Chapter-26:

An Introduction to Chromatographic Separations

Introduction to Chromatography

Definition

Chromatography is a separation technique based on the different interactions of compounds with two phases, a *mobile phase* and a *stationary phase*, as the compounds travel through a supporting medium.

Components:

Mobile phase: a solvent that flows through the supporting medium

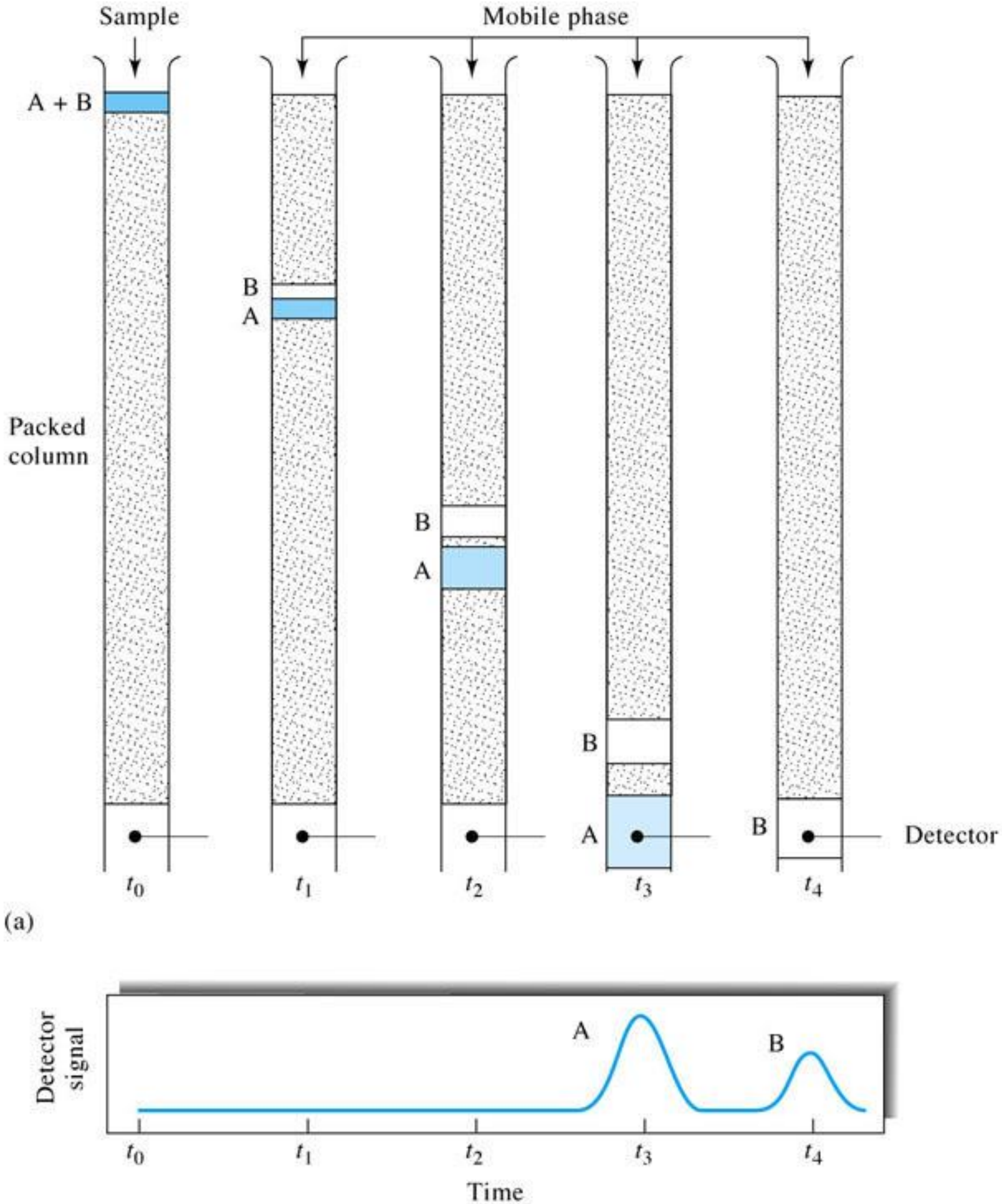
Stationary phase: a layer or coating on the supporting medium that interacts with the analytes

Supporting medium: a solid surface on which the stationary phase is bound or coated

Uses for Chromatography

Real-life examples of uses for chromatography:

- Pharmaceutical Company – determine amount of each chemical found in new product
- Hospital – detect blood or alcohol levels in a patient's blood stream
- Law Enforcement – to compare a sample found at a crime scene to samples from suspects
- Environmental Agency – determine the level of pollutants in the water supply
- Manufacturing Plant – to purify a chemical needed to make a product



The analytes interacting most strongly with the stationary phase (B) will take longer to pass through the system than those (A) with weaker interactions.

These interactions are usually chemical in nature, but in some cases physical interactions can also be used.

Types of Chromatography

1) The primary division of chromatographic techniques is based on the type of mobile phase used in the system:

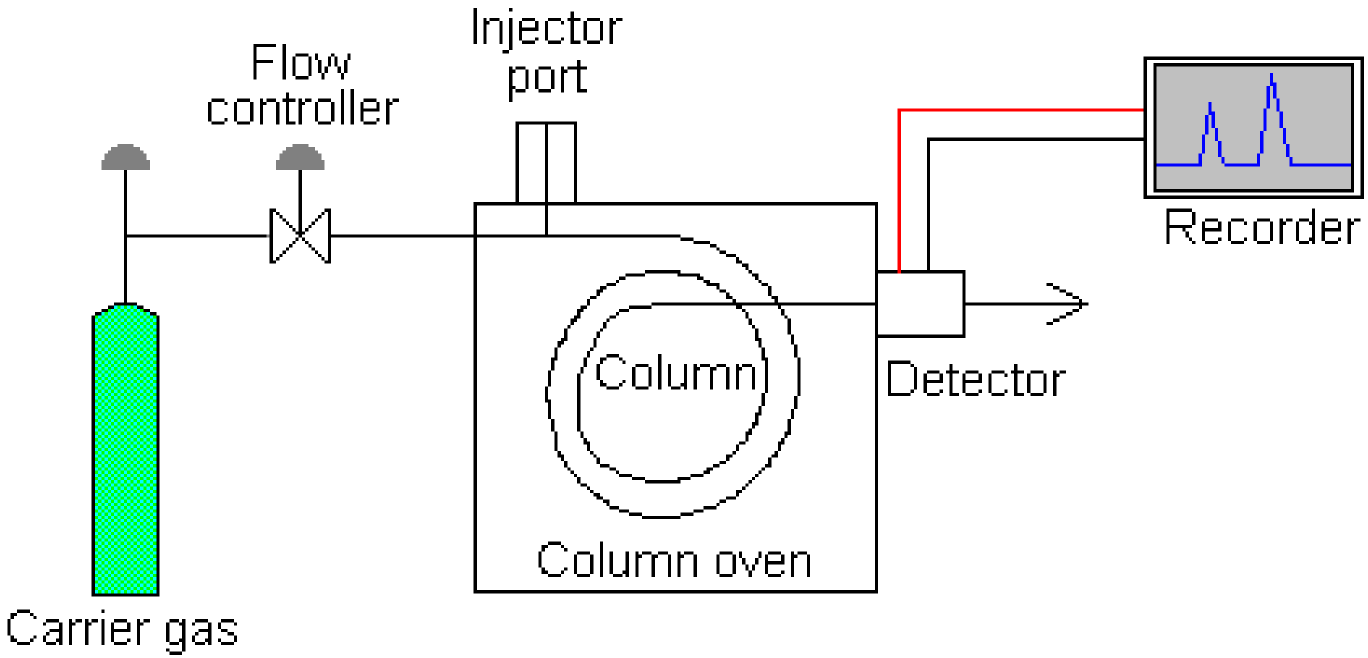
Type of Chromatography	**Type of Mobile Phase**
Gas chromatography (GC)	gas
Liquid chromatograph (LC)	liquid

Types of Chromatography

2) Further divisions can be made based on the type of stationary phase used in the system:

