Module ChE 311 Biochemical Engineering

Downstream processing
Lecture 4B – Chromatography basics

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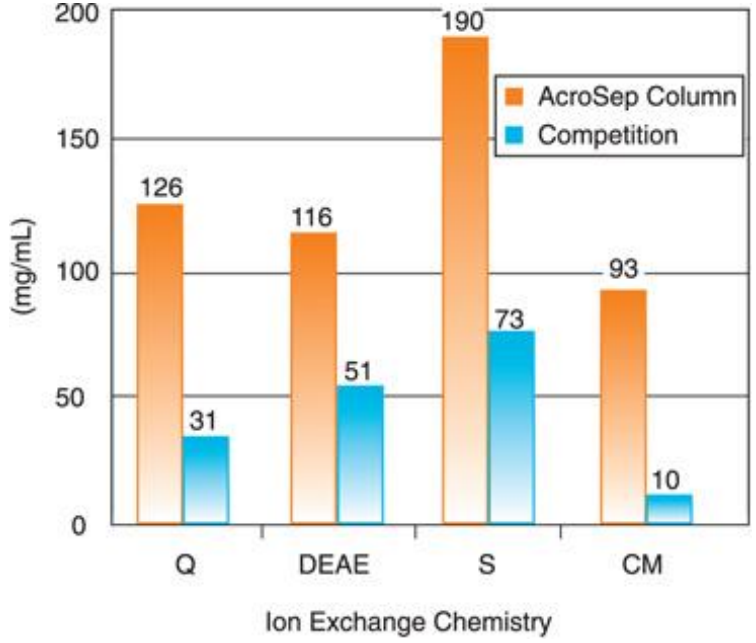
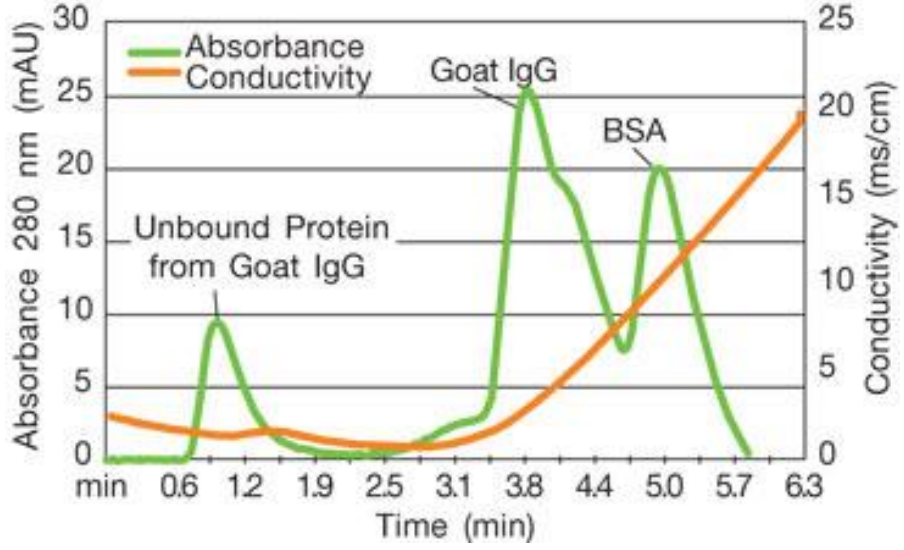
Chromatography (well, mostly protein chromatography)

