Characterization and Optimization of a Protein Recovery Process of Interferon-α-2b of an *E.coli* Fermentation

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Declaration of originality

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Abstract

The primary protein recovery process of an industrial *E.coli* fermentation at large scale was characterized and optimized based on the total protein, total interferon-α-2b and interferon-isoform concentrations. Mass balances for total protein and interferon-α-2b were set up for each centrifugation step throughout the primary recovery process to identify critical process steps. With the characterization of the recovery process a loss of 90% of the total protein and a loss of 80% in total interferon-α-2b was observed, occurring at two main critical process steps (CPS). The majority loss of proteins was identified during critical process step one, due to insufficient solubility and low protein saturation levels in water.

Following the characterization, CPS-1 was optimized by the addition of detergents. Two different detergents, Sarkosyl and Zwittergent 3-14 were tested and their impacts on the following recovery steps as well as on the first down-stream step were studied. A new purification method was developed in order to remove one of the detergent (Sarkosyl) from process samples prior to the down-stream process With the addition of Zwittergent 3-14 during CPS-1 the recovery of interferon-α-2b at the end of the protein recovery process was successfully increased 4-fold compared to the previous protein recovery and no negative impact on the first down-stream step was observed.

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Abbreviations

aa	amino acid
AB	antibody
abs	absorbance
AGG	aggregation number
AMC	Anti-Mouse IgG Fc Capture
ANOVA	analysis of variances
AP	alkaline phosphatase
BCA	Bicinchoninic acid
BET	Brunauer, Emmet and Teller surface area
BLI	Bio-Layer Interferometry
bp	base pairs
BSA	Bovine Serum Albumiun
CCF-design	central composite face-centered design
cFDA	5(6)-carboxylfluorescein
cmc	critical micelle concentration
cmt	critical micelle temperature
CPS	Critical Process Steps
DCU	Dublin City University
DF	degrees of freedom
DI-water	Deionized-water
DoE	design of experiments
DTT	Dithiothreitol
DV	diafiltration volume
E.coli	*Escherichia coli*
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

EDTA	Ethylene Diamine Tetraacetic Acid
EOF	End of Fermentation
FCM	Flow-Cytometer
FDA	Food and Drug Administration
h	hour
HCl	Hydrochloric acid
HCP	Host cell proteins
HPLC	High-performance-liquid-chromatography
HRP	horseraddish peroxidase
IEC	Ion-exchange chromatography
IEF	Iso-Electric Focussing
IFN	Interferon
IgG	Immunoglobulin G
Iso	Isoforms
kDa	kilodalton
kHz	kilohertz
L	Liters
LOD	limit of detection
LOF	lack of fit
LOQ	limit of quantification
M	Molar
min	minute
mL	milliliter
MM	molar mass
MMW	average micelle molecular weight
mS/cm	milli Siemens per centimeter
MW	molecular weight
MWCO	molecular weight cut-off
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NHS	N-hydroxysuccinimide
NIBRT	National Institute of Bioprocessing Research & Training
nm	nanometers
OD	optical density
PAGE	Polyacrylamide Gel Electrophoresis
PBS-buffer	Phosphate buffered saline

PES	polyethersulfone
PEGylated	polyethylene glycol polymer
pI	isoelectric point
PTM	Posttranslational Modifications
PVDF	polyvinyliderne difluoride
QS	quantity sufficient
RF	refractive index
RP	Recovery Process
RP-HPLC	reversed phase HPLC
rpm	rounds per minute
RT	Room Temperature
RtT	retention time
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SP	sulphopropyl
SPR	surface-plasmon resonance
TBST	Tris-Buffered Saline and Tween 20 buffer
TCA	Trichloroacetic Acid
TFA	Trifluoroacetic Acid
TFF	tangential flow filtration
TMP	transmembrane pressure
UF/DF	ultrafiltration/ diafiltration
UPLC	Ultra-high-performance-liquid-chromatography
UP-water	Ultra-pure water
UV	Ultraviolet
WB	Western Blot

all amino acids:

Ala	Alanine
Arg	Arginie
Asn	Asparagine
Asp	Aspartic Acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutaminc Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophane
Tyr	Tyrosine
Val	Valine

Symbols

β	theoretical protein solubility	mol/L
c	speed of light in vacuum	m/s
C_0	concentration of protein in feedstock	mg/ml
C_S	molar salt concentration	mol
γ	shear rate	cycle/sec
ΔG_D	free energie of protein stability	J/mol
G_N	free energie of native protein	J/mol
G_U	free energie of unfolded protein	J/mol
K_S	salting-out constant	
P/V	specific power input	W/m^3
Q_S	specific impeller pumping rate	
R^2	statistic coefficient of determination	
S	solubility of the protein	mol/L
v	speed of light in substance 'n'	m/s
V_0	void volume of column	ml
V_L	volume loaded up to the breakthrough point	ml
V_{tip}	impeller tip speed	m/sec
vvm	volume of gas per volume & minute	

Chapter 1

Introduction

1.1 Thesis aims and scopes

The primary recovery of proteins at the end of a fermentation process can be considered as a keystone for the overall protein loss during purification. Recombinant proteins are often produced in batch and fed-batch fermentations at industrial scale since the early 1980s using *Escherichia coli (E.coli)* or other microbial cells. The main challenge remains in the isolation and recovery of the protein from the fermentation broth at the end of the fermentation. The primary recovery of proteins after a microbial fermentation is usually composed of several centrifugation steps to separate the protein from the fermentation broth and to reduce the volume before starting the final purification steps during downstream processing. During the centrifugation steps different set-points of pH, conductivity and volume will be adjusted which can affect the product quality and quantity. Therefore the characterization of the primary protein recovery process is indispensable to maintain product quality and quantity, especially at industrial scale.

The recombinant protein interferon-α-2b is produced at manufacturing scale at 40'000L in an *E.coli* batch fermentation since the early 1980s. The fermentation is followed by a primary protein recovery process in order to harvest the recombinant protein and separate it from the fermentation broth prior to the final purification steps. This recovery process was identified as the primary source of the overall protein loss during the production of the drug substance interferon-α-2b. In order to optimize this primary recovery process a research collaboration was formed in 2011 between the industrial partner and NIBRT/ DCU with the aim to enhance the overall process

yield.

The primary focus of this project is to characterize the protein recovery process and to identify critical process parameters in order to optimize and minimize the protein loss during the recovery process. In a first step protein mass balances for total protein and total interferon-α-2b need to be set up throughout the recovery process to identify the critical process steps (CPS). Following the characterization, identified CPS can be investigated and optimized in order to increase the overall process yield.

In a second step, identified critical process steps will be optimized using design of experiment and optimized conditions will be applied to the protein recovery process in a small scale study. Following the optimization, the impact of the optimized process on the first down-stream step will be investigated.

This project is done in a research collaboration between an industrial partner and NIBRT/DCU, therefore all information given in this manuscript is confidential!

1.2 Thesis outline

This thesis is divided into twelve chapters, whose structure is described in the following paragraphs:

Chapter 2 - Project Outline

The first part, chapter 2, provides information about the project outline itself. It contains detailed plans about the manufacturing process, as well as sampling systems that were put in place through the manufacturing process.

Chapter 3 - Simulation of the protein recovery process

Chapter 3 describes the development of a protein assay using a small scale model of the manufacturing recovery process. Since different media components do interfere with some protein assays it is important to develop a robust method with a minimum of interference from media or cell components.

Beside this, chapter 3 also provides information about some characteristics of the recovery process itself as well as the application of the small scale model of the recovery process at lab scale.

Chapter 4 - Process Characterization - total protein mass balances

Chapter 4 is the first of three parts about the characterization of the protein recovery process. Total protein mass balances are set up throughout the entire RP in order to identify critical process steps for protein recovery.

Chapter 5 - Process Characterization - total Interferon mass balances

This chapter is the second part about process characterization. After setting up mass balances for total protein in chapter 4, chapter 5 contains mass balances for total interferon-α-2b. Critical process steps are identified for IFN losses throughout the recovery process and compared to the CPS's identified for total protein.

Chapter 6 - Process Characterization - Isoform mass balances

Chapter 6 is the last part about the characterization of the protein recovery process and can be divided into three sections. The first section focuses on the development and optimization of a HPLC method for the quantification of interferon-isoforms. The second section discusses the development of a purification and concentration technique for the recovery process samples in order to purify interferon-α-2b and its isoforms from host cell proteins prior to analysis. The last section of this chapter covers the setting up of mass balances for the interferon-isoforms. Identified critical process steps are compared with the findings from chapter 4 and 5.

Chapter 7 - Process Optimization - protein dilution study

Chapter 7 is the first of two chapters focussing on the optimization of the identified critical process step 1. A dilution study is performed around cycle 1 of the protein recovery process to increase protein solubility. The optimal dilution factor is applied to the protein recovery process in a small scale study and the impact of the optimized recovery process on the first down-stream step is investigated.

Chapter 8 - Process Optimization - Solubility study with detergents

This chapter is the second part focussing on the optimization of the critical process step 1. Different detergents are tested for their potential to improve the interferon-α-2b solubility during CPS-1. Two detergents are selected and implemented in the protein recovery process in a small scale study. The impact of the presence of the detergents on the following recovery process steps is studied.

Chapter 9 - Detergent Removal from the Recovery Process

Chapter 9 focuses on the removal of the two detergents from process samples. First, two analytic methods are developed for the quantification of the two detergents selected in chapter 8. Following, different techniques are studied for their potential to remove the detergents from process samples while keeping interferon-α-2b in solution.

Chapter 10 - Impact of optimized recovery process on Down Stream Process

This chapter studies the impact of the presence of detergents on the first down-stream step, an ion-exchange chromatography method. First, the ion-exchange method is characterized and mass balances for interferon-α-2b and total protein are being set-up throughout the chromatographic procedure. The second part studies the impact of the two detergents on the interferon-α-2b purification with the ion-exchange method.

Chapter 11 - Summary chapter - Optimized Protein Recovery Process

Chapter 11 is summarizing the findings of chapter 7, 8, 9 and 10 and proposed three new optimized protein recovery processes with an enhanced interferon-α-2b recovery. Following, advantages and disadvantages of each optimized process are discussed and the most efficient and applicable optimized recovery process is presented, considering interferon recovery and process time and cost.

Chapter 12 - Conclusion and Outlook

This chapter gives a conclusion of the achieved results of all ten chapters, focusing on the characterization of the primary protein recovery process, the optimization of identified critical process steps and the impact of the optimized processes on the first down-stream step. Chapter 12 also contains an outlook of potential following steps in order to increase the interferon-α-2b productivity and to enhance the overall process yield.

Chapter 2

Project Outline

2.1 Introduction

Interferon-α-2b is produced at manufacturing scale since 1983. The industrial partner is a leader in the pharmaceutical industry in developing and producing therapeutic proteins. Their facility in Ireland includes large-scale fermentation, purification, sterile manufacturing and quality control operations.

On site analysis in the years up to 2011 showed an overall yield of only 5% of the interferon-α-2b production process. The main loss of interferon of around 75% was detected in the primary protein recovery process prior to the purification process. These findings made the recovery process priority number 1 for the research collaboration between the industrial partner, NIBRT and DCU. The collaboration started in summer 2011 with the aim to characterize and optimize the primary recovery process for interferon-α-2b to enhance the overall process yield.

This chapter will provide more detailed information about the structure and outline of this research collaboration and gives a guidance on the different steps of this project.

2.2 Interferon-α-2b drug substance production

Interferon and cytokines are an important part of our immune system to protect it from pathogens such as viruses, bacteria, parasites or tumour cells [17]. Therefore they are nowadays often used in cancer treatment or for immune diseases. Interferon can be divided into two main categories:

type I and type II. Type I interferon (IFN-α, -β and -ω) are important in the anti-viral response and activity against tumours [18, 19]. Type II interferon (IFN-γ) is active against bacterial infection as well as in tumour control [20].

Interferon-α-2b is an important drug for the treatment of viral diseases, especially Hepatitis C [21, 22]. Up until today there are three companies who are producing IFN-α-2 at manufacturing scale [23, 24]. Figure 2.1 shows the molecular structure of the recombinant protein interferon-α-2b.

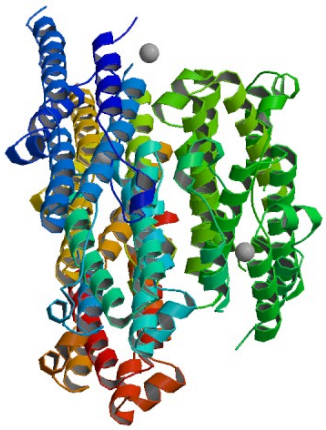

Fig. 2.1: Molecular structure of interferon-α-2b [1]

Interferon-α belongs to the large class of glycoproteins but studies have shown that the glycan-profiles are not mandatory to achieve biological activity of IFN-α in cancer and Hepatitis-C treatment [25]. Hence, interferon-α is produced by bacterial fermentation to avoid glycan variability, that can occur during production by mammalian cells.

At manufacturing scale interferon-α-2b is produced by a modified *E.coli* strain in a batch fermentation. *Escherichia coli* K-12/P1 strain using a EK1 *E.coli* host, bears the plasmid KMAC-43 to produce interferon-α-2b. The plasmid itself contains of 7882 bp including the human interferon-α-2b gene, a tetracycline resistance region and a temperature-inducible runaway origin of replication, which allows the plasmid to accumulate to a high copy number following a temperature up-shift during fermentation. The production occurs at a 32000L working volume, at 70 rpm and pH 7.0. The pH is maintained by the addition of NaOH. Two temperature shifts are necessary during the production. The first one is from 30 to 37°C and the second one is from 37 down to 25°C. In the first phase of the fermentation at 30°C cell growth of the *E.coli* cells occurs. The short second

phase at 37°C is the induction phase for interferon-α-2b, whose genome sequence is located on a temperature controlled vector. The last phase at 25°C is for protein/interferon production and maintenance of cell viability.

Producing recombinant proteins in bacterial cells has the advantage of no glycosylation of the protein of interest. However, there is also a large disadvantage, proteins are generally not secreted into the medium but remain within the cytoplasm or periplasmic space of the bacterial cell. Hence, cells need to be lysed in order to release the protein of interest and to harvest it. This happens in the primary protein recovery process which will be described in more detail in the next section. After the recovery process the actual purification steps follow. The first step for interferon-α-2b is an ion-exchange chromatography using a cation-exchange column. This step is followed by several chromatographic and filtration steps until the pure active drug substance of interferon-α-2b remains.

The overall process yield is around 5%, with the major loss during the primary recovery process. Therefore this part was chosen to be the most important process to be characterized and optimized to enhance the overall process yield.

2.3 Recovery Process for Interferon-α-2b

The primary recovery process post fermentation has two main purposes. First, break up the bacterial cells to release the proteins and secondly to separate the proteins from the fermentation broth. For the recovery of interferon-α-2b the recovery process is comprised of four steps, each separated by a centrifugation step. An overview of all four steps can be seen in figure 2.2.

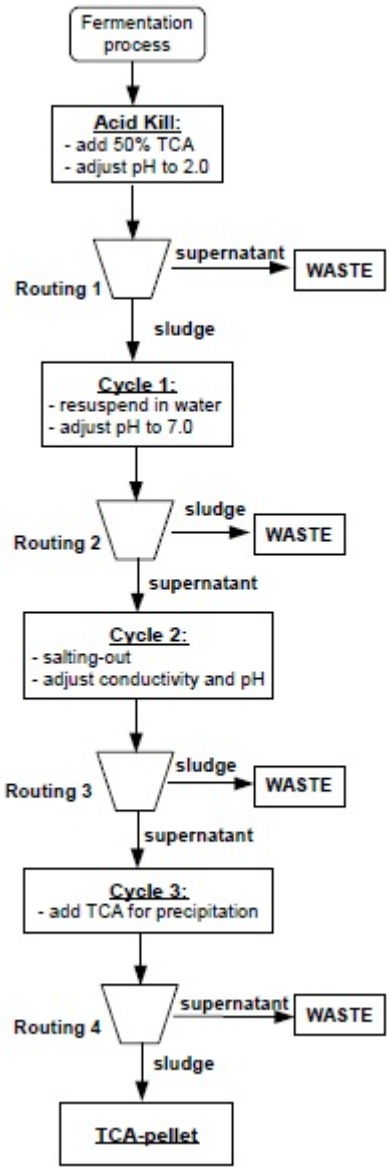

Fig. 2.2: Overview of the primary protein recovery process for interferon-α-2b

Step 1 is called the "acid kill", which happens in the production fermenter. At the end of the fermentation 50% TCA is added to the fermentation broth to a final concentration of 1.5% to break up the cells. The pH is lowered to 2.0 by the addition of 75% Phosphoric Acid. After 30 minutes agitation all cells are lysed, proteins released and due to low pH environment proteins precipitated. The pellet of the following centrifugation step continues in cycle 1, the second step of the recovery process.

In this cycle the pellet is redissolved in water and the pH is set to 7.0 due to the addition of

NaOH. Agitation for four hours under these conditions re-suspends the precipitants. After the 2nd centrifugation step the supernatant is recovered which enters cycle 2, step number 3 of the recovery process. This cycle is also called the "Salting-Out" step with the purpose to salt-out host cell proteins and to keep interferon in solution. This is achieved by adjusting conductivity and pH due to the addition of NaCl and HCl.

Again the supernatant is recovered during the centrifugation step 3 entering the last cycle 3. In this step 50% TCA is added to a final concentration of 3% in order to precipitate all proteins again. After a holding time of 10 - 24 hours to flocculate precipitants, a final centrifugation step is performed where the sludge is collected in the TCA-pellet.

In previous studies a loss of up to 70% of the interferon was discovered during this process making the recovery process the main important part for characterization and optimization.

2.4 Project set-up

The whole project was divided into three main work packages. The first one is defined as the setup of the analytical toolkit in which different analytical methods, needed for the characterization of the primary recovery process, were included. The second work package contains the characterization of the primary recovery process on the basis of mass balances. The third and last work package includes the optimization of the recovery process, starting from the critical process steps identified during the characterization. Table 2.1 gives a more detailed plan about different steps of this project.

Chapter 3 discusses the set up and the selection of analytical methods in order to follow the protein amount throughout the recovery process using a small scale model of this process. Chapter 4, 5 and 6 dispute the characterization of the primary protein recovery process using mass balances for total protein, total interferon-α-2b and interferon-isoforms to identify critical process steps. Chapter 7 and 8 focus on the optimization of the identified critical process steps of the recovery process using a dilution study and the application of different detergents to increase protein solubility. Chapter 9 discusses several techniques to remove the detergents from process samples at the end of the protein recovery process prior to the down-stream process. And finally in chapter 10 the impact

Tab. 2.1: Project-outline and objectives

Work package	**description of the objectives**	
Analytical Toolkit	total protein	protein-assays
	total interferon	SDS-PAGE
		Western Blot
		Biacore, BLItz
	Isoforms	RP-HPLC
		sample purification & concentration
Process Characterization	total protein	mass balances
		identification of CPS
	total interferon	mass balances
		identification of CPS
	Isoforms	mass balances
Process Optimization	Solubility study	Dilution experiment
		detergents addition
	Detergent removal	Sarkosyl
		Zwittergent 3-14
	impact on DSP	Sarkosyl
		Zwittergent 3-14

of different detergents on the first down-stream step, an ion-exchange method, is investigated.

2.5 Implementation of sampling system

In order to characterize the primary protein recovery process a sampling system was put in place for the manufacturing process by the industrial partner. Samples were taken at different time-points throughout the process in order to analyse these sample for total protein and total interferon-α-2b and to set-up protein mass balances. Figure 2.3 shows the sampling plan, indicating all sample time points that were implemented by the industrial partner.

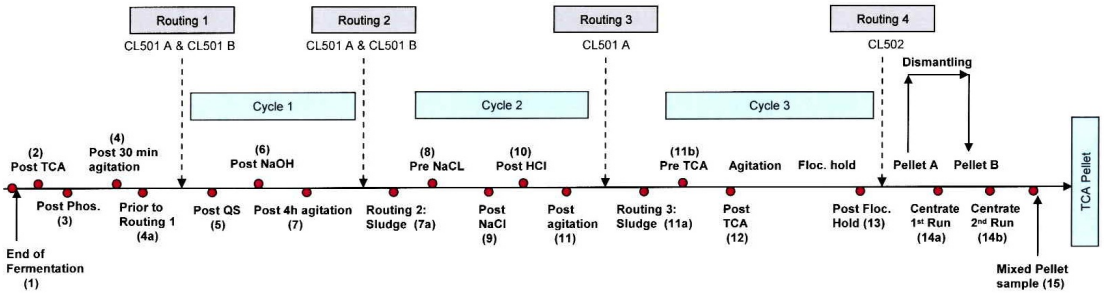

Fig. 2.3: Overview of all sample time points throughout the whole primary recovery process

Each red dot symbolizes a sample taken at this stage of the recovery process. Later on in chapter 4 and 5 all samples are only named after their number, related to the sample time point taken from the process. Samples 14a and 14b are both from the supernatant of Routing 4 that goes into the waste stream. For this last routing two separate runs are required in order to process all material from cycle 3. Hence, sample 14a is the supernatant from the first run and sample 14b the one from the second run. Sample 15 contains a mixed sample of both pellets received from run 1 and 2 of routing 4 together.

Samples 4a, 7a, 11a and 11b were added to the sampling plan at later stage. Accordingly the first two batches 1-AVAF-211 and 1-AVAF-212 do not contain these samples and the whole sampling system consists only of 16 samples. From batch 2-AVAF-201 all samples were included into the sampling and one batch consists of 20 samples. Table 2.2 gives an overview about the different batches performed on sight by the industrial partner during this project and indicates which of these batches was analysed.

Batches 2-AVAF-202, -203 and -205 were not analysed, since problems occurred during the shipment from the manufacturing site to the university. Samples taken at the manufacturing site were stored at -80°C immediately after sampling and shipped on dry ice to the university, where they were stored again at -80°C prior to analysis.

Tab. 2.2: Different batches of the primary protein recovery process for interferon-α-2b

batch-number	year	no. of samples taken	missing samples	analysed
1-AVAF-211	2011	16	4a, 7a, 11a & b	yes
1-AVAF-212	2011	16	4a, 7a, 11a & b	yes
2-AVAF-201	2012	19	2	yes
2-AVAF-202	2012	20	none	no
2-AVAF-203	2012	20	none	no
2-AVAF-204	2012	0	all	no
2-AVAF-205	2012	18	2 &8	no
2-AVAF-206	2012	20	none	yes
2-AVAF-207	2012	20	none	yes
2-AVAF-208	2012	20	none	yes
2-AVAF-209	2012	18	5 & 6	yes
2-AVAF-210	2012	20	none	yes
2-AVAF-211	2012	20	none	yes
2-AVAF-212	2012	20	none	yes

2.6 Conclusion

This PhD-thesis focuses on the optimization of the primary protein recovery process for interferon-α-2b to increase the IFN recovery and the overall process yield. The work is divided into three work packages, whereas package one and two covers the setup of several analytical techniques in order to characterize the primary protein recovery process of the manufacturing site from the industrial partner (compare chapter 3). Process samples taken from the manufacturing process (see section 2.5) are analysed for total protein, total interferon-α-2b and interferon-isoforms in order to set up mass balances and to identify critical process steps (compare chapter 4, chapter 5 and chapter 6).

Work package three focuses on the optimization of the identified critical process steps. A dilution study is performed to enhance protein solubility (chapter 7) and different detergents are tested for their potential to increase interferon-α-2b solubility (chapter 8). In a last step, methods are developed to remove detergents from process samples (chapter 9) and the impact of remaining detergents on the first down-stream step is investigated (chapter 10).

Chapter 3

Simulation of the Protein Recovery Process in a Small Scale Model

3.1 Introduction

A priority step for process optimization is the understanding of each step during the process. Therefore it is necessary to characterize the process with respect to proteins and the effect of each process step on the different proteins, i.e. host cell protein and drug substance.

To detect the total amount of protein throughout the protein recovery process, it is necessary to find a protein assay that is able to quantitatively measure proteins at all stages of the recovery process without interference by the fermentation broth components.

The industrial protein recovery process from the industrial partner will be analysed and characterized during this study. During this process strong acids, bases and salts are added, which can interfere with any protein assay and falsify the results. Therefore it is necessary to examine the influence of these components during the process on the selected protein methods to find the method which shows the least interference.

To examine the influence of different components on several protein assays, a simulation of a small-scale model of the protein recovery process is performed. For the simulation of the recovery process two proteins with known concentration dissolved in water or fermentation medium are used to perform the protein recovery process instead of using the end of fermentation broth

(EOF).

Besides the selection of an adequate protein assay, the simulation of the recovery process will also provide information about the characteristics of the process itself. Due to changes of certain parameters during the recovery process simulation (protein, medium, volume) several different informations should be gained during this simulation study.

3.1.1 Characteristics of BSA and IFN

Bovine Serum Albumin (BSA) is a commonly studied protein and is widely used as a reference standard in several protein assays. It has a molecular weight of 66.5kDa and consists of 607 amino acids [26]. The secondary structure of BSA is composed of 67% α-helix, but it can undergo modifications dependent on pH, temperature and various kinds of denaturants [27]. BSA is an universally accepted reference protein for total protein quantification, since its amino acid composition represents an average over most host cell proteins [10]. Table 3.1 gives an overview of the amino acid composition of Bovine Serum Albumin.

Tab. 3.1: Amino Acid Composition of BSA [10]

Ala 48	Cys 35	Asp 41	Glu 58
Phe 30	Gly 17	His 16	Ile 15
Lys 60	Leu 65	Met 5	Asn 14
Pro 28	Gln 21	**Arg 26**	Ser 32
Thr 34	Val 38	Trp 3	Tyr 21

Arginine (Arg) is one of the amino acids that is detected during the Bradford protein assay, in order to quantify the total amount of protein. Since BSA has a total of 607 amino acids, the fraction of arginine represents 4.28%.

Interferon-α-2b on the other hand is a much smaller protein with only 165 amino acids and a molecular weight of 19.265kDa [6]. Its amino acid composition is shown in table 3.2.

Tab. 3.2: Amino Acid Composition of IFN-α-2b [6]

Ala 8	Cys 4	Asp 8	Glu 14
Phe 10	Gly 5	His 3	Ile 8
Lys 10	Leu 21	Met 5	Asn 4
Pro 5	Gln 12	**Arg 10**	Ser 14
Thr 10	Val 7	Trp 2	Tyr 5

The fraction of arginine present in interferon-α-2b equals to 6.06%. Hence, the arginine fraction is 1.78% higher in IFN-α-2b than it is in BSA which can have a significant impact during the total protein quantification using protein assays as the Bradford method.

3.1.2 Small-scale models

Small scale models are commonly used in biotechnology to simulate a culture process at smaller scale [28]. Process characterization studies are mainly performed with small scale models, especially during cell culture to understand culture performance for culture growth and productivity and final product quality [29].

The most common scaling criteria for small-scale models of fermentations is a constant specific power input (P/V) [30] and a constant VVM [31]. But also other scale down strategies are described in literature for example impeller tip speed (V_{tip}), shear rate (γ) and specific impeller pumping rate (Q_S) [32].

In this study a small scale model is used to simulate the protein recovery process which includes 4 centrifugation steps. Between the centrifugation steps different acids, bases and salts are added at certain flow-rates to the process in order to set the pH or conductivity. The amount of the acids, bases or salts were either scaled-down in a linear relation to the working volume or they were dependant on the pH/ conductivity setting.

The biggest challenge in the scale-down model of the protein recovery process is the simulation of the four centrifugation steps which will be discussed in the following section.

Centrifugation

The centrifugation step is the most challenging part during the scale-up or scale-down of a recovery process. At manufacturing scale continuous centrifuges are used to separate cells and particles from the fermentation broth. For the performance of a continuous centrifuge several litres of fermentation broth are required. At lab-scale the fermentation broth volume is often less than a litre and therefore discontinuous centrifuges are used. The efficiency of clarification is highly dependent on the capacity of the separator or centrifuge [33]. The smaller the capacity of the centrifuge the higher is the efficiency. Hence, separation of precipitants from the supernatant performed with small volumes in a discontinuous centrifuge is more efficient than at a large scale in a continuous centrifuges. The next paragraphs explains the differences in performance between continuous and discontinuous centrifuges.

Most continuous centrifuges at large scale are either separator or decanter. Both systems function according to a similar mechanism. Figure 3.1 shows the mechanism from the inside of a separator.

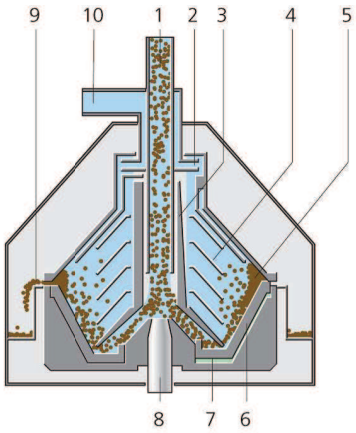

Fig. 3.1: Mechanism of continuous centrifuge; Separator from Westfalia: 1: Product feed, 2: Centripetal pump, 3: Distributor, 4: Disc stack, 5: Solids holding space, 6: Sliding piston, 7: Closing chamber, 8: Spindle, drive, 9: Solids ejection port, 10: Discharge, clarified phase [2]

The fermentation broth enters through the product feed (1). In the rising channel the product is split up over a large number of disk inter-spaces (4). Due to higher density the solids stream against the conical discs and slide to the solid holding space (5). The sliding piston (6) opens and closes hydraulically and provides a gap for discharging the accumulated solids. The clarified liquid streams to a central discharged channel towards the centripetal pump (2) and is discharged under pressure through the discharged line (10). A large clarification area compared to sedimen-

tation by gravity is realized by the conical discs and high g-forces and allows to process large amount of volumes [2].
Discontinuous centrifuges are used on a daily basis in laboratories as bench-top or floor-centrifuges. The separation occurs through sedimentation by gravity and centrifugal force (g-force) and is based upon behaviour of particles in the applied centrifugal field. Discontinuous centrifugation can be separated into two main types: differential centrifugation and density gradient centrifugation. Density gradient separation can either separate per size (rate zonal separation) or per density (Isopycnic separation). Differential centrifugation is based on the size and mass of particles and separates a solution into pellet and supernatant [34].
All centrifugation steps performed during the small scale model of the primary recovery process used differential centrifugation.

3.1.3 Goals/Aims

The simulation of the protein recovery process in a small scale model is set up and performed in order to achieve the following aims:

- selection of protein assay to determine total protein amount in recovery process samples throughout the entire process
- impact of reduced process volume and variable protein concentration on the recovery process
- specificity of the recovery process for certain proteins

In order to characterize the primary recovery process a protein assay needs to be put in place that allows the determination of total protein in process samples without interfering with different components added throughout the fermentation and recovery process. Using samples from the small scale model of the recovery process, the protein assay with the best criteria can be selected. The other information regarding protein specificity or impact of variable protein concentration will provide fundamental information in order to proceed with process characterization at manufacturing scale.

3.2 Materials and Methods

3.2.1 List of Materials

Tab. 3.3: Materials and suppliers

materials	supplier
Total protein kit, Micro Lowry	Sigma Aldrich
Pierce® BCA Protein Assay Kit	Thermo Scientific
Bradford Reagent	Sigma Aldrich
Bovine serum albumin (BSA)	Sigma Aldrich
interferon-α-2b drug substance	industrial partner
fermentation medium	industrial partner
Trichloroacetic acid solution (TCA)	Sigma Aldrich
Phosphoric Acid (H_3PO_4)	Sigma Aldrich
NaOH	Merck KGaA
Sodium Chloride (NaCl)	VWR
HCl	Fisher Scientific

3.2.2 Protein assays

Protein assays are used to determine the amount of protein in fermentation or culture samples and to follow protein production throughout a process. The most sensitive and accurate method would be to perform acid hydrolysis followed by amino acid analysis. Since this method is very time consuming and challenging to perform, several other protein assays are available which are easier and faster in performance.

Lowry-method

The Lowry method is a protein assay based on the Biuret reaction and the Folin - Ciocalteau reaction. In the Biuret reaction the peptide bonds of the protein react with copper under alkaline conditions to produce Cu^{2+}. This reacts in the second reaction with the Folin reagent to oxidize

aromatic amino acids [35]. Therefor the sensitivity of the Lowry method is dependent on the amino acid composition of the protein, especially the aromatic amino acids tryptophan and tyrosine. The reaction results in a strong blue or purple colour and can be detected at a suitable wavelength between 500 and 800 nm [11].

For the performance of the Lowry assay the different steps listed in table 3.4 have to be followed.

Tab. 3.4: Performance of Lowry method [11]

component	required volume [mL]
standard/ sample	0.500
Lowry reagent (Na_2CO_3, $CuSO_4$, NaOH and $KNaC_4H_4O_6-4H_2O$)	0.500
incubate for 20 min at RT	
Folin Reagent ($C_{10}H_5NaO_5S$)	0.250
incubate for 30 min at RT measure absorbance between 550 and 750 nm	

BCA-method

The BCA method is as well as the Lowry method based on the Biuret reaction followed by the detection of the formed cuprous cations (Cu^{1+}). The colorimetric detection of Cu^{1+} uses bicinchoninic acid (BCA). Two molecules of BCA chelate with one molecule of Cu^{1+} to form a water soluble complex with an absorbance maximum at 562nm [12].

As well as the Lowry method, the BCA method is sensitive to the amino acid composition of the protein. The BCA method detects the presence of the amino acids Tyrosine and Tryptophan, as well as Lowry, but it is also depending on the macromolecular structure of the protein and the number of peptide bonds.

For the performance of the BCA assay the different steps listed in table 3.5 have to be followed.

Tab. 3.5: Performance of the BCA method [12]

component	required volume [mL]
standard/ sample	0.050
BCA reagent (Na_2CO_3, $NaHCO_3$, BCA, $Na_2C_4H_4O_6$ and $CuCO_4$)	1.000
incubate for 30 min at 37°C	
cool down to RT	
measure absorbance at 562nm within 10 min	

Bradford-method

The Bradford method is the most popular dye-based protein assay to quantify proteins in solution. It is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution with an absorbance maxima at 595nm [35]. The anionic blue dye binds most readily to the amino acids Arginine, Histidine and Lysine. This leads to variances in the response of the assay to different proteins which have different amino acid distributions [35]. This has to be kept in mind by selecting an adequate protein standard.

The advantage of the Bradford method compared to Lowry and BCA assay is that it is simpler, faster and more sensitive. The Bradford method shows also less interference with common reagent and non-protein components of biological samples [35].

For the performance of the Bradford assay the different steps listed in table 3.6 have to be followed.

Tab. 3.6: Performance of the Bradford method [13]

component	required volume [mL]
standard/ sample	0.050
Bradford reagent (Brilliant Blue G, Phosphoric Acid and Methanol)	1.500
incubate for 10 min at RT	
measure absorbance at 595nm within 20 min	

3.2.3 Simulation of Protein Recovery Process

The protein recovery process is based on the primary recovery process from the industrial partner to recover proteins, especially interferon, from the fermentation broth. The simulation was performed with a small scale model of the original process. The goal of this simulation is to identify the most suitable protein assay to detect the amount of protein throughout the whole process and to identify first characteristics of this primary recovery, to get a better understanding of the whole process.

3.2.3.1 Down-Scale model

The primary recovery process performed at manufacturing scale has the aim to recover interferon-α-2b and its isoforms (-2, 3 and 4) from the fermentation broth. Another aim is to reduce the volume and to remove cell compartments as well as nucleic acids from the fermentation broth before it goes into the purification process.

The elementary recovery process starts with a volume of up to 40'000L which is reduced to 8'000L. These volumes were scaled down to a starting volume of 40mL and 500mL which were reduced to 20mL and 150mL during the process.

To work as closely as possible to the original recovery process most of the parameters were kept the same, only some concentrations of added base or acids had to be reduced at small scale. Besides reduced acid and base concentrations the biggest difference between the original process and the small scale model are the centrifugation steps. At large scale a continuous centrifuge has been used, whereas in the small scale model a discontinuous centrifuge was used to separate the precipitations from the supernatant. The separation of a discontinuous centrifuge works more efficiently than a continuous one which makes it challenging to achieve similar process conditions in these steps (compare section 3.1.2).

The different volumes and acid/ base concentrations used for the elementary process, as well as for the small scale models are listed in table 3.7.

Tab. 3.7: Overview of the original primary recovery process and two small scale versions

process step	original process		40mL small scale model		500mL small scale model	
	process volume [L]	acid/ base concentration [M/%]	process volume [mL]	acid/ base concentration [M]	process volume [mL]	acid/ base concentration [M]
Acid Kill:						
EOF / start volume	32'500		40		500	
TCA addition	1'020	50%	1.20	50%	15.0	50%
set pH to 2.0	1'300	75% H_3PO_4	0.0		0.0	
water QS + agitation	QS 40'000		0.0		0.0	
centrifugation step 1:						
NaOH addition:						
collected pellet	2'600 kg		0.78 g		12.8 g	
redissolve pellet	9'500	water	20.0	water	150.0	water
set pH to 7.0	90.0	19.4M NaOH	1.0	0.1M NaOH	2.0	1.0M NaOH
agitation for 4 h						
centrifugation step 2:						
Salting Out:						
collected supernatant	7'400		19.7		160.0	
set conductivity to 20mS/cm	350.0	2M NaCl	8.5	1M NaCl	43.0	1M NaCl
set pH to 4.5	120.0	4M HCl	0.10	0.2M HCl	1.4	0.2M HCl
agitation for 1 h						
centrifugation step 3:						
TCA-pellet:						
collected supernatant	7'000		25.0		190.0	
add TCA	460.0	50%	1.60	50%	12.50	50%
hold for 10 - 24 h						
centrifugation step 4:						
collect TCA-pellet:	75.0 kg		0.70 g		14.50 g	

At large scale a continuous centrifuge from Westfalia CRA-160 at 5,800 rpm with a flow rate of 0.9 - 5.4 m3/h was used during centrifugation step 1,2 and 3. Step 4 involves a continuous separator from Westfalia BKB-45, which is run at 5'800 rpm, inflow rate of 0.9 - 5.4m^3/h and a back-pressure of 3 bar. For the down scale models a discontinuous bench-top centrifuge was used with the following parameters: 40ml volume was centrifuged at 7500rpm (8175 g) in a fixed bucket, at 4°C for 30 minutes, and 500mL volume was centrifuged at 3500 rpm (1780 g) in a swing out rotor, at 4°C for 30 minutes.

To identify different characteristics of the protein recovery process, certain parameters were changed in the scale-down models. First of all different starting volumes of the scale-down model were used to identify the effect of reduced volume to the process, 40mL versus 500mL (see also table 3.1.2). Secondly different protein concentrations were tested at the beginning of the small scale model, to identify the impact of variances of protein production during fermentation on the recovery process. The protein concentration at the beginning of the small scale model varied between 0.07 g/L to 2.0 g/L. The third characteristic to be analysed with these small scale models was the protein specificity of the recovery process. Therefore different proteins were used to perform the down-scale model, BSA, IFN and BSA + IFN together.

3.3 Results and Discussion

3.3.1 Protein assays

Three different protein assays were performed and analysed after the following criteria: sensitivity, accuracy, pH-dependency, interference of media components, time factor and the handling. All experiments were performed using BSA (Bovine serum albumin) as the reference standard.
The different advantages and disadvantages of each method are listed in the table 3.8 below.

Tab. 3.8: Advantages and disadvantages of three protein assays

		detection range					
method	sample volume [μL]	min mg/mL	max mg/mL	standard deviation	pH-dependency	ferm. media dependency	process time [h]
Lowry	500	0.05	0.4	$\pm$2.4%	-	-	2h00
BCA	50	0.03	0.5	$\pm$0.5%	none	yes	1h30
Bradford	50	0.05	1.0	$\pm$0.4%	none	none	1h00

The Lowry assay was performed following the instructions listed in table 3.4 and described in section 3.2.2. All experiments were performed in DI-water and BSA was used as the reference standard.
Looking at the different criteria for the Lowry assay, it can be seen that this assay is not favourable compared to other assays. The required sample amount is with 500 μL very high, which means that a lot of standard material would be required to measure a reference standard in triplicate with each set of samples to be analysed. Although the detection range of 0.05 - 0.4mg/mL would be sufficient, BCA and Bradford do provide an even bigger detection range, with a lower standard deviation than the Lowry-assay. The last disadvantage of Lowry over the other assays is the long process time, see table 3.8. The average required time for Lowry is twice as long as for Bradford. Since the Lowry method showed already disadvantages in the first 4 criteria compared to BCA and Bradford, the last two criteria were not studied for Lowry.

The BCA-assay was performed following the instructions listed in table 3.5 and described in section 3.2.2. All experiments were performed in DI-water besides the study on interferences with fermentation media. These experiments were performed in fermentation media.
In some of the studied criteria the BCA-assay showed advantages over the other tested assays.

Hence, the BCA assay required only a tenth of sample volume than the Lowry assay (50 μL). The biggest advantage of the BCA-assay is the accurate detection of very low protein concentration, down to 0.03mg/mL with a standard deviation of only 0.5%, see table 3.8. There was no pH dependency detectable, which is important for analysing samples throughout a recovery process where different pH-conditions are present. Unfortunately the BCA-assay showed a high interference with the pure fermentation media. To avoid these interferences high dilutions of the media and samples would be necessary, which would interfere with the accuracy of this assay and the limit of detection. Another big disadvantage of the BCA-assay is the required process time and the handling of the assay performance itself. With a process time of 1.5 hours the BCA assay is in the middle of the three tested assays, but during the performance an incubation time at 37°C is necessary. This gives an additional factor in which variations can occur during assay performance and increases the risk of appearance of handling mistakes.

The Bradford-assay was performed following the instructions listed in table 3.6 and described in section 3.2.2. All experiments were performed in DI-water besides the study on interferences with fermentation media. These experiments were performed in fermentation media.
The Bradford assay shows, in most of the tested criteria, advantages over the other two protein assays, see table 3.8. It requires only a small amount of sample which is important when the available amount of standard material is limited. The working range, in which an accurate linear relation exists between the detected absorbance and the protein amount, is very wide. This is especially an advantage when the analysed samples have big variations in their protein amount. The Bradford assay showed also no pH dependency over the whole range from pH 1.0 to pH 12 and the interference of media components was very low. A more detailed discussion about the pH dependency can be found at later stage of this section.

A big advantage of the Bradford method over the other two protein assays is the short process time. The whole assay can be performed in less than an hour and it is easy to handle. No preparation of reagents is required, the Bradford-reagent can be bought as a ready to use reagent without any further preparations required.

Three protein assays listed in table 3.8 were compared regarding the aim to find an assay which gives accurate protein predictions over a wide range, shows no interference with different pH-values and media components and has a short process time with easy handling. The comparison

showed that the Bradford assay is the most suitable method to perform accurate protein measurements in solution over a wide range with a minimum expenditure of time.

To determine the pH dependency of the Bradford-assay two BSA stock solutions at 0.3mg/mL were prepared, one diluted with DI-water and the other one diluted with PBS-buffer (1X). Both BSA stock solutions were divided into 9 fractions. Each fraction was set to a certain pH-unit between 1.0 - 12.0 using either NaOH or HCl. The Bradford-assay was performed and the absorbance values at 595nm were detected and plotted versus the pH values as shown in figure 3.2. For the BSA-sample in DI-water, DI -water was used to deduct the background noise from the measurements. On the other hand PBS-buffer was used to deduct the background noise from the BSA-samples diluted in PBS-buffer.

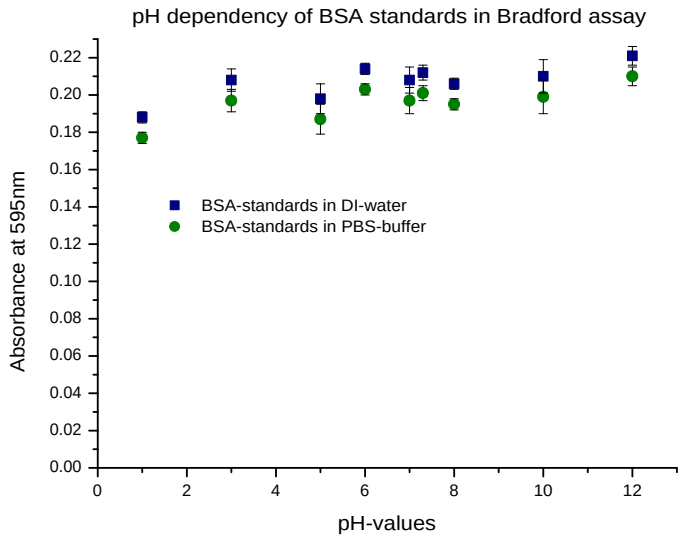

Fig. 3.2: pH dependency of BSA standards during Bradford assay

Looking at the absorbance values at 595nm for 0.3mg/mL BSA in DI-water compared to the one in PBS-buffer, no significant difference can be detected between both buffers. This shows that PBS-buffer does not interfere with the Bradford-assay.

Some slight variations can be detected between the absorbance of 0.3mg/mL BSA-sample at different pH-values. These small variations can be explained by dilutions, which occurred during the addition of NaOH or HCl while setting the pH.

Overall no significant pH-dependency on the Bradford assay was detectable. This is an important

result, since the pH varies a lot during the recovery process. Knowing that the pH does not have any impact on the assay avoids further sample preparations for pH-adjustment, before analysing recovery process samples from different stages of the process.

3.3.1.1 Protein Reference Standards

Protein assays do not detect the whole protein, but they detect certain amino acids or peptide bonds between the amino acids as described in section 3.2.2. Therefore it can be expected that proteins with different size and different amino acid distributions will have differences in their limit of detection as well as in the absorbance rate.

Two proteins were tested in the following experiment in two different buffers to study the impact of their size and amino acid distribution on the Bradford assay. Interferon-α-2b and BSA were analysed with the Bradford assay at different concentrations in DI-water and in fermentation media.

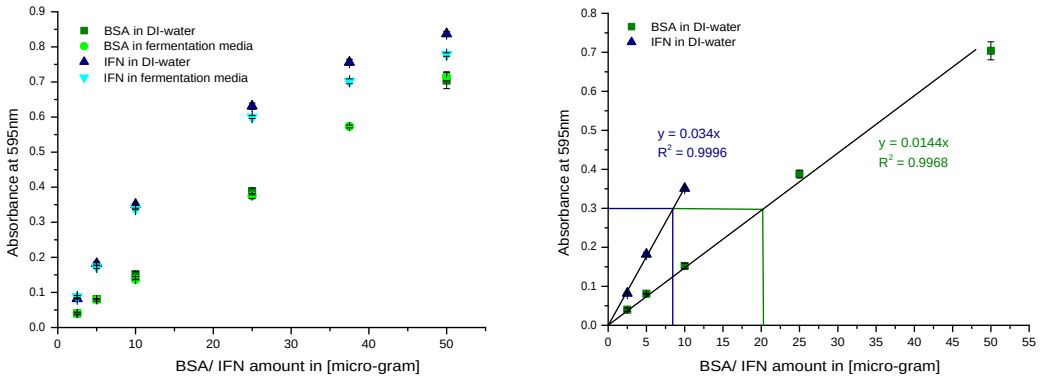

Fig. 3.3: The significance of using the correct protein standard for each Bradford-assay

The graph on the left of figure 3.3 shows the absorbance at 595nm of IFN-α-2b and BSA in DI-water and fermentation media over the amount of protein. No significant difference can be seen between the absorbance of IFN-α-2b in DI-water and fermentation media nor for BSA in DI-water and fermentation media. In all four cases the appropriate blank was used to subtract the background noise. This shows that the influence of media components on the detection of BSA and IFN-α-2b with the Bradford assay is not significant and can be negligible. However, comparing pure fermentation media with DI-water showed, that the fermentation medium already

contains 0.06g/L of total protein due to the presence of yeast extracts in the media. This shows the importance of selecting the correct blank value to deduct the present background noise.
A distinct difference can be seen in the absorbance at 595nm between IFN-α-2b and BSA for the same protein concentrations. The absorbance of IFN-α-2b is always higher compared to BSA. Looking at the right graph of figure 3.3 shows that the detection range of the Bradford assay differs for IFN-α-2b and BSA. BSA can be detected in an accurate linear range between 0.0 - 50.0μg (0.05 - 1.0g/L) with a R^2=0.9968. On the other hand IFN-α-2b can only be detected in an accurate linear range between 0.0 - 10.0 μg (0.05 - 0.20g/L) with a R^2=0.9996. Therefore the limit of detection is five times higher for BSA than for IFN-α-2b and IFN-α-2b has a higher absorbance than BSA. As mentioned in the paragraph above the differences in the absorbance and the limit of detection can have two reasons. First, the size of the proteins. BSA is composed of 607 amino acids, whereas IFN-α-2b comprises only 165 amino acids (see section 3.1.1). Therefore BSA is 3.6-fold bigger than IFN-α-2b, meaning for each single protein there are more peptide bonds and amino acids present to react with the Bradford reagent. Hence, BSA needs to be more concentrated to reach a saturation point with the Bradford reagent compared to a smaller protein. The second reason is the distribution of the amino acids in the protein. Arginine is the primary reactant of the Bradford reagent (compare section 3.2.2). The percental amount of Arginine in IFN-α-2b is 1.78% higher than in BSA (see 3.1.1), meaning one single protein of IFN-α-2b can interact with more Bradford reagent than one BSA-protein. Summarizing, IFN-α-2b is 3.6-fold smaller than BSA but contains 1.42x more Arginine, hence, the limit of detection of IFN-α-2b for Bradford assay is 5-fold lower than of BSA. It needs to be kept in mind that this calculation only looks at the naked amino acid chain. However, it does not take the tertiary structure of both proteins into account where some of the Arginine aminoc acids might be hidden in the inside of the protein.
Hence, the right graph in figure 3.3 gives an example that illustrate the importance of selecting the correct reference standard. Having an unknown process sample with the measured absorbance at 595nm of 0.30 abs: using BSA as the reference standard but IFN-α-2b is present in the process sample would result in an overestimation of the protein concentration. However, using IFN as the reference standard when BSA is present in the process sample would result in an underestimation of the protein.

After showing the importance of selecting the correct reference standard to determine the protein

amount in an unknown sample, the next question would be which standard to choose if you have a mixture of proteins in your process sample. Therefore BSA and IFN-α-2b were analysed together in a mixture with different portions of IFN-α-2b, see table 3.9 below.

Tab. 3.9: Analysing a mixed protein sample using Bradford assay

Experiment	theoretical protein concentration [g/L]	IFN fraction [%]	analysed protein concentration [g/L]]
1	total: **0.60**		total: **0.791**
	IFN: **0.30**	50.0	IFN: **0.373**
2	total: **0.60**		total: **0.738**
	IFN: **0.20**	33.3	IFN: **0.348**
3	total: **0.60**		total: **0.681**
	IFN: **0.10**	16.7	IFN: **0.322**

The second column of table 3.9 shows the theoretical concentration of total protein (BSA + IFN-α-2b) and pure IFN-α-2b for each experiment. The fourth column lists the detected protein concentrations using the Bradford assay. For total protein determination, BSA was used as a reference standard and for IFN-α-2b determination, IFN-α-2b was used as the reference standard.

Table 3.9 shows, that using IFN-α-2b as the reference standard to determine the total protein concentration in a mixture of proteins results in underestimation of the concentration. This confirms the observations in the section above. However, IFN-α-2b can be used as a reference standard to determine the IFN-α-2b concentration in a protein mixture, as long as the IFNα-2b portion represent more than 50% of total protein mixture.

Using BSA as a reference standard to determine the total protein concentration in a mixture of proteins, the total protein amount will be overestimated, especially if non-host cell proteins are present in the mixture. IFN-α-2b does not belong to host cell proteins of *E.coli* and is different in its amino acid distribution. However, if the IFN-α-2b portion is below 17% of the mixture, the prediction of total protein concentration becomes accurate using BSA as the reference standard.

Concluding, for a heterogeneous sample containing different kinds of protein it is more accurate to use BSA as the reference standard, as long as proteins with high Arginine levels, such as IFN, are below 20%. If the IFN-α-2b fraction represents more than half of the total amount of protein, it is more precise to use IFN-α-2b as reference to determine the IFN-α-2b concentration in a mixture but not for total protein determination.

3.3.2 Simulation of Recovery Process

3.3.2.1 Recovery Process buffer

Simulation of the recovery process was performed in a small scale model as listed in table (3.7). The total protein of 2.0 g/L at the beginning, was determined at different stages throughout the process using the Bradford-assay and BSA as a reference standard.

The impact of different components added during the recovery process (TCA, NaOH, NaCl or HCl) on the Bradford assay was studied. It showed that the addition of these components does not have a significant impact on the Bradford assay, as long as the simulation is performed in DI-water. The Blank-values vary between 0.424 - 0.458. These variations (Δ 0.034) are acceptable, since similar variations were observed due to small differences in the DI-water itself and due to different batches of Bradford reagent.

Performing the simulation in fermentation media showed that the blank value of pure fermentation media does change significantly throughout the process. The Blank values of fermentation media varied between 0.534 - 0.468 through the recovery process simulation. These variations (Δ 0.066) are twice as high as observed with DI-water and need to be considered for protein analysis. Therefore a negative control of pure fermentation media but without any additional protein was performed for the whole process simulation with all experiments using fermentation media (data not shown).

Figure 3.4 shows the recovered total protein amount in % throughout the recovery process simulation using BSA at 2.0g/L in DI-water and fermentation media at 40mL. All protein concentrations were detected via Bradford assay using BSA as a reference standard and DI-water or fermentation media as blank.

Figure 3.4 shows no significant difference between the simulated recovery of BSA in DI-water and fermentation media. The main difference between both simulations were the necessary addition of H_3PO_4 during the acid kill using fermentation media to set the pH down to 2.0. This addition was not necessary using DI-water as a buffer, since the pH was already below 2.0 after adding TCA. This indicates that the fermentation media gives a higher buffered environment compared to DI-water. However, the overall recovery of BSA throughout the recovery process was identical in DI-water and fermentation media, with up to 80%. The biggest loss of BSA was observed between

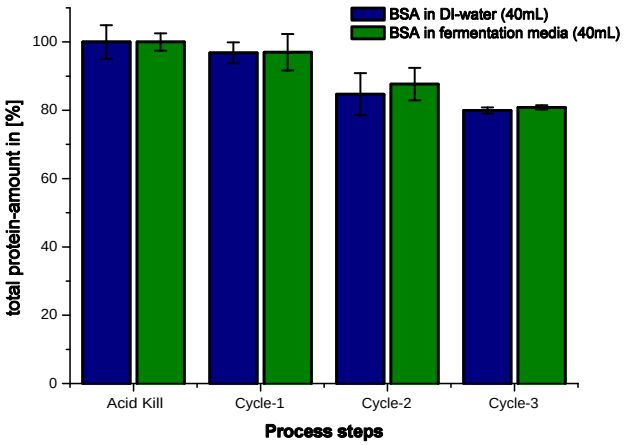

Fig. 3.4: Buffer dependency on protein recovery throughout recovery process

cycle 1 and 2 of around 18%, compare figure 3.4. The detected reduction in total protein in step 3 of the recovery process could not be confirmed with the protein mass balances. The protein amount lost during step 3 could not be detected in the sludge recovered from centrifugation step 3. This indicated that the protein either degrades due to the addition of NaCl or HCL or this reduction is due to handling errors.

No loss of total protein was detected between step 1 and 2 neither in DI-water nor in fermentation media. Addition of TCA and NaOH do not have an impact on the protein recovery. It has to be pointed out that the concentration of NaOH used in this small scale process is much lower than the one used at production scale (0.1M versus 19.4M). The much higher NaOH concentration can have a different impact on the protein recovery especially in the protein conformation and function.

Concluding, performing the recovery process simulation with BSA in DI-water or fermentation media does not show any significant differences. Both simulations showed the main reduction of BSA during step 3 while adding NaCl and HCl. The loss of BSA could not be confirmed by mass balances. The reduced amount of BSA was not found in the sludge recovered at this step. This indicated either protein degradation, meaning the protein was not lost but not detectable via Bradford assay. Alternatively there may be handling mistakes, to reach the conductivity set point of 20mS/cm a high amount of NaCl-solution was necessary to add. The increment of the volume could falsify the results.

3.3.2.2 Volume effect

The simulation of the recovery process at 40mL and 500mL in DI-water was performed as listed in the table 3.7 with a starting concentration of 2.0g/L for both conditions. For both simulations the total protein amounts were analysed via Bradford assay using BSA as a reference standard and DI-water as the blank.

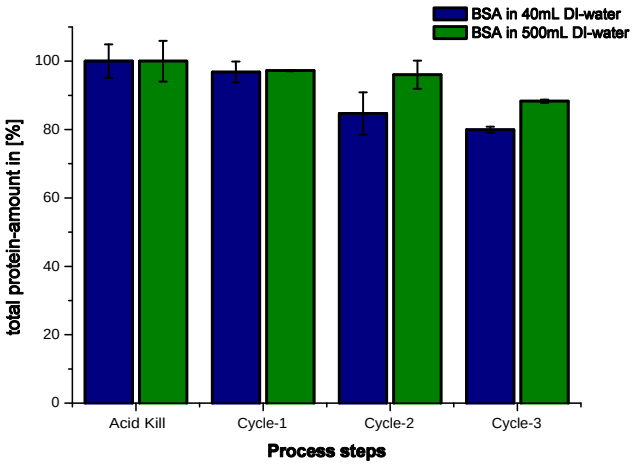

Fig. 3.5: Volume dependency on protein recovery throughout recovery process

Figure 3.5 shows the simulation of the recovery process at 40mL compared to the simulation at 500mL. It can be seen that the recovery is dependent on the volume. The protein loss of around 20% detected with the small scale model at 40mL could not be confirmed with the small scale model at 500mL. This indicates that the detected protein loss at 40mL was only due to handling errors or imprecise determinations of the volume in cycle 2, which led to the reduced protein amount. Performing the simulation at a larger scale of 500mL showed, that no significant protein loss occurred during the simulation. The overall loss of BSA during the whole recovery process was less than 10%. Due to changes in the blank-value, Bradford reagent and dilution errors the overall acceptable deviation of this assay is $\pm$5.0%. This means that the detected protein loss of 10% is in between the acceptance criteria of variations and is therefore not significant.

Concluding, simulation at higher scale showed that the detected loss of total protein in the previous section was due to handling mistakes and imprecise determinations of the volume. At higher volume no significant protein loss was detectable throughout the whole process for BSA.

3.3.2.3 Protein starting concentration

Simulation of the recovery process was performed with different protein starting concentrations (2.0g/L versus 1.07g/L) to study the impact of the starting concentration on the recovery process. Similar changes could occur due to different protein production amounts during the fermentation process.

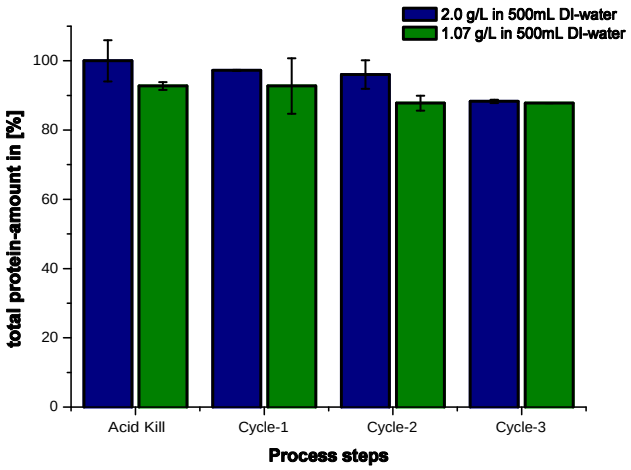

Fig. 3.6: Protein starting concentration dependency on protein recovery throughout recovery process

The results above in figure 3.6 show that different protein starting concentrations at the beginning of the recovery process have no significant impact on the protein recovery throughout the process. The slight changes between the simulation at 2.0g/L compared with the simulation containing only 1.07g/L at different steps of the process are less than 5% and therefore in between the acceptable deviations of the Bradford assay. Therefore it can be said that the protein starting concentration has no influence on the efficiency of the protein recovery process.

3.3.2.4 Protein specificity

For this experiment three different pools of proteins were prepared to perform the simulation of the recovery process. The first one contained 2.0 mg/mL of BSA diluted in DI-water. The second pool consisted of 1.0mg/mL BSA + 0.07mg/mL IFN-α-2b. A similar proportion can be expected at the end of the fermentation process before it enters the recovery process. The last pool only consists of 0.07mg/mL IFN-α-2b diluted in DI-water.

All samples were analysed with the Bradford assay using DI-water as a blank throughout the recovery process. For pool 1 and 2 BSA was used as a reference standard, since in pool 2 IFN-α-2b represents less than 16.7%. For pool 3 IFN-α-2b was used as a reference standard, since no other protein was present.

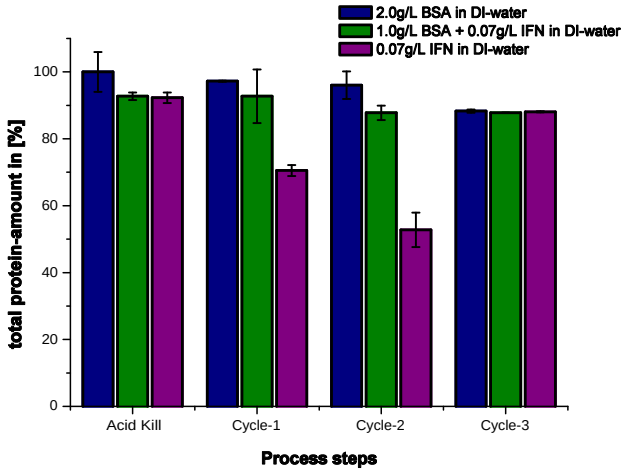

Fig. 3.7: Different protein dependency on protein recovery throughout recovery process

Comparing only the first two pools with each other in figure 3.7 it can be seen that there are no significant differences between the recovery of BSA at 2.0mg/mL and BSA+IFN at 1.07mg/mL, as already discussed in the previous section. Comparing these results with the recovery of pure IFN at 0.07mg/mL significant changes in the protein recovery can be observed in the first three steps, leading to the conclusion that the protein itself and its concentration do have an impact on the protein recovery. However, looking more closely into these results it can be seen that the reduction in the amount of IFN-α-2b between the acid kill and cycle 1 is only 7.55mg and between cycle 1 and 2 only 4.55mg. Due to previous experiments, variations of $\pm$5.0mg of proteins are very likely due to changes in the blank values as well as dilution errors. This means that the obviously significant reduction in IFN-α-2b during the first three steps of the recovery process is not significant but is caused by variations in the Bradford assay. This would also explain why the IFN-α-2b amount in cycle 3 increased again to a final protein recovery of 90%. All observations lead to the conclusion that the IFN-α-2b remained constant throughout the recovery process and that the reduced IFN amounts in cycle 1 and 2 were due to errors and imprecise measurements in the Bradford assay.

Comparing all three simulations with each other it can be said that the recovery process is not specific for any particular protein. For all three combinations the process showed the same efficiency. It can also been said that the protein concentration has no impact on the efficiency of the process. Between 0.07mg/mL and 2.0mg/mL the recovery of proteins resulted in comparable results. However, it can be said that at small volumes and low protein concentrations the accuracy of the small scale model is reduced and can lead to incorrect results.

3.4 Conclusion

The performance of the simulation of the primary protein recovery process had several aims to accomplish. First goal was the selection of a protein assay to follow the amount of total protein throughout the recovery process. The Bradford assay showed the highest potential for the quantification of total protein in process samples. It is the most accurate assay which shows no or only low interference to different media components and no pH dependency. Therefore the Bradford assay was chosen to quantify total protein in recovery process samples.

In order for the selection of the most suitable reference standard for the Bradford assay, BSA was selected as long as the other proteins with a high Arginine level, as interferon-α-2b, are less than 20% of the protein mixture. If these proteins with high Arginine levels represents more than 50% of the protein mixture one of those proteins need to be selected as the reference standard, in order to quantify the correct total protein amount.

A second aim of this chapter was the analysis of certain parameters on the protein recovery. The results show that neither different buffers, as media or water, nor the protein starting concentration have an impact on the protein recovery during this process. However, it could be seen that the reduction of the process volume in a small scale model does influence the accuracy of the performance. Hence, results achieved with the small scale model at 500mL give a more representative picture of the manufacturing process than the 40mL small scale model.

The last aim of the recovery process simulation was to research if the process is specific for any particular protein. It was shown that different types of proteins as BSA or interferon behaved similar throughout the entire process. However, both proteins showed no significant loss during the entire process simulation. Due to previous studies performed on sight of the manufacturing recovery process, a loss of at least 50% was expected in the interferon-α-2b at the end of the process. A reason why this protein loss was detected on sight at the manufacturing scale but not during the simulation in DCU could be as follows. First, the loss was only detected in interferon amount and not in total protein. Hence, it is possible that the loss only appears in IFN, however, other studies of this chapter indicate that the process is not protein specific. Second, the simulation of the recovery process differs to the one from the industrial partner in the concentrations of NaOH, NaCl and HCl. More concentrated bases and acids can have a stronger impact on the protein stability and

recovery than less concentrated form. The biggest differences between the small scale model and the manufacturing process are the centrifugation steps. The industrial partner is using continuous centrifuges which are less effective in separation than discontinuous centrifuges used in DCU for the small scale model.

In summary, these results provide already considerable information about the characteristics of the primary recovery process, although the simulation of the recovery process in a small scale model has its limits. To obtain further characteristics about total protein and interferon throughout the process, process samples need to be analysed and protein mass balances need to be set up. These results can be found in the following chapter 4, 5 and 6.

Chapter 4

Process Characterization - Total Protein Mass Balances

4.1 Introduction

The characterization of the primary recovery process is indispensable to maintain product quality and quantity, and to gain better understanding of single process steps. The primary recovery of proteins after a microbial fermentation is usually composed of several centrifugation steps to separate the protein from the fermentation broth and to reduce the volume before starting the final purification steps during down-stream processing. In order to characterize the process, mass balances of total protein have been set up for different centrifugation steps. Using the mass balances, critical process steps can be identified and selected for further studies or optimization.

In this chapter samples derived from the manufacturing primary recovery process were analysed for their total protein quantities and mass balances were set up for all four centrifugation steps performed during the primary recovery. A detailed plan of the sampling points throughout the process has been discussed in chapter 2 and can be seen in figure 2.3. In order to set up full mass balances, samples from the waste-stream were also required. These sample points were only put in place and included into the sampling plan from batch 2-AVAF-201. For two batches, 1-AVAF-211 and 1-AVAF-212, these samples were not available and therefore the setup of a complete mass balance was not possible for these batches.

4.1.1 Cell lysis

The majority of microbial produced products are of intracellular type and retained within the cells, like interferon-α-2b. In order to isolate and harvest intracellular products, microbial cells must be disintegrated by physical, chemical or mechanical means [36]. At small or lab scale this step can easily be achieved by mechanical cell disruption using liquid shear methods such as ultrasonic or high-pressure homogenization [37]. However, at large or manufacturing scale this step can be more challenging. The choice of cell disruption method is often dependent on the type of product to recover. To receive high throughput of large volumes, the cell disruption method is either a high-pressure homogenizer, solid shear using a bead mill or non-mechanical but chemical disruption with acids or alkalis [37].

For harvesting sensitive proteins, as enzymes, cell disruption is often achieved using bead mills or high-pressure homogenization since the addition of strong acids or alkalis could lead to damage of the protein. For more robust proteins, chemical cell lysis can be used to achieve product release but also cell inactivation. Due to low pH by acid addition, proteins precipitate within this step [37].

During the primary protein recovery process for interferon-α-2b, cell lysis is performed by chemical cell disruption due to the addition of trichloroacetic acid (TCA) and phosphoric acid. This step therefore called acid kill. Under low pH conditions the cell wall and membrane breaks up and released proteins precipitate. On the other hand, for analysis of proteins in the end of fermentation sample, sonication is used to disrupt the cells. The ultrasound of a sonicator with frequency higher than 15 - 20 kHz causes disruption of microbial cells in suspension [37].

4.1.2 Flow Cytometry

Flow Cytometry (FCM) is a method of enumerating and analysing cells which has been utilized since the 1970's [38]. In conjunction with the use of fluorescent dyes, flow cytometry has become an increasingly important tool in the screening of cells of all kinds [39]. The basic idea of the method is that cells will pass in single file through the measuring device of the system in a fluid stream [40]. With the use of fluorescence dyes it is not only possible to distinguish between living and dead microorganism but also to differentiate between intermediate physiological states of the

cells. For example, a cell may be metabolically active but may or may not have the capability to replicate itself [41]. With the selection of different dyes certain different extrinsic parameters of the cells can be analysed, for example: DNA, chromatin structure, RNA and total and basic proteins [40].

4.1.3 Protein stability

Protein stability and solubility is highly important during preparation, sterilization, shipping and storage of pharmaceutical proteins. Therefore a lot of studies focus on the understanding of protein stability and solubility and the parameters interfering with it [42]. The following two sections describe the dependency of protein stability and how to increase the protein solubility in solutions. Protein stability can be described by the energy change involved in unfolding the three dimensional structure of a protein to the polypeptide chains. This energy change is called the standard Gibbs energy ΔG_D [3] and can be described by the following formula 4.1.

$$\Delta G_D = G_U - G_N \tag{4.1}$$

ΔG_D is the free energy of the protein stability, G_U stands for the free energy of the unfolded state and G_N for the energy of the folded, native state [43].
The free energy levels of folded and unfolded proteins can be seen in figure 4.1.

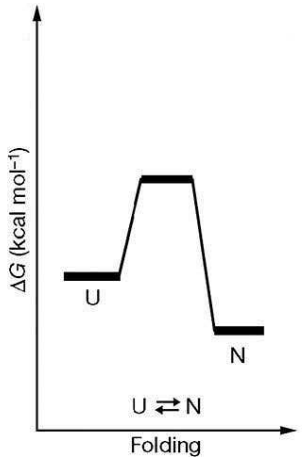

Fig. 4.1: Kinetic stability of a protein that folds to a stable native state through a free-energy barrier [3]

The larger and more positive the free Gibbs energy ΔG_D of a protein, the more stable the protein

is to denaturation. ΔG_D is highly dependent on temperature as well as on the pH of the protein solution. It is well known that changes in the pH and salt concentration interfere with the unfolding free energy of proteins [44]. Far from their pI, proteins accumulate large net charges which increase their free energy, thus lower ΔG_D and the protein stability. Studies have shown that proteins at low pH of 4.0 have higher risks for degradation compared to proteins at a pH around 6.0 or higher [45]. The impact of salt on protein stability and solubility will be discussed in the following section.

Besides pH and salt concentration, also other parameters affect protein stability and can lead to protein degradation like temperature or oxygen radicals. Most proteins are highly instable at high temperatures and lead more easily to degradation at temperatures over 37°C [45]. Also the presence of oxygen radicals can modify proteins in their tertiary and secondary structure [46].

For drug delivery, protein stability plays also an important role. Some protein-drugs have a fast metabolic degradation activity. To reduce this metabolic degradation in the human body the protein is PEGylated at the end of the purification process. PEGylation reduces the metabolic degradation and improves the drug delivery in the human body [47].

4.1.4 Protein solubility and Salting-out of proteins

Protein solubility is dependent on various interactions as protein-protein, protein-water, protein-ions and ion-water. Besides these interactions other factors such as ionic strength, solution composition, pH and temperature interfere with the protein solubility as well [42]. In this section we will focus on the impact of salt on protein solubility in water.

Salt can either stabilize or destabilize proteins dependent on their concentration and the type of salt. Salt ions affect the protein stability by changing the hydrogen-bonding properties of water. Known as the Hofmeister Series, the preferential hydrations of salt anions increase in the following order: $Cl^- < CH_3^- < COO^- < SO_4^{2-}$ whereas cations following a different order: $Ni^{2+} < Ca^{2+} < Ba^{2+} < Mg^{2+} < Na^+$ [48].

Two terms are used when talking about protein solubility and the impact of salt: salting-in and salting-out. Salting-in describes the stabilization of proteins in water due to low salt concentrations. Salt is increasing the surface tension of a medium and hence increases non-specific electrostatic interactions by new formations of protein - salt ion pairs. These new ion pairs enhance

the water binding ability of the protein surface. Salt concentrations used for salting-in are usually between 0-0.3M [42]. Table 4.1 gives some examples for salts commonly used for salting-in and salting-out of proteins.

Tab. 4.1: Salts commonly used for Salting-In and Salting-Out of proteins [14]

Salting-In	Salting-Out
$BaCl_2$	$MgSO_4$
$CaCl_2$	NaCl
$MgCl_2$	Na_2SO_4
$MnCl_2$	$Mg(OAc)_2$
	$Ca(OAc)_2$

Salting-out on the other hand describes the destabilization and precipitation of proteins in solution. At high salt concentrations proteins are less soluble in water [14]. Salt ions, especially sodium ions, lead to preferential hydrations, meaning water molecules are attracted by salt ions. Hence, the number of water molecules available to interact with the protein surface decreases and leads to protein precipitation formations [14]. Different proteins need different amount of salts to precipitate. Therefore the protein solubility in the salting-out state can be described as in equation 4.2:

$$logS = \beta - K_s C_s \tag{4.2}$$

S stands for the solubility of the protein, β is the theoretical solubility of the protein with zero salt, K_s is the salting-out constant and C_s is the molar salt concentration [42]. With this equation it is possible to calculate the required concentration from a particular salt in order to precipitate a certain type of protein.

The salting-out characteristics of proteins is often used for concentrating and purifying proteins selectively from solution [49]. A comparable approach is used during the primary protein recovery process characterized in this chapter. Sodium chloride and hydrochloric acid are added during the process with the aim to salt-out different proteins.

4.1.5 Goals and Aims

The aim of this chapter is to characterize the primary protein recovery process performed by the industrial partner on the basis of total protein amount throughout the process. Samples taken from the manufacturing process at different time-points of the process are analysed for their total protein amount via Bradford assay. Using these results, mass balances for total protein are set up for each step throughout the recovery process and critical process steps can be identified.
Hence, the three main aims of this chapter are as follow:

- characterize primary protein recovery process
- set up of mass balances for total protein throughout recovery process
- identification of critical process steps

4.2 Materials and Methods

4.2.1 List of Materials

Tab. 4.2: Materials and suppliers

materials	supplier
Recovery Process samples	industrial partner
Trizma Base	Sigma Aldrich
EDTA tetrasodium salt	VWR
Bradford Reagent	Sigma Aldrich
Bovine serum albumin (BSA)	Sigma Aldrich
Acetic Acid (glacial) 100%	VWR
BD FACScalibur flow cytometer	Teagasc
5(6)-carboxyfluprescein diacetate	Sigma Aldrich
Ethanol	Sigma Aldrich

4.2.2 Sample preparation

Different sample preparations need to be performed on samples received from the manufacturing protein recovery process, see sampling plan in figure 2.3.

End of fermentation sample

This sample preparation is only necessary for the EOF-sample (End of fermentation sample), since all *E.coli* cells are still intact and most proteins are kept inside the cell. To analyse total protein produced by *E.coli*, cell lysis needs to be performed. This is established by using a sonicator UO-100H (Hielscher, Teltow, Germany).

