A Techno-Economic Framework for Assessing Manufacturing Process Changes in the Biopharmaceutical Industry

A thesis submitted to University College London

for the degree of

DOCTOR OF ENGINEERING (EngD)

by

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October 2009

Abstract

Industry pressures encourage and sometimes ‘force’ biopharmaceutical companies to implement process changes throughout a product’s lifecycle, so as to enhance yields, purity, robustness and cost-effectiveness. However, making a change involves technical, regulatory, and clinical risks. Possible changes to a product’s quality mean that all changes must be backed-up either with non-clinical bioequivalence studies or with lengthy and costly clinical trials and approved by regulatory authorities. These hurdles combined with the upfront costs can results in a tendency to avoid changes, whereas they may represent economic opportunity if evaluated holistically. This thesis explores the possibility of creating a systematic evaluation framework that captures the technical and regulatory activities involved in process changes to rapidly gauge the potential cost and risk implications.

Fundamentaldrivers and consequencesof making bioprocesses changes were benchmarked in a survey to help create the framework model. Key technical activities were captured, namely development, manufacturing, retrofitting and validation at all stages of development. Impacts of changes were linked to regulatory activities needed to assess comparability. Resulting uncertainties such as the likelihood of repeating clinical trials, market losses, delays to market from retrofit, revalidation, or regulatory approval disruptions, and the costs involved in proving product equivalence were captured. The framework was translated into Microsoft Excel with macros for Monte Carlo simulations to account for the uncertainties.

Minor and major change scenarios based on the purification of polyclonal IVIG by means of a blood-plasma fractionation process were used to demonstrate the usefulness of the proposed framework. The impact of ‘forced’ and optional changes were compared at different stages of development. Changes made during late-phase development resulted in market share losses and delays that outweighed any yield improvement modifications. The model predicted that it would be more profitable to make process modifications either during early phase development or post-product approval assuming stockpiling of approved product was feasible.

The feasibility of purifying a new product, alpha-1 antitrypsin (AAT) from a waste fraction, Fraction IV precipitate, was another process change scenario explored using scale-down studies. Experimental trials of the preliminary filtration and anion exchange purification steps were carried out, yielding low recoveries of AAT. Ciphergen®’s SELDI-TOF-MS ProteinChip technology was used to investigate the value of using a high throughput optimisation method to improve the isolation of AAT. Quantitative analysis of the protein samples using the Ciphergen® was compared to well-established protein concentration determination methods, eliminating variability in samples and differences in MS intensity by normalising the data.

The work in this thesis has demonstrated the usefulness of a combined business, technical and risk approach for evaluating the risks and benefits of implementing process changes.

Acknowledgements

There are a many people who have given me advice, guidance and support during my EngD years, at UCL, BPL and elsewhere.

I am particularly indebted to Suzy Farid, my supervisor and friend, for her patience, guidance, encouragementand resolutesupport throughout the “lifetime” of my EngD.

I am grateful to Mark Bulmer for his guidance, for teaching me all things “purification”and for going well out of his way to find accurate source data that I could use in my simulation work, as well as always supporting me during the time spent at Elstree.

I am thankful to Nigel Titchener-Hooker for his advice and for going out of his remit to give me the fullest opportunity to complete my doctorate.

I am extremely grateful to John More for all his advice during my time at BPL.

I am grateful to Bill Hornby for his guidance in preparing the benchmarking survey work.

I would like to thank Alex Berrill for hishelp and support in designing the SEDLI-TOF-MS experiments. I would like to extend that thanks to Sunil Chhatre also.

I am grateful to all who those worked in Lab 4 during my time at BPL, especially Tara Dolan for her clear and practical advice when it came to designing filtration and ion exchange experiments, to Martyn Paddock for his help onimmunoelectrophoresis analysis and to Vicky, Myf,Vatsala, Neal, Rob, and James for allowing me to use their bench/fridge space and to pick their brains when things were not running smoothly.

Many thanks to Ez for proof reading partsof this thesis for me.

I would like to thank the EPSRC and BPL for funding my research work.

Finally, and by no means least, I would like to express my sincerest gratitude to my family and friends for their genuine and unfaltering support always- especially my mother, father, Dena, Sal and Ez.

Contents

List of Tables

List of Figures

NOMENCLATURE & ABBREVIATIONS

ABBREVIATIONS	
A280	Absorbance at 280 nm
AAT	Alpha 1-antitrypsin
AUC	Area under the curve
BLA	Biological License Application
BCA	Bicinchoninic acid
BPL	Bio Products Laboratory
CBER	Centre for Biologics Evaluation and Research
CDER	Centre for Drug Evaluations and Research
cGMP	Current Good Manufacturing Practice
COG	Cost of Goods
CRP	C reactive protein
DI	Deionised water
DTT	Dithiothreitol
EAM	Energy absorbing molecule
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Evaluation Agency
FA+1	Fraction A+1 of the Cohn fractionation process
FDA	United States Food and Drug Administration
FIV	Fraction IV of the Cohn fractionation process
FV	Fraction V of the Cohn fractionation process
F B+1	Fraction B+1 of the Cohn fractionation process
GC globulin	Gc globulin/Vitamin D binding protein(DBP)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IND	Investigational New Drug
MBL	Mannose Binding Lectin
MHRA	Medicines and Healthcare products Regulatory Agency
NDA	New drug application
NPV	Net Present Value
OD	Optical density
R&D	Research and Development
S/D Treatment	Solvent/Detergent Treatment
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization mass spectrometry
TMAE	Trimethylaminoethyl
TRIS	Tris(hydroxymethyl)aminomethane
TCEP	Tris(2-carboxyethyl)phosphine
UCL	University College London

NOMENCLATURE

COG/g	Cost of goods per gram	$/g
f	Lang factor	-
FCI	Fixed capital investment	$

Chapter 1

Background and Thesis Scope

1.1 INTRODUCTION

The development and manufacture of biopharmaceutical drugs is a complex and heavily regulated process. At any stage of a drug's life cycle cost reduction, yield improvement, technological advancements, and regulatory requirements can lead companies to make regular changes to manufacturing processes. These changes can have a detrimental or indeed positive effect on product quality and safety, and so every change must be carefully monitored and approved by the appropriate regulatory authorities. This thesis explores the management of process changes in the biologics sector, looking at the different strategies used by companies to cope with change. The possibility of capturing all manufacturing and regulatory consequences of manufacturing process changes in a framework is proposed. Case studies based on blood-plasma fractionation are used to demonstrate the impact of such changes.

In this chapter, background to this study and the scope of the thesis is provided. Section 1.2 gives an overview of the biopharmaceutical industry, and the challenges it faces during development and manufacture. Section 1.3 provides background to the blood-plasma fractionation industry and the manufacture of plasma-derived products, of which the case study examples in this thesis are based. The economics of the plasma protein therapeutic industry is described in section 1.4. Section 1.5 gives a brief description of Bio Products Laboratory, UK. Methods for cost analysis and specific examples found in literature are discussed in section 1.6, and the incorporation of risk analysis is given section 1.7. Finally, the contributions, aims, and organisation of the thesis are given in section 1.8.

1.2 THE BIOPHARMACEUTICAL INDUSTRY

The biopharmaceutical sector is the fastest growing segment of the pharmaceutical industry, growing at an annual rate of around 15 percent(PharmaVision, 2009) and has a market size estimated to be worth in excess of $40 billion in 2009 (Carlson, 2009;Georg, 2005) compared to $12 billion in 2003 (Walsh, 2003) and some $33 billion in 2004 (Walsh, 2006).

By 2006, there were 165 approved biopharmaceutical products, these include recombinant proteins, monoclonal antibodies and nucleic acid–based (Walsh, 2006), and account for approximately 10 percent of the total expenditure for marketed drugs (PharmaVision, 2009).

1.3 CHALLENGES WITHIN THE BIOPHARMACEUTICAL INDUSTRY

The process of bringing these products to the market is costly and risky. Biopharmaceutical companies incur colossal research and development (R&D) costs in getting a therapeutic product to market, in developing the product and allowing for the numerous failed drug candidates. A survey of 10 pharmaceutical firms concluded that the average out-of-pocket costs per new drug to the point of marketing approval was on average US$ 802M (DiMasi *et al.*. 2003).Given the uncertainty associated with drug development, biopharmaceutical companies will typically juggle a pipeline of drugs to remain profitable (Rajapakse, 2005).

The significant revenue potential in successful biotherapuetic drugs weighs up against the enormous cost risks involved in development. Some of the challenges will be in attempting to shorten the development time to market, reduce production costs, maximise process robustness and potential, and improve product quality. These aims can be conflicting and often improving one can only happen at the expense of the others. Product development therefore will often focus on achieving whichever of these is considered to be of most importance; every company has a different strategy.

1.4 UNCERTAINTY DURING DEVELOPMENT

Changes are made to processes at all stages of a product's life cycle, during development and commercial stages. The need for changes to be made will decrease as a drug goes through development and processes are generally set at Phase III clinical development stage. The impact of implementing the changes, which can be measured by the costs accrued indirectly and directly and time delays, will increase as a drug is further developed and closer to filing for regulatory approval and can increase the risk of repeating clinical trials, clinical failure, and losses in market share. During Early phase clinical development there is the highest risk of clinical failure (Werner, 2004) and so companies may not be willing to spend money on making changes. At the end of Phase III clinical development there is the highest risk of losing market share; it is likely that there will be a lengthy delay to prove product equivalence. Post- product approval, there is a lower risk of losing market share, as the product is already in the market and

the changes can be made in parallel to processing the product. This is summarised in Figure 1.

Table 1.1 Impact of making process changes at different points in a drug's life cycle.

Stage of Drug Development	**IND**	**Phase I**	**Phase II**	**Phase III**	**Post-product Approval**
Risk of clinical failure*	(71%)	High (77%)	(47%)	Medium (45%)	Low (5%)

* Percentages give risk of clinical failure in the development of Monoclonal Antibody products. (Werner, 2004.)

1.5 REGULATORY AUTHORITIES

If the drug candidate successfully completes phase III clinical trials then the company can submit a new drug application (NDA) to the regulatory authorities. This will consist of all the data from pre-clinical and clinical trials along with details of the manufacturing process and proof that the process will consistently produce a pure, safe and reliable drug. The regulatory authorities will then review the application and if they are satisfied with the quality and quantity of data supplied the drug will be approved for sale. The harmonization of regulatory processes among the three main national regulatory agencies (the EU, the US and Japan) is making it easier for biopharmaceutical companies seeking to establish a global presence for their products (Walsh, 2006).

1.6 OPPORTUNITIES AND FUTURE PROSPECTS

Annual biopharmaceutical R&D expenditure is roughly $19–$20 billion with pipelines largely dominated by biotech-based. There are an estimated 2,500 biotech drugs in the discovery phase, 900 in preclinical trials and over 1,600 currently are in clinical trials (Walsh, 2006). Cancer indications are the most common targets for biopharmaceuticals development, whilst the most significant products are monoclonal antibodies (mAbs) and vaccines. Annual sales of approved biopharmaceuticals in were estimated at $33 billion. Sales values of therapeutic mAbs are expected to reach $ $33 Billion by

2012(Research and Markets Report, 2009). In total, the total biopharmaceutical market should approach or perhaps exceed $70 billion by the end of the decade.

1.7 COLD ETHANOL PRECIPITATION

In the 1940's a method for precipitating proteins from human plasma by varying the pH and adding ethanol was developed, this method became known as the Cohn fractionation process (Cohn, 1946). The method is used to purify a number of proteins from human plasma including albumin, immunoglobulins and the various prothrombin complexes. The method of cold ethanol precipitation is robust, well characterised, cost effective and has an excellent safety record hence it has been used more widely than any other technique . The original method has undergone many changes since its initial development but the main principles of the process remain the same and are still being used by plasma fractionators today.

Alcohol water mixtures tend to have a lower dielectric constant than water alone, which increases the force of molecular interactions. However it was demonstrated that the low temperatures of the Cohn process cause the dielectric constant of the medium to remain largely unchanged by alcohol addition and the real driving force to be the dehydration of proteins by alcohol (Reynolds, 2004). The dehydrated proteins then become strongly attracted to each other and begin to form agglomerates. Another parameter that has an influence on the solubility of the proteins is pH. At pH values above or below the isoelectric point the net charge of the protein moves away from zero making the protein more soluble.

The use of cold ethanol precipitation to fractionate blood plasma is based upon the varying solubilities of the different plasma proteins. By manipulating the five key variables; ethanol concentration, pH, temperature, ionic strength and protein concentration, selected proteins can be made to precipitate thus enabling their separation from the others by either filtration or centrifugation. The purity and yield of each precipitated protein is a function of all the variables above. The complex interactions involved and the interdependence of the variables makes mathematical modelling of the process problematic. For this reason the operating conditions of large scale fractionation processes tend to be carefully selected based on a combination of experimentation, knowledge and experience (Stryker, 1985).

1.8 COST DRIVERS-IVIG, ALBUMIN, OTHER PRODUCTS

The demand for purified Human Normal IgG (HNIG) from blood plasma has risen significantly and there is a current shortage as the demand exceeds supply (Lebing*et al.*, 2003). Some of the major indications are listed in Table 1.2.

The industrial scale production of human immunoglobulins has been taking place since the late 1940's. The product has an excellent track record in terms of safety and efficacy, which goes some way to explaining why the technology used in production has only begun to change significantly over the last two decades (More and Harvey, 1991). Several techniques such as chromatography have been successfully developed in producing high purity IgG from plasma (Lebing*et al.*, 2003; Li*et al*, 2002). A number of other methods have combined chromatography with the traditional method of cold ethanol precipitation. This approach combines the safety, and robustness of the traditional process with the high product purities achievable using chromatography.

Table 1.2 Some major uses of intravenous imunoglobulin (IVIG). *HyperimmuneIgGs are not included.*

Neurology	**Haematology**	**Immunology**	**Dermatology**	**Nephrology rheumatology, opthalmology and other**
GuillainBarre syndrome (RCT and CR)	Immune thrombocytopenia (RCT)	Primary antibody deficiencies (XLA, CVID, HIGM, WASand others)	Kawasaki syndrome (RCT)	Vasculitis (RCT)
Multifocal motor neuropathy (RCT)	Post bone marrow transplant (RCT)	Secondary antibody deficiencies (myeloma, CLL (RCT), drugsand other causes)	Dermatomyo sitis (RCT)	Sysytemic lupus erythematosis
Chronic inflammatory demyelinating polyneuropathy (RCT)	Myeloma and chronic lymphocytic leukaemia (RCT)		Toxic epidermal necrolysis	Streptococcal toxic shock syndrome
Dermatomyositis and inflammatory myopathies (RCT)	Parvovirus B19-associated aplasia		Blistering diseases*	Birdshot retinochoroidopathy
Myasthenia gravis (RCT)	Immune neutropenia		Immune urticaria	Autoimmune uveitis
Lambert–Eaton syndrome (RCT)	Immune haemolytic anaemia		Atopic dermatitis	Mucous membrane pemphigoid

1.9 THE VALUE OF USING SIMULATION TOOLS TO MODEL ASSESS PROCESS CHANGES

Simulation models are useful to predict and understand the impact of changes to a manufacturing system. They can be extremely useful as a means to 'analyze, communicate, and document' process changes (Harrison *et al.,* 2003). Modelling a bioprocess can provide a common reference and evaluation framework to facilitate process development. The impact of process changes can be readily evaluated and documented in a systematic way. Once a reliable model is available, it can be used to identify cost-sensitive areas of a process. These are usually capital intensive process steps or operating costs and also low yieldsor production throughputs (Petrides, 1994). Modelling results can then be used in conjunction with scaled down and pilot plant studies in order to optimize those portions of the process, leading to reduced developments times. Hollocks*et al.* (1995) also noted the benefits to manufacturing processes through simulation. These were found to be a reduction in operating costs, reduction in throughput time, faster implementation of plant changes reduction in capital costs, reduction in design-to-market time, reduction in risk and greater understanding of process. As production technologies for new drugs become progressively more complex, seamless process development is crucial to maintaining and improving manufacturing operations and in order to shorten timelines and reduce cost of goods (Byrom*et al.*, 2000).

Modelling drug development and manufacturing processes enables the interactions between the different bioprocess activities and the resource demands to be captured and quantified. Bioprocess modelling has previously been used to explore the cost-effectiveness of manufacturing options and to aid decision-making (Farid*et al..*, 2000; Lim *et al.,* 2005; Mustafa *et al.*,2005; Rajapakse*et al.* 2005; Biwer*et al.* , 2005). In these past investigations decision-support tools have been used to measure the cost of goods in biopharmaceutical manufacture and in some cases the quantify impact of manufacturing decisions on development timelines and costs. In addition, simulating the drug development process and a portfolio of drugs with their development activities has been explored. This provides management with the capacity to investigate several strategies and to use the insight gained to make real-life decisions that would add value in both the short and long term to the portfolio (Rajapakse, 2005).

Currently there are no tools available for implicitly assessing a change in a manufacturing bioprocess. However, Farid (2001) and Lim (2005) have both

demonstrated tools for simulating processes catered specifically for the bioprocess industry. Therefore, there is an increasing need for adequate modelling and simulation tools for the design and analysis of implementing changes to processes. In the present contribution, hierarchical modelling strategies have been used to simulate detailed manufacturing processes. Detailed activity based costing methods are used accounting for labour, cost of material and other ancillary task resources (Farid, 2000; Lim, 2005; Mustafa, 2005; Rajapakse, 2005).

A tool which takes into account all technical and business aspects involved in making changes is required to be able to assess the various strategy options for implementing process changes. Previous work by Lim (2005) has seen the start of the use of such work, where Quality Control and Quality Assurance based activity resources and costs were estimated and implemented into a manufacturing model. However, a much more accurate simulation of the activities involved is required. In this paper a hierarchical framework is proposed that accommodates all manufacturing, business and regulatory activities involved when implementing a change. The software approach detailed in this paper would be useful in a company for ensuring adequate linkage between process and business decisions. An example would be the choice of manufacturing route to adopt e.g. in-house versus contract manufacturing, and the implications of this on the key performance metrics.

1.10 BIO PRODUCTS LABORATORY (BPL)

Bio Products Laboratory, Herts, UK run a large-scale plasma fractionation scheme from which a wide range of therapeutic products are extracted. Plasma is sourced exclusively from the US Plasma Collection Centre and the finished products supplied competitively to the NHS. Where surplus of product exists it is supplied to selected overseas market. Case studies used to give examples of process change in this thesis are based on BPL's fractionation process.

1.11 PROCESS CHANGE MANAGEMENT IN THE BIOLOGICS INDUSTRY

There are many economic and regulatory safety drivers that lead to changes being made in the biopharmaceutical industry. Traditionally in the past, there was a reluctance to make process changesowing to regulatory hurdles and costs. But now increased pressures mean it is necessary to make changes to remain profitable.

1.11.1 CHALLENGES IN THE BIOPHARMACEUTICAL INDUSTRY

In the past, small differences in the production process of biologics have yielded different products, and can affect the safety and immunogenicity of the product (Wagner, 2005). However, the positive impact of making changes to process steps, especially with new emerging technologies (Tetzlaff, 2005), can be hugely significant, and so a series of tradeoffs must be evaluated when choosing which, if any process changes to implement. Results of a survey (summarised in Chapter 3) on the impact of changes made to processes made in the biopharmaceutical industry showed that nearly half of respondents believed that five or more changes are made to a single process in its lifetime; demonstrating its importance in this industry.

Increased pressures in the industry mean that it is necessary to make changes to remain profitable. There are two general approaches to manufacturing. Some companies, such as Eli Lilly adopt a more 'aggressive' form of development; by using more generic, well-established processes, which are more likely to be approved more quickly, rather than extensively refining the process at early stages of development. Other companies will make changes along the way, if they realise that a particular process step is not going to be economically feasible. An example of this is Protherics, UK, who were producing a septic shock drug, and during development removed a protein affinity chromatography step as they found the overall running costs over time would be in the scale of between $500M-$1 billion. The 'aggressive' approach companies tend to have an emphasis on speed of entry into market, thus, enabling the capture of a larger percentage of the market share. This can inevitably lead to processes that are not running at their optimum potential with low productivity levels and process inefficiencies. Therefore, to remain real competitors, these companies will eventually need to retrofit and modify their processes post product approval. The alternative is to prolong development until the process is well defined and optimised allowing for enough slack for process changes. These companies may make significant manufacturing changes from early to the later stages of clinical development, if they realise that a particular process step is not going to be economically feasible. However, these delays can cause the company to lose a considerable percentage of their market share to competitors, which can result in considerable loss in sales revenue. A day's delay in gaining regulatory approval and product availability could be worth approximately US$ 1 million (Clemento*et al.* , 1999). However, such delays in the time to market are more often due to the deficiencies in manufacturing rather than to the

scientific or clinical sections in the biotechnology industry (Fisher &Pascucci, 1996), and so implementing changes early on can be beneficial.

However, it is often updates in regulations that force companies to implement changes to their processes, as well as the need to run more efficient processes. The impact of making a change to a manufacturing process is not trivial; in the biologics sector it is often quoted that "the product is defined by its manufacturing process". Even small differences to a process can have a huge impact on product quality and product stability; however, this depends on the molecular complexity of the drug. These process change challenges have been summarised in Figure 1.1.

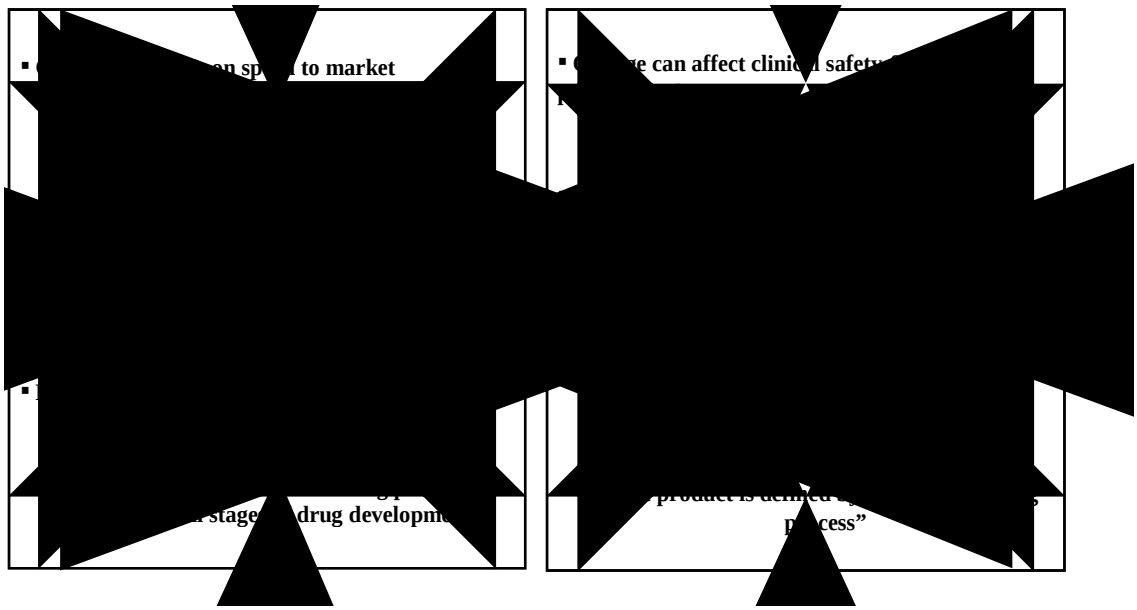


Figure 1.1 Challenges in the biopharmaceutical industry that lead to process changes

Some examples of manufacturing changes that have an impact on the process are summarised in Table 1.3; these may have a direct impact on the process description, or could be equipment related changes or based on analytical techniques utilised. Multiple changes of different magnitudes are normally made to a single process in its lifetime. All these challenges highlight the requirement for adequate software tools to aid the design and analysis of implementing changes to processes and to provide a systematic way of evaluating their economic impact.

Table 1.3 Examples of some of the more common types of biopharmaceutical process changes (Agalloco and Carleton, 2007)

Type of Change	Examples
Direct process changes	▪ A change in batch size or process parameter such as an increase in fermentation culture growth time, which would result in and increase cell proliferation. ▪ New or revised procedures ▪ A change in formulation chemistry ▪ A more moderate process change would be a change in cleaning procedure or cleaning agents.
Equipment related change	▪ A major equipment change, such as the scale-up of a fermentation process ▪ Converting a single-product facility into a multi-product facility ▪ The addition of new but comparable equipment, which has no effect on the process such as a new fermentation train, would be classed as a more moderate change.
Analytical based changes	▪ A change in control methods, such as the deletion of a method specification or an analytical method. The addition of new control methods which requires no deletion of current methods would be classed as a more moderate change. ▪ The extension of expiration dating, or a change in stability storage method. ▪ A change in site location of testing facilities is another example of moderate analytical change.

1.11.2 INDUSTRIAL EXAMPLES OF BIOPROCESS CHANGES

1.11.2.1 THE DEVELOPMENT OF AAT

The example of developing alpha1-antitrypsin (AAT, an alpha1-proteinase inhibitor) can be used to portray the complex issues arising from making changes to a plasma fractionation process.This protein is used to treat hereditary AAT-deficiency (hereditary emphysema), asthma, chronic bronchitis, cystic fibrosis, and neonatal respiratory distress syndrome (RDS) (Curling, 2002).

AAT is currently isolated from Cohn Fraction IV-1 using a process in which the starting fraction contains only 31% of the protein (Mattes *et al.*. 2001). In this method devised by Coan*et al.*(1985), the paste is dissolved, subjected to fractional precipitation with

PEG, DEAE Sepharose chromatography, diafiltration,and ultrafiltration to yield sterile filtered product, which contains 50% of the starting AAT(Coan *et al.*. 1985). The specific activity of the product is ≥ 0.35 mg of functional protein/mg protein with "small amounts of other plasma proteins". This process has been modified by Bayer using anion-exchange and cation-exchange steps, incorporating Solvent/Detergent (S/D) treatment, reactant removal in a third cation-exchange step, and terminal dry heat treatment. This method improves the yield to 64%–70%, and in some cases achieves a purity of 95% (Curling, 2002).

1.11.2.2 HUMANIZED MONOCLONAL ANTIBODY (SYNAGIS®)

Monoclonal antibodies (MAbs) are complex biomolecules composed of protein and carbohydrate moieties. During manufacturing changes, there are many opportunities for posttranslational modifications such as changes in carbohydrate structure or deamidation, which could introduce microheterogeneity(Schenerman *et al.*. 1999).

Synagis® is a humanized monoclonal antibody directed against the F protein on Respiratory Syncytial Virus (RSV). Schenerman*et al.* compared the product Synagis® manufactured following changes in scale and facility was evaluated using a broad range of product characterization methods. The Synagis® manufactured following the process changes was compared to the product used in pivotal clinical studies.

1.11.3 POTENTIAL FOR CHANGE IN BLOOD-PLASMA INDUSTRY

Plasma protein fractionation is the largest industry segment in global therapeutic protein manufacture. Currently in source plasma, (collected by plasmapheresis: the removal, treatment, and return of (components of) blood plasma through blood circulation) about 7g/L of IgG is available and processing yields are on average between 2.5 and 4.5 g/L. Optimising such process would improve yields by another 1g/L. However, if major changes are made to the process steps, using higher yielding unit operations then an increase in yield of 70% or more could be seen.(Curling, 2002)

Plasma is a unique source of multiple products, it contains about 60g/L of protein of which approximately 57 grams are used for different therapeutic products, all with a wide concentration range. Any change in the unit's operational sequence will affect all the products downstream of the change, and so it is typical that large-scale plasma fractionators leave the bulk of their processes unchanged.

1.11.4 CHANGE CONTROL

A formal change control system is normally established to evaluate all changes that may affect the production process. Written procedures are provided for the identification, documentation, appropriate review, and approval of changes in raw materials, specifications, analytical methods, facilities, support systems, equipment (including computer hardware), processing steps, labelling and packaging materials, and computer software. It is necessary that any proposals for GMP relevant changes are drafted, reviewed, and approved by the appropriate organisational units, and reviewed and approved by the quality control departments. The potential impact of proposed changes on the product quality is assessed using scale-down studies and lot-to-lot testing prior to changes being implemented.

1.11.5 PROCESS CHANGE CLASSIFICATION

A classification procedure may help in determining the level of testing, validation, and documentation needed to justify changes to a validated process. For example, the FDA classes changes as minor or major depending on the nature and extent of the changes, and the effects these changes may impart on the process. Scientific judgement determines what additional testing and validation studies are appropriate to justify a change in a validated process. When implementing approved changes, measures are taken to ensure that all documents affected by the changes are revised. After the change has been implemented, there is normally an evaluation of the first batches produced or tested under the change. The potential for critical changes to affect established retest or expiry dates is also evaluated. If necessary, samples of the intermediate or approved product is produced by the modified process is placed on an accelerated stability program and/or can be added to the stability monitoring program.

1.11.6 PRODUCT EQUIVALENCE STUDIES.

Demonstration of comparability is a sequential process, beginning with quality studies and supported, as necessary, by non-clinical, clinical and/or pharmacovigilance studies. If a manufacturer can provide evidence of comparability through physico-chemical and biological studies, then non-clinical or clinical studies with the post-change product are not warranted. In other cases, additional non-clinical and/or clinical data will be required. The need, extent and nature of non-clinical and clinical comparability studies can be determined on a case-by-case basis in consideration of various factors that may be associated with risk. The EMEA have summarised these factors concisely in their ‘Guideline on Comparability of Biotechnology-Derived Medicinal Products After a

Change in the Manufacturing Process: Clinical and Non Clinical Issues'(European Medicines Agency (EMEA), 2007) as follows:

• The process complexity, the nature of the change, the potential impact on the molecule structure and on the final product profile. The nature and extent of differences demonstrated by the physico-chemical and quality related biological characterisation, including product-related substances, impurity profile, stability and excipients. Thus, well-characterised differences may provide a background for a rational and focused approach with respect to the need for non-clinical and clinical studies.

• Product complexity, including heterogeneity and higher order structure and the availability, capabilities and limitations of analytical tests. If the analytical procedures used are not sufficient to discern relevant differences that can impact the safety and efficacy of the product, additional non-clinical and/or confirmatory clinical testing may be necessary.

• Structure-activity relationship and strength of the association of quality attributes with safety and efficacy;

• Relationship between the therapeutic protein and endogenous proteins and the severity of (potential) consequences for immunogenicity; e.g. risk of autoimmunity

• Mode of action: unknown or multiple modes of action complicate the evaluation of the impact of changes

• Therapeutic indications/target patient groups - The impact of possible differences can vary between the target populations covered by the different indications.

• Posology, e.g., dosing regimen and route of administration, for instance, repeated administration via the subcutaneous route is more likely to be associated with immunogenicity than intravenous administration of a single dose

• The therapeutic window/dose-response curve

• Previous experience, e.g., immunogenicity, safety. Experience with the pre-change product or with other products in the same class can be relevant. However, biotechnology-derived proteins should be considered individually.

For products in development, all these points above should be taken into consideration. However, the extent of the comparability studies will likely increase if manufacturing changes are introduced at the later stages of clinical development. A change after conduct of confirmatory efficacy and safety studies represents the most challenging

situation. The selection of non-clinical and clinical studies is product-driven, i.e. a strategy for comparability testing should be chosen that best predicts and detects clinically relevant differences with sufficient accuracy.

1.12 CONCLUSIONS

For companies to remain economical and to uphold to the stringent regulatory system they must continuously improve processes by maximising yield potential, whilst minimising resources, utility usage and operating costs throughout product development, post-approval and marketing (Chirino and Mire-Sluis, 2004).

All these requirements and pressures drive companies in the industry to consider making changes to manufacturing processes throughout all stages of a drug's life cycle (Werner, 2004). At earlier stages of drug development, there is an emphasis on speed of product entry to market. Many companies will opt for the more 'aggressive' approach to development by emphasising their efforts in rushing to market with a more or less generic process (Sofer and Hagel, 1997). This will help companies capture a larger share of their target market, nevertheless, in many cases this will lead to alterations being made to improve the process at later stages of development, including post-product approval. Bringing a therapeutic to market six months early or six months late can lead to a one-third increase or decrease, respectively, in its lifetime profitability(Forgione and Van Trier, 2006).

1.13 CONTRIBUTIONS, AIMS AND ORGANISATION OF THESIS

The aim of this thesis is to explore the consequences of making manufacturing changes to processes within the biologics sector. This includes the description of a framework to encapsulate possible technical, financial, and regulatory outcomes of such changes. This allows for informed decision-making, and strategic planning when managing alternative change scenarios. To demonstrate the usefulness of this method, examples of possible financial, regulatory and experimental outcomes have been provided.

In Chapter 2, a survey portraying the results of a survey designed to benchmark the key drivers and implications of making changes to bioprocesses in biotechnology companies is provided. More specifically statistics are presented on the most common types of manufacturing changes made, the most important reasons companies cite for making these changes, their frequency and timing in a product's lifecycle and the typical delays and costs incurred to demonstrate product equivalence and satisfy regulatory authorities.

In Chapter 3 a framework to help gauge the technical, regulatory and financial activities involved in making such bioprocess changes and their implications is suggested. The framework set-up and methods used to calculate the cost of a process change activity is provided. The inputs required are described along with the outcomes used to measure the change. Data collected throughout the study together with general assumptions required to model the implications of a change is also presented

A case study utilising the framework to investigate the consequence of making process changes, whether these are forced or are made to enhance productivity is explored in Chapter 4. The study looks at the impact of making process changes of varying magnitude and type to an IVIG fractionation stream at different stages of product development including post-product approval.

In Chapter 5, another process change scenario investigates the purifying a new product from a current waste fraction. The potential in purifying AAT from Fraction IV (FIV) precipitate at BPL is explored. The method trialled is based on a process designed by Kee*et al.*, 2004. Laboratory scale experiments are used to assess whether there is sufficient AAT in FIV paste and whether the purification process suggested by Kee*et al.* can be applied to BPL's fractionation process. The experiments involved mimicking the first two isolation steps in the process.

In Chapter 6 a high throughput optimisation method using Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology is used to improve the low recovery of AAT obtained with anion exchange chromatography, as step highlighted in Chapter 5. The usefulness of analysing samples using Ciphergen® non-selective (NP20) chips is also compared to well-established concentration determination methods, such as ELISA methods.

Chapter 2

Process Changes: Benchmarking Industry Drivers

2.1 INTRODUCTION

The biopharmaceutical industry faces mounting competitive pressure to reduce costs of manufacturing whilst increasing speed to market (Kleyn and Kitney, 2007;Rajapakse *et al.*. 2004). Product competition, governing regulatory bodies and customer requirements dictate a need for companies to strive for enhanced product purity and process robustness (Narhi and Nordstrom, 2005). The manufacture of biological products is a complex process. For companies to remain economical and uphold to the stringent regulatory system they must regularly improve processes by maximising yield potential, whilst minimising resources, utility usage and operating costs throughout product development, post-approval and marketing (Chirino and Mire-Sluis, 2004;Lim *et al.*. 2006;Mustafa, 2006;Werner, 2004).

All these requirements and pressures drive companies in the industry to consider making changes to manufacturing processes throughout all stages of a drug's life-cycle (Werner, 2004). At earlier stages of drug development, there is an emphasis on speed of product entry to market. Many companies will opt for a more "aggressive" approach to development. In a rush to get a product to market before their competitors, process optimization can be sacrificed for speed (Sofer and Hagel, 1997). Given that bringing a therapeutic to market six months early can lead to a one-third increase in its lifetime profitability and in the past it has even been quoted that getting a new product to market as little as one month early was typically worth more to an organization than the same product's entire research and development costthere is a real incentive in "aggressive" development (Forgione and Van Trier, 2006). However, a hastily assembled process may pose scale-up challenges or not be cost-effective at a commercial scale. Some companies prefer to take this approach, and then later redesign the process post-approval. Others design robustness and validation into the process at very early stages of development using established guidelines(Sofer and Hagel, 1997).

The impact of making a change to a manufacturing process is not trivial; in the biologics sector it is often quoted that "the product is defined by its manufacturing process"(Wagner, 2005). Even small differences to a process can have a huge impact on product quality and product stability (Wagner, 2005) and so companies require

regulatory approval to continue with clinical trials or commercial production (English, 2007). Although such changes are often intended to benefit the patient, it is essential that any change in the manufacturing process does not adversely affect the safety or efficacy of the product (Chirino and Mire-Sluis, 2004).

In the past, small differences in the production process of biologics have yielded different products, and can affect the safety and immunogenicity of the product (Wagner, 2005). However, the positive impact of making changes to process steps, especially with new emerging technologies (Tetzlaff, 2005), can be hugely significant, and so a series of tradeoffs must be evaluated when choosing which, if any, process changes to implement.

In section 2.2 a description of the survey methodology is given. In section 2.3 the survey results look at the reasons for making process changes, the types of changes, how frequently they are made, the timing and implications of such changes, why changes are not made, and if it is easier to make changes now or not.

2.2 SURVEY METHODOLOGY

A web survey was set-up so as to gain an insight into why changes are made to manufacturing processes, the types of changes made, the frequency and timing of process changes, the cost and time delay implications of changes made and reasons why companies do not carry out proposed changes. It consisted of 13 multiple-choice, Percentage breakdown, rating and fill-in type questions. The survey took approximately 10- 15 minutes to complete. The survey was emailed to over 500 employees of biopharmaceutical and biotechnology companies worldwide including subscribers of the journal Bioprocess International in April 2006. 81 responses were received. The profile of respondents is indicated in Figure 2.8.

2.3 RESULTS

2.3.1 WHY ARE PROCESS CHANGES MADE?

Firstly, the principal motivations that drive biopharmaceutical companies to make manufacturing changes were determined. Respondents were asked to choose the five most common reasons for implementing a manufacturing change out of a choice of ten (Figure 2.1). Industry responses suggest that the most likely reason for making process

changes is to reduce batch-to-batch variability and hence achieve a more robust and stable process. The remaining top four motives are: to increase yield or purity; to manage a change in raw material supply; to reduce costs and to replace outdated equipment or equipment with insufficient capacity.

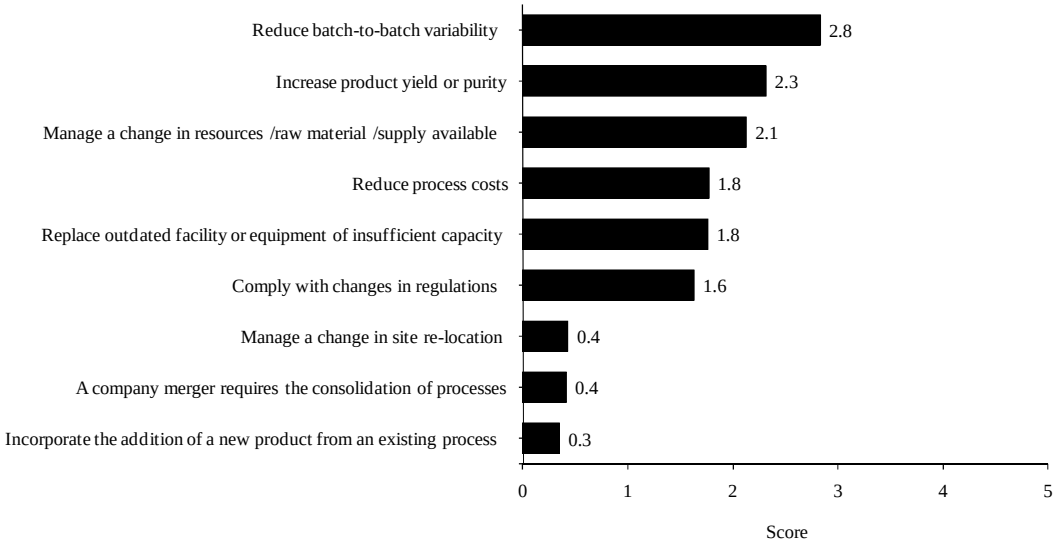


Figure 2.1 The key reasons for implementing manufacturing process changes in the biopharmaceutical industry ranked in order of their average scores. The maximum score of 5 indicates the most likely reason for making a change and 1 indicates the least likely reason.

Reducing batch-to-batch variability is key to satisfying the stringent regulatory requirements where the process must operate within validated limits and meet predefined acceptance criteria. It is common for the process to be refined as clinical development proceeds so as to increase process robustness as the product gets closer to market (Chan& Jensen, 2004). These are usually minor changes and hence tend to only require comparability assays to prove equivalence.

Making changes so as to increase product yield or purity also scored highly since process improvements process improvements can make a significant contribution to lowering the cost of goods, while saving investments and freeing up capacity for new business. Such process improvements can be realized by ongoing improvements with no major regulatory impact or by step-wise and significant enhancements requiring regulatory measures. Werner (2004) provides an example of the financial impact of yield improvements; a10-fold increase in fermentation titre coupled with a 30% increase in yield resulted in a 6-fold reduction in the annual cost of goods.

For example, process optimisation and intensification in the traditional blood plasma fractionation industry have the potential to increase yields of IgG by 1g/L, a 14% improvement in yield (Curling, 2002). On the other hand, major changes such as replacing precipitation units with high yielding chromatography steps offer the possibility of yields reaching values of 70% or more (typical precipitation yields are on average 46%) (Curling, 2002)at the expense of increased equivalent and efficacy studies.

Yield or purity enhancement changes will generally occur during the development stage and it is less likely to be implemented post-approval, as it is likely to have a significant effect on the product stability. If the product stability or efficacy is affected, then a company will need re-approval from the regulatory agencies, and in some cases may need to repeat clinical trials.

The need to switch to alternative raw material or utility supplies also forces companies to make changes. This can arise from the availability of a cheaper or more reliable supplier, the vendor discontinuing their line of supply in favour of organisations or countries that are more profitable, or the vendor going out of business. The other key reason given was to replace an outdated facility or equipment of insufficient capacity. Companies looking to expand their market size may want to use larger equipment or switch to newer units of operation that offer greater efficiency. While this type of process change may reduce overall costs in the long run, the disruptions during implementation of the change and immediate costs from resulting regulatory activities, may radically increase short-term costs. Hence, decreasing the cost of implementing the process change will inevitably be a fundamental issue when choosing alternative process options for making the change.

2.3.2 HOW FREQUENTLY ARE PROCESS CHANGES MADE?

The number of changes made to processes can influence the process scheduling patterns, the manufacturing costs, and can cause major delays in the development of a product. Respondents were asked to indicate the average number of changes that are made to a single process throughout a product's life span (Figure 2.2). For companies with multi-product facilities, the number of products in their portfolio that were subject to change was also determined (Figure 2.2). Across the whole sample, nearly half the respondents (45%) estimated that more than five changes are made to a single process throughout a drug's lifecycle. The bulk of the remaining respondents (36%) believed that on average 2 to 5 changes are made to a single process. This implies that 81 % of

respondents believe that on average 2 or more changes are made to every process throughout development. For companies with multi-product facilities this can initiate substantial costs and may result in scheduling problems. Only 7% of respondents stated that no changes were made.

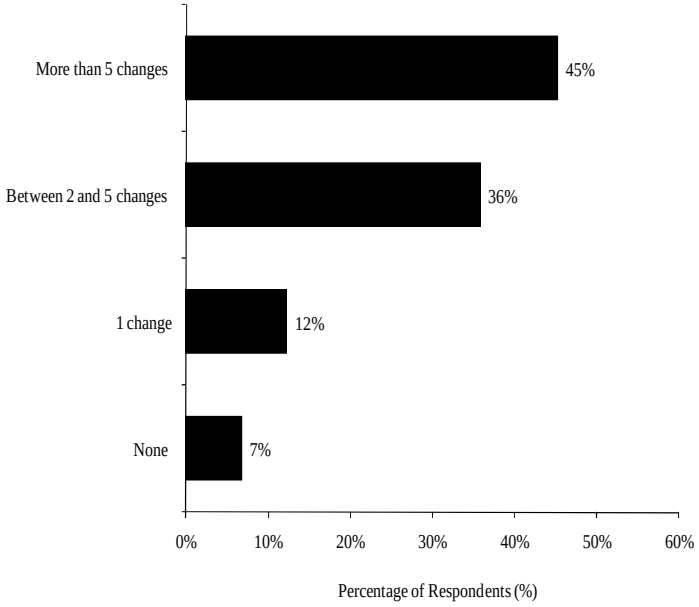


Figure 2.2 Percentage distribution of industry responses by the number of changes that are typically made to a single process in a drug's life cycle.

This is important in not only assessing the costs involved in making the change to a process, but also the types of studies that will be involved in assessing product

Table 2.1 lists the percentage of the products in a portfolio of drugs that have changes made to them as estimated by respondents. Nearly two-thirds of the respondents (65%) estimated that more than 50% of the drug products in their company's portfolio had changes made to them. This clearly shows that process changes are inevitable in bioprocess manufacturing.

2.3.3 TYPES OF CHANGES MADE TO BIOPROCESSES

In this section, the nature of the changes most frequently made, for example an addition or removal of a unit operation are explored. The magnitude of the changes made, specifically the Percentage of the changes that respondents considered to be 'major' or 'minor' was also established.

This is important in not only assessing the costs involved in making the change to a process, but also the types of studies that will be involved in assessing product

Table 2.1 Percentage distribution of industry responses by proportion of portfolio products experiencing process changes.

Percentage portfolio products with process	**% Respondents**
More than 50%	67
Between 10% and 50%	17
Less than 10%	15
0%	1
Total	100
Number of respondents	81

equivalence. Regulatory agencies such as the US FDA, categorise manufacturing process changes into three categories: major, moderate, and minor.

A major change is one that has substantial potential to have an adverse effect on the identity, strength, quality, purity, or potency of a product as they may relate to the safety or effectiveness of the product. A major change requires the submission of a supplement and approval by FDA prior to distribution of the product made using the change. This could be a change in operating step for example, a change from filtration to centrifugation in recovery. A minor change is one that is considered to have minimal potential to have an adverse effect on the identity product characteristics e.g. changes to equipment of the same design and operating principle or changes in scale.(FDA Guidance Document, 1998).

Respondents were asked to estimate the Percentage of changes that involved replacing a unit operation, substituting two or more steps with a single new step e.g. the use of expanded-bed adsorption, the addition of one or more new steps (with no elimination involved) or an elimination of one or more steps (involving no addition of new steps). The industry responses were averaged for each type of change and the results are summarised in Table 2.2.

On average, respondents estimated that nearly 30% of changes that occur involve the replacement of a manufacturing step from a process. This often includes the scaling up of equipment, which should have minimal impact on the final product as noted by the respondents.

Table 2.2 Percentage distribution of the different types of change.

Type of change	**% Breakdown**
Replacing 1 step with a new step	29
Elimination of steps (with no addition of new steps)	17
Addition of new steps (with no elimination)	16
Replacing 2 or more steps with a new single step e.g. use of EBA	16
Other	23
Total	100
Number of respondents	81

The addition of new steps (16%) and the elimination of steps (17%) occur less frequently according to respondents. A large proportion of respondents (23%) cited other reasons for introducing changes to a process. Most respondents noted that the most frequent change made to processes were modifications to existing process parameters to yield better purities and efficiency, as other types of changes are more complicated to manage from a regulatory perspective. Contract manufacturers will make changes regularly to transfer to a new customer process. Many of the respondents noted that they were forced to replace process steps to improve process safety or due to changes in regulations. An example of this is the removal of animal-derived raw materials from processes. Changes in formulation and a change of expression system to provide higher expression levels were also prominent.

Generally, the respondents believed that only one-third of the changes made to a single process in its lifecycle are “major” changes. Many respondents stated that “major” changes were avoided where possible. New steps are added to increase product purity in a scenario where perhaps a clinical adverse reaction has occurred in patients and there is no other choice, otherwise, the risk is not taken as highlighted in Table 2.3. One respondent highlighted that in their company minor changes that are made post-approval are typically made for cleaning steps (non-product contact) to improve yields (e.g. affinity column cleaning) that requires minimal regulatory approval.

Table 2.3 Percentage distribution of process changes by their magnitude.

Magnitude of change	**% Breakdown**
Minor	67
Major	33
Total	100
Number of respondents	81

2.3.4 TIMING OF MAJOR PROCESS CHANGES

The regulatory consequences of manufacturing changes will depend not only upon their nature but also on the stage of clinical development. The stages of a drug's life-span where "major" changes are most frequently made was established. The results in Figure 2.3 show that changes that are more significant are made at the earlier stages of development. More than half (59%) of all changes are made at the preclinical stage or at early phase product development. At this stage, the process may not be fully defined and so changes are inevitable; also, the changes made here are prior to large clinical trials and so it will not be costly to repeat any regulatory activities required or a repeat may not be necessary to at all. On average 30% of changes are made during the later clinical phases and 21% post-market approval. Typically, changes made during pivotal studies or just prior to submission of a marketing application require more data to support product comparability than manufacturing changes made during earlier phases of clinical development. It may be riskier to implement a "major" change during the later stages of development than post-product approval, because any necessary regulatory activities cause a delay to market. Once the product has gone commercial, the manufacturers can generate their product in parallel to introducing changes.

2.3.5 IMPLICATIONS OF MAKING PROCESS CHANGES

Major changes, such as modifications in the expression system or in a process sequence of a given product can influence the levels of both product-related substances (e.g. those derived from anticipated post-translational modification, with properties comparable to the desired product) and impurities related to the product (molecular variants of the desired product that do not share the comparable efficacy and safety)(Weinberg, 2005). Therefore, once a change has been implemented, regulatory authorities demand that proof of comparability or dedicated clinical comparative efficacy and safety studies are carried out. Changes in production methods of a biological product may necessitate an

assessment of comparability to ensure that these manufacturing changes have not affected the safety, identity, purity, or efficacy of the product. This assessment typically

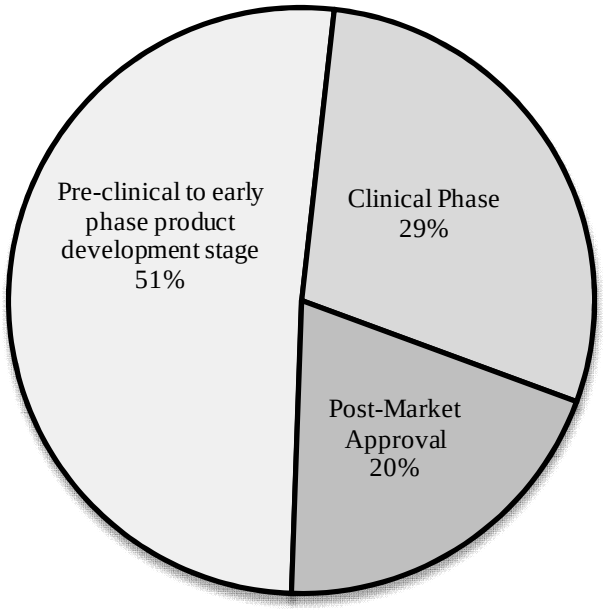


Figure 2.3 Timing of "major" process changes that occur throughout a drug's lifecycle. The values represent an average of the percentage breakdowns provided by respondents.

consists of a hierarchy of sequential tests in analytical testing, preclinical animal studies and clinical studies (Chirino and Mire-Sluis, 2004). Comparability studies require that physicochemical properties, biological activity, and immunochemical properties are highly similar for pre-change and post-change products, and that where physicochemical differences are detected, these changes have no adverse impact upon the safety or efficacy of the product (Weinberg, 2005).

