Basics of chromatographic Techniques

Course 1

Kannan R., Ph. D.

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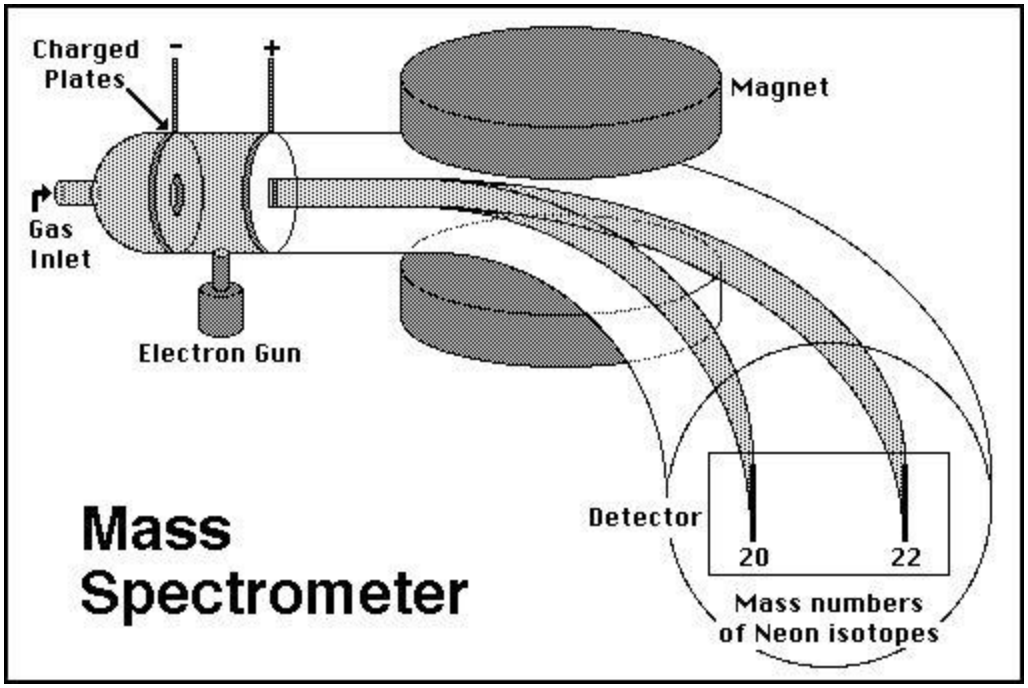
Mass spectrometer: 100 years after J.J. Thomson invented the first mass spectrometer

parabola spectrograph



"I feel sure that there are many problems in chemistry which could be solved with far greater ease by this than by any other method"

http://masspec.scripps.edu/mshistory/perspectives/sborman2.php

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Historical Developments in MS

Investigator(s)	Year	Contribution
Thomson	1899–1911	First mass spectrometer
Dempster	1918	Electron ionization and magnetic focusing
Aston	1919	Atomic weights using MS
Stephens	1946	Time-of-flight mass analysis
Hipple, Sommer, and Thomas	1949	Ion cyclotron resonance
Johnson and Nier	1953	Double-focusing instruments
Paul and Steinwedel	1953	Quadrupole analyzers
Beynon	1956	High-resolution MS
Biemann, Cone, Webster, and Arsenault	1966	Peptide sequencing
Munson and Field	1966	Chemical ionization
Dole	1968	Electrospray ionization
Beckey	1969	Field desorption MS of organic molecules
MacFarlane and Torgerson	1974	Plasma desorption MS
Comisarow and Marshall	1974	FT-ICR MS
Yost and Enke	1978	Triple quadrupole MS
Barber	1981	Fast atom bombardment (FAB)
Tanaka, Karas, and Hillenkamp	1983	Matrix-assisted laser desorption/ionization
Fenn	**1984**	**ESI on biomolecules**
Chowdhury, Katta, and Chait	1990	Protein conformational changes with ESI MS
Mann and Wilm	1991	MicroESI
Ganem, Li, and HenionChait and Katta	1991	Noncovalent complexes with ESI MS
Pieles, Zurcher, Schär, and Moser	1993	Oligonucleotide ladder sequancing
Henzel, Billeci, Stults, Wong, Grimley, and Watanabe	1993	Protein mass mapping
Siuzdak, Bothner, Fuerstenau, and Benner	1996–2001	Intact viral analysis

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History

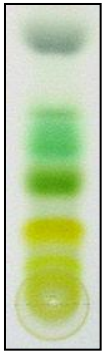
Mikhail Tswett, Russian, 1872-1919

Botanist

In 1906 Tswett used to chromatography to separate plant pigments

He called the new technique chromatography because the result of the analysis was 'written in color' along the length of the adsorbent column

Chroma means “color” and graphein means to “write”



Thin layer chromatography is used to separate the colorful components of a plant extract

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Historical Developments in Chromatography

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Investigator(s)	Year	Contribution
Way and Thompson	1848	Recognized the phenomenon of ion exchange in solids.
Runge, Schoenbein, and Goeppelsroeder	1850-1900	Studied capillary analysis on paper.
Lemberg	1876	Illustrated the reversibility and stoichiometry of ion exchange in aluminum silicate minerals.
Reed	1892	First recorded column separation: tubes of kaolin used for separation of $FeCl_3$ from $CuSO_4$.
Tswett	**1903-1906**	**Invented chromatography with use of pure solvent to develop the chromatogram; devised nomenclature; used mild adsorbents to resolve chloroplast pigments.**
Karrer, Kuhn, and Strain	1930-1932	Used activated lime, alumina and magnesia absorbents.
Holmes and Adams	1935	Synthesized synthetic organic ion exchange resins.
Reichstein	1938	Introduced the liquid or flowing chromatogram, thus extending application of chromatography to colorless substances.
Izmailov and Schraiber	1938	Discussed the use of a thin layer of unbound alumina spread on a glass plate.
Brown	1939	First use of circular paper chromatography.
Tiselius	1940-1943	Devised frontal analysis and method of displacement development.

Historical Developments in Chromatography

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Investigator(s)	Year	Contribution
Martin and Synge	1941	Introduced column partition chromatography.
Consden, Gordon, and Martin	1944	First described paper partition chromatography.
Boyd, Tompkins, et al	1947-1950	Ion-exchange chromatography applied to various analytical problems.
M. Lederer and Linstead	1948	Applied paper chromatography to inorganic compounds.
Kirchner	1951	Introduced thin-layer chromatography as it is practiced today.
James and Martin	**1952**	**Developed gas chromatography.**
Sober and Peterson	1956	Prepared first ion-exchange celluloses
Lathe and Ruthvan	1956	Used natural and modified starch molecular sieves for molecular weight estimation.
Porath and Flodin	1959	Introduced cross-linked dextran for molecular sieving.
J. C. Moore	1964	Gel permeation chromatography developed as a practical method.

Importance

Chromatography has application in every branch of the physical and biological sciences

12 Nobel prizes were awarded between 1937 and 1972 alone for work in which chromatography played a vital role

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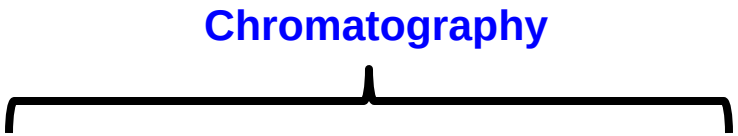
Chromatography

The substances in a mixture are not chemically combined, so therefore they can be separated through some physical process.

chromatography, technique for separating the components, or solutes, of a mixture on the basis of the relative amounts of each solute distributed between a moving fluid stream, called the mobile phase, and a contiguous stationary phase. The mobile phase may be either a liquid or a gas, while the stationary phase is either a solid or a liquid.

Chromatography is the ability to separate molecules using partitioning characteristics of molecule to remain in a stationary phase versus a mobile phase. Once a molecule is separated from the mixture, it can be isolated and quantified.

