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Ministry of Higher Education and Scientific Research
Abderrahmane Mira University of Bejaia
Faculty of Natural and Life Sciences
Department of Food Sciences

Course handout

Speciality:

L 3 CQAA

Liquid chromatography methods

Presented by:

Dr MEKHOUKHE AIDA

Teacher-Researcher

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Preamble

Chromatography is one of the most versatile and essential analytical techniques in modern science. It serves as a cornerstone for separating, identifying, and quantifying components in complex mixtures. Since its inception by Mikhail Tswett in the early 20th century, the method has undergone significant advancements, evolving into a wide array of techniques such as gas chromatography (GC), liquid chromatography (LC), and high-performance liquid chromatography (HPLC), among others.

This technique finds its application in numerous fields, including pharmaceuticals, food safety, environmental monitoring, and biochemistry, where precision and accuracy are paramount. By exploiting differences in the physical or chemical properties of substances, chromatography provides invaluable insights into the composition and behavior of samples, thus advancing both scientific research and industrial applications.

This document is very useful for all undergraduate students, especially those in the CQAA Bachelor's program (Quality Control and Food Analysis).

Liquid chromatography methods are essential analytical tools in the field of quality control and food analysis. They enable the separation, identification, and quantification of components in complex mixtures such as food, beverages, pharmaceuticals, and cosmetics.

For CQAA students, mastering these techniques is crucial because:

- They are widely used in laboratories to monitor food safety and ensure regulatory compliance;
- They provide accurate and reproducible results essential for quality assurance;
- They help detect contaminants, additives, and active compounds in various products;
- They are valuable skills in both academic research and industrial careers.

Objective of this Document

The primary objective of this document is to equip students with the knowledge and skills necessary to understand and apply chromatographic methods effectively. It seeks to :

- Introduce the basic principles of chromatography in an engaging and student-friendly manner;
- Highlight the different types of chromatographic techniques with clear examples;
- Provide practical insights into the applications of chromatography in both academic and professional contexts;
- Explain the role of chromatography in quality control, research, and industry;
- Compare and contrast different chromatographic methods, emphasizing their advantages, limitations, and ideal applications;
- Familiarize students with the instrumentation and technical setups used in chromatography laboratories.

List of Abbreviations

AAL : Aleuria Aurantia lectin

ADME : Absorbed Distributed Metabolized and Excreted

APIs : Aactive Pharmaceutical Ingredients

AC :Affinity chromatography

BAC : Boronate Affinity Chromatography

CC :Column chromatography

CF : Chromato Focusing

CM: Concentration of analyte A in the mobile phase.

CGL : Gas liquid chromatography

CIM : Convective Interaction Media

CP : Chromatography on paper

CPV : Column Peak Variance

CS: Concentration of analyte

DLS : Dynamic Light Scattering

DAD : Diode-Array Detector

DBC : Dynamic Binding Capacity

DEAE : Diethylaminoethyl Cellulose

2D-IEX : Ion Exchange Chromatography with 2D Chromatography

ECD : Extra-Column Dispersion

EDMA Ethyleneglycol DimethAcrylate

FPLC : Fast Protein Liquid Chromatography

FTIR : Fourier Transform Infrared

GF : Size Gel filtration

GFC Gel-Filtration Chromatography

GLC : Gas-Liquid Chromatography

GMA : Glycidyl MethAcrylate

GPC :Gas chromatography

GPC Gel Permeation Chromatography

GSC : Gas solid chromatography

HCD : Host Cell DNA

HCP : Host Cell Proteins

HETP : theoretical equivalent plate height

HIC : Hydrophobic interaction chromatography

HPLC: High-Performance Liquid Chromatography

HPAC : high-performance affinity chromatography

HPIEX : High-Performance Ion Exchange Chromatography

HPLAC : high-performance liquid affinity chromatography

h-HST : Hydrophilic Hybrid Surface

IAC : Immunoaffinity chromatography

IAM : Immobilized Artificial Membrane

IC : Ion Chromatography

ICP: Inductively coupled plasma mass spectrometry

IEX : Ion exchange chromatography

IEX-MS : Ion Exchange Chromatography Coupled with Mass Spectrometry

ILC : Immobilized Liposome Chromatography

IMAC : Immobilized Metal Ions Amino acids

IMAC : Metal-Ion Affinity Chromatography

IUPAC : International Union of Pure and Applied Chemistry

LAC : Lectin Affinity Chromatography

LC: Liquid Chromatography

LC-MS : Liquid Chromatography Mass Spectrometry

LC-MS/MS : Liquid chromatography–mass spectrometry

LLC: liquid–liquid chromatography

LPC : Liquid phase chromatography

LSAC: Liquid-solid adsorption chromatography

LSC : Liquid solid chromatography

MALS : Multi-Angle Light Scattering

MIPs : Molecularly imprinted polymers

MOAC : Metal Oxide Affinity Chromatography

MS : Mass Spectrometry

NPC: normal-phase chromatography

NPLC normal phase liquid chromatography

PACHA : Paper chromatography hybridization assay

PAHs : Polycyclic Aromatic Hydrocarbons

POPs : Persistent Organic Pollutants

PEEK : PolyEther Ether Ketone

RID : Refractive Index Detector

RPC: reversed-phase chromatography

SCMA : Sulfated Cellulose Membrane Adsorbers

SEC : Size-exclusion chromatography

SPC : Supercritical phase chromatography

TLC :Thin layer chromatography

THF : Tetrahydrofuran

RI : Refractive Index

VLPs : Virus-like particles

VOCs : Volatile Organic Compounds

WGA : Wheat Germ Agglutinin

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I.1 Definition

Chromatography is essentially a physical separation method in which the components of a gaseous, liquid or solid mixture are separated by their distribution between two phases: one of these phases in the form of a porous bed, bulk liquid, layer or film is generally immobile (stationary phase), while the other is a fluid (mobile phase) which percolates through or over the stationary phase.

IUPAC defines chromatography as a physico-chemical process for separating substances in a mixture. This separation is made possible by the distribution of these substances between the mobile phase and the stationary phase.

I.2 Principle

Chromatography is based on the entrainment of a dissolved sample by a mobile phase through a stationary phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), separation (liquid-solid) and affinity or differences between their molecular weights. As a result of these differences, some components of the mixture remain in the stationary phase for longer and move slowly through the chromatography system, while others move quickly into the mobile phase and leave the system more quickly.

I.3 Nernst distribution coefficient

This is the basic physico-chemical parameter in chromatography which quantifies the concentration ratio of each compound between the two phases present.

The values of distribution coefficient (K) vary widely. They are large (1000, for example) when the mobile phase is a gas, and small (2, for example) when the two phases are in a condensed state. As each compound occupies only a limited space in the column, and moreover with a variable concentration, the true values of Concentration of analyte in the mobile phase (Cm) and concentration of analyte in stationary phase (Cs) are not accessible, but their ratio is constant.

$$A_{\text{Mobile phase}} \rightleftharpoons A_{\text{Stationary phase}}$$

$$K = {}^{Cs}/_{Cm} \qquad (1)$$

The greater K, the greater the uptake of the compound in the stationary phase and the greater the retention, and vice versa.

Separation is therefore based on the differential entrainment of the constituents of the mixture. These components pass through the stationary phase with times that are proportional to their intrinsic properties (size, structure, etc.) or their affinity with the stationary phase (polarity, etc.).

I.4 Types of chromatography

There are several variants of chromatographic techniques, classified in several different ways: (Figure 1 and 2) according to the:

1. Nature of the phases (mobile and stationary).
2. Operating procedure.
3. Principle of the phenomena involved in the separation type of equilibrium

Classification according to the physical nature of the phases

- A distinction is made according to the nature of the mobile phase:

Liquid phase chromatography (LPC)

Gas chromatography (GPC)

Supercritical phase chromatography (SPC)

- Depending on the nature of the stationary phase, a distinction is made between :

Liquid/solid chromatography (LSC)

Liquid/liquid chromatography (LLC)

Gas/solid chromatography (GSC)

Gas/liquid chromatography (CGL). - Depending on the chromatography support, a distinction is made between:

Planar chromatography: the stationary phase is present on the surface of a flat support - there are two types

Chromatography on paper (CP): a flat surface of cellulose is used as the support and holds a liquid stationary phase by imbibition.

Thin layer chromatography (TLC): the stationary phase is spread over a flat surface (planar chromatography).

Column chromatography (CC): the stationary phase is held in a narrow tube and the mobile phase moves through it by gravity or under the action of an HPLC pressure difference.

Classification according to the principle of chromatographic phenomena

Chromatography can be classified according to the separation phenomena or mechanisms involved. The factors influencing the partitioning of molecules between the stationary and mobile phases include their solubility in a liquid solvent, molecular size and shape, polarity, electric charge, and the presence of specific atomic groups forming particular binding sites. Table I summarizes the characteristics of different types of chromatography.

Table I: Main characteristics of the different chromatographic modes and their applications

Chromatographic mode	Stationary phase	Mobile phase	Applications
Size exclusion (SEC) or Gel filtration (GF)	Particles of well-defined size, of different sized pores	Aqueous buffer	Desalting, buffer exchange Determination of molecular weight Final polishing
Ion exchange (IEC)	Particles coated with anion or cation exchanger functionalities	Aqueous buffers, containing salts for elution	Separation of charged molecules Good choice for protein separation on preparative scale
Affinity (AC)	Covalently immobilized affinity ligand on particles	Aqueous buffers	Good choice for capture of target molecule at low concentrations in sample
Hydrophobic interaction (HIC)	Particles of well defined size, coated with small hydrophobic ligands C_2—C_4	Saline aqueous solutions	Well developed for protein separation No prior desalting necessary, good after IEC
Reversed phase (RPC)	Particles of well-defined size, coated with hydrophobic ligands C_4—C_{18}	Water, buffers of low molarity, and organic solvents for elution	Indicated for neutral and uncharged molecules, soluble in aqueous/organic mixtures
		Presence of salts problematic above 10 mM	Excellent for analytical HPLC, seldom preparative (proteins denatured by organic solvents)

I.5 Recent Developments in Chromatographic Methods

More recent separation processes, such as supercritical fluid chromatography, chromatographic separation by paper chromatography hybridization assay, etc., are gaining in importance as new separation processes. These techniques include:

1. **Paper chromatography hybridization assay (PACHA)**

This is a DNA hybridization method based on the chromatographic relocation of DNA onto a nitrocellulose strip that passes through an immobilized test area.

2. **Hydrophobic interaction chromatography**

works on the same principles as ion exchange and steric exclusion chromatography. When sample molecules with hydrophobic and hydrophilic zones are applied to an HIC section in a high salt buffer, the solutes being tested are less likely to survive.

3. Optical force chromatography

The balance between optical and liquid drag power acting on particles is required for optical force chromatography. Chromatographie haute performance et immuno-affinité ; La chromatographie d'immuno-affinité combine l'utilisation de la chromatographie liquide avec la restriction spécifique des anticorps ou d'autres agents spécialisés. Il est utilisé pour filtrer et centraliser les données avant une analyse plus approfondie à l'aide d'une autre méthode.

4. Mixed-mode chromatography

Multimodal chromatography is another name for mixed-mode chromatography. Mixed-mode media combine complementary chromatography methods within the same medium, reducing the number of segmental processes required throughout the purification process.

5. Dye and ligand chromatography

This is a purification method with a high degree of selectivity and purification rate. The ability of several compounds to purify cibacron blue nucleotides is needed to improve this procedure.

I.6 Fundamental concepts

I.6.1 Retention quantities

Historically, retention parameters were required for sample identification and characterisation of the adsorbents and solvents used for elution. These parameters have also been found useful for studying the separation process, including peak broadening. Good separation of compounds present in a mixture is linked to a series of parameters shown in Table II

Table II. The main parameters of chromatographic separation.

Parameter	Symbol	Definition
Retention time	t_R	time taken by an analyte to elute from a chromatographic column and reach the detector
Dead time of the column	t_0	time taken by an unretained compound, which does not interact with the stationary phase,
Reduced retention time	$(t_R - t_0)$	the subtraction between retention time and dead time of the column
Retention factor	k	the ratio of reduced retention time to the dead time of the column
Selectivity (separation factor)	α	the ratio of retention factors of two separated compounds
Resolution	R	ability to separate two chromatographic peaks
Plate number	N	the number of small layers, along the chromatographic column, in which an equilibration of the sample distribution between stationary and mobile phase takes place

I.6.2 Gaussian elution peak

When a small quantity is injected, each eluted compound produces a symmetrical Gaussian-shaped peak (Figure. 1), allowing the definition of the following parameters.":

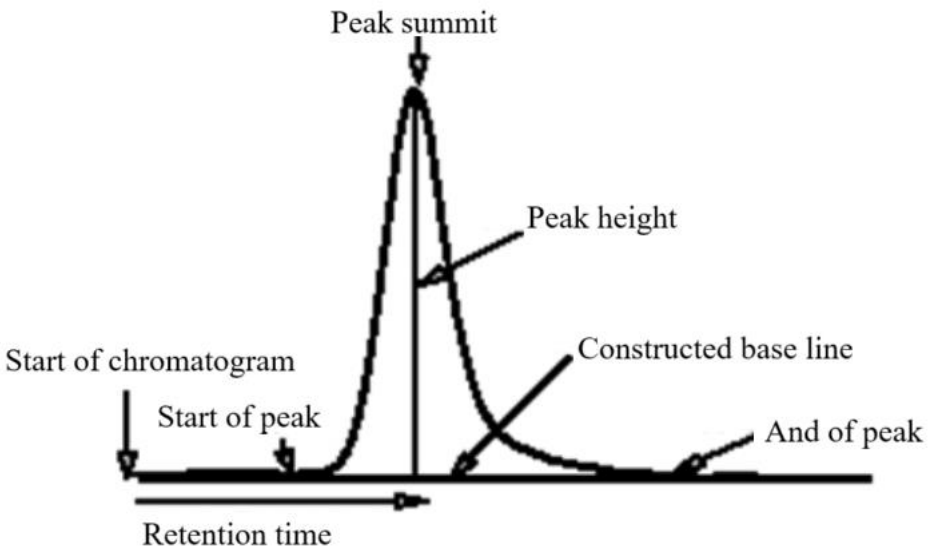


Figure 1: Representation of a chromatographic peak

I.6.3 Notion of time

Dead time: also known as Tm, is the time taken for a compound not retained by the stationary phase to pass through the column (time spent in the mobile phase). (Figure.2)

Retention time: The retention time (tr) is the time taken for the molecules of a compound to be analysed (solute) to travel between the inlet and the outlet of the column.

Reduced retention time: Reduced retention time Tr' is time spent by a solute in the stationary phase, i.e.

T'r = Tr -Tm (2)

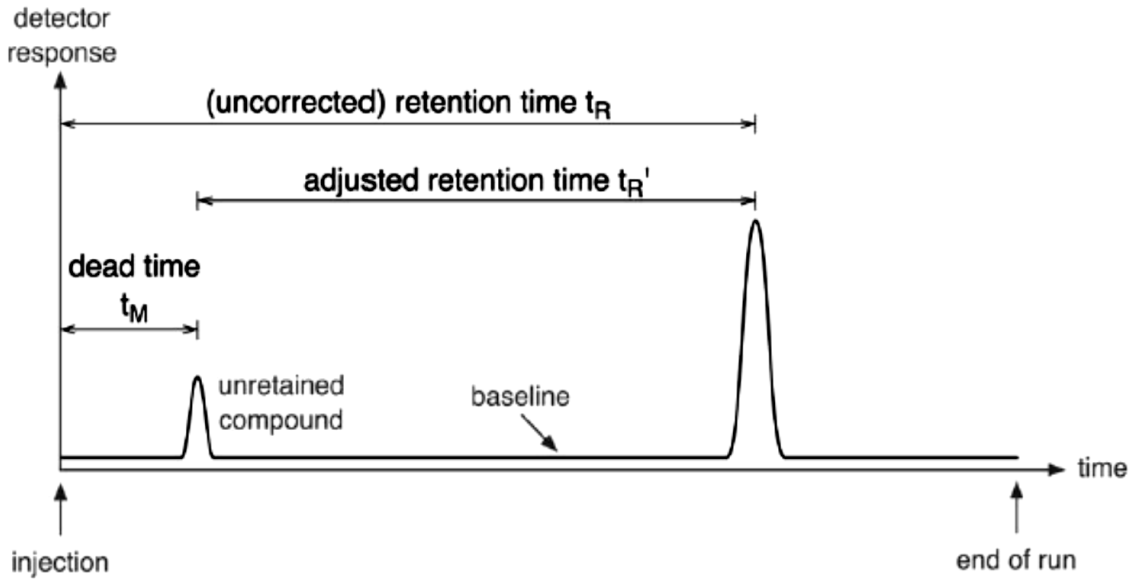


Figure 2: Illustration of retention parameters

I.6.4 Notion de volume

Void volume (Vm): This is the volume of the mobile phase contained within the chromatographic column. If *D* represents the (assumed constant) flow rate of the mobile phase, then**: Vm = Tm × D** **(3)**

Mobile phase flow: rate D = volume of mobile phase circulating through the chromatographic column per unit time (ml.min-1).

- **Retention volume Vr** (elution volume of mobile phase required to make the solute migrate through the stationary phase.

Vr = Tr × D **(4)**

Reduced retention volume (VR′): By analogy with the reduced retention time, the reduced retention volume is defined by the following equation:

V'r = Vr – Vm **(5)**

where:

- *VR* is the retention volume of the compound
- *VM* is the void (dead) volume of the column

I.6.5 Linear mean speed of displacement

-Linear mean velocity of the moving phase : (species not selected)

u = L / Tm

-Average linear velocity of the solute (species retained)

V= L/Tr

I.7 Capacity factor

The capacity factor (or retention factor), denoted *k′*, is defined as the ratio of the retention time of the analyte to that of a non-retained compound. A non-retained compound has no affinity for the stationary phase and elutes with the solvent front at a time *tm*, also referred to as the dead time or hold-up time.

This value gives an indication of how long each component is retained on the column (i.e. how many times the component is delayed by the stationary phase compared with the time it spends in the mobile phase). K is independent of flow rate and column dimensions. High K values for highly retained compounds.

$$k' = \frac{tr - tm}{tm} \qquad (6)$$

It can also be expressed as the ratio of the amount of solute in the stationary phase to the amount of solute in the mobile phase. When a compound of total mass mT is introduced into the column, it is split into two quantities: mM in the mobile phase and mS in the stationary phase. If the operating conditions are not changed, these two quantities remain constant during its migration through the column. Their ratio, called the retention factor, is independent of mT.

$$K' = \frac{ms}{mM} = \frac{Cs \times Vs}{CM \times VM} \qquad (7)$$

ms: mass of the solute in the stationary phase

m*M*: mass of solute in the mobile phase

- A high K' value indicates that the sample is highly retained and has spent a significant proportion of the interaction with the stationary phase.
- K values should be between 1 and 10 for good separations.

I.8 Selectivity (separation) factor

Selectivity is denoted by (α) and gives a measure of the chromatographic system's ability to chemically distinguish the components of the sample (to characterise the distance

separating the peaks of two peaks). This factor is equal to the ratio of the capacity factors of two solutes from which separation is to be achieved (Figure. 3).

$$\alpha = \frac{(tr2 - tm)}{(tr1 - tm)} = \frac{k'2}{k'1} \quad (8)$$

- k2‘: capacity factor of compound 2; k1’: capacity factor of compound 1; α= 1 no separation takes place so the retention time is the same. Selectivity must be greater than 1.

Two compounds cannot be separated unless they have k'≠ 0

- α is always greater than 1 because when it is equal to one the peaks are co-eluting i.e. there is no space between the peaks of the two peaks.

- High α values indicate good separation power and good separation between the peaks. Effect of α on resolution: modification with the greatest impact on resolution. α can be modified using the following

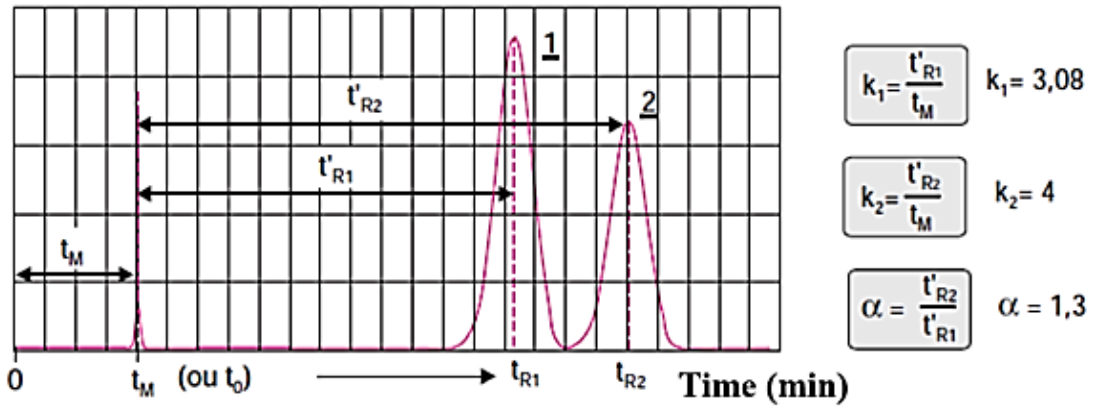


Figure 3 : Retention and separation factors between two adjacent compounds

Each compound has its own retention factor. An alone does not indicate whether separation is really possible. In this figure, the separation factor is approximately 1.3.

I.9 Resolution factor (separation)

The resolution of two neighbouring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. It is calculated using the following formula (Figure.4).

$$R = 2\frac{tr2 - tr1}{w1 + w2} \quad (9)$$

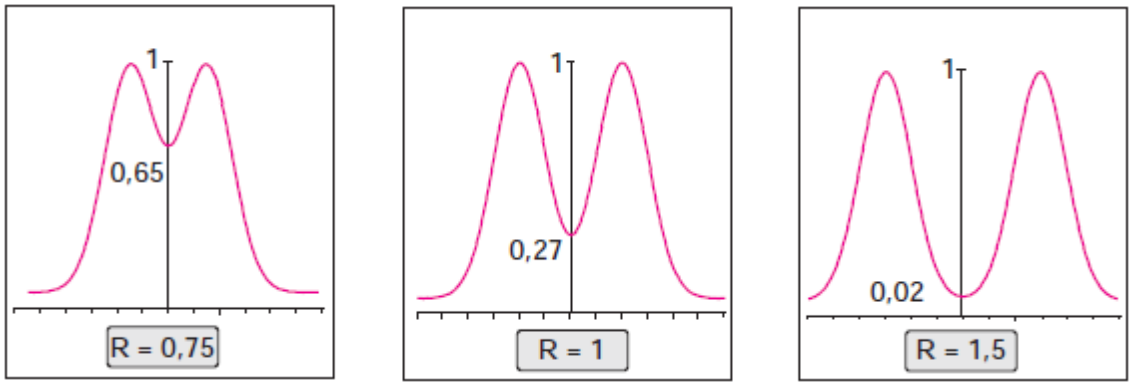


Figure 4: Resolution factor

- When the resolution (R) is 1, there is still a slight overlap of peaks—about 2%. As the resolution improves to R = 1.5, the contribution of one peak's area to the next is reduced to approximately 0.03%, which corresponds to an essentially complete separation. If the peaks are of equal size, this small overlap is generally negligible. However, if one of the eluting peaks is present only in minor proportions, the contribution of the larger peak's overlap to the height and area of the smaller peak can become significant.

I.10 Column efficiency

The efficiency of a chromatographic column is measured, for each compound, by the number of theoretical plates N in the column and the equivalent height of a theoretical plate H. This theory arose from the search for a static model to describe the operation of a chromatographic column in the same way as a distillation column. Instead of considering the real, continuous movement of the mobile phase, it is assumed that it progresses by successive jumps and comes into equilibrium with the stationary phase between two transfers, which makes it possible to fictitiously divide the column into a certain number of zones in which equilibria are achieved and which are called theoretical plateaus (Figure. 5).

The concept of theoretical plates is often used to analyse zone broadening in chromatography. The chromatography column is considered to be made up of a certain number of segments or plates of height H; the size of H is of the same order as the diameter of the resin particles. Equilibrium is assumed to exist within each segment.

The elution peaks can be likened to Gauss curves. The geometric characteristics of the Gauss curve (Fig.5) allow us to calculate N for a given solute from the chromatogram

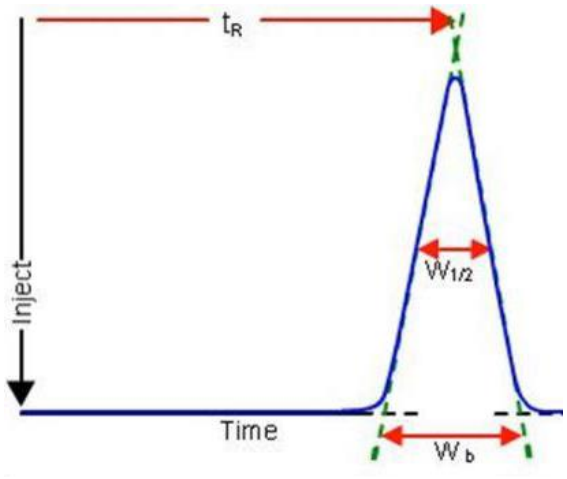


Figure 5: Theoretical plateau number

N= 16(tr/wb)2 (10)

N=5.54(tr/(w1/2)) 2 (11)

w1/2 = Peak width at 1/2 peak height.

wb = Peak width at base.

tr = Peak retention time or elution volume.

The higher the number of plates, the more efficient the column. The plate number generally depends on the length of the column: the longer the column, the larger the plate number. Consequently, column efficiency can also be expressed in terms of plate height (h) or theoretical equivalent plate height (TEHP).

HETP=L/N (12)

Where, L = length of column

N or plates/metre N = plate number.

Several factors have a negative impact on maximum efficiency

I.11 Influence of the speed of the mobile phase

In all of the above, particularly in the various expressions characterising separations, the speed of the mobile phase in the column is not taken into account. Clearly, this speed must have an effect on the progress of the analytes through the column, and therefore on their dispersion, in short on the quality of the analysis in progress.

I.12 Van Deemter equation

The simplified form, proposed by this author in 1956, is well known in gas chromatography for packed columns. It relates H (HEPT) to the average linear velocity of the mobile phase u in the column (Fig.6.):

$$H = A + B/U + C \times U \quad (13)$$

The curve representing the Van Deemter equation is therefore a hyperbola where A, B and C are constants.

U: average velocity of the moving phase (gas vector).

A: is the influence of turbulent diffusion due to heterogeneities in the flow.

B: is a factor of molecular diffusion in the mobile phase.

C: depends on the resistance to mass transfer in the liquid phase.

This equation shows that there is an optimum flow rate for each column, corresponding to the minimum of H, as predicted by the curve in equation 1.30. The decrease in efficiency as the flow rate increases is a result that everyone has discovered to their cost when trying to speed up a chromatographic separation by increasing the flow rate of the mobile phase. What is less intuitive, however, is the loss of efficiency due to a flow rate that is too slow. To explain this phenomenon, we need to go back to the origin of terms A, B and C, each of which has an area of influence that can be seen on the graph (Figure. 6).

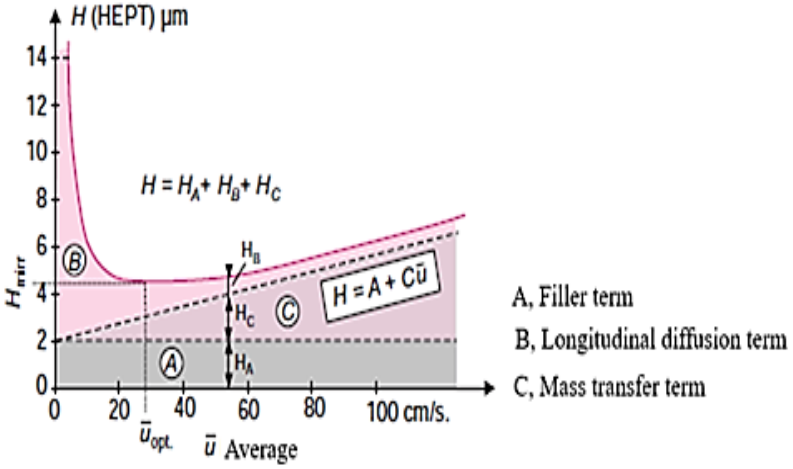


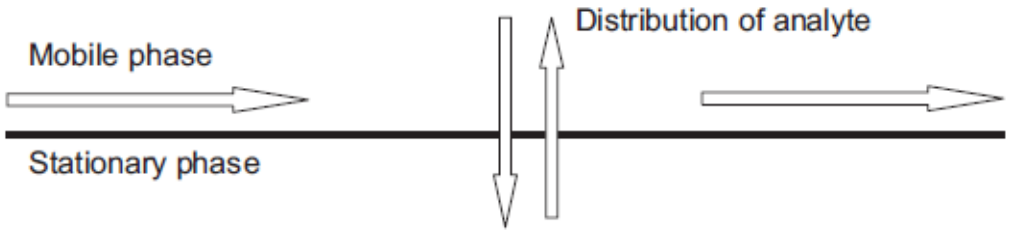
Figure 6: Van Deemter curve in gas chromatography

II.1 Historical and General concepts

Liquid Chromatography LC is a chromatographic technique in which the mobile phase is a liquid. Is a specific type of chromatography technique that plays an essential role in research across multiple scientific disciplines. It involves the distribution of the mixture between two phases, one stationary, and one mobile, leading to differential migration and subsequent separation of the components.

LC is a type of chromatography in which the mobile phase is a liquid. Separations in LC are based on the distribution of compounds between a liquid mobile phase and a stationary phase. This technique was first used by Tswett when he began practicing column chromatography in 1903. Although LC was primarily a preparative tool until the 1960s, it has since become the dominant form of chromatography for chemical analysis in clinical and biomedical laboratories. A key advantage of LC over gas chromatography (GC) is its ability to work directly with liquid samples, such as those commonly encountered in clinical or biological specimens. Before the mid-1960s, the supports used in LC columns consisted of large, irregularly shaped particles, similar to those used in packed columns for GC.

The separation takes place in a column, which contains a stationary phase. The mobile phase transports a pulse of the mixture of analytes to be resolved through the column. The analytes are separated into individual species by a selective distribution between the mobile and stationary phase and leave the column outlet as narrow bands, which they can be detected (Figure.7)



Major phenomena: Mass transfer kinetics and phase equilibria between the mobile and stationary phase

Figure 7: How does LC function

These supports were useful in preparative work but were not suitable for many analytical applications because they tended to result in broad peaks and separations with low

resolution. In addition, these supports generally had limited mechanical stability and could be used only at relatively low operating pressures.

Significant developments began in the 1960s to produce smaller, more mechanically stable, and more efficient supports for LC, along with instrumentation compatible with these advanced materials. This led to the emergence of high-performance liquid chromatography (HPLC). The use of these more efficient supports allowed for narrower peaks, improved separations, and lower limits of detection in LC.

These improvements, combined with the ability to automate HPLC, have made it the method of choice for most routine chemical separations and analyses in modern laboratories, including clinical settings. Other advantages of HPLC and LC include the wide variety of separation mechanisms, stationary phases, solvents, and detectors that can be utilized, offering great flexibility for diverse analytical needs.

II.2 Purpose

The purpose of Liquid Chromatography (LC) is to separate, identify, and quantify the individual components within a complex mixture. It is used to analyze substances that are dissolved in liquids, providing detailed information about the composition of the mixture. The main goals of LC are Separation; Identification and Quantification.

II.2.1 Separation of mixtures

The primary purpose of liquid chromatography is to separate different substances within a mixture. Separation occurs because the components in the mixture interact differently with the stationary phase and mobile phase. Components that interact more strongly with the stationary phase will move more slowly. Components that have weaker interactions with the stationary phase and a higher affinity for the mobile phase will move faster. The separation allows scientists to study complex mixtures by isolating individual components.