The received EOF-pellet is redissolved in 200mL 20mM Tris, 1mM EDTA buffer and stirred for 30min until homogeneity is achieved. 5mL of this solution are taken for sonification. The sonicator UO-100H is set to the following parameters: cycle = 1 and amplitude = 90%. The sonotrode is inserted into 5mL EOF solution and the sonicator is switched on for 60sec followed by a 20sec

break. This cycle needs to be repeated 18 times. Keep EOF solution on ice during the whole sonification process.
The 20mM Tris, 1mM EDTA buffer will be used as the blank-solution for total protein determination of the EOF sample, diluted with DI-water with the same dilution factor that is used for the EOF-sample itself.

Samples containing precipitations

This sample preparation is necessary for all recovery process samples containing protein precipitations. This applies for all samples between S-2 and S-13, see sampling plan in figure 2.3.
Samples were thawed in a water-bath at 37°C for 2 hours until the whole sample volume was thawed. Samples were mixed well before transferring the whole volume into 50mL centrifuge tubes.
5mL of each sample will be centrifuged at 4000rpm at 4°C for 30min in a discontinuous centrifuge FL40R (Thermo Scientific, Dublin, Ireland). The volume of the supernatant is been collected and samples are stored at 4°C until proceeding with protein determination. The unused sample volume was stored back at -80°C after sample preparation was finished.
The sludge is redissolved in 5mL of 20mM Tris, 1mM EDTA buffer (pH 8.0) on an orbital shaker at 600rpm for 2.0 hours. After redissolving, the pH is set to 7.5$\pm$0.5 with either 1M tris or 4M acetic acid. After collecting the final pH and volume of the redissolved sludge the samples are mixed one more hour on the orbital shaker at 600rpm before storing them at 4°C until proceeding with protein determination.
For all supernatants the blank solution, to be used for total protein determination, will be pure fermentation medium, diluted with DI-water with the same dilution factor that is used for the supernatant samples themselves. For all redissolved sludge samples, 20mM Tris, 1mM EDTA buffer (pH 8.0) will be used as the blank solution for total protein determination, also diluted with DI-water with the same dilution factor that was used for the sludge samples themselves.

TCA-pellet

This sample preparation is only necessary for the TCA-pellet, S-15, since this is the only sample that comes in a pellet form.

Approximately 10g of TCA-pellet are redissolved in 100mL of chilled 10mM acetic acid and stirred for 10min until solution is homogenized. The redissolved pellet is centrifuged in the FL40R centrifuge (Thermo Scientific, Dublin, Ireland) at 4000rpm for 60min at 4°C. After centrifugation the supernatant is discard and the pellet is redissolved in 500mL chilled 20mM tris, 1mM EDTA buffer (pH 8.0) for 20min. After redissolving the pH is set to 8.0±0.1 with either 4M acetic acid or 1M tris, followed by 2 hours of gently mixing. At the end the pH is set to 5.5±0.05 with 4M acetic acid followed by a last centrifugation at 3500rpm for 20min at 4°C. The volume of the collected supernatant is recorded and stored at 4°C until proceeding with protein determination. The sludge can be discard.

The 20mM tris, 1mM EDTA buffer (pH 8.0) is used as the blank-solution for total protein determination of the TCA-pellet, diluted with DI-water with the same dilution factor that is used for the TCA-pellet itself.

4.2.3 Total protein determination

After different sample preparations are performed all samples are analysed for total protein with the Bradford assay, see section 3.2.2 and table 3.6. The reference standard is BSA in DI-water, since the expected IFN-α-2b portions of total protein concentration is weigh below 16.7% (compare section 3.3.1.1 and table 3.9).

The blank value for the reference standard is always DI-water. However, the blank value for the recovery process samples differ from sample to sample. More information is given in each sample preparation section.

4.2.4 Flow Cytometry analysis

Protein recovery process samples S-1 to S-4 are analysed for their vital physiological cell state with a BD FACScalibur flow cytometer (Teagasc, Carlow, Ireland) which is equipped with a 15-mW 488nm argon laser and a 635nm diode laser. Samples S-1 to S-4 derived from the manufacturing

recovery process are diluted with phosphate-buffered salin to an OD_{600} of 0.100 and stained with 5(6)-carboxyfluorescein diacetate (cFDA) to a concentration of 5mg/l. The sample is incubated at 37°C for 10 minutes followed by an incubation on ice for 10 minutes.

After injecting the sample containing fluorescence dye onto the flow cytometer, the fluorescence signal emitted is collected in four optical channels, FL1 (515 to 545nm), FL2 (564 to 606nm), FL3 (>650nm) and FL4 (>670nm). The BD software (BD Biosciences) is used in the cytometer to complete the data analysis.

Sample-1 untreated and sample-1 treated with 90% ethanol are used as a positive and negative control to find the best discrimination between the subpopulations of active and dead cells.

4.3 Results and Discussion

All samples analysed in this section were taken from the manufacturing protein recovery process according to the sampling plan in section 2.5. Samples were taken from four different batches. Two of them performed in 2011 (1-AVAF-211 and 1-AVAF-212) and the other batches from 2012 (2-AVAF-201 and 2-AVAF-206). The samples were frozen at -80°C immediately after sampling and shipped on dry ice from the industrial partner to DCU, where they were stored at -80°C until analysis was performed.

4.3.1 Characterization of 4 Recovery Process Cycles

Batch 2-AVAF-206 was selected as an example of all 4 analysed batches of the protein recovery process in this section. Figure 4.2 shows the detected total protein amount throughout the protein recovery process, after different sample preparations were performed (see section 4.2.2). The four red arrows indicate four centrifugation cycles which separated the four different cycles of the recovery process from each other. S-1 to S-4 represent the acid kill cycle, S-5 to S-7 are in cycle-1, S-8 to S-11 are part of cycle-2 , S-11b to S-13 represent cycle-4 and S-15 stands for the final TCA-pellet. All samples indicated with a red 'W', are samples collected from the waste stream for the particular centrifugation cycle.
Bars in blue represent the protein fractions analysed in the sludge, meaning these proteins were in precipitated form at this stage during the recovery process. Bars in green, on the other hand, represent the protein fractions analysed in the collected supernatant, meaning these proteins were in solution at the particular stage.

Figure 4.2 represents the total protein amount throughout the protein recovery process of batch 2-AVAF-206. During the acid kill cycle, *E.coli* cells are disrupted to release the proteins due to the addition of TCA and H_3PO_4. The addition of acid also leads to the precipitation of the proteins, therefore the main protein fraction of S-2 to S-4 can be find in precipitated form. S-1 represent the EOF (end of fermentation) sample in which all proteins are still in solution. The total protein amount at the end of the fermentation was expected to be similar to sample S-4. The difference between S-1 and S-4 can be explained due to the lack of analysing the supernatant of the fermentation broth. S-1 represents only the spun down cell pellet at the end of the fermentation. Proteins

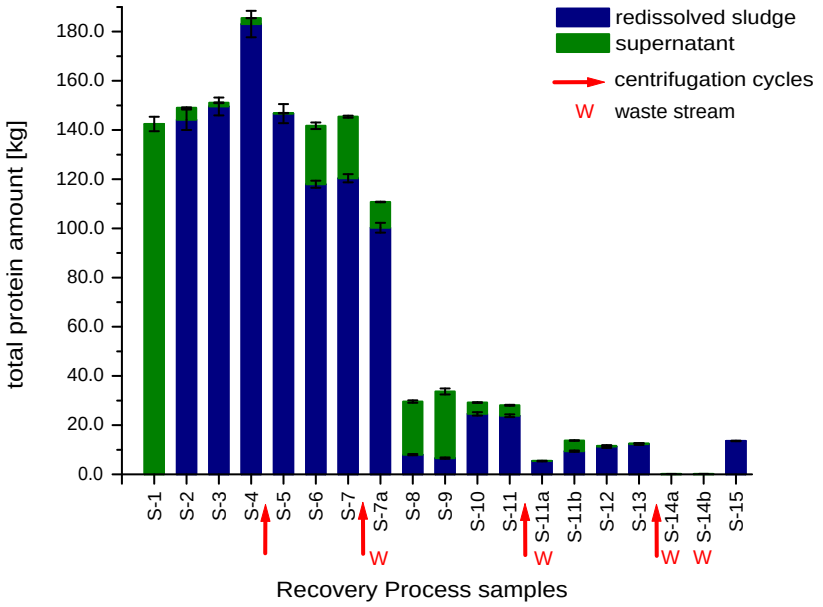

Fig. 4.2: Total protein amount throughout recovery process of batch 2-AVAF-206

released to the supernatant during fermentation are not included in this analysis. Due to analysis of batches 1-AVAF-211 and 1-AVAF-212, where both, supernatant and cell pellet, were available, the total protein amount released to the supernatant corresponds to 20.0 - 23.0kg (data not shown). This corresponds to the detected difference in total protein between S-1 and S-4 of this batch. Since S-1, where cell lysis was performed using a physical disruption, does not show significantly higher protein levels than S-4, where cell lysis was achieved using chemical disruption (see section 4.1.1), the acid kill step results in an efficient disruption of *E.coli* cells and release of proteins in precipitated form, a more detailed discussion about the characterization of the acid kill step can be found in section 4.3.1.1.

During cycle-1 of the recovery process (S-5 to S-7), the recovered protein pellet needs to be resolubilised. This should be achieved due to the addition of UP-water and pH setting to 7.0 by addition of NaOH. Figure 4.2 shows that only a small protein percentage ($\approx$18%) gets resolubilised under these conditions and the majority of total protein remains in precipitated form.

Cycle-2 (S-8 to S-11) is a 'salting out step' to selective recovery the protein of interest (compare section 4.1.4). For this purpose the conductivity is set to 20mS/cm by the addition of NaCl, followed by pH setting to 4.5 due to the addition of HCl. With the reduction of the pH in sample 10

more than 80% of total protein precipitate and only half of the total protein amount gets recovered during the following centrifugation step. It needs to be investigated if this 'salting out step' is protein specific and only *E.coli* host cell proteins are salted-out and IFN-α-2b is recovered (compare chapter 5). Due to previous results of the small scale model of the recovery process (compare chapter 3), no major specificity in the recovery of different proteins is expected. It is more likely that the protein of interest IFN-α-2b shows similar characteristics during the recovery process and the 'salting-out step' to the host cell proteins.

The last cycle, cycle-3 (S-11b to S-15), ends with the formation of the TCA-pellet. Due to the addition of TCA the remaining proteins precipitate and the precipitants are recovered in the TCA-pellet. The protein precipitation works efficient, since only small amounts of total protein were detected in solution in sample 13 (see figure 4.2).

Summarizing, the characteristics of the recovery process cycles are as follow: the acid kill efficiently disrupts *E.coli* cells to release and precipitate total proteins. Solubilisation of the protein precipitants should take place during cycle-1, but as seen in figure 4.2 the yield of solubilisation is only 20% of total protein. During cycle-2 proteins are efficiently salted-out, with a yield of 50%. It still needs to be characterized if this salting-out is protein specific and is not affecting IFN recovery. Finally in cycle-3 the recovered proteins precipitate with a yield of more than 95% and are recovered in the TCA-pellet.

4.3.1.1 Characterization of the Acid Kill step with flow cytometry

The previous section of this chapter discussed already the protein recovery of the *E.coli* cells throughout the acid kill step. The protein recovery from the end of fermentation sample with physical cell disruption using a sonicator was compared to sample 4 from the recovery process where cell lysis was achieved by chemical disruption with acid addition. The following graph shows more detailed information about the physical cell disruption of the *E.coli* cells using a sonicator.

Figure 4.3 shows the protein recovery from *E.coli* cells of the EOF sample in dependency on different sonification cycles. It can be seen that the recovery of total protein is increasing with an increase in sonification cycles. This is as expected. However, after 16 cycles the product recovery stagnates and no significant increase can be seen compared to 18 cycles. These results

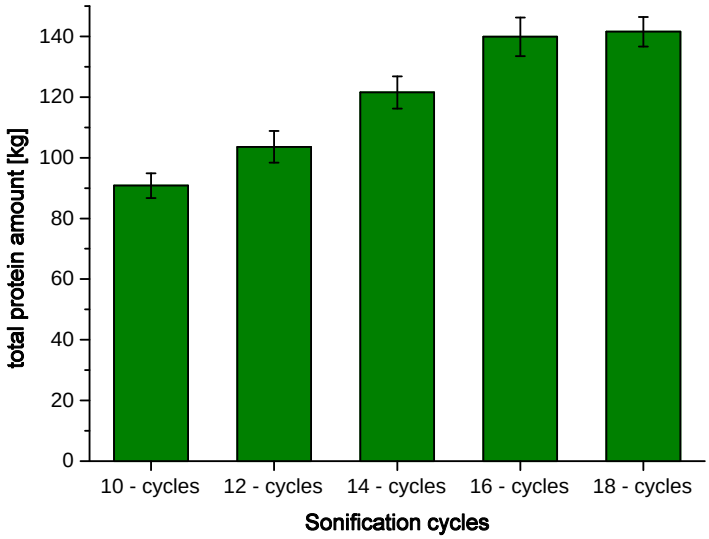

Fig. 4.3: Total protein recovery from *E.coli* cells of the EOF sample after physical cell disruption with a sonicator

show that with 16-18 sonification cycles the cell lysis of *E.coli* cells is complete and all proteins are recovered.

In order to analyse the acid kill step at the beginning of the primary protein recovery process for its capability for complete cell lysis flow cytometry was used. During the acid kill, cell lysis of *E.coli* cells should take place and proteins are released into the supernatant. In order to ensure a complete disruption of all cells at the end of the acid kill step, samples were analysed for their physiological state using the flow cytometer. During sample treatment, cells were stained with a fluorescence dye which stains the cells depending on the condition of the cell membranes. With the flow cytometer each individual cell can be detected by a laser at a different wavelength at which the UV reading is dependent on the staining of the cell (see section 4.1.2). In this way the flow cytometer can distinguish between living cells with intact membranes, injured cells with partly intact membranes and dead cells with complete disrupted membranes. In order to have a complete cell lysis and to recover all proteins from the *E.coli* cells, all cells need to be 'dead' with completely disrupted membranes.

Recovery process samples S-1 to S-4 were analysed with the flow cytometer as described in section 4.2.4. The results obtained for sample-1, the end of fermentation sample, is depicted in figure 4.4.

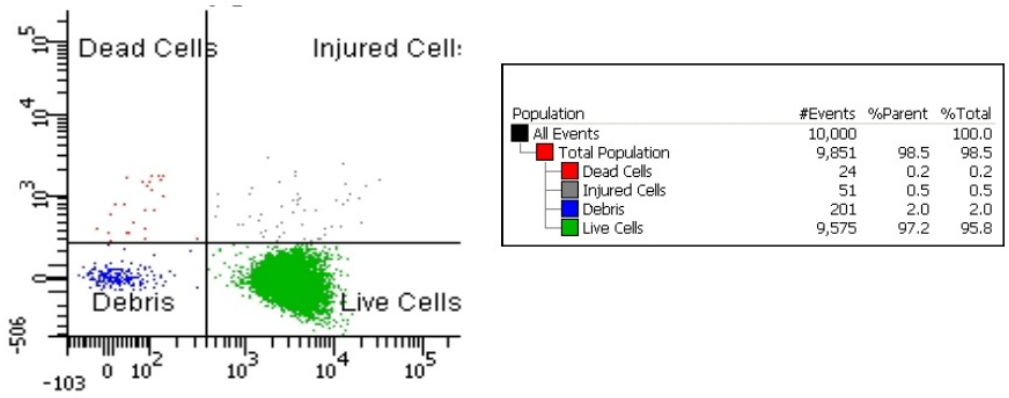

Fig. 4.4: Results of flow cytometric analysis of the end of fermentation sample (before acid kill) of the recovery process of batch 2-AVAF-206

This figure shows the results of the flow cytometry analysis of the end of fermentation sample (before the acid kill). It can be seen that over 96% of the cells are in an active and viable physiological state, which is to be expected at the end of the fermentation. No cells (< 1.0%) were identified as injured or dead.

Analysing samples 2 and 3 of the recovery process, which were taken during the acid kill step after the addition of TCA and phosphoric acid, showed an increase in injured and dead cells (data not shown). Sample 2 had 40% dead cells and 10% injured and sample 3 showed 80% dead and 5% injured cells. This observation indicates that the addition of acid and lowering the pH to 2.0 results in disruption of the *E.coli* cells.

Sample 4 of the recovery process is the last sample taken during the acid kill, after 30 minutes of agitation at pH 2.0. The flow cytometer results of this process time point are shown in figure 4.5.

It can be seen that over 98% of cells were detected as dead cells with completely disrupted membranes at sample time point 4. This observation indicates that the acid kill step works efficiently in lysing and disrupting the *E.coli* cells to release all proteins into the supernatant. These data confirm the findings observed during the total protein quantification of samples 1 to 4 of the previous section 4.3.1.

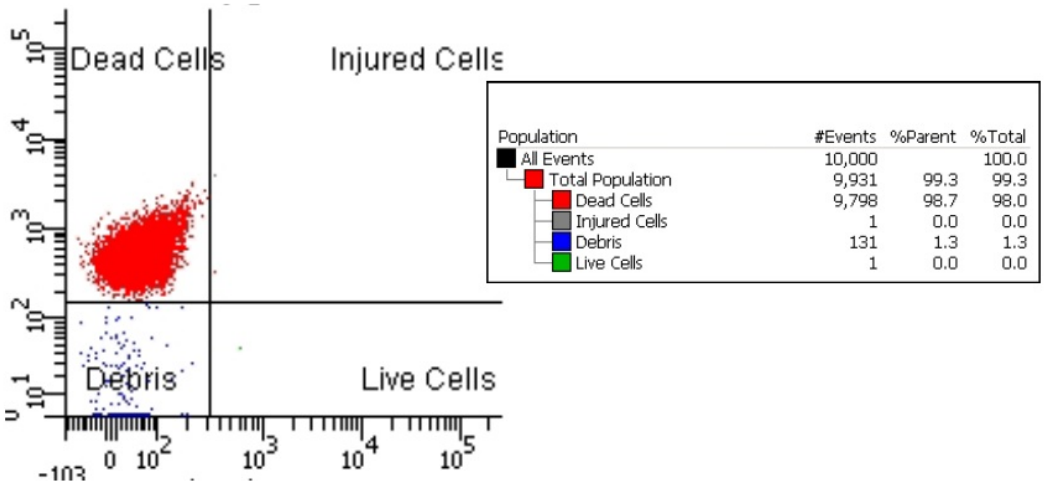

Fig. 4.5: Results of flow cytometric analysis of sample 4 (end of acid kill) of the recovery process of batch 2-AVAF-206

4.3.2 Protein Mass Balances throughout the Recovery Process

To gain further information about the characteristics of the recovery process and to identify critical process steps, mass balances were set up for each of the four centrifugation cycles.

Acid Kill → Cycle 1

The first mass balance is between the acid kill and cycle-1. The formula 4.3 illustrates the samples of the recovery process that have been used for this balance.

$$S-4 \implies S-5 + waste \tag{4.3}$$

Inserting the analysed total protein quantities for each sample results in mass balance 4.4.

$$185.5kg \pm 5.41kg \implies 146.9kg \pm 3.97kg + not\ measured\ (38.6kg) \tag{4.4}$$

Since a sample of the waste stream of the first centrifugation cycle is not available, it is not possible to set up a complete protein mass balance for this step. However, due to the analysed protein

amount in S-5 and S-4, theoretically around 38kg of total protein end up in the waste stream during this centrifugation. This corresponds to 80% recovery of total protein and 20% loss in total protein. Since the yield of the protein precipitation during the acid kill is over 95%, the protein loss during this centrifugation can be explained by the inefficiency of the centrifugation process itself. As described in chapter 3 the efficiency of a continuous centrifuge is not comparable to a discontinuous centrifuge. Hence, the separation of precipitations from the supernatant is only around 80%.

Cycle 1 → Cycle 2

The next mass balance needs to be set up between cycle 1 and 2. Formula 4.5 gives information about the recovery process samples that are needed for this balance.

$$S-7 \implies S-8 \ + \ waste\ (S-7a) \tag{4.5}$$

Inserting the analysed total protein quantities for each sample results in mass balance 4.6.

$$145.4kg \pm 2.04kg \implies 29.6kg \pm 0.75kg \ + \ 110.7kg \pm 2.1kg \tag{4.6}$$

The mass balance 4.6 between cycle 1 and 2 results in an even balance for total protein. The 5.1kg (3.1%) that are missing from a complete mass balance are in between the standard deviations of the sample analysis. Hence, more than 75% of total protein can be found in the waste stream and only 20% of total protein is recovered during this centrifugation cycle. The reason therefore can be found in the insufficient resolubilisation of protein precipitations during cycle 1. Only 18% of total protein is resolubilised during cycle 1 which is recovered in the supernatant after centrifugation cycle 2. Most proteins that remain in precipitations are not recovered (80%) and can be found in the waste stream of this centrifugation.

Cycle 2 → Cycle 3

The third mass balance is between cycle 2 and 3. The formula 4.7 illustrates the samples of the recovery process that are been used for this balance.

$$S-11 \implies S-11b \ + \ waste\ (S-11a) \tag{4.7}$$

Inserting the analysed total protein quantities for each sample results in mass balance 4.8.

$$28.1kg \pm 0.74kg \implies 13.7kg \pm 0.39kg \ + \ 5.5kg \pm 0.12kg \tag{4.8}$$

The mass balance 4.8 between cycle 2 and 3 does not result in a complete mass balance, 8.9kg of total protein (31.7%) were neither detected in the recovered supernatant nor in the waste stream. The reason therefore could lie in the imprecise determination of the process volume for the different steps through the volumetric flow rate. Since the waste stream is very concentrated with respect to total protein (up to 25.0g/L), small changes in the volume can lead to significant changes in the determination of total protein.

Another explanation for an incomplete mass balance is protein degradation. Protein degradation can lead to modifications in the chemical structure (amino acids chain) or shape (disulphide bonds) of the protein (compare section 4.1.3). The change of the chemical structure can lead to changes in the detection of the total protein amount, as described in chapter 3. Hence, the undetected total protein amount of ≈9.0kg can be due to modification of the protein structure which falsifies the protein measurements.

However, even with the incomplete mass balance it can been seen that only 50.0 - 60.0% of proteins are recovered in this centrifugation cycle. This is due to the salting-out of protein in cycle 2. It still needs to be investigated if the salting-out of proteins only affects *E.coli* host cell proteins or interferon-α-2b as well (see chapter 5).

Cycle 3 → TCA-pellet

The last mass balance needs to be set up between cycle 3 and the TCA-pellet. Formula 4.9 gives information about the recovery process samples that are needed for this balance.

$$S-13 \implies S-15 \ + \ waste\ (S-14a/b) \tag{4.9}$$

Inserting the analysed total protein quantities for each sample results in mass balance 4.10.

$$12.6kg \pm 0.38kg \implies 13.6kg \pm 0.12kg \ + \ 0.3kg \pm 0.03kg \tag{4.10}$$

The mass balance 4.10 between cycle 3 and the TCA-pellet results in a complete balance for total protein. Only Δ1.3kg (10.3%) are exceeding the complete even mass balance which is in between the standard deviations of the sample analysis. Hence, a complete recovery of total protein is achieved during the last centrifugation cycle.

Summarizing all four mass balances throughout the recover process, the centrifugation between cycle 1 and 2 can be identified as the main critical process step with a loss of more than 75% in total protein. The centrifugation between cycle 2 and 3 is another critical step, with only 50% recovery of total protein.

4.3.3 Comparison between 4 batches of the Recovery Process

An important factor for industrial processes and process characterization is the robustness and reproducibility of a process. For this reason four different batches of the recovery process were analysed for their total protein behaviour throughout the process.

Figure 4.6 shows the analysed total protein quantity for different time points throughout 4 different batches of the protein recovery process. To prepare the fermentation media different lots of raw material were used for the different batches. Batch 1-AVAF-211 and 1-AVAF-212 had similar lots of raw material to produce the fermentation media. However, batch 2-AVAF-201 and 2-AVAF-206

used different lots of raw-material for their fermentation media preparation.

Sample S-7a, S-11a and S-11b were only available for batches 2-AVAF-201 and 2-AVAF-206 but not for batches 1-AVAF-211 and 1-AVAF-212.

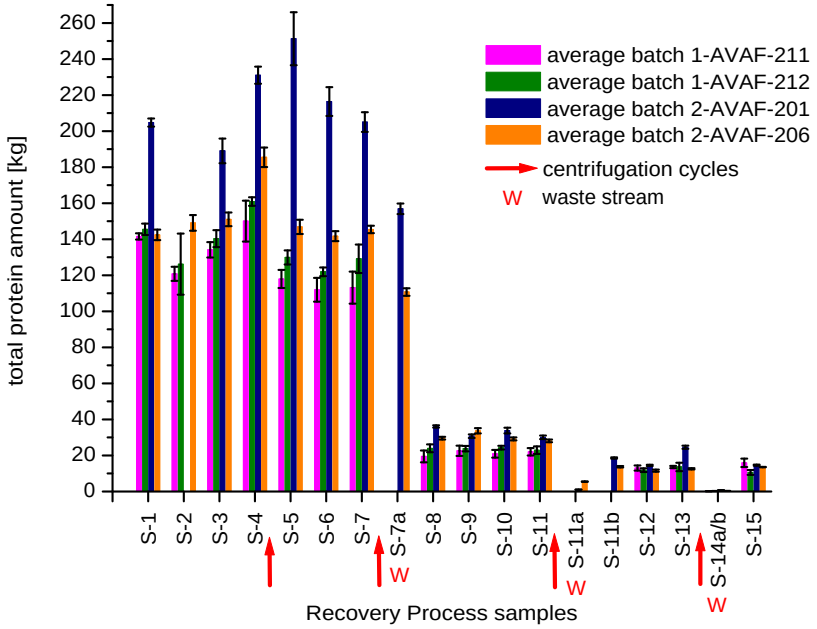

Fig. 4.6: Total protein amount throughout recovery process of 4 batches: 1-AVAF-211, 1-AVAF-212, 2-AVAF-201 and 2-AVAF-206

It can be seen in figure 4.6 that the produced amount of total protein at the end of the fermentation differs between the 4 analysed batches dependent on the raw material that was used for the media preparation. Batch 1-AVAF-211 and 1-AVAF-212 which had the same raw material for media preparation show no significant difference in their total protein production at end of the fermentation (compare S-1 in figure 4.6).

It has to be kept in mind that sample 1 represents different protein pools for the different batches. For batch 1-AVAF-211 and 1-AVAF-212 S-1 represent the total produced protein amount, meaning protein kept in the intracellular compartments of the *E.coli* cells, as well as the protein fraction that was released into the supernatant. However, for batch 2-AVAF-201 and 2-AVAF-206 S-1 represents only the protein pool that was kept in the intracellular compartments of the *E.coli* cells. Meaning the total produced protein amount during fermentation is precisely represented by S-4 for these batches. Hence, comparing S-4 from batch 2-AVAF-201 with S-1 from batches 1-AVAF-

211/212 it can be seen that the total protein production during the fermentation is more than 40% higher. Also for batch 2-AVAF-206, the total protein production is up to 20% higher than in batches 1-AVAF-211/212. Since the focus in this chapter is on the protein recovery process and not on the fermentation or raw-material side this observation will not be discussed any further at this stage.

Besides the difference in the total produced protein amount between the different batches, the characteristics of the 4 cycles of the recovery process are very alike between all batches. For all 4 batches the acid kill cycle resulted in efficient cell disruption to release all total protein (refer to section 4.3.1), followed by a 10 - 17% loss in total protein due to insufficient centrifugation cycle (refer to section 4.3.2). During cycle 2 the solubilisation of total protein is a key factor in all four batches. Independent of the total protein amount at the beginning of this cycle (S-5), the amount of resolubilised protein and therefore recovered protein in cycle 3 (S-8) is very similar in all four batches. The variations between batch 2-AVAF-201 and 1-AVAF-211 in S-5 corresponds to Δ80kg, but in S-8 this variation is reduced to Δ14kg. This indicates that the insufficient protein solubilisation in cycle 1 is due to a protein saturation in S-7. Independent of the protein amount entering cycle 1, if the protein saturation level of water is reached no more protein can be solubilised. This observation in cycle 1 corresponds with the total protein amounts detected for the following cycles in all four batches. The big variations in total protein amount observed in the acid kill and cycle 1 due to different produced amount of total protein during fermentation, cannot be observed in cycle 2 and 3. The total protein amount detected in samples of cycle 2 and 3 are comparable between all four batches.

Since the total recovered protein amount at the end of the recovery process is independent of the total protein amount at the end of the fermentation, this recovery process is the key factor in optimizing and enhancing the overall process yield. An increase in protein production during the fermentation would not lead to an enhanced protein amount at the end of the recovery process due to the limited saturation level of protein solubilisation in cycle 1.

4.3.4 Critical process steps & protein mass balances

To identify the overall critical process steps of the protein recovery process, the average of the percental total protein amount of all four analysed recovery process batches was determined and is

shown in figure 4.7. The total protein amount quantified in S-4 was set to 100% since this sample represents the total protein amount for all four batches (compare section 4.3.3). The percentages of all other samples were calculated on the basis of sample 4.

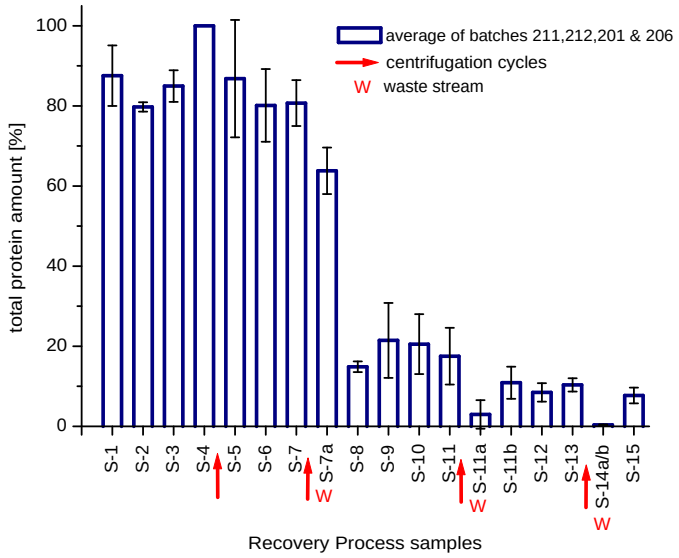

Fig. 4.7: Total protein amount throughout recovery process of 4 batches in percentage; S-4 was set to 100%

Figure 4.7 shows the characteristics of the total protein quantity throughout the recovery process in percentage of four analysed batches. Two critical process steps can be identified, between cycle 1 and 2 (S-7 to S-8) and between cycle 2 and 3 (S-11 to S-11b). The mass balances for these two critical process steps are listed below in formula 4.11 and 4.12.

Critical Process Step 1: Cycle 1 → Cycle 2

$$80.7\% \pm 5.7\% \quad \Longrightarrow \quad 14.9\% \pm 1.3\% \; + \; 63.8\% \pm 5.8\% \tag{4.11}$$

In the critical process step 1 more than 80% of total protein ends up in the waste stream and only 20% is recovered. The reason for this significant protein loss is insufficient protein solubilisation in cycle 1 due to low saturation levels (compare section 4.3.2 and 4.3.3).

Critical Process Step 2: Cycle 1 → Cycle 2

$$17.5\% \pm 7.1\% \implies 10.9\% \pm 4.0\% + 3.0\% \pm 3.5\% \tag{4.12}$$

The second critical process step shows a protein decrease of more than 40% and only 60% of total protein is recovered. This loss in total protein occurs during the salting-out step of proteins in cycle 2. At this stage it is not identified yet, if this loss occurs only in host cell proteins or as well in the protein of interest interferon-α-2b (4.3.2). Further studies in the characteristics of IFN-α-2b itself during this recovery process are necessary, which will be discussed in chapter 5.

Summarizing these results, two critical process steps were identified in the protein recovery process. The first step showed a total protein loss of up to 80% and the second one showed a protein loss of up to 40%. Overall, the total protein loss during the whole recovery process is up to 90%, meaning that the process yield is only 10% for total protein.

4.4 Conclusion

In this chapter process characterization of the primary protein recovery using total protein mass balances were performed. An overall loss in total protein of more than 90% was identified throughout the recovery process. The loss occurs due to several reasons. First, insufficient separation of precipitations from supernatant using continuous centrifuges. Second, insufficient resuspension of precipitated proteins during cycle 1 due to low saturation levels of proteins in water. And third, salting-out of proteins during cycle 2. Hence, two main critical process steps were identified: between cycle 1 and 2 and cycle 2 and 3.

An open question at this stage is: does interferon-α-2b behave in a similar way to total protein throughout the recovery process? In particular, for the second identified critical process step, the salting-out step, it is of high interest whether interferon-α-2b is salting-out as well or is this step protein specific and only affects host cell proteins. Due to previous results discussed in chapter 3, it can be assumed that interferon-α-2b behaves in a similar way to total protein. The characteristics of total interferon-α-2b will be discussed in the following chapter 5.

Chapter 5

Process Characterization - Total Interferon Mass Balances

5.1 Introduction

In chapter 4 the primary protein recovery process was characterized using total protein mass balances. As mentioned in section 4.4 a next step is to verify the characteristics of total protein for the protein of interest interferon-α-2b.

This chapter now will focus on the characterization of the primary protein recovery process on the basis of total interferon-α-2b amount throughout the process. The same set of samples derived from the manufacturing process as described in section 4.1 are analysed for their total interferon-α-2b amount using SDS-PAGE.

After identifying critical process steps for the loss of total protein it is indispensable to investigate if the protein of interest follows the same characteristics. Especially for the salting-out step, whose purpose it is to salt-out host cell proteins but not the recombinant protein.

5.1.1 SDS-PAGE

Polyacrylamide-Gel Electrophoresis (PAGE) is an important tool in protein characterization for more than forty years [50]. There are four different forms of PAGE: Native-PAGE, SDS-PAGE, 1D-PAGE and 2D-PAGE. Native-PAGE detects native, non-denatured proteins and separates a

protein mixture depending on their charge and mobility in the gel [35]. SDS-PAGE on the other hand analyses denatured proteins and separates them as a function of molecular weight. Due to the addition of sodium dodecyl sulphate (SDS) the denatured protein is negatively charged and thus the original charge has no impact on the separation [35]. 1D-PAGE is the performance of just a native or SDS-PAGE. 2D-PAGE on the other hand has an IEF-separation prior to the SDS-PAGE. IEF, iso-electric focusing, separates the protein mixture after their pI. In this way a protein mixture can be separated as a function of pI and molecular weight and provides much more information about single proteins [51]. In this section we will focus only on the procedure of a SDS-PAGE.
A SDS-PAGE consists of six major steps: sample preparation, polyacrylamide gel, buffer system, electrophoresis, staining and analysis which will be described in more details in the next paragraphs.

Sample preparation

The sample preparation is very dependent on the type of PAGE that needs to be applied. For a SDS-PAGE the important ingredients are SDS and a reducing agent like β-Mercaptoethanol or Dithiothreitol (DTT). Due to the application of heat (70°C) for 10 minutes the reducing agent reduces disulphide bonds of the native protein. SDS coats the polypeptides chain of the protein with a negative charge [52].

Polyacrylamide Gel

The gel is made out of acrylamide, whose concentration is highly important for a successful separation of a protein mixture. Using a high concentration of acrylamide results in a gel with very small pores, hence only small proteins can be separated and large proteins remain in the upper part of the gel during electrophoresis. Low concentrations of acrylamide on the other hand result in gels with large pores and only large proteins can be separated [4]. Figure 5.1 gives an example of the same sample analysed with different gels of different concentration of acrylamide.

Besides different acrylamide concentration, many gels consist of a stacking and a running gel. The stacking gel is usually made of a different buffer and a much lower concentration of acrylamide than the running gel. In this way protein can get stacked according to their size before entering the

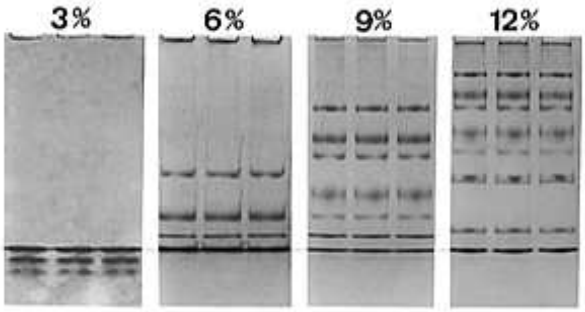

Fig. 5.1: Dependency of acrylamide concentration on successful separation of different protein mixtures [4]

running gel. More information can be found in the following section buffer system.
Nowadays most gels are gradient gels. They consist of different concentrations of acrylamide and allow to separate proteins with a wider molecular weight range [53]. These gels can be purchased commercially to increase reproducibility.

Buffer System

There exist two different systems to run a SDS-PAGE. The first and older one is the continuous system. The same running buffer is used for the inner and outer chamber of the PAGE-unit. This system is easy to perform but it is low in protein separation and resolution [52].
The newer system is the discontinuous system also called Laemmli-system [54]. Different buffers are used for the inner and outer chamber with different pHs. Due to the presence of different ions and the different pHs, an ion gradient is formed in the stacking gel. This ion gradient is forming a successive front that runs towards the sample molecules, which become stacked between the ion gradient according to their electrophoretic mobility. Hence, small molecules at the bottom and larger on the top. As soon as this front is entering the running gel, the pH changes and the ion gradient is eliminated. Due to the pre-selective ordering of the proteins according to their size, separation and resolution is increased [55].
A known problem nowadays with this Laemmli-system is the working pH. At pH 8-9 disulphide bonds start to form again between cysteine due to their pKa of 8-9. Therefore an optimized discontinuous buffer system is used with a Bis-Tris running buffer operating at pH 6.8. A reducing agent,

sodium bisulfide, is also added to the inner chamber that moves into the gel ahead to maintain a reducing environment for the proteins [56]. Especially for small proteins this system can provide an improvement in resolution [57].

Electrophoresis

There are three modes of operation of the electrophoresis: constant voltage, constant current or constant power input. In most cases a constant voltage is used to run electrophoresis. Usually the voltage is set at the beginning to provide a current of 50-60mAm per gel, which is around 112-120V. If the voltage is set higher the electrophoresis will be shorter but the gels can get too hot during the run, which can destroy the gels. The same can happen if the voltage is set too low and the run time is increased too much [57].

Staining

There are two widely-used techniques available for staining gels: coomassie-blue or silver staining. Coomassie blue staining is the more practical version of the two, since it involves only two steps. The gels are incubated in coomassie blue stain for one hour and then de-stained in a methanol/ acetic acid solution overnight. Nowadays there are even commercialized coomassie blue stains available that allow detaining with pure DI-water [58].
Silver staining is more difficult because it requires additional steps and some experience to perform successfully. However, it is more sensitive and detects also smaller bands with low protein concentrations [58].

Analysis

To receive information about the molecular weight of an unknown protein, gels can be scanned to obtain pictures. After that the molecular weight can be determined using the molecular weight marker as a reference [50].
To determine the protein quantity of single bands, densitometry is needed. The density analysis of bands of a reference standard can be correlated to the density of unknown bands in the gel.

However, it needs to be kept in mind that the SDS-PAGE is not a very accurate quantitative method and is more used in protein characterization.

In this chapter pre-cast gradient gels are used with an acrylamide concentration from 4-12% and a stacking gel. The used buffer system is the new Laemmli-buffer system with a Bis-Tris buffer at pH 7.0, since the protein of interested has a low molecular weight of 19kDa. After electrophoresis with constant voltage supply, gels are stained using coomassie blue staining, followed by quantification via densitometry. More details about the procedure can be found in the material and method section of this chapter.

5.1.2 Western Blot

Western blotting (WB), was developed in the early 1970 and describes the transfer of proteins from polyacrylamide gel onto a membrane. The Western Blot, also called protein blotting or immunoblotting, consists of several different steps which need to be considered in assay development or optimization. The most important parameters to be considered throughout western blotting are: selection of membrane, transfer technique, blocking agent, antibody concentrations and the antigen detection [59]. Nitrocellulose and PVDF (polyvinylidene difluoride) are the most common membranes used in western blotting. PVDF membranes have the advantage of high protein binding capacity, physical strength and chemical stability. Nitrocellulose membranes, on the other hand are more widely used although they are poor in binding small proteins and an additional membrane incubation step needs to be performed. Both membranes bind the proteins through primarily hydrophobic interactions [60].

Three different techniques can be used to transfer the proteins onto the membrane: simple diffusion, vacuum blotting and electro-blotting. Electro-blotting is the most common technique in protein blotting due to its advantages in speed and completeness of transfer. Two different electroblotting techniques are available: wet transfer and semi-dry transfer. In a wet transfer, the sandwich of gel, membrane and filter paper is placed vertical in a buffer tank with platinum wire electrodes. The semi-dry transfer on the other hand, places the sandwich horizontal between two stainless steel or graphite-carbon plate electrodes which are clamped together. Each layer of the sandwich is well soaked in the transfer buffer which provides the connection for the electrophoretic transfer. The semi-dry transfer has the advantage of blotting several membranes simultaneously,

cheaper equipment and less required power input [60].

The following step after the transfer of the proteins onto the membrane is the blocking of the membrane. This is necessary to mask any potential non-specific binding sites on the membrane itself and to promote renaturation of antigen sites on the proteins. The blocking agent is usually 5% BSA or 5% non-fat milk powder in a Tris buffered saline containing 0.1% Tween-20 (TBST). After blocking the membrane, primary and secondary antibodies needs to be selected and their concentration optimized. Optimization of the correct antibody concentration should be utilized to ultimately provide the best signal to noise ratio and can be achieved conducting a dot blot analysis. Besides the concentration of the antibody a decision between polyclonal and monoclonal antibodies need to made. Polyclonal antibodies have the advantages of having many antibody molecules that bind the target protein antigen and hence resulting in stronger signals. On the other hand this can lead to protein binding which are unrelated to the protein of interest. In this case monoclonal antibodies are favourable, since they are more sensitive and specific for the protein antigen of interest. However, a disadvantage of monoclonal antibodies can be that the specific antigenic site is affected (denatured) by the electrophoresis and no or less antibody binding can occur [60].

The last step in western blotting is the detection of the antigen, mainly using a secondary antibody. This detection can be over immunodetection as chemiluminescent detection or fluoresces labelling. Chemiluminescense detection usually contains an enzyme linked reagent using alkaline phosphatase (AP) or horseradish peroxidase (HRP). The enzyme is coupled with the secondary antibody and catalyses the reaction with a substrate (HRP catalysis the oxidation of luminol), which light output can then be detected on film or by a camera equipped documentation system [61]. If the signal is detected by film no quantitative information can be gained. However, chemiluminescense detection is usually the most sensitive detection technique in western blotting. Another common method is the labelling of secondary antibodies with fluorescence molecules. These techniques have the advantage of being faster and more robust since they eliminate the steps associated with substrate incubation, washing and film exposure [60].

5.1.3 Biacore and BLItz

The Biacore bio-sensor instruments were launched in the 1990 and with a label-free surface plasmon resonance (SPR) technology [62]. The SPR-technology is an optical method, measuring the

refractive index near a sensor surface. The detector measures the changes in the refractive index that occur due to mass variations at the sensor surface when molecular complexes form or break. One binding partner of the molecular complex is bound to the sensor surface, the other one is flowing in a flow cell over this surface to allow binding [62][63]. Figure 5.2 shows a basic set-up of the surface plasmon resonance technology of a Biacore system.

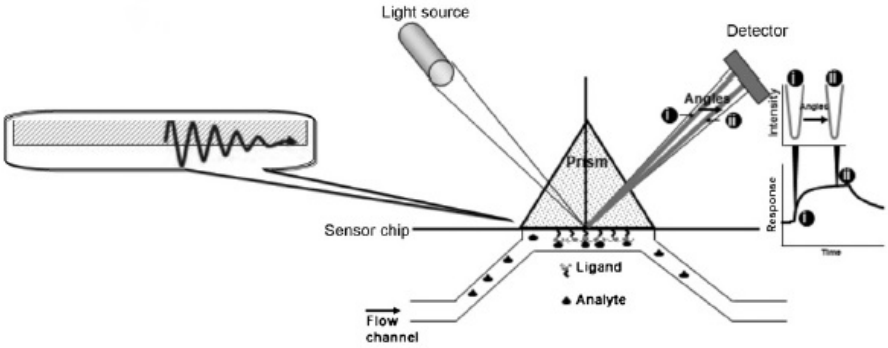

Fig. 5.2: Basic set-up of the surface plasmon resonance technology of a Biacore system. Changes of the refractive index are symbolized with little arrows and indicated with the numbers one and two. These changes are than displayed by the computer software as a change in resonance over time [5].

As it can be seen in figure 5.2, the SPR-technology in the Biacore system is based on a sensor chip which are immobilized with a ligand specific to the target analyte which flows through the flow channel. Several different pre-immobilized chips are available from Biacore, the most common ones are CM4 and CM5 sensor chips. These chips are pre-immobilized with carboxylmethylated dextran on their gold surface. These dextran groups can be attached to different ligands, such as antibodies, protein A or others. In order to immobilize these ligands onto the sensor surface, the dextran groups need to be activated by the addition of N-hydroxysuccinimide (NHS) and N-ethyl-N-(3-dimethylaminopropyl)carbodiimde hydrochloride (EDC). Following the activation, the specific ligand can be immobilized onto the chip surface and remaining binding sites are blocked with ethanolamine [5]. Due to its flexibility in the chip-surface, the Biacore can be used for several application: analysis of kinetic constants, measurement of analytes' concentration, protein-protein interaction and the affinity of an interaction [62].

The BLItz^{TM} system is based on the biosensor technology, similar to the Biacore. Instead of using surface plasmon resonance technology the BLItz system uses label-free bio-layer interfrometry

technology (BLI). Different analyte-specific 'Dip and Read' bio-sensors are pre-immobilized available with different capture molecules, such as Protein A, Anit-Mouse-Capture (AMC) and several more. The biosensor is selected after the affinity to the recombinant protein to be analysed, for example Protein A to determine IgG [64].

A label-free BLI technique is used to measure protein interactions in real time. White light is emitted down the biosensor which is reflected back from the tip surface to the BLItz instrument. The reflected wavelengths are dependent on the coating of the biosensor, resulting in a shift in the wavelength if the target protein is bound to the sensor surface. The wavelength shift, due to the binding of proteins, is collected over a certain amount of time between 30 - 120 seconds and re-calculated in a binding rate in nm/sec. The binding rate can be correlated directly to the specific protein concentration present in the sample [65].

5.1.4 Interferon and its isoforms

This chapter focuses on the characterization of total interferon-α-2b. As mentioned in chapter 2 interferon-α-2b has four known isoforms which are Iso-2, Iso-3, Iso-4-P and Iso-4-R. The total interferon-α-2b amount in process samples includes all fours isoforms and the active drug substance altogether.

A more detailed description of the chemical structure of the different isoforms can be found in the following chapter 6.

5.1.5 Goals and Aims

The aim of this chapter is to characterize the primary protein recovery process on the basis of total interferon amount throughout the process. Samples taken from the manufacturing process at different time-points of the process are analysed for their total IFN amount using SDS-PAGE. Using these results, mass balances for total interferon are set up for each step throughout the recovery process and critical process steps can be identified. The identified critical process steps can then be compared with the findings of chapter 4 to see, if total protein and total interferon behave in a similar way throughout the recovery process.

Hence, the three main aims of this chapter are as follow:

- set up of mass balances for total interferon throughout recovery process
- identification of critical process steps
- comparison between total protein and total interferon characteristics throughout the process

5.2 Materials and Methods

5.2.1 List of Materials

Tab. 5.1: Materials and suppliers

materials	supplier
Recovery Process samples	industrial partner
Trizma Base	Sigma Aldrich
EDTA tetrasodium salt	VWR
Acetic Acid (glacial) 100%	VWR
Methanol	Sigma Aldrich
interferon-α-2b drug substance	industrial partner
NuPAGE® Novex Bis-Tris Gel 4-12%	Invitrogen
NuPAGE® LDS Sample Buffer (4x)	Invitrogen
NuPAGE® Reducing Agent (10x)	Invitrogen
NuPAGE® MES SDS Running Buffer (20x)	Invitrogen
NuPAGE® Antioxidant	Invitrogen
prestained protein ladder, 10-170kDa	Fisher BioReagent
EZ-Run Protein Gel Staining Solution	Fisher
Fast semi dry transfer buffer (10x)	Invitrogen
2.5mm thick blotting paper	Whatman
Nitrocellulose Blotting membrane NOVEX	Invitrogen
TBST-buffer (1X TBS + 0.05% Tween-20)	Sigma Aldrich
anti-IFN-α-2b, mouse monoclonal	Santa-Cruz
anti-IFN-α-2b [9D3], mouse monoclonal	abcam
IRDye 800CW goat anti-mouse IgG (H+L)	LI-COR Biosciences
CM-5 chips	GE Healthcare
Amine Coupling Kit	GE Healthcare
HBS-EP+-buffer 10x	GE Healthcare
Anti-mIgG Fc Capture (AMC) sensors	forte BIO (PALL Life Sciences)

5.2.2 Sample preparation

All sample preparations are performed as described in chapter 4 in section 4.2.2. Only the EOF-sample was analysed in two different forms. First, after sonification to perform cell lysis before starting SDS-PAGE method (followed protocol in section 4.2.2). And second, without performing any cell lysis prior to SDS-PAGE method. In this case, cell lysis was only achieved due to heating and the addition of SDS during performance of SDS-PAGE method (compare following section).

5.2.3 SDS-PAGE method

SDS-PAGE stands for Sodium Dodecyl Sulphate Polyacryl Amide Gel Electrophoresis and separates proteins by size (refer to section 5.1.1). In this experiment pre-cast gels from NuPAGE (Invitrogen by life technologies, Dublin, Ireland) are used to increase reproducibility. Novex ® Bis-Tris gels (Invitrogen by life technologies, Dublin, Ireland) are used with a polyacrylamide gradient of 4-12% for optimal separation of small and medium sized proteins (compare section 5.1.1). The SDS-PAGE method follows mainly the suggested protocol steps from Life Technology-NuPAGE, [15], using a discontinuous buffer system (compare section 5.1.1). The SDS-PAGE protocol followed in these experiments is listed in table 5.2.

Tab. 5.2: Performance of sample preparation for SDS-PAGE with NuPAGE Novex Bis Tris gels 4-12% [15]

component	required volume [μL]
standard/ sample	5.0
NuPAGE® LDS Sample Buffer (4x)	5.0
NuPAGE® Reducing Agent (10x)	2.4
Deionized water	7.6
• vortex samples and incubate at 70°C for 10min (do not boil)	

After sample preparation for SDS-PAGE the following steps are required:

- load 10μL of each standard/ sample to one well of NuPAGE® Novex® Bis Tris gels
- fill inner buffer chamber with 200mL 1X NuPAGE® MES SDS Running Buffer containing 500μL NuPAGE® Antioxidant
- fill outer buffer chamber with 800mL 1X NuPAGE® MES SDS Running Buffer

- run 2 gels at constant voltage, 110V (current $\approx$ 130mA) for 90 minutes

Each gel contains one well loaded with a molecular weight marker, pre-stained protein ladder for 10-170kDa (Fisher BioReagents, Dublin, Ireland), as well as 3 to 4 IFN-α-2b reference standards. The reference standards were diluted with DI-water to concentrations between 1.0 to 0.05g/L.
After the electrophoresis run, four more steps need to be followed:

- place gels in fixing solution (7% acetic acid in 40% (v/v) methanol) for 1h at RT
- stain gels in Coomassie staining solution (EZ-Run Protein Gel Staining Solution, Fisher Scientific, Dublin, Ireland)
- incubate at RT for 45-60min
- destain gels in DI-water over night at RT
- scan and analyse gels, using LI-COR scanner (LI-COR Biosciences, Nebraska, USA)

5.2.4 SDS-PAGE-quantification

After destaining gels in DI-water over night, gels need to be scanned using a LI-COR scanner (LI-COR Biosciences, Nebraska, USA). With the software Odyssey 3.0 scanned gels can be analysed for their densitometry of different protein bands (compare section 5.1.1).
The analysed density of the IFN-α-2b reference standards (in K-points) are used to perform a linear standard curve in the range of 0.05 - 1.0g/L of IFN-α-2b. The determined slope of this standard curve is used to calculate IFN-α-2b amounts of the recovery process samples using their analysed density of the IFN-α-2b band at 19kDa.

5.2.5 Western Blot method

The Western Blot is performed following the electrophoresis run, before fixing and staining of the gels (compare previous section). In this experiment a semi-dry transfer is performed using a nitrocellulose membrane (Invitrogen by life technologies, Dublin, Ireland).
After the completion of the electrophoresis run the gel needs to equilibrate in 1X fast semi dry transfer buffer (Invitrogen by life technologies, Dublin, Ireland) for 10 min, followed by the transfer onto the membrane. Therefore a blotting sandwich needs to be put together following the order displayed in figure 5.3:

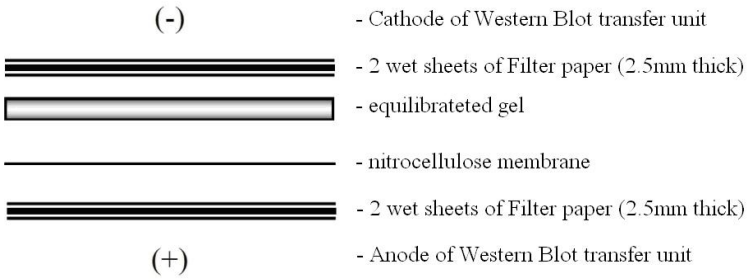

Fig. 5.3: Order of blotting sandwich for semi-dry transfer

The transfer runs at constant voltage (9.0V) for 17min (expected current: 400mA).

After the transfer following steps need to be performed:

- blocking of unspecific binding sites in 5% BSA in TBST (3h at RT)
- 3 washes with TBST for each 10min
- incubation in primary antibody (1/5000 in TBST) over night at 4°C
  use: anti-IFN-α-2b, mouse monoclonal (Santa-Cruz, Texas, USA)
  or: anti-IFN-α-2b [9D3], mouse monoclonal (abcam, Cambridge, England)
- 4 washes with TBST for each 10min
- incubation in secondary antibody (1/10000 in TBST) for 2h at RT
  use: IRDye 800CW goat anti-mouse IgG (H+L) (LI-COR Biosciences, Nebraska, USA)
- 6 washes with TBST for each 10min
- scan membrane, using LI-COR scanner (LI-COR Biosciences, Nebraska, USA)

Western Blot-quantification

After scanning the membranes with the LI-COR scanner (LI-COR Biosciences, Nebraska, USA) and optimization of the intensity levels of the 700 and 800 channels, the software Odyssey 3.0 (LI-COR Biosciences, Nebraska, USA) is used to measure the fluorescence signal of each band. The analysed fluorescence signal of the IFN-α-2b reference standards (in K-points) is used to perform a linear standard curve in the range of 0.05 - 0.5g/L of IFN-α-2b. The determined slope of this standard curve is used to calculate IFN-α-2b amounts of the recovery process samples using their analysed fluorescence signal of the IFN-α-2b bands.

5.2.6 Biacore and BLItz method

Biacore system

The Biacore T100 (GE Healthcare, Buckinghamshire, England) is used for all analysis. The surface of a CM-5 chip (GE Healthcare, Buckinghamshire, England) is activated with an amine coupling kit (GE Healthcare, Buckinghamshire, England) and Protein A is immobilized onto the chip surface. Following the immobilization, the sample analysis is performed is the following order:

- capture IFN-antibody (abcam, Cambridge, England) at 10mg/ml
  flow-rate: 10μL/min, contact time: 120sec
- inject interferon-α-2b reference standard or process sample
  concentration: 0.05-1.0mg/ml, at 10μL/min and 120sec contact time
- regenerate chip with 10mM glycine at pH 2.0 for 120 seconds

The analysed RU signals of the IFN-α-2b reference standards are used to perform a linear standard curve in the range of 0.05 - 1.0g/L of IFN-α-2b. The determined slope of this standard curve is used to calculate IFN-α-2b amounts of the recovery process samples using their analysed RU signals.

BLItz system

The BLItz system (forte BIO, Portsmouth, UK) is used with anti-mIgG Fc Capture (AMC) sensors to quantify interferon-α-2b reference standards. In a first step the anti-IFN antibody (abcam, Cambridge, England) at 0.2mg/ml is immobilized onto the sensor surface with a contact time of 120 seconds. In a second step the interferon-α-2b reference standards are captured by the immobilized antibody at concentration from 0.075 - 0.1mg/ml and a contact time of 120 seconds. The difference between the binding signal of the antibody and the IFN reference standard can be correlated to the interferon-α-2b concentration.

5.3 Results and Discussion

5.3.1 Interferon characteristics throughout Recovery Process

The total interferon-α-2b (total IFN) amount, including IFN-α-2b and all isoforms, was quantified in recovery process samples via SDS-PAGE as described in sections 5.2.3. Figure 5.4 shows the total IFN-α-2b in soluble (green) and in insoluble (blue) fractions throughout the whole primary recovery process. All samples indicated with a red 'W' are samples collected from the waste stream for the particular centrifugation step, which are indicated through an arrow. Standard deviations are calculated from an average error of reference standards that were performed in triplicates and applied for the process samples.

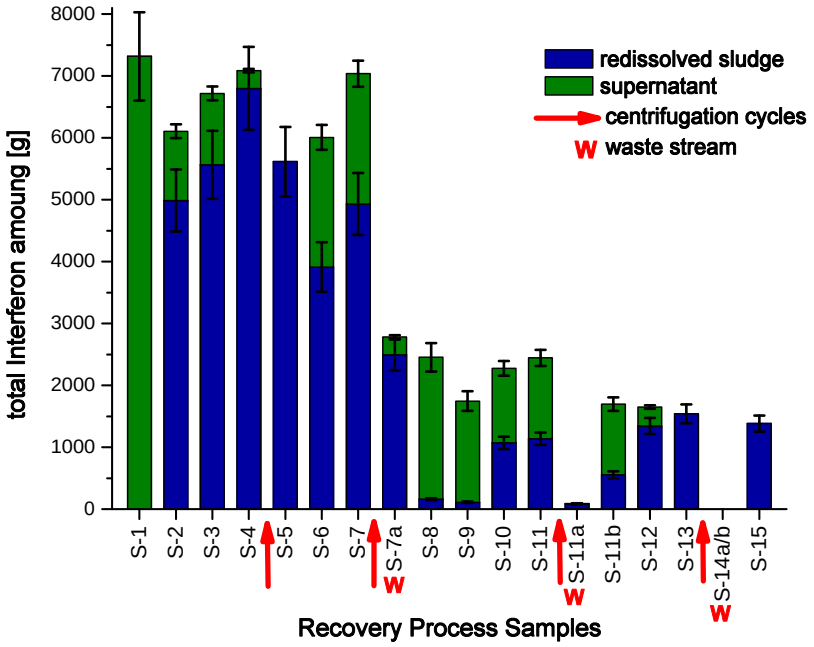

Fig. 5.4: Interferon-α-2b amount in soluble and insoluble fractions throughout the recovery process

Samples S-1 to S-4, representing the acid kill step of the recovery process, illustrate that the functions of the acid kill, to disrupt cells and release whole proteins, are fulfilled. Since the end of fermentation sample S-1 shows similar total IFN amount than the last sample of the acid kill step (S-4), all IFN was released from the cells during the acid kill. Another purpose of the acid kill is to precipitate all proteins, which also worked efficiently since more than 97% of total IFN

in S-4 was found in the insoluble fraction in precipitations. Sample 2 and 3 show lower total IFN amounts compared to S-1 and S-4, which is not as expected. Usually a lower protein amount in these samples indicates that the disruption of the cells and the release of proteins is not fully completed at this stage. Due to the sample preparation for SDS-PAGE, complete cell lysis should occur during the addition of SDS and incubation at 70°C, meaning that similar amounts in total IFN were expected for S-1, S-2, S-3 and S-4. A reason for the lower total IFN amounts in S-2 and S-3 can be found in the imprecise quantification method of densitometry. Keeping in mind that the standard deviation for interferon reference standards is in average 10%, the total IFN amounts of S-1 - S-4 are within this variation.

Looking at the total IFN amount in cycle-1 (S-5 - S-7) similar unexpected variations between the samples can be seen. Due to inefficient continuous centrifugation a decrease of around 15-20% between S-4 to S-5 in total IFN amount was expected (compare chapter 4, section 4.3.1), which remained constant in samples 6 and 7. However, including the 10% variations into the calculations all three samples result in a constant total IFN amount with around 15% decrease compared to sample 4. The distribution of soluble and insoluble fractions in total IFN in sample 6 and 7 are as expected. Only up to 35% of total IFN gets resolubilised during cycle-1, leaving 65% of total IFN remaining in precipitated form.

The insufficient solubilisation of total IFN in cycle-1 explains the low amount of recovered total IFN in cycle-2, which represents only 40% (compare S-8 in figure 5.4). This indicates that only the total IFN in the soluble fractions was recovered during the centrifugation cycle. The total IFN in the insoluble fractions was detected in the waste stream in S-7a. During cycle-2 the salting-out of *E.coli* host cell proteins should take place. However, as it can be seen in S-10 and S-11 in figure 5.4, precipitation also occurs in total IFN of up to 50%. This indicates that the salting-out step is not protein specific for interferon-α-2b.

In cycle-3 a reduction of around 30% in total IFN is detected compared to the end of cycle-2. The reduction can be explained due to the precipitates of total IFN formed in cycle-2. The majority of the precipitates of total IFN end-up in the waste stream during the centrifugation cycles and were partly detected in S-11a. During cycle-3 a precipitation of all proteins should occur due to the addition of TCA. This process is efficient, since all detected total IFN was in precipitated form in sample 13. Due to the efficient precipitation of total IFN in cycle-3 the majority is being recovered

in the TCA-pellet at the end of the recovery process.

5.3.2 IFN mass balances throughout the Recovery Process

All mass balances of total IFN throughout the recovery process were performed using the results of batch 2-AVAF-206 shown in figure 5.4. Since the analysis was performed only once, no standard deviations are available.

Acid Kill $\rightarrow$ Cycle 1

The first mass balance is between the acid kill and cycle-1. The formula 5.1 illustrates the samples of the recovery process that have been used for this balance.

$$S-4 \implies S-5 + waste \tag{5.1}$$

Inserting the analysed total interferon-α-2b quantities for each sample results in mass balance 5.2.

$$7\,086.6g \implies 5\,614.4g + not\,measured\,(1\,472.2g) \tag{5.2}$$

Only 80% of total IFN was recovered during centrifugation cycle-1, indicating that around 20% ends up in the waste stream. Unfortunately no sample is available from this waste stream to proof this assumption. However, as already mentioned in the previous sections a reduction in protein of 15-20% was expected due to insufficient separation of particles and supernatant in a continuous centrifuge as it has been used during this centrifugation cycle.

Cycle-1 → Cycle-2

The next mass balance needs to be set up between cycle 1 and 2. Formula 5.3 gives information about the recovery process samples that are needed for this balance.

$$S-7 \implies S-8 \; + \; S-7a \tag{5.3}$$

Inserting the analysed total interferon-α-2b quantities for each sample results in mass balance 5.4.

$$7\,037.38g \implies 2\,453.2g + 2\,780.6g \tag{5.4}$$

In formula 5.4 35% of total IFN was recovered after centrifugation cycle 2 but only 40% of total IFN was detected in the waste stream. Leaving an uneven mass balance with 25% of total IFN undetected. As described in the section above, the quantification via SDS-PAGE has a mean standard deviation of 10%. Due to variations in total IFN amount in the three samples of cycle-1 (compare figure 5.4) it is likely that S-7 contains only 6000g of total IFN instead of 7000g. This would lead to a 40% recovery of IFN in S-8 and up to 48% of IFN in the waste stream (S-7a). The difference of 12% to an even mass balance is within the standard deviation of the method.

Cycle-2 → Cycle-3

The third mass balance is between the cycle 2 and cycle-3. The formula 5.5 illustrates the samples of the recovery process that are been used for this balance.

$$S-11 \implies S11b \; + \; S-11a \tag{5.5}$$

Inserting the analysed total interferon-α-2b quantities for each sample results in mass balance 5.6.

$$2\,444.6g \implies 1\,695.4g + 91.8\,g \tag{5.6}$$

The mass balance 5.6 indicates that only 70% of total IFN is recovered during centrifugation cycle 3. Hence, 30% of total IFN is lost in this step but only 4% could be detected in the waste stream. This shows that only 74% of total IFN was detected after the centrifugation cycle 3. The missing difference of 24% to a complete mass balance can be explained by the significant standard deviations of the quantitative method.

Cycle-3 $\rightarrow$ TCA-pellet

The last mass balance needs to be set up between cycle 3 and the TCA-pellet. Formula 5.7 gives information about the recovery process samples that are needed for this balance.

$$S-13 \implies S-15 \;+\; S-14a/b \tag{5.7}$$

Inserting the analysed total interferon-α-2b quantities for each sample results in mass balance 5.8.

$$1\,539.9g \implies 1\,382.3g + 0\,g \tag{5.8}$$

The last balance for total IFN of the recovery process results in an even mass balance, since 90% of total IFN was recovered in the TCA-pellet. Including the significant standard deviations of the SDS-PAGE method of 10%, this recovery can be add up to a 100%. This would also explain that no IFN was detected in the waste stream of this centrifugation cycle. Besides a complete recovery of total IFN in the TCA-pellet, the untraceable IFN in the waste stream can be below the detection limit of this method. Ten percent of total IFN corresponds to 160g of IFN resulting in a concentration 0.02g/L at this stage of the process. The limit of detection of IFN using SDS-PAGE

is 0.05g/L.

5.3.3 Comparison of IFN-amount between 4 batches

To study the reproducibility of interferon-α-2b recovery throughout the recovery process, four different batches of recovery process samples were analysed via SDS-PAGE for their total IFN amount and the average in percentage was calculated for each sample. The average total IFN amount of four batches for each sample of the recovery process can be seen in figure 5.5.

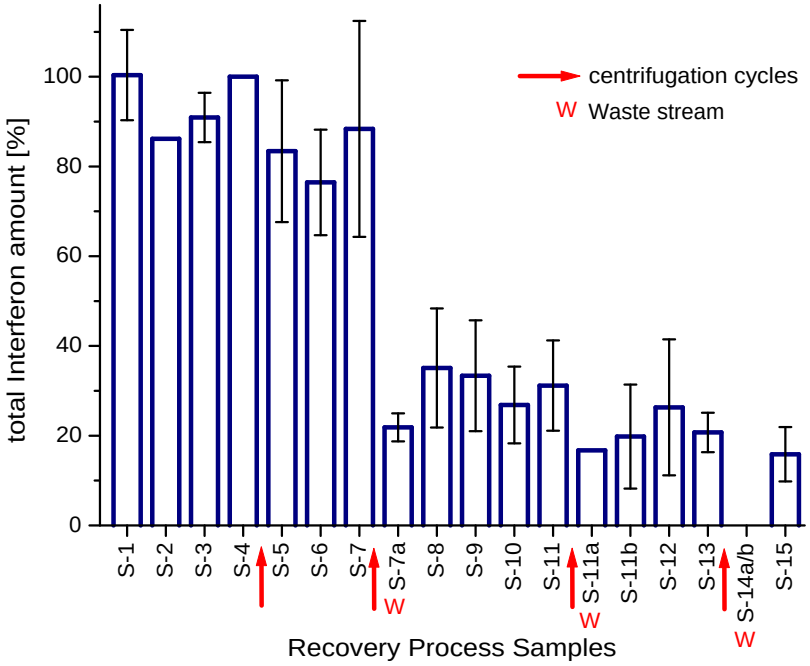

Fig. 5.5: Average of interferon-α-2b amount of 4 different batches throughout the recovery process

Figure 5.5 indicates that the characteristics of total IFN throughout the recovery process is consistent. Two critical process steps (CPS) can be identified. The critical process step one is between cycle 1 and 2 where more than 60% of total IFN is lost in the waste stream (S-7a) and only 40% is recovered (S-8). The second critical process step is between cycle 2 and 3. After this centrifugation cycle 70% of total IFN is recovered (S-11b) and up to 30% ends up in the waste-stream (S-11a). As it can be seen in figure 5.5 this IFN reduction in CPS-2 is indeed in between the standard deviations of sample 11 and 11b, meaning the detected changes are not significant. However,

since samples 8, 9 and 10 result in comparable results to S-11 and on the other hand samples 13 and 15 result in comparable IFN amounts to S11b, the reduction in total IFN between these two sample sets can be seen as significant.

The significant big standard deviations of these results do not necessarily indicate big variations in total IFN between the different analysed batches. It is more likely that these deviations are due to an imprecise quantitative method. Besides the common errors that affect the standard deviation, such as dilution errors and performance errors of the method, the SDS-PAGE method also has an important error due to the quantification via densitometry (compare section 5.2.4). After scanning the gels, every single band appearing at 19kDa needs to be marked with a square border, before the band density is determined. Besides execution errors during this analysis, two other factors can falsify the analysis at this stage.

The IFN-samples with known concentration that are used as a reference standard contain only purified IFN material, meaning the detected density of the band is only due to interferon. However, the recovery process samples contain less than 4% IFN and over 96% of the total amount of proteins are host cell proteins (HCP) from *E.coli*. Hence, marking the band at 19kDa with a square border can lead to the contaminent of other proteins into the analysis of the band-density. Figure 5.1.1 shows the scanned-image of a SDS-PAGE with a molecular weight marker in lane 1, the purified interferon standard material at two concentrations in lane 2 and 3, and 9 different recovery process samples.

In order to reduce the influence of host cell proteins on the interferon quantification, sample dilutions are necessary. However, since the IFN amount is less than 4% of the overall total protein amount, high dilutions would lead to very low IFN concentrations. Due to repeated analysis of purified interferon material, standard deviations of 8.5% were detected for IFN concentrations between 0.5 - 1.0g/L and up to 20% deviation for IFN concentrations between 0.05 - 0.1g/L. This indicates that the selected dilution of each sample needs to be balanced to minimize the influence of host cell proteins on the analysis but also to maximize the IFN-concentration in the assay.

On the other hand, since more than 96% of total protein represent host cell proteins from *E.coli*, it is possible that there are other proteins beside interferon with a molecular weight of 19kDa. As described in section 5.6, SDS-PAGE separates proteins based on molecular weight. If a protein mixture contains more than one protein with the same or similar molecular weight, no differences

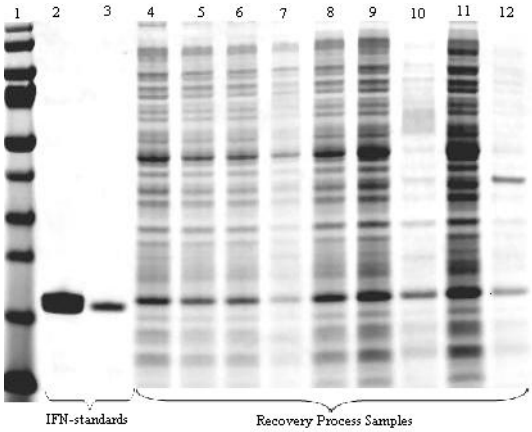

Fig. 5.6: Scanned SDS-PAGE image of purified interferon standard material and different recovery process samples

can be detected between these proteins. Meaning all proteins with the same molecular weight appear only in one band on the SDS-PAGE. In our case it would lead to an overestimation of the total interferon in the recovery process samples.

To confirm the detected total IFN amounts throughout the recovery process a more specific analytical method needs to be performed, for example Western Blot or HPLC/ UPLC.

5.3.4 Comparison of total protein and total Interferon characteristics

The detected amount of total protein throughout the recovery process discussed in chapter 4 is compared to the analysed amount of total IFN in the recovery process samples. Figure 5.7 shows the comparison between total protein and total IFN in percentage throughout the whole recovery process, whereas sample 4 was set to 100%.

Both, total protein and total IFN, follow a similar characteristic throughout the recovery process with two major critical process steps. The first critical process step was identified between cycle 1 and 2 for total protein as well as for total interferon and also the second critical process step is identical for total protein and total IFN between cycle 2 and 3.

The loss in interferon in both critical process steps is not as distinctive as it is in total protein. Table 5.3 lists the average losses in total protein and total IFN during the two identified critical process steps as well as the overall loss throughout the recovery process.

The overall loss throughout the recovery process of 90% in total protein and 80% in total IFN

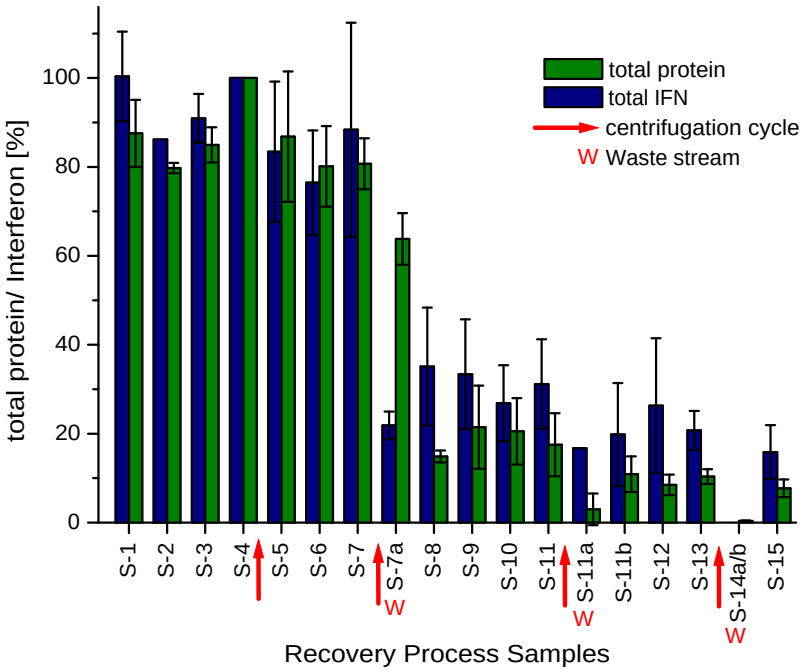

Fig. 5.7: Total interferon-α-2b characteristics compared to total protein amount throughout the recovery process

indicate that this primary recovery process is not protein specific for interferon-α-2b. Two critical process steps were identified throughout the primary protein recovery process for total protein (compare chapter 4) and total interferon-α-2b (compare section 5.3.3).

5.3.5 Alternative methods for total Interferon-α-2b quantification

Until now, all results for total interferon-α-2b have been quantified using SDS-PAGE. As explained before in section 5.1.1, SDS-PAGE does not specifically detect interferon but distinguishes between proteins only by size. It is possible that several proteins with the same or similar molecular weight than interferon are present in the recovery process samples. Accordingly, the SDS-PAGE detects only the mixture of proteins with the same molecular weight and thus, it may overestimate the amount of interferon in the recovery process samples.