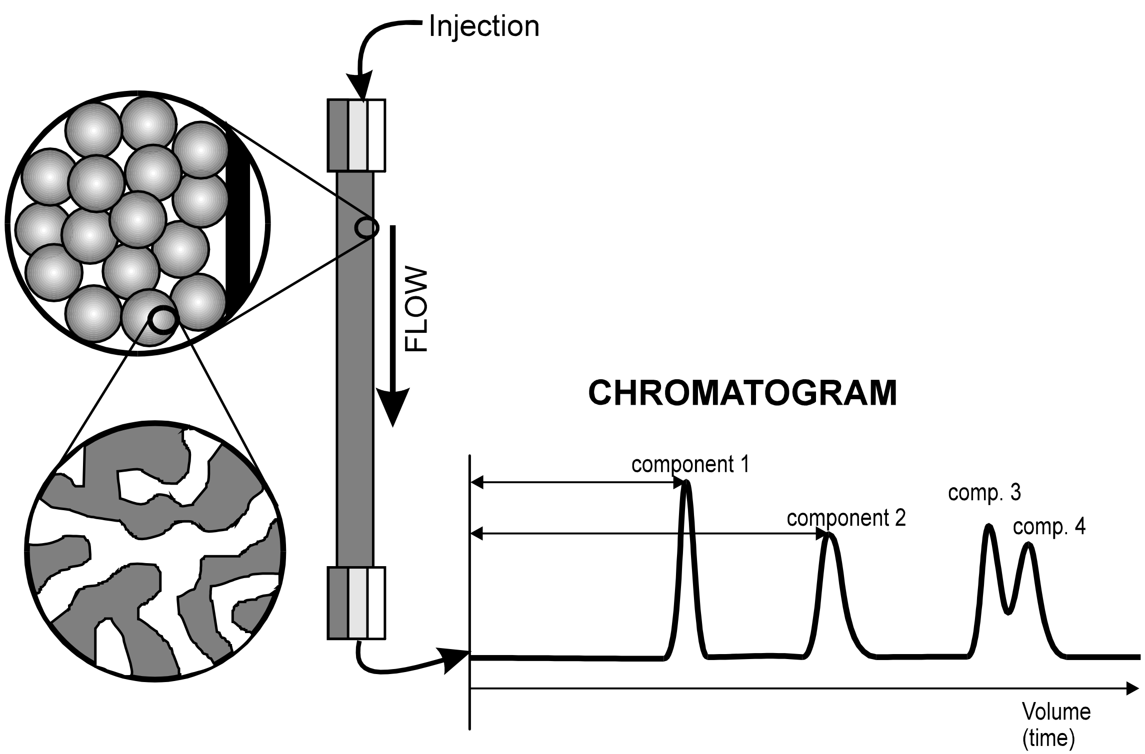
Gas Chromatography (GC)

Name of GC Method	Type of Stationary Phase
Gas-solid chromatography	solid, underivatized support
Gas-liquid chromatography	liquid-coated support
Bonded-phase gas chromatography	chemically-derivatized support



Liquid Chromatography (LC)

Name of LC Method	Type of Stationary Phase
Adsorption chromatography	solid, underivatized support
Partition chromatography	liquid-coated or derivatized support
Ion-exchange chromatography	support containing fixed charges
Size exclusion chromatography	porous support
Affinity chromatography	support with immobilized ligand

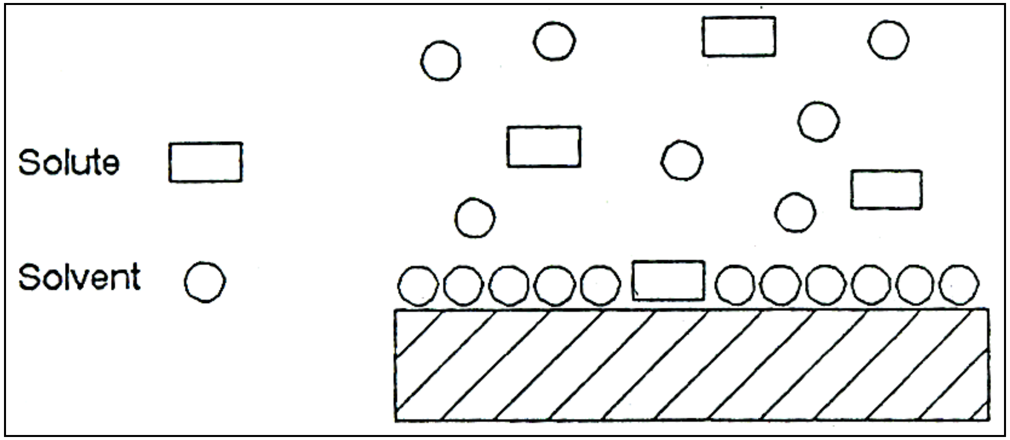


Adsorption Chromatography

Separation based on their adsorption onto the surface of solid (stationary phase).

Normal phase-like separation

Nonpolar mobile phase for polar, non-ionic compounds

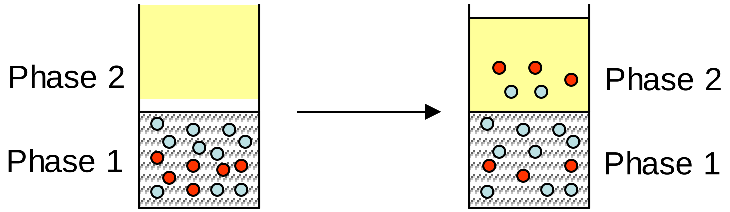


Ex; Column chromatography TLC, HPLC

Partition Chromatography

Solute are separated based on their partition between a liquid mobile phase and a liquid stationary phase coated on a solid support.

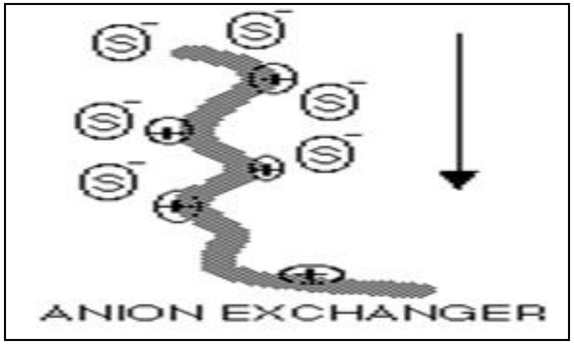
- Normal – analyte is nonpolar organic; stationary phase MORE polar than the mobile phase
- Ex : TLC, Paper Chromatography



- Reverse – analyte is polar organic; stationary phase LESS polar than the mobile phase
- Ex : HPLC

Ion Exchange Chromatography

- Use ionic stationary phase
  - ions separated on the basis of their tendency to displace counter ions adsorbed on stationary phase (Depends on charge, hydration, “solubility”
  - Used for analysis of aminoacids and its base pair.



- Anionic stationary phases: used for cation separation
- Cationic stationary phases : for anion separation for ionic compounds
- - Ex : HPLC

Size Exclusion Chromatography

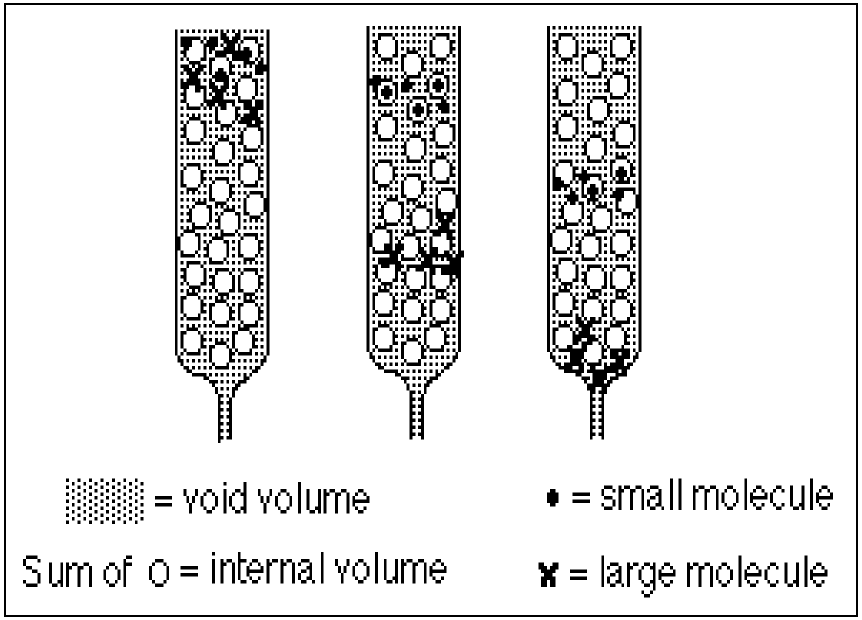
- Separation is a result of "trapping" of molecules in the pores of the packing material