Different Chromatographic Techniques



Stationary Phase

1.Thin Layer Chromatography
2. Paper Chromatography
3. Column Chromatography

Mobile Phase

1. Liquid chromatography
2. Gas Chromatography

Classification according to the force of separation

1- Adsorption chromatography.
2- Partition chromatography.
3- Ion exchange chromatography.
4- Gel filtration chromatography.
5- Affinity chromatography.

Thin Layer Chromatography

TLC is a method for identifying substances and testing the purity of compounds.

TLC is a useful technique because it is relatively quick and requires small quantities of material.

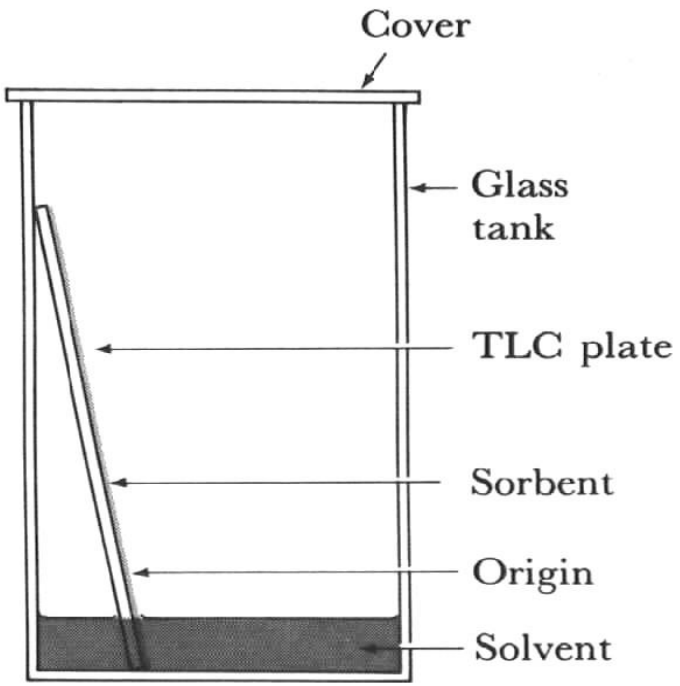
Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase.

The stationary phase: is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate.

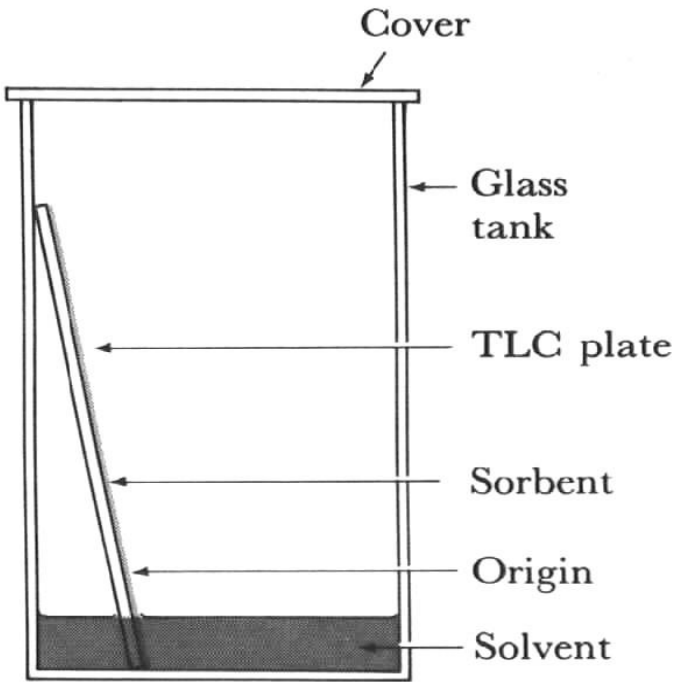
The mobile phase: is a developing liquid which travels up the stationary phase, carrying the samples with it.

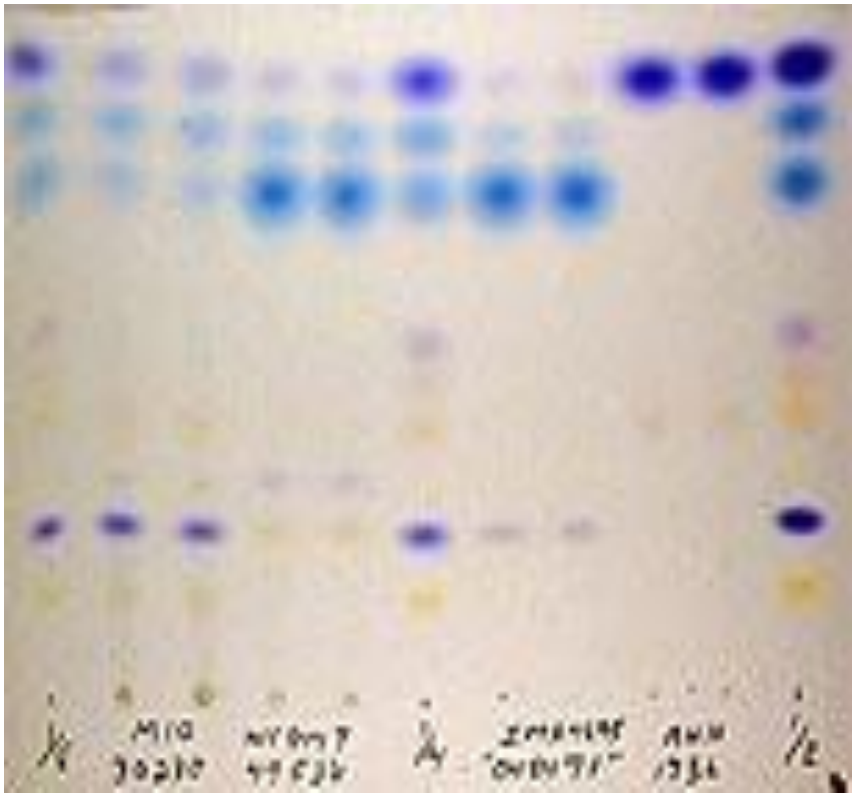
Components of the samples will separate on the stationary phase according to how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.

Thin Layer Chromatography

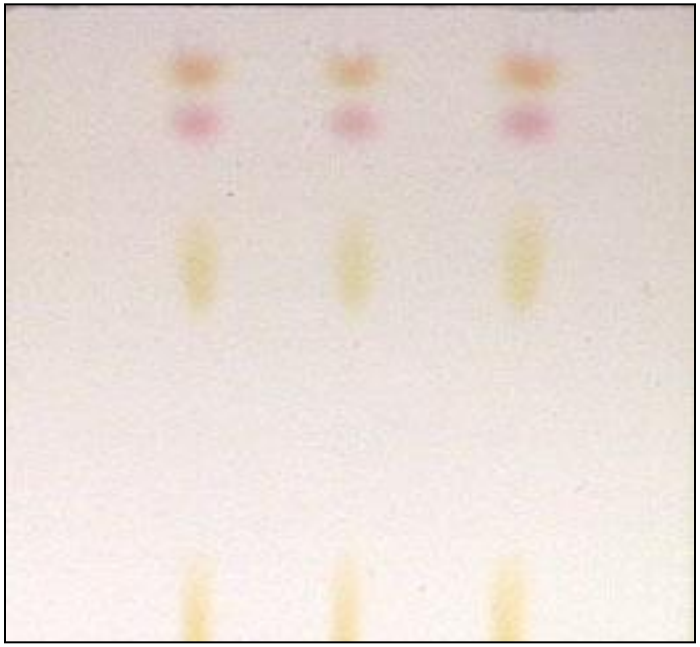


Thin Layer Chromatography





Identifying the Spots (visualization)



If the spots can be seen, outline them with a pencil.

If no spots are obvious, the most common visualization technique is to hold the plate under a UV lamp.

