Separation and analysis of biological probes: methods and instruments

Basic principles of chromatography techniques

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Темы лекций

1. Методы центрифугирования
2. Основные принципы хроматографии
3. Жидкостная хроматография
4. Электрофорез
5. Капиллярный электрофорез

Basic principles of chromatography

Chromatography is a set of laboratory techniques for the separation of **analyte**, i.e. a mixture of compounds/particles present in gases or in liquids.

Chromatography is a physical method of separation that is based on the distribution of components between **two phases**, **stationary phase** and **the mobile phase** moving in a definite direction.

Stationary phase is usually a solid substance (often called sorbent) or a liquid film immobilized by adsorption on a solid support matrix.

Mobile phase is a liquid or gas moving through a stationary phase

Chromatography techniques allows **separation** of multicomponent mixtures, identification of components and their **quantification**.

Classification of chromatography techniques

By aggregation state:

gas chromatography
- gas-liquid chromatography
- gas-solid phase chromatography

liquid chromatography
- liquid-liquid chromatography
- liquid-solid phase chromatography
- liquid-gel chromatography

Classification of chromatography techniques

By interaction mechanism between sorbent and analyte:

Distribution chromatography (Распределительная хроматография) separates molecules according to their solubility in the stationary phase (gas-liquid chromatography) or according to their solubility in both mobile and stationary phases. Стационарная фаза химически связана с основой/субстратом. Подвижная фаза – жидкость или газ. Разделение за счет различий в полярности веществ. Частный случай - обращённо-фазная хроматография.

Size-exclusion chromatography (гель-фильтрация или эксклюзионная хроматография) separates molecules according to their size (or more accurately according to their hydrodynamic diameter or hydrodynamic volume). Вещества разделяются по размеру за счёт их разной способности проникать в поры неподвижной фазы. Стационарная фаза инертна и с разделяемыми веществами не взаимодействует.

Classification of chromatography techniques

By interaction mechanism between sorbent and analyte:

Ion exchange chromatography (Ионообменная хроматография, разновидность ионной хроматографии**)** uses an ion exchange mechanism to separate analytes based on their respective charges. Разделяет практически любые заряженные молекулы, включая белки, нуклеотиды и аминокислоты. Часто это первый этап очистки белков.

Adsorbtion chromatography (Адсорбционная хроматография) разделяет соединения за счёт их различной способности сорбироваться (физисорбция или хемисорбция) и десорбироваться на поверхности адсорбента с развитой поверхностью, например, силикагеля.

Affinity chromatography (Аффинная хроматография) is based on selective non-covalent interaction between an analyte and specific molecules. Например, высокоспецифичные взаимодействия антигена и антитела, фермента и субстрата, рецептора и лиганда, белка и нуклеиновой кислоты.

Classification of chromatography techniques

По типу субстрата/основы:

Column chromatography - the stationary bed is within a tube
The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole volume of the tube (packed column)
or be concentrated on or along the inside tube wall leaving a free channel for the mobile phase along the axis of the tube (open tubular column).

Planar chromatography is a separation technique in which the stationary phase is present as a plane or applied on a plane.
The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (**paper chromatography**)
or a layer of solid particles spread on a support such as a glass plate (**thin-layer chromatography**).

Glossary for liquid chromatography

Term	Definition
Mobile phase or carrier	solvent moving through the column/plane substrate
Stationary phase or adsorbent	substance that stays fixed inside the column/on the surface of plane substrate
Eluent	fluid entering the column/plane substrate
Eluate	fluid exiting the column (that is collected in flasks)
Elution	the process of washing out a compound through a column (plane substrate) using a suitable solvent
Analyte	mixture whose individual components have to be separated and analyzed

Classification of chromatography techniques

<u>Special techniques</u>:

Reversed-phase chromatography is any liquid chromatography procedure in which the mobile phase is significantly more polar than the stationary phase. (in normal-phase liquid chromatography, the mobile phase is significantly less polar than the stationary phase).

Two-dimensional chromatography - when one chromatographic mode is insufficient to separate all compounds

In planar chromatography - The sample is spotted at one corner of a square plate, developed, air-dried, then rotated by 90° and usually redeveloped in a second solvent system.

In column chromatography - a series of unresolved peaks eluted from the first column is directed onto a second column with different physico-chemical properties.

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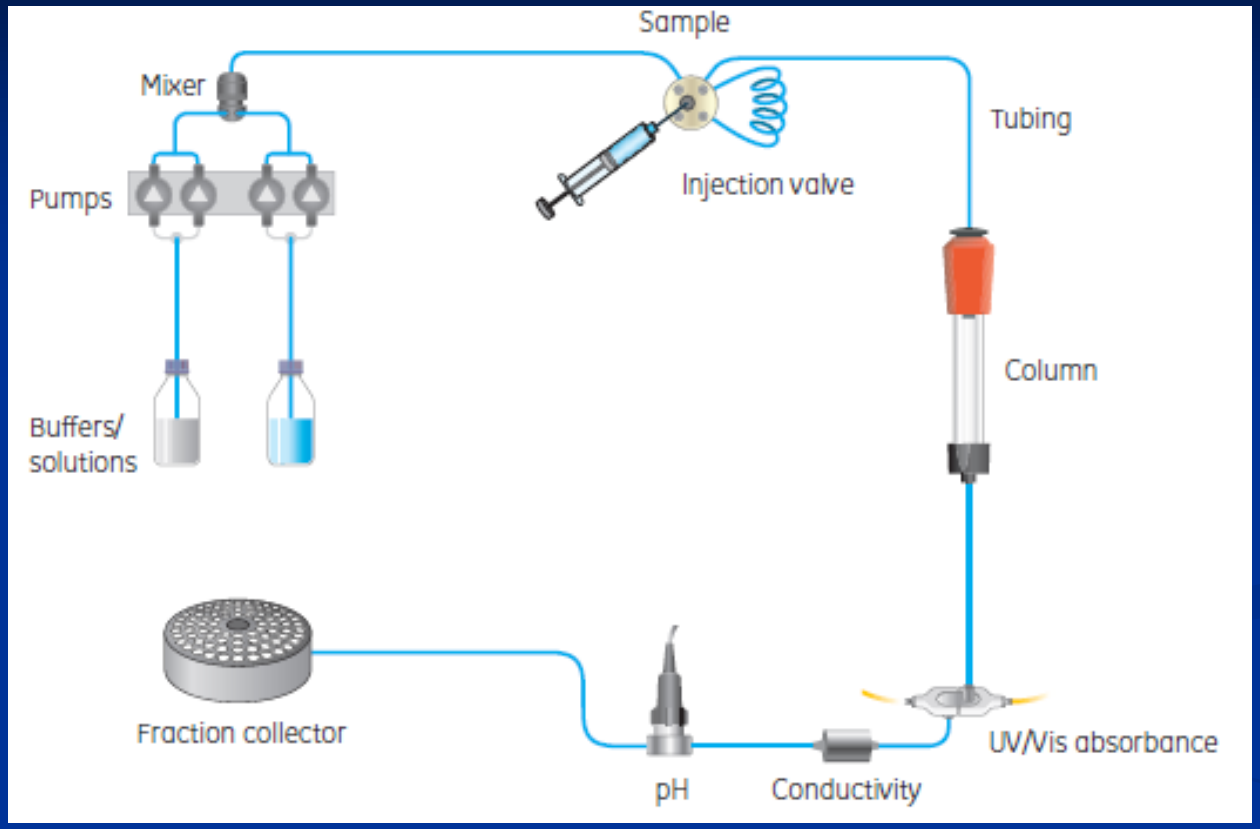
Liquid column chromatography



Typical flow path for a chromatography system.

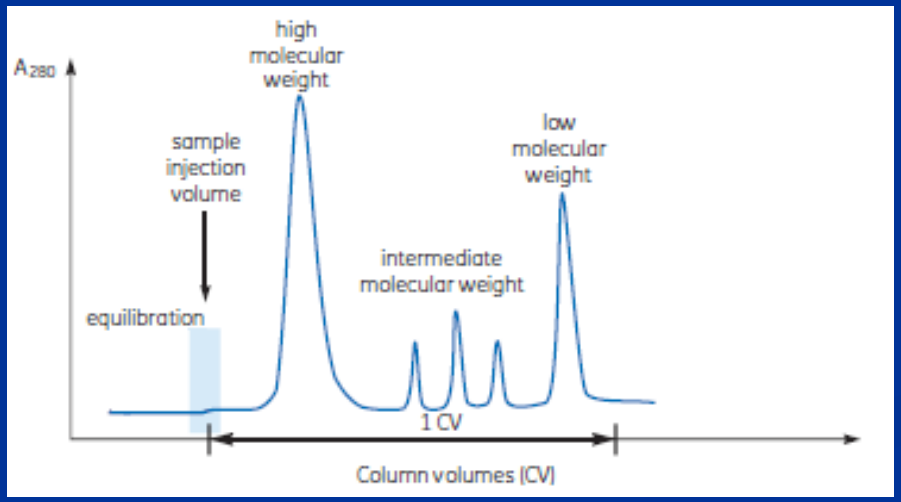
Size exclusion chromatography (SEC)/Гель-фильтрация или эксклюзионная хроматография

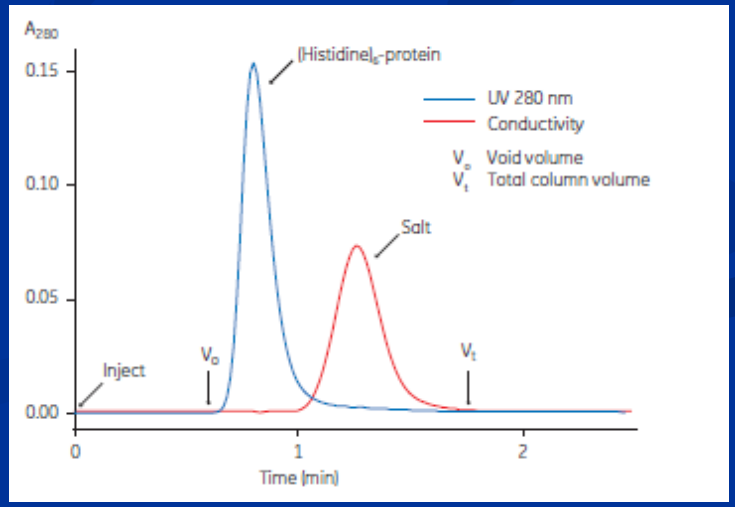
SEC allows separation of substances with differences in molecular size, under mild conditions.

The technique can be used for protein purification or for group separation where the sample is separated in two major groups.

Group separation is mainly used for desalting and buffer exchange of samples.

Sample components are eluted isocratically (single buffer, no gradient). Separation can be performed within a broad pH, ionic strength, and temperature range.
The medium accepts a variety of additives: cofactor, protein stabilizers, detergents, urea, and guanidine hydrochloride.



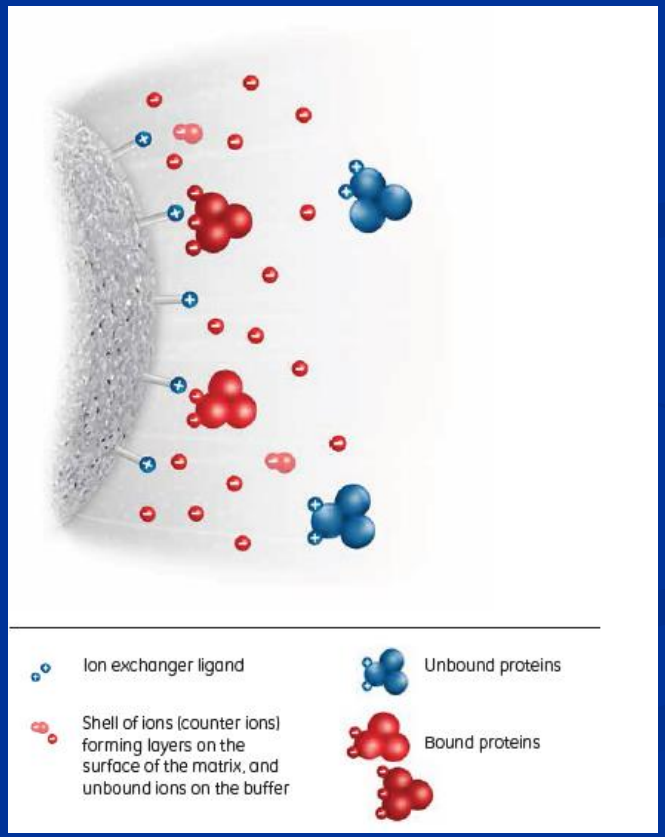



Large molecule that cannot enter the pores of chromatography beads
Salt or other low-molecular-weight substances that can use the entire pore volume of the beads
Target protein that can use a fraction of the pore volume of the beads

Ion exchange chromatography (IEX)

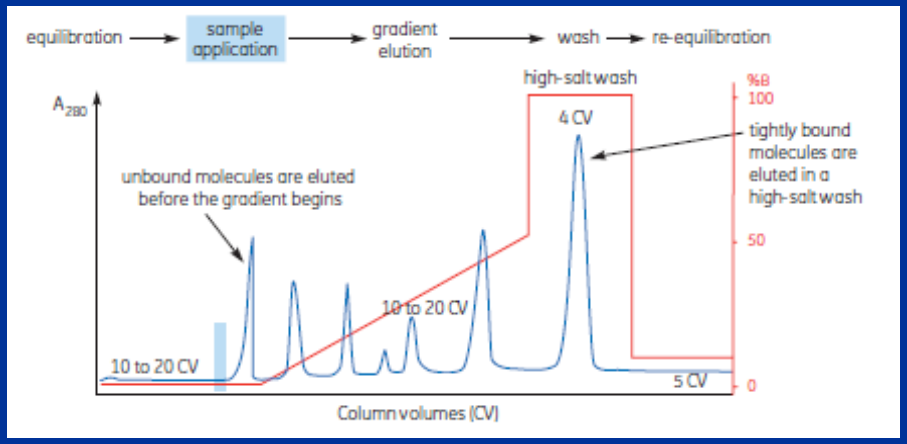
IEX separates proteins with differences in surface charge to give high-resolution separation with high sample-loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged medium.

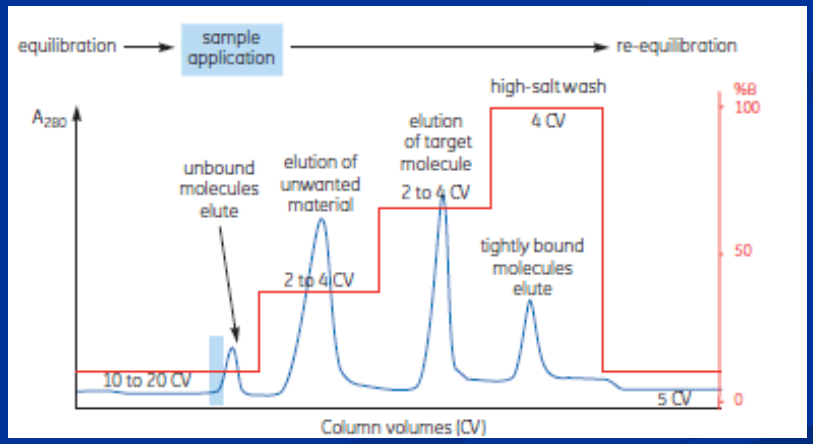
Target proteins are concentrated during binding and collected in a purified, concentrated form. IEX media can be used at high flow rates because binding kinetics for IEX are fast, and rigid chromatography particles can be used.



Ion exchange chromatography (IEX)

Proteins bind as they are loaded onto a column at low ionic strength. The conditions are then altered so that bound substances are desorbed differentially. Elution is usually performed by increasing salt concentration or changing pH in a gradient, or stepwise. The most common salt is NaCl, but other salts can also be used to modulate separation, for example, salts containing K^+, Ca^{2+}, Mg^{2+}, $CH3COO^-$, SO_4^{2-}, I^-, or Br^- ions. The buffer used can also impact separation. Ions that bind to the protein might change its behavior in IEX.



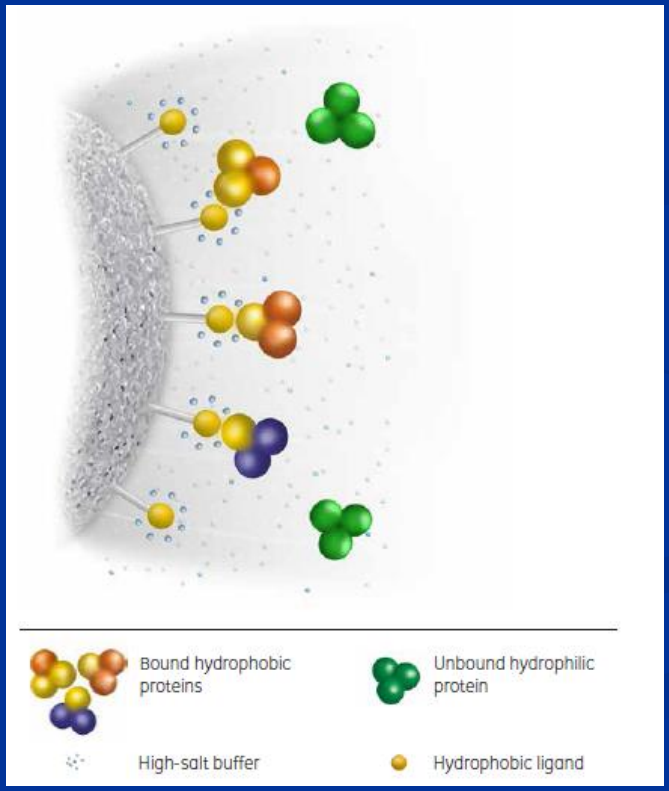


Hydrophobic interaction chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The method is well-suited for the capture or intermediate step in a purification protocol.

Separation is based on the reversible interaction between a protein and the hydrophobic surface of a chromatography medium.

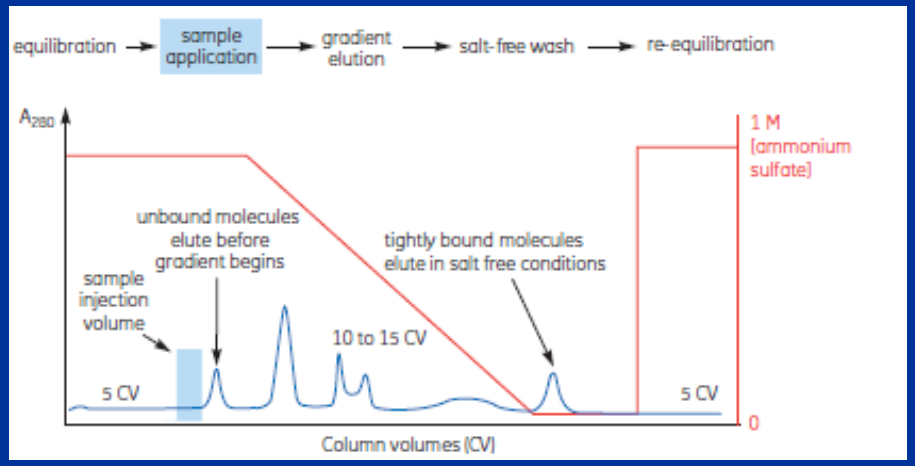
This interaction is enhanced by high ionic-strength buffer.

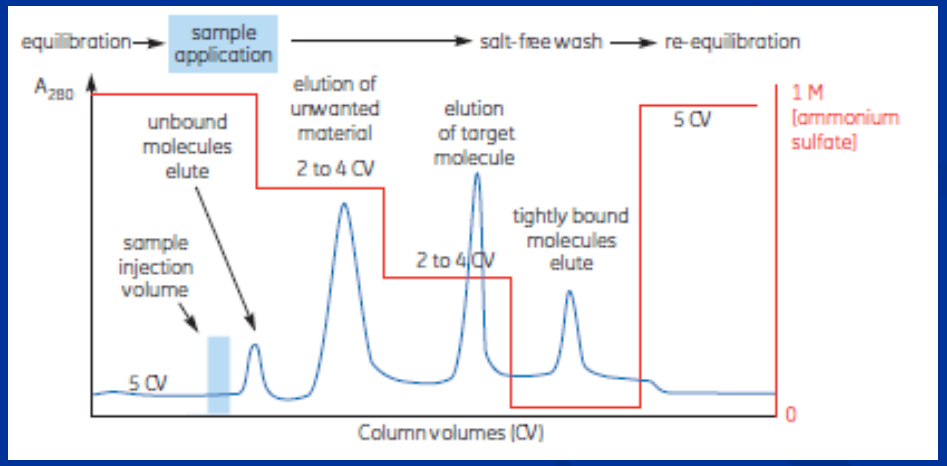


Hydrophobic interaction chromatography (HIC)

Many sample components bind a HIC column in high ionic-strength solution, typically 1 to 2 M ammonium sulfate or 3 M NaCl. Conditions are then altered so that the bound substances are eluted differentially.

Elution is usually performed by decreasing the salt concentration.





Reversed phase chromatography (RPC)

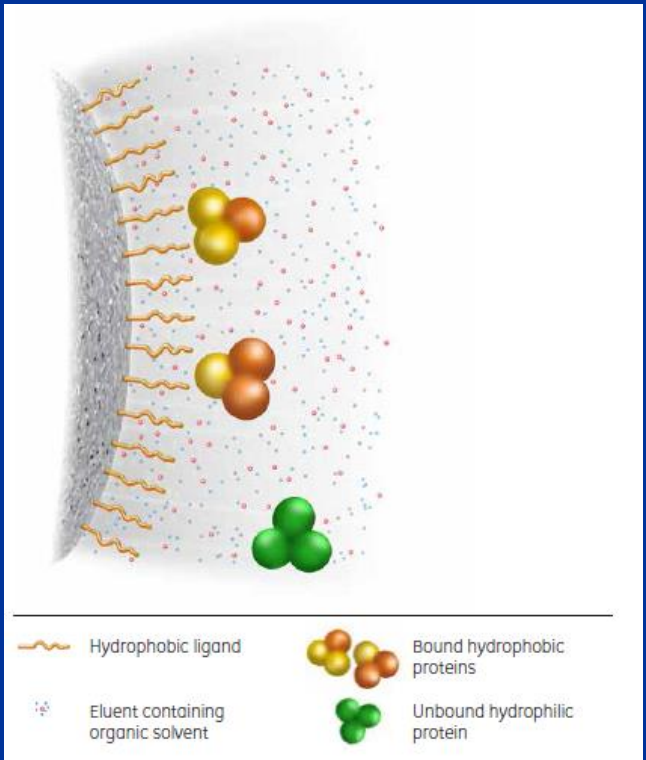
RPC separates proteins and peptides on the basis of hydrophobicity.

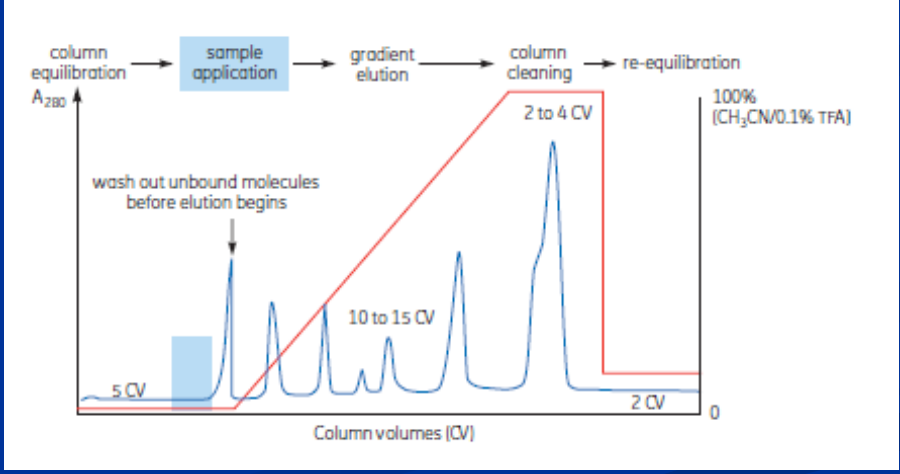
RPC is a high-resolution method, requiring the use of organic solvents.

RPC is excellent, particularly for small target proteins that are less commonly denatured by organic solvents.

Sample components bind as they are loaded onto the column. Conditions are then altered so that the bound substances are eluted differentially.

Due to the nature of the reversed-phase matrices, binding is usually very strong.





Chromatofocusing (CF)

CF separates proteins according to differences in their isoelectric point (pI). It is a powerful method and can resolve very small differences in pI (down to 0.02 pH units) and thus separate very similar proteins. However, the capacity of the method is low; CF should preferably be used for partially pure samples.

A pH gradient is generated on the column as buffer and medium interact. The medium is a weak anion exchanger, and the buffer is a polyampholyte elution buffer containing a mixture of polymeric buffering species that buffers a broad pH range.

Proteins with different pI values migrate at different rates down the column as the pH gradient develops, continually binding and dissociating while being focused into narrow bands and finally eluted.

CF is useful for high-resolution analytical separations and in preparative purification if IEX or other methods do not give a satisfactory purification.

Affinity chromatography

Affinity chromatography is a method of separating biochemical mixture based on a highly specific interaction between antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid.

The high selectivity of affinity chromatography is caused by allowing the desired molecule to interact with the stationary phase and be bound within the column in order to be separated from the undesired material which will not interact and elute first.

The desired molecules are let go in the presence of the eluting solvent (of higher salt concentration). This process creates a competitive interaction with the immobilized stationary molecules and the desired molecules are released in highly purified form.

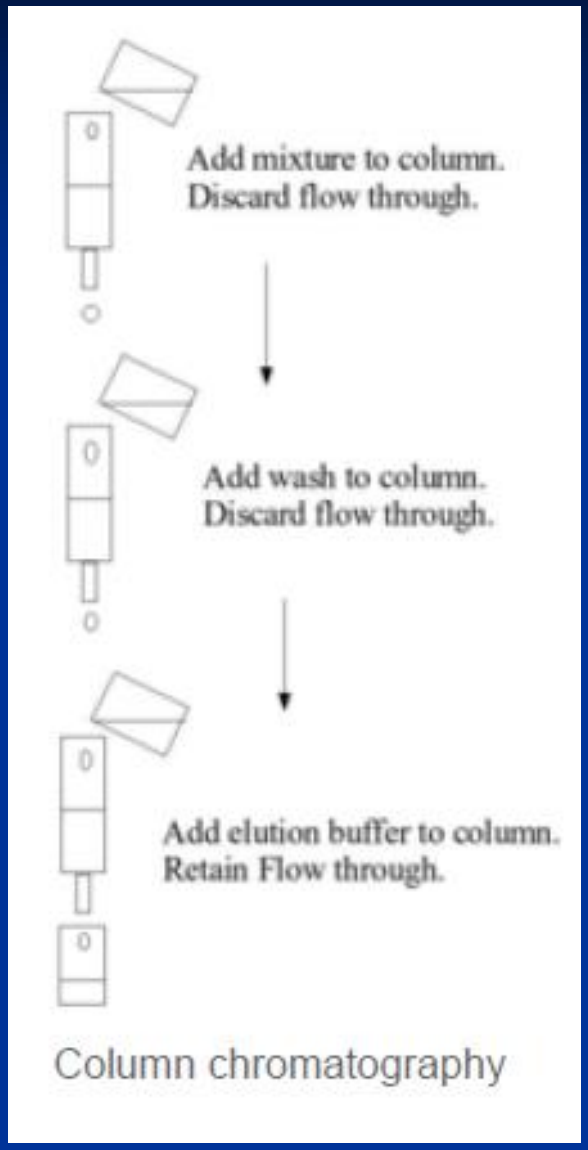
Molecules bound to affinity sorbent can be eluted by changing salt concentrations, pH, pI, charge and ionic strength directly or through a gradient to resolve the particles of interest.

Affinity chromatography is an excellent choice for the first step in purifying a protein or nucleic acid from a crude mixture.

Typical Biological Interaction Used in Affinity Chromatography

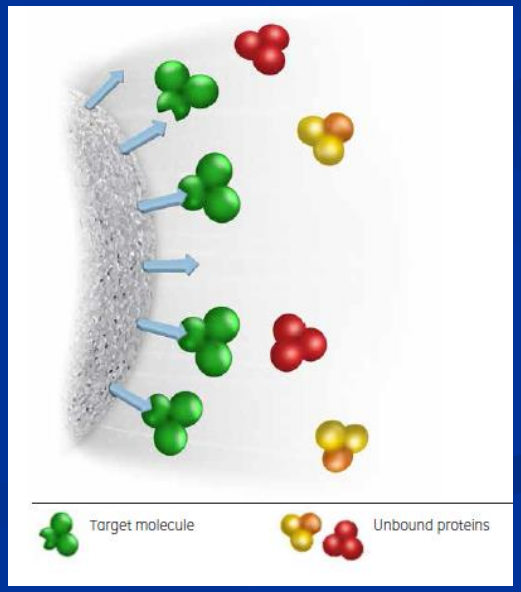
	Types of Ligand	Target Molecule
	Enzymes	Substrate analogue
	Antibody	Antigen
	Lectin	Polysaccharide
	Nucleic acid	Complementary base sequence
	Hormone	Receptor
	Avidin	Biotin
	Calmodulin	Calmodulin binding molecule
	Poly-A	RNA contating poly (U) sequence
	Glutathione	GST fusion protein
	Proteins A and G	Immunoglobulins
	Metal ions	Poly fusion protein

Batch and column setups



Batch treatment:

- Add the initial mixture to the solid phase in a vessel and mix,
- Separate the solid phase, remove the liquid phase by centrifugation,
- Wash and centrifuge,
- Add the elution buffer, re-centrifuge and removing the elute.



It works usually at a normal pressure

Immobilized metal ion affinity chromatography is based on the specific coordinate covalent bond of some molecules or molecular groups to metals.

Immobilized metal ions, such as cobalt, nickel, copper bind histidine containing proteins or peptides.
Elution of bound proteins is performed using a gradient of imidazole from 100 to 500 mM or by step elution. Gradient elution often gives two peaks, an early peak corresponding to naturally binding proteins in the lysate and a later peak corresponding to the histidine-tagged protein, which has higher affinity for the medium.

Recombinant DNA technology is used His-tag sequence into the relevant gene.

Immobilized metal ions, such as iron, zinc or gallium – bind phosphorylated proteins or peptides.
Methods used to elute the protein of interest include changing the pH, or adding a competitive molecule

A chromatography column containing nickel-agarose beads used for purification of proteins with histidine tags

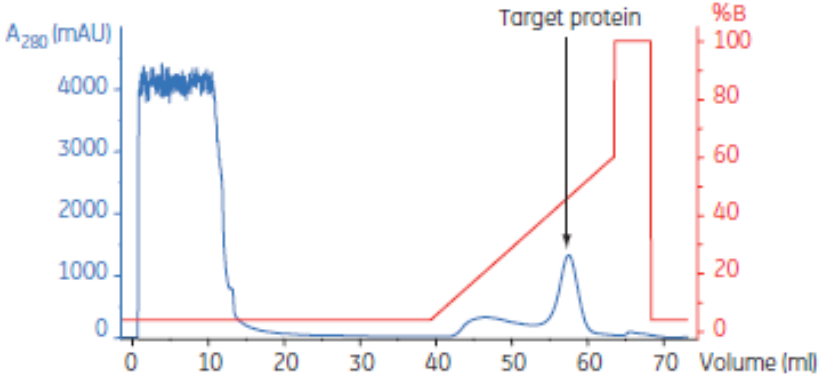
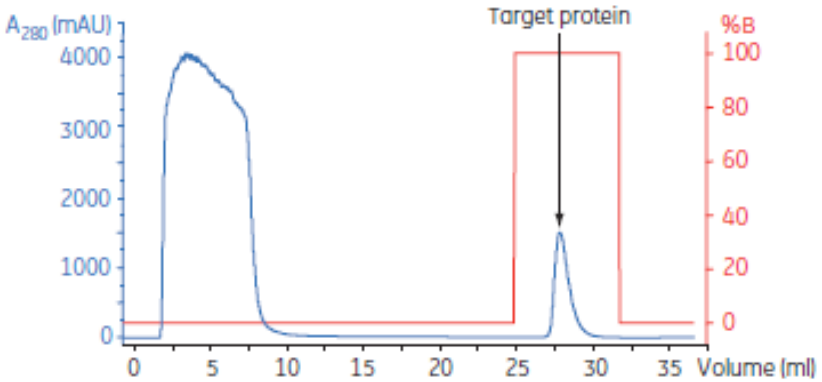


Fig A7.3. Typical IMAC purification with gradient elution.

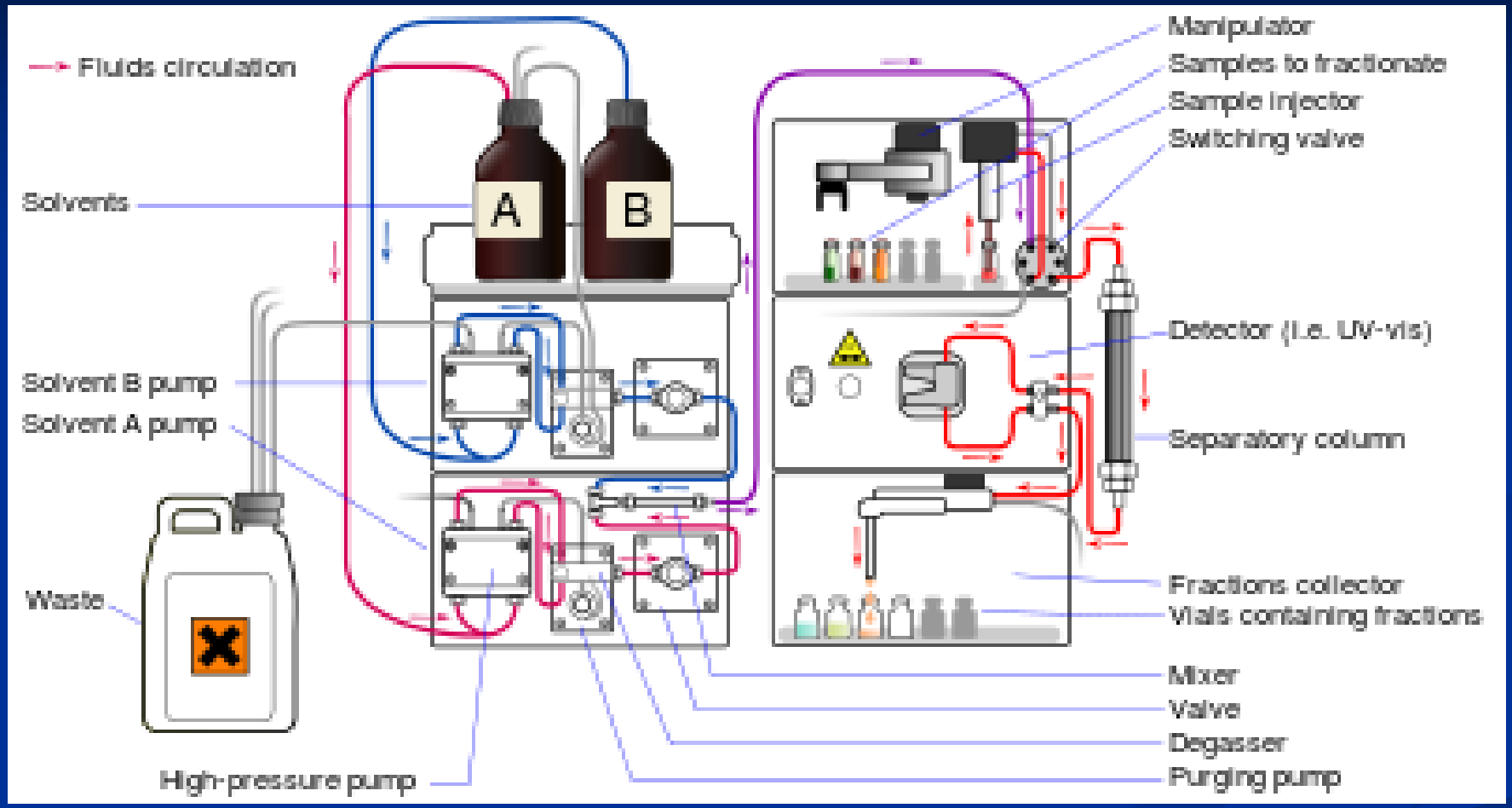


Gradient vs. one step elution

High-performance liquid chromatography (HPLC; formerly referred to as **high-pressure liquid chromatography**) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture.

It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

Many different types of columns are available, filled with adsorbents varying in particle size, and in the nature of their surface ("surface chemistry"). The use of smaller particle size packing materials requires the use of higher operational pressure and typically improves chromatographic resolution (the degree of peak separation between consecutive analytes emerging from the column). Sorbent particles may be hydrophobic or polar in nature.


Fluids circulation
Solvents
A
B
Solvent B pump
Solvent A pump
Waste
High-pressure pump
Manipulator
Samples to fractionate
Sample injector
Switching valve
Detector (i.e. UV-vis)
Separatory column
Fractions collector
Vials containing fractions
Mixer
Valve
Degasser
Purging pump