To my parents, for getting me this far.
Expanded bed adsorption: a study of bed behaviour during the recovery of a typical bioproduct

A thesis submitted to the University of London for the degree of DOCTOR OF PHILOSOPHY by Nicholas Allen Willoughby B.Eng. (Hons.)

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Abstract

The majority of work in this Thesis will investigate the use of expanded beds for different separations in the field of Biotechnology and consider how best to utilise expanded beds most efficiently and effectively. The aims of the project were to develop a novel affinity purification from initial method scouting to pilot plant expanded bed level, and then to consider and develop a more effective and faster method of monitoring and control of and expanded bed than traditionally used off-line manual techniques.

A protocol was successfully developed for the purification of yeast alcohol dehydrogenase (ADH) from unclarified homogenate using immobilised metal ion affinity chromatography in a STREAMLINE™ expanded bed adsorption system, demonstrating high levels of recovery of the target protein.

A novel system of breakthrough monitoring and control was developed using a combination of in-bed sampling and a rapid stopped flow analysis system. This enabled accurate control of product breakthrough at different levels when purifying ADH from crude homogenate feedstock. In addition this monitoring technique demonstrated variation in matrix utilisation with axial position, providing scope for the final section of work noted here. In-bed sampling also allowed the physical properties of the matrix to be studied and experimental radial and axial particle size distributions during expansion were obtained, along with the development of a method for measuring voidage in short sections of the bed under expansion.

Finally, matrix utilisation was studied using segregated beds of varying particle size ranges in order to establish dynamic binding capacities and hence matrix productivity. Determination of dynamic capacities at different breakthrough levels for beds consisting of smallest and largest particles showed that, while smaller particles have more than double the total binding capacity of larger particles, at low breakthrough levels the larger particles have a higher dynamic binding capacity and hence a higher productivity.
Acknowledgements

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Introduction

This short section outlines the objectives of the work undertaken for this thesis, describing the initial aims of the work and how these aims link the results Chapters together. As will become apparent, the scope of this thesis changed somewhat with time, reflecting the insight gained from earlier studies. The thesis is arranged to show the progression of this work.

This thesis had the following initial objectives:

- The development of a new chromatographic protocol suitable for control and monitoring studies, and the transfer of this protocol to an expanded bed adsorption system
- The development of a fast monitoring system for improved control and utilisation of expanded bed matrices
- Demonstration of this fast monitoring system using realistic feedstocks

The degree to which these objectives have been met will be discussed in the final conclusions Chapter, Chapter 7
Chapter 1 General Principles of Chromatography and Expanded Bed Adsorption

The following Chapter will give an overview of chromatographic principles and the development and operation of expanded bed adsorption (EBA) columns.

1.1 Introduction

Chromatography describes many different processes in both the chemical and biological fields. The broadest definition would be to say that chromatography is the separation of a substance (or substances) from a mobile phase by attachment onto a stationary phase. Separation of coloured dyes on filter paper by speed of travel, is perhaps the simplest form of chromatography, and is known as one-dimensional chromatography. It is this principle that is still used for purification of proteins in biotechnology. The addition of a second “dimension” results in the mobile phase passing through a stationary column of adsorbent, where the cross-section of the column represents the second dimension. Most chemical and biological chromatographic purifications utilise a column of adsorbent or matrix of some kind and so are classed as two-dimensional chromatography. Two-dimensional chromatography has the major advantage of being able to handle large quantities of mobile phase.

Chromatography is widely used as a late or final stage in the purification of fine chemicals or biological products since as a process step it can achieve very high
degrees of purity of final product. In addition chromatography does not place the substances involved under any high shear forces or create problems with heat generation or dissipation and as such can be considered a gentle process step.

1.2 Basic chromatography theory

Products and contaminants are separated as solutes in the mobile phase according to their interaction with the stationary phase (the matrix). If they have no affinity to the matrix, i.e. remain partitioned in the solvent of the sample, then they will pass straight through the column with no retardation. If they have a high affinity for the matrix they may totally adsorb onto it, and be impossible to remove without causing damage to the solute. These two scenarios are of little practicable use, except if the solute in question is an impurity in which case the first situation may be desirable. There are two further possibilities; firstly the situation where the substance has an affinity for the matrix so mild that it is simply slowed down in its passage down the column, thus delaying its elution long enough for totally non-binding impurities to be washed off. This is the basis of some forms of chromatography such gel filtration. In the cases of the fields being studied here however, the situation of interest is that where the solute binds to the matrix such that it is effectively stationary while the solvent used for loading is flowing through, but where conditions can be easily modified to elute the target solute when so desired.
1.3 Basic Principles

The following sections will give an overview of the basic requirements of a chromatographic stationary phase and describe the principles of affinity chromatography in general. Immobilised metal-ion affinity chromatography and hydrophobic interaction chromatography, the two types of chromatography presented in this thesis, will be described in Chapters 2 and 3 respectively.

1.3.1 The Matrix (adsorbent or stationary phase)

Almost all matrices used nowadays for fixed bed chromatography are porous structures. In operation the pores are filled with solvent. The matrix is usually composed of hydrophilic polymer, and the solvent is usually an aqueous buffer system. In addition to this, certain other properties are desirable in the matrix. As well as hydrophilicity, an absence of groups to which proteins can spontaneously bind is desirable. A degree of physical and chemical stability is also required to cope with certain processes such as sanitisation (often 1M or stronger NaOH) and regeneration and repacking of columns after loss of activity. Other properties depend specifically on the process under consideration (see later).

Final important considerations include ensuring that the matrix material is porous enough to allow proteins to penetrate to an internal network of pores, thus ensuring maximum surface area for adsorption with minimum volume of matrix.
1.3.2 Affinity chromatography

Affinity chromatography is unique within the field of separation technology in that it is the only separation method that utilises the biological function of the target molecule by causing the molecule to bind to a complementary ligand on the surface of the chromatography matrix. Because of this use of biological function, the specificity of these separations is very high allowing separations from complex broths, and purifications of the order of several thousand fold are common.

Affinity chromatography was first recognised as a biological purification tool (Axen et al, 1967) to bind primary amino groups to polysaccharide matrices activated by cyanogen bromide. Cyanogen bromide activation is now the most generally used method to attach ligands to matrices in affinity chromatography.

Nowadays affinity chromatography is the method of choice for the purification of monoclonal antibodies and antigens (the specific antibody - antigen interaction makes affinity chromatography ideal for the purification of either), DNA-binding proteins and receptors. It is also a effective method of purification for enzymes, nucleic acids and even whole cells. A list of binding substrates and respective ligands are shown on the next page:
Enzyme - Substrate analogue, inhibitor, cofactor
Antibody - Antigen, virus, cell
Nucleic acid - Complementary base sequence, histones, nucleic acid polymerase, binding protein
Hormone, vitamin - Receptor, carrier protein
Cell - Lectin, cell surface specific protein.

The term “affinity chromatography” has been taken to mean different things by different authors. In the extreme it is taken to mean all adsorption chromatography methods bar ion exchange. However for the purposes of this introduction it will be taken to include ligands based on specific functional pairs only. In the extreme, affinity chromatography is taken to refer to separations based on biological affinity only. Within the context of the work presented here, specific chemical interactions are considered “affinity” steps. (note - there exists a separate category of biomimetic ligands such as dyes which bind to the active sites of functional enzymes, but cannot be classes as biologically active, which nevertheless are usually included in the above “biologically functional ligands” group).

Since affinity chromatography relies on function properties, active and inactive forms of the target can be separated. This also makes affinity chromatography a very powerful purification tool. Within the context of this project, the specific area of interest will be the use of immobilised metal ion affinity chromatography (IMAC) to separate a target enzyme from a crude broth. IMAC is a form of chromatography relying on separating target proteins on a column containing
immobilised transition metal ions. Proteins are separated on the basis of electron density and this is determined by the amino acid arrangement coupled with the tertiary and quaternary protein structure. Although IMAC may not distinguish between active and inactive molecule it can separate native and unfolded protein and separates by biological differences, so for the purposes of this work is defined as an affinity separation. This will be described and discussed further in detail in Chapter 2.

1.4 Chromatographic Techniques

A typical chromatography process consists of four steps - loading (adsorption), washing, elution and column regeneration. These steps are similar in almost all forms of chromatography, but with variations depending on specific processes. A general description of these processes is given below, but more specific details depend on the exact process.

1.4.1 Sample Preparation

For efficient adsorption the physiological conditions (pH, ionic strength) of the sample and the column must allow effective binding. Ideally the conditions should be optimal for the interaction between the substrate and the ligand. This can be achieved by various methods varying from simple pH alteration through desalting and chemical additions to complex modifications such as dialysis and buffer exchange. Although other primary recovery operations may well be necessary in order to clarify samples destined for fixed bed chromatography it is hoped that these will not be necessary when the sample is introduced to an
expanded bed, with the possible exception of initial treatments to release intracellular products.

1.4.2 Adsorption of sample

The sample is usually loaded onto a pre-prepared column (the column will have been equilibrated in buffer prior to the load step commencing), the length of load period depending on the specific conditions required. In some cases samples may be loaded until a specific percentage breakthrough of target substance is achieved, in other cases simply a fixed volume may be loaded. When the load step is complete the column is washed.

1.4.3 Washing

This step is carried out to remove any unbound or weakly bound contaminants from the column. Equilibration buffer is run through the column until all waste material is removed from the system.

1.4.4 Elution

After washing the required products and any remaining contaminants are removed from the column. This step is known as elution. In the event of other contaminants being still present on the column then the elution is carried out in such a way as to ensure the product is contaminant free (i.e. multiple step elutions). The method of elution varies depending on the method of
chromatography but buffer changes, pH changes and salt gradients are common methods.

1.4.5 Column Regeneration

After elution the column must be cleaned, usually with NaOH solution, although less robust matrices are often cleaned with specific reagents (see later sections). If the column is to be immediately reused then it is usually regenerated by washing with equilibration buffer specific to the type of chromatography being carried out. Otherwise cleaning buffers are washed out of the system with water and the column stored in 20% ethanol or sodium azide solution to prevent bacterial growth.

All of the above steps are usually carried out with liquid flow in a downward direction, at a flowrate appropriate to the scale of the system and the separation being carried out. Often the elution is carried out at a lower flowrate than the other steps since this minimises peak width.
1.5 Expanded Bed Adsorption - General process theory

Expanded bed adsorption is a single pass operation in which products (usually proteins) are recovered from a crude feedstock. In this respect the operation seeks to combine the specificity of separation and overall volume reduction of chromatography with the clarification efficiency of a centrifugation and filtration system into one discrete step. This would be hoped to reduce the need for costly and time-consuming prior clarification steps such as centrifugation and filtration. Typical required stages in protein purification are illustrated in Figure 1.1 below.
Expanded bed adsorption is intended to replace all stages within the capture section of the process.

Expanded bed adsorption is suitable for the recovery of both extracellular and intracellular products; the former by direct capture from fermenter or cell culture broth and the latter after homogenisation or cell lysis. As an effective operation it “came of age” in 1992 with the introduction of the STREAMLINE process.
range of expanded beds and matrices produced by Pharmacia Biotech AB, Uppsala, Sweden. The following text seeks to introduce and detail the key aspects of expanded bed operation.

1.5.1 Fluidised Bed Adsorption

The problem to which expanded bed operation became a solution was the requirement to combine three key aspects of the capture stage of a process – clarification, concentration and product stabilisation. Although filtration and centrifugation systems can achieve the first two objectives to varying degrees, some form of more specific separation is required to remove such things as proteases. If these are not removed then the product could be at risk and time constraints are enforced between the capture and later purification steps.

Initial attempts to combine both crude separation techniques and some form of chromatographic separation were hampered by the approaches adopted. Overly complex methods relied on keeping the adsorbent particles stationary at all times - in effect a packed bed but with greater voidage. In order to achieve this a good deal of work [Chetty and Burns 1991; Terranova et al 1991] was carried out considering stabilising ferromagnetic adsorbent beads in an external magnetic field. This method has had a good deal of success with virtually no mixing or movement of the particles and thus plug flow is achievable. However the drawbacks are great - the need for expensive and heavy-duty magnetic equipment coupled with the problem of heat generation, especially in systems which require the temperature to be kept low to prevent biodegradation.
Dreager and Chase (1991) looked at the possibility of using conventional packed bed matrices expanded in an column. Although they found that the matrix could be expanded to 2-3 times its settled height, this was achieved at very low flowrates thus limiting the possible applications to systems requiring very low throughput. In addition this can cause serious problems because the uniform density of the matrix beads causes inherent instability in the bed, resulting in turbulent flow and the accompanying eddies, backflow and dead spaces. Since plug flow through a packed bed maximises the number of theoretical plates in the system (N), then an expanded bed exhibiting turbulent flow will have an inferior adsorption performance to an equivalent packed bed exhibiting plug flow. This greatly increases the amount of time needed to perform the separation and also the amount of debris retained by the column, often resulting in fouling of the matrix or the column. Although Chase (1994) has shown that when a bed is expanded where the solid-liquid density difference is minimal there is visually little mixing and the bed retains a uniform character during expansion, stability and complete plug flow would be unlikely. In addition a minimal density difference between the two phases precludes the use of high flowrates, or feedstocks of high viscosity, as the bed expands proportionally more under these situations.

Dreager and Chase (1991) also considered the adsorption characteristics of both Q-Sepharose (strong anion exchanger) for BSA solutions and Protein A Sepharose CL-4B (affinity) for human immunoglobulin G. They noted that, while solutions of pure protein were used the breakthrough characteristics were
similar to that of the matrix in packed bed mode, when cells or cell debris were introduced into the sample the adsorption performance greatly deteriorated. In addition the increase in viscosity of the sample caused over-expansion of the bed due to minimal density difference between the solid and liquid phases.

Consequently, in order for the true potential of expanded bed adsorption as a downstream processing operation to be realised, a method was needed to stabilise the bed under adsorption and ensure that the flow through the bed remained as close as possible to plug flow. Several methods have been suggested to aid this. The inclusion of baffles around the side of the column [Buijs et al. 1980] is said to stabilise the bed when used in conjunction with standard packed bed matrices, but this does not solve the problem of small density differences between the solid and liquid phases, and so flowrates used were still minimal.

The mathematical descriptions regarding bed fluidization are not referred to in this thesis however, to present a complete picture, are included as an appendix for reference.

1.5.2 STREAMLINE expanded bed matrices

The most simple and effective of the methods tried to stabilise an expanded bed is that use by Pharmacia in their STREAMLINE columns. By grading the adsorbent beads with a distribution of size or density, the system is most effective - the density and size gradient means that upon expansion the bed is stable as individual beads adopt a stable height in the bed based upon their
density, and the heavy degree of matrix mixing observed in previous attempts is all but eradicated. To achieve the necessary particle gradients the beads are quartz-cored, thus giving a higher average density to the matrix than would normally be observed with fixed bed chromatography media (see table 1.1 below). This has the additional benefit of allowing the system to support either higher flowrates or higher feedstock viscosities, as an increase in either of these tends to cause the bed to expand further but an increase in density of the matrix would result in a drop in expanded height.

Table 1.1 – Illustration of densities and particle sizes for various types of STREAMLINE expanded bed adsorption matrices (Amersham Pharmacia Biotech, 1997)

<table>
<thead>
<tr>
<th>Base Matrix</th>
<th>Ligand</th>
<th>Mean Density (kg/m^3)</th>
<th>Particle Size Distribution (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose-quartz DEAE</td>
<td>1.15</td>
<td>100-300</td>
<td></td>
</tr>
<tr>
<td>Agarose-quartz SP</td>
<td>1.15</td>
<td>100-300</td>
<td></td>
</tr>
<tr>
<td>Agarose-quartz IDA</td>
<td>1.15</td>
<td>100-300</td>
<td></td>
</tr>
<tr>
<td>Agarose-quartz Heparin</td>
<td>1.15</td>
<td>100-300</td>
<td></td>
</tr>
<tr>
<td>Agarose-metal Protein A</td>
<td>1.3</td>
<td>80-165</td>
<td></td>
</tr>
</tbody>
</table>

The physical properties under expansion of both of the ion exchange matrices listed above have been reviewed by Chang and Chase (1993 et al, 1994) when compared to equivalent Sepharose Fast Flow matrices. They found that, under conditions of equal bed expansion, the flowrate in the STREAMLINE matrix was considerably higher.
1.5.4 STREAMLINE 25 Expanded Bed

Experiments carried out for this thesis were conducted using STREAMLINE 25 and STREAMLINE 50 columns supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). These are two of the smallest of the presently available expanded beds the company produce, and are intended for use in process development and method optimisation. The two columns have, respectively, 25 and 50 mm internal column diameters but are both 1 m in length.

The extra equipment required to run the STREAMLINE 25 and 50 columns is very similar to that required for standard packed bed chromatography. The system requires pumps, valves, fraction collection and on line spectroscopy / pH measurement / conductivity measurement as required. The STREAMLINE 50 system is shown in exploded view in figure 1.2. Diagrams of the system layouts specific to the different sections of work performed can be found in the later Chapters of this thesis.
Figure 1.2 – An exploded diagram of the principal components of the 0.05m diameter STREAMLINE column (ST-50):

1,14,19,22,25 O-rings; 2 connector; 3 distributor plate; 5 bottom net; 6 stand feet; 7 washer; 8 nut; 9 stand; 10 bottom flange; 11 adaptor plate; 12 adaptor distributor plate; 13 adaptor net; 15 adaptor; 16 rod piston; 17 column tube; 18 gasket; 20 upper flange; 21 rod sealing; 23 lid, 24 domed nut
Note that the upper adaptor in this system is hydraulic and can be raised or lowered by means of a pump feeding to a reservoir above the adapter. This allows for simple alteration of the adapter height, which is important as bed height can vary considerably during a run and the bed is functioning most efficiently when the upper adapter is positioned as close as possible to the top of the bed to minimise dead space. In addition to this, the hydraulic adapter opens up the possibility of eluting bound particles in the reverse direction (i.e. downwards) and therefore eluting the bed in packed mode rather than expanded mode (see later).

In order to ensure uniform expansion, STREAMLINE expanded beds are fitted with special flow adapters underneath the bottom mesh net of the column. These ensure the flow of liquid into the column is distributed evenly across the entire cross-section of the column. In addition the holes in the adapter and the mesh are large enough to allow the passage of particulate matter, even whole cells, but are small enough to confine the matrix within the column in settled or downward elution mode. The design of the adapter is thought to be crucial to the stability of flow through the column. In addition to the STREAMLINE adapter design, the system produced Bioprocessing Limited (Comsett, UK) under the name PROSEP® recently is of interest (see later).
1.5.5 Stability of expanded bed adsorption

An expanded bed that exhibits stable behaviour under fluid flow conditions (such as a STREAMLINE system) can allow certain conclusions to be drawn concerning the hydrodynamic behaviour of the bed under expansion. [de Luca et al. 1994] considered the effect of the design of the liquid distributor in terms of the effect on the bed expansion and on the hydrodynamic properties. (de Luca, 1994) found that the value \( n \) in the Richardson-Zaki equation (Equation 11 in the appendix) depended on the design of the distributor, but also that the height of the bed upon expansion was reduced by having a lower settled bed height. This is not to be expected since the stable position (in terms of height) of any particle in an expanded bed depends on the density and size of the particle and the linear velocity of the fluid in the column but not on its starting height. This lead researchers to look more closely at the particle behaviour and draw the conclusion that the design of distributors was often causing channelling of liquid, especially in beds with low initial height, causing uneven expansion. (de Luca, 1994) stated that, to ensure channelling did not effect the stability of the bed, the distributor should be of a design such that fluid flow is even over the entire cross-section of the bed, and also that the height of the settled bed should be at minimum double the value of the diameter of the column. In general also it was noted that bed stability upon expansion increases with settled bed height, but in addition as settled bed height is increased constraints are placed on the maximum liquid linear velocity in the column and also the use of high viscosity solutions, both for reasons of excessive bed expansion.
1.5.6 STREAMLINE adsorbents available

Pharmacia have widened greatly the varieties of STREAMLINE adsorbents available in the last few years. Initially only low specificity ligands such as ion exchangers and HIC groups were produced, because of concerns relating to the more harsh cleaning and regeneration steps required for processes which handle crude feedstocks. However, these ligands do not possess the high specificity for the separation product which is often required nowadays for maximum purity. Thus several new adsorbents have been developed based on the STREAMLINE system and some of these were used in the course of this project.