The implications of making process changes can be established by measuring accrued cost, time delay and the severity of the regulatory activities involved. In this section, the respondents were questioned on the types of studies that they utilised to assess product equivalence at the later stages of development and post-market approval and the length of time taken to complete them. This is illustrated in Figure 2.4 where the regulatory studies involved are compared for phase III of process development and post-market approval. Industry responses suggest that most changes made during phase III process development or post-approval only require bioequivalence or comparability studies (37% and 47% respectively) rather than a repeat of phase I (12%, 13%) or full-scale

trials (13%, 18%). This is probably a reflection of the industry's reluctance to make major changes at late stage of development as highlighted earlier in Figure 2.3. Respondents estimated that on average 24% of changes during phase III development demanded re-validation of process and equipment, in contrast to 10% post-approval. This correlates with comments from many of the respondents, who stated that the majority of changes made post-approval were to modify existing equipment, thus reducing the extent of re-validation. At the phase III development stage, all assays and methods should have already been validated and should not require major process changes.

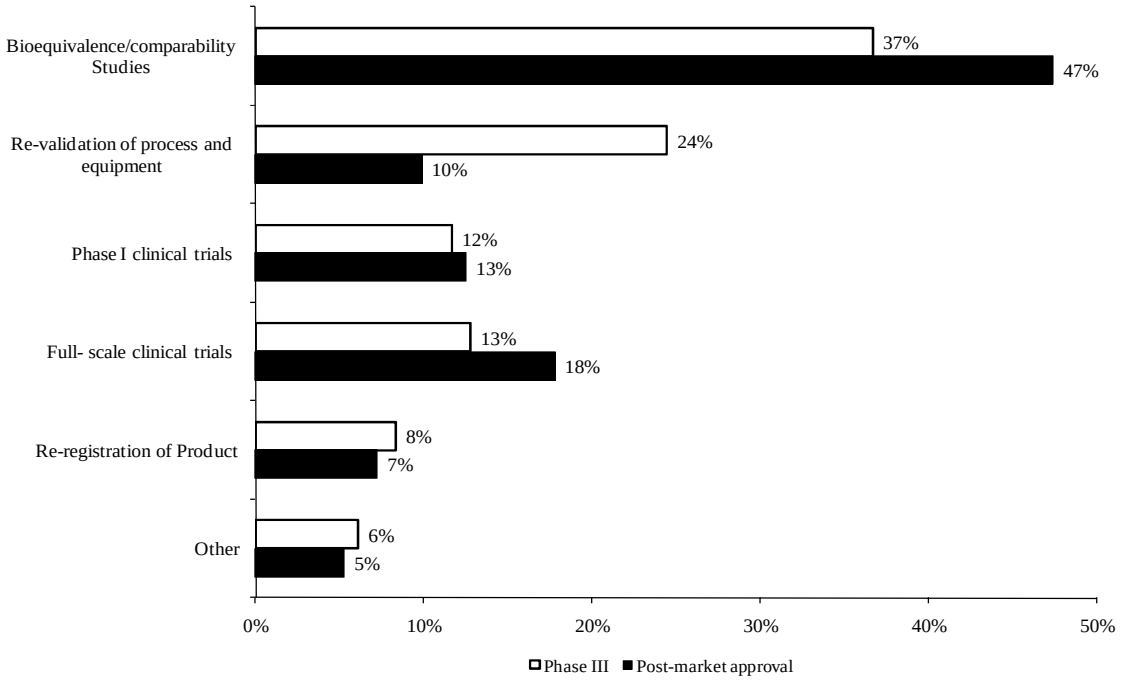


Figure 2.4 The proportion of regulatory activities involved in assessing product equivalence for process changes made during phase III of process development (black bars) and post-market approval (white bars).

Based on the responses, the most likely durations of each stage are summarised in Figure 2.5. Bioequivalence studies typically take up to 3 months and 3-12 months. In general, the analytical studies involved in these activities can be performed in-house or they can be outsourced and completed parallel to the production of lots for comparability testing, avoiding lengthy delays. At the other end of the spectrum, a repeat of full scale clinical trials can cause lengthy delays of over 18 months, according to 68% of respondents, while a repeat of phase I clinical trials or re-registration of product are typically quoted as requiring 12-18 months. The typical costs that are amassed when demonstrating product equivalence was also analysed.

Figure 2.6 shows the Percentage breakdown of the typical costs that are accumulated when process changes require only bioequivalence studies and with those that need a repeat of clinical trials. Over 70% of respondents estimated that bioequivalence studies incurred costs under $500,000, and over 64% of respondents believed that the cost of also repeating clinical trials was greater than $2M. To determine a more accurate figure, a subgroup of the respondents (57%) were asked to choose from a range of costs in these two categories. The subgroup estimated that bioequivalence studies typically cost

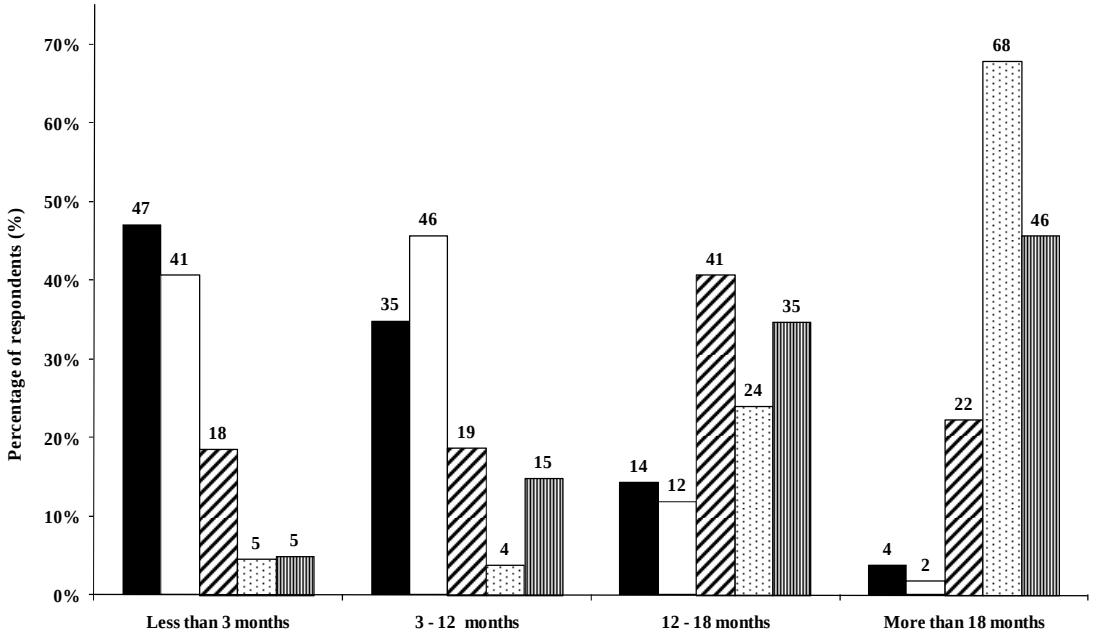


Figure 2.5 Thepercentage distribution of industry responses of typical durations of a) bioequivalence studies (■), b) revalidation of process and equipment (□), c) a repeat of clinical trials (▨), d) a repeat of full-scale clinical trials (▩), e) product re-registration (▥).

below $250,000 (70%) and a repeat of clinical trials typically cost $2M-$15M (38%). Comparability studies have made it much easier to prove product equivalence, but only covers changes that do not affect the product stability. A summary of these implications is listed. In Table 2.4.

The top three factors influencing the likelihood of repeating clinical trials are the nature and hence extent (44%) followed by regulatory requirements (25%) and the phase of development (17%). Several respondents claimed that they would not implement any changes that would require repeated clinical studies post-product approval, which may explain why many respondents ranked phase of development last. This can explain the

relatively low percentage estimation of stage of product development (17%) as a factor in determining the re-use of clinical trials.

The scheduling strategy used to retrofit a process is essential in determining any delays that may occur. The respondents were asked to choose the average length of shutdown periods, when post-approval changes are required, as well as the methods utilised to keep a continuity of supply. Table 2.5 lists the respondents estimation of the length of time a facility is usually shutdown to accommodate the changes made and to revalidate the process, specifically for post-approval changes.

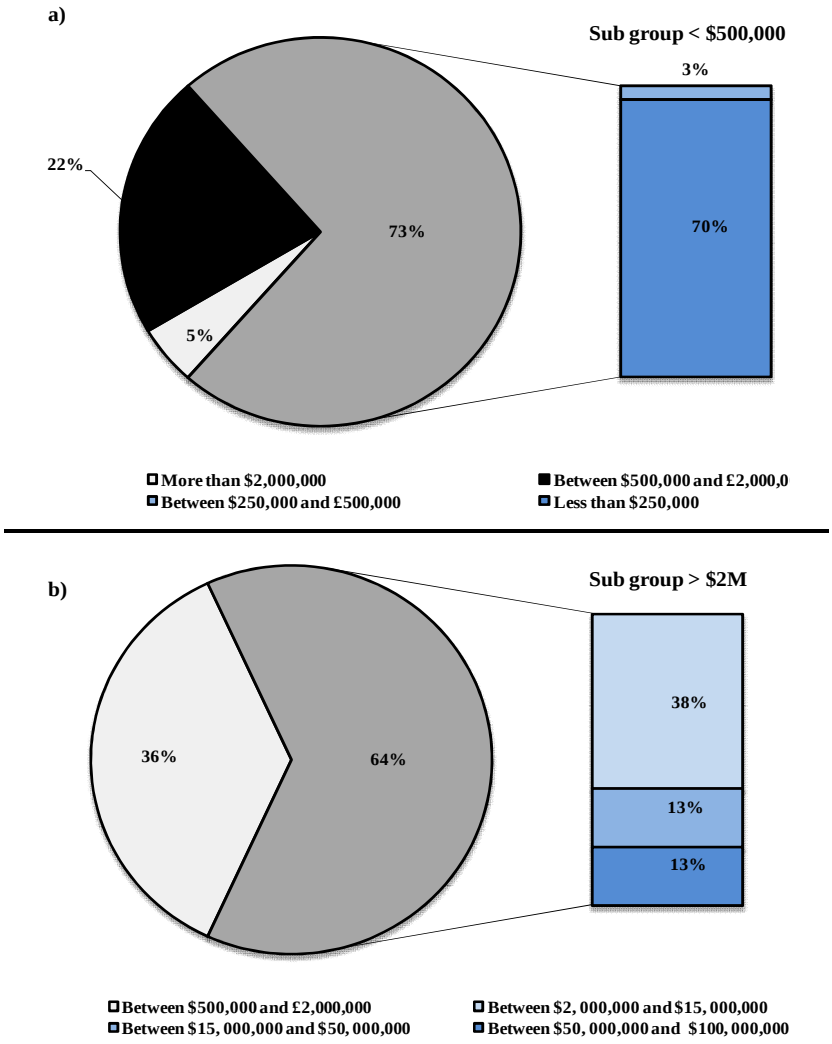


Figure 2.6 Percentage distribution of industry responses by typical costs that incur when process changes require a) only bioequivalence studies or b) also a repeat of clinical trials. The pie chart breakdown represents the total sample. The subset group breakdown, represents 57% of total responses.

Table 2.4 Typical durations and costs of product equivalence studies. The values in brackets indicate the Percentage of respondents selecting the category shown. A breakdown of the typical duration results is shown in Appendix Chapter 2.

Product Equivalence Study	Typical Duration	Typical Cost
Bioequivalence/comparability studies	Less than 3 months (47%)	Up to $250000 (70%)
Re-validation of process and equipment	3-12 months (46%)	
Repeat of phase I clinical trials	12-18 months (41%)	$2M-$15M
Repeat of full- scale clinical trials	More than 18 months (68%)	(38%)
Number of respondents	81(subgroup = 15)	

The majority of respondents indicated that seamLess integration of changes was possible, with no shutdown periods (44%,) or if there were shutdown periods they typically lasted for 3 months (41%).

Table 2.5 Percentage distribution of industry responses by shutdown durations during a post-approval process change

	% Respondents
No shut-down periods are required	43
0-3 months	38
3-6 months	17
More than 6 months	2
Total	100
Number of respondents	65

In Table 2.6 a list of the methods used by companies to maintain their continuity of product supply. It can be seen that 49%of respondents run processes in parallel and 39% maintain that they stockpile their product in advance. However, only 7% of companies choose to outsource to interim manufacturing organisations, to keep their supply continuous. 5% of respondents said they do not use any of the above approaches when implementing a manufacturing change and subsequently experience delays.

Clearly, the overall basis for making a change is to enhance overall profits. We tried to establish some cost financial benefits of making the process change; however, only a few respondents (15) provided data for this questions. These are summarised in Table 2.7, and provide an indication that process changes maybe expected to typically yield a 16% decrease in Cost of Goods (COG).

Table 2.6 Percentage distribution of industry responses toapproaches used to maintain a continuity of product supply during process change.

	% Respondents
Product is stockpiled in advance	52
Processes are scheduled to run in parallel	38
Outsource to interim manufacturing organisations	6
Other	4
Total	100
Number of respondents	69

Table 2.7 Average estimates of long-term financial benefits resulting from process changes.

	Average Percentage Change
Net present value, NPV gain	19
Return on Investment, ROI	29
Profit margin*	22
Mark-up*	33
Change in operating costs	16
Total	100
Number of respondents	15

**Although mark-up and profit margin are often used interchangeably they are in fact different, mark-up percentage is the percentage difference between the actual cost and the selling price, while gross margin percentage is the percentage difference between the selling price and the profit.*

2.3.6 REASONS FOR NOT MAKING PROCESS CHANGE

It is clear that the cost of implementing manufacturing changes and lengthy regulation paperwork and approval from governing bodies stop many biopharmaceutical companies from going ahead with process changes. Consequently, manufacturers think twice about upgrading equipment or revising processes, even if it means forgoing opportunities to modernize outdated systems and improve formulations (Wechsler, 2007) . In this section the reasons for not making process changes is explored. Respondents were asked to give the five most likely reasons for choosing not to make a manufacturing process change out of the seven reasons given in Figure 2.7.

The top five reasons for not making changes are: the costs involved, the delay in production, the requirement of regulatory approval, delay in product launch, and the risk of having to prove equivalence. The largest number of respondents chose the cost of process change as the most likely reason for not implementing change. This includes validation studies and the possibility of repeating clinical trials. This again confirms that whilst process changes may lower long-term costs, the delays and short-term costs from regulatory activities prevent many improvements from being made.

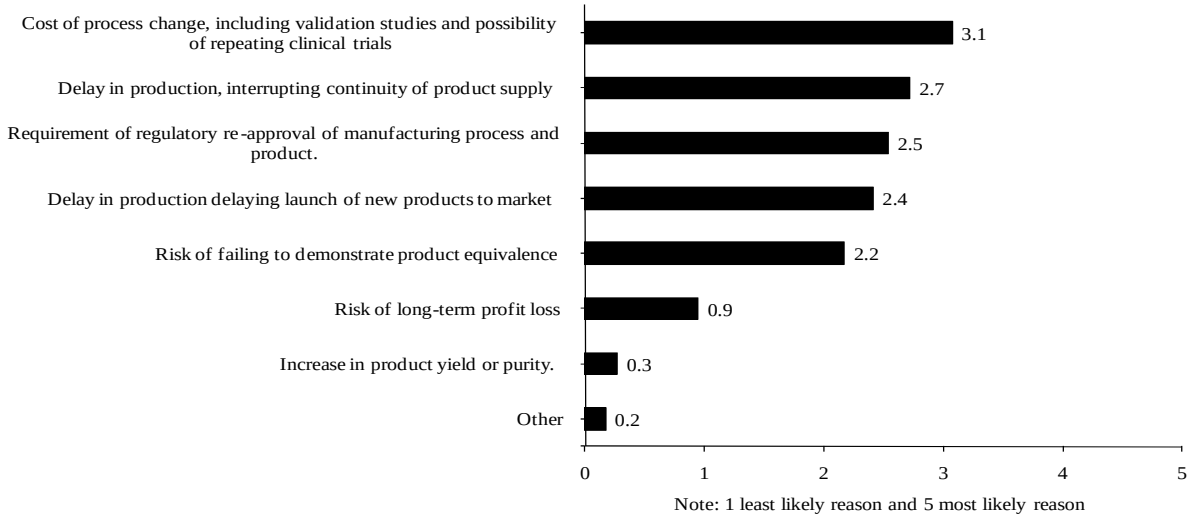


Figure 2.7 The key reasons for rejecting manufacturing process change proposals in the biopharmaceutical industry ranked in order of their average scores. Note score of 5 indicates most likely reason to make a change and 1 indicates least likely reason.

The prospect of repeating clinical studies is a big deterrent, because of the costs and time-delays it poses. Delays in process already in production, which could interrupting continuity of supply was a key reason for not making changes to processes. Another probable reason for not making change is the delay to market of a process still in development. Some companies rush to market with a generic process, without refining it or making changes during early stages of development, as this will increase their market share. The requirement of regulatory approval, which could include re-registration, is another major reason for opting not to make changes to a process.

2.3.7 IS IT EASIER TO MAKE PROCESS CHANGES NOW?

Respondents were asked to give their opinion on whether it is now easier to make a change in a process now than 10 years ago. The results suggest that there is not an obvious answer; 51% of respondents said yes, making changes to process is easier now, but 49% thought it was harder than 10 years ago. Those who said it was more difficult to make changes reasoned that regulations are increasingly stringent and lengthier to get through the requirements of regulations such as the introduction of 'Guidance for Industry Part 11, Electronic Records; Electronic Signatures — Scope and Application' CFR part 11 (FDA Guidance Document, 2003) in the last 10 years(Wechsler, 2007). However, those who think it may be easier said that regulatory bodies generally have a greater understanding of many biologicals such as monoclonal antibodies, which are considered "well characterised proteins", and so companies are able to place more emphasis on showing equivalency through analytical methods versus clinical data. For highly glycosylated products and viral products the experience and tools still require more development to give scientists the confidence to make process changes. There are also better analytical techniques available now to aid in gaining regulatory acceptance. Although regulatory agencies say they are considering changes on a risk basis, they do not appear to have decreased the data requirements for making a change or significantly decreased review times. Patent expiries, the prospect of 'generic' biologics, long regulatory review periods and limitations to the number of changes that regulatory authorities will consider per year all remain hurdles which deter companies from making changes.

2.4 CONCLUSIONS

From this survey, we can conclude that changes are frequently being made to processes in the biopharmaceutical industry, at all stages of product development. The main reasons for change are to reduce batch-to-batch variability. Major changes are not frequently made once process have been approved for production; the major deterrents are the costs and delays to production. If a repeat of clinical studies is required to demonstrate product comparability after a change is made, then it is likely that the proposal for change will be rejected. Knowledge of the product and process, an understanding of the capabilities and limitations of the analytical methods, and a definition of the relationship between the quality attributes and the safety and efficacy of the product are critical for appropriate assessment of the impact of a manufacturing change. Where quality attributes have not been linked to safety and efficacy or where

physiochemical differences observed have been known to impact the clinical properties of the biological product, comparability of pre-change and post-change product may need to include a combination of quality, non-clinical, and/or clinical studies (Weinberg, 2005) .

This survey gives an overview of the general position and direction of the industry. Answers given are a matter of opinion; however, senior professionals in the biopharmaceutical industry were targeted (see profile of respondents in Figure 2.8). .

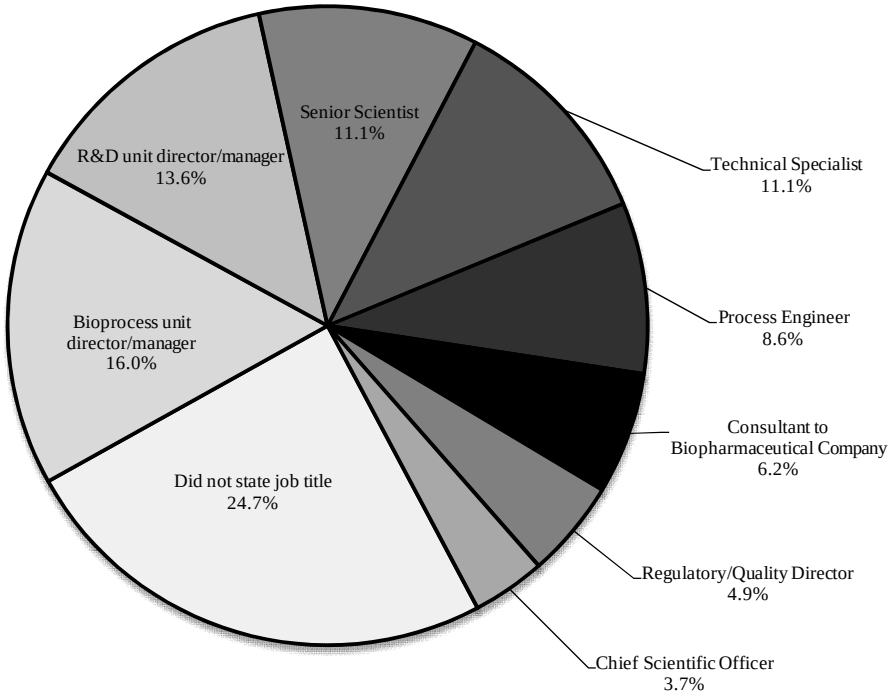


Figure 2.8 The overall profile of respondents

Chapter 3

A Framework to Gauge the Technical, Regulatory and Financial Implications of Bioprocess Changes

3.1 INTRODUCTION

As indicated in the preceding chapters, during the development phase biopharmaceutical companies will often try to reduce the cost of manufacturing whilst increasing speed to market, resulting in less than efficient processes. In general, forced and unforced bioprocess changes can transpire from excessive costs, product competition, regulatory rules, and customer requirements, amongst other reasons. In this chapter, a conceptual framework to assess the economic and manufacturing impacts of making process changes in the manufacture of biologics is presented. The impact of uncertainties when changes are made is also explored. This is then followed by a description of the implementation of the framework into a decision-making tool.

In section 3.2 a detailed description of the biologics industry and process change domain is that is addressed by the framework is given. In section 3.3 a description of the scope of the model basis is presented. The process change activity framework is detailed in section 3.4, and the modelling approach utilised is provided in section 3.5.

3.2 DOMAIN DESCRIPTION

The key features involved in process change management of biologics manufacturing are identified in this section. As described in Chapters 2 and 3, implementing a process change, no matter how small involves an inherent risk. Biological products are more complex than pharmaceutical drugs, and so the demonstration of comparability is more difficult and any changes manufacturing and scale-up can impede regulatory approval. The change will have a direct and indirect impact on cost, time to market (if in development phase), and approval from regulatory authorities. A method to capture the tasks, resources, business issues and uncertainties involved in process change development and implementation can be used as part of a company's process management strategy. The framework should be able to capture the variety of scenarios that can result in a process change and the different outcomes when implemented. Some of these possible scenarios are described here.

3.2.1 TYPES OF PROCESS CHANGE

3.2.1.1 FORCED CHANGES

Not all changes are made to solely to improve process efficiency or purity or to reduce costs. As suggested in the introduction in the biopharmaceutical industry manufacturers are frequently required to make changes to their processes as enforced by regulatory bodies, such as the US based Food and Drug Administration (FDA) or the European Regulations European Agency for the Evaluation of Medicinal Products (EMEA) or occasionally to meet new customer demands. Companies that do not comply with the suggested changes may eventually be forced to shut down or they could lose a large proportion of their market capture to competitors. The forced modifications have been classified into categories of 'minor' and 'major' changes. The FDA define different types of manufacturing changes into similar classifications. A major change is defined as having substantial potential to have an adverse effect on the identity, strength, quality, purity, or potency of a product as they may relate to the safety or effectiveness of the product will eventually require an applicant to submit and receive FDA approval of a supplement before distribution of the product with the manufacturing change. Minor changes will require notifying FDA of the changes only in an annual report (U.S.Food and Drug Administration, 1997).

3.2.1.2 MAJOR YIELD-IMPROVEMENT CHANGES

Process performance can also be improved with a cost reduction or yield enhancement. Minor changes usually increase the cost-versus yield performance of a process. Only a major technological change has the potential to drastically change the cost-yield ratio, potentially providing a company with a significant competitive advantage (Baker and Wheelwright, 2004). This is highlighted in Figure 3.1. As this figure highlights most changes increase both cost and yield, and the only way to jump back across curves is if you have a change that results in a major increase in yield that outweighs the implementation costs. The changes should results in either large reductions in the cost of recovery or large increases in yield, each with little or no negative impact on the alternative measure. Thus, given a defined a defined recovery and purification process for any product, yield enhancements within existing unit operation should shift the cost relative to yield.

3.2.2 TIMING OF CHANGES

Changes are made to processes at all stages of a product's life cycle, during development and commercial stages. The need for changes to be made will decrease as a drug goes through development and processes are generally set at Phase III clinical development stage. The impact of implementing the changes, which can be measured by

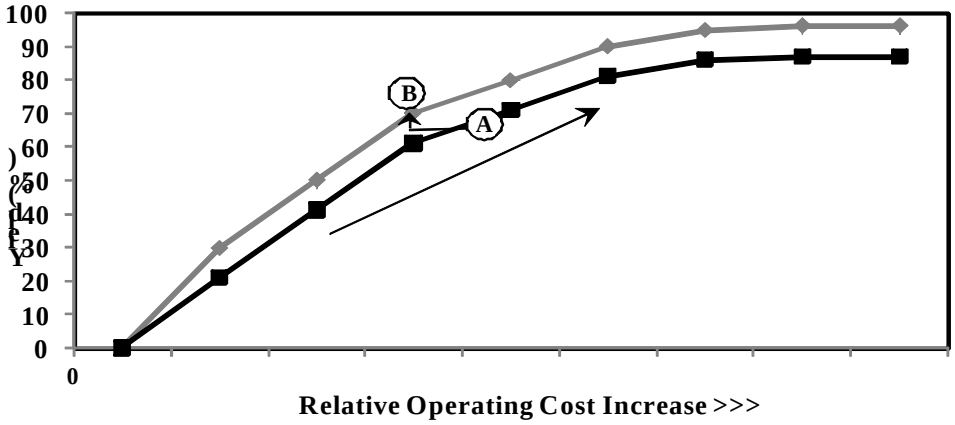


Figure 3.1A hypothetical 'yield versus operating cost' curve for any recovery process. Effective implementation of major changes are jumps in performance independent of cost see arrow A to B. *Adapted from (Baker and Wheelwright, 2004)*

the costs accrued indirectly and directly and time delays, will increase as a drug is further developed and closer to filing for regulatory approval and can increase the risk of repeating clinical trials, clinical failure, and losses in market share. During early phase clinical development there is the highest risk of clinical failure (Werner, 2004) and so companies may not be willing to spend money on making changes. At the end of Phase III clinical development there is the highest risk of losing market share; it is likely that there will be a lengthy delay to prove product equivalence. Post- product approval, there is a lower risk of losing market share, as the product is already in the market and the changes can be made in parallel to processing the product. This is summarised in Figure 3.2.

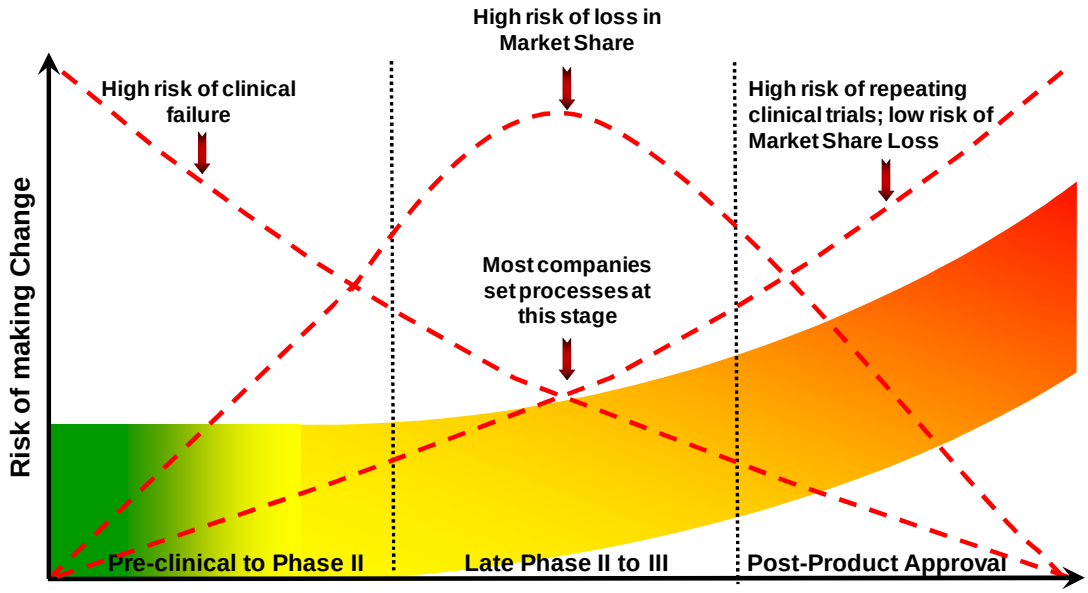


Figure 3.2 The risk of implementing process changes at different stages of a drug's development cycle.

3.2.3 OUTCOME OF PROCESS CHANGES

There are different possible product outcomes when making manufacturing process changes:

- No difference may be seen as a result of the change, in this case where no further studies to prove equivalence are necessary.
- Differences may be seen but can be justified; again, no further studies will be necessary.
- Differences with limited analytical discernment is shown. This time further studies will be necessary.
- Differences are great enough to have an impact on clinical efficacy, that they cannot be excluded. Further studies are necessary. The result of this study can be positive and thus acceptable, or negative and thus rejected.

The next section describes the process change framework, which should be able to capture the variety of backgrounds and results of a bioprocess change.

3.3 SCOPE OF MODEL

The purpose of this model is to assess the full impact of making different possible process changes, at all stages of development and under a variety of circumstances. More specifically, the scope of the framework is defined as follows:

- To assess the full impact of making process changes in biomanufacturing.

The tool attempts to cover the full economic and manufacturing impacts involved in any process change project, including the effect of time delay.

- To capture the technical and regulatory activities in a generic framework for dealing with process changes at any stage of a drugs life cycle.

  The assessment should cover the different scenarios that might occur throughout the different developmental phases, including post- product approval.

- To conduct profitability and risk analysis.

  The implementation of a process change could be strategic in that it will increase company profits, or if it is a forced change, then it may have a short- or long-term detrimental impact on cost. The risk involved in such process changes is quantified, and the critical ranges in key parameters involved are defined, allowing for the alternatives being explored to be compared economically.

- To be able to assess industrially relevant case studies to demonstrate the use of the framework.

  The framework should be generic to the biopharmaceutical industry, allowing for a speedy assessment of any type of change. As aforementioned, in this thesis, example case studies centred on a human-derived plasma fractionation process based at Bio Products Laboratories (Herts, UK) are employed to demonstrate the usefulness of this tool. The use of the company-derived data also gives the research a greater sense of commercial reality than previous works have.

In summary, the framework captures the risk and the rewards of making biomanufacturing process changes and provides a rational basis for confident decision-making in biopharmaceutical drug development and process optimisation. The following section gives a detailed description of the framework and approach used to model bioprocess changes.

3.4 PROCESS CHANGE ACTIVITY FRAMEWORK

A structured model modelling approach is used in order to capture all the activities that are affected by making a bioprocess change and new activities that transpire when accommodating the change. The framework is based on a hierarchical structure, which encompasses all the possible technical and business related activities that may be involved. The theoretical framework incorporates manufacturing, resource-allocation, and regulatory associated tasks. All of these tasks will affect the strategic process change decision-making. The tool structure clearly covers the key tasks and resources

involved when arranged in this hierarchical, task- oriented manner. This methodology has been used previously in The Department of Biochemical Engineering, University College London to model the manufacture of biopharmaceuticals by researchers (Farid, 2001;Lim *et al.*2004) and to model the phases involved in the process of drug development (Rajapakse, 2005;Karri *et al.*2001). The framework in this paper extends the hierarchy further to incorporate the extra activities involved when making changes to a bioprocess such as comparability studies and process validation, see Figure 3.3. The hierarchical breakdown proves useful in providing maximum flexibility, as it allows processes to be simulated at various levels of details. Modelling at the higher levels gives an overview of the entire process with its key operational and economic parameters. Subsequent details of each higher-level activity can obtained by breaking them down into sub-tasks. The more detailed levels of modelling at a lower level and give more accurate statistics and results. For example, changes to product yield can be modelled in the manufacturing blocks in addition to the influence of regulatory hurdles by accounting for comparability or product equivalence studies. As a result, manufacturing alternatives or modifications can be evaluated in terms of process economics, time, yield and resource utilisation.

As portrayed in the framework, at the highest level a 'process change activity' is modelled, this is then broken down into the key activities that may be involved in such a change, these are manufacturing, development, validation, product equivalence study, and market activities. At a greater level of detail, these tasks can be broken down again. For example, manufacturing is broken down into the process stream and its associated ancillary tasks. This can be broken down further into each process unit, and its associated ancillary tasks. For example for the each process unit, there will be labour requirements and there may be associated Cleaning in Place (CIP), and Quality Control and assurance (QC/QA) tasks. Each activity will have different inputs of time and cost and will vary according to the process change type and magnitude. Lower level frameworks for validation and product equivalence activities show how the tasks can be split further to gauge the impact of a process change more accurately (Figure 3.4 and Figure 3.5.).

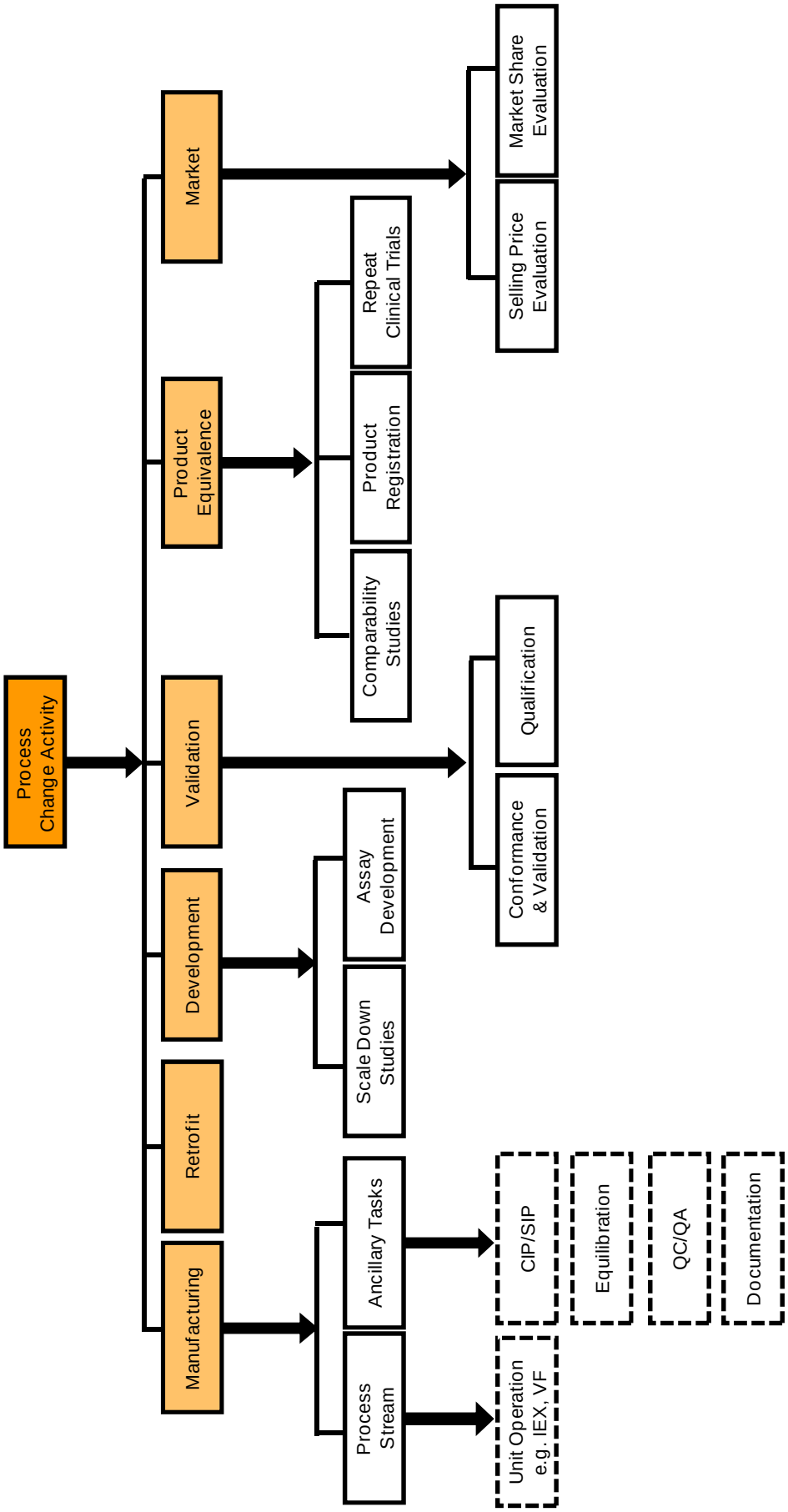


Figure 3.3A hierarchical framework capturing the activities involved in implementing a process change.

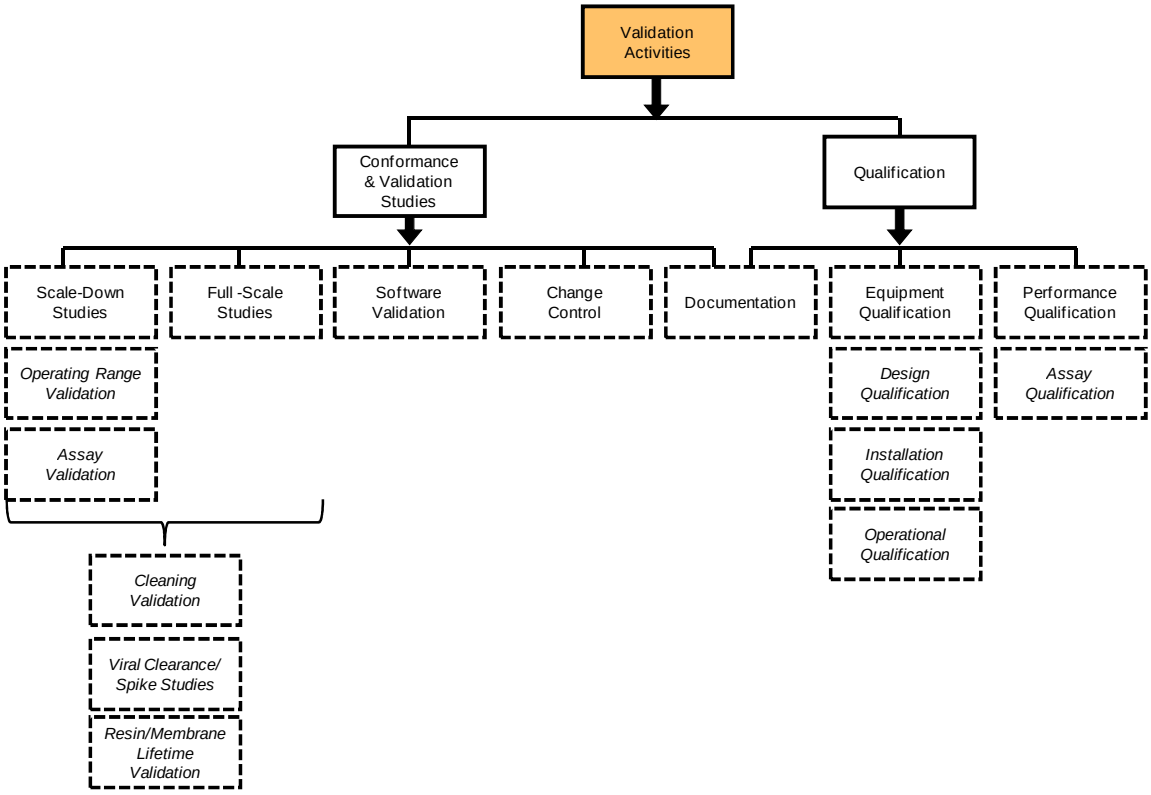


Figure 3.4A framework to model the validation activities that might occur when implementing a process change activity

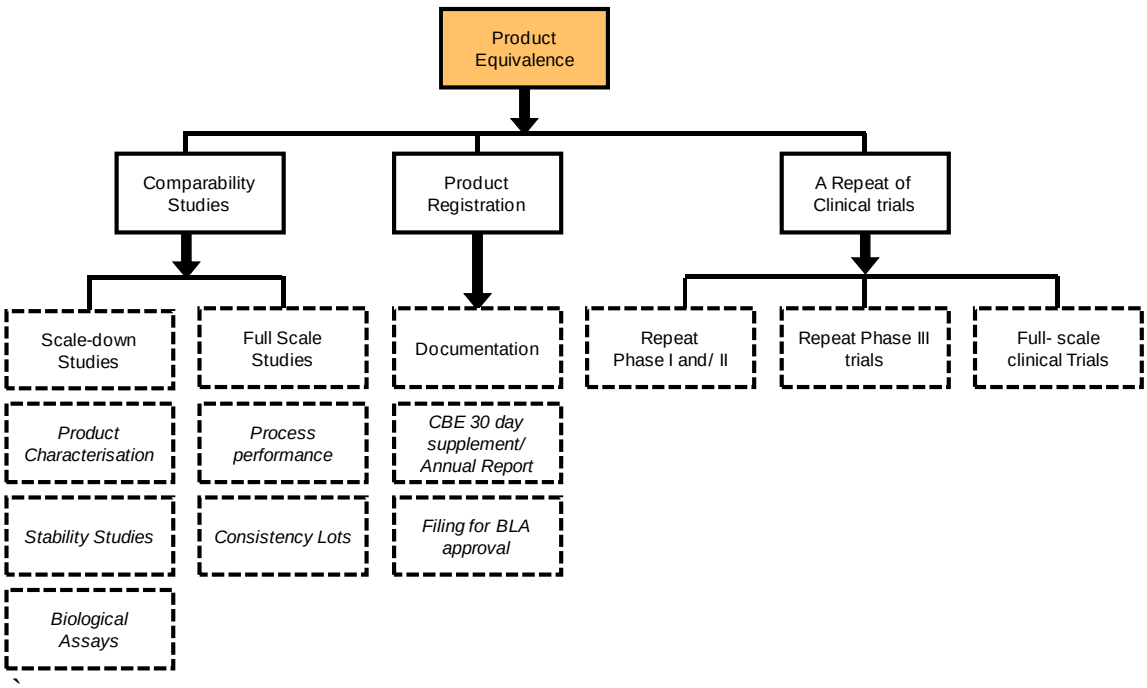


Figure 3.5A framework to model the 'product equivalence' activities that might occur when implementing a process change activity

3.4.1 KEY PROCESS CHANGE ACTIVITIES

In this section an outline of the fundamental activities involved when making a process change is described.

3.4.1.1 MANUFACTURING

The manufacturing task is the operative section in the 'process change activity' framework; this is where the actual change is made and subsequently shapes all the other activities. It comprises of two sub sections; a process stream, in which a proposed process change is being made and its associated ancillary tasks. The process stream comprises the product manufacture activities (e.g. chromatography, filtration), and the ancillary tasks comprise of process-related activities. These activities can be equipment or material preparation such as Cleaning-In-Place (CIP) of reusable unit operations or preparations, or regulatory-compliant activities in cGMP manufacturing plants, such as QC/QA, lot reviews and batch documentation. Including these support activities is necessary to improve the accuracy of cost and time-delay calculations.

3.4.1.2 RETROFIT

The retrofitting of the new process step or modification must be taken into account. The chief risk will be the delay in development or the risk and length of a shutdown period when modifications are made to the plant, as well as the installation of new equipment. Labour costs should be taken into account; this may be outsourced in this task. Regulatory activity involved in a retrofit task, such as validation is considered in a separated section.

3.4.1.3 DEVELOPMENT

A process change activity may transpire either as a result of new advancements stemming from a company's research and development (R&D) laboratories or from external factors such as suboptimal recoveries or regulatory influences. Therefore, prior to a change being made there will have been 'development activity' where the proposed change is analysed and optimised at laboratory scale. Although, R&D work is ongoing in any successful biopharmaceutical company, the development cost and time should still be included in the process change economics. This has been divided into scale down experiments and assay development work.

3.4.1.4 VALIDATION

Validation is described in the US Food and drug Administration (FDA) as the "establishment of documented evidence" conveying " a high degree of assurance" that

a "specific process" , pertaining to a particular product of interest, consistently produces a product that fulfils pre-determined criteria and quality attributes (Center for Drug Evaluation and Research, 2009). It is a requirement for cGMP compliant processes , so as to obtain and gain and maintain a product license in the United States, European Union amongst other regions. The studies must span all stages of development and will take into account variations in raw materials (e.g. different plasma sources in blood-fractionation process), and operating variables (Sofer and Zabriskie, 2000). This is work is typically carried out in manufacturing and QC/QA sections of a company. In this study, the validation task has been divided into two key sections 'Qualification' and 'Conformance and Validation Studies'.

3.4.1.4.1 QUALIFICATION

Re-qualification activities may arise from change controls arising from process changes. Prior to the initiation of process validation, the utilities, equipment and software (or computer) systems need to meet certain criteria or 'be qualified'. The qualification task is split into six main activities: Design Qualification (DQ), Factory Acceptance Tests (FAT), Site Acceptance Tests (SAT),Installation Qualification (IQ), Operation Qualification (OQ), and Performance Qualification (PQ).

- DQ is a preliminary step in equipment qualification. It involves the affirmation that pre-determined equipment requirements have been established and detailed designs completed. This is prior to any construction or production that takes place in a current good manufacturing practise (cGMP) dedicated facility area. Documented assurance that these stipulated requirements will typically include engineering drawings, process and instrumentation diagrams, process flow documents, air flow and instrumentation diagrams.
- IQ is documented verification that systems (equipment, facilities, and utilities) its components comply are included, properly installed, and fulfil design qualification and manufacturer specifications. Calibration of equipment and utilities will be performed too.
- OQ certifies that all the components of a system operate together as specified in DQ. Tests are performed on the critical parameters of the system and or the process. These are usually the independent of manipulated variables associated with the equipment. All test data and measurements are documented in order to set a baseline for the equipment or process.

- PQ is performed on the manufacturing process as a whole; it ensures that the total system performs as intended within specified operating ranges. The system includes all hardware and software components, associated equipment, labour and procedures that make up the system. Individual components of the system are not tested independently. Various parameters in a system such as speed, response, capacity, power are typically measured. The ability for a process to perform over long periods of time within tolerances deemed acceptable is verified (Agalloco and Carleton, 2007;Center for Drug Evaluation and Research, 2009;Sofer and Zabriskie, 2000).

3.4.1.4.2 PROCESS VALIDATION STUDIES

Any significant change to a process will result in the need for subsequent re-validation. Process validation will ensure a robust product that is highly reproducible over time. The manufacturing process, in addition to the individual equipment, must be validated. The goal is to create a robust manufacturing process that consistently produces a drug product with acceptable variation that adheres to quality criteria of purity, identity, and potency. A validation plan for the manufacturing process should be drafted and executed by engineers in order to satisfy guidelines. It involves the assessment of scale-down and large-scale manufacturing validation studies.

- Scale-down study activities will include laboratory-scale validation of cGMP, operating criteria applicable to yield and purity rather than equipment efficiency as seen in PQ, assays developed in the 'development activity', and system cleaning protocols. The validation of operating ranges will demonstrate that a product manufactured within set operating criteria meets release specifications. Critical operating parameters include temperature, pH, and composition of raw materials amongst others.
- Full-scale studies will involve the validation of scalability studies between full-scale, pre-clinical, clinical and small-scale process runs
- Cleaning validation should ensure the cleaning effectiveness of new equipment and post-batch cleaning for non-disposable units. Cleaning methods are developed and qualified to prove the removal of residuals or by-products from manufacturing and cleaning activities. At points identified in the cleaning validation protocol, swab and rinse samples are collected and analysed using qualified methods. Results from three 'conformity' batches are required.

- Viral clearance and spiking studies involving a process where change is implemented will require the demonstration that the removal or inactivation of impurities is still sufficient within acceptable levels. Viral clearance studies are performed in scaled-down model systems within biologically contained environments. Other clearance studies can be performed at full-scale.
- Lifetime studies of membrane and resins, where the maximum number of resin cycles allowed before a resin is discarded commonly established by laboratory-scale studies. They are designed to show consistent product recovery and purity. Defined maximum resin lifetimes specific to the process under review, rather than generic claims without supporting data are required by regulatory agencies, as there is variability, for example in the plasma source and in chromatography regeneration conditions (temperature, solute concentrations, volumes and resin contact time). Current regulatory guidance calls for evaluation of the virus removal capability of resins at the beginning and end of their lifetimes. Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary (Kelley *et al.*. 2008).
- Computer validation of changes to the computerized system are made according to a change procedure and are formally authorized, documented and tested. Records of all changes, including modifications and enhancements made to the hardware, software and any other critical component of the system are made, demonstrating that a validated state has been maintained.
- Change control is a 'formal system by which qualified representatives of appropriate disciplines review proposed or actual changes that might affect validated states' (International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2009). Once the process and its equipment have been qualified and the product and process consistency validated, then it is necessary to ensure that the process has not been deliberately changed even in a minor form, without prior authorisation by appropriate individuals. The scale of change control will depend on the process' stage of development, and more documentation will be required if a process has already been fully validated, i.e. at the late phase of development and once a product has gone to market.
- Documentation of all qualification and validation activities is required. All documents are prepared, reviewed, approved and distributed according to written procedures. This will be spread across all departments within a biopharmaceutical

company (Agalloco and Carleton, 2007;Center for Drug Evaluation and Research, 2009;Sofer and Zabriskie, 2000).

