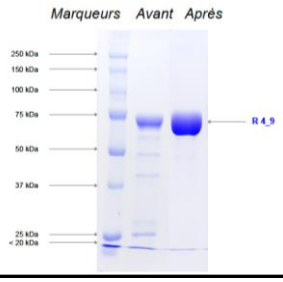
Separation/Purification of Biomacromolecules

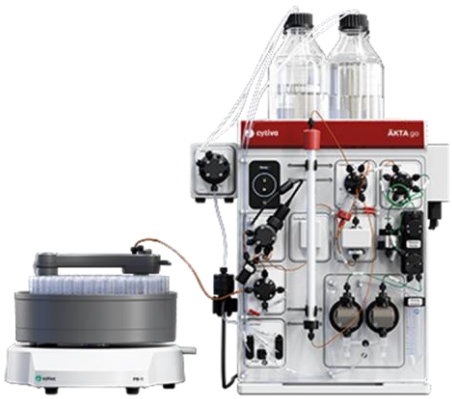
Master I Applied Microbiology

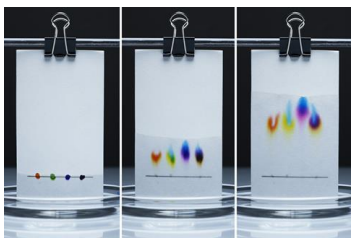
University of Jijel

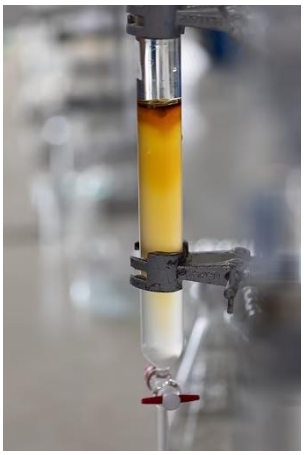
Pr H Ouled Haddar



Chapter 5. Chromatography techniques







Introduction

Chromatography is a laboratory technique for the separation of a mixture into its components.

The mixture is dissolved in a fluid solvent (gas or liquid) called the mobile phase, which carries it through a system (a column, a capillary tube, a plate, or a sheet) on which a material called the stationary phase is fixed.

Because the different constituents of the mixture tend to have different affinities for the stationary phase and are retained for different lengths of time depending on their interactions with its surface sites, the constituents travel at different apparent velocities in the mobile fluid, causing them to separate.

The separation is based on the differential partitioning between the mobile and the stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation

Partition coefficient

A partition coefficient or distribution coefficient is the ratio of concentrations of a compound in a mixture of two immiscible solvents at equilibrium. This ratio is therefore a comparison of the solubilities of the solute in these two liquids.

K_D= concentration of solute in the stationnary phase / concentration of solute in the mobile phase

Chromatography may be preparative or analytical.

The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification.

Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture.

The two types are not mutually exclusive.

Chromatography, is derived from Greek (χρῶμα) chroma, which means "color", and (γράφειν) graphein, which means "to write".

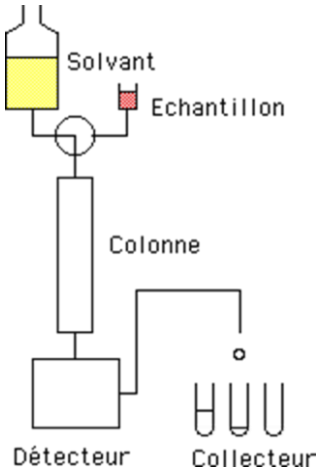
The combination of these two terms was directly inherited from the invention of the technique first used to separate pigments,

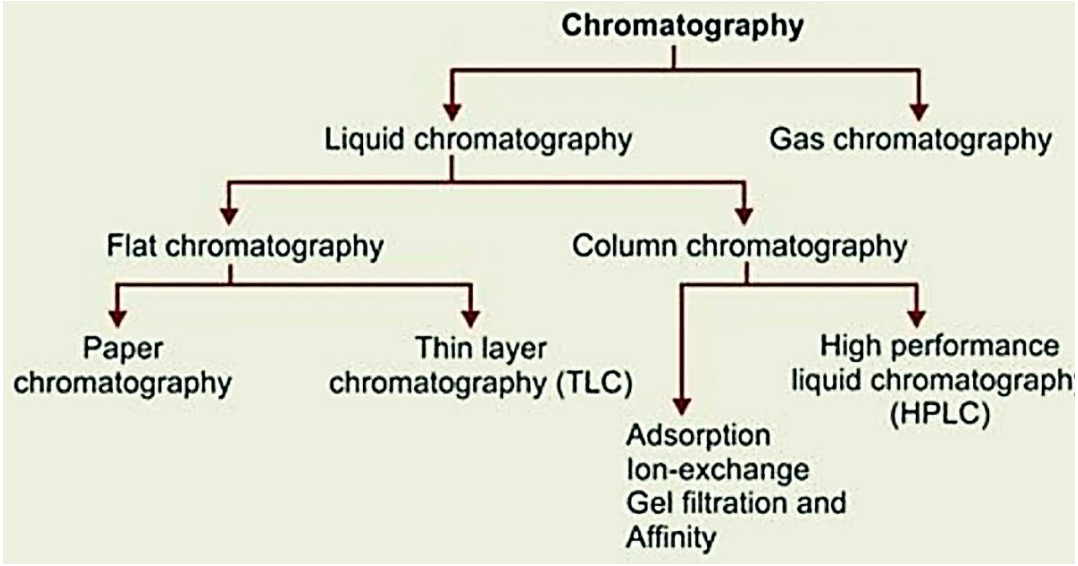
Chromatography was first devised at the University of Kazan by the Italian-born Russian scientist Mikhail Tsvet in 1900. He developed the technique and coined the term chromatography in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls.

Since these components separate in bands of different colors (green, orange, and yellow, respectively) they directly inspired the name of the technique.

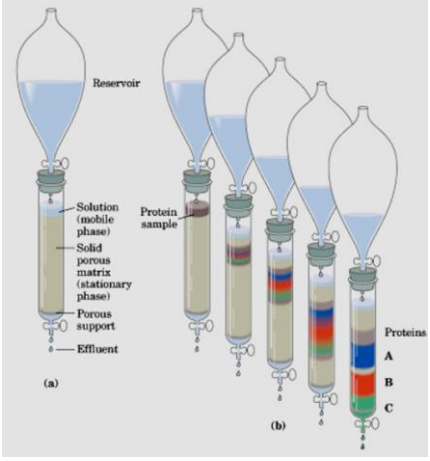
Researchers found that the main principles of Tsvet's chromatography could be applied in many different ways, resulting in the different varieties of chromatography.

Advances are continually improving the technical performance of chromatography, allowing the separation of increasingly similar molecules..

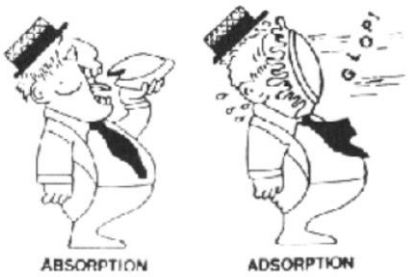




Different types of chromatography



1. Adsorption chromatography



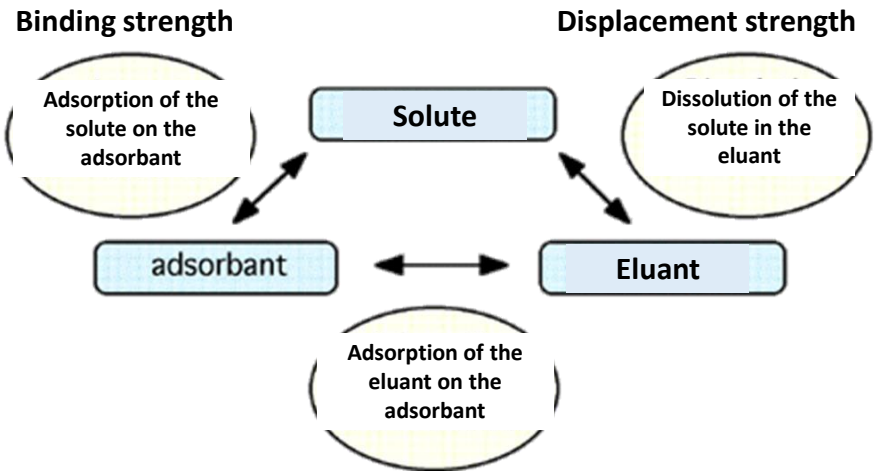
Adsorption chromatography is the oldest types of chromatography technique. It makes use of a mobile phase which is either in liquid or gaseous form. The mobile phase is adsorbed onto the surface of a stationary solid phase

In this technique chemicals are retained based on their adsorption and desorption at the surface of the support, which also acts as the stationary phase.

Retention in this method is based on the competition of the analyte with molecules of the mobile phase as both bind to the surface of the support.

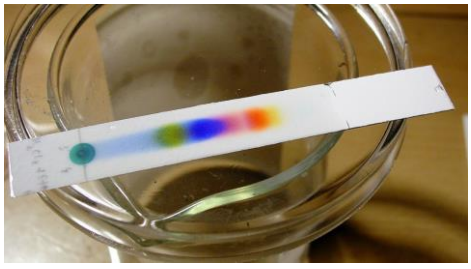
The degree of a chemical's retention in adsorption chromatography will depend on:

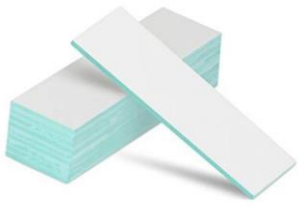
- the binding strength of this chemical to the support,
- the surface area of the support,
- the amount of mobile phase displaced from the support by the chemical, and
- the binding strength of the mobile phase to the support



Some commonly used adsorbents

Silica gel, hydrated silica gel, microcrystalline cellulose, alumina, modified silica gel, calcium carbonate, charcoal….