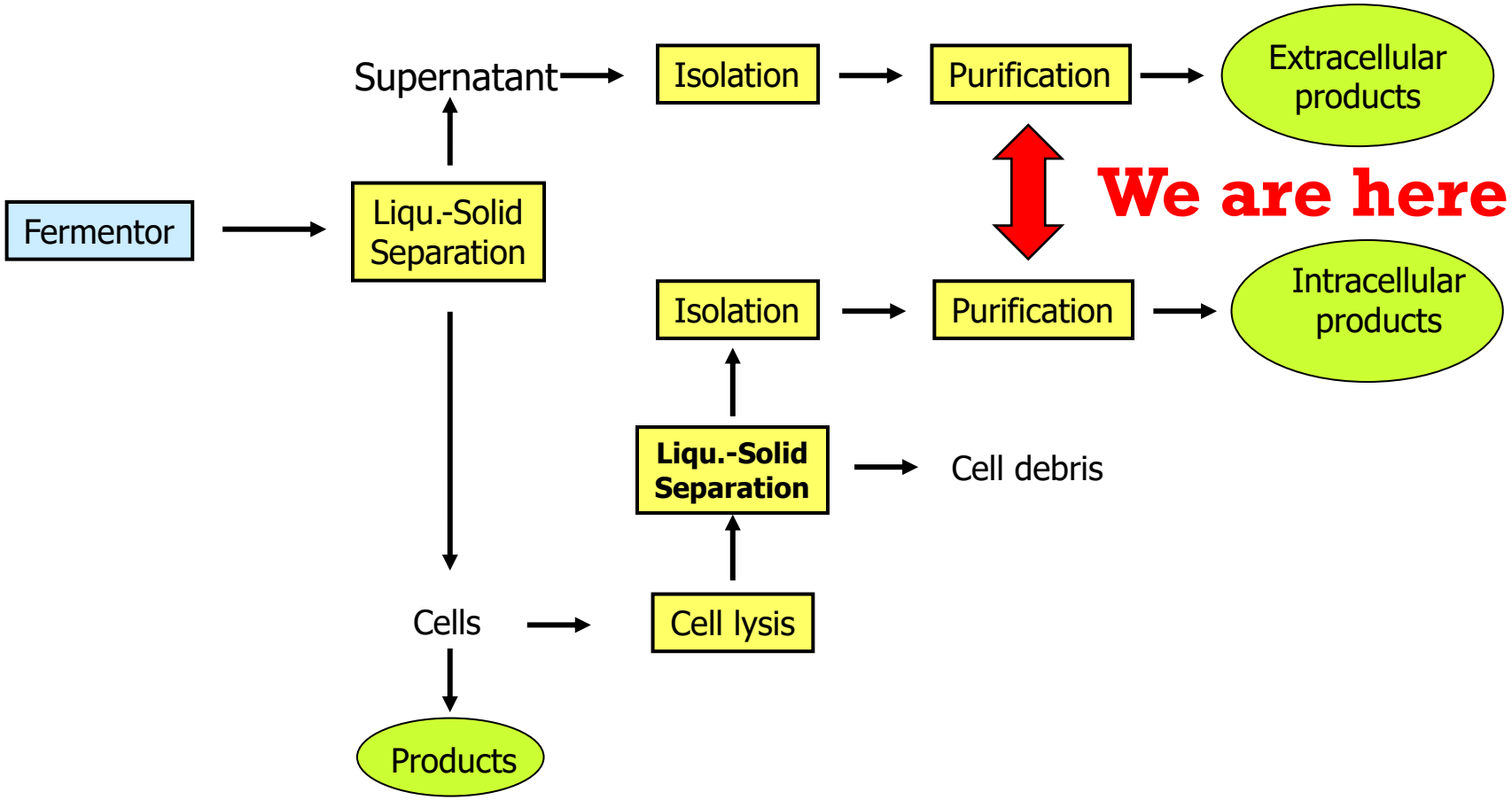




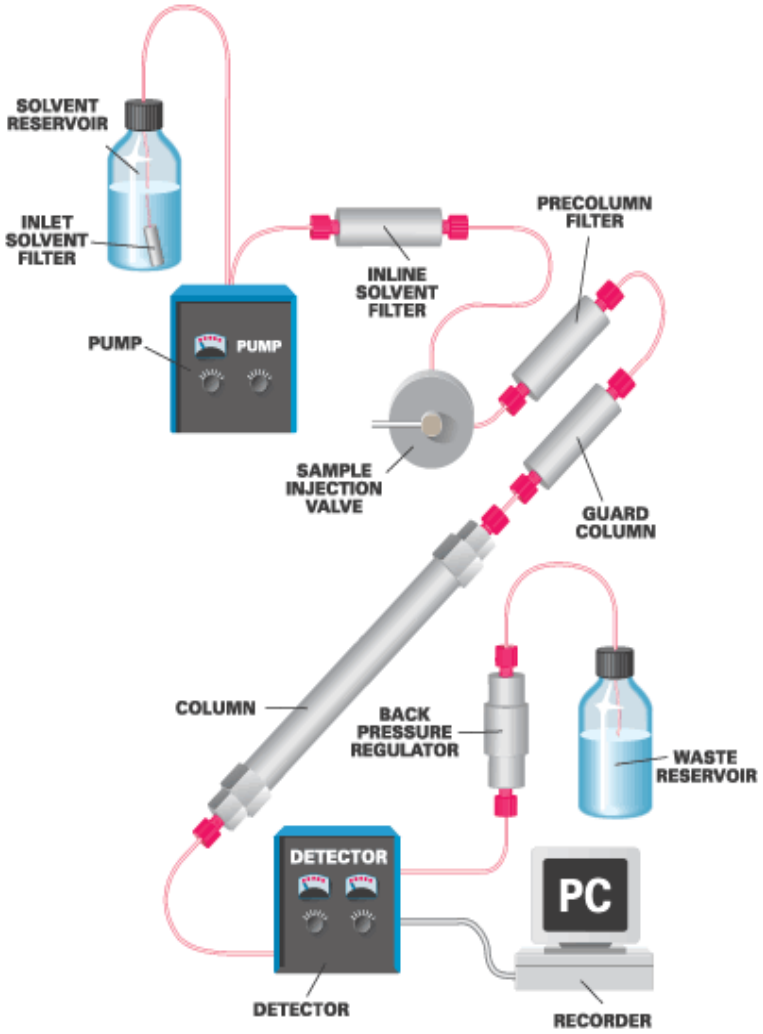
WARNING

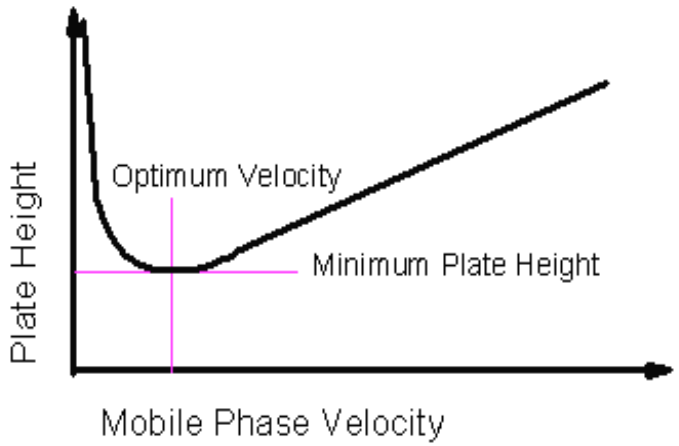
- The theory of chromatographic separations spans over an extremely large number of topics ranging from the types of chromatographies, the design of the resins, the characterization of the column behavior, the optimization of the separation conditions etc …
- We will limit ourselves to the most important of these subjects
- Here we will exclusively discuss **preparative chromatography**

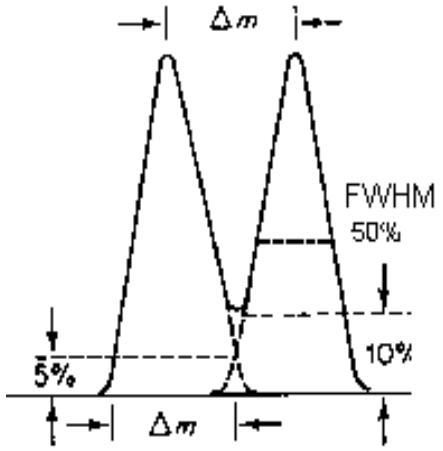
Common pathway for a purification protocol



4.1 A quick reminder



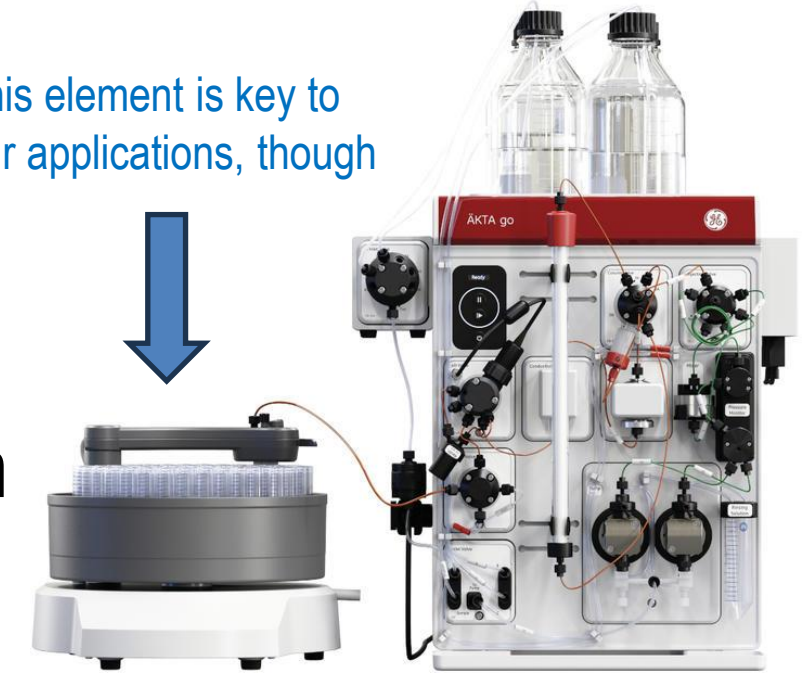




Same components** are found in chromatographs at all scales

- Pumps
- Pipes, tubing, valves
- Injection/loading system
- Stationary phase
- Detector

