Handbook of Process Chromatography
A Guide to Optimization, Scale-up and Validation

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High-resolution techniques such as HIC, [EC, immobilized metal affinity chromatography, RPC or AC] may be found at this stage, preferentially selected to be complementary to the technique used for capture.

The final polishing step, when required, may employ media with particle sizes ranging from 15 to 100 μm. This step is often required to provide the requisite purity for therapeutics by removing any aggregates that form during processing or any trace impurities (e.g., product variants) that have copurified with the product. GP and RPC are the most common polishing steps, but other techniques such as HIC and ion exchange can also be used. In some cases, this final chromatography step is also used to transfer product into a formulation solution.

In the capture, intermediate and polishing scenario described above, the particle size range included 100 μm for all cases. In some firms, one particle size is used for all chromatography steps, with excellent results. However, as the requirement for resolution increases, the need for smaller particle size media generally increases. Fortunately, since smaller sample volumes are to be processed at the later stages, the cost of media and equipment may still be acceptable.

Once the basic design of the purification process has been decided, the optimization of the individual purification steps, and finally the entire process, may start.

OPTIMIZATION OF THE PURIFICATION STEP

Optimization goals

Optimization of the purification step includes the arrangement of the order of techniques used and optimization of the running conditions to yield a product of sufficient purity, and dimensioning of the substeps to cope with the processing volumes of earlier steps. The building blocks commonly used for chromatography are based upon separation by size (GE, charge [EC], hydrophobicity [HIC], lipophilicity [RPC], and affinity [AC]. The discriminating power increases in the order GE < IECE < HIC, RPC < AC. AC is traditionally rarely seen in the early stages of the purification scheme where the liability of fouling is high, which leads to a short lifetime of the relatively expensive affinity media, and also due to the fact that capacity of affinity media traditionally has been lower than, for example, ion exchangers. However, this trend has recently been broken, and successful initial purification with Protein A STREAMLINE has been demonstrated. Of course, using a media of high discriminating power early in the purification scheme will result in a process of fewer steps, which is advantageous. The important objectives for optimization are high purity, recovery, and capacity, leading to high productivity.

Purity

To achieve high purity, the most important parameter of the chromatographic step is selectivity. A high-selectivity chromatography medium is employed to give a high resolution between the product and closely eluting impurities. Since resolution is such a fundamental measure of degree of separation, the meaning of resolution for some different cases, and the effect on purity and/or yield, is illustrated in Figure 5.5. It is seen that a purity of 99% at 95% yield requires a resolution of 1.0 between the product and an impurity composing 10% of the start material, provided that the peaks are symmetrical (this is seldom the case at high loads – cf. Chapter 12). The resolution factor 1.5 is used as a reference value here since it corresponds, in practice, to a complete separation of peaks [i.e., a purity of 100% at 99% yield of a 50/50 mixture] and provides a certain safety margin in case of variation of the composition of the feedstream and sample volume. However, it must be noted that a resolution factor of 1.5 is no measure of purity – the purity of the fraction collected needs to be confirmed by complementary assays (see Chapter 9). The resolution factor needed is related to the purity and yield required and the type of impurities that are present. The goal for optimization of each individual step is to achieve sufficient resolution of the product from impurities at the working conditions. What counts is the total result of the process, which means that the requirement for resolution in

Figure 5.5 Recovery as a function of final purity at various chromatographic resolution factors for a sample containing 10% impurities.
different steps may differ considerably and also that the separation strategy may be dictated by the relative ease of achieving high resolution using different techniques, i.e. a resolution factor of 1.5 is clearly not the ultimate goal for every step. Resolution is achieved by a large separation factor (peak-to-peak distance) and/or a low dispersion factor (peak width), as shown in Figure 5.6. The peak-to-peak distance is affected by the inherent selectivity of the chromatography medium, the slope of the gradient, and the column length. The dispersion factor is affected by the flow rate, particle size of the chromatography medium, retention time, and the diffusivity of the solute. Extra-column effects (e.g., large dead volumes, mixing chambers, etc.) will also cause broadening of zones.

Recovery
Recovery of active product is the prime goal of the separation. The recovery is dependent upon the resolution attained and the requirement for purity. In some cases, material may be irreversibly lost on the chromatography medium or denatured, leading to a low recovery of active material even though the purity is high and the capacity is high. Thus, control of recovery during optimization is essential. It should also be noted that loss of active product may be due to long hold-up times in the system or the bed, and this parameter may therefore need to be controlled, especially when system configuration will vary.

Capacity
The capacity of the chromatography medium for the target solute may be estimated by determination of the breakthrough capacity (see Chapter 12). This will be the maximum applicable amount of material the chromatography medium can adsorb before material leaks through the bed under the running conditions used, e.g., pH, ionic strength, and concentration of interfering substances. The capacity per unit bed volume is limited by the surface area of the sorbent that is sterically available for the solute and the ligand concentration.

The association constant will affect the degree of utilization of the capacity (i.e., a low association constant in affinity chromatography will result in low capacity, cf. Figure 5.24). This effect may also result from an inappropriate choice of adsorptive buffers in IEC, HIC, or RPC, which causes the solute to be retarded and not fully retained.

The operational level of capacity utilization will be dictated by the amount and type of impurities that will bind more strongly to the chromatography medium than the target solute. Therefore, binding conditions which will favor a higher proportion of adsorbed target solute as compared to impurities may be optimal, although this may correspond to lower degree of utilization if the target solute was to be adsorbed from a pure solution. Thus, using a realistic feed for the optimization is very important.

Material may be lost at very high loadings, and it is therefore recommended to check the recovery of active material as a function of loading. Also, since peak shape generally becomes more asymmetrical at high loads, i.e., showing tailing (see Chapter 12), which will reduce the resolution, it may be advantageous to operate at a slightly lower loading. However, sample displacement effects may sometimes sharpen the solute zones. Symmetrical peaks were found at column loads up to 80% of the maximum capacity.

In summary, the preferred way to determine the capacity is to use a representative feed (including impurities) to obtain the 5% breakthrough level, check the recovery of active product, and calculate the material balance to assure quantitative recovery at the selected conditions. The variability of feed properties need also to be taken into account.

Productivity
The throughput is equal to the amount of purified product per unit time and the productivity is the throughput per volume of chromatography medium (i.e., gram per liter and hour). The amount of purified product per unit bed volume is given by utilized capacity times relative recovery.