II.2.2 Identification

Once the components are separated, the next step is to identify them. Liquid chromatography (LC) separates compounds based on their chemical properties, such as:

- **Polarity:** Polar molecules interact differently with the stationary and mobile phases compared to nonpolar molecules.
- **Size:** Larger molecules can be separated from smaller ones, especially in size-exclusion chromatography.
- **Ion Charge:** In ion chromatography, charged molecules are separated based on their interactions with the stationary phase.

Detectors are then used to identify each component as it elutes (exits) the LC column. Common detectors include :

- **UV/Vis Detectors:** Measure the amount of light absorbed by a substance, providing information about its concentration.
- **Liquid Chromatography-Mass Spectrometry (LC-MS):** Offers detailed identification by determining the molecular weight and structure of the components.

These detection methods allow for both qualitative and quantitative analysis of the separated compounds.

II.2.3 Quantification

Liquid chromatography can also quantify the amount of each component in a mixture. by comparing the intensity of the signal from the detector to known standards, the concentration of each separated substance can be calculated accurately.

This quantification is important in quality control, for example, to ensure drugs contain the correct dosage of active ingredients or to measure the levels of pollutants in environmental samples.

II.3 Fundamental Principle of Separation in Liquid Chromatography

Revolves around the differential interactions between the components of a mixture (analytes), the mobile phase (a liquid solvent), and the stationary phase (a solid or liquid phase). These interactions dictate how quickly or slowly different components move through the chromatographic system, leading to their separation. Liquid chromatography can be performed in a variety of modes (Figure.8).

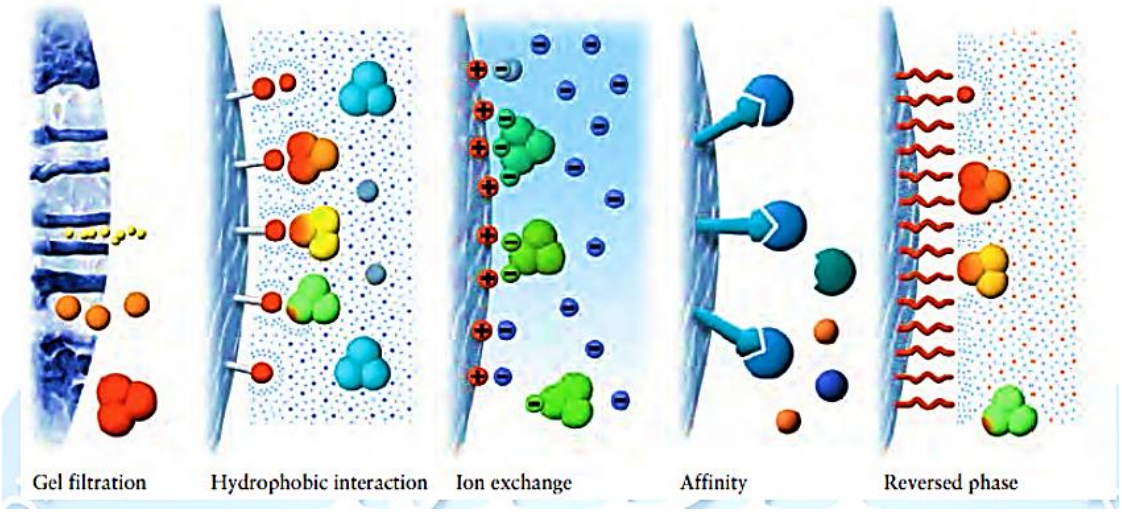


Figure 8 : Principale techniques separation of biomolecules by chromatography

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties, as shown in Figure 8. Ion exchange chromatography (IEX) separates biomolecules according to differences in their net surface charge. Property Technique Charge Ion exchange chromatography (IEX), chromatofocusing (CF) Size Gel filtration (GF), also called size exclusion Hydrophobicity Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC) Biorecognition (ligand specificity) Affinity chromatography (AC). Separation principles in chromatographic purification

II.4 Factors Affecting Separation

The factors affecting chromatographic separation include:

- **Polarity:** Non-polar compounds interact more strongly with a non-polar stationary phase, while polar compounds interact more with a polar stationary phase. This principle forms the basis of normal-phase and reverse-phase chromatography.
- **Size:** In size-exclusion chromatography, separation is based solely on molecular size. Larger molecules elute faster because they are excluded from the pores of the stationary phase, while smaller molecules enter the pores and elute later.
- **Charge:** In ion-exchange chromatography, molecules are separated according to their charge. Oppositely charged molecules are attracted to the charged functional groups on the stationary phase.
- **Specific interactions:** In affinity chromatography, the stationary phase contains ligands that specifically bind to the target molecule, allowing unbound molecules to pass through.

II.5 Components of a Liquid Chromatography System

In LC, each component of the system plays a crucial role in ensuring accurate and efficient separation, identification, and quantification of compounds. Below is a more detailed overview of the key components (Figure. 9)

II.5.1 Pump

The pump is responsible for delivering the mobile phase at a controlled and constant flow rate through the system, typically under high pressure. The role of the pump is crucial in maintaining consistent flow rates and ensuring that the mobile phase moves through the column with sufficient force to achieve good separation.

- **Pressure**: In HPLC systems, pumps typically operate at pressures ranging from 40 bar (600 psi) to over 400 bar (6000 psi), depending on the column type and mobile phase.

- **Flow rate**: The pump controls the flow rate, which is critical for optimizing resolution. Typical flow rates range from 0.1 to 10 mL/min.
- **Gradient control** : Pumps in gradient HPLC systems vary the composition of the mobile phase over time to improve the separation of compounds with different polarities. In isocratic systems, the mobile phase composition remains constant.

II.5.2. Injector

The injector introduces the sample into the flow of the mobile phase. This step must be precise to avoid errors in sample volume, which could affect the results.

Types of injectors :

- **Manual injectors** : These require the operator to manually inject the sample using a syringe, usually via a valve that introduces the sample into the mobile phase stream.
- **Autosamplers** : Automated injectors that can handle multiple samples in sequence. They are often used in high-throughput systems for increased efficiency and reproducibility. Autosamplers also allow for precise control over sample volume.

II.5.3 Column

The column is where the actual separation of compounds occurs, making it the heart of the LC system. It's packed with the stationary phase, which interacts differently with the components of the sample, leading to separation. Types of columns are :

- **Reverse-phase columns:** These are packed with hydrophobic materials such as C18 (octadecylsilane) or C8 (octylsilane), which retain non-polar compounds, allowing polar compounds to elute more quickly.
- **Normal-phase columns**: Contain a polar stationary phase, often silica, which retains polar compounds while non-polar ones elute faster.
- **Ion-exchange columns**: Contain charged resins that attract and retain oppositely charged ions. These columns are widely used for the separation of proteins, peptides, and amino acids.
- **Size-exclusion columns** : Separate molecules based on their size, with larger molecules eluting faster as they do not enter the pores of the stationary phase.

The main column parameters include the column length, internal diameter, particle size of the stationary phase, and pore size, all of which influence the efficiency, resolution, and retention time in liquid chromatography.

- **Length and diameter**: Longer columns provide better separation (higher resolution) but also increase the run time. Typical lengths range from 50 mm to 250 mm, with diameters around 4.6 mm for analytical purposes.
- **Particle size** : Smaller particles in the stationary phase improve resolution but increase pressure. Typical particle sizes range from 3 to 5 µm in HPLC columns.

II.5.4. Detector

The detector identifies the compounds as they elute from the column by measuring a property related to their concentration. The detector sends signals to a data system, which records the response and generates a chromatogram. Common types of detectors are presented in Table.III

- **UV-Visible (UV-Vis) Detector**: The most common type, it measures the absorption of UV or visible light by compounds. Different wavelengths can be used depending on the compound's characteristics. Sensitive and compatible with many compounds. Only works with compounds that absorb UV/visible light.
- **Diode-Array Detector (DAD)**: A type of UV-Vis detector that can scan across a range of wavelengths simultaneously, providing more detailed information about the compounds.
- **Fluorescence Detector**: Measures the fluorescence emitted by compounds upon excitation with a specific wavelength of light. Highly sensitive, useful for detecting trace amounts. Only works with compounds that fluoresce.
- **Refractive Index (RI) Detector**: Measures changes in the refractive index of the mobile phase as compounds elute. Can detect compounds that don’t absorb UV light. Less sensitive than UV detectors and cannot be used with gradient elution.
- **Mass Spectrometry (MS)** : Provides information about the molecular weight and structure of compounds by ionizing them and measuring the mass-to-charge ratio of the ions. Very sensitive and capable of identifying unknown compounds. More expensive and complex than UV detectors.

Table. III : Common detectors for liquid chromatography.

Detector name	Compounds detected	Detection limit[a]
Refractive index detector	General: all compounds	0.1–1 μg
UV–vis absorbance detector	Compounds with chromophores	0.1–1 ng
Evaporative light scattering detector	Nonvolatile compounds	0.1 μg
Fluorescence detector	Fluorescent compounds	1–10 pg
Conductivity detector	Ionic compounds	0.5–1 ng
Electrochemical detector	Electrochemically active compounds	0.01–1 ng
Mass spectrometry	General: full-scan mode	0.1–1 ng
	Selective: SIM mode	

II.5.5 Mobile Phase Reservoirs

The mobile phase reservoir holds the solvents used in chromatography. The choice of mobile phase depends on the type of LC being performed and the chemical properties of the compounds to be separated. Key considerations for the mobile phase include:

- **Solvent composition**: Common solvents include water, methanol, acetonitrile, and buffers (e.g., phosphate buffer). In gradient systems, the composition of the mobile phase changes during the run.
- **Solvent purity** : Impurities in the solvent can interfere with the separation and cause background noise in the detector. High-purity solvents are typically used in LC systems.
- **Degassing** : Dissolved gases in the mobile phase can form bubbles that disrupt the flow and interfere with detection. Degassing methods (e.g., helium sparging, vacuum degassing) are often used to remove gases from the solvent.

II.5.6 Column Oven

The column oven is used to maintain a stable temperature during separation. Temperature affects both the viscosity of the mobile phase and the interaction between the compounds and the stationary phase, so it’s crucial for reproducibility and resolution. The role of temperature control is :

- **Higher temperatures** : Can decrease the viscosity of the mobile phase, allowing for faster flow rates and improved peak shape.

- **Lower temperatures** : May improve separation for heat-sensitive compounds but slow down the process.

II.5.7 Data Acquisition System (Chromatography Software)

The data acquisition system (also called chromatography software) collects and processes the signal from the detector, converting it into a readable form (typically a chromatogram). The chromatogram displays the detector response (e.g., UV absorption) over time, showing peaks for each separated compound. Functions (Figure.9) :

- **Peak identification** : Helps identify compounds based on retention times and detector response.
- **Quantification** : Measures the area under each peak, which is proportional to the concentration of the compound.
- **System control** : Modern chromatography systems are often fully automated, with the software controlling pump flow rates, gradients, and detector settings.

II.5.8 Waste Reservoir

Once the mobile phase has passed through the column and detector, it is collected in a waste reservoir. Proper disposal of the waste is essential, particularly if hazardous chemicals are used.

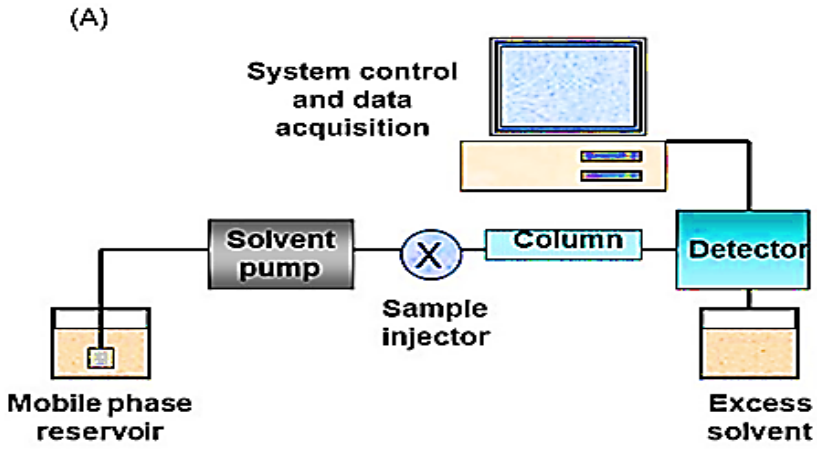


Figure 9: A system for performing high-performance liquid chromatography

II.6 Types of liquid chromatography

Liquid chromatography (LC) comprises various types, each designed to separate compounds based on different principles such as polarity, charge, size, or specific molecular interactions. The choice of method depends on the nature of the sample and the objective of the analysis. The following section outlines the main types of liquid chromatography.

III.1 Introduction

Chromatography is a powerful technique used to separate the components of complex mixtures. Among its different types, adsorption chromatography is one of the earliest and most fundamental. It plays a crucial role in analytical and preparative chemistry, especially in the purification of natural products, pharmaceuticals, and organic compounds. This method relies on the interactions between the sample components and a solid stationary phase, allowing the separation based on differences in polarity and adsorption strength.

III.2 Principle

Adsorption chromatography is a type of LC in which chemicals are retained based on their adsorption and desorption at the surface of the support, which also acts as the stationary phase (adsorbent) (see Figures. 10 and 11). This method is also sometimes referred to as liquid-solid chromatography. 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 (1) the binding strength of this chemical to the support, (2) the surface area of the support, (3) the amount of mobile phase displaced from the support by the chemical, and (4) the binding strength of the mobile phase to the support.

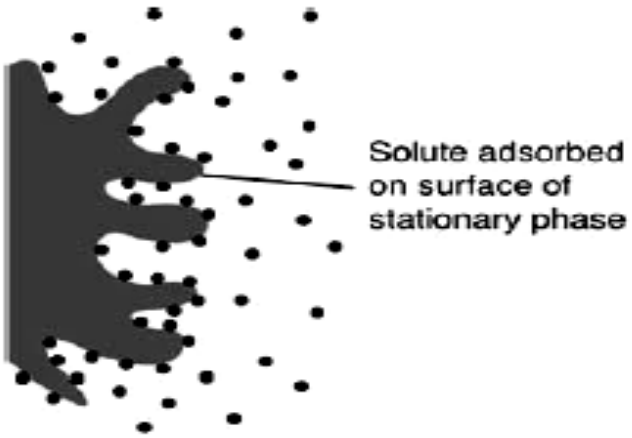


Figure. 10 Adsorption chromatography principle

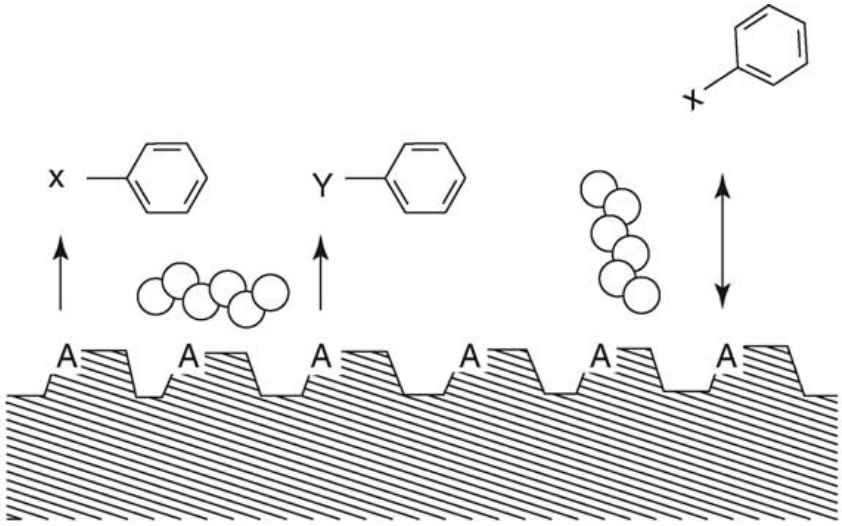


Figure. 11: Separation principles of Adsorption chromatography
the adsorptive sites of the stationary phase are symbolized by A; the solute molecules interact with their polar groups X or Y; the mobile phase drawn is hexane, which can also interact weakly with A.

III.3 Adsorption

Adsorption may be defined as the concentration of solute molecules at the interface of two immiscible phases. In liquid-solid adsorption chromatography (LSAC) the mobile phase is a liquid while the stationary phase is a finely divided, usually porous solid. The atoms in the bulk of the solid are subjected to equal forces in all directions, whereas the surface atoms experience unbalanced forces which can attract molecules from the surrounding solution to restore the balance.

III.3.1 Adsorption isotherms

An adsorption isotherm describes the equilibrium relationship between the concentration of a solute in the adsorbed phase and its concentration in the unadsorbed (or bulk) phase at a given temperature. It is typically represented as a plot of the amount of solute adsorbed onto a solid surface versus its equilibrium concentration in the solution. The adsorption of a solute from a solvent onto a solid surface can be classified according to different types of isotherms, as illustrated in Figure. 12.

1. The S-shape isotherm represents the situation in which, as adsorption proceeds, it becomes easier for the solute molecules to be adsorbed; those already adsorbed on the surface at the most active sites assist further adsorption by intermolecular bonding. It is found that such isotherms are generally, but not always, given by flat molecules standing edge-on to the adsorbent surface, e.g., phenol adsorbed on alumina, where the hydroxyl group probably

forms a hydrogen bond to surface oxygen atoms on the alumina, and the aromatic nucleus associates with other solute molecules.

2. The *L* (or normal Langmuir) isotherm is the most common one met with in LSAC. As adsorption proceeds, the most active sites are first covered by adsorbate and the ease with which adsorption takes place decreases until finally the monolayer is complete, and all the adsorption sites are occupied. This type of isotherm is usually obtained when molecules are adsorbed flat and when there is no intermolecular bonding.

3. The *H* (high-affinity) isotherm starts at a positive value on the ordinate axis, showing that all the solute has been removed from dilute solution. This isotherm is typical of chemisorption.

4. The C (constant-partition) isotherm is linear. This indicates that, as adsorption proceeds, the ease with which it takes place remains constant. This type of isotherm, though common in partition chromatography, is rarely observed in LSAC.

Adsorption isotherms can be further subdivided according to subsequent inflections and plateaus. Usually, a further rise folIowing an initial plateau, or merely an inflection, indicates the formation of a second layer on top of the first, or in some cases, a reorientation of the first layer.

Maxima have been observed in some isotherms, mainly in the *Land H* classes. The solutes involved associate in solution and it has been suggested that at high concentrations the association may draw some of the adsorbed solute back into solution.

Further discussion of nonlinear isotherms and their practical significance follows in the section concerned with sample size in adsorption chromatography.

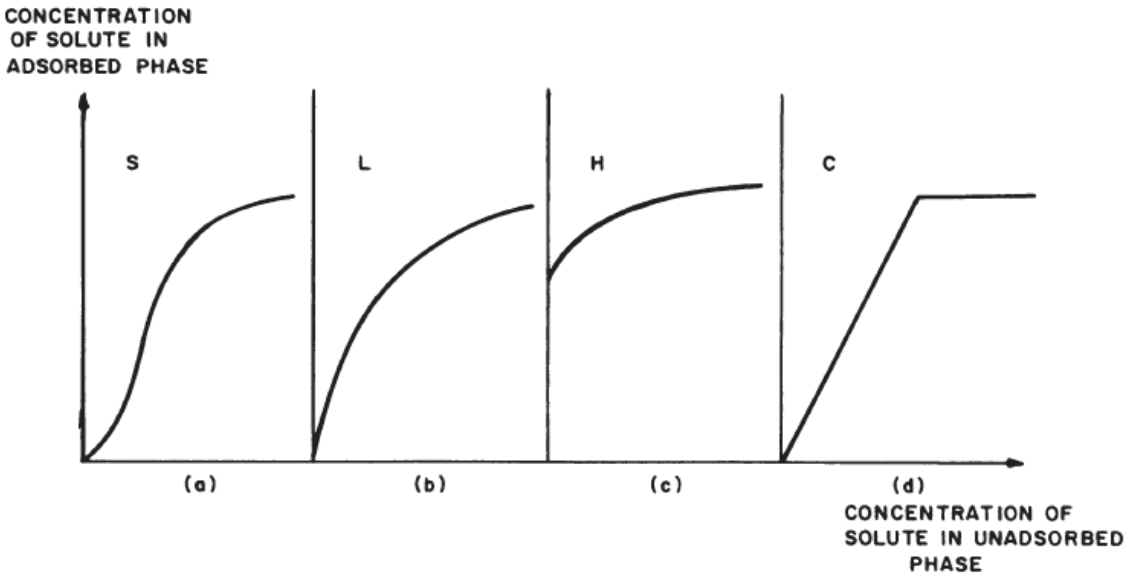


Figure.12: Basic types of adsorption isotherms between a liquid and a solid surface

III.3.2. Nature of adsorption forces

In adsorption chromatography, the forces responsible for the retention of compounds on the stationary phase can be classified into the following categories:

III.3.2.1 Van der Waals Forces (London Dispersion Forces)

These are intramolecular forces which hold neutral molecules together in the liquid or solid state. They are purely physical in character and do not involve the formation of any chemical bonds. Adsorption of this type is known as physical adsorption, and is characterized by low adsorption energies leading to the rapid establishment of equilibria and hence good chromatographic separation. Dispersion forces account for the entire adsorption energy in the case of nonpolar solutes adsorbing onto nonpolar adsorbents—for example, hydrocarbons on graphite. However, Snyder (2) has shown that the contribution of dispersion forces to adsorption on alumina varies significantly: it is around 100% for saturated hydrocarbons but drops to less than 50% for polar molecules such as acetone or methanol.

III.3.2.2 Inductive Forces

These exist when a chemical bond has a permanent electrical field associated with it, e.g., a C-CI or C-NO2. Under the influence of this field, the electrons of an adjacent atom, group, or molecule are polarized so as to give an induced dipole moment. It seems (2) that induction forces make a major contribution to the total adsorption energy on alumina but not on silica.

III.3.2.3 Hydrogen Bonding

These forces make an important contribution to the adsorption energy between solutes having a proton-donor group and a nucleophilic polar surface possessed by, for example, silica or alumina, which is normally covered with hydroxyl groups. Similarly, the hydroxyl groups on the surface may react with other weakly electrophilic groups such as ethers, nitriles, or aromatic hydrocarbons.

III.3.2.4 Charge Transfer

This could occur, for example, when an electron is transferred from a solute S to a surface site *A* to form an adsorbed complex of the type $S^+ A^-$. However, it has been shown (2) that forces due to charge transfer make an insignificant contribution to the adsorption energy of most compounds.

III.3.2.5 Covalent Bonding (Chemisorption)

This occurs when chemical bonds are formed between solute and adsorbent. These relatively strong chemical forces give rise to *H-type* isotherms and generally lead to poor separation in elution chromatography.

Chemisorption is often exploited for the selective retention of certain compound types, e.g., the adsorption of amines by cation-exchange resins, the adsorption of olefins by silver nitrate-impregnated silica. On the other hand, *H-type* isotherms are not uncommon in high-efficiency elution chromatography.

They can be attributed to the chemisorption of certain solutes onto those active sites on the surface of the adsorbent that have not been fully deactivated. For example, silica surfaces may contain some residual acidic sites which chemisorb bases. Similarly, alumina contains basic sites which strongly chemisorb acids. F10risil (magnesium silicate) also contains strong acidic sites and has been observed to chemisorb a wide variety of compounds, including aromatic hydrocarbons, basic nitrogen compounds, and esters, while magnesia chemisorbs polynuclear aromatic hydrocarbons.

As a consequence of chemisorption in columns, certain solutes give rise to strongly tailing elution bands, resulting in incomplete resolution and sample recovery, while in TLC part of the sample is seen to remain behind as a spot at the point of application.

III.4 Adsorbent

III.4.1 Definition

An adsorbent is a material, typically solid and highly porous, with a large surface area that can attract and retain other substances (gases, liquids, or dissolved solids) on its surface through intermolecular forces. Adsorbents are widely used because of their high capacity and selectivity for adsorption. Its main characteristics include the following:

✓ A material having capacity or tendency to adsorb another substance. ✓ A substance that is used to adsorb another substance onto its surface. The stationary phase in chromatography. In thin layer chromatography, the paper of the chromatographic plate is the adsorbent. In column chromatography, a substance such as silica gel is the adsorbent. ✓ A substance that is able to take up a gas, liquid or dissolved substance on a surface in a condensed layer. ✓ A highly porous solid with the ability to concentrate and hold gasses and vapors in contact with the solid. This includes moisture, as well as many other organic and inorganic molecules. ✓ A

material, usually solid, capable of holding gases, liquids and suspended matter at its surface and in exposed pores.

III.4.2 Choice of Adsorbent

III.4.2.1 General Properties of Adsorbents

Adsorbents used for LSAC are finely divided, porous solids with a surface area usual greater than 50 m2 g-l. Table.IV shows the more common materials used as adsorbents, placed approximately in order of increasing strength. Strong adsorbents, i.e., those adsorbents with a relatively high concentration of strongly active sites, are preferred for the separation of weakly adsorbed, chemically inert compounds such as hydrocarbons, while weak adsorbents are preferred for the separation of labile or strongly adsorbed compounds. Although the nature of the adsorbent surfaces has been indicated, this is frequently modified by the presence of free acid or base left over from the manufacturing stage or by the deliberate addition of buffering agents.

Other materials that have been used as adsorbents include calcium sulfate, talc, polyamide, organo-clays, and molecular sieves. A number of group-selective adsorbents have been prepared by impregnating an adsorbent with a material that wiIl form a complex with a specific organic functional group. For example, silver nitrate-impregnated silica gel has been used for the separation of unsaturates.

Salica gel and alumina are by far the two most common adsorbents in use today. It is not now necessary for one to prepare one's own adsorbent since they are readily available commercially. In fact, one can go even further, since precoated TLC plates are now widely used and prepacked, high efficiency columns are gradually being introduced. For a list of suppliers of chromatographic silica and alumina. Table. IV lists some suppliers of other adsorbent materials referred to in this chapter.

In selecting a suitable adsorbent, one needs to consider "adsorbent type" (i.e., strength, polar or nonpolar, surface *pH)* and the surface area and pore diameter. These factors will now be considered in turn.

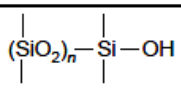
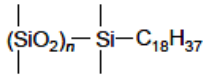
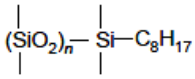
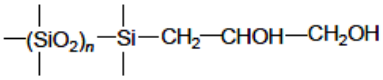
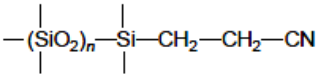
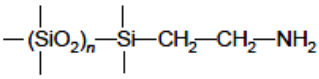
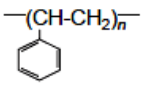
Table.IV: Activated Adsorbents in Approximate Order of Increasing "Strength"

Adsorbent	Nature of active sites
Sucrose	Neutral
Starch	Neutral
Kieselguhr	Neutral
Silica	Acidic
Magnesium silicate	Acidic
Alumina	Acidic and basic
Fuller's earth	Acidic
Magnesia	Basic
Charcoal	Neutral and acidic
Ion-exchange resins	Acid or basic species

III.4.2.2 Adsorbent Type

Different types of adsorbents display varying selectivities toward distinct classes of compounds. Polar adsorbents (such as metal oxides and magnesium silicate) preferentially adsorb unsaturated, aromatic, and polar molecules, including alcohols, amines, and acids. These polar adsorbents can be further classified as acidic, basic, or neutral, depending on the surface pH. For instance, silica, magnesium silicate, and cation-exchange resins exhibit acidic properties and therefore chemisorb basic compounds. Table V presents some important stationary phases used in both GLC and HPLC.

Table V: Important stationary phases for GLC and HPLC

GC		
Silicones		With the proper choice of R and R' a wide range of polarities and special functionalities is available
Polyglycols	$-(O-CH_2-CH_2)_n-OH$	Polar stationary phase: *n* ranges from 4 to 800
LC		
Silica		Three-dimensional network
Octadecyl silica		Reversed phases
Octyl silica		
Diol silica		Polar bonded phases
Nitrile silica		
Amino silica		
Polystyrene		Three-dimensional network due to cross-linking with divinylbenzene
Strong cation exchanger Weak cation exchanger Strong anion exchanger Weak anion exchanger	• $-SO_3^-H^+$ • $-COO^-H^+$ • $-NR_3^+OH^-$ • $-NH_3^+OH^-$	• Can be silica or polystyrene

- **Inorganic adsorbents**

a) Silica gel

Silica or silicon dioxide is the most popular type of adsorbent for LC that is due to simple synthesis of high purity materials with established porous structure and well-defined surface chemistry. The active sites (centers) of silica include five types of weakly acidic silanol groups as shown in Figure. 13.

Thus, silica is considered as a polar stationary phase. In the case of normal phase liquid chromatography (NPLC), these sites interact with the polar analyte functional group/s in nonpolar mobile phase. The properties of specific silica depend on its type (quartz, silica gel, aerosol etc.) and the number of active sites, which is defined by activation treatment procedure such as heating or acid wash. Silica gel is most commonly used to separate amino acids, alkaloids, fatty acids, lipids, steroids, essential oil, sugars, terpenoids etc.

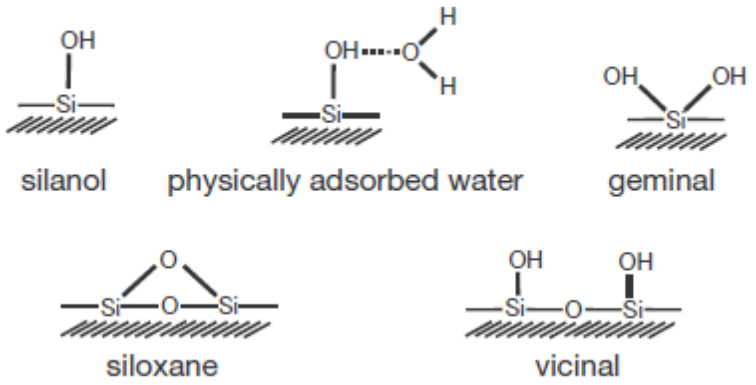


Figure 13: Active adsorption sites on the silica surface

b) Alumina (Al_2O_3)

The reactive sites of alumina are the aluminium cations (Al3þ) and oxygen anions (O_2_). Adsorbents such as alumina are known for their large surface area, high mechanical properties, and good resistivity to thermal degradation. Aluminum oxide or alumina (Al_2O_3) is a solid white ceramic material. Thus, this adsorbent works as polar stationary phase with amphoteric properties. Alumina is a popular stationary phase in TLC.

c) Kieselguhr

Kieselguhr (Diatomaceous earth) has neutral pH. Known also as diatomaceous earth and kieselguhr, diatomite is the classic material for use either as a precoat or as a filter aid. Is the fossilized remains of microscopic algae, several million years old, of which over 10 000 varieties have been recorded. The special properties of diatomite, i.e., light weight (low in density), high porosity and high absorptivity have facilitated its application as filter medium and absorbents. It is chemically inert & highly stable. Diatomite consists of approximately 90 per cent silica and the remainder consists of compounds, such as, aluminium and iron oxides.

d) Magnesia

Magnesia (MgO) often replace alumina. It is too finely divided to allow filtration and can be mixed with filter aid. Active magnesia is obtained by dehydration of the content

e) Polar-Bonded Silica

The polarity of silica surface can be attuned by chemical modification by reaction of silanol groups with various reagents. Chemically bonded silica with immobilized polar and nonpolar functional groups can be prepared. These modified silicas are especially useful for LC applications.

