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Hydrophobic interaction chromatography for removal of antibody aggregates

Master's degree project

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Abstract

The purpose of this master's thesis was to screen a number of different HIC media including existing products, products from a competing company and new prototypes in order to find the most suitable media and parameters for aggregate removal in purification of a MAb. Samples with high aggregate content, approximately 15% and 93% were used. Screening in the 96 well filter plate format was performed followed by aggregate analysis and verification in the column format. Different salts, efficiency and the antibody binding capacity were investigated for one prototype media, B1 – Phenyl (20µmol/ml). The effects of salt content, pH, incubation time and sample dilution on antibody binding capacity were also tested for the prototype medium as well as a competitor medium. The results showed that 96 well filter plate screening can give a lot of information about the nature of HIC media. There was an observed correlation between the plate format and the column format regarding the salt concentration at which the sample eluted and a poorer correlation of the aggregate content between the two formats. B1 – Phenyl (20µmol/ml) is a promising prototype which reduces aggregate levels at low salt concentrations. The approximate maximum antibody binding capacity for B1 – Phenyl (20µmol/ml) in the 96 well filter plate format was 12 mg/ml$_{resin}$ and it suggestively has highest antibody binding capacities at high sodium sulphate concentrations and at low pH values.

Keywords

HIC, MAb, aggregate, ligand, 96 well filter plate format, ammonium sulphate

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Hydrophobic interaction chromatography for removal of antibody aggregates

Helena Nilsson

Populärvetenskaplig Sammanfattning

Olika typer av antikroppar används idag som läkemedel för en rad sjukdomar såsom till exempel rheumatoid artrit. Antikroppar som ska användas till läkemedel kan tillverkas med hjälp av celler framtagna från olika cellinjer. Antikropparna måste sedan renas fram och uppfylla olika renhetskrav för att de ska kunna injiceras i människor. Vid rening av antikroppar kan en teknik som heter hydrofob interaktionskromatografi (HIC) användas som ett steg i en reningsprocess. Antikroppar har en tendens att binda till varandra och bilda aggregat och HIC är en metod som brukar användas för att minska halten av aggregat. När man renar antikroppar med hjälp av HIC så använder man ett material, en HIC-gel, som aggregaten och antikropparna binder in till vid höga halter av vissa typer av salt. Aggregaten binder hårdare till HIC-gelen och kan då separeras från de enkla antikropparna.

I detta examensarbete har en rad olika typer av HIC-geler undersökts med avseende på hur bra de kan minska aggregathalten i ett antikroppsprov. Ett antal olika parametrar har studerats bland annat olika saltsorter, salthalter, pH värden och inkubationstider. En intressant prototypgel som undersöktes kunde minska aggregathalten vid relativt låga halter av inbindningssaltet, vilket är bra ekonomiskt och för stabiliteten av antikroppen.

Examensarbete 30hp
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Abbreviations

BHK = Baby Hamster Kidney (Cell Line)
CCF = Central Composite Face Design
CHO = Chinese Hamster Ovary (Cell Line)
CIP = Cleaning in Place
COS = Monkey Kidney (Cell Line)
CV = Column Volume
DoE = Design of Experiments
Fab = Fragment Antigen-Binding
Fc = Fragment Crystallizable
HCP = Host Cell Protein
HIC = Hydrophobic Interaction Chromatography
Ig = Immunoglobulin
MAb = Monoclonal Antibody
MLR = Multiple Linear Regression
NaCl = Sodium Chloride
NaOH = Sodium Hydroxide
NS0 = Mouse Myeloma (Cell Line)
SEC = Size Exclusion Chromatography
Sp2/0 = Mouse Myeloma (Cell Line)

1. Introduction

In modern healthcare monoclonal antibodies (MAbs) are used in a number of areas for example treatments of anti-inflammatory diseases such as rheumatoid arthritis and different types of cancer [1-2]. The MAb industry is constantly growing and is estimated to reach US$16.7 billion dollars in 2008 [3]. Competition contributes to constant improvement and the market demands production of higher titres at a lower cost. Higher titres however result in higher purification costs and optimizing the purification process is just as important as producing high titres [2].

The antibody production process consists of an upstream cell culture process and a downstream purification process. The downstream purification process includes different chromatographic steps, viral inactivation and different filtration steps [4].

The chromatographic purification of MAbs is normally done with three chromatography steps in the following order; one capture step, one intermediate step and one polishing step. For IgG, a Protein A affinity purification step is commonly used as the capture step. One or more ion exchange chromatography steps can be used as the intermediate step and as the polishing step ion exchange chromatography or hydrophobic interaction chromatography (HIC) can be used [2]. HIC separates proteins based on their different surface hydrophobicity and can be used in a MAb purification process to remove final impurities, especially antibody aggregates [2-3].

1.1 Background chemical/technical

1.1.1 Antibodies

Antibodies or Immunoglobulins are a part of the adaptive immune system and have several functions. Their main function is to bind foreign antigens, and antibodies have specificity towards different groups of antigens. When antibodies bind to microbes it leads to neutralization of the microbe and in some cases engulfment of the microbe by macrophages or monocytes. Antibodies also activate the complement system which responds to extra cellular microbes such as bacteria. Activation of the complement system leads to bacterial lysis, inflammation and in some cases engulfment of the bacteria [5]. Conclusively antibodies play an important part in the body's immune system and the biopharmaceutical industry is developing methods to make use of their versatility.

There are five different classes or isotypes of antibodies; IgA, IgD, IgE, IgG, IgM. There are also subclass divisions for IgA and IgG, two and four respectively. Structurally all antibody classes contain similar core structures containing constant and variable regions held together by disulfide bonds. The variable regions are the ones binding to the various antigens and responsible for the antibody's specificity. All antibodies contain two or more sites that bind to antigen, so called fragment antigen binding (Fab) sites and one or more crystallisable fragment (Fc). IgG is the most common antibody in humans and comprise 70-75% of all immunoglobulin present in the blood. IgG consist of two heavy chains and two light chains linked together with disulphide bonds (Figure 1). [5-6]

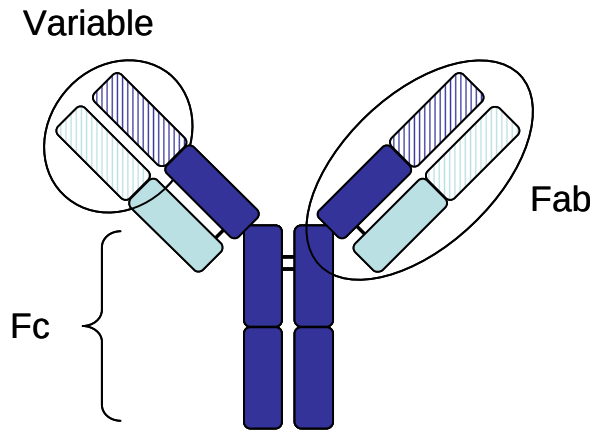

Figure 1. The structure of IgG, adapted from [5]. Fab and Fc fragments as well as the variable region are illustrated in the picture. The dark blue colour represents the heavy chain, the light blue colour represents the light chain and the broken lines represent the variable regions.

MAbs are identical antibodies from one clone and the cell lines that produce them are called hybridoma cell lines. These cell lines are produced by fusion of a normal B cell with a cancer cell (myeloma cell) creating an immortal antibody producing cell, a hybridoma. The hybridoma that has the same specificity as the original B cell is then selected, giving rise to an immortal cell line producing only one type of antibody [5]. Some common mammalian cell lines used for MAb production are Chinese Hamster Ovary (CHO), Mouse Myeloma (NS0, Sp2/0), Monkey Kidney (COS) and Baby Hamster Kidney (BHK) [2].

1.1.2 Chromatography

Chromatography is a common technique used in protein separation. There are different variants of chromatography which are all based on different substance's distribution between two immiscible phases, one mobile and the other stationary. Different substances will distribute differently between the two phases and the distribution can be described by the distribution coefficient K_d, a quotient between the concentration in phase A and the concentration in phase B. The different distribution coefficients for the substance to be purified and the other substances in the solution are what results in the separation. [7]

Column chromatography is one common mode of chromatography. The stationary phase is fixed in a column and the mobile phase flows through the column (Figure 2). [7]

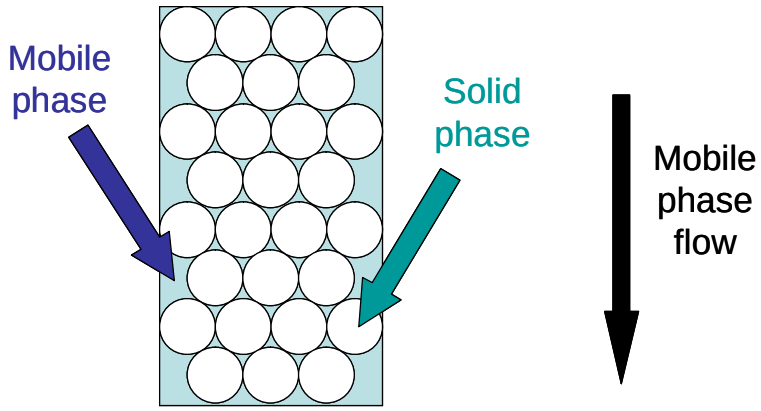

Figure 2. Schematic picture of a column including the mobile and solid phase as well as the mobile phase flow direction.

1.1.2.1 Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography (HIC) is a chromatography method where the hydrophobic interaction between proteins and the HIC media in an aqueous solvent are used in order to purify proteins (Figure 3). HIC media consist of hydrophobic ligands, attached to a spherical porous matrix. There are several types of ligands used in HIC for example phenyl, butyl and methyl. Certain salts, so called cosmotropic salts, enhance the hydrophobic effects and this makes it more thermodynamically favourable for hydrophobic patches on proteins to adsorb to the hydrophobic ligand. A negative salt gradient from high to low cosmotropic salt is used for elution of the protein. [8]

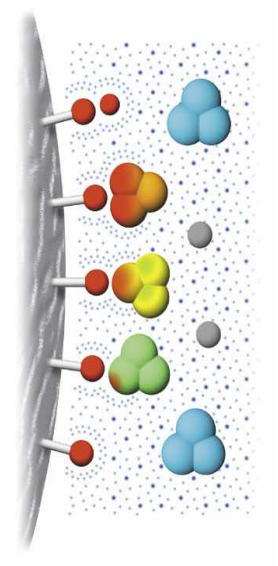

Figure 3. Schematic picture of adsorption in hydrophobic interaction chromatography. Red patches symbolise hydrophobic regions. [9] Picture reproduced with kind permission from GE Healthcare Biosciences AB.

Different ions have different effects upon protein precipitation and therefore affect the hydrophobic interactions differently. The Hofmeister series describes different ions' salting-in vs salting-out effect (Figure 4). A chaotropic ion creates disorder in the water structure and therefore also has a salting-in effect and decreases the hydrophobic effect. A cosmotropic ion has the opposite effects. [9]

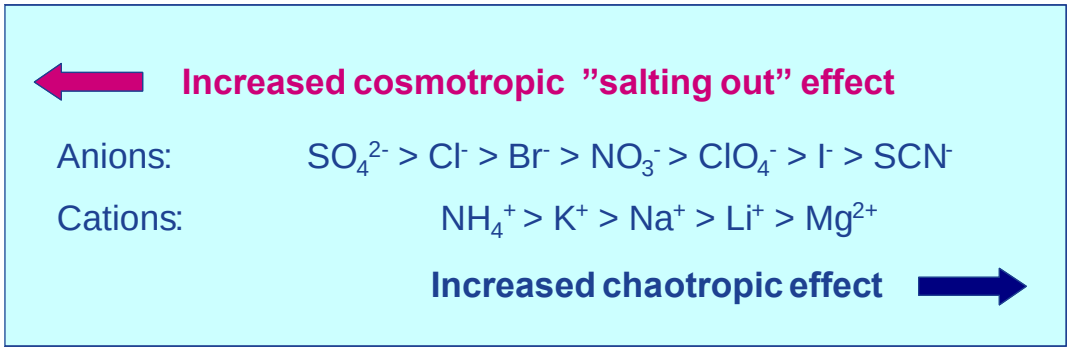

Figure 4. The Hofmeister series [9]

1.1.2.2 Size exclusion chromatography (SEC)

SEC is a chromatography method which separates proteins by size. The chromatographic matrix contains a defined pore size and proteins are retained differently in the pores and this result in separation. The larger proteins pass through the column unretained and smaller proteins are retained in the pores [8]. SEC is commonly used for analysis of antibody aggregate content.

1.1.3 96 well filter plate format

Figure 5. Example of 96 well filter plates, pre-filled and sold under the name PreDictor™. Picture reproduced with kind permission from GE Healthcare Biosciences AB.

The 96-well filter plate format enables a high throughput screening (Figure 5) where a large number of conditions can be investigated simultaneously thus saving a lot of time. The chromatography media (solid phase) is present in the wells used and the liquid phase is added to the wells and removed by vacuum or centrifugation. The same chromatography steps that are used in a column run are used during a plate run. A schematic overview of the chromatographic steps for each well in a 96 well plate can be seen in Figure 6. [10]

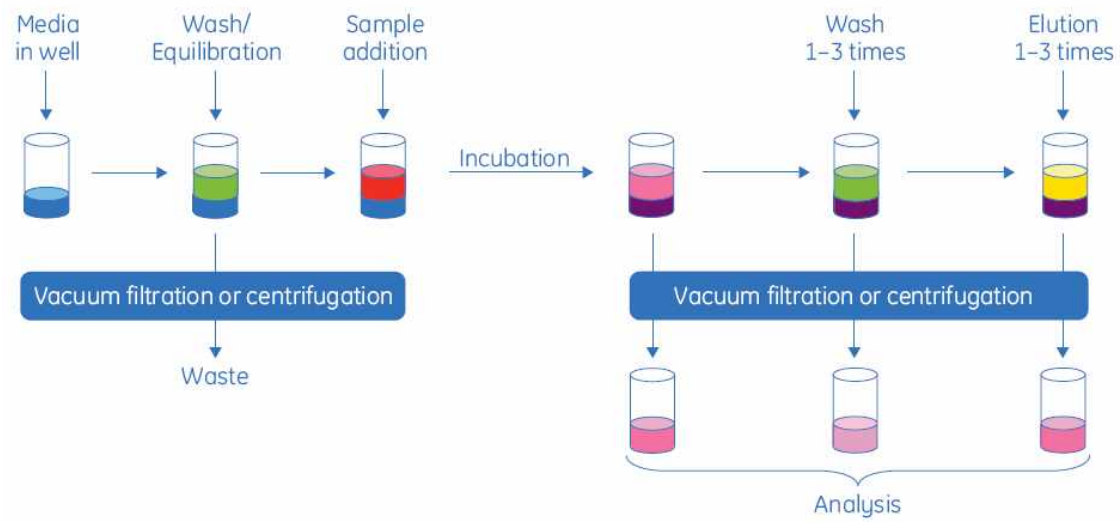

Figure 6. An overview of one well in the 96 well plate and the chromatographic steps. [11] Picture reproduced with kind permission from GE Healthcare Biosciences AB.

1.1.4 Adsorption isotherm

An adsorption isotherm describes the sample concentration at equilibrium in the solid phase (C_S) as a function of the sample concentration in the mobile phase (C_M). The adsorption isotherm curve can be plotted using the Langmuir model. Langmuir's adsorption equation is described as:

$$C_S = \frac{(Q \times K \times C_M)}{(1 + C_M)}$$

where Q is the total number of binding sites per unit surface area and K is the association constant. [8]

An adsorption isotherm can be used in order to get an idea of the chromatography medium's protein surface concentration in a given environment. By varying the sample concentration and using a fixed amount of chromatography media or vice versa and allowing it to reach equilibrium, one could get an idea of the maximum surface concentration of the chromatography media also called the maximum binding capacity. Figure 7 shows an example of an adsorption isotherm with various sample concentrations and a fixed chromatography medium volume.

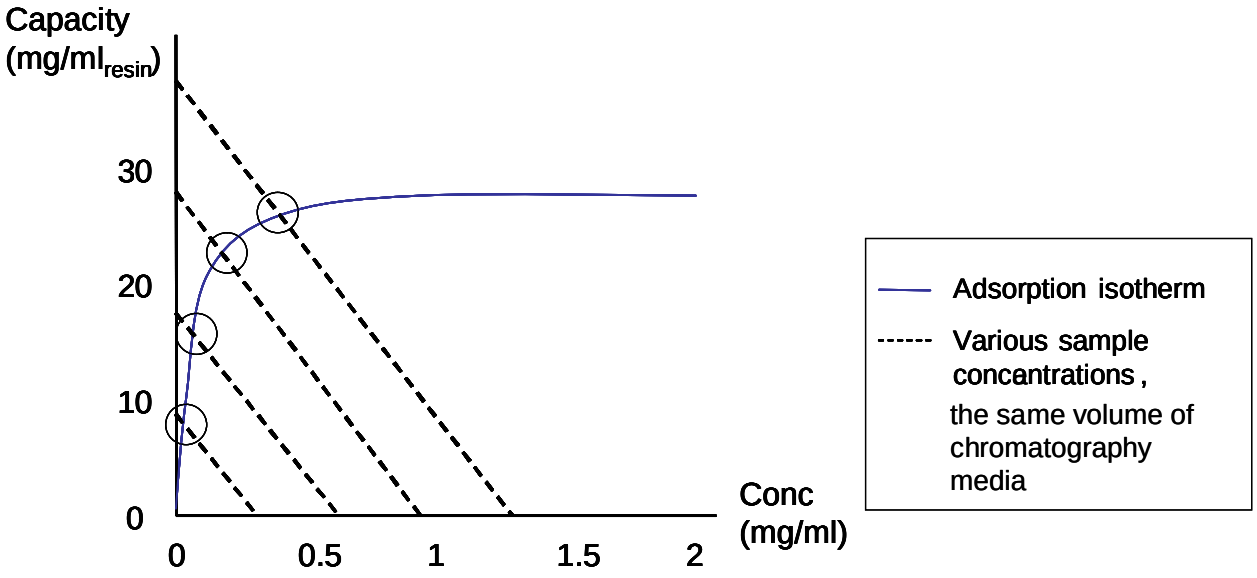

Figure 7. Illustration of an adsorption isotherm. Blue solid line represents the adsorption isotherm and the dotted lines represent various sample concentrations with the same chromatography media volume. By varying the initial sample concentrations, the adsorption isotherm can be plotted and hence the capacity can be estimated. The dotted lines represent the phase relation between the sample concentration and the gel volume. The equations for these lines originate from the equation

$$C_S = (C_{ini} - C_{end}) \times \left(\frac{V_{lq}}{V_{resin}} \right)$$

where;
C_S = the protein concentration in the solid phase (capacity) at equilibrium
C_{ini} = the initial sample concentration,
C_{end} = the sample concentration in the liquid phase at equilibrium,
V_{lq} = the sample volume
V_{resin} = the volume of the resin.

1.1.5 Design of Experiments (DoE)

In order to investigate several factors and their influence upon one or several responses, a Design of Experiments can be conducted. This can be done in a number of different ways and normally a reference experiment, called the center-point, together with a number of experiments arranged symmetrically around the center-point are performed. There are a number of different types of experimental designs such as e.g. different factorial and composite designs. Factorial designs are normally used for screening purposes and composite designs for optimization. [12]

Based on the investigated factors, the response factor or factors and the selected intervals the software MODDE from Umetrics creates an experimental design. After the experiments have been performed, the results are evaluated by regression analysis, creating a model for the data. This model is evaluated in different ways and two important parameters used in the evaluation of the model are R2 and Q2. R2 is called goodness of fit and describes how well the regression model can be made to fit the raw data. The range for R2 is 0-1 where 1 represents a perfect fit. Q2 stands for goodness of prediction and this is an estimation of how well the model can predict new experiments. The range of Q2 is (-∞)-1 where 1 is a

perfect prediction and negative values represent poor predictions. In the case of a good model the values of R2 and Q2 should be high and not differ from each other much more than 0.2-0.3 units. [12]

The term model validity describes how valid the model is. If the model validity has a value of 1 the model is perfect and with values lower than 0.25 there is a significant lack of fit of the model. If the values are below 0.25 the error in the model is significantly larger than the pure error. The reproducibility describes a comparison of how the response varies under the same conditions (pure error) and the total variation of the response. If the reproducibility has a value of 1, the variation is very low, and the reproducibility is perfect. [13]

1.2 Purpose

The purpose of this master's thesis was to screen a number of different HIC media, including a number of prototypes in order to find the most suitable media and parameters for aggregate removal in purification of a MAb. Previous similar experiments have been performed on a MAb sample containing a low amount of aggregates (approximately 2%) which made it difficult to see significant differences in aggregate content [14]. In this master's thesis material with higher aggregate content, approximately 15% and 93%, has been used which has enabled a more in-depth analysis.

2. Materials and methods

2.1 Samples

Two samples with the same IgG, derived from Chinese hamster ovary (CHO) cells:

1. Approximately 15% aggregate determined by an analytical SEC column (Superdex™ 200 5/150 GL), Protein A purified by an XK26/20 column packed with MabSelectSuRe™, concentration: 6.26 mg/ml measured by an analytical column (HiTrap™, 1ml) packed with MabSelectSuRe. pH adjusted to 6.0 after elution with 100mM sodium citrate buffer during Protein A purification (Table 1). The sample was during this master's thesis filtrated when appearing opalescent. Both 0.45 µm and 0.22 µm filters were used. The sample also had a tendency to precipitate when mixed with the binding buffers. The samples were in those cases filtrated with filters with a diameter of 0.22 µm.
2. Approximately 93% aggregate determined by an analytical SEC column (Superdex 200 10/300 GL), Protein A purified by an XK26/20 column packed with MabSelectSuRe and SEC purified by a HiLoad™ 16/60 column packed with Superdex 200, concentration 1.6 mg/ml calculated from absorbance measurements at 280 nm (using an extinction coefficient of 1.5 [15]), at the approximate pH of 6.5 [16]. Sample in 10 mM ammonium acetate buffer.

2.2 Methods

2.2.1 Buffer and solution preparation

2.2.1.1 Sample preparation

Binding buffer: 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS)
Elution buffer: 60 mM sodium citrate, pH 3.4
Strip buffer: 100 mM sodium citrate, pH 3.0
CIP buffer: 0.5 M NaOH

2.2.1.2 96 well filter plate screening

Stock solutions:

- 1.6 M ammonium sulphate, 50 mM sodium phosphate, pH 7.0
- 50 mM sodium phosphate, pH 7.0

Running buffers, prepared from stock solutions:

- 0.8 M ammonium sulphate, 50 mM sodium phosphate, pH 6.6
- 0.6 M ammonium sulphate, 50 mM sodium phosphate, pH 6.6
- 0.5 M ammonium sulphate, 50 mM sodium phosphate, pH 6.6
- 0.4 M ammonium sulphate, 50 mM sodium phosphate, pH 6.6
- 0.3 M ammonium sulphate, 50 mM sodium phosphate, pH 6.7
- 0.2 M ammonium sulphate, 50 mM sodium phosphate, pH 6.7

2.2.1.3 Column packing and testing

Packing buffer: 0.1 M potassium phosphate, pH 6.6
Testing buffer 1: 0.4 M NaCl
Testing buffer 2: 0.8 M NaCl

2.2.1.4 Column verification

Stock solutions:

- 1.6 M ammonium sulphate, 50 mM sodium phosphate, pH 7.0
- 50 mM sodium phosphate, pH 7.0

Binding buffer, prepared from stock solutions: 0.8 M ammonium sulphate, 50 mM sodium phosphate, pH 6.6
Cleaning in Place solution: 30% propanol

2.2.1.5 Different salts

Binding buffers:

- 2.5 M NaCl, 50 mM sodium phosphate, pH 7.0
- 0.5 M Sodium sulphate, 50 mM sodium phosphate, pH 7.0
- 0.6 M sodium citrate buffer, 50 mM sodium phosphate, pH 6.0

Elution buffer: 50 mM sodium phosphate, pH 7.0
Cleaning in Place solution: 30% propanol

2.2.1.6 Adsorption isotherm study

Binding buffer: 0.25 M sodium sulphate, 50 mM sodium phosphate, pH 6.0
Elution buffer 1: 50 mM sodium phosphate, pH 6.0
Elution buffer 2: 50 mM sodium phosphate, 20% ethylene glycol, pH 6.0

2.2.1.7 Design of Experiments

Binding buffer	**Elution buffer**
0.5 M sodium sulphate, 50 mM sodium citrate, pH 4.0	50 mM sodium citrate pH 4.0
0.25 M sodium sulphate, 50 mM sodium citrate pH 4.0	50 mM sodium citrate pH 4.0
50 mM sodium citrate pH 4.0	50 mM sodium citrate pH 4.0
0.5 M sodium sulphate, 50 mM MES, pH 6.0	50 mM MES, pH 6.0
0.25 M sodium sulphate, 50 mM MES, pH 6.0	50 mM MES, pH 6.0
50 mM MES, pH 6.0	50 mM MES, pH 6.0
0.5 M sodium sulphate, 50 mM Tris/HCl, pH 8.0	50 mM Tris/HCl, pH 8.0
0.25 M sodium sulphate, 50 mM Tris/HCl, pH 8.0	50 mM Tris/HCl, pH 8.0
50 mM Tris/HCl, pH 8.0	50 mM Tris/HCl, pH 8.0

2.2.2 Sample preparation

Protein A purification

An XK26/20 column packed with MabSelect SuRe (10 cm bed height) was used for the Protein A purification with a linear flow of 250 cm/h. An overview of the method can be seen in Table 1. After Protein A purification the pH was adjusted to 6.0 using NaOH.

