An Investigation into Process Related Fouling of Chromatographic Supports

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Doctor of Philosophy

by

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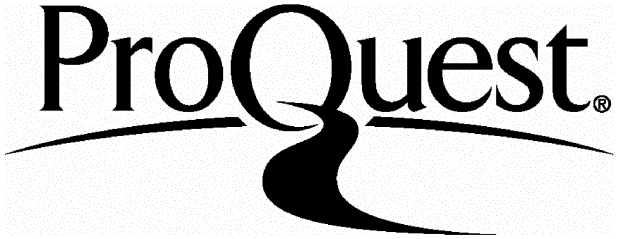

To my Parents, and Karen.

"Keep thy foot out of brothels, thy hand out of plackets,
Thy pen from the lenders' books, and defy the foul fiend"

King Lear, **III, iv.**

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ABSTRACT

This work focuses on the integration of chromatography, specifically ion exchange chromatography (IEC), within a process. Chromatography is commonly used after pre-treatment of the crude process stream by at least one processing step designed to remove foulants and competitors which significantly alter the separation and may irreversibly damage the support. Since chromatography represents a considerable proportion of process costs, significant effort is made to reduce the amount of contaminants reaching the chromatographic stage by the use of pre-treatment, incurring a penalty in reduced product yield, increased process time and increased capital/operating costs. The selection of the appropriate pre-treatment is restricted by a lack of knowledge concerning the sensitivity of the chromatography to key contaminants. The development of methods for evaluating the susceptibility to specific foulants is the aim of this work.

Initially a test separation of an intracellular enzyme, yeast alcohol dehydrogenase (ADH), was used to determine the changes in chromatographic separations with a variety of process stream compositions. By monitoring the yield and purity of the ADH , the performance was shown to be load and time dependant. This approach was unable to provide a general quantitative analysis: other techniques were used to elicit how the fouling occurred.

Frontal analysis was used to relate changes in the chromatographic performance to a decrease in the effective diffusivity of the ion exchange support. The technique was rapid and could be used to define the limit of the feed stream loading. Moment analysis was used to determine a change in the effective diffusivity and the packed column axial dispersion for both functional ion exchange and the base matrices, but required rigorous experiments to obtain consistent results. Results indicated particulate fouling to be a major source of performance change, and that fouling species acted together to produce effects absent when the fouling species was present on it's own.

The methods described are generic in application and could provide a basis for improving the integration of chromatography into processes.

CONTENTS

1. INTRODUCTION

1.1 General Background

In biologicals processing, chromatography unit operations are critical. No other unit operation has the selectivity to remove a single species from a mixture with very similar properties, combined with straightforward scaling (Hammond, 1989). The 20th Century has seen phenomenal growth in the biotechnology industry, and ever increasing emphasis on product safety through purity. As a result, the commercial importance of preparative chromatography is assured. A recognition of the technique's effectiveness is that it is the exception to find a biologicals process that does not contain at least one chromatography stage.

Inspite of the widespread application of chromatography in biologicals processing, the design and operation of large-scale beds is still based on dogma. As noted by Draeger (1991), much of the research effort in biologicals processing has been placed in improving the productivity of the fermentation process, often with little or no regard to the problems that may be introduced in downstream processing. The effect of this focus has been to dramatically increase fermentation yields, with the result that the major cost in the process is loaded towards the purification (Fish, 1984). Chromatography equipment and packing represents a significant fraction of this cost, and the optimum design and operation is therefore crucial to the process economy.

One of the problems associated with large-scale column chromatography is that of fouling. Because of the expense of the column packing, significant effort is placed into preventing any potentially damaging contaminants reaching the bed. The aim of this work is to examine the effects of a realistic process stream on the performance of column based chromatography. It is hoped the results can add to the knowledge of processing biological streams, and be useful in characterising the likely fouling properties of a particular type of process stream. In this chapter, the fundamental

processes involved in chromatography will be reviewed. The effect of fouling on the purification of enzymes is examined in the subsequent chapter, followed by a description of the effects of fouling on fundamental adsorption and transport parameters in packed beds.

1.1.1 Commercial Importance of Chromatography to Biotechnology

With the exception of industrial enzymes and vaccines, products of the biotechnology industry have traditionally been low molecular weight compounds such as antibiotics, amino acids and other food additives. With the advent of reliable and straightforward genetic engineering techniques, coupled with advancements in automated screening technologies, the scope for making products that have useful and novel biological activities from biological systems has been realised.

Table 1.1: Top 10 Biotechnology Drugs

(*The Economist*, February 25th 1995)

Company	Distributor	Product	Sales Revenue ($m)
Amgen	Amgen	Neupogen	719
Amgen	Amgen	Epogen	587
Biogen	Schering-Plough	Intron A	572
Genentech	Eli Lilly	Humulin	560
Amgen	Ortho Biologics	Procrit	500
Genentech	SKB	Engerix-B	480
Chiron	Merck	R- NAK HB	245
Genentech	Genentech	Activase	236
Genentech	Genentech	Protropin	217
Genentech	Hoffman La Roche	Roferon-A	172
Total Sales			4,288
Total Industry Sales			7,700

The global market for healthcare products is large and valuable, and consequently the emphasis on product development has been targeted in this direction. Table 1.1 shows the value of the top ten drugs marketed in 1993. All of these drugs are proteins, all involve production in a fermentation system, and all involve several chromatography stages in their purification.

Development and competition among companies in the biotechnology sector is intense, since the financial rewards of arriving first to the market with a novel drug are immense. By 1995, the FDA had approved 24 biotechnology drugs, and 180 products were in phase II or III clinical trials, and at least double this number were entering Phase I clinical trials in the US alone (Genetic Engineering News, August 1995). The majority of these new products were biologically active peptides or proteins, again requiring at least one chromatographic purification in their production.

It is difficult to find any reference to the size of the market in process scale chromatography, although the size may be inferred from the biotechnology industry drug sales and the research and development effort. Richards (1989) estimated the value of the market to be $200 million. Today the market must be considerably more valuable.

Column chromatography has so far been proved to be the best and most economical way of achieving the product specifications demanded by licensing organisations. Globally, governments are searching for ways to reduce public spending, and the human healthcare market has come under severe pressure to reduce costs, whilst maintaining product quality. In the future, chromatography will be forced to compete with alternative technologies, such as selective membranes, process-scale electrophoresis and field-flow fractionation. Understanding the basis for designing and operating columns efficiently will be essential if chromatography is to maintain its commercial importance in the industry.

1.1.2 Chromatography and Biologicals Processing Strategy

The work discussed in this thesis will concentrate on the processing of protein products. The justification for this is not just the value of the market, but also that process development tends to be state-of the-art for such products, because they are new. This is not to deny the importance of chromatography in the more mature food and chemical industries, but because of the widespread use of chromatography, it is essential to narrow the focus in a study such as this.

For the production of protein products, all the common fermentation systems have been used including microbial, animal and plant cell culture. The problems posed in processing such products are severe, since they are typically produced at low concentration compared to contaminants, and product and contaminants have very similar physical and chemical properties. Products intended for human healthcare have an absolute regulatory requirement to be pure and homogeneous, containing proscribed maximum levels of contaminating species such as DNA or endotoxins. Since 1992 such regulations have been extended to cover animal healthcare products in Europe (EC Directive 91/341/EEC), and the trend in regulatory standards is clear.

To achieve the kind of purity required, the biologicals process strategy must make use of several processing steps acting in concert, since it is rare to find a single unit operation that produces a one-stage purification to final product. Furthermore, many of the expression systems used are novel, and process design based on the depth of experience available in the more traditional chemical industries is not currently available to biotechnologists.

Figure 1.1 shows one of the possible process flowsheets for the production of an intracellular protein. The design involves selecting the unit operations and their sequence, and optimising the operating conditions for each, accounting for the desired

purity, quantity and formulation of the final product, together with an appreciation of the economic yield.

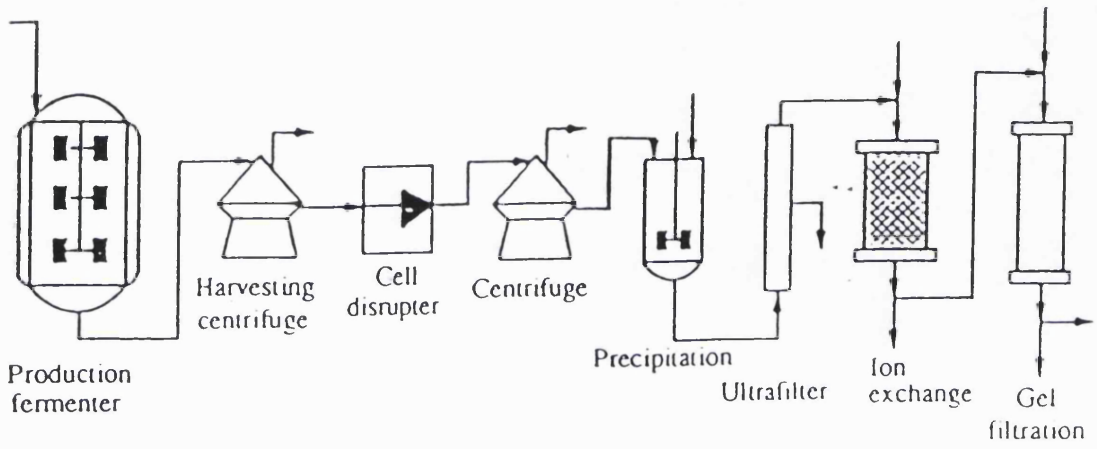

Figure 1.1: *An example of a possible route for the production of an intracellular protein product (from Aguilera Soriano, 1995).*

A typical biological process will normally consist of the stages discussed below. Each stage can be achieved through a variety of unit operations, and the skill in the design is to select the most appropriate. After primary production (normally fermentation, but potentially involving plant tissue, animal tissue or even mammalian milk), there is normally a gross isolation or harvesting step. This step will separate contaminants from product on the basis of very differing physiochemical properties, for example size or density. Options include continuous centrifugation and microfiltration. At this point, product is normally dilute and the next stage typically involves concentration through precipitation, phase separation, flotation or batch adsorption. Next a high resolution purification is used, capable of selectively purifying the product away from contaminants very similar in size and chemistry. Currently, this is exclusively achieved

by chromatography, using one or several of the available types to achieve the specified purity. It is almost *de rigeur* to finish processing with a gel filtration step, to remove product multimers and isomers, whilst simultaneously achieving a buffer exchange for formulation.

From this discussion it is apparent that chromatography provides the separating power in the process design, and this is why it is so central to the process. The widespread acceptance of the efficiency of the technique is illustrated by the FDA's insistence that all therapeutic agents must have at least one chromatographic step in their production (Jagschies, 1988; Sofer & Nystrom , 1989).

Design of processes for the purification of biological materials tends to be heuristic; unit operations are selected according to previous experience, the available literature or the prevailing dogma. A method for aiding in the selection of process unit operations has been suggested by Lightfoot (1987), based on an activity-concentration diagram (Figure 1.2). Any number of paths could be taken across the diagram from the feed to the final product, but consideration of the two extreme routes illustrated is instructive. The first path is to purify the product to its desired activity, and then concentrate. This means processing large volumes, with associated high handling costs, large quantities of solvents or buffers, and high capital investment in holding and preparation vessels. The second strategy is to concentrate and raise the activity to that required. This is more economically attractive, than the first strategy. Adsorption chromatography provides an opportunity to follow the second route, adsorbing the molecule of interest, and eluting in a smaller volume. However, to achieve this requires a thorough understanding of the chemistry of the system. Lightfoot acknowledges that most biologicals processing systems are closer to the first extreme than the second, presumably because of a paucity of knowledge surrounding the complexities of biological process streams.

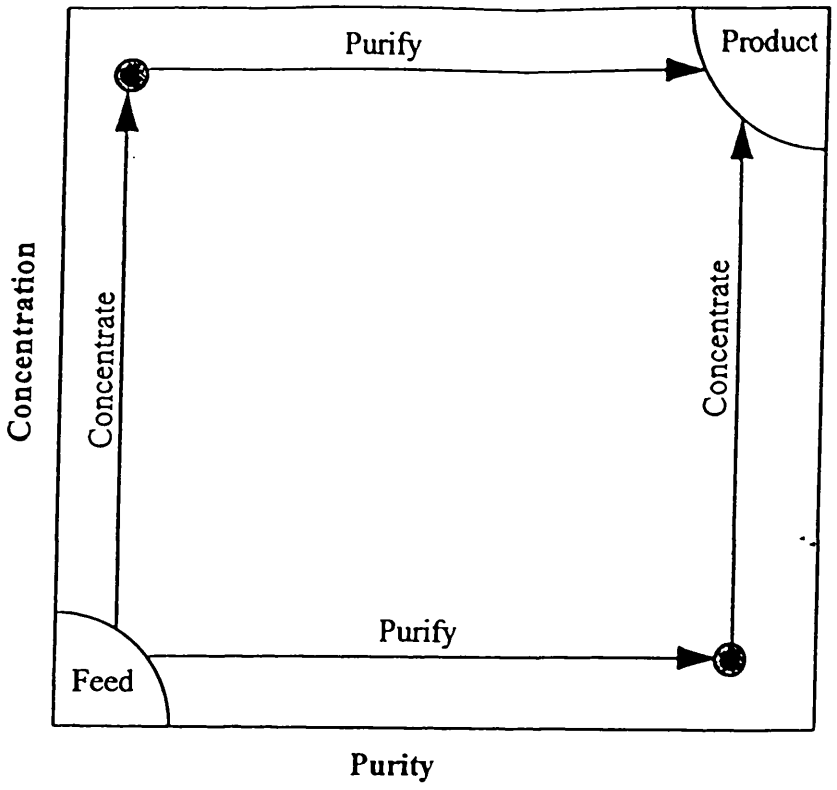

Figure 1.2: *A schematic activity-concentration diagram (Lightfoot, 1987).*

Ho (1990) illustrates Lightfoot's approach, suggesting that process designs employ highly specific techniques (immuno-affinity adsorption) early in the process to produce the concentrated product stream. However, there is an unconfirmed, but generally perceived drawback owing to the expense of such highly specific adsorbents. As a result, several unit operations may be introduced into the design, with the goal to remove contaminants that are *thought* to be involved in fouling the adsorbent. This then produces a process completely opposite to that which was intended, and this despite an almost complete absence of any data in the literature to support the causes of biological fouling of chromatographic matrices. It is in this context that the work presented here is set.

1.2 A Brief History of Preparative Liquid Chromatography

Chromatography was first described by Mikhail Tswett at the beginning of this century. He used the technique to purify pigments from plants, and coined the term chromatography. Although Tswett used the technique for purifying compounds, it was not until the 1930's that chromatography was used as a preparative tool. In 1941 Martin & Synge (1941) put forward the first theoretical treatment of the process, based on the theoretical plate concept used in distillation columns. Tiselius (1956) classified chromatography into three modes of operation; isocratic, elution and displacement chromatography. Other workers were confirming that chromatography was a useful preparative tool, and could provide a selectivity unrivalled by other techniques. Driven by the requirement for rare-earth metals, Spedding and co-workers developed a chromatography system capable of producing large quantities of rare earth oxides to purity. A pilot plant consisting of sixty-eight four and six inch diameter columns was eventually built (Spedding , 1955).

Whilst the initial matrices consisted of natural clays, Adams and Holmes (1935) demonstrated that ground phonograph records would exhibit ion-exchange properties, a discovery that led to a systematic study of organic resins resulting in the huge diversity of solid supports available at the current time. Inspite of this, it was not until 1956 that (Sober, 1956; Peterson, 1956) diethylaminoethyl and carboxymethyl derivatised cellulose matrices were produced and used to purify protein mixtures. Since then chromatography has steadily developed to it's current pre-eminence in the science of protein purification.

1.3 Chromatography Techniques

1.3.1 Definition of Chromatography

Chromatography is the name given to a group of separation techniques that are characterised by a differential partitioning of molecules between a stationary and a mobile phase. The type of chromatography associated with protein purification is termed liquid chromatography, because the mobile phase is liquid. There are a large number of possible configurations for chromatography, but in the preparative case the predominant configuration is a solid phase consisting of closely packed spherical beads held in a cylindrical bed. The mobile phase is passed through the bed along the longitudinal axis of the column. This is the type of chromatography described in this thesis.

1.3.2 Mode of Operation

Tiselius (1956) defined three distinct chromatographic modes of operation which he called isocratic, elution and displacement chromatography.

In isocratic chromatography, sample is loaded continuously to the column. Since the components in the feed stream have different affinities for the solid matrix, the component with the lowest affinity will appear in the column effluent first followed by successively mixed bands in the order of affinity for the solid phase from lowest to highest. This is a very efficient way of loading a column for separation of binary mixtures. When the second component starts to appear in the effluent, this is recycled after the column is washed, and the process repeated.

In elution mode, a band of sample is applied to the top of the column. The conditions of the mobile phase are changed with time, such that the affinity of the solutes for the solid phase are decreased either by a continuos gradient with increasing power to displace the solutes, or by step changes in the eluting power of the mobile phase. From a preparative

point of view, step-wise elution is more economical in use of materials, and can also provide a good separation, gradients being more suitable for analytical applications. The technique with a step elution is to achieve a concentration of displacer in the mobile phase that just fails to remove the solute of interest. A small step-change in the displacer concentration should just remove the solute of interest.

In displacement chromatography, a solute with a higher affinity for the solid phase than any of the solutes in the sample (the terminal displacer) is used to drive the other solutes ahead of it. A train is formed moving at the velocity of the terminal displacer, the lowest affinity solute being eluted first followed by the others in increasing order of affinity for the solid phase.

These three modes can be operated at process scale, although the vast majority of processes for protein purification use elution chromatography. The work presented here thus concentrates on liquid chromatography operating in the elution mode.

1.3.3 Types of Chromatography for Protein Purification

There are an a very diverse range of chromatography techniques available, and in the following sections the major techniques that have found success in preparative separations will be discussed. The structure of the solid phase used for separation is probably as critical to the success of the separation as is the type of chromatography, because of the complex structure of proteins related to their function. Loss of structure as a result of the protein contacting with a poorly designed solid phase equates to loss in yield. Therefore it is worth looking at the properties of solid phases for protein purifications.

1.3.3.1 Stationary Phase Structure

The stationary phase is at the heart of the chromatographic process. Clonis (1987) has discussed the properties of a matrix ideal for preparative liquid chromatography, and these features are discussed below.

The matrix should be highly porous to minimise the resistance of diffusing molecules to reach the stationary phase. The degree of porosity has a major effect on the capacity and efficiency of the separation, since transport within the bead exclusively relies on diffusion of the solutes. Recently, matrices have been produced that reportedly allow convective transport in the matrix (perfusion chromatography). The matrix should be highly hydrophilic, to allow good wetting, and to minimise non-specific adsorption of solutes. The matrix must be sufficiently stable to withstand chemical derivatisation, regeneration and sanitisation when in use, and have sufficient mechanical strength to allow economical flowrates. Additionally, the matrix should be available at an economical price, and be re-useable to prevent the need for re-packing.

Most of the commercially available matrices are spherical beads, a geometry that gives good flow characteristics. Bead sizes typically range from 5 to 200 μm. Since the adsorption process relies on diffusion, small bead diameters have an advantage over larger diameters in possessing a larger surface area per unit volume, and smaller distances for solutes to diffuse. However, pressure drop increases inversely with particle diameter, and packing costs also tend to be higher for smaller beads. As a result, preparative packings normally consist of beads in the 30 to 90 μm range.

The range of particle size distribution of the packing is also important. Monodispersed beads will have the most stable packing and the best flow characteristics. However monodispersed packings, although available, are very expensive, and their use is restricted to analytical applications where extremely high efficiency of the column is required (Ugelstad et al (1983)). For polydispersed beads, Unger and Janzen (1986)

quote a particle size distribution where the ratio d_{p90}/d_{p10} is 1.5 to 2 of the cumulative distribution as being an acceptable figure (d_{p90} is the particle diameter below which 90 % of the particles in the distribution are found, and d_{p10} is the particle diameter below which 10 % of the particles in the distribution are found).

Whilst there are a very large range of matrices available, a very significant factor in selecting matrices is cost. Price is determined by the bead shape, the size distribution and the quoted size. Spherical particles are more expensive than irregular particles, and the smaller the bead size, and the narrower the size distribution, the higher the costs. Ultimately, however, the cost of the matrix is determined by how much product can be produced over the column lifetime, not by the capital cost of purchasing the packing.

The number of matrices available for protein chromatography are extensive and bewildering. Porous silica, glass and hydroxyapatite inorganic matrices, polyacrylamide, polymethylmethacrylate and polystyrene synthetic matrices have all found applications. Nevertheless, polysaccharide matrices, cellulose, dextran and agarose matrices are by far the most commonly used matrix for protein separations due to their hydrophilicity (Janson & Jonsson , 1989; Verzele 1990).

Polysaccharide matrices are normally described as gels, referring to the large quantity of imbibed immobile solvent (usually aqueous buffer), which typically accounts for 90 % of the bead volume. The extreme hydrophilicity of these gels prevents non-specific irreversible adsorption and denaturation of proteins that is experienced on many inorganic matrices. However, the mechanical strength of such matrices is low, which presents problems in achieving high flowrates, and can cause bed compression if the column is fouled. Attempts to increase the mechanical strength of gels has included encapsulation of the gel in a rigid macroporous bead (Macrosorb), or cross-linking of the polysaccharides to increase rigidity of the bead (Sepharose Fast Flow). Nonetheless, such structures are less rigid than the incompressible inorganic matrices, such as silica. Although ideal in terms of rigidity, few protein separations use silica as a matrix

because of it's high non-specific adsorption, which tends to cause denaturation. The other major drawback to silica is it's instability to alkaline pH, preventing sanitisation and cleaning using caustic solutions. Attempts to stabilise the surface of silica by coating with zirconium oxide (Stout & DeStefano, 1985) have not been entirely successful, and Janson and Jonsson (1989) note that the need for rigid packings like silica as a protein purification matrix have stimulated manufacturers to develop the range of rigid synthetic organic polymers available today.

In conclusion, the number and type of matrices available for protein separations is enormous. Generally preferred matrices are based on polysaccharide or synthetic organic gels. There are available stationary phases based on 10 μm, 30 μm and 90 μm average diameter beads that have the mechanical strength, porosity and stability required to cover the demands of protein separations from microgram to kilogram quantities.

1.3.3.2 Types of Adsorption Chromatography

The work presented in this thesis centres on adsorption chromatography. Gel filtration chromatography is never used early in a process, because the sample is always diluted in the run, and sample loads are practically limited to 10 % of the column volume for there to be any resolution. It's most common application is as a polishing step at the end of the process, as discussed in Section 1.1.2. Gel filtration is a rather special case of chromatography, since separation is by exclusion of the molecule from a proportion of the liquid phase, rather than by adsorption to a stationary phase. A full discussion on the theory and practice of gel filtration is given by Hagel (1989).

There are a large number of adsorption chromatography techniques, the selectivity of each technique being reliant on different types of molecular interactions. The choice of the technique is dependent on the process stream conditions. In a review of 100 protein separation methods (Bonnerjea et al., 1986), the most commonly used first

chromatography step in such processes was ion-exchange chromatography. The popularity of this technique is it's high capacity and (relatively) low cost, as well as it's general ease of application.

The adsorption of proteins to ligands on an inert matrix is due to one or several of the following forces:

(i) ion-ion or ion-dipole bonding
(ii) hydrogen bonding
(iii) van der Waals forces
(iv) π - π electron interactions
(vi) metal-ligand bonding
(vii) covalent bonding

Although these interactions determine the strength of the adsorption, the specificity of a ligand is due to stearic factors. Proteins are large three dimensional structures, and the exact interaction between ligand and molecule has proved difficult to predict in the absence of detailed protein structure and conformation.

Whilst a large number of supports have been developed, it is not always certain, even to the designer, which of the above forces are dominant in the adsorption, and such is the diversity of protein structure, screening prospective adsorbents for separation is not a trivial task.

1.3.3.2.1 Ion Exchange Chromatography (IEC)

The basis of IEC selectivity is the ionic interaction between charged residues on the protein and the matrix. The adsorption is mild and predictable based on the charge of the protein, which is easily determined. The method is generally applicable, since all proteins carry ionisable residues, and it is the cheapest of the matrices available. The

disadvantage of the technique is it's low selectivity and the requirement for low ionic strength samples.

It's high capacity, low cost have made it a popular process scale substrate (Sofer & Nystrom , 1989).

1.3.3.2.2 Hydrophobic Interaction Chromatography (HIC)

Like IEC, HIC is applicable to a wide range of protein separations. Selectivity is based on interaction between hydrophobic residues on the protein, and immobilised hydrophobic ligands on the matrix. The capture step involves adsorption from a sample high in salt concentration, and eluting by decreasing ionic strength, making a logical case for a HIC step following ammonium sulphate precipitation.

1.3.3.2.3 Hydroxyapaptite

Hydroxyapatite is a crystalline calcium phosphate adsorbent. The mechanism of protein adsorption is contentious, and not really understood. It is a low cost material, and it is comparable to IEC, although applications are not common. It has a very strong avidity for DNA, and has found applications where removal of DNA from a product is critical to meet the purity specification.

1.3.3.2.4 Affinity Chromatography

Affinity chromatography is the most selective of adsorption techniques for proteins. Purification factors of several thousand are possible (Bonnerjea et al., 1986). The selectivity is based on the specificity of biological molecules for their substrate, to which they bind with high affinity, but to which contaminants have little or no affinity. The extreme version of this is immuno-affinity chromatography, where the ligand is a monoclonal antibody exquisitely selective for an epitope on the desired product.

In practice, it is rare for affinity steps to be applied to crude process streams because of the potential for fouling, and packing is expensive (a scan of a manufacturers price list reveals costs up to 120 times that of a comparable ion-exchange packing (Pharmacia LKB Biotechnology)). Nonetheless there are several commercially successful processes using affinity chromatography, one of the best known being protein A purification of IgG. In conclusion, affinity chromatography is justified for high value, low volume product where high purification factors are desired.

1.3.3.2.5 Metal Chelate Chromatography

The basis for selectivity in this method is the adsorption of proteins by co-ordination to immobilised metal ions, usually zinc(II) or copper(II). Binding occurs through metal-ion co-ordination to histidine, cysteine or tryptophan residues on the protein. It is reportedly a very selective method, and has been used to purify genetically engineered products with poly-histidine tails (Brewer, 1990). Adsorption is independent of the ionic strength of the sample, but product will contain the metal-ions, usually necessitating a buffer exchange step to be incorporated.

1.3.3.2.6 Covalent Chromatography

Seldom used for protein chromatography, this type of chromatography works by reaction of immobilised thiols with cysteine residues on the target protein. A major disadvantage of the technique is that the reacted thiols need to be regenerated, a process that takes hours, and is best performed in batch operation.

1.3.4 Definition of Process-Scale Chromatography

There is a distinction to be made between chromatography for analytical purposes and chromatography for the production of a pure compound for study or sale. In analytical

chromatography, the *raison d'etre* is the information gathered about the sample. The sample itself is not of interest, and once chromatographed, it is essentially waste. Throughput is then determined on a cost per sample per time basis, the cost for a fully resolved chromatogram.

Whilst some workers have considered preparative and process chromatography to be interchangeable (Guiochon & Katti , 1987), others have chosen to make a distinction between preparative and process chromatography that upon inspection appears to be a valid and useful distinction (Knox & Pyper, 1986). Preparative chromatography is concerned with isolating a product to a previously defined specification. The product is intended for study, for example in efficacy trials or structure analysis. Here, the crucial factor is purity - cost and yield are secondary considerations. On the other hand, process chromatography is concerned with producing commercially successful products for sale. Cost per unit of product is the sole driving optimisation parameter. Whilst Knox and Pyper have also considered the scale of operation, this author considers that for protein purification, process chromatography is related to the intended final use of the product, not the quantity of product (which may be only gram quantities per annum), or the scale of the equipment required to prepare it.

Although analytical chromatography will not be discussed further, it is worth mentioning that this chromatography is a very useful tool in providing analysis for biological industrial processes. Use of high speed, on-line HPLC systems for fermentation control is of great research interest, and it is likely that this technique will be employed in on-line monitoring of downstream processing, including preparative chromatography, in the near future.

1.4 The Theory Of Chromatography Separations

In this section a review of the main concepts used in describing chromatographic performance will be made. These consists of descriptions of the typical

chromatographic peak produced by a solute eluted from a packed bed, and the band spreading associated with it.

1.4.1 The Theoretical Plate

The model of the theoretical plate was first expounded by Martin & Synge (1941), who adopted the theory used for distillation columns. The chromatographic bed is considered to consist of a series of hypothetical plates stacked perpendicular to the direction of flow. In each plate, the solute is considered to be in equilibrium between the mobile and stationary phase, and the thickness of each plate is called "the height equivalent to a theoretical plate" (HETP or H).

Explicitly, HETP can be expressed by Equation 1.1,

$$H = \frac{\sigma_L^2}{Rut_R} = \frac{\sigma_L^2}{L} \qquad 1.1$$

here, R is the retardation factor, t_r is the retention time, u the interstitial fluid velocity , σ_L is the standard deviation of the eluted solute concentration profile in column length terms, and L is the length of the bed (Giddings 1965).

By inspection of 1.1, it follows that a small value of H leads to a smaller standard deviation per unit length, that is, the concentration profile of an eluting solute is sharp. H is therefore often quoted as column efficiency - the smaller H, the better the column efficiency.

Standard deviation of the solute elution concentration profile can also be expressed in time or volume units,

$$\sigma_L = \sigma_t Ru = \frac{\sigma_V Ru}{Q} \qquad 1.2$$

here Q is the volumetric flowrate of the mobile phase. By substitution, plate height is given by Equation 1.3

$$H = \frac{\sigma_t^2 Ru}{t_R} = \frac{\sigma_V^2 Ru}{V_R Q} \qquad 1.3$$

here V_R is the retention volume.

The number of theoretical plates in a packed column is the bed length, L, divided by the HETP, H (Equation 1.4),

$$N = \frac{L}{H} \qquad 1.4$$

The number of plates is proportional to the column length, so all things being constant, an increase in bed length will increase the number of plates. High values of N will produce sharp peaks of solutes. Because N is approximately the same for different solutes under the same operating conditions, the number of plates is often quoted as a useful measure of a column's separating ability.

1.4.2 Solute Dispersion in Chromatography

When a solute is injected onto a column, the width of the solute band continually increases with distance owing to a number of dynamic processes in the column, systematically described and analysed by van Deemter (1956).

Solute band broadening (increasing variance) occurs as a result of inhomogeneities in the streamline flow of the mobile phase, deviations in the adsorption equilibrium due to mass transfer resistance between the mobile and stationary phase, and axial molecular diffusion driven by solute concentration gradients. These three effects are independent,

and the total variance of a band of solute is made up of the sum of the three variances for each of these processes. This variance can be expressed as plate heights, so that the plate height is made up of the sums of the three processes,

$$H = H_{flow} + H_{diffusion} + H_{mass\ transfer} \qquad 1.5$$

In a packed bed, streamlines must find their way around the packing, and the flowpaths are tortuous and indirect. Molecules in the streamlines will move ahead or behind the average concentration depending on the distance travelled. As a result, the band is spread. van Deemter called this type of dispersion eddy diffusion. The magnitude of the effect is determined by packing geometry, and is independent of the flow velocity,

$$H_{flow} = 2\lambda d_p = A_0 \qquad 1.6$$

here λ is a geometrical constant determined by the column packing. Giddings (1965) notes that molecules would also be subject to diffusion across streamlines, so that in a real system molecules would sample more streamlines than if they were fixed to only one. The longer the column residence time, the more chance the molecules will differing streamlines, and therefore there will be a weak dependence on flow, and shown to be approximated in experiments by Knox (1982) as Equation 1.7,

$$H_{flow} = A u^{1/3} \qquad 1.7$$

In practice, values of A reflect the column packing. Well packed columns typically have values of A from 0.5 to 1, whereas poorly packed columns have values as high as 5.

The second effect contributing to band broadening is diffusion in the axial direction of the column. Solute molecules in the mobile phase will tend to diffuse in all directions until there is no concentration gradient. However, uniform distribution of the solute

molecules will not occur because the mobile phase is moving , and the solutes spend a proportion of their time on the stationary phase. The band spreading attributable to this effect is a direct function of the diffusivity of the solute in the mobile and stationary phases, and to the interstitial tortuosity. The relationship was defined by van Deemter (Equation 1.8).

$$H_{diffusion} = \frac{2\gamma D_m + 2\gamma_s D_s k}{u} = \frac{B_0}{u} \qquad 1.8$$

where D_s is the solute diffusivity in the stationary phase, k is the capacity factor, γ and γ_s are obstruction factors to account for tortuosity. van Deemter et al (1956) give values for γ and γ_s between 0.5 and 1, depending on the packing.

The final contribution to the band broadening is the resistance to mass-transfer. Since the system consists of a mobile (liquid phase) and a stationary (solid) phase, the transfer of solute from one to the other is not instantaneous. Equilibrium cannot occur locally, since the mobile phase is continuously moving, and thus the liquid phase concentration of solute is continuously changing. For the porous chromatography matrices most widely used in protein purification, the mobile phase is considered to be stagnant within the pores. Solute transport into and out of the pores is by diffusion. Molecules diffusing a short distance into then out of the pores will spend more time in the mobile phase than those molecules diffusing further into the pores. This results in the solute band broadening. Additionally, the time then spent on the stationary phase by molecules penetrating further into the pores will be longer, and this further contributes to band broadening.

The analysis of dispersion as a result of mass-transfer is rigorously treated by Giddings(1965). Equation 1.9 is the relationship derived by Giddings for the H contribution by mass-transfer.

$$H_{mass\text{-}transfer} = \frac{R(1-R)d_p^2}{30\gamma_s D_m} \bullet u = C_0 u \qquad 1.9$$

As discussed, the height equivalent to a theoretical plate, H, is then given by the sum of Equations 1.6, 1.8 and 1.9.

$$H = A_0 + \frac{B_0}{u} + C_0 u \qquad 1.10$$

Equation 1.10 is referred to as the van Deemter Equation. This equation is a fundamental description of the chromatography process, it's importance stemming from the relationship it gives between the column packing, the column operation and the efficiency of the resulting separation.