![Figure 5.6](image-url)
Gel filtration

The differences in size between molecules is the basic principle utilized for separation in gel filtration, also known as size exclusion chromatography (SEC). The GF mechanism was observed for the first time in the early 1960s. The technique rapidly became popular due to the ease of operation and the high resolution it provides. The most important parameters for optimization of preparative chromatography are summarized in Table 5.5.

Table 5.5: Important factors to consider in optimizing different preparative chromatographic steps

<table>
<thead>
<tr>
<th>Molecular characteristic</th>
<th>Chromatographic technique</th>
<th>Features</th>
<th>Limitations</th>
<th>Important factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>GF (Size exclusion chromatography, SEC)</td>
<td>Insensitive to buffer composition, Uncomplicated</td>
<td>Dilutes the sample, Limited resolution and sample volume</td>
<td>Pore size and pore volume of chromatography, Bed height, Flow rate</td>
</tr>
<tr>
<td>Charge</td>
<td>IEC</td>
<td>Concentrates sample, High sample capacity</td>
<td>Low salt for adsorption, Sample eluted in high salt</td>
<td>pH, Gradient slope, Sample load</td>
</tr>
<tr>
<td>Lipophilicity</td>
<td>RPC</td>
<td>High resolvability, especially for small solutes, Concentrates sample</td>
<td>May denature the sample, Medium sample capacity, Organic solvents needed</td>
<td>Media backbone, Gradient slope of modifier, Sample load</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>HIC</td>
<td>High resolvability, Sample eluted in low salt, Concentrates sample</td>
<td>High salt for adsorption, Sample solubility</td>
<td>Hydrophobic ligand type, Choice of salt concentration, Gradient slope</td>
</tr>
<tr>
<td>Biospecific sites</td>
<td>AC</td>
<td>High discrimination factor, Step elution, Concentrates sample</td>
<td>Preparation of ligand, Medium sample capacity</td>
<td>Association constant, Sample residence time, Elution conditions</td>
</tr>
</tbody>
</table>

It is tempting to optimize this relationship in different steps. For example, (a) select a chromatography medium that maximizes the capacity per bed volume, (b) maximize the resolution per cycle time, and (c) choose conditions that maximize the productivity per cycle time. However, maximizing the productivity does not necessarily imply that the overall process will be more efficient. The key is to find the right balance between resolution and productivity that best suits the specific requirements of the purification process.
[often amino] groups attached to the chromatography matrix. This type of chromatography medium will retain polynucleotides (due to the negatively charged phosphate groups) and proteins and peptides at a pH above the isoelectric point where these solutes are negatively charged. If the charged groups on the ion exchanger are titratable, e.g. secondary or tertiary amines as DEAE (diethylaminoethyl), the ion exchanger is said to be 'weak' whereas if the charge of the ion exchanger is independent of pH over the range commonly used, as QAE [quaternary aminoethyl], the exchanger is said to be 'strong'. Thus, the ion exchanger being classified as weak or strong has nothing to do with the strength of the interaction. The other type of ion exchangers are cation exchangers for which the negative charge will attract proteins and peptides below their isoelectric point. Common charged groups for cation exchangers are CM [carboxymethyl], giving a weak ion exchanger, and SP [sulphopropyl], giving a strong ion exchanger. To enhance the availability of the ion exchange ligand for larger macromolecules the group is often attached to the matrix through a spacer arm.

**Optimization of resolution in IEC**

The discrimination power of different anionic or cationic charged ligands is generally not very different and therefore the most powerful way to alter the selectivity is to change the charge of the solute, i.e. by varying the pH. This is illustrated in Figures 5.11 and 5.12. As expected, the retention time increases with increasing pH (and negative charge) for proteins above the isoelectric points being chromatographed on an anion exchanger. The opposite is true for proteins below their isoelectric point chromatographed on a cation exchanger. However, there is no one-to-one relationship between the net charge and the retention time in IEC of proteins due to the complex distribution of charges over the molecular surface (see Chapter 11). The variation of charge with pH is most powerfully exploited in isoelectric focusing or chromatofocussing [e.g. see Janson and Ryden 67].

Changing the speed with which the mobile phase composition is changed (i.e. the gradient) will result in a change of the spacing of solutes and thus the resolution as shown in Figure 5.13. The gain in peak-to-peak distance is larger than the loss in zone broadening, which will result in a net gain in resolution. This is one of the most common parameters used for regulating the separation in adsorption chromatography, and best results are expected to be found in the retention range of 5–20 column volumes as evident from Figure 5.13 [cf. Chapter 12]. The actual chromatogram, reproduced in Figure 5.14, shows that peaks 2 and 3 are not separated. One way to improve a separation is to decrease the gradient slope, i.e. increase the number of column volumes in the gradient and/or reduce the gradient range (i.e. if peak 1 is uninteresting.

Another way will be to decrease the sample load (cf. Figures 5.13c and d). In this case the best solution would probably be to vary the pH (provided that the pH was not optimized for this separation in the first place), or select another chromatography medium (e.g. test a DEAE ion exchanger).

The third important parameter is the sample load. A large sample load will occupy a large zone in the bed leading to a broader peak. Under overload conditions, the elution volume will decrease with load and the load will also produce pronounced tailing of the peak leading to contamination of successive peaks (see Chapter 12). At very high loads another phenomenon called sample displacement will occur, which may sharpen the solute zones but overlap between zones may reduce yield and/or purity. Working at overload conditions (and sacrificing yield) is sometimes suggested in process chromatography in order to maximize productivity. However, overload effects may be avoided by restricting the load to approximately one-third of the maximum load (illustrating the importance of using chromatography media of high capacity for the one might test a gradient from 45% B to 85% B in 10 column volumes).

![Figure 5.11 Selectivity change as a function of pH for an anion exchanger. Separation of chymotrypsinogen A (isoelectric point 9.0), cytochrome c (9.4), lysozyme (11.0), transferrin (6.0), ovalbumin (4.7), and β-lactoglobulin (5.1) on Mono Q (a quaternary ion exchanger). (Courtesy of A. Ljungdahl, Pharmacia Biotech AB.)](image-url)
product. Under these conditions the concentration of solutes adsorbed will vary approximately linearly with the concentration of solutes in the mobile phase (working under these conditions is called linear elution chromatography, cf. Chapter 12) and peaks will be fairly symmetrical, as shown by Figure 5.14 where 12 mg of protein per 1 ml of sorbent is loaded. Still, the zone broadening will decrease with load, leading to increased resolution as illustrated in Figure 5.15. This figure also shows the influence from the flow rate, and as found for Gf, the relative influence from zone broadening due to high flow rate is low when running at high sample loads.