Table 1. Method overview of the chromatographic steps in the Protein A purification, CV = column volume.

Step	**Step length**	**Buffer composition**
Equilibration:	4 CV	20 mM sodium phosphate 150 mM NaCl, pH 7.4
Sample loading:	Approx chr 1: 2850 ml chr 2: 2950 ml	Filtered Feed
Wash:	5 CV	20 mM sodium phosphate 150 mM NaCl, pH 7.4
Elution:	3 CV	60 mM sodium citrate, pH 3.4
Strip:	2 CV	100 mM sodium citrate, pH 3.0
CIP:	10 min contact time	0.5 M NaOH
pH regeneration:	10 CV	20 mM sodium phosphate 150 mM NaCl, pH 7.4

2.2.3 96 well filter plate screening

Stepwise elution in the 96 well filter plate format for 18 different HIC media was performed. In Table 2 the binding buffers and correlating step elution buffers investigated are summarized. Duplicate samples for each HIC medium and binding buffer were investigated. Ten control wells per 96 well filter plate that did not contain any HIC media were treated with binding buffer, sample addition and elution buffer. For an overview of the number of controls per ammonium sulphate see Table 3.

Table 2. The different binding buffers and the different elution buffers investigated. All binding and elution buffers also contained 50 mM sodium phosphate.

Binding buffer (ammonium sulphate concentration)	Elution buffers used together with the binding buffer stated in the left column (ammonium sulphate concentration)
0.8 M	0.6 M, 0.5 M, 0.4 M, 0.3 M, 0.2 M, 0 M
0.6 M	0.5 M, 0.4 M, 0.3 M, 0.2 M, 0 M
0.5 M	0.4 M, 0.3 M, 0.2 M, 0 M
0.4 M	0.3 M, 0.2 M, 0 M
0.3 M	0.2 M, 0 M
0.2 M	0 M

Table 3. The total number of controls for all three 96 well filter plates investigated. The binding buffer also contained 50 mM sodium phosphate.

Ammonium sulphate concentration	Number of controls in total for the three 96 well plates
0.8 M	6
0.6 M	2
0.5 M	4
0.4 M	6
0.3 M	4
0.2 M	2
0 M	6

For the HIC prototypes screened (stated in the list below) the following details can be specified:

- B1: Base matrix 1; High Flow Agarose, porosity 1,
- B2: Base matrix 1; High Flow Agarose, porosity 2.
- Porosity 2 > Porosity 1
- After the base matrix code the name of the ligand is stated followed by the ligand concentration in µmol/ml

HIC media screened

1. Phenyl Sepharose™ 6 Fast Flow hs
2. Phenyl Sepharose 6 Fast Flow ls
3. PlasmidSelect
4. Competitor 1 = Toyopearl® Phenyl-650 M (Tosoh)
5. Competitor 2 = Toyopearl® Phenyl-650 C (Tosoh)
6. B1 – Butyl (24µmol/ml)
7. B1 – Butyl (36µmol/ml)
8. B1 – Butyl (44µmol/ml)
9. B1 – Phenyl (11µmol/ml)
10. B1 – Phenyl (20µmol/ml)
11. B1 – Phenyl (31µmol/ml)
12. B1 – Phenyl (37µmol/ml)
13. B1 – Octyl (3.5µmol/ml)
14. B2 – Butyl (36µmol/ml)
15. B2 – Phenyl (17µmol/ml)
16. B2 – Phenyl (27µmol/ml)
17. B2 – Phenyl (42µmol/ml)
18. B2 – Methyl (81µmol/ml)

Preparation of the 96 well filter plates
A HIC media slurry of approximately 14% for each HIC medium was prepared. The slurry was created by using 15 ml Falcon tubes and centrifugation. A 96 well filter plate was filled with 200 µl of HIC media slurry for each used well. This gave rise to approximately 28 µl of HIC media in each well. The plate was sealed with aluminium foil.

Sample used
The MAb sample containing approximately 15% aggregate (See chapter 2.1 Samples) was diluted 1:4 with different amounts of the two stock solutions of ammonium sulphate giving rise to different binding buffer concentrations of ammonium sulphate. The two stock solutions used were: 1.6 M ammonium sulphate, 50 mM sodium phosphate, pH 7.0 and 50 mM sodium phosphate pH 7.0 without ammonium sulphate. The Binding buffer concentrations of ammonium sulphate are shown in Table 2.

Workflow for the 96 well filter plate screening [10]

1. The top aluminium foil was removed while holding the filter plate against the collection plate.
2. The bottom seal was peeled off and the filter plate was placed in an up-right position on a collection plate.
3. **The storage solution was removed:** The plates were centrifuged for 1 minute at 370g.
4. **Equilibration:** 200 µl of binding buffer/well was added. The plates were centrifuged for 1 minute at 370g. The bottom of the plate was blotted on a soft paper tissue to remove drops of binding buffer that may have occurred on the underneath of the plate. (This step is important to prevent the plate from leakage.) This was repeated three times. A micro plate foil was put on the bottom of the plate.
5. **Sample loading:** 200 µl of clarified samples with different concentrations were added to the appropriate wells.
6. **Incubation:** The top of the plate was covered by using a micro plate foil. The plate was then incubated for 60 minutes on a plate shaker at mixing intensity 1100 rpm.
7. **Removal of flow through:** The foil cover was removed and then the plate was centrifuged at 1 minute at 370g. The fractions were collected for analysis.
8. **Washing out unbound sample:** 200 µl of binding buffer was added to each well. The plate was centrifuged at 370 x g for 1 minute. This was repeated three times. The fractions were collected for analysis.
9. **Elution:** 200 µl of elution buffer was added to each well. The plate was then left standing for approximately 2 minutes. The plate was centrifuged at 370g for 1 minute. The fractions were collected for analysis. This was repeated at two times for each elution buffer times except for eluting at 0 M where an extra elution step was added.
10. **Analysis:** The absorbance at 280 nm was read on a plate reader. A buffer plate was read with triplicates for each binding end elution buffer used and due to their similarity an average was taken for all the buffers. This value was subtracted from all obtained absorbance values. The wash and elution fractions from each elution buffer for six selected HIC media were pooled and analysed by an analytical SEC column. The SEC column used was a Superdex 200 5/150 GL and an ÄKTAexplorer™ 10 system was used for the analysis. The monomer and aggregate peak were integrated in order to determine the aggregate content. The selected HIC media were PlasmidSelect, Competitor 2, B1 – Butyl (36µmol/ml), B1 – Phenyl (11µmol/ml), B1 – Phenyl (20µmol/ml) and B1 – Phenyl (31µmol/ml).

2.2.4 Column packing and testing

Column packing

The six selected HIC media from the 96 well filter plate screening were packed in Tricorn 5/100 columns using an ÄKTAexplorer 100 system. PlasmidSelect and Competitor 2 were transferred to the packing buffer (0.1 M potassium phosphate) prior to packing the HIC media. B1 gels were packed in 20% ethanol. A top column was used for all the HIC media. PlasmidSelect and Competitor 2 were packed with a down flow in the following way: after the set flows had been applied the top column was removed and the pack flows were applied. The adaptor was then lowered. The four B1 HIC Media were packed with an up flow and the extra column on the bottom in the following way: after the set flows had been applied the top column was removed and the pack flows 1 and 2 were applied. The adaptor was then lowered and pack flow 3 was applied. An overview of different HIC media and the parameters for packing can be seen in table 4.

Table 4. The different HIC media with the parameters for packing.

HIC media	Parameters	Bed height after packing
PlasmidSelect	Set & Pack flow: 489 cm/h The set flow was applied until the gel was set and the pack flow was applied until the gel was stabilized.	10.9
Competitor 2	Set flow: 30.6 cm/h, applied until the gel was set Pack flow 1: 306 cm/h, until the gel was stabilized	11.0
B1 – Butyl (36µmol/ml)	Set flow: 500 cm/h (7 min) Pack flow 1: 1500 cm/h (5 min) Pack flow 2: 2500 cm/h (3 min) Pack flow 3: 3000 cm/h (3 min)	10.5
B1 – Phenyl (11µmol/ml)	Set flow: 500 cm/h (7 min) Pack flow 1: 1500 cm/h (5 min) Pack flow 2: 2500 cm/h (3 min) Pack flow 3: 3000 cm/h (3 min)	10.8
B1 – Phenyl (20µmol/ml)	Set flow: 500 cm/h (7 min) Pack flow 1: 1500 cm/h (5 min) Pack flow 2: 2500 cm/h (3 min) Pack flow 3: 3000 cm/h (3 min)	10.3
B1 – Phenyl (31µmol/ml)	Set flow: 500 cm/h (7 min) Pack flow 1: 1500 cm/h (5 min) Pack flow 2: 2500 cm/h (3 min) Pack flow 3: 3000 cm/h (3 min)	10.6

Column testing

The packing qualities of the columns in Table 4 were analysed. Each column was equilibrated with 0.4 M NaCl and a pulse containing 50 µl 0.8 M NaCl was used as sample. The flow was 20 cm/h. The conductivity peak derived from the test was integrated and analysed for asymmetry and plates/m. Acceptance criteria: asymmetry between 0.8-1.8 and number of plates/m >3700.

2.2.5 Column verification

Column verification overview:

Column verification tests at a flow of 200 cm/h were performed for the HIC media displayed in Table 4. The sample used was the same that was used for the 96 well filter plate screening containing approximately 15% aggregate (See chapter 2.1 Samples). The column confirmation method is presented in Table 5. 2 ml fractions were collected and analysed by an analytical SEC column (Superdex 200 5/150 GL together with an ÄKTAexplorer™ 10 system). The monomer and aggregate peak were integrated in order to determine the aggregate content.

Table 5. An overview of the chromatographic steps for the column confirmation.

Step	Volumes	Buffer composition
Equilibration:	5 CV	0.8 M ammonium sulphate, 50 mM sodium phosphate, pH 6.6
Sample loading:	20 ml	Filtered feed, diluted 1:4 in binding buffer
Wash:	3 CV	0.8 M ammonium sulphate, 50 mM sodium phosphate, pH 6.6
Gradient Elution:	15 CV	50 mM sodium phosphate, pH 7.0
Wash	5 CV	50 mM sodium phosphate, pH 7.0
CIP:	~5 CV	30% propanol

2.2.6 Adsorption isotherm study

Sample transfer to the binding buffer

The MAb sample used was the one containing approximately 93% aggregate (See chapter 2.1 Samples). In order to transfer the sample to the binding buffer (0.25 M sodium sulphate, 50 mM sodium phosphate, pH 6.0) a pre-packed NAP™-10 column containing Sephadex® G-25 medium was used. After removal of the top and the bottom cap the column was equilibrated with approximately 15 ml of the binding buffer. After the binding buffer had completely entered the gel bed, 1 ml of sample was added. The sample was eluted with 1.5 ml of elution buffer. The column was re-equilibrated with approximately 15 ml of the binding buffer and another 1 ml of sample was added and eluted with 1.5 ml of elution buffer. The sample concentration before the buffer transfer was 1.6 mg/ml and after the buffer transfer 1 mg/ml. This was calculated from absorbance measurements at 280 nm (using an extinction coefficient of 1.5 [15]).

Sample parameters

An of HIC media slurry of 5% in 20% ethanol was prepared for the prototype B1 – Phenyl (20µmol/ml). A 96 well filter plate was filled with 100 µl of HIC media slurry for each used well. This gave rise to 5 µl gel/well. Five different sample concentrations were used: 1 mg/ml, 0.667 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml. The samples were diluted in the plate and triplicate samples and duplicate controls for each dilution were used. The controls were treated the same way as the samples and containing chromatography media.

Workflow for the Adsorption Isotherm plate performance [10]

1. The top aluminium foil was removed.
2. The bottom seal was removed.
3. **Removal of storage solution:** The storage solution was removed by centrifugation for 1 minute at 500g (rcf).
4. **Equilibration:** 200 µl of binding buffer (0.25 M sodium sulphate, 50 mM sodium phosphate, pH 6.0) was added to each well that was going to be used (including the controls). The plate was centrifuged for 1 minute at 500g. This step was performed three times. The bottom of the plate was covered with an aluminium foil.

5. **Sample loading**: (Dilution in the plate)
   - The buffer was added to the plate first, this took approximately 8 minutes.
   - The sample was then added, this took approximately 20 minutes.
   - The plate was sealed with a plastic micro plate foil.
6. **Incubation:** The plate was put on incubation on shaker for 6 hours at 1100 rpm approximately 30 minutes after the first sample was added to the plate.
7. The top plastic foil was removed.
8. The bottom aluminium foil was removed.
9. **Removal of flow through:** The supernatant was removed by centrifugation for 1 minute at 500g. The fractions were collected in a 96 well UV plate for further analysis.
10. **Wash out of unbound sample:** 200 µl of equilibration buffer was added to each well used. The plate was centrifuged for 1 minute at 500g. The fractions were collected in a 96well UV plate for further analysis. This step was performed three times.
11. **Elution:** 200 µl of elution buffer 1 (50 mM sodium phosphate, pH 6.0) was added to each well used. The plate was centrifuged for 1 minute at 500g. The fractions were collected in a 96 well UV plate for further analysis. This step was performed three times.
12. **Final elution:** 200 µl of elution buffer 2 (50 mM sodium phosphate, 20% ethylene glycol, pH 6.0) was added to each well used. The plate was centrifuged for 1 minute at 500g. The fractions were collected in a 96 well UV plate for further analysis. This step was performed three times.
13. **Analysis:** The plates were read at 280 nm and 310 nm by a plate spectrophotometer. The values from the 280 nm were the ones used in the calculations; the measure at 310 nm was to check for possible light scattering effects. A buffer plate was read containing 16 replicates for each binding and elution buffer. The average of the replicates was taken and this was subtracted from the absorbance values. The capacity was calculates by taking the average of the control wells for each sample concentration and subtracting the average buffer control value and finally subtracting the average of the flow through values for each sample concentration.

2.2.7 Design of Experiments

Sample transfer to binding buffers

A PD MultiTrap™ G-25 plate was used in order to transfer the sample into the different buffers with the different pH and salt content (for design conditions see Table 6). The media in the MultiTrap plate was resuspended on a plate shaker at 1100rpm for 2 minutes upside down and then 2 minutes the right way up. The top and bottom seals were removed and the MultiTrap plate was placed on a collection plate. The storage solution was removed by centrifugation at 800g. After this 300 µl of equilibration buffer was added, followed by centrifugation for 1 minute at 800g. The equilibration was performed in total 5 times. 120 µl of sample was added and eluted in a UV plate by centrifugation at 800g for 2 minutes.

The MAb sample used contained approximately 93% aggregate (See chapter 2.1 Samples). The sample concentration before the buffer transfer was 1.6 mg/ml and after the buffer transfer 1.3 mg/ml. This was calculated from absorbance measurements at 280 nm (using an extinction coefficient of 1.5 [15]). Two pooled samples from 11 wells each containing the same elution buffer were measured after the buffer transfer and both were very similar (1.95 and 1.97 AU). After the sample transfer to the new buffers the samples were then diluted in the different buffers in order to give rise to the three different dilutions 1.3 mg/ml, 0.65 mg/ml and 0.16 mg/ml. Duplicate controls for the centre point buffer (0.25 M sodium sulphate, 50 mM MES, pH 6.0) for the three concentrations were used in the experiment.

5% of HIC media slurry was prepared for two prototype resins, B1 – Phenyl (20µmol/ml) and Competitor 2. For Competitor 2 there were some difficulties in determining the gel slurry and the estimation was that the slurry for Competitor 2 was around 4.75%. Each used well in a 96 well filter plate was filled with 100 µl gel slurry, giving rise to 5 µl gel/well.

Table 6. The different parameters used in the Design of Experiments.

pH	Load salt concentration (M)	Dilution factor	Initial sample concentration (mg/ml)	Time (min)
4	0	8	0.16	10
4	0	1	1.3	10
4	0.5	8	0.16	10
4	0.5	1	1.3	10
6	0.25	2	0.65	10
8	0	8	0.16	10
8	0	1	1.3	10
8	0.5	8	0.16	10
8	0.5	1	1.3	10
4	0.25	2	0.65	30
6	0	2	0.65	30
6	0.25	8	0.16	30
6	0.25	2	0.65	30
6	0.25	2	0.65	30
6	0.25	2	0.65	30
6	0.25	1	1.3	30
6	0.5	2	0.65	30
8	0.25	2	0.65	30
4	0	8	0.16	90
4	0	1	1.3	90
4	0.5	8	0.16	90
4	0.5	1	1.3	90
6	0.25	2	0.65	90
8	0	8	0.16	90
8	0	1	1.3	90
8	0.5	8	0.16	90
8	0.5	1	1.3	90

Workflow for the Design of Experiments plate performance [10]

1. The top aluminium foil was removed.
2. The bottom aluminium foil was removed.
3. **Removal of storage solution:** The storage solution was removed by centrifugation for 1 minute at 500g (rcf).
4. **Equilibration:** 200 µl of binding buffer was added to each well that was going to be used. (A1-10, B1-B10, C1-10, D1-10, E1-10, F1-10 see Appendix Figure 51 and 52. The plate was centrifuged for 1 minute at 500g. This step was performed three times.
5. **Blotting:** The plate was blotted against a soft paper tissue and placed on an empty UV-plate.
6. **Sample loading 1:** 120 µl of the sample was added to each well. Since incubation time also was a factor in the experiment, Sample was added first to rows named T3 which were rows: 1, 4, 7, 10 (see Appendix Figure 51 and 52). A plastic foil was placed on top of the plate.
7. **Incubation 1:** The plate was put on incubation on plate shaker for 60 min at 1100 rpm
8. **Sample loading 2**: The plastic foil was removed. 120 µl of the sample was added to each well to rows called T2 which were rows 2, 5, 8 (see Appendix Figure 51 and 52). A plastic foil was placed on top of the plate.
9. **Incubation 2:** The plate was put on incubation on plate shaker for another 20 min at 1100 rpm

10. **Sample loading 3**: The plastic foil was removed. 120 µl of the sample was added to each well to rows called T1 which were rows 3, 6, 9 (see Appendix Figure 51 and 52). A plastic foil was placed on top of the plate. (There were some drops left in the pipette after the sample addition, this regards sample added to wells: A9 & C6)
11. **Incubation 3:** The plate was put on incubation on plate shaker for another 10 min at 1100 rpm
12. The top plastic foil was removed.
13. **Removal of flow through:** The supernatant was removed by centrifugation for 1 minute at 500g. The fractions were collected in the 96 well UV plate and saved for further analysis.
14. **Wash out of unbound sample:** 200 µl of equilibration buffer was added to each well used. The plate was centrifuged for 1 minute at 500g. The fractions were collected in a 96 well UV plate for further analysis. This step was performed three times.
15. **Blotting:** The plate was blotted against a soft paper tissue and placed on an empty UV-plate
16. **Elution:** 120 µl of elution buffer was added to each well used. The plate was covered with a plastic lid and shaken on a plate shaker for 10 min at 1100 rpm. The plate was centrifuged for 1 minute at 500g. The fractions were collected in a 96 well UV-plate for further analysis. The plate was blotted against a soft paper tissue and placed on an empty UV-plate. This step was performed three times. (The second and the third time the rpm were 900. Note: There was no blotting performed after the third elution)
17. **Final elution:** 120 µl of 20% ethylene glycol in 50 mM MES buffer pH 6 was added to each well used. The plate was covered with a plastic foil and shaken on a plate shaker for 10 min at 900 rpm. The plate was centrifuged for 1 minute at 500g. The fractions were collected in a 96 well UV plate for further analysis. The plate was blotted against a soft paper tissue and placed on an empty UV-plate. This step was performed three times. (After the third elution there was no blotting since no further elution was performed)
18. **Analysis:** The plates with fractions collected were read at 280 nm and 310 nm by a plate spectrophotometer. A buffer plate containing 120 µl and 200 µl of the different binding buffers in quadruplicate were also read on plate reader at 280 nm and 310 nm. The values from the 280 nm were the ones used in the calculations; the measure at 310 nm was to check for possible light scattering effects. The buffers were absorbance-wise very similar and therefore averages for all buffers containing 120 µl and 200 µl respectively were calculated. (An outlier was excluded from the 120 µl buffers, due to being so different compared to the others. 0.20 AU, while the other buffer values lied between 0.15-0.17). The average value from the duplicate controls for the accumulated fractions flow through, wash 1, wash 2 and wash 3 was calculated for each concentration respectively subtracting the buffer controls for these fractions (120 µl buffer average for the flow through fraction and 200 µl buffers average for the three washes respectively), this was then used in the calculation as the initial sample concentration. The capacities were calculated through subtracting the flow through values from the different initial sample concentrations applied to the wells and finally by subtracting the average buffer value for the 120 µl buffers.

2.3 Temperature

All experiments were performed in room temperature.

2.4 Chemicals

Table 7. The chemicals used.

