EPFL minor in Biotechnology

Module ChE-437
Part 4 - Chromatography

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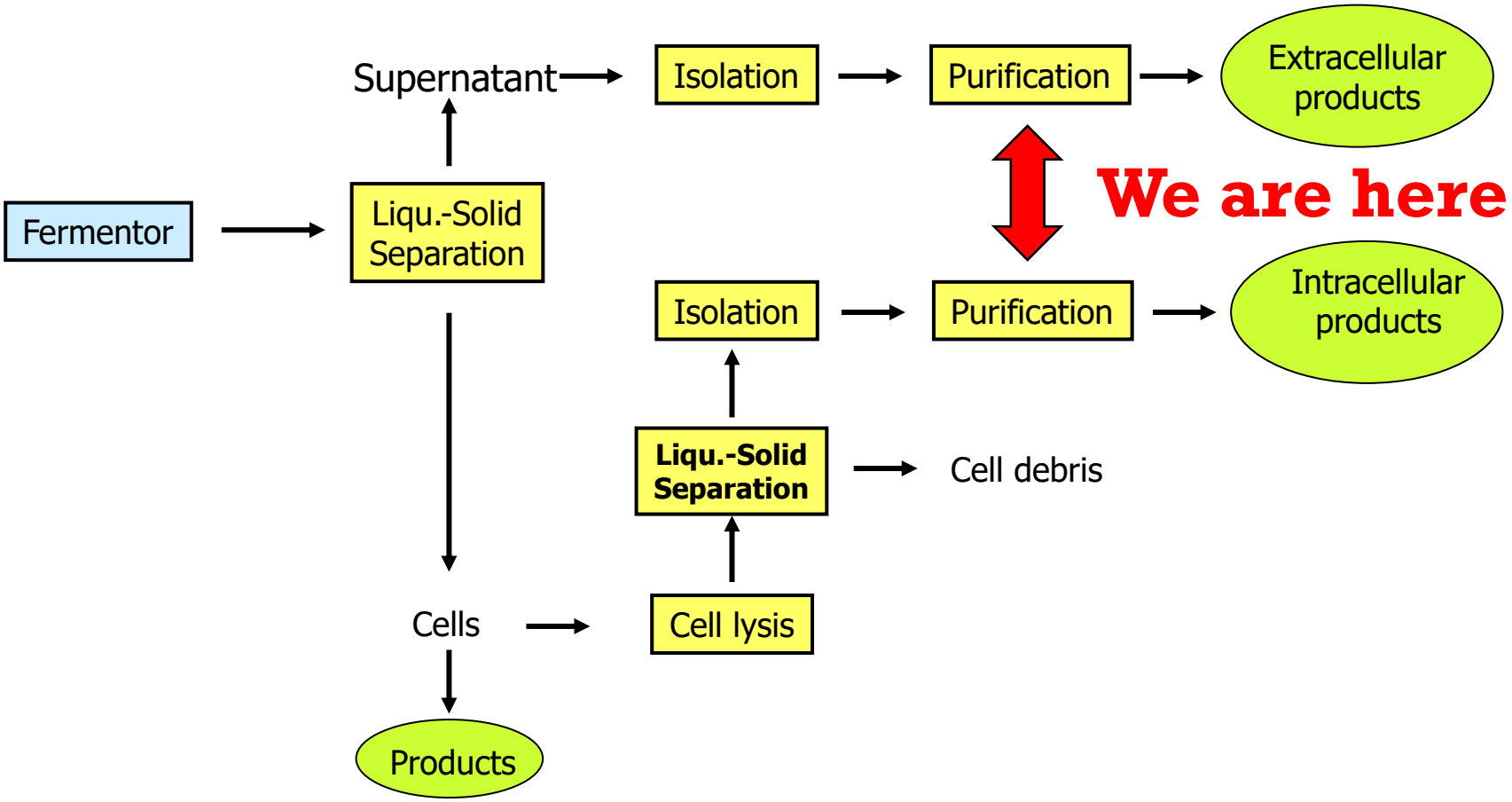
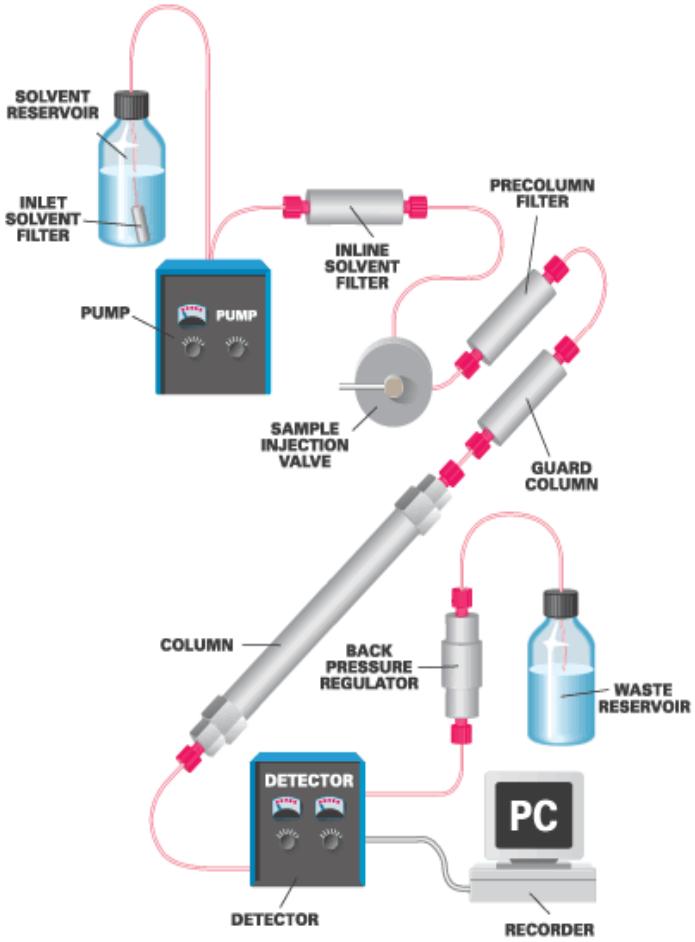
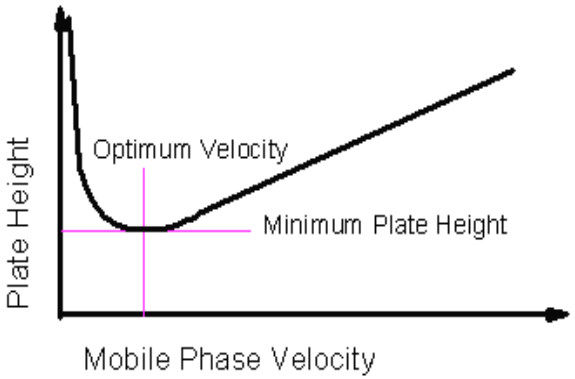
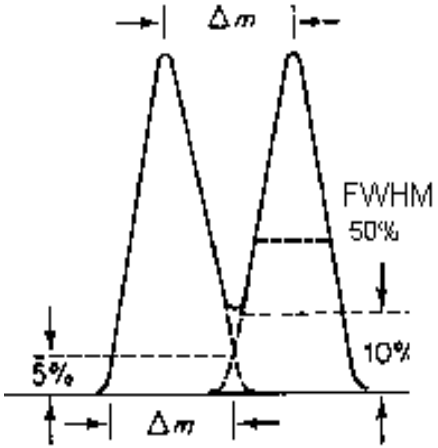
Common pathway for a purification protocol



6.4.1 A quick reminder







1. Chromatography is a **thermal technique**

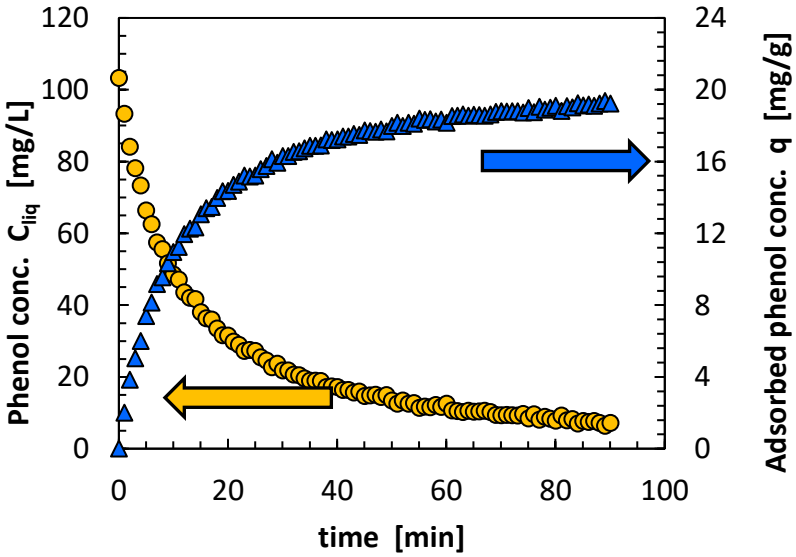
- It is based on the (selective) **adsorption** of solutes (the target molecule) on an **adsorbent** (the chromatography resin). One exception: Size Exclusion Chromatography (SEC)
- The **affinity** of the target molecule for the resin is dictated by thermodynamics
- The **adsorption isotherm** gives the adsorbed concentrations as a function of the residual solute concentration in the liquid phase **at equilibrium**. Answers the question «**how much** can I adsorb»? Reflects the resin **adsorption (static or equilibrium) capacity**
- Adsorption is not instantaneous and follows kinetics of various forms. Answers the question «**how fast** do I adsorb»?

Determination of adsorbed concentration q

- A volume of solution V_{liq} with a concentration C_0 [mg/mL] of adsorbate is contacted with a mass m_{resin} [g] of adsorbent (e.g. chromatography resin)
- As the solute adsorbs on the resin, the concentration C(t) in the liquid phase will decrease until it reaches an end value C_{eq} once equilibrium is reached
- At any time, the adsorbed concentration of solute q(t) [mg/g] can be determined by a mass balance: the quantitiy of solute that was removed from the solution is now adsorbed on the solid.
- Hence:

$$q(t) = \frac{V_{liq} \cdot (C_o - C(t))}{m_{resin}}$$

Time course of phenol adsorption on activated charcoal
V_{liq} = 250 mL, C_0 = 100 mg/L, m_{resin} = 1.25 g

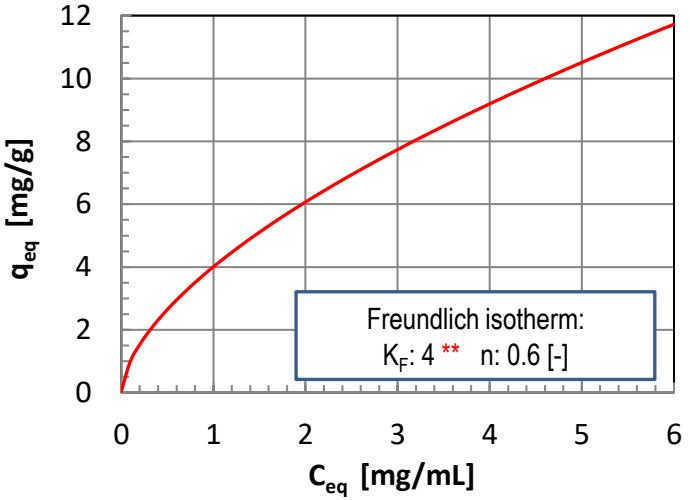


Adsorption isotherms: two popular models

Freundlich isotherm

$$q_{eq} = K_F \cdot C_{eq}^{n}$$

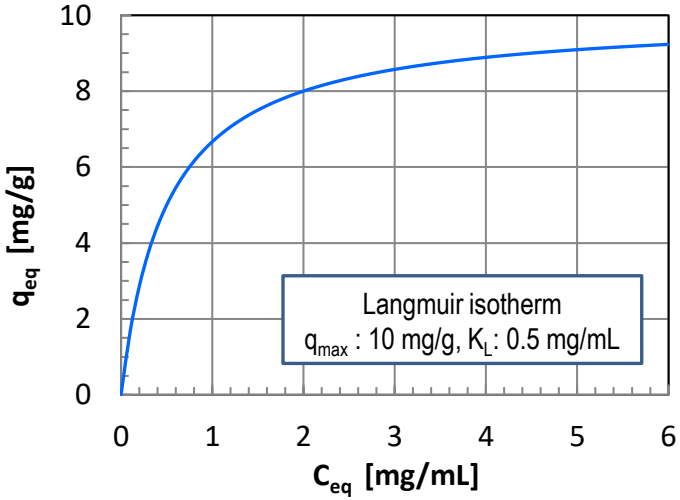
** Please note that the measuring units of K_F dépend on the value of n
Here, since n=0.6, K_F=4 [$mg^{0.4} \cdot mL^{0.6}/g$]

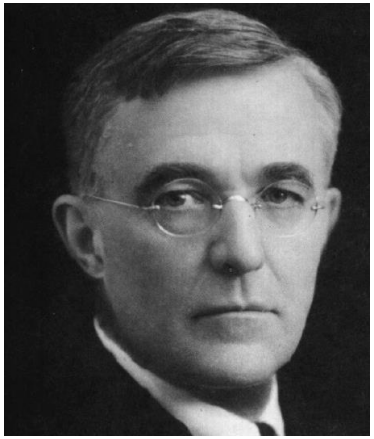


Herbert Max Finlay Freundlich
(1880-1941)

Langmuir isotherm

$$q_{eq} = q_{max} \cdot \frac{C_{eq}}{K_L + C_{eq}}$$





Irving Langmuir (1881-1957)
Chemistry Nobel Prize, 1932

NB: there are countless other models, but these two cover our basic needs

Determination of the model parameters

- In both cases, estimation of the model parameters can be done visually, by linearization or by curve-fitting
- Langmuir isotherm can be linearized in three different way. The Hanes-Woolf plot, i.e. (C_{eq}/q_{eq} vs C_{eq}) is recommended:

$$\frac{C_{eq}}{q_{eq}} = \frac{K_L}{q_{max}} + \frac{1}{q_{max}} \cdot C_{eq}$$

- Freundlich isotherm is linearized by plotting $\ln(q_{eq})$ as a function of $\ln(C_{eq})$

$$\ln(q_{eq}) = \ln(K_F) + n \cdot \ln(C_{eq})$$

- **Non-linear regression (curve-fitting)** should however be preferred

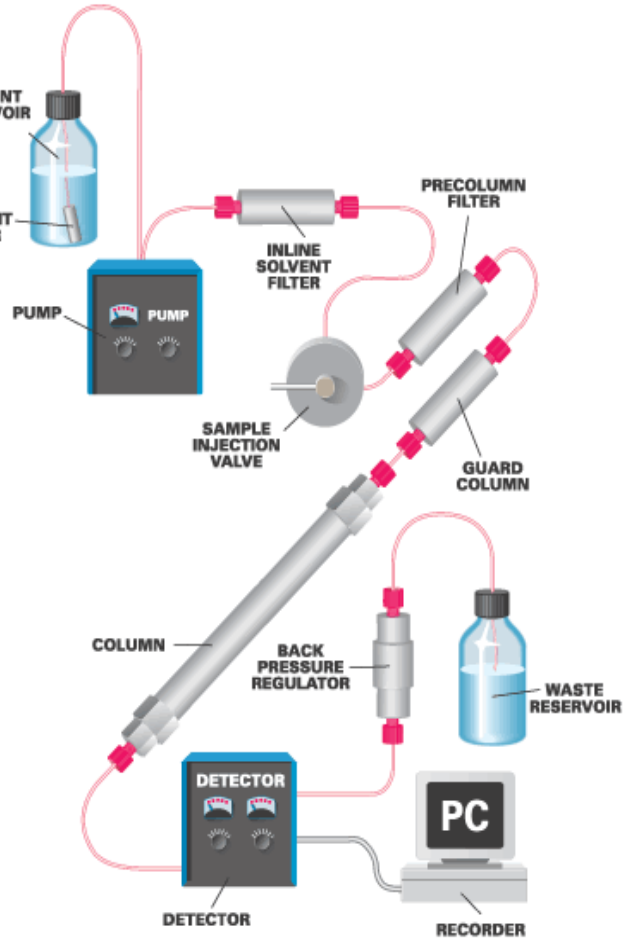
2. Preparative, bind & elute chromatography

- We will discuss only **preparative**, industrial scale chromatography
- In preparative chromatography, interesting **eluted fractions are collected** and further processed/purified
- As opposed **to isocratic elution**, **bind & elute chromatography** relies on adsorbing **(binding)** target molecules and releasing **(eluting)** them in an ordered time sequence by altering a parameter (pH, ionic strenght …)
- This type of chromatography goes through the following stages: **load, wash, elution and regeneration** before starting again
- Chromatography is performed **batchwise**. Its capacity is usually limited but its resolution power can be extremely high. Yield and speed are usually good

Components of chromatography equipment …

… are the same at small and large scale

- Pumps and eluents
- Pipes, tubing, valves
- Injection/loading system
- Stationary phases (resins)
- Detector(s)
- Fraction collector is specific to preparative chromatography



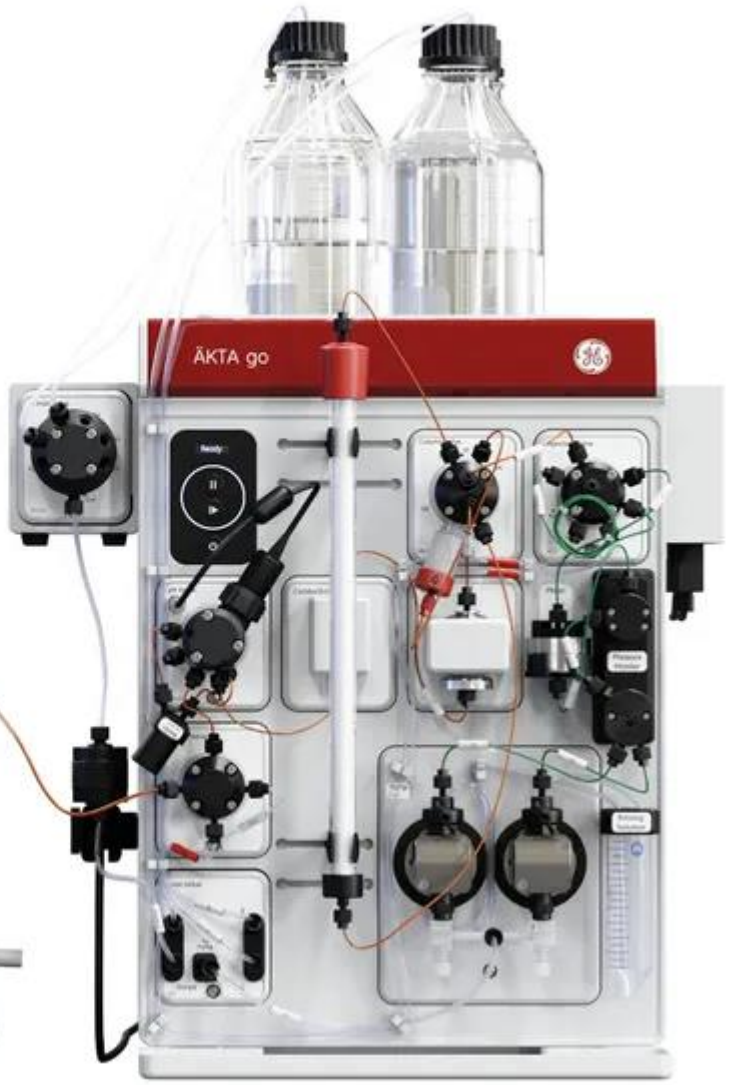
A great development tool …

This range of preparative chromatography devices is a standard in the biophyrmaceutical industry.

Its different models are all running on the same operating system/platform.

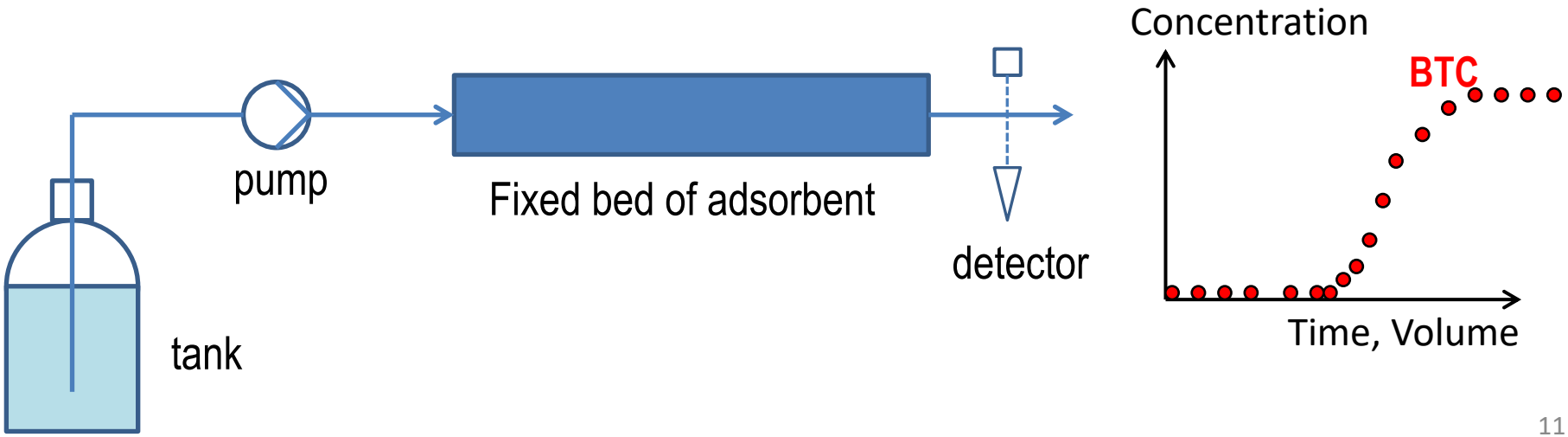
This enables a rapid and smooth development of chromatography purification protocols from bench- to production scale.

Check the fraction collector !!!



Breakthrough curve, static and dynamic capacity

- A solution of adsorbate is pumped continuously through a column filled with adsorbent
- The solute gradually adsorbs on the solid phase, until it can be detected in the exiting stream.
- The measure of the exit concentration as a function of time is called a **breakthrough curve (BTC)**
- The experimental set-up is usually as follows:



Breakthrough curve, static and dynamic capacity

Static capacity L_t

$$L_t = Q \cdot C_0 \cdot \int_0^{\infty} \left(1 - \frac{C}{C_0}\right) \cdot dt \qquad (4.1)$$

Since flow rate Q is constant:

$$L_t = C_0 \cdot \int_0^{\infty} \left(1 - \frac{C}{C_0}\right) \cdot dV \qquad (4.2)$$

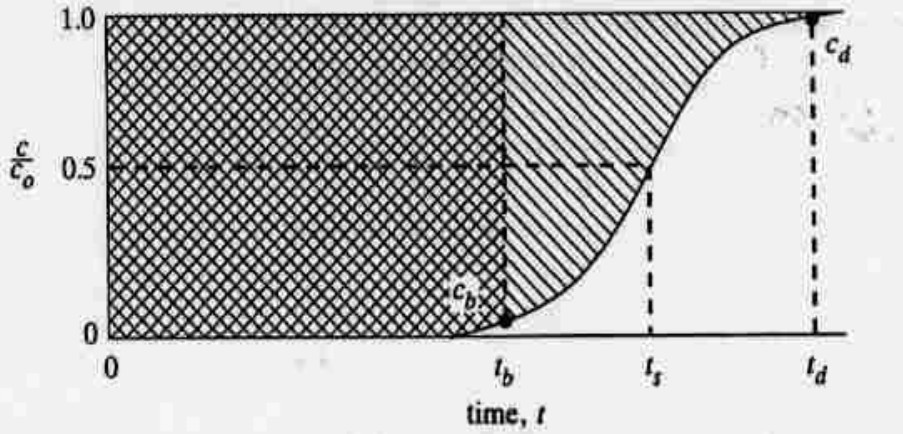


FIGURE 12.3-2. *Determination of capacity of column from breakthrough curve.*

Dynamic capacity L_u

$$L_u = Q \cdot C_0 \cdot \int_0^{t_b} \left(1 - \frac{C}{C_0}\right) \cdot dt \qquad (4.3)$$

Or also:

$$L_u = C_0 \cdot \int_0^{t_b} \left(1 - \frac{C}{C_0}\right) \cdot dV \qquad (4.3_{bis})$$

Remark: since flow rate is assumed constant, the X axis can be time, volume, quantity of solute or quantity of solute divided by adsorbent mass that has been pumped through the column

Types of (preparative) chromatography

- Cation exchange chromatography (CEX)
- Anion exchange chromatography (AEX)

IEX

- Hydrophobic interaction chromatography (HIC)
- Mixed mode chromatography (MMC)
- Affinity chromatography (AC)
- Size exclusion chromatography (SEC)

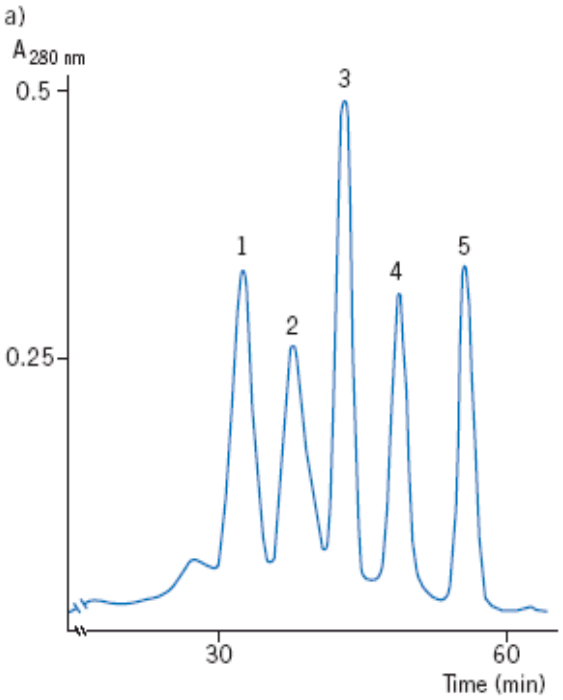
A chromatogram conveys a lot of information

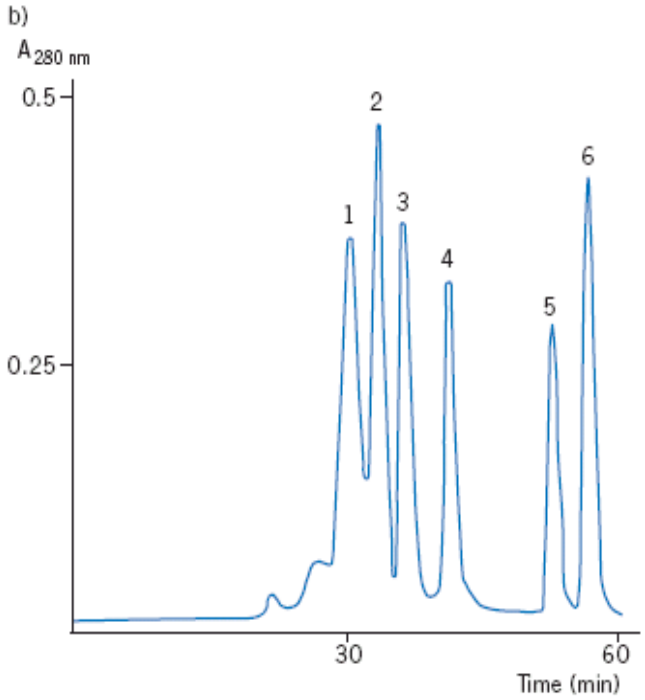
Column: Superose 6 HR 10/30
Sample: 100 µl solution containing:
1. Thyroglobulin (M_r 669 000), 5 mg/ml
2. Ferritin (M_r 440 000), 0.3 mg/ml
3. Bovine serum albumin (M_r 67 000), 8 mg/ml
4. Ribonuclease A (M_r 13 700), 5 mg/ml
5. Glycyl tyrosin (M_r 238), 0.6 mg/ml

Buffer: 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.0
Flow: 0.4 ml/min

Column: Superose 12 HR 10/30
Sample: 100 µl solution containing:
1. IgG (M_r 160 000), 2.5 mg/ml
2. BSA (M_r 67 000), 8 mg/ml
3. β-lactoglobulin (M_r 35 000), 2.5 mg/ml
4. Cytochrome C (M_r 12 400), 1 mg/ml
5. Vitamin B12 (M_r 1 355), 0.1 mg/ml
6. Cytidine (M_r 243), 0.1 mg/ml

Buffer: 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.0
Flow: 0.4 ml/min





Column?
Stationary phase(s)?
Detector(s)?
Eluent(s)?
Flow rates?
Gradients?
Temperature?
pH?
Ionic strength?