Thus, optimizing IEC involves, first, choosing pH to achieve the most favorable selectivity [getting the solute of interest first in the gradient reduces the risk for contamination but increases the risk for the solute being displaced by more strongly retained solutes]. The second parameter to optimize is the salt gradient of the mobile phase, which should be within the range of 5–20 column volumes to get maximum resolution. Thirdly, utilize this resolution to increase the sample load to the resolution limit that is acceptable with respect to purity, yield, and robustness. The interplay between sample load, flow rate, and gradient volume is illustrated in Figure 5.16. Once again, it is seen that optimization should be focused on increasing the gradient volume (i.e., decreasing the gradient slope, percent B per column volume), while keeping the flow rate at a reasonably high level. The gradient time is regulated by the flow rate (i.e., number of column volumes per unit time).

It is assumed that the residence time of the sample, i.e., the time allowed for the sample to equilibrate with the sorbent, is sufficient for quantitative binding. The required residence time is a function of solute pore diffusivity, solvent viscosity, and particle size, and is regulated by the eluent velocity during sample load.

Gradient elution may sometimes not be feasible for large-scale processes. The optimized gradient conditions need, in this case, to be transferred to a series of steps to first elute less retained solutes, and then elute the product, and finally desorbing all tightly bound solutes in a wash step. The resolution cannot be expected to be as good in a step elution as in a gradient run but may be sufficient to yield a product of required purity.

**Productivity in IEC**

The productivity, expressed as amount product per unit time and unit chromatography medium, is determined by the breakthrough capacity of the ion exchanger, $Q_B$, the recovery of active product, and the cycle
time. The operational capacity of the chromatography medium for the solute of interest in the sample mixture, i.e., the breakthrough capacity, is determined by frontal analysis as described in Chapter 12.

One needs to distinguish between two different situations where ion exchange is employed. One being in the early purification stage where capacity is of major concern and purity is of less concern. In this case, applying sample close to the maximum load is possible, depending upon feedstream variability. The sample volume and concentration may be very large since all product is adsorbed, unless the solution contains a high concentration of competing ionic substances (e.g., has a high ionic strength). As a practical rule it is recommended to keep total sample loading below 80% of the maximum capacity of the ion exchanger. However, at high concentrations the system is operated in the non-linear region of the isotherm, and severe tailing of the peaks will be observed, something that may decrease the purity and/or yield of product (see Chapter 12). The risk of losing material due to aggregation or precipitation at high concentrations must also be considered.

The other situation occurs when quantitative recovery and high purity are the objectives. In this case, the load may be limited to 80% of the capacity of the chromatography medium, as shown in Figures 5.13 and 5.14. The productivity may, at this load, be calculated to 1–25 g protein per liter of chromatography medium an hour, depending upon the difficulty of the separation (e.g., peak 1 or peak 2 in Figure 5.14).

After the breakthrough capacity has been determined for the actual feed and eluent conditions it is wise to apply the intended load, and check the purity, yield and activity of the collected fraction.

Reversed-phase chromatography

In RPC, hydrophobic substances dissolved in a polar solvent are separated due to their preferential interaction with non-polar ligands attached to a chromatography medium. The liquid phase is composed of an aqueous buffer containing a water-soluble organic modifier, and this modifier will also constitute the liquid interface at the non-polar ligands.

The model presently proposed is a solvophobic model where the solute is forced into the stationary phase due to the strong mutual interaction of the molecules in the mobile phase (thus 'excluding' the solute from
the mobile phase). However, this model has been challenged by one with a more direct surface-to-surface interaction between the solute and the chromatography medium, and the dominating mechanism in RPC is probably different for small organic molecules, polar compounds, and macromolecules (see Chapter 12).

The first RPC separations appeared in the late 1940s when polar solutes were separated on chemically modified silica gels. The use of RPC was applied to the purification of polypeptides in the late 1970s and has since then achieved considerable interest due to the high resolving power of the technique. For biomolecules, RPC has been applied primarily for separation of peptides, including large-scale preparative use. The high surface coverage of the stationary phase in RPC will cause strong interactions with the solute. This may cause disruption of the tertiary structure of proteins which leads to denaturation and loss of activity. Therefore, the application of RPC for preparative protein purification is generally limited to stable solutes or substances that may be renatured. The use of organic modifiers in the solvent at levels of up to 80% will also put restrictions on the applicability to large purifications (e.g., explosion-proof equipment is needed and costs for solvent may add significantly to the production cost).

**Reversed phase media**

Reversed phase media are composed of a base matrix to which or ligands, commonly n-alkyl chains, are attached. In some cases, the matrix is sufficiently non-polar to provide the lipophilic environment required for interactions, and no substitution is made. The base matrix used include silica, zirconium oxide, polystyrene-divinylbenzene other types of organic polymers. It is generally found that the type of matrix used will influence the separation, probably due to the influence of surface interactions (see Chapter 12). Whereas silica has been successfully used as a base matrix for analytical RPC, the lipophilic surface or coating will attract organic modifier molecules that will form the adsorbed liquid phase. Different ligand coating silica are used, and butyl, octyl, or octadecyl groups (denote C₈, and C₁₈, respectively) are common. For larger molecules, the ligand density and properties of the matrix influence the retention, primarily governed by the ligand density and properties of the matrix. The shorter ligands are commonly used for protein separation and the longer for peptide separation.

**Optimization of resolution in RPC**

Since the retention in RPC is due to surface interactions between solute and the chromatography medium, the premier action for aiding selectivity is to screen different media. The choice of ligand is from the following prerequisites: the interaction should not be so strong as to denature the solute (meaning that shorter lengths are used for example, proteins), and the interactions should not be too strong to avoid extreme pl conditions. It may also be noted that the ligand matrix may affect the separation due to the close contact with the surface. Pearson et al. found in an evaluation of RPC of protein: the most determining factor for resolution was the type of silica. The empirical influence of the type of chromatography matrix is treated in Figure 5.17, where the separation of a synthetic peptide mixture on two silica-based materials and one polymeric material is shown.
A rational design of a purification processes is based upon experience and/or practical implications of chromatography theory. Understanding the relationships governing the separation is, of course, also of use when trouble-shooting the process.