In order to obtain more precise information of the actual quantity of interferon-α-2b in recovery process samples several different techniques were tested for potential to quantify total interferon. These techniques are: Western Blot, Biacore and BLItz and their results are discussed in this sec-

Tab. 5.3: Summarising total protein loss and total interferon loss throughout the entire recovery process

critical process step (CPS)	total protein loss [%]	total IFN loss [%]
CPS-1 (Cycle-1)	80.0%	60.0%
CPS-2 (Cycle-2)	40.0%	30.0%
overall loss in PRP	**90.0%**	**80.0%**

tion.

The first method used for a more specific quantification of interferon-α-2b in recovery process samples is the Western Blot. The Western Blot separates the protein mixture by size, followed by a protein-specific quantification using an antibody specific for interferon-α-2b (compare section 5.1.2).

All Western Blot quantifications were performed as described in section 5.2.5. Figure 5.8 shows the linear relationship between interferon-α-2b and the densitometric area of the fluorescence signal analysed using Western Blot between 0.5 and 4.0μg interferon.

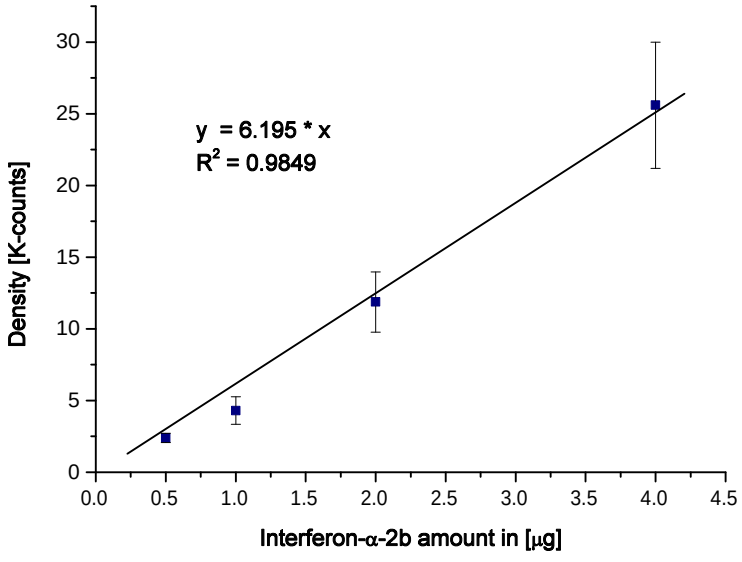

Fig. 5.8: Linear regression of total interferon-α-2b reference standards analysed with Western Blot

Figure 5.8 shows a linear relationship between interferon and fluorescence intensity with a R^2-value of 0.9849. The slope of the linear regression is 6.195 [K-count/μg], which can be used to

determine interferon in process samples over the measured fluorescence intensity. Results also indicate an increase in the variation of the interferon quantification for higher protein amounts. Hence, this quantification method is more precise for IFN below 2.0μg which corresponds to an IFN concentration of 0.2mg/ml. If required, process samples should be diluted to a final interferon concentration below 0.2mg/ml with DI-water.

Observations throughout the method development showed that the measured fluorescence intensities are highly dependent on the incubation time of the IFN-antibody and on the protein transfer from gel to membrane. Hence, it is important to run 3 to 4 IFN reference standards with each membrane and perform a new linear regression for each run to quantify IFN in process samples more accurately.

Overall, this Western Blot can be used to quantify total interferon-α-2b in process samples, however, the method is not very accurate. It shows high variations with errors of up to 15%, especially at higher interferon concentrations (>2μg). Consequently, 5 samples of the recovery process batch 2-AVAF-206 were analysed for their total amount of interferon-α-2b with this Western Blot method. Each sample was run in quadruplicates on the same membrane with 4 IFN reference standards that were used for the IFN quantification.

Figure 5.9 shows the results of total interferon-α-2b for the 5 five samples of the recovery process batch 2-AVAF-206 quantified by Western Blot and by SDS-PAGE.

The interferon-α-2b results quantified with the Western Blot show significantly lower values of IFN compared with the SDS-PAGE method throughout the recovery process. In the end of fermentation sample 3'200g of total interferon-α-2b were quantified, which is reduced throughout the primary recovery to yield 800g of interferon. The majority of this loss occurs during cycle-1 (between S-7 and S-8), where 65% of the initial IFN is lost. This process step was identified as critical process step 1 from previous results for total protein and total interferon. Between S-11 and the final TCA-pellet (S-15), an additional 30% loss in interferon-α-2b was detected with the Western Blot results, which is the critical process step 2. Overall, a 75% loss in interferon-α-2b was detected with the Western Blot results throughout the primary protein recovery process.

These findings are consistent with the SDS-PAGE results for interferon-α-2b. However, comparing the interferon results quantified with SDS-PAGE with the results quantified with Western Blot (figure 5.9), it can be seen that the SDS-PAGE overestimates the amount of interferon in recovery

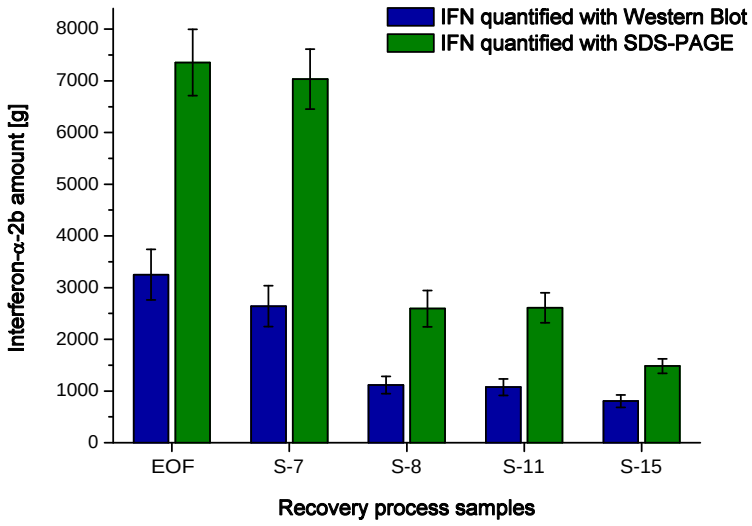

Fig. 5.9: Total interferon-α-2b quantified with Western Blot and SDS-PAGE in recovery process samples of batch 2-AVAF-206

process samples. The SDS-PAGE consistently quantifies twice as much interferon-α-2b compared to the Western Blot. These findings were not unexpected, since SDS-PAGE only distinguishes proteins by size such that host cell proteins with a similar molecular weight than interferon would falsify the SDS-PAGE results. The Western Blot results should only detect the interferon which interact with the specific antibody for interferon-α-2b. Accordingly, these results should be more precise and should give more reliable information about the actual quantity of interferon-α-2b in recovery process samples. However, it is not clear if all isoforms of interferon-α-2b bind to the specific antibody. If some isoforms do not bind to the antibody for interferon-α-2b, Western Blot results would underestimate the total interferon amount in recovery process samples.

In summary it was seen, that the SDS-PAGE results did overestimate the amount of interferon-α-2b in recovery process samples. Western Blot results for interferon were generally only half the amount of the SDS-PAGE quantities. However, the percentage reduction of interferon throughout the recovery process remained the same. During the critical process step 1 in cycle 1, over 65% of the initial interferon-α-2b is lost and an overall loss of 75% in interferon-α-2b was detected throughout the whole protein recovery process.

Besides Western Blot, two other techniques were tested for their potential to quantify total interferon-

α-2b in recovery process samples. These techniques are the Biacore and the BLItz system. Both techniques are based on the interaction of a specific interferon antibody to interferon-α-2b. The IgG-antibody itself is immobilized on the chip or sensor surface where it interacts with Protein A or an anti-mouse Fc-capture-ligand (compare section 5.1.3). Experiments with the Biacore and BLItz system were carried out as described in section 5.2.6 with the interferon-α-2b drug substance as the reference standard.

During the method development for both techniques, Biacore and BLItz, an unstable baseline of the antibody - interferon complex was observed. This indicates that the affinity between the antibody and interferon-α-2b is not sufficient and the binding-interaction is very weak. Due to this observation four different commercialised antibodies for interferon-α-2b from different suppliers were tested (Santa-Cruz, Sigma and Abcam). The antibody, which showed the highest binding affinity towards the interferon-α-2b drug substance was used for further experiments (interferon-α-2 antibody [4E10] from Abcam).

Another observation made during the method development is the poor resolution of both methods. Both methods quantify the proteins over a shift in the refractive index (Biacore) or wavelength (BLItz) due to the binding of molecules on the chip/ sensor surface. The immobilization of the specific IgG-antibody results in a significant shift in the refractive index or wavelength of the Biacore/ BLItz system, due the molecular weight of the IgG-AB of $\approx$150kDa. Unfortunately the binding of interferon-α-2b, which is only 19kDa, does not result in a significant shift in the refractive index/ wavelength, especially at lower IFN concentrations. Hence, the limit of detection for the Biacore is 0.3g/l which is higher than the average concentration of interferon-α-2b in recovery process samples. The BLItz system showed a limit of detection of 0.08g/l but the upper limit of detection was identified at 0.1g/l due to saturation of the sensor surface. This results in only a small detection range, which makes it challenging for protein quantification of process samples.

Due to the two observations made during method development, no increase in accuracy and resolution for total interferon-α-2b quantification in recovery process samples was expected with the Biacore or BLItz system compared to the existing results with SDS-PAGE and Western Blot. Hence, no further analysis was carried out with the Biacore and neither the BLItz system.

5.4 Conclusion

This chapter studied the characteristics of total interferon-α-2b throughout the primary recovery process. An overall loss of up to 80% was detected, which is 10% less than detected for total protein (see chapter 4). Two main critical process steps (CPS) were identified throughout the process which confirm the findings of total protein mass balances. CPS-1 is between cycle 1 and 2 due to insufficient protein resuspension. Critical process step 2 is between cycle 2 and 3 where salting-out of protein occurs not only in host cell proteins but also in interferon-α-2b. Since total protein and total interferon-α-2b show very similar characteristics throughout the entire primary recovery process, it can be concluded that the process is not protein specific.

Since the method used for interferon quantification is known not to be very accurate for specific protein quantification, some samples of the recovery process were quantified with an alternative method, the Western Blot. Results showed that the SDS-PAGE method indeed overestimated the IFN amounts in recovery process samples of about 50%. However, the characteristics of interferon-α-2b throughout the recovery process were similar to the findings with SDS-PAGE. The overall loss in interferon quantified with the Western Blot method was 75% with the majority loss occurring during cycle 1, which was identified as critical process step 1.

The SDS-PAGE and Western Blot methods only quantify the total interferon-α-2b amounts but do not distinguish between the different isoforms of interferon that are present in recovery process samples. Hence, in a next step the characteristics of the four different IFN isoforms throughout the protein recovery process need to be investigated. These results can be found in the following chapter 6.

Beside the characteristics of the IFN isoforms, a next step is the optimization of identified critical process steps of the primary protein recovery process. Since CPS-1 was shown to have a bigger impact on the overall loss of interferon, this steps needs to be investigated and optimized first. The main problem in cycle 1 in an insufficient resuspension of interferon in water. Hence, a solubility study should be performed to ascertain the potential to increase interferon solubility during this cycle. The results of the solubility study and other optimization steps of the protein recovery process can be found in chapters 7 and 8.

Chapter 6

Process Characterization - Interferon-Isoform Mass Balances

6.1 Introduction

This chapter focuses on the characterization of the primary protein recovery process of interferon-α-2b looking at the different interferon isoforms present. Mass balances throughout the entire recovery process need to be set up to investigate critical process steps for different interferon isoforms, as well as transformations from one isoform to the other.
The first part of this chapter focuses on the development and optimization of quantification methods to distinguish between the different interferon isoforms. Quantification of low protein concentrations in the presence of host cell proteins can be challenging and often requires purification and concentration steps prior to the analysis. Hence, the second part of this chapter focuses on the development of a purification and concentrating step for interferon isoforms in recovery process samples prior to the protein quantification. The last part discusses the obtained characteristics of interferon isoforms throughout the primary protein recovery process.

6.1.1 Interferon-α-2b and its isoforms

As mentioned in chapter 2, during the *E.coli* fermentation at manufacturing scale, not only the active drug substance interferon-α-2b is produced, but also 4 different isoforms. These isoforms

are called Iso-2, Iso-3, Iso-4-P and Iso-4-R. Isoform 4 can be divided into Iso-4-R and Iso-4-P with Iso-4-P as the pyruvated form of isoform 4 and Iso-4-R as the non-pyruvated form. The chemical structure of isoforms 2, 3 and 4 can be seen in figure 6.1.

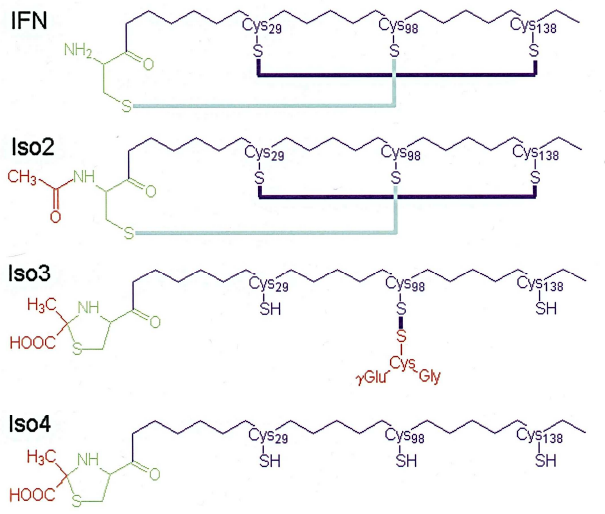

Fig. 6.1: Interferon-α-2b and three of its four Isoforms [6]

The differences between the isoforms and the active drug substance are in their number of disulphide bonds and post-translational modifications (PTM) in the characteristic groups. The PTMs for Iso-2 are an acetylation and oxidation at the N-terminal of Cys^{1}, for Iso-3 and Iso-4 the disulphide bridge between Cys^{29} and Cys^{138} is partial reduced and the disulphide bridge between Cys^{1} and Cys^{98} is fully reduced. The N-terminal of Cys^{1} is bonded to a pyruvic acid. Iso-3 also has a Glutathione attached at Cys^{98}.

Due to previous studies it is known that during the fermentation the isoforms are produced rather than the active drug substance. Isoforms such as Iso-4 can be transformed into the active drug substance at a later stage of the down-stream process. This conversion can be seen in figure 6.2. Therefore it is of high interest to follow the isoform distribution throughout the production and recovery process prior to down-stream processing.

This chapter focuses on the characteristics of the different interferon isoforms throughout the primary protein recovery process. Mass balances on each isoform should be set-up throughout the recovery process to identify transformations of one isoform to the other or increased protein loss of one particular isoform at different steps of the process.

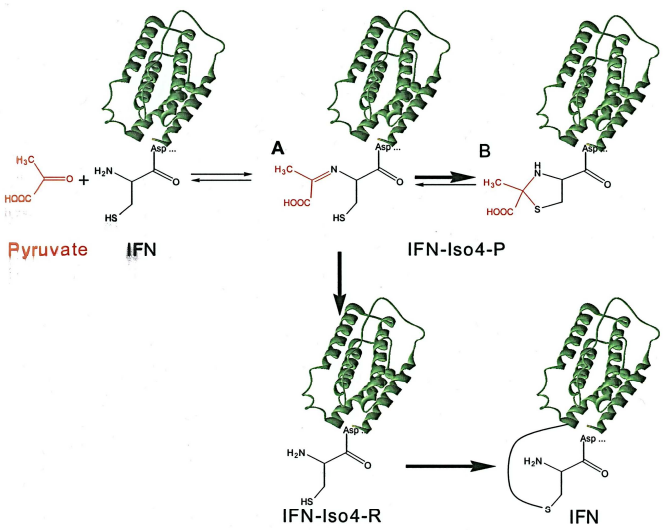

Fig. 6.2: Conversion of Iso-4-P and Iso-4-R into the active drug substance interferon-α-2b [6]

6.1.2 Reverse phase high pressure liquid chromatography

High performance liquid chromatography, or HPLC, is an analytical technique widely used in chemistry, biology and numerous other research fields. It is a major tool for analyses of biomolecules and it can be used for a wide range of protein types and sizes [66]. Its principle lies in liquid solid interactions where the molecules of interest interact with the solvent and the solid phase of a column. Depending on the affinity of the molecules towards the mobile phase or the column, the compound will pass through the column slower or faster. In the end, separated compounds reach the end of the column where they are detected at different time points [66][67].

A wide variety of columns exists to exploit different physical and chemical properties. The most popular one for protein separation and quantification is reverse phase HPLC (RP-HPLC). This technique separates molecules by polarity using a stationary phase covered with hydrocarbon chains (typically C-8 or C-18) [68]. Affinity with the mobile phase is set using a mix between water and on organic solvent (typically methanol or acetonitrile). Compounds are thus separated according to their hydrophobicity [69]. Reverse phase HPLC is also known to distinguish between closely related molecules with similar chemical structures as protein isoforms [70].

The resolution, sensitivity and reproducibility of a reverse phase HPLC run is dependent on several different factors, such as mobile phase, flow rate, column temperature, injection volume and protein load [70].

The mobile phase is composed of water and organic solvents. During isocratic elution mode, the composition of the mobile phase is constant. An alternative is gradient elution with varying mobile phase composition throughout the elution. Ion pairing agents such as TFA (trifluoroacetic acid) are commonly added to the mobile phase in order to improve the separation and to buffer the mobile phase [71].

The flow rate is defined as the linear velocity of the mobile phase through the column. The increase of flow rate is limited by the maximal pressure capacity supported by the column. An increase in the flow rate has a significant impact on the retention time of an analyte, since it increases the dynamics and kinetics of the interaction between the analytes and the mobile phase [72].

Column temperature modifies the chemical kinetics, therefore affects sample affinity to the column and to the mobile phase [73]. An increase in the column temperature also decreases the mobile phase viscosity and therefore facilitates the movement of samples through the column. High column temperature can also cause protein denaturation, modification or degradation and thus modifies its behaviour in the column [70].

The injection volume and the protein load determine the actual protein amount injected onto the column for analysis. If the column load exceeds the limits of the dynamic capacity of the column, this leads to local saturation of the column. Hence, the protein load and injection volume need to be adjusted to each other to avoid overloading of the column [67].

6.1.3 Design of Experiments - MODDE software

With the still increasing effort but the limiting factor of time and supplies, it is getting more important to have an established plan and a structured analysis of different experiments. It is important to receive the maximum information out of the experiments about a system with a minimal labour input. In this case it is helpful to use the Design of Experiment (DoE). One example is the optimization of analytical methods such as HPLC methods, where several different factors have to be considered on the same time. The Design of Experiments is used to plan the amount of experiments and to analyse them afterwards in an appropriate way. Depending on the amount of input variables (factors) and the amount of different levels of the input variables the DoE arranges different experimental designs. The goal of the DoE is to determine the significant input variables and their interactions and to describe the process output with a mathematical equation depending

on the significant input variables. With the received information out of the DoE-analysis the dependency of the inputs and outputs are statistically evaluated and the effect of the input variables and their interactions to the output are quantifiable.

6.1.3.1 Different experimental designs

The basic design of experiments is simple and straightforward in that the effects of multiple input factors are varied and investigated in relation to response variables. The basic designs are called full factorial designs. A full design applies a lot of experiments in order to cover every possibility. To reduce the number of experiments a fractional factorial design can be performed first, which provides sufficient information about which factor is significant or insignificant. In order to use design of experiments as an optimization tool, more information about the different interactions between the input variables and the output are required. In this case a central composite factorial design (CCF-design) can be used. For this design different experiments will be added at a third level (the center level). Figure 6.3 shows an example of a face centred central composite factorial design of three factors [7].

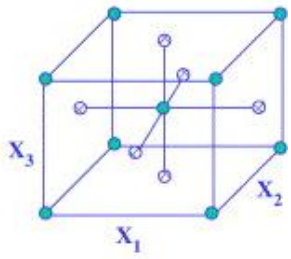

Fig. 6.3: A central composite factorial design for three factors [7]

Depending on the amount of input variables and their interactions to the output the Design of Experiment supports to plan the experiments and to analyse the results in an appropriate way to receive as many information as possible.

6.1.3.2 Linear regression models

The concept of linear regression or regression analysis deals with finding the best possible relationship between a response and the independent variables of a system. This type of analysis

also aims to determine the strength of this relationship and allows for greater predictions of responses. Simple linear regression predicts the best fitting line between response data and a single independent variable [74]. The use of more than one independent variable is called multiple linear regression, however, this section will focus on simple linear regression.
In the age of powerful computing a regression line between data points is usually done using a software. Simple linear regression is a method of determining the best possible fit of the data in relation to the desired response [75].

Evaluation of the model quality can be made using several factors. R^2, Q^2, model validity and reproducibility are given by the 'summary of fit'. These values can determine if the model is valid and can also explain some variations between the different models and the actual defined working parameters after optimization.

R^2 and Q^2

R^2, the least square coefficient, is one of the most common ways to evaluate a model. It is the fraction of the variations of actual values from predicted values.
It is given by:

$$R^2 = \frac{SS - SS_e}{SS} \tag{6.1}$$

With:

$SS = \sum_i Y_i^2$	or the sum of the square of the values for the model (sum of squares).
$SS_e = \sum_i \left(Y_i - \hat{Y}_i\right)^2$	or the sum of the square of the difference between real and predicted values (sum of squares of residuals).

Thus, R^2 corresponds to the difference between the values given for the creation of the model and the model itself. A perfect model gives no difference between predicted and actual values, thus $SS_e = 0$, and $R^2 = 1$ [74].

Q^2, or cross-validation, defines the prediction ability of a model. It is a fraction of an actual value of the prediction value, but the value itself is not part of the values used to create the model [75].

It is given by:

$$Q^2 = \frac{SS-PRESS}{SS} \tag{6.2}$$

with PRESS as the prediction residual sum of squares. As for R^2, it is a value ≤ 1, with 1 as the best value [76].

Comparison of these coefficients gives an excellent indication on the quality of a model. Since R^2 usually overestimates the fitting, and is well complemented with Q^2. Thus, a good model implies close R^2 and Q^2, with a generally admitted difference of less than 0.2-0.3 [76].

Model validity

This value based on the F-test of $MS_{model\ error}/MS_{pure\ error}$ indicates the validity of the model, if the model error is in the same range as the variations of the values [76]. A value over 0.25 indicates no lack of fit. It needs replicates to be calculated, and in the case where replicates are not in a sufficient number and/or show a lot of variation, this value can be less considered than R^2 and Q^2 [75]. It can be noticed that the F-test, which is based on the model validity, is dependent on the degrees of freedom (DF), itself related to the number of runs and replicates. It is also related to the number of independent variables of the model: for instance, 2 parameters A and B in a quadratic model, lead to the independent variables A, B, AB, A^2 and B^2. However, during the creation of the model, some of these variables are not significant and are excluded. Although this can improve the quality of the model, this costs degrees of freedom (DF) [74]. Negative values can appear with an insufficient number of runs, replicates and/or degrees of freedom. These can also appear if the replicates are too close to each other. Indeed, this causes a lack of fit of 0, and is reflected in the model validity calculation by a negative value.

Reproducibility

Reproducibility is given by:

$$reproducibility = 1 - \frac{MS_{pure\ error}}{MS_{total\ corrected}} \tag{6.3}$$

With: MS = $\frac{1}{N}\sum_i \left(\hat{Y} - Y\right)^2$, the mean square [74]. This value assesses the variation of points at the same conditions, replicates or center points, compared to the general variation of the model. A value below 0.5 indicates the pure error is important and the control on the procedure is poor [76].

6.1.3.3 ANOVA, F-Test and Lack of Fit

Analysis of variance (ANOVA) is a statistical technique used to examine and model the relationship between a response variable and one or more independent variables. ANOVA can also be used to compare two alternative models (one of which may have been refined) to evaluate the performance [76]. One way to calculate the analysis of variances is the F-test.

The F-test compares the model variance with the residual variance and calculates if the model-coefficients are adequate and significant. If the variances are similar (close to one) it is less likely that any of the variables have a significant effect on the response [75].

Usually the calculated F-value will be compared with a theoretical value of the F-distribution which is listed in statistic tables. To avoid this step, there is the possibility to calculate a p-value. This p-value calculates the proportion of the area under the curve of the F-distribution that lies beyond the observed F-value. The p-value has to be smaller than 0.05 (with a 95% confidence interval) to assure the model-coefficients have a significant effect on the response and the model or the coefficient is significant. If the model is not significant there is the possibility to perform some replication measurements to improve the model. If any model-coefficients are not significant these variables can be removed from the model [74].

The next important statistic parameter is the Lack of Fit (LOF). The LOF is the variation of the data around the fitted model. Meaning the variation of the replicates about their mean values should be less than the variation of the design points about their predicted values. If this statement applies the LOF is greater than 5.0 % and the Lack of Fit is not significant. A significant Lack of Fit does not mean that the whole model does not fit well to the model. It is always important to check the experimental conditions. Were the replicates performed independently? If they were just repeated measurements it is likely that the error has been underestimated and the LOF is artificially small. In this case the LOF is no longer a valid test. It is also important to look at the Degrees of Freedoms which are left over to calculate the LOF. As more leftover degrees of freedom exist to

calculate the Lack of Fit as more precise will be the result of the LOF. There are so many Degrees of Freedom as experiments were performed. The calculation of every model-coefficient needs one DF and two for the calculation of the total error [75][74].

For this work all design and experiments and statistical analysis were carried with the support of the software MODDE 10.0 from Umetrics.

6.1.4 Purification and concentrating techniques for protein mixtures

Production of recombinant proteins with bacterial cells result in the presence of a large fraction of host cell proteins at the end of the fermentation and the beginning of the down-stream process. This can lead to a decrease of the detection sensitivity of the analytical method. Furthermore the concentration of recombinant proteins are very low at this stage of the process and are often at the limit of quantification. For that reason certain purification and concentrating techniques for protein mixtures are available in order to separate the recombinant protein from host cell proteins to increase the sensitivity level of the analytical methods.
The following sections describe some potential purification and concentrating techniques for the separation of the recombinant protein from host cell proteins.

6.1.4.1 Ion-exchange chromatography

Ion-exchange chromatography, or IEC, is a type of separation based on the charges of a molecule. In other terms, the separation occurs thanks to the electrostatic interactions between a charged molecule and an immobilized material of the opposite charge, the ion-exchanger [77]. A cation-exchanger binds positively charged molecules and an anion-exchanger binds negatively charged molecules [78]. For protein separation using IEC, pH is extremely important. Depending on the pH of its environment and its pI, a protein will have a positive or negative net charge. At a specific pH, name the isoelectric point (pI), the net charge is zero, meaning that all positive charges of the various amino acids compensate the negative charges [79]. This point is very important when working with proteins (or any other zwitterions). The pI generally corresponds to the minimum of solubility of zwitterions, and precipitation often occurs at this pH. Moreover, if the pH is below the

pI net charge of the molecule is positive, and negative if the pH is above the pI. With knowledge of this point, it is possible to estimate if a molecule carries a positive or a negative net charge at a given pH [78].

The conductivity of the medium, closely related to the salt concentration, also plays an important role in ion-exchange chromatography. The strength of the binding of the surface will depend on the charges present. A high conductivity implies that more charges of the surface of the exchanger are involved with ions in the medium. It is then generally advised to have low conductivity in the medium in order to improve the binding of the analytes on the column [80].

The isoelectric point of interferon-α-2b is at 6.2, thus depending on the sample pH its net charge can be positive or negative. For this reason different resins were tested for their potential to bind interferon-α-2b, which are described in the following sections.

SP Sepharose

SP Sepharose is a strong cation-exchange resin on highly cross-linked 34μm agarose beads, which allows high resolution and high-capacity separations of protein mixtures. This resin is available in prepacked columns, such as HiPrep^{TM} and HiTrap^{TM} columns (GE Healthcare, Buckinghamshire, UK), and laboratory packs up to 10.0l. The resin matrix is composed of 6% spherical, cross-linked agarose with sulphopropyl (SP) as the functional groups. It has a binding capacity of 55mg ribonuclease A/ml medium, which results in a total binding capacity of 275mg ribonuclease A for 5ml columns [81].

Dowex

Dowex ion exchange resin, produced by Dow water Solutions, are a leader in separation technologies. It was the first manufacture for ion-exchange resins in 1983 and is still today one of the most common resin producer in the world [82]. Dow offers a wide range of different resins for chemical and pharmaceutical column separations from weak anion-exchanger to strong cation-exchanger [83].

Dowex® 1x4 chloride form (20-50 mesh) is a strong anion-exchanger, which is composed of

styrene divinylbenzene and a particle size of 20 - 50 mesh. Its binding capacity is roughly 35mg protein/ml and it meets the FDA food additive regulations. The Dowex® 50Wx4 hydrogen form (50-100 mesh) is a strong cation-exchanger with sulfonic acid as the active group and a particle size of 50 - 100 mesh. Its binding capacity is roughly 20mg protein/ml and it meets the FDA food additive regulations [83].

6.1.4.2 Hydroxyapatite

Mixed-mode chromatography combines at least two modes of interactions simultaneously. The use of multiple mechanisms of interactions on one stationary phase allows a much wider range of application. Mixed-mode chromatography has its benefits in analysing complex mixtures of proteins due to the 2D selectivity on a single run [84]. One example for mixed-mode chromatography is the application of hydroxyapatite resin.

Hydroxyapatite resin is considered to be a 'pseudo-affinity' chromatography or 'mixed-mode' ion-exchange. The functional groups consists of positively charged pairs of calcium ions and clusters of six negatively charged oxygen atoms associated with phosphates. Hence, it is a combination of cation-exchanger and metal affinity chromatography. Protein amino groups interact with the negatively charged phosphates and get repelled by positively charged calcium groups. Both interactions affect the protein binding and can be disrupted by the addition of salt or increasing pH. The carboxyl clusters and/ or phosphoryl groups on proteins interact with the hydroxyapatite through calcium affinity which is 15 to 60 times stronger than the ionic interaction alone. This metal affinity is not affected by ionic strength. However, it can be dissociated by the addition of phosphate [85].

The hydroxyapatite resin used for the experiments included in this study has a binding capacity of 25mg lysine/g CHT and a nominal pore size of 600-800 $\mathring{A}$ [86].

6.1.4.3 Ultrafiltration spin tubes

Another technique used for protein purification and concentration is ultrafiltration. Process samples from fermentation broth or the primary protein recovery often require sample purification prior to protein quantification due to the presence of large numbers of impurities [87]. Ultrafil-

tration techniques are successfully applied in large-scale processes as well as in laboratories for protein purification. Especially the purification of low concentrated proteins in protein mixtures is challenging with chromatographic techniques, and ultrafiltration is certainly beneficial. The impact of pH and salt concentrations on the purification of proteins with ultrafiltration was studied by Ehsani et al. 1996 [88]. Salt concentration and pH showed an impact on the hydrodynamic radius of the proteins and thus interfere with the purification by size.

Ultrafiltration at laboratory scale is performed with ultrafiltration spin tubes, which can be used for purification and concentration. The separation technique is controlled by the selection of the membrane and its pore size. A membrane pore size that retains the product while passing low molecular weight contaminants result in a concentration step, whereas membrane pore size that passes the product but retains any larger molecular weight contaminants results in a purifying step. The molecular weight cut-off of the membrane in protein purification should be three to six times smaller or larger than the molecular weight of the product [89].

Besides the pore size of the membrane, the membrane material is another important factor which needs to be considered during ultrafiltration. Different membrane materials, such as polyethersulfone, nylon or polypropylene, have different key characteristics, for example: low DNA or protein binding, antifoam resistance or specific chemical and physical properties. For the separation of protein mixtures in the fermentation broth a membrane with low protein binding and high flow rates and throughputs is beneficial. Hence, a hydrophilic polyethersolfone membrane is commonly used to separate such process samples [89].

A disadvantage of the purification of protein mixtures using ultrafiltration is the risk of membrane fouling by larger proteins than the protein to purify or by protein adsorption to the membrane [89]. In this case protein recovery is reduced due to the fouling of the membrane pores by larger proteins. Size exclusion chromatography can be an alternative method for the separation of protein mixtures and is described in the following section.

6.1.4.4 Size exclusion chromatography

Size exclusion chromatography (SEC), or gel filtration, separates a mixture of molecules by their molecular weight and the shape of the molecules. Molecules diffuse through the SEC matrix, which consists of a range of beads with slightly different pore sizes. The separation of the protein

mixture depends on the ability of different proteins to enter all or some of the channels inside the beads. Hence, smaller proteins have more potential channels to access and diffuse slower through the column, whereas larger proteins are excluded from the channels and pass quickly through the column [90]. In 'ideal' size exclusion chromatography, the separation of the protein mixtures is only dependent on the hydrodynamic volume of the protein, which is dependent on the molecular weight and the shape of the protein. Unfortunately, 'ideal' SEC is only achieved when the stationary phase of the column is neutral and the protein polarity similar to the mobile phase [91].
Previous studies showed that a decrease in the pH of the mobile phase results in an increase in the retention volume of the target protein, whereas a decrease in the ionic strength of the mobile phase results in a decrease of the retention volume. Several different reasons have been suggested for this observation, which are changes in the protein conformation, alteration of the pore size of the resin, protein adsorption or protein exclusion due to intermolecular electrostatic interactions [92]. In order to run the size-exclusion chromatography under 'ideal' conditions the mobile phase should be at neutral pH and contain an ionic strength of 0.15M salt to avoid pH dependent non-ionic interactions with the stationary phase.

6.1.4.5 Controlled protein precipitation

Precipitation methods are cheap and simple methods that are commonly used in purification processes. They are generally easily scalable and comprise a variety of techniques that can suit depending on the protein, media and conditions involved. The general idea of protein precipitation consists in the aggregation of proteins into clusters that can be separated from the rest of the solution by centrifugation. Proteins can then be resuspended into smaller volumes. This can be for instance a common and easy way to separate proteins from their media after production [93].
As already explained in section 4.1.4 proteins are soluble in water because of their electrostatic interactions with water. Since the electrostatic interaction of a protein is dependent on the pH, its solubility is therefore also dependent on the pH. This explains also why their lowest solubility occurs when the pH equals the pI, where their net charge is 0 and therefore their activity with water is the weakest. Studying the influence of pH will be therefore a major point of all investigations [94].

One typical method is called 'salting-out', which acts on the free water of the solution. It is one of

most commonly used methods, especially in early stages of purification processes [93]. In order to be soluble in water, a molecule has to be in electrostatic interaction with a certain number of water molecules. If salt is added, it will interact with water molecules. If enough salt is added, water molecules will no longer interact with the protein but with the salt with which it has a higher affinity. Eventually, free water molecules will not be in sufficient number to keep the protein in solution, in other terms, protein will precipitate [95]. Detailed information about the salting out of proteins can be find in section 4.1.4.

Precipitation with water-miscible organic solvents, like ethanol, or for this work acetone, is another commonly used protein precipitation technique. These solvents will interact with water molecules, which will group themselves around solvent molecules. This results in a decrease of water activity. Non-polar proteins can become more soluble, where water-soluble proteins solubility decreases. They tend then to aggregate together due to an energetically more favourable situation. In other terms, they will precipitate [96].

6.1.5 Goals and Aims

The overall goal of this chapter is the set-up of mass balances of each interferon isoform throughout the entire primary protein recovery process. In order to achieve this aim, an analytical method for interferon isoform quantification needs to be developed.
Existing quantification methods for interferon isoforms will be optimized for their resolution and sensitivity followed by the development of a purification and concentrating step of the recovery process samples prior to interferon isoform quantification. Hence, the three main goals for this chapter are as follow:

- Optimization of RP-HPLC methods for interferon isoform quantification
- Development of purification and concentration methods prior to isoform quantification
- Set up of mass balances of interferon isoforms through the primary protein recovery process

6.2 Material and Methods

Tab. 6.1: Materials and suppliers

materials	supplier
Recovery Process samples	industrial partner
interferon-α-2b drug substance	industrial partner
interferon-isoforms reference standard	industrial partner
Sodium phosphate monobasic	Merck
Sodium phosphate dibasic	Merck
Sodium chloride	Merck
YMC ODS-A 5ţm 4.6x100mm Column 300$\mathring{A}$	Waters
Zorbax 300SB C18 rapid res HPLC column	Agilent
Aeris WIDEPORE 3.6u XB-C18 New column 150 x 4.6mm	Phenomenex
Acetonitrile	Fisher Scientific
Trifluoroacteic acid (TFA)	Fisher Scientific
Ultrafiltration spin tubes	Sartorius
Zwittergent 3-14	Sigma Aldrich
Sarkosyl (30%)	Sigma Aldrich
Dowex® 1x4 chloride form (20-50 mesh)	Sigma Aldrich
Dowex® 50Wx4 hydrogen form (50-100 mesh)	Sigma Aldrich
hydroxyapatite with < 200 nm particle size (measured by BET)	Sigma Aldrich
Acetone	Sigma Aldrich
Ammonium sulphate	Sigma Aldrich
Trizma Base	Sigma Aldrich
EDTA tetrasodium salt	VWR
HiPrep 16/60 Sephacryl S-100 HR column	GE Healthcare
Bradford Reagent	Sigma Aldrich
Pierce® BCA Protein Assay Kit	Thermo Scientific
Bovine serum albumin (BSA)	Sigma Aldrich

6.2.1 Interferon-α-2b Isoform detection with RP-HPLC

Interferon-α-2b and its isoforms are quantified by three different RP-HPLC methods. All three methods have a reversed phase C-18 column but differ in the pore size and technology. The columns are used on an Agilent HPLC system 1100 with UV detector (Agilent, Cork, Ireland). Two of the three methods, Bakerbond and Zorbax, originate from the industrial partner and the existing SOPs were transferred to the lab in DCU.

For all methods, the drug substance of interferon-α-2b, provided by the industrial partner was used as the reference standard for all quantifications. In order to examine the retention times for the different isoforms, the DEAE feed-sample provided by the industrial partner was used, which derives from the industrial downstream process for interferon-α-2b production.

All samples and standards were diluted, if required, with a phosphate buffer (0.02M phosphate buffer, 0.22M NaCl, pH 8.0) to the appropriate concentration and filtered through a 0.45μm PVDF filter (Millipore, Billerica, USA).

6.2.1.1 RP-HPLC with Bakerbond column

The Bakerbond method uses a C-18 column, 5μm particles, 300 $\AA$ pore size and 4.6 x 100mm column size (Waters, Massachusetts, USA). Two different mobile phases were used: mobile phase A with 0.1% (v/v) trifluoroacteic acid (TFA) aqueous solution filtered through Whatman 0.45μm cellulose nitrate membrane filter (Whatman/GE Healthcare, Buckinghamshire, UK) and mobile phase B with acetonitrile / 1.0% aqueous TFA (90:10, v/v) filtered through Millipore 0.45μm Durapore membrane filter (Millipore, Billerica, USA). The gradient method started at 53% mobile phase A to 47% mobile phase B and ran over 52 minutes with an increase in mobile phase B. Flow rate was constant at 3.2ml/min and column temperature was controlled at 35°C. Standard injection volume was 20μl with interferon-α-2b concentrations of 0.05 - 4.0mg/ml.

6.2.1.2 RP-HPLC with Zorbax column

The Zorbax method uses a C-18 column, 3.5μm particles, 80 $\AA$ pore size and 4.6 x 150mm column size (Agilent, Cork, Ireland). Two different mobile phases were used: mobile phase A with 0.2% (v/v) trifluoroacteic acid (TFA) aqueous solution filtered through Whatman 0.45μm cellu-

lose nitrate membrane filter (Whatman/GE Healthcare, Buckinghamshire, UK) and mobile phase B with acetonitrile / 2.0% aqueous TFA (90:10, v/v) filtered through Millipore 0.45μm Durapore membrane filter (Millipore, Billerica, USA). The gradient method started at 50.5% mobile phase A to 49.5% mobile phase B and ran over 72 minutes with an increase in mobile phase B. Flow rate was constant at 1.0ml/min and column temperature was controlled at 35°C. Standard injection volume was 20μl with interferon-α-2b concentrations of 0.05 - 4.0mg/ml.

6.2.1.3 RP-HPLC with Aeris column

The Aeris method, developed in the lab at DCU, uses a C-18 Aeris widepore core-shell column, 3.6μm particles, 100 $\AA$ pore size and 4.6 x 150mm column size (Phenomenex, Cheshire, UK). Two different mobile phases were used: mobile phase A with 0.1% (v/v) trifluoroacteic acid (TFA) aqueous solution filtered through Whatman 0.45μm cellulose nitrate membrane filter (Whatman/GE Healthcare, Buckinghamshire, UK) and mobile phase B with acetonitrile / 1.0% aqueous TFA (90:10, v/v) filtered through Millipore 0.45μm Durapore membrane filter (Millipore, Billerica, USA). The gradient method started at 53% mobile phase A to 47% mobile phase B and ran over 35 minutes with an increase in mobile phase B. Flow rate was constant at 1.0ml/min and column temperature was controlled at 45°C. Standard injection volume was 20μl with interferon-α-2b concentrations of 0.05 - 4.0mg/ml.

6.2.2 Design of Experiment study

For the optimization of RP-HPLC methods, design of experiment (DoE) was used. Four factors, column temperature, flow rate, injection volume and interferon load, were analysed for two responses, peak width and retention time. A CCF design was used at two levels and 3 center points giving 27 experiments per method. The full list of experiments and factor levels can be found in the appendix A.

For the design and statistical analysis the software MODDE 10.0 from Umetrics was used.

6.2.3 Purification and Concentration of Interferon-α-2b

6.2.3.1 Ultrafiltration spin tubes

Ultrafiltration spin tubes (Sartorius, Goettingen, Germany) were used for concentrating or purifying samples, dependent on the molecular weight cut-off of the polyethersulfone membrane (PES). A molecular weight cut-off (MWCO) of 5kDa was used for concentrating samples, whereas 50kDa and 100kDa cut-offs were used for sample purification.

The upper chamber of the spin tube was filled with 5mL of the process sample, in the presence or absence of a detergent (1.0% Zwittergent 3-14 or 3.0% Sarkosyl).

For concentrating samples, tubes were centrifuged at 7500rpm at room temperature for 30 minutes, until approximately 1.0ml was left in the upper chamber. The recovered volume of process samples in the upper and lower chamber were recorded and a sample taken from both compartments and stored at 4°C for further analysis. The aim of this concentrating step was to recover all proteins in the upper chamber at a higher concentration than the untreated samples. No proteins were expected to be found in the lower chamber.

For purification of process samples, tubes were centrifuged at 7000rpm (7122g) at room temperature for 40 minutes, until three quarters of the volume had passed through the membrane into the lower chamber. The volume of recovered process sample in the upper and lower chambers were recorded and samples from both chambers were taken for mass balances and stored at 4°C for further analysis. The aim of the purification step was to recover all interferon in the lower chamber, whereas the larger host cell proteins should remain in the upper chamber.

6.2.3.2 Ion-exchange methods

Four different ion-exchange materials were tested to purify recovery process samples. The first one, using Sepharoses derived from the industrial partner. The other three with Dowex and hydroxyapatite were developed as part of this work.

SP Sepharose in HiTrap column

The purification method for recovery process samples is the same method used during the industrial downstream processing and more detailed information on sample preparations and the method can be found in chapter 10 section 10.2.2.
This purification was carried out with 5ml HiTrap SP HP columns (GE Healthcare, Buckinghamshire, UK) controlled on an AKTA Explorer (GE Healthcare, Buckinghamshire, UK). All samples taken throughout the method are stored at 4°C for further analysis.

Dowex and Hydroxyapatite

To perform ion-exchange chromatography in order to separate interferon from host cell proteins, the chromatographic procedure was simulated in a small 2.0mL Eppendorf tube, instead of using a column. Between 100 - 200mg of the specific resin was added to the eppendorf tube, followed by the addition of 1.0mL of the process sample containing interferon and host cell proteins. After 2.0 hours of continuously mixing, the samples were centrifuged at 10'000rpm (12'200g) for 15min at room temperature (Eppendorf centrifuge, Hamburg, Germany). The supernatant (0.8mL) was recovered and stored at 4°C for further analysis.
The resins to be investigated for this study were: Dowex® 1x4 chloride form (20-50 mesh), Dowex® 50Wx4 hydrogen form (50-100 mesh) and hydroxyapatite with < 200 nm particle size (measured by BET).

6.2.3.3 Controlled protein precipitation with acetone or ammonium sulphate

For controlled protein precipitation, 2mL of the recovery process sample was poured into a 50mL centrifuge tube. With continuous stirring of the sample, 2mL of acetone or ammonium sulphate was gently added with a pipette. Samples were then centrifuged for 30 minutes at 7500rpm (FL40R centrifuge, Thermo Scientific, Dublin, Ireland). After having collected the supernatant, pellets were re-suspended in 2mL of extraction buffer (20mM Tris, 1mM EDTA buffer, pH 8.0). Samples were taken before precipitation (starting samples), after centrifugation (supernatant) and after resuspension of the pellets and stored at 4°C for further analysis.

6.2.3.4 Size-Exclusion Chromatography

For sample purification with size-exclusion chromatography a HiPrep 16/60 Sephacryl S-100 High Resolution column (GE Healthcare, Buckinghamshire, UK) was used. Column flow rate was controlled constantly at 1.0ml/min with an AKTA Explorer (GE Healthcare, Buckinghamshire, UK). All solutions were filtered through 0.21μm filter (Millipore, Billerica, USA) prior to injection onto the column. Equilibration was performed with 0.05M Na_2HPO_4, 0.15M NaCl at pH 7.20 for two column volumes. The recovery process sample of 1.0ml was directly injected onto the column with a separate pump at 1.0ml/min. Elution of the sample was performed for 160 minutes at 1.0ml/min with the equilibration buffer.
Different fractions throughout the elution were collected to concentrate the recovered protein in this fraction with ultrafiltration spin tubes as described in the method above.
The column was washed with deionized water for 1 column volume prior to equilibration and new sample injection.

6.2.4 Total protein quantification

Total amounts of protein were quantified using the BCA assay as described in section 3.2.2 and table 3.5 or Bradford assay as described in section 3.2.2 and table 3.6. Samples without a detergent present were analysed using the Bradford assay, whereas samples with a detergent present were analysed using the BCA-assay. Sample pellets were dissolved in EDTA-Tris buffer (20mM Tris, 1mM EDTA, pH 8.0) prior to protein analysis. BSA dissolved in water was used as a reference standard for all total protein quantifications. The blank was chosen according to the sample to be analysed, from water, EDTA-Tris buffer or detergents.

6.2.5 Total Interferon-α-2b quantification

Total interferon-α-2b amounts were quantified with SDS-PAGE as described in chapter 5.2.3 and 5.2.4. Sample pellets were dissolved in EDTA-Tris buffer (20mM Tris, 1mM EDTA, pH 8.0) prior to analysis as mentioned in the section above. No further sample treatment was required. The drug substance of interferon-α-2b was used as a reference standard for all quantifications.

6.3 Results and Discussion

This discussion section can be divided into three main parts. The first part focuses on the optimization of quantification methods for interferon-α-2b and its isoforms. The second part discusses possibilities to purify and concentrate process samples prior to quantification to enhance accuracy and resolution of the quantitative methods. The last and third part looks into the mass balances of interferon isoforms throughout the primary protein recovery process.

6.3.1 Quantification of Interferon-α-2b Isoforms

In order to quantify interferon and its isoforms in recovery process samples, analytical methods are required. This section compares three available RP-HPLC methods with each other followed by an optimization of the methods to increase their resolution and accuracy using a design of experiment (DoE).

6.3.1.1 Comparison of different RP-HPLC methods

Three different RP-HPLC methods are compared for their resolution and accuracy to quantify interferon and its isoforms. Two methods were developed by the industrial partner and existing SOPs were transferred to the labs in DCU. The third method was developed as part of this work in relation to the existing two methods. Retention times for all isoforms needed to be re-established due to a change in the HPLC manufacturer. The new re-established retention times of all isoforms for all three methods are shown in table 6.2.

Tab. 6.2: Retention times [min] of all 4 isoforms for all three RP-HPLC methods

methods	IFN-α-2b	Iso-2	Iso-3	Iso-4
Bakerbond	6.51	8.06	10.27	12.61
Zorbax	15.17	16.95	19.47	22.95
Aeris	12.67	14.58	16.99	18.89

The Zorbax and Aeris method can also distinguish between two different forms of isoform 4, the pyruvated form (Iso-4P) and the non-pyruvated form (Iso-4R). The pyruvated form elutes only a few seconds after the non-pyruvated form, which leads to an overlap of both peaks. Complete separation of both peaks could not be achieved with any of the three RP-HPLC methods. However, the utilization of UPLC methods might improve the resolution and lead to a complete peak separation.

Figure 6.4 shows the UV-chromatogram of a DEAE-feed sample from the down-stream process of interferon-α-2b for all three RP-HPLC methods. The DEAE-feed sample contains interferon-α-2b and its three to four isoforms. The elution sequence of the isoforms as well as the chemical structure of each isoform were published by Yan-Hui Lui 2010 [6].

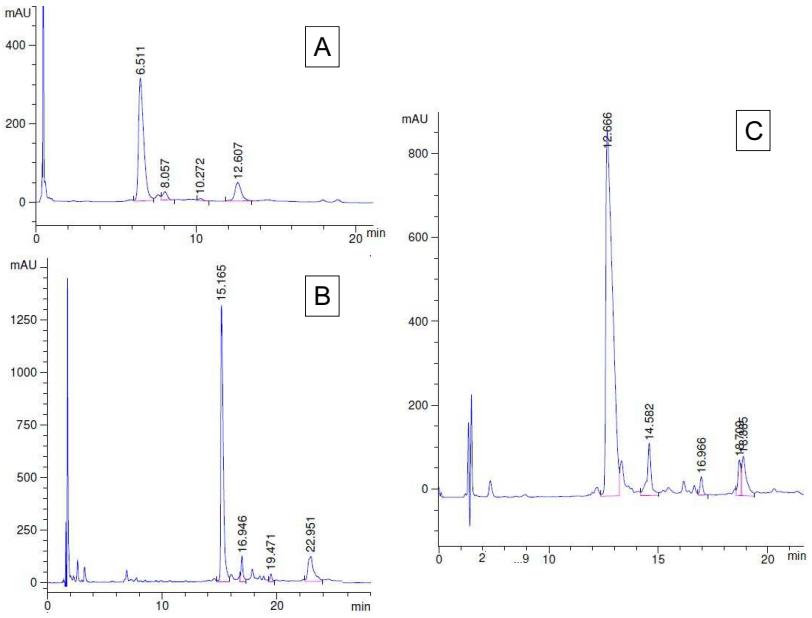

Fig. 6.4: UV-chromatogram of RP-HPLC analysis of DEAE-feed sample, showing interferon-α-2b and its 3/4 isoforms: A) chromatogram of bakerbond column; B) chromatogram of zorbax column; C) chromatogram of aeris column

Window A in figure 6.4 shows the chromatogram of the Bakerbond column with interferon-α-2b at a retention time of 6.5 minutes. Limit of detection (LOD) for IFN-α-2b is 1.5μg and limit of quantification (LOQ) is 10.0μg. Window B shows the chromatogram of the Zorbax column, which is much more sensitive compared to the Bakerbond column. The LOD for IFN-α-2b is 0.2μg and the LOQ is 1.0μg. The disadvantage of the Zorbax method is the low resolution at low concentrations due to large interferences of other proteins in the sample, which makes it difficult

to distinguish between different peaks, especially for isoform-2 and -3. These isoforms are often at very low concentrations and therefore difficult to identify. Window C in figure 6.4 shows the chromatogram of the Aeris column. The sensitivity is slightly lower compared to the Zorbax method, with a LOD of 0.2μg and a LOQ of 2.0μg. However, the Aeris column provides the best separation of the two forms of isoform-4.

Besides sensitivity, two other parameters have been investigated during the comparison study of the three methods: the drift in retention time and the peak-width. The drift in retention time needs to be as small as possible in order to detect and quantify interferon and its isoforms in unknown process samples. The peak-width of a peak should be as thin as possible to avoid fronting and tailing which can falsify the peak area and hence the protein quantity.

Table 6.3 shows the retention time drifts for interferon-α-2b and the peak-width for IFN-α-2b and isoform-4 for all three methods. The drift in retention time is calculated from the analysed retention times of three runs of the interferon-α-2b drug substance with a constant IFN load.

Tab. 6.3: Retention time drift and peak width of all three RP-HPLC methods

	RtT drift [min]	Peak width [min]	
method	IFN-α-2b	IFN-α-2b	Isoform 4
Aeris	0.69	0.69	0.92
Bakerbond	1.35	0.48	0.57
Zorbax	0.27	0.23	0.60

It can be seen, that the Zorbax method has the lowest retention time drift and the smallest peak-width for interferon-α-2b, followed by the Aeris method. The Bakerbond method has the smallest peak-width for isoform-4 since this method cannot distinguish between the two forms of isoform-4 and both elute under the same peak. In order to optimize the retention time drift and the peak-width of interferon-α-2b a design of experiment study was performed, the results of which are shown and discussed in the following section.

6.3.1.2 Optimization of RP-HPLC methods with Design of Experiment

To optimize the retention time drift and the peak-width of the three methods for interferon-α-2b a CCF design (Central Composite Face centred design) for four factors was used, giving 27 experiments for each method. The four factors investigated are flow rate, injection volume, IFN-load and column temperature. The previous method conditions were generally set as the center points, if this was applicable. The full design plan of all experiments with all factor values can be found in the appendix A. The development of the models and the statistical analysis were done with the software MODDE 10.0 from Umetrics.

Table 6.4 gives an overview of the different parameters that were investigated throughout the design of experiments study to optimize the three HPLC methods.

Tab. 6.4: Design of Experiment parameters for the optimisation of all three HPLC methods

method	injection volume [μL]	IFN - load [μg]	flow-rate [ml/min]	temperature [°C]
Aeris	10 - 40	2 - 20	0.5 - 2.5	25 - 55
Bakerbond	10 - 40	2 - 20	1.0 - 3.2	35 - 55
Zorbax	10 - 40	2 - 20	0.5 - 2.0	25 - 55

Six different models were developed, for each response (retention time and peak-width) for each of the three methods. The models for retention time gave good statistic results for all methods with Q^2 and R^2 always above 0.97, which indicates a good correlation between the actual and the predicted values (6.1.3.2). For all three models only three of the four factors are statistically significant, which indicates their p-value of less than 0.05 (see appendix A). These three factors are: column temperature, flow-rate and IFN-load. The impact of the injection volume is statistically insignificant for the retention time. However, most of the factor-factor interactions are not significant either, with the exception of the interaction between flow-rate and temperature.

For all methods the injection volume, IFN-load and flow rate have a negative impact on the retention time, meaning the larger these factors get the shorter the retention time will be. This observation is as expected. More protein loaded onto the column results in lower binding affinity

as described in section 6.1.2. And a higher flow rate results in a reduced binding time and a shorter retention time of the protein. The column temperature has a positive impact on the retention time which results in longer retention times at higher temperatures. This result indicates that the column temperature has a positive impact on the binding affinity of the protein to the column, which increases with higher temperature.

The most significant impact on the retention time is the flow-rate which is indicated through the highest factor value in all model equations. This impact can also be seen in figure 6.5 in the image on the left, which shows a contour plot of the retention time of the Aeris method dependent on the column temperature and the flow rate.

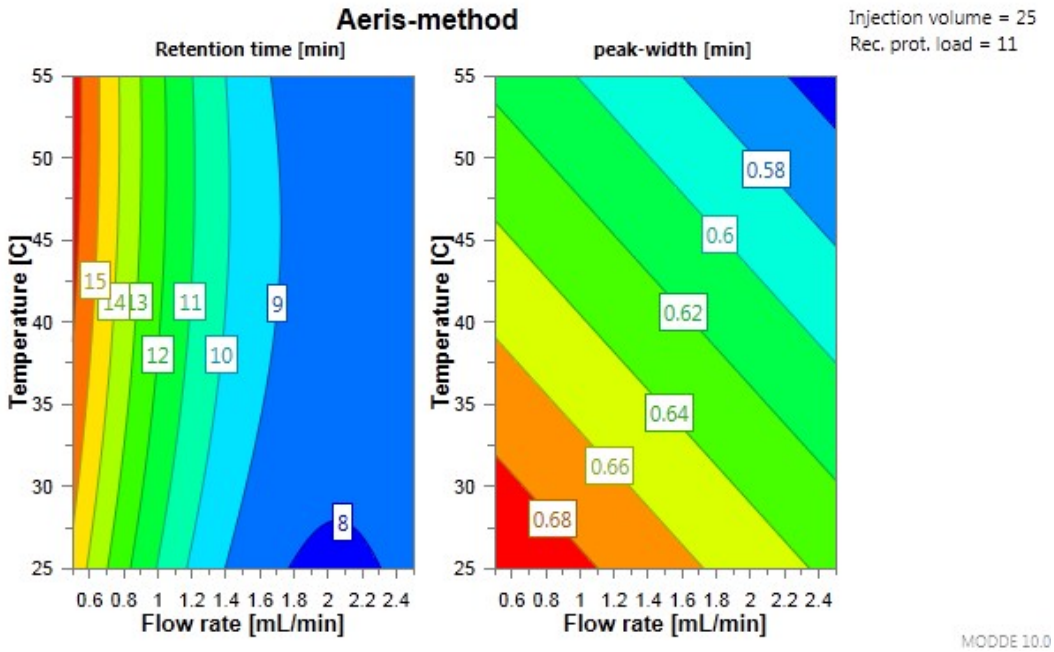

Fig. 6.5: Contour plot of the retention time and peak-width of Aeris method dependent on column temperature and flow-rate. IFN-load is set constant to 11μg and injection volume is constant at 25μl. Left: retention time [min], right: peak width [min]

The image on the left in figure 6.5 shows the high dependency of the retention time on the flow-rate for the Aeris method, especially at lower flow rates up to 1.2ml/min. The impact of the column-temperature is less significant compared to the injection volume and the IFN-load.

The models for the peak-width were slightly less statistically quantitative compared to the models for the retention time. The Q^2 and R^2 are between 0.86 and 0.97 for the Aeris and Zorbax methods respectively and even below 0.55 for the Bakerbond method. Values above 0.85 still indicate a

good correlation between predicted and actual values but a Q^2 of less than 0.55 should be handled carefully. Similar to the retention time models, only the column temperature, flow rate and IFN load have a significant impact on the peak-width but not the injection volume (see p-values in the appendix A).

The injection volume, flow rate and column temperature have a negative impact on the peak width, meaning with an increase in those factors, the peak width is decreasing. This observation is as expected. With an increased flow rate the elution of a protein is shorter compared to a lower flow-rate. The IFN load is the only factor with a positive impact on the peak-width. Higher protein amounts loaded onto the column results in wider peak-width which is as expected. The image on the right in figure 6.5 shows the dependency of flow rate and column temperature on the peak-width of IFN during the Aeris method. It can be seen that with an increase in flow rate and column temperature the peak-width is decreasing. Contour plots for the Bakerbond and Zorbax method, as well as all statistic parameters are shown in the appendix A.

To optimize the retention time drift and the peak-width of IFN-α-2b, the following criteria were set: retention time (RtT) has to be within two minutes of the average RtT and the peak-width has to be lower than a certain maximum value. The two criteria for all three methods can be seen in table 6.5.

Tab. 6.5: Criteria for retention time and peak-width optimization for all three HPLC methods

method	retention time [min]	Peak width [min] IFN-α-2b
Aeris	9 - 13	< 0.55
Bakerbond	9.5 - 12	< 0.64
Zorbax	15 - 19	< 0.25

With the two criteria for the retention time and the peak-width a sweet spot plot can be designed which highlights the areas where both responses are within the specified ranges. Figure 6.6 shows the sweet spot plot for the Aeris method. The green area indicates the sweet spot where both

criteria meet, the blue area highlights the areas where only one criteria meets and the white areas indicate that no criteria meets. On the inner axis are the injection volume and the IFN-load and on the outer axis are the flow-rate and the column temperature.

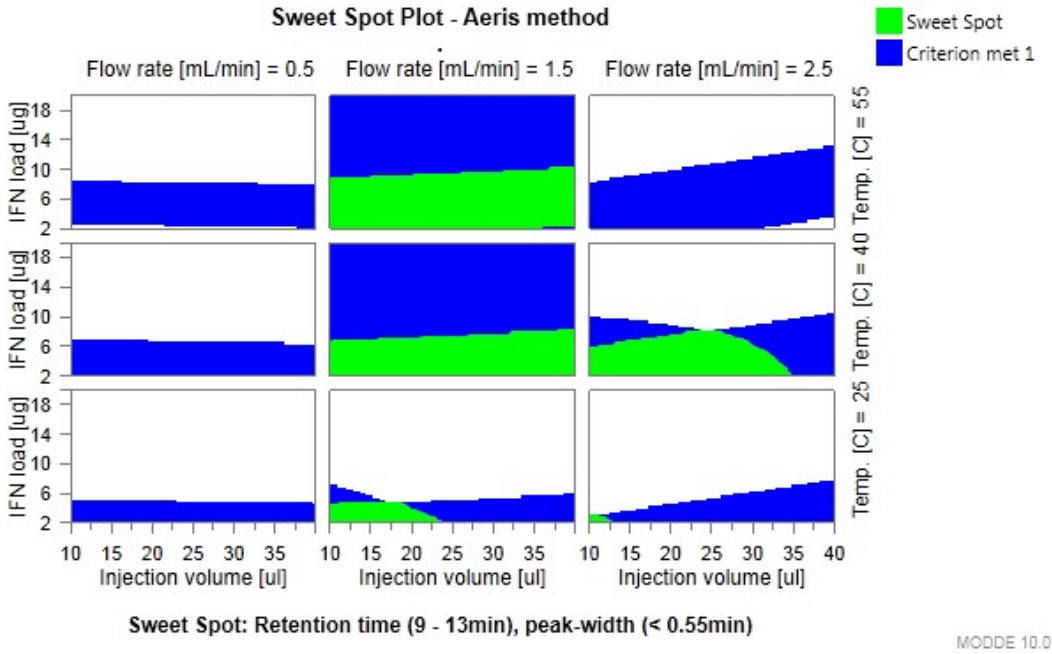

Fig. 6.6: Sweet Spot plot from Aeris method: Green area meets both criteria, Retention time between 9-13 min and peak width less than 0.55min. Blue area meets only of those two criteria

As discussed earlier in this section, the flow rate and column temperature have the biggest impact on the retention time and the peak-width. Both criteria, retention time between 9-13 minutes and peak-width less than 0.55 minutes, are only fulfilled at a flow-rate higher than 1.5mL/min and a column temperature of more than 40°C. In order to receive the biggest possible sweet spot area but keeping the column temperature as low as possible to avoid possible protein degradation, the flow rate was set to 1.5ml/min and the column temperature to 40°C. To be within the sweet spot area, the IFN load onto the column has to be below 6μg. Since the injection volume has no significant impact on either the retention time nor the peak-width, the volume of the injected sample can be adjusted with respect to the protein concentration to keep the actual IFN load below 6μg.

Similar procedures were performed for the Zorbax and Bakerbond method and the new optimized RP-HPLC method conditions are shown in table 6.6. The sweet spot plot for the Zorbax method can be found in the appendix A.

Tab. 6.6: Optimized RP-HPLC conditions for Aeris, Bakerbond and Zorbax method

method	column temp. [°C]	flow rate [ml/min]	IFN load [μg]	Inj. volume [μl]
Aeris	40	1.50	< 6.0	11 - 37
Bakerbond	35	2.05	< 18.0	11 - 37
Zorbax	40	1.25	< 8.0	11 - 37

The new optimized factor conditions for all RP-HPLC methods were validated with each six experiments. Three interferon-α-2b concentrations between 0.1 and 0.5 mg/ml were selected and the IFN load and injection volume were adjusted to be first within the new optimized criteria of each method and in the second run to be outside the criteria. All six validation runs for each method were performed in triplicate and each run fulfilled the criteria. These results validated the optimized RP-HPLC methods which are improved in their accuracy of the retention time and peak-resolution. A comparison between the retention time drifts and the peak-width before and after the optimization study can be seen in table 6.7.

Tab. 6.7: Comparison of retention time drifts and peak widths before and after optimization

	Retention time drift [min]		Peak width [min]	
Method	before optim.	after optim.	before optim.	after optim.
Aeris	0.69	0.19	0.69	0.53
Bakerbond	1.35	0.26	0.48	0.37
Zorbax	0.27	0.11	0.23	0.21

The retention time drift of the Aeris method decreased 3.6-fold and the peak-width reduced by 25%. The optimization of the Bakerbond method is even more drastic with a 5.2-fold reduction in retention time drift as well as a 25% reduction in peak-width. The Zorbax method shows less change with the optimized method. Only a 2.5-fold decrease was seen for the retention time drift while the peak-width was reduced by 10%. Even though, Zorbax showed the smallest changes

due to the optimization, this method is still the most accurate one with the highest resolution followed by the Aeris method and the Bakerbond. Keeping in mind that only the Aeris method was able to distinguish between the two forms of isoform-4, this method was selected as the best method to quantify interferon-α-2b and its isoforms in process samples. Another advantage of the Aeris method compared to the Zorbax and Bakerbond methods is the short run time of only 35 minutes and thus the smallest consumption of mobile phase with 52.5ml per run. Hence, all further analysis for interferon-α-2b and its isoforms with RP-HPLC was performed with the optimized Aeris method.

6.3.2 Purifying and concentrating of recovery process samples

One major problem with the quantification of interferon-α-2b and its isoforms in recovery process samples is the presence of a large amount of host cell proteins. More than 95% of total proteins are HCP and only 5% are interferon. The second problem is the low concentration of interferon and its isoforms in process samples, which makes it difficult to quantify IFN accurately. To enhance the accuracy and resolution of the quantification of interferon and its isoforms in recovery process samples, different purification and concentrating methods were tested to first purify IFN from host cell proteins, followed by a concentrating step. The aim of each purification and concentrating method is to receive at least 95% recovery of interferon to avoid large errors within the quantification results.

6.3.2.1 Ion-exchange purification with HiTrap columns

One purification and concentrating method was already put in place by the industrial partner, which is the same ion-exchange method as the first step of the industrial down-stream process. This method was transferred to the labs in DCU and adjusted to some samples throughout the recovery process. Sample preparation and the ion-exchange chromatography were performed as described in section 6.2.3.2. For quantification of IFN-isoforms the elution fractions 7-12 were selected which are the fractions with the highest UV-280 values. The elution fractions were analysed for interferon-α-2b and isoforms with the Aeris RP-HPLC method and SDS-PAGE as described in sections 6.2.1.3 and 6.2.5. Further to these samples, the starting sample, flow-through and wash

sample were also analysed for interferon with the SDS-PAGE.

SDS-PAGE results of the starting and flow through sample showed that 50% of loaded interferon was found in the flow through and only 40-50% were recovered in the elution fractions which were used for quantification. These findings indicate that interferon was underestimated in recovery process samples on site with the industrial partner when using the HiTrap method as purification and concentrating step prior to the quantification analysis.

To avoid this loss in interferon during the purification step, some samples were injected directly onto the RP-HPLC column. Figure 6.7 shows the comparison between the quantified interferon-α-2b and its isoforms in the elution fractions 7-12 after the HiTrap method and the quantified IFN in samples directly injected onto the HPLC column without purification. The figure also shows the comparison of IFN quantified with SDS-PAGE in the elution fractions and without purification.

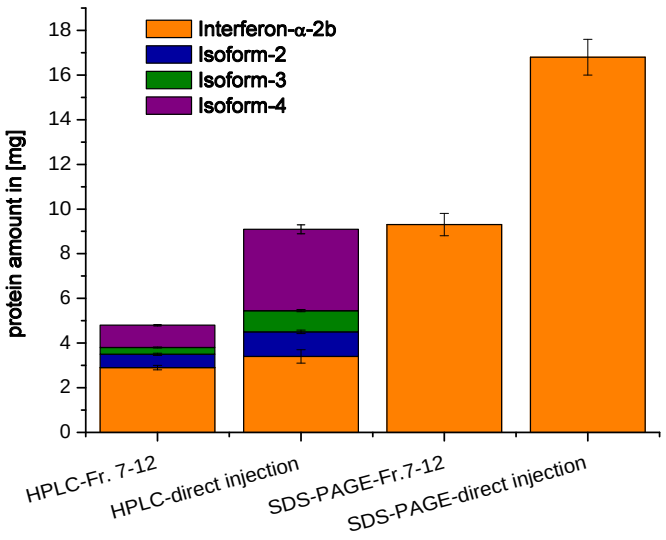

Fig. 6.7: Comparison between interferon and its isoforms quantification of HiTrap fractions 7-12 and after direct injection without purification quantified with RP-HPLC and SDS-PAGE

The results for interferon and its isoforms quantified by HPLC and SDS-PAGE show both that only 50% of the loaded interferon was detected in the elution fractions. The IFN in the samples analysed without any purification step is for both methods, HPLC and SDS-PAGE, twice as much as the interferon detected in the purified sample. These findings confirm the previously results of IFN detected in the flow through of the ion-exchange method. A closer look to the isoforms indicates, that the majority of this loss is in isoforms-4. Isoform-4 is the only isoform without any

disulphide bonds, hence its tertiary structure will be different compared to the interferon-α-2b protein. A change in the tertiary structure can lead to a change in the net-charge of the protein. Since ion-exchange chromatography purifies proteins as a result of charge it is possible that isoform-4 binds with a much lower affinity to the HiTrap column than IFN and the other two isoforms (section 6.1.1 and 6.1.4.1).

Comparing the quantified total interferon-α-2b by RP-HPLC with the results obtained with SDS-PAGE shows that the SDS-PAGE overestimates the IFN in recovery process samples. This result confirms the findings from chapter 5, where the SDS-PAGE results were compared to Western Blot results which is more protein specific. SDS-PAGE only distinguishes by molecular weight and it is very likely that other host cell proteins with the same molecular weight than interferon are present in the recovery process samples.

Hence, the most precise results for interferon-α-2b were quantified by RP-HPLC without any purification. However, the big disadvantage of this procedure is the low accuracy and low resolution of the UV-280 chromatogram. Figure 6.8 shows such a chromatogram of a TCA-pellet directly injected onto the Aeris column without any sample purification or concentration.

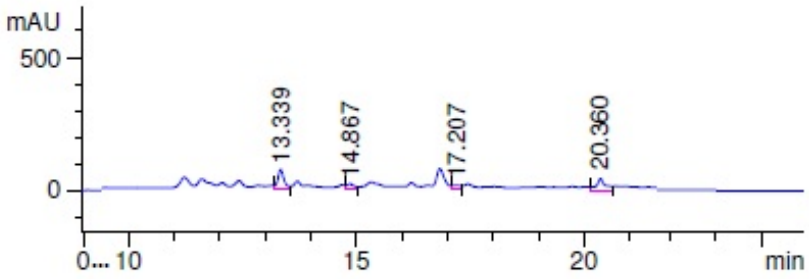

Fig. 6.8: UV-chromatogram of a direct injection of TCA-pellet onto the Aeris column without any sample purification

The chromatogram shows that it is very difficult to distinguish between the different peaks and to select the correct peak for each isoform. Especially isoform-2 and -3 are so low in concentration that no peak is visible without amplification. Thus, analysing all recovery process samples with this procedure can easily lead to false interferon quantities due to errors during peak selection and imprecise peak-areas. Hence, a purification and concentrating method prior to the interferon quantification with RP-HPLC would increase the accuracy and sensitivity.

The ion-exchange chromatography method with HiTrap column showed only a 50% recovery of

purified interferon and its isoforms which makes it unfeasible for the purification of recovery process samples prior to IFN quantification. However, another observation was made during the comparison of purified and unpurified samples. Analysing the end of fermentation (EOF) at different pH for their isoform distribution showed a strong dependency of isoform-3 and -4 on the pH value of process samples. Figure 6.9 shows the pH dependency of the isoform distribution in EOF samples.

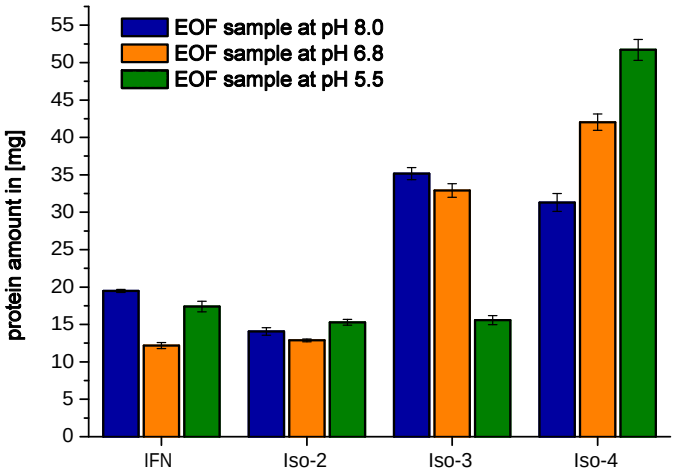

Fig. 6.9: pH dependency on isoform distribution in end of fermentation sample

Three different pH values were tested: 5.50, 6.80 and 8.00. At the end of fermentation the pH of the fermentation broth is around 7.0, for the purification with ion-exchange the pH was reduced to 5.50. As it can be seen in figure 6.9 the reduction of pH leads to a shift between isoform -3 and -4. With a reduction in the pH, isoform-3 is converted to isoform-4. Interferon-α-2b and isoform-2 are less affected by the pH, which could be due to their disulphide bonds and thus stronger tertiary structure. The pH dependency of the isoform distribution in recovery process samples indicates the importance of purifying, concentrating and quantifying the process samples without changing the pH of the sample. Hence, the ion-exchange method did not only underestimate the interferon-α-2b and its isoforms, it also gave misleading results about the isoform distribution in the analysed samples.

Summarizing the results of this section shows that the existing purification method followed by RP-HPLC quantification of interferon, underestimated the total interferon-α-2b and falsifies the

isoform distribution. Hence, a new purification and concentration method needs to be developed, which recovers interferon to a minimum of 95% and requires no pH adjustment prior to the purification, concentrating or quantification step.

6.3.3 Other purification methods

The previous section showed that the accurate quantification of interferon isoforms requires a purification and concentration step to separate interferon-α-2b from host cell proteins. The method in place, an ion-exchange method, demonstrated to underestimate interferon-α-2b and falsifies the results for the isoform distribution. Hence, this section focuses on the development of a new purification and concentrating method. Seven different techniques have been tested to purify the recovery process samples. All tested techniques were assessed on two criteria: first, an interferon-α-2b recovery yield of at least 95% and second, no necessity for pH-adjustment during the purification.

6.3.3.1 Ultrafiltration with spin tubes

The ultrafiltration with spin tubes is the first technique used to purify and concentrate recovery process samples. The principle of this method is to separate a mixture of molecules based on molecular weight. Hence, no pH-adjustment seems to be required in order to purify or concentrate the recovery process samples.