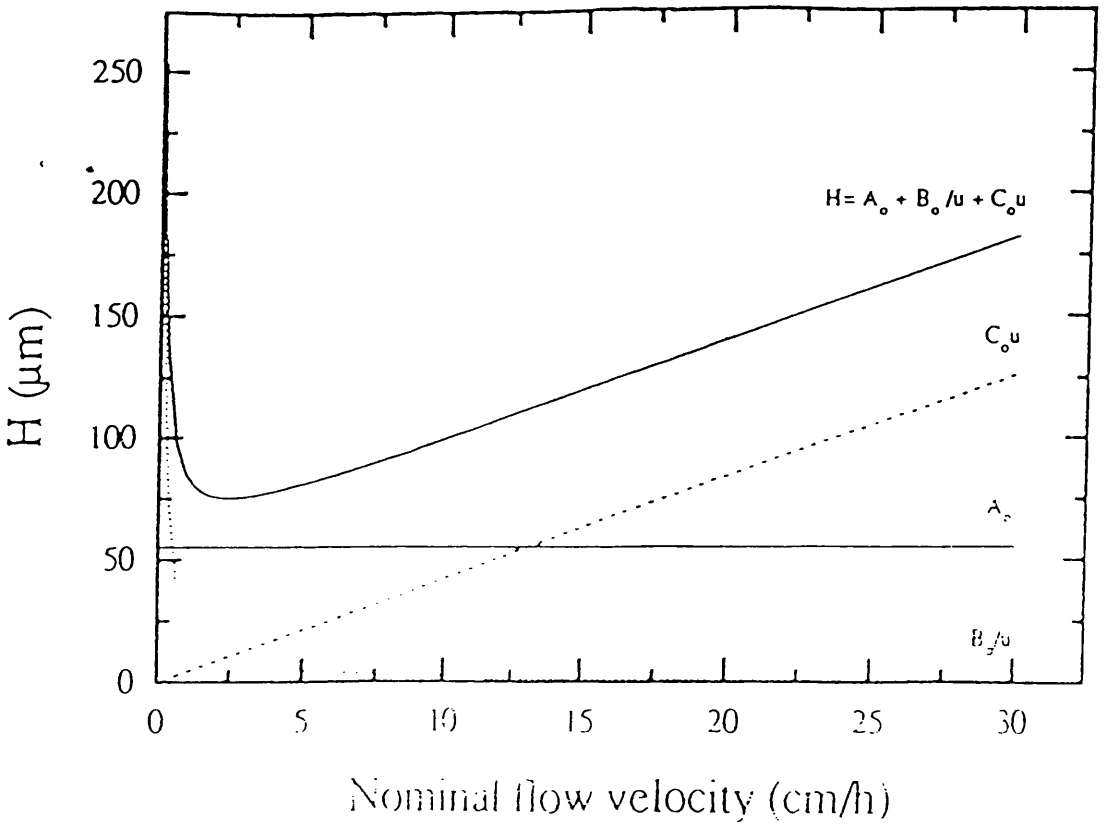

Figure 1.3: *The effects of eddy diffusion, molecular diffusion and mass-transfer on plate height, H. A_0, B_0 and C_0 are the terms of the van Deemter Equation. A_0 = 55 µm, B_0 = 200 µm²/s and C_0 = 0.5 s (from Aguilera Soriano 1995).*

Figure 1.3 shows a typical van Deemter plot for a large molecule (eg a protein). For large molecules, the contribution of diffusion is small. This is because of the low diffusivity of large molecules. Small value of H mean large numbers of theoretical plates for a unit length of packing, giving the high efficiencies sought in column chromatography. H is reduced when the values A_0, B_0 and C_0 are minimised. Furthermore, the velocity of the mobile phase can also be selected to give a minimum H value.

1.4.3 Adsorption

In adsorption chromatography the solute is distributed between the mobile and stationary phase. The equilibrium distribution of the solute in the mobile and stationary phase for varying solute concentrations at constant temperature is called the adsorption isotherm. Isotherms are characteristic for solute and stationary phase under a given set of mobile phase conditions (pH, ionic strength, temperature and so on). The simplest type of adsorption is the linear isotherm where the equilibrium relationship between solute in the mobile phase and solute in the stationary phase is directly proportional to the mobile phase solute concentration. This type of adsorption isotherm is normally only found for low solute concentrations, and is predominant in analytical applications.

More complex isotherms are exhibited at the higher solute concentrations required in preparative chromatography. The most widely used isotherm for describing the adsorption characteristics of protein molecules is the Langmuir isotherm (Langmuir ,1918). The original work was developed as an analysis of gas chromatography, but it has been shown by experiment that the model is a good fit for many protein adsorption processes. The assumptions made by Langmuir are that the solute binding sites are thermodynamically homogeneous, and that the molecules are adsorbed at discrete attachment points, adsorption of the molecule has no effect on the energy of adjacent sites, adsorption is as a monolayer, reversible and desorption is dependent only on the quantity of adsorbed species.

Given these assumptions, the Langmuir isotherm is then given by the relationship of Equation 1.11,

$$q^* = \frac{q_m c^*}{K_d + c^*} \qquad 1.11$$

Where q* and c* are the solute concentrations in the stationary and mobile phase respectively, K_d is the dissociation constant for the solute from the stationary phase, and q_m is the maximum capacity of the stationary phase for the solute.

The use of the Langmuir isotherm as a description of protein adsorption has been criticised by Velayudhan and Horvath (1988), since it can be demonstrated that the assumptions of Langmuir do not hold for proteins. van der Weil (1989) has shown that serum albumin binds to ion-exchangers in a bi-layer, and stearic hindrance of binding of proteins to adsorbents is well known (Chicz and Regnier, 1990). Nevertheless, the Langmuir isotherm models experimental data very well, and numerous authors have used the description (van der Weil , 1989; Chase , 1984;Wells, 1989).

An alternative model is that of the Freundlich isotherm, which accounts for a inhomogeneity in the energy of the available binding sites,

$$q^* = K(c^*)^{1/n} \qquad 1.12$$

Here, K and n represent the Freundlich constant and the Freundlich index. A criticism of the isotherm is that there is no account of the maximum capacity of the stationary phase for the solute. Jones and Do combined features of the Langmuir isotherm to Freundlich isotherms to account for this (Jones & Do , 1991).

Despite this, and accepting it's limitations, most workers have found the description of the Langmuir isotherm to be a simple and adequate description of protein adsorption.

1.4.4 Adsorption in Fixed Beds

The adsorption of a solute to a stationary phase in chromatography is governed by three processes; film mass transfer, pore diffusion and adsorption kinetics. This is illustrated schematically in Figure 1.4.

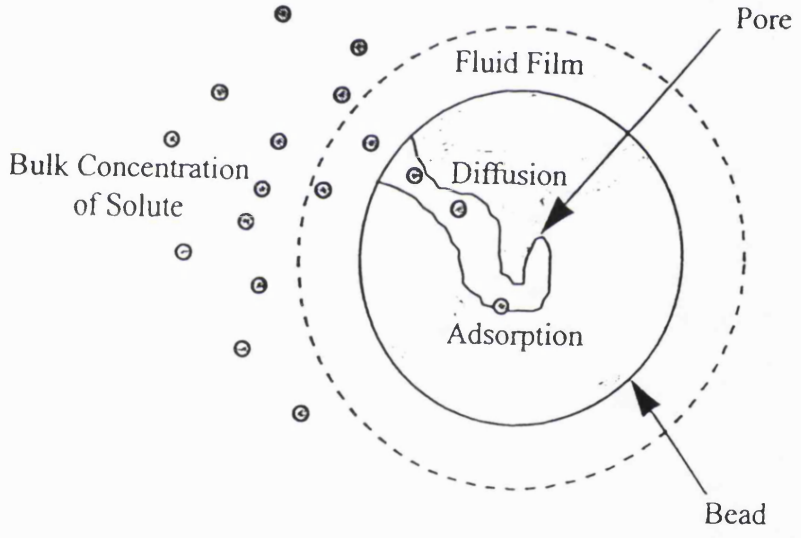

Figure 1.4: *Transport processes in liquid chromatography.*

Film mass transfer assumes a linear driving force approximation with the rate of mass transfer of the solute proportional to the difference in concentration from the bulk solute concentration to the surface solute concentration. The rate is governed not only by the concentration, but also by a fluid film transfer coefficient, k_f.

After transport to the surface of the bead, the solute diffuses into the pores of the bead. Two models have been used to describe diffusion in the pores of particles. The homogeneous surface diffusion model (Hand et al, 1983). In this model it is assumed

that diffusion of the solute on the surface of the pore is dominant. This is true for a homogeneous model with strongly adsorbed solutes. The porous particle model offers a better description of the sorts of gels used in protein chromatography, where the pores are large enough to accommodate proteins freely. Here, solute diffuses down pores and is adsorbed on to the pore walls, liquid diffusion dominating. The diffusivity of a solute within the pores is referred to as the effective diffusivity. Effective diffusivity is related to the solute free diffusivity by Equation 1.13,

$$D_e = \frac{D_{AB}}{\psi} \qquad 1.13$$

Here D_e is the effective diffusivity, D_{AB} is the free diffusivity, and ψ is a tortuosity value accounting for the pore twists and turns. In practice, ψ will always be greater than 1, and typically occupies the range between 2 and 6 (Cussler, 1984).

The surface reaction between the adsorbate and the solute is normally considered to be reversible and described by a second order rate reaction. In models of adsorption, the rate of adsorption is considered to be very rapid, with fluid film mass-transfer and diffusivity dominating the mass transfer rate, and such models have been shown to accurately fit experimental data (Horstmann and Chase, 1989).

1.5 Fouling of Chromatography Matrices by Biological Processes

It should be noted that novel processes using primary production in whole plants and animals are starting to be commercialised, and the challenges to downstream processing presented by such primary production routes have yet to be taken seriously. Anyone familiar with cooking will know the potential fouling properties of materials like milk. Nevertheless, most commercial biotechnology processes today use fermentation as the primary production stage.

Inspite of the importance of chromatography to industrial biologicals processing, there have been very few studies specifically related to the effects and causes of fouling on chromatography matrices. In this section, an attempt will be made to review the relevant literature, as well as to list the possible contaminants from fermentation that might foul chromatography matrices.

There are an enormous range of molecules in a biological process, and the list below has no pretensions to its being exhaustive. The general conclusion from an inspection of such a list is that, in isolation, a knowledge of the properties of the fouling moiety will give an indication as to it's likely action. Therefore, a hydrophobic molecule will rob the capacity of a HIC column, whilst interacting with an ion exchange column in a non-specific fashion. However, the synergistic fouling activity of mixtures of these classes of contaminants is probably impossible to predict, and can only be determined through experiment.

1.5.1 Fouling Contaminants in Chromatography

Biological systems are beautiful structures containing thousands of molecules that can be released by processing. Each and every one of these molecules has the potential to react with the column matrix, and any interactions that are not directly involved with the target product could be considered fouling. The following list considers these molecules as large classes, grouped by their structures. This is admittedly crude, but arises by necessity. Also considered here are the additives to the primary production stage that might be expected to find their way to the chromatography step.

Where the effects of column cleaning are irreversible and cumulative, column lifetime will be reduced. Even when a cleaning routine is successful, repeated cleaning must also affect the column through leaching of ligand, or degradation of the matrix structure.

1.5.1.1 Proteins

Proteins are macromolecules carrying both charged, hydrophilic amino acids, and hydrophobic amino acids. Proteins are thus well equipped to adsorb to all the commonly used matrices given the right physical conditions. Sources of protein from eukaryote systems also tend to be highly modified after transcription, to include significant quantities of sugars (glycoproteins) or lipids (lipoproteins). Such modifications are generally on the surface of proteins, and thus may be expected to take a significant role in the interaction with the column.

Any protein that interacts with the matrix and is not the target protein can be said to be fouling the column. The most likely effect is a reduction in the capacity. In some instances, protein may precipitate on the column, reducing the pore size, and restricting diffusivity. This is most likely to happen when buffers are changed, and clean-in -place (CIP) protocols must bear this in mind, particularly when an organic solvent wash is considered (Sofer & Nystrom, 1987). Sodium hydroxide CIP routines are normally very effective in removing protein from columns.

A common experience in processing biologicals is a haze of colloidal proteinaceous material, which may physically block the column leading to bed compression and failure of the bed.

1.5.1.2 Nucleic Acids

Nucleic acids are polyanionic polymers which may account for up to 14% of cell dry weight depending on the culture conditions. Nucleic acids will bind with high affinity to anion exchangers at pH>3. DNA tends to be more significant than RNA. RNA has a lower molecular weight in general, and therefore does not bind with such a high affinity. Binding of DNA is reportedly very strong so that even sodium hydroxide at 1 M strength will not remove it (Sofer and Nystrom, 1987). This is particularly a problem for products where DNA must be demonstrated to be below a maximum value (usually

in picogram amounts per dose); DNA on a column is always in the position to leach off into product.

Nucleic acids also increase the viscosity of the process stream. According to Darcy's law, pressure drop is directly related to viscosity. For this reason nucleic acids are usually removed by precipitation or by nuclease digestion.

1.5.1.3 Lipids

Lipids are a wide group of compounds characterised by their sparing solubility in water. They may represent up to a quarter of cell dry weight in cells like yeast. The range of lipids in eukaryotic cells is much greater than that in prokaryotes. There is a wide range of structural diversity, ranging from amphoteric molecules with hydrophilic heads of phosphate or sugar moieties, to highly hydrophobic steroids.

Because of this structure, the possibilities of lipids interacting with matrices either through their hydrophobic or hydrophilic areas are great. They are also smaller molecules than proteins, with the result that they will have greater diffusivity, and access to more of the matrix bead. Biologically lipids are excellent at forming bi-layers into which proteins are inserted, and it seems reasonable to expect that formation of coats on the matrix will provide further opportunities for attachment of other fouling species.

1.5.1.4 Whole Cells and Cell Debris

Following disruption of a cell, the process stream will contain fragments of cells from sub-micron to whole ghost cells, or even cells that have escaped disruption. The blanket term for this is "cell debris". Most cell walls tend to be hydrophilic and carry a negative charge. The composition of the cell wall depends on the type of cell. Gram negative bacteria have a rigid peptidoglycan wall sandwiched between an inner and outer membrane; gram positive bacteria have a much thicker peptidoglycan wall, but the outer membrane is absent; Yeast cells have a cell membrane surrounded by a thick wall

of cross-linked glucose, mannose and protein polymers. Eukaryotic animal cells and plant protoplasts have no solid wall, the cells being bounded by a lipid bi-layer.

Disruption of cells on an industrial scale is almost always by mechanical means for reasons of economy. The result is a process stream that consists of cell wall fragments with lipid attached, or lipid free in self organising small vesicles. Inspite of the interest in cell disruption, descriptions of the structure of cell debris beyond particle size distributions, are non-existent, and must be inferred from the source of the starting material.

It is dogmatic that the addition of process stream containing even small concentrations of cell debris will result in the column quickly blocking. For this reason , cell debris is removed from process streams by extensive treatment by centrifugation or filtration or a combination of these. Industrial scale centrifugation is unlikely to remove all the cell debris (Tinnes et al, 1992), whereas microfiltration may be successful in removing all but the finest debris. However, it is this debris that may be the cause of fouling, and it is reportedly common practice to use dead end filtration to as low as 0.2 μm to clarify pre-chromatography (Sofer & Nystrom, 1987). This is expensive, and may account for up to 50 % of the process cost.

1.5.1.5 Fermentation Components

The components of fermentation broths may be carried down as far as the chromatography stage. Pirotta (1985) and Petterson (1989) note some of the possible broth components that may foul chromatographic matrices.

Antifoam agents consist of silicone, animal or vegetable oils. Most of these compounds are hydrophobic and are excellent at coating surfaces. Antifoam is a potential disaster in cross-flow membrane filtration, where the hydrophobicity of the membrane is readily coated by the antifoam (Capanelli, 1990). The chief interactions of antifoams with chromatographic matrices should therefore also be through hydrophobic interactions. Silicone antifoams have been shown to bind to strongly to chromatographic matrices,

although there is no report in the effect on the performance of the subsequent chromatography (Lilly, 1979; Darbyshire, 1981).

The remaining contaminants considered are polyuronic acids and polyphenolic acids. These carry strong negative charges, and are often seen bound to the top few centimetres of a column as a brown band. The removal of such colourants is a challenge in this author's experience, and the consequences for the chromatography are probably a reduction in capacity.

1.5.2 Chromatography Fouling Studies

There are very few studies of biologicals fouling in chromatography. Such studies have tended to be qualitative rather than quantitative.

Petterson (1989) has approached fouling from the perspective of cleaning a column. He observes that most of the fouling contamination occurs in the top 5 to 10 centimetres of a packed bed. The column can be cleaned by back-flushing the column, or by using a CIP protocol of ever increasing complexity, targeting each type of contaminant with caustic, detergent or organic solvent washes. When all else fails, re-packing the top 5-10 cm of the column is suggested.

Tice et al (1987) have studied the effect of repeated injections of a protein mixture (ovalbumin, conalbumin and soybean trypsin inhibitor) on the separation of these proteins. They measured capacity and resolution as a performance indicator, and over ten cycles found the resolution to be reduced. A similar approach was taken by Johansson for cation, anion and hydrophobic interaction and gel filtration chromatography (Johansson, 1984; Johansson, 1985; Johansson 1986). Over 1000 cycles he found that the retention volume and peak height of a sample was not affected. A criticism of both these approaches is that small sample loads were used of relatively pure proteins, a situation that is not normally found in process separations.

Draeger (1991) has reported the effect of whole cells on the performance of ion-exchange batch adsorption and expanded bed adsorption of serum albumin. A

reduction in capacity was noted, presumably as a result of cell adsorption to the matrix. It should be noted, in some applications cells/cell debris may have a positive effect; Shaewitz (1989) has reported a useful co-purification of yeast enzymes with yeast cell walls, theorising that the cell walls act as a functional ion-exchange matrix.

Kril et al. (1987) have reported the reduction of the dynamic capacity of ion-exchange resins and activated charcoal beds as a result of microbial fouling in studies of waste-water treatment.

1.5.3 Models of Fouling

There are no models for fouling of chromatography matrices with biological process streams. However, the literature holds a considerable amount of work on the fouling of membrane processes by chromatography. Fanes and Fells (1987) have reviewed models for fouling of ultrafiltration by protein solutions. The emphasis of the models is on the evaluation of membrane permeability as a function of time. Mechanistic models discussed consider protein to concentrate at the membrane surface, where it aggregates to form a deposit reducing the membrane voidage and decreasing the membrane permeability. Such a model might be applicable to fouling where aggregates of fouling moieties may form both in the interstices of the packing and within the bead itself, driven by concentration gradients.

The other major field of investigation into fouling is that of catalysts. Models have been developed to account for loss of catalytic activity by coating of active sites and by pore restriction of the porous catalyst support (Beckman and Froment, 1979). Here the catalyst support could be considered equivalent to a chromatography matrix, and the active sites equivalent to the ligand. Catalyst deactivation has been shown to be related to the quantity of foulant trapped.

It may be hypothesised that the fouling effects in chromatography are most likely to be based on coating and pore blockage of the matrix.

1.6 Scope of the Thesis

Chromatography is the only process scale technique that can achieve purification of a biological product to homogeneity. The economical performance of chromatography unit operations are fundamental to the success of a product. The effects of biological species (lipids, proteins, cell debris etc.), on the performance of chromatographic separations have never been systematically investigated inspite of the knowledge that such species will cause separation difficulties. It is the aim of this project to provide a first step in the quantification of the effects of fouling on chromatographic matrices. It is hoped that this information can be used to provide more efficient separations, and economical processes.

In this work, efforts have concentrated exclusively on ion-exchange resins. This decision is based solely on the popularity of the technique as a chromatography processing step. It is hoped that the method of approach described here can enable the investigation of other systems and process conditions.

Chapter 2 investigates the purification of a yeast alcohol dehydrogenase from a yeast cell homogenate prepared using different concentrations and operating conditions to give a range of fouling conditions. The effect on yield and purity of the enzyme in relation to the processing conditions is discussed.

Chapter 3 provides a more quantitative analysis of the effects of fouling using frontal analysis of a solution of bovine serum albumin to analyse the condition of packed beds after fouling with a defined process stream. The effects of fouling on column capacity, and the effective diffusivity of the albumin are described.

Chapter 4 uses the method of moments to estimate the effects on the transport parameters of the column as a result of fouling and Chapter 5 assesses the significance of the results.

Finally, Chapter 5 will draw together the main conclusions of the thesis, and suggest future work that could add to that presented here.

2. CHROMATOGRAPHY OF YEAST HOMOGENATE

2.1 INTRODUCTION

The discussion in Chapter 1 concluded that the technique of chromatography is currently unique in the processing of biological products, in being the only high resolution technique that is scaleable. It can selectively purify a desired product from a multitude of contaminants with similar properties, under non-denaturing conditions and without introducing high shear forces. Introduction of the first chromatography step at the earliest possible stage in a process provides significant economic benefits by minimising the number of processing steps. Furthermore, not only is purification achieved, but the desired product is effectively concentrated by being eluted in a fraction of the volume of feed stream loaded to the column. As the concentration of a product is dominant from a process cost viewpoint, because separations costs are strongly related to the materials handling (Lightfoot, 1990), there are clear advantages to concentrating at the earliest opportunity.

The most common form of chromatography used in biologicals processing is elution chromatography, and for the purpose of this work, wherever chromatography is mentioned, it implies packed bed column chromatography operating in the elution mode as defined by Tiselius (1956).

In order to evaluate the performance of chromatography, it is important to consider the way in which preparative chromatography is used, and what the aims of such a separation are. When a chromatography step is used in a process, it must consistently produce a product of a pre-defined purity and yield. Practically, the chromatography process consists of loading a feed stream at the top of the column and taking fractional cuts of the effluent as it leaves the column, retaining those fractions with the required product purity and discarding or recycling the other fractions. What affects the yield and purity of the product is the degree of dispersion of the product, that is, how much the fractions containing the product overlap with contaminants. It is accepted dogma that column chromatography must be susceptible

to compression and clogging due to fouling species in the process stream. Accordingly many processes are designed to remove the maximum amount of fouling materials from the process stream prior to chromatography to protect the expensive chromatography media from degradation in performance, and the need for frequent replacement. The potential disadvantage in over-processing a process stream prior to the chromatography is loss of product yield and associated fluid handling and capital costs. The work presented here was intended to elucidate the effect of processing large quantities of crude feed streams on the performance of the chromatography to better understand the integration of chromatographic unit operations in biologicals processing.

There are a large number of chromatography matrices available, reflecting the diverse nature of possible protein separations. In a review of 100 protein purification papers by Bonnerjea et al. (1986), the most commonly used first chromatography step was found to be ion-exchange chromatography (IEC). Ion-exchange has found this popularity because it is relatively cheap and robust, although not as selective as some affinity methods. Owing to the popularity of IEC as a first chromatographic step, it was felt appropriate to conduct this work concentrating on an ion-exchange matrix.

The philosophy behind this work was to use a real process system that would illustrate the problems associated with processing biologicals using high resolution chromatography. The system selected was to purify alcohol dehydrogenase (E.C. 1.1.1.1) from *Saccharomyces cerevisiae*. Selection of this system was based on consideration of the following reasons. Firstly, the yeast model is of great importance commercially in the biotechnology industry for a range of products, most recently in genetically engineered systems, where the glycosylation of expressed proteins give superior biological performance compared to bacterial systems, and very high yields compared to cell or tissue culture systems. Secondly, processing an intracellular protein product such as alcohol dehydrogenase (ADH) represents one of the most challenging separation processes in biologicals processing, because of the large quantity, and diverse range of contaminants

released during the cell disruption. Finally, Brewer's yeast is commercially available as a quality controlled product that gives a readily available supply of a standard material for processing, without the need to resort to in-house fermentation for fresh material. An additional advantage of yeast is that it is a well understood processing model at University College London, and the data derived from this study can draw on, and be extrapolated to, previous and future work at this institution.

Yeast ADH was purified from a variety of process streams containing different quantities of fouling species. Sample was injected onto the column and the fractions collected using ADH as a marker of the column performance, through measurement of the purification factor (ratio of specific activity post-chromatography to specific activity in the injected sample) and yield of the ADH. In order to have a standard measure of the purification, a fractionation diagram was constructed to allow a purification factor to be calculated against constant yield. This approach had first been applied to the salt precipitation of proteins by Richardson et al. (1990). The construction of the fractionation diagram is shown in Figure 2.1.

The fraction of ADH and protein eluted was determined by collecting fractions and assaying for total protein and ADH activity. Total ADH eluted was then plotted against total protein eluted to give a curve that was typically sigmoid. The steeper the curve, the narrower the ADH peak, and therefore the better the purification achieved. Constructing the tie-lines indicated by the dashed lines in Figure 2.1 allowed a purification factor to be worked out for any given yield. The quantity (B-A) was the yield of ADH, and the gradient (B-A)/(D-C) gave the purification factor achieved at this yield. From such a diagram it was possible to maximise the gradient (and, therefore, the purification factor) for any given yield. For this work, a standard yield of 95 % was arbitrarily chosen, and the maximum purification factor achieved at this yield was determined from the constructed fractionation diagram.

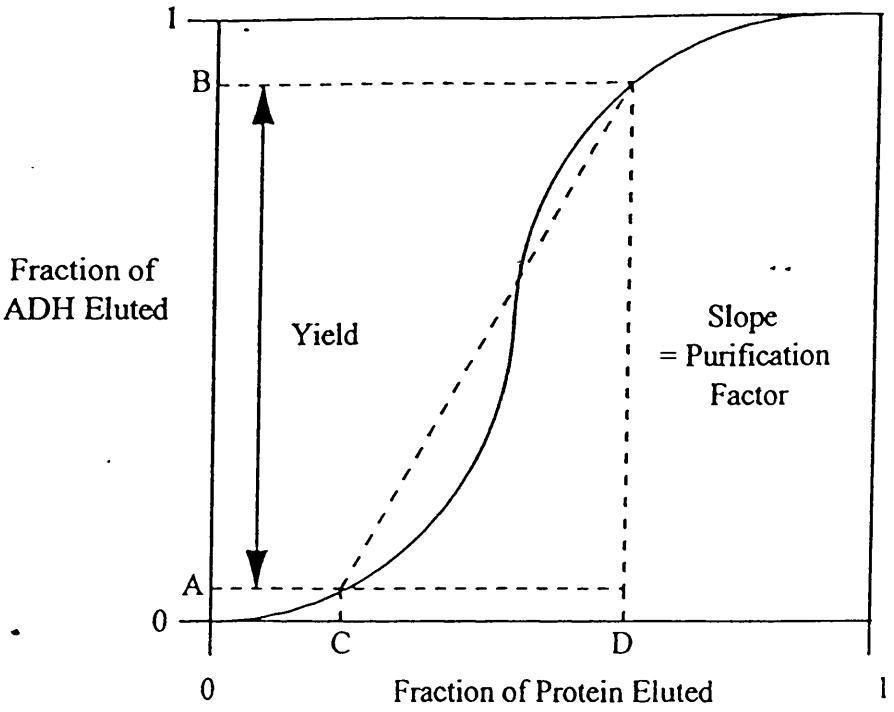

Figure 2.1: *The construction of the fractionation diagram. Dashed lines are constructed tie-lines to allow the yield and purification factor to be calculated as described in the main text.*

The work in this chapter describes the use of ion-exchange chromatography to purify ADH from variously treated yeast homogenates, and what effect the process stream has on the separation performance.

2.2 MATERIALS AND METHODS

2.2.1 CHROMATOGRAPHY SYSTEM

2.2.1.1 Chromatography Matrix

The chromatography matrix used in this series of experiments was DEAE Sepharose Fast Flow. This is an anion exchange matrix produced by Pharmacia LKB Biotechnology. The ion exchanger is based on Sepharose 4B, which is prepared by cross-linking agarose with 2,3-dibromopropanol and desulphating the resulting gel by alkaline hydrolysis under reducing conditions. Diethyl-aminoethyl (DEAE) groups are attached to monosaccharide units of the gel by ether linkages to give the functional ion-exchanger. Agarose based beads cross-linked in this fashion are macroporous and allow good diffusion of macromolecules, combined with very low non-specific interactions as a result of the gel's hydrophilicity.

Fast Flow gels have average diameters of beads of 90 μm with a size range of 45 - 165 μm making the gels suitable for high flowrate applications, ideal for preparative separations.

The chemical, physical and thermal stability of the gels allows them to be used in all solvents in the pH range 3-10 (Pharmacia Handbook-In Exchange Chromatography, 1991) - prolonged exposure of DEAE Sepharose to very alkaline solutions should be avoided because of the instability of DEAE as a free base.

2.2.1.2 Chromatography System

The system used for the chromatography work is illustrated in Figure 2.2.

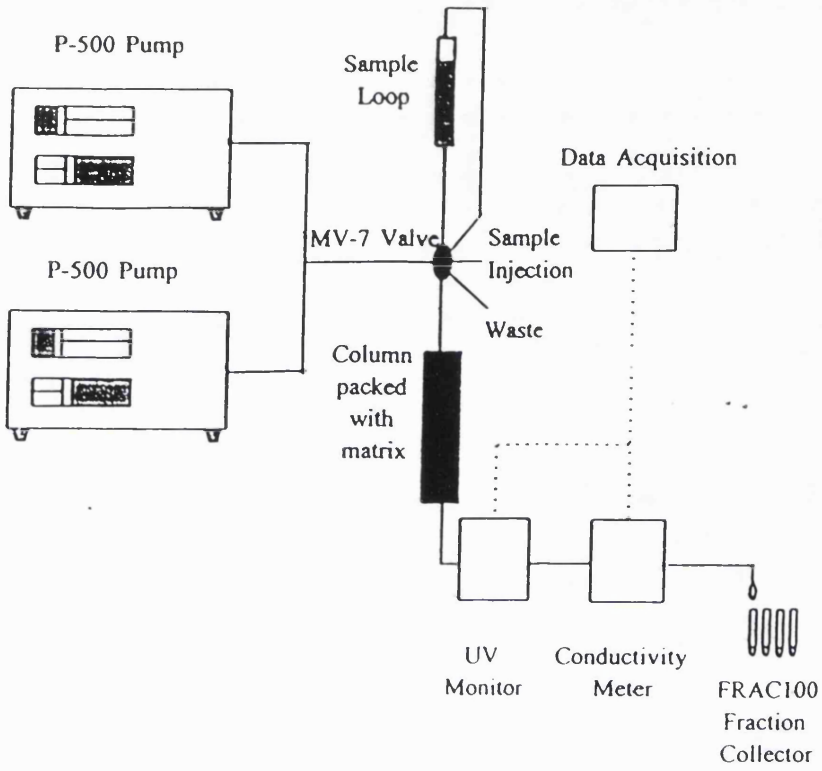

Figure 2.2: *The Pharmacia FPLC™ System*

The chromatography system used in this work was the FPLC™ system supplied by Pharmacia LKB Biotechnology. The system consisted of the following components:

(a) LCC 500 Programmable Controller.

(b) Two P500 pumps - Capable of delivering constant flow in the range 1 - 499 mL/hr up to a maximum pressure of 4 MPa. Both pumps were supplied with a pressure transducer with a 0-20 mA output suitable for a chart recorder or an A/D converter for computer logging of pressure data.

(c) Valve MV-7 - A three-position valve used as an automatic injection valve under the control of the LCC-500.

(d) UV-1 Ultraviolet Spectrophotometer/Monitor - 280 nm fixed wavelength spectrophotometer with a single path flow cell.

(e) Conductivity meter.

(f) Selection of sample injection loops from 50 μL to 50 mL.

(g) Frac100 Programmeable Fraction Collector - Capable of taking a minimum 0.1 mL fraction in volume or time mode.

Output signals from the UV-1, the conductivity meter and the pressure transducers could be captured via a chart recorder, or a PE Nelson 970 A/D converter coupled to a microcomputer (PS/2 IBM 386) running the software package Turbochrom (V 2.1) from Perkin Ellmer.

2.2.1.3 Columns

The majority of the experiments were run using a Pharmacia XK16 column, a 16 mm internal diameter glass chromatography column with a low pressure jacket for temperature control by water circulation. However two other types of column were used - the C10 and XK26 columns, again both supplied by Pharmacia. The columns were pressure rated up to a maximum 0.3 Mpa.

The columns were used with the supplied net frits. The frits might be expected to have an effect on the blockage of the column, but in practice, no cell debris was found to collect preferentially at the frit surface. Other manufaturer's column designs may use a different type of frit which may prove to contribute to fouling. This is not considered in this work, where the chromotography substrate is the material of interest.

2.2.2 EXPERIMENTAL PROCEDURE

2.2.2.1 Preparation of Fouling Stream

The fouling stream was prepared from a starting material of packed Baker's yeast (*Saccharomyces cerevisiae*) supplied by J. W. Pike Ltd. The fouling stream was prepared

by suspending the yeast in the following proportions with buffer: 1.3 Kg of yeast per litre of 20 mM phosphate buffer at pH 7 to give a yeast suspension of 600 g yeast /L on a wet weight basis. Typically 12 Kg of the yeast cake were added into 9.2 L of buffer to give 20 L of yeast suspension, but this volume was adjusted depending on the requirements for the experiment. The yeast suspension was homogenised by 5 discrete passes through a Manton-Gaulin type homogeniser at a pressure of 500 Bar. Two models of homogeniser were used, having the same principal of disruption, differing only in capacity - the APV K3 and the APV Lab 60 models. During the homogenisation, the process stream was maintained at 4 °C.

The resulting homogenate was then passed through a disc stack centrifuge (Westfalia Separator SAOOH 205) at a flowrate of 40 L/hr. The centrifuge used 37 separating discs, and gave a Q/Σ value of 1.9×10^{-8} m/s (Trowbridge, 1962).

For some of the fouling experiments, the disc stack centrifuge centrate was used with no further treatment, except dilution, as explained in the Section 2.3. In other experiments, the centrate was further treated in order to remove selectively particulates and/or lipids from the process stream. This was achieved using a Beckman L7 Ultracentrifuge running at 40,000 rpm for 1 hour (~160,000g). This had the effect of sedimenting the solid particulates and leaving lipid accumulated at the surface of the supernatant. Highly clarified homogenate could be withdrawn from the supernatant for comparison to the disc stack centrifuge centrate (Section 3.3).

The fouling stream preparation route is shown diagramatically in Figure 2.3. In all, the preparation time of the fouling stream was from 2.5 to 4 hours. Temperature of the homogenate was maintained at 4 °C in temperature controlled equipment, and held on ice after processing. As sterile processing was not a practical option, and to ensure fresh material, this preparation route was followed for every individual experiment - a maximum

of 8 hours lifetime was given to the processed homogenate. This processing route was strictly adhered to to ensure equivalence of material between individual experiments.

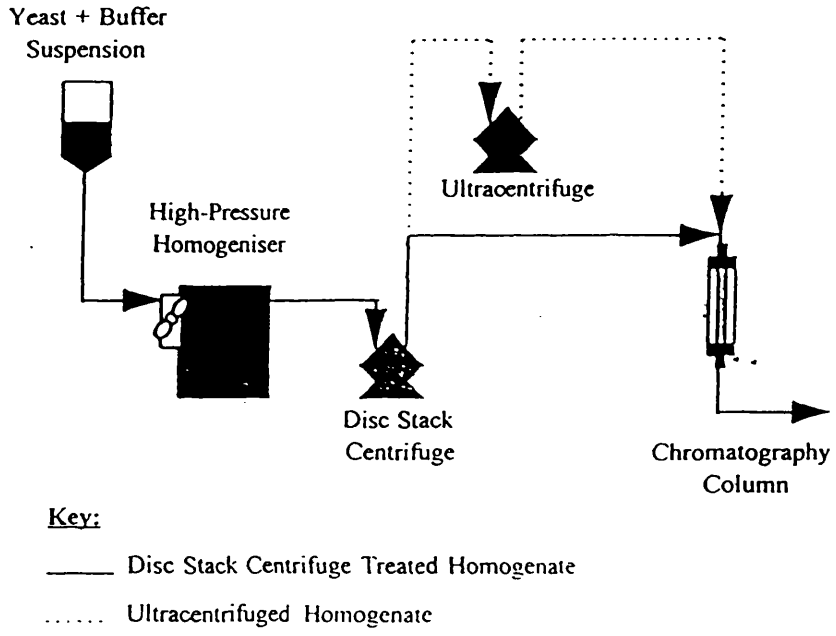

Figure 2.3: *The process for preparing yeast homogenate sample for injection onto the test column.*

2.2.2.2 Column Packing

The column packing procedure used was that described by Andersson (personal communication). This method was adhered to in all the experiments described in this section. To reduce the quantities of protein and adsorbent required, relatively short columns were preferred in these studies, column dimensions were 5.0 cm ± 0.1 cm and 1.6 cm in diameter. The required volume of DEAE Sepharose slurry was de-gassed under

vacuum at room temperature. After eliminating air from the column frits, the gel slurry was poured down the side of the glass column . The column was then filled to the top with de-ionised water and the top adapter fitted to the column. The P-500 FPLC pumps were then used to pack the slurry at a flowrate of 14 mL/minute until a constant bed height was achieved in the column. Next the adapter was brought to the surface of the gel and manually compressed by about 0.5 cm to provide the packed bed.