Chemical	Quality	Supplier
20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS)	Not specified	Elsichrom AB
Ammonium sulphate	Pro analysis	Merck
Citric acid	Pro analysis	Merck
Ethanol	Not specified	Kemetyl
Ethylene glycol	Pro analysis	Merck
MES buffer	Not specified	Sigma
Othophosphoric acid	Pro analysis	Merck
Propanol	Not applicable	Unknown
Sodium chloride	Pro analysis	Merck
Sodium hydroxide	Not applicable	Unknown
Sodium phosphate	Pro analysis	Merck
Sodium sulphate	Pro analysis	Merck
Tris buffer	Pro analysis	Merck

2.5 Equipment

Desalting: NAP™ -10 column, GE Healthcare Biosciences AB**;** PD MultiTrap™ G-25, GE Healthcare Biosciences AB
HIC & Protein A purification: ÄKTAexplorer™ 100, GE Healthcare Biosciences AB; Tricorn 5/100 column, GE Healthcare Biosciences AB**;** XK26/20 column, GE Healthcare Biosciences AB
Analytical SEC & Analytical Protein A purification: ÄKTAexplorer 10, GE Healthcare Biosciences AB**;** Superdex 200 5/150 GL, GE Healthcare Biosciences AB**;** Superdex 200 10/300 GL, GE Healthcare Biosciences AB**;** MabSelect SuRe HiTrap™ 1 ml, GE Healthcare Biosciences AB
96 well filter plates: available internally, GE Healthcare Biosciences AB
UV plates: 96 well flat bottom 3635, Costar
Plate shaker: MTS 2/4 digital, IKA
Centrifuge: 5810R, 5811 no: 0037021, Eppendorf ; rotor: A-2-DWP, Eppendorf
Plate readers: SPECTRAmax plus, Molecular Devices
Spectrophotometer: Ultrospec 6300 pro, GE Healthcare Biosciences AB
Transparent microplate foil: Microplate Foil (96-well) BR-1005-78, GE Healthcare Biosciences AB
Aluminium microplate foil: 'F' Film PCR Sealers, Bio-Rad
Syringe Filter: 0.45µm Filtropur S, Sarstedt; 0.2µm, Filtropur S, Sarstedt
Filter: ULTA™ prime CG, GE Healthcare Biosciences AB

3. Results

3.1 96 well filter plate screening

3.1.1 Absorbance

The absorbance results for the different HIC media from the 96 well plate screening are shown in Figures 8, 9 and 10. The Binding buffer and the wash buffer consisted of 0.8 M ammonium sulphate, 50 mM sodium phosphate. The elution buffers all contained 50 mM sodium phosphate as well as 0.6 M, 0.5 M, 0.4 M, 0.3 M, 0.2 M and 0 M (none) ammonium sulphate respectively. The MAb sample used contained approximately 15% aggregate and the load was approximately 11 mg/ml$_{resin}$. For some low absorbance values the result after subtracting the buffer controls were slightly below zero. The negative concentrations were adjusted to zero when presenting the results in Figures 8, 9 and 10. (The most negative value for binding buffer 0.8 M ammonium sulphate, 50 mM sodium phosphate was -0.00912 AU).

The calculated approximate yield for PlasmidSelect is 81.4%, Competitor 2 is 81.7% and B1 – Phenyl (20µmol/ml) is 51.6% (Figure 8). The percentage is based upon the total amount of recovered protein for the different HIC media compared to an average control value (1.90 AU) from the ten control wells in this plate. The control value for each of the ten control wells was based on the accumulated fractions from flow through, wash 1, wash 2 and wash 3 with the average buffer value subtracted from each fraction respectively. (The control wells contained no gel, were equilibrated with different binding buffers and sample was applied to these wells.)

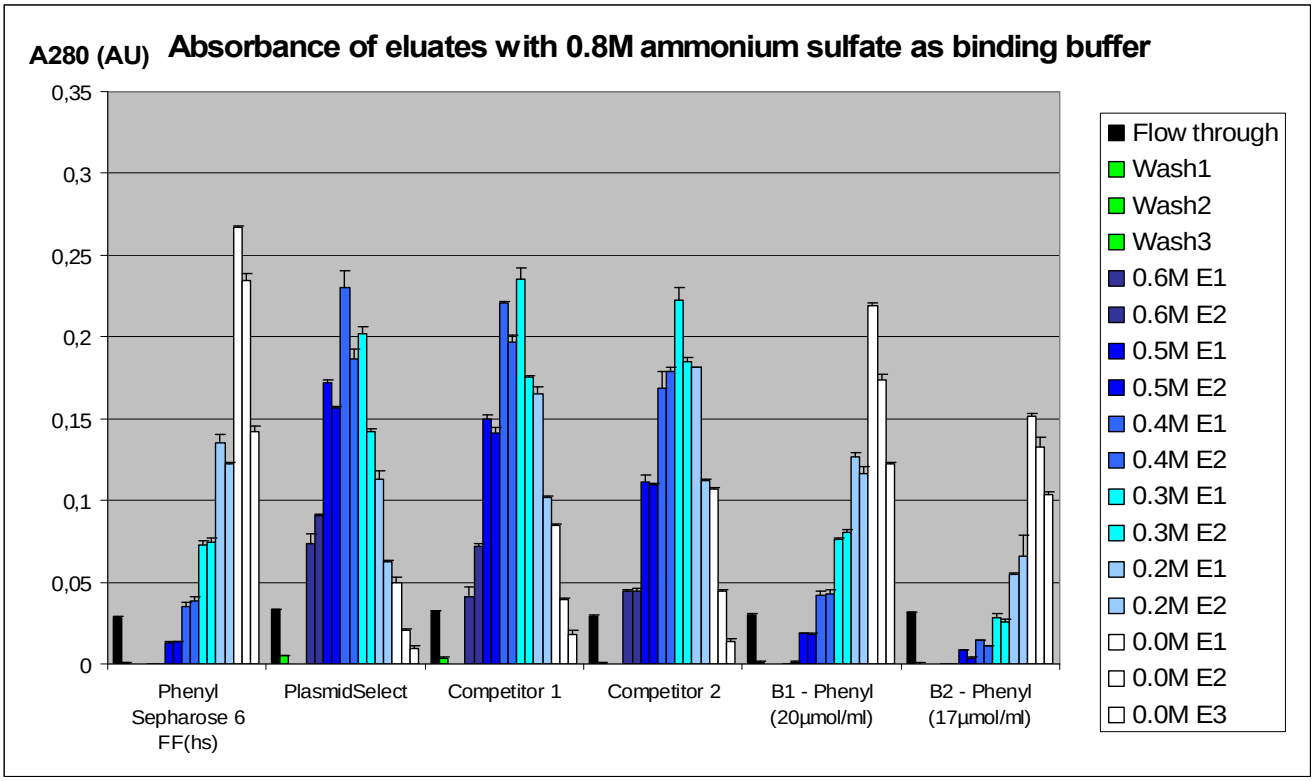

Figure 8. Flow through, wash and elution fractions measured in absorbance for the following chromatography media: Phenyl Sepharose 6 FF(hs), PlasmidSelect, Competitor 1, Competitor 2, B1 – Phenyl (20µmol/ml) and B2 – Phenyl (17µmol/ml). The elution fractions in the figure are called for example 0.6M E1 which means that this is the first elution with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). The variations between the duplicates are presented by y error bars.

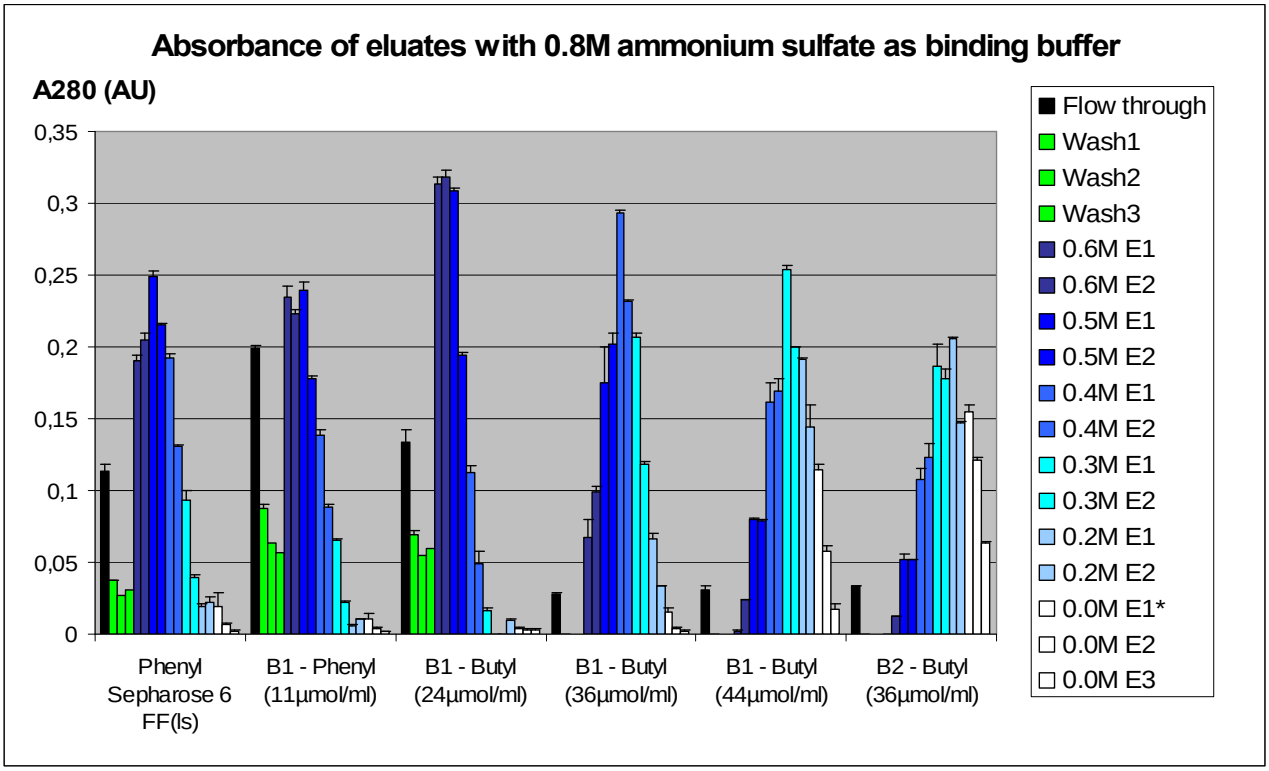

Figure 9. Flow through, wash and elution fractions measured in absorbance for the following chromatography media: Phenyl Sepharose 6 FF(ls), B1 – Phenyl (11µmol/ml), B1 – Butyl (24µmol/ml, B1 – Butyl (36µmol/ml), B1 – Butyl (44µmol/ml) and B2 – Butyl (36µmol/ml. The elution fractions in the figure are called for example 0.6M E1 which means that this is the first elution with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). The variations between the duplicates are presented by y error bars. *This elution plate got bumped causing a splash and probable cross contamination between some wells in this elution.

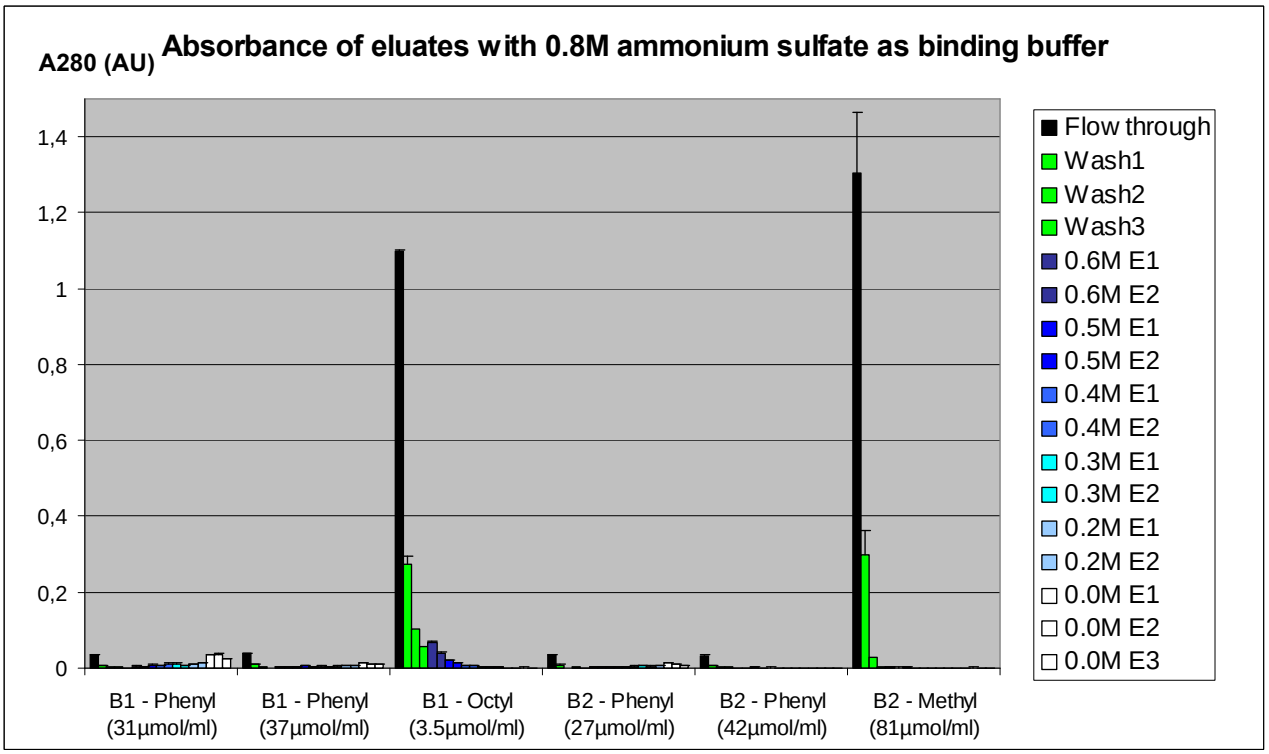

Figure 10. Elution fractions measured in absorbance for the following chromatography media: B1 – Phenyl (31µmol/ml), B1 – Phenyl (37µmol/ml), B1 – Octyl (3.5µmol/ml), B2 – Phenyl (27µmol/ml), B2 – Phenyl (42µmol/ml) and B2 – Methyl (81µmol/ml). The elution fractions in the figure are called for example 0.6M E1 which means that this is the first elution with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). The variations between the duplicates are presented by y error bars.

3.1.2 Aggregate content

The aggregate content as well as the absorbance at 280 nm for the selected HIC media with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer is presented in Figures 11, 12, 13, 14, 15. The elution fractions all consisted of 50 mM of sodium phosphate as well as 0.6 M, 0.5 M, 0.4 M, 0.3 M, 0.2 M and 0 M (none) ammonium sulphate respectively. (The selected HIC media were PlasmidSelect, Competitor 2, B1 – Butyl (36µmol/ml), B1 – Phenyl (11µmol/ml), B1 – Phenyl (20µmol/ml) and B1 – Phenyl (31µmol/ml)). The MAb sample used contained approximately 15% aggregate.

HIC media B1 – Phenyl (31µmol/ml) resulted in only one data point due to too little protein for the SEC analysis and was therefore not plotted as a graph. The aggregate content was 5.5% at 50 mM sodium phosphate without ammonium sulphate as elution buffer.

The fractions that contained too little protein for SEC analysis are presented as black triangles in the graphs and with zero aggregate but nothing can be said about the aggregate content in these samples. For some low absorbance values the result after subtracting the buffer controls the absorbance values were slightly below zero. (The lowest value was -0.00463 AU). The negative concentrations were put to zero when presenting the results in Figures 12, 14 and 15.

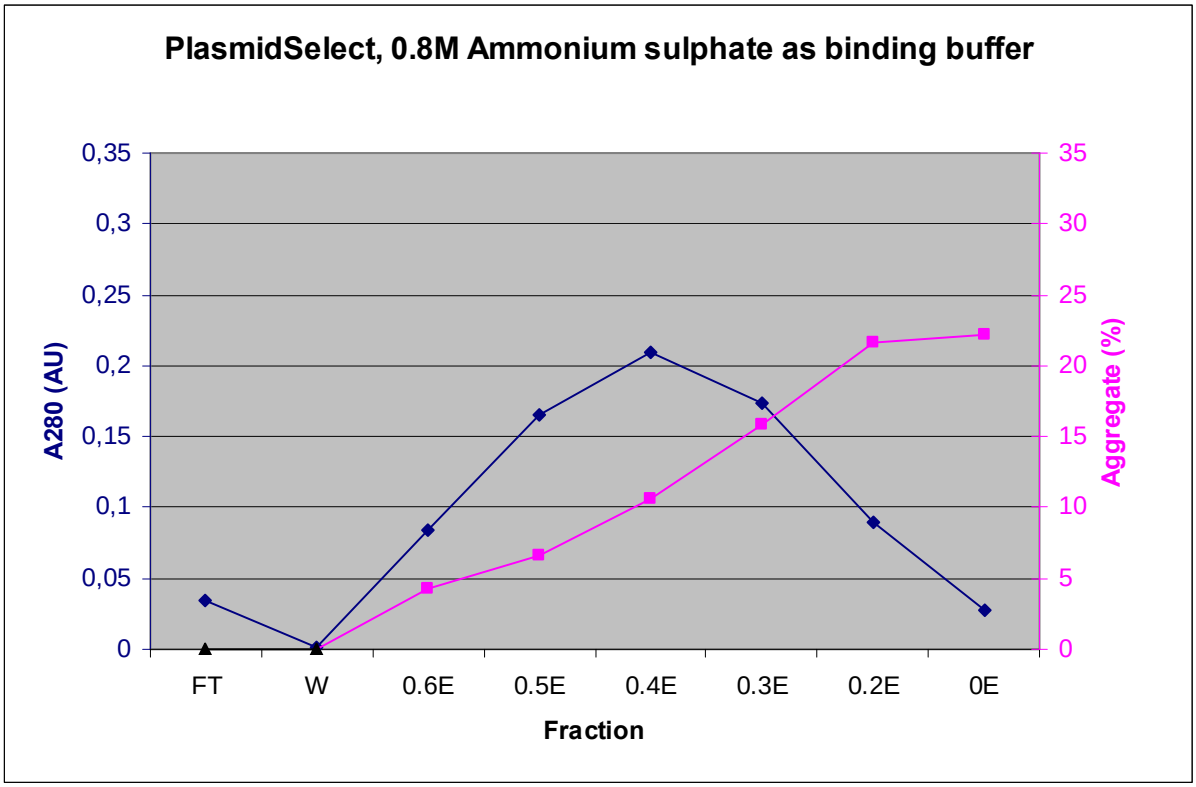

Figure 11. Aggregate content (%, right axis) and absorbance at 280 nm (AU, left axis) for PlasmidSelect with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer. The flow through (FT), wash (W) and elution fractions are shown in the figure. The elution fractions in the figure are called for example 0.6E which means that this is the fraction eluted with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). As also can be seen in Figure 8, a great amount of the protein seemed to elute for concentrations of 0.5 M to 0.3 M ammonium sulphate. The aggregate content at the highest absorbance value was 10.6%. The absorbance in AU is presented by blue colour and tilted squares. The aggregate in % is presented by pink colour and by squares.

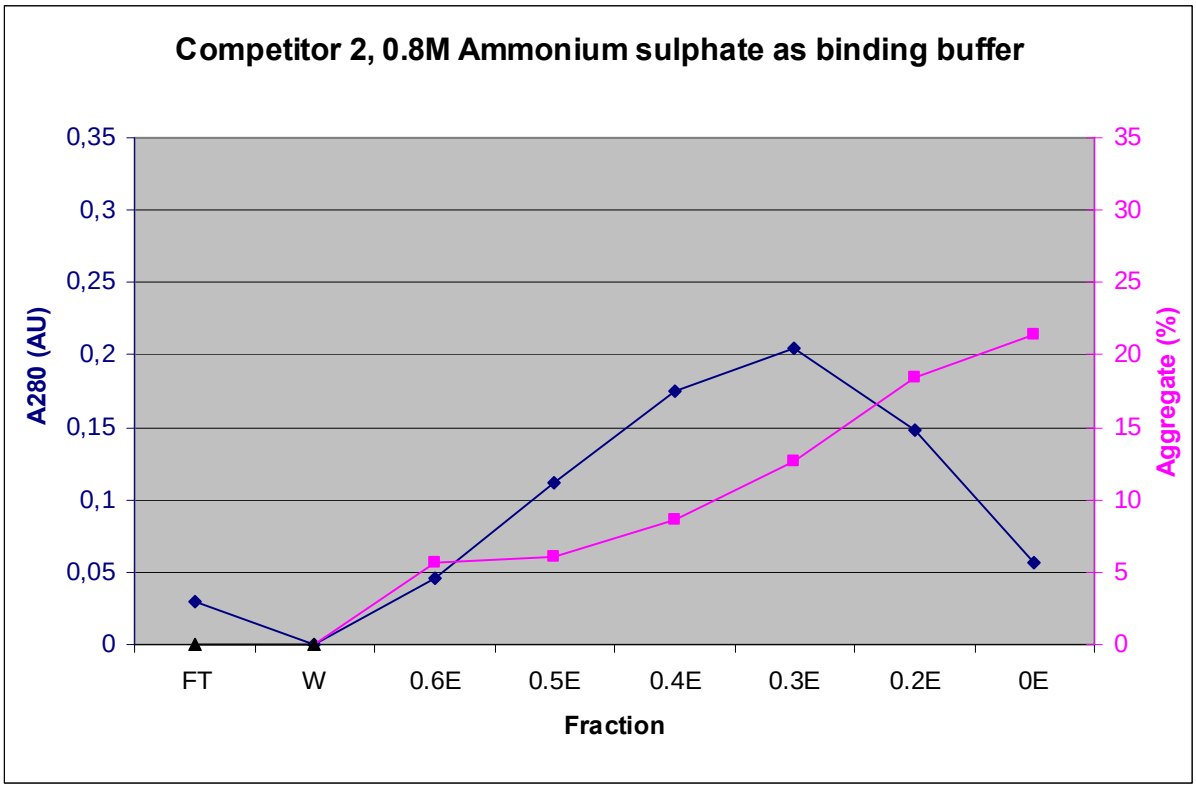

Figure 12. Aggregate content (%, right axis) and absorbance at 280 nm (AU, left axis) for Competitor 2 with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer. The flow through (FT), wash (W) and elution fractions are shown in the figure. The elution fractions in the figure are called for example 0.6E which means that this is the fraction eluted with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). As also can be seen in Figure 8, a great amount of the protein seemed to elute for concentrations of 0.4 M to 0.2 M ammonium sulphate. The aggregate content at the highest absorbance value was 12.7%. The absorbance in AU is presented by blue colour and tilted squares. The aggregate in % is presented by pink colour and by squares.

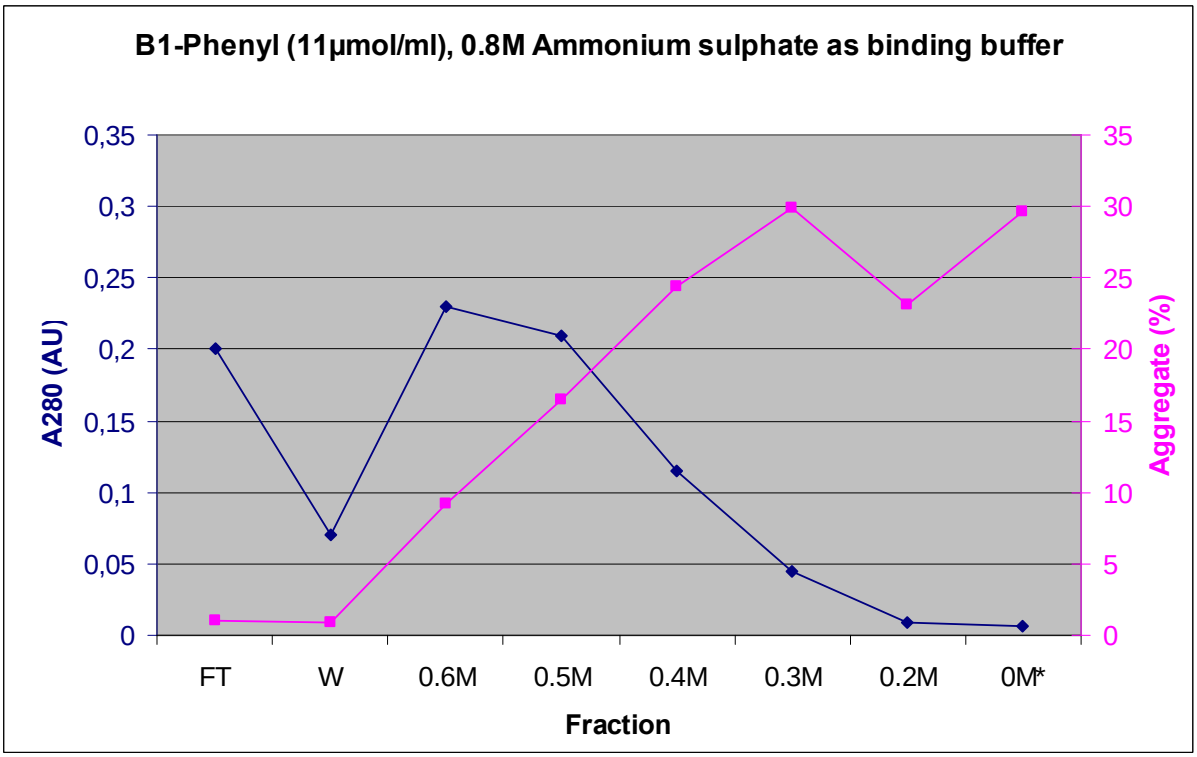

Figure 13. Aggregate content (%, right axis) and absorbance at 280 nm (AU, left axis) or B1 – Phenyl (11µmol/ml) with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer. The flow through (FT), wash (W) and elution fractions are shown in the figure. The elution fractions in the

figure are called for example 0.6E which means that this is the fraction eluted with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). As also can be seen in Figure 9, a lot of the protein came out in the flow through and also in the wash fractions. However the aggregate content was low in these fractions so not much monomer was lost. The aggregate content at the highest absorbance value was 9.2%. The aggregate peak was well separated from the absorbance peak. The absorbance in AU is presented by blue colour and tilted squares. The aggregate in % is presented by pink colour and by squares. *This elution plate got bumped causing a splash and probable cross contamination between some wells in this elution.

