HÖGSKOLAN I BORÅS
INSTITUTIONEN INGENJÖRSHÖGSKOLAN

Characterising of chromatography gels for purification of erythropoietin

Karakterisering av kromatografigeler för rening av erytropoietin

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Examensarbetet omfattar 15 högskolepoäng och ingår som ett obligatoriskt moment i Högskoleingenjörsexamen i Kemiingenjör med inriktning bioteknik, 180 högskolepoäng
Nr K4/2008

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Ämne: Teknik

Serie och nummer: K4/2008

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Datum: 2008-09-08

Nyckelord: Erythropoietin, chromatography, protein purification

Acknowledgements

This 15 credit thesis work was executed in collaboration with Instituto Superior Politécnico José Antonio Echeverría (CUJAE) and Centro de Inmunología Molecular (CIM). It was performed at CUJAE situated in Havana, Cuba, during the period April-June 2008.

We were kindly received at both CUJAE and CIM. We would like to thank following persons for their help, knowledge, advice and patience: Lourdes Zumalacárregui de Cárdenas (CUJAE), Yanet Borrego (CIM) and José Ameneiros Martinez (CUJAE). We would also like to thank everyone else who has been involved in any way. We are grateful for all the help that we have received. It contributed to the completion of this thesis work.

Thank you!

Abstract

Erythropoietin is a human natural hormone which task is to regulate the amount of red blood cells in the body. At Centro de Inmunología Molecular, situated in Havana, erythropoietin is produced by recombinant DNA-technique. The protein is purified through several chromatography steps.

Among other things, Centro de Inmunología Molecular uses affinity chromatography and ion exchange chromatography. To both of these chromatographic methods, gel is used as stationary phase.

The aim of this study was to investigate and determine parameters for characterising of two gels, this because Centro de Inmunología Molecular have to exchange the gels. The reason for the gel exchange is that the currently used gels will not be manufactured any more.

The gel used in the affinity chromatography is Chelating Sepharose Fast Flow and the gel used in the ion exchange chromatography is Q Sepharose Fast Flow. For both of this gels kinetic parameters and isotherm parameters were determined by experiments. The isotherm parameters q_{max} and K_d were calculated from an adsorption isotherm.

To be able to calculate q_{max} and K_d for both Q Sepharose Fast Flow gel and Chelating Sepharose Fast Flow gel different experiments were made. A kinetic adsorption and an isotherm adsorption were made on each gel. The kinetic adsorption was made in due to find out how long the two different processes were supposed to run and to understand which part of the mass transfer that is controlling the rate. There is no use to let the process to be in progress any longer than until the adsorption ceases. For the Q Sepharose Fast Flow gel this was after 200 seconds. The adsorption with the Chelating Sepharose Fast Flow gel never ceased completely, but after 1000 seconds the adsorption was so slow that it would be no use to continue the process. If the processes continue after the calculated times only money, hours and recourses will be wasted.

The data that were achieved was plotted in two different isotherm adsorption models both the Freundlich- and the Langmuir model, this to determine which model that had the best fit. One could see that the Q Sepharose Fast Flow gel was following the model of Langmuir better and because of this the Langmuir equation was used to calculate q_{max} and K_d. The q_{max} for the Q Sepharose Fast Flow gel agreed a lot with the value that Centro de Inmunología Molecular had assumed. When it came to the Chelating Sepharose Fast Flow gel, the same kind of plotting was made. But one could see that this time the data was following the model of Freundlich much better. Therefore a calculation of the desired q_{max} was impossible. Only the value of K_d was calculated. Because the company Centro de Inmunología Molecular still needed the value of q_{max} an assumption that the gel was following the model of Langmuir was made. q_{max} was calculated but without any satisfied results. The programs Excel, Statgraphic and Matlab have been used in all calculations.

Sammanfattning

Erytropoietin är ett mänskligt hormon som finns naturligt i kroppen och används för att reglera mängden röda blodceller. På Centro de Inmunología Molecular i Havanna produceras erytropoietin med recombinant-DNA-teknik. Proteinet renas efter framställning genom flera kromatografi steg.

På Centro de Inmunología Molecular används bland annat affinitetskromatografi och jonbyteskromatografi och till båda metoderna används gel som stationär fas.

Syftet med detta examensarbete var att karaktärisera två geler då de ska bytas ut mot nya likvärdiga geler eftersom de gamla inte ska fortsätta att produceras.

Gelen som används till affinitetskromatografin är Chelating Sepharose Fast Flow och gelen som används till jonbyteskromatografin är Q Sepharose Fast Flow. För båda dessa geler skulle kinetiska och isotermiska parametrar fastställas genom experiment. De isotermiska parametrarna q_{max} och K_d beräknades utifrån en adsorptionsisoterm.

För att kunna beräkna q_{max} och K_d på både Q Sepharose Fast Flow gel och Chelating Sepharose Fast Flow gel gjordes olika experiment. Både en kinetisk adsorption och en isotermisk adsorption utfördes på vardera gel. Den kinetiska adsorptionen utfördes för att ta reda på hur länge de olika processerna ska köras samt för att bestämma vilken del av masstransporten som är hastighetsbestämmande. Det är ingen mening att låta processen pågå längre än tills adsorptionen upphör. För Q Sepharose Fast Flow gelen var detta efter 200 sekunder. Vid experimentet med Chelating Sepharose Fast Flow gelen avstannade inte adsorptionen helt men efter 1000 sekunder var adsorptionen så långsam att en fortsatt process skulle vara onödig. Om processerna får fortsätta efter det att dessa tider uppnåtts för vardera gel så kommer endast pengar, tid och resurser att slösas.

De data som ficks fram från den isotermiska adsorptionen plottades i två olika isotermiska adsorptionsmodeller, både Freundlich- och Langmuirs modell. Detta för att kunna avgöra vilken modell som passade bäst. För Q Sepharose Fast Flow gelen kunde man se att den följde Langmuirs modell bäst och därför användes denna ekvation för att beräkna q_{max} och K_d. q_{max} för Q Sepharose Fast Flow gelen stämde mycket väl överens med vad Centro de Inmunología Molecular hade antagit. När det gällde Chelating Sepharose Fast Flow gelen gjordes samma plottning men här visade det sig att den följde Freundlich bättre. Därför kunde inte det önskade värdet på q_{max} beräknas utan endast K_d. Då företaget Centro de Inmunología Molecular behöver värdet på q_{max} antogs gelen följa en Langmuir modell och q_{max} beräknades, men utan goda resultat. I alla beräkningar har programmen Excel och Statgraphic använts.

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1. Introduction

Erythropoietin is a protein that controls the growth of red blood cells. Today it is used in hospitals to treat anaemia for example caused by cancer. Erythropoietin is a human natural hormone that can be produced by recombinant DNA-technique.

At Centro de Inmunología Molecular, situated in Havana, erythropoietin is produced and purified. The purification process demands several steps to achieve the high desired purity. By using the principals of affinity chromatography, ion exchange chromatography and gel filtration, the purification process is designed to follow a strategy called Three Phase Purification Strategy.

The Three Phase Purification Strategy consists of three phases called capture, intermediate purification and polish. Affinity chromatography is used in the capture phase first with Blue Sepharose Fast Flow gel and then Chelating Sepharose Fast Flow gel. In the intermediate purification, ion exchange chromatography is used with Q Sepharose Fast Flow gel. The last phase is polishing which is done with gel filtration.

Centro de Inmunología Molecular needs to change the gel used in the second part of the capture phase, Chelating Sepharose Fast Flow gel, and the gel used in the intermediate purification phase, Q Sepharose Fast Flow gel. The reason for the gel exchange is that the currently used gels will probably not be manufactured any more.

Production of pharmaceuticals must be done with permission. Centro de Inmunología Molecular has permission to produce and purify erythropoietin with the gels that are currently used. In order to change the gels, the company are obligated to prove that the new gels are equal to the old gels for each process. It is a requirement to prove that the final product is completely unchanged to maintain the license.

To be able to compare the new gels with the old gels a number of parameters that characterise the gels must be determined. The gels that are currently used at Centro de Inmunología Molecular in the purification process of erythropoietin have never been studied and characterised regarding these parameters.

The aim of this study was to investigate and determine parameters for the characterising of the Chelating Sepharose Fast Flow gel and the Q Sepharose Fast Flow gel that are currently used at Centro de Inmunología Molecular. Kinetic parameters and isotherm parameters should be determined with the help of two different experiments for each gel, one kinetic adsorption experiment and one adsorption isotherm experiment. All experimental data should be analysed with the computer programs Statgraphics and Excel. By applying the kinetic data in Matlab the parameters k and k' could be determined. From the adsorption isotherm experiment it could be decided whether the gel follows a Langmuir adsorption isotherm or a Freundlich adsorption isotherm. From the best fitting isotherm the parameters K_d and q_{max} or n could be calculated. The parameters from the two experiments can then be used for further calculations in a program in Matlab by Centro de Inmunología Molecular. The program achieves a complete characterisation of the gels that can be used for comparing the new gels with the old gels.

From the experimental work it should be possible to achieve enough data to determine the parameters. Hopefully the parameters can be used by Centro de Inmunología Molecular to successfully compare the new gels with the old gels.

This report has a part of theoretical review which treats the characteristics of erythropoietin, the theories of the different chromatography methods, the theory of mass transfer and the theory of adsorption isotherms. In the chapter Materials and Methods, the performance of the experiments is described with the equipment and chemicals that were used. In the Result and Discussion chapter the analysed data is presented together with some small discussions. The final part consists of conclusions and recommendations. The primary data, models from Statgraphics and Matlab plus calculations are presented in appendixes.

2. Characteristics of erythropoietin

2.1 Structure of erythropoietin

Erythropoietin, EPO, is a human natural hormone which is produced mostly by kidney cells but a small amount is produced in liver cells. It is a growth hormone that stimulates the development of stem cells in the bone marrow to ripe red blood cells (Hoffmann, 2008).

Erythropoietin is a glycoprotein that consists of 166 amino acid residues. The structure of the protein is formed with internal disulfide bounds and has four oligosaccharide chains. The two disulfide bonds are located between Cys29-Cys33 and between Cys7-Cys161. The last one is necessary for the biological activity. Three of the oligosaccharide chains are linked with nitrogen at the position 24, 38 and 83 to the amino acid asparagine and the fourth is linked with oxygen to the amino acid serine at position 126.

Erythropoietin consists from the beginning of 166 amino acid residues but just after the protein synthesis is done the amino acid residue of arginine at the C- terminal is cut off. The total molar mass of the complete protein is approximately 30-34 kDa. The polypeptide chain contributes with 60 percent of the molar mass (18 kDa) and the oligosaccharides with the remaining 40 percent. The variation in the composition of the carbohydrates in the protein presents different isoforms in the nature. The carbohydrates are necessary for the biological activity, especially the nitrogen linked carbohydrates (Hellström, Karlsson & Larsson).

EPO is formed in four anti parallel α-helixes with a part of primary structure between the helix A and B and between C and D (Fig. 2-1) (Boissel et al, 1993).

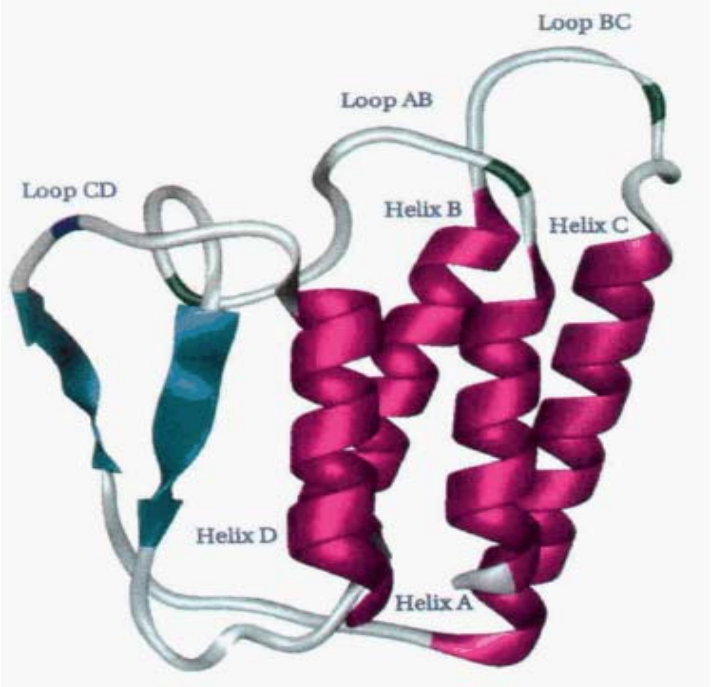

Figure 2-1. The structure of erythropoietin with the four anti parallel α-helixes[1].

[1] Figure 2-1: (Electronic). <http://www.jbc.org/cgi/reprint/268/21/15983.pdf l>. (2008-09-04)

2.2 The function of erythropoietin

Bone marrow is divided in red and yellow bone marrow. It is the red marrow that produces the red and white blood cells. The ratio between red and yellow can be changed depending on the amount of blood that needs to be produced. When blood needs to be produced the yellow marrow is changed in to red marrow (Nationalencyklopedin, 2008c).

Red blood cells, also known as erythrocytes, are transporting oxygen from the lungs to the tissues of the body and carbon dioxide from the tissues back to the lungs. Erythrocytes do not have nucleus or cell organelles but consists of haemoglobin instead to which the oxygen is bound. Because of the fact that they do not have nucleus or organelles, the cells do not have the capability to maintain alive or repair themselves. Therefore they have a very limited life span of about 120 days (Bassett, 2005).

If the amount of erythrocytes is decreasing or they are not functioning, the amount of oxygen that arrives to the tissues is diminished. When the kidney experiences decreased oxygen level it starts to produce erythropoietin (Bassett, 2005). The main task of EPO is to raise the total amount of erythrocytes in the blood (Sparks, 1987). The protein is secreted to the blood vessel (Hoffmann, 2008) and transported to the bone marrow. EPO binds to specific receptors on the erythroid progenitor cell and the complex of EPO and its receptor starts a cascade of signals (Storey, 1998). The signals initiate the process of differentiation and proliferation of stem cells to mature erythrocyte (Stein, 2004). This process is called eryhropoiesis (Fig.2-2) (Bassett, 2005).

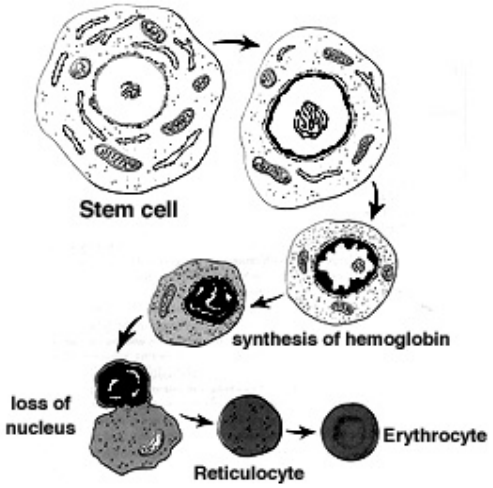

Figure 2-2. The erythropoiesis from bone marrow stem cell to mature erythrocyte[2].

2.3 Erythropoietin therapy

Anaemia is a disease where the patient has a lack of erythrocytes. The minimized amount of erythrocytes results in decreased amount of haemoglobin in the blood which leads to a decreasing absorption of oxygen. The patient contracts fatigue, sleepiness, dizziness, difficulties of concentration, decreasing sexual drive and other symptoms, related to the lack of oxygen (Hoffmann, 2008).

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[2] Figure 2-2: (Electronic). <http://people.eku.edu/ritchisong/301notes4.htm>. (2008-07-08)

Anaemia can be caused by kidney failure which results in a reduced EPO production. Anaemia can also occur from chemotherapy in cancer treatment or chronic infections such as HIV (Ng, 2005). Anaemia was treated with repeated blood transfusions and iron supplementation in the 1970s and early 1980s. In the late 1980´s a genetically created version of the hormone, recombinant human erythropoietin (epoetin), started to be used (Lameire, 2000). Instead of using other peoples erythrocytes, epoetin treated anaemia by stimulating the growth of the patients own blood cells (Hoffmann, 2008). This method was both more effective and more lenient than the previous ones and has resolved in a more satisfactory treatment of anaemia (Lameire, 2000).