Common solvents

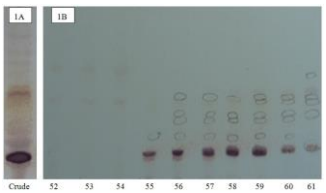
Solvent	Eluotropic value (ε^0) Al_2O_3
Alkanes and Cyclohexane	<0.1
Toluene	0.29
Dichloromethane	0.42
Tetrahydrofuran	0.45
Acetone	0.56
Ethyl acetate	0.58
Acetonitrile	0.65
Isopropanol	0.82
Ethanol	0.88
Methanol	0.95
Water	>1

Example of adsorption chromatography: Thin layer chromatography

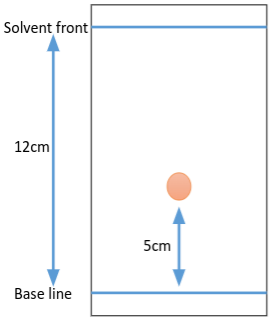
Thin Layer Chromatography (TLC) is a chromatographic technique used for the separation and analysis of mixtures.

In TLC, a stationary phase, typically a thin layer of adsorbent material (such as silica gel or alumina), is coated onto a flat support, and a liquid mobile phase is used to move the sample mixture across the stationary phase.

As the components of the mixture interact differently with the stationary phase, they separate into distinct bands, allowing for qualitative and quantitative analysis of the individual substances.



How to calculate the R*f* value

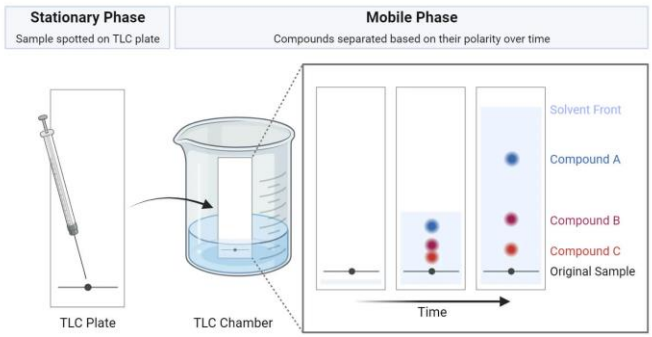


$$Rf = \frac{distance\ moved\ by\ substance}{distance\ moved\ by\ solvent}$$

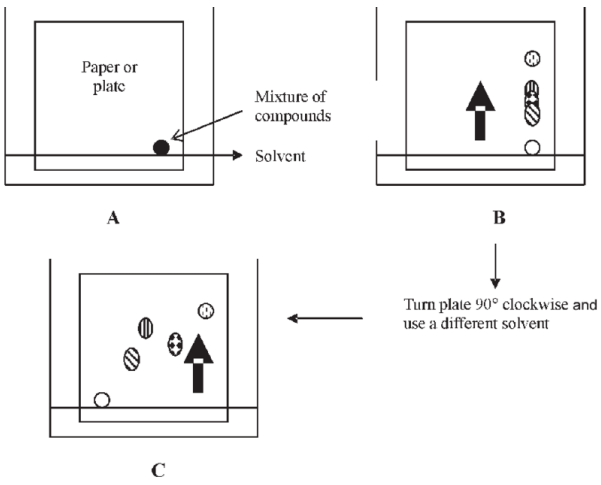
$$Rf = \frac{5cm}{12cm}$$

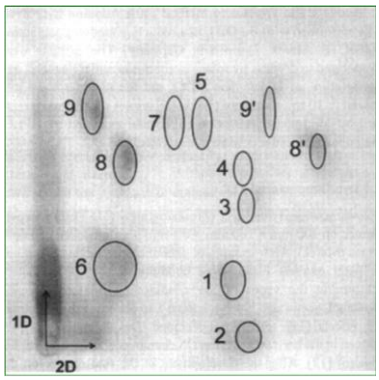
$$Rf = 0.42$$

Thin Layer Chromatography



Bidimensional thin layer chromatography





Thin Layer Chromatography (TLC) finds various applications in different fields. Some of the most important applications include:

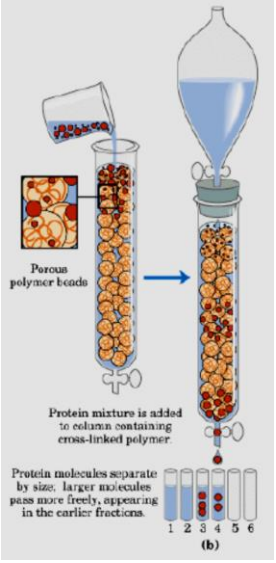
- Pharmaceutical Analysis: analysis of drug purity, identification of components, and monitoring reactions during drug synthesis.
- Forensic Science: analysis of substances such as drugs, toxins, and biological fluids, aiding in criminal investigations.
- Environmental Analysis: analysis of environmental samples to identify and quantify pollutants, pesticides, and other contaminants in air, water, and soil.
- Food and Beverage Industry: quality control and monitoring of food and beverage products, helping to detect and quantify additives, contaminants, or adulterants.

- Botanical Analysis: identification and characterization of plant constituents, such as alkaloids, flavonoids, and essential oils, in botanical and herbal products.
- Clinical Chemistry: In clinical laboratories, TLC is applied for the separation and analysis of complex biological samples, such as blood or urine, assisting in disease diagnosis and monitoring.
- Chemical Synthesis: in organic chemistry laboratories to monitor the progress of reactions, assess the purity of synthesized compounds, and isolate individual components from reaction mixtures.
- Biochemistry: separating and analyzing biomolecules, such as amino acids, nucleic acids, and lipids, in biochemical research.
- Quality Control in Industry: Various industries, including petrochemical and cosmetic industries, use TLC for quality control purposes to assess the composition and purity of raw materials and finished products.

2. Gel filtration chromatography

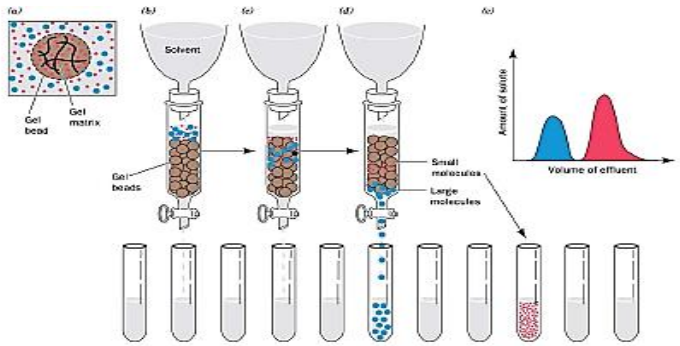
Size Exclusion Chromatography (SEC), also known as gel filtration chromatography or gel permeation chromatography, is a chromatographic technique used for the separation of macromolecules based on their size.

In SEC, a porous gel matrix is used as the stationary phase.
Larger molecules are excluded from the pores and pass through the column more quickly, while smaller molecules enter the pores and experience a longer retention time.
This method is particularly useful for separating and analyzing polymers, proteins, and other biomolecules based on their molecular size in solution.



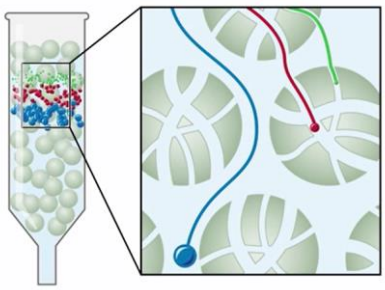
The stationary phase consists of polysaccharide beads ("Sephadex™" or "Sepharose™" type) swollen in the solvent used for elution:

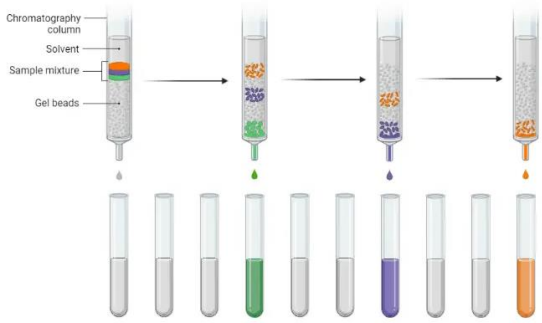
- the volume of the beads is very finely calibrated
- thus molecules whose volume is higher than that of the beads cannot penetrate and are eluted quickly;
- on the other hand, molecules whose volume is less than that of the beads penetrate there and undergo friction which delays them,



GF steps:

1. Deposition of a mixture of molecules (large and small) on a column filled with Sephadex gel.
2. Small molecules can penetrate Sephadex beads because their diameter is smaller than that of the gel pores. Large molecules cannot due to their large size; they are therefore excluded from the gel (hence the name exclusion chromatography).
3. Large molecules have a shorter path to travel to reach the bottom of the column; they are therefore eluted first.
4. Small molecules are eluted next because they have a greater distance to travel to reach the bottom of the column





There are 2 types of gels:

- **<u>Hydrated gels</u>**

they become porous after swelling in water,

ex: Sephadex (dextrans), fixes up to 10 times their water mass (water gain = 10g of water/g of dry support)

Insoluble in water and saline solutions

Stable in alkaline or weakly acidic solutions

Completely hydrolyzed by strong acids

They must be protected from bacterial contamination (sodium azide, etc.)