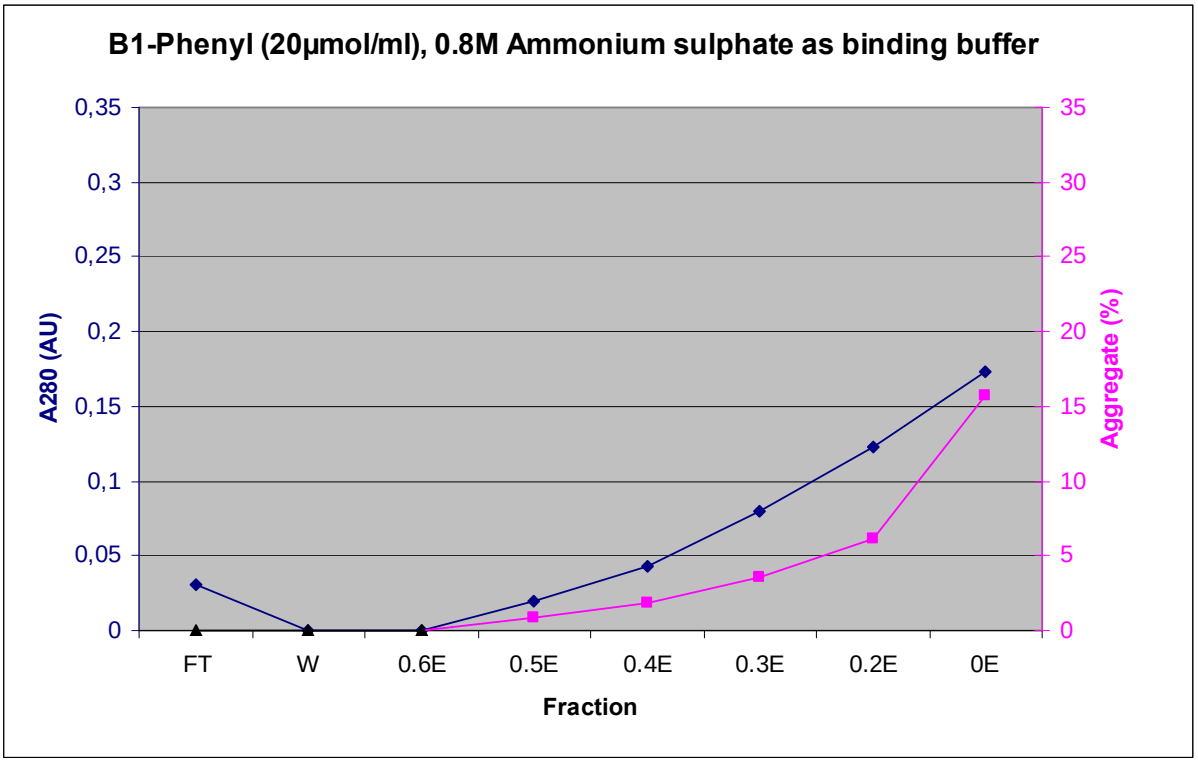

Figure 14. Aggregate content (%, right axis) and absorbance at 280 nm (AU, left axis) for B1 – Phenyl (20µmol/ml) with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer. The flow through (FT), wash (W) and elution fractions are shown in the figure. The elution fractions in the figure are called for example 0.6E which means that this is the fraction eluted with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). As also can be seen in Figure 8, the protein content was low and even though it increases towards lower salt concentrations a lot of the material did not elute. The aggregate content at the highest absorbance value was 15.6%. The absorbance in AU is presented by blue colour and tilted squares. The aggregate in % is presented by pink colour and by squares.

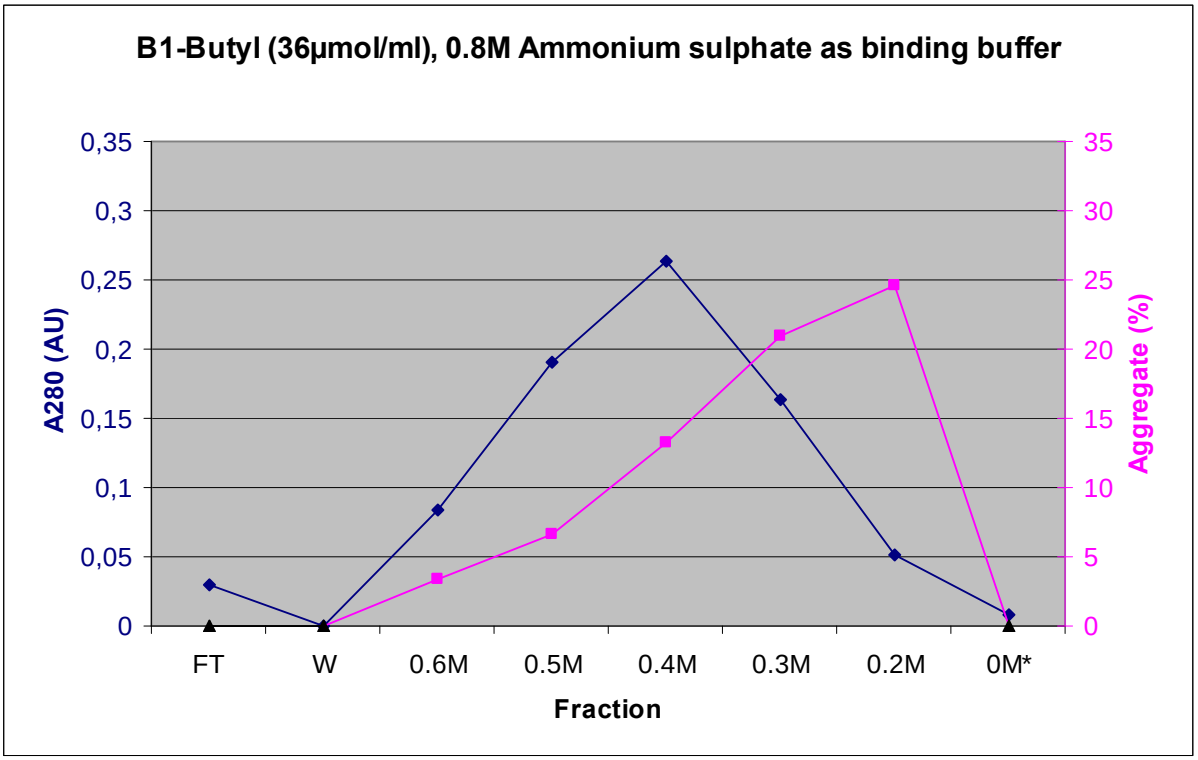

Figure 15. Aggregate content (%, right axis) and absorbance at 280 nm (AU, left axis) for B1 – Butyl (36µmol/ml) with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer. The flow through (FT), wash (W) and elution fractions are shown in the figure. The elution fractions in the figure are called for example 0.6E which means that this is the fraction eluted with 0.6 M of ammonium sulphate (and 50 mM sodium phosphate). As also can be seen in Figure 9, a great amount of the protein seemed to elute for concentrations of 0.5 M to 0.3 M ammonium sulphate. The aggregate content at the highest absorbance value was 13.2%. The aggregate peak was well separated from the absorbance peak. The absorbance in AU is presented by blue colour and tilted squares. The aggregate in % is presented by pink colour and by squares. *This elution plate got bumped causing a splash and probable cross contamination between some wells in this elution.

3.2 Column test

The six selected HIC media from the 96 well filter plate experiment were packed in columns and the results from the column verification are concluded in Table 8. All HIC media had acceptable plates/meter and asymmetry.

Table 8. The results from the column test in plates/meter and peak asymmetry.

HIC media	Plates/meter	Asymmetry
B1 – Butyl (36µmol/ml)	6234	0.97
B1 – Phenyl (11µmol/ml)	5181	0.88
B1 – Phenyl (20µmol/ml)	5652	0.93
B1 – Phenyl (31µmol/ml)	4847	0.84
PlasmidSelect	5006	0.82
Competitor 2	4806	0.89

3.3 Column verification

The results from the column runs are described in Figures 16, 17, 18, 19, 20 and 21. The MAb sample used contained approximately 15% aggregate and the load was approximately 16 mg/ml$_{resin}$. For prototype B1 – Phenyl (11µmol/ml) the sample eluted early in the conductivity gradient while for prototype B1 – Phenyl (20µmol/ml) the sample eluted late in the gradient. Regarding PlasmidSelect, B1 – Butyl (36µmol/ml) and Competitor 2 the sample eluted approximately in the middle of the conductivity gradient. Finally for prototype B1 – Phenyl (31µmol/ml) the sample did not elute in significant amounts.

The aggregate levels as well as the absorbance at 280 nm at the absorbance peaks are described in Table 9. In Table 9 there is also a comparison between the column and plate runs regarding the following parameters at the absorbance peaks; aggregate level, absorbance and the ammonium sulphate concentration. The ammonium sulphate concentrations at the aggregate peaks for chromatograms displayed in Figures 16-21 and the ammonium sulphate concentration for the fractions in Figure 22 were calculated in the following way:

$$c_{peak(frac)} = \left(\frac{Cond_{peak(frac)}}{(Cond_{\max} - Cond_{\min})} \right) \times c_{bb}$$

where;

$c_{peak\backslash frac}$ = the ammonium sulphate concentration at the aggregate peak (Figures 16-21) or at the centre of the elution fraction (Figure 22)

$Cond_{peak\backslash frac}$ = the conductivity at the absorbance peak (Figures 16-21) or at the centre of the elution fraction (Figure 22)

$Cond_{max}$ = the conductivity maximum in the chromatogram

$Cond_{min}$ = the conductivity minimum in the chromatogram

c_{bb} = the concentration of the binding buffer (0.8 M ammonium sulphate)

The ammonium sulphate concentration at which the protein elutes for all HIC media seemed to be similar between plate and column. The aggregate concentrations at the absorbance peak were similar between plate and column for HIC media PlasmidSelect and Competitor 2 and different for HIC media B1 – Butyl (36µmol/ml) and B1 – Butyl (20µmol/ml). Figure 22 shows prototype B1 – Butyl (36µmol/ml) as an example where the SEC chromatograms for the elution fractions are incorporated into the absorbance chromatogram from Figure 17 in order to graphically visualize the increase in aggregate content. The aggregates are withheld by the HIC media and are eluted when the conductivity decreases. The aggregate level is also concentrated from the original 15% to 37% at the highest level.

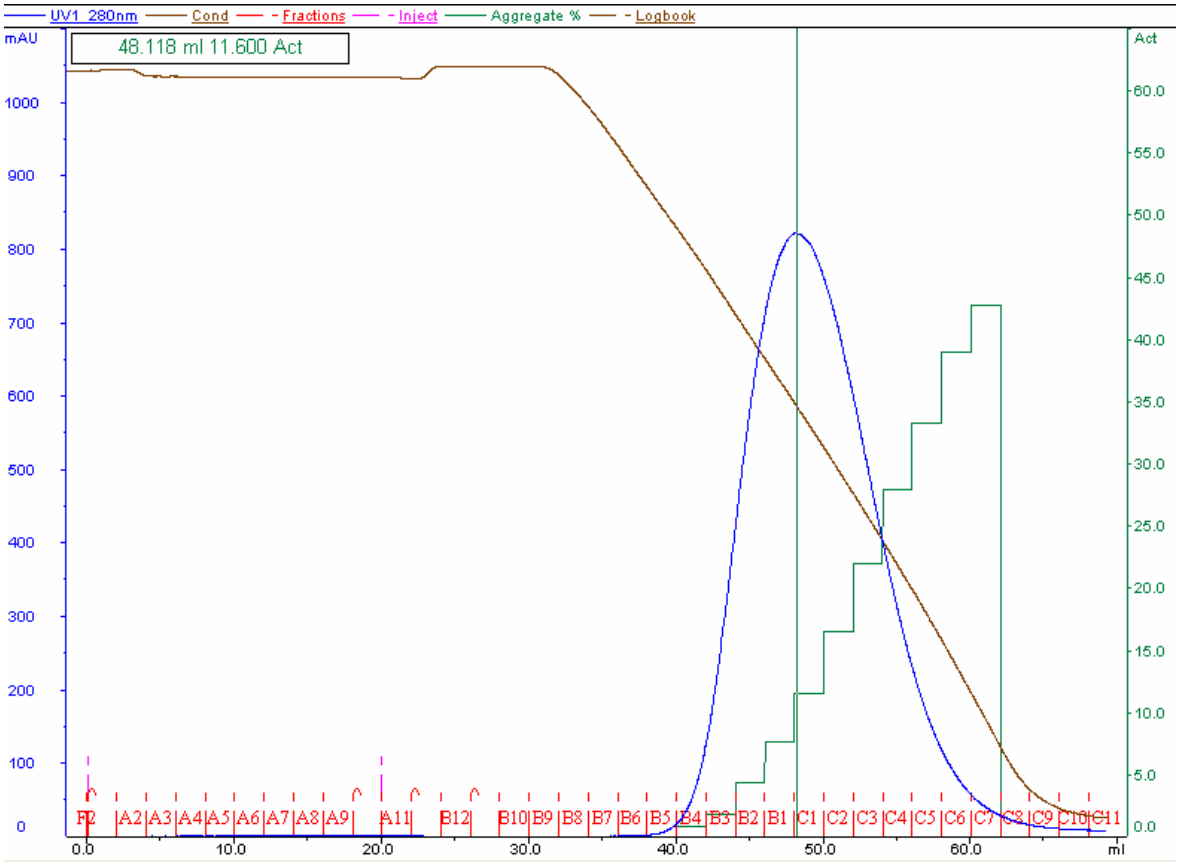

Figure 16. The chromatogram for PlasmidSelect with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer and 50 mM sodium phosphate without ammonium sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. PlasmidSelect eluted at an approximate concentration of 0.5 M ammonium sulphate. The aggregate content at the absorbance peak was 11.6%.

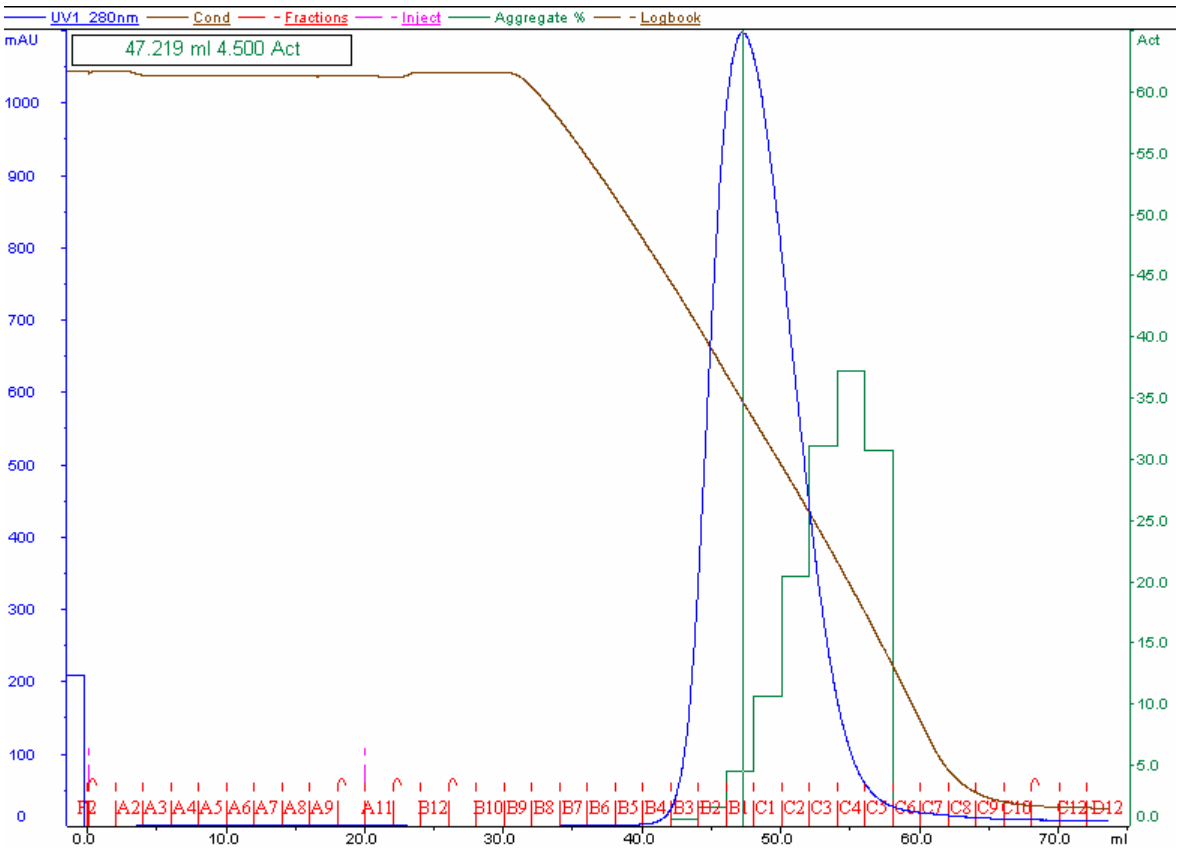

Figure 17. The chromatogram for B1 – Butyl (36µmol/ml) with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer as binding buffer and 50 mM sodium phosphate without ammonium

sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. B1 – Butyl (36µmol/ml) eluted at an approximate concentration of 0.5 M ammonium sulphate. The aggregate content at the absorbance peak was 4.5%.

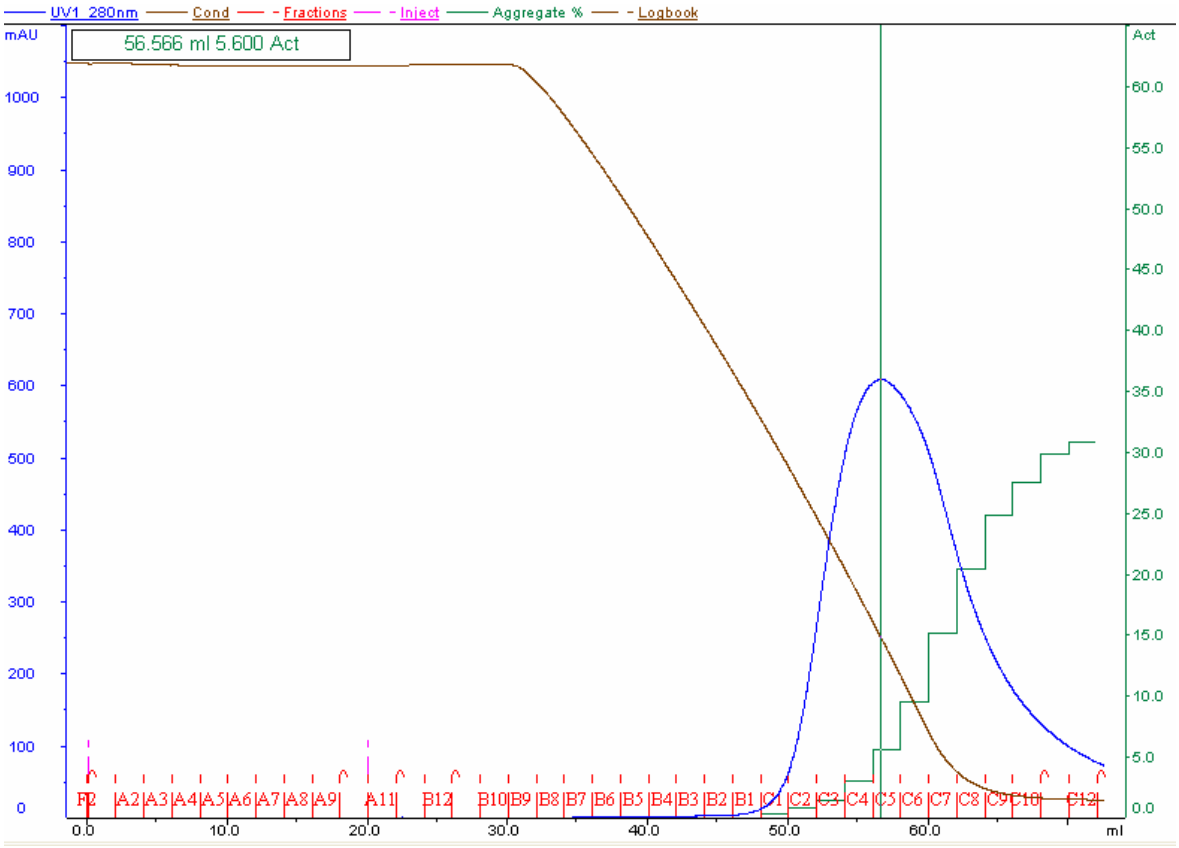

Figure 18. The chromatogram for B1 – Phenyl (20µmol/ml) with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer as binding buffer and 50 mM sodium phosphate without ammonium sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. B1 – Phenyl (20µmol/ml) eluted at an approximate concentration of 0.2 M ammonium sulphate. The aggregate content at the absorbance peak was 5.6%.