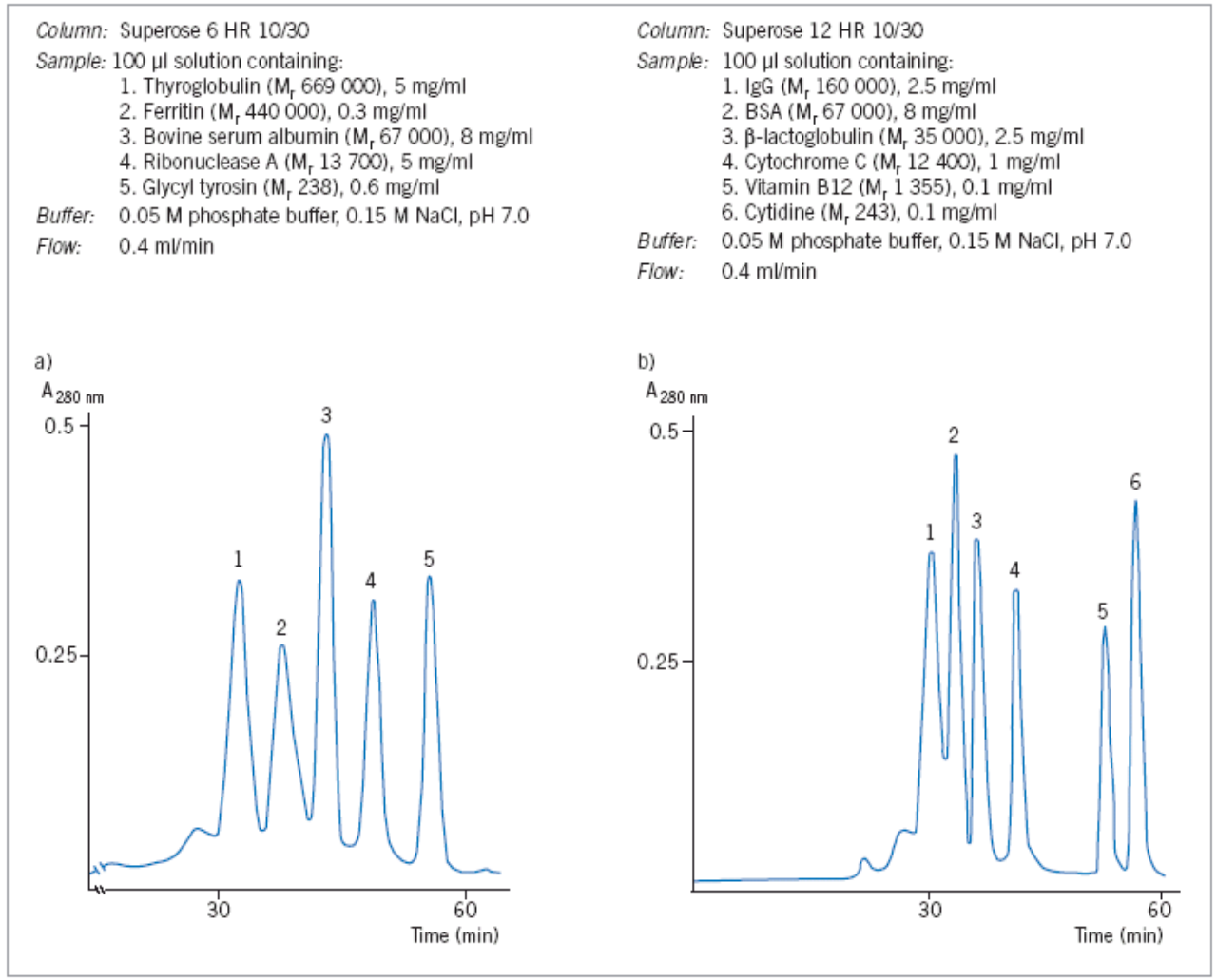
This element is key to our applications, though



Alliance HPLC System.

NB: the pictures are not to scale (far from it!)

A chromatogram conveys a lot of information



However, **identification** of the peak corresponding to the target molecule requires some specific analytical method

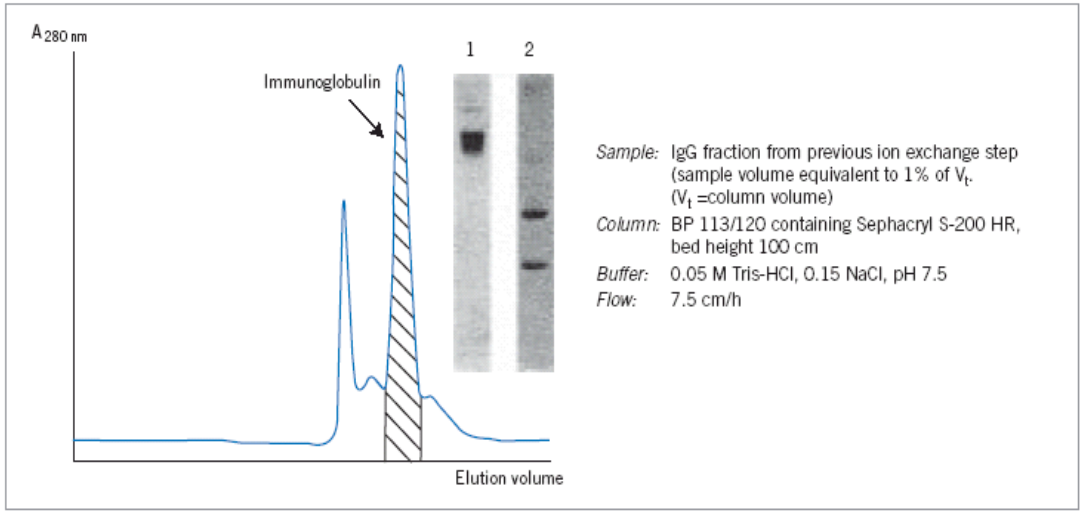


Fig. 34. Purification of monoclonal antibodies on Sephacryl S-200 HR. Inset shows analysis by gradient SDS-PAGE of the immunoglobulin pool. Lane 1, native sample; lane 2, sample reduced with 2-mercaptoethanol.

Production-scale columns

Importance of column packing

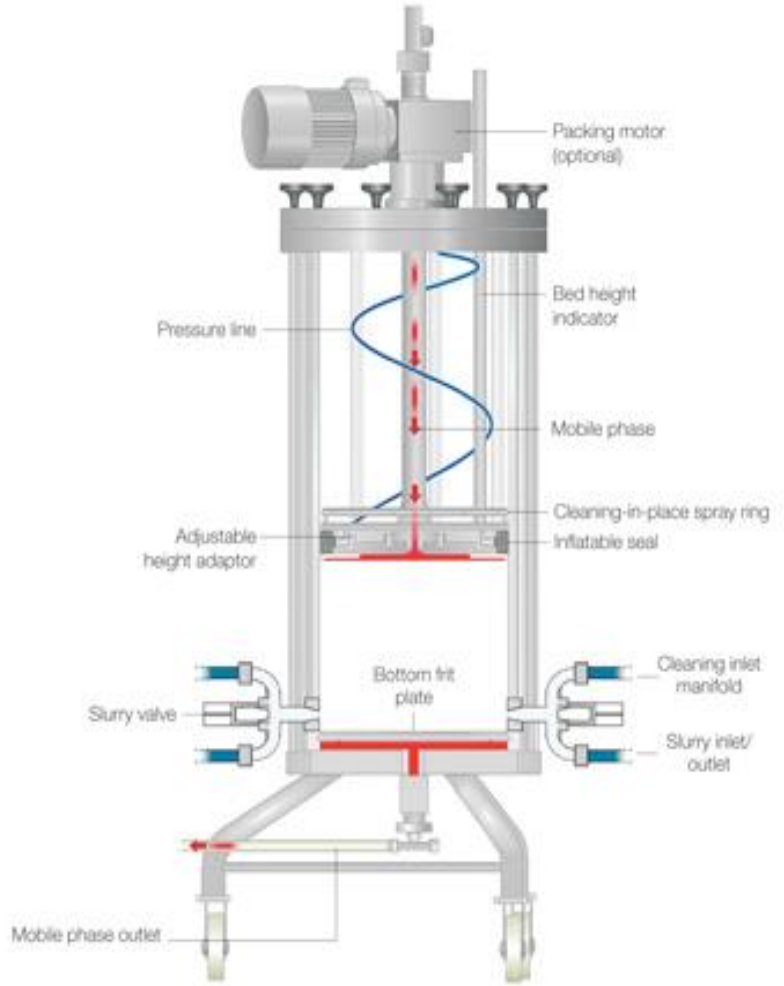
- The quality of column packing is key to the separation efficiency
- Beads should be distributed in a highly homogenous manner and the bed should be devoid of cracks and channels
- Packing can be done manually for lab-scale columns, but has to be automatized for production-scale systems
- There are several experimental techniques to assesss the quality of a packing. For instance, breakthrough curves or pulse injection of an inert tracer.
- In a well-packed column, the height of a theoretical plate H is about 3 times the diameter of the resin particles.