These stationary phases can be obtained by treatment of silica with chloro- and alkoxy-silanes, which can react with silanol groups and provide stable coating of silica with an organic layer as shown in (1).

$$\equiv Si - OH + R_1R_2R_3Si(CH_2)_nF \;=\; \equiv Si - O - Si(R_2R_3)(CH_2)_nF + R_1H \qquad (1)$$

where R1, R2 and R3 are either reactive (chloride-, alkoxy- and others) or alkyl groups, while at least one of them should be reactive to silanol, n –is number of methylene groups or length of linker between surface and terminal functional group F. In the case of polar bonded silica diol-(–CH(OH)–CH2OH), primary amino (–NH2), thiol (–SH) and cyano (–CN) groups are the most common functional groups. The typical representatives of polar bonded silicas are aminopropylsilica, cyanopropylsilica and mercaptopropylsilica.

If terminal functional group is methyl- or phenyl- the resulting stationary phase is nonpolar and it is suitable for reversed phase LC (RPLC).

f) Nonpolar-Bonded Silica

RPLC represents the most applicable chromatographic mode, which is the opposite to NPLC in terms of polarity, namely the use of a polar mobile phase and a nonpolar stationary phase. The separation in RPLC is based on hydrophobic interactions between analytes and nonpolar surface of silica obtained by covalent immobilization of alkyl- or phenylalkyl-molecules. The typical hydrophobisation of silica surface includes reaction of reactive silanol groups with alkylchlorosilanes as shown in (1). The typical moieties grafted to silica are octyl- (–C_8H_{17}) or octadecyl -(–$C_{18}H_{37}$). The notations RP-8 and RP-18 mean that the stationary phases are silica with octyl- and octadecyl- groups covalently bound to the surface.

The stationary phase for liquid–liquid chromatography (LLC) is usually silica coated with a thin film of liquid, which operates as stationary phase itself. The coating can be performed by treatment of the silica by a solution of this liquid in a suitable solvent and subsequent displacement of the solvent by mobile phase. Depending on polarity the prepared liquid stationary phase can be used both in normal phase (NP-LLC) and reversed phase (RP-LLC) modes.

- **Organic adsorbents**: Some organic adsorbents used in TLC are:

a) Cellulose and its acetylates

These adsorbents are fibrous and can be used with relative advantages over paper as the flow is more even and there is less diffusion of the dissolved substances. The flow is also faster. Modified cellulose powders are used to obtain ion-exchange separation in TLC and can

be used with or without binder. Cellulose adsorbed water which brings separation by partition mechanism. These materials are commonly used for separating hydrophilic substances like amino acids, sugars etc.

b) Charcoal and Activated carbon

Charcoal has the specific property of adsorbing strongly aromatic substances. Adsorptive property of activated carbon can be modified by depositing on it a film of a non-electrolyte or a fatty acid.

Surface Area and Pore Diameter

The surface area and pore diameter of a given adsorbent vary widely with the method of manufacture. Probably no two commercial manufacturers produce silicas of the same surface area and pore diameter. The variation in properties of different batches of the same grade of adsorbent from one manufacturer is usually not great. Dramatic changes do occasionally occur, however, presumably due to alterations in process conditions. Attention must be drawn to those instances where the same manufacturer operates plants in more than one location. The properties of the adsorbents from the alternative locations are rarely identical. As a result, the reader is recommended to pay particular attention to the properties of his adsorbent and to standardize on one particular grade where possible.

Adsorbents for chromatography are porous solids with high specific surface areas usually in excess of 50 m^2 g-l to provide high sample capacity.

The surface area increases as the porosity increases and the average pore diameter decreases. The linear adsorption coefficient *KO* of a solute is independent of both these parameters provided the solute molecule is small enough to enter the pores unimpeded and provided the nature of the active surface sites is independent of the pore diameter. We shall see that in the case of silica gel, for example, large differences in pore diameter correspond to differences in surface structure.

III.5 Choice of Mobile Phase

The choice of the mobile phase is critical to achieving efficient separation of components in a mixture. The mobile phase in this technique moves through a stationary phase, and the interactions between the analytes, the mobile phase, and the stationary phase determine how quickly each component moves.

III.5.1 Polarity of the Mobile Phase

There are various degrees of polarity, ranging from non-polar to highly polar. The choice of mobile phase depends on matching its polarity with that of the analytes and the stationary phase.

- **Polarity Matching**: The mobile phase should be chosen based on the polarity of the analytes and the stationary phase. A polar mobile phase competes with the analyte for adsorption sites on the stationary phase.
    - **Polar Stationary Phase** (e.g., silica gel): Use a less polar mobile phase (e.g., hexane, toluene) to allow non-polar analytes to interact strongly with the stationary phase and separate well.
    - **Non-polar Stationary Phase**: A polar mobile phase (e.g., methanol, water) is used to interact with polar analytes.
- **Gradient Elution**: Sometimes, a combination of solvents is used, starting with a less polar solvent and gradually increasing the polarity (e.g., in liquid chromatography, switching from hexane to acetone or methanol) to allow better separation over time.

III.5.2 Strength of Solvent

The strength of the solvent depends on:

- **Eluting Power**: The eluting power of a solvent refers to its ability to move the analyte through the stationary phase.
    - **Weak Solvent**: A weaker solvent results in longer retention times because the analyte strongly interacts with the stationary phase.
    - **Strong Solvent**: A stronger solvent reduces retention time, pushing the analytes through the stationary phase faster by weakening interactions with the stationary phase.
- **Eluotropic Series**: Solvents are often ranked in terms of their eluting power in an **eluotropic series** (e.g., for silica, pentane is weak, while methanol is strong).

III.5.3 Viscosity of the Mobile Phase

In adsorption chromatography, the viscosity of the mobile phase is an important factor that affects how efficiently compounds are separated. When a solvent is highly viscous, it moves more slowly through the column, which can lead to several problems. First, it increases the backpressure in the system, which may require more effort to maintain a steady flow and could even damage the equipment. Second, it slows down the movement of analytes, resulting in longer analysis times. Finally, it can cause the separation peaks to become wider and less distinct, reducing the overall resolution of the chromatogram. Common mobile phases like methanol, acetonitrile, or mixtures of water and acetonitrile offer low viscosity, ideal for many chromatography systems.

III. 5.4 Solubility of the Analytes

The mobile phase must adequately dissolve the analytes to allow them to be transported through the column. If analytes are not soluble in the mobile phase, they will not migrate and cannot be separated effectively.

III.5.5 Compatibility with Detection Methods

The choice of the mobile phase must also be compatible with the detection method used in the chromatographic system. For example:

- **UV Detection**: Avoid solvents that absorb strongly in the UV region.
- **Mass Spectrometry**: Volatile solvents like acetonitrile or methanol are preferred to prevent contamination of the ion source.

III. 5.6 pH and Buffering

Choosing the appropriate buffer system and pH range is essential to optimize selectivity and peak shape in chromatographic analysis.

- **pH Control**: The pH of the mobile phase can influence the ionization of analytes, affecting their interaction with the stationary phase. Adjusting the pH can optimize separation for weak acids or bases.
- **Buffers**: In some cases, buffers (e.g., phosphate, acetate) are added to the mobile phase to maintain a consistent pH throughout the separation process.

III.6 Common Mobile Phases

The choice of mobile phase is critical to achieving effective separation. Common mobile phases include :

- **Non-polar solvents**: Hexane, heptane, cyclohexane (used for non-polar analytes).
- **Moderately polar solvents**: Ethyl acetate, dichloromethane, toluene (used for medium-polarity compounds).
- **Polar solvents**: Methanol, ethanol, water, acetonitrile (used for polar analytes).

VI.1 Introduction

Size-exclusion chromatography (SEC), also known as molecular exclusion, gel permeation (GPC), and gel-filtration chromatography (GFC), is probably the easiest mode of chromatography to perform and to understand. The fourth general type of LC is size-exclusion chromatography (SEC). This is a liquid chromatographic technique that separates substances according to their size. This technique is based on the different ability of analytes to access mobile phase within the pores of a support. No true stationary phase is present in this system. Instead, the mobile phase in the pores acts as the stationary phase. SEC uses a support that has a certain range of pore sizes. As solutes travel through this support, small molecules can enter the pores while large molecules cannot. Larger molecules can enter a smaller volume of the column, so they elute before smaller molecules. The result is a separation based on size or mass.

It is widely used in the biological sciences for the resolution of macromolecules, such as proteins and carbohydrates, and also is used for the fractionation and characterization of synthetic polymers. Unfortunately, nomenclature associated with this separation mode developed independently in the literature of the life sciences and in the field of polymer chemistry, resulting in inconsistencies. In the ideal SEC system, molecules are separated solely on the basis of their size ; no interaction occurs between solutes and the stationary phase. In the event that solute/support interactions do occur, the separation mode is termed nonideal SEC.

VI.2 Principle separation

The main principle is the separation mainly based on the sizes and shapes of the molecular sieves with porous material that is gels are commonly employed Figure.30. High molecular weight compounds are eluted first and the low molecular weight compounds are eluted later.

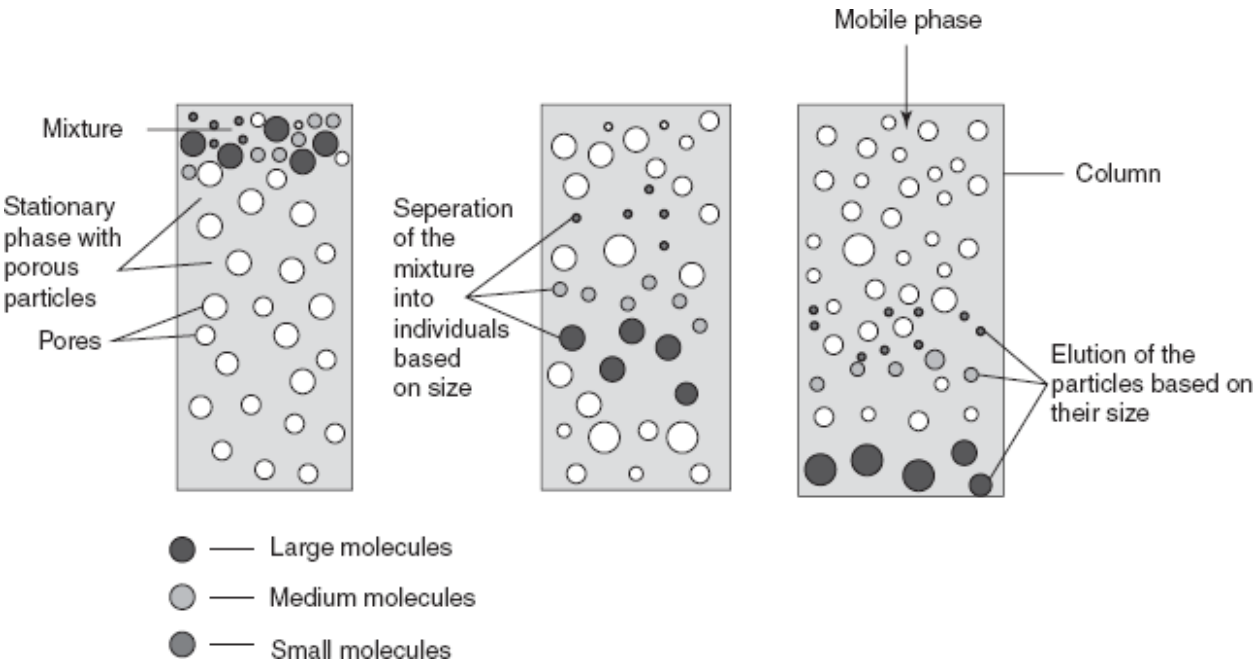


Figure. 30 : Separation technique

VI.3 Basic theory

This section describes some theory and expressions used to define operating parameters in SEC. For a more extensive description, see reference. Results from SEC experiments are usually expressed as an elution profile or chromatogram that illustrates the variation in concentration of eluted sample components (for proteins typically shown as UV absorbance, at wavelength 280nm) as they elute from the column in order of their apparent size. Figure. 31, shows a hypothetical chromatogram of a SEC fractionation. The total volume of a column, CV, can be divided in different partial volumes which are conceptually depicted in Fig. 31.

Molecules that are too large to enter pores in the matrix are eluted together in the void volume, V0 (Figures. 31, 32), as they pass directly through the column at the same speed as the flow of eluent. For a well-packed SEC column, V0 is approximately 30% of the CV. Molecules with partial access to the pores of the matrix, elute from the column in order of decreasing size, that is, the smaller the molecule, the larger the accessible pore volume and the later the elution. Small molecules such as salts that have full access to the intraparticle pores, move through the column, but do not separate from each other. These molecules elute at the total liquid volume CV−Vs=Vt (Figure. 31), just before one CV for resins that have a low matrix content (i.e., a very high proportion of intraparticle pore volume, Vi, relative to particle volume, Vp).

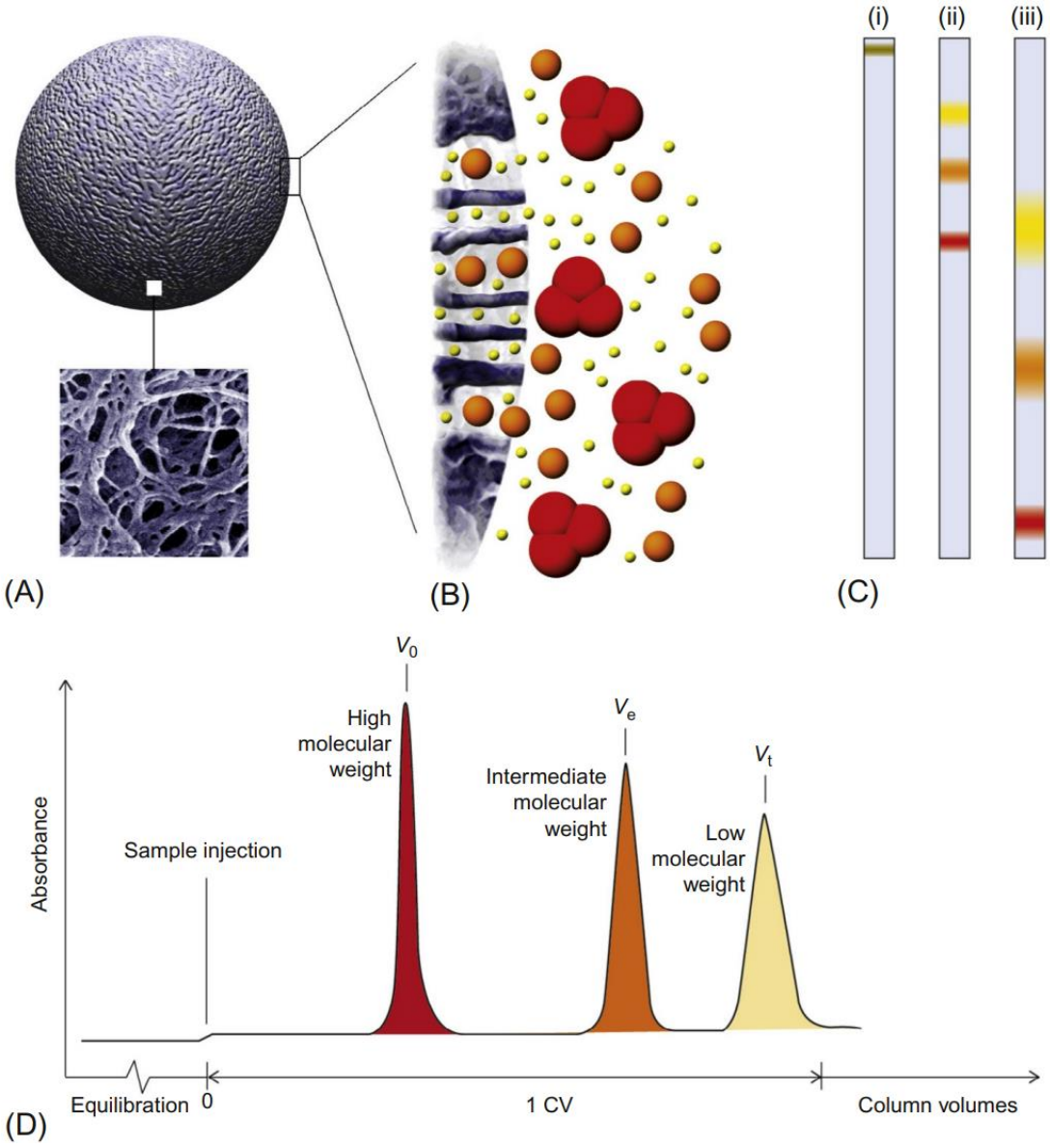


Figure. 31 : Size-exclusion chromatography principle

(A) Schematic picture of a chromatography resin bead with an inserted electron microscopic enlargement. (B) Schematic drawing of sample molecules diffusing into or being excluded from the bead pores. (C) Graphical description of separation: (I) sample applied on the column; (II) the smallest molecule (yellow) is more delayed than the largest molecule (red); (III) the largest molecule is eluted first from the column. Band broadening causes significant dilution of the protein zones during SEC. (D) Hypothetical chromatogram. Courtesy of GE Healthcare. Size Exclusion Chromatography : Principles and Methods, GE Healthcare Life Sciences, 18-1022-18.

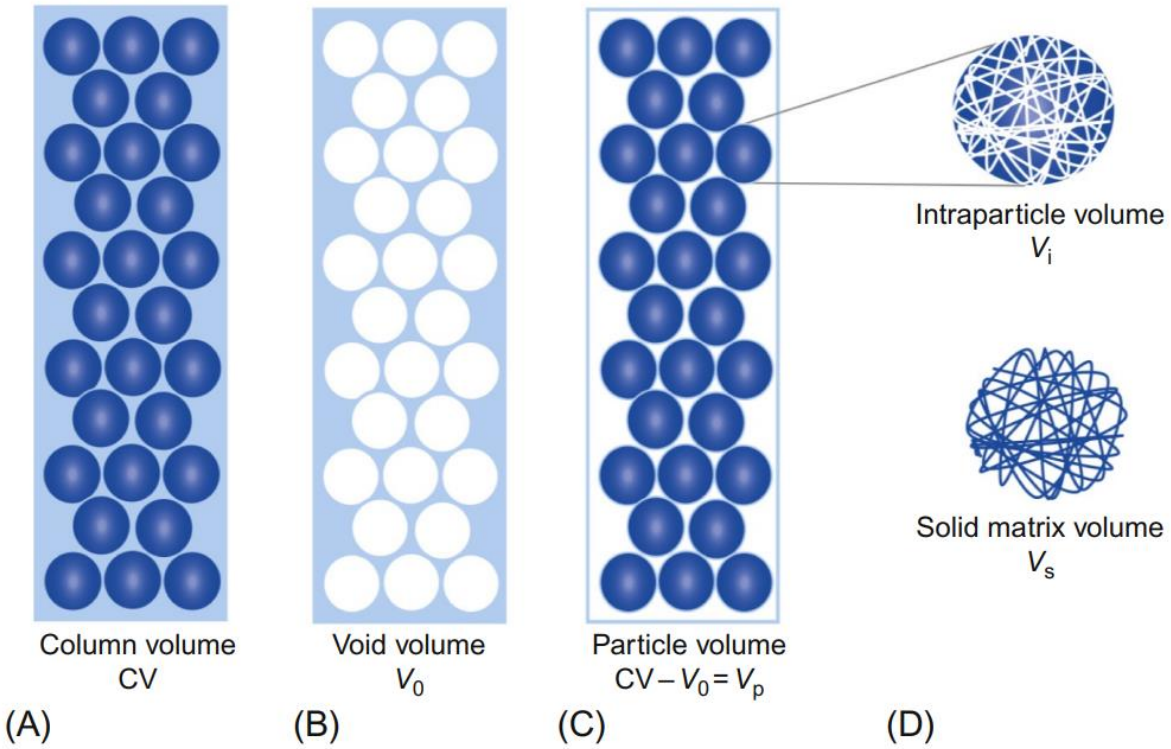


Figure. 32 : Diagrammatic representation of a column and its subset of volumes indicated in blue.

From left to right, the total column volume, CV (A), the void volume, V0 (B), and the resin particles volume, Vp (C), where Vp is made up of the intraparticle pore volume, Vi (D upper), and the volume of the solid matrix, Vs (D lower). Courtesy of GE Healthcare, adapted from L. Fischer, An introduction to gel chromatography, Laboratory Techniques in Biochemistry and Molecular Biology, vol. 1 part II, North Holland Publishing Company, Amsterdam, 1969

The partition ratio between the stationary phase and mobile phase is defined by the distribution coefficient (K_d)

$$(K_d) = (V_e - V_o) / V_i$$

Where V_e is the elution volume that is volume of the solvent between the injection and elution ; V_o is the void volume that is the porous particles occupied volume ; V_i is the inner volume that is the volume of the solvent held in the packing material.

The total volume is given by the following equation :

$$V_t = V_g + V_i + V_o$$

Where V_t is the total volume ; V_g is the volume of the polymer gel; V_i is the volume of solvent held in the pores; V_o is the free volume which is present outside of the particles.

In the ideal SEC system, molecules are separated solely on the basis of their size ; no interaction occurs between solutes and the stationary phase. In the event that solute/support interactions do occur, the separation mode is termed nonideal SEC.

The stationary phase in SEC consists of a column packing material that contains pores comparable in size to the molecules to be fractionated. Solutes too large to enter the pores travel with the mobile phase inthe interstitial space (between particles) outside the pores. Thus, the largest molecules are eluted first from an SEC column. The volume of the mobile phase in the column, termed the column void volume, Vo, can be measured by chromatographing a very large (totally excluded) species, such as Blue Dextran, a dye of MW = 2 × 106.

VI.4 Stationary phases

Stationary phases in SEC can be based on porous silica but also on other inorganic materials and very commonly on organic polymeric materials. Polymeric materials for making the stationary phase are more frequently used in SEC than in other HPLC procedures. In size exclusion chromatography, both classical particulate and monolithic packing materials are used.

Monoliths are novel materials for this chromatographic technique. The advantage of monolithic stationary phases is that they contain a significant amount of small pores within their pore size distribution. In each column filled with porous particles, intergranular pores are formed, through which the mobile phase flows and fills a large number of open micropores inside the grains. In monolithic columns, the filling contains both large-sized mesopores and micropores within the “solid matrix”, though the liquid hardly flows through the micropores.
The pores within the particulate column packing material, or the micropores in the monolithic columns, always have different diameters to some extent The column matrix also known as resin consists of microscopic beads of inert material. In size exclusion chromatography columns, the mean value and the characteristics of the pore diameter distribution are selected based on the hydrodynamic radius of the separation particles. This is related to the molecular weight distribution of the molecules in sample. The stationary phases, used in SEC (Table. X), are divided into the following types :

1. Organic and non-organic—based on the chemical structure;

2. Wet swollen and hard—relates to the interactions with the mobile phase;

3. Lipophilic and hydrophilic—based on the properties of the substances to be separated.

Table X : Classification and characterization of stationary phases used in size exclusion chromatography (SEC)

Types of stationary phases for SEC				Characteristic
	Feature	Name	Examples	
Classical packing material	Chemical structure	Organic	Dextran based, e.g. Sephadex LH-20, Sephacryl	Physically stable
			Agarose based, e.g. Sepharos, Biogel A, Ultragel A, Work Beads 17 SEC, Superose 12 HR	Wide pore distribution
			Acrylate, methacrylate, e.g. Biogel P, Ultragel ACA	
			Polystyrene—divinylbenzene, e.g. PS-DVB, Lichrogel PS 1-4000, TSK gel G1000-G7000	
			Branched polymers	
		non-organic	Silica, e.g. Lichrospher SI 100-4000, TSK-GEL SW 2000-4000	Deactivated surface sorption
			Porous glass, e.g. CPG 40-3000	High physical stability
			Zirconium oxide aluminum oxide	
	Interaction with mobile phase	Wet swollen	Polymethacrylate, e.g. Toyopearl HW-40	Requires preparation before use—swelling
			Hydroxypropylated dextran, e.g. Sephadex LH-20	High mass transfer rate Low back pressure
		Hard	Methacrylate co-polymer, e.g. Toyopearl HW-40	Deactivated surface sorption (blocks strongly polar groups, e.g. −OH and =O, on the surface)
			Poly(styrene-co-divinylbenzene, e.g. TSK gel $H_{HR}GMH_{HR}$	
			Silica	
			Porous glass, e.g. BioGlas	
			Zirconium oxide	
	Properties of the substances forseparation	Lipophilic	Dextran based, e.g. Sephadex LH-20	High resolution separation
			Poly(styrene-co-divinylbenzene, e.g. TSK gel $H_{HR}GMH_{HR}$	Excellent reproducibility, excellent durability
		Hydrophilic	Silica	Deactivated surface sorption (blocks the strongly polar −OH group)
			Porous glass, e.g. BioGlas	
			Methacrylate, e.g. Biogel	
Monolithic packing material	Chemical structure	Organic	Polymeric monolith, e.g. methacrylate – based, polystyrene-co-divinylbenzene	Highly porous
		Non-organic	Silica monolith, e.g. Chromolith	Highly homogeneous More suitable for high resolution Exhibit a distinct bimodal pore volume distribution with macropores and mesopores—fast separations at very low column back pressures High column performance

VI.5 Pore size

Another important parameter for column selection is the proper choice of sorbent porosity. The molar mass range of the samples to be investigated determines the column porosity. The larger the pores, the higher molar mass samples can be characterized. Unfortunately, there is no general nomenclature, which will allow easy selection of column pore sizes. Each manufacturer has its own system for pore size designation.

In general, SEC columns can be either single porosity columns with narrow pore size distribution or linear (also called mixed-bed) or multipore columns with a very broad pore size distribution (Figure.33). SEC separation capacity is limited by the available pore volume and depends on sorbent type, column dimensions and the slope of the calibration curve. The highest selectivity for a separation is determined by the lowest slope of the calibration curve.
For single porosity columns the separation capacity is concentrated in a narrow molar mass range. This yields a calibration curve with a flat or shallow slope in this region. Therefore, single porosity columns have a limited molar mass separation range, but a high resolution in that range. In contrast to that, columns with a broad pore size distribution provide a larger separation range and the calibration curve has a steeper slope and therefore less resolution.

Often linear or mixed-bed columns are either used in QC for fast screening experiments or to identify the molar mass range of a sample, so that it can be investigated on a matching single porosity column bank with higher precision.

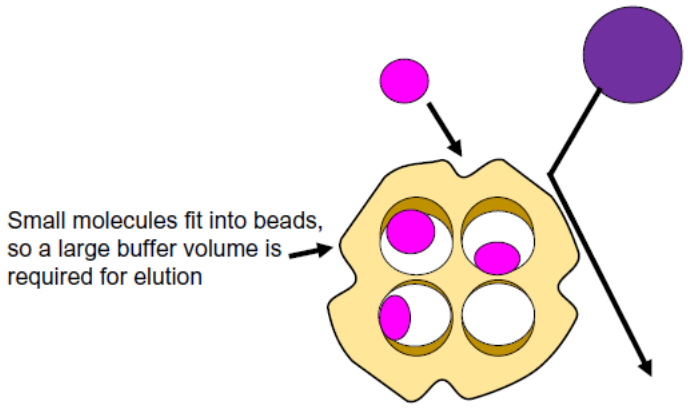


Figure. 33 : Size exclusion chromatography column.
Larger molecules are excluded from gel beads and emerge from the column sooner while smaller molecules must navigate the intricate network of pores and exclude later**.**

VI.6 Mobile Phase

A prerequisite for size exclusion chromatography is that the mobile phase (the eluent) be a good solvent for the analyte. The mobile phase must be selected to avoid destroying the column packing material, and the elution strength should be sufficient to eliminate adsorption of the polymer molecules to the surfaces of the packing material. Selection of the mobile phase determines what would be an appropriate stationary phase. Generally, the mobile phases can be divided into two basic groups, depending on the separation conditions :

a. Non-aqueous, i.e. lipophilic conditions

Under non-aqueous, lipophilic conditions, solvents such as tetrahydrofuran, dioxane, tetrachloroethylene, chlorobenzene, dichlorobenzene, toluene, and xylene are commonly used. As stationary phases, copolymers of styrene and divinylbenzene or polyesters are preferred due to their resistance to organic solvents. This system is used to determine the molecular weight and molecular weight distribution of polymers with low to medium polarity that are soluble in non-polar solvents (e.g., polystyrene in toluene), as well as lipids, phospholipids, waxes, and other non-polar substances of natural origin.

b. Aqueous solutions or strongly polar non-aqueous (hydrophilic) eluents

Eluents such as dimethylformamide, methanol, acetonitrile, and their mixtures with water, as well as aqueous solutions of salts, acids, and bases, are commonly used. As stationary phases, polar materials such as polydextranes and other polysaccharides, polycarbonate, porous glass,

or silanized silica gel are employed. These systems are used for the separation and characterization of molecular weight distributions of polar polymers (e.g., polyethylene glycol in water), particularly biopolymers such as polysaccharides, proteins, and nucleotides.

VI.7 Ideal Size Exclusion Mechanism

The principal mechanism involved in separation by exclusion chromatography is not very complicated. The analytes are separated between the mobile and the stationary phase based on their size difference. In this system, the mobile phases carries some of the components in the injected sample and delivers them to the stationary phase. The latter is characterized by a porous structure, which in turn determines the nature of separation mechanism.

In exclusion chromatography separation is based on differences in flow delays for molecules with different hydrodynamic radius, which in practice means molecular weight. This is a result of molecules diffusing into the solvent trapped in the pores of the stationary phase (Figure.34). Particles that are larger than the pore size (with the largest hydrodynamic radius) will not diffuse to the pores, but pass in the space between them. This condition is called exclusion. Total exclusion (exclusion limit) occurs for particles that do not pass into any of the pores. This process occurs rapidly for molecules with the largest molecular weight or molecular size which means that they are eluted in the beginning of the separation process and constitute the first peak in the chromatogram. The smaller are the particles, larger is the available pore volume, which extends the flow path through the column. If the molecules are small enough to penetrate into all the pores of the stationary phase, then the entire mobile phase volume is available for non-limited diffusion, and this is called the penetration limit. Between exclusion limit and penetration limit, the specific size exclusion region (separation region) exists. It is characteristic for each SEC chromatographic column, which allows to determine the molecular weight from the largest molecule that is fully included in the pore-volume to the smallest molecule that is fully excluded.

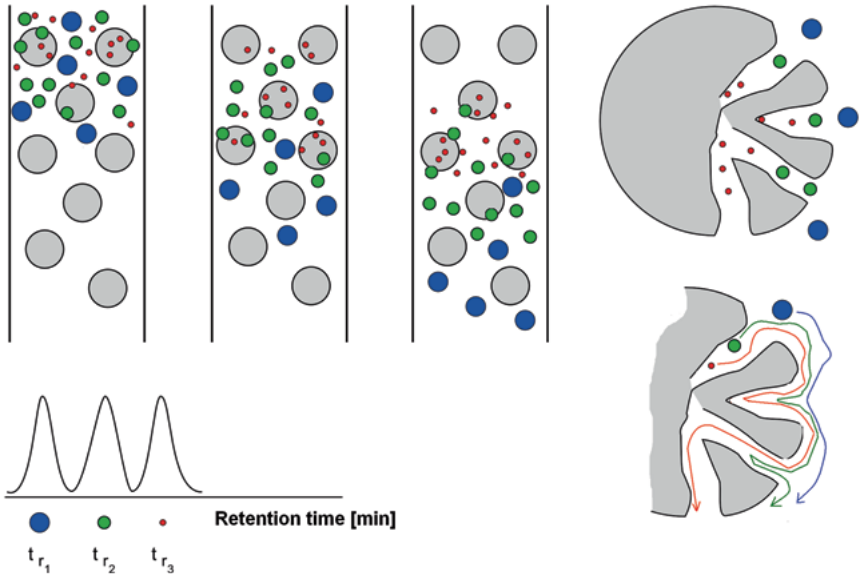


Figure.34 : Principal mechanism for separating molecule mixtures by size exclusion chromatography

VI.8 Non-Ideal Size Exclusion Mechanism

Even though size exclusion is the main retention mechanism in SEC, it is not the only one. Generally, the goal is to eliminate sorption interactions between the surfaces of the column packing particles and the separated substances, but this is not always possible. Then the separation mechanism becomes thermodynamically more complicated. Sorption interactions are primarily associated with adsorption phenomena between the analyte and stationary phase packing. This is defined as the mechanism of adsorption and surface interaction. In this mechanism, the adsorbate- adsorbent interaction relies on Van der Waals forces or formation of hydrogen bonds. The Van der Waals interaction is important for the creation of the so-called instantaneous dipole moment, or London dispersion forces.