1.5.6.1 STREAMLINE chelating fast flow

This adsorbent, to be used for immobilised metal ion affinity chromatography, is based on macroporous cross-linked 6% agarose containing crystalline quartz, with attached iminodiacetic acid groups on spacer arms as the ligand for forming chelates with free metal ions. At the time of preparing this section, the adsorbent is still considered to be a prototype and as such binding capacity data etc. is unknown.

1.5.6.2 STREAMLINE rProtein A

The most recent (at time of writing) of the STREAMLINE matrices, the packed bed derivative of this adsorbent is considered ideal for the purification of monoclonal antibodies and antibody fragments. As such the STREAMLINE
version must be considered for any expanded bed purification of antibody fragments as is being considered in a section of this project. The matrix is based on highly cross-linked 4% agarose around a metal core, with an attached recombinant protein A ligand from *E. coli*, with a ligand density of approximately 6 mg/ml matrix.

According to Pharmacia, the matrix has a specially designed open pore structure and a higher density core then that of other STREAMLINE matrices to allow for improved mass transfer of more bulky molecules. In addition the recombinant protein A has been engineered to include a C-terminal cysteine, with the matrix epoxy chemistry favouring a thioether coupling to provide a single point C-terminus attachment of the recombinant protein A. This orientation of the ligand produces a much more favourable binding site for antibodies.

1.5.7 PROSEP fluidised bed adsorption

PROSEP matrices have been used for some time in conventional chromatography applications. They are derived from a basic porous glass matrix and posses the required attributes of durability, stability of ligand attachment, incompressibility and wide range of available pore size. Fluidisation interest however lies in its high density relative to water (between 1.3 and 1.5) thus ensuring that the, during any expansion, the bed height does not increase greatly at low flowrates thus precluding the use of high viscosity feeds. These properties are very similar to that of the STREAMLINE matrices, but the difference with
the PROSEP system is that, rather than using a dedicated system, it is designed to function using existing chromatography equipment.

The principle is that, rather than manufacturing a dedicated distributor for liquid flow to be placed under the bottom adapter of the column, by removing the adapter net completely and simply replacing it with a layer of solid glass beads good stable liquid flow and distribution can be achieved. The beads have undergone surface derivitisation to prevent non-specific interactions with proteins and other particles, and because the particles are solid glass they are considerably more dense then even the PROSEP matrix. This means that the matrix stays separate from the glass beads, preventing the matrix from leaking out of the bottom of the column and the glass beads ensure even fluid flow across the diameter of the bed and therefore stable bed expansion. The PROSEP matrix is intended for use as an affinity chromatography reagent, with a wide range of possible ligand attachments.

1.5.8 Stages in a typical expanded bed run

This section is not intended to replace the materials and methods sections of later Chapters but rather to provide the reader with an introduction to the general context of the operation. Various stages in the operation of bed are illustrated in figure 1.3
1.5.8.1 Packing

In the initial stages the bed must be prepared and fully expanded and tested before any sample can be loaded onto the adsorbent. Detailed instructions for this can be found in the Pharmacia booklet “STREAMLINE 25 Column - User Manual” and will also be described later in this work in terms of specific experiments. This process will be briefly discussed here in relation to the STREAMLINE 25 column. The bed is intended to be run at a settled bed height of approximately 15cm (Pharmacia recommendations). Hence for a STREAMLINE 25 column this requires approximately 75 ml of settled matrix. The bed must then be slowly expanded up to a linear flowrate of approximately
400 cm hr\(^{-1}\). This allows all the particles to rearrange to their proper position in relation to their size/density and ensures that, once settled again the bed will be stable in future expansions. In addition it is often necessary, especially with prototype adsorbents, to elutriate the material during expansion to remove any excessive fine particles that may be present and that will prevent a sharp boundary on expansion between the bed and the liquid. This procedure is described in detail in the STREAMLINE 25 user manual.

1.5.8.2 Bed Expansion and Equilibration

Initially the bed is expanded in and upwards direction using the equilibration buffer. According to Chase and Chang (1993) and Pharmacia recommendations the bed should be expanded to approximately 2-3 times the settled height. Hjorth (1997) suggests that for most adsorbents and buffers with viscosities in the order of that of water, this expansion can be achieved with a linear liquid flowrate of 300 cm/h.

1.5.8.3 Loading of sample

When the bed is fully expanded at the required liquid flowrate and has stabilised, the liquid being passed through the column is switched to the load sample. Some degree of disagreement about methods of loading occurs at this point. Since the load samples are often of high viscosity relative to the buffers used then the bed expansion in the load step will be higher than in any other step. Consequently the liquid flowrate may need to be altered. Chang and Chase (1996) advocate
keeping the bed height and therefore degree of expansion constant at all times by varying the liquid flowrates and show higher productivity can be achieved using these methods. On the other hand, Barnfield-Frej (1994) suggests that the liquid flowrate should be kept constant allowing the bed expansion to vary according to the viscosity of the liquid being loaded. This has the advantage that the bed height does not need to be monitored constantly, which is difficult when loading opaque liquids such as yeast homogenate or where other than borosilicate glass columns are used.

1.5.8.4 Washing

When the load step is satisfactorily complete (often judged by percentage breakthrough in the output stream of target material) the bed must be washed to remove traces of cell debris and non-binding substances such as contaminating proteins. In general washing is carried out using equilibration buffer for simplicity and ease of preparation. Draeger and Chase (1991) studied the effect of highly viscous wash buffers on volumes of liquid required and effects on elution and found that, while the addition of glycerol to wash buffers to increase viscosity reduced the wash volume required it also appeared to have a detrimental effect on elution.

1.5.8.5 Elution

Elution from an expanded bed is frequently carried out with the bed in a packed configuration, i.e. the bed is allowed to settle after washing and the upper
adapter lowered to the top of the settled bed. Elution is then most commonly carried out in a downward direction although either direction is possible. Elution can be carried out using a step or gradient protocol as with packed chromatography, although scale requirements mean that step elutions are favoured.

1.5.8.6 CIP

CIP (Clean-in-place) procedures for STREAMLINE™ matrices given in literature vary according to the type of matrix, usually due to the varying chemical stabilities of the different matrix functional groups. However most are based around a caustic wash, although strengths of NaOH solutions used tend to vary in the order of 0.1-1M NaOH. In addition some workers advocate an alternating acid/alkali wash (usually acetic acid) for enhanced cleaning, and storage is invariably in 20% ethanol. Specific matrices such as rProtein A are cleaned using agents such as 6M Gu.HCl since the immobilised protein A would be damaged by the action of NaOH.
1.6 Choice of Yeast Alcohol Dehydrogenase as a target

In order to study the characteristics of a purification process, it is necessary to select a target to be used. This choice necessitates a molecule that is easily monitored and sourced from a readily available feedstock, but must demonstrate properties inherent to the challenges provided by a typical biological separation. A generic protein stable at a reasonably wide range of conditions such as temperature and pH (such as albumin) would not present a fair test of a novel purification approach directly from crude feedstock such as that presented in this thesis. However it is also impractical, particularly for reasons of cost, to attempt to purify a true biological or therapeutic target protein, such as a monoclonal antibody. With this in mind an enzyme was chosen that, while not exhibiting all the characteristics of a target such as an antibody, does share some common properties such as rapid degeneration outside the physiological ranges of pH and temperature, with this degeneration measurable by loss of enzymic activity.

The enzyme alcohol dehydrogenase, sourced from bakers' yeast (*Saccharomyces cerevisiae*), has previously been widely used for generation of generic information regarding downstream processing operations such as homogenisation (Hetherington *et al*, 1971) and centrifugation (Clarkson *et al*, 1995) as well as expanded bed adsorption itself (Smith, 1997). The molecule is used in medical and forensic science, primarily for the determination of alcohol concentration, but also for the direct measurement of NAD$^+$ levels.
The enzyme has a molecular weight of 150 kDa, consisting of 4 subunits each with a molecular weight of 36 kDa (Kagi and Vallee, 1960). It also contains 4 tightly bound Zinc ions (Zn$^{2+}$); (Vallee and Hoch, 1955). The molecule suffers an irreversible loss of activity when exposed to pH levels below 6 (Hoch and Vallee, 1956) and under these conditions the molecular weight drops from 150 to 36 kDa. This indicates the Zinc ions are vital for maintaining the integrity of the quaternary structure.

In addition to this information, work (Smith, 1997) has shown that the activity of the enzyme in yeast homogenate reduced by, on average, 50% over a 24 period even when kept on ice at 4°C. This demonstrates the instability of the enzyme generally, making it a good model in terms of these characteristics for unstable high-value therapeutic proteins.
1.7 Conclusions

This Chapter has presented the theory of expanded bed adsorption and chromatographic separation in general. The characteristics of the experimental system to be used in this study have been outlined in terms of the column, the matrix and the biological feedstock. The following Chapter details the development of a novel expanded bed adsorption separation from a crude feedstock that provides the necessary basis for the later studies of bed performance and of bed characteristics.
Chapter 2 - Development of Immobilised Metal Ion Affinity Chromatography for ADH separation

Immobilised metal ion affinity chromatography was used to develop an expanded bed method for the affinity purification of an enzyme, alcohol dehydrogenase (ADH), from bakers' yeast. A small scale packed bed protocol was developed using pure ADH and this was scaled up into larger packed beds using clarified yeast homogenate. Finally this method was transferred into a STREAMLINE 25 expanded bed, where the protocol was successfully performed over three cycles, yielding in excess of 80% product recovery upon elution in all cases.

2.1 Introduction

Immobilised metal-ion affinity chromatography, or IMAC, is an important specific form of affinity chromatography which was applied in this section of the project to enable the purification of alcohol dehydrogenase directly from yeast homogenate using expanded bed technology.

The IMAC process for separation of proteins was first proposed more than 20 years ago under the name 'metal chelate affinity chromatography' [Porath et al. 1975]. The basis for the 'normal' form of IMA-adsorption ('reversed' IMAC can be achieved using metalloproteins) metal ions are immobilised onto the supporting matrix. This event occurs (in the form of chelation) because metal ions in water can be considered Lewis acids due to coordination of water molecules resulting in high solvation of the metal ion. Since the water molecules are acting as Lewis bases in the solution, they can be preferentially be replaced by a stronger electron pair donor such as a Nitrogen, Sulphur or Oxygen atom with a free electron pair. In this instance a metal complex forms and the electron
pair donor is considered to be a ligand. The process is referred to as ligand exchange. When a single ligand donates an electron pair to the complex, this is known as a monodentate ligand but a complex of two or more donor atoms (a polydentate ligand) and a metal ion becomes a metal chelate. In a chelate the binding of the metal ion is much stronger than that of a complex due to the higher level of electron pair interaction.

IMAC relies on the differences in affinities of proteins for metal ions immobilised on to a matrix in the form of chelates. All amino acids will form chelates with metal ions due to amino groups and carboxyl groups acting as further electron pair donors. However certain residues such as histidine, cysteine and tryptophan (Porath's triad [Porath et al. 1975]) have electron donor atoms in their side chains. Therefore these three amino acids should form more stable chelates than others due to the participation of electron donating side chains.

There are relatively few commonly used chelating ligands for metal ion attachment to matrices. Iminodiacetic acid (IDA) is perhaps the most common, but the small size of the molecule necessitates binding it to the matrix using a spacer arm to prevent steric hindrance affecting the system. Also used is tris(carboxymethyl)ethylenediamine (TED) which results in a matrix that retains the metal ion more strongly than IDA-ME⁺ but consequently means proteins bind to the system less strongly. This is due to the third carboxylic acid group acting as an electron donor in TED-Me⁺, which is not present in IDA-Me⁺

Metal ions used are most commonly first row transition metals (Ni²⁺, Zn²⁺, Cu²⁺ and Fe³⁺). Although quite a large variety of metal ions were considered for chelate binding, the electronic structure of the above set makes them particularly useful and hence they are now the main metals used.

Problems with the use of this method are likely to occur however in the production of therapeutic drugs since these metal ions are susceptible to leaching during the elution phase of the protocol, and must be regarded as highly toxic contaminants. Hence care must be taken to monitor for metal ions in these cases. Monitoring is easily conducted using atomic adsorption (AA)
spectroscopy of samples, although provided that the purification protocol of the protein concerned contains further column steps it is unlikely that any metal ions leached from the column will be co-purified with the product in any other form of chromatography.

2.1.1 Protein binding influences

As mentioned above, it has been suggested that histidine, cysteine and tryptophan are residues most likely to provide the necessary electron pair donation to attach a protein to chelate metal ions (under condition of neutral or near-neutral pH; at extreme pH's other residues become charged and affect the interactions). Other parts of peptides such as amino terminal and backbone nitrogen, and backbone carbonyl oxygen may also contribute to this effect. Recent work [Yip et al. 1989] suggests however that the most significant interactions are those between histidine / cysteine and the chelate metal ions. However this does not mean that a protein with high concentration of any of these two amino acids will automatically attach itself to a chelate metal ion. In point of fact, since most proteins exist in tightly folded or coiled three-dimensional structures, and the even the smaller IDA-Me+ chelate is very bulky, then only accessible surface residues are likely to be able to form complexes with the chelate metal ions. This means that, even though the full three-dimensional structure of the protein may be known, it is likely to be impossible to predict totally the binding characteristics, and these can only be determined experimentally. In addition, although the thiol sulphur in cysteine has a strong affinity for immobilised metals (especially Zn^{2+}) it is very rare to find free surface cysteine residues since they tend to oxidise on the surface in the presence of metal ions to form disulphide linked dimer pairs. In general it can be considered that the strength of attachment of a protein to a metal chelate is likely to be proportional to the number of free surface histidines in the protein structure. Further slight variations in affinity of the protein for the metal ion occur depending on the location of the histidines. For example, if the two residues are sequential in the protein structure then in may not be spatially possible for both to interact with available metal ions at the same time.
2.1.2 Methods in IMAC

The IMAC process basically consists of two important steps; adsorption and elution. The conditions of both of these steps must be carefully considered if the separation is to work effectively. Since the binding of a protein to a metal chelate requires a free electron pair, the adsorption step must be carried out under conditions where this is true of the amino acids in question. Normally this means ensuring that the electron donor groups are unprotonated, or at least partially so. This would encourage the use of higher than neutral pH’s for such processes and this is often the case, but care must be taken to ensure that the pH is not increased to such an extent that other, normally non-donating amino acids become charged and cause binding of non-required proteins to the column. In addition it is usual to add a non-buffering salt (e.g. 0.5M NaCl) as this will tend to inhibit non-specific electrostatic interactions that may otherwise interfere with protein binding. In addition it is vital that the actual buffer used does not compete with the protein for access to the immobilised metal ions. Usually if alkali conditions are required then a phosphate buffer will be used, and if acidic conditions are preferable an acetate buffer would usually be used but both buffers are considered acceptable.

Elution is usually carried out in one of three ways; protonation, ligand exchange or chelate breakage.

Protonation is considered to be the most common method for elution of bound protein on an IMAC system [Sulkowski 1985] due to its relative simplicity. Protonation of the electron donor groups on the surface of the protein molecule will reverse the interaction with the chelated metal ion and cause elution. This can be achieved quite simply by a gradient or step decrease in the pH of the buffer being used. There are three important considerations to this method however. Firstly, the metal chelate itself must remain stable on lowering of pH, and this is a significant problem with chelate zinc ions specifically. Secondly the protein required should not be affected by lowering the pH of the system, and
many proteins do irreversibly lose activity below pH 4-5. Finally care must be taken to avoid isoelectric precipitation of the proteins in question as this can lead to poor recovery and, more importantly at least in terms of fixed bed chromatography, blocking of the column.

Ligand exchange is the second method utilised for elution of bound proteins. The protein required can be easily eluted at near neutral pH (important if the protonation method above is unsuitable for the separation planned) by the introduction of a competing electron donor in the elution buffer, for example imidazole. Imidazole forms a stable complex with metal chelates which does mean that the column must first be saturated with the compound and equilibrated before the protein sample can be allowed to bind, or the experiment is unlikely to be successful or valid. Once equilibrated however, proteins can be bound and then successfully eluted using an imidazole gradient.

The final choice of method for elution is the breakage of the chelate itself. This can be carried out using a mild chelating agent such as histidine itself, which if introduced in a higher concentration than is present in the protein sample causes the protein to break away from the metal ion and be eluted. Stronger agents such as EDTA also perform this task successfully but care must be taken with proteins with structural metal ions as these may also be lost if EDTA is used at too high a concentration. In addition this method is non-specific and thus should only be used if the protein required is the sole bound protein on the column. However careful use of an EDTA gradient may bypass this problem.

2.1.3 Review of Literature

There has been considerable work in the field of purification of proteins and enzymes using immobilised metal-ion affinity chromatography since the initial work of Porath [1975]. In 1984, [Chadha et al.] patented the purification of human fibroplasts and leucocyte interferons using copper chelate affinity chromatography. However, their results showed a tendency for the target molecules to bind irreversibly to the copper chelate (bis-carboxyemthyl amino
ligands) and could only be partially recovered even when the bed was only partially saturated with copper. This results in a process which, while effective at binding is of little use unless the target molecules could be easily removed. The work concluded that the binding of the interferons to copper was too strong for an overall effective process, and better results may be obtained by the use of a zinc chelate. This was however not possible in the system used.

A review of the work carried out in the field was published [Sulkowski 1985] including a discussion of the chemistry basis for IMAC, and consideration of the relationship between protein structure and possible binding to metal chelates, with particular reference to the effect of tertiary protein structure on the possible attachment sites. In addition to this, summaries of proteins successfully attached to metal chelates were given. The paper concluded that one freely accessible surface histidine residue was sufficient to cause attachment to metal chelates, although multipoint attachment by two or more surface histidines resulted in stronger retention. Histidine was found to contribute more strongly to a bond forming than tryptophan, and the effect of cysteine residues could not be determined. This in any case is of less importance since free cysteines (those that have not formed disulphide linkages) are very rare in proteins.

Work in the field of IMAC began to increase towards the end of the 1980's, with [Rassi et al, 1986] producing work on the purification of muscle enzymes and human growth hormone. Recoveries on elution in excess of 80% were obtained from immobilised ferric ions of for both glycogen phosphorylase and lactate dehydrogenase, along with data demonstrating the relative strength of protein binding to different metal ions. More general work in the field was carried out by [Hochuli et al, 1987] and [Hutchens et al, 1989] looking at new adsorbents and base matrices to use in the field, along with ligand choices and, in the case of Hutchens, analysis of equilibrium partition data.

In the early 1990's, further work was published investigating the method in more detail. [Belew et al, 1990] looked at the effect of solute structure and ligand density on the retention of peptides, finding that there is an optimum
concentration for metal ions forming chelates on the matrix (in this case Cu(II) ions). The effect of the nature of the gel matrix was found to be difficult to predict. Further reviews on the subject generally have been published [Beitle et al, 1991] and [Arnold, 1991], discussing and demonstrating the importance and relevance of IMAC as a protein purification tool.

IMAC is ideally suited to the downstream processing operation of expanded bed adsorption, being a relatively robust affinity chromatography step. Most forms of affinity chromatography have relatively sensitive ligands, and as such are not suitable for even moderately harsh clean-in-place (CIP) protocols such as 0.5 – 1 M NaOH. IMAC however utilises the less sensitive IDA ligand that can be cleaned more easily. Since expanded bed adsorption, by its nature, uses crude feedstock, more robust CIP cycles are a requirement. Consequently this section studies the development of an expanded bed adsorption protocol using immobilised metal ion affinity chromatography.
2.2 Process Development

2.2.1 Choice of feedstock and target

A typical traditional route for the purification of an intracellular enzyme from whole cells compared to an expanded bed route for the same purification is shown in Figure 2.1:

Figure 2.1 - Comparison of different stages involved in expanded bed and packed bed processes

In this case the enzyme of choice was Alcohol dehydrogenase (ADH) from pressed baker’s yeast. Previous investigations have successfully purified the enzymes Glucose-6-phosphate dehydrogenase (G6PDH) (Chang et al, 1996) and ADH (Smith, 1997) from the same source, suggesting that the material is a
convenient source for this work. The purification of ADH using hydrophobic interaction chromatography has been the basis of much work at University College London including projects using expanded bed adsorption systems. The combination of the ease of preparation of the feedstock along with the presence of large quantities of contaminating proteins, nucleic acids and debris gives a convenient but realistic feedstock for process development. In addition, since IMAC utilises surface exposed histidine and tryptophan residues, it is reasonable to suppose that ADH will be a suitable target molecule as its tertiary structure indicates several surface histidine residues.

