Module ChE 311 Biochemical Engineering

Downstream processing
Lecture 5 - Process chromatography

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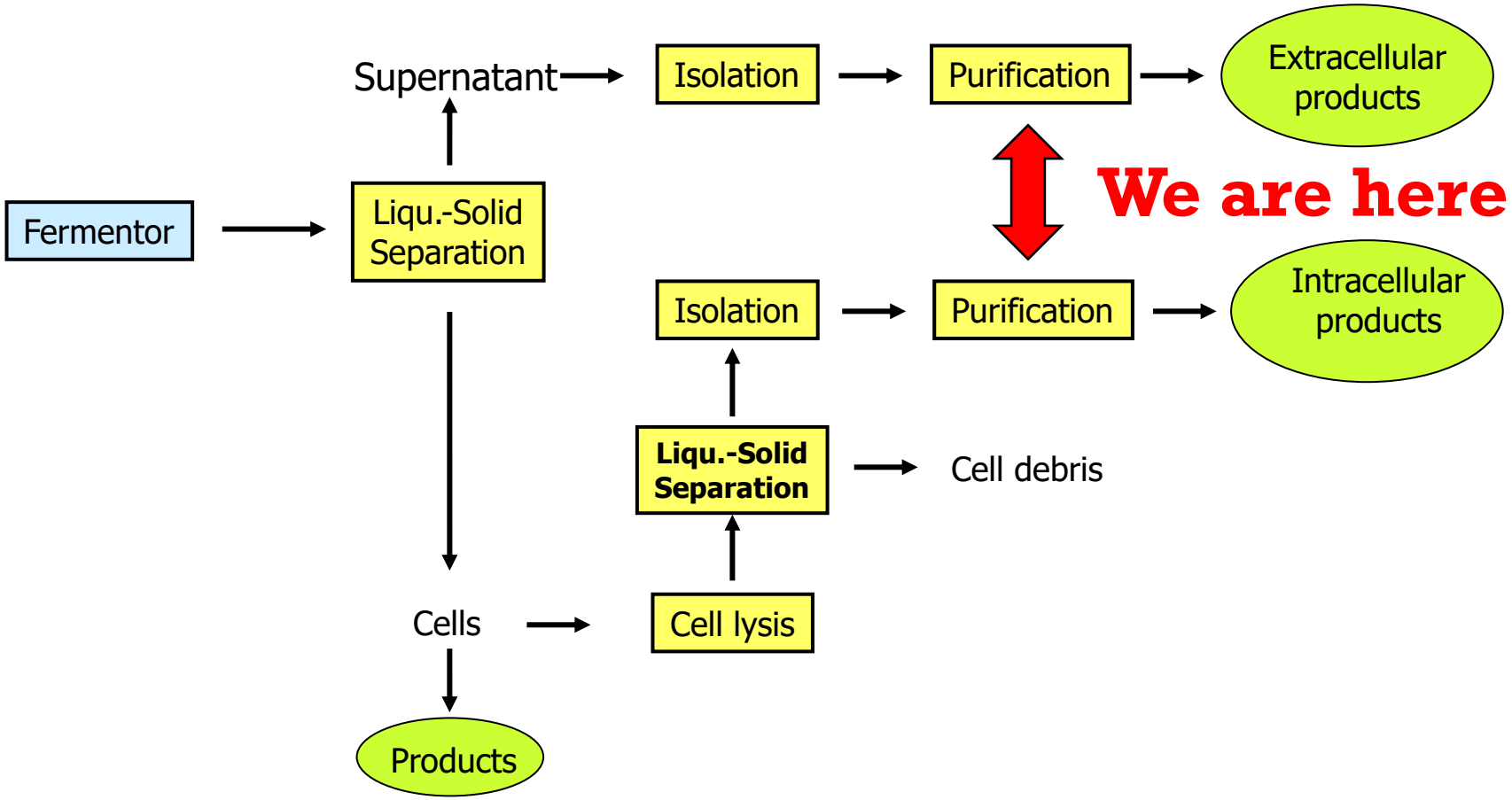
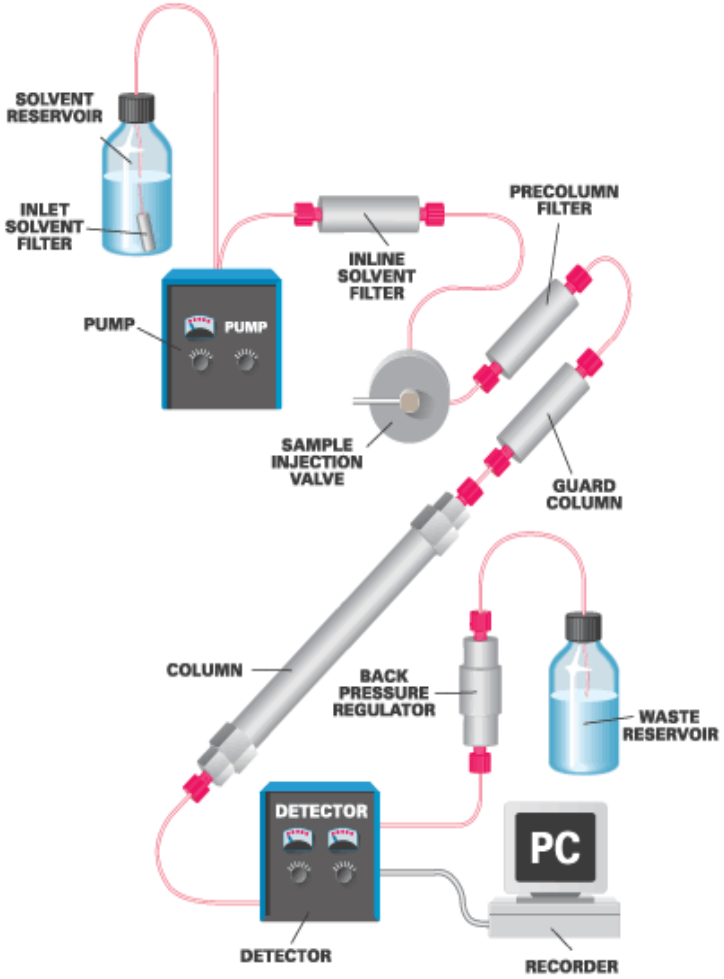
WARNING

- 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**

Source: Bioprocess International

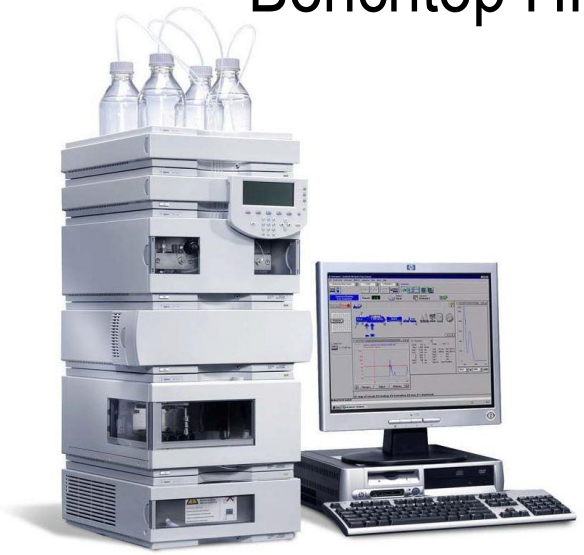
Common pathway for a purification protocol





There is chromatography … and then there is **process chromatography**

Benchtop HPLC



Benchtop **preparative LPLC**

Bioprocess chromatographic purification

Do you observe similitudes, differences?

Production-scale columns

How do you fill a large scale column with resin?

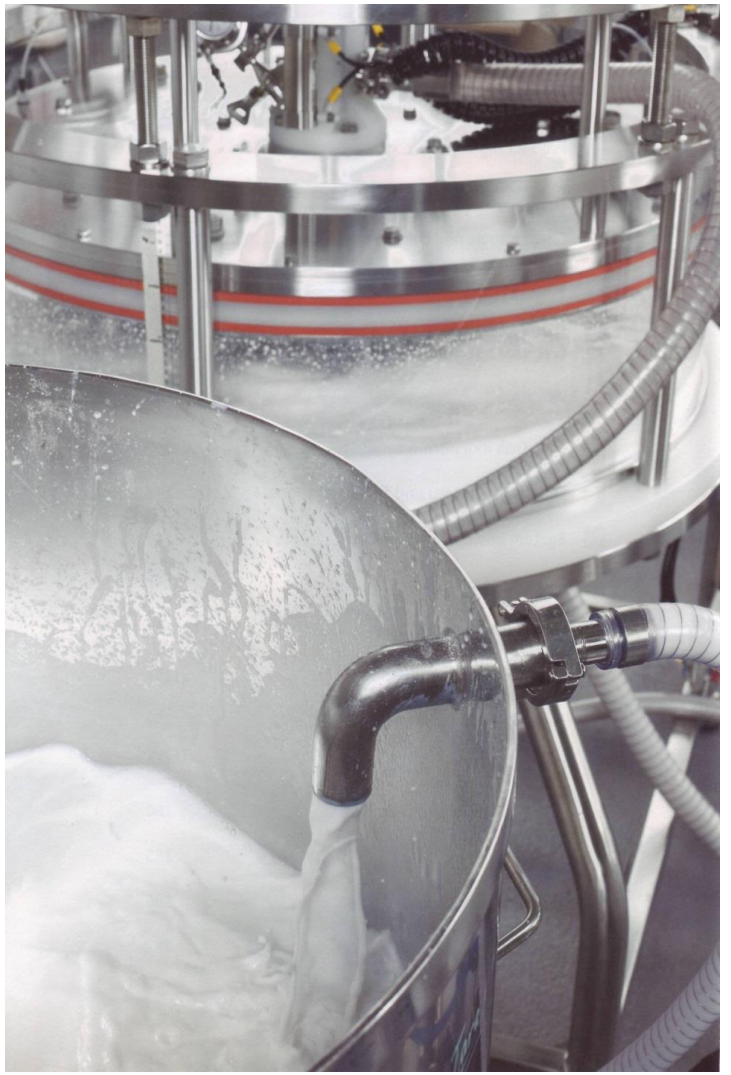
But then, how do you check it has been packed correctly?

- Columns of all sizes can be purchased pre-packed from the suppliers
- However, manual packing is still performed in many different settings
- The packing should be uniform and enable an even flow and distribution of the eluent
- Manual packing of a lab-scale column is relatively straightforward
- Packing of a large-scale, production column is usually done with a large degree of automation (see video posted on Moodle)

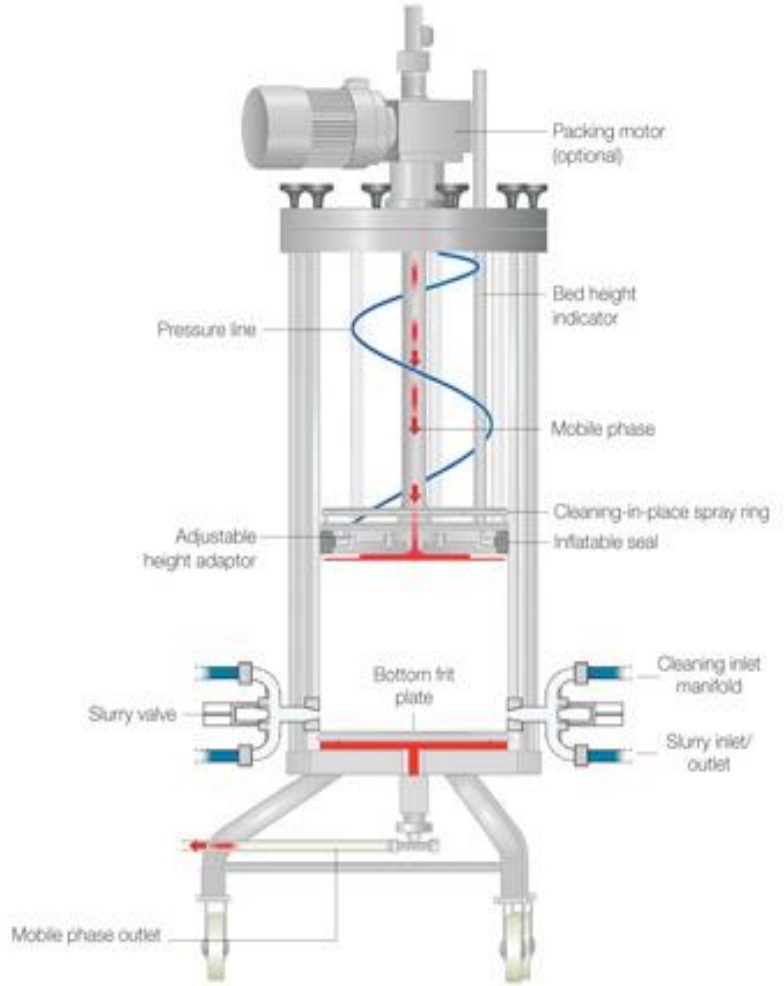
Range of OPUS® prepacked chromatography column. Source: Merck Millipore

Importance of column packing

- The quality of column packing is key to the separation efficiency
- Beads should be homogeneously distributed 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 assess the quality of a packing. For instance, pulse injection of an inert tracer or breakthrough curves
- In a well-packed column, the height of a theoretical plate H is about 3 times the diameter of the resin particles



4.11 Column filling and packing



Schematic drawing of Bio-Rad InPlace column.

Bio-Rad EasyPack Column

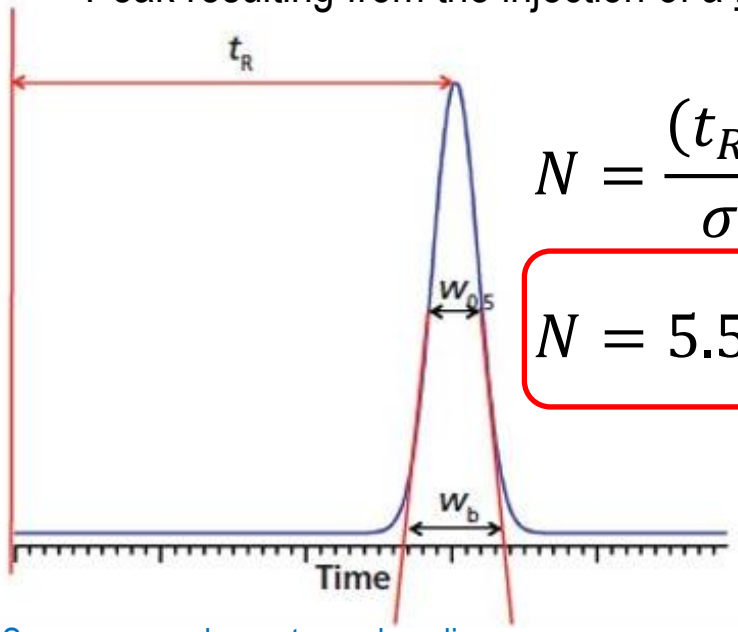
Bio-Rad InPlace Column and Control Console

BIO-RAD

Vidéo sur le remplissage de colonnes industrielles

Number of theoretical plates N and asymmetry factor F_a

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



Source: www.chromatographyonline.com

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

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

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

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

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

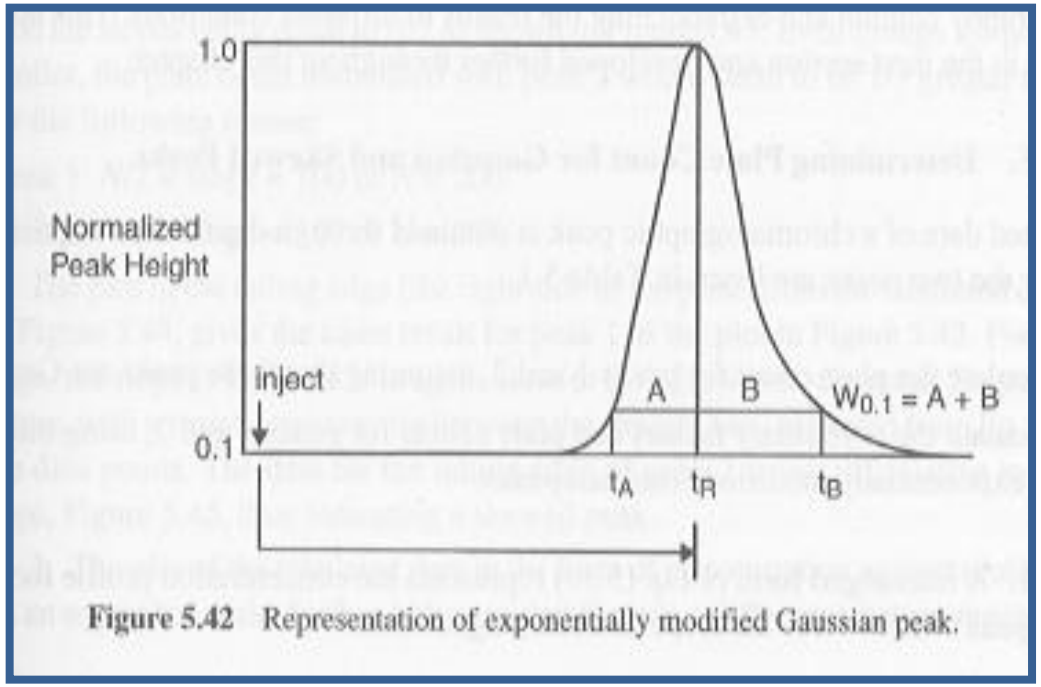


Figure 5.42 Representation of exponentially modified Gaussian peak.