Concentrating

In a first step, the ultrafiltration spin tubes were used to concentrate interferon-α-2b in the recovery process samples. IFN has a molecular weight of around 19kDa, hence a molecular weight cut-off of 5kDa was selected to concentrate interferon.
The experiment was performed as described in section 6.2.3.1 and the agitation time was adjusted to receive the desired concentration. For example reducing the volume from 5ml to 1ml leads to a 5-fold concentration from a protein concentration at 1mg/ml to 5mg/ml. Figure 6.10 shows the results for interferon before and after the concentrating step with ultrafiltration spin tubes at a cut-off of 5kDa.

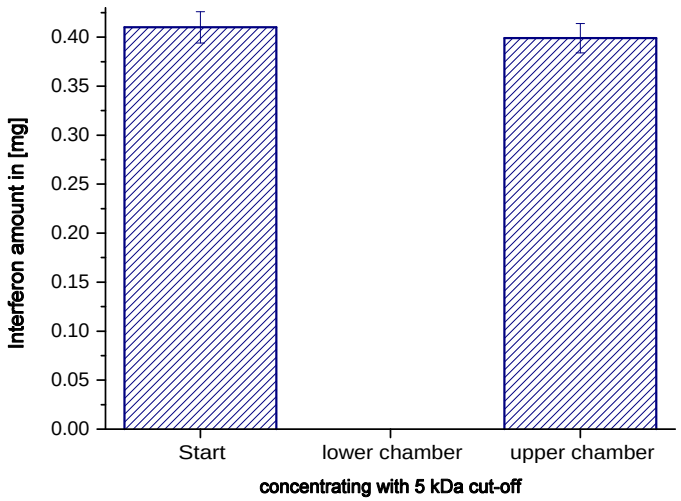

Fig. 6.10: Concentrating of recovery process samples with ultrafiltration spin tubes and 5kDa cut-off. Lower chamber gets discard and upper chamber is concentrated sample

The sample concentrated in this experiment was a TCA-pellet from the manufacturing process and no additional sample treatment was performed prior to this method. Interferon-α-2b was quantified by SDS-PAGE as described in section 6.2.5. It can be seen that all interferon was recovered in the upper chamber of the spin tube and no IFN was found in the lower chamber. Since the volume in the upper chamber reduced throughout the centrifugation and the interferon amount remained constant, the protein concentration increased. In this example the IFN concentration increased from 0.082g/l to 0.399g/l which is a concentration factor of 5.

These results indicate that interferon-α-2b can be successfully concentrated in recovery process samples using ultrafiltration spin tubes with a cut-off of 5kDa. No pH setting is required for this method and the interferon recovery was > 95%, hence both basic criteria are fulfilled.

Purification

In a second step, the ultrafiltration spin tubes were used to separate interferon-α-2b from host cell proteins in recovery process samples. As mentioned before, IFN has a molecular weight of 19kDa, hence molecular weight cut-off of 50 and 100kDa were selected for the purification. Usually a cut-off of at least three times the molecular weight of the purified molecule is selected (section 6.1.4.3).

All experiments were performed as described in section 6.2.3.1 using a TCA-pellet from the manufacturing process. Figure 6.11 shows the results of the TCA-pellet purification with ultrafiltration spin tubes and a 100kDa cut-off in the presence and absence of 3% Sarkosyl.

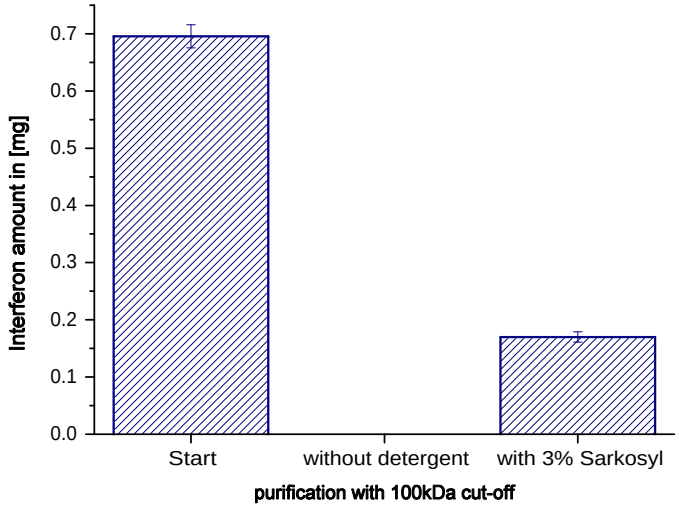

Fig. 6.11: Purification of recovery process samples with ultrafiltration spin tubes and 100kDa cut off in the presence and absence of 3% Sarkosyl. The image shows the starting sample and the recovery of interferon-α-2b in the lower chamber of the ultrafiltration spin tubes.

The interferon-α-2b was quantified by SDS-PAGE as described in section 6.2.5. Figure 6.11 shows the recovery of purified interferon-α-2b in the lower chamber of the ultrafiltration spin tubes. As it can be seen, in the absence of a detergent no IFN was detected in the purified fraction, neither with 50kDa nor 100kDa cut off. This observations led to the conclusion, that interferon interacts with other larger host cell proteins and the formed protein complex is too big to pass through the ultrafiltration membrane. Accordingly, a detergent was added to the starting sample in order to avoid protein-protein interactions. Two different detergents were tested, Sarkosyl at a concentration of 3.0% and Zwittergent 3-14 at a concentration of 1.0%. The bar to the right in figure 6.11 shows the recovery of interferon in the purified sample in the presence of 3% Sarkosyl. It can be seen that the recovery of IFN in the purified fraction in the presence of 3% Sarkosyl did improve compared to the experiment without a detergent. However, the interferon recovery is less than 30%, meaning that 70% was lost during this purification, which is not acceptable for a pre-treatment prior to a quantification. An explanation for the low IFN recovery could be that larger host cell proteins foul the pores of the membrane and hinder the small interferon molecules

passing through.

Using the addition of 1.0% Zwittergent 3-14 to the starting sample resulted in only 2% recovery of the initial interferon in the purified sample (data not shown). This observation indicates that Sarkosyl has a higher potential to break-up protein-protein interactions than Zwittergent 3-14.

In summary, the separation of interferon from host cell proteins in recovery process samples with ultrafiltration spin tubes did not result in a sufficient recovery yield. The low interferon recovery yield of less than 30% is probably due to larger host cell proteins fouling the membrane pores and hindering the passage of smaller molecules. An inverted method might result in higher IFN recovery yields, where larger molecules pass through first and the elution of smaller molecules is delayed. An example of such a method would be size exclusion chromatography, the results of which are discussed later in this section.

6.3.3.2 Ion-exchange and precipitation methods

Besides using ultrafiltration spin tubes for the purification of recovery process samples prior to interferon quantification, several other techniques were also investigated. This section describes briefly the application of three other techniques which are: cation- and anion-exchange resin, mix-mode resin and controlled protein precipitation. For all experiments the TCA-pellet of the manufacturing process was used.

Dowex resin

The hitherto existing method used for the purification of recovery process samples prior to isoform quantification, is an ion-exchange method with cationic SP Sepharose resin. Due to the nature of a cation-exchange method it requires a controlled pH environment below the pI of the protein to be purified (section 6.1.4.1). Section 6.3.2.1 showed that the adjustment of the pH of the sample influences the distribution of the interferon isoforms. Instead of setting the pH of the process sample, an idea was to use a cationic resin for pH below 6.2 and an anionic resin for sample pH above 6.2. The resins used for this experiment are Dowex® 1x4 chloride form as the anionic resin and Dowex® 50Wx4 hydrogen form as the cationic resin. The experiments were performed as described in section 6.2.3.2 and total protein and total interferon-α-2b were quantified in the

supernatant using the BCA-assay and SDS-PAGE according to sections 6.2.4 and 6.2.5.
The results showed that, independent of the conductivity value of the sample and independent of the presence or absence of a detergent, both resins have a stronger binding affinity at pH values around 5.0. It was also observed that interferon and host cell proteins show similar binding affinity towards both resins, which makes it impossible to separate one from the other. In summary, neither of the Dowex resins showed potential to separate interferon from host cell proteins with or without pH adjustment.

Hydroxyapatite

The second technique tested for the purification of interferon in recovery process samples is the use of a mix-mode resin as hydroxyapatite. This type of resin involves non-specific interactions between positively charged calcium ions and negatively charged phosphate ions, thus it should bind positively charged molecules as well as negatively charged molecules (section 6.1.4.1). The experiment was performed as described in section 6.2.3.2.
Unfortunately results showed that hydroxyapatite has a similar binding affinity towards interferon and host cell proteins present in the recovery process samples. The impact of different pH, conductivity and the presence and absence of two detergents on the binding of hydroxyapatite to interferon and host cell proteins were tested. None of these factors showed a significant improvement on the separation of interferon from host cell proteins. Hence, the mix-mode resin hydroxyapatite cannot be used to separate interferon-α-2b from host cell proteins in recovery process samples prior to IFN quantification.

Protein precipitation

The last technique tested in this section is protein precipitation with ammonium sulphate and acetone. The aim of this technique is to perform a selective protein precipitation by the controlled addition of either ammonium sulphate or acetone to the recovery process samples. Different proteins have different precipitation points due to the addition of salt or organic solvents (section 6.1.4.5). Accordingly, this phenomenon is also called salting-out of proteins. The idea is to salt-out host cell proteins while maintaining interferon-α-2b in solution. The experiments were performed as

described in section 6.2.3.3.

Similar to the results obtained with Dowex and hydroxyapatite, protein precipitation with ammonium sulphate and acetone showed no significant difference between the precipitation point of interferon and host cell proteins. Both groups of proteins precipitated at similar time points throughout the addition of ammonium sulphate and acetone. This led to the conclusion that the characteristics of both protein groups are too similar in order to separate them efficiently with neither of the above methods.

In summary, all three different techniques tested for the purification of interferon from host cell proteins showed that both protein groups have similar characteristics in their binding affinity towards ion-exchange resins and mix-mode resins and in their precipitation points. The remaining characteristic that differ between both protein groups is their molecular weight. Hence, the following section focuses on the IFN purification from process samples using size-exclusion chromatography.

6.3.3.3 Size-exclusion chromatography

As a last technique to purify interferon-α-2b from host cell proteins in recovery process samples, size-exclusion chromatography was tested. Results of the previous section showed that many protein characteristics of interferon and host cell proteins are very similar and accordingly this makes it challenging to separate one from the other efficiently. One characteristic that distinguishes between interferon and host cell proteins is molecular weight. Interferon-α-2b has a molecular weight of approximately 19kDa, whereas most host cell proteins have a molecular weight between 60 and 100kDa. Results with ultrafiltration spin tubes in section 6.3.3.1 showed, that a separation of IFN and HCP by size is possible, but larger proteins tend to block the membrane pores and interfered with this technique. With size-exclusion chromatography larger molecules should elute first and the elution of smaller molecules is delayed due to partitioning with the stationary phase of the column (section 6.1.4.4).

For the purification of interferon from host cell proteins in recovery process samples, a HiPrep 16/60 Sephacryl S-100 High Resolution column was used with a separation range of 1 - 100kDa and 120ml column volume. The experiments were performed as described in section 6.2.3.4.

In order to identify the elution time of interferon, a sample of the pure drug substance of interferon-

α-2b in phosphate buffer was injected onto the column. Figure 6.12 shows the elution chromatogram at UV-280 for interferon-α-2b in phosphate buffer at a concentration of 3.40g/l.

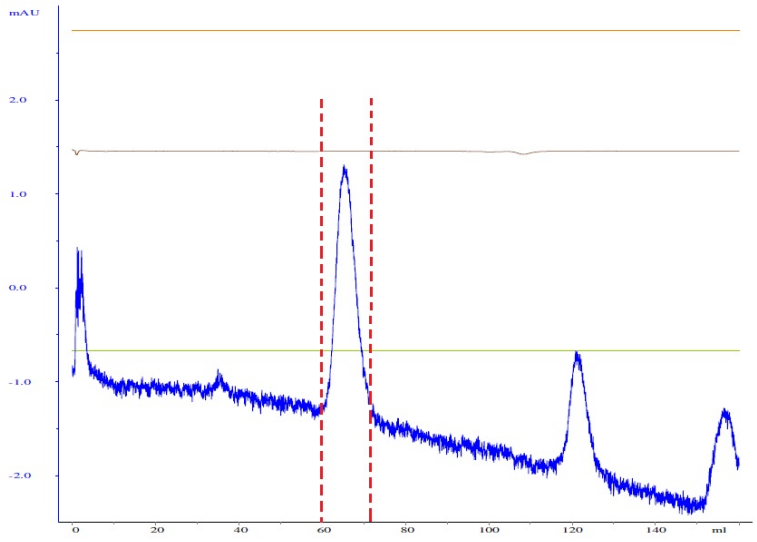

Fig. 6.12: UV chromatogram of size exclusion purification of interferon-α-2b drug substance in phosphate buffer. Interferon elutes between 60 and 72ml at a flow rate of 1ml/min

Two elution peaks can be seen in the elution chromatogram, the first with an elution time of 60 - 72 minutes and the second at 115 - 125 minutes. The first peak is expected to be interferon with an increase in the UV-280 reading of Δ2.50mAU. The second, smaller peak is probably due to impurities in the phosphate buffer. Both peaks were collected as separate fractions, as well as the eluate between and before the peaks. Thus, four different fractions were collected for different protein analysis. These fractions were analysed for total protein using the Bradford assay and for total interferon-α-2b using SDS-PAGE as described in sections 6.2.4 and 6.2.5.

No proteins, neither total protein nor interferon, were detectable in any of the collected fractions. The reason therefore is probably the significant dilution performed throughout the size-exclusion chromatography. One millilitre was injected onto the column and was recovered in fractions between 10 - 60ml. Hence, the collected fractions were concentrated prior to their analysis with ultrafiltration spin tubes and a 5kDa cut-off as described in section 6.2.3.1 and discussed in section 6.3.3.1. Unfortunately after the concentration only 15% of the initial interferon amount was found in the fraction of peak 1 (60-72min). In all other fractions no protein was detected which was as expected. It is inexplicable what happened to the remaining 80% of the initial interferon, other than that dilution resulted in the concentration falling below the detection limit of the assay

system. Results of section 6.3.3.1 showed that the concentrating step with ultrafiltration spin tubes has a recovery yield for interferon of >95% and no protein was detected in the flow through of the concentrating step.

Figure 6.13 shows another UV-280 chromatogram of a size-exclusion purification. The sample injected onto the column contained 0.5g/l BSA and 0.6g/l IFN (drug substance) diluted with fermentation media.

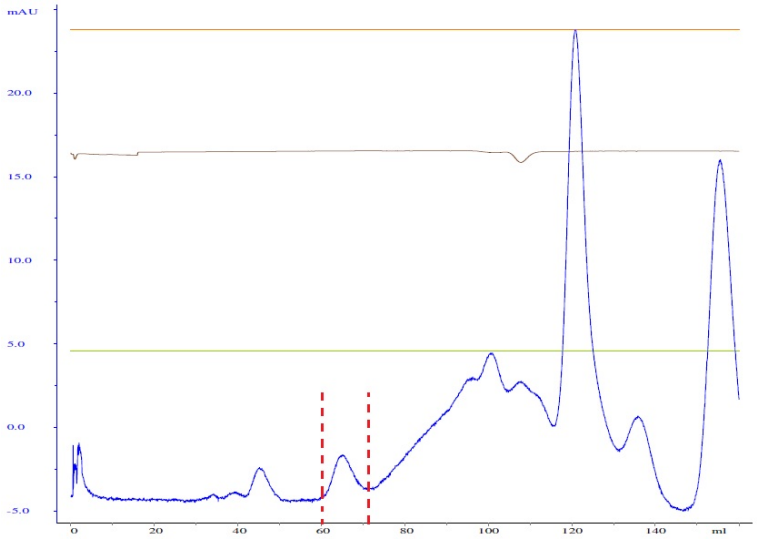

Fig. 6.13: UV chromatogram of size exclusion purification of a mixture of interferon-α-2b drug substance and bovine serum albumin (BSA) in process media. Interferon elutes between 60 and 72ml at a flow rate of 1ml/min

Two elution peaks can be seen in the first half of the chromatogram, the first peak between 43 to 50ml and the second peak between 60 to 72ml. The first peak is expected to be BSA, since this protein is larger than interferon and should elute first and the second peak is expected to be interferon with the same elution time than detected during the first experiment. The peaks eluted after interferon are expected to be media components from the fermentation media and are collected in one fraction.

The chromatogram shows a complete separation between both peaks which indicates a complete separation of the protein mixture between BSA and IFN. However, concentrating and analysing both fractions for total protein and interferon-α-2b again resulted in only 10 - 15% recovery of the initial injected concentration of BSA and IFN. These results are unexpected. Theoretically, all eluted proteins recovered in the different fractions should add up to the initial protein loaded onto

the column. There is no reasonable explanation why more than 70 - 80% of the initial protein load is not detectable in any of the collected fractions.
Since it is not explicitly possible to determine where the proteins were lost throughout this method (either throughout the SEC or the concentrating step), no further optimization steps were performed. In summary, the SEC showed potential to separate interferon-α-2b from other proteins by size without any additional pH settings but the recovery yield was less than 20%. This yield is too low and insufficient for a purification method prior to a quantification. Hence, the size-exclusion chromatographic method cannot be used to separate interferon from host cell proteins in recovery process samples prior to the IFN isoform quantification.

6.3.3.4 Summary of all purification and concentrating methods

Recovery process samples have two challenging characteristics for protein quantification, first the interferon-α-2b is at a very low concentration of less than 0.3g/l and second interferon-α-2b represents less than 5% of the total protein with 95% being host cell proteins. Quantifying these recovery process samples directly with the RP-HPLC methods for their IFN isoforms result in low resolution and poor accuracy. Accordingly, a purification and concentrating method was required in order to receive higher resolutions and increase the accuracy of the RP-HPLC quantification.
First the existing ion-exchange HiTrap method was tested which showed an IFN recovery of only 50% and a falsified isoform distribution due to required pH settings. Next, ultrafiltration spin tubes were tested for their potential to purify and concentrate interferon in recovery process samples. An efficient method was developed for concentrating interferon with ultrafiltration spin tubes and 5kDa cut-off membranes with a IFN recovery yield of >95%. The purification with 100kDa cut-off membrane resulted only in 30% interferon recovery, which is not sufficient. Furthermore, three other techniques with ion-exchange resin, mix-mode resin and protein precipitation were tested for their potential to separate interferon from host cell proteins. None of these methods met the criterion of 95% recovery yield. At last a size-exclusion chromatographic method was investigated. SEC uses the inverted technique compared to ultrafiltration spin tubes. Instead of the filtration of smaller molecules through a membrane, larger molecules are eluting first followed by the smaller ones. The SEC successfully separated interferon from other larger proteins but unfortunately only with a recovery of <20%.

Another possible method for the purification of interferon from host cell proteins would be immunoaffinity chromatography with specific interferon-α-2b antibodies on the stationary phase. However, results with the Biacore and BLItz (compare section 5.3.5) showed a very low binding affinity between interferon and commercialized antibodies. And it is not certain if all isoforms bind to the same antibody for interferon-α-2b since there exists no pure reference standard or specific antibody for each isoform. Hence, the immunoaffinity chromatographic method is not expected to have any potential to purify interferon from process samples with a recovery yield of >95%.

None of the tested purification methods were able to fulfil both criteria of an IFN recovery yield of >95% and no requirement for pH adjustment. Hence, the only way to get an idea about the mass balances of IFN isoforms throughout the recovery process is the direct sample injection onto the RP-HPLC column and accept the low resolution and poor accuracy.

6.3.4 IFN isoforms distribution throughout the primary recovery process

This section focuses on the distribution of the different interferon isoforms throughout the recovery process. Unfortunately, no complete mass balances could be performed due to the lack of a suitable purification method or more sensitive quantification methods for the isoforms.

As discussed in section 6.3.3.4, the only existing method to quantify IFN isoforms in recovery process samples is the direct injection of the sample onto the RP-HPLC column. The Aeris column was used for all quantifications, since this column is most sensitive and accurate in the quantification of all isoforms as described in section 6.3.1.2.

Results of chapter 4 and 5 showed that total protein and total interferon-α-2b exist in precipitated form and in solution throughout the recovery process. To avoid the necessity to separate precipitants from isoforms in solution and hence reducing the interferon concentration in each fraction even further, only samples of the recovery process were analysed that have simply proteins in solution and no protein precipitant fractions. These samples are S-1 (EOF) and S-8 (compare figure 5.4) and S-15 (TCA-pellet), where all precipitants can easily be solubilized in extraction buffer (20mM Tris, 1mM EDTA buffer, pH 8.0).

The isoform results of these three samples, quantified by Aeris RP-HPLC method as described in section 6.2.1.3, are shown in figure 6.14

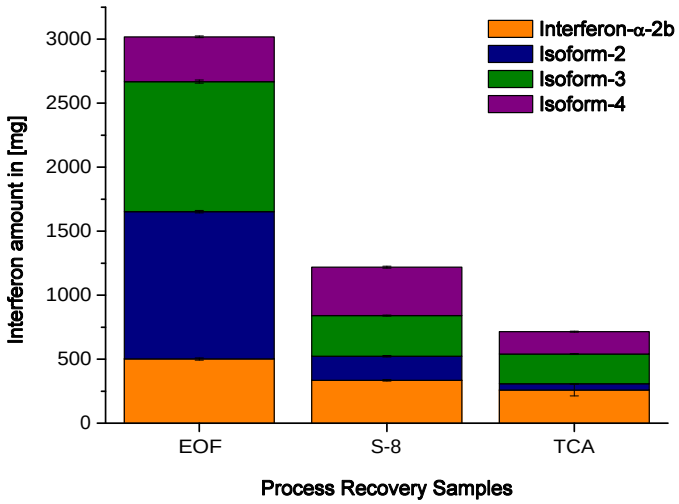

Fig. 6.14: Interferon isoform distribution in the recovery process samples EOF, S-8 and TCA-pellet in total grams quantified with RP-HPLC and Aeris column

It can be seen in figure 6.14 that the total amount of interferon-α-2b, including all isoforms, is reducing throughout the recovery process. From the end of fermentation to S-8 (cycle-2) more than 58% of total interferon is lost, which is consistent with the findings of total IFN quantified by SDS-PAGE discussed in chapter 5. From sample 8 to the final TCA-pellet of the recovery process further 40% in total interferon-α-2b is lost, which results in an overall loss of more than 75%. These findings are consistent with the mass balances set up in chapter 5. Comparing the actual quantified interferon from the RP-HPLC analysis with the SDS-PAGE quantification of chapter 5 it can be observed that with RP-HPLC only half the IFN is quantified compared with SDS-PAGE. This observation is consistent with the findings of section 5.3.5. It was shown that SDS-PAGE overestimates the total interferon-α-2b due to interferences of host cell proteins with the same or similar molecular weight than interferon. However, the findings of figure 6.14 show the total interferon-α-2b was overestimated but the percentage reduction and IFN loss throughout the recovery process remains the same.

Looking at the distribution of the isoforms, figure 6.14 shows that the majority of interferon at the end of fermentation is composed of the isoforms 2 and 3 and only smaller portions of isoform 4 and interferon-α-2b. Throughout the recovery process interferon-α-2b and isoform 4 show only minor reductions of less than 40% whereas isoform 2 and 3 are reduced by 75 - 95%. With the available

information it is not clear whether isoform 2 and 3 were lost to higher fractions throughout the recovery process or if some of these isoforms were converted to isoform 4 or interferon-α-2b. In order to draw these conclusions a full mass balance of all isoforms throughout every step of the recovery process would be required.

Results in figure 6.15 show the interferon isoform distribution as a percentage for each of the three analysed recovery process samples.

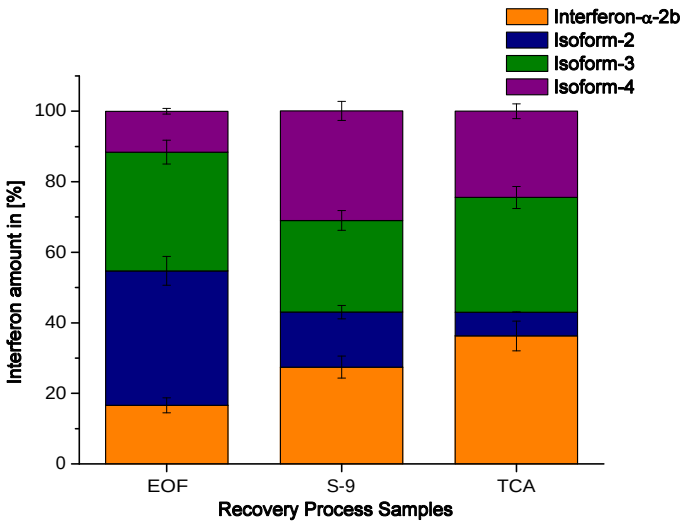

Fig. 6.15: Interferon isoform distribution in the recovery process samples EOF, S-9 and TCA-pellet in total percentage quantified with RP-HPLC and Aeris column

As was already seen in figure 6.14, interferon-α-2b and isoform 4 increase throughout the recovery process and isoform 2 and 3 decrease. In the end of fermentation the majority of total interferon is isoform 2 and 3 with more than 40 and 32%. Interferon-α-2b and isoform 4 represent only 15 and 12%. This proportion changes throughout the recovery process and in the TCA-pellet interferon-α-2b and isoform represent 36 and 25%. Isoform 2 and 3 on the other hand represent only 8 and 31%.

This observations is important, since only interferon-α-2b is the actual drug substance and isoform 4 can be converted to interferon-α-2b during the down-stream process. This means with 27% of interferon-α-2b and isoform 4 in the EOF sample only 810g of the 3000g total interferon are expedient for the drug substance. In the TCA-pellet interferon-α-2b and isoform 4 represent 61% together which are 450g of the 750g total interferon. Hence, more than 50% of the expedient

fraction of total interferon is recovered throughout the recovery process, which is twice as much as the 25% recovery in total interferon.

In summary, the results for interferon isoforms quantified with RP-HPLC showed a loss of 75% in total interferon-α-2b throughout the protein recovery process. Looking at the expedient fraction of interferon-α-2b and isoform 4, only 50% reduction was observed throughout the process. The major loss was detected between the EOF and sample 8, which confirms the findings with the SDS-PAGE of chapter 5.

It has to be kept in mind to treat these results with caution. Quantification with RP-HPLC showed very low resolution and poor accuracy due to low protein concentrations and interference from a large number of host cell proteins present in the recovery process samples.

6.4 Conclusion

Concluding the findings observed during this chapter, this section is divided into four parts. The first part will summarize the results of the characterization and optimization of the three RP-HPLC quantification methods for interferon isoforms, followed by the summary of the findings for a purification and concentration method for recovery process samples prior to the RP-HPLC quantification. The third part focusses on the conclusion of the isoform distribution throughout the recovery process analysed with the RP-HPLC methods. The last part of this section gives an overall conclusion for this chapter and an outlook for next possible steps.

6.4.1 Quantification of interferon isoforms

Three RP-HPLC methods for interferon isoform quantification were characterized and optimized with design of experiments to receive higher accuracy and enhanced resolution. The Zorbax method was identified to have the best accuracy and highest resolution for the quantification of interferon-α-2b. However, the Aeris method showed the best accuracy and resolution in order to quantify all interferon isoforms and interferon-α-2b. Hence, the Aeris method was selected as the method of choice for further interferon isoform quantification.

6.4.2 Purification and concentrating recovery process samples

Since recovery process samples have very low concentrations of interferon and its isoforms, while the presence of host cell proteins interferes with the RP-HPLC quantification, a purification and concentrating step prior to quantification was required. The method currently used by the industrial partner, an ion-exchange method with HiTrap columns, showed only a 50% recovery in interferon and the pH required adjustment during sample preparation resulted in changes in the isoform distribution and hence falsified the results. Other techniques were tested for purifying and concentrating recovery process samples to receive a recovery yield of at least 95% and no pH adjustment is required during sample preparation.

Ultrafiltration spin tubes with a 5kDa cut-off showed good results to concentrate interferon in recovery process samples with an IFN recovery yield of over 95%.

For purifying recovery process samples, ultrafiltration spin tubes, different ion-exchange resins,

mix-mode resins, protein precipitation and size-exclusion chromatography were tested. However, none of these techniques resulted in a recovery yield of 95%. SEC showed the highest potential and successfully separated interferon from host cell proteins. Unfortunately the recovery was only 20%.

6.4.3 Isoform distribution throughout the recovery process

Since no purification technique had a 95% recovery for interferon, selected samples of the recovery process were analysed for their isoform distribution directly with the RP-HPLC method and Aeris column without any pre-purification steps. Results confirmed the findings of the SDS-PAGE with 75% loss of total interferon throughout the recovery process. The main reduction occurred in isoform 2 and 3, whereas isoform 4 and interferon-α-2b showed only a 50% reduction throughout the process. To draw final conclusions as to whether the reduction in isoform 2 and 3 is due to losses throughout the recovery process or due to conversions into isoform 4 and interferon-α-2b, more recovery process samples need to be analysed for their isoform distribution with more accurate quantification methods.

6.4.4 Overall conclusion and Outlook

The findings of this chapter showed that no method is in place to accurately quantify the interferon isoforms throughout the entire recovery process. The existing RP-HPLC methods can distinguish between the isoforms if the process samples reach a certain purity and concentration. Without purification the existing quantification methods are not accurate enough and are too poor in resolution in order to obtain reliable results. The purification of the recovery process sample showed to be more challenging than expected. Several known techniques for protein purification were tested, however none of them met the criteria to purify the recovery process samples successfully.

In order to set up an accurate and robust method for the quantification of interferon isoforms throughout the recovery process a more sensitive quantification method needs to be developed. Possible methods known from the literature could be an UPLC method or a LC-MS method. However, the literature showed that the presence of large amounts of host cell proteins interferes with these quantification methods. Hence, the development of a purification method prior to the

interferon quantification seems to be indispensable. With the current protein purification techniques available, it was not possible to find a method for interferon purification in the recovery process samples.

Furthermore, recovery process samples directly analysed with the low accurate RP-HPLC quantification methods showed a loss of more than 75% of the total interferon throughout the recovery process with the main loss in interferon 2 and 3. The critical process point was identified before cycle-2 which confirms the findings of the two previous chapters. Hence, the next step is to optimize the recovery process in order to minimize the loss of interferon-α-2b and its isoforms.

Chapter 7

Process Optimization - Protein Dilution Study

7.1 Introduction

Chapters 4, 5 and 6 have shown two critical process steps (CPS) throughout the primary protein recovery process, with an overall loss of 80% in the total interferon-α-2b and 90% in total protein. CPS-1 was identified during cycle-1 with an interferon loss of 60% due to insufficient protein solubilization and results indicated a protein saturation problem.

This chapter will focus on the optimization of the primary critical process step by diluting the protein concentration during cycle-1 of the protein recovery process in order to eliminate the saturation problem and to increase the interferon-α-2b recovery.

7.1.1 Protein solubility levels

The solubility levels of proteins in water can vary from completetly insoluble to hundreds of milligrams per milliliter, for example the protein crambin is completely insoluble in water [97] whereas serum albumin has a solubility of more than 500mg/ml [98]. Protein solubility is a thermodynamic parameter which is influenced by a number of extrinsic and intrinsic factors. Most important extrinsic factors are: pH, ionic strength, temperature and the presence of solvent additives such as detergents [99]. On the other hand intrinsic factors are mainly defined by the charged

groups (side chains) on the protein surface, thus the folding and packing of the polypeptide chain. The proportion of charged side chains on the protein surface is determined by 4 main physical forces: conformational entropy, charge-charge interactions and salt bridges, hydrophobic interactions and hydrogen bonding and polar group burial [100].

Free rotation of protein bonds provide strong entropic driving forces which can lead to unfold a protein. However, protein charges on the surface are more attractive than repulsive interactions at neutral pH. Hence, protein can be stabilized by reducing the surface charges due to the addition of small salt concentrations (salting-in, see section 4.1.4). Salt ions reduce the electrostatic attractions between neighboring protein molecules. Hydrophobicity and hydrogen bonding as well as van-der-Waals interactions are also important for the protein stability and solubility such as polar group burial [100].

Proteins interact with water molecules in order to stay in solution. If too many proteins are present for too few water molecules the solution is saturated and proteins start to aggregate and become insoluble. Studies have shown that this precipitation can be partly reversed by the addition of further buffer or water [99].

Protein solubility is a function of ionic strength. With the addition of salt at low ionic strength, protein solubility can be increased; this is termed salting-in. However, the addition of salt at high ionic strength leads to a decrease in protein solubility and protein precipitation; this is termed salting-out. Protein stability is also dependent on the pH. The stability decreases at lower pH, acidic environment, and at the pI of the protein [101].

7.1.2 Protein precipitation and salting-out

Protein precipitation or salting-out is one of the oldest techniques to separate proteins from a solution mixture [101]. Precipitation of proteins due to the addition of salt was already briefly described in section 4.1.4. This section will focus more on a macromolecular level during the salting-out.

Grover et al. [102] describes salting-out as a precipitation of less soluble material and proteins, from a solution such as water, in which it is mixed with other substances. As already mentioned in section 4.1.4 and equation 7.1, the salting-out effect can be mathematically described by the

Setschenow equation [102]:

$$\log S_o/S = k * C_s \tag{7.1}$$

With:

- S_o = solubility of the protein in water
- S = solubility of salt
- C_s = salt concentration in mol/L
- k = salting out constant (dependent on the salt)

If the salting-out constant is a positive value, it indicates salting-out (precipitation) and a negative value indicates an increase in protein solubility. With a constant protein solubility value in water (S_o) it follows from equation 7.1, that the added amount of salt is directly proportional to the amount of protein precipitationed. The salting constant 'k' is the sum of cationic and anionic salt ions, and different salt ions have different salting constants. Hence, various combinations of salt ions, such as different salts as NaCl and $MgCl_2$, lead to different properties to cause salting-out.
The physical and thermodynamic reactions/ processes during salting-out is very complex and still not completely understood. Several different types of intermolecluar interactions and forces occur between protein and water, water and salt and protein and salt [102]. However, the salting-out effect occurs mainly due to the electrostatic effects and the competing between proteins and salt ions over water molecules. The electrostatic effects are dependent on the size, structure, charge density and hydration of the protein and the salt ions itself.
In particular the size, structure and shape of the protein have an important role during salting-out, as was shown by Chane et al. [101]. At low pH and ionic strength the proteins form derivates from the sphericity which become more voluble and hence less stable and precipitation occurs. Non-spherical proteins have a larger contact surface for interactions with salt ions or other proteins, which leads to an increase in the formation of precipitants.
Protein-protein interactions in protein mixtures can also lead to co-precipitation of proteins [103]. Co-precipitation occurs due to isoelectric coagulation of proteins and sometimes even disulfide bond formations between cystein groups [104]. Especially in the presence of calcium salts, co-precipitation of proteins occurs. This can lead to a salting-out of proteins with high solubility

levels at lower ionic strength or salt concentrations than usually required, due to protein-protein interaction of a protein with a lower solubility level.
Grover and Ryall [101] also describe the salting-out effect by an internal pressure theory. According to this, the salting-out effect increases with a decrease in the compressibility of the solution. This explains the low protein solubility levels in water, since water has a very low compressibility factor. This study also shows that the salting-out effect of proteins is less effective in the presence of low protein concentrations.
In summary, the salting-out of proteins depends on several factors such as, electrostatic effects, protein shape, protein-protein interactions and protein concentration.

7.1.3 Goals and Aims

The aim of this chapter is the optimization of the critical process step one of the primary protein recovery process by the implementation of a dilution step during cycle-1 to eliminate the protein saturation problem and to enhance interferon-α-2b recovery.
First, a screening step of several different dilution factors is performed to identify the optimal dilution condition for maximal interferon-α-2b recovery and minimal process volume. Secondly, the optimal dilution factor is applied to the primary protein recovery process in a small scale study to investigate the impact of increased protein solubilization in cycle-1 on the following recovery process steps. Finally, the impact of the increased protein recovery during the recovery process on the first step of the down-stream process, an ion-exchange method, is investigated.

7.2 Material and Methods

7.2.1 List of Materials

Tab. 7.1: Materials and suppliers

materials	supplier
Recovery process samples (EOF, and Sample-4)	industrial partner
interferon-α-2b - drug substance	industrial partner
NaOH	Sigma Aldrich
EDTA tetrasodium salt	VWR
Trizma Base	Sigma Aldrich
Bovine serum albumin (BSA)	Sigma Aldrich
Bradford Reagent	Sigma Aldrich

7.2.2 Dilution experiments

The dilution experiments were performed using recovery process samples from the manufacturing process. Sample time point 4 was selected for this purpose (figure 2.3 in chapter 2.5). The sample 4 was thawed in a waterbath at 37°C and centrifuged in a bench top centrifuge (FL40R centrifuge, Thermo Scientific, Dublin, Ireland) at 7500rpm for 30 minutes at 4°C. Supernatant was discharged and the pellet was dissolved in water accordingly to the different dilution factors. Dilution factor one correlates to the original volume of the small scale model (table 3.7). After setting the pH to 7.0 with NaOH the sample was mixed for 4 hours constantly. To collect the resuspended proteins, the sample was centrifuged again under the same conditions as mentioned earlier and both fractions, supernatant and pellet, were recovered and stored for protein analysis at 4°C. Collected pellets were dissolved in EDTA-Tris extraction buffer (1mM EDTA, 20mM Tris, pH 8.0) prior to analysis.

7.2.3 Simulation of the Protein Recovery Process

The laboratory-scale simulation of the recovery process (RP) is based on the industrial-scale primary protein recovery process as described in chapter 3.2.3. The industrial process is performed with a working volume of 32'500L which was scaled down to 0.04L and 0.5L for the laboratory RP (table 3.7).

For the performance of the process simulation two types of samples are used. The first type is an End of Fermentation (EOF) sample, orginated from a fermentation at manufacturing scale, which was shipped on dry ice to DCU. The second type of sample is an End of Fermentation pellet, originated from a small scale fermentation run performed from the industrial partner. This pellet was resuspend in an EDTA-Tris-extraction buffer (1mM EDTA, 20mM Tris, pH 8.0) in the labs in DCU to proceed with the simulation of the recovery process.

In order to determine the impact of a dilution-step on the recovery process and the recovery of interferon at the end of the RP-process, the amount of water added to the process in cycle-1 after the first centrifugation was adjusted according to the dilution factor. A two-fold dilution factor correlates to a two-fold increment in the water-volume added to the process.

Throughout the simulation of the recovery process, samples were taken at several different time points as implemented on site with the industrial partner for the manufacturing process (chapter 2.5 and figure 2.3). Samples taken from the simulation process were stored at 4°C until protein analysis was performed.

7.2.4 Simulation of Down Stream method - Ion Exchange Chromatography

The simulation of the first step of the down-stream process, an ion-exchange chromatography method, was performed as described in chapter 10 in section 10.2.2.

7.2.5 Total protein quantification - Bradford

Total protein was quantified using the Bradford assay as described in chapter 3.2.2 and table 3.6. Sample pellets were dissolved in EDTA-Tris buffer (1mM EDTA, 20mM Tris, pH 8.0) prior to protein analysis. BSA dissolved in water was used as a reference standard for all total protein

quantifications. The blank was chosen accordingly to the sample to be analysed, water, EDTA-Tris buffer (1mM EDTA, 20mM Tris, pH 8.0) or diluted fermentation media.

7.2.6 Total Interferon-α-2b quantification

Total interferon-α-2b was quantified using SDS-PAGE as described in chapter 5.2.3 and 5.2.4. Sample pellets were dissolved in EDTA-Tris buffer (1mM EDTA, 20mM Tris, pH 8.0) prior to analysis as mentioned in the section above. No further sample treatment was required. The interferon-α-2b drug substance was used as a reference standard for all quantifications.

7.3 Results and Discussion

7.3.1 Dilution factor study

Chapter 4 and 5 discussed the characteristics of the primary protein recovery process performed by the industrial partner. Two critical process steps (CPS) were discovered for total protein and total interferon-α-2b recovery. CPS-1 was identified during cycle 1 of the recovery process and is believed to be due to insufficient resuspension of precipitated proteins (HCP and IFN) due to the low solubility of the proteins in water. CPS-2 was identified in cycle 2, where salting-out of protein occurs not only in host cell proteins but also in interferon-α-2b. CPS-1 has been selected as the more critical process step with a loss of 80% in total protein and 60% in interferon-α-2b. Hence, a first step in the recovery process optimization is to look at the critical process step 1 and to enhance the protein solubility in water.

Since the protein recovery process is performed at a manufacturing scale of 40,000L the optimization steps need to be applicable to large scale and GMP production conditions. One relatively straight forward method to increase the protein solubility is to dilute the samples by increasing the volume. Accordingly, different dilution factors were tested to enhance the interferon-α-2b solubility in water. Keeping the process volumes at manufacturing scale in mind, a two or three fold dilution would be the highest realistic volume to be implemented on site with the industrial partner.

Figure 7.1 shows the resuspended amounts of total protein and total interferon-α-2b in water during cycle-1 of the recovery process for different dilution factors. Dilution factor 1 represents the actual conditions of the manufacturing process in cycle-1. Hence, a two-fold dilution correlates to a doubling in volume.

The dilution experiment was performed as described in section 7.2.2. Each dilution factor was performed in triplicate and figure 7.1 shows the average of these three runs for the collected supernatant. The results show the protein fraction in % that was resolubilized during the experiment.

The total amount of interferon-α-2b (IFN), analysed with SDS-PAGE (section 7.2.6), shows an increase from 32% at dilution factor of 1 up 50% for a dilution factor of 2. Thus the recovery of IFN during CPS-1 can be improved from 32% up to 50% by simple dilution. However, it can be seen

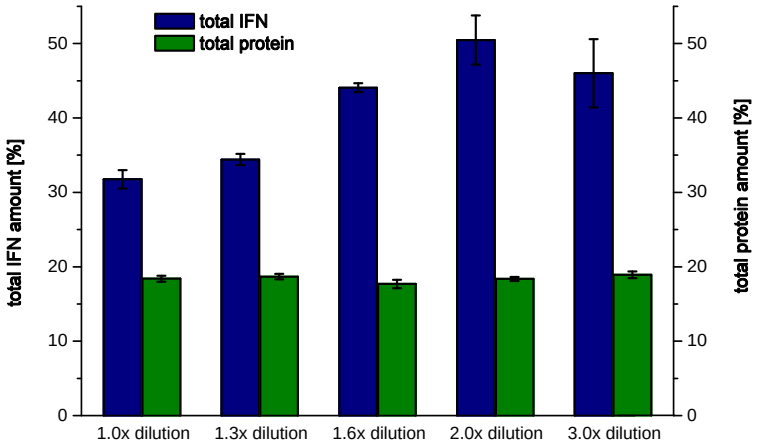

Fig. 7.1: Recovered amount of solubilized interferon-α-2b and total protein for different dilution factors during Cycle 1 of the Recovery Process

that the increase in IFN-solubility is not linearly correlated with the dilution factor, as would have been expected. The reason for the non-linear relationship may be due to protein-protein interactions. Host cell proteins (HCP) represent a large fraction of total protein in the process samples and therefore non-specific interactions with IFN proteins is likely. Some HCP have a lower solubility limit in water compared to IFN, which might interfer with the solubilty of IFN, by co-precipitation effects (section 7.1.2). This hypothesis is further supported by the detected IFN fraction at a 3-fold dilution factor, where no increase in resolubilized IFN was observed even though the volume was increased with corresponding decrease in concentration. This indicates that all 'free' IFN-proteins (those not interacting with HCP) are in solution at a 2-fold dilution factor, and the remaining IFN protein interacts with HCP which have a much lower solubility limit in water. Hence these IFN proteins remained in a precipitated form even though the volume was further increased.

The total amount of protein, analysed using the Bradford assay (section 7.2.5), remained constant over the whole range of dilution factors at around 18 - 20%. This result is unexpected. From the observations in chapter 4 and 5, it was expected that the solubility of total protein and interferon would be similar, since they are following the similar concentration profiles throughout the recovery process. However, this is not the case, which supports the hypothesis, that some host cell proteins have a much lower solubility limit in water. Host cell proteins with a high solubility limit in water are resolubilized completely at a 1x dilution factor (section 7.1.1). The remaining 80%

of HCP appears to have a much lower solubility limit in water and thus remained in precipitated form even after the water level was increased.

One may wonder why the increment of interferon at different dilution factors cannot be seen in the total protein results. The reason is probably due to the small amount of IFN compared to total quantity of proteins, with interferon representing less than 5% of total protein. Hence, the small changes in the amount of interferon would not be significant compared to the total protein concentration.

Since the IFN solubility increases with a doubling of the dilution factor, whereas the total amount of protein remained constant, the ratio of HCP to IFN is changing. At a dilution factor of 1-fold, which represents the working conditions at manufacturing scale, interferon-α-2b represents only 8.5% of total protein. With an increment in IFN due to an increase in the dilution factor, this ratio between HCP and IFN changes and interferon represents 13.4% of total protein at a 2-fold dilution factor. This means that interferon represents a higher fraction of the total amount of protein and it needs to be investigated how this interfers with the subsequent salting-out step of the recovery process. This impact will be investigated in a simulation of the recovery process in a small scale model in the following section.

7.3.2 Small scale recovery process model

In order to verify the observed results from the previous section, a simulation of the protein recovery process in a small scale model at 0.04L was performed as described in section 7.2.3. The simulation was performed four-times and all samples taken throughout the recovery process were analysed using SDS-PAGE for total interferon-α-2b (7.2.6) and Bradford assay for amount of total protein (7.2.5). Cycle-1 to cycle-4 in the following figures represent the four different centrifugation cycles throughout the recovery process (c.f. figure 2.2 of the primary protein recovery process). The detected amount of protein at each cycle is the recovered protein fraction after the centrifugation cycle.

Figure 7.2 shows the total amount of interferon-α-2b throughout the recovery process simulation (steps cycle 1 to cycle 4) at small scale (0.04L) for the undiluted (1x dilution) and the twice diluted samples (2x dilution).

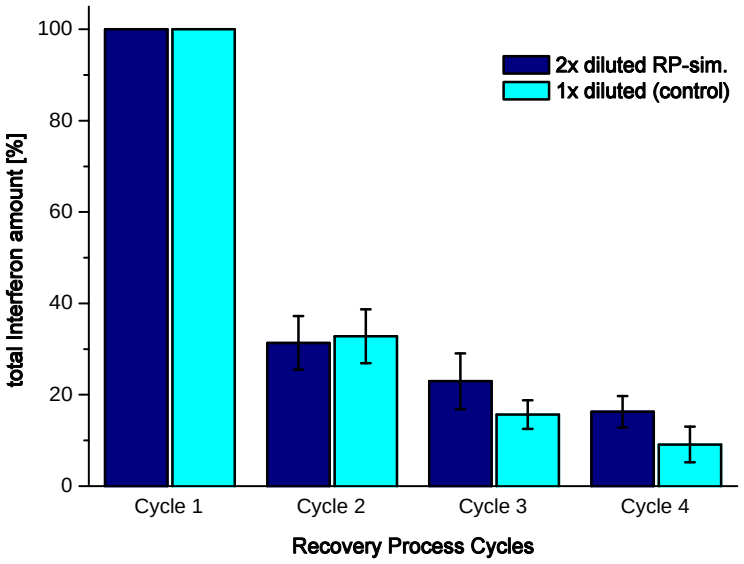

Fig. 7.2: The amount of total interferon-α-2b recovered throughout the simulated recovery process in a small scale model at 0.04L-scale

The IFN results in figure 7.2 show an approximate 32% recovery after resolubilization in water (cycle-2) for both, 2x and 1x diluted simulation runs. Hence, no improvement in protein solubility could be observed due to increasing the volume. This result is not consistant with the observation from section 7.3.1, where an increment of 18% in IFN recovery was observed due to dilution of the sample two-fold. One reason for this inconsitency in the amount of IFN may be due to the quantification method to determine total IFN using SDS-PAGE. The results in chapter 5 have shown that the average error in quantifying IFN reference standards with SDS-PAGE can be up to 10%. Consequently it is possible that in the case of the simulation of the recovery process, the recovered amount of IFN after resolubilization actually increased in the 2x diluted run compared to the 1x diluted run, however the error in quantitative estimation using the SDS-PAGE method resulted in an apparent lack of improvement.

Following resolubilization is the salting-out step in which protein recovery is represented by cycle-3, figure 7.2. The results indicate a reduced loss of IFN in this step in the 2x diluted simulation run. Although the amount of interferon is the same in cycle 2, the volume is doubled, cosequently the IFN concentration is only half of the concentration as in the 1x diluted run. A lower protein concentration can have an impact on the salting-out of proteins, where proteins precipitate due to

protein-protein interactions (section 7.1.2). A lower protein concentration results in fewer protein-protein interactions and consequently reduced protein precipitation. The observed enhanced IFN recovery in the 2x diluted run can also be seen at the end of the recovery process in the TCA-pellet (cycle-4), where 17% of total IFN was recovered in the 2x diluted simulation compared to only 10% recovery of IFN in the 1x diluted run. However, care should be taken in interepreting this result, since the error bars, which result from the four performed iterations of the recovery process simulation overlap each other. This indicates that the detected differences are probably not significant. The challenge with the small scale model is the very small volume of 0.04L. At this scale small errors can have a big impact on the results, as has been seen previously in chapter 3. Increasing the volume of the recovery process simulation, would be expected to increase the precision of the results. To verify the significance of the findings of this section, the process simulation needs to be performed at larger scale (see section 7.3.3).

Figure 7.3 shows the total amount of protein throughout the recovery process simulation at small scale (0.04L) for the undiluted (1x dilution) and twice diluted samples (2x dilution).

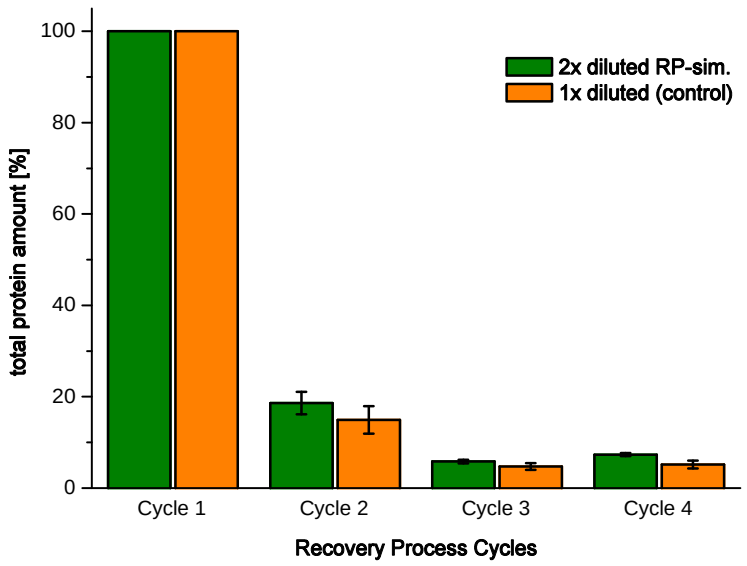

Fig. 7.3: Recovered amount of total protein throughout the simulated recovery process in a small scale model at 0.04L scale

The recovered amount of total protein after the resolubilization step (in cycle-2) is 18 - 20%

for both the 2x and 1x diluted simulation. These results are consistant with the findings of the previous section 7.3.1. Consequently no significant improvement was observed in the recovery of total protein due to the doubling of the volume, i.e. reducing the protein concentration to a value potentially below the solubility limit. A similar result was observed for total protein results for the salting-out step (cycle-3), where no significant differences were observed between the 2x and 1x diluted runs. A similar amount of total protein precipitated during the salting-out step regardless of the protein concentration. Overall, at the end of the recovery process simulation, the detected total protein recovery is 7.3% in 2x diluted and 5.2% in 1x diluted run, which is not considered to be significantly different.

Since the recovery process simulation at 0.04L scale demonstrateed an increase in total IFN recovery at the end of the process through a two-fold increase of the volume, a change in the HCP to IFN ratio might be expected between the 2x and 1x diluted runs. However, the ratio of HCP to IFN is in both cases comparable with a factor of 8.5 for 2x diluted simulation and a factor of 8.6 for the 1x diluted run. One reason for the similar ratio of HCP to IFN between both simulations, is that the measured amount of total protein in 2x and 1x diluted run is similar. The amount of interferon is only a small fraction of the total protein, hence the detected changes in IFN have no significant impact on the HCP to IFN ratio.

Summarising the results of the recovery process simulation at 0.04L scale with a 2x and a 1x dilution shows that while there is potential for an increase in IFN recovery through dilution in cycle-1 no definite conclusion can be drawn, due to the large errors associated with the small scale at which this simulation was performed. In order to further explore the potential to increase IFN recovery through process sample dilution, a simulation at larger scale, with a working volume of 0.5L should provide more reliable and reproducible results.

7.3.3 Pilot Scale model at 500mL

Since the results of the recovery process simulation at 0.04L scale showed some inconsistencies, the simulation with a 2x and 1x dilution was repeated at the larger scale of 0.5L in order to determine wether more reliable and reproducible results could be determined. The recovery process simulation was performed as described in section 7.2.3. Total interferon-α-2b was quantified using SDS-PAGE and total protein was quantified using the Bradford assay (see section 7.2.6 and

7.2.5). The simulation was performed three times and the results shown in figures 7.4 and 7.5 are the average values of these runs. As already described in section 7.3.2 above, in figure 7.4 and 7.5, cycle-1 to cycle-4 stand for the four different centrifugation cycles throughout the recovery process (see figure 2.2 of the primary protein recovery process). The detected amount of protein at each cycle represents the recovered protein fraction after the centrifugation.

Figure 7.4 shows the total amount of interferon-α-2b throughout the recovery process simulation at 0.5L for the original volume ratio (1x dilution) and the double volume ratio (2x dilution).

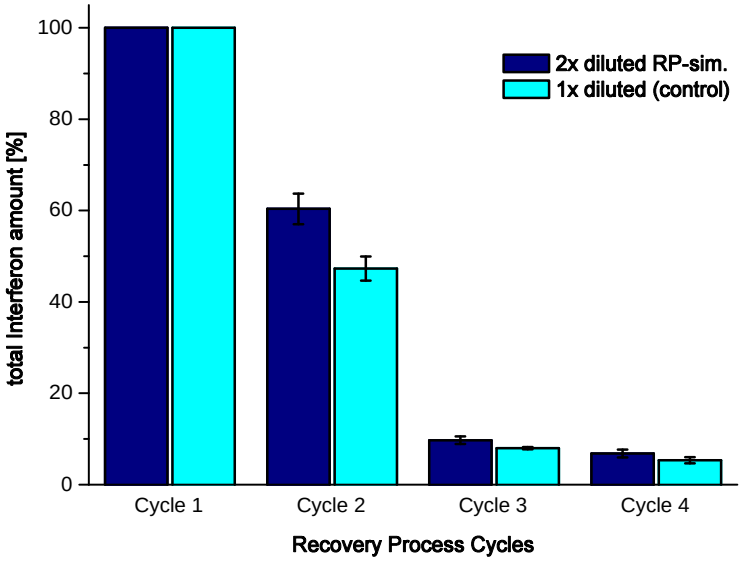

Fig. 7.4: Recovered total interferon-α-2b amount throughout the simulated recovery process in a small scale model at 0.5L

The simulation at large scale (0.5L) shows an increase in IFN recovery after the second centrifugation step due to a two-fold dilution of the process samples. After cycle-2, 60% of IFN was recovered in the 2x dilution run compared with only 47% of IFN in the 1x dilution run. These findings are consistant with the results from the dilution study (section 7.3.1), where a duplication of the volume resulted in an increase of IFN solubility of up to 15%, however, they are not consistant with results received in the simulation runs at 0.04L scale. Since a large error (more than $\pm$6%) is associated with the IFN recovery in the simulation at 0.04L scale, an increase in IFN recovery of 15% at this point would be within the standard variations of the small scale simulation.

Hence, the findings of the recovery process simulation at 0.5L are more reliable.
An unexpected result is the loss of interferon in the salting-out step for both dilution runs (1x and 2x), as seen for cycle-3 in figure 7.4. The loss of IFN in the 2x dilution run (50%) is even higher than the IFN loss of the 1x dilution run (37%). Both runs had a similar salt concentration during the salting-out step at a conductivity of ≈10mS/cm. Since the 2x diluted simulation was less concentrated in interferon (0.25g/L) than the 1x diluted run (0.32g/L), the salting-out of proteins should have been less efficient (section 7.1.2). In order to understand this phenomenon fully, the salting-out step during the protein recovery process needs to be characterized in more detail. For example, the impact of salt concentration, pH and agitation time on the salting-out behaviour of proteins. The results in chapters 4 and 5, showed that the salting-out had only a minor importance in the optimization of IFN recovery and therefore the further characterization was not considered to be a high priority.
The overall IFN recovery in both simulations is around 10%, without a significant difference between 0.04L and 0.5L scales. Hence, if the recovery process is performed with all 4 cycles, the doubling of the volume during cycle 1 is not considered to be beneficial. However, one alternative would be to perform only 3 cycles and leave out the salting-out step, which should improve the IFN recovery of 15% when doubling the volume.

Figure 7.5 shows the total amount of protein throughout the recovery process simulation at 0.5L scale for the original volume ratio (1x dilution) and the double volume ratio (2x dilution).

From figure 7.5 it can be seen that the amount of total protein and total interferon varried in a similar way between the 2x diluted and 1x diluted runs. After the second centrifugation (cycle-2), up to 50% of total protein was recovered in the 2x diluted simulation, which is a 20% increase compared to the 1x dilution run. Hence, as a result of a two-fold dilution the recovery of total protein increased at this stage of the process. This finding is inconsistant with the previous findings in the dilution study (7.3.1) or in the simulation at 0.04L scale (7.3.2). A reason for this may be the low working volumes at 0.04L for the simulation at small scale and 0.005L for the dilution experiments. As the results have shown (chapter 3), the accuracy and reproducibility of the recovery process simulation increases with higher working volumes. Hence, the results obtained in this section with the simulation at 0.5L working volume provide more reliable and reproducible results

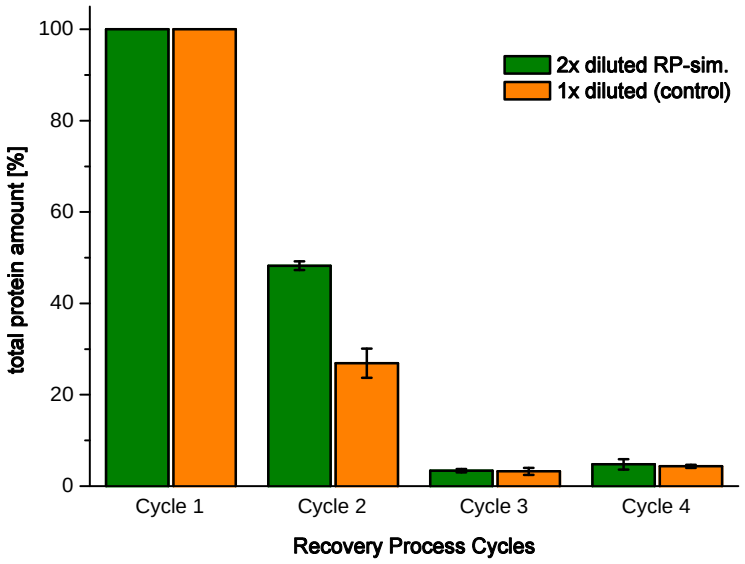

Fig. 7.5: Recovered total protein amount throughout the simulated recovery process in a small scale model at 0.5L

for total protein.

During the salting-out step a similar observation was made for total protein and total interferon. More protein salted-out in the less concentrated 2x diluted run, which is unexpected. The 2x diluted simulation run lost up 45% of total protein during the salting-out step and the 1x dilution run lost up to 25% of total protein. As explained in the paragraph above this phenomenom needs to be characterized in more detail in order to understand it completely. At the end of the recovery process in the TCA-pellet the 2x and 1x diluted run recovered both around 5% of total protein with no significant difference between them.

Looking at the ratio between host cell protein (HCP) and recombinant protein (IFN), there is no significant difference detectable between the 2x and 1x dilution runs. The host cell protein behaves in a similar way to the doubling of the volume as the recombinant protein, hence the same ratio of HCP to IFN is generated throughout the simulation process.

Summarising the findings of the simulation of the recovery process, the duplication of the volume during the resolubilization of precipitated protein (cycle-1) does improve the protein solubilization. However, this enhancement in protein solubility is not protein specific and appears for the

recombinant protein interferon-α-2b as well as for the host cell proteins of *E.coli*. The increased loss of both IFN and HCP in the salting-out step due to increased volume, is unexpected. In order to gain an increase in protein recovery throughout the recovery process, the process would need to be shortened and performed without the salting-out step. This step should provide an improvement of up to 15% in interferon recovery.

7.3.3.1 Recovery process simulation without the salting-out step

Section 7.3.3 showed an 15% increase in interferon solubility due to a two-fold dilution in cycle-1 of the recovery process. Unfortunately this increase got lost during the salting out step. Accordingly it was suggested to perform the recovery process without the salting-out step. This section discusses the results of the recovery process simulation without the salting-out step for a two-fold dilution during cycle-1. Figure 7.6 showes the recovered protein fractions for the recovery process simulation at 0.5l without the salting-out step for a two-fold and a one-fold dilution during cycle-1.

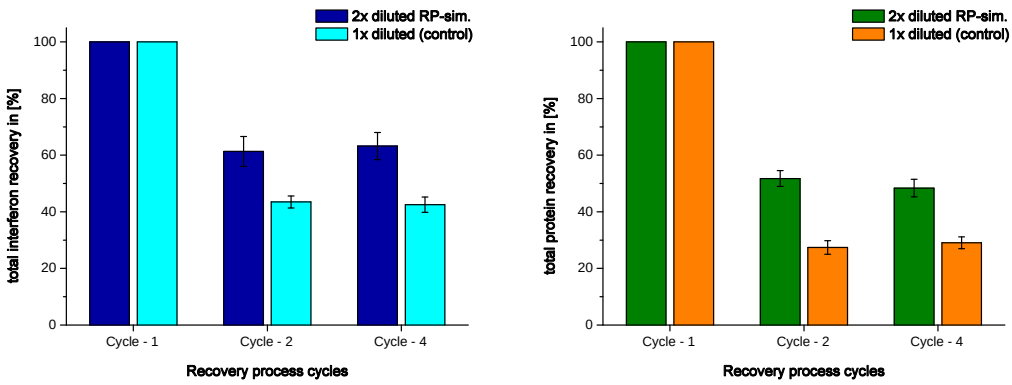

Fig. 7.6: Recovery process simulation in a small scale model at 0.5L with a 2-fold dilution or 1x dilution (control). Left side, shows the recovery of interferon throughout the simulation. Right side, shows the recovery of total proteins.

Total proteins were quantified with Bradford assay as described in section 7.2.5 and total interferon-α-2b was quantified with SDS-PAGE as described in section 7.2.6. As it can be seen in the left image of figure 7.6, the recovery of interferon-α-2b gets improved from 40 to 60% recovery due to a two-fold dilution in cycle-1. This observation confirms the findings of the previous section. The improvement in interferon recovery persists until the end of the recovery process due to the elimination of the salting-out step. Hence, the total interferon-α-2b recovery increased of 20% at

the end of the recovery process in the presence of a 2-fold dilution in cycle-1 and in the absence of the salting-out step.

The right image of figure 7.6 shows the total proteins recovered throughout the recovery process simulation without the performance of the salting-out step for a two-fold and one-fold dilution in cycle-1. Similar to the interferon results, total protein solubility is improved during cycle-1 from 30 to 50% due to a two-fold dilution in this cycle. These results confirm the findings from the previouse section. The improvement in total protein recovery also persist until the end of the recovery process in the absence of the salting-out step. Hence, the TCA-pellet shows a 20% increase in total protein recovery due to a two-fold dilution in cyle-1 and the elimination of the salting-out step.

In summary, the total interferon-α-2b and total protein recovery can be improved up to 20% at the end of the recovery process due to a two-fold dilution during cycle-1 and the elimination of the salting-out step. With the absence of the salting-out step, the ratio between host cell proteins and interferon in the final TCA-pellet did change. More host cell proteins are present in relation to interferon-α-2b, which can have an impact on the down-stream process and it needs to be further investigated.

7.3.4 Impact on Down-Stream Processing (DSP)

In section 7.3.3 the impact of a two-fold dilution in cycle-1 of the recovery process on the interferon recovery was investigated. In the presence of the salting-out step no significant improvement in IFN recovery was detectable. However, in the absence of the salting-out step a 20% improvement in the interferon recovery at the end of the recovery process was detected. Due to the elimination of the salting-out not only the interferon recovery was improved but also the recovery of total protein and thus host cell proteins. The presence of higher fractions on host cell proteins can interfer with the down-stream purification methods. Hence, this section focusses on the impact of a two-fold dilution during cycle 1 of the recovery process and the absence of the salting-out step on the first step of the down-stream process.

The first step of the down-stream process is an ion-exchange method with a SP sepharose column. All experiments were performed as described in section 7.2.4. The TCA-pellets of the recovery process simulation at 0.5l with a 2x and 1x dilution during cycle-1 and in the absence of the salt-

ing out step, were used for the purifications with the ion-exchange method. The recovered total interferon-α-2b and total proteins in the different fractions are shown in figure 7.7. Total interferon was quantified by SDS-PAGE as described in section 7.2.6 and total protein was quantified by Bradford assay as described in section 7.2.5.

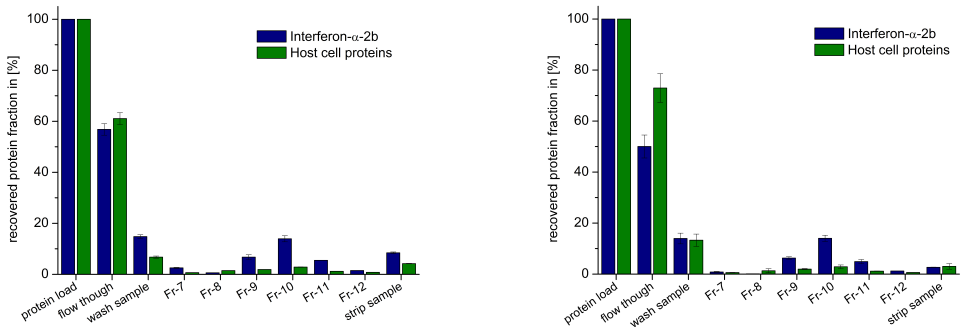

Fig. 7.7: Recovered total interferon-α-2b throughout the simulation of the first down stream step, an IEC method. TCA-pellet used for this purification derived from RP-simulation without the performance of the salting-out step. Left side: RP-simulation with a 2-fold dilution factor; right side: RP-simulation without dilution, control

The left image of figure 7.7 shows the total interferon and total protein recovered in the different fractions of the purification run with the TCA-pellet of the recovery process simulation and a two-fold dilution in cycle 1. It can be seen that over 50% of the initial interferon loaded onto the column does not bind to the column and is detected in the flow through. Further 20% are detected in the wash- and strip-sample and only 30% of the inital loaded interferon-α-2b is recovered in the elution fractions 7-12.

Comparing these findings with the results obtained with the TCA-pellet of the recovery process simulation with a one-fold dilution in cycle 1 and the absence of the salting-out step, no significant differences can be observed (see right image of figure 7.7). This indicates that the addition of a two-fold dilution during cycle-1 of the recovery process has no significant impact on the first purification step of the down-stream process.

The TCA-pellets received from the recovery process simulation with a 2x and 1x dilution during cycle-1 and in the presence of the salting-out step were also purified with the ion-exchange method. Results of recovered interferon and total protein showed comparable results to the findings of figure 7.7. Hence, the absence of the salting-out step has no significant impact on the first purification step of the down-stream process.

In summary, the addition of a two-fold dilution during cycle 1 of the recovery process and the absence of the salting-out step showed no significant impact on the interferon purification during the first step of the down-stream process. In the elution fractions 7-12 over 30% of the initial amount of interferon loaded onto the column is recovered, which is comparable to the findings with the control sample (no dilution and salting-out step present).

7.4 Conclusion and Outlook

In this chapter the optimization of the primary protein recovery process with a dilution step during cycle 1 was discussed. The findings between the simulation runs of the recovery process at 0.04L and 0.5L were inconsistant. Experiments operated at smaller scales (below 0.1L) show larger variations in reproducibility and the results are therefore considered less reliable. Hence, the simulation was performed at a larger scale of 0.5L which resulted in more reliable and reproducible results with smaller variations.

An improvement of up to 15% in total interferon-α-2b and total protein solubility during cycle-1 was detected in the simulation run at 0.5L due to a two-fold dilution. However, the increased protein recovery during cycle-1 is subsequently lost during the salting-out step. Hence, in order to achieve an optimized process with increased interferon recovery, the process needs to be shortened and the salting-out step discarded. As a result an increase in interferon and total protein recovery should be achieved due to dilutions at cycle-1.

The addition of a two-fold dilution during cycle 1 in the absence of the salting-out step resulted in a 20% increase in the interferon recovery at the end of the recovery process compared to a 1x dilution during cycle-1. Hence, the elimination of the salting-out step and the addition of a two-fold dilution in cycle-1 results in 60% recovery of the initial interferon-α-2b amount at the end of the recovery process.

The impact on the first step of the down-stream process of a two-fold dilution during cycle-1 of the recovery process and in the presence or absence of the salting-out step was investigated. None of the factors, neither the two-fold dilution nor the absence of the salting-out step, showed a significant impact on the first purification step for interferon-α-2b. Thirty percent of the initial interferon-α-2b loaded onto the column was recovered in the elution fractions for all samples.

The next step in the recovery process optimization with a two-fold dilution is the investigation of an impact of the absence of the salting-out step on the interferon isoform distribution. Only interferon-α-2b and isoform-4 can be used as the final drug substance at the end of the process. Hence, the impact on the isoform distribution needs to be analysed. However, a more accurate quantification method with higher resolution needs to be developed first (as discussed in chapter

6).

Furthermore, a next step in the process optimization is the implementation at large/ manufacturing scale. So far all experiments were performed at small scale of 0.500l. In a next step the two-fold dilution during cycle-1 needs to be applied at pilot scale between 50 - 300 liters scale followed by the implementation at manufacturing scale at 9000 liters. Due to the optimized recovery process, the TCA-pellet should be more concentrated in interferon-α-2b. Hence, the impact of a more concentrated TCA-pellet on the down-stream process needs to be investigated. If the chromatographic columns of the purification steps are overloaded due to higher concentrations of interferon-α-2b, the TCA-pellet can be split in half and the down-stream process is performed twice. That way no additional changes need to be implemented for the down-stream process.

Chapter 8

Process Optimization - Solubility Study with Detergents

8.1 Introduction

This chapter focuses on the application of different detergents to improve the protein solubility throughout the primary protein recovery process of interferon-α-2b. Previous chapters (4 and 5) have shown that the low recovery yield of IFN at the end of the recovery process is mainly due to low solubilisation levels of the protein during cycle-1 of the process. In order to improve the protein solubility and hence the protein recovery, different detergents will be tested for their potential to increase interferon-α-2b solubility.

8.1.1 Detergents

Detergents are water-soluble amphiphiles, containing hydrophilic and lipophilic parts, which have the capacity to solubilize lipids, proteins and other hydrophobic molecules. In this section the different characteristics of detergents will be described, as well as their role in protein solubilisation followed by the application of specific detergents such as Sarkosyl and Zwittergent 3-14.

8.1.1.1 Detergent Characteristics

Detergents belong to the class of surfactants which decrease the surface-pressure at the air-water interface when added to an aqueous medium. Detergents usually have a polar head group and a hydrophobic chain (tail). This structure is the key for their function as solubilisation agent. In aqueous solution they form spherical micelle structures, with the hydrophobic chains facing away from the water [105]. The polar head is interacting with the water and the hydrophobic tails aggregates, leaving a highly organized structure [106]. Detergents were originally developed as cleaning agents as soap [8]. In the last century they also found their application in the biochemistry of proteins [8].

Detergents can be classified according to their chemical structure which are divided into four major groups: ionic detergents, bile acid salts, non-ionic detergents and zwitterionic detergents. Ionic detergents have a net charge on their head group which is either positive or negative. They are generally used for the complete disruption of cellular structures and denaturation of proteins. Bile acid salts are a type of ionic detergents but without a hydrophobic chain. Their backbone consist of rigid steroidal groups. Instead of spherical micelles, they form kidney-shaped aggregates [105][107].

Non-ionic detergents consist of an uncharged hydrophilic head and hydrophobic chain (tail). They are considered to be mild and non-denaturing and used for breaking protein-lipid interactions rather than protein-protein interactions. Non-ionic detergents with short hydrophobic chains can lead to protein deactivation and some non-ionic detergents do precipitate in the presence of certain salt-ions and are pH sensitive [105][107].

Zwitterionic detergents combine the characteristics of non-ionic and ionic detergents and are usually non-denaturing and not pH-sensitive. The synthetic zwittergents are known as sulfobetains who differ in their hydrophobic tail length [105][106].

Each detergent, independent of chemical structure, has five important characteristics which help to select the most suitable detergent. These are: critical micelle concentration (cmc), critical micelle temperature (cmt), kraft point, cloud point and aggregation number [105]. The cmc is the minimal detergent concentration required to form micellar structures. The critical micelle concentration is dependent on the length of the hydrophobic alkyl chain. With an increase in the chain length, the cmc decreases. The cmc of a detergent is needed to identify the correct amount of detergent to be

added to a protein solution in order to achieve efficient protein solubilisation. Too low detergent concentrations, below the cmc, result in insufficient solubilisation of proteins and too high detergent concentrations can interfere during further purification steps [106].

The cmt gives the minimum required temperature for the detergent to form micelles. Most detergents have a very low cmt, blow 0°C, hence they do not precipitate in the cold. However, the cmt increases with long hydrophobic alkyl chains [107].

The kraft point is also dependent on the temperature. This point defines the temperature at which micelles are in equilibrium and the solubility becomes equal to the cmc. In most cases the kraft point and cmt are identical or similar [105].

The cloud point defines the temperature at which non-ionic detergents separate as a pure phase from an aqueous solution. A low cloud point can be an advantage in the separation of the detergent-protein complex and other cell and media components [105].

The aggregation number of a detergent is known as the number of monomers contained in a micelle and can be calculated by [106]:

$$Aggregation\,No. = \frac{micellar\,MW}{monomeric\,MW} \tag{8.1}$$

The aggregation number is not just dependent on the molecular weight of the detergent but also on the ionic strength of the buffer. With an increase in ionic strength the aggregation number is increasing as well [107]. Hence, with an increase of salt in the buffer more detergents monomers are required to form micelles and the solubilisation property of the detergent is decreasing.

Certain different factors influence the characteristics of the detergents, especially the salt concentration and pH-value of the detergent buffer. An increase in the ionic strength of the detergent buffer leads to a decrease of the cmc while the micellar size increases. Ionic detergents are more sensitive to variations in the salt concentration than non-ionic and zwitterionic detergents. The pH value of the buffer affects the micelle size of the detergent and their stability. Especially ionic detergents tend to protonate in acidic pH environment which can lead to precipitation of the detergent [107].

