

## LARGE SCALE PROTEIN SEPARATIONS: ENGINEERING ASPECTS OF CHROMATOGRAPHY

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### ABSTRACT

The engineering considerations common to large scale chromatographic purification of proteins are reviewed. A discussion of the industrial chromatography fundamentals is followed by aspects which affect the scale of separation. The separation column geometry, the effect of the main operational parameters on separation performance, and the physical characteristics of column packing are treated. Throughout, the emphasis is on ion exchange and size exclusion techniques which together constitute the major portion of commercial chromatographic protein purifications. In all cases, the state of current technology is examined and areas in need of further development are noted.

The physico-chemical advances now underway in chromatographic separation of biopolymers would ensure a substantially enhanced role for these techniques in industrial production of products of new biotechnology.

### KEYWORDS

Chromatography, protein purification, ion exchange, gel filtration.

### INTRODUCTION

Biologically active proteins - enzymes, hormones, antibodies and biopharmaceuticals - are in continually increasing commercial demand for industrial catalysis, diagnostics and medical therapy. Typically, a product is isolated and purified from a complex mixture containing solids, other proteins, carbohydrates, lipids and other chemicals. Some examples of such mixtures are fermentation broths of whole or disrupted recombinant microorganisms, tissue culture and body fluids, for example, blood. The separation protocol requires a sequence of several distinct unit operations (e.g., solid-liquid separations, cell disruption, liquid-liquid extraction, ultrafiltration, salt or alcohol fractionations), and it usually leads to an often usable, but still somewhat crude, protein fraction with the desired biological activity. For some products, especially the injectable biotherapeutic agents, further purification is necessary. It is accomplished by one or more

"polishing" steps - usually some form of chromatography. A few examples of injectable protein products which rely on chromatographic process to attain the necessary purity are given in Table 1.

**TABLE 1. SOME PROTEIN PRODUCTS WHICH REQUIRE CHROMATOGRAPHY PROCESSING**

• Vaccines (viral and bacterial)	Immunization
• Epidermal growth factor (EGF)	Treatment of burns, corneal wounds and ulcers
• Human growth hormone (hGH)	Pituitary dwarfism
• Superoxide dismutase (SOD)	Heart attack, stroke, kidney disease
• Interleukin-2 and other lymphokines	Anticancer agents
• Interferons	Certain cancers
• Insulin	Diabetes
• Factor VIII	Hemophilia
• Tissue plasminogen activator (tPA)	Control of heart attack while in progress

Although relatively new to large scale protein purification, industrial chromatography has long been used in separations of low molecular weight, non-protein substances (Wankat, 1986), e.g. in water purification by ion exchange and the purification of high fructose corn syrup. The nature of proteins, however, poses some unique process engineering problems in chromatography. Some problems of design and scale-up are examined in this review with a stocktaking of areas where deficiencies need to be tackled. The detailed techniques for purification of specific bioactive proteins are not treated; many references to these techniques have been cited by Sene and Boschetti (1988), among others.

#### Chromatography

Chromatographic separations most commonly employ cylindrical columns packed with a particulate chromatographic medium (or gel, matrix, adsorbent, support, etc.). Separation depends on physicochemical interactions between the dissolved components of a mixture and the stationary phase - the solid medium or a liquid supported on it. The mixture is applied to the column as a small volume of solution. The column is then washed (or eluted, or developed) with a solvent which is the mobile phase (or eluent). The composition of the mobile phase may be held constant in time, as in isocratic elution; or more often, the composition may be

varied either linearly (linear gradient elution) or in steps. Different components of the mixture come off the column at different times, depending on how strongly a particular component interacts with the stationary phase, and on the conditions of elution (solvent composition and flowrate, pH, temperature). Hence, the components are separated.

The nature of interaction between the components of the mixture and the column matrix is one way of classifying (Lesins and Ruckenstein, 1988) the chromatographic techniques: ion exchange, gel filtration, hydrophobic interaction chromatography (Roettger and Ladisch, 1989), affinity chromatography, and others. For protein purifications, ion exchange chromatography is by far the most common followed by gel filtration; these are the techniques emphasized in this review. The very powerful technique of affinity chromatography, which depends on biospecific interactions (e.g., enzyme-substrate, antibody-antigen) between the protein in solution and its interacting protein immobilized on the support, is similar to ion exchange in terms of operational and engineering concerns. The affinity media are more expensive however, due to added costs of immobilized protein and the immobilization process. On large scale, the quantity of column medium requirements make affinity chromatography a particularly capital intensive technique relative to other, already expensive, chromatography procedures. Further, affinity media are less robust since the immobilized protein may be easily denatured. Cleaning and operational regimes must take due account of low medium stability to avoid frequent medium replacement. Despite limitations, the affinity technique is gradually coming into industrial use; it is capable of producing a very pure product from a dilute mixture in a single step. Product recovery from an affinity column may be complicated by the tight binding of the required protein to the support.