Detection of proteins is often done at 280 nm

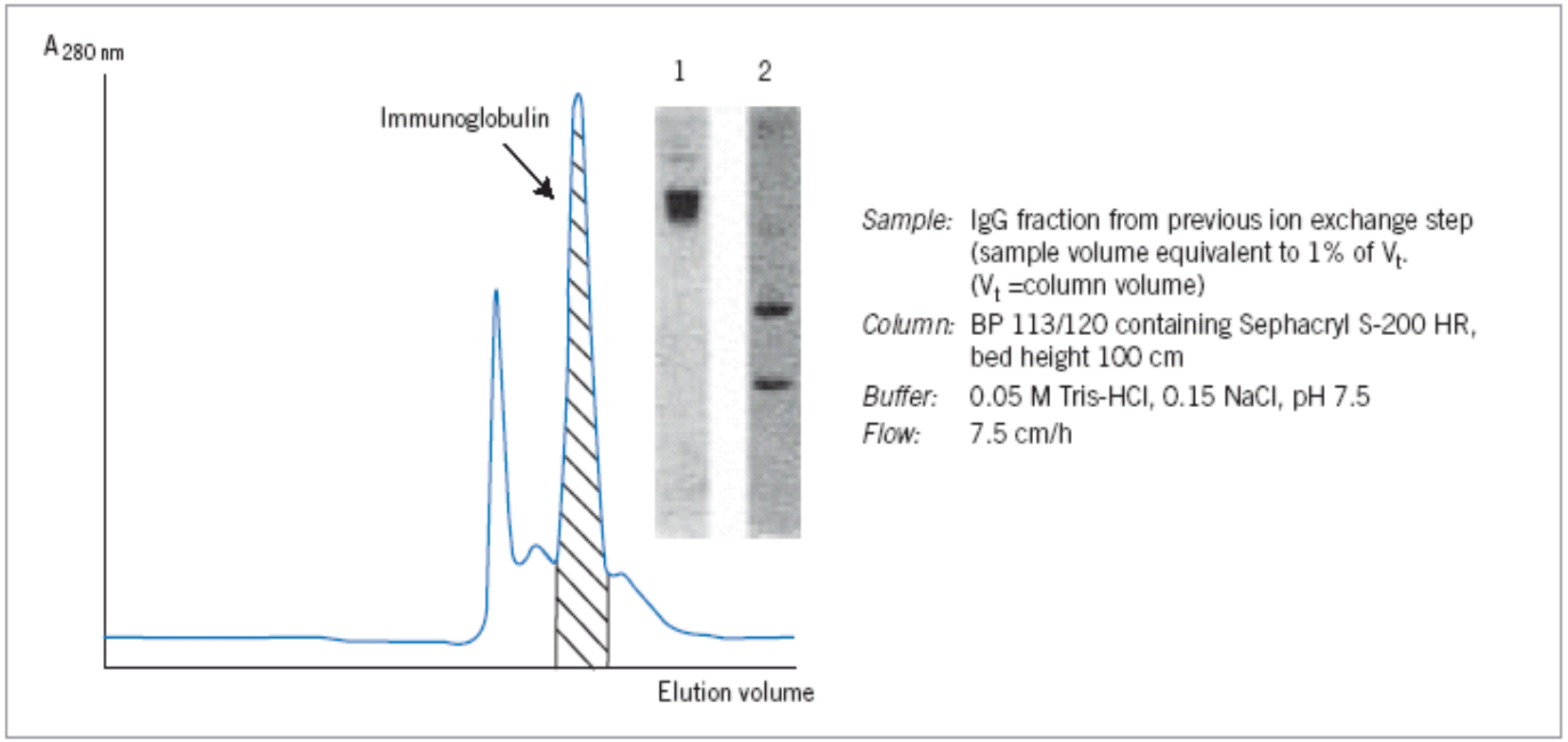


Fig. 34. Purification of monoclonal antibodies on Sephacryl S-200 HR. Inset shows analysis by gradient SDS-PAGE of the immunoglobulin pool. Lane 1, native sample; lane 2, sample reduced with 2-mercaptoethanol.

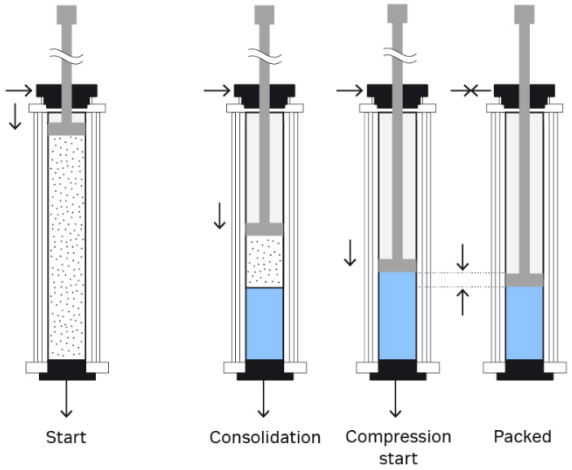
However, **identification** of the peak corresponding to the target molecule requires some specific analytical method

Industrial columns: design aspects

- Most of the time, the resin bed height is kept constant
- It is the column diameter, on the other hand, that is increased by a large factor for scale up
- Flow rate is then set to keep the superficial velocity of the eluent constant

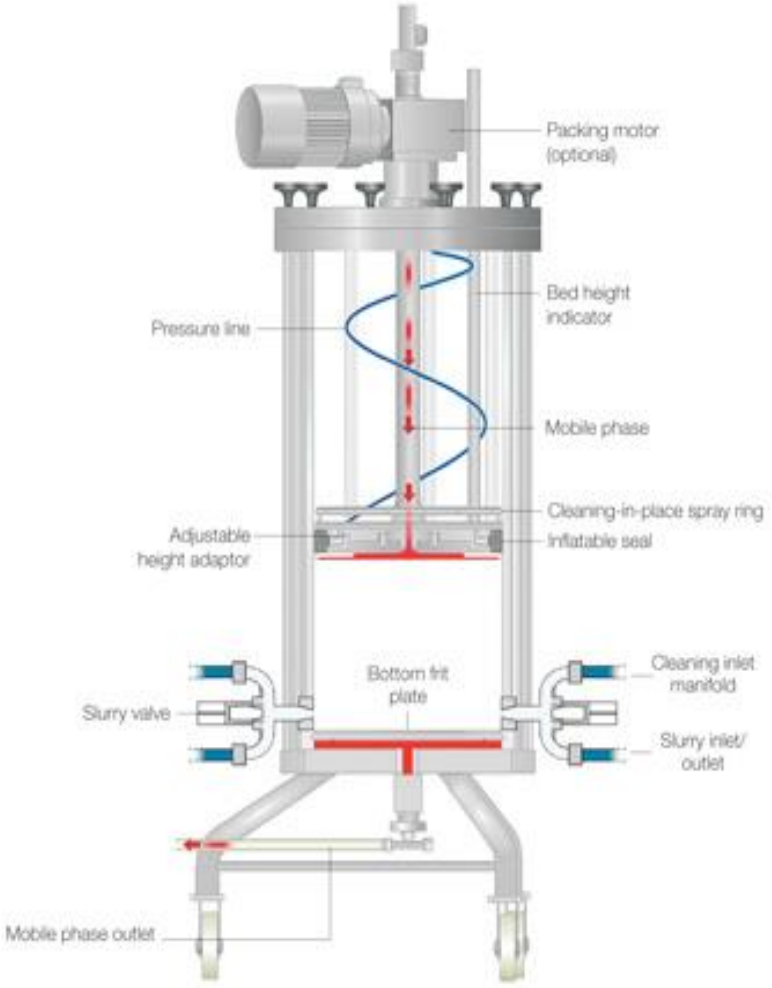
Packing of an industrial column

- How is it done?
- What should be checked?



Source: Cytiva

Column filling and packing



Schematic drawing of Bio-Rad InPlace column.

Bio-Rad EasyPack Column

Bio-Rad InPlace Column and Control Console

BIO-RAD

Video on packing protocols for pilot and industrial columns

Importance of column packing

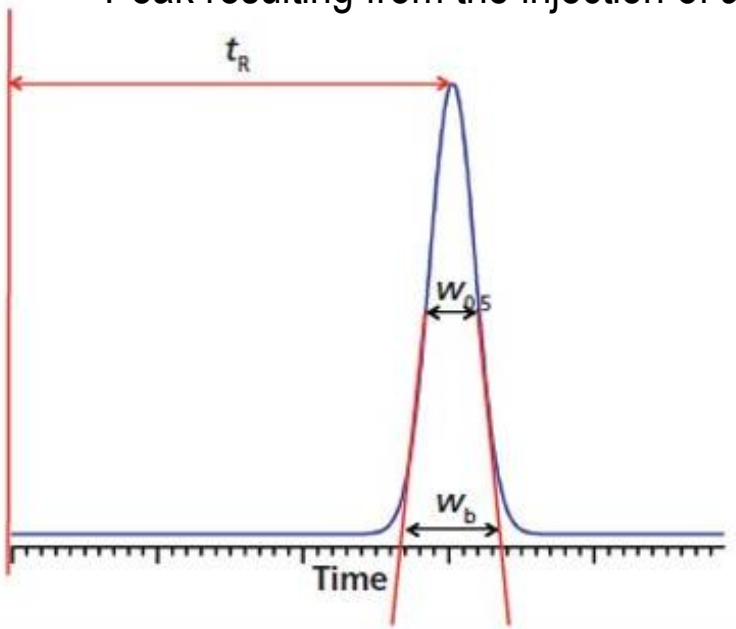
- The quality of column packing is key to the separation efficiency
- Beads should be distributed in a highly homogenous manner and the bed should be devoid of cracks and channels
- Packing can be done manually for lab-scale columns, but has to be automatized for production-scale systems
- There are 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

Properties of the resin's matrix material

- Highly porous matrices offer high adsorption surface and capacity
- Non-porous matrices are better suited to high resolution separations
- Inert matrices reduce the risk of non-specific interactions with solutes
- A good physical stability ensure that the volume of the fixed bed does not change even in the case of large pH or ionic strength variations
- Good mechanical rigidity and uniformly sized particles allow high flow rates, in particular during the cleaning and re-equilibration steps
- Good chemical stability allows cleaning of the resin even at extreme pH values or in the presence of organic solvents
- Like for adsorption, most stationary phases are based either on synthetic polymers (e.g. polystyrene / DVB) or on natural polymers (e.g. agarose)

Number and equivalent height of theoretical plates

Peak resulting from the injection of a **non-adsorbing marker**



Source: www.chromatographyonline.com

$$N = \frac{(t_R)^2}{\sigma^2} \qquad (4.4)$$

- There are various formulas to calculate N from the peak geometry. They all derive from the basic properties of Gaussian peaks
- The formulas of equation 4.5 are the most commonly used

$$N = 5.54 \cdot \left(\frac{t_R}{W_{0.5}}\right)^2 = 16 \cdot \left(\frac{t_R}{W_b}\right)^2 \qquad (4.5)$$

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

$$H = \frac{L}{N} \qquad (4.6)$$

Peak asymmetry

- 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 the peak height:

- Asymmetry factor

$$F_a = \frac{B}{A} \qquad (4.7)$$

- Tailing coefficient

$$F_t = \frac{A+B}{2A} \qquad (4.8)$$

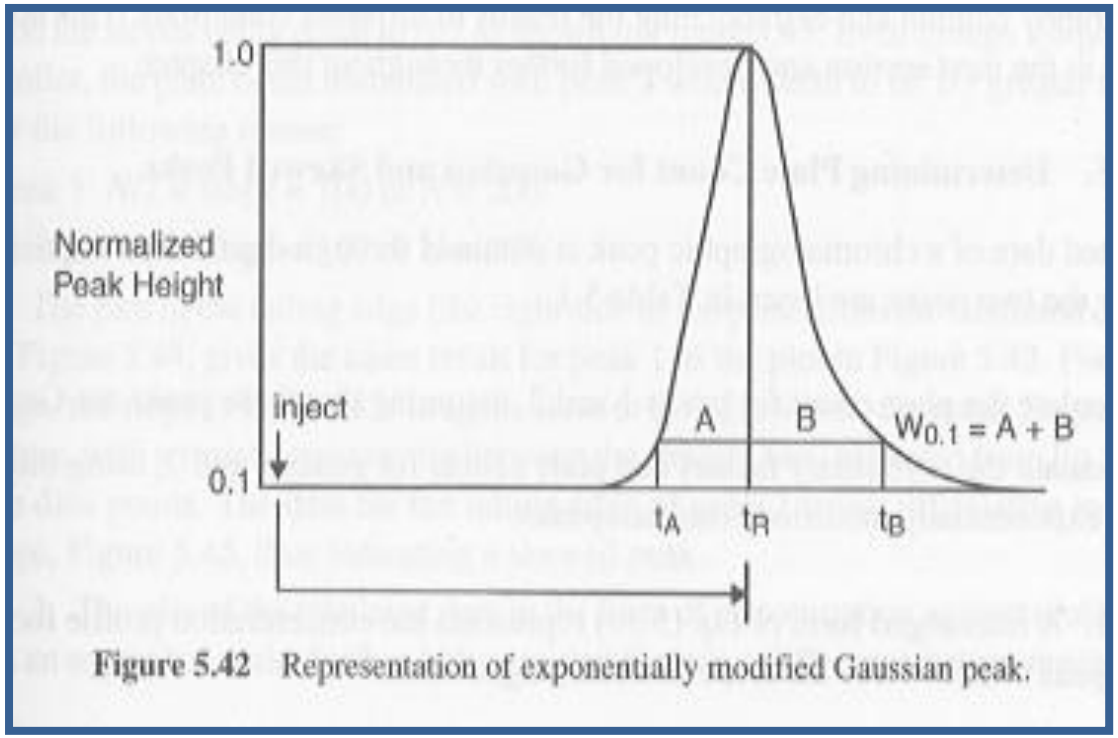
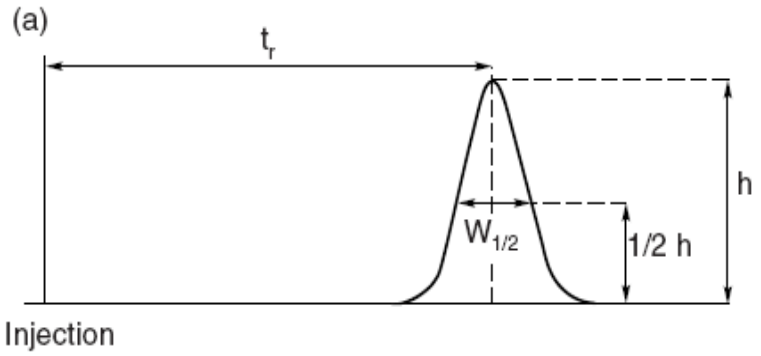


Figure 5.42 Representation of exponentially modified Gaussian peak.

Packing quality



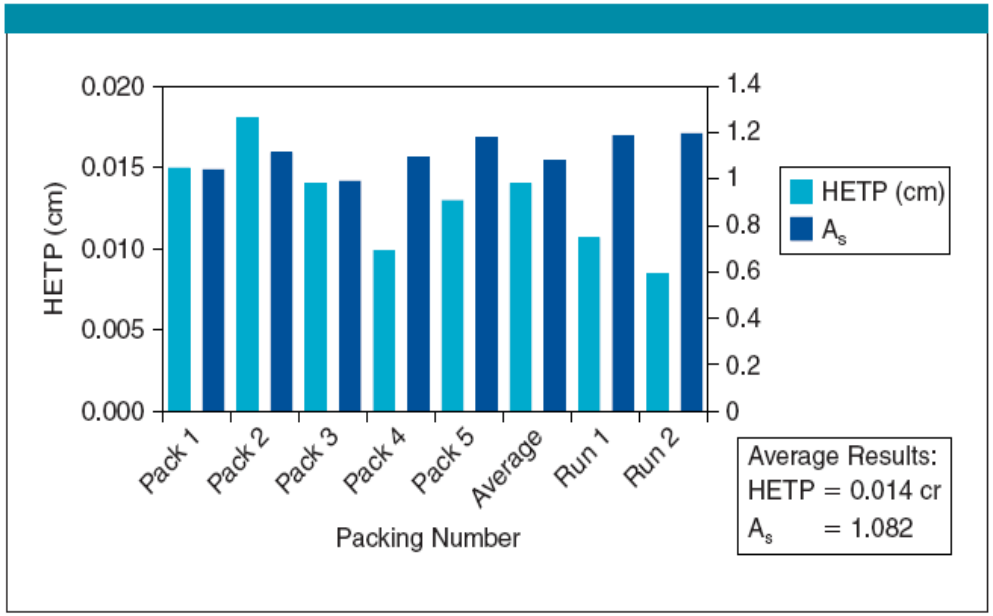


Figure 2. Summary of Sephacryl S-200 HR packing qualification

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

Influence of the liquid distribution system

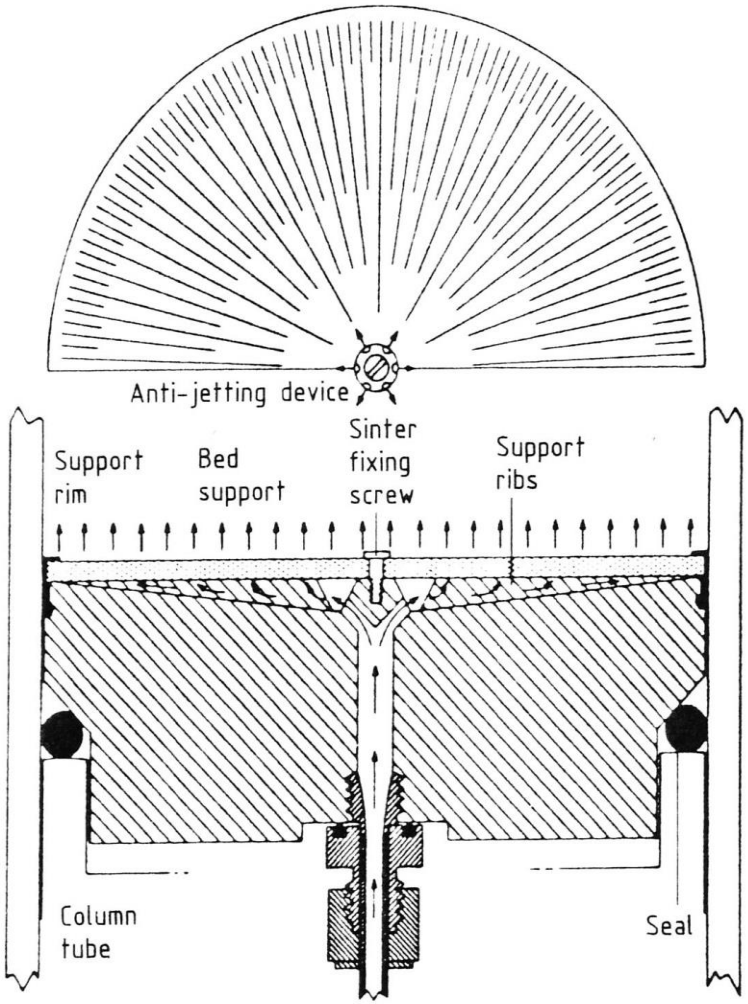
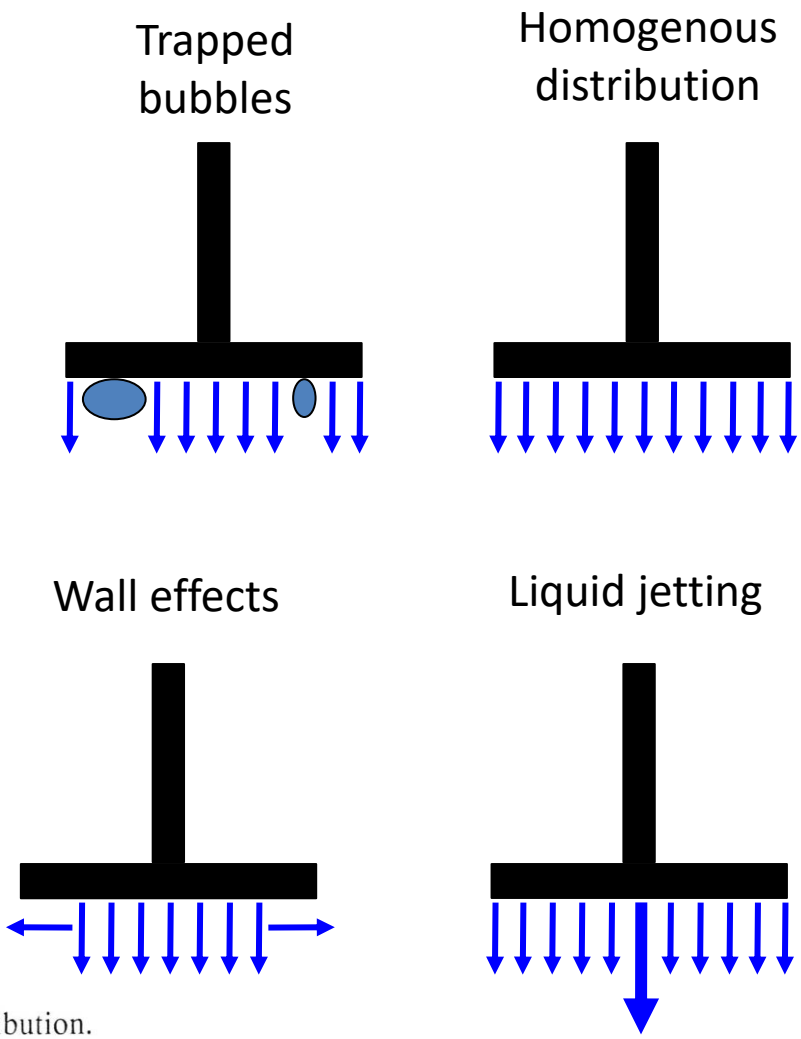
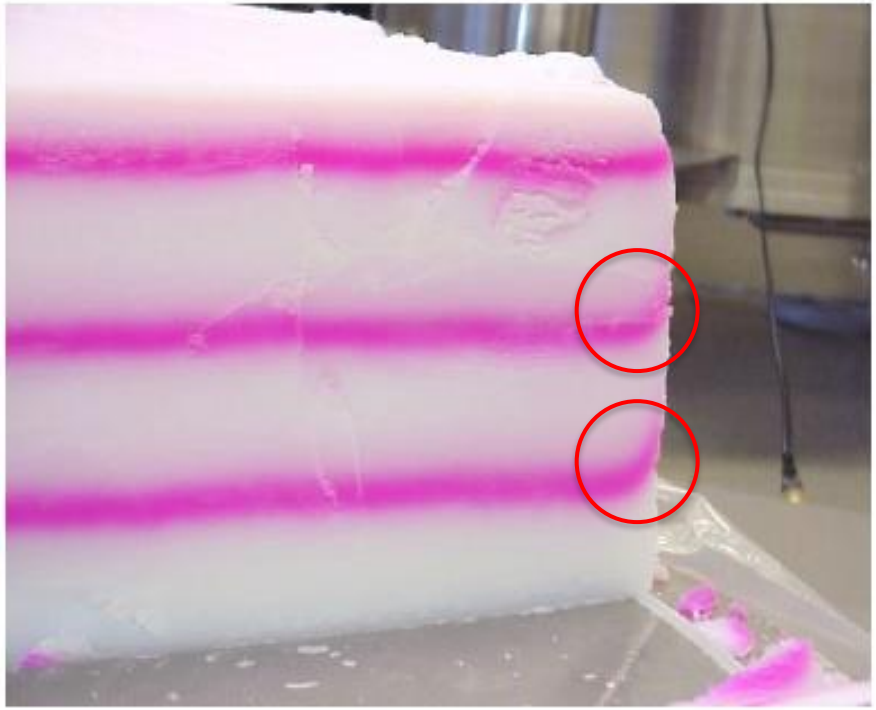


Fig. 1-3. Example of column end cell design providing uniform flow distribution.