Source: https://biopharma-asia.com/

A brief check list (before you do anything stupid)

- **Industrial chromatography columns** are usually not stored for extended periods
- They have to be filled with resin and packed on a regular basis
- It is then important to assess the **quality of the packing** before starting any purification campaign
- Parameters that are typically measured include **pressure drop, peak symmetry** (or lack thereof) and **number of theoretical plates (NTP)**
- Please check the **Bio Rad video** on the **packing of industrial columns**

4.2 Column filling and packing



Schematic drawing of Bio-Rad InPlace column.

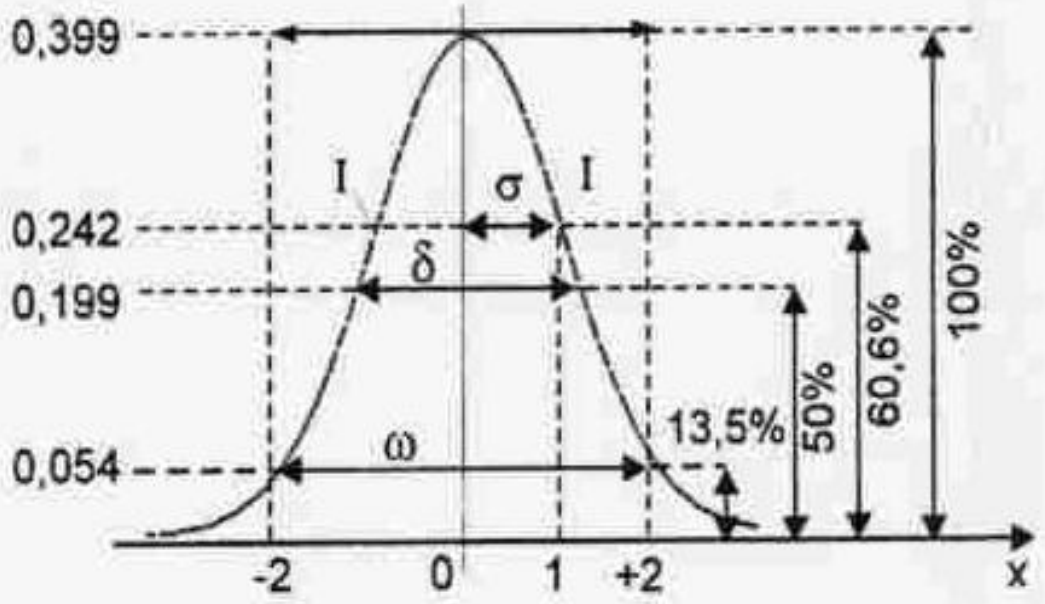
Bio-Rad EasyPack Column

Bio-Rad InPlace Column and Control Console

BIO-RAD

Vidéo sur le remplissage de colonnes industrielles

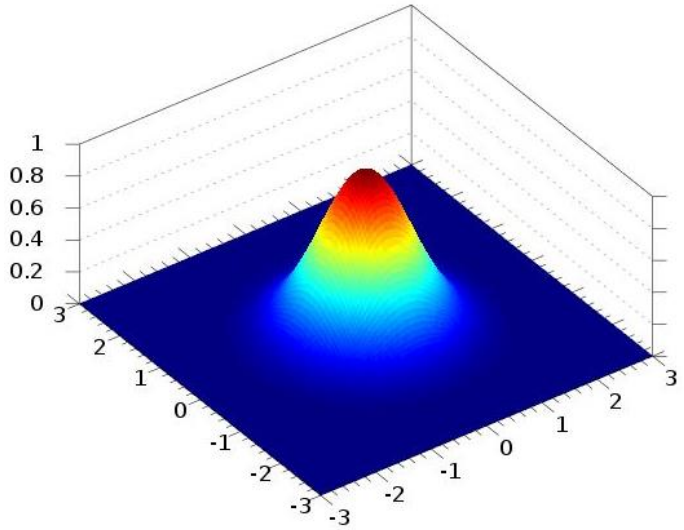
Properties of Gaussian peaks



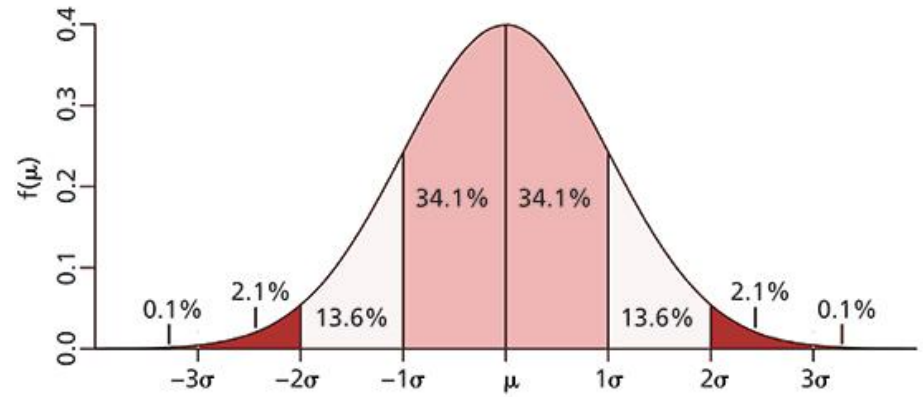
$\delta = 2{,}35\,\sigma$
$\omega = 4\,\sigma$
$\omega = 1{,}7\,\delta$

(6.4-1)

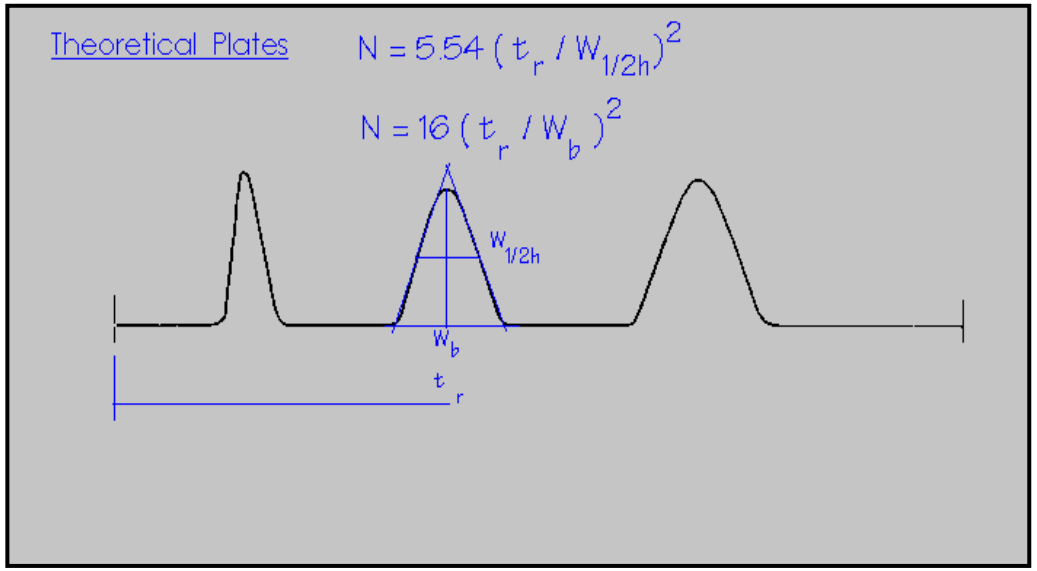
l'aire comprise entre -2 et +2 vaut 95,4% de l'aire totale comprise entre la courbe et l'axe des x



- σ is the standard deviation
- σ^2 is the variance



Number N and equivalent height H of theoretical plates



The number of theoretical plates is an expression of the quality of the column packing and the homogeneity of the flow through it.