#### Scale of Separation

The scale of separation depends on the quantity of the material to be purified. The loading capacity of the ion exchange matrix, i.e., the quantity of protein that may be adsorbed, governs the size of the separation column. Loadings of 50-100 kg protein per m<sup>3</sup> of ion exchanger are possible. The ionic strength of protein solution during the loading phase must be low, therefore pretreatment (desalting) of mixture may be necessary. The loading capacity of ion exchange media in manufacturers' catalogues given in terms of density of charged groups per unit weight of the medium, is not necessarily an indicator of the protein binding capacity. Only a small proportion of this capacity may be actually accessible to the protein depending on the hydrodynamic size of the protein, and the support pore diameter. Loading capacity varies with ionic strength and pH of solution because these parameters affect the dimensions of the hydrophillic, porous support media.

These swelling characteristics of the media are structure dependent: more rigid, highly cross linked media show less variation in volume; however, they also, usually have lower loading capacities.

At present, the loading capacity of a medium for protein has to be determined empirically. Methods for predicting the loading capacity on the basis of support structure, support charge density, the hydrodynamic size of the protein and its charge characteristics, need to be developed. Ideally, the loading capacity should be high, elution should be easy under mild conditions and the required resolution should be achievable. Laboratory experiments are indispensable to packing selection; resolving power, in particular, can be demonstrated only through experiments.

Processes requiring large variations in ionic strength or pH of the eluent, should be conducted in media which are dimensionally stable: expansion and contraction of an initially well-packed column introduces flaws into the packed section and the performance deteriorates. More rigid, but highly porous media which have good geometric stability (Bite et al. 1987) are now coming into use.

#### Dimensions of Column

Once the loading capacity of the selected chromatography matrix is known, the dimensions of the column need to be determined. In ion exchange chromatography, an adsorbed protein displaced by alteration of elution conditions undergoes a change in its surface charge characteristics. The protein then moves down the column with little further interaction (the "on-off" mode of operation). Long columns have no major impact on resolution. Therefore, ion exchange columns tend to be short, up to 1m in height. Columns are scaled up in diameter to accommodate the quantity of matrix needed for the separation, the height is held constant. For good resolution only a fraction (2-5%) of the total loading capacity is used in sample application. The necessity to uniformly distribute the flow over the entire cross section of the column may place technical limits on diameter. Thus, larger separations need multiple columns running in parallel.

A chromatography separation unit is made up of several components. Apart from the chromatographic columns, there are tubings, tanks, gradient mixers, valves, pumps, in-line filters, flow and sample detectors, among other possible pieces. Careful design of this hardware, and its arrangement in relation to the chromatographic column is essential to successful separation and purification. The separation achieved in the column should not be lost due to mixing elsewhere in the system (Johansson et al., 1988). A major preoccupation with scale-up is to ensure that the laboratory scale separation efficiency is retained upon scale-up. A good scale-up is achieved if the HETP (Height Equivalent of a Theoretical Plate) of the

total full scale system (i.e., column, tubing, etc.) equals or betters the HETP of the laboratory system for a well defined mixture (Johansson et al., 1988). Regular evaluation of HETP of a process column is also an index of its continued effective performance.

The number of theoretical plates (or ideal equilibrium stages) available for the separation of a particular protein  $i$ , can be calculated using

$$N_i = 16 \left( \frac{t_i}{W_i} \right)^2 \quad (1)$$

where  $t_i$  is the retention time and  $W_i$  the basal peak width for the protein (Figure 1). For a column of length  $L$ , the separation efficiency parameter, HETP, is

$$\text{HETP} = \frac{L}{N_i} \quad (2)$$

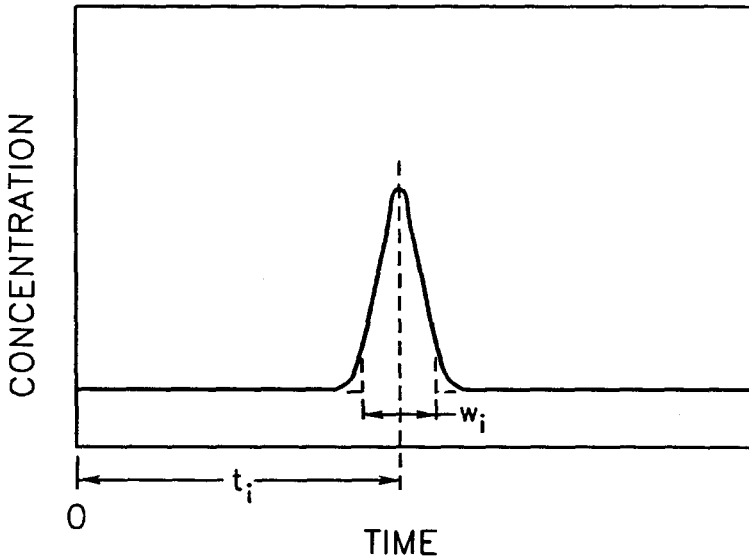


FIGURE 1. Concentration-Time elution profile for a component  $i$ : retention time and the basal peak width.