3.4.1.4.3 PRODUCT EQUIVALENCE

Typically, product equivalence is a stepwise procedure, beginning with limited or comprehensive quality studies and then, as necessary, supported by non-clinical, clinical and or pharmacovigilance data. If a manufacturer can provide evidence of comparability through physico-chemical and biological studies, then non-clinical or clinical studies with the post-change product are not warranted. The need, extent and nature of non-clinical and clinical comparability studies will be determined on a case-by-case basis in consideration of various factors that may be associated with risk, such as: Non clinical and clinical equivalence studies. This includes all experimental assays used to prove product equivalence as well as the cost of producing enough material for comparability lot to lot testing. Here the activities have been divided as follows:

- Comparability studies can involve small scale analytical and biological or bioassay methods, as well as full scale conformity batches:
  i. Analytical tests include both chemical and physical assays. Methods include assays routinely carried out on all production lots: those initially used to fully characterize product structure and identity, establish product consistency from one production lot to another, and new tests if applicable.
  ii. Bioassays are functional tests which assess the activity or potency of the product. These tests may also serve as measurements of the biological integrity, e.g. the correct conformation of the product and thus complement other analytical measurements. Both assay types are validated and have a specific range of acceptable values for defining product activity; this is included under the ‘validation’ task.
  iii. Stability studies identify whether a product’s stability has been affected post change, this can occur even with slight modifications to the production. Any change not readily detectable by the characterization studies and with the potential to alter protein structure or purity and impurity profiles is evaluated for its impact on stability. Accelerated and stress stability studies are often used to establish degradation profiles and a direct comparison of pre-change and post-change products.

iv. 'Conformance lots' also known as 'consistency' or 'qualification' batches at a commercial scale. In the United States a minimum of three consecutive lots are required, whilst for European filings it is common practise to perform up to five manufacturing runs. Comparability is not just about meeting specifications, post-change material may be better, and thus efficacy must be proven(Sofer and Zabriskie, 2000).

- Non-clinical 'bridging' studies which include *in vitro* studies and *in vivo* studies, obtain preliminary efficacy, toxicity, pharmacokinetic (PK), and pharmacodynamic (PD) information. *In vitro* Studies entail quality-related bioassays of pre- and post-change products that are tested concurrently using a comparative study design. With *invivo* studies, one or more relevant species or properly validated animal models are used to resolve uncertainties vis-à-vis pharmacokinetic parameters and pharmacodynamic effects relevant to the clinical application, and safety (Sofer and Zabriskie, 2000).
- Clinical studies are the only method of determining the true potency of a product, and clinical 'bridging' studies may be required from as early as developmental phases I-II. At all phases, trials are designed to assess the safety (pharmacovigilance), tolerability, pharmacokinetics, and the pharmacodynamics of a drug. These studies are normally randomized controlled trials, and as a minimum should have adequate power to demonstrate non-inferiority in terms of protective immune response and to detect common adverse events for the tested. From late phase II to post BLA filing, dosing studies, clinical efficacy studies, specific safety studies, and immunogenicity studies will be determined. Typically, changes made during pivotal studies or just prior to submission of a marketing application require more data, and thus resources, time and cost, to support product comparability than manufacturing changes made during earlier phases of clinical development. The extent of the trial depends on the type of change made. In this study, the clinical bridging studies have been split into small-scale, and large-scale activities (Figure 3.5).
- Regulatory reporting of all the product equivalence study protocols and results is mandatory. The FDA has varying report types depending on the magnitude of change and where in a drug's life cycle the change is made. Minor process changes can be implemented without approval; these are documented in full detail in the 'Annual Report'. Moderate changes, must be filed under either the 'Changes Being

Effected CBE-0' file, which allows the immediate distribution of the product once the supplement has been submitted to FDA or CBE-30 requires that you must wait 30 days wait preceding to distribution. Major changes will require FDA approval before any distribution of product, and this is filed under the 'Prior Approval Supplement' (PAS) (Center for Biologics Evaluation and Research (CBER), 2001;FDA Guidance Document, 1998).

3.4.1.4.4MARKET

The process change can have an advantageous impact on the products selling price, but it can also cause significant delays in going to market. This can significantly reduce the market share captured. An evaluation of the impact of cost and time on potential profit before a change is made will need to be carried out.

3.5 MODELLING APPROACH

A structured approach is used in order to facilitate the calculation of the impact of making a process change within a biologics manufacturing process. Figure 3.6 provides a simplified schematic of the proposed framework modelling methodology. The framework has four elements: the process change activity, which includes a detailed biomanufacturing process model and all validation activities; a profit and loss model; a risk model, and a set of criteria used to distinguish between the strategic process change options. The figure shows some of the main input and output parameters used in the calculation. The outputs of the model include the cost, delay and risk of making a process change. The cost analysis was extended to include profitability indicators such as the Net Present Value (NPV) and Cost of Goods (COG).The cost of the manufacturing process is calculated pre- and post-change implementation. The model framework allows for interactions between activities such as, manufacturing mass balance, resource utilisation, costing with validation, product equivalence, retrofit and development activities. The method also looks at implementing any of these changes at various stages of a drug's life cycle, from pre-development phase to post-product approval. This is described in section 4. This approach combined with the

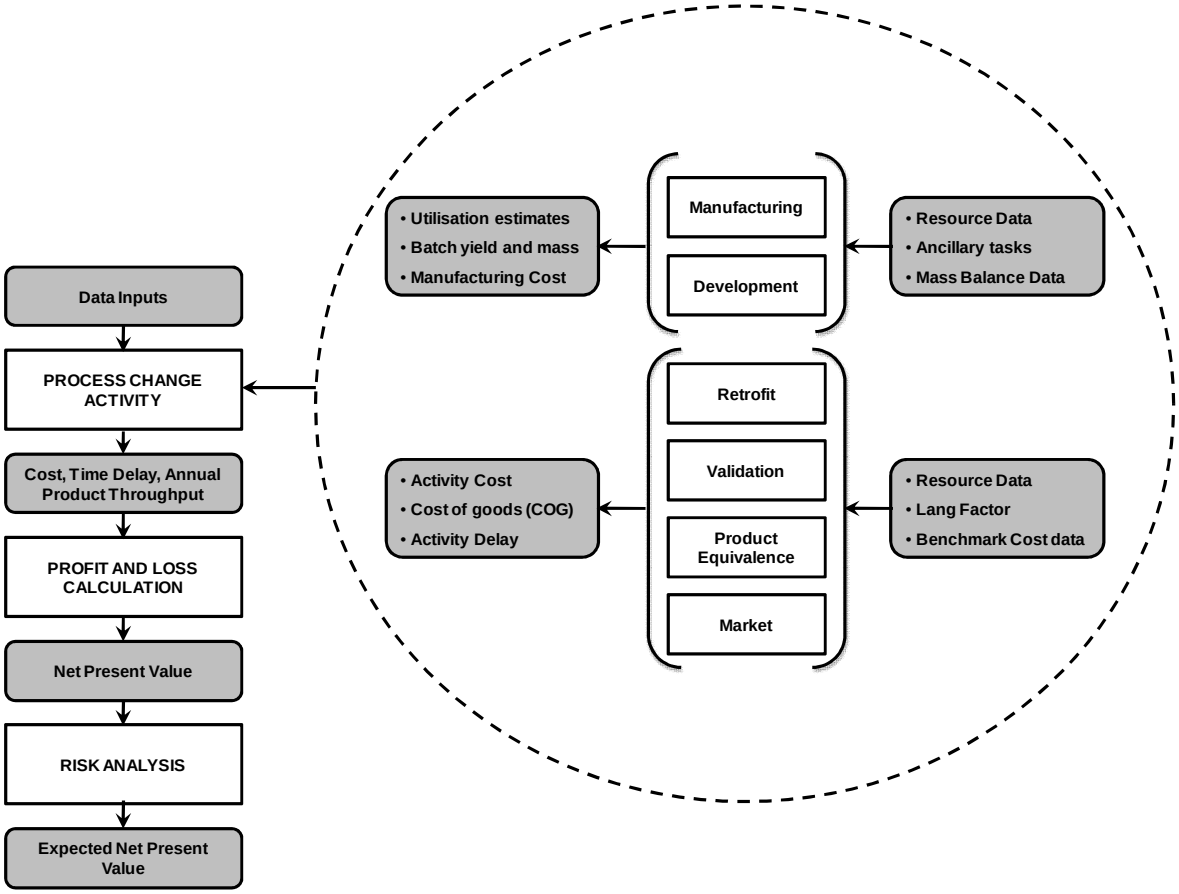


Figure 3.6A Simplified schematic of the main inputs and outputs of the proposed framework.

hierarchical 'Process change activity' framework' enables the calculation of different scenarios rapidly and allow the assessment of whether the process change alternative is feasible in terms of cost and time-delay. Different outputs can also be accessed. The costs and duration of each task is available for analysis and comparison.

3.5.1 GENERIC ATTRIBUTES

3.5.1.1 RESOURCES

Each activity in the process change framework will utilise a range of resources. Much of these are involved in biopharmaceutical development and in the everyday running of biological processes. These include both renewable, e.g. labour, and facility, and non-renewable which includes some materials and costs.

3.5.1.2 COST

The costs considered include the capital expenditure and investments that are accrued at all stages of drug development. General biologics development costs will conclude

capital costs for facility building, manufacturing for clinical trials and commercial manufacturing. In costing for manufacturing both for clinical trials and the market, the cost of goods per gram (COG) was calculated per batch. Other costs include process development, and validation studies. These were collected through, a survey on process changes (Chapter 3), literature (Rajapakse, 2005;Farid, 2001;George *et al.*. 2007;Lim *et al.*. 2004) and through conversations with industrial experts (Bio Products Laboratories). A good indication of the direct impact is to look at the installed cost, working capital, and operating cost pre- and post-change. This alongside validation, product equivalence, retrofit and market costs will give a good indicator of the impact of the change.

3.5.1.3 DURATION

The duration of each task was either calculated based on inputs or distributions. The durations of each task was inserted into the profit and loss model, and changes in cost and time to market were computed. Again, duration estimates were taken from the survey on process changes, literature and industrial expertise.

3.5.2 MANUFACTURING

The manufacturing process is at the core of the process change execution, other process change tasks, such as validation, and equivalence studies are consequences of this change in manufacturing. Therefore, while it was important to model the biomanufacturing process in detail, it was more important to capture the strategically important costs and risks of the manufacturing change. The scale of manufacturing will vary depending on the stage of development. For this study, assumptions have been made concerning the scaling at each stage, with a focus on methods used at Bio Products Laboratory, UK; however, the approach and scaling used will vary from company to company, as some companies scale-up processes at very late stage of development or even after BLA (Biological license application) approval. There are some advantages to keeping everything scaled-down through Phase III, but this means an enormous gamble of scale-up and comparability assessment success. Zeid, 2005 cites that companies such as Bayer use this strategy in developing Kogenate, a recombinant coagulation factor VIII, as did Genentech with Activase, a recombinant tissue plasminogen activator approved to treat acute heart problems such as myocardial infarction (AMI) and acute ischemic strokes.

There have been several publications on successful biomanufacturing modelling at UCL(Chattre, 2008;Farid, 2001;Farid *et al.*. 2007;George *et al.*. 2007;Lim *et al.*. 2004) to name but a few. Therefore, it was decided that these method would be employed to model the manufacturing task to a sufficient degree of detail. The model concepts and equations used are described in subsequent chapters, these were primarily focused on the models created by Farid, 2001 (SimBiopharma), and by Lim, 2004 (Biopharmkit).

3.5.2.1 INTRODUCTION

The manufacturing task comprises operational tasks (e.g. filtration, chromatography), ancillary tasks such as general equipment preparation (Cleaning-In-Place (CIP), and Steaming-In-Place (SIP)), and specific equipment preparation (e.g. chromatography column re-generation, equilibration), and regulatory compliance (QC/QA activity and batch documentation) and the resource data required for each task: equipment, operators, renewable materials, non-renewable materials, and utilities (Lim, 2005). Process stream compositions are determined using a mass balance, based on equations centred on the law of conversion of mass suggested by Farid, (2001, 2007).

In this study, the manufacturing model is centred on process streams, which are part of a plasma fractionation process used at Bio Products Laboratories, Herts, UK. A database of equipment costs, process step durations, materials utilisation, and global input parameters to the model such as annual demand and overall product yield are collected and verified by industrial experts. Each unit operation has a process model comprising of the design equations and mass balances. These are used to size equipment, determine the composition of the output streams and the amount of materials required (e.g. chromatography buffers). Equipment sizes are determined by matching processing requirements such as volume to a database of equipment dimensions available at the time of writing. In some instances, since this work is based on a real process, if exact unit sizes or other resource data is already known, then it is inserted directly rather than calculated. A summary of these inputs and outputs has been presented in, as has been previously employed by Farid, 2001, and Lim. 2004.

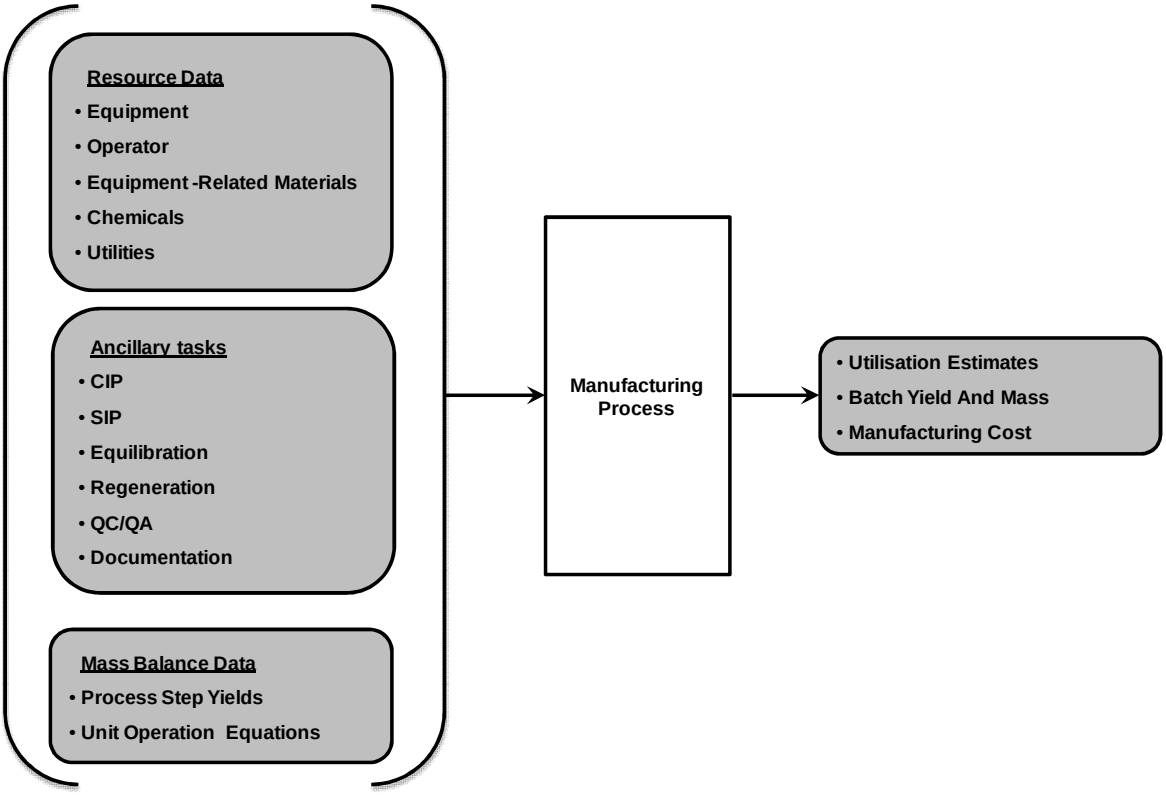


Figure 3.7 The key components of the manufacturing model

3.5.2.2 PROCESS MASS BALANCE CALCULATIONS

As previously mentioned, the mass balance calculation used to compute process stream compositions were based on those derived by Farid, 2001. This model was chosen, as it does not require complex input data, and also provides simple outputs that allow for the rapid assessment of manufacturing scenarios. Each unit operation is modelled by a set of equations that calculate the compositions of the process streams, as well as certain process variables. The general mass-balance equation for operations is based on the law of conservation of mass:

$$\sum_{i}^{n} m_{in_i} = \sum_{i}^{n} m_{out_i} \qquad \textbf{3.5.1}$$

Where *m* is the mass of components *i*to n. The basis for the process models the mass balance models derived by Farid have been included in Appendix Chapter 3for reference purposes.

3.5.3 DEVELOPMENT

Development is made up of manufacturing activities for non-clinical and clinical trial phases. To model various scenarios, cost and duration assumptions have been used,

these are described in more detail in the case study in Chapter 5. Normally, processes are optimized just prior to phase III development (Agalloco and Carleton, 2007), and so it is assumed that pilot-scale manufacturing was required for Phases I-II, and full-scale manufacturing was required for phase III. Different companies have different strategies regarding timing of scale-up, some even waiting to scale up after acquiring a BLA from the FDA. Pilot-scale manufacturing for development is calculated using the process flowsheet method described in section 3.5.2, other assay development costs are approximated based on literature references, and the costs provided by assay development outsourcing companies.

3.5.3.1 CLINICAL TRIALS

Clinical trial costs are based on the number of patients and durations. In this case costs and durations used, are based on the EMEA note for guidance on 'The clinical investigation of Human normal immunoglobulin for intravenous administration (IVIG)' (EMEA: Committee for Proprietary Medicinal Products, 2000), which gives indication on number of patients and duration that are very different to costs and durations required for the development of for example monoclonal antibody products. Plasma-derived products clinical trials require fewer patients than monoclonals; their toxicity is not an issue considering their human origin. In addition, the clinical trial process is different in that volunteer studies are not required and so trials start in patients as phase I/II. A number of plasma products are also considered to be orphan drugs, which again limit the extent of clinical trials and the number of patients necessary. (Personal communication, Thierry Burnouf, Human Protein Process Sciences, Lille, France)

3.5.4 MEASURING THE COST OF PROCESS CHANGES

3.5.4.1 BIOPROCESS PLANT FIXED CAPITAL INVESTMENT

A factorial method for capital cost estimation is often used in process engineering (Farid, 2007). In an approach initially proposed by Lang (1948) for chemical engineering plants, the fixed capital investment (FCI) can be calculated by multiplying the equipment cost by a "Lang" factor. The estimates are based on historical cost data and connect the total capital cost of the biomanufacturing plant to the cost of the equipment utilised. The Lang factor is dependent upon the type of process plant being used (Novais *et al.*. 2001). The specific value for such a factor applicable to bioprocessing plants is the total summation of individual factors that constitute the fixed capital investment. The equipment costs are based on conversations with industrial

experts at BPL or from literature (Farid, 2001;George *et al.*. 2007;Lim *et al.*. 2004;Mustafa *et al.*. 2005). Equipment sizes are determined by matching processing requirements such as volume to a known equipment dimensions.

This method provides an order-of-magnitude estimate of the fixed capital cost. In addition to the equipment cost, the Lang factor accounts for cost factors relating to such as piping, instrumentation, electrical work, buildings, utilities and site preparation, as well as design and engineering costs and contractor's fees. The value of the factor depends on the type of plant. A cost equation summarising this technique is given:

$$= \cdot = \left(\sum_{=1}^{10} \right)$$

3.5.2

Where, FCI = fixed capital investment, E = total equipment purchase cost, and L = "Lang factor" for the plant. The factors f_1 to f_{10} relate to $E_{conv}$to give the cost of process and utilities equipment (f_1, f_1 4 1), pipework and installation (f_2), process control (f_3), instrumentation (f_4), electrical power (f_5), building (f_6), detail engineering (f_7), construction and site management (f_8), commissioning (f_9), and validation (f_{10}). A contingency factor, c, is also normally included. This is summarised in Table 3.1. For a bioprocess plant, a Lang factor value of 8.13 has been recommended by Novais*et al.*, 2001.

3.5.4.2 COST OF GOODS MODEL

The manufacturing cost is calculated based on the direct operating costs from the process flowsheet. The costs of the QC/QA labour are also calculated as a function of utilisation. The cost of staff per hour is inputted, and for each unit operation in the process flowsheet, the requirements of the QC/QA and batch documentation activities are specified. The remaining costs are calculated as percentages of the direct operating labour or fixed capital investment. As well as the fixed capital investment, the other manufacturing plant output used to measure costs is the Cost of goods (COG).

Table 3.1 Capital investment factors for bioprocessing plants and corresponding "Lang" factors as suggested by Novais, 2001

Description	*f* i
f1 - Equipment and utilities	1.00
f2 - Pipework and installation	0.90
f3 - Process control	0.37
f4 - Instrumentation	0.60
f5 - Electrical power	0.24
f6 - Building	1.66
f7 - Detail engineering	0.77
f8 - Construction and site management	0.40
f9 - Commissioning	0.07
f10 - Validation	1.06
c - contingency factor	1.15
Lang Factor	**8.13**

The COG model employed is shown in Table 3.2. This is frequently used in bioprocessing and includes costs associated with cGMP biopharmaceutical plants (Farid, 2001). The direct or variable costs are computed based on the utilisation of the material, utilities and staff resources. The indirect costs or fixed overheads are derived from the capital investment. Staff costs are based on their utilisation rather than considering them as a fixed annual salary-based cost, as has previously been used by Farid, 2001, Lim, 2004, George, 2007. A cost category termed "general utilities" accounts for ongoing utility charges, such as HVAC systems. The cost is derived as a function of the facility size or floor area.

3.5.4.3 ADDITIONAL PROCESS CHANGE ACTIVITY INVESTMENT

The assessment of all process change activity costs and times, include not only the development cost, but also the cost and time required to meet regulatory requirements inherent to changing a manufacturing process in a regulated environment. Collectively, these values characterize each alternative. The implementation of a process change can result in the new investment of equipment. If this is the case then validation of the equipment and revalidation of the process will be required. The retrofitting of the new equipment will include new piping installations, design validation, building construction, electrical supply and instrumentation.

Table 3.2 Cost of goods model breakdown, *adapted from(Mustafa et al.. 2005)*

Cost category		Value
Direct cost of goods	Direct raw materials	f (utilisation)
	Miscellaneous materials	0.5 * Direct raw materials
	Direct utilities	f (utilisation)
	Operating labour	f (utilisation)
	Supervisors	0.2 * Operating labour
	Quality Control & Quality Assurance	f(utilisation)
	General management	1.0 * Operating labour
Indirect cost of goods	Maintenance	0.1 * FCI * Y
	Local taxes	0.02 * FCI * Y
	Insurance	0.01 * FCI * Y
	Depreciation	FCI / Depreciation period * Y
	General utilities	Cost per unit area per year * Facility size * Y
Total cost of goods	-	Direct COG / Indirect COG
Total cost of goods per gram (COGs)	-	Total COG / Annual production output

Where FCI is the fixed capital investment and Y the project duration in years.

Within a process change activity, the new revalidation costs and retrofit costs are required to calculate the inclusive new equipment investment costs. The Lang factor method has been employed to calculate this by calculating the new fixed capital investment. The new equipment costs are sourced from literature or industrial experts at BPL and Jacobs Engineering Group Inc, UK. The new retrofit cost are calculated using the following equation:

$$= [\quad * \quad = \quad ( ] \ * \ -1)$$

3.5.3

where is the retrofit cost if >1, otherwise:

$$[= \quad ( ] \ *)$$

3.5.4

The new revalidation cost is calculated in the same fashion:

$$= \quad * \quad [= \quad ( ] \ * \ -1)$$

3.5.5

where is the revalidation cost if >1, otherwise:

3.5.6

and so, the new fixed capital investment is calculated as follows:

3.5.7

where, E_n = total equipment purchase cost, and L_n = the additional Lang factors, i.e. the summation of the retrofit factor (f_{Rf}) and revalidation factor (f_{Rv}).

As suggested previously, the type of change made can be categorized into two groups of minor and major. The cost impact of a major change on revalidation and retrofitting costs will differ to that of a minor change; this has been captured in the revalidation and retrofit calculations (Table 3.3 and Table 3.4).

Table 3.3 Capital Investment Lang factors to calculate retrofit and revalidation costs of a 'minor' bioprocess change

Description	***f*i**
New Capital investment	1
New Pipework and installation	0.9
New equip design and engineering	0.77
New electrical supply	0.24
New Instrumentation	0.6
Retrofit	**3.51**
Revalidation	**1.06**
Contingency factor	*1.15*
Total Process Change Lang Factor	**5.26**

Table 3.4 Capital Investment Lang factors to calculate retrofit and revalidation costs of a 'major' bioprocess change

Description	***f*i**
New Capital investment	1
New Pipework and installation	0.9
New equip design and engineering	0.77
New Building (extension)	1.66
New electrical supply	0.24
New Instrumentation	0.6
Retrofit	**5.17**
Revalidation	**1.06**
Contingency factor	*1.15*
Total Process Change Lang Factor	**7.16**

The new development costs will include scaled down manufacturing and assay development. The new scaled down manufacturing activity is calculated as a function of the new manufacturing cost, as has been done for general biopharmaceutical development manufacturing in section 3.5.3. Any new assay development pre-process change implementation has been estimated as a percentage of the cost for assay development of a new drug, based on the project duration. These costs were verified by industrialists, through personal communications at BPL. Development durations were taken from benchmark data from the survey results in Chapter 2, from literature or based on the 'real' industrial case examples at Bio Products Laboratory.

Comparability study costs are based on those from the manufacturing lots, and QC/QA effort required to analyse and verify the necessary batches. The clinical bioequivalence study cost estimates are dependent on the type of change made and stage the change is made in, and these are classified as extensive or of small scale. These cost estimates have been taken from benchmark data from the survey in Chapter 2, and literature estimates.

As summary of the costing methods used to capture the process change activities is portrayed in Table 3.5. The features of this costing method are further explored in the case study presented in chapter 5.

Table 3.5A Summary of the Process Change Costing Methods

Item	Basis
Manufacturing Costs	f (Detailed process flowsheet)
Revalidation Costs	f (New equipment cost, validation Lang factor)
Retrofit Costs	f (New equipment cost, retrofit Lang factor*)
Comparability Studies	f (Consistency batches, QC/QA)
Clinical Trials Costs	f (Phase of development, type of change)
Development Costs	f (Detailed process flowsheet**, assay development cost)

*Retrofit Lang factor is the summation of capital investment, new pipe work and installation, new equipment design and engineering, new electrical supply, and new instrumentation; ** Indicates pilot scale manufacturing.

3.5.5 PROFIT

Many financial performance metrics can be used to measure the profitability or the potential detrimental costs of implementing a process change proposal. The most widely used techniques are: Payback time, with or without interest, return on investment (ROI), interest of rate of return (IRR), and net present value (NPV) (Humphreys and Wellman, 1996). NPV is used as it gives a good indication of the layout of investments, future costs and revenue outcomes across all phases of development through to commercial phase (Rajapakse, 2005). In this case NPV has been used as an indicator of how much value an investment or project adds or loses as the case may be for a process change. The Net Present Value (NPV) of a project or investment is defined as the sum of the present values of the annual cash flows minus the initial investment. The net or annual cash flows are discounted or adjusted by incorporating the uncertainty and time value of money. The calculation of NPV involves identifying the size and timing of the expected future cash flows generated by the project or investment, determining the discount rate or the estimated rate of return for the project, and calculate the NPV using the equations shown in Table 3.6.

A project should only really be invested in, if the NPV is greater than or equal to zero. If the NPV is less than zero, the project will not provide enough financial benefits to justify the investment, since there are alternative investments that will earn at least the rate of return of the investment. In theory, a company will select all the projects with a positive NPV. However, because of capital or budget constraints a percentage change in NPV can be used to compare process change options. This is determined as follows:

$$= \left(\frac{-}{} \right) x$$

3.5.8

If $NPV_{old}>1$, otherwise

$$= \left(\frac{-}{} \right) x$$

3.5.9

Table 3.6 The steps are used to calculate the portfolio NPV for each year of operation, a*dapted from (Rajapakse, 2005;Rajapakse, 2004)*

Category	**Year (t)**				
	0	**1**	**2**	**…**	**n**
A. Total capital investment					
B. Revenue					
C. Running costs (without depreciation)					
D. Profit (B-C)					
E. Depreciation					
F. Taxable profit (D-E)					
G. Tax (33% of F)					
H. Net cash flow (-A+B-C-G)					
I. Discount factor $(\frac{1}{(1+r)^t})$*					
J. Annual present value (H*I)					
K. Net present value $(\sum_{t=0}^{n} J_t)$					

*r is the discount rate, and t is the year.

3.5.6 RISK

A great deal of uncertainty and risk is present implementing a process change. Factors such as process yields, costs, delays, regulatory approval, pricing, and market share capture all involve a factor of risk. By incorporating the effects of risk, the functionality of the framework was enhanced as it enabled the certainty associated with output measures to be expressed. Once the key uncertainties are identified, probability distributions are assigned in order to reflect the risk of a proposed strategy. Expert opinion was used to identify suitable distributions. Monte Carlo simulation technique is used to determine resulting frequency distributions of the output measures using the '@Risk 5.0 for Excel' risk analysis software (Palisade Corporation, Newfield, NY, USA). The software allows a wide variety of distributions, both continuous as well as discrete to be applied. For each of the distributions, the mean and standard deviations are set before the simulation starts. This allowed the possibility of setting the type of distribution that best described each parameter during simulation studies. For example, the product equivalence study could be described using a discrete distribution, e.g. either with clinical trials or without situation, whereas the variation of the cost of process validation or retrofitting could be specified using a triangular distribution.

Probability descriptions of input variables and Monte Carlo sampling together provide a practical method of finding the distribution of the desired output given the various random and deterministic input variables (Farid, 2001;Rajapakse, 2004).

3.6 CONCLUSIONS

This chapter has provided a hierarchical framework that captures all activities involved in introducing process change activities; capturing both the technical and regulatory activities involved. An overall discussion of the framework set-up and methods used to calculate the cost of a process change activity has been provided. Inputs into the model and the outputs from the tool have been summarised to provide an understanding of the capabilities of this method. Much work has been put into collecting data that can be used as default data for the simulations. These have been presented along with other assumptions made.

The application of the tool is demonstrated through a case study in the next chapter

Chapter 4

Evaluating the Implications of Making Process Changes Throughout a Drug's Lifecycle

4.1 INTRODUCTION

As described in previous Chapters, forced and unforced bioprocess changes can emerge as a cost-reduction exercise, or result from product competition, regulatory rule changes, or to satisfy new customer needs. In this chapter, the conceptual framework and methods suggested in the preceding chapter will be used to assess the economic and manufacturing impacts of making different types of process changes at different stages of drug development. The impact of uncertainties when the changes are made is also explored. The case study in this chapter is based upon a plasma-fractionation process, producing intravenous immunoglobulin (IVIG).

4.1.1 OPPORTUNITIES FOR CHANGE IN PLASMA-DERIVED IVIG FRACTIONATION

An industry where process change modelling is highly relevant is the human plasma fractionation sector. Plasma protein fractionation is by far the largest industry segment in global therapeutic protein manufacture. More than 500 metric tons (about 492 imperial tons) of human serum albumin (HSA) and more than 60 tons of intravenous immunoglobulins (IVIG) are produced annually from more than 22 million litres of source and recovered plasma(Curling, 2002). This $6.9 billion industry supplies products to more than one million patients each year.

The Cohn–Oncley 'backbone' fractionation processes as described in the Chapter 2, was primarily designed to purify albumin. Processes for other plasma proteins have been developed either by addition of (cryo-)precipitation or adsorptive technologies before using ethanol fractionation or by mainly chromatographic processing of fractions of the Cohn system, and so consequently IVIG production is far from optimised. By implementing minor changes and attempting to optimise their current processes, fractionators have the scope to increase yields by another one g/L (Curling, 2002). However, major improvements where process changes and the implementation of high yielding units may also increase yields and purity of the final product significantly, an increase in yield of 70% or more may be seen amongst all products derived from plasma fractionation, as suggested by Curling, 2002. In general, major losses of IVIG of up to

25% (approximately 1.6g/L) commonly occur during Fractionation III or B+1 precipitation stages (Curling, 2002;Teschner *et al.*. 2007).

High pathogen safety and high yields have also become the dominant goals of the plasma fractionation industry, and thus improvements have frequently been made and in some cases still can be enhanced (Buchacher and Iberer, 2006).

The purification principles of IVIG have not changed dramatically in the last two decades (Buchacher and Iberer, 2006). Manufacturers operations have focused on the prevention and removal of aggregates when it turned out that aggregates are responsible for product related side effects. Demand for IVIG has escalated over the past 20 years and currently exceeds availability (Lebing *et al.*. 1999). This growth in the market has come about as a direct result of increased usage in healthcare procedures. The market potential for IVIG is large, but it is virtually impossible for new plasma fractionators to set up because of the huge costs involved, or for existing fractionators to increase their overall production capacity due to a lack of a source of 'processable' human plasma. IVIG yields, however, can be optimised by making changes to existing processes, and there are several possibilities for improving process yields (Lebing*et al.*, 2003; Curling, 2002).

In fractionation circles, regulators still hold the philosophy that 'the process defines the product' and many companies are still reluctant to make any changes. With the framework presented in this chapter, companies can make a more informed choice based on costs, revenues, delays, and uncertainties involved.

The remainder of this chapter is structured as follows. Section 4.2 describes the background to the case study and the process change scenarios investigated. Section 4.3 summarises the methodology and case study assumptions for the initial deterministic analysis. Section 4.4 presents the results of the deterministic comparison of process changes made pre and post-approval. Section 4.5 and 4.6 summarise the methodology results and discussion of the risk analysis and cost where the impact of the technical and regulatory uncertainties on the attractiveness of the process change scenarios was evaluated.

4.2 CASESTUDY BACKGROUND

4.2.1 BPLIVIG PURIFICATION

The IVIG production process at BPL is based upon cold ethanol fractionation. Fresh frozen plasma is purified to selectively precipitate major proteins in plasma, using

variations in concentration of ethanol, salt, temperature. The exact conditions used are a combination of those from Cohn -Method 6 (See Appendix 4- Figure 1) and those developed by Kitsler and Nitchman (1962). Albumin and IgG are largely separated by the second precipitation step (the Fraction A+1 stage) and then undergo a series of further precipitations to remove impurities.Final purification is achieved using ion exchange chromatography with DEAE-Sephadex media to capture impurities. After chromatography the process stream is practically pure IgG (>99%), this is processed further to remove adventitious agents such as viruses and is then adjusted to the desired concentration, formulated and sterile filled ready for distribution (Reynolds, 2004). A basic process flow sheet depicting the steps involved in the IVIG production stream is shown in Figure 4.1.

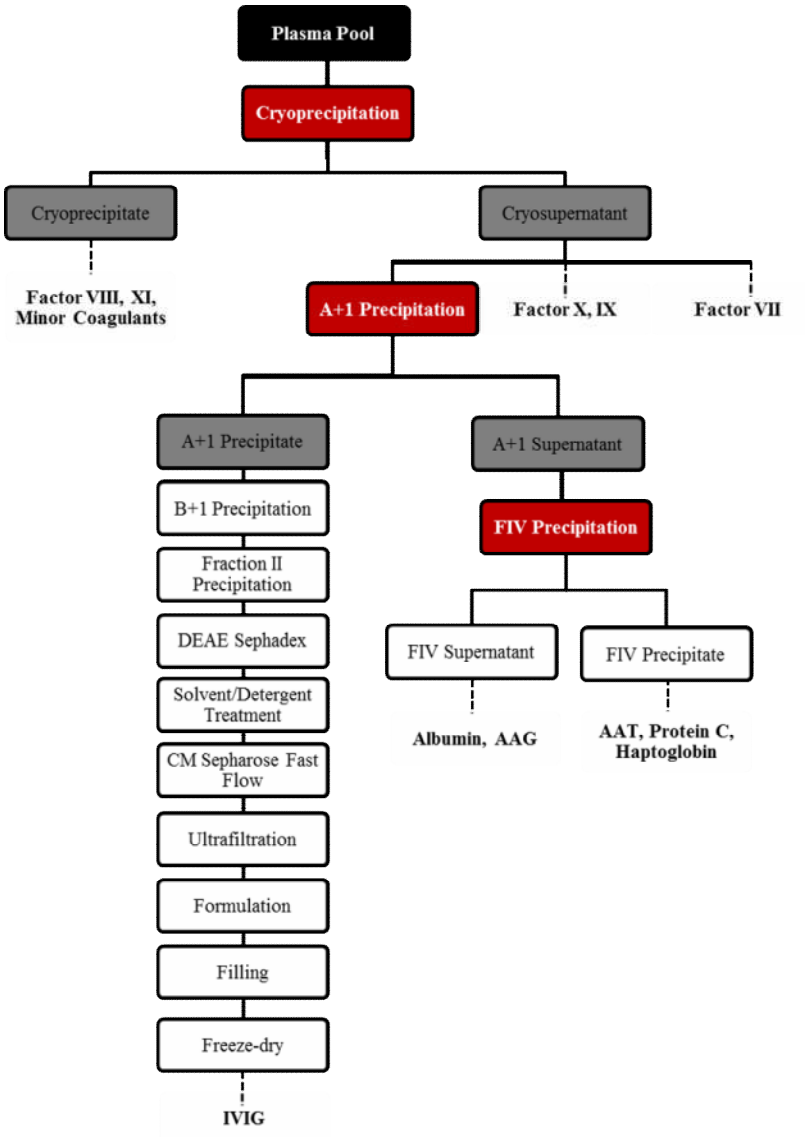


Figure 4.1 A process flow sheet depicting and example of a plasma fractionation scheme based on a combination of Cohn (Method 6) and Kitsler and Nitchmanmethods, with a focus on the IVIG production stream.

4.2.2 SCENARIOS

The use of this framework for assessing the impact of making manufacturing changes on strategic technical and business indicators is exhibited via a case study. The case study is based on a 'real' industry scenario where different changes have been implemented to the IVIG processing stream of a plasma fractionation process over a number of years. The aim of these examples are to illustrate how the decision-support software can be used by biopharmaceutical companies to investigate the effects of optimising their processes to increase the cost-effectiveness of their process, prior to committing to a particular option.

4.2.2.1 FORCED CHANGES SCENARIOS

The IVIG purification process at BPL has undergone a number of changes over the years. Two examples of process changes that were 'forced' upon the company are described in this section. Forced changes could be set by the regulatory authorities or set by customer demand. The 'forced' changes have been categorized as a 'minor' and a 'major' change. The 'forced minor' change scenario was the addition of a viral filtration step to the IVIG process. This step was added to complete a three step viral log reduction, in adherence with newer regulatory requirements, and to satisfy customer preference, see Table 4.1.

If the change was not made, the company could lose a percentage of their market capture, as it is a customer preference, but also it is foreseen that the addition of this step will eventually become obligatory and so, if the change is not made, they could lose all the market.

The 'forced' major change scenario is a set of formulation modifications. Firstly, the final product is modified from a solid freeze-dried form to a liquid formulation. This mainly consists of the removal of a lyophilisation step. Liquid formulations are preferred to those that are freeze-dried, again because of patients, pharmacists and doctor preference. Secondly, the formulation composition was changed, to using Sorbitol and Polysorbate 80 as a replacement stabiliser to sucrose and albumin. Advanced studies have shown that the old formulation containing albumin and sucrose was not tolerated by all patients. Again, this is considered a 'forced' change, because without the modifications, the company risk losing their customers and maybe even

approval, and thus lose a share of their market capture to other IVIG fractionators. Also, the use of albumin as the major stabiliser significantly added to the manufacturing costs of the product, as it is a product itself, and any reduction in such costs is as advantageous.

The risks of having to repeat clinical studies to prove efficacy increases with major changes. In reality, the changes were made at various stages of development and some changes were made simultaneously. However, to gauge the full impact of every change, the changes are modelled separately and at all stages of a drug's life cycle.

4.2.2.2 A MAJOR YIELD-IMPROVING CHANGE SCENARIO

One type of change that has not been implemented to IVIG process stream, is one that would a greatly enhancing the product yield. Typical processing yields purify IVIG to 2.5–4.5 g/L. As stated earlier, minor process optimization can improve yields by approximately1 g/L (Curling, 2002). Only major modifications to the process stream can increase yield to up to 70% or more. These changes need to come from the implementation of high yielding unit operations. At BPL, losses of 25% (approximately1.6g/L) are identified at B+I fraction stage (seeFigure 4.2), whilst moderate or low losses are also seen at other stages (BPL Internal Presentation, 2005). There is good evidence to suggest that high recoveries can be achieved by replacing fractionation steps with chromatographic processes (Curling *et al.*. 2005). The replacement of this ethanol fractionation step may improve selectivity and specificity to yield a product with a higher purity. Enhanced binding capacity may allow for downsizing the following separation steps and improving productivity (Burnouf, 1995; Lebing, 2003) suggest that chromatographic steps replacing the traditional ethanol fractionation steps decrease overall production time, increase IVIG yield from plasma, improve physiological IgG and purity. Therefore a proposal has been made to establish chromatography-based purification stages, downstream process from Fraction II to bulk formulated product. This change will lead to modifications that will require an adjustment of the subsequent process steps. One such example of the replacement of 'B+1' fractionation and possible subsequent steps has been suggested at BPL(BPL Internal Presentation, 2005), and is shown in Figure 4.2 d). This modification will require extensive validation to demonstrate the quality and safety of the final purified product, which will be costly and time consuming. Again, as this is a major change full-scale clinical efficacy studies may be necessary, if insufficient product and process

comparability is proven through bioequivalence studies alone. This is the riskiest process change suggested, but if successful, could reap long-term profits.

Table 4.1 Minor and major manufacturing changes that were implemented to the IVIg production stream of a plasma fractionation process, the reasons for the change and the foreseeable impact.

Change Type	Change Description	Reason for change	Negative Impact	Positive Impact
'Forced' Minor Change	Addition of new viral inactivation step.	FDA requirement: modification is made to improve virus safety. This is a patient/doctor preference. Without the additional virus inactivation step the product will not be licensable in UK, US and Europe in the future.	Direct and Indirect costs. Retrofit (Shutdown) delay Potential yield decrease Proof of comparability required.	This enables the company to maintain it's market share capture. Premium pricing can be adopted.
'Forced' Major Change	Formulation changes: Product changed from lyophilised (solid) to liquid form. The product is formulated with sorbitol and polysorbate 80 instead of sucrose and albumin.	The liquid form product is a patient/doctor preference. Easier room-temperature storage The new formulation (minus sucrose) is tolerated by more patients.	No significant capital investment required No significant shutdown period. No impact on yield Efficacy studies are required, because the product is considered to be a new product.	This enables the company to maintain it's market share capture. Premium pricing can be adopted. No significant capital investment is required.
Major Yield-Enhancing Change	Replacement of B+1 precipitation with QXL Fast flow Chromatography.	Enhanced productivity Improved process robustness	Direct and indirect costs. Expensive column Retrofit (shutdown) delay Greatest risk of new clinical efficacy study data, because the product is considered to be a new product.	Yield improvement. Potentially reduced direct cost of goods per gram.

This yield-enhancing change suggestion as well as the forced change scenarios have all been modelled at early phase and late phase development, as well as after the product has been commercialised. The scenarios have been summarised in Table 4.1, and a comparison of the different IVIG purification streams is shown in Figure 4.2.

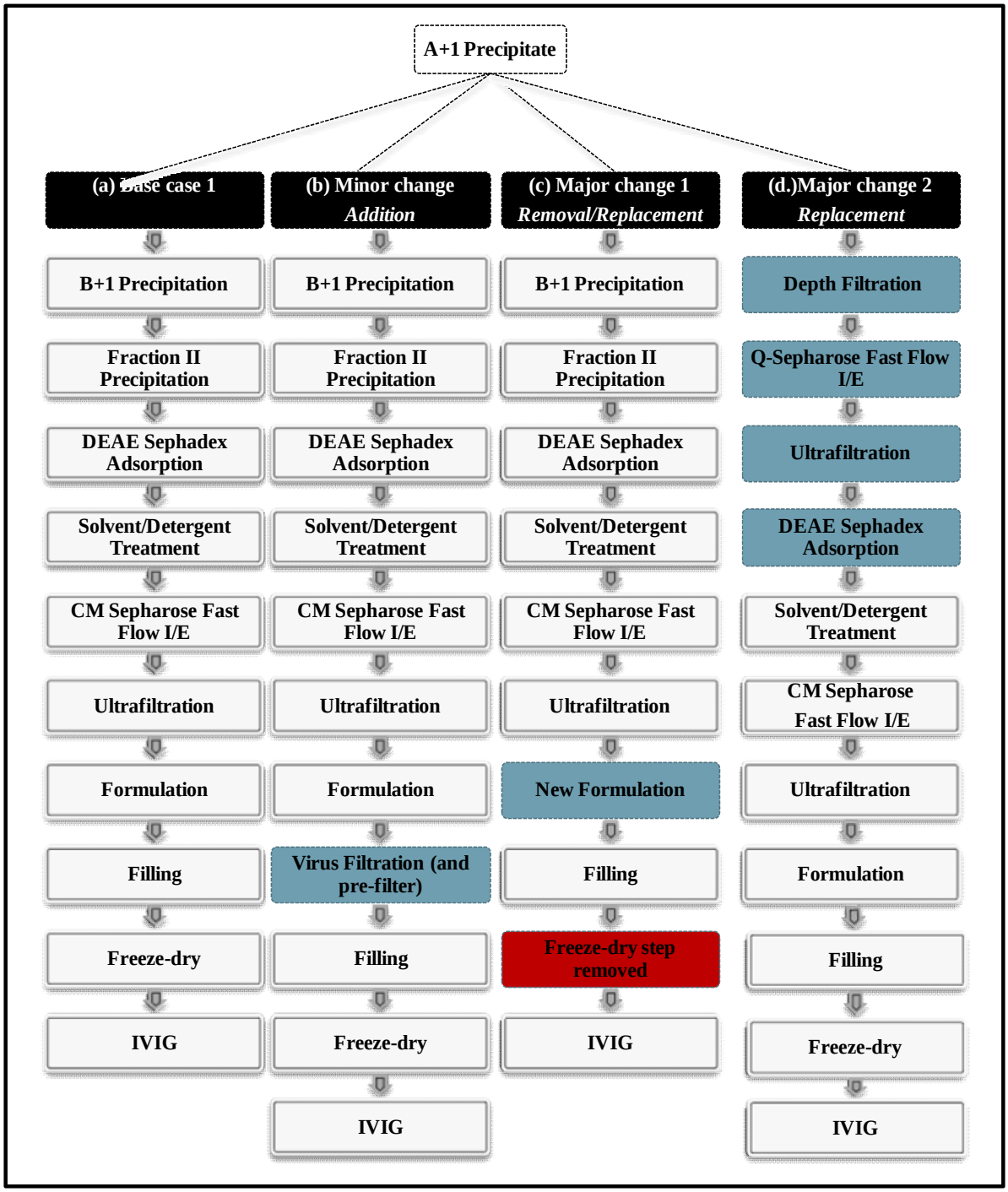


Figure 4.2. Process change scenariosinvestigated: (a) the base case (b) a minor change with the an additional virus inactivation step, (c) a major change where the formulation process is modified and the freeze dryer step is removed, and (d) a major change where Fraction B+1 precipitation is replaced with a chromatography-based purification

step**.***Note. The formulation steps refers to the addition of either sucrose and albumin or sorbitol and Polysorbate 80.*

4.3 METHOD- DETERMINISTIC ANALYSIS

4.3.1 DATA COLLECTION

Part of the work involved in preparing a tool for prototyping drug development is to collect data to populate the model and verify the outputs. In this study, BPL's IVIG manufacturing process was modelled, attempting to keep equipment sizes, and costs, and resources utilised as realistic as possible, so as to be able to verify whether process change activities simulated were acceptable or not. Therefore, the bulk of the data used was obtained from personal communications at BPL, such as, facility size, material costs, equipment sizes, plasma costs. However not all data was readily available, and the remainder of costs were obtained from literature, such as clinical trial costs, risks and durations (DiMasi *et al.*. 2003;EMEA: Committee for Proprietary Medicinal Products, 2000;Rajapakse, 2004), typical unit yields (Curling, 2002;Farid, 2001). Other costs for equipment were obtained from Jacobs Engineering Group Inc., UK. Process change activity costs and durations were obtained from benchmark data resulting from the survey in Chapter 3. Some costs, such as those for process validation and assay development were given 'ballpark' costs obtained from outsourcing websites, such as Immunochemistry technologies LLC, and verified with industrial experts at UCL and BPL. Probability distributions used to simulate the risk involved in the process change activities were approximated based on typical scenarios situations and again validated via discussions with industrialist experts. While sensible inputs were sought, the prime target was to demonstrate the application of the framework to capture all the activities involved in a process change scenario.

4.3.2 MANUFACTURING AND FACILITY ASSUMPTIONS AND INPUTS

A summary of the default input values used in the model is given in Table 4.2 and Table 4.3. As described in the previous chapter, the method for calculating the fixed capital investment was obtained by multiplying the total equipment purchase cost by a factor, traditionally termed the Lang factor. The Lang factor was assumed to have a base value of 8.13 according to Novais*et al.*. (2000). The default value for the annual facility cost of general utilities per unit floor area was assumed to be \$300/m^2(Lim*et al.*, 2004*)*.

The costs for known resources were inputted as raw data, these costs were sourced from communications at BPL, and were based on their true sizes. However, some of the data costs acquired from literature and Jacobs engineering Inc, were estimated using a ratio of their sizes, raised to an index value.

This is shown in the following equation:

$$= \left(\frac{\quad}{\quad}\right)$$

4.3.1

Where n is the index value, 0.6 (based on the six-tenth rule used by process engineers (Sinnott, 1993). Some of the inputs, such as the plasma source, and initial process steps are used to manufacture other plasma-derived products such as albumin, and the clotting factor proteins, and so these costs and resources are divided amongst all the products. IVIG, the current cost-driver in the fractionation process will typically use 40% of the resources shared amongst the whole plant, and use 50% of resources shared solely between IVIG and albumin.