• **Permanent gels**

characterized by a reticulated structure (permanent porosity)

Organic gels:

formed from copolymers, porosity is produced during copolymerization

High chemical and mechanical stability, withstands high pressure

Mineral gels:

these are silica gels which have a lacunar structure with large pores

Do not swell

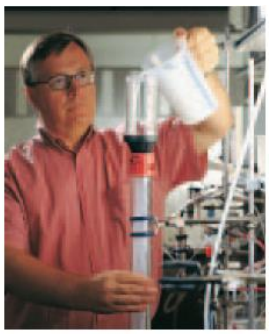
A slight adsorption effect exists which adds to molecular sieving

Characteristics of a gel

- Water gain
- The diameter of the pores (A° or in nm)
- The gel exclusion limit (in g or dalton) PM markers
- Specific surface area (m^2/g)
- Granule diameter (mm or mesh)

Examples of gels

Gel	Bead size (µm)	Fractionation range (Globular proteins)
Sephacryl S-100 HR	25-75	1 000-10 0000
Sephacryl S-500 HR	25-75	40 000-20 000 000
Superdex 75	11-15	3 000-70 000
Superdex 200	11-15	10 000- 60 0000
Sephadex G10	40-120	-700
Sephadex G50 (moyen)	50-150	1 500- 30 000
Sephadex G200	40-120	5 000- 60 0000
Sepharose 6B	45-165	10 000- 4 000 000
Sepharose 4B	60-200	70 000- 40 000 000



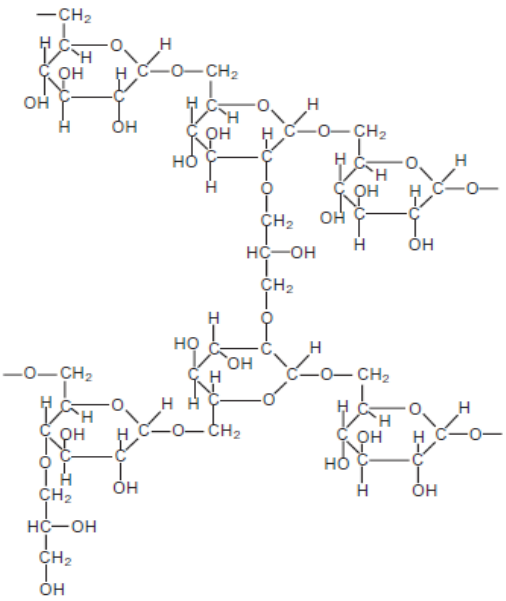


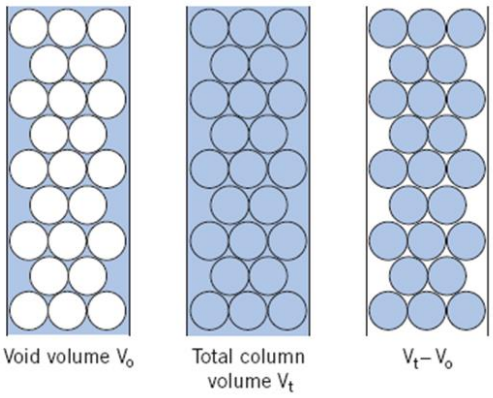
Fig. 41. Partial structure of Sephadex.

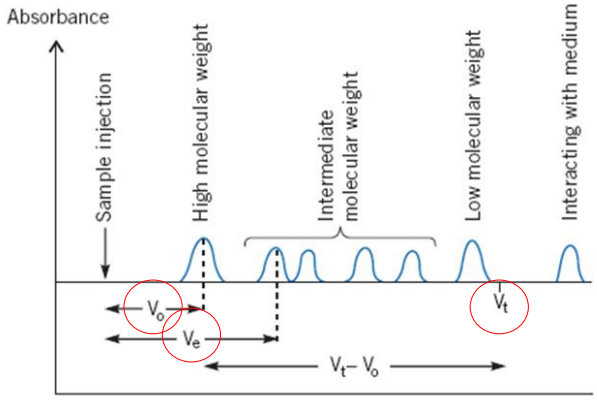
Applications

- This technique is widely used for the separation or elimination of salts or small molecules in protein solutions: DESALTING
- This technique is also applied to the FRACTIONATION OF MIXTURES OF MACROMOLECULES and to the (approximate) DETERMINATION OF THE MOLAR MASS of proteins.
- In the latter case, it is first necessary to calibrate the column with proteins of known molar mass, draw the standard curve, then carry out a graphical determination.

Determination of the molecular mass of a protein

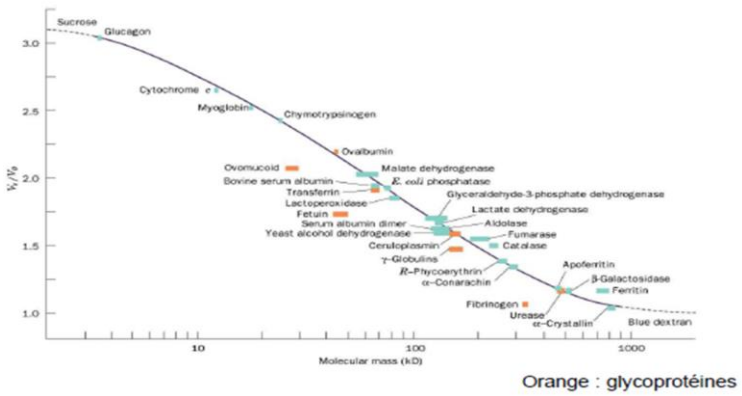
- A mixture of molecules called standards, of known molar masses, is separated by gel filtration chromatography
- each molecule is eluted with an elution volume Ve
- moreover, the exclusion volume of the gel (V_0) is the elution volume of a molecule not delayed by the gel, that is to say a molecule of size greater than that of the beads and which is not diffused. Very often, we use dextran blue, an ose polymer with a molar mass greater than $2{,}10^6$ Da to determine V_0.
- finally, the total volume of the gel (Vt) is the sum of the volume of the beads and the volume external to the beads: it is given by the elution volume of a molecule which diffuses completely in the beads and which is therefore totally delayed.





We determine the partition coefficient

$$K_D = \frac{V_e - V_0}{V_t - V_0}$$



Orange : glycoprotéines

we draw the standard line: log (molar mass) = f (KD) (or molar mass = f (KD) on a semi-logarithmic scale)

the elution volume of a molecule X is determined under the same chromatography conditions as for the standards

we report the KD of the molecule X on the linear part of the standard line and thus we determine its molar mass

Exclusion limit of a gel

If we know the molecular mass of the molecule that we want to separate from a mixture by this technique, we must choose the best suited gel: it is the one whose fractionation range (exclusion limit) is i.e. the range of molar masses for which there is a partial or a total diffusion, includes the molar mass of the molecule to be purified,

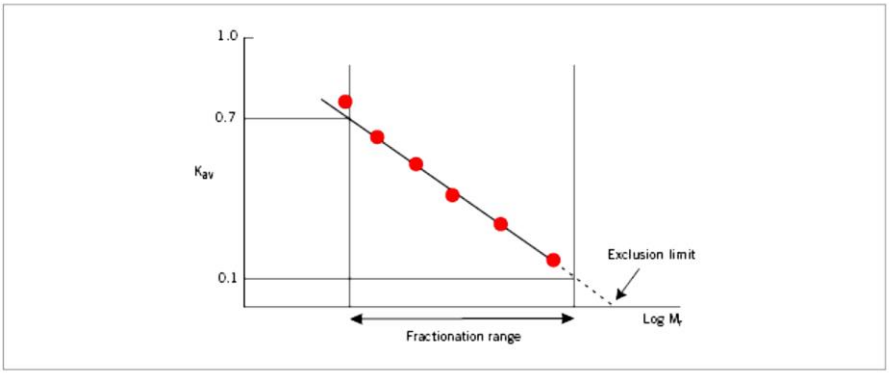
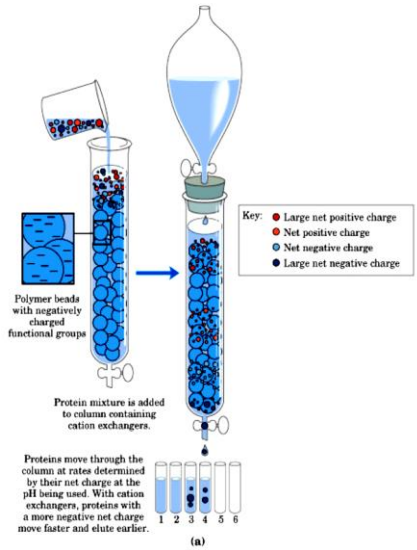


Fig. 10. Defining fractionation range and exclusion limit from a selectivity curve.

3. Ion exchange chromatography

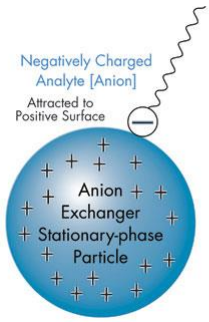
Ion exchange chromatography (IEC) is a separation technique in which charged ions in a sample are selectively retained or eluted based on their interactions with charged functional groups immobilized on a solid support material, typically a resin or gel matrix.

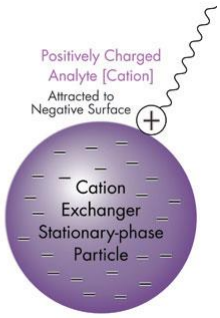


The principle behind ion exchange chromatography relies on the reversible binding of ions to charged sites on the stationary phase material.

The stationary phase typically consists of a resin or gel matrix containing charged functional groups, such as positively charged groups (anion exchange) or negatively charged groups (cation exchange).

These charged groups interact with ions of opposite charge in the sample solution.