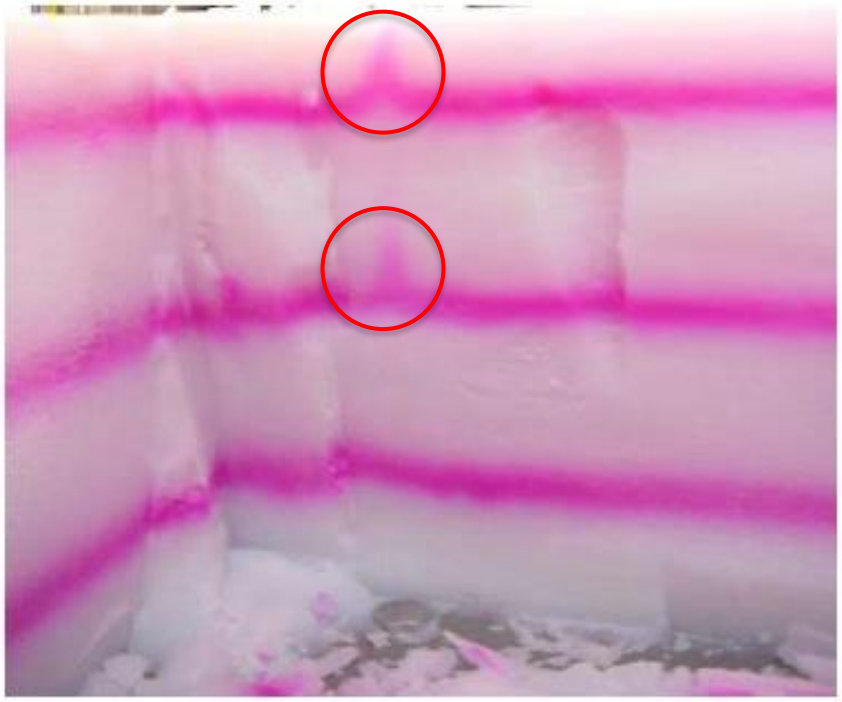


Measurement of column performance (dye test) (1/2)

Wall Effect

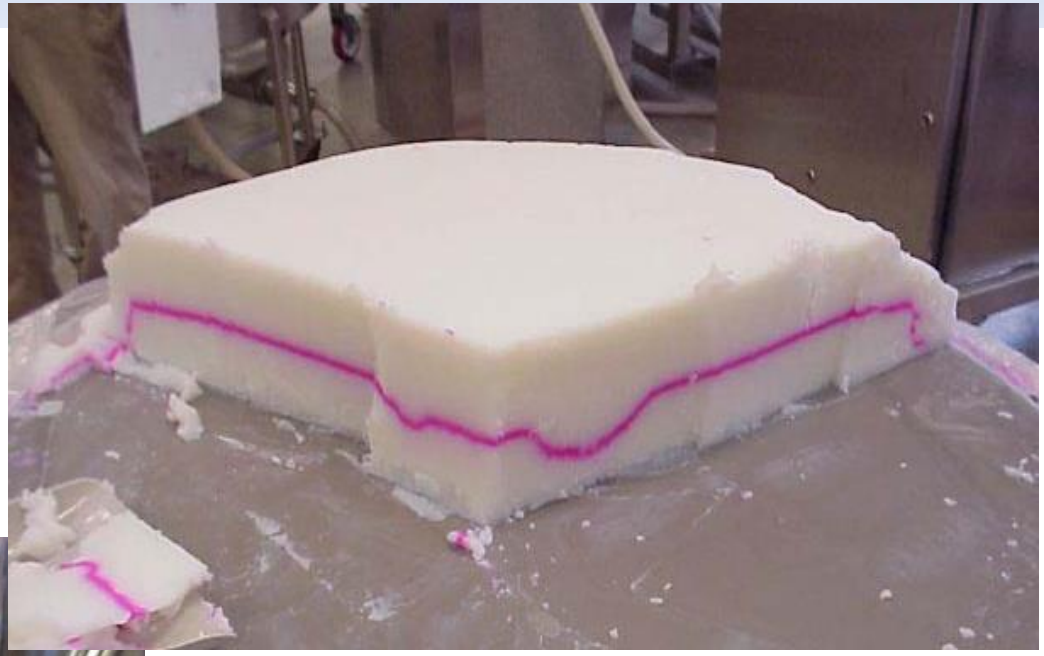


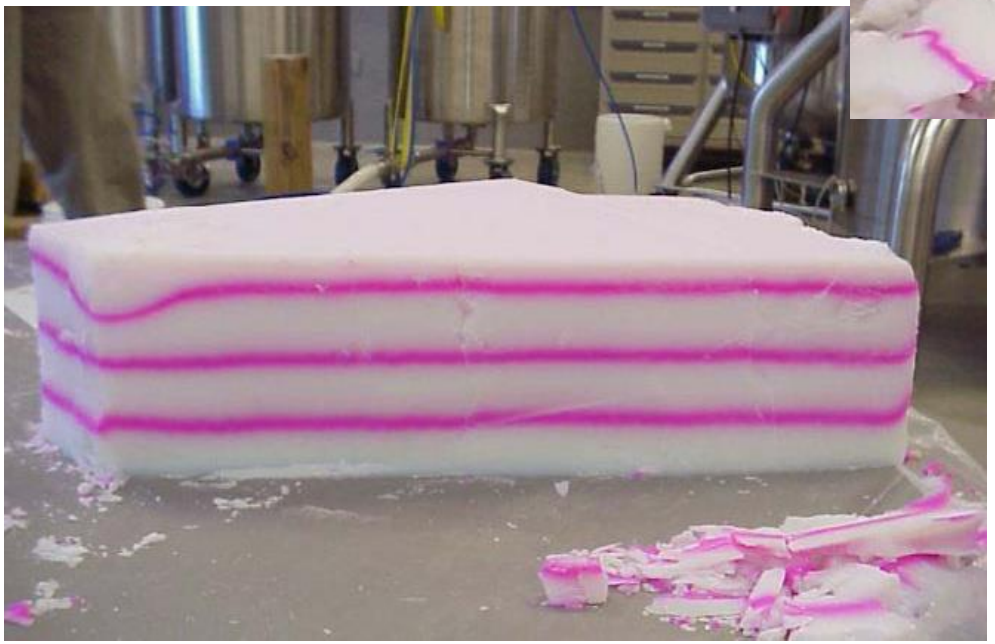
Screen Retainer Bolts



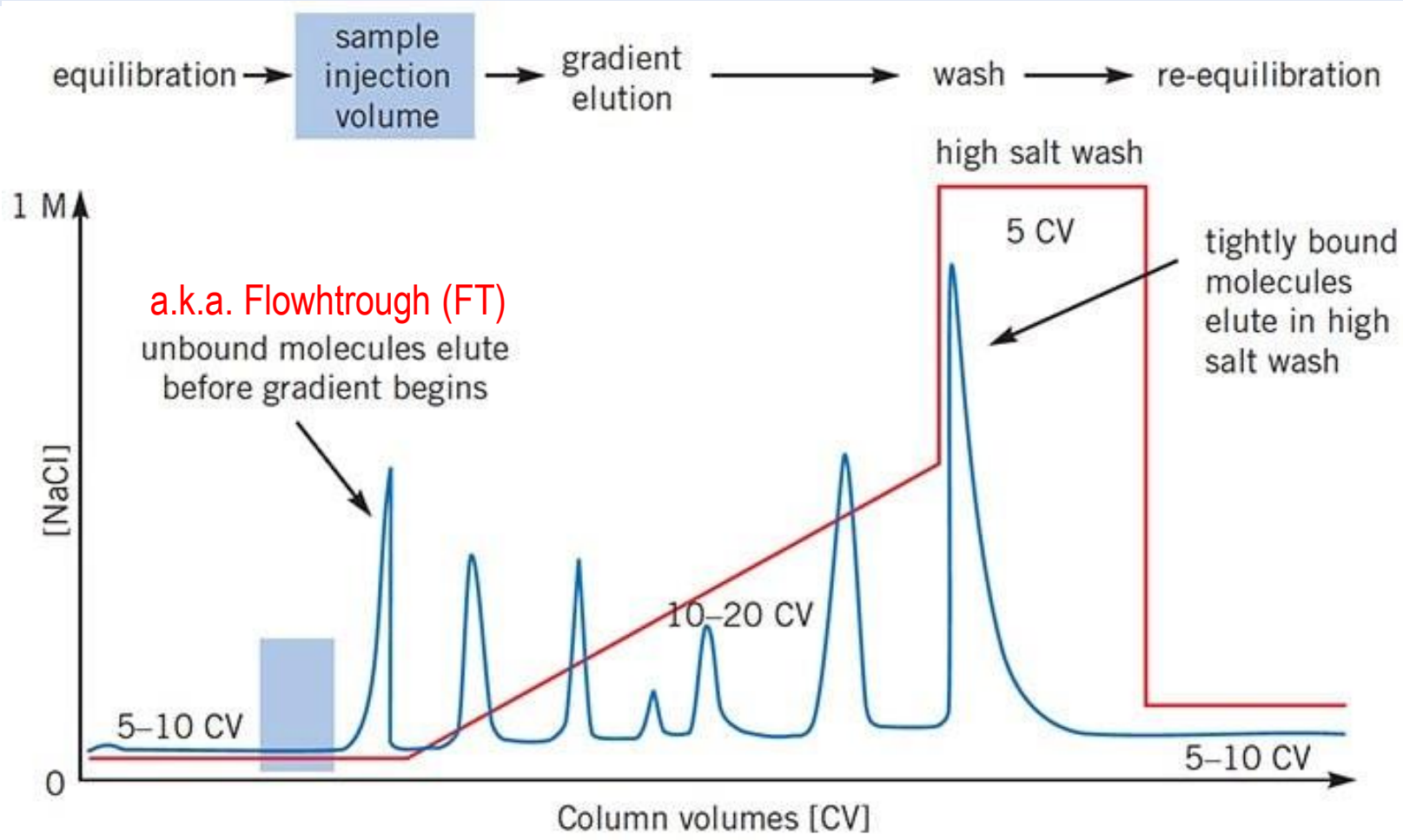
The repeated injection of a dye tracer allows a direct, visual evaluation of the column behaviour and performance

Measurement of column performance (dye test) (2/2)



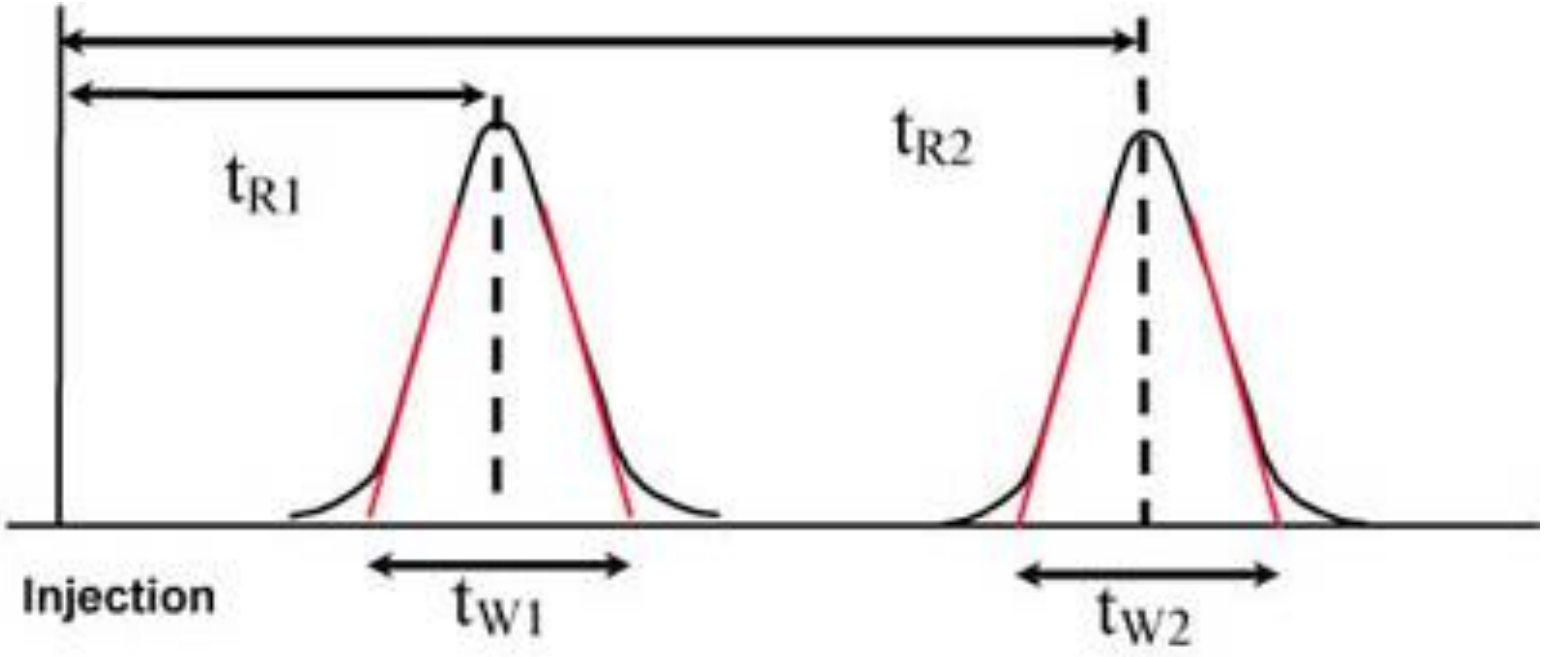


The different steps in a typical separation



Any idea on the type of chromatography being depicted here?

Resolution between two peaks



- This parameter is an expression of how efficiently two compounds are separated

$$R = 2 \cdot \frac{t_{R2} - t_{R1}}{t_{W1} + t_{W2}} \qquad (4.9)$$

Resolution: example for two identical peaks

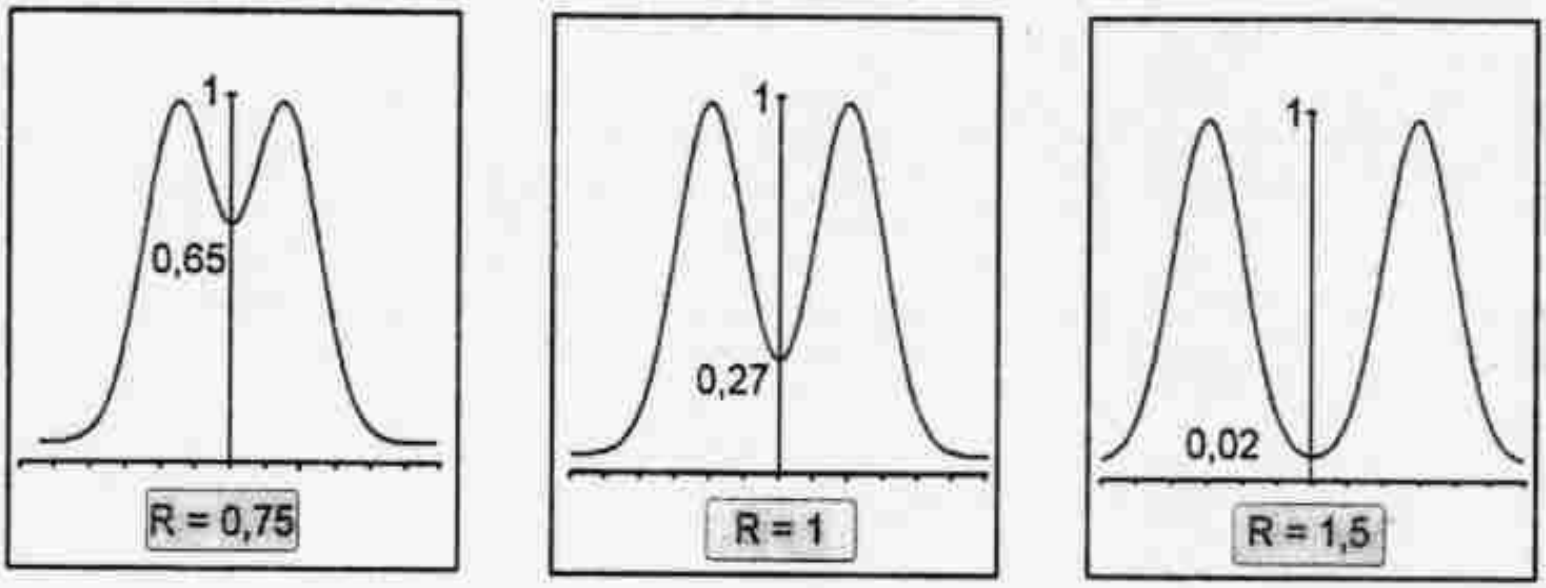
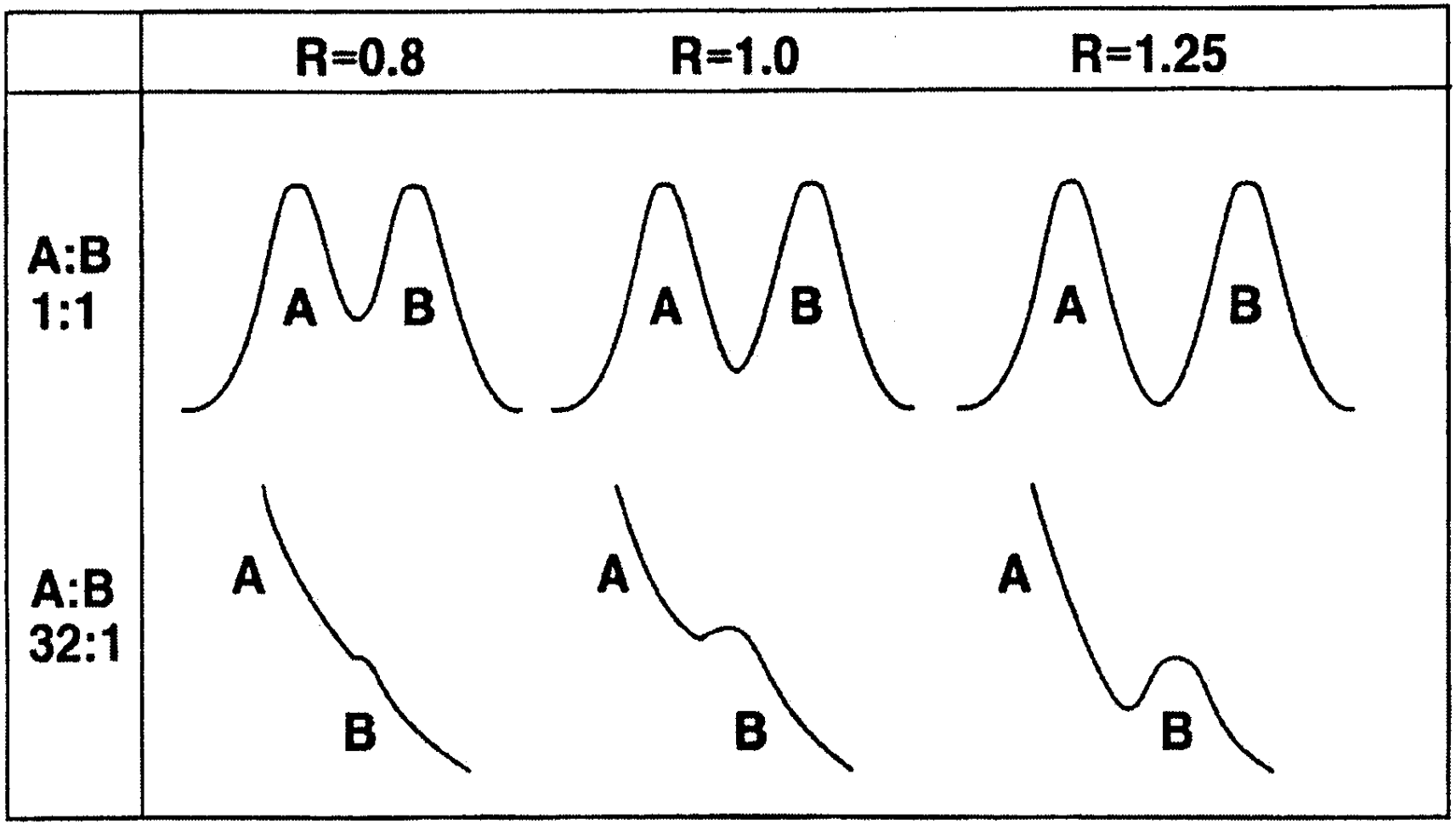


Figure 1.8 – *Facteur de résolution*. Simulation de pics chromatographiques par juxtaposition plus ou moins rapprochée de 2 courbes gaussiennes identiques. Aspect visuel correspondant aux valeurs de R indiquées sur les diagrammes. A partir de R = 1,5 on considère que les pics sont résolus, la vallée entre les pics étant d'environ 2 %.

Influence of relative quantities on resolution



Influence of the relative amounts of separated components on peak resolution

The van Deemter equation $H = A + \frac{B}{u} + C \times u$

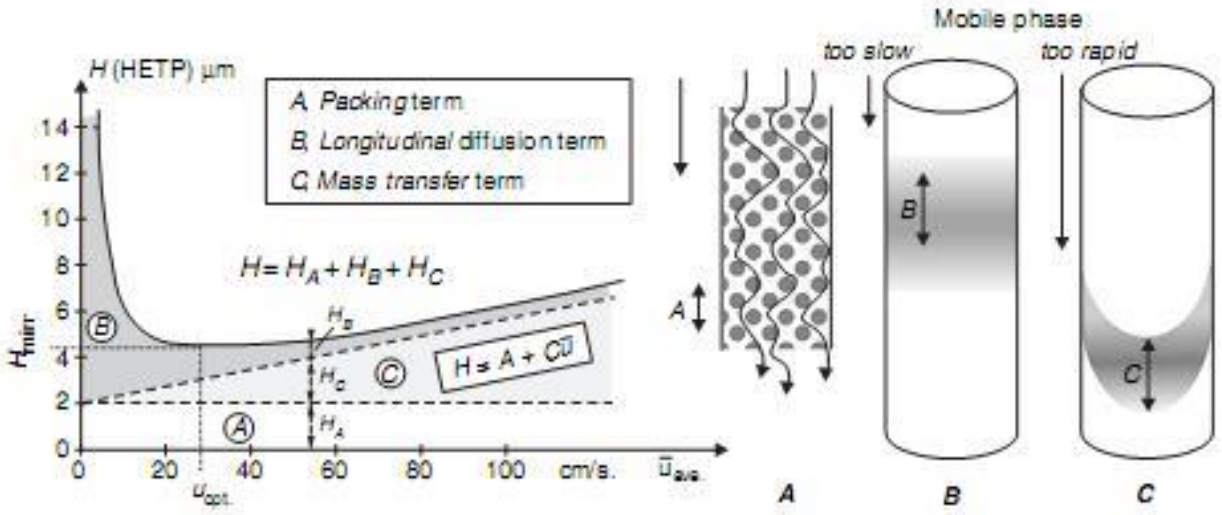
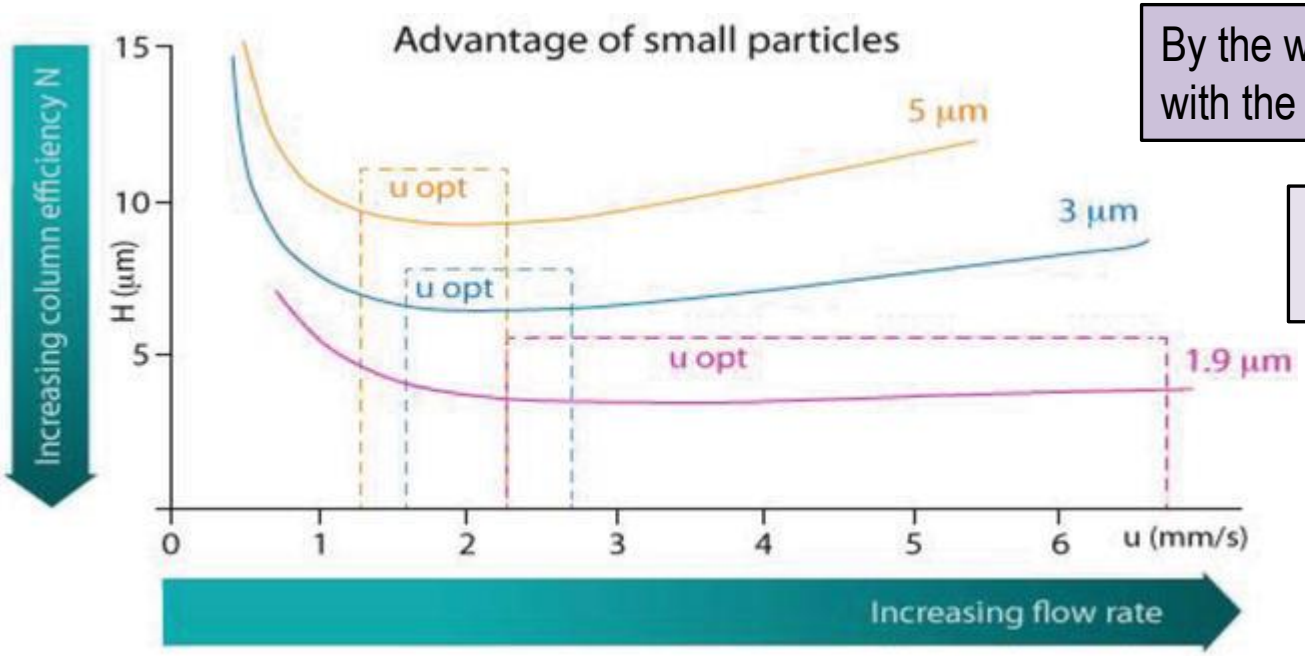


Figure 1.11 *Van Deemter's curve in gas chromatography with the domains of parameters A, B and C indicated.* There exists an equation similar to that of Van Deemter that considers temperature: H = A + B/T + CT.