Elution from an IMAC matrix is normally carried out in one of three ways (Beitle, 1991); by lowering the pH of the system, addition of competing agents such as imidazole or histidine, or finally by the addition of a strongly chelating agent such as EDTA. In this case pH elution is not a favourable option since although a drop to a pH of 4 should be sufficient to release ADH (Arnold, 1991), the enzyme activity would be impaired at such a value, reducing the process yields. Competitive elution or the use of EDTA would be the methods of choice in this situation. In this thesis the use of EDTA elution was studied, since EDTA does not interfere with the standard methods of monitoring the system. Both histidine and imidazole interfere with the system monitoring at UV 280 nm since both adsorb at this frequency, and imidazole was shown to affect the protein assays of the feedstock (average protein concentration 10 mg/ml feed) by 5-10% when imidazole was added in the concentration range 0-0.5 M. Since the Biorad assay reagent used measures average histidine levels to calculate protein
concentration, then histidine as an elution agent will interfere heavily with assay results.
2.3 Experimental Work

2.3.1 Materials

Packed bed chromatography was carried out using 5 mL Chelating HiTrap columns and XK16/20 glass columns (1.6 cm diameter, 15 cm packed matrix height) packed with approximately 30 mL of Chelating Sepharose FastFlow, all provided by Amersham Pharmacia Biotech AB (Uppsala, Sweden) on a Pharmacia FPLC system consisting of an LCC-500 controller, two P-500 pumps and a Frac-100 fraction collector. The output from the column was monitored for UV-absorbance at 280 nm and 254 nm, and these values logged using a PE Nelson 900 interface and Turbochrom v4 (both Perkin Elmer Nelson Systems Inc., California, USA).

The expanded bed separations were carried out using a STREAMLINE 25 expanded bed (2.5 cm diameter, 15 cm settled matrix height) filled with 75 mL of STREAMLINE chelating matrix (gifts from Amersham Pharmacia Biotech). Peristaltic pumps (model 505-S, Watson-Marlow Ltd., Cornwall, U.K.) were used for buffer and feed application. UV absorbance at 280 and 254 nm was monitored using the system described above.

For all experiments, fractions collected were analysed for ADH activity and protein concentration. These analyses were conducted using a UVIKON 922 UV/VIS spectrometer (Kontron Instruments S.p.A., Milan, Italy).

Bakers' yeast was obtained in the form of blocks (DCL Ltd., Clackmannanshire, U.K.). All other chemicals used were laboratory grade (Sigma Aldrich Fluka, Poole, Dorset, U.K.).
2.3.2 Off-line ADH Assay

Alcohol dehydrogenase (ADH) activity was determined following the method of Bergmeyer (1979). ADH catalyses the reaction below:

\[
\text{Ethanol} + \beta \text{NAD}^+ \xrightleftharpoons{ADH} \rightarrow \text{Acetaldehyde} + \beta \text{NADH} + H^+
\]

The formation of acetaldehyde from ethanol may be monitored by measuring the increase in absorbance at 340nm due to the formation of \(\beta\text{NADH}\). The reaction mixture consisted of 600mM ethanol (Fluka), 1.0 mM glutathione (Sigma), 0.62mM semicarbazide HCl (Sigma), 1.8mM NAD\(^+\) (Sigma) in 50mM Tris HCl (Sigma) buffer at pH 8.8. Semicarbazide inhibits the reverse reaction.

One unit of enzyme activity is defined as the amount of ADH necessary to catalyse the conversion of 1µmol of ethanol to acetaldehyde per minute at 25°C. All assays were performed in 1cm path length cuvettes and the reaction was started by the addition of the enzyme. Potassium dihydrogen phosphate buffer (100mM, pH 6.5) was used to dilute samples to produce a linear change in absorbance less than 0.5 A.U/min. Assays were performed in duplicate with a reproducibility of ±5%.
2.3.3 Total Protein Assay

Total protein concentration was determined using the Bradford assay (Bradford, 1976). The assay is based on the shift in absorbance from 465nm to 595nm which occurs when Coomassie Blue G-250 dye binds to proteins in acidic solution. The colour change from pale orange to blue is relatively linear and thus permits quick and easy protein determination. In this study a commercially available dye was used (Bio-rad Protein Assay reagent, Bio-rad, Hemel Hempstead, UK).

Samples to be assayed were diluted to within the range 0.01 to 1.0mg.mL\(^{-1}\) protein using potassium dihydrogen phosphate buffer at (100mM, pH 6.5). Reagent and dilute sample were added to the cuvette and the absorbance at 595nm read after 10 minutes. A standard curve produced using bovine serum albumin (Sigma) for each batch of dye reagent, enabled protein determination to be made directly from the absorbance reading of the unknown sample. Assays were performed in duplicate with a reproducibility of ±5%.

2.3.4 Methods

2.3.4.1 Initial method scouting - Determination of binding and elution characteristics

For initial testing, pure ADH (Sigma, 340 units/mg solid, EC 1.1.1.1) at 2mg solid / mL buffer was used. Initial trials were carried out using a 5 mL HiTrap in order to determine the binding and elution characteristics of pure
ADH to immobilised Zn$^{2+}$, Cu$^{2+}$ and Ni$^{2+}$ ions. For all the experiments the feed was loaded at 200 cm/h and elution was carried out at 100 cm/h to ensure consistency on scale-up.

5 mL of metal ions in a 5000 ppm solution were loaded onto the column from a superloop. The column was then washed in buffer A (0.02 M KH$_2$PO$_4$/0.5 M NaCl, 10 column volumes). Pure ADH solution (5 mL) was loaded onto the column followed by another wash in buffer A (5 column volumes). A gradient elution of 0 to 100% of buffer B (buffer A plus 0.05 M EDTA) was then carried out over 6 column volumes. This procedure was repeated using all three metals and in duplicate. These traces were monitored for UV absorbance at 254 and 280 nm and samples were taken at 2 min. intervals and assayed for ADH and total protein content. As a control the same experiment was performed with no metal ions loaded onto the column.

After a suitable metal was chosen, step EDTA elution trials were run in order to determine exact EDTA elution conditions.

### 2.3.4.2 Packed Bed Method Development

From the results of the HiTrap trials, an XK16/20 packed bed was used to determine if the behaviour of ADH in clarified yeast homogenate differed from that of pure ADH. For these trials, bakers' yeast was disrupted in a high pressure homogeniser (Lab 60, APV Ltd., Crawley, U.K.) fitted with a restricted orifice discharge valve for 5 discrete passes at 500 bar and 5°C and then solid KH$_2$PO$_4$ and NaCl added to make the solution up to the concentration of buffer.
A (0.02M KH₂PO₄/ 0.5M NaCl). This was clarified using a Beckman J2-MI centrifuge (Beckman Instruments Inc., California) at 16000 g for 30 mins.

The effect of other components in homogenised yeast on the elution conditions for ADH determined in the HiTrap trials was studied. This was done using a Pharmacia XK16/20 column packed to a bed height of 15 cm with Chelating Sepharose Fast Flow matrix. This was then loaded with one column volume (approximately 30 mL) of Zn²⁺ ions in solution at 5000ppm. After washing (5 column volumes buffer A) one column volume of clarified yeast homogenate diluted to 10mg/mL protein content was loaded onto the column. Again the column was washed in buffer A and a step elution protocol was run using EDTA concentrations of 0.003M, 0.006M and 0.05M in buffer A. Samples taken at 2 minute intervals throughout the load, wash and elution were assayed for ADH activity and protein concentration.

2.3.4.3 STREAMLINE Method Development

The developed packed bed protocol was run in replicate on the STREAMLINE 25 expanded bed in order to ensure reproducibility of results. For this work, unclarified homogenate as before was diluted to 10 mg overall protein / mL homogenate in order to ensure consistent load conditions between runs and also to lower the viscosity of the feed to a level where bed height was not greatly increased by the change from buffer to feedstock.
In the case of the expanded bed elution was carried out in a downwards direction with the bed in packed mode to attempt to minimise the ADH peak width and hence improve purification and concentration of the product.

Following the packed bed method scouting, several runs were carried out using the STREAMLINE 25 expanded bed system. The layout of the system used is shown in Figure 2.2. The column was filled with 75 mL of STREAMLINE chelating matrix. This was fully expanded in both water and buffer A in order to determine the expansion characteristics of the matrix.
Figure 2.2 - Schematic layout of expanded bed system showing valves, piping and monitoring equipment:

Key to symbols:
- EB: 2.5cm Expanded Bed
- Bubble Trap
- 4port-4way Jacobo Valve
- 4port-2way Jacobo Valve
2.3.4.4 Residence time distribution experiments

Residence time distribution experiments (RTD's) were carried out using a step change from buffer A to buffer A plus 0.25% v/v acetone, monitored using UV 280nm absorbance. This was conducted in order to check the stability of the bed on expansion and to ensure that the flow through the bed was approximately plug-like.

2.3.4.5 Experimental procedure

Prior to loading the yeast homogenate, the matrix was activated by loading 75 mL of 5000 ppm Zn$^{2+}$ in solution. The bed was expanded at a linear velocity of 200 cm/h in buffer A until the height was stable (typically 0.5 h). Yeast homogenate, diluted to an overall protein concentration of 10 mg/mL, was loaded onto the column until the ADH activity in the outlet stream reached 5% of the activity in the load sample (5% breakthrough). The bed was then washed, again at 200 cm/h, until the UV 280nm absorbance returned to the baseline level. The wash was then stopped and the bed allowed to settle. The direction of flow was then reversed, and the bound ADH eluted at 100 cm/h using the following protocol:

1 column volume buffer A
2 column volumes buffer B (A + 0.003M EDTA)
3 column volumes buffer C (A + 0.006M EDTA)
2 column volumes buffer D (A + 0.05M EDTA)
The system was cleaned after being re-expanded in buffer A. The initial CIP procedure consisted of a water wash and then 10 column volumes of 1M warm NaOH. (approximately 50° C), then a second water wash followed by 20% v/v ethanol for storage. Following further trials, a more rigorous CIP procedure was adopted using 1M NaOH followed by 30% v/v isopropyl alcohol, 25% v/v acetic acid and then 20% v/v ethanol, with water washes in-between each step.

Trials were carried out in triplicate to ensure reproducibility of results.
2.3 Results

2.3.1 Determination of binding and elution characteristics

The experimental chromatograms for the trials using the three different metal ions are shown in figures 2.3, 2.4 and 2.6. The trace for the no metal ion trial is shown in figure 2.7. The mass balance data for these experiments is shown in table 2.1.

Figure 2.3 – Binding of ADH on a Cu-charged HiTrap column

![Diagram showing binding and elution characteristics of ADH](image_url)
Figure 2.4 - Binding of ADH on a Ni-charged HiTrap column

5 ml 2 mg/ml ADH in buffer A onto 5 ml chelating Ni
charged column - 0-50mM EDTA elution
Figure 2.5 - Binding of ADH on a Zn-charged HiTrap column

5 ml 2 mg/ml ADH in buffer A onto 5 ml chelating Zn$^{2+}$ charged column - 0-50 mM EDTA elution
Figure 2.6 - Binding of ADH on an uncharged HiTrap column

Table 2.1 - Mass balance data for HiTrap binding and elution trials

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>Load (Units ADH)</th>
<th>Wash (Units ADH)</th>
<th>Elute (Units ADH)</th>
<th>% Recovery on elution</th>
<th>% Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu²⁺</td>
<td>2750</td>
<td>0.2</td>
<td>5.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>3420</td>
<td>0.2</td>
<td>1594</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>2310</td>
<td>4.5</td>
<td>2090</td>
<td>91</td>
<td>91</td>
</tr>
<tr>
<td>None</td>
<td>2880</td>
<td>2162</td>
<td>0.1</td>
<td>0.005</td>
<td>75</td>
</tr>
</tbody>
</table>
Using Zn\textsuperscript{2+} as the immobilised metal ADH was eluted at approximately 5mM EDTA and could be recovered in excess of 90% of loaded activity. In contrast, using Ni\textsuperscript{2+} the ADH could only be recovered at less than 50% of the total loaded activity, and was eluted at approximately 15mM EDTA. The ADH could not be eluted from the Cu\textsuperscript{2+} charged column before the Cu\textsuperscript{2+} ions themselves were stripped from the column by the chelating action of the EDTA. On the basis of these results it was decided that both larger scale packed bed trials and expanded bed work would be carried out using immobilised Zn\textsuperscript{2+} ions. The trials using no metal ion showed that ADH did not bind to the column under the conditions utilised.

Step elution trial results using Zn\textsuperscript{2+} are shown in figures 2.7 and 2.8.
Figure 2.7 – Step elution of ADH from 1mL Zn\(^{2+}\) Column using EDTA

5ml 2mg/ml ADH in buffer A onto 5ml chelating Zn\(^{2+}\) charged column - 0 to 50mM step elution
Figure 2.8 – Step elution of ADH from a 5 mL Zn$^{2+}$ column using EDTA

2.3.2 Packed bed experiments

The elution trace from the small packed column showed ADH eluted during the 0.006M EDTA step, demonstrating that any effects created by components in clarified yeast homogenate other than ADH did not significantly affect the elution conditions required. Consequently this elution regime was considered suitable for experiments using the STREAMLINE 25 expanded bed with unclarified homogenised yeast.
The total binding capacity of the STREAMLINE chelating matrix was estimated with 30mL of the matrix packed in an XK16/20 column. The total capacity of the matrix at 100% ADH breakthrough was found to be 1075 U(ADH)/mL matrix.

2.3.3 Expanded bed experiments

The results of the residence time distribution curves (shown in figure 2.9) were used to determine the stability of the bed on expansion. From these traces the number of theoretical plates in the system was found to be in the range 32 - 39. The values obtained are in the range typical of EBA and show a variation of 18% across the range of results obtained. This is within the maximum variation of 20% for bed stability recommended by Pharmacia (STREAMLINE® 50 handbook) and it is reasonable to assume that the bed is stable upon expansion and the flow through it is approximately plug-like.
The results of the packed bed trials predicted that the ADH peak would occur within the buffer C (buffer + 6mM EDTA) step. Protein and ADH assays were carried out in the load, wash and elution at 3 minute intervals. Values for protein concentration and ADH activity were calculated and plotted using Microcal Origin (Microcal Software, Northampton, MA).

Dynamic binding capacity was calculated in the STREAMLINE 25 column in expanded mode at 10% total ADH breakthrough (clarified yeast homogenate at 10mg/mL total protein content) using a flowrate of 200 cm/h.
The dynamic capacity of the bed was calculated as the number of units of ADH per mL of settled matrix bound before 10% breakthrough of ADH occurs (Angal, 1989) and was estimated from the amount of enzyme contained in the fractions up to 10% ADH breakthrough. In this case the dynamic capacity of the STREAMLINE® matrix was found to be 235 U(ADH)/mL matrix. This value, along with that of the dynamic capacity given earlier, compares favourably with values for STREAMLINE® Phenyl low-sub (a hydrophobic interaction matrix) of 260 U(ADH)/mL dynamic capacity and 750 U(ADH)/mL total capacity (Smith, 1997).

The expanded bed experiment described in the methods section above was carried out in triplicate in order to check for reproducibility. A typical experimental trace is shown in Figure 2.10, along with data for ADH and protein concentrations and yield values in Table 2.2.
Figure 2.10 - An example of ADH and protein concentrations in column outflow during expanded bed experiment, along with buffer step changes as shown by the EDTA trace

Table 2.2 - Load, wash and elution sample pool data from a sample expanded bed run showing yield and purification factor for each stage

<table>
<thead>
<tr>
<th></th>
<th>Volume (mL)</th>
<th>ADH (units)</th>
<th>Protein (mg)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification Factor</th>
<th>Yield (ADH%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load</td>
<td>360</td>
<td>25300</td>
<td>3070</td>
<td>8.24</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Wash</td>
<td>973</td>
<td>6690</td>
<td>2490</td>
<td>2.69</td>
<td>0.3</td>
<td>21.1</td>
</tr>
<tr>
<td>Elution (Buffer A)</td>
<td>70</td>
<td>65</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>0.2</td>
</tr>
<tr>
<td>(Buffer B)</td>
<td>154</td>
<td>97</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>0.3</td>
</tr>
<tr>
<td>(Buffer C)</td>
<td>323</td>
<td>24748</td>
<td>366</td>
<td>67.6</td>
<td>8.2</td>
<td>78.2</td>
</tr>
<tr>
<td>(Buffer D)</td>
<td>222</td>
<td>98</td>
<td>7</td>
<td>14</td>
<td>1.7</td>
<td>0.3</td>
</tr>
</tbody>
</table>
The initial run on the expanded bed showed an ADH recovery on elution of 99% and a purification factor with respect to contaminating protein of 11. Although these are very positive results, subsequent experiments showed a reduced recovery on elution to a steady state of approximately 60% yield. This was believed to be due to initial bed contamination not being completely removed and hence a more rigorous CIP protocol was adopted (see experimental methods in this Chapter and also Chapter 3). In addition, it was noted that the ADH peak was crossing over the 6mM EDTA to 50mM EDTA step boundary, and this was likely to effect recovery values since ADH in 50mM EDTA will be inactive due to subunit separation and therefore will not register on rate assays. Consequently the EDTA concentration employed in the elution was gradually increased in an attempt to contain the ADH peak entirely in the third step. It was noted that, although 6mM EDTA eluted the ADH, increasing the EDTA concentration resulted in earlier elution of the ADH peak. The EDTA concentration was increased to 20mM before the peak was clearly contained in the third step. After these changes the recovery was stable at 80% or greater over multiple runs, and the improved CIP appeared effective at removing contaminants. In addition further stability work showed that ADH maintained 90-95% initial activity in 20mM EDTA solution for at least 2 hours at room temperature, longer if kept at 4°C.

The IMAC results compare reasonably favourably with more common, less specific chromatographic methods such as ion exchange or hydrophobic interaction chromatography, although the purification factor is quite low for an affinity separation. This is thought to be due in part to the use of EDTA as an
elution mechanism. Since EDTA in high concentrations (such as the 50mM in the final elution step) removes the metal ions from the matrix, the system becomes more irreversible in nature and prevents re-binding of contaminating proteins and the development of a chromatographic profile. As a result the purification suffers because all proteins bound to the matrix will tend to co-elute. This can be improved with an accurate step elution protocol but the nature of the EDTA elution mechanism is always likely to cause problems in this area.

In addition the binding and elution of ADH was found to be extremely susceptible to small variations of pH since the choice of pH 7.2 for the buffers is extremely close to the pKₐ of histidine, affecting the charge on the imidazole ring and hence the ability to bind. Consequently extreme care has to be taken in selecting the pH of buffers to ensure consistent results. Further work is considering the possibility of modifying buffer pH to ensure more consistent binding without affecting enzyme activity.

The major concern with the work lies in the purification factor with respect to contaminating proteins, which although acceptable for a less specific method such as ion exchange is quite low for what is essentially an affinity separation. The nature of the EDTA elution is likely to contribute to lower purification factors, since EDTA strips off the metal ions from the matrix thus preventing further binding to the column. Consequently anything bound to the column runs the risk of being eluted in one step if the metal ions are detached in this step. This can be minimised by ensuring that the ADH is eluted at as low an EDTA concentration as possible.
2.4 Conclusions

The work performed here shows that yeast ADH can be effectively purified from unclarified homogenates of pressed baker's yeast using immobilised metal-ion affinity chromatography in an expanded bed. The recovery of ADH on elution from the expanded bed compares favourably with other results shown in literature, although concerns about the long term stability of the matrix must be addressed before more detailed conclusions can be drawn. The introduction of a more rigorous CIP protocol into the system should increase the life of the matrix. The concern over the relatively low purification factor achieved indicates that careful control to narrow the elution peak or lower the amount of unwanted protein in the elution through the use of a tight EDTA step elution is necessary for such systems.
Chapter 3 - Hydrophobic Interaction Chromatography

Although the IMAC method proved effective, concerns about the performance of the method over many cycles (robustness) and possible interference of leached ions with some of the monitoring techniques described in Chapter 4, along with the more complex nature of the experimental protocol lead to the decision being taken to use hydrophobic interaction as the basic protocol instead. Primary in this decision being taken was the requirement for a simple, rapid and easily repeatable protocol to ease the development of monitoring systems. This Chapter details the principles behind the chromatographic method, along with reproducibility data and CIP method development performed to ensure the reliability of the method over many cycles.