Valuable insights into which parameters are the important ones and to what degree these parameters will influence the separation can be gained from varying the values of these parameters and studying the theoretical results obtained. This type of calculation is facilitated by the use of software routines, and some selected applications that may be found useful for elucidating the effects of various parameters in liquid chromatography are supplied with this book.

Liquid chromatographic separations are based upon the different degrees of interaction of the dissolved substances with a chromatographic medium. Thus, in order to separate the target substance from impurities by chromatography, conditions that favor interaction with the target substance but not with the impurities or vice versa must be established. This important property of the matrix is loosely referred to as the selectivity (which is also influenced by the solvent and the solute). The solutes are physically separated by passing a solution through the packed bed that will transport solutes confined in the extraparticle space. The concentration of solutes in the extraparticle space will be inversely related to their degree of interaction with the chromatography medium, and desorption is regulated by an eluent. Thus, molecules will be eluted in increasing order of affinity to the chromatographic medium.

**BASIC RELATIONSHIPS**

The purpose of process chromatography is to separate, i.e. resolve, one target component from impurities. The resolution is achieved by selectively retarding the target component or the impurities to different extents while keeping the dispersion of solute bands as small as possible. There are a few relationships that are fundamental to all types of chromatography and from which basic parameters regulating the retention and zone broadening of solutes may be described and the resolution calculated. The complete chromatographic process may be described by the material balance of the system.

**Retention**

The time spent in the extraparticle space is determined by the retention factor, $k'$ (the retention factor is sometimes denoted $k$, see Ref. 1), which is given by the amount [or rather, number of moles] of solute in the stationary phase, $W_s$, as compared to that in the mobile phase, $W_m$,

$$k' = \frac{W_s}{W_m} \quad (12.1)$$

The relative migration of a solute will be equal to the relative amount found in the mobile phase and the retention volume of the solute, $V_R$, will be related to the retention factor by

$$V_R = V_M + k'V_M \quad (12.2)$$

where $V_M$ is the mobile phase volume. The retention factor is related to the distribution coefficient, $K_{11}$, expressing the concentration of solute in the stationary phase, $C_s$, over that in the mobile phase, $C_M$, by

$$k' = K_{11}V_s/V_M \quad (12.3)$$

where $V_s$ is the volume of the stationary phase. It is important to note that the retention factor is proportional to the phase ratio, $V_s/V_M$, of the chromatographic medium (e.g. phase ratios may vary with specific surface area of materials). It is also seen from Equation (12.3) that the retention factor (and the retention volume) is only constant when the distribution coefficient is constant. This is not the case for chromatography in the nonlinear mode, which is common in preparative separations (see below). Thus, varying the sample concentration may result in variations, or even shifts, in retention volumes of solutes due to influences of the isotherm on the distribution coefficient.

The definitions of mobile phase volume and stationary phase volume need some consideration. The mobile phase volume is equal to the elution volume of the solute under non-retentive conditions. Thus, while the total liquid volume (i.e. the extraparticle and intraparticle volume) of the column may be equal to the mobile phase volume of small solutes, this is certainly not true for large solutes, which are excluded from a fraction of the intraparticle volume. It is in most cases
not possible to assign a stationary phase volume to the chromatographic medium [except in gel filtration where the stagnant phase corresponds to the stationary phase]. In some cases it may be more appropriate to talk in terms of surface area.\(^3\)

The selectivity factor, \(\alpha\), for two solutes is affected by the chromatography material under the experimental conditions chosen (i.e., mobile phase composition, temperature, etc.), and is expressed by the relative retention of the solutes as

\[
\alpha = \frac{k_2'}{k_1'}
\]  \hspace{1cm} [12.4]

While \(k'\) may vary due to the differences in phase ratio of different materials the separation factor is not affected.

**Zone broadening**

The peak width is primarily affected by the zone broadening in the column and the variance of the zone, \(\sigma^2\), is proportional to the distance traveled by the zone, \(z\). The zone broadening per unit length is called the plate height\(^2\) and is denoted \(H\) [or HETP, height equivalent to a theoretical plate]

\[
H = \sigma^2 / z
\]  \hspace{1cm} [12.5]

Conversion from length to volume, and setting \(z = L\), where \(L\) is the column length, yields the familiar relationship

\[
H = L / (V_R/\sigma)^2
\]  \hspace{1cm} [12.6]

The number of plates per column, \(N\), is given by \(L/H\), and thus

\[
N = (V_R/\sigma)^2
\]  \hspace{1cm} [12.7]

One description of the variables influencing the plate height of the column is given by the van Deemter equation\(^4\)

\[
H \approx A + \frac{B}{u} + C u = 2 \lambda_d p + \frac{2}{u} \left[ 0.6 D_M + D_S \left( \frac{V_R}{V_0} - 1 \right) \right] + \frac{V_0}{V_R} \left( \frac{1 - V_0}{V_R} \right) \delta_p^2 + \frac{30 D_S}{u}
\]  \hspace{1cm} [12.8]

where \(\lambda\) is a geometric factor of order unity, \(d_p\) is the particle size, \(D_M\) and \(D_S\) are, respectively, the diffusion coefficient of the solute in the mobile phase and intraparticle phase, \(u\) is the mobile phase velocity, and \(V_0\) is the void volume. \(A\) is related to eddy dispersion, \(B\) to molecular diffusion and \(C\) to mass-transfer resistance. For small particle sized media or solutes of high diffusivity the effect of eddy dispersion is reduced by molecular diffusion, which leads to an extension of the \(A\) term to incorporate this coupling.\(^5\) However, this effect may be neglected in preparative purifications of biomacromolecules. Equation (12.8) may be written using so-called reduced parameters, i.e. the reduced plate height, \(h\), given by

\[
h = \frac{H}{d_p}
\]  \hspace{1cm} [12.9]

and the reduced velocity, \(v\), given by

\[
v = \frac{ud_p}{D_M}
\]  \hspace{1cm} [12.10]

giving the following simplified equation:

\[
h = 2 \lambda_d + \frac{2}{v} \left[ 0.6 + \frac{V_0}{V_R} \left( \frac{1 - V_0}{V_R} \right) \right] + \frac{V_0}{V_R} \left( \frac{1 - V_0}{V_R} \right) \delta_p^2 = 1 + \frac{1.5}{v} + 0.04 v
\]  \hspace{1cm} [12.11]

where \(\delta_s\) is a factor to account for the restricted diffusion in the intraparticle space \((\delta_s = D_s/D_M\) and is typically 0.05--0.2 for macromolecules\).