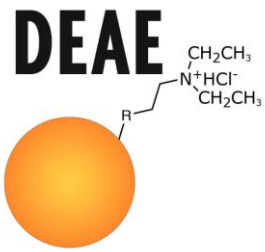
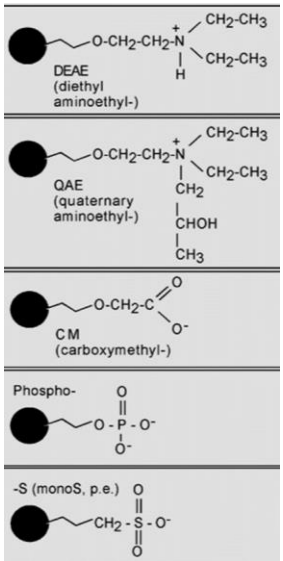


Table 12.5 Examples of Common Ion-Exchange Resins

Type	Functional Group	Examples
strong acid cation exchanger	sulfonic acid	$-SO_3^-$ $-CH_2CH_2SO_3^-$
weak acid cation exchanger	carboxylic acid	$-COO^-$ $-CH_2COO^-$
strong base anion exchanger	quaternary amine	$-CH_2N(CH_3)_3^+$ $-CH_2CH_2N(CH_2CH_3)_3^+$
weak base anion exchanger	amine	$-NH_3^+$ $-CH_2CH_2NH(CH_2CH_3)_2^+$



Firstly, the resin is equilibrated in a buffer with a pH in which the group carried by the ion exchanger is ionized:

- in the case of a base (anion exchanger), the pH should be lower than the pKa of the ionizable group (example: the pKa of the diethylaminoethylammonium group is 9.5);
- in the case of an acid (cation exchanger), the pH should be higher than the pKa of the ionizable group (example: the pKa of the carboxymethyl group is 4).

$$pH = pK_a + \log\frac{[base]}{[acid]}$$

The molecules to be separated are in the same buffer (therefore at the same pH) and depending on their isoelectric point (pI), they carry a net charge:

- negative (the pI of bovine serum albumin is 4.9, this protein is negatively charged at pH 7)
- neutral ;
- positive (the pI of cytochrome c is 10.7, this protein is positively charged at pH 7)

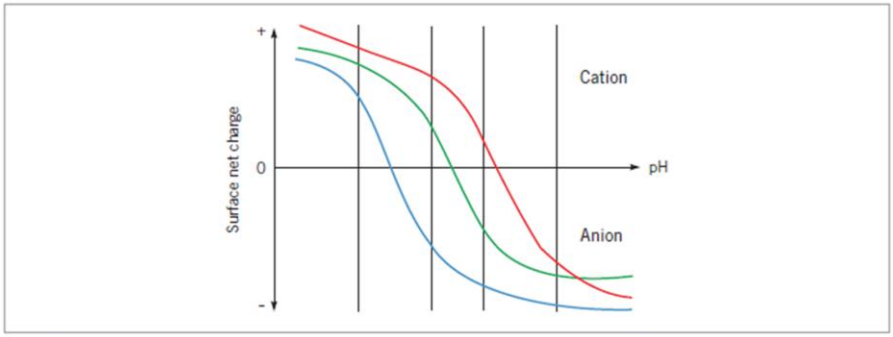


Fig. 2. Theoretical protein titration curves, showing how net surface charge varies with pH.

The sample solution is applied to the IE column, and the ions in the sample are selectively retained to the stationary phase based on their charge and affinity for the functional groups on the resin.

The degree of binding is influenced by factors such as the ionic strength, pH, and composition of the elution buffer.

After loading the sample, the column is washed with a buffer solution to remove unbound or weakly bound components.

The bound ions are then eluted from the column using :

- ✓a gradient of increasing ionic strength,
- ✓pH changes, or
- ✓specific elution conditions tailored to release the bound ions of interest.

- By **modifying the pH** of the mobile phase such that the molecules which are charged are no longer charged (or they carry a charge of the same sign as the ion exchanger); there is then no longer any electrostatic interaction between the molecules and the charged group carried by the resin and the molecules are eluted.
- By **adding a salt** (at an increasing concentration) which necessarily provides an ion with the same charge as the molecules attached to the resin: this ion is called a **"counter-ion"**.

The order of increasing effectiveness of counterions for cation exchange resins is as follows:

- **Monovalent cations : $Li^+ < H^+ < Na^+ < NH_4^+$**
- **Divalents cations : $Cd^{2+} < Mn^{2+} < Mg^{2+} < Zn^{2+} < Cu^{2+} < Ca^{2+}$**
- **moreover, efficiency increases with charge valence: $K^+ < Ca^{2+} < Al^{3+}$**

Elution by a continuous gradient of salt

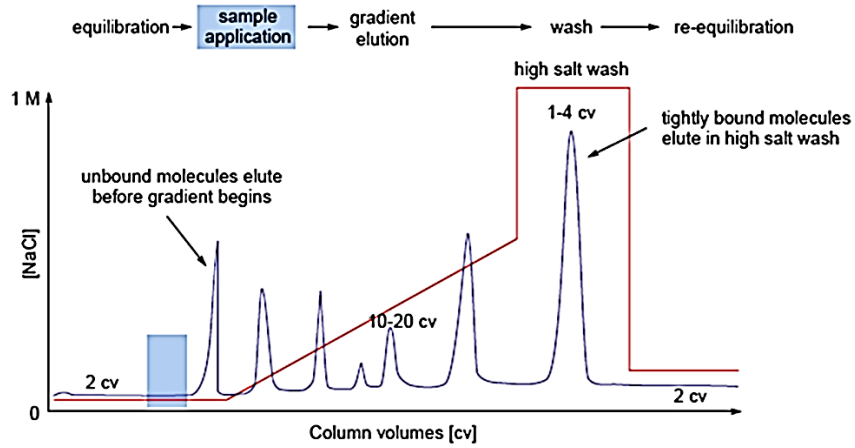
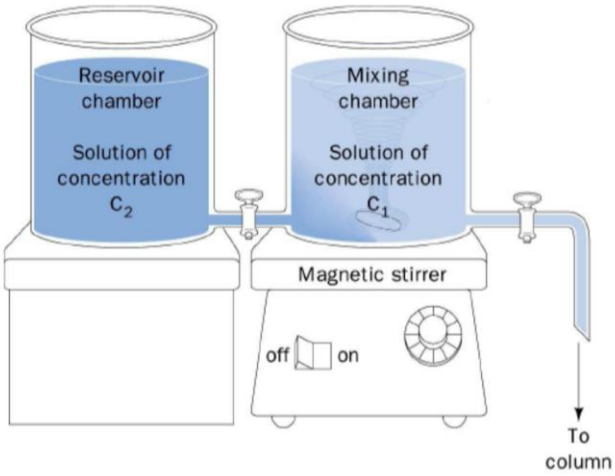


Fig. 31. Typical IEX gradient elution.

The continuous gradient of salt solution improves the resolution of the chromatographic separation



$$c = c_2 - (c_2 - c_1)f$$

f is the residual fraction of the two volumes combined divided by the initial volume =

$$(Vol1+2)/(Vol1+2_{ini})$$

Stepwise elution

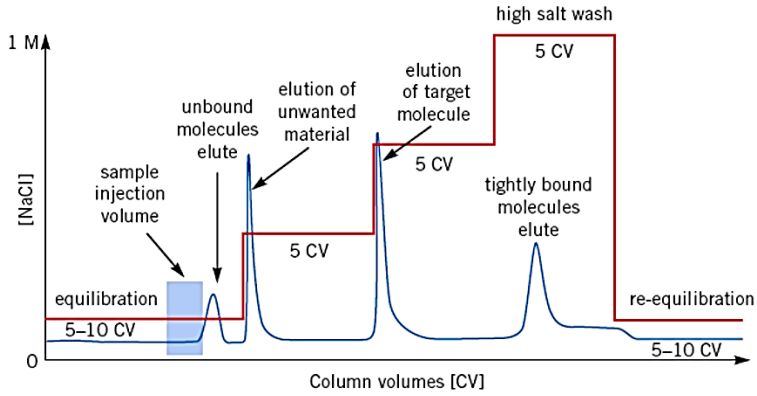


Fig. 10b. Typical IEX separation using step elution (25–30 column volumes).

In chromatographic separation, "resolution" refers to the degree of separation between two adjacent peaks in a chromatogram.
It measures the ability of the method to distinguish between different analytes or components in a mixture.

A higher resolution indicates better separation, enabling more accurate identification and quantification of individual substances.

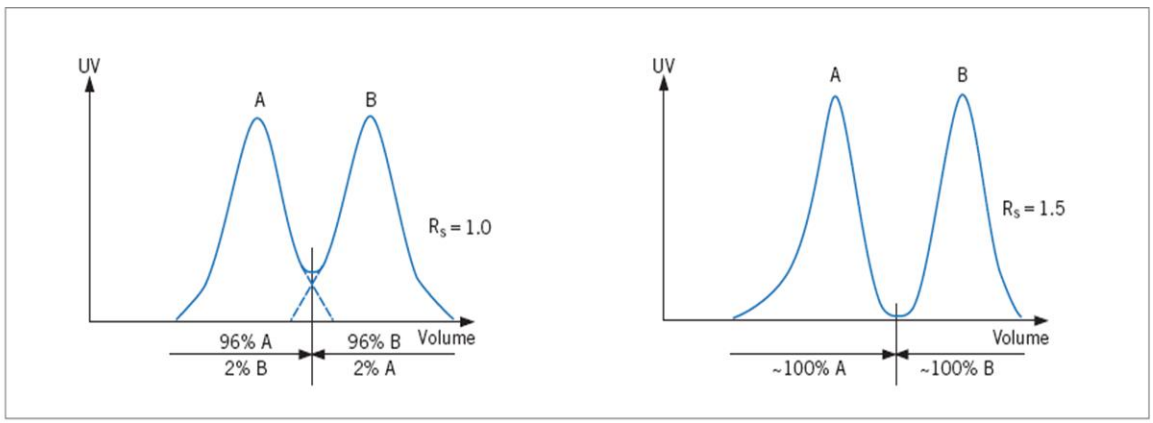
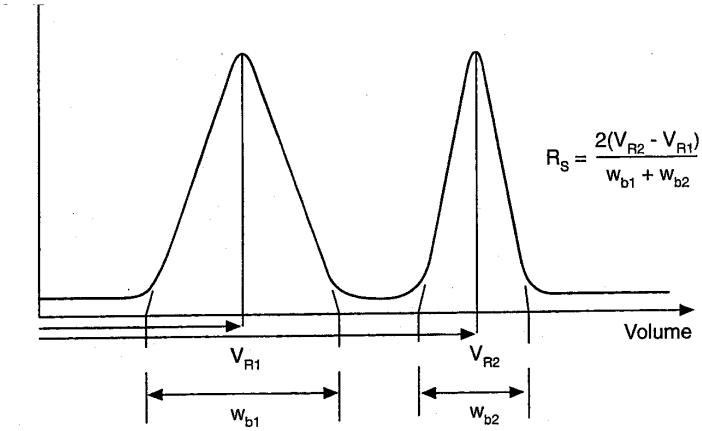


Fig. 5. Separation results with different resolutions.