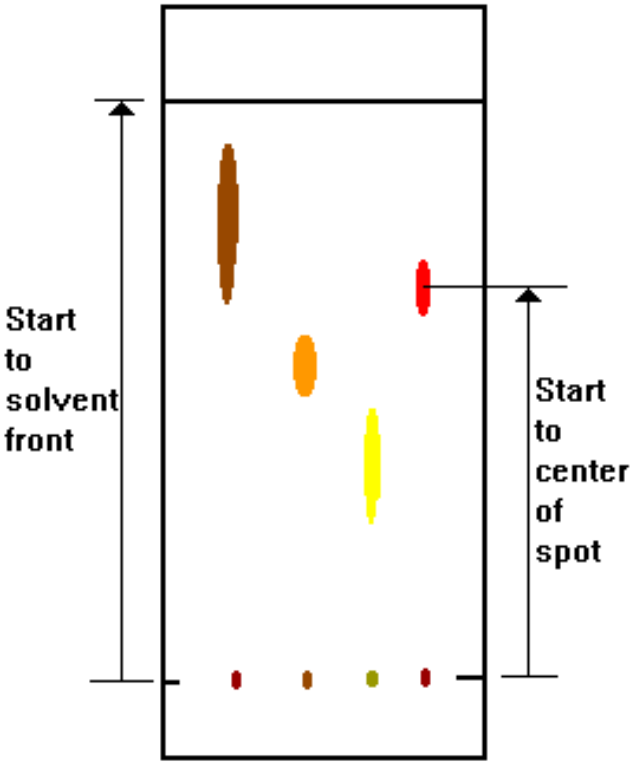
Many organic compounds can be seen using this technique, and many commercially made plates often contain a substance which aids in the visualization of compounds.

Visualizing Agents

Reagents	Compounds
Iodine	Aromatic compounds
UV light	Unsaturated compounds
p-Anisaldehyde	Carbohydrate
Bromocresol green	carboxylic acid
2,4-dinitrophenylhydrazine	Mainly for aldehydes and ketones
Ninhydrin	Good for amines
Sulfanilic Acid Reagent (Diazotized), Pauly's Reagent	phenolic compounds turn orange or yellow with this reagent sprayed on the TLC
Sulfuric acid	Sugar
Aniline phthalate	Cardiac glycosides
Antimony trichloride	Alkaloids
Dragendorff’s reagent	

Interpreting the Data

$$R_f = \frac{\text{Distance from start to center of substance spot}}{\text{Distance from start to solvent front}}$$



The R_f (retention factor) value for each spot should be calculated.

It is characteristic for any given compound on the same stationary phase using the same mobile phase for development of the plates.

Hence, known R_f values can be compared to those of unknown substances to aid in their identifications.

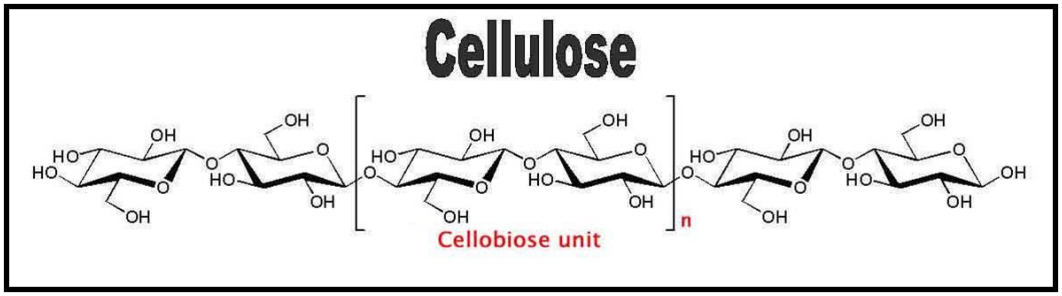
Paper Chromatography

A method of partition chromatography using filter paper strips as carrier or inert support.

The factor governing separation of mixtures of solutes on filter paper is the partition between two immiscible phases.

One is usually water adsorbed on cellulose fibres in the paper (stationary phase).

The second is the organic solvent flows past the sample on the paper (stationary phase).



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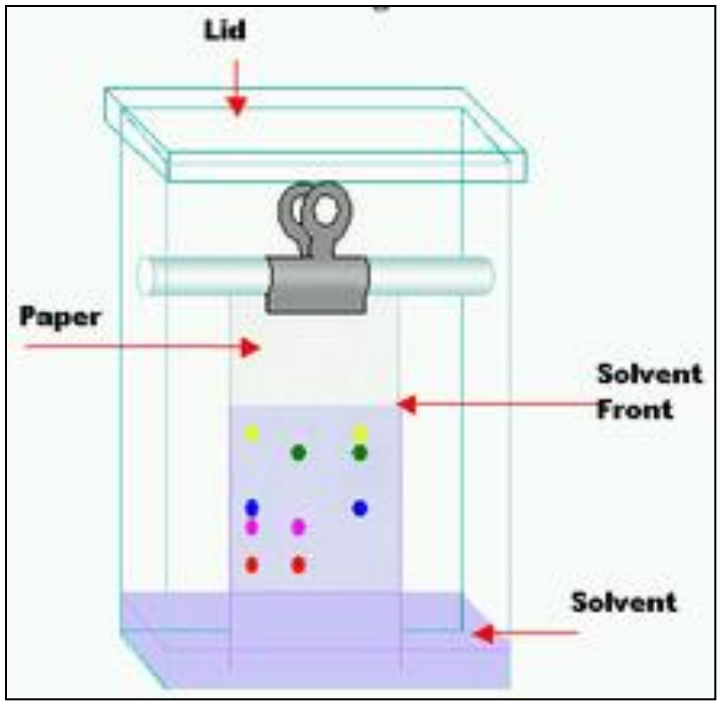
Paper Chromatography



$$K = \frac{c(stationary)}{c(mobile)}$$

A method of partition chromatography using filter paper strips as carrier or inert support.

The factor governing separation of mixtures of solutes on filter paper is the partition between two immiscible phases.

One is usually water adsorbed on cellulose fibres in the paper (stationary phase).

The second is the organic solvent flows past the sample on the paper (stationary phase).

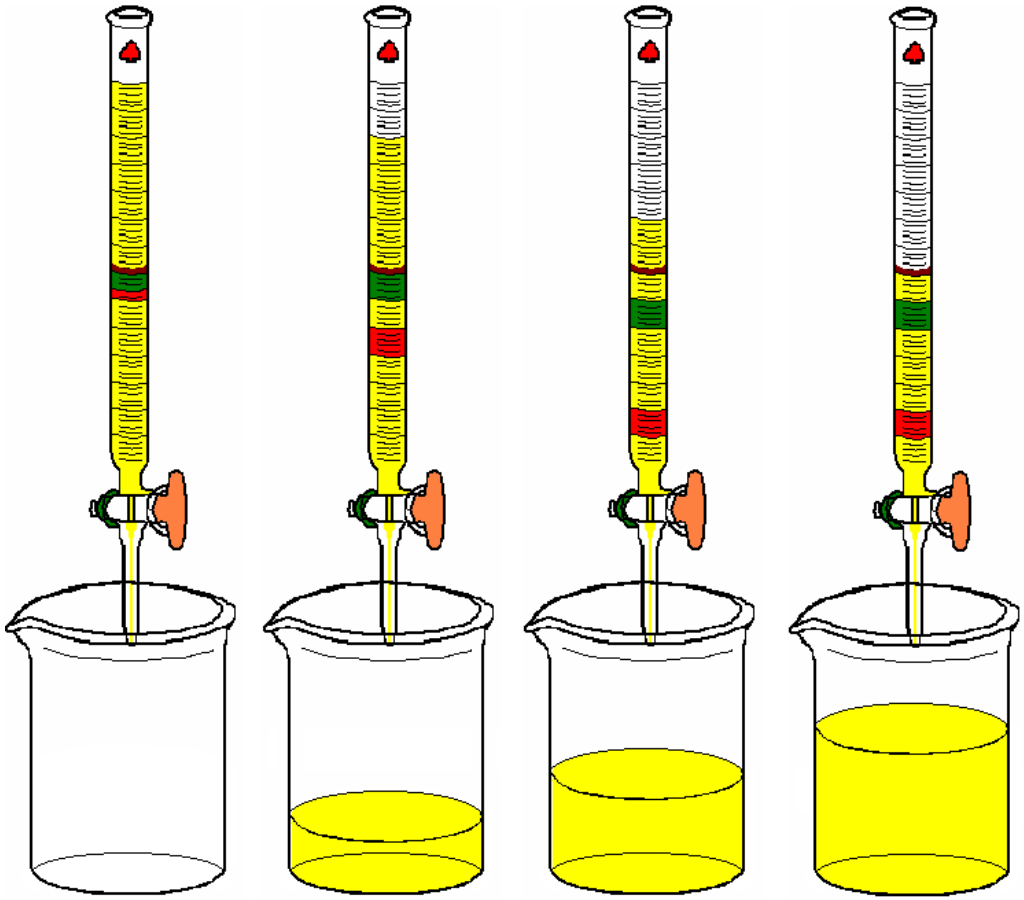
Partition occurs between the mobile phase and the stationary aqueous phase bound by the cellulose.