The right-hand side of the equation comes from the assumption that the relative mobility of the sample zone \(V_0/V_R = 0.5\) and that \(\delta_s = 0.2\). Equation (12.11) may be used for a general, qualitative description of the different contributions to column zone broadening, as illustrated in Figure 12.1. It is shown that the \(B\) term has only influence at very low flow rates and for fast diffusing solutes, and that the dominating term at high flow rates and for slow diffusing solutes is the \(C\) term. The restricted diffusion will have a large impact on the slope of the \(C\) term. It may be noticed that \(A\) in some cases may be dependent upon the flow rate (i.e., leading to coupling, as mentioned above) and also that convective transport may reduce the \(C\) term at very high flow rates. However, Equation (12.8) has been used successfully in qualitative predictions in preparative-scale gel filtration, ion exchange chromatography and reversed-phase chromatography.\(^3,7,8\)

Another equation that has been found useful for calculating the plate height is the empirical Knox equation, where the coupling between eddy dispersion and longitudinal diffusion is taken care of in the \(A\) term according to

\[
h = A' v^\beta + \frac{B'}{v} + C' v
\]  \hspace{1cm} [12.12]

Bristow noted that \(A' = 1, B' = 2\) and \(C' = 0.05\) for well-packed high-performance liquid chromatography [HPLC] columns.\(^9\) This is in good agreement with the van Deemter equation [i.e., the right-hand side of Equation (12.11)].
The right-hand side of the equation is obtained from Equations (12.2) and (12.7) under the assumption that the mobile phase volumes and the plate numbers for the two solutes are identical. By setting \( k'_2 + k'_1 = 2k' \) the equation may be rearranged to the often used expression

\[
R_S = \frac{1}{4} \alpha \frac{1}{\alpha} \frac{k'_2}{1 + k'} \sqrt{N} \tag{12.14}
\]

which separates the effects from selectivity, the retention factor, and the column efficiency (in some textbooks \( k' \) is arbitrarily set to \( k'_2 \)).

A resolution factor of 1.5 yields, in practice, a complete separation of two solutes having Gaussian peak shapes, cf. Figure 5.5 (the effect of different resolution factors on the yield and purity may be simulated by the accompanying software routine - see the section 'Modeling the effects of basic parameters below').

The graph in Figure 12.2 illustrates that the most important single parameter affecting the resolution by far is the selectivity factor. For instance, the gain in resolution from increasing the plate number 20-fold (e.g. by increasing the column length 20-fold) may be achieved by increasing the selectivity factor from 1.01 to 1.05 (as calculated from Equation (12.14)).

**Mass transfer**

The transport of solute through the column is dependent upon the concentration of solute in the mobile phase, \( C_m \), compared to that in the

\[
R_S = \frac{2(V_{k'2} - V_{k'1})}{w_{k'2} + w_{k'1}} = \frac{k'_2 - k'_1}{2(2 + k'_2 + k'_1)} \sqrt{N} \tag{12.13}
\]
stationary phase, \( C_s \), and the interstitial velocity of the mobile phase, \( u \).

The width of the solute zone is affected by dispersion. These factors may be combined to give the following expression for the material balance in the column:

\[
\frac{\partial C_M}{\partial t} - \frac{\partial^2 C_M}{\partial z^2} - u \frac{\partial C_M}{\partial z} - \frac{V_s}{V_m} \frac{\partial C_s}{\partial t} = 0
\]  

(12.15)

Thus, for an infinitesimal small segment within the column the change of concentration of solute in the mobile phase per unit time is given by the changes in concentration due to dispersion [first term on the right-hand side], and transport of molecules in the mobile phase [second term], and as a result of the adsorption/desorption process [third term on the right-hand side]. Dispersion arises from axial diffusion, eddy dispersion and non-equilibrium as described by the van Deemter equation. In theory, film diffusion (i.e. diffusion of molecules through the stagnant solvent layer around particles) will also contribute to dispersion, but this effect is normally small and often ignored. Convective flow transport of molecules through the particles will enhance mass transport and reduce the dispersion caused by long-range intraparticle diffusion. The recent review of mass transfer in chromatographic separation by Li et al. can be recommended for further reading.

Unfortunately there is no analytical solution to Equation (12.15) for gradient elution chromatography and results must be calculated numerically. This together with approximations needed for dispersion of zones and for adsorption/desorption equilibrium at high sample loading common in process chromatography have resulted in different approaches for theoretical simulations of chromatographic purifications.

The use of chromatography theory for modeling chromatographic separations is discussed in Chapter 13.

Flow resistance of packed beds

Sometimes factors other than the chromatographic ones need to be addressed. For instance, even though the separation factor is large enough to allow for a decrease in separation time, the pressure drop over the packed bed at elevated eluent velocities may exceed the pressure rating of the pump or of the chromatographic medium. In that case a decrease in column length may be a better solution (the resolution is proportional to the square root of the column length, cf. Equation (12.13)).

The pressure drop over a packed bed may be calculated from the Hagen-Poiseuille equation, as adapted to packed beds by Blake, Kozeny and Carman,

\[
\Delta P = \frac{\pi L}{4 d_p^2 (1 - \varepsilon)^2} \frac{(1 - \varepsilon)^2}{\varepsilon} 36 k = \frac{L}{4 d_p^2} \frac{180(1 - \varepsilon)^2}{\varepsilon^4}
\]  

(12.16)

The first term of Equation (12.16) is a conversion from interstitial liquid velocity, \( u \), to nominal liquid velocity with the aid of the void fraction \( \varepsilon = V_d/V \), where \( V_d \) is the geometric bed volume. The pressure drop is given in Pascal, \( Pa = N \text{m}^{-2} \), if the viscosity of the solvent, \( \eta \), is expressed in second newtons per square meter (\( s \text{N m}^{-2} \)), the velocity, \( u \), in centimeters per second, and the column length, \( L \), and the particle size, \( d_p \), in centimeters [see Appendix A for conversion factors]. The aspect factor, \( k \), depends upon the shape of the particles, and is close to 5 for spherical beads. The last term of Equation (12.16) is called the flow resistance parameter, and is used to compare the packing density and permeability of packed beds.

