

Kód předmětu: C8980



MASARYKOVA UNIVERZITA

Protein expression and purification

- VIII. Protein purification chromatography

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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.



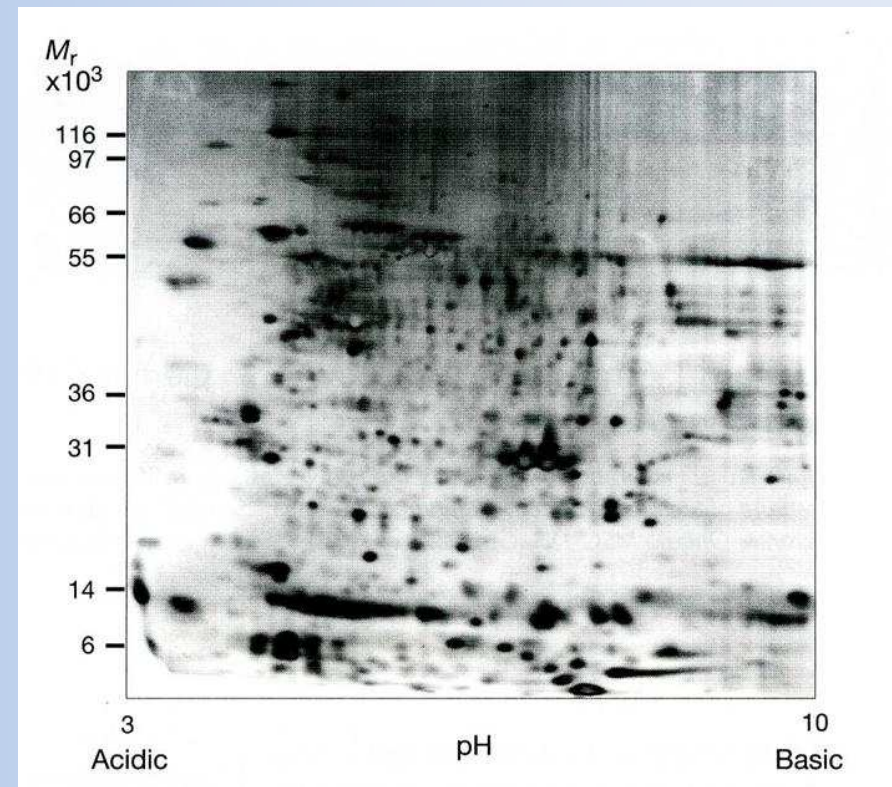
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

VIII. Protein purification chromatography

8.1. Protein abundance in the cell

The challenge of protein purification becomes self-evident when one considers the complex mixture of macromolecules present in a biological matrix such as a cell or tissue extract.

- Several thousand other proteins with different properties are present in any given cell type (~5,000–8,000 proteins)
- Nonproteinaceous materials
 - DNA
 - RNA
 - Polysaccharides
 - Lipids
- Actin – 1,000,000 molecules per cell
- Transcription factor – 10 molecules per cell



VIII. Protein purification chromatography

8.2. How much protein is needed and what level of purity is required?

Application	Amount required	Purity required
Identification	0.002-0.2 μg	high (>95%)
Immunology	μg -mg	medium to high
Enzymology	1-5 mg	high (>95%)
Biophysical analysis	mg-g	high (>95%)
3D structure	10-20mg	high (>95%)
Pharmaceutical	mg-kg	high (>99.9%)

Most sensitive instruments: Electrospray ionization (ESI)

Matrix-assisted laser desorption/ionization (MALDI)

0.2–1 pmol (25 kDa ~ 5–25 $\mu\text{g}/\text{ml}$)

Ion trap ~ 1 fmol

VIII. Protein purification chromatography

8.2. How much protein is needed and what level of purity is required?

Converting moles to micrograms (http://molbiol.edu.ru/eng/scripts/01_04.html)

Protein molecular mass (daltons)	Amount of protein per nmole	Number of moles in 1 µg of protein
1,000	1 µg	1 nmole or 6×10^{14} molecules
10,000	10 µg	100 pmoles or 6×10^{13} molecules
20,000	20 µg	50 pmoles or 3×10^{13} molecules
50,000	50 µg	20 pmoles or 1.2×10^{13} molecules
100,000	100 µg	10 pmoles or 6×10^{12} molecules
200,000	200 µg	5 pmoles or 3×10^{12} molecules

One mole of a protein is the amount that contains 6.023×10^{23} molecules of that protein, which is known as Avogadro's number. The weight of a mole of a protein in grams (g) is the same as its molecular mass. For example, for a protein with a molecular mass of 20,000 daltons, the weight of 1 mole of protein is 20,000 g.

$$1 \text{ mmole} = 10^{-3} \text{ moles}$$

$$1 \text{ µmole} = 10^{-6} \text{ moles}$$

$$1 \text{ nmole} = 10^{-9} \text{ moles}$$

$$1 \text{ pmole} = 10^{-12} \text{ moles}$$

$$1 \text{ fmole} = 10^{-15} \text{ moles}$$

VIII. Protein purification chromatography

8.3. Using recombinant proteins often simplifies the purification process

Purifying rare proteins from natural biological sources is often extremely difficult, requiring extremely large quantities of starting material and a 1–2-million-fold purification to achieve homogeneity (growth factors, receptors or transcription factors).

Protein	Source	Yield (μg)	References
Platelet-derived growth factor (PDGF)	human serum (200 L)	180	Heldin et al. (1981)
Coelenterate morphogen	sea anemone (200 kg)	20	Schaller and Bodenmuller (1981)
Peptide YY (PYY)	porcine intestine (4,000 kg)	600	Tatemoto (1982)
Fibroblast growth factor (FGF)	bovine brain (4 kg)	33	Gospodarowicz et al. (1984)
Transforming growth factor- β (TGF- β)	human placenta (8.8 kg)	47	Frolik et al. (1983)
Human interferon	human leukocyte-conditioned medium (10 L)	21	Rubinstein et al. (1979)
β_2 -adrenergic receptor	rat liver (400 g)	2	Graziano et al. (1985)
Tumor necrosis factor (TNF)	HL60 tissue culture medium (18 L)	20	Wang and Creasy (1985)

VIII. Protein purification chromatography

8.3. Using recombinant proteins often simplifies the purification process

In contrast, purifying over-expressed recombinant proteins in milligram to kilogram quantities has been greatly simplified by the ability to produce target proteins containing a fusion partner (or “a purification handle”) designed to facilitate protein purification.

The tumor necrosis factor (TNF- α) is a multifaceted polypeptide cytokine known to be a mediator of inflammation and is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. TNF- α secreted by the macrophage causes blood clotting which serves to contain the infection. TNF- α has been detected in synovial fluid of patients with rheumatoid arthritis.

TNF (HL60 tissue culture medium) 18 L – 20 μ g of protein (Wang and Creasy, 1985)

TNF (*E. coli*) ? L medium Pkg. size: 50 μ g – USD 625

VIII. Protein purification chromatography

8.4. Proteins can be separated on the basis of their intrinsic properties

Many proteins and peptides of biological interest are of very low abundance, often constituting $<0.1\%$ of the total cellular proteins.

- Large quantities of source material required ($>0.1\%$).
- Availability of separation facilities (e.g. instrumentation).
- Physical constraints of chromatographic resin support capabilities.

To fully exploit the chemical and physical properties of a target protein in designing an appropriate strategy for its purification, the following parameters for the target protein should be obtained in initial pilot studies:

- molecular weight (e.g., by SDS-PAGE, size-exclusion chromatography or analytical centrifugation),
- pI (e.g., by isoelectric focusing), and
- stability with respect to pH, salt, temperature, proteases, inclusion of additives to protein solvents to maintain biological activities (e.g. detergents, thiol reagents, and metal ions).

VIII. Protein purification chromatography

8.4. Proteins can be separated on the basis of their intrinsic properties

8.4.1. Exposed amino acid side chains determine protein solubility

The protein-protein variation in solubility is due to the differences in the ratio of solvent-exposed charged (i.e., polar) and hydrophobic amino acids on protein surfaces.

Parameters that influence the solubility of a protein include:

- solvent pH
- the ionic strength and nature of the buffer ions
- solvent polarity
- temperature

Because it is not possible to predict with accuracy the solubility properties of proteins, much of the skill in purification comes from experience in handling proteins under a variety of conditions.

Proteins tend to precipitate differentially from aqueous solution upon addition of:

- neutral salts (ammonium sulfate)
- polymers (polyethylene glycol)
- organic solvents (ethanol, acetone)

VIII. Protein purification chromatography

8.4. Proteins can be separated on the basis of their intrinsic properties

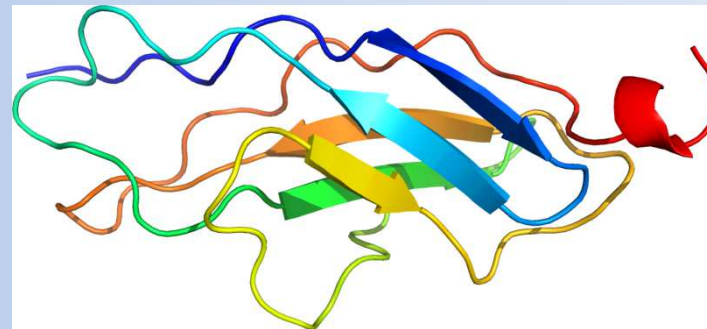
8.4.2. The size and shape of proteins affect their movement through liquids and gels

Proteins vary markedly in size, ranging from a few amino acid residues of a few hundred daltons to more than 10,000 amino acids with a molecular mass in excess of 1,000,000 daltons.

However, the molecular mass of most proteins falls in the range of 6 kD to 200 kD.

In the purification techniques of **size-exclusion chromatography**, a protein solution is passed through a column of porous beads. The internal diameter of the pores are such that large proteins do not have access to the internal space of the bead, whereas small proteins have free access.

Titin is the largest known protein. Its human variant consists of 34,350 amino acids, with the molecular weight of the mature protein being approximately 3,816,188.13 Da. Its mouse homologue is even larger, comprising 35,213 amino acids with a MW of 3,906,487.6 Da.



In **SDS-PAGE**, proteins are denatured and fully coated with the negatively charged detergent SDS, such that they migrate in electrophoretic gels on the basis of their molecular weight.