- 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
- The «C-term» is the most influential in protein chromatography !!!

Influence of particle size on plate height



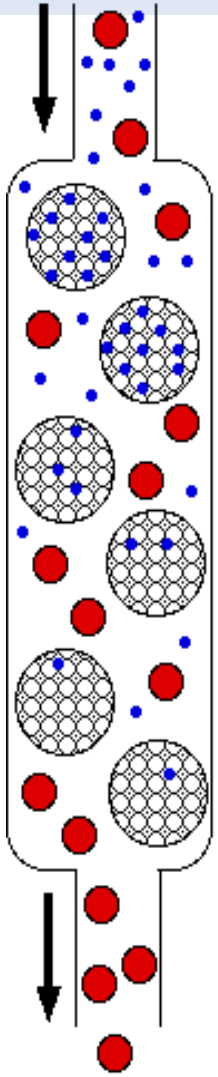
By the way, how will Δp increase with the linear velocity of the liquid?

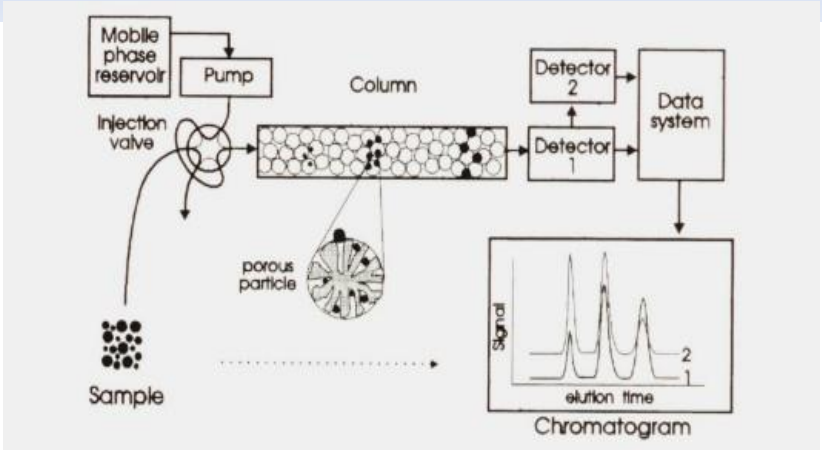
The **Ergun equation** shall bring you the answer

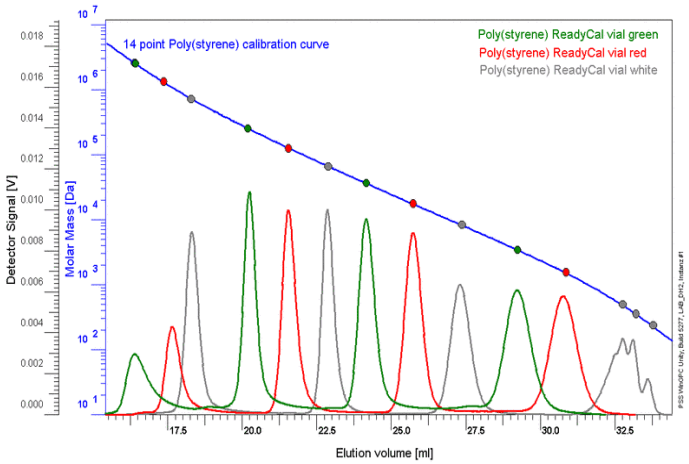
Beware!

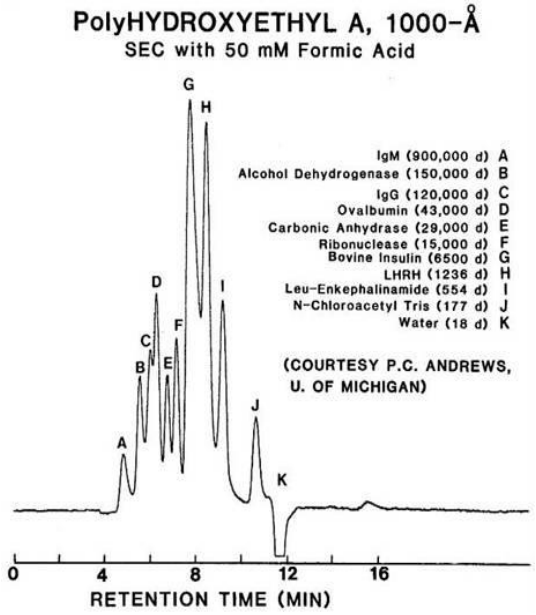
1. The van Deemter equation is valid only for an isocratic elution of the solutes
2. Although it leads to lower H values, one cannot decrease the resin particle size indefinitely because Δp will increase dramatically

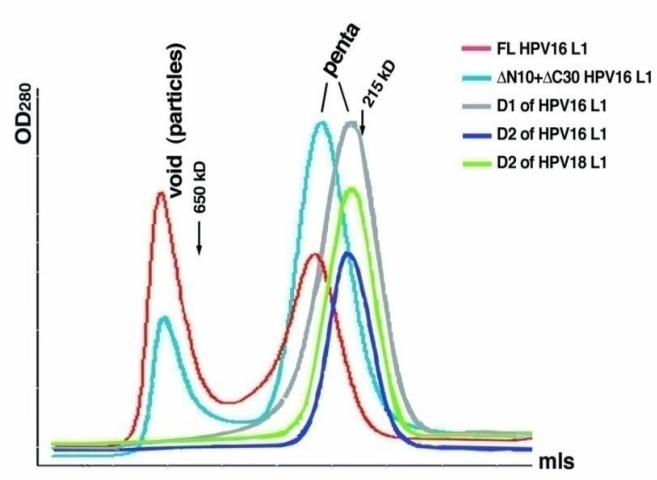
6.4.2 Size exclusion chromatography





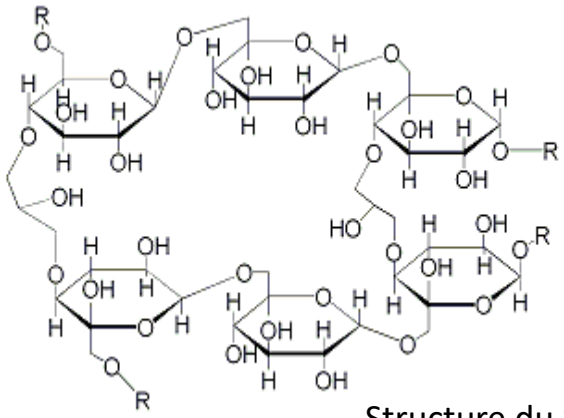




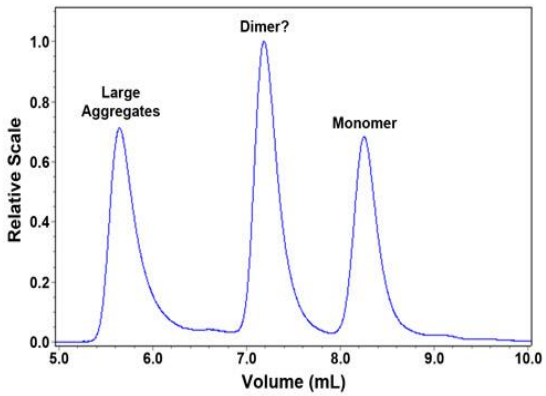


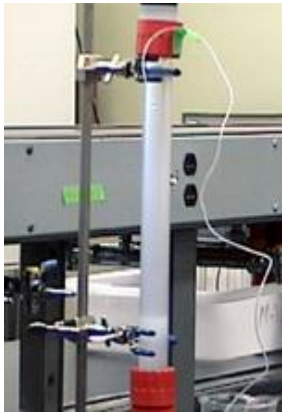
Many names for one single technique

- Porath & Flodin (1959): Gel Filtration Chromatography
- Pedersen (1962): Size Exclusion Chromatography
- Hjertén & Mosbach (1962): Molecular Sieve Chromatography
- Moore (1964): Gel Permeation Chromatography

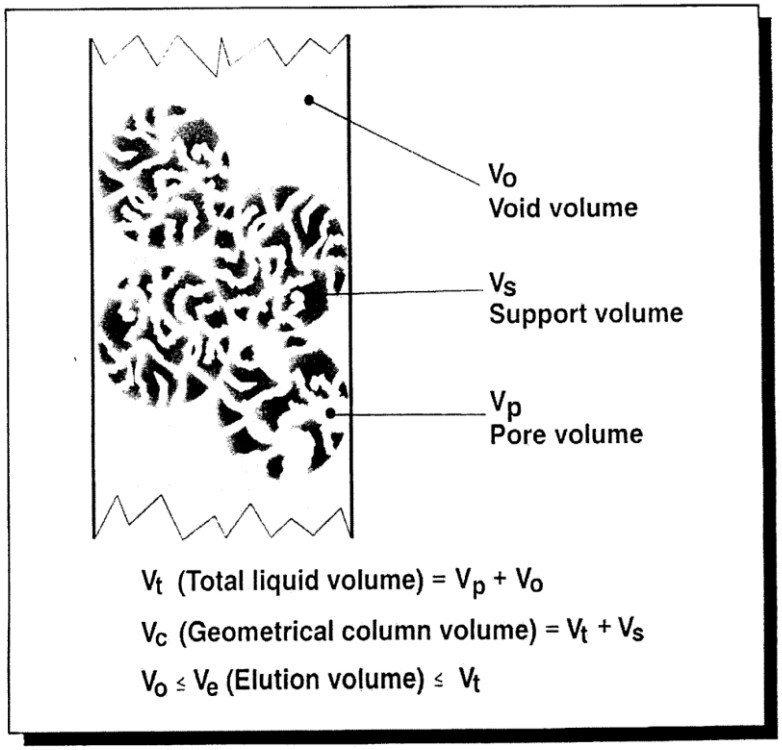


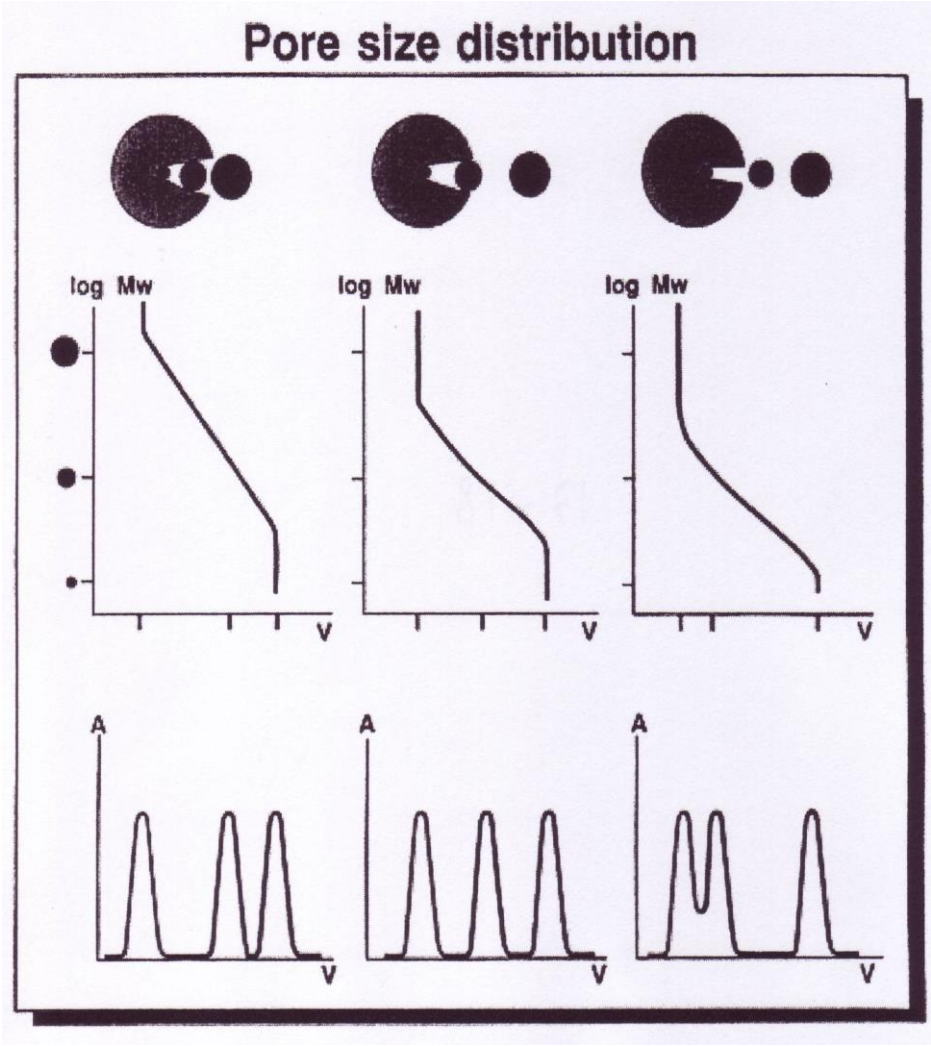
Structure du Sephadex ®





SEC: principle for separation

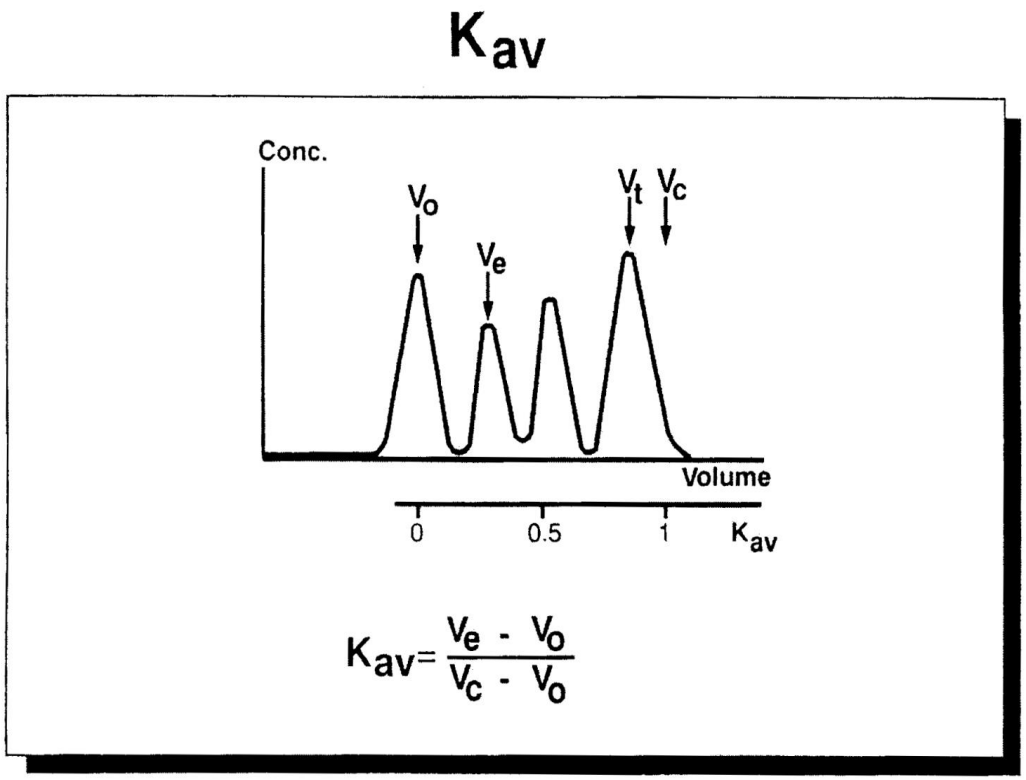




With SEC, separation is only possible between V_0 and V_t.

With elution chromatography techniques, volumes are very often much larger than V_t.

How results are expressed



V_0 = Void volume

V_e = Elution volume of the target molecule

V_t = Elution volume of salts and other small molecules

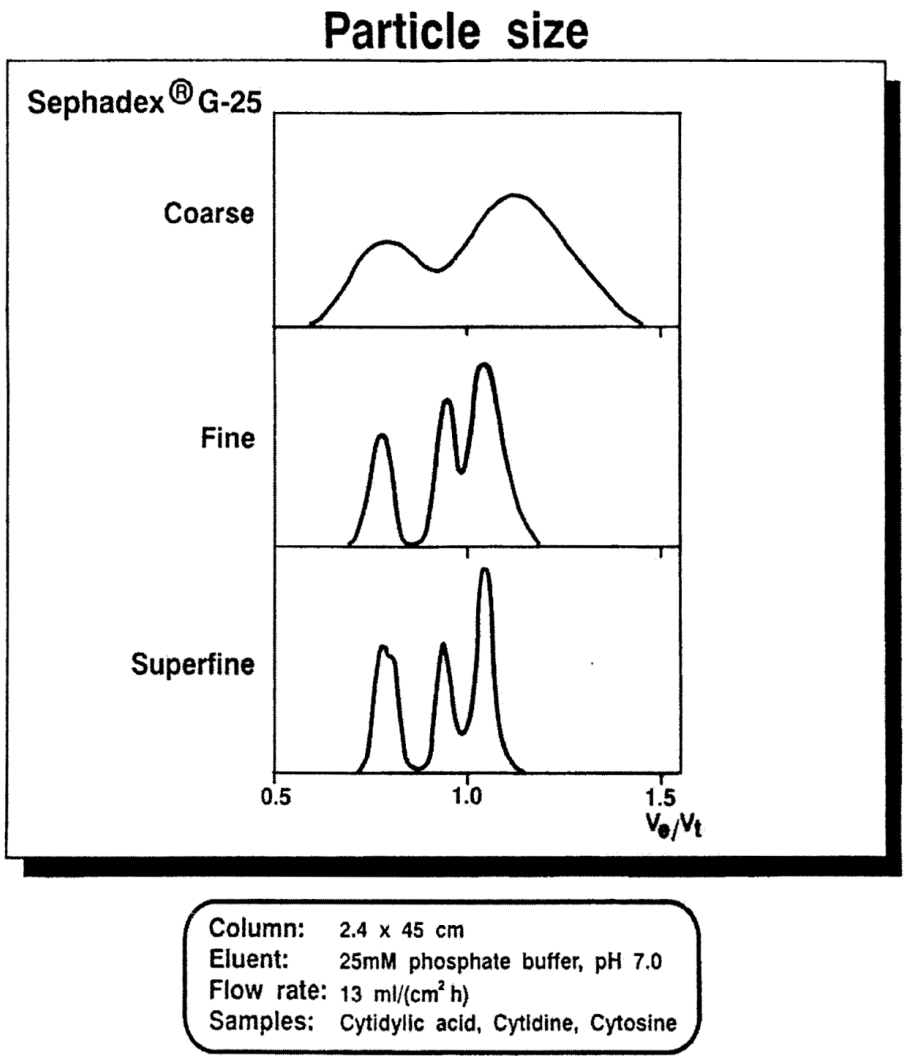
V_c = Geometric volume of the column

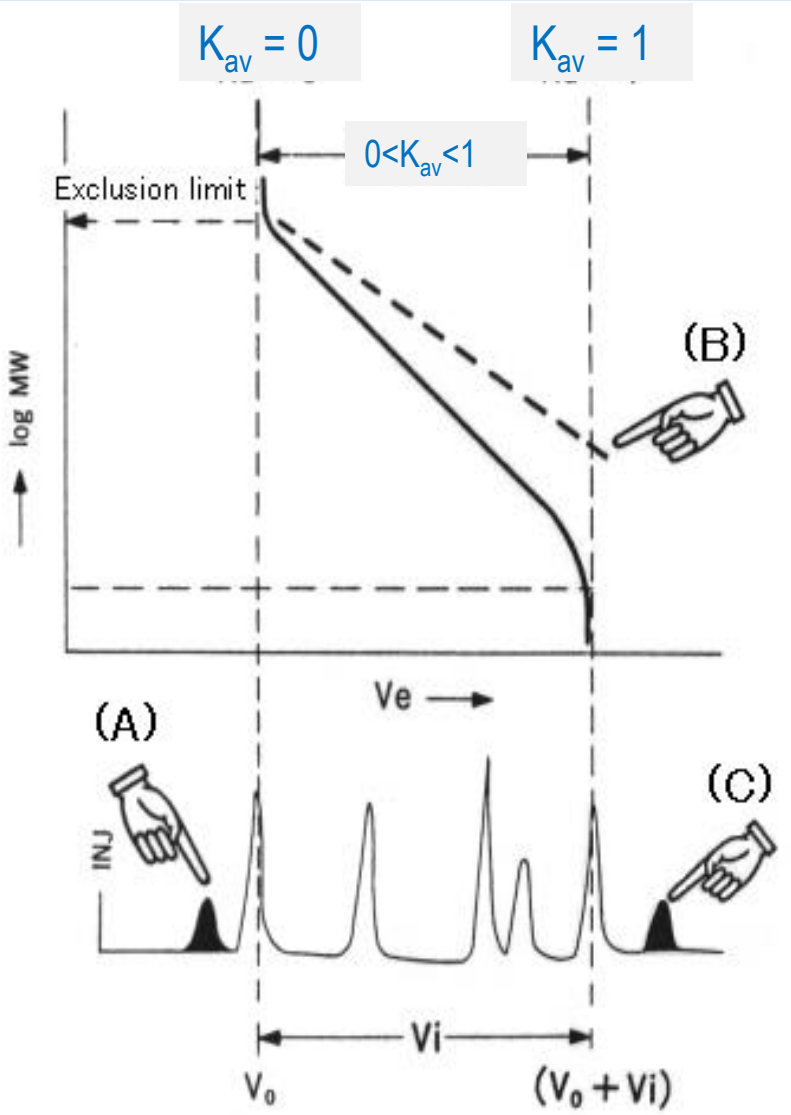
K_{av} = Partition Coefficient

Every protein (or DNA or RNA) elutes from a specific SEC column in a reproducible manner based on its Stokes Radius, which is related to its mass.

The measure of this elution behavior is expressed as its Partition Coefficient, K_{av}.