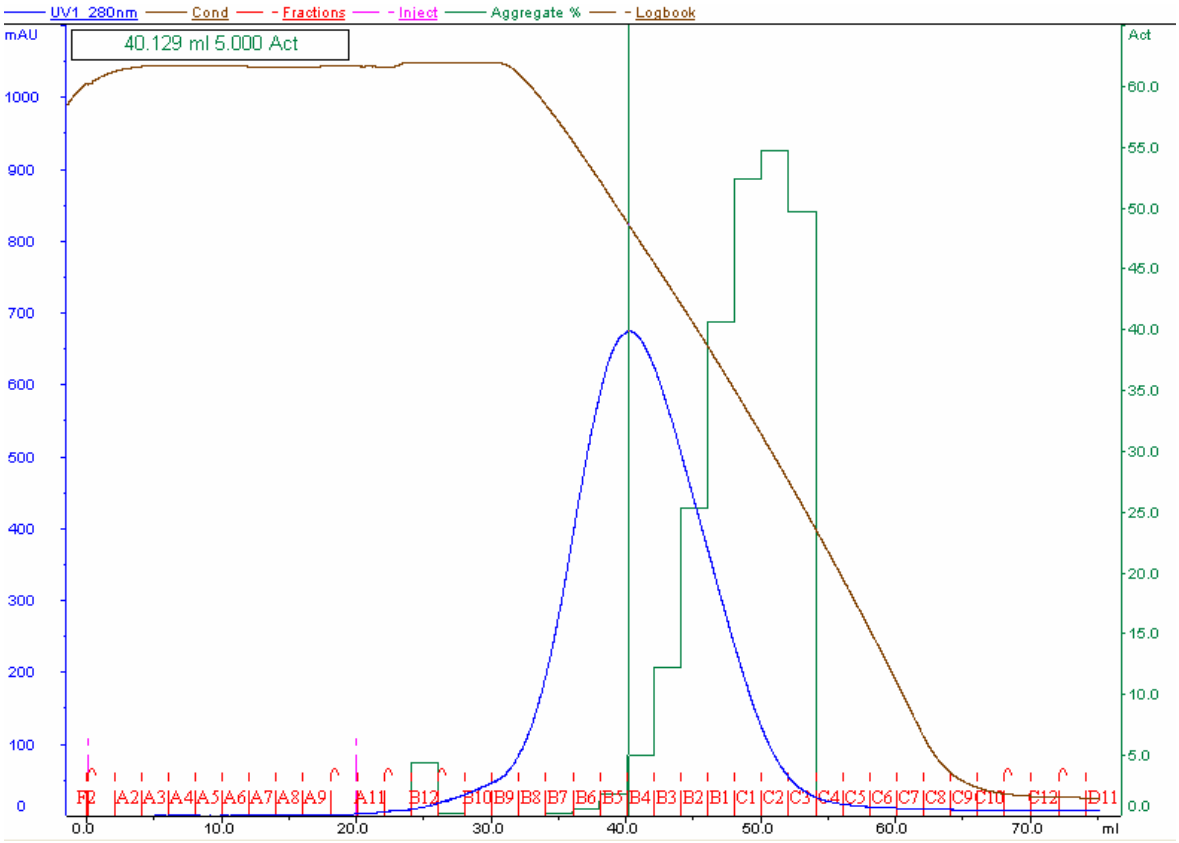

Figure 19. The chromatogram for B1 – Phenyl (11µmol/ml) with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer as binding buffer and 50 mM sodium phosphate without ammonium sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. B1 – Phenyl (11µmol/ml) eluted at an approximate concentration of 0.7 M ammonium sulphate. The aggregate content at the absorbance peak was 5%.

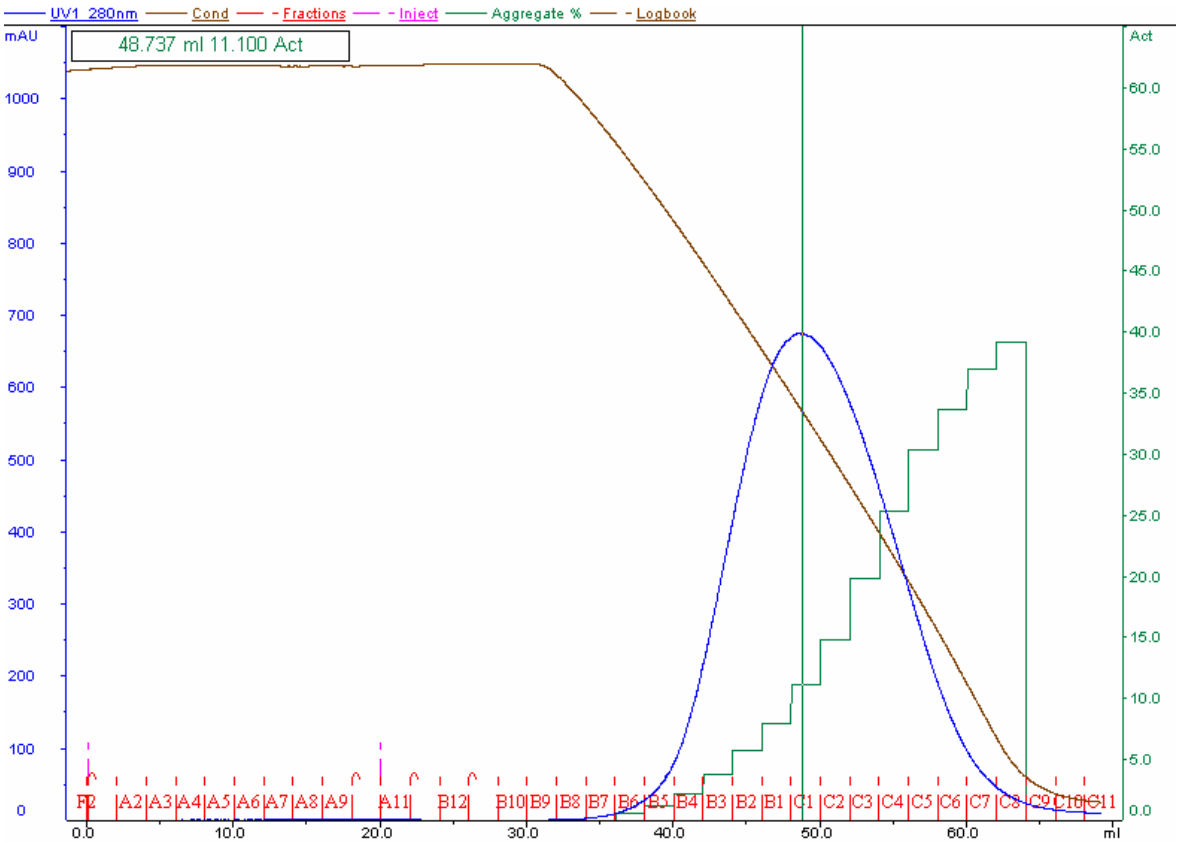

Figure 20. The chromatogram for Competitor 2 with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer as binding buffer and 50 mM sodium phosphate without ammonium sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU,

right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. Competitor 2 eluted at an approximate concentration of 0.5 M ammonium sulphate. The aggregate content at the absorbance peak was 11.1%.

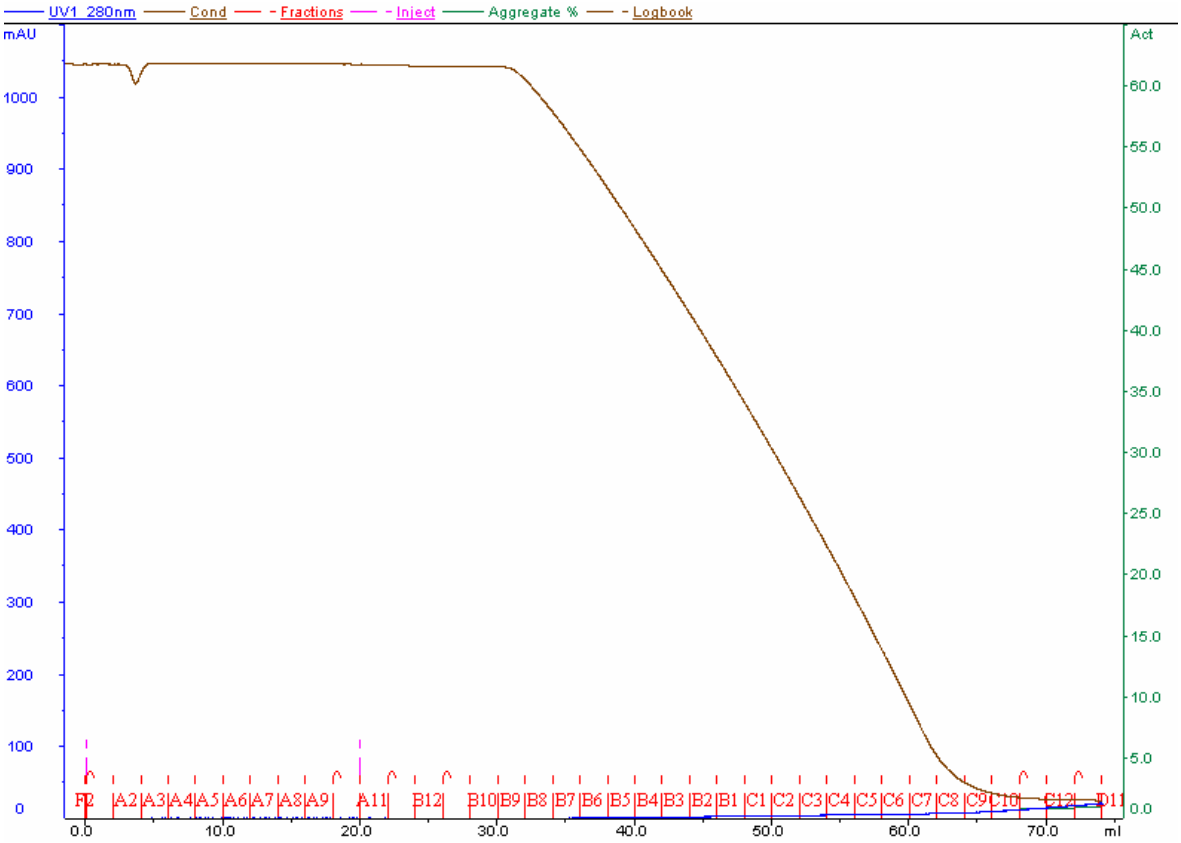

Figure 21. The chromatogram for B1 – Phenyl (31µmol/ml) with 0.8 M ammonium sulphate, 50 mM sodium phosphate as binding buffer as binding buffer and 50 mM sodium phosphate without ammonium sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. The protein did not elute in significant amounts.

Table 9. A comparison of the results from the plate experiment and the column experiment. The table describes the values at the absorbance peaks for following parameters: ammonium sulphate concentration, aggregate level and absorbance values. The ammonium sulphate concentration at where

	Ammonium sulphate concentration at the absorbance peak at 280 nm		**Aggregate level at the absorbance peak at 280 nm**	
	Column	**Plate**	**Column**	**Plate**
HIC media				
PlasmidSelect	**~ 0.49 M**	**0.4 M**	11.6%	10.6%
Competitor 2	**~ 0.47 M**	**0.3 M**	11.1%	12.7%
B1 – Butyl (36µmol/ml)	**~ 0.49 M**	**0.4 M**	4.5%	13.2%
B1 – Phenyl (11µmol/ml)	**~ 0.67 M**	**0.6 M**	5%	9.2%
B1 – Phenyl (20µmol/ml)	**~ 0.22 M**	**0 M**	5.6%	15.6%

the protein elutes seems to be similar between plate and column experiments for all of the HIC media. The aggregate content seems to be similar between plate and column experiments for PlasmidSelect and Competitor 2. The aggregate content between plate and column for B1 – Butyl (36µmol/ml), B1 – Butyl (20µmol/ml) were different.

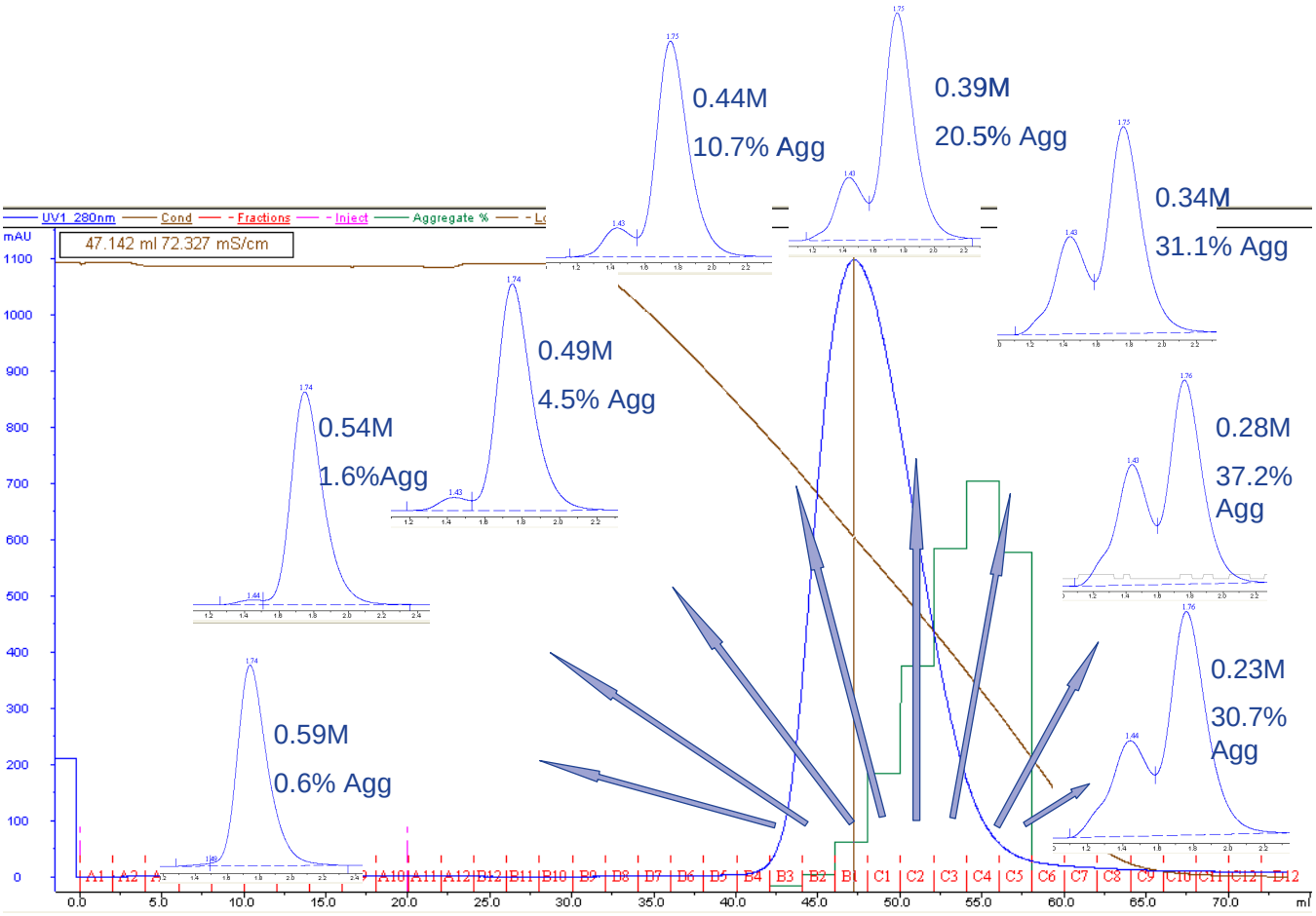

Figure 22. Prototype B1 – Butyl (36µmol/ml) is used as an example and illustrates the aggregate and the ammonium sulphate concentration at the elution fractions from the column run. Figure 17. is used as background of this picture and SEC chromatograms for the eight elution fractions are incorporated into the absorbance chromatogram in order to graphically visualize the increase in aggregate content. In the small chromatograms the aggregate peak is the smaller left peak next to the larger monomer peak to the right. The molarity next to each elution peak represents the calculated approximate concentration of ammonium sulphate and the percentage stands for the aggregate content in that fraction (Agg is abbreviation for aggregate)

3.4 Different salts

One chromatography medium, B1 – Phenyl (20µmol/ml), was investigated and analysed using four different salts; ammonium sulphate, sodium citrate, sodium sulphate and sodium chloride. The MAb sample used contained approximately 15% aggregate and the load was approximately 16 mg/ml$_{resin}$.. Binding concentrations for the respective salts were 0.8 M for ammonium sulphate, 0.6 M for sodium citrate, 0.5 M for sodium sulphate and 2.5 M for sodium chloride. All the different salts also contained 50 mM of sodium phosphate. The chromatograms for the different salts are shown in Figures 23, 24, 25 and 26. The aggregate content at the absorbance peak at 280 nm were 5.6% for ammonium sulphate, 9.4% for sodium citrate, 5.3% for sodium sulphate and 14.3% for sodium chloride.

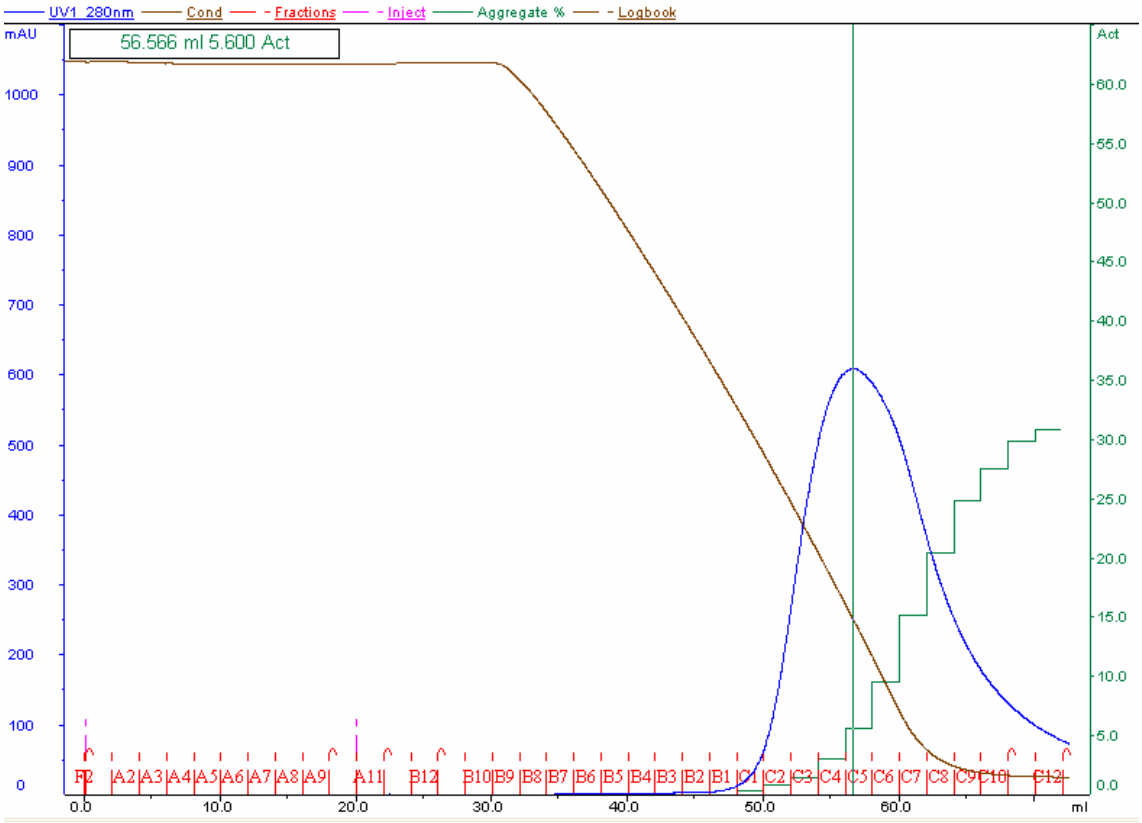

Figure 23. B1 – Phenyl (20µmol/ml) was tested using 0.8 M Ammonium Sulphate, 50 mM sodium phosphate as binding buffer and 50 mM sodium phosphate without ammonium sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. The aggregate peak at the absorbance peak at 280 nm was 5.6%.

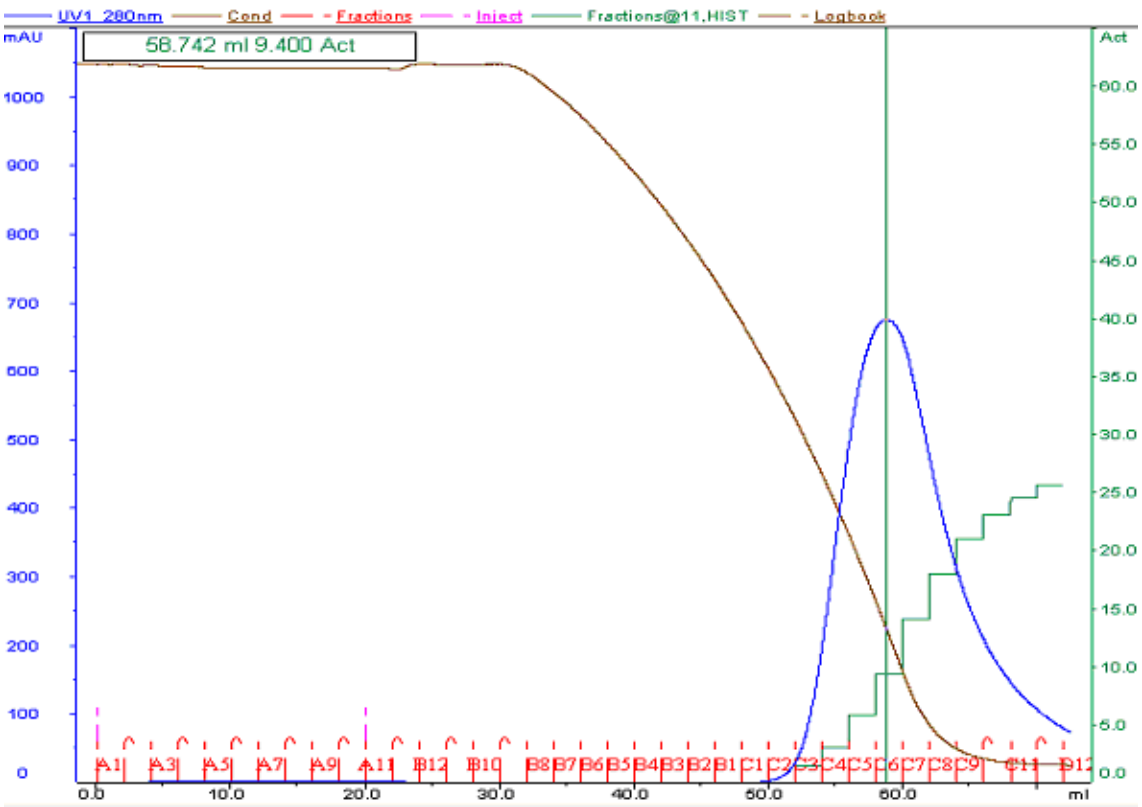

Figure 24. B1 – Phenyl (20µmol/ml) was tested using 0.6 M sodium citrate, 50 mM sodium phosphate as binding buffer and 50 mM sodium phosphate without sodium citrate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. The aggregate peak at the absorbance peak at 280 nm was 9.4%.

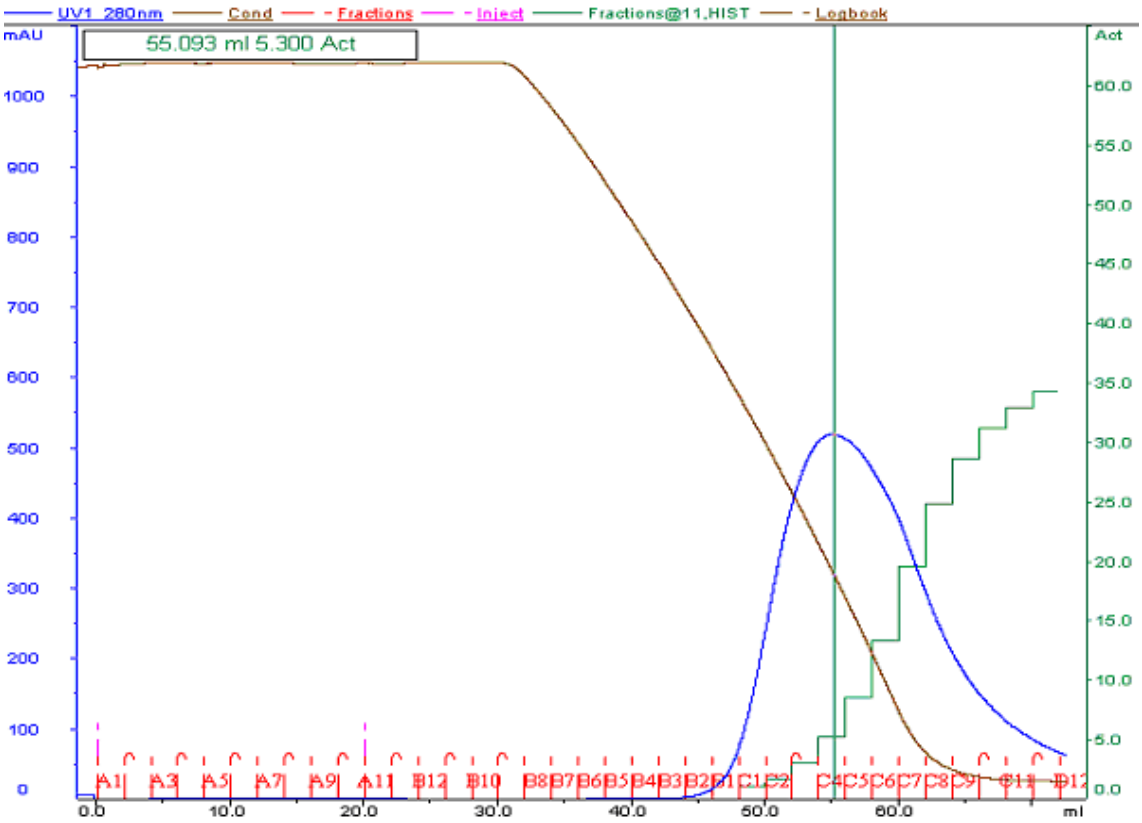

Figure 25. B1 – Phenyl (20µmol/ml) was tested using 0.5 M sodium sulphate, 50 mM sodium phosphate as binding buffer and 50 mM sodium phosphate without sodium sulphate as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. The aggregate peak at the absorbance peak at 280 nm was 5.3%.