Large amount of EPO is produced with recombinant gene technology for biopharmaceutical purposes (Ng, 2005). It is one of the more successfully commercially human hormones that have been developed with this technique (Campell & Farrell, 2003).

The ability to raise the amount of erythrocytes in the blood has made EPO desirable as a drug. It contributes to an improved oxygen carrying capacity of the blood and therefore an increased performance (Nationalencyklopedin, 2008e). EPO is classified as a performance-enhancing drug and banned for use in the sport area (Ng, 2005). Several cases of drug use with erythropoietin have been reported in both skiing and cycling (Hellström, Karlsson & Larsson)

3. Purification of protein

3.1 Purification of protein in general

Proteins can be useful in many fields. Depending on what it is going to be used for, different levels of purity is required. Depending on what purity is desired different methods can be used. When it comes to pharmaceutical preparation almost a 100% of purity is required, while the requirements of purity in the food industry is not as high. To express purification of proteins the Three Phase Purification Strategy can be used (Amersham Biosciences AB, 2001).

3.1.1 Three Phase Purification Strategy

The phases of Three Phase Purification Strategy are capture, intermediate purification and polishing.

The aim of the capture phase is isolation, concentration and stabilisation of the desired protein. However the main task is to remove critical contaminations such as proteases and glycosidase rapidly. This is done to avoid destruction of the proteins and preserve the proteins activity intact. Even during harsh cleaning conditions it is easy to obtain the capture phase and in that case then with ion exchange chromatography. The capture phase consists of sequential separation steps often made by affinity or ion exchange chromatography. In the first step both clear and crude material can be used. If the material is crude, focus should be stability and simplicity to get rid of the most dangerous contaminations. This makes it easy to use large volumes and high speed.

The intermediate purification phase is used to eliminate almost all bulk impurities in a minimized number of steps with different techniques. Impurities can be other proteins, nucleic acids, endotoxins and/or viruses. The speed is not as important in the intermediate purification phase as in the capture phase since all destructive impurities should already have been eliminated in the latter, but a high resolution is needed. The high resolution is achieved with a continuous gradient or with a multi step elution. After the intermediate purification phase all impurities should have been eliminated. The only substances that might still exist in the sample, except the desired protein, are trace impurities.

Trace impurities, among others substances close related with the product, should be removed in the polishing phase to obtain the desired purity. High resolution is required to obtain the desired purity. It might be needed to sacrifice an amount of the product in order to achieve the desired purity. Because of the high concentration of the protein the loss at this point is more costly than in the other phases. Therefore it is important to get the highest possible recovery (Amersham Biosciences AB, 2001).

3.1.2 Chromatography in general

Chromatography is a method used for separation. The main basis is equilibrium between two phases, stationary phase and mobile phase. The stationary phase can be solid particles, a gel or a liquid. The mobile phase is either a gas or a liquid which runs though the stationary phase to enable equilibrium for the components in the impure sample. The different components distribution in the two phases is described by the coefficient K_d (Harris, 2007).

The first chromatography was done by M. S. Tsvet when he separated plant pigment with liquid chromatography 1903. The technique got its name because of the coloured substance of the chlorophyll and other plant pigments that was in his experiment. A fundamental study of

liquid chromatography was published in 1940 by Richard Synge and Archer Martin. They were awarded with the Nobel Prize in Chemistry in 1952 for their work. In chromatography the two main groups are liquid and gas chromatography. It is the mobile phase that makes the difference, if it is liquid or gas. Gas chromatography can only be used for volatile substances. Liquid chromatography can be divided in many different types depending on what type of stationary phase that is used.

- Affinity chromatography, by using the affinity between the stationary phase and the target protein.
- Ion exchanging chromatograph, separation by using the advantages of ion forces.
- Gel filtration, separation by size in a column filled with porous gel marbles.
- Thin layer chromatography. *TLC*, the stationary phase is a thin adsorbing layer. Often used is a plate of glass or aluminium foil with a layer of silicone gel or an aluminium oxide (Nationalencyklopedin, 2008g).

What these liquid chromatography types have in common is the liquid mobile phase.

Today liquid chromatography is very important in the pharmaceutical industry, biotechnology, biochemistry and in medicine. Even if the foundations of the methods are the same, liquid chromatography has strongly developed since 1970 and the equipment has become more automatic and computerized. In the liquid chromatography the stationary phase is usually packed in a column and can consist of an ion exchanger, a gel or a chemical substrate that has been modified. The mobile phase is called eluent and is generally water based salt solutions. The eluent is pumped through the column (Nationalencyklopedin, 2008g).

There are three possibilities for separating a substance. Depending on different substances tendency to be adsorbed by the stationary phase the velocity through the column will be different. If the substance is totally adsorbed by the stationary phase it will be completely fixed in the column. If it does not get adsorbed the substance will travel at the same velocity as the mobile phase. When the substance is partly adsorbed it will stay longer in the column which will increase the dwell time. The dwell time depends on the distribution between the amount in the stationary phase and the mobile phase. When the substance is bound to the stationary phase it is still but in the mobile phase it will transport through the column. This is described with the distribution coefficient, K. Depending on different substances distribution coefficient they will achieve various velocity through the column. This makes it possible to separate substances according to their interaction with the stationary phase (Nationalencyklopedin, 2008g).

3.1.3 Adsorption chromatography in general

Adsorption is described as a process where a solid substance binds another substance to its surface. Attraction forces are formed between the molecules, in the liquid, and the solid surface. Adsorption on a solid surface can be divided in two types, chemical and physical adsorption. At the chemical adsorption the molecules bind to the surface with strong chemical bonds. These bonds are very hard to break and it takes rough methods to desorb the molecules. When it comes to desorbing molecules at a physical adsorption it is much easier. The molecules will bond to the solid surface with weak forces that are much easier to break

by washing or by rising the temperature. The amount of adsorbed molecules will diminish when the temperature is rising (Jonsson, 2008).

The distinction between different substances possible to be adsorbed to one and the same adsorbent is used in separation, purification and analyses. Adsorption is used in chromatography to separate and analyse components in mixtures. To obtain high efficiency and a high capacity a solid phase with a big surface should be used (Jonsson, 2008).

An adsorption isotherm is an expression which shows the relationship between the amount of adsorbed substance to a surface and the concentration of the same substance in the solution at constant temperature and equilibrium (Jonsson, 2008). There are a number of available adsorption isotherms for use at different occasions. This thesis discusses two adsorption isotherms that are well known and often used in biotechnical context (Harrison et al, 2003).

Langmuir
Langmuir adsorption isotherm is a theoretically derived expression. This expression gives the relationship between the amount of adsorbed substrate to a solid surface and the concentration of the same substance in a solution of gas or liquid (Nationalencyklopedin, 2008i). The Langmuir adsorption isotherm was developed by Irving Langmuir (1881–1957) who was an American scientist working at the American company General Electric. At the company he was, among other things, working with the development of gas filled light bulbs (Nationalencyklopedin, 2008h). In his work he studied the adsorption of various gases on metal surfaces and he was so successful in this field that he won the Nobel Prize in Chemistry 1932. Langmuir observed that the amount of adsorbed substrate rose quickly in the beginning and then slowly tended to level out. After this observation he derived an equation which is based on a simple model of surface behaviour, known as the Langmuir adsorption isotherm (Logan, 1996). The isotherm is limited to monolayer adsorption and assumes that:

1. There are no interactions between the molecules adsorbed to the surface.
2. It is the same energy of adsorption all over the surface.
3. The molecules adsorb to one site and then it does not move over the surface. (Richardson, Harker & Backhurst, 2002)

The Langmuir adsorption isotherm:

$$[CS] = \frac{K_{eq} \cdot S_{tot} \cdot [C]}{1 + K_{eq} \cdot [C]}$$

$[CS]$ =Concentration of chemical species adsorbed to an adsorption site
K_{eq} =Equilibrium constant
S_{tot} =Total concentration of adsorbent sites
$[C]$ =Concentration of chemical species in the mobile phase (Harrison et al, 2003)

In discussions about the Langmuir adsorption isotherm in the remaining thesis $[CS]$ will be written as q and S_{tot} as q_{max}.
The plot of the Langmuir adsorption isotherm equation is a curve which is concave downward (Fig.3-1). The curve has a linear slope in the low concentration area and a plateau where the surface sites become saturated (Harrison et al, 2003).

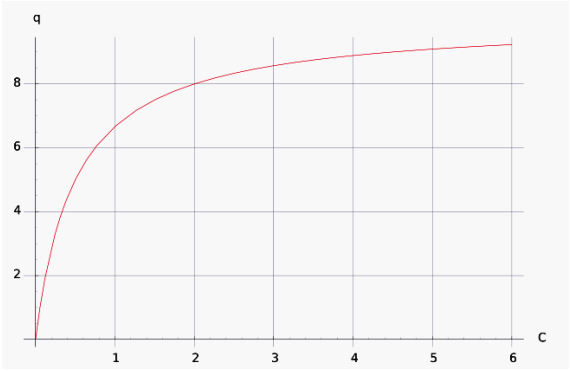

Figure 3-1. The shape of the Langmuir adsorption isotherm[3].

Unlike the most simple linear adsorption isotherm, which assumes that the amount of adsorption sites is a lot larger than the concentration of dissolved species, the Langmuir adsorption isotherm calculates with $q_{max.}$ The possibility to use the value of q_{max} in calculations and comparisons is useful for companies using preparative or industrial scale adsorption and chromatography. To get the most efficient production it is necessary to use as many adsorption sites as possible and then you can not ignore the empty adsorption sites available (Harrison et al, 2003).

Freundlich

The Freundlich adsorption isotherm is another isotherm that is well known and often used in biotechnology. This isotherm is also limited to monolayer. The difference between the Freundlich adsorption isotherm and the Langmuir adsorption isotherm lays in the adsorbing surface. In the Freundlich adsorption isotherm it assumes that the energy of adsorption is heterogeneously spread over the surface (Harrison et al, 2003). Herbert Max Finlay Freundlich (1880–1941) was a German–American physical chemist (A Dictionary of Scientists, 1999). Freundlich developed his isotherm empirically in 1926 when he was working with adsorption of organic compounds from aqueous solutions on to charcoal. Although the isotherm originally was developed empirically it has been shown that the Freundlich adsorption isotherm have some thermodynamic justification (Richardson, Harker & Backhurst, 2002).

The Freundlich adsorption isotherm:

$$[CS] = K_{eq} \cdot [C]^{1/n} \qquad n > 1$$

$[CS]$ =Concentration of chemical species adsorbed to an adsorption site
K_{eq} =Equilibrium constant
$[C]$ =Concentration of chemical species in the mobile phase
n =A constant usually greater than 1 (Harrison et al, 2003)

[3] Figure 3-1: (Electronic). <http://commons.wikimedia.org/wiki/Image:Langmuir_sorption_isotherm.svg>. (2008-07-10)

In discussions about the Freundlich adsorption isotherm in the remaining thesis $[CS]$ will be written as q.

When n > 1 Freundlich isotherm is concave downward just like the Langmuir adsorption isotherm (Harrison et al, 2003). But even if they both are concave downward they are not the same, the Freundlich adsorption isotherm does not have a linear slope in the low concentration area and it never levels out and will never reach a q_{max} (Fig.3-2).

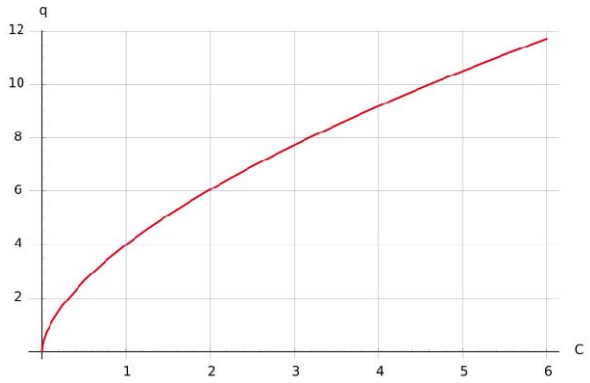

Figure 3-2. The shape of Freundlich adsorption isotherm[4].

Kinetic of adsorption
Centro de Inmunología Molecular wants to use an equation for understanding which mechanism of mass transfer that is controlling the rate. They have chosen to use an equation they call Daniels equation. The equation is from the book Adsorption of Microorganisms to Surfaces. In a chapter written by Stacy L. Daniels the equation is described (Bitton & Marshall, 1980). Even if the equation is from a book about microorganisms, Centro de Inmunología Molecular think that it can be used with erythropoietin.

The equation: $\log\left(\frac{c}{c_0}\right) = k \cdot t + k' \cdot \sqrt{t}$

The first part of the equation, $k \cdot t$, seems to be a linear relationship like Fick's first law of diffusion. The law tells us that the diffusion flux is proportional to the concentration gradient (Nationalencyklopedin, 2008d). Substances moves from an area with higher concentration to an area with lower concentration. The erythropoietin in the liquid phase diffuses through the film around the porous gel beads because of the difference in concentration.

The second part of the equation, $k' \cdot \sqrt{t}$, can show us a situation where the protein diffuse into a porous homogeneous medium. Chromatography gel beads are an example of a homogeneous medium. In a gel bead no stirring will take part and the protein will only move by diffusion. The protein's totally traveled distance from the starting point increases only as the square root of the time (Atkins & de Paula, 1992)

[4] Figure 3-2: (Electronic). <http://commons.wikimedia.org/wiki/Image:Freundlich_sorption_isotherm.svg>. (2008-07-10)

3.2 Affinity chromatography

If two substances are susceptibility to react with each other they have a big affinity (Nationalencyklopedin, 2008a). Affinity chromatography makes it possible to separate biopolymers such as antibodies, antigens, hormones, or other proteins in an efficient way. Biopolymers have the possibility to recognise different chemical structures with high selectivity and then bind specific the structure. The separation process can be compared with the "lock-and-key" mechanism (Weston & Brown, 1997). This separation becomes efficient because of the bio specific bindings (Nationalencyklopedin, 2008b)

Because the method is simple, affinity chromatography is used where other techniques take a lot of time or maybe not even work (GE Healthcare, 2007). The substance that is supposed to be separated, the ligate, has a specific binding surface. On the matrix that is the solid surface there are leaches that have small ligands which has the same kind of specificity as the ligate which makes the ligate easy to adsorb (Fig.3-3). When the ligate has been adsorbed it is easy to rinse away those particles that did not get adsorbed. The ligate is then recovered with a specific desorbing agent (Encyclopedia of Chemical Technology, 1979). One can also recover the ligate by changing pH or to increase the salt concentration (Harrison et al, 2003). The bonds between the ligate and the ligand can be electrostatic, hydrophobic interactions, van der Waals' forces or hydrogen bonding. The ligand must be of a reversible kind so that when recovering the ligate the bond between them breaks (GE Healthcare, 2007).

From the beginning the matrix was based on activated Sepharose but today there are many different types of matrix that which also tolerates the high pressures that are used in HPLC (Patel, 1997). The positive with using affinity chromatography is that theoretically only one purity step is needed. Because of this the yield can be raised. If the yield is 80 % in one step where the total process needs 7 steps of purity, the yield will only be 21 % in the end which of cause is a lot less. If only one step is needed a yield of 80 % will be achieved. Unfortunately it can be very difficult to find the right ligand because it has to be so specific (Encyclopedia of Chemical Technology, 1979).

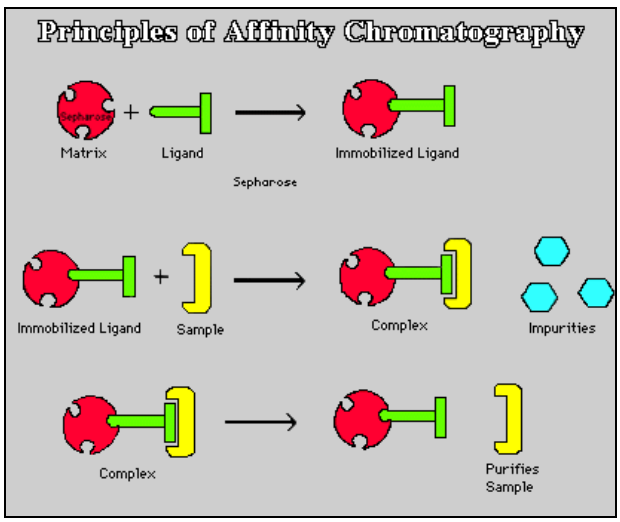

Figure 3-3. Principles of affinity chromatography[5].