Influence of Sephadex gel particle size and calibration of a column



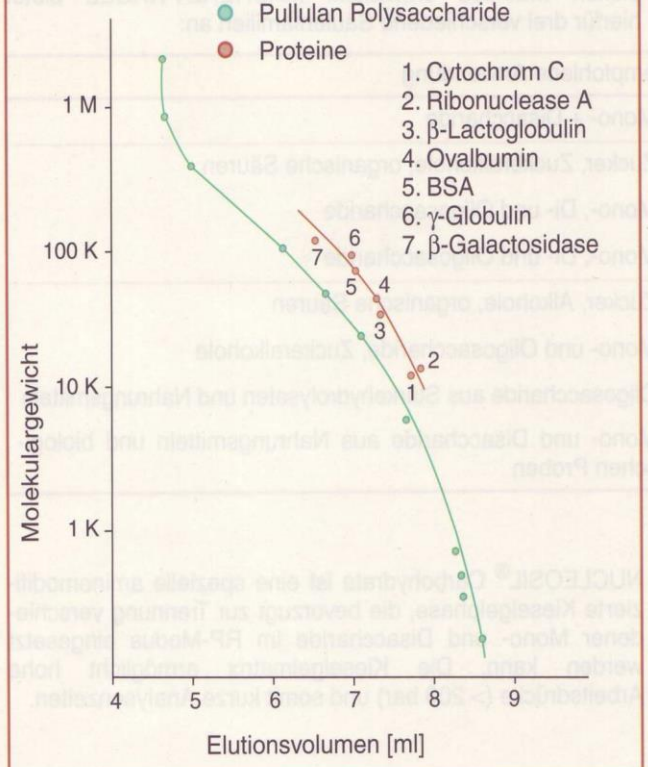


Calibration of SEC separations

Gelfiltrations-Säulen für biochemische Anwendungen

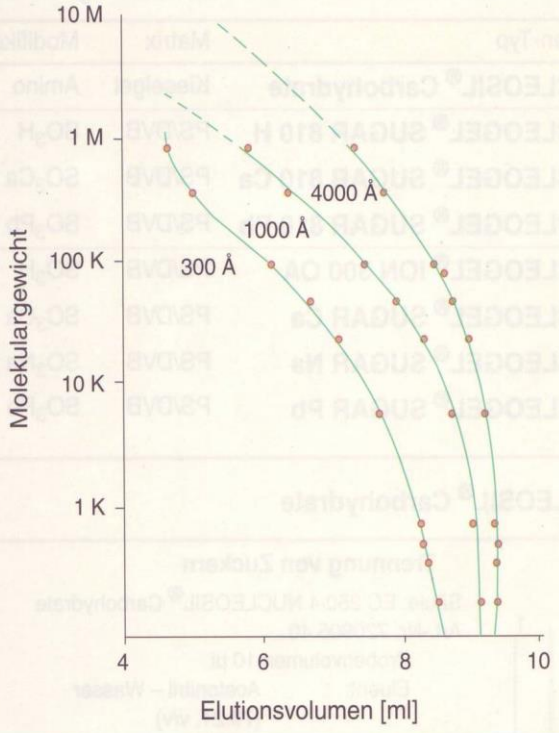
Eichkurven für Polysaccharide und Proteine auf NUCLEOGEL® GFC 300-8

Säule: VA 300/7.7 NUCLEOGEL® GFC 300-8, 300 x 7,7 mm ID, Art.-Nr. 719447
Eluent: 0,02M NaH_2PO_4, 0,2 M NaCl, pH 7,0
Flussrate: 1,0 ml/min
Detektion: UV 280 nm



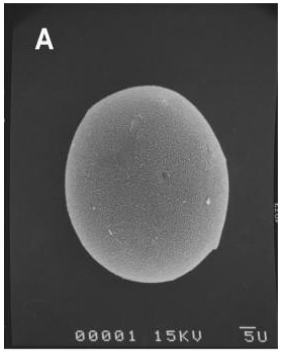
Eichkurven für NUCLEOGEL® GFC Säulen verschiedener Porenweiten

Säulen: VA 300/7.7 NUCLEOGEL® GFC
Eluent: Wasser
Flussrate: 1,0 ml/min
Raumtemperatur
Eichsubstanzen: Pullulan Polysaccharide



Sephadex®

- **Sephadex** is the tradename of a cross-linked dextran used mainly for SEC
- It is however also possible to chemically modify the chains of this polysaccharide to allow cation or anion exchange chromatography separations
- This stationary phased has been developed by Jerker PORATH and Per FLODIN, two Pharmacia scientists. It has been commercialized by the same company in 1959 (a.k.a Amersham Bioscience, GE Healthcare)
- The name stems from **SE**paration **PHA**rmacia **DEX**tran
- Sephadex is produced under the form of small beads (diam. between 20 and 300 μm). By varying the degree of cross-linking it is possible to influence the separating properties of the gel



Porath J. & Flodin P. (1959): Gel filtration: A method for desalting and group separation. *Nature* 183, 1657-1659.

Properties of Sephadex®

Table 5. Properties of Sephadex.

Gel type	Dry bead size µm	Fractionation range Globular proteins	Fractionation range Dextrans	Swelling factor ml/g
Sephadex G-10	40 – 120	– 700	– 700	2 – 3
Sephadex G-15	40 – 120	– 1 500	– 1 500	2.5 – 3.5
Sephadex G-25 Coarse	100 – 300	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-25 Medium	50 – 150	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-25 Fine	20 – 80	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-25 Superfine	10 – 40	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-50 Coarse	100 – 300	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-50 Medium	50 – 150	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-50 Fine	20 – 80	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-50 Superfine	10 – 40	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-75	40 – 120	3 000 – 80 000	1 000 – 50 000	12 – 15
Sephadex G-75 Superfine	10 – 40	3 000 – 70 000	1 000 – 50 000	12 – 15
Sephadex G-100	40 – 120	4 000 – 150 000	1 000 – 100 000	15 – 20
Sephadex G-100 Superfine	10 – 40	4 000 – 100 000	1 000 – 100 000	15 – 20
Sephadex G-150	40 – 120	5 000 – 300 000	1 000 – 150 000	20 – 30
Sephadex G-150 Superfine	10 – 40	5 000 – 150 000	1 000 – 150 000	18 – 22
Sephadex G-200	40 – 120	5 000 – 600 000	1 000 – 200 000	30 – 40
Sephadex G-200 Superfine	10 – 40	5 000 – 250 000	1 000 – 150 000	20 – 25

Example 1 : desalting / buffer exchange

Small scale-up using buffer exchange, up to 60 ml sample

Fig 18A: Five HiTrap Desalting columns connected in series

Sample: 2 mg/ml BSA in 50 mM sodium phosphate, 0.5 M NaCl, pH 7.0
Sample vol.: 28 % of column volume (1.4, 4.3 and 7.1 ml respectively)
Flow rate: 5 ml/min

Column: HiTrap Desalting, 1 × 5 ml, 3 × 5 ml, 5 × 5 ml
Buffer: 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0
System: ÄKTA$_{FPLC}$

Fig. 18B: HiTrap Desalting 1X5 ml in series

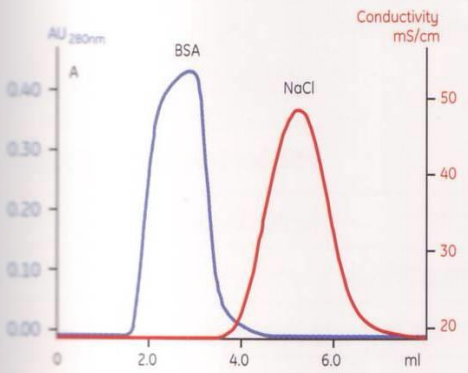


Fig. 18C: HiTrap Desalting 3X5 ml in series

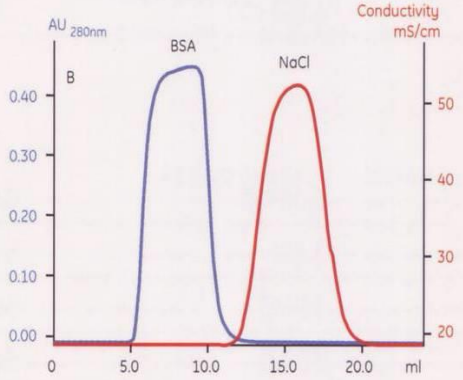
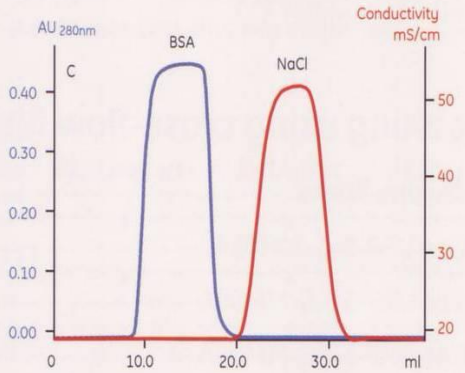


Fig. 18D: HiTrap Desalting 5X5 ml in series



Conclusions

Fig. 18B–D shows results using HiTrap Desalting columns from sample volumes of 1.4, 4.3 and 7.1 mL. Connect HiTrap Desalting columns in series for fast and simple scale-up, without back pressure problems.

About HiTrap Desalting and HiPrep Desalting

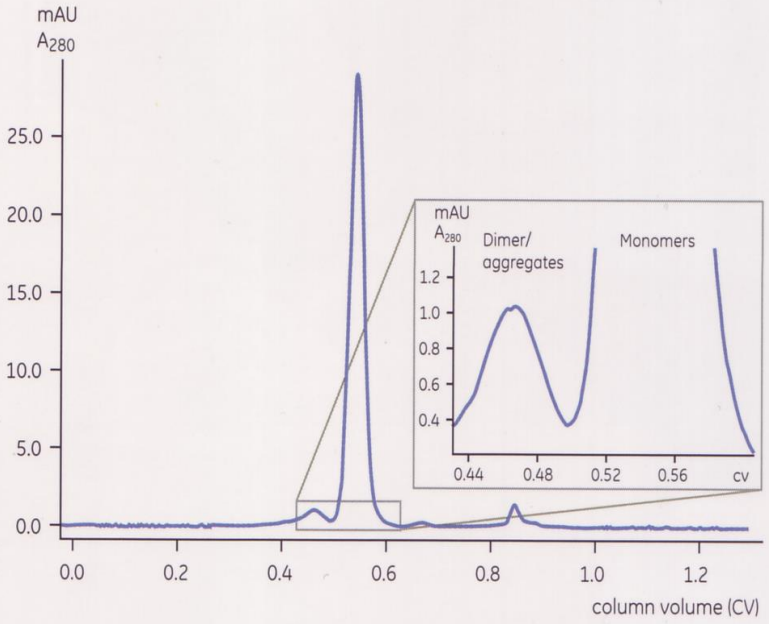
HiTrap Desalting and HiPrep Desalting are prepacked with Sephadex G-25 for fast, simple desalting and buffer exchange. Using four HiPrep 26/10 Desalting in series desalt 60 ml of sample in a very short time.

Example 2 : monomer/dimer separation

Monomer/dimer separation of a MAb

Fig. 7B: Polishing of Protein G purified IgG_1 antibody using gel filtration

Column: Superdex 200 10/300 GL
Buffer: PBS, pH 7.2
Flow rate: 0.7 ml/min
System: ÄKTA$_{FPLC}$



Conclusions

In most antibody preparations there is a possibility that IgG aggregates and dimers are present. Therefore, it is essential to include a gel filtration polishing step to get pure, homogenous MAbs. Superdex 200 gel filtration is an excellent medium for this purpose, as can be seen in Fig. 7B.

About Superdex 200

Superdex 200 medium is ideal for polishing and removing aggregates and dimers in MAb purification because:

- Separations in the range from M_r 10 000 up to 600 000 (globular proteins)
- Easy and predictable scale-up
- Excellent reproducibility and durability
- Available in expertly prepacked columns and as bulk media

Rapid three-step purification of a labile, oxygen-sensitive enzyme

Fig. 11A: Capture: anion exchange chromatography

Sample: 40 ml clarified *E. coli* extract of DAOCS, kept on ice

Column: HiPrep 16/10 Q XL

Start buffer (A): 50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 M benzamidine-HCl, 0.2 mM PMSF, pH 7.5;

Elution buffer (B): A + 1.0 M NaCl

Gradient: 0 % B in 5 column volumes, 30 % B in 5 column volume, 100 % B in 5 column volumes (step gradient)

Flow: 10 ml/min (300 cm/h)

System: ÄKTAFPLC

Fig. 11B: Intermediate purification: hydrophobic interaction chromatography

Sample: 40 ml DAOCS pool from HiPrep 16/10 Q XL, kept on ice

Column: SOURCE 15ISO, packed in HR 16/10 column

Start buffer (A): 1.6 M ammonium sulphate, 10 % glycerol, 50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5

Elution buffer (B): 50 mM Tris-HCl, 10 % glycerol, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5

Gradient: 0–16 % B in 4 column volume, 16–24 % B in 8 column volume, 24–35 % B in 4 column volume, 100 % B in 4 column volume

Flow: 5 ml/min (150 cm/h)

System: ÄKTAFPLC

Fig. 11C: Polishing: gel filtration

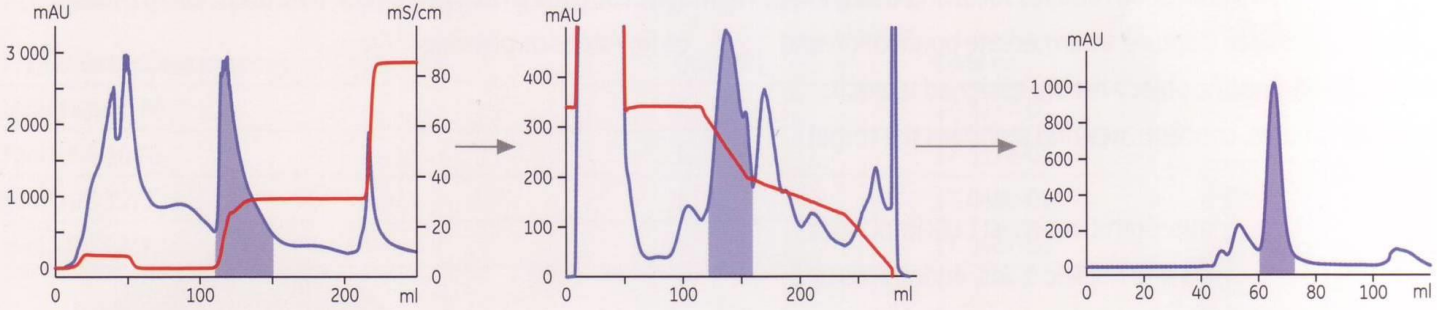
Sample: 3 ml DAOCS pool from SOURCE 15ISO, kept on ice

Column: HiLoad 16/60 Superdex 75 prep grade

Buffer: 100 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 mM benzamidine-HCl, 0.2 mM PMSF, pH 7.5

Flow: 1 ml/min (30 cm/h)

System: ÄKTAFPLC



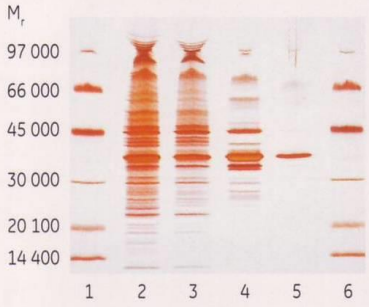


Fig. 11D: SDS-PAGE, silver stained

Lane 1: LMW

Lane 2: Start material *E. coli* extract

Lane 3: DAOCS pool from Q Sepharose XL

Lane 4: DAOCS pool from SOURCE 15ISO

Lane 5: DAOCS pool from Superdex 75 pg

Lane 6: LMW

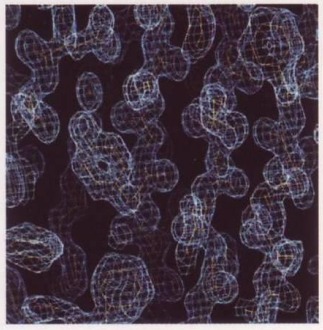
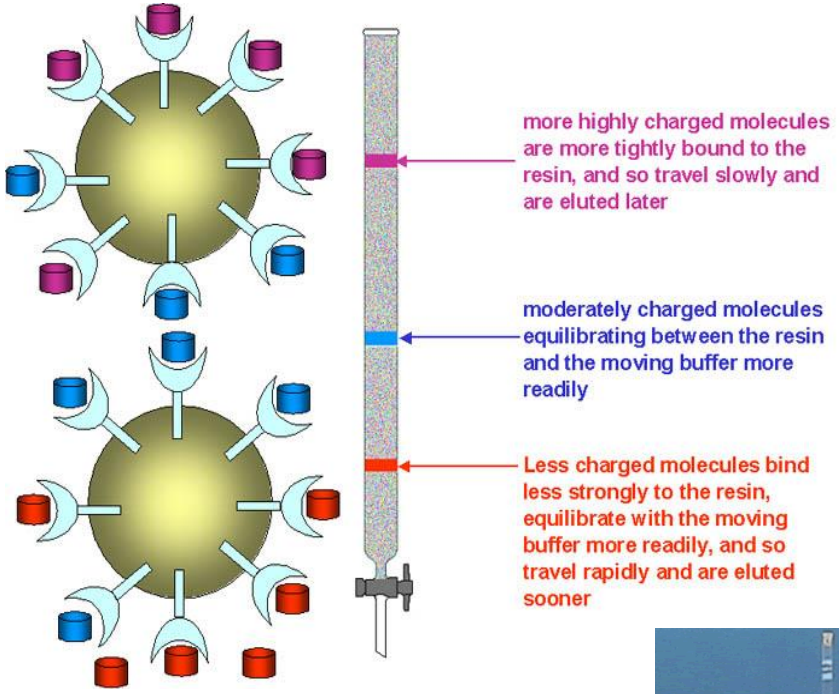
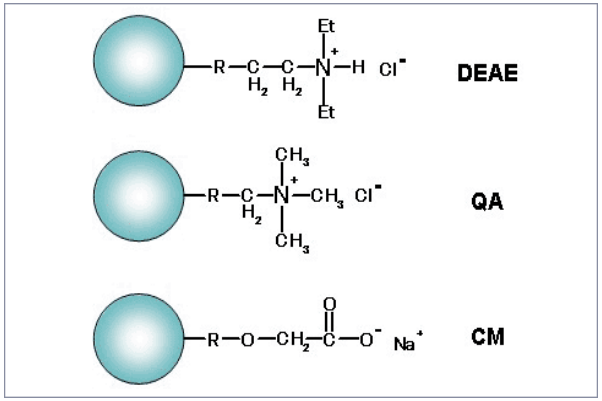
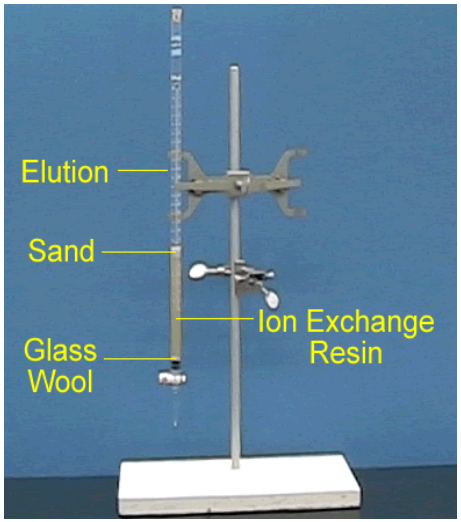


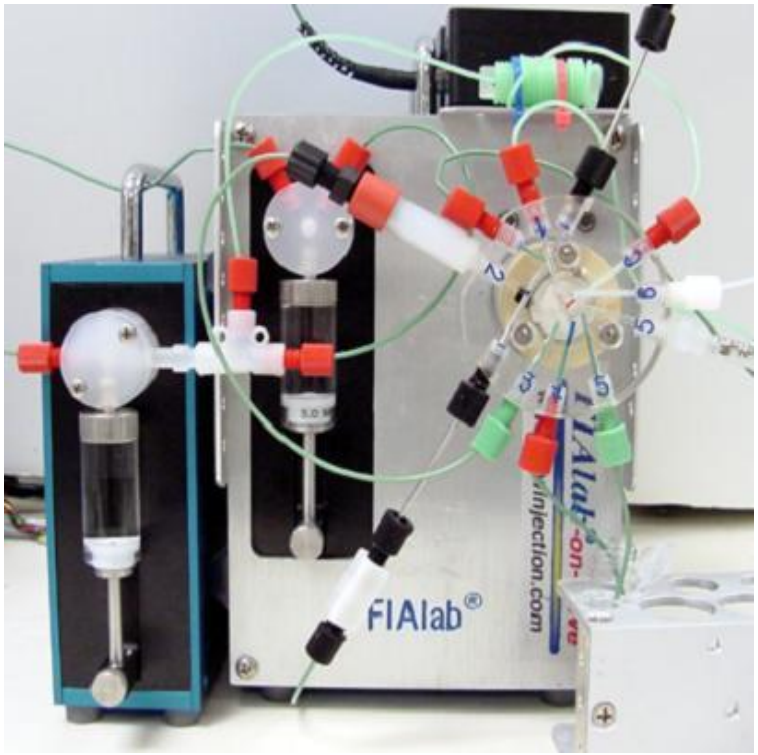
Fig. 11E: High resolution density map of purified DAOCS

Ion exchange chromatography (IEC)

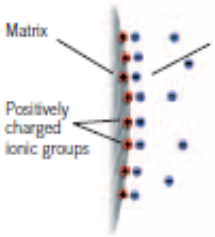






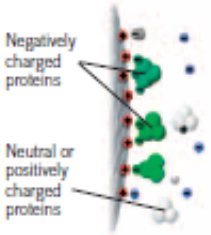


6.4.2 IEX: principle and sequence of steps

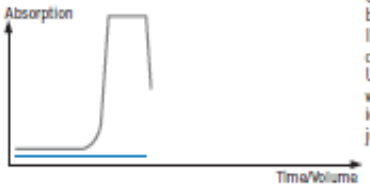


Equilibration

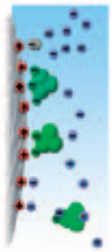
IEX medium equilibrated with start buffer.



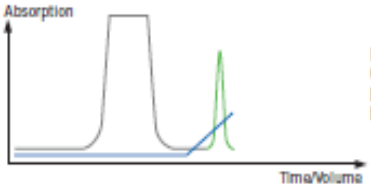
Sample application



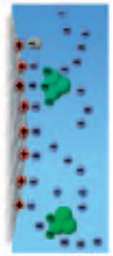
Oppositely-charged proteins bind to ionic groups of the IEX medium, becoming concentrated on the column. Uncharged proteins, or those with the same charge as the ionic groups, elute during or just after sample application.



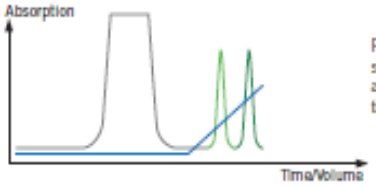
Elution 1



Increasing ionic strength (using a gradient) displaces bound proteins as ions in the buffer compete for binding sites.

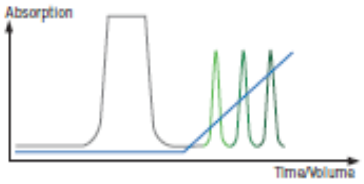


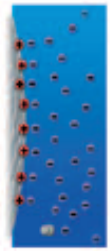
Elution 2



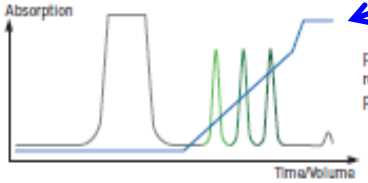
Further increases in ionic strength displace proteins that are more highly charged (more tightly bound)

Elution 3





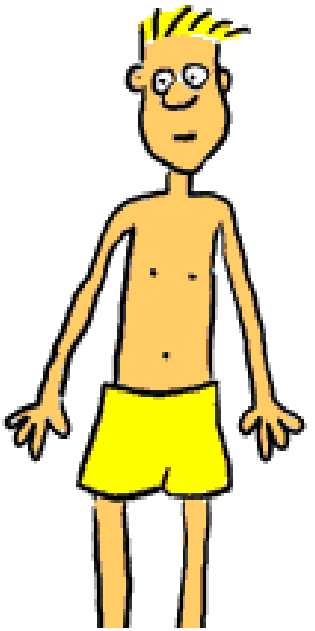
Wash



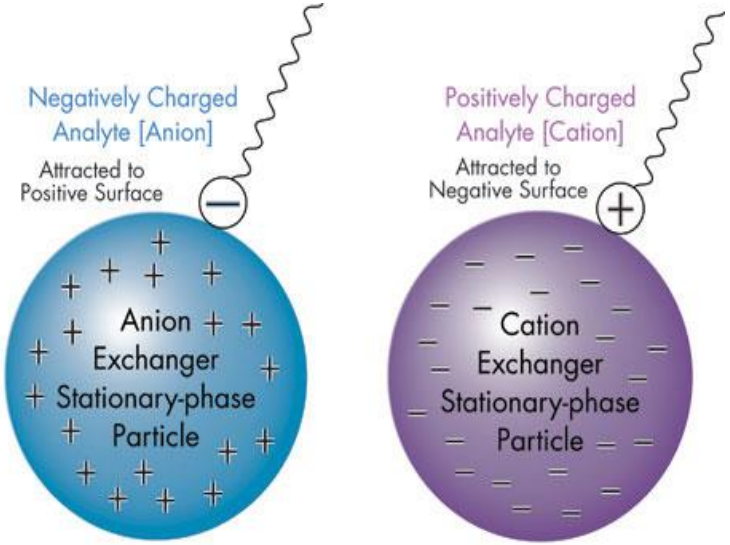
Final high ionic strength wash removes any ionically bound proteins before re-equilibration

Please note the final washing step at the end of the cycle. It is done at an even higher ionic strength than for elution, in order to desorb the last molecules

Strong and weak ion exchangers



- The strength has nothing to do with adsorption capacity, but with the degree of ionization of the functional groups
- Strong exchangers remain ionized over a broad pH range
- Weak exchangers are on the other hand only partially ionized at the extremities if the working pH range



Ion Exchange Sorbents & Structure

Sorbent	Structure		pKa
Anion Exchangers			
Aminopropyl (1º amine)	$-Si-(CH_2)_3NH_3^+$		9.8
N-2 Aminoethyl (1º & 2º amine)	$-Si-(CH_2)_3NH_2^+(CH_2)_2NH_3^+$		10.1, 10.9
Diethylamino (3º amine)	$-Si-(CH_2)_3NH^+(CH_2CH_3)_2$		10.6
Quaternary Amine Chloride	$-Si-(CH_2)_3N^+(CH_3)_3$	Cl^-	always charged
Quaternary Amine Hydroxide	$-Si-(CH_2)_3N^+(CH_3)_3$	$CH_3CO_2^-$	always charged
Quaternary Amine Acetate	$-Si-(CH_2)_3N^+(CH_3)_3$	OH^-	always charged
Quaternary Amine Formate	$-Si-(CH_2)_3N^+(CH_3)_3$	CHO_2^-	always charged
Polyimine	$-Si-(CH_2)_3R-[NHCH_2CH_2]_x$		
Cation Exchangers			
Carboxylic Acid	$-Si-CH_2COOH$		4.8
Propylsulfonic Acid	$-Si-(CH_2)_3SO_3H$		<1
Benzenesulfonic Acid	$-Si-(CH_2)_2-C_6H_4-SO_3H$		always charged
Benzenesulfonic Acid High-Load	$-Si-(CH_2)_2-C_6H_4-SO_3H$		always charged
Triacetic Acid	$-Si-(CH_2)_3NH-(CH_2)-N(CH_2COOH)_2$ | CH_2COOH		

NOTE: In an un-ionized state, these sorbents are hydrophilic (polar) sorbents.