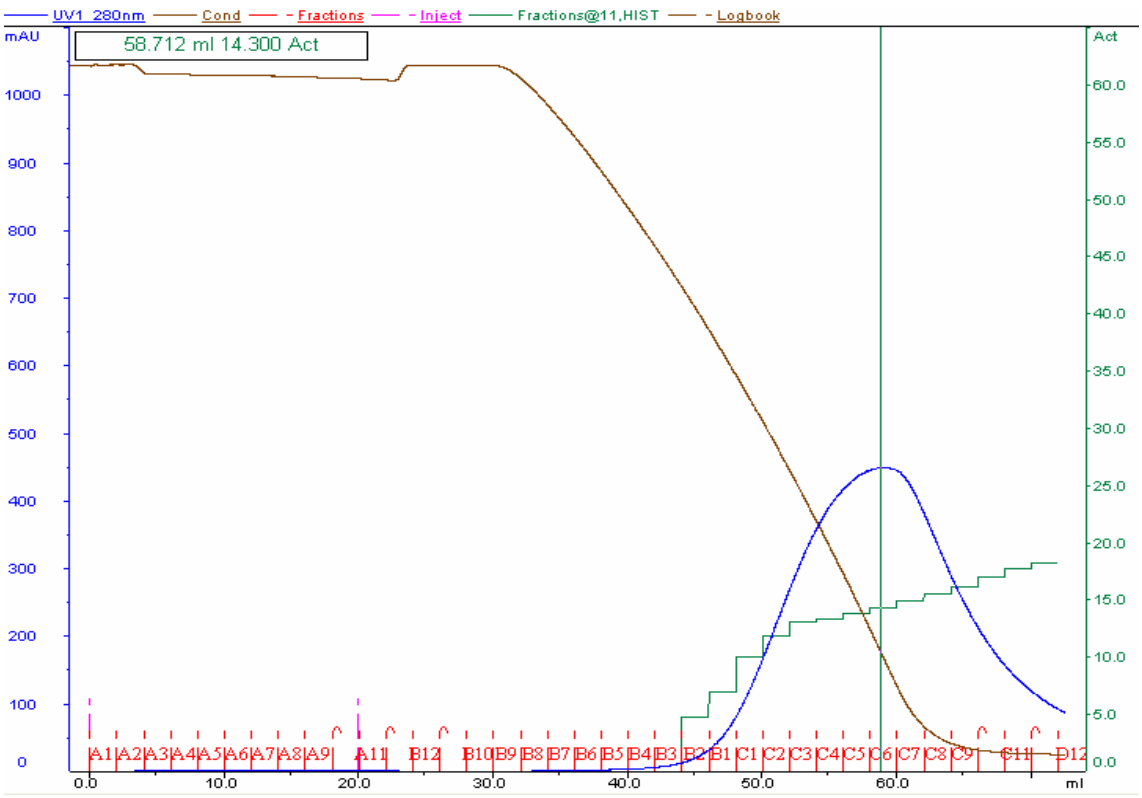

Figure 26. B1 – Phenyl (20µmol/ml) was tested using 2.5 M sodium chloride, 50 mM sodium phosphate as binding buffer and 50 mM sodium phosphate without sodium chloride as elution buffer. Left axis and blue colour curve represents the absorbance at 280 nm in mAU, right axis and green colour curve represents the aggregate content in % and brown colour represents the conductivity. The aggregate peak at the absorbance peak at 280 nm was 14.3%.

3.5 Adsorption isotherm study

Prototype B1 – Phenyl (20µmol/ml) was chosen for an isotherm study in the 96 well filter plate format. The MAb sample used contained approximately 93% aggregate. The purpose of the study was to estimate the resin's capacity of purifying MAbs. The obtained antibody binding capacities were plotted against the five applied sample concentrations (1 mg/ml, 0.667 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml) in Figure 27. A Langmuir model fit was used in order to approximate the total antibody binding capacity of the resin resulting in an approximately 12 mg/ml_{resin} in the 96 well filter plate format. The results for the isotherm study were based of the flow through values and the measured initial sample concentrations only. The protein was very difficult to elute at the conditions used.

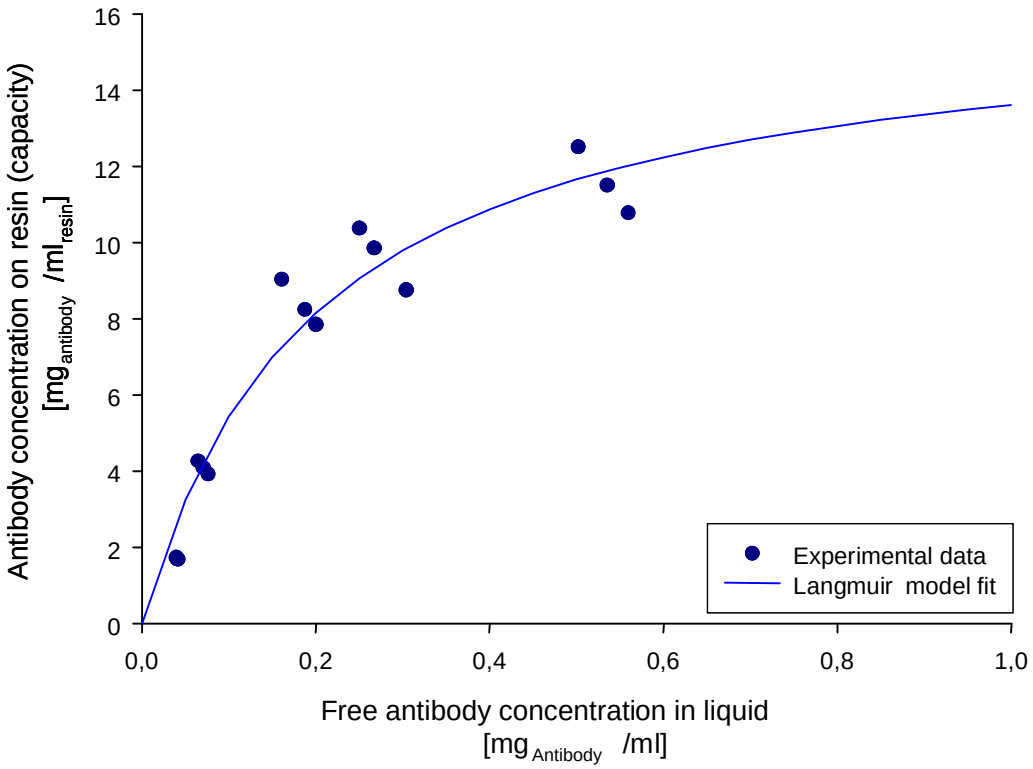

Figure 27. The antibody binding capacity results from the adsorption isotherm study for prototype B1 – Phenyl (20µmol/ml) are plotted against the different sample dilutions that were used. A Langmuir model fit was used in order to approximate a total antibody binding capacity of the HIC media. The approximate total antibody binding capacity in the 96 well filter plate format is 12 mg/ml_{resin}.

3.6 Design of Experiments

Prototype B1 – Phenyl (20µmol/ml) and Competitor 2 were chosen for an evaluation regarding antibody capacity in a Design of Experiments using a central composite face design (CCF) containing 24 experiments + 3 center-points. The chosen varied factors were loading pH and salt, incubation time and sample concentration and the response factor was antibody binding capacity mg/ml_{resin}. The sample used was the MAb sample containing a high amount of aggregate, 93%. With the conditions tested, Competitor 2 did not have binding capacities large enough for evaluation of the results in the software MODDE. On the other hand, Prototype B1 – Phenyl (20µmol/ml) had higher binding capacities with the conditions tested and could therefore be evaluated using MODDE. Due to asymmetrical data, since the chosen center-point was not in the centre, a Box-Cox transformation was performed making the data more normal distributed. An estimated lambda max of 0.33 was used, and all capacity values were transformed by using the power of the estimated lambda max value [12]. After evaluation using the MODDE software, the optimal binding capacity values in the results were re-transformed by using the power of (1/0.33). Before the transformation the model validity was negative, but the transformation resulted in a positive model validity and a more true evaluation in MODDE.

Figure 28 shows the coefficient plot where high sample concentrations, high salt concentrations and low pH in the loaded sample resulted in higher binding capacities. Incubation time only seems to have a slight impact on the binding capacity. There is an interaction coefficient between pH and salt which results in high binding capacities. Figure 29 shows the summary of fit from the regression analysis. The high and similar values for R2 and Q2 show that there is a good summary of fit to the model. The Reproducibility is very high, which means that the variation of the response under the same conditions is very small. The model validity in figure 29 has a value below 0.25 which means that there is a significant lack of fit to the model and that the error in the model is significantly larger than the pure error [13]. The capacity contour plot in Figure 30 shows how the antibody capacities at the longest incubation time (90 min) and the highest sample concentration (1.3 mg/ml) based upon the results are predicted to vary with different pH and salt content.

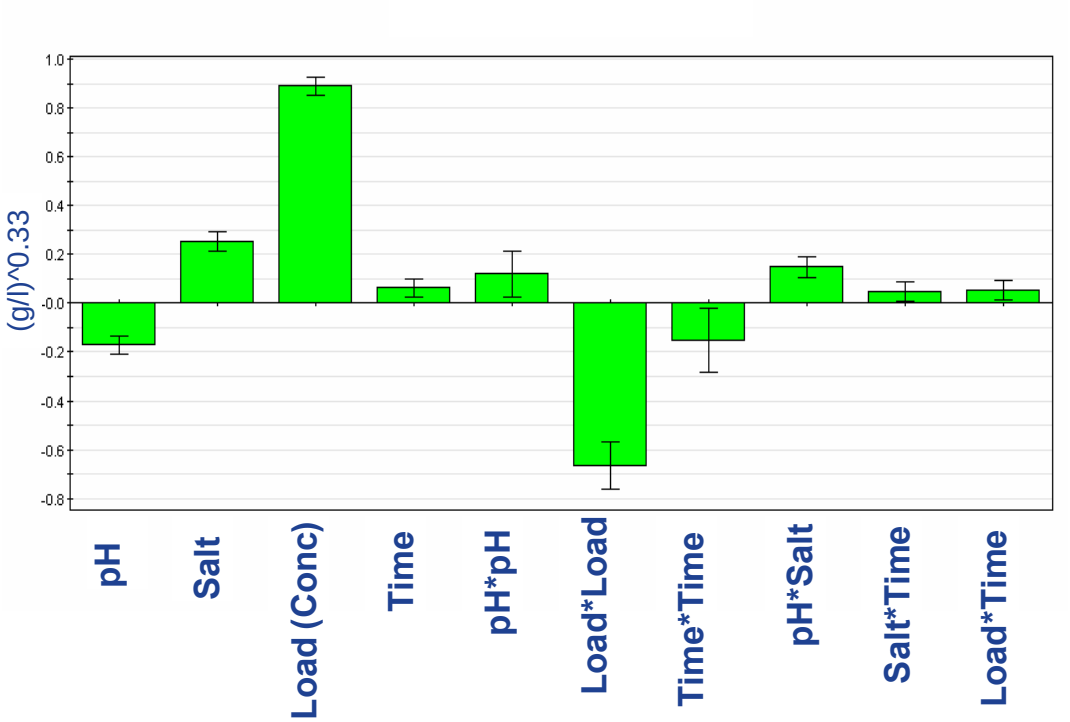

Figure 28. The coefficient plot for prototype B1 – Phenyl (20µmol/ml) after removal of the insignificant coefficients.

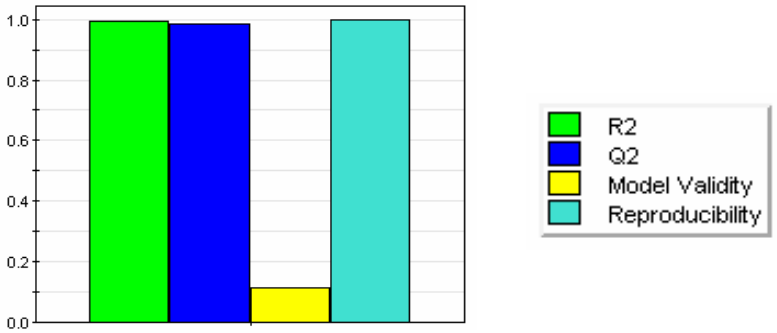

Figure 29. The summary of fit (Multiple linear regression) shows high positive values for R2, Q2, and reproducibility. The model validity has a value under 0.25 which shows a significant lack of fit to the model.

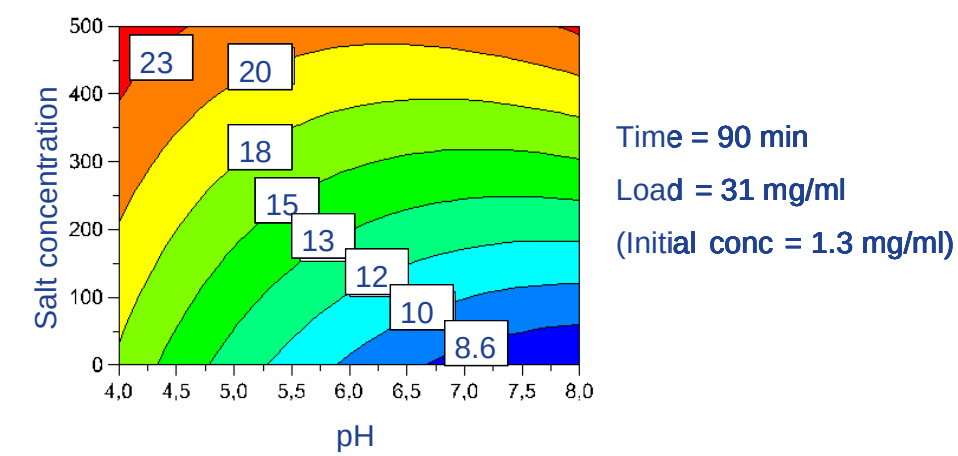

Figure 30. Contour plot of the antibody binding capacity for prototype B1 – Phenyl (20µmol/ml). The plot shows how the binding capacity in mg/ml_{resin} varies with pH and salt concentration after 90 minutes sample incubation and an initial concentration of 1.3 mg/ml. High salt concentrations and low pH result in higher binding capacities.

4. Discussion

96 well plate format

Screening in the 96-well filter plate format gives much information about the nature of HIC media in terms of hydrophobicity and different elution conditions. This is an efficient way to simultaneously collect a large amount of data when screening and benchmarking different media and elution conditions. The results from the 96 well plate experiment that are concluded in Figures 8, 9 and 10 showed significant differences between eluate fractions for the different types of HIC media respectively. The slurry concentration was misjudged and can therefore vary between the different HIC media. (The lowest grading on the Falcon tube was mistaken for 1 ml when it was in fact 1.5 ml, giving rise to an approximate HIC media slurry of 14% instead of the planned 10%). However, the elution pattern is likely to stay similar even if the HIC media volume varies. In figures 8, 9 and 10 one can compare different HIC media's hydrophobicity and where most of the protein elutes. The less amount of ligand the less hydrophobic and the weaker is the binding between the HIC media and the sample. For example for base matrix B1 with phenyl ligand with different ligand concentrations: 11, 20, 31 and 37 µmol/ml one can see in Figure 9 that B1 – Phenyl (11µmol/ml) is not particularly hydrophobic. Considerable amount of material comes in the flow through and the wash fractions. Increasing the ligand concentration to 20 µmol/ml, the media becomes more hydrophobic and material elutes late in the concentration gradient. Not all material seems to elute at this ligand concentration. Increasing the ligand concentration even further to 31 and 37 µmol/ml the media becomes so hydrophobic that only a small amount of the material elutes. A similar pattern can be seen for base matrix B1 with butyl ligand. In Figure 9 three different ligand concentrations are displayed all following the same pattern. Based on these results 96 well filter plate screening seems to be an efficient way to compare different HIC media's hydrophobicity.

Column format

The prototype B1 – Phenyl (20µmol/ml) performed best in the column experiments reducing the aggregate levels at low salt concentrations (Figure 18). Analyzing the results from the column confirmation two things were desirable; a low aggregate content at the absorbance peak and a good separation between the absorbance and aggregate peak. HIC media B1 – Phenyl (11µmol/ml) and B1 – Phenyl (20µmol/ml) fulfilled both a low aggregate content and a good separation between the absorbance peak and the aggregate peak. Prototype B1 – Phenyl (20µmol/ml) eluted the protein late in the gradient enabling binding of the sample at lower salt concentrations than B1 – Phenyl (11µmol/ml). This is favourable both due to costs of salt and also that the MAb had a strong tendency to precipitate at high salt concentrations.

Comparing the plate and column format

Comparing the results from the plate and column format there is a good correlation between the salt concentrations where the sample eluted but not such a good correlation between the aggregate levels at the absorbance peaks. However, there are some differences between the plate and column format such as for example that the elutions in the plate format are performed stepwise and not as a continuous gradient that is used in the column format. Another reason for the poor correlation for the aggregate contents measured in the plate and column format could be the difference in load. The miscalculation of the HIC media slurry concentration in the 96 well filter plate experiment resulted in a lower load for the plate experiment aimed for. For the plate screening the load was approximately 11 mg/ml$_{resin}$ compared to the column experiments where the load was approximately 16 mg/ml$_{resin}$. This could explain the poor correlation between the plate and column format regarding the aggregate levels at the absorbance peaks and that prototype B1 – Phenyl (20µmol/ml) performed so much better in the column format than in the plate format. In the plate experiment the incubation time was one hour which also could be enough for a strongly hydrophobic media to bind to and interact with the protein so strongly that a deformation of the protein could take place. This would result in difficulties to elute the protein. In the column there is a constant flow through the column and the sample interacts with the HIC media for a shorter amount of time. In order to make a proper comparison between the plate performance and column performance,

the plate experiment could be repeated using a more exact way to determine the gel slurry concentration and using the same load of 16 mg/ml$_{resin}$.

Salt study

Ammonium sulphate and sodium sulphate performed in a relatively similar way, resulting in approximate aggregate contents of 5.6% and 5.3% respectively with broad absorbance peaks (Figures 23 and 25). Sodium citrate did not perform as well resulting in 9.4% aggregate at the absorbance peak (Figure 24). Sodium chloride performed very poorly in aggregate removal having an approximate 14.3% aggregate at the absorbance peak (Figure 26). Sodium sulphate was chosen as the salt to continue with for the last two experiments. Since high pH values around 8 were to be used sodium sulphate was more appropriate than ammonium sulphate which has a tendency to create gaseous ammonia at those pH values.

Adsorption isotherm study

The results from the isotherm study showed an approximate antibody binding capacity in the 96 well filter plate format for the prototype media B1 - Phenyl (20µmol/ml) of 12 mg/ml$_{resin}$ (Figure 27). Keeping in mind that this capacity was based on a sample containing approximately 93% aggregate this is a fairly high capacity. It would be of interest to examine the binding capacity of this promising prototype on a column and comparing it to Competitor 2 using a sample containing an aggregate level of approximately 15%. It would also be interesting to test how well prototype B1 - Phenyl (20µmol/ml) purifies the sample from HCP and possible Protein A leakage. If to be repeated the elutions could be performed for an extended period of time in order to try to elute the bound protein.

Design of Experiments

The results from the Design of Experiments showed high antibody binding capacities for high concentrations of sodium sulphate and high capacities for low pH values at pH 4. A high binding capacity for high salt concentration is expected but the high binding capacity at pH 4 is less obvious since the MAb would be strongly charged and should not interact as strongly with the ligand. One possibility to the high capacity at pH 4 could be that the structure of the MAb no longer is intact and that hydrophobic parts are exposed, creating a stronger interaction with the hydrophobic ligand. There was a significant lack of fit of the model and the results from the Design of Experiments need to be completed with further tests in order to draw any definite conclusions. Another Design of Experiments might be studied with a constant sample concentration and incubation time while only salt concentration and pH is varied. Instead of diluting the sample, the gel volume in each well could be varied giving a similar effect as a sample dilution. In order to instead investigate different loads, which can be of interest, different volumes with the same original sample concentrations would be applied to a set gel volume. It could also be of interest to, at a constant load and where the HIC media is overloaded, examine different sample concentrations' effect on the binding capacity of the HIC media.

Concluding remarks

B1 – Phenyl (20µmol/ml) is a promising prototype which could elute the investigated MAb at low salt concentrations. The maximum antibody binding capacity in the 96 well filter plate format for a 93% aggregate sample is approximately 12 mg/ml$_{resin}$. This prototype would be interesting to investigate further in terms of capacity in the column format and further benchmarking.

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I want to thank my scientific reviver Karin Caldwell for her support and feedback regarding my report. I truly am inspired by her passion for hydrophobic interaction chromatography and it has been a delight to have had such an enthusiastic and skilled scientific reviewer. Last but not least I also want to thank my opponents Moa Hellberg and Kaleb Eriksson for their input and feedback regarding my report.

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Appendix

96 well filter plate screening

The absorbance values in % of total added protein for flow through, wash and eluate fractions at all binding buffers; 0.8M, 0.6M 0.5M 0.4M, 0.3M and 0.2M ammonium sulphate (which all also contained 50 mM sodium phosphate respectively); are presented in Figures 31-48. Each Figure represents one HIC media and the results from the elutions at all the different binding buffers tested. The percentage is based upon the total amount of recovered protein for the different HIC media compared to an average control value from the ten control wells in this plate. The control value for each of the ten control wells was based on the accumulated fractions from flow through, wash 1, wash 2 and wash 3 with the average buffer value subtracted from each fraction respectively. (The control wells contained no gel, were equilibrated with different binding buffers and sample was applied to these wells.) Control value was 1.90 AU for HIC media displayed in Figures 31-36, 1.81 AU for HIC media displayed in Figures 37-42 and 1.78 AU for HIC media displayed in Figures 43-48.

The plate design for the 96 well filter plate screening is shown in Figures 49, 50 and 51.