VIII. Protein purification chromatography

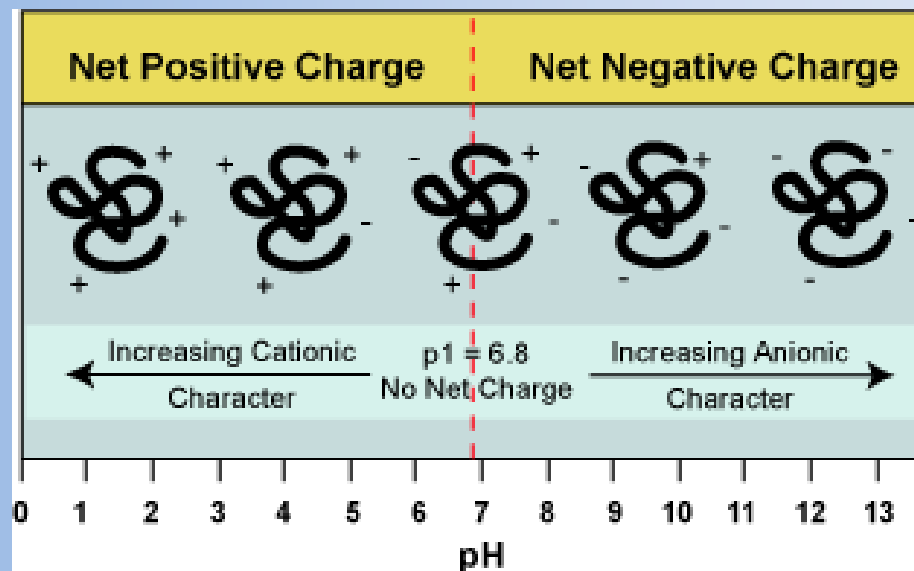
8.4. Proteins can be separated on the basis of their intrinsic properties

8.4.3. Differences in the surface charge of proteins are exploited in ion-exchange chromatography

The net charge on a protein is the sum of the positively and negatively charged amino acid residues, at the pH of the solvent.

- Basic proteins (having net positive charge at neutral pH) have a majority of basic amino acids (e.g., arginine, lysine, and histidine).
- Acidic proteins (having net negative charge at neutral pH) have a majority of acidic amino acids (e.g., aspartic acid, glutamic acid).

The pH at which the net charge of a protein is zero is referred to as the isoelectric point (pI).



VIII. Protein purification chromatography

8.4. Proteins can be separated on the basis of their intrinsic properties

8.4.4. Ligand-binding proteins may be purified by affinity chromatography

Most proteins exert their biological function by specifically interacting with some other cellular component. Enzymes bind to substrates, cofactors, activators, inhibitors and metal ions.

- Hormones bind to receptors.
- Transcription factors bind to nuclear locations, export signals and DNA templates.
- The exquisite specificity of antibody-antigen interactions forms the basis for immunoaffinity chromatography
- Metal atoms attached to a chromatographic support (immobilized metal affinity chromatography IMAC)



VIII. Protein purification chromatography

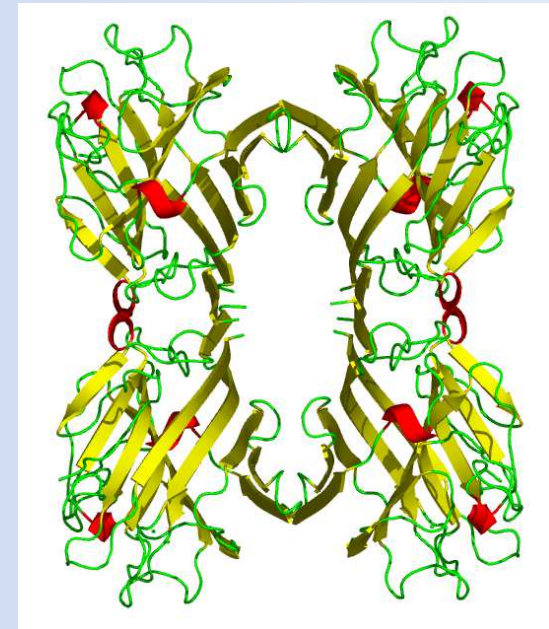
8.4. Proteins can be separated on the basis of their intrinsic properties

8.4.5. Posttranslational modifications provide additional opportunities for purification by affinity chromatography

Posttranslational modifications are fundamental to processes controlling cellular behavior, including cell signaling, growth, and transformation.

- Addition of carbohydrates to form glycoproteins
- Addition of phosphates to form phosphoproteins
- Addition of lipids to form lipoproteins

- Glycoproteins can be captured using immobilized lectins.
- Phosphoproteins can be captured using immobilized antibodies directed against phosphotyrosine or, alternatively, using IMAC.



Leucoagglutinin, a toxic phytohemagglutinin found in raw *Vicia faba* (Wikipedia)

VIII. Protein purification chromatography

8.4. Proteins can be separated on the basis of their intrinsic properties

8.4.6. Thermostable proteins can often be purified easily

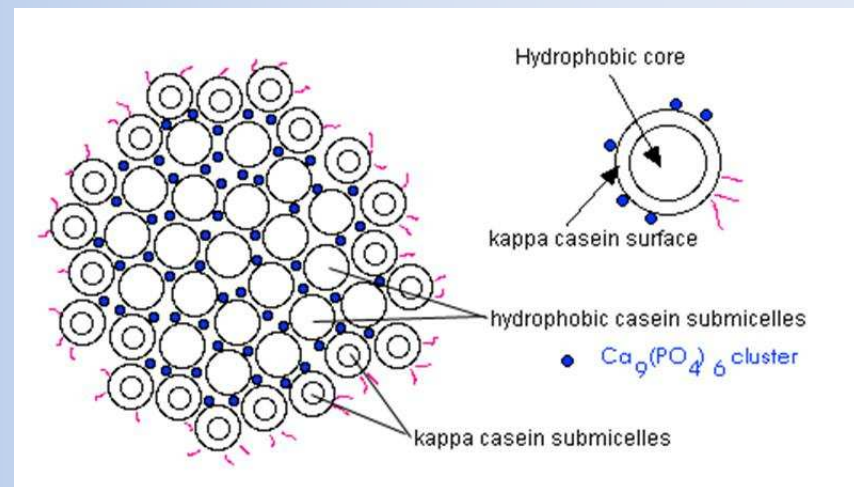
Proteins are typically inactivated and precipitate if heated to 95°C.

However, some proteins exhibit a remarkable degree of thermoresistance:

- Stathmin (mammalian intracellular regulatory protein)
- Muscle phosphatase inhibitor I
- Alkaline phosphatase (innate resistance to digestion with proteases)

Very often proteins with intrinsically disordered structure are thermoresistant:

- Tau (protein associated with microtubules)
- Casein (major milk major protein; 80%)



8.5. Devising strategies for protein purification

Before attempting to design a purification scheme, it is always worthwhile to carry out pilot experiments on the crude extract to determine whether the target protein possesses any unusual chemical properties that might be exploited in a purification strategy.

- Molecular weight
- pI
- Degree of hydrophobicity
- Presence of carbohydrate (glycoprotein)
- Phosphate modification
- Free sulfhydryl groups
- Stability with respect to:
 - pH
 - Salt
 - Temperature
 - Proteolytic degradation
 - Mechanical shear
- Bioaffinity for heavy metals

If the nucleotide sequence is known, much of this information might be obtained by close inspection of the deduced amino acid sequence.

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.1. Is retention of biological activity essential?

An important consideration is whether it is essential to retain biological activity of the target protein during purification.

Most proteins retain activity at:

- Low temperature
- Neutral aqueous buffers
- Stabilizing additives (glycerol, detergent)

Chromatography techniques use incompatible conditions:

- Organic solvents (acetonitrile)
 - Ion-pairing acids (TFA)
 - HCl (10 mM)
 - NaOH (10 mM)
 - MgCl₂ (3 M)
 - Glycine buffer (pH 2.3)
- } Reversed-phase column
- } Immunoaffinity columns

To limit the losses of biological activity of labile target proteins during purification, it is important to:

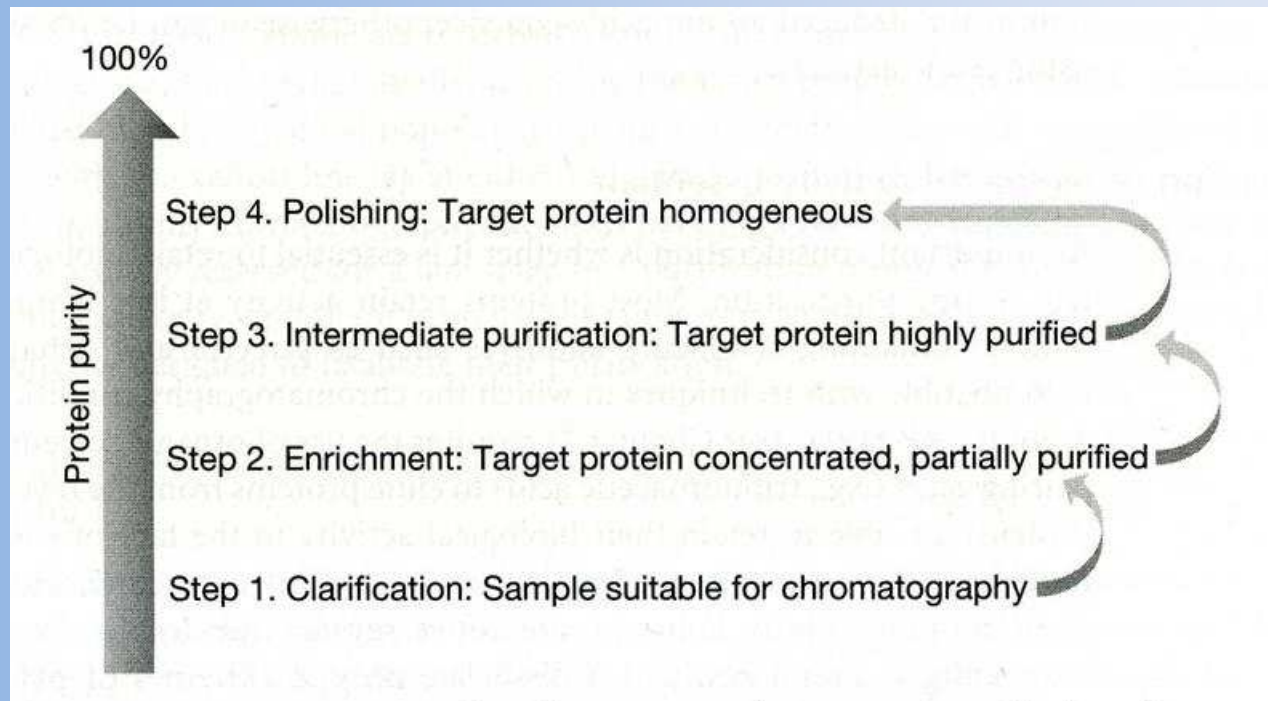
- minimize the number of steps in the purification protocol,
- avoid the need for buffer exchange between steps, and
- discriminate between losses of biological activity due to denaturation and physical losses caused by irreversible adsorption to the chromatographic support or by proteolytic degradation.

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

The average number of steps necessary to purify proteins to homogeneity is four, with an overall yield of 28% and a purification factor of 6380, corresponding to an average ninefold purification and 73% yield per step.