- Very large molecules can't get into the pores – unretained

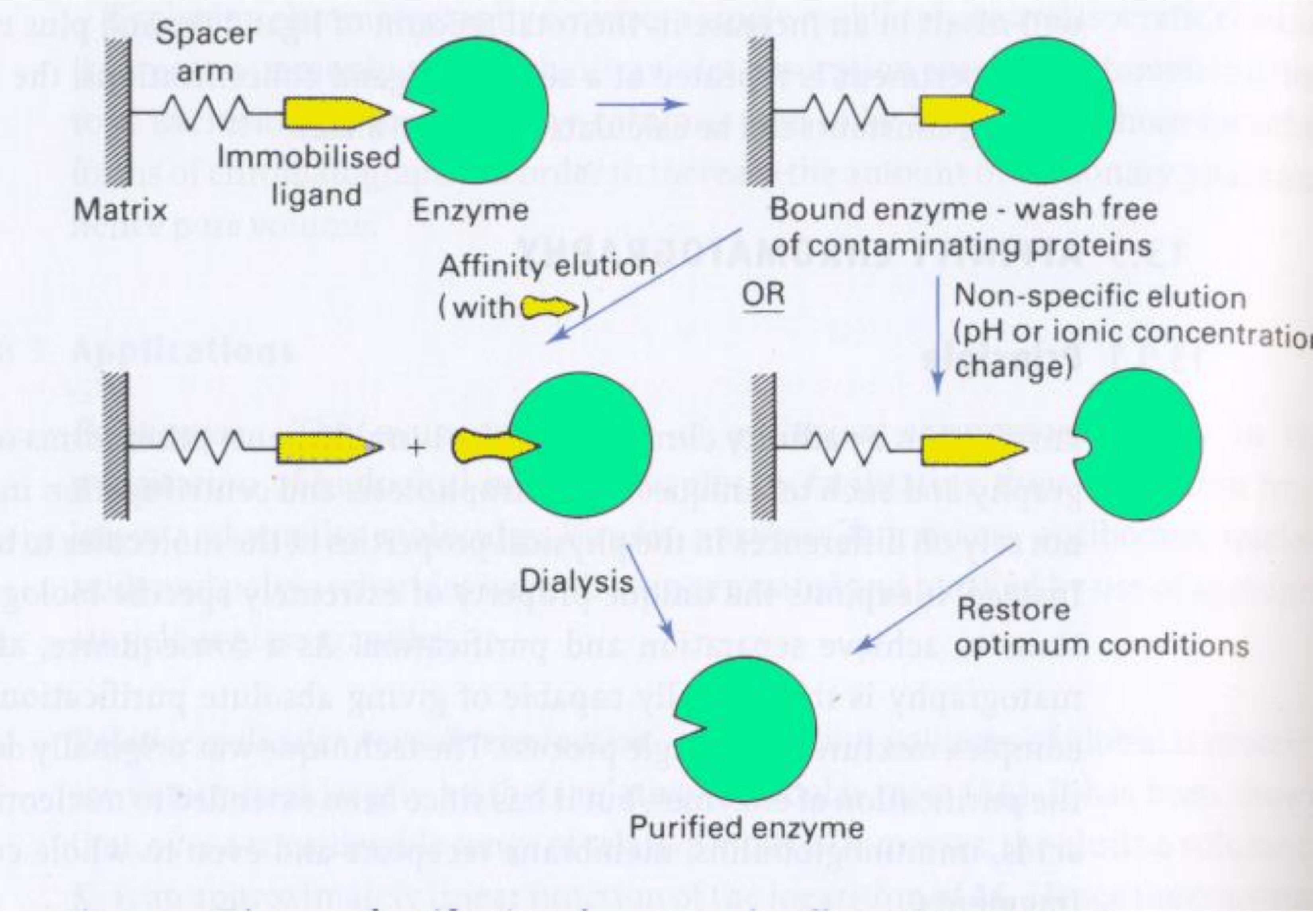
- Very small molecules get hung up in to pores for a long time - most retained – longest retention time

- stationary phase is a porous matrix

- Ex: GC, HPLC



Affinity chromatography

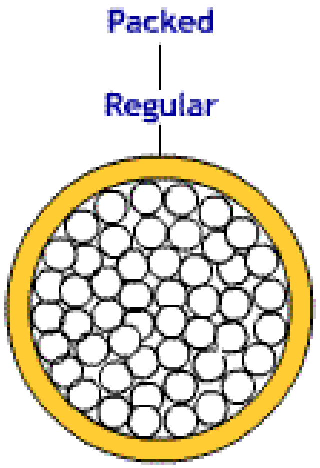


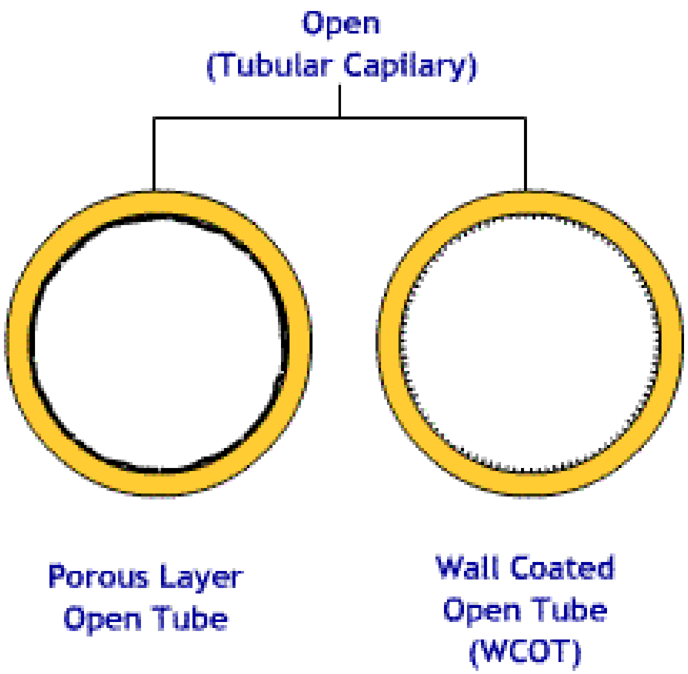
3) Chromatographic techniques may also be classified based on the type of support material used in the system:

Packed bed (column) chromatography

Open tubular (capillary) chromatography

Open bed (planar) chromatography





Some chromatography terms

Analyte

•Substance that is to be separated during chromatography

Immobilized phase

•Stationary phase which is immobilized on the support particles or on the inner wall of the column tubing

Mobile phase

•Phase which moves in a definite direction. (liquid/gas/fluid).

•Consists of the sample being separated/ analyzed and the solvent that moves the sample through the column.

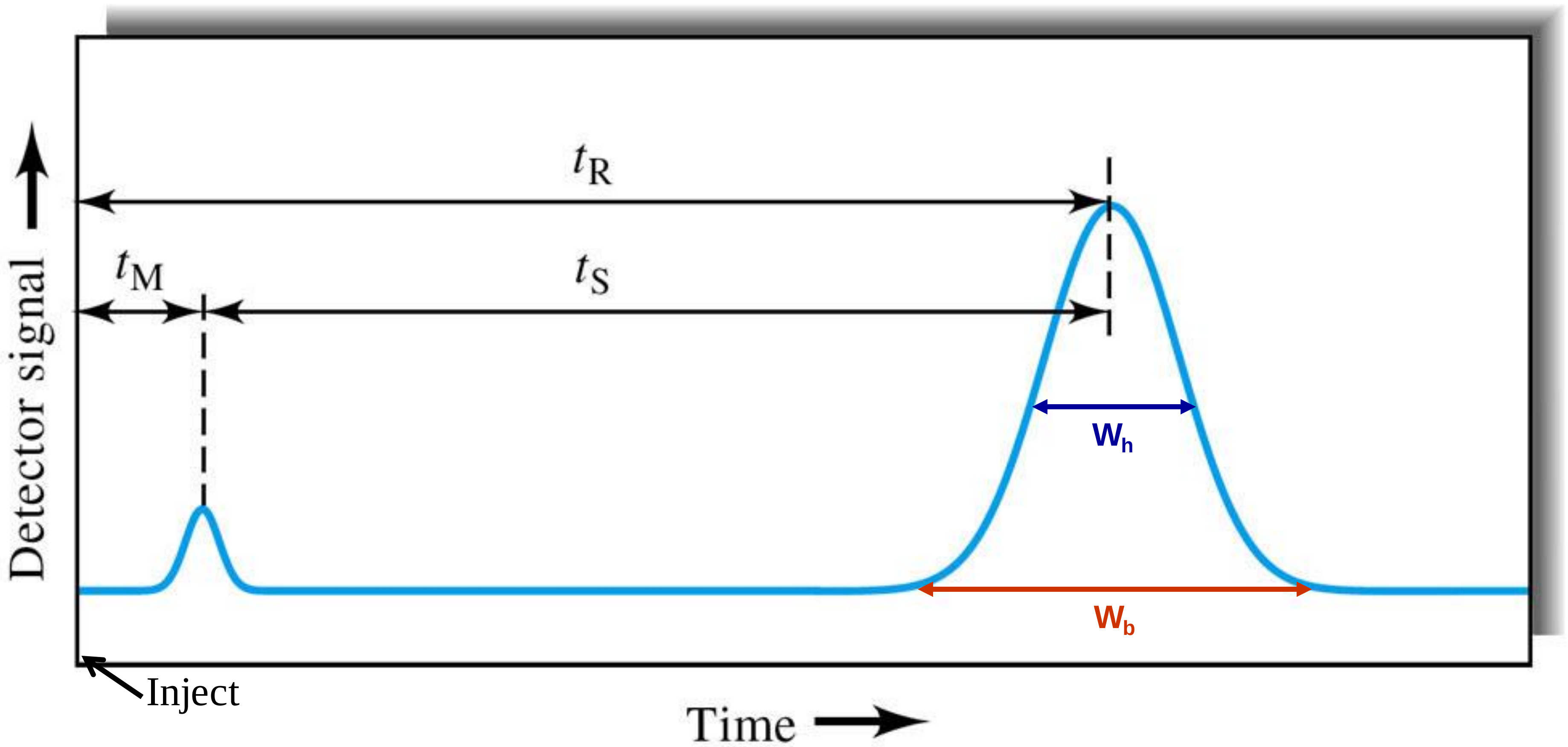
Effluent

•Mobile phase leaving the column.