3.1 Hydrophobic interaction chromatography.

Although a successful method, as explained in earlier Chapters, had been developed for the immobilised metal ion affinity purification of yeast alcohol dehydrogenase, a decision was taken, primarily for reasons of simplification, to utilise a previously developed protocol (M. Smith, 1997) for the purification of yeast ADH using hydrophobic interaction chromatography. Although the method used here had been almost completely defined, various results are included with which to demonstrate the methods reproducibility, maximum number of efficient cycles of the matrix that can be achieved, and the
development of a more robust Clean-in-Place cycle for efficient matrix rejuvenation.

3.1.1 Hydrophobic interaction theory

Hydrophobic molecules in aqueous solvents will self-associate due to hydrophobic interaction. In biological systems, hydrophobic interaction is of great importance as the folding of globular proteins, association of protein subunits and many other biological processes rely on hydrophobic interaction for their correct function. Hydrophobic interaction chromatography utilises the fact that the surfaces of globular proteins have extensive hydrophobic patches in addition to the expected hydrophilic groups. These hydrophobic regions bind to the hydrophobic ligands, alkyl or aryl side chains on the gel matrix under conditions favouring interaction for example aqueous solutions with high salt concentrations.

The first attempt to synthesis hydrophobic adsorbents was made by Yon (1972) followed by Er-el et al. (1972), Hofstee (1973) and Shaltield and Er-el (1973). Characteristically these early adsorbents exhibited a mixed ionic-hydrophobic character (Wilchek and Miron, 1976). Despite this Halperin et al. (1981) claimed that protein binding to such adsorbents was predominately of a hydrophobic nature. Later, Porath et al. (1973) and Hjerten et al. (1974) succeeded in synthesising charge-free hydrophobic adsorbents and demonstrated that the binding of proteins was enhanced by high concentration of neutral salts, confirming the earlier observation of Tiselius (1948), and that elution of the
proteins from the matrices could be achieved by washing the column with salt
free buffer or by decreasing the polarity of the eluent (Hofstee, 1973; Porath et
al., 1973).

The first commercially produced hydrophobic adsorbents were the Octyl and
Phenyl Sepharose Cl-4B matrices (Janson and Laas, 1978) of the charge free
type. The commercial availability of new matrices, opened up the application of
hydrophobic techniques, to include a wide variety of biomolecules such as serum
proteins (Hrkal, Rejnkova, 1982), receptors (Kuehn et al., 1980) and cells
(Hjerten, 1981).

A large number of theories have been proposed to explain hydrophobic
interaction chromatography. However most of the theories are essentially based
upon the interaction of hydrophobic solutes and water (Tanford, 1973;
Creighton, 1984). None of the theories have enjoyed universal acceptance but
common to them all is the central role of structure-forming salts and the
influence they exert on the solute, solvent and adsorbent within the
chromatography system. Porath (1986) proposed “salt-promoted adsorption” as a
general concept for HIC and other types of solute-adsorbent interactions
occurring in the presence of high concentration of neutral salts.
Hofstee (1973), and later Shaltield and Er-el (1973), proposed the theory of
“hydrophobic chromatography” with the implicit assumption that the mode of
interaction between proteins and immobilised hydrophobic ligands was similar
to the self-association of small aliphatic organic molecules in water. Porath et al.
(1973) suggested a salting out effect in hydrophobic adsorption, extending the
earlier observation of Tiselius (1948). They suggested that "...the driving force is the entropy gain arising from structure changes in the water surrounding the interacting hydrophobic groups". This concept was further extended and formalised by Hjerten (1981) who based this theory on the well known thermodynamic relationship:

$$\Delta G = \Delta H - T\Delta S$$

which describes the change in free energy of a process. He proposed that the displacement of the ordered water molecules surrounding the hydrophobic ligands and proteins led to an increase in entropy ($\Delta S$) resulting in a negative value for the change in free energy of the system. This implied that the hydrophobic ligand-protein interaction was thermodynamically favourable. It is worth noting at this point, that both enthalpy and entropy change with temperature for hydrophobic interactions. Theoretical treatments are complicated but one can conclude that the strength of hydrophobic interactions should increase with an increase in temperature (Janson and Ryder, 1989). Another popular theory is based on the correlation of the effect of neutral salts in salting out (precipitation) and hydrophobic interaction chromatography (von der Harr 1976; Melander and Horvath, 1977). Melander and Horvath, (1977) suggested that hydrophobic interaction was accounted for by an increase in the surface tension of water arising from the structure forming salts dissolved in it. They stated that the salt that increased the surface tension the most, gave the strongest interaction. Also the higher the salt concentration, the stronger was the interaction. A combination of the two mechanisms has long been exploited even
before hydrophobic adsorbents were synthesised (Hoffman and McGivern, 1969).

van der Walls attraction forces have also been used to explain the interaction between proteins and hydrophobic ligands (Srinivasan and Ruckenstein, 1980). The basis for this theory is that the van der Walls attraction forces increases as the ordered structure of water increases in the presence of salting out salts.

3.1.2 Effect of type and concentration of salt on hydrophobic interaction.

The type and concentration of salt are two factors of great importance in hydrophobic interaction chromatography, as are additives that change the polarity of the solvent. The latter is exemplified by ethylene glycol or propan-2-ol, both of which decrease the interaction between the HIC gel and proteins by changing the overall structure of water slightly towards a structure resembling an organic solvent.

The influence of salt on hydrophobic interaction follows the well known Hofmeister (lyotropic) series (Pahlman et al., 1977). Salts that promote hydrophobic interaction are to the left in the series. The salts to the right of the series, $ClO_4^-$, $I^-$, $SCN^-$, are called chaotropic. Hydrophobic interactions are increased in the presence of salts such as ammonium sulphate to a greater extent than salts lower down the Hofmeister series (shown on next page).
Anions:

\[ SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^- \]

Cations:

\[ NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} \]

3.1.3 Hydrophobicity of amino acids and proteins.

As one might expect for stable ternary protein structures, most hydrophobic amino acids are buried within the interior of the protein, with hydrophilic amino acids mostly populating the protein surface. However some hydrophobic amino acids also appear on the surface and the hydrophobicity of a protein is taken as the sum of the hydrophobicity of the exposed amino acids and parts of the backbone.

Two methods for the calculation of the hydrophobicity of amino acids have been presented. One is based on the direct measurement of the solubility of individual amino acids in water and organic solvents (Nozaki and Tanford, 1971; Jones, 1975). A hydrophobicity scale for the different amino acids was constructed on the basis of the free energy transfer from the amino acids from ethanol or dioxane to water.

The second approach was based on an empirical inspection of known protein structures (Cornette et al. 1987). Here several hydrophobicity scales are based on the environment of the different amino acids, the fraction of amino acids buried within the protein and other parameters.
Between the two approaches there were some striking differences (Janson and Ryden, 1989). Proline is a rather hydrophobic amino acid, however its secondary structure-breaking properties make it appear in bends, typically on the surface of proteins. Lysine, another hydrophobic residue on the solubility scale was found to be the most exposed of the amino acids using the second method (Comette et al. 1987).

The amino acids composition of a protein can be seen to have a major influence on the hydrophobicity of the molecule and knowledge of the primary structure of the protein may shed important insight into the behaviour of a target molecule on a hydrophobic support relative to other known proteins.
3.1.4 Interaction between protein and matrix.

Studies have shown that more than one ligand on the adsorbent must be involved in the binding of proteins to hydrophobic supports (Hjerten et al., 1974). This is known as multi-point attachment. Further studies of the kinetics of protein binding have shown that adsorption proceeds via a multi-stage reaction (Jennissen, 1986). The rate-limiting step for the binding of phosphorylase \( b \) to Butyl-Sepharose was shown to be the slow conformation change or re-orientation of the protein on the HIC gel rather than the collision between the protein and the amphiphilic gel. Hydrophobic interaction chromatography is, in general, a mild method due certainly to the stabilising influence of salts and the recoveries are often high (Scopes 1994).

3.1.5 Matrices and functional groups for hydrophobic interaction chromatography.

The remaining factors that should be considered when selecting HIC media and optimising HIC separations are:

- Ligand type and degree of substitution.
- type of base matrix.
- pH.

The type of immobilised ligand primarily determines the selectivity of the adsorbent. Straight chain alkyl (hydrocarbon) ligands show purely hydrophobic characteristics while aryl ligands exhibit mixed mode behaviour. It has been
shown that at a constant degree of substitution, protein binding capacities of HIC adsorbents increase with increased alkyl chain length (Hofstee and Otillio, 1978). The charge free Octyl and Butyl Sepharose FF adsorbents from Pharmacia are based on the glycidyl ether coupling procedure. The short spacer introduced during this coupling has minimal effect on the hydrophobic character of the matrices since the hydrophobic nature of the short carbon backbone is compensated for by the hydrophilic –OH group of the ether. The Phenyl Sepharose matrices have the potential for π-π interactions via the phenyl group to promote a slightly different type of hydrophobic interaction.

In addition to these matrices, others are available such as the Toyopearl range of Hydrophobic matrices from TosoHaas (Germany). These are based upon an inert methacrylate base with ligands coupled via inert linkages. Ligands available in this range include ether, phenyl and butyl, with respectively increasing hydrophobicity. These matrices are primarily used where non-specific interaction between the target molecule and the base matrix is suspected as methacrylate is almost totally inert to ionic and hydrophobic effects. These effects have been described (Builder, 1993) but generally it is accepted that in order to change from one matrix type to another, modification of binding and elution conditions will be required, and in the case of very strongly hydrophobic proteins, it may prove impossible to use carbohydrate base matrices (e.g. agarose) at all.

In addition rigid copolymer matrices (e.g. methacrylate) generally have a higher degree of robustness and can be used for more cycles without replacement. In
addition, they can be packed at significantly higher flowrates than gel based matrices.

As the degree of substitution of HIC adsorbents increases so to does the protein binding capacity. There is evidence to suggest that a limit to the binding capacity is reached at very high degrees of substitution (Rosengren et al., 1975), which coupled with an increase in the strength of interaction can result in circumstances from which elution is difficult (Jennisson, 1978).

The effect of pH is complicated. In general an increase in pH was found to decrease hydrophobic interaction (Porath et al., 1973) probably as a result of the titration of charged groups leading to the increased hydrophilicity of the proteins. Other reports suggest that hydrophobic interaction increases as pH decreases (Halperin et al., 1981). No real generic rules were found in the literature to predict the behaviour of proteins on HIC matrices as a function of pH and information for individual proteins should be found experimentally.

3.2 Basic Experimental Protocol

Detailed here is the experimental protocol as initially used (Smith, 1997) for ADH purification from a homogenised suspension of bakers' yeast using an expanded bed adsorption system employing hydrophobic interaction. Method development had showed that a matrix utilising phenyl groups as the base ligand performed well at separating ADH from contaminating proteins in a STREAMLINE system. The experiment detailed here was performed repeatedly to verify the suitability of the protocol as a reproducible basis for future work.
All experiments were conducted using prepared bakers’ yeast as a source of ADH and captured in a STREAMLINE 50 EBA system (Amersham Pharmacia Biotech, Uppsala, Sweden) filled with STREAMLINE Phenyl hydrophobic interaction matrix.

3.2.1 Feedstock

Bakers’ yeast was made up into suspension at 250g/L (wet weight) in 0.1M potassium phosphate buffer. This was then disrupted in a high pressure homogeniser (Lab 60, APV Ltd., Crawley, UK) fitted with a restricted orifice discharge valve for 5 discrete passes at 500 bar with the temperature maintained at 5°C. The homogenised feed was then assayed for ADH activity and diluted to 100 U/mL ADH activity (±50%). In the process, the feed was also made up to 0.78M Ammonium sulphate for optimum binding to the matrix (Smith, 1997).

Bakers’ yeast was obtained in the form of pressed blocks (DCL Ltd., Clackmannanshire, UK.). All other chemicals used were laboratory grade (Sigma Aldrich Fluka, Poole, Dorset, UK).

3.2.2 Chromatographic Methods

Peristaltic pumps (model 505-S, Watson-Marlow Ltd., Cornwall, UK.) were used for buffer and feed application and to raise and lower the column hydraulic adaptor using 20% v/v ethanol as the hydraulic fluid. All valves and equipment (see Figure 3.1) were from the Amersham Pharmacia Biotech range.
The automated CIP cycle (see CIP development section) was controlled via an LCC 500 Plus controller and a P-6000 pump (Amersham Pharmacia Biotech). The column was filled with 300mL of STREAMLINE Phenyl matrix (Amersham Pharmacia Biotech) to give a settled bed height of approximately 15
cm. System monitoring was carried out using a Pharmacia UV1 monitor measuring absorbance at 280 nm and a conductivity monitor (Model pH/ion Multiplexing monitor, Sepracor, MA 01752, USA). Both of these were connected to a PC using a PE 900 interface and logged using Turbochrom version 3.0 (Perkin Elmer Nelson Systems Inc., California, USA).

Fractions were collected every 2 minutes during load, wash and elution stages (Model SuperFrac, Amersham Pharmacia Biotech, Sweden) and assayed for protein and ADH as described in Chapter 3. Flow reversal in the system was facilitated by the inclusion of a 4-way manual valve as illustrated in figure 4.1 and a custom-made quick-fit glass bubble trap was inserted into the system upstream of the four-way valve to prevent air entering the column.

Equilibration, loading and washing of the column were carried out in an upwards direction at a linear velocity of 200 cm/h. Elution was carried out in a downwards direction with the bed settled at a linear velocity of 75 cm/h. The column was equilibrated with 0.02 M KH2PO4 + 0.78M (NH4)2SO4 for the equivalent of 5 expanded bed volumes to ensure that the bed expansion had reached a stable level. At this point the adjustable hydraulic adapter was positioned approximately 5cm above the top of the matrix to allow for slight further bed expansion caused by the higher viscosity of the feedstock as compared to the equilibration buffer. The feedstock was then applied onto the column, and loaded based upon a matrix dynamic capacity at 10% product breakthrough (Smith, 1997) of 275 U/mL matrix. In this case, for 300mL of matrix, approximately 900 mL of feedstock was loaded, or 3 settled bed
volumes. During multiple experimental repeats this quantity typically varied from 750 to 1000 mL.

After loading the column was washed using 0.02 M KH₂PO₄ + 0.78M (NH₄)₂SO₄ until the UV 280 nm absorbance trace had returned to baseline. Once all particulate material had been washed from the column, flow was stopped and the bed allowed to settle under gravity. The upper adapter was then lowered onto the surface of the settled bed to reduce the dead volume of the system. A step decrease in salt concentration using buffer B (KH₂PO₄, 0.02M, pH7) was applied to the column at 75cm/h in the downward direction, to elute bound ADH and protein from the column.

3.2.3 Clean-in-Place

Initial development of this protocol had used a simple 1M NaOH clean-in-place step, however this was seen to have limited effectiveness over repeated cycles. Consequently it was necessary to develop a more effective cleaning protocol for the system if the matrix were to be used for multiple repeats without compromising adsorption / desorption performance. This will be described later in the Chapter.
3.3 Results

3.3.1 C.I.P method development

Since a simple NaOH clean step was not effective for regenerating the bed after multiple runs, extra CIP steps were required. Consultations with Pharmacia suggested that the introduction of steps to sanitise as well as clean the bed would assist in regeneration, and steps to specifically remove lipids as well as debris, protein and nucleic acids would prove valuable. STREAMLINE Phenyl matrix is able to tolerate various cleaning agents including acetic acid, urea, guanidine hydrochloride, isopropyl alcohol (propan-2-ol) and NaCl. Within the scope of requirement for improved bed sanitation along with removal of lipid contaminants, acetic acid and isopropyl alcohol were chosen as extra cleaning agents to be tested as part of the CIP cycle for improved bed regeneration. Hence a modified CIP cycle was developed as follows:

1M NaOH (200 cm/h, upflow, 10 column volumes)
DI H2O (200 cm/h, upflow, 3 column volumes)
25% v/v Acetic Acid (200 cm/h, upflow, 5 column volumes)
DI H2O (200 cm/h, upflow, 3 column volumes)
30% v/v isopropyl alcohol (200 cm/h, upflow, 5 column volumes)
20% ethanol (200 cm/h, upflow, 5 column volumes)
This process was controlled using the LCC-500 controller and a P-6000 pump with the system programmed to run the CIP overnight to improve turnaround time for the bed.

3.3.2 Robustness and Reproducibility

The experimental method, as described above, with the modified CIP protocol, was run 10 times consecutively with the same matrix to check the reproducibility of the method. Quantity of ADH loaded, recovery on elution and relative product purity with respect to contaminating protein were all plotted against cycle number to check for any matrix deterioration. The results are presented in Table 3.1. and show reasonable consistency of matrix performance over multiple cycles.

Table 3.1 - Variation in quantity ADH bound and breakthrough level reached with cycle number

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Units ADH bound</th>
<th>Relative Purity of eluate (wrt protein)</th>
<th>%Breakthrough level reached</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>87000</td>
<td>11.9</td>
<td>10.9</td>
</tr>
<tr>
<td>2</td>
<td>77000</td>
<td>8.6</td>
<td>4.8</td>
</tr>
<tr>
<td>3</td>
<td>100775</td>
<td>12.9</td>
<td>13.5</td>
</tr>
<tr>
<td>4</td>
<td>97050</td>
<td>16.4</td>
<td>10.4</td>
</tr>
<tr>
<td>5</td>
<td>111250</td>
<td>9.9</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>78200</td>
<td>10.7</td>
<td>2.6</td>
</tr>
<tr>
<td>7</td>
<td>100800</td>
<td>10.2</td>
<td>14.6</td>
</tr>
<tr>
<td>8</td>
<td>206900</td>
<td>12.8</td>
<td>48</td>
</tr>
<tr>
<td>9</td>
<td>169260</td>
<td>15.6</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>82120</td>
<td>9.14</td>
<td>12.2</td>
</tr>
</tbody>
</table>
In addition the UV and conductivity traces for cycles 1, 5 and 10 are shown in Figure 3.2 (a,b and c) for direct comparison.

Figure 3.2 – Logging traces for cycles 1, 5 and 10 (a, b and c respectively)

a) Experimental Trace – Cycle 1
b) Experimental Trace – Cycle 5

![Graph showing conductivity, activity, and protein levels over volume.](image-url)
3.3.3 Clean-in-Place work

The modified CIP process appeared effective in maintaining matrix performance after multiple cycles, however it was still noted that flow appeared to be partially disrupted after several cycles, with bed channelling occurring despite the absence of air under the adaptor. Upon dismantling the bed it could be seen that a heavily viscous, white jelly-like substance could be seen under the flow distributor of the column, blocking several of the holes in the distributor and hence channelling the fluid flow. This was suspected to be nucleic acids collecting under the distributor during load steps and not being effectively broken down by the CIP. Hence a hold step was introduced midway through the
1M NaOH stage of the CIP to give a 2 hour contact time for the NaOH on the column and on the matrix. After further runs it could be seen that this was successfully removing the white jelly and flow was no longer affected.

3.4 Conclusions

3.4.1 Robustness and reproducibility

As can be seen in Table 3.1, repeated use of the matrix up to 10 cycles shows little deterioration in performance in terms of product recovery or product purity. Consequently, where this protocol is to be used in future studies, a maximum number of cycles of 10 was imposed to ensure no matrix deterioration problems affected results. After this number of cycles, the matrix was replaced with fresh from the same production batch to ensure consistency.

From these results it could be concluded that the hydrophobic interaction chromatography step could be successfully used over multiple cycles for the purification of ADH from yeast homogenate, and hence was selected as the basis for further work. This, coupled with the relative simplicity compared to IMAC and the removed risk of either leached zinc or EDTA interfering with monitoring processes, meant that HIC was chosen as the basic method for on-line monitoring work described in Chapter 4.
Chapter 4 – Online monitoring

The work detailed in this Chapter investigates the use of a novel rapid online monitoring system, using in-bed sampling, to facilitate more effective control of an expanded bed.

