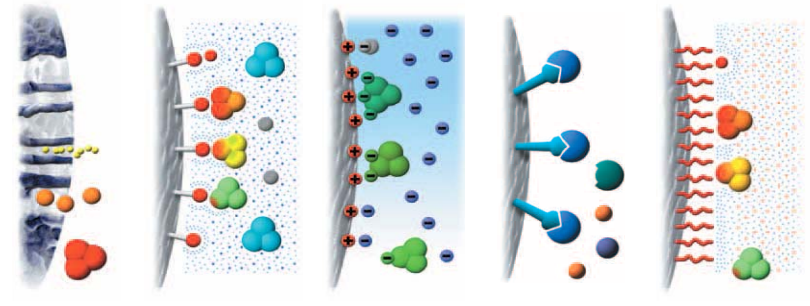
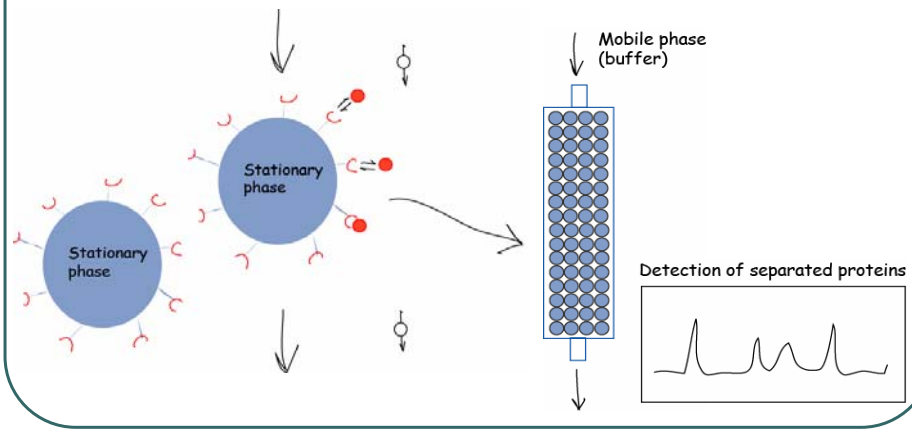
Protein Purification and Analysis

Liquid chromatography

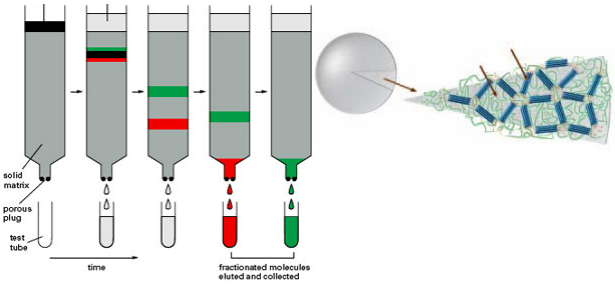
Liquid chromatography

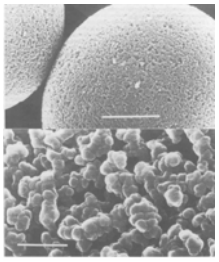


Basic principle



The stationary phase





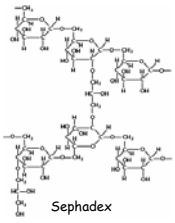
- Hydrophilic
- No spontaneous binding to proteins.
- Should contain functional groups
- Chemically and physically stable
- Rigid to allow a high linear flow-rate

The stationary phase

Matrix materials

dextran (α(1->6) linked glucose residues)	Sephadex Sepharose Sephacryl
agarose	Sepharose Aca
polystyrene/divinyl benzene	MonoBeads
polyacrylamid	Bio-Gels
silica	HPLC columns
cellulose (β(1->4) linked glucose residues)	"not any more"