Elution: **is the process of removing analyte from stationary phase by mobile phase**

<u>1) Typical response obtained by chromatography</u> (i.e., a chromatogram):

chromatogram - concentration versus elution time



Where:

t_R = retention time t_S = solute retention time

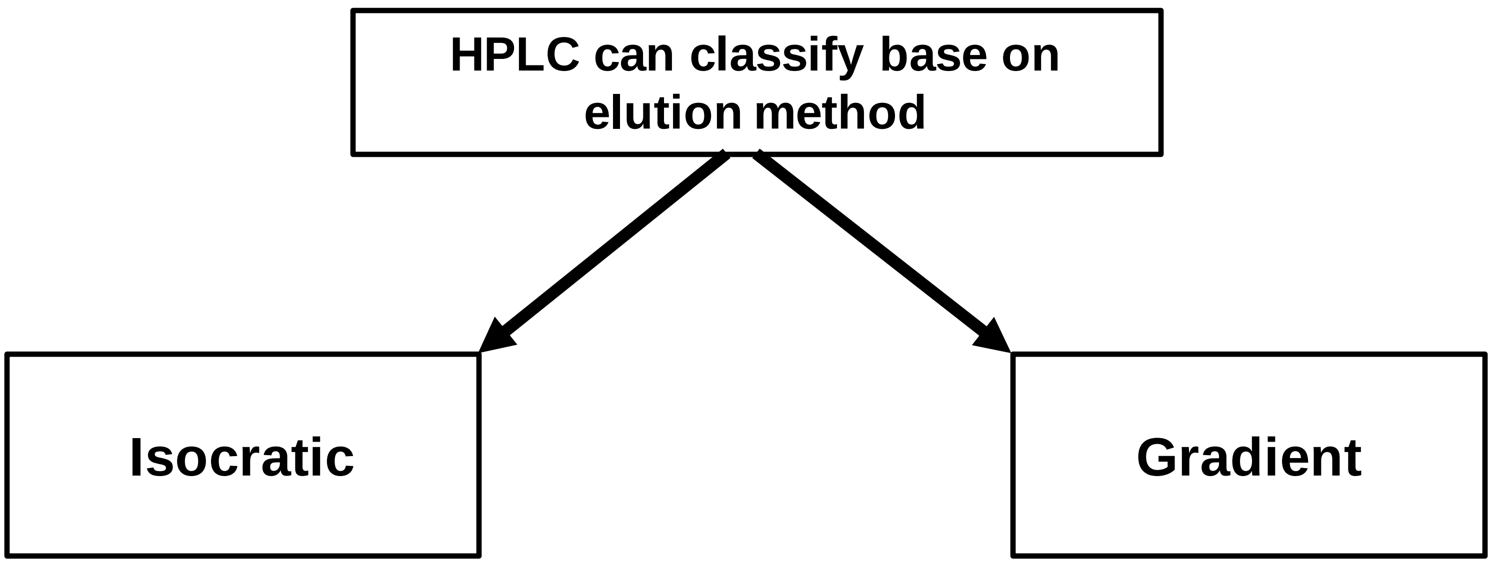
t_M = void time

W_b = baseline width of the peak in time units

W_h = half-height width of the peak in time units

High performance liquid chromatography

- HPLC is an extension of conventional liquid chromatography.
- Powerful tool in analytical techniques
- Columns are tightly packed, and the eluent is forced through the column under high pressure(up to 5,000 psi) by a pump.
- Allows to use a very smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing through it.
- Allows a much better separation of the components of the mixture.



Liquid Chromatography: Adsorption chromatography

Isocratic

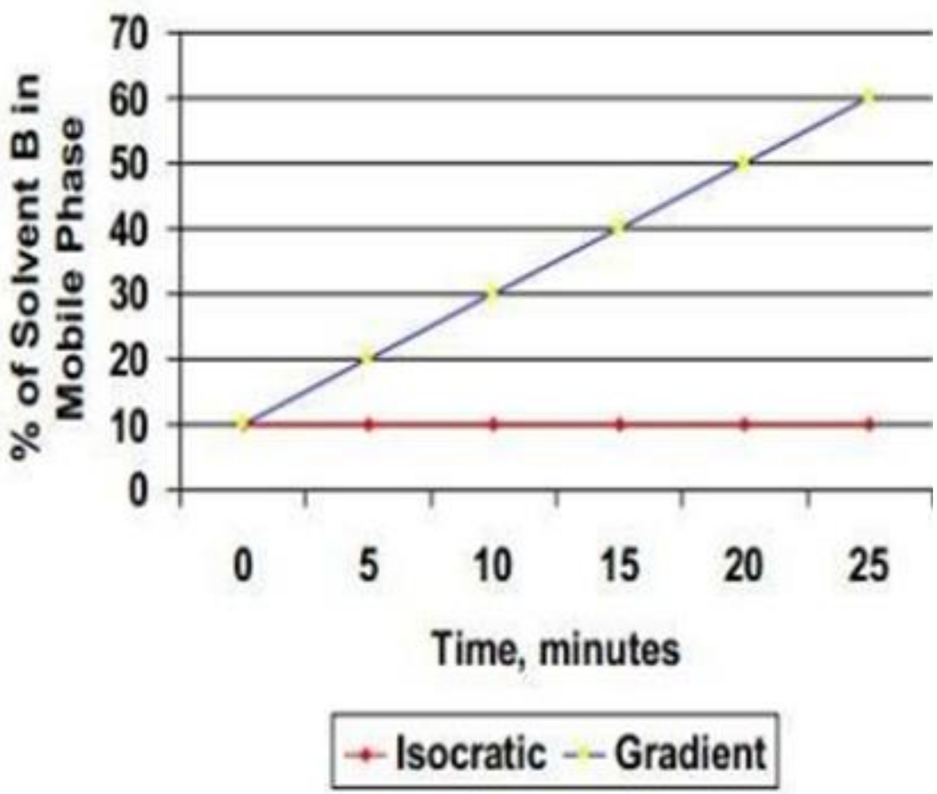
mobile phase solvent composition remains constant with time

- Best for simple separations
- Often used in quality control applications that support and are in close proximity to a manufacturing process

Gradient

mobile phase solvent ("B") composition increases with time

- Best for the analysis of complex samples
- Often used in method development for unknown mixtures
- Linear gradients are most popular (for example, the "gradient" shown at right)

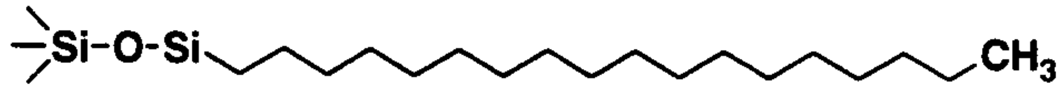


HPLC can be classified based on separation mode to

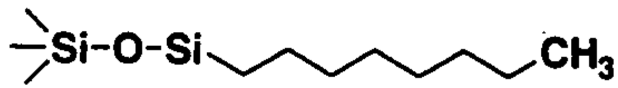
- **1- Normal Phase;** when the stationary phase is more polar than the mobile phase
- **2- Reverse Phase;** when the mobile phase is more polar than the stationary phase
- **Reverse phase chromatography;** The silica gel is polar and to be used for the reverse phase separation, its polar surface has to be changed. This can be done by attaching different functional groups such as hydrocarbons mostly C-8 and C18 (none polar).
- As a result we create a none polar phase. This type is used more than the Normal Phase, and the reason why it is more popular is that its weak mobile phase is the high polar water, therefore, the samples are applied in this weak mobile phase i.e applied in aqueous status such as biological compounds.
- This makes it especially attractive in clinical chemistry for drug confirmation, amino acid analysis and hormone separations.