For a given protein, and other operating conditions, the HETP depends on the flow rate through the column. Generally, the lower the flow rate, the smaller the HETP. However, low flow rates limit throughput, and some compromise between throughput and resolution needs to be established. Quantitative optimization schemes should aid

process development here. The fundamentals of chromatographic efficiency parameters and their measurement have been discussed recently (Mori, 1988).

#### Physical Characteristics of Media

Ideally, best resolution is attained with highly uniform packing media with small bead diameter. For example, in HPLC (High Performance Liquid Chromatography) [which is otherwise the same as any other form of chromatography] the typically good resolution is due to very fine media ( $\leq 10 \mu\text{m}$  diameter). Large pressure losses, vanishingly low flow rates and the possibility of blockage mitigate the use of very small particles in large-scale industrial ion exchange chromatography of proteins. Usually, the particle size used is larger ( $> 40 \mu\text{m}$ ) - sometimes sufficiently so that a small amount of cell debris can be satisfactorily handled in initial separations in the column.

Selection of the optimum particle size remains a matter of empirical judgement. Rules, based on adsorption-desorption kinetics, mass transfer around and into the particle, and pressure drop considerations, are needed to optimise matrix dimensions for a given resolution. Also, while particles with an extremely narrow size distribution improve packing characteristics, they tend to be more expensive. How wide a particle size distribution is tolerable without significant deleterious effect on resolution, remains to be addressed (Hedman et al., 1988). A similar question may be raised on pore size distribution.

The pressure drop ( $\Delta P$ ) through a packed column of spherical, incompressible particles with diameter  $d_p$ , and the effluent flow velocity ( $U_L$ ) through the column are related according to Kozeny-Carman equation.

$$U_L = \frac{\phi d_p^2 \Delta P}{L \mu_L} \cdot \frac{\epsilon_s^3}{(1 - \epsilon_s)^2} \quad (3)$$

where  $\phi$  is a proportionality constant. Equation (3) applies to laminar flow as would normally occur in a chromatography column. The equation indicates the effects of bed voidage ( $\epsilon_s$ ), pressure drop ( $\Delta P$ ), bed height ( $L$ ), effluent viscosity ( $\mu_L$ ) and the particle size ( $d_p$ ) on superficial velocity of the eluting medium. In compressible beds the linear relationship between flow and pressure drop breaks down (Janson and Dunnill, 1974) and the flow drops off rapidly beyond a certain pressure drop (Figure 2). A hysteresis effect is often observed with compressible packings - the bed does not fully recover the flow rate even though the pressure drop which initially caused the compression is released (Figure 2).

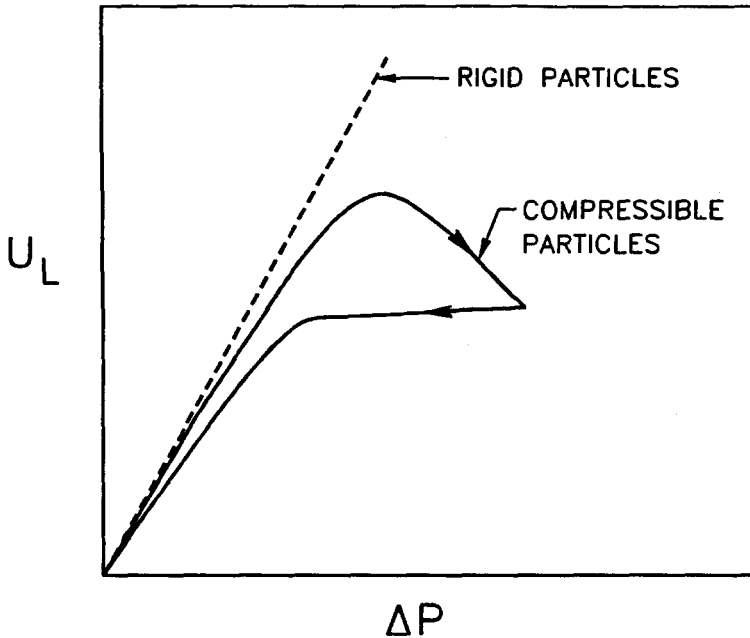


FIGURE 2. Listed flow velocity through the bed vs. pressure drop across the bed for rigid and compressible particles.