The peak is obtained by injecting a small amount of a tracer that does not interact with the chromatography resin.

$$N = \frac{t_r^2}{\sigma^2} \quad (6.4\text{-}2)$$

$$N = 5.54 \cdot \left(\frac{t_r}{W_{0.5h}} \right)^2 = 16 \cdot \left(\frac{t_r}{W_{base}} \right)^2 \quad (6.4\text{-}3)$$

- There are various ways to calculate N from the peak geometry. They all derive from Equ. 6.4-1
- The formulas of equation 6.4-3 are most often used

$$H = L/N \quad (6.4\text{-}4)$$

The equivalent height of a theoretical plate, H, corresponds to the total column length, L, divided by N

Peak asymmetry F_a and resolution R

- Various factors (inhomogenous packing, channelling) can change the peak appearance into a non-Gaussian geometry
- The observed asymmetry is characterized by two parameters, each of them measured at 10 % of peak height:

The **resolution R** is an expression of how efficient the separation is between two components

$$R = 2 \cdot \frac{t_2 - t_1}{W_1 + W_2}$$

Asymmetry factor $F_a = \frac{B}{A}$ (6.4-5)

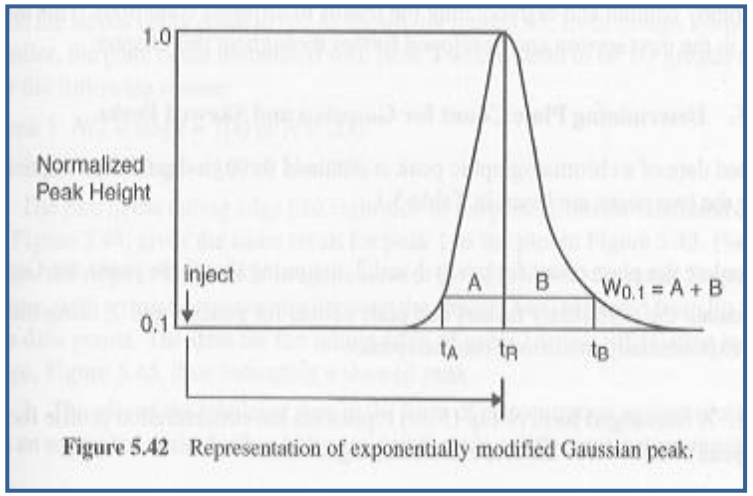
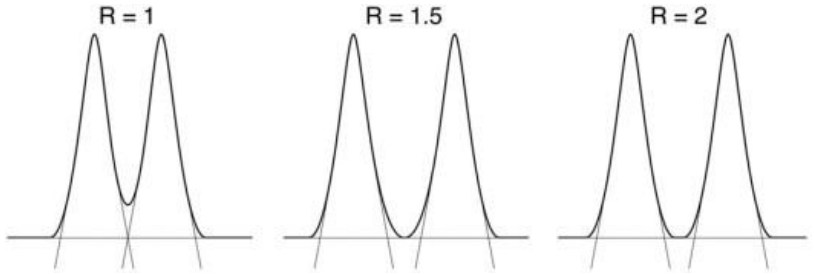
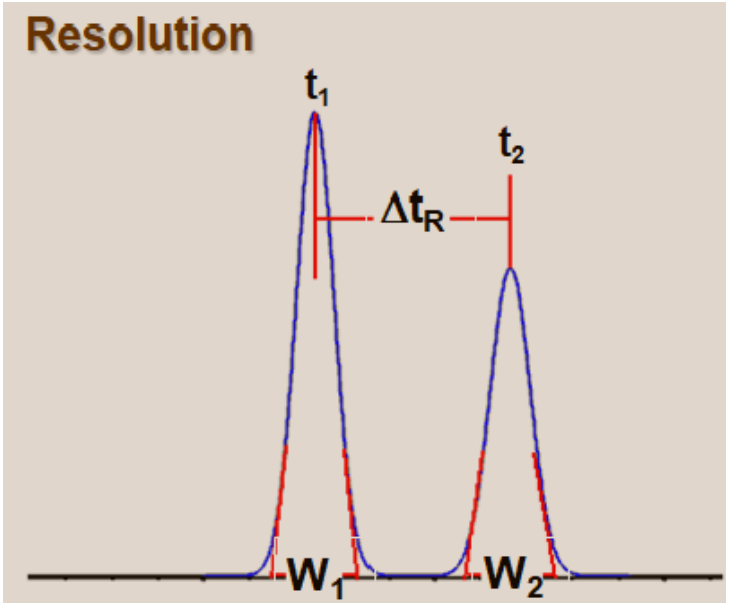


Figure 5.42 Representation of exponentially modified Gaussian peak.





Influence of the liquid distribution system

HPLC column: one entry point but small diameter: no liquid distribution issue

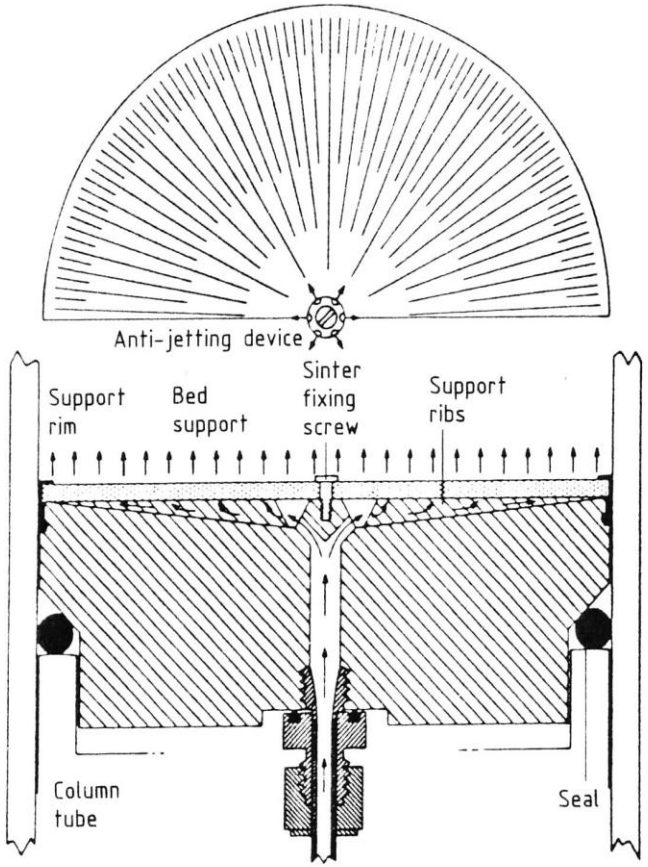
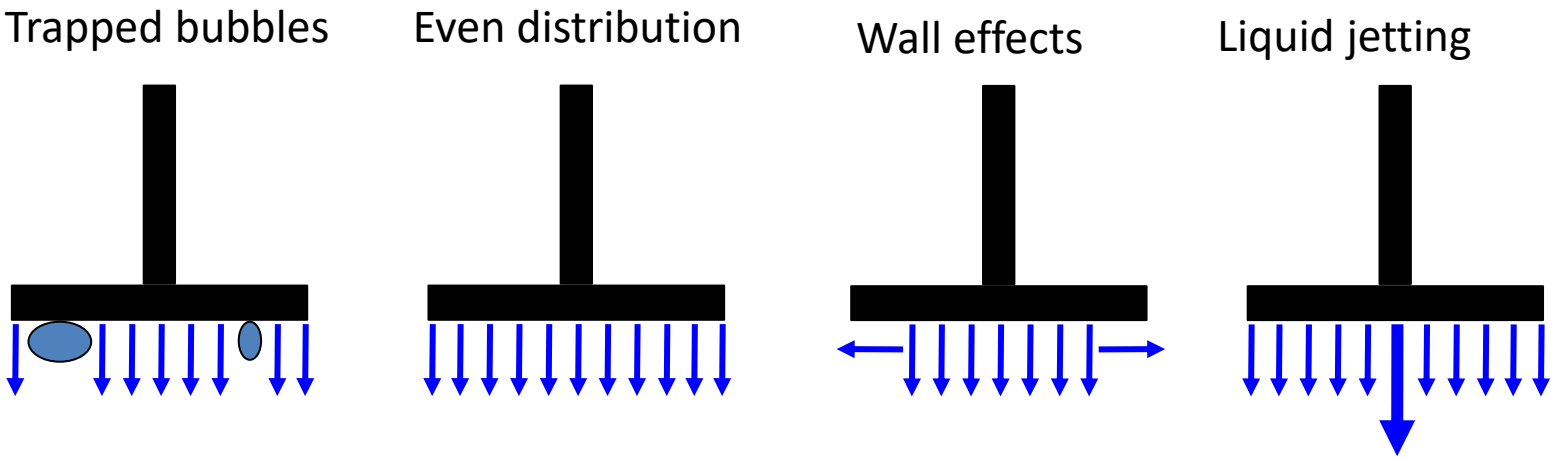
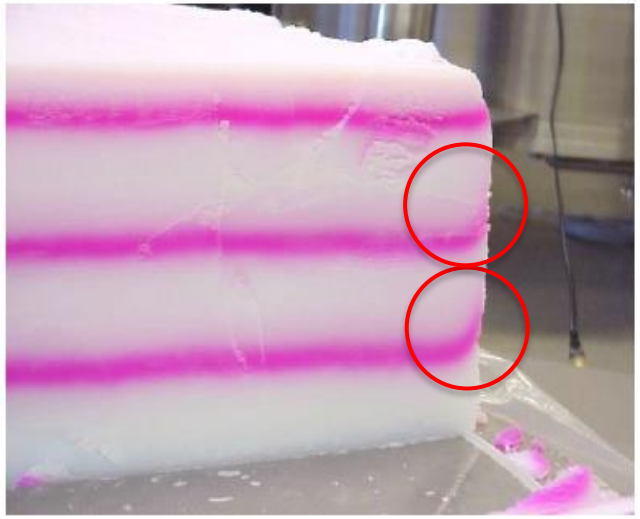