Table 4.2A summary of some of the default model inputs

Default Inputs	
IVIG or Plasma Input	**Value**
Average plasma start pool weight (kg)	av. 6200
Cost of plasma ($/g)	130
Max number of batches/Week	2
Typical number of batches/Year	80
Market patient size in the UK	3000
IVIG Market Capture (%)	40%
Av. Vol Blood Plasma/ Batch (L)	6200
IVIG selling price ($)	45
Facility Input	**Value**
Lang Factor	8.1
Depreciation Period (%)	10
Facility Size for IVIG manufacture (m^2)	7604
Equipment cost estimation factor	0.6
General Utilities Cost Per Unit Area ($)	300
NPV Input	
Project Duration (Yr)	12
Depreciation (%)	10
Interest rate (%)	10
Discount factor (%)	10
Tax (%)	33
Exchange rate	1GBP=1.6USD (2009) 1EUR=1.5USD (2009)

Table4.3A summary of some of the resource cost data collected for the manufacturing calculations

Manufacturing Resources		
Labour		**Cost ($/hr)**
Operator		30
section manager		60
QC/QA Staff		50
Utilities		**Cost ($)**
WFI		0.032/L[1]
Steam		0.0144/Kg
Cooling Water		0.001/L
DEMIN		0.01/Kg
Chemicals and Biochemcials		**Cost ($)**
Sodium Hydroxide		4/Kg
Sodium Chloride		1.5/Kg
Polysorbate 80		50/Kg
Sucrose		9/Kg
Albumin		3000/Kg[2]
TnBP (tri(n-butyl)Phosphate detergent)		51/L
Glycine		16/L
Soy bean oil		55/L
Glacial Acetic Acid		5/L
20% Ethanol		670/tonne
Sorbitol		59/Kg
Filter Aid (Kg) Hyflo Super-Cel		944/Kg[3]
Consumables	**Size**	**Cost ($)**
Ultrafiltration modules	1m^2	1,500
Virus removal pre-filter cartridge	1 m^2	800
Virus removal 50nm Virus filter	36 m^2	6,300[4]
Membrane Cassettes ($/unit)	0.5 m^2	2,100
Lenticular depth filter sheet	3.2 m^2	240
depth filter (0.5μm)	10"	128
Plate and frame sheets	-	2
0.22 Micron Cartridge Filter () ($/unit)	0.05 m^2	75
DEAE - Sephadex matrix($/Kg)	1kg	1,838
Ion Exchange Matrix ($/L) (reusibility * 250)	1L	420

1.(WFI costs varied from £5-16/m3)
2.£15000/Kg
3.£8850/15Kg
4. £3960/Filter

Manufacturing Resources		
Equipment	**Size**	**Cost ($)**
Cost of Balance (weighing)	-	5,000
Cost of crusher	-	5,000
Plasma thawing tank	-	36,000
Holding Tank	1000L	37,500
Jacketed Fraction Vessel	500L	32,000
Disk Stack Centrifugation	65,00rpm	160,000
Heat Exchanger	$100m^2$	10,579
DEAE Sephadex adsorption Tank	600L	16,000
Vibromixer	-	8,000
Silverson Mixer	-	16,000
CM Sepharose Fast Flow chromatography column	1.6m diameter	208,000
Virus Inactivation Tank	$0.1m^3$	2,750
Plate and frame filter press system	-	16,000
Cuno Housing Filtration system2 housings/3trolleys	-	1,320,000[1]
Depth Filtration Housing (Fraction B+1)	-	9,600
Virus Filtration housing	14.5m by 10m	112,000
Filtration system	-	320,000
Millipore UF unit	14.5m by 10m	700,000
Depth Filtration unit	-	320,000
Dead-End Filtration unit	-	75,000
CIP Vessel (mobile)	-	120,000
Large Scale Chromatography System with Process Control	-	1,000,000
Chromatography column, Height = 20cm, D= dm	0.7	120,000
	0.8	165,000
	1	200,000
	2	550,000
Chromatography Rig	-	250,000
jacket 500L vessel cost- 20K	500	32,000
Mobile vessel	500	80,000
Freeze dryer	-	320,000
Filter press	-	400,000
Mobile vessel	500	80,000
Bottle & Stopper filling system	-	6,400,000

1- £35,000 per trolley

4.3.3 IVIG CLINICAL TRIALS

In general, clinical trial studies for plasma-derived products require fewer patients than other therapeutics, as their toxicity is not an issue considering their human origin. The studies do not involve a volunteer phase (normally phase I), but rather begin in patients

as phase I/II. Also, a number of plasma products are considered to be 'orphan drugs', which again limit the extent of clinical studies required and the number of patients involved (EMEA: Committee for Proprietary Medicinal Products, 2000; personal communication with Thierry Burnouf, Human Protein Process Sciences, Lille, France).

IVIG is primarily used to treat patients with antibody deficiencies, as described in Chapter 1, of which there are many indications. It is assumed that two primary studies took place: a primary antibody deficiencies (PAD) trial, this covers congenital agammaglobulinaemia,hypogammaglobulinaemia, and common variable and severe combined immunodeficiencies, and ITP trials (Idiopathic Thrombocytopenic Purpura) which also covers, Kawasaki disease and Guillain-Barré Syndrome indications. The costs, durations, and patient numbers used to calculate clinical study costs in this study are given in Table 4.4.

Table 4.4 Resource assumptions for Clinical Trial of plasma derived IVIG

Phase of Clinical trial (IVIG)	Costs ($)	Duration (months)	Number of Patients
Pre-clinical Studies	$0.16M	72	-
Phase II Studies	$0.4M	2	15-50
Phase III Studies	$3M	15	50-100

4.3.4 NET PRESENT VALUE

The drug life cycle is modelled from the pre-clinical study phase through to commercial phase, and then for another 12 years. A typical drug life cycle runs 12–15 years after its launch (Pandey, 2003). A depreciation value of 10% was used for the calculations, the tax was set at 33% and a discount factor of 10% was used. At the end of each year the model outputs the year's expenses and revenues in order for the NPV calculation for that particular year to be performed. A payback period of ten years was used for loans. The interest was set at 10%.

4.4 RESULTS- DETERMINISTIC ECONOMIC EVALUATION

4.4.1 COST OF GOODS

Cost comparisons of the base case, a 'forced' minor change, an addition of a virus inactivation step, a 'forced' major change where the formulation is modified, and a major change- where B+1 precipitation is replaced with a chromatography-based purification step is shown in Figure 4.3. In the plasma fractionation industry direct costs are much higher than that of typical pharmaceutical manufacturing process, mainly due

to the exceptionally high raw material cost of plasma source at approximately $150/L(Burnouf, 2005;Waller, 2005).The plasma source makes up 33% of costs in the base case, with 29% making up the remainder of the direct costs. In industry, the plasma costs have been shown to make up 22%-50% of total costs depending on the scale of operation (Curling *et al.*. 2005;Curling *et al.*. 2009). The minor change shows an increase in raw material cost, and this changes the cost percentage breakdown somewhat. The major formulation change shows a significant decrease in raw material costs, mainly from the removal of albumin in the product (IVIG) formulation. Albumin is itself a product and has a retail price of $3/g, this is removed along with sucrose at $0.01/g, and both replaced with Polysorbate 80 at $0.05/g and Sorbitol at $59/Kg. This 'forced' change example gives a beneficial outcome when evaluating the cost of goods alone. The major chromatography process change in Figure 4.3 d) portrays a significant reduction in raw material usage, decreasing the percentage costs from 13% to 7%. There is also a considerable increase in indirect costs mainly from the new capital invested in new equipment.

Figure 4.4 shows the total annual cost of goods per gram categorised into direct and indirect costs of the different process change scenarios. The variation in total cost of goods per gram is principally from the direct costs. Only the major chromatography change shows a significant increase in indirect cost of goods from $17/g to $20/g. Here the minor change shows an increase in direct costs mainly arising from $30,000 in additional direct raw material costs, this will in turn impacts on the miscellaneous raw material costs calculated and the total direct costs. The slight decrease in variable and fixed costs seen in the major formulation change is due to the removal of a freeze dryer unit and a decrease in raw materials costs described earlier. The total cost of goods per gram remains similar at $43/g, $47/g, and $42/g for the base case, the minor change and

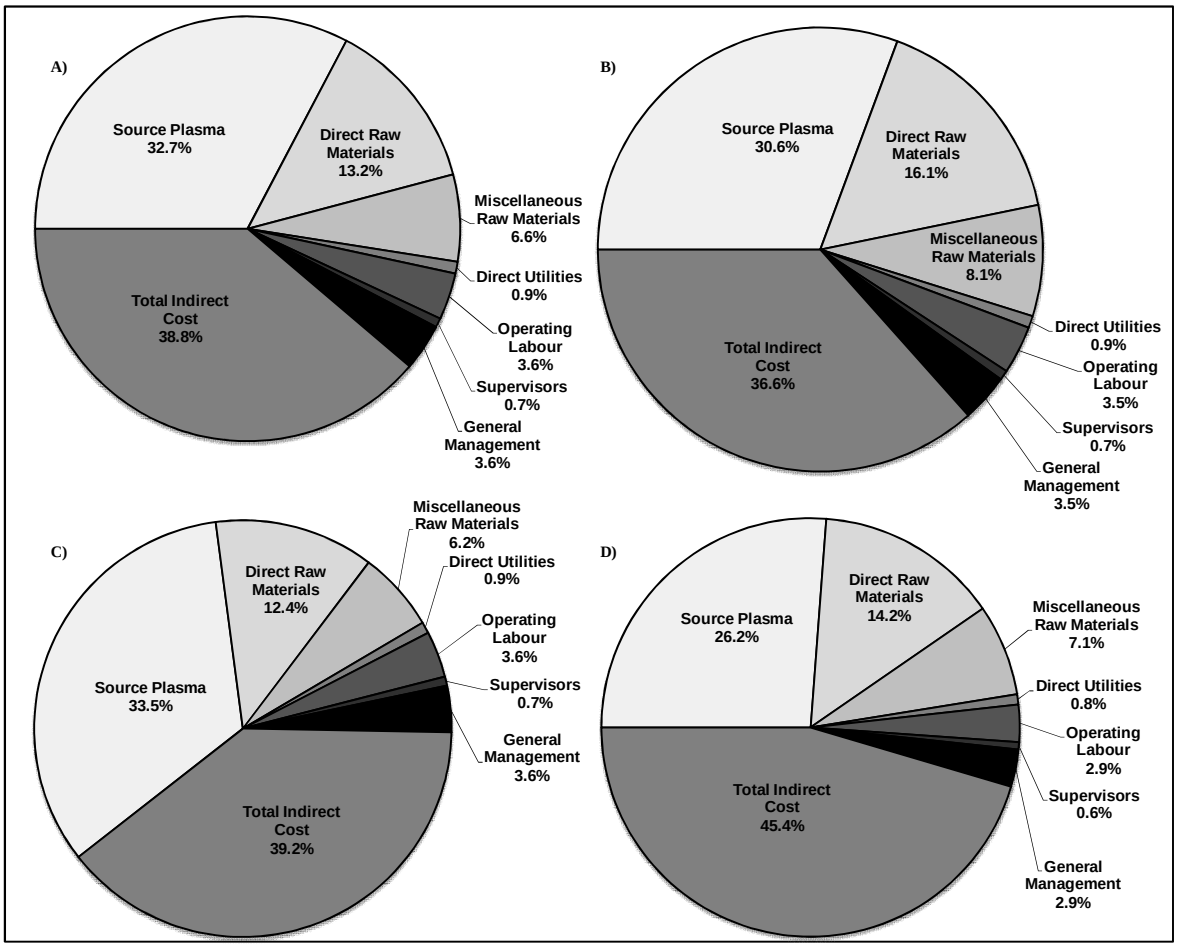


Figure 4.3 Process change scenario cost comparisons of (A) the base case, (B) a 'forced' minor change -an addition of a virus inactivation step, (C) a 'forced' major change where the formulation is modified (D) a major change- where B+1 precipitation is replaced with a chromatography-based purification step.

the formulation change respectively. It is however, significantly decreased to $32/g for the chromatography replacement option, making it a desirable option. This however does not take into account 'process change activity' costs.

4.5 METHOD- RISK ANALYSIS

For every scenario, the process change framework was used to capture all the subsequent activities that may be encountered. All three scenarios were implemented at early phase clinical trials, late phase development, and post product approval. To be able to compute the impact of a process change scenario, a number of cost, duration, and uncertainty assumptions had to be made. The uncertain inputs are assigned probability distributions and calculated using Monte Carlo simulations.

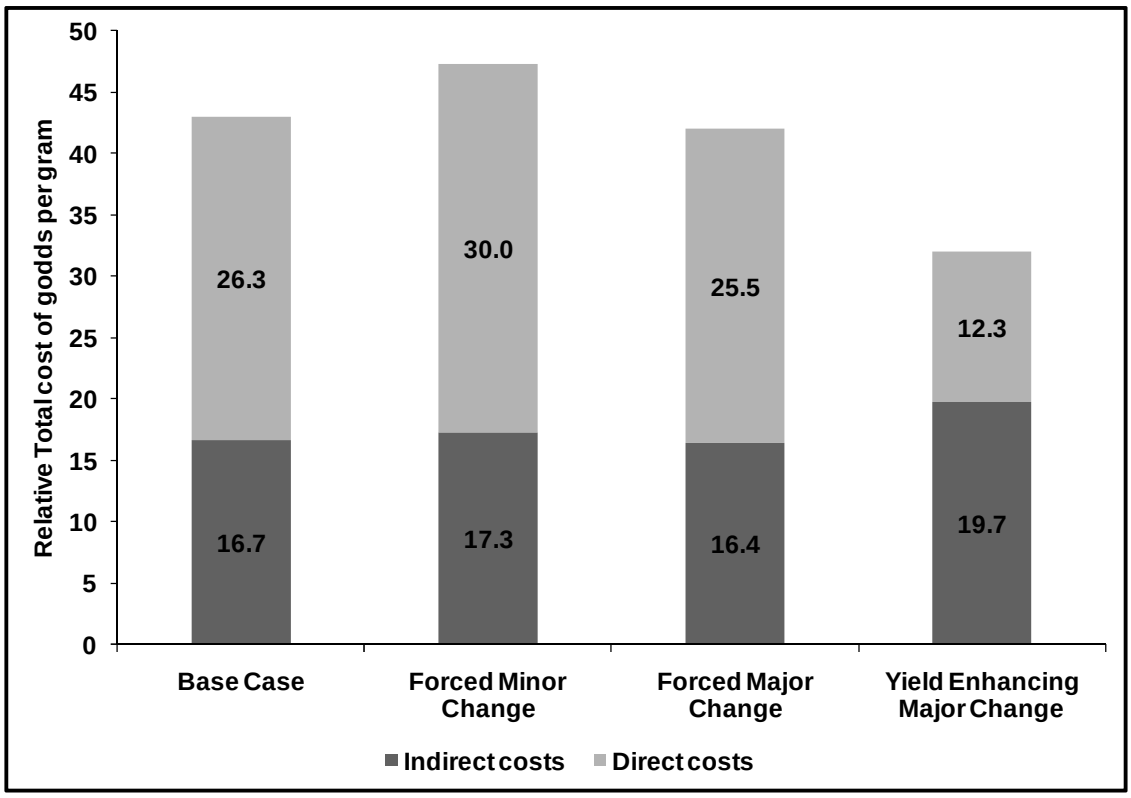


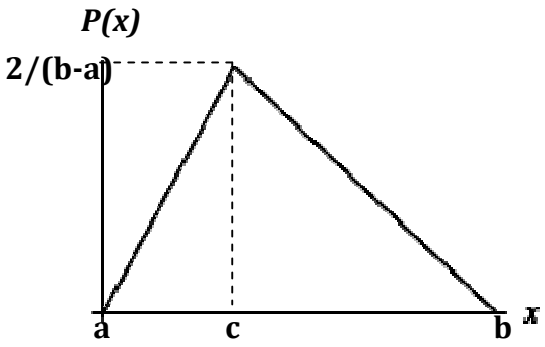
Figure 4.4 The total annual COG/g (direct and indirect costs) comparison of different process change scenarios

4.5.1 PROBABILITY DISTRIBUTIONS

Probability distributions describe the range of possible values that a random variable can attain and the probability that the value of the random variable is within any measurable subset of that range. The distribution types used in the scenarios are described next.

4.5.1.1 TRIANGULAR DISTRIBUTIONS

In probability theory and statistics, the triangular distribution is a continuous probability distribution with lower limit (a), mode (b) and upper limit (c).



Where the probability is:

$$( ) = \begin{cases} \frac{(-)}{(-)(-)} & \leq \leq \\ \frac{(-)}{(-)(-)} & \leq \leq \end{cases}$$

4.5.1

For each triangular probability distributions the lower limit, mode and upper limit represent the worst case, base case and best-case scenarios, respectively. For discrete distributions, the probability of occurrence is given. In this study triangular distributions have been used to model uncertainty in costs, such as revalidation and retrofit costs.

4.5.1.2 DISCRETE DISTRIBUTIONS

A discrete distribution describes the probabilistic properties of a random variable that takes on a set of values that are discrete, i.e. separate and distinct from one another. Discrete values are separated only by a finite number of units. In this study it has been used in situations where there is an 'either/or' scenario. For example, product equivalence activities will entail either non-clinical comparability studies, or clinical bridging studies.

4.5.2 MONTE CARLO SET-UP

To incorporate the risk of uncertainty in key input factors, the inputs were assigned probabilities of occurrence and distributions, and Monte Carlo simulations were performed. The Monte Carlo method relies on repeated random sampling to compute the results.

4.5.3 SCENARIO ASSUMPTIONS

Table 4.5shows a list of the key input values, assumptions and probabilities used to model the process change scenarios. Assumptions for the key activities involved in implementing the change, their costs, durations and risk of occurrence have been suggested for the three different stages of development, as well as for the scenario possibility of no change being made, this is considered the base case option.

- Batch number - the number of batches was kept the same throughout all process change scenarios. The batch size is determined by the plasma pool donated, on average 6200L per batch, with a capacity to run 80 batches per year. However, to account for possible batch losses or potential increased pool sizes, a triangular probability distribution with the possibility of ± 5 batches was used.

- Selling price - it is assumed that the selling price will not vary with the process changes. Uncertainty in the pricing from competition and fluctuations in the market have been reflected with a ±10% triangular distribution.
- Annual cost of goods – the cost of goods may vary for the base case and with the minor and major formulation changes mainly from direct cost uncertainties such as raw material costs and utility usage. For the major yield improvement change, the costs are more uncertain, due to new equipment costs, as well as direct cost uncertainties.
- Retrofitting and revalidation costs - these have been given a ±25% triangular distribution.
- Product equivalence activities – this will either consist of solely non-clinical comparability work or will also include clinical studies. This has been given a discrete probability of occurrence. The likelihood of clinical trials occurring will increase with the size of the change being made as well as the timing. The later the stage of development, the more likely clinical trials will need to be repeated.
- The cost of product equivalence - this also increases with the change magnitude and timing. This is also uncertain and has been given a ±25% triangular distribution.
- Delays to market– delays to the market may occur whilst a change is being implemented. This disruption includes retrofitting, revalidation ad regulatory approval d setbacks. Once a product is approved and is commercial, the product can be stockpiled whilst a change is being made, and so will result minimal losses in revenue.
- Market share – the company may see market share losses if customer requirements are not met, i.e. if the 'forced' changes are not made. Therefore, losses are more likely to occur in the 'no change' case. The scenarios are investigated with and without market share loss considerations. The demand of IVIG normally surpasses the demand, (although this may be changing with increased competition), and so the market share may not be affected. Although, if regulatory recommendations are not satisfied, it will be affected.

Table 4.5 Inputs, assumptions and probabilities used to model the process change scenarios. For each triangular probability distributions the first, second and third numbers represent the worst case, base case and best-case scenarios, respectively. For discrete distributions, the probability is of occurrence is given. Where only one number is present, that value remains constant through all scenarios.

Input	Probability Distribution	Uncertainty Variable	No Change	Early phase development	Late phase development	Post-product approval
Number of batches (#)	Triangular	No. Of batches +/- 5	Tr(75,80,85)	Tr(75,80,85)	Tr(75,80,85)	Tr(75,80,85)
Selling Price/gram ($)	Triangular	Selling price, +/-10%	Tr(37,45,47)	Tr(37,45,47)	Tr(37,45,47)	Tr(37,45,47)
Minor change Scenario						
Manufacturing						
Annual Cost of goods ($)	Triangular	Annual cost of Goods +/- 5%	Tr(-5%,78, +5%)	Tr(-5%,84, +5%)	Tr(-5%,84, +5%)	Tr(-5%,84, +5%)
Retrofit ($ in millions)	Triangular	Revalidation cost +/- 25%	-	Tr(-25%,0.02M,+25%)	Tr(-25%,0.02M,+25%)	Tr(-25%,0.02M,+25%)
Revalidation ($ in millions)	Triangular	Retrofit cost +/- 25%	-	Tr(-25%,0.3M,+25%)	Tr(-25%,0.3M,+25%)	Tr(-25%,0.3M,+25%)
Development ($ in millions)	-	-		0.05M	0.05M	0.05M
Product Equivalence study	Discrete	Comparability or clinical bridging study cost	-	Discrete(0.9,0.1)	Discrete(0.8,0.2)	Discrete(0.5,0.5)
Clinical bridging study cost ($ in millions)	Triangular	Comparability cost +/- 25%	-	Tr(-25%,0.29M,+25%)	Tr(-25%,0.58M,+25%)	Tr(-25%,0.58M,+25%)
Comparability study ($ in millions)	Triangular	Clinical study cost +/- 25%	-	Tr(-25%,0.58M,+25%)	Tr(-25%,0.58M,+25%)	Tr(-25%,1.58M,+25%)
Market						
Regulatory Activity delay (Years)	Discrete	Comparability or clinical bridging study delay	-	Discrete(0.5,1)	Discrete(1.5,2)	Discrete(0.75,1.5)
Market Share loss (%)	*Triangular*	-	*Tr(0,50,70)*	*IF comparability study, Tr(0,5,50)*	*IF comparability study, Tr(0,10,50)*	*IF comparability study, Tr(0,10,50)*
	Triangular	-		*IF clinical study, Tr(0,10,50)*	*IF clinical study, Tr(0,25,50)*	*IF clinical study, Tr(0,15,50)*

Input	Probability Distribution	Variable	Early phase development	Late phase development	Post-product approval
Major formulation change Scenario					
Manufacturing					
Annual Cost of goods ($)	Triangular	calculated annual Cost of Goods +/- 5%	Tr(-5%,77, +5%)	Tr(-5%,77, +5%)	Tr(-5%,77, +5%)
Retrofit ($ in millions)	-	-	-	-	-
Revalidation ($ in millions)	-	-	-	-	-
Development ($ in millions)	-	-	0.05M	0.05M	0.05M
Product Equivalence ($ in millions)	Discrete	Comparability studies or clinical bridging studies	Discrete(0.9,0.1)	Discrete(0.3,0.7)	Discrete(0.3,0.7)
Clinical bridging study cost ($ in millions)	Triangular	Comparability cost +/- 25%	Tr(-25%,0.29M,+25%)	Tr(-25%,0.58M,+25%)	Tr(-25%,0.58M,+25%)
Comparability study ($ in millions)	Triangular	Clinical study cost +/- 25%	Tr(-25%,1.58M,+25%)	Tr(-25%,2.58M,+25%)	Tr(-25%,3.58M,+25%)
Market					
Regulatory Activity delay (Years)	Discrete	Comparability study delay or clinical bridging study delay	Discrete(1,1.5)	Discrete(2,3)	Discrete(1.5,2.5)
Market Share loss (%)	*Triangular*	-	*IF comparability study, Tr(0,5,50)*	*IF comparability study, Tr(0,30,50)*	*IF comparability study, Tr(0,5,70)*
	Triangular	-	*IF clinical study, Tr(0,15,50)*	*IF clinical study, Tr(0,50,50)*	*IF clinical study, Tr(0,15,70)*

4.6 RESULTS- RISK ANALYSIS

In addition to the cost of goods, Net Present Value (NPV) is used to assess the impact of making the different process changes. This is where the process change activities have been taken into account.

When the key uncertainties are incorporated, the range of possible expected NPV values

Input	Probability Distribution	Variable	Early phase development	Late phase development	Post-product approval
Major Chromatography Yield Enhancement Change Scenario					
Manufacturing					
Annual Cost of goods ($)	Triangular	Calculated annual Cost of Goods +/- 5%	Tr(-10%,73, +10%)	Tr(-10%,73, +10%)	Tr(-5%,73, +10%)
Retrofit ($ in millions)	Triangular	Calculated revalidation cost +/- 25%	Tr(-25%,1.1M,+25%)	Tr(-25%,1.1M,+25%)	Tr(-25%,1.1M,+25%)
Revalidation ($ in millions)	Triangular	Calculated retrofit cost +/- 25%	Tr(-25%,18.8M,+25%)	Tr(-25%,18.8M,+25%)	Tr(-25%,18.8M,+25%)
Development ($ in millions)	-	-	0.15M	0.15M	0.15M
Product Equivalence ($ in millions)	Discrete	Comparability studies or clinical bridging studies	Discrete(0.9,0.1)	Discrete(0.3,0.7)	Discrete(0.3,0.7)
Clinical bridging study cost ($ in millions)	Triangular	Comparability cost +/- 25%	Tr(-25%,0.29M,+25%)	Tr(-25%,0.58M,+25%)	Tr(-25%,0.58M,+25%)
Comparability study ($ in millions)	Triangular	Clinical study cost +/- 25%	Tr(-25%,1.58M,+25%)	Tr(-25%,2.58M,+25%)	Tr(-25%,3.58M,+25%)
Market					
Regulatory Activity delay (Years)	Discrete	Comparability study delay or clinical bridging study delay	Discrete(1,1.5)	Discrete(2,3)	Discrete(1.5,2.5)
Market Share loss (%)	*Triangular*	-	*IF comparability study,Tr(0,5,50)*	*IF comparability study, Tr(0,30,50)*	*IF comparability study, Tr(0,5,70)*
	Triangular	-	*IF clinical study,Tr(0,15,50)*	*IF clinical study, Tr(0,50,50)*	*IF clinical study, Tr(0,15,70)*

and their likelihood can be calculated. The expected NPV(ENPV), i.e. the mean of each distribution, was computed for each scenario based on the inputs and assumptions shown inTable 4.5, firstly, without considering market share loss uncertainties and then including market share losses.

4.7 ENPV WITHOUT THE UNCERTAINTY OF MARKET SHARE LOSSES

Figure 4.5 shows the distribution of thenet present value for the 'no change scenario' compared to the 'forced' minor change scenario implemented at the various stages of development: early phase development, late phase development, and post-product approval. Making no change seems to be the most attractive option, with the most positive percentagechange in ENPV over a 13 year time period, taking all uncertainties into account . The early and late phase changes shift the distribution curves significantly to the left indicating that the model predicts that making changes during development significantly reduces the likelihood of it being profitable. Post-approval and early phase changes give a bimodal distribution depending on whether a repeat of clinical trials is required or not, but theENPV is more attractive for the change made post-product approval.

Figure 4.6 and Figure 4.7portray the distribution of the net present value in for the 'forced' major change, and the major yield-enhancing change. There is not a significant difference in NPV distribution between the two major change scenarios. In both cases, the change made at early phase development is less profitable than the minor change, but has a similar ENPV to the post-product approval change. Late phase changes again show the least desirable outcomes, with an even greater decrease in ENPV. Again, a binomial distribution is portrayed, this is dependent on whether clinical trials or comparability studies are necessary, clinical trials giving the most undesirable NPV. For the minor changes, we do not see binomial distributions, as the NPV is most sensitive to the delays to market caused by the change; as the comparability study costs will be lower, as in most cases there will be no requirement for a repeat of clinical trials.

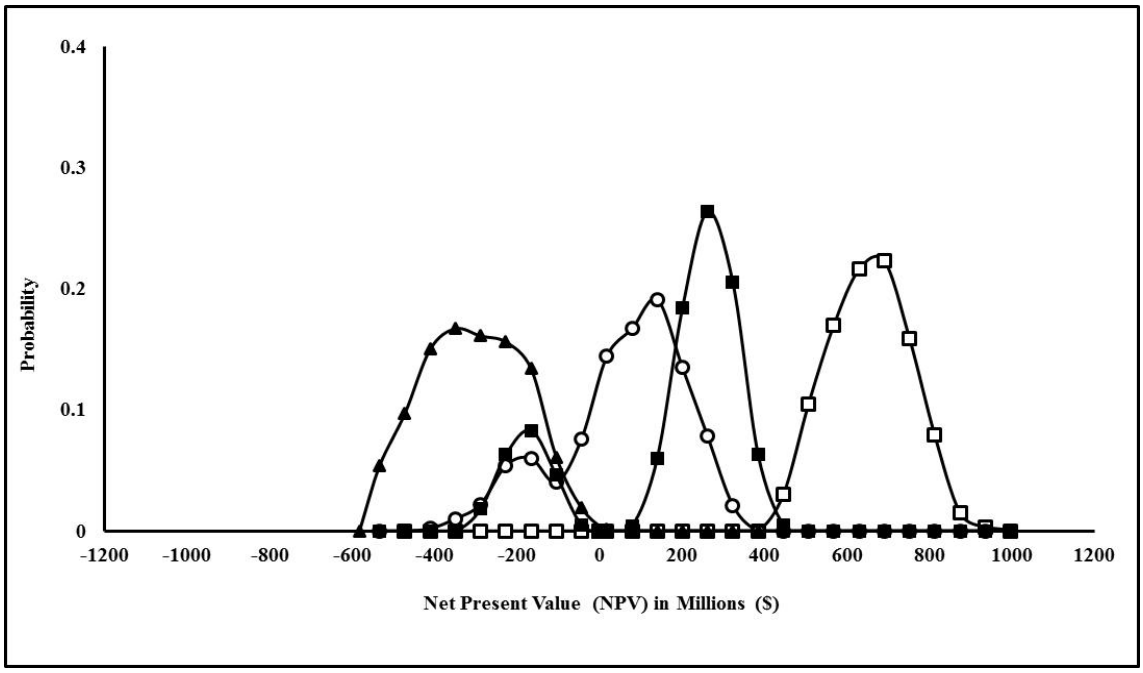


Figure 4.5 Distribution of thenet present value in for a 'forced' minor change scenario at the various stages of development: no change (□), early phase development (○), late phase development (▲), and post-product approval (■).*The risk of potential market share losses is not taken into account.*

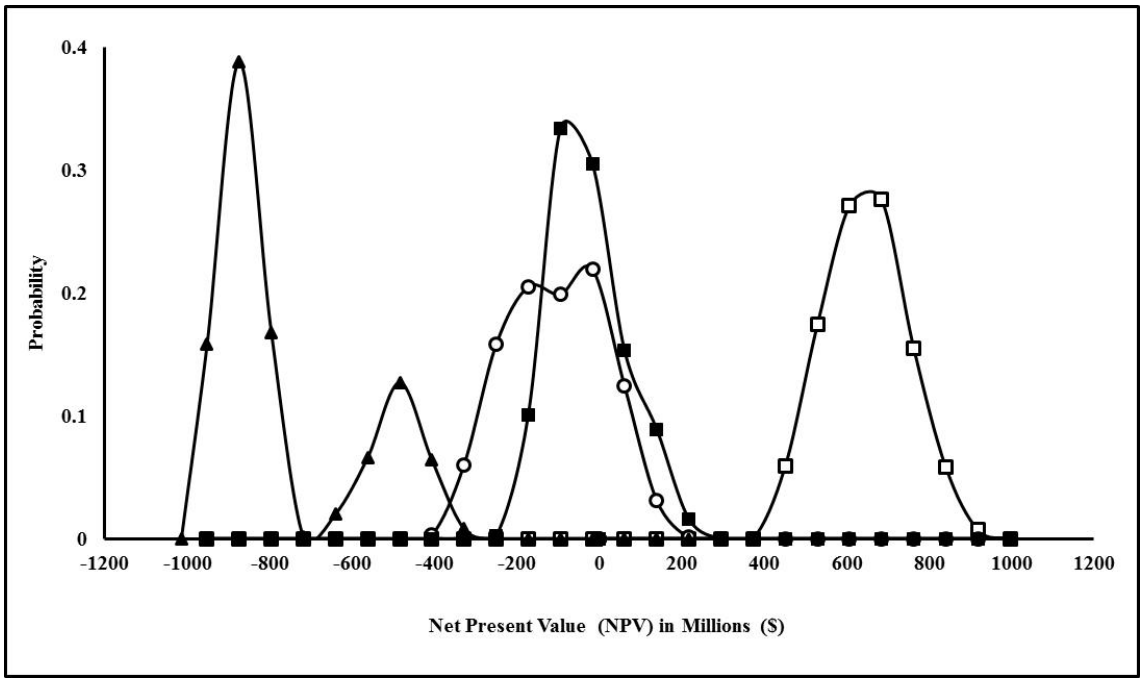


Figure 4.6 Distribution of the net present value in for a 'forced' major change scenario at the various stages of development: no change (□), early phase development (○), late phase development (▲), and post-product approval (■).*The risk of potential market share losses is not taken into account.*

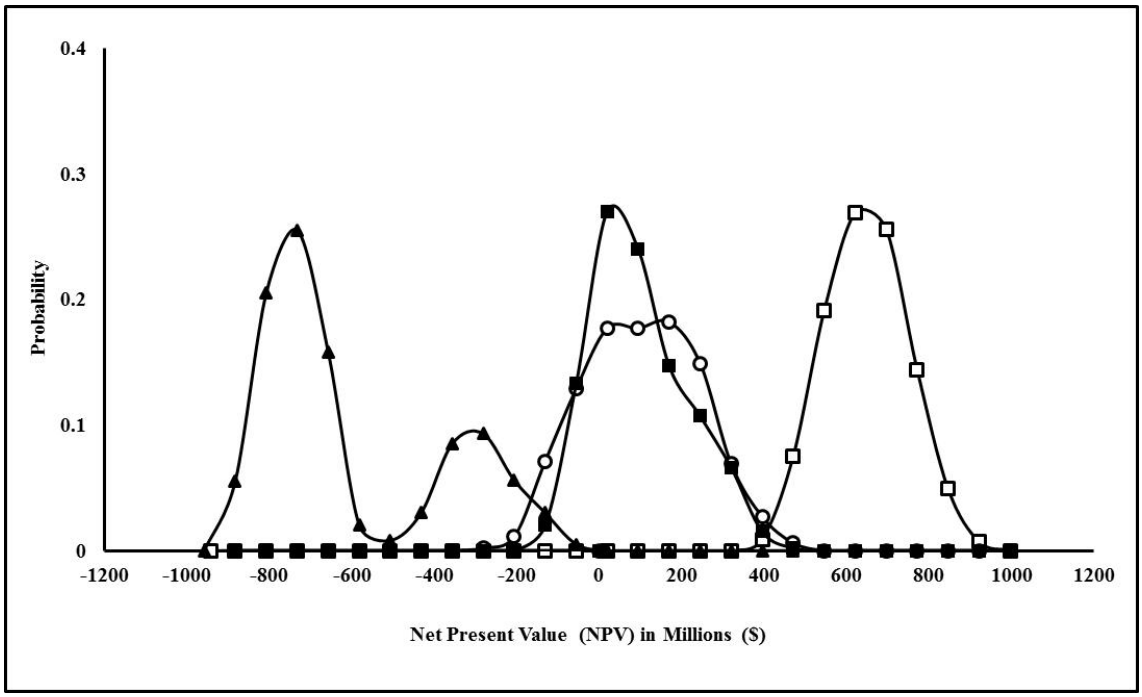


Figure 4.7 Distribution of thenet present value in for a major yield improving change scenario at the various stages of development: no change (□), early phase development (○), late phase development (▲), and post-product approval (■).*The risk of potential market share losses is not taken into account.*

The percent change in expected net present value from the base case of all the minor and major changes at all stages of development is portrayed in Figure 4.8. This shows that the best option is to not make any changes, as the percentage changes are all negative. However, the 'forced' changes have to be made and so if there is an option in timing then the optimal time to make that change, seems to be at early phase development if it is a minor change but a major change with no great improvement in COG or in particular an enhancement in annual throughput makes it the worst scenario for a forced major change. The least profitable scenario is to make a minor change at late phase III development, which tends to incur the biggest delays with the biggest risk of having to repeat larger clinical efficacy and comparability bridging studies before going to market. In this case the major forced formulation changes have caused an improvement in percentage ENPV at late stage development, possibly due to the lack of retrofit cost required and shortening of the processing stream (the removal of freeze drying step). The major yield improving change gives more positive percentage changes than both the minor and major changes that do not have an impact on yield if made at early phase development or post-product approval if product stockpiling is factored in However, if the change is made at late phase development, the NPV seems to be more

sensitive to the delays to market caused by the change, and thus gives the least profitable outcome.

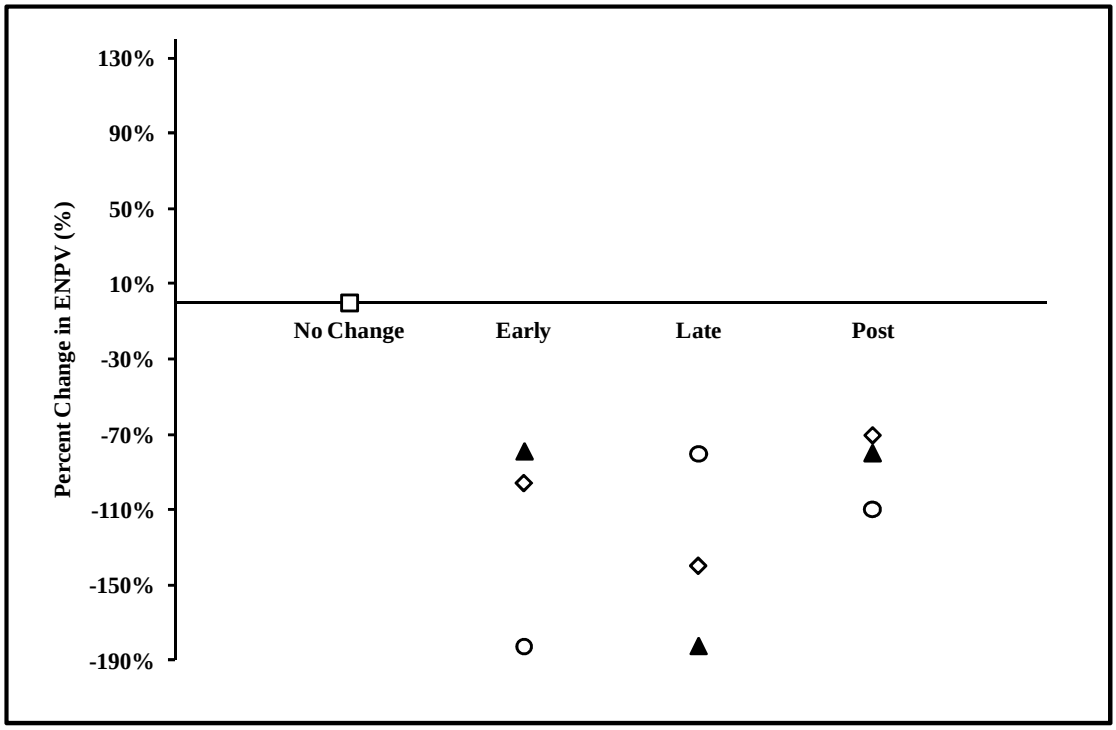


Figure 4.8 The percent change in expected net present value from the base case (no change (□) set-up when implementing three change scenarios: a 'forced' minor change (◇), a 'forced' major change (○), and a major yield-improving change (▲). *The risk of potential market share losses is not taken into account*.

4.7.1 ENPV INCLUDING THE UNCERTAINTY OF MARKET SHARE LOSSES

The impact of market share uncertainty on the process change scenarios is considered next. The potential for loss in product market share is directly proportional to the reduction in NPV. The bigger the loss in market share the bigger the overall loss in NPV. Figure 4.9 shows the NPV distribution for a minor change. The ENPV is greatest when the change is made post-product approval and at early phase development. Changes at late phase again show the least profitable outcomes. The foremost difference here is in the scenario of 'no change', which now has a less attractive ENPV than early phase development and post-product approval changes. The potential for market share losses in this scenario are significant. If the product improvement changes are not made, then the product may not be approved, either in Europe or internationally, depending on EMEA and FDA regulations. In addition, the company may lose a share to competitor products of a better quality. The same patterns are seen in the 'forced' major change and

major yield change distributions Figure 4.10 and Figure 4.11, although the mean NPV estimates have shifted further to the left,

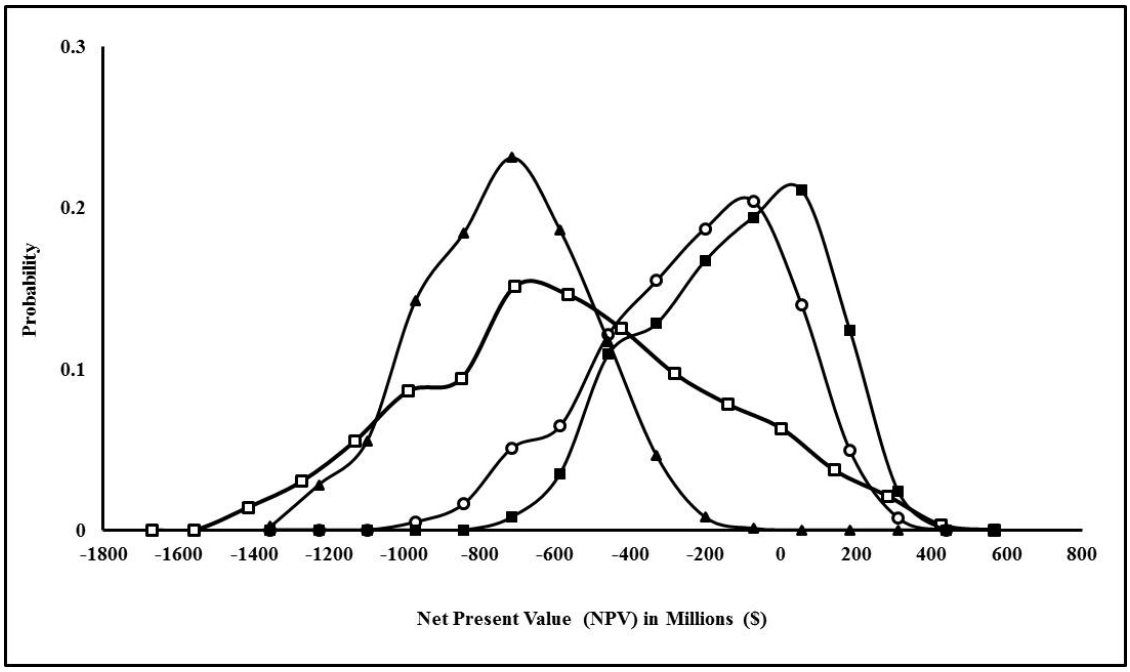


Figure 4.9 Distribution of the net present value with the addition of market share loss uncertainties for a 'forced' minor change scenario at the various stages of development: no change (□), early phase development (○), late phase development (▲), and post-product approval (■).

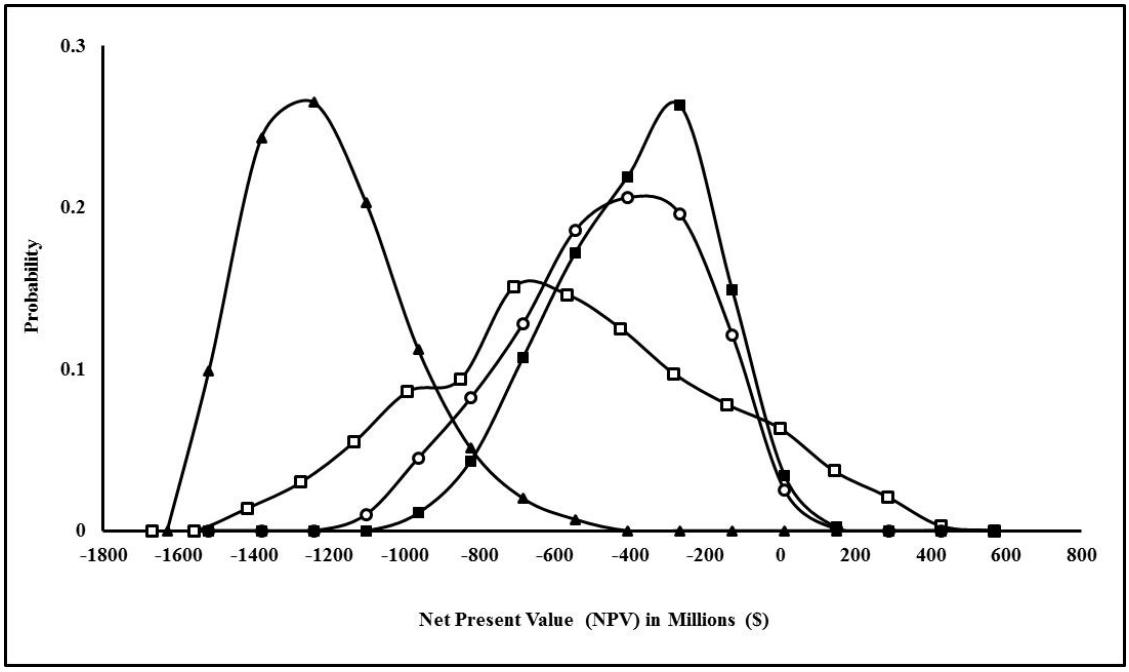


Figure 4.10 Distribution of thenet present value with the addition of market share loss uncertainties for a 'forced' major change scenario at the various stages of development: no change (□), early phase development (○), late phase development (▲), and post-product approval (■).

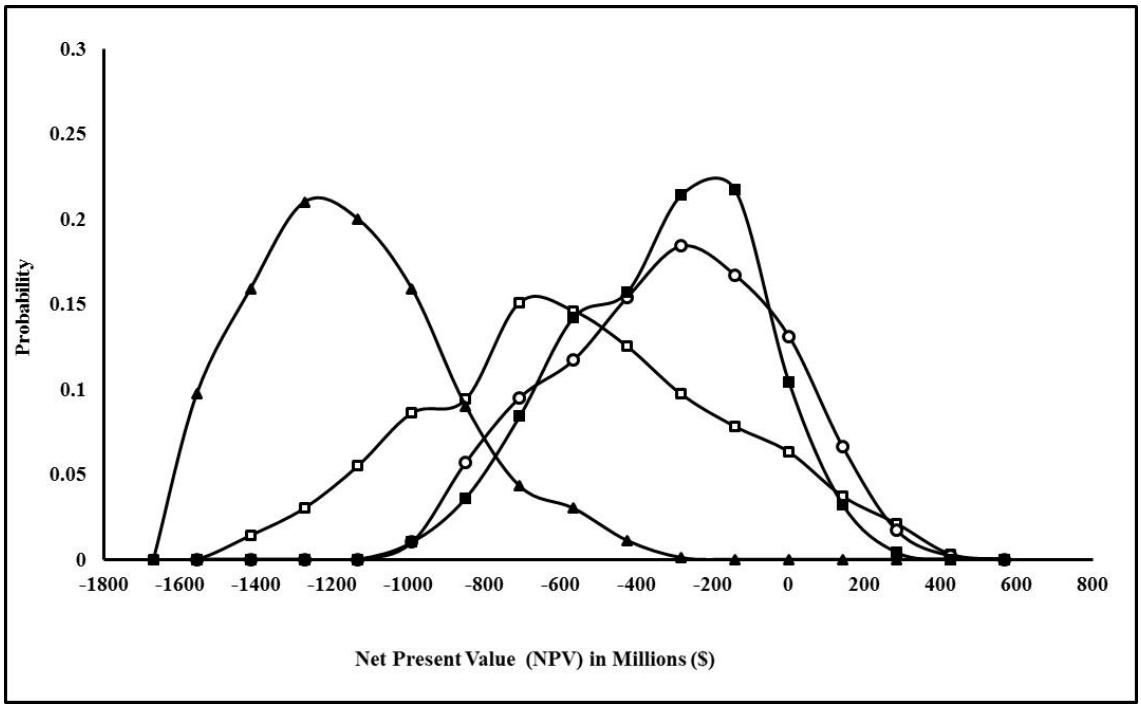


Figure 4.11 Distribution of the net present value with the addition of market share loss uncertainties for a major yield improving change scenario at the various stages of development: no change (□), early phase development (○), late phase development (▲), and post-product approval (■).

with more negative mean values. This shows that ENPV is are more sensitive to market share capture and so should be accounted for. Figure 4.12 shows the percent change in expected net present value of the process change scenarios from the base case of the 'no change' scenario with market share uncertainty incorporated. For all the changes, the NPV is most negative when the change is made at late phase development. At early phase development, the major yield-enhancement looks to more profitable than the 'forced' minor and major changes.

In this case, the annual throughput has a greater impact than product equivalence studies. The major yield improvement step also results in higher NPVs than the major formulation change at late phase development and at commercial phase. The best stage to make a minor or non-yield enhancing change is post-product approval, when product can be stockpiled. Although, the yield improvement change is better than the minor and major changes at commercial phase, it has a more positive change in NPV at early phase

development, where the risk of market share loss and clinical study repetition is the lowest.

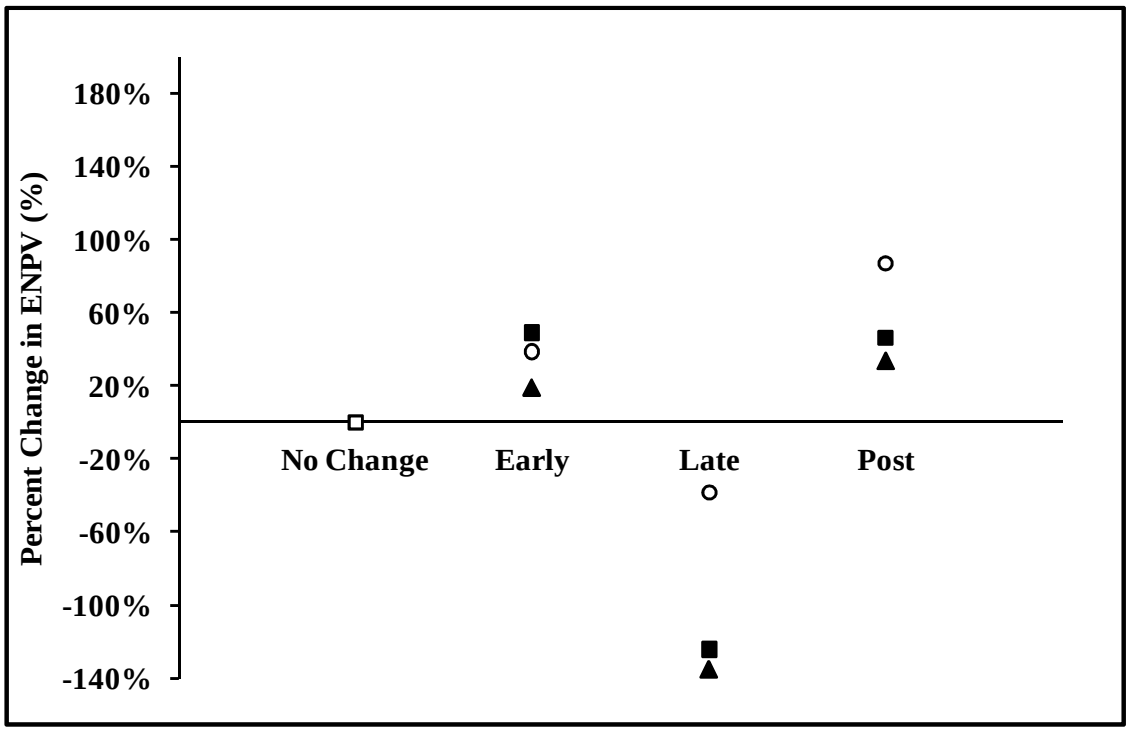


Figure 4.12 The percent change in expected net present value from the base case (no change (□) set-up when implementing three change scenarios: a 'forced' minor change (○), a 'forced' major change (▲), and a major yield-improving change (■), including the risk of market share losses.