It is important to notice the large influence of the void fraction, \( \varepsilon \), on the flow resistance and the calculated permeability. For instance, a bed of hexagonal close-packed uniform spheres has a void fraction of 0.26, and gives a pressure drop of six times that of a bed of randomly packed spheres, having a void fraction of 0.40. It may be noted that the void fraction of silica-type materials is often in the range of 0.42-0.45, that of mono-sized synthetic polymers is around 0.36-0.40 and that of non-rigid polymers is in the range 0.30-0.33. A higher void fraction yields lower pressure drops but a larger contribution to the non-separating volume of the system.

The influence of viscosity on flow resistance needs to be considered when applying viscous samples or adding viscous modifiers to the eluent, and when transferring separation methods to cold room.

Changing the particle size from 100 to 10 \( \mu m \) increases the flow resistance 100 times. This requires the use of high-pressure systems for running chromatography media of small particle size (e.g. from the 5 bar system used for standard chromatography to the 100 bar system for HPLC) even though the column lengths for HPLC are normally shorter than those used for standard chromatography. The large influence of void fraction on pressure drop, as illustrated in Figure 12.2, shows that an inhomogeneously packed column will yield a larger pressure drop than expected from the measured void fraction, thus unrealistic pressure drops may be indicative of column deterioration. Equation (12.16) is useful for determination if a packed bed is compressed or not, i.e. if the flow resistance is substantially larger, e.g. 50%, than expected from particle size, length, viscosity, void fraction, and the contribution from system factors (i.e. connectors, tubing, frits, etc.). The flow resistance caused by the system may be determined by replacing the packed column with an empty column of the same type.
contribute to the total peak width as described in Equation (12.19). The
injector-dependent constant, $K_{\text{injector}}$, has been found to be close to 5 for
ordinary laboratory injectors and approaching 12 for optimal injectors
and large sample volumes where the injection profile is a square wave. At
very large sample volumes the load will be the limiting factor for peak
width and thus resolution.

An optimal sample volume when processing large feeds may be
calculated with the aid of Equation (12.19). This optimum will balance
the detrimental effects of a large sample volume [i.e. running few cycles]
and the zone broadening when running at high flow rates [i.e. splitting
the sample into many cycles]. A guide to the optimal sample volume is
given by

$$V_{\text{injector, optimal}} = \left( \frac{V_{\text{feed}} K_{\text{injector}} V_c V_p Q_m^{1/2}}{15 D_M} \right)^{1/3}$$  (12.22)

where $V_{\text{feed}}$ is the amount of sample (milliliters) to be processed per hour.
The equation was found to support the general rule of processing a
volume equal to 2% to 6% of the column volume each cycle at cycle
times of 5 h to 1 h, respectively.7

The sample concentration that is applicable is restricted by the vis-
cosity of the injected sample plug as compared to the viscosity of the
eluent. The general rule is that the relative viscosity of the sample plug
should be less than 1.5. This corresponds to a sample concentration of
70 mg ml$^{-1}$ of a globular protein such as serum albumin.6 High vis-
cosity of the sample will cause a distorted rear zone of the elution band.21
This may be avoided by using an eluent of matching viscosity
(though not generally applicable, see below) or reducing the viscosity
effects by special column constructions.22

**Ion exchange chromatography**

The interaction in ion exchange chromatography has been described by a
stoichiometric model where the solute will displace a number of counter-
ions from the surface equating the number of interacting sites of the
solute.23,24 This model has recently been questioned, and a model
where general electrostatic interaction theory for charged surfaces is
used to explain the retention has been presented.25 An evaluation of the
two concepts using a weakly charged chromatography media gave results
in favor of the electrostatic interaction theory.26 However, it was sug-
gested that the two models would describe different extremes of ion
exchange chromatography, and further investigations must be performed
before a conclusive statement can be made.26 Since the stoichiometric
displacement model (SDM) currently provides the basis for ion exchange
theory, it will be used in this section.

The stoichiometric model has recently been refined to incorporate the
steric shielding of ion exchange groups by large solutes. This model is
called the steric mass action (SMA) model, and was used for modeling
ion exchange chromatography in overload mode.27

**Retention**

The retention factor in ion exchange chromatography is a function of the
concentration of salt in the mobile phase, $c$, and the properties of the
solute and adsorbent according to

$$k' = k_0 e^{-z}$$  (12.23)

where $k_0$ is related to the ion exchange capacity of the medium, $Q$, ($k_0$
is proportional to $Q$), and $z$ is the interacting charge of the solute. This
equation is given for the stoichiometric model (in the electrostatic model in $k'$
is proportional to $I^{-1/2}$, where $I$ is the ionic strength).22 The
relationship between the retention factor and the mobile phase concen-
tration is shown in Figure 12.6. The retention varies drastically with $c$
and $z$. It is seen that the retention will be sensitive to the ionic strength
only in a limited region, the elution window, and that keeping the ionic
strength constant will separate molecules, differing only slightly in $z$, far
apart. On the other hand, separation of mixtures of solutes varying
substantially in $z$ requires a continuous change in $c$, i.e. gradient elution.

![Figure 12.6 Influence of mobile phase salt concentration and characteristic charge on retention in ion exchange chromatography.](image-url)
However, such separations (i.e., simultaneous determination of several components) are only of primary concern in analytical applications. Typical values for the characteristic charge for proteins in ion exchange chromatography are in the range of 3.6–8.2, \(^{24}\) and 4.8–7.5, \(^{29}\) although this will vary with pH (see Chapter 11).

It may be noticed that Equation (12.23) predicts that all solutes will move along the column bed and that this effect is not negligible unless \(k'\) is large. It is therefore recommended that the sample be applied in a low ionic strength buffer. For solutes that are strongly adsorbed, the mobile phase concentration may need to be increased considerably to desorb the solute. In order to reduce separation times (and excessive dilution of sample zones) the concentration is, in elution chromatography of complex mixtures, varied either continuously (gradient chromatography) or stepwise. For special cases, combination of isocratic, step elution and gradient elution may be favorable.