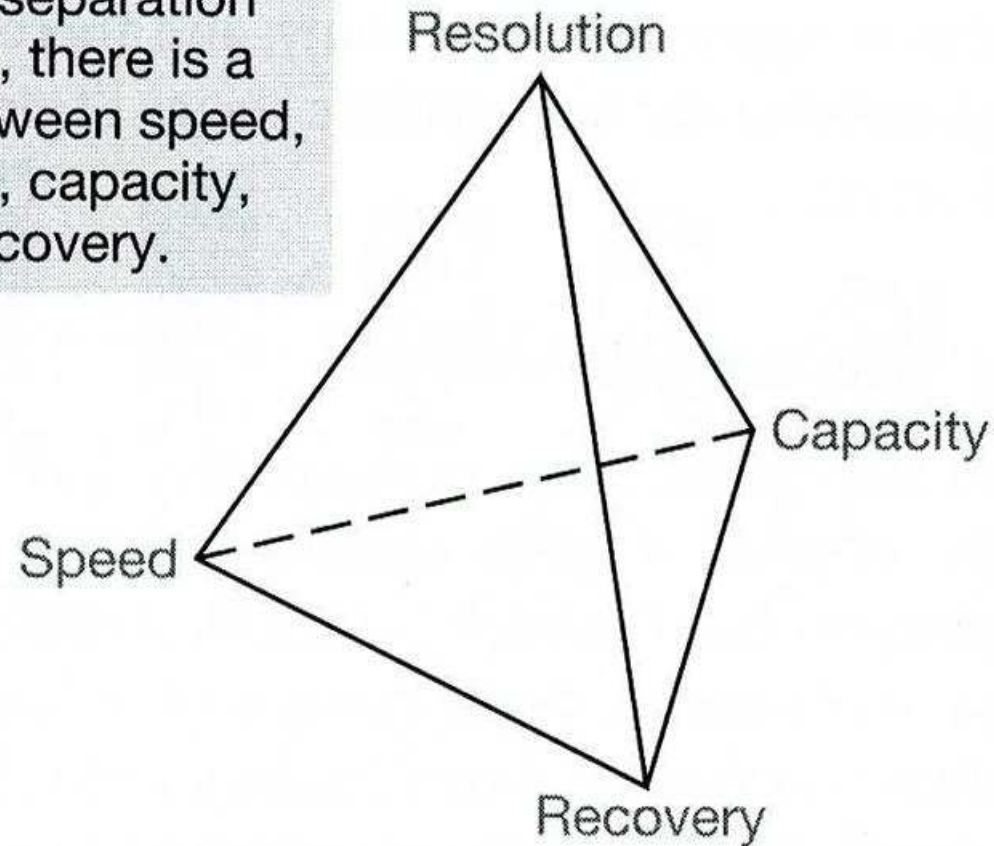
It is generally recognized that with most conventional chromatographic supports, there are compromises among **speed, resolution, recovery, and capacity**.

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

For every separation technique, there is a balance between speed, resolution, capacity, and recovery.



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

Enrichment (capture) stage: Rapid/high-capacity, low-resolution modes

Chromatography:

ion-exchange
hydrophobic
interaction
affinity:

charge
hydrophobicity

DNA
dye

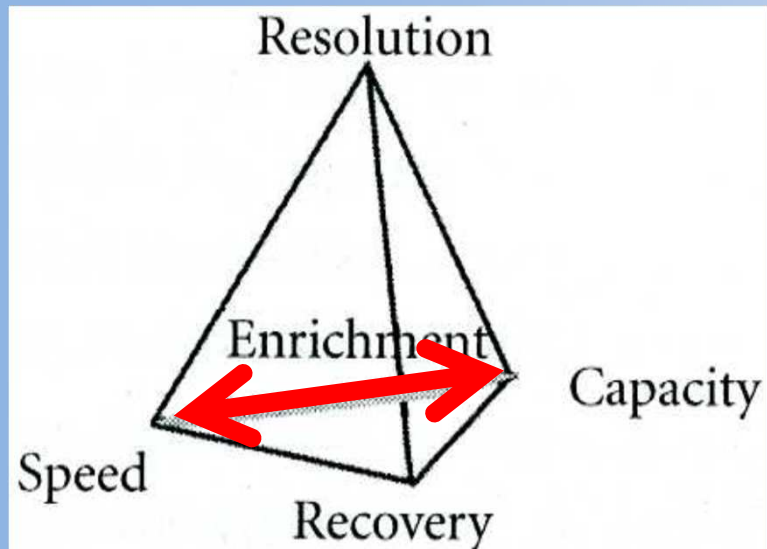
DNA binding
specific dye-binding affinity

substrate
lectin

ligand binding site
carbohydrate content and
type

IMAC
immunoaffinity

metal binding
specific antigenic site



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

Intermediate purification stage: High-capacity, low-resolution modes

Chromatography:

ion-exchange

hydrophobic

interaction

size exclusion

chromatofocusing

charge

hydrophobicity

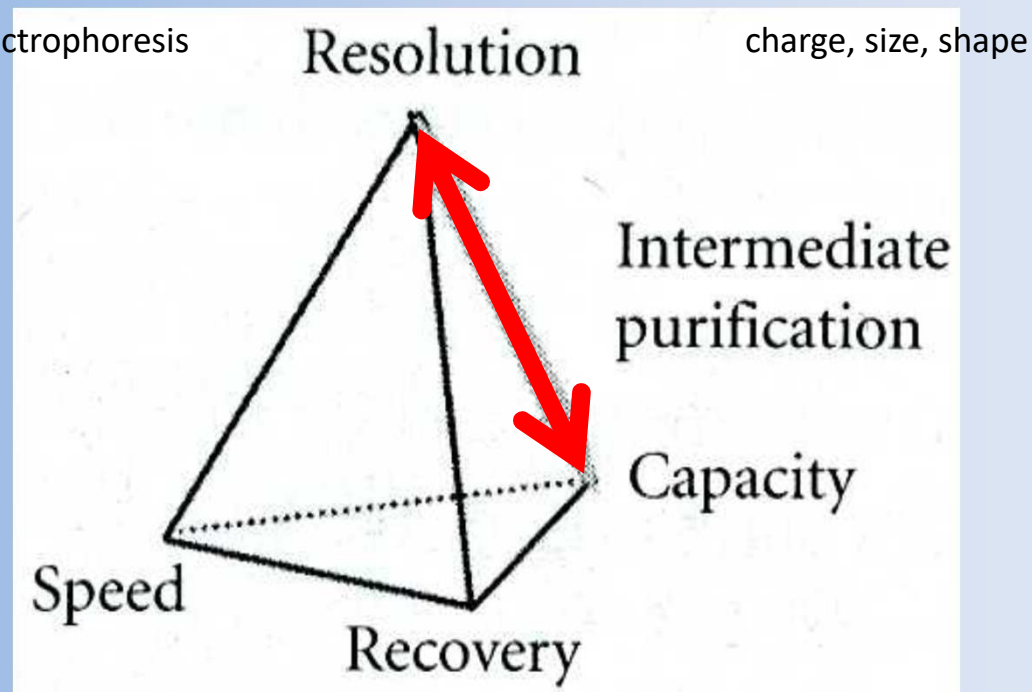
size, shape

pI

Electrophoresis

gel electrophoresis

charge, size, shape



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

Final polishing stage: Low-capacity, high-resolution modes

Chromatography:

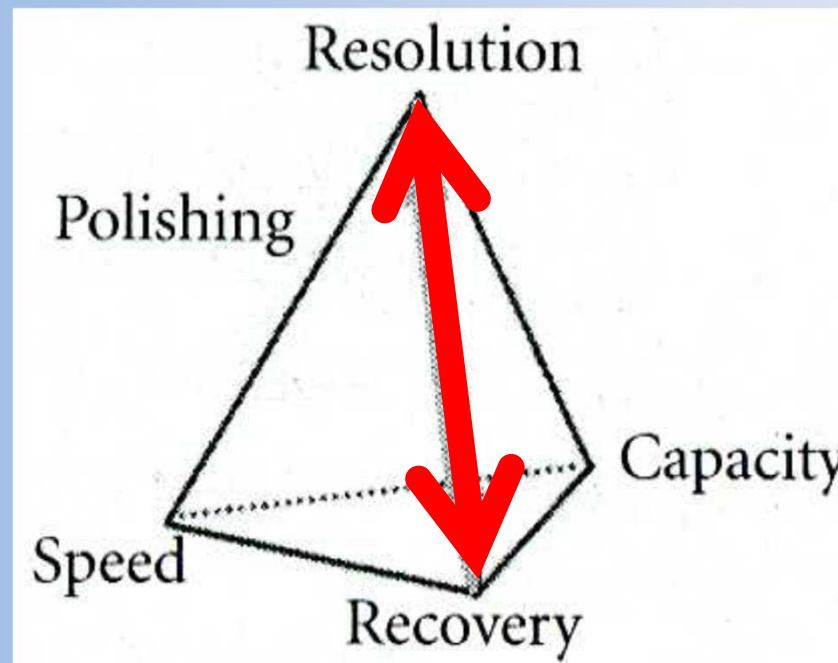
reversed-phase HPLC
size exclusion

hydrophobicity, size
size, shape

Electrophoresis

gel electrophoresis
isoelectric focusing
free-flow electrophoresis

charge, size, shape
pI
charge, size, shape



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

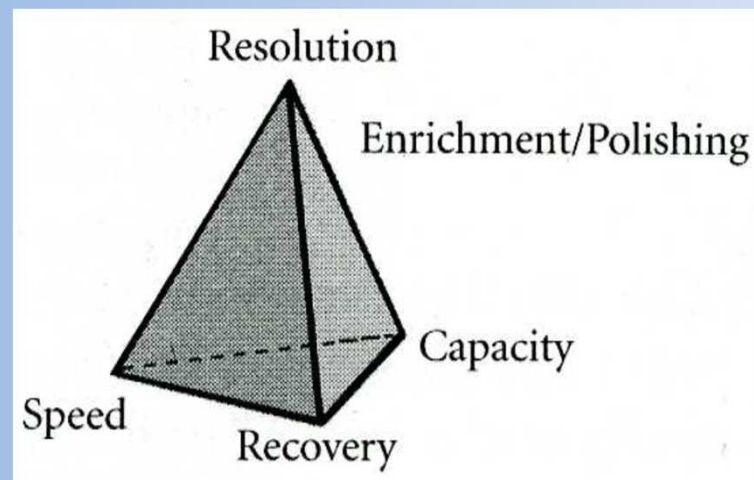
8.5.2. How many purification steps are necessary?