The process change option proposed to enhance process yield gives positive percentage changes in NPV at early and post-product approval phases, making them more desirable options in comparison to making no process change. However, the mean NPV values are in reality largely negative as can be seen in Figure 4.11. The major yield-improving step of substituting B+1 fractionation for QXL chromatographic step does not result in a high enough yield to be a risk-free profitable option. Further analysis involved increasing the IVIG yield per batch on ENPV for the major yield-enhancing process change (Figure 4.13). This only showed early phase development changes to be profitable. There have been many proposals in the industry for the substitution of traditional fractionation-based purification of IVIG for chromatographic based steps (Burnouf, 1995;Curling *et al.*. 2005), citing total process yield improvements of up to 20%. Such a change would necessitate new clinical trials and new product registration

in all of the countries in which the products are licensed. This is a high price to pay for possible yield increases of an already safe product. Only a company without a manufacturing history, building a new facility on a "greenfield" site, will be able to implement this process improvement,

despite the significant advantages of modern membrane and chromatographic technologies in widespread use throughout the biotechnology industry.

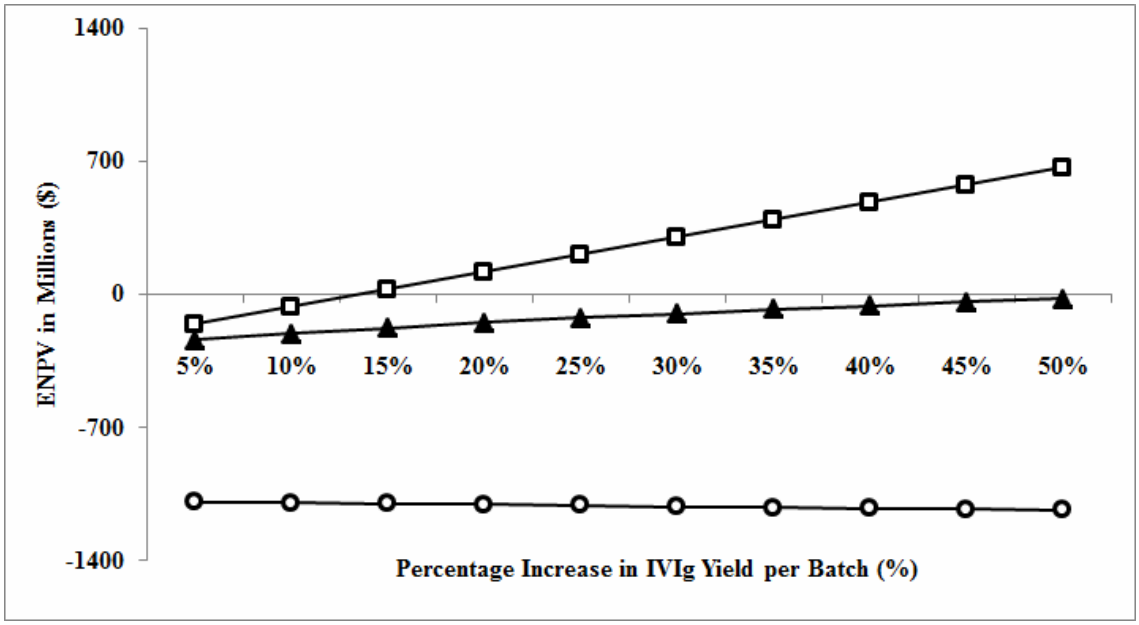


Figure 4.13 The impact of percentage increase in IVIG yield per batch on ENPV at early phase development (□), late phase development (○), and post-product approval (▲).

4.8 CONCLUSIONS

In this chapter, a case study illustrating the implementation of various changes to a plasma fractionation process is investigated. Process changes of varying magnitude and type, were explored at different stages of product development including post-product approval. The example illustrates how the framework described in Chapter 4 can be used to investigate the effects of making process changes, whether these are forced upon them or are made to enhance productivity.

The scenario results show that the stage of implementation is far more significant than the process change type; it is not economically advisable in the three cases investigated to make a change at late phase clinical development. The best approach would be to implement that change once a product is approved and commercial or once the company

are able to stockpile sellable product, to minimise the impact of process change delays. Major yield enhancing changes made to traditional fractionation processes are not economically feasible, cost and delays involved in undertaking clinical trials re-registration the 'new' product in all of the countries in which the products are licensed is too high a cost to pay for relatively small yield enhancements.

Chapter 5

Purifying a New Product from a Side-fraction: The Feasibility of Purifying Alpha 1-Antitrypsin from Fraction IV Precipitate

5.1 INTRODUCTION

Biopharmaceutical companies are driven to maximising plant output potential whilst minimising resources, utility usage and operating costs throughout all stages of drug cycle, so as to remain economical and uphold to the stringent regulatory requirements (Chirino and Mire-Sluis, 2004). They also typically need to have a portfolio of drugs in development to remain successful (Rajapakse, 2005).

The plasma fractionation process is unique in that its starting material plasma is a source of multiple products. Most fractionators have not maximised their product portfolio potential. Newer product purification streams were based on older fractionation processes created to purify initially only albumin and a few clotting factors, and so subsequent product yields of newer products are far from optimal. One reason for this is that any change in the unit operation's sequence will affect all products downstream of the modification. This distinguishes the design of fractionation processes from more typical processes that recover single therapeutic entities from microbial or transgenic sources (Curling, 2002). One way to increase portfolio without having a direct impact on products already in the market is to purify material from a side (waste) stream.

One such side stream in the plasma fractionation process is Fraction IV (FIV) precipitate. At Bio Products Laboratory, Herts, UK (BPL), a large-scale fractionator of human plasma-derived products, FIV precipitate is currently a side fraction that is currently disposed of or a small-unprocessed volume is sold as a by-product. The precipitate is currently unexploited, but it still contains a number of proteins, although whether these proteins are damaged or are present in a useful form is largely unknown. The research and development team at BPL wanted to investigate whether there was any potential to purify sufficient protein from the precipitate. The fraction would be treated as a new starting material for a purification process. This is advantageous in that it would not affect the current plasma fractionation process or any other derived

products that are already in the market. This may have created complicated regulatory issues.

Alpha 1-antitrypsin (AAT or A1-AT or α1-antitrypsin), also known as alpha-1 proteinase inhibitor (α1-P1) is one such potential protein present in FIV precipitate or FIV paste, see Figure 4.1 and Figure 5.1. Plasma-derived α1-antitrypsin (AAT) has a significant market potential and is currently marketed by several companies for the treatment of hereditary emphysema and generates over $200 M in revenues annually (alphaMed Pharmaceuticals Corporation, 2008). However, it is marked by significant shortages, and it is often quoted that the demand is far greater than the current supply(Karnaukhova *et al.* 2006;Mattes *et al.*2001).

The potential in purifying AAT from FIV precipitate at BPL is investigated in this chapter. The method trialled is based on a process designed by Kee*et al.*, 2004 (Figure 5.1). Laboratory scale experiments are used to assess whether there is sufficient AAT in FIV paste and whether the purification process suggested by Kee*et al.* can be applied to BPL's fractionation process. The experiments involved mimicking the first two isolation steps in the process.

5.2 ALPHA 1-ANTITRYPSIN

5.2.1 PROTEIN DESCRIPTION

AAT is a glycoprotein synthesised in the liver normally present at 2000mg/L in serum. It is the most abundant of the serine proteinase inhibitor (SERPIN) family in human plasma (Chen, 1998;Karnaukhova *et al.*. 2006). It consists of a single polypeptide chain and has a molecular weight of 52 kDa. This protein's primary function is the protection of lung tissue (Chen, 1998;U.S.Congress: Office of Technology Assessment, 1985). It is secreted into the blood circulation and diffuses into tissue space, inhibiting a wide range of serine proteases, however, its main physiological role is in not as an anti-trypsin but in inhibiting the enzyme neutrophil elastase (NE); a potent protease that degrades structural proteins (Travis, 1988). It does this by forming extremely stable complexes that are rapidly removed from circulation (Beatty, 1980;Chen, 1998). In addition to inhibiting elastase, AAT is capable of inhibiting a number of proteases including serine, trypsin, chymotrypsin, collagenase, thrombin, kalikerin and plasmin (U.S.Congress: Office of Technology Assessment, 1985).

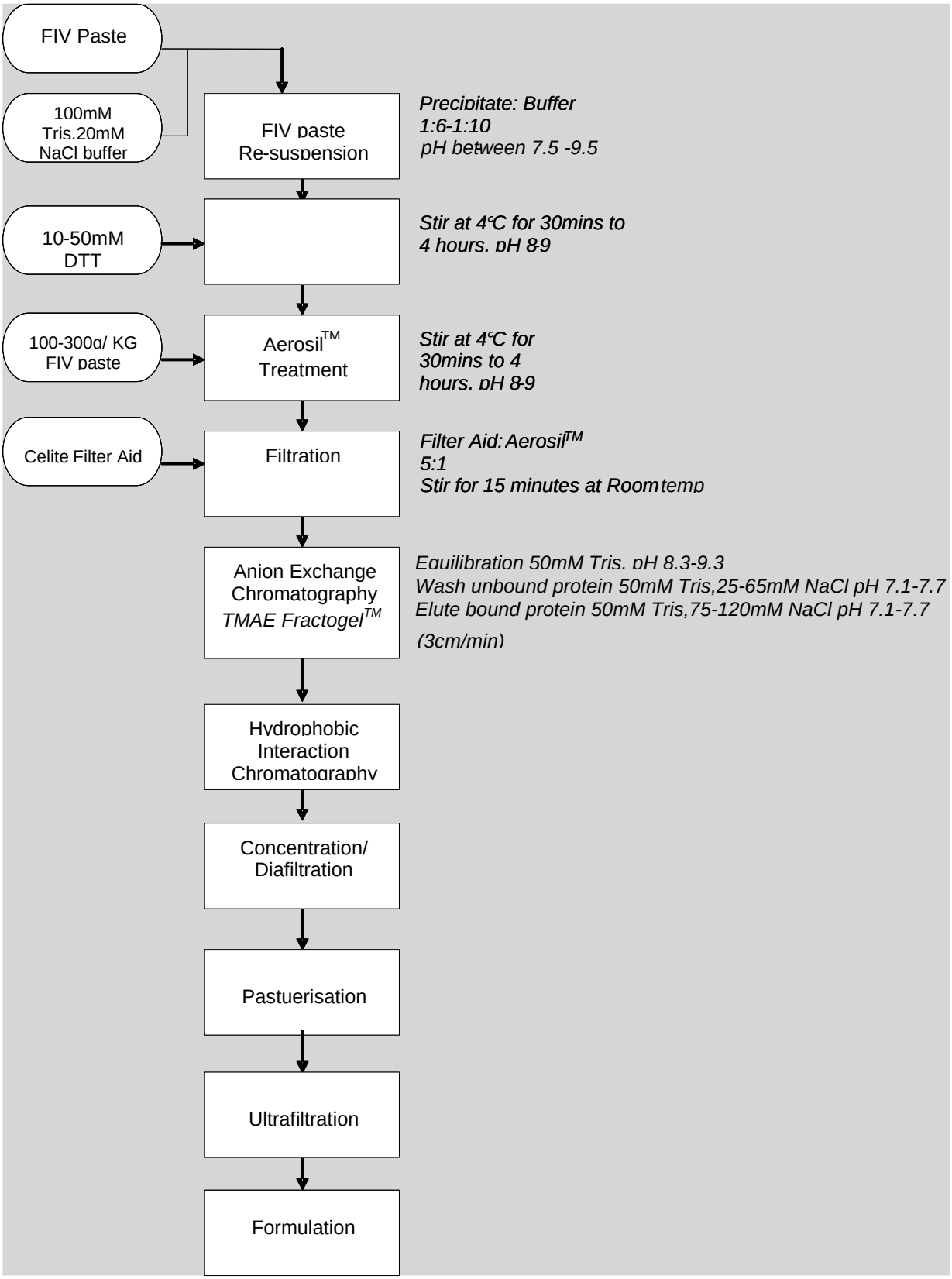


Figure 5.1 Kee*et al.* Method for the purification of AAT from Human Plasma FIV precipitate.

AAT is naturally folded in a metastable structure (Karnaukhova *et al.*. 2006), thermodynamically this is not the most stable form, and therefore, the protein is prone to conformational modifications and aggregation(Lomas, 1993).

5.2.2 INDICATION AND DOSAGE

AAT is currently only used as a replacement therapy to treat patients with genetic AAT deficiency (AAD). AAD is a hereditary disorder which causes chronic obstructive pulmonary disease (COPD), principally emphysema, in the early stages of adult life from 30-40 years.

Emphysema is a chronic, hereditary, autosomal recessive disorder of the alveolar structure associated with enlargement of the distal air spaces, accompanied by the destruction of their walls. It is usually fatal in the majority of deficient individuals, and greatly accelerated in cigarette smokers The average level of AAT needed for adequate anti-elastase activity in the lung is 0.8g/L (Ho and Gibaldi, 2003). Patients with a deficiency of serum AAT have little or no anti-elastase activity in their lower respiratory tract (Gadek, 1981). Replacing AAT levels has been shown to effectively increase levels in the serum and lung fluid, slowing the progression of emphysema related changes in patients. However, treatment is limited to patients with early evidence of the disease, and those with particular phenotypes (Ho and Gibaldi, 2003).

The second, more frequent, manifestation is disease of the liver that can affect newborn babies, children and adults. Less frequent is an inflammatory disease of the skin called necrotizing panniculitis. AAT may also be used as a medication for the treatment of fibromyalgia (FM), a syndrome characterized by chronic generalized musculoskeletal pain (Blanco B.I., 2007) and in the localised treatment (inhalation) of AAT to treat cystic fibrosis; by again inhibiting excess elastase due to the chronic inflammation, causing subsequent lung tissue damage(EMEA: Committee For Orphan Medicinal Products, 2008).

Currently licensed treatments of pulmonary emphysema involve the intravenous infusion of the plasma-derived AAT preparations, with a recommended dose of 60 mg of active AAT per kg of body weight administered once weekly. To maintain a threshold level of AAT of 11 μMol, AAT deficient-patients should receive augmentation therapy for the duration of their lives, which slows down the progression of emphysema.

5.2.3 MARKET FOR AAT

The rate of prevalence of the syndrome is from 1.5 to 3% of the population of Europe and North America (Blanco B.I., 2007) However recent publications indicate that it is widely under- and misdiagnosed (de Serres, 2003;Karnaukhova *et al.*. 2006). WHO estimate that less than 4% of individuals with AAT deficiency have been diagnosed and only a portion of them are receiving treatment (World Health Organization, 1996). This may be because even those with very low levels of the protein do not necessarily exhibit problems. North-western Europeans are most likely to carry a mutant AAT gene. Recent research reveals that clinicians are improving in time to making the diagnosis. However, the delay is still significant especially in older patients and women. Furthermore, manifestation of the disease is a mixture of genetic predisposition and environmental factors. For example, a person who is heterozygous may simply have a predisposition to COPD if they smoke.

AAT has received orphan drug designation for a variety of indications from the EMEA and FDA. An aerosolized form of AAT has been given orphan drug status for TalecrisBiotherapeutics, Inc. for the treatment of AAD; Kamada, Israel for the treatment of Bronchiectasis and BCG (Europe) Ltd, United Kingdom, for the treatment of cystic fibrosis amongst others. So-called "fast-track approval" guidelines set out by the FDA are designed to encourage the development of therapeutics for these diseases, and to bring them as quickly as possible to market. Although patient populations are small the revenue from orphan drugs can be immense. The main product competitors are listed in Table 5.1.

Table 5.1 Major Alpha-1 antitrypsin products in the market

Trade name	Company	Primary Indication	Stage in Drug Cycle	Source	Dosage	Purity
Prolastin	Talecrisbiotherapeutics	Augmentation / Replacement therapy	Commercial	Human-derived plasma	60 mg active AAT/kg body	62%*
Aralast	Baxter Healthcare Corp.	Augmentation / Replacement therapy	Commercial	Human-derived plasma	61 mg active AAT/kg body	70%*
Zemaira	CSL Behring	Augmentation / Replacement therapy	Commercial	Human-derived plasma	62 mg active AAT/kg body	99%*
Trypsone	Grifols Institute	Augmentation / Replacement therapy	Approved	Human-derived plasma	63 mg active AAT/kg body	-
Alfalastin	LFB Biomedicaments	Augmentation / Replacement therapy	Approved	Human-derived plasma	33.33 mg / ml	-
Alpha-1 antitrypsin	Kamada (Israel)	Bronchiectasis	Orphan drug /Clinical Trials	Human-derived plasma	-	-
Alpha-1 antitrypsin	BCG (Europe) Ltd	Cystic fibrosis	Orphan drug /Clinical Trials	Human-derived plasma	-	-
rhAAT	GTC biotherapeutics	Augmentation / Replacement therapy	Orphan drug /Clinical Trials	Milk (Transgenic goat)	-	-
rAAT	Baxter Healthcare Corp.	Augmentation / Replacement therapy	Orphan drug /Clinical Trials	Recombinant yeast	-	-
Alpha-1 antitrypsin	Other groups	Augmentation / Replacement therapy	Early stage research/clinical trials	Transgenic animals, Rice, expression of AAT in various hosts (Kaunaukhov*æt al*, 2006)	-	-

*(de Serres, 2003)

5.3 METHOD AND MATERIALS

5.3.1 INTRODUCTION

An attempt to purify AAT from Cohn Fraction IV-1 precipitate (Bio Products Laboratory plasma fractionation process) is provided. The method used is based on the ZLB Behring, patent by Kee*et al.* (Publication Number: WO/2004/060528): 'Method for Purification of Alpha-1-Antitrypsin'. Based on the invention, protein impurities are destabilized by cleavage of disulfide bonds with a reducing reagent, such as a dithiol, which does not affect AAT. The destabilized proteins are then adsorbed on a solid protein-adsorbing material, without the addition of a salt as a precipitant. Separation of the solid adsorbent from the solution results in a purified AAT suspension that is

suitable for chromatographic purification. Only the first filtration and anion exchange chromatography steps were attempted.

5.3.2 CHEMICALS

All chemicals were of analytical grade quality, and were obtained from Fisher-Scientific UK (Loughborough, Leicestershire, UK) unless stated otherwise.

Sodium monobasic Phosphate (NaH_2PO_4), and sodium dihydrogenPhosphate (Na_2HPO_4) were purchased from BDH Limited, VMR International Ltd., (Poole Dorset, England, UK). Buffers were prepared using deionised water, and were stored at room temperature for a maximum of one month. AAT standard was a product of Sigma-Aldrich Co. Ltd (Poole, UK).

5.3.3 SAMPLE COLLECTION

The FIV precipitate used throughout the experiment (Batch number LC5622) was removed from centre of a disk-stack centrifuge (wetter paste) on a single day, and frozen in a -40^{o}C freezer. For each experiment, the frozen FIV paste (10-30g) was allowed to thaw at room temperature. The paste was then re-suspended to 10% w/v inPhosphate buffer (10mM $Na_2H_2PO_4$, 10mM NaOH), pH 6.2. The suspension was homogenized at low setting to minimise foaming for 20 minutes at 2-8°C. The solution was adjusted to a temperature of 2-8°C and pH 7.5. The suspension was stirred for approximately 20 minutes. At a high pH (above 8) the protein becomes less stable and below pH 6.0, alpha-1-antitrypsin tends to denature and aggregate (Kee and Cook, 2004).

5.3.4 DISULPHIDE BRIDGE REDUCTION

Dithiothreitol (DTT) is used to reduce protein disulphide bonds, but not reduce other protein bonds such as peptide linkages or other groups which would cause a fragmentation of the protein (Glaser, 1982). In this case the method takes advantage of the unusual disulfide bond in AAT, which consists of a single cysteine residue in the polypeptide chain bound to a free pendant cysteine. In contrast to other circulating plasma proteins, the disulfide bridge in AAT does not add to its structural stability. Therefore, other plasma proteins in the solution are precipitated out in the presence of the reductant, achieving a better subsequent separation of AAT(Glaser *et al.*. 1982), mainly from AAT which has similar properties to AAT in size and isoelectric point.

DTT was added to the solution to a final concentration of 0.03M. It was stirred for 15 mins at room temp and then stirred for 2 hours at 2-8°C. If necessary the pH was adjusted to 7.5 again.

5.3.5 PRECIPITATING OUT IMPURITIES FROM THE REDUCED SUSPENSION

Contacting the reduced suspension with an insoluble protein-adsorbing material

Aerosil™ 380, an insoluble silica adsorbent was added to the suspension at 16.7g/L suspension and stirred for 1 hour at 2-8°C to precipitate the DTT-disrupted proteins.

5.3.6 FILTRATION

Celite™ 1000 was added as a body feed to the suspension at a rate of 5 parts Celite™ to 1 part Aerosil and the solution was stirred at 2-8°C. The solution was filtered under pressure at 2 mpa maximum. Firstly, through a 44mm diameter glass filter (Sartorius AG, Goettingen). 50 mL of sample was filtered at one time and pooled. The remaining residue debris of the FIV paste not yet filtered was dissolved in an additional 100mL of dissolution buffer; this was also filtered and added to the pooled filtrate. The filtrate was then filtered through two sets of cellulose acetate filters, both 44mm in diameter: a 0.45μm and a 0.2 μm cut-off point filter stacked on top of each other. The samples were filtered in 50mL batches and pooled. The filter was changed between samples as it blocked each time. No washes were taken. This step is intended to filter out the disrupted proteins.

5.3.7 PREPARATIVE ANION EXCHANGE CHROMATOGRAPHY

Chromatography was performed on an ÄKTA Prime (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equipped with a fraction collector. Two different strong anion exchange chromatography resins were tested for the separation of AAT from the remaining proteins present in the 'Fraction IV' filtrate:

1. Fractogel® TMAE (M) "Q-type" (EMD Chemicals Gibbstown, NJ, USA), which consists of a Methacrylate matrix, a hydrophilic synthetic vinyl polymer with the functional group, trimethylammoniumethyl 'CH2-CH2-$N^+(CH3)_3$', and bead particle sizes in the range of 40 – 90μm.
2. HiTrap Q Sepharose Fast flow (G.E. Healthcare), which consists of media packed with macroporous, cross-linked agarose beads (particle size 45–165 μm) with the functional group '$CH2N^+(CH3)_3$' attached.

Both were pre-packed 1mL columns. The filtrate is applied directly onto the chromatography columns containing an anion exchange resin. Column fractions were collected in plastic tubes using a FRAC-100 fraction collector (Amersham Biosciences), Absorbance, pH, salt concentration and pressure were monitored on-line. This step is used to isolate AAT from remaining Albumin, impurities.

5.3.8 BUFFER AND SAMPLE CONDITIONS

The conditions set in the Kee*et al.*, 2004 patent describes the Fractogel TMAE (M) column being equilibrated with an equilibration buffer of 50 mMTris and a pH of about 8.6-8.9, and then loaded to approximately 50-70% of a pre-determined protein capacity with the AAT final filtrate. Contaminants are then be removed from the column by washing the column with an wash buffer (approximately 50 mMTris, about 25-65 mMNaCI, and pH about 7.1-7.7), and AAT is subsequently eluted using an elution buffer (approximately 50 mMTris, about 70-120 mMNaCI, and pH about 7.1-7.7). However, the attempt at using these conditions yielded product peaks of extremely low absorbance (~500 mAu), and 1% Agarose and SDS gel analysis revealed no AAT in the peak. Therefore, alternative buffer conditions were investigated so as to obtain any product recovery (Table 5.2).

The Q Sepharose fast flow and Fractogel® TMAE columns were equilibrated with 20mM Phosphate buffer (NaH_2PO_4:Na_2HPO_4) or with 20mM Phosphate buffer 30mM NaCl at pH 6.2, these conditions are based on a method for AAT isolation using anion exchange by Kumpalume(Kumpalume *et al.*. 2007;Kumpalume *et al.*. 2008). FIV filtrate was loaded onto the column (4.5 or 9mL) at 1.5mL/min. Unbound protein was removed by washing with the equilibration buffer. Bound protein was eluted with 20mM Phosphate pH 6.2 containing 1M NaCl at a gradient or step elution over 10 column volumes. The column was then washed with 2 column volumes of buffer containing20mM Phosphate 2M NaCl, pH 6.2 to see if anymore protein would be eluted from the column, the column was then sanitised with 0.5M NaOH to remove any denatured proteins. The column was also sanitised with 20% ethanol to remove any lipoproteins and lipids between runs. Eluted fractions were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western Blotting, total protein and ELISA assays.

Table 5.2 Initial anion exchange run conditions including resin type, load volume, equilibration, wash, and elution buffer compositions.

Run	Resin Type	Load Volume (mL)	Equilibration and Wash Buffer	Salt (1M NaCl) Elution Type
A	Fractogel®	4.5	20mM Phosphate, pH 7	Gradient 0-1 M NaCl (10 CVs)
B	Fractogel®	9	20mM Phosphate, pH 7	Gradient 0-1 M NaCl (10 CVs)
C	Fractogel®	9	20mM Phosphate, pH 7	Step to 1M NaCl
D	Q Sepharose FF	9	20mM Phosphate, pH 7	Step to 1M NaCl
E	Fractogel®	9	20mM Phosphate, 30mM NaCl, pH 7	Step to 1M NaCl
F	Q Sepharose FF	9	20mM Phosphate, 30mM NaCl, pH 7	Step to 1M NaCl
G	Q Sepharose FF	9	20mM Phosphate, pH 7	Gradient 0-1 M NaCl (10 CVs)

5.3.9 PROTEIN CONTENT ANALYSIS

5.3.9.1 SDS PAGE ANALYSIS

SDS-PAGE was performed using a Bio-Rad Mini-PROTEAN II system (BioRadInc). The samples were diluted to approximately 1 mg/mL in deionised water and reducing buffer containing 62.5 mmol/L Tris-HCL, 2 %(w/v), 10% Glycerol(v/v), SDS, 0.03%(w/v) Bromophenol Blue, 5%(v/v) β-Mercaptoethanol. Samples were heated at 95°C for 5 minutes, and centrifuged for 5mins at 13,000 revolutions per minute (RPM). A 10-µL volume of each sample was applied to the gel (4–20% gradient SDS polyacrylamide gel was used, Pierce ThermoScientific). Run conditions were 180 Volts for 45 minutes. Standards run to allow identification of protein bands included protein marker, and AAT (Sigma–Aldrich). After electrophoresis, gels were washed in dH_2O and then fixed and stained using 40% v/vmethanol 10% v/vacetic acid 0.15% v/vCoomassie brilliant blue R-250 for 20 minutes. Followed by de-staining (overnight)

using 30% v/vmethanol, 10% acetic acid. Gels were stored in 75% Acetic acid and scanned.

5.3.9.2 WESTERN BLOT

Hybond ECL Nitrocellulose Membrane sheets and Hybond™ Blotting Paper were obtained from GE Healthcare (Little Chalfont, UK). Gels were run as in section 5.3.9.1, and captured proteins were transferred to a nitrocellulose membrane Hybond-ECL nitrocellulose membrane. The nitrocellulose membrane was blocked for 60 min at room temperature in 10 mMPhosphate buffer (5 mM NaH_2PO_4, 5 mM Na_2HPO_4), 125 mMNaCl, pH 6.8 Phosphate-buffered saline (PBS) containing 1.25% w/vpowdered milk (PBS plus milk). Specific antibodies (either enzyme conjugated or unconjugated) against a single protein were added to PBS plus milk protein and incubated with the membrane for 1 h at room temperature. Three 5 min PBS washes, were followed by detection with horseradish peroxidase. Where the specific antibodies were not enzyme conjugated, a second enzyme conjugated antibody (from Sigma–Aldrich), was employed, followed by PBS washes and detection. Antibodies against AAT (Abcam ab7633-1000, Cambridge, UK), were used.

5.3.9.3 TOTAL PROTEIN (BICINCHONINIC ACID) ASSAY

Total protein concentration was determined using the Bicinchoninic Acid (BCA) assay kit from Pierce (Rockford, IL) in a 96-well format. This procedure is very applicable to microtitre plate methods. BCA reacts with complexes between copper ions and peptide bonds to produce a purple end product. The advantage of BCA is that the reagent is fairly stable under alkaline conditions, and can be included in the copper solution to allow a one-step procedure. A molybdenum/tungsten blue product is produced.

The assay was calibrated with bovine serum albumin, BSA (It is noted that a calibration using AAT would have been preferable, but constraints in supply was an obstacle) using a curve from 50 to 2000 μg/mL. Calibration standards and samples were diluted in 10 mMPhosphate buffer, pH 7 . Samples are spun at 14000 rpm for 4 mins. 25uL of each standard and unknown sample are aspirated into a microplate well . Working reagent is added (200μL) to each well and mixed thoroughly on a horizontal plate shaker for 30s. The plate was then covered plate and incubated at 37°C for 30 minutes. The samples are then immediately read at 562 nm using a BMG Fluorostar (type) microplate reader.

5.3.9.4 TOTAL PROTEIN (BCA™) MICROPLATE REDUCING AGENT COMPATIBLE ASSAY

Samples are diluted and prepared as in 5.3.9.3. A sample control was created by diluting the original sample buffer with reducing agent. 9 uL of standards, sample, and control are transferred in triplicate to a 96 well plate (flat bottom). 4 uL of Compatibility Reagent are added to each well. The plate is covered, mixed on a plate shaker for 1 minute and incubated at 37°C for 15 minutes. Working reagent is added at 260 uL per well.The plate is then covered, mixed for 1 minute using a plate shaker, and incubated at 37°C for 30 minutes. Absorbance at 562 nm is measured.

5.3.9.5 ALPHA 1-ANTITRYPSIN ELISA QUANTIFICATION

The Enzyme-linked-Immuno-Sorbent-Assay (ELISA) (Immunodiagnostik, Germany) is used for the quantitative determination of AAT in serum or plasma. The assay utilises the sandwich technique with 2 selected polyclonal antibodies that bind to AAT. Standards, controls, and prediluted samples are added to wells of a microplate coated with a high affinity polyclonal anti-human AAT antibody. During the first incubation step, AAT is bound by the immobilised antibody. Then peroxidase-conjugated polycloncal anti-human AAT is added into each microtitre well and a sandwich of capture antibody-hAAT -peroxidase-conjugate is formed.TMB is used as a peroxidase substrate. Finally, an acidic stop solution is added to terminate the reaction. The colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of AAT. A dose response curve plotting optical density at 450nm against concentration is generated, using the values obtained from the standard. AAT concentration present in samples is generated from this curve.

Prior to use, all reagents samples were warmed to room temperature and mixed well. All microtitre wells are washed 5 times by dispensing 250mL of diluted wash buffer into each well. After the final wash step, the residual buffer is removed by tapping the plate on adsorbent paper. 100µL of standards, control and unknown samples were added in duplicate to each well. The plate was tightly covered and incubated at room temperature for 1 hour on a horizontal mixer. The contents of each well were discarded and washed 5 times by dispensing 250µL of diluted wash buffer into each well. 100µL of conjugate was then added to each well and again incubated for 1 hour at room temperature on a horizontal mixer. The contents were discarded and washed 5 times.

100µL of substrate was added into each well and incubated in the dark for approximately 10 minutes. Once a good colour differentiation is observed, 50µL of stop buffer is mixed into each well. Absorption was read at a wavelength of 450nm (reference 620nm).

5.3.9.6 TURBITIMER ASSAY: ALBUMIN AND TRANSFERRIN CONCENTRATION

The concentration of albumin and Transferrin contaminants in the samples were measured using turbidimetry, using the Turbitimer(R) apparatus from Siemens (formerly DADE-Behring, Germany). Albumin, Transferrin and protein standard turbiquantsare all products of Siemens.

5.4 RESULTS AND DISCUSSION

5.4.1 PROCESS TRIAL

The first process attempt was prepared based on conditions described by Kee*et al.* (2004) process (Figure 5.1) up until anion exchange chromatography stage. The depth filter provided ineffective recovery (Table 5.3). The process also produced anion exchange product peaks of extremely low absorbance (~500mAu), and 1% Agarose and SDS gel analysis revealed no AAT in the product peak (Figure 5.2). This was also confirmed using BCA Total protein and AAT ELISA assays (Table 5.3)

Alternative buffer conditions were investigated so as to obtain any product recovery (Table 5.2). The total protein loading capacity was found to reach breakthrough at 4.5mL protein load for Fractogel and, 5mL for QFF. Figure 5.3A) corresponds to conditions A set in Table 5.2, and gives the elution profile for FIV filtrate on a 1mL pre-packed Fractogel EMD TMAE column at maximum loading capacity of 4.5mL. Peak 1 represents the wash flow-through of unbound protein with 20mM Phosphate at pH 7, Peak 2 shows product elution at a 10-column volume salt gradient elution (0-1 M NaCl); Peak 3 is a 0.5M NaOH wash. The product eluate (peak 2) was of a low total protein concentration and so the column was saturated at a load of 9mL seen in Figure 5.3 B) and G) for Fractogel TMAE and Hitrap Q Sepharose FF respectively again corresponding to conditions set in Table 5.2 B) and G). Relative to the wash and NaOH peaks, the product eluate peak still gave of a low UV absorbance reading. SDS page and 10% Agarose gel analysis revealed that AAT is mostly removed in the elution peak, but so does many other proteins especially Albumin. Separation of albumin from AAT is

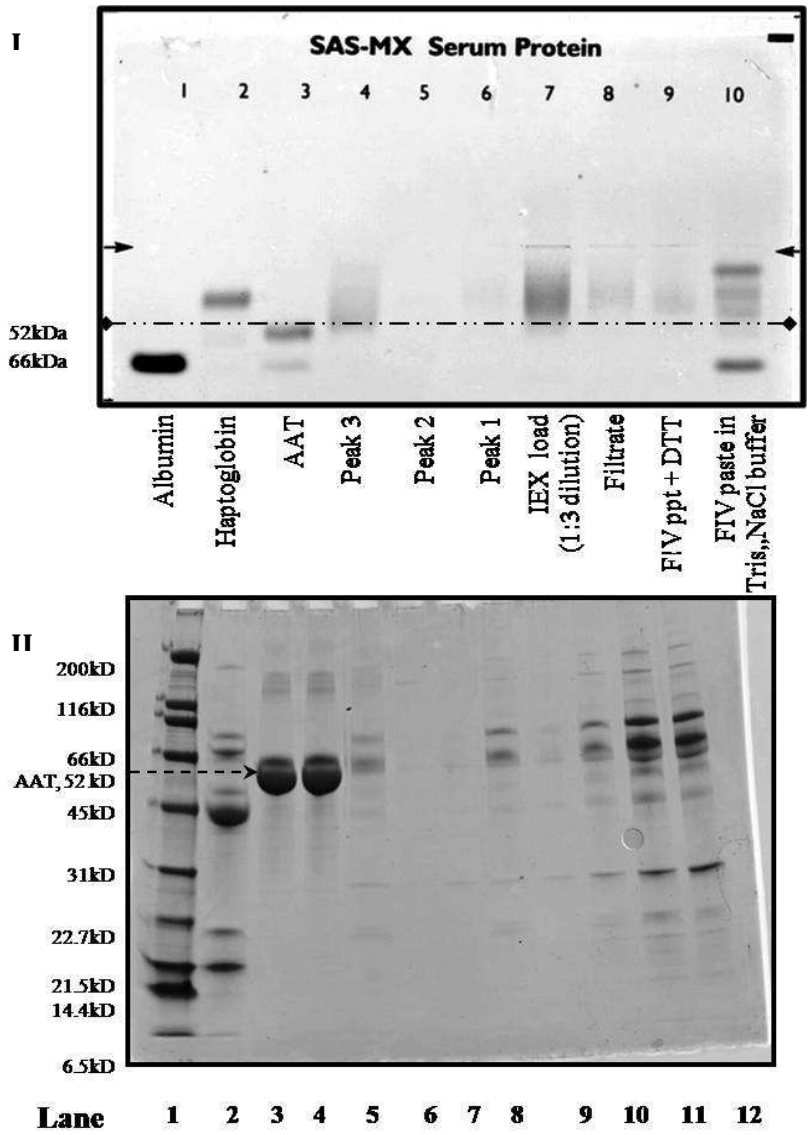


Figure 5.2 Initial process trial, based on Kee*et al.* conditions: Analysis of process samples for the presence of AAT from successive steps in the purification of AAT from Cohn FIV precipitate by I) electrophoresis on a 1% agarose gel. II) SDS-gel electrophoresis. Samples (4-20% Pierce gel, Coomassie blue stain).

SDS Gel : Molecular weight marker (lane 1), Haptoglobin (lane 2), AAT standard (lane 3), AAT standard (lane 4), FIV paste dissolved in Tris, NaCl buffer (lane 12), FIV paste plus 30mm dithiothreitol (lane 11), FIV paste + wash (lane 10), Ion exchange* Run 1: peak 1 (lane 9), peak 3 (lane 8), Run 2: peak 1(lane 7), peak 2 (lane 6), peak 3 (lane 5).

difficult because both proteins are similar in size (AAT 52 kDa and Albumin 67 kDa), and in charge (AATpI 5.2 and albumin pI 4.8). There also seems to be AAT present in some of the NaOH wash peaks. and so, in order to maximise product recovery in the elution peak a step elution to 1M NaCl was also taken (Figure 5.6).

Table 5.3 Total protein content and AAT content was measured for all process samples using the Pierce microplate reducing agent compatible BCA™ total protein assay and an AAT Elisa kit (Immundiagnostik) respectively. All samples were desalted using AmiconCentricon YM-3 regenerated cellulose filter devices, MWCO 3,000 to remove DTT from samples prior to assays.

	FIV paste in Phosphate buffer (10%)	Post Reduction (DTT addition (2 hours)	Aerosil addition (1 hour)	Celite addition	Filtrate
Total Sample Volume(mL)	302.0	303.4	307.0	327.1	169.2
Total Protein (g/L)	30.0	28.8	26.8	*	3.9
Total Protein Mass (g)	9.1	8.7	8.2	*	0.7
Total Protein Yield %	100%	96%	91%		7%
AAT (g/L)	-	-	-	-	0.018
Mass AAT (mg)	-	-	-	-	2.97
Mass AAT (g)	-	-	-	-	0.003
Purity (%)	-	-	-	-	0.44%

* Celite interferes with absorbance reading.

The profiles show elution with an initial flow-through wash of unbound protein with 20mM Phosphate at pH 7 and 20mM Phosphate, 30mM NaCl at pH 7. With no salt in the wash a much bigger and sharper product peak is seen for both the Fractogel and Q-Sepharose gels.

The Fractogel run also seems to give a much smaller peak when the column is washed with caustic soda. The gel analysis reveals that AAT is present in all the unbound wash samples, and in all NaOH washes except for the Run D: QFF with no salt in wash. This is also confirmed in the western blots (Figure 5.13**).**AAT is present in all peaks, but it is more highly concentrated in the product peak. In general, protein concentration after anion exchange is very low, mainly because the filtration step prior to it was ineffective

in that it yielded almost no protein and thus AAT recovery, see Table 5.3. Recovery and purity of AAT post-anion exchange for each run is summarised in Appendix Chapter 5. The purity of AAT post Anion exchange is extremely low at all conditions (≤ 5%), with the QFF column and no salt wash giving the maximum purity. In this case, the DTT disruption, and AerosilTM precipitation steps do not remove of the surrounding protein impurities as suggested by Glaser (Glaser, 1982;Kee and Cook, 2004)

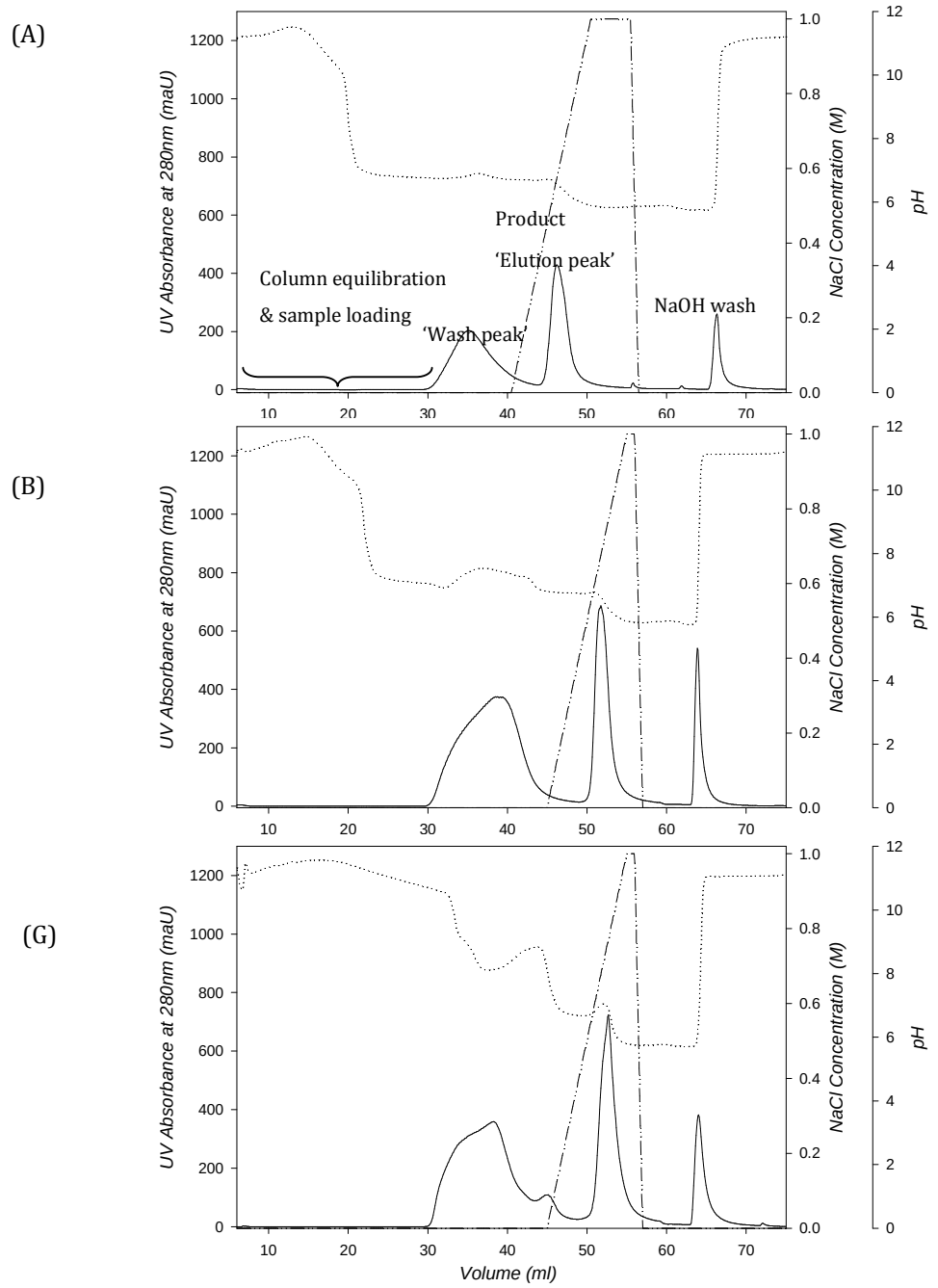


Figure 5.3 Elution profiles of Fraction IV filtrate on 1mL pre-packed columns: (A) & (B) Fractogel EMD TMAE column, and (G) Hitrap Q Sepharose FF. Peak 1 represents the wash of unbound protein with 20mM Phosphate at pH 7, Peak 2 shows a 10 column volume salt gradient elution (0-1 M NaCl), Peak 3 is a 0.5M NaOH wash. (A) represents a load of 4.5mL and (B)/(G) a 9mL load. Symbols are: (—) Absorbance, (-··-) NaCl concentration, (···) pH.

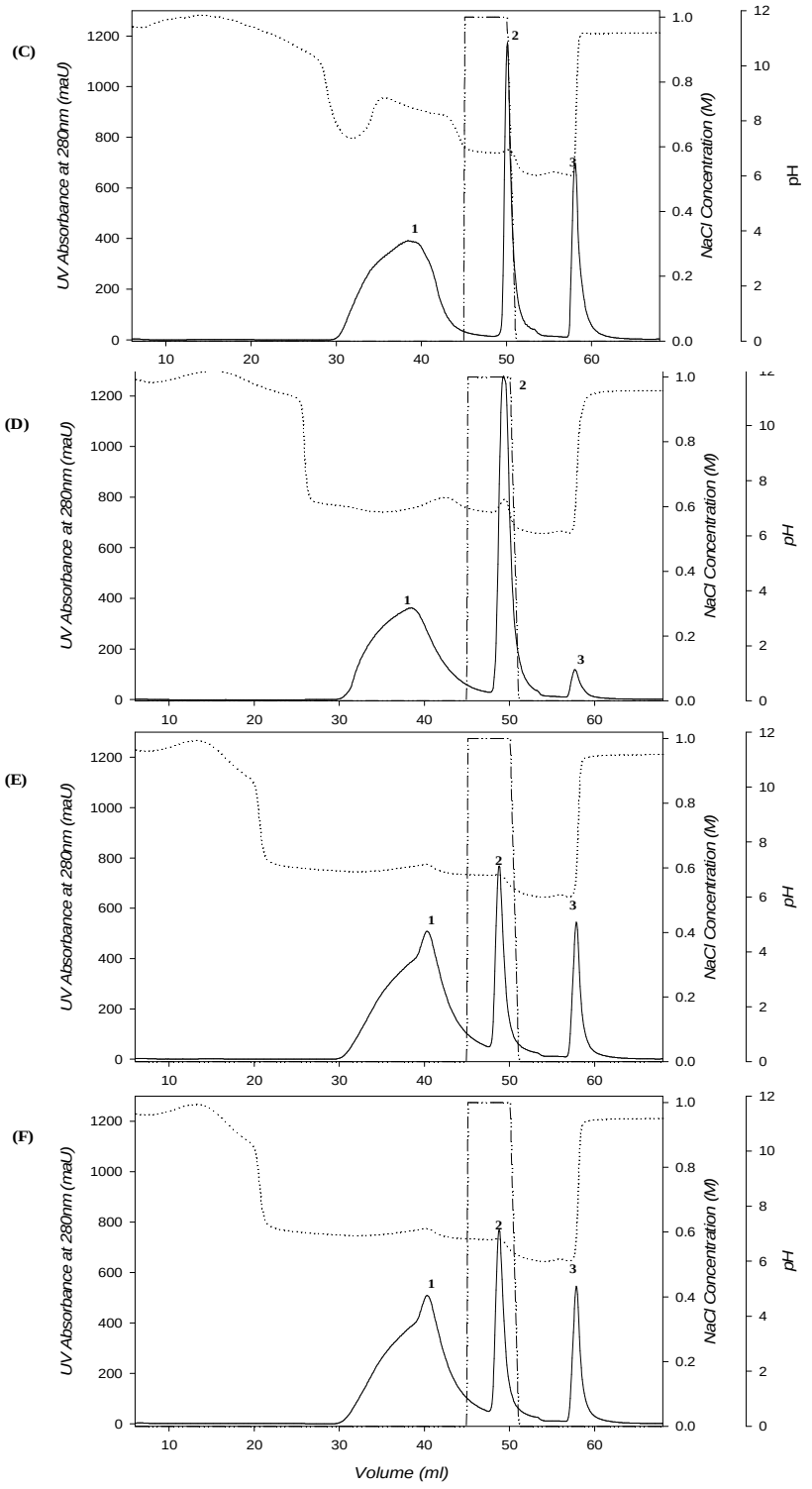


Figure 5.4 Elution profiles of Fraction IV filtrate on 1mL pre-packed columns: (C) & (E) Fractogel EMD TMAE column, and (D) & (F) Hitrap Q Sepharose FF. Peak 1 represents the wash of unbound protein with 20mM Phosphate at pH 7 in profiles(C) & (D); 20mM Phosphate, 30mM NaCl at pH 7 in profiles (E) & (F). Peak 2 shows a salt step elution to 1 M NaCl, Peak 3 is a 0.5M NaOH wash. Symbols are: (—) Absorbance, (-·-·-) NaCl concentration, (···) pH.