Many authors have drawn attention to the phenomenon of adsorption, and thermodynamic imbalance by entropy (ΔS) and enthalpy (ΔH) changes during the separation. Adsorption is a spontaneous process and is associated with thermodynamic changes (thermodynamic functions). To describe the effects associated with the adsorption energy, several thermodynamic terms have to be used, e.g. free enthalpy of adsorption, adsorption entropy, and adsorption enthalpy. Therefore, in size exclusion chromatography the entropic and enthalpic mechanisms exist. The entropic retention mechanism relates to the size exclusion that results from the variation in particle size of molecules. Changes in entropy are caused by concentration gradients, the flow within the column, and the diffusion process. This results in partial or full exclusion of macromolecules from the pores or from the outer surface

of the sorbent particles. This entropic mechanism is recognized as the basic retention mechanism in SEC. If there are interactions between the analyte and the stationary phase, or between the mobile and stationary phase, this adds an additional enthalpic retention mechanism.

Additional retention mechanisms can add undesirable effect, e.g: lower selectivity of column and the erroneous molar weight estimation. Some examples to cite are

• Peak broadening due to inappropriate choice of column ;

• Diffusion effects ;

• Inadequate separation of peaks in the mixture ;

• Illogical elution order of the components in the mixture (polymer molecules that strongly bind to the sorbent will leave the column later regardless of their size).

To avoid damaging adsorption phenomena, one can try to change either the elution strength or the flow rate of the mobile phase, or the entire column.

VI.9 Applications

SXC has been used as a purification technique for a variety of molecules. The targets that were purified are listed in Table XI, including process performance.

Table XI: Characteristics of targets that have been purified by SXC with respective process performance.

Target	Size	Shape	Maximum Recovery	Impurity Removal
Bacteriophage M13KO7	916 × 7.2 nm	rod	>90%	>99% HCP, 93% DNA
IgM	25–35 nm diameter [4]	radial pentamer	≤90%	95% HCP
IgG	12 nm diameter [5]	Y-shaped	87%	99% HCP
γ-globulin	12–35 nm diameter	various	n.a.	n.a.
Influenza A virus	80–120 nm diameter	spherical, sometimes filamentous	>95%	>99% HCD, 92% HCP
ssRNA dsRNA	700–6374 nt and 500–6374 bp length	various	>90% retained on the column	n.a.
Baculovirus [1]	200–300 nm × 30–60 nm	rod	91%	>99% total protein, 85% total DNA
Parapoxvirus ovis (Orf)	220–300 nm × 140–200 nm	rod	67% to >90%	>98% total protein, >60% total DNA
Hepatitis C virus	30–80 nm diameter	spherical	>97%	>99% total protein, 84% total DNA
OP7 [2]	80–90 nm diameter	spherical	n.a.	89% total protein
Adeno-associated virus (AAV)	20 nm diameter	near spherical	>95%	≥80% total protein, ≥94% total DNA
Lentiviral vector	120 nm diameter	spherical	≥86%	80% total protein and total DNA
Latex particles [3]	190 nm diameter	spherical	≤21% for CPS, ≤7% for CPH	n.a.

Abbreviations: HCP—host cell proteins, HCD—host cell DNA, n.a.–not applicable.

The overall process performance for all targets was, except for the latex particles, very good, yielding recovery of 85% or higher under optimized conditions. Here, it must be noted that the use of latex particles was intended to serve as a model for biological nanoparticles of

comparable size, as this would enable faster process development. However, the latex particles remained largely in the stationary phase during elution and were therefore not suited as a model. Impurity removal is another important aspect of the purification method and demonstrates the selectivity to retain mainly the target. By this, 80% or more of the contaminating proteins were removed, often reaching the detection limit of the analytical protein assays. The removal of DNA was 60% or higher (see table XI).

Steric exclusion chromatography (SXC) has emerged as a promising technique for purifying large, fragile biomolecules such as enveloped viral vectors. Unlike conventional methods like anion exchange chromatography (AEX), which often reduce virus infectivity due to harsh elution conditions (e.g., high salt or pH changes), SXC operates under mild conditions that preserve viral integrity. Its size-dependent separation mechanism, which does not rely on electrostatic interactions, also facilitates protocol transfer to a variety of targets, including viruses, extracellular vesicles, and virus-like particles. Given the structural diversity of viruses, SXC offers a valuable alternative in viral bioprocessing, although challenges remain due to the lack of a universal purification strategy.

For antibodies, which were used as a target species in the very first SXC publications, other chromatography techniques such as protein A affinity chromatography and ion exchange chromatography are well established, and hence present no need to establish SXC to purify antibodies. We therefore assume that no further research on γ-globulin purification by SXC was published since then.

Recently, a study was published using SXC as a sample preparation technique for physicochemical characterization (including size distribution, electrophoretic mobility, and visual appearance by electron microscopy) of Orf viral vectors. It was reported that sample preparation with SXC was superior to commonly performed ultracentrifugation methods, which are time-consuming and costly. Hence, SXC might offer the opportunity to prepare a desired target of any size and shape for subsequent characterization methods such as electron microscopy, which requires purified samples.

SXC is a versatile platform technology that can be applied to a wide range of targets with varying sizes, shapes, and surface properties, thanks to its non-chemical, size-based separation principle. This makes it especially suitable for sensitive biomolecules such as enveloped viruses, virus-like particles (VLPs), and exosomes, as it enables gentle elution. However, the industrial implementation of SXC is still in its early stages. Most studies have been conducted at the research scale, with only one reported large-scale application—for an Orf vaccine—which was unsuccessful. Scalability remains a key challenge.

SXC can be used at different stages of downstream processing (DSP) for viral vectors, typically after clarification, and does not require feed material pretreatment like other chromatography methods. Sometimes, tangential-flow filtration (TFF) is used before SXC to concentrate the product. After SXC, additional purification steps such as SCMA, Capto™ Core 700, HIC, or AEX may be applied, and a combination of two chromatography steps may be enough for regulatory approval, depending on the dose. However, residual PEG from SXC must be removed, usually by ultrafiltration and diafiltration, although the effectiveness of PEG removal needs further verification. High PEG levels in the eluate are a concern due to potential immunogenicity, so measuring residual PEG before drug release is important. Although rare, PEG allergies have been reported, such as with the Comirnaty® vaccine, but PEG is generally considered safe and not a barrier to market approval.

VI.10 Recent advances in SEC

VI.10.1 Narrow-bore and micro-bore SEC columns

There is a growing demand in the pharmaceutical and biotechnology industries for smaller, more sensitive SEC (Size Exclusion Chromatography) techniques to reduce sample use and enhance compatibility with mass spectrometry. This has led to efforts to develop SEC columns with smaller diameters. However, because SEC columns have very low column peak variance (CPV), extra-column dispersion (ECD) can greatly affect their efficiency. Since CPV decreases with smaller column volumes, using larger columns can help maintain efficiency by increasing CPV, despite the trend toward miniaturization.

There is a known challenge in size-exclusion chromatography (SEC): while small column volumes are desirable for improved sensitivity and reduced sample consumption, miniaturized columns often suffer from extra-column dispersion (ECD), which negatively impacts performance. Studies, including those by Eksteen, have shown that reducing the internal diameter (ID) of SEC columns (from 4.6 mm to 1 mm or even 300 μm) significantly enhances detection sensitivity, particularly useful for analyzing small protein quantities and antibody fragments. Capillary and microflow SEC columns have also demonstrated potential in coupling with native mass spectrometry (MS), yielding up to 100-fold increases in MS sensitivity. However, issues such as tubing and system dispersion still limit chromatographic efficiency at these scales. Recent work on 2.1 mm ID columns reinforces the importance of minimizing system dispersion for successful SEC miniaturization.

A 150 × 2.1 mm SEC column offers a promising compromise between separation efficiency, low sample consumption, and compatibility with mass spectrometry (MS). On

commercial UHPLC systems, it is possible to achieve up to 50% of the column's theoretical efficiency. Additionally, past estimates of SEC column performance may have underestimated intrinsic efficiency due to band broadening from non-specific interactions with column hardware, particularly frits. Recent advancements in low-adsorption hardware have reduced these interactions, allowing for lower dispersion measurements and more accurate modeling of column efficiency.

VI.10.2 Low-adsorption ("bioinert") SEC column hardware

Undesired secondary interactions, particularly with column hardware, can significantly impact SEC separations. Recent studies have highlighted the adsorptive loss of negatively charged analytes to stainless steel surfaces, which are present in both column components and instrument flow paths. While alternative materials (like titanium or nickel-cobalt alloys) and mobile phase additives (e.g., chelators such as medronic acid or EDTA) have shown some improvements, they do not fully eliminate these interactions. Surface masking with protective layers has proven more effective. Polyether ether ketone (PEEK) is commonly used for this purpose and generally improves analyte recovery (see Table XII). However, due to its hydrophobic nature, PEEK can itself lead to increased adsorption of certain analytes—particularly in aqueous SEC applications involving large biopharmaceuticals and aggregates.
Hybrid Surface Technology (HST), featuring vapor-deposited hybrid silica with exposed ethylene bridges and partially capped silanols, was found to be suboptimal for SEC separations due to secondary interactions. To improve compatibility with aqueous chromatography, a hydrophilic modification was introduced, resulting in hydrophilic HST (h-HST) hardware. Columns made with h-HST demonstrated improved recovery and peak shape, particularly for high-molecular-weight species (HMWS), due to reduced electrostatic and hydrophobic interactions. While other low-adsorption column coatings exist, many are not specifically designed for aqueous SEC. A recent comparative study confirmed h-HST columns as the most effective at minimizing analyte interactions. As biopharmaceuticals continue to increase in complexity, low-adsorption hardware such as h-HST is expected to play an increasingly critical role in successful SEC separations.

Table XII : Overview of low-adsorption SEC columns and used technology currently avail able on the market.

Manufacturer	Column Example	Technology	Details
Waters	XBridge Premier BEH SEC	h-HST	C, O hydrophilically modified ethylene-bridged hybrid surface
Phenomenex	Biozen SEC	BioTi	Titanium lining and frit
Tosoh	TSKgel BioAssist SEC	PEEK	PEEK housing
Sepax	Zenix and SRT SEC	PEEK	PEEK housing
YMC	YMC-Pack Diol SEC	PEEK	PEEK lining and frit
Agilent	AdvanceBio SEC	PEEK	PEEK lining and frit

Technical specifications (hardware and/or frit material) were taken from publicly available sources on manufacturers' websites or brochures

VI.10.3 Ultra-wide pore SEC columns for large molecules

Ultra-wide pore Size Exclusion Chromatography (SEC) columns are specialized tools designed for separating and analyzing very large molecules, such as high molecular weight polymers, proteins, and biopolymers. These columns have exceptionally large pores, typically ranging from 1000 Å to over 10,000 Å, to accommodate the dimensions of macromolecules without exclusion.

Applications :

Separation of ultra-high molecular weight compounds. Analysis of aggregates or conjugates (e.g., protein-protein interactions or protein-polymer complexes). Characterization of synthetic polymers, polysaccharides, and biopolymers.

Stationary Phase :

Typically composed of silica, polymethacrylate, or agarose, depending on the application.

Designed to be inert and provide minimal interaction with analytes.

Mobile Phase :

Commonly aqueous or organic solvents, tailored to the solubility and stability of the molecules analyzed.

Buffers may be added to maintain pH and ionic strength for biomolecules.

VI.10.4 Recycling SEC

Recycling SEC (or recycling gel filtration chromatography) is an advanced technique that enhances resolution by repeatedly passing the sample through the same SEC column. Achieving enhanced resolution in SEC analysis is challenging due to the fact that selectivity

is driven almost exclusively by the mean pore size and pore size distribution of the column. So, the only parameter that has an impact on resolution and can be improved is efficiency. Reducing particle size is an attractive option to increase resolution, but this also leads to increased pressure and stronger shear forces.

Reducing the mobile phase flow rate to reach optimal plate height can improve resolution in SEC, but it significantly increases analysis time. Similarly, using longer columns enhances separation but also leads to longer run times and higher-pressure requirements. An alternative solution, developed in the 1960s, is recycling chromatography, which involves repeatedly passing the sample zone through the column to enhance separation without increasing system pressure. This method, particularly effective with small injection volumes and limited compound numbers, achieves separation by cycling until peak widths match the column length, typically using just two columns in series.

VI.10.5 Coupling SEC with MS SEC

Separates molecules based on their hydrodynamic size, while MS provides information about molecular mass and structural details. Coupling SEC with MS provides a powerful analytical tool for the comprehensive characterization of complex biopharmaceutical products. It provides information about the distribution of molecular sizes within a sample and it can facilitate the determination of protein oligomeric states, post-translational modifications and identification of associated ligands or contaminants. However, SEC typically involves using aqueous buffers and mild conditions to maintain the native state of biomolecules, while MS often requires volatile solvents and acidic conditions for ionization. Older MS instruments were unable to tolerate high salt concentrations, while SEC columns lacked the necessary inertness, resulting in generally suboptimal peak shapes, particularly when volatile salts were involved. Therefore, indirect coupling of SEC to MS was performed by the manual collection of SEC fractions that were analysed by direct infusion native MS (nMS), after tedious desalting protocols to switch to MS-compatible volatile buffers.

VI.10.6 Coupling SEC with new detectors

Coupling Size Exclusion Chromatography (SEC) with modern or advanced detectors enhances its analytical power by providing additional insights into the molecular properties of analytes.

Increased Data Depth : In addition to molecular weight distribution, coupled detectors can provide insights into molecular size, shape, aggregation, and conformation.

Enhanced Accuracy : Reduces reliance on molecular weight calibration standards, especially for complex or irregular molecules.

Broader Applications : Enables characterization of diverse analytes such as proteins, polymers, nanoparticles, and polysaccharides.

VI.11 Common Detectors for Coupled SEC Systems

A. Multi-Angle Light Scattering (MALS)

Measures scattered light intensity at multiple angles to determine absolute molecular weight (Mw) and radius of gyration (Rg). **Applications** : Determining molecular weight without calibration standards ; Studying macromolecular conformation (e.g., globular vs. elongated shapes) ; Characterizing aggregation in proteins or biopolymers.

B. Refractive Index (RI) Detector

Measures the refractive index difference between the mobile phase and eluent to quantify analyte concentration. **Applications** : Universal detection of molecules lacking UV chromophores ; Often used alongside MALS or viscometry for molecular weight analysis.

C. Viscometry Detector

Measures intrinsic viscosity to provide information on molecular size, shape, and branching.

Applications: Characterizing polymer branching and chain flexibility ; Studying structural changes in proteins or polysaccharides.

D. Dynamic Light Scattering (DLS)

Measures fluctuations in scattered light due to Brownian motion to estimate hydrodynamic radius (Rh). **Applications** : Characterizing size distributions of nanoparticles, micelles, or large biomolecules. Complementary to MALS for detailed size analysis.

E. Fluorescence Detector

Detects fluorescence emission from analytes with intrinsic or labeled fluorophores. **Applications** : Sensitive detection of low-concentration biomolecules ; Studying specific interactions, such as protein-ligand binding.

F. Mass Spectrometry (SEC-MS)

Directs eluent to a mass spectrometer for molecular weight determination and structural analysis. **Applications** : Identifying and quantifying oligomeric species ; Analyzing complex mixtures like biopharmaceuticals or polymer blends.

G. Online Fourier Transform Infrared (FTIR) Detector

Detects characteristic vibrational modes of analytes for compositional analysis. **Applications** : Studying chemical composition and structural features of polymers ; Analyzing carbohydrate or lipid content in biopolymers.

H. Ultra-High-Resolution UV-Vis Detector

Monitors absorption at multiple wavelengths to track specific analytes or monitor changes in chromophores. **Applications** : Tracking protein unfolding by monitoring aromatic residues. Studying mixtures of biomolecules with distinct UV signatures.

VI.10.6.2 Coupling Multiple Detectors

Many SEC systems combine multiple detectors for comprehensive analysis. For example :

SEC-MALS-RI : Provides absolute molecular weight, size, and concentration in a single run.

SEC-DLS-MALS : Combines hydrodynamic and gyration radius measurements for detailed size characterization.

SEC-MS-UV: Enables detailed structural and compositional analysis, particularly for synthetic polymers or biologics.

V.1 Introduction

Ion-exchange chromatography (IEC) is part of ion chromatography which is an important analytical technique for the separation and determination of ionic compounds, together with ion-partition/interaction and ion-exclusion chromatography. Ion chromatography separation is based on ionic (or electrostatic) interactions between ionic and polar analytes, ions present in the eluent and ionic functional groups fixed to the chromatographic support Figure.22. Ion-exchange chromatography uses a resin to separate proteins according to their surface charges. This type of column contains a resin bearing either positively or negatively charged chemical groups. Resins containing positively charged groups attract negatively charged solutes and are referred to as anion-exchange resins. Resins with negatively charged groups are cation exchangers. In low-salt solutions, proteins with a negative surface charge will bind more stronglyto positively charged anion-exchange columns. Likewise, proteins with a positive surface charge will bind to negatively charged cation-exchange columns. Later, higher salt concentration buffer is applied to the column so it can compete with the resin for the bound proteins, and the bound proteins can then be eluted through the high-salt buffer.

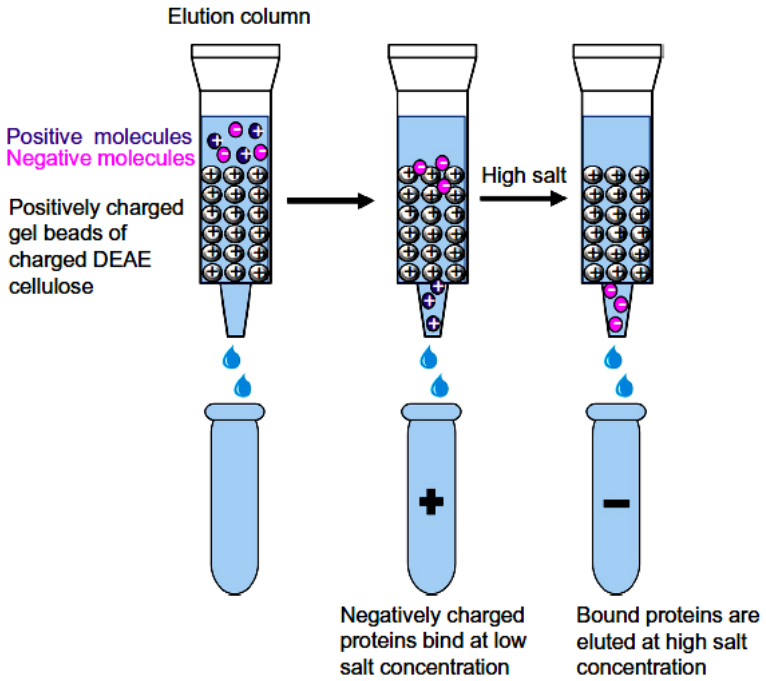


Figure 22 : Ion exchange chromatography separates molecules based on positive and negative charges. If the beads in column are positively charged, then negatively charged proteins will bind to column matrix at low salt as a result of ionic interactions. Proteins can then be induced to dissociate with high salt.

Two distinct mechanisms as follows ; ion exchange due to competitive ionic binding (attraction) and ion exclusion due to repulsion between similarly charged analyte ions and the ions fixed on the chromatographic support, play a role in the separation in ion chromatography. Ion exchange has been the predominant form of ion chromatography to date. This chromatography is one of the most important adsorption techniques used in the separation of peptides, proteins, nucleic acids and related biopolymers which are charged molecules in different molecular sizes and molecular nature. The separation is based on the formation of ionic bonds between the charged groups of biomolecules and an ion-exchange gel/support carrying the opposite charge. Biomolecules display different degrees of interaction with charged chromatography media due to their varying charge properties.

V.2 Principle

IEC can be considered a type of adsorption chromatography in which the interactions between the solute and the stationary phase are primarily electrostatic. The stationary phase, known as the ion exchanger, contains fixed functional groups that carry either negative or positive charges, allowing for the selective retention of oppositely charged ions from the mobile phase. (Figure.23 A). Exchangeable counterions preserve charge neutrality. A sample ion (or charged sites on large molecules) can exchange with the counterion to become the partner of the fixed charge. Ionic equilibrium is established as depicted in Figure. 23B. The functional group of the stationary phase determines whether cations or anions are exchanged. Cation exchangers contain covalently bound negatively charged functional groups, whereas anion exchangers contain bound positively charged groups. The chemical nature of these acidic or basic residues determines how stationary-phase ionization is affected by the mobile-phase pH

IEC is based on the formation of ionic or electrostatic interactions between analyte ions or highly polar molecules and an oppositely charged stationary phase. There are two basic types known as cation-exchange and anion-exchange. In cation exchange mode the stationary phase is negatively charged and analytes that are positively charged interact with the stationary phase. In anion exchange mode the stationary phase is positively charged and interacts with negatively charged analytes. Overall electrostatic charge, charge density, and surface charge distribution of the analyte all play an important role in the mechanism of retention.[18] Typically, during chromatographic separation, the ionic strength of the mobile phase eluent is gradually increased to displace charged analytes that are ionically interacting

with the stationary phase. The elution times of individual ions are determined by the strength of the ionic interactions between the analyte and stationary phase (Figure. 23 A).

Hence, coupling IC with mass spectrometry, especially high-resolution instruments, enables ions to be both separated and have their mass-to-charge ratio measured on a continuous basis. There is some overlap with HILIC-MS and IP-MS in terms of compatible analytes, but the mechanism of retention in IEC leads to a unique analyte separation profile, particularly for ionic compounds as illustrated in Figure. 23 B.

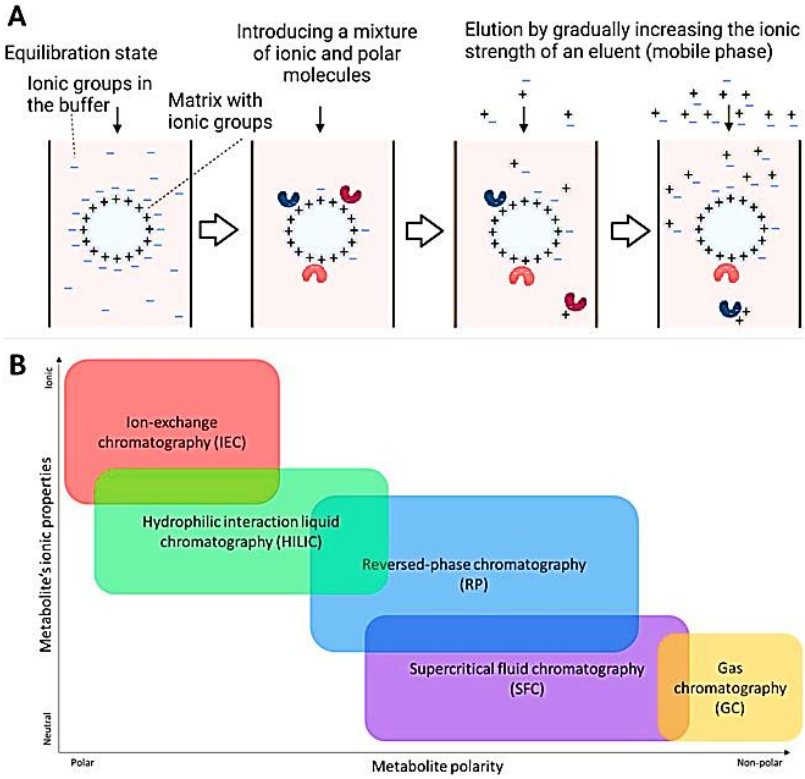


Figure 23 : Principle base in Ion-Exchange Chromatography (IEX)

IEC separates highly polar and ionic compounds providing a unique separation space compared to other chromatography types. (A) A schematic representation of the mechanism of anion-exchange chromatography. The column's stationary phase is positively charged and equilibrated with a mobile phase containing negative ions (e.g., OH^- or another anion) at a minimal concentration. When a sample is introduced with negatively charged or polarizable analytes, the charged analytes displace the negative ions (e.g., OH^- ions) from the stationary phase and bind instead, therefore being retained. Analyte ions have differential affinity for the stationary phase depending upon their charge ; the affinity is directly determined by Coulombic force. The ionic strength of the mobile phase is usually increased for a gradient elution where the concentration of ions in the mobile phase (e.g., OH^-) is gradually increased

until the analyte ions are displaced by the increasing ion concentration in the mobile phase (isocratic elution is also sometimes used). (B) Indicative illustration of chromatographic separation space showing how ion-exchange chromatography extends the separation space beyond reversed-phase chromatography (RP-LC) and hydrophilic interaction liquid chromatography (HILIC) for highly polar and ionic molecules.

V.3 Theory of ion exchange

Purification using ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. Most ion exchange experiments are performed in five main stages. These steps are illustrated schematically.

The first stage is equilibration in which the ion exchanger is brought to a starting state, in terms of pH and ionic strength, which allows the binding of the desired solute molecules. The exchanger groups are associated at this time with exchangeable counter-ions (usually simple anions or cations, such as chloride or sodium). The second stage is sample application and adsorption, in which solute molecules carrying the appropriate charge displace counter-ions and bind reversibly to the gel. Unbound substances can be washed out from the exchanger bed using starting buffer. In the third stage, substances are removed from the column by changing to elution conditions unfavourable for ionic bonding of the solute molecules. This normally involves increasing the ionic strength of the eluting buffer or changing its pH. In Figure.24 desorption is achieved by the introduction of an increasing salt concentration gradient and solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first.

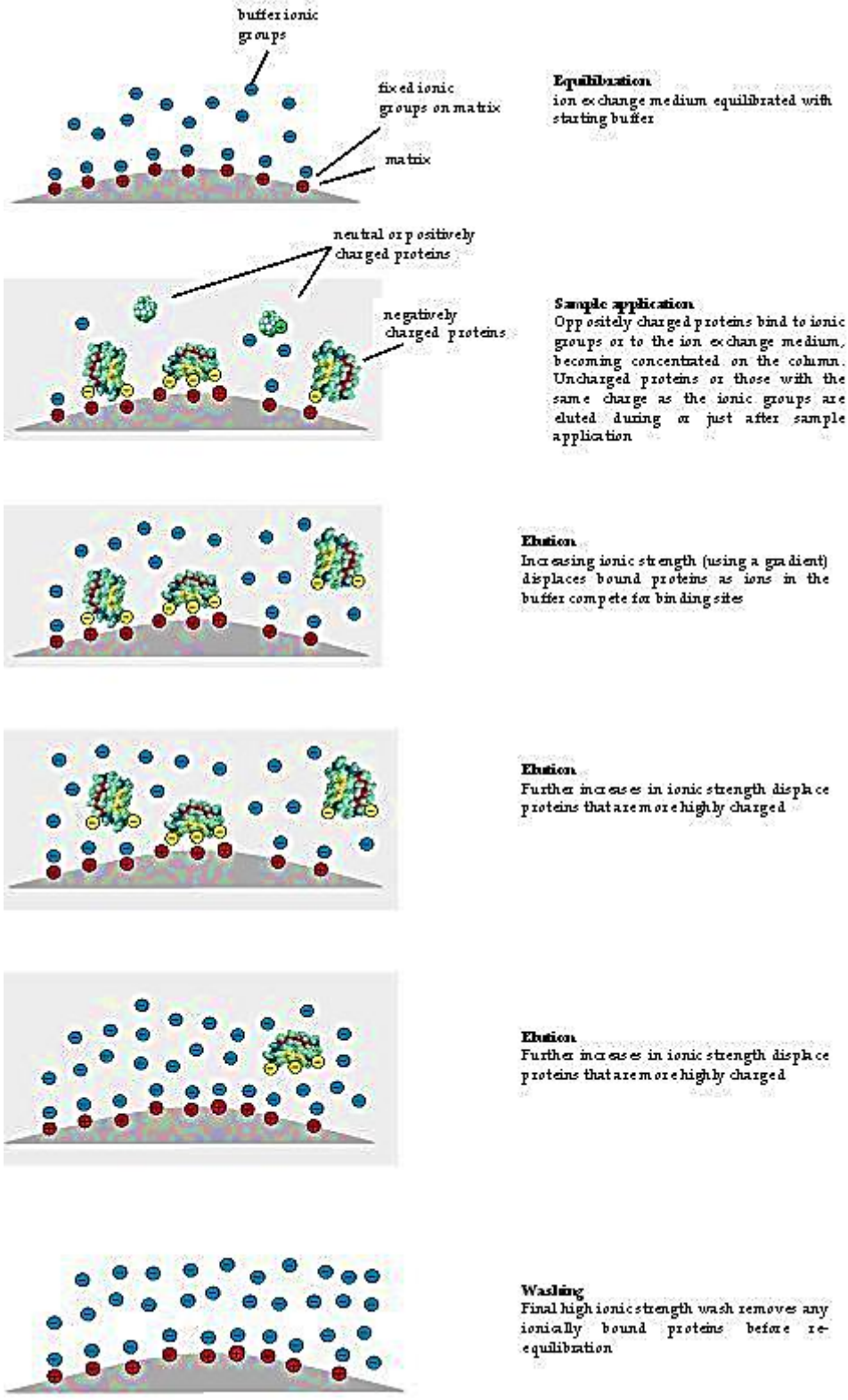


Figure. 24 : The principle of ion exchange chromatography (salt gradient elution).

The fourth and fifth stages are the removal from the column of substances not eluted under the previous experimental conditions and re-equilibration at the starting conditions for

the next purification. Separation is obtained since different substances have different degrees of interaction with the ion exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces. These interactions can be controlled by varying conditions such as ionic strength and pH.

The differences in charge properties of biological compounds are often considerable, and since ion exchange chromatography is capable of separating species with very minor differences in properties, e.g. two proteins differing by only one charged amino acid, it is a very powerful separation technique. In ion exchange chromatography one can choose whether to bind the substances of interest and allow the contaminants to pass through the column, or to bind the contaminants and allow the substance of interest to pass through. Generally, the first method is more useful since it allows a greater degree of fractionation and concentrates the substances of interest. In addition to the ion exchange effect, other types of binding may occur. These effects are small and are mainly due to van der Waals forces and non-polar interactions. Ion exchange separations may be carried out in a column, by a batch procedure or by expanded bed adsorption. All three methodologies are performed in the stages of equilibration, sample adsorption etc. described previously.

V.4 Ion exchange mechanism

IEC which is designed specifically for the separation of differently charged or ionizable compounds comprises from mobile and stationary phases similar to other forms of column based liquid chromatography techniques. Mobil phases consist an aqueous buffer system into which the mixture to be resolved. The stationary phase usually made from inert organic matrix chemically derivative with ionizable functional groups (fixed ions) which carry displaceable oppositely charged ion. Ions which exist in a state of equilibrium between the mobile phase and stationary phases giving rise to two possible formats, anion and cation exchange are referred to as counter ion (Figure.25) . Exchangeable matrix counter ions may include protons (H+), hydroxide groups (OH^-), single charged mono atomic ions (Na^+, K^+, Cl^-), double charged mono atomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}) as well as organic bases (NR_2H^+) and acids (COO^-).