The isolation depends on partition coefficient of the solute.

Column Chromatography

Column Chromatography

Stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or under the effect of gravity



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Factors affecting solutes separation in CC

Factor	Effect
Particle size of solid stationary phase (or of support)	Decrease of size improves separation (but very small particles need high pressure).
Column dimensions	Efficiency increases as ratio length / width increases.
Uniformity of packing	Non uniform packing results in irregular movement of solutes through column & less uniform zone formation, (i.e. band broadning or tailing).
Column temperature	Increase in column temperature results in speed of elution but does not improve separation (tailing).
Eluting solvent	Solvents should be of low viscosity (to give efficient resolution) & high volatility (to get rapid recovery of the substances).
Solvent flow rate	Uniform & low flow rate gives better resolution.
Continuity of flow	Discontinuous flow disturbs resolution
Condition of adsorbent	Deactivation of adsorbent decreases separation.
Concentration of solutes	Substances of high concentration move slowly.

Mode or type	Stationary phase	Mobile phase	Mechanism
Adsorption Chromatography	**Solid that attracts the solutes**	**Liquid or gas**	**Solutes move at different rates according to the forces of attraction to the stationary phase.**
Partition Chromatography	**Thin film of liquid formed on the surface of a solid inert support**	**Liquid or gas**	**Solutes equilibrate between the 2 phases according to their partition coefficients**
Ion Exchange Chromatography	**Solid resin that carries fixed ions & mobile couterions of opposite charge attached by covalent bonds**	**Liquid containing electrolytes**	**Solute ions of charge opposite to the fixed ions are attracted to the resin by electrostatic forces & replace the mobile counterions.**
Molecular Exclusion Chromatography	**Porous gel with no attractive action on solute molecules**	**Liquid**	**Molecules separate according to their size:** **1.Smaller molecules enter the pores of the gel, and need a larger volume of eluent.** **2.Larger molecules pass through the column at a faster rate.**
Affinity Chromatography	**Solid on which specific molecules are immobilized**	**Liquid or gas**	**Special kind of solute molecules interact with those immobilized on the stationary phase**

Elution techniques

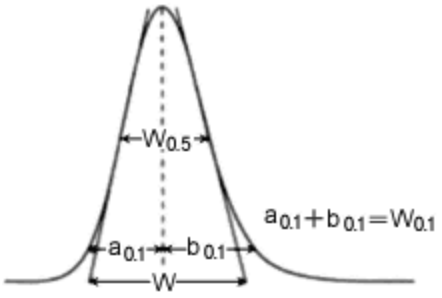
Technique	Procedure
Isocratic elution	**Addition of solvent mixture of fixed composition during the whole process.**
Gradient elution	**<u>Continuous or linear elution</u>: in which there is continuous change in the composition of the mobile phase over a period of time (e.g. polarity, pH or ionic strength).**
	<u>Step wise or fractional elution</u>: in which the change is not continuous i.e. a sudden change in the composition of the mobile phase is followed by a period where the mobile phase is held constant.

Number of Theoretical Plates (N)

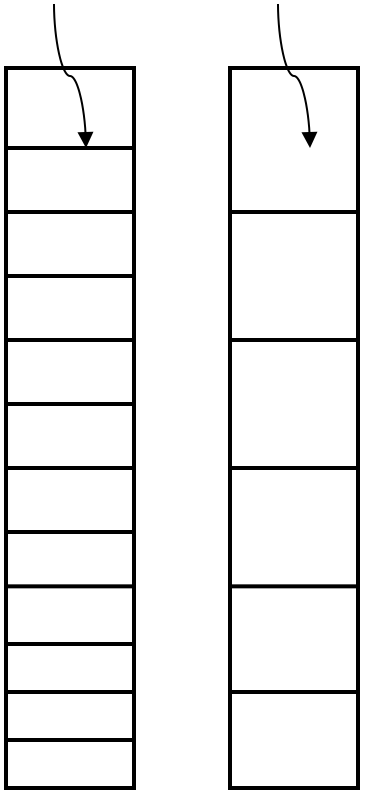
H = Theoretical Plate Height
L = Length of the Column.

$$N = L / H$$

As HETP decreases efficiency of the column increases.



$$N = L\,(tr/W)^2$$



Adsorption Column Chromatography

Adsorbents:

The most common are **Alumina** & **Silica gel** in which the **interactions** with solute molecules is **due to OH groups present on their surface**.

More polar molecules are **adsorbed more strongly** & thus, will **elute more slowly**

Strength of adsorption of polar groups (solutes) on polar support is in the following order:

-C=C- < O-CH3 < -COOR < >C = O < -CHO < -NH2 < -OH < -COOH

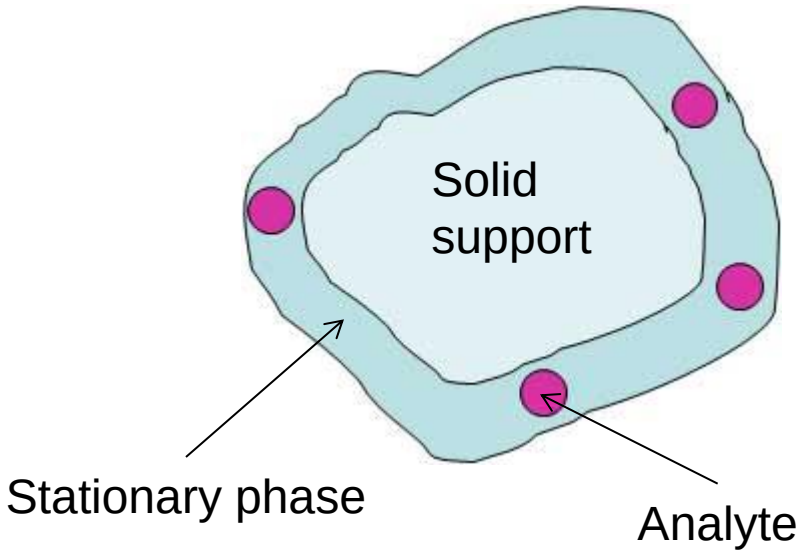
Olefins < Ethers < Esters < Lactones < Aldehydes < Amines < Phenols < Acids.

Partition Chromatography

In partition chromatography a solid support with a high surface area such as crushed firebrick or keiselguhr is coated with a high boiling liquid which acts as the stationary phase. Separation occurs because of the differences in solubility for the analytes in the stationary and mobile phases.

The partition coefficient is defined as:

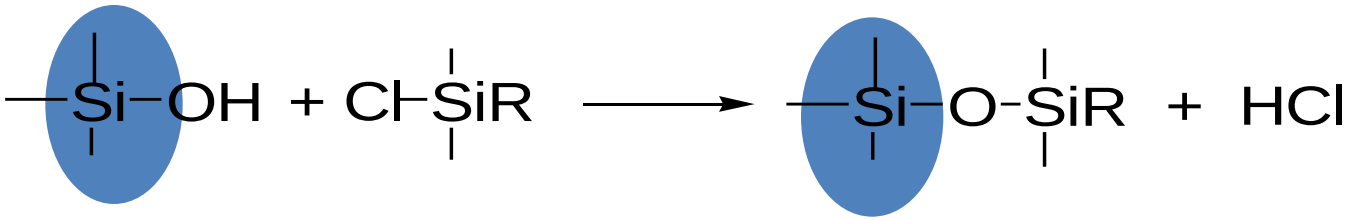
$$K = \frac{\text{Conc}^{n}.\text{ in stationary phase}}{\text{Conc}^{n}.\text{ in mobile phase}}$$



coated support particle

Bonded phase chromatography

In bonded phase chromatography, the molecule acting as the stationary phase is chemically bonded to the solid support.