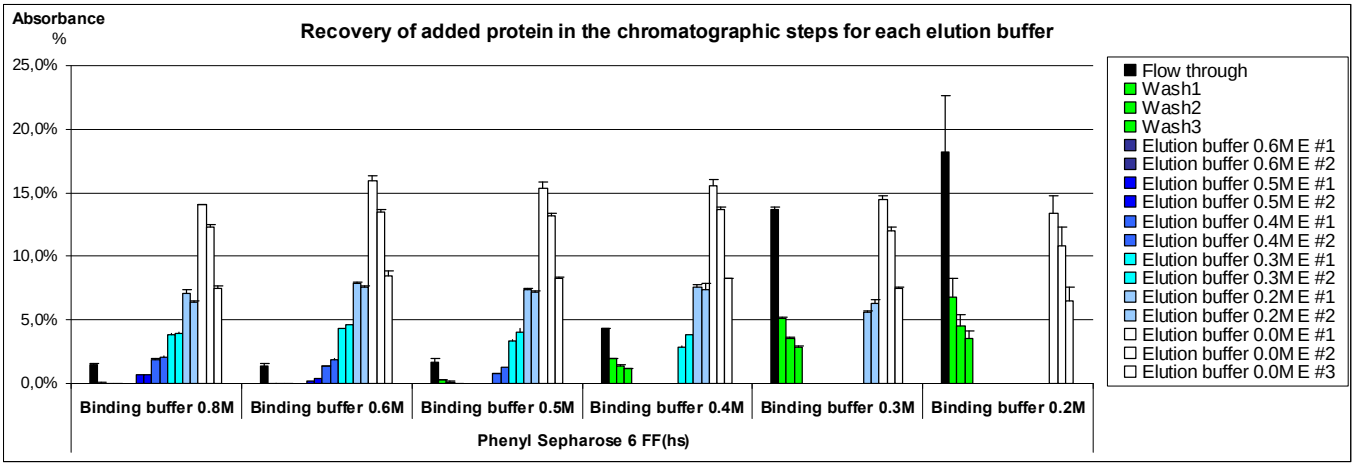

Figure 31. Absorbance eluates for Phenyl Sepharose 6 FF(hs) in % of total amount of applied sample.

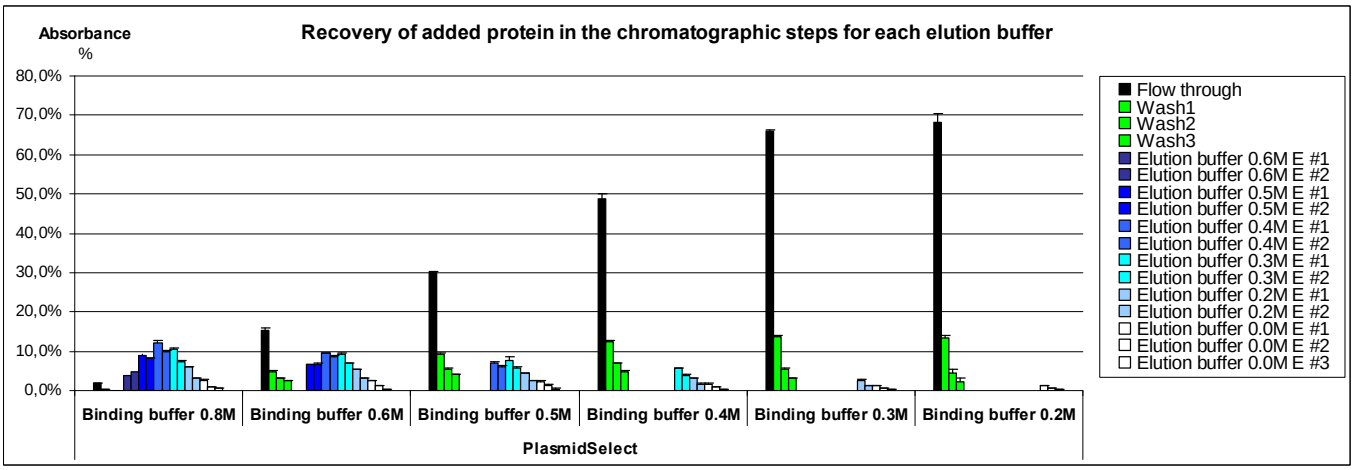

Figure 32. Absorbance eluates for PlasmidSelect in % of total amount of applied sample.

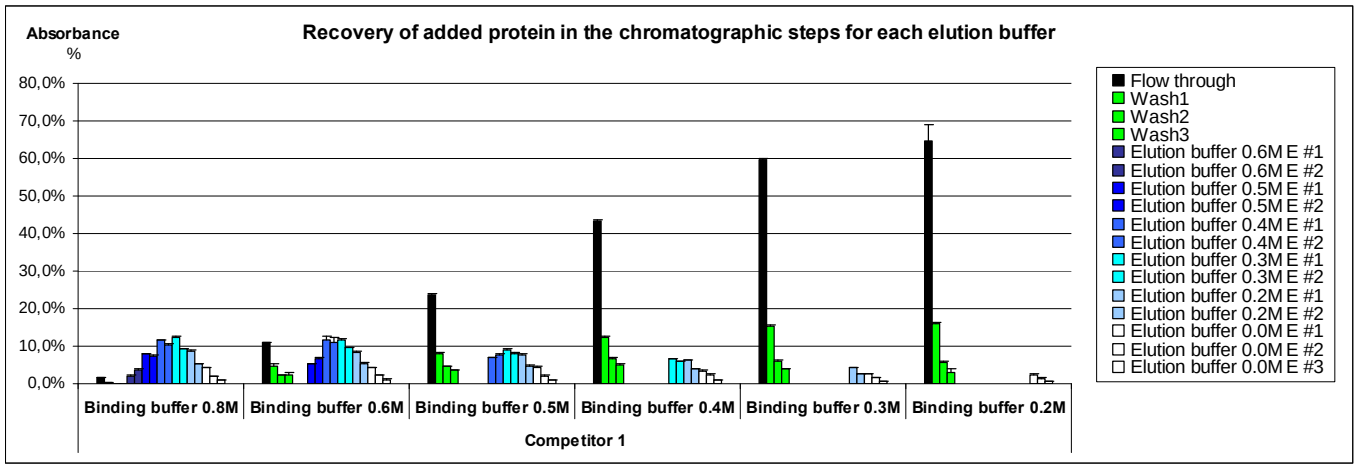

Figure 33. Absorbance eluates for Competitor 1 in % of total amount of applied sample.

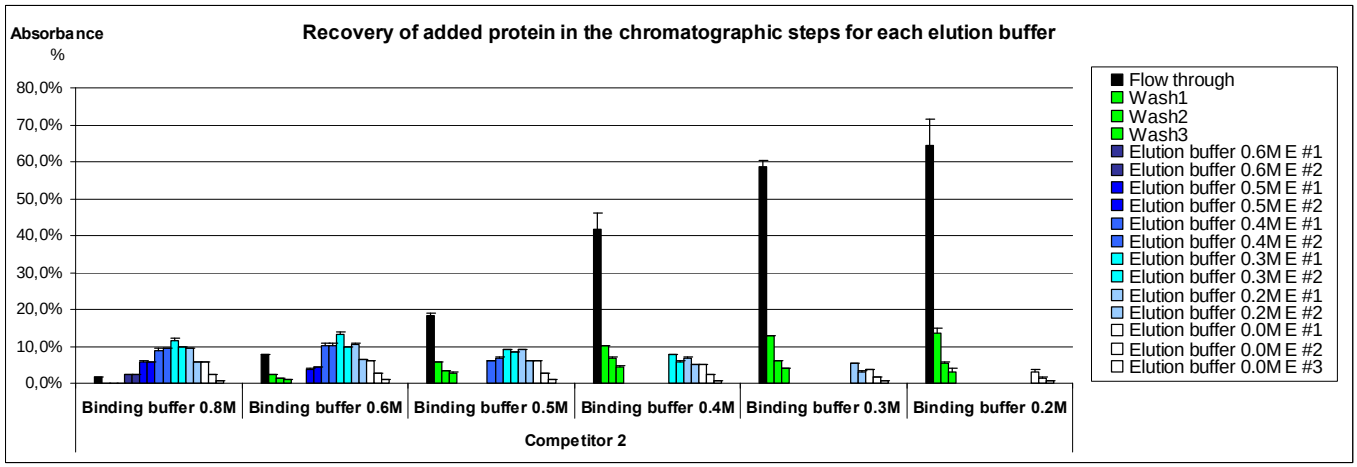

Figure 34. Absorbance eluates for Competitor 2 in % of total amount of applied sample.

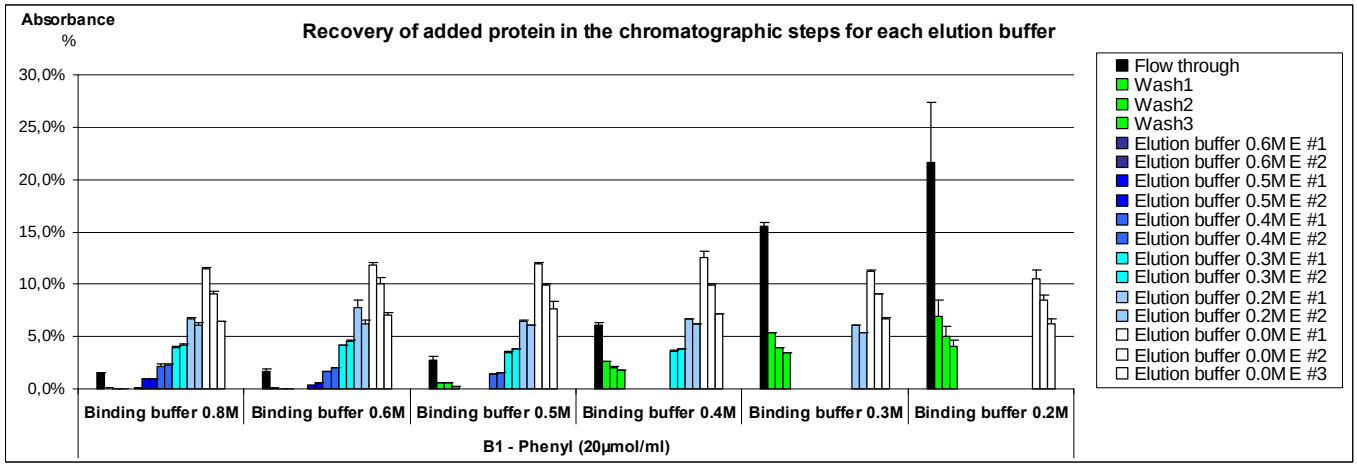

Figure 35. Absorbance eluates for B1 – Phenyl (20µmol/ml) in % of total amount of applied sample.

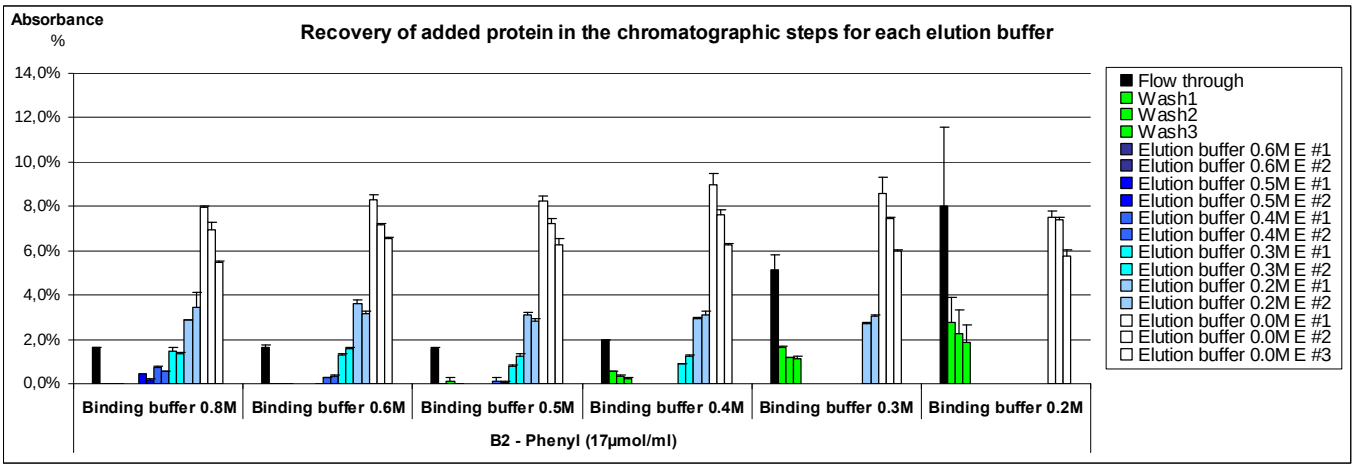

Figure 36. Absorbance eluates for B2 – Phenyl (17µmol/ml) in % of total amount of applied sample.

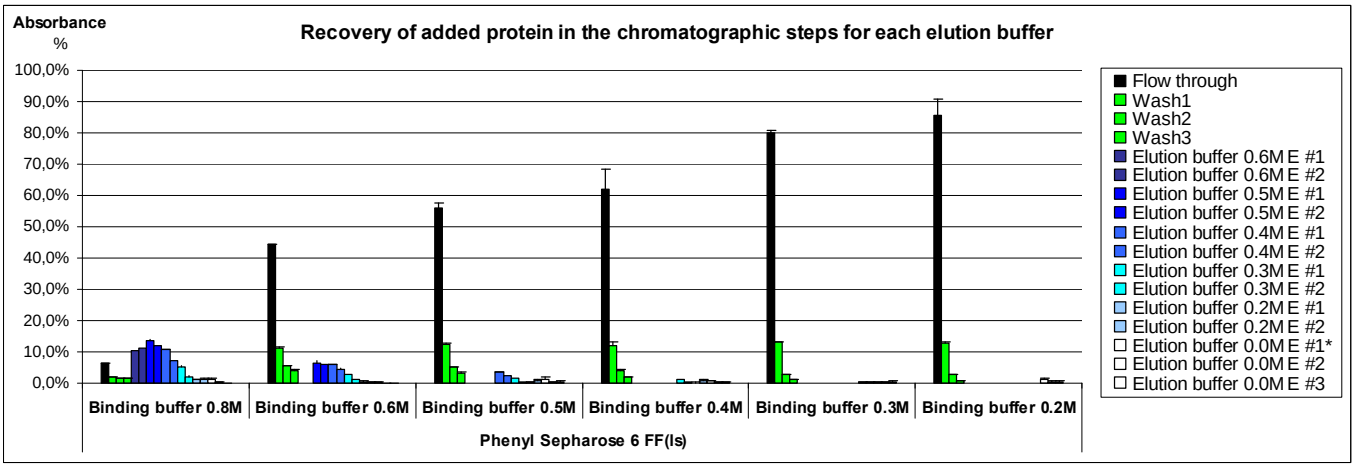

Figure 37. Absorbance eluates for Phenyl Sepharose 6 FF(ls) in % of total amount of applied sample.

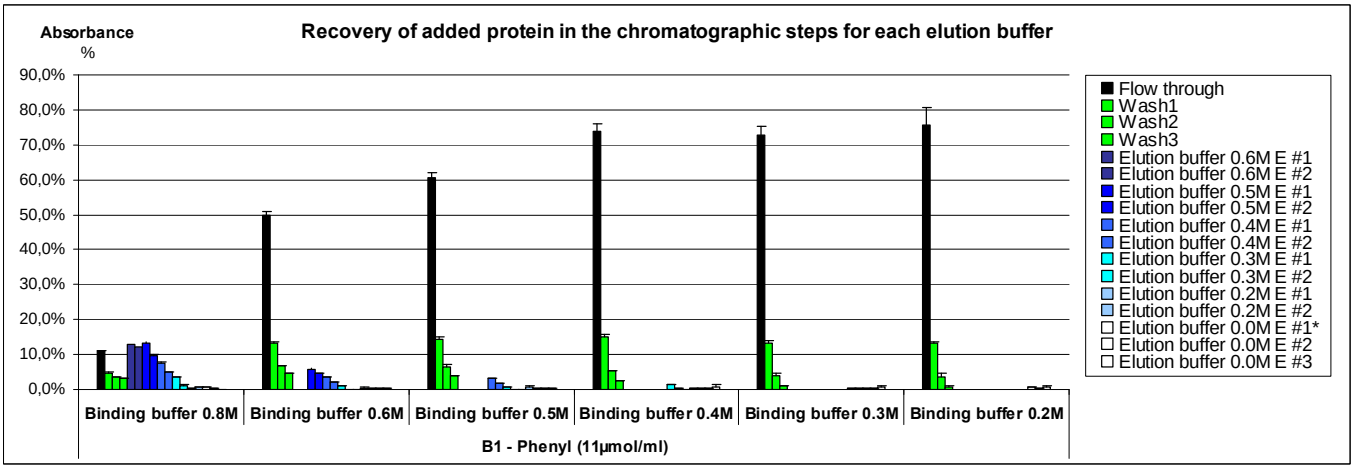

Figure 38. Absorbance eluates for B1 – Phenyl (11µmol/ml) in % of total amount of applied sample.

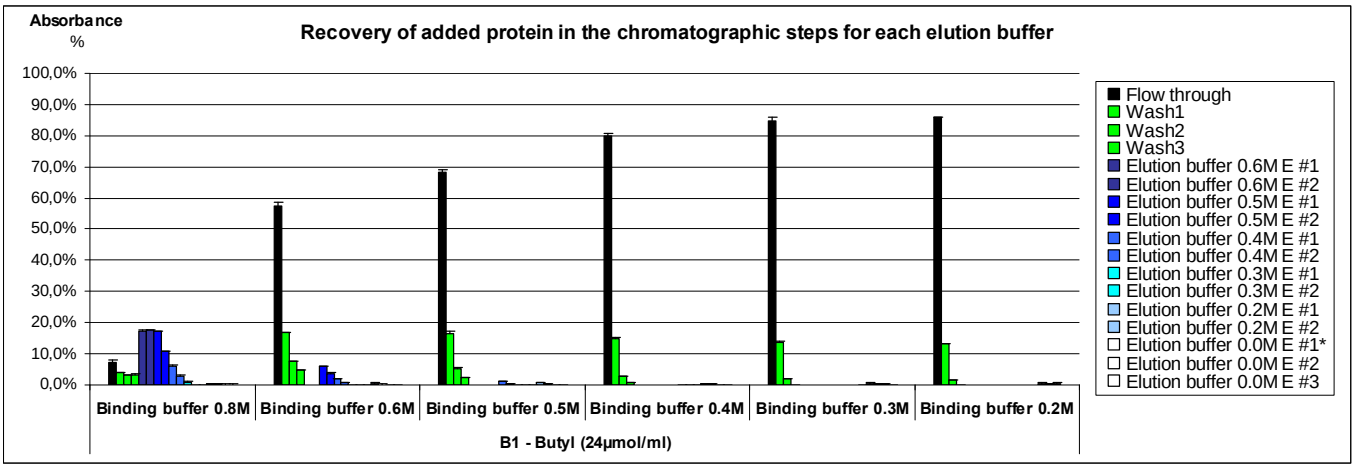

Figure 39. Absorbance eluates for B1 – Butyl (24µmol/ml) in % of total amount of applied sample.

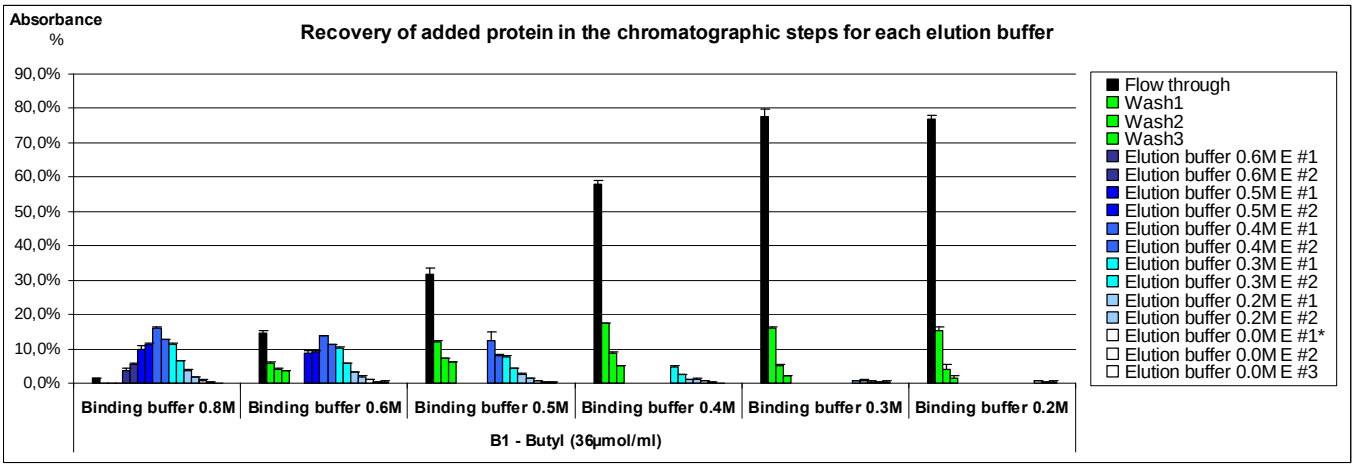

Figure 40. Absorbance eluates for B1 – Butyl (36µmol/ml) in % of total amount of applied sample.

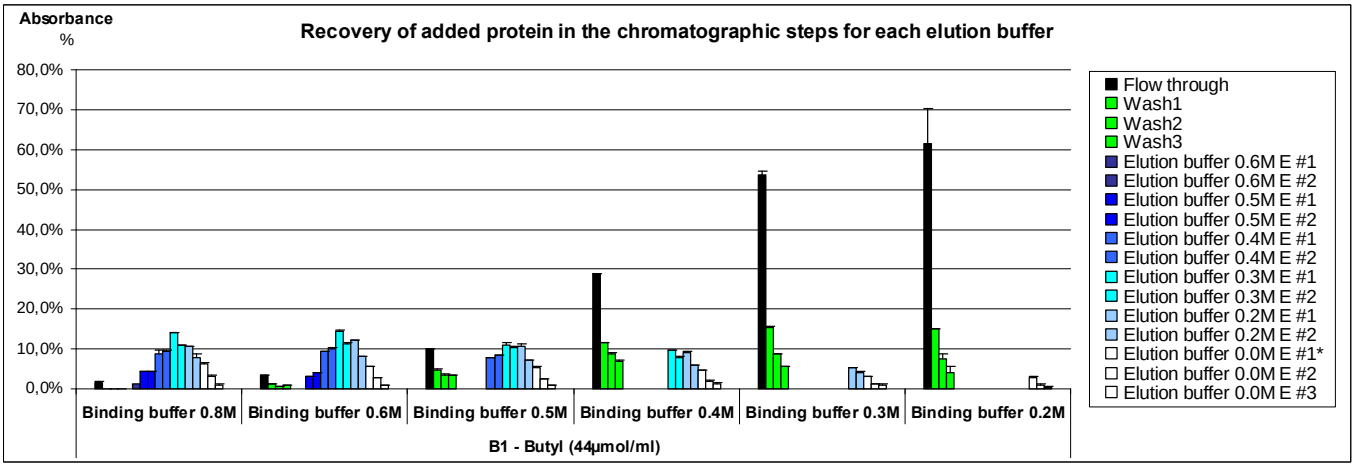

Figure 41. Absorbance eluates for B1 – Butyl (44µmol/ml) in % of total amount of applied sample.

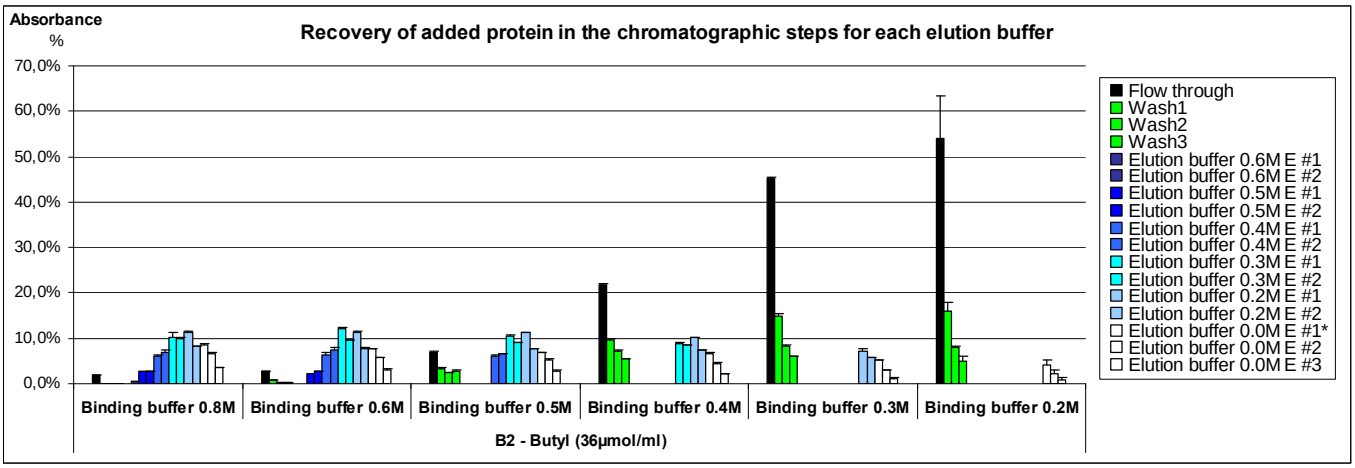

Figure 42. Absorbance eluates for B2 – Butyl (36µmol/ml) in % of total amount of applied sample.