Cations are separated on cation-exchange resin column and anions on an anion exchange resin column. Separation based on the binding of analytes to positively or negatively charged groups which are fixed on a stationary phase and which are in equilibrium with free counter ions in the mobile phase according to differences in their net surface charge (Figure.25).

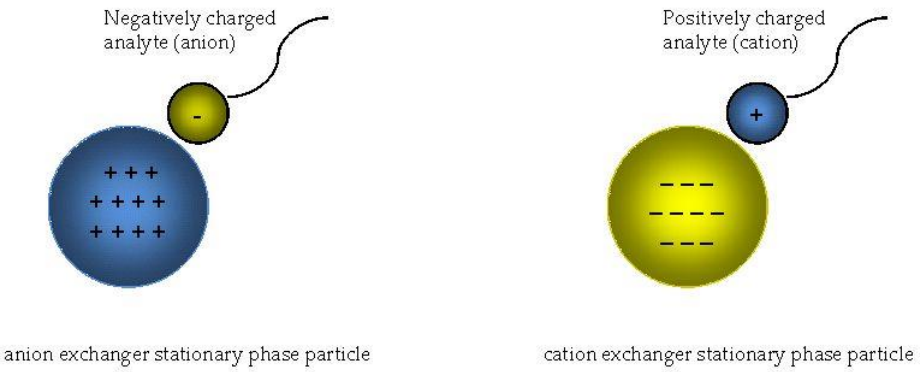


Figure 25 : Types of ion exchangers

Ion exchange chromatography involves separation of ionic and polar analytes using chromatographic supports derivatized with ionic functional groups that have charges opposite that of the analyte ions. The analyte ions and similarly charged ions of the eluent compete to bind to the oppositely charged ionic functional group on the surface of the stationary phase. Assuming that the exchanging ions (analytes and ions in the mobile phase) are cations, the competition can be explained using the following equation :

$S\text{-}X^-C^+ + M^+ \leftrightarrow S\text{-}X^-M^+ + C^+$

In this process the cation M+ of the eluent replaced with the analyte cation C^+ bound to the anion X- which is fixed on the surface of the chromatographic support (S).

In anion exchange chromatography, the exchanging ions are anions and the equation is represented as follow :

$S\text{-}X^+A^- + B^- \leftrightarrow S\text{-}X^+B^- + A\text{-}S^-X + A^-$

The anion B- of the eluent replaced with the analyte cation A- bound to the positively charged ion X+ on the surface of the stationary phase. The adsorption of the analyte to the stationary phase and desorption by the eluent ions is repeated during their journey in the column, resulting in the separation due to ion-exchange.

Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography support according to differences in their overall charge, charge density and surface charge distribution.

Net surface charge of all molecules with ionizable groups is highly pH dependent. Therefore pH of the mobile phase should be selected according to the net charge on a protein of interest within a mixture is opposite to that of matrix functional group, that it will displace the functional group counter ion and bind the matrix. On the other hand oppositely charged proteins will not be retained. Adsorbed protein analytes can be eluted by changing the mobile phase pH which effect the net charge of adsorbed protein, so its matrix binding capacity.

Moreover increasing the concentration of a similarly charged species within the mobile phase can be resulted in elution of bound proteins. During ion exchange chromatography for example in anion exchange as illustrated in Figure.25, negatively charged protein analytes can be competitively displaced by the addition of negatively charged ions. The affinity of interaction between the salt ions and the functional groups will eventually exceed that the interaction exists between the protein charges and the functional groups, resulting in protein displacement and elution by increasing gradually the salt concentration in the mobile phase.

Complex mixtures of anions or cations can usually be separated and quantitative amounts of each ion measured in a relatively short time by ion exchange chromatography. In classical ion-exchange chromatography separations have been performed in the open-column mode. Column which is loosely packed with stationary phase as small particles made of 1-2 cm diameter glass. The mobile phase or eluent contains the competing ion and is passed continuously into the column and percolates through it under gravity. Sample mixture is applied to the top of the column and allowed to pass into the bed of ion- exchange material. Eluent flow is then resumed and fractions of eluent are collected at regular intervals from the column outlet. Open column ion-exchange chromatography is very slow due to low eluent flow-rates. Increasing flow rate may result in deteriorated separation efficiency (Figure.23). In modern ion-exchange chromatography the usage of high efficiency ion exchange materials combined with flow-through detection have overcome of these challenges. Separations are performed on the column which is filled with ion-exchanger as particles in uniform size. The particles of ion-exchange material are generally very much smaller than those used for classical open column ion-exchange chromatography. However ion-exchange resins used in modern chromatography have lower capacity than older resins. The eluent must be pumped through the column due to the small particle size of stationary phase. The sample mixture is applied into eluent by the injection port. Finally the separated ions are detected with a flow-through detection instrument.

V.5 Key features of Ion exchange chromatography

V.5.1 Stationary Phase (Resin)

The stationary phase in ion exchange chromatography (IEX) is the most critical component, as it is responsible for the selective separation of charged molecules. The stationary phase consists of a matrix or resin that carries ionizable functional groups. These groups interact with oppositely charged molecules in the sample, allowing for separation based on differences in charge Table VII.

V.5.2 Types of Stationary Phases

There are various types of ion exchange resins based on the strength of the ion exchange interaction, the structure of the matrix Figure.26, and the charged functional groups. Here are the main categories (see Table VII) :

V.5.2.1Weak Ion Exchange Resins

Weak ion exchange resins are types of ion exchange materials that contain functional groups whose charge can be affected by the pH of the mobile phase. These resins are characterized by their relatively limited pH range, where they maintain ion exchange properties, meaning they lose their ion exchange capability when the pH is outside this range.

There are two main types of weak ion exchange resins : weak cation exchangers and weak anion exchangers. Each has a distinct functional group that can be protonated or deprotonated based on the pH, affecting their ability to bind oppositely charged molecules.

- ***Weak Cation Exchange Resin***

Weak cation exchange resins have negatively charged functional groups that interact with positively charged molecules (cations). The most common functional group is the carboxyl group ($-COO^-$), which can be protonated or deprotonated depending on the pH of the mobile phase. They work effectively in the pH range of about 4 to 7. Outside this range, they lose their charge.

At low pH (acidic conditions), the carboxyl groups are protonated and cannot bind cations effectively.

At higher pH (more basic), the carboxyl groups are deprotonated and negatively charged, which allows them to bind positively charged cations.

Examples of Weak Cation Exchangers

CM (Carboxymethyl) Cellulose : A cellulose-based weak cation exchange resin containing carboxyl ($-COO^-$) groups. It is widely used in protein purification because of its mild separation conditions.

CM-Sepharose : Another example of a weak cation exchange resin that contains carboxymethyl groups attached to a Sepharose matrix, useful in biomolecule separation.

- ***Weak Anion Exchange Resin***

Weak anion exchange resins have positively charged functional groups that interact with negatively charged molecules (anions). The most common functional group is the amine group ($-NH_2$), which can be protonated or deprotonated based on the pH. These resins work well in the pH range of about 7 to 9. Outside this range, they lose their ability to bind anions.

At low pH, the amine groups are protonated ($-NH_3^+$), which gives them a positive charge and enables binding with negatively charged anions.

At higher pH, the amine groups are deprotonated and neutral, making them unable to bind anions effectively.

Examples of Weak Anion Exchangers :

DEAE (Diethylaminoethyl) Cellulose : A weak anion exchange resin that contains diethylaminoethyl ($-NH(CH_2CH_3)_2$) functional groups. It is a popular choice for separating biomolecules such as proteins and nucleic acids.

DEAE-Sepharose : Another commonly used weak anion exchange resin with a DEAE group attached to a Sepharose matrix.

V.5.2. 2 Strong Ion Exchange Resins

Strong ion exchange resins are types of ion exchangers that contain permanently charged functional groups. Unlike weak ion exchangers, strong ion exchange resins maintain their charge over a wide range of pH, making them highly effective for separating charged molecules in conditions that vary in pH or ionic strength. Their robustness and high binding capacity make them widely used in applications where consistent ion exchange properties are essential.

There are two main types of strong ion exchange resins : strong cation exchangers and strong anion exchangers, each of which interacts with oppositely charged molecules.

- ***Strong Cation Exchange Resins***

Strong cation exchange resins have negatively charged functional groups that interact with positively charged molecules (cations) regardless of the pH. The most common functional group in strong cation exchangers is the sulfonate group ($-SO_3^-$). These groups are fully ionized across a wide pH range, ensuring that the resin remains negatively charged at almost any pH.

Examples of Strong Cation Exchangers

SP (Sulfopropyl) Sepharose: A commonly used strong cation exchange resin containing sulfonate groups attached to a Sepharose matrix. It is widely applied in protein purification.

Sulfopropyl (SP) Resins : General strong cation exchange resins that use sulfonate groups and are effective across a broad pH range.

- ***Strong Anion Exchange Resins***

Strong anion exchange resins have positively charged functional groups that interact with negatively charged molecules (anions), and they maintain their positive charge over a wide

pH range. The most common functional group in strong anion exchangers is the quaternary ammonium group ($-N^+(CH_3)_3$), which remains positively charged at all pH values, allowing it to bind negatively charged molecules under almost any condition.

Examples of Strong Anion Exchangers

*Q (Quaternary) Sepharo*se: A widely used strong anion exchange resin with quaternary ammonium functional groups. It is frequently used for the separation of nucleic acids and proteins.

Quaternary Ammonium (Q) Resins: General strong anion exchange resins that utilize quaternary ammonium groups, effective in a wide range of pH values.

V.5.2.3 Soft Matrix Resins

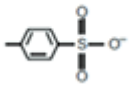
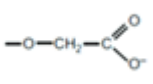
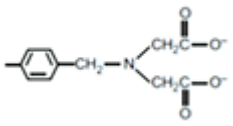
Made from hydrophilic materials like agarose or cellulose. These matrices are suitable for biomolecules like proteins and DNA because they provide a gentle environment.

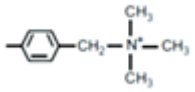
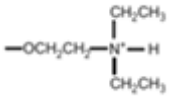
Soft resins typically have lower mechanical strength, limiting their use in high-pressure systems.

V.5.2.4 Rigid Matrix Resins

Made from synthetic polymers such as polystyrene or methacrylate, rigid resins are mechanically strong and capable of withstanding the high pressures used in liquid chromatography (HPLC). These materials are commonly employed in industrial settings or in high-resolution applications (see TableVII).

Table VII : Cation- and Anion-Exchange Resins Commonly Used for Biochemical Separations

Ion exchange media	**Structure**
Cation exchange media	
Strongly acidic, polystyrene resin	
Weakly acidic, carboxymethyl (CM) Cellulose	
Weakly acidic, chelating, polystyrene resin (Chelex-100)	

Anion exchange media	
Strongly basic, polystyrene resin (Dowex-1)	
Weakly basic, diethylaminoethyl (DEAE) cellulose	

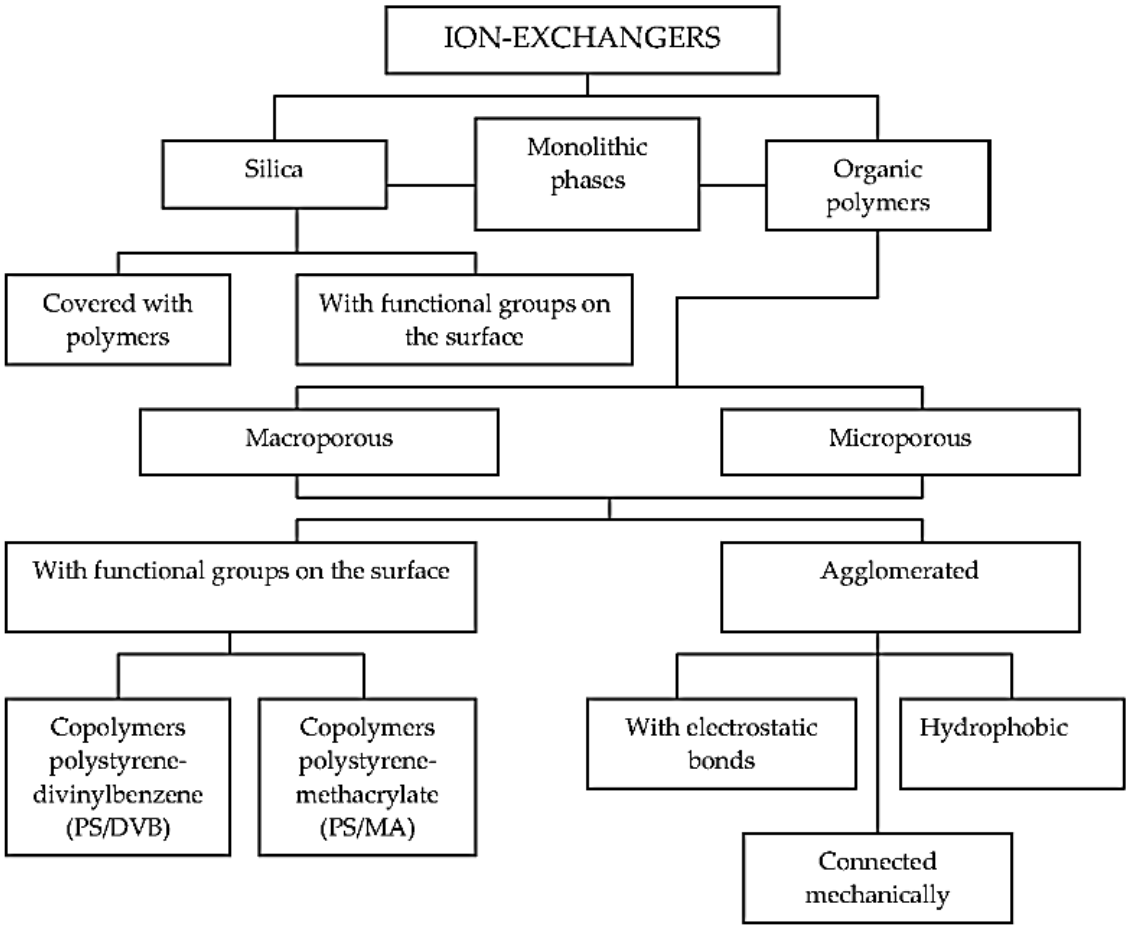


Figure 26. Stationary phases used in ion chromatography**.**

V.5.3 Physical properties of resins

1. ***Particle size*** : They are available as fine powder of uniform particle size from 50 – 200 mesh. They should allow free and uniform flow of mobile phase. They should contain more exchangeable functional groups.

2. ***Cross linking and swelling*** : when more cross linking agent is present, they are more rigid, but swell less. When swelling is less, separation of ions of different sizes is difficult as they

cannot pass though the pores present. When less cross linking agent is present, they are less rigid, but swell more. When swelling is more, separation will not be efficient as exchange of functional group does not takes place due to wide pore. Hence an optimum quantity of cross linking agent should be added to the polymeric ion exchange resin for the separation to be effective.

V.5.4 Mobile phase

The mobile phase in ion exchange chromatography (IEX) is a liquid buffer solution that flows through the column, carrying the sample along with it. The primary role of the mobile phase is to facilitate the interaction between the charged analytes (molecules in the sample) and the charged groups on the stationary phase (resin), as well as to control the binding and elution of those analytes.

In ion exchange chromatography generally eluents which consist of an aqueous solution of a suitable salt or mixtures of salts with a small percentage of an organic solvent are used in which most of the ionic compounds are dissolved better than in others in. Therefore the application of various samples is much easier. Sodium chloride is probably the most widely used and mild eluent for protein separation due to has no important effect on protein structure. However NaCl is not always the best eluent for protein separation. Retention times, peak widths of eluted protein, so chromatographic resolution are affected by the nature of anions and cations used. These effects can be observed more clearly with anion exchangers as compared to cation exchangers. The salt mixture can itself be a buffer or a separate buffer can be added to the eluent if required. The competing ion which has the function of eluting sample components through the column within reasonable time is the essential component of eluting sample. Nature and concentration of the competing ions and pH of the eluent are the most important properties affecting the elution characteristics of solute ions.

The eluent pH has considerable effects on the functional group which exist on the ion exchange matrix and also on the forms of both eluent and solute ions. The selectivity coefficient existing between the competing ion and a particular solute ion will determine the degree of that which competing ion can displace the solute ion from the stationary phase. As different competing ions will have different selectivity coefficients, it follows that the nature of competing ion will be an important factor in determining whether solute ions will be eluted readily. The concentration of competing ion exerts a significant effect by influencing the position of the equilibrium point for ion-exchange equilibrium. The higher concentration of the competing ion in the eluent is more effectively displace solute ions from the stationary

phase, therefore solute is eluted more rapidly from the column. Additionally elution of the solute is influenced by the eluent flow-rate and the temperature. Faster flow rates cause to lower elution volumes because the solute ions have less opportunity to interact with the fixed ions. Temperature has relatively less impact, which can be change according to ion exchange material type. Enhancement of the temperature increases the rate of diffusion within the ion-exchange matrix, generally leading to increased interaction with the fixed ions and therefore larger elution volumes. At higher temperatures chromatographic efficiency is usually improved.

V.6 Ion Exchange Capacity

Capacity refers to the number of charged sites available for interaction with the target molecules. There are two types of capacity :

- **Total Capacity :** Refers to the maximum number of exchangeable sites on the resin.
- **Dynamic Binding Capacity (DBC) :** Refers to the number of exchange sites that are effectively used under specific flow conditions.

For preparative purposes, it's important to match the resin's capacity with the amount of sample to ensure efficient separation.

V.7 Application

Table VIII. Separation methods and their applications in ion chromatography

Method	Separation Mechanism	Functional Group	Typical Eluents	Selected Analytes	Detection Modes
Anion-exchange chromatography (suppressed and non-suppressed)	Ion-exchange	$-NR_3^+$	Suppressed ion chromatography: Na_2CO_3 + $NaHCO_3$, NaOH, KOH Non-suppressed ion chromatography: benzioc acid, phtalic acid, aromatic and aliphatic carboxylic acids, sulfonic acid	F^-, Cl^-, Br^-, I^-, ClO_2^-, ClO_3^-, ClO_4^-, BrO_3^-, HPO_4^{2-}, SCN^-, CN^-, $P_2O_7^{4-}$, NO_2^-, NO_3^-, S^{2-}, SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, AsO_3^{2-}, WO_4^{2-}, MnO_4^{2-}, CrO_4^{2-}, SiO_3^{2-}, SeO_3^{2-}, SeO_4^{2-}, SiF_6^-, $Cr_4O_7^{2-}$, BF_4^-, carboxylic acids,	Conductivity, UV-VIS, amperometric, MS, ICP-MS
Cation-exchange chromatography (suppressed and non-suppressed)	Ion-exchange	$-SO_3^-$	Suppressed ion chromatography: H_2SO_4, HCl, HNO_3, methylosulfonic acid (MSA) Non-suppressed ion chromatography: HNO_3, tartaric acid, dipicolinic acid (DPA)	Rb^+, Cs^+, Li^+, Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, aliphatic amines	Conductivity, UV-VIS, MS, ICP-MS
		$-SO_3^-/NR_3^+$	2,4-pyridinedicarboxylic acid (PDCA), oxalic acid	Cu^{2+}, Ni^{2+}, Cd^{2+}, Pb^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Sn^{2+}, Zn^{2+}, Co^{2+}, Sn^{4+}, Cr^{3+}, As^{3+}, As^{5+}, UO_2^{2+}, La^{3+}, Ce^{3+}, P^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+}, V^{4+}, V^{5+}, Gd^{3+}, Tb^{3+}, Dy^{3+}, Tm^{3+}, Yb^{3+}, Ho^{3+}, Er^{3+}, Lu^{3+}, Am^{3+}, Pu^{3+}	UV-VIS, MS, ICP-MS
Ion-exclusion chromatography	Ion-exclusion	$-SO_3^-/NR_3^+$	Water, diluted mineral acids,	ClO_4^-, I^-, BrO_4^-, Cl^-, NO_2^-, NO_3^-, carboxylic acids, aldehydes, silicates, amines, carbohydrates	Conductivity, UV-VIS, amperometric
Anion-pair chromatography	Ion-pairs	Neutral	NH_4OH, tetramethylammonium hydroxide (TMAOH), tetrapropylammonium hydroxide (TPAOH), tetrabutylammonium hydroxide (TBAOH)	F^-, Cl^-, Br^-, I^-, SCN^-, CN^-, ClO_3^-, ClO_4^-, BrO_3^-, NO_2^-, NO_3^-, SO_3^{2-}, SO_4^{2-}, SeO_3^{2-}, SeO_4^{2-}, anionic surfactants, metal complexes, aromatic carboxylic acids	Conductivity, UV-VIS
Cation-pair chromatography		Neutral	aliphatic sulfonated hydrocarbons, (C_2–C_{10}), selected inorganic anions (e.g., PF_6^-, complexing agents)	Li^+, Na^+, K^+, NH_4^+, Rb^+, Cs^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, alkylamines, alkanolamines, cationic surfactants, sulfonic and phosphonium compounds	Conductivity, UV-VIS

V.8 Detection methods

The conductivity detector is still the most popular, but other types of detection modes (e.g., UV-VIS, amperometric, spectrometric ones) can also be applied for different analytes. An overview of detection methods is given in Table VIII.

Figure.27 shows a classification of the detection methods commonly used in ion chromatography. The most popular conductometric detector can be applied to determine all anions and cations of strong acids and bases (e.g., F–, Cl^-, NO^{3-}, PO_4^{3-}, SO_4^{2-}, Na^+, K^+, NH^{4+}, Mg^{2+}, Ca^{2+}). The UV-VIS detection is a popular detection mode for high-performance liquid chromatography (HPLC), but its application in ion chromatography is limited because only a few inorganic ions have an appropriate chromophore.

The UV-VIS detection methods can be divided into direct and indirect modes. For the direct UV-VIS detection, the molar absorption of co-ions should be zero. The direct UV-VIS detection is applied for determinations of NO^{2-} and NO^{3-}, as well as Br^- and I^- in the presence of high Cl^- concentrations. Moreover, sulfide, chromate, thiocyanate, thiosulfate, and selected metal chloro- and cyano-complexes can be determined in wastewater with this

detection method. The UV-VIS detection with post-column reactions is a versatile technique that combines enhanced sensitivity and selectivity for specific applications.

The chemiluminescence detection is generally performed in a post-column reaction mode. In ion chromatography, the fluorescence detection is rarely used as a detection method, because only few ions fluoresce. Furthermore, the amperometric detection can be used for samples with pK values > 7.

The amperometric detection method application helps in determining ions such as I^-, S^{2-}, $S_2O_3^{2-}$, SCN^-, CN^-, or heavy and transition metals. Due to the poor selectivity and sensitivity, the refractive index detection is very rarely used in ion chromatography. The most powerful detection method (used not only in ion chromatography) is mass spectrometry. Although this detection method is helpful in wastewater analysis, it is not popular in ordinary laboratories due to the high apparatus cost and complex requirements to be met by the method operator.

Table VIII : Detection methods applied in ion chromatography

Detection Mode	Principle	Example Applications
Conductivity	Electrical conductivity	Anions and cations with pK_a or $pK_b < 7$
Direct and indirect UV-VIS	UV-VIS light absorption	UV-active anions and cations, heavy and transition metals after post column derivatisation reaction
Fluorescence	Excitation and emission	Ammonium, amino acids, and selected amines after post column derivatisation
Amperometry	Oxidation or reduction	Anions and cations with pK_a or $pK_b > 7$
MS	Electrospray ionization	Hyphenation technique for structural characterization of organic anions and cations
ICP-AES, ICP-MS	Atomic emission	Hyphenation techniques for species analysis of metal and metalloids
Refractive index	Change in refractive index	High concentrated samples

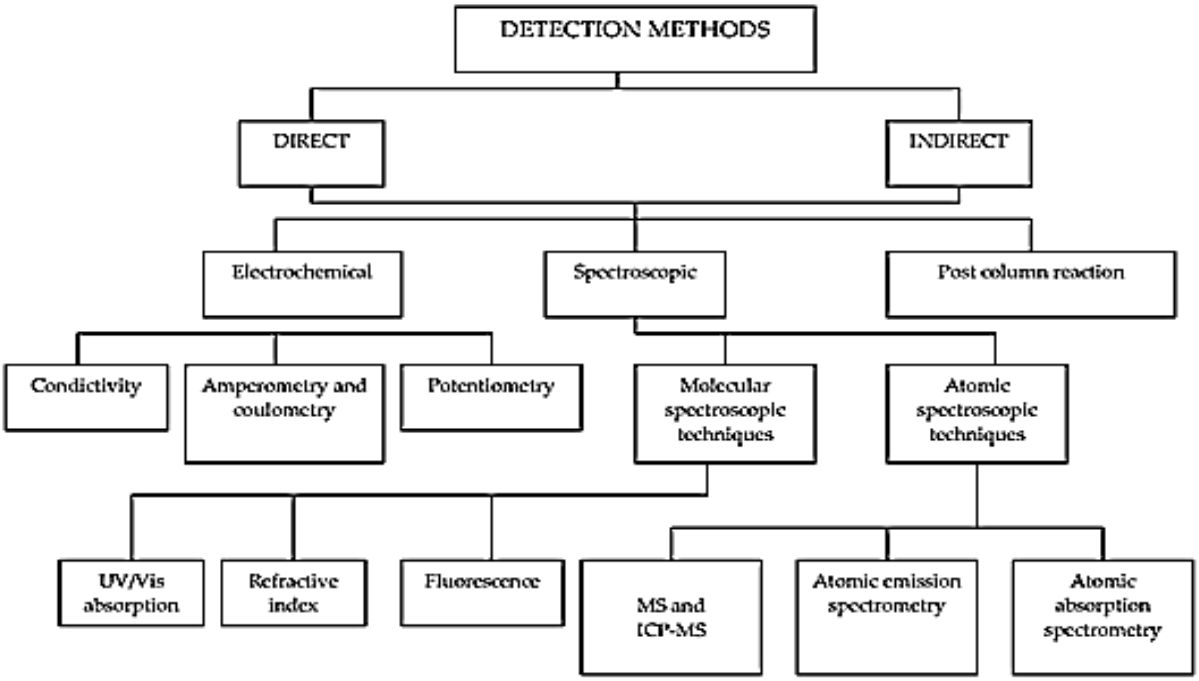


Figure 27 : Division of detection methods for ion chromatography

V.9 Basic process steps

Eluent loading, sample injection, separation of sample, elution of analytes A, and elution of analyte B, shown and explained below. Elution is the process where the compound of interest is moved through the column. This happens because the eluent, the solution used as the solvent in chromatography, is constantly pumped through the column. The chemical reactions below are for an anion exchange process Figure. 28.

1. Step 1

The eluent loaded onto the column displaces any anions bonded to the resin and saturates the resin surface with the eluent anion. This process of the eluent ion (E^-) displacing an anion (X-) bonded to the resin can be expressed by the following chemical reaction: $Resin^+ -X^- + E^- <=> Resin^+ -E^- + X^-$

2. Step 2

A sample containing anion A and anion B are injected onto the column. This sample could contain many different ions, but for simplicity this example uses just two different ions ready to be injected onto the column.

3. Step 3

After the sample has been injected, the continued addition of eluent causes a flow through the column. As the sample elutes (or moves through the column), anion A and anion B adhere to the column surface differently. The sample zones move through the column as eluent gradually displaces the analytes The continued addition of the eluent causes a flow through the column. As sample elutes, anion A and anion B adhere to the column surface differently. The sample zones move through the column as eluent gradually displaces the analytes.

4. Step 4

As the eluent continues to be added, the anion A moves through the column in a band and ultimately is eluted first. This process can be represented by the chemical reaction showing the displacement of the bound anion (A^-) by the eluent anion (E^-). $Resin^+ -A^- + E^- <=> Resin^+ -E^- + A^-$

5. Step 5

The eluent displaces anion B, and anion B is eluted off the column.

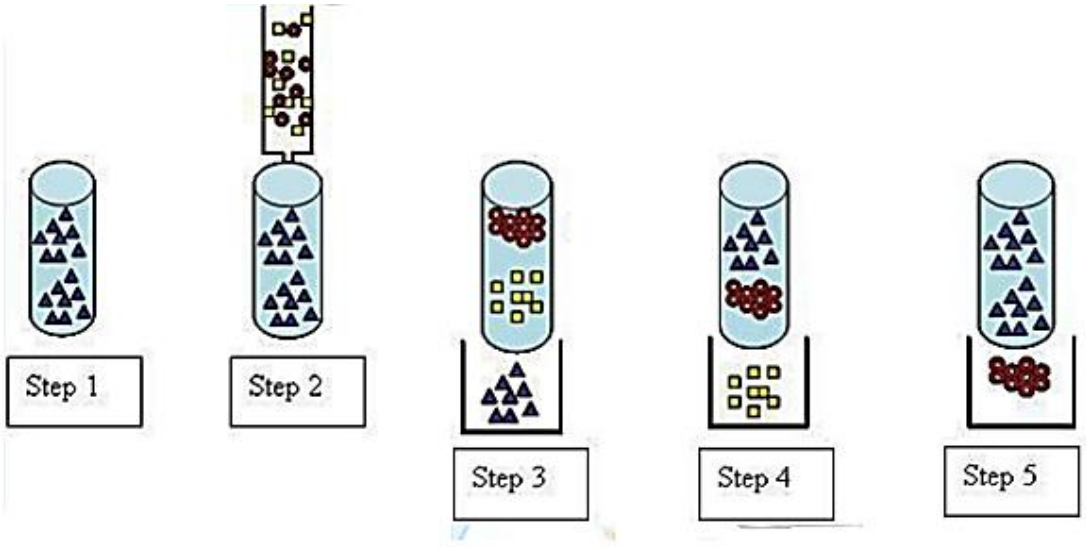


Figure 28 : The basic process steps

V.10 Recent trends in ion exchange chromatography

Among the various recent advances in ion exchange chromatography, particulary:

V.10.1 Stationary-Phase Architecture

Stationary-phase construction for IC columns comprises nine basic architectures: silane-based modification of porous silica substrates, electrostatic-agglomerated films on nonporous substrates, electrostatic-agglomerated films on ultrawide-pore substrates, polymer-grafted films on porous substrates, chemically derivatized polymeric substrates, polymer-

encapsulated substrates, ionic molecules adsorbed onto chromatographic substrates, step-growth polymers on polymeric substrates, and hybrid materials based on a combination of a silane-modified silica substrate with a polymeric exterior surface coating. Five of these — electrostatic-agglomerated films on ultrawidepore substrates, polymer-grafted films on porous substrates, chemically derivatized polymeric substrates, polymer-encapsulated substrates, and step-growth polymers on polymeric substrates see Figure.29: Ion chromatography stationary-phase architectures most widely used in recently introduced phases: (a) electrostatic agglomerated ultrawide-pore substrates, (b) polymer-grafted film on porous substrates(c) chemically derivatized polymeric substrates, (d) polymerencapsulated substrates, and (e) step-growth polymers on polymeric substrates. Electrostatic agglomerated films on ultrawide-pore substrates : For the most part, electrostatic agglomerated films on nonporous substrates have been largely supplanted by higher capacity versions utilizing ultrawide-pore substrates. By using an architecture similar to that based on nonporous substrates

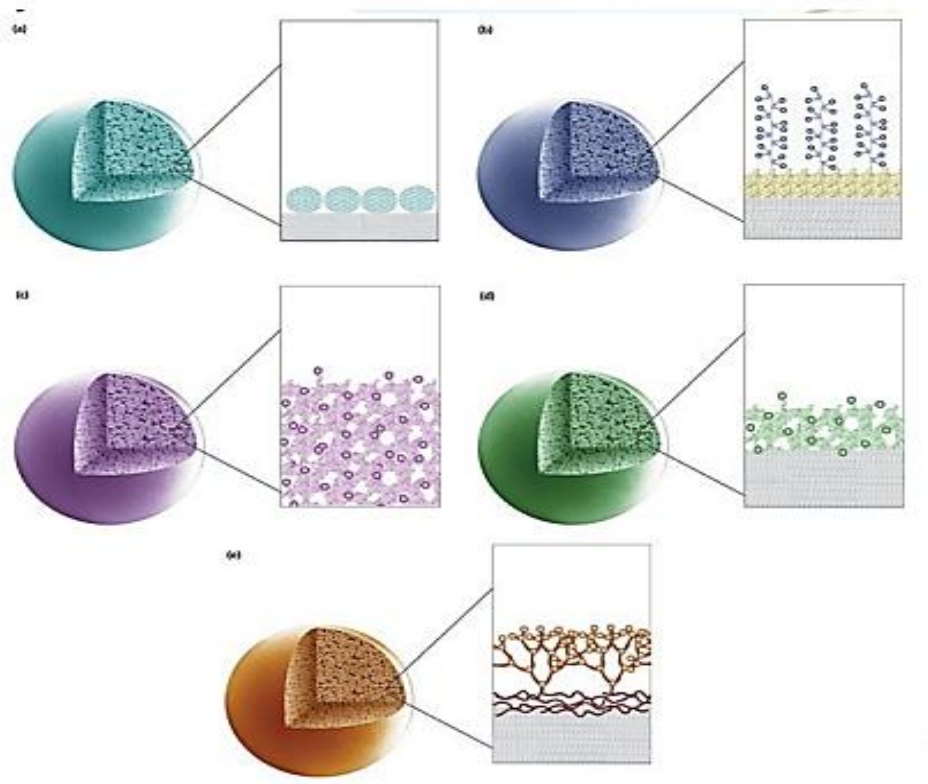


Figure 29: Ion chromatography stationary-phase architectures most widely used in recently introduced phases: (a) electrostatic agglomerated ultrawide-pore substrates, (b) polymer-grafted film on porous substrates(c) chemically derivatized polymeric substrates, (d) polymerencapsulated substrates, and (e) step-growth polymers on polymeric substrates.