Recombinant proteins with a fusion tail (or "TAG")

Combined enrichment/polishing purification stage: High-capacity, rapid/high-resolution modes

Chromatography:

affinity	substrate lectin IMAC immunoaffinity	enzyme/ligand-binding site carbohydrate-binding domain metal binding antigenic epitopes
hydrophobic interaction	hydrophobic aa tails (e.g., poly[Phe])	hydrophobicity
ion/exchange	charged aa tails (e.g., poly[Arg])	charge (or precipitation)



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

1. Clarifying the starting material

In purification protocol, it is always important to include steps for removing insoluble residues (lipid droplets causing column blockage) and that the initial clarification/concentration step be as rapid as possible (proteolytic degradation):

- **Differential centrifugation** (awkward)
- **Filtration** through a plug of glass wool or fine mesh cloth (less efficient)
- **Fractional precipitation** (salts, polymers, organic solvents) – very high (80%) average yield and able to gently concentrate large volumes
- **Ultrafiltration** with a variety of molecular-mass cutoff limits (1,000–300,000 daltons) – relatively slow

TABLE 1.4. Procedures for clarifying and concentrating large-volume protein samples

Concentration Process	Basis of Method
Precipitation	
ammonium sulfate (including “salting-out” chromatography)	differential solubility
polyethylenimine	forms insoluble complexes with acidic macromolecules (i.e., acidic proteins, DNA, RNA)
ethanol	differential solubility
Phase-partitioning (e.g., polyethylene glycol)	differential solubility
Ultrafiltration	size and shape

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

2. Capturing the target protein

For native proteins, enrichment is best accomplished using high-capacity/low-resolution chromatographic procedures:

- **Hydrophobic interaction (HIC)**
- **Anion-exchange chromatography**
- **Nonbiospecific affinity chromatography** (triazine dye chromatography)
- **Immunoaffinity chromatography** (high cost)

For recombinant proteins, several fusion systems are developed:

- **Oligohistidines (IMAC)**
- **Antigenic epitopes (Mab)**
- **Carbohydrate-binding proteins** (domains recognized by lectins)
- **Biotin-binding domain** (affinity to avidin or streptavidin)

If required, a specific protease cleavage site can be engineered into the fusion protein.

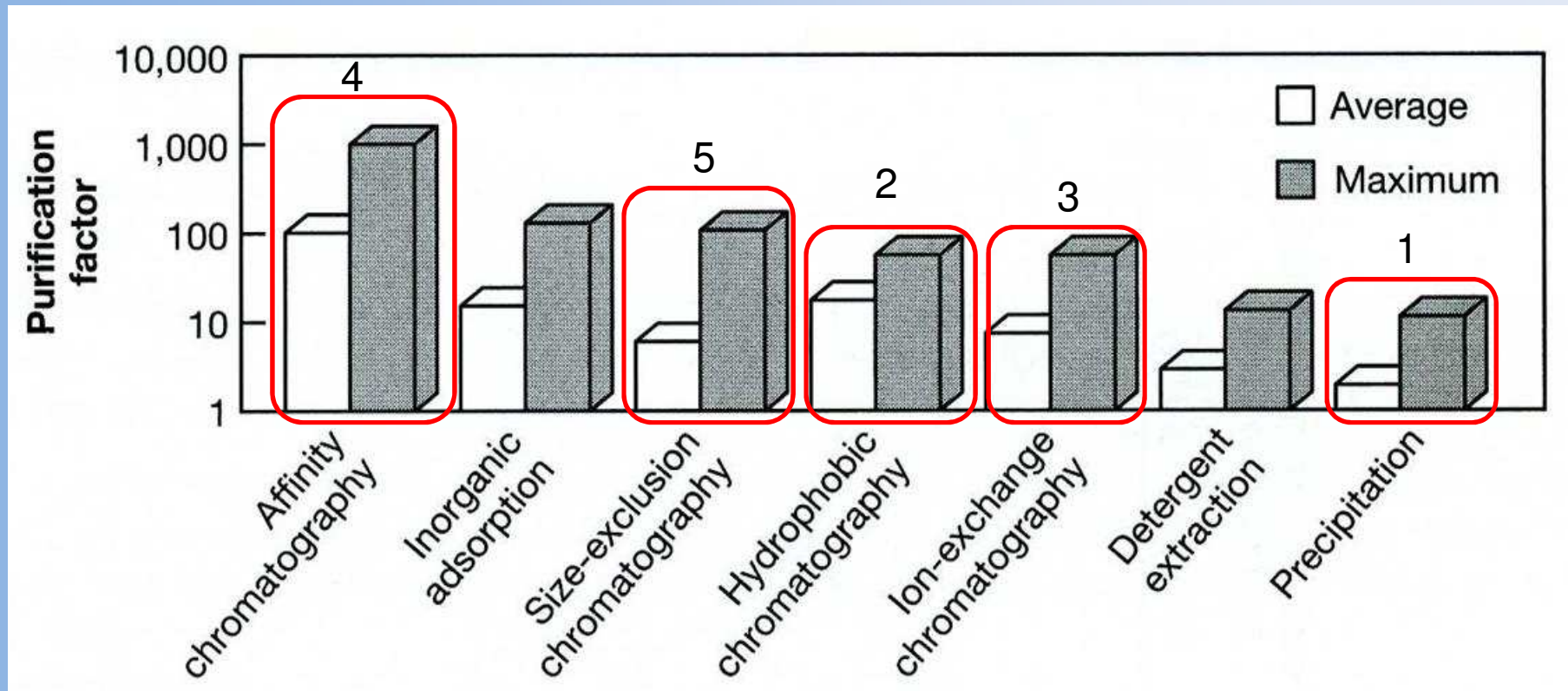
VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

3. Purifying and concentrating-intermediate steps

This step should be designed to provide further purification and reduction of sample volume, and it is best accomplished using intermediate capacity/intermediate- to high-resolution chromatography.



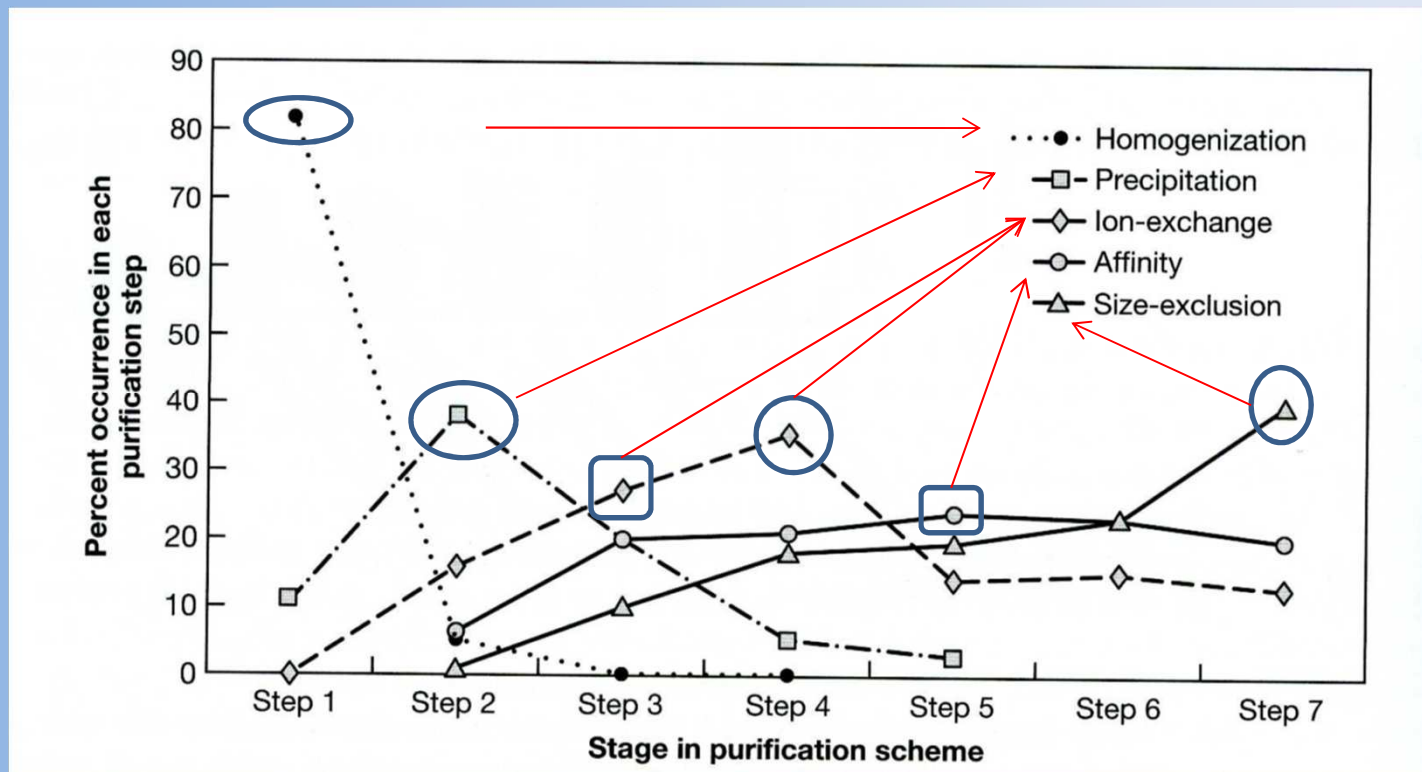
VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

4. Final polishing

The purpose of the final polishing step(s) is to remove any minor contaminants remaining, to remove possible aggregates, and to prepare the homogenous target protein for its intermediate use or for storage.



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

Which order of steps is best?

According to an analysis of successful purification methods by Bonnerjea et al. (1986):

- homogenization
- clarification/fractional precipitation
- anion-exchange chromatography
- affinity separation
- SEC

An important consideration in designing the order of purification steps is to minimize buffer-exchange steps:

- Fractional precipitation using ammonium sulfate
- Hydrophobic interaction chromatography
- Ion-exchange chromatography
- SEC, dialysis, or membrane ultrafiltration

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

Checklist for protein purification:

[Redacted]

[Redacted]

[Redacted]

[Redacted]

[Redacted]

[Redacted]




[Redacted]

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.2. How many purification steps are necessary?