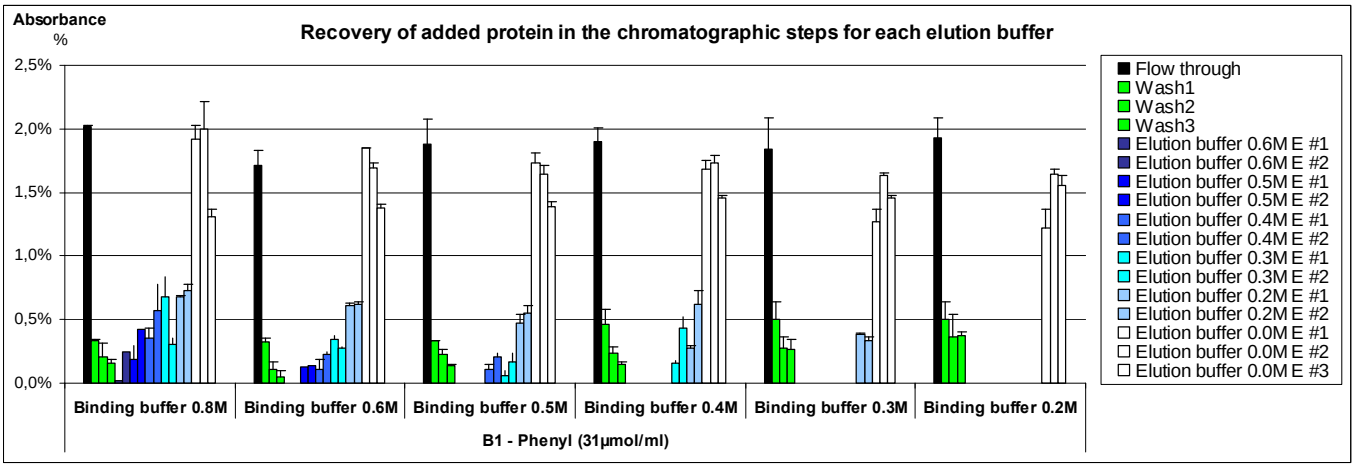

Figure 43. Absorbance eluates for B1 – Phenyl (31µmol/ml) in % of total amount of applied sample.

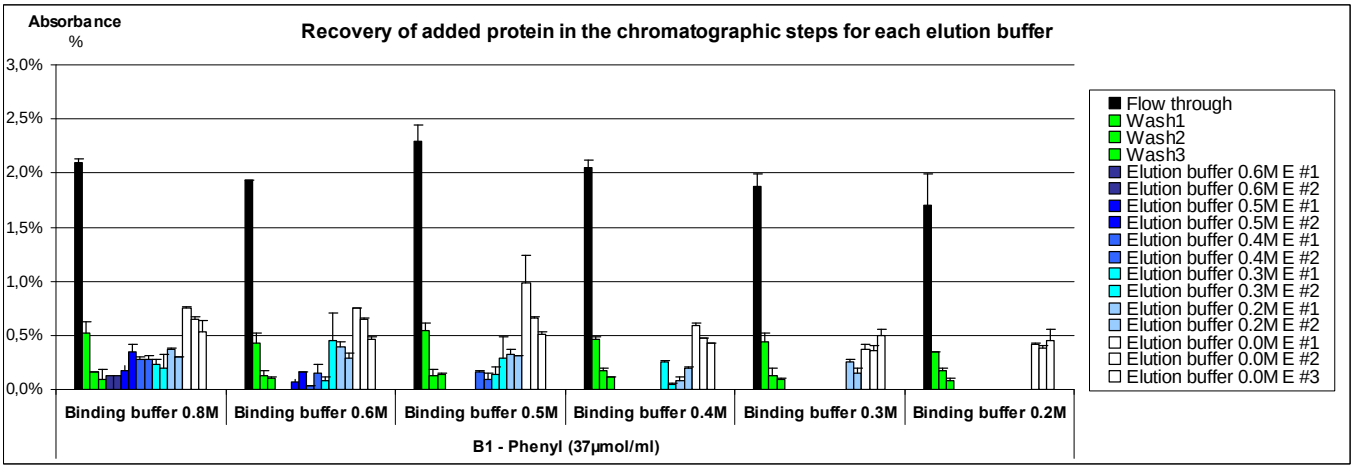

Figure 44. Absorbance eluates for B1 – Phenyl (37µmol/ml) in % of total amount of applied sample.

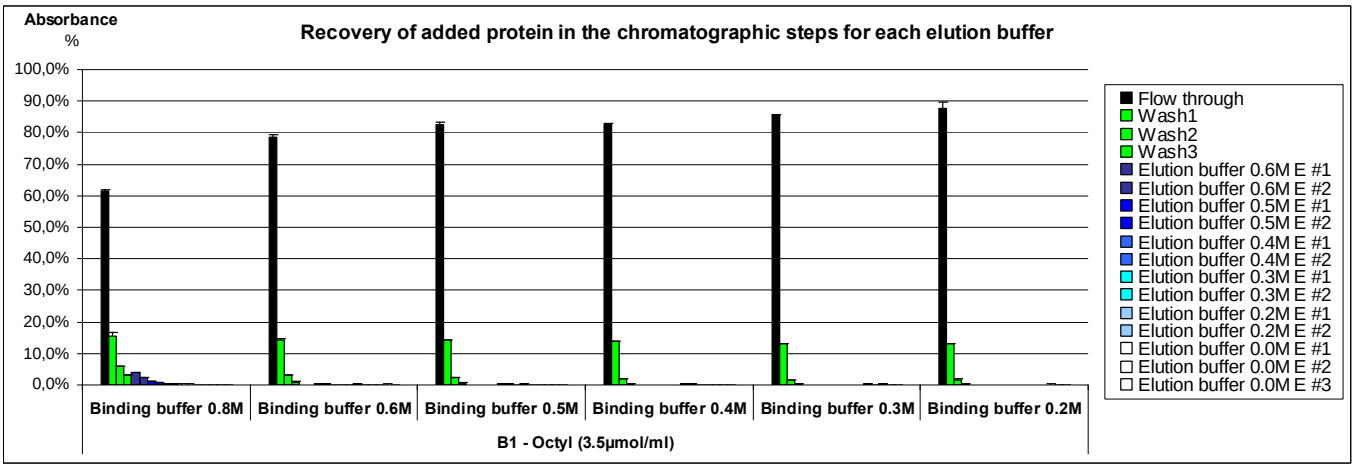

Figure 45. Absorbance eluates for B1 – Octyl (3.5µmol/ml) in % of total amount of applied sample.

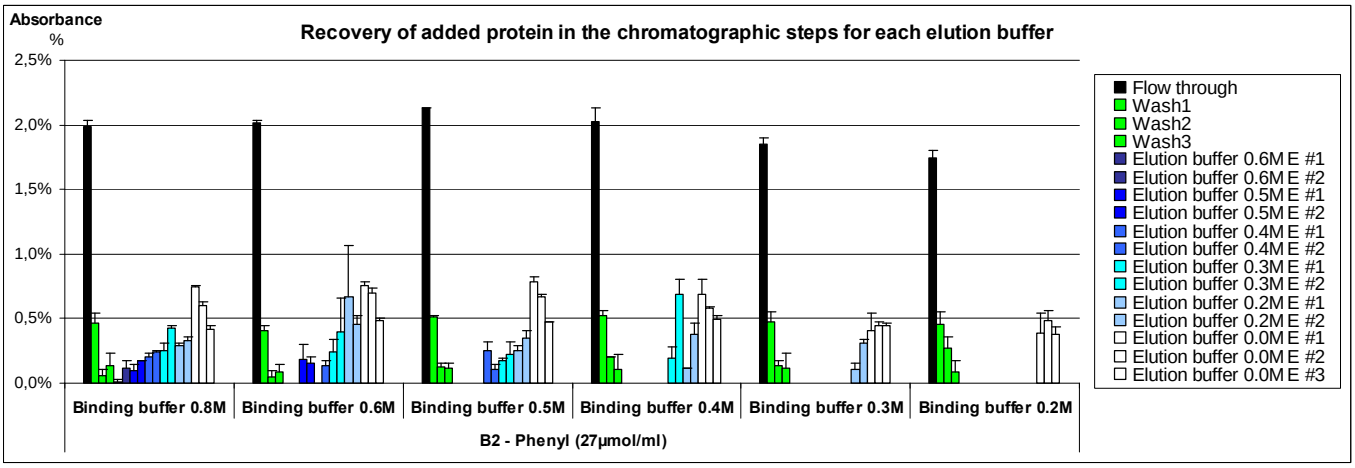

Figure 46. Absorbance eluates for B2 – Phenyl (27µmol/ml) in % of total amount of applied sample.

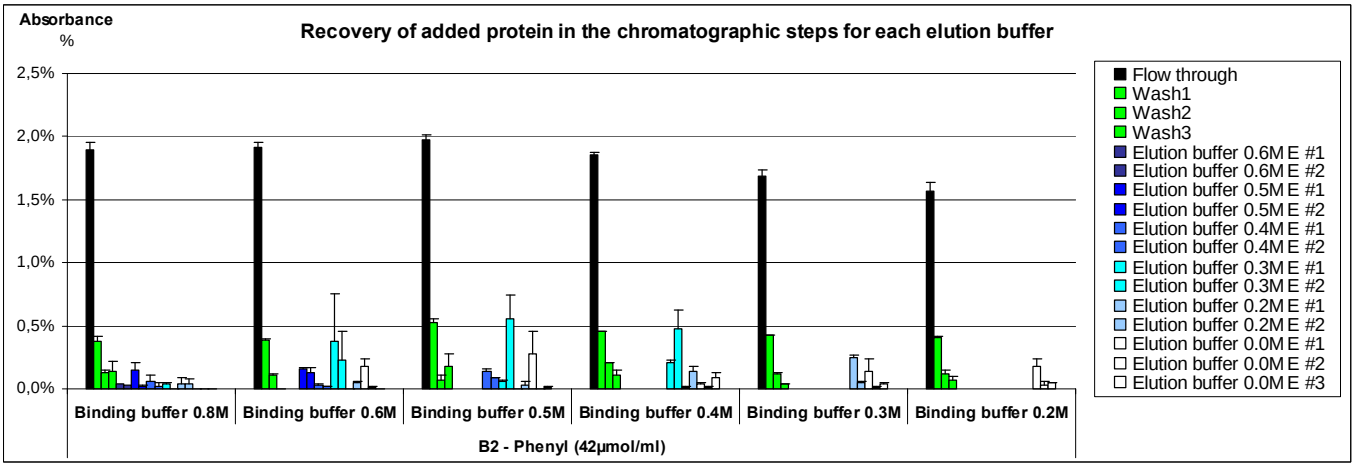

Figure 47. Absorbance eluates for B2 – Phenyl (42µmol/ml) in % of total amount of applied sample.

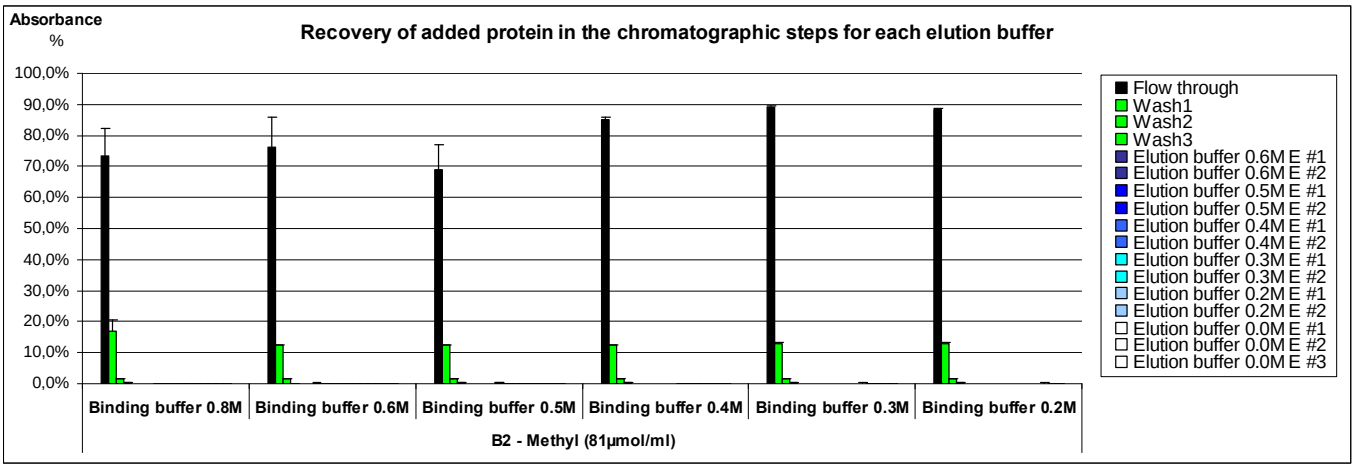

Figure 48. Absorbance eluates for B2 – Methyl (81µmol/ml) in % of total amount of applied sample.

Ammonium sulphate concentration in the binding buffers		**Media A**		**Media B**		**Media C**		**Media D**		**Media E**		**Media F**	
		1	2	3	4	5	6	7	8	9	10	11	12
0 M	A												
0.2 M	B												
0.3 M	C												
0.4 M	D												
0.5 M	E												
0.6 M	F												
0.8 M	G												
Controls containing no media with following ammonium sulphate concentration as binding buffer	H	0 M	0 M	0.2 M	0.2 M	0.3 M	0.3 M	0.4 M	0.4 M	0.8 M	0.8 M	Empty	Empty

Figure 49. The plate design from the 96 well filter plate experiment, plate 1 (corresponding to figure 8 and 31-36). Row A contained binding buffer with 50 mM sodium phosphate without ammonium sulphate, row B contained binding buffer with 0.2 M ammonium sulphate, 50 mM sodium phosphate and so on. The HIC media were placed in two columns each in rows A-G. The controls with no media and the different binding buffers tested are displayed in row H wells 1-10.

Ammonium sulphate concentration in the binding buffers		Media M		Media N		Media O		Media P		Media Q		Media R	
		1	2	3	4	5	6	7	8	9	10	11	12
0 M	A												
0.2 M	B												
0.3 M	C												
0.4 M	D												
0.5 M	E												
0.6 M	F												
0.8 M	G												
Controls containing no media with following ammonium sulphate concentration as binding buffer	H	0 M	0 M	0.3 M	0.3 M	0.4 M	0.4 M	0.5 M	0.5 M	0.8 M	0.8 M	Empty	Empty

Figure 50. The plate design from the 96 well filter plate experiment, plate 2 (corresponding to figure 9 and 37-42). Row A contained binding buffer with 50 mM sodium phosphate without ammonium sulphate, row B contained binding buffer with 0.2 M ammonium sulphate, 50 mM sodium phosphate and so on. The HIC media were placed in two columns each in rows A-G. The controls with no media and the different binding buffers tested are displayed in row H wells 1-10.

Ammonium sulphate concentration in the binding buffers		Media G		Media H		Media I		Media J		Media K		Media L	
		1	2	3	4	5	6	7	8	9	10	11	12
0 M	A												
0.2 M	B												
0.3 M	C												
0.4 M	D												
0.5 M	E												
0.6 M	F												
0.8 M	G												
Controls containing no media with following ammonium sulphate concentration as binding buffer	H	0 M	0 M	0.4 M	0.4 M	0.5 M	0.5 M	0.6 M	0.6 M	0.8 M	0.8 M	Empty	Empty

Figure 51. The plate design from the 96 well filter plate experiment, plate 3 (corresponding to figure 10 and 43-48). Row A contained binding buffer with 50 mM sodium phosphate without ammonium sulphate, row B contained binding buffer with 0.2 M ammonium sulphate, 50 mM sodium phosphate and so on. The HIC media were placed in two columns each in rows A-G. The controls with no media and the different binding buffers tested are displayed in row H wells 1-10.

Adsorption Isotherm study

In figure 52 the isotherm design in terms of initial sample concentrations were investigated for possible adsorption isotherm curves using the Langmuir model.

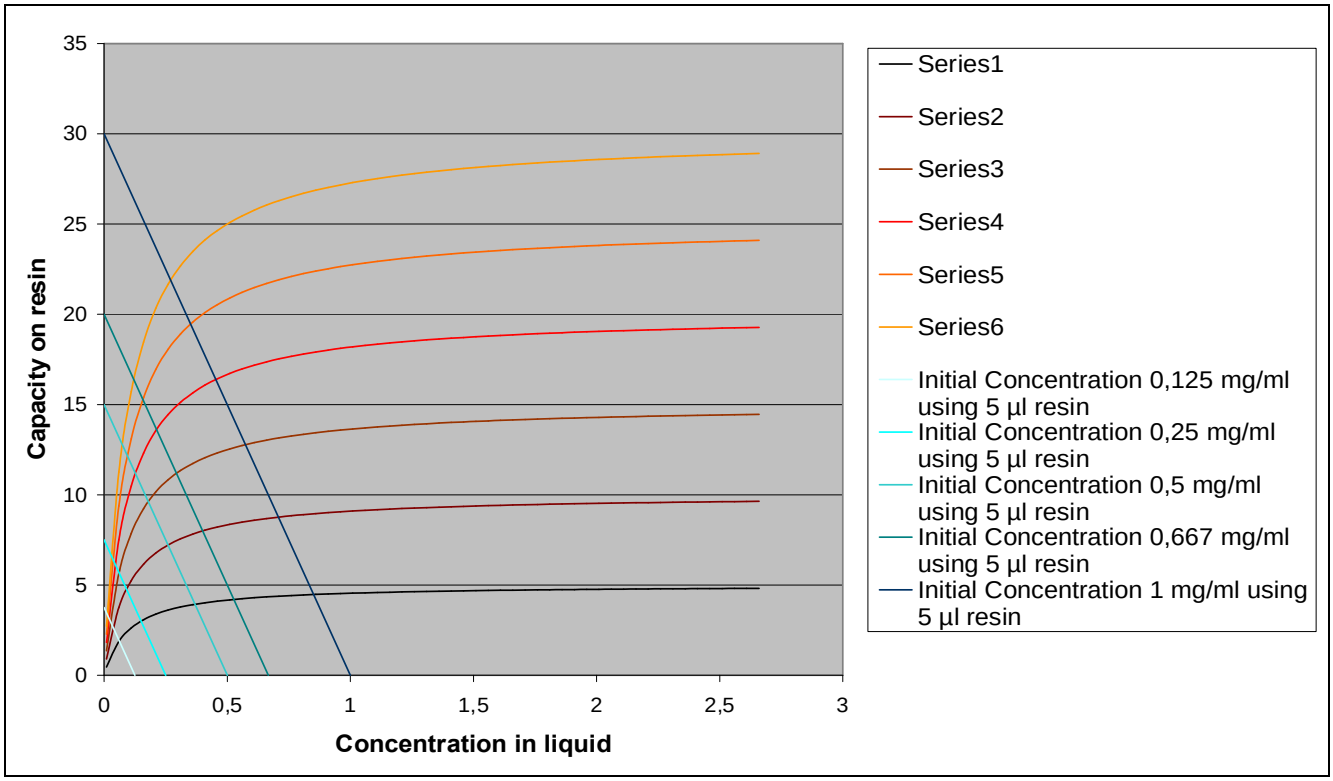

Figure 52. Adsorption isotherm design where possible cases of isotherm curves were plotted in order to make sure that the sample concentrations chosen would be able to give an approximation of the total capacity.

Design of Experiments

In Figure 53 the plate design for the Design of Experiments is shown with the colour explanations in Figure 54. T1, T2, and T3 stands for the different times tested 10, 30 and 90 minutes respectively. In each square in row A-F wells 1-10 in Figure 53 three things are stated separated by a comma. First the pH of the buffer, then ammonium sulphate concentration in the buffer (A more detailed buffer content is described in Figure 54) and last the dilution. (Dilution 1 = 1.3 mg/ml, dilution 2 = 0.65 mg/ml and dilution 8 = 0.16 mg/ml)

		T3	T2	T1	T3	T2	T1	T3	T2	T1	Control (T3)
		1	2	3	4	5	6	7	8	9	10
Media 1	A	pH 4, 0, 8	pH 4, 0.25, 2	pH 4, 0, 8	pH 4, 0.5, 1	pH 6, 0.25, 8	pH 4, 0.5, 1	pH 8, 0, 1	pH 6, 0.5, 2	pH 8, 0, 1	pH 6, 0.25, 8
	B	pH 4, 0, 1	pH 6, 0.25, 2	pH 4, 0, 1	pH 6, 0.25, 2	pH 6, 0.25, 2	pH 6, 0.25, 2	pH 8, 0.5, 8	pH 6, 0.25, 2	pH 8, 0.5, 8	pH 6, 0.25, 8
	C	pH 4, 0.5, 8	pH 6, 0, 2	pH 4, 0.5, 8	pH 8, 0, 8	pH 6, 0.25, 1	pH 8, 0, 8	pH 8, 0.5, 1	pH 8, 0.25, 2	pH 8, 0.5, 1	pH 6, 0.25, 2
Media 2	D	pH 4, 0, 8	pH 4, 0.25, 2	pH 4, 0, 8	pH 4, 0.5, 1	pH 6, 0.25, 8	pH 4, 0.5, 1	pH 8, 0, 1	pH 6, 0.5, 2	pH 8, 0, 1	pH 6, 0.25, 2
	E	pH 4, 0, 1	pH 6, 0.25, 2	pH 4, 0, 1	pH 6, 0.25, 2	pH 6, 0.25, 2	pH 6, 0.25, 2	pH 8, 0.5, 8	pH 6, 0.25, 2	pH 8, 0.5, 8	pH 6, 0.25, 1
	F	pH 4, 0.5, 8	pH 6, 0, 2	pH 4, 0.5, 8	pH 8, 0, 8	pH 6, 0.25, 1	pH 8, 0, 8	pH 8, 0.5, 1	pH 8, 0.25, 2	pH 8, 0.5, 1	pH 6, 0.25, 1

Figure 53. The plate design for the Design of Experiments. The colour explanation is described in figure 54. Wells 1-9 in Rows A, B and C contains a plate design for media 1 and wells 1-9 in rows D, E and F contains a plate design for media 2.

pH 4, 0 M Sodium sulphate, 50mM citric acid	pH 4, 0.25 M Sodium sulphate, 50mM citric acid	pH 4, 0.5 M Sodium sulphate, 50mM citric acid	pH 6, 0 M Sodium sulphate, 50mM MES	pH 6, 0.25 M Sodium sulphate, 50mM MES	pH 6, 0.5 M Sodium sulphate, 50mM MES	pH 8, 0 M Sodium sulphate, 50mM Tris/HCl	pH 8, 0.25 M Sodium sulphate, 50mM Tris/HCl	pH 8, 0.5 M, Sodium sulphate, 50mM Tris/HCl

Figure 54. Colour explanation for the Design of Experiments plate design displayed in figure 53.