Electrostatic agglomerated films on ultrawide-pore substrates

Electrostatic agglomerated films on nonporous substrates have largely been replaced by higher-capacity materials built on ultrawide-pore substrates (Figure. 29a). These substrates, with pore sizes ranging from 100–300 nm, allow ion-exchange colloids to coat both the internal and external surfaces, significantly increasing capacity. With an optimal pore-to-particle size ratio, these materials can achieve 6–8 times the capacity of their nonporous counterparts (i.e., 30–150 µEq/mL vs. 5–30 µEq/mL). Due to the growing demand for high-capacity materials in ion chromatography (IC) and the availability of high-capacity suppressors, this architecture has gained widespread use in recent years.

Polymer-grafted films on porous substrates

The material shown in Figure. 29b is commonly used to produce high-capacity chromatographic packings without the need for crosslinking to control selectivity. These materials are synthesized by grafting polymer strands onto a substrate surface, either through pre-existing polymerizable groups, surface modification to introduce such groups, or the addition of an initiator. However, crosslinking monomers cannot be used, as they would cause gel formation, which restricts control over selectivity. This grafted architecture is mainly used in applications requiring high-capacity and high-water content stationary phases. Although they can be made from both polymer- and silica-based substrates, they are typically polymer-based in practice.

Chemically derivatized polymeric substrates

Some modern chromatographic materials (Figure. 29c) are produced using proprietary synthesis methods, making the exact derivatization chemistry unknown in commercial products. These materials often have high capacity, as functional groups are not confined to the surface. However, if reactions occur deep within the dense polymer matrix, mass transport becomes slow, leading to poor chromatographic performance. While early versions of this architecture showed limited performance, newer materials like the IC SI-52 4E column (Showa Denko) demonstrate that high-performance stationary phases can be achieved using this approach.

Figure. 29d shows another type of material—polymer-encapsulated substrates, developed by Professor Gerard Schomburg. This method involves coating substrates (e.g., alumina) with a preformed polymer containing residual double bonds, followed by curing at

high temperature to form a crosslinked polymer film that permanently encapsulates the substrate, making it suitable for reversed-phase chromatography.

The main advantage of this architecture is that it does not require covalent attachment to the substrate surface, making it suitable for inorganic materials that cannot be chemically modified. Initially developed for reversed-phase chromatography on alumina, the method was later adapted by Schomburg's group to create weak cation-exchange phases using a butadiene–maleic acid copolymer. This innovation marked a major shift in the design of stationary phases for inorganic cation separation, moving from strong-acid to weak carboxylic acid-based exchangers. However, drawbacks include the potential for swelling or shrinking during gradient or temperature changes, and alkaline degradation of the underlying silica, even with an intact coating.

V.11 Avantages and desavantages of ion exchange chromatography

Ion exchange chromatography is a very powerful separation technique that is used not only for preparative chromatography but also for analytical chromatography. However, like all other chromatography modes, IEX does have some limitations. One of the main disadvantages of ion exchange chromatography is its buffer requirement : because binding to IEX resins is dependent on electrostatic interactions between proteins of interest and the stationary phase, IEX columns must be loaded in low-salt buffers. For some applications, this restriction may require a buffer exchange step prior to ion exchange chromatography. Conversely, its requirement for loading samples in buffers of low ionic strength makes ion exchange chromatography an excellent second purification step after hydrophobic interaction chromatography (HIC). Ion exchange chromatography, unlike some other chromatography methods, also permits high flow rates, which in some cases can be crucial to the recovery of active protein. Finally, a limitation of weak ion exchangers is their pH dependence. When working outside of their optimal pH range, these resins rapidly lose capacity, and more importantly, resolution (see Table IX).

Table IX : Avantages and disavantages of ion exchange chromatography

IEX Pros	IEX Cons
Permits high flow rate	Sample must be loaded at low ionic strength
Concentrates samples	Clusters of positively charged residues can cause a net-negatively charged protein to bind a cation exchanger, and vice versa
High yield	Small changes in pH can greatly alter binding profile of IEX resin
Buffers are nondenaturing	Particle size greatly influences resolution

V.12 Advanced techniques in Ion Exchange Chromatography (IEX)

Advanced techniques in IEX focus on enhancing separation efficiency, improving selectivity, and combining IEX with other chromatographic or analytical methods. These advanced approaches are especially useful for complex mixtures, challenging separations, or high-throughput needs. Here's a detailed exploration of advanced IEX techniques **:**

1. High-Performance Ion Exchange Chromatography (HPIEX)

HPIEX is an advanced form of ion exchange chromatography designed for high-resolution, high-speed separations. It typically uses smaller particle sizes in resins, higher pressures, and optimized flow rates to improve performance.

Applications

- Ideal for high-throughput analyses in pharmaceutical quality control.
- Frequently used in the analysis of complex biological samples like protein digests or nucleic acids.

2. Fast Protein Liquid Chromatography (FPLC)

FPLC is an automated form of IEX often used for protein purification. It allows precise control over flow rates, buffer gradients, and detection, typically running at low to moderate pressures.

Applications

- Highly effective in purifying recombinant proteins, antibodies, and enzymes.
- Commonly used in the pharmaceutical industry for scalable protein and biologic purification.

3. Mixed-Mode Ion Exchange Chromatography

Mixed-mode chromatography combines ion exchange with hydrophobic interaction or affinity-based separation in a single chromatographic step. This enhances selectivity and allows the separation of molecules based on multiple properties simultaneously.

Applications

- Purification of proteins that are difficult to separate based on charge alone.
- Used in the downstream processing of biologics where multiple impurities must be removed.

4. Ion Exchange Chromatography Coupled with Mass Spectrometry (IEX-MS)

IEX is coupled to mass spectrometry (MS) for high-resolution separation followed by detailed structural or quantitative analysis of the separated compounds.

Applications

- Detailed analysis of post-translational modifications in proteins (e.g., phosphorylation, glycosylation).
- Identification and quantification of low-abundance peptides or metabolites in complex samples.

5. Ion Exchange Chromatography with 2D Chromatography (2D-IEX)

Two-dimensional chromatography (2D-IEX) enhances separation by using two orthogonal chromatographic techniques in sequence, such as IEX followed by reversed-phase (RP) chromatography.

Applications

- Widely used in proteomics to separate and identify proteins from complex biological samples.
- Often applied in metabolomics to resolve complex mixtures of small molecules.

6. Monolith Ion Exchange Chromatography

Monolith chromatography uses a continuous stationary phase (monolith) instead of traditional resin beads. Monolithic columns provide high flow rates and low backpressure, ideal for high-throughput processing.

Applications

- Purification of large biomolecules like viruses, plasmid DNA, and even whole cells.
- Used in biopharmaceutical industries for large-scale production of therapeutic proteins.

7. Ion Chromatography (IC)

Ion chromatography (IC) is a subset of liquid chromatography, specifically used for the separation of ions and polar molecules based on their charge. It can involve suppressor technology for enhanced sensitivity in detecting ions.

Applications

- Water quality analysis (e.g., testing for nitrates, phosphates, and other anions).
- Pharmaceutical industry for monitoring ionic impurities in drug formulations.

8. Capillary Ion Exchange Chromatography

Capillary IEX involves the use of capillary columns, which are smaller in diameter (usually less than 1 mm), leading to more efficient separations in terms of both speed and resolution, with minimal sample volumes.

Applications

- Used in proteomics and metabolomics for analyzing limited sample amounts.
- Suitable for trace analysis in environmental and clinical samples.

9. Membrane Chromatography (IEX Membranes)

Membrane chromatography uses ion exchange membranes rather than packed columns for separations. This allows for faster flow rates and lower pressure drops, making it ideal for large-scale operations.

Applications

- Widely used in the production of monoclonal antibodies and vaccines.
- Ideal for polishing steps where fast flow rates are required.

IV.1 Introduction

Partition chromatography is one of the fundamental techniques in separation science. This technique was first introduced in the early 20th century and has since become a foundational method in analytical and preparative chemistry for separating mixtures of closely related compounds. This method is particularly useful for separating mixtures of substances based on their distribution between two liquid phases. It has been widely applied in analytical chemistry, especially for the separation of polar and semi-polar compounds. Partition chromatography is commonly used in techniques such as paper chromatography, thin-layer chromatography, and liquid-liquid chromatography.

Partition chromatography can be performed in various forms, including liquid-liquid partition chromatography, where both phases are liquids, or gas-liquid chromatography (GLC), where the mobile phase is a gas and the stationary phase is a liquid. Regardless of the form, the technique allows precise separation of substances in complex mixtures.

IV.2 Principle

The principle of partition chromatography is based on the differential solubility of components of a mixture between two immiscible phases: a stationary phase and a mobile phase. The key idea is that when a mixture of substances is introduced into the chromatography system, each component will partition itself between these two phases in accordance with its solubility, leading to their separation Figure.14

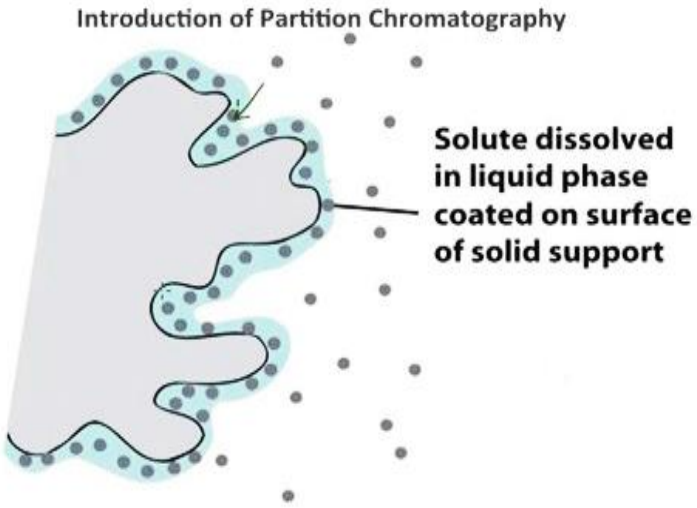


Figure 14: Partition chromatography

IV.3 Immiscible Phases

Partition chromatography involves two immiscible phases:

- *Stationary phase*: This phase is typically a liquid that is immobilized (e.g., on a solid support). This liquid acts as the medium where the solutes dissolve.
- *Mobile phase*: This can either be a liquid or a gas that flows through or along the stationary phase and carries the solutes. The mobile phase acts as a carrier that moves the components through the column.

IV.4 Partitioning of Solutes Between Two Phases

The principle behind partition chromatography can be understood as the repeated transfer (or partitioning) of solutes between the stationary and mobile phases. This transfer is governed by the partition coefficient Ķ, which reflects how much of a particular solute prefers to dissolve in the stationary phase compared to the mobile phase. The partition coefficient is given by the equation :

$$K = \frac{c_{\mathrm{s}}}{c_{\mathrm{m}}}.$$

Here

K: Partition coefficient (repartition coefficient)

C_s : Substance concentration in the stationary phase

C_m: Substance concentration in the mobile phase

Each component of a mixture will have a different partition coefficient, meaning some components will spend more time in the stationary phase (moving slowly), while others will prefer the mobile phase (moving faster). The difference in movement through the stationary phase leads to separation of the mixture's components.

IV.5 Key Features of Partition Chromatography

The main characteristics of partition chromatography are:

- **Stationary Phase**

Typically, a liquid that is immobilized on a solid support or material. The liquid stationary phase can be either water or an organic solvent, depending on the type of chromatography being performed.

- **Mobile Phase**

Usually a liquid or gas that carries the mixture through the stationary phase. The mobile phase flows through or over the stationary phase and is responsible for carrying the components of the mixture.

IV.6 Types of Partition Chromatography

Partition chromatography comes in different variations, depending on the nature of the phases and the setup.

IV.6.1 Liquid-Liquid Partition Chromatography

In this classic version, both the stationary and mobile phases are liquids. Here, the stationary phase is typically a liquid that has been adsorbed onto a solid support (like silica gel). The mobile phase is another liquid, which flows over the stationary phase.

Example: Separation of non-volatile organic compounds.

Mechanism: The components of the sample partition between the stationary liquid phase and the flowing mobile liquid phase.

IV.6.2 Gas-Liquid Chromatography (GLC)

This method uses a gas as the mobile phase and a liquid as the stationary phase. GLC is particularly suited for the separation of volatile compounds.

Stationary Phase: A liquid is coated on an inert solid support, such as diatomaceous earth, inside a column. Mobile Phase: A carrier gas (e.g., nitrogen or helium).

Application: Separation of volatile organic compounds like hydrocarbons, alcohols, and esters. GLC operates on the principle that volatile compounds, when carried by the gas phase, will interact with the stationary liquid phase to different extents, leading to their separation.

IV.6.3 Counter-Current Chromatography

In this advanced technique, both phases are liquids, but they are continuously and dynamically flowing. The stationary phase remains in the system by virtue of its higher viscosity or density, while the mobile phase flows through it. The two liquids are immiscible.

Application: Often used for the purification of natural products and biomolecules.

IV.7 Factors Affecting Separation

Several factors influence how effectively components are separated in partition chromatography:

- **Solubility**: The relative solubility of the components in the mobile and stationary phases is the most important factor. A slight change in solvent composition can drastically affect the separation.
- **Temperature**: Particularly in gas-liquid chromatography, the temperature can affect the volatility of the components, impacting their retention time.
- **Flow Rate of the Mobile Phase**: A higher flow rate means faster elution, which may result in poorer separation, as there may not be enough time for partitioning between the phases.
- **Column Length**: A longer column provides a greater surface area for interaction between the solutes and stationary phase, improving separation.

IV.8 Variant of partition chromatography

In partition chromatography, two important variants are normal-phase chromatography (NPC) and reversed-phase chromatography (RPC). These techniques differ primarily in the polarity of their stationary and mobile phases, and they are widely used for the separation of compounds based on polarity.

IV.8.1 Normal-Phase Chromatography (NPC)

Normal phase LC involves a combination of a polar stationary phase and a less polar (or even nonpolar) mobile phase. Normal phase was one of the first developed separation methods and, for this reason, reversed phase LC was labeled just that, as it involves stationary and mobile phases with the reversed polarities. Today, a polar functional group is bonded to silica, which is advantageous when analytes that are insoluble in polar solvents need to be separated. In regard to elemental speciation analysis by ICP spectrometry, normal phase LC has very limited use. This is partially due to the incompatibility of the mobile phase with the conventional ICP but also because reversed phase LC can do many of the separations as well. Often, normal phase LC is used in combination with size exclusion chromatography. Although not as popular today, normal phase LC is still a corner stone of LC.

In NPC, the stationary phase is polar while the mobile phase is non-polar. The retention of analytes occurs by the interaction of the stationary phase's polar functional groups with the polar groups on the particles' surfaces, and they elute from the column by

addition of the low polarity compound followed by other compounds of increasing polarity (Figure. 15. This method is widely used to separate analytes with low to intermediate polarity.

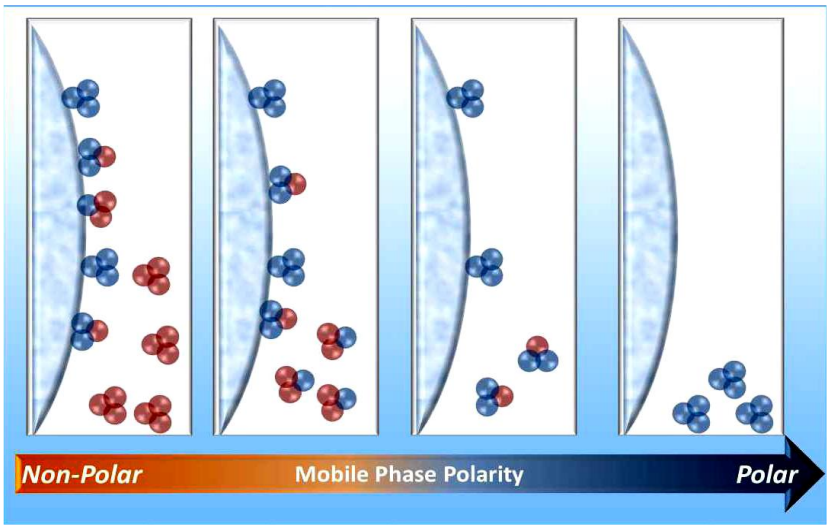


Figure. 15: Diagram of normal-phase chromatography separation.

The stationary phase is polar and retains the polar molecule (blue) most strongly. The relatively non-polar molecules (redcircles) are quickly eluted by the mobile phase, a non-polar solvent. An increase in mobile phase polarity will move polar molecules through the column.

IV.8.1.1 Key Features of Normal-Phase Chromatography

8.1.1.1 Stationary Phase

In NPC, the stationary phase is more polar than the mobile phase. As illustrated in Figure 16, the retention increases as the polarity of the mobile phase decreases and thus polar analytes are more strongly retained than nonpolar analytes, the opposite of that in reversed-phase liquid chromatography (RPLC). The column packing is either an inorganic adsorbent (silica gel or, less often, aluminum, titanium, or zirconium oxides) or a moderately polar bonded phase (cyanopropyl, $–(CH_2)^3–CN$; diol,$–(CH_2)^3–O–CH_2–CHOH–CH_2–OH$; or aminopropyl, $–(CH_2)^3–NH_2$) chemically bonded on a suitable support material, which is usually silica gel. The mobile phase is usually a mixture of two or more organic solvents: nonpolar solvent and strongly or weakly polar solvents, or a mixture of water and an organic solvent (usually acetonitrile) in aqueous normal-phase chromatography (ANP). Moderately polar bonded phases may be used either in the normal-phase mode with organic mobile phases, such as nhexaneþ2-propanol, or in the reversed-phase mode with aqueous–organic mobile phases. However, normal-phase behavior can also be sometimes observed in nonaqueous RPLC with chemically bonded C18, C8, or other alkyl-bonded phases.

The reason is the activity of polar residual silanol groups that remain on the support surface after incomplete reaction of silica gel with organosilanes. Thus, such a stationary phase may behave as a deactivated polar adsorbent in nonpolar or weakly polar organic solvents. As the retention on inorganic adsorbents originates due to the interactions of the polar adsorption centers on the surface with the polar functional groups of the analytes, this mode was also called adsorption or liquid–solid chromatography.

The stationary phases used in normal phase chromatography are usually silica or alumina and they are polar as a result of hydroxyl groups (-OH) (Figure. 16). The surface hydroxyl groups interact with the functional groups on the solute molecules and depending on the strength of this interaction preferentially absorb one solute relative to another (absorb the more polar compound relative to the less polar). In case the solute is a neutral molecule that has a permanent dipole or if a dipole can be induced on it, then it will be attracted by dipole-dipole interaction to the stationary phase.

A mechanism that describes the adsorption process in normal phase liquid chromatography is shown in Figure. 16.

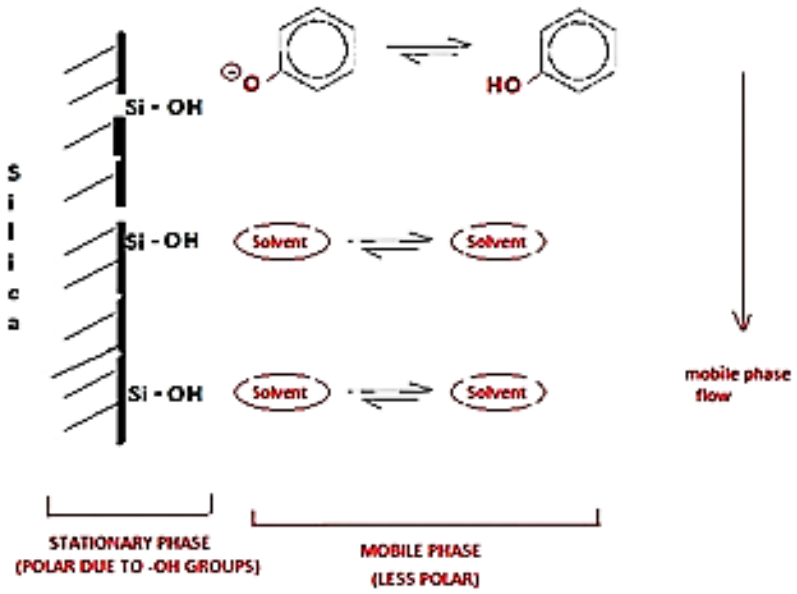


Figure. 16: The mechanism of retention of the solute Ph-OH is shown in a typical normal phase chromatography separation.

An equilibrium is established between the charged and the protonated form of the solute. The charged form binds to the surface of the polar stationary phase and competes for the same positions with solvent molecules. If the polarity of the solvent is increased then more

solvent molecules bind to the surface of the stationary phase and the solute (PhOH) elutes faster since it remains relatively unretained.

8.1.1.2 Mobile phase

In NPC, the mobile phase is non-polar or weakly polar. As it flows through the chromatographic column, it carries the components of the sample mixture. Separation occurs based on the differential interactions of the compounds with the non-polar mobile phase and the polar stationary phase. Compounds that are more polar tend to interact more strongly with the stationary phase and are retained longer, while less polar compounds are eluted more quickly with the mobile phase.

A) Characteristics of the Mobile Phase in Normal-Phase Chromatography

Non-Polar Nature

The mobile phase is typically composed of non-polar or weakly polar solvents. The low polarity ensures that polar compounds in the sample have a higher affinity for the stationary phase (which is polar), while non-polar compounds will prefer the mobile phase and move more quickly through the column.

Common Solvents

Hexane: One of the most commonly used non-polar solvents in NPC. It is a hydrocarbon and highly non-polar, often used as a base solvent.

***Chloroform*:** Moderately non-polar, it is sometimes mixed with other solvents to adjust the polarity of the mobile phase.

Diethyl Ether: A weakly polar solvent that is used when a slightly more polar mobile phase is needed for better separation.

***Toluene*:** Another non-polar solvent often used in combination with other solvents to achieve desired separation characteristics.

Mixtures of solvents: NPC often uses a combination of solvents to fine-tune the eluting power (polarity) of the mobile phase. For example, a mix of hexane and diethyl ether can be used to achieve intermediate polarity.

IV.8.2 Role in Separation

Non-polar compounds in the sample will have a higher affinity for the non-polar mobile phase, meaning they will partition into the mobile phase more readily and move

quickly through the column. Polar compounds, on the other hand, will interact more with the polar stationary phase and be retained longer, causing them to move more slowly.

IV.8.2.3 Factors Affecting Separation

The efficiency and resolution of chromatographic separation depend on several key factors namely:

- **Polarity** of the analytes and the phases is the key factor in determining how well a compound will be retained in the column.
- **Mobile Phase Composition**: The choice of non-polar solvent and its strength influences how quickly the compounds elute.
- **Column Temperature**: This can affect the solubility and interaction of the analytes with the stationary phase.
- **Stationary Phase Material**: The type of polar material (e.g., silica gel) and its surface chemistry will determine the strength of interaction with polar compounds.

IV. 8.2.4 Applications of Normal-Phase Chromatography

Normal-phase chromatography (NPC) has several important applications in fields such as organic chemistry, pharmaceuticals, natural products, and analytical chemistry. Its ability to effectively separate polar compounds makes it valuable for both analytical and preparative purposes. NPC is particularly useful when dealing with compounds that are difficult to retain in reversed-phase systems, offering an alternative approach for the purification and analysis of polar mixtures.

1. Separation of Natural Products

- **Plant Extracts**: NPC is widely used in the isolation and purification of natural products such as alkaloids, terpenoids, flavonoids, and phenolic compounds from plant extracts. Many of these compounds are polar, and NPC can effectively separate them based on their polarity.
- **Lipids and Phospholipids**: Natural lipid classes like phospholipids, glycolipids, and fatty acids are often separated using NPC, where the polar stationary phase interacts with polar functional groups in lipids, allowing for fine separation.

2. Pharmaceuticals and Drug Development

- **Purification of Drug Compounds**: Many pharmaceutical compounds contain polar functional groups (e.g., alcohols, amines, acids), and NPC is used for their purification, especially in early stages of drug development and research.
- **Separation of Drug Metabolites**: NPC is often employed to separate drug metabolites from biological samples due to its ability to retain and separate polar compounds, which are often produced as metabolites in the body.
- **Quality Control**: Pharmaceutical companies use NPC for quality control during drug manufacturing processes, ensuring that impurities and unwanted byproducts are separated and removed.

3. Separation of Isomers and Enantiomers

- **Chiral Separations**: NPC is widely used to separate **enantiomers** (chiral compounds) due to its ability to resolve small differences in the interactions of chiral molecules with the stationary phase. This is crucial in the pharmaceutical industry, where enantiomeric purity can have significant biological and therapeutic implications.
- **Geometric Isomers**: NPC is also used to separate geometric isomers (e.g., **cis-trans isomers**) because of the differences in their interaction with the polar stationary phase

4. Fatty Acid and Oil Analysis

- **Separation of Triglycerides and Fatty Acids**: In food chemistry and nutritional science, NPC is frequently used to analyze the fatty acid composition of oils and fats. It helps in separating different types of fatty acids and triglycerides, which is essential for determining the nutritional content and quality of food products.
- **Phospholipid Separation**: NPC is also valuable in separating **phospholipids** from complex mixtures of lipids, especially in biochemical and medical research.

5. Environmental Testing

- **Pesticides and Pollutants**: NPC is used to separate and analyze polar pesticides, herbicides, and contaminants in environmental samples such as soil, water, and air. The separation of polar organic compounds is important in environmental monitoring and ensuring regulatory compliance for pollutant levels.
- **Water Contaminants**: For detecting organic pollutants in water, NPC can effectively isolate and measure these substances due to their polar nature.

6. Chemical Synthesis and Purification

- **Organic Synthesis**: In organic chemistry labs, NPC is a common technique for purifying reaction products, especially when the products are polar and need to be separated from non-polar byproducts or solvents.
- **Synthetic Polymers**: NPC can be applied in the analysis and purification of certain polar polymers and their monomers, especially when functional groups such as hydroxyl, carboxyl, or amine are present.

7. Food and Beverage Industry

- **Flavor and Aroma Compounds**: NPC is used to separate polar compounds responsible for flavors and aromas in foods and beverages, such as esters, phenols, and organic acids.
- **Vitamins and Nutritional Components**: The technique is also useful in separating polar vitamins (e.g., vitamin C, B vitamins) and other nutritional components from food matrices during quality control testing.

8. Biological Sample Analysis

- **Amino Acid and Peptide Separation**: NPC is employed for the separation and analysis of amino acids, peptides, and other polar biomolecules in biological samples like **blood**, **urine**, and **tissue extracts**. This is critical in biomedical research, clinical diagnostics, and metabolomics.
- **Nucleotides and Nucleosides**: NPC can separate nucleotides, nucleosides, and other biomolecules that play a role in biochemical pathways and genetics research.

9. Forensic Science

- **Toxicology Testing**: NPC is used in forensic toxicology to detect and analyze polar drugs and metabolites in biological samples (e.g., blood, urine) during investigations related to drug use or poisoning.
- **Analysis of Explosives and Hazardous Chemicals**: Certain explosives and hazardous materials that contain polar functional groups can be separated and identified using NPC in forensic investigations.

IV.8.2 Reversed-Phase Chromatography (RPC)

Reversed-phase chromatography (RPC) is the most widely used form of liquid chromatography, particularly in high-performance liquid chromatography (HPLC). In RPC, the stationary phase is non-polar (hydrophobic), and the mobile phase is polar. This is the opposite of normal-phase chromatography, which is why it’s called "reversed-phase."

In RPC, components of the mixture are separated based on their hydrophobicity (non-polarity). Polar compounds are eluted faster, while non-polar compounds are retained longer due to stronger interactions with the non-polar stationary phase.

IV.8.2.1 Reversed-phase chromatography-how does it work

RPC is a separation of molecules based on their hydrophobicity. Hydrophilic molecules elute first from a reversed-phase column, strongly hydrophobic molecules last. The most common chromatographic mode used for reversed-phase separations of peptides and proteins is gradient elution, using two different mobile phases. In this operation mode the sample, dissolved in a hydrophilic, typically aqueous eluent, will be injected onto a column which is equilibrated with the same hydrophilic eluent. A hydrophobic molecule will bind to the column, because the affinity of the molecule to the hydrophobic stationary phase is larger than to the hydrophilic mobile phase. After the sample is loaded, a gradient will be started with a continuously increasing concentration of a hydrophobic eluent. The molecule elutes when the affinity of the molecule to the liquid phase is larger than that to the stationary phase. When ionic solutes are to be separated, the addition of a counter ion (ion pair reagent) will improve the retention of the ionic species. The non-polar stationary phase of a reversed-phase packing is usually an alkyl bonded phase based on a silica support. It is prepared by covalently attaching an alkyl group to the surface silanol group of silica gel via a siloxane linkage. The non-silica based reversedphase sorbents can be divided into packings based on inorganic matrices and packings bases on polymeric matrices

IV.8.2.2 Principle

Molecules are separated in reversed-phase chromatography by partitioning in the mobile phase and stationary phase. The partitioning is governed by an equilibrium, specific for the solute interacting with the mobile phase and stationary phase.

As the mobile phase moves the solute down the column, there is a constant movement of the solute from the mobile phase to the stationary phase and vice versa. The more hydrophobic the solute, the higher is the affinity of the solute to the stationary phase, the more time the solute spends in the stationary phase and the later it leaves the column. As a result, molecules with different equilibrium constants elute at different times and are separated. Despite numerous studies concerning the theory of retention in reversed-phase chromatography there is still considerable uncertainty as to the mechanism of the overall process.