[5] Figure 3-3: (Electronic). <http://www.bio.davidson.edu/Courses/Molbio/MolStudents/01grnoland/affinchr.html>. (2008-07-10)

3.2.1 Immobilized Metal Affinity Chromatography, IMAC
Some proteins have a higher affinity to different metals. The affinity depends either on the structure, such as metalloproteins, these proteins requires a metal center, or on that proteins with amino acid residues, such as histidine and cysteine, easily binds to some specific metals such as nickel and copper (Harrison et al, 2003). The amino acids form complex with the metal that is bound to the matrix via chelation. The matrix is a Chelating Sepharose and at natural pH (6-8) complexes with His and Cys is formed (GE Healthcare, 2007).

3.2.2 Chelating Sepharose
Sepharose (Fig. 3-4) is a trade name for a bead formed agarose (Wikipedia, 2008). Chelate means claw like (Nationalencyklopedin, 2008f). Chelating Sepharose is a metal chelate forming ligand which is bound to Sepharose (GE Healthcare, 2007). First the matrix has to be loaded with metal ions that will be able to bind to the target protein. One can use metal ions such as Ni^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+} or Fe^{2+}. The bindings between the target protein and the matrix are dependent on the pH. To elute the target proteins after the process is finished one often reduce the pH or rinse with EDTA (GE Healthcare, 2007).

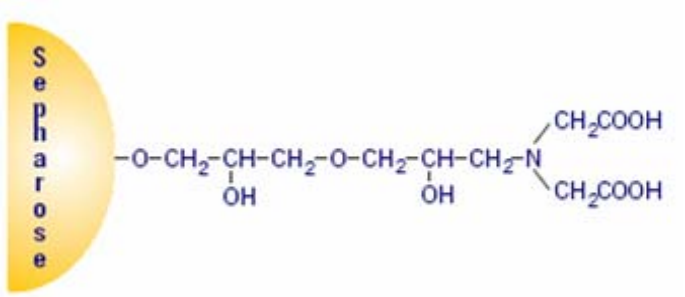

Figure 3-4. Partial structure of Chelating Sepharose High Performance and Chelating Sepharose Fast Flow[6].

3.3 Ion exchange chromatography

The principle behind the ion exchange chromatography, IEX, is the interactions between charged molecules in a solution and molecules of opposite charge immobilized in the matrix (Amersham Biosciences Limited, 2004). The separation is based on the reversible and selective adsorption of charged molecules (desired solute) to an immobilized ion exchanger with opposite charge (adsorbent) (Holme & Peck, 1993). The ion exchanger consists of a matrix composed by non soluble pearls, in which charged functional groups have been bonded covalently (Stigbrand, 1990).

[6] Figure 3-4: (Electronic). <http://teachline.ls.huji.ac.il/72682/Booklets/PHARMACIA-AffinityManual.pdf>. (2008-07-15)

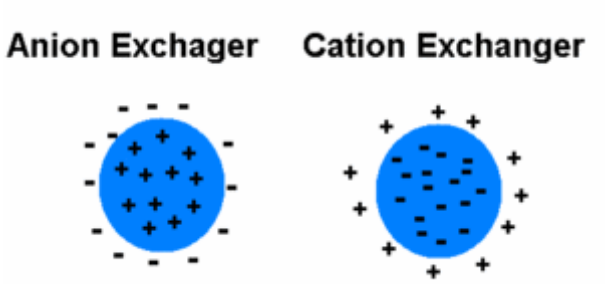

Figure 3-5. The anion exchanger and cation exchanger with exchangeable counter-ions[7].

Ion exchange chromatography can be used with both negative and positive ions (Fig. 3-5). If the ions that are suppose to be exchanged, the ions to be adsorbed, are negative, anions, the ions in the matrix will be positive and have exchangeable counter ions that are negative charged. This is called an anion exchanger. The other way around will be a cation exchanger (Amersham Biosciences Limited, 2004).

Different molecules integrate to the ion exchanger with different strength. How strong they integrate depends on their type of charge, the strength of the charge and how the charge is spread over the molecules surface. By varying the pH in the mobile phase one can control the molecules integrations with the solid matrix. With IEX, molecules with a very small difference in net charge can be separated from each other. This is true even for proteins where the difference in charge only arise because of one charged amino acid more or less. This quality makes IEX an excellent separation method (Amersham Biosciences Limited, 2004).

The largest application area of ion exchange chromatography is the separation of biopolymers such as nucleic acids and proteins (Neue, 1997). Proteins are built up by amino acids as a string of pearls. There are 20 different amino acids and their various combinations gives the proteins their function and structure. The string of pearls folds in different ways which gives it a unique three dimensional structure (Sjöström, 2008). The characteristics of the side chain in some amino acids may result in charged groups in the protein (Campell & Farell, 2003). Proteins are ampholytes, having both positive and negative charge (Flickinger & Drew, 1999).

The amino acids residues aspartic acid and glutamic acid are negative charged meanwhile lysine and arginine have positive charges under physiological conditions. Slightly below neutral pH histidine is protonated and cysteine is deprotonated slightly above neutral pH. A negative charge can be contributed by tyrosine, threonine and serine if they are phosphorylated. Threonine and serine can also contribute as well as asparagine with a negative or positive charge from glycosylation. Negative charge from glycuronates or sulfonated sugars and positive charge from deacetylated amino sugars. By deamination the amino acids asparagine and glutamine forms aspartate and glutamate which is negatively charged (Swadesh, 1997).

The total charge of the protein is depending of the pH of the surroundings and is zero when the amount of negative and positive charges in the protein is equal. This stage is called the

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[7] Figure 3-5: (Electronic). <http://en.wikibooks.org/wiki/Proteomics/Protein_Separations_-_Chromatography/Ion_exchange >.
(2008-07-16)

isoelectric point, pI (Amersham Biosciences Limited, 2004). It is not only the net charge that is important for the interaction with the matrix. The distribution of the charges on the surface may result in patches of positive and negative charge and the protein may even interact with the matrix at pI (Flickinger & Drew, 1999).

IEX is often performed in several steps (Fig. 3-6).

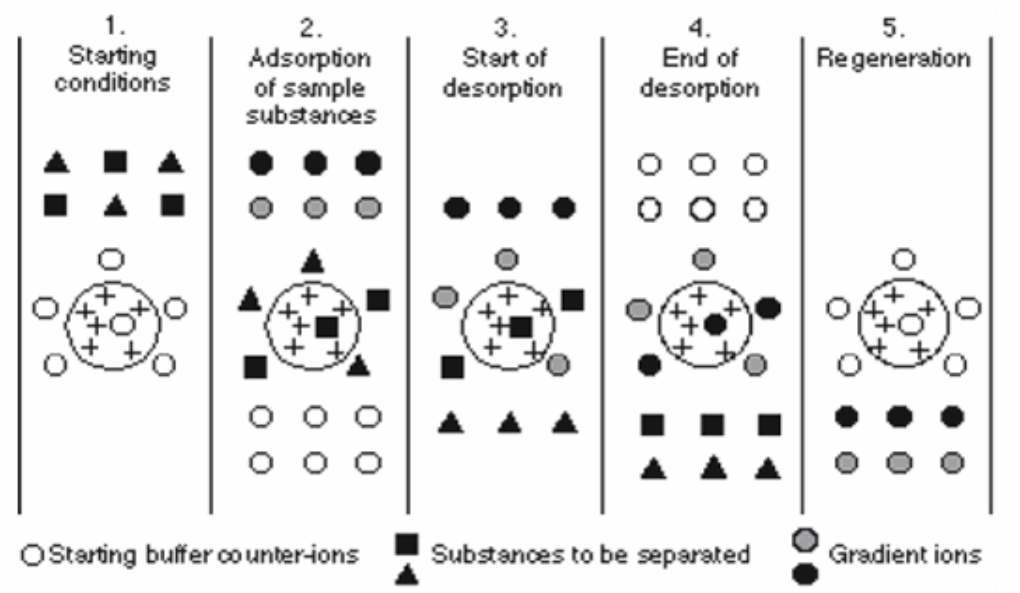

Figure 3-6. Ion exchange chromatography in five steps[8].

The first step is to make the solid phase, the matrix, susceptible to the molecule that is supposed to be adsorbed. To offer the right conditions for the molecule to bond in a satisfied way, the ion exchanger needs to be equilibrated. The right conditions such as pH and temperature has to fit the charge of the molecule. Ions, counter ions, with the same charge as the desired molecule is connected on the ion exchangers sites.

The next step is to add the sample. The molecules will be adsorbed by changing place with the counter ions and bind to the matrix reversible. All the molecules that did not bind will be flushed away with the initial buffer.

Separation of the molecules that has bound to the matrix is made by applying an elution gradient or multi step elution. This is the third step. The bonds between the molecules and the matrix will not be as strong when the ion strength is changing. This because, increased ion strength changes the charge of the molecules and the bonds will cease to exist. The weaker bond between the molecules and the matrix the faster the molecules will elute.

In the last two steps the molecules will elute depending on their charge and the elution gradient, and then a new equilibrium is made on the column to prepare it for a new separation (Amersham Biosciences Limited, 2004). In ion exchange separations of protein gradient

[8] Figure 3-6: (Electronic). Modified figure from:
Amersham Pharmacia Biotech AB (2002). *Ion Exchange Chromatography- Principals and Methods*. Pdf-document. (2008-07-16)

elution is used nearly every time. Often the pH remains the same and the ionic strength is increased in the buffer (Neue, 1997).

The manufacture of the Q Sepharose Fast Flow gel, which was examined in this thesis, is Amersham Biosciences AB, located in Uppsala, Sweden. According to their handbook "Ion Exchange Chromatography and Chromatofocusing" the gel is based on cross-linked agarose that forms 90 µm agarose beads to which the functional group Quaternary ammonium is bound.

$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$

The choice of matrix is important to achieve a good separation. In the same handbook they are mentioning several aspects to consider when choosing matrix for a separation. Most often one chooses to bind the desired protein to the solid matrix and let the impurities pass with the eluent. When the purification is done like that, a larger amount of purified protein can be obtained. But it is also possible to do the purification process in the opposite way and let the protein pass with the eluent (Amersham Biosciences Limited, 2004).

The strength on the ion exchanger and if it will be an anion or a cation is depended on the type of functional group the matrix contains. The capacity of the ion exchanger is depending on the amount and the accessibility of functional groups. There are many different functional groups that can be used in an ion exchanger, e.g. Quaternary ammonium in Q Sepharose Fast Flow gel.

The choice of ion exchanger should be considered depending on if the molecules in the sample are more stabile under or over their pI-value. A cation exchanger is used when the molecules are stabile under their pI and an anion exchanger is then used when the molecules are more stabile over its pI-value. If the molecules have stability over a wide range both anion and cation exchanger can be used. The choice between anion- and cation exchanger can be controlled by choosing a buffer with suitable pH which controls the charge on the molecule. But the choice of the ion exchanger is limited by the proteins characteristics. To keep the activity of the protein and not make it denaturant the pH has to lie between some boundaries (Amersham Biosciences Limited, 2004).

The manufactures also points the following factors to take under consideration when choosing a matrix for a process. They indicate that the Q Sepharose Fast Flow gel is working well for these conditions.

- The ion exchanger must be able to match the charge of the molecule in order to perform a good separation. The charge of the molecule can be changed with different pH on the buffer. The ion exchanger has to work in the pH-range where the charge of the molecule is stable. The Q Sepharose Fast Flow gel is able to work in the pH-range from 2 to 12.

- The ion exchanger can either be strong or weak which refers to that a strong ion exchanger can operate in a larger pH range then a weak one. A weak ion exchanger can with high and low pH lose its charge, which lowers its capacity in sample volume, the strong will not. The Q Sepharose Fast Flow gel is a strong ion exchanger.

- To achieve a good appropriate cycle time and improved productivity the flow rate of the process may be taken in consideration. The Q Sepharose Fast Flow gel has good

flow properties that come from the cross-linking. The high throughput of the gel makes it useful in industrial processes.

- A process is often first tested in laboratory scale to achieve the optimal conditions for the separation. If the procedure is to be scaled up the ion exchanger should be able to perform in the same way in a larger scale without the need to change any parameters.

- The ability to wash and regenerate the matrix for use in further separations is an important economical aspect. The reproducibility is depending on the fact that the matrix does not change its volume during the separation. The Q Sepharose Fast Flow gel is tolerant to changes in pH and ionic strength without changing its size (Amersham Biosciences Limited, 2004).

4. Material and Methods

4.1 Kinetic adsorption experiment of Q Sepharose Fast Flow gel

4.1.1 Equipment
Scale: Sartorius, 1700

Ionometer: Crison, GLP 22

Agitator with screw: Janke & Kunkel Ika® – Labortechnik, RW 10 R

Agitator with magnet: MLW, RH3

Spectrophotometer UV: Shimadzu, UVmini 1240

Centrifuge: HITACHI, HIMAC centrifuge

Micropipettes: Rongtai, 200-1000 µl, 20-200 µl, 5-50 µl

Funnel: Pyrex no 3

Measuring flask: 1000 ml

Measuring cylinder: 50 ml, 10 ml

Beaker: 25 ml

Vials Eppendorf: 1.5 ml

Quartz Cuvette: 1 ml

Aluminium foil

4.1.2 Chemicals
Gel of Q Sepharose Fast Flow: Handled by Centro de Inmunología Molecular

Hr EPO solution 15,212 mg/ml: Handled and distributed by Centro de Inmunología Molecular

Sodium phosphate monobasic, NaH_2P0_4(s), Sigma-Aldrich

Sodium phosphate dibasic, Na_2HPO_4(s), Sigma-Aldrich

Distilled water

4.1.3 Method
An equilibrium buffer with pH 6 was made. 0.257 g Na_2HPO_4 and 2.181 g NaH_2P0_4 were weight on aluminium foil. The chemicals were poured into a measuring flask that then was filled up with distilled water to 1 litre. The mixture was put on agitation with magnet until it was dissolved. The pH-value was measured to 5.95.

20 ml of the Q Sepharose Fast Flow gel was measured in a cylinder and transferred to a funnel. 300 ml of the made equilibrium buffer with pH 5.95 was added to the gel during agitation with a screw. The pH-value was measured on the last leaving buffer to 5.95. This procedure was done to equilibrate the gel.

1.972 ml EPO solution with concentration 15.212 mg/ml was transferred, with a micropipette, to a 25 ml beaker. 18.028 ml equilibrium buffer pH 5.95 was measured with a 50 ml measuring cylinder and a micropipette. The equilibrium buffer was transferred to the 25 ml beaker with EPO to reach the concentration of 1.5 mg EPO/ml. 1 ml of the equilibrated gel was measured in a 10 ml measuring cylinder. The gel was transferred to the beaker at the same time as the beaker was put on agitation with magnet. After 0.5 minute a 1.000 ml sample of the suspension was taken and transferred with a micropipette to a vial. The vial was centrifuged at 900 rpm for 1 minute. The clear supernatant was transferred with micropipette to a 1 ml cuvette and the absorbance was measured. After the first sample the time between sampling was extended to 1.5 minutes. This was done with the 8 following samplings. Thereafter time was extended to 3 minutes and 5 additional samples were taken. Absorbance of all samples was measured with the same method, as described for the first sample. Before every measurement the absorbance of the spectrophotometer was adjusted to zero with buffer in the cuvette.

The results were put together in a table and diagrams were made.

4.2 Adsorption isotherm experiment of Q Sepharose Fast Flow gel

4.2.1 Equipment
Scale: Sartorius, 1700

Spectrophotometer UV: Shimadzu, UVmini 1240

Centrifuge: HITACHI, HIMAC centrifuge

Thermomixer: Eppendorf AG Thermomixer compact TCEPPE01, calibrated 21/2 -08

Vortex: Janke & Kunkel IKA-WERK, VIBROFIX VF1 Electronic

Micropipettes: Rongtai, 200-1000 µl, 20-200 µl, 5-50 µl

Falcon tubes: 15 ml

Vials Eppendorf: 1.5 ml

Quartz Cuvette: 1 ml

4.2.2 Chemicals
Gel of Q Sepharose Fast Flow: Handled by Centro de Inmunología Molecular. Equilibrated in the kinetic adsorption experiment of Q Sepharose Fast Flow gel

Hr EPO solution 15,212 mg/ml: Handled and distributed by Centro de Inmunología Molecular

Equilibrium buffer: Made in the kinetic adsorption experiment of Q Sepharose Fast Flow gel.

