Optimisation and scale-up of preparative ion-exchange chromatography

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Abstract
Protein mixtures are sometimes separated using preparative ion-exchange chromatography. Preparative chromatography is optimised to give high yield, purity and productivity. Often the process is optimised in a small scale and then the optimum point is scaled-up to production scale. Different reasons may require the process to be scaled-up both axially and radially in one step, and then a method must be used which can predict the separation and productivity in a simple way. The aim of this thesis is to optimise the separation of insulin from BSA on a strong anion-exchange column and then find a simple method for scale-up of the separation.

The result of experiments with columns with different dimensions shows that an increased width not influences the separation but a larger length gives a better separation even if the gradient is compensated for increased length. An important result from the optimisation, which was performed in small scale, is that more protein than expected could be separated. The conclusion is that a self-displacement effect probably is an important mechanism of the separation. The gel used in all experiments is Sepharose Q FF.

Introduction
The thesis was performed at the Department of Chemical Engineering where research associated to preparative chromatography is conducted. The results from the optimisation in small scale were used to calibrate models in this research. The optimisation was made towards high yield and productivity and adjusting the gradient length controls the separation in the large-scale experiments.

Chromatography is a separation technique commonly used to purify or analyse complex mixtures. One important difference between preparative and analytical chromatography is that the loading factor is much larger in preparative chromatography, the sample can be about 10-20 % of the column volume [1]. Other differences is that it is optimised towards high yield instead of high selectivity or resolution and that the same additives sometimes cannot be used in the mobile phase because it can be difficult to get rid of [2].

Theory
Many purification methods are optimised on low-volume columns. Preparative chromatography is optimised to give high yield and productivity with the desired purity of the product. During the optimisation often some separations are fractionated to be able to calculate purity in each point in the chromatogram.

After the optimisation it is important to be able to transfer the method to larger scale with the same quality of the product. This is not always trivial since column properties and therefore the separation conditions like dimensions and mobile phase distribution always changes when a larger column is used. A good approach to reach a method for scale up is to change the gradient time. Yamamoto proposed the following formula for linear gradients with the same concentration difference and stationary phases with the same porosity:

\[
t_{g,2} = t_{g,1} \left( \frac{V_{v,2}}{V_{v,1}} \right) \left( \frac{F_1}{F_2} \right) \left( \frac{L_1}{L_2} \right)
\]

\(V_v\) is void volume, \(F\) is volume flow and \(L\) is length of respective column. [3, 5]

Materials and methods
The proteins used are insulin and BSA [4] and the optimisation is made to give high yield and
productivity of insulin, which is eluted at the end of every separation.

The optimisation was performed on a bed with 9 mm diameter and 34 mm height and the instrument was an ÄKT A from Amersham Pharmacia biotech. The gel used was Sepharose Q FF, a strong anion exchanger. The pH chosen for gradient and loading optimisation was 9.0. 20 mg of respective protein was loaded for 0-1 M NaCl gradients between 5 and 40 column volumes (CV). The flow was 1 ml/min and the buffer was 20 mM Tris-HCl.

For the loading optimisation 30 CV gradients and twice as much BSA as insulin was used. Between 0.25 and 60 mg insulin was loaded on the column in volumes between 0.1 and 12 ml. The larger volumes were loaded with superloops from Pharmacia biotech.

A gradient optimisation with high loading, 60 mg insulin and 120 mg BSA was also performed to find out how much longer gradient was needed when there is more protein.

Experiments with 0.3 and 0.6 ml/min flow were also accomplished. The loading was then 10 mg insulin and 20 mg BSA.

One of the separations was fractionated and the fractions was analyzed with a gel filtration column and 10 mM Tris-HCl pH 7.3, 0.5 M NaCl buffer. The purity, yield and productivity could then be calculated. The purity 97 % insulin was chosen and the result was transferred to the other successful separations by visual estimation.

Another bed with equal dimensions was made to evaluate the reproducibility of the packing procedure.

For the scale up experiments first a bed with equal height but 50 mm diameter was made and then a bed with 9 mm diameter and 6.6 cm height was made. The same amount of protein per ml of bed was used as in the experiment, which was fractionated during the optimisation. Also the scale up experiments were fractionated and analyzed with gel filtration.

**Results**

The gradient optimisations were predictable, the separation was faster with a shorter gradient and a longer gradient was required with higher loading. For low loading 10 CV was chosen while 20 CV gradient was chosen for high loading.

The loading optimisation was performed to give high productivity × yield at 97 % purity. The result was that the highest loading 60 mg insulin and 120 mg BSA was to be used. That is 180 mg total protein, which is surprisingly high for a bed with the volume 2.16 ml. It is 83 mg protein per ml bed, and the separation is as good as the other ones with low load. The separation is much better at medium and high loading than at low loading, see figures 1 and 2. Notice the much higher absorbance at the high load, which means that the productivity is much larger. The result means that there is probably a self-displacement effect which means that the insulin ions forces the BSA ions to leave the column as soon as there are enough free insulin ions [4].

![Figure 1](image1.png)  
**Figure 1. Description of the separation at low loading.**

![Figure 2](image2.png)  
**Figure 2. Description of the separation at high loading.**
The results from the experiments where the flow was varied shows that lower flow than 1 ml/min gives better separation and yield, but it gives so much poorer productivity and therefore low productivity $\times$ yield. The most important reason for the low productivity is the regeneration and equilibration time with the buffer on the column. [4]

Two experiments with equal parameters were performed on two different beds with the same dimensions, and the result is shown in figure 3. The result is good enough, the differences in the figure is largely due to difference in the amount of protein loaded. Integration showed that in one of these experiments the amount of loaded protein was 16 % less.

![Figure 3](image)

**Figure 3. Description of the reproducibility of the bed.**

The experiment in which the diameter of the bed was increased and other parameters were constant verified that the separation is not influenced by a larger diameter. Only small differences appeared, and they cannot be considered significant. The results are compared in figure 4 below. [4]

![Figure 4](image)

**Figure 4. Description of an experiment with larger bed width.**

One of the experiments was performed on a bed with increased height, 6.6 cm. The results are shown in figures 5 below.

![Figure 5](image)

**Figure 5. Description of an experiment with larger bed height. The gradient was compensated for increased height.**

The difference between the two experiments is small; the separation was only slightly better with the higher bed. [4]

**Conclusions**

The most important conclusion from the optimisation was that a surprisingly large amount protein could be separated. The interpretation is
that the proteins affect each other at the high concentration so that a self-displacement effect appears. Another conclusion was that a slightly flatter gradient is required with a larger loading.

The conclusion from the experiments with greater width was that the most important principles described works well for the system used, which is not a surprise.

From the experiment with larger height the expected conclusion was received that the separation is not much changed. It is only slightly better with a gradient compensated for the different column dimensions [4].

References

2. Schmidt-Traub (Ed.) (2005), Preparative Chromatography


4. A. Nilsson (2006), Optimering och uppskalning av preparativ jonbyteskromatografi