Can you identify the weak and strong ion exchangers in the above table?

Cation exchangers

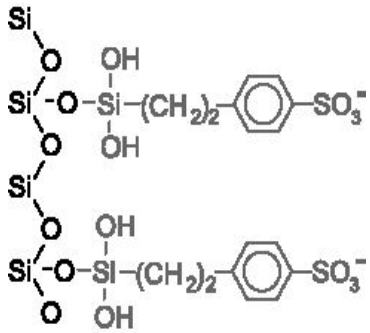
Strong cation exchangers

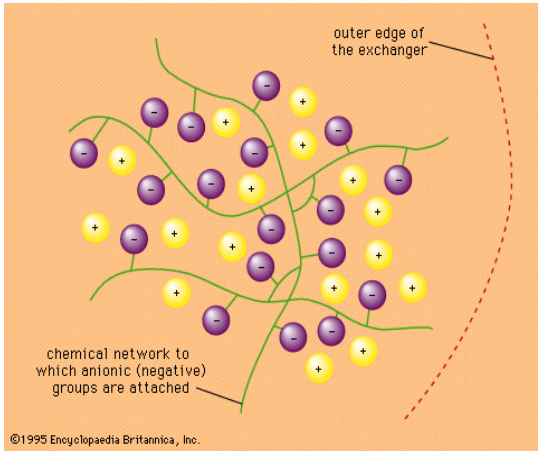
- Sulfopropyl (SP)
- Methyl sulfonate (S)

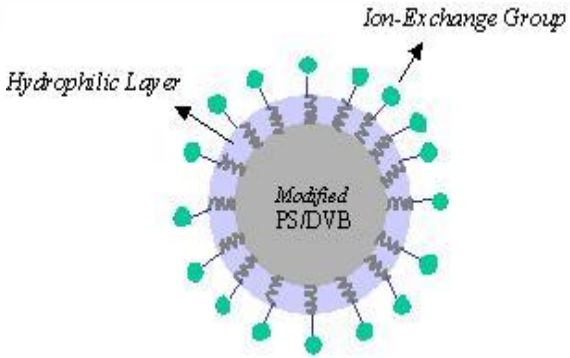
Weak cation exchangers

- Carboxymethyl (CM) -O–CH_2COO^-

S-cation exchanger CM-cation exchanger

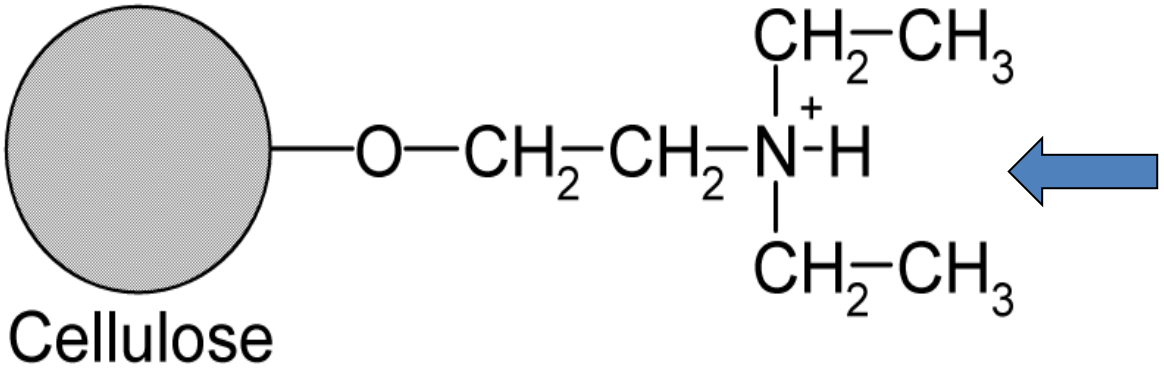






Anion exchangers

- Large selection of AEs on the market
- Based most often on amines with various degrees of susbtitution
- The more substituted the ammonium, the more basic it is and the stronger the exchanger is
- **Quaternary amines** are always strongly basic
- **Tertiary amines** are the most commonly used



A weak anion exchanger, DEAE (diethyl aminoethyl), grafted on cellulose

Some functional groups for anion exchangers

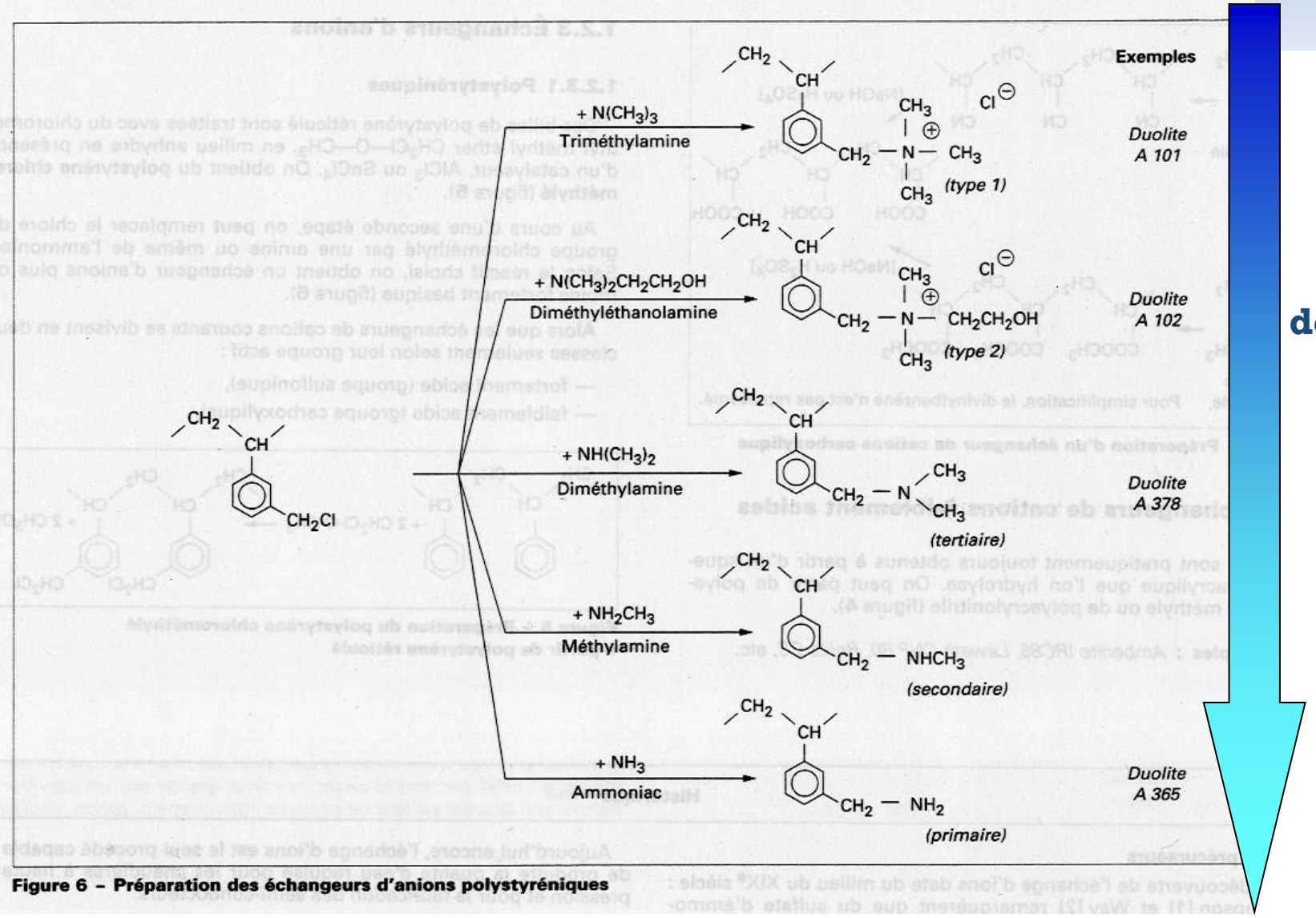
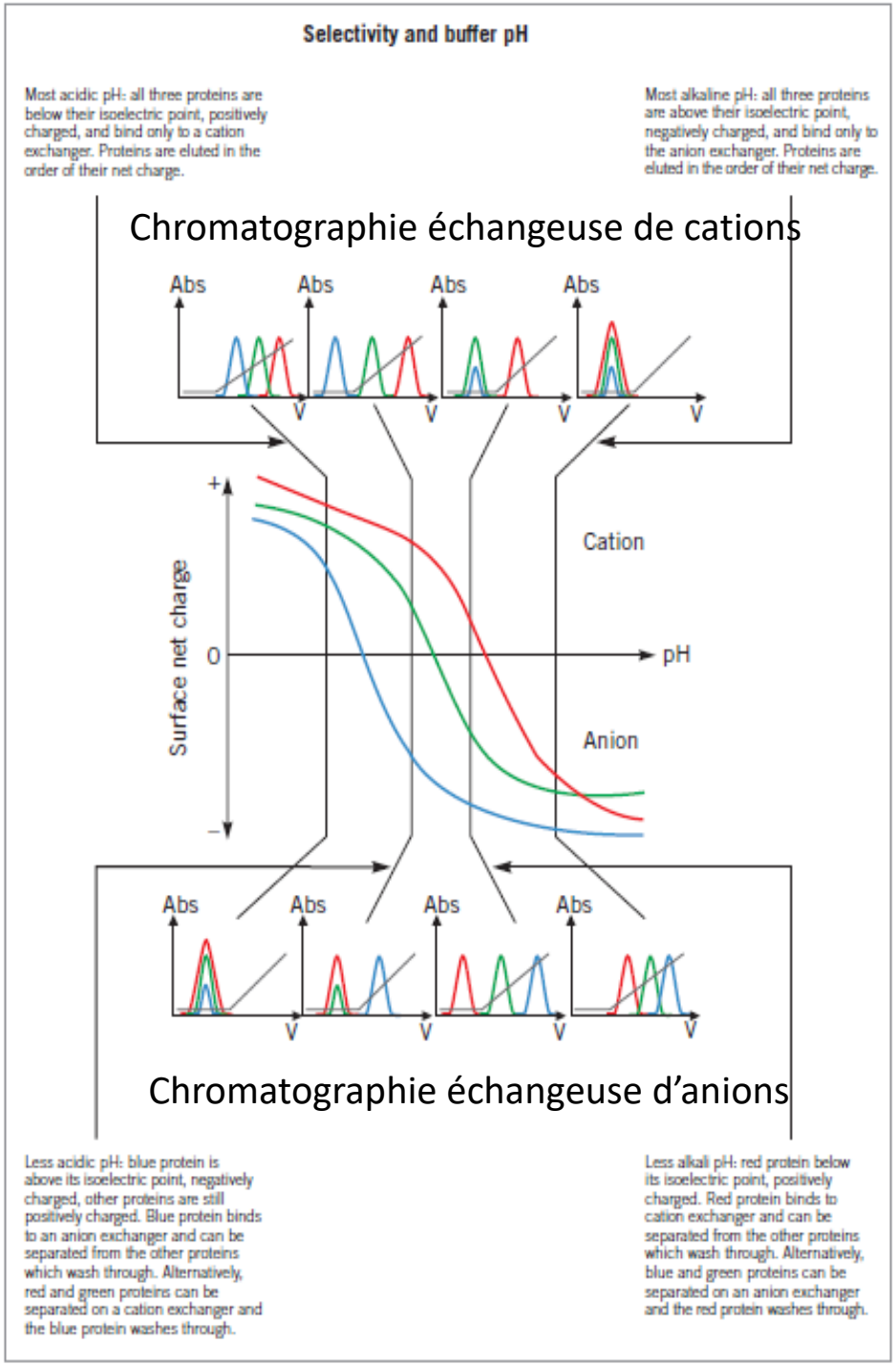
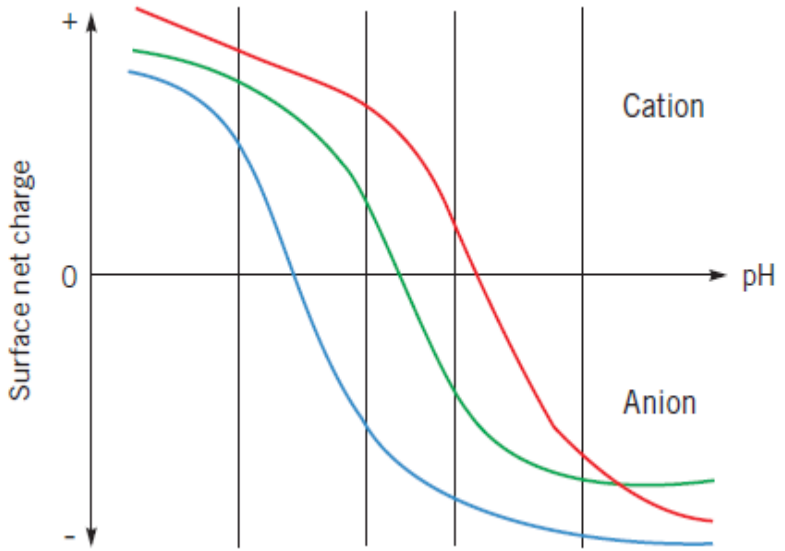


Figure 6 – Préparation des échangeurs d'anions polystyréniques

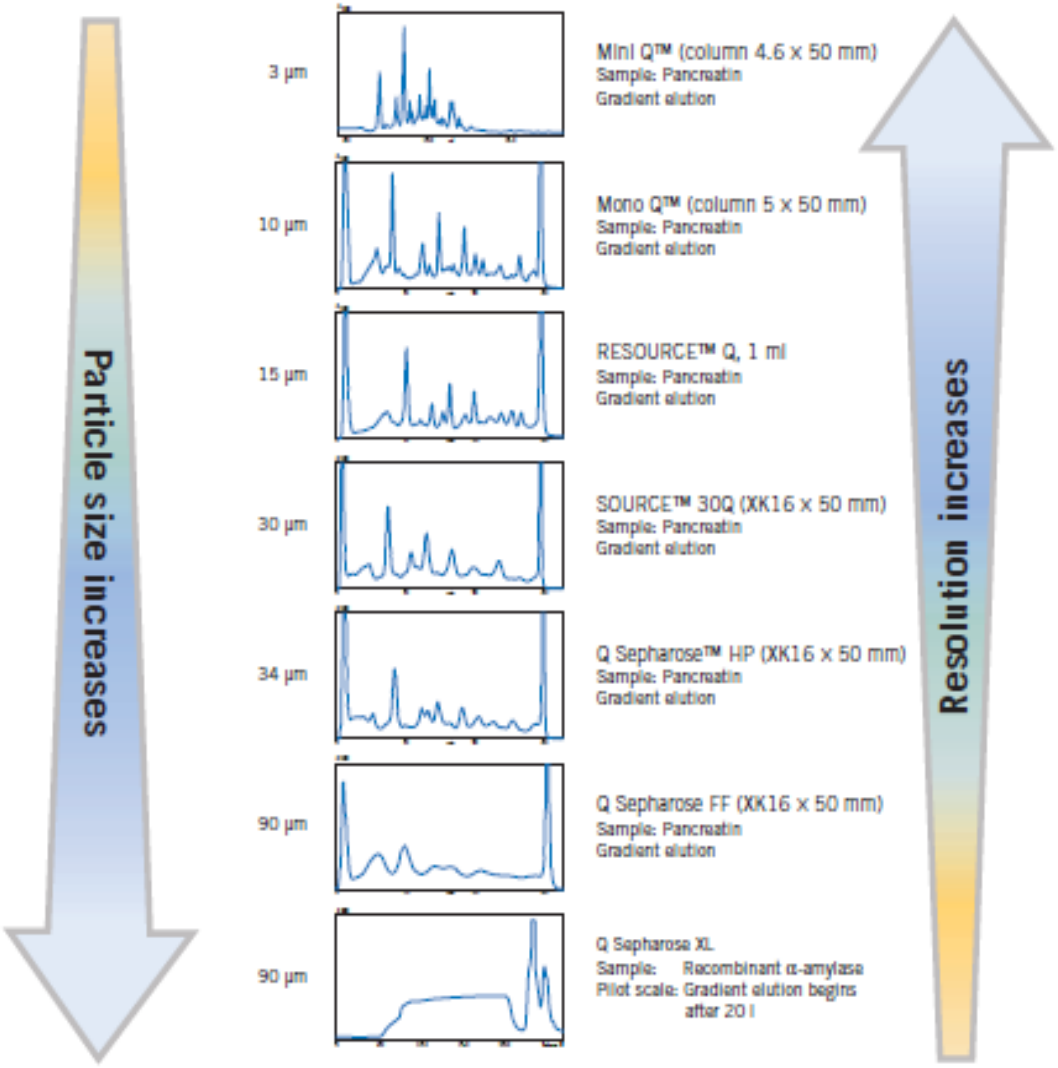
decreasing basicity and strength

Selectivity and pH of buffer

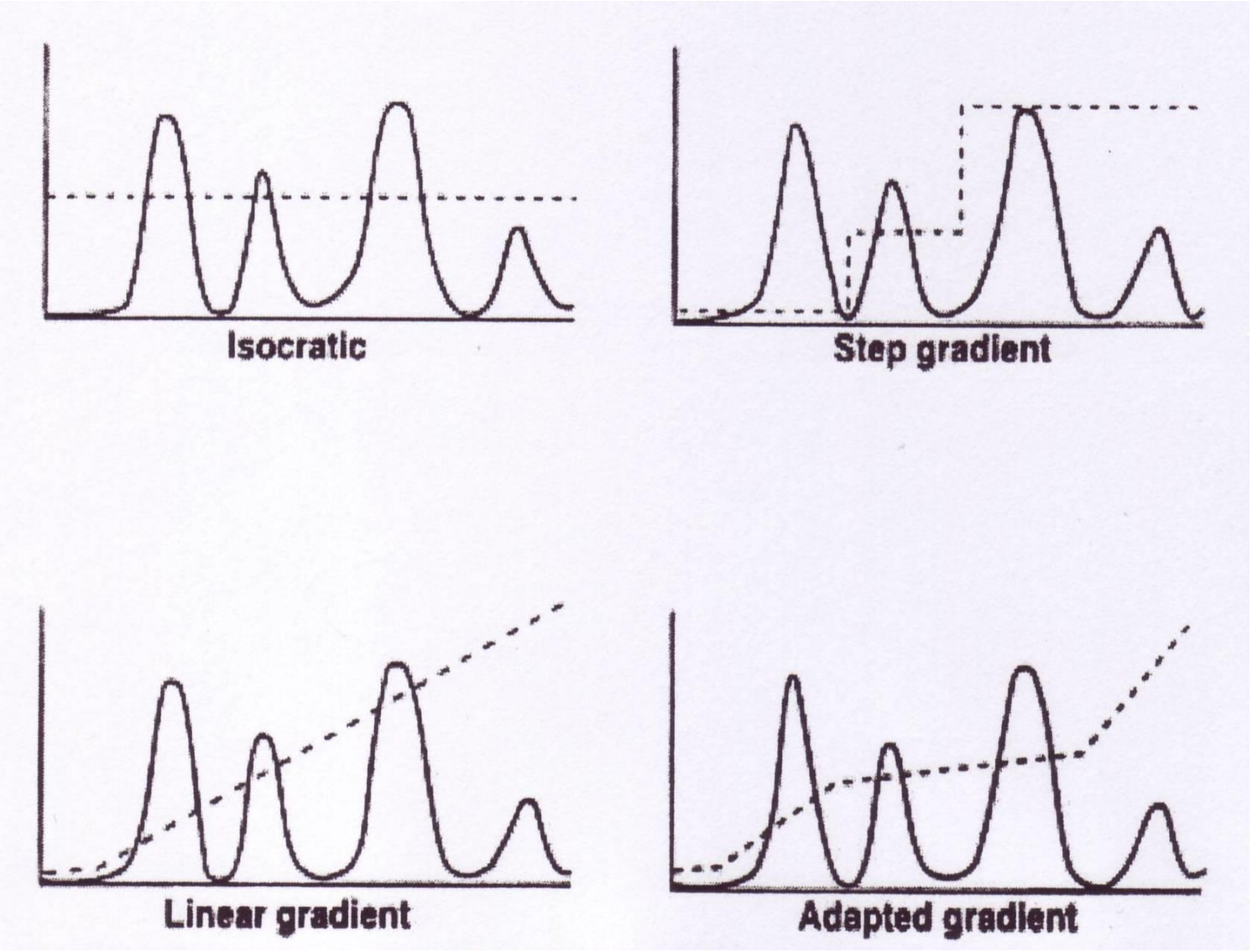




Influence of particle size and selectivity on resolution



The different elution modes



Selectivity and elution mode (1/2)

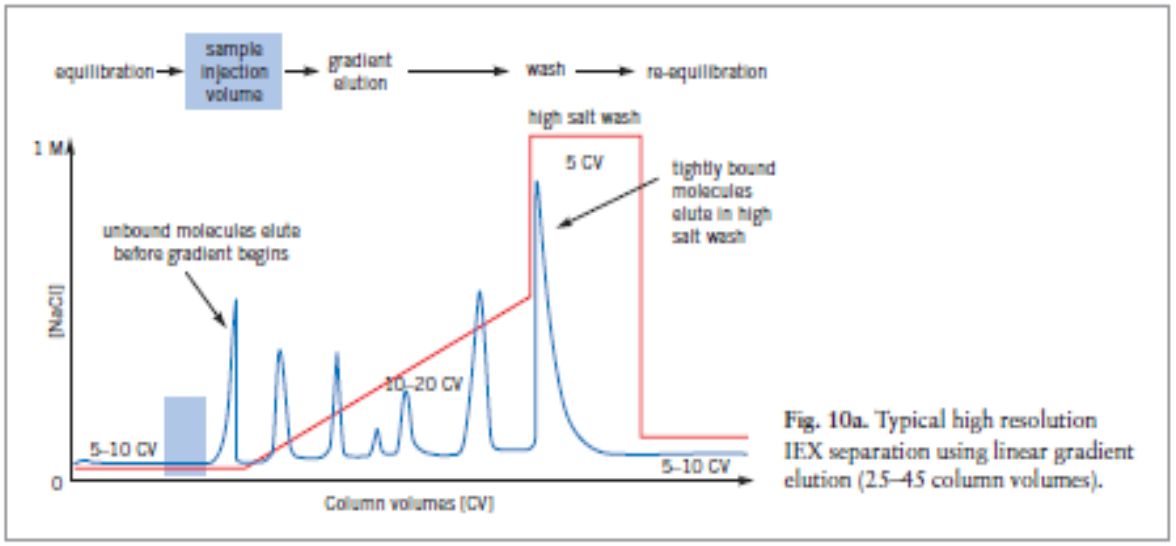


Fig. 10a. Typical high resolution IEX separation using linear gradient elution (25–45 column volumes).

Elution by a linear gradient. Often used for unknown samples, or for separations where a high resolution is needed (including analytics).

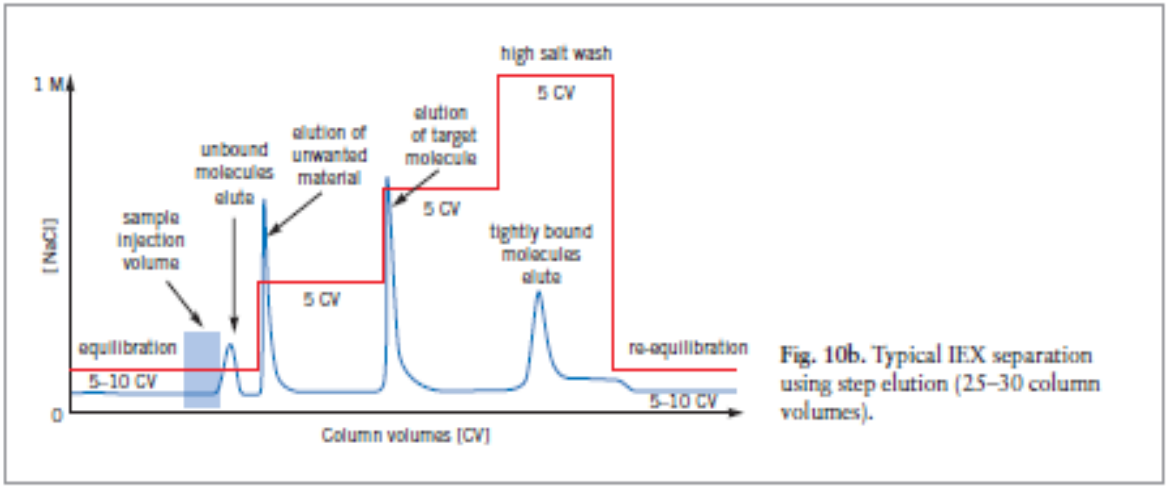


Fig. 10b. Typical IEX separation using step elution (25–30 column volumes).

Stepwise elution. Applied once the separation was optimized with a linear gradient. Saves eluent and reduces time for separation whil enabling a high degree of purity.