4.2.3 Method
12 vials were filled with 0.400 ml of equilibrated Q Sepharose Fast Flow gel each. The vials were centrifuged at 2800 rpm for 2 minutes. The supernatants were removed. All vials were weighed to control equal amount of gel.

In 6 Falcon tubes, 6 different concentration of EPO solution were prepared. The preparations were done after the table below. The volumes were taken with micropipettes.

Table 4-1. The volumes of EPO solution and buffer that were mixed in falcon tubes are shown together with the obtained concentrations.

Expected Concentration of EPO (mg/ml)	EPO solution volume (ml)	Buffer Volume (ml)	Obtained Concentration of EPO (mg/ml)
12	2,37	0,63	13,151
10	1,97	1,03	11,016
8	1,58	1,42	8,387
6	1,18	1,82	6,263
4	0,79	2,21	4,142
2	0,39	2,61	1,987

The absorbance of the 6 solutions was measured and the concentrations are showed in the table above. Before every measurement the absorbance of the spectrophotometer was adjusted to zero with buffer in the cuvette.

All the vials were shaken by a vortex. To each vial 1 ml of EPO solution was added with micropipette. Each concentration was represented in 2 vials. The vials were turned by hand to mix the solution with the gel and then put in a thermo mixer for 1.5 hours. The thermo mixer was set to room temperature (25°C approx) and a speed of 1400 rpm.

Afterwards, the gel in the vials had some time to sediment before the absorbance of the clear supernatant was measured. The supernatant was carefully transferred to a 1 ml cuvette with micropipette. The absorbance was measured with a spectrophotometer at 280.0 nm. Before every measurement the absorbance of the spectrophotometer was adjusted to zero with buffer in the cuvette.

The results were put together in a table and diagrams were made.

4.3 Kinetic adsorption experiment of Chelating Sepharose Fast Flow gel

4.3.1 Equipment
Scale: Sartorius, 1700

Ionometer: Crison, GLP 22

Agitator with screw: Janke & Kunkel Ika® – Labortechnik, RW 10 R

Agitator with magnet: MLW, RH3

Spectrophotometer UV: Shimadzu, UVmini 1240

Centrifuge: HITACHI, HIMAC centrifuge

Micropipettes: Rongtai, 200-1000 µl, 20-200 µl, 5-50 µl

Funnel: Pyrex no 3

Measuring flask: 1000 ml, 100 ml

Measuring cylinder: 50 ml, 10 ml

Beaker: 25 ml

Vials Eppendorf: 1.5 ml

Quartz Cuvette: 1 ml

Aluminium foil

4.3.2 Chemicals
Gel of Chelating Sepharose Fast Flow: Handled by Centro de Inmunología Molecular

Sodium phosphate monobasic, $NaH_2P0_4(s)$, Sigma-Aldrich

Sodium phosphate dibasic, $Na_2HPO_4(s)$, Sigma-Aldrich

Sodium Chloride, NaCl(s), UNI-CHEM®

Cupric sulphate anhydrous, $CuSO_4$, Fluka AG

Equilibrium phosphate buffer pH 7.2: Handled and distributed by Centro de Inmunología Molecular

EPO solution, approximately 11.5 mg/ml: Handled and distributed by Centro de Inmunología Molecular

Distilled water

4.3.3 Method
A buffer was made in a 1000-ml measuring flask. 0.02 g of Na_2HPO_4, 15.57 g of NaH_2P0_4 and 29.22 g of NaCl were weight on aluminium foil and added to a 1000 ml measuring flask. The flask was filled with distilled water to the 1000 ml mark and turned by hand until the solid particles had dissolved. The pH-value was determined to 4.27 which is acceptable even if the value in fact should have been 4.1.

A copper sulphate solution with a concentration of 0.1 mol/l was made by weighting 2.49 g $CuSO_4$ in a 100 ml measuring flask and then filling it up with distilled water to the mark. The flask was turned by hand until the solid particles had dissolved.

10 ml of Chelating Sepharose Fast Flow gel was measured in a 10 ml measuring cylinder and poured into a funnel. The gel was stirred by an agitator with a screw. One at a time different volumes of solutions were added. They were run through the gel very slowly to make sure the equilibrium was correct. The volumes and solutions were 50 ml of distilled water, 20 ml of copper sulphate solution, 50 ml distilled water, 20 ml phosphate buffer pH 4.27 and a volume of phosphate buffer pH 7.2 large enough to make the pH-value of the last leaving buffer to 7.2. This was done to equilibrate the gel.

The exact concentration of the EPO solution, with a concentration of approximately 11.5 mg/ml, was determined to 11.417 mg/ml by measuring the absorbance in a spectrophotometer at 280 nm. 1.314 ml of EPO solution with concentration of 11.417 mg/ml was transferred with a micropipette to a 25 ml beaker. 18.686 ml phosphate buffer pH 7.2 was measured with a 50 ml measuring cylinder and micropipette. The two solutions were mixed in the 25 ml beaker to reach the concentration of 0.75 mg EPO/ml. The concentration of this solution was checked by measuring the absorbance with spectrophotometer.

1 ml of the equilibrated gel was measured in a 10 ml measuring cylinder and transferred to the 25 ml beaker with the EPO solution. The suspension was put on agitation with a magnet. After 0.5 minute a 1.000 ml sample of the suspension was taken and transferred with a micropipette to a vial. The vial was centrifuged at 2800 rpm for 1 minute. The clear supernatant was transferred with micropipette to a 1 ml cuvette and the absorbance was measured. After the first sample the time between sampling was extended to 1.5 minutes. This was done with the 11 following samplings. Thereafter, time was extended to 3 minutes and 4 additional samples were taken. 1 more sample after 10 minutes, 20 minutes and 30 minutes. The absorbencies of all samples were measured with the same method, as described for the first sample. Before every measurement the absorbance of the spectrophotometer was adjusted to zero with buffer in the cuvette.

The results were put together in a table and diagrams were made.

4.4 Adsorption isotherm experiment of Chelating Sepharose Fast Flow gel

4.4.1 Equipment
Scale: Sartorius, 1700

Spectrophotometer UV: Shimadzu, UVmini 1240

Centrifuge: HITACHI, HIMAC centrifuge

Thermomixer: Eppendorf AG Thermomixer compact TCEPPE01, calibrated 21/2 -08

Agitator with screw: Janke & Kunkel Ika® – Labortechnik, RW 10 R

Ionometer: Crison, GLP 22

Micropipettes: Rongtai, 200-1000 µl, 20-200 µl, 5-50 µl

Measuring flask: 100 ml

Funnel: Pyrex no 3

Falcon tubes: 15 ml

Vials Eppendorf: 1.5 ml

Quartz Cuvette: 1 ml

4.4.2 Chemicals
Gel of Chelating Sepharose Fast Flow: Handled by Centro de Inmunología Molecular. Equilibrated in Kinetic adsorption experiment of Chelating-gel

Hr EPO solution 13.522 mg/ml: Handled and distributed by Centro de Inmunología Molecular

Hr EPO solution 8.326 mg/ml: residue from the adsorption isotherm experiment of Q Sepharose Fast Flow gel, concentration checked with spectrophotometer

Hr EPO solution 5.866 mg/ml: residue from the adsorption isotherm experiment of Q Sepharose Fast Flow gel, concentration checked with spectrophotometer

Hr EPO solution 4.142 mg/ml: residue from the adsorption isotherm experiment of Q Sepharose Fast Flow gel, concentration checked with spectrophotometer

Equilibrium buffer 7.2: Handled and distributed by Centro de Inmunología Molecular

Equilibrium buffer 4.27: Made in the kinetic adsorption experiment of Sepharose Fast Flow gel

EDTA solution: Handled and distributed by Centro de Inmunología Molecular

Sodium Chloride, NaCl(s), UNI-CHEM®

Distilled water

4.4.3 Method

A sodium chloride solution with a concentration of 0.5 mol/l was made by weighting 2.922 g NaCl in a 100 ml measuring flask and then filling it up with distilled water to the mark. The flask was turned by hand until the solid particles had dissolved.

10 ml of Chelating Sepharose Fast Flow gel was measured in a 10 ml measuring cylinder and poured into a funnel. The gel was stirred by an agitator with a screw. One at a time different volumes of solutions were added to clean the gel. The volumes and solutions were 20 ml of EDTA solution and 30 ml of sodium chloride solution.

To equilibrate the clean gel in the funnel different volumes of solutions were added. They were run through the gel very slowly to make sure the equilibrium was correct. The volumes and solutions were 50 ml of distilled water, 20 ml of copper sulphate solution, 50 ml distilled water, 20 ml phosphate buffer pH 4.27 and a volume of phosphate buffer pH 7.2 large enough to make the pH-value of the last leaving buffer to 7.2.

14 vials were filled with 0.400 ml of equilibrated gel each. The vials were centrifuged at 2800 rpm for 2 minutes and then the supernatants were removed. All vials were weighed to control equal amount of gel.

The concentrations that should be used were prepared from four different EPO solutions. From one solution with an EPO concentration of 13.522 mg/ml two samples with concentration approximately 10 mg/ml and 4 mg/ml were made. From a second solution with an EPO concentration of 8.326 mg/ml two samples with concentration approximately 2 mg/ml and 0.4 mg/ml were made. From a third solution with an EPO concentration of 4.142 mg/ml one samples with concentration approximately 1 mg/ml were made. At last two samples with concentration approximately 0.8 mg/ml and 0.6 mg/ml were made out of a solution with an EPO concentration of 5.866 mg/ml. The volumes used are shown in the table below. Totally a volume of 2.5 ml each was made.

Table 4-2. The volumes of EPO solution and buffer that were mixed in falcon tubes are shown together with the obtained concentrations.

Expected Concentration of EPO (mg/ml)	EPO solution Volume (ml)	Buffer Volume (ml)	Obtained Concentration of EPO (mg/ml)
10	1,85	0,65	9,724
4	0,74	1,76	4,205
2	0,60	1,90	1,864
1	0,60	1,90	0,959
0,8	0,34	2,16	0,763
0,6	0,26	2,24	0,615
0,4	0,12	2,38	0,366

The absorptions of all new EPO solutions were measured to control the initial concentration of the experiment. The spectrophotometer was adjusted to zero between each measurement. To each vial, with 0.400 ml of equilibrated gel, 1 ml of EPO solution was added by micropipette. Each concentration was represented in 2 vials. The vials were turned by hand to mix the solution with the gel and then put in a thermo mixer for 2 hours. The thermo mixer was set to room temperature (25°C approx) and a speed of 1400 rpm.

The vials were then centrifuged for 2 minutes at 2800 rpm to make the gel sediment. The clear supernatants were transferred to new vials which then were centrifuged again at 2800 rpm for 2 minutes. The new clear supernatants were carefully transferred to a 1 ml cuvette with micropipette. The absorbencies were measured with a spectrophotometer at 280.0 nm.

The results were put together in a table and diagrams were made.

5. Results and discussion

5.1 Kinetic adsorption of Q Sepharose Fast Flow gel

In the figure below (Fig. 5-1) it is easy to see that the concentration of EPO is decreasing very rapidly in the beginning and reach equilibrium where almost no change in concentration accrues. This is a typical picture for purification of proteins with chromatography gel.

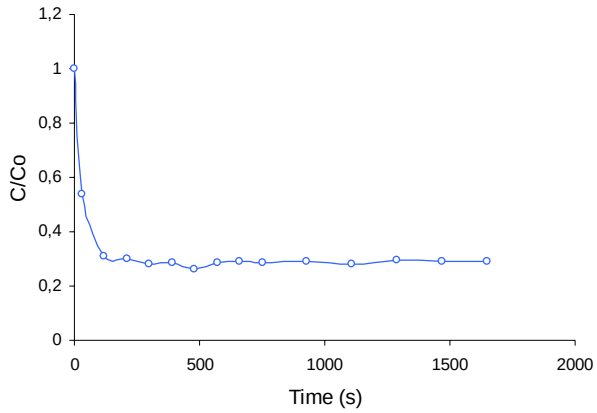

Figure 5-1. Chart from Excel with data from the Q-gel kinetic adsorption experiment.

In this chart one can see that the adsorption of EPO to the gel comes to a standstill after no more than approximately 200 seconds. From this chart it is not possible to calculate any constants. If the data instead is adjusted to the Daniels equation the results become more useful (Fig. 5-2).

$$\log\left(\frac{c}{c_0}\right) = k \cdot t + k' \cdot \sqrt{t}$$

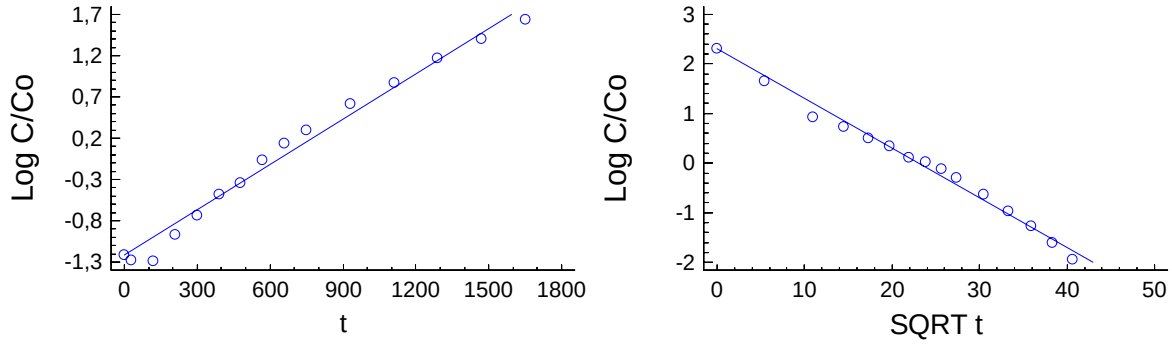

Figure 5-2. Daniels equation chart from Statgraphics with data from the Q-gel kinetic adsorption experiment.

Daniels equation gives the coefficients k and k'. With our data k = 0.002 ± 3.72 x 10^{-4} s^{-1} (Standard error = 0.000; P = 0.000) and k'= -0.101 ± 1.21 x 10^{-2} $s^{-0.5}$ (Standard error = 0.006; P = 0.000) with a confidence interval at 95 %.

It is easy to see that the straight line of Daniels equation follows the data points well. Both for t and square root of t the adjusted model lies slightly above the points until about 400 seconds (20 $s^{0.5}$). Afterwards, the model gives lower values than what the experimental data shows. The last two values show that the model ones again probably will be too high for following experimental data but the experiment was finished before this could be confirmed.

If the two linear graphs are put together it will be possible to read-out which of the two that will be the determining coefficient at which time (Fig. 5-3). The point where the curve intersects the x-axis can be decided either graphically or by multiplying the absolute value of k and k' by different t and practice the trial and error method. We have chosen the first of these two methods and the result was that k' was the determining coefficient in the beginning up until about 3025 s (55 $s^{0.5}$) where the curve intersect and k becomes the determining coefficient. The result was then verified mathematically. Total calculations in Appendix 3.

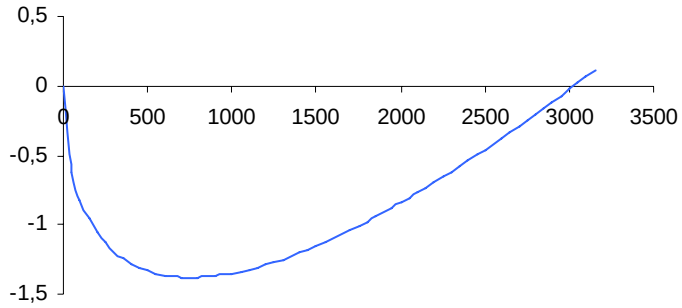

Figure 5-3. Daniels equation with values of k and k' for decision of the determining coefficient.