Cross-linked agaros



Sephadex

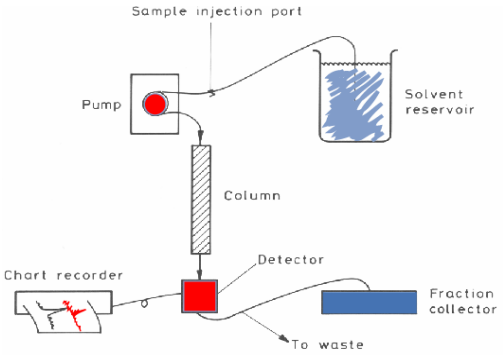
The stationary phase

Table. Characteristics of common separation media

Separation technique	Characteristics	Sample composition		Cost of matrix SEK/litre	Stage of purification
		Before	*After*		
Ion exchanger	High capacity; used in batch or column mode	Low ionic strength; correct pH	High ionic strength; Change in pH	3,000	Variable but especially early
Hydrophobic interaction	High capacity; useful after salt precipitation	High ionic strength	Low ionis strength and/or change in pH	6,000	Variable but especially early
Hydroxylapatite	Mild separation conditions	Neutral pH; low phosphate	Neutral pH; high phosphate concentration	4,200	Variable
Metal chelate	Not sensitive to ionic strength	Absence of chelators	Altered pH; presence of chelators	15,000	Variable
Covalent	Matrix requires lengthy regeneration	Neutral to mildly acidic pH	Presence of low molecular weight thiols	30,000	Late
Chromatofocusing	High resolution	Low ionic strength	Presence of amphoteric buffer; pH close to protein pI	6,500	Late

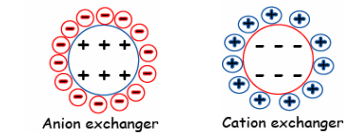
The mobile phase is different for different stationary phases.

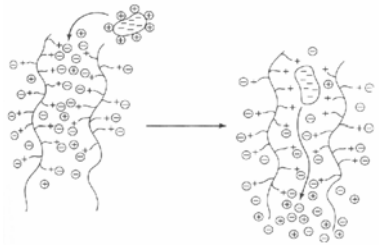
Chromatographic equipment



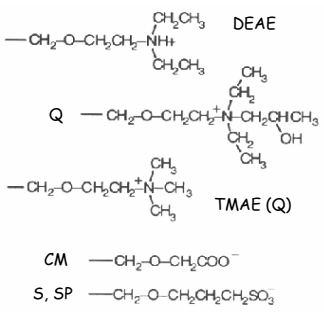
- A pump to give an even flow of liquid
- A column in which the protein separation occurs
- A detector to provide a continuous measurement
- A recorder to give a visual and permanent read-out of the detector

Ion exchange chromatography





Stationary phases

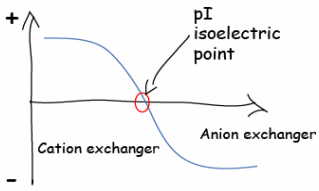


Ion exchange chromatography

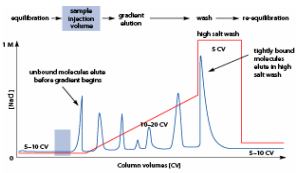
Mobile phases

Table. Suitable buffer compositions for ion-exchange chromatography

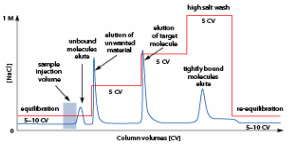
For anion exchange			For cation exchange		
temp (°C)	pH	buffer	temp (°C)	pH	buffer
20 0	8.5 - 9.2 9.0 - 9.7	Diethanolamine or 2-amino-2methyl-1,3-propandiol	0 - 30	3.6 - 4.3	Lactic acid
			0 - 30	4.3 - 5.2	Acetic acid
20 0	7.8 - 8.5 8.2 - 8.9	Tris [tris(hydroxymethyl)-aminomethane]	0 - 30	4.7 - 5.4	Pivalic acid
			0 - 30	5.2 - 5.8	Picolinic acid
20 0	7.4 - 8.0 7.8 - 8.4	Triethanolamine	20 0	5.8 - 6.6 6.0 - 6.8	Mes
20 0	6.6 - 7.3 7.0 - 7.7	Imidazole	20 0	6.3 - 6.9 6.5 - 7.1	Ada
20 0	6.2 - 6.8 6.5 - 7.1	Bis-tris[bis-(2-hydroxy-ethylimino-tris-(hydroxy-methyl-methane)]	20 0	6.8 - 7.5 7.1 - 7.8	Mes
20 0	5.6 - 6.3 5.9 - 6.6	Histidine	20 0	7.2 - 7.8 7.6 - 8.2	Tes
20 0	4.9 - 5.6 5.2 - 5.9	Pyridine	20 0	7.8 - 8.5 8.2 - 8.9	Tricine



Ion exchange chromatography

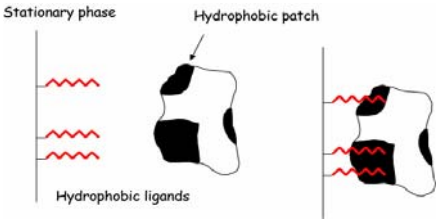


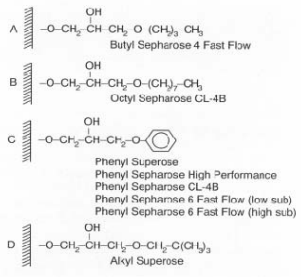
- Binding buffer: 20 mM Tris pH 8.0.
- Elution buffer: 20 mM Tris pH 8.0, 1 M NaCl
- Step-wise elution works well when large changes in ionic strength are applied!!!