The column was run for 1 hour at 14 mL/min to ensure that the bed height remained at a constant value, and the column end pieces adjusted if any further settling occurred. If the final packed bed fell outside the specification for length, the column was re-packed to avoid possible stratification of bead sizes in the bed that might occur if small amounts of gel were added to, or removed from, the bed.

2.2.2.3 Elution Chromatography of Yeast Homogenate

Yeast alcohol dehydrogenase was purified from crude yeast homogenate using a packed bed of DEAE Sepharose Fast Flow. The chromatography used two buffers, buffers A and B. Buffer A was prepared with di-sodium hydrogen orthophosphate and sodium dihydrogen orthophosphate AR grade or equivalent (eg BDH) to give a final phosphate concentration of 20 mM. pH was adjusted to 7 using 1 M sodium hydroxide AR grade or equivalent (eg BDH) as required. Buffer B consisted of the same 20 mM phosphate buffer as buffer A, with the addition of 1 M sodium chloride AR grade or equivalent (eg. BDH). Buffers were prepared freshly each day and de-gassed at room temperature by vacuum filtration through a 0.2 μm filter.

Method development for the purification of the yeast ADH is described in Section 2.3.1. The final method developed is given below.

A freshly packed bed (Section 2.2.2.2) was initially equilibrated with a minimum 5 column volumes of buffer A before an homogenate sample was loaded to the bed. Once the sample was loaded, the bed was washed with 2 column volumes of buffer A to remove unbound material. The column was then developed by using a linear 0 - 1 M sodium chloride gradient over 10 column volumes using a mixture of buffers A and B, this gradient was under the control of the FPLC™ LCC500 controller. The experiments were normally run at ambient temperature, 18 - 22 °C.

Fractions of the column eluent were collected in the FRAC 100 fraction collector; each fraction collected was 20 % of the column volume (generally 2 mL). Collected fractions were stored on ice and assayed as soon as possible for total protein content and ADH activity. From these assays the fractionation diagram was constructed.

2.2.3 ASSAY TECHNIQUES

2.2.3.1 Alcohol Dehydrogenase (ADH) Activity Assay

The assay for ADH was based on the method of Bergmeyer (1983). The assay measures the reduction of nicotinamide adenine dinucleotide (NAD) by ADH catalysing the formation of ethanal from ethanol. The rate of reduction of NAD is followed spectrophotometrically by an increase in absorbance at 340 nm. The reaction is first order with respect to ADH, and therefore the concentration of ADH is proportional to the reaction rate.

The assay reagents were as follows:

Tris Buffer	5.64 g/L
Semi-Carbizide Hydrochlride	0.67 g/L
Glutathione	0.312 g/L
Nicotinamide Adenine Dinucleotide	1.42 g/L

Ethanol 35 mL/L

The above reagents were combined in distilled water and adjusted to pH 8.8 with 4 M sodium hydroxide. This gave a mixture referred to as ADH reagent. All reagents were analytical grade or better obtained form Sigma Chemical Company. The solution was stable for up to 7 days at 4 °C.

The ADH reaction was measured at 25 °C. ADH reagent was incubated in a water bath at this temperature prior to use. Samples for assay were pre-incubated for 10 minutes at 25 °C prior to use. The spectrophotometer was not temperature controlled, but assay time was from 30 seconds to 2.5 minutes, and temperature changes were therefore considered to be insignificant to the precision of the assay. The assay was performed by adding 10 µL of sample to 1.5 mL of the ADH reagent in a cuvette. Once the sample was added, the cuvette was rapidly inverted three times, and placed in the spectrophotometer (Beckman DU-64, Beckman Instruments). The rate of absorbance change was measured, and the enzyme activity calculated from Equation 2.1.

$$\text{Activity} = (1/\varepsilon_{340}\, L)(\Delta A/t)(V_c/V_s) \qquad 2.1$$

where ε_{340} is the micromolar extinction coefficient of NADH at 340 nm (6.22 $cm^2/\mu mol$), L is the light path length (1 cm), $\Delta A/t$ is the rate of absorbance change, V_c is the cuvette volume and V_s is the volume of sample added. The resultant activity is in U/mL where U is the SI unit for enzyme activity defined as the amount of enzyme required to convert 1 µmol of substrate in 1 minute.

The reproducibility of this assay was tested by making repeated measures of the ADH activity in a sample of homogenate. Reproducibility was between ± 5 % for four repeats of the same sample. Spiking pure ADH (Sigma) into a sample of homogenate showed there to be no activation or inhibition of the measured activity of ADH in the presence of yeast

homogenate. Likewise, measurements of the ADH activity on the presence of sodium chloride up to 1 M concentration showed no effect.

2.2.3.2 Protein Assay

The protein assay was a commercially available protein assay kit from Bio-Rad. The assay is based on a shift in absorbance maximum of an acidified solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm when this dye complexes with protein. The assay is based on the principal described by Bradford (1976).

For this assay, 50 μL of sample was mixed with 1.5 mL of assay reagent in a cuvette and allowed to react for a period between 5 and 30 minutes before absorbance was measured at 595 nm. The resulting absorbances were converted to protein concentrations using a standard curve prepared with dilutions of bovine serum albumin over a range 0.1 to 1 mg/mL.

Reproducibility of the assay was typically ± 5 % for duplicate samples. There was no effect of salt concentration on the precision of the assay. When particulate suspensions were present it was necessary to spin down the sample by centrifugation at 16,000 rpm in a bench top centrifuge (MSE Microcentaur) for 15 minutes, and then assay the supernatant.

2.2.3.3 DNA Assay

The assay for DNA was the method of Leyva and Kelly (1974). 2-deoxyribose reacts with a chromogenic reagent to give a coloured product with an absorbance maximum at 600 nm. The chromogenic reagent was prepared as follows. 1.5 g of diphenylamine (Sigma) was dissolved in 100 mL of glacial acetic acid (Sigma). To this was added 1.5 mL of concentrated sulphuric acid (Sigma). This formed reagent A. 0.5 mL of acetaldehyde were

mixed with 24.5 mL of distilled water to form reagent B. The chromogenic reagent was freshly prepared before use by the addition of 0.1 mL of reagent B to 20 mL of reagent A.

200 µL of the samples for assay were prepared by precipitation with an equal volume of 0.4 M perchloric acid at 4 °C for 30 minutes. Precipitate was collected by sedimentation in a bench top centrifuge (MSE Microcentaur) at 16,000 rpm for 10 minutes. The resulting pellets were incubated with 200 µL of 1 M perchloric acid at 70 °C for 30 minutes. The samples were cooled and 0.5 mL of chromogenic reagent was added. Samples were read at 600 nm after overnight incubation at 37 °C.

Sample concentration was determined against a standard of highly polymerised calf thymus DNA (BDH) dissolved in 10 mM Tris/0.5 mg/mL bovine serum albumin buffer at pH 7.4. Standards were measured in the range 0 to 1250 µg/mL DNA.

Reproducibility of the standards was ± 15% for duplicate samples.

2.2.3.4 Lipid Assay

The amount of lipid in the samples of yeast homogenate was assayed by chloroform extraction and weighing the dried extract. The method used for the chloroform extraction was that of Bligh & Dyer modified by Kates (1986). 1 mL of sample was extracted at ambient temperature with a 2:1 v/v mixture of chloroform:methanol for 2 hours with occasional agitation. After 2 hours the sample was centrifuged (MSE Europa 24) at 4000 rpm at 4 °C in stoppered glass vessels. The supernatant was reserved and 4.75 mL of methanol:chlorofrom:water in the ratio of 2:1:0.8 v/v was added to the pellet. This was resuspended and left to extract for a further 2 hours. The centrifugation was repeated and the supernatant added to that previously reserved. To the combined supernatants, a further 2.5 mL each of chloroform and water were added and the mixture agitated. The resulting

emulsion was broken by centrifugation for 5 minutes at 4000 rpm and 4 °C. The lower chloroform phase was drawn off and evaporated to dryness in a rotary vacuum evaporator. The residue was taken up in 2 mL of 1:1 chloroform:methanol v/v, and placed in a weighing bottle. A further 2 mL of the chloroform - methanol mixture was used to wash the evaporator flask and this was added to the first 2 mL in the weighing bottle. The weighing bottle was dried in a desiccator over potassium hydroxide and under nitrogen to constant weight (± 0.0005g). All reagents were analytical grade or better (eg BDH).

Reproducibility of the samples was ± 20 % for duplicate samples. The method was long, and the scope for introducing further errors through partitioning, and loss of phases on the equipment was high.

2.2.3.5 Determination of Solids Dry Weight

Total dry weight of samples could be determined by drying 5 mL of the sample in a pre-weighed foil boat in an oven at 80 °C to constant weight (± 0.0005g). Solids particulate content was estimated by difference of the assayed amount of soluble protein, lipid and sodium chloride. Typically, variation between samples was ± 10 %.

Nucleic acids and other components were not considered and would have introduced a small error into the estimated solids content.

2.3 RESULTS AND DISCUSSION

2.3.1 Development of ADH Chromatographic Method

According to Okuma et al. (1991), ADH has a rather narrow range of stability between pH 5.5 and pH6.5. In the same paper, the pI of the yeast enzyme was determined as 5.5, a value agreed with by Hoffstetter-Kuhn (1990).

The pH stability of the enzyme was measured in it's purified state (Sigma Product # 3263, Lot # 50H80551) and also in raw homogenate prepared as described in Section 2.2.2.1. Measurement of the enzyme stability was made by comparing the initial activity values for ADH to the activity determined after incubation at 25 °C at a constant pH for a given time. The results are shown in Figure 2.4 a) and 2.4 b) for the change in activity with time for the ADH in homogenate and the pure ADH in phosphate buffer. It was found that there was no significant difference in the rate of decay of activity for the homogenate compared to the pure ADH solution, and the enzyme retained an activity independent of pH in the range pH 6.7 to pH 4.5 with a half-life in excess of 100 minutes. Outside this range, ADH activity reduced rapidly with a half-life of about 5 minutes. Because there was no significant difference between the purified ADH and the homogenate it was concluded that there was no proteolytic activity in the homogenate over this pH range.

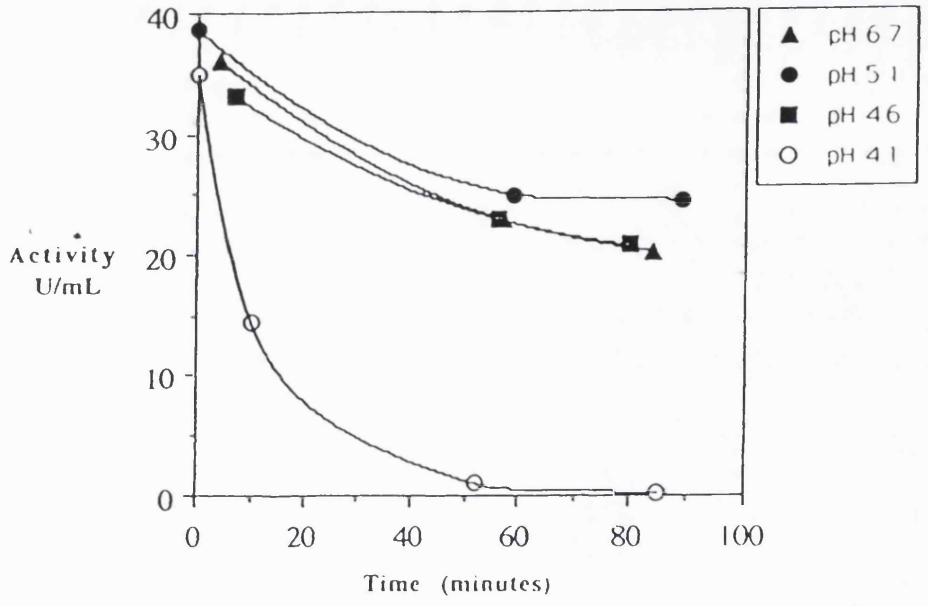

Figure 2.4 (a): *The effect of pH on the activity of ADH at different pH values.*

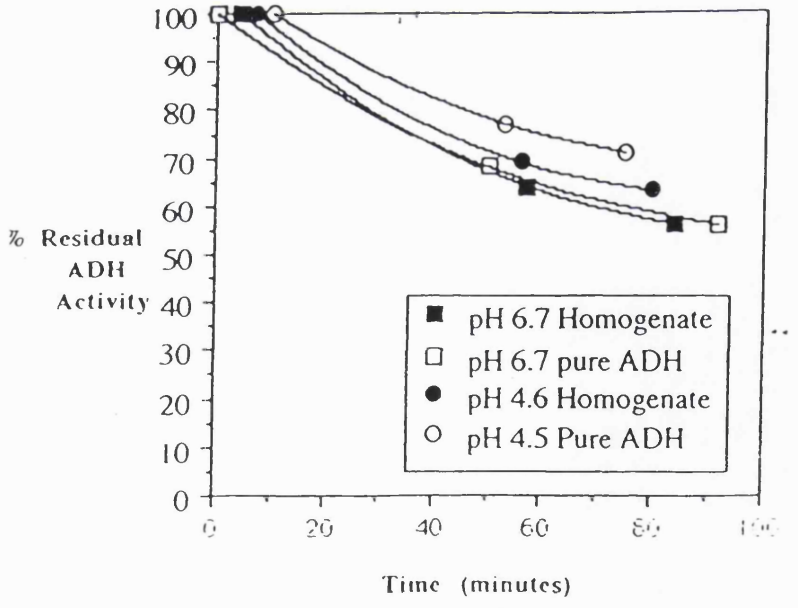

Figure 2.4 (b): *Stability of pH in homogenate and phosphate buffer.*

The ADH stability range allows either anion or cation exchange resins to be used in the purification as the pI of the enzyme is quoted as 5.5. The stability work showed that anion exchange could be used for purifying ADH in buffers from a pH greater than the pI of 5.5 up to the stability limit of pH 6.7

The method development for the anion exchange chromatography used pure ADH (Sigma) to run a series of experiments at different loading levels and pH values from 5.5 to 7.0 in 0.5 pH steps. The development of the elution step used 0 to 1 M sodium chloride gradients from 2 to 10 column volumes. The final method developed (Section 2.2.2.3) was then used to purify ADH from the homogenate preparation clarified by ultracentrifugation as described in Section 2.2.2.1. The column was a 10 mL XK26 column packed as described in Section 2.2.2.2, and run at a linear flowrate of 0.8 cm/minute, a flowrate consistent with a preparative production process. A total 200 mg of protein in a 10 mL volume was loaded to the column and the resulting chromatogram is shown in Figure 2.5.

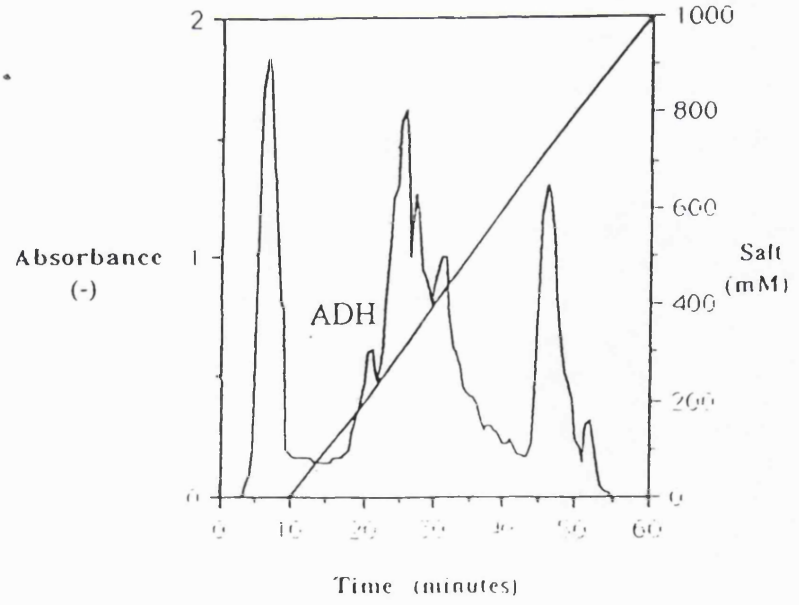

Figure 2.5: *Method for ADH purification from yeast homogenate, A280 absorbance versus retention volume.*

Two peaks of ADH activity were found in the assayed fractions, although only one peak could be determined on the A_{280} trace. The first peak was eluted during the column washing step and the second peak eluted at 180 - 200 mM sodium chloride. The purification factor achieved in the second peak was a modest 2-fold, and the yield was 70 %, the remaining ADH being accounted for in the first activity peak.

This chromatography method served to provide the method against which the different fouling streams were compared.

2.3.2 Equilibrium Binding of ADH to DEAE-Sepharose Fast Flow

The chromatography development runs showed that the DEAE-Sepharose Fast Flow had a rather low capacity for the yeast ADH. An experiment was performed to determine the adsorption isotherms for both the pure ADH and the ADH in homogenate binding to the DEAE-Sepharose Fast Flow matrix to try to understand what was causing this.

DEAE-Sepharose Fast Flow gel was equilibrated in 20 mM phosphate buffer at pH 6.5 (Section 2.2.2.2) by washing several times in the buffer, and then overnight equilibration in the buffer at room temperature. The settled gel volume was measured. Equal volumes of gel to homogenate were combined at a range of homogenate dilutions. These were incubated at 25 °C for 24 hours with continuous agitation using a bacterial shaker incubator. After this time the supernatant was removed and assayed for ADH activity. The quantity of ADH bound to the gel was measured by difference between the original homogenate activity and the final supernatant activity. A control of homogenate alone was set up to account for the loss of activity due to thermal or proteolytic activity. The initial and final activity of the control was measured and this value for activity loss was used to correct the activity assayed in the other samples. The experiment was repeated for the pure

ADH in phosphate buffer at pH 6.5. It was expected that ADH would bind with the DEAE-Sepharose to give a favourable isotherm of the Langmuir type.

Langmuir, 1918, described the analysis that gave this type of favourable isotherm it's name, and the analysis has been extensively applied to adsorption because of it's simplicity. The assumptions made are discrete solute attachment points, isothermal attachment, maximum adsorption corresponds to a monolayer, reversible adsorption and desorption is dependant only on the amount of material attached to the surface of the adsorbent. The application of the Langmuir isotherm to protein adsorption has been criticised by many workers as one or several of these assumptions has been shown invalid for several proteins (Velayudhan and Horvath, 1988; Gosling, 1985; van der Weil , 1989).

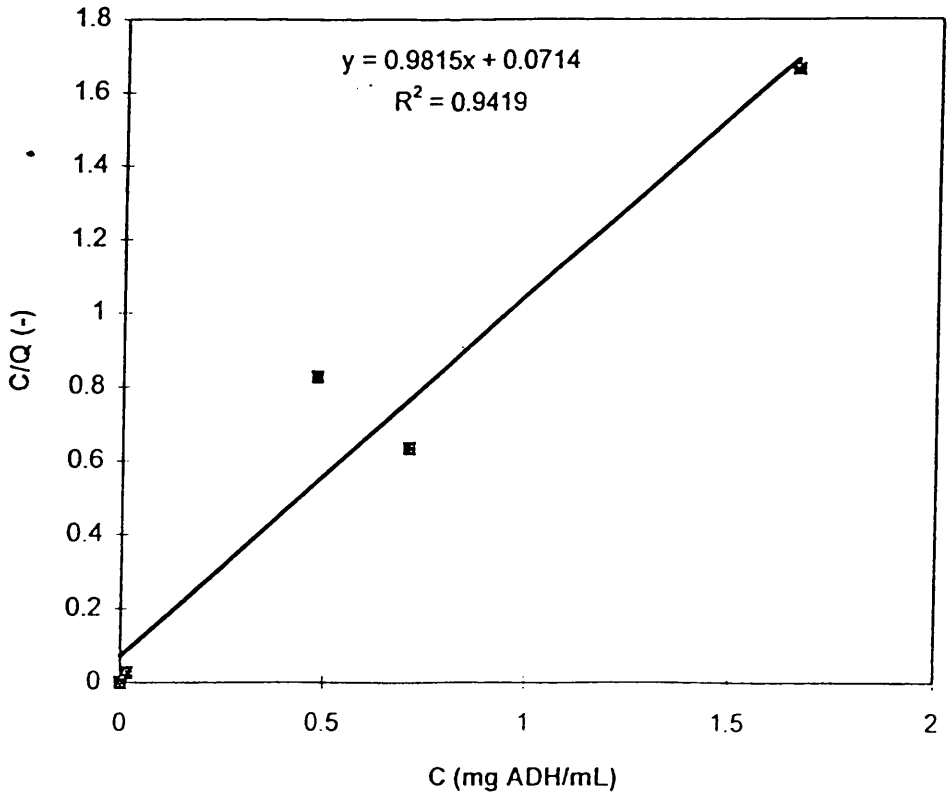

Figure 2.6: *Eadie-Hoftsee plot for the binding of pure ADH to DEAE-Sepharose FF at pH 6.5*

A typical Langmuir isotherm was determined for the pure ADH at pH 6.5. The Eadie-Hoftsee plot for the isotherm for pure ADH is shown in Figure 2.6, where 1 mg of protein is equivalent to 350 Units of ADH activity. This gives a maximum equilibrium capacity for ADH of 356 U/mL of gel. The dissociation constant was determined as 0.0714 mg/mL gel. It should be noted that the volume used here is the Units of protein adsorbed per mL of adsorbent, which also includes interstitial volume of the adsorbent.

Figure 2.7 and Figure 2.8 shows the equilibrium isotherm of the ADH in the presence of homogenate at pH 6.5 and pH 7.5 respectively, and it is immediately apparent that the expected Langmuir adsorption isotherm was not followed by ADH.

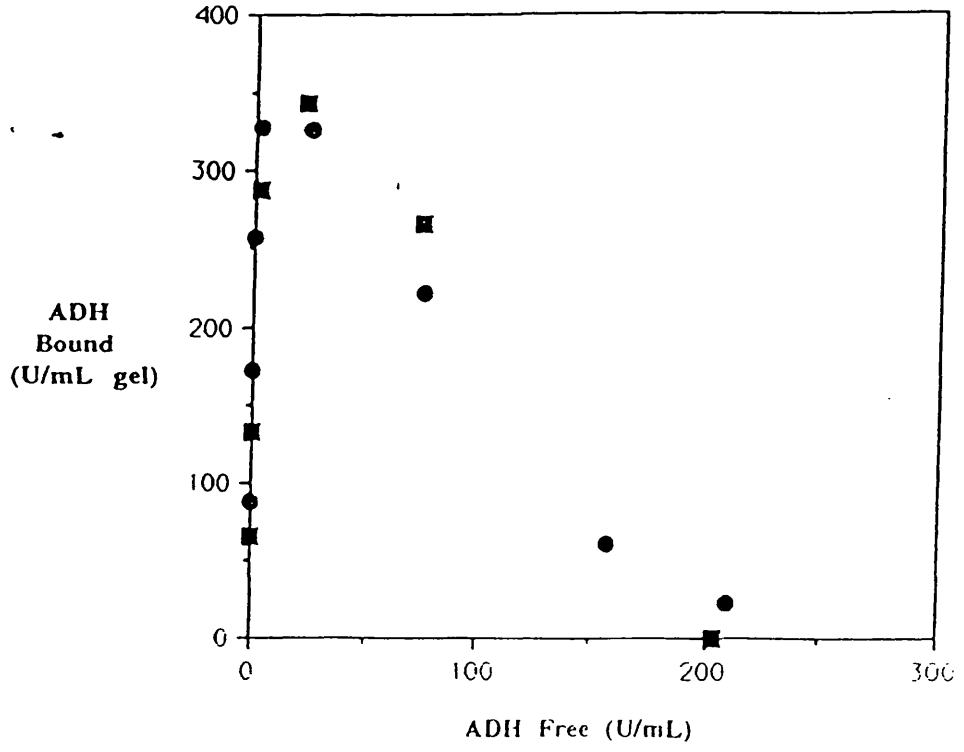

Figure 2.7: *Isotherm for equilibrium binding of ADH in homogenate to DEAE-Sepharose FF at pH 6.5.*

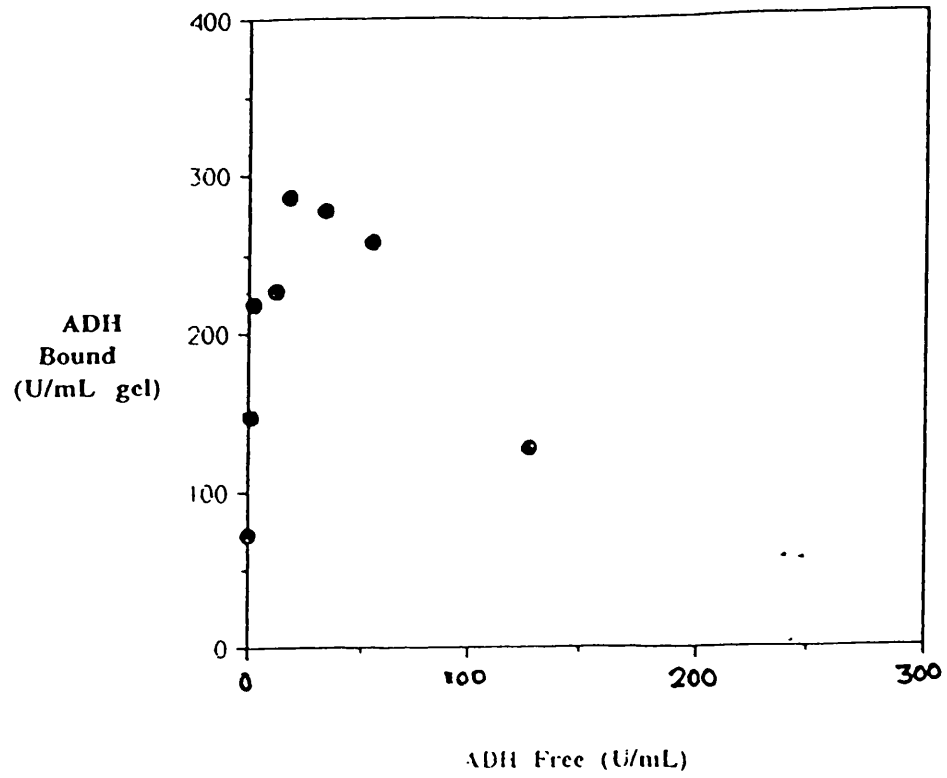

Figure 2.8: *Isotherm for equilibrium binding of ADH in homogenate to DEAE-Sepharose FF at pH 7.5.*

It has been observed (Chicz & Regnier, 1990) that three dimensional structure sterically determines which surface residues on the protein contact the stationary phase. Even a small protein will have hundreds of charged residues capable of interacting with a charged surface, and it is likely that the protein will be absorbed at more than one site. For a protein such as ADH, the molecule has a molecular weight of approximately 141 kD and is made up of four identical subunits (Buhner and Sund, 1969), interactions between the matrix and the molecule, and molecule to molecule will be complex. For a mixture of proteins and fouling species the binding of ADH would seem to be affected by competition for it's binding sites, and ADH is more easily dissociated from the matrix by the contaminants present in the homogenate. A maximum ADH concentration that can be bound to the DEAE-Sepharose is of the order of 300 to 340 U/mL (corresponding to about 1 mg

ADH/mL) of the absorbent matrix, in the presence of 6 mg/mL of total protein. Beyond this concentration, the ADH is displaced, probably by protein with a higher affinity for the matrix.

A consequence of this isotherm behaviour is that the chromatography matrix DEAE-Sepharose exhibited a low capacity for ADH in homogenate, and the separation was correspondingly more difficult. Although an attempt was made to increase the affinity of the matrix for ADH by shifting the pH further from the pI of the protein, the effect was to correspondingly reduce the capacity of the anion exchanger, where the maximum adsorption capacity is typically observed at pH 6 (van der Weil, 1989).

Despite the low levels of capacity, it was decided to pursue the ion-exchange route, as it provided a reproducible test-bed with which to develop an engineering approach to the analysis of column fouling.

2.3.3 Effect of Repeated Fouling on the Column Chromatography of ADH

The effect of fouling on the chromatography performance was examined by measuring the purification achieved at a set yield for several chromatographic cycles of loading-eluting-regenerating the column. For this work ten column cycles were performed using the yeast homogenate clarified by ultracentrifugation, as described in Section 2.2.2.1. A freshly packed column of DEAE Sepharose Fast Flow column was produced using the method described in Section 2.2.2.2. The fractionation diagrams of protein versus ADH activity were constructed for the first, fifth and tenth chromatographic cycles are shown in Figure 2.9.

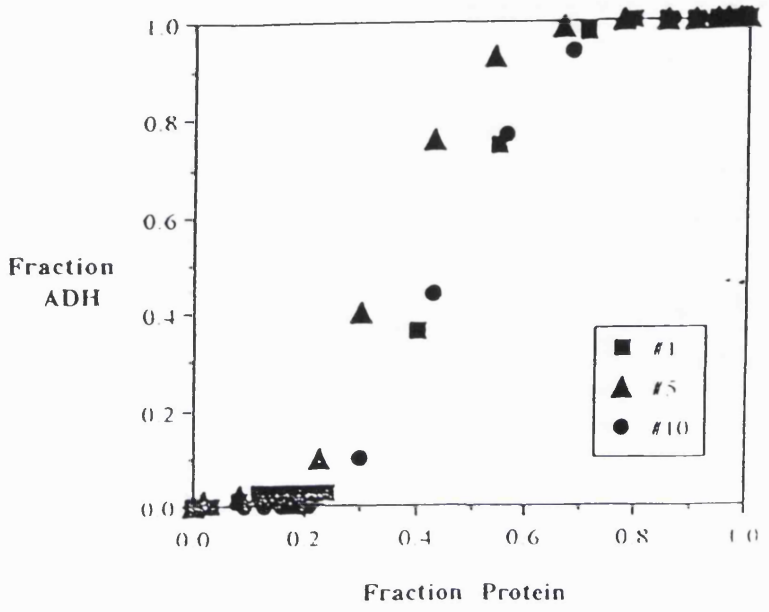

Figure 2.9: *Fractionation diagrams for the first, fifth and tenth cycles in a series of ten chromatography cycles. The column was treated with 2 mL of ultracentrifuge clarified homogenate in each cycle.*

The columns were run using the method described in Section 2.2.2.3, and the column regenerated using 1 M sodium chloride followed by equilibration in a 20 mM phosphate buffer at pH6.5.

The purification factor achieved at a 95 % yield of ADH is shown in Table 2.1.

Table 2.1: The purification factor achieved for 10 cycles of ADH purification from ultracentrifuged homogenate.

Cycle Number	ADH Specific Activity *	Purification Factor at 95 % Yield
1	7.95	2.1
2	8.10	1.9
3	7.80	2.2
4	7.92	2.1
5	9.86	2.5
6	8.91	2.2
7	7.56	2.0
8	7.76	2.1
9	7.89	2.3
10	7.64	2.1

* The amount of protein loaded was 150 mg , determined for the bulk sample. ADH specific activity was repeatedly determined, values for each sample shown to illustrate that the specific activity did not vary greatly over the time course of the experiments.

The maximum achieved purification factors achieved for these runs was the 2.5 fold purification for cycle number 5. The range was from 1.9 to 2.5 times. This was a rather modest increase in purity, however, it should be remembered that the purpose of this work was to demonstrate the change in performance of the chromatography with increased fouling. A review of the A280 absorbance pattern for the ten cycles, showed the chromatography to be very similar, even after ten cycles. This was reflected in the nature of the fractionation diagrams which could be more or less overlaid for each of the cycles.

Mass balances were attempted for material loaded and removed from the column. These could be closed for the amount of protein and ADH loaded. Because the material was ultracentrifuged, it was inferred that the size of any particulates would be very small, and size particle analysis using the Brookfield DCP 1000 Photosedimentometer (Brookhaven Instruments Corporation) detected no particles below 1 micron. Likewise, free lipid from cell membranes was removed in the ultracentrifuge, as it creamed at the top of the supernatant and could therefore be removed. No lipid could be detected in the sample loaded to the column using the assay described in Section 2.2.3.4. Whilst it was difficult to achieve reproducible results between the amount of (non-protein) solids loaded to the column and solids in the effluent, there was no evidence for large levels of particulate entrapment in the column. Again the results would tend to suggest that no gross changes in column performance were discernible over the 10 cycles, and from this it was inferred that there was no significant fouling of the column.

The ten cycle experiment was repeated, but this time using the disc stack centrifuge clarified yeast homogenate as the sample as described in section 2.2.2.1. Samples were loaded to carry the same amount of soluble protein as for the ultracentrifuged samples. A fresh column was packed for this work, and no cleaning was performed between the cycles , other than to re-generate the column. Table 2.2 gives the results for the first, fourth, fifth and tenth cycles.

The purification diagrams for the corresponding cycles are shown in Figure 2.10.

Table 2.2: The purification factor achieved for 10 cycles of ADH purification from disc stack centrifuged homogenate.

Cycle Number	ADH Specific Activity	Purification Factor at 95 % Yield
1	6.88	3.2
4	7.21	2.4
6	7.00	4.1
10	6.98	11.9

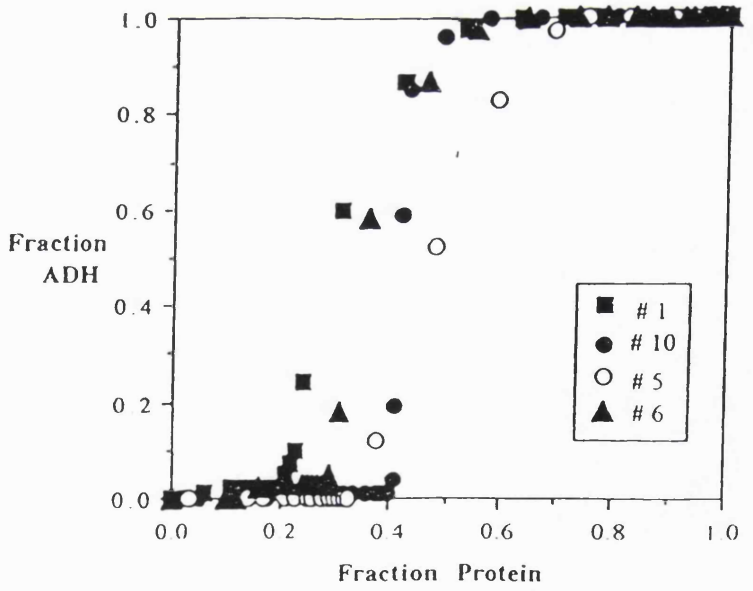

Figure 2.10: *Fractionation diagrams for the first, fifth, sixth and tenth cycles in a series of ten chromatography cycles. The column was treated with 2 mL of disc stack centrifuged clarified homogenate in each cycle.*

There was a clear upward trend in the purification factor for the column fouled using the disc stack centrifuged material. As before, an equivalent amount of soluble protein was loaded for each cycle (150 mg) to allow comparison to the work using the disc stack centrifuged homogenate. Once more assay results allowed protein and ADH to be completely accounted for by a component mass balance between quantities of protein and ADH loaded to and recovered from the column, whereas solids component balances were difficult to achieve reproducibly. The A_{280} spectrophotometer trace for the disc stack centrifuged material showed an extra peak as compared to the ultracentrifuged A_{280}. This peak had a turbid nature, suggesting that it contained most of the insoluble cell debris material loaded to the column. Analysis of the size distribution of the particles passing through the bed as compared to the sample loaded showed there to be no difference (Figures 2.11 (a) and 2.11 (b)). Determination of the particle size was by a Brookfield photosedimentometer. This determined cell debris size by measuring the time taken for the debris to settle under an applied centrifugal force asuming that Stoke's law applies. Values for the cell debris density were obtained from Olbrich (1989). The experimental parameters are listed below:

Disc Speed	3999.47 rpm
Particle Density	1.068 g/cm
Spin Fluid	10 % Sucrose
Spin Fluid Density	1.038 g/cm
Spin Fluid Volume	15 mL
Spin Fluid Viscosity	1.34 cp

The size data, coupled with the solids mass balance, indicated that most of the particulates loaded were able to pass through the bed without retention. It is possible that sub-micron range solids were entrapped in the bed, as such particles were outside the detection range of the Brookfield Photosedimentometer.