R can be a C_{18} alkane chain or an amine (NH_2) or cyano (CN) group or some other group. The nature of R determines the types of analytes which can be separated.

The theories of partition and adsorption chromatography are both used to describe this mode of chromatography although it is often classified as a partition technique.

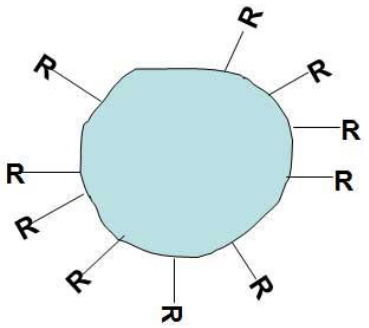


diagram of phase bonded to silica

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Types Partition Chromatography

There are two types of partition chromatography **normal** phase and **reversed** phase, they are defined by the relative polarities of the mobile and stationary phases

Normal phase

The stationary phase is polar eg: R = cyano group and the mobile phase is non-polar eg: petroleum ether or hexane.

For this reason, the use of silica (a polar molecule) as the stationary phase (as in adsorption chromatography) is also considered to be a normal phase separation method.

Reversed phase

The mobile phase is comparatively polar eg: methanol, water, acetonitrile and the stationary phase is non-polar eg: R = C_{18} bonded phase.

Because of its versatility and wide range of applicability, reversed-phased chromatography is the most frequently used hplc method.

Gel Permeation Chromatography (GPC)

This type is also known as:

Size Exclusion Chromatography (SEC)

Molecular Exclusion Chromatography (MEC)

Molecular Sieve Chromatography (MSC)

Gel Filtration Chromatography (GFC)

Gel Chromatography.

Stationary phase

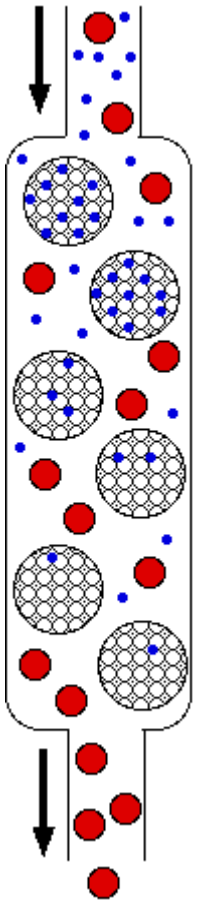
Porous polymeric matrix: formed of spongy particles, with pores completely filled with the liquid mobile phase **(gel)**.

The gels (polymers) consist of **open**, **three-dimensional networks** formed by cross-linking of long polymeric chains.

The **pore size** varies with the degree of cross-linking.

The **diameter of the pores is critical as** separation is based on that **molecules above certain size are totally excluded from the pores because they can not enter the gel**.

The **interior of the pores is reached**, partially or wholly, **by smaller molecules**.



Mobile phase

Mobile phase

Mobile phase is a liquid as **water** or **dilute acohol**

Separation mechanism

Based on difference between the solutes molecular weights.

Molecules will distribute themselves outside & inside the pores according to their size.

Larger are excluded, **medium sized** enter half-way & **smallest** permeate all the way.

Applications of GPC to natural products

Determination of M. wt. of peptides, proteins & polysaccharides.

Desalting of colloids e.g. desalting of albumin prepared with 2% $(NH_4)_2SO_4$.

Separation of mixture of mono- & polysaccharides.

Separation of amino acids from peptides & proteins.

Separation of proteins of different molecular weights.

Separation of mucopolysaccharides & soluble RNA.

Separation of myoglobin & haemoglobin.

Separation of alkaloids & purification of enzymes.

Affinity Chromatography

The five steps in affinity chromatography

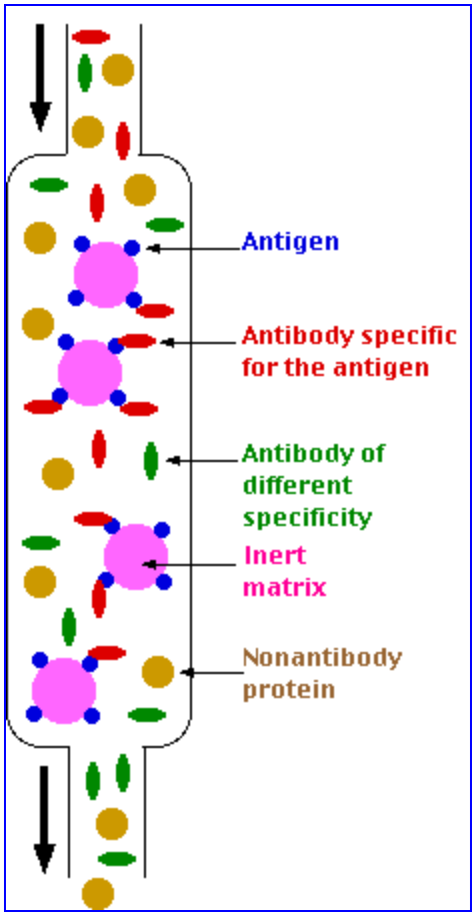
Activation- the ligand is bound to the chromatographic solid support.

Loading- the analytes to be separated are introduced into the mobile phase stream.

Binding- the analytes of interest are retained due to Interaction with the ligand of the stationary phase.

Washing- unwanted analytes are eluted from the column.

Elution- the analyte(s) of interest are washed from the column by changing the mobile phase composition.



Application:
-Purification of proteins
-Study of drug and hormone interactions with proteins
-Immunoassays

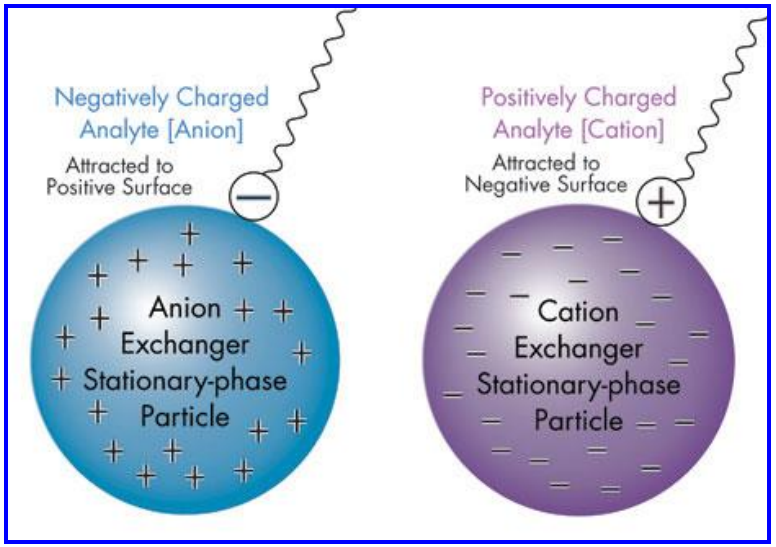
Ion Exchange Chromatography

Stationary phases for ion-exchange separations are characterized by the nature and strength of the acidic or basic functions on their surfaces and the types of ions that they attract and retain.

Cation exchange is used to retain and separate positively charged ions on a negative surface.

Conversely, anion exchange is used to retain and separate negatively charged ions on a positive surface.

With each type of ion exchange, there are at least two general approaches for separation and elution.