Selectivity and elution mode (2/2)

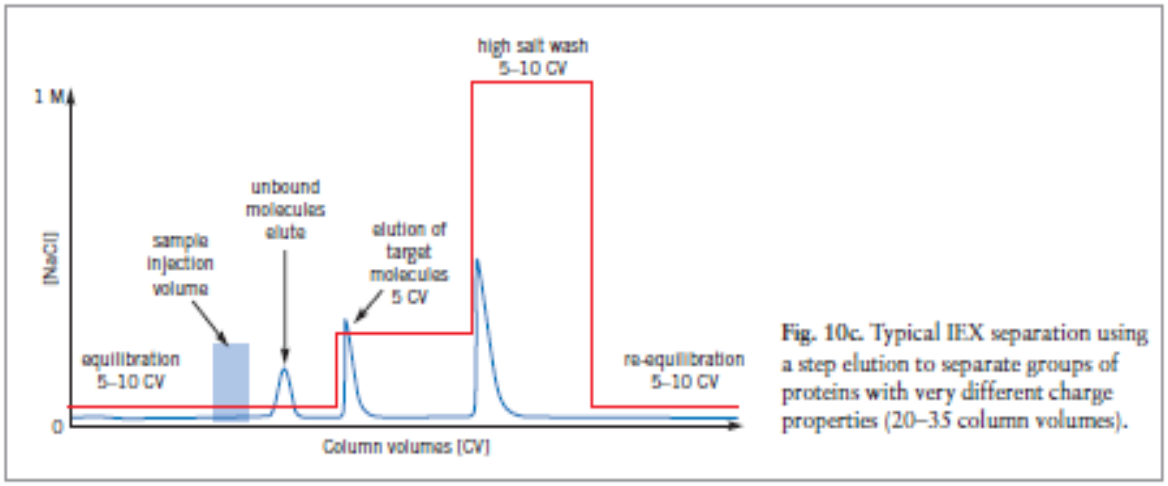


Fig. 10c. Typical IEX separation using a step elution to separate groups of proteins with very different charge properties (20–35 column volumes).

Stepwise elution can also be used for a separation by groups. The target molecule can thus quickly isolated from contaminant proteins, and under a concentrated form.

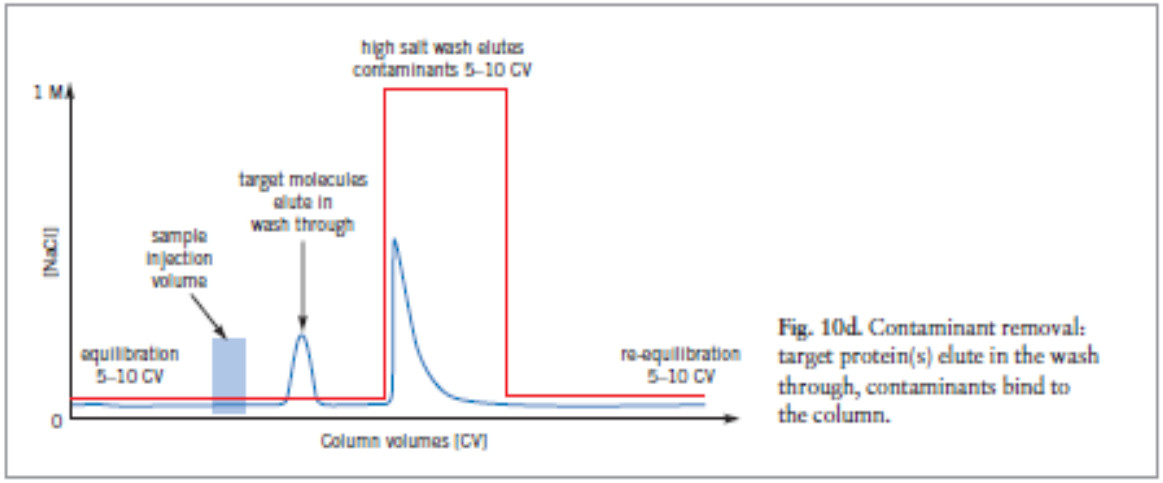


Fig. 10d. Contaminant removal: target protein(s) elute in the wash through, contaminants bind to the column.

Occasionnally, a resin can be chosen so that retains as much of the contaminants as possible while letting the target molecule flow through. The contaminants are then eluted in one block at high salt concentration. This approach is known as **negative chromatography**

Optimisation of conditions (pH, ionic strength)

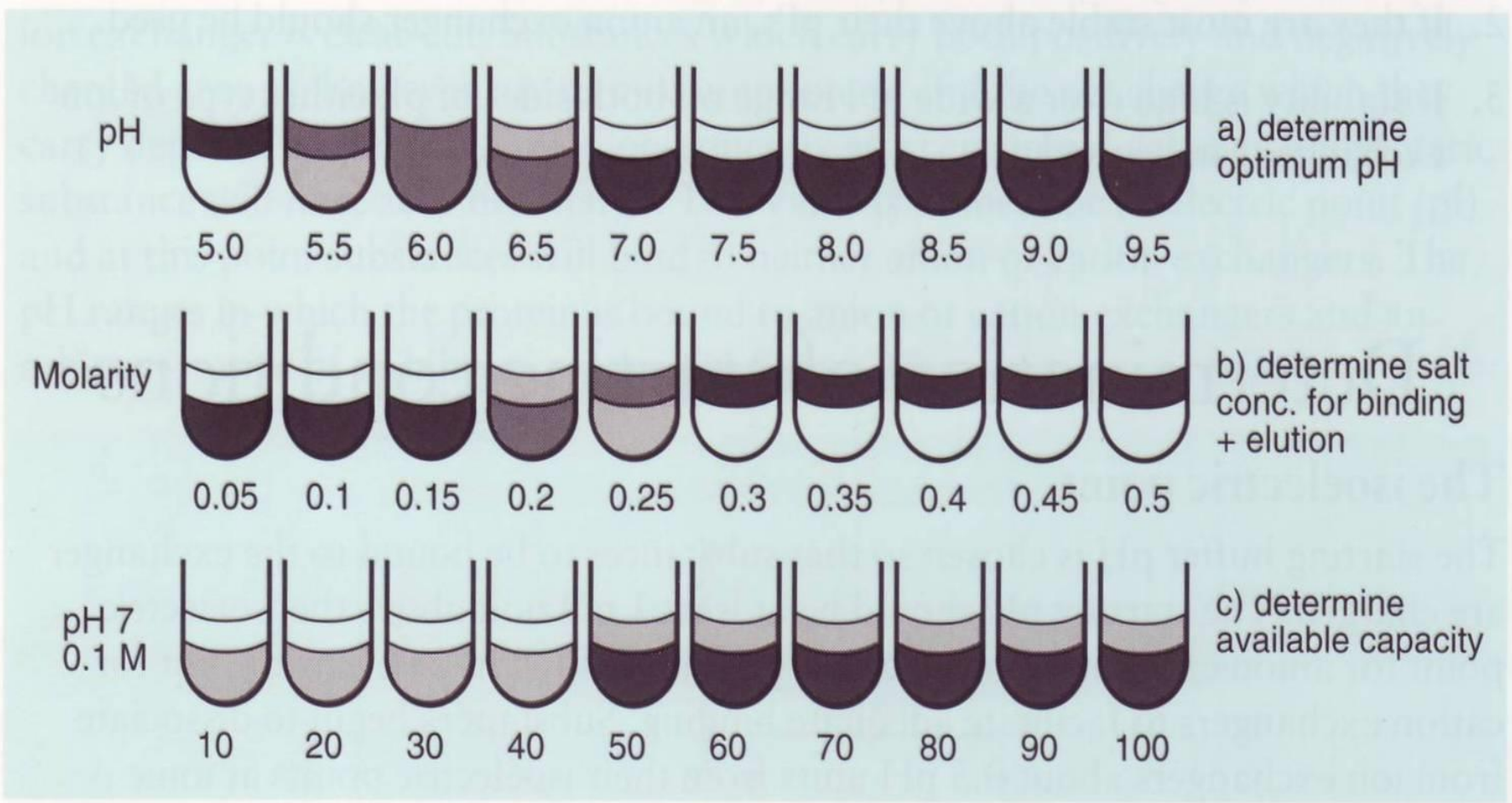


Fig. 38. Test-tube methods for selecting ion exchange conditions.

Condition screening in 96 wells formate

PreDictor (GE Healthcare)

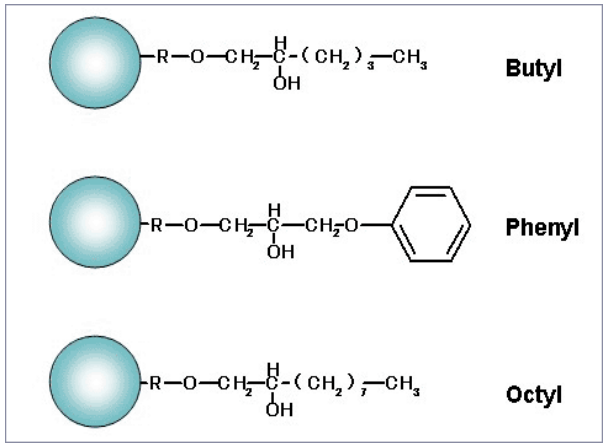
Le produit est constitué de plaques à 96 puits contenant une phase stationnaire (différentes sortes sont disponibles).

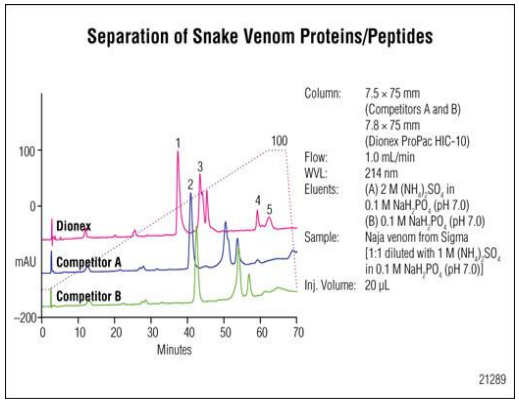
L'adsorption, le lavage et l'élution se font par pipetage et vidange des puits sous vide à l'aide d'un équipement spécial.

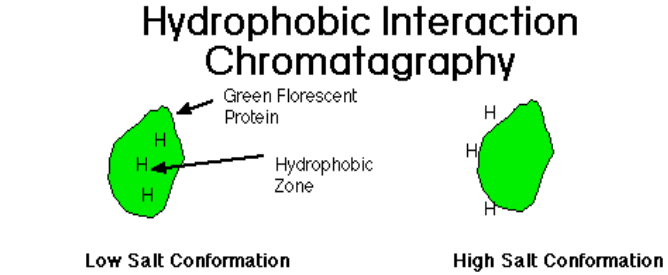
On peut ainsi optimiser les conditions de fixation et d'élution, évaluer la sélectivité, mesurer les isothermes.

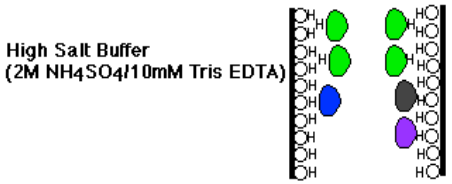
Demonstration video at the following URL:
http://video.google.com/videoplay?docid=8779429940227942623#

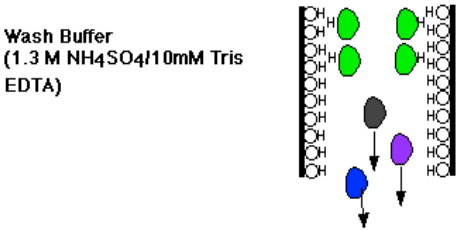
6.4.2 Hydrophobic Interaction Chromatography

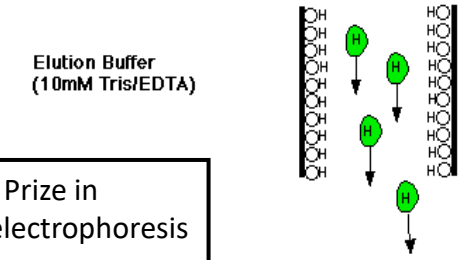












Arne Tiselius – 1948 Nobel Prize in chemistry for his work on electrophoresis and adsorption

First observations and separation principle

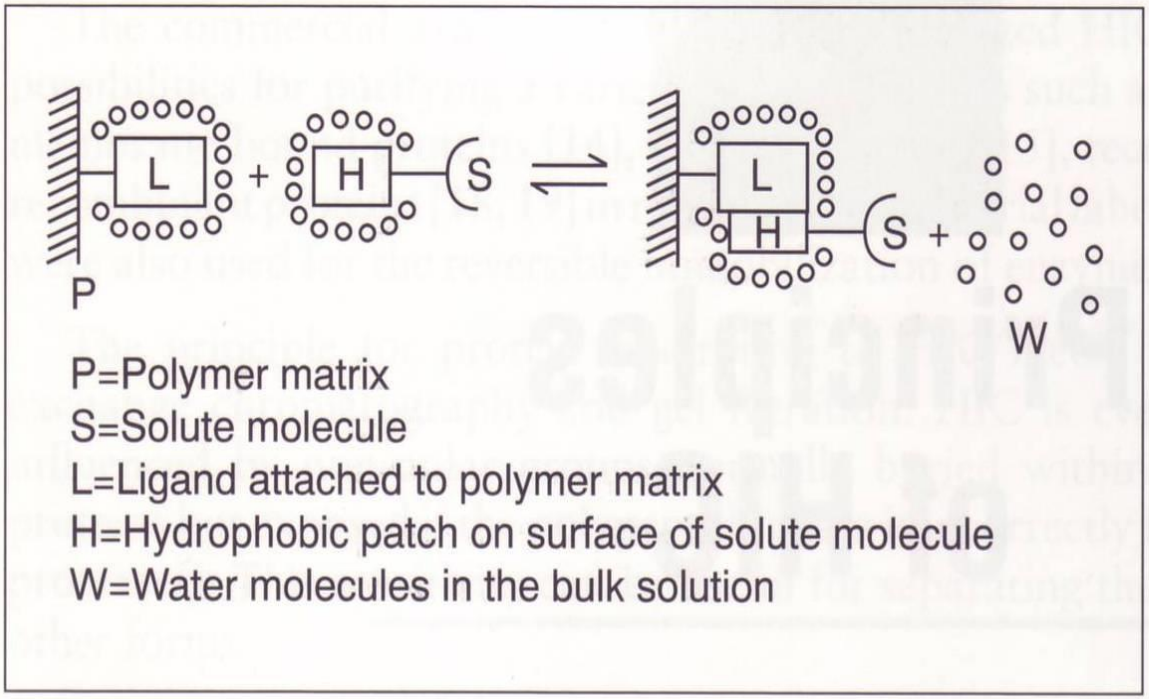
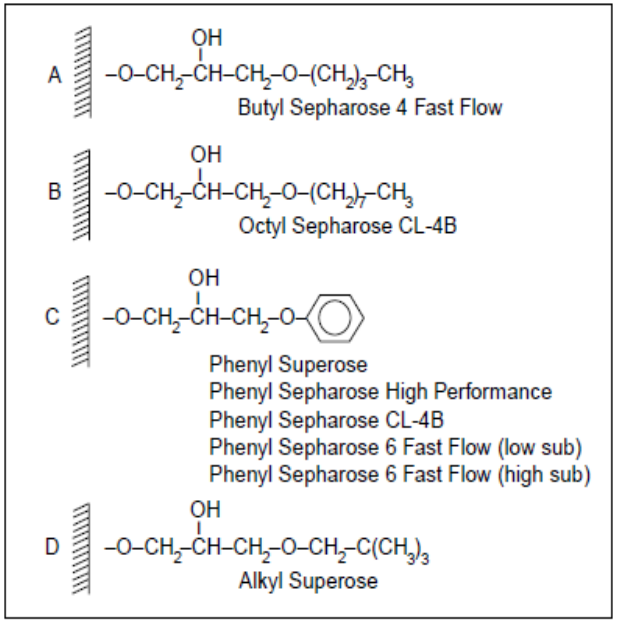


Fig. 1.
Close to the surface of the hydrophobic ligand and solute (L and H), the water molecules are more highly ordered than in the bulk water and appear to "shield off" the hydrophobic ligand and solute molecules. Added salt interacts strongly with the water molecules leaving less water available for the "shielding off" effect, which is the driving force for L and H to interact with each other.

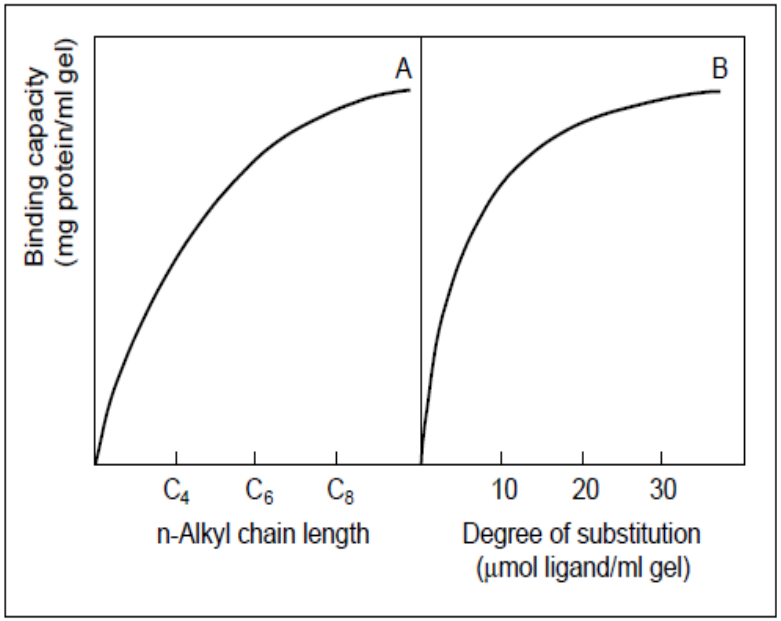
Tiselius, A. (1948): Adsorption separation by salting out. *Arkiv för Kemi, Mineralogi Geologi* 26B, 1–5

"...proteins and other substances which are precipitated at high concentrations of neutral salts (salting out), often are adsorbed quite strongly already in salt solutions of lower concentration than is required for their precipitation, and that some adsorbents which in salt-free solutions show no or only slight affinity for proteins, at moderately high salt concentrations become excellent adsorbents"

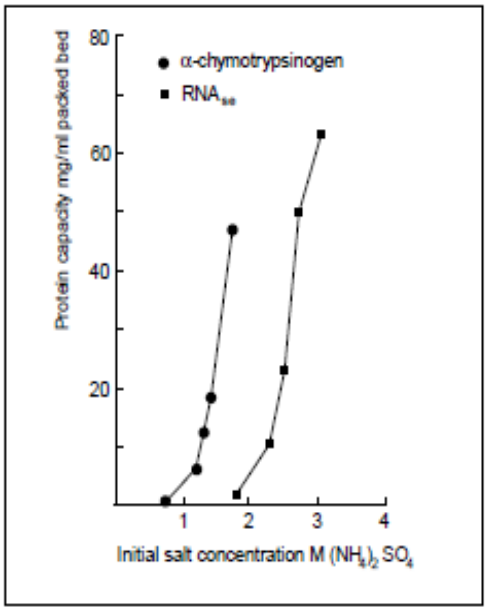
Hydrophobic ligands, chain length, degree of substitution and salt concentration



The various types of ligands



Influence of alkyl chain length and degree of substitution on binding capacity



Influence of salt concentration on adsorption capacity

Effect of salt type

Since HIC separation is based on the same principle as precipitation, a positive influence is identified for the same ions as in the **Hofmeister series.**

← Increasing precipitation ("salting -out") effect

Anions: PO_4^{3-}, SO_4^{2-}, $CH_3 \cdot COO^-$, Cl^-, Br^-, NO_3^-, CLO_4^-, I^-, SCN^-

Cations: NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}

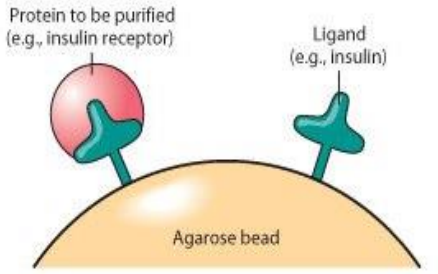
Increasing chaotropic ("salting-in") effect →

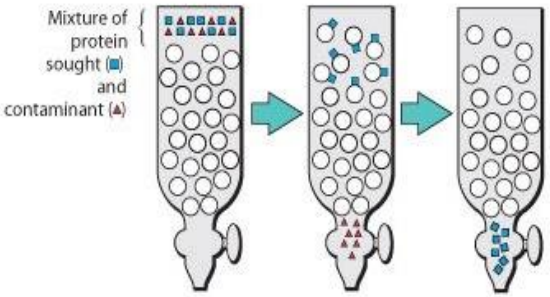
Table 1.
The Hofmeister series on the effect of some anions and cations in precipitating proteins.

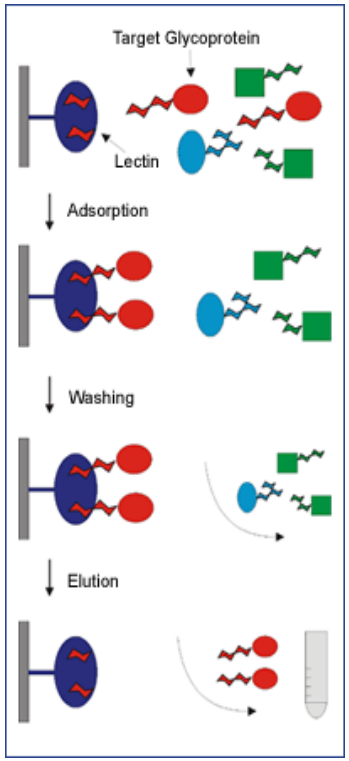
$Na_2SO_4 > K_2SO_4 > (NH_4)_2SO_4 > Na_2HPO_4 > NaCl > LiCl \ldots > KSCN$

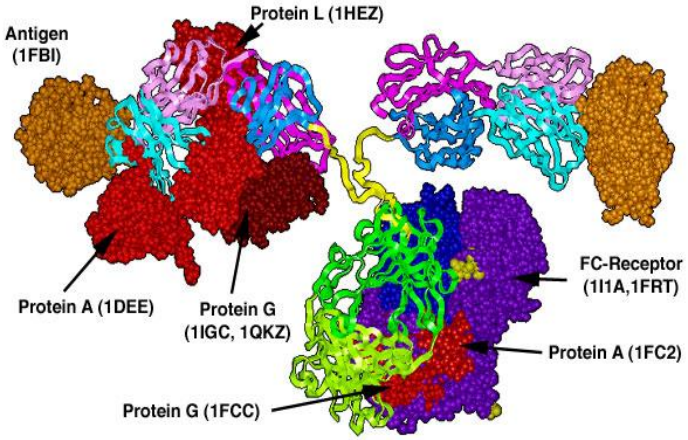
Table 2.
Relative effects of some salts on the molal surface tension of water.