Work was performed using the yeast alcohol dehydrogenase / hydrophobic interaction STREAMLINE protocol discussed in Chapter 3. Using a computer controlled stopped flow analysis system previously developed at University College London for other applications (Habib et al, 1997; Richardson et al, 1996) coupled to a novel automated in-bed sampling system, accurate control of product breakthrough was demonstrated to various breakthrough levels. In addition, the system facilitated monitoring of contaminants, enabling more effective control of wash steps in the process. Finally, monitoring of the bed during elution showed that the capacity of the bed for product binding was being utilised more heavily in the bottom sections of the bed than in the top sections, and this lead to further work in this thesis examining bed productivity.

4.1 Theory

Monitoring and control of all biotechnological process systems has been a much neglected area of downstream processing for many years. Fermentation and upstream processes have seen developments in control, with simplistic controls of physiological condition and off-line monitoring of cell growth being replaced
with on-line monitoring of cell biomass and product levels. In the case of downstream processing operations, poor controls result in lower process yields, longer process times, less efficiency and sometimes, in the case of upstream operations such as fermentations, cell death resulting in total product loss. This means that improvements in control and monitoring of products and contaminants can result in improvements in product recovery and reductions in process time.

For the vast majority of chromatographic steps in particular, control is carried out using off-line monitoring systems only. Even when monitoring is carried out on-line, it is usually monitoring of a general parameter, such as UV absorbance, and is usually downstream of the chromatography column. This introduces an inherent lag time into the whole system (the time taken for a change in column conditions, such as a buffer change, to register on system detectors) and hence can affect the final productivity of the process. This lag time can be minimised by reducing the distance between the column itself and the monitor, and is usually quite short in the case of packed bed systems, as the system will tend to have a limited dead volume.

Significant control issues exist in the operation of EBA systems however. The lag time of the system is high, usually of the order of minutes for a 50mm diameter bed at normal liquid velocities (2-300cm/hr) (Pharmacia STREAMLINE 50 operators handbook). This delay is particularly problematic in the load and wash steps of process – while the column is running in expanded mode, during which a significant void is maintained between the top of the
matrix and the top adaptor of the column. Where control to a particular level of product breakthrough is sought the significant dead volume in the bed makes control of the breakthrough to a given set point level difficult. In the past workers have relied upon historical trends to determine loading conditions. This is non-reproducible and not robust as it is prone to changes both in feedstock content and in the matrix capacity each of which can occur over time (Willoughby et al, 2000). Consequently accurate prediction of the end of the load, wash and elution steps may all be impaired, resulting in the consumption of larger process volumes and hence lower product concentration factors and higher buffer demands. All of the above problems are exacerbated when a product assay that takes more than a few seconds is needed for monitoring since this adds an additional time delay. In the case of expanded bed adsorption, the ability to monitor quickly and accurately for products and contaminants from within the bed rather than in the eluent stream would represent a considerable advantage and improvement.

Recently workers have reported on the use of in-column sampling for EBA control (Chase et al, 1999). In this case the breakthrough was very slow (over 4 hours for C/C₀=0.1 at the top of column loading at 200 cm/hr) which enabled a small-scale packed bed chromatographic analysis to be used for monitoring of the product and control of the EBA. For the work studied in this thesis, applications where feedstock control is more demanding were considered. In this case, the yeast homogenate / alcohol dehydrogenase / HIC system was chosen (see Chapter 3). This particular system is a particular challenge from a monitoring and control point of view since the ADH breakthrough curve is very steep. This makes accurate control using off-line assay methods difficult and the
need for constant attention and manual sampling is both time consuming and costly from an industrial production point of view. As the expanded bed purification of this enzyme is well characterised, robust and reproducible, this allowed for the monitoring and control of the system to be studied without concerns about inherent process variability.

The system developed for this analysis work was based around a stopped flow analyser (SFA) system, developed earlier at UCL (Richardson et al, 1986; Habib et al, 1997) for the rapid development of precipitation processes, linked to an expanded bed equipped with sample ports along the expanded axis of the bed. Using this system samples could be extracted on-line and these assayed automatically. Analysis was not confined to the ADH product but included a capacity for monitoring key contaminants; protein, RNA and cell debris, whose levels, both total and relative to the product ADH, are essential in determining the efficiency of the process and as determinants of key set-points in the EBA process (for example end of load step, end of wash step). This equipment was used to examine whether such a system could be used to facilitate faster and more accurate control of the load, wash and elution steps of expanded bed operation. [In addition the expanded bed equipped with side ports was designed to be used separately from the stopped flow analysis system to study hydrodynamic parameters and other effects such as particle size distribution variations in the bed and voidage distributions during bed operation. These experiments will be described in the following Chapters.]
4.2 Equipment Used – Hardware

4.2.1 Column modification

The modified expanded bed used for these experiments was based around the frame of a STREAMLINE ST-50 column as described previously. However the glass column was replaced by another manufactured in the Chemistry Department at University College London. The dimensions of the column itself were as that of the STREAMLINE column – 1m length and 5cm internal diameter. However the column had a number of small side ports blown into the wall. These were positioned along the column axis, at 5cm intervals, with the initial port being 5cm above the column base. The top port was at an axial height of 50cm. These ports were all 0.5cm in diameter, designed to fit tightly 0.65cm diameter rubber bungs. Illustrations of the column and port design are shown in figure 4.1
This ported column was designed to be used for on-line monitoring of both adsorption / desorption behaviour and system hydrodynamics. As such various sampling methods were considered and developed.

4.2.2 Sampling systems

The work on column hydrodynamics and on adsorption / desorption behaviour required sampling systems which could perform different tasks. The particle size
characterisation studies, for instance, required a sampling method that could remove a representative matrix sample from the column while the system was running. In contrast, studies of voidage and of product binding required effectively the opposite, a system that would remove fluid from the process stream, including all debris, products and contaminants, but leave matrix particles within the column.

Initial studies focused on the use of needles of varying bore to select samples on the basis of size with matrix particles, being the larger than any component in the process stream, being retained selectively by the sampling device if a small enough bore needle was utilised. Removal of matrix particles for size distribution determination could be carried out by utilising a needle of a bore larger than the diameter of the largest matrix particles. Although this approach proved useful for that part of the work (Chapter 5) removal of any matrix particles was undesirable for the online monitoring work, and it proved difficult to use a needle of any diameter without blockage or accidental removal of fine particles occurring. It was decided that a better sampling method was required for this work and hence specific requirements were considered. The most obvious choice of material for allowing the contents of the process stream, but not the matrix particles, to be removed is the top and bottom mesh nets from the expanded bed itself as these are designed specifically for this function. However it proved initially difficult to get this mesh into a small enough sampling device to fit into the ports in the expanded bed. This problem was overcome by designing a short stainless steel tube, of internal diameter ~ 1.5mm and external diameter ~ 3mm, and length 20 mm. Approximately 5 mm of the end of the tube
was milled to reduce the external diameter to ~2.25 mm and then a circle cut from a spare lower adaptor net of diameter ~5 mm was placed on the narrow end of the tube. This was then held in place by forcing a narrow piece of steel over the end of the pipe, bringing the diameter of the narrow section back to ~3 mm. This pipe was then fitted into a bored rubber plug, of diameter ~6.5 mm, as mentioned earlier in the Chapter. This design is illustrated in Figure 4.2.

Figure 4.2 – Illustration of modified sampling device

In order to allow monitoring from more than one position in the bed, a multiple position programmable valve (Pharmacia IMV-8, Amersham Pharmacia Biotech, Uppsala, Sweden) was utilised, allowing instantaneous switching between up to eight monitoring points. This valve was controlled by an FPLC
LCC-500 controller (Amersham Pharmacia Biotech). The output from this valve was then connected to the stopped flow analysis system in the case of on-line product and contaminant monitoring work.

4.2.3 Stopped Flow Analysis System (hardware)

4.2.3.1 Stopped flow analyser

The stopped flow analyser (SFA) was built in-house (K. Yeung and I. Holwill). It consisted of two 8 roller peristaltic pumps, one 3 roller peristaltic pump, a 2-way solenoid valve and a 3-way solenoid valve, housed in a steel case with dimensions L = 400mm, W = 350mm, D = 200mm illustrated in figure 4.3. The design of the SFA was based on the one applied by Chard (Chard 1997), however 8-roller peristaltic pumps were introduced to reduce the errors during dilution.

The two 8-roller peristaltic pumps were used for diluting the sample and mixing the reagent at appropriate levels before sending diluted samples to the appropriate destinations (ADH-spectrophotometer and diode-array spectrophotometer). The 3-roller pump was used to pump wash buffer around the various tubes in the cleaning step. The 2-way solenoid valve enabled the user to switch from reagent mix to wash buffer during the cleaning step. The 3-way solenoid valve was also used in the cleaning cycle. The sampling and assaying sequence can be seen illustrated in figure 4.4
The dilution levels were controlled by choosing different tube diameters for sample and buffer. The scheduling of pump and valve actions were determined by knowing the, flow-rate, the length and diameter of the tubes for the different components. Table 4.1, shows the different tube sizes which were available and the measured flow-rates. The relative error in flowrate was found using an electronic balance to measure the weight of deionised water passing through the system per unit time. The peristaltic pumps and solenoid valve were controlled by a supervisory computer through 7 digital signals (0-5V) (National Instruments Corporation, AT-DIO-32F, 32-bit digital I/O interface).
Table 4.1 - Flow-rates for the different tube diameters

<table>
<thead>
<tr>
<th>Internal Diameter (mm)</th>
<th>Length (cm)</th>
<th>Volume (cm³)</th>
<th>Flow-rate (ml.min⁻¹)</th>
<th>Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>20</td>
<td>0.04</td>
<td>1.95</td>
</tr>
<tr>
<td>2</td>
<td>0.76</td>
<td>20</td>
<td>0.09</td>
<td>4.10</td>
</tr>
<tr>
<td>3</td>
<td>1.52</td>
<td>20</td>
<td>0.36</td>
<td>12.6</td>
</tr>
<tr>
<td>4</td>
<td>2.29</td>
<td>20</td>
<td>1.22</td>
<td>32</td>
</tr>
</tbody>
</table>
Figure 4.3 illustrates the stopped flow analyser (SFA) schematically. Two 8 roller peristaltic pumps (1, 3) were for sample dilution and reagent mixing. The wash pump (2) has only 3 rollers as accuracy of flow is not as critical during a wash. The two-way valve (4), allows the user to switch from reagent to buffer when the washing sequence was initiated. The three-way valve (5) was used to switch from supernatant sample to buffer to enable the washing of the tubing and flow-cells.
Figure 4.4 - Stopped flow analyser sample preparation and wash cycles

Sample Preparation:

Diode-array spec & waste

Sample stream

Buffer

Pump 1

Pump 3

Waste

flow cell for ADH assay

Wash:

Buffer

Sample stream

Buffer

Pump 1

Pump 3

Waste

flow cell for ADH assay

Valve

Figure 4.4, The top diagram illustrates the sample dilution and reagent mixing sequence. The sample is diluted with buffer after Pump 1 and a “t-piece” splits the stream so that initially the diluted sample is sent to the diode-array spectrophotometer for spectral scanning. Pump 3 draws off a fraction of the diluted sample and mixes it with the reagent, which is sent to the spectrophotometer for ADH assaying. The bottom diagram illustrates the wash cycle. Buffer is pumped (pump 2), initially to the diode-array spectrophotometer to wash out the flow-cell and then via Pump1 and Pump 3 to the ADH flow-cell. Hence all the tubes are washed.
The above wash cycle is altered slightly during the monitoring of the chromatography work, whereby no buffer is washed back through the sample tube.

4.2.3.2. Spectrophotometer

A spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, Upsala, Sweden) with a flow cell was utilised for at-line monitoring of ADH activity levels. The spectrophotometer was placed after the SFA, allowing simultaneous measurement of ADH levels and RNA/protein/debris levels (measured by diode array spectrometer, see later in this Chapter). Flow cells with 10mm and 1mm path lengths were included to allow for rapid online switching of dilution levels without re-programming of the system. The ADH assay was performed following the same principle as the off-line assay described in Chapter 3 but the assay time was limited to 15 seconds before the SFA was switched to wash and the assay cycle began again.

4.2.3.3 Diode-array spectrophotometer

The diode-array spectrophotometer was built in-house (K.Yeung and I.Holwill). It consisted of a diode–array spectrometer (Carl Zeiss, Jena, Germany), a deuterium light source, the appropriate lenses to focus the light on to the spectrometer and a fan to prevent over-heating housed in a steel case with dimensions L = 400mm, W = 350mm, D = 200mm. This
was used for the monitoring of RNA, protein and debris using a rapid wavelength scan between 190-700 nm.

4.3 Experimental Methods

4.3.1 Feedstock

Bakers' yeast was made up into suspension at 250g/L (wet weight) in 0.1M potassium phosphate buffer. This was then disrupted in a high pressure homogeniser (Lab 60, APV Ltd., Crawley, UK) fitted with a restricted orifice discharge valve for 5 discrete passes at 500 bar with the temperature maintained at 5°C. The homogenised feed was then assayed for ADH activity and diluted to 100 U/mL ADH activity (±50%). In the process, the feed was also made up to 0.78M Ammonium sulphate for optimum binding to the matrix (Smith, 1997). Protein, RNA and cell debris levels were seen to vary by up to 75% for the different feedstock batches.

Bakers' yeast was obtained in the form of pressed blocks (DCL Ltd., Clackmannanshire). All other chemicals used were laboratory grade (Sigma Aldrich Fluka. Poole, Dorset, UK).
4.3.2 Off-line sampling and monitoring

The output from the column was monitored for UV-absorbance at 280nm and 254nm, and these values logged using a PE Nelson 900 interface and Turbochrom v4 (both Perkin Elmer Nelson Systems Inc., California, USA).

Fractions were collected using a Superfrac 100 (Amersham Pharmacia Biotech) and were analysed for ADH activity and protein concentration. These analyses were conducted using a UVIKON 922 UV/VIS spectrometer (Kontron Instruments S.p.A., Milan, Italy) as described in Chapter 3.

4.3.3 On-line Assays

4.3.3.1 ADH

On-line product monitoring was based on the initial rate of adsorbance change at 340nm as ADH catalysed the reduction of NAD\(^+\) (as detailed in the off-line assay methods). The assay was carried out using a custom built stopped-flow analyser (SFA) connected to a computer-controlled spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with controllable twin cuvettes (with 1mm and 10mm pathlengths respectively). ADH concentration was determined using an initial rate assay carried out for 15 seconds after which the flowcells were automatically washed and the next sample analysis began. Full details of the SFA method can be found in other publications (Habib et al, 1997; Richardson et al, 1986). The total system time was 40 seconds and assays were carried out to a duplicate accuracy of ±5%.
4.3.3.2 Contaminants

The concentration levels of key contaminants (RNA, protein and debris) were determined using a diode-array spectrometer and fibre optic probe (Carl Zeiss, MMS polychromater, Jena, Germany) fitted into a custom built system (University College London, UK) for rapid ultraviolet-visible (UV-VIS) spectral acquisition in the wavelength region of 200-700nm (filtered spectral scan every 1.5 seconds). Diluted samples (dilution factor of 17) were introduced and removed from the diode-array spectrophotometer automatically via a 1mm pathlength flowcell (Helma, Essex, UK). Contaminant levels of ribonucleic acid (RNA), protein and cell debris were predicted from the analysis of spectral data between 220-500nm. A calibration model was created through the use of a factorial experiment applying known standards of RNA (Sigma Chemical, Poole, UK), bovine serum albumin (BSA, fraction V, Sigma Chemical, Poole, UK) and washed Baker's yeast cell debris. A multivariate partial least squares (PLS) method was employed to regress the known levels of contaminant levels to the main features of their spectral data to form the calibration model, which was subsequently adopted for contaminant prediction. The prediction errors were 0.11 mg mL⁻¹, 0.41 mg mL⁻¹ and 0.14 Au or 0.091 mg mL⁻¹ (dry weight) for RNA, protein and cell debris respectively. Full details of the calibration method have been reported elsewhere (Habib et al., 1997).
4.3.4 Expanded Bed Operation

The expanded bed was set up to run conventionally, as described in Chapter 3, and then the stopped flow analysis system was arranged to remove samples from within the column. For both on and off line monitoring work the column was filled with 300mL of STREAMLINE Phenyl matrix (Amersham Pharmacia Biotech, Uppsala, Sweden).
The layout of the system is represented in Figure 4.5:

Figure 4.5 – Layout of In-bed monitoring system:

4.3.5 Conventional eluate monitoring

The bed was expanded in 0.1M potassium phosphate / 0.78M ammonium sulphate at 65.3 mL/min (equivalent to a liquid velocity of 200 cm/h) until the system was stable and the bed height constant (this took approximately 30 mins). The homogenate feed was then applied to the bed and the system monitored until an outlet ADH concentration equivalent to 10% breakthrough (C/Co = 0.1) was achieved as recorded on the on-line monitoring equipment. At this point the system was switched back into equilibration buffer and washed until the on-line debris level returned to the initial base line. At this point the system was stopped and the bed allowed to settle. The direction of liquid flow was then reversed and
the adaptor lowered to the top of the settled bed. Elution was carried out in a downward direction at a flowrate of 25.8 mL/min (equivalent to a liquid velocity of 75 cm/h) with the bed packed and monitored until the on-line monitoring system showed that the ADH concentration in the eluant had reached baseline.

The system was cleaned using an automated CIP cycle of 10 expanded column volumes 1M NaOH, 5 expanded column volumes of both 30% v/v isopropanol and 25% v/v acetic acid (with 5 column volume water washes in between). Finally the column was equilibrated with 10 expanded column volumes of 20% v/v ethanol. This cycle ensured both effective cleaning and sanitization. No change in the bed capacity was observed throughout the experimental period (approximately 10 cycles of operation).

Each experiment was repeated at least twice in order to ensure reproducibility of results to ±10% in terms of yield and peak height.

4.3.6. Operation using Stopped Flow Analysis of in-bed samples

Samples were taken from the ported column, using a Gilson Minipuls multiroller peristaltic pump (Gilson, Villiers-le-Bel, France) at a flowrate of 2 ml/min. This flowrate was selected to provide an adequate supply of fresh material for analysis without disturbing the bed stability. Residence time distribution (RTD) tests were carried out to determine the effects of constant sample removal on the hydrodynamic performance of the bed. RTDs were performed at 65.3 mL/min flowrate using a step-down method at UV 280nm absorbance. The buffer step down chosen was from 20 mM phosphate at pH 7 to 20 mM phosphate + 0.5% v/v acetone, at pH 7. Bed stability was calculated from an NTP (Number of
Theoretical Plates) value measured from the step trace as described in Chapter 3 and both experiments (sampling on and sampling off) were performed in duplicate.

Sampling at different points up the bed axis was facilitated through automated switching valves. The stopped flow analyser operated on a split stream from the 2mL/min sample line and enabled the removal of a small sample of liquid (approximately 1 mL) for analysis every 40 seconds. Dilution of the samples was achieved in the SFA delivery system prior to analysis. The more concentrated samples are diluted 1 in 236 and the more dilute 1 in 17. The sample was then split and one part sent to the diode array spectrophotometer for RNA, debris and protein analysis. Further dilution for the product (ADH) rate assays can be achieved by using different cuvette path lengths. The results were then logged by PC and plotted on screen to allow on-line operator monitoring of the system. A similar system to that described above has been used for monitoring selective flocculations (Habib et al. 1997) and for fractional precipitation work (Richardson et al. 1996). All instruments were linked to a computer through serial RS232 and A/D connections to facilitate data logging and instrument control. The software used for these purposes was based on LabVIEW (National Instruments).

4.3.7 In-bed product analysis

The breakthrough of ADH through the expanded bed was monitored by in-bed sampling up the axis of the bed. A sequence of sample points was selected
starting 10cm axial height above the lower adapter. During loading 5 samples at this height were removed followed by 5 samples at 25 cm axial height and then finally a further 5 samples were removed at a height of 40 cm (approximately the top of the bed when expanded at the loading flowrate of 65.3 mL/min). Monitoring continued at this height for the remainder of the duration of the load and wash. In the elution phase the sampling switched between three points: 5 and 10 cm axial height and the column eluent. Samples were removed from each location in turn and at 40-second intervals in order to obtain three distinct elution traces reflecting the material bound in the top third, two-thirds and the total volume of the packed bed.