The negative coefficient of k' makes the curve go towards zero when t goes towards eternity. In order to adjust the curve to the equilibrium the model tries to compensate with a positive value of k'. This may work for small values of t when the curve is dominated by the term $k' \cdot \sqrt{t}$, but for larger values of t the curve will increase and pass the logarithm value of $\frac{c}{c_0} = 1$. This mathematically model does not consider the fact that it is physical impossible for the concentration of EPO to cross the initial concentration. The model is probably not designed to handle a lapse towards an equilibrium concentration.

By plotting the logarithm of the concentration ratio (with correction to the equilibrium concentration of EPO reached in the solution) against time, hopefully a model could be achieved which fits the experimental data better.

An assumption was made, that the ratio of $\frac{c}{c_0}$ tended to go towards the value of 0.263 in the equilibrium state. The program Matlab was used for the calculations. The equation $\log(\frac{c - c_\infty}{c_0 - c_\infty}) = k' \cdot t^\alpha$ was fitted to the experimental data.

Through calculations by Matlab the values of the coefficient k' and the exponent α were achieved. The value of α was found to 0.2335 and the value of k' was calculated to -0.7546. As can be seen in the figure below (Fig. 5-4) the adjusted model (the line) does not fit the experimental data (marked with rings) well.

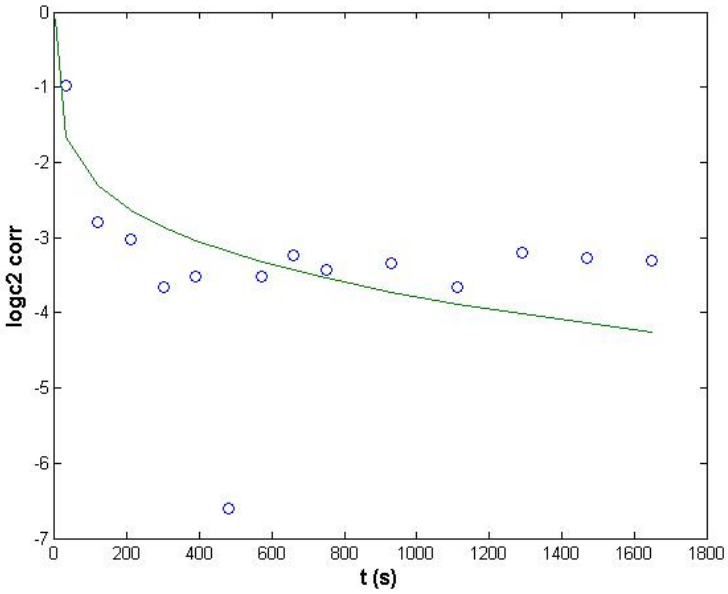

Figure 5-4. . The figure shows the adjusted model as a line and the experimental data as rings. The label for the y-axis is logc_corr which means logarithm of the ratio c/c_o with the correction to the equilibrium ratio.

The rapid adsorption in the beginning of the experiment gives only a few experimental values before the equilibrium is reached. It is difficult to adjust the model to the experimental data with a small number of values. The adsorption reached equilibrium already after the first four experimental values. To achieve a better and more correct adjustment of the model, more experimental points are needed in the beginning of the adsorption. The calculations from Matlab are shown in Appendix 4.

5.2 Adsorption isotherm of Q Sepharose Fast Flow gel

In this report the value from the sample with lowest concentrations is excluded to achieve a better fit of the equation.

The data were plotted in a Freundlich adsorption isotherm model (Fig. 5-5). R-square, adjusted for the degree of freedom, was calculated to 95,083 %. This is a good value. Theoretically this model is a fit. The complete model with values is shown in Appendix 2.

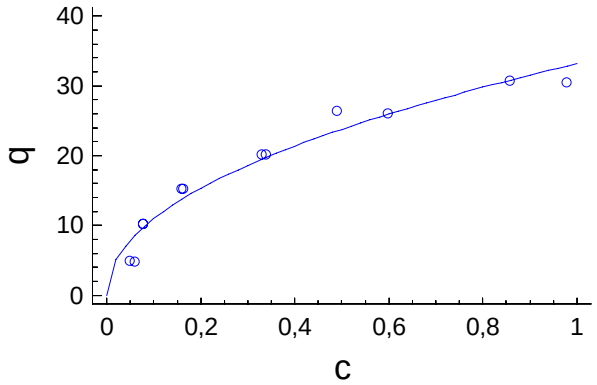

Figure 5-5. Freundlich adsorption isotherm chart from Statgraphics with data from the Q-gel adsorption isotherm experiment.

The data was also plotted in the Langmuir adsorption isotherm model and the R-square, adjusted for the degree of freedom, was calculated to 97,736 %. This is an even better fit (Fig. 5-6). The data was homogenously distributed around the curve which indicates that the model corresponds to the reality. The complete model with values is shown in Appendix 2.

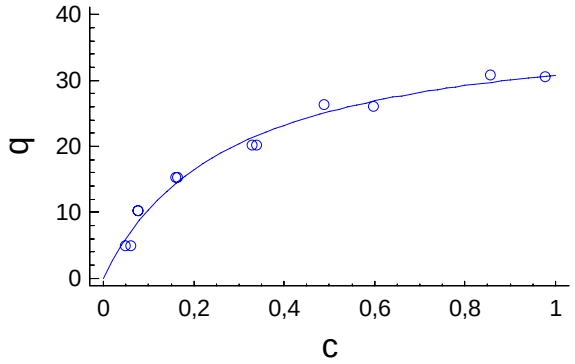

Figure 5-6. Langmuir adsorption isotherm chart from Statgraphics with data from the Q-gel adsorption isotherm experiment.

Because the Langmuir had a better R-square percentage and a good distribution it was the better isotherm, therefore our decision was to continue with this model.

From Langmuir adsorption isotherm equation, three different linear equations can be derived. This is a method to calculate K_d and q_{max}. By plotting the experimental data using the different linear equations, the best equation can be decided.

Scatchard equation: $\frac{q^*}{c^*} = -\frac{1}{K_d} \cdot q^* + \frac{q_{max}}{K_d}$

Double reciprocal: $\frac{1}{q^*} = \frac{K_d}{q_{max}} \cdot \frac{1}{c^*} + \frac{1}{q_{max}}$

Semi reciprocal: $\frac{c^*}{q^*} = \frac{1}{q_{max}} \cdot c^* + \frac{K_d}{q_{max}}$

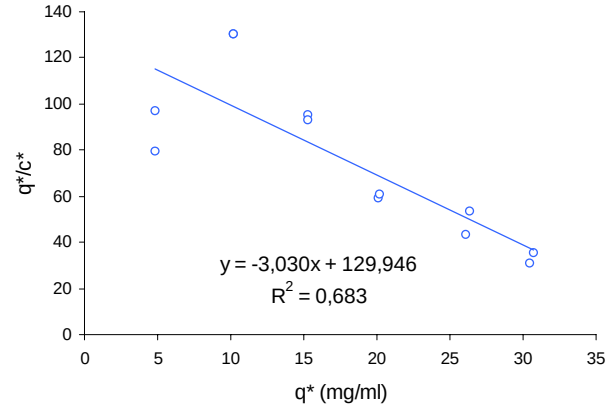

Figure 5-7. The Scatchard chart from Excel with data from the Q-gel adsorption isotherm experiment.

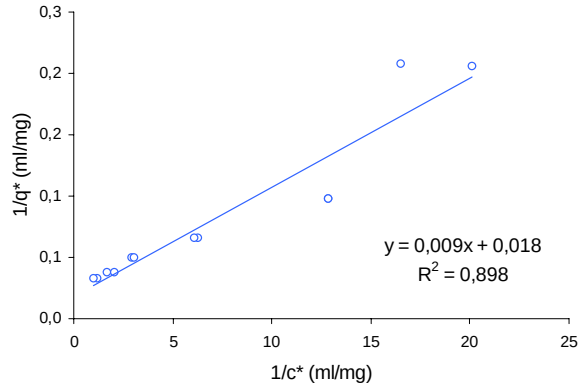

Figure 5-8. The double reciprocal chart from Excel with data from the Q-gel adsorption isotherm experiment.

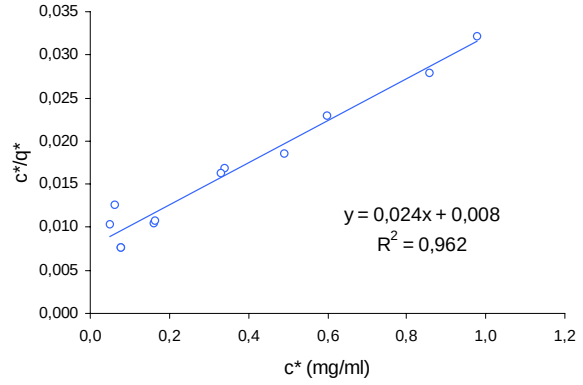

Figure 5-9. The semi reciprocal chart from Excel with data from the Q-gel adsorption isotherm experiment.

The Scatchard has a good homogenously distributed data but such a low R-square value that the model is not interesting to continue with (Fig. 5-7).

Both the double reciprocal (Fig. 5-8) and the semi reciprocal (Fig. 5-9) had high R-square values, the double reciprocal with a value of 89.8 % and the semi reciprocal with a value of 96.2 %. There is not such a big difference between the two values. The semi reciprocal was the equation with the most homogenously distributed data, and therefore the best fit. Semi reciprocal was then used in Statgraphics to calculate K_d and q_{max}. The semi reciprocal chart from Statgraphics with data from the Q-gel isotherm experiment is shown below in figure 5-10.

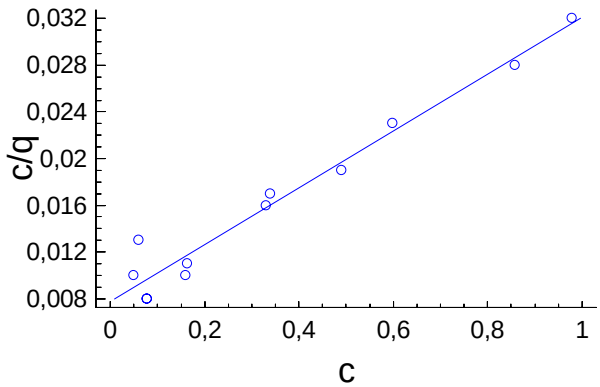

Figure 5-10. The semi reciprocal chart from Statgraphics with data from the Q-gel adsorption isotherm experiment.

Statgraphics shows the values of the semi reciprocal equation with their different statistic parameters. From these results q_{max} and K_d can be determined. The slope coefficient, which equals $\frac{1}{q_{max}}$, was found to 0.024 ± 0.003 ml/mg (Standard error = 0.002; P = 0.000) with a confidence interval at 95 %. The intercept of y-axis, which equals $\frac{K_d}{q_{max}}$, was found to 0.008 ± 0.002 (Standard error = 0.001; P = 0.000) with a confidence interval at 95 %. The value of q_{max} was calculated to 41.137 mg/ml and the value of K_d was calculated to 0.320 mg/ml. The result of Statgraphics is shown in Appendix 2 and the calculations in Appendix 3.

According to Yanet Borrego and David Curbelo at CIM the expected value of q_{max} was between 30 and 50 mg/ml. With our result from the isotherm experiment we could conclude a value of approximately 41 mg/ml. With this value we can assume that their expectations agreed with reality.

5.3 Kinetic adsorption of Chelating Sepharose Fast Flow gel

The chart below (Fig. 5-11) is very much alike the chart for the kinetic of the Q-gel. Just like the other one it shows that the concentration decrease very rapidly in the beginning and then reach equilibrium.

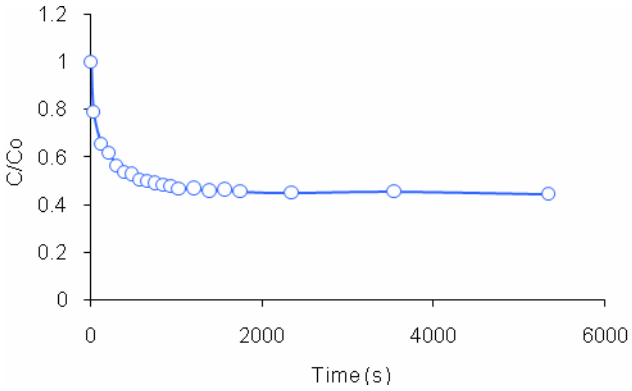

Figure 5-11. Chart from Excel with data from the CH-gel kinetic adsorption experiment.

In this chart one can see that the adsorption of EPO to the gel never comes to a standstill in this experiment. But just before 1000 seconds one can see that the curve planes down thoroughly. From this chart it is not possible to calculate any constants. If the data instead is adjusted to the Daniels equation, the results become more useful as shown in figure 5-12.

This chart does not give any usable parameters and therefore the data were put in the Daniels equation.

$$\log\left(\frac{c}{c_0}\right) = k \cdot t + k' \cdot \sqrt{t}$$

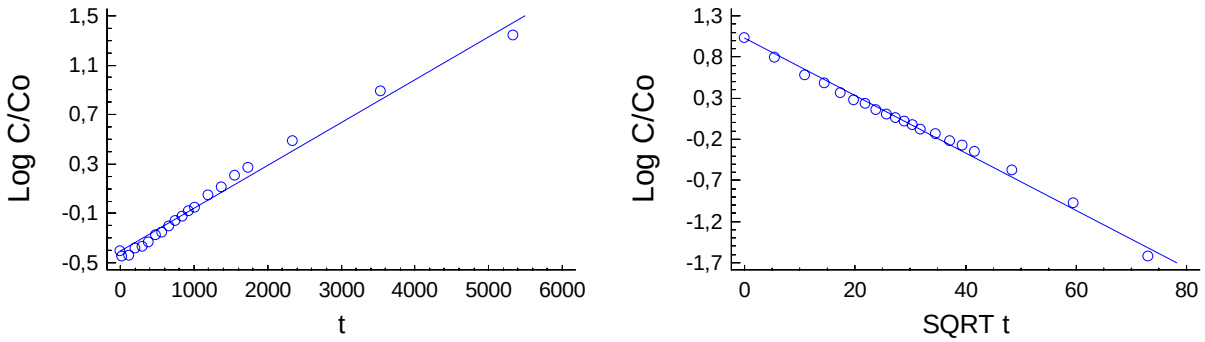

Figure 5-12. Daniels equation chart from Statgraphics with data from the CH-gel kinetic adsorption experiment

Daniels equation gives the coefficients k and k'. With our data k = 3.47 x 10^{-4} ± 4.48 x 10^{-5} s^{-1} (Standard error = 0.000; P = 0.000) and k'= -3.50 x 10^{-2} ± 2.26 x 10^{-3} $s^{-0.5}$ (Standard error = 0.001; P = 0.000) with a confidence interval at 95 %.

It is easy to see that the straight line of Daniels equation follows the data points well. Both for t and square root of t the adjusted model lies slightly above the points until fully 1000 seconds (31.6 $s^{0.5}$). Afterwards, the model gives lower values than what the experimental data shows. The last value shows that the model ones again might be too high for following experimental data but the experiment was finished before this could be obtained.

To determine the point where the curve intersects the x-axis once again we have chosen a graphical solution (Fig. 5-13). The result was that k' was the determining coefficient up until about 10130 s (100.6 $s^{0.5}$) all times. The result was then verified mathematically. Total calculations are in Appendix 3.

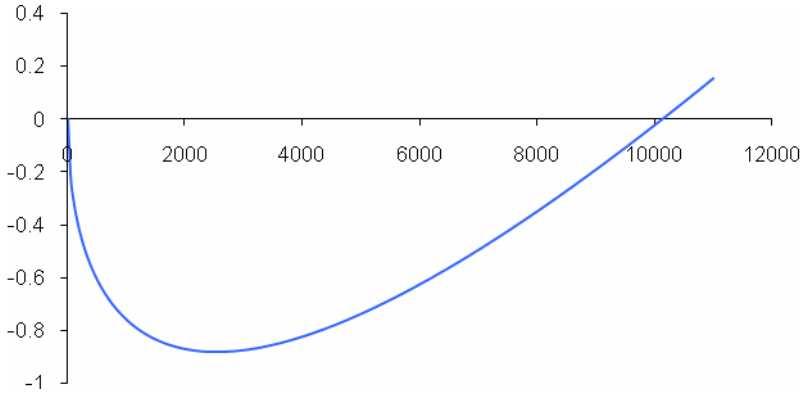

Figure 5-13. Daniels equation with values of k and k' for decision of the determining coefficient.