Hydrophobic Interaction Chromatography (HIC)

Stationary phases





Hydrophobic Interaction Chromatography (HIC)

← 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 →

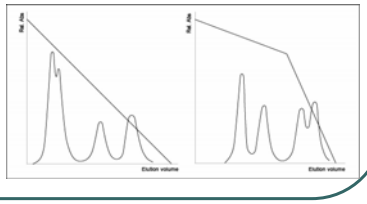
The Hofmeister series on the effect of some anions and cations in precipitating proteins

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

Relative effects of some salts on the molal surface tension of water

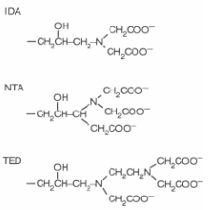
Sodium, potassium or ammonium sulphates produce relatively higher "salting-out" (precipitation) or molal surface tension increment effects.

$NH_4(SO_4)_2$ is probably the most common mobile phase in HIC



Immobilized metal affinity chromatography (IMAC)

Stationary phases



IDA (iminodiacetic acid) is tridentate
NTA (nitrilo triacetic acid) is tetradentate
TED (tris(carboxymethyl ethylene diamide) is pentadenate

- These chelating ligands bind divalent ions such as Fe, Co, Ni, Cu, Zn but also some trivalent ions, Fe, Al
- Mainly histidine but also tryptophan and cystein residues can bind
- Residues must be on the surface of the protein, and the strength of the binding depends on the number of residues
- In practice, the bulk of proteins do not bind but certain specific proteins do
- His-tag !!!!!

Immobilized metal affinity chromatography (IMAC)

Mobile phase

- Usually a buffer with high ionic strength (up to 1 M NaCl) to avoid non-specific ionic interactions
  Binding buffers have usually pH 6-8, (not histidine as a buffer)

Elution

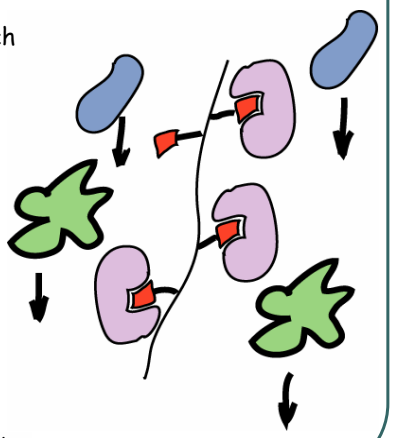
Displacement of proteins by:

- 1) imidazole
- 2) EDTA
- 3) pH, below pH 6 histidine usually becomes protonated

Affinity chromatography

The use of an immobilized natural ligand which specifically holds back the desired protein.

Application	Group to be coupled/ ligand	Product
ATPases	Bovine testis calmodulin	Calmodulin Sepharose
Deoxyribonucleases	Denatured calf thymus DNA	DNA-agarose
Lipases Coagulation factors Growth factors Lipoproteins	Heparin	Heparin-Sepharose
NAD+- and NADP+ dependent enzymes	Cibacron Blue 3GA	Hitrap Blue
Biotinylated substances	Streptavidin	Hitrap Streptavidin HP

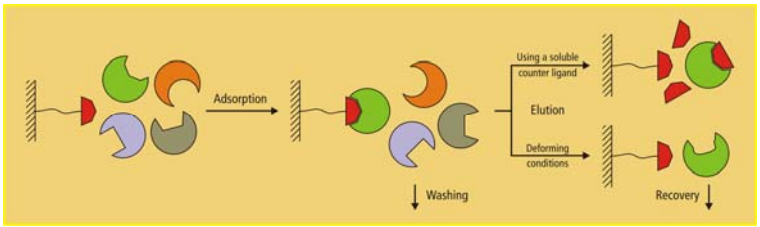


There is no limit, only your knowledge

Affinity chromatography

Specific interaction of the solute with an immobilized affinity ligand, leading to its binding to the immobilized ligand.
A washing step, where non-bonded solutes are rinsed off.
An elution step, where the selectively sequestered molecules are recovered by elution

with a competing substrate, or by applying conditions that lead to an altered conformation of the attached macromolecule that results in loss of specific interaction.



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