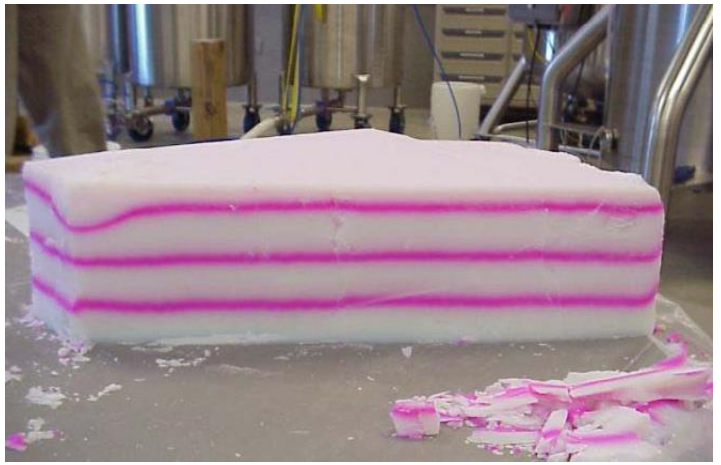
Fig. 1-3. Example of column end cell design providing uniform flow distribution.



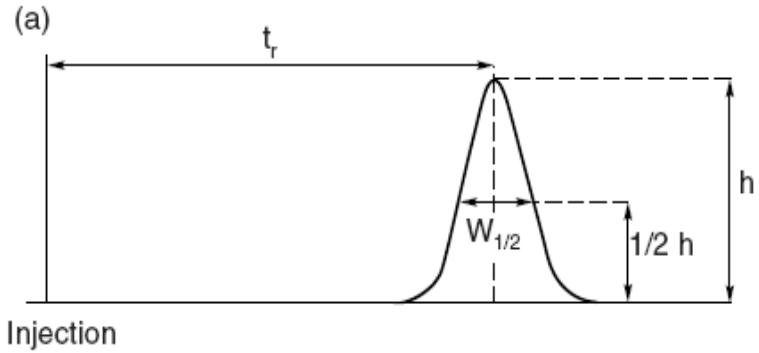
Wall Effect



Liquid jetting



Packing quality: number of theoretical plates, peak (a)symmetry



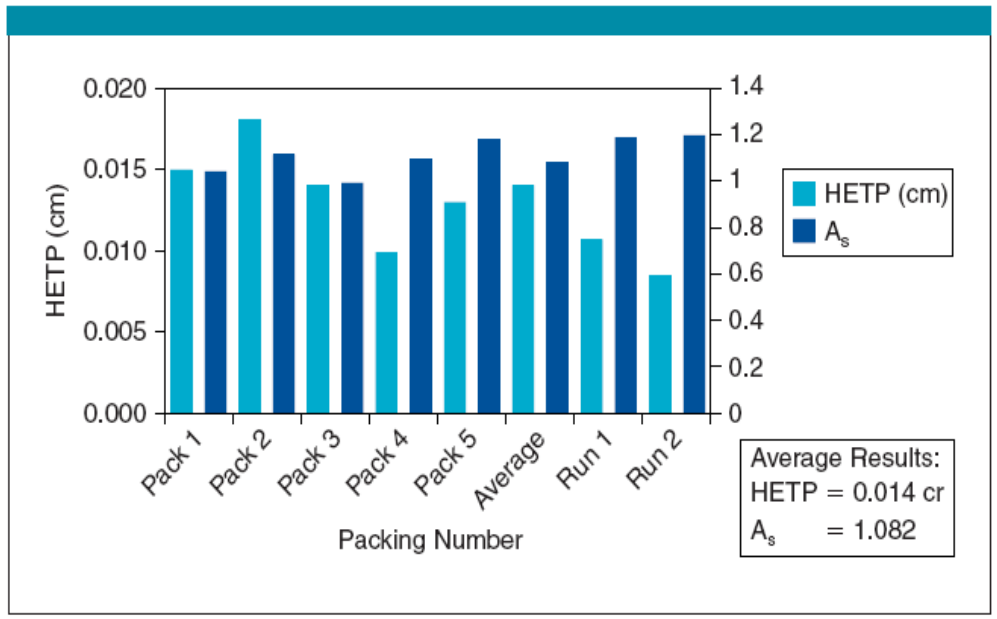
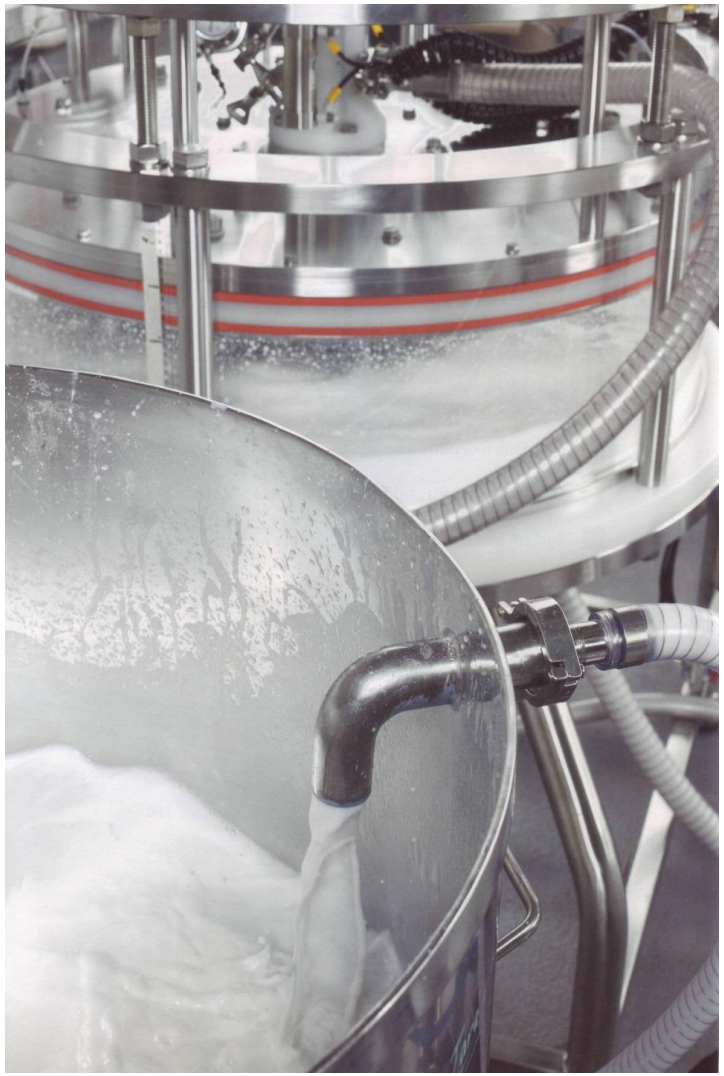