RPC has become a powerful tool widely used in the analysis and purification of biomolecules because of the high resolution provided by the technique. It is considered a very versatile technique because it can be used for non-polar, polar, ionizable, and ionic molecules. In RP-HPLC, the separation principle is based on the hydrophobic interaction between the analytes and non-polar groups bound on the stationary phase. Silica is the most common material used for column packing, which consists mainly of silicon dioxide (SiO_2) and has octadecyl (hydrocarbons having 18 carbonatoms) and octyl (hydrocarbons having 8 carbon atoms) groups chemically bound to the surface. The mobile phase composition is usually water or a water-miscible organic solvent (methanol, acetonitrile). The analytes adsorbed on the hydrophobic surface remain bound until the higher concentration of the organic solvent promotes the desorption of the molecules from the hydrophobic surface (Figure.17). More hydrophobic analytes are eluted slower than are the hydrophilic analytes.

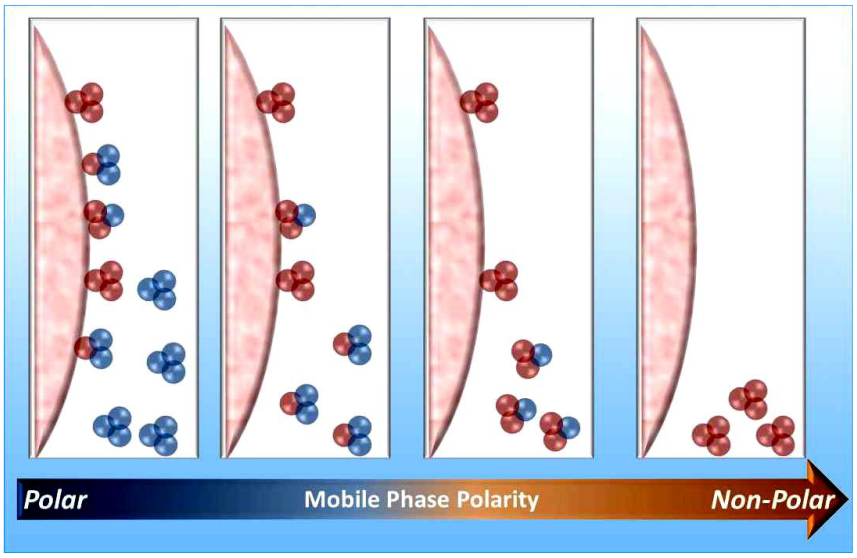


Figure. 17: Diagram of Reverse-phase chromatography separation.

The stationary phase is nonpolar and retains the non-polar molecule (red) most strongly. The relatively polar molecules (blue circles) are quickly eluted by the mobile phase, a polar solvent. A decrease in mobile phase polarity will move non-polar molecules through the column.

IV.8.2.3 Theory of reversed phase chromatography

In reversed phase chromatography, separation relies on hydrophobic interactions between the solute in the mobile phase and the hydrophobic stationary phase. Although the exact nature of this interaction is debated, it is generally thought to be driven by a favorable increase in entropy. Initially, both the solute and stationary phase are surrounded by structured water.

When they interact, the exposed hydrophobic surfaces decrease, reducing the structured water and increasing entropy, which makes the binding energetically favorable.

8.2.2.1 Matrix

The stationary phase is a critical component in RPC, influencing the separation mechanism and performance of the column. Here are detailed aspects of the stationary phase in RPC: A reversed phase chromatography medium consists of hydrophobic ligands chemically grafted to a porous, insoluble beaded matrix. The matrix must be both chemically and mechanically stable. The base matrix for the commercially available reversed phase media is generally composed of silica or a synthetic organic polymer such as polystyrene. Figure.18 shows a silica surface with hydrophobic ligands.

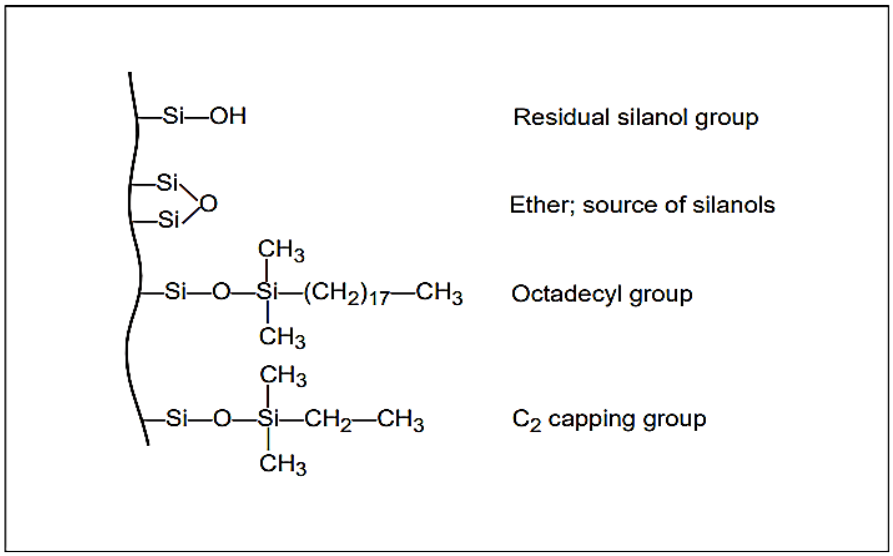


Figure. 18 : Some typical structures on the surface of a silica-based reversed phase medium. The hydrophobic octadecyl group is one of the most common ligands

Silica was the first polymer used as the base matrix for reversed phase chromatography media. Reversed phase media were originally developed for the purification of small organic molecules and then later for the purification of low molecular weight, chemically synthesised peptides. Silica is produced as porous beads which are chemically stable at low pH and in the organic solvents typically used for reversed phase chromatography. The combination of porosity and physical stability is important since it allows media to be prepared which have useful loading capacities and high efficiencies. It is worth noting that, although the selectivity of silica-based media is largely controlled by the properties of the ligand and the mobile phase composition, different processes for producing silica-based matrices will also give media with different patterns of separation. The chemistry of the silica gel allows simple derivatisation

with ligands of various carbon chain lengths. The carbon content, and the surface density and distribution of the immobilised ligands can be controlled during the synthesis.

The primary disadvantage of silica as a base matrix for reversed phase media is its chemical instability in aqueous solutions at high pH. The silica gel matrix can actually dissolve at high pH, and most silica gels are not recommended for prolonged exposure above pH 7.5.

The most important feature of silica-based reversed-phase packings is the interaction of analytes with the surface silanols (Figure.19). There are about 8 mmol m 2 silanols on a surface of a fully hydroxylated silica, and at most about 50% of them can be removed with bonding reactions. A very important effect is the ion exchange of protonated bases with ionized silanols, which is most relevant at neutral pH, but often not negligible at acidic pH either. The combination of reversedphase interaction with silanol interaction often results in strongly tailing peaks, a phenomenon not uncommon if the analyte contains basic functional groups.

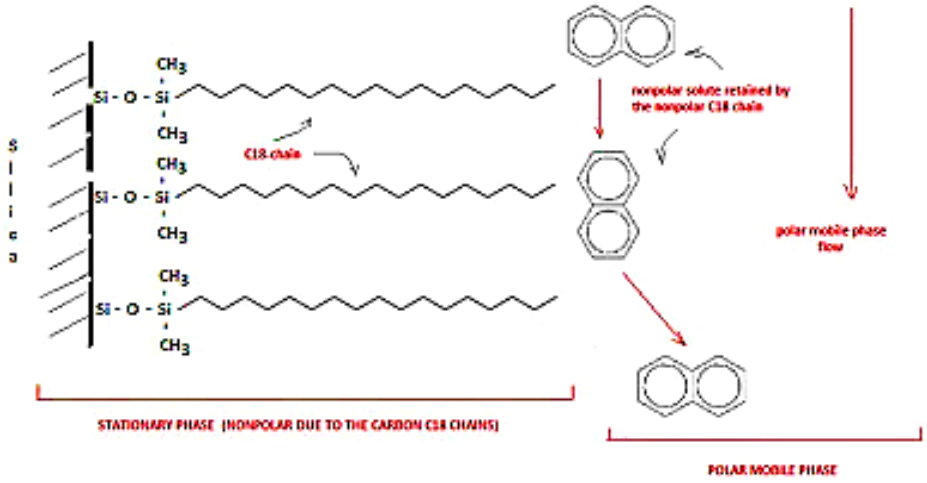


Figure. 19 : Stationary reversed phase (silica resin with C18 chain)

8.2.2.2 Mechanism of Separation

In RPC, polar compounds move faster through the column because they have a higher affinity for the polar mobile phase, whereas non-polar compounds interact more strongly with the hydrophobic stationary phase and thus elute more slowly. This causes the separation of compounds based on their hydrophobic interactions.

- **Hydrophobic modifications**

Hydrophobic interaction or the hydrophobicity of a packing is the primary driver of retention in reversed-phase chromatography. Steric and polar interactions modify the retention and the selectivity of a separation. The different polar interactions are the ion exchange mostly with ionized silanols on the surface, the hydrogen bonding with silanols or with other polar functional groups embedded in the ligand or otherwise incorporated into the packing, electron– donor and –acceptor interactions with aromatic groups on the surface, and dipole–dipole interactions. We will discuss each of these in more detail below.

The retention factor of simple hydrophobic compounds can be used to judge the hydrophobicity of a packing. The dominant factor that influences the hydrophobicity is the chain length of the ligand. The methylene-group selectivity (i.e., the relative retention of hydrocarbons that differ from each other by a methylene group in the chain) is useful for measuring the hydrophobic selectivity of a packing. Typically, C18 packings have a larger methylene-group selectivity than C8 packings, but the surface coverage plays a role as well. This is due to steric influences stemming from the crowding of the ligand. For polynuclear aromatic hydrocarbons it was found that the 'shape selectivity' increases with the liganddensity of polymeric bonded phases. On the other hand, packings with a low ligand density such as Waters Atlantiss dC18 have been found to exhibit a better retentivity than highly coated phases on a similar silica. The better availability of the ligand for interaction with analytes for a packing with a low ligand density is the cause of this phenomenon. On a densely covered C18, not all of the ligand is available for interaction with the analyte.

The silica particles are chemically modified with hydrophobic groups, typically long-chain hydrocarbons. Common modifications include :

1. Alkyl-Bonded Phases

These are the most common stationary phases in reversed-phase chromatography, where hydrophobic alkyl chains are bonded to the silica surface.

- **C18 (Octadecylsilane, ODS)**

Consists of an 18-carbon alkyl chain (CH_3-$(CH_2)_{17}$-Si-) see (Figure.20). The most hydrophobic stationary phase. Strong hydrophobic interactions, retaining non-polar to moderately polar compounds for longer periods. Suitable for a broad range of applications, including pharmaceuticals, environmental samples, and peptides. Provides the best retention for non-polar compounds and separates complex mixtures effectively. Widely used in HPLC for separating small molecules, drug formulations, and lipids.

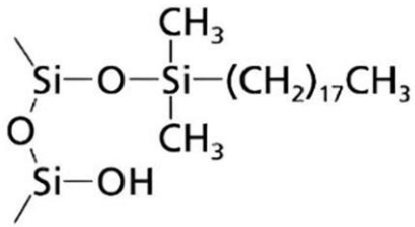


Figure. 20 : **Structure of C18**

- **C8 (Octylsilane)**

Consists of an 8-carbon alkyl chain (CH_3-$(CH_2)_7$-Si-) see Figure.21. Provides moderate hydrophobicity, with weaker interactions compared to C18. Faster elution of non-polar compounds due to reduced retention. Suitable for compounds that are less hydrophobic or require faster separation. Often used for compounds that elute too slowly on C_{18} columns. Peptide separations, organic acids, and moderately polar pharmaceuticals.

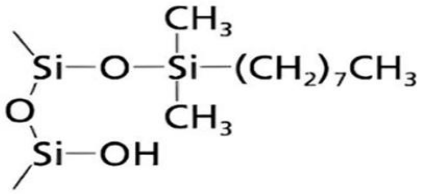


Figure.21 : Structure of C8

- **C4 (Butyl)**

Contains a 4-carbon alkyl chain (CH_3-$(CH_2)_3$-Si-).Even shorter alkyl chains, like C4, provide less hydrophobic interaction and are sometimes used for very large or hydrophobic analytes like proteins and peptides.Structure: A 4-carbon alkyl chain. Shorter alkyl chains lead to even weaker hydrophobic interactions. Faster elution for highly hydrophobic compounds or large biomolecules like proteins and peptides. Ideal for analytes where over-retention on C18 or C8 columns is problematic. Applications: Proteins, large peptides, and bio-molecules, where reduced retention is necessary to avoid degradation during prolonged runs.

- **C1 (Methylsilane)**

A single carbon alkyl chain (CH_3-Si-). The least hydrophobic stationary phase, offering minimal retention of non-polar compound. Useful when separation of very polar analytes is required. Interactions are dominated by other mechanisms (e.g., polar interactions) rather than hydrophobicity.

- **Phenyl Bonded Phase**

A phenyl group (C_6H_5) bonded to the silica surface offers both hydrophobic interactions and π–π stacking interactions. While it provides weaker hydrophobicity compared to alkyl chains such as C_{18}, it offers unique selectivity for analytes containing aromatic rings. **Applications:** Suitable for the separation of aromatic compounds, polyaromatic hydrocarbons, certain pharmaceuticals, and other compounds requiring specific aromatic interactions.

8.2.2.3 Mobile phase

The mobile phase in reversed-phase chromatography is a mixture of water or buffer with a polar organic solvent such as methanol, acetonitrile, isopropanol (IPA), or tetrahydrofuran (THF).

The mobile phase significantly affects the solute separation process. In most cases, binary mixes of water and an organic solvent are employed, although ternary or quaternary mixtures of water and two or three different organic solvents can also be utilized to regulate the strength and selectivity of elution. The degree of interaction between the solutes and the stationary phase is determined by the mobile-phase composition, which includes solvents, buffers, and other additives. To maximize each of these elements a variety of strategies can be used. Theoretically, a large range of water-miscible organic solvents can be employed as modifiers, however, only a few of them are used in RPLC, including acetonitrile, methanol, ethanol, tetrahydrofuran, and isopropanol.

The primary objective of utilizing these organic solvents is to retain the mobile phase's polarity low enough to dissolve the partially hydrophobic solute yet high enough to enable the solute's binding to the reverse-phase phase chromatographic matrix. The process of changing the number of organic solvents in the mobile phase to isolate a molecule of interest is known as a gradient elution.

8.2.2.4 Key Characteristics of the Mobile Phase in Reversed-Phase Chromatography

Reversed-phase chromatography (RPC or RP-HPLC) is a widely used technique in analytical chemistry, especially for separating compounds based on hydrophobicity. The mobile phase in reversed-phase chromatography plays a crucial role in determining the efficiency and selectivity of the separation. Here are the key characteristics of the mobile phase in RPC :

1. Polarity

- The mobile phase in reversed-phase chromatography is generally polar compared to the non-polar stationary phase. It is often a mixture of water or aqueous buffer and a less polar organic solvent (such as methanol, acetonitrile, or tetrahydrofuran).
- As the polarity of the mobile phase decreases (more organic solvent), non-polar compounds elute faster, since they interact less with the stationary phase.

2. Composition

The mobile phase is usually a mixture of water (as the polar component) and organic solvents such as : Methanol, Acetonitrile and Ethanol. The composition of the mobile phase can be adjusted to enhance separation. Higher organic solvent content results in faster elution of hydrophobic compounds.

The proportion of organic solvent (like methanol or acetonitrile) in the mobile phase influences the retention time of analytes. Increasing the amount of organic solvent in the mobile phase typically reduces the retention of hydrophobic compounds, causing them to elute faster.

3. pH of the Mobile Phase

The **pH** is an important factor, particularly when separating compounds with ionizable groups (like acids or bases). The pH can influence the ionization state of both the analytes and the stationary phase, altering their interactions.

Buffers (e.g., phosphate or acetate buffers) are often added to the aqueous part of the mobile phase to maintain a stable pH, improving reproducibility and resolution.

4. Organic Solvent Percentage

The percentage of organic solvent in the mobile phase affects the separation.

A higher percentage of organic solvent decreases retention times for hydrophobic analytes, causing them to elute faster. A lower percentage of organic solvent increases retention times, as analytes interact more with the hydrophobic stationary phase.

5. Gradient vs. Isocratic Elution

- **Isocratic elution**: The mobile phase composition remains constant throughout the run. This is useful for simple separations.
- **Gradient elution**: The composition of the mobile phase changes gradually (usually increasing the organic solvent content). This method is ideal for separating complex mixtures with analytes of varying polarity

6. Viscosity

- The viscosity of the mobile phase affects the column backpressure and, consequently, the flow rate. A higher concentration of organic solvent usually lowers the viscosity compared to a pure aqueous mobile phase.
- The mobile phase's viscosity also influences peak broadening and separation efficiency.

7. Solubility of the Analytes

The analytes must be soluble in the mobile phase. Poor solubility can lead to precipitation and poor chromatographic performance. The mixture of water and organic solvent is often optimized to ensure solubility of all components.

8. Selectivity

- The choice of organic solvent, its percentage, and any additives significantly impact the selectivity of the separation.
- By modifying these parameters, analysts can optimize the separation of compounds with similar retention times or polarity.

9. UV Transparency

- Since reversed-phase chromatography is commonly coupled with UV detection, the mobile phase should be transparent at the wavelengths used for detection. Solvents like acetonitrile and methanol are frequently used because of their low absorbance in the UV range (190-220 nm).

10. Volatility

- In preparative chromatography or when using mass spectrometry (MS) as a detection method, the volatility of the mobile phase is important. Volatile solvents like acetonitrile or methanol are preferred, as they can easily be removed by evaporation or ionization in MS.

IV. 8.2.3 Applications

RPC has a broad range of applications across various scientific fields, due to its versatility in separating both small molecules and large biomolecules. Here are some detailed applications across industries:

1. Pharmaceutical Industry

It is applied mainly in:

- **Drug Purity and Impurity Profiling**

Reversed-phase chromatography (RPC) is a key tool in ensuring the purity of active pharmaceutical ingredients (APIs). It is widely used to separate and quantify impurities, including degradation products and contaminants, that may affect the safety or efficacy of pharmaceutical compounds. RPC allows for accurate determination of the concentration of the main active compound while also enabling the detection and quantification of trace-level impurities. Its high resolution and reproducibility make it essential in quality control and regulatory compliance within the pharmaceutical industry.

- **Bioavailability Studies**

RPC is used to analyze drug concentrations in biological fluids (like blood, plasma, or urine) to understand how drugs are absorbed, distributed, metabolized, and excreted in the body (ADME studies).

- **Metabolite Analysis**

In pharmacokinetics, RPC is employed to separate and identify drug metabolites. It is frequently coupled with mass spectrometry (LC-MS) for identifying and quantifying metabolites in complex biological matrices.

- **Peptide and Protein Purification**

Peptides and proteins used in biologic drugs or research can be separated and purified using RPC. The technique helps in ensuring that biologics have the required purity and composition.

2. Proteomics and Biotechnology

RPC plays a critical role in proteomics and biotechnology for the separation, purification, and analysis of peptides and proteins.

- **Protein Separation**

RPC plays a critical role in proteomics (the large-scale study of proteins). It is used for protein identification, quantification, and separation based on hydrophobicity.

Coupled with techniques like **LC-MS/MS**, it allows for the separation of complex protein mixtures and analysis of post-translational modifications.

- **Peptide Mapping**

This technique is widely used for verifying the sequence of peptides in protein drugs and biopharmaceuticals. Peptides produced from enzyme digestion of proteins are separated via RPC and analyzed to ensure correct sequence and structure.

- **Monoclonal Antibody (mAb) Purification**

In the production of monoclonal antibodies (used in cancer treatment and immune therapy), RPC is used for the final purification steps, ensuring that the antibodies are of the correct form and free from contaminants.

3. Food and Beverage Industry

Is widely used in the food and beverage industry for the analysis of food components, additives, contaminants, and natural compounds. It is particularly useful for :

- **Food Additive Analysis**

RPC is applied to detect and quantify food additives, preservatives, colorants, and flavoring agents in various food products. It helps ensure that these additives are within legal and safe limits.

- **Fatty Acids and Lipids**

Separation of complex mixtures of fatty acids and lipids from food samples is commonly performed using RPC. This is particularly important for understanding the nutritional content and for quality control.

- **Pesticide Residue Detection**

In fruits, vegetables, and grains, RPC is used to detect pesticide residues, ensuring that levels are below harmful thresholds set by regulatory agencies.

- **Natural Product Analysis**

Many plant-derived bioactive compounds (like polyphenols and flavonoids) are analyzed using RPC to assess their health benefits or to confirm the authenticity of functional foods.

4. Environmental Monitoring

- **Water Quality Testing**

RPC is crucial for detecting pollutants, pesticides, and organic compounds in water samples. This includes tracking hazardous chemicals like polycyclic aromatic hydrocarbons (PAHs) and pharmaceuticals that may enter water systems from wastewater.

- **Soil and Air Pollution Analysis**

Complex mixtures of environmental contaminants, such as volatile organic compounds (VOCs), persistent organic pollutants (POPs), and other toxic chemicals, can be effectively separated and quantified using RPC.

5. Clinical and Biomedical Research

- **Therapeutic Drug Monitoring (TDM)**:

RPC is employed in clinical labs for therapeutic drug monitoring, where the concentration of drugs in patient samples (blood, serum, plasma) is measured to optimize drug dosing and minimize toxicity.

- **Biomarker Discovery**

In biomarker discovery and clinical diagnostics, RPC is often used to separate and identify proteins, peptides, and small molecules that can serve as disease markers.

- **Hormone and Vitamin Analysis**

It is used to measure concentrations of hormones (like steroids) and vitamins (like Vitamin D metabolites) in clinical and research settings.

6. Forensic Science

- **Toxicology**

In forensic toxicology, RPC is applied to detect and quantify drugs, poisons, and other toxic substances in biological samples like blood, urine, or tissues.

- **Illicit Drug Testing**

RPC is used to identify and quantify illicit drugs and their metabolites in forensic samples.

- **Post-mortem Analysis**

In cases of suspected drug overdose or poisoning, RPC helps in identifying toxic agents in post-mortem samples.

7. Cosmetic Industry

- **Quality Control of Ingredients**

The purity of cosmetic ingredients, such as preservatives, fragrances, and active compounds (like retinoids or sunscreens), is evaluated using RPC. This ensures that the products meet safety and quality standards.

- **Analysis of Essential Oils**

Essential oils, which are often used in perfumes, skincare, and other cosmetics, are composed of complex mixtures of volatile compounds. RPC is employed to analyze and confirm their composition.

8. Agriculture

- **Analysis of Plant Extracts**

In agricultural research, RPC is used to identify bioactive compounds in plant extracts that may have herbicidal, pesticidal, or pharmaceutical properties.

- **Veterinary Medicine**:

RPC is applied to analyze drug residues in animal products (meat, milk, eggs), ensuring that veterinary drugs are used safely and within acceptable limits.

- **Pesticide and Herbicide Monitoring**

Pesticides and herbicides used in crops are monitored using RPC to ensure that residue levels in food products are below the regulatory limits.

9. Polymer Chemistry

- **Polymer and Plastic Additive Analysis**:

Additives in plastics and polymers, such as antioxidants, stabilizers, and UV protectants, are analyzed using RPC. This ensures the quality and longevity of plastic products and materials.

- **Polymer Purification**

RPC can also be used in the purification of synthetic polymers to ensure their homogeneity and performance characteristics.

IV. 8.2.4 Detection Systems

Reversed-phase chromatography is often coupled with various detection techniques include:

- **UV-Vis Detection**: The most common, used for detecting compounds that absorb UV light.
- **Fluorescence Detection**: Used when compounds are fluorescent or derivatized to become fluorescent.
- **Mass Spectrometry (LC-MS)**: Provides both separation and identification by detecting the mass of molecules, making it extremely powerful for complex mixtures like proteins and metabolites.

VII.1 Introduction

Is a powerful and highly selective separation technique widely used in biochemical and biotechnological fields. Its unique advantage lies in its ability to isolate and purify specific biomolecules—such as proteins, enzymes, antibodies, or nucleic acids—from complex mixtures with high specificity. Due to its effectiveness and precision, affinity chromatography has become an essential tool in areas such as pharmaceutical development, diagnostic research, and protein engineering.

VII.2 Principle

Affinity chromatography separates target proteins based on reversible interactions between the protein and a specific ligand immobilized on the chromatographic matrix. Many proteins naturally possess recognition sites that allow selective binding to appropriate ligands. These interactions mimic biological processes, such as enzyme–substrate or antigen–antibody binding. In this technique, one molecule of the interacting pair (the affinity ligand) is immobilized on a solid support and placed in a column, serving as the stationary phase. When the sample is passed through the column, only molecules complementary to the ligand bind, while others are washed away. The bound target is then eluted by changing the mobile phase. The column can be reused after re-equilibration with the initial conditions Figure.35.

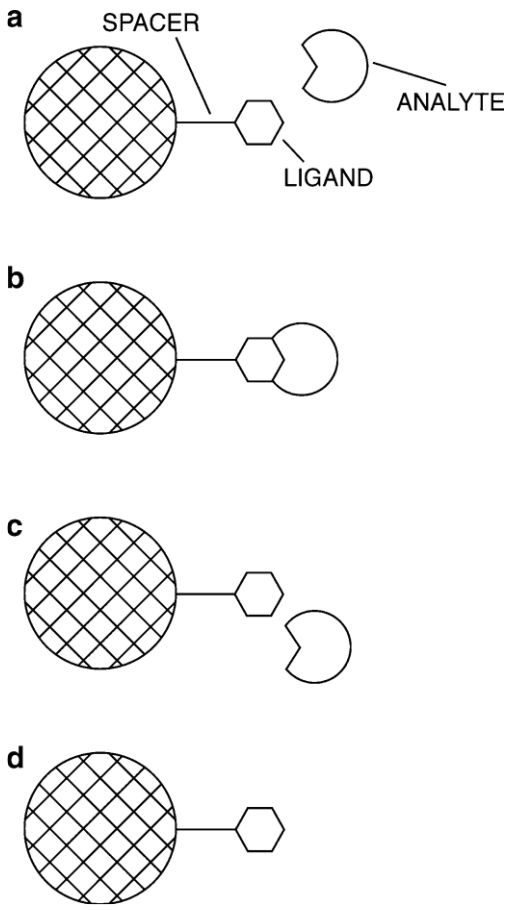


Figure 35. Principle of bioselective affinity chromatography. (**a**) The support presents the immobilized ligand to the analyte to be isolated. (**b**) The analyte makes contact with the ligand and attaches itself. (**c**) The analyte is recovered by the introduction of an eluent, which dissociates the complex holding the analyte to the ligand. (**d**) The support is regenerated, ready for the next isolation.

VII.3 Basic Principles of Affinity Separations

Affinity separation techniques exploit the specific and reversible interactions between biological molecules. By immobilizing a recognition agent (the affinity ligand) onto a solid support, a target molecule can be selectively captured and separated from complex mixtures. The strength of this interaction is quantified by the association constant (**Ka**) or its inverse, the dissociation constant (**Kd**), with strong interactions typically having $\mathbf{Ka \geq 10^6\ L \cdot mol^{-1}}$. Most applications require a change in buffer conditions to release the bound target, although weak affinity chromatography can use milder, isocratic elution. Affinity ligands can be biological (e.g., antibodies, lectins) or synthetic (e.g., biomimetic dyes, immobilized metal ions), depending on the application. Examples of such ligands are listed in Table VIII.

Affinity chromatography typically follows the "on/off" elution scheme, as illustrated in Fig ure36. In this process, a target molecule binds specifically to an immobilized ligand within the column while non-target compounds are washed away. The target is then eluted using a specific buffer. The column can be regenerated by cleaning and reapplying the application buffer, making it ready for reuse in future cycles.

The scheme shown in Figure. 36 is commonly used with affinity ligands that have relative strong binding for their targets (i.e., Ka>105 -106 l mol1). Under these conditions, the affinity ligand has high retention for its target and will result in long elution times under the sample application conditions. This is the reason why a separate elution buffer or solution is later applied to desorb the target. This desorption may be accomplished by either displacing this target with a competing agent, in a method known as biospecific elution, or by lowering the binding strength of the target for the affinity ligand by changing a property such as the pH, ionic strength or polarity of the mobile phase, giving a technique known as non-specific elution.

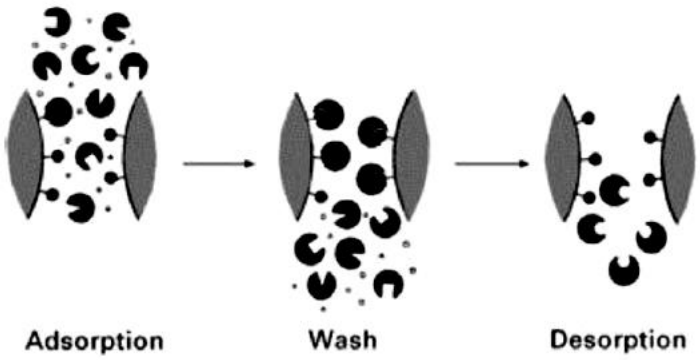


Figure 36 : A typical application and elution scheme for affinity chromatography.

VII.4. Components of affinity medium

When affinity chromatography is used for the purification and separation of large biomolecules from complex mixtures, the support (matrix), spacer arms, and ligand must be considered (see Figure.37 and Table XIII).

VII.4.1 Supports and Matrices

Various materials and matrices have been used as supports in affinity chromatography. Examples of these supports, and an indication of their relative extent of use, are given in Fig 35.

Agarose

Has remained a popular support for affinity chromatography since the work by Cuatrecasas et al. in 1968. Advantages of using agarose as a support for affinity chromatography are its low cost, its large pore size (e.g., for biomolecule separations or immobilization), its low non-specific binding for many biological agents, and its good stability over a broad pH range. These features have made agarose a common material in affinity chromatography for both the large- and small-scale purification of targets, as indicated in Figure 37. However, agarose has limited mechanical stability at high operating pressures. This last factor tends to limit the use of agarose as a support in analytical-scale separations based on high performance liquid chromatography (HPLC).

Cellulose

As shown in Figure. 37, various carbohydrates, including cellulose, have been used as supports in affinity chromatography. Cellulose was widely employed in early antibody and enzyme purifications during the 1950s–60s. Although it is now less common than agarose, cellulose remains useful in membrane-based affinity separations due to its low backpressure and suitability for high flow rates. Despite its lower surface area and mechanical stability, it performs well in preparative settings. Carbohydrate-based supports have also been incorporated into hybrid materials with dense cores (e.g., quartz), as seen in expanded-bed adsorbents, which help prevent clogging by forming a fluidized bed during sample loading.

In the late 1970s and early 1980s, researchers began developing supports to integrate affinity chromatography with HPLC, leading to the emergence of high-performance affinity chromatography (HPAC). Modified porous silica particles and glass beads with hydrophilic groups (e.g., diols) became widely used. These materials offered several advantages: customizable pore and particle sizes, strong mechanical stability under HPLC conditions, ease of ligand immobilization, and versatility across applications from purification to biomedical analysis. However, they also presented drawbacks, including limited pH stability and higher non-specific binding in their unmodified forms compared to carbohydrate-based supports.

Organic polymers

A variety of organic polymers have also been used as supports for affinity chromatography. Many of these organic polymers have been based on polystyrene or polymethacrylate. Native polystyrene has a hydrophobic backbone which could lead to high levels of non-specific interactions in affinity chromatography. However, hydrophilic coatings

can be placed on this material, thus allowing use of polystyrene supports for affinity chromatography based on perfusion media or other separation formats. Polymethacrylates are more hydrophilic in nature and can be used in either their original or modified forms for affinity separations. Organic polymers based on coated polystyrene or polymethacrylates have been used in HPAC. Other examples of organic polymers that have been used in affinity chromatography are polysulfones and polyamides, which have been employed in membrane supports. Like agarose, many of these organic polymers can be used over a large pH range and have good biocompatibility.