As for the Q Sepharose Fast Flow gel the model must be corrected towards equilibrium.

Once again a plot was made with the logarithm of the concentration ratio (with correction to the equilibrium concentration of EPO reached in the solution) against time.

The assumption that the ratio of $\frac{c}{c_0}$ tended to go towards the value of 0.4449 as an equilibrium ratio was made. The program Matlab was used for the calculations. The equation $\log(\frac{c-c_\infty}{c_0-c_\infty}) = k' \cdot t^\alpha$ was fitted to the experimental data.

Through calculations by Matlab the values of the coefficient k' and the exponent α were achieved. The value of α was found to 0.5078 which is very close to the definition of the square root. The value of k' was calculated to -0.0849. The figure below (Fig. 5-14) shows that the adjusted model (the line) fits well to the experimental data (marked with rings). In opposite to the Q Sepharose Fast Flow gel,

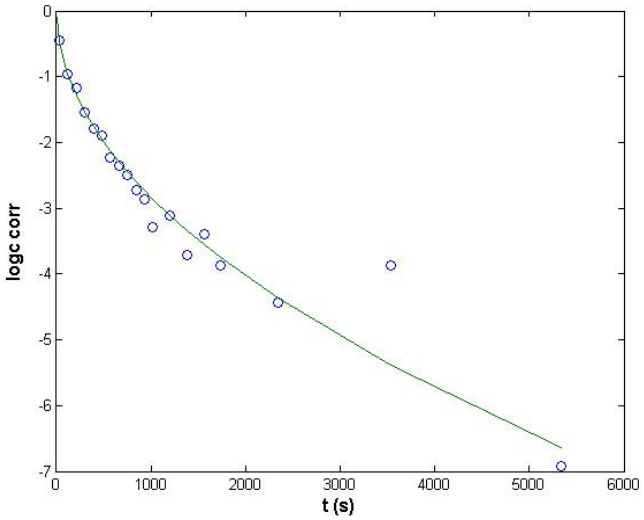

Figure 5-14. The figure shows the adjusted model as a line and the experimental data as rings. The label for the y-axis is logc_corr which means logarithm of the ratio c/c_o with the correction to the equilibrium ratio.

The conclusion of this was that $k' \cdot \sqrt{t}$ is the determining term for this experiment. According to the theory behind the equation, the adsorption process of EPO to Chelating Sepharose Fast Flow gel is controlled by the diffusion into a porous homogeneous medium. The calculations from Matlab are shown in Appendix 4.

5.4 Adsorption isotherm of Chelating Sepharose Fast Flow gel

The value from the sample with lowest concentrations is excluded to achieve a better fit of the equation.

The data was plotted in a Freundlich adsorption isotherm model (Fig. 5-15). R-square, adjusted to the degree of freedom, was calculated to 94,665 %. The model has a quite good homogenously distribution of data around the curve. The complete model with values is shown in Appendix 2.

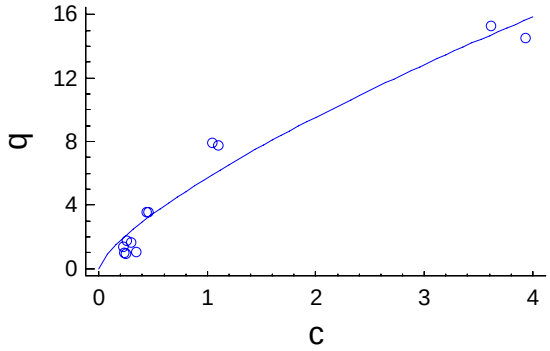

Figure 5-15. The Freundlich adsorption isotherm chart from Statgraphics with data from the CH-gel adsorption isotherm experiment.

The same data was put in a Langmuir isotherm equation to see if the fit was better (Fig. 5-16). For Langmuir R-square, adjusted to the degree of freedom, was calculated to 96,907 %. This R-square value shows an even better fit compared to the Freundlich isotherm but they are still very close to one another. The complete model is shown in Appendix 2.

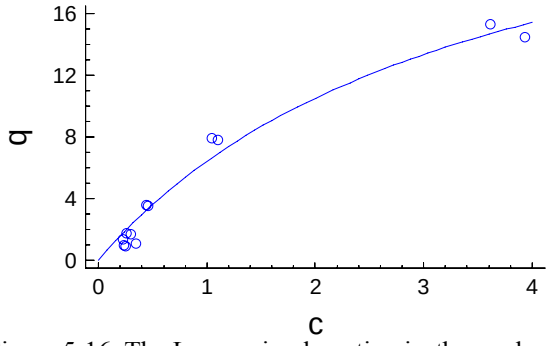

Figure 5-16. The Langmuir adsorption isotherm chart from Statgraphics with data from the CH-gel adsorption isotherm experiment.

The difference between the two models lies in the distribution of data. In the Langmuir the data is considerably less homogenously then it is in the Freundlich. Because of this, a comparison between the two isotherm models is necessary. Our decision was to compare the linear derives of the two models. All charts are shown below in figure 5-17 and figure 5-18.

See chapter 5.2 Adsorption isotherm of Q Sepharose Fast Flow gel for the expression of the linear Langmuir equations.

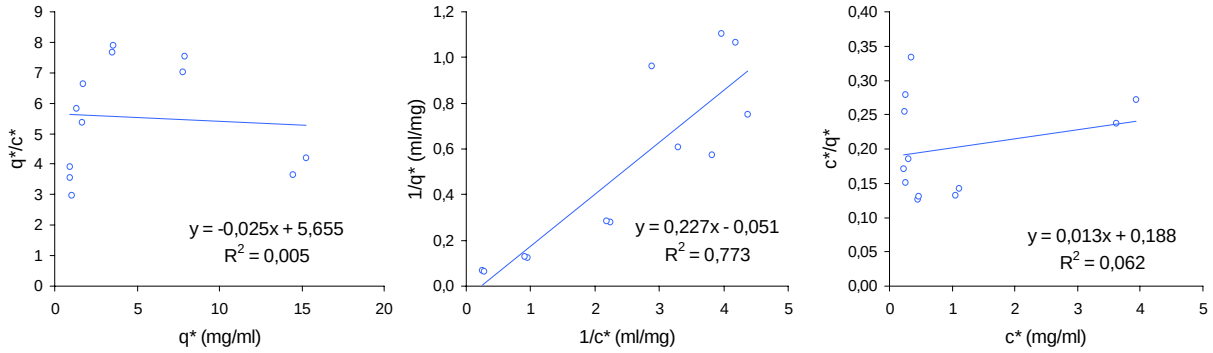

Figure 5-17. From Excel, the three different linear equations of Langmuir adsorption isotherm with their respective R-square value. From the left Scatchard, double reciprocal and semi reciprocal.

Linear Freundlich: $\log q^* = \frac{1}{n} \cdot \log c^* + \log K_a$

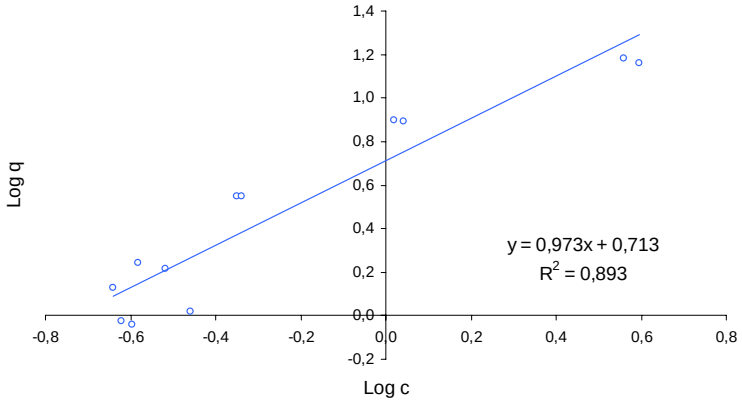

Figure 5-18. The linear equation of the Freundlich adsorption isotherm chart from Excel with data from the CH-gel adsorption isotherm experiment.

The linear equations of the Langmuir isotherm have low R-square values. The data does not correspond at all with a straight line. This makes these equations useless for further calculations. Instead the linear Freundlich isotherm was chosen because it's higher R-square value. Even though this is not a great fit it is still the best model. This was then used in Statgraphics to calculate K_a and n (Fig. 5-19).

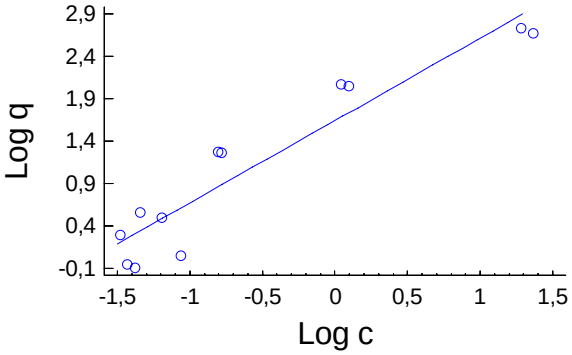

Figure 5-19. The linear Freundlich adsorption isotherm chart from Statgraphics with data from the CH-gel adsorption isotherm experiment.

Statgraphics shows the values of the linear Freundlich equation with their different statistic parameters. From these results K_a and n can be determined. The slope coefficient, which equals $\frac{1}{n}$, was found to 0.973 ± 0.237 (Standard error = 0.106; P = 0.000) with a confidence interval at 95 %. The intercept of y-axis, which equals $\log K_a$, was found to 1.643 ± 0.267 (Standard error = 0.120; P = 0.000) with a confidence interval at 95 %. The value of n was calculated to 1.028 and the value of K_d was calculated to 0.193. The result of Statgraphics is shown in Appendix 2 and the calculations in Appendix 3.

Because CIM needed a value on q_{max} for the Chelating Sepharose Fast Flow gel as well, this was calculated even if our conclusion was that the Freundlich isoterm was the best fit. To calculate the q-max, the linear Langmuir equation with the highest r-square value was used. This was the double reciprocal equation which had an r-square value on 77,3 %. The double

reciprocal chart from Excel with data from the CH-gel adsorption isotherm experiment is shown in figure 5-17.

Double reciprocal: $\frac{1}{q^*} = \frac{K_d}{q_{max}} \cdot \frac{1}{c^*} + \frac{1}{q_{max}}$

The double reciprocal data was then used in Statgraphics to calculate K_d and q_{max}. The double reciprocal chart from Statgraphics with data from the CH-gel isotherm experiment is shown below (Fig. 5-20).

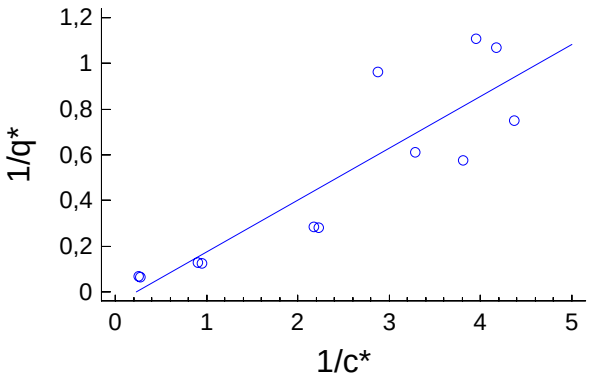

Figure 5-20. The double reciprocal chart from Statgraphics with data from the CH-gel adsorption isotherm experiment.

Statgraphics shows the values of the double reciprocal equation with their different statistic parameters. From these results q_{max} and K_d can be determined. The slope coefficient, which equals $\frac{K_d}{q_{max}}$, was found to 0.227 ± 0.086 ml/mg (Standard error = 0.039; P = 0.000) with a confidence interval at 95 %. The intercept of y-axis, which equals $\frac{1}{q_{max}}$, was found to -0.051 ± 0.246 (Standard error = 0.111; P = 0.652) with a confidence interval at 95 %. The value of q_{max} was calculated to -19.471 mg/ml and the value of K_d was calculated to -4.414 mg/ml. The result of Statgraphics is shown in Appendix 2 and the calculations in Appendix 3.

It is totally unreasonable to have a negative value of q_{max} which is just another acknowledgement for that the data follows the Freundlich isotherm and not the Langmuir isotherm.

6. Conclusions and Recommendations

From the results of the kinetic adsorption experiments we have drawn the conclusion that it is no meaning to let the adsorption process continue longer than until the point where the adsorption cease. For the Q Sepharose Fast Flow gel this is a time of 200 seconds. When it comes to the Chelating Sepharose Fast Flow gel, our experiment does not show a total ceasing of the adsorption process. But we have observed that after 1000 seconds the adsorption process is very slow. Therefore we think that this is the right point to end the process. Our recommendation is that it is a waste of money, time and resources to let the processes proceed beyond these points.

From Daniels equation k and k' were calculated. For the Chelating Sepharose Fast Flow gel the rate of the adsorption is controlled by the diffusion into the pore. It is not possible for us to determine which part of the transport that is controlling the rate for the Q Sepharose Fast Flow gel.

Our recommendation is to make new experiments with more values from the beginning of the adsorption. To be able to achieve this, it might be necessary to change the method.

The remaining results of the adsorption isotherm experiments have been examined separately. q_{max} for the Q Sepharose Fast Flow gel and n for the Chelating Sepharose Fast Flow gel have been compared with the expected respective values from CIM. For the Q Sepharose Fast Flow gel the expected and the determined value of q_{max} corresponds very well. CIM has not given us any expected value of n for the Chelating Sepharose Fast Flow gel probably because they thought that the gel was following a Langmuir adsorption isotherm which is not true according to our experiment.

Our recommendation is to make the same adsorption isotherm experiments with new potential usable gels, to be able to compare new values with our values from this report. The comparison is necessary to find equal chromatographically material for further production and purification of EPO.

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Appendix 1: Primary Data

Kinetic adsorption experiment of Q Sepharose Fast Flow gel

Time (s)	Absorbance (280 nm)
0,0	-
30,0	0,601
120,0	0,344
210,0	0,333
300,0	0,315
390,0	0,318
480,0	0,294
570,0	0,318
660,0	0,326
750,0	0,320
930,0	0,322
1110,0	0,315
1290,0	0,327
1470,0	0,324
1650,0	0,323

Adsorption isotherm experiment of Q Sepharose Fast Flow gel

C_0 (mg/ml)	Absorbance (280 nm)
1,987	0,037
1,987	0,045
4,142	0,058
4,142	0,058
6,263	0,119
6,263	0,122
8,387	0,253
8,387	0,245
11,016	0,445
11,016	0,364
13,151	0,638
13,151	0,727

Kinetic adsorption experiment of Chelating Sepharose Fast Flow gel

Time (s)	Absorbance (280 nm)
0	0,559
30	0,441
120	0,367
210	0,345
300	0,315
390	0,300
480	0,295
570	0,282
660	0,278
750	0,274
840	0,269
930	0,266
1020	0,260
1200	0,262
1380	0,256
1560	0,259
1740	0,255
2340	0,252
3540	0,255
5340	0,249

Adsorption isotherm experiment of Chelating Sepharose Fast Flow gel

C_0 (mg/ml)	Absorbance (280 nm)
0,366	0,340
0,366	0,254
0,615	0,188
0,615	0,178
0,763	0,258
0,763	0,170
0,959	0,226
0,959	0,195
1,864	0,333
1,864	0,341
4,205	0,779
4,205	0,821
9,724	-
9,724	-

Appendix 2: Statgraphics

Kinetic adsorption of Q Sepharose Fast Flow gel

Kinetic adsorption described by Daniels equation

Multiple Regression Analysis

Dependent variable: LOG(C:Co)

Parameter	Estimate	Standard Error	T Statistic	P-Value
t	0,00182742	0,000171983	10,6256	0,0000
SQRT(t)	-0,10052	0,00558392	-18,0017	0,0000

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	20,3055	2	10,1527	577,92	0,0000
Residual	0,228382	13	0,0175679		
Total	20,5338	15			

R-squared = 98,8878 percent
R-squared (adjusted for d.f.) = 98,8022 percent
Standard Error of Est. = 0,132544
Mean absolute error = 0,0982044
Durbin-Watson statistic = 0,536412
Lag 1 residual autocorrelation = 0,668532

The StatAdvisor

The output shows the results of fitting a multiple linear regression model to describe the relationship between LOG(C:Co) and 2 independent variables. The equation of the fitted model is
LOG(C:Co) = 0,00182742*t - 0,10052*SQRT(t)

Since the P-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level.