Common Reverse Phase (RP) Packings

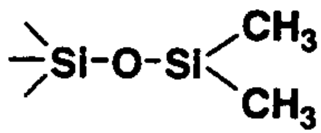
Octadecyl -



Octyl -



Dimethyl -



NORMAL PHASE:

Cyanopropyl -

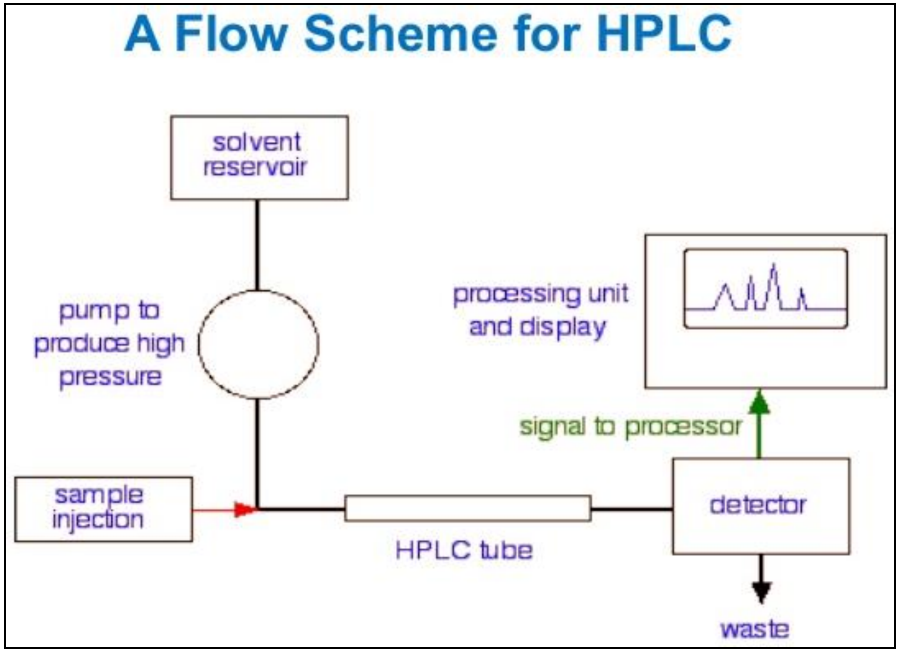
Aminopropyl -

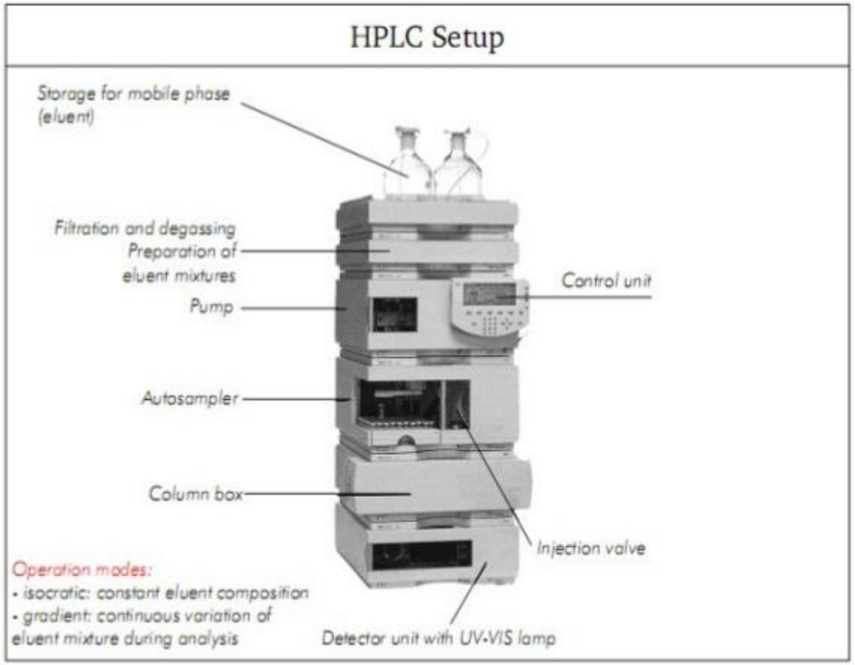
HPLC Technique

- Utilizes liquid mobile phase to separate the mixture
- Analytes are first dissolved in a solvent then through the column under high pressure of up to 400 atm
- Mixture is resolved into its components in the column
- The total separation time is often 5 or 10 minutes rather than hours or even days required for some separations by gravity flow with the larger systems.