8.1.1.2 Detergents in protein solubilisation

For almost 40 years detergents and surfactants are used in solubilizing membrane proteins. The detergent used most often in membrane studies is the non-ionic polyoxyethylene detergent Triton X-100 [108].

Proteins with a high level of hydrophobic parts, such as membrane proteins, have a low solubility level in water. They aggregate in order to protect their hydrophobic parts. In the presence of detergents, the hydrophobic tail of the detergent interacts with the hydrophobic domain of the protein and protects it from aggregation. A detergent-protein complex is formed [106].

Different detergents have different abilities to solubilize certain proteins. Some detergents are known to be more harsh and even denature proteins (ionic detergents), whereas other detergents are milder and preserve the biological activity of the protein [109]. Figure 8.1 shows the tendency of detergents to denature proteins dependent on the detergent charge, size and length of the alkyl chain.

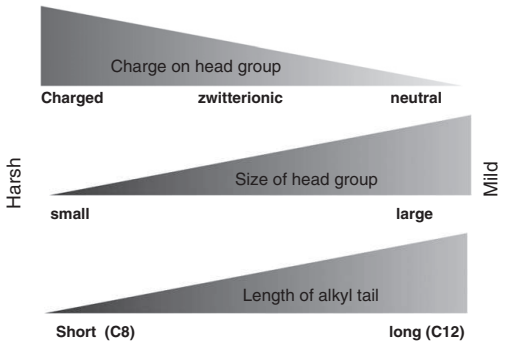

Fig. 8.1: The tendency of a detergent to denature membrane proteins can be qualitatively understood by considering the size and charge of the polar head group, as well as the length of the alkyl tail [8].

Figure 8.1 shows that with a decrease in size of the head group and length of the alkyl tail and an increase in charge the detergent becomes more harsh and lead to protein denaturation. With an increase in the length of the alkyl tails (hydrophobic) the detergent is milder and the ability of protein solubilisation is increasing. Alkyl tail length of C-8 to C-14 are commonly used for protein solubilisation [8]. With an increase in the alkyl tail length the critical micelle concentration is decreasing. Detergents with smaller cmc have usually a larger average micellar molecular weight which makes them more challenging for removal [110].

Several hundreds of detergents are available nowadays for the solubilisation of proteins (membrane proteins) and each of them shows different potential to solubilize different proteins. A few characteristics should be considered by selecting the detergent to use. First, if the detergent needs to be removed post-solubilisation step, detergents with high cmc and low molecular weight are favourable. Those detergents are more easily to be removed by dialysis or ultrafiltration. However, if the cmc is too high, the required detergent concentration added to receive solubilisation is increasing as well [107]. Another factor is the detergent property to absorb A-280. This might be beneficial for detergent quantification but it can also interfere with the protein-analysis. Nevertheless, it should be considered during the selection for the right detergent [107].
If the solubilisation step follows an ion-exchange chromatographic or electrophoresis step, charged detergents from the ionic-family should be avoided and non-ionic or zwitterionic detergents are favourable [105]. The same applies for process steps requiring strong acidic environments with low pH. In this case anionic detergents should be avoided since they form insoluble complexes (precipitates) at low pH [105]. Other parameters that should be considered are temperature and ionic strength of the buffer. Some detergents are unstable at low temperature and the solubility potential of detergents is usually decreasing by the addition of large salt concentrations [105].
After selecting the best possible detergent, the detergent-protein ratio needs to be determined. In general, to receive successful protein solubilisation, the detergent concentration is operated near or at the critical micelle concentration of the detergent [105]. However, as a guide a concentration of 1.0mg/ml of detergent is sufficient to solubilize 1.0mg/ml of protein [111].

Summarizing, the selection of the best detergent for the solubilisation of different proteins is dependent on several factors. Sometimes more than one detergent fulfils the criteria and different detergents need to be tested for their solubilisation potential in a small scale study.

8.1.1.3 Specific detergent for protein solubilisation

Five different detergents from all four groups were tested for their solubilisation potential of interferon-α-2b during the primary protein recovery process. These are Triton X-100 (non-ionic), Sodium-Deoxycholate (bile-acid salt), CHAPS (zwitterionic), Lauroylsarcosinate (anionic) and Sulfobetains 3-08 - 3-16 (zwitterionic).
Triton X-100 is the detergent most widely used in protein membrane solubilisation. It has the

advantage of a low critical micelle concentration, hence only small amount of Triton are required to enhance protein solubility. On the other hand a low cmc results in high aggregations numbers and average micellar weight (MMW) which makes it more difficult to remove the detergent from process samples. Sodium-deoxycholate is an ionic detergent and more precise a bile acid-salt detergent. Its cmc is 4-8mM, more than 5 times higher than the cmc of Triton but consequently the aggregation number and the MMW are reduced which makes them easier to remove from process samples. CHAPS, chemical name 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, is a zwitterionic detergent, meaning it combines both characteristics of ionic and non-ionic detergents. The physical properties, however, are similar to the detergent Deoxycholate. The properties of Sarkosyl (Lauroylsarcosinate) and Zwittergent 3-14 will be discussed in more details in the following two sections. Table 8.1 contains the physical properties of all five detergents for the molar mass (MM), critical micelle concentration (cmc), aggregation number (AGG) and the average micellar molecular weight (MMW). All values are given for room temperature between 20 and 25°C [16].

Tab. 8.1: Overview of physical properties of five different detergents. All values are given for room temperature between 20 - 25°C, [16]

Detergent	MM [g/mol]	cmc [mM]	AGG	MMW [Da]
Triton X-100	625.0	0.2 - 0.9	100 - 155	80'000
Deoxycholate	414.6	4 - 8	4 - 10	1'700 - 4'200
CHAPS	614.9	6 - 10	4 - 14	6'150
Sarkosyl	293.4	14.6	2.0	600
Zwittergent 3-14	363.6	0.1 - 0.4	83	30'000

Sarkosyl

N-laurylsarcosine (Sarkosyl) is an anionic detergent which is used for over 20 years in the solubilisation of membrane proteins. Sarkosyl has a high critical micelle concentration of 14.6mM, which has the consequence of high detergent concentrations in the process samples of up to 10% Sarkosyl (340.8mM) [112]. However, due to the high cmc the average micellar molecular weight

is very low (600Da) and thus the detergent is easily removed from process samples by dialysis or ultrafiltration.

Leinwand et al (1991) [113] described the use of Sarkosyl in generating soluble proteins after bacterial expression. Sarkosyl shows high potential to disrupt coaggregates between the target recombinant protein and membrane proteins and to prevent those formations. However, Sarkosyl has the disadvantage of having a carboxylic acid function. Hence it will protonate quickly at relatively week acidic pH values [107].

Zwittergents

Zwittergent 3-14 belongs to the synthetic zwitterionic detergents, which are known as sulfobetaines [106]. Several different sulfobetaines exist with different length in their alkyl chain. Most common ones are SB-3-08, SB-3-10, SB-3-12, SB-3-14 and SB-3-16. The potential for protein solubilisation is increasing with the length of the alkyl chain, however, with an increase in the alkyl chain the detergent stability is decreasing. Zwittergent 3-14 has a low critical micelle concentration with only 0.1 - 0.4mM, thus the average micellar molecular weight is quite high with 30'000Da and the detergent is difficult to remove from process samples [16].

Croze et al (1995) [114] used Zwittergent 3-14 throughout the purification of recombinant human interferon-β. The author could show, that Zwittergent 3-14 is a nondenaturing and pH insensitive detergent and the target protein remained its bioactivity at the end of the purification step. For solubilisation purposes Zwittergent 3-14 was used at a concentration of 2.5% (68.76mM), which is more than 150 times higher than the critical micelle concentration.

8.1.2 Design of Experiment

Design of Experiment was used for the optimization of protein solubility in the presence of detergents. The software MODDE 10.0 from Umetrics supported the selection of the best design and in the statistical analysis of the data. More information on the statistic background and different experimental designs can be find in section 6.1.3.

8.1.3 Aim and goals

The aim of this chapter is the optimization of the interferon-α-2b recovery throughout the primary protein recovery process, by the addition of a detergent in cycle-1 to increase protein solubility. First, five different detergents will be tested for their potential to improve the solubility of interferon-α-2b. Second, the optimal concentration of selected detergents, process volume and agitation time will be determined using Design of Experiment and the software MODDE 10.0 from Umetrics. In a final step, the two selected detergents will be added to the primary recovery process at the optimal determined concentration and the impact of the following recovery process steps will be investigated in a small scale model. A discussion of the impact of the addition of the detergents on interferon-α-2b and total protein recovery throughout the different steps of the recovery process will be followed.

8.2 Material and Methods

8.2.1 List of Materials

Tab. 8.2: Materials and suppliers

materials	supplier
Recovery Process samples (EOF, and Sample-4)	industrial partner
interferon-α-2b drug substance	industrial partner
NaOH	Sigma Aldrich
Trizma Base	Sigma Aldrich
EDTA tetrasodium salt	VWR
Bovine serum albumin (BSA)	Sigma Aldrich
Sarkosyl	Sigma Aldrich
Zwittergent 3-14	Sigma Aldrich
Triton-X-100	Sigma Aldrich
CHAPS	Sigma Aldrich
Na-Deoxycholate	Sigma Aldrich
Zwittergent 3-08 - 3-16	Merck
Bradford Reagent	Sigma Aldrich
Pierce® BCA Protein Assay Kit	Thermo Scientific

8.2.2 Protein solubilisation with detergents

The protein solubility experiments with detergents were performed using recovery process samples from the manufacturing process. Sample time point 4 was selected for this purpose (compare figure 2.3 in chapter 2.5). The sample 4 was thawed in a water bath at 37°C and then centrifuged in a bench top centrifuge (FL40R centrifuge, Thermo Scientific, Dublin, Ireland) at 7500rpm for 30 minutes and 4°C. Supernatant was discharged and the pellet was dissolved in the detergent solution according to the volume ratio of the manufacturing process (table 3.7). The detergent

solution was prepared with water and the appropriate concentration of the different detergents. After setting the pH to 7.0 with NaOH the sample was mixed for 4 hours constantly. To collect the resuspended proteins, the sample was centrifuged again at 7500rpm for 30min and 4°C and both fractions, supernatant and pellet, were recovered and stored for protein analysis at 4°C.
All experiments were performed in 50mL centrifuge tubes at a working volume of 5mL for the resolubilization part.

8.2.3 Simulation of the Protein Recovery Process

The simulation of the recovery process (RP) is based on the primary protein recovery process from the industrial partner as described in chapter 3.2.3. The original process is performed with a working volume of 32'500L which was scaled down to 0.04L and 0.5L (table 3.7).
For the performance of the process simulation an end of fermentation (EOF) sample was used. The EOF pellet originated from a small scale fermentation run performed from the industrial partner and was shipped on dry ice to DCU. This pellet was resuspend in an EDTA-Tris-extraction buffer (1mM EDTA, 20mM Tris, pH 8.0) in DCU to proceed with the simulation of the recovery process. In order to implement the addition of a detergent into the original recovery process, the water was replaced by the appropriate detergent solution in cycle-1 in order to resolubilize the protein precipitates from the acid kill. The detergent solution was prepared with water and the required concentration of detergent.
Throughout the simulation of the recovery process samples were taken at different time points as it has been implemented on site with the industrial partner for the manufacturing process (chapter 2.5 and figure 2.3). Samples taken from the simulation process were stored at 4°C until protein analysis was performed.

8.2.4 Total protein quantification - BCA

Total protein was determined using the BCA assay as described in chapter 3.2.2 and table 3.5. Sample pellets were dissolved in EDTA-Tris buffer prior to protein analysis. BSA dissolved in water was used as a reference standard for all total protein quantifications. The blank was chosen according to the sample to be analysed, which differs between water, EDTA-Tris buffer (1mM

EDTA, 20mM Tris, pH 8.0), detergents or diluted fermentation media.

8.2.5 Total Interferon-α-2b quantification

Total interferon-α-2b was quantified using SDS-PAGE as described in chapter 5.2.3 and 5.2.4. Sample pellets were dissolved in EDTA-Tris buffer (1mM EDTA, 20mM Tris, pH 8.0) prior to analysis as described in section 8.2.4. No further sample treatment was required. The interferon-α-2b drug substance was used as a reference standard for all quantifications.

8.2.6 Detergent quantification

Sarkosyl and Zwittergent 3-14 were quantified with RP-HPLC and UV-280 detection for Sarkosyl and RI detection for Zwittergent 3-14. More details for the quantification procedure can be found in section 9.2.5.

8.3 Results and Discussion

8.3.1 Different detergents

In chapters 4 and 5, two critical process steps from the primary recovery process were identified. CPS-1 was identified to have the biggest impact on the interferon loss throughout the process, due to low protein solubility in water. In chapter 7, an increase of the volume during CPS-1 was investigated in order to improve the protein solubility. Results showed an improvement from 32% to 47% in interferon recovery after CPS-1 due to a two-fold dilution.

The enhancement in IFN solubility of about 15% due to dilution requires further improvement. Detergents are well described in the literature to improve protein solubility, particularly for membrane proteins or several recombinant proteins (section 8.1.1.1). As described in section 8.1.1.1, detergents form micelles and interact with the protein to form a protein-detergent complex. Therefore 5 different detergents, known from the literature to improve protein solubility, were investigated and tested for their potential to improve interferon-α-2b solubility.

Figure 8.2 shows the amount of interferon resolubilized after the addition of the different detergents.

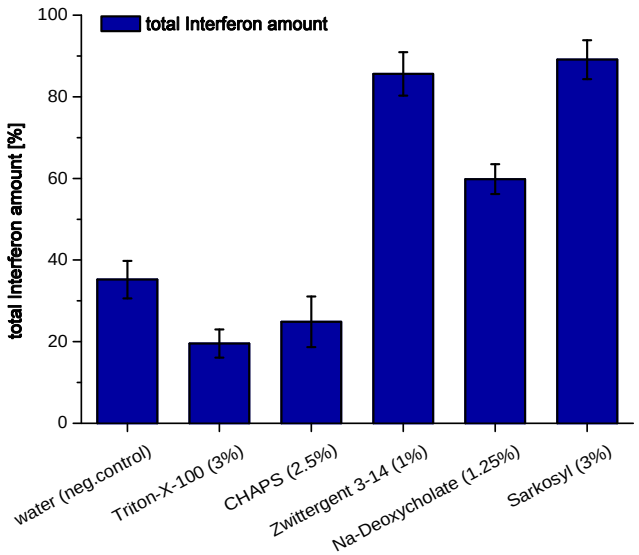

Fig. 8.2: Interferon-α-2b solubility in different detergents: non-ionic (Triton X-100), zwitter ionic (CHAPS and Zwittergent) and an-ionic (Na-Deoxycholate and Sarkosyl)

The detergent solubility experiment was performed as described in section 8.2.2 and total interferon-α-2b was quantified using SDS-PAGE as described in section 8.2.5. Different types of detergents were tested: non-ionic detergents (Triton X-100), zwitter ionic detergents (CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and Zwittergent 3-14), and an-ionic detergents (Na-Deoxycholate and Sarkosyl [Sodium lauroyl sarcosinate]). All selected detergents were found in literature to improve protein solubility (section 8.1.1.3), at the concentrations used. All detergent concentrations are well above their cmc (critical micelle concentration) to ensure micelle formations (section 8.1.1.3, table 8.1). As can be seen from figure 8.2 some detergents have a higher potential to improve interferon solubility than others. Triton X-100 and CHAPS have no positive impact on IFN solubility. Indeed the results for 20% Triton and 25% CHAPS showed that the IFN solubility was even below the solubility level of the negative control (32%; IFN solubility in water without detergent present). Sodium-Deoxycholate, on the other hand, does improve the IFN solubility to a value of 60%, which is an enhancement of 28% compared to the negative control. The highest potential to improve interferon solubility was demonstrated by Zwittergent 3-14 and Sarkosyl which showed more than 80% solubility of IFN, representing a 3-fold increase compared to the negative control. Since Zwittergent 3-14 is a zwitter ionic detergent and Sarkosyl an anionic detergent, the capability of detergents to improve the IFN solubility appears to be independent of the net charge. Zwitter ionic detergents have no net charge and anionic detergents have a negative net charge (section 8.1.1.1).

This leaves the question as to why do some detergents have a higher ability to improve the IFN solubility than others? From literature studies (section 8.1.1.1), the chemical structure of the detergents does appear to have an important role in their function and capability to improve protein solubility. Figure 8.3 shows the different chemical structures of the 5 detergents tested in figure 8.2.

Looking at the chemical structures of the detergents in figure 8.3, some parallels can be seen between the structure of Zwittergent 3-14 and Sarkosyl, the detergents showing the highest potential to improve IFN solubility. In the literature (section 8.1.1.1) it is suggested that the chemical structure of the hydrophobic region of detergents, affects the structure of the micelles, which are an important detergent characteristic in protein solubility. Compared to the other three detergents,

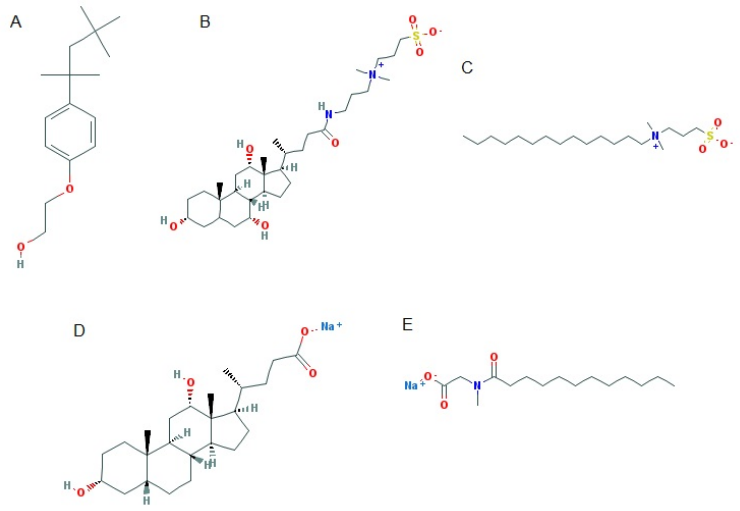

Fig. 8.3: Chemical structure of different detergents: A) Triton X-100 - non-ionic; B) CHAPS - zwitter-ionic; C) Zwittergent 3-14 - zwitter-ionic; D) Na-Deoxycholate - an-ionic; E) Sarkosyl - an-ionic

Zwittergent 3-14 and Sarkosyl have a much longer hydrophobic carbon chain (tail). This tail seems to be indispensable to improve interferon solubility. As discussed in section 8.1.1.1, an increase in the length of the hydrocarbon chain results in an increase in the micelle size and a decrease of the critical micelle concentration (cmc). This means that fewer detergent molecules are needed to construct the micelle and increase protein solubility.

To investigate the dependency of the hydrocarbon length on interferon solubility, different Zwittergents (all at 1%) which only differ in their hydrocarbon length were tested for protein solubility. The results can be seen in figure 8.4.

Zwittergent 3-08 means that this Zwittergent has a hydrocarbon chain composed of 8 carbon-atoms, Zwittergent 3-10 chain has 10 carbon atoms and so on. As seen in figure 8.4 the IFN solubility increases with an increase in the hydrocarbon chain of the Zwittergent. This result confirms the suggestion, that the hydrocarbon chain has an important role in IFN solubility, which was mentioned earlier in this section. A short hydrocarbon tail of only 8 or 10 carbon atoms appears to have no positive impact on the IFN solubility. However, by increasing to 12 carbon atoms in the hydrocarbon chain the IFN solubility was enhanced up to 70%. With 14 or 16 carbon atoms the IFN solubility could be increased even further to 90%, representing a 3-fold increment in IFN solubility compared to the negative control without detergent.

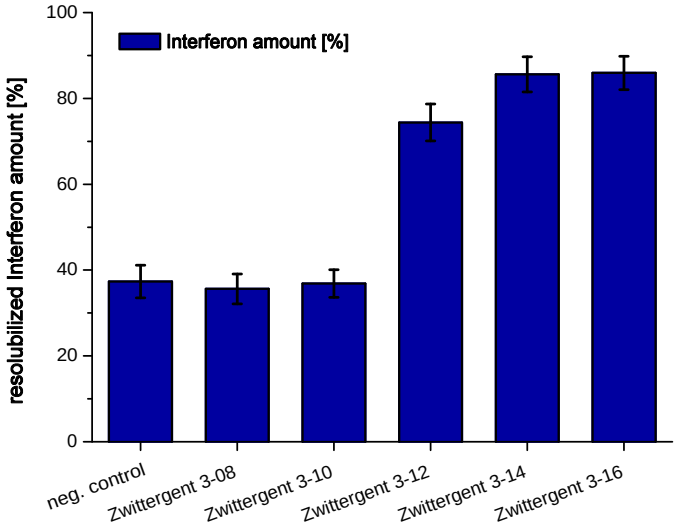

Fig. 8.4: Interferon-α-2b solubility in Zwittergent-solutions dependent on the length of the detergent's carbon chain (tail)

The study with different detergents has shown that Zwittergent 3-14 (at 1% concentration) and Sarkosyl (at 3% concentration) have the highest potential to improve interferon-α-2b solubility by up to 90%. The ability of detergents to increase protein solubility was indeed shown to be dependent on chemical structure and length of the hydrophobic hydrocarbon chain. It was demonstrated that for Zwittergents, a minimum of 12 carbon atoms in the hydrocarbon chain are required to achieve sufficient interferon solubility. Sarkosyl has 11 carbons in its hydrocarbon chain.
In conclusion, an addition of either Zwittergent 3-14 or Sarkosyl during cycle-1 (CPS-1) of the protein recovery process was shown to increase the interferon solubility from 32% to 90%.

8.3.2 Optimization of Interferon solubility with Sarkosyl

8.3.2.1 Design of Experiment Study for Sarkosyl

The results from section 8.3.1 showed that Sarkosyl has a high capability to improve the interferon-α-2b solubility during cycle-1 of the recovery process. The next step was to identify the optimal process conditions during cycle-1 for successful IFN solubilisation. Three factors were selected for investigation: detergent concentration, process volume and agitation time. The optimal de-

tergent concentration should be as low as possible but high enough to achieve at least 75% IFN solubility.

In order to investigate the impact of the detergent concentration, agitation time and process volume on interferon solubility, a design of experiment (DoE) study was performed with Sarkosyl. With a DoE investigation the influence of each factor onto the different responses as well as on each other can be investigated (section 8.1.2). A central composite face-centred design with 2 levels and 3 factors was chosen for this investigation. The different parameters that were investigated for the optimisation of the interferon solubility with design of experiment can be found in table 8.3 and the full DoE worksheet, which lists all experiments performed for this design can be found in the appendix A.

Tab. 8.3: Design of Experiment paramters for the optimisation of interferon solubility

detergent	detergent concentration [%]	process volume [ml]	agitation time [h]
Sarkosyl	0.1 - 3.0	10 - 45	2 - 8

The experiments for DoE were performed as described in section 8.2.2, the amount of total interferon-α-2b was quantified using SDS-PAGE as described in section 8.2.5 and total amount of protein was analysed using the BCA assay as described in section 8.2.4.

Figure 8.5 shows a response surface plot of the interferon solubility as a function of Sarkosyl concentration and process volume, generated with the statistic software MODDE 10.0. Factor 1 represents the Sarkosyl concentration in % shown on the x-axis, factor 2 illustrates the process volume, plotted on the y-axis and the third factor describes the agitation time which is set to a fixed value of 5 hours in figure 8.5. The value of 100% volume represents the working conditions in the recovery process at manufacturing scale. Hence, 150% of the volume is equivalent to a 1.5-fold dilution factor as described in chapter 7. Two responses were measured during this DoE investigation: solubilized amount of total interferon (z-axis) and the solubilized amount of total protein (data not shown). The model used to generate figure 8.5 is dependent on the detergent concentration, the process volume, the quadratic detergent concentration and the interaction between detergent concentration and process volume. The goodness of fit is 0.987, the goodness of

prediction equals to 0.973 and the model validity is at 0.730. All these parameters indicate that the model is robust and useful to provide reliable estimations of the interactions between detergent concentration, process volume and interferon solubility (section 6.1.3.2). More details about the statistics of this model can be found in the appendix A.

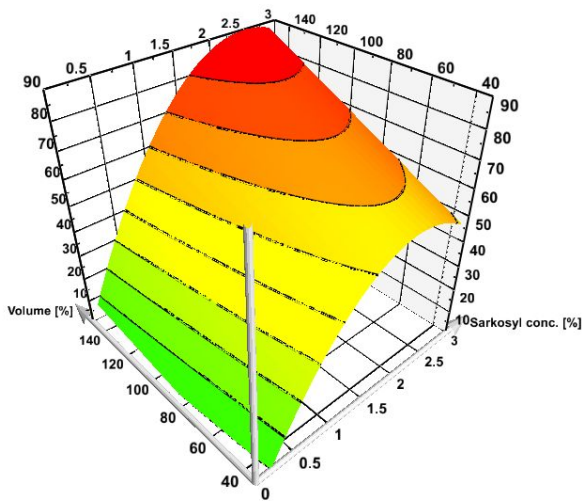

Fig. 8.5: Interferon-α-2b solubility as a function of Sarkosyl concentration [%] and process volume [%] with a constant agitation time of 5.0h. Data generated with MODDE software 10.0

As seen in figure 8.5 the interferon solubility is highly dependent on the Sarkosyl concentration. An increase in the detergent concentration results in an increase in the IFN solubility, with a maxima at 2.7% of Sarkosyl and 90% IFN solubility. The IFN solubility is also dependent on the process volume. As discussed in chapter 7, an increase in process volume results in an enhancement of the IFN-solubility. The impact of the volume increases with detergent concentration. The impact of Sarkosyl concentration on IFN solubility is expected, since with higher detergent concentrations more micelles can be formed which avoids or breaks-up protein aggregations to enhance protein solubility.

The agitation time was shown to have no significant impact on the IFN solubility and even could be removed from the model. This means that the agitation time during cycle 1 of the recovery process (figure 2.2) can easily be shortened to 2 hours without a significant reduction in interferon solubility.

All data were also analysed for total protein and the results show similar interactions of detergent concentration, process volume and agitation time on total protein solubility as they show for total

IFN (data not shown). The DoE results and statistics for the total protein model can be found in the appendix A.

In summary, the optimal conditions for maximal interferon solubility are an increased process volume of 150% (dilution factor 1.5) and a Sarkosyl concentration of 2.7 - 3.0%. The next question is what impact has the presence of Sarkosyl on the salting-out step of the recovery process? Hence, a simulation of the recovery process at small scale was performed with the addition of Sarkosyl during cycle-1. This will be discussed in the following section 8.3.2.2.

8.3.2.2 Process simulation with 3.0% Sarkosyl

In the previous section the optimal process conditions for cycle 1 were identified in order to improve interferon-α-2b solubility to enhance the overall IFN recovery in the primary recovery process. Agitation time and process volume were identified to be less important and therefore set to their working conditions at the manufacturing process. The detergent concentration showed the biggest impact on IFN solubility. The optimal Sarkosyl concentration was identified to be at 3.0%. In this section the impact of the addition of Sarkosyl during cycle-1 on the recovery process will be investigated.

The simulation of the recovery process was performed as described in section 8.2.3, total IFN was quantified using SDS-PAGE as described in section 8.2.5 and total protein was quantified using the BCA assay as explained in section 8.2.4. All results of different simulations of the recovery process with Sarkosyl were performed at a small scale of 0.5L in triplicate.

Figure 8.6 shows the results for the amount of recovered interferon throughout the recovery process simulation. The different cycles stand for the 4 centrifugation cycles performed throughout the process (figure 2.2). The negative control was performed following the working conditions of the manufacturing recovery process without any addition of detergent.

As can be seen from figure 8.6 the interferon recovery after centrifugation cycle 2 can be increased 3-fold, to 85% due to the addition of 3% Sarkosyl during cycle-1. Only 28% of IFN was recovered at this point in the negative control. An interesting observation was made for CPS-2, the salting-out step, in the presence of Sarkosyl. More than 50% of the recovered interferon from cycle-1 was lost during the salting-out step and only 30% of IFN was recovered after the third centrifugation step. Due to this enormous loss of interferon during the salting-out step, only 20% of overall interferon

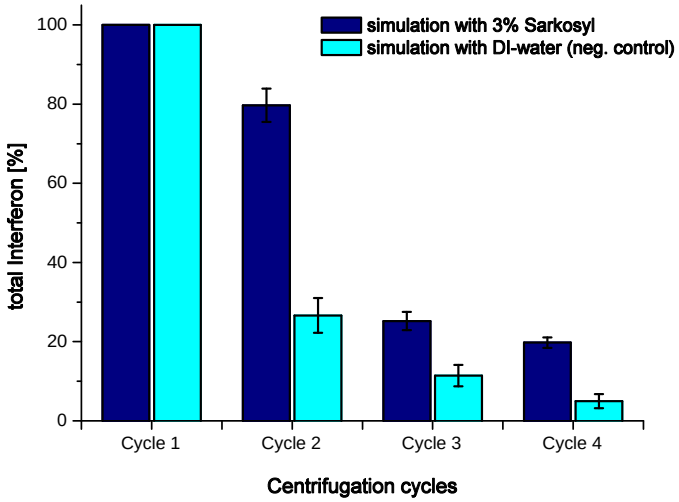

Fig. 8.6: Interferon-α-2b amount throughout the recovery process simulation at 0.5L with and without the addition of 3% Sarkosyl

was recovered at the end of the recovery process in which 3% Sarkosyl was present. However, this is still 3 times higher than for the negative control with only 7% of total IFN recovery in the TCA-pellet.

Since the major loss of IFN was detected during the salting-out step, the impact of CPS-2 on interferon and Sarkosyl was analysed in more detail. Experiments and a literature survey (section 8.1.1.3) showed that Sarkosyl is highly unstable in the presence of chloride ions. They also showed that the pI of Sarkosyl is at 5.0, consequently at a pH of 5.0 and below, Sarkosyl precipitates and with it a lot of proteins. Changing the acid used during the salting-out step from HCl to acetic acid to avoid the addition of chloride ions gave no improvement in the overall IFN recovery due to the low pH set point of 4.5 (data not shown).

It was also tested whether a lower starting concentration of Sarkosyl may minimize the interference during the salting-out step. Hence, instead of using 3% of Sarkosyl during cycle-1, only 1% of Sarkosyl was added, however no improvement during the salting-out step was observed (data not shown).

From the total protein behaviour throughout the simulation in the presence of 3% Sarkosyl, it can be seen (figure 8.7) that they follow the same characteristics as for total interferon.

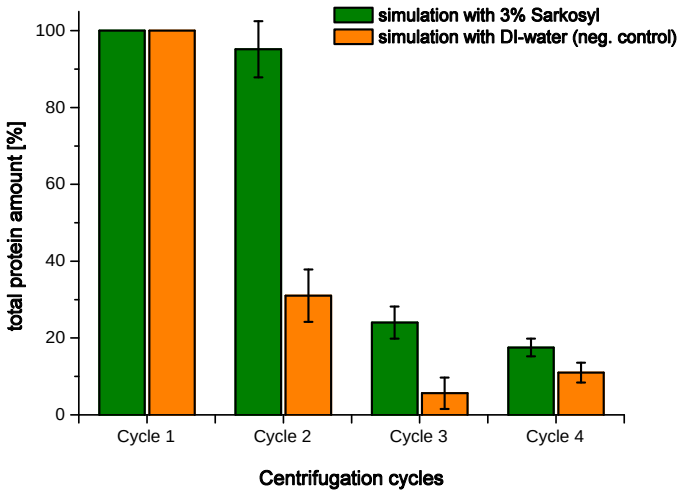

Fig. 8.7: The amount of total protein throughout the recovery process simulation at 0.04L in the presence and absence of 3% Sarkosyl

The results seen in figure 8.7 are as expected since, as discussed in section 8.3.1 and 8.3.2.1, the addition of Sarkosyl does not improve the solubility of a specific type of protein but affects the host cell protein solubility in the same way as it does for interferon. Therefore no difference in the amount of total protein throughout the recovery process was expected compared to the amount of IFN. Since total protein and interferon behave in a similar way throughout the recovery process in the presence of Sarkosyl, the HCP/ IFN ratio remained the same at the end of the process.

The process characterizations in chapter 4 and 5 have shown that the salting-out step has a less significant role in the protein recovery process, than the other three cycles. Since the salting-out step results in the biggest loss in interferon and total protein in the presence of 3% Sarkosyl, a simulation of the recovery process was performed in the absence of the salting-out step. Results of this simulation for total interferon and total protein are shown in figure 8.8.

Both graphs in figure 8.8, for total interferon and for total protein, show that without performing the salting-out step (centrifugation cycle 3), the protein recovery at the end of the process could be increased 2- to 3-fold, by the addition of Sarkosyl. More than 75% of total interferon and up to 90% of total protein was recovered at the end of the recovery process in the presence of 3% Sarkosyl but in the absence of the salting-out step. In the absence of the salting-out the ratio of host cell proteins to interferon also changed. Without the salting-out step and Sarkosyl present the

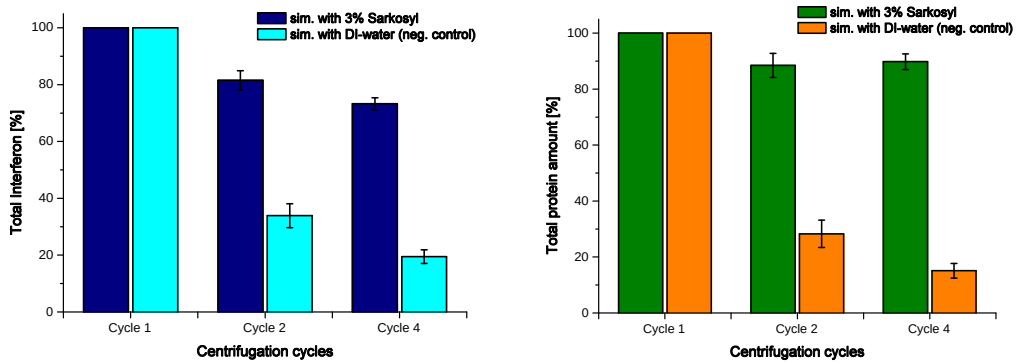

Fig. 8.8: The amount of interferon-α-2b (left) and total protein (right) throughout the recovery process simulation without salting-out step at 0.04L in the presence and absence of 3% Sarkosyl

ratio of host cell protein to IFN increased to approximately 25%, showing that a higher fraction of host cell proteins are present. A shift of 25% in the protein distribution may have an impact on the downstream purification and need to be considered later.

Summarizing, an increase in IFN recovery to 20% at the end of the recovery process can be accomplished by the addition of 3% Sarkosyl during cycle-1 of the process. However, the addition of Sarkosyl only improves the critical process step 1 and does not improve the CPS-2 (salting-out). Due to the addition of Sarkosyl this critical process step is even impaired and more than 55% of total IFN is lost. Total protein behaves in a similar way to IFN throughout the recovery process with Sarkosyl. Performing the recovery process without the salting-out step but with addition of Sarkosyl, gives an interferon recovery of more than 75% and up to 90% in total protein. The ratio of HCP to IFN changes by up to 25%, which may have a significant impact on DSP.

Sarkosyl characteristics throughout the recovery process simulation

The Sarkosyl concentrations throughout the recovery process simulation after the addition in cycle-1 were quantified using the RP-HPLC method with UV detector as described in section 8.2.6. During cycle-1 of the recovery process 3.0% Sarkosyl is added to improve the protein solubility at this stage of the process.

Results of the recovery process simulation in the absence of the salting-out step showed a 95% recovery of Sarkosyl in the TCA-pellet, the last sample of the recovery process (data not shown). Only minor amounts of Sarkosyl were detected in the waste streams. These results indicate, that

Sarkosyl has a strong interactions with the proteins present in the recovery process, since they follow both the same characteristics throughout the recovery process. The final Sarkosyl concentration in the TCA-pellet is dependent on the volume used for solubilizing the pellet.

8.3.3 Optimization of Interferon solubility with Zwittergent 3-14

8.3.3.1 Design of Experiment Study for Zwittergent 3-14

The DoE investigation with Sarkosyl in section 8.3.2.1 showed that the agitation time has no significant impact on the IFN solubility and the impact of the process volume is very little compared to the detergent concentration. Hence, the DoE-design for the Zwittergent 3-14 investigation was reduced to only one factor. Only the Zwittergent 3-14 concentration itself was taken into account and varied between 0.009% and 2%. Figure 8.9 shows that the amount of solubilized interferon is dependent on the concentration of Zwittergent 3-14. Several different concentrations were tested in order to find the optimum to reach an IFN solubility of at least 75%. The results were compared to the negative control, IFN solubility in DI-water, which represents 0.0% of Zwittergent 3-14.

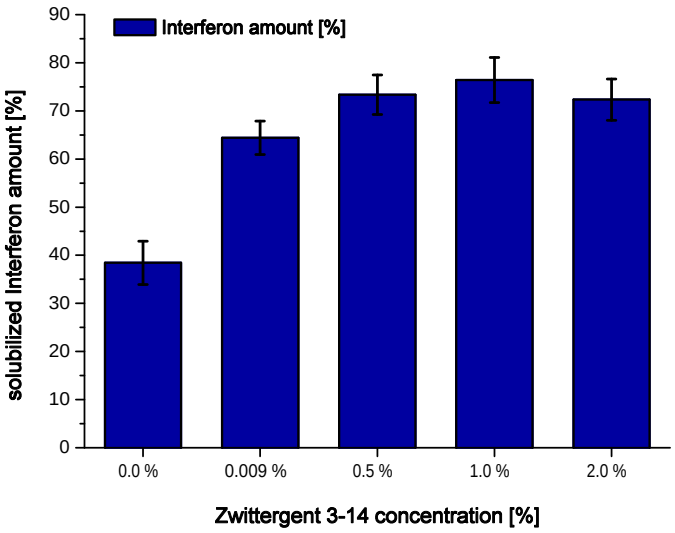

Fig. 8.9: Interferon-α-2b solubility as a function of Zwittergent 3-14 concentration [%]; cmc = 0.011%

As expected from previous results, the IFN solubility increases with increasing Zwittergent con-

centration, with a maximum between 0.5 - 1.0%. These results show that a maximum IFN solubility can be achieved with a lower Zwittergent 3-14 concentration than with Sarkosyl. This can be explained by the critical micelle concentration (cmc) of both detergents. The cmc of Sarkosyl is 0.42% (14.6mM) which is much higher than the cmc of Zwittergent 3-14 with 0.011% (0.4mM) (section 8.1.1.3). A higher cmc means that higher detergent concentrations are required to achieve the micelle formations which are important for protein solubilisation.
An unexpected result was the high IFN solubility at only 0.009% of Zwittergent 3-14 which is below the cmc value. This result shows, that even without formation of micelles, Zwittergent 3-14 has a high capability to improve the IFN-solubility. Increasing the detergent concentration above 1.0% did not result in any significant improvement in IFN solubility, leaving the optimal conditions for Zwittergent 3-14 between 0.5 - 1.0% for maximal interferon solubility.

8.3.3.2 Recovery Process simulation with Zwittergent 3-14

Results from section 8.3.1 showed that Zwittergent 3-14 also showed a high potential to improve interferon solubility. In section 8.3.3.1 the impact of the detergent concentration on IFN solubility was investigated to find the optimal conditions to improve CPS-1 from the recovery process simulation. The high solubility values of IFN for very small Zwittergent concentrations was unexpected. Therefore the recovery process simulation was performed with 5 different Zwittergent concentrations (2.0%, 1.0%, 0.5%, 0.1% and 0.001%) in a small scale model at 0.04L, as described in section 8.2.3. Total interferon (section 8.2.5) and total protein (section 8.2.4) were analysed for all sample time points taken throughout the simulation. The total amount of interferon in each sample taken after a centrifugation step of the simulation are shown in figure 8.10.

Each of the simulation runs at 0.04L scale, shown in figure 8.10, are performed at least twice and the average values are presented, except for simulation with 2.0% Zwittergent which was performed only once. The results show that all Zwittergent concentrations, even those below the cmc, gave a significant improvement in interferon solubility and recovery after the second centrifugation step compared to the negative control without detergent. For 0.001% of Zwittergent 3-14 an increase of more than 20% in IFN solubility was found compared to the negative control. These results confirm the findings from section 8.3.3.1. However, the increased IFN recovery during

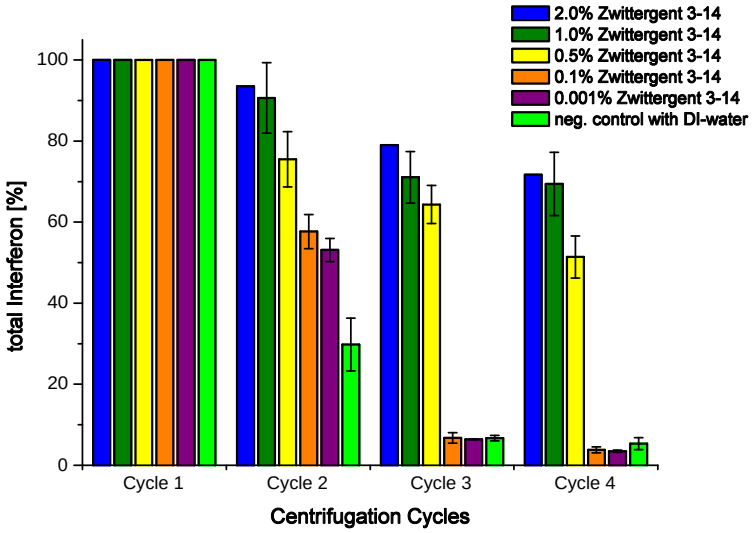

Fig. 8.10: The amount of interferon-α-2b throughout the recovery process simulation at 0.04L scale with the addition of Zwittergent 3-14 during cycle-1 at 5 different concentrations

cycle-1 (CPS-1) is lost during the salting-out step (CPS-2) for simulations with Zwittergent 3-14 concentrations less than 0.5%. Due to the addition of salt (NaCl) and reduction of the pH to 4.5, the solubility of interferon (as well as all other proteins) decreases, since salt-ions interact with the water molecules (section 7.1.2). Detergent molecules also interact with the salt-ions and their potential for protein solubilisation is reduced (section 8.1.1.2). Zwittergent 3-14 concentrations less than 0.5% are too low to compensate the interference of salt ions and to keep the proteins (IFN) in solution (section 8.1.1.2). Hence, interferon precipitates during the salting-out step if the Zwittergent 3-14 concentration present in solution is below 0.5%. For simulation runs with a Zwittergent 3-14 concentration above 0.5% the loss of interferon during the salting-out was only around 20%. Hence, more than 70% of total IFN was recovered at the end of the process simulation with 1.0% or 2.0% of Zwittergent 3-14 present.

The previous simulation runs were only performed in a small scale model at 0.04L scale. Results have shown that this scale provides less accurate and less robust results for the simulation of the recovery process. Hence, a simulation of the recovery process was also performed at 0.5L scale which should provide more accurate results. Since the simulation at 0.04L scale showed that 1.0% Zwittergent 3-14 is the lowest detergent concentration with a high interferon recovery of at least 60%, this concentration was selected for the simulation at larger scale.

Both simulations, with 1.0% Zwittergent and negative control, were performed in triplicate and samples were analysed at each sample time point for total interferon and total protein. Figure 8.11 shows the results of total IFN and total protein after each centrifugation cycle of the recovery process simulation.

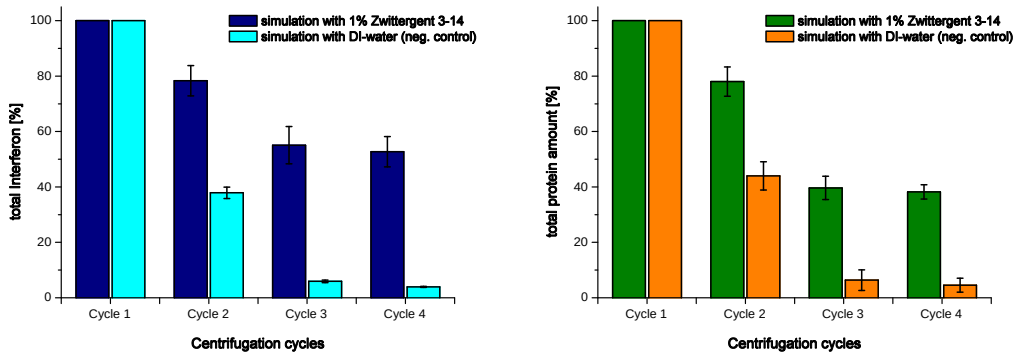

Fig. 8.11: Interferon-α-2b (left) and total protein (right) throughout the recovery process simulation at 0.5L in the presence and absence of 1% Zwittergent 3-14

The recovery process simulation at 0.5L scale confirms the findings of the simulation at 0.04L scale with 1.0% Zwittergent 3-14. Around 80% of total interferon was recovered after the 2nd centrifugation step, which is consistent with the findings in section 8.3.3.1. Due to the addition of the detergent Zwittergent 3-14 during cycle-1 the interferon solubility is increased which results in twice as much IFN recovery at the end of CPS-1 due to the Zwittergent addition. The next step, salting-out step, was identified as the CPS-2 during the process characterization in chapters 4 and 5. In the absence of detergent up to 25% of total interferon is lost during this step. In the presence of 1.0% Zwittergent 3-14 the loss in IFN remains constant and does not increase, as observed during simulations with Sarkosyl (see section 8.3.2.2). At the end of the recovery process simulation with 1.0% Zwittergent more than 50% of total interferon was recovered, and only 7 - 8% with no detergent present. Hence, the addition of 1.0% Zwittergent during cycle-1 of the recovery process improved the total interferon recovery at the end of the process by more than 6-fold.

Comparing the results of total interferon with total protein throughout the recovery process, it can be seen (figure 8.11) that total protein follows the same characteristics throughout the recovery process with and without 1.0% Zwittergent present. More than 80% of total protein was solu-

bilized during cycle-1 due to the addition of detergent, however more than half of this was lost during the salting-out step. Hence, less than 40% of total protein was recovered at the end of the recovery process with 1.0% Zwittergent present. This corresponds to a 5-fold increase in total protein recovery due to the addition of a detergent in cycle-1.

Since more total proteins are salted-out during the salting-out step than total interferon, the ratio between HCP and IFN changes when Zwittergent is present at the end of the recovery process. In the absence of a detergent, only 8.5% of total protein represents interferon. However, with the Zwittergent 3-14 present interferon represents up to 22% of the total protein. This ratio between host cell proteins and recombinant proteins can have an impact on the subsequent purification during the down-stream processing and variations in this ratio need to be considered at that stage.

The simulation with the addition of 1.0% Zwittergent 3-14 was also performed with a shortened recovery process, without performing the salting-out step. Results showed a recovery of up to 70% in total interferon at the end of the recovery process and a recovery of 65% in total protein (data are not shown). In the absence of the detergent 25% of IFN and 22% of total protein were recovered. Hence, eliminating the salting-out step results in an even higher recovery of total interferon at the end of the recovery process, but also in an increased recovery in total protein, the majority of which are host cell proteins. The presence of increased host cell proteins might interfere with the down-stream processing. Therefore it must be investigated whether the exclusion of the salting-out step is beneficial for the interferon purification and the overall process yield.

During the characterization of the recovery process in chapters 4 and 5 significant variations were detected in the production of proteins (HCP and IFN) during the fermentation process. These variations can reach up to $\pm$20% in total protein and total interferon. Therefore it is important to consider these variations during process optimization. The recovery process was simulated using the twice the amount of total protein (and IFN) at the beginning of the recovery process simulation (2x protein). The simulation (at 0.04L scale) was performed with the addition of 0.5% and 1.0% of Zwittergent 3-14 during cycle-1 as well as with a negative control, and can be seen in figure 8.12.

The results for total interferon throughout the recovery process in figure 8.12 show that with the addition of 1.0% Zwittergent 3-14 during cycle-1 up to 55 - 60% of IFN is recovered at the end of the recovery process even with a double protein concentration (2x) at the end of the fermentation.

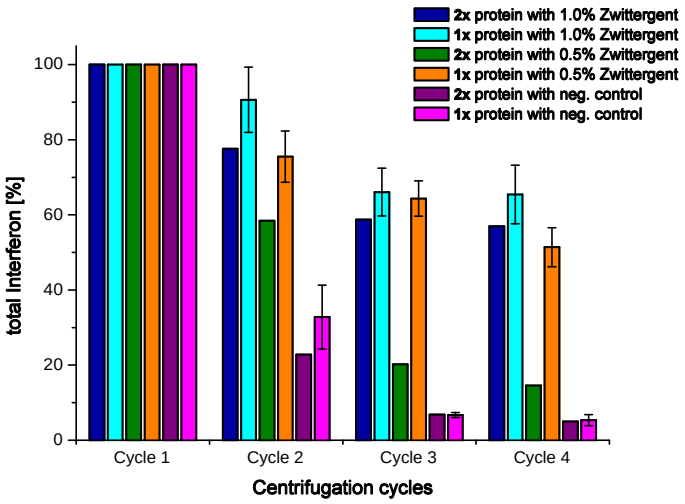

Fig. 8.12: The amount of interferon-α-2b throughout the recovery process simulation at 0.04L scale with double and single amounts of protein at the beginning of the process and the addition of different concentrations of Zwittergent 3-14

It can also be seen that with only 0.5% Zwittergent 3-14 present, the recovery of IFN is reduced at the end of the recovery process, if the protein concentration is twice as high. With a double protein concentration and addition of 0.5% Zwittergent 3-14, only 15% of total interferon is recovered at the end of the recovery process. This is 40% less compared to the single protein concentration. These findings confirm those from the beginning of this section. Results from figure 8.10 showed, that if the detergent concentration present during the salting-out step is too low to compensate the interference from the salt-ions and to keep all interferon in solution, a large amount of IFN is salted-out. If the protein concentration is doubled at the beginning of the recovery process, 0.5% Zwittergent is insufficient to keep all interferon in solution and protect them from the interference from the salt-ions. However, the addition of 1.0% Zwittergent provides a sufficient concentration to recover a similar percentage of the total interferon irrespective of protein concentration at the beginning of the recovery process.

In summary, this section has shown that with the addition of 1.0% Zwittergent 3-14 during cycle-1 of the recovery process, the interferon recovery increased from 8% up to 52% at the end of the recovery process, after all 4 cycles of the manufacturing process. This enhancement is independent of the protein concentration at the beginning of the recovery process, which can differ due to variations in the protein production during fermentation. A reduction of the process cycles, hence,

elimination of the salting-out step, results in an even higher interferon recovery of 70%.

Zwittergent 3-14 characteristics throughout the recovery process simulation

The Zwittergent 3-14 concentrations throughout the recovery process simulation after the addition in cyle-1 were quantified using the RP-HPLC method with RI detector as described in section 8.2.6. During cycle-1 of the recovery process 1.0% Zwittergent 3-14 is added to improve the protein solubility at this stage of the process.
Figure 8.13 shows the recovered Zwittergent 3-14 fractions throughout the recovery process simulation at a scale of 0.5l.

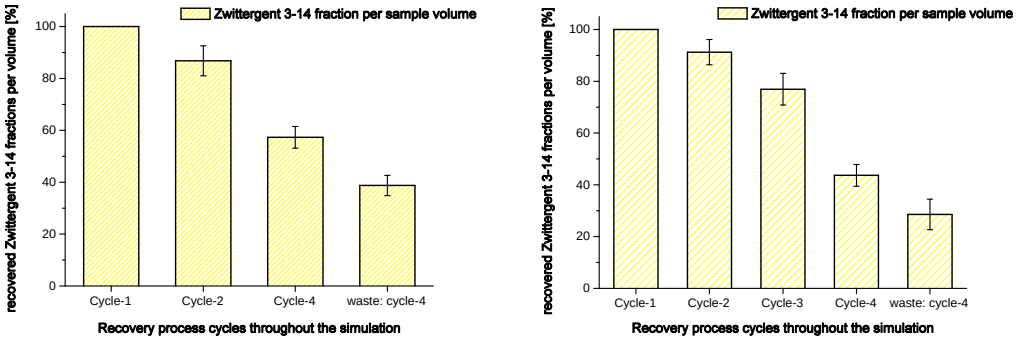

Fig. 8.13: Recovered Zwittergent 3-14 fractions throughout the recovery process simulation at a scale 0.5l. Left: process simulation in the absence of the salting-out step. Right: process simulation in the presence of the salting-out step

The left image of figure 8.13 shows the Zwittergent 3-14 characteristics throughout the recovery process in the absence of the salting-out step. More than 90% of the initial Zwittergent 3-14 is recovered after the centrifugation step post cycle-1. This is as expected, since the detergent was added in order to keep the proteins in solution and to recover them in the supernatant after the centrifugation. During the next centrifugation step only 60% of the Zwittergent 3-14 is recovered in the TCA-pellet and 40% of the initial Zwittergent 3-14 remains in solution and is detected in the supernatant. These findings indicate that the interaction between proteins and detergent gets disturbed by the addition of TCA and only 60% of the present detergent concentration is precipitating with the proteins. However, the results can also indicate that only 60% of the overall present detergent molecules are in interactions with the proteins and only that detergent fraction

gets precipitated.

Anyhow, only 60% of the Zwittergent 3-14 added during cycle-1 is found in the TCA-pellet at the end of the recovery process in the absence of the salting-out step. The final detergent concentration in the TCA-pellet is dependent on the volume used for solubilizing the pellet.

The right image of figure 8.13 shows the Zwittergent 3-14 characteristics throughout the recovery process in the presence of the salting-out step. More than 90% of the Zwittergent 3-14 added during cycle-1 is recovered after the centrifugation in cycle-2. This observation confirms the findings of the Zwittergent 3-14 characteristics in the absence of the salting-out step. Further, during the salting-out step in cycle-3 an additional 20% loss in Zwittergent 3-14 was observed. This reduction in Zwittergent 3-14 corresponds to the loss in interferon and total protein detected during the salting-out step in the presence of Zwittergent 3-14, as discussed in the previous section. It is likely that the lost Zwittergent 3-14 molecules during the salting-out step precipitated due to interactions with the proteins that were salted-out.

In the last step of the recovery process, an additional 30% loss of the initial Zwittergent 3-14 was detected due to the TCA addition. This observations corresponds again with the findings of the Zwittergent characteristics in the absence of the salting-out step. Over 25% of the initial Zwittergent 3-14 did not precipitate in the presence of TCA and was found in the waste stream of cycle-4. However, only 40% of the Zwittergent 3-14 added during cycle-1 was recovered in the final TCA-pellet. The final detergent concentration in the TCA-pellet is dependent on the volume used for solubilizing the pellet.

In summary, 60% of the Zwittergent 3-14 added during cycle-1 of the recovery process is recovered in the absence of the salting-out step and only 40% is recovered in the presence of the salting-out step. However, the final detergent concentration in the TCA-pellet is dependent on the volume used for solubilizing the TCA-pellet. The possibility of having the Zwittergent 3-14 at different concentrations in the TCA-pellet needs to be kept in mind for the investigation of the impact of the optimized recovery process on the down-stream process.

8.4 Conclusion

In this chapter process optimization using different detergents to improve protein solubility was discussed. Two detergents were identified to have the highest potential for improving interferon-α-2b solubility, Sarkosyl and Zwittergent 3-14. An investigation, using design of experiments was used to determine the impact and significance of different factors on the critical process step 1 (CPS-1) of the recovery process. It was shown that the detergent concentration has the biggest impact on the protein recovery during CPS-1. Sarkosyl at 3.0% resulted in a 90% recovery of IFN during CPS-1 and addition of 1.0% Zwittergent 3-14 had a 80% recovery in interferon.
Recovery process simulations in a small scale model were performed to study the impact of detergent addition during CPS-1 on the remaining steps of the recovery process. Simulations were performed following all 4 cycles of the process and with the elimination of the salting-out step. Results are gathered together in table 8.4.

Tab. 8.4: Summary of Recovery Process simulation results with the addition of detergents

		full recovery process		**without salting-out**	
		detergent present	control	detergent present	control
Sarkosyl 3%	**interferon**	20%	7%	**75%**	20%
	tot. protein	20%	8%	90%	18%
Zwittergent 1%	**interferon**	**55%**	7%	**70%**	25%
	tot. protein	40%	8%	65%	22%

As can be seen in table 8.4, the highest interferon-α-2b recovery was achieved with 3% Sarkosyl present and in the absence of the salting-out step. This condition results in a 10-fold increase in interferon recovery at the end of the process compared to the original process conditions at manufacturing scale. Due to the addition of Zwittergent 3-14 at 1%, the IFN recovery can be enhanced 7-fold compared to the recovery in the original process. Excluding the salting-out step, enhances the IFN recovery further and gives a 10-fold improvement, similar to the results with Sarkosyl.

Both options, addition of 3% Sarkosyl or 1% Zwittergent 3-14, result in a large enhancement in interferon recovery throughout the recovery process. Hence, there are four potential optimized

recovery processes available: addition of 3% Sarkosyl, or addition of 1% Zwittergent 3-14 to the process, which is performed in the presence or absence of the salting-out step. In order to identify which of these 4 processes is the most suitable and convenient one to be scaled-up and implemented at manufacturing scale, the impact on the down-stream process needs to be considered.

The investigation of this chapter did not study the impact of the detergent on the interferon-isoform distribution. It is possible that the different interferon-isoforms have different solubility levels, hence, the isoform distribution at the end of the recovery process can be different in the presence or absence of a detergent. In order to analyse the impact of a detergent on the interferon-isoform distribution, complete mass balances of each isoform need to be set-up throughout the protein recovery process. As discussed in chapter 6 no analytical method is currently available that provides the accuracy and robustness to quantify interferon-isoforms at all stages of the recovery process. Hence, a more accurate and suitable method for interferon-isoform quantification needs to be developed first, in order to give a statement about the impact of the detergents on the interferon-isoform distribution.

The findings of this chapter also did not cover the impact of the addition of a detergent on the bio-activity of interferon-α-2b. Before implementing the addition of a detergent to the protein recovery process at manufacturing scale, the bio-activity of interferon-α-2b at the end of the recovery process or down-stream process needs to be proven. However, findings in literature showed, that the addition of Zwittergent 3-14 had no impact on the bio-activity of the analysed protein [114].

In a next step the impact of the proposed optimized recovery processes on the first step of the down-stream process will be investigated. Four different aspects need to be considered: the impact of the presence of a detergent, the impact of variations in the ratio between host cell proteins and interferon, the impact of possible variations in total protein levels at the end of the recovery process and the impact of a different interferon-isoform distribution on the binding affinity towards the ion-exchange column. The removal of the detergents from the process needs to be investigated as well. At the end of the production process, the pure drug substance interferon-α-2b should be present without contamination by a detergent. Hence, the detergent needs to be removed, either through existing down-stream methods or through an additional step between the recovery and down-stream process. The removal of the detergents is discussed in the following chapter 9 and the impact of the detergents on the down-stream process will be discussed in chapter 10.

Chapter 9

Detergent Removal from the Recovery Process

9.1 Introduction

This chapter will focus on different techniques in order to remove detergents from recovery process samples. In chapter 8 two different detergents, Sarkosyl and Zwittergent 3-14, were added during cycle-1 to the primary protein recovery process in order to increase the protein solubility and protein recovery. These detergents need to be removed at the end of the primary protein recovery process (TCA-pellet) prior to further steps of the down-stream process.

9.1.1 Quantification of detergent

The addition of a detergent to a bioprocess in order to improve protein solubility requires the availability of a quantitative method for the detergent. Several different techniques for the quantification of ionic, non-ionic and zwitterionic detergents have been described in the literature. These techniques are spectrophotometric, potentiometric, titrimetric and labelling methods [115]. A colorimetric method is described by Haeuw et al. [116] which is based on the classical Lowry method. Another method based on the titrimetric principle, detects zwitterionic detergents by titration with ammonium sulphate in the presence of Triton X-100 [116]. Rajasekharan et al. [117] described a spectrophotometric methods for the quantification of non-ionic, ionic and zwitterionic

detergents by the principle of binding oil (triolein) to the hydrophobic tail of the detergent and measuring the turbidity changes. Unfortunately, all these methods are indirect methods and based on the detection of reaction products, what makes them less precise and less sensitive [118].

A more accurate and faster method is the usage of high pressure liquid chromatography (HPLC). Detergents are amphiphilic, hence contain hydrophobic parts which bind to reversed phase C-18 columns [116]. Some detergents containing chromaphores, such as Sarkosyl, absorb ultraviolet light and can be detected with a UV-detector [119][120]. However, other detection method with RP-HPLC are described in literature as well, which can be used for wider range of different detergents (ionic, non-ionic and zwitterionic). These detection methods are: evaporative light scattering (ELSD) [119], chemiluminescent nitrogen detection (CLND) [118] and charged aerosol detection (CAD) [121]. A disadvantage of these three detection methods is that the detectors are no standard lab equipment and often not available.

An alternative to UV, ELS, CLN and CA detection can be the refractive index detector (RID)[116]. The RI quantification is not limited to sugar containing detergents, neither does it require high detergent concentrations [122]. Hence, it can be used for the detection of ionic, non-ionic and zwitterionic detergents.

The refractive index describes how light propagates through a medium, or more specifically, it measures how much light is deviated or refracted when entering a material. It than compares this measurement to a reference medium [123]. The ratio between the speed of light of the reference medium and the substance n is given by: [72]

$$n = \frac{c}{v} \tag{9.1}$$

With:

- n = refractive index of substance 'n'
- c = speed of light in vacuum [m/s]
- v = speed of light in substance 'n' [m/s]

A disadvantage of the RI detection is its incompatibility with gradient solutions which can be problematic if several detergents need to be separated and analysed on the same time [116].

9.1.2 Removal of detergents from process samples

The presence of detergents in process samples can unfortunately interfere with further purification steps, such as ion-exchange chromatography steps or disrupt analytical work. Hence, in many situations the detergent needs to be removed from the process samples at small scale for analytical purposes or even at large scale for further purification steps [105]. Several different detergent removal methods are described in literature which range from physical methods such as diafiltration, resin adsorption, gel chromatography and dialysis to chemical methods such as co-precipitation [124].

Some detergents are easier to remove than others. For example detergents with a high cmc value are more easily removed than detergents with low cmc because they bind less tightly to the protein. Another detergent parameter affecting the removal process is the average micellar molecular weight (MMW). Detergents with a large average MMW are more difficult to remove by dialysis or ultrafiltration than detergent with a small MMW. Most non-ionic detergents have a large MMW, however, these detergents can be removed by ion-exchange chromatography since they are not known to interfere with the resin [107].

One of the most common methods for detergent removal is dialysis, especially for detergents with a high cmc. Detergent-protein solutions are diluted below the critical micelle concentration in order to break-up detergent micelles. The smaller detergent monomers can than easily be removed from the solution by dialysis due to the diffusion of the detergent molecules to a lower concentrated area [105]. Unfortunately this technique is not suitable for large scale applications in which case an ultrafiltration or UF/DF method would be used.

Other detergent removal techniques include hydrophobic adsorption, gel chromatography, ion-exchange chromatography and nickel columns with His tags. Some of these techniques are explained in more detail in the following sections.

9.1.2.1 Ion-exchange chromatography

Section 8.1.1.1 described the different characteristics of detergents which could be divided in non-ionic, ionic and zwitterionic subclasses. These characteristics of having positively or negatively charged groups can be used for the removal of the detergent.

Ion-exchange chromatography binds either anionic or cationic molecules, whereas non-ionic molecules do not bind at all [105]. Hence, non-ionic or zwitterionic detergents can be removed by IEC because they do not bind to the resin and the detergent pool can be found in the flow through or wash fraction of the column [125]. On the other hand, ionic detergents can be removed by IEC because they do bind to some of the resins and the protein is either found in the flow-through or can be washed off from the column by extensive washing [126].
In the following sections three different resins will be described in more detail for their potential to remove detergents from process samples. These resins are: Dowex, hydroxyapatite and detergent removal resin.

Dowex and Hydroxyapatite

Dowex ion-exchange resins are leaders in separation technology. Dowex resin find its application in water treatment, power generation, food application, chemical process and bioprocess purification. There are over 60 different Dowex resins available from Dow, which last from weak and strong anion resins to weak and strong cation resins over mixed-bed resin and anion- and cation-exchange resins [82]. Dowex meets the FDA food additive regulations as already discussed in section 6.1.4.1.
Dowex® 1x4 chloride form is an anion-exchange resin, hence it will bind negatively charged molecules such as the anionic detergent Sarkosyl [127]. New data from Dow showed the successful application of Dowex® 1x4 chloride resins for the removal of the zwitterionic detergent CHAPS while recovering over 80% of the protein contents [128].
Many detergents, especially zwitterionic detergents are stable over a wide pH range and their positive or negative charge is independent on the pH of the buffer. Hence, the binding of the detergent to the resin is most likely independent on the pH. However, the net-charge of proteins is highly dependent on the pH of the buffer. Thus, the pH may have an important role in the removal of detergents from protein mixtures. The conductivity value is always an important factor in ion-exchange chromatography and needs to be considered throughout all studies with IEC [129][130].

Hydroxyapatite is a mixed-mode resin which combines two modes of interactions simultaneously: cation-exchanger and metal affinity chromatography [85]. More detailed information about the

characteristics of hydroxyapatite can be find in section 6.1.4.2.

Most detergents, either non-ionic or ionic showed no binding affinity towards hydroxyapatite. Hence, the detergent should be removed by hydroxyapatite chromatography and found in the flow through of the column. Penin et al. [126] developed a detergent exchange assay for SDS (sodium dodecyl sulphate) using hydroxyapatite chromatography, where the protein-detergent complex bound to hydroxyapatite and the detergent was removed through extensive washing with the loading buffer. Similar to the Dowex resin, pH and conductivity have an impact on the protein binding to the hydroxyapatite resin and need to be considered in the investigation for detergent removal using hydroxyapatite [131].

Detergent removal resin

Detergent removal resin is a commercial product from Thermo Scientific, specially developed for the removal of detergents from protein-containing process samples. This resin combines the separation techniques of size-exclusion and affinity chromatography. The porous resin matrix allows only small molecules of less than 10kDa to enter and to get in contact with the detergent affinity resin. Hence, large molecules such as proteins, do not get in contact with the affinity resin and pass through the column [132].

The binding affinity of the detergents is independent of salt concentration and pH, however, it is dependent on the micelle molecular weight (MMW). Detergents with a large MMW (>10kDa) will not easily get in contact with the affinity resin due to the cut-off of the porous resin matrix. Studies showed that the pH of a detergent solution can have an impact on the average micelle molecular weight. Dependent on the detergent the MMW is either increasing or decreasing with a change in the pH [133]. The presence of salt is as well affecting the MMW of detergents. With an increase in the salt concentration the micelle molecular weight is increasing as well. Thus, it will be more difficult for the detergent to pass through the porous resin matrix [107].

These facts show that even though the binding of detergents to the affinity resin is independent of the pH and conductivity, these two parameters need to be considered throughout the detergent removal assay due to their impact on the micelle molecular weight of the detergent.

9.1.2.2 Ultrafiltration spin tubes

Ultrafiltration separates the detergent-protein mixture by size, thus this method is only effective if the average micelle molecular weight differs from the molecular weight of the target protein. Ionic detergents in particular, are known to have a small micelle molecular weights and therefore are suitable for removal with ultrafiltration. However, other studies showed potential to use ultrafiltration for the removal of detergents with larger micelle molecular weights (MMW) [134]. Detergents with large MMW, larger than the cut-off pore size of the ultrafiltration membrane, form a two-phase region on the membrane surface. In this dense micelle rich phase, the detergents are present as surfactant monomers and slowly pass through the membrane. Studies showed a removal yield of the detergent of 98%, but unfortunately also a protein loss of more than 50% [134].
Besides the micelle molecular weight of the detergent, the removal yield of detergent from process samples by ultrafiltration is also highly dependent on three other parameter: membrane material, temperature and salt concentration. Several studies showed that the best separation properties were obtained with polysulfone or polyethersulfone membranes [134][135][136]. An increase in temperature throughout the ultrafiltration has a positive impact on the permeate flux due to the decrease in solution viscosity and thermal expansion of the membrane material. On the other hand, with an expansion of the membrane material and thus an increase of the pore-size, the protein loss through ultrafiltration might increase as well [136]. During the removal of detergents with large MMW, an increase in the temperature has a negative impact. With increasing temperature and salt concentration the flux through the membrane is decreasing [134]. This can be explained by the impact on the micelle molecular weight of the detergent. As described in section 8.1.1.1, the MMW is increasing with an increase in temperature and ionic strength, especially for non-ionic detergents. Hence, the removal of the detergent with ultrafiltration will be slowed down.
Franzreb et al. [134] also showed that eight exchange sample volumes are required in order to receive an 98% removal of the detergent. Hence, a sample volume of 5ml in ultrafiltration spin tubes, needs to be washed with 40ml of exchange buffer in order to remove 98% of the detergent.

UF/DF system - scale up

A UF/DF system operates with a tangential flow system which is the major difference to the dead-end filtration in ultrafiltration spin tubes. In order to scale up a UF/DF system certain different parameters are important, which are: membrane type, protein concentration, process volume and process time. In general the first decision has to be made for the membrane type and the TFF (tangential flow filtration) module. Several different membrane materials are available, the most common ones at large scale are: polysulfone, polyethtersulfone and cellulose. Cellulose has the lowest protein adsorption whereas polyethersulfone is known to show the best separation techniques for detergent removal. The TFF type needs to be selected dependent on the process volume to be processed and on the available space. Spiral wound and flat plate are known to be more efficient for larger process volumes, since they can be operated with increase turbulence compared to hollow fibre [9].

The next key parameter to be selected in the membrane flux, which is highly dependent on the trans-membrane pressure and the protein concentration of the solution to be filtered. Figure 9.1 shows a typical trend of flux versus trans-membrane pressure for low and high protein concentrations.

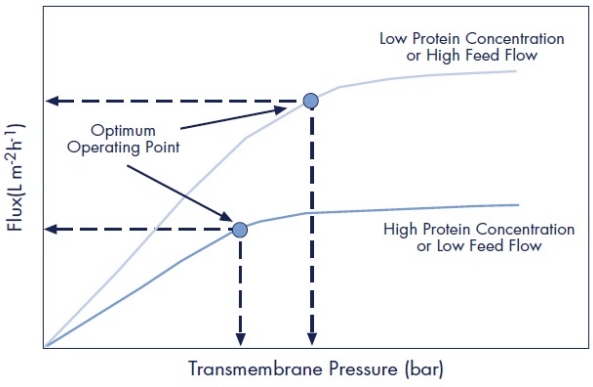

Fig. 9.1: A typical trend of flux versus trans-membrane pressure for low and high protein concentrations [9]

For a low protein concentration of around 5mg/ml a flux value of approximately 150 L/m^2/h and a trans-membrane pressure of 30psid is usually selected [137]. The next step is the calculation of the required membrane area, which is dependent on the membrane flux and the process volume to

be processed. The membrane area can be calculated following equation 9.2:

$$area\left[m^2\right] \quad = filtrate\,volume\,[L] \div \left(Flux\left[L * m^{-2} * h^{-1}\right] * process\,time\,[h]\right) \tag{9.2}$$

With the membrane area and the membrane flux the required pump feed rate can be calculated as follow:

$$Pump\,feed\,rate[L/min] \quad = Flux\left[L * m^{-2} * h^{-1}\right] * Area\left[m^2\right] \tag{9.3}$$

With these two equations the required membrane area, buffer volume, feed rate and process time can be calculated dependent on the process volume and the present protein concentration.

9.1.3 Goals and Aims

The aim of this chapter is the successful removal of different detergents from the process samples at the end of the primary protein recovery process prior to further down-stream processing. In a first step, quantification methods for the two detergents, Sarkosyl and Zwittergent 3-14, are being developed in order to quantify the reduced detergent levels during the removal steps. In a second step different techniques such as ion-exchange chromatography and ultrafiltration are tested for their potential to remove the two detergents from process samples.

9.2 Material and Methods

9.2.1 List of Materials

Tab. 9.1: Materials and suppliers

materials	supplier
Recovery Process samples (TCA-pellet)	industrial partner
Dowex® 1x4 chloride form (20-50 mesh)	Sigma Aldrich
Hydroxyapatite with < 200 nm particle size (BET)	Sigma Aldrich
Sarkosyl	Sigma Aldrich
Zwittergent 3-14	Sigma Aldrich
Detergent Removing Resin	Thermo Scientific
Pierce® Detergent Removal Spin Columns	Thermo Scientific
Vivaspin 6 centrifugal concentrator	Sartorius
Trizma Base	Sigma Aldrich
EDTA tetrasodium salt	VWR
Aeris WIDEPORE 3.6u XB-C18 column 150 x 4.6mm	Phenomenex
Glycerol triolein	Sigma Aldrich
Bovine serum albumin (BSA)	Sigma Aldrich
interferon-α-2b drug substance	industrial partner
Acetonitrile	Fisher Scientific
Trifluoroacteic acid (TFA)	Fisher Scientific
Triolein	Sigma Aldrich

9.2.2 Detergent removal using Ion-exchange resins

To perform ion-exchange chromatography in order to remove detergents from process samples, the chromatographic procedure was simulated in a small 2.0mL Eppendorf tube, instead of using a column. Between 100 - 200mg of the specific resin was added to the Eppendorf tube, followed by

the addition of 1.0mL of the process sample containing detergent (either Sarkosyl or Zwittergent 3-14). After 2.0 hours of continuously mixing, the samples were centrifuged at 10'000rpm (12'200g) for 15min at room temperature (Eppendorf centrifuge, Hamburg, Germany). The supernatant (0.8mL) was recovered and stored at 4°C for further analysis.

The resins to be investigated for this study were: Dowex® 1x4 chloride form (20-50 mesh) and hydroxyapatite with < 200 nm particle size (BET).

9.2.3 Detergent removal using Size exclusion and affinity chromatography

This method combines both, size exclusion and affinity chromatography, using a detergent removing resin (Thermo Scientific, Dublin, Ireland). This resin allows small molecules (< 10kDa), like detergent molecules, to enter the affinity matrix and to bind to a specially developed ligand. Hence, larger molecules (>10kDa), such as interferon-α-2b, pass through the column without interacting with the affinity matrix, which avoids non-specific binding.

For the removal of the detergents, Pierce® Detergent Removal Spin Columns were used, following the manufacturer's instruction manual [132].

Samples were taken from the recovered flow-through and stored at 4°C for further analysis.

9.2.4 Detergent removal using Ultrafiltration/Diafiltration

Detergent removal with Ultrafiltration/Diafiltration was simulated at small scale with ultrafiltration spin tubes (Sartorius, Goettingen, Germany). These spin tubes contain a Polyethersulfone membrane (PES) with a molecular weight cut off (MWCO) of 5kDa and 10kDa.

The process sample (5mL) containing detergent were filled in the upper chamber of the spin tube. Tubes were centrifuged at 7000rpm (7122g) at room temperature for 40 minutes, until half of the volume had passed through the membrane into the lower chamber. The upper chamber volume was top up (to 5mL) with extraction buffer (20mM Tris, 1mM EDTA buffer, pH 8.0) and centrifugation continued. This cycle was repeated a minimum of 5 times.