The R-Squared statistic indicates that the model as fitted explains 98,8878% of the variability in LOG(C:Co). The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 98,8022%. (Note: since the model does not contain a constant, you should be careful in interpreting the R-Squared values. Do not compare these R-Squared values with those of models which do contain a constant). The standard error of the estimate shows the standard deviation of the residuals to be 0,132544. This value can be used to constructprediction limits for new observations by selecting the

Reports option from the text menu. The mean absolute error (MAE) of 0,0982044 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file.

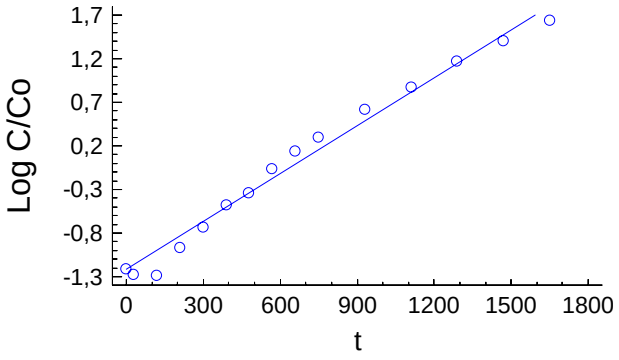
In determining whether the model can be simplified, notice that the highest P-value on the independent variables is 0,0000, belonging to t. Since the P-value is less than 0.01, the highest order term is statistically significant at the 99% confidence level. Consequently, you probably don't want to remove any variables from the model.

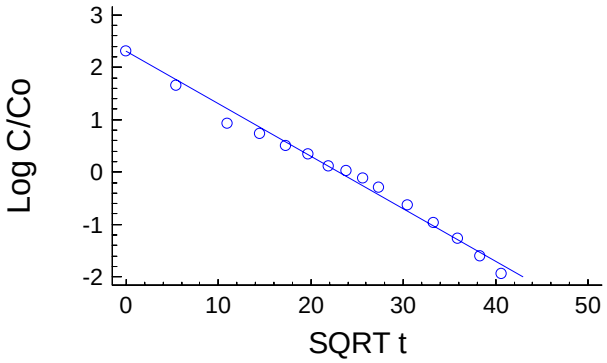
95,0% confidence intervals for coefficient estimates

Parameter	Estimate	Standard Error	Lower Limit	Upper Limit
t	0,00182742	0,000171983	0,00145587	0,00219897
SQRT(t)	-0,10052	0,00558392	-0,112583	-0,0884564

The StatAdvisor

This table shows 95,0% confidence intervals for the coefficients in the model. Confidence intervals show how precisely the coefficients can be estimated given the amount of available data and the noise which is present.





Adsorption isotherm of Q Sepharose Fast Flow gel

Freundlich adsorption isotherm

Nonlinear Regression

Dependent variable: q
Independent variables: c

Function to be estimated: a*c^n
Initial parameter estimates:
  a = 33,0
  n = 4,0

Estimation method: Marquardt
Estimation stopped due to convergence of residual sum of squares.
Number of iterations: 8
Number of function calls: 35

Estimation Results

Parameter	Estimate	Asymptotic Standard Error	Asymptotic 95,0% Confidence Interval Lower	Upper
a	33,1642	1,36357	30,126	36,2024
n	0,480397	0,0414166	0,388115	0,572679

Analysis of Variance

Source	Sum of Squares	Df	Mean Square
Model	4728,06	2	2364,03
Residual	42,2098	10	4,22098
Total	4770,27	12	
Total (Corr.)	944,333	11	

R-Squared = 95,5302 percent
R-Squared (adjusted for d.f.) = 95,0832 percent
Standard Error of Est. = 2,0545
Mean absolute error = 1,41094
Durbin-Watson statistic = 0,99118
Lag 1 residual autocorrelation = 0,329258

Residual Analysis

	Estimation	Validation
n	12	
MSE	4,22098	
MAE	1,41094	
MAPE	16,1008	
ME	-0,14178	
MPE	-8,91973	

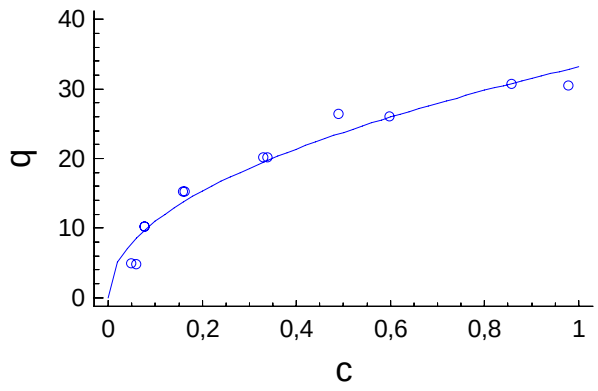
The StatAdvisor

The output shows the results of fitting a nonlinear regression model to describe the relationship between q and 1 independent variables. The equation of the fitted model is 33,1642*c^0,480397

In performing the fit, the estimation process terminated successfully after 8 iterations, at which point the estimated coefficients appeared to converge to the current estimates.

The R-Squared statistic indicates that the model as fitted explains 95,5302% of the variability in q. The adjusted R-Squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 95,0832%. The standard error of the estimate shows the standard deviation of the residuals to be 2,0545. This value can be used to construct prediction limits for new observations by selecting the Forecasts option from the text menu. The mean absolute error (MAE) of 1,41094 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file.

The output also shows aymptotic 95,0% confidence intervals for each of the unknown parameters. These intervals are approximate and most accurate for large sample sizes. You can determine whether or not an estimate is statistically significant by examining each interval to see whether it contains the value 0.0. Intervals covering 0.0 correspond to coefficients which may well be removed form the model without hurting the fit substantially.



Langmuir adsorption isotherm

Nonlinear Regression

Dependent variable: q
Independent variables: c

Function to be estimated: a*c/(1+b*c)
Initial parameter estimates:
  a = 100,0
  b = 1,0

Estimation method: Marquardt
Estimation stopped due to convergence of residual sum of squares.
Number of iterations: 5
Number of function calls: 17

Estimation Results

Parameter	Estimate	Asymptotic Standard Error	Asymptotic 95,0% Confidence Interval Lower	Upper
a	141,013	11,9033	114,491	167,535
b	3,57764	0,466016	2,53929	4,61599

Analysis of Variance

Source	Sum of Squares	Df	Mean Square
Model	4750,83	2	2375,42
Residual	19,4331	10	1,94331
Total	4770,27	12	
Total (Corr.)	944,333	11	

R-Squared = 97,9421 percent
R-Squared (adjusted for d.f.) = 97,7363 percent
Standard Error of Est. = 1,39403
Mean absolute error = 1,17224
Durbin-Watson statistic = 1,38658
Lag 1 residual autocorrelation = 0,272146

Residual Analysis

	Estimation	Validation
n	12	
MSE	1,94331	
MAE	1,17224	
MAPE	11,3718	
ME	-0,0185407	
MPE	-3,20931	

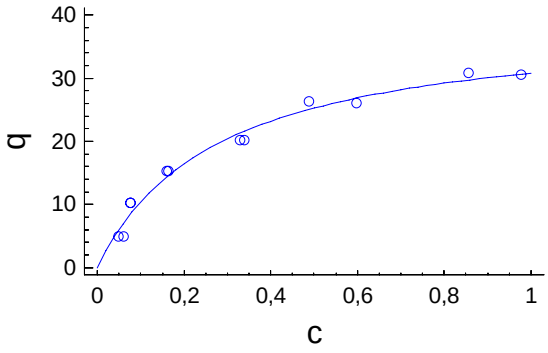
The StatAdvisor

The output shows the results of fitting a nonlinear regression model to describe the relationship between q and 1 independent variables. The equation of the fitted model is 141,013*c/(1+3,57764*c)

In performing the fit, the estimation process terminated successfully after 5 iterations, at which point the estimated coefficients appeared to converge to the current estimates.

The R-Squared statistic indicates that the model as fitted explains 97,9421% of the variability in q. The adjusted R-Squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 97,7363%. The standard error of the estimate shows the standard deviation of the residuals to be 1,39403. This value can be used to construct prediction limits for new observations by selecting the Forecasts option from the text menu. The mean absolute error (MAE) of 1,17224 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file.

The output also shows aymptotic 95,0% confidence intervals for each of the unknown parameters. These intervals are approximate and most accurate for large sample sizes. You can determine whether or not an estimate is statistically significant by examining each interval to see whether it contains the value 0.0. Intervals covering 0.0 correspond to coefficients which may well be removed form the model without hurting the fit substantially.



Linear Langmuir adsorption isotherm

Multiple Regression Analysis

Dependent variable: c:q

Parameter	Estimate	Standard Error	T Statistic	P-Value
CONSTANT	0,00777017	0,000711197	10,9255	0,0000
c	0,0243091	0,00153175	15,8701	0,0000

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0,000665814	1	0,000665814	251,86	0,0000
Residual	0,0000264357	10	0,00000264357		
Total (Corr.)	0,00069225	11			

R-squared = 96,1812 percent
R-squared (adjusted for d.f.) = 95,7993 percent
Standard Error of Est. = 0,00162591
Mean absolute error = 0,00117635
Durbin-Watson statistic = 1,67703 (P=0,1725)
Lag 1 residual autocorrelation = 0,138097

The StatAdvisor

The output shows the results of fitting a multiple linear regression model to describe the relationship between c:q and 1 independent variables. The equation of the fitted model is c:q = 0,00777017 + 0,0243091*c

Since the P-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level.

The R-Squared statistic indicates that the model as fitted explains 96,1812% of the variability in c:q. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 95,7993%. The standard error of the estimate shows the standard deviation of the residuals to be 0,00162591. This value can be used to construct prediction limits for new observations by selecting the Reports option from the text menu. The mean absolute error (MAE) of 0,00117635 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the P-value is greater than 0.05, there is no indication of serial autocorrelation in the residuals.

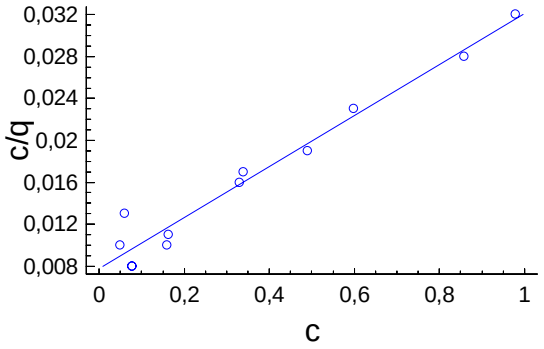
In determining whether the model can be simplified, notice that the highest P-value on the independent variables is 0,0000, belonging to c. Since the P-value is less than 0.01, the highest order term is statistically significant at the 99% confidence level. Consequently, you probably don't want to remove any variables from the model.

95,0% confidence intervals for coefficient estimates

Parameter	Estimate	Standard Error	Lower Limit	Upper Limit
CONSTANT	0,00777017	0,000711197	0,00618552	0,00935482
c	0,0243091	0,00153175	0,0208961	0,0277221

The StatAdvisor

This table shows 95,0% confidence intervals for the coefficients in the model. Confidence intervals show how precisely the coefficients can be estimated given the amount of available data and the noise which is present.



Kinetic adsorption of Chelating Sepharose Fast Flow gel

Kinetic adsorption described by Daniels equation

Multiple Regression Analysis

Dependent variable: LOG(C:Co)

Parameter	Estimate	Standard Error	T Statistic	P-Value
t	0,000347272	0,0000213029	16,3017	0,0000
SQRT(t)	-0,0349545	0,00107686	-32,4597	0,0000

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	8,96552	2	4,48276	1303,75	0,0000
Residual	0,0618904	18	0,00343835		
Total	9,02741	20			

R-squared = 99,3144 percent
R-squared (adjusted for d.f.) = 99,2763 percent
Standard Error of Est. = 0,0586375
Mean absolute error = 0,0470916
Durbin-Watson statistic = 0,649666
Lag 1 residual autocorrelation = 0,57777

The StatAdvisor

The output shows the results of fitting a multiple linear regression model to describe the relationship between LOG(C:Co) and 2 independent variables. The equation of the fitted model is
LOG(C:Co) = 0,000347272*t - 0,0349545*SQRT(t)

Since the P-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level.

The R-Squared statistic indicates that the model as fitted explains 99,3144% of the variability in LOG(C:Co). The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 99,2763%. (Note: since the model does not contain a constant, you should be careful in interpreting the R-Squared values. Do not compare these R-Squared values with those of models which do contain a constant.) The standard error of the estimate shows the standard deviation of the residuals to be 0,0586375. This value can be used to construct prediction limits for new observations by selecting the Reports option from the text menu. The mean absolute error (MAE) of 0,0470916 is the

average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file.

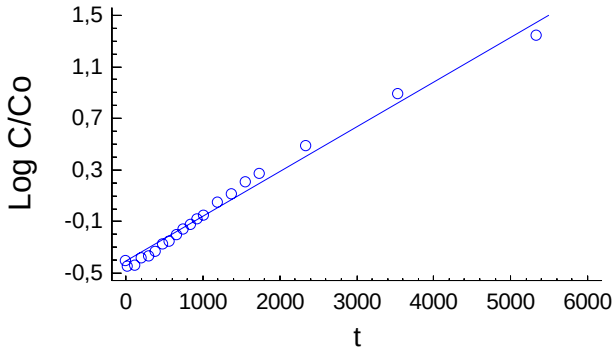
In determining whether the model can be simplified, notice that the highest P-value on the independent variables is 0,0000, belonging to t. Since the P-value is less than 0.01, the highest order term is statistically significant at the 99% confidence level. Consequently, you probably don't want to remove any variables from the model.

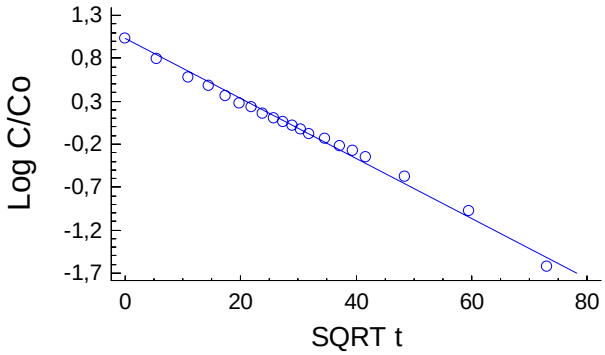
95,0% confidence intervals for coefficient estimates

Parameter	Estimate	Standard Error	Lower Limit	Upper Limit
t	0,000347272	0,0000213029	0,000302516	0,000392028
SQRT(t)	-0,0349545	0,00107686	-0,0372169	-0,0326921

The StatAdvisor

This table shows 95,0% confidence intervals for the coefficients in the model. Confidence intervals show how precisely the coefficients can be estimated given the amount of available data and the noise which is present.