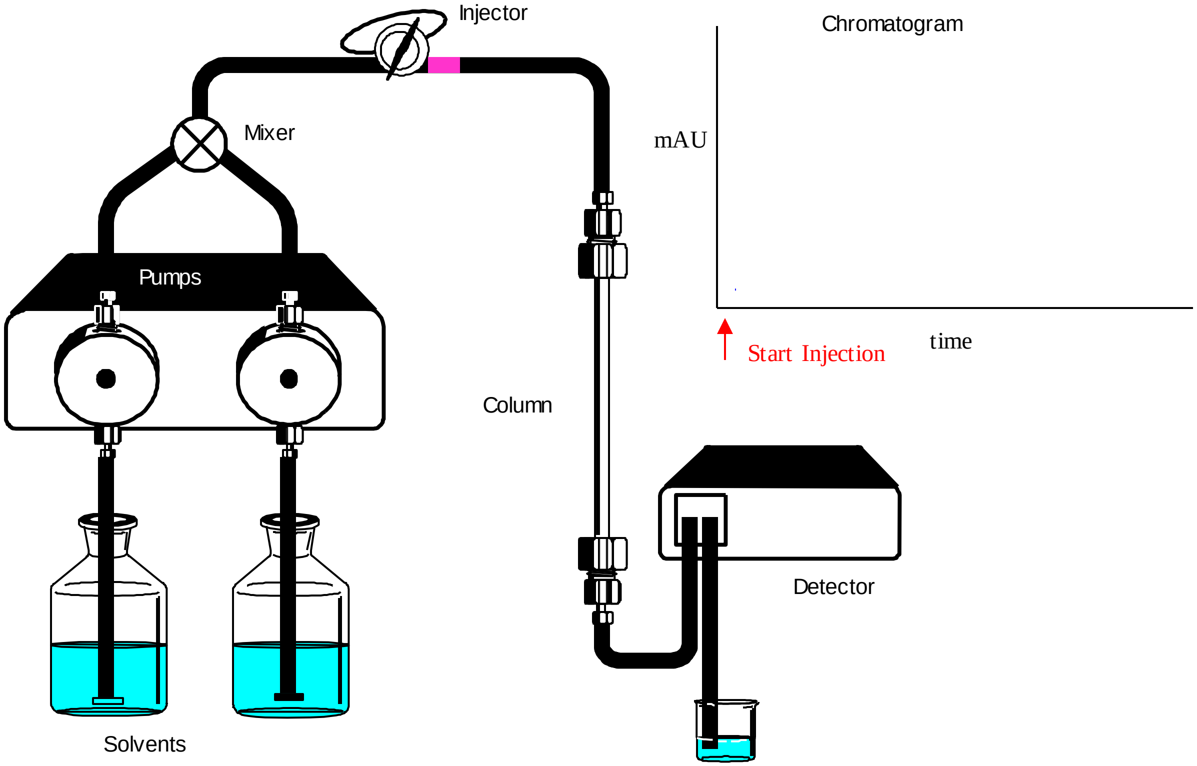
Components of HPLC

- Pump
- Injector
- Column
- Detector
- Recorder or data system



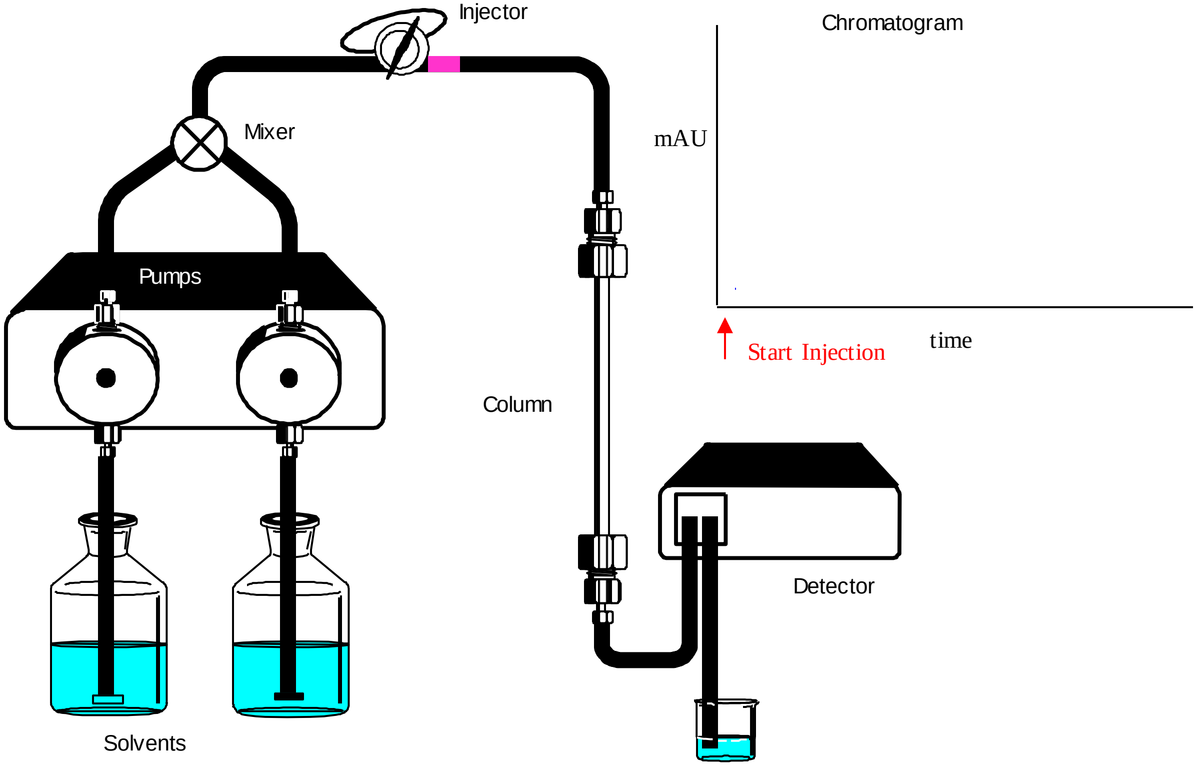


Separations

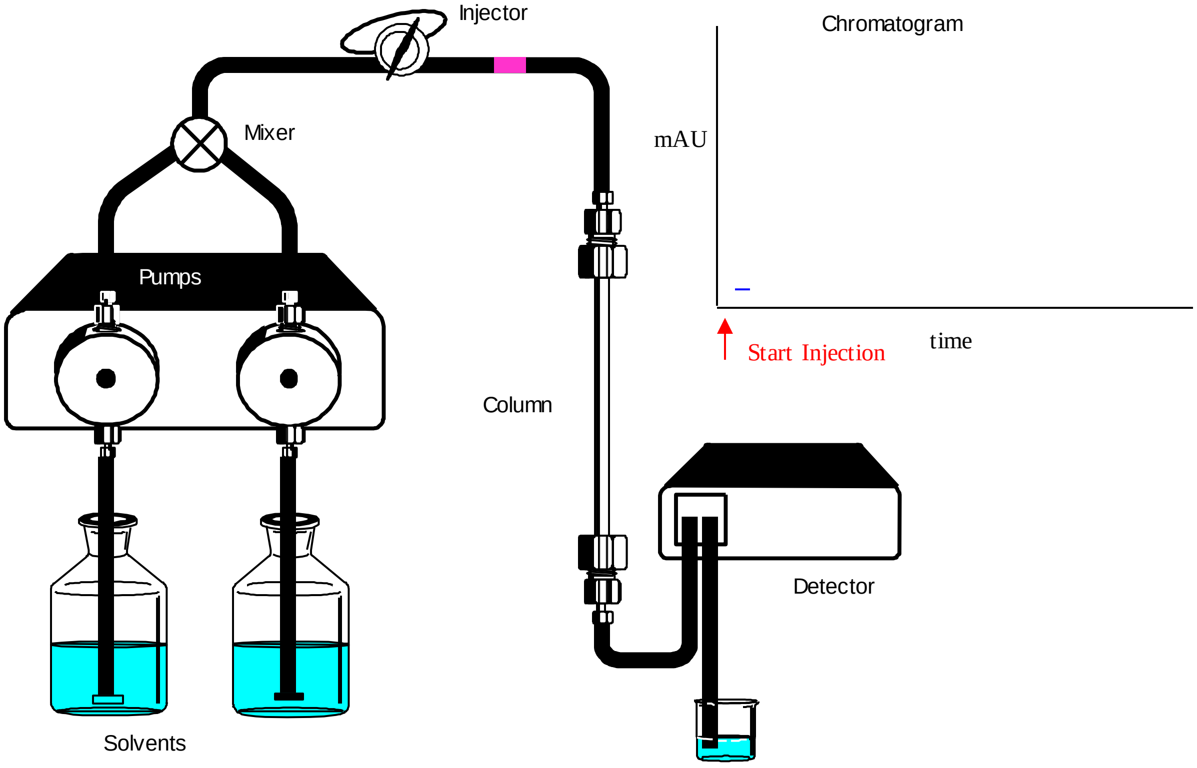


High Performance Liquid Chromatograph

Separations



Separations



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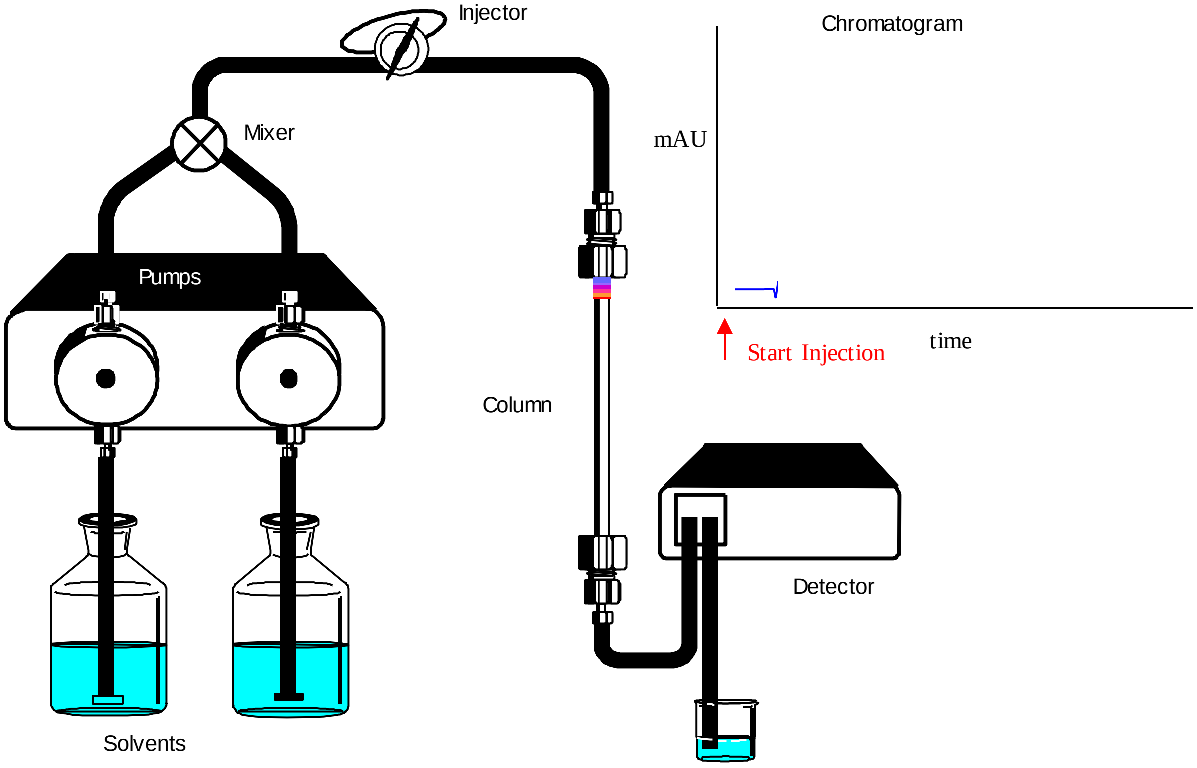