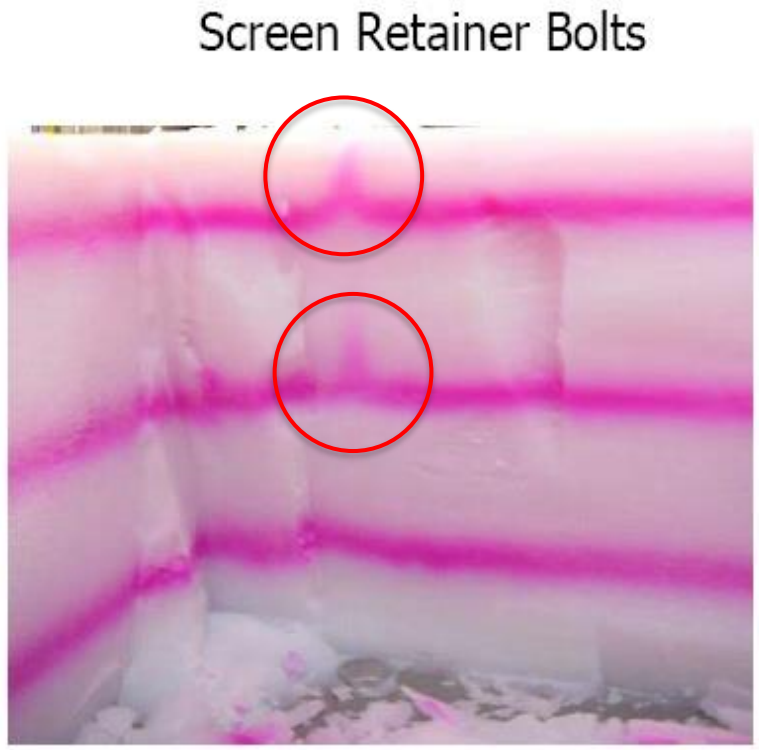


Figure 2. Summary of Sephacryl S-200 HR packing qualification

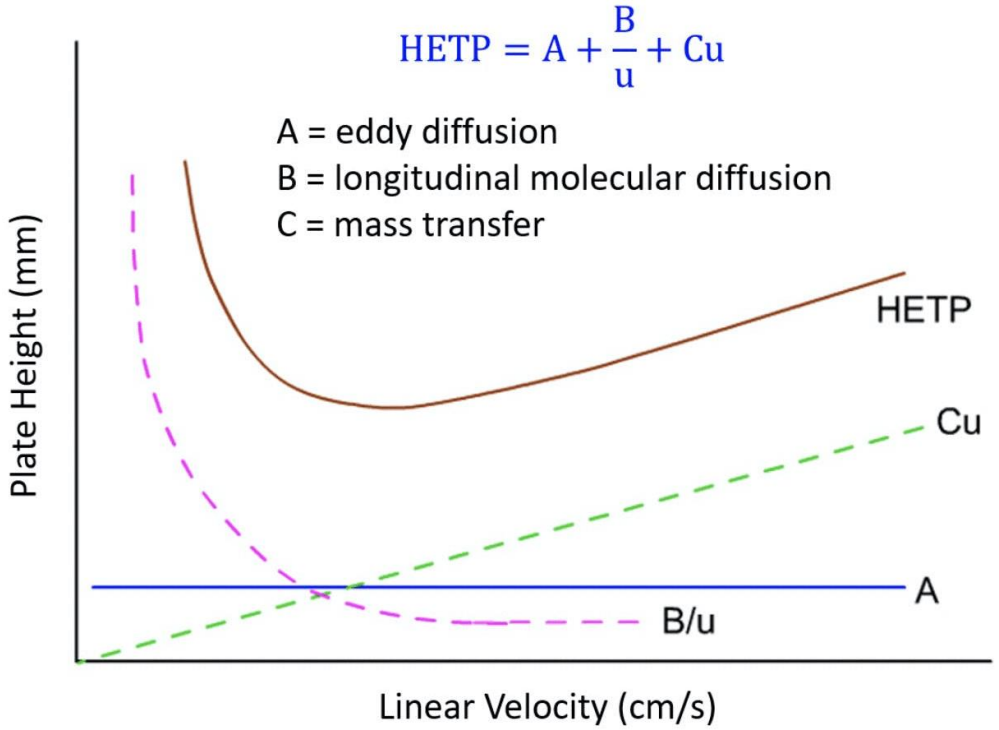




A.S. Rathore et al., BioPharm International, March 2003, 30-40

The van Deemter equation

$$H = A + \frac{B}{u} + C \times u$$

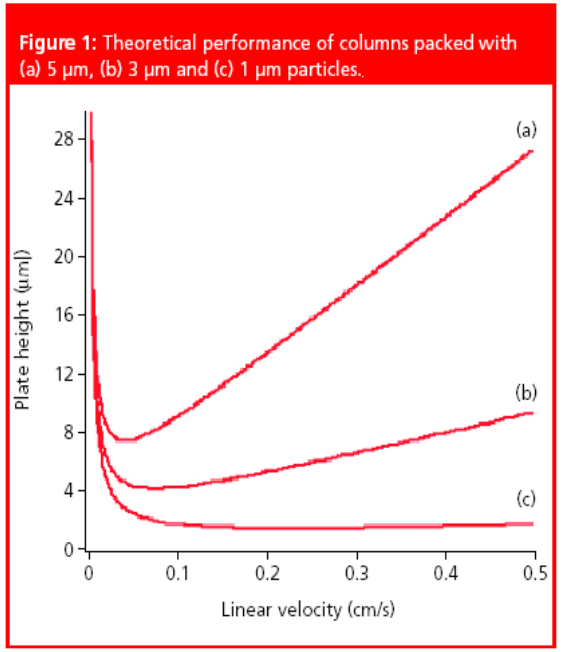


Beware!