Resolution

Resolution (R_s) is defined as the distance between peak maxima compared with the average base width of the two peaks.

R_s = 2 x (distance between peaks)/(average peak width)
= 2 (VR2 - VR1) / (wb1 + wb2)



Applications:

Ion exchange chromatography is used to separate ionizable molecules, regrdless of their size: mineral ions, amino acids, peptides, proteins, nucleotides, nucleic acids, ionized carbohydrates and ionized lipids.

Chromatofocusing

Chromatofocusing is a chromatographic technique used for separating and purifying proteins based on their isoelectric points (pI).

It involves the use of a column with a pH gradient, where proteins are initially adsorbed onto the column matrix and then eluted based on their pI values as the pH gradient is applied.

This technique allows for the separation of proteins with small differences in pI values.

A chromatographic column is filled with a matrix that has a pH gradient.
At one end of the column, the pH is lower, and it gradually increases towards the other end.

Proteins in the sample mixture are loaded onto the column under conditions where their charges are reversible, typically at a pH above or below their respective pI values.

As the proteins move through the column, they encounter regions of pH where they become increasingly charged or neutral depending on their pI values.

Proteins with higher pI values will bind strongly to the column matrix in the lower pH regions, while those with lower pI values will elute later as the pH increases, when their charge becomes less positive.

The result is a separation of proteins based on their pI values, with each protein eluting at a specific pH corresponding to its pI.

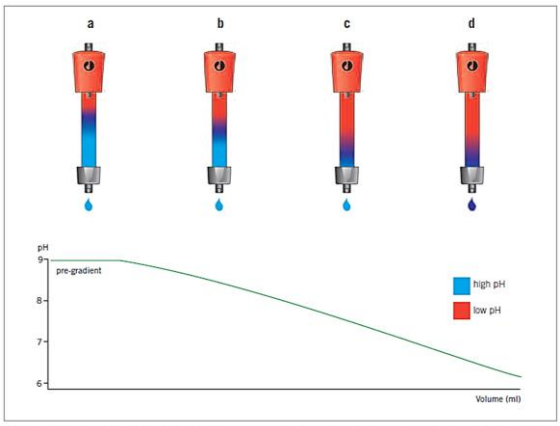
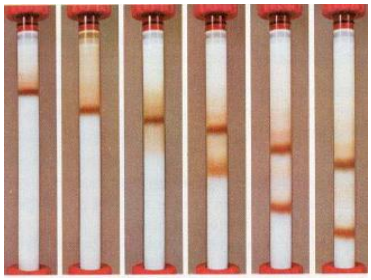


Fig. 77. Development of a pH gradient in a chromatofocusing column. The column is pre-equilibrated with start buffer at high pH (a) and elution with Polybuffer at low pH (b,c,d,) generates a descending linear pH gradient.

Proteins that have high pI are eluted first and proteins that have low pI are eluted last



Horse myoglobin pI 7.4

Sperm whale myoglobin pI 8.2

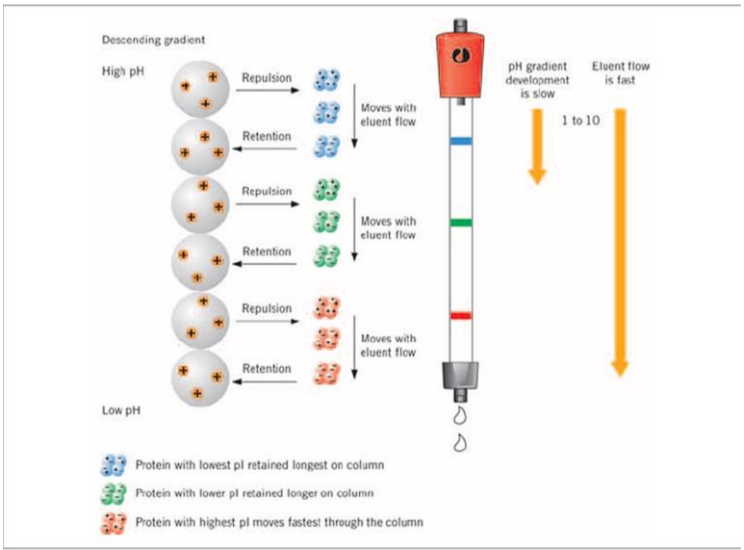


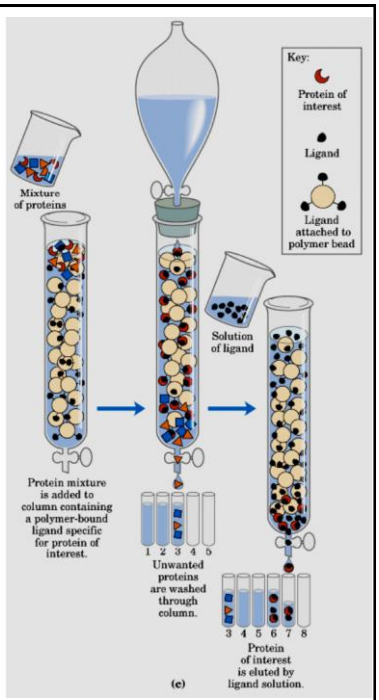
Fig. 78. Proteins with different pIs separate as they pass through the column. Molecules with the same isoelectric point are focused in narrow bands during the separation.

4. Affinity chromatography

Affinity chromatography is a chromatographic technique used for separating and purifying biomolecules based on their specific interactions with ligands immobilized on a chromatography matrix.

These interactions typically involve biological binding partners such as antibodies, enzymes, receptors, or other proteins, allowing selective capture and purification of the target molecule from a complex mixture.

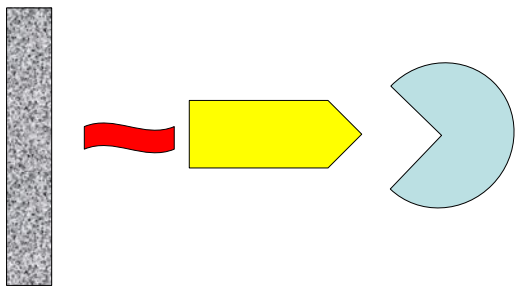
This is the most selective type of chromatography, as a protein can be purified by a factor of 10^3 to 10^4 in a single run.



Types of affinity interactions

Affinity interaction type	Principle	Example
Antigen-Antibody interactions	specific binding between an antigen and its corresponding antibody. Antibodies are immobilized onto the chromatography matrix, and the target antigen in the sample selectively binds to these antibodies.	Immobilized protein A/G for antibody purification (Fc region of immunoglobulins)
Ligand-Receptor Interactions	binding between a ligand (small molecule) and its receptor (protein). The ligand is immobilized on the chromatography matrix, and the receptor in the sample selectively binds to it.	Immobilized lectin (Concanavalin A, Wheat Germ Agglutinin) for glycoprotein purification
Enzyme-Substrate Interactions	the enzyme is immobilized onto the chromatography matrix, and its substrate in the sample selectively binds to it.	Purification of alkaline phosphatase using a chromatography matrix with immobilized p-nitrophenyl phosphate
Nucleic Acid-Protein Interactions	specific binding between nucleic acids and their complementary binding partners, such as transcription factors or DNA-binding proteins.	chromatin fragments containing specific histone modifications or DNA sequences recognized by chromatin-associated proteins are immobilized onto the chromatography matrix.
Metal-Ion Affinity Interactions	Certain proteins or peptides have specific affinity for metal ions such as nickel, zinc, or copper. Metal ions are immobilized onto the chromatography matrix, and the target protein containing a metal-binding site selectively binds to these ions.	Purification of a recombinant histidine-tagged protein using nickel-charged chromatography resin.
Covalent Interactions	The ligand is covalently attached to the chromatography matrix, and the target molecule selectively binds to it through covalent bonding.	Immobilized reactive groups for thiol-containing proteins (maleimide or iodoacetyl groups)
Nucleic acids-complementary sequence		

Components of affinity chromatography columns



Matrix **spacer** **ligand** **substrate**

To make certain ligands accessible to the binding site on the molecule with which they interact, it is necessary to increase the length of the carbon chain at the end of which the activated group is located. This additive chain is called a "spacer arm".