- For various reasons, the might feature a non-Gaussian geometry
- The observed asymmetry is characterized by the factor F_a, measured at 10 % of the peak height:

$$F_a = \frac{B}{A}$$

Fa > 1 : tailing
Fa < 1 : fronting

The van Deemter equation

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

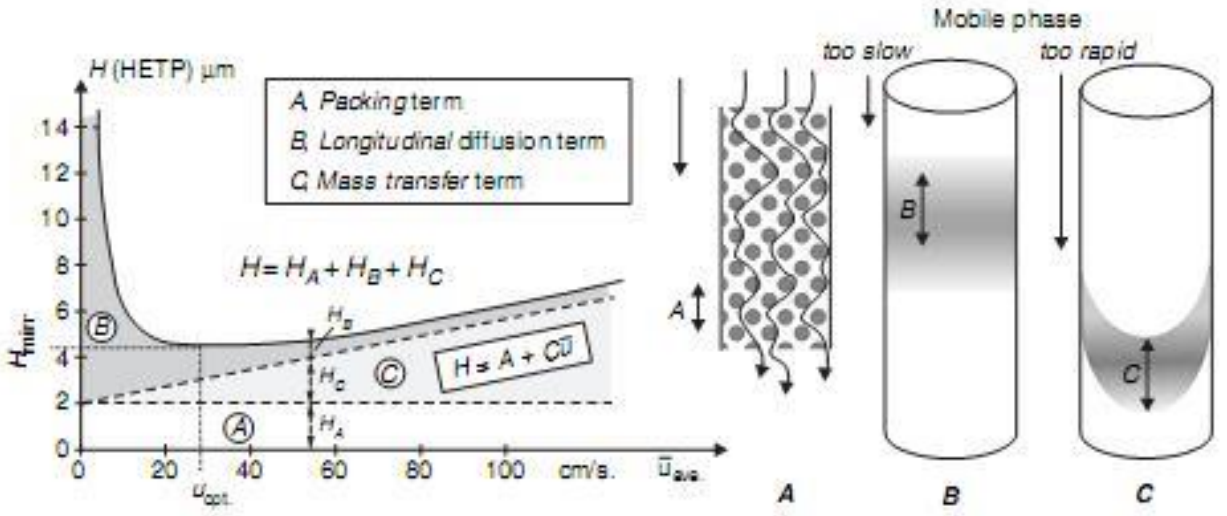
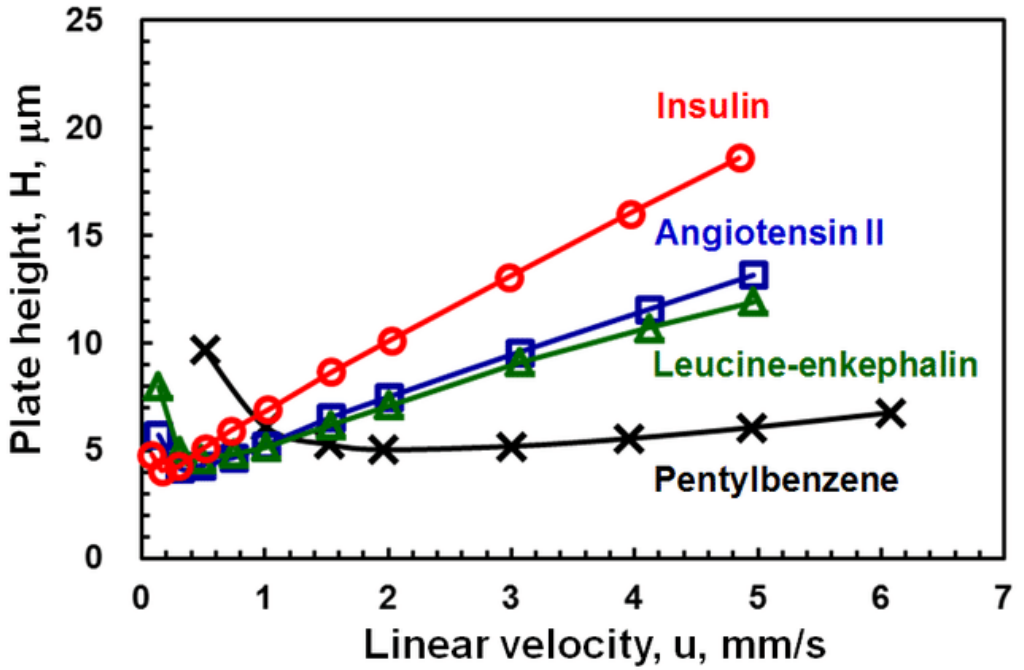


Figure 1.11 *Van Deemter's curve in gas chromatography with the domains of parameters A, B and C indicated.* There exists an equation similar to that of Van Deemter that considers temperature: H = A + B/T + CT.



- 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 !!!

Van Deemter curves for molecules of different sizes, ranging from insulin (5808 g/mol) to pentylbenzene (148.2 g/mol)

The influence of a lower diffusion coefficient on axial dispersion (B-term) and mass transfer (C-term) is clearly visible

Control of packing quality and resolution between peaks

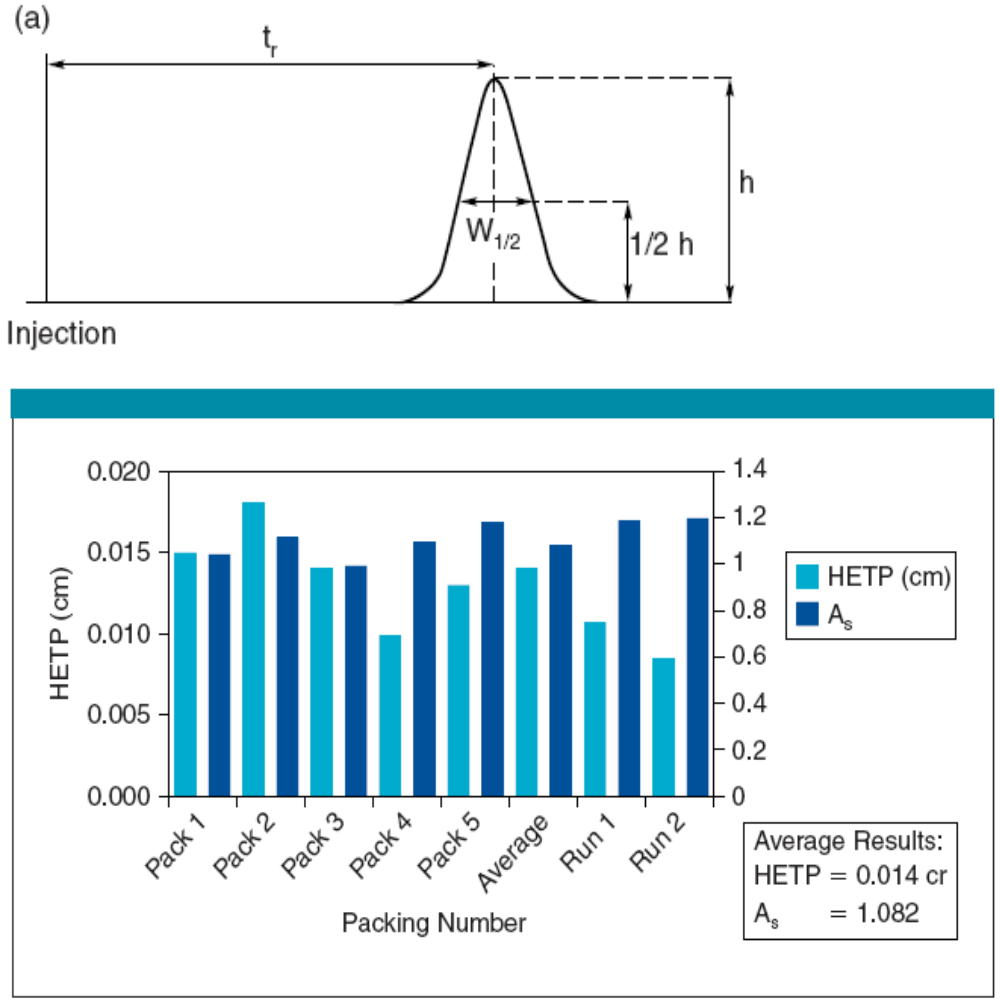
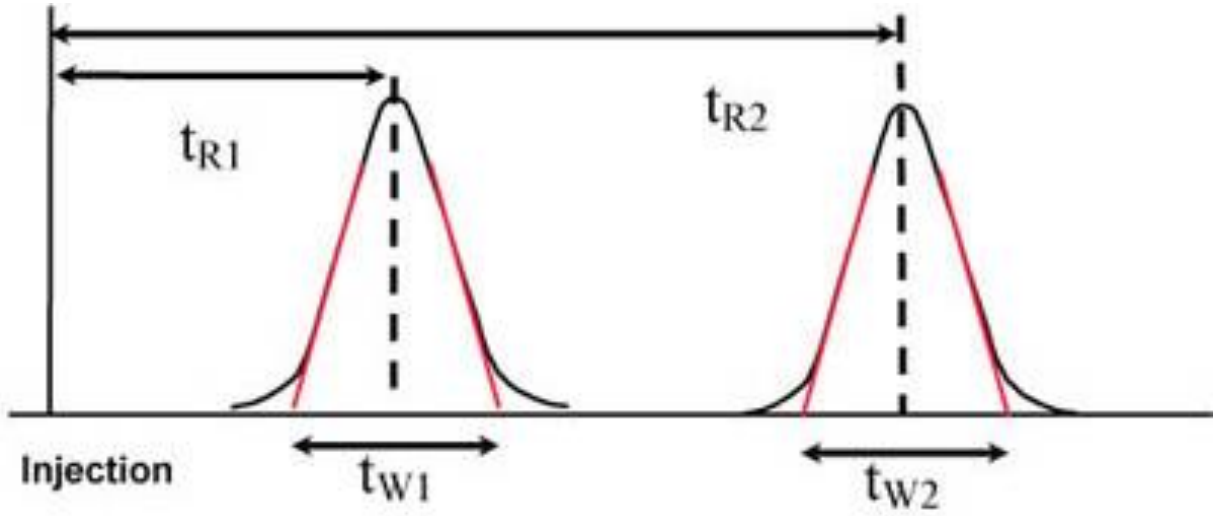


Figure 2. Summary of Sephacryl S-200 HR packing qualification

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



- The resolution factor R is an expression of separation efficiency between two compounds

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

Resolution for identical and for differently-sized peaks

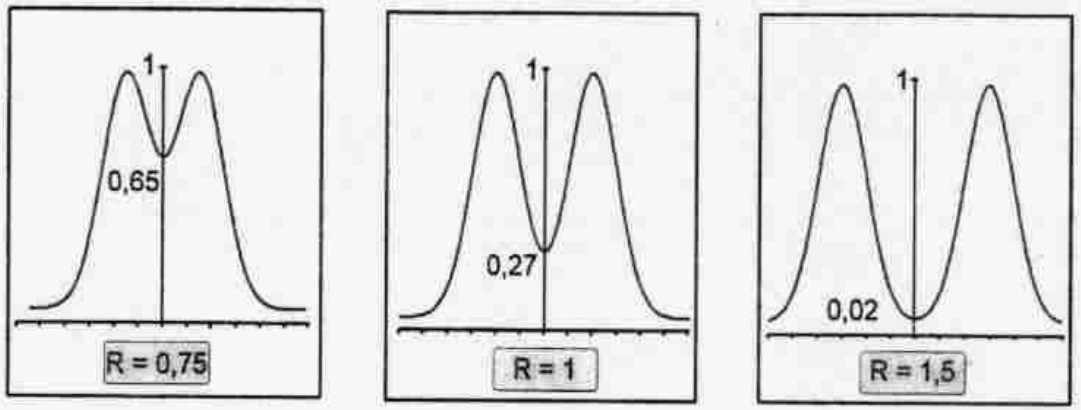
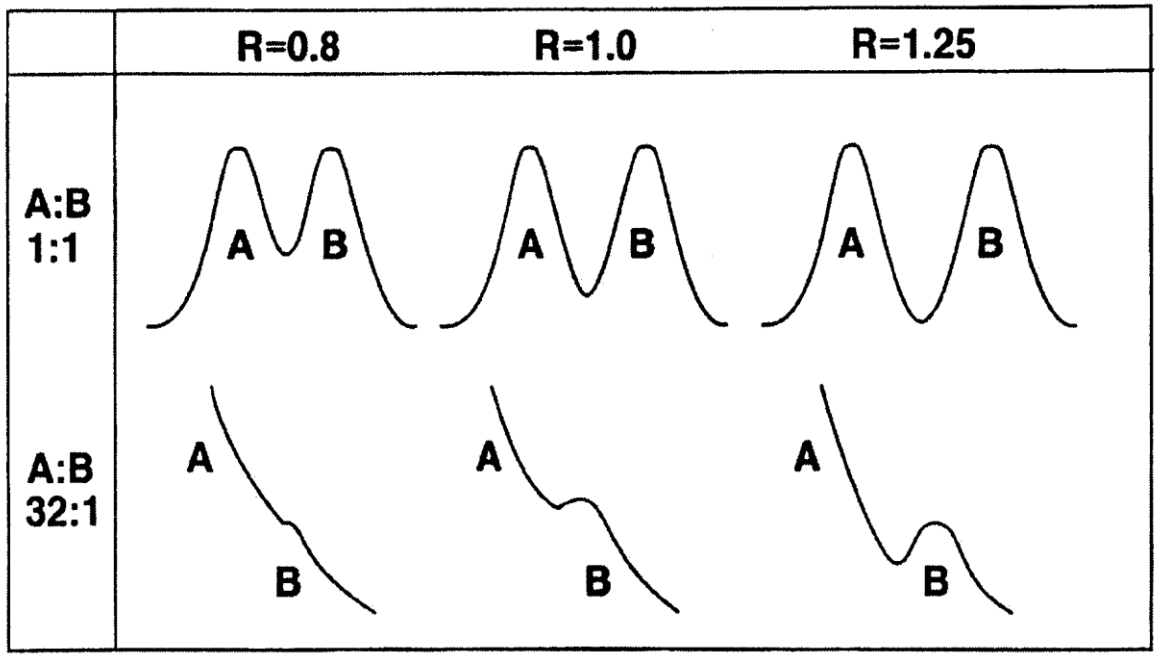


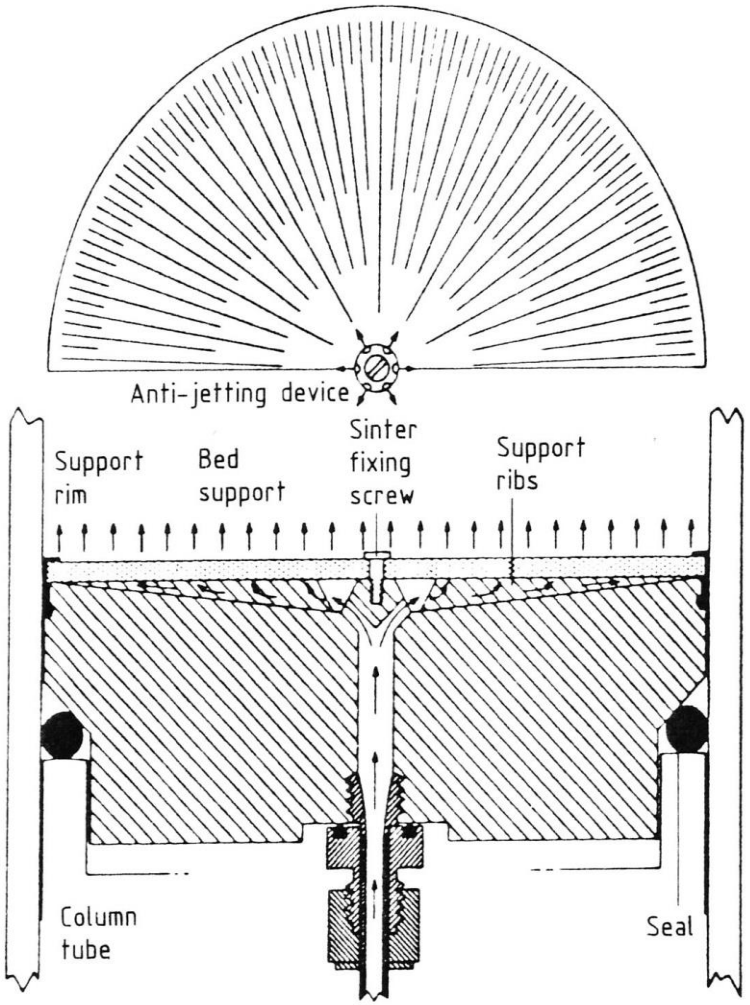
Figure 1.8 – *Facteur de résolution*. Simulation de pics chromatographiques par juxtaposition plus ou moins rapprochée de 2 courbes gaussiennes identiques. Aspect visuel correspondant aux valeurs de R indiquées sur les diagrammes. A partir de R = 1,5 on considère que les pics sont résolus, la vallée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

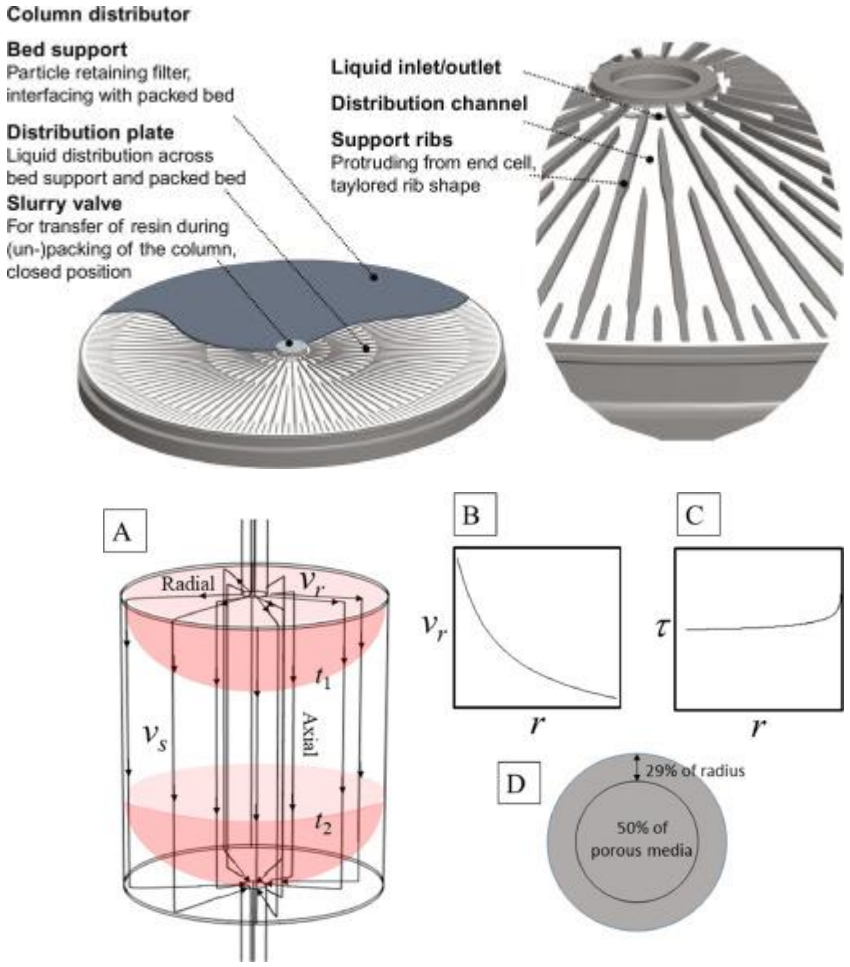
This picture gives a visual impression of the separation efficiency between two peaks of identical size (A:B = 1:1) for different values of the resolution R

This picture gives a visual impression of the separation efficiency between two peaks of identical size (A:B = 1:1) and two peaks where the concentration of compound A is 32 times higher than that of compound B



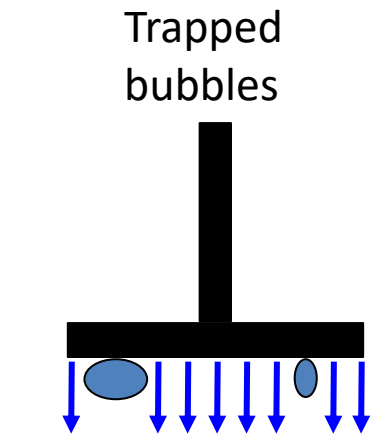
Influence of the liquid distribution system

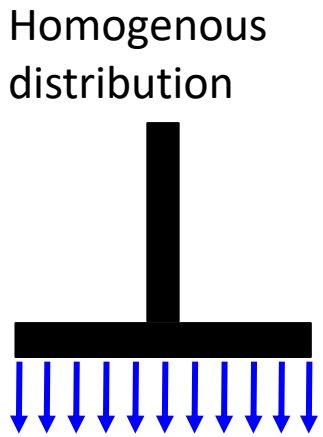


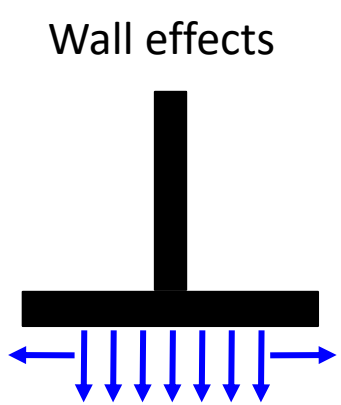


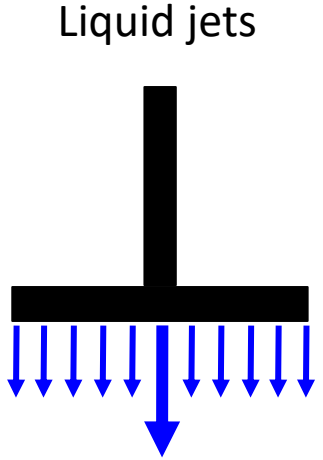
R. Ghosh et al., Journal of Chromatography A, 1618 (2020), 460892