In addition to running the system to a product breakthrough level of 10%, the system was also run to a level of 30% product breakthrough in order to consider more fully breakthrough characteristics.

4.4 Results

4.4.1 Residence Time Distribution results

RTD tests performed showed only a 6% change in number of theoretical plates (NTP) values calculated from bed residence time distribution tests while the system was expanded and samples being removed on line compared to a standard STREAMLINE 50 column, confirming that disruption to the bed caused by the in-bed sampling method was minimal. RTD curves performed
with the sampling system turned on and off are shown in figures 4.6 (a and b respectively):

Figure 4.6 – RTD traces from modified expanded bed system – large downward spikes indicate switch from buffer + acetone to buffer

a) With on-line monitoring on
4.4.2 Off-line versus On-line monitoring

Illustrated in figure 4.7 is a comparison between the ADH eluate peak obtained from the on-line monitoring system and conventional off-line assays, demonstrating the consistency between the two techniques. Error bars are not shown in this case as they would be too small to be seen behind the plot points.
4.4.3 Bed Breakthrough Characteristics

After the stability of the system during monitoring had been established, experiments were carried out to monitor and control the breakthrough of ADH during the load phase of the run. Control was achieved to 10% product breakthrough by monitoring at various points in the bed. Illustrated below are the ADH breakthrough curves for different sampling points monitored during a 10% breakthrough control experiment (Figure 4.8). As can be seen from the 40 cm axial position points (green triangles in figure 4.8), ADH breakthrough peaked at a little over 11% after switching from load to wash at 10% product breakthrough (switching point was 12 minutes into load). This represents very
rapid and effective control considering the rapid nature of the breakthrough curve (around 1% increase in product concentration per minute)

Figure 4.8 – ADH breakthrough characteristics at different axial positions within an expanded bed
Figure 4.9 below shows two breakthrough curves on one plot, with the stopped flow analyser being used to control the product breakthrough to 10% and 30% respectively. This illustrates the capability of the system to control to multiple breakthrough levels as required.

Figure 4.9 - Product breakthrough control using the SFA system: 30% ADH breakthrough compared to 10% ADH breakthrough (monitored at an axial position of 40 cm)

4.4.4 Contaminant Monitoring

In addition to the on-line monitoring of products, the SFA system can be used to track various contaminants and other parameters – for this work the SFA also monitored overall protein, cell debris and RNA levels. Breakthrough curves for these are shown in figure 4.10 (a, b and c). In these examples, traces were taken from the same run illustrated in the ADH breakthrough curve in Figure 4.8, with
monitoring carried out at the same axial positions of 10, 25 and 40 cm above the bottom adaptor of the expanded bed.

Figure 4.10 – Contaminant monitoring from within an expanded bed
a) Total protein (from left, points 1-6 measured at 10 cm axial position; points 7-12 measured at 25 cm axial position; points 13-18 measured at 40 cm axial position).
b) RNA (from left, points 1-6 measured at 10 cm axial position; points 7-12 measured at 25 cm axial position; points 13-18 measured at 40 cm axial position)
c) Cell debris (from left, points 1-6 measured at 10 cm axial position; points 7-12 measured at 25 cm axial position; points 13-18 measured at 40 cm axial position)

As can be seen from the above traces, curves have been fitted to the protein and RNA traces to correspond to the three different monitoring positions using the non-linear curve fitting function on Microcal Origin. However this function could not be reasonably fitted to the debris trace. In all cases the curve follows the same mathematical function, based upon a Boltzmann distribution:

\[ y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/\lambda}} + A_2 \]

fitted as copies from the first part of the trace.
4.4.5 Monitoring during elution

In addition to product monitoring during the load and wash phase, on-line sampling was also carried out during the elution phase while the bed was settled, with samples being taken at 5 and 10 cm axial height and also at the column outlet. These traces are illustrated in figure 4.11 below:

Figure 4.11 – On-line monitoring of the elution phase at various positions together with the ADH eluted in each peak
4.5 Discussion

4.5.1 Product monitoring

Results from initial experiments were from data monitored off-line only. The level of agreement between the manual assays and stopped flow analysis was very good, with a variation of approximately 5% over triplicate assay repeats for ADH and approximately 10% for protein. The larger discrepancy with protein values is likely to be due to the differing basis of the monitoring techniques employed, hence while the stopped flow analyser performs a faster version of the manual ADH assay, the protein levels are measured by an absorbance scan in the stopped flow analyser but by dye absorbance shift methods in the manual assay. These data confirm the suitability of the SFA method for the monitoring function.

From the samples taken at a height of 40cm (approximately equivalent to the height of the top of the bed during the load step) very accurate control of the system can be maintained. This is because the SFA is “seeing” the column exit conditions with minimal lag time. The lag time between an event occurring within the bed and the control computer registering this fact is corresponding only to the length of time required to sample and assay using the SFA. The stopped flow analyser used here was capable of performing a complete step from taking a sample to making data available within 40 seconds. When the column is operating at a standard load flowrate of 200cm/h the column-to-sample lag is approximately 5 minutes plus a minimum of 1 minute for a manual ADH assay.
for an off-line manual system. The experimental data showed that with such a configuration the EBA system could be accurately controlled to 10% breakthrough of ADH during experimental repeats even though the feed conditions could vary in terms of amount of debris present and protein concentration by up to 50%. The variations were achieved by varying the time interval between feedstock homogenisation and loading onto the column, and utilising the proportionally faster proteolytic degradation of ADH when compared to other proteins in the homogenate. Additionally, even though the ADH concentration in the feed was constant, experimentally the time taken to reach 10% breakthrough varied by as much as 15%. This feature of biological variability is inherent and demonstrates further the value of on-line rather than predictive control.

In-bed monitoring provided valuable insights into the adsorption processes occurring during expanded bed operation. The development of the product (ADH) breakthrough can be seen in Figure 4.8. (Similar breakthrough curves were observed for protein, RNA and debris in Figure 4.10 a, b and c). While the characteristics of the breakthrough curves for debris and RNA are similar at all of the three monitoring points during the load, those of the ADH decrease in steepness further up the column. This is most likely due to the effective reduction in ADH concentration driving force in the bed. As the feedstock front boundary passes up the column and ADH is bound, the quantity of ADH available for binding is reduced.

In addition this system could be used during the wash to monitor debris and nucleic acids back to baseline. This would provide more accurate information to be obtained, and hence allow more effective control then simply
monitoring the UV absorbance at 280nm, as is traditionally used in chromatography.

The elution of the product and total protein were monitored at three equispaced points along the axis of the packed bed and gave three similar but differently sized peaks (figure 4.11). These peaks can be considered as follows; that obtained from monitoring the packed bed at 10cm axial height gives the quantity of ADH eluted from the top third of the bed, that obtained at 5cm axial height gives the cumulative eluted from the top two-thirds of the bed and that obtained from the column exit stream (elution) gives the total quantity of ADH and protein eluted from the whole bed. This separation of the bed into sections allows bed utilisation and binding characteristics to be studied and enables the extent of bed utilisation to be determined together with estimates of bed productivity and buffer consumption. Such information is potentially very powerful for specifying the operating conditions needed to maximise EBA productivity given the need to achieve high bed utilisation with what are expensive chromatographic media.

Elution results showed that the top third of the bed contained 11600 units of ADH, the top two-thirds 24000 units and the whole bed 87000 units. Hence 60% of the eluted ADH came from the bottom third of the bed while the utilisation of the top third of the bed was very limited in that it accounted for less than 15% of the total bed capacity. These data show that there might be room for improvements regarding bed productivity, yields and buffer consumption based on varying the particle size distribution and bed height. The practical investigation of this forms the basis of the remainder of this thesis.
4.6 Conclusions

The work in this Chapter demonstrates that the stopped flow analysis system can be used to control accurately product breakthrough, even in cases such as these where the rate of breakthrough is very rapid. In addition, the monitoring of key contaminants is vital for the minimising of both time and buffer consumption during wash and CIP stages of a process.

Monitoring of product binding and elution in different areas of the bed has shown that bed utilisation varies considerably with axial position for a typical, 10% product breakthrough run. Further studies in this area are contained within later Chapters.
Chapter 5 – Bed hydrodynamics

The work in this chapter considers matrix bead distribution and voidage variations with axial height within the expanded bed system. The specially modified expanded bed system as described in Chapter 4 was used to facilitate the removal of particles and fluid at different axial positions and hence the particle size distribution and voidage could be considered at different flowrates. This in turn could provide key information regarding bed stability to be considered in the final part of the thesis regarding matrix utilisation.

5.1 Mathematical Theory

As discussed in the attached appendix, particle size distributions have an effect on the behaviour of the bed, both in terms of its rate of expansion and the degree of expansion. Mathematically it is simpler to model an EBA system as containing particles of a fixed size. Variation in particle size introduces more variables into the system. However, with the use of STREAMLINE matrices in expanded bed adsorption systems, this problem of particle size distribution must be considered since these matrices have a relatively wide particle size distribution, especially when compared to equivalent packed bed matrices. This is of course inherent in their nature to ensure stable expansion of the system to create a true expanded bed, rather than an unstable fluidised bed. Work (Wen and Yu, 1966) has stated that, provided the condition \[
\frac{\text{largest particle size}}{\text{smallest particle size}} \leq 1.3
\]
is adhered to, then the system can be regarded as containing particles of a single
size. However in the case of STREAMLINE matrices, this condition cannot be met - consequently another approach must be taken in order to model hydrodynamic behaviour. This Chapter therefore seeks to practically investigate particle size distribution and voidage effects within an expanded bed during operation.
5.2 Particle Size Distribution Studies

5.2.1 Materials and Methods

The work detailed here was performed using the modified STREAMLINE 50 expanded bed column fitted with a glass column modified with side ports (as described in Chapter 4). These ports were plugged with rubber bungs and samples of matrix were removed using 1.1mm x 50mm needles pierced through the rubber bungs as required. Samples of 1-2mL (matrix and buffer combined) were collected in 10mL tubes. Samples were obtained by piercing the bung at the required position and allowing the pressure inside the column to drive sample out through the needle.

Peristaltic pumps (model 505-S, Watson-Marlow Ltd., Cornwall, UK.) were used for buffer and feed application and to raise and lower the column hydraulic adapter. The layout of the system used for the experimental work is illustrated in figure 5.1. The column was packed with 300mL of STREAMLINE Phenyl matrix, giving an initial settled bed height of 15cm.
Particle size distribution measurements were carried out on a Malvern 3600 E laser particle sizer (Malvern Instruments, Malvern, UK) fitted with a 300mm focal length lens, scanning in the range 5.8 to 564 microns (particle diameters). Samples were added to a stirred cell within the sizer and diluted with distilled water as necessary to obtain optimal readings.

In order to illustrate that the sampling method was not preferentially selecting for matrix beads of a certain size, a sample of well-mixed matrix was taken prior to filling the column. This was scanned for particle size distribution, and compared with samples taken at different axial positions while the bed was expanded. A combination of samples taken from the top, middle and bottom axial positions of an expanded bed was found to have a similar particle size distribution to the sample of the well-mixed matrix.
5.2.1.1 Radial particle size distribution

Radial particle size distributions were measured in order to ensure that the bed was exhibiting stable expansion characteristics across its entire cross-sectional area. The bed was initially expanded at 200 cm/hr in water until stable (≈ 30 mins.). A needle was then inserted into the bung at a 25cm axial height and samples were taken traversing the column internal diameter at 10 mm intervals. The particle size distributions of these samples were measured using the Malvern sizer. This sampling was repeated (again at 25cm axial height) for liquid flowrates of 250 and 300 cm/hr respectively and in triplicate, with 3% variation in values of the numeric diameter ratios $d_{10}$, $d_{50}$ and $d_{90}$. These are defined as the point on the particle distribution where, respectively, 10%, 50% and 90% of the particles (by number) are smaller than the stated diameter. The $d_{10}$ statistic is an indicator of the proportion of fines in a particle size distribution, whilst $d_{50}$ gives the volume mean and $d_{90}$ a measure of the proportion of large particles present. These statistics were selected in order to characterise the sample particle distributions.
5.2.1.2 Axial particle size distribution

The bed was expanded in water at 200 cm/hr until the expansion was deemed stable on visual inspection (~ 30 minutes). A needle was then inserted approximately 2.5 cm (radial midpoint of the column) into the bottom rubber bung (axial height = 5 cm) and a small sample of liquid and matrix withdrawn (~1 ml). This was repeated in each of the sampling ports until an axial height of 30 cm was reached. The particle size distribution of each sample was determined using the Malvern sizer. These experiments were repeated at velocities of 100 and 300 cm/hr respectively. The process was repeated in triplicate to ensure consistency. Results were ± 3% in terms of particle size distribution parameters (d10, d50 and d90) at each position and condition tested.

5.2.2 Particle Size Distributions - Results and Conclusions

5.2.2.1 Radial particle distribution

Radial particle size distributions were determined at an axial height of 25 cm. The radial distribution of particle size is examined in Figure 5.2 for both the d50 and d90 diameters as functions of radial position and at differing liquid flowrates. The d90 statistic is a useful measurement of the content of large particle in the size distribution and was used to indicate the spread of particle sizes.
5.2.2.2 Axial particle distribution

Results showing the variation of particle size distributions with axial position (at 200cm/h linear velocity) are shown in figure 5.3. The results are expressed in the form of a decumulative number-based distribution demonstrating the decrease in particle size with increasing axial height.
Figure 5.3 – Decumulative particle size distribution variation with axial position
The variation in volumetric mean particle diameter ($d_{50}$) versus axial position for the normal operating flowrate of 200 cm/hr is shown in figure 5.4, along with variation in $d_{10}$ and $d_{90}$ with axial position.

Figure 5.4 – Variation in Particle mean diameter with axial position

5.2.2.3 Conclusions

The measurements of radial particle size variation showed that the size distribution was constant within the accuracy of the experimental method ($\pm 5\%$ for triplicate repeats). This demonstrated that the bed was stable under expansion and that the bed exhibited characteristics of constant stratification, with any wall affects infringing less than 5mm into the column. This is a strong indication that
the flow in the column is of plug nature, which is a condition of many of the mathematical assumptions used in the voidage work in this volume.

Measurement of the axial particle size distribution showed the expected decrease in average particle size with increasing axial height, with the largest particles located in the lower axial portions of the bed and the smallest particles adopting high axial positions. This is the behaviour expected on expansion but this is the first time it has proved possible to confirm this behaviour experimentally.

5.3 Voidage Studies

5.3.1 Methods

Bed voidage was estimated from a system of residence time distribution (RTD) curves. For this the expanded bed was fitted with a specially designed liquid sampling device in place of the rubber bungs. This device was designed to remove only the process liquid from within the bed and to leave the matrix particles in the column. This was achieved by the inclusion of a filter mesh constructed from the expanded bed adapter screens mounted across the needle bore. This device was specially designed for the on-line monitoring work discussed in Chapter 4 and is illustrated in 4.2. Samples were taken from the ported column, using a Gilson Minipuls multiroller peristaltic pump (Gilson, Villiers-le-Bel, France) at a flowrate of 2 mL/min. The flow from this pump was monitored for UV-absorbance at 280nm using a UV-1 monitor (Amersham Pharmacia Biotech), and these values logged using a PE Nelson 900 interface
and Turbochrom v4 (both Perkin Elmer Nelson Systems Inc., California, USA). A second UV monitor was placed at the base of the column in the inlet stream. Simultaneous monitoring of the column inlet stream and at the axial sampling point enabled the effects of mixing in the inlet piping before the column on the RTD traces to be accounted for and eliminated.

Voidage experiments were carried out by recording the UV trace resulting from a step change from both water + 0.5 g/L blue Dextran 2000 (Sigma-Aldrich, Poole, Dorset, UK) and water + 0.5%v/v acetone (Sigma-Aldrich) to water. A step change rather than a pulse method was adopted due to technical difficulties in administering a pulse to the expanded bed with the experimental layout used. Measurements were carried out at liquid velocities of 150, 200 and 250 cm/hr and at axial positions of 5-35 cm at 5 cm intervals. Acetone, having a low molecular weight, penetrates both the inter- and intra-particle free spaces, and so should be seen to give a slightly higher voidage reading than blue dextran. Blue dextran was used as this has a very high molecular weight and theoretically does not penetrate the interior of the matrix particles at all, hence only passing through the voids between the particles. This was considered important in terms of viewing the matrix particles as solid spheres to ensure that only the inter-particle void was measured. In order to ensure that only the inter-particle void was being seen while using blue dextran, complete bed voidage trials were carried out at linear liquid flowrates from 100-400cm/h.

Voidage for short sections of bed was hence calculated: Since a fixed axial distance separates any two sampling points in the column the material has to
pass through a known total column volume between these points. This volume is equal to the fluid space plus that occupied by the particles in that section. Subtracting the two RTD traces obtained from two such sampling points then allows the delay time between the two points to be measured and, since the volumetric flowrate is known, the volume of liquid passing between the points can then be calculated. The average voidage, $\varepsilon$, between the points was then estimated using a modification of equation 5.1 below, shown in equation 5.2:

$$
\varepsilon = \frac{V_l}{V_c}
$$

Where $V_c$, the column volume, is redefined as the volume between the two sampling points (a and b) under consideration, with axial heights $h_a$ and $h_b$ in a column of diameter $d$, and $V_l$ is the liquid volume between points a and b (the volume not occupied by matrix particles). The voidage can then be calculated from:

$$
\varepsilon = \frac{V_l}{\pi \left( \frac{d}{2} \right)^2 (h_a - h_b)}
$$

Furthermore, the liquid volume, $V_l$, can be defined by the flowrate, $Q$ and $\Delta T$, delay time between the two axial positions in the column at which samples are removed, obtained from the RTD curves.
Hence the voidage for any section of the bed between two sampling points can be determined. The variables for the equation defined above are shown for clarification in Figure 5.5:

Figure 5.5 – Variables measured in bed voidage calculation

This method relies upon the assumption that the fluid in the column is exhibiting plug flow characteristics and that the tracer only sees the voidage that is exterior to the particles. The extent to which the former is an acceptable assumption can be determined by the calculation of the axial dispersion coefficient in that region of the bed that gives a measure of the extent of mixing occurring within the column volume. The axial dispersion coefficient was calculated from the RTD curve at the sampling points for the section under
consideration using the dimensionless dispersion number, $N_D$, a function of Peclet number, as defined below:

$$N_D = \frac{D_{ax}}{u_e L} \quad 5.4$$

Where $D_{ax}$ is the axial dispersion coefficient, $u_e$ is the interstitial liquid velocity and $L$ is the axial length of the section of bed under consideration, in effect $h_a - h_b$. The dispersion number was calculated using an axial dispersion model for closed vessel boundary conditions (Levenspiel, 1972):

$$\sigma_\theta^2 = 2[N_D] - 2[N_D]^2 \left(1 - e^{-\frac{1}{N_D}}\right) \quad 5.5$$

Where $\sigma_\theta^2$ is the dimensionless standard deviation of the RTD curve. A dispersion number of approaching zero indicates plug flow, and one approaching infinity indicates fully turbulent flow. The above equation was solved for $N_D$ where $\sigma_\theta^2$ was found from the RTD trace. Numerical solutions were found by iteration using Microsoft Excel solver to $\pm 10^{-7}$, and then $D_{ax}$ values were calculated from these. In order to assume the closed vessel boundary conditions used here, the bed must be regarded as a series of discrete “packets” with no transfer of particles from one section to another, and no back mixing of fluid into a section once it has left that section. While these assumptions are not guaranteed to be valid within the bed, the excessive mathematical complexity required to solve the iterations generated by the use of the strictly more accurate
open vessel boundary conditions (Levenspiel, 1972) takes the work beyond the scope of this thesis since the axial dispersion coefficients are only presented here for proof of principle rather than to be used for further calculations.

The second assumption, that the Dextran tracer sees only the column voidage is reasonable given its large molecular diameter compared to the matrix pores. Although the Dextran preparation itself contains some smaller particles that could access the interior of the beads, pulses of Dextran solution produced Gaussian peaks, suggesting that interior penetration does not occur to any significant level.

Measurements of bed settled voidage were carried out by extracting a 1 mL sample of well-mixed settled matrix and then removing the liquid using vacuum filtration, leaving a matrix cake, which was then weighed. Voidage was then calculated based on an assumption of an average particle density of 1.22 g/cm$^3$ (De Luca et al, 1994).