Ionizable group	pKa	pH 2	3	4	5	6	7	8	9	10	11	pH 12
C-terminal (COOH)	4.00	+	+	-	-	-	-	-	-	-	-	-
Aspartate (COOH)	4.50	+	+	-	-	-	-	-	-	-	-	-
Glutamate (COOH)	4.60	+	+	-	-	-	-	-	-	-	-	-
Histidine (imidazole)	6.20	+	+	+	-	-	-	-	-	-	-	-
N-terminal (amino)	7.30	+	+	+	+	-	-	-	-	-	-	-
Cysteine (SH)	9.30	+	+	+	+	+	+	-	-	-	-	-
Tyrosine (phenol)	10.10	+	+	+	+	+	+	+	-	-	-	-
Lysine (amino)	10.40	+	+	+	+	+	+	+	+	-	-	-
Arginine (guanidino)	12.00	+	+	+	+	+	+	+	+	+	+	+

 + charge
 - charge
 Zero charge

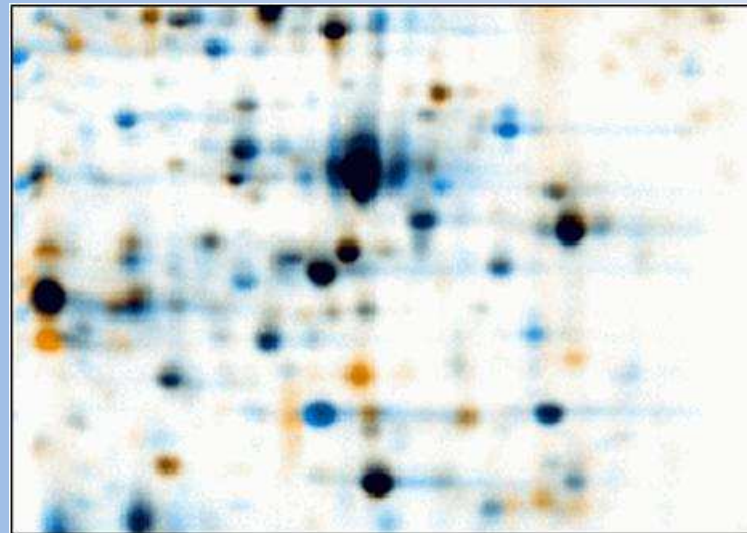
VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.3. Enrichment of low-abundance proteins by preparative electrophoresis

The dynamic range of protein abundance in a biological sample can be 1,000,000 copies per cell for the cytoskeletal proteins that maintain cellular architecture. On the other hand, we can work with a transcription factor ranging from 10 copies per cell.

2D electrophoresis can separate only a subset of a total proteome, at best 1,500–2,000 proteins.



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

Chromatography (Greek: color writing) – technique for separating the component of a mixture by allowing the sample to distribute between two phases – one remains stationary (stationary phase), while the other moves (mobile phase).

- A packed bed of solid material in a column (**liquid chromatography**)
- Spread as a thin layer or film on flat plat (**thin-layer chromatography**)
- Paper (**paper chromatography**)

Liquid chromatography:

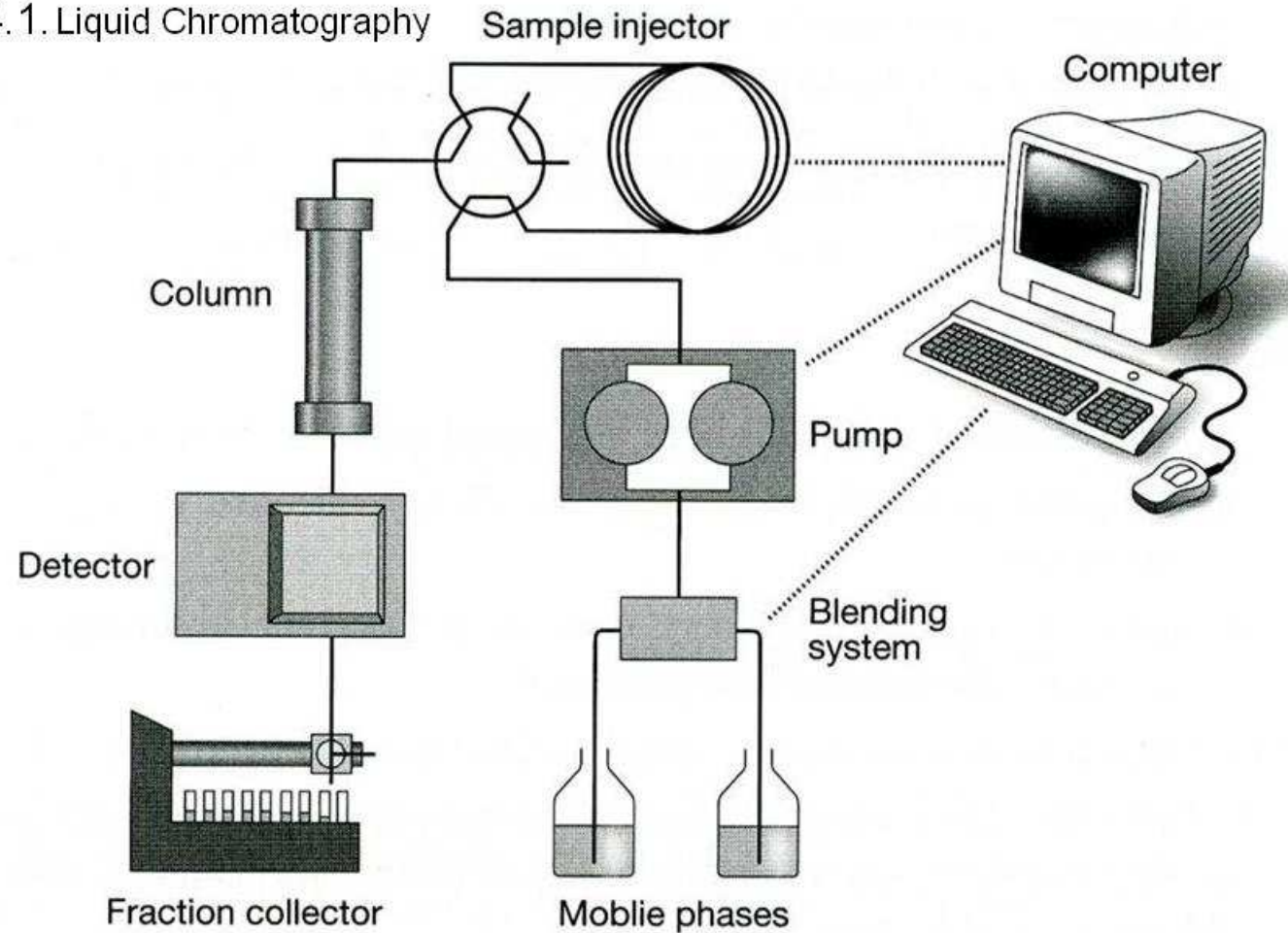
- **A stationary phase** (with controlled structure and surface chemistry).
- **A column** (packed with stationary phase).
- **A mobile phase(s)** or solvent(s) of controlled chemical composition that moves the solute through the column.
- **Chromatography equipment** capable of accurately delivering the sample to the column.
- **Software programs** for blending the mobile phase(s).

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

8.5.4.1. Liquid Chromatography

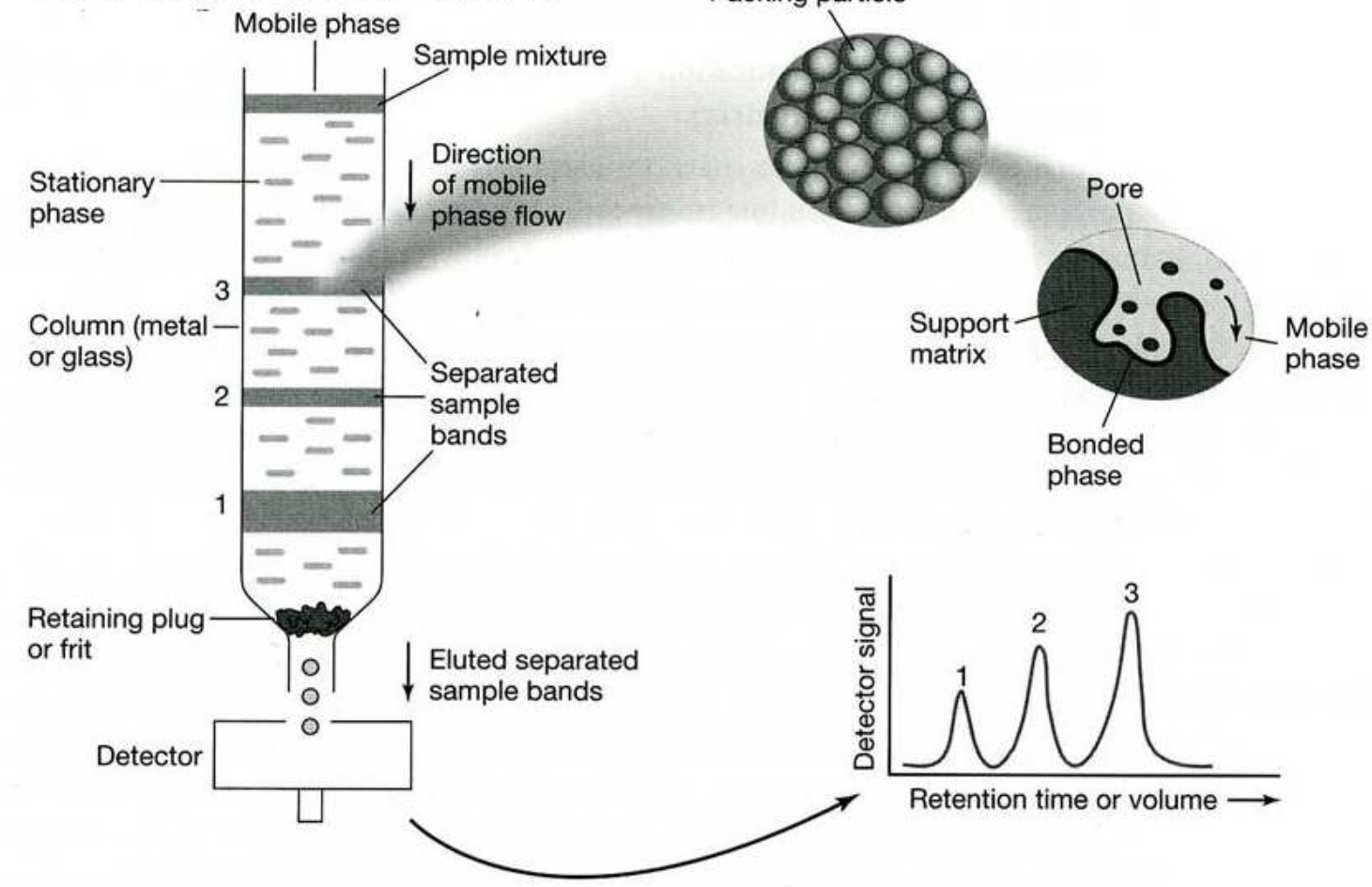


VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

8.5.4.1. Liquid Chromatography



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

8.5.4.1. Liquid chromatography – stationary phase

TABLE 1.5. Liquid chromatography methods for separating proteins and peptides

Principle of Chromatographic Separation	Type of Chromatography
Net charge	Ion-exchange chromatography (Chapter 5)
Size and shape	Size-exclusion chromatography, also referred to as gel-filtration or gel-permeation chromatography (Chapter 6)
Hydrophobicity	Hydrophobic interaction chromatography Reversed-phase high-performance liquid chromatography (RP-HPLC) (Chapter 7)
Biological function (bioaffinity)	Affinity chromatography (Chapter 8)
Carbohydrate content	Lectin chromatography (Chapter 8)
Antigenicity	Immunoaffinity chromatography (Chapter 8)
Metal binding	Immobilized metal ion affinity chromatography (Chapter 9)