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



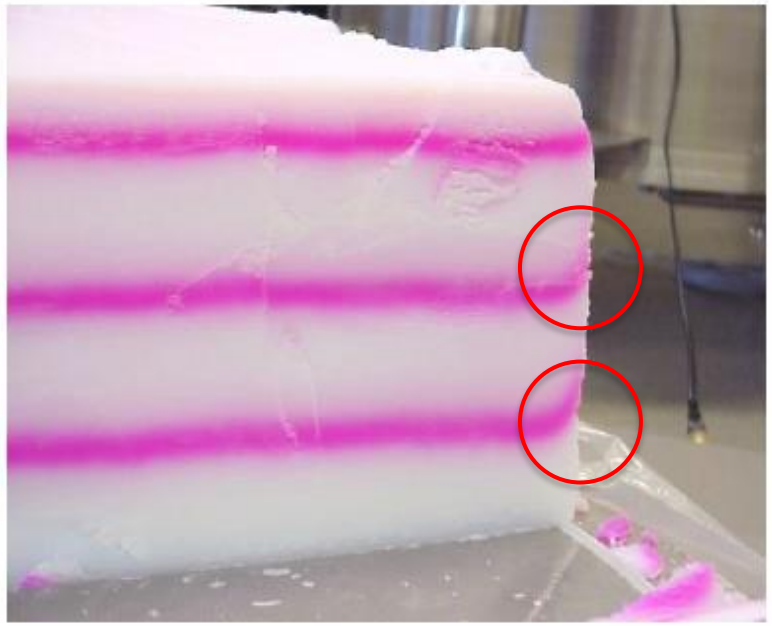




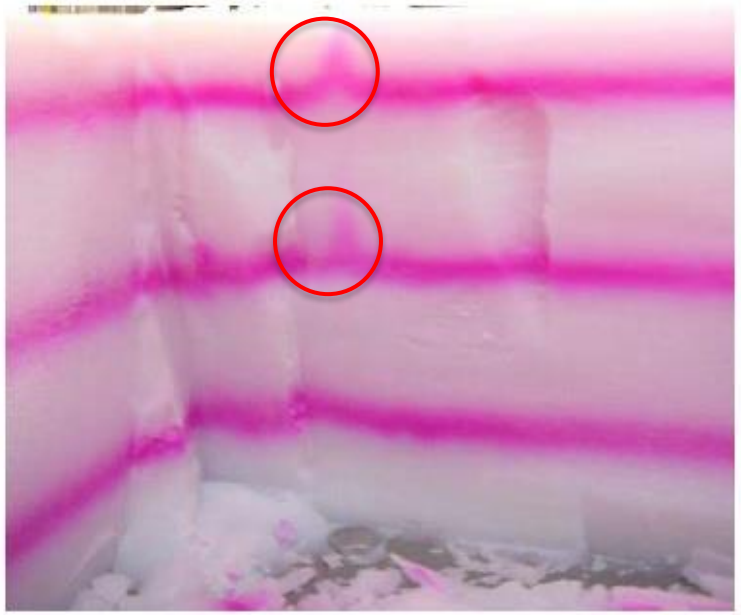


Measurement of column performance (dye test)

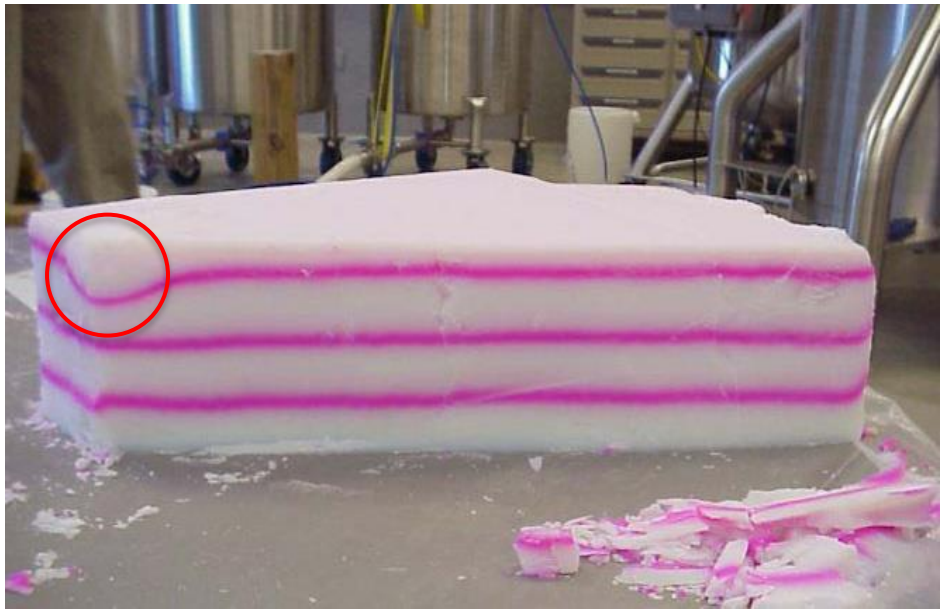
Wall Effect



Screen Retainer Bolts



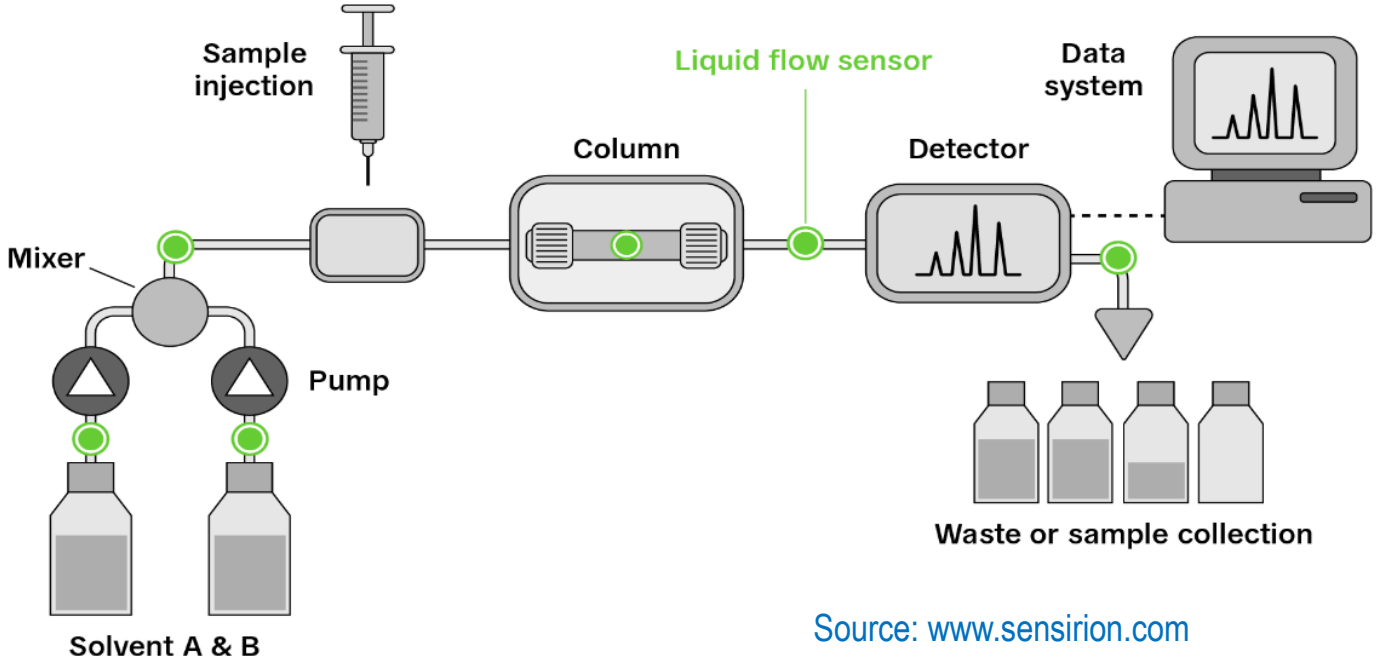
Liquid jetting in the center



The repeated injection of a dye tracer gives a direct, visual evaluation of the column performance

The component are the same for analytical, preparative and production chromatography

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



Source: www.sensirion.com

- Detection is achieved most of the time by UV-VIS, fluorescence, conductometry, pH, or refractive index measurement
- The different types of chromatography resins will be discussed next

Types of (preparative) chromatography

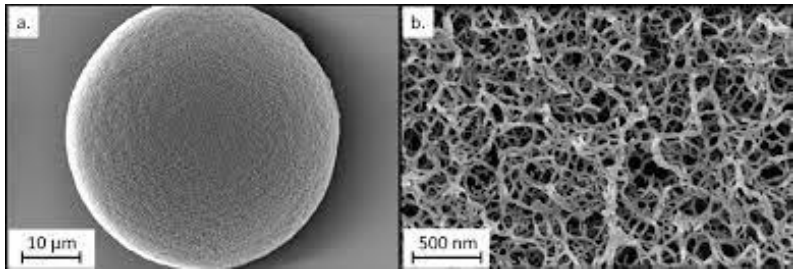
- Cation exchange chromatography (CEX)
- Anion exchange chromatography (AEX)

IEX

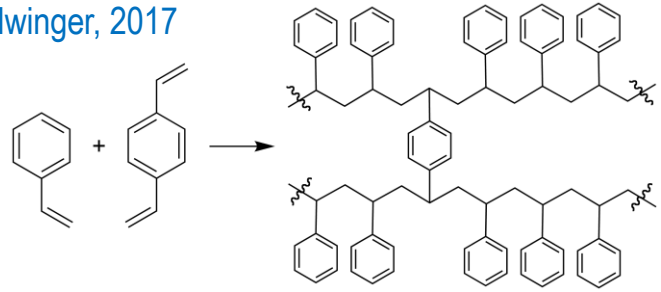
- Hydrophobic interaction chromatography (HIC)
- Mixed mode chromatography (MMC)
- Affinity chromatography (AC)
- Size exclusion chromatography (SEC)

Chromatography resins; materials and properties

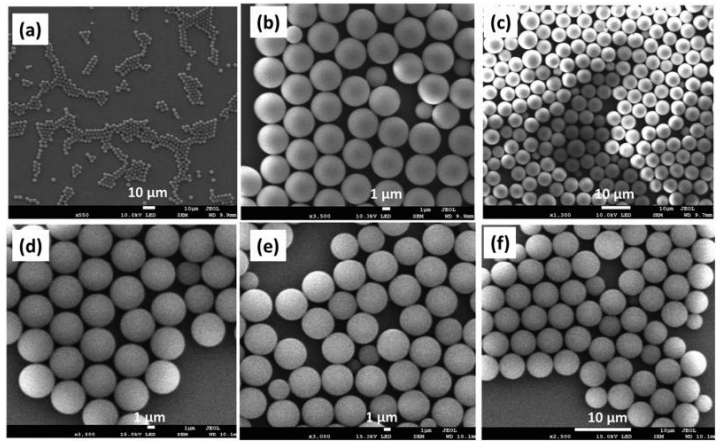
- Most stationary phases are based either on synthetic polymers (e.g. polystyrene / DVB, polymethacrylate / DVB) or on natural polymers (e.g. cross-linked agarose)
- These matrices are then functionalized by coupling with various groups
- Generally speaking, resin particles should induce very little non-specific adsorption
- They should be highly porous, chemically inert and mechanically resistant
- They should be spherical and with a narrow particle size distribution



MabSelect TM resin particle © F. Elwinger, 2017

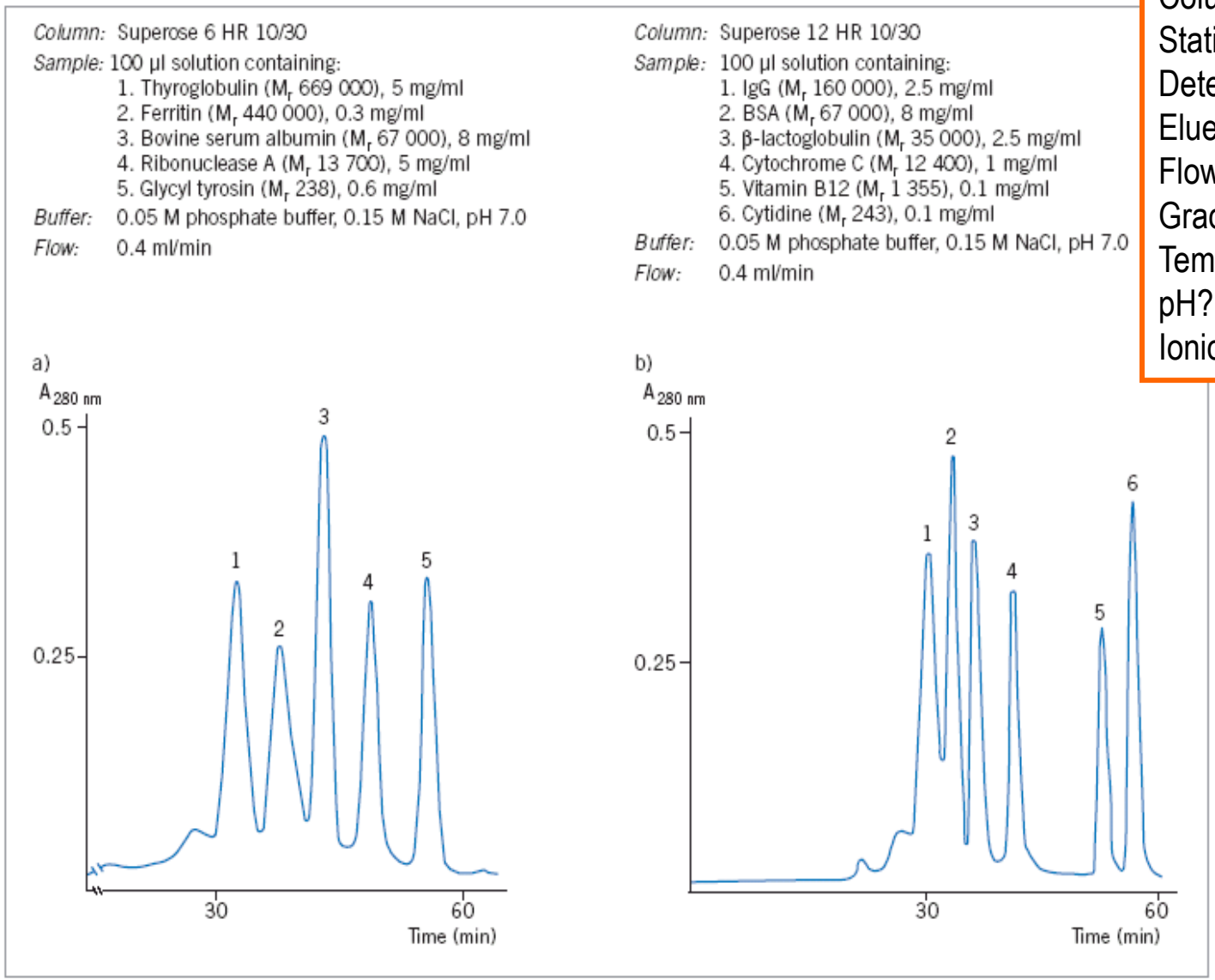


Polystyrene/Divinylbenzene polymer



J. Xiao et al., Reactive and Functional Polymers 178, September 2022, 105357

A chromatogram conveys a lot of information



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

Absorbance at 280 nm corresponds to aromatic rings and serves as a generic protein detector.

The peak/fraction containing the target molecule must be identified with a specific technique

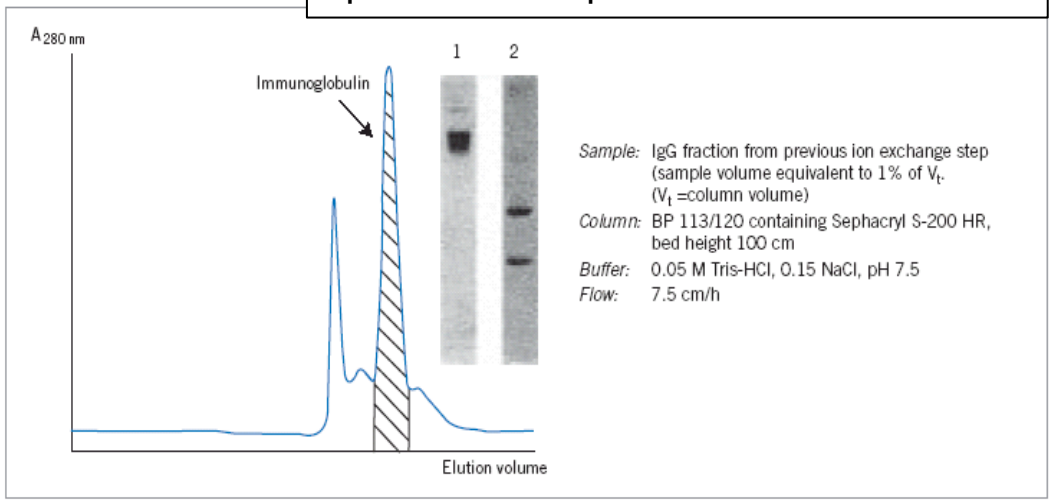
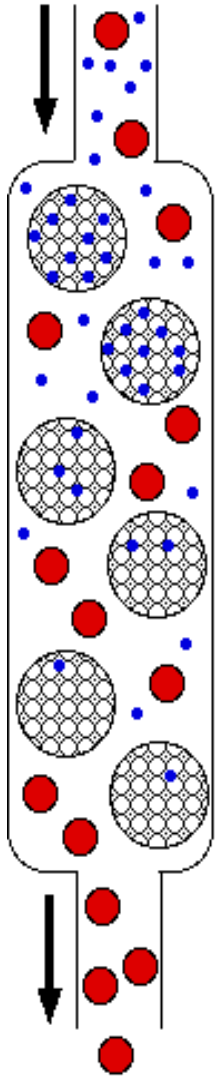
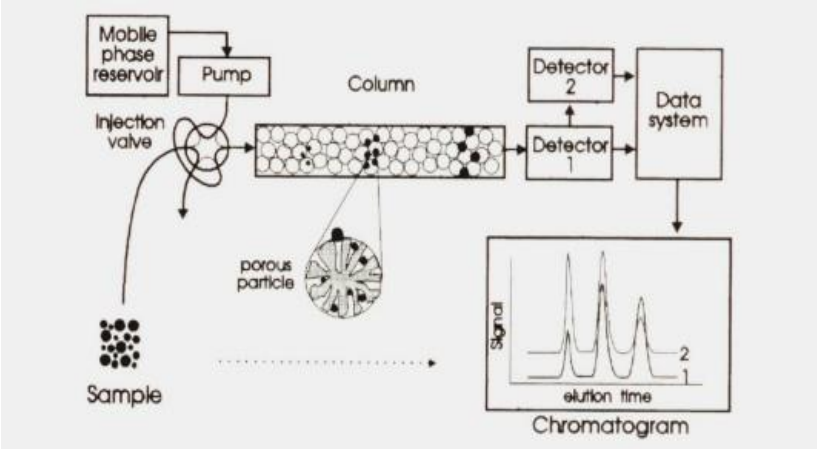
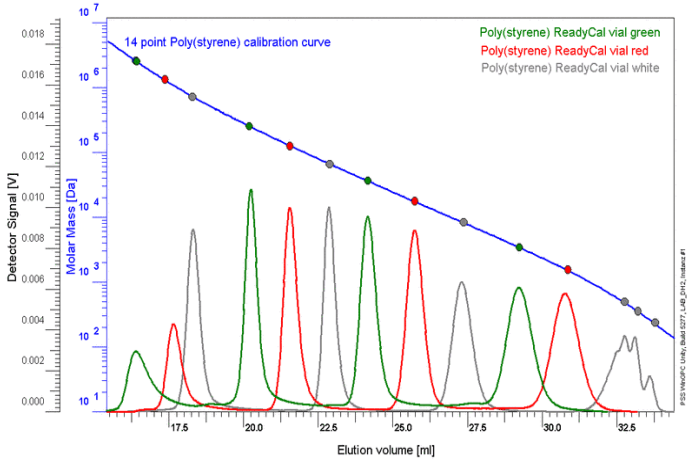


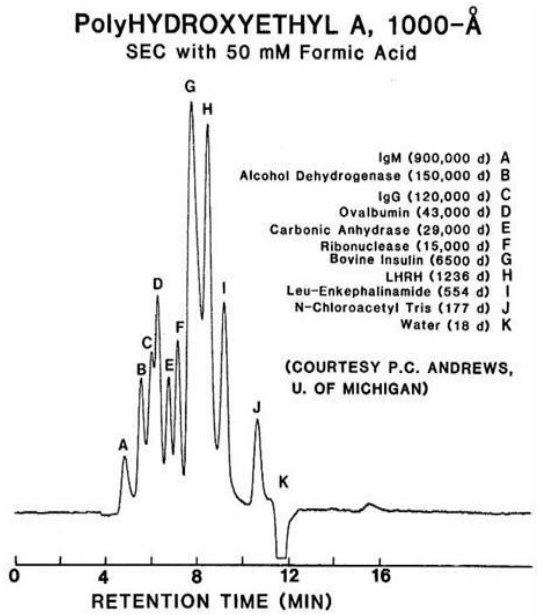
Fig. 34. Purification of monoclonal antibodies on Sephacryl S-200 HR. Inset shows analysis by gradient SDS-PAGE of the immunoglobulin pool. Lane 1, native sample; lane 2, sample reduced with 2-mercaptoethanol.