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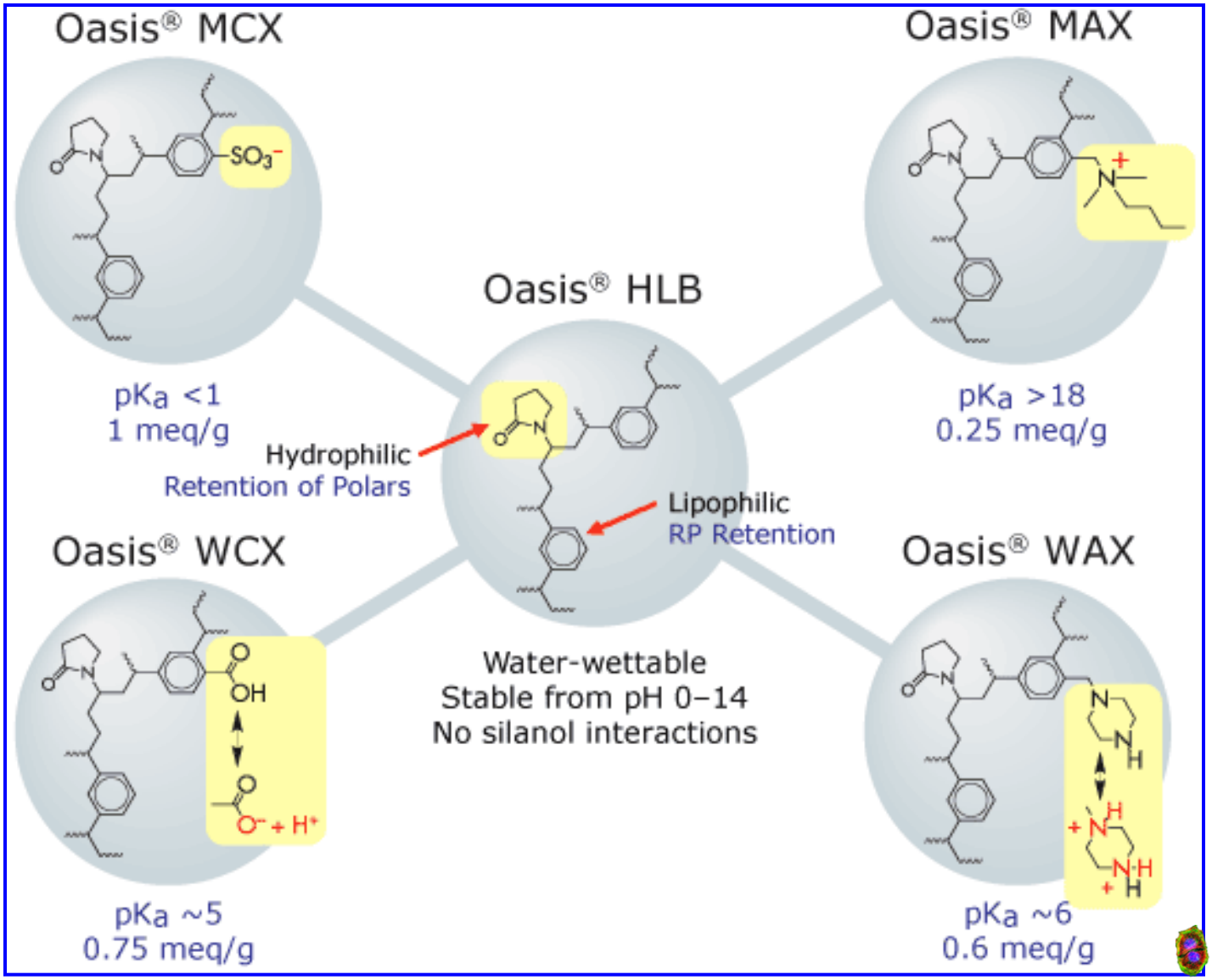
Different kinds of Ion Exchange Chromatography



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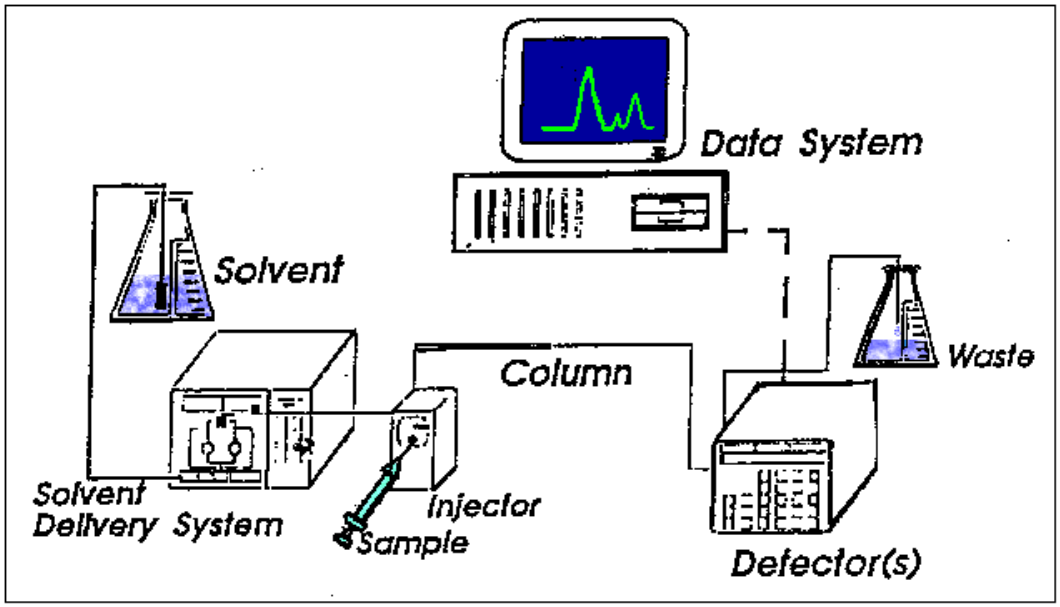
HPLC



More common HPLC Detectors

- UV-Visible
- Fluorescence
- PDA
- Light Scattering

Less sensitive (microgram level)

- **Mass Spectrometry**

Highly sensitive

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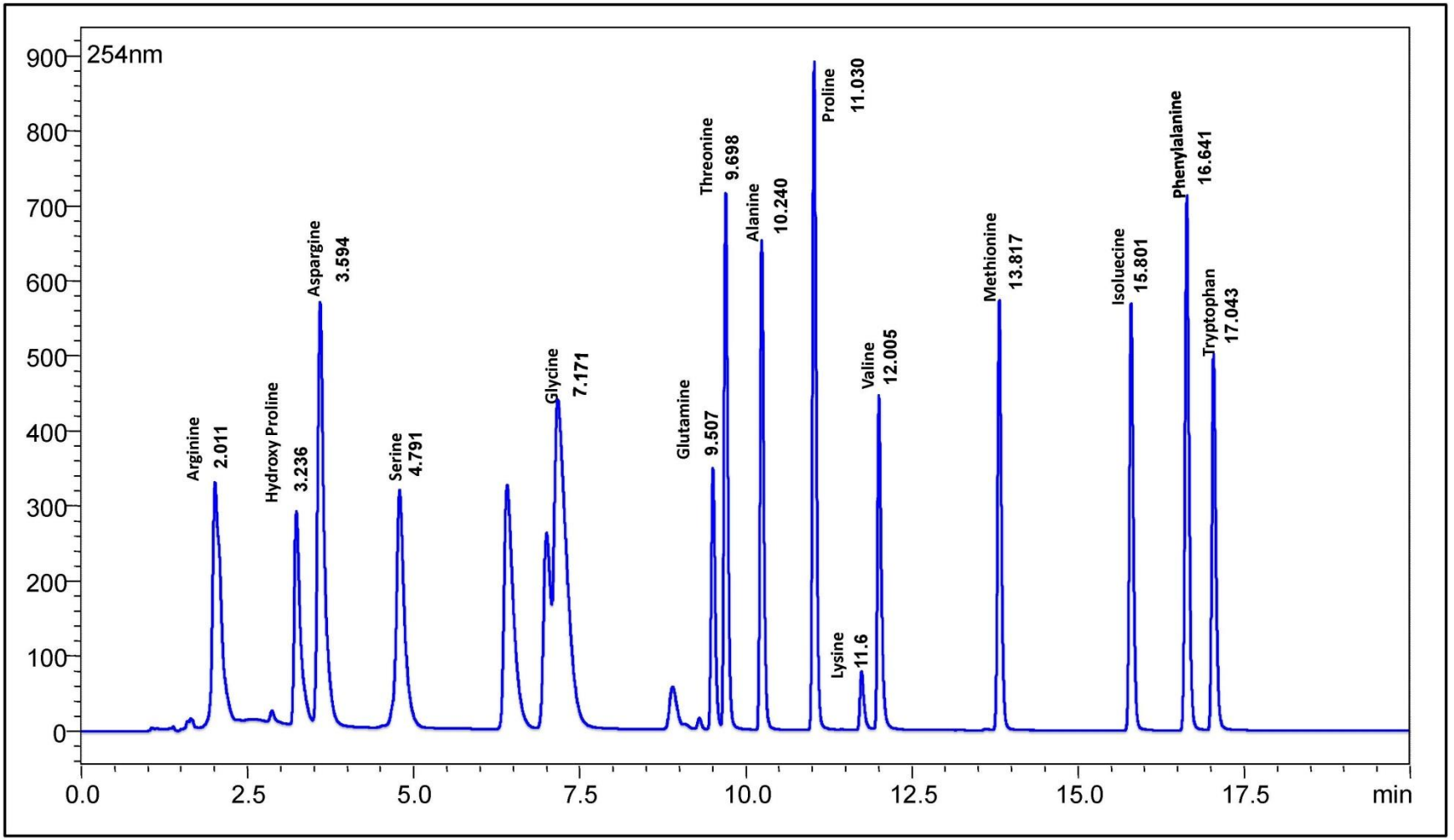
Chromatogram of Amino acid derivatives