The retention volume is given by Equation (12.2) [i.e., \(V_R = V_M + k'V_M\)]. This equation is valid only for conditions where the retention factor is constant, i.e., under isocratic conditions. When the concentration of the mobile phase is changed (i.e., as in gradient elution) the retention factor is also continuously changed. The apparent retention factor calculated from the retention volume in gradient elution does not have any physicochemical meaning.\(^{4}\)

If the chromatographic system contains large extracolumn dead volumes, these will be added to the retention volume (but these should not be incorporated into the calculation of \(V_M\), see the section 'Experimental determination of basic parameters' below).

**Zone broadening**

The zone broadening in isocratic elution will be affected by the same factors as noted for gel filtration. If the adsorption–desorption kinetics is not fast, this factor will also contribute to zone broadening. However, in gradient elution a sharpening effect from the gradient is obtained (i.e., molecules at the front of the zone sense a lower ionic strength and thus a higher retention factor than molecules at the rear of the zone). This means that a steady state regarding zone broadening will be reached and, furthermore, all sample zones will attain the same (narrow) width on the column, provided the elution conditions (e.g., column length, and gradient conditions) are sufficient to promote this steady state. The degree of zone sharpening will depend upon the slope of the gradient (higher degree of sharpening for a steeper gradient) and also on the relationship between \(k'\) and ionic strength for the solute. For traditional media, peak widths of 50–80% of those calculated from Equation (12.8) may be expected.\(^{8,29}\) This sharpening effect is, together with the possibility to regulate \(k'\), the main advantages of gradient elution.

**Resolution**

The resolution in isocratic elution ion exchange is given by Equation (12.14). A plot of resolution versus \(k'\) shows that the effect of \(k'\) is low for \(k'\) above 10 (see Figure 12.2). Also, the largest effect on the resolution by far comes from the differences in \(k'\) between the two solutes, i.e., the selectivity.

Since \(k'\) varies during gradient elution, Equation (12.14) may not be used for calculation of resolution in this elution mode. Yamamoto and co-workers found that the following equation was useful for predicting the influence of experimental parameters on the resolution in linear gradient elution of proteins in ion exchange and hydrophobic interaction chromatography.\(^{25}\)

\[
R_s = \frac{L}{\sqrt{gV_bH}}
\]  \hspace{1cm} (12.24)

where \(L\) is the column length, \(g\) is the gradient slope (mole/liter per liter gradient volume), \(V_b\) is the interstitial void volume, and \(H\) is the plate height. The plate height will be slightly lower as calculated from Equation (12.8) due to the zone-sharpening effect of the gradient provided the sample load is low and that the desorption kinetics is fast. At high flow rates the plate height will be proportional to the \(G\) term. The influence of the column length may be eliminated from Equation (12.24) by setting \(L = V_c/A_c\), where \(A_c\) is the cross-sectional area of the column and \(V_c\) is the geometric column volume.

**Experimental parameters**

The retention in ion exchange is determined by the charge of the solute and the exchanger, and the ionic strength of the buffer. The particle size and por dimensions, and flow rate, will affect the mass transfer, and the resolution will be limited by the sample load. The gradient slope is important in gradient elution.

**Properties of the solute**

For amphipatic solutes (i.e., solutes whose charge is pH-dependent) or weak exchangers, the pH of the buffer is of great importance. To ensure complete ionization the pH should differ by at least one unit from the \(pK_a\) of the charged groups. For proteins and oligopeptides it may be noticed that the isoelectric point is equivalent to the pH where the net charge of the biomolecule is zero. However, there may exist areas of positive charge above the isoelectric point and areas of negative charge below the isoelectric point that will promote interactions with cation and anion exchangers (effects of pH have been noted as far as 4 units from the isoelectric point, see Chapter 11). The pH will affect the
characteristic charge and thus have a large influence on the retention factor and resolution [cf. Figure 12.2]. For acidic proteins separated on an anion exchange the retention will increase with increasing pH. For basic proteins separated on a cation exchanger the retention will decrease with an increase in pH [cf. Figures 5.11 and 5.12]. The magnitude of the change will be dictated by the slope of the retention curve [a plot of charge versus pH] for each protein. If the slope is similar (which is often the case), then all proteins will be affected in a similar way by a change in pH and no gain in selectivity will be obtained.

It must be noted that specific solute properties might cause shift in elution positions when the mobile phase composition or sample load is changed. This may be due to different charge–pH relationships, influence from solute size on \( k' \), or an effect of crossing isotherms [see below].

Properties of the mobile phase

The influence of the type of counter ions on selectivity has been discussed [e.g. see Kopciwicz et al.24]. From recent work it may be concluded that specific effects of the salt used are not to be expected.30

The governing parameter is the elution strength of the salt, and the effect of one type of salt may be obtained by another type of salt by adjusting the concentration. The relative elution strengths of different ions are listed in Table 12.1.

The retention factor is dependent upon the ionic capacity of the gel raised to the power of \( z \) [Equation (12.23)]. However, by keeping \( Q / c \) constant, \( k' \) is also kept constant.3 Thus if the ionic strength needed to desorb a substance is unsuitable, an alternative may be to use a chromatography medium having another ionic capacity.

Optimization of the starting conditions [i.e. the ionic strength of the start buffer] and the gradient slope are important for the resolution in gradient elution (i.e. to affect the peak-to-peak distance and keep the dispersion low). In laboratory preparations, different shapes of the gradient have been elaborated, but this is complicated for large scale purposes. For reasons of robustness, industrial-scale purifications by ion exchange chromatography are preferentially based upon step elution of the target solute. However, since gradient elution generally offers higher resolution of solutes having similar affinities for the adsorbent, this elution principle is gaining in popularity, especially as equipment for reliable large-scale gradient elution is becoming available [see Chapter 14]. Gradient elution will also produce more concentrated zones of eluted product due to the sharpening effect. Furthermore, the influence of interacting charge on \( k' \) [see Figure 12.6] makes gradient or step elution necessary for elution of large molecules [e.g. proteins] while small solutes [e.g. peptides] may be separated under isocratic conditions.