Matrixes

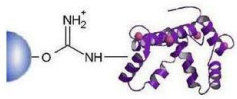
- Agarose (sepharose)
- Polyacrylamide
- Glass beads of controlled porosity

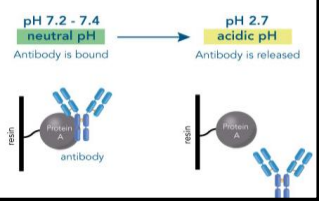
Ligands

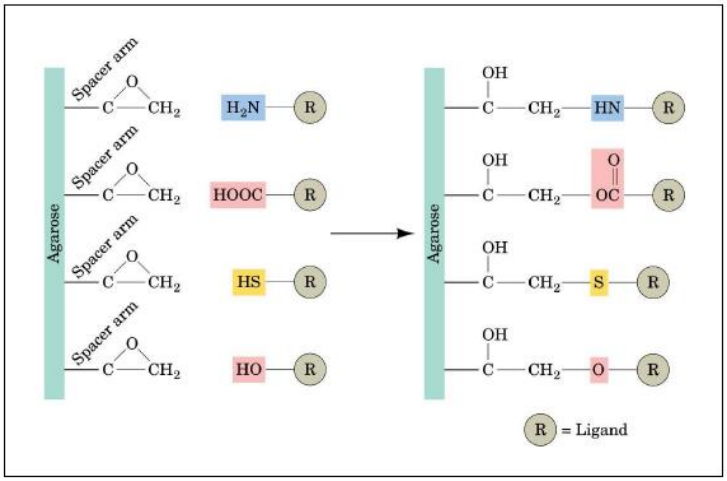
- Should have **specific** and **reversible** binding affinity to the substance to be separated
- Presence of chemically modifiable groups which allow binding with the matrix without altering the specific affinity

Examples of specific ligands

Ligands	Specificity
2'5' ADP	$NADP^+$ enzymes
5'AMP	NAD^+ enzymes and ATP-dependant kinases
Arginine	Proteases and zymogenes (prothrombine…)
Benzamidine	Proteases (trypsine, urokinase…)
Cibacron blue	Enzymes with nucleotide cofactors , serum albumine
Calmodulin	Kinases, phosphodiesterases, cyclases…
ConA (concanavalin A)	α–D glycopyranosyl, α-D mannopyranosyl residues …
Gelatine	Fibronectine
Helix promatia lectin	N-acétyl-α-D-galactosaminyl residues
Lysine	Plasminogene, ribosomal RNA
PolyA	Nucleic acids with polyT sequence, proteins
G protein	Fc region of IgG

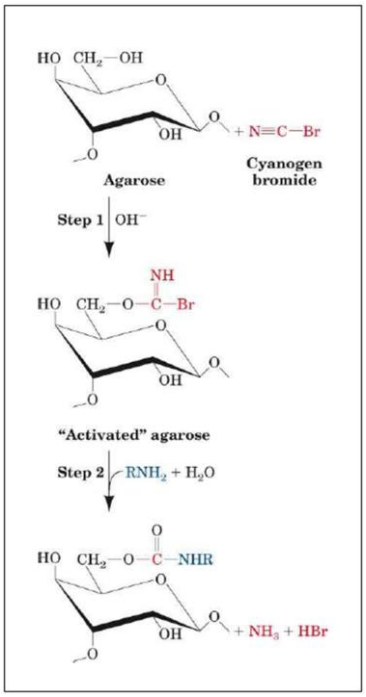






Ligand-matrix coupling

Immobilization of the ligand on the matrix requires its **pre-activation**, that is to say, **reaction sites** must be created which allow covalent bonds to be established.



Agarose activation using cyanogen bromide

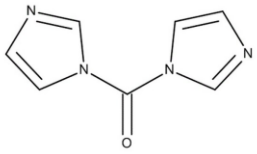
Activation of the hydroxyl groups of the support (agarose for example) by cyanogen bromide in a solution at pH 8.3

Imidocarbonate groups are formed to which ligands containing free amines, for example a protein, can be attached via the side chain of its lysines.

Unreacted hydroxyl groups are blocked by the action of ethanolamine or glycine;

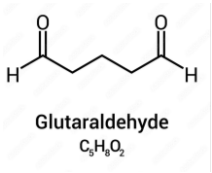
Other activation methods

- The hydroxyl groups of the support can be also activated by carbonyl diimidazole;

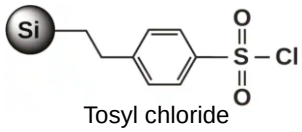


Carbonyl diimidazole

- Activation of the support can be carried out with glutaraldehyde which polymerizes and forms 5-carbon chains which constitute attachment points at a certain distance from the matrix;



- Tosyl chloride allows the coupling of molecules which have an amino, thiol or phenol group.



Tosyl chloride

Affinity chromatography steps

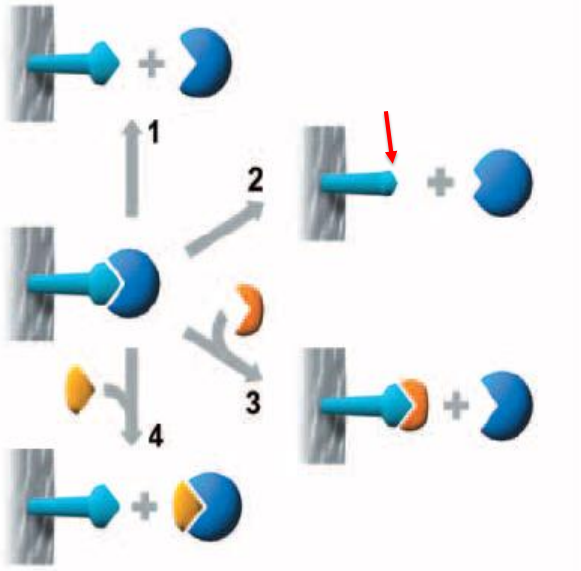
1. Affinity medium is equilibrated in binding buffer.

2. Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.

3. Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.

4. Affinity medium is re-equilibrated with binding buffer.

Elution



Method 1
The simplest case. A change of buffer composition elutes the bound substance without harming either it or the ligand.

Method 2
Extremes of pH or high concentrations of chaotropic agents are required for elution, but these may cause permanent or temporary damage.

Methods 3 and 4
Specific elution by addition of a substance that competes for binding. These methods can enhance the specificity of media that use group-specific ligands.

Fig. 5. Elution methods.

Typical affinity purification chromatogram

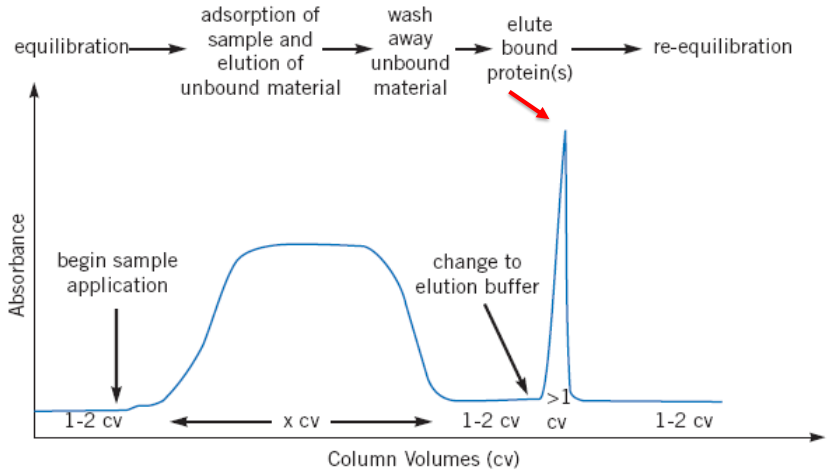


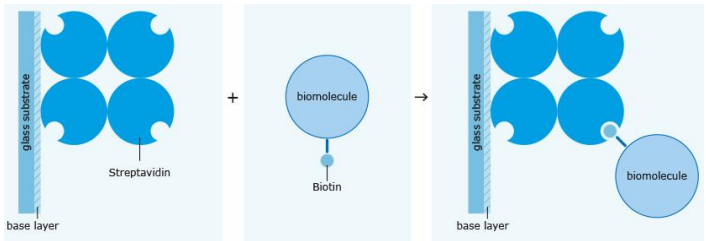
Fig. 2. Typical affinity purification.

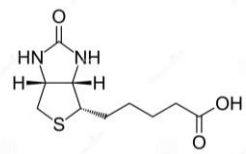
Applications

Streptavidin-biotin affinity chromatography is based on the strong and specific interaction between biotin, a small molecule, and streptavidin, a protein.
The principle revolves around utilizing this interaction to capture and purify biotinylated molecules from a complex mixture.

- ***Immobilization of Streptavidin***: Streptavidin, a tetrameric protein derived from *Streptomyces avidinii*, is immobilized onto a solid support matrix, such as agarose beads or a chromatography column. This immobilization can be achieved through covalent attachment or other suitable methods.
- ***Binding of Biotinylated Molecules***: The sample containing biotinylated molecules is applied to the streptavidin column. These biotinylated molecules can be proteins, nucleic acids, peptides, or other biomolecules that have been labeled with biotin.
- ***Specific Interaction***: Biotin binds to streptavidin with extremely high affinity, forming a stable complex. This interaction is one of the strongest non-covalent interactions known in nature. Each streptavidin tetramer can bind up to four biotin molecules simultaneously.

- ***Washing***: After application of the sample, unbound or weakly bound molecules are washed away with a buffer solution. Since the streptavidin-biotin interaction is highly specific and strong, the bound biotinylated molecules remain tightly attached to the column.
- ***Elution***: Finally, the specifically bound biotinylated molecules are eluted from the column using competitive displacement or by disrupting the streptavidin-biotin interaction using high concentrations of free biotin or denaturing agents. This allows for the isolation and purification of the biotinylated target molecules.