Samples were taken after every centrifugation cycle from the upper and lower chamber for mass balances and stored at 4°C for further analysis.

9.2.5 Detergent quantification

Sarkosyl quantification - RP-HPLC

The amounts of Sarkosyl in process samples were quantified using RP-HPLC with a UV detector (Agilent, Cork, Ireland) at 280nm and a C-18 column with a particle size of 3.6μm (Phenomenex, Cheshire, UK). A gradient method was used for separation, with mobile phase A containing 0.1% (v/v) trifluoroacetic acid (TFA) aqueous solution, and mobile phase B consistent of acetonitrile (ACN) / 1.0% aqueous TFA (90:10, v/v). Column temperature was operated at 45°C and the flow-rate set constant to 1.0 mL/min. The gradient method is identical to the method for interferon-isoform quantification with RP-HPLC described in chapter 6.
The retention time for Sarkosyl under the described conditions is between 5.2 and 5.4 minutes.

Zwittergent 3-14 quantification - spectrophotometric

Detection of small traces of Zwittergent 3-14 in process samples was performed with a spectrophotometric method with Triolein. Five μL of 1.015M Triolein was pipetted into a 1.5mL Eppendorf tube and 1.0mL of process sample added on top. The solution was mixed for exactly 15 seconds and the absorbance/ turbidity measured at 660nm immediately (compare [117]).
An absorbance reading above 0.0 indicates the presence of detergent traces.

Zwittergent 3-14 quantification - RP-HPLC-RID

Quantification of Zwittergent 3-14 was performed using a reverse-phase HPLC method (Agilent, Cork, Ireland) with refractive index detector [122] and a C-18 column with a particle size of 3.6μm (Phenomenex, Cheshire, UK). An isocratic method was used with acetonitrile (ACN) / 1.0% aqueous TFA (45:55, v/v) for detergent detection, followed by a regeneration step of the column with acetonitrile / water solvent (80:20, v/v). Column temperature was operated at 35°C and the flow-rate set to a constant volume of 1.0 ml/min for detection and column regeneration.
The retention time for Zwittergent under the described conditions is between 2.3 and 2.6 minutes.

9.2.6 Total protein quantification - BCA

The total amounts of protein were quantified using the BCA assay as described in section 3.2.2 and table 3.5. Sample pellets were dissolved in EDTA-Tris buffer (1mM EDTA, 20mM Tris, pH 8.0) prior to protein analysis. BSA dissolved in water was used as a reference standard for all total protein quantifications. The blank was chosen according to the sample to be analysed, and included water, EDTA-Tris buffer, detergents or diluted fermentation media.

9.2.7 Total Interferon-α-2b quantification

The total amounts of interferon-α-2b were quantified using SDS-PAGE as described in section 5.2.3 and 5.2.4. Sample pellets were dissolved in EDTA-Tris buffer prior to analysis as mentioned in the section above. No further sample treatment was required. The interferon-α-2b drug substance was used as a reference standard for all quantifications.

9.3 Results and Discussion

This result and discussion section focuses on the removal of the detergent from recovery process samples. If a detergent is added to the recovery process to improve protein solubility and total interferon-α-2b yield, it needs to be ensured that this detergent can be removed from the process during the DSP steps prior to the final filling of the drug substance is reached. The following sections discuss the removal of two detergents, Sarkosyl and Zwittergent 3-14 from recovery process samples. Four different techniques have been tested to remove both of the detergents and the results are presented and discussed here. The first section focusses on the development of quantification methods for both detergents in order to quantify the level of removal.

9.3.1 Development of quantification methods for Sarkosyl and Zwittergent 3-14

The first step in developing a method for detergent removal, is the development of quantification methods for these detergents, otherwise it is impossible to determine to which level the detergent has been removed. Hence, this section focuses on the development of quantification methods for Sarkosyl and Zwittergent 3-14. First, the focus will be on Sarkosyl, followed by the results of Zwittergent 3-14.

Figure 9.2 shows the linear regression between Sarkosyl concentration (%) and the peak area (mAu*s). Results were obtained using the RP-HPLC method as described in section 9.2.5 under Sarkosyl quantification. Detergents are amphipathic (section 8.1.1.1), thus their hydrophobic part binds to C-18 columns. Sarkosyl, an an-ionic detergent, also has the characteristics to absorb UV light at wavelengths between 250 - 280nm.

A linear relationship between Sarkosyl and peak area (as seen in figure 9.2), was observed for the range 0.0 - 3.0% of Sarkosyl. Sarkosyl elutes with a retention time of 5.3min, thus it does not interfere with the elution of interferon-α-2b or its isoforms. Standards of Sarkosyl were prepared in DI-water, as well as in process media to investigate the impact of process samples on the quantification method. Results show that the presence of process media had no significant impact on the Sarkosyl quantification. The difference between the two slopes, one determined with Sarkosyl in DI-water (74'244), the other with Sarkosyl in process media (77'385), is less than 4% and therefore considered negligible. Hence, the RP-HPLC method with UV detector can be used to

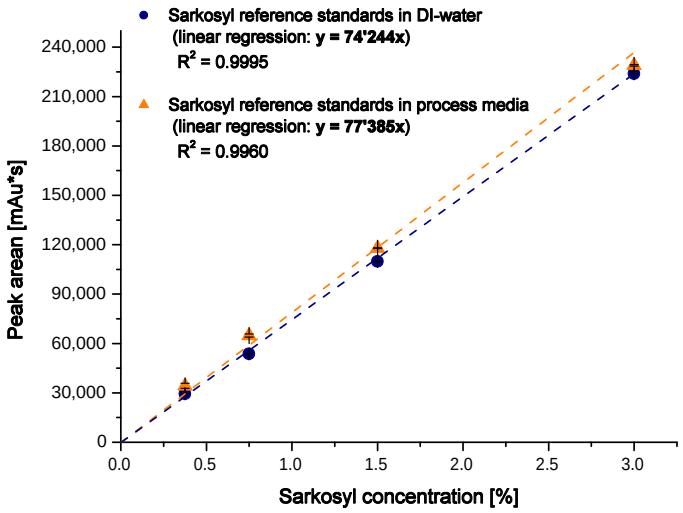

Fig. 9.2: Linear regression of Sarkosyl reference standards between 0.0 and 3.0%

quantify Sarkosyl in process samples at concentrations from 0.0 to 3.0%.

The quantification of Zwittergent 3-14 is more challenging. This detergent belongs to the category of zwitter-ionic detergents which show no absorbance to UV light. In literature several different indirect quantification methods are described for Zwittergents (section 9.1.1), one of which is turbidity measurements at 660nm with Triolein. Figure 9.3 shows three linear regressions of Zwittergent 3-14 reference standards between 0.0 - 0.10% concentration.

All three linear regressions shown in figure 9.3 were obtained following the procedure explained in section 9.2.5 under spectrophotometric detection, using Zwittergent 3-14 dissolved in DI-water as the reference standard. The variation between the different slopes is greater than 38%. These large variations can be explained by the nature of this method. The added Triolein forms a complex with Zwittergent 3-14 which shows a turbidity reading at 660nm. This complex is highly unstable, thus the absorbance values are dependent on the measuring time point. Due to significant variations in this method, it is not suitable for quantification but only for the detection of traces of Zwittergent 3-14. Besides high variations, this method also showed interference with the process media (results not shown), hence quantitative detection of Zwittergent 3-14 in process samples requires process media as a blank to eliminate interference.

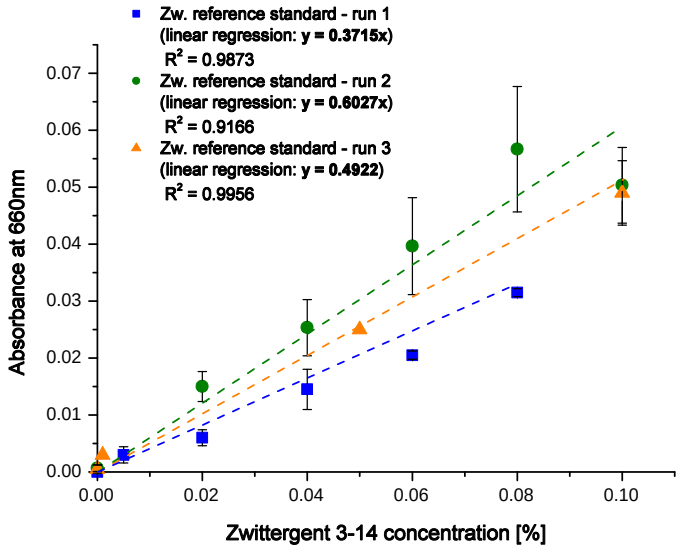

Fig. 9.3: Linear regression of Zwittergent 3-14 reference standards between 0.0 and 0.10%

In order to quantify Zwittergent 3-14, a more accurate method is required. In the literature several quantification methods are described using HPLC with a CAD detector (light scattering) [122]. Unfortunately such a device was not available for this study. An alternative was found using a RI detector. The refractive index describes how light propagates through a medium, or more specifically, it measures how much light is deviated or refracted when entering a material, compare section (section 9.1.1). In this way the refractive index detector also detects all non-sugar-containing detergents, such as Zwittergent.

Figure 9.4 shows the linear regression model between the peak area of the refractive index signal and the Zwittergent 3-14 concentration for three different injection volumes into the HPLC. All measurements were performed following the method described in section 9.2.5 under Zwittergent quantification with RP-HPLC and RID.

Figure 9.4 shows that a linear relation exists between the peak area and the Zwittergent 3-14 concentration for 0.05 - 1.0% of Zwittergent with a R^2-value between 0.990 - 0.994. It also shows that the slope is dependent on the injection volume of the Zwittergent. Hence, the injection volume needs to be kept constant throughout different measurements. In order to increase the sensitivity for small Zwittergent concentrations of around 0.05%, the injection volume was set to 60μL.

Process medium was shown to have a significant impact on the quantification of Zwittergent with

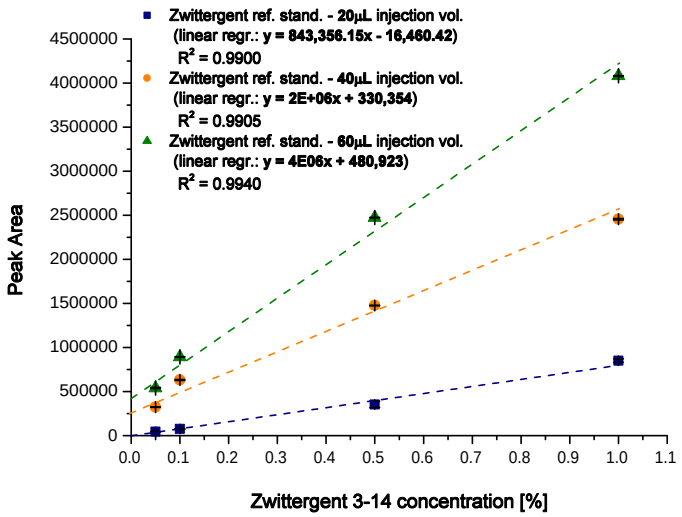

Fig. 9.4: Linear regression of Zwittergent 3-14 reference standards in process media between 0.05 and 1.0% detected with RP-HPLC and RI detector

this RP-HPLC method, hence all reference standards were prepared in process media to have similar conditions than the process samples to be analysed.

The variations in peak area between triplicates are less than $\pm 5.0\%$ over the whole detection range. Hence, this method is an accurate method to quantify Zwittergent 3-14 in process samples between 0.05 - 1.0% of Zwittergent.

9.3.2 Removal of Sarkosyl from process samples

Sarkosyl was added during cycle 1 of the recovery process in order to improve protein solubility and increase the recovery of interferon-α-2b (chapter 8). The following sections focus on the removal of Sarkosyl from this recovery process. Four different techniques will be discussed: an ion-exchange method, a mixed-mode method, a combination of affinity and size exclusion and an ultrafiltration spin-tube method.

9.3.2.1 Sarkosyl removal using ion-exchange resin

Ion-exchange resins can be used for removal from detergents of process samples due to the different charged groups on detergents (section 9.1.2.1). Two different resins have been tested for the

removal of Sarkosyl, an anionic (Dowex) and a mixed-mode resin (hydroxyapatite).

Dowex 1x4 chloride form

Dowex is one of the first manufactured ion-exchange resins and is still of high interest for application at industrial scale (section 9.1.2.1). Dowex resin is available with certain different characteristics: weak and strong acid cation exchanger, weak and strong basic anion exchanger, mixed bed resin, fine mesh resin or resins with different polishing. For the removal of Sarkosyl an anionic fine mesh resin was used (Dowex 1x4 chloride form, 20-50 mesh).
Figure 9.5 shows the obtained results for the unbound fraction of Sarkosyl, in green, and total interferon, in blue. The aim of this experiment was to have all Sarkosyl molecules bound to the resin while unbound total interferon-α-2b remained as high as possible in solution.

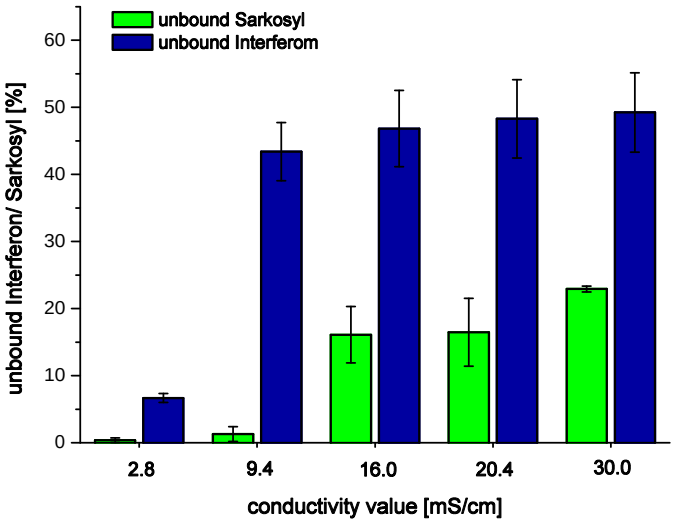

Fig. 9.5: Recovery of unbound interferon and Sarkosyl after treatment with Dowex resin as a function of conductivity of the process sample (pH constant at 7.0)

The experiment was performed as described in section 9.2.2. Total interferon was quantified with SDS-PAGE, compare section 9.2.7 and Sarkosyl was quantified with RP-HPLC and UV detection as described in section 9.2.5. Figure 9.5 shows the recovery of unbound Sarkosyl and interferon after treatment with Dowex resin as a function of conductivity. It can be seen that at low conductivity, 2.8mS/cm, the affinity of Sarkosyl and interferon to the Dowex resin was very high and more than 98% of Sarkosyl and 95% of interferon of the initial concentration bound to the resin. As the

conductivity increased the affinity of Sarkosyl and interferon for the Dowex resin decreased. This observation is as expected. The affinity of proteins or detergents to resins is highly dependent on conductivity, as explained in section (section 9.1.2.1).

The optimal condition was identified at 9.4mS/cm, where still more than 97% of Sarkosyl bound to the resin, but only 50-55% of interferon. Hence, more than 45% of total interferon was recovered, while removing 97% of Sarkosyl from the process sample. This result shows that Sarkosyl has a higher affinity to bind to the Dowex-resin than interferon under these operating conditions.

The impact of pH on the binding affinity of Sarkosyl and interferon to Dowex resin was also studied. The pH showed no significant impact on the binding affinity of either Sarkosyl or interferon (data not shown). Hence, only the conductivity needs to be controlled for this method.

In summary, Sarkosyl can be removed from process samples using Dowex anionic exchange resin, with a yield of more than 97% removal of Sarkosyl and over 40% recovery of interferon. However, more than 60% of interferon was lost during this step, hence, other possibilities to remove Sarkosyl from process samples will be investigated.

Hydroxyapatite

Hydroxyapatite is a mixed-mode resin, which involves non-specific interactions between positively charged calcium ions and negatively charged phosphate ions, thus it should bind positively charged molecules as well as negatively charged molecules (section 9.1.2.1).

The aim of this experiment was to develop a method where Sarkosyl binds to the hydroxyapatite, while the recombinant protein interferon-α-2b remained in solution. Figure 9.6 shows the recovered Sarkosyl and interferon-α-2b fractions in process samples after exposing these samples to hydroxyapatite as described in section 9.2.2. Different pH and conductivity values were chosen to examine the impact of these parameters on the binding affinity of Sarkosyl or IFN towards hydroxyapatite resin.

Sarkosyl and total interferon-α-2b concentrations in figure 9.6 were quantified using RP-HPLC and SDS-PAGE as described in section 9.2.5 and 9.2.7. The results show that Sarkosyl (in green) has a high affinity towards hydroxyapatite resin which is independent of pH or conductivity. The result for Sarkosyl for pH 7.5 and conductivity of 2.8mS/cm is considered as an outlier, since no explicable reason could verify this high recovery of Sarkosyl. Usually the affinity of molecules

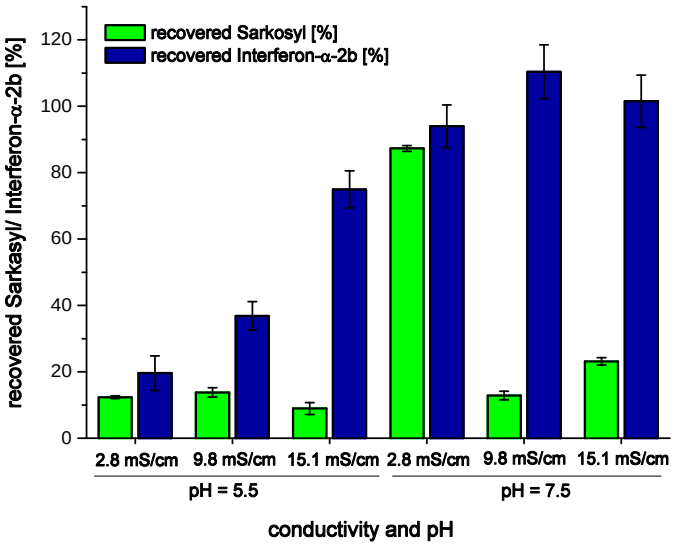

Fig. 9.6: Recovery of interferon-α-2b and Sarkosyl after treatment with hydroxyapatite resin dependent on different pH and conductivity values

for the resin increases with lowering the conductivity and not the other way around. However, ignoring the single outlier, Sarkosyl bound to hydroxyapatite independently of pH or conductivity and 80 - 90% of the Sarkosyl could be removed.

On the other hand, the affinity of interferon-α-2b (in blue) towards hydroxyapatite resin shows a high dependency on the pH and conductivity values. With low pH (5.5) and low conductivity (2.8mS/cm) the affinity towards hydroxyapatite is increased and only 20% of the original IFN amount was found in solution. With increasing pH and conductivity this affinity decreased and at pH 7.5 and a conductivity greater than 10mS/cm only very little binding of IFN towards hydroxyapatite occurred.

Hence, the best conditions to remove Sarkosyl from process samples, while keeping interferon-α-2b in solution using hydroxyapatite, is at neutral pH (7.5) and high conductivity (> 10.0mS/cm). Under these optimal conditions 75% of Sarkosyl can be removed from the process samples while recovering > 95% of interferon-α-2b in solution.

9.3.2.2 Sarkosyl removal using size-exclusion and affinity resin in spin columns

Since pure ion-exchange resin binds both the detergent and protein of interest, interferon-α-2b, a combined technique of size exclusion and affinity chromatography was tested to remove the detergent from process samples. The 'Detergent Removing Resin' from Thermo Scientific is a resin only developed for the removal of unwanted detergents from process samples containing proteins or other macromolecules (section 9.1.2.1). The resin matrix allows only small molecules, less than 10kDa, to enter and come into contact with a specially developed detergent ligand. Larger molecules pass through the column without entering the resin matrix and without coming into contact with the detergent ligand. Hence, separation of detergent and proteins should be improved and higher protein recovery should be achieved.

Experiments performed in this section were performed as described in section 9.2.3. Spin columns were filled with the detergent removal resin as described in the kit manual [132]. Figure 9.7 shows the recovered Sarkosyl concentration (in green) and total interferon fraction (in blue) in the flow through of the spin columns as a function of pH and conductivity of the process sample.

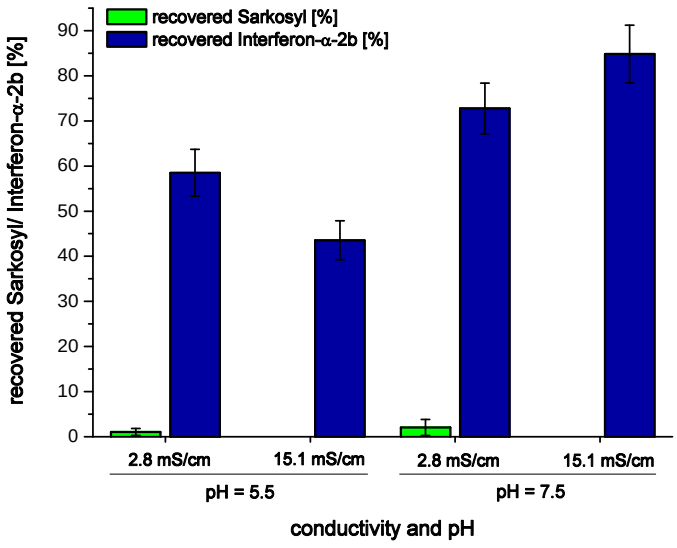

Fig. 9.7: Recovery of interferon and Sarkosyl in flow through of spin column packed with 'Detergent Removal Resin' at different pH and conductivity values

Total interferon-α-2b was quantified by SDS-PAGE as described in section 9.7 and Sarkosyl was quantified with the RP-HPLC method as described in section 9.2.5. Figure 9.7 shows that Sarkosyl

can be successfully removed from process samples while recovering more than 85% of interferon-α-2b. Due to the limited size of the spin columns, a maximum of 1.0 - 1.5% of detergent could be removed from process samples before the column reached saturation. Process samples used for the experiments of figure 9.7 contained 1.0% of Sarkosyl at the beginning, hence a complete removal Sarkosyl is possible with these small spin columns. However another experiment was performed using process sample containing up to 2.0% of Sarkosyl. In this experiment only 80% of the Sarkosyl was removed by the detergent removal resin due to overloading of the column (data not shown). In order to remove more than 1.0 - 1.5% of Sarkosyl, larger columns would be required or several cycles with fresh detergent removal resin need to be performed.

The data shown in figure 9.7 also show the impact of pH and conductivity on the binding affinity of Sarkosyl and interferon-α-2b towards the resin. No significant impact of either pH or conductivity can be observed for the binding of Sarkosyl towards the detergent removal resin. Under all 4 tested conditions, Sarkosyl bound to the resin successfully and more than 98% of Sarkosyl was removed from the process sample. On the other hand some effects of the pH on the interferon-α-2b interaction with the detergent removal resin were observed. The results in figure 9.7 show the affinity of interferon towards the resin is stronger at acidic pH (5.5) than it is at neutral pH (7.5). Theoretically interferon with a molecular weight of 19kDa, should not come into contact with the detergent removal resin, since only small molecules of less than 10kDa should pass through the pores to the resin (section 9.1.2.1). However, this suggest that there is a broad pore-size distribution and it is possible that due to minor changes in the packing of the column some of these pores are larger than others and also molecules with 19kDa get in contact with the detergent removal resin. For this case a working pH of 7.5 is the optimum and results in more than 85% recovery of interferon-α-2b. The conductivity shows no significant impact on the affinity of interferon towards the detergent removal resin and can therefore be considered negligible.

In summary, using spin columns filled with detergent removal resin, successfully removes > 98% of Sarkosyl from process samples while recovering > 75% of interferon-α-2b at a working pH of 7.5.

A negative aspect of this method is the scaling-up. So far the spin columns were loaded with 100μL of settled resin, which can remove 1.0% of Sarkosyl from 100μL sample (section 9.1.2.1). In order to remove 3.0% of Sarkosyl from 9'000L at manufacturing scale, more than 27'000L of

resin would be required. These large amounts are not available so far, thus this method is only interesting at small scale of up to 100mL of resin.

9.3.2.3 Sarkosyl removal using Ultrafiltration/Diafiltration with spin tubes

As seen in previous sections no method tested so far for the removal of detergents from process samples fulfilled all required criteria: detergent removal >95%, IFN recovery >60% and application at large scale. Either the recovery of interferon was very low (see section 9.3.2.1) or the application at large scale is impossible (see section 9.3.2.2). In this section the focus is on Ultrafiltration/Diafiltration which separates the detergent from the process samples by size. In order to simulate the UF/DF at small scale, spin tubes with different cut-offs were used as explained in section 9.2.4. Since the protein of interest, interferon-α-2b, has a molecular weight of 19kDa, membrane cut-offs of 10kDa and 5kDa were selected. This allows the protein to remain in the retentate, whereas the detergent should be found in the flow-through of the spin tubes (permeate). Figure 9.8 shows the recovered Sarkosyl (orange), total protein (green) and total interferon-α-2b (blue) fractions after each cycle 1 - 6 with spin tubes having a 10kDa cut-off membrane.

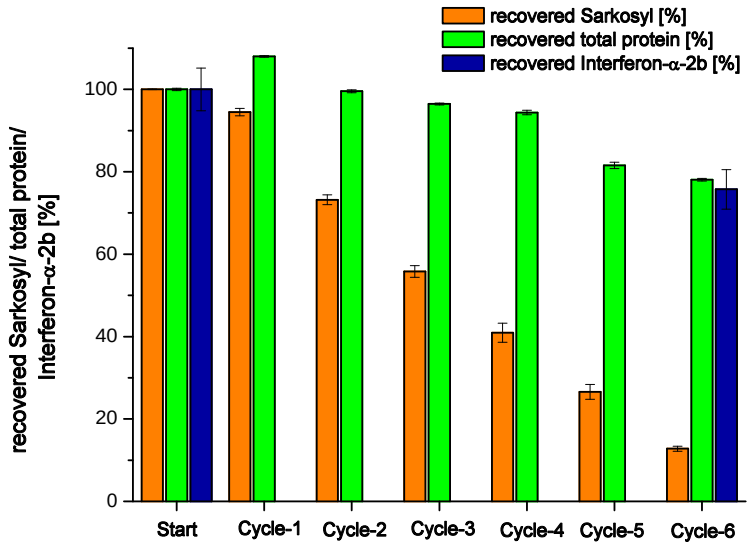

Fig. 9.8: Recovery of interferon and Sarkosyl in upper chamber (retentate) of spin tubes with 10kDa cut-off (pH=7.0, C=10.0mS/cm): after each cycles the upper chamber was filled up with extraction buffer and the centrifugation repeated as before

Sarkosyl concentrations of figure 9.8 were quantified using RP-HPLC as described in section 9.2.5

and total protein and total interferon-α-2b were quantified using the BCA-assay and SDS-PAGE as described in section 9.2.6 and 9.2.7. Figure 9.8 shows the recovered detergent and protein fractions after 6 performed cycles with ultrafiltration spin-tubes. One cycle involves a centrifugation run of the spin tube, filled with the process sample. After each cycle the volume in the upper chamber (retentate) of the spin-tube was topped-up with EDTA-Tris buffer (1mM EDTA, 20mM Tris, pH 8.0) and the contents of the lower chamber (permeate) was discharged (compare 9.2.4).
The results in figure 9.8 show a continuous, almost linear decrease in Sarkosyl concentration over the 6 cycles, with a removal of more than 80% of the initial Sarkosyl concentration. Hence, with a starting concentration of 3% Sarkosyl and 80% removal, only 0.6% of the Sarkosyl remains at the end of cycle 6. Due to the addition of further cycles (10 instead of 6), the removal of Sarkosyl can be further improved. In a new experiment 10 cycles were performed and Sarkosyl was removed by more than 95% (data not shown).
Looking at the protein recovery throughout the cycles, it can be seen in figure 9.8 that total protein and interferon-α-2b are recovered to 80% after 6 cycles, hence only 20% of the initial protein was lost. This result is as expected, due to the molecular weight of interferon with 19kDa, it should remain in the retentate. Analysing the different flow-through fractions of each cycle resulted in no findings of interferon or any other proteins. It is therefore possible that the missing protein fraction (20%) is trapped inside the membrane pores. The centrifugation of the spin-tubes is a dead-end centrifugation system and not a continuous one, hence all molecules get drawn-down into the membrane. However, if the g-force of the centrifuge is too high more proteins become trapped within the membrane, thus reducing the g-force may improve the interferon recovery.
The addition of further cycles in order to improve the Sarkosyl removal will decrease the protein recovery. Results after the performance of 10 cycles showed only 60% recovery of interferon (data not shown). Consequently it is very important to choose the right g-force (pressure for UF/DF-system) in order to minimize the loss of proteins which become trapped in the membrane pores (due to high g-force or pressure).
In summary, using spin-tubes with a 10kDa cut-off for detergent removal results in 80 - 95% removal of Sarkosyl and 80 - 60% recovery of interferon-α-2b dependent on the amount of cycles performed and the rate of g-force selected for the centrifugation cycles.
It has to be kept in mind that the spin-tubes were only used to explore the potential of a UF/DF

system to remove Sarkosyl (detergents) from process samples. Due to the limited available sample volume, the spin-tubes were used as a simulation of a UF/DF system at small scale. At larger scale (process scale) the UF/DF system would find its application.
Main differences between both techniques (spin-tubes and UF/DF) are the continuous flow in the UF/DF system which is not possible with the spin-tubes. All molecules of all sizes are drawn towards the membrane and some molecules become trapped inside, fouling the pores and hindering smaller molecules passage through. This can be a big problem for the process samples used in this study. It is known that the process samples contain a large fraction of host cell proteins which have an average size of more than 100kDa. Hence, it is likely that these proteins will found the membrane pores and interfere with the Sarkosyl removal. If a UF/DF system with a continuous flow were used this phenomenon would be reduced. Larger molecules who do not pass through the membrane would be carried on in the flow and do not found the membrane pores. Hence, using a UF/DF system instead of spin-tube would be expected to improve Sarkosyl removal as well as protein recovery.

In addition to spin-tubes with a 10kDa cut-off, spin-tubes with only 5kDa cut-off were also tested for the removal of Sarkosyl from process sample. Due to the smaller pore size protein recovery should be increased. Figure 9.9 shows the recovered fractions of Sarkosyl (orange), total protein (green) and total interferon-α-2b (blue) after each cycle.

The explanation for the term cycles in this experiment have already been explained above, as well as the quantification methods used for Sarkosyl, total protein and interferon. The results shown in figure 9.9 are as expected. Sarkosyl was removed from the process samples at a much slower rate compared to results with a 10kDa cut-off. After 6 cycles only 30% of the original Sarkosyl concentration had been removed. On the other hand, the protein recovery was slightly improved compared to the spin-tubes with 10kDa cut-off. Between 85-90% of protein was recovered after 6 cycles. The disadvantage of using only 5kDa cut-off instead of 10kDa, is the increased expenditure of time (i.e. lower productivity) that is required with the 5kDa cut-off to reach a detergent removal yield of 95%. The 5kDa cut-off membrane takes more than 2.5 times longer in order to remove the same amount of detergent of the samples. This can have a big impact on the process cost especially at industrial scale. It therefore needs to be considered in the decision which situ-

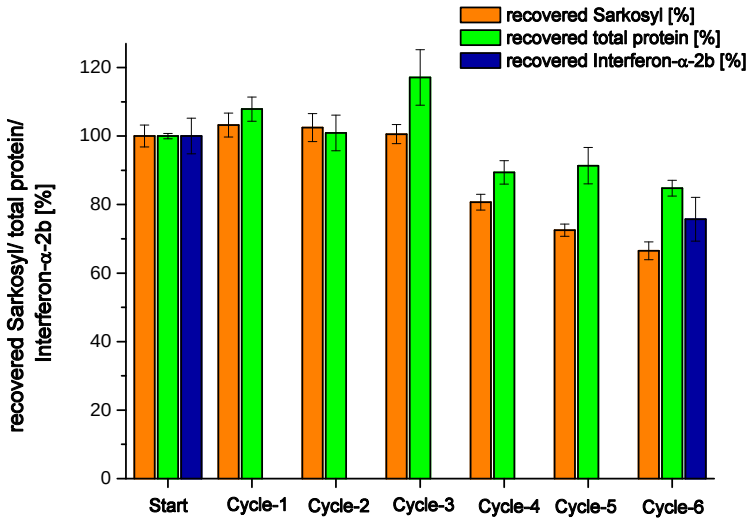

Fig. 9.9: Recovery of interferon and Sarkosyl in upper chamber of spin tubes with 5kDa cut-off (pH=7.0, C=10.0mS/cm): after each cycles the upper chamber was filled up with extraction buffer and the centrifugation repeated as before

ation is more beneficial, longer process time and more buffer consumption, or a slightly reduced protein recovery but shorter process time.

In summary, the UF/DF system and membranes with 10kDa cut-off show the highest potential to successfully remove Sarkosyl from process samples compared to all methods. It is the only method that meets all three criteria: it removes Sarkosyl to a yield of 95 - 100%, it recovers interferon-α-2b to a level of 60% or greater and it is straight forward to apply at large scale. At large-scale the results are expected to improve further.

9.3.3 Removal of Zwittergent 3-14 from process samples

In addition to the detergent Sarkosyl the detergent Zwittergent 3-14 was also proposed to be added to the primary protein recovery process in order to improve protein solubility (section 8.3.3.1). Hence, a method needs to be developed in order to remove Zwittergent 3-14 from the process. In a similar way to the method for Sarkosyl removal, the method for Zwittergent removal should fulfil three criteria: first, to remove Zwittergent from process samples with a yield of 95% or higher. Second, to recover interferon-α-2b to a level of at least 60% or higher and third, the method needs

to be feasible for large scale.

Keeping these goals in mind, the same three techniques tested for Sarkosyl removal were investigated for their potential to remove Zwittergent 3-14: ion-exchange resin (Dowex and hydroxyapatite), size exclusion and affinity resin and ultrafiltration/diafiltration. Since Zwittergent is an zwitter-ionic detergent and not an anionic detergent (as Sarkosyl), the characteristics and behaviour can be different. Therefore it is mandatory to investigate once again all three different techniques for their potential to remove now Zwittergent 3-14 from process samples. All results are shown and discussed in the following sections.

9.3.3.1 Zwittergent removal using Ion-exchange resin

Comparable to the experiments performed for Sarkosyl removal from process samples, two different resins were tested for the removal of Zwittergent 3-14. First an anionic resin (Dowex) and second a mixed mode resin (hydroxyapatite).

Dowex

Dowex 1x4 chloride resin is an anion-exchanger which is used for purification of fine pharmaceuticals, as it meets requirements of the FDA Food Additive Regulation (section 9.1.2.1). As described in section 9.2.2, Dowex resin was used to remove the detergent Zwittergent 3-14 from process samples. The aim is to have all detergent molecules bound to the resin, while keeping the protein of interest, interferon-α-2b, in solution.

Figure 9.10, shows the recovery of Zwittergent 3-14 and interferon-α-2b after treatment with Dowex resin as a function of pH and conductivity.

Zwittergent 3-14 was quantified with RP-HPLC and RI detector as described in section 9.2.5 and total interferon-α-2b was quantified by SDS-PAGE as described in section 9.2.7. Figure 9.10 shows that both Zwittergent 3-14 and interferon-α-2b bound to the Dowex resin with a similar affinity. Between 80 to 20% of Zwittergent 3-14 and 80 to 50% of interferon bound to the Dowex resin depending on conductivity and pH of the sample. Hence, the binding of Zwittergent-14 and total interferon-α-2b towards the Dowex resin is dependent on the conductivity and pH. As the conductivity and pH are lowered, the stronger is the binding of Zwittergent 3-14 and interferon

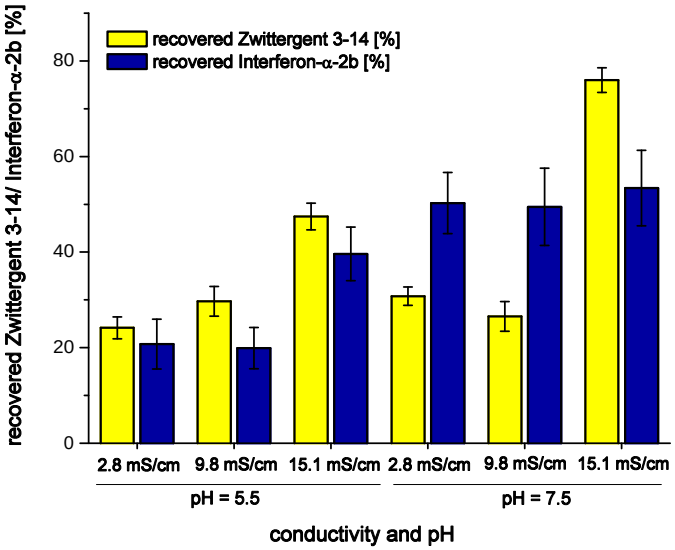

Fig. 9.10: Recovery of interferon-α-2b and Zwittergent 3-14 after treatment with Dowex resin dependent on different pH and conductivity values

to the resin. The impact of the conductivity is as expected. As explained in section 6.1.4.1, the binding of molecules to resins is highly depending on the salt concentration (ionic strength) of the sample. However, the impact of pH is unexpected. A pH of 5.5 is below the pI of interferon which gives the protein a positively charge (compare section 6.1.4.1). Hence, the protein should not be attracted to the negatively charged resin. It is possible that interferon binds through the detergent to the resin and the interaction between detergent and protein increases with lower pH. This would explain the reduces interferon recovery at lower pH values.

Unfortunately both protein and detergent bind with a similar affinity to the Dowex resin under all conditions, which makes it impossible to separate one from the other with this technique. The best separation was observed at pH 7.5 and a conductivity value of 9.8mS/cm. Under this condition 70% of Zwittergent 3-14 was removed with 50% recovery of interferon-α-2b. However, 70% removal of Zwittergent 3-14 is not sufficient and does not meet the aim of at least 95% removal yield.

Hydroxyapatite

The anion-exchange resin Dowex showed no potential for a separation of the Zwittergent 3-14 and interferon-α-2b, for that reason the mixed-mode resin hydroxyapatite was also tested for its potential to remove Zwittergent 3-14 from process samples. Mixed-mode resins have non-specific interactions with positively and negatively charged molecules (compare section 9.1.2.1), which may be beneficial in order to separate the detergent Zwittergent 3-14 from process samples containing different kind of proteins.

Figure 9.11 describes the recovery of Zwittergent 3-14 and interferon-α-2b after treatment with hydroxyapatite resin dependent on different pH and conductivity values.

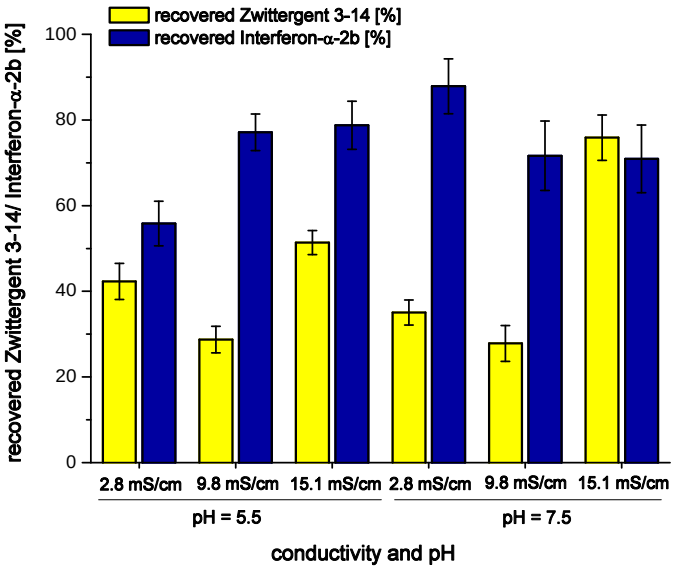

Fig. 9.11: Recovery of interferon-α-2b and Zwittergent 3-14 after treatment with hydroxyapatite resin as a function of pH and conductivity

Zwittergent 3-14 was quantified using RP-HPLC and RI detector as described in section 9.2.5 and total interferon-α-2b was quantified by SDS-PAGE as described in section 9.2.7. It can be seen in figure 9.11 that Zwittergent 3-14 and total interferon bound both to hydroxyapatite resin with different affinities at different pH and conductivity levels. However, the binding of Zwittergent 3-14 and interferon to hydroxyapatite, showed no direct correlation with conductivity or pH. This observation is unexpected, especially in regards to the observations made during the Sarkosyl removal from process samples discussed in section 9.3.2.1. In the presence of Sarkosyl, the binding

of interferon-α-2b to hydroxyapatite showed a strong dependence on the conductivity and pH of the process sample. This dependency of interferon cannot be seen with Zwittergent 3-14. This leads to the conclusion that the affinity of interferon-α-2b towards hydroxyapatite is influence by the detergent present in the sample. Accordingly, in the presence of Zwittergent 3-14 the recovery of interferon is improved compared to the presence of Sarkosyl, with a yield of 55 to 90%.
The highest removal of Zwittergent 3-14 was achieved at a conductivity of 9.8mS/cm independent on the pH with 70% removal. This yield is not sufficient and does not meet the target of at least 95% removal of Zwittergent 3-14. Accordingly this technique will not find application in the removal of Zwittergent 3-14 from process samples.
In summary, both resins Dowex and hydroxyapatite do not have the potential to remove Zwittergent 3-14 from process samples and reach the yield of 95% detergent removal, while recovering at least 60% of interferon-α-2b. Hence, different techniques other than ion-exchange need to be investigated.

9.3.3.2 Zwittergent removal using size-exclusion and affinity resin in spin columns

The 'Detergent Removal Resin' from Thermo Fisher is, as described in section 9.3.2.2, a combined technique of size-exclusion and affinity chromatography. The matrix allows only small molecules to permeate and come in contact with a specially developed detergent ligand, whereas larger molecules pass through the column without coming into contact with the ligand. The molecular weight cut-off of the matrix is 10kDa. An expected difficulty for the removal of Zwittergent 3-14 from process samples, is the average micelle size of Zwittergent 3-14, of 30kDa which is larger than the molecular weight cut-off of the 'Detergent Removal Resin' matrix.
The experiments with the 'Detergent Removal Resin' were performed with small spin columns as described in section 9.2.3. Figure 9.12 shows the recovery of interferon-α-2b and Zwittergent 3-14 in the flow-through of the spin columns packed with 'Detergent Removal Resin' as a function of pH and conductivity.

Zwittergent 3-14 was quantified using RP-HPLC and RI detector as described in section 9.2.5 and interferon-α-2b was quantified using SDS-PAGE as described in section 9.2.7. The results in figure 9.12 show that Zwittergent 3-14 could be removed from process samples with a yield of 80 - 20% depending on pH and conductivity. The initial concentration of Zwittergent 3-14 was 1.0%,

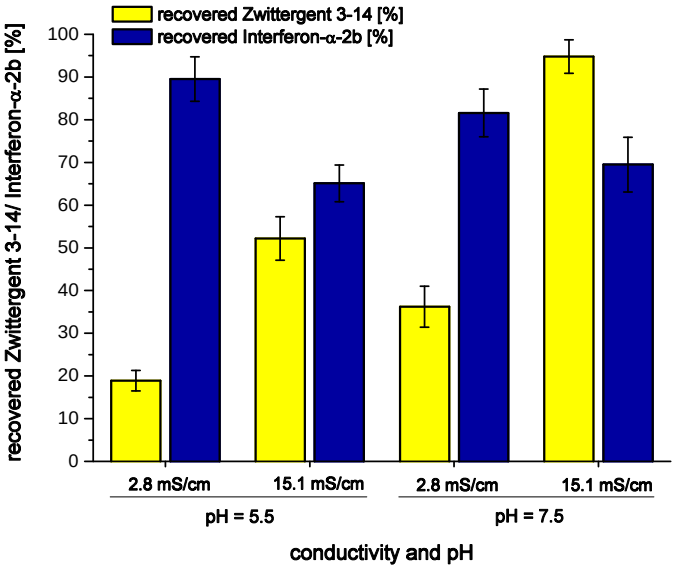

Fig. 9.12: Recovery of interferon and Zwittergent 3-14 in flow through of spin column packed with 'Detergent Removal Resin' at different pH and conductivity values

hence 0.8 - 0.2% of Zwittergent 3-14 concentration could be removed with the spin columns.

The results indicate that the binding of the detergent to the 'Detergent Removal Resin' is dependent on the pH and on conductivity of the process sample. With lower pH and lower conductivity the affinity of the detergent towards the resin increased, hence the removal of the detergent improved. This observation was unexpected, since the affinity of molecules to the resin is independent on the pH and conductivity. However, the micelle molecular weight (MMW) of the detergent is affected by the presence of salt-ions (section 8.1.1.1). With increasing ionic-strength, the MMW of the detergent is increasing as well. Hence, with increasing conductivity less detergent micelles can pass through the porose resin matrix and bind to the affinity resin.

The dependency on the pH and conductivity of the binding from the detergent to the resin was not detectable during the Sarkosyl removal from process samples discussed in section 9.3.2.2. All Sarkosyl bound completely to the 'Detergent Removal Resin' independent of pH and conductivity. The difference in affinity towards the resin of both detergents could be due to their different molecular weights and different detergent characteristics. The average micelle size of Sarkosyl is only 0.l6kDa whereas the average micelle size for Zwittergent 3-14 is 30kDa. The formation of micelles and their size is dependent on pH, conductivity and temperature (section 8.1.1.1). Hence

it is possible, that the affinity of Zwittergent 3-14 towards the resin is independent of pH and conductivity, but the micelle size of the detergent changes with different pH and conductivity values. Smaller micelles can more readily pass through the matrix pores than larger one. Accordingly, detergents with smaller micelles can be removed more efficiently with this 'Detergent Removal Resin' than detergents with larger micelles.

The recovery of interferon-α-2b is consistent and between 70 - 85% of the initial amount, independent of pH and conductivity. During the removal of Sarkosyl a slight dependency on pH of the resin affinity to interferon-α-2b was observed. This dependency cannot be seen throughout the Zwittergent 3-14 removal. The difference between the affinity of interferon-α-2b to the resin in the presence of Sarkosyl and Zwittergent 3-14 could be due to interactions between the detergent and the protein. Detergents and proteins do interact with each other in solution and this interaction is affected by pH (section 8.1.1.2), as well as the detergent characteristics. The interaction between Sarkosyl and interferon appears to be stronger and affected by pH more than the interaction between Zwittergent 3-14 and interferon. Hence, the recovery of interferon-α-2b is dependent on pH in the presence of Sarkosyl due to this interaction, but is not dependent on pH in the presence of Zwittergent 3-14.

In summary, with the 'Detergent Removal Resin' in spin columns more than 80% of the initial Zwittergent 3-14 concentration could be removed at low pH (5.5) and low conductivity (<2.8mS/cm), with a recovery of interferon-α-2b of 80%. The combination of size-exclusion and affinity resin showed a higher potential for the removal of Zwittergent 3-14 than the usage of ion-exchange resin on its own. However, removal of 80% of the initial detergent concentration is still not sufficient to find application at industrial scale. As mentioned in section 9.3.2.2, the scale up of this method for a large scale of 9'000L is impossible for the moment, since the 'Detergent Removal Resin' is only available in small amounts up to 100mL.

9.3.3.3 Zwittergent removal using Ultrafiltration/Diafiltration with spin tubes

The last technique tested for the removal of Zwittergent 3-14 from process samples is ultrafiltration/diafiltration which separates a mixture of molecules based on size depending on the molecular weight cut-off of the membrane. For the removal of Zwittergent 3-14 two different molecular weight cut-offs were tested, 10kDa and 5kDa. The experiments were performed as described in

section 9.2.4 using spin-tubes with different cut-offs to simulated the ultrafiltration/diafiltration process at lab-scale.

Figure 9.13 shows the recovered fraction of interferon-α-2b, total protein and Zwittergent 3-14 in the upper chamber (retentate) of the spin-tubes for a molecular weight cut-off of 10kDa.

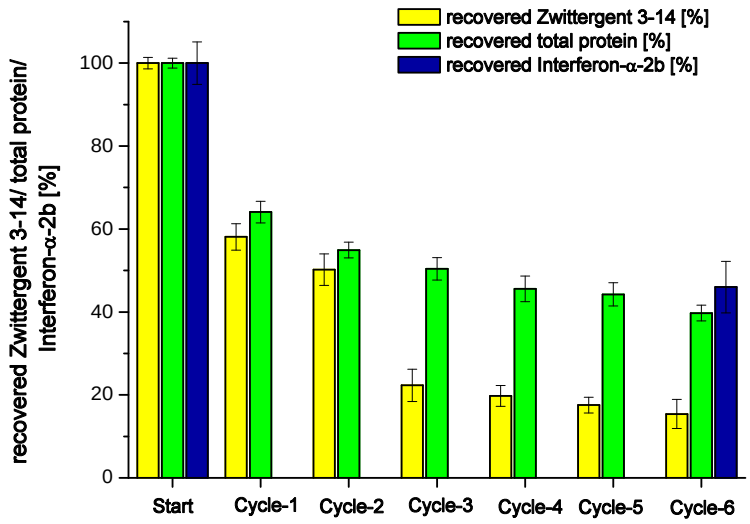

Fig. 9.13: Recovery of interferon, total protein and Zwittergent 3-14 in upper chamber (retentate) of spin-tubes with 10kDa cut-off (pH=7.0, C=10.0mS/cm): after each cycle the upper chamber was filled-up with extraction buffer and the centrifugation step repeated as before

Zwittergent 3-14 was quantified by RP-HPLC and RI detector as described in section 9.2.5 and total protein and total interferon-α-2b were quantified using the BCA-assay and SDS-PAGE as described in sections 9.2.6 and 9.2.7. The different cycles shown in figure 9.13 stand for the centrifugation cycles performed. As mentioned in section 9.3.2.3 for Sarkosyl removal, the volume in the upper chamber was topped-up with EDTA-Tris buffer (1mM EDTA, 20mM Tris, pH 8.0) after each cycle.

The results in figure 9.13 show that Zwittergent 3-14 can be removed from process samples using a spin column. After 6 cycles 80% of the initial Zwittergent 3-14 concentration had been removed. Unfortunately, the removal rate of the detergent was reduced in the last cycles (4 - 6) and almost stagnates, consequential it is unlikely that the addition of more cycles would improve Zwittergent 3-14 removal. The reason for this slow removal of Zwittergent 3-14 in the last cycles, is probably due to the large average micelle size (30kDa) of the detergent. During each centrifugation cycle

the volume in the upper chamber of the spin-tube decreases, hence, the concentration of detergent and/or proteins increases. At higher concentrations the formation of micelles of the detergent increased and the risk of these micelles fouling the membrane and blocking pores is enhanced. The fouling of the membrane by detergent micelles or other large molecules should be reduced in a UF/DF system, particularly using a TFF system. In a TFF ultrafiltration/diafiltration system a continuous flow is applied across the membrane which reduces membrane polarisation and fouling. Hence, the removal of Zwittergent 3-14 would be expected to improve in a TFF operated UF/DF system compared to a dead-end centrifugation system using spin-tubes.

The recovery of total protein and total interferon-α-2b decreases constantly throughout the different centrifugation cycles, with a recovery of 50% of the initial total protein and total interferon. The addition of more cycles would most likely lead to further loss in interferon-α-2b. Compared to the UF/DF results for Sarkosyl removal (section 9.3.2.3), where no interferon was found in the flow-through of the spin-tubes, all lost interferon could be detected in the flow-through for the removal of Zwittergent 3-14. This result is unexpected. Interferon-α-2b has a molecular weight of 19kDa, hence little should pass through the membrane pores with a cut-off of 10kDa. A reason for this unexpected observation could be that the g-force of the centrifugation cycles was too high creating a high transmembrane pressure and possible membrane distortion, consequently larger molecules were pulled through the pores. A reduction of the g-force would decrease this loss of interferon throughout the cycles, but would also reduce the removal of Zwittergent 3-14.

Another option to reduce the loss of interferon is the reduction of the pore size of the membrane. Thus same experiment was performed with spin-tubes with a cut-off of only 5kDa. Figure 9.14 shows the recovered fractions of Zwittergent 3-14, total protein and total interferon in the retentate of the spin-tubes with 5kDa cut-off.

It can be seen in figure 9.14 that the recovery of total protein and total interferon-α-2b throughout the 6 cycles increased compared to the spin-tubes with 10kDa cut-off. After 6 cycles more than 80% of total protein and total interferon was recovered, hence only 20% of the initial interferon was lost. However, it can also be seen that the removal of Zwittergent 3-14 is reduced and stagnates at 50% removal for the last 4 cycles. Thus, no improvement in the Zwittergent removal is expected due to addition of further cycles.

In summary, membranes with a cut-off of 10kDa show potential for the removal of Zwittergent

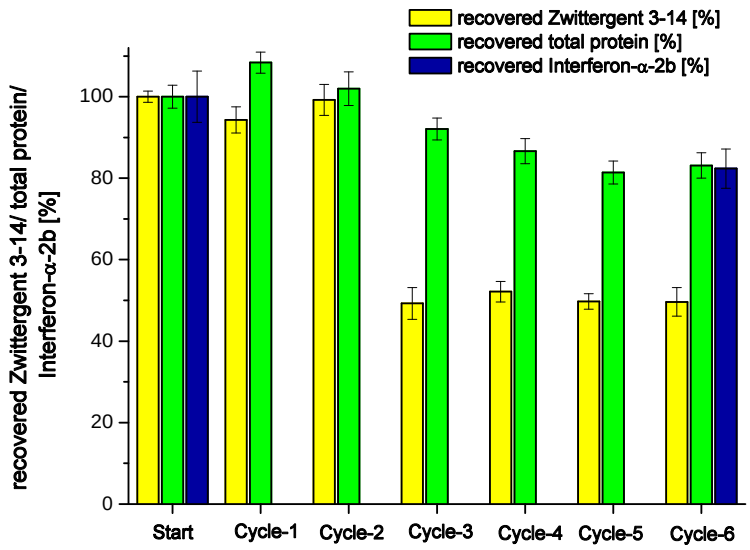

Fig. 9.14: Recovery of interferon, total protein and Zwittergent 3-14 in upper chamber (retentate) of spin-tubes with 5kDa cut-off (pH; 7.0, C; 10.0mS/cm): after each cycle the upper chamber was filled with extraction buffer and the centrifugation step repeated as before

with a yield of 80% while recovering 50% of interferon-α-2b. The removal of detergent and the recovery of interferon is likely to be improved using an ultrafiltration/diafiltration system and a selection of a suitable value for g-force or pressure. On the other hand membranes with only 5kDa cut-off did not show significant potential for the removal of Zwittergent 3-14 since only 50% of the initial Zwittergent concentration could be removed.

9.3.4 Theoretical scale-up of detergent removal methods

Of the studied methods the most promising one for detergent removal was ultrafiltration/diafiltration using spin-tubes. At large-scale a continuous TFF operated UF/DF system would be used. As described in section 9.1.2.2 several parameters are important to scale-up an ultrafiltration method. The most important parameters for the selection of the correct membrane-size are: filtrate volume [L], flux or flow rate [$L*m^{-2}*h^{-1}$], process time [h], protein concentration [g/L] and membrane type. With this information the required membrane area can be calculated using equation 9.2.
For the application of a UF/DF system for the removal of detergents from the protein recovery process, the following parameters were assumed:

filtrate volume:	9'000 L
protein concentration:	5.0 g/L
membrane type:	regenerated cellulose
flux:	150 L/m^2/h
process time:	4 hours

With a low protein concentration of 5 g/L higher filtration flux rates are required. The flux decreases with an increase in protein concentration. Using the above parameters the required membrane area can be calculated as follows using equation 9.4:

$$\begin{aligned} area\left[m^2\right] &= filtrate\,volume\,[L] \div \left(Flux\left[L * m^{-2} * h^{-1}\right] * process\,time\,[h]\right) \\ &= 9'000\,[L] \div \left(150\left[L * m^{-2} * h^{-1}\right] * 4\,[h]\right) \\ &= 15\left[m^2\right] \end{aligned} \tag{9.4}$$

In order to have a safety margin for process time and filtrate volume an extra 20% is included for the membrane area calculation. With the 20% safety margin a 18 m^2 membrane area is required to process 9'000L in 4 hours with an initial protein concentration of 5.0g/L. The regenerated cellulose membrane has been chosen due to its very low protein binding characteristics (section 9.1.2.2).
The next step is the calculation of required feeding buffer and the trans-membrane pressure (TMP). The TMP is dependent on the protein concentration and on the flux. With low protein concentration and high flux rates, a high trans-membrane pressure is required of around 30 psi. The required feeding buffer volume is dependent on the necessary diafiltration volume (DV). One DV is processed when the filtrate volume collected equals the starting retentate volume. Results from the experiments with the spin-tubes showed that 6-10 cycles were required to remove the detergents up to 95%. One cycle corresponds to $\frac{1}{2}$ DV, meaning 3-5 diafiltration volumes are required. With one DV corresponding to 9'000L a feeding buffer volume between 27'000 and 45'000L will be required to remove the detergents from process samples.
Since the continuous UF/DF is expected to be much more efficient than a dead-end filtration system with spin tubes, these calculations should represent the worst-case scenario.

9.4 Conclusion

In this chapter 3 different techniques were investigated for the removal of two different detergents, Sarkosyl and Zwittergent 3-14, from process samples. The techniques were: ion-exchange resin, size exclusion with affinity resin in combination and ultrafiltration/diafiltration.

The first two sections of this chapter focused on the quantification of the detergent Sarkosyl and Zwittergent 3-14. Results have shown that RP-HPLC with UV detector can be used to quantify Sarkosyl in process samples in the presence of different proteins. Since Zwittergent 3-14 does not absorb UV light, a different method was required for its quantification. RP-HPLC with RI detector showed good and accurate results for the quantification of Zwittergent 3-14. With these two methods it was possible to quantify Sarkosyl and Zwittergent 3-14 in the process samples.

The next sections focused strictly on the removal of the different detergents. All results are summarized and conclusion are drawn in the following two sections for the removal of Sarkosyl and the removal of Zwittergent 3-14.

9.4.1 Removal of Sarkosyl

Five different techniques were tested for their potential to remove Sarkosyl from process samples while recovering most of the interferon-α-2b. The first method was an ion-exchange method with a Dowex resin, which successfully binds Sarkosyl but also interferon-α-2b. The conductivity of the process sample has a significant impact on the binding affinity of Sarkosyl and interferon towards the resin. A conductivity of 10mS/cm was shown to represent a reasonable compromise in order to remove more than 97% of the initial Sarkosyl concentration while recovering 40% of the interferon.

The next method tested was again an ion-exchange method with a mixed mode resin, hydroxyapatite. Data showed that Sarkosyl and interferon-α-2b bind to this resin depending on the conductivity and pH of the process sample. The best working conditions were found at a pH of 7.5 with a conductivity of 10mS/cm. Under these conditions 75% of the initial Sarkosyl concentration could be removed while keeping more than 95% of interferon-α-2b in solution.

The third method tested was a combination of size exclusion with affinity resin in spin columns. Results show that Sarkosyl can be removed successfully by more than 98% of the initial concen-

tration while recovering over 75% of interferon-α-2b. No significant impact of the conductivity was observed and only a minor impact of the pH could be detected. Hence, performing the method at a neutral pH is beneficial for the interferon recovery.
The fourth and fifth techniques were ultrafiltration/diafiltration methods performed with spin-tubes and two different cut-offs. A membrane with 10kDa pore size showed high potential for the successful removal of Sarkosyl from process samples. Up to 80 - 95% of the initial Sarkosyl concentration was removed from process samples, while recovering between 80 - 60% of interferon-α-2b. The use of membranes with smaller pore size (5kDa) resulted in a slight improve in protein recovery but only 60% removal of Sarkosyl.

9.4.2 Removal of Zwittergent 3-14

The same five techniques tested for Sarkosyl removal were also tested for Zwittergent 3-14 removal from process samples. The ion-exchange method with Dowex-resin showed a dependency of pH and conductivity on the binding of Zwittergent and interferon to the resin. The best compromise was found to be at a pH of 7.5 with a conductivity of 10.0mS/cm which resulted in 70% removal of the initial Zwittergent 3-14 concentration while recovering 50% of interferon-α-2b.
The second ion-exchange method was performed with the mixed-mode resin hydroxyapatite. Neither the Zwittergent 3-14 nor interferon binding to hydroxyapatite showed an obvious impact on the pH or conductivity values. However, the optimum working conditions were observed at a conductivity of 10.0mS/cm, independent on the pH. At these conditions 70% of the initial Zwittergent 3-14 concentration was removed while keeping 75% of interferon-α-2b in solution.
The combination of size exclusion and affinity resin was studied for Zwittergent 3-14 removal as the third method. No effect of pH or conductivity was observed on the binding affinity of interferon. However, the binding affinity of Zwittergent towards the resin was dependent on the pH and the conductivity. Hence, optimal working conditions were observed at a pH of 5.5 and a conductivity value of 2.8mS/cm. Under these conditions 80% of the initial Zwittergent 3-14 concentration was removed while recovering 80% of interferon-α-2b.
The last two techniques tested for Zwittergent 3-14 removal were ultrafiltration/diafiltration methods performed with spin-tubes and two different cut-off sizes. With a 10kDa cut-off 80% of the

initial Zwittergent 3-14 concentration was removed while 50% of interferon-α-2b was recovered. The addition of further cycles might improve the removal of Zwittergent even further but also reduce the recovery of interferon. The use of a smaller cut-off membranes (5kDa) did not show any beneficial results. The recovery of interferon was improved to 80% but only 50% of the initial Zwittergent concentration could be removed.

9.4.3 Overall conclusion and outlook

Comparing all five methods with each other, the most promising method for successful removal of the detergent while recovering as much interferon as possible is the combined technique of size exclusion with affinity resin. Unfortunately this method finds its application only at small-scale up to 100mL and is not suitable for large scale application up to 9'000L. Hence, an alternative method is the ultrafiltration/diafiltration method using spin-tubes with a cut-off of 10kDa. Up to 80 - 95% of the initial Sarkosyl concentration could be removed while recovering between 80 - 60% of interferon. Also for Zwittergent 3-14 more than 80% of the initial concentration was successfully removed while recovering 50% of interferon-α-2b. The application of this technique with a TFF operated UF/DF system at large-scale should even improve the yield for both detergents removal as well as the recovery of interferon-α-2b.
In summary the different studies showed that the removal of Zwittergent 3-14 from process samples is more challenging than the removal of Sarkosyl. The reason is the average micelle size of the two detergents, which is much smaller for Sarkosyl than for Zwittergent 3-14.

A next step will be to look at the scale-up of the ultrafiltration/diafiltration method with a TFF operated UF/DF system to test the theoretical estimated parameters of membrane size, pressure and flow-rate for accuracy and functionality. It also needs to be tested what impact the new protein recovery process samples, containing small amounts of detergents, have on the down-stream process. The impact of different concentrations of both detergents on the first step of the down-stream process will be investigated and discussed in chapter 10.

Chapter 10

Impact of Optimized Recovery Process on Down-Stream Process

10.1 Introduction

Chapter 8 focused on the application of two detergents, Sarkosyl and Zwittergent 3-14, throughout the primary protein recovery process to improve the interferon-α-2b solubility and overall recovery. In chapter 9 several different techniques were tested to remove the detergents from process samples to a yield of 95%. This chapter will now focus on the impact of these two detergents on the down-stream process. Therefore the first step of the down-stream process of interferon-α-2b, a cation-exchange chromatography method, will be characterized for its ability to purify interferon from host cell proteins. In a second step different concentrations of the two detergents will be investigated for interference with IFN purification using the IEC method. The different detergent concentrations derived from addition during the primary recovery process in chapter 8 and the remaining concentration after the detergent removal study from chapter 9.

10.1.1 Down-stream process for interferon-α-2b

The down-stream process for the production of the drug substance interferon-α-2b follows the primary protein recovery process and consists of several purification steps, involving extraction, conversion, three chromatographic steps, crystallization and ultrafiltration techniques. The last

sample of the primary protein recovery process (compare figure 2.2 in chapter 2), the TCA-pellet, is extracted and injected onto an ion-exchange column for purification and concentration of interferon-α-2b. The first down-stream step is followed by a conversion of the IFN isoforms and an ultrafiltration step for further concentration of the protein.

The remaining steps of the down-stream process are of less interest, since the focus in this chapter is mainly on the first down-stream process, ion-exchange chromatography which is explained in more detail in the following section.

10.1.1.1 Ion-exchange chromatography with SP Sepharose column

The first step in the down-stream process of interferon-α-2b is an ion-exchange chromatography. In order to process the last sample of the primary protein recovery process, the TCA-pellet, IFN and all related isoforms are extracted from the pellet by resolubilizing the pellet in an extraction buffer (375mM Tris, 19mM EDTA at pH 8.0). After solubilisation for 120 minutes the pH is adjusted to 5.45 with acetic acid and the solution is filtered through a 0.5μm filter. The extraction of the TCA-pellet results in approximately 3'100 l which is the feed volume for the ion-exchange method.

The ion-exchange chromatography step is a cation-exchanger with a 75l SP Sepharose fast flow (FF) column, which objective is to separate interferon and its isoforms form host cell proteins (HCP). The column is equilibrated with the equilibration and wash buffer, 10mM sodium acetate at pH 5.5 followed by feed injection of the extracted TCA-pellet (3'100l) at a constant flow rate of 4500ml/min and a controlled temperature between 2-8°C. After a wash step the interferon is eluted from the column with a gradient from 0.0M NaCl to 0.42M NaCl over 4 hours at 4500ml/min flow rate and a pH of 5.5. Only 20% of the elution volume is pooled together for further purification steps ($\approx$ 220l) which is dependent on the highest UV-280 reading. After elution the column is regenerated in 0.5M NaOH and 1M NaCl for further purifications.

The 3'100l feed volume containing interferon-α-2b and its isoforms from the TCA-pellet are eluted into 1'080l elution volume of which only 220l contain interferon and its isoforms. This means that through the cation-exchange method the process volume is reduced 14 fold from 3'100l down to 220l for further purification.

10.1.2 Cation-exchange chromatography

The purification of recombinant proteins from multicomponent protein mixtures throughout the down-stream process involves usually a series of different types of liquid chromatography which includes size exclusion, ion-exchange, adsorption and affinity chromatographic processes [138]. Ion-exchange chromatography (IEC) is the most common and widely used liquid chromatography technique in industrial purification due to its large sample-handling capacity, broad applicability and ease of scale-up and automation abilities [78].

IEC separates multicomponent protein mixtures primarily through electrostatic interactions between the charged amino acid side chains and the surface charge on the ion-exchange resin [139]. Proteins have ionisable chemical fragments which positive or negative charge enhancement is a function of the proteins pI and the surrounding pH [140]. As described in section 6.1.4.1 the pI (isoelectric point) of the protein is the pH at which the net charge on the protein is zero. If the pH of a solution is below the pI of the target protein, the net charge on the protein becomes positively charged and the protein will bind to a cation-exchange resin and vice versa [138].

The cation- or anion-resins exist with several different chemical properties such as matrix composition, pH and pressure stability, density of charged groups and ligand chemistry [141]. An important aspect for a successful separation of protein mixtures by ion-exchange chromatography is the selection of an optimal ion-exchange resin which is mainly based on the desired resin charge and strength and the sample properties. Various resins are available with positive and negative functional groups as strong and weak ion-exchangers. Strong ion-exchangers are fully ionized over a broad pH range, whereas weak ion-exchanger are partially ionized over a narrow pH range and thus their charge can vary significantly with the pH. Weak ion-exchange resins are commonly more flexible in selectivity and require milder elution conditions than strong ion-exchange resins [78][142]. Sulfopropyl (SP) functional groups are strong cation-exchangers (exchange capacity: -0.22mmol/ml)[143] and are conventional resins in the use of protein purification [141]. SP-Sepharose cation-exchange resins are widely used in combination with sodium acetate buffers at pH levels between 5.5 to 6.0, due to the pKa value of sodium acetate of 4.75 (calculated with the Henderson Hasselbalch equation [144]). The buffer strength should be minimal at a concentration of 10mM, in order to provide sufficient buffer capacity (10mM sodium acetate with 8.6mM Na-acetate and 1.4mM acetic acid results in a pH of 5.53). However, buffers with a high buffering

strength (50 - 100mM) can also interfere with the binding of proteins to the resin and reduce the binding capacity [78].

The binding affinity of proteins towards the resin is mainly dependent on the chemical and physical properties of the protein itself. However, other factors can affect the protein binding as well, which are conductivity, temperature, flow-rate and the dynamic binding capacity of the column. If the ionic strength of the protein sample or the loading buffer is too high, salt ions bind to the binding sites of the column resin and decrease the binding capacity of the column [78]. The impact of temperature on the protein binding affinity is less significant and hence, ion-exchange chromatography is often carried out at room temperature. However, temperature can have an impact on the protein structure and thus on the protein binding to the resin. Some studies have shown that the protein binding to the column resin is improved at lower temperature (10°C) [145].

The flow-rate is directly correlated to the contact or residence time between protein and resin. With an increase in the flow-rate the residence time is decreasing. In general, the protein binding is decreased with an increase in the flow-rate. However, low flow-rates lead to an increase in the elution volume and thus to protein dilution [146]. The binding capacity of a column has also a significant impact on the protein binding. If the column is overloaded no additional protein binding can occur. The dynamic binding capacity of a column can be characterized by the performance of breakthrough curves and calculated using the following equation:

$$dynamic\,binding\,capacity \quad = C_0 * (V_L - V_0) \tag{10.1}$$

With:

V_L = volume loaded up to the breakthrough point

V_0 = void volume of the column

C_0 = concentration of protein in the feedstock

The shape of the breakthrough curve provides information about the column efficiency, as the sharper the curve is the higher is the column efficiency [146].

The elution condition and format varies from protein to protein and is dependent on protein stability. More detailed information is described in the following section.

10.1.2.1 Gradient versus isocratic elution

The final step during chromatography is the elution of the protein from the column. In ion-exchange chromatography the protein elution can be achieved by varying either the pH or the ionic strength of the elution buffer. Most commonly used is elution by changing the ionic strength. Ion-exchange columns are highly dependent on salt concentration. Hence, a small change in the ionic strength will have a large effect on the retention of the protein [147]. Zhang et al. [148] have shown that the pH elution can provide better resolution than the salt elution. However, using a pH elution provides higher risks of protein precipitation at the proteins pI. On the other hand, an increased salt concentration in the elution fraction can interfere with further down-stream steps. Hence, it is dependent on the protein properties and the down-stream process itself which of the two elution conditions is more favourable.

Besides the elution conditions, the elution format needs to be selected as well. Two main different elution formats are commonly used: isocratic elution and gradient elution. Isocratic elution uses only one elution buffer at a constant ionic strength and pH value. This method is less complex, easy to develop and easy to transfer from one system to another. However, the isocratic elution is usually not useful to separate multi-component samples. In this case a gradient method is much more beneficial, which provides a higher peak capacity [149]. Throughout the gradient elution either the pH or the ionic strength is varied in a continuous or discontinuous (stepwise) approach [78].

10.1.2.2 Interference of host cell proteins

A challenge in recombinant protein production with microbial cells, especially in hosts such as *E.coli*, is the purification of the recombinant protein from host cell proteins. Cation-exchange chromatography is a common technique widely used to remove host cell proteins from the fermentation broth [150]. However, host cell proteins are also known to interfere with the purification of recombinant proteins due to interaction with the stationary phase or protein-protein interactions. Especially the purification of small recombinant proteins with a low molecular weight can be affected by the presence of host cell proteins.

10.1.2.3 Interferences of detergents

Detergents are amphiphilic molecules as described in section 8.1.1.1 and they contain a hydrophilic head and a hydrophobic tail. They are widely used to increase protein solubility (8.1.1.2) and can be divided into four major groups: ionic detergents, bile salts, non-ionic detergents and zwitterionic detergents. Detergents of categorizes a and b are known to interfere with purifications with ion-exchange chromatography. For example anionic detergents, such as Sarkosyl, will bind to anion-exchange columns and thus interfering with the protein binding to the column. In cation-exchange chromatography, anionic detergents inhibit the binding of proteins to the resin by masking their negative charges [151]. Non-ionic and zwitterionic detergents have no net charge, thus they should not interfere with the purification by ion-exchange chromatography. Some studies show, that also non-ionic and zwitterionic detergents can partially mask the native charges of the proteins, which results in a decreased binding affinity of the protein towards the ion-exchange resin [151]. However, studies from Dencher et al. [152] showed an increased binding of the protein to the column in the presence of zwitterionic detergents.
The impact of detergents on ion-exchange chromatography can be dependent on several characteristics of the detergent, for example: cmc, micellar molecular weight, cloud point and UV-transparency. It was shown that detergents with high cmc are less suitable for the application in ion-exchange chromatography than detergents with lower critical micelles concentrations [153].

10.1.3 Goals and Aims

The aim of this chapter is the investigation of the impact of two detergents, Sarkosyl and Zwittergent 3-14, on the first down-stream step for interferon-α-2b, an ion-exchange chromatography method. This investigation is carried out in three main steps. First, the ion-exchange chromatography method is characterized for its interferon-α-2b recovery and certain parameters such as flow-rate, protein load and the presence of host cell proteins are investigated for their level of interferences throughout the purification. Second, the presence of the anionic detergent, Sarkosyl, during the purification is studied at different concentrations of the detergent to find the minimal acceptable detergent concentration at which interferences on the IEC method are negligible. In a last step the impact of the zwitter-ionic detergent, Zwittergent 3-14, is investigated on the ion-exchange

method and the recovery of interferon-α-2b. The IEC method is also studied for its potential to remove the detergent from process samples throughout the purification step for interferon-α-2b, in order to avoid interferences with further purification methods during the down-stream process.

10.2 Material and Methods

10.2.1 List of Materials

Tab. 10.1: Materials and suppliers

materials	supplier
Recovery process samples	industrial partner
Trizma Base	Sigma Aldrich
interferon-α-2b drug substance	industrial partner
EDTA tetrasodium salt	VWR
Sarkosyl	Sigma Aldrich
Zwittergent 3-14	Sigma Aldrich
Acetic Acid (glacial) 100%	VWR
Sodium Acetate	Merck
HiTrap SP HP or FF 5ml column	GE Healthcare
Sodium chloride	Merck
Pierce® BCA Protein Assay Kit	Thermo Scientific
Bovine serum albumin (BSA)	Sigma Aldrich

10.2.2 Ion-Exchange Chromatography - HiTrap cation exchanger

This method was developed by the industrial partner and protocols were applied to the systems available in the labs in DCU. Only minor changes had to be made, which are discussed in the result section.

Sample preparation

Samples used for the ion-exchange chromatography are called the TCA-pellet, which is the last sample of the protein recovery process. Ten gram of this pellet were dissolved in 100mL of 10mM acetic acid solution and homogenized for 10 minutes for an acid wash. After homogenization the solution was centrifuged at 4000rpm for 45min at 4°C with a FL40R centrifuge (Thermo

Scientific, Dublin, Ireland). The supernatant was discharged and the pellet was solubilized in 500mL of extraction buffer (20mM Tris, 1mM EDTA, pH 8.0) and homogenized for 20 minutes. pH was set to 8.0 with either 4M acetic acid or 1M Tris and agitated gently for 2 hours until all precipitants were solubilized. Next, conductivity was reduced below 2mS/cm with 5mM sodium acetate (pH 5.5) and pH was adjusted to 5.5 with 4M acetic acid. In a last step, the solution was re-centrifuged at 4000rpm for 15min at 4°C using a FL40R centrifuge (Thermo Scientific, Dublin, Ireland). The pellet was discharged and the supernatant was filtered through a 0.45μm PVDF filter (Millipore, USA).