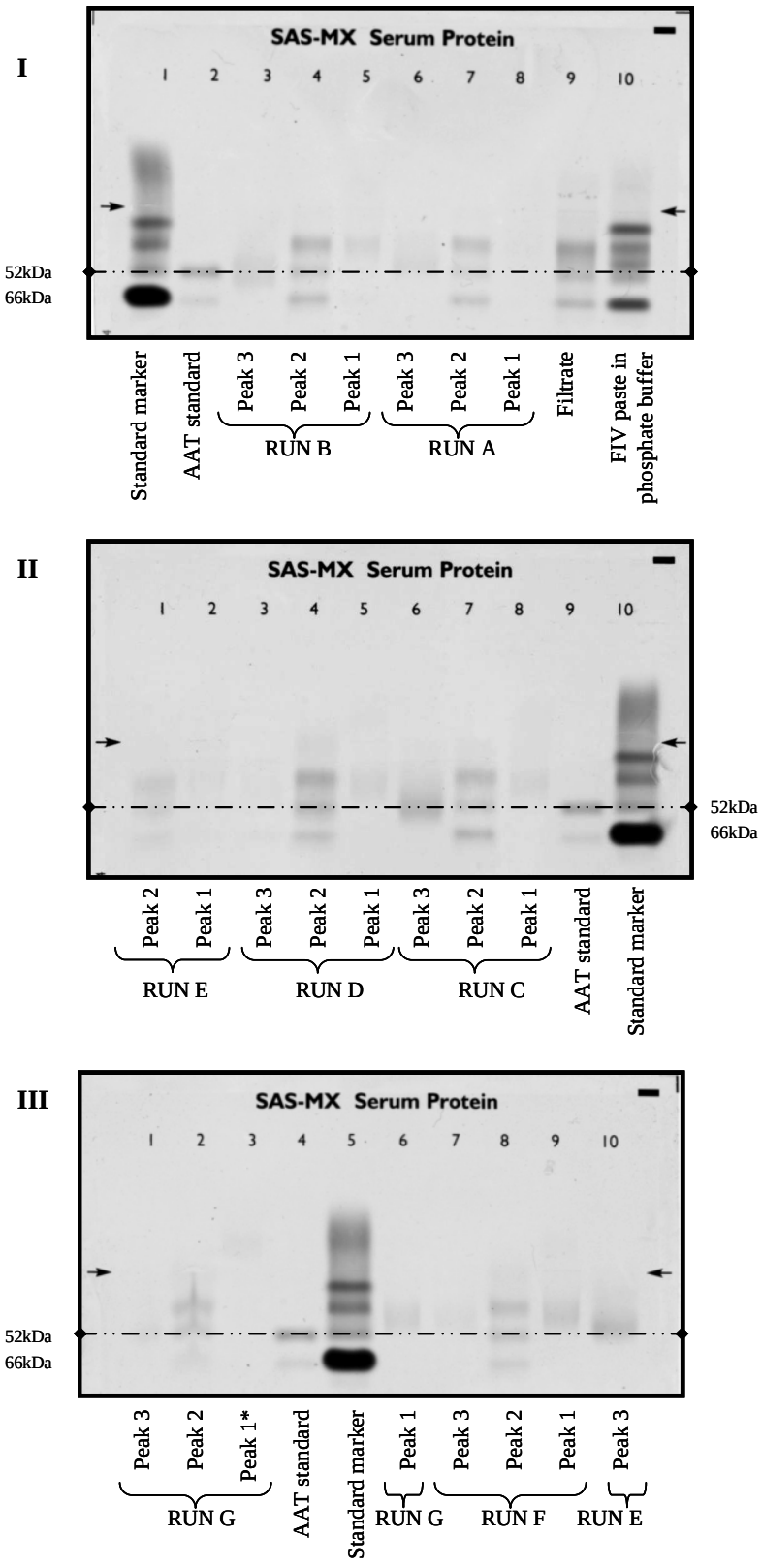


Figure 5.5 Analysis of process samples for the presence of AAT by electrophoresis on a 1% Agarose gel. Standard marker used is N/T protein Control LC® (Dade-Behring). Samples were electrophoresed at 80V for 30 minutes.

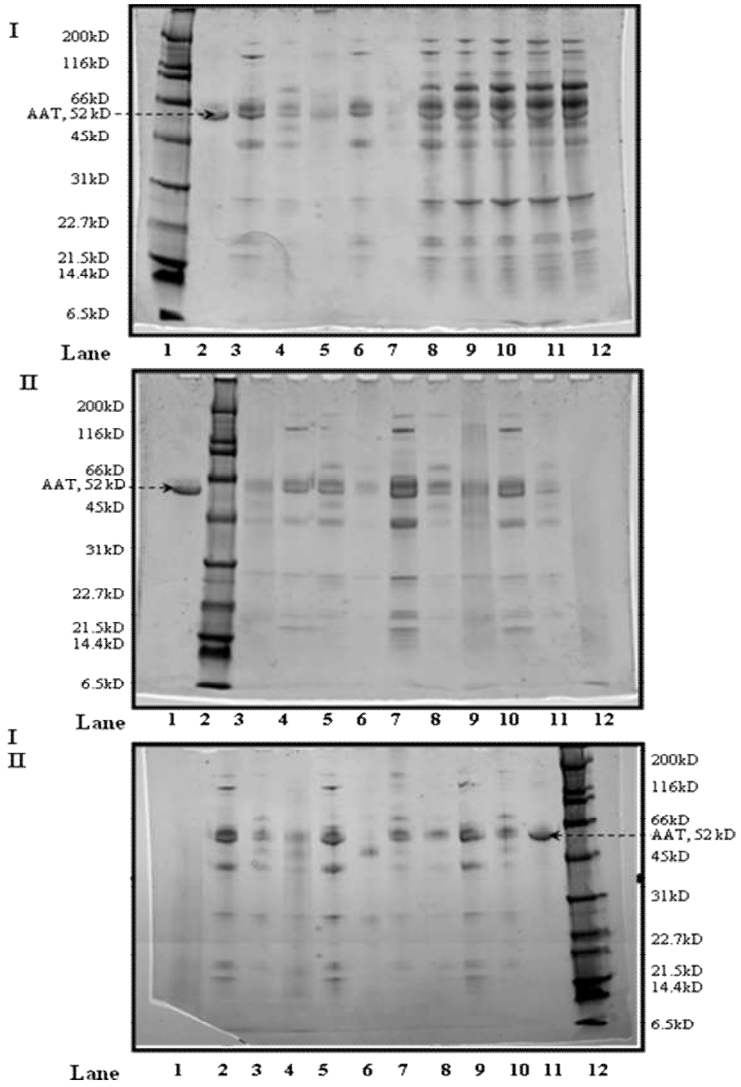


Figure 5.6 Analysis of samples from successive steps in the purification of AAT from Fraction IV precipitate, and preparative anion exchange runs using SDS-gel electrophoresis. Samples were applied to a 4-20% Pierce gel and electrophoresed at 120V for 45 minutes. The gel was stained with Coomassie blue.

Gel I: molecular weight marker (lane 1), AAT standard marker (lane 2), FIV paste dissolved in Phosphate buffer *10%* (lane 12), FIV paste plus 30mm dithiothreitol (lane 11), FIV paste plus 2% Aerosil (lane 10), FIV paste plus 8% celite (lane 9), FIV filtrate (lane 8), ion exchange* Run A: peak 1 (lane 7), peak 2 (lane 6), peak 3 (lane 5) , ion exchange Run B: peak 1(lane 4), peak 2 (lane 3), *peak 3 (see GEL II).*

Gel II: molecular weight marker (lane 2), AAT standard marker (lane 1), Run B: peak 3 (lane 12), Run C: peak 1 (lane 11), peak 2 (lane 10), peak 3 (lane 9), Run D: peak 1 (lane 8), peak 2 (lane 7), peak 3 (lane 6), Run E: peak 1 (lane 5), peak 2 (lane 4), peak 3 (lane 3).

Gel III: molecular weight marker (lane 12), AAT standard marker (lane 11), Run F: peak 1 (lane 10), peak 2 (lane 9), peak 3 (lane 8), Run G: peak 1 (lane 7), peak 1* (lane 6), peak 2 (lane 5), peak 3 (lane 4), Run B: peak 1 (lane 3), peak 2 (lane 2), peak 3 (lane 1). *(Peak 1 is unbound wash, peak 2 is salt elution, and peak 3 is NaOH wash)

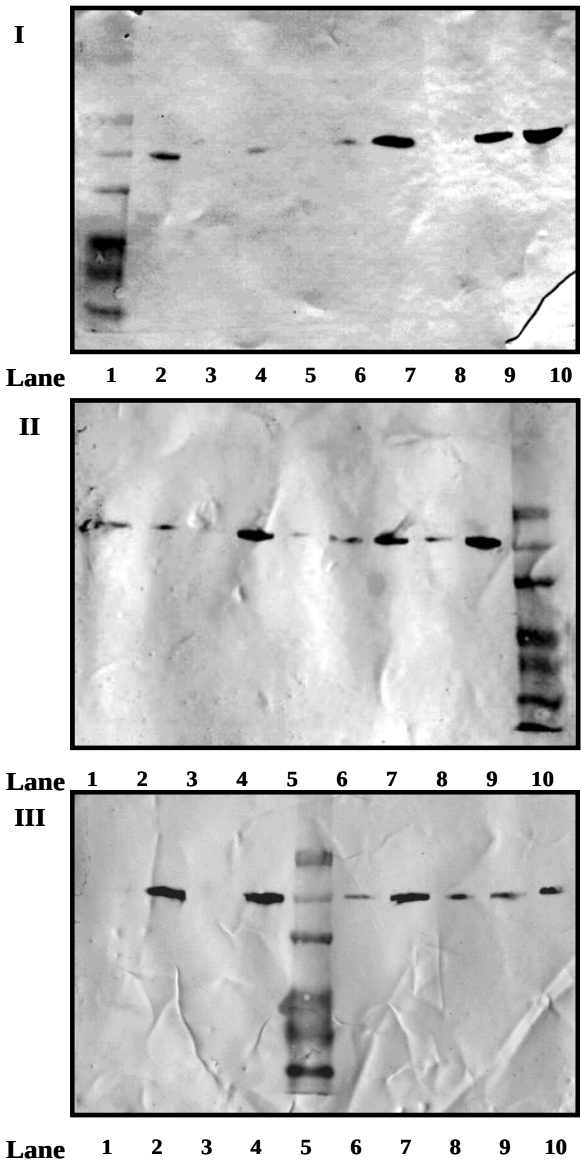


Figure 5.7 Analysis of samples from successive steps in the purification of AAT from Fraction IV precipitate, and preparative anion exchange runs using Western blotting method. Samples were applied to a 4-20% Pierce gel and electrophoresed at 120V for 45 minutes.

Gel I: molecular weight marker (lane 1), AAT standard marker (lane 2), FIV paste dissolved in Phosphate buffer *10%* (lane 10), FIV filtrate (lane 9), ion exchange* Run A: peak 1 (lane 8), peak 2 (lane 7), peak 3 (lane 6) , ion exchange Run B: peak 1(lane 5), peak 2 (lane 4), peak 3 (lane 3).

Gel II: molecular weight marker (lane 10), AAT standard marker (lane 9), Run C: peak 1 (lane 8), peak 2 (lane 7), peak 3 (lane 6), Run D: peak 1 (lane 5), peak 2 (lane 4), peak 3 (lane 3), Run E: peak 1 (lane 2), peak 2 (lane 1), *peak 3 (see GEL III).*

Gel III: molecular weight marker (lane 5), AAT standard marker (lane 4), Run E: peak 3 (lane 10), Run F: peak 1 (lane 9), peak 2 (lane 8) peak 3 (lane 7), Run G: peak *1 (lane 6), peak 1 (lane 3), peak 2 (lane 2), peak 3 (lane 1). *(*Peak 1 is unbound wash, peak 2 is salt elution, and peak 3 is NaOH wash*)

5.4.2 IMPROVING PROTEIN RECOVERY FROM FIV PRECIPITATE DEPTH FILTRATION

The resulting elution profiles, and gel analyses from the process run trial indicate that the conditions which give the highest protein content is with an initial wash the unbound protein was with 20mM Phosphate buffer, no salt at pH 6.2. Bound protein should then be eluted with 20mM Phosphate pH 6.2 containing 1M NaCl using a step elution. There is no need to increase salt concentration as no more protein was eluted at 2M salt prior to the 0.5M NaOH column wash. The filtration stage showed an extremely poor recovery total protein and AAT. To improve the recovery of FIV paste filtration step, body feed addition and type was investigated. A very poor step yield of 8% total protein is recovered, and a poor isolation of 0.003% AAT is attained after the depth filtration step.

In this section, an improvement in filtration is attempted at this stage by assessing the impact of body feed, and reducing agent on protein and AAT recovery.

5.4.3 BODY FEED FILTER AID TYPE

Various body feed types were mixed in with the sample prior to depth filtration. The filtration method is described in section 5.3.6. The filter aid is added in order to increase flux rates and improve cake permeability. These are inert, rigid powders with 25-30 μm pores to trap solids while encouraging the formation of open flow channels for the liquid(Reynolds, 2004). At this stage no reducing agent or precipitating agent (Aerosil^{TM}) was added. The body feeds tested are: Celpure^{TM} 100, Celpure^{TM} 300, Celpure^{TM} 1000, Celpure^{TM} HYFLO NF- an acid washed type , currently discontinued at a pharmaceutical grade, (World Minerals Inc, Santa Barbara, CA, USA). The body feed percentages and mixing times analysed are show inTable 5.4.

The suspension is added to a Sartorius SM16249 filter housing (Max pressure:10 bar, max volume:220mL) (filter at 2 bar pressure) , with a 47mm Seitz K900 Depth filter (already with a pre-coat) . The total protein recovery is measured crudely at absorbance A280, and AAT concentration is measured using an Elisa kit.

The impact on body feed alone is portrayed in Figure 5.8. The maximum percentage recovery of protein is 45%, using a 3%w/v, Celpure^{TM} HYFLO NF mixture, an average of 28% protein is recovered amongst all aids and concentrations. This may mean that

the bulk impurities are retained. Percentage body feed and mixing times were tested using Celpure™ 1000 and Celpure™ HYFLO NF , as they showed the highest

Table 5.4A summary of all the body feed types, mixing times, and concentrations investigated using a K900 Seitz 47mm filter using a Sartorius SM16249 filter housing at RT, slow mixing.

Body Feed Type	Body feed (w/v) %	Mixing time (min- max)
Celpure 100	3	15mins+
Celpure 300	3	15mins+
Celpure 1000	3	15mins+
Celite HYFLO NF	3	15mins+
Celpure 100	10	15mins+
Celpure 300	10	15mins+
Celpure 1000	10	15mins+
Celite HYFLO NF	10	15mins+
Celpure 100	3	overnight
Celpure 300	3	overnight
Celpure 1000	3	overnight
Celite HYFLO NF	3	overnight
Celpure 100	10	overnight
Celpure 300	10	overnight
Celpure 1000	10	overnight
Celite HYFLO NF	10	overnight

recoveries. The impact of body feed type, content and along with the mixing time length is shown in Figure 5.9. The longer mixing times and lower percentage body feed content gave higher overall recoveries. This is however, measured very crudely using absorbance at A280nm. AAT concentration is measured for all the conditions and the yields calculated (Table 5.5). A very poor yield of 10-13% AAT is recovered under all conditions through the single depth filtration step. Since there is not much change in AAT yield under all the conditions tested, the removal of impurities at this stage is more important at this stage. Therefore, the lower total protein recovery may be more desirable. The cumulative mass of filtrate collected was recorded against time and filtration curves for a range of pressures in the batch filter are shown in Figure 5.10. These curves show how the mass of filtrate builds up over time. It can be seen that using the Celite HYFLO NF^{TM} at 3% body feed enables a fixed amount of filtrate to be collected in a shorter time. The filtration curves all exhibit the same initial steep slope gradually levelling out into a plateau. This shape is a result of the increasing resistance of the filter cake as more solids are deposited and the depth of the cake grows making it more difficult for the liquid to flow through. The filters quickly became blocked under all conditions except with Celpure 1000^{TM}, 3% body feed. This build up of cake usually has the effect of improving the clarification with time since the cake captures a greater proportion of the solids and may be more cost-effective.

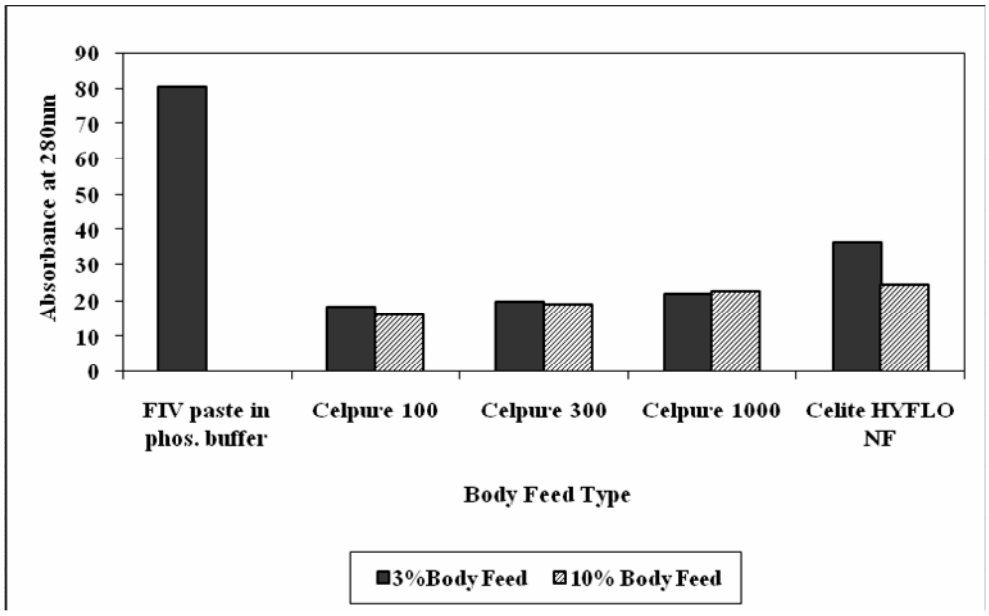


Figure 5.8 The impact of filter aid body feed type and percentage body feed on protein concentration. 20g of FIV paste was filtered through a K900 Seitz filter using aSartorius

SM16249 filter housing. The filtration process was stopped when the filtrate started to foam.

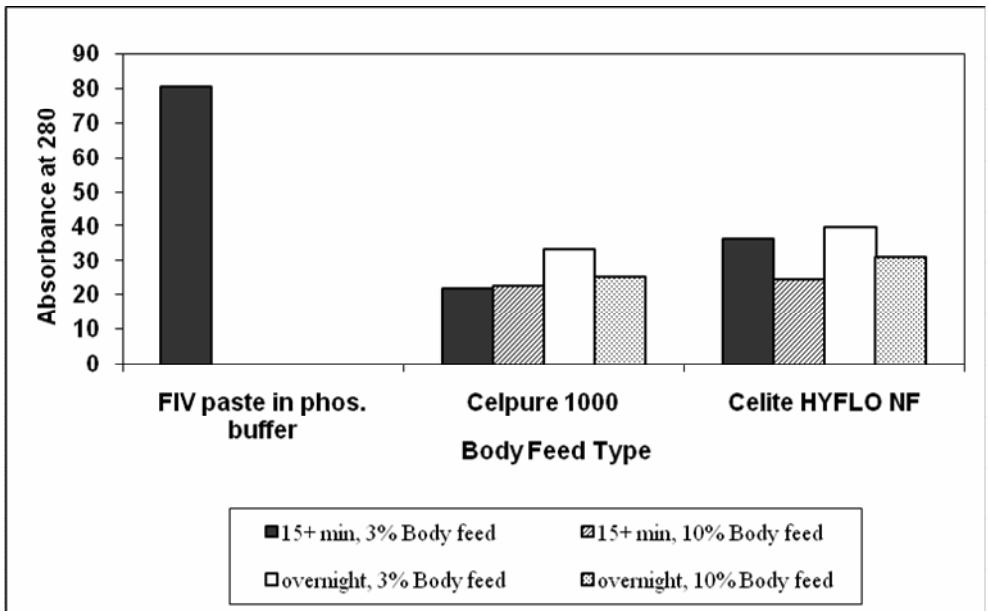


Figure 5.9 The impact of filter aid body feed type, percent body feed, and mixing time on protein concentration. 20g of FIV paste was filtered through a K900 Seitz filter using a Sartorius SM16249 filter housing. The filtration process was stopped when the filtrate started to foam.

Table 5.5 This table shows the concentration of AAT in FIV paste, and the recoveries using various body feed types and conditions.

Body Feed Type	Body feed % (w/v)	Mixing time	AAT concentration (g/L)	AAT Yield (%)
FIV paste in phosphate buffer	N/A	N/A	0.392	N/A
Celpure 100	3	15 mins*	-	-
Celpure 300	3	15 mins	-	-
Celpure 1000	3	15 mins	0.040	10%
Celite HYFLO NF	3	15 mins	0.050	13%
Celpure 100	10	15 mins	-	-
Celpure 300	10	15 mins	-	-
Celpure 1000	10	15 mins	0.040	10%
Celite HYFLO NF	10	15 mins	0.042	11%
Celpure 100	3	overnight	-	-
Celpure 300	3	overnight	-	-
Celpure 1000	3	overnight	0.049	12%
Celite HYFLO NF	3	overnight	0.046	12%
Celpure 100	10	overnight	-	-
Celpure 300	10	overnight	-	-
Celpure 1000	10	overnight	0.052	13%
Celite HYFLO NF	10	overnight	0.051	13%

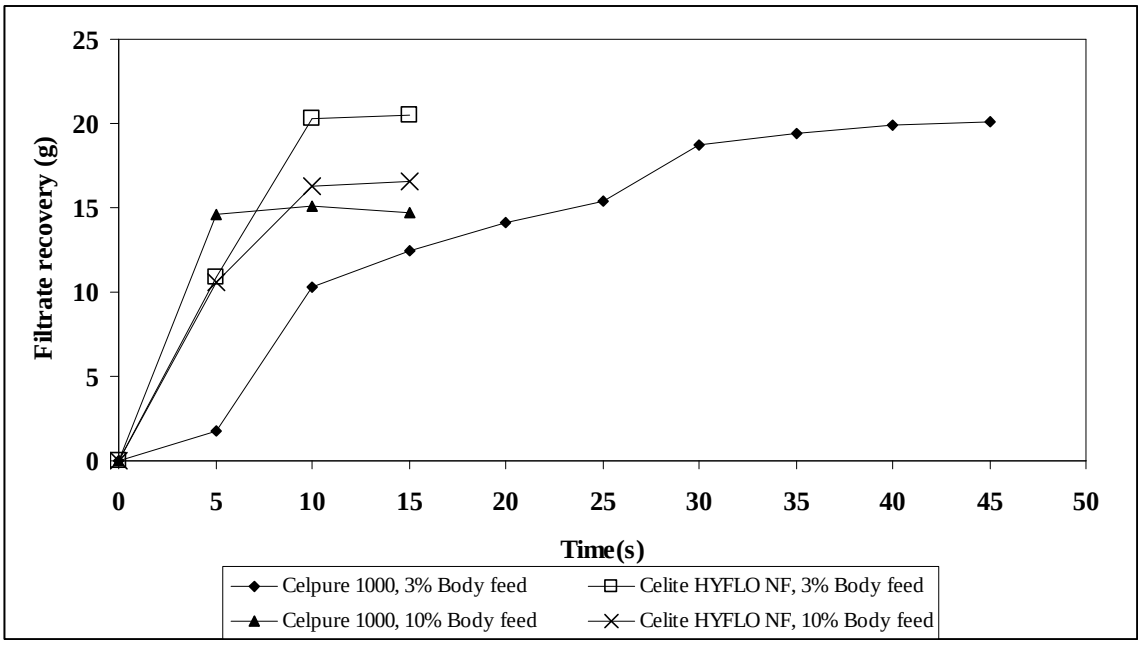


Figure 5.10 Fraction IV paste filtrate recovery over time(s). 20g of FIV paste was filtered through a K900 Seitz filter using a Sartorius SM16249 filter housing. The filtration process was stopped when the filtrate started to foam.

5.4.4 THE IMPACT OF REDUCING AGENT ON FIV PRECIPITATE FILTRATION

The impact of adding the reducing agents with body feed prior to depth filtration was also investigated. A FIV paste suspension was made up in BIS-TRIS buffer (20mM, pH 7.1). The pH was monitored adjusted back to pH 7.1 throughout the experiment.

The impact of adding the reducing agents: DTT, TCEP and no reducing agent with and without Celpure^{TM}1000 body feed was investigated. Initially, a crude measure of absorbance at 280nm was used to estimate total protein recovery. The reducing agents disrupt the absorbance reading and so the yield calculation was based on the separate filtrate feeds with its appropriate reducing agent. The highest recovery was observed when no reducing agent or feed was added, and the lowest total protein recovery when DTT was added to the FIV solution (Table 5.6). To eliminate the error in absorbance reading caused by the reducing agents, a BCA^{TM}protein assay-reducing agent compatible kit(Pierce) was used to also measure total protein (Table 5.7). It shows a similar result in that a FIV dissolution with no body feed or reducing agent yielded the highest recoveries, but adding TCEP to the solution would seem to give the lowest recoveries. Since AAT yield was shown not to be too variable with body feed or it may be more appropriate to choose the condition that resulted in the lowest overall protein recovery. This should signify the highest removal of impurities; however, more detailed analysis of the samples can reveal what exactly has been removed. Also, at a larger scale, the difference in 10-13% yield could translate to a much greater product mass loss. The addition of the reducing agents to a commercial process could prove laborious in that the removal of excess denaturants would be required by either dilution or a buffer-exchange step, such as dialysis, diafiltration, gel-filtration chromatography or immobilization onto a solid support. For commercial applications the need for additional steps would be costly. The cumulative mass of filtrate collected was again recorded against time and filtration curves for the range of body feed and reducing agent conditions Figure 5.11. The FIV paste suspension with no additions of body feed or reducing agent yield higher flux rates enabling a fixed amount of filtrate to be collected in a shorter time.

Table 5.6 The impact of adding a reducing agent on filtrate recovery (Total protein recovery %) prior to filtering Fraction IV paste dissolution. FIV paste was filtered through a K900 Seitz filter using a Sartorius (SM16249) filter housing. The filters were washed with dissolution buffer (20mM BIS-TRIS)

Sample	Total Protein Recovery in Filtrate with no Body feed	Total Protein Recovery in Filtrate with Celpure1000
FIV paste in No reducing agent (1 hour)	43%	40%
FIV paste in DTT(1 hour)	32%	28%
FIV paste in TCEP(1 hour)	35%	34%

Table 5.7 Analysis of total protein using the BCA™ Reducing protein assay-reducing agent compatible kit (Pierce) to assess the impact of adding a reducing agent prior to filtering a Fraction IV paste dissolution on percent total protein recovery FIV paste was filtered through a K900 Seitz filter using a Sartorius SM16249 filter housing. The filter was washed with dissolution buffer (20mM BIS-TRIS) and added to the filtrate.

Sample	Concentration (g/L)				Filtration step Protein Yield (%)
	Feed	(+/-SEM), n=3	Filtrate	(+/-SEM), n=3	
FIV ppt in no reducing agent no body feed	26.2	1.51	14.5	1.05	55
FIV ppt in DTT, no body feed	43.3	7.66	13.8	1.66	32
FIV ppt in TCEP, no body feed	39.7	10.94	8.2	0.89	21
FIV ppt in no reducing agent plus C1000*	84.1	7.76	13.0	1.63	49
FIV ppt in DTT plus C1000*	70.5	6.25	15.5	2.71	36
FIV ppt in TCEP plus C1000*	60.8	17.37	7.1	0.57	18

* Celite interference in absorbance reading

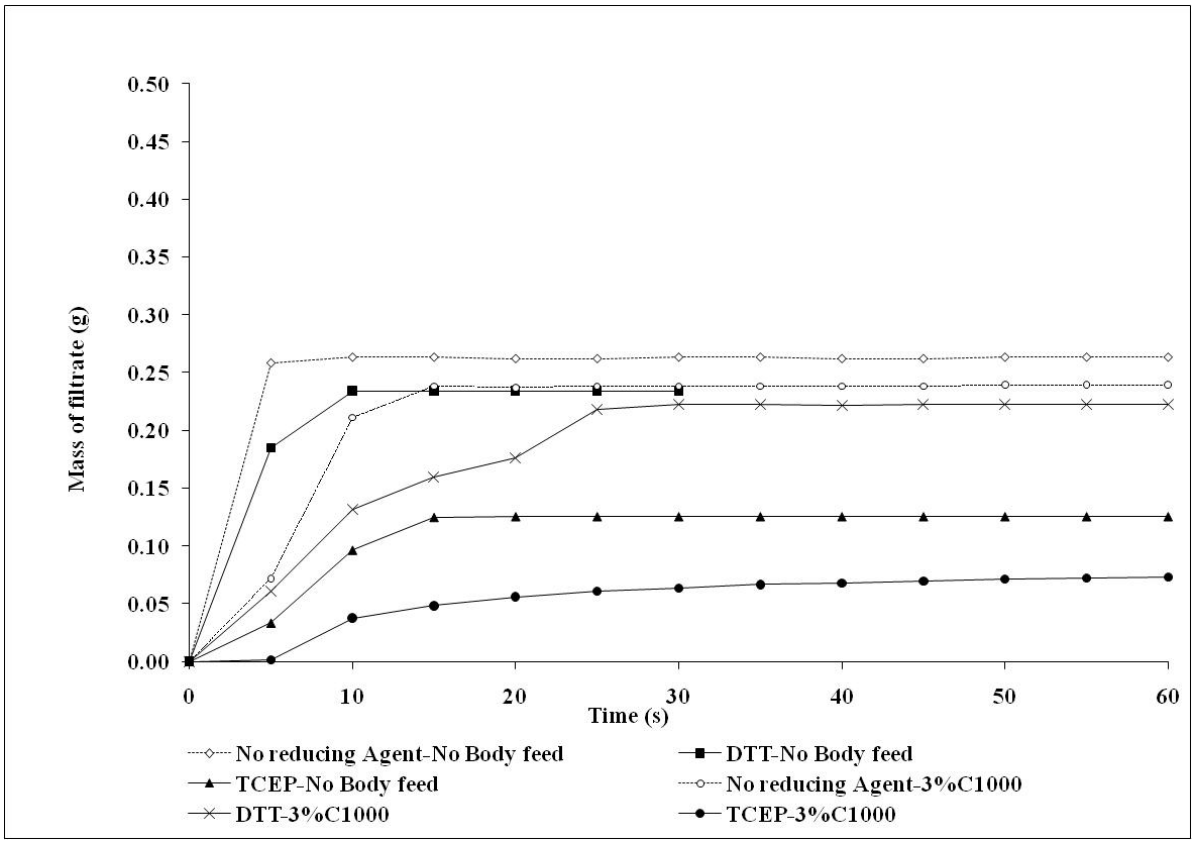


Figure 5.11 Filtration curves for the recovery of Fraction IV filtrate over time(s). 20g of FIV paste was filtered through a K900 Seitz filter using a Sartorius SM16249 filter housing.

5.4.5 IMPROVED PROCESS RUN

Based on the results from the previous sections an improved process is set. The process is a lot simpler than the process set by Kee*et al.*, 2004, in that no reducing agents, precipitating agent or body feedis added to FIV the dissolution prior to filtering. The conditions used are Q Sepharose or Fractogel TMAE columns, pH adjusted to 6.2, equilibrated and washed with 20mM Phosphate buffer at pH7, and product is recovered using a step elution to 1M NaCl over 10 column volumes. Good elution profiles are achieved when using the Hitrap QFF and Fractogel TMAE columns (Figure 5.12).

Very little protein is removed from the column after the caustic soda wash when using the Q Sepharose column. SDS page gel and western blot analysis (Figure 5.13) show that for Q-Sepharose run indeed there is little , if any protein in peak 3 (NaOH wash)

(lane 12), but there is still Albumin in the product peak (lane 11). The conditions run using the Fractogel column gives a purer product peak (lane 7) with less obvious Albumin bands present. This is also shown in the total protein and AAT concentration assays, with a purer product peak when using the Fractogel column. However, the purity content of AAT is so small at 1-2% of total protein that it is difficult to compare accurately. A maximum anion exchange step yield is achieved is with the Q-Sepharose column at 51% . This is very low for ion exchange chromatography, and there is still a great deal of room for improvement. The depth filtration step has been improved to give a yield of 83% AAT recovery.

Table 5.8 Total protein and AAT content of samples recovered from all stages of the AAT purification process from the improved process run. Anion exchange data is for Fractogel, and Q Sepharose FF

Sample type	Average Total Protein mass (mg)	Average AAT Mass (mg)	Protein Step Yield (%)	Protein Overall Yield (%)	AAT Step Yield (%)	AAT Overall Yield (%)	AAT Purity (%)
FIV paste							
FIV paste dissolved in phosphate buffer	3229.8	46.1	-	-	-	-	1%
Depth filtrate + wash	1912.7	38.5	65%	65%	83%	83%	2%
Membrane Filtrate + wash	1976.2	20.3	95%	62%	53%	44%	1%
Membrane Filtrate + wash pH adjusted to pH 6.2 (load)	1228.5	20.3	64%	39%	100%	44%	2%
Fractogel Run A Peak 1	817.8	6.5	-	-	-	-	-
Fractogel Run A Peak 2	365.1	8.4	29%	11%	41%	18%	2%
Fractogel Run A Peak 3	60.4	0.3	-	-	-	-	-
Q Sepharose FF, peak 1	1270.0	7.4	-	-	-	-	-
Q Sepharose FF, peak 2a	31.4	0.1	58%	23%	51%	23%	1%
Q Sepharose FF, peak 2b	710.4	10.3					
Q Sepharose FF, peak 3	38.8	0.1	-	-	-	-	-

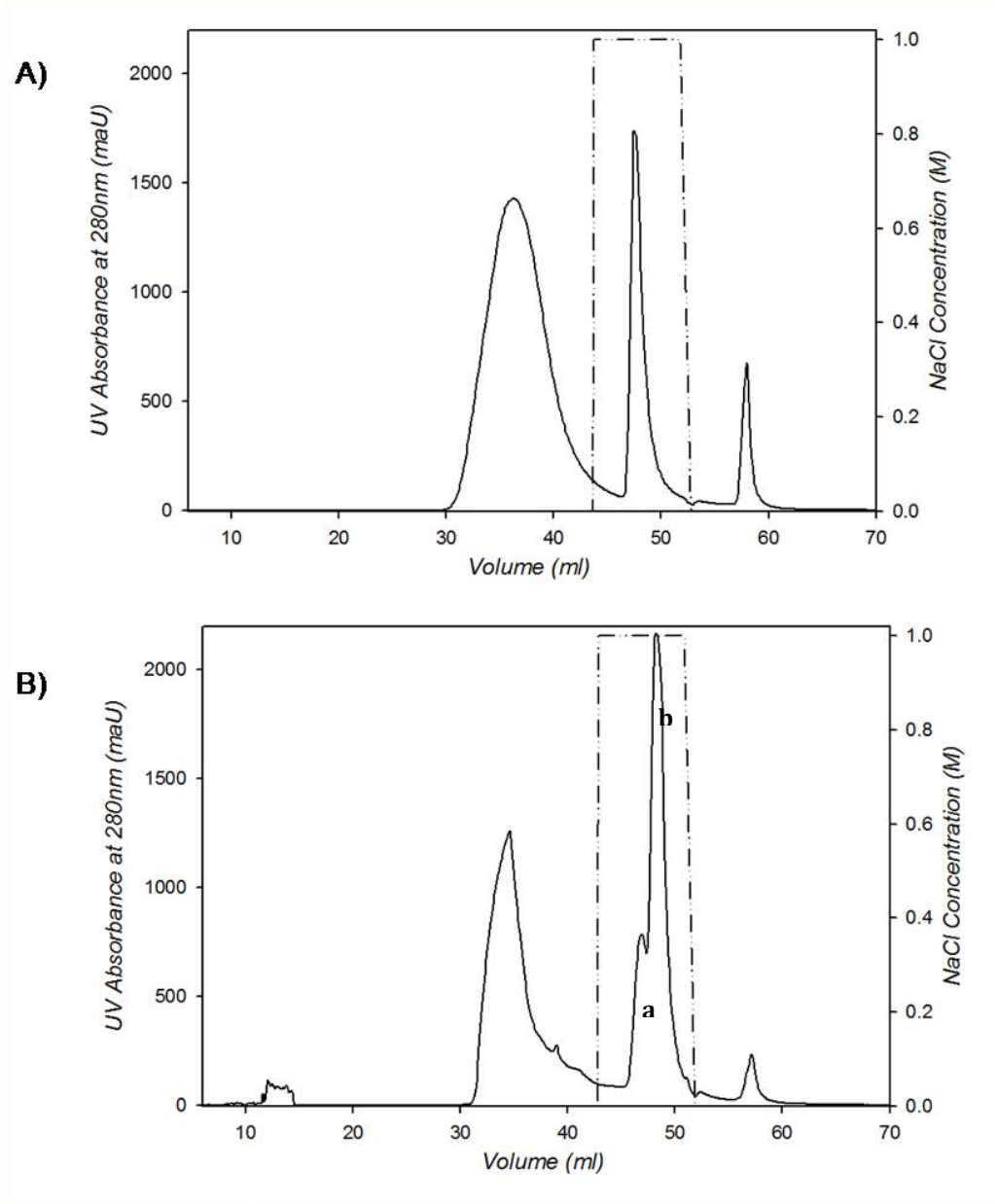


Figure 5.12 Elution profiles of Fraction IV filtrate on 1mL pre-packed columns: (A) Fractogel EMD TMAE column, and (B) Hitrap Q Sepharose FF. Columns are equilibrated to pH 7 with 20mM Phosphate buffer, protein is loaded at pH 6.2, Peak 1 represents the wash of unbound protein with 20mM Phosphate at pH 7, Peak 2 (a-b) shows a step elution to 1 M NaCl, Peak 3 is a 0.5M NaOH wash. Symbols are: (—) Absorbance, (- -) NaCl concentration

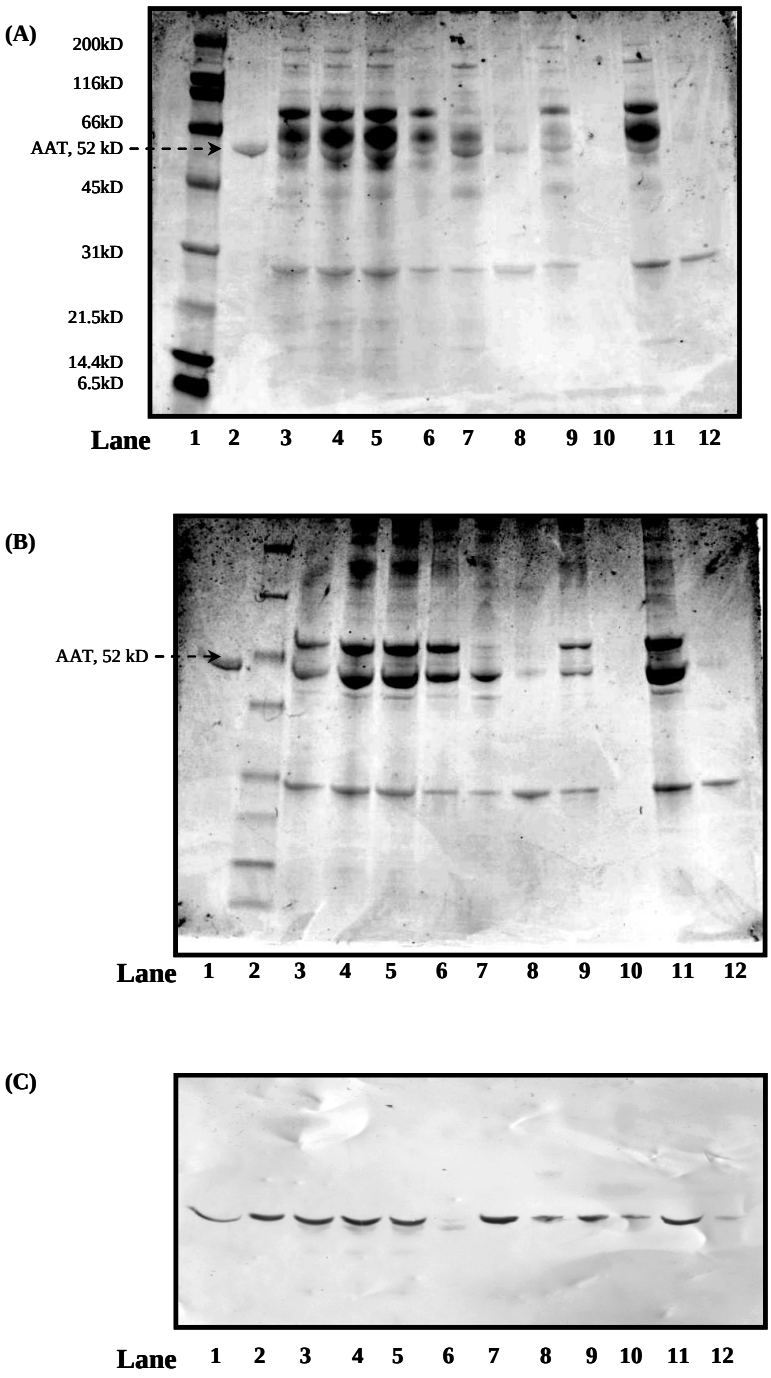


Figure 5.13 Analysis of samples from successive steps in the purification of AAT from Fraction IV precipitate using SDS-gel electrophoresis and Western blot analysis. Samples were applied to a 4-20% Pierce gel and electrophoresed at 120V for 45 minutes. For SDS PAGE analysis the gels were stained with Coomassie blue.

(A): Reduced SDS PAGE gel: molecular weight marker (lane 1), AAT standard marker (lane 2), FIV paste dissolved in Phosphate buffer *10%* (lane 3), Filtrate+ Wash (lane 4), 6.2 pH adjusted load (lane 5), ion exchange* Fractogel : peak 1 (lane 6), peak

2(lane 7), peak 3 (lane 8), ion exchange* Q Sepharose peak 1 (lane 9) : peak 2a(lane 10), peak 2b (lane 11) peak 3(lane 12).

(B): Non-reduced SDS PAGE gel: AAT standard marker (lane 1), molecular weight marker (lane 2), FIV paste dissolved in Phosphate buffer *10%* (lane 3), Filtrate+ Wash (lane 4), 6.2 pH adjusted load (lane 5), ion exchange* Fractogel : peak 1 (lane 6), peak 2(lane 7), peak 3 (lane 8), ion exchange* Q Sepharose peak 1 (lane 9) : peak 2a(lane 10), peak 2b (lane 11) peak 3(lane 12).

(C): Western Blot analysis. The blot is probed with a polyclonal goat alpha 1-antitrypsin antibody conjugated with Horse radish Peroxidase (HRP): AAT standard (lane 1), FIV paste dissolved in Phosphate buffer *10%* (lane 2), Depth filtrate+ Wash (lane 3), Membrane Filtrate (0.45µm and 0.2µm filters) + Wash (lane 4), 6.2 pH adjusted load (lane 5), ion exchange* Fractogel : peak 1 (lane 6), peak 2(lane 7), peak 3 (lane 8), ion exchange* Q Sepharose peak 1 (lane 9) : peak 2a (lane 10), peak 2b (lane 11) peak 3 (lane 12).

*(Peak 1 is unbound wash, peak 2 is salt elution, and peak 3 is NaOH wash)

5.5 CONCLUSIONS

In this Chapter an attempt was made to purify AAT from FIV precipitate of a plasma fractionation scheme at BPL. Results show that whilst AAT can be purified from FIV precipitate, the process based on the conditions set by Kee*et al.*, did not prove successful for the FIV precipitate from the fractionation process at BPL. The addition of a reducing agent such as DTT to F IV to disrupt the protein structure and precipitate out surrounding impurities did not prove valid here. The major impurity of Albumin was still present at high concentrations post filtration, and so this step was also removed. Anion exchange chromatography and depth filtration steps were effectively modified and resulted in a fully functional process. The recovery of AAT filtrate from depth filtration was improved greatly. The filtration performance was also improved by removing all filter aids and reducing agents. The final recoveries of AAT are very low at 18% or 23% when using Fractogel or Q-Sepharose media respectively. FIV precipitate may not be the ideal start material for the purificationof AAT , as the start concentration of AAT is far too low. However, the product is in demand, so a cost-benefit trade-off for selling it as a cheap by product or purifying AAT from the paste a

new product needs to be analysed further. Other proteins are present in FIV and may be present at higher concentrations and so may be more suited to a purification from this stage, this also needs to be assessed. Alternatively, AAT can also be purified from a higher process fraction (Fraction A+1), but this was would have an impact on approved products such as Albumin. This has been investigated by Kumpalume (2007, 2008).

Chapter 6

Improving 'Q Sepharose' Step Isolation of Alpha 1-antitrypsin using the SELDI-TOF-MS Technology: A High Throughput Method

6.1 INTRODUCTION

In the previous chapter, a process to purify AAT from FIV precipitate of a human plasma fraction process (a combination of the Cohn (Method 6) and those developed by Kitsler and Nitchman (1962)) is given at laboratory scale. The first isolation step used was strong anion exchange chromatography in the form of Merck's Fractogel EMD TMAE (M)and GE Healthcare's 'Q Sepharose Fast Flow' media. Successful AAT isolation was achieved using 1mL columns under the following conditions:columnequilibration with 20mM Phosphate buffer (NaH_2PO_4:Na_2HPO_4) at pH 6.2, FIV filtrate loading at 1.5mL/min, washing to remove unbound protein with the equilibration buffer, and elution of bound protein using 20mM Phosphate 1M NaCl, pH 6.2 step elution over 10 column volumes. The column was then sanitised with 0.5M NaOH to remove any protein stuck on the column. The average step-yield was low at 33% AAT recovery, with a purification factor increase of 1.74 based on AAT and total protein concentrations. Analysis shows that there is AAT present at the wash, elution and sanitisation stages. Protein lost during the initial no-salt wash stage may be a result of column overloading or weak binding or a combination of both. Some protein may also be bound too tightly to the column, and only detached during the caustic wash stage. AAT and Total protein concentration, SDS-gel, and 10% Agarose gel analysis also revealed that there were impurities in the salt elution step. The main impurities were Albumin and Transferrin. An attempt to improve AAT recovery and reduce impurities binding to the column is described in this chapter.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) ProteinChipArray (CiphergenBiosystems, Inc., Fremont, CA) technology is used to mimic the isolation of AAT from FIV filtrate at microchip scale using the 'Q-chip' array. This array carries the same functional group [$CH_2N+(CH_3)_3$] as the Q-Sepharose resin. The purpose of this work is to determine the usefulness of using this high throughput technique to optimise the anion exchange step suggested. Sample analysis using the SELDI-TOF-MS and Ciphergen® NP20 chips was also

investigated to compare its usefulness against well-established concentration determination methods, such as ELISA methods.

6.1.1 ION EXCHANGE CHROMATOGRAPHY

The principle of ion exchange chromatography is based upon the interaction between charged groups on the surface of proteins (the mobile phase) and those of oppositely charged groups on a chromatographic matrix (the stationary phase). The protein dipolar ion displaces any counter ions e.g. sodium or chloride ions from the matrix's functional group, and then will be displaced by an increasing amount of counter ion. This can be done by adding an increasing amount of salt to an elution buffer i.e. a salt gradient elution. Alternatively, a pH gradient can be used, so that the net charge on the adsorbed protein decreases. The pH of the solution at which the overall charge is neutral (the isoelectric point or p*I*of the molecule) will not interact with a charged medium. At a pH lower than the p*I*, the molecule will have a net positive charge; and if its pH is higher the net charge is negative. When a mixture of proteins is added to a column, the starting conditions of buffer, pH, and ionic strength will change the net charge of the protein of interest, and so can be manipulated to interact with the matrix. As a result, either everything passes through except the protein of interest or in some cases impurities are bound to the column, either way successfully separatesthe protein of interest. It is important to find the optimum combination of mobile phase conditions and matrix to maximise recovery.

The matrix comprises spherical particles substituted with ionic groups that are either negatively (cationic) which bind to positively-charged molecules or positively (anionic) charged which bind to negatively-charged molecules. Proteins bound to an anion exchanger will elute as pH is decreased, and will elute as pH is increased when bound to a cation exchanger(GE Healthcare Handbook, 2002).

In general, matrices differ in the type of ligand used and the density of its binding sites. The matrix is usually porous to give a high internal surface area. This medium is packed into a column to form a 'packed bed'. The bed is then equilibrated with buffer, which fills the pores of the matrix and the space in between the particles (Reynolds, 2004).

Ion exchange chromatography is the most broadly used chromatography method, and practically all industrial purification processes comprise one or more ion exchange step (Staby, 2000). In general, the matrices are not as costly as other types of matrix, such as those used in affinity chromatography. The process also has a high capacity and a

consistent concentrating effect and so the process is suited to large-scale operations, such as plasma fractionation.

6.1.2 Q SEPHAROSE FAST FLOW

Q Sepharose Fast Flow is a commonly used strong anion exchanger from GE Healthcare. The ion exchange group is a quaternary amine group:

$$–O–CH_2CHOHCH_2OCH_2CHOH\ \mathbf{CH_2N+(CH_3)_3}$$

Strong ion exchangers are fully charged over the total pH range normally applicable to proteins and peptides. It has a highly cross-linked agarose base matrix, which gives the media its chemical and physical stabilities.

6.1.3 SELDI-TOF-MS

6.1.3.1 INTRODUCTION

Surface Enhanced Laser Desorption/Ionisation Time-of-Fight Mass Spectrometry (SELDI-TOF-MS) is a commercialised ionisation method in mass spectrometry that is used for the analysis of protein mixtures (Tang, 2004), that employs chromatographic surfaces. This combination of specificity and reproducibility of mass spectrometry, allows for a high-throughput analysis of a wide variety of biological samples, rapidly producing protein profiles (Woolley and Al Rubeai, 2009). The SELDI-TOF-MS ProteinChip Reader PCS series 4000 apparatus was employed for this particular study.

The SELDI-TOF-MS is made up of three key components: the ProteinChip array, a mass spectrometer and the data collection and analysis software (Lin *et al.*. 2004;Schipper *et al.*. 2007). Each chip has an array of 8 -16 spots. The spots are composed of different chromatographic surface type such as hydrophobic, hydrophilic, ion exchange or immobilised metallic ion chromatographic matrices(Lin *et al.*. 2004). This platform is attractive for studying a wide range of conditions quickly, as it requires small sample sizes (from as little as 1μL) and minimal or no sample preparation of even crude protein extracts, because of the equipment's retentate chromatography feature(Lin *et al.*. 2004;Panicker *et al.*. 2009;Woolley and Al Rubeai, 2009). Effectively, an experiment is performed on the chip surface immediately before analysis. This reduces sample loss and allows smaller amounts of proteins to be analysed. The ionisation of some proteins are suppressed by the presence of others; proteins present in higher concentrations suppress the ionisation of proteins of lower abundance, allowing for straightforward quantification of protein concentration. However, Dijkstra*et al.* (2007)

also point that this can be a source of variation in sample analysis, reducing mass peak areas.

6.1.3.2 ADVANTAGES AND LIMITATIONS OF SELDI-TOF-MSTECHNOLOGY

As its name suggests, SELDI-TOF-MS can be considered an enhanced form of the conventional Matrix-assisted Laser Desorption/Ionisation Time-of-Fight Mass Spectrometry (MALDI TOF MS). The main difference is in the target surfaces to which proteins are applied, which are coated with various activated 'planar chromatographic chemistries' (Voderwulbeke *et al.*. 2005). With the SELDI-TOF-MS technology it is possible to fractionate a protein mixture, or separate particular classes of proteins on the chip or array surface. It is also separated from potentially interfering salts and other sample contaminants by subsequent on-spot washing with appropriate buffers. The respective chemical properties of each array make it possible to focus the analysis to either negatively or positively charged proteins, and allows for targeting of specific metal binding or phosphoproteins.