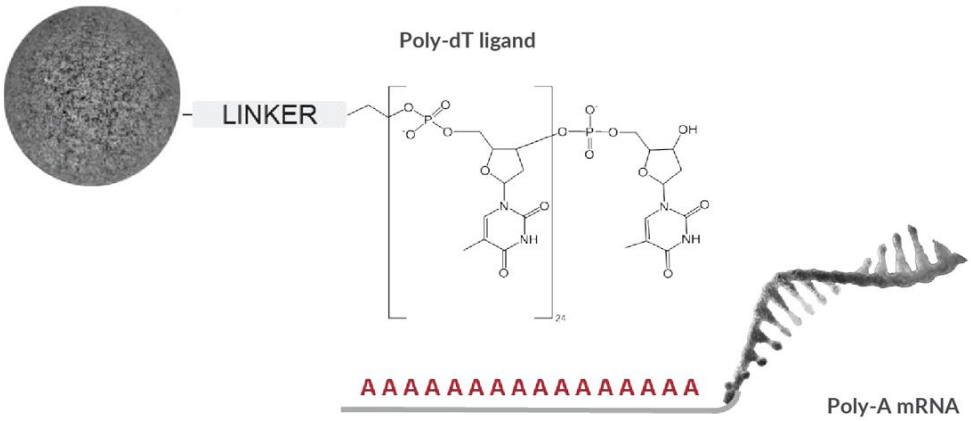


Biotin
Vitamin B_7

$C_{10}H_{16}N_2O_3S$

Mechanism of action of an Oligo (dT)25 affinity resin

Oligo dT affinity chromatography is a technique used primarily for the purification of mRNA (messenger RNA) from a mixture of RNA molecules. The principle of oligo dT affinity chromatography is based on the specific hybridization between oligo(dT) sequences and the poly(A) tails present at the 3' end of eukaryotic mRNA molecules.

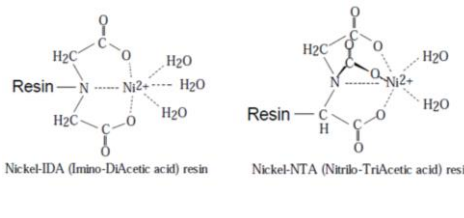


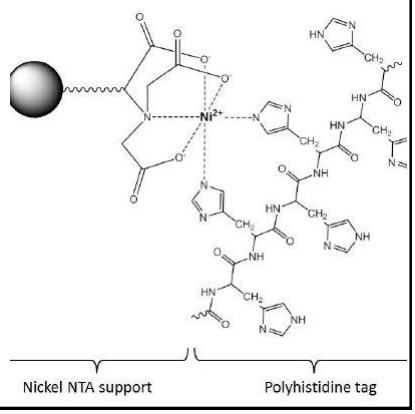
The resin is comprised of a 50µm porous poly(styrene-co-divinylbenzene) base bead with a polydeoxythymidine (poly-T) 25-mer (dT-25) conjugated to the surface using a proprietary linker.

Metal chelate affinity chromatography

The principle of metal chelate affinity chromatography involves the selective binding of proteins containing specific amino acid sequences, typically polyhistidine tags, to **metal ions** immobilized onto a solid support matrix

- Metal ions such as nickel (Ni^{2+}), cobalt (Co^{2+}), or copper (Cu^{2+}) are immobilized onto a solid support matrix, usually agarose beads or chromatography columns by chelating agents, such as nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA), which are covalently linked to the matrix
- The sample containing the protein of interest, which has been genetically engineered to contain a polyhistidine (His-tag) sequence, is applied to the metal chelate column. His-tags are short peptide sequences consisting of six to ten consecutive histidine residues, which have a strong affinity for metal ions
- The specifically bound polyhistidine-tagged protein is then eluted from the column using elution buffers containing competitive chelating agents or imidazole. These compounds disrupt the protein-metal ion interaction, resulting in the release of the purified protein from the column.

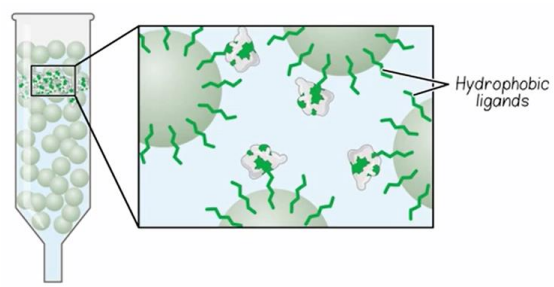




5. Hydrophobic interaction chromatography

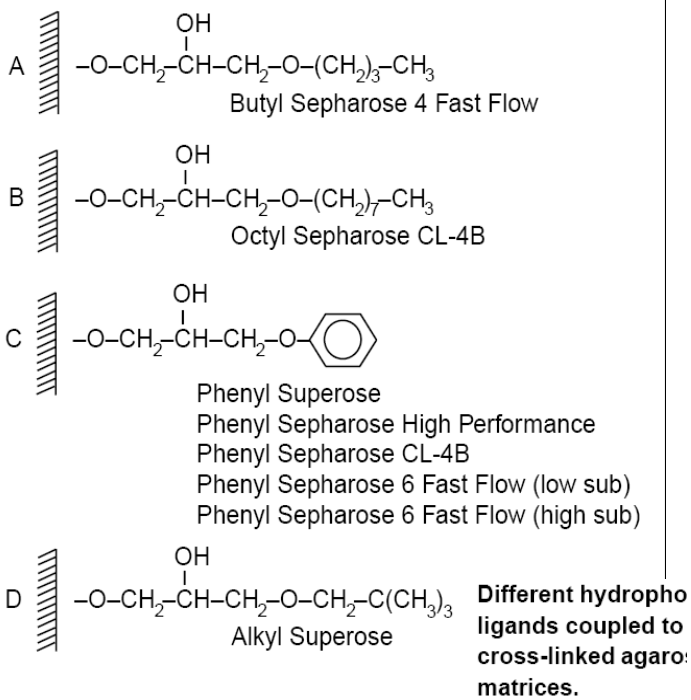
Hydrophobic interaction chromatography (HIC) is a technique used for the separation and purification of biomolecules based on differences in their hydrophobicity.

The principle of HIC relies on the reversible interactions between hydrophobic regions of the biomolecules and hydrophobic ligands immobilized on a solid support matrix.



Hydrophobic ligands, such as alkyl chains (e.g., octyl, butyl, or phenyl groups), are covalently attached or immobilized onto the surface of a solid support matrix, typically agarose or silica beads.

These ligands create a hydrophobic stationary phase on the chromatography column.



Different hydropho ligands coupled to cross-linked agaro matrices.

Principle and steps

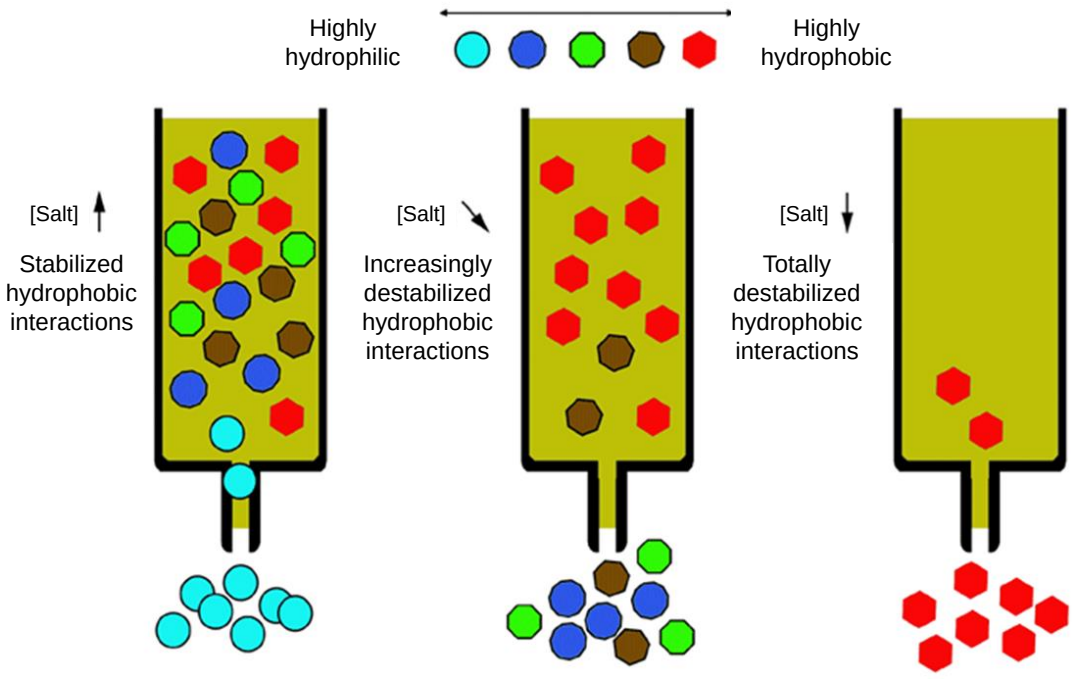
Biomolecules with higher hydrophobicity tend to interact more strongly with the hydrophobic ligands on the column, leading to their retention on the stationary phase.

The strength of interaction depends on factors such as the size and shape of the hydrophobic regions on the biomolecules, as well as the concentration and type of salts in the mobile phase.

After sample application, a gradient of decreasing hydrophobicity, typically achieved by decreasing the concentration of a salt (e.g., ammonium sulfate) or an organic solvent (e.g., acetonitrile or ethanol), is applied to the column.

This gradient elution allows for the stepwise release of the retained biomolecules based on their decreasing affinity for the hydrophobic ligands.