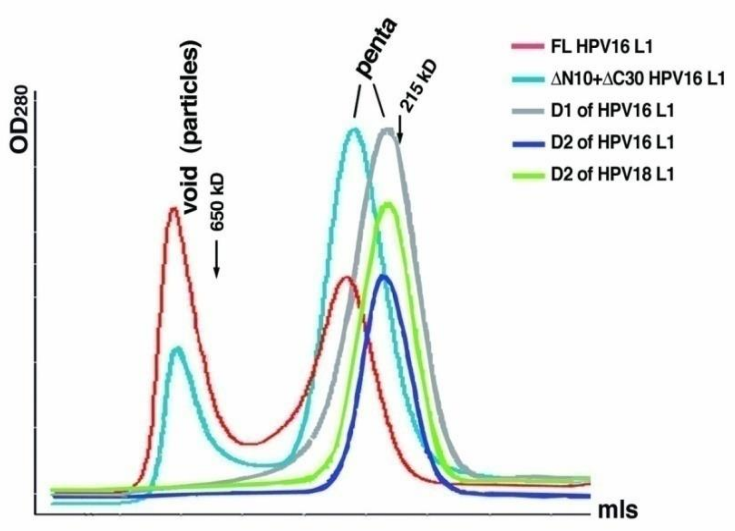
4.2 Size exclusion chromatography

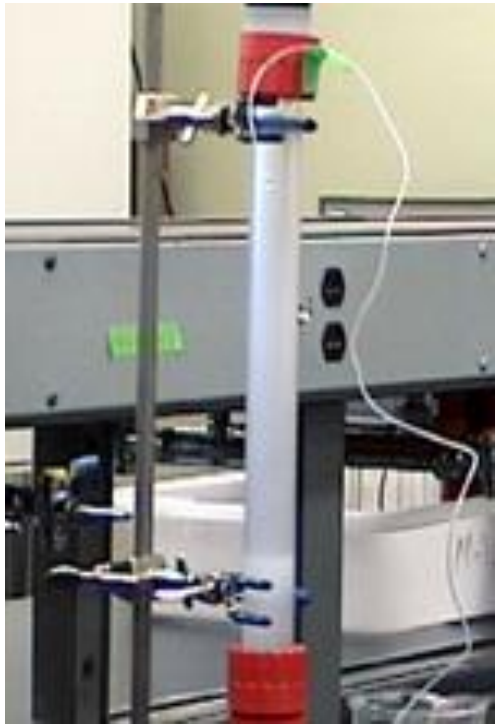






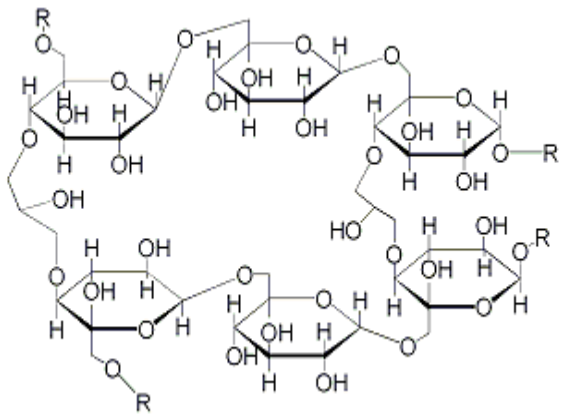




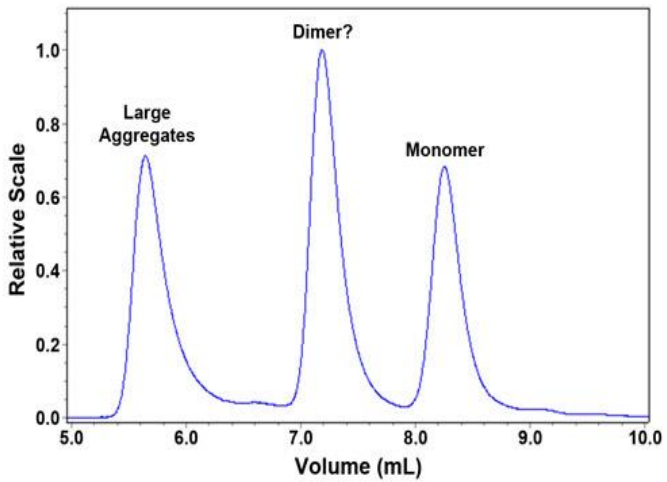


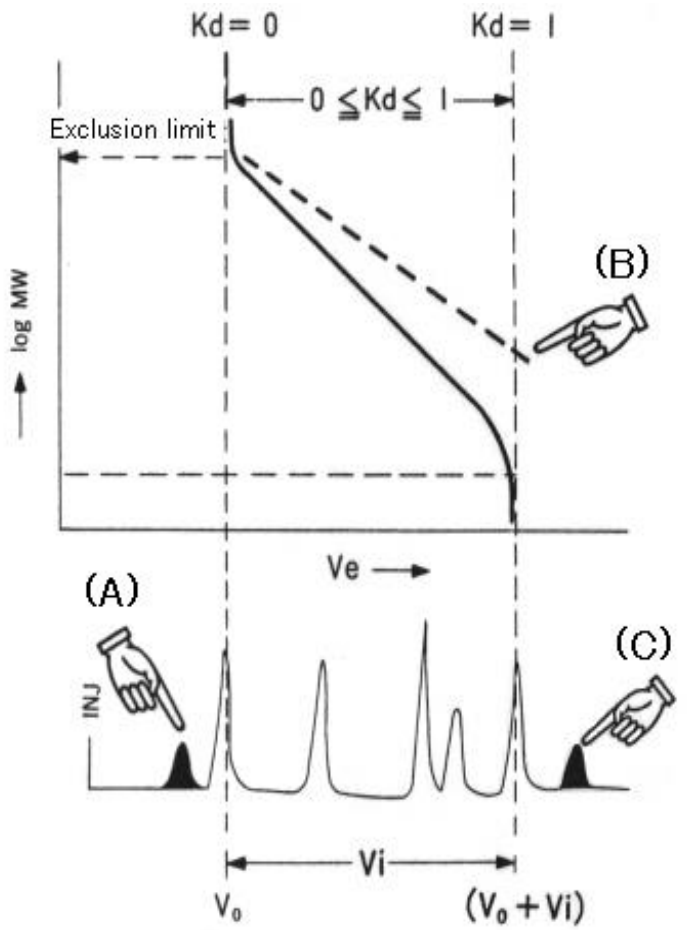
Many names for one single technique

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

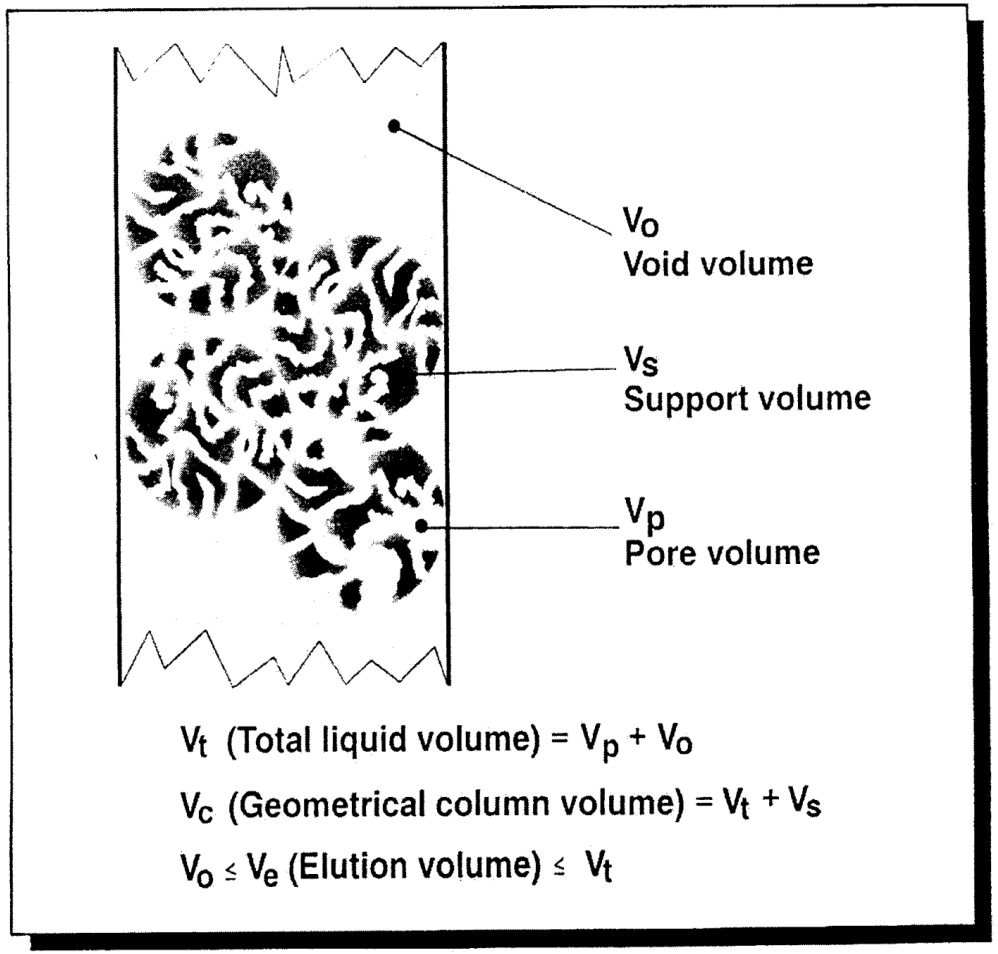


Structure of Sephadex ®



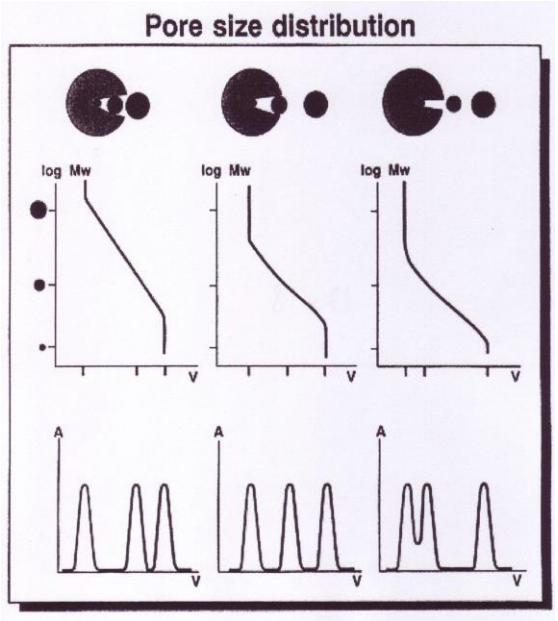


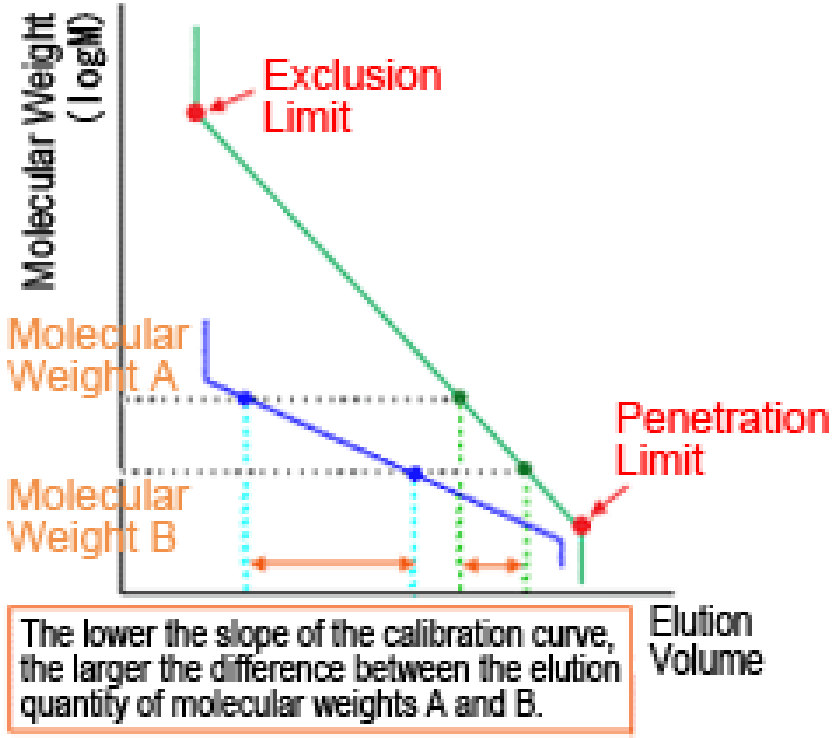
SEC: principle for separation



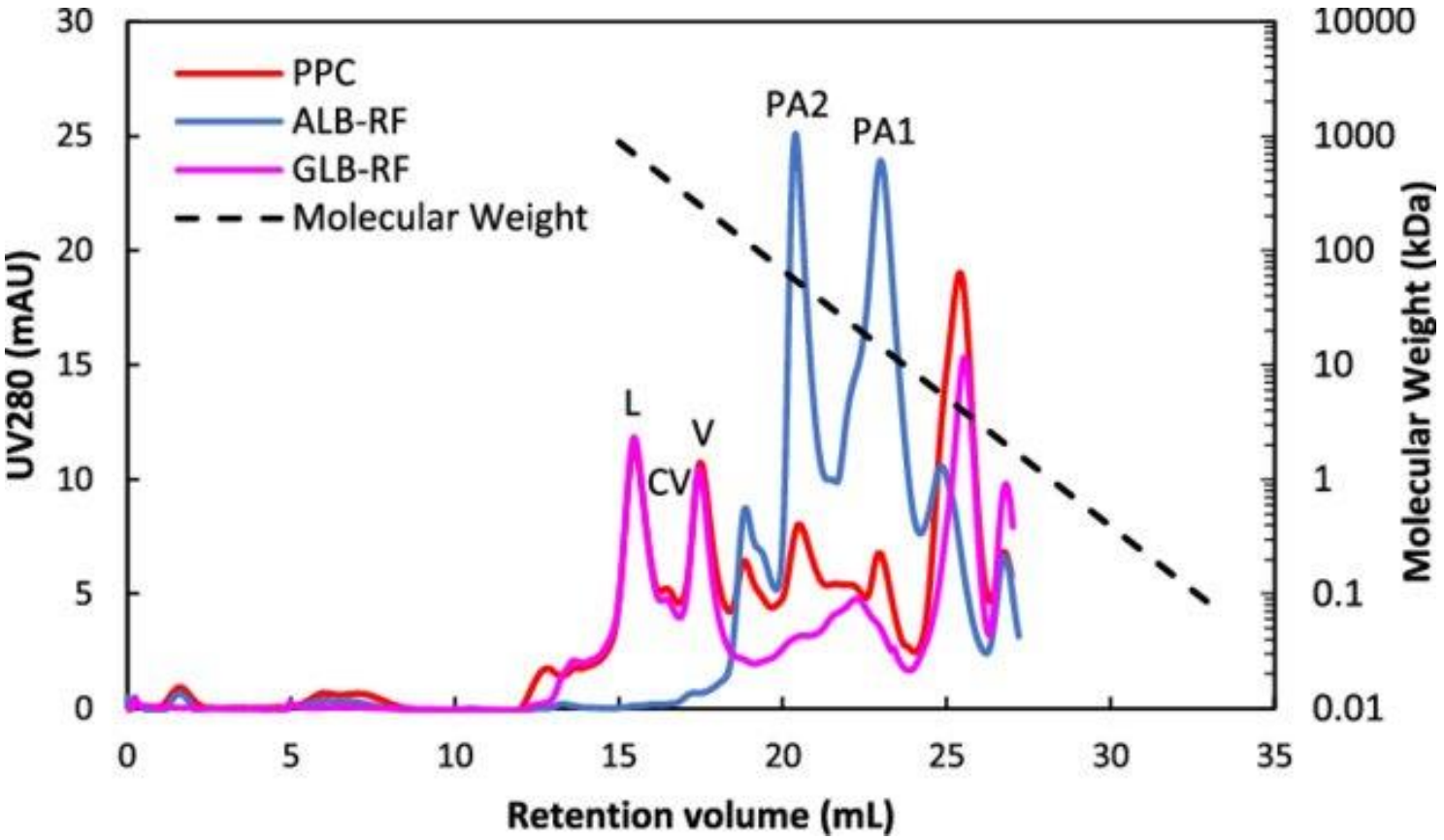
With SEC, separation is only possible between V_0 and V_t.

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





An example: SEC chromatogram of pea proteins



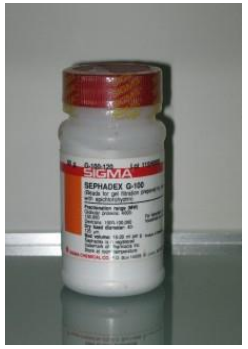
This chromatogram shows the elution profile of a pea protein concentrate both in terms of elution volume and of molecular weights

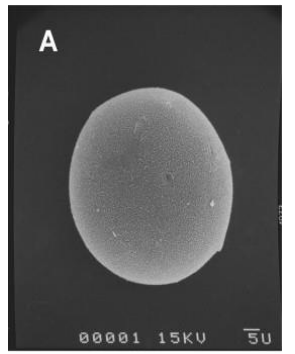
R. Kornet et al., Food Hydrocolloids 2022
https://doi.org/10.1016/j.foodhyd.2021.107456

Legend: (PPC), albumin-rich fraction (ALB-RF) and globulin-rich fraction (GLB-RF), measured from 5 g/L protein solutions at pH 7.0. L = legumin, CV = convicilin, V = vicilin, PA1 and PA2 = albumins PA1 and PA2. For the UV detector a wavelength of 280 nm was used, and the signal intensity (arbitrary units) is shown on the y-axis. The black dashed line represents the molecular weight as function of retention volume.