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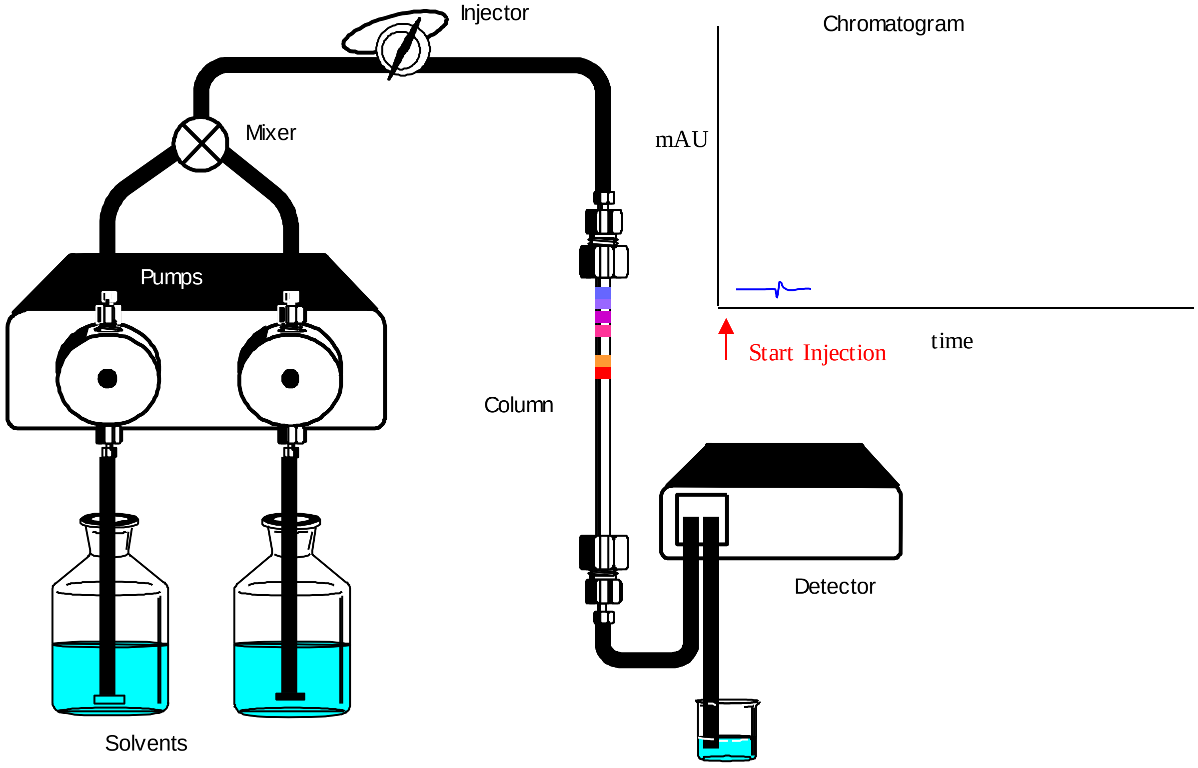

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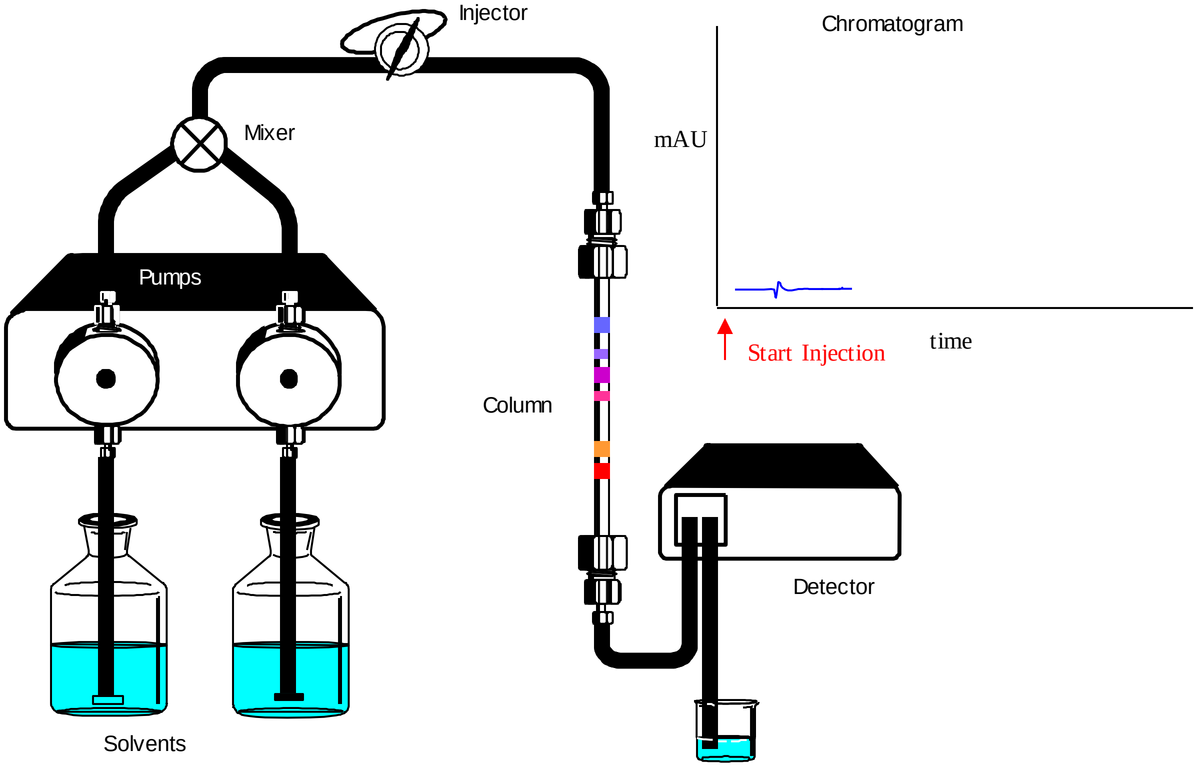

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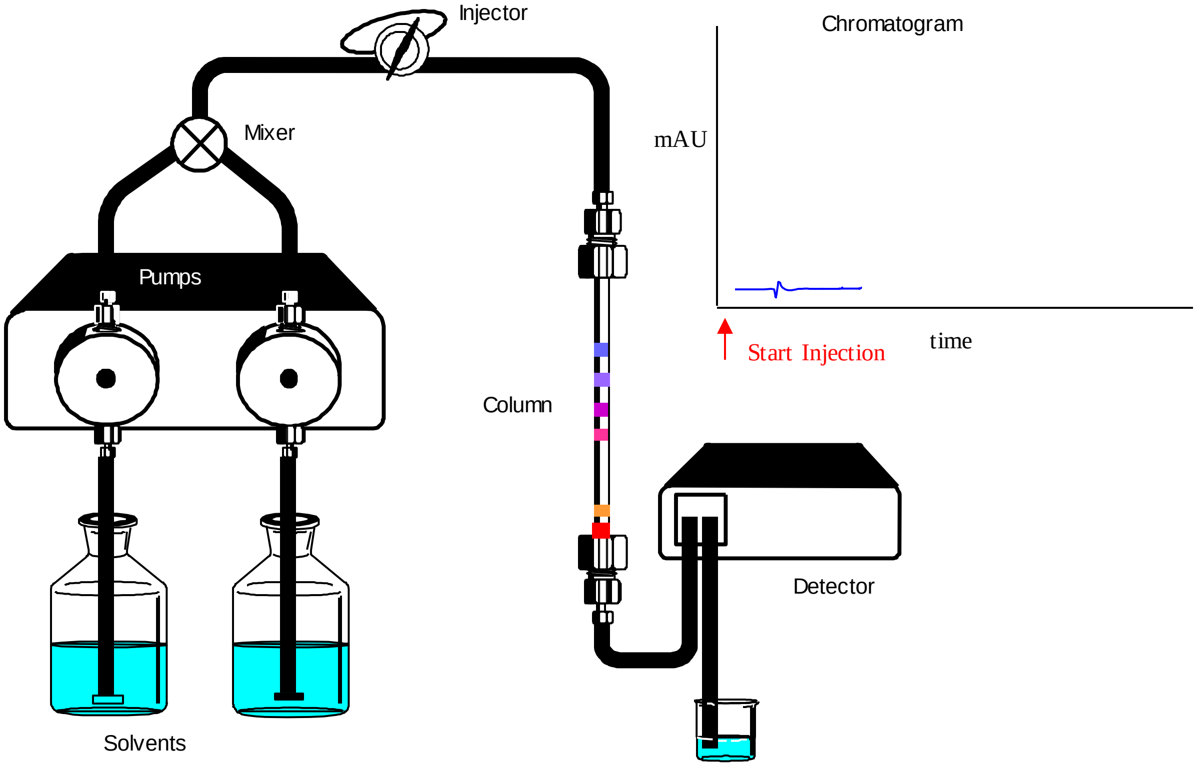