- *Support matrix:* rigid solids, hard gels, or soft gels.
- *Particle size (d_p) and structure:* spherical versus irregular particles.
- *Pore structure:* porous versus pellicular and superficially porous particles.
- *Bonded phase.*

VIII. Protein purification chromatography

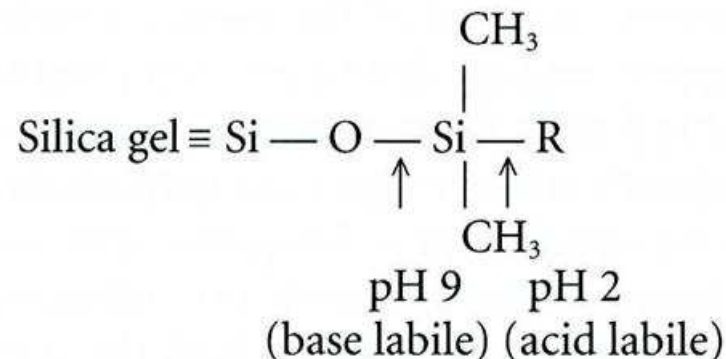
8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

8.5.4.2. Liquid chromatography – support matrix

- **Rigid solids** – inorganic materials (porous silica, controlled pore glass, hydroxyapatite, alumina and zirconium) (4,000–6,000 psi; 26–39 MPa)
- **Hard gels** – synthetic organic polymers (polystyrene-divinylbenzene, polyacrylamide, polyvinyl alcohols, and polymethacrylate)
 - POROS or SOURCE (2,000–5,000 psi; 13–35 MPa)
- **Soft gels** – natural polymers such as cellulose, dextran, and agarose.

In most cases, support matrices used in protein and peptide applications are hydrophilic, charge-neutral, and have low nonspecific binding characteristics.



VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

8.5.4.3. Liquid chromatography – particle size and structure

Particle size (d_p) – critical determinant in LC influencing the chromatographic efficiency in a given separation (mechanical stability – column lifetime; surface area – analyte capacity factor k').
(POROS/SOURCE: $d_p = 3\text{--}20\ \mu\text{m}$ in diameter)

Particle shape – better to have narrow range of particle sizes (packed columns with very broad particle distribution are inefficient and less permeable)

Purpose of LC	Particle Size
Analytical applications	3–10 μm diameter ^a
Preparative separations	10–40 μm diameter
Low-pressure/large-scale applications	40–150 μm diameter
Very large-scale operations	~300 μm diameter

^aMedia made from particles with a $d_p < 1\text{--}3\ \mu\text{m}$ have proven to be impractical because of inherent problems with packing and the need for very high operating pressures.

VIII. Protein purification chromatography

8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

8.5.4.4. Liquid chromatography – pore structure (accessible surface area)

Typically, the pore diameter must be 5 times the size of molecules being purified to permit them to access all of the pores via molecular diffusion.

- Macroporous packings contain pores ranging from 1,000 to 10,000 Å (IEC).
- Mesoporous packings (wide-pore) contain pore diameters of 180–500 Å (HIC).
- Microporous packings have 60–120 Å pore diameters (RP-HPLC).

Stationary phase vs. bonded phase

Whereas the column packing matrix provides the chemically inert “skeleton” for the stationary phase, the bonded phase provides the functional groups, which are designed to selectively bind solute molecules.


VIII. Protein purification chromatography

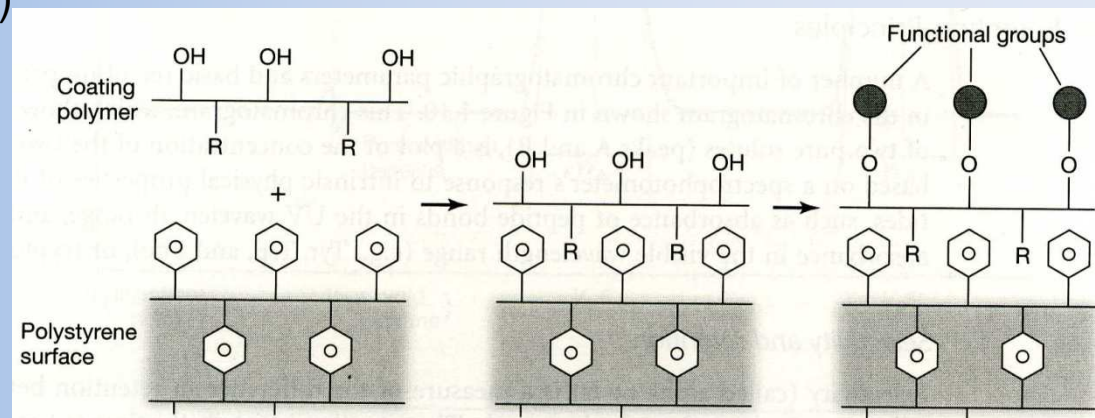
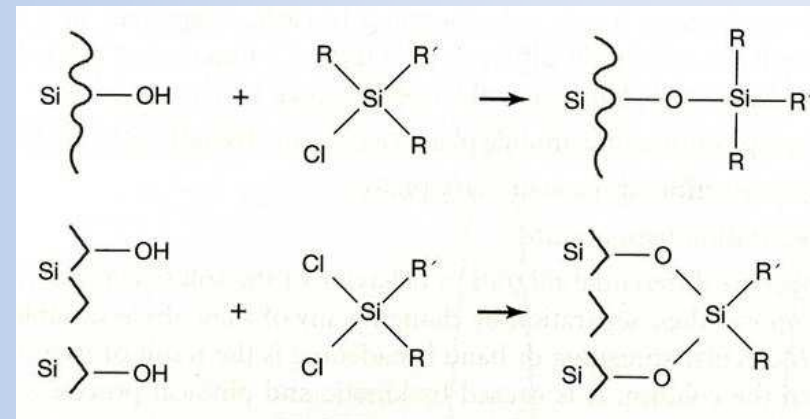
8.5. Devising strategies for protein purification

8.5.4. Strategies based on chromatographic methods for protein and peptide purification

8.5.4.5. Liquid chromatography – bonded phase

Functional R groups are usually:

- Methyl (CH₃) groups
- Hydrocarbon chains (C₆, C₈ or C₁₈)
- Nitrile group (–C≡N)
- Amino group (–NH₂)
- Carboxylic group (–COO⁻)
- Sulphate group (–SO₃⁻)
- Phenyl group (–)



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8.5.4.6. Liquid chromatography – chromatographic performance

The separation of charged molecules, such as peptides and proteins, as they move down a column is affected by differential migration of solutes and their spreading or dispersion (peak or band broadening).

When the interaction of a solute with the stationary phase is very strong, it is retained to a greater extent, and thus will move more slowly.

Equilibrium distribution coefficient $K_D = S_S / S_M$

S_S – concentration of a solute in the stationary phase

S_M – concentration of same solute in the mobile phase

The migration behavior is influenced by three major variables:

- composition of the mobile phase (pH, ionic strength),
- composition of the stationary phase, and
- separation temperature.

Molecular spreading is the result of dilution of a solute band as it moves down the column (kinetic and physical processes versus thermodynamic processes).

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8.5.4.7. Liquid chromatography – basic retention principles

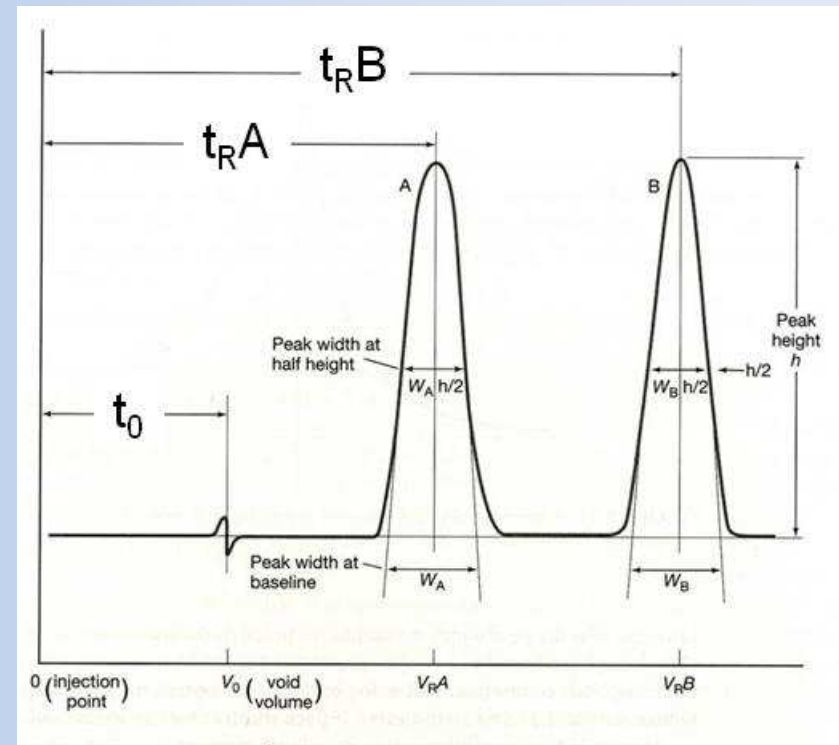
Selectivity (α) is a measure of the difference in retention between the solute of interest and other solutes in the sample.

Retention is simply the time (t_r) or volume (v_r) it takes for a solute to move through the column.

The capacity factor (k' , or the **retention factor**) is the number of column volumes required to elute a particular solute, and t_0 represents the void time.

$$k' = (t_r - t_0) / t_0$$

Selectivity is sometimes expressed as the ratio of the capacity factors k' of two solutes being separated: $\alpha = k'_2 / k'_1$



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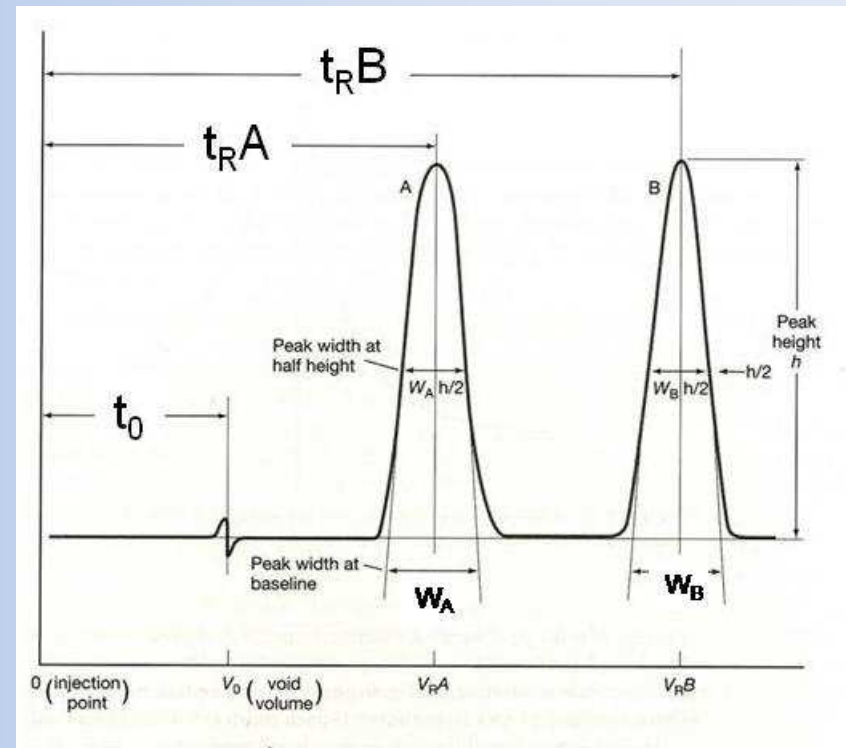
8.5.4.7. Liquid chromatography – basic retention principles

Selectivity and retention

Efficiency $N = 16 (t_R / W)^2 = 5.54 (t_R / W_{h/2})^2$

Resolution $R_s = (t_{RB} - t_{RA}) / (W_A + W_B)^{1/2}$
 $= (t_{RB} - t_{RA}) / (W_{Ah/2} + W_{Bh/2}) 0.85$
 $= 1/4 (\alpha - 1) (N^{1/2}) (k' / 1 + k')$

N = theoretical plate number

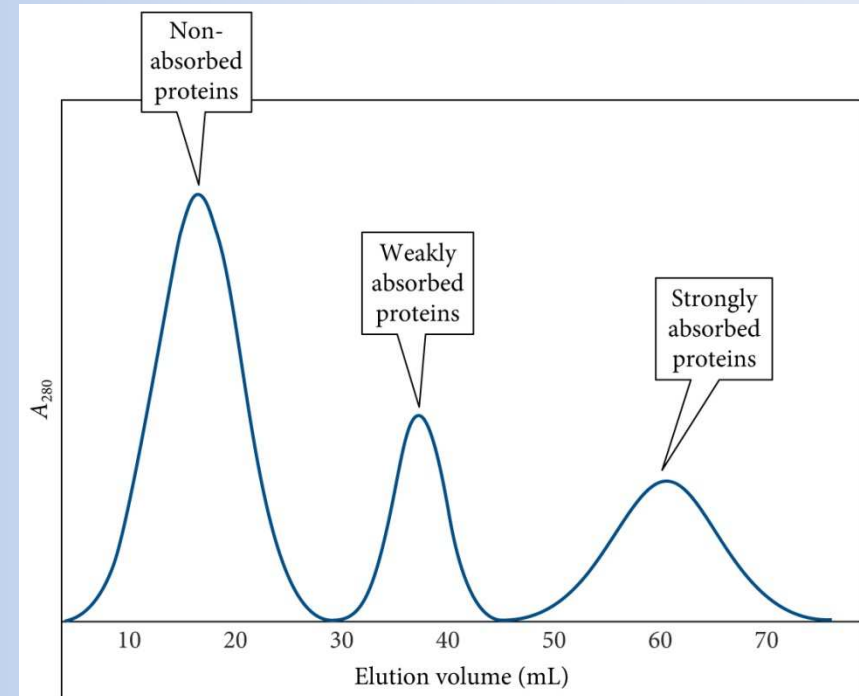
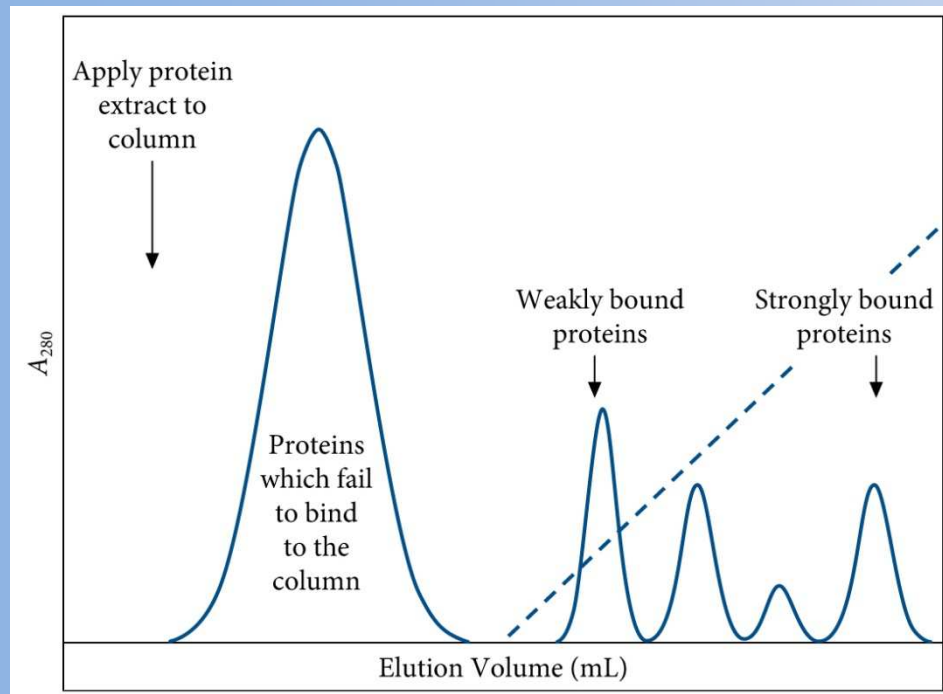


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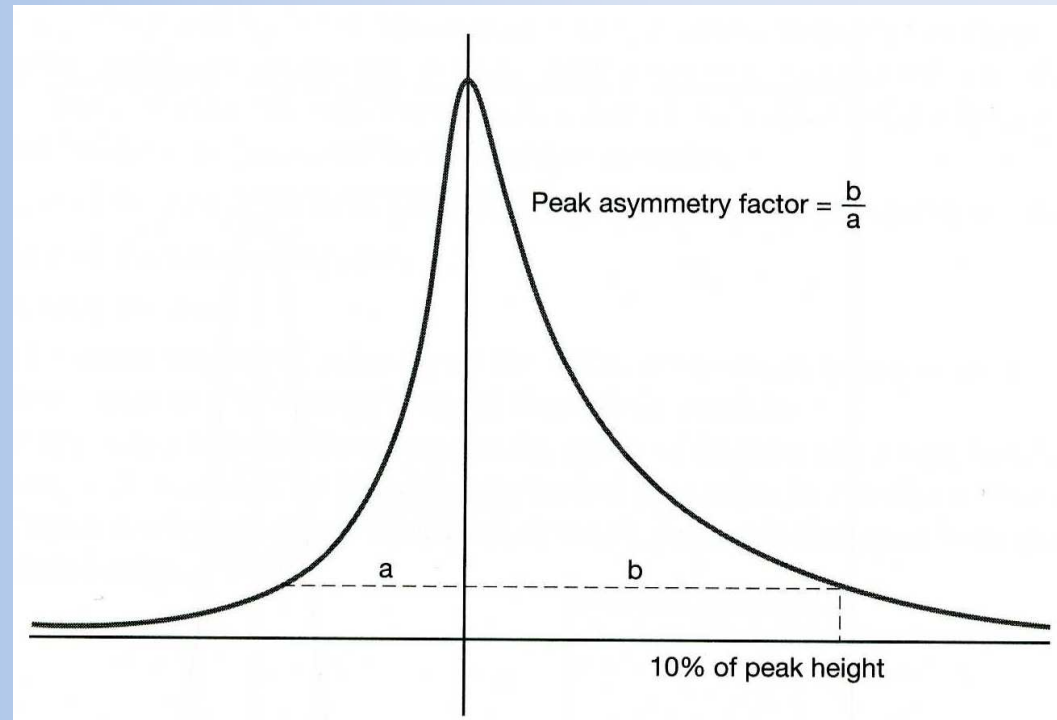
8.5. Devising strategies for protein purification

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8.5.4.7. Liquid Chromatography – basic retention principles

Band broadening and efficiency

Asymmetric peaks. A value >1 is a tailing peak (commonly caused by sites on the packing with a stronger than normal retention of the solute).



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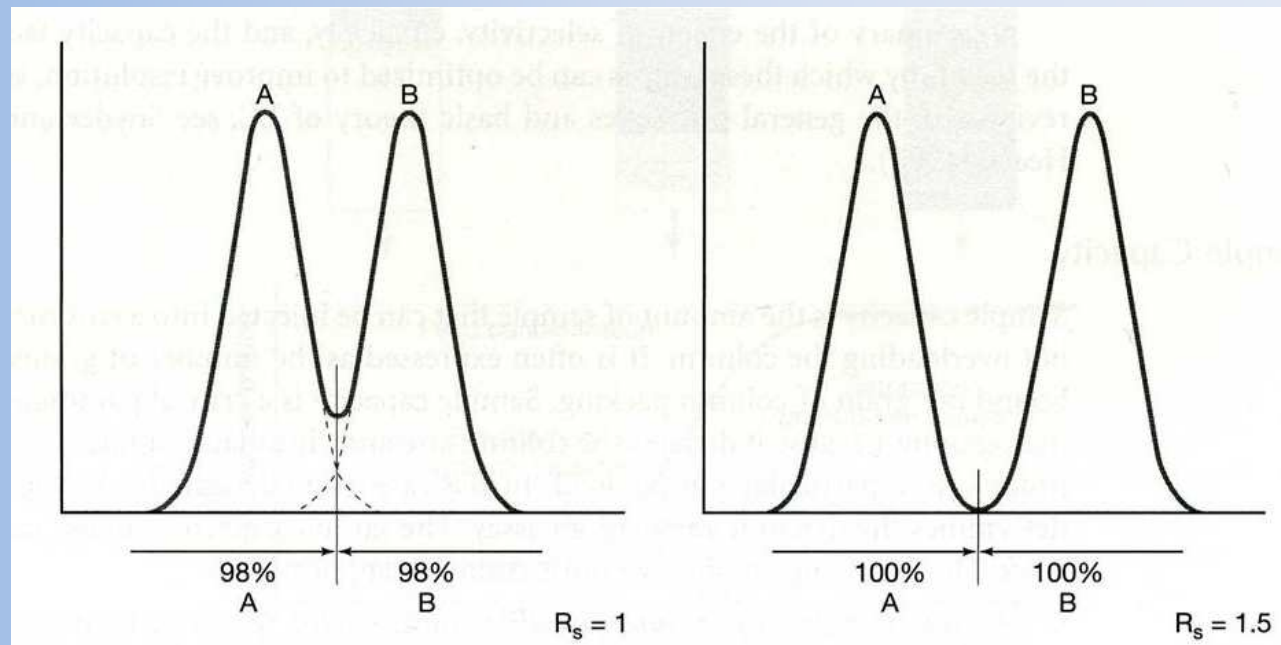
8.5.4.7. Liquid chromatography – basic retention principles

Resolution

Resolution (R_S) is defined as the extent of separation between two chromatographic peaks.

$$R_S = 2 (t_{rB} - t_{rA}) / (W_A + W_B)$$

Resolution is a composite function of both thermodynamic and kinetic parameters and is expressed in terms of an equation that includes the selectivity factor α , the capacity factor k , and the plate number N .



$$R_S = \frac{1}{4}(\alpha - 1)(N)^{1/2} \left[\frac{1}{1 + k} \right]$$

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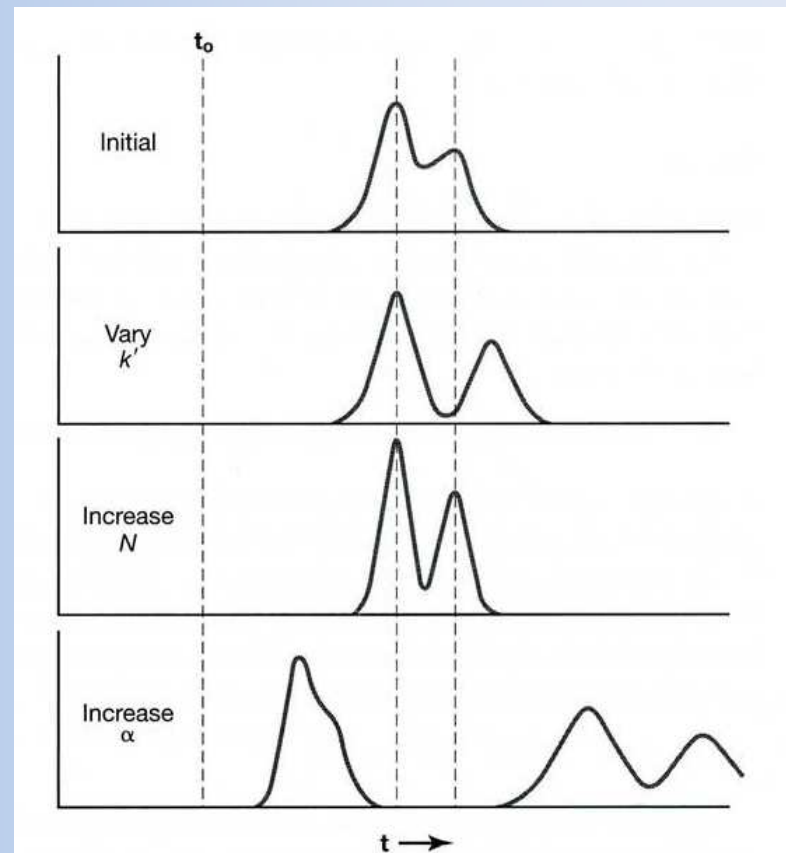
8.5.4.7. Liquid chromatography – basic retention principles

Resolution

$$R_S = \frac{1}{4}(\alpha - 1)(N)^{1/2} \left[\frac{1}{1 + k'} \right]$$

k' - Capacity is directly related to the distribution coefficient of a solute between the mobile and stationary phases.

α - **Selectivity** is affected by the surface chemistry of the column packing, the nature and composition of the mobile phase, the nature of the stationary phase and the gradient shape.



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8.5.4.7. Liquid chromatography – basic retention principles

Resolution

$$R_S = \frac{1}{4}(\alpha - 1) \times N^{1/2} \times \frac{k'}{1 + k'}$$

Resolution = selectivity factor × efficiency factor × retention factor

Factor	Effect on R_S	How to Improve R_S
Selectivity factor ($\alpha = k_2/k_1$)	For closely spaced peaks, α is close to 1.0, so <i>small</i> changes in α have <i>large</i> effects on resulting resolution.	Alter composition of mobile phase (e.g., organic modifier pH, buffer salt), stationary phase, and/or temperature
Efficiency factor $N = 5.54 (t_r/W_{h/2})^2$	Since R_S is a function of the square root of N , large changes in N are required to make small changes in resolution.	Increase column length, decrease particle size of column packing, or decrease flow rate. Minimize extra-column dead volume.
Retention factor ($k' = [t_r - t_o]/t_o$)	When k' is small (<1), R_S increases rapidly with an increase in k' . However, beyond a k' value of 5, R_S increases very little with further increases in k' . Separations that involve k' values >10 result in long separation times and excessive band broadening.	Alter the eluent strength. Values of k' can be increased and/or decreased by using so-called weaker and stronger solvents, respectively (see Table 7.2).

VIII. Protein purification chromatography

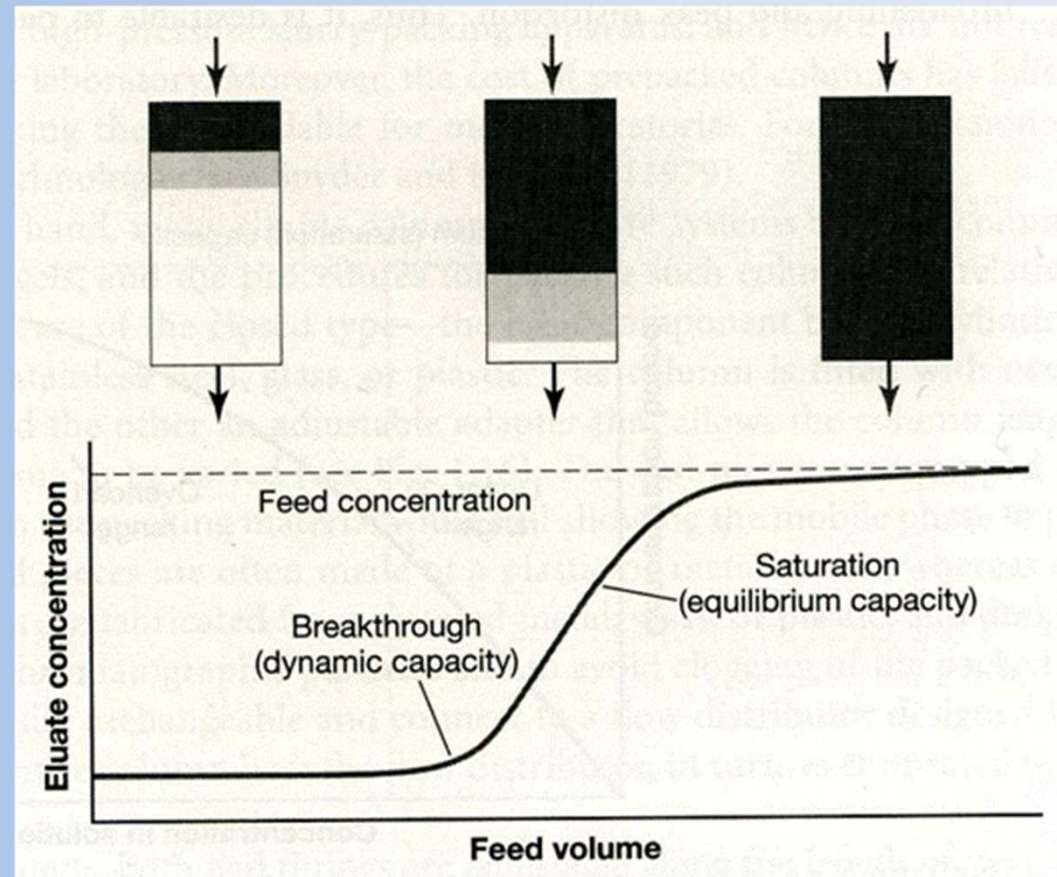
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8.5.4.8. Liquid chromatography – sample capacity

Sample capacity is the amount of sample that can be injected into a chromatographic system without overloading the column (the number of grams of sample per gram of column packing).

- Measurement of saturation or equilibrium capacity
- Frontal adsorption analysis



VIII. Protein purification chromatography

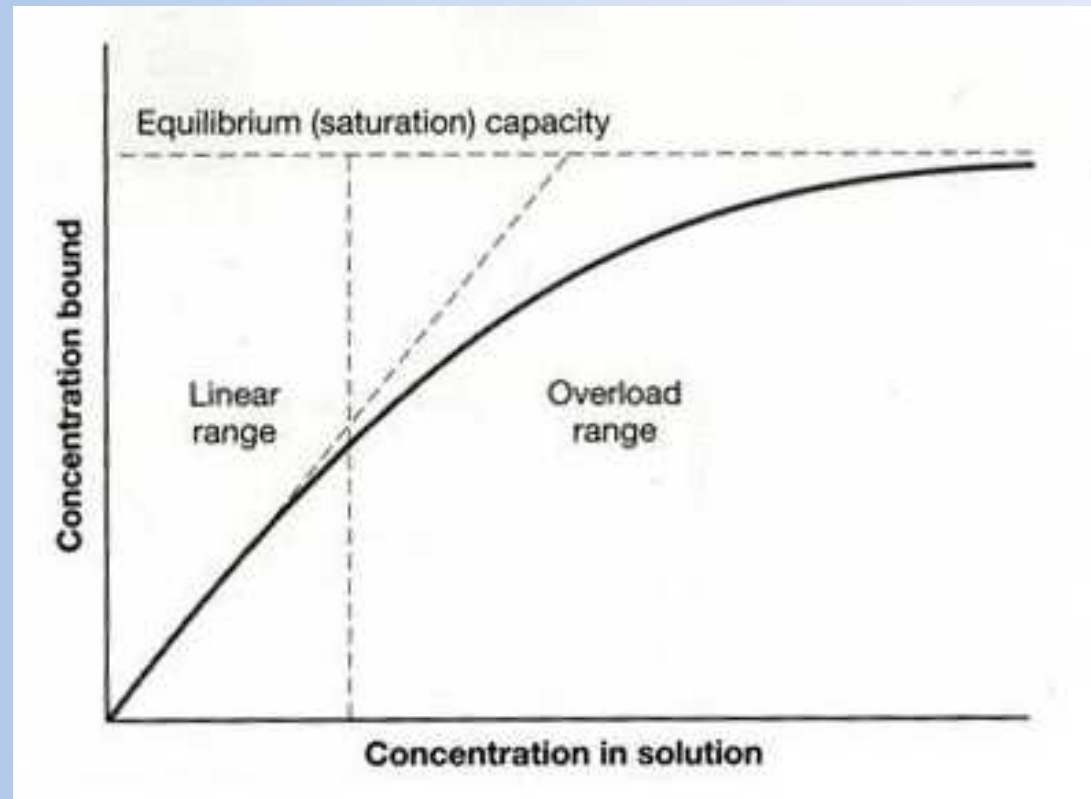
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8.5.4.8. Liquid chromatography – sample capacity

Column loadability

For optimal chromatographic performance and to achieve the greatest resolution, column loadability is critical parameter.



VIII. Protein purification chromatography

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8.5.4.8. Liquid chromatography – packing a column