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

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

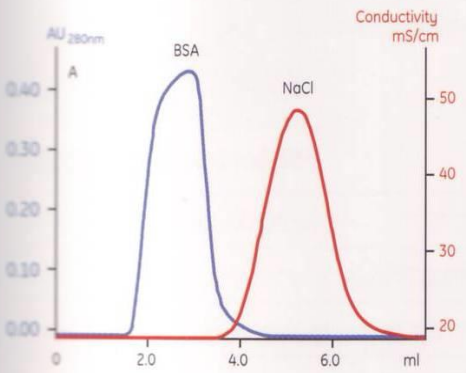


Fig. 18C: HiTrap Desalting 3X5 ml in series

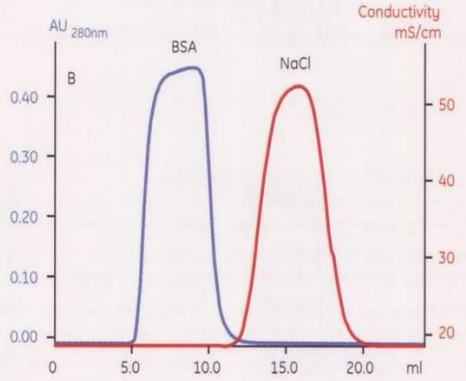
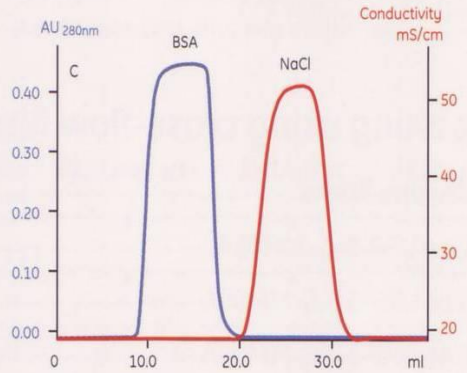


Fig. 18D: HiTrap Desalting 5X5 ml in series



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.

For desalting applications, the sample can represent up to 25-30% of the column volume

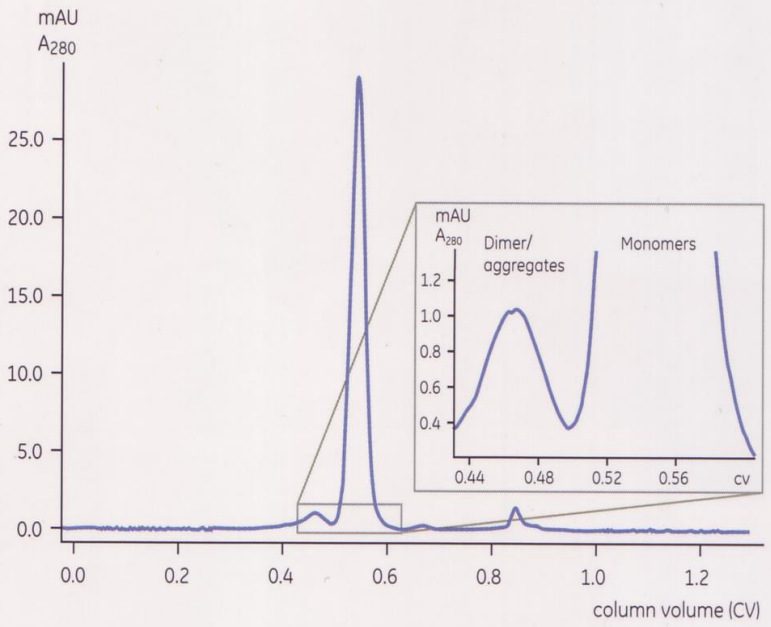
This separation is made easy by the extremely large size difference between protein molecules and ions

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:

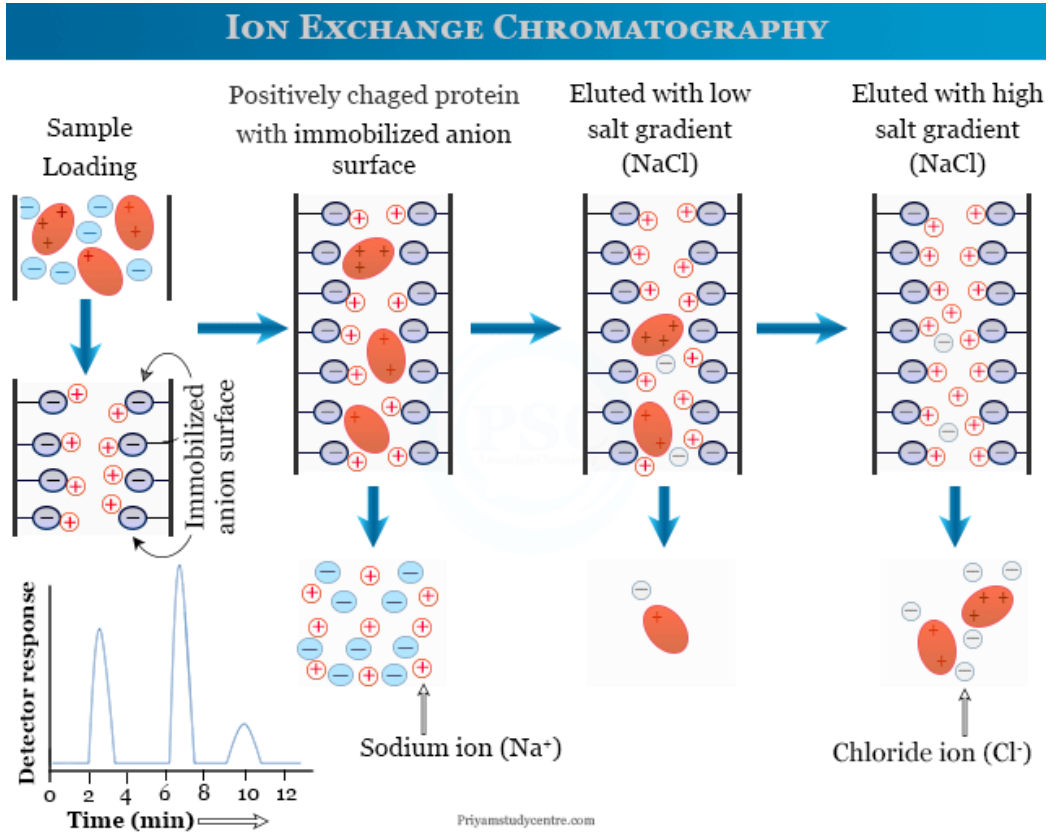
- Separations in the range from M_r 10 000 up to 600 000 (globular proteins)
- Easy and predictable scale-up
- Excellent reproducibility and durability
- Available in expertly prepacked columns and as bulk media

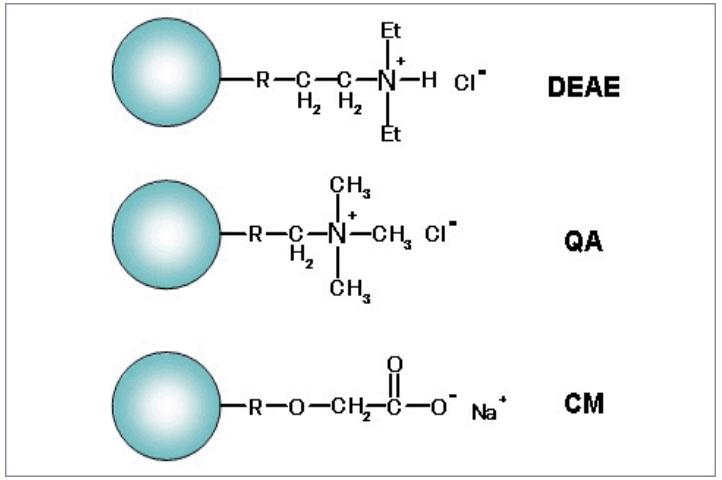
Purified antibodies such as IgG have a tendency to form aggregates which are not acceptable in the final formulation.

SEC with a properly selected separation range can help remove these larger species from the purified antibody.

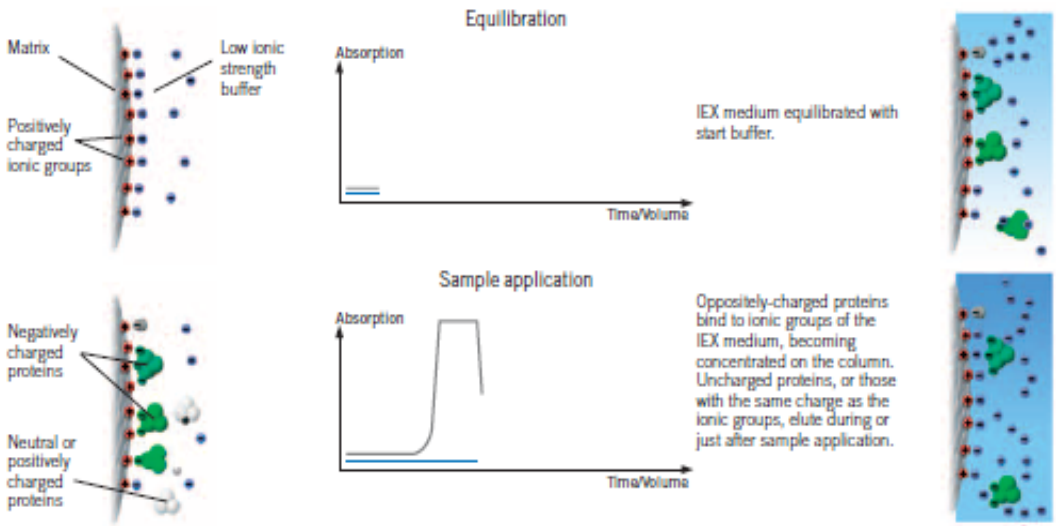
However for such separations it is not possible to load more than 2-3% of the column volume.

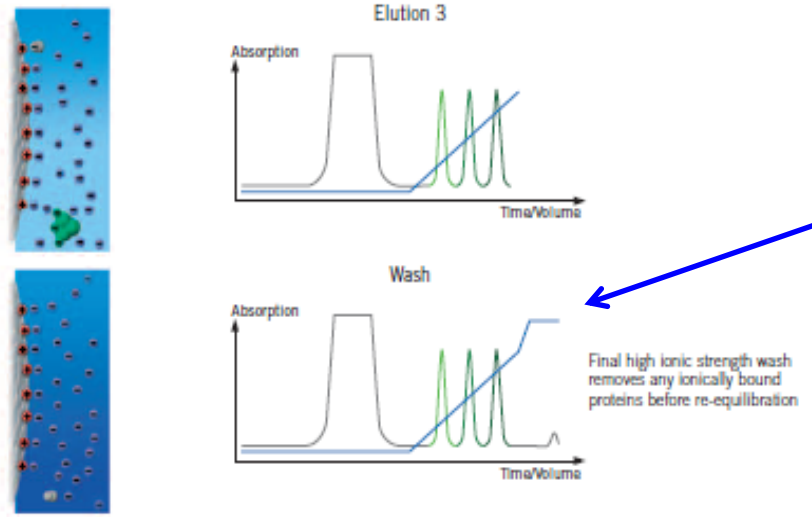
4.3 Ion exchange chromatography (IEX)

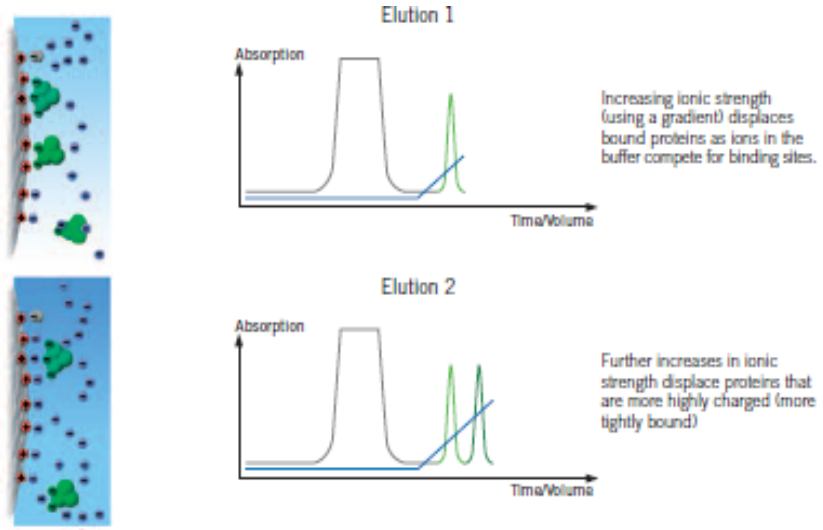


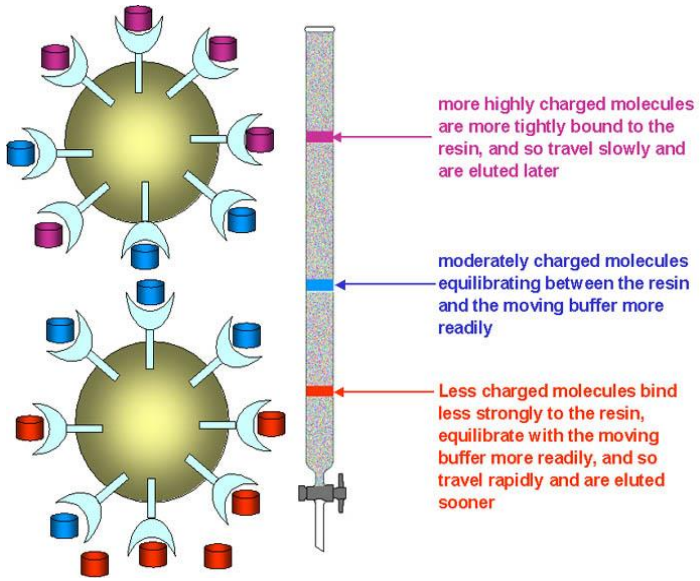


IEX: principle and sequence of steps



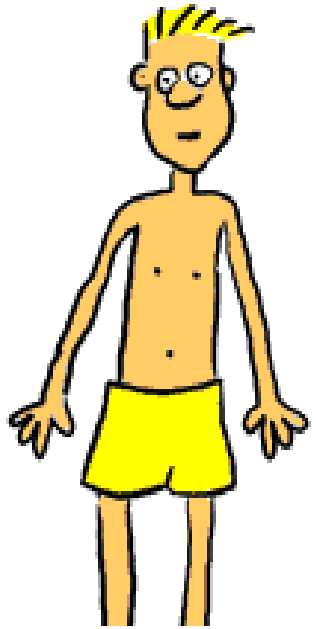




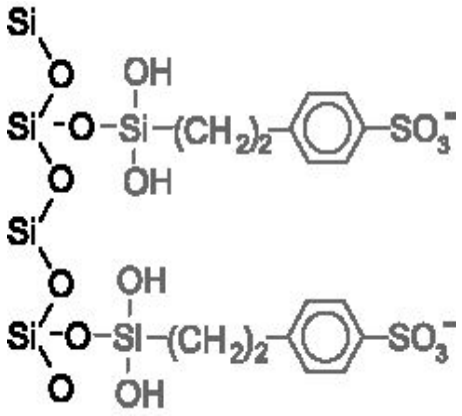


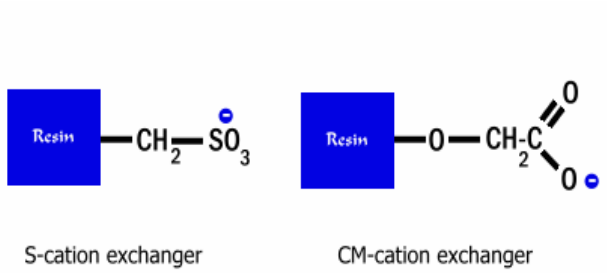
Please note the final washing step at the end of the cycle. It is done at an even higher ionic strength than for elution, in order to desorb the last molecules

Strong vs weak cation exchangers (CEX)



- 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





Strong cation exchangers

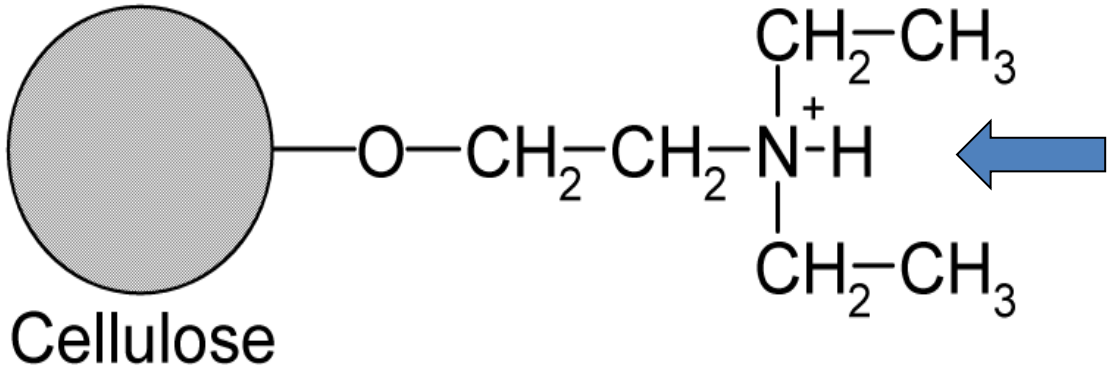
- Sulfopropyl (SP)
- Methyl sulfonate (S)

Weak cation exchangers

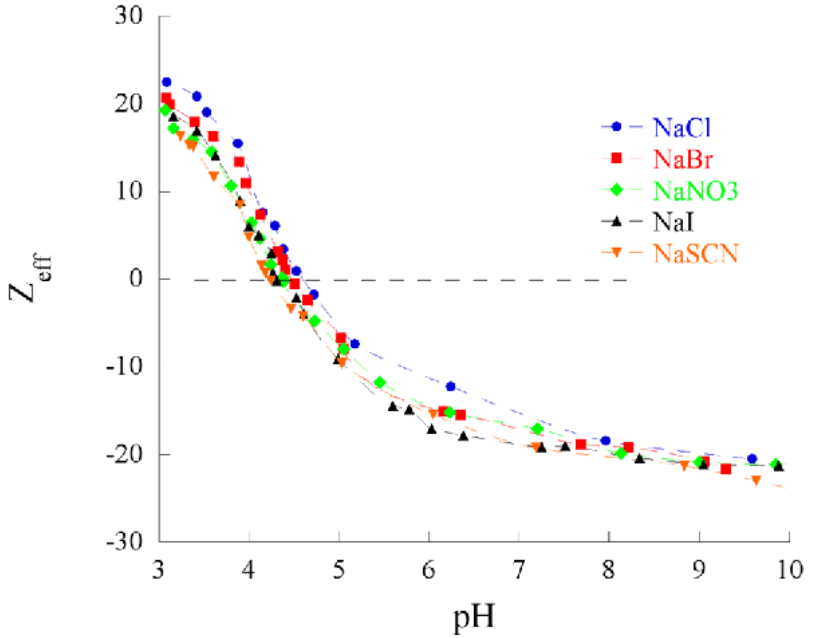
- Carboxymethyl (CM) $-O–CH_2COO^-$

Anion exchangers (AEX)