When used with a robotics system, SELDI-TOF MS is a high throughput technique that allows hundreds of samples to be tested in a relatively short time. Sample requirements are very low and can be directly applied without pre-treatment (Seibert *et al.*. 2004). Issaq*et al.*. (2003) estimated the cost of the protein chip arrays to be $75/chip for 8 spots, which although high is economical when the savings in time and labour and the wealth of information generated is considered.

Numerous summaries of the SELDI-TOF-MS technology's usefulness and recent developments have been reviewed (Issaq*et al.*. 2003; Kiehntopf*et al.*. 2007; Merchant and Weinberg, 2000; Seibert *et al.*. 2004; Vorderwulbecke*et al.*. 2005).

As with all analytical methods, SELDI-TOF MS has its limitations. Although it can rapidly generate proteome profiles from complex mixtures, and is chiefly successful at discovering proteins in the low-molecular-weight range, it is not routinely reproducible. In general it has low resolution and mass accuracy, coupled with the inability to successfully profile high-molecular-weight proteins (Issaq *et al.*. 2003).

Many of limitations that apply to mass spectrometry in general, apply to SELDI-TOF-MS. This is true of biases based on analysing multiple analytes and the relatively low sensitivity when compared to immunoassays such as ELISA (Grizzle *et al.*. 2005).

There are also several problems, which may be unique to the SELDI-TOF-MS system marketed by Ciphergen®. Dijkstra*et al.*. (2007) writes about many of sources of variation states in the SELDI-TOF-MS, and how this can affect the resulting spectra. For example, the competition for sites in the matrix, the washing stage(see 6.3.3) which can remove weakly bound proteins, all can reduce peak area size. One drawback that is often stated, is the bias towards peptides and smaller proteins (proteins <30 kDa), and that the sensitivity and resolution of the Time of Flight (TOF) analyser falls off markedly above 30kDa. However, with the optimisation of the Energy Absorbing Molecule (EAM) mixture (see 6.3.3) larger molecules do give a good resolution. In general, proteins present in high concentrations may also suppress the ionisation of less concentrated proteins and post-translationally modified proteins such as glycoproteins or phosphoproteins, by competing for binding places or ionization energy, which can be misleading (Engwegen *et al.*. 2007;van Breemen *et al.*. 2006). This is very true with blood serum or plasma, which is which contains highly abundant proteins such as albumin and immunoglobulin (Whelan *et al.*. 2008). These proteins, which account for 97% of all proteins, suppress middle and low-abundance proteins in the sample. Grizzle *et al.* (2005) states that one weakness is the relatively low resolution (± 0.2%) of the bundled mass spectrometer which may cause problems with analysis of data. This can make unconditional "peak" determination difficult if a peak matching approach is used in analysis, and may also potentially represent multiple proteins. For very sensitive analysis, it has been reported that peaks may vary slightly in location due to instrumental drift (Grizzle *et al.*. 2005).

SELDI-TOF-MS as a method has also been criticized because of the lack of data reproducibility that is seen. This is mainly due to lack of standardization of pre-analytical and analytical phases and has been reported extensively (Diamandis and Van Der Merwe, 2005;Dijkstra*et al.*. 2007b;Kiehntopf*et al.*. 2007;Liggett *et al.*. 2004;Panicker*et al.*. 2009).

6.2 EXPERIMENTAL OUTLINE

A Q SepharoseFast Flow HiTrap column has been used to isolate AAT from FIV filtrate, using the binding conditions of: pH 6.2, 0mM salt. An improvement in the binding conditions of AAT is attempted using the on the 'Q-chip' (Q Sepharose mimic). Due to the nature of the SELDI-TOF-MS technology, these experiments explore the most favourable binding conditions only. Good eluting conditions can be based on

conditions that show poor binding. However, elution has not been explored in this study, for if it is possible to isolate the protein of interest in a single step, then subsequently it should be suitable to elute everything off the column. Also, the SELDI-TOF-MS method is limited, as it does not allow for differing binding, and eluting buffers to be used. A matrix of 16 conditions based on pH and salt concentration were initially tested. These are summarised inTable 6.1.

Table 6.1A summary of the initial scouting conditions set to improve isolation of AAT from FIV filtrate using the SELDI-TOF-MS 'Q-10' chip

pH	**NaCl concentration (mM)**			
6.2	0	50	100	150
6.8	0	50	100	150
7.4	0	50	100	150
8	0	50	100	150

Additional conditions were then set-upbased on the initial scouting results, to investigate whether the improved data could be further enhanced and to discover the limits of AAT binding to the 'Q-10' chip. These conditions were of a lower pH and increased salt concentration, and are summarised in Table 6.2. These experiments were run on separate days, and so different preparations of Energy Absorbing Molecule (EAM) were used, as it expires after 24 hours. This has an impact on resulting MS intensities, andit is not possible to compare the raw data directly if using different preparations of EAM. Nine of the binding conditions tested at 'micro-scale' were then run at a larger scale, using1mL GE HiTrap QFF columns to validate the use of the SELDI-TOF-MS results. The conditions tested at the larger scale are summarised inTable 6.3. Breakthrough curves were run for all the conditions to further explore the impact of the binding condition. For each condition tested, the column was loaded to 5% breakthrough. Protein isolation was analysed using the following assays: Normal phase (NP20): a non-selective surface chip, SDS page, AAT specific ELISA, BCA total protein, and the turbitimer assay for Albumin, Transferrin concentrations. These methods are explained in section 6.3.

Table 6.2A summary of all the conditions tested to establish better isolation of AAT from FIV filtrate using the SELDI-TOF-MS 'Q-10' chip.

pH	NaCl concentration (mM)				
5.1	0	50	100	150	200
5.6	0	50	100	150	200
6.2	0	50	100	150	200
6.2	0	50	100	150	200
6.8	0	50	100	150	200
7.4	0	50	100	150	200
8	0	50	100	150	200

Note. The greyed boxes were run on a separate day, and with a different EAM mixture. A citrate buffer was used for pH 5.1 to 5.6. A Phosphate buffer was used for pHs 6.8 to 7. Both a citrate buffer and Phosphate buffer were used at pH 6.2 to ensure there was no variation caused by the buffer type.

Table 6.3A summary of the conditions tested at the larger scale of 1mL using a GE HiTrap QFF column, to validate the SELDI-TOF-MS runs

pH	NaCl concentration (mM)		
5.6	0	100	150
6.2	0	100	150
8	0	100	150

6.3 MATERIALS AND METHODS

6.3.1 CHEMICALS

All chemicals were of analytical grade quality, and were obtained from Fisher-Scientific UK (Loughborough, Leicestershire, UK) unless stated otherwise. Sodium monobasic Phosphate (NaH_2PO_4), and sodium dihydrogenPhosphate (Na_2HPO_4) were purchased from BDH Limited, VMR International Ltd., (Poole Dorset, England, UK). Buffers were prepared using deionised water, and were stored at room temperature for a maximum of one month. The composition of all the buffers used during SELDI-TOF-MS and column chromatography experiments is given in 5.2Table 5.1. AAT standard was a product of Sigma-Aldrich Co. Ltd (Poole, UK).

6.3.2 SAMPLE COLLECTION

The protein solution used in all chromatography runs was derived from frozen FIV paste. The paste (10-30g) was allowed to thaw at room temperature, and then dissolved in 10mM Phosphate buffer. The paste was then suspended at 10% w/v in a Phosphate buffer (10mM $Na_2H_2PO_4$: 10mM NaOH), pH 6.2. The suspension was then gently homogenized using a Silverson mixer (Silverson Machines LTD, Chesham, UK) with a 44mm disintegrating head (designed to break up solid materials into small pieces) for approximately 20 minutes at a temperature of 2-8°C. The solution was adjusted to a pH 7.5. A body feed of 3% w/v Celite^{TM} 1000 (Advanced Mineral Corporation, Goleta, CA) was then added to the suspension and stirred at room temperature. The solution was filtered using an air pressure stainless steel filtration device (SM 16249 housing, Sartorius, Gottingen, Germany) of 47 mm internal diameter (active area of 10x3 m^2). The suspension was firstly filtered through a Seitz K900 depth filter (Seitz-Filter-Werke,BadKreuznach, Germany), and then through two stacked cellulose acetate filters: a pre filter with a 0.2 μm pore size (Sartorius, Gottingen, Germany) and a second filter of pore size 0.45μm (Whatman International Ltd,Maidstone, UK). In all cases, a maximum pressure of 2MPa was used. This process step is described in Chapter 5 in detail.

6.3.3 SELDI-TOF MS METHODS

6.3.3.1 GENERAL SELDI-TOF MS METHOD

The analysis of proteins by the SELDI-TOF-MS was carried out in four distinct stages;

1. The purification or enrichment of the proteins of interest on the array or chip surface (a pre-coated stainless steel slide). The 'chip type' was determined by its coating, which 'enhances' the surface to bind preferentially to a specific group of proteins established on their physiochemical properties.
2. The second step was the creation of charged ions. The sample was further mixed with a UV energy absorbing matrix (EAM) such as (α-cyano-4-hydroxycinnamic acid for proteins less than 20 kDa and sinapinic acid for proteins greater than 20kDa in mass), which caused the entire mixture to crystallize as it dried. It was then placed into the mass spectrometer, and a fast UV laser pulse (~4ns) was fired at the array surface. The matrix allows a single

proton to be added (or in some cases subtracted) from the peptide or protein and causes the molecule to become charged or ionised. The charged protein(s) were then accelerated out of the source (receiving constant kinetic energy) using a series of oppositely charged metal plates and travel into the mass analyser section of the mass spectrometer.

3. The firing of a laser at the sample and matrix mixture.
4. Determination of the masses of the proteins in that sample, analysis and comparison of individual samples.

6.3.3.2 'Q10' CHIP PROTOCOL

Q10 ProteinChip arrays and sinapinic acid were obtained from BioRad Laboratories, Inc. (Hemel Hempstead, UK). This strong anion exchanger encompasses active spots that contain cationic, quaternary ammonium groups that interact with the negative charges on the surface of the target proteins. The surface binds proteins that are negatively charged at a given pH. It works in the same manner as a strong anion exchange media such as Q Sepharose.

FIV filtrate samples are prepared as described in section 5.3.3.

All samples were diluted 20 fold in 10mM Phosphate buffers, with varying salt concentrations and pH values as shown inTable 6.2. The same buffers were used for equilibration and washing steps. The Q10 chips were equilibrated in the appropriate buffers, by adding 150µL of each buffer into deep-well plates attached to the chips and placed on a horizontal plate-shaker (600rpm) at room temperature for 5 minutes. This step is repeated. The reconstituted sample (150µL) is then loaded onto the chip and mixed at room temperature for 30 minutes. The sample is removed by aspiration and chips are then washed in the appropriate buffers (150µL) three times, each time they are placed on the horizontal plate shaker for 5 minutes. This is followed by a quick wash in deionised water (150µL), to ensure no interference from the Phosphate buffer. The surfaces were air-dried (approximately 30 minutes) and loaded with 1µL of EAM solution, a saturated solution of aSPA , made up of 5mg of sinapinic acid (SPA) reconstituted in 200µLof 100% acetonitrile, and 200µL of 1% v/v Trifluoroacetic acid (TFA). This step was repeated once more after air-drying. Proteins bound to the Q10 ProteinChip arrays were analyzed by means of a ProteinChip PCS4000 (BioRad Inc. (Hercules, CA, USA) mass spectrometer, Enterprise version. Instrument and data collection were controlled through the CiphergenExpress v3.0.6 software interface.

Overall instrument settings were in positive mode for the ion source at 25 kV and a digitizer rate at 800 MHz.The instrument settings were determined separately for the low mass and high mass range of the protein profile. Data collection was set to 150 kDa optimized for m/z between 2–30 kDa for the low mass range and 30–100 kDa for the high mass range. For the low mass range, the laser intensities ranged from 185–200 with a detector sensitivity of 8 and number of shots averaged at 180 per spot for each sample. For the high mass range, the laser intensities ranged from 230–240 with a detector sensitivity of nine and the number of shots averaged at 150 per spot.

6.3.3.3 'NP20' CHIP PROTOCOL

Normal phase ProteinChips (NP20) (BioRad Laboratories, Inc. (Hemel Hempstead, UK) were used as an analytical tool to qualitatively and quantitatively assess AAT standard samples (Sigma), and samples from column chromatography runs, including load, wash and eluate samples. The NP20 is a non-selective ProteinChip and so all proteins in a tested sample should bind to the array. The active spots contain silicon dioxide, which allows proteins to bind via serine, theronine, or lysine (BioRad Inc., 2004).

All samples were diluted 10-fold, and 5μL is applied directly to an NP20 spot surface. The spots were air dried for 20-30minutes, and then the surfaces were washed three times with 5uL deionised water to remove proteins that were not bound to the surface. The surfaces were briefly air dried (approximately 10 minutes) and loaded with 1uL of EAM solution: again a saturated solution of 5mg sinapinic acid (SPA) reconstituted in 200μL 100% acetonitrile, and 200μL of 1% TFA. This step was repeated once more after air-drying. The arrays were analysed using the PC4000 reader (see section 6.3.3.2).

6.3.3.4 HITRAP QFF

All chromatographic procedures were carried out using pre-packed 1mL (7mm by 25mm) HiTrap QSepharose Fast Flow (QFF) columns (GE Healthcare). The mean particle size was approximately 95μm and the particle size range is 45–165μm as quoted by the manufacturer. All experiments were performed on an ÄKTA Prime Plus (GE Healthcare) equipped with a fraction collector. FIV filtrate was prepared as described in section5.3.3.

6.3.3.5 PROTEIN QUANTIFICATION

For a description of the protein quantification methods used, i.e. SDS page (reducing), western blot, Total protein (BCA) assay, Alpha 1-antitrypsin ELISA quantification and Turbitimer assay in determining albumin and transferrin concentrations see Chapter 5.

6.3.3.5.1 SELDI-TOF-MS NP20 ANALYSIS

The method is described in section 6.3.3.3

6.4 RESULTS AND DISCUSSION

6.4.1 'Q10 PROTEINCHIP' MS INTENSITY PROFILE DATA

The Q10 ProteinChipsresults from the initial scouting AAT binding conditions (Table 6.1) are shown in Figure 6.1. The conditions set were based on data from Glaser (1982),Kee and Cook (2004), Kumpalume*et al.*. (2007) who also looked at purifying AAT from FIV paste, using of an anion exchange isolation step.

AAT binds at 50.1kDa/e; this is confirmed by running an AAT standard (Sigma-Aldrich) on an NP20 chip and is shown in Figure 6.2. It shows the same mass as seen in the 'Q10' chips, but they have slightly different ionizations and peak shapes owing to the differing chips, and decreased competition of proteins in the AAT standard mixture. There is competition amongst different proteins and salts in any specimen for ionization, a general phenomenon in mass spectrometry termed 'ion suppression' which lowers the yield of specific ions and reduces the sensitivity of detection (Hortin, 2006).

An increase in pH from 6.2-8.0 at 0mM NaCl does not appear to have an impact on AATbinding;a slight reduction in low mass impurity levels is seen.However, when salt concentration is increasedfrom 0mM to 150mM NaCl fewerlow and high mass impuritiesare bound (Data collection was set to 150 kDa optimized for m/z between 2–30 kDa for the low mass range and 30–100 kDa for the high mass range); this can clearly be seen with the impurity Transferrin at approximately 79kDa/e, which gradually decreases as salt concentration is increased at all the pH values tested. Albumin is much harder to remove and is present under the majority of conditions tested, even some of the high salt concentrations, except atpH 6.2 and 6.8 at 150mM NaCl, where binding is reduced significantly. Therefore, based purely on impurity binding it seems that AAT is best isolated at pH 6.2 or 6.8, with a salt concentration of 150mM.

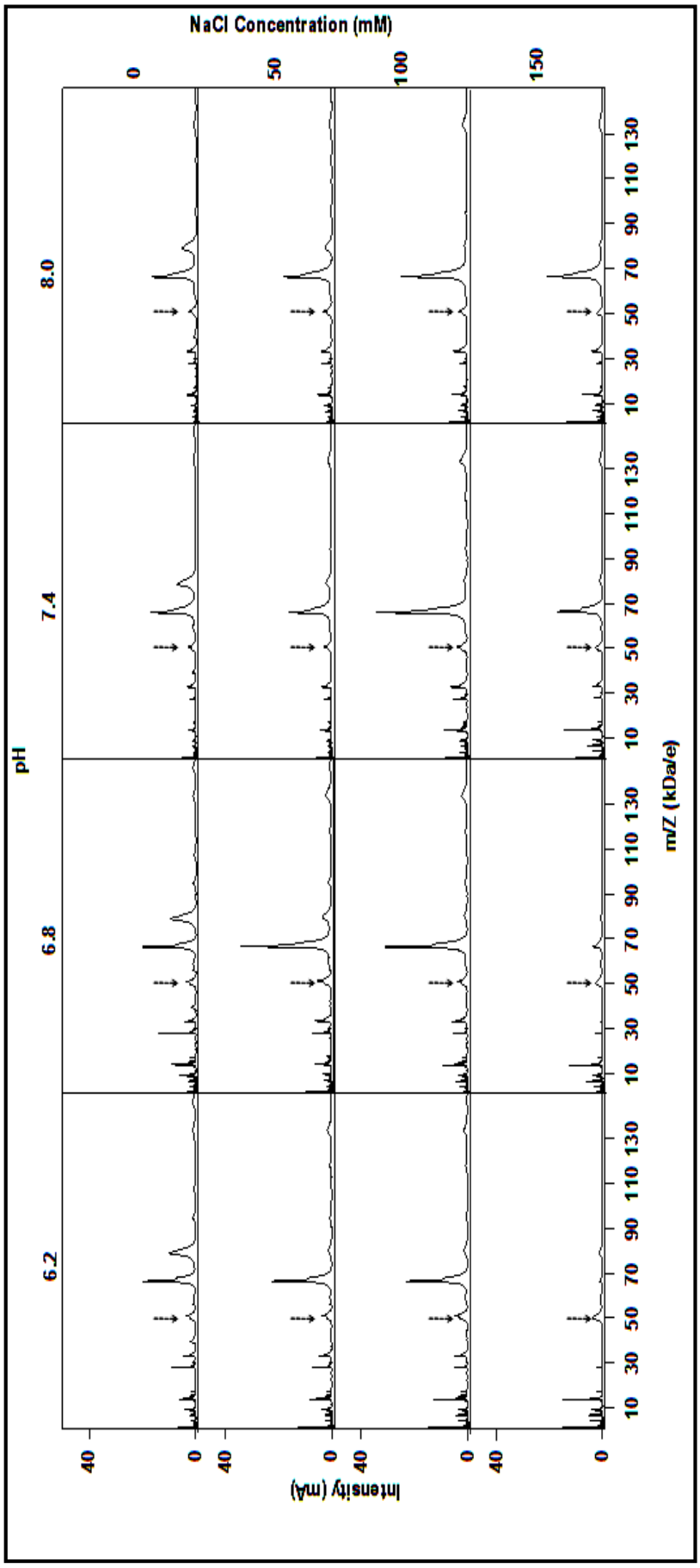


Figure 6.1SELDI-TOF-MS spectra of protein samples fractionated on a 'Q10' ProteinChip. AAT is fractionated from FIV filtrate samples under a variety of binding conditions, which contains a large number of plasma-derived impurities. A matrix of 16 conditions (pH 6.2,6.8,7.4 and 8,NaCl concentrations of 0mM, 50mM, 100mM, 150mM were analysed. The arrow identifies the AAT peak which appears at 50.1kDa/e; (Albumin) at 66kDa/e and Transferrin at 79kDa/e. SELDI-TOF-MS results are recorded by summing the signal intensities between ± 0.3% of the mass

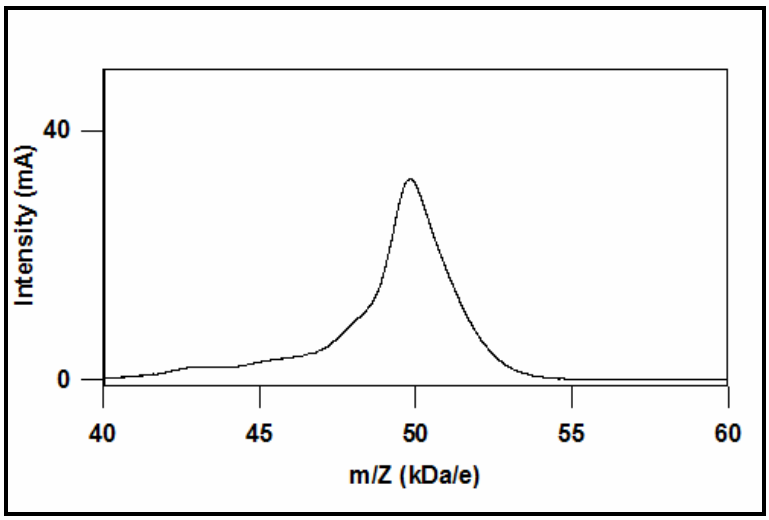


Figure 6.2SELDI-TOF-MS (NP20 ProteinChip) spectrum portraying the peak location of Alpha 1-antitrypsin (approximately showing at 50kDa/e) from an AAT standard purchased from Sigma-Aldrich. There were impurities present in the standard, but these have not been shown.

However, the spectra plots do not portray any information on protein concentration; one of the flaws in the SELDI-TOF-MS design is that product samples cannot be taken for analysis post SELDI-TOF-MS processing. Protein intensity or the area under the curve (AUC) of each protein peak can be used as measure of the amount of protein bound onto the ProteinChip or in other words protein abundance or concentration (Grizzle *et al.*. 2005; Meleth *et al.*. 2005). For this study each protein peak was identified at 50.1kDa/e, 66kDa/e and 79kDa/e for AAT, Albumin, and Transferrin respectively, by summing the signal intensities based on mass resolution (± 0.3% of the mass) and the area under the curve or 'amplitude' was measured.

In this study, two methods have been used to portray this data effectively: contour plots and data bars. The contour plots were plotted using SigmaPlot 10.0 (Systat, San Jose, CA). The contour plots are more descriptive and have been used to depict the binding intensity of each protein over the range of conditions, effectively showing regions of very high or low binding, see Figure 6.3. The data bars were plotted in Microsoft

Excel, 2007. They are calculated based on the highest and lowest values across the whole data set (of all three proteins, and all buffer conditions), and so each data bar is comparable to each other. The data bar summary allows for easy comparison across the whole range of data, gives the exact AUC value, and also allows for any outliers to be quickly observed, see Figure 6.4.

Protein	NaCl Concentration	Protein I
		pH 6.2
AAT	0mM NaCl	100
	50mM NaCl	15
	100mM NaCl	7
	150mM NaCl	96
Albumin	0mM NaCl	1515
	50mM NaCl	1657
	100mM NaCl	1611
	150mM NaCl	57
Transferrin	0mM NaCl	20
	50mM NaCl	2
	100mM NaCl	2
	150mM NaCl	2

Figure 6.4 Data bars portray a summary of the abundance of AAT, Albumin, and Transferrin based on peak area under the curve (AUC) at 50.1kDa/e, 66kDa/e and 79kDa/e respectively from the SELDI-TOF-MS intensity profiles. Results from

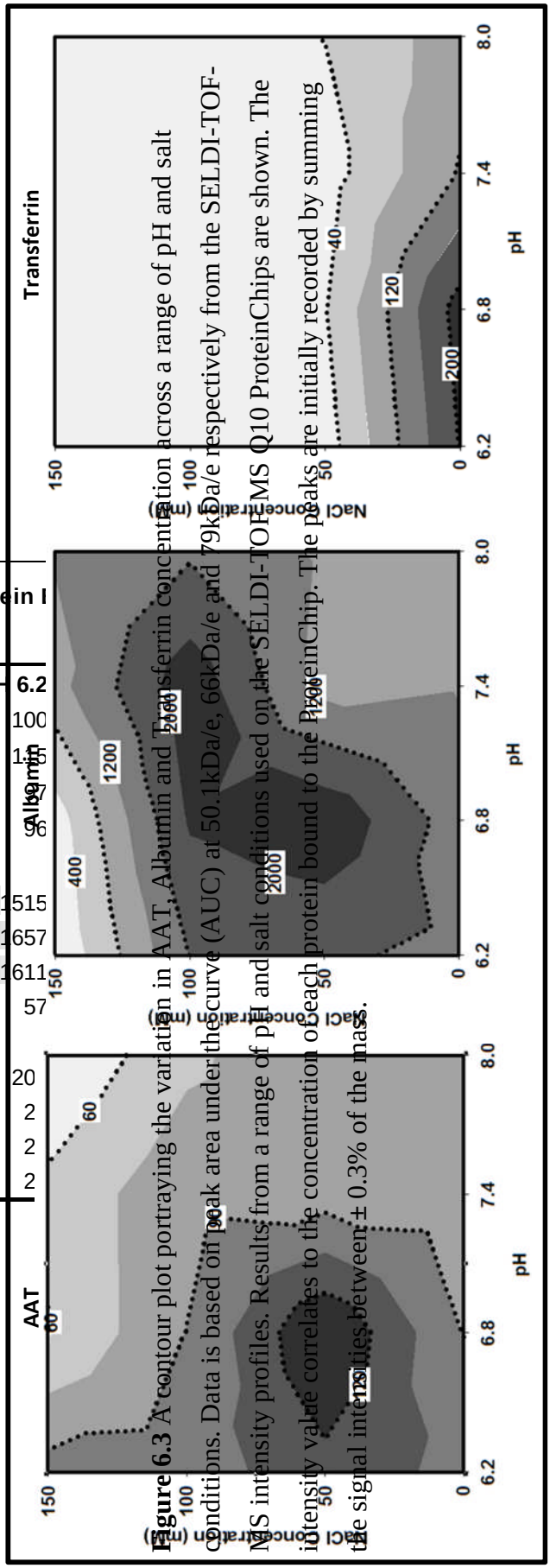


Figure 6.3 A contour plot portraying the variation in AAT, Albumin and Transferrin concentration across a range of pH and salt conditions. Data is based on peak area under the curve (AUC) at 50.1kDa/e, 66kDa/e and 79kDa/e respectively from the SELDI-TOF-MS intensity profiles. Results from a range of pH and salt conditions used on the SELDI-TOF-MS Q10 ProteinChips are shown. The intensity value correlates to the concentration of each protein bound to the ProteinChip. The peaks are initially recorded by summing the signal intensities between ± 0.3% of the mass.

a range of pH and salt conditions used on the SELDI-TOF-MS Q10 ProteinChips are shown. The intensity value correlates to the concentration of each protein bound to the ProteinChip. The peaks are initially recorded by summing the signal intensities between ± 0.3% of the mass. Optimal conditions are highlighted in red.

The contour plot shows AAT to bind with higher intensities at lower pH values of 6.2 and 6.8, and with the highest intensity at a salt concentration of 50mM. However, the data bar summary reveals that although AAT concentration is at its highest at 50mM NaCl with peak areas of 115mAs and 135 mAs for pH 6.2 and 6.8 respectively, it is not significantly higher than the other salt concentrations at pH 6.2 (which range from 96-100 mAs). The biggest impurity present in the samples is Albumin. Both the contour and data plots show reduced Albumin binding under buffer conditions of 150mM salt and at the low pH values of 6.2 and 6.8. Transferrin does not bind well at the majority of conditions tested. However, increased binding to the Q10' ProteinChip is seen at 0mM NaCl, and at its highest at pH 6.2-6.8. Therefore, using the results from the SELDI-TOF-MS spectra data and the protein intensity plots, it can be deduced that the optimum buffer conditions for isolating AAT using Q-Sepharose is at pH 6.2 for the increased AAT binding, and using 150mM NaCl for reduced impurity binding.

To investigate whether AAT isolation and binding could be improved further, buffers were prepared at lower pH conditions, and at increased salt concentrations. These are summarised in Table 6.2. The spectra resulting from these conditions can be seen in Appendix 5. In this instance, all for the extended data set covered a lower absorbance intensity range of approximately 0-5 mA intensity range, compared to 0-40mA intensity range seen in the initial 'Q10' ProteinChip plots. These experiments were run on two separate days, and different preparations of Energy Absorbing Molecule (EAM) were used, as it expires after 24 hours. This affects the resulting mass spectra intensities, making it impossible to compare the results from the two data setsdirectly. Other possible sources of variation have been described in detail by Dijkstra*et al.*. (2007a). It has been suggested (Baggerly *et al.*. 2004) that the use of data processing should be employed to compare different SELDI-TOF-MS data sets. Therefore, before comparison all the spectra data have been normalized to be in the same [0, 1] intensity range using the following equation:

The normalized intensity (NVi) is given,

$$= \frac{-}{-}$$

6.4.1

Where, for a single spectrum, Vi denotes the raw intensity at the i-th m/z value, and Vmin and Vmax denote the smallest and largest observed intensities in the spectrum, respectively. This method of normalisation was applied to the SELDI-TOF-MS processed data initially used to plot the MS spectra, and so the whole data set using different EAM samples could be compared. This is shown in Figure 6.5.The additional buffer conditions show a similar trend to the initial results: at pH 5.1 and 5.6 AAT is shown to be better isolated from the impurities at higher salt concentrations of 150mM, and 200mM NaCl. However, buffer conditions at pH 5.1 shows an apparent decrease in

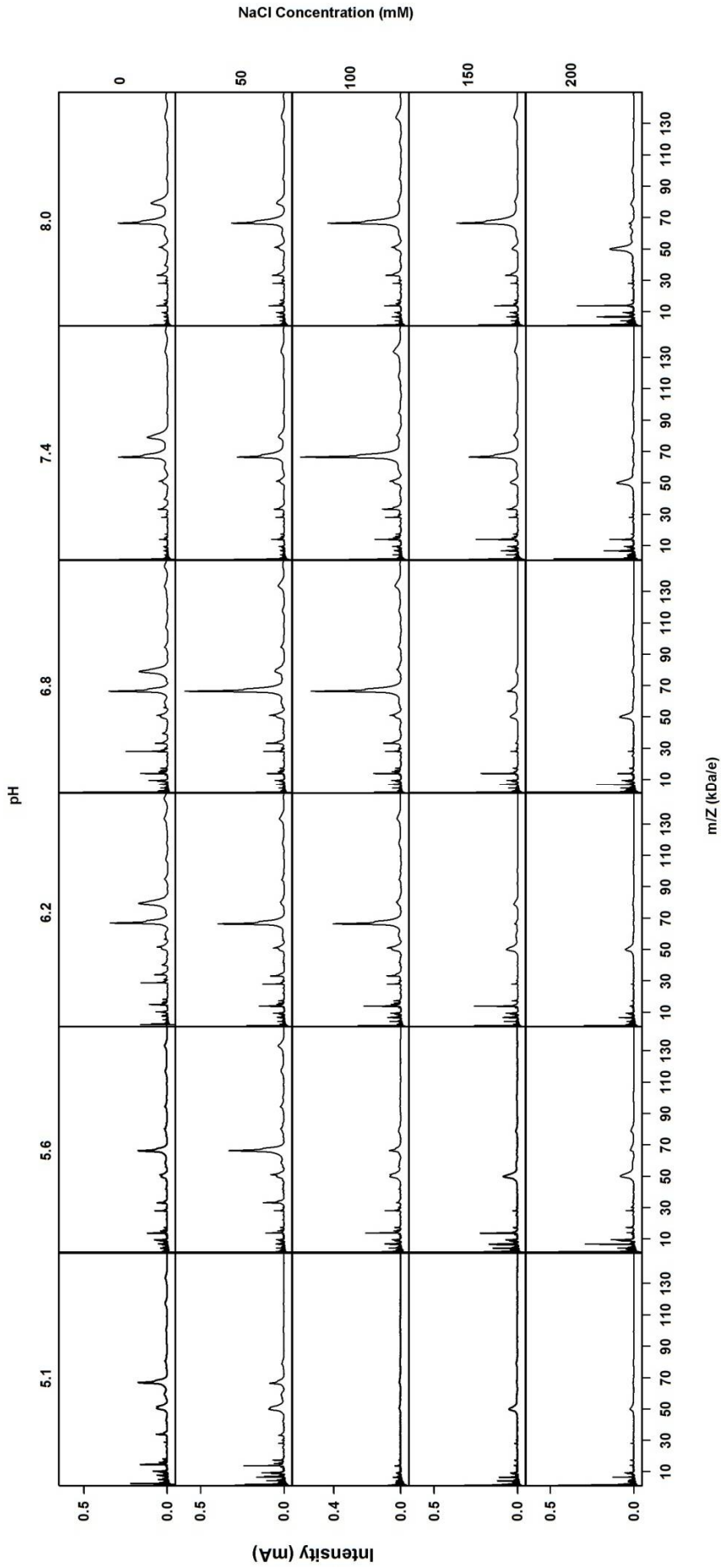


Figure 6.5 Normalised SELDI-TOF-MS spectra based on SELDI-TOF-MS processed data that is baseline corrected. The protein profiles portray the binding intensity and isolation of proteins present in FIV filtrate fractionated using the 'Q10' ProteinChip under a variety of buffer conditions: pH 5.1-8.0, 0-200mM NaCl. Based on Baggerly,(2007) method.

AAT intensity at the increased salt conditions. Work by Glaser *et al.*. (1982) suggests that alpha-l-antitrypsin may begin to denature and aggregate at pH values much lower than pH 6, which will have an impact on protein intensity. A smaller data set is investigated in this instance and so it is not possible to create a suitable contour plot based on the raw intensity data, to show the impact on protein binding. Consequently, only the data bar summary method has been utilised to look at the binding intensity ofAAT, Albumin, and Transferrin (Figure 6.6). There is not an obvious trend at pH 5.1 and 5.6 over the salt concentration range. There may be some AAT denaturation as explained previously, and total impurity removal is again improved under higher salt concentrations. The highest binding of AAT is seen at pH 5.6, 50mM NaCl, but acceptable levels are achieved across the salt concentration range, and so it may be more efficient to implement conditions with better impurity removal. At 200mM NaCl, there is a general increase in AAT binding as pH is increased, aside from an outlying low value at pH 6.2.

Protein	NaCl Concentration	Protein Bound based on Peak Area (AUC) (mAs) (+/-0.3%)					
		pH 5.1	pH 5.6	pH 6.2	pH 6.8	pH 7.4	pH 8.0
AAT	0mM NaCl	29	24	-	-	-	-
	50mM NaCl	20	41	-	-	-	-
	100mM NaCl	2	22	-	-	-	-
	150mM NaCl	20	33	22	-	-	-
	200mM NaCl	8	27	18	32	39	57
Albumin	0mM NaCl	143	154	-	-	-	-
	50mM NaCl	63	285	-	-	-	-
	100mM NaCl	5	52	-	-	-	-
	150mM NaCl	4	1	87	-	-	-
	200mM NaCl	4	11	2	3	5	22
Transferrin	0mM NaCl	2	4	-	-	-	-
	50mM NaCl	4	4	-	-	-	-
	100mM NaCl	1	4	-	-	-	-
	150mM NaCl	3	5	3	-	-	-
	200mM NaCl	2	8	2	5	3	7

Figure 6.6 Data bars portray a summary of the concentration of AAT, Albumin, and Transferrin based on peak area under the curve (AUC) at 50.1kDa/e, 66kDa/e and 79kDa/e for the additional buffer condition: pH 5.1 and pH 5.6, 0-200mM NaCl, and pH 6.2, 6.8, 7.4, 8.0 at [200mM NaCl]. The intensity value correlates to the concentration of each protein bound to the ProteinChip. The peaks are initially recorded

by summing the signal intensities between ± 0.3% of the mass. Optimal conditions are highlighted in red.

An additional FIV filtrate sample at pH 6.2, 150mM NaCl was also analysed along with this new data set. The ratio of AAT to the impurities in both samples (see pH 6.2, 150mM NaCl in Figure 6.4 and Figure 6.6) are very different, with more Albumin being present in the second sample. The second sample also has a much lower AATpeak area, which is likely to have been suppressed by the increased Albumin content in the sample competing for binding places or ionization energy.

6.4.2 PURITY OF AAT FROM 'Q10 PROTEINCHIP' MS INTENSITY PROFILE DATA

As seen in the previous section, given that the range of buffer conditions was investigated in two separate sets of experiments, the intensity or abundance of the protein in the 'Q10' fractionates cannot be compared directly. This is chiefly attributable to the difference in intensity range, but also to variations in the FIV precipitate samples. Determining the purity of the protein in the samples is another method that can be used to normalise the data. The method used to calculate the purity is described below:

The area under the profile peaks from the intensity plots were quantified for AAT and the two significant impurities: Albumin (ALB) and Transferrin (Tf). For clarity, the sum of these relevant proteins is labelled as Total Protein.

Purity of AAT is given by:

$$\left[\frac{\qquad}{\qquad}\right] \qquad \textbf{6.4.2}$$

Where Total Protein $_{\text{Peak Area}}$ is:

$$\left[\quad +\quad +\quad\right] \qquad \textbf{6.4.3}$$

The dataset is effectively normalised by taking the ratio of AAT to Total Protein in each set. The optimum binding conditions are the regions with the highest AAT: Total Protein ratio, where the most AAT and the lowest totalimpurity intensities are observed. Figure 6.7 displays a contour plot portraying the purity of AAT across the data range. As expected, AAT purity is at its highest levels when buffers of high salt [200mM NaCl] concentrations are used; this is true for the full range of pH levels tested. The plot also shows that decreasing the pH allows for good purity levels to be achieved for wider

range of salt concentrations ranges; at [150mMNaCl], AATshows good purity levels at the low pHs of 5.1-6.2.

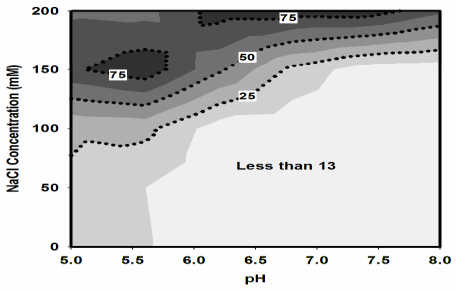


Figure 6.7 The percentage purity of AAT (AAT/ Total Protein) when fractionated using the SELDI-TOF-MS 'Q10' ProteinChip under a range of pHs and NaCl concentrations.

However, some information is lost here, as the plot does not show the impact on Albumin and Transferrin individually. Figure 6.8 displays a data bar summary of the percentage purity of all the proteins. This shows that the increase in purity of AAT is mostly due to the decrease in Albumin concentration. There is no significant trend in Transferrin purity across all conditions, although the highest levels of Transferrin "purity" appear at low buffer pHs of 5.1-6.2, with the highest salt concentrations (150mM-200mM). However, this does not appear to have a major impact on AAT purity; as it is present in minor concentrations compared to Albumin. The original AAT MS intensity contour and data plots had shown the best AAT binding at asalt concentration of 50mM NaCl, but a combined improvement of purity and yield only achieved at higher salt concentrations.

6.4.3 HITRAP 'SCALE-UP' DATA

The data generated using the SELDI-TOF-MS is known to be variable (Dijkstra *et al.*. 2007). Therefore, a range of conditions using larger strong anion exchange columns (1mL, GE HiTrap Q Sepharose Fast Flow) is used to test the 'Q10' chip

Protein	NaCl Concentration	Protein Purity based on Peak Area data (%)					
		pH 5.1	pH 5.6	pH 6.2	pH 6.8	pH 7.4	pH 8.0
AAT	0mM NaCl	17%	13%	5%	5%	6%	6%
	50mM NaCl	23%	13%	6%	5%	7%	7%
	100mM NaCl	27%	29%	6%	4%	4%	4%
	150mM NaCl	73%	84%	53%	21%	6%	4%
	200mM NaCl	60%	58%	81%	81%	83%	66%
Albumin	0mM NaCl	82%	85%	83%	82%	85%	86%
	50mM NaCl	72%	86%	92%	93%	91%	90%
	100mM NaCl	65%	66%	93%	95%	95%	95%
	150mM NaCl	17%	4%	32%	76%	92%	96%
	200mM NaCl	28%	25%	10%	7%	10%	26%
Transferrin	0mM NaCl	1%	2%	11%	13%	9%	8%
	50mM NaCl	5%	1%	1%	2%	2%	3%
	100mM NaCl	9%	5%	1%	1%	1%	1%
	150mM NaCl	10%	12%	16%	3%	2%	1%
	200mM NaCl	12%	17%	9%	12%	7%	8%

Figure 6.8A data bar summary of the percentage purity of AAT, Albumin and Transferrin when fractionated using the SELDI-TOF-MS 'Q10' ProteinChip under differing pH values and NaCl concentrations. Optimal conditions are highlighted in red.

generated data trends. 1mL pre-packed scale columns have been successfully used to reliably scoutappropriate buffer conditions and matrix types to use at larger scale. Other studies have shown the usefulness of using 1mL pre-packed columns, for example to explore a range of hydrophobic interaction media preceding a more comprehensive study of expanded bed separation on the most suitable choice (Smith et al.. 2002).

For each condition tested, the column was equilibrated, FIV filtrate was loaded, and unbound protein was washed off using the same buffer, just as the SELDI-TOF-MS samples were processed. The initial 'wash peak' fractions were collected and pooled. 20mM Phosphate, 2M NaCl was used to remove all bound protein from the column in the 'elution peak' , which was also collected in fractions and then pooled (Figure 6.9).Protein intensity and mass were determined using the non-selective SELDI-TOF-

MS NP20 ProteinChips, in conjunction with an ELISAkit to measure AAT concentration or the Siemens Turbitimerapparatus to measure Albumin and Transferrin concentrations.

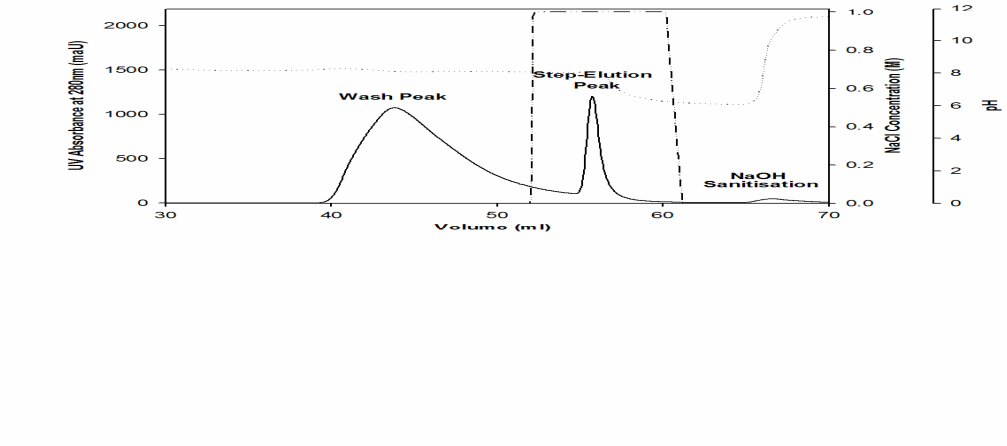


Figure 6.9 An example of the elution profiles from the Hitrap Q Sepharose FF columns. Fraction IV filtrate is loaded at pH 8, 150mM NaCl on to 1mL pre-packed columns: The first peak represents the wash of unbound protein with 20mM Phosphate, 150mM NaCl at pH 8, The second peak shows a salt step elution to 2M NaCl, and the third peak is a 0.5M NaOH wash. Symbols are: (—) Absorbance, (-.-.-) NaCl concentration, (……) pH

Figure 6.10 and Figure 6.11 show the SELDI-TOF-MS intensity profiles for the eluate and wash peaks respectively. The eluate profile shows that pH 6.2 is most favourable in removing the impurities, primarily Albumin. However, salt concentration at pH 6.2 does not seem to have an obvious impact on impurity binding as seen in the 'Q10' chip experiments. At pH 8, there are fewer impurities bound at the highest salt concentration of 200mM. The wash profile is clearer in showing impurity removal; there are more impurities present in the flow-through wash when using conditions of higher salt concentrations, illustrating that they are not binding to the column.

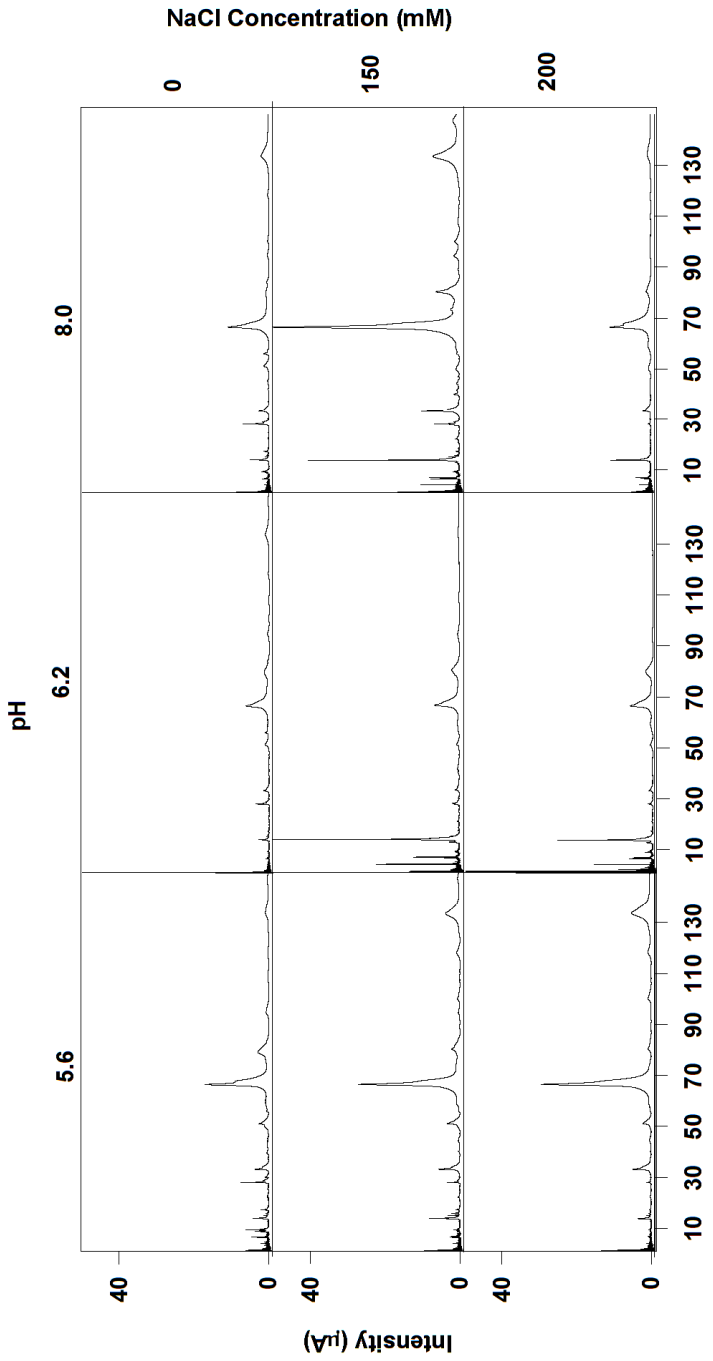


Figure 6.10SELDI-TOF-MS spectra representing protein profiles of Fraction IV eluate samples. FIV filtrate were loaded onto a HiTrap Q Sepharose FF column, under the conditions shown in the figure . Loading, equilibration and binding were kept under the same conditions to mimic the SELDI-TOF-MS Q10 technique used earlier. A step elution (2M NaCl) was used to remove all the protein bound onto the column, and so the profiles represent the binding capacity of the matrix. The profiles were produced using the non-selective SELDI-TOF-MS 'NP20' ProteinChip. A matrix of 9 conditions (pH 5.6, 6.2 and 8,NaCl concentrations of 0mM, 150mM, and 200mM) were analysed at 1mL column scale. The AAT peak is shown at a 52kDa/e; Albumin at 66kDa/e and Transferrin at 79kDa/e.

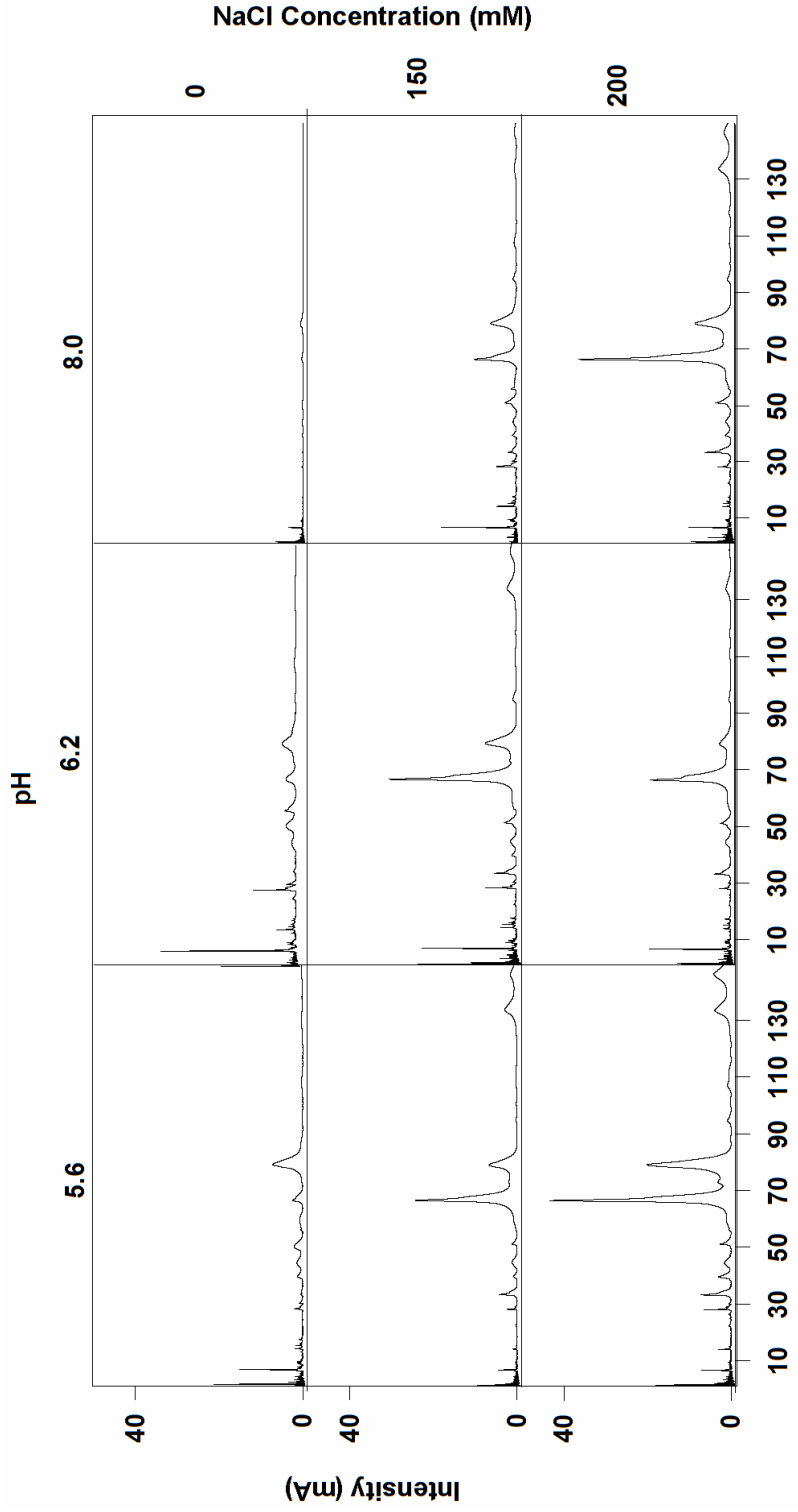


Figure 6.11SELDI-TOF-MS spectra representing wash peaks from Fraction IV filtrate samples after they were loaded onto a HiTrap Q Sepharose FF column, under the conditions shown in the figure load and wash conditions were the same . **The profile shows protein that did not bind to the column.** The profiles were produced using the non-selective SELDI-TOF-MS 'NP20' ProteinChip. A matrix of 9 conditions (pH 5.6, 6.2 and 8,NaCl concentrations of 0mM, 150mM, and 200mM) were

At pH 8, 0mM NaCl very little or what appears to be no protein is present in the wash, indicating that all protein, AAT and impurities are binding to column. At lower pH levels and high salt concentrations more impurity peaks can be seen in the wash profiles; these proteins are not binding to the column. If the load and wash procedures are suitably optimised then it is possible there will be no requirement for product elution optimisation; after washing off unbound proteins, all product could then be removed from the column using a highly concentrated salt step-elution.