Purification with IEC

Ion-exchange chromatography was performed with a cation exchange column (GE Healthcare, Buckinghamshire, UK), HiTrap SP HP or FF, with a 5mL column volume. The column is connected to a chromatography system AKTA Explorer (GE Healthcare, Buckinghamshire, UK) which controlled the salt gradient and solvent flow-rate. Two mobile phases were used for the separation: mobile phase A was 10mM sodium acetate at pH 5.5 and mobile phase B was 10mM sodium acetate, 0.7M sodium chloride at pH 5.5. The separation was performed at room temperature (no temperature control) and with a continuous flow rate of 5.0mL/min. During elution 3 parameters were monitored: UV at 280nm, conductivity and the gradient between mobile phase A and B.

Before sample injection, the HiTrap column was equilibrated with mobile phase A for 20 min at 5mL/min, followed by flushing the system (by-passing the column) with the sample solution for 6.0min. One hundred millilitre of the sample were injected onto the column at a flow-rate of 5.0mL/min and the flow through collected for further analysis. After sample injection the column was washed with 8 column volumes (40mL) of mobile phase A to remove all non-bound proteins from the column. The waste from this step was collected for further analysis and labelled as 'wash' sample. After the wash step the elution of the sample followed, which was done using two different operations:

First, a gradient method, mobile phase B was increased continuously from 0% to 60% in 18 minutes at a flow rate of 5.0mL/min. The eluent was collected using a fraction collector which collected a new fraction every minute. Alternative a second operation was used based on an iso-

cratic method. The ratio between mobile phase A and B was set to a fixed value and elution was performed at this ratio for a defined time with a flow-rate of 5mL/min. The specific ratio and duration of the elution was defined for each experiment individually.
Following elution the column was cleaned by stripping the column with 100% mobile phase B for 5min at 5ml/min. These fractions were collected for further analysis and labelled as 'strip' sample. In a last step the column was regenerated with 50mL of mobile phase B at 5.0ml/min before equilibration in preparation for the next sample. Each column could be used at least 5 times without losing resolution in the purification.
The complete methods for equilibration, sample load, column wash, elution and column regeneration can be found in the appendix B.

10.2.3 Detergent quantification

Sarkosyl and Zwittergent 3-14 were quantified following the methods described in section 9.2.5.

10.2.4 Total protein quantification

Total protein was quantified using the BCA assay as described in section 3.2.2 and table 3.5 or the Bradford assay as described in section 3.2.2 and table 3.6. Samples without a detergent present were analysed using the Bradford assay, whereas samples with a detergent present were analysed using the BCA-assay. Sample pellets were dissolved in EDTA-Tris buffer (20mM Tris, 1mM EDTA, pH 8.0) prior to protein analysis. BSA dissolved in water was used as a reference standard for all total protein determinations. The reagent blank was chosen according to the sample to be analysed, and included water, EDTA-Tris buffer or detergents.

10.2.5 Total Interferon-α-2b quantification

Total interferon-α-2b was quantified using SDS-PAGE as described in chapter 5.2.3 and 5.2.4. Sample pellets were dissolved in EDTA-Tris buffer (20mM Tris, 1mM EDTA, pH 8.0) prior to analysis as mentioned in the section above. No further sample treatment was required. The interferon-α-2b drug substance was used as a reference standard for all quantifications.

10.2.6 Interferon-Isoform quantification

Interferon-isoforms were quantified using the RP-HPLC method as described in section 6.2. Reference standards of interferon-α-2b were prepared in phosphate buffer as well as in phosphate buffer containing 3% Sarkosyl and 1% Zwittergent 3-14.

10.3 Results and Discussion

This result and discussion section is divided into three main parts: the first focuses on the characterization of the ion-exchange chromatographic method and the investigation of the interference of host cell proteins on the interferon-α-2b purification. The second and third parts concentrate on the impact of the two detergents, Sarkosyl and Zwittergent 3-14, on the purification of interferon-α-2b using the ion-exchange method.

10.3.1 Characterization of Ion-Exchange Chromatography with HiTrap column

The first step of the down-stream process for the industrial manufacture of interferon-α-2b is an ion-exchange-chromatographic step. As described in section 10.1.1.1, this cation exchanger binds the positive charged interferon-α-2b in order to purify it from host cell proteins. The TCA-pellet, derived from the primary protein recovery process, was therefore re-dissolved in an extraction buffer (375mM Tris, 19mM EDTA, pH 8.0), pH adjusted to 5.45, conductivity set below 2mS/cm and filtered through 0.45μm pore size. Four x 800L were loaded onto a 75L column (SP Sepharose Fast Flow) and 240L (7.5%) were collected for further purifications. Figure 10.1 shows a flow-chart of the cation-exchange chromatography method.

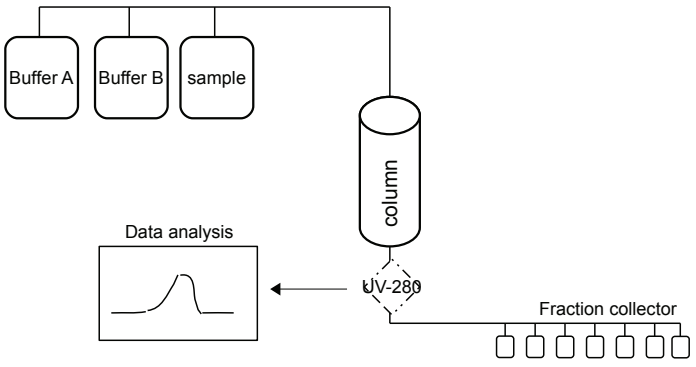

Fig. 10.1: Flow-chart of the ion-exchange chromatography method

This first DSP step was simulated at small scale using a 5mL HiTrap SP Sepharose HP or FF column. Sample preparations and chromatography were performed in a similar way to the manufac-

turing process as described in section 10.2.2. Figure 10.2 shows the chromatogram of a TCA-pellet derived from the manufacturing process and extracted at small scale in the labs in DCU.

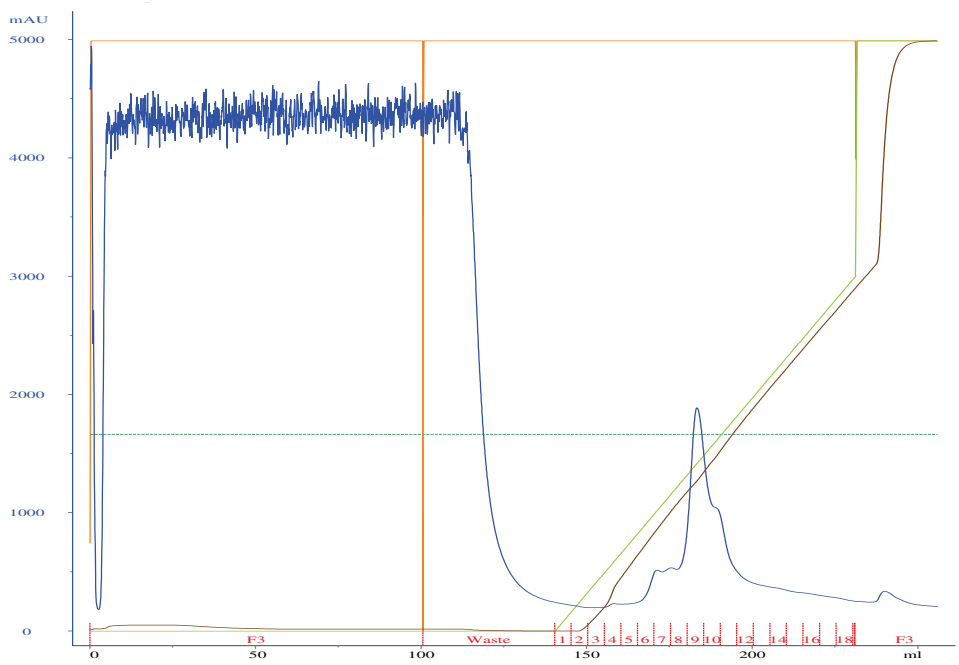

Fig. 10.2: AKTA chromatogram of purification of TCA-pellet: negative control

Figure 10.2 shows the UV reading at 280nm (blue), the conductivity reading (brown) and the gradient of mobile phase B (green) from the sample load, the wash step, the elution in 18 fractions and the stripping of the column. The loading of the sample corresponds to the first 100mL in the chromatogram with a UV-280 reading of around 4000mAU, followed by the wash step for 40mL with the UV-280 reading dropping to 200mAU. The third part, shown in the chromatogram in figure 10.2 is the elution of interferon-α-2b collected in 18 fractions each with 5mL volume. The UV-280 reading usually reaches its maximum at 1300 - 1500mAU in fraction 9 and drops back down to 300mAU in fraction 13 or 14. The last step is the stripping of the column with 25mL, which usually shows a small peak in the UV-280 reading with a maximum of 500mAU.

The selection criteria for the fractions to be pooled for further purification is the highest UV-280 reading ± 2 fractions on each side, which would mean fraction 7-11 with the maximum UV reading being in fraction 9. Some purification chromatograms showed a remote tailing in the elution peak which lead to the decision to take fractions 7-12 for further purification as a default setting. Beside this selection criteria all fractions, sample load, wash-step, elution-fractions and strip-sample,

were analysed for total protein using the Bradford assay and for total interferon-α-2b using SDS-PAGE. Fractions 7-12 from the elution were also analysed for interferon-α-2b and its isoforms with RP-HPLC.

The chromatogram in figure 10.2 and the quantified amounts of protein in each fraction are classified as the negative control sample for all further runs performed with this method.

Figure 10.3 shows total protein and total interferon-α-2b in the different collected fractions of the TCA-pellet purification using the ion-exchange method and HiTrap column. The results correspond to the chromatogram shown in figure 10.2.

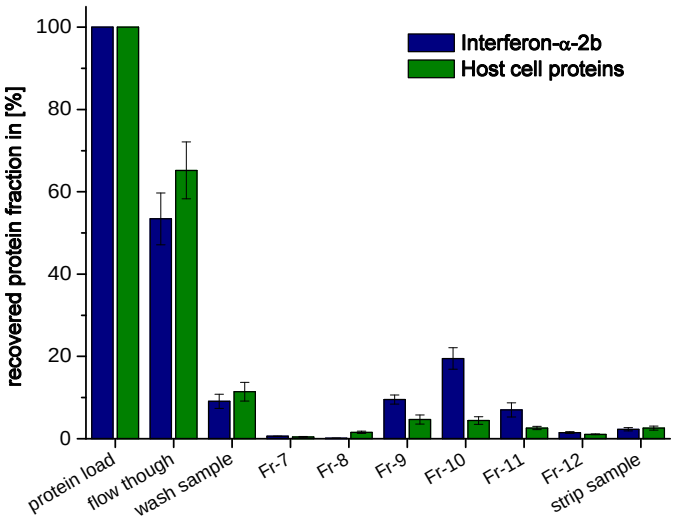

Fig. 10.3: Interferon-α-2b and total protein fractions quantified in samples of the purification of a TCA-pellet (negative control) with the HiTrap column

Total protein (in green) was quantified by the Bradford assay as described in section 10.2.4 and total interferon-α-2b (in blue) was quantified by SDS-PAGE as described in section 10.2.5. Figure 10.3 shows that 50% of the loaded interferon-α-2b was found in the flow through and does not bind to the column and around 10% were found in the wash- and strip-sample. This means that only 40% of the loaded interferon-α2b was found in the eluted fractions. Nearly all of these 40% are found in fractions 7-12, with a maximum of 20% in fraction 10. This low yield in IFN recovery is unexpected and leads to the conclusion that the column was overloaded. Roughly 40mg of interferon-α-2b was loaded onto the 5mL HiTrap column. The theoretical dynamic binding capacity of this column is 55mg ribonuclease/ml medium at 5ml/min, which would mean 275mg

ribonuclease for the 5ml column. Hence, it is very unlikely that the column is overloaded with 40mg interferon. Other reasons for low protein binding to the column are: high flow-rate, column temperature, binding-buffer or low binding affinity of the protein to the column. Each of these reasons was investigated and discussed in more detail later in this section.

Looking at the total protein in the different fractions of the TCA-pellet purification it can be seen that almost 70% of the loaded total protein was found in the flow-through, 10% in the wash- and strip-sample and only 20% in the eluted fractions. These findings are as expected, as the aim of this purification method was, to purify interferon from host cell proteins. Figure 10.3 also shows that the maximum of eluted total protein is in fraction 9 and not fraction 10 as for interferon-α-2b. The maximum in fraction 9 corresponds to the observed maximum in the UV-280 reading seen in figure 10.2. This indicates that the UV-280 in the chromatogram is mainly dictated by the elution of total protein and does not correspond to the interferon-α-2b elution. The shift between the elution of total protein and interferon also indicates that total protein is eluting earlier from the column at a lower salt concentration than interferon. Hence, the binding affinity towards the column should be less than the binding affinity of interferon.

Figure 10.3 shows only the recovered fractions of total protein and interferon, expressed as a percentage of the protein loaded. However, looking at the actual amount of total protein and interferon (shown in the first row of table 10.5) it can be seen that interferon represents only a very small fraction of the total proteins. Interferon-α-2b is less than 9% of the protein in the loaded sample and only 20% of the protein eluted from the column. This means that each eluted fraction (7-12) contains around 4-5 times more total protein (in mg) than interferon-α-2b. This also explains why the UV-280 reading of figure 10.2 corresponds more to the quantified total protein and less to interferon-α-2b.

Interferon-α-2b was also quantified using RP-HPLC as described in section 10.2.6. Fractions 7-12 were injected directly onto the RP-HPLC column and quantified over the peak-area from the UV-280 readings. The start sample of the TCA-pellet was first filtered through a 0.22μm filter and then directly injected on the RP-HPLC column without any pre-treatment. Due to the presence of several different host cell proteins in the TCA-pellet, the HPLC chromatogram shows over 60 small peaks in less than 20 minutes. This makes the quantification and selection of the correct peak very challenging and results have to be treated with caution. The quantification of

the TCA-pellet resulted in 17.16mg total interferon-α2b, meaning all isoforms were measured together. This is only half the amount determined by SDS-PAGE (35.86mg). The total interferon-α-2b in fraction 7-12 quantified by RP-HPLC resulted in 4.8mg, compared to 14.5mg quantified by SDS-PAGE. The comparison between results obtained by SDS-PAGE and RP-HPLC indicates an overestimation of interferon quantification using SDS-PAGE, a result similar to that seen and discussed in chapter 5 and 6. However, the proportion between interferon load and recovery is comparable. Results from the RP-HPLC show a 30% recovery of interferon, and SDS-PAGE results show a 40% interferon recovery. It is likely that the amount of interferon in the TCA-pellet was overestimated by RP-HPLC due to interference by host cell proteins. Thus, with less interferon load the recovered portion in fractions 7-12 would be higher than 30%. Hence both quantification methods give similar results for the proportion between interferon load and recovery, consequently all further analysis for IFN will be done by SDS-PAGE quantification.

Both interferon quantification methods indicated a loss of 60% of interferon-α-2b during the purification step with the ion-exchange Sepharose column. Most of the lost interferon was found in the flow through, thus it did not bind to the column. In order to investigate the low IFN recovery during this step, several factors were examined. First, two factors to be investigated were overloading of the column and the flow-rate. Is the flow rate too high during protein loading, some proteins may flushed through the column without giving them enough time to bind to the column. Hence, protein load and flow-rate during protein load were reduced by 50% for a purification run. The third factor is the column temperature. At manufacturing scale this ion-exchange method is performed at 4°C. During small scale performance the column temperature was uncontrolled and therefore at room temperature. As described in section 10.1.2, lower temperatures can be beneficial for protein binding to the column. Due to difficulties in the implementation of setting-up the whole AKTA system in a cold room, only the column was put on ice during the purification and both mobile phases were kept refrigerated until use. The fourth factor investigated was the salt concentration of the binding buffer (mobile phase A). If the salt concentration is too high it can interfere with the binding of proteins to the column. Hence, the sodium acetate concentration was reduced from 10mM to 5mM for one purification run. The last factor investigated was a low binding affinity of interferon-α-2b towards the Sepharose column. Thus, the same sample was loaded twice onto the same column. The flow-through of the first sample load was recovered and directly

loaded onto the column a second time without performing the elution or wash-step in between. All results on the impact of the five described factors on the interferon recovery during the ion-exchange method are gathered together in table 10.2. The table shows measured amounts for total protein and total interferon for sample load, flow through, wash- and strip-sample and the elution fractions 7-12 in mg and in percentage in relation to the sample load. It also shows the proportion factor between total protein and interferon in the starting sample.

All experiments were performed with the TCA-pellet from the manufacturing process and sample preparation was performed as described in section 10.2.2. Total protein was quantified using the Bradford assay as described in section 10.2.4 and total interferon was quantified using the SDS-PAGE as described in section 10.2.5.

The first row in table 10.2 shows the results of the negative control from the TCA-pellet purification following SOP instructions from the industrial partner as described in section 10.2.2. These are also the same results shown in figure 10.2 and 10.3. All other results will be compared and assessed in relation to this negative control.

The second row shows the results after reducing the protein load onto the column. Instead of 35mg of interferon-α-2b only 12mg were loaded onto the column at the same flow rate to investigate a potential overload of the column. As can be seen in the last column of table 10.2, the reduction of the protein load showed no significant impact on the interferon recovery in fractions 7-12 of the column-elution. The recovery was even slightly lower with 34.33% of the loaded interferon compared to 40% recovery in the negative control. The reduced IFN recovery indicates a low binding affinity of interferon towards the column or an increased interference of other molecules. With a reduced protein load, less interferon molecules are available to interact with the binding sites of the column.

The next row lists the results for a reduced flow-rate of 2.5ml/min during the protein load. Fifty percent of the loaded interferon were recovered in elution fractions 7-12 which is slightly higher than the control run. Hence, it can be assumed that the reduction of the flow-rate during protein load results in improvement of the recovery of interferon from 40 to 50%. However, more than 35 - 40% of interferon was still detected in the flow-through and did not bind to the column. Hence, other factors must interfere with the binding between interferon and Sepharose column.

Tab. 10.2: Characterisation of Ion-Exchange Chromatography - 1st DSP step

	protein load - START			**protein amount in WASTE**				**recovered protein**	
sample description	protein load total IFN	protein load total protein	total protein/ IFN fraction	flow-through total IFN	flow-through total protein	wash + strip total IFN	wash + strip total protein	**fractions: 7 - 12 total IFN**	fractions: 7 - 12 total protein
negative control in [%]	35.86mg (100%)	421.7mg (100%)	11.7	16.1mg (44.8%)	253.5mg (60.1%)	4.9mg (13.9%)	54.7mg (12.0%)	**14.5mg (40.36%)**	76.19mg (18.1%)
1/2 protein load in [%]	12.20mg (100%)			6.25mg (51.2%)		2.03mg (16.7%)		**4.19mg (34.33%)**	
flow-rate 2.5mL/min in [%]	24.59mg (100%)			9.16mg (37.27%)		4.23mg (17.2%)		**12.31mg (50.06%)**	
cold column temperature in [%]	29.78mg (100%)	368.6mg (100%)	12.4	17.89mg (60.07%)	237.56mg (64.45%)	3.58mg (12.02%)	51.02mg (13.84%)	**10.15mg (34.08%)**	76.53mg (20.76%)
mobile phase A reduced salt in [%]	21.64mg (100%)	312.74mg (100%)	14.4	9.65mg (44.58%)	193.63mg (61.92%)	3.41mg (15.75%)	36.35mg (14.82%)	**7.82mg (36.12%)**	43.7mg (13.97%)
re-load flow through (FT-1) in[%]	25.77mg (100%)	306.66mg (100%)	11.9	10.93mg (42.41%)	181.23mg (59.1%)				
FT-1 re-loaded in[%]	10.93mg (100%)	181.23mg (100%)	16.58	12.13mg (47.08%)	194.42mg (63.4%)	2.69mg (10.44%)	34.04mg (11.1%)	**10.15mg (39.39%)**	53.66mg (17.5%)
purified sample in [%]	11.10mg (100%)	28.88mg (100%)	2.6	0.04mg (0.38%)	1.81mg (6.3%)	0.02mg (0.2%)	1.0mg (3.4%)	**10.12mg (91.2%)**	25.07mg (86.8%)
purif. sample + 50% HCP in [%]	23.60mg (100%)	235.03mg (100%)	9.96	7.53mg (31.9%)	120.38mg (51.22%)	2.90mg (12.3%)	32.19mg (13.7%)	**12.96mg (54.9%)**	60.84mg (28.9%)

In row 4 of table 10.2 the impact of a reduced column temperature is shown. Results show no significant difference compared to the control sample. However, it has to be kept in mind that the temperature was not controlled nor monitored, meaning the actual column temperature during the purification is unknown. It is possible that controlling the column and buffer temperature at 4°C has a more significant positive impact on the IFN recovery. However, a 80% IFN recovery simply through controlling the column temperature at 4°C is unlikely, since the cooling of the column and mobile phases showed no positive impact at all.

The next factor investigated was a reduced salt concentration in the binding buffer, as seen in row 5. No significant impact on the interferon-α-2b recovery was detected compared to the control run (36% to 40%). The original salt concentration of the binding buffer, 10mM sodium acetate, was already at the lower levels. If the salt concentration is too low the buffer loses its potential to control the pH and can have a negative effect on protein binding.

Row 6 of table 10.2 shows the results to investigate a potential low binding affinity of interferon to the column. The flow through was collected and directly re-loaded onto the column without any wash- or elution-step. Results show that due to re-loading of the flow-through no improvement in IFN recovery was achieved. Hence, no further IFN bound to the column after the first loading run. If interferon-α-2b has only a low binding affinity towards the column, more IFN should have bound to the column in the second loading run. Since the observation showed no additional IFN binding in the second loading run, it is very likely that other molecules present in the sample interfere with the binding of interferon to the column. Most likely these molecules are host cell proteins, which represent a high fraction of the total protein amount of the TCA-sample.

In order to investigate a potential interference of host cell proteins with the binding of interferon to the column, a purified sample was tested for its binding affinity to the column. The purified sample derived from collected elution fractions 7-12 from different runs of the TCA-pellet. Row 7 of table 10.2 shows the recovered interferon at the different stages during the ion-exchange method. More than 90% of the loaded interferon was recovered in the elution fractions 7-12. This result shows, if less host cell proteins are present in the sample (only 60% compared to 90% in control sample), the purification is more efficient and the recovery yield of interferon is over 90%. In a next step the purified sample was mixed 50:50 with the unpurified control sample to increase the amount of host cell protein in the starting sample. Row 8 of table 10.2 shows the results. The increase in

host cell proteins decreased the recovered interferon in elution fractions 7-12 from 90 to 55%. The interferon loss in the flow through did increase from 0.4 to 32% due to the increase in host cell proteins. These results confirm the assumption that host cell proteins interfere with the binding of interferon to the column and therefore reduce the recovery yield of IFN in the elution fractions. A next step will be to investigate how the host cell proteins interfere with the interferon-α-2b binding to the column. This will be discussed in more detail in the following section.

In summary the results of the first DSP step, an ion-exchange method, showed that interferon-α-2b was purified from host cell proteins from 8.5% purity to 19%. Unfortunately only 40% of the loaded interferon was recovered in the purified fractions and over 50% was found in the flow through of the column. Several different factors were investigated for their potential to improve the interferon recovery. Only a reduced flow-rate during protein load showed a small positive impact on the IFN recovery. The largest impact was shown to be the absence or a reduction in host cell protein in the starting sample. This led to the conclusion that host cell proteins interfere with the binding of interferon to the column, which will be investigated and discussed in more detail in the following section.

10.3.1.1 Interferences of host cell proteins with the interferon purification

The previous section showed that host cell proteins have a major impact on the binding of interferon to the ion-exchange column. In order to investigate how the host cell proteins interfere with the IFN binding, different potential scenarios of the interaction were gathered together. Figure 10.4 gives a schematic overview of 4 potential cases of how host cell proteins can interfere with interferon binding to the column. In all 4 cases, the black vertical line represents the ion-exchange column, the red v-shapes illustrate the binding sites on the column, the small blue figures represent interferon-α-2b and the large green figures are host cell proteins. In case one, some of the interferon binds to the column, whereas other interferon bind to host cell proteins which prevent them from binding to the column. In scenario two, host cell proteins interact with interferon molecules which are bound to the column. Hence, larger host cell proteins block potential binding sites for other interferon molecules. Case three describes the case where, due to the high levels of HCP, the host cell proteins bind directly to the column such that only some interferon attaches to the column through interactions with the host cell proteins. The last scenario 4, assumes that interferon and

host cell proteins both bind to the column, but larger host cell proteins block potential binding sites for other interferon, thereby reducing the capacity.

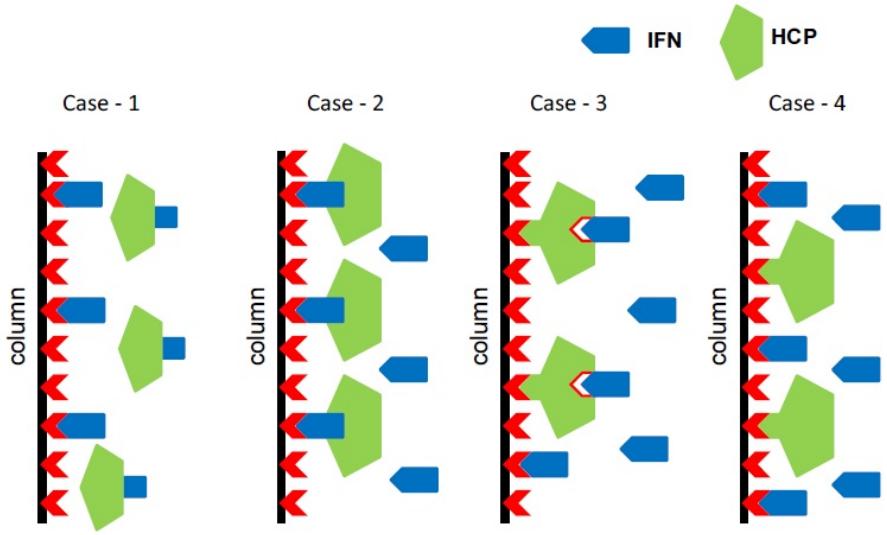

Fig. 10.4: Possible interferences of host cell proteins with the binding of interferon-α-2b to the ion-exchange column - 4 scenarios: 1.) some IFN bind to HCP and prevent binding of IFN to the column. 2.) HCP interacts with IFN bound to the column and blocks potential binding sites for other IFN molecules. 3.) HCP binds directly to the column and only some interferon can attach to the column through HCP. 4.) IFN and HCP bind both to the column, but larger HCP blocks potential binding sites for further interferon to bind, i.e. HCP reduces the column capacity.

In order to investigate which of the described scenarios occurs during the binding of interferon to the column, two different experiments were performed. The first experiment investigated whether scenario 2, 3 or both occur during HCP interactions. In both cases either host cell proteins or interferon bind to another protein through protein-protein interactions at the surface of the column. Detergents break-up protein-protein interactions, hence, a second wash-step was implemented following the original wash-step with binding buffer (10mM sodium acetate). The additional wash-step was performed with 1.0% Zwittergent 3-14 in 10mM sodium acetate. If protein-protein interactions occurred in the column, the unbound protein should be eluted with the detergent wash-step.

Figure 10.5 shows the AKTA chromatogram of the purification of a TCA-pellet with an additional wash step containing 1.0% Zwittergent 3-14 following the original wash step with 10mM sodium acetate.

The red circle in figure 10.5 shows a clear peak in the additional wash step, indicating that either

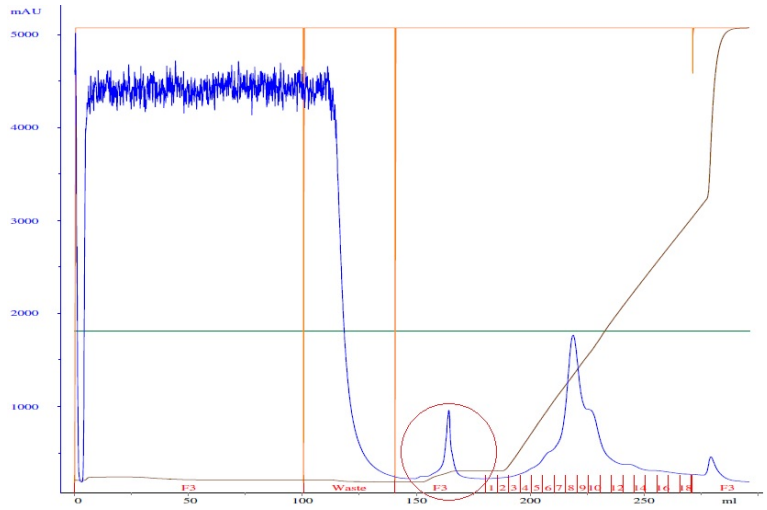

Fig. 10.5: AKTA chromatogram of sample purification with an additional wash step with detergent: red circle

host cell proteins or interferon were indeed attached to the column by protein-protein interactions. Analysing the additional wash step using the BCA assay for total protein and SDS-PAGE for total interferon-α-2b showed only host cell proteins were present in this sample and no interferon was detectable. This clearly shows that scenario 3 of figure 10.4 is negligible and case 2 is likely to be the prominent interference mechanism.

In order to examine if case 1 and 4 are existent or negligible one further experiment was performed. The recovered flow-through of a TCA-pellet purification was injected onto a fresh, regenerated column. If interferon molecules were not attached to any host cell proteins (case 4), these interferon molecules should bind to the fresh regenerated column. However, if interferon is attached to host cell proteins no further binding of interferon to the fresh column should occur.
Figure 10.6 shows the results for total protein and total interferon of the different steps throughout the ion-exchange purification. The graph on the left shows the results of purification run 1 and the graph on the right shows the results of purification run 2 using the flow through of run 1 as starting sample. Total protein was quantified using the Bradford assay as described in section 10.2.4 and total interferon-α-2b was quantified using the SDS-PAGE as described in section 10.2.5.
The left graph of figure 10.6 shows the TCA-pellet purification with standard operating conditions as described in section 10.2.2. The obtained results for total protein and total interferon are comparable to the negative control sample, with 50% of loaded interferon found in the flow-through

of the column and 38 - 40% of interferon recovered in the elution fractions 7-12. These results are as expected, if scenario one in figure 10.4 is the predominant mechanism for IFN binding interference.

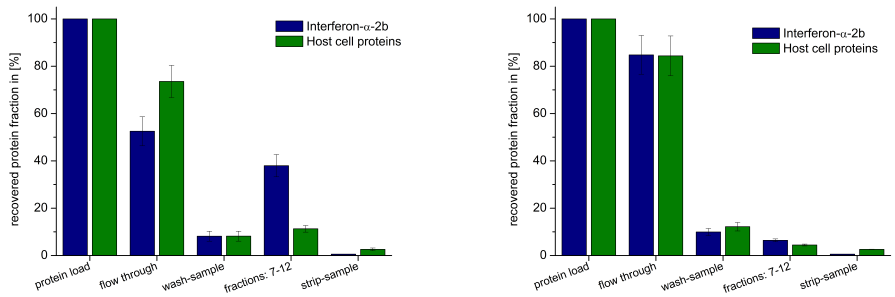

Fig. 10.6: Interferon-α-2b and total protein fractions quantified in samples of the purification of a TCA-pellet with the HiTrap column: left, purification of TCA-pellet; right, purification of flow through from previous purification

The graph on the right of figure 10.6 also shows a purification with standard operating conditions except that the starting sample is not TCA-pellet but the flow-through of the purification run 1 (graph to the left). It can be seen that the recovery of interferon decreased to less than 10%, and more than 80% of the loaded interferon was found in the flow-through, not binding to the column. These results lead to the conclusion that it is more likely that scenario 1 (from figure 10.4) occurs within the column and interferon and host cell proteins interact with each other. Depending on how the proteins interact, interferon can either still bind to the column (case 2) or the binding site of interferon to the column is blocked by host cell proteins and no binding occurs (case 1). Scenario 4 cannot be eliminated completely. Some host cell proteins will also bind directly to the column, since host cell proteins are also found in the purified elution fractions.

In summary, results have shown that the main interferences of host cell proteins with the binding of interferon to the column are due to interactions between interferon and host cell proteins as described in scenarios one and two in figure 10.4. Some interference will also result from host cell proteins blocking potential binding sites of interferon as described in case four. To avoid these interferences, protein-protein interactions need to be eliminated. This can be achieved by the addition of a detergent in the starting sample. On the other hand, the detergent itself can have a major impact on protein binding to the column itself.

However, it was not the primary focus of this study to optimize the ion-exchange purification step

but more to characterize it and to apply it to samples obtained from the recovery process simulation from chapter 8. These results can be found in the following section 10.3.2.
For all results obtained for the investigation of host cell protein interferences it needs to be kept in mind that interferon was always quantified by SDS-PAGE which separates proteins only by molecular weight. Hence, it is possible that some interferon detected in the starting or flow through samples are not interferon-α-2b but only host cell proteins with the same molecular weight or some isoforms of interferon with different characteristics. Even though RP-HPLC confirmed the findings of the SDS-PAGE results in the negative control sample at the beginning of this section, this fact cannot be completely neglected and has to be considered for further optimization steps of the purification method.

10.3.1.2 Isocratic elution of interferon-α-2b

The original SOP for the ion-exchange purification follows a gradient elution from 0 - 60% mobile phase B (elution buffer) in 18 minutes with a flow rate of 5ml/min (compare section 10.2.2). This corresponds to a salt concentration of 0 - 0.42M NaCl. It was shown in figure 10.2, that most proteins and interferon-α-2b elute after 9 - 10 minutes which corresponds to a salt concentration of 0.21M NaCl (30% mobile phase B). To simplify the method and to reduce the salt concentration in the recovered fraction, an isocratic approach was tested for the elution of interferon-α-2b from the column.
The TCA-pellet was prepared and the ion-exchange purification was performed as described in section 10.2.2, except for the elution-phase. After the wash-step with the binding buffer, the method was stopped and isocratic elution was conducted manually, by increasing the salt concentration step wise, starting at 0.147M NaCl (21% mobile phase B), which corresponds to elution fraction 6. The aim of this experiment was to find the lowest possible salt concentration for interferon-α-2b elution.
Figure 10.7 shows interferon-α-2b and total protein fractions throughout different steps of the ion-exchange purification with a step-wise isocratic elution at 21%, 25%, 28% and 31% of mobile phase B.

Total protein was quantified using the Bradford assay as described in section 10.2.4 and total interferon-α-2b was quantified using the SDS-PAGE as described in section 10.2.5. Figure 10.7

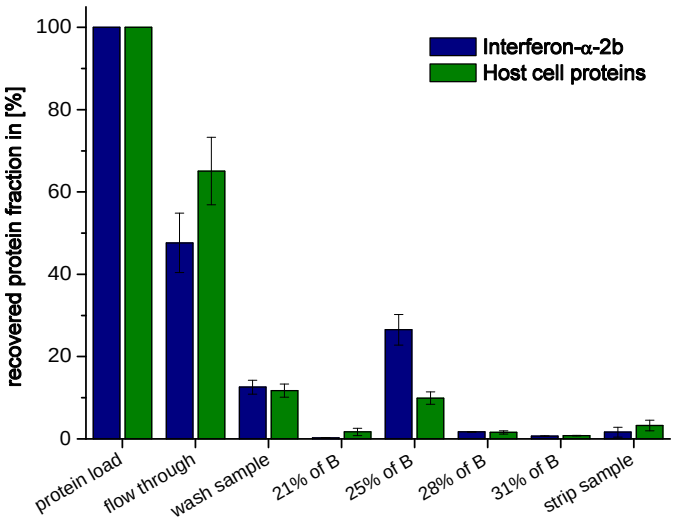

Fig. 10.7: Interferon-α-2b and total protein fractions quantified in samples of the purification of a TCA-pellet with the HiTrap column and isocratic elution: 25% of B represents 0.175M NaCl which corresponds to fraction 7 during the gradient elution

shows that the majority (>95%) of interferon-α-2b bound to the column elutes at a salt concentration of 0.175M NaCl (25% mobile phase B), which corresponds to elution fraction 7. The elution conditions for 25% mobile phase B were 5ml/min for 3.3min giving a collection volume of 16.5mL. No interferon was detected after the elution condition of 21% mobile phase B (0.147M NaCl - elution fraction 6) at 5ml/min for 5 minutes. And only a minor percentage of interferon was detected after the elution conditions of 28% mobile phase B (0.196M NaCl - elution fraction 8). These results indicate that all proteins elute at a similar salt concentration of 0.175M NaCl and no gradient elution is required for elution of interferon from the column. Hence, with isocratic elution at 25% mobile phase B (0.175M NaCl), all column bound interferon can be recovered in less than 20mL. Compared to the results obtained with gradient elution the recovered volume is reduced from 25mL to 20ml, meaning interferon is more concentrated, and the salt concentration in the recovered fraction is also reduced from 0.21M to 0.175M NaCl.

Looking at the recovery of interferon-α-2b under isocratic elution, no significant difference can be observed compared to the gradient elution. In the negative control, with gradient elution, 40% of the loaded interferon was recovered in elution fractions 7-12, 50% IFN was found in the flow through and 10% was detected in the wash- and strip-sample. Under isocratic elution 33% of

the loaded interferon-α-2b was recovered in the elution at 25% of mobile phase B, 50% IFN was found in the flow through and 11% was detected in the wash- and strip-sample. The slightly reduced amount of interferon recovered in the elution between gradient and isocratic is not significant. Given that the flow-through and wash/strip sample resulted in similar results, the difference between the recovered interferon in the elution fractions was almost certainly due to variations in the quantification method, namely SDS-PAGE.

In summary the isocratic elution of interferon-α-2b at 25% mobile phase B (0.175M NaCl) is an efficient and simplified alternative to the gradient elution method. Interferon-α-2b will be more concentrated due to a smaller elution volume and the salt concentration in the recovered elution fraction will also be reduced compared to the gradient elution fraction. In the event of further optimization studies of this ion-exchange purification method, the implementation of an isocratic elution should be considered.

10.3.2 Impact of detergents on Ion-Exchange Chromatography step

This section discusses the impact of the two different detergents, Sarkosyl and Zwittergent 3-14, on the ion-exchange purification, which is the first DSP-step following the primary protein recovery process. Both detergents were used during the recovery process to improve protein solubility (chapter 8), hence the impact of their presence in the final TCA-pellet on the purification needs to be examined.

First, the impact of Sarkosyl on the ion-exchange method will be discussed followed by the results of the impact of Zwittergent 3-14.

10.3.2.1 Impact of Sarkosyl on IEC

The impact of Sarkosyl on the ion-exchange purification was tested for different concentrations of the detergent. First the impact of Sarkosyl concentration present throughout the recovery process was examined, followed by smaller detergent concentrations, which would be present after the performance of a detergent removal step.

In order to improve protein solubility during the primary protein recovery process, Sarkosyl was added at a concentration of 3.0% during cycle-1. Results of chapter 8 showed that at the end of

the optimized recovery process with Sarkosyl, the detergent was still present at a concentration of 2.4 - 2.6%. Hence, the impact of the presence of 3.0% Sarkosyl in the TCA-pellet on the first step of the down-stream process needs to be investigated.

At first, a TCA-pellet of the manufacturing process containing 3% Sarkosyl was used for interferon-α-2b purification with the ion-exchange method, as described in section 10.2.2. It was not possible to bring this purification to an end, since loading a TCA-pellet containing 3% Sarkosyl onto the Sepharose column, was impossible. Sarkosyl is an anionic detergent, which is known to interfere with some ion-exchange resins. In the literature most described interactions are between Sarkosyl and anion exchangers (section 10.1.2.3) but apparently Sarkosyl also interacts with the cation-exchanger Sepharose. This observations lead to the conclusion that Sarkosyl needs to be removed from the recovery process sample prior to the down-stream process.

In chapter 9 different techniques were proposed for the removal of Sarkosyl from process samples. Over 95% - 98% of the initial 3% Sarkosyl could be removed with some of the techniques. Hence, the next experiment was performed with a Sarkosyl concentration of 0.15% which corresponds to 95% removal. The sample preparation was the same as in the previous test, a TCA-pellet from the manufacturing process was spiked with 0.15% of Sarkosyl and interferon purification was performed with the ion-exchange method as described in section 10.2.2. First observations made during the performance was the successful loading of the sample. However, after quantifying total interferon-α-2b in the collected samples, no interferon was detectable in the elution fractions 7-12 (data not shown). On the contrary, 90% of the loaded interferon was detected in the flow-through and 10% in the wash- and strip-sample. This leads to the conclusion that the presence of a Sarkosyl concentration of 0.15% prevents the binding of interferon to the column completely. The binding of total protein to the column was less affected than interferon. Over 10% of the original loaded total protein was detected in the elution fractions 7-12 (data not shown), 80% in the flow through and 10% in the wash- and strip-sample. These results lead to the conclusion that the presence of 0.15% Sarkosyl in the TCA-pellet has a significant negative impact on the purification steps. Hence, the Sarkosyl concentration in the TCA-pellet needs to be reduced further.

The next experiment was performed with a TCA-pellet containing a Sarkosyl concentration of 0.06%, which corresponds to 98% removal. The IFN purification was performed under standard conditions as described in section 10.2.2. Figure 10.8 shows the AKTA chromatogram of the

TCA-pellet purification containing a Sarkosyl concentration of 0.06% (on the left side) and the control sample without detergent present (on the right side).

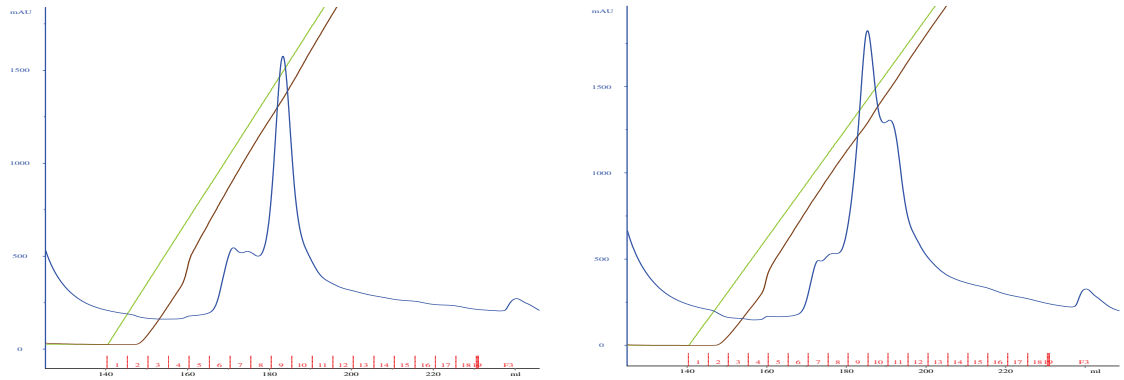

Fig. 10.8: AKTA chromatogram from TCA-pellet purifications containing a Sarkosyl concentration of 0.06% (left) or without Sarkosyl (right)

The chromatograms of both purifications, with and without Sarkosyl at 0.06%, look very similar. However, analysing the collected samples for the TCA-pellet containing 0.06% Sarkosyl, resulted in only 6% recovery of the loaded interferon in the elution fractions 7-12, 80% in the flow through and 15% in the wash- and strip-sample. The interferon recovery of the control sample without Sarkosyl present was as expected: 38% recovery of the loaded IFN in the elution fractions 7-12, 50% in the flow through and 12% in the wash- and strip-sample. These big differences between control and Sarkosyl-containing sample were unexpected since the elution chromatograms were very similar in their UV-280 profile. Having a closer look at the chromatograms in figure 10.8, one major different can be seen. The elution peak of the control sample has a small shoulder on the right side. This shoulder indicates the presence of the majority of interferon in the elution fractions 10 and 11 of the control sample. The other small difference between both chromatograms is the height of both peaks. The control sample reaches a slightly higher UV-280 value of 1750mAU, compared to 1600mAU for the TCA-sample with a Sarkosyl concentration of 0.06% present. The difference in the peak height can be explained by the reduced recovery of total protein in the elution fractions. The TCA-pellet containing 0.06% of Sarkosyl resulted in 12% recovery of the loaded total protein in the elution fractions 7-12, whereas the negative control without Sarkosyl showed 17% recovery in total protein.

However, with only 6% recovery in interferon-α-2b, the impact of Sarkosyl at a concentration of 0.06% is still significant. Hence, the Sarkosyl concentration was reduced once more to 0.03%

which corresponds to 99.0% removal.

In a last experiment a TCA-pellet derived from the manufacturing process was spiked with Sarkosyl to a concentration of 0.03% and used for interferon purification with the ion-exchange method as described in section 10.2.2. Results of the quantified interferon-α-2b and total protein in the different collected samples throughout the purification, are shown in figure 10.9.

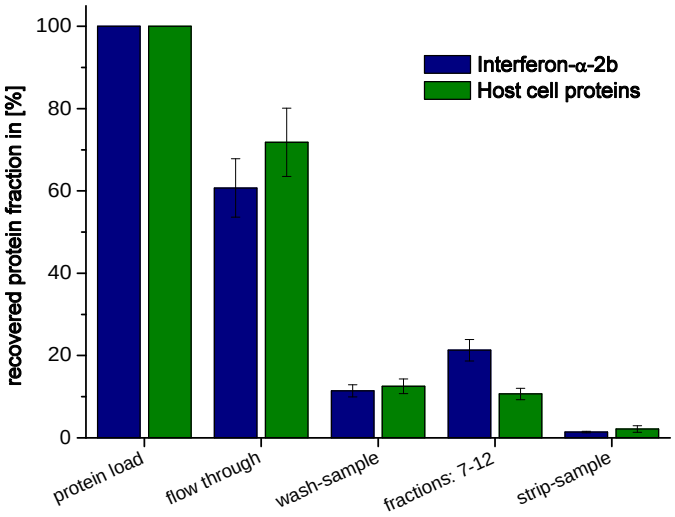

Fig. 10.9: Interferon-α-2b and total protein fractions quantified in samples after the purification of a TCA-pellet containing 0.03% Sarkosyl

The interferon recovery in the elution fractions 7-12 is 23% of the initial loaded IFN, which is a significant improvement compared to the IFN recovery with 0.06% Sarkosyl present. However, the IFN recovery is still 1.6 times less than in the negative control without any Sarkosyl present. The total protein recovery with 0.03% Sarkosyl present, is also lower than in the control sample, 13% recovery in the elution fractions 7-12 with 0.03% Sarkosyl compared to 18% in the control sample. These results show, that even with a low concentration of 0.03%, Sarkosyl has a significant impact on interferon purification. However, achieving a Sarkosyl removal of more than 99% is very challenging, as shown in chapter 9. Hence, the presence of Sarkosyl in the primary recovery process results in maximal 20-25% recovery in interferon-α-2b throughout the first down-stream step, if 99% of Sarkosyl has been removed prior to the purification method.

In summary, the results showed that the presence of Sarkosyl in primary recovery process samples has a major impact on the first purification step. Even after 99% detergent removal, the impact

on interferon-α-2b recovery throughout the DSP-step was still significant. However, looking on the following steps throughout the down-stream process, it is important to know how the Sarkosyl concentration behaves through the first DSP-step and how much of the Sarkosyl concentration gets carried onto the next DSP-step. Figure 10.10 shows the Sarkosyl concentration in all fractions of the ion-exchange purification with the TCA-pellet containing Sarkosyl at 0.06%.

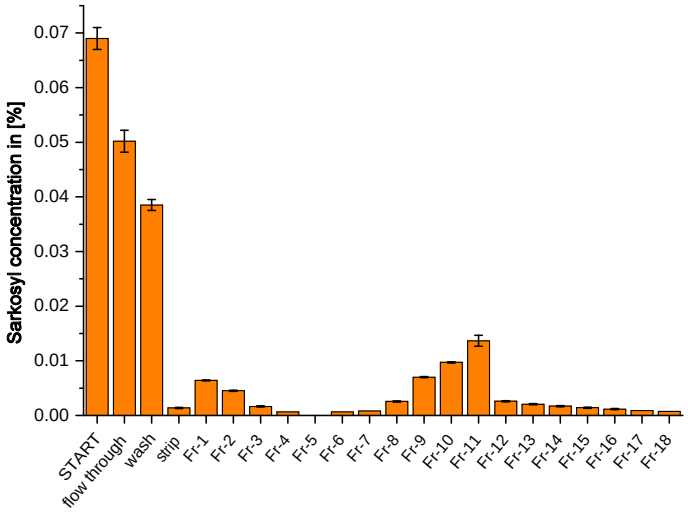

Fig. 10.10: Sarkosyl concentration quantified in each sample of the TCA-pellet purification containing 0.06% Sarkosyl

The amount of Sarkosyl was quantified by RP-HPLC and UV detection as described in section 10.2.3. The Sarkosyl concentrations shown in figure 10.10 are the absolute values quantified in each fraction independent on their fraction-volume. It can be seen in figure 10.10 that the majority of the Sarkosyl is found in the flow-through and wash sample, both of which go to the waste stream. The concentration of Sarkosyl detected in the elution fractions is very low between 0-0.015%. However, the Sarkosyl concentration elutes in a similar profile to interferon, with the maximum in fractions 10 and 11. This leads to the conclusion that some detergent molecules and proteins still interact with each other throughout the ion-exchange method. For the following DSP steps, elution fractions 7-12 will be collected together and the Sarkosyl concentration of all 6 fractions gives an average concentration of 0.007%, which should have no significant impact on the second DSP-step, a UF/DF-method.

10.3.2.2 Impact of Zwittergent on IEC

The impact of the presence of Zwittergent 3-14 on the first down-stream process step, an ion-exchange method, was examined. Zwittergent 3-14 was utilized to improve protein solubility during the primary protein recovery process (chapter 8). The first part of this section focuses on the impact of Zwittergent 3-14 at 1.0%, which is the detergent concentration added during the recovery process, on the ion-exchange purification followed by the impact of reduced Zwittergent concentrations, which would be present after a detergent removal step.

To investigate the impact of Zwittergent 3-14 being present at the end of the recovery process on the ion-exchange purification, a TCA-pellet of the manufacturing process was prepared as described in section 10.2.2. The TCA-pellet solution was than spiked with Zwittergent 3-14 at a concentration of 1.0% and the ion-exchange purification was performed as usual.
Figure 10.11 shows the AKTA chromatogram of the whole purification method for the TCA-pellet containing 1.0% Zwittergent.

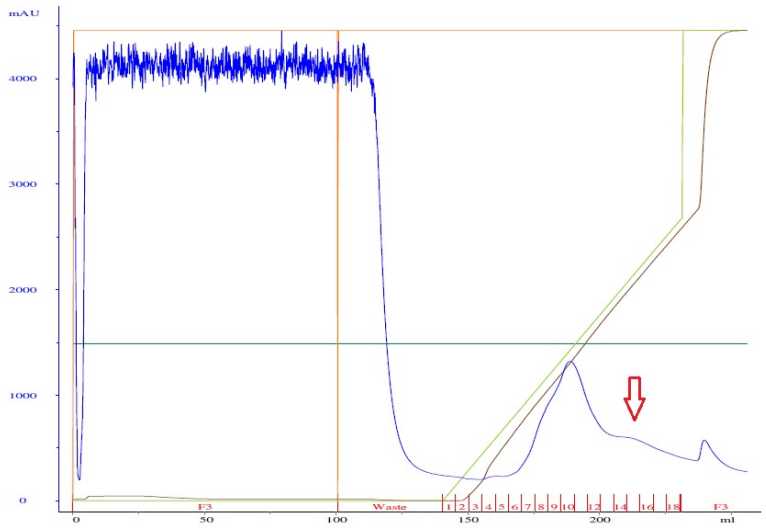

Fig. 10.11: AKTA chromatogram of sample purification with 1.0% Zwittergent 3-14 present

The chromatogram looks very similar to the chromatogram of the control sample without detergent (see figure 10.2). Only in the elution fraction after fraction 11 or 12 some minor differences are visible (red arrow). The UV-280 peak is wider and prolate compared to the negative control and the UV-280 reading does not return to the start value (200mAU) but stagnates between 600 and 400mAU. These results indicate that the presence of Zwittergent at 1.0% has an impact on

interferon-α-2b purification.

In order to gain more information as to how Zwittergent 3-14 affects IFN purification, all samples collected during the purification run were analysed for total interferon and total protein. The results of these quantifications are shown in figure 10.12.

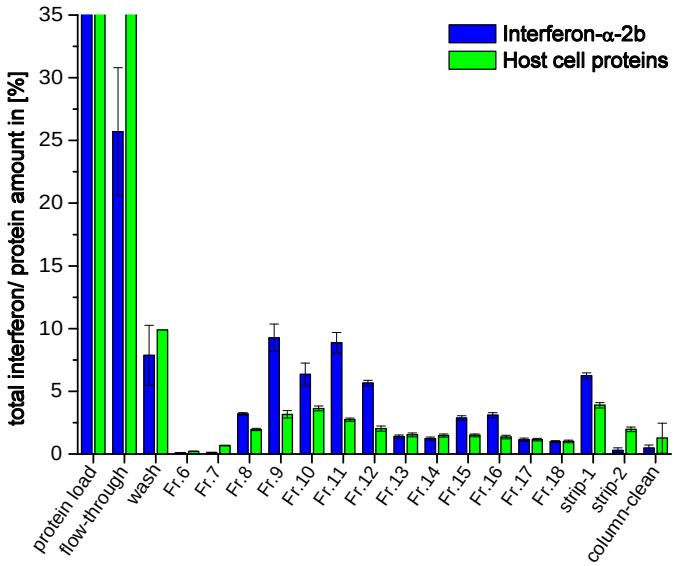

Fig. 10.12: Total interferon and total protein recovery after sample purification with 1.0% Zwittergent 3-14 present

Total protein was analysed using the BCA-assay as described in section 10.2.4 and total interferon-α-2b was quantified using the SDS-PAGE as described in section 10.2.5. The results in figure 10.12 indicate that the presence of 1.0% Zwittergent 3-14 in the TCA-pellet has no impact on the binding of interferon-α-2b to the ion-exchange column. Only 25-30% of the loaded interferon was found in the flow-through, which is 15-20% less than observed in the control sample without detergent (45% of IFN in flow through). This improvement of the interferon binding to the column correlates with the observation made throughout the study of host cell protein interference on IFN binding to the column (section 10.3.1.1). The results indicated that host cell proteins interact with interferon and thus prevent interferon from binding to the column (scenario 1 in figure 10.4). To avoid or reduce this interference, the suggestion was made to add a detergent to the process sample prior to purification. The observations in this section confirm this suggestion. With the addition of Zwittergent 3-14 prior to the purification the binding of interferon to the column improved to

15-20%.

Looking at the recovered interferon detected in the elution fractions it can be seen that the elution is protracted, which corresponds to the observation made in figure 10.11. The elution fractions 7-12 contained 33.5% of the loaded interferon, but an additional 11% was found in the last elution fractions 13-18 and 7% in the strip-sample. These results indicate that the binding of interferon-α-2b to the column is stronger in the presence of 1.0% Zwittergent 3-14 and a stronger salt gradient is required to elute all IFN from the column. With the original salt gradient only 33% of the loaded interferon is recovered for further down-stream steps. However, a stronger salt gradient might increase the interferon recovery to 45 - 50%, which is higher than the recovery of the control sample without Zwittergent (38%). Instead of changing the salt gradient, an isocratic elution mode might be even more beneficial in order to improve the interferon recovery. This option will be discussed in one of the following section 10.3.2.3.

The impact on the interferon elution profile of Zwittergent 3-14 also indicates that some detergent is still present during the elution. Hence, Zwittergent 3-14 was quantified in all collected fractions of this purification experiment and results are discussed in the following section.

The binding and elution of total protein towards the column in the presence of 1.0% Zwittergent 3-14 was less affected. 62% of the loaded total protein was found in the flow through, 9% in the wash-sample, 20% in the elution fractions and 7% in the strip-sample. This proportion is comparable to the findings of the control sample without detergent present, except for the 7% in the strip-sample. The increased amount in the strip sample is the only indication that the binding of total protein to the column is slightly stronger in the presence of Zwittergent 3-14 than without it.

In summary, the presence of 1.0% Zwittergent 3-14 in the process sample showed a positive impact on the binding of interferon-α-2b to the column. However, besides the impact on the binding, an impact on the elution of IFN from the column was also observed. In the presence of Zwittergent 3-14 the interferon affinity is increased and stronger salt gradients would be required to remove all bound interferon, otherwise only 33.5% of the loaded interferon-α-2b is recovered for further purification steps.

Zwittergent 3-14 removal during DSP

In order to investigate the behaviour of the Zwittergent 3-14 itself during the interferon purification with the ion-exchange method, all purification fractions were analysed for their Zwittergent 3-14 concentration. Figure 10.13 shows the Zwittergent 3-14 recovery after purification of the TCA-pellet containing 1.0% Zwittergent 3-14.

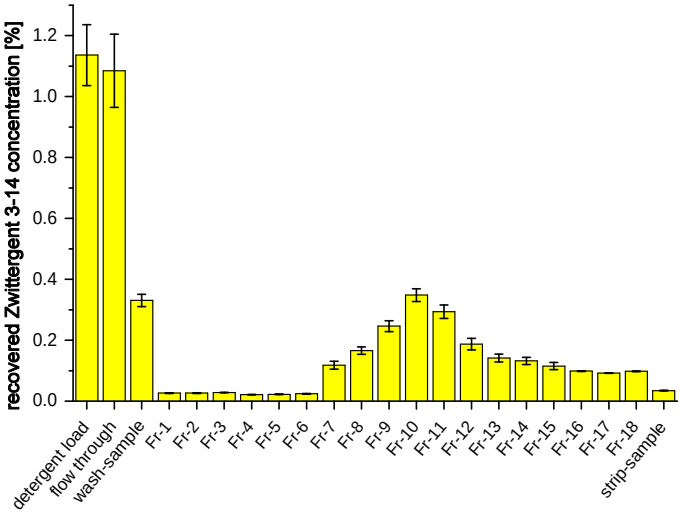

Fig. 10.13: Zwittergent 3-14 recovery after purification of TCA-pellet containing 1.0% Zwittergent 3-14

Zwittergent 3-14 was quantified with RP-HPLC and RI detector as described in section 10.2.3. Similar to the presentation of the Sarkosyl results, the Zwittergent 3-14 concentrations shown in figure 10.13 are the absolute values quantified in each fraction independent on their fraction-volume. It can be see that the majority of Zwittergent 3-14 is found in the flow through of the column and does not interact with the column. However, it is also observed that the Zwittergent 3-14 concentration in the elution fractions proceeding proportional to the interferon results. No interferon nor Zwittergent 3-14 is detected in elution fractions 1-6. From elution fractions 7-10 both amounts increase and from elution fractions 11-18 they decrease with a higher concentration in the last fraction compared to the first fraction. These results indicate that Zwittergent 3-14 is interacting in some way with the column, either directly or through interactions with the proteins. Injecting 1.0% Zwittergent 3-14 on the column without any proteins being present resulted in a 100% recovery of the injected detergent in the flow through and no binding or interactions were

observed between Zwittergent 3-14 and the column itself (data not shown). This leads to the conclusion that Zwittergent 3-14 does not bind directly to the column but through interactions with proteins, interferon and or host cell proteins.

Calculating the average Zwittergent 3-14 concentration between elution fractions 7-12 results in a detergent concentration of 0.226% in 30mL purified sample. This shows, that Zwittergent 3-14 is removed through the ion-exchange purification by 90% of its initial starting concentration referring to the recovered average detergent concentration of the starting volume. However, due to reduction of the volume, the detergent is concentrated to the final concentration of 0.226% Zwittergent 3-14, which will be present and needs to be taken into account for the next purification step of the down-stream process.

10.3.2.3 Isocratic elution of interferon-α-2b containing 1% Zwittergent 3-14

Results in the last section showed, that interferon-α-2b binds stronger to the ion-exchange column in the presence of 1.0% Zwittergent 3-14. In order to remove all interferon from the column the suggestion was made to increase the salt concentration during the elution. In this experiment an isocratic elution mode at different salt concentrations starting from 0.147M (21% mobile phase B) to 0.35M (50% mobile phase) was investigated. During the elution the alterations in the UV-280 reading was observed. As soon as the UV-280 reading showed no further significant increase or decrease, the next salt concentration was adjusted.

A TCA-pellet from the manufacturing process was prepared as described in section 10.2.2 and spiked with 1.0% Zwittergent 3-14 followed by the ion-exchange purification. The method was performed as described in section 10.2.2 except the elution phase. This part was performed manually with isocratic elution at different salt concentration as explained above.

Figure 10.15 shows total interferon-α-2b and total protein in collected samples after purification with isocratic elution at different salt concentrations of the TCA-pellet containing 1.0% Zwittergent 3-14.

Total protein was quantified using the BCA-assay as described in section 10.2.4 and total interferon-α-2b was quantified using SDS-PAGE as described in section 10.2.5. Results in figure 10.15 confirm the findings of the previous section of an increased interferon binding to the column in the presence of 1.0% Zwittergent in the TCA-pellet. Only 33% of interferon-α-2b was found in

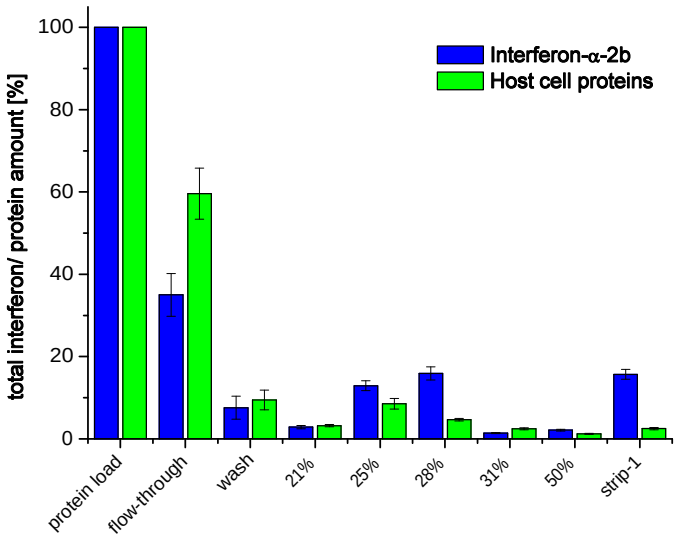

Fig. 10.14: Total interferon-α-2b and total protein recovery after sample purification with 1.0% Zwittergent 3-14 present and isocratic elution at different salt concentrations

the flow-through, which is less than in the control sample without detergent. Following the results of the flow-through and the wash-sample, are the results for the different isocratic elution fractions with increasing salt concentrations, starting at 0.147M NaCl corresponding to 21% of mobile phase B (elution fraction 6). Less than 5% of the loaded interferon was detected in this fraction after 5 minutes of constant elution at 5ml/min and no significant increase in the UV-280 reading was observed. The next fraction was collected at an isocratic elution at 0.175M NaCl (25% of mobile phase B, elution fraction 7) for 3 minutes at 5ml/min. Over 15% of the loaded IFN was detected in this fraction with a UV-280 maximum at 1000mAU. In the third fraction, at 0.196M NaCl (28% mobile phase B, elution fraction 8), 18% of the loaded interferon was detected which was unexpected. The UV-280 reading showed no increment but only continuous decreasing from 500mAU down to 100mAU over the whole 4 minutes at 5ml/min. The last two fractions at 0.217M NaCl (31% mobile phase B) and 0.35M NaCl (50% mobile phase B) showed only very little interferon recovery and constant UV-280 reading below 100mAU for 5 and 4 minutes at 5ml/min. These findings suggest that more than 38% of the loaded interferon-α-2b can be recovered with an isocratic elution at 0.196M NaCl (28% mobile phase B). Without detergent present all interferon eluted at 0.175M NaCl (25% mobile phase B) as results showed in section 10.3.1.2. This is another confirmation of the observation that interferon-α-2b has a higher affinity for the

ion-exchange column in the presence of 1.0% Zwittergent 3-14.

However, looking at the last fraction in figure 10.15 it can be seen that 15% of the loaded interferon was still recovered in the strip-sample, which goes to the waste stream. In order to recover this IFN portion in the elution fraction, the salt concentration would need to be increased to 0.4M NaCl or higher. However, a high salt concentration can have a negative impact on other purification methods or on the protein itself (section 10.1.2), hence the interferon elution should be operated at the lowest salt concentration as possible.

In order to confirm the findings of isocratic interferon elution at 0.196M NaCl the experiment was repeated with only one isocratic elution step at 0.196M NaCl (28% mobile phase B). The UV-280 reading and quantified interferon results indicated a very slow elution of IFN from the column (data not shown). This leads to a big dilution of the interferon in the recovered fraction. Hence, a higher salt concentration (0.217M NaCl) was selected for a faster elution of interferon from the column and a more concentrated IFN in the recovered fraction.

Figure 10.15 shows an AKTA chromatogram of the isocratic elution profile at 0.217M NaCl corresponding to 31% mobile phase B (elution fraction 9) for a TCA-pellet containing 1.0% Zwittergent 3-14.

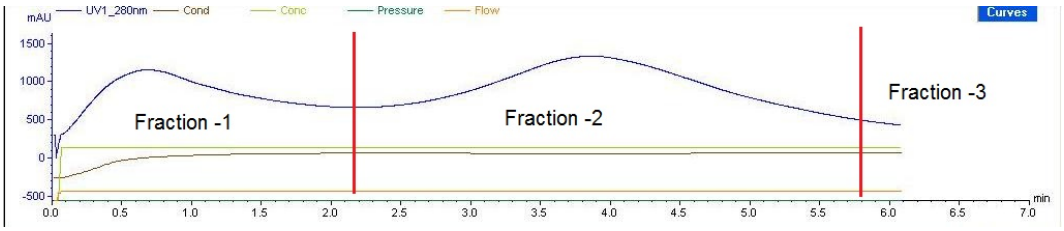

Fig. 10.15: AKTA chromatogram of an isocratic elution at 31% mobile phase B of TCA-pellet containing 1.0% Zwittergent 3-14 (31% mobile phase B equals to 0.217M NaCl which corresponds to fraction 9 of a gradient elution)

The isocratic elution chromatogram of interferon-α-2b at 0.217M NaCl shows two maxima in the UV-280 reading. The first maximum was reached after 0.6min elution at 5ml/min with a UV-280 reading of 1100mAU. The second maximum reached a UV-280 reading of 1400mAU after 3.9 minutes of 5ml/min elution. This observation of two maxima was unexpected and needs to be further investigated. Each maximum was collected as a separate fraction and analysed for interferon and total protein.

The results of the analysed fractions as well as the other collected samples of the purification of

a TCA-pellet containing 1.0% Zwittergent 3-14 are shown in figure 10.16. The ion-exchange purification was performed as described in section 10.2.2 except the elution, which was performed manually with isocratic conditions at 0.217M NaCl. During the isocratic elution 4 different fractions were collected. Fraction zero is called 31%-prime during which the AKTA system and the column were flushed with 31% mobile phase B (0.217M NaCl) at 5ml/min until the conductivity reading was constant. Fraction 1 was operated under similar conditions to fraction zero and all following fractions. Eluent was collected for 2 minutes until UV-280 reading reached its first minimum. Fraction 2 was collected for 4 minutes at 5ml/min and fraction 3 for 2.5 minutes.
Figure 10.16 shows the recovery of total interferon-α-2b, total protein and Zwittergent 3-14 in the collected samples throughout the purification of a TCA-pellet containing 1.0% Zwittergent 3-14 with isocratic elution at 0.217M NaCl.

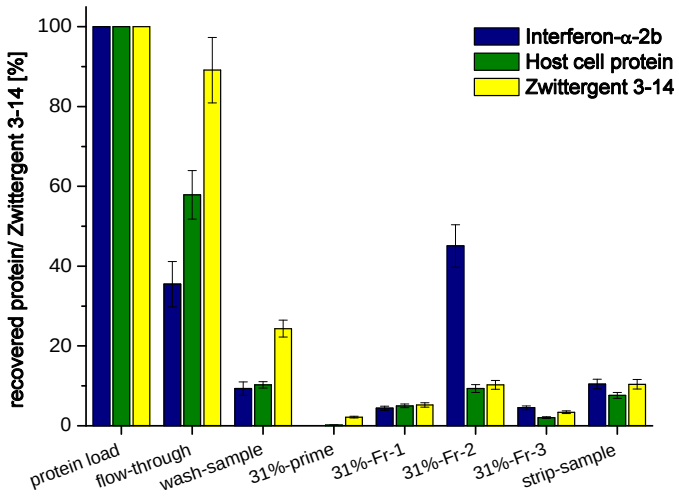

Fig. 10.16: Total interferon-α-2b, total protein and Zwittergent 3-14 recovery after purification of TCA-pellet with 1.0% Zwittergent 3-14 present. Isocratic elution at 31% of mobile phase B (31% mobile phase B equals to 0.217M NaCl which corresponds to fraction 9 of a gradient elution)

Total protein was quantified using the BCA-assay as described in section 10.2.4, total interferon-α-2b was quantified using SDS-PAGE as described in section 10.2.5 and Zwittergent 3-14 was quantified by RP-HPLC and RI detector as described in section 10.2.3. It can be seen in figure 10.16 that only 35% of loaded interferon-α-2b was detected in the flow through. This finding is as expected and was already seen in previous purifications of TCA-pellets containing 1.0% Zwitter-

gent 3-14. No interferon was detected in the 31%-prime fraction which was as expected. Flushing the system and column with elution conditions has the only purpose to adapt the column environment to the elution conditions. That way the elution of interferon starts without delay when collecting the elution fractions and big dilutions can be prohibited.

Only a small portion of less than 5% of the loaded interferon was detected in the elution fraction 1. This was unexpected. Due to the high UV-280 reading with a maximum at 1100mAU a higher IFN proportion was expected. However, the UV-280 reading is not protein specific and also represents all host cell proteins eluted at that time. The majority of interferon-α-2b was recovered in the second elution fraction. More than 40% of the loaded IFN was recovered during these 4 minutes. Elution fraction 3 shows only a small portion of interferon of less than 5% which was as expected. The UV-280 reading was constantly decreasing during this fraction from 400mAU down to 200mAU.

The strip sample still contains 10% of the loaded interferon which is higher than the control sample. However, as mentioned previously, in order to reduce this portion it would require a much higher salt concentration during the elution which can have an negative impact on further purification steps.

The host cell proteins showed no significant changes compared to the negative control without any detergent present except the slightly higher protein portion in the strip sample. This phenomenon was already discussed before in the previous section.

In summary, using an isocratic elution at 0.217M NaCl for TCA-pellet purification containing 1.0% Zwittergent 3-14 results in an interferon-α-2b recovery of 50% in 30ml fraction volume. The gradient elution of the TCA-pellet containing 1.0% Zwittergent 3-14 resulted in 33.5% recovery of the loaded interferon in the elution fractions 7-12. Hence, due to isocratic elution the IFN recovery could be improved from 33.5 to 50% in the same fraction volume. And the interferon recovery of TCA-pellet with 1.0% Zwittergent 3-14 present is even improved compared to the negative control without a detergent which results only in 40% recovery of the loaded interferon.

Besides interferon and total protein recovery, figure 10.16 also shows the recovery of Zwittergent 3-14 in the collected samples after purification with isocratic elution of the TCA-pellet containing 1.0% Zwittergent. In contrast to previous Zwittergent results, these data are not the absolute concentrations of Zwittergent but the proportion of detergent per volume recovered in each fraction

in relation to the starting concentration of 1.0%. The results show that the majority of Zwittergent 3-14 (>90%) does not interact with the column and is collected in the flow through and the wash-sample. Only small levels of less than 10% were found in elution fractions one, two and three. Under consideration of the volume of elution fraction 1 and 2, the absolute concentration of Zwittergent 3-14 in both fractions together would be around 0.3%. These results illustrate that in fact 90% of the Zwittergent 3-14 was removed by the ion-exchange purification but the recovered 10% became 3 times concentrated in the elution fraction. This results in 0.3% Zwittergent 3-14 in the recovered fractions for further down-stream process steps. These findings confirm the previous observations from section 10.3.2.2, which showed 0.23% Zwittergent 3-14 in the recovered fraction for further purification steps.

Impact of the Recovery Process simulation samples on DSP

Throughout the primary protein recovery process optimization with Zwittergent 3-14 (chapter 8) two different optimized processes with Zwittergent 3-14 were proposed. The first includes the addition of Zwittergent during cycle 1 but otherwise following the steps of the original process, including the salting-out step. The second optimized recovery process involves the addition of Zwittergent 3-14 during cycle 1 but in the absence of the salting-out step. Due to the removal of the salting-out step the proportion between host cell proteins and interferon increased. Results of section 10.3.1.1 showed that host cell proteins do interfere with the interferon purification by ion-exchange chromatography. Hence it is important to investigate the impact of the different proportions between HCP and IFN on the ion-exchange purification.

The TCA-pellets recovered after the large scale simulation at 0.5L of the protein recovery process with 1.0% Zwittergent in the presence and absence of the salting-out step were prepared for the ion-exchange purification as described in section 10.2.2. The purification was performed under standard conditions and an isocratic elution at 31% mobile phase B (0.217M NaCl). Obtained results for interferon-α-2b and total protein showed for both pellets that only a small portion of the loaded interferon bound to the column (15%) and the majority was found in the flow through (75%). The reduced interferon recovery of both pellets can be explained by the reduced Zwittergent 3-14 concentration in the TCA-pellets after the recovery process simulations. Results in chapter 8 showed that the Zwittergent 3-14 concentration is decreasing throughout the recovery

process simulation below 0.5%. It is possible that below a certain concentration, Zwittergent 3-14 loses its ability to prohibit interactions of host cell proteins with the interferon binding to the column and start to interfere with the IFN-affinity itself. For that reason, the Zwittergent 3-14 concentration in both TCA-pellets derived from the recovery process simulation in absence and presence of the salting-out step was adjusted to 1.0% prior to the ion-exchange purification.
Repeating the purification with the TCA-pellets and adjusted Zwittergent 3-14 concentrations to 1.0%, resulted in comparable results for both TCA-pellets than previously obtained with the TCA-pellet from the manufacturing process. One third (35%) of loaded interferon-α-2b was found in the flow through, 40-45% of interferon was recovered in the isocratic elution fraction and 15-20% of IFN was detected in the wash- and strip-sample (date not shown). These results indicate that the changes in the proportion between host cell protein and interferon due to the presence or absence of the salting-out step has no significant impact on the recovery of interferon during the ion-exchange purification. The results also showed that a decrease in the Zwittergent 3-14 concentration from 1.0% to 0.5% has a negative impact on interferon purification and the detergent concentration needs to be adjusted to 1.0% prior to purification. Another option besides adjusting the Zwittergent 3-14 concentration would be the removal of the detergent from the process sample prior to purification. The impact of process samples on the purification post Zwittergent 3-14 removal is discussed in the following section 10.3.2.4.