- 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



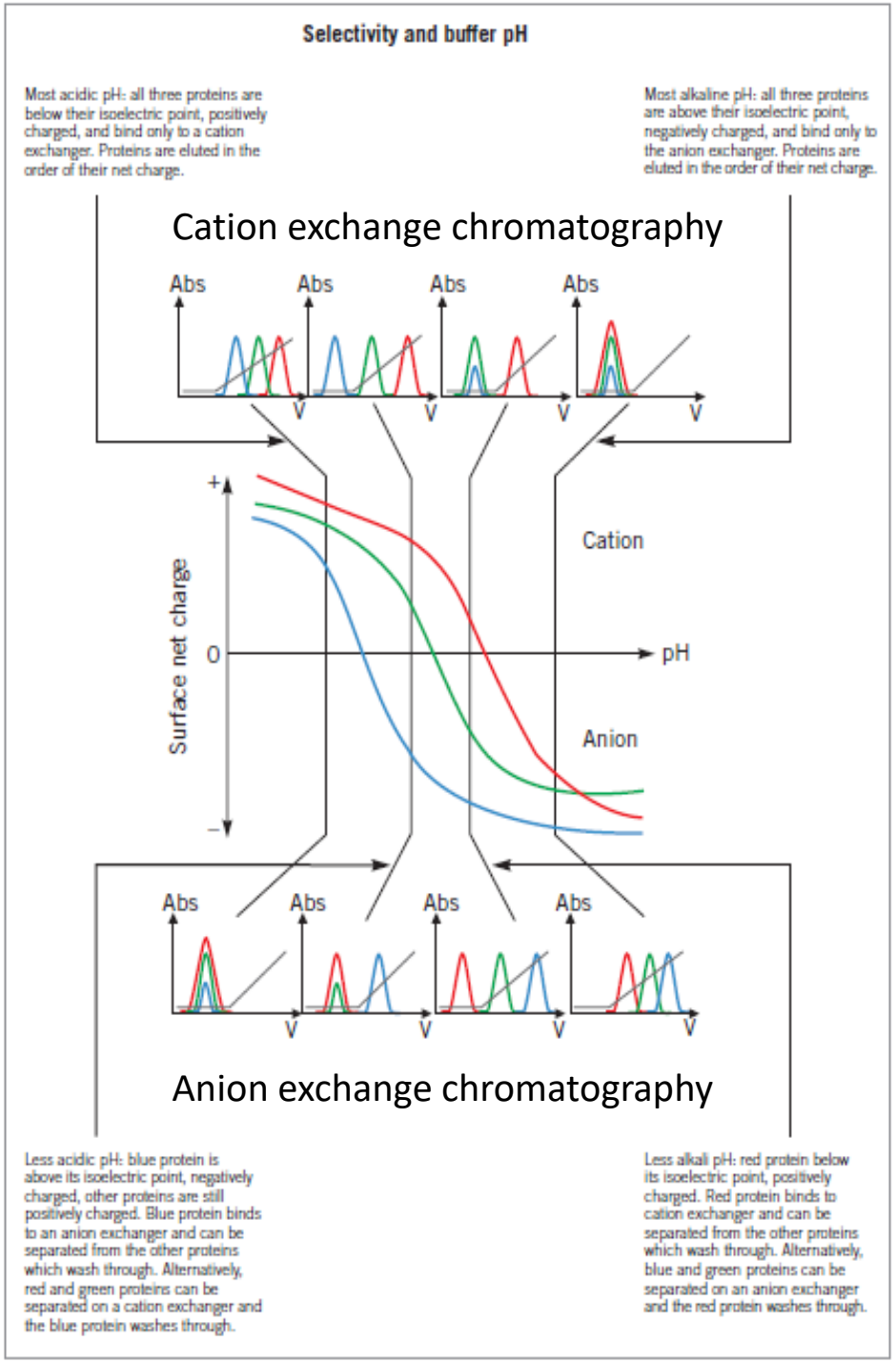
A weak anion exchanger: DEAE (diethyl aminoethyl), grafted on cellulose

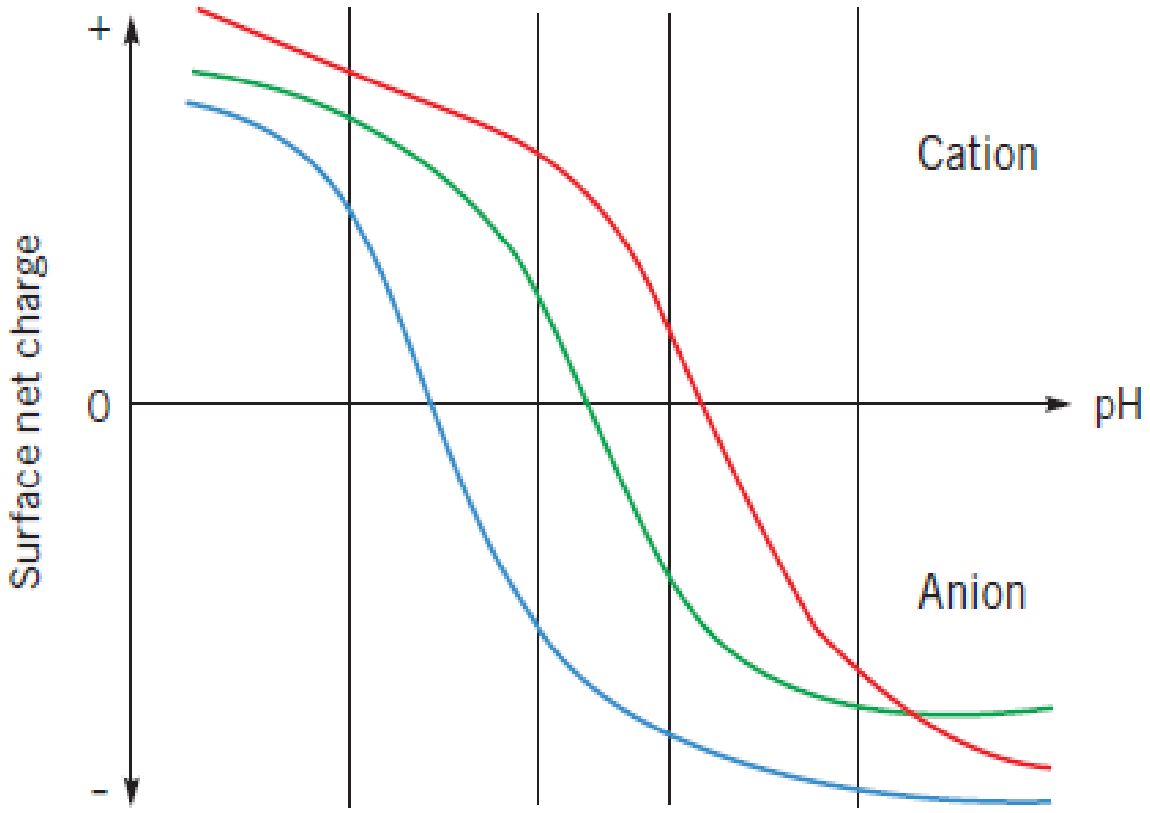


Titration curve (i.e. charge vs pH) for BSA.

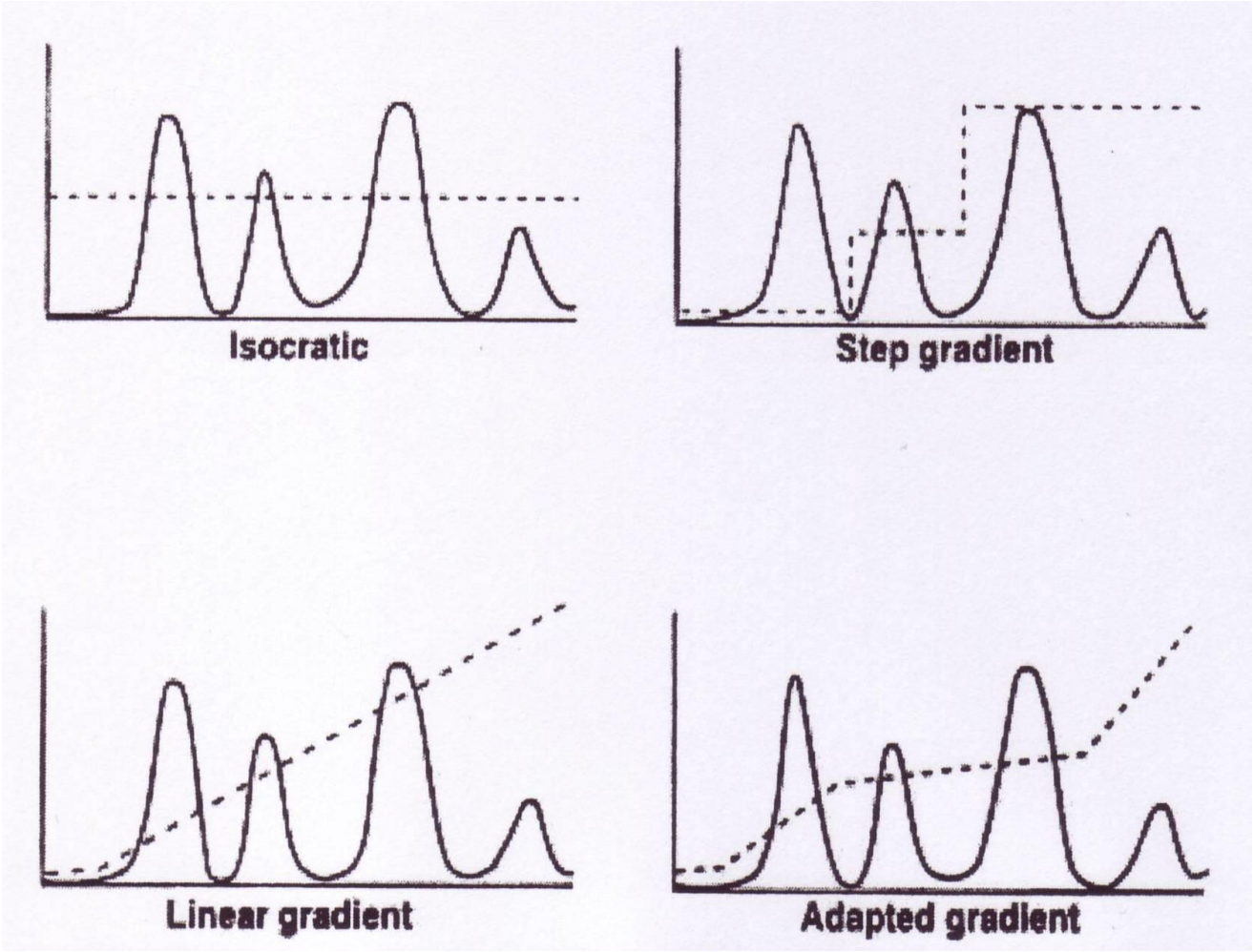
Adsorption on an anion exchange resin can be achieved at pH values above 4.7 (BSA's pI)

Selectivity and pH of buffer

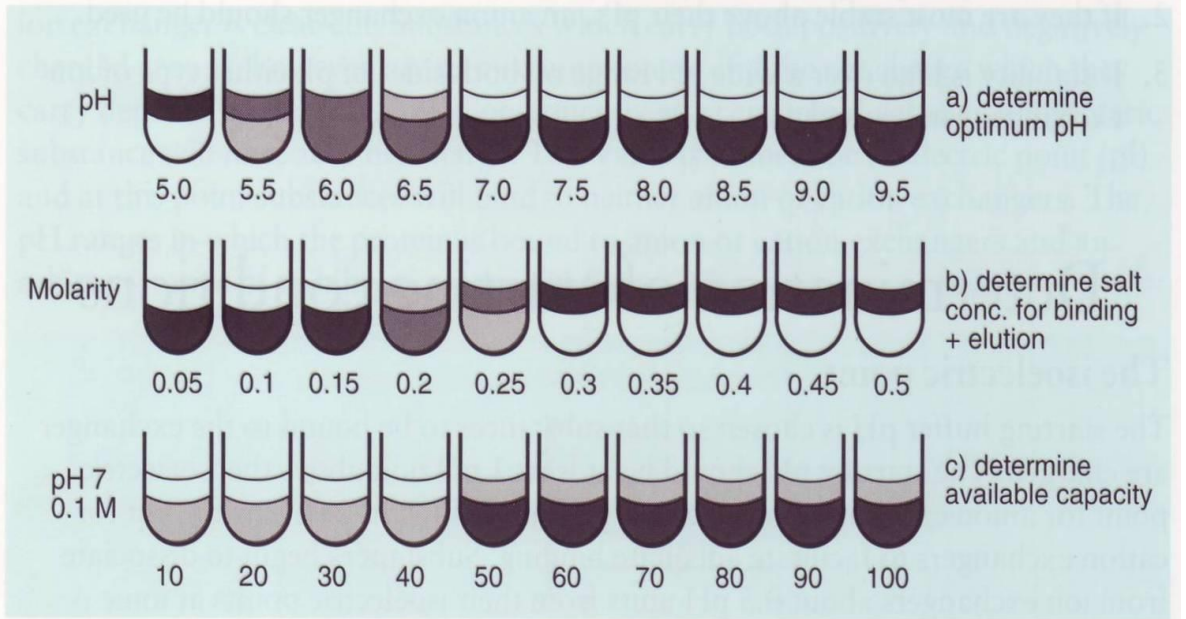




The different elution modes



Optimizing binding conditions



Here pH for binding, salt concentrations for minimal non-specific adsorption and elution as well as resin loading were optimized.

What do you think of this optimization protocol? Could you propose an alternative approach?

PreDictor (Cytiva): Condition screening in 96 wells format

The product consists of 96-well plates containing a stationary phase (different types are available)

Adsorption, washing and elution are performed by pipetting and emptying the wells under vacuum, using special equipment

This makes it possible to optimize binding and elution conditions, assess selectivity and measure isotherms.

- Initial media and condition screening in a parallel format will enable a rapid way of investigating many parameters and efficiently screen for the required selectivity.
- An elution study with varied elution conditions may thus reveal conditions where more target protein is eluted then HCPs.

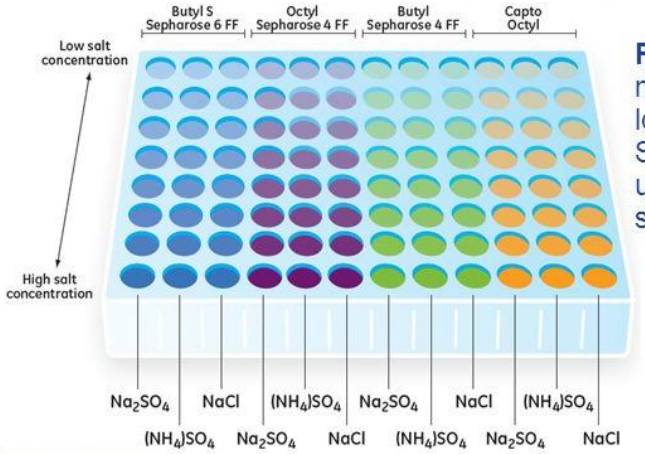
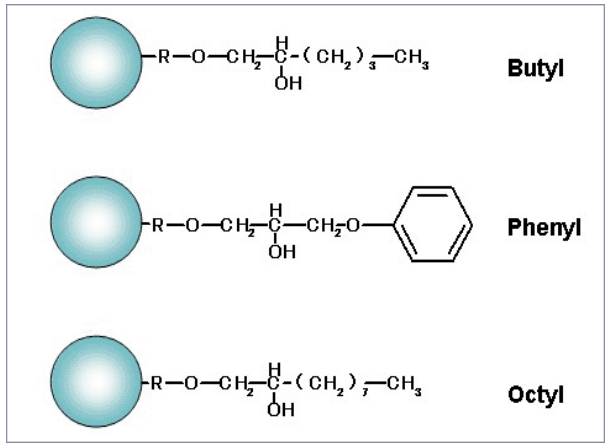


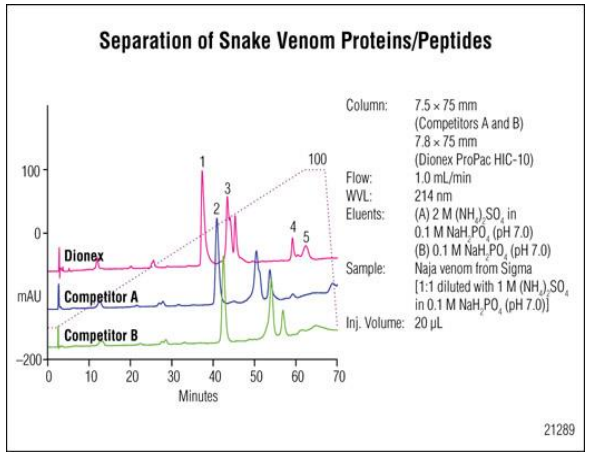
Fig 2. Plate design for screening media and salt type viewed for the low hydrophobicity screening plate. Same distribution of factors were used for the high hydrophobicity screening plate.

imagination at work

8 /High-throughput screening of HIC media in PreDictor™ plates for capturing a recombinant protein from *E.coli*

4.4 Hydrophobic Interaction Chromatography (HIC)



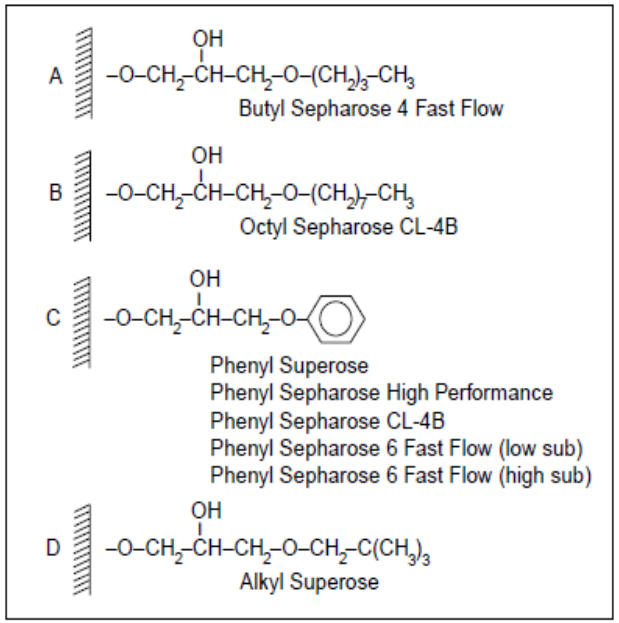


Arne Tiselius – 1948 Nobel Prize in chemistry for his work on electrophoresis and adsorption

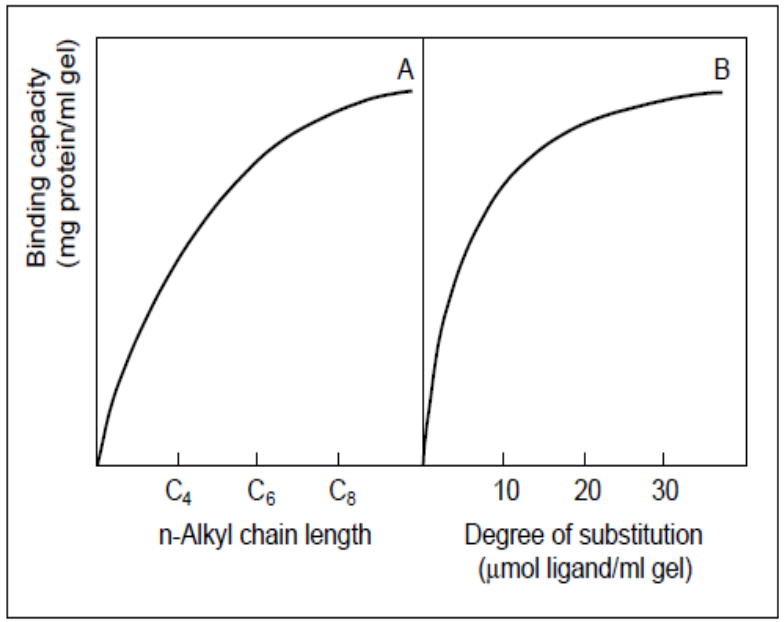
Tiselius, A. (1948): Adsorption separation by salting out. *Arkiv för Kemi, Mineralogi Geologi* 26B, 1–5