5.3.2 Results

5.3.2.1 Measurement of voidage variation with axial position

Voidage was calculated from RTD traces obtained at axial positions from 5-25 cm and at linear flowrates of 150, 200 and 250 cm/h. Voidage of a section of the bed was calculated from the RTD traces at either end of the section. For the purposes of graphical representation, the voidage calculated was assumed to be the point voidage at the axial midpoint of the section of the bed being
considered. Illustrated in figure 5.6 below is voidage versus axial position for flowrates of 150, 200 and 250 cm/h.

Figure 5.6 – Voidage versus axial position for a range of linear liquid flowrates

In addition, the axial dispersion coefficients at each axial position and flowrate are illustrated in figure 5.7 below. Axial dispersion coefficients were obtained by numerical iteration using Microsoft Excel solver to obtain solutions of equation 5 listed in the method section.
Figure 5.7 – Variation in axial dispersion coefficient with axial position and flowrate

Figure 5.6 demonstrates the increase in voidage with axial position. The values of voidage, particularly at the highest position measured (between 20 and 25 cm axial height) seem to be quite high (the maximum value for voidage, that for a sample of solid dispersed in an infinitely large liquid volume, would be unity). However, the voidage of a settled bed can be assumed to be roughly 0.4 (the voidage value for a volume of uniform spheres) and so all voidage values must be between 0.4 and 1. In the case of the 250 cm/h data in figure 5.6, the overall height of the expanded bed at this flowrate was approaching 50 cm. This would correspond to an AVERAGE voidage (for the entire bed) of approximately 0.82 (settled volume = 300mL, so with a voidage of 0.4, = 180 mL matrix in a total expanded volume of 980 mL). While this suggests that the voidage values of 0.8 – 0.95 measured in the bed could be 5% higher than this average would suggest,
this is still a reasonable error margin and is indicated by the error bars on figure 5.6.

5.3.3 Conclusions

This work demonstrates that the voidage of small sections of the expanded bed can be determined using a system of residence time distribution-type step down curves at various axial positions in the bed. The voidage data obtained show that voidage increases with both axial position and linear liquid flowrate within an expanded bed.

Voidage can be seen to approach unity asymptotically at high liquid flowrates and axial positions – however no fit is shown for this data, as a suitable mathematical expression to match the experimental data cannot be found. Shown in Figure 5.8 below is a plot of voidage versus linear flowrate for different axial positions in the column.
It proved problematic to fit any form of theoretical curve to this data accurately – as voidage approaches unity at high flowrates and axial positions the fitted curves agreed less with the experimental data. The further consideration of this issue, along with continuing theoretical mathematical modelling of voidage within an expanded bed could be the subject of future work.

These data do however demonstrate that, since both particle size distribution and voidage vary with axial position in the bed, then chromatographic performance is also likely to vary, since particle size affects specific surface area available for adsorption and both this and voidage are important parameters in chromatographic kinetics. This information, coupled with the variable utilisation...
within the bed demonstrated in Chapter 4, leads to the final section of work in
the thesis on matrix utilisation and productivity.
Chapter 6 – Investigation of Matrix Utilisation

The work in this Chapter focuses on studies of matrix utilisation. Since previous work had showed that the utilisation of the matrix varied at different positions in the bed during a typical run, the matrix was separated into fractions, and those comprising the smallest and largest particles were studied. These fractions were run as discrete expanded beds. Results showed that, at product breakthrough levels of 30% or less, the larger particles were utilised in a far more efficient manner than the smaller particles. The smaller particles however, having a larger total binding capacity, would become more effective at higher product breakthrough levels.

6.1 Introduction

Results shown in previous Chapters demonstrate a variation in the utilisation of the matrix with axial position in the expanded bed. Facilitation of internal sampling during load, wash and elution stages has shown that, for a typical breakthrough level of ADH C/C₀ = 0.1, 60% of the product is bound in the bottom 33% of the settled bed. While this would be expected, since the direction of the load is upwards, thus exposing the bottom sections of the bed to higher concentration driving forces than the upper sections, the differences in levels of discrepancy between matrix utilisation in the bottom third of the settled bed (60 %) and the top third (13%) is still very pronounced. This lead to consideration of the possibility of separating the bed into sections of varying
particle size ranges, corresponding as closely as possible with the thirds of the settled bed mentioned above and studied during on-line monitoring of system elution described previously. These sections could then be studied separately and described in terms of binding and elution characteristics, and hence binding capacities (both total and dynamic) and eventually, productivity.

6.2 Methods

6.2.1 Bed Segregation

In order to consider the bed as separate sections of varying particle size distribution, it was necessary to separate the bed into sections that as closely as possible correspond to the sections shown in the on-line monitoring sections earlier, i.e. the top, middle and bottom thirds of the bed during settled elution.

To achieve this, the bed was expanded at such a flowrate as to allow sampling points to correspond to the chosen divisions between these three sectors. During early attempts, a liquid linear flowrate of between 200 and 300 cm/h (using either buffer or 20 mM phosphate buffer) was employed to give an expanded height of approximately 45 cm. This allowed the sampling points at 15 and 30 cm to be used to divide the three defined sectors of the bed. It soon became apparent that this was likely to result in a proportionally larger bottom third, where the voidage was lower, and vice versa in the top third. Sampling positions had to be altered to compensate for this, resulting in the use of ports at 10 cm and 25 cm to separate a 45 cm expanded bed. This produced three beds of
approximately the same settled volume (100mL ± 5%) and was the most accurate separation that could be achieved using the fixed position sampling ports.

The bed was then split by simply removing the plug in the sampling point at the chosen height and collecting the liquid and matrix forced out by a combination of gravity and the upflow of liquid in the column. Removing the plug at 25 cm axial height for example would allow all the liquid and matrix from above that point to be collected – in effect the top third of the bed while expanded. This process was then repeated at the 10 cm axial position to collect the middle third of the bed. The bottom third was then removed from the bed using the conventional unpacking method and collected.

This method relies on removal of a variable volume of matrix and process fluid to result in a constant settled volume of matrix.

These separated thirds were utilised for a series of experiments to study capacity and breakthrough characteristics in order to determine possible variations due to particle size. The beds so formed are termed “segregated beds” from now on.

6.2.2 Particle Size Ranges

These segregated partial beds were studied to discover the size range of particles contained within each section. Particle size measurements were carried out in a Malvern 3600 E Laser Sizer (Malvern Instruments, Great Malvern, UK) and the
particle size distribution curves were quantified using the measurements $D_{10}$, $D_{50}$ and $D_{90}$, these being; respectively, the particle diameter value at which 10%, 50% and 90% of the number of particles in the sample are smaller than the given diameter. $D_{50}$ is equivalent to the medial particle diameter for the sample on a number basis, and was used as the main description for these systems. However $D_{10}$ and $D_{90}$ give respectively measures of the proportion of very small and very large particles present in the system – since particle settling velocity and hence bed expansion depend on $(\text{particle diameter})^2$ as described in Chapter 6, then knowledge of the variations in particle distribution at the extremes of size ranges present would give useful information regarding bed expansion behaviour.

6.2.3 Bed Breakthrough Behaviour

Studies were carried out on the breakthrough behaviour of the enzyme ADH from the Yeast/Hydrophobic Interaction system used for work earlier in this thesis. Work was carried out using packed beds of the separate matrix samples to assess the total binding capacity of the different matrix sections and expanded bed work was carried out to assess breakthrough characteristics and hence dynamic binding capacity.

6.2.3.1 Packed Bed Experiments – Determination of Total Binding Capacity

Packed bed chromatography was used to determine total binding capacity of each of the separated sections of the expanded bed. Matrix was packed into XK16/20 columns (Amersham Pharmacia Biotech) to a volume of 20 mL (10 cm
packed height). Packing was carried out using an FPLC system, consisting of an LCC-500 controller, two P-500 pumps and a Frac-100 fraction collector. The output from the column was monitored for UV-absorbance at 280nm, and these values logged using a PE Nelson 900 interface and Turbochrom v4 (both Perkin Elmer Nelson Systems Inc., California, USA). Columns were packed at up to a linear liquid flowrate of 300 cm/h, to prevent further bed compression while running at 200 cm/h.

Samples were assayed for ADH content only, using the off-line method detailed in Chapter 2 for these trials, bakers' yeast was disrupted in a high pressure homogeniser (Lab 60, APV Ltd., Crawley, U.K.) fitted with a restricted orifice discharge valve for 5 discrete passes at 500 bar and 5°C and then a concentrated solution of KH₂PO₄ and (NH₄)₂SO₄ added to make the solution up to the concentration of 0.02M KH₂PO₄/0.78M (NH₄)₂SO₄. This was clarified using a Beckman J2-MI centrifuge (Beckman Instruments Inc., California) at 16000 g for 30 minutes.

Columns were equilibrated in Buffer A (0.02M KH₂PO₄/0.78M (NH₄)₂SO₄) for 5 column volumes and then the clarified feedstock was loaded onto the columns at 200 cm/h linear velocity. Loading continued until 100% ADH breakthrough was noted by off-line assay and then the column was washed using buffer A until a UV 280 nm baseline was reached. The ADH bound to the matrix was then eluted using buffer B (0.02M KH₂PO₄) at 75 cm/h and the eluate peak collected and assayed for ADH content. This method was performed using
representative samples of all 3 separated sections of the expanded bed. Total
binding capacity can then be calculated from the quantity of ADH eluted and the
matrix volume in the column.

6.2.3.2 Expanded Bed Experiments – Determination of breakthrough
Characteristics and Dynamic Binding Capacity

Experiments were carried out using the separated sections of the expanded bed
described above, replaced in the modified STREAMLINE 50 bed and run under
previously described conditions (Chapter 4). On-line monitoring was used to
control the breakthrough of the target protein to different levels, and thus the
quantity of ADH bound at different breakthrough levels could be assessed for
different sections of the bed. This enabled dynamic binding capacities to be
calculated using the method described below.

By plotting breakthrough levels against process volume, the area under the curve
can be integrated as illustrated in figure 6.1 below:
**6.2.3.3 Determination of matrix productivity**

In order to consider further the use of beds consisting of segregated particles, consideration was given to the productivity of each of the smaller beds, based upon productivity versus matrix volume.

Productivity with respect to matrix volume is the same in effect as dynamic binding capacity at a given breakthrough level – units on product bound per mL of matrix used.
6.3 Results

6.3.1 Particle size distributions

Samples were taken from each of the three segregated beds, along with a representative sample of the original STREAMLINE Phenyl matrix as from which the segregated beds were generated. These were sized in a Malvern Laser sizer and decumulative particle size distributions were created. From this information, the characterising values $d_{10}$, $d_{50}$ and $d_{90}$ could be calculated and are noted below in Table 6.1:

Table 6.1 – Particle characterisations for segregated beds

<table>
<thead>
<tr>
<th></th>
<th>Bottom third (&quot;boulders&quot;)</th>
<th>Middle third (&quot;fines&quot;)</th>
<th>Top third (&quot;fines&quot;)</th>
<th>Standard matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{10}$</td>
<td>180</td>
<td>149</td>
<td>131</td>
<td>155</td>
</tr>
<tr>
<td>$d_{50}$</td>
<td>236</td>
<td>184</td>
<td>178</td>
<td>198</td>
</tr>
<tr>
<td>$d_{90}$</td>
<td>320</td>
<td>239</td>
<td>232</td>
<td>260</td>
</tr>
</tbody>
</table>
6.3.2 Bed breakthrough characteristics

6.3.2.1 Packed bed – Total binding capacity

Total binding capacity was found by loading packed beds of each of the sections of segregated matrix to 100% ADH breakthrough. Total capacities are listed in Table 6.2 below. Total binding capacities in mg/mL were calculated from those in U/mL using the activity of pure yeast ADH (Sigma), given as 340 U/mg protein.

Table 6.2 – Total binding capacities of segregated beds

<table>
<thead>
<tr>
<th></th>
<th>Bottom third (“boulders”)</th>
<th>Middle third (“fines”)</th>
<th>Top third</th>
<th>Standard matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Binding Capacity</td>
<td>661</td>
<td>1235</td>
<td>1322</td>
<td>1050</td>
</tr>
<tr>
<td>(U/mL matrix)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Binding Capacity</td>
<td>1.9</td>
<td>3.6</td>
<td>3.9</td>
<td>3.1</td>
</tr>
<tr>
<td>(mg/mL matrix)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.3.2.2 Expanded bed – Breakthrough curves

Illustrated in Figure 6.2 below are the breakthrough curves for the bottom and top thirds of the bed (two sections of equal settled matrix volume - the “boulders” and “fines” respectively) up to 30% ADH breakthrough, showing the differences between the two in terms of product breakthrough characteristics:

Figure 6.2 – ADH breakthrough curves for beds of equal settled volume but variable particle size distribution (“boulders” and “fines”)

![Breakthrough Curves Graph]

- ■ Small particles ($d_{50} = 150 \mu m$)
- ○ Large particles ($d_{50} = 182 \mu m$)
6.3.2.3 Expanded bed – Dynamic binding capacity

Dynamic binding capacity was calculated by integrating the area under the breakthrough curves for fines and boulders respectively, using Microcal Origin, and is expressed per mL of matrix. The results are illustrated in Figure 6.3 below:

Figure 6.3 – Dynamic binding capacities of ADH per mL of matrix as a function of breakthrough levels for beds of equal settled volumes but different particle size distributions
6.3.3 Matrix utilisation and productivity

Productivity of the expanded bed can be expressed with respect to several process factors – in this work the productivity is expressed as quantity of ADH purified with respect to volume of matrix used. Productivity with respect to matrix volume is effectively the same as dynamic binding capacity, and hence can be calculated from the area under a breakthrough curve. Hence the productivity of the segregated beds (beds smallest and largest particles only) is shown in Table 6.3 below:

Table 6.3 – Productivity of segregated beds with respect to volume of matrix used (expressed in mg protein purified/mL matrix used) at different breakthrough levels

<table>
<thead>
<tr>
<th>Segregated bed designation</th>
<th>ADH breakthrough level (C/C₀)</th>
<th>Productivity (mg ADH/mL matrix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smallest Particles</td>
<td>0.05</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.8</td>
</tr>
<tr>
<td>Largest Particles</td>
<td>0.05</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>1.8</td>
</tr>
</tbody>
</table>
6.4 Discussion

As can be seen clearly from the dynamic binding capacity data illustrated in Figure 6.4 and Table 6.3, the larger particles in the bed have a much higher binding capacity at low levels of ADH breakthrough when compared to that of the smaller particles. This is of great interest because the majority of industrial uses of chromatographic separations operate in the low breakthrough range, most particularly when dealing with a product of very high value such as a therapeutic protein. The dynamic binding capacities of the segregated beds can be compared with that of a full bed of STREAMLINE Phenyl matrix as supplied. The dynamic capacity at 5% ADH breakthrough (Smith, 1997) was found to be 0.95 mg/mL of matrix and so it can be seen that, at low breakthrough levels, the vast portion of the binding capacity of the bed is being provided by the lower part of the bed. This correlates well with the results presented in Chapter 4 of this thesis, demonstrating again that, under conditions of low product breakthrough, the lower portions of the bed are well utilised but the smaller, higher expanding particles have little or no effect on the capacity. In-bed monitoring of the elution phase of a run where a full bed was controlled to 10% ADH breakthrough showed limited utilisation of the upper sections of the matrix when compared to the lower sections.

In addition the shapes of the traces in figure 6.4 is of considerable interest – while the that of the larger particles is hyperbola-like, tending asymptotically to approximately 1.9 mg/mL matrix, that of the smaller particles seems to be becoming more exponential at higher breakthrough levels. This again compares
well to the total binding capacities of the two particle size ranges. Although lack of data means dynamic binding capacities at higher breakthrough levels can only be predicted, it appears that the larger particles have virtually reached 100% binding capacity of 1.9 mg/mL matrix by approximately 30% ADH breakthrough. In contrast the small particles have only just reached 30% of their total binding capacities. The mechanism and reasons behind this cannot be fully explained without considerable further modelling and experimental work, but the most likely explanation would seem to be related to collision frequency - the bed of larger particles has a considerably smaller expanded volume at the operating flowrate then that of the smaller particles, and hence has a smaller voidage (~0.5 at 200 cm/h compared to 0.8 for smaller particles). This means a smaller amount of free space for product molecules to pass through, increasing the likelihood of collisions with matrix particles in the more compact bed. This in turn should lead to a faster utilisation of available binding. The smaller particles in comparison, having a larger specific surface area by virtue of their reduced average diameters, have a considerably higher total binding capacity than the larger particles, but take longer to reach that capacity.

With respect to productivity, it can be seen that the productivity varies considerably with particle size – this is due to a combination of the higher dynamic capacity at low breakthrough levels of the larger particles, coupled with the lower expanded bed volume shown by a bed consisting of larger particles. Since most phases of expanded bed operation are performed on a column volume basis, a smaller overall column volume is bound to result in a lower buffer consumption. In addition a shorter column results in a shorter process
time. Previous work has always been based on studies indicating that shorter beds have lower stability and hence reduced chromatographic separation performance. This, coupled with the reduced specific surface area of larger particles, has meant that the only significant attempts to reduce bed expansion have been made by attempting to increase the density of the matrix and hence allow higher linear flowrates while maintaining bed stability. While this approach is highly valid, the risk is that an increased linear flowrate may impinge on the kinetics of some systems, particularly those that are affinity ligand based.

The issue of chromatographic resolution has less relevance in the field of expanded bed adsorption as compared to conventional packed bed chromatography. Expanded bed systems are rarely used in anything other than capture steps, and as such are not usually required to perform, say, the separation of two similar components during an elution. Capture steps are concerned primarily with clarification and concentration, along with the removal of major contaminants. Because of this, the segregated beds demonstrated here could be of great importance in the area of tailoring downstream purification operations to suit individual purifications. While narrower particle size ranges and lower bed heights may reduce the separation efficiency of the bed, this will not cause problems in capture steps and the advantages of improved productivity, lower operating times and lower buffer consumption should far outweigh any loss in chromatographic performance.
This work is to be considered only partially completed, to the degree that the timescale of this study permitted. Significant further progress could be made along these lines, and will be discussed in Chapter 7

6.5 Conclusions

The work conducted in this Chapter has demonstrated the variation in experimental performance of both the smaller and larger ends of an expanded bed matrix particle size distribution. Larger particles would seem to perform more efficiently at lower breakthrough levels and hence lower dynamic capacity requirements, whereas smaller particles contribute to the high binding capacity required at higher breakthrough levels. This of course leads to the suggestion that, pending a more in depth study of this work, the possibility exists to customise expanded bed matrix particle size ranges to suit the different requirements of different processes.
Chapter 7 – Conclusions and Future Work

The objectives of this work were described in the introduction at the start of this thesis. They were threefold: Firstly, the development of a new chromatographic protocol suitable for control and monitoring studies, and the transfer of this protocol to an expanded bed adsorption system. Secondly, the development of a fast monitoring system for improved control and utilisation of expanded bed matrices, and finally, the demonstration of this fast monitoring system using realistic feedstock.

The extent to which this has been met will be discussed in this Chapter and summarised at the end.

7.1 IMAC

7.1.1 Conclusions

The aim of this part of the project was to develop a novel affinity purification protocol from initial scouting right through to pilot scale expanded bed level. This work demonstrates that yeast ADH can be effectively purified from unclarified homogenates of pressed baker’s yeast using immobilised metal-ion affinity chromatography in an expanded bed. The recovery of ADH on elution from the expanded bed compares favourably with other results shown in the literature, although concerns about the long-term stability of the matrix must be addressed before more detailed conclusions can be drawn. It can be concluded,
however that IMAC, as far as has been investigated, fits the required criteria for an initial capture step for the target protein. The protein is both captured from a crude feedstock, concentrated and cell debris removed, with the additional benefit of a large (ten-fold) decrease in the contaminating protein burden. Since the initial requirements of a preliminary purification step are simply capture, clarification and concentration (as would be achieved by more traditional engineering steps such as centrifugation or filtration) then the benefit of removal of protein contaminants means that the burden on further purification steps will be greatly reduced. Consequently it can be stated that expanded bed adsorption using an IMAC matrix is an effective initial step in a purification scheme for ADH from crude yeast homogenate.