1. The van Deemter equation is valid only for an isocratic elution of the solutes
2. Although it leads to lower H values, one cannot decrease the resin particle size indefinitely because of the corresponding increase in pressure drop

In protein chromatography, the C term rules!!!

- The van Deemter equation illustrates the difficulty to combine a low value for H (efficient separation) with a high flow rate/liquid velocity
- The A, B and C terms of the equation are related to packing quality, axial diffusion and mass transfer, respectively



Effect of flow rate and loading on resolution (in protein chromatography)

Column: SOURCE 30Q, 10 mm i.d. × 50 mm (4 ml)
Sample: Mixture of lactoglobulin B and amyloglucosidase
Sample load: 1 mg/ml bed volume
Start buffer: 20 mM BIS-TRIS PROPANE, pH 7.0
Elution buffer: 0.5 M sodium chloride, 20 mM BIS-TRIS PROPANE, pH 7.0
Flow: a) 4 ml/min (300 cm/h)
b) 13 ml/min (1000 cm/h)
Gradient: 0–100% elution buffer, 20 CV

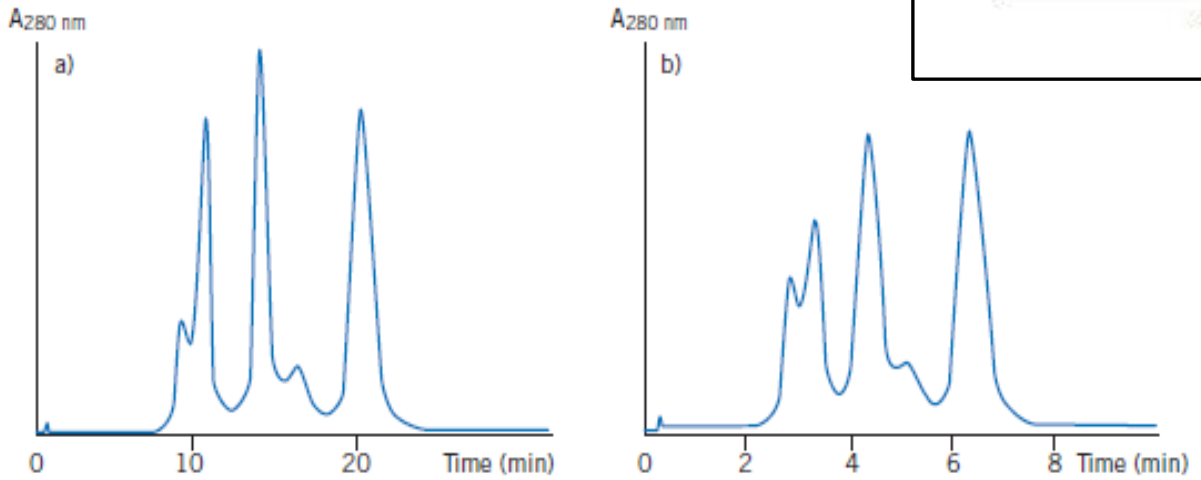
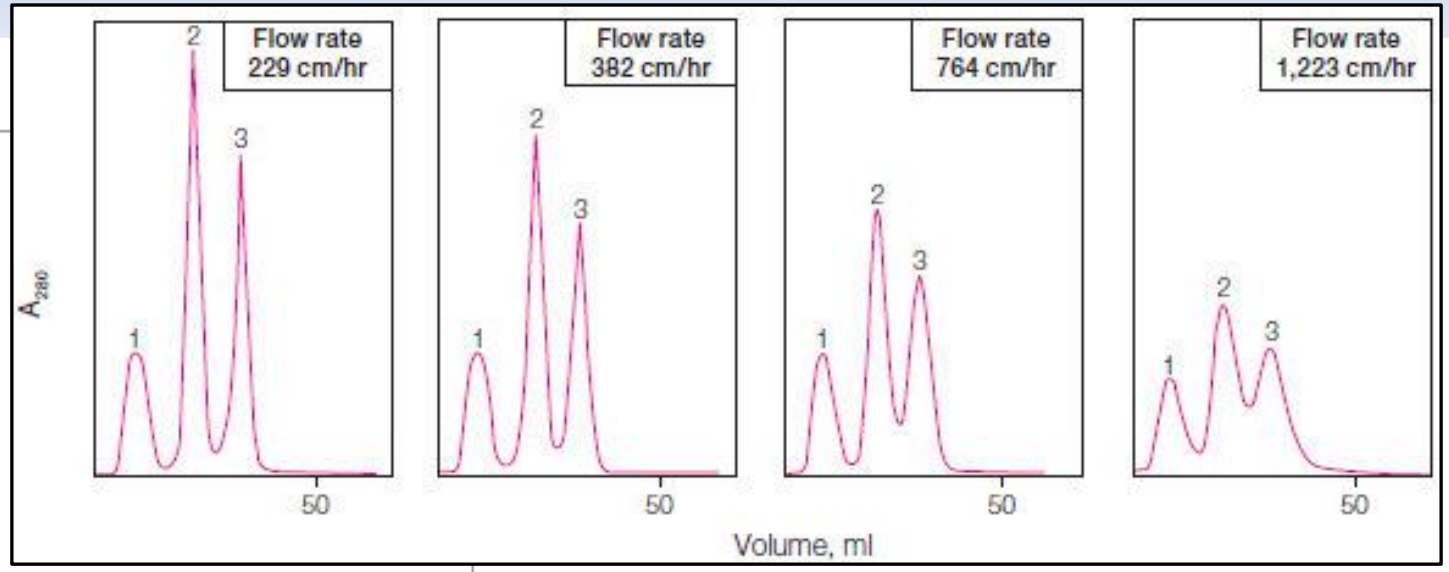
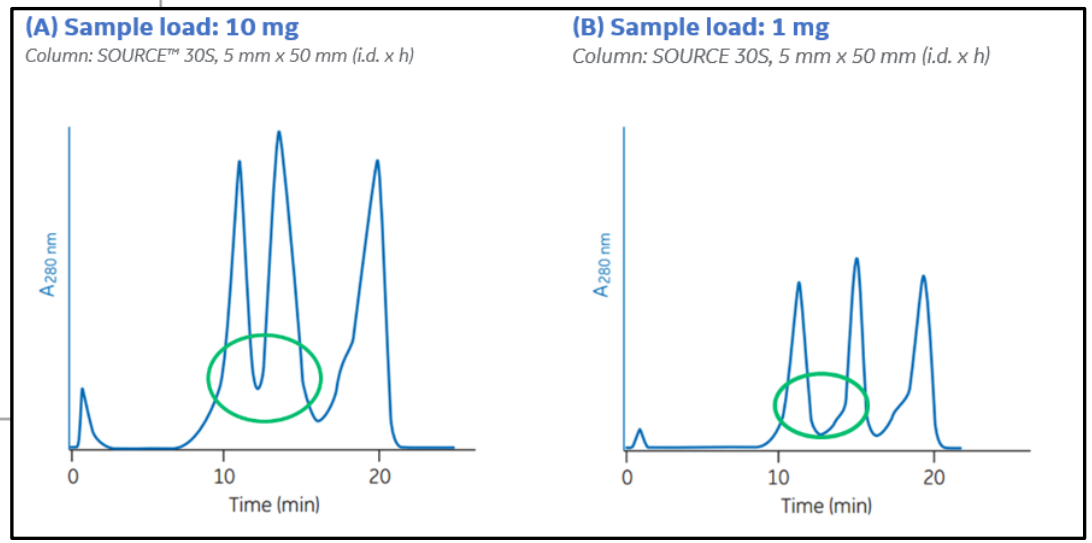


Fig. 26. Influence of increasing flow rate on resolution.





Source: Cytiva