"...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"

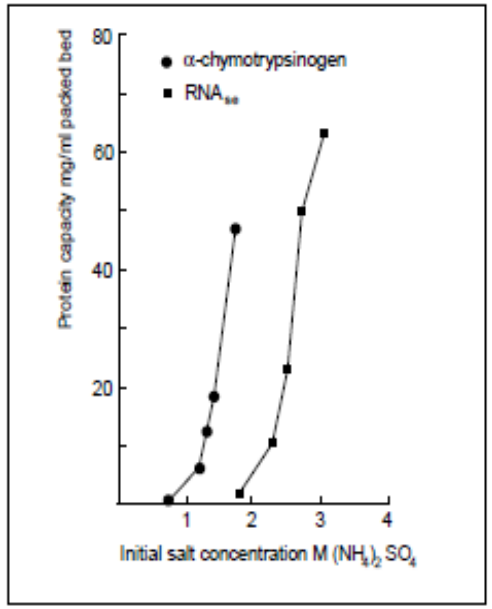
Ligands, chain length, degree of substitution and salt concentration



The various types of ligands

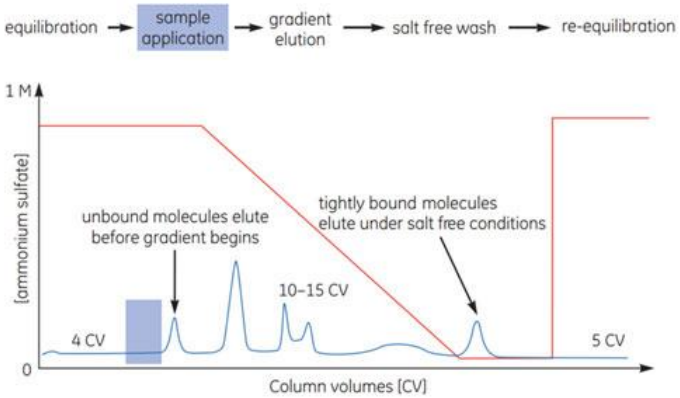


Influence of alkyl chain length and degree of substitution on binding capacity



Influence of salt concentration on adsorption capacity

Typical HIC chromatogram
Note the loading of the column at high salt concentration and the decreasing salt concentration during the elution phase (red line)



Effect of salt type: go for the salting-out electrolytes

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+}

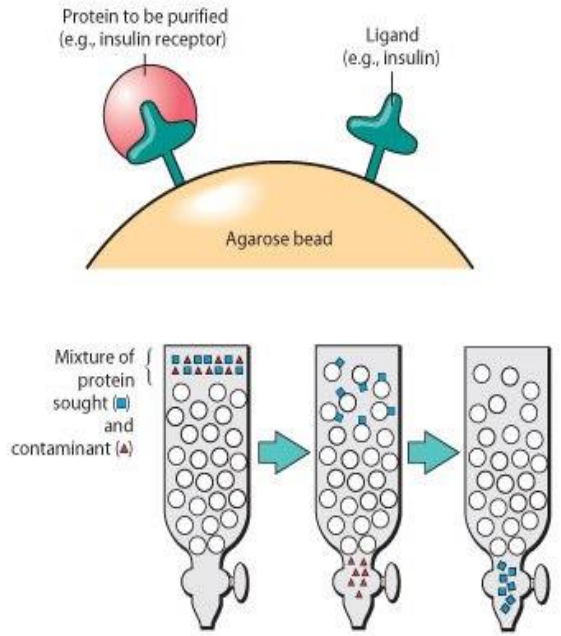
Increasing chaotropic ("salting-in") effect →

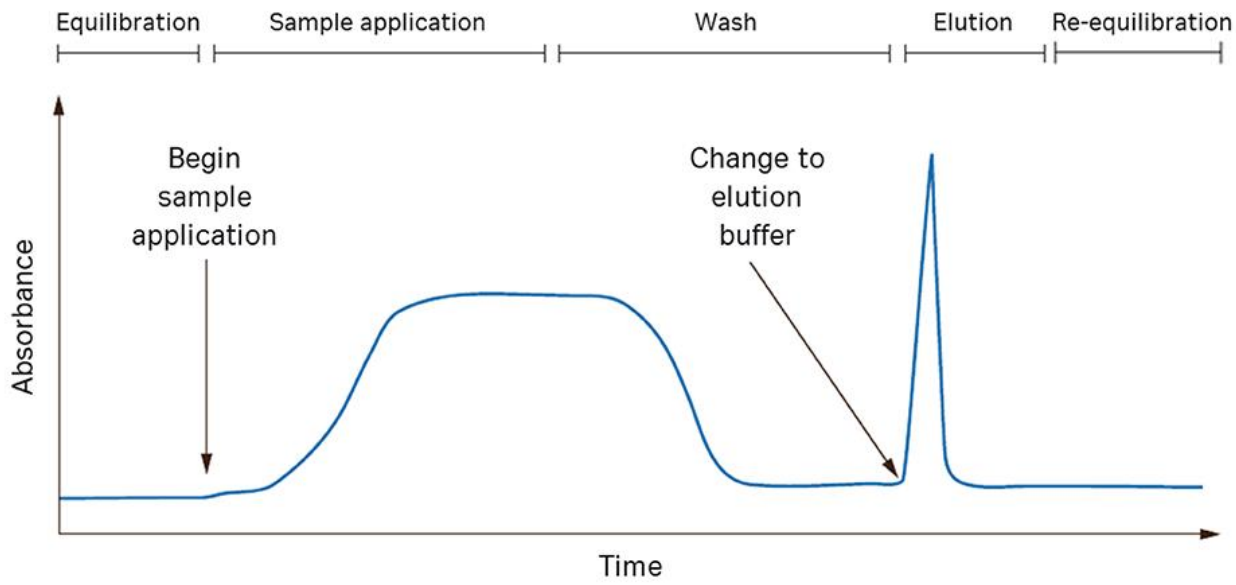
Table 1.
The Hofmeister series on the effect of some anions and cations in precipitating proteins.

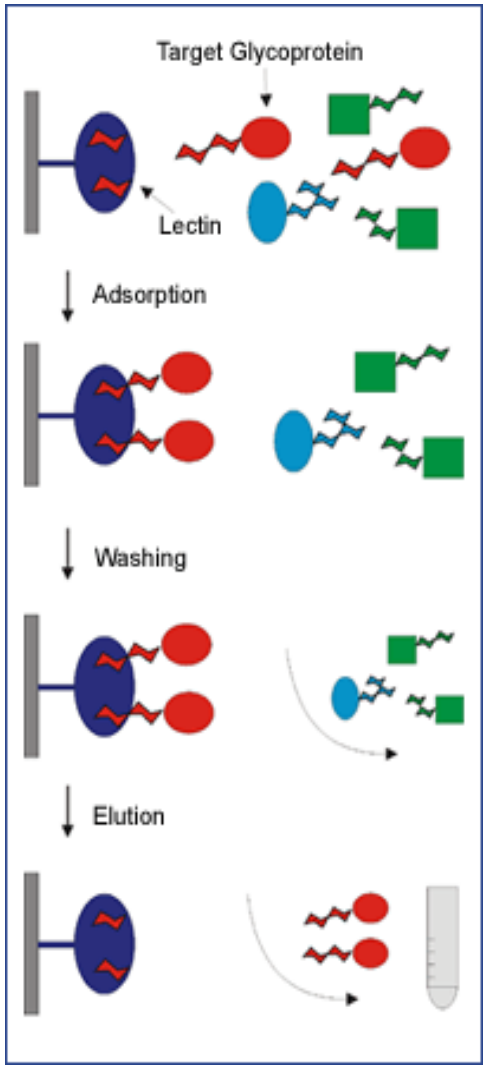
$Na_2SO_4 > K_2SO_4 > (NH_4)_2SO_4 > Na_2HPO_4 > NaCl > LiCl \ldots > KSCN$

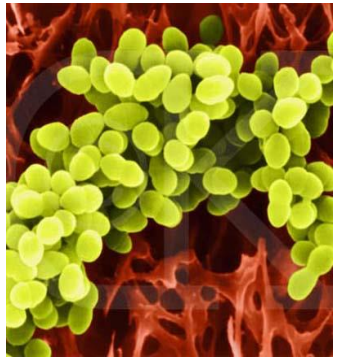
Table 2.
Relative effects of some salts on the molal surface tension of water.

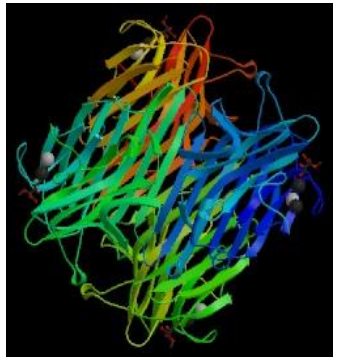
4.9 Affinity Chromatography (AC)

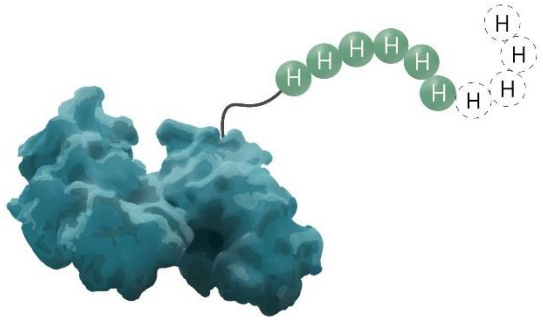






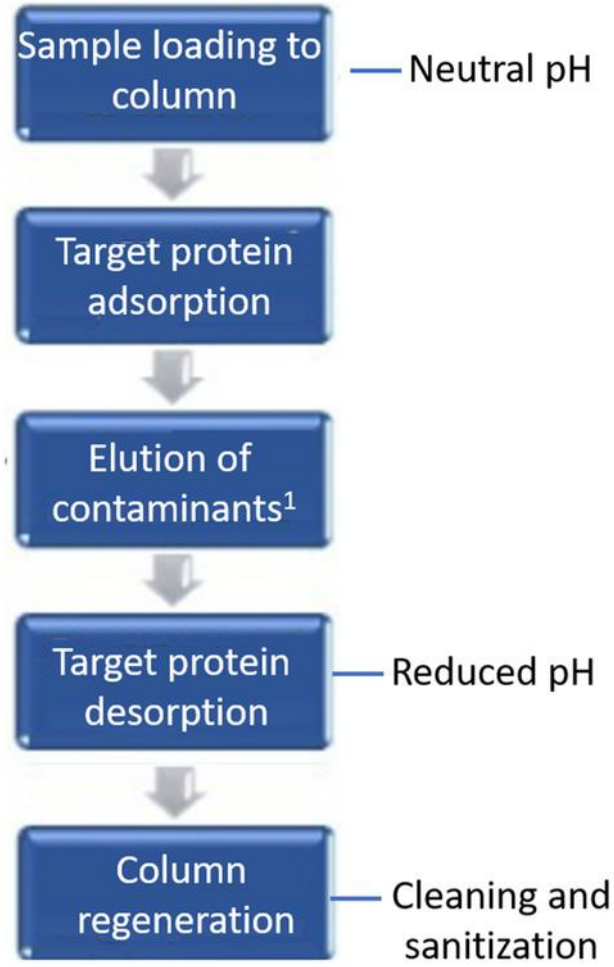






General observations

- 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 concentrating 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 polyrity
- AC can sometimes be used to remove specific contaminants (e.g. using Benzamidine Sepharose FF (high sub)) to remove serine proteases (**a.k.a. negative chromatography**)



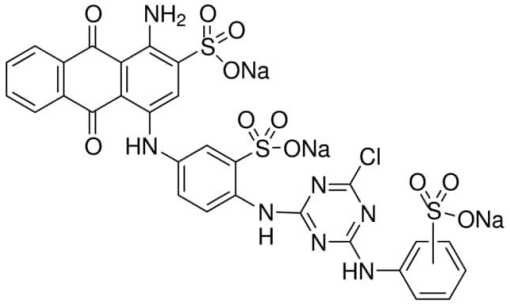
[1]Product isoforms, DNA, RNA, leached protein A, cell culture medium components, host cell proteins, dimers, aggregates, product fragments.

Affinity ligands and corresponding solutes

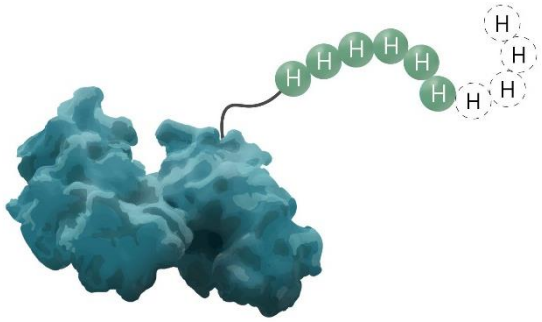
Ligand	Solute
2’,5’ ADP	Enzymes with $NADP^+$ as cofactor
5’ AMP	Enzymes with $NADP^+$ as cofactor, and ATP-dependent kinases
Arginine	Proteases and zymogens (including prothrombin, prekallikrein, clostripain)
Benzamidin	Proteases (including trypsin, urokinase, kallikrein, prekallikrein)
Cibacron Blue, Procion Red	Large selection of enzymes having nucleotides as cofactors
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

Affinity ligands and corresponding solutes

Ligand	Solute
Lectin of *Helix pomatia*	N-acétyl-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	Fc region of IgG and similar molecules
Protein G	Similar to those of Prot. A, but different affinity for IgG of other species
Ni-NTA, Co-NTA	Recombinant proteins with a His-tag
Wheat germ lectin	N-acetyl-D-glucosamine



Cibacron Blue Source: Sigma Aldrich

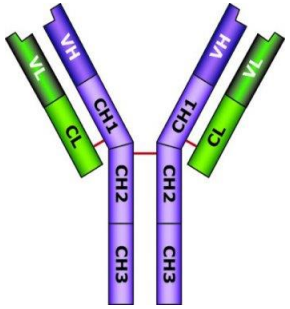


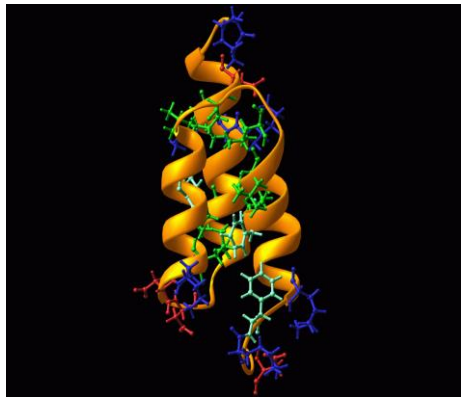
Protein with $(His)_{6-10}$ tag

Pierce™ High Capacity Ni-IMAC Resin, EDTA compatible
Source: Thermo Fisher

Protein A

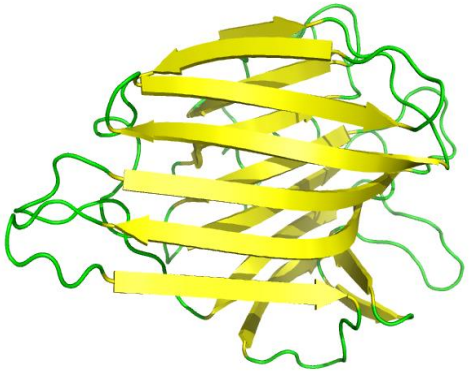
- Protein extracted from the Cowan strain of *Staphylococcus aureus*
- Capable of binding to the constant regions of human, murine and rabbit IgG heavy chains
- Can be used in place of the secondary antibodies for ELISA and Western blotting
- Main usage is for industrial chromatography purification of antibodies
- The high cost of such resins (ca. 10’000 CHF/kg) is balanced by its extreme selectivity



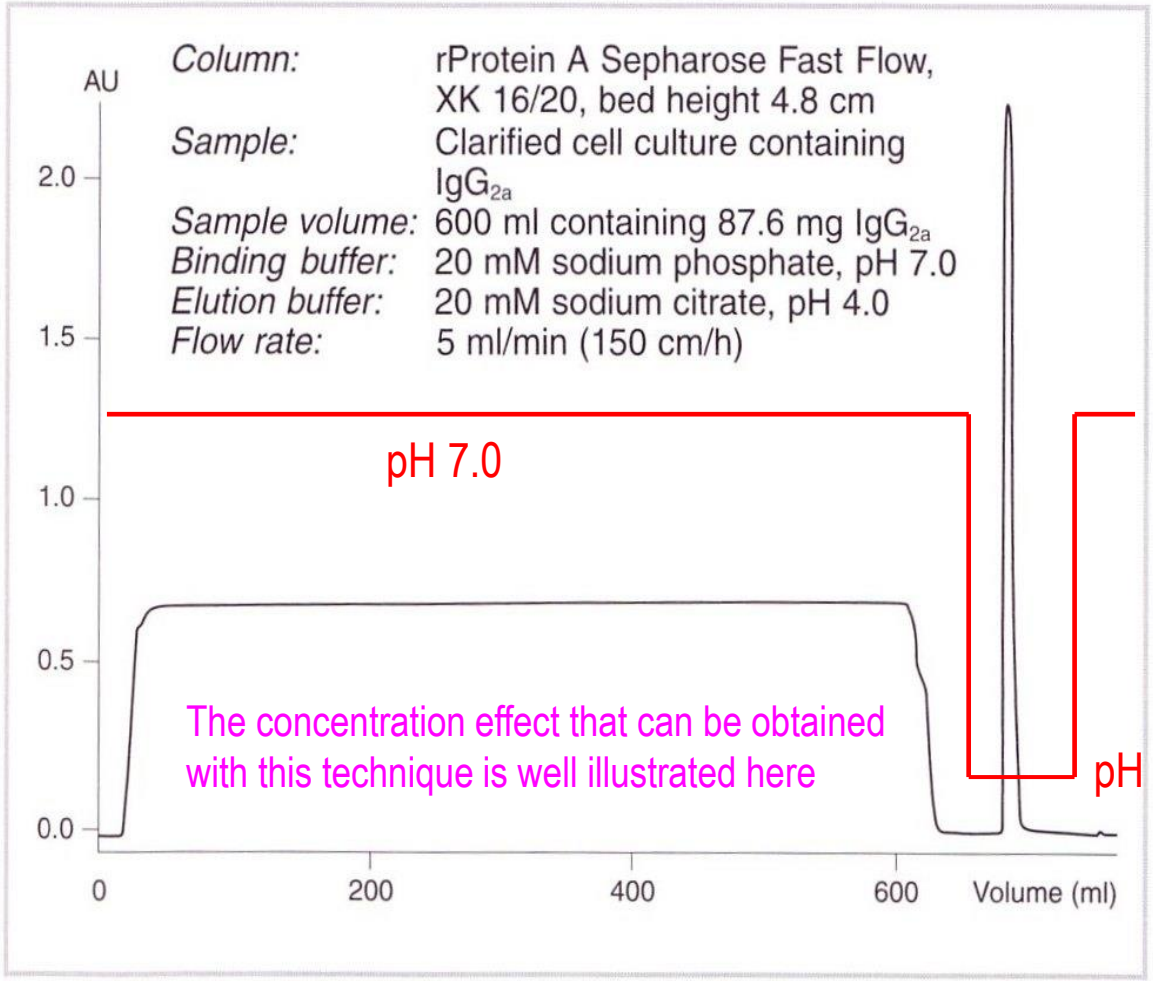


Concanavalin A

- Concanavalin A (Con A) is a lectin type protein isolated from leguminous plants such as jackbean (Canavalia ensiformis).
- Con A has a MW of 25.5 kDa and a pI between 4.5 and 5.5.
- It specifically binds mannose and exists under different oligomeric forms: a dimer at pH 5.5. and a tetramer above pH 7
- 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