7.1.2 Future work

The major concern and therefore source for possible future study in this area lies with the long-term stability and therefore reproducibility of the protocol. A more rigorous examination of the CIP procedure may allow longer matrix life in this process. In addition, while the purification factor with respect to contaminating protein achieved by this method is good for an initial capture step, further work on the elution protocol may allow even greater improvements in purity of the eluted product. This would increase the effectiveness of this step in a purification protocol.
7.2 On-line monitoring and control

7.2.1 Conclusions

With this section of the project, the initial target was to develop a rapid monitoring system capable of controlling an expanded bed running with a realistic crude feedstock. This was achieved using a combination of in-bed sampling and a stopped flow analysis technique, allowing fast and accurate control of the breakthrough of ADH during its purification from yeast homogenate. In addition, in-bed monitoring of the elution phase of the purification provided information regarding distribution in matrix utilisation (with respect to axial position), which led onto the final section of work regarding matrix utilisation.

Compared with more traditional off-line monitoring approaches this work shows the value of reducing the time between an event occurring and the data from that event becoming available to the user. In cases such as are shown in this thesis where the breakthrough of product is very rapid during the loading phase, the loss of an expensive target (for instance, a therapeutic such as an antibody fragment) can be minimised by rapid monitoring and control. At the same time, productivity of a piece of chromatographic equipment can be maximised. This happens because it removes the need for safety margins to prevent product loss. Off-line methods in a situation where any product loss due to breakthrough is economically unsound would have to be based on previous data as to when breakthrough would occur during the load step. Then, any protocol would have to be based on a worse case scenario – that is, where the initial breakthrough had
occurred at the earliest time point in previously acquired data. This, in turn, could lead to under-utilisation of the beds capacity and hence economic inefficiency. Using on-line rapid monitoring from around the top of the bed (while expanded) would allow the load to be stopped accurately at the point of product breakthrough regardless of any run-to-run variations.
7.2.2 Future Work

Although the aims of this section were met, there are still areas that would merit further study. Repeating the work performed here with a different target protein and different feedstock would further confirm the validity of the approach adopted here. In addition, for industrial applications, a less intrusive sampling system would be of benefit however to achieve this without compromising the effectiveness of the monitoring system would require considerable work.

7.3 Particle size distribution and voidage work

7.3.1 Conclusions

The methods adopted in this work demonstrated experimental determination of both radial and axial particle size distributions, along with a method developed for the determination of the voidage of short sections of expanded bed. The results presented correlate well with available mathematical descriptions for similar systems and allowed a greater insight than has previously been demonstrated into the hydrodynamic behaviour of a bed under expansion.

Work previously performed (De Luca, 1994 and Thömmes, 1997) has studied the areas of bed hydrodynamics and particle size distribution but a study of particle behaviour carried out from within the bed has not been conducted prior to the work presented here. The advantage of internal monitoring is that it allows
a much more detailed picture of the spread of particles of differing sizes within the bed to be carried out. This work has shown that the bed remains stable in terms of particle size distribution in both the radial and the axial plane, which is vital in the maintenance of stable, plug flow in the column. Although this behaviour can be predicted mathematically this experimental verification is critical for a plug flow assumption to hold during operation.

7.3.2 Future work

The experimental results shown here can be correlated to available mathematical expressions; however, the development of a unified particle size/voidage model specifically for expanded bed adsorption would allow the experimental data to be confirmed in more detail, along with enabling more accurate prediction of bed behaviour under representative varying conditions of process operation.

7.4 Matrix Utilisation

7.4.1 Conclusions

This was the final work undertaken, and stemmed from observations during earlier parts of the project. Results demonstrated that the dynamic binding capacity of the matrix varied considerably with particle size, such that, at low breakthrough levels, larger matrix particles had much higher dynamic binding capacities than smaller particles. Smaller particles had higher total binding
capacities however, as would be expected due to their higher specific surface area.

This suggests that, when operating at low breakthrough levels, a segregated bed consisting of the larger particles only would have a higher productivity based on units of product processed per unit of matrix volume. This, coupled with the shorter process time and lower buffer consumption that comes from a lower expansion height at a given flowrate, would produce a more efficient and productive bed.

7.4.2 Future work

As mentioned in Chapter 7, this work must only be considered partially complete, and there are several possible areas that could be investigated in the future. 100% breakthrough curves could be used to complete the characterisation of matrix particle properties, and demonstrate productivities at higher breakthrough levels than were considered in this study. In addition, more complete consideration of bed height would allow process time and buffer consumption to be investigated in more detail. This work only considers shorter segregated beds when information regarding segregated beds of the height recommended by Pharmacia (15cm settled height) would enable further insight into bed performance.
7.5 Final Conclusions

In respect to the initial objective of this work, the development of a protocol suitable for monitoring and control studies, this has only been partially achieved. Although a successful IMAC protocol was developed and optimised, concerns regarding the complexity of the protocol as well as limited knowledge regarding the long-term reproducibility of the experiments led to the adoption of a more well characterised, robust, HIC protocol.

This protocol was successfully adapted for use in the monitoring and control studies and, along with an adaptation of a stopped flow analysis system previously developed at UCL (Habib et al, 1997), a system was developed to facilitate the rapid monitoring and analysis required for the control studies planned. In this respect the second objective of the thesis has been met fully.

The final objective, the demonstration of the validity of this rapid monitoring using a realistic feedstock, was also carried out successfully using Bakers’ yeast homogenate.

In addition, the work described here went still further, investigating binding behaviour within the expanded bed, and demonstrating variable binding performance in different regions of the bed seemingly dependant on particle diameter.
In these respects, the initial aims of the thesis have been met for the most part, and in some areas they have been surpassed.
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Appendix: Expanded Beds - Fluidisation theory

Introduction

Fluidisation is the process whereby a continuous stream of fluid (gas or liquid) is allowed to pass through a bed of solid particles, with the separation of the particles caused by the fluid flow itself. Fluidised reactors and beds have been used in many applications in the chemical industry for well over half a century, and their use is well documented in such applications as catalytic cracking (where a stream of hydrocarbon gas is allowed to contact a solid catalyst to force a splitting reaction creating smaller molecules). This is one example of gas-solid fluidisation. However, the less common (at least in the chemical industry) process of liquid-solid fluidisation is the basis of this research. In the field of Biotechnology, chromatographic separations have been successfully carried out using expanded beds, and a dedicated series of chromatography columns and matrices are available from Pharmacia Biotech (STREAMLINE™ components) solely for expanded bed operations.

Fluidisation theory for liquid-solid systems

Liquid can flow either up or down through a bed packed with solid particles. In term of fluidisation, flow downward through a bed is of little interest since the
bed cannot become fluidised in this state. However flow downwards in the bed is important for other reasons and will be discussed elsewhere.

When liquid flows upwards through the bed, pressure drop from along the length of the bed will be directly proportional to the flowrate of the liquid at low flowrates, and the bed remains in a “packed” configuration. However as flowrate increases, and the apparent weight of the particles becomes equal to the frictional drag exerted on them by the fluid flow (the apparent weight is the actual weight of the particle minus the buoyancy of the particle in the liquid concerned) then the particles begin to move and rearrange themselves so as to offer less resistance to liquid flow - the bed is beginning to expand. This point is known as the fluidising point. This process continues with increasing liquid flowrate until the particles reach the state where they are offering the least possible resistance to fluid flow. This is the situation where the pressure drop is highest, since further increases in liquid flow result in the particles becoming further separated, but since the resistance to flow remains approximately the same so does the pressure drop. At this stage the bed is said to be fluidised.

In the fluidisation of a particulate bed, it is vital that the fluidisation is as good as possible (free of channelling, regions of backmixing, etc.). This is achieved with plug flow of liquid through the bed. This is very difficult with a bed of solid particles, especially if the are of irregular shapes, or if they aggregate easily. Hence the study of flow patterns is important to achieve good fluidisation.

By plotting log $\Delta P$ (pressure drop along column) against log $U$ (superficial liquid velocity) as shown in figure A1 the effects described above are shown graphically. Log $\Delta P$ increases linearly until the point A (the fluidising point).
The pressure drop continues to increase until it reaches a maximum (point B) before falling slightly and becoming independent of superficial velocity and hence independent of flowrate. (region CD). Note that if velocity were reduced again the pressure drop would remain constant until the particles were just touching one another (point E - the point of maximum stable porosity for a fixed bed). The bed would in theory remain in this state as flowrate was further lowered but in practice any external vibrations would cause particle rearrangement and a return to the line OA rather than the theoretical line EF, with lower pressure drop for a given superficial velocity.

Figure A1 – Illustration of different stages of particle fluidisation

In order to relate the pressure drop to the difference in density of the solid and liquid phases, it is necessary to perform an unsteady state momentum balance. Given that a fluidised bed can be assumed to be a two phase closed system, bounded by the bottom of the bed vessel (plane b) and the upper surface of the particles (plane t) then a momentum balance, after converting vectors to scalars for simplicity, yields:
\[
\frac{\partial}{\partial t} P_{\text{tot}} = \rho_f \left( u^*_f \right) S - \rho_f \left( u^*_f \right) S + p_i S - p_t S - F - mg
\]  

(1)

In the above expression, \( P_{\text{tot}} \) is the total momentum of the system, \( S \) is the cross-sectional area of the column and \( u \) is the local velocity at any given point. The terms on the right hand side of the system represent, respectively, the rate of momentum in to the system by bulk motion, the rate of momentum leaving the system by bulk motion and the pressure forces exerted on the system by the bottom and top planes. \( F \) is the force exerted by the surfaces of the solid in the system on the liquid and \( mg \) is the force on the liquid due to gravity. Since in an expanded bed of solid particles, the surface area of particles is generally much greater than the internal surface area of the containing column then the latter can be neglected hence \( F \) is the force exerted by the surface area of the entrained particles only, and is therefore equal to the weight of these particles.

\[
F = (1 - e)SH\rho_s g
\]  

(2)

Where \( H \) is the height of the bed and \( e \) is the voidage, or the fraction of liquid contained in the bed. At steady state \( \partial / \partial t (P_{\text{tot}}) = 0 \).

Assuming constant velocity profiles across the bed, and that the liquid in the system is effectively incompressible, \( \rho_f = \rho_{f,0} \) and \( \left( u^*_f \right) = \left( u^*_f \right) \)
The force acting on the fluid due to gravity, $mg$, can be converted to a more manageable form by replacing the mass term thus $mg = e \rho_f S H g$

If all these terms are substituted into the fundamental momentum balance it becomes

$$\quad (e - 1)H \rho_s g - e \rho_f H g = p_t - p_b$$

(3)

Now defining $P = p - \rho_f g h$ where $h$ is distance to any plane (so if $h$ is distance to plane $t$ then $(H-h)$ is distance to plane $b$) then by substitution and rearrangement,

$$-\Delta P = P_t - P_b = (1 - e)(\rho_s - \rho_f)H g$$

(4)

This expression holds while the bed is in the fluidised regime.

Minimum fluidisation velocity

As the upward flow of liquid through a packed bed of uniformly shaped particles increases, a certain point is reached when the particles are just freely supported in the liquid. This is known as the point of incipient fluidisation. At this point a corresponding minimum fluidisation velocity $u_{mf}$ can be calculated based on the
Carman-Kozeny equation for the voidage in a fixed bed. This equation is only valid given STREAMLINE flow in a bed packed with uniform spheres of diameter $d$, but these are the conditions that apply in a well-packed expanded bed utilising a standard beaded matrix. The Carman-Kozeny equation is based on the theory that the pressure drop in a fixed bed is equal to the buoyant weight of the solid particles supported in it. This holds until the transition to fluidisation.

The equation below is obtained when the voidage at the point of incipient fluidisation ($e_{mf}$) is substituted into the Carman-Kozeny equation:

$$u_{mf} = 0.0055 \frac{e_{mf}^3}{1 - e_{mf}} \frac{d^2(\rho_s - \rho_f)g}{\mu} \quad (5)$$

Although the voidage at the point of incipient fluidisation depends a great deal on the particle size, shape and even density, for uniform spherical particles it would be assumed to be about 0.4. Thus substituting this into the above equation:

$$\left( u_{mf} \right)_{e_{mf}=0.4} = 0.00059 \frac{d^2(\rho_s - \rho_f)g}{\mu} \quad (6)$$

Under some conditions, such as when the particles are too large for STREAMLINE flow to take place at the point of fluidisation, a more general
equation based on the pressure gradient in the bed (for example the Ergun equation):

\[-\Delta P = 150 \left(1 - \frac{e^2}{e^3}\right) \frac{\mu u_{mf}}{d^2} + 1.75 \left(\frac{1 - e}{e^3}\right) \frac{\rho_f u_{mf}^2}{d}\]

(7)

Thus by substituting $e = e_{mf}$ at the point of incipient fluidisation and $-\Delta P$ from equation (4) the equation can be expressed in terms of $u_{mf}$:

\[(1 - e_{mf})(\rho_s - \rho_f)g = 150 \left(1 - \frac{e_{mf}}{e^3}\right)^2 \frac{\mu u_{mf}}{d^2} + 1.75 \left(\frac{1 - e_{mf}}{e^3}\right) \frac{\rho_f u_{mf}^2}{d}\]

(8)

Then by multiplying both sides by $\frac{\rho_f d^3}{\mu^2 (1 - e_{mf})}$ to express the equation in terms of dimensionless numbers:

\[
\frac{\rho_f (\rho_s - \rho_f) g d^3}{\mu^2} = 150 \frac{1 - e_{mf}}{e^3} \frac{u_{mf} d \rho_f}{\mu} + 1.75 \left(\frac{u_{mf} d \rho}{\mu}\right)^2
\]

(9)

In this equation several dimensionless numbers can be inserted for ease of expression. For instance, $\frac{d^3 \rho_f (\rho_s - \rho_f) g}{\mu^2}$ is the Galileo number $Ga$, and $\frac{u_{mf} d \rho_f}{\mu}$ is a modified form of the Reynolds number and in this case will be designated the Reynolds number at minimum fluidisation velocity or $Re_{mf}$.

This allows equation (9) above to be simplified to:
\[ Ga = 150 \frac{1 - e_{nf}}{e_{nf}^3} Re_{nf} + \frac{1.75}{e_{nf}^3} Re_{nf}^2 \]  

And since by definition \( u_{nf} = \frac{\mu}{d \rho_f} Re_{nf} \) and the voidage at minimum fluidisation can be predicted or measured then the minimum fluidising velocity for a given system can be obtained.

Models for describing bed expansion

In order to meet the initial aims of the thesis, it is important to consider the behaviour of the individual particles within and expanded bed as a route to predicting the behaviour of the whole bed. To this aim, this section introduces published mathematical models for describing bed behaviour.

There are two main methods for describing the behaviour of a bed of particles during fluidisation. In general liquid-solid fluidised systems are characterised by the regular expansion of the bed as velocity increases above the minimum fluidising velocity (but below terminal particle falling velocity). The general relationship between liquid linear velocity and voidage is very similar to the relationship between sedimentation velocity and particle volumetric concentration in a solid-liquid suspension. This is perhaps not too surprising as the systems are hydrodynamically very similar, with the “stationary” phase being the solid particles in the case of an expanded bed and the liquid in the case
of a suspension. The utilisation of this fact in a model to predict behaviour was first postulated by Wilhelm and Kwauk (1948) and further developed by Richardson and Zaki (1954) and was dubbed 'the cell model' as it treats one particle as being limited to movement within a fixed cell. The cell is taken to consist of two concentric spheres, the radius of the inner being that of the particle. It was noted by these workers that:

\[ \frac{u_e}{u_i} = e^n \]  

(11)

Where \( u_e \) is the fluidisation velocity, \( u_i \) is the corresponding velocity at infinite dilution, \( e \) is the voidage and \( n \) is an index. This was seen by plotting particle Reynolds number against bed voidage over a range of conditions during fluidisation. Values of \( n \) were found to range from 2.4 to 4.8 and can be found for know Galileo number \( Ga \) given the expression

\[ \frac{4.8 - n}{n - 2.4} = 0.043Ga^{0.57} \left[ 1 - 1.24 \left( \frac{d}{d_i} \right)^{0.27} \right] \]  

(12)

Although the conditions are similar for both sedimentation and fluidisation, Richardson and Zaki found that while \( u_i \) and \( u_o \) (free settling velocity of a particle in infinite medium) are very similar for sedimentation they were found to vary in fluidisation and proposed the following equation:

\[ \log_{10} u_o = \log_{10} u_i + \frac{d}{d_i} \]  

(13)
Since $d/d_t$ is the ratio of particle diameter to tank diameter, it would in all probability be very small in a sedimentation tank but may be significant in a fluidised column. This is believed to account for the difference noted by Richardson and Zaki. More recently modifications of the above correlation have been developed [Khan and Richardson 1989] to allow for the influence of the walls of the vessel during fluidisation. When voidage is plotted against superficial liquid velocity on logarithmic co-ordinates, the voidage remains constant at low flowrates (fixed bed regime) and then increases in a linear fashion with velocity (fluidised regime).

The second and more widely used model is based on viewing the bed as a series of bundles of tangled tubes for fluid flow. If these tubes could be viewed as individual straight tubes, then the bed could be modelled based on this. Based on a derivation of the Hagen-Poiseulle equation but replacing the mean tube velocity $u$ in the equation with the interstitial velocity $u/e$ and the tube diameter with the hydraulic diameter $D_H$ gives the following modified version of the Hagen-Poiseulle equation:

$$\Delta P = \frac{2(36)\mu u H(1-e)^2}{d^2 e^3}$$

(14)

This model is however flawed slightly as it would be erroneous to assume that the bed consists of straight-pathed tubes as modelled by the above equation. In order to take account of this the value 2 at the start of the equation is replaced
with the value $25/6$. This results in the Blake-Kozeny equation. The modification of the value 2 is known as the tortuosity factor $\tau$ where:

$$\tau = \frac{\text{average path length}}{\text{bed height}}$$

(15)

This model can be used to predict the behaviours of both fluidised and fixed beds, but it must be noted that the tortuosity varies for fluidised beds but remains constant for fixed beds. A completely fluidised bed will have a tortuosity value of approaching unity.

Effect of particle size distribution on fluidisation velocity

In general it can be assumed that, if the particles being fluidised have a uniform size then the transition between fixed and fluidised bed will be categorised by a discrete step on the pressure drop-liquid velocity curve (figure 1.5). However as the distribution of particle sizes becomes more spread, the transition tends more towards a smooth curve, thus creating three discrete regions on the pressure drop-fluidisation curve; the fixed bed region, the fully fluidised region and a transition region separating the two. In this case there is no specific minimum fluidising velocity, but a small range of velocities from the velocity at the onset of fluidisation (often known as $u_{mf}$) and the velocity at which the bed is totally fluidised (known as $u_f$). In this case the value for minimum fluidisation velocity must be approximated, and can be obtained from equations such as (14) using a mean particle diameter value (often approximated to diameter at 50%
total frequency on a particle diameter vs. frequency plot) so long as the value for
the voidage at minimum fluidisation velocity is known. This can usually be
approximated to $e_{mf} = 0.4$ although [Wen and Yu 1966] found that
$e_{mf} = 0.42$ gave improved bed stability.

Effect of particle size distribution on voidage

In a fluidised bed with uniform sized particles, uniform voidage would also be
expected. However in the case of a bed with a size distribution, the particles will
segregate according to their terminal falling velocity in the fluid, and as such the
voidage will vary along the length of the bed as the particles are segregated. This
presents problems with respect to system modelling since the void fraction is
variable with height in the bed.

This problem has been considered by [Wen and Yu 1966] who studied binary
systems and stated that, if the ratio $\frac{\text{largest particle size}}{\text{smallest particle size}} \leq 1.3$ then all particles
would form a single layer in the system. Thus the expansion characteristics of
the bed could be defined by equation (16) below where the particle diameter is
replaced by an equivalent

$$e^{4.7} \frac{dg(\rho_s - \rho_f)\rho_f}{\mu^2} = 18 \text{Re} + 2.7 \text{Re}^{1.687}$$

(16)

Under the conditions $0.01 \leq \text{Re} \leq 1000$ for the system studied (water) and where
$d = d_e$ as defined:
\[ d_\varepsilon = \left( \sum_{i=1}^{n} \frac{x_i}{d_i} \right)^{-1} \] (17)

Where \( x_i \) is the fraction of the total weight of the particles of all particles with diameter \( d_i \). In the case of particle size distributions larger than those covered by the above equations, the particles were seen to segregate and the voidage could be determined by summing the voidage of individual layers of the system.