10.3.2.4 Impact on DSP post Zwittergent 3-14 removal

One possibility to reduce the impact of Zwittergent 3-14 on the down-stream process is the removal of the detergent prior to the DSP. Chapter 9 discussed different techniques for the removal of Zwittergent 3-14. This section discusses the impact on the ion-exchange purification of small remaining traces of Zwittergent 3-14 after the detergent removal step. It is assumed that 95% of the initial Zwittergent 3-14 concentration could be removed, which leaves only 0.05% of Zwittergent left in the process sample.
A TCA-pellet from the manufacturing process was spiked with 0.05% Zwittergent 3-14 and ion-exchange purification was performed as described in section 10.2.2 with the standard gradient elution method. Figure 10.17 shows recovered interferon-α-2b and total protein after purification of the TCA-pellet containing 0.05% Zwittergent 3-14.

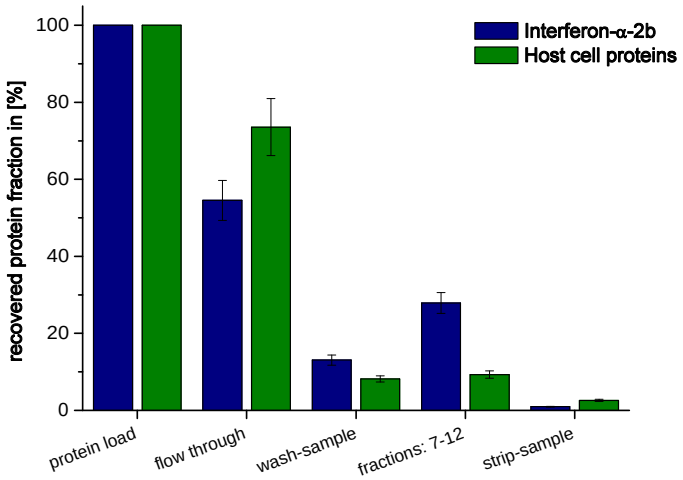

Fig. 10.17: Total interferon-α-2b and total protein recovery after purification of TCA-pellet with 0.05% Zwittergent 3-14 present. Gradient elution from 0 - 60% mobile phase B

Total protein was quantified using BCA assay as described in section 10.2.4 and total interferon-α-2b was quantified using SDS-PAGE as described in section 10.2.5. Results show a similar recovery of interferon than analysed in the negative control sample without detergent present, with a slightly higher interferon portion in the flow-through. More than half (55%) of the loaded IFN was detected in the flow through which is 5-10% higher than the control sample. 35% of loaded interferon was analysed in the elution fractions 7-12, which is slightly lower than the control sample with 38-40% IFN recovery. Previous results with 1.0% showed an improved interferon binding to the column but also a delayed and declined IFN recovery after column elution. With 0.05% Zwittergent 3-14 no impact on the column elution was observed, however, a minor negative impact on the interferon binding to the column was detected. These findings confirm the observation from the previous section where a reduced Zwittergent 3-14 concentration showed interference with the interferon binding to the column. The results indicate that the presence of 0.05% Zwittergent 3-14 has a minor negative impact on the interferon purification but results for the IFN recovery yield in elution fractions are still satisfying.

The amount of total protein showed a very similar trend compared to interferon. A minor increase of total protein in the flow-through and a small decrease in the elution fractions 7-12.

Zwittergent 3-14 was quantified as well in all fractions with the RP-HPLC method and RI detector. However, no detergent could be detected in any of the elution fractions. This shows that the

majority of the 0.05% Zwittergent 3-14 was detected in the flow-through and wash-sample and the concentration in the elution fractions is below 0.005%.

In summary, the presence of 0.05% Zwittergent 3-14 in process samples showed only a minor impact on the interferon purification with the ion-exchange method. Only 32% of the loaded interferon-α-2b was recovered in the elution fractions 7-12 for further purification steps which is slightly slower compared to the control sample without detergent present with 38-40% IFN recovery. However, the IFN recovery yield was still sufficient (35%), hence, the impact was negligible.

10.4 Conclusion

This chapter focused on the impact of the presence of two different detergents in recovery process samples on the first purification step of the down-stream process. At first the purification step, an ion-exchange method, was characterized, followed by the study of the impact of Sarkosyl on the DSP step and finally the impact of Zwittergent 3-14.
In order to summarize results and draw conclusions this section is divided into four parts: first the characterization of the IEC, second the impact of Sarkosyl and third the impact of Zwittergent 3-14 followed by the final overall conclusion and outlook.

10.4.1 Characterization of IEC

The purification with the ion-exchange method of a TCA-pellet from the manufacturing process results in only 38-40% recovery of the loaded interferon-α-2b and 45-50% were detected in the flow through. Difference parameters, such as flow-rate, IFN load, column temperature or loading buffer were analysed for their impact on the low interferon recovery during this purification. None of them showed a significant impact. The main impact on the poor interferon recovery yield is due to the presence of host cell proteins. Investigation of host cell protein interference indicated that HCP interact with interferon and hence, interfere with the IFN binding to the column. To reduce this interference the suggestion was made to add a detergent to the process samples which avoids protein-protein interactions.
In conclusion, host cell proteins interfere with the interferon binding to the column due to protein-protein interactions for which reason the recovery yield of interferon-α-2b of the ion-exchange purification is only 38-40%.

10.4.2 Impact of Sarkosyl

Investigating the impact of Sarkosyl on the ion-exchange purification showed, that Sarkosyl has a major impact on this first down-stream step. Without removal, at 3% Sarkosyl, no loading of the sample onto the column was possible, which leads to the conclusion that Sarkosyl interacts with the column and blocks the pores and/or binding sites. Even after 99% removal of detergent, the remaining 0.03% Sarkosyl still had a significant impact on the purification and only 20-25%

of the loaded interferon was recovered in the elution fraction for further down-stream steps. The recovery of interferon during the first down-stream step in the present of small traces of Sarkosyl could be improved by equilibrating the ion-exchange column with the Sarkosyl concentration being present in the process sample. This needs to be further investigated before applying the Sarkosyl addition at manufacturing scale.

Results also showed, that with the ion-exchange method leftover traces of Sarkosyl from the detergent removal step do further decrease with a removal yield of 90%.

This leads to the conclusion that even after successful removal of Sarkosyl (yield 99%) the maximal interferon recovered from the purification is only 20-25% of the initially loaded IFN.

10.4.3 Impact of Zwittergent 3-14

The investigation of the impact on the ion-exchange purification of 1.0% Zwittergent 3-14 in process samples showed a distinct effect on the interferon binding to the column. Due to the presence of Zwittergent 3-14 more interferon bound to the column and only 30-35% of loaded IFN was found in the flow-through. However, the elution of interferon was delayed and resulted only in a recovery yield of 33%. In order to improve the IFN elution, higher salt concentrations were required. An isocratic elution mode was applied at constant 0.217M NaCl, which resulted in 40-45% recovery of interferon-α-2b. Zwittergent 3-14 was also removed from the process sample through the ion exchange method. Over 90% of the initial Zwittergent 3-14 concentration were removed from 1.0% in 100mL down to 0.3% in 30mL fraction volume.

Smaller concentrations of Zwittergent 3-14, less than 1.0%, were shown to have a negative impact on interferon recovery during the purification. This led to the conclusion to either adjust the detergent concentration back to 1.0% prior to purification or remove the Zwittergent further with detergent removal steps. At least 95% of the 1.0% Zwittergent 3-14 needs to be removed to be below 0.05% in the process sample prior to the purification. Zwittergent 3-14 at 0.05% showed a small but negligible impact on the IFN purification with a IFN recovery yield of 32%.

It can be concluded that Zwittergent 3-14 at 1.0% has a positive impact on the purification and interferon recovery can be improved to 40-45% with the implementation of an isocratic elution. The ion-exchange method can also be used to remove the Zwittergent from process samples with a yield of 90%.

It still needs to be investigated if the implementation of the isocratic elution has an impact on the IFN isoform distribution. It is possible that some isoforms have a higher binding affinity to the column than others and elute only at salt concentrations higher than 0.22M NaCl. These isoforms would elute in the strip sample with the isocratic elution at 0.217M NaCl.

10.4.4 Overall Conclusion and outlook

It was shown that the first step of the down-stream process, an ion-exchange method, has only an interferon-α-2b recovery yield of 38-40% which is unsatisfying. In order to improve the IFN recovery two possibilities are available. First, addition of a detergent prior to the purification which improves the binding of interferon to the column or second addition of another purification step to remove host cell proteins from the process sample.

The presence of a detergent at the end of the recovery process, showed to have significant impact on the interferon purification with the ion-exchange method. Sarkosyl needs to be removed completely from the process sample prior to purification to avoid any interference with the IFN binding to the column. Even at a removal yield of 99% (0.03% Sarkosyl) only 20-25% of the loaded interferon is recovered during the purification which is 13-20% less than the recovery of the negative control without any detergent.

The presence of Zwittergent 3-14 on the other hand showed a positive influence on the IFN purification. With the implementation of an isocratic elution, 40-45% of the loaded interferon-α-2b was recovered during the ion-exchange purification while removing 90% of the 1.0% Zwittergent concentration from the process sample.

A next step would be the application at large-scale for the two detergents present during the ion-exchange purification. In particular the implementation of the isocratic elution conditions with 1.0% Zwittergent 3-14 needs to be tested at larger scale.

Chapter 11

Summary Chapter - Optimized Protein Recovery Process

11.1 Introduction

This chapter will summarize the results obtained throughout the optimization of the primary protein recovery process in chapters 7, 8, 9 and 10 and propose three new optimized recovery processes for interferon-α-2b with an increased recovery yield.

The primary protein recovery process for interferon-α-2b was characterized setting-up mass balances for total protein, total interferon-α-2b and interferon-isoforms throughout the whole recovery process. Two critical process steps (CPS) were identified in chapters 4, 5 and 6, with CPS-1 during cycle 1 and CPS-2 during cycle-2 with an overall loss in total interferon-α-2b of 80%. The majority of this loss (60%) was found to be during cycle-1 (CPS-1) due to insufficient protein solubilisation. Further results led to the conclusion of a protein saturation problem during this step of the process. With CPS-1 being responsible for over 60% loss of interferon-α-2b, this step was the primary focus throughout the process optimization study.

The following sections in this chapter will propose three optimized recovery processes for interferon-α-2b by discussing the protein recovery and economic factors such as cost and time reduction. Possible advantages and disadvantages of each optimized process compared to the original process will be discussed in order to select the optimal protein recovery process for interferon-α-2b.

11.2 Optimized Recovery Processes

This section will focus on the illustration of three optimized recovery processes for interferon-α-2b including the first down-stream step. The critical process step 1, characterized in chapters 4, 5 and 6 showed a 60% loss in interferon due to insufficient protein solubilisation and saturation problems. Hence, a dilution study was performed around the CPS-1 in order to improve the interferon solubility, as discussed in chapter 7. Section 11.2.1 will summarize these results including the impact on the down-stream process and propose an optimized protein recovery process.
A different approach to increase protein solubility was the addition of detergents throughout cycle 1 of the protein recovery process. Sarkosyl and Zwittergent 3-14 showed the highest potential to solubilize interferon-α-2b as discussed in chapter 8. After the successful implementation of the addition of a detergent in a small scale study of the recovery process, the removal of those detergents needed to be investigated. Two different approaches were available for the removal of the detergents: first, developing a new method for the removal and second, using the first down-stream step for the detergent removal. Chapter 9 discussed the potential of several different techniques to remove the detergents, Sarkosyl and Zwittergent 3-14, at the end of the recovery process prior to purification steps. Chapter 10 shows the impact of the two different detergents on the first down-stream step at different concentrations and discussed the potential of the ion-exchange step to remove the detergents from the process. The following two sections 11.2.2 and 11.2.3 summarize the results for Sarkosyl and Zwittergent 3-14 and propose the optimal recovery process for interferon-α-2b considering additional detergent removal steps and the impact on the down-stream process.

Current primary protein recovery process

The current primary protein recovery process, as described in section 2.3 and displayed in figure 2.2, exist out of 4 centrifugation steps with a total process time between 16 and 20 hours. The interferon-α-2b recovery at the end of the process in the TCA-pellet is 20% with the major loss of 60% during cycle-1. Throughout the first down-stream step, an ion-exchange method, only 40% of the interferon is recovered in the elution fraction for further purification steps. Hence, only 8% of the initial produced interferon-α-2b is remaining after the recovery process and the first down-

stream step. Assuming, 7'000g of total interferon-α-2b are produced throughout the fermentation process, only 560g of IFN are recovered during the recovery process and first down-stream step, with the majority loss of 5'600g throughout the primary protein recovery process.

End of Fermentation		protein recovery process cycles	IFN recovery		ion-exchange step runs	IFN recovery		overall recovery of IFN
100%	$\rightarrow$	4 cycles	20%	$\rightarrow$	1 run	40%	$\rightarrow$	8%
7'000g	$\rightarrow$		1'400g	$\rightarrow$		560g	$\rightarrow$	560g

11.2.1 Optimized Recovery Process with two-fold dilution

The two-fold dilution during cycle-1 was performed in the presence or absence of the salting-out step (cycle 3), as discussed in chapter 7. In order to achieve a significant improvement in interferon recovery at the end of the protein recovery process, the salting-out step needs to be eliminated from the process. Hence, the new optimized recovery process would exist only out of 3 cycles (process time 15 hours), with a double process volume during cycle-1 and an overall interferon-α-2b recovery of 60%. Results of chapter 7 showed that the two-fold dilution has no significant impact on the first down-stream step, hence, the ion-exchange step can be performed following the standard protocol. However, with a three-fold increase in interferon-α-2b recovery at the end of the primary protein recovery process, the ion-exchange column would be overloaded with only one purification run. Thus, the TCA-pellet needs to be divided into two parts and each fraction is purified separately by the down-stream process. With a 40% recovery of interferon in each elution fraction of the ion-exchange runs, 24% of the initial produced interferon-α-2b is recovered after the first down-stream step. Assuming, 7'000g of total interferon-α-2b are produced throughout the fermentation process, 1'680g of IFN are recovered during the recovery process and first down-stream step. Compared to the current protein recovery process, this is a three-fold improvement in the interferon-α-2b recovery. However, at least the first down-stream step needs to be performed twice due to the increased interferon-α-2b amount in the TCA-pellet.

End of Fermentation		protein recovery process cycles	IFN recovery		ion-exchange step runs	IFN recovery		overall recovery of IFN
100%	→	3 cycles	60%	→	2 runs	40% each	→	24%
7'000g	→		4'200g	→		840g each	→	1'680g

11.2.2 Optimized Recovery Process with 3% Sarkosyl

The addition of 3% Sarkosyl to the primary protein recovery process to improve protein solubility was discussed in chapter 8. Results showed that the presence of Sarkosyl interferes with the salting-out step due to instability of the detergent below a pH of 5.0. Hence, the protein recovery process would need to be performed in the absence of the salting-out step. Results from chapter 10 showed a significant impact of Sarkosyl-containing process samples on the first down-stream step, an ion-exchange method. Hence, it was concluded that Sarkosyl needs to be removed from the process prior to the down-stream process. Chapter 9 discussed several different techniques for the removal of Sarkosyl from the process and the UF/DF-method showed the most efficient removal rate while recovering interferon-α-2b. The implementation of the UF/DF step is most beneficial after the solubilisation step of the protein recovery process (cycle-1). This results in the elimination of the TCA-precipitation step at the end of the recovery process and replacing it with the UF/DF step.

Accordingly, the optimized protein recovery process exists out of two cycles (process time 5 hours) with the addition of 3% Sarkosyl during cycle 1 and an interferon-α-2b recovery of 80%. The UF/DF method removes more than 95% of Sarkosyl while recovering more than 60% of interferon-α-2b with a process time of 4-5 hours. After the removal of the detergent from the process the first down-stream step is performed following the standard protocol. Results showed (chapter 10) that even traces of Sarkosyl (<0.03%) have a significant impact on the purification with the ion-exchange method and only 25% of interferon-α-2b is recovered in the elution fraction for further purifications. With 60% recovery during the UF/DF step and only 25% interferon recovery throughout the ion-exchange step, the overall recovery in interferon-α-2b from the initial produced amount is only 12%. Assuming, 7'000g of interferon-α-2b are produced during the fermentation process, only 840g will be recovered after the first down-stream step, which is only 1.5-fold higher compared to the current recovery. Due to the addition of 3% Sarkosyl the interferon solubility increased four-fold, however, due to detergent removal steps and the reduced

recovery throughout the purification in the presence of detergent traces, the overall improvement in interferon-α-2b recovery is only 1.5-fold.

End of Ferment.		recovery process cycles	IFN-yield		UF/DF IFN-yield		ion-exchange step runs	IFN-yield		overall IFN-yield
100%	$\rightarrow$	2	80%	$\rightarrow$	60%	$\rightarrow$	1	25%	$\rightarrow$	12%
7'000g	$\rightarrow$		5'600g	$\rightarrow$	3'360g	$\rightarrow$		840g	$\rightarrow$	840g

11.2.3 Optimized Recovery Process with 1% Zwittergent 3-14

The addition of 1.0% Zwittergent 3-14 during cycle-1 of the protein recovery process improved the protein solubility as discussed in chapter 8. Results showed, that Zwittergent 3-14 is less pH-sensitive than Sarkosyl, thus the protein recovery process can be performed in the absence or presence of the salting-out step. In the presence of the salting-out step the interferon-α-2b recovery at the end of the recovery process was 55%. Results during the characterization showed no significant impact of the salting-out step on the interferon-α-2b recovery and neither on the interferon-isoform distribution. **Hence, the salting-out step can be eliminated from the recovery process which results in a 70% recovery of interferon-α-2b due to the addition of Zwittergent 3-14.**

Results of the first down-stream step in chapter 10 showed that due to the presence of Zwittergent 3-14 the binding of interferon towards the ion-exchange column is stronger and an isocratic elution mode is more beneficial. Hence, with 1% Zwittergent 3-14 present in the process sample and an isocratic elution mode, more than 50% of interferon-α-2b is recovered throughout the first down-stream step. However, a reduced Zwittergent 3-14 concentration in the process samples indicated a negative impact on the purification by ion-exchange. Thus, the Zwittergent 3-14 (SB 3-14) concentration needs to be adjusted to 1.0% at the end of the protein recovery process prior to the down-stream process. The ion-exchange method also showed potential to remove Zwittergent 3-14 successfully from process samples with 75% removal, leaving Zwittergent 3-14 at a concentration of 0.25% in the elution fraction. Since the 70% of interferon-α-2b recovery, rather than the 8% of the standard recovery process, would almost certain exceed the capacity of the ion-exchange column, the final material from the primary recovery process would need to be divided into two fractions and the ion-exchange cycle operated twice.

Performing the protein recovery process in the absence of the salting-out step, but in the presence of 1.0% Zwittergent 3-14, followed by the ion-exchange step with an isocratic elution mode, results in 35% recovery of interferon-α-2b for the further purification steps. Assuming, 7'000g interferon-α-2b are produced during the fermentation, 2'450g are recovered throughout the protein recovery process and the first down-stream step. **This is more than a 4-fold improvement in the interferon recovery compared to the current recovery process.**

End of Ferment.		recovery process cycles	IFN-yield		SB 3-14 adjustment		ion-exchange step runs	IFN-yield		overall IFN-yield
100%	$\rightarrow$	3	70%	$\rightarrow$	100%	$\rightarrow$	2	50%	$\rightarrow$	35%
7'000g	$\rightarrow$		4'900g	$\rightarrow$	4'900g	$\rightarrow$		1'225g	$\rightarrow$	2'450g

11.3 Advantages and disadvantages of the optimized process

The previous sections proposed three different optimized recovery processes for interferon-α-2b. This section will discuss some advantages and disadvantages of each of the proposed processes to finally select the optimal recovery process.

The first optimized recovery process, using a two-fold dilution during cycle-1 of the recovery process in order to improve the protein solubility was proposed in section 11.2.1. A three-fold increase in total interferon-α-2b recovery was achieved after the first down-stream step, from 8% recovery to a 24% overall recovery. However, a negative aspect and challenging problem is the increase in process volume in cycle-1 due to the dilution step. In order to implement this process change, the availability of a larger reactor needs to be assured. Another problem for the implementation of this optimized process at manufacturing scale can be the capacity of the final centrifugation step of the primary recovery, which will need to process twice as much volume than before. This separator run needs to be performed twice in order to process the entire process volume, which increases process time and cost.

In summary, the optimized recovery process using a two-fold dilution gives a three-fold increase in interferon-α-2b recovery, however the implementation at large scale can be challenging, although productivity and yield would increase significantly compared to the standard recovery process.

The optimized recovery process using an addition of 3% Sarkosyl during cycle-1 of the protein recovery process resulted in only a 1.5-fold increase in interferon-α-2b recovery after the first down-stream step. However, these calculations are worst case scenarios and it is very likely that the recovery, especially during the UF/DF step, will be improved. Using 3% Sarkosyl during the recovery process resulted in the highest interferon-α-2b solubility of all three processes. However, due to the characteristics of the detergent, the implementation of a new detergent-removal method would be indispensable. The removal of the detergent, followed by the first down-stream step result in an increased loss of interferon-α-2b and only 12% overall recovery. Another negative aspect of this optimized recovery process is the three-fold increase in process volume after the UF/DF step which needs to be processed by the ion-exchange method. Unfortunately a reduction of volume is not possible as it would increase the detergent concentration.
In summary, the addition of Sarkosyl did result in the highest interferon-α-2b solubility but due to unavoidable implementation of a detergent removal method, the overall interferon-α-2b is only 1.5-fold increased compared to the current recovery. Hence, it is not worth to continue with the implementation of this optimized recovery process at pilot scale or manufacturing scale.

The last proposed optimized recovery process was the addition of 1.0% Zwittergent 3-14 during cycle-1 of the recovery process to increase protein solubility. This optimized process results in more than four-fold increment in the overall interferon-α-2b recovery. The application of this optimized process at larger scale is the less challenging one without any significant volume increments or implementation of new methods. Only small changes, such as the addition of the detergent and the elimination of the salting-out step, need to be adjusted for the optimized process. Also the performance of the ion-exchange method was simplified by using an isocratic elution mode instead of a gradient elution. However, due to the increase in interferon recovery throughout the protein recovery process, the first step of the down-stream process needs to be performed twice, which results in an increase in process time and cost.
In summary, the addition of 1.0% Zwittergent 3-14 during cycle-1 of the recovery process and the elimination of the salting-out step results in the optimal protein recovery process with a four-fold increase in the interferon-α-2b recovery.
Another point which needs to be considered with the optimized protein recovery process is the

variation in protein production throughout the fermentation. As shown in chapter 4 the amount of protein at the end of the fermentation can vary $\pm 20\%$. So far these variations in total interferon-α-2b could not be observed in the final TCA-pellet at the end of the recovery process (due to protein saturation problems in cycle-1) and thus had no impact on the down-stream process. However, with the enhanced protein solubility in cycle-1 due to the addition of Zwittergent 3-14 these variations in interferon-α-2b will be detectable in the final TCA-pellet and need to be considered during the down-stream process. In order to ensure and maintain reproducibility throughout the entire down-stream process, the interferon-α-2b concentration in the feed-volume of the first ion-exchange method should be kept constant with a tolerance level of $\pm 5\%$ and if necessary the down-stream process needs to be performed twice or three times.
A schematic illustration of the most efficient optimized protein recovery process can be seen in figure 11.1.

The optimized protein recovery process for interferon-α-2b in figure 11.1 shows the addition of 1.0% Zwittergent 3-14 during cycle 1 and the elimination of the salting-out step resulting in a four-fold increase in interferon recovery after the first down-stream step.
Throughout this study the impact of Zwittergent 3-14 on the protein activity of interferon-α-2b was not investigated. However, Croze et al. [114] have shown that interferon-β retained its full bio-activity in the presence of 2.5% Zwittergent 3-14. These results suggest that interferon-α-2b would almost certain retain its bio-activity in the presence of Zwittergent 3-14.
Besides the bio-activity of interferon-α-2b, the impact of Zwittergent 3-14 on the interferon-isoform distribution was not completely investigated. Results with the currently available method indicated no significant difference in the isoform distribution due to solubilisation in the presence of Zwittergent 3-14, however further studies with complete mass balances need to be performed using a more accurate analytical method than currently available.

In summary, the optimized protein recovery process for interferon-α-2b with the addition of 1.0% Zwittergent 3-14 resulted in a four-fold increase in the interferon-α-2b recovery. The process time of the of the recovery process itself was reduced from 16 to 14 hours. However, the down-stream process time was increased due to the performance of two or three purification runs of the same TCA-pellet. Since the majority of the process costs and process time is during the fermen-

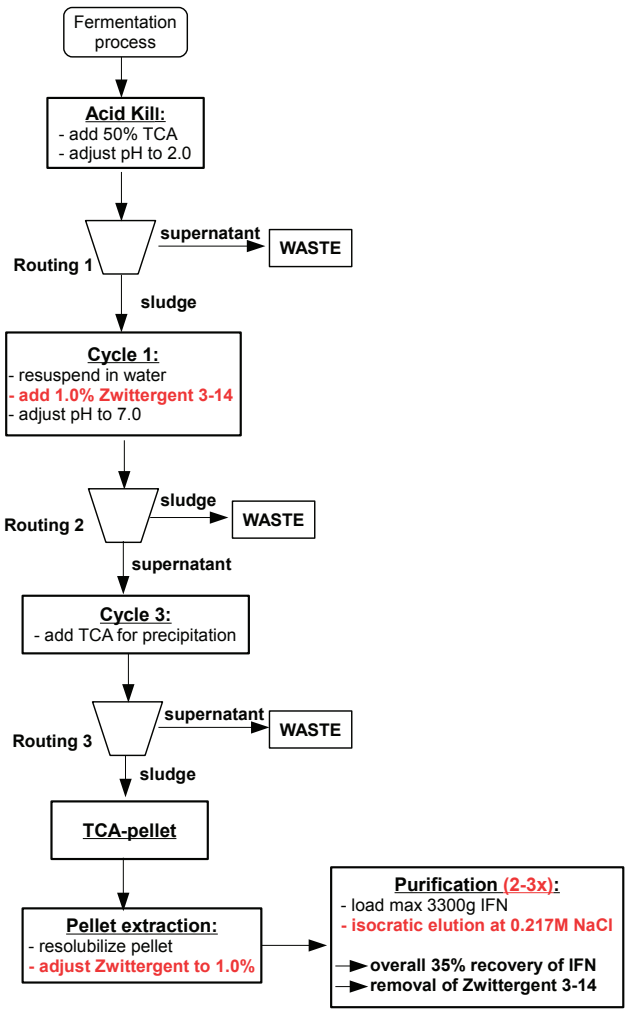

Fig. 11.1: The optimized primary protein recovery process for interferon-α-2b with 1.0% Zwittergent 3-14 addition during cycle-1 and in the absence of the salting-out step. First down-stream step, an ion-exchange method, is performed with isocratic elution. Overall IFN recovery is 35% which is a four-fold increase to the current recovery.

tation process, it is beneficial to perform the fermentation once, recover an increased amount of interferon-α-2b and perform the down-stream process two or three times as necessary.

11.4 Conclusion

This chapter summarized all findings of the previous chapters 7, 8, 9 and 10 and proposed three optimized protein recovery processes using a two-fold dilution, addition of 3% Sarkosyl and addition of 1% Zwittergent 3-14 in cycle-1 of the recovery process. The interferon-α-2b recovery throughout the recovery process and until the first down-stream step was analysed and compared to the current process performance. A two-fold dilution resulted in a three-fold increase in IFN recovery, the addition of 3% Sarkosyl resulted only in 1.5-fold increment in IFN and the addition of 1% Zwittergent 3-14 resulted in more than four-fold increase in the interferon recovery after the first down-stream step.

Following the proposed optimized recovery processes, advantages and disadvantages were discussed for each of the optimized recovery processes in order to find the most efficient and applicable protein recovery process. The addition of 1.0% Zwittergent 3-14 during cycle-1 of the recovery process turned out to be the most applicable and efficient optimized process for an increased interferon-α-2b recovery. The optimized recovery process is very similar to the current process which makes it easier for the implementation of the new process at larger scale. On the other hand, this optimized process resulted in the highest interferon-α-2b recovery throughout the recovery process and the first down-stream step.

The next step is the implementation of the optimized recovery process and the first down-stream step at manufacturing or pilot scale. However, prior to this step three other aspects of the optimized process need to be verified first. The impact of Zwittergent 3-14 addition on the bio-activity on interferon-α-2b and on the interferon-isoform distribution. And the impact of possible variations in the protein production on the optimized recovery process as well on the down-stream process.

Chapter 12

Summary, Conclusion and Perspective

12.1 Introduction

The results obtained in chapters 3 to 11 will first be summarized before comparing them to the different objectives of this thesis. Following the gathering of the results, conclusion will be drawn and an outlook will be given for the next steps. Table 12.1 summarizes the objectives of this thesis, as they were already listed in table 2.1 in section 2.4.

Tab. 12.1: Thesis objectives

Work package	Objectives
1.) Analytical Toolkit	• total protein (Bradford, BCA-assay) • total interferon-α-2b (SDS-PAGE, Western Blot, Biacore) • isoforms (RP-HPLC & purification, concentration)
2.) Process Characterization	• total protein (mass balances & CPS) • total interferon-α-2b (mass balances & CPS) • isoforms (mass balances)
3.) Process Optimization	• solubility study (dilution experiment, detergents) • detergent removal • impact on DSP

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12.2 Summary

Chapter 3 showed the successful set-up of a small scale model of the industrial primary protein recovery process. The performance of simulation runs varying different parameters such as volume, protein concentrations and type of protein, provided first information about the characteristics of this process and the small scale model. The simulations were also used for the selection and set-up of analytical methods.
The Bradford assay was identified as the most accurate method in order to detect the amount of total protein in recovery process samples using BSA as the reference standard. The simulations also showed that the model provides more reliable results as the working volume increases i.e. with higher scales. A last result was the finding of the recovery process non-specificity to different types of proteins.

In chapter 4 the primary recovery process was characterized and protein mass balances have been set-up throughout the entire recovery process. An overall loss of up to 90% in total protein was identified throughout the recovery process. This loss occurred mainly in two identified critical process steps (CPS). Cycle 1 was selected as the critical process step 1 with a loss of more than 80% of total protein. The main problem of CPS-1 is the poor solubility of proteins and low saturation levels of proteins in water. The second critical process step was identified during cycle 2, with a total protein loss of 40% due to salting-out by the addition of NaCl and HCl.

Chapter 5 contained the setting-up of mass balances for total interferon-α-2b throughout the primary recovery process. An overall loss of 80% in interferon-α-2b was detected. The same critical process steps as identified for total protein were identified for interferon-α-2b. CPS-1, during cycle 1, showed a loss of 60% interferon, and CPS-2, during cycle 2, a loss of up to 30%.

The first part of chapter 6 focused on the development and optimization of quantification methods for interferon-isoforms. Three RP-HPLC were successfully optimized with design of experiment for their accuracy and resolution. However, accuracy and resolution were not sufficient to quantify interferon-isoforms in all recovery process samples. Host cell proteins and low interferon-isoform concentration were interfering with the analytical methods. Hence, purification and concentration methods for interferon-isoforms were investigated to purify interferon prior to the quantification. However, no technique met the required criteria to purify interferon-isoforms successfully.

Selected samples of the recovery process were analysed anyway for the interferon-isoform distribution with the optimized RP-HPLC methods. Results were less accurate and robust but confirmed the findings of chapters 4 and 5 of two critical process steps and a total interferon loss of 75%. The main reduction was identified in isoform-2 and isoform-3, whereas isoform-4 and interferon-α-2b showed only a 50% loss throughout the recovery process.

Chapter 7 discussed the dilution study around the critical process step 1 in order to increase protein solubility and to prevent protein saturation. A two-fold dilution during cycle 1 of the protein recovery process resulted in 15% increase of interferon-α-2b solubility. However, this improvement was not detectable at the of the recovery process due to increased losses throughout the critical process step 2 (salting-out step). An elimination of the salting-out step resulted in 15-20% improvement in interferon-α-2b recovery at the end of the process compared to a one-fold dilution. At the end of this chapter the impact of a two-fold dilution during cycle 1 of the recovery process on the first down-stream step was investigated. No significant impact of an increase in interferon-α-2b recovery on the ion-exchange method was detected.

In chapter 8 different detergents were tested to increase interferon-α-2b solubility throughout the critical process step 1. Two detergents, Sarkosyl and Zwittergent 3-14 were identified as having the highest potential for improving IFN solubility. Sarkosyl at a concentration of 3% resulted in 90% solubility of interferon-α-2b during CPS-1. However, this increment was not detectable at the end of the recovery process due to an increased loss of interferon throughout the salting-out step. In the absence of the salting-out step more than 75% of interferon-α-2b was recovered at the end of the recovery process. Zwittergent 3-14 at a concentration of 1.0% resulted in 80% IFN solubility during the critical process step one and an overall IFN recovery of 55% at the end of the recovery process. However, in the absence of the salting-out step this IFN recovery could be improved to 70%, which is a 3.5-fold improvement compared to the original recovery process.

Chapter 9 focused on the removal of the two detergents, Sarkosyl and Zwittergent 3-14, from process samples. In a first step two RP-HPLC methods were developed to quantify Sarkosyl and Zwittergent 3-14 in recovery process samples. In a second step five different techniques were tested for their potential to remove Sarkosyl and/ or Zwittergent 3-14 from process samples. Under consideration of the application at large-scale, the detergent removal yield and interferon-α-2b recovery, an ultrafiltration/ diafiltration technique showed the most promising results. Using

a membrane with a 10kDa cut-off, more than 80-95% of Sarkosyl could be removed from the samples while recovering between 80-60% of interferon-α-2b and 80% of Zwittergent 3-14 was removed while recovering 50% of the interferon.

In chapter 10 the impact of the presence of the two detergents, Sarkosyl and Zwittergent 3-14 on the first down-stream step was investigated. In a first step the ion-exchange chromatography method was characterized and results showed that only 38-40% of the initial interferon-α-2b was recovered in the elution fractions of the column. After the investigation of several different factors, the presence of host cell proteins was identified as the biggest impact on poor interferon-α-2b recovery throughout this purification step.

In a second step the impact of the presence of Sarkosyl and Zwittergent 3-14 on the interferon purification with the ion-exchange method was investigated. The presence of 3% Sarkosyl resulted in a blockage of the column which led to the conclusion that Sarkosyl needs to be removed from the process prior to the down-stream step. However, even a 99% removal of Sarkosyl, leaving the detergent at a concentration of 0.03%, resulted in a significant impact on the interferon purification and only 20-25% of the initial loaded interferon-α-2b was recovered in the elution fractions.

The presence of Zwittergent 3-14 in the process samples had less impact on the down-stream step. In the presence of 1.0% Zwittergent 3-14 the binding affinity between interferon and the column was increased and the elution of IFN was delayed. Changing the elution mode to an isocratic elution at 0.217M NaCl resulted in 40-45% recovery of the initial loaded interferon-α-2b in the elution fraction. In parallel more than 90% of the detergent concentration was removed throughout this ion-exchange method which resulted in a Zwittergent 3-14 concentration of 0.25% prior to the next purification step. However, reduced Zwittergent 3-14 concentrations of less than 1.0% resulted in a negative impact of the interferon-α-2b purification unless the detergent concentration was below 0.05%.

Chapter 11 summarized all findings from chapters 7, 8, 9 and 10 and proposed three new optimized recovery processes including the first down-stream step. After discussion of advantages and disadvantages of each optimized process the addition of 1.0% Zwittergent 3-14 in the absence of the salting-out step and the implementation of an isocratic elution during the ion-exchange method resulted in the most applicable and efficient optimized recovery processes with a four-fold increase in interferon-α-2b recovery.

The following table 12.2 summarizes the results achieved in the optimization of the primary protein recovery process (chapters 7 and 8) and shows the losses in interferon-α-2b and total protein throughout the primary protein recovery process in its current version and from the optimized processes.

Tab. 12.2: Summary of the recovery of total protein, total interferon-α-2b and interferon-isoforms throughout the primary protein recovery process of the manufacturing process, 2-fold dilution process, with 3% Sarkosyl and with 1.0% Zwittergent 3-14

process description			**protein losses in [%]**				**recovery in [%]**
process	salting-out pres/ abs	protein analysed	Acid Kill	Cycle-1 solubility	Cycle-2 salting-out	Cycle-3 TCA-pellet	overall recovery
manufacturing process	present	total prot.	20%	75%	40%	<1%	10%
		total IFN	20%	65%	30%	<1%	20%
		isoforms	-	58%	40%	-	25%
2x dilution process	present	total prot.	-	50%	80%	<1%	5%
		total IFN	-	40%	80%	<1%	8%
	absent	total prot.	-	50%	-	<1%	50%
		total IFN	-	40%	-	<1%	**60%**
3.0% Sarkosyl process	present	total prot.	-	10%	70%	<5%	20%
		total IFN	-	20%	65%	<5%	20%
	absent	total prot.	-	10%	-	<5%	90%
		total IFN	-	20%	-	<5%	**75%**
1.0% Zwitter-gent 3-14 process	present	total prot.	-	22%	50%	<1%	40%
		total IFN	-	20%	30%	<1%	**55%**
	absent	total prot.	-	22%	-	<5%	65%
		total IFN	-	20%	-	<5%	**70%**

Table 12.2 shows that almost all modified recovery processes show an increase in interferon-α-2b recovery. However, as discussed in chapter 11 the implementation of a two-fold dilution during the recovery process can be challenging in its implementation at large scale and the presence of Sarkosyl had a negative impact on the interferon-α-2b purification. Hence, the most applicable

and efficient optimized recovery process is the addition of 1.0% Zwittergent 3-14 in the absence of the salting-out step and implementation of an isocratic elution mode during the ion-exchange purification step.

12.3 Conclusion

In this PhD-thesis the primary protein recovery process was characterized for total protein, total interferon-α-2b and interferon-isoforms and two critical process steps were identified during cycle 1 and 2 for all types of proteins. Since total protein and interferon-α-2b both followed similar characteristics throughout the recovery process, these results confirmed the findings of chapter 3, where no protein specificity of the recovery process could have been seen in the small-scale study of the primary protein recovery process. CPS-1 was identified with the majority of protein loss, which makes this step as the number one priority for optimization studies. Interferon-α-2b was detected in the waste stream of the centrifugation step post cycle 1 due to insufficient IFN solubility and low saturation levels in water.

Table 12.1 shows the objectives of this project divided into three work packages and the majority of work package one and two have been successfully completed and discussed during chapter 3, 4, 5 and 6. The methods of the analytical toolkit have been set up and were used in order to achieve the aims of work package 2, characterization of the primary protein recovery process. During the characterization, mass balances have been set-up and critical process steps could be identified for total protein as well as total interferon-α-2b.

The results for work package three were discussed in chapter 7, 8, 9 and 10. A protein solubility study was performed, with the application of a dilution and the addition of two different detergents in order to improve interferon-α-2b solubility and to increase the IFN recovery at the end of the process. Furthermore, methods for the removal of those detergents have been developed and the impact on the first down-stream step of the optimized recovery processes was investigated.

With the findings of this thesis it can be concluded that the primary protein recovery process for interferon-α-2b was successfully optimized and the interferon recovery four-fold increased due to the addition of Zwittergent 3-14 during cycle 1 of the recovery process. This optimized primary protein recovery process, as discussed in chapter 11 can be seen in figure 12.1.

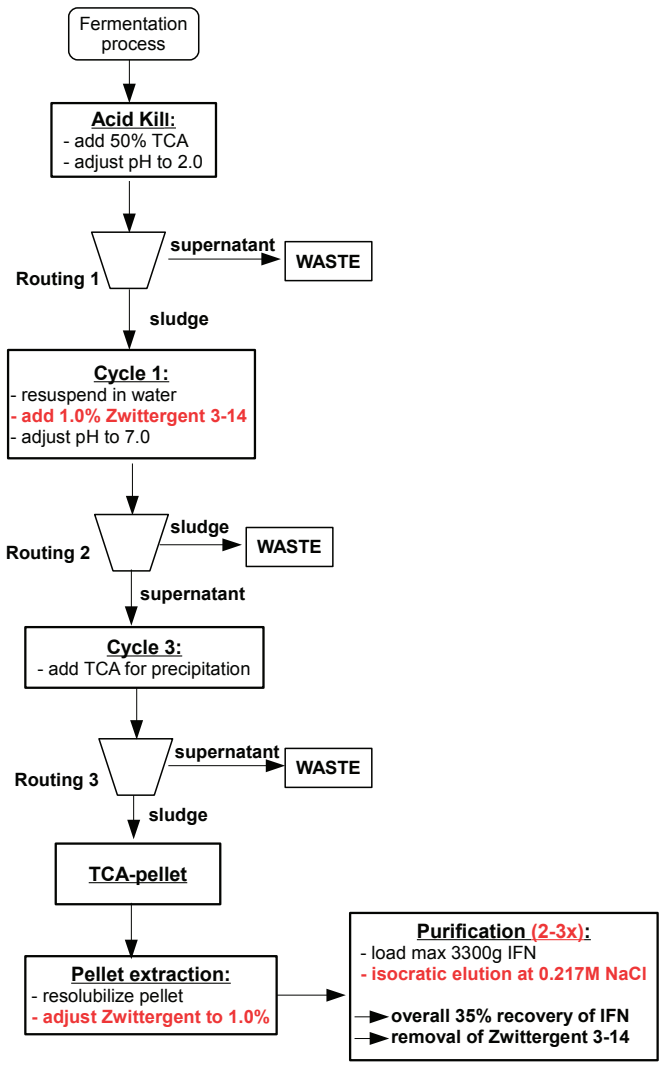

Fig. 12.1: The optimized primary protein recovery process for interferon-α-2b with 1.0% Zwittergent 3-14 addition during cycle-1 and in the absence of the salting-out step. First down-stream step, an ion-exchange method, is performed with isocratic elution. Overall IFN recovery is 35% which is a four-fold increase to the current recovery.

12.4 Perspective

As discussed in the previous section, all objectives of the three work packages were successfully achieved throughout this project. However, further tasks need to be carried out for the successful application of the optimized recovery process. An obvious step is the implementation of the optimized recovery process at a pilot scale using continuous centrifugation systems to simulate similar process conditions at manufacturing scale. Furthermore, the application of the isocratic

elution mode of the ion-exchange method needs to be tested in a pilot scale or manufacturing scale to verify the findings of the small scale process. To improve the overall process yield further the interferon-α-2b recovery of the first down-stream step can be enhanced by optimizing IFN purification using ion-exchange method.

Besides the implementation of the optimized recovery process at large scale, the impact of the addition of Zwittergent 3-14 on the protein bio-activity and the interferon-isoform distribution needs to be investigated. Hence, an analytic method for interferon-α-2b bio-activity is required and the protein activity needs to be tested in the presence and absence of the detergent and at the end of the down-stream process. In order to study the impact of Zwittergent 3-14 on the interferon-isoform distribution a more accurate analytical method is required to quantify all different isoforms in recovery process samples. This could be achieved by the implementation of a UPLC method or a LC-MS method or the development of efficient purification methods to purify interferon from host cell proteins prior to quantification.

Another step to increase the overall interferon-α-2b yield is the optimization of the fermentation process to increase the protein productivity. So far a simple batch process is used with two uncontrolled temperature shifts. To optimize the interferon productivity the temperature shifts can be controlled throughout the process with the application of calorimetric technology and the implementation of a feeding strategy throughout the bio-process, using a fed-batch process can improve the interferon-α-2b productivity even further.

Besides the improvement in protein productivity, the optimization of the fermentation process can also resolve the problem with variations in the protein production throughout the fermentation. Having a more stable and robust protein production would reduce the variations throughout the primary protein recovery process and the down-stream process.

12.5 Final Conclusion

This project collaboration between the industrial partner and NIBRT/ DCU has shown that with strategic planning, process optimization can be achieved which results in an increase of the overall process yield. First, the setting-up of an analytical toolkit provides robust and reliable methods in order to carry out process characterizations and to identify critical process steps by setting-up mass balances. In a second step, these critical process steps can be optimized with the help of design of experiments in order to increase the overall process yield. In a last step, the impact of the optimized process on following process steps needs to be investigated and considered for the selection of the most efficient and applicable optimized process.

In this project this strategic planning resulted in an optimized primary protein recovery process with a four-fold increase in interferon-α-2b recovery due to the addition of a detergent and the elimination of a process step.

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Appendix A

Statistics for design of experiment

A.1 Statistics for RP-HPLC optimization

Bakerbond-method

Exp No	Exp Name	Run Order	Incl/Excl	Injection Volume	IFN Load	Flow Rate	Temperature	Retention time	Area	Peak width
1	A	3	Incl	10	2	1	35	13.528	2019.1	0.7336
2	A	16	Excl	40	2	1	35	9.855	482.22	0.6682
3	A	14	Incl	10	20	1	35	12.513	23015.6	0.6056
4	B	27	Incl	40	20	1	35	12.385	22808.5	0.4442
5	B	28	Incl	10	2	3.2	35	8.791	720.803	0.5243
6	A	26	Incl	40	2	3.2	35	9.855	482.22	0.6682
7	A	19	Incl	10	20	3.2	35	8.794	7071.06	0.5807
8	A	20	Incl	40	20	3.2	35	8.83	6530.6	0.6053
9	A	9	Incl	10	2	1	55	13.363	2268.39	0.5962
10	B	13	Incl	40	2	1	55	12.599	1973.71	0.3864
11	A	11	Incl	10	20	1	55	12.435	25285.7	0.5688
12	A	2	Incl	40	20	1	55	12.532	22761.5	0.5785
13	A	18	Incl	10	2	3.2	55	9.515	605.265	0.617
14	A	1	Incl	40	2	3.2	55	9.592	546.666	0.6216
15	B	29	Incl	10	20	3.2	55	8.315	6855.89	0.4561
16	A	4	Incl	40	20	3.2	55	8.875	6329.14	0.6227
17	C	6	Incl	10	11	2.1	45	9.674	5322.93	0.423
18	C	8	Incl	40	11	2.1	45	9.668	6353.13	0.42
19	C	21	Incl	25	2	2.1	45	9.99	962.059	0.3969
20	C	7	Incl	25	20	2.1	45	9.536	10042.6	0.4363
21	C	12	Incl	25	11	1	45	12.732	11723.6	0.4108
22	C	5	Incl	25	11	3.2	45	8.569	3572.96	0.4384
23	C	23	Incl	25	11	2.1	35	9.525	5507.69	0.4321
24	C	22	Incl	25	11	2.1	55	9.133	5563.78	0.4242
25	A	15	Incl	25	11	2.1	45	9.685	5908.6	0.3787
26	A	25	Incl	25	11	2.1	45	9.655	5836.8	0.3798
27	A	10	Incl	25	11	2.1	45	9.618	5809	0.3778
28	B	24	Incl	25	11	2.1	45	9.727	5514.77	0.4274
29	C	17	Incl	25	11	2.1	45	9.777	5522.26	0.4207

Fig. A.1: The full list of experiments, factor levels and results for all three responses for the design of experiment of the bakerbond column: Experiments in the categories A and B were performed first. In order to improve the developed model experiments from category C were added.

Retention time	Coeff. SC	Std. Err.	P	Conf. int(±)
Constant	9.63527	0.0752218	7.16906e-032	0.156433
Injection Volume	0.0227579	0.0612295	0.713853	0.127334
IFN Load	-0.334424	0.0612295	2.03156e-005	0.127334
Flow Rate	-1.88626	0.0612295	5.78162e-019	0.127334
Temperature	-0.0479801	0.0612295	0.442018	0.127334
F*F	1.2661	0.0969916	1.51897e-011	0.201706
Inj*F	0.191147	0.0652582	0.00801781	0.135712
N = 28	Q2 =	0.964	Cond. no. =	2.975
DF = 21	R2 =	0.982	RSD =	0.2495
	R2 adj. =	0.977		
			Conf. lev. =	0.95
Peak width	**Coeff. SC**	**Std. Err.**	**P**	**Conf. int(±)**
Constant	0.386163	0.0179513	2.72818e-014	0.0377143
Injection Volume	-0.014469	0.0134772	0.297188	0.0283145
IFN Load	-0.00799768	0.0134772	0.560281	0.0283145
Flow Rate	0.0173468	0.0134772	0.214358	0.0283145
Temperature	-0.0122476	0.0134772	0.37548	0.0283145
Inj*Inj	0.0442669	0.0341588	0.211376	0.0717649
Load*Load	0.0393669	0.0341588	0.264206	0.0717649
F*F	0.0473669	0.0341588	0.182479	0.0717649
T*T	0.0509169	0.0341588	0.153384	0.0717649
Inj*F	0.0585526	0.0143645	0.000708786	0.0301787
N = 28	Q2 =	0.534	Cond. no. =	6.456
DF = 18	R2 =	0.818	RSD =	0.05489
	R2 adj. =	0.728		
			Conf. lev. =	0.95

Fig. A.2: The full list of coefficients for the models retention time and peak width of the bakerbond column, including the p-values of ANOVA. The list also provides the numbers of experiments included (N), the degress of freedom (DF), the R^2 and Q^2 values of each model and the condition number (cond.no)

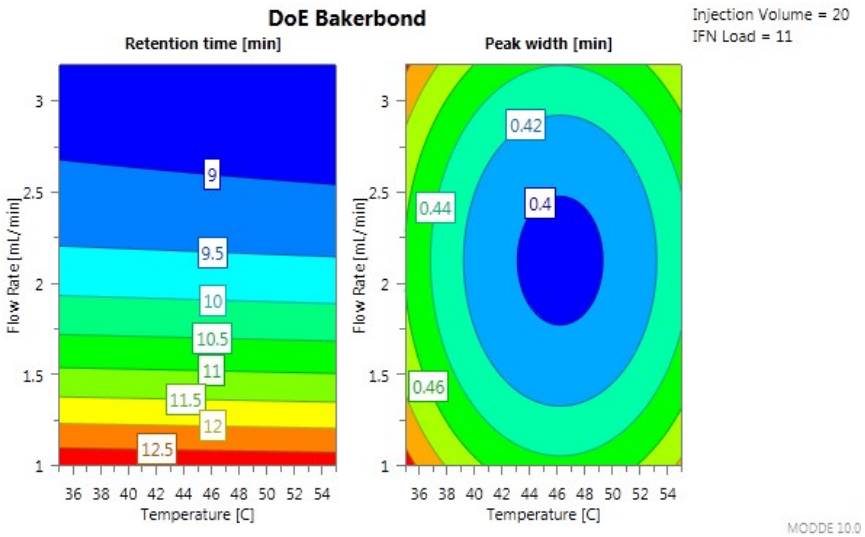

Fig. A.3: Response Surface Plot of the DoE investigation for the bakerbond-method. The retention time (left) and peak width (right) dependent on the flow rate and column temperature. Injection volume is set to constant at 20μL and interferon load is set constant to 11μg.

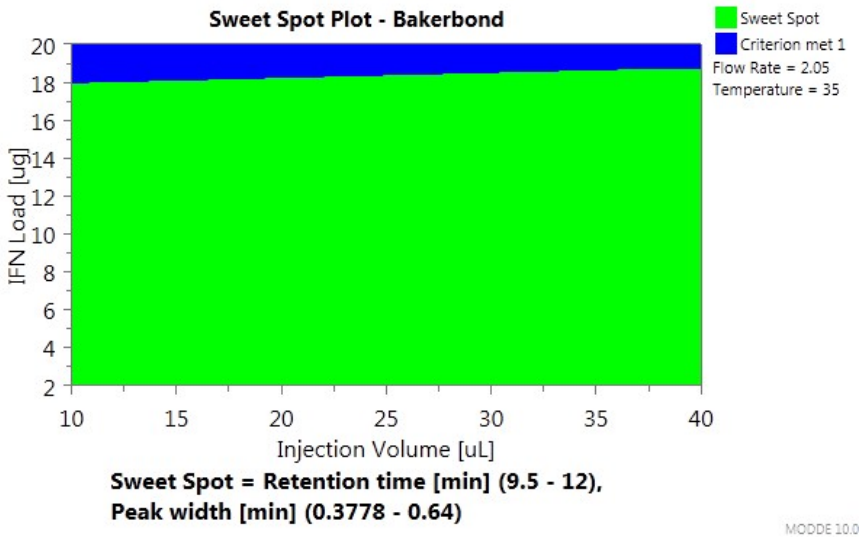

Fig. A.4: Sweet-Spot-Plot of the DoE investigation for the bakerbond-method. The retention time and peak width in dependency on the IFN load and injection volume with column temperature and flow-rate set constant at 35°C and 2.05ml/min. The sweet spot (green area) indicates where both criteria are fullfilled: retention time between 9.5 - 12min and peak width <0.64min.

Zorbax-method

Exp No	Exp Name	Run Order	Incl/Excl	Injection vol.	IFN load	Flow rate	Temp.	Retention time	Area	Peak width
1	N1	20	Incl	10	2	0.5	25	20.375	4875.1	0.3192
2	N2	2	Incl	40	2	0.5	25	20.329	3800.8	0.3003
3	N3	9	Excl	10	20	0.5	25			
4	N4	16	Incl	40	20	0.5	25	20.177	39998.6	0.4145
5	N5	14	Incl	10	2	2	25	14.116	1131.11	0.2363
6	N6	23	Incl	40	2	2	25	14.07	1005.79	0.2342
7	N7	5	Incl	10	20	2	25	13.787	13178.2	0.294
8	N8	25	Incl	40	20	2	25	13.757	20472.6	0.3299
9	N9	13	Incl	10	2	0.5	55	22.072	4520.14	0.2863
10	N10	19	Incl	40	2	0.5	55	22.117	3063.72	0.2712
11	N11	6	Incl	10	20	0.5	55	21.811	45358.1	0.4155
12	N12	7	Incl	40	20	0.5	55	21.871	38788.1	0.3934
13	N13	27	Incl	10	2	2	55	13.757	1079.67	0.1871
14	N14	8	Incl	40	2	2	55	13.684	889.658	0.1811
15	N15	15	Incl	10	20	2	55	13.495	12583.9	0.2959
16	N16	21	Incl	40	20	2	55	13.445	10231.9	0.2753
17	N17	26	Incl	10	11	1.25	40	15.608	10351.4	0.2558
18	N18	3	Incl	40	11	1.25	40	15.651	11085	0.2686
19	N19	1	Incl	25	2	1.25	40	15.714	1681.83	0.2324
20	N20	11	Incl	25	20	1.25	40	15.54	19833.7	0.3356
21	N21	10	Incl	25	11	0.5	40	21.986	25565.9	0.3536
22	N22	4	Incl	25	11	2	40	14.463	6418.98	0.2387
23	N23	17	Incl	25	11	1.25	25	14.53	10395	0.2437
24	N24	12	Incl	25	11	1.25	55	14.963	10666.8	0.2621
25	N25	24	Incl	25	11	1.25	40	15.662	10086.1	0.2565
26	N26	22	Incl	25	11	1.25	40	15.607	10080.2	0.2586
27	N27	18	Incl	25	11	1.25	40	15.646	10312.1	0.2656

Fig. A.5: The full list of experiments, factor levels and results for all three responses for the design of experiment of the zorbax column: Experiment number 3 was excluded from the model, since no results were generated for this HPLC-run.

Retention time	Coeff. SC	Std. Err.	P	Conf. int(±)
Constant	15.6206	0.0227405	0	0.047776
Injection vol.	-0.00456105	0.0157258	0.775104	0.0330388
IFN load	-0.121605	0.0157258	3.96181e-007	0.0330388
Flow rate	-3.68478	0.0157258	8.09361e-033	0.0330388
Temp.	0.32844	0.0157258	4.55421e-014	0.0330388
F*F	2.6456	0.0351869	6.07208e-024	0.0739249
T*T	-0.8324	0.0351869	5.2126e-015	0.0739249
F*T	-0.511057	0.016761	6.018e-017	0.0352135
N = 26	Q2 =	0.999	Cond. no. =	5.176
DF = 18	R2 =	1.000	RSD =	0.06406
	R2 adj. =	1.000		
			Conf. lev. =	0.95
Peak width	**Coeff. SC**	**Std. Err.**	**P**	**Conf. int(±)**
Constant	0.260946	0.00466316	7.84574e-019	0.00993924
Injection vol.	-0.00152032	0.00327429	0.649087	0.00697895
IFN load	0.0506537	0.00327429	1.25472e-010	0.00697895
Flow rate	-0.0492925	0.00327429	1.84452e-010	0.00697896
Temp.	-0.0116648	0.00327429	0.00283533	0.00697895
Load*Load	0.0151922	0.00722256	0.0527128	0.0153944
F*F	0.0273422	0.00722256	0.00179509	0.0153944
Inj*T	-0.00546464	0.00349584	0.138858	0.00745118
Load*F	-0.00598536	0.00349585	0.107462	0.00745118
Load*T	0.00626463	0.00349585	0.09332	0.00745118
F*T	-0.00510216	0.00349584	0.165054	0.00745118
N = 26	Q2 =	0.913	Cond. no. =	5.185
DF = 15	R2 =	0.972	RSD =	0.01313
	R2 adj. =	0.953		
			Conf. lev. =	0.95

Fig. A.6: The full list of coefficients for the models retention time and peak width of the zorbax column, including the p-values of ANOVA. The list also provides the numbers of experiments included (N), the degress of freedom (DF), the R^2 and Q^2 values of each model and the condition number (cond.no)

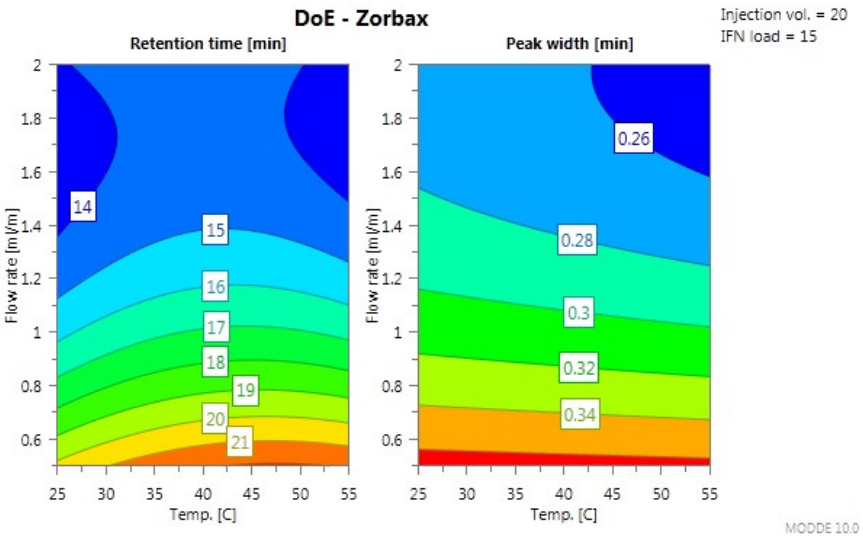

Fig. A.7: Response Surface Plot of the DoE investigation for the zorbax-method. The retention time (left) and peak width (right) dependent on the flow rate and column temperature. Injection volume is set to constant at 20μL and interferon load is set constant to 15μg.

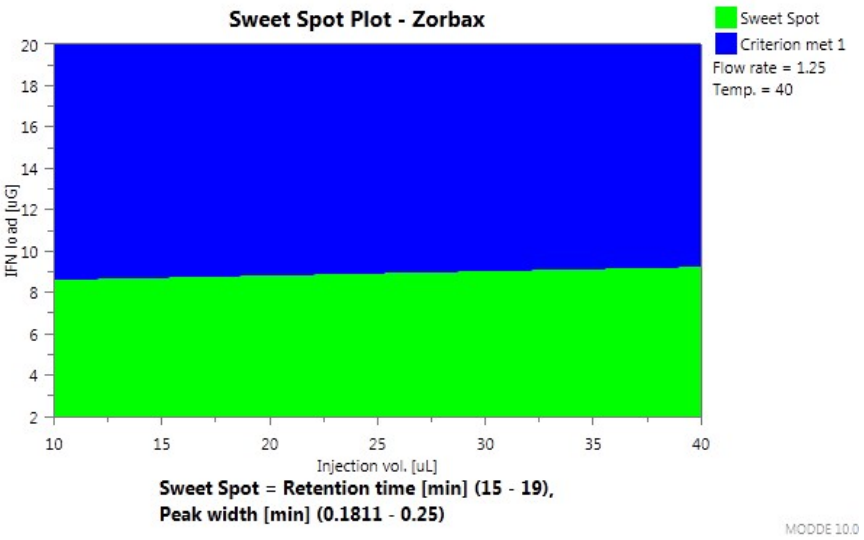

Fig. A.8: Sweet-Spot-Plot of the DoE investigation for the zorbax-method. The retention time and peak width in dependency on the IFN load and injection volume with column temperature and flow-rate set constant at 40°C and 1.25ml/min. The sweet spot (green area) indicates where both criteria are fullfilled: retention time between 15 - 19min and peak width <0.25min.

Aeris-method

Exp No	Exp Name	Run Order	Incl/Excl	Injection volume	IFN load	Flow rate	Temp.	Rt	Area	Width
1	N1	24	Incl	10	2	0.5	25	15.708	2501.39	0.4374
2	N2	20	Incl	40	2	0.5	25	14.627	2551	0.4211
3	N3	2	Incl	10	20	0.5	25	14.19	32987.2	0.8899
4	N4	12	Incl	40	20	0.5	25	14.386	33054.7	1.0103
5	N5	11	Incl	10	2	2.5	25	8.81	796.99	0.6185
6	N6	19	Incl	40	2	2.5	25	8.383	681.832	0.4016
7	N7	3	Incl	10	20	2.5	25	8.137	10641.8	0.8087
8	N8	5	Incl	40	20	2.5	25	8.109	10508.7	0.7637
9	N9	26	Incl	10	2	0.5	55	16.471	2500.95	0.4215
10	N10	17	Incl	40	2	0.5	55	16.49	2660.28	0.4044
11	N11	15	Incl	10	20	0.5	55	15.985	32644.3	0.8371
12	N12	27	Incl	40	20	0.5	55	17.241	31741.5	0.8114
13	N13	10	Incl	10	2	2.5	55	8.702	622.378	0.3958
14	N14	9	Incl	40	2	2.5	55	8.612	612.236	0.3924
15	N15	8	Incl	10	20	2.5	55	8.616	9764.85	0.7205
16	N16	23	Incl	40	20	2.5	55	8.288	8096.84	0.6842
17	N17	25	Incl	10	11	1.5	40	9.622	6246.05	0.6534
18	N18	1	Incl	40	11	1.5	40	9.676	6664.97	0.6035
19	N19	14	Incl	25	2	1.5	40	9.784	1040.63	0.4008
20	N20	6	Incl	25	20	1.5	40	9.371	11486.4	0.6966
21	N21	22	Incl	25	11	0.5	40	15.738	16748.8	0.7354
22	N22	7	Incl	25	11	2.5	40	9.01	3999.22	0.6039
23	N23	21	Incl	25	11	1.5	25	8.803	7212.59	0.6647
24	N24	13	Incl	25	11	1.5	55	9.136	6296.23	0.591
25	N25	18	Incl	25	11	1.5	40	9.658	6487.52	0.6237
26	N26	16	Incl	25	11	1.5	40	9.503	6482.59	0.6216
27	N27	4	Incl	25	11	1.5	40	9.697	6563.85	0.6682

Fig. A.9: The full list of experiments, factor levels and results for all three responses for the design of experiment of the aeris column.

Rt	Coeff. SC	Std. Err.	P	Conf. int(±)
Constant	9.57798	0.102257	2.37017e-023	0.216775
Injection volume	-0.0238339	0.0679202	0.730234	0.143984
IFN load	-0.181333	0.0679202	0.0167772	0.143984
Flow rate	-3.56494	0.0679202	2.43332e-019	0.143984
Temp.	0.466	0.0679202	3.82779e-006	0.143984
F*F	2.92857	0.157832	3.03135e-012	0.334589
T*T	-0.475934	0.157832	0.00821068	0.334589
Inj*Load	0.167187	0.0720403	0.033835	0.152718
Inj*T	0.137313	0.0720403	0.0747682	0.152718
Load*T	0.160063	0.0720403	0.041066	0.152718
F*T	-0.406063	0.0720403	3.71367e-005	0.152718
N = 27	Q2 =	0.981	Cond. no. =	5.301
DF = 16	R2 =	0.995	RSD =	0.2882
	R2 adj. =	0.992		
			Conf. lev. =	0.95
Width	**Coeff. SC**	**Std. Err.**	**P**	**Conf. int(±)**
Constant	0.625233	0.00981248	1.41469e-024	0.0204685
Injection volume	-0.0161222	0.0120178	0.194784	0.0250687
IFN load	0.184939	0.0120178	1.49991e-012	0.0250687
Flow rate	-0.0321778	0.0120178	0.014472	0.0250687
Temp.	-0.0420889	0.0120178	0.0022436	0.0250687
Inj*F	-0.0226812	0.0127468	0.0903819	0.0265894
Load*F	-0.0434688	0.0127468	0.00277547	0.0265894
N = 27	Q2 =	0.863	Cond. no. =	1.299
DF = 20	R2 =	0.932	RSD =	0.05099
	R2 adj. =	0.911		
			Conf. lev. =	0.95

Fig. A.10: The full list of coefficients for the models retention time and peak width of the aeris column, including the p-values of ANOVA. The list also provides the numbers of experiments included (N), the degress of freedom (DF), the R^2 and Q^2 values of each model and the condition number (cond.no)

A.2 Statistics for Sarkosyl optimization

Exp No	Exp Name	Run Order	Incl/Excl	Detergent conc	Volume	Agitation time	IFN conc	Total Protein conc
1	N1	5	Incl	0.1	33.33	2	0	10.87
2	N2	15	Incl	3	33.33	2	55.02	46.48
3	N3	9	Incl	0.1	150	2	0	16.66
4	N4	16	Incl	3	150	2	78.8	67.57
5	N5	2	Incl	0.1	33.33	8	0	11.7
6	N6	13	Incl	3	33.33	8	45.9	48.1
7	N7	8	Incl	0.1	150	8	2.55	18.44
8	N8	12	Incl	3	150	8	82.88	68.71
9	N9	11	Incl	0.1	91.665	5	0	15.39
10	N10	3	Incl	3	91.665	5	68.52	82.71
11	N11	17	Excl	1.55	33.33	5	17.47	36.17
12	N12	6	Incl	1.55	150	5	86.52	68.88
13	N13	14	Incl	1.55	91.665	2	61.4	51.05
14	N14	1	Incl	1.55	91.665	8	66.34	58.17
15	N15	7	Incl	1.55	91.665	5	67.2	51.7
16	N16	10	Incl	1.55	91.665	5	70.4	55.37
17	N17	4	Incl	1.55	91.665	5	73.6	59

Fig. A.11: The full list of experiments, factor levels and results for all three responses for the design of experiment of the interferon-α-2b solubility study with Sarkosyl. Experiment number 11 was excluded from the model, since it was identified as an outlier in the normal distribution of the residuals,

IFN conc	Coeff. SC	Std. Err.	P	Conf. int(±)
Constant	69.4209	1.89352	7.49285e-013	4.16756
Detergent conc	32.857	1.45307	1.42677e-010	3.19814
Volume	8.93434	1.54605	0.000123052	3.4028
Det*Det	-36.0539	2.3868	1.05855e-008	5.25325
Det*Vol	7.27625	1.62458	0.000933174	3.57564
N = 16	Q2 =	0.973	Cond. no. =	3.062
DF = 11	R2 =	0.987	RSD =	4.595
	R2 adj. =	0.982		
			Conf. lev. =	0.95
Total Protein conc	**Coeff. SC**	**Std. Err.**	**P**	**Conf. int(±)**
Constant	56.1211	3.02957	1.21412e-009	6.66795
Detergent conc	24.051	2.32486	5.26165e-007	5.11692
Volume	7.44321	2.47363	0.0118867	5.44436
Det*Det	-17.4581	3.8188	0.000801249	8.40503
Det*Vol	3.64625	2.59928	0.188267	5.7209
N = 16	Q2 =	0.867	Cond. no. =	3.062
DF = 11	R2 =	0.928	RSD =	7.352
	R2 adj. =	0.902		
			Conf. lev. =	0.95

Fig. A.12: The full list of coefficients for the models interferon-α-2b solubility and total protein solubility, including the p-values of ANOVA. The list also provides the numbers of experiments included (N), the degress of freedom (DF), the R^2 and Q^2 values of each model and the condition number (cond.no)

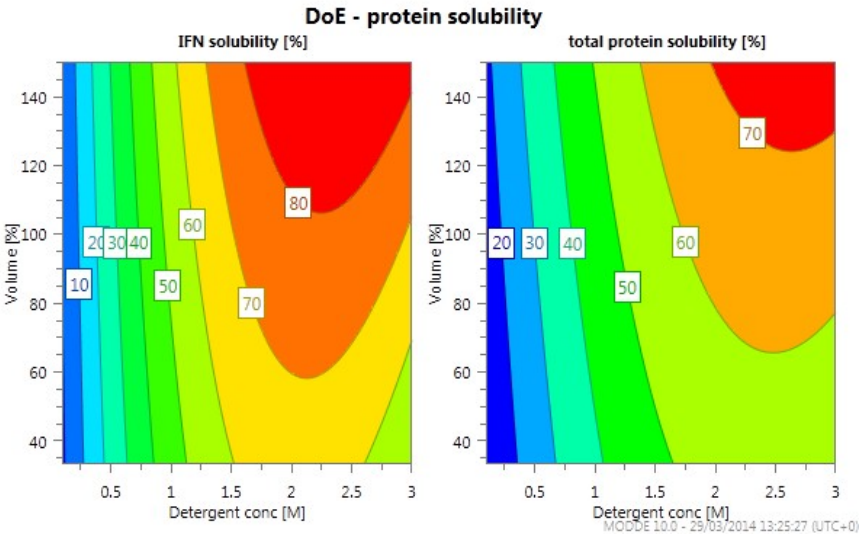

Fig. A.13: Response Surface Plot of the DoE investigation for the protein solubility study. The interferon-α-2b solubility(left) and total protein solubility (right) dependent on the process volume and Sarkosyl concentration.

Appendix B

Ion-exchange purification - HiTrap method

```
UNICORN V3.10
User: MSD    27.04.2012 15:52:10
Method file: c:\...\MSDmethod\HiTrap Equilibrate Wash and Equilibrate

Method Information
Method name: HiTrap Equilibrate Wash and Equilibrate
Method created by: MSD
Date of creation: 03.11.2011 17:09:58
Target system: System 1
Method last modified by: MSD
Date of last modification: 03.11.2011 17:09:58
Strategy name: E100 110
Strategy date: 19.06.1999 13:57:16
Strategy size: 1012080 bytes

Variables
Main method:
  Main
  0.00  Base Volume 5.03 {ml} HiTrap_SP_1x5_ml
  0.00  Flow 5.00 {ml/min}
  0.00  PumpBInlet B1
  0.00  ColumnPosition Position2
  0.00  OutletValve WasteF1
  0.00  BufferValveA1 A11
  0.00  Block  Remove Storage_Solution
    Remove_Storage_Solution
    0.00  Base SameAsMain
    0.00  BufferValveA1 A11
    25  End_block
  0.00  Block  Regenerate
    Regenerate
    0.00  Base SameAsMain
    0.00  Gradient 100 {%B} 0.00 {base}
    50  End_block
  0.00  Block  Equilibrate
    Equilibrate
    0.00  Base SameAsMain
    0.00  Gradient 0 {%B} 0.00 {base}
    90  AutoZeroUV
    100.00  End_block
```

Fig. B.1: AKTA-Explorer method for the equilibration of the cation-exchange column HiTrap SP HP with 5ml column volume (GE Healthcare)

```
UNICORN V3.10
User: MSD    27.04.2012 15:53:22
Method file: c:\...\MSDmethod\HiTrap IFN Purification

Method Information
Method name: HiTrap IFN Purification
Method created by: Sean
Date of creation: 29.08.1982 17:34:04
Target system: System 1
Method last modified by: MSD
Date of last modification: 01.11.2011 10:16:20
Strategy name: E100 110
Strategy date: 19.06.1999 13:57:16
Strategy size: 1012080 bytes

Variables
Main method:
  Main
  0.00  Base CV 5.03 {ml} HiTrap_SP_1x5_ml
  0.00  Flow 5.00 {ml/min}
  0.00  ColumnPosition Position2
  0.00  Wavelength 280 {nm} 254 {nm} 260 {nm}
  0.00  Block  Sample_Load
    Sample_Load
    0.00  Base Volume
    0.00  ColumnPosition Position2
    0.00  BufferValveA1 A18
    0.00  OutletValve F3
    100.00  End_block
  0.00  Block  Wash
    Wash
    0.00  Base SameAsMain
    0.00  PumpWash A11 OFF OFF OFF
    0.00  BufferValveA1 A11
    0.00  OutletValve WasteF1
    5.00  End block
  0.00  Block  Elution
    Elution
    0.00  Base SameAsMain
    0.00  Gradient 60 {%B} 18.00 {base}
    0.00  OutletValve FracF2
    0.00  FractionCollector 5.00 {ml}
    18.00  FractionationStop
    18.00  End block
  0.00  Block  Strip
    Strip
    0.00  Base SameAsMain
    0.00  OutletValve F3
    0.00  Gradient 100.00 {%B} 0.00 {base}
    5.00  End_block
```

Fig. B.2: AKTA-Explorer method for the purification of interferon-α-2b using cation-exchange column HiTrap SP HP with 5ml column volume (GE Healthcare)

Appendix C

Oral presentations/ poster

At the following conferences, oral presentations/ posters have been given:

- 16th European Congress on Biotechnology (ECB) Conference, Edinburgh; *Optimization of an industrial primary protein recovery process by Design of Experiment*; poster, July (2014)
- 3rd Annual Limerick Postgraduate Research Conference, Limerick; *Application of Design of Experiment for Optimization of a protein recovery process*; oral presentation, May (2014)
- Research Day of School of Biotechnology, Dublin City University, Dublin; *Optimization of an industrial primary recovery process using Design of Experiments*; oral presentation, February (2014)
- 33rd International Symposium on the Separation of Proteins, Peptides & Polynucleotides Group (ISPPP), Boston; *Purifying and concentrating recovery process samples for recombinant protein purification*; oral presentation, July (2013)
- Research Day of School of Biotechnology, Dublin City University, Dublin; *Characterization and optimization of an industrial primary recovery process to enhance the protein yield*; poster, January (2013)
- 9th European Society of Biochemical Engineering Sciences (ESBES) Conference, Istanbul; *Characterization and optimization of an industrial primary recovery process to enhance the protein yield*; poster, September (2012)
- Research Day of School of Biotechnology, Dublin City University, Dublin; *Optimization of a protein recovery of Interferon by analyzing and controlling the protein recovery process*; oral presentation, January (2012)