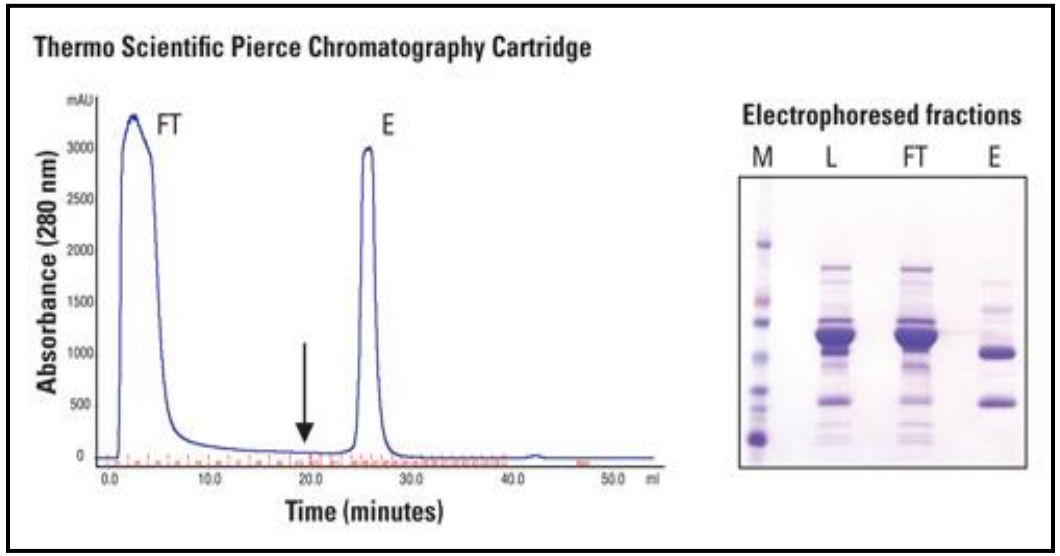


Affinity Chromatography (AC), example 1



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

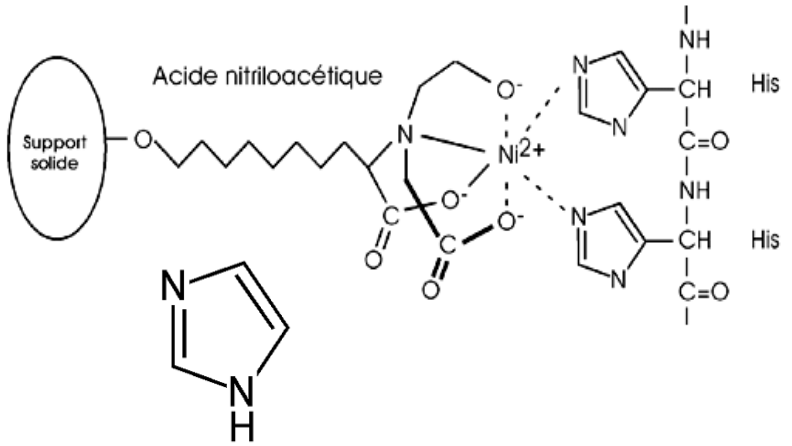
Typical AC chromatograms where a very large volume of liquid is charged on the column, followed by a washing step and the quick elution of a single product peak



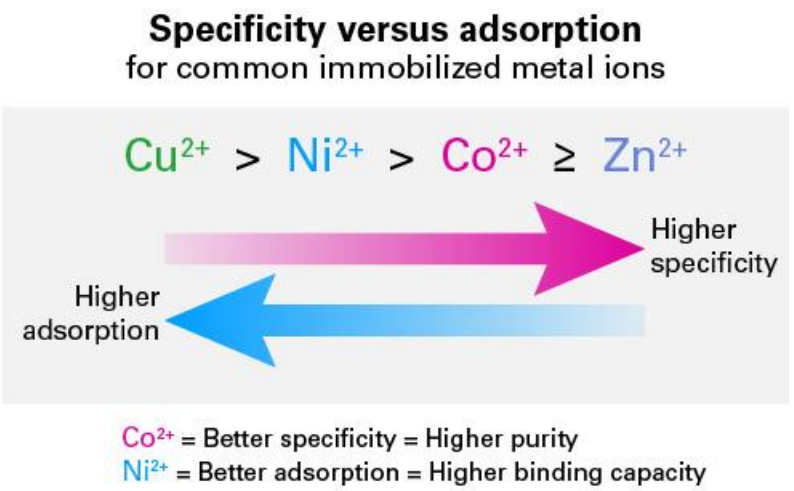
AC, Example 2: His-tag™ et IMAC

(IMAC = Immobilized Metal Affinity Chromatography) (His-tag is a trademark deposited by EMD Bioscience)

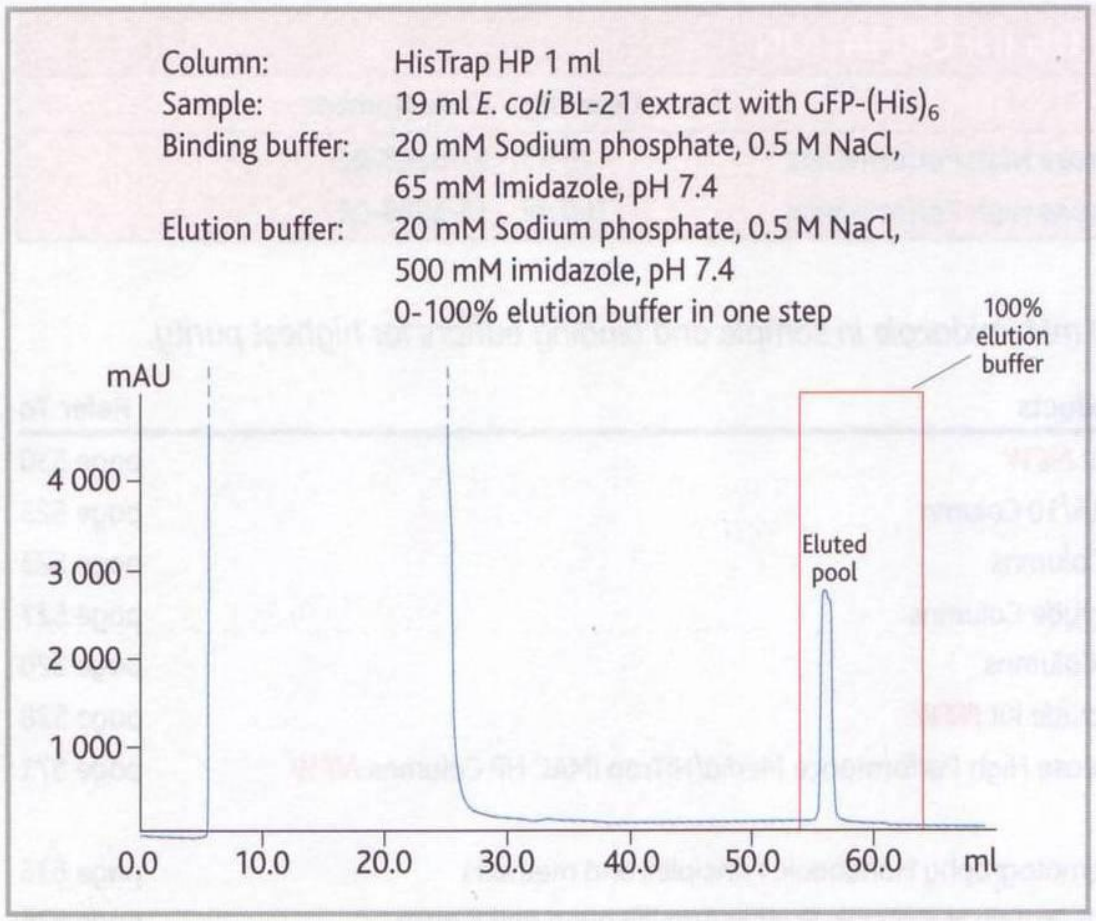
- **His-tag** consists in adding a short sequence of 6 (or 10) Histidine residues at one extremity of the recombinant protein
- The tag was invented at Roche and its vectors are being distri-buted (among others) by Qiagen, Sigma, Thermo Scientific, GE Healthcare
- Use of the vectors is free of charge 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



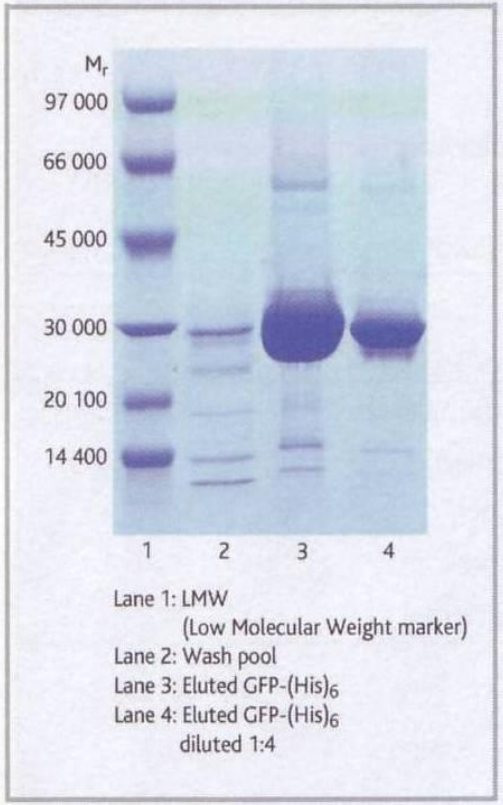
imidazole



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.



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.

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)

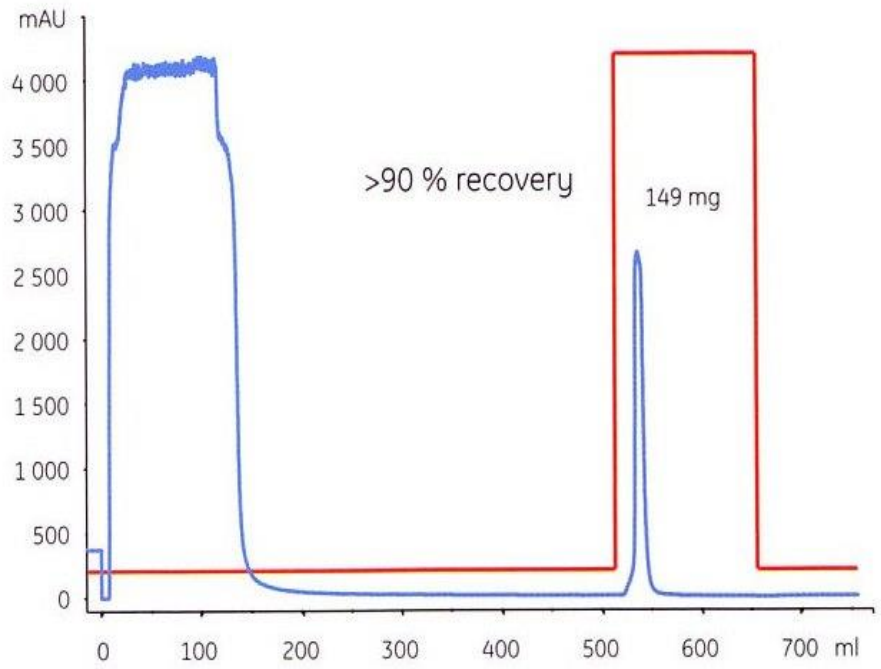
Columns: HisTrap FF 1 ml, HisTrap FF 5 ml, HisPrep™ FF 16/10 20 ml. All columns are prepacked with Ni Sepharose 6 Fast Flow.

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



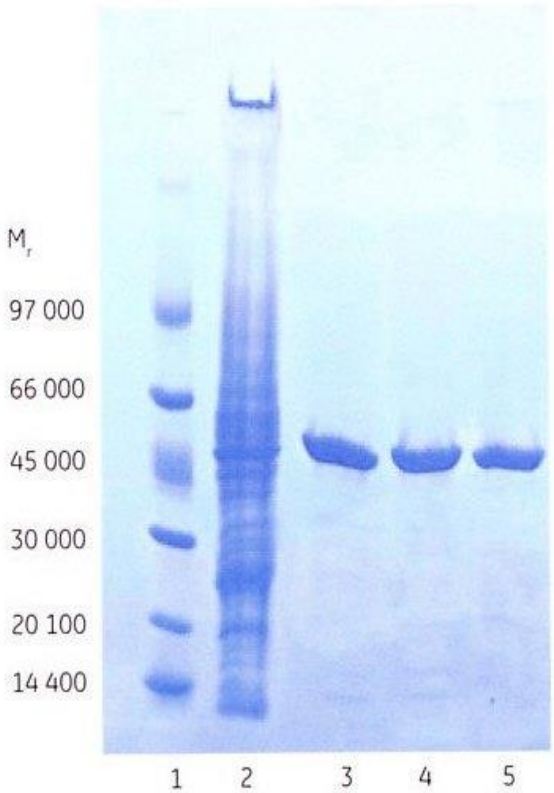


Fig. 2B: SDS-PAGE

Lane 1: LMW

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

Lane 3: Eluted pool, HisTrap FF 1 ml

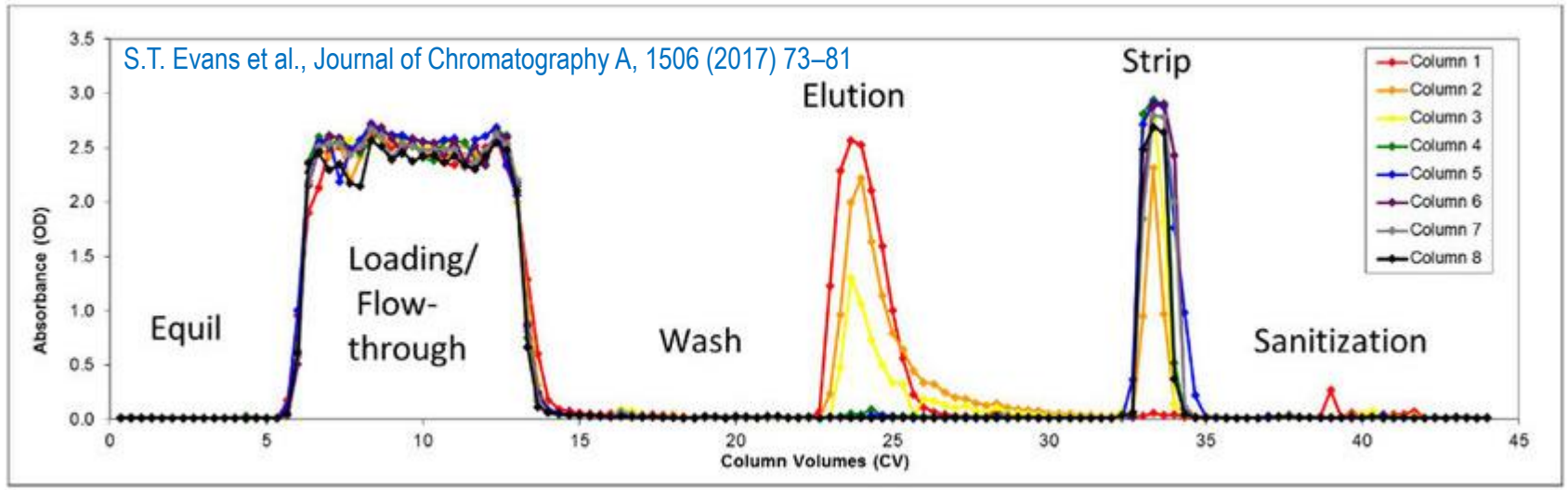
Lane 4: Eluted pool, HisTrap FF 5 ml

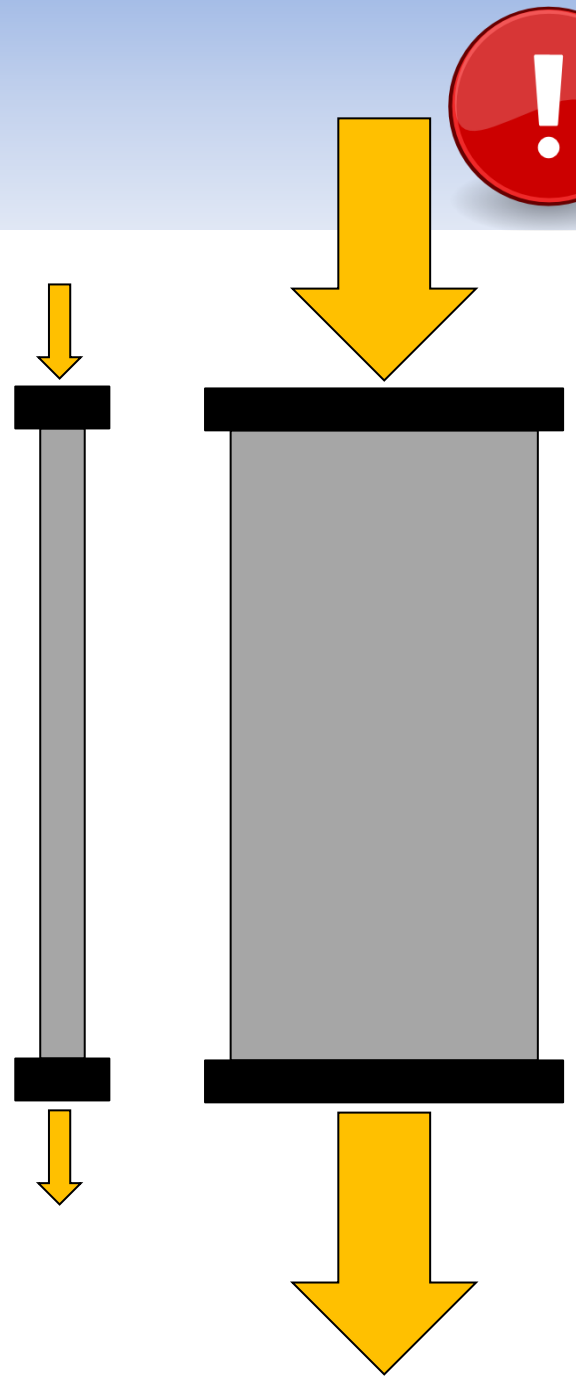
Lane 5: Eluted pool, HisPrep FF 16/10 (20 ml)

Scale-up

4.10 Scale-up in chromatography

- One goal in the scale-up is to conserve the operating conditions and elution profile and retention times that were obtained at lab-scale
- This can be achieved by keeping the fixed bed length and the liquid superficial velocity constant (there are other ways, though).
- Increase in capacity is thus obtained by simply increasing column diameter.
- Hence to obtain a 100-fold increase in capacity, one will have to multiply column diameter by a factor of 10 = $(100)^{1/2}$





Production-scale columns