Analysis of amino acids using LC-UV after derivatizing with AQC . Chromatogram of 15 amino acid mix in reverse phase column.

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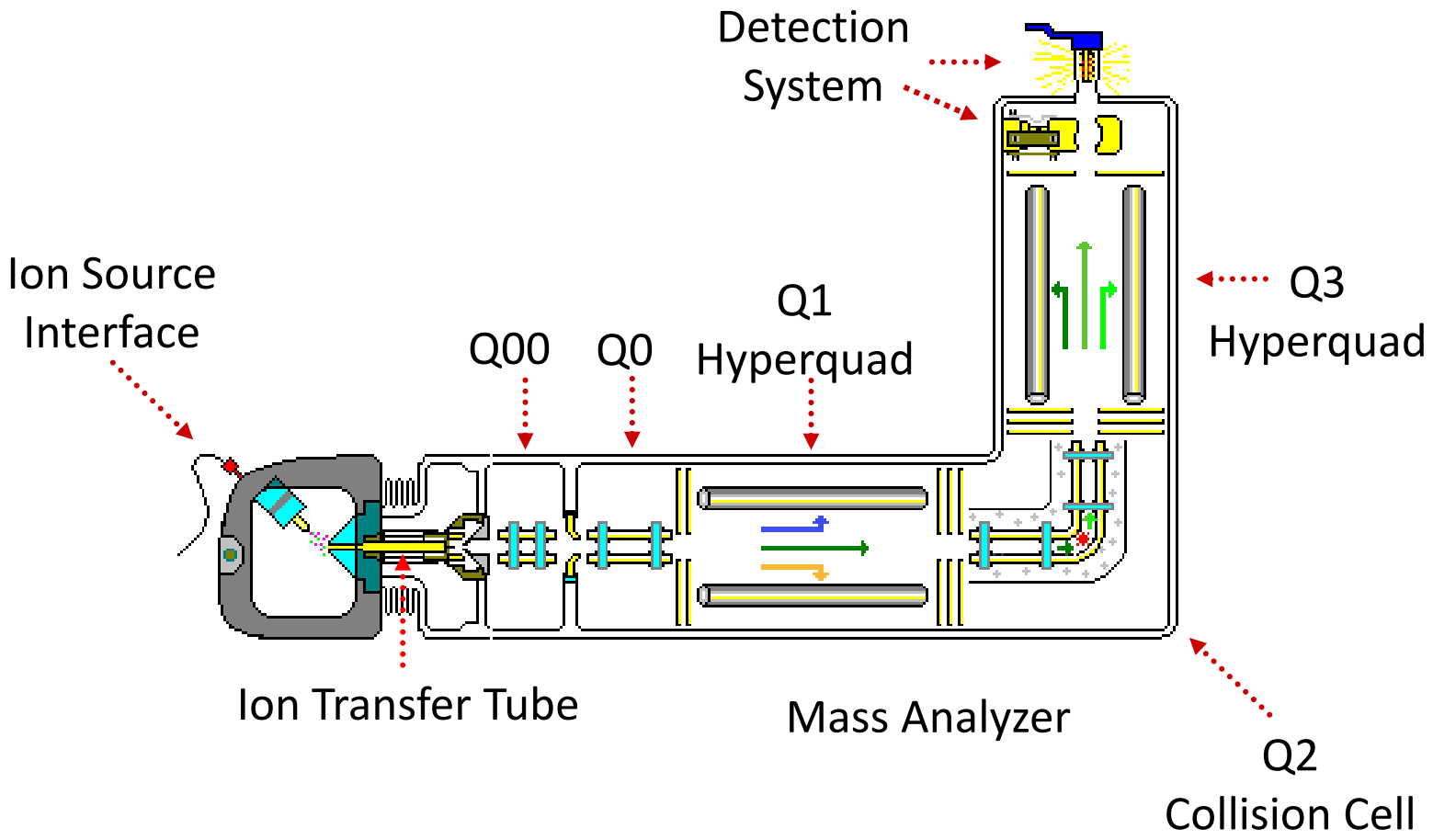
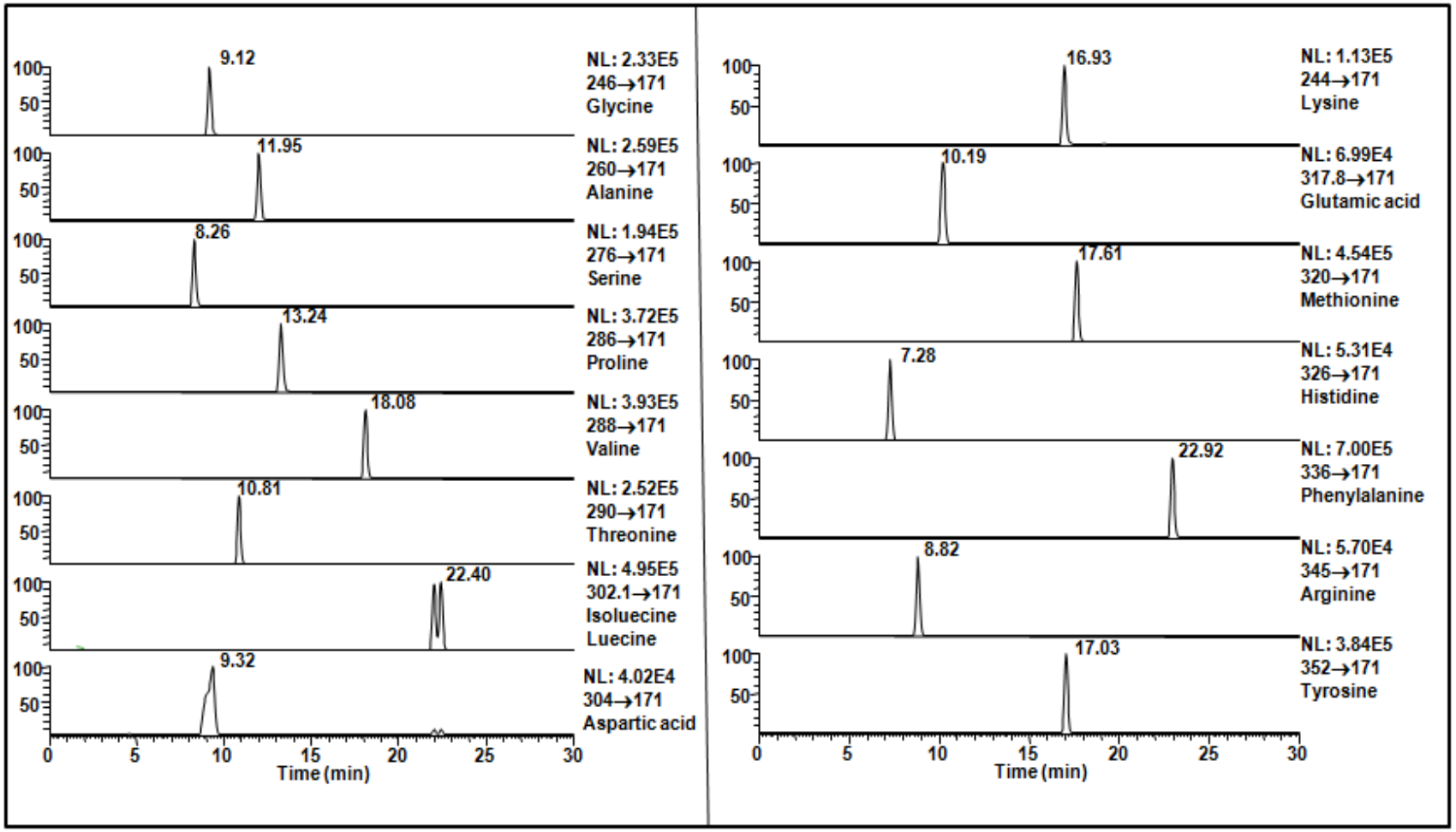
LC-MS System