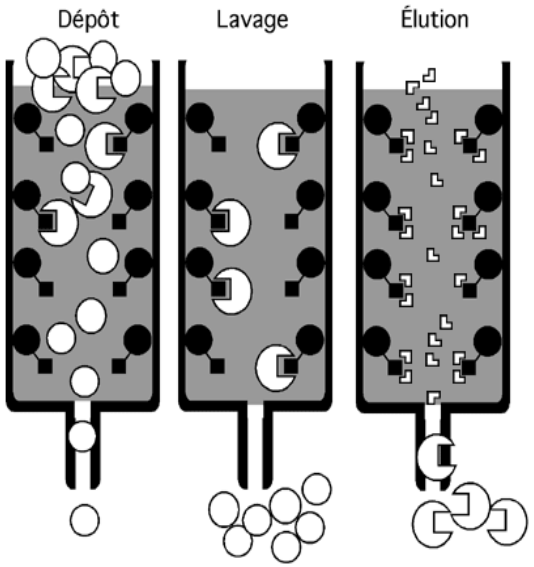
6.4.2 Affinity Chromatography (AC)

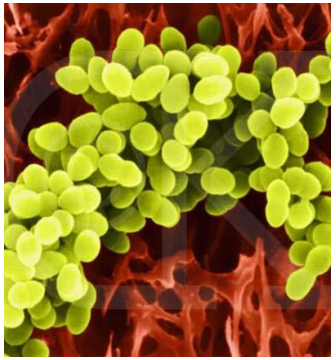


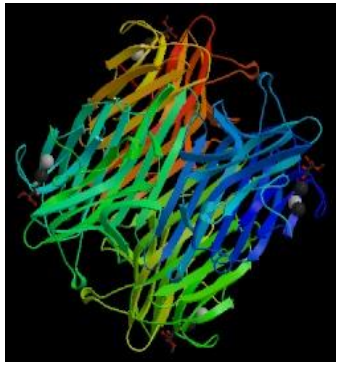












General observations

- Affinity chromatography (AC) separates molecules based on specific, reversible interactions between proteins and a specific ligand attached to the matrix. All other molecules do not bind and are directly eluted in the flowthrough
- AC allows to concentrate samples, sometimes by a large factor
- Desorption is obtained either in a specific manner by using a competitive ligand, or in a non-specific manner by modifying pH, ionic strength or polarity
- AC can sometimes be used to remove specific contaminants (e.g. using Benzamidine Sepharose FF (high sub)) to remove serine proteases

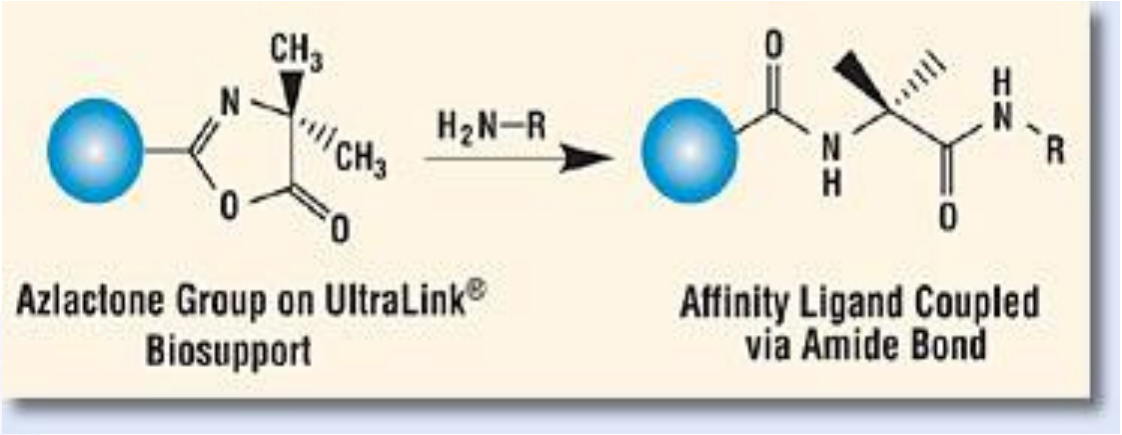
Affinity ligands and corresponding solutes

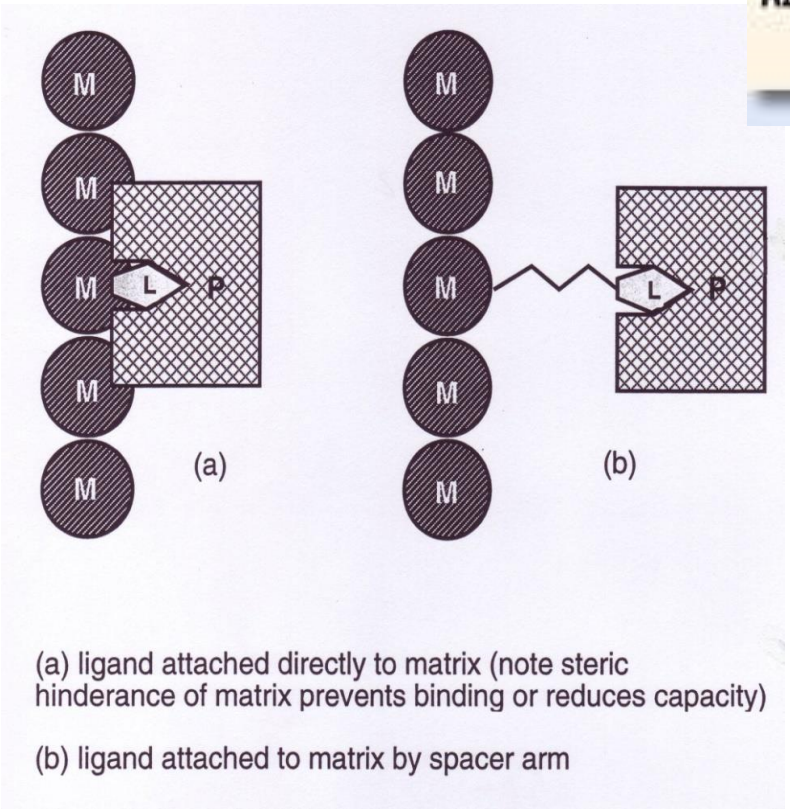
Ligand	Solute
2',5' ADP	Enzymes with $NADP^+$ as cofactor
5' AMP	Enzymes with $NADP^+$ as cofactor, and ATP-dependent kinases
Arginine	Proteases and zymogens (including prothrombin, prekallikrein, clostripain)
Benzamidin	Proteases (including trypsin, urokinase, kallikrein, prekallikrein)
Cibacron Blue	Large selection of enzymes having nucleotides as cofactors; serum albumin
Concanavalin A	**Terminal chains with residues such as α-D-glucopyranosyles, α-D-mannopyranosyles or sterically similar species**
GammaBind G, type 2	F_c region of IgG and molecules similar to protein G
Gelatin	F_c region of IgG and molecules similar to protein G
Ni-NTA, Co-NTA	**Recombinant proteins fitted with a $(His)_6$ tag**

Affinity ligands and corresponding solutes

Ligand	Solute
Lectin of *Helix pomatia*	N-acetyl-α-D-galactosaminyl residues
Heparin	Growth and coagulation factors, restriction endonucleases and other nucleic acid-binding proteins
Lentil lectin	Affinity similar to concanavalin A, but weaker affinity for simple sugars
Lysine	Plasminogen, ribosomal RNA
Poly(A)	Nucleic acids and oligonucleotides containing poly(U) sequences; RNA-specific proteins
Poly(U)	Nucleic acids, especially mRNA containing poly(A) sequences; poly(U)-binding proteins
Protein A	**F_c region of IgG and similar molecules**
Protein G	Similar to those of Prot. A, but different affinity for IgG of other species
Procion Red	Large selection of enzymes having nucleotide-type cofactors
Wheat germ lectin	N-acetyl-D-glucosamine

Spacer arm, for an easier access to the ligand





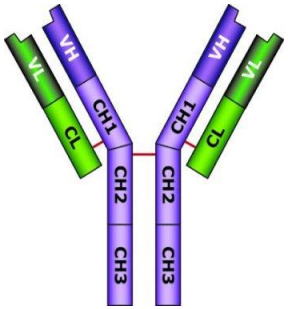
(a) ligand attached directly to matrix (note steric hinderance of matrix prevents binding or reduces capacity)

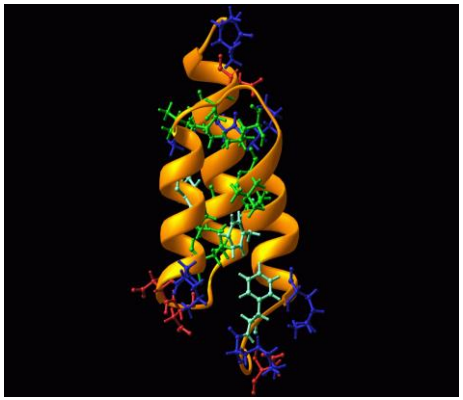
(b) ligand attached to matrix by spacer arm

Thermo Scientific UltraLink Biosupport immobilization chemistry. Azlactone groups of the activated resin react spontaneously with primary amines on proteins or other ligands to form highly stable amide bonds, conjugating the molecule by a 5-atom spacer arm.

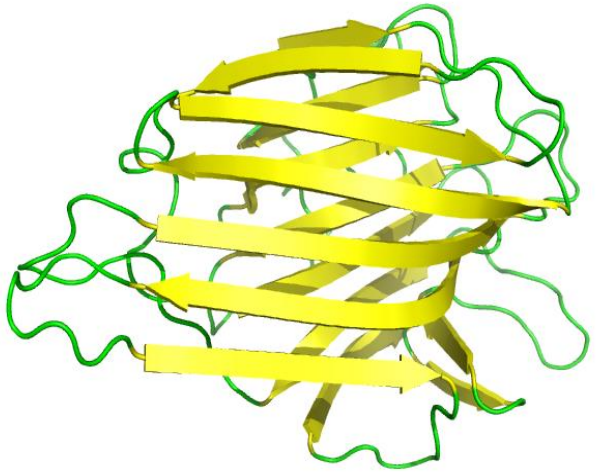
Protein A

- Protein extracted from the Cowan strain of *Staphylococcus aureus*
- Capable of binding to the constant regions of human, murine and rabbit IgG heavy chains
- Can be used in place of the secondary antibodies for ELISA and Western blotting
- Main usage is for industrial chromatography purification of antibodies



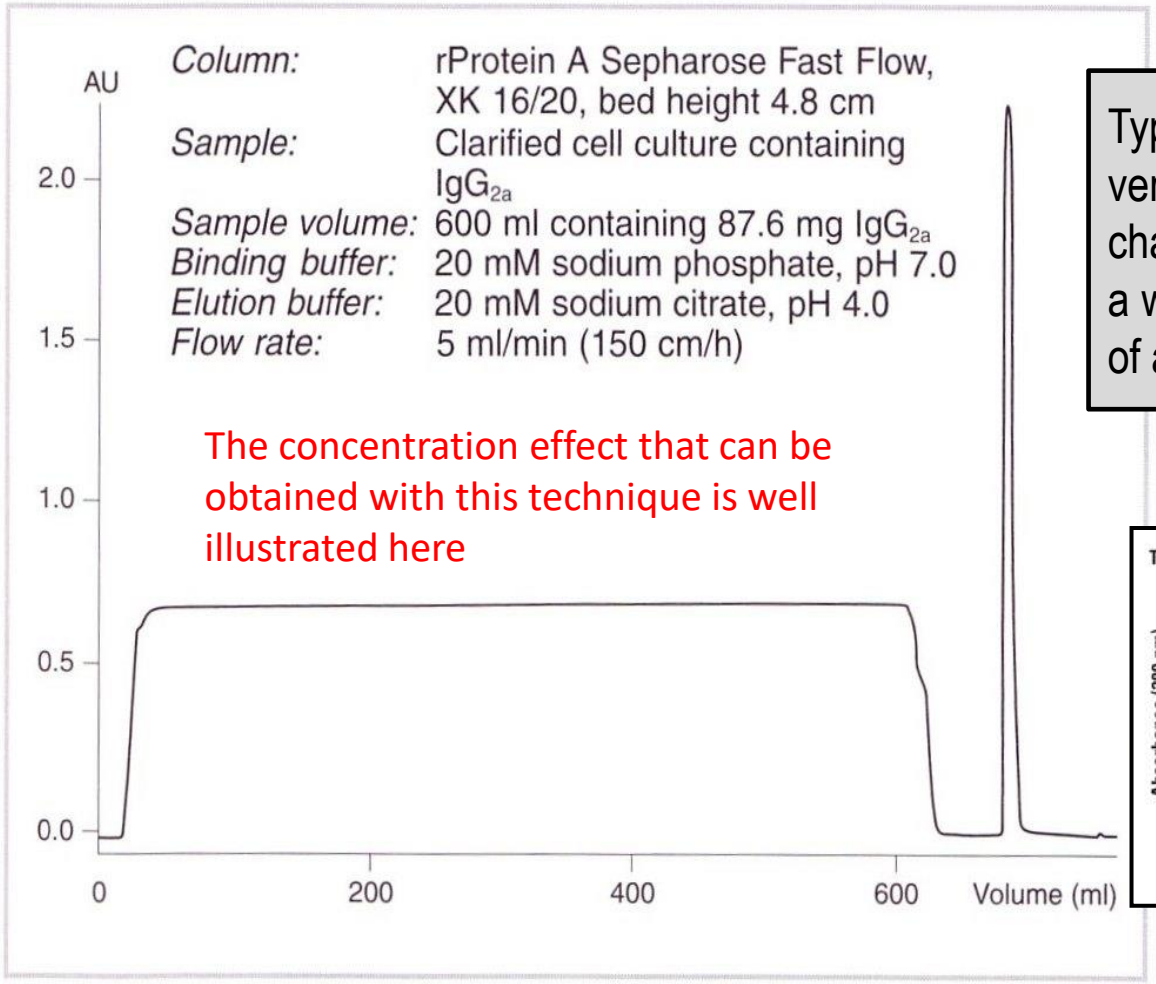


Concanavalin A



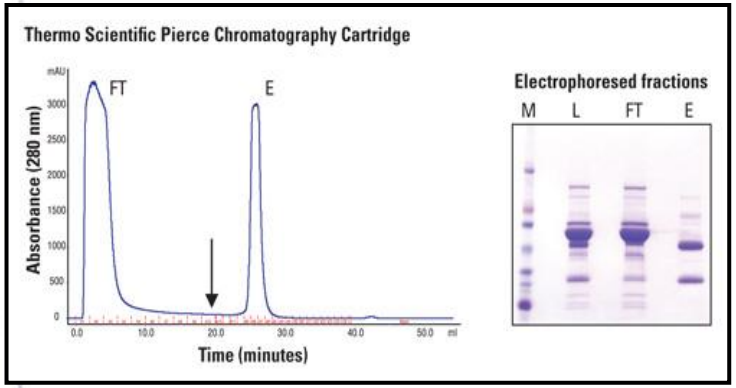
- Concanavalin A (Con A) is a lectin type protein isolated from leguminous plants such as jackbean (*Canavalia ensiformis*).
- Con A specifically binds mannose and exists under different oligomeric forms (dimer, tetramer) depending on pH value
- The mannose-Con A interaction has been investigated and characterized by a variety of techniques.
- The adsorption sites of Con A have also been characterized by crystallography

Affinity Chromatography (AC), example 1



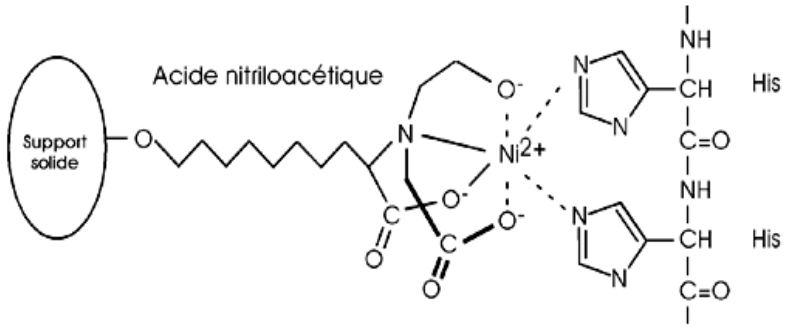
Purification of a monoclonal IgG_{2a} from clarified cell culture on rProtein A Sepharose Fast Flow.

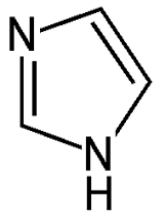
Typical AC chromatogram where a very large volume of liquid is charged on the column, followed by a washing step and the quick elution of a single product peak



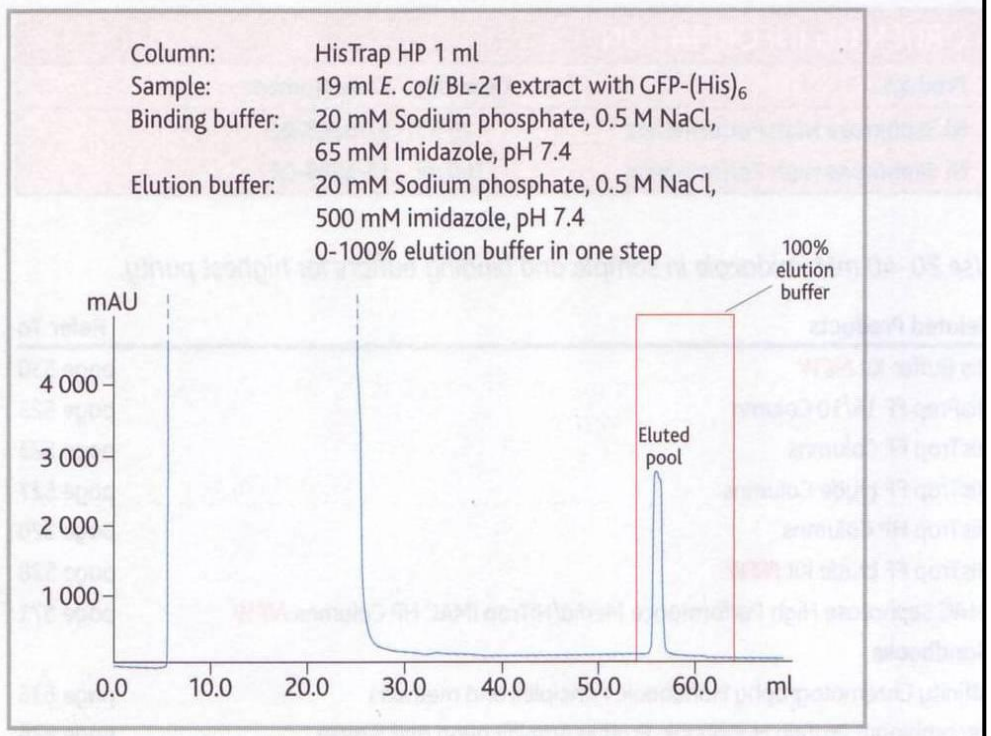
AC, Example 2: His-tag™ and IMAC (Immobilized Metal Affinity Chromatography) (His-tag is a trademark deposited by EMD Bioscience)

- **His-tag** consists in adding a short sequence of 6 (or 10) Histidine residues at one extremity of the recombinant protein
- The tag was invented at Roche and its vectors are being distributed (among others) by Qiagen, Sigma, Thermo Scientific, GE Healthcare
- Use of the vectors is free of charge for academic research, but royalties have to be paid to Roche for any commercial application
- **IMAC** uses the great affinity of histidine residues for chelated transition metal ions such as Ni^{2+} or Co^{2+}
- Elution can be performed at pH values below 6 (Histidines residues partially reduced), but displacement using imidazole is a milder approach

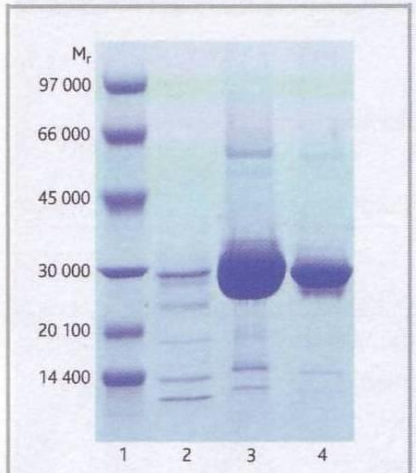




Example 3: IMAC purification of a recombinant GFP



Purification of GFP-(histidine)$_6$ on HisTrap HP 1 ml. Due to the small bead size of Ni Sepharose High Performance (34 µm) the eluted pool was 2 ml, resulting in a concentrated eluted target protein.



Lane 1: LMW (Low Molecular Weight marker)
Lane 2: Wash pool
Lane 3: Eluted GFP-(His)$_6$
Lane 4: Eluted GFP-(His)$_6$ diluted 1:4

SDS-PAGE of pooled fractions. Inclusion of 65 mM imidazole in the sample and during the wash provided the highest possible purity with no loss of target protein.

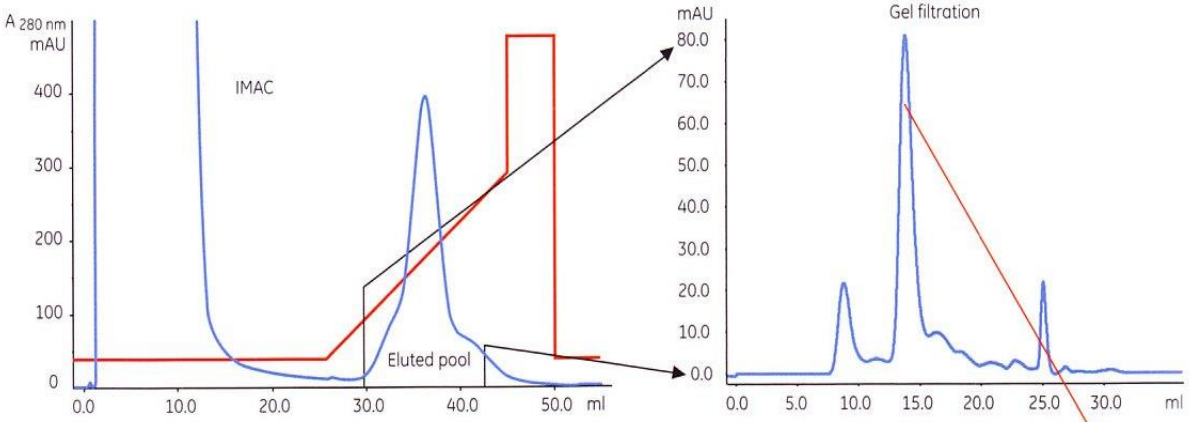
Two-step purification of a high molecular weight histidine-tagged protein

Fig. 1A: First purification step with IMAC

Sample:	10 ml *E. coli* extract with low-level expression of a histidine-tagged mannanase, Man 26A, from *Cellulomonas fimi* (M_r ~ 100 000)
Column:	HisTrap™ HP 1 ml
Binding buffer:	20 mM sodium phosphate, 30 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4
Gradient:	25 ml linear gradient 30–300 mM imidazole
Flow rate:	1 ml/min
System:	ÄKTAexplorer™ 100

Fig. 1B: Second step with gel filtration

Sample:	0.5 ml concentrated sample from IMAC step
Column:	Superdex™ 200 10/300 GL
Buffer:	PBS, pH 7.5
Flow rate:	0.5 ml/min
System:	ÄKTAexplorer 100



Conclusions

- The high molecular weight protein histidine-tagged mannanase Man 26A was purified in its enzymatically active form
- Excellent binding properties of Ni Sepharose™ High Performance (HP)
- 60 mg of purified protein in a single run
- A second purification step using gel filtration with Superdex 200 was added for high purity needs of 95 %

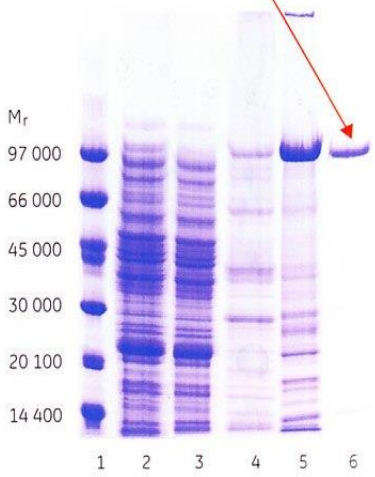


Fig. 1C: SDS-PAGE

Lane 1:	LMW
Lane 2:	*E. coli* extract
Lane 3:	IMAC flow-through
Lane 4:	Early IMAC fraction
Lane 5:	IMAC eluted pool
Lane 6:	Gel filtration pool

Scale-up of an IMAC chromatography step

Fig. 2A: Scale-up purificaiton of a histidine-tagged protein

Sample: Histidine-tagged Maltose binding protein in *E. coli* extract (samples loaded contained 8, 40 and 160 mg, respectively)

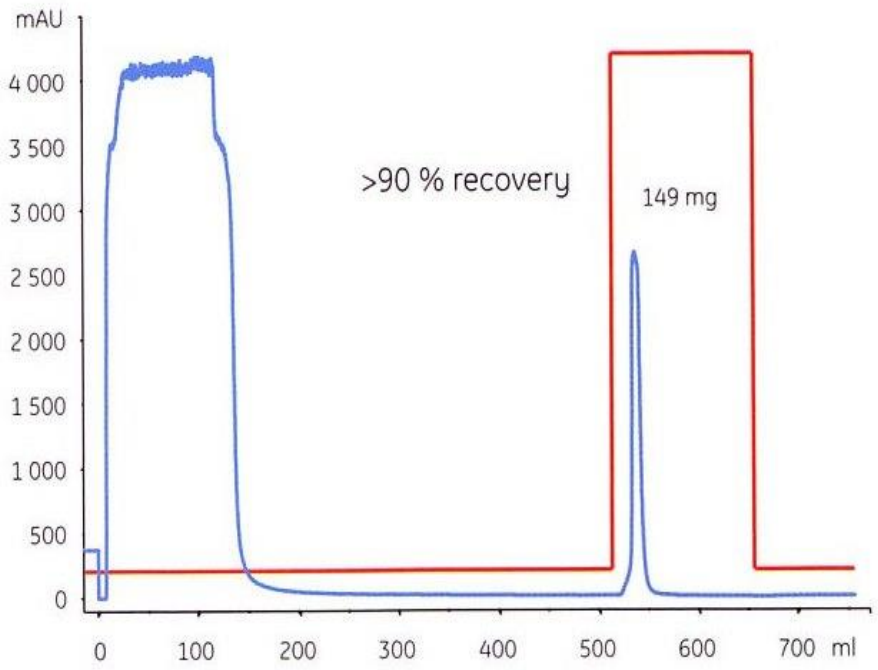
Columns: HisTrap FF 1 ml, HisTrap FF 5 ml, HisPrep™ FF 16/10 20 ml. All columns are prepacked with Ni Sepharose 6 Fast Flow.

Binding buffer: 20 mM sodium phosphate, 25 mM imidazole, 500 mM NaCl, pH 7.4

Elution buffer: 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4

Flow rates: HisTrap FF 1 ml: 1 ml/min; HisTrap FF 5 ml: 5 ml/min; HisPrep FF 16/10: 5 ml/min

HisPrep FF 16/10, 20 ml



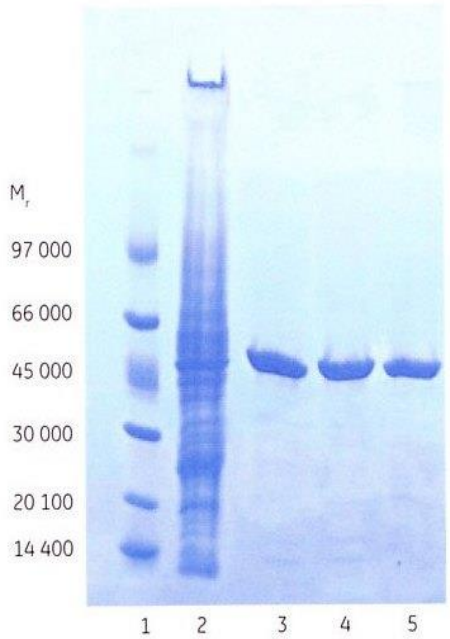


Fig. 2B: SDS-PAGE

Lane 1: LMW

Lane 2: Start material, *E. coli* extract

Lane 3: Eluted pool, HisTrap FF 1 ml

Lane 4: Eluted pool, HisTrap FF 5 ml

Lane 5: Eluted pool, HisPrep FF 16/10 (20 ml)

How are chromatography separations scaled-up?

- Industrial chromatography is scaled-up in a simplified way:
  - The bed height is kept constant
  - The fluid superficial velocity is kept constant
  - The flow rate (and hence the capacity) is increased by the desired factor F_{su}
  - Hence, the column diameter must be increased by ……..