Monolith

One area of growth in affinity chromatography over the last 15–20 years has been in the creation and use of monolith supports. Originally developed in the 1990s for other forms of chromatography, monolith supports offer several advantages over traditional particulate supports. These advantages include their low backpressures, high permeability, good separation efficiencies, and ability to be made in a variety of sizes and shapes. There are several types of monoliths that have been employed in affinity chromatography, ranging from organic polymers to silica, agarose, and cryogels. Many monoliths that have been used in affinity chromatography are polymers based on glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA), which have been used with immobilized agents that include antibodies, enzymes, and peptides. This includes polymethacrylate monoliths known as convective interaction media (CIM), which have been employed in applications that range from the purification of large biological agents (e.g., DNA, proteins, and viruses) to antibody-based separations and immobilized metal-ion affinity chromatography (IMAC).

Titania

Other alternative materials have been explored in recent years as supports for affinity chromatography. One of these materials is titania (TiO_2). Titania-based supports have been mostly used for the isolation of phosphopeptides in a method that has been referred to as metal oxide affinity chromatography (MOAC). Titania nanoparticles and dendritic polyglycerol-coated chitosan nanomaterials containing Ti^{+4} have also been employed in IMAC for the separation and purification of phosphopeptides and glycopeptides.

There are many ways in which supports may be employed in affinity chromatography, as demonstrated in Figure.37. A packed column is the most common of these formats. This is not surprising given this approach was used in the early work with affinity chromatography,

spanning from the studies by Starkenstein in 1910 through the beginning of the modern era of affinity chromatography in 1968.

As shown in Figure.37, packed columns have remained the main support format in affinity chromatography, being utilized in more than 90% of the reported applications in this field over the last 50 years. This has included both work with low-to-medium performance supports like agarose or cellulose and supports for HPAC, such as silica or glass beads.

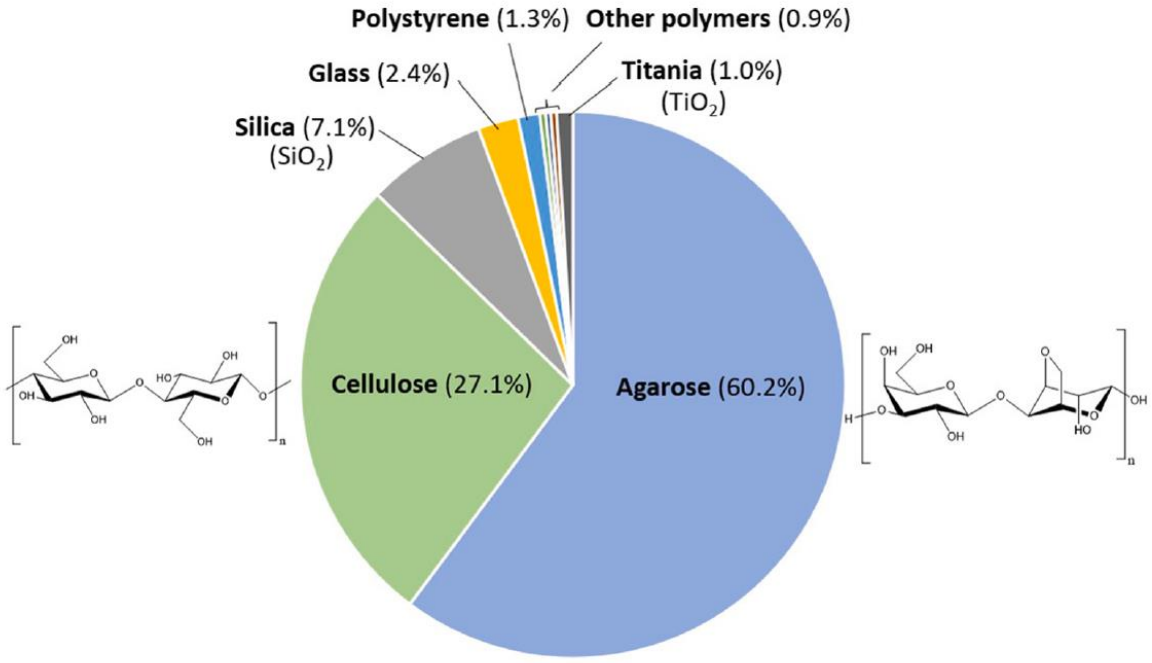


Figure. 37 : Use of various supports in affinity chromatography

Table XIII Common supports for affinity chromatography

Support matrix	*Usable pH range*
Agarose	2–14
Cellulose	1–14
Dextran	2–14
Silica	2-8
Glass	2-8
Polyacrylamides	3–10
Polyhydroxymethacrylates	2–12
Oxirane–acrylic copolymers	0–12
Styrene–divinylbenzene copolymers	1–13
Polyvinyl alcohols	1–14
N-Acryloyl-2-amino-2-hydroxy-1,2-propane	1–11

VII.4.2 Ligands used in affinity chromatography

One way of categorizing the various types of affinity chromatography is by the type of binding agent that is used for the separation. Many binding agents have been utilized as

stationary phases in affinity chromatography. As shown in Figure. 38, this method has made use of both naturally-occurring binding agents and non-biological ligands. Natural binding agents employed in affinity chromatography have included enzymes, antibodies, antigens, immunoglobulin-binding proteins, biotin plus avidin or streptavidin, lectins, serum proteins, carbohydrates, lipids, and nucleic acids. Non-biological ligands have included aptamers, dyes, metal ion chelates, MIPs, and boronates.

VII.4.2.1 Biological binding agents

The use of a naturally-occurring, biological binding agent in an affinity column is often referred to as bioaffinity chromatography or biospecific adsorption.

- ***Enzymes***

Affinity chromatography remains a key method for isolating enzymes. While non-biological agents like dyes are commonly used, biological agents such as immobilized inhibitors, substrates, and cofactors are still applied. Enzymes themselves can also be immobilized in columns to separate inhibitors and other binding solutes. Examples include the use of enzymes like Penicillin G acylase, glucoamylases (G1 and G2), cellobiohydrolase I, trypsin, α-chymotrypsin, lysozyme, and pepsin for the separation of drugs, inhibitors, and other compounds.

- ***Antibody***

This is also the type of affinity chromatography that was used in most of the early work in this field for enzyme and antibody isolation and by Cuatrecasas et al in 1968 for staphylococcal nuclease, α-chymotrypsin and carboxypeptidase A on columns that contained immobilized nuclease inhibitors. Bioaffinity chromatography has remained one of the most common forms of affinity chromatography over the last five decades.

Immunoaffinity chromatography (IAC) is by far the most common form of bioaffinity chromatography, as demonstrated in Figure.38. In this approach, the binding of an antibody with its corresponding target, or antigen, is used as the basis for the affinity separation. Antibody-antigen interactions are strong and highly selective and can involve many types of compounds as the antigen. These properties have long made IAC a popular and powerful tool for the capture and isolation of specific targets from complex samples. IAC methods have since been reported for a large array of targets that span from hormones and toxins to peptides, antibodies, enzymes, recombinant proteins, and viruses. The same properties that have made IAC valuable as a purification tool have also made it attractive as a means for sample preparation and analysis.

- ***Immunoglobulin-binding proteins***

Immunoglobulin-binding proteins are a third class of biological binding agents that are often used in affinity chromatography. As indicated by Figure.38, this combination is the second most-reported form of bioaffinity chromatography. Protein A is a bacterial cell wall protein from *Staphylococcus aureus;* it is commonly employed in the detection or purification of many subclasses of immunoglobulins from various species. Protein G is produced by group G Streptococci and can have different selectivity than protein A for some immunoglobulin subclasses and species. Protein L is another binding agent in this group ; this agent is derived from *Peptostreptococcus magnus* and has strong binding with human IgG and IgA. Mixtures of immunoglobulinbinding proteins or recombinant forms such as protein A/G and protein G/L have also been used in affinity methods. These immunoglobulin- binding proteins can be used to capture and purify antibodies, as well as to measure immunoglobulins in samples.

- ***Interactions of biotin with proteins***

Another form of bioaffinity chromatography is based on the interactions of biotin with proteins such as avidin and streptavidin for immobilization or to carry out separations. Biotin is also known as vitamin H or vitamin B7. Streptavidin is produced by *Streptomyces avidinii*, while avidin is a glycoprotein found in egg whites. Both avidin and streptavidin exhibit strong binding towards biotin and have association equilibrium constants in the range of 1013 to 1015 M^{-1} for this binding. A common example of these type of interactions is used to capture a biotinylated target on a streptavidin or avidin support, as was demonstrated for biotin-labeled peptides and proteins in the mid-1970s.

Avidin has recently been used with biotin and affinity columns in various formats to isolate proteins under native elution conditions. Streptavidin-biotin interactions have also been employed in miniaturized affinity systems to identify and characterize ligands for nanodisc-embedded G-protein coupled receptors.

- ***Lectins***

Lectins are another group of binding agents that are often used in bioaffinity chromatography. The resulting method is sometimes known as lectin affinity chromatography (LAC). Lectins are non-immune system carbohydrate-binding proteins that are capable of binding reversibly to targets that contain sugar moieties. A common example of a lectin that is often used as a support in affinity chromatography is concanavalin A (Con A). This lectin binds to high-mannose type glycans and glycans with mannose branching**.** Wheat germ agglutinin (WGA) is another type of lectin that has been employed as an affinity ligand in chromatography.

It is able to bind to N-acetyl-D-glucosamine and sialic acid. Recent work has used serial lectin affinity chromatography, in which lectin columns are coupled together, to characterize glycans on the same glycoconjugate by arranging the columns in various orders.

There have also been studies in which lectins have been used with monolith supports containing immobilized Con A and WGA and affinity microcolumns that contain Con A or *Aleuria Aurantia* lectin (AAL).

- ***Serum proteins***

Serum proteins have been used as binding agents in many reports employing bioaffinity chromatography. Two examples are the transport proteins BSA and HSA. These are the most abundant proteins in bovine and human plasma, respectively, and have weak-to-high strength interactions with a variety of drugs, hormones, and fatty acids. Another example of a serum protein that has often been used in bioaffinity chromatography is AGP. AGP is an acute phase protein with a high carbohydrate content that binds to many basic, neutral, and cationic drugs. Each of these serum proteins has been used as a chiral stationary phase and to study how factors such as solute structure, temperature, and composition or pH of the mobile phase affect the resulting chiral separations. Columns containing these transport proteins have also been employed to examine the affinities and binding sites of numerous drugs with these proteins, as well as to characterize allosteric interactions and the effects of protein modifications on drug binding. In addition, affinity microcolumns containing serum transport proteins have recently been used in the method of ultrafast affinity extraction to characterize drug interactions with soluble binding agents.

- ***Carbohydrate***

Carbohydrate-based ligands have also been employed in bioaffinity chromatography. Some examples are the use of amylose, cellulose, and their derivatives for chiral drug separations. The carbohydrates α-, β-, and ϒ-cyclodextrin have been frequently used in chiral separations. This latter group of carbohydrates are composed of 6-to-8 glucopyranose units that are arranged in a circular polymer with a hydrophobic cavity and a hydrophilic exterior. Chiral selection of a drug can occur with these binding agents through the formation of an inclusion complex with hydrophobic cavity of cyclodextrin and differential binding of the drug to groups located at the mouth of the cavity. Cyclofructans, which are macrocyclic oligosaccharides based on D-fructofuranose, are a related group of carbohydrates that have been employed as stationary phases for chiral separations.

- ***Lipids***

Lipids are another class of biological agents that have been employed in bioaffinity chromatography. For instance, lipids have been used in immobilized artificial membrane (IAM) chromatography to determine the partition coefficients of drugs for cell membranes. Monolayers of phospholipid analogs such as phosphatidyl choline, sphingomyelin, and choline have all been used as biological ligands in this method. IAM has been employed for examining drug interactions with immobilized receptors and transporters. In the method of immobilized liposome chromatography (ILC), liposomes or lipid bilayers have been placed on chromatographic supports and utilized to study drug-membrane interactions.

ILC has been utilized to screen membrane penetrable components and bioactive ingredients in traditional medicine. Lipids such as phosphatidylcholine and cholesterol have been immobilized on monolithic supports for use in IAM or related applications.

- ***Nucleic acids***

Nucleic acids can also be used as ligands for bioaffinity chromatography. This is the case in DNA affinity chromatography, which is a technique used to retain and purify DNA-binding proteins. This method first appeared in the late 1960s and early 1970s. DNA-binding proteins that have been isolated by this approach include DNA or RNA polymerases, DNA repair proteins, helicases, histones, primases, restriction enzymes, teleomerases, topoisomerases, and transcription factors. In non-specific DNA affinity chromatography, a general preparation of fragmented nuclear DNA (e.g., calf thymus DNA) is used in a column to separate DNA-binding proteins from other proteins and sample components that do not bind DNA. In specific DNA affinity chromatography, a particular section of DNA is used as the affinity ligand. The ligand in this case is chosen to have a sequence, structure, or restriction site that can be used to capture a protein that will interact specifically with this DNA segment.

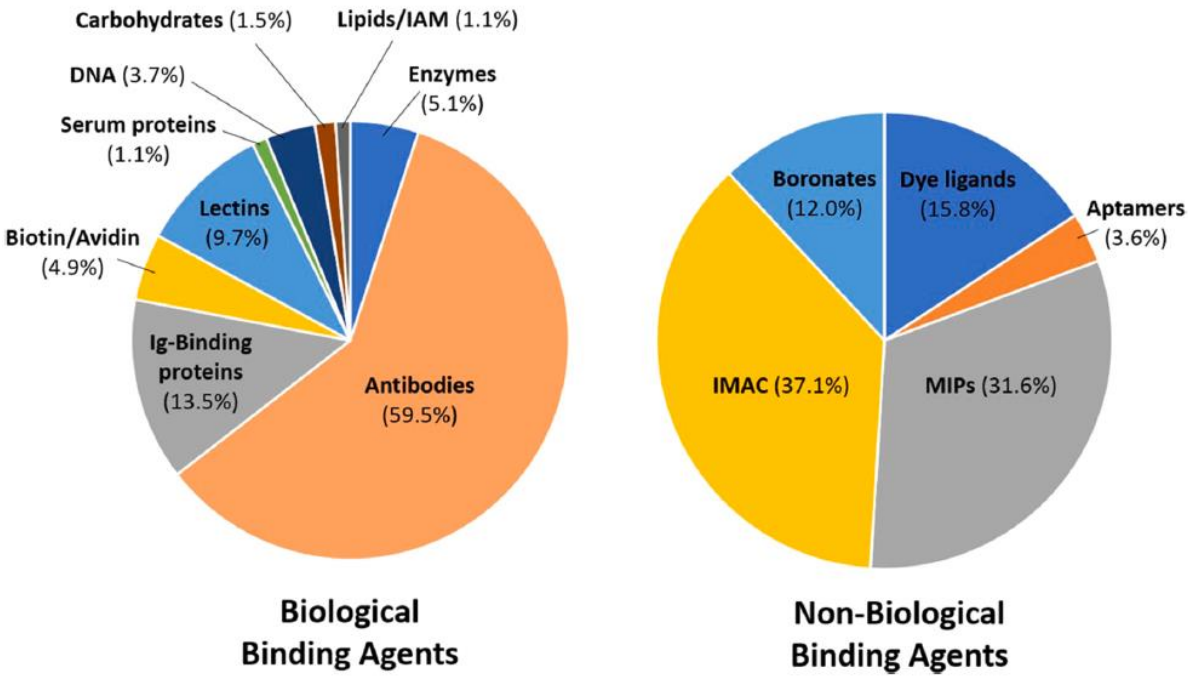


Figure. 38 : Extent of use of representative biological and non-biological binding agents in affinity chromatography

VII.4.2.2 Non-biological binding agents

The use of non-biological agents in affinity chromatography began in the early 1970s, soon after the emergence of modern affinity chromatography. One area that appeared during this time was dye-ligand affinity chromatography.

- ***Dye-ligand***

Most of the binding agents used in this method are triazine dyes or related compounds. These dye-ligands contain a chromophore and reactive group for covalent coupling to a support. Chromophores that are present on these dyes, and which are used for dyeprotein binding, have included anthraquinone, azo, and phathalocyanine groups. Further modifications to the structures of these dyes have been made over the years to improve their specificity in interacting with a given target. The low cost, ease of immobilization onto supports, and stability of dye-ligands have made these binding agents popular in affinity separations, as demonstrated in Figure.38, and especially for large-scale processes. Examples of proteins that have been purified through the use of dye-ligands are HSA, lysozyme, fucoidan, lactoferrin, α-chymotrypsin, and IgG.

Dye-ligand affinity chromatography is part of a broader range of methods known as biomimetic affinity chromatography.

This area includes the use of dyes as ligands along with binding agents that are produced by means of combinatorial synthesis or selection from large libraries of potential affinity ligands (e.g., using phage display or ribosome display techniques).

- ***Aptamers***

Aptamers are important examples of binding agents that are developed through the screening of libraries containing potential ligand candidates. Aptamers are synthetic oligonucleotide sequences, generally based on DNA or RNA, that are screened and chosen for their binding to specific target molecules. Aptamers have been popularized through the availability of a screening and amplification method known as the systematic evolution of ligands by exponential enrichment (SELEX), which was first reported in 1990. The three-dimensional structure of an aptamer can lead to relatively strong and specific noncovalent complexes with its binding target. Aptamers have been used in a number of studies as alternatives to antibodies due to the good specificity of aptamers, their low immunogenicity, and their ability to be produced without the need for a biological system. Applications of aptamers in affinity chromatography have included their use to bind small molecules such as ochratoxin A, cocaine, and diclofenac ; aptamers have also been used to bind larger targets such as proteins and cells. Aptamers have been immobilized onto both organic and inorganic supports, including monoliths, and have been used in various formats that range from columns to microfluidic systems.

- ***MIPs***

MIPs are a group of polymeric supports that are prepared to contain cavities or pockets that can bind to a given target. Affinity-based applications for this type of support include chiral separations and solid-phase extraction. Reports in which MIPs are used in some form with chromatography, including as tools for sample preparation, now make up one of the three main groups of affinity applications that employ non-biological binding agents (see Figure. 39). Target compounds for these supports are often low-mass solutes. However, methods are now available for the use of MIPs with large targets like proteins. Most of the early work with MIPs made use of organic polymers to create this type of medium, but other materials such as monoliths, hybrid materials, and membrane-based MIPs have also been reported.

As shown in Figure.39, metal ion chelates are another important example of non-biological binding agents that can be used in affinity chromatography.

- ***IMAC***

IMAC generally relies on the interaction between immobilized metal ions and amino acids on the target analyte that can act as electrondonating groups, such as cysteine, histidine, or tryptophan residues. Iminodiacetic acid, nitrilotriacetic acid, carboxymethylated aspartic acid, diethylene triamine pentaacetate, and tris(carboxymethyl) ethylene-diamine are examples of chelating ligands that have been used in IMAC to contain metal ions. These chelating ligands have been immobilized onto several types of supports, including silica, agarose, and cryogels. Various metal ions have also been used in IMAC. Metal ions such as Cu^{+}, Ag^{+}, Pd^{2+}, Pt^{2+}, Cd^{2+}, and Hg^{2+} tend to bind targets that contain sulfur, while metal ions such as Ni2+, Cu2+, and Zn2+ tend to coordinate with targets having accessible groups that contain nitrogen, sulfur, or oxygen. Protein purification is a common use of IMAC. For instance, this method has been employed in purifying natural proteins such as HSA, immunoglobulins, lysozyme, and α-amylase. IMAC has also been used to isolate histidine-tagged recombinant proteins, DNA-based aptamers, and phosphopeptides.

- ***Boronic acid***

Boronic acid and its derivatives are nonbiological binding agents used in boronate affinity chromatography (BAC), a technique first introduced in 1970 for retaining nucleosides and sugars. BAC is primarily used to separate compounds containing cis-diol groups, such as glycoproteins, catechols, nucleotides, and carbohydrates. It works through a reversible esterification reaction between boronic acids and cis-diols, which is pH-dependent. This allows controlled capture and release of target molecules. BAC has been applied to enrich glycoproteins, glycopeptides, and nucleosides from complex samples. Common binding agents include 3-aminophenylboronic acid and 4-vinylphenylboronic acid, with newer agents offering lower pKa values for use in neutral or acidic conditions.

VII.5 Process

Affinity chromatography relies on the specific recognition that occurs between many biological molecules, such as the binding of an antibody with an antibody or the interaction of a hormone with its receptor. These interactions are used in affinity chromatography by permanently bonding (or ‘immobilizing’) onto a solid support an appropriate binding agent. This immobilized agent is known as the affinity ligand and represents the stationary phase for this method. Once the affinity ligand and its support have been placed within a column, this column can be used to retain any substance that will form a strong but reversible complex with the ligand. The optimum association constant (Ka) for such a system in purification work is generally 104 –108 l mol 1 . However, stronger and weaker interactions can be used in

analytical applications of affinity columns. The most common format employed in affinity chromatography is given in Figure. 39. In this format, a solution containing the target of interest is passed through a column containing an immobilized ligand capable of binding to the target. This step is performed in the presence of an application buffer that allows such binding to occur. Because the resulting interaction is usually selective in nature, the ligand will recognize and retain the target while allowing other compounds in the sample to pass through the column in the nonretained peak. However, due to the strong binding that is often present between the target and ligand, the target is held within the column until the mobile phase or chromatographic conditions are varied.

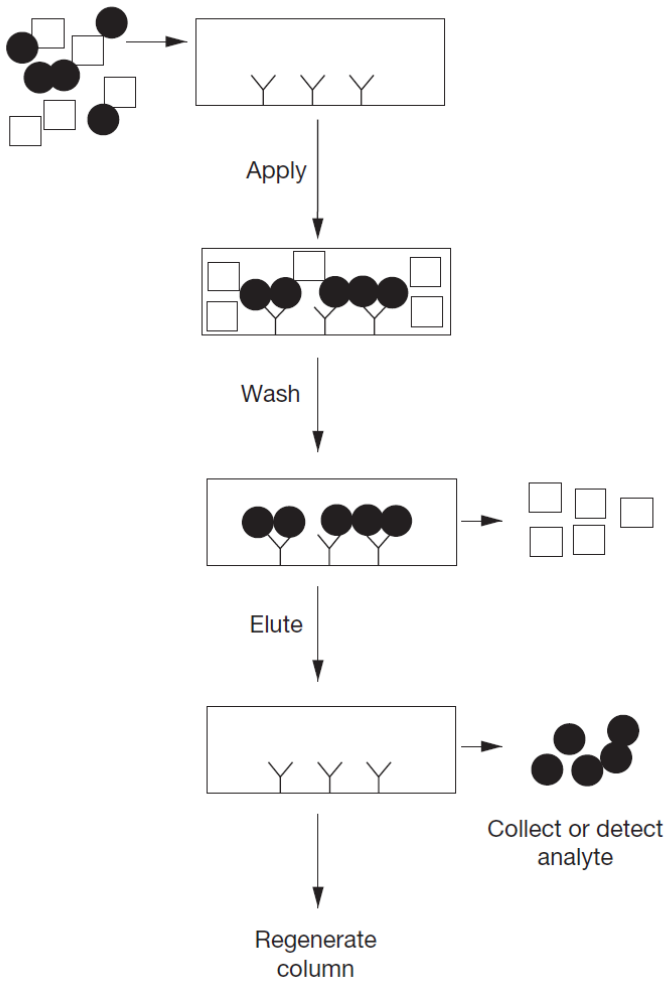


Figure 39: Typical separation scheme for affinity chromatography, showing the steps for sample application, column washing, analyte/target elution, and column regeneration.

This variation in conditions is often accomplished by passing an elution buffer through the column. As the target elutes from the column, the target is captured for further use or

monitored by an online detector. The column is then cleaned, allowed to regenerate in the application buffer, and used for application of the next sample. As this scheme suggests, there are several factors to consider in the design of an affinity separation. These factors include the ligand, support material, immobilization method, and application or elution conditions.

VII.6 Applications of modern affinity chromatography

There are many ways in which affinity chromatography can be used for chemical or biochemical separations, purification, analysis, orcharact erization. Figure. 40 shows some fields in which affinity chromatography has been used on a frequent basis, based on research publications that have used this method. Many of these applications have been in the fields of biochemistry, biochemical research and molecular biology (a combined total of 49.2% for the papers examined in Fig. 39). This is not surprising in that the affinity chromatography was originally developed to meet the need for specific separations in these fields.

In a similar manner the areas of biotechnology, microbiology, cell biology, and immunology also employ this separation method on a routine basis (combined total of 22.7%).

VII.5.1 Analytical chemistry

Analytical chemistry and other areas of chemistry (e.g., environmental chemistry, clinical chemistry, multidisciplinary chemistry) make up another important set of applications for affinity chromatography (17.7%) along with pharmacology and pharmaceutical science (4.2%). These applications emerged in the early 1970s and saw rapid growth after the development of high-performance affinity methods in the late 1970s and early 1980s. The use of affinity chromatography in biophysical studies of biological systems (4.2%) is another consistent area of applications, with work based on this combination first appearing in the mid-1970s.

Many of the applications that are represented by Figure. 40 involve the use of affinity chromatography as a method for the purification of biomolecules.

This type of application makes use of the ability of affinity chromatography to provide both high selectivity and strong binding for a given target, making it possible to often isolate this target in only one or a few steps even when it is present in a complex matrix. Important examples of these applications are the use of affinity chromatography for both the smallscale and large-scale purification of enzymes, native proteins, and recombinant proteins, such as by using dye-ligand or biomimetic affinity chromatography. Common examples of popular

small-scale applications are the isolation of his-tag proteins by IMAC, and the isolation of specific antibodies or antigens by IAC.

VII.5.2 Cell affinity chromatography

Cell affinity chromatography is a specialized application of affinity-based separation used to isolate specific cell types. It relies on interactions between cell surface components—such as receptors or glycoproteins—and immobilized ligands that selectively bind these targets. Common ligands include lectins, which bind glycoproteins, and antibodies, which target specific surface proteins. This method was first explored in the 1970s, notably for purifying insulin receptors and isolating tumor cells using Con A. Since then, it has been applied to separate various cells, including thymocytes, red blood cells, lymphocytes, sperm, and cancer cells. Techniques have evolved to include columns, capillaries, microarrays, cryogels, microfluidic devices, and monoliths.

Over the past forty years, affinity chromatography has evolved into a powerful analytical technique, especially with the development of high-performance formats compatible with HPLC systems. Its common "on/off" elution mode offers simplicity, speed, and ease of automation. Analytical applications include methods based on antibodies, antigens, protein A/G, immobilized metal affinity chromatography (IMAC), boronates, and lectins. These systems often allow direct detection of target compounds through online fluorescence, absorbance, mass spectrometry, or post-column reactions. Affinity columns have also been integrated with other techniques—such as reversed-phase chromatography, gas chromatography, and capillary electrophoresis—to enable multi-dimensional analysis. Indirect detection strategies, like chromatographic immunoassays, are also used, where labeled analogs or agents compete with or are displaced by the analyte. Both competitive and immunometric (e.g., sandwich) assay formats can be applied to measure specific targets.

Affinity chromatography is widely used in analytical workflows for sample pretreatment. One example is the use of molecularly imprinted polymers (MIPs) in solid-phase extraction. Another common approach is immunoextraction, where immobilized antibodies in an affinity column selectively capture target compounds before analysis by techniques such as liquid chromatography, capillary electrophoresis, mass spectrometry (online or offline), or gas chromatography (offline). Affinity chromatography can also be used to selectively remove interfering substances from a sample, allowing improved analysis of the remaining components.

Immunodepletion, which uses immobilized antibodies to remove abundant proteins before analyzing low-abundance proteins, is now common in proteomics. Affinity chromatography is also widely used for chiral separations with chiral stationary phases (CSPs), including enzymes, proteins, and antibodies, playing a key role in drug development. Additionally, affinity chromatography is valuable for studying biological interactions—such as protein-protein or drug-protein binding—by measuring binding strength and site characteristics, often using zonal elution techniques. This method helps analyze the nature and strength of various molecular interactions.

VII.5.3 Frontal analysis

A second method that is often used in affinity chromatography for binding studies is frontal analysis. Frontal analysis is performed by continuously applying a known concentration of the analyte onto the affinity column until a front, or breakthrough curve, is formed. The position and shape of the breakthrough curve can then be used to determine the equilibrium constants and number of binding sites of the applied analyte for the affinity ligand.

VII.5.4 Biological interaction

Affinity chromatography is also used to study the kinetics of biological interactions. several methods have been developed for this purpose, including:

- Band-broadening measurements
- Peak-fitting methods
- Split peak effect
- Peak decay analysis

These techniques have been applied to various systems, such as protein-protein, drug-protein, antibody-antigen, and aptamer-target interactions.

A more recent advancement is ultrafast affinity extraction, which utilizes a microscale affinity column with an immobilized binding agent to rapidly isolate the unbound form of a drug from a sample. By adjusting the extraction time (from seconds to milliseconds), researchers can determine both the equilibrium binding constants and dissociation rates for drug-protein complexes.

Additionally, affinity chromatography has inspired the development of other techniques for studying binding and kinetics, notably modern biosensor platforms like surface plasmon resonance (SPR) spectroscopy. In SPR, the interaction between a solute and an immobilized

agent is monitored in real time within a flow-based chamber, allowing detailed kinetic analysis.

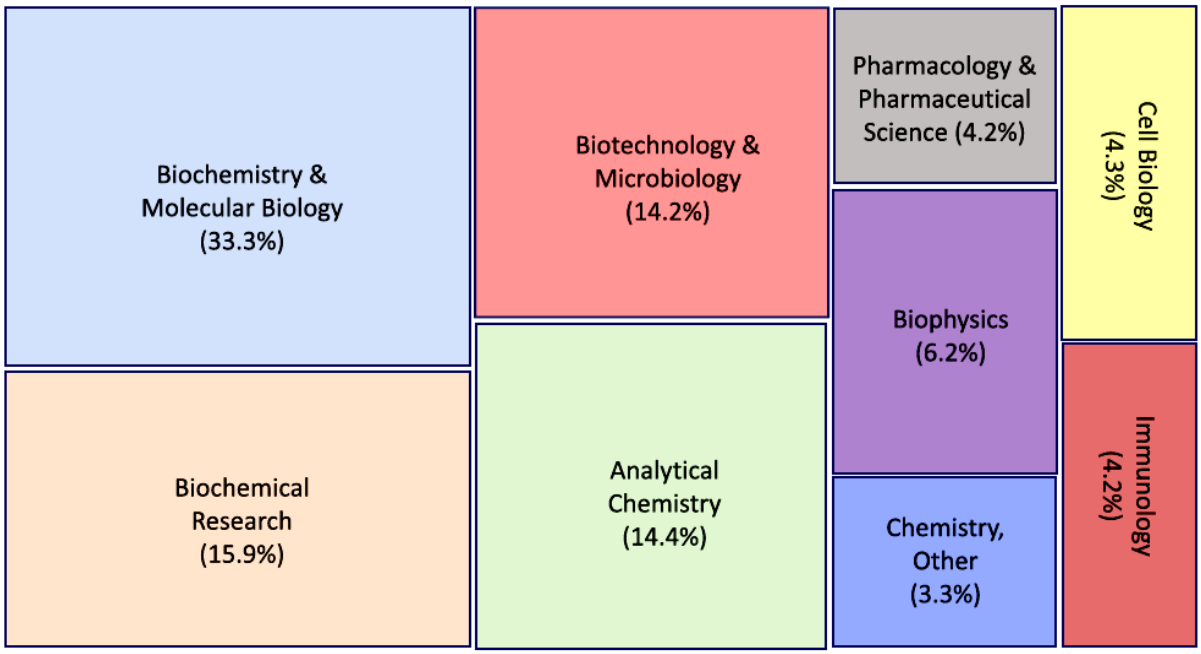


Figure. 40 : Fields in which affinity chromatography is often used in chemical or biochemical separations, isolation, analysis, or characterization

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