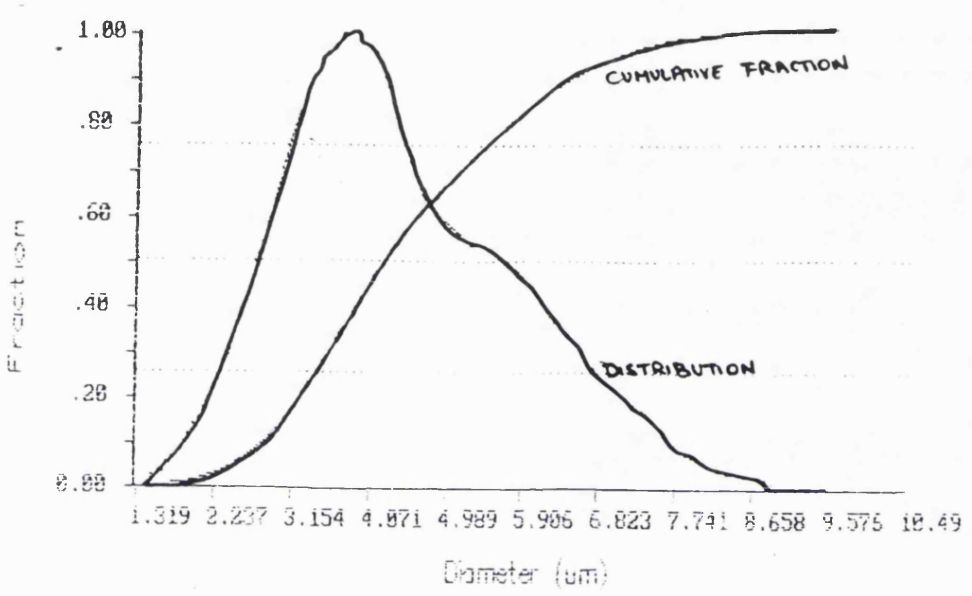

Figure 2.11 (a): *The particle weight distribution of yeast cell homogenate clarified through a disc stack centrifuge as determined by Brookfield photosedimentometer.*

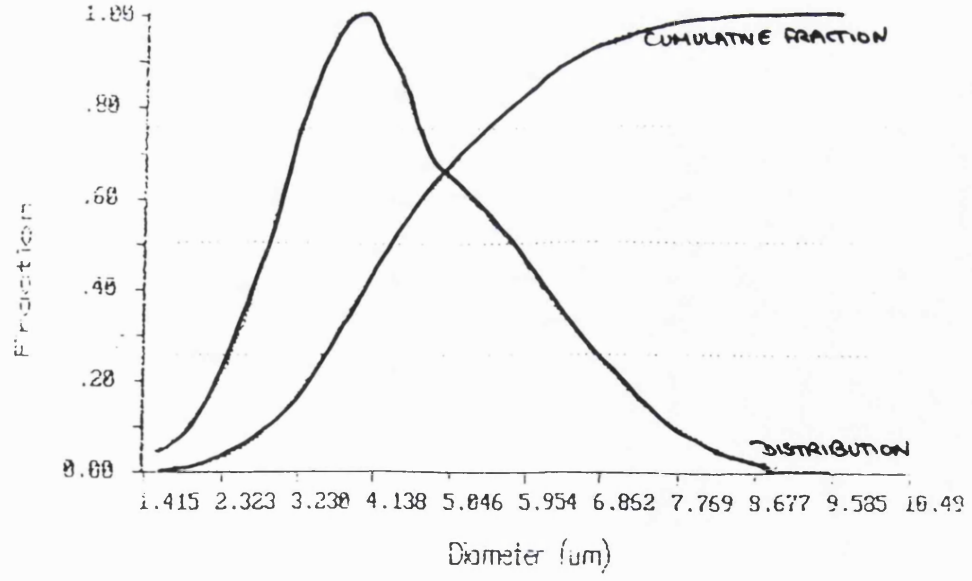

Figure 2.11 (b): *The particle weight distribution of yeast cell homogenate after passage through the chromatography bed.*

The question must be asked as to why there is a marked increase in the purification factor of the ADH. This must be indicative of a process of fouling that is occurring, all be it that the effect is to increase the performance of the separation rather than to degrade it. There is a report in the literature of *S.cerevisiae* cell wall debris separating proteins by an ion-exchange mechanism (Shaewitz et al, 1989). Thus, a plausible hypothesis for the increase in the purification factor is that there was entrapment of solids (cell wall debris) in the bed, and that these solids served to act as a functional ion-exchanger with a higher selectivity for the ADH than the matrix itself. It is compatible with this hypothesis that the more sub-micron solids entrapment that occurs, the higher the purification factor. This was observed. In Shaewitz et al's paper, ADH is one of the proteins studied, showing an apparent peak adsorbance of ADH at pH 6 to 6.5, consistent with the pH at which these experiments were conducted, that is pH 6.5.

This work illustrates the use of the fractionation diagram as a method for determining the efficiency of a separation. The diagram has been used here to take a purification factor at a standard arbitrary yield of 95 % of the activity of ADH recovered after chromatography. This allowed a comparison between experiments; using the diagram as a design tool can quickly show at which point yield can be sacrificed for purification factor. Higher purification factors can be achieved if yields can be sacrificed, and it is possible to determine the pay-off between purity and yield for a complete process with the aid of fractionation diagrams constructed at each purification stage.

The encouraging aspect of this work is that a difference in column chromatography is detectable using ADH purification as a marker, where performance is related to the degree of clarification in the process stream and the number of cycles to which the column has been subjected. However, this difference is system specific, leading to an increase in purification factor for ADH, which will not necessarily be seen for a different protein purification. The small capacity of the matrix for ADH was also a problem, limiting the

amount of homogenate that could be loaded in a sample, since eventually ADH is removed from the column by displacement of competing species in the homogenate, and it is then not possible to construct the fractionation diagram.

2.3.4 Effect of High Solids Loading on Chromatographic Performance

The aim of this work was to determine the effect of loading solids on the purification of the ADH. As was commented in the previous section, it was not possible to purify directly ADH from homogenate if quantities of total protein exceeding about 200 mg were loaded to the 10 mL packed beds, since the ADH would be competitively displaced by other species. It was of interest to push the packed bed to the limit of its performance, that is to the point where it would just start to be clogged by the solids loaded. As a result it was decided to use homogenate to foul the bed, and then determine the separation performance of the bed by using a test loading of ultracentrifuged homogenate from which ADH was purified. This is a similar approach to that taken by Tice et al, 1987, where repeated fouling of the column by a synthetic mixture of three proteins was tested over several cycles, and the performance determined by the chromatographic resolution between the proteins after they had been chromatographed.

Columns were packed as described in Section 2.2.2.2. The performance of freshly packed columns was determined by injecting a sample of ultracentrifuged homogenate (2ml containing 80 mg of protein, and ADH specific activity 6 U/mL). Purification of the ADH was performed as described in Section 2.2.2.3, and the purification measured by constructing a purification diagram. The column was then fouled with a quantity of disc stack centrifuged homogenate ($Q/\Sigma = 1.9 \times 10^{-8}$ m/s), washed with a 0 to 1 M sodium chloride gradient, re-equilibrated with phosphate buffer and re-tested with the same ultracentrifuged homogenate to determine purification of the ADH after the bed had been fouled.

The columns tested were fouled with four different loads of homogenate from 10 mL to 100 mL. After each experiment, a fresh column was packed. Columns were badly discoloured after fouling, and no attempt was made to clean the columns post-fouling, since there was a chance of bed compression and packing instability or channelling as a result of the history of the column. There was some stratification of the discoloration in the bed, with columns typically showing a dense brown discoloration over the first centimetre, followed by two progressively lighter zones of equal length (2 cm).

The change in pressure over the course of the sample loading was measured using the pressure transducers in the P-500 pumps. It was found that there was a significant increase in pressure during sample loading by up to 0.05 MPa, but this was transient, and reduced after the sample was loaded and buffer was washed through the column. The pressure rise was presumed to be due to the viscosity of the sample and blockage of the column interstitial spaces by particulate solids. However, as the pressure drop was transient, it can be inferred that no permanent blockage of the interstitial space occurred as a result of the sample loaded. The total dry weight of the loaded material was compared to the total dry weight found in the column effluent in order to estimate the extent of solids deposition within the column. Results for the estimated amount of material trapped in the column compared to the purification factor derived from the fractionation diagrams pre- and post-fouling are shown in Table 2.3.

The values in Table 2.3 indicate that there is a small amount of solids trapped in the bed during the fouling process, and this has a generally negative effect on the separation of ADH. There was no improvement in the separation of the ADH with increasing solids loading. This is probably as a result of a different fouling mechanism. When the column was repeatedly recycled after loading 2 mL of homogenate in 2.3.3, an amount of the fouling species would be removed during the regeneration process - for this experiment, the purification would experience the full effect of all the foulants on the column, with the result that the purification factor was reduced rather than increased. It may be that the

improved purification was still present, but it's effect is cancelled out by the increased fouling.

Table 2.3: Effect of Solids Retention on Purification Factor

Volume of Yeast Homogenate Challenge (mL)	Sedimented Solids Loaded (mg)	Estimated Solid Deposition in Column (mg)	Purification Factor @ 95% Yield (-)
0*	0	0	2.30
10	700	10	1.12
0*	0	0	2.10
25	1740	31	1.65
0*	0	0	2.05
50	3480	23	1.42
0*	0	0	2.20
100	6960	500	1.50

* *Denotes freshly packed column*

The low purification factors achieved, even with a fresh column, makes comparison difficult, and the results achieved are quite marginal. The fractionation diagrams show very little difference between clean and fouled columns. This is due to the size of the fractions taken, that is 2 mL. To take smaller volume samples would have improved the resolution of the fractionation diagram, and this may have been more diagnostic for the fouling.

The yield and purification factor were determined for each of the fractions taken in each run, a total of 26 fractions. The yield was multiplied by the purification factor in each fraction to give a yield factor, and this was plotted as a function against the column retention volume. It was found that this pattern was more revealing about the degree of

fouling that occurred after addition of the crude homogenate to the bed than the fractionation diagram. The results are shown in Figures 2.12 (a) and 2.12 (b).

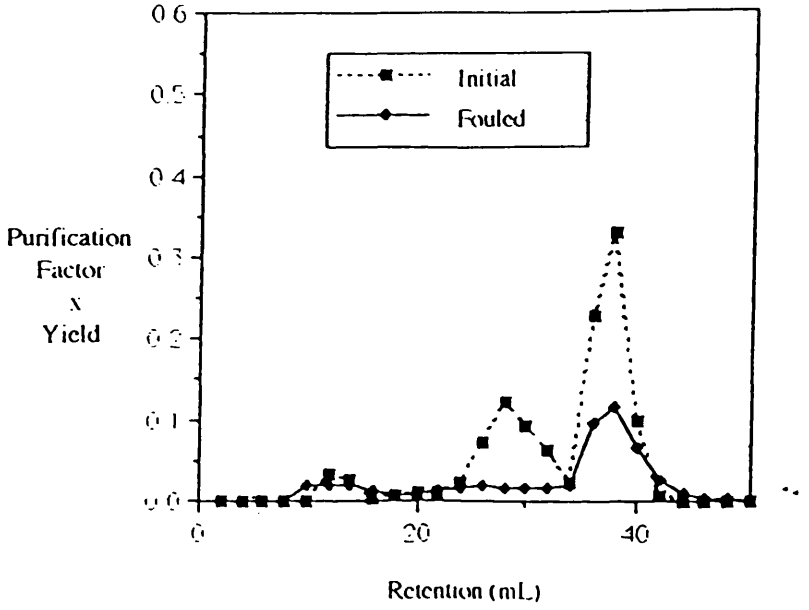

Figure 2.12 (a): *The yield factor for a clean and a fouled column treated with 10 mL of disc stack centrifuged yeast homogenate. Yield factor is defined as the product of the yield and purification factor of the ADH for each of the 26 fractions collected in the experiment.*

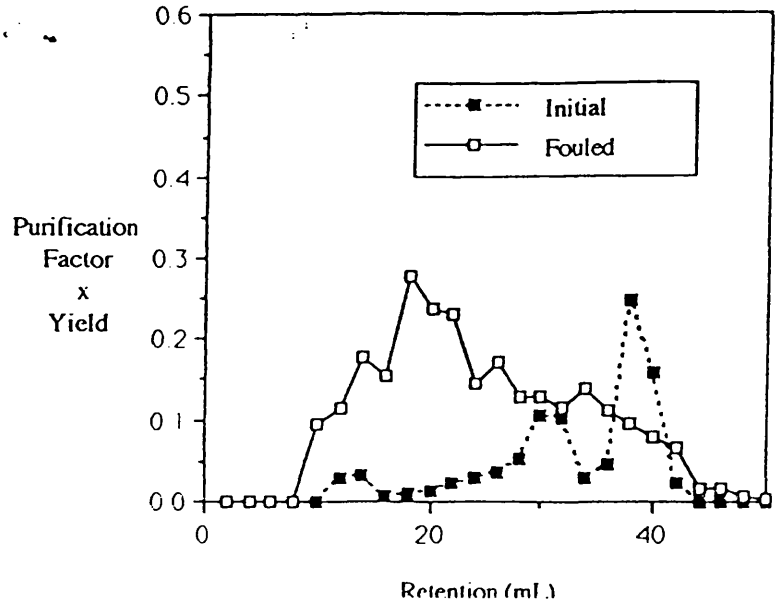

Figure 2.12 (b): *The yield factor for a clean and a fouled column treated with 100 mL of disc stack centrifuged yeast homogenate.*

It should be noted that for the fresh column, there are three peaks of ADH activity. The first peak corresponds to column flow through of unbound material. The second two were probably isozymes of ADH, resolved by the DEAE-Sepharose. It has been reported that there are two isozymes of ADH in yeast termed ADH 1 and ADH 2, and these have been separated by DEAE-Toyopearl 650 M ion-exchange (Okuma et al., 1991) - the method used was similar to the chromatographic method reported here.

It is immediately obvious that three peaks are reduced to two for the case of the 10 mL homogenate fouled column, corresponding to unbound ADH and a single peak of ADH activity eluted, an indication of loss of resolution. When the extreme case of 100 mL of homogenate is loaded to the column, the separation of the ADH is much changed, and there is a displacement and spreading of the activity peak. Although the overall yield is high, the purification is very low.

2.4 CONCLUSIONS

ADH in cell homogenates is competitively displaced from anion exchange columns, probably by competing proteins. As a result, it was not possible to load a large amount of protein to the column with the system chosen. Interestingly, hydrophobic interaction chromatography (M. Smith, 1997) has been shown to behave in the opposite way, ADH being bound preferentially as protein loading is increased. This is very diagnostic of the structure of the ADH molecule, and it would appear that the exterior of the molecule has a high degree of hydrophobic groups which explains the low interaction with the anion exchanger.

It is apparent that there is an effect on the separation performance after columns have been fouled. The fractionation diagram was not very useful for describing this effect. There are a couple of reasons for this. Firstly , the resolution of the diagram is restricted to the number

of fractions taken. In these experiments, 2 mL fractions were collected. The ultimate resolution would be a continuous sampling device capable of identifying the difference between the ADH and contaminants (preferably individual fouling species). A diode array spectrophotometer may have helped resolve this, although the process stream may be to complicated to allow the fine resolution required. The use of a more easily detetable tracer is a second approach, for example a dye or radioactive tracer, but these were not considered practical the time. The second problem with the fractionation diagram is that where the purification factor achieved is very small, the fractionation diagram is never going indicate a significant difference. Additionally, volume eluted is not part of the plot, so even although the ADH peak may be retarded or not retained, the fractionation diagram will not show this. A conventional chromatogram may be more useful as an indicator of relative separation performance. However when a significant purification is achieved, then fractionation diagram will indicate this difference as the results of Section 2.3.3 show. The power of the fractionation diagram is that it can maximise the yield versus purity relationship, but only where significant purification occurs.

The goal of purification for commercial purposes is to achieve a specified purity at an economical yield. For a very crude process stream, it is difficult to analyse the chromatogram to determine mechanisms of fouling. Thus, although purity and yield will show the effects of fouling, this is the symptom rather than the cause of the fouling. There is an additional challenge with all biological systems, in that in choosing one type of protein to purify, unique properties will almost certainly be encountered that produce surprises in its purification. For this reason test systems will always be very specific.

This work has shown that the amount of particulates present in the feed stream to a chromatography column will exert an effect on the purification. Interestingly for small amounts of solids loading, where the amount of solids trapped in the column was not detectable by solids mass balance (as assayed by dry weight), there was actually an improvement in the column separation that could best be explained by an ion-exchange

action of the sub-micron cell debris, and was a very significant effect. For larger quantities of solids, there was a degradation in the purification factor, but because the purification factor was so small (even for the freshly packed column) it was difficult to see if the effect was cumulative with the amount of solids loaded. No attempt was made to analyse the fouling components further than to determine solids deposition, because it seemed unlikely that the use of purification factors could be used to determine the mechanism of fouling as discussed previously. It is highly likely that there is a synergistic effect between classes of contaminants in the crude homogenate, and this will be investigated in subsequent chapters.

3. FRONTAL ANALYSIS

3.1 INTRODUCTION

The operation and economy of a packed bed adsorber is dependant on the capacity for the solute of interest. Capacity is therefore a critical parameter when considering the design of an adsorption step, and how the process stream affects the capacity of the adsorbent has implications for the economy of the adsorption step. Frontal analysis when applied to packed bed adsorbers, refers to the pattern of solute breakthrough that is observed when solute is continuously fed to the packed bed. As solute is loaded onto an adsorbent, solute binds to the column and, eventually, starts to breakthrough in the column outflow. The point at which solute just starts to appear in the column effluent is referred to as breakthrough, and under normal operating conditions defines the capacity of the column - to continue loading solute under such conditions, although using more of the available capacity of the bed, loses solute in the column effluent , and thus adversely affects the economics of the process. It should also be noted that the point at which breakthrough occurs is dependant on the flowrate used for the adsorption, and the capacity will be somewhat less than the equilibrium capacity for the same quantity of adsorbent - for this reason the capacity at the initial breakthrough is often referred to as the dynamic capacity of the column.

If, instead of stopping the flow at the first sign of solute breakthrough, solute is continued to be fed to the column, a typical breakthrough pattern is seen in the solute concentration in the column effluent. If sufficient volume of solute is applied to the packed bed, the concentration of solute in the column effluent reaches the concentration in the feed, and the column is then saturated with solute and has achieved it's maximum capacity. The variation of the concentration profile of the solute in the effluent stream as a function of time, or volume of solute added to the column, is known as the breakthrough curve. The

process of measuring such a curve is referred to as frontal analysis (Cooney, 1990; Chase, 1984; Cooney 1993). Figure 3.1 shows a comparison of a typical breakthrough curve.

The shape of the breakthrough curve is characteristic of the processes that are occurring in the column as solute binds to the adsorbent. In this study, the shape and nature of the curve has been used to diagnose the condition of the column, and to infer the values of key chromatographic transport parameters that govern the column performance. The shape and position of the breakthrough curve are dependant on the capacity of the adsorbent, the equilibrium characteristics of the solute for the adsorbent and the mass transfer characteristics of the adsorbent. Accordingly a comparison of the breakthrough curves for columns fouled using different fouling streams and conditions will give a strong indication of the fouling that has occurred, and importantly, an indication of the mechanism of fouling.

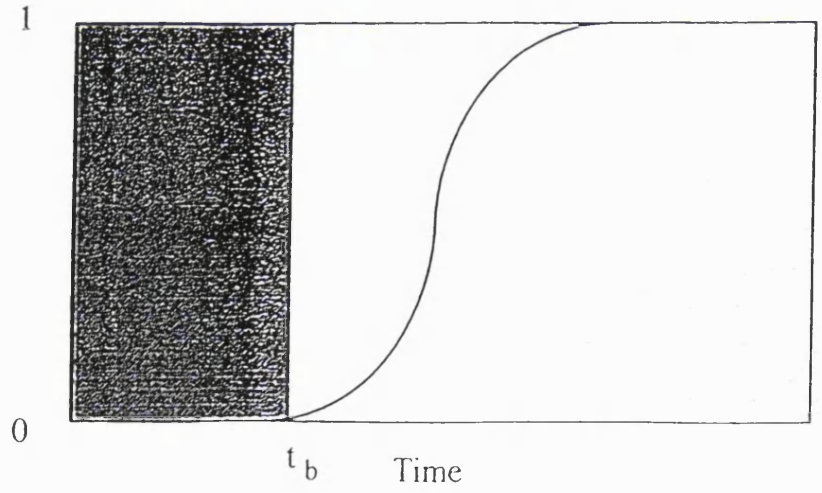

Figure 3.1: *Typical breakthrough curve. The hatched area represents the capacity of the column at 1 % breakthrough which is characteristic at a given flowrate.*

There is a lot of literature on models to predict adsorber bed concentration profiles (Chase, 1984). Typically for protein adsorption chromatography the equilibrium binding isotherms are normally favourable. For favourable isotherms, a concentration profile is quickly established in the packed bed, and this progressively moves down the length of the bed as time (volume of solute loaded) increases. The concentration profile is referred to as a constant pattern front (CPF) (Figure3.2).

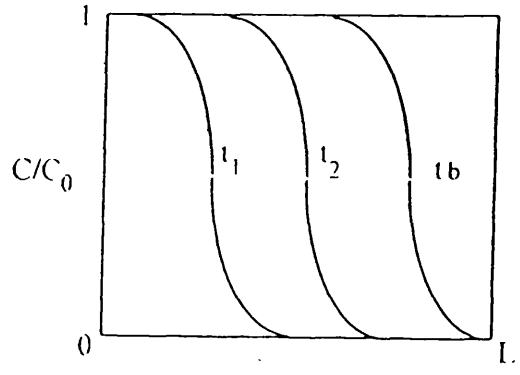

Figure 3.2: *Constant pattern front development in packed beds*

Examples for the solution of this type of CPF are given by Hand et al (1984).

Horstmann and Chase (1989) have developed a model to determine breakthrough curves considering both the external liquid film resistance and pore diffusion resistance to be important. The analysis of Cooney is instructive in qualitatively analysing the shape of breakthrough curves. The approximation of adsorber breakthrough curves derived by Cooney (Cooney 1990; Cooney, 1993) is presented here for completeness. The work provides a simple approximation to the breakthrough pattern asuming a constant pattern adsorption of a solute, as would be theoretically observed in a column of infinite length. The validity of the approximation has been shown by Cooney by a comparison to work performed by Hand et al (1984). Although the approximation coud be used to derive values

for diffusivity and fluid film mass trnsfer, the approach taken here is to make a visual judgement of the dominant effects on adsorption breakthrough caused by fouling

The model described by Cooney is presented here for completeness. Cooney assumes a fluid film transfer model, with solute transfer through a stagnant film governed by a fluid film mass transfer coefficient and a linear concentration difference between the film solute concentration and the bulk fluid solute concentration

Solid Phase $$\frac{dY}{dt} = \frac{15D_S}{R^2(Y_i - Y)} \qquad 3.1$$

Fluid Phase $$\frac{dY}{dt} = \frac{k_f S_0}{K(X_i - X)} \qquad 3.2$$

, where Y is the dimensionless solute concentration in the solid phase, Y_i is the interfacial value of the solute at the solid-liquid boundary, D_s is the solute diffusivity in the stationary phase, R is the particle radius, S_0 is the feed solute concentration, k_f is the liqiud film mass transfer coefficient, K is the ratio of the feed solute concentration to the equilibrium concentration of the solute in the stationary phase, X is the dimensionless solute concentration in the liquid phase, X_i is the value of X at the solid liquid interface and t is time. For the CPF situation, Y=X, and Cooney suggests a Freundlich isotherm, (although a Langmuir isotherm is equally acceptable) so that $Y^* = X_i^m$, where m = 1/n, the inverse of the Freundlich index.

Substituting for Y gives

$$\beta (X_i^m - X) = (X - X_i) \qquad 3.3$$

where $\beta = 15D_S K/R^2 k_f S_O$

From this result the breakthrough curve is approximated using the following two relationships,

$$X = (\beta X_i^{\,m} + X) / (\beta + 1) \qquad 3.4$$

and

$$t = \frac{(\varepsilon + (1-\varepsilon)K)}{\varepsilon VL} + \frac{K}{k_f S_0} \int_{x0}^{x} \frac{dx}{(X_i - X)} \qquad 3.5$$

The parameter affecting the shape of the breakthrough curve is determined by Cooney to be the Biot number, the ratio of the fluid film mass transfer coefficient to the effective solute diffusivity given by β. A symmetrical breakthrough curve that has a steep gradient indicates a sharp isotherm and a similar magnitude of internal diffusion control and external fluid film control. Broadening of the breakthrough curve is observed when the isotherm is less sharp. Early breakthrough is caused by increasing dominance of the external fluid film resistance. Tailing at the top end of the breakthrough curve is due to increased dominance of the diffusivity of the solvent. This is illustrated in Figure 3.3.

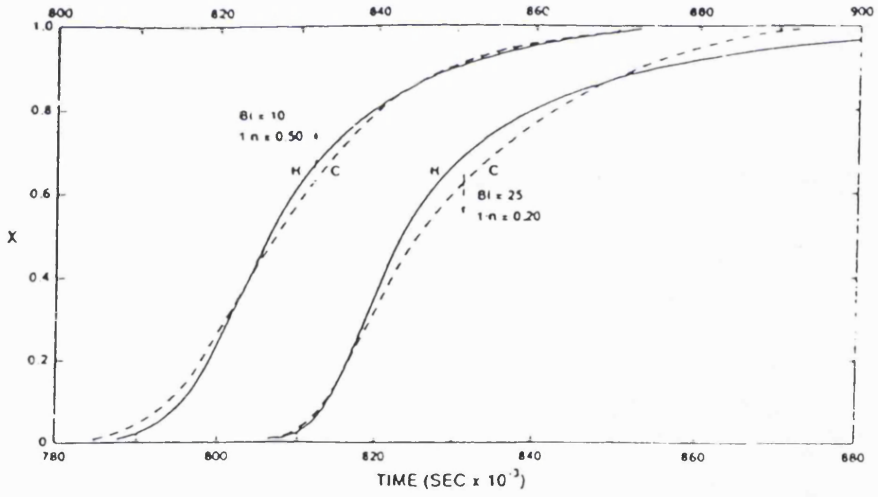

Figure 3.3: *As the Biot number decreases, the "leakage" (early breakthrough) increases, and conversely tailing decreases. Leakage represents dominance of the external fluid film mass transfer, whereas tailing represents dominance of solute diffusivity (reproduced from Cooney, 1993).*

The analysis presented here will form the basis for the assessment of the changes occurring during chromatographic support fouling. The remainder of this chapter goes on to describe

how the breakthrough curves can be used to determine the change in the performance of a packed bed. The fouling stream used is designed to be both realistic and highly challenging for a packed bed to handle, having very high particulate concentrations typical of that which occurs when purifying an intracellular protein from a whole cell homogenate that has been partially or extensively treated by industrial-scale centrifugation.

3.2 MATERIALS AND METHODS

3.2.1 CHROMATOGRAPHIC SYSTEM

3.2.1.1 Chromatography Matrix

The experiments describe were all performed with DEAE Sepharose Fast Flow. This is an anion exchange resin, the structure of which is described elsewhere (Section 2.2.1.1).

3.2.1.2 Buffers

The experiments were carried out using 20 mM phosphate buffer, and ionic strengths were varied up to 1 M using sodium chloride. Phosphate buffers were prepared using di-sodium hydrogen orthophosphate and sodium dihydrogen orthophosphate AR grade or equivalent (eg BDH). pH was adjusted to 7 using 1 M sodium hydroxide AR grade or equivalent (eg BDH) as required. All buffers were prepared freshly each day and de-gassed at room temperature by vacuum filtration through a 0.2 μm filter.

3.2.1.3 Chromatography System

All chromatography hardware was supplied by Pharmacia LKB Biotechnology, and the system used was the FPLC ™ system which has been described elsewhere (Section 2.2.1.2). In summary the system consisted of a programmable controller, two pumps, an automatic injection valve, a fixed wavelength UV spectrophotometer equipped with a flow cell measuring absorbance at 280 nm and sample injection loops of various volumes up to 50 mL. The output from the UV monitor and the pressure transducers attached to the system pumps were logged to a PC using Perkin Elmer Turbochrom 2.1 and using a PE Nelson 900 Series A/D interface to capture the data.

3.2.2 EXPERIMENTAL PROCEDURE

3.2.2.1 Introduction

In the experiments a freshly packed bed was challenged with a fouling stream of either constant volume containing varying concentrations of fouling species, or of different volumes containing the same concentrations of fouling species. Different masses of foulants were therefore injected onto the packed bed in each experiment. To assess the impact of the foulant on the column performance, the breakthrough curve of the packed column was measured before and after the fouling challenge, and the changes in the pattern of the breakthrough curves then evaluated. In most instances, it was not appropriate to re-use the columns since, particularly at the higher concentrations of foulants used, the structure of the bed may have been disturbed which could produce artifacts. However, a clean-in-place (CIP) protocol was tested that proved to be very effective at returning the column to it's original state. Nonetheless it was considered appropriate to play safe and always use freshly prepared columns for each experimental series.

Breakthrough curves were experimentally determined for fresh and fouled columns using bovine serum albumin (BSA) prepared by ethanol precipitation (Cohn fraction V, eg Sigma product A9418). The breakthrough curves were measured by the A_{280} absorbance , and the curves analysed to determine the effect of fouling on the adsorption isotherm, the fluid phase mass transfer coefficient, k_f, and the effective diffusivity, D_e, of the BSA in the packed bed.

As with previous experiments, a real process stream was used made from a yeast homogenate (Section 2.2.2.1). This was produced fresh for each series of experiments to a standard protocol ensuring reasonable similarity between experiments. Process fouling streams were characterised using a variety of assays, namely protein, DNA, lipid, total

organic carbon and dry weight in order to mass balance fouling species that encountered the column (Section 2.2.3).

3.2.2.2 Description of Fouling Stream

The fouling stream was always prepared from a starting material of caked Baker's yeast (*Saccharomyces cerevisiae*) (J.W. Pike Ltd.). The preparation of the fouling stream is as described in Section 3.2.2.2. Great care was used to ensure a standard production protocol. In summary, 12 Kg of yeast was suspended in 9.2 L of 20 mM phosphate buffer at pH 6.5 to 7 to give a 20 L volume of yeast at 600 g/L wet weight. This concentration was chosen as it approached the maximum processing capabilities of the homogenisers used. The yeast suspension was homogenised using 5 discrete passes through a Manton-Gaulin high pressure homogeniser (Model K3, APV) at 500 bar. Temperature was maintained at 4 °C. The resulting homogenate was partially clarified by passage through a disc stack centrifuge (SAOOH 205, Westfalia Separator Ltd) at a flowrate of 40 L/hr.
The resulting centrate was used directly to foul columns or was further processed in laboratory centrifuges to remove selectively more particulate and lipid material to give a different fouling stream.

3.2.2.3 Column Packing Procedure

The column packing procedure used was that described by Andersson (personal communication). This method was adhered to in all the experiments described in this section. To reduce the quantities of protein and adsorbent required, relatively short columns were preferred in these studies, column dimensions were 5.0 cm ± 0.1 cm and 1.6 cm in diameter. The required volume of DEAE Sepharose slurry was de-gassed under vacuum at room temperature. After eliminating air from the column frits, the gel slurry was

poured down the side of the glass column . The column was then filled to the top with de-ionised water and the top adapter fitted to the column. The P-500 FPLC pumps were then used to pack the slurry at a flowrate of 14 mL/minute until a constant bed height was achieved in the column. Next the adapter was brought to the surface of the gel and manually compressed by about 0.5 cm to provide the packed bed.

It should be mentioned that the packing procedure has an effect on the performance of the column, so again the importance of using a standard protocol to pack the columns was not ignored. Nevertheless there are some reservations about the last part of the packing procedure, to whit, the manual compression of the bed which would have caused some inevitable variance in the packing of individual columns.

3.2.2.4 Column Fouling Procedure and Determination of Breakthrough Curves

The following method was used to determine the breakthrough curves in all the experiments described. Before use, freshly packed columns were equilibrated with 20 mM phosphate buffer. 2 mg/mL BSA in 20 mM phosphate buffer was then pumped onto the column at a flowrate of 2 mL/minute. The BSA was continually pumped onto the column until the effluent concentration equalled the input concentration, at which point the column was washed with 20 mM phosphate buffer. When no further protein was washed from the column, a step change of 1 M sodium chloride in 20 mM phosphate buffer was introduced onto the column removing the bound BSA. Once again, when no more protein was removed from the column as shown by the absorbance at 280 nm, the column was re-equilibrated with 20 mM phosphate buffer. Now the column was challenged with a fouling stream produced as described in Section 3.2.2.2. The fouling material was of varying volume and concentration depending on the experiment. The maximum fouling mass challenge used in any of these experiments was 100 mL of the 600 g/L (wet weight) homogenate treated with the disc stack centrifuge alone. All the fouling material was

applied to the column at a flowrate of 2.6 mL/minute - use of higher flowrates was found to risk clogging of the packed bed and bed compression.

Following the foulant challenge, the column was once more washed using a gradient of sodium chloride from 0M to 1M to 0 M over 3 column volumes, producing a saw tooth salt gradient. This was a necessary requirement to remove protein from the column that would otherwise interfere with the reading of the breakthrough curve. It should be noted that in one series of experiments, purified cell particulates were used, and in this instance removal of the protein using the sodium chloride gradient was unnecessary.

Finally, the capacity of the column was once more measured to determine the change in the column performance as a result of the fouling species entering the column.

3.2.2.5 Clean-in-Place (CIP) Protocol

The cleaning-in-place protocol was carried out to the following method communicated by Jagerston (1991). The column was initially washed with 20 mM phosphate buffer, and then treated with 1 M sodium hydroxide at ambient temperature at 0.1 mL/minute. The flowrate was designed to keep the column in contact with sodium hydroxide for a long period, whilst maintaining a flushing flow to remove dissolved foulants. This procedure was carried out for 5 hours. The column was then flushed with 20 mM, 1 M sodium chloride phosphate buffer until the inlet and outlet pH of the buffer was the same.

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of Repeated Fouling on Column Breakthrough

Results for the breakthrough curves are presented graphically as the ratio of output concentration of BSA to input concentration of BSA (C/C_0) versus time. A freshly packed column was packed as described in Section 2.2.2.2. Yeast homogenate was prepared, and further processed by ultracentrifugation to remove solid particulates (Section 2.2.2.1). This column was fouled by repeatedly loading 2 mL quantities of yeast homogenate to the same column. After each fouling cycle the capacity of the column was determined using BSA at 2 mg/mL. Each cycle consisting of loading the column with 2 mL of yeast homogenate, testing the breakthrough curve with BSA, regenerating the matrix, and then repeating the procedure for a total of ten cycles. The breakthrough curves for the initial, fifth and tenth cycle are shown in Figure 3.4. The corresponding dynamic capacity values for the breakthrough curves are shown in Table 3.1.