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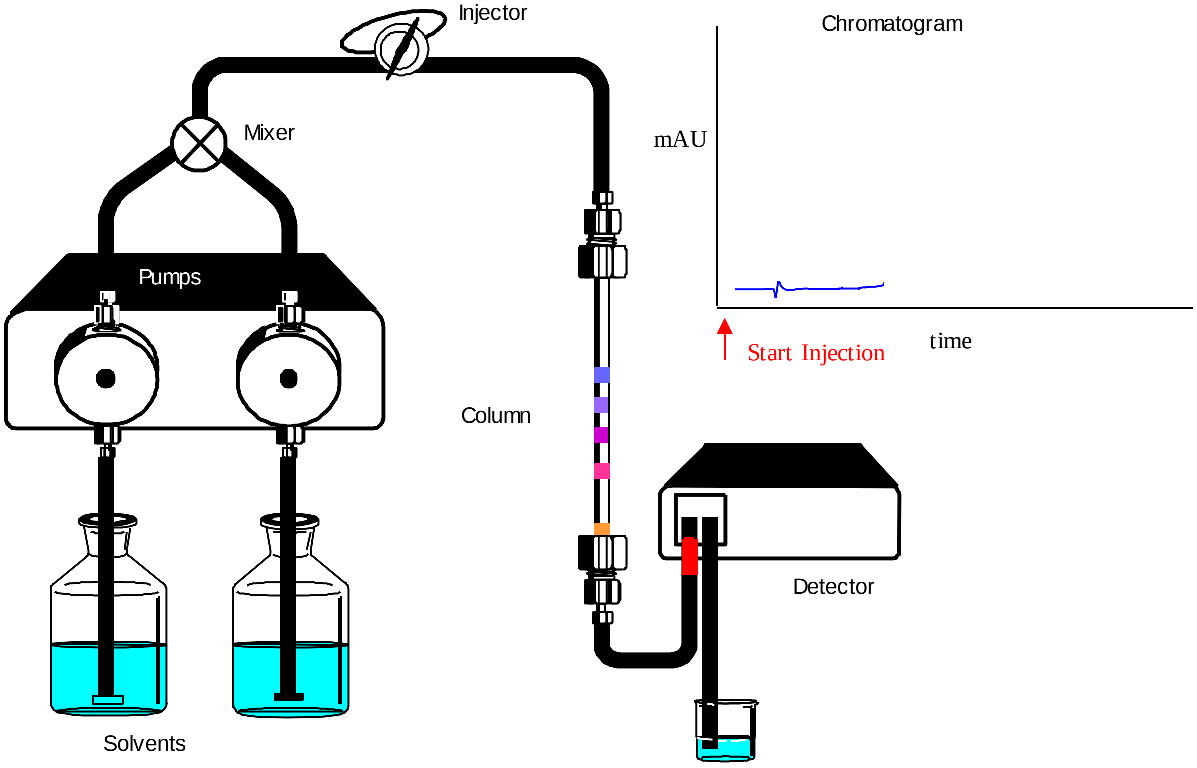

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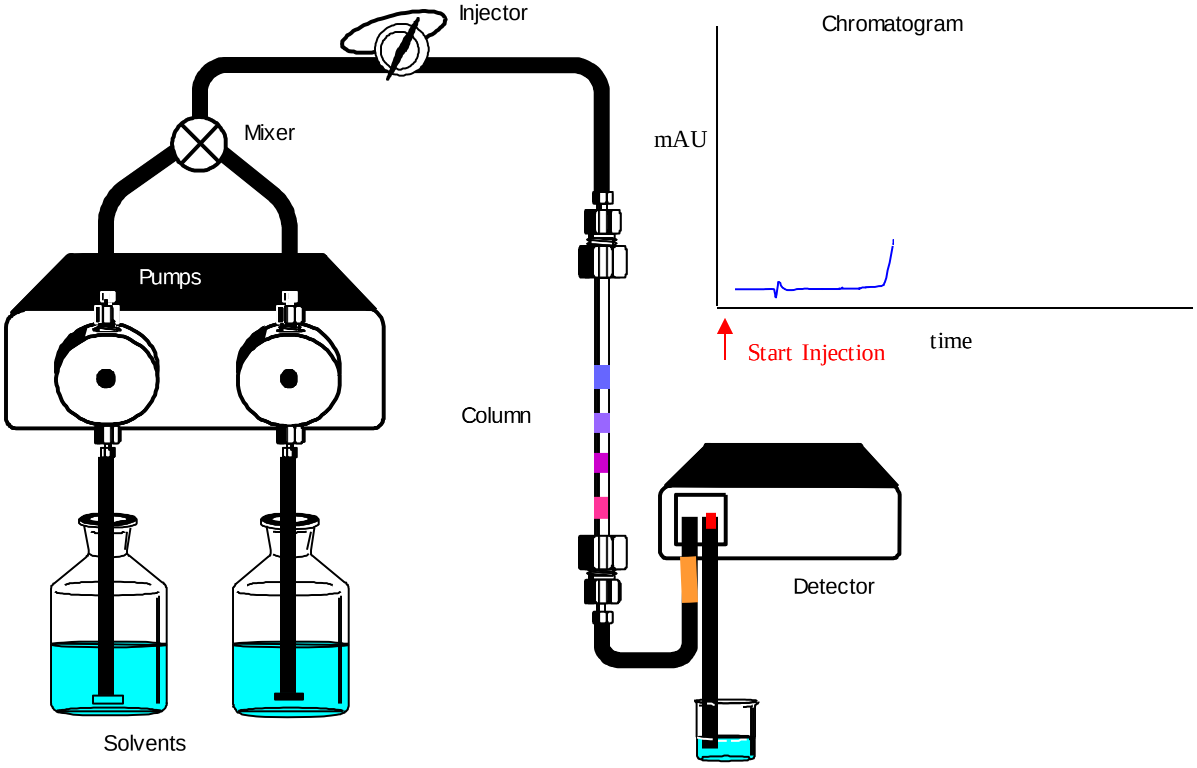

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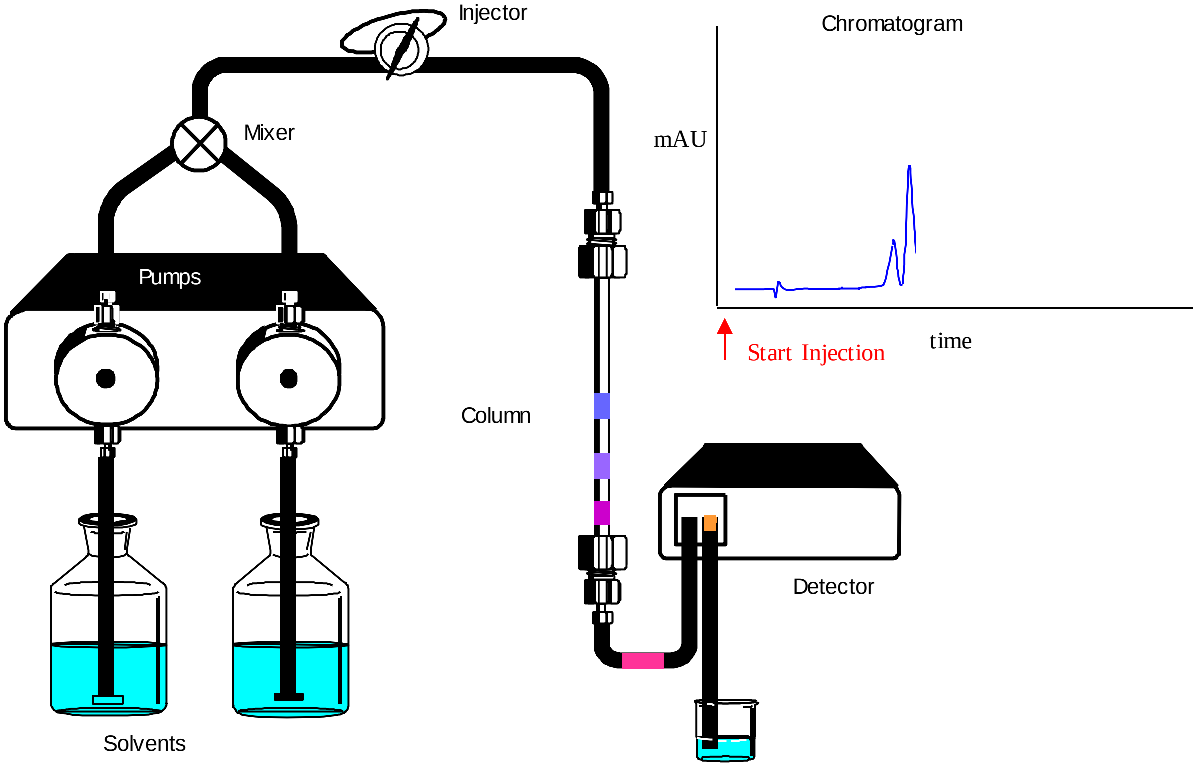

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