Adsorption isotherm of Chelating Sepharose Fast Flow gel

Freundlich adsorption isotherm

Nonlinear Regression

Dependent variable: q
Independent variables: c

Function to be estimated: a*c^n
Initial parameter estimates:

a = 0,1
n = 0,1

Estimation method: Marquardt
Estimation stopped due to convergence of residual sum of squares.
Number of iterations: 12
Number of function calls: 45

Estimation Results

Parameter	Estimate	Asymptotic Standard Error	Asymptotic 95,0% Confidence Interval Lower	Upper
a	5,72684	0,465078	4,69058	6,7631
n	0,734722	0,0661554	0,587319	0,882126

Analysis of Variance

Source	Sum of Squares	Df	Mean Square
Model	585,523	2	292,762
Residual	14,5349	10	1,45349
Total	600,058	12	
Total (Corr.)	299,698	11	

R-Squared = 95,1501 percent
R-Squared (adjusted for d.f.) = 94,6652 percent
Standard Error of Est. = 1,20561
Mean absolute error = 0,962955
Durbin-Watson statistic = 1,09699
Lag 1 residual autocorrelation = 0,393251

Residual Analysis

	Estimation	Validation
n	12	
MSE	1,45349	
MAE	0,962955	
MAPE	48,8989	
ME	-0,170414	
MPE	-37,6612	

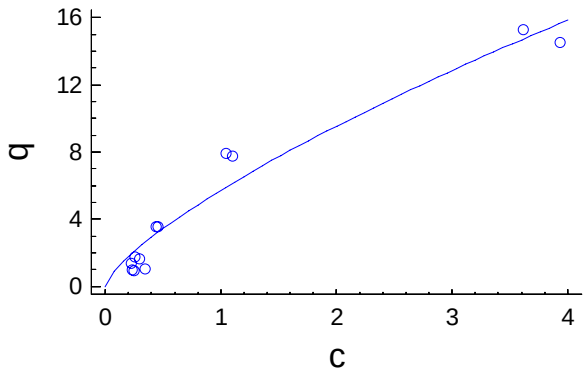
The StatAdvisor

The output shows the results of fitting a nonlinear regression model to describe the relationship between q and 1 independent variables. The equation of the fitted model is 5,72684*c^0,734722

In performing the fit, the estimation process terminated successfully after 12 iterations, at which point the estimated coefficients appeared to converge to the current estimates.

The R-Squared statistic indicates that the model as fitted explains 95,1501% of the variability in q. The adjusted R-Squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 94,6652%. The standard error of the estimate shows the standard deviation of the residuals to be 1,20561.This value can be used to construct prediction limits for new observations by selecting the Forecasts option from the text menu. The mean absolute error (MAE) of 0,962955 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file.

The output also shows aymptotic 95,0% confidence intervals for each of the unknown parameters. These intervals are approximate and most accurate for large sample sizes. You can determine whether or not an estimate is statistically significant by examining each interval to see whether it contains the value 0.0. Intervals covering 0.0 correspond to coefficients hich may well be removed form the model without hurting the fit substantially.



Langmuir adsorption isotherm

Nonlinear Regression

Dependent variable: q
Independent variables: c

Function to be estimated: a*c/(1+b*c)
Initial parameter estimates:
  a = 10,0
  b = 0,1

Estimation method: Marquardt
Estimation stopped due to convergence of residual sum of squares.
Number of iterations: 5
Number of function calls: 17

Estimation Results

Parameter	Estimate	Asymptotic Standard Error	Asymptotic 95,0% Confidence Interval Lower	Upper
a	8,26448	0,98967	6,05935	10,4696
b	0,285766	0,073959	0,120975	0,450558

Analysis of Variance

Source	Sum of Squares	Df	Mean Square
Model	591,631	2	295,816
Residual	8,42683	10	0,842683
Total	600,058	12	
Total (Corr.)	299,698	11	

R-Squared = 97,1882 percent
R-Squared (adjusted for d.f.) = 96,907 percent
Standard Error of Est. = 0,917978
Mean absolute error = 0,733281
Durbin-Watson statistic = 0,988793
Lag 1 residual autocorrelation = 0,421284

Residual Analysis

	Estimation	Validation
n	12	
MSE	0,842683	
MAE	0,733281	
MAPE	41,7586	
ME	-0,226773	
MPE	-34,8023	

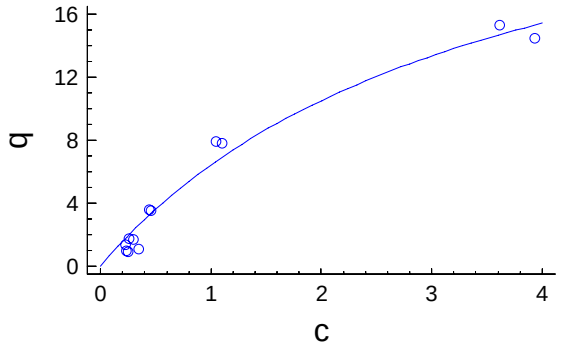
The StatAdvisor

The output shows the results of fitting a nonlinear regression model to describe the relationship between q and 1 independent variables. The equation of the fitted model is 8,26448*c/(1+0,285766*c)

In performing the fit, the estimation process terminated successfully after 5 iterations, at which point the estimated coefficients appeared to converge to the current estimates.

The R-Squared statistic indicates that the model as fitted explains 97,1882% of the variability in q. The adjusted R-Squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 96,907%. The standard error of the estimate shows the standard deviation of the residuals to be 0,917978. This value can be used to construct prediction limits for new observations by selecting the Forecasts option from the text menu. The mean absolute error (MAE) of 0,733281 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file.

The output also shows aymptotic 95,0% confidence intervals for each of the unknown parameters. These intervals are approximate and most accurate for large sample sizes. You can determine whether or not an estimate is statistically significant by examining each interval to see whether it contains the value 0.0. Intervals covering 0.0 correspond to coefficients which may well be removed form the model without hurting the fit substantially.



Linear Freundlich adsorption isotherm

Multiple Regression Analysis

Dependent variable: LOG(q)

Parameter	Estimate	Standard Error	T Statistic	P-Value
CONSTANT	1,64277	0,11979	13,7138	0,0000
LOG(c)	0,972947	0,106309	9,1521	0,0000

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	10,9367	1	10,9367	83,76	0,0000
Residual	1,3057	10	0,13057		
Total (Corr.)	12,2424	11			

R-squared = 89,3346 percent
R-squared (adjusted for d.f.) = 88,268 percent
Standard Error of Est. = 0,361345
Mean absolute error = 0,294379
Durbin-Watson statistic = 0,75746 (P=0,0016)
Lag 1 residual autocorrelation = 0,547603

The StatAdvisor

The output shows the results of fitting a multiple linear regression model to describe the relationship between LOG(q) and 1 independent variables. The equation of the fitted model is LOG(q) = 1,64277 + 0,972947*LOG(c)

Since the P-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level.

The R-Squared statistic indicates that the model as fitted explains 89,3346% of the variability in LOG(q). The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 88,268%. The standard error of the estimate shows the standard deviation of the residuals to be 0,361345. This value can be used to construct prediction limits for new observations by selecting the Reports option from the text menu. The mean absolute error (MAE) of 0,294379 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the P-value is less than 0.05, there is an indication of possible serial correlation. Plot the residuals versus row order to see if there is any pattern which can be seen.

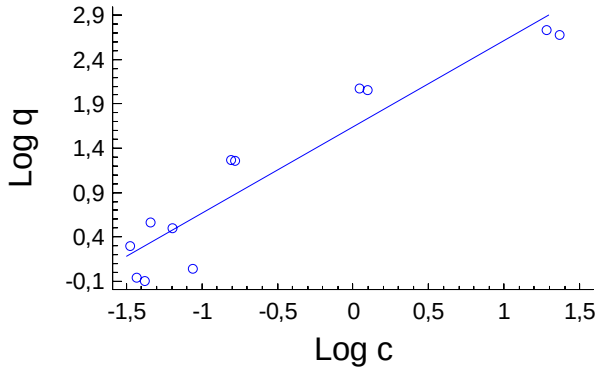
In determining whether the model can be simplified, notice that the highest P-value on the independent variables is 0,0000, belonging to LOG(c). Since the P-value is less than 0.01, the highest order term is statistically significant at the 99% confidence level. Consequently, you probably don't want to remove any variables from the model.

95,0% confidence intervals for coefficient estimates

Parameter	Estimate	Standard Error	Lower Limit	Upper Limit
CONSTANT	1,64277	0,11979	1,37586	1,90967
LOG(c)	0,972947	0,106309	0,736076	1,20982

The StatAdvisor

This table shows 95,0% confidence intervals for the coefficients in the model. Confidence intervals show how precisely the coefficients can be estimated given the amount of available data and the noise which is present.



Appendix 3: Calculations

Daniels equation of Q Sepharose Fast Flow gel

$$Log\left(\frac{c}{c_0}\right) = k \cdot t + k' \cdot \sqrt{t}$$

$$Log\left(\frac{c}{c_0}\right) = 0.00182742 \cdot t - 0.10052 \cdot \sqrt{t}$$

Confidence Interval:

$$\text{k limits} = \frac{0.00219897 - 0.00145587}{2} = \pm 3.72 \times 10^{-4}\ s^{-1}$$

$$\text{k' limits} = \frac{0.112583 - 0.0884564}{2} = \pm 1.21 \times 10^{-2}\ s^{-0.5}$$

c = equilibrium concentration of solute in the mobile phase

c_0 = start concentration of solute in the mobile phase

k = coefficient

k'= coefficient

t = time

Verifying of demanding coefficient

$$0.00182742 \cdot 3025 - 0.10052 \cdot \sqrt{3025} = -0.0006$$

Langmuir Semi reciprocal equation of Q Sepharose Fast Flow gel

$$\frac{c^*}{q^*} = \frac{1}{q_{\max}} \cdot c^* + \frac{K_d}{q_{\max}}$$

$$\frac{c^*}{q^*} = 0{,}0243091 \cdot c^* + 0{,}00777017$$

$$q_{\max} = \frac{1}{0{,}0243091} = 41{,}137\ mg/ml$$

$$K_d = 0{,}00777017 \cdot 41{,}137 = 0{,}320\ mg/ml$$

c* = equilibrium concentration of solute in the mobile phase

q* = equilibrium concentration of solute in the stationary phase

q_{max} = total concentration of adsorbent sites

K_d = dissociation constant

Confidence interval:

$$\frac{1}{q_{\max}}\ \text{limits} = \frac{0.0277221 - 0.0208961}{2} = \pm\ 0.003\ ml/mg$$

$$\frac{K_d}{q_{\max}}\ \text{limits} \frac{0.00935482 - 0.00618552}{2} = \pm\ 0.002$$

Daniels equation of Chelating Sepharose Fast Flow gel

$$Log\left(\frac{c}{c_0}\right) = k \cdot t + k' \cdot \sqrt{t}$$

$$Log\left(\frac{c}{c_0}\right) = 0.000347272 \cdot t - 0.0349545 \cdot \sqrt{t}$$

Confidence Interval:

$$\text{k limits} = \frac{0.000392028 - 0.000302516}{2} = \pm\, 4.48 \times 10^{-5}\ s^{-1}$$

$$\text{k' limits} = \frac{0.0372169 - 0.0326921}{2} = \pm\, 2.26 \times 10^{-3}\ s^{-0.5}$$

Verifying of demanding coefficient

$$0.000347272 \cdot 10130 - 0.0349545 \cdot \sqrt{10130} = -0.0002$$

Freundlich linear equation of Chelating Sepharose Fast Flow gel

$$Log\ q^* = \frac{1}{n} \cdot Log\ c^* + Log\ K_a$$

$$Log\ q^* = 0.972947 \cdot Log\ c^* + 1.64277$$

$$K_a = e^{1.64277} = 5.169469$$

$$K_d = \frac{1}{K_a} = \frac{1}{5.169469} = 0.1934435 \approx 0.193$$

$$n = \frac{1}{0.972947} = 1.027805 \approx 0.103$$

Confidence Interval

$$\frac{1}{n}\ \text{limits} = \frac{1.20982 - 0.736076}{2} = \pm\, 0.236872 \approx 0.237$$

$$Log\ K_a\ \text{limits} = \frac{1.90967 - 1.37586}{2} = \pm\, 0.266905 \approx 0.267$$

Langmuir double reciprocal of Chelating Sepharose Fast Flow gel

$$\frac{1}{q^*} = \frac{K_d}{q_{\max}} \cdot \frac{1}{c^*} + \frac{1}{q_{\max}}$$

$$\frac{1}{q^*} = 0.226712 \cdot \frac{1}{c^*} - 0.0513584$$

$$q_{\max} = -\frac{1}{0.0513584} = -19.471\ mg/ml$$

$$K_d = 0.226712 \cdot -19.471 = -4.414,\ mg/ml$$

c* = equilibrium concentration of solute in the mobile phase

q* = equilibrium concentration of solute in the stationary phase

q_{max} = total concentration of adsorbent sites

K_d = dissociation constant

Confidence interval:

$$\frac{1}{q_{\max}}\ \text{limits} = \frac{0.194992 - (-0297709).}{2} = \pm 0.246\ ml/mg$$

$$\frac{K_d}{q_{\max}}\ \text{limits} \frac{0.313141 - 0.140283}{2} = \pm 0.086$$

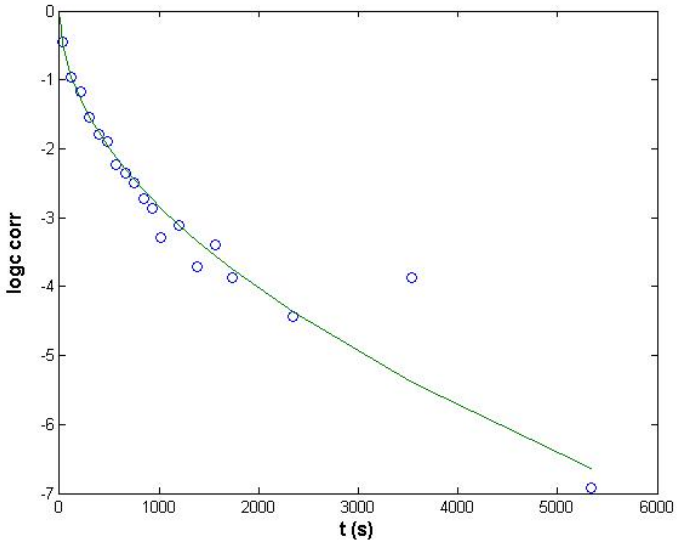
Appendix 4: Matlab Calculations

Chelating Sepharose Fast Flow gel

The data input and the results displayed in Matlab. The matrix t represents the time and the matrix c represents the ratio c/co. The data for the matrix is the experimental data from the kinetic adsorption experiment of Chelating Sepharose Fast Flow gel.

```
>> t=[0 30 120 210 300 390 480 570 660 750 840 930 1020 1200 1380 1560 1740 2340 3540
5340]';

>> c=[1 0.798 0.657 0.617 0.564 0.537 0.528 0.504 0.497 0.490 0.481 0.476 0.465 0.469
0.458 0.463 0.456 0.451 0.456 0.445]';

>> plot(t,c)

>> logc=log(c/c(1));

>> plot(t,logc,'o')

>> logc_corr=log((c-0.44445)/(c(1)-0.44445))

logc_corr =

         0

   -0.4519

   -0.9608

   -1.1693

   -1.5362

   -1.7922

   -1.8945

   -2.2331

   -2.3582

   -2.5011

   -2.7213

   -2.8684

   -3.2971

   -3.1192

   -3.7136
```

```
   -3.3995
   -3.8733
   -4.4405
   -3.8733
   -6.9178
>> p=polyfit(log(t(2:20)),log(-logc_corr(2:20)),1)
p =
    0.5078   -2.4664
>> alpha=p(1)
alpha =
    0.5078
>> k=-exp(p(2))
k =
   -0.0849
>> plot(t,logc_corr,'o',t,k*t.^alpha)
```



Q Sepharose Fast Flow gel

The data input and the results displayed in Matlab. The matrix t2 represents the time and the matrix c2 represents the ratio c/co. The data for the matrix is the experimental data from the kinetic adsorption experiment of Q Sepharose Fast Flow gel.

```
>> t2=[0 30 120 210 300 390 480 570 660 750 930 1110 1290 1470 1650]';

>> c2=[1 0.539 0.308 0.299 0.282 0.285 0.264 0.285 0.292 0.287 0.289 0.282 0.293 0.291
0.290]';

>> plot(t2,c2)

>> logc2=log(c2/c2(1));

>> plot(t2,logc2,'o')

>> logc2_corr=log((c2-0.263)/(c2(1)-0.263))

logc2_corr =

         0

   -0.9822

   -2.7959

   -3.0191

   -3.6581

   -3.5115

   -6.6026

   -3.5115

   -3.2353

   -3.4245

   -3.3445

   -3.6581

   -3.2014

   -3.2704
```

```
   -3.3068
>> p=polyfit(log(t2(2:15)),log(-logc2_corr(2:15)),1)
p =
    0.2335   -0.2816
>> alpha=p(1)
alpha =
    0.2335
>> k=-exp(p(2))
k =
   -0.7546
>> plot(t2,logc2_corr,'o',t2,k*t2.^alpha)
```