The flow rate is not critical in ion exchange chromatography as long as the residence time (i.e. the time allowed for the sample to equilibrate with the gel phase) is sufficient. In most cases, the kinetics is sufficiently fast to allow very high flow rates to be used in gradient or step elution. The dispersion that will take place during elution (i.e. due to gel filtration of the solute) will dilute the zones somewhat, but the effect on resolution is compensated for by regulating the selectivity [also, there is a zone-sharpening effect in gradient elution]. Therefore ion exchange chromatography is conducted at high flow rates as compared to gel filtration. However, as evident from Equation (12.24) the resolution will decrease with increasing values of \( H \), and \( H \) will predominantly be affected by the \( C \) term, i.e. proportional to the flow rate, at high flow rates. On the other hand, keeping the separation time constant means that \( g \) will be inversely proportional to the flow rate and, thus, the net effect on resolution will be nil.

Properties of the chromatography medium

The selectivity factor is dependent upon the type of charged group and the number of charges but is not generally influenced by the matrix as such, unless secondary interaction mechanisms [e.g. hydrophobic interactions or size exclusion] are influencing. Thus the separation pattern on an HPLC type of media, e.g. Mono Q, was found to be very similar to that on Q Sepharose Fast Flow [having the same functional group] which facilitated scale-up from laboratory conditions.32

The particle size will influence the plate height the same way as for gel filtration, though the effect may not be so dramatic due to the zone-sharpening effect of gradient elution. The effect of particle size on resolution of proteins in gradient elution was calculated from Equation (12.24), and good correlations to experimental results were found.33

Smaller particles generally show better mass transfer due to shorter diffusion paths.

The pore size and pore structure of the gel will have an effect on the accessibility of adsorptive sites for the solutes and the kinetics of the adsorption/desorption process. Thus, for large molecules, wide pore gels having an open gel structure to provide fast access to adsorptive sites and minimizing the risk of blocking the pathways will be preferential.

---

Table 12.1: Elution strength of different ions

| Anion exchange chromatography | Acetate < formate < chloride < bromide < sulfate < citrate |
| Cation exchange chromatography | Lithium < sodium < ammonium < potassium < magnesium < calcium |
Results from comparisons of gels of different pore structure indicate that macroporous gels are favorable. In most cases the adsorptive site is made more accessible by attachment to the gel surface via a spacer arm, which will have a positive effect on available capacity and adsorption kinetics.

The diffusive mass transport of solutes is the primary limiting factor provided that the adsorption/adsorption kinetics is fast (which is often the case). As noted for catalysts and chromatography media, the mass transport may be enhanced by promoting convective flow through the chromatographic particles.\textsuperscript{34,36} This is achieved by increasing the pore dimensions of the particles to be of the same order as that of the void channels between the particles, to decrease the flow resistance of the porous phase (cf. Equation \{12,16\}). Applications of large pore size chromatography media based on synthetic as well as natural polymers to the ion exchange chromatography of proteins have been described.\textsuperscript{37-39} Superporous media are characterized by higher resolution at elevated flow rates as compared to traditional chromatography media as a result of the increase in mass transfer.\textsuperscript{39} The trade-off for speed is the reduced capacity caused by the reduction in the chromatographic matrix (i.e., the superpores). It was recently shown that the productivity of conventional media may in some situations exceed that of superporous media due to this loss in capacity.\textsuperscript{40,41} In another comparison, a gel composite media, where transport is reported to take place through surface diffusion, was found to give high capacity at high flow rates and also compared favorably to the properties of superporous media.\textsuperscript{22} This illustrates that there is a continuous development of process chromatography media for ion exchange, and, furthermore, tools for the correct evaluation of the properties of these media as compared to traditional alternatives are essential for the practitioner in the field.

**Sample load**

In isocratic elution the sample volume will contribute to the zone broadening as for gel filtration, and it was concluded that concentration overload provides maximal throughput.\textsuperscript{25} The sample volume is a critical factor in gradient elution ion exchange chromatography unless the sample solution is of high ionic strength (e.g., has a high salt content). Under unfavorable conditions the sample may be diluted during the sample application, either as a result of isocratic elution (e.g., due to too high a salt content) or frontal chromatography (i.e., other components of the sample are more strongly retained). This should be checked by determining the break-through capacity of the target solute in the feed solution (see below).

The total load (i.e., concentration times sample volume) is limited by the amount of sample that may be applied, which in turn is determined by the capacity of the gel for the solute (and the influence from contaminating solutes). The sample concentration as such is often low, an ion exchange chromatography is a very efficient concentration step, in addition to provide purification from other solutes.

The sample load will influence the resolution since the band will occupy a finite width. Restricting the load to less than 30% of the maximal load will normally be sufficient to avoid overlapping, and the influence on peak width or retention time will be small.\textsuperscript{4} In practice this means that 25% of the column is used for sample loading while 75% of the column is used for the separation (obviously there is a lower limit to the column length for this rule of thumb to be valid).

The effect of overload mode, as a result of volume or concentration overload, is discussed below (see the section on non-linear chromatography).

The capacity is, together with the quantitative recovery of solute, the most important feature of the gel and experimental conditions chosen. For example, some gels may show very high capacity but low yield, and therefore a careful examination of the properties (e.g., breakthrough capacity and material balance calculations) of the gel under the experimental conditions chosen is important.

**Reversed-phase chromatography**

The retention mechanism in reversed-phase chromatography is still not fully understood.\textsuperscript{44} Retention has been explained from a solvophobic model where the solute is forced into the stationary phase due to the strong mutual interaction of the molecules in the mobile phase (thus 'excluding' the solute from the mobile phase). The retention is in this model related to the solubility parameters of the solute and the components of the mobile phase, though the relationship is complicated and only qualitative information is given from applying the theory.\textsuperscript{3,45} Another model discussed early on was based upon liquid-liquid partitioning of the solute between the mobile phase and the stationary phase or the stationary liquid interface, but such effects have not been shown experimentally. However, experimental evidence supporting a partitioning process between small apolar solutes and the stationary phase has recently been presented.\textsuperscript{44,46} On the other hand, it was found that polar solutes behaved differently from apolar solutes.\textsuperscript{46} It may also be expected that large solutes will behave differently from small solutes. Therefore, in this chapter, implications of reversed-phase chromatography theory will be made with reference to the existing theory (e.g., see Jander and Churáček).