For the purposes of this work it must be remembered that the important feature to determine is the change in the shape of the breakthrough curve as a result of the amount of fouling material that has been injected onto the column over the course of the experiment. The results show that after 5 cycles of fouling, there is very little change in the performance of the column. This has been shown previously in Section 2.3.3 for the chromatographic purification of alcohol dehydrogenase (ADH) in an exactly analogous series of experiments. The values for the capacity at 1 % breakthrough are very similar throughout the series of experiments. This represents the effective usable capacity in a process. It could therefore be concluded that the fouling undertaken in this series of experiments has no effect on the column performance. However, if the figures for the total capacity of the column are examined, there is a definite upward trend with cycle number. This is very obvious in the breakthrough curves when the initial run is compared to the final

breakthrough curve after ten cycles have been undertaken. The reason for the increase is a slight shift in the position of the breakthrough curve combined with an increase in the tailing of the curve. This increased tailing is not a function of the column packing since the same column was used in all the experiments, and it can therefore be concluded that the increased tailing is due to an increase in the effective diffusivity of the BSA as a direct result of the fouling that has occurred. The gradient of the breakthrough curve in the mid-part of the curve is the same for all the curves, so there is no apparent change in the equilibrium binding of the BSA. Likewise the fluid film mass transfer is unaffected by the fouling as the breakthrough curves all follow the same initial run pattern.

Table 3.1: Capacity values for the repeated fouling of a DEAE-Sepharose FF column (Ultracentrifuge prepared homogenate)

Cycle Number	Capacity at 1 % Breakthrough (mg BSA)	Capacity at 100 % Breakthrough (mg BSA)
1	314.5	525.7
2	310.1	510.0
3	320.5	515.2
4	317.0	512.1
5	332.5	505.3
6	319.0	519.4
7	328.5	521.6
8	315.1	514.2
9	320.1	531.2
10	300.8	550.3

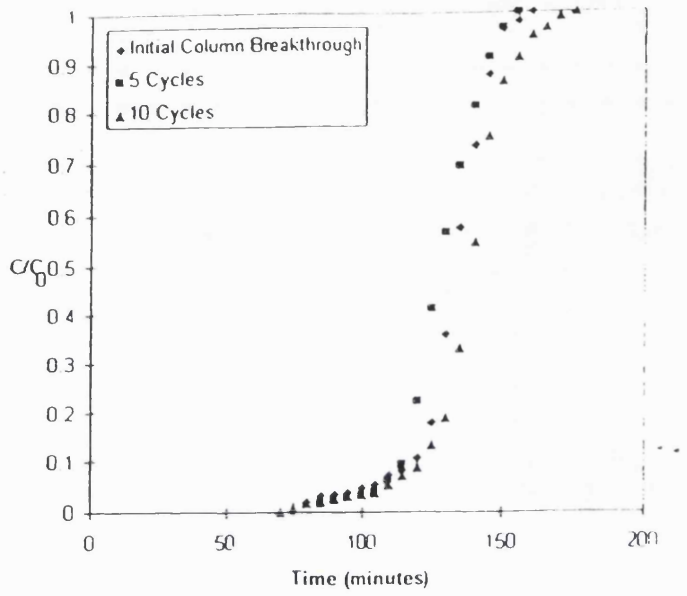

Figure 3.4: *The effect of repeatedly fouling a DEAE-Sepharose Fast Flow column with ultracentrifuged yeast homogenate. 2 mL of homogenate were loaded for ten cycles, initial, fifth and tenth cycle breakthrough curves shown here.*

As has been discussed previously in Chapter 2, it proved very difficult to use the assays described to get meaningful answers to the mass balance of material being loaded to and from the column. This was partly due to the small quantities of material loaded and partly due to the reproducibility of the assays. In general it was not possible to close the mass balances for solids, as sometimes more material appeared in the column effluent than was loaded to the column. When mass balances appeared to give less material leaving than loaded, it was nevertheless rather small amounts of material that were left bound to the column. Accordingly, the type of foulants interacting with the column could be inferred from the method of preparation of the fouling stream Hence an ultracentrifuged yeast homogenate largely free of particulate and lipid matter - was considered to contain only sub-micron sized particulates. The remainder of the foulants would then be soluble

proteins, lipoproteins, carbohydrates and nucleic acids. For this series of experiments, there was not a large effect due to these moieties interacting with the column.

The series of fouling experiments was now repeated using the disc stack centrifuged yeast homogenate prepared as described in Section 3.2.2.2. As before, a freshly packed column was fouled by repeatedly loading 2 mL quantities of the yeast homogenate to the column. The initial capacity of the column was determined using 2 mg/mL BSA and then the column went through ten fouling cycles using the disc stack centrifuge prepared homogenate. The results are once more presented as C/C_0 versus time for the initial, fifth and tenth fouling cycles in Figure 3.5.

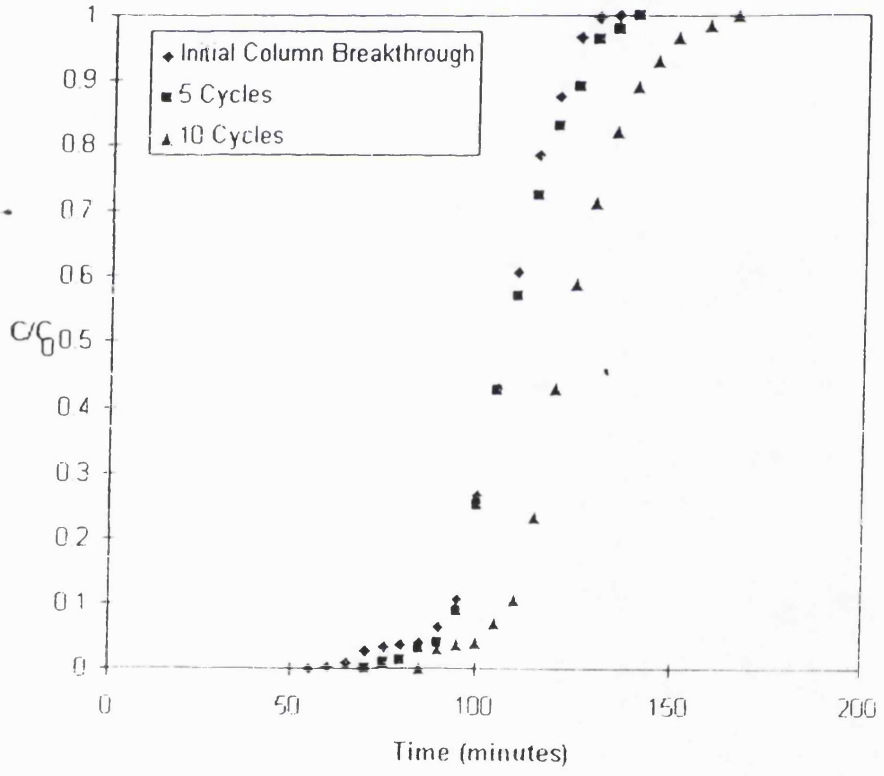

Figure 3.5: *The effect of repeatedly fouling a DEAE-Sepharose Fast Flow column with disc stack centrifuged yeast homogenate. 2 mL of homogenate were loaded for ten cycles, initial, fifth and tenth cycle breakthrough curves shown here.*

The capacity data for the ten runs are shown in Table 3.2.

From the results it is apparent that there is a very discernible change in the 1 % breakthrough capacity of the column as well as the 100 % breakthrough capacity of the column, and this is clearly related to the number of cycles of material passed through the column. This series of experiments is exactly the same as a series of experiments performed in Section 2.3.3, where the purification of alcohol dehydrogenase was increased from about 2 fold at the start to 11.4 fold at the end of the ten cycles of fouling with disc stack centrifuged homogenate. It was surmised then that the reason for the increased purification may well be as a result of foulants binding to the column and serving to act selectively in concert with the DEAE groups on the column to improve the column's performance. Certainly the evidence is shown here for an increase in column capacity at 1 % breakthrough, which is the figure of interest for the operation of the column economically - in effect the column performance has been significantly improved.

As expected, the shape of the breakthrough curves shows that the position after ten cycles of the breakthrough curve has been shifted quite significantly, although the gradient at 50 % breakthrough remains constant. There is no evidence of the columns exhibiting increasing leakage with increasing number of cycles, but after ten cycles there is once more evidence of some tailing, although this is not large.

The typical quantities of lipid, protein and solid particulates used in the disc stack centrifuge material is shown in Table 3.3.

Table 3.2: Capacity values for the repeated fouling of a DEAE-Sepharose FF column.(Disc stack centrifuge prepared homogenate)

Cycle Number	Capacity at 1 % Breakthrough (mg BSA)	Capacity at 100 % Breakthrough (mg BSA)
1	242.9	425.8
2	230.8	461.7
3	245.7	439.6
4	263.6	450.0
5	278.1	414.6
6	290.3	465.5
7	289.9	480.1
8	323.3	475.6
9	339.6	497.8
10	361.9	509.1

Table3.3: Quantities of Lipid, Protein and Solids Loaded per Cycle

Assayed Material	Quantity (per 2 mL)
Protein	40 mg
Lipids	6.26 mg
Particulate Solids	696 mg

When the ultracentrifuged material was used as the fouling challenge, there was less change in the capacity than is apparent with the disc stack centrifuged homogenate. It is apparent from the figures in Table 3.3 that the quantity of solid particulate material is the largest contributor to the mass of the fouling stream for the disc stack centrifuged material. It is very probable that the change in capacity is as a result of the presence of an amount of the particulate solids being trapped on the column and serving to act in a functional manner to increase the column capacity for BSA.

The overall conclusions from this work are that the shape of the breakthrough curves is not greatly affected by the amount of fouling material loaded to the column in these experiments. More specifically the equilibrium parameters are not affected as the gradients of the curve at 50 % breakthrough are the same, and thus the isotherms can be judged to be unaffected. The columns do not show any increased leakage with increasing number of cycles, thus implying that the fluid film mass transfer coefficient of BSA for the adsorbent has not been affected at this level of fouling. There is some evidence for tailing on the breakthrough curves, indicating that the diffusivity of the BSA has been affected and is a function of the number of cycles and thus presumably the quantity of fouling material injected onto the column. The tailing seems to be comparable for both the case of the ultracentrifuged material and the disc stack centrifuged foulants.

When solid debris is present, however, there is a significant increase in the capacity of the column at both 1% breakthrough and total capacity. After ten cycles the 1 % breakthrough capacity of the column has increased by 50 %. It has also been found previously that the purification of alcohol dehydrogenase is very significantly increased (over 5 times) after ten cycles of fouling with disc stack centrifuged material. This is strong evidence for the particulates to be exhibiting an ion exchange function as they become trapped on the column. A similar, although smaller effect is seen with the ultracentrifuged material, as can be seen from the shift in position of the breakthrough curve after ten cycles. This result

shows the importance of the selection and the choice of operating parameters of unit operations on the subsequent performance of the adsorption step. Although there is very little change in the equilibrium and mass transfer characteristics of the column, the change in capacity when particulates are present is significant.

3.3.2 Effect of Loading Large Quantities of Particulate Solids to Packed Beds

It has been shown in section 3.3.1 that there is relatively small effect on the mass transport and equilibrium parameters of repeatedly loading small quantities of yeast homogenate onto a packed bed. However, the presence of particulates did cause an effect, although one that actually worked in favour of an improved separation rather than against it. The series of experiments in this section is aimed at loading much larger quantities of fouling material to the column. It was hypothesised that for very high solids-containing homogenate the column structure would be altered due to severe deposition of solids, eventually leading to clogging of the column and bed compression. The aim of this series of experiments was to go as close to this limit as possible, and then determine what effect this had on the performance of the column.

In this set of experiments, a freshly packed column was challenged with high concentrations of yeast homogenate prepared by disc stack centrifuge (Section 3.2.2.2). Columns were used only once because of the expected damage done to the bed structure at these extremes of loading

An inspection of the breakthrough curves shows very marked changes in the shape according to the amount of solids loaded. For 10 mL of disc stack centrifuged yeast homogenate, there is a marked decrease in the capacity of the column. This then represents the whole of the amount of material added in one go compared to five cycles of fouling in Section 3.3.1. The effect of adding the 10 mL of fouling material in one go is quite

Section 3.3.1. The effect of adding the 10 mL of fouling material in one go is quite different to the effect achieved when the column is recycled. Not only is the capacity of the column reduced, but also the shape of the breakthrough curve is modified compared to the initial (freshly packed) column. There is still no evidence of increased leakage in the column, which leads to the conclusion that the fluid mass transfer coefficient is still unaffected at this level of fouling. However the breakthrough curve exhibits a long tail, indicating that the effective diffusivity of the BSA has been reduced.

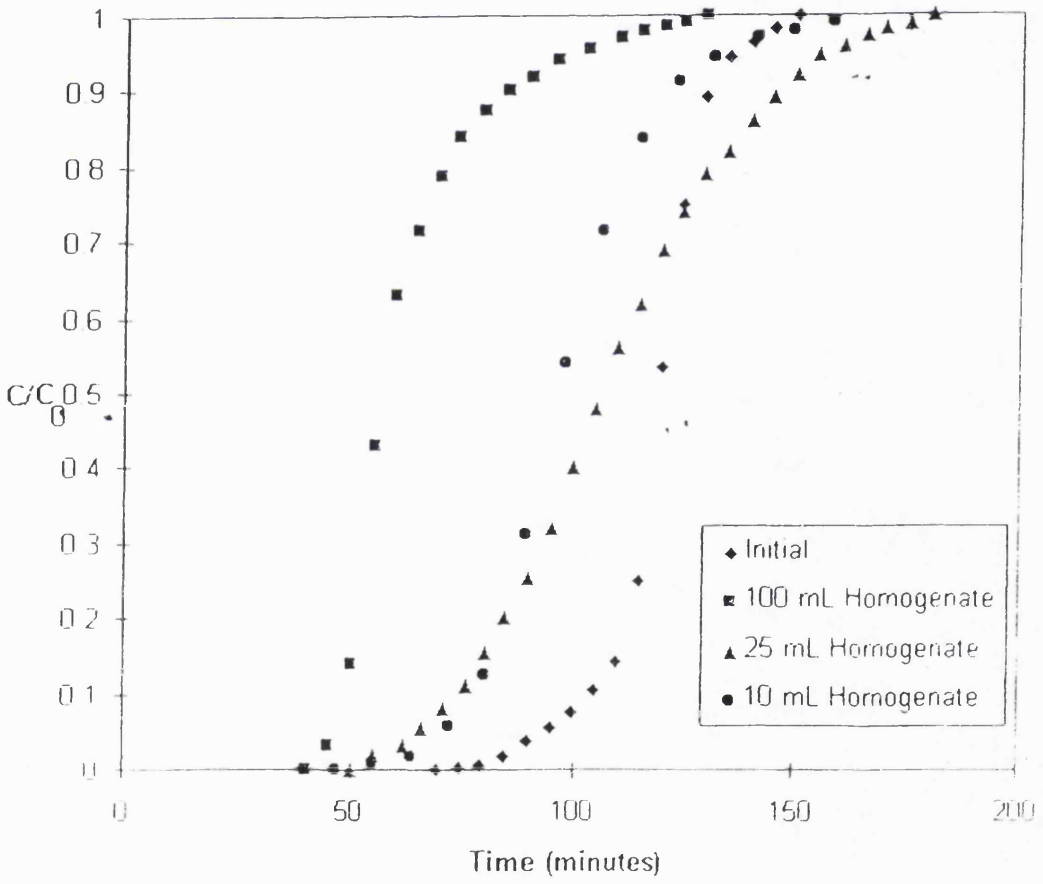

Figure 3.6: *The effect of fouling a DEAE-Sepharose Fast Flow column with disc stack centrifuged yeast homogenate. The effect of single loadings of 10 mL, 25 mL and 100 mL compared to the fresh column breakthrough curves are shown. Table 3.4 gives the values of solids retained by the column after each fouling cycle. The fresh breakthrough curve*

Table 3.4: Effect of loading high concentrations of particulate solids to a packed bed of DEAE-Sepharose FF.

Volume of Yeast Homogenate Challenge (mL)	Sedimented Solids Loaded (mg)	Estimated Solid Deposition in Column (mg)	1 % Breakthrough Capacity (mg BSA)	100 % Breakthrough Capacity (mg BSA)
0*	0	0	319.0	495.7
10	700	10	171.0	225.0
0*	0	0	453.6	533.2
25	1740	31	165.0	250.3
0*	0	0	320.0	430.0
50	3480	23	158.0	243.2
0*	0	0	320.0	430.0
100	6960	500	105.0	159.3

The extreme case represented here is for the loading of 100 mL of yeast homogenate to the packed bed. One of the problems with using such a high concentration of solids to challenge a packed bed was to avoid bed clogging and compression as this would confuse the results. Pressure drop during loading of the column was therefore recorded, and the pressure drop versus flowrate of each column was tested for fresh and fouled columns. This relationship is shown in Figure 3.7.

The plot of flowrate versus pressure indicates that a small amount of clogging did occur, but in each of the cases investigated, the pressure drop was never sufficient to cause visible bed compression, and by mass balance, most of the particulate solids appeared to flow straight through the column. Injecting solids onto the column did cause a transient increase in pressure drop which can probably be ascribed to two factors of the fouling homogenate.

Firstly the viscosity of the homogenate was higher than the buffers used. Secondly the particulates would cause an increase in pressure over the time they occupied the interstices of the packed bed. This pressure drop was transient, and the flowrate versus pressure drop shows only a slight increase in pressure drop after fouling. This is evidence for the majority of the fouling to be inside the beads rather than external, since a major deposition of solids in the column interstitial space would interfere with convective flow, which simply was not seen according to the pressure drop data.

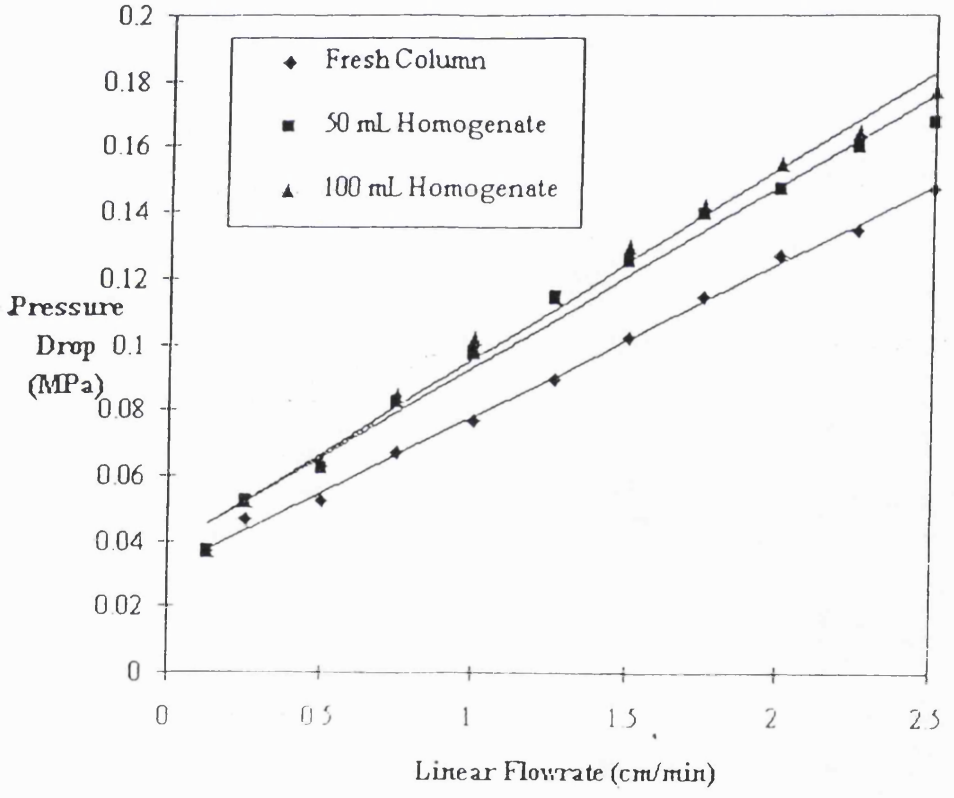

Figure 3.7 *Pressure drop as a function of flowrate for a freshly packed column and columns fouled with 50 and 100 mL of disc stack centrifuge homogenate. There was a slight hysteresis on increasing versus decreasing flowrate which has been omitted for clarity.*

For the cases where 25 mL and 50 mL of homogenate are loaded to fresh columns the change in capacity is intermediate, and increases with the amount of material loaded. Again, the major change in the breakthrough curve is associated with tailing, indicative of a change in the diffusivity of the BSA within the column.

For this series of experiments, the increase in capacity that had been observed when repeatedly loading 2 mL of foulant to the column was not seen, even when the total quantity of foulant added should have been the same. This must be due to a different fouling mechanism arising when the foulant was loaded as one shot, and this is not unreasonable to suppose. For the case where 2 mL of homogenate was loaded, the column was recycled, thus removing a large proportion of protein and other charged moieties that would contribute to fouling the column. In the case where a single large slug of homogenate was applied to the column, it may be supposed that interaction between the foulants may have caused a sort of "snowball" effect, and hence the effectiveness of the recycling prior to measuring the capacity was much less than for the case where smaller amounts of homogenate were repeatedly loaded.

A comparison was also made between loading 100 mL of the ultracentrifuged yeast homogenate compared to 100 mL of the disc stack centrifuged homogenate to determine whether the major cause of fouling was the particulate cell debris, or whether a very clarified homogenate loaded as a single slug would also cause a large reduction in the column capacity. The result is illustrated in Figure 3.8 for the effect of fouling using such an ultracentrifuged fouling stream.

It is very obvious from this result that the presence of solid particulates has a very marked effect on both the capacity and the diffusivity of the BSA. When essentially particulate free homogenate alone is applied to the column, there is very little difference between the fouled

and the fresh column, just a slight tailing in the fouled condition, as was observed for the repeated cycles with 2 mL of the yeast homogenate (Section 2.3.3).

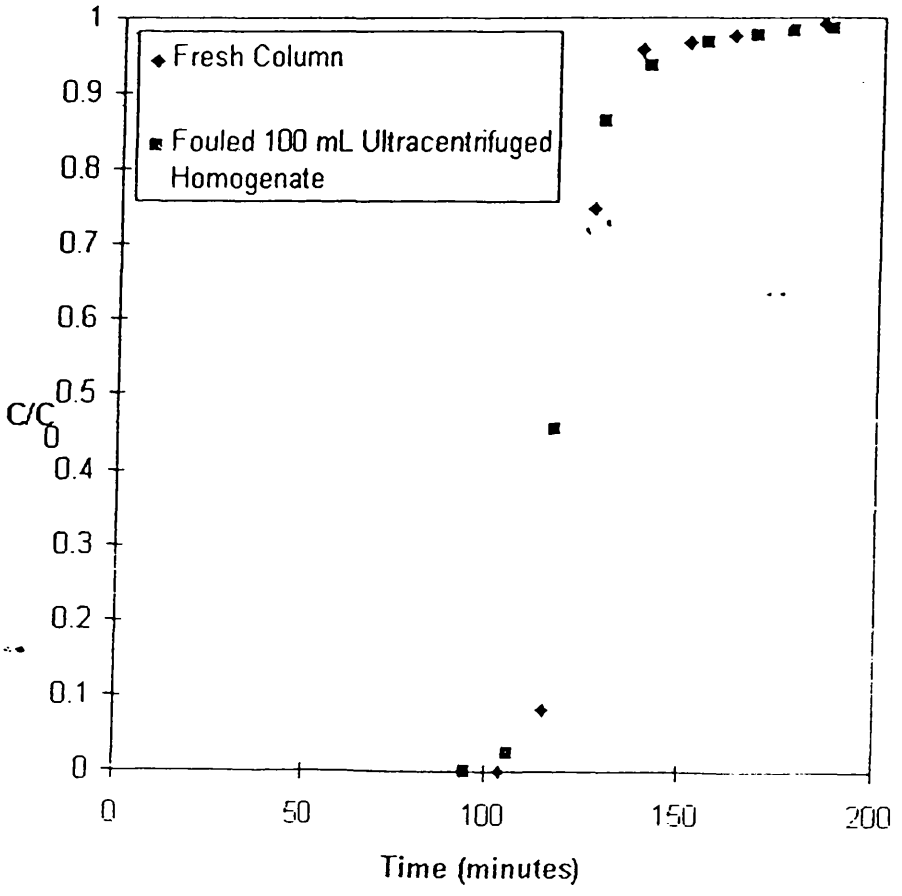

Figure 3.8: *Effect of loading 100 mL of ultracentrifuged yeast homogenate to a freshly prepared column. Graph shows breakthrough curves for the freshly packed and the fouled column.*

3.3.3 Comparison of a Model for Breakthrough Curves

The effect on column performance has been determined by capacity and by qualitatively analysing the breakthrough curves in order to determine the change in the equilibrium and mass transfer characteristics pre- and post-addition of foulant streams. The results show that the changes are manifested by a reduction in capacity and generally a decrease in the effective diffusivity of the BSA within the matrix. In order to quantify this reduction, the model developed by Horstmann (1989) was used. The model includes the effects of external film transfer and pore diffusion, assuming that the rate of the surface reaction is sufficiently rapid to be ignored.

3.3.3.1 Description of the Model

For completeness a description of the model developed by Horstmann is givenm here. For diffusion within a particle, concentration of a solute at a given point is described by (Horstmann, 1989):

$$\varepsilon p \frac{dci}{dt} + (1 - \varepsilon p) \frac{dqi}{dt} = \varepsilon p De \nabla^2 ci \qquad 3.6$$

, where c and q are the liquid and solid solute concentrations respectively and the subscript i denotes the interfacial value. ε_p is the intraparticle inclusion porosity, and De is the solid phase diffusion coefficient. The surface diffusion is either not considered, or lumped with the pore diffusion in the effective diffusivity. The rate of mass transfer through the external film of the adsorbent particle relates the bulk liquid solute concentration to the pore concentration at the particle surface, characterised by a linear driving force model (Horstman, 1989).

$$\left(\frac{\partial ci}{\partial r}\right)_{r=R} = \frac{kf}{De\varepsilon p}(c - ci)r = R \qquad 3.7$$

where r is the radial distance from the centre of the particle, and R is the particle radius. At the particle centre:

$$r = 0 \qquad \frac{\partial c_i}{\partial r} = 0 \qquad 3.8$$

Surface reaction between the solute and the adsorbent is described by a second order reaction, is isothermal and completely reversible. The adsorption reaction is then given by:

$$\frac{\partial q_i}{\partial t} = k_{ad}c_i(q_m - q_i) - k_{de}q_i \qquad 3.9$$

which is the Langmuir isotherm at equilibrium, where $K_d = \frac{k_{de}}{k_{ad}}$, and q_m is the maximum capacity.

$$q = \frac{q_m c_i}{(K_d + c_i)} \qquad 3.10$$

The equation of continuity for a packed bed adsorber is given by:

$$DL\frac{d^2c}{dx^2} - l\frac{dc}{dx} - \Pi_i = \frac{dc}{dt} \qquad 3.11$$

where D_L is the axial dispersion coefficient, and l is the length in an axial direction along the packed bed. The rate expression is given by:

$$\Pi i = \frac{3}{R}\frac{(1-\varepsilon)}{\varepsilon} De(\frac{\partial ci}{\partial r})r = R \qquad 3.12$$

The transport within the particle is described by equation 3.1. At each point in the column the concentration outside the fluid film is related to the liquid phase concentration at the particle surface of the particle by:

$$(\frac{\partial ci}{\partial r})r = R = \frac{kf}{De\varepsilon p}(c - ci)r = R \qquad 3.13$$

The solution of the model was achieved by a finite difference method (Horstmann et al., 1989). The equation of continuity in the bed for each cell, neglecting axial dispersion is given by:

$$\frac{\partial \omega j}{\partial t} = \frac{l}{\delta x}(\omega j\text{-}1 - \omega j) \qquad 3.14$$

The boundary conditions for the bed are the concentration of solute in the bed is zero at time zero, and the concentration of solute at the inlet is given by:

$$c(0,t) = C0 \qquad t \geq 0 \qquad 3.15$$

3.3.3.2 Description of Experiments

The aim of this set of experiments was to determine a value for the decrease in performance of the packed bed with increasing solids loading. In order to detect the effect of only the solids, a solid particulate preparation was made by centrifuging yeast homogenate (prepared

as described in Section 3.2.2.2) in an ultracentrifuge at 250,000g for 30 minutes. The solids were then resuspended, washed with buffer and the process repeated. After each wash cycle, the supernatant was tested for the presence of protein, and after four washes, 98 % of the soluble protein was found to be removed as measured by Bradford assay (Section 2.3.3). The solids were then resuspended in their original starting volume in phosphate buffer and used to foul freshly packed beds as described in Section 3.2.2.4. As before the breakthrough curve was determined after each amount of solids had been loaded, and the model was used to determine the effective diffusivity of the BSA by fitting the experimental breakthrough curve.

The amount of solids loaded in each of the six runs treated is shown in Table 3.5. The amount of solids trapped in the column is added for comparison, but there does not seem to be a relationship between the amount of solids retained in the column and the amount of solids loaded. The most likely reason for this is the difficulty in achieving reproducible results for the dry weight measurements, coupled with the observation that relatively tiny amounts of these particulates appear to be retained. A less plausible reason for the lack of relationship between the solids loaded and those retained is that, during passage through the column, channelling occurs which allows particulates to pass through the column without being retained. This would result in obvious column deterioration in efficiency, and this was not seen.

Table 3.5: Solids loaded and retained in fouling packed bed columns with particulate solids.

Experiment Number	Solids Loaded (mg)	Solids Retained (mg)
1	0	0
2	208	0
3	435	73
4	870	80
5	1305	55
6	1680	63

The parameters used for the model are shown in Table 3.6. Breakthrough curves were generated by the model using these parameters and fitting the curve to the experimental data by varying the effective diffusivity of the BSA. The experimental and model breakthrough curves are shown in Figures 3.9 to 3.15.

Table 3.6: Model Parameters

Parameter	**Value Used**
Column Length	5.0 cm
Diameter	1.0 cm
Adsorbent Mass	3.85 g
Fluid Film Mass Transfer Coefficient	5×10^{-3} ms^{-1}
Interstitial Porosity	0.34
Intraparticulate Porosity	0.89
Maximum Adsorbent Capacity	33 mg/mL adsorbent
Particle Diameter	93 μm

The parameters were from values measured by Draeger (1991), and are assumed to be realistic for the system used (Chase, 1994). Since the expected results are dependent on the diffusivity, the values of the parameters chosen would not affect the comparison between the model results.

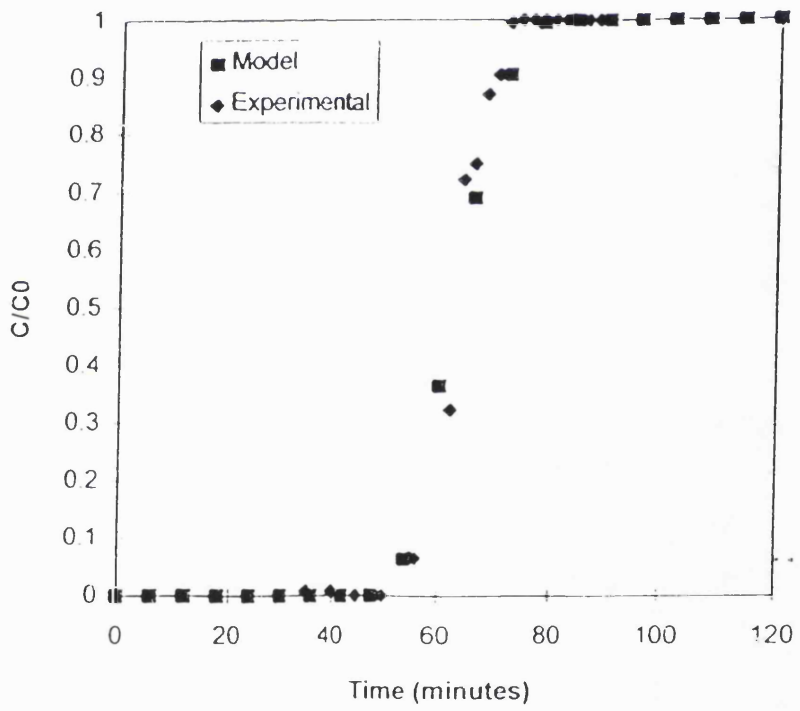

Figure 3.9: *Breakthrough curve for the freshly packed column, run number 1.*

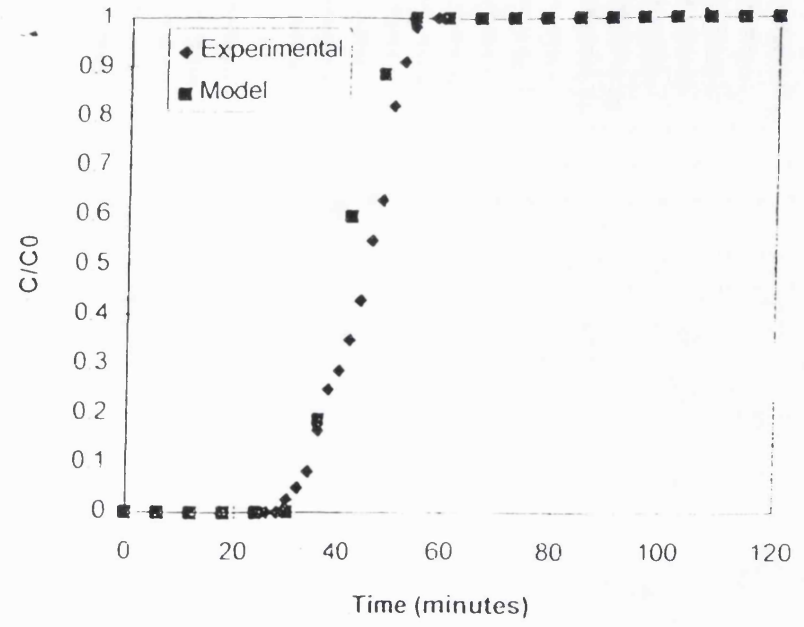

Figure 3.10: *Breakthrough curve for run number 2, addition of 208 mg of solid particulates to the column.*

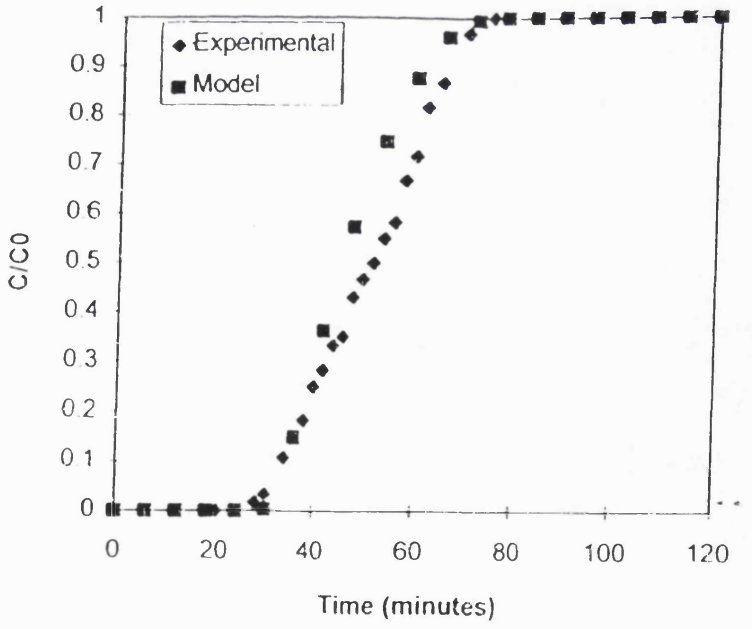

Figure 3.11: *Breakthrough curve for experiment 3, 435 mg of solids loaded to the column*

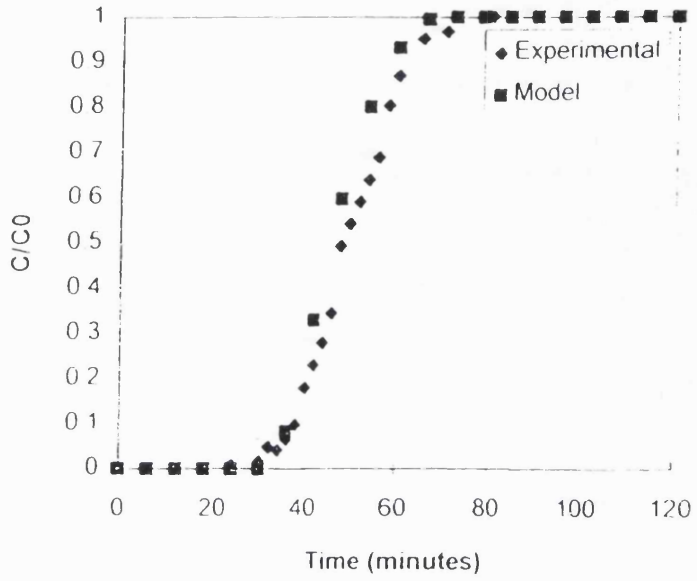

Figure 3.12: *Breakthrough curve for experiment 4, 870 mg of solids loaded to the column.*

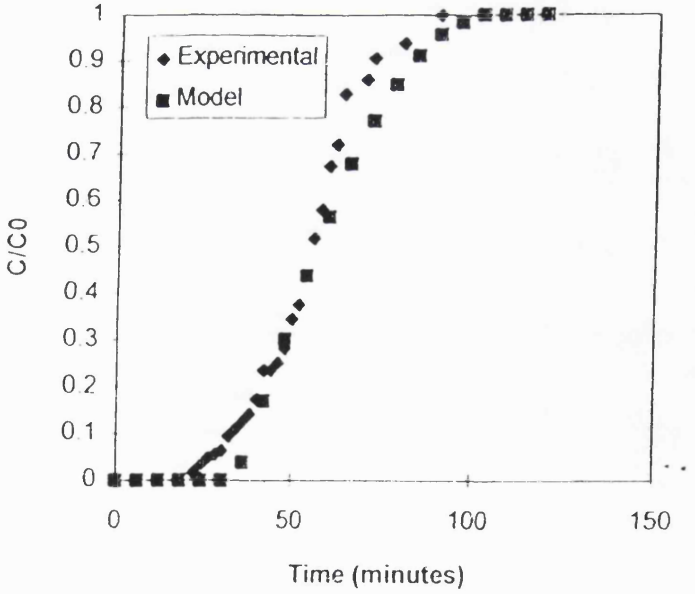

Figure 3.13: *Breakthrough curve for experiment 5, 1305 mg of solids loaded to the column.*

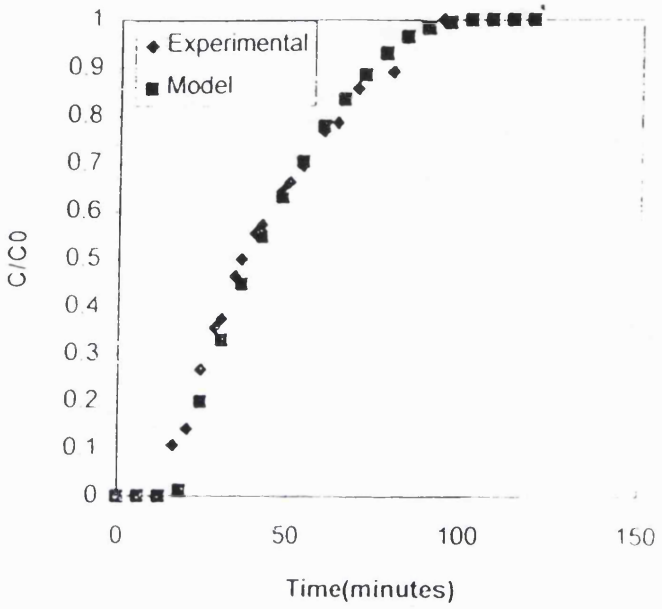

Figure 3.14: *Breakthrough curve for experiment 6, 1680 mg of solids loaded to the column.*

The observation of increased tailing of breakthrough curves with the degree of fouling suggests that the major change in the columns occurring during this particular type of process is one of a reduction in the effective diffusivity. It is therefore reasonable to use the effective diffusivity as a varying parameter in order to fit the model to the experimental data. The model achieved close fits to the experimental data using the effective diffusivity as the sole modified parameter. The value of free diffusion of BSA can be calculated from the semi-empirical equation of Polson (Horstmann, 1989), which gives a value of 7.4×10^{-11} $m^2\ s^{-1}$ for BSA. The effective diffusivity in the fast flow matrix is about an order of magnitude less than this, at $0.85 - 1.0 \times 10^{-11}$ $m^2\ s^{-1}$. The results are plotted as the reduction

in the effective diffusivity of the BSA compared to the unfouled column (which is therefore a value of 1) versus the amount of solids loaded, and are shown in Figure 3.15.

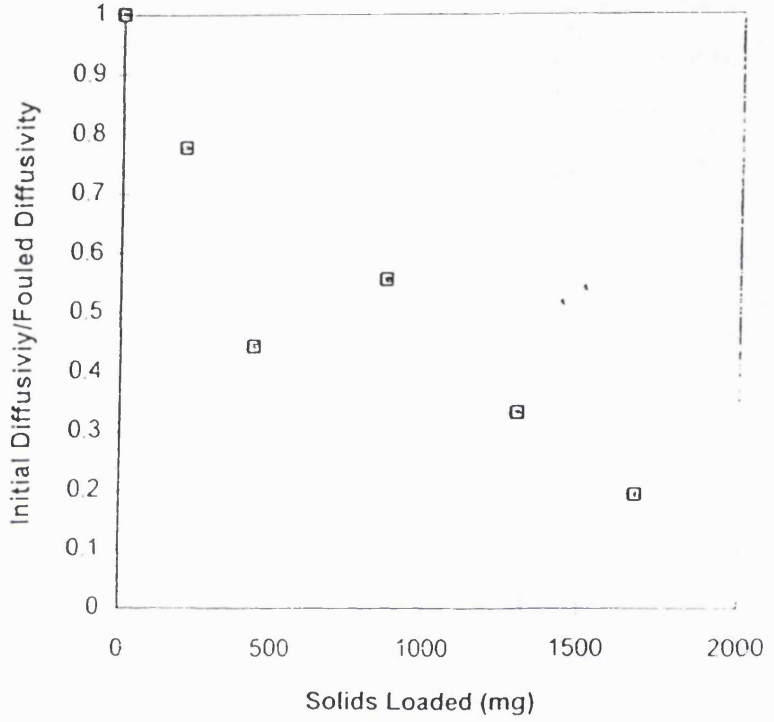

Figure 3.15: *Reduction in effective diffusivity as a function of solid particulates loaded to the column. Fresh column effective diffusivity = 1 for no fouling.*

Assuming that it is valid to use the effective diffusivity as a way of fitting the experimental data, it is clear from Figure 3.15 that the reduction in effective diffusivity in the column is directly related to the amount of solids loaded. Unfortunately, the amount of solid particulates trapped does not seem to show the same dependence, probably due to the difficulty in assay reproducibility for dry weights. The effective diffusivity is reduced by 80 % of it's starting value when 1.68 g of solids have been loaded to the column.

3.3.4 Effect of pH on Fouling of Packed Beds

The aim of this series of experiments was to determine the effect of pH on the breakthrough curves after fouling had been performed at the same pH. For this series of

experiments a constant value of solid particulates was used (500 mg) prepared as described in Section 3.3.3.2. This was loaded to the column in 25 mL of phosphate buffer and the breakthrough curve determined pre- and post-fouling at the appropriate pH. Four pH values were examined; pH 6, pH 7, pH 8 and pH 9.

Table 3.7: Capacity Data for Fouling at Different pH

pH	Column State	1 % Breakthrough Capacity (mg BSA)	Total Capacity (mg BSA)
6	Fresh	168.7	335
6	Fouled	290.8	392.2
7	Fresh	157.3	266.5
7	Fouled	78.5	255
8	Fresh	178.4	321
8	Fouled	159.4	301
9	Fresh	185.4	316.8
9	Fouled	32.21	214.3

The results for the capacities of the columns at each pH, pre-and post-fouling are shown in Table 3.7. Comparison of the breakthrough curves pre- and post-fouling are shown at each pH in Figures 3.16 to 3.19.

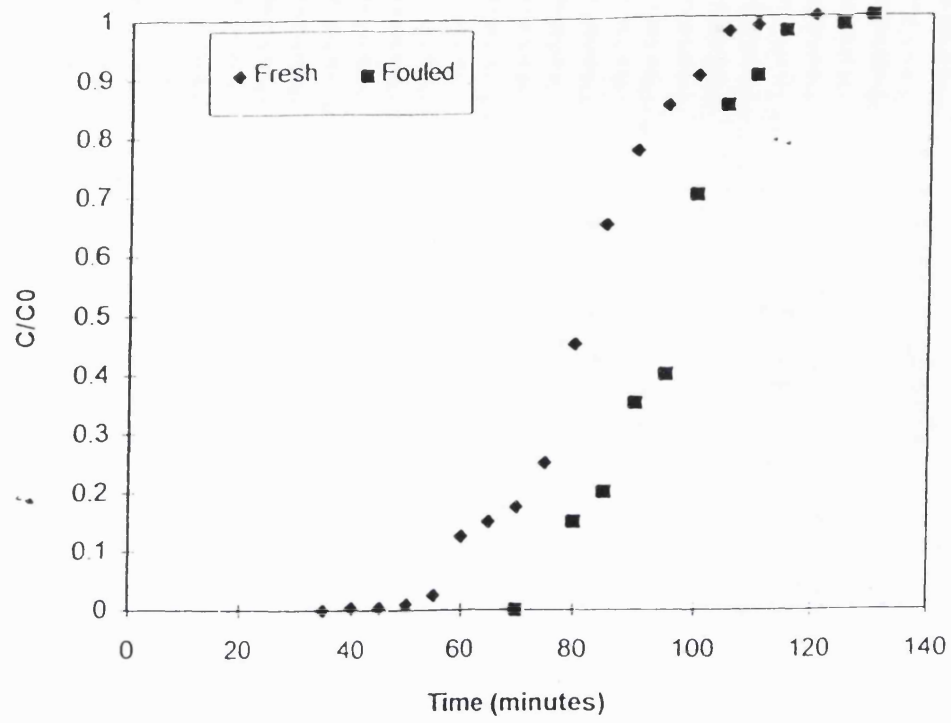

Figure 3.16: *Effect of solids particulate loading (500 mg) on breakthrough curves for BSA at pH 6.*

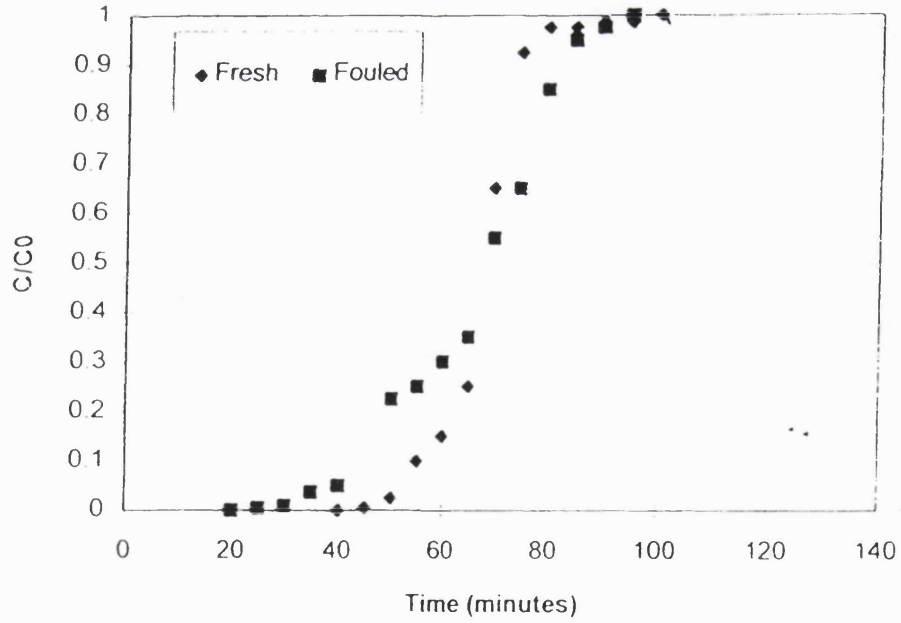

Figure 3.17: *Effect of solids particulate loading (500 mg) on breakthrough curves for BSA at pH 7.*

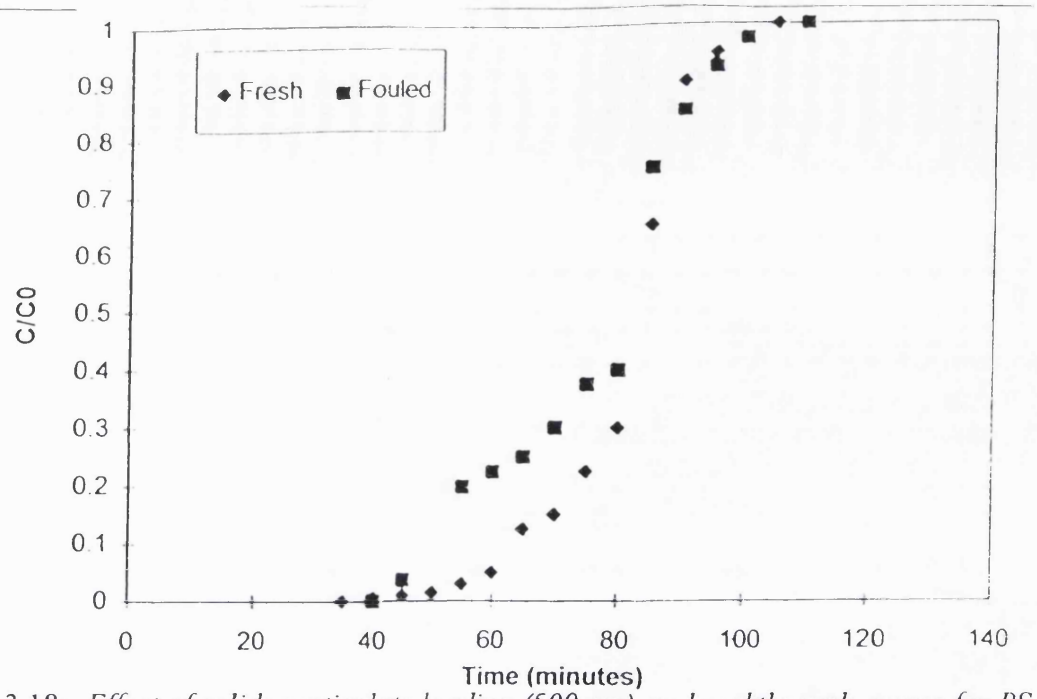

Figure 3.18: *Effect of solids particulate loading (500 mg) on breakthrough curves for BSA at pH 8*

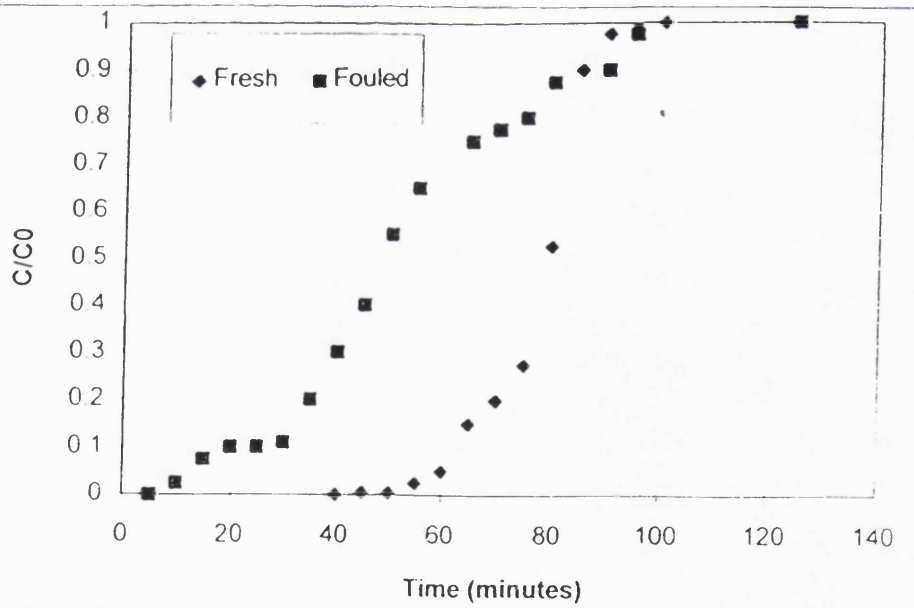

Figure 3.19: *Effect of solids particulate loading (500 mg) on breakthrough curves for BSA at pH 9.*

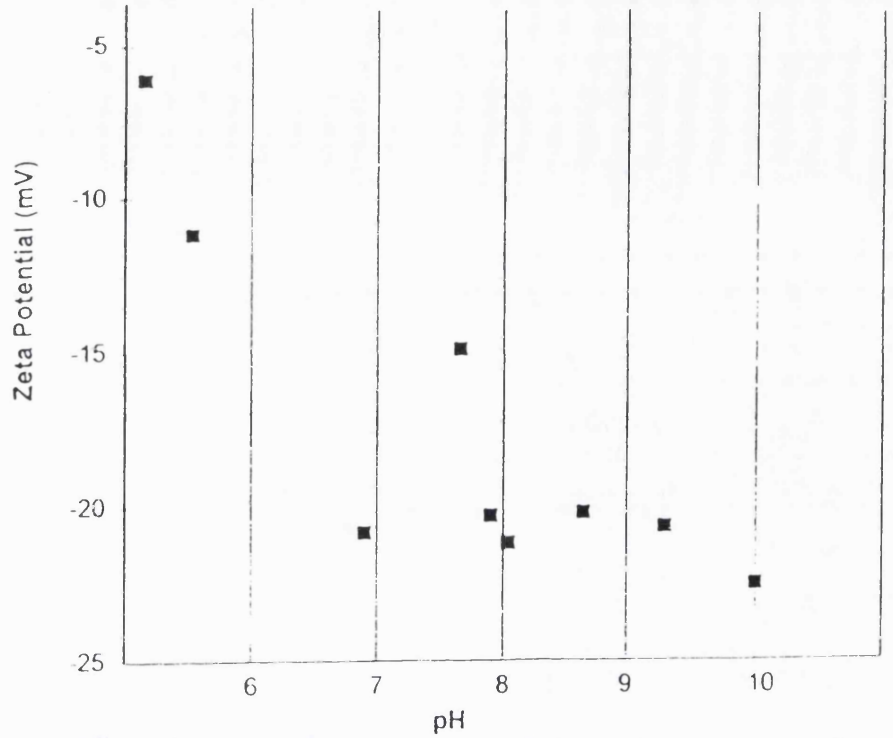

Figure 3.20: *Effect of pH on cell debris charge. Cell debris was prepared as described in the text, and suspended in buffer at the appropriate pH in order to measure the Zeta potential.*

The first thing to note about these results is that the important feature is the change observed from the fresh case to the fouled case. The capacities of the columns are not directly comparable from run to run since experiments for the binding of BSA are performed at different pH's, and in each case a fresh column was prepared to make the initial pre-fouling measurement of capacity. It would be expected that the amount of BSA binding to the adsorbent would decrease with increasing pH since there would be a decreased protein-adsorbent interaction. BSA forms dipoles in acidic buffers and carries a substantial negative charge, and hence would be expected to bind strongly to anion exchangers at low pH above the isoelectric point of the protein. This is not seen probably due to differences in the beds arising during packing of the columns.

The negative potential of the solid particulates decreases with pH, and is very reduced at pH 6. Accordingly it would be expected that the fouling effect of the particulates is less at pH 6, and the capacity of the column for BSA appears to increase after fouling. This has been

seen in Section 3.3.1. A possible explanation is that there is physical entrapment of a small amount of solids in the column, and that this has a functional anion exchange action to serve to increase the capacity of the columns. Potentially this effect is present in all the fouled columns, but it is masked by a greater concentration of foulants becoming entrapped, this time by ion-exchange as well as physical entrapment which offsets any advantage gained with a small amount of fouling, and leading to an overall decrease in column performance.

At pH 9, the column appears to be severely affected by the loading of solids particulates, and this would fit an explanation of more negatively charged cell debris interacting strongly with the adsorbent, and leading to quite extreme change in the breakthrough curve characteristics. For pH 7 and pH 8, the change in performance pre-and post-fouling is rather similar. It can be said that the effect on capacity is less than for pH 9, but greater than for pH 6.

The amount of solids trapped in the columns was again not related to the amount of change seen in the breakthrough curves, although it is obvious that the column characteristics have been changed by injecting solid particulates onto the column.

3.3.5 Efficiency of Clean-in-Place (CIP) Treatment of Columns

The ability to be able to clean a column *in-situ* without the requirement to re-pack is an important consideration for the economics of a process involving packed bed chromatography. One of the advantages of the Sepharose gels is their relative resistance to alkali attack, and reasonably harsh caustic wash treatments can be performed in order to remove foulants from the bed and re-generate the original characteristics of the bed.

For this series of experiments a column was fouled with yeast homogenate prepared through a disc stack centrifuge and then cleaned using the CIP method communicated by Jagerston (1991), and described in Section 3.2.2.5. The column breakthrough curve was

measured pre- and post-fouling and post CIP to ascertain the effect. The amount of homogenate used was 50 mL to reflect a severe fouling event. The results are shown in Figure 3.21. The caustic wash was shown to be very effective, and there was really no significant difference between the fresh column and the fouled column. It must be noted that this is only for one cycle of cleaning. In operation a column may be expected to go through hundreds of cycles in it's lifetime, and a caustic wash must have an effect on the stability and lifetime of the matrix. There is no way to test this other than to do this number of cycles. The information obtained here is thus limited to the efficiency of cleaning based on one cycle.

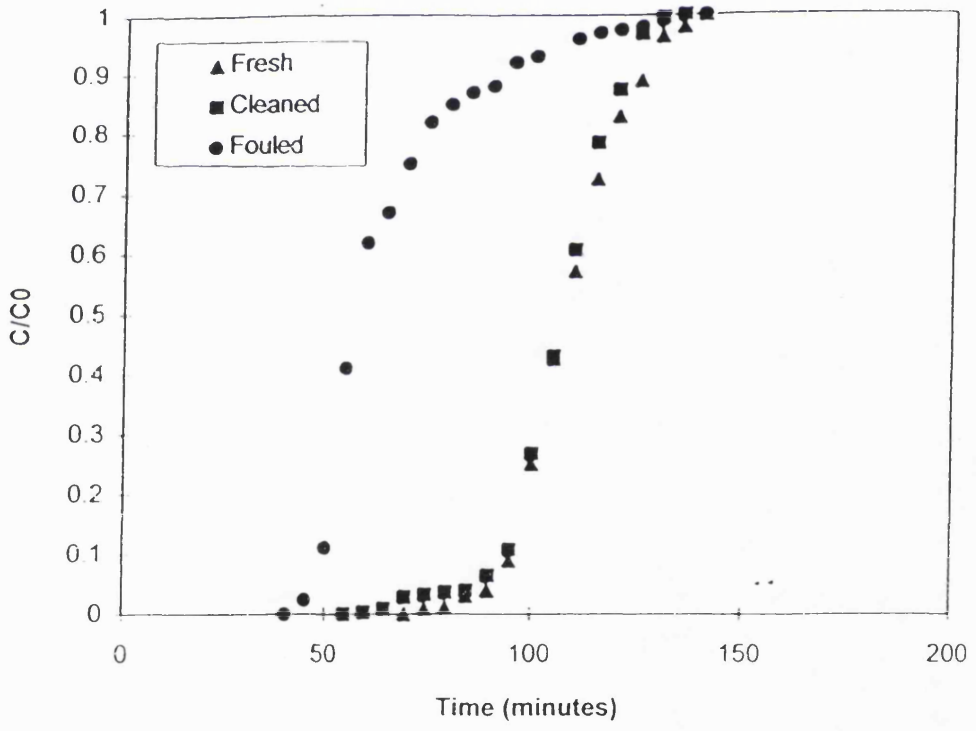

Figure 3.21: *Illustration of efficacy of CIP treatment using caustic wash and sodium chloride gradients to clean a column fouled with 50 mL of yeast homogenate prepared in a disc stack centrifuge.*

3.4 CONCLUSIONS

This work has centred on the fouling of packed beds using particulate solids from yeast homogenate. Comparison of the breakthrough curves produced when a column is fouled with a highly clarified homogenate to a stream containing a high proportion of solids, shows that the solids are responsible for a considerable change in the column breakthrough characteristics, and furthermore that this change is related to the absolute amount of solids loaded. If the change in performance is due to solids deposition in the bed, which would seem a reasonable hypothesis, it has not been possible to quantify the amount of deposition or whether deposition is localised in one area of the packed bed (for example, the top 5 cm of the column as reported by Petterson, 1989). From the mass balance data it is possible to determine that the amount of solids deposition is small in relation to the solids that manage to pass through the bed - it has not been possible to show that there is a class of material that is preferentially bound to the column (Section 2.3.3, Figures 2.12 (a) and (b)). From the clean-in-place work, it is clear that there is material on the column that degrades the column performance, and that such material appears to be readily removed with a caustic wash.

The presence of yeast cell debris in the process stream decreases the capacity of the bed in most cases. However, when a relatively small amount of homogenate is loaded to the column, there appears to be an increase in the capacity. It is possible that this occurs with all the fouling experiments, but is masked by more dominant effects when heavy loading of homogenate is applied to the column. The charge on the surface of the cell debris is clearly important in the fouling process, and this leads to the conclusion that at least some of the change in capacity is due to ionic interactions, thereby competing with the protein for adsorption and leading to the observed decrease in capacity.

4. PULSE RESPONSE ANALYSIS

4.1 INTRODUCTION

In Chapter 3, the effects of fouling on transport and adsorption parameters was examined by the use of frontal analysis. It was concluded that the adsorption was affected in terms of the dynamic capacity, and the diffusivity of the matrix. In this chapter, a different quantitative approach was taken to quantify the changes in the transport parameters associated with fouling of packed beds; the so-called pulse response technique.

In a pulse response experiment, a sample of a test substance is applied to the column and the shape of the concentration profile of the test substance as it leaves the column is diagnostic of the transport parameters for the packing in the column.

The parameters investigated here were intraparticle diffusivity, axial dispersion and the intraparticle void volume (the fraction of the bead volume available for the diffusing component). Since axial dispersion is strongly dependent on the bed structure, it follows that any change in the bed caused by compression, channelling or solids deposition in the packing interstices will be detected through changes in this parameter's value. In contrast, the internal fouling of the beads will be characterised by a change in the diffusivity and the intraparticle void volume. The fluid film transfer coefficient is generally considered not to dominate in the range of fluid velocities experienced in chromatography, and it's value was estimated using the correlation of Ohashi (1981). The work on frontal analysis (Chapter 3) showed that fouling exerts an effect on the adsorption to reduce the capacity of the adsorbent. For simplicity of analysis, adsorption was not considered in the pulse response analysis - the matrices used were in effect run as size exclusion chromatography (SEC), and the pH and ionic strength was selected in order to prevent adsorption..

Several studies have employed pulse response techniques to determine transport parameters in chromatography beds, including macromolecules (Ching et al 1989; Boyer and Hsu, 1992; Ming and Howell 1993). The method is straightforward to apply, and requires no more sophisticated equipment than a standard chromatography system as described in Figure 2.2. However the pulse response technique has been criticised by Lenhoff (1987) for lack of control over errors and baseline estimations. For this reason, the experimental method and data analysis must be performed carefully so as to minimise errors.

Work reported in previous chapters has shown that particulates have a profound effect on the performance of the separation. In this work an attempt was made to separate the fouling occurring due to the structure of the base matrix Sepharose 4 FF, and that due to the functional DEAE-Sepharose Fast Flow matrix. DEAE-Sepharose Fast Flow might be expected to show additional fouling over the base matrix as a result of interactions between the functional ligands and fouling species in the process stream.

The aim of this work was to establish a relationship between the degree of fouling to which a column had been exposed by the monitoring the change in the transport parameters that occurred after fouling. An attempt was made to isolate the effects of different classes of contaminants, notably lipids and particulates on the fouling process.

4.2 MATERIALS & METHODS

4.2.1 CHROMATOGRAPHIC SYSTEM

The chromatographic system used in this series of experiments was the same as that described in Section 2.2.1 with the following changes.

4.2.1.1 Chromatographic Matrices

Two chromatographic matrices were used for the work. The first matrix was the functional ion-exchanger, DEAE-Sepharose Fast Flow as described in Section 2.2.1.1. The second matrix used was the base matrix, Sepharose 4 Fast Flow, the base matrix that is substituted to produce the range of functional Fast Flow gels available, including DEAE-Sepharose Fast Flow. This base matrix is not normally available as a commercial gel, but was kindly supplied by Pharmacia LKB Biotechnology, Uppsala.

In order to use the pulse analysis, a particle diameter needed to be established for the matrices under test. The particle size distribution was measured using an image analyser (Magiscan 2A, Joyce-Loebl Ltd., Dukesway, Gateshead NE11 0PZ, UK). A sample of beads were placed in an Improved Neubauer type haemocytometer, and the diameter of 300 beads measured manually using the point to point function of the analyser, the edge of each particle being selected manually.

The particle diameter is a sensitive parameter in the moment analysis (Section 4.2), and the calculation of the mean diameter value from the distributions measured is therefore important. Previous workers have reported that the most appropriate value is the surface average value for particle diameter (Rumpf & Gupte 1971; Dullien 1979).

The surface average diameter is defined in Equation 4.1.

$$d_{p_{mean}} = \frac{\left(\int_0^\infty d_p(d_p^{\,2})n(d_p)\,\mathrm{d}d_p\right)}{\left(\int_0^\infty d_p^{\,2}n(d_p)\mathrm{d}d_p\right)} \qquad 4.1$$

Here $n(d_p)$ is the particle size distribution. The average particle radius can be calculated from the measured particle size distribution using equation 4.2, which is the discrete form of 4.1 for the radius of the bead, R_p, rather than the diameter of the bead (Rasmuson, 1985).

$$R_p = \frac{\sum_{i=1}^{N} R_{p_i}^3}{\sum_{i=1}^{N} R_{p_i}^2} \qquad 4.2$$

Values for R_p for the matrices were found to be 47.8 and 46.4 μm for the two batches of Sepharose 4 FF and 46.8 μm for the single batch of DEAE Sepharose FF used in the experiments reported here. This compares well with the manufacturer's literature, which cites a range for the diameter of FF gels between 45 and 165 μm, and an average particle diameter of 90 μm (R_p = 45 μm).

4.2.1.2 Chromatographic System

The chromatographic system used was described in Section 2.2.1.2. The system was modified to include two P-6000 pumps and the UV-1 spectrophotometer was replaced with a variable wavelength spectrophotometer (Dynamax, Rainin Instrument Co., USA) capable of measuring absorbance at 420 nm.

The reason for adding in these components was to ensure that the concentration profile measured for the test proteins was not affected by contaminants leaking off the column after the fouling event. The design of both the P-500 and P-6000 pumps is one of a reciprocating piston action with two cylinders. One cylinder fills as the other cylinder provides flow to the system. When the output cylinder is empty, a valve switches to allow the second cylinder to provide flow as the first cylinder fills. At the point when the valve switches between the cylinders, there is a momentary and unavoidable

interruption in the flow that causes a pulse in the column, visible as a peak on the concentration profile of any test protein. This peak introduces an error into the peak analysis. This peak artefact could be avoided by using the larger capacity P-6000 pumps. The P6000 pumps were capable of delivering sufficient volume from one cylinder to perform a whole experiment and thus avoid the formation of extraneous pulses.

The variable wavelength spectrophotometer was added to the system to avoid interference in the absorbance of the test protein by contaminants present in the test protein solutions and foulants leaching from the column. Absorbance was measured for haem containing proteins (haemoglobin and cytochrome c) at the absorbance maximum of 420 nm. This was far removed from the 280 nm absorbance maximum typical of the protein and nucleic acid contaminants present in the fouled bed and possible impurities in the test samples.

The temperature was carefully controlled by means of a circulating water bath to ensure that buffers, samples and packing were maintained at 20 °C ± 0.5 °C throughout the pulse response analysis experiments.

4.2.1.3 Column Packing

Chromatography columns were packed as described in Section 2.2.2.2, to the same dimensions and tolerances (5.0± 0.1 cm bed length x 1.6 cm diameter).

The packed bed void volume was required in the analysis of the data, and this was estimated using Blue Dextran 2000 (Pharmacia LKB Biotechnology) for the Sepharose 4 FF gel. The void volume was estimated from the apex of the peak absorbance. Since the dextran molecule is completely excluded, the retention time of a small sample of Blue Dextran injected onto the column gives an accurate measure of the column interstitial volume.

Blue Dextran is irreversibly bound to ion-exchange matrices, and it as therefore necessary to use an alternative method to estimate the column voidage of a packed bed of DEAE Sepharose FF. This was achieved by injecting a sample of amylopectin (A-7780 Sigma) to the column, collecting fractions and measuring the apex of the peak using iodine to give a blue colour reaction with collected amylopectin, and a spectrophotometer to determine the peak. As the minimum practical fraction volume was 0.1 mL, this restricted the resolution of the method. However, retention times measured by both methods in the Sepharose 4 FF showed that the method was reliable to the precision of ± 0.1 mL.

4.2.2 EXPERIMENTAL METHOD

4.2.2.1 Treatment of Data for Pulse Analysis Using Moments

The pulse analysis method is based on the statistical moments theory of Kubin (1965) and Kucera (1965). Schneider and Smith (1968) developed the chromatographic model from which the equations were derived relating the first absolute and second central moment of the chromatographic peak to the parameters governing the transport of molecules in chromatography. In the pulse analysis method used for this work, a step pulse of a solute is injected into the packed bed, and the statistical moments of the solute output concentration profile were determined from which the axial dispersion, effective diffusivity and the porosity (equilibrium distribution) of a protein in the support were calculated.

The application of the moment analysis of a pulse of solute has been used by several workers to obtain the transport parameters of proteins in a variety of chromatographic systems (Arnold ,1985); Ching, 1989; Boyer & Hsu, 1992; Ming & Howell, 1993). This is despite the acknowledged errors that can accrue, particularly in estimating the

second central moment (Lenhoff, 1987). In particular the second absolute moment is prone to variations arising from small amounts of impurities in the pulsed sample, difficulties in estimating the start and end of the pulse, noise and extra-column effects (Chesler & Cram , 1971). The reason that the method has found wide use is it's general applicability to any chromatographic peak shape (making it ideal for use in a fouled column) and the lack of any requirement for very specialised equipment and analytical tools.

For this work, adsorption was not considered in order to simplify the experimental method and data analysis. The effect of fouling on the adsorption process is discussed in Chapter 3.

The average retention time of a concentration peak of a solute is given by the first absolute moment, μ_1

$$\mu_1 = \frac{\int_0^\infty C(L,t)t\,dt}{\int_0^\infty C(L,t)\,dt} \qquad 4.3$$

$$\mu_1 = \frac{L}{u}\left[1 + \frac{(1-\varepsilon)}{\varepsilon}\bullet\varepsilon_p\right] + \frac{t_0}{2} \qquad 4.4$$

Here L is the bed height, u is the interstitial fluid velocity, ε is the column void fraction, ε_p is the inclusion porosity, and t_0 is the input pulse time.

The peak variance is given by the second central moment, μ'_2 , expressed as,

$$\mu_2' = \frac{\int_0^\infty C(L,t)\,(t-\mu_1)^2\,dt}{\int_0^\infty C(L,t)\,dt} \qquad 4.5$$

$$\mu_2' = \frac{2L}{u}\left[\frac{(1-\varepsilon)}{\varepsilon}\bullet\frac{R_p^2\,\varepsilon_p^2}{15}\bullet\left(\frac{1}{D_e}+\frac{5}{k_f R_p}\right)+\frac{D_L}{u^2}\left(1+\frac{(1-\varepsilon)\varepsilon_p}{\varepsilon}\right)^2\right]+\frac{t_0^2}{12} \qquad 4.6$$

Here the D_L is the axial dispersion coefficient, R_p the particle radius, k_f the external fluid film mass transfer coefficient.

The first absolute moment is used to estimate the value of the bead inclusion porosity, which is the volume fraction of intraparticle space available to the diffusing solute injected in the pulse. From consideration of equation 4.4, a plot of $\mu_1 - t_0/2$ versus L/u yields a straight line. The gradient of this line can then be used to find a value for the inclusion porosity if the void volume of the bed is known (this can be readily found using the average retention of a large molecule such as dextran).

The second absolute moment allows estimation of the axial dispersion, D_L and the intraparticle diffusion coefficient, D_e. Equation 4.6 is rearranged to give 4.7.

$$\frac{\mu_2' - \frac{t_0^2}{12}}{\frac{2L}{u}} = \frac{(1-\varepsilon)}{\varepsilon}\bullet\frac{R_p^2\,\varepsilon_p^2}{15}\bullet\left(\frac{1}{D_e}+\frac{5}{k_f R_p}\right)+\frac{1}{u}\left(\frac{D_L}{u}\left[1+\frac{(1-\varepsilon)\varepsilon_p}{\varepsilon}\right]^2\right) \qquad 4.7$$

A plot of the left-hand side of equation 4.7 versus the reciprocal interstitial velocity (1/u) yields a linear function, the slope of which is related to the axial dispersion and the y-intercept is related to the external fluid film transfer coefficient and the effective diffusivity of the solute.

It has been noted that the method of moments analysis applied to chromatographic systems is prone to errors. The length of tubing connecting the column and detector was kept to as short as possible to minimise the pulse dispersion before the column and detector. The first moment (average retention time) was corrected to include the volume of the tubing connecting the column to the sample, and for the column end pieces according to equation 4.6 (Suzuki , 1974).

$$\mu_1 = (\mu_1)_{measured} - \frac{V_d}{Q} \qquad 4.8$$

Here V_d is the dead space volume calculated from the length and internal diameter of connecting tubing and fittings, and Q is the volumetric flowrate. The correction was confirmed using a pulse of blue dextran in which the column end pieces were pushed together. Likewise, the second moment analysis took into account the dispersion of the pulse of solute by the tubing and fittings. The second moment was calculated for the tubing and fittings without the column. The second moment represents the peak variance, and, since the chromatographic variance is additive, the second moment measured for the packed beds was the sum of the variance in the column plus the variance in the tubing and fittings. Thus the second central moment in the experiments was determined by measuring the second central moment and subtracting the second central moment due to the extra-column effects.

The numerical integrations used to determine first and second moments were performed using the trapezoidal rule and the following equations (Grushka et al., 1969).

$$\mu_1 = \frac{\sum_{all\ t} t_i y_i}{\sum_{all\ t} y_i} \qquad 4.9$$

$$\mu_2' = \frac{\sum_{all\ t} (t_i - \mu_1)^2 y_i}{\sum_{all\ t} y_i} \qquad 4.10$$

here y_i is the peak height at the ith interval. The moments were determined by a computer program written in ProMatlab version 3.5i (The Mathworks Inc.) by G. A. Soriano (1995), to carry out the determination of the limits of integration, to correct for any baseline drift and to perform the integration and evaluate the first absolute and second central moments of the concentration peaks. The limits of integration were based on the criteria established by Dyson (1990). The program was run on a SUN SPARC station 1 (Sun Microsystems Europe Inc.).

4.2.2.2 Test Proteins

The two test proteins used in this work were cytochrome c and haemoglobin. Selection of the proteins was based on their absorbance maxima at 420 nm which prevented interference from fouling species, their molecular weight range and their relative cost and availability. Cytochrome c was from horse heart (Sigma C-7752), molecular weight 12 kD, and a quoted purity of 99 %; haemoglobin was from a bovine source (Sigma H-2500), molecular weight 64 kD and a quoted purity of 99 %.

4.2.2.3 The Pulse Analysis Method

The pulse analysis method consists of analysing the concentration profile of a sample as it leaves the bed for a range of different flowrates. As for previous experiments, columns were always freshly packed for each series of experiments, except where clean-in-place (CIP) methods were investigated.

Freshly packed columns were equilibrated with 20 mM phosphate buffer (prepared as described in Section 2.2.2.3) with 0.5 M sodium chloride. The sodium chloride was added to this concentration to minimise ionic and hydrophobic interactions between the test proteins and the packing matrix. Once equilibrated, samples of protein were injected onto the column at constant flowrate from 1 to 5 mL/minute in 1 mL/minute steps. Sample volumes and concentrations were 200 μL and 2 mg/mL for both the test proteins used.

The concentration profile of the protein was detected using the spectrophotometer at 420 nm. The bed voidage was determined as described in 4.2.1.3. These conditions were then the base case of the fresh column.

Next, the bed was equilibrated with 20 mM phosphate buffer to provide conditions for adsorption of the fouling stream. A fouling stream was prepared as described in Section 2.2.2.1. This stream was diluted to a range of concentrations from 10 % to 100 % of processed yeast homogenate. In each case the 50 mL of the process stream was used to foul the column. For loading process stream, a flowrate of 2.6 mL/minute was used; higher flowrates risked bed compression.

Three types of fouling experiments were performed. Firstly the effect of fouling on the unsubstituted base-matrix, Sepharose 4 FF was examined. This base matrix had no functional ligands, and consequently the fouling that occurred should be dependant on the physical filtration-type mechanisms provided by the matrix. It was recognised that

there was the possibility of interactions with the bed due to hydrophobic interaction; such effects have been reported (Swergold, G.D. and Rubin, C.S., 1983; Meredith, S.J. J.Biol. Chem., 1984). However the Sepharose gel is known to be highly hydrophilic, so it was to be expected that such effects would be negligible.

The second type of experiment used the substituted DEAE Sepharose FF gel. This should have the same fouling properties as the base matrix, with the addition of fouling species interactions with the DEAE ligands. By comparison of the base matrix to the functional gel, the effects due to the ligands could be inferred. The assumption made here is that the chemistry involved in substituting the base matrix with ligand did not significantly affect the bead structure.

The final series of experiments aimed to understand the action of two major classes of contaminants in the process stream, namely lipid and cell particulate debris. Lipids are often quoted as a major source of fouling in column chromatography (Petterson, 1989). A lipid-depleted process stream was compared to a solids-depleted fouling stream to separate their effects. Solids-depleted process streams were produced simply by ultracentrifuging the disc stack centrifuge processed material and leaving the solids in the pellet, as described in Section 2.2.2.1. Lipid-depleted process stream was prepared as described in Section 3.3.3.2.

As before, CIP operation was tested using the protocol described in 3.2.2.5. This had proved very successful when judged by the pre and post-fouling breakthrough curves. Because it is so important to the process economics to be able to re-use a column, it was interesting to see whether the method was as successful when the transport parameters discussed here were used as a criterion for whether or not the column was clean after a CIP treatment.

Following fouling, the column was washed using a 0 to 1 M sodium chloride gradient in phosphate buffer followed by a 1 to 0 M sodium chloride gradient to give a saw-tooth

concentration profile of sodium chloride. This procedure was carried out over 8 column volumes, and the columns were then re-equilibrated with the 0.5 M sodium chloride in phosphate buffer over a further two column volumes.

The pulse analysis was then repeated for the cytochrome c and haemoglobin and void volume was measured again.

4.2.2.4 Extra-column Peak Dispersion Effects

The effect of the tubing, connectors, column end-pieces was determined by measuring the eluted peak concentration for the system with no packing, and the column end-pieces pushed together, as advocated by Arnold et al (1985). The pulse experiments were conducted as described in Section 4.2.2.2. The dispersion determined here could be used to correct the values obtained when the packing was present, and thus give a value for the dispersion due to the packing alone (Section 4.2.2.4). It should be noted that the band width of the sample during injection is also accounted for in this measurement.

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of Process Stream Concentration on Sepharose 4 FF Base Matrix

It was found that the maximum loading that could reliably be achieved with the base matrix was a 1 in 5 dilution of disc stack centrifuged material (Section 2.2.2.1), termed 20 % homogenate in this study. Loading higher concentrations was not reproducible, in that the bed would collapse unpredictably during the pulse response experiments, presumably as a result of blocking the column with solids debris.

In Section 4.2.2.4, a condition of the data analysis is that there is no adsorption of the test proteins to the matrix, either before or after fouling. The buffer was chosen to contain sodium chloride at 0.5 M strength in order to minimise any adsorption. The retention times determined for the test proteins before and after fouling were very similar; adsorption of the test proteins would have led to significant variation in the retention times. In fact, retention times were generally observed to show a slight increase after the fouling event, suggesting a deposition of foulants in the bed reduced the void volume available.

The results of the moment analysis for the base matrix are summarised in Table 4.1. The intraparticle void volume or the inclusion porosity, is a measure of the volume available for the diffusing solute inside the bead. Thus the cytochrome c molecule at 12.4 kD would be expected to show a higher value for the inclusion porosity than the larger haemoglobin molecule at 64 kD. This result was confirmed in the base matrix. Figure 4.1 shows the effect of increased process stream loading on the inclusion porosity for both cytochrome c and haemoglobin.

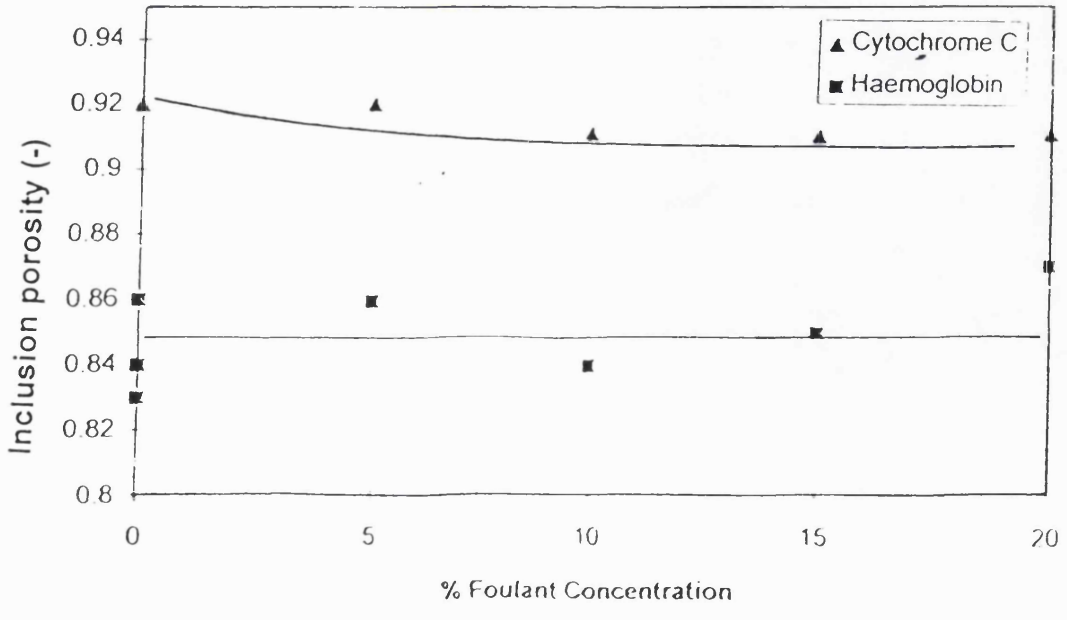

Figure 4.1: *The change in the intraparticle void volume measured using cytochrome c and haemoglobin on Sepharose 4 FF.*

There is possibly a small change in the intraparrticle void volume measured for cytochrome c as the solid loading reaches 20 %. The same effect is not apparent for the haemoglobin molecule, and there is a degree of scatter in the initial values for the inclusion porosity. A significant decrease in the intraparticle void volume would signal a deposition of foulants in the bed. There is conceivably evidence to support a small degree of deposition, since the cytochrome c molecule might be expected to be more sensitive to very low levels of fouling compared to the haemoglobin, as a consequence of their relative sizes. However, the indication is that foulants do not deposit to any significant extent within the internal pore structure of the matrix. The hypothesis could be further tested using a larger range of molecular sizes.

The axial dispersion of the bed is related to it's structure. The quoted value in this work is the ratio of the axial dispersion coefficient to the interstitial velocity, D_L/u. The justification for using this ratio is that the dispersion Péclet number ($Pe = ud_p /D_L$) is weakly dependent on the interstitial velocity, u, for the range of operation considered, 1<1ReSc <100, (Figure 4.2), and therefore the ratio D_L/u can be considered constant.

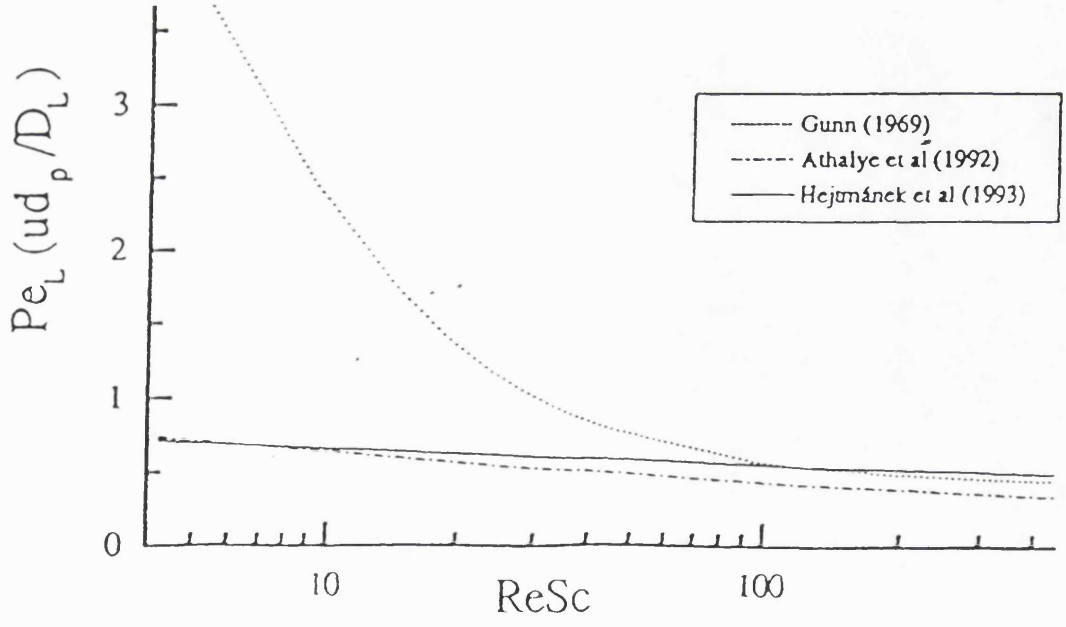

Figure 4.2: *Comparison of correlations for the prediction of the axial dispersion coefficient within the range of chromatographic operation, Péclet number as a function of reduced velocity Re Sc (From Soriano (1995)).*

The results for D_L/u are shown in Figure 4.3. It is apparent that the axial dispersion shows a significant increase as a function of solids loading. At 10 % solids loading, there is a rather large change in the axial dispersion figure. Table 4.1 shows the column voidage, ε, to be lower than the other columns used. As a result the 10 % fouled column shows the highest increase in axial dispersion. The general trend is an upward increase in the axial dispersion, but the relationship is not solely dependent on solids concentration, but also how the column has been packed. This illustrates the difficulty in relating experiments using different columns for each points, since even with careful attention to detail, subtle differences in packing are discernible in this analysis.

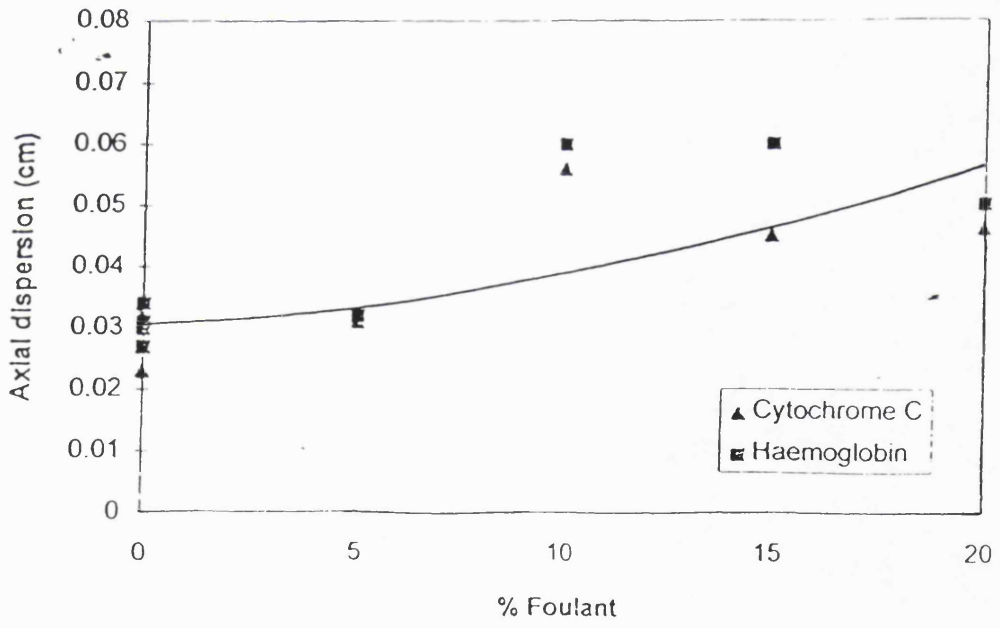

Figure 4.3: *The effect of increased process stream loading on D_L/u for cytochrome c and haemoglobin on Sepharose 4 FF*

Table 4.1: Summary of Transport Parameters for Sepharose 4 FF

Column Number	Protein	Bed Length (cm)	Void Volume, ε (-)	Freshly Packed Column Parameters			Process Stream	Fouled Column Parameters		
				ε_p (-)	D_L/u (cm)	D_e (cm^2/s)		ε_p (-)	D_L/u (cm)	D_e (cm^2/s)
1	Cytochrome c	4.95	0.35	0.92	0.027	3.32×10^{-7}	10 % Whole Homogenate	0.92	0.031	2.73×10^{-7}
2	Cytochrome c	4.85	0.32	0.92	0.023	3.41×10^{-7}	20 % Whole Homogenate	0.91	0.056	2.78×10^{-7}
3	Cytochrome c	5.05	0.35	0.92	0.030	3.23×10^{-7}	50 % Whole Homogenate	0.92	0.045	2.28×10^{-7}
4	Cytochrome c	4.90	0.34	0.92	0.032	3.82×10^{-7}	100 % Whole Homogenate	0.91	0.046	2.47×10^{-7}
1	Hemoglobin	4.95	0.35	0.86	0.027	2.17×10^{-7}	20 % Lipid Depleted	0.86	0.032	1.94×10^{-7}
2	Hemoglobin	4.85	0.32	0.84	0.031	2.21×10^{-7}	100 % Lipid Depleted	0.84	0.060	1.89×10^{-7}
3	Hemoglobin	5.05	0.35	0.83	0.027	2.15×10^{-7}	20 % Solids Depleted	0.85	0.060	1.80×10^{-7}
4	Hemoglobin	4.90	0.34	0.86	0.034	2.25×10^{-7}	100 % Solids Depleted	0.87	0.050	1.60×10^{-7}

The effective diffusivity of the cytochrome c and the haemoglobin are shown in Figure 4.4, where the values are shown as the measured effective diffusivity as a ratio of the free diffusivity (D_e/D_m). It was found that there was a decrease in the effective diffusivity, and that this was a direct function of the amount of fouling process stream added to the column. This would tend to support the small change in the intraparticle void volume seen with the cytochrome c. Chapter 3 suggests that there are indeed major changes in the effective diffusivity as a result of loading fouling streams to columns, confirming the observations made in these experiments.

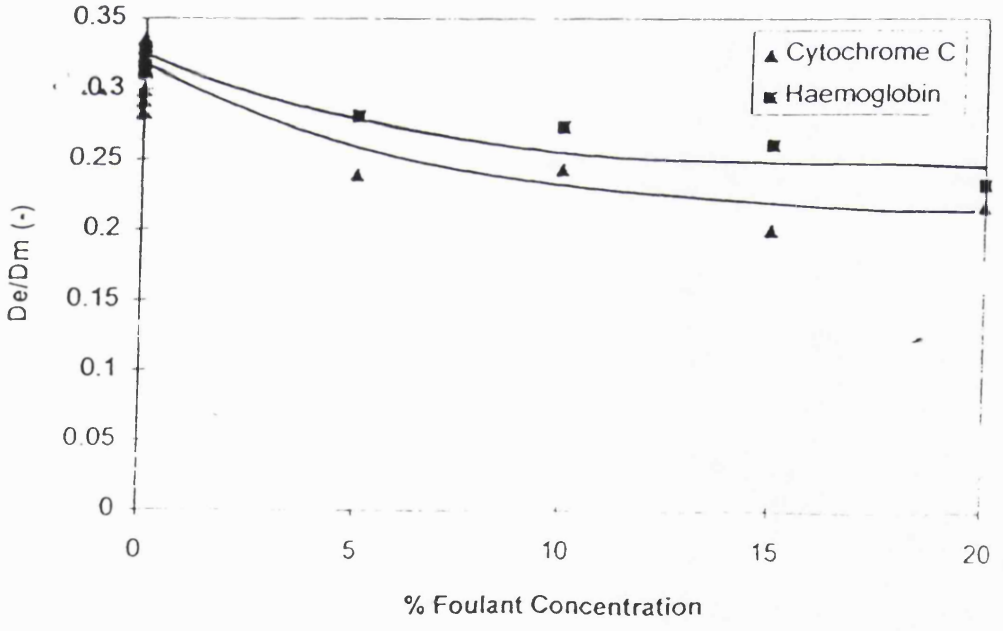

Figure 4.4: *The change in axial dispersion with increased process stream loading for cytochrome c and haemoglobin on Sepharose 4 FF.*

4.3.2 Effect of Process Stream Concentration on DEAE Sepharose FF

It was found that the DEAE Sepharose FF matrix was capable of taking a higher process stream loading than the base matrix without collapsing. This was also observed in the frontal analysis work of Chapter 3, where the 600 g/L preparation of yeast homogenate passed through a disc stack centrifuge could be loaded at full strength onto a packed bed.

The results obtained for the DEAE Sepharose FF column are summarised in Table 4.2. Haemoglobin was not used as a test pulse in this work because the results in Section 4.3.1 showed that differences between the transport properties of the bed compared for the two proteins were at best small, and probably not significant. The effect of the fouling stream on the column inclusion porosity for cytochrome c is shown in Figure 4.5.

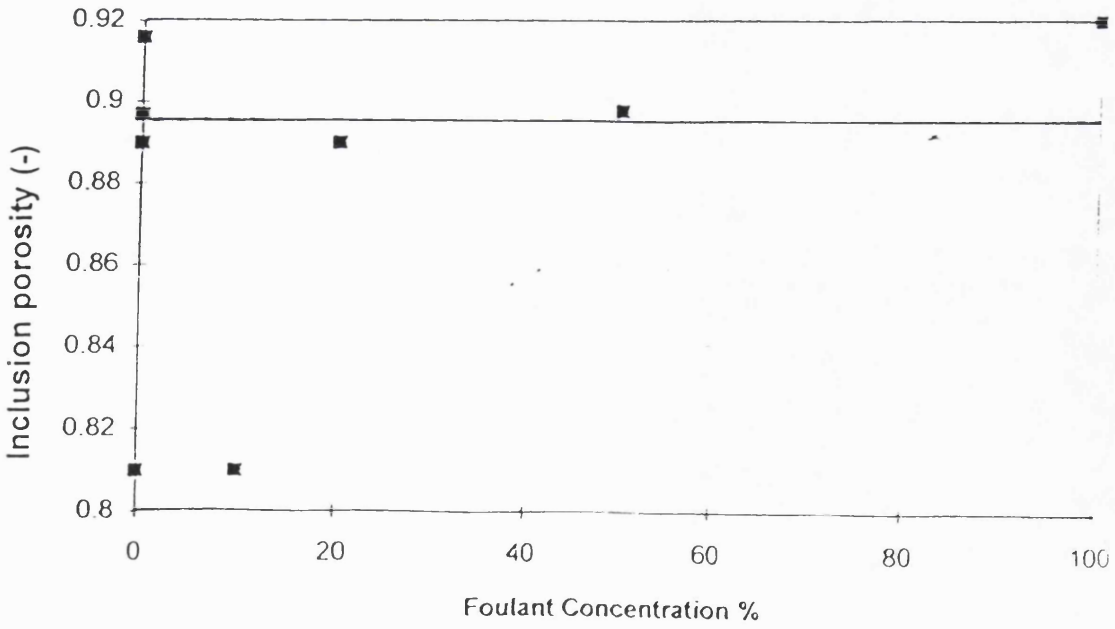

Figure 4.5: *The change in the intraparticle void volume measured using cytochrome c on DEAE Sepharose FF.*

There is no apparent decrease in the inclusion porosity as was observed for the base matrix. There is a great deal of scatter in the results, and the reason for this may be due to the method used for estimating the column void volume. In the case of the base matrix, values for the void volume were measured for the fresh and fouled column using Blue Dextran 2000. After fouling, the void volume was measured once more. It was found that there was no difference detected in the inclusion porosity after fouling, inspite of the fact that there was a significant change in the axial dispersion. The reason for this is the difficulty in estimating the apex of the peak, especially after the fouling event, when the increased dispersion causes tailing, decreasing the sharpness of the eluted band. This effect is compounded when amylopectin is used in the case of a DEAE Sepharose FF bed, since the resolution of the peak is already reduced to ± 2 % due to the 0.1 mL fractions taken. The inclusion porosity value is dependant on the accurate determination of the column void volume; the scatter in the results is probably a reflection of this.

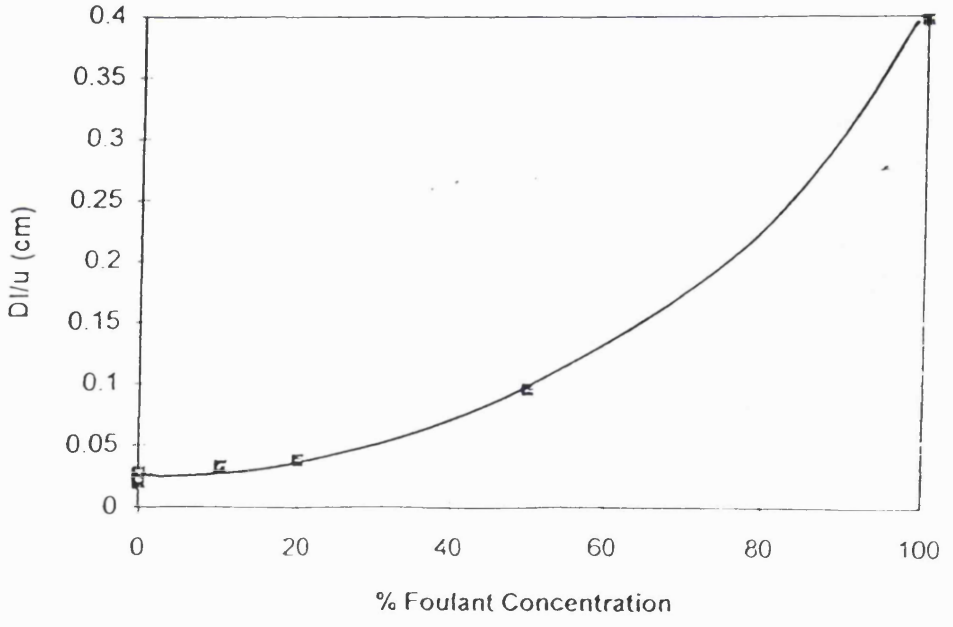

Figure 4.6: *The effect of increased process stream loading on D_t/u for cytochrome c on DEAE Sepharose FF*

The effect of the fouling stream on the axial dispersion is shown in Figure 4.6. There is an apparently direct relationship between the axial dispersion value obtained and the increase in axial dispersion up to a value of 20 % (1 in % dilution) of homogenate.

Unlike the previous set of experiments, the measured inclusion porosity was similar for 10 %, 20 % and 100 % process stream loadings. Only the column void volume for the bed loaded with 50 % concentration of process stream solids is the bed void volume slightly lower, indicating a more compressed column. The results described in Section 4.3.1 would suggest that there would be a larger increase in the axial dispersion for such a bed, and this is confirmed in Figure 4.6 for 50 % process stream loading. However, at 100 % loading, the column shows a very large increase in axial dispersion. The suggestion is that there is a linear fouling process up to a certain value of fouling (in this case 20 % of the process stream concentration), beyond which the process of fouling enters a run-away exponential increase, finally resulting with the bed compressing. Previous observations would agree with this (see Section 3.3.2). The effect of the fouling process stream on the effective diffusivity is shown for cytochrome c in Figure 4.7.

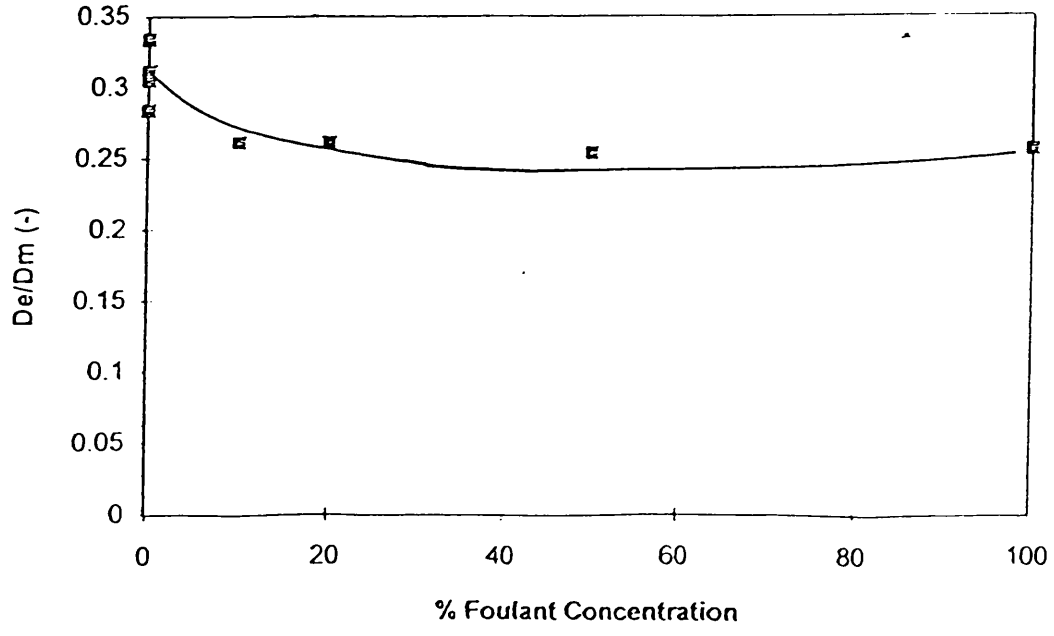

Figure 4.7: *The change in axial dispersion with increased process stream loading for cytochrome c on DEAE Sepharose FF.*

Table 4.2: Summary of Transport Parameters for DEAE Sepharose FF

Column Number	Protein	Bed Length (cm)	Void Volume, ε (-)	Freshly Packed Column Parameters			Process Stream	Fouled Column Parameters		
				ε_p (-)	D_L/u (cm)	D_e (cm^2/s)		ε_p (-)	D_L/u (cm)	D_e (cm^2/s)
1	Cytochrome c	4.95	0.37	0.81	0.020	3.81×10^{-7}	10 % Whole Homogenate	0.81	0.033	2.98×10^{-7}
2	Cytochrome c	5.00	0.38	0.89	0.026	3.24×10^{-7}	20 % Whole Homogenate	0.89	0.038	2.97×10^{-7}
3	Cytochrome c	4.95	0.35	0.90	0.020	3.55×10^{-7}	50 % Whole Homogenate	0.90	0.096	2.89×10^{-7}
4	Cytochrome c	5.05	0.38	0.92	0.028	3.48×10^{-7}	100 % Whole Homogenate	0.92	0.397	2.92×10^{-7}
5	Cytochrome c	4.90	0.36	0.94	0.022	3.82×10^{-7}	20 % Lipid Depleted	0.87	0.032	3.38×10^{-7}
6	Cytochrome c	5.05	0.37	0.92	0.028	3.56×10^{-7}	100 % Lipid Depleted	0.91	0.077	3.21×10^{-7}
7	Cytochrome c	5.00	0.37	0.91	0.023	3.86×10^{-7}	20 % Solids Depleted	0.91	0.022	3.15×10^{-7}
8	Cytochrome c	4.95	0.38	0.86	0.036	3.85×10^{-7}	100 % Solids Depleted	0.88	0.074	2.81×10^{-7}

The effective diffusivity is seen to decrease as a function of the amount of process stream added to the bed up to 20 % loading, after which the effective diffusivity to free diffusivity ratio reaches an asymptotic value of 0.25. This effect is similar to that seen with the axial dispersion, and it is probable that the two effects are related. When the amount of fouling deposition is relatively small there is a decrease proportional to the loading - this is in agreement for observations made of the base matrix in Section 4.3.1. After a certain quantity of process stream has been added (in this case 20 %), the major effect is deposition in the bed interstices, observed here in the increase in axial dispersion (Figure 4.6). At this point there is no further change in the effective diffusivity, presumably because there is severe fouling that will very rapidly lead to bed compression. Certainly at the loading reported here, compression is a possibility.

4.3.3 Effect of Solids Depleted Process Stream Versus Lipids Depleted Process Stream

The effects of two major putative fouling components was separated by purifying the process stream so that either lipids (lipid-depleted stream) or solids (solid-depleted stream) were selectively removed as described in 4.2.2.2. Experiments were once more conducted with cytochrome c alone.

Figure 4.8 shows the relative effects of a whole process stream, a solids enriched process stream and a lipid enriched process stream on the axial dispersion of the column. It is apparent that there is an increase in the axial dispersion for both the lipid and solid depleted streams, with the increase in axial dispersion being marginally higher for the lipid depleted stream. The change in axial dispersion is never seen to be as great for either of the solids or lipid depleted streams as compared to the whole homogenate, which leads to the possibility of the fouling being a synergistic effect of both the lipid and particulate solids. It would have been revealing to push the solids loading even higher to determine if there would be a sudden large increase in axial dispersion, but this was not possible owing to the difficulty in preparing sufficient quantities of material.

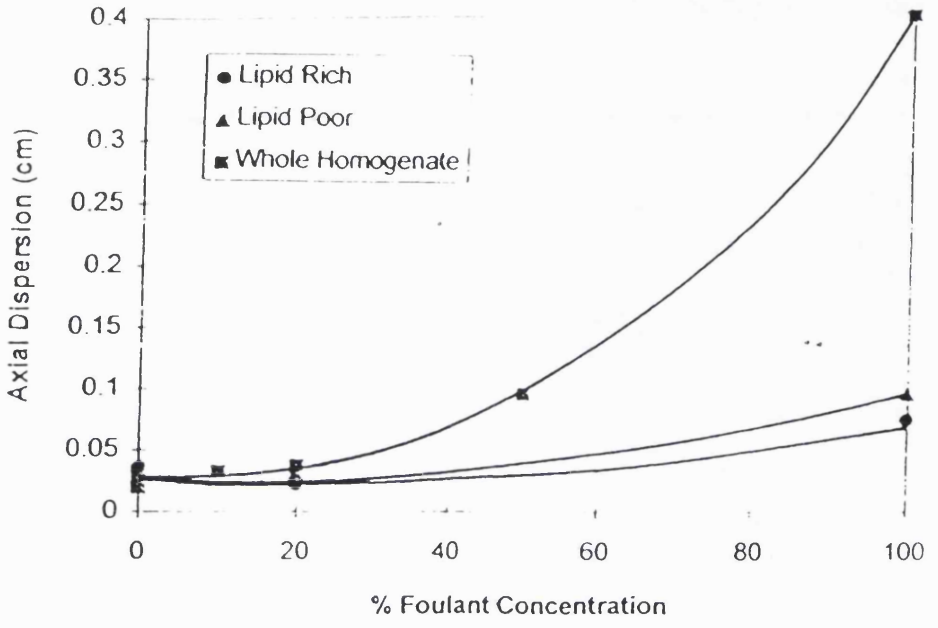

Figure 4.8: *Comparison of axial dispersion for whole homogenate, solids depleted homogenate and lipid depleted homogenate on DEAE Sepharose FF.*

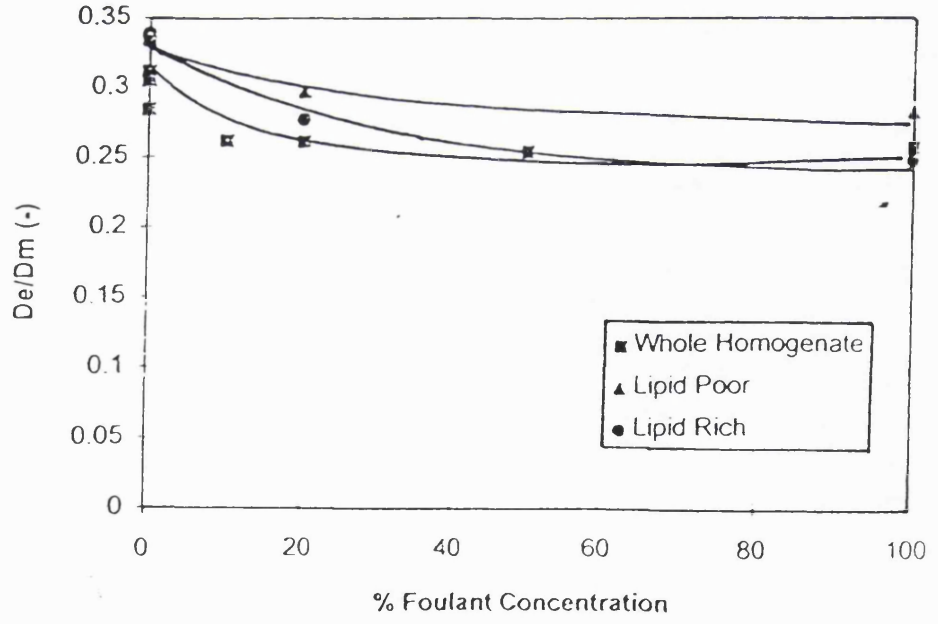

Figure 4.9: *Comparison of effective diffusivity for whole homogenate, solids depleted homogenate and lipid depleted homogenate on DEAE Sepharose FF.*

Figure 4.9 shows the effect of solids and lipids on the diffusivity. Although changes are small, it is apparent that the lipid (enriched in the solid-depleted stream) seems to exert

a similar effect on the diffusivity as the whole homogenate, whereas the lipid-depleted (solids rich) stream shows a lesser effect. It is possible that the intraparticle fouling could be largely controlled by the amount of lipid present, with a relatively smaller contribution from the solid particulates. This is not an unreasonable hypothesis, since lipids would be able to diffuse much more readily into the pores of the beads compared to the solids. It could be envisaged that only a certain size range of solids could have access to the bead interior due to stearic hindrance, and thus the effect of solids on the effective diffusivity should be less than that of the lipids. It is also possible that a limit is reached to the diffusivity of the proteins as a result of coating of the bead. Major classes of amphipathic lipids are found in the cell membranes of yeast notably phosphoglycerides and sphingolipids. Although water insoluble, these large molecules have polar head groups, and such molecules are able to pack themselves closely around hydrophobic/hydrophilic areas. A possible reason for the change in diffusivity reaching a constant value with the solids loaded may be due to lipid coating the available spaces up to a point where no further coating is possible.

This experiment is intriguing in that it hints at possible mechanisms for fouling. However more data needs to be gathered to confirm these observations and hypotheses.

4.3.4 Clean-In-Place of Fouled Columns

The effect of CIP on the axial dispersion and the effective diffusivity are summarised in Table 4.3 for a variety of process stream loadings. In general, where the highest loadings were applied, the less likely the axial dispersion was to return to it's original value, although in all cases the CIP treatment was effective in improving significantly the axial dispersion. Likewise the effective diffusivity is generally increased after fouling by the CIP protocol.It was concluded that the relatively simple sodium hydroxide wash was very effective in cleaning even quite severely fouled columns.

Table 4.3: Effect of CIP on Axial Dispersion and Effective Diffusivity

Column	D_L/u (cm)	Effective Diffusivity (cm^2/s)	Ratio of Effective Diffusivity to Free Solute Diffusivity
Fresh	0.020	3.81×10^{-7}	0.33
10 % Yeast Homogenate	0.033	2.98×10^{-7}	0.26
CIP	0.028	3.48×10^{-7}	0.30
Fresh	0.026	3.24×10^{-7}	0.28
20 % Yeast Homogenate	0.038	2.94×10^{-7}	0.26
CIP	0.018	3.70×10^{-7}	0.32
Fresh	0.020	3.55×10^{-7}	0.31
50 % Yeast Homogenate	0.096	2.89×10^{-7}	0.25
CIP	0.031	3.70×10^{-7}	0.32
Fresh	0.028	3.48×10^{-7}	0.31
100 % Yeast Homogenate	0.397	2.92×10^{-7}	0.26
CIP	0.036	3.85×10^{-7}	0.33

4.4 CONCLUSIONS

The technique of moments is useful in determining transport parameters in both fresh and fouled columns. It is interesting that both the base matrix and the DEAE Sepharose FF showed similar changes following fouling, although the DEAE Sepharose was able to take a higher level of fouling than the Sepharose 4 FF. Presumably this was as a result of the chemistry involved in introducing ligands.

It was difficult to determine the void volume of the column after fouling had occurred, because the markers were subject to band broadening and tailing caused by fouling. This in turn affected the value of the inclusion porosity. This would also impact on the value estimated for the diffusivity and the axial dispersion.

The aim of this work was to determine the effect of the different fouling species on the transport parameters governing solute behaviour in chromatography. It would have been convenient to separate out which of the possible fouling species were responsible for the effects determined here. Mass balances were performed on all the major classes of likely fouling species, that is cell debris particulates, lipids, nucleic acids and protein, using the assays described in Section 2.2.3. Generally it was found that the mass balances were too variable to be of much use in determining which of these species were responsible for fouling the column. This is why the results are quoted as percentage v/v dilutions of the 600 g/L yeast homogenate processed by disc stack centrifugation. The assay systems used were difficult to perform reliably, and the errors introduced in running them were of the order, or greater than the quantity of material likely to be entrapped in the column.

In this work, the external fluid film mass transfer coefficient, k_f, was estimated by the correlation of Ohashi, 1981. It was assumed that this value would remain constant throughout the fouling since it would take a large amount of fouling around the whole of the exterior of the beads to affect the fluid film. The evidence from the frontal analysis (Chapter 3) was that k_f was not significantly affected by the fouling conditions used, and that the assumption of a constant value is valid for the amount of fouling demonstrated here.

There is some evidence that there are two distinct fouling regimes occurring. Up to a 20 % process stream concentration, there is an apparently linear decrease in the diffusivity and linear increase in the axial dispersion. Beyond this point, the reduction in the

diffusivity reaches a saturation value, possibly due to coating of the available sites by foulants deposition internally in the bead. This was also seen with a lipid-rich process stream, inferring that this process was largely due to lipids. The effect was less pronounced with the solids-enriched stream. After this point there is a huge increase in the axial dispersion, as the bed structure is changed due to foulant deposition in the column interstices. This is typical of the sort of behaviour expected in dead end filtration, where the filter is suddenly blinded and overcome with the amount of solids. When either solids or lipids are present on their own, the axial dispersion does not increase at the 100 % loading of the process stream, indicating that these two species have a synergy in their fouling action.

A further point to arise out of this work, is the importance of packing on bed behaviour. A small change in the column void volume, indicating a more compressed bed, was sufficient to cause a significant change in axial dispersion, over and above what might have been predicted from the rest of this work. This has important consequences for a process where a bed is re-packed periodically. Clearly there will be an optimum packing procedure to produce the correct compression in the bed. Too soft will reduce the column chromatographic efficiency, too hard and the bed will foul more readily and it's lifetime will be reduced.

Despite the acknowledged difficulty of the pulse response analysis, the results presented here show that it is a useful tool. This work must be seen as a very preliminary exercise, which had the virtue of using a real, and challenging, process model. However real process streams are complex, and this lead to an impossible task in assaying the possible fouling species, other than in very general terms. A less complex, artificial process stream containing one, or a mixture of test fouling species would be very interesting. and would help to further evaluate the causes of fouling and it's mechanism.

5. DISCUSSION OF EXPERIMENTAL ISSUES, LIMITATIONS OF MODELS AND ERRORS

5.1 Experimental Issues

5.1.1 Introduction

The work described in this thesis attempts to develop practical methods for determining the causes of fouling in packed bed chromatography, an industry wide and economically important separation process. Such methods must be easy to apply and give reproducible answers if they are to be of utility in obtaining design information. There have been no systematic studies of process fouling of chromatography matrices reported in the literature, inspite of the importance of the chromatography unit operation. The following sections go on to describe some of the problems encountered in the course of this work, and attempts to critically examine the utility or otherwise of the methods used and the results reported here.

5.1.2 The Yeast ADH System

Many of the problems experienced with measuring the performance of the chromatography through the purification factor were as a result of the yeast system chosen. This system had the virtue of being a natural system, and one that was readily available through commercial sources as a standardised starting material. However, the enzyme ADH is very susceptible to oxidation, and is easily inactivated/activated depending on its physicochemical environment. This restricted the use of chromatography substrates - both weak and strong cation exchangers and strong anion exchangers caused loss of enzyme activity, and a similar effect was seen with hydrophobic interaction substrates.

5.1.3 The Chromatography System

The method of the fractionation diagram was limited because even with anion exchange matrices, the yield of ADH was low. The low ADH yield was due to competitive

binding of other process components, and a loss of enzyme activity through non-specific binding.

In addition, the use of an enzyme assay of fractions eluted from the column restricted the resolution of the diagram. Likewise, the capacity analysis relied on a picture of the column after it had been fouled, not during the fouling process itself. The problem in both cases is one of detection of a specific component from a mixture. This could be addressed in two ways. Firstly by using a marker protein that can be detected without interference . It is possible that a dye could be used to allow the protein to be determined using a visible wavelength spectrophotometer, for example, Coomassie blue. However, anion exchangers will bind dyes with high avidity, and such an approach is of dubious utility. Radioactive marking is also a possibility, but would involve working in a radiation laboratory with dedicated equipment. In addition the method has to be cheap and reliable. A second possibility is to use a synthetic fouling process stream to reduce the complexity of the material to be analysed. However, such an option makes interpretation of results and comparisons with feedstocks more difficult and was therefore not pursued.

5.1.4 Process Stream Characterisation

Characterisation of the fouling stream was difficult to perform. The methods chosen were difficult to apply and rather time consuming. It is obvious that the specificity of the conclusions drawn in this work are limited by the relatively poor characterisation of the process stream. To characterise the process stream is not a trivial task; however the usefulness of the data to designers would be immense, and this would be a worthwhile area of research.

5.2 Limitations of Models

The models presented in this thesis for the breakthrough of solute are based on a consideration of the physical transfer mechanisms governing transport of the solute from the liquid bulk through to the site of adsorption within the individual beads of the matrix. Essentially the models are based on the same mass balancing of solute over a

finite element of the column. the Cooney (1990) model differs from that of Horstmann (1989) in assuming a constant pattern breakthrough of the solute forming at every point in the column allowing the dimensionless concentration of solute in the liquid phase to be equal to the dimensionless concentration of solute in the solid phase. Transport of solute to the surface of the bead is considered to occur through a stagnant liquid film, and the rate of transport is governed by a fluid film transfer coefficient and a linear concentration difference between the stagnant film and the bulk liquid. At the surface of the bead, transport through the pores of the bead is assumed to be governed exclusively by diffusion of the solute to the final site of adsorption where reaction takes place. This is assumed to be so rapid in comparison to the diffusion that it can be ignored from the analysis.

In practice the above assumptions have been shown to be valid in numerous research publications (Chase, 1984; Guiochon, 1987; Horstmann, 1989; Draeger, 1991; Cooney, 1993). The Cooney model is an approximation, as the constant pattern front assumption is an ideal situation. However, the comparison of the approximate solution to a much more rigorous treatment by Hand (Hand at al, 1984), showed the differences to be less than 5 %. In the work presented here, the model of Cooney was used to make a visual determination of the effect of fouling by inspection of the shape of the breakthrough curves. The conclusions were that fouling acted to reduce the diffusion of solute in the beads rather than by affecting the fluid film mass transfer. The model of Horstmann was used to fit experimental data using the diffusion of the solute as the only variable parameter, based on the observation that the fluid film transfer is unaffected by fouling. The fit was again judged by visual inspection, and the results are presented in Chapter 3 as discussed. The experimental and model curves can be almost overlaid, and the fit was found to be greater than 96% in all cases.

Limitations of the models in practice are imposed by the paucity of data on the physical constants involved in the adsorption. Values for the fluid film mass transfer were estimated from correlations in the literature (e.g. Ohashi, 1981). Values for the

interstitial porosity, intraparticle void volume and adsorption capacity were taken from a library of data based on similar gels held by Chase, and made available for this work.

The model used for the pulse analysis work is based on the statistical theory of moments. This relates the retention time (first absolute moment) and variance (second central moment) of a chromatographic peak to the physical parameters of intraparticle void volume (the proportion of the bead internal volume available for the diffusing solute), effective diffusivity and axial dispersion of the solute. Again, the fluid film mass transfer was estimated by means of engineering correlations.

The method is limited by the scarcity of existing measurements. The method is time consuming, and close attention must be paid to errors to get meaningful results. However the experimental data are fit very well by the model as shown in the following discussion giving credence to this approach.

5.3 Discussion of Errors

The method of moments must be used in conjunction with tight error controls. The problems of estimating peak start and finish were achieved using a differentiation test, and baseline drift was compensated for by manual adjustment of the baseline. As sources of error, these can therefore be eliminated. In Table 1, a list of the measured variables is shown. These were constant values, and could therefore be ignored - any error in their measurement is constant between the fouled and fresh column, and although having an effect on the absolute values of the reported parameters in Tables 4.1 and 4.2, the comparison between values is still valid. Pump flowrate calibration was checked after every experiment, and found to be invariant. All experiments were performed at constant temperature.

Table 1: Measured variables that were constant

Variable
Particle radius
Buffer viscosity
Buffer density
Bulk solute diffusion coefficient
Pump flowrate
System tubing volume

Table 2 shows those values that varied from experiment to experiment.

Table 2: Values that varied from experiment to experiment

Variable
Column volume
Void fraction

A sensitivity analysis was applied to the variables in Table 2, varying their measured value by ± 2 %. The justification for this is that this is the accuracy possible using a ruler to measure a length to an accuracy of 0.5 mm in 5 cm (1 %) and to measure a peak retention time from either a trace length 10 cm (2 %) or a 0.2 mL fraction from a 10 mL column (2 %). The figures presented in Table 3 are for a column fouled with 10 % homogenate from Table 4.1 for cytochrome c, and are typical of the results obtained.

Table 3: Sensitivity Analysis of Variable Parameters

Variable	% Error			Intraparticle Intraparticle Void Volume (-)			D_L/u (cm)			Effective Diffusivity ($cm^2/s \times 10^7$)		
	+2	-2	0	+2	-2	0	+2	-2	0	+2	-2	0
Void Volume	0.36	0.34	0.35	0.91	0.91	0.91	0.031	0.032	0.031	2.68	2.77	2.73
Column Volume	10.1	9.7	9.9	0.91	0.91	0.91	0.031	0.031	0.031	2.73	2.73	2.73

The results of this analysis show the effective diffusivity to be most sensitive to errors in measuring the void volume whilst a 2 % error in measuring the column volume had no effect on the values of the measured variables to the precision quoted.

The other major measurements were determination of the gradient from a plot of first moment versus column length divided by interstitial fluid velocity, and the gradient and intercept of a plot of second moment versus the reciprocal of the fluid velocity. The fit of these plots to a linear function was a measure of the goodness of fit of the experimental data to the model. For all experiments, the coefficient of variance, r^2, was greater than 0.95 - a value of less than 0.95 meant that the data was rejected and discarded, or more data was gathered to improve the fit. An example of the two plots is shown in Figure 1 and 2 for the 10 % fouling experiment described in table 4.1 for cytochrome c. These graphs are typical for the results presented.

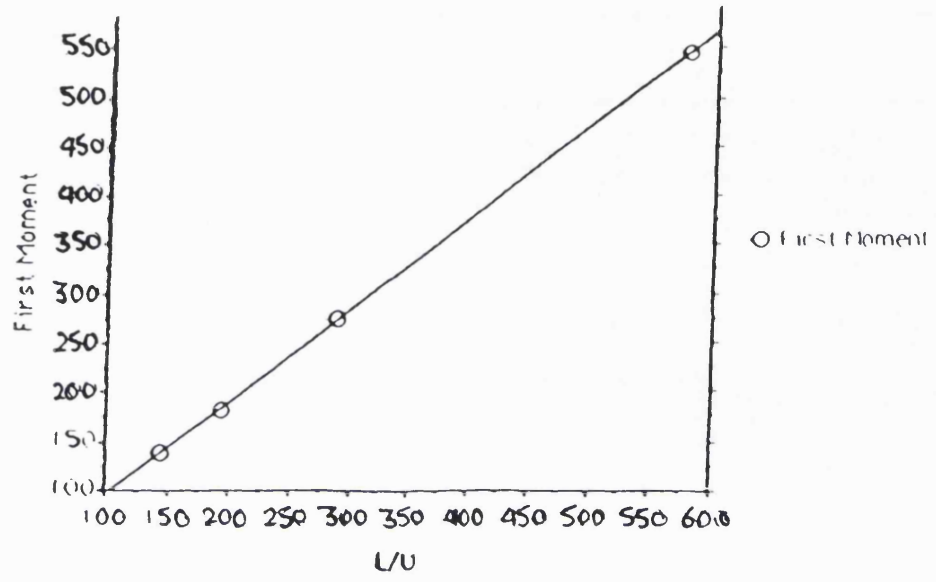

Figure 1: *First moment plotted as a function of L/U for 10 % fouled column in table 4.1 with cytochrome c as the test material (r^2=1).*

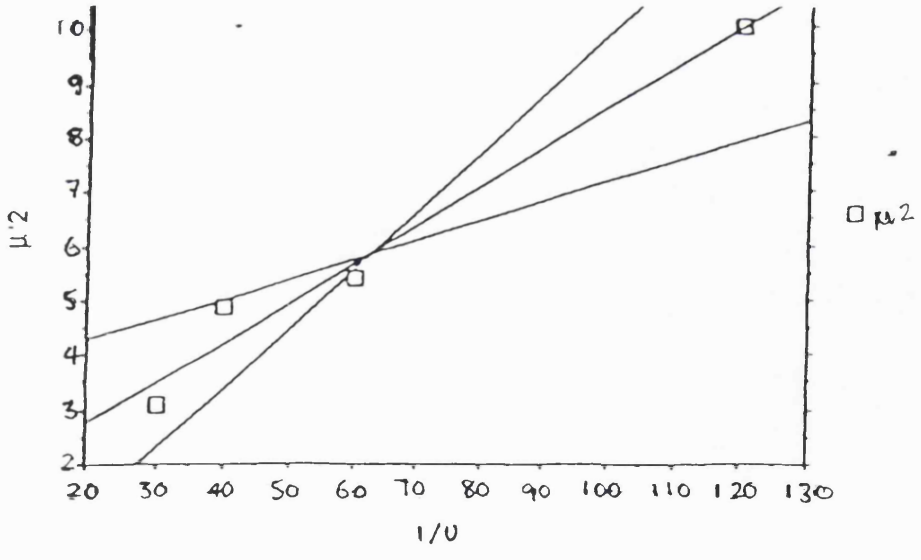

Figure 2: *Second moment plotted as a function of 1/U for 10 % fouled column in table 4.1 with cytochrome c as the test material (r^2=0.96).*

A sensitivity analysis was applied to the same data allowing for a 5% error in the gradient of the first moment plot, and a 5 % error in both the slope and gradient of the second moment plot. The results are shown in Table 4.

Table 4. Sensitivity Analysis of Linear Regression for First and Second Moments

Variable	% Error			Intraparticle Intraparticle Void Volume (-)			D_L/u (cm)			Effective Diffusivity ($cm^2/s \times 10^7$)		
	+5	-5	0	+5	-5	0	+5	-5	0	+5	-5	0
First Moment Gradient	0.99	0.89	0.94	0.98	0.84	0.91	0.028	0.035	0.031	3.17	2.31	2.73
Second Moment Gradient	.063	.057	.060	0.91	0.91	0.91	0.032	0.030	0.031	2.73	2.73	2.73
Second Moment Intercept	2.84	2.57	2.70	0.91	0.91	0.91	0.031	0.031	0.031	2.60	2.87	2.73

In Tables 4.1 and 4.2, the initial values of the cytochrome c and haemoglobin intraparticle void volume and diffusivity can be used to determine the standard deviation for these values. For the diffusivity, the cytochrome c standard deviation is 0.21×10^{-7} cm^2/s and for the haemoglobin the standard deviation is 0.04×10^{-7} cm^2/s. A comparison between the column's fresh and fouled state indicate that the measurements made show a significant difference, and occur as a result of column fouling. The results of the sensitivity analysis show the first moment gradient to be the most sensitive to errors in the estimation of the gradient, leading to a 16% error in the diffusivity. The minimum difference measured in the diffusivity is 20%, and thus even allowing for such an error, the results presented in Tables 4.1 and 4.2 would be significant..

No conclusions on the intraparticle void volume have been drawn, although fouling should act to decrease this value; the measurements made do not show a significant difference between the fresh and the fouled state. It is possible that a greater amount of

fouling may show up a significant intraparticle void volume change, but in these experiments, axial dispersion changes as a result of solids disruption of the bed structure may mask any effect of change in intraparticle void volume.

The axial dispersion value is dependant on the bed structure, so a comparison of the initial measurements of axial dispersion in Tables 4.1 and 4.2 is not appropriate. Even so, the sensitivity analysis shows that errors of 5 % in measurement have only a small effect on the axial dispersion, and the axial dispersion differences between the fresh and fouled columns shown in Tables 4.1 and 4.2 are significant. It is probably more appropriate to plot change in axial dispersion (fresh versus fouled column) rather than absolute axial dispersion, since the bed structure affects this value, and no two packed beds will have an identical structure. This would be a recommendation for future work.

Errors in the values of the second moment gradient and intercept have less effect on the diffusivity, axial dispersion and intraparticle void volume values calculated, although the same quality criterion was placed on the linear regression of the second moment as for the first moment, that is, $r^2 > 0.95$.

In conclusion, the method of moments is sensitive to errors and steps must be taken to control these. These include control of the measurements taken, and checks on the fit of the experimental data to the model as have been applied in this study. If this is undertaken, the results obtained are significant and meaningful, and the method is a useful tool for measuring the fouling at process scale.

6. CONCLUSIONS AND FUTURE WORK

6.1. Introduction

In the biotechnology industry, chromatography operations are critical to the economic success of a process. No other scaleable technique has been shown to provide the separation power required to achieve pure, homogeneous biological products. In the case of therapeutic products, regulatory authorities may require there to be at least one chromatography step in the process.

Inspite of the almost universal application of chromatography in biological processing, there remain design, operation and scale-up problems requiring attention. One of the problems for design and operation of a process is the fouling of chromatographic supports, leading to a degradation of the separation performance. Although the effect of fouling is well-known to practitioners of chromatography, there have been very few systematic studies of the effect of fouling by biological process streams on chromatography matrices. Column performance after fouling can be partially re-gained by cleaning, but harsh cleaning regimes will also have an impact on the column performance and lifetime. Consequently, the design of many processes incorporates steps specifically designed to remove contaminants in the process feed that are believed to be responsible for degrading the performance of the chromatography. This affects the cost and yield of the process if the steps are not required. Clearly there is an optimum amount of feed treatment that allows the product specification to be met, without the need for aggressive clean-in-place protocols of the chromatographic matrix.

The aim of this work was to provide a starting point for the systematic evaluation of the effect of fouling contaminants on the performance of separation, developing a methodology that could be used to determine the effects of fouling, as well as providing results that can be used to understand the likely consequences of a feed stream composition on a chromatography process. The following sections summarise the results, highlighting their contribution to the understanding of fouling of chromatographic matrices, and suggesting areas for future study.

6.2 Chromatography of Yeast Homogenate

The effect of particulate fouling on the performance of the chromatography step was demonstrated using the intracellular enzyme alcohol dehydrogenase, using the yield and purity of this enzyme as a marker of performance.

The work concentrated on the effect of particulates on the performance of the column, since particulates are considered to be a source of severe fouling for chromatography columns. It was found that for repeated loadings of small amounts of particulate containing feed, there was an improvement in the purification factors achieved, and this was related to the number of cycles of the column. After 10 cycles the best purification factors were achieved, and these were 5 times greater than for the fresh column. It was hypothesised that this effect was due to ion-exchange activity of yeast cell debris trapped in the column, as reported by Shaewitz et al (1989). For this to be the case, the particles would have to be sub-micron, and contribute very little to the total mass of the particulates in the process stream. When a similar mass of particulates was loaded as a single shot, the effect on the purification factors was not repeated, and instead, a reduction in separation performance was observed. This is evidence for two different mechanisms of fouling. In the first case, the case of ten repeated cycles, the column went through a regeneration stage after each fouling. In the case of a single loading, the column was exposed in a single cycle to the same equivalent mass of particulates.

Use of the fractionation diagram as an estimator of column performance was disappointing. The method would probably have worked better with a system in which higher purification factors could be achieved. The ADH separation suffered from low affinity of the enzyme for the matrix, and the resolution achieved in the fractionation diagram, using the 2 mL fractions, was not sufficient to show the effects of fouling. The untested power of the diagram is to be able to define the cuts at which the highest purity

at a given yield can be taken. In practice, it was found that the A_{280} trace was a better indication of the separation performance from a qualitative point of view.

This work gives similar information to the work reported by Tice et al. (1987), and Johansson and co-workers, (1984, 1985, 1986). In this work they looked at resolution as a measure of chromatographic performance, but the results are the effects of fouling and give no measure of the fouling mechanism. Since all such work will be system dependent, the applicability to other process schemes is limited.

6.3 Frontal Analysis

Like the chromatography, frontal analysis of the breakthrough of BSA for a series of columns could be shown to be affected by the treatment of the column. Unlike the chromatography, it was possible to make an analysis of the mechanisms of fouling.

Particulate concentration was shown to have an effect on the capacity of the column. For repeated cycles, the dynamic capacity increased with the number of cycles for the disc stack centrifuged feed stream, and to a lesser extent for the ultracentrifuged feed stream. The reason for this was hypothesised to be due to the same ion-exchange effect of cell debris as seen in the chromatography of ADH. Like the chromatography, this effect was masked or absent when loading of the column was as a single shot. The fouling mechanism is thus shown to be dependent on cycle number and degree of loading of solids.

It was observed that the fouling also caused a reduction in the effective diffusivity, D_e, of the matrix, as evidenced by tailing in the breakthrough curves. When fouling was very severe, breakthrough curves could still be reasonably approximated using a model where the effective diffusivity was the only varied parameter. This implies that fouling by particulates is largely occurring inside the beads of the matrix. If fouling was

occurring as a result of coating the external surface of the bead, a decrease in the fluid mass transfer coefficient , k_f, would have been expected, but this was not observed.

The charge carried by the cell debris is clearly important to the fouling process, indicating that the mechanism of fouling is at least partially caused by ionic interaction of cell debris with the matrix, and not simply physical pore blocking.

Frontal analysis was found to be a very quick and reproducible method for estimating the fouling effects. There are models in the literature (Chase, 1984; Horstmann and Chase, 1989) which can be used to fit the chromatographic transport parameters to determine the effect of foulants as described in this thesis. However, it must be remembered that the fouling mechanism is a time dependant process. Interaction of fouling species with other fouling species to cause a self-amplifying effect on the fouling is a possibility, and this cannot be tracked using this method. What is required is an on-line method. Spiking BSA into a fouling feed and then following it's breakthrough, or better still using the breakthrough of the product of interest in the feed, is a better experiment. However, this does require the ability to precisely determine the breakthrough of the solute of interest, and the assays to do this were not available, too variable or too time intensive to consider. Non-invasive analysis by infra-red spectrophotometry, or diode array spectrophotometry seems a promising way of analysing breakthrough, and would be an interesting path to pursue.

6.4 Pulse Response Analysis

The pulse response analysis using the method of moments was used to determine the effect of different levels of foulants on the transport parameters involved in the chromatographic process.

The value of the inclusion porosity for the gel tested did not change with the level of foulants used. However a decrease was observed for the effective diffusivity, De, and this was dependant on the amount of solids loaded, inferring fouling within the bead.

The effect of lipid foulants alone was not as great as the effect of particulates on the effective diffusivity, suggesting particulates actually had a greater access to the internal structure of the bead than lipids, possibly assisted by ionic interactions between debris and matrix.

Fouling of the bed by deposition in the column interstices was shown by an increase in the column axial dispersion. The increase in axial dispersion when a feed stream contained lipids and solids was greater than for feed streams containing either solids, or lipids on their own.

A column that was shown to be compressed during packing by a reduced void volume (compared to the other columns), was found to foul more severely. This is an important result, since inconsistencies in column packing could potentially have a large effect on the predicted column lifetime. Process scale columns can be difficult to pack, and the design of the column hardware that allows a consistent packing protocol could be critical to the economic success of a process.

Frontal analysis and pulse response analysis were shown to be very sensitive to foulants, and were able to detect the change in a column after CIP. This would allow CIP cycles to be investigated for their efficiency, and for their effect on the column lifetime. However, such experiments involve running hundreds of cycles, which was outside the scope of this work.

A drawback of the pulse response analysis is the extreme amount of time needed to get results. Each experiment had to be run under very controlled conditions and with great attention to detail. Nevertheless, the technique is very sensitive, requires no special equipment or analytical tools, and thus has great potential for evaluating the causes and mechanisms of fouling in chromatographic matrices.

6.5 Assay Results

Throughout this work, assay results have been disappointing. The methods discussed in Chapter 2, with the exception of the protein assay, require a great deal of time to perform, and are very variable. As the columns used were small, the amounts of foulants trapped were also small, and were generally undetectable as they fell within the variability of the assay. For this reason it has been impossible to define the absolute quantities of material fouling the column. A further problem was the system chosen. Whilst the process feed had the virtue of being a genuine process stream, it also caused a problem because of the consequent complexity and range of fouling species. A model process stream containing just lipid, or nucleic acid, or solid particulates would be a fruitful line of research to follow.

The use of microscopy to examine matrix post-fouling was considered, but the risk of introducing artefacts during sample preparation is great with most microscopic techniques. NMR spectroscopy, a non-invasive and sensitive method has been applied to studies of transport phenomena (Ilg et al, 1992), and this could be an interesting route to follow.

6.6 Hydrodynamics

The column hydrodynamics have not been studied to any great extent in this work. Transient pressure drops were observed when loading the process stream to the column, but after fouling, the pressure drop returned to the pre-fouling value. Permeability experiments showed there to be no evidence of pore blockage.

The fouling studies presented here used a single flowrate and temperature for fouling. It is very probable that fouling will be flowrate dependant, as has been shown for fouling of membrane filters (Blatt, 1971). Temperature is also important in biological process systems. Whilst viscosity increases with temperature, the fluidity of lipid and protein molecules decreases. Viscosity increase will cause a pressure drop increase, but in

filtration processes, it is known that filtration at 4 °C suffers less from fouling, more than offsetting the higher pressure drop. It is for this reason that materials such as blood serum are cold filtered (Todd, 1997).

Aguilera Soriano (1995) has developed models for the compression of chromatography matrices under different flow conditions, and this work would provide an excellent starting point for the effects of fouling on the hydrodynamics of packed beds.

NOMENCLATURE

A	Bed cross-sectional area	cm^2
A_0	A constant in the van Deemter equation	cm
B_0	A constant in the van Deemter equation	cm^2/s
C_0	A constant in the van Deemter equation	s
C_0	Inlet solute concentration	mg/mL
C	Solute concentration in mobile phase	mg/mL
c_i	Point concentrationof fluid inside particle	mg/mL
c*	Equilibrium fluid phase concentration per bed volume	mg/mL
D_{AB}	Diffusion coefficient in free solution	cm^2s^{-1}
D_e	Effective solid phase diffusion coeffcient	cm^2s^{-1}
D_m	Diffusion coefficient in free solution	cm^2s^{-1}
D_L	Convective axial dispersion coefficient	cm^2s^{-1}
d_p	Stationary phase particle diameter	cm
D_s	Solute diffusivity in stationary phase	cm^2s^{-1}
H	Height equivalent to a theoretical plate (HETP)	cm
k_f	liquid film mass transfer coefficient	ms^{-1}
K	Constant equal to Q_0/C_0	$mLmL^{-1}$
K_d	Dissociation constant	$mgmL^{-1}$
L	Bed length	cm
N	Number of theoretical plates	-
n	Freundlich constant	cm
q*	Equilibrium adsorbed phase concentration	$mgmL^{-1}$
q_i	Point concentration of solute in adsorbent	$mgmL^{-1}$
q_m	Maximum adsorbent capacity	$mgmL^{-1}$
Q	Solute concentration in solid phase	$mgmL^{-1}$
Q	Volumetric flowrate	cm^3s^{-1}
Q_0	Concentration of solute on adsorbent in equilibrium with feed	$mgmL^{-1}$
R	Radius of particle	cm
r	Radial distance coordinate for particle	cm

R_p	Radius of particle	cm
t_R	Retention time	s
u	Interstitial fluid velocity, based on εA	cms^{-1}
V	Bed volume	cm^3
V_d	Dead volume of chromatography system	cm^3
V_R	Retention volume	cm^3
X	Dimensionless solute concentration in liquid phase, C/C_0	-
X_i	Interfacial value of X at solid-liquid boundary	-
Y	Dimensionless solute concentration in solid phase, Q/Q_0	-
Y_i	Interfacial value of Y at solid-liquid boundary	-

Greek Symbols

β	Parameter equal to 5/Bi	-
γ	Obstruction factor to diffusion in the interparticle space	-
γ_s	Obstruction factor to diffusion in the stationary phase	-
ε	Interparticle void fraction in a bed	-
ε_p	Intraparticle inclusion porosity of a solute	-
λ	Flow-geometry dependent constant	-
μ1	First absolute moment	s
μ'2	Second central moment	s2
σ_L	Peak standard deviation in column length units	cm
σ_V	Peak standard deviation in volume	cm3
σ_t	Peak standard deviation in time	cm
ψ	Tortuosity constant	-

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