Biomolecules with lower hydrophobicity, which interact less strongly with the stationary phase, elute earlier, while those with higher hydrophobicity elute later



Typical HIC separation profile

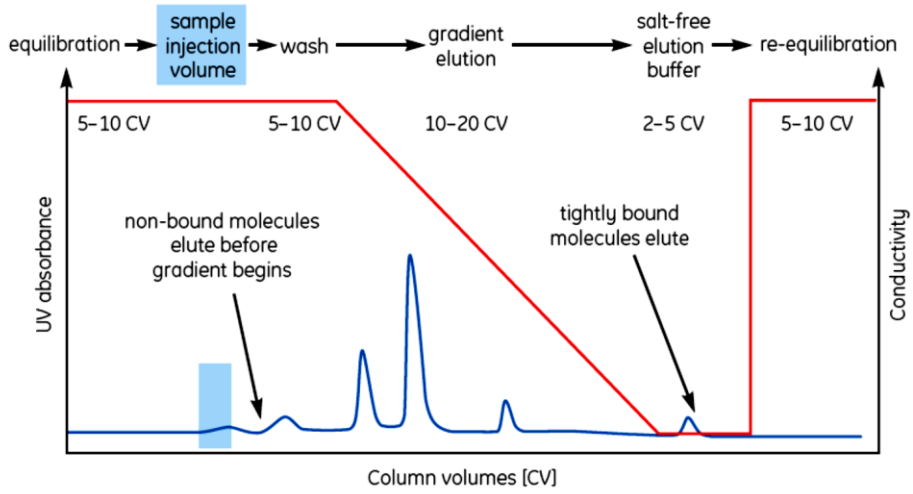
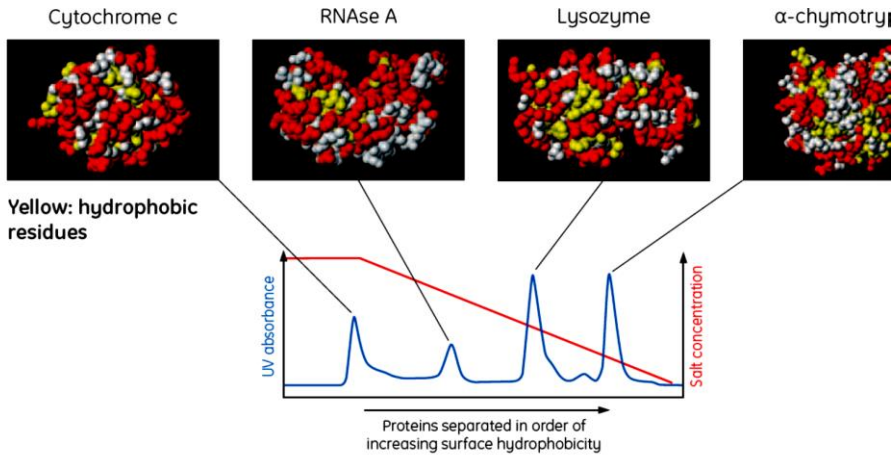


Fig 15. Typical high-resolution, HIC separation using linear gradient elution.



Column:	Phenyl Sepharose High Performance packed in Tricorn 10/100 column
Sample:	Cytochrome c, RNAse A, lysozyme, α-chymotrypsin
Start buffer:	1.7 M ammonium sulfate, 0.02 M Tris-HCl, pH 7.5
Elution buffer:	0.02 M Tris-HCl, pH 7.5
Gradient:	0–100% elution buffer in 10 CV
Flow:	1 ml/min, 76 cm/h

Fig 2. Proteins are separated according to differences in their surface hydrophobicity (yellow indicates hydrophobic and red hydrophilic amino acid residues), as shown in this separation of standard proteins on Phenyl Sepharose High Performance.

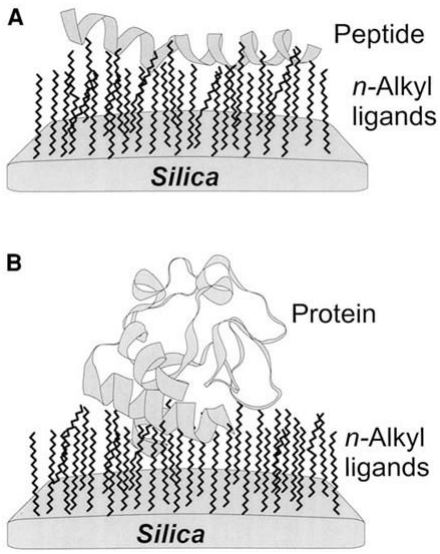
6. Reversed phase chromatography

Reverse phase chromatography (RPC) is a technique used for the separation and purification of molecules based on their hydrophobicity.

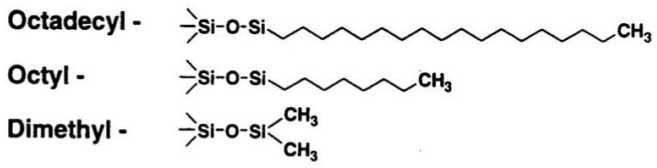
The principle of reverse phase chromatography involves the use of a hydrophobic stationary phase and a polar mobile phase

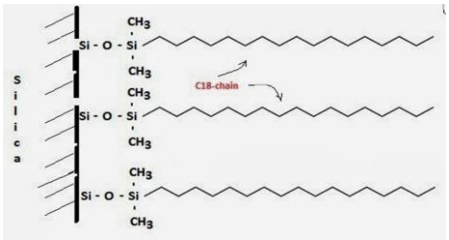
The stationary phase is nonpolar, often composed of hydrophobic ligands bonded to a solid support

The mobile phase is polar, usually consisting of water or a water-miscible organic solvent like methanol or acetonitrile.

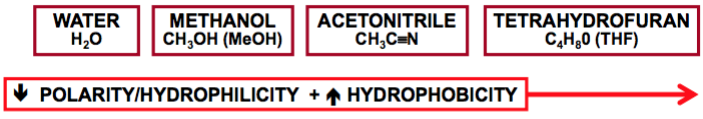


Hydrophobic Stationary Phase: the stationary phase is typically composed of hydrophobic materials such as alkyl chains (e.g., C18, C8) bonded to a solid support matrix, commonly silica gel or polymer beads.
These hydrophobic ligands create a non-polar environment that facilitates the retention of hydrophobic molecules.





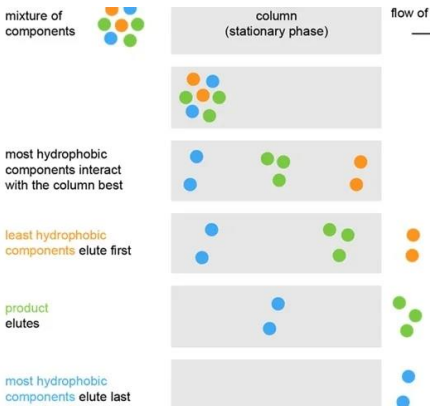
Polar Mobile Phase: The mobile phase used in RPC is typically an aqueous solution containing polar solvents such as water or a water-miscible organic solvent like methanol or acetonitrile.
The polarity of the mobile phase facilitates the elution of polar molecules.



Hydrophobic molecules tend to interact more strongly with the hydrophobic stationary phase and are therefore retained longer, while polar molecules interact less strongly and are eluted more rapidly.

To elute the retained molecules from the column, a gradient of increasing hydrophobicity is applied to the mobile phase.

This gradient is typically achieved by increasing the concentration of the organic solvent (e.g., methanol or acetonitrile) in the mobile phase over time.



As the hydrophobicity of the mobile phase increases, hydrophobic molecules are gradually desorbed from the stationary phase and eluted from the column.

The retention time of each molecule depends on its degree of hydrophobicity: **more hydrophobic molecules elute later, while less hydrophobic or polar molecules elute earlier.**

Typical RPC separation

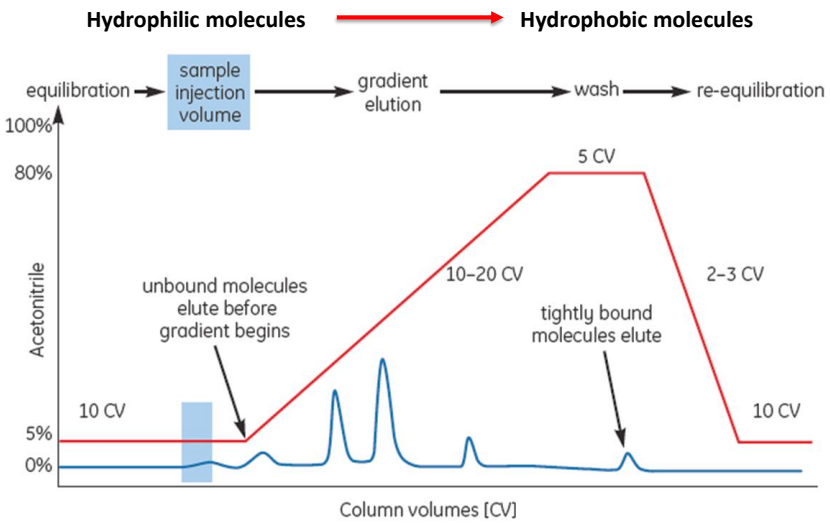


Fig 75. Typical high-resolution, RPC separation using gradient elution.

High-Performance Liquid Chromatography (HPLC) is a powerful separation technique used to separate, identify, and quantify components in a mixture. The principle of HPLC involves the use of a liquid mobile phase to carry the sample through a chromatographic column packed with a stationary phase at high pressure.

Gas chromatography (GC) is a separation technique used to separate, identify, and quantify components in a mixture based on their distribution between a stationary phase and a mobile phase. The mobile phase is an inert gas, typically helium or nitrogen, which flows through the chromatographic column. The mobile phase carries the sample components through the column.

Supercritical fluid chromatography (SFC) is a separation technique that combines the principles of gas chromatography and liquid chromatography. The principle involves using supercritical fluids, typically carbon dioxide (CO_2), as the mobile phase, instead of traditional liquid solvents, and a stationary phase packed into a chromatography column.
A supercritical fluid is a substance that is at a temperature and pressure above its critical point, where it exhibits properties of both a gas and a liquid.