Figure 6.12 A) shows a data bar summary of the concentration of proteins present in the product eluate, and in the wash peak. The concentrations are determined using ELISA methods for AAT, and the Siemens Turbitimer method for Albumin and Transferrin. This eluatedata shows that there is no major change in AAT concentration across all conditions, and Albumin removal is best at the low pH and high salt concentrations; these trends are inline with of the initial 'Q10' chipresults. Transferrin is present in higher amounts than was seen in'Q10' chips fractions, and so may require an additional removal step at a later stage. The wash data again confirms a similar trend in AAT concentration, and almost no AAT is present all across the sample range. More Albumin is present at high salt concentrations indication non-binding, and is best removed at pH 6.2. There is not much variation in Transferrin concentration. The data bar summaries also portray the improved removal of impurities at lower pH levels and higher salt concentration more clearly than in the MS plots.

Figure 6.12 B) shows a data bar summary of the concentration data quantified using SELDI-TOF-MS NP20 chips. Peak area (mAs) is quantified from the intensity profiles and is relative to protein abundance. The chips are non-selective and thus should confirm everything present in the sample. The product eluate samples showAAT to have the greatest peak area at pH 5.6 and the best Albumin removal at pH 6.2 for all salt concentrations. Transferrin concentration varies, but no obvious trend is seen; the lowest concentrations are at outliers of pH8, 0mM NaCl, and pH 5.6, 200mM NaCl. In the wash peak, the lowest AATlosses are seen at 0mM NaCl. More Albumin is lost at high concentrations of salt as seen in Figure 6.12 A); the protein concentration wash data. No obvious trends are seen in Transferrin removal over the buffer condition range tested, but there is more Transferrin present in the wash peaks than the eluate peaks.

A)

Protein	NaCl Concentration	Protein Bound (g/L) Eluate Peak			Unbound Protein (g/L) Wash Peak		
		pH 5.6	pH 6.2	pH 8	pH 5.6	pH 6.2	pH 8
AAT	0mM NaCl	0.04	0.04	0.05	0.00	0.004	0.007
	100mM NaCl	0.03	0.03	0.01	0.00	0.006	0.014
	200mM NaCl	0.01	-	0.00	0.00	0.012	0.008
Albumin	0mM NaCl	1.25	1.47	1.33	0.03	0.11	0.03
	100mM NaCl	0.59	0.04	1.12	0.41	0.59	0.30
	200mM NaCl	0.09	0.04	0.28	0.29	0.79	0.40
Transferrin	0mM NaCl	0.49	1.10	0.68	0.24	0.30	0.40
	100mM NaCl	0.08	0.52	0.42	0.44	0.33	0.57
	200mM NaCl	0.06	0.02	0.21	0.29	0.56	0.44

B)

Protein	NaCl Concentration	Protein Bound (mAs) Eluate Peak			Unbound Protein (mAs) Wash Peak		
		pH 5.6	pH 6.2	pH 8	pH 5.6	pH 6.2	pH 8
AAT	0mM NaCl	98.53	51.44	58.07	52.06	31.55	6.46
	100mM NaCl	168.91	32.74	25.58	70.46	126.31	105.09
	200mM NaCl	100.40	26.83	14.99	141.50	82.89	151.96
Albumin	0mM NaCl	837.64	253.66	438.59	113.38	90.83	20.31
	100mM NaCl	1256.91	277.16	2306.77	1027.79	1313.32	440.19
	200mM NaCl	1127.90	240.10	459.31	1880.12	804.10	1518.61
Transferrin	0mM NaCl	80.74	24.59	6.32	219.48	84.26	16.74
	100mM NaCl	26.58	47.14	61.06	185.18	231.18	212.48
	200mM NaCl	9.67	56.42	16.21	685.65	67.72	269.60

Figure 6.12A data bar summary showing the concentration of AAT, Albumin and Transferrin present in the 'eluate' (bound protein) and 'wash' peaks (unbound protein) (see **Figure 6.9**) from a HiTrap Q Sepharose Fast flow separation step over a range of conditions (pH 5.6-8, [NaCl] 0-200mM). In A) the data is determined using ELISA (AAT) and Siemens Turbitimer (Albumin and Transferrin) methods; in B) the data shows the Peak Area (± 0.003) from the intensity profiles produced using the non-selective SELDI-TOF-MS 'NP20' ProteinChip. The AAT peak is measured at a 50.85kDa/e; Albumin at 66kDa/e and Transferrin at 79kDa/e. Optimal conditions are highlighted in red.

Overall, the NP20 chips do provide similar trends to that seen using standard concentration determination methods. This is far more obvious in the wash data results, in the MS profiles and in the data bar summaries; this may be due to the slightly lower overall protein content in the wash samples. There are also more dissimilarities in trends between wash and eluate data when determining the concentration using the NP20 chips, than seen with the ELISA and Turbitimer methods.

Variability in the plasma-derived FIV samples was seen and this may have affected the subsequent purification results.Therefore, to eliminate this variability a yield of eluate or wash sample from protein load was used to re-evaluate the ELISA and Turbitimer concentration data (Figure 6.13). The eluate yield data showed similar trends to that seen in the concentration data (Figure 6.12) except in the case of Transferrin concentration: where this result suggests that more of it is removed at the highest salt concentration of 200mM NaCl, across all pH values (Figure 6.13). This is also in line with AAT binding and Albumin removal; providing an optimal buffer condition. It is also reflects the results predicted in the 'Q10' chip data.

Protein	NaCl Concentration	Protein Bound (%Yield) Eluate Peak			Unbound Protein (% Yield) Wash Peak		
		pH 5.6	pH 6.2	pH 8	pH 5.6	pH 6.2	pH 8
AAT	0mM NaCl	59%	99%	63%	0%	9%	8%
	100mM NaCl	71%	94%	19%	0%	16%	30%
	200mM NaCl	90%	-	0%	0%	29%	30%
Albumin	0mM NaCl	56%	42%	49%	1%	3%	1%
	100mM NaCl	37%	2%	38%	25%	27%	10%
	200mM NaCl	9%	1%	14%	28%	25%	20%
Transferrin	0mM NaCl	37%	100%	50%	18%	30%	29%
	100mM NaCl	11%	51%	32%	60%	33%	44%
	200mM NaCl	3%	2%	18%	15%	46%	37%

Figure 6.13A data bar summary of percentage protein yield in the 'eluate'(bound protein) and percentage yield loss in the 'wash'(unbound protein) peaks (see **Figure 6.9**) from a HiTrap Q Sepharose Fast flow separation step over a range of conditions (pH 5.6-8, [NaCl] 0-200mM). This is based on the concentration data(**Figure 6.12**) determined using ELISA methods for AAT, and the Siemens Turbitimer method for Albumin and Transferrin. Optimal conditions are highlighted in red.

6.4.4 PURITY OF AAT IN HITRAP QFF SAMPLES

To compare the purity results from the 'Q10' chip SELDI-TOF-MS analysis, the purity of the HiTrap samples is calculated usingboththe concentration and yield data, again this is normalised using the method described in section 6.4.2. The eluate peak data analysed using ELISA and Turbitimer methods exhibit similar AAT trends: better purity of AAT at low pH values and high salt concentrations in the product (Figure 6.7.14 A).No obvious trend is seen in the case of Albumin and Transferrin. The improved purities of AAT and Transferrin are mainly due to the low concentrations of Albumin originally observed at pH 5.6, 200mM NaCl and at pH 6.2, 100-200mM NaCl(Figure 6.12 A). The purity data calculated using the peak areas from the NP20 chip samples do not show obvious trends. AAT has improved purity levels across the range of conditions tested expect at pH 8,100-200mM NaCl. The NP20 MS profiles as seen in Figure 6.10 and Figure 6.11 portray a profile of almost no impurities present in the product peak at pH 5.6,200mM NaCl. However, upon analysisthe purity of AAT is calculated to be only 8% when using this normalisation method, and this is compared to Albumin and Transferrin solely. Therefore, the NP20 MS profile does not accurately reflect column performance, and should be used in conjunction with a quantitative method, such as that described in section 6.4.2.

The wash peakpurity data shows a better correlation between the ELISA/Turbitimer and NP20 data. Minimal AAT is present in wash fraction at pH 5.6-6.2, and Albumin is present in greater quantities at increased salt conditions. More Transferrin appears to be removed in the wash fraction at 0mM NaCl at all pH levels. In this case, NP20 Chip can be used to successfully analyse data if there is low sample variation and if the data set is normalised.

A)

Protein	NaCl Concentration	Protein Bound (%Purity) Eluate Peak			Unbound Protein (%Purity) Wash Peak		
		pH 5.6	pH 6.2	pH 8	pH 5.6	pH 6.2	pH 8
AAT	0mM NaCl	2%	2%	2%	0%	1%	2%
	100mM NaCl	4%	6%	1%	0%	1%	2%
	200mM NaCl	8%	-	0%	0%	1%	1%
Albumin	0mM NaCl	70%	56%	64%	11%	26%	7%
	100mM NaCl	84%	7%	72%	48%	63%	34%
	200mM NaCl	54%	-	57%	49%	58%	47%
Transferrin	0mM NaCl	28%	42%	33%	89%	73%	91%
	100mM NaCl	12%	87%	27%	52%	36%	64%
	200mM NaCl	38%	-	43%	51%	41%	52%

B)

Protein	NaCl Concentration	Protein Bound (%Purity) Eluate Peak			Unbound Protein (%Purity) Wash Peak		
		pH 5.6	pH 6.2	pH 8	pH 5.6	pH 6.2	pH 8
AAT	0mM NaCl	10%	16%	12%	14%	15%	15%
	100mM NaCl	12%	9%	1%	5%	8%	14%
	200mM NaCl	8%	8%	3%	5%	9%	8%
Albumin	0mM NaCl	82%	77%	87%	29%	44%	47%
	100mM NaCl	87%	78%	96%	80%	79%	58%
	200mM NaCl	91%	74%	94%	69%	84%	78%
Transferrin	0mM NaCl	8%	7%	1%	57%	41%	38%
	100mM NaCl	2%	13%	3%	14%	14%	28%
	200mM NaCl	1%	17%	3%	25%	7%	14%

Figure 6.14 The purity of AAT, Albumin and Transferrin in the 'eluate' (bound protein) and 'wash' (unbound protein) peak fractions from a HiTrap Q Sepharose Fast flow separation based on **A)** Elisa methods for AAT and Turbitimer concentration estimates for Albumin and Transferrin; **B)** AUC (Area under the curve of target peaks) from NP20 chip SELDI-TOF-MS profiles. Total protein is considered to be the summation of AAT, Albumin and Transferrin concentrations. The Peak Area (± 0.003) is calculated from the intensity profiles produced using the non-selective SELDI-TOF-MS 'NP20' ProteinChip. The AAT peak is measured at a 50.85kDa/e; Albumin at 66kDa/e and Transferrin at 79kDa/e.

6.5 CONCLUSIONS

Process step optimization can be time-consuming and costly. High-throughput technology allows scientists to screen a large number of conditions at reduced time-scales. The SELDI-TOF-MS has been used to improve the isolation of alpha 1-antitrypsin (AAT) in FIV filtrate with 'Q Sepharose' anion media, by speedily trialling a variety of buffer conditions. A total of 21 buffer conditions were tested, varying pH, and salt concentrations. A number of these conditions were mimicked at 1mL scale to validate the method.

This chapter has highlighted that SELDI-TOF-MS 'Q10' technology can be used to predict the general trends in binding condition of a HiTrap Q-Sepharose FF column. The 'Q10' chips and HiTrap QFF data both successfully showed improved isolation of AAT at lower pH values of 5.6-6.2, and with the highest salt concentrations tested (150-200mM NaCl), when measured using the NP20 chips and more traditional protein determination methods, such as ELISA analysis.

The HiTrap QFF data shows that yields of 94-99% AAT can be achieved but with a compromise in impurity removal. The optimal condition for the isolation of AATwas found to be at pH 5.6, 200mM NaCl, where a 90% yield can be achieved, but more work would need to be done, to determine whether AAT is stable or has been denatured at this low pH value. This result is reiterated in the SELDI-TOF-MS NP20 profiles, where very little impurities are seen. A larger data set would be required to determine the exact conditions to use. However, a large discrepancy in the purity levels was seen between the Q10 chips and the HiTrap QFF columns. The SELDI-TOF-MS 'Q10' fractionation predicted purities > 50% under optimal conditions; in contrast the purities seen with the HiTrap QFF columns was <10% under the optimal conditions. However, the very low concentrations of AAT, in this relatively impure FIV filtrate sample does make the analysis more troublesome.

A fully automated approach would allow for the screening a large number of conditions at short time-scales. Analysis has shown that variability in samples and differences in MS intensity can be eliminated by normalising the data.

Analysis of the larger-scale material was carried out using the SELDI-TOF-MS NP20 chips, Elisa, Total protein. NP20 chip MS analysis was found to comparable to ELISA and Turbitimer immunoassay methods when the dataset was normalised.

Other work by (Gupta and Gowda, 2008) have shown a proportional reduction in AAT activity as pH is lowered much below pH 7, and so further work on the stability ofAATunder the conditions suggested in this chapter will need to be analysed in conjunction with this work.

6.6 INTRODUCTION

Process changes are inevitable in the manufacture of biologics. Changes that improve yields are vital in order for companies to remain competitive, and for the processes to remain robust and economical. The impact of making a change to a manufacturing process is not trivial; in the biologics sector it is often quoted that "the product is defined by its manufacturing process". Product purity, quality and efficacy cannot be compromised and any changes directly affecting the manufacturing stream or the analyticalactivities associated will need to be made to remain in line with the rigid regulatory protocol, where proof of these factors can be timely and costly.However, the positive impact of making changes to process steps, especially with new emerging technologies, can be hugely significant, and so a series of trade-offs must be evaluated. This chapter summarises the research in this thesis to enable process changes to be evaluated from both a manufacturing and development lifecycle perspective. It also describes future work activities that can build on this research.

6.7 OVERALL CONCLUSIONS

This thesis explores the impact of both positive and detrimental results of process changes in the biopharmaceutical industry, with a particular focus on biologics. A systemic evaluation framework has been created to capture the technical and regulatory activities involved in process changes so as to rapidly gauge the potential cost and risk implications.

Chapter 2 describes a survey that was carried out to benchmark typical costs and durations, as well as to gauge industry attitudes to implementing changes to their processes. The findings suggested that the majority of changes are made to reduce batch-to-batch variability and improve product yield or purity. Major changes, that require thorough efficacy studies, are not frequently made once processes have been approved for production; the main deterrents being the vast costs and delays in production where they cannot be stockpiled. Feedback from the survey respondents suggested that regular communication with regulatory authorities before and during change implementation is key to avoiding significant future costs and delays in production. The survey results showed that ifa proposed manufacturing change during

the development stage or post productapproval is likely to instigate a repeat of lengthy clinical trials, then companies are likely to reject the proposal for change.

Chapter 3 describes the modelling approach adopted in this thesis to evaluate the potential of process changes. Key technical activities were captured, namely development, manufacturing, retrofitting and validation at all stages of development. The impacts of changes were linked to regulatory activities needed to assess comparability.Development and product equivalence activities were based on benchmark costs and time spans taken from the survey, which also helped create the framework of the model.Mass balance calculations were used to compute process stream compositions to calculate the manufacturing process yield. Strategicuncertaintieswere accounted for in the model such as the likelihood of repeating clinical trials, the market share losses, delays to market from retrofit, revalidation, or regulatory approval disruptions, and the costs involved in proving product equivalence. The activities were measured in cost and time delay, and eventually converted into a single measure of profit: Net Present Value. Incorporating the risks involved at each step enabled scenarios to be evaluated based on the Expected Net Present Value and the probability, where p(NPV>0). The entire framework was translated into Microsoft Excel with macros for Monte Carlo simulations to account for the uncertainties.

A case study illustrating the implementation of various changes to a plasma fractionation process was then explored in Chapter 4. Process changes of varying magnitude and type, were explored at different stages of product development including post-product approval. The example illustrates how the framework described can be used to investigate the effects of making process changes, whether these are forced upon them or are made to enhance productivity. The scenario results showed that the stage of implementation is far more significant than the process change type; it is not economically possible in the three cases investigated to make a change at late phase clinical development. The best approach would be to implement that change once a product is approved and commercial or once the company is able to stockpile sellable product, to minimise the impact of process change delays. Major yield enhancing changes made to traditional fractionation processes were not found to be economicallyfeasible. Costs and delays involved in undertaking clinical trials as well as re-registration of the ‘new’ product in all of the countries in which the products are licensed were too high a cost to pay for relatively small yield enhancements.

Another process change case study presented in Chapter 5,looked at the purification of a new product from a waste fraction, however, this time with an experimental outlook. The protein Alpha 1-antitrypsin (AAT), which is effective in the treatment of hereditary AAT-deficiency, amongst other indications, was purified from FIV precipitate of a plasma fractionation scheme using a process suggested by Kee*et al.* (2004)in a patent. The initial purification stages were mimicked at laboratory scale, but proved unsuccessful using FIV precipitate. However, by making changes to the template process,AAT was successfully purified from FIV precipitate. The addition of a reducing agent such as DTT to FIV to disrupt the protein structure and precipitate out surrounding impurities caused filtration problems, and did not provide better yields. Additional precipitating agents and body feed additions to the FIV dissolution prior to filtering as suggested in the Kee*et al.* (2004) patent were also removed, as the filtration step efficiency was not improved and thebiggest impurityalbumin, was still present at very high concentrations. Depth filtration and anion exchange chromatography steps were effectively modified and resulted in a fully functional process. The recovery of AAT filtrate from depth filtration was greatly improved in this way. The filtration performance was also improved by removing all filter aids and reducing agents. The final process recoveries of AATwere very low at 18% or 23% when using Fractogel or Q-Sepharose media respectively, suggesting that FIV precipitate may not be the ideal starting material for the purificationof AAT.

Chapter 6 presented an example of process step optimization using the SELDI-TOF-MS technology to improve the isolation of alpha 1-antitrypsin (AAT) in FIV filtrate with ‘Q Sepharose’ anion mediaby speedily trialling a variety of buffer conditions. SELDI-TOF-MS fractionation technology (in this case the ‘Q10’ chip) was found to be useful at predicting trends in binding efficiencies over a range of pH and salt concentrations forHiTrap Q-Sepharose FF columns. Improved isolation of AAT was achieved using ‘Q10’ chips at lower pH values of 5.6-6.2, and with the highest salt concentrations tested (150-200mM NaCl),this was qualified using more robust 1mLHiTrap columns. The HiTrap QFF data showed that yields of 94-99% AAT can be achieved but with a compromise in impurity removal. The optimal condition for the isolation of AAT was found to be at pH 5.6, 200mM NaCl, where a 90% yield was achieved. However, more work would need to be done, to determine whether AAT is in a stable form or has been denatured at this low pH value. The HiTrap result seems to coordinate with the SELDI-TOF-MS NP20 profiles, where almost no impurities were present in the product peak at

at pH 5.6, 200mM NaCl, although, a larger data set would be required to determine the exact conditions to use. However, upon further inspection, when the purity of AATwas quantified, based on the ratio of AAT to total protein an undesirable percentage purity of only 8% is observed – this is based solely on the two main impurities present: Albumin and Transferrin. This would determine that SELDI-TOF-MS 'Q10' fractionation overestimates the purity that can be achieved at large-scale. However, the very low concentrations of AAT, in this relatively impure FIV filtrate sample does make the analysis more troublesome. Analysis has shown that variability in samples and differences in MS intensity can be eliminated by normalising the data. Analysis of the larger-scale material was carried out using the SELDI-TOF-MS NP20 chips, ELISA, Total protein. NP20 chip MS analysis was found to comparable to ELISA and Turbitimer immunoassay methods when the dataset was normalised.

6.8 FUTURE WORK

The work in this thesis presents a holistic framework for assessing the potential of process changes using both process economic models embracing development, manufacturing, and regulatory costs as well as scale-down models for rapid identification of optimal process conditions. It also provides a strong base for further work; several examples have been highlighted below.

Whilst the survey work provided valuable insight into the industry attitudes and practices concerning the implementation of process changes, this could be updated to discover how the use of the now more mainstream use of Process Analytical Technology (PAT), and the implementation Quality by Design (QbD) would affect the responses. In practise, PAT and QbD should afford companies much more process flexibility and a larger design space, even between development stageswithout affecting the product quality, and more importantly less involvement from the regulatory authorities during these critical stages. Technically, this may result in fewer "major" process changes, and less need for filing for 'Prior Approval Supplementation', often seen as a burden and can prevent manufacturers from implementing continuous improvement or introducing technological advances.It may also mean that companies will possibly have to make larger investments earlier in the product lifecycle during process development in advance of approved commercial operations, moving away from the more "aggressive" manufacturing approach. On top, whether this really does provides any "regulatory relief" and faster approval of new product applications and process changes, and or just "regulatory flexibility" would be useful to find out.A

secondary survey used to benchmark the costs and time spans of more specific process change activities, such as validation and retrofittingwould be useful to refine the correlations used in this thesis.

The systematic framework used in this thesis to capture the activities involved in process changes is modelled in Microsoft Excel with macros for Monte Carlo simulations to account for the uncertainties, and is modelled on an individual case basis. It may also be useful to model the framework dynamically to incorporate the analysis of process variations with time. The use of commercially available packages such as Aspen Engineering Suite (Aspen Technology; Cambridge, Massachusetts), and gPROMS (PSE; London, UK), could be useful in modelling the manufacturing activity, but would not include the key regulatory and clinical development activities. The use of a more generic software package such as Extend (Imagine That; San Jose, California) can be used to model discrete events that can be customized for manufacturing and development activities. The scenarios used to demonstrate the usefulness of the framework were based on the purification of polyclonal IVIG by means of a blood-plasma fractionation process.Further process change scenarios of interest could involvethe replacement of an earlier fractionation step, and to observe the impact on the multiple products that are derived from human-derived plasma.

As has previously beensuggested, the experiment to purify AAT from FIV paste yielded low final recoveries of AATand so FIV precipitate may not be the ideal starting material. However, the product is in demand, so a study to assess the trade-off of selling FIV precipitate as a cheap by-product or of purifying AAT from the paste as a new product needs to be analysed further. Other proteins, such as GC globulin, Protein C, Mannan binding lectin and C-reactive Protein are present in FIV and may be present at higher concentrations and so may be more suitable to purify from this fraction point. To assess this thoroughly, the differing commercial, development, and technical attributes must be compared. Commercial attributes will include, the potential market demand (dose per patient and per annum), the presence of direct competitors and indirect competitors, the possible selling prices, the potential to patent the purification process, and estimated regulatory approval rates. Development issues should cover the ease of process development (based on scalability, and the presence of similar processes in industry), processing time, initial concentration in FIV precipitate, the complexity of the protein structure, potential overall yield, the final purity, and of course the process change activities, which will include validation, the risk and cost of clinical trials, and

assay development. Manufacturing issues to consider will be a comparison of the initial capital investments as well as the annual cost of goods per gram, as well as perhaps the utilisation of disposables.

AAT can also be purified from an earlier process fraction (Fraction A+1), which has an impact on approved products such as Albumin. This has been investigated experimentally by Kumpalume*et al.* (2007, 2008).An economic comparison of the purification of AAT from FIV paste and from Fraction A+1 including all process change activities, such as the risk of repeating clinical trials would provide a valuable insight on the potential new products may have in the traditionally unchanged blood-plasma fractionation process.

The use of Ciphergen®'s SELDI-TOF-MS ProteinChip technology to investigate the value of using a high throughput optimisation method and to improve the isolation of AAT would be more valuable if a fully automated approach was used. This would allow for the screening of a large number of conditions in short time-scales, and would further showcase the usefulness of the SELDI-TOF-MS by improving its ability to predict the results of scaled-up conditions.

It would be valuable to look at further complementary experiments analysing AAT stability upon reaching the Fraction IV precipitate stage and also during the AAT purification process suggested in this thesis. The stability of AAT at the initial FIV precipitate stage could be compared to Kumpalume's suggested starting point, Fraction A+1, to assess which was the most economically valid pool. Following on from this, it would be beneficial to also look at protein re-folding methods that might be useful in "recapturing" any AAT denatured by some of the harsh fractionation process methods utilised.

In conclusion, the future work outlined draws upon the background survey, the framework, and methods utilised in this thesis. The development of more sophisticated models, increased benchmarked data, and the incorporation of PAT and QbD will increase the accuracy of prediction of this type of work. With an industry push towards better global harmonization in product development, regulatory submission and quality, it will mean it is even more relevant in the future to model process change activities using a generic systematic approach such as that described in this thesis.

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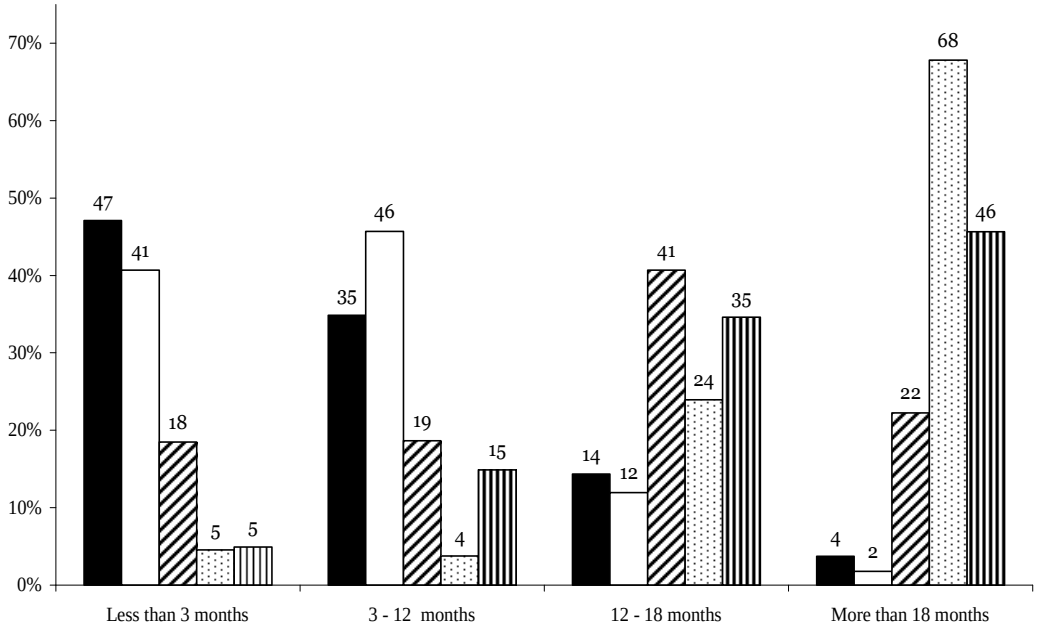
APPENDIX

Appendix: Chapter2



Appendix 2- Figure 1 The Percentage breakdown of respondents who estimated the durations of post change activities to be less than 3 months; 3-12 months; 12-18 months; or more than 18 months, n=81. Legend: Bioequivalence/comparability studies (■), Revalidation of process and equipment (☐), Phase I clinical trials (▨), full-scale clinical trials (▩), re-registration of product (▥)

Appendix: Chapter 3

Appendix 3- Table 1

Unit operation	Basis – simple models	Key outputs
Centrifugation	Solids carry-over Solids volume-fraction in sediment	Sediment composition Supernatant composition
Microfiltration	Flux Rejection coefficients	Permeate composition Retentate composition Membrane area *or* Concentration factor *or* Processing time
Diafiltration	Flux Rejection coefficients Number of diafiltration volumes *or* Contaminant removal-fraction	Permeate composition Retentate composition Membrane area *or* Concentration factor *or* Processing time Number of diafiltration volumes *or* Contaminant removal-fraction
Dead-end filtration	Flux Rejected particle-fraction, Particle volume-fraction in retentate	Permeate composition Retentate composition Membrane area *or* Processing time
Chromatography	Flowrates, yields	Product stream composition Waste stream composition Processing time Buffer volumes

Appendix 3- Table 2 Examples of the Farid, 2001 mass balance models used to model the manufacturing process task in the process change activity framework.

Inputs	**Outputs**	**Mass Balance Calculations**
Centrifugation Solids-carry-over-fraction, S Solid-volume-fraction in sediment, $v_{s_{sed}}$ Total feed stream component masses, $m_{in_{tot}}$ Total volume of feed stream, $V_{in_{tot}}$ Solid density, ρ_s * Assumption: Only 1 solid component	Solids removal fraction, R Supernatant and sediment solid component masses, $m_{s_{sup}}$, $m_{s_{sed}}$ Supernatant and sediment liquid component masses, $m_{l\,i_{sup}}$, $m_{l\,i_{sed}}$	1. R $R = 1 - S$ 2. $m_{s_{sed}}$, $m_{s_{sup}}$ $m_{s_{sed}} = R * m_{s_{in}}$ $m_{s_{sup}} = m_{s_{in}} - m_{s_{sed}}$ 3. $\rho_{l_{in}}$ $m_{l_{in}} = m_{in_{tot}} - m_{s_{in}}$ $V_{l_{in}} = V_{in_{tot}} - V_{s_{in}}$ $\rho_{l_{in}} = \frac{m_{l_{in}}}{V_{l_{in}}}$ 4. $m_{l_{sed}}$ $V_{s_{sed}} = \frac{m_{s_{sed}}}{\rho_s}$ $V_{tot_{sed}} = \frac{V_{s_{sed}}}{v_{s_{sed}}}$ $V_{l_{sed}} = V_{tot_{sed}} - V_{s_{sed}}$ $m_{l_{sed}} = V_{l_{sed}} * \rho_{l_{in}}$ 5. $m_{l\,i_{sed}}$, $m_{l\,i_{sup}}$ $m_{l\,i_{sed}} = \frac{m_{l\,i_{in}}}{m_{l_{in}}} * m_{l_{sed}}$ $m_{l\,i_{sup}} = m_{l\,i_{in}} - m_{l\,i_{sed}}$

Inputs	**Outputs**	**Mass Balance Calculations**
Membrane filtration Calculation mode: Membrane area per unit, A / Processing time per cycle, t Average flux, J Volume of feed stream, V_{in} No of cycles, n Concentration factor, CF Rejection coefficient, RC	Output mode: Membrane area per unit, A / Processing time per cycle, t Total processing time, T Permeate and retentate stream component masses, $m_{i\,perm}$, $m_{i\,ret}$	1. A, t $A = \frac{V_{in}(1-CF^{-1})}{J*n*t}$ or $t = \frac{V_{in}(1-CF^{-1})}{J*n*A}$ 2. T $T = n*t$ 3. V_{ret} $V_{ret} = \frac{V_{in}}{CF}$ 4. $m_{i\,ret_{RC\neq 0}}\ (0 < RC_i \leq 1)$ $m_{i\,ret_{RC\neq 0}} = m_{i\,in} * CF^{(RC_i - 1)}$ $V_{i\,ret_{RC\neq 0}} = \frac{m_{i\,ret_{RC\neq 0}}}{\rho_i}$ 5. $m_{i\,ret_{RC=0}}\ (RC_i = 0)$ $V_{ret_{RC=0}} = V_{ret} - \sum V_{i\,ret_{RC\neq 0}}$ $V_{i\,ret_{RC=0}} = \frac{V_{i\,in}}{V_{in_{RC=0}}} * V_{ret_{RC=0}}$ $m_{i\,ret_{RC=0}} = V_{i\,ret_{RC=0}} * \rho_i$ 6. $m_{i\,perm}$ $m_{i\,perm} = m_{i\,in} - m_{i\,ret}$

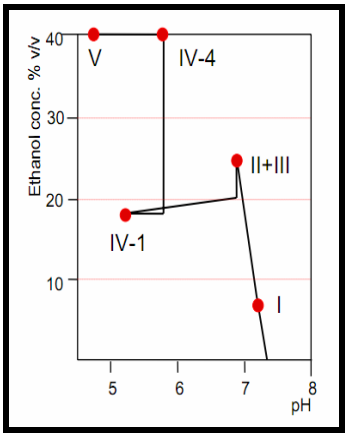
Inputs	**Outputs**	**Mass Balance Calculations**
Diafiltration Calculation mode: Membrane area per unit, A / Processing time per cycle, t Average flux, J No of cycles, n Rejection coefficient, RC No of diafiltration volumes, D Volume of components in tank, V_o Component masses in tank, m_o	Output mode: Membrane area per unit, A / Processing time per cycle, t Total processing time, T Permeate and retentate stream component masses, $m_{i\ perm}$, $m_{i\ ret}$	1. A, t $A = \frac{D * V_o}{J * n * t}$ or $t = \frac{D * V_o}{J * n * A}$ 2. T $T = n * t$ 3. V_{ret} $V_{ret} = V_o$ 4. $m_{i\ ret_{fromV_o}}$ $m_{i\ ret_{fromV_o}} = m_{i\ o} * e^{-D(1-RC_i)}$ $V_{i\ ret_{fromV_o}} = \frac{m_{i\ ret_{fromV_o}}}{\rho_i}$ 5. $m_{i\ ret_{fromV_{buff}}}$ $V_{ret_{fromV_{buff}}} = V_{ret} - \sum V_{i\ ret_{fromV_o}}$ $V_{i\ ret_{fromV_{buff}}} = \frac{V_{i\ buff}}{V_{buff}} * V_{ret_{fromV_{buff}}}$ $m_{i\ ret_{fromV_{buff}}} = V_{i\ ret_{fromV_{buff}}} * \rho_i$ 6. $m_{i\ ret}$ $m_{i\ ret} = m_{i\ ret_{fromV_o}} + m_{i\ ret_{fromV_{buff}}}$ 7. $m_{i\ perm}$ $m_{i\ perm} = m_{i\ in} - m_{i\ ret}$

Inputs	**Outputs**	**Mass Balance Calculations**
Chromatography Column height, H No of column volumes, CV Linear flow rates, u Column volume, V_{col} No of cycles, n Yield fraction, y Product-stream-column-volumes, CV_{prod}	Processing time per cycle, t Buffer volumes required, V Product and waste stream masses, $m_{i\,prod}$, $m_{i\,waste}$	1. t_r, $\quad r = L, W, E$ $t_r = \frac{H * CV_r}{u_r}$ 2. V_r, $\quad r = L, W, E$ $V_r = CV_r * V_{col} * n$ 3. $V_{prod} = CV_{prod} * V_{col}$ $m_{i\,prod_{from\,L}} = m_{i\,L} * y_i$ $V_{i\,prod_{from\,L}} = \frac{m_{i\,prod_{from\,L}}}{\rho_i}$ $V_{prod_{from\,E}} = V_{prod} - \sum V_{i\,prod_{from\,L}}$ $V_{i\,prod_{from\,E}} = \frac{V_{i\,E}}{V_E} * V_{prod_{from\,E}}$ $m_{i\,prod_{from\,E}} = V_{i\,prod_{from\,E}} * \rho_i$ $m_{i\,prod} = m_{i\,prod_{from\,L}} + m_{i\,prod_{from\,E}}$ $m_{i\,waste} = m_{i\,L} + m_{i\,W} + m_{i\,E} - m_{i\,prod}$ From gel filtration, the equations were similar except there were no terms related to the wash step.

b

Inputs	**Outputs**	**Mass Balance Calculations**
Dead-end filtration Calculation mode: Membrane area per unit, A / Processing time per cycle, t Average flux, J Rejection factor, RF Particle-volume fraction, $v_{p\,ret}$	Output mode: Membrane area per unit, A / Processing time per cycle, t Permeate and retentate stream component masses, $m_{i\,perm}$, $m_{i\,ret}$	1. $m_{i\,ret}\,(0 < RF_i \leq 1)$ $m_{i\,ret_{RF\neq 0}} = RF * m_{i\,in}$ $V_{i\,ret_{RF\neq 0}} = \frac{m_{i\,ret_{RF\neq 0}}}{\rho_i}$ 2. $m_{i\,ret_{RF=0}}\,(RF_i = 0)$ $V_{ret} = \frac{\sum V_{i\,ret_{RF\neq 0}}}{v_{p\,ret}}$ $V_{ret_{RF=0}} = V_{ret} - \sum V_{i\,ret_{RF\neq 0}}$ $V_{i\,ret_{RF=0}} = \frac{V_{i\,in}}{V_{in_{RF=0}}} * V_{ret_{RF=0}}$ $m_{i\,ret_{RF=0}} = V_{i\,ret_{RF=0}} * \rho_i$ 3. $m_{i\,perm}$ $m_{i\,perm} = m_{i\,in} - m_{i\,ret}$ 4. A, t $V_{perm} = V_{in} - V_{ret}$ $A = \frac{V_{perm}}{J * t}$ or $t = \frac{V_{perm}}{J * A}$
Viral clearance No of virus units in inlet stream, vr_{in} Log clearance factor, LCF	No of virus units in outlet stream, vr_{out}	1. vr_{out} $vr_{out} = vr_{in} * 10^{LCF}$

Appendix: Chapter 4

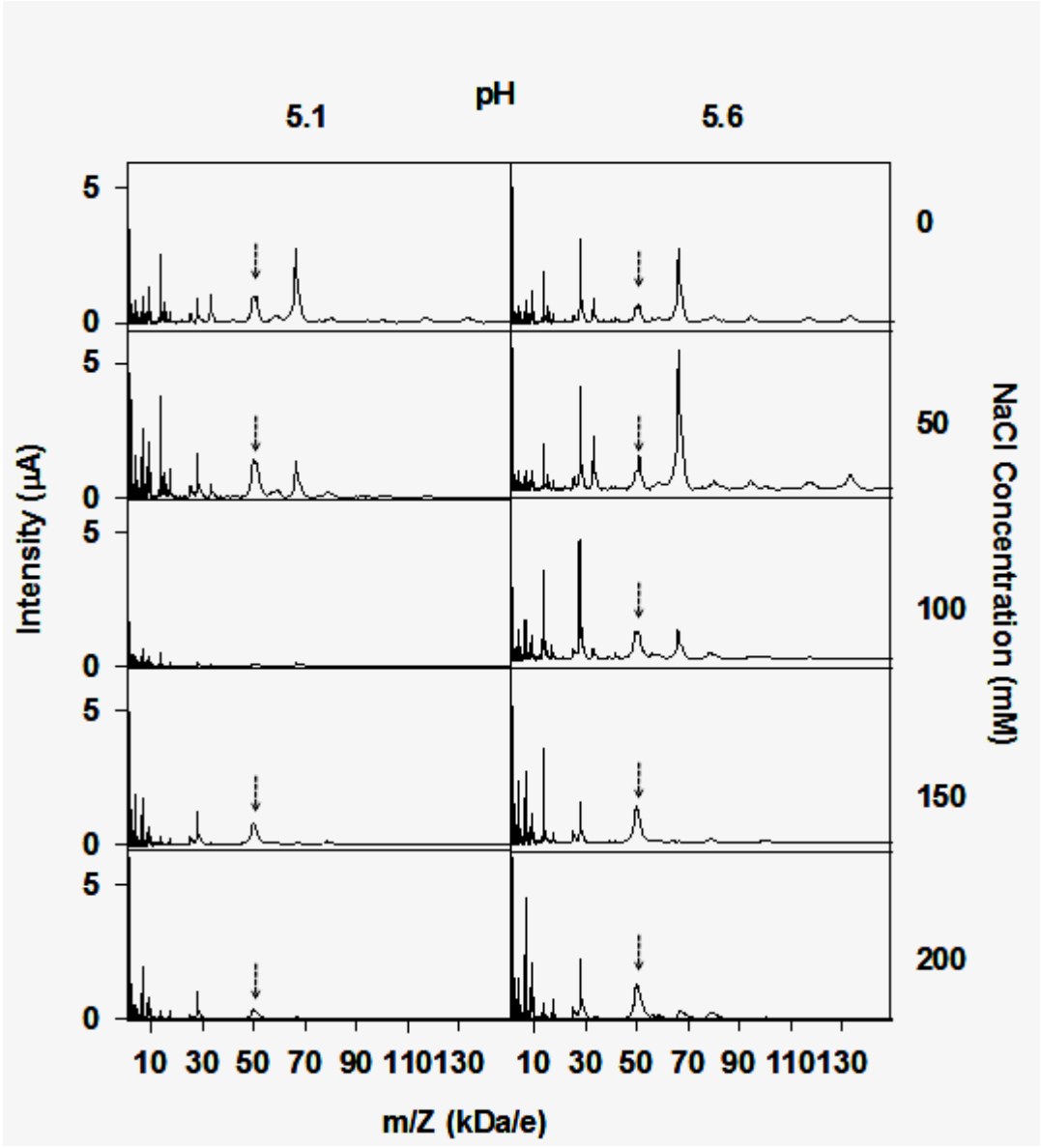


Appendix 4- Figure 1 A depiction of the Cohn (Method 6) adapted from Curling *et al.* 2005. 'A comparative study of Cohn and chromatographic fractionation using a novel affinity "Cascade Process"'

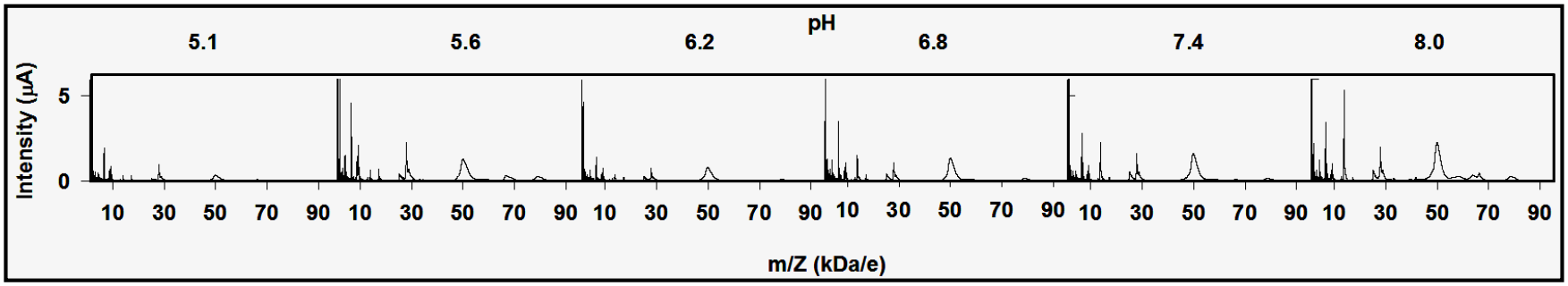
Appendix 4- Table 1 Total Protein and AAT content of samples recovered from the anion exchange step from the process trial run based on the Kee*et al.*, 2004 process for Fractogel, and Q Sepharose FF under various buffer conditions (**Table 5.2)**. Samples were measured using the Pierce microplate reducing agent compatible BCA™ total protein assay and an AAT Elisa kit (Immundiagnostik) . All samples were desalted using AmiconCentricon YM-3 regenerated cellulose filter devices, MWCO 3,000 to remove DTT from samples prior to analysis.

		Total Protein Mass (mg)	Total Protein Yield (%)	Mass AAT (mg)	AAT Yield (%)	AAT purity (%)
Fractogel	**Run A**					
	Peak 1	429	5%	negligible	-	-
	Peak 2	323	4%	negligible	-	-
	Peak 3	93	1%	negligible	-	-
Fractogel	**Run B**					
	Peak 1	295	3%	0.7	25%	0.2%
	Peak 2	228	3%	2.6	86%	1.1%
	Peak 3	73	1%	0.1	5%	0.2%
Fractogel	**Run C**					
	Peak 1	303	3%	0.8	27%	0.3%
	Peak 2	140	2%	1.9	64%	1.3%
	Peak 3	136	2%	0.1	2%	0.1%
Q Sepharose FF	**Run D**					
	Peak 1	247	3%	0.1	2%	0.0%
	Peak 2	271	3%	2.1	70%	0.8%
	Peak 3	43	0%	0.0	1%	0.1%
Fractogel	**Run E**					
	Peak 1	349	4%	0.2	6%	0.1%
	Peak 2	124	1%	1.4	46%	1.1%
	Peak 3	108	1%	0.1	3%	0.1%
Q Sepharose FF	**Run F**					
	Peak 1	353	4%	0.2	8%	0.1%
	Peak 2	135	1%	7.9	-	5.9%
	Peak 3	55	1%	0.0	1%	0.0%
Q Sepharose FF	**Run G**					
	Peak 1	282	3%	0.6	19%	0.2%
	Peak 2	250	3%	13.0	100%	5.2%
	Peak 3	97	1%	0.1	2%	0.1%

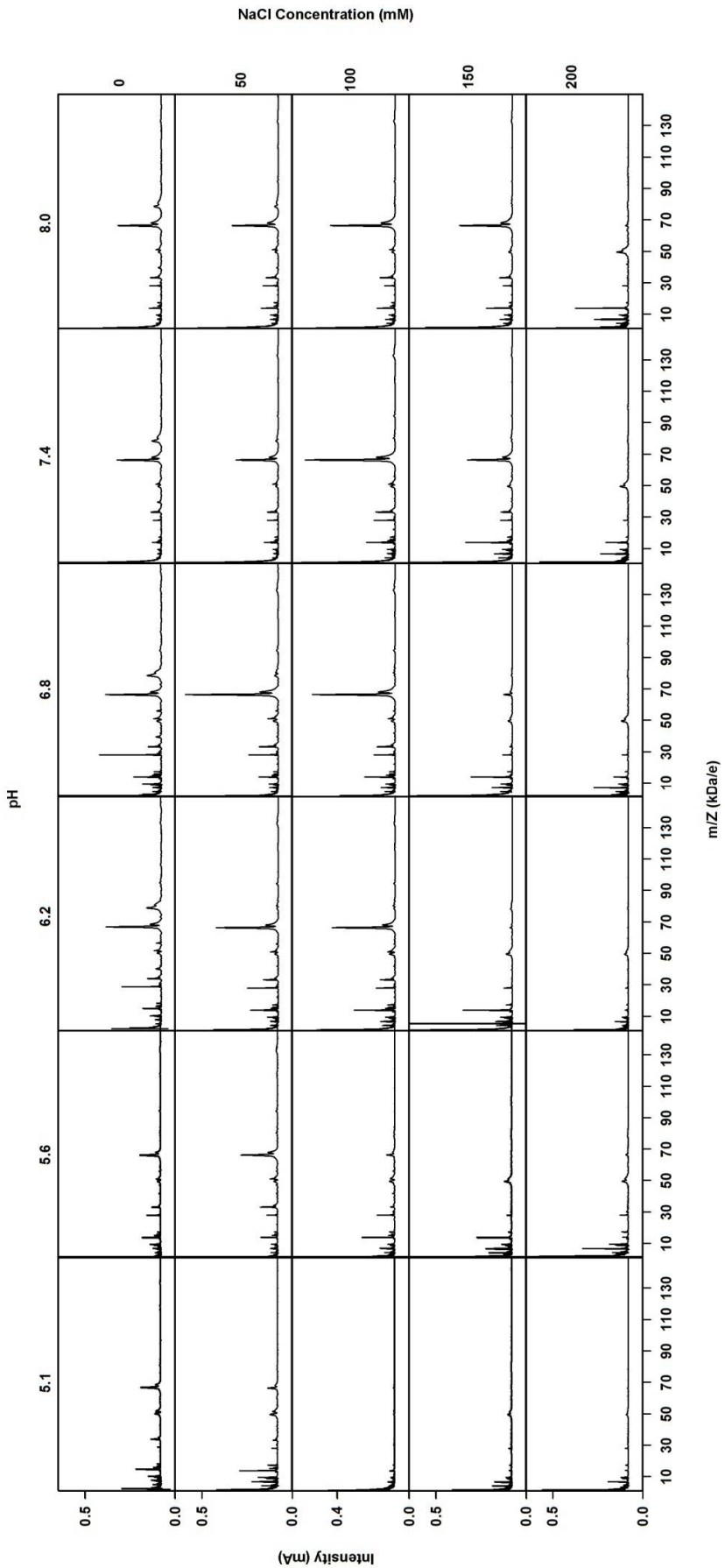
Appendix: Chapter 5



Appendix 5 - Figure 1SELDI-TOF-MS spectra representing protein profiles portraying the impact of further buffer conditions on the binding intensity and isolation of AAT using the 'Q10' ProteinChip. The buffer conditions shown are a combination of pH 5.1-5.6 and 0-200mM NaCl.



Appendix 5- Figure 2SELDI-TOF-MS spectra representing protein profiles portraying the impact of further buffer conditions on the binding intensity and isolation of AAT. AAT is fractionated using the 'Q10' ProteinChip from FIV filtrate samples under a variety of buffer conditions: pH 5.1-8, 200mM NaCl.



Appendix 6 - Figure 3 Normalised SELDI-TOF-MS spectra based on baseline corrected data. The protein profiles portray the binding intensity and isolation of proteins present in FIV filtrate fractionated using the 'Q10' ProteinChip under a variety of buffer conditions: pH 5.1-8, 0-200mM NaCl. Based on Baggerly, 2007 method