Triple Stage Quadrapole (TSQ)



LC-MS Chromatogram of Amino acid derivatives



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Different Kinds of Experiments

- Full Scan (MS)
- MS/MS Scan (Product Ion Scan)
- Parent ion scan (Precursor scan)

Less sensitive (lower ng)

- Selected Reaction Monitor (SRM) scan
- Multiple reaction monitor (MRM) scan

More sensitive (fg)

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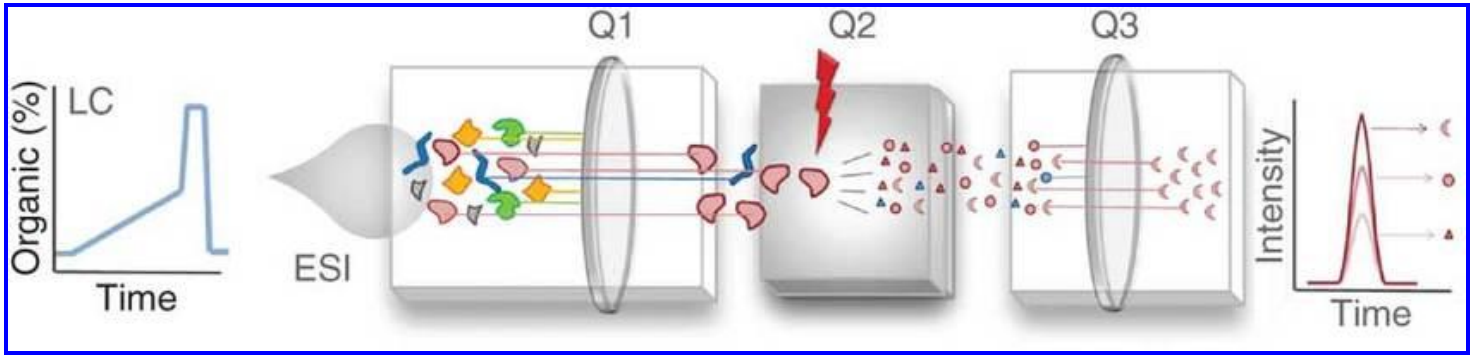
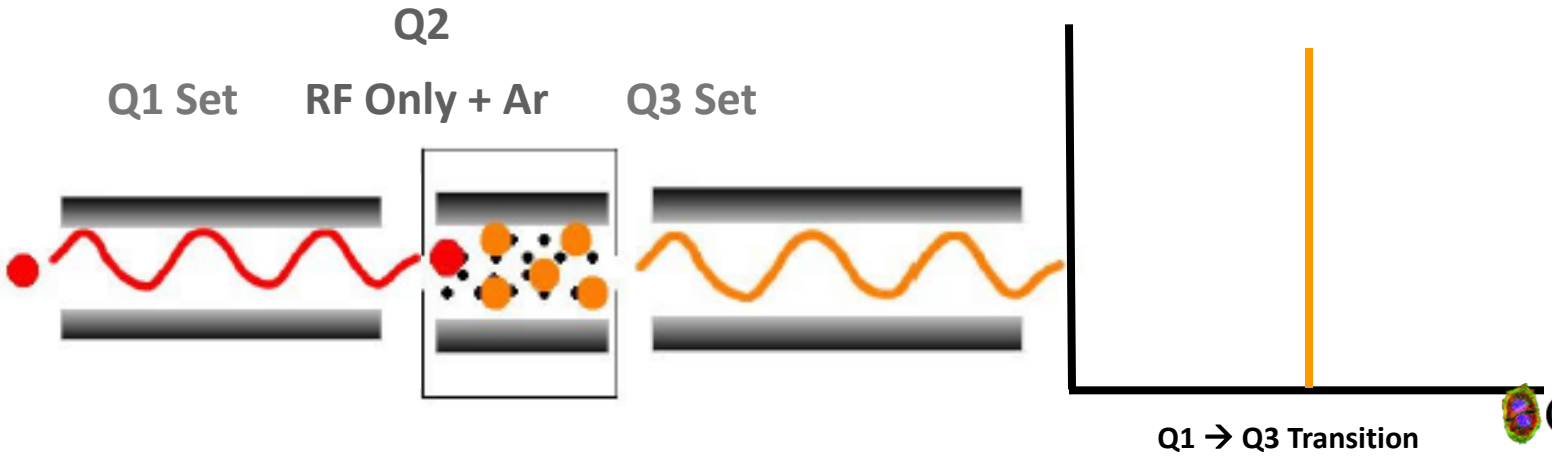
Selected Reaction Monitoring (SRM) method



Nature Methods 9, 555–566 (2012)



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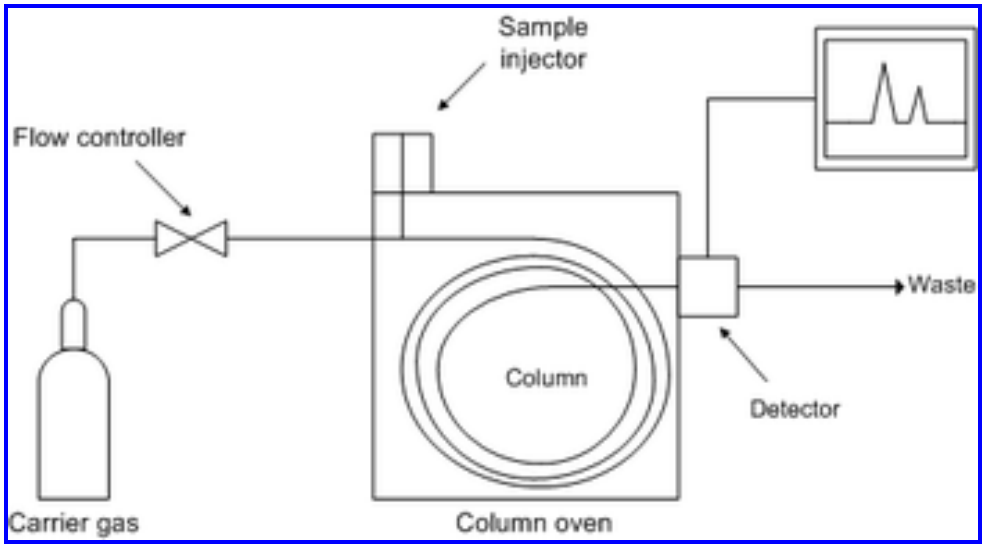
Gas Chromatography



"Without Chromatographic separation it is impossible to analyze complex samples by using Mass spec"

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