Apart from the more recent use of incompressible packings, several techniques have been available to get around the bed compression problem encountered with softer packings (Janson and Dunnill, 1974). Thus, longer columns with perforated subdividing baffles have been used as well as columns containing baffles located parallel to the column axis. The most commonly encountered arrangement, however, is that of stacked column - a column consisting of several short, fat columns, each with its own distributor and collector, connected in series through short, narrow bore tubes. The effluent from the top column section is fed to the one below and so on. This arrangement has the advantage that the slower moving products may be withdrawn from intermediate stages in the column (Janson and Dunnill, 1974). The multiplicity of flow collection and redistribution steps necessitate careful engineering of hardware to minimize mixing during these steps. Stacked columns are necessary usually only in gel filtration chromatography.

The operational costs of a chromatographic column are determined in part by the pressure drop required for a given flow rate. The power ( $P_o$ ) dissipated in overcoming the pressure drop ( $\Delta P$ ) at a superficial flow velocity ( $U_L$ ) through a column of cross section area  $A$  is:

$$P_o = U_L A \Delta P \quad (4)$$

Pressure losses in the pipework, valves, etc. are easily calculated using techniques given in chemical engineering literature.

While most chromatography is performed in cylindrical beds, schemes which confine the support material between two concentric porous cylinders with radial flow through the packing have also been proposed (Saxena and Dunn, 1989). The radial configuration may have certain advantages over the conventional column design.

Another attractive area for development is the possibility of operating chromatography as a continuous processing step. Some of the earlier proposals on this form of operation have been reviewed by Wankat (1986) in a general context; a more recent option is due to Siegell et al. (1986).

#### Gel Filtration or Size Exclusion Chromatography (SEC)

Gel filtration or size exclusion chromatography is probably the most mild protein purification technique since the protein-support interaction is largely of physical-hydrodynamic nature. The separation is based on the hydrodynamic size of the protein; the size or radius of the protein molecule depends on the molecular weight according to a power law relationship, the radius varies with environmental conditions (solvent, ionic strength, pH).

SEC is one of the more predictable chromatographic techniques and it is possible to calculate selectivity and separation range from the pore size distribution of a support; of course, the hydrodynamic characteristics of the proteins of interest need to be known. These may be determined, for example, by comparing the chromatogram of the mixture on a well characterized column with the chromatogram of a mixture of known hydrodynamic characteristics on the same column. Effects of pore size, size distribution, support morphology and characterization of morphology, have been discussed by Hagel (1988). Despite relative predictability, experimentation on small-scale is useful to scale-up of SEC processes. Much of this type of work is done on laboratory scale prepacked packed columns because SEC is somewhat more susceptible to packing variation. Small prepacked SEC columns have been reviewed (Makino and Hatano, 1988).

In SEC columns the resolution is usually directly related to the square root of the column length; however, Montelaro (1988) has cited work in which longer columns led to poorer resolution. Similarly, there is some evidence that a slight degree of packing compression may improve resolution by virtue of decreased voidage. Use of rigid, silicon based packings in aqueous SEC has been examined by Unger and Kinkel (1988). A good SEC packing should minimize non-physical, secondary interactions (e.g., ionic interactions, hydrophobic effects, adsorption, etc.)

between the support and the protein. In such columns, the height equivalent of a theoretical plate is usually of the order of three support particle diameters or better.

#### Other Design Considerations

Apart from the physicochemical and hydrodynamic aspects of separation, the chromatography process engineering is influenced by the requirements of Good Manufacturing Practice. Features for in-place cleaning, sterilization, depyrogenization, and containment of dangerous material should be engineered into the process at the design stage. The process must be capable of validation through all stages of operation. It is worth noting that the high degree of process instrumentation commonly employed on the developmental stages of a chromatography process is often unnecessary on the production scale.

#### CONCLUSION

Some of the major engineering considerations encountered in design and scale-up of chromatography processes for protein purification have been reviewed. The current status of process technology has been examined, and areas in particular need of further research have been pointed out.

#### NOMENCLATURE

A	Cross sectional area of bed ( $m^2$ )
$d_p$	Support particle diameter (m)
HETP	Height equivalent of a theoretical plate (m)
L	Column length (m)
$N_i$	Number of theoretical plates for $i$ th component (-)
$P_o$	Power (W)
$\Delta P$	Pressure drop through the column (Pa)
SEC	Size exclusion chromatography
$t_i$	Retention time of $i$ th component (s)
$U_L$	Superficial liquid velocity ( $m \cdot s^{-1}$ )
$W_i$	Basal peak width for $i$ th component (s)
$\epsilon_s$	Bed voidage (-)
$\mu_L$	Viscosity of solution ( $Pa \cdot s$ )
$\phi$	Proportionality constant (-)

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