular stress.

Another possible approach could be by investigating cell cycles in order to characterise the cell populations undergoing various phases of the cell cycle throughout the culture. Previous research (Dutton et al., 2006) suggests that cultures which have fewer cells in the G0-G1 phase of the cell cycle early on (up to day 3) go on to have higher peak cell counts and higher titres than those with a higher % in G0-G1 at the same time points. This could be utilised to increase titres by holding cells in G0-G1 at the start of a run.

Another strong benefit of HCP profile characterisation by mass spectrometry is the identification of HCP species which are particularly problematic to remove from the final drug product and which are known to compromise patient safety by causing adverse immune reactions. Tools like CHOPPI (Bailey-Kellogg et al., 2014) can be used to estimate potential immunogenic effects of residual HCPs on patient safety. Being aware of such proteins facilitates attempts to prevent their production altogether by genetic engineering of the gene in question. Chiu et al. (2017) described such an approach - they created knock-out CHO cells without lipoprotein lipase, an HCP species that negatively affects product stability if it remains in the final drug product.

Most importantly, this research will establish a base understanding about the cells we are using in the biopharmaceutical industry which will be crucial as other technological advances mean we are likely to see significant changes in upstream and downstream processing, such as a switch from fed-batch to continuous perfusion biomanufacturing in upstream, and multi-column continuous chromatography or new resins in downstream, as well as generally more complex therapeutic protein structures as opposed to relatively well established monoclonal antibody structures.

7.6 Final word

What has started as a very open project to study the interface between upstream and downstream processing has quickly become a more specialised investigation. Upstream and downstream processing of biopharmaceuticals consists of many different steps and parameters as well as various important quality attributes that could all be explored in great detail to provide a better understanding of the full picture. The upstream and downstream process parameters and quality attributes that were assessed in this project do not form an exhaustive list by any means as there are many other parameters and attributes that might be of interest to a bioengineer, some of which have been mentioned in the conclusion of this thesis.

But the parameters and attributes that were examined here were of special interest to me and were relevant based on previous literature – literature about host cell proteins that demonstrated we still have a lack of understanding about them and which provided motivation to research HCPs further; but also literature about other quality attributes that highlighted the fact that other research groups were focusing on these other areas such as primary recovery (e.g. filtration) or product aggregation. Overall, the fact that host cell proteins affect not just the efficiency of the process but are also a major concern for patient safety, yet are still quite poorly understood, made them a focus of this thesis.

While there is still a lot to be learned about host cell proteins and what they can tell us about cells during mAb production, the discoveries made and presented in this thesis are very useful with regards to how upstream and downstream processing are linked. The holistic approach used during these studies should be adopted for future research. Since upstream process development includes the performance of several DoE studies for the identification of optimal operating conditions, these small-scale studies should be expanded to include a downstream processing perspective and determine the effects on product quality attributes. High-throughput small-scale unit operations and assays are available for such studies and statistical analysis of DoE data allows for the creation of feasible operating windows with overlaid upstream and downstream responses. Furthermore, implementation of improved mass spectrometry analysis can serve as an orthogonal assay to HCP-ELISAs which will increase confidence in quantification results as well as provide information about specific species that are present at certain conditions. Increased publications of HCP species identification data create useful reference points and can be used to compare mass spectrometry results with published literature to identify HCP species that pose a risk to patient safety.

In summary, the integration of upstream and downstream process development can provide significant insight into biopharmaceutical production, and the HCP species

identification presented in this thesis offers a new level of insight into HCPs that are retained during mAb purification which may be used to aide process development strategies.

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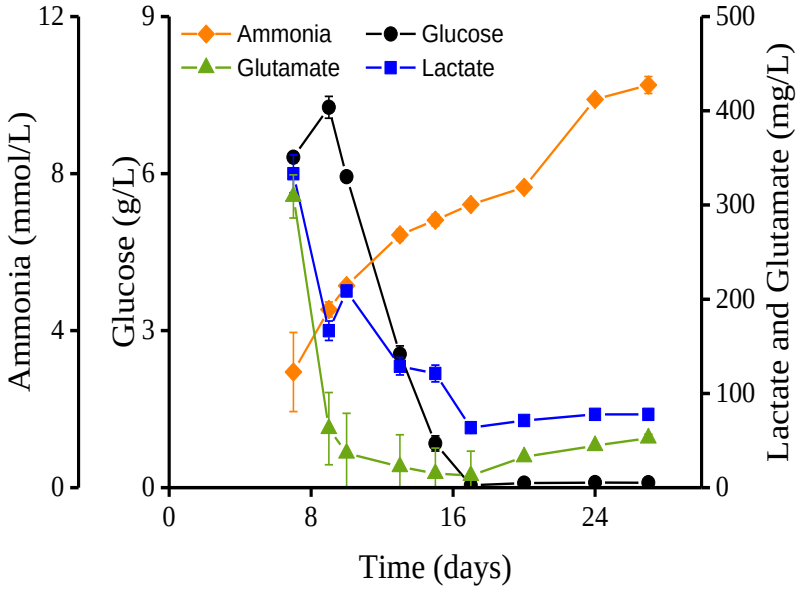
9 APPENDIX

Culture	Temp	pH	Seed density	Mode	Harvest day
1	+ 3	0	+ 5	Fed-Batch	14
2	- 3	0	- 5	Batch	12
3	- 3	+ 0.15	0	Fed-Batch	21
4	+ 3	- 0.15	0	Batch	12
5	+ 1	+ 0.15	- 5	Batch	17
6	+ 1	- 0.15	+ 5	Fed-Batch	14
7	- 3	+ 0.15	+ 5	Fed-Batch	18
8	+ 3	- 0.15	- 5	Batch	18
9	+ 3	+ 0.15	+ 5	Batch	10
10	- 3	- 0.15	- 5	Fed-Batch	14
11	+ 3	+ 0.15	- 5	Fed-Batch	17
12	- 3	- 0.15	+ 5	Batch	12
13	+ 1	0	0	Batch	12
14	+ 1	0	0	Batch	12
15	+ 1	0	0	Batch	12
16	+ 1	0	0	Fed-Batch	17
17	+ 1	0	0	Fed-Batch	17
18	+ 1	0	0	Fed-Batch	17
19	+ 3	0	- 6.3	Batch	18
20	+ 3	0	- 6.3	Batch	18

Table 14. Harvest days for each culture of the mAb 1 ambr250 DoE. 0 = middle point of each parameter range, numbers indicate relative values based on 0. Redacted seed density values represent ranges in units of 10^6 viable cells/mL. Cultures were harvested when less than 150 mg/L of antibody was produced over 2 days, although cultures that never reached this rate were harvested discretionally to gather data for comparison. Cultures 13-18 are centre point repeats and 19-20 are replicates of the operating conditions usually used for mAb 1 production.

(A)



(B)

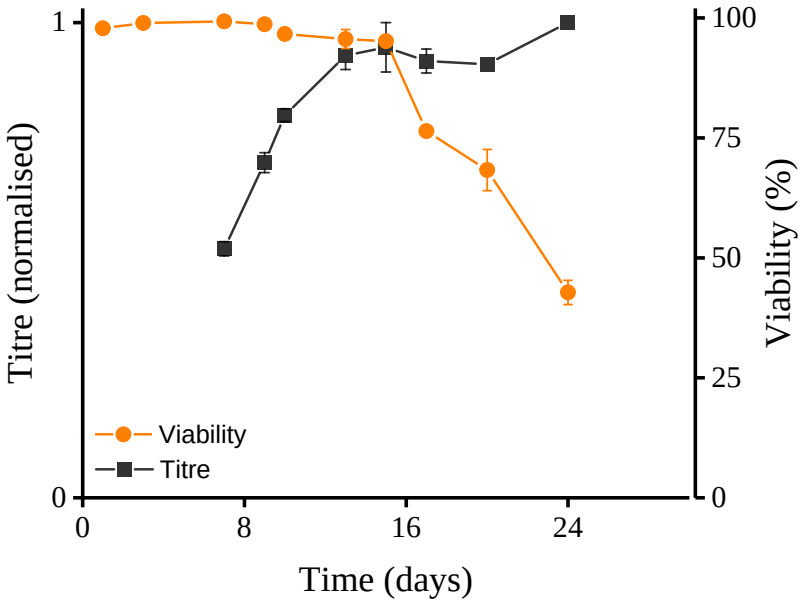


Figure 87. (A) Metabolite profiles as well as (B) culture viability and titre of all shake flasks used in shear study I were measured prior to pooling material together. Error bars are based on 29x replicate shake flask cultures. Results demonstrate that all shake flasks behaved similarly.

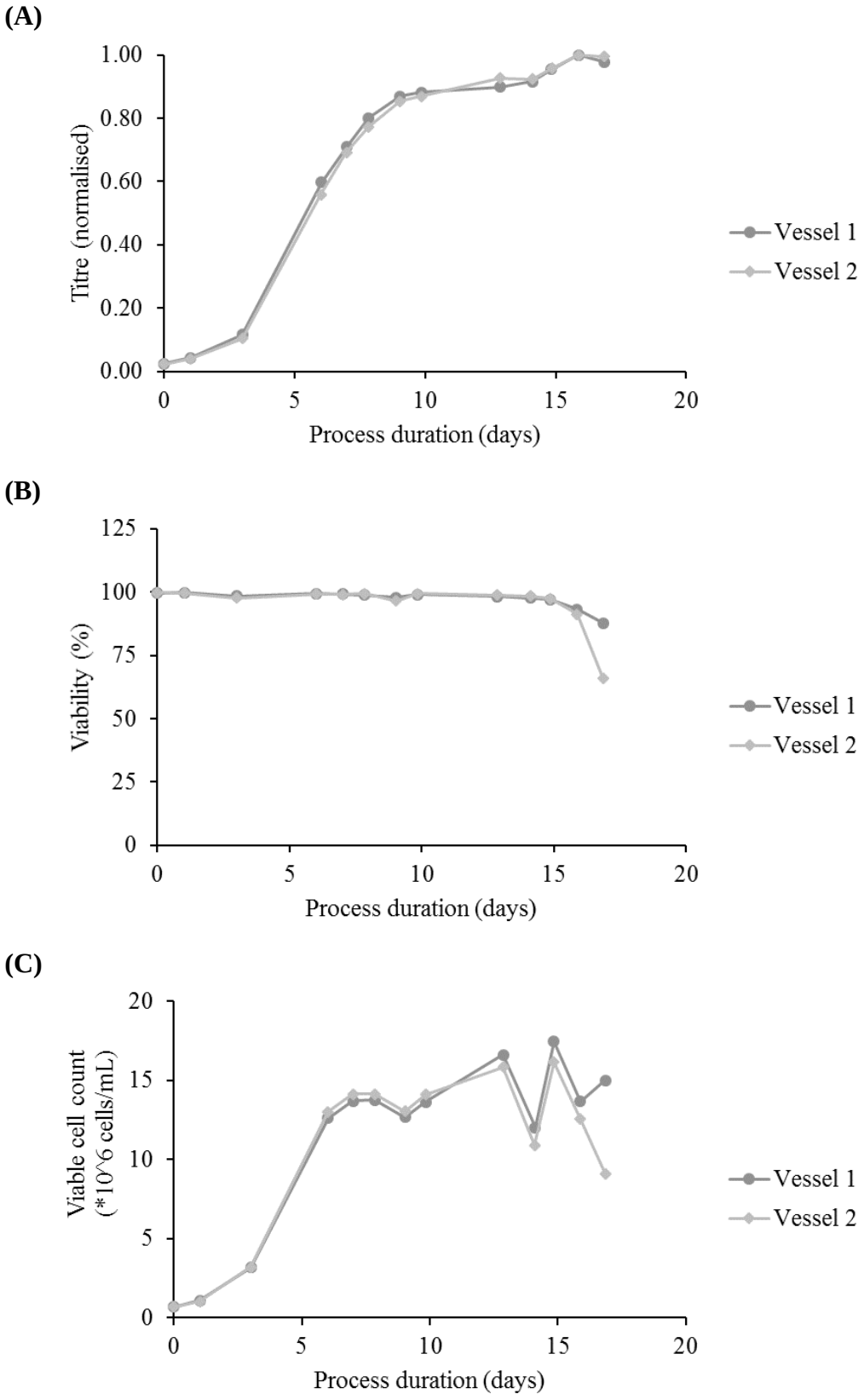


Figure 88. Shear study II bioreactor data. Note that the inconsistency in viable cell counts at day 13 is likely due to poor sampling technique as suggested by the rapid restoration of the viable cell count back to the expected range, which was most likely caused by insufficient flushing of the sample tube prior to sampling.

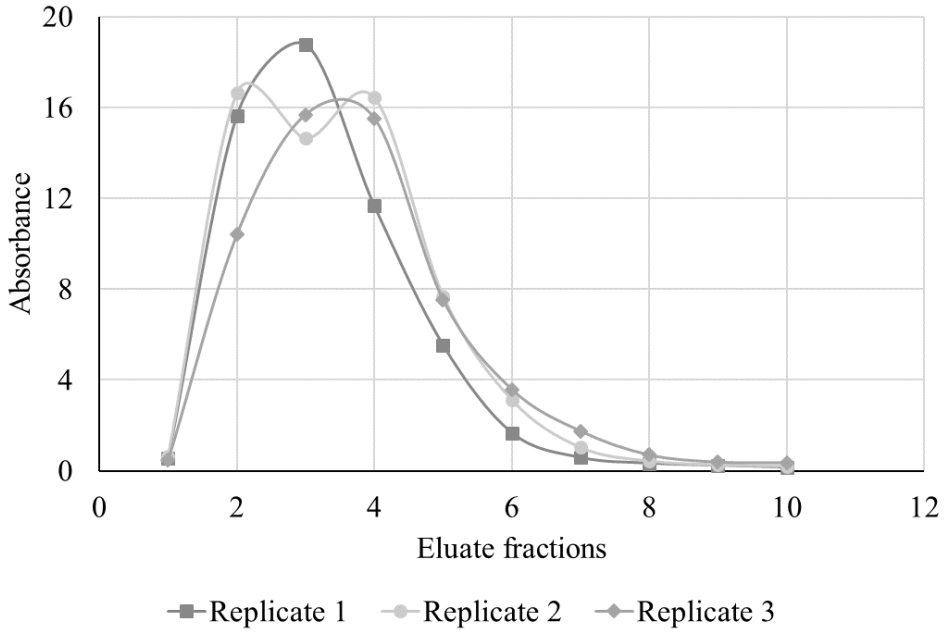


Figure 89. Pseudo-chromatogram of three replicate Tecan purifications (mAb 4).

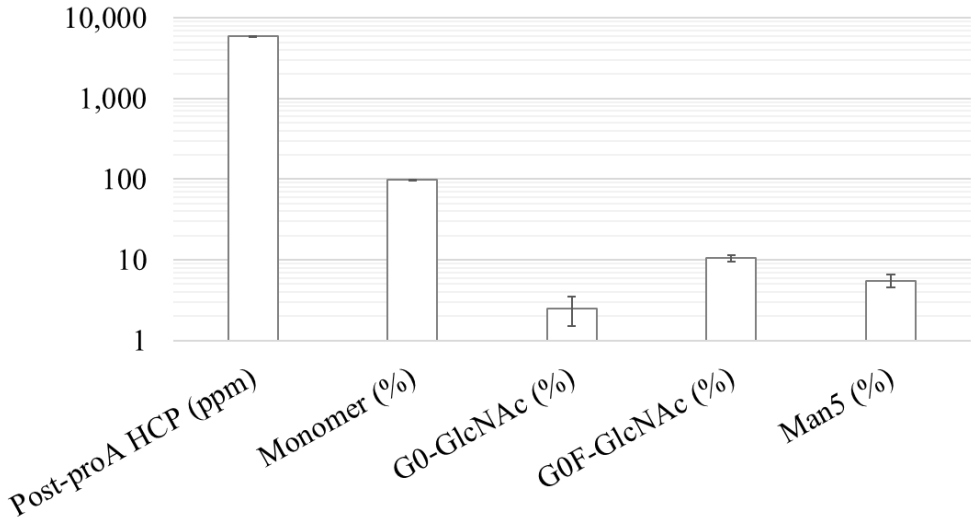


Figure 90. Product quality attribute results for three Tecan purification replicates (mAb 4).

	Average	STDV	% CV
Post-proA HCP (ppm)	5911.0	1312.6	22.2
Monomer (%)	97.4	0.1	0.1
G0-GlcNAc (%)	2.5	0.7	26.1
G0F-GlcNAc (%)	10.6	0.5	4.7
Man5 (%)	5.5	1.1	19.9

Table 15. Product quality data for three Tecan purification replicates (mAb 4). STDV = standard deviation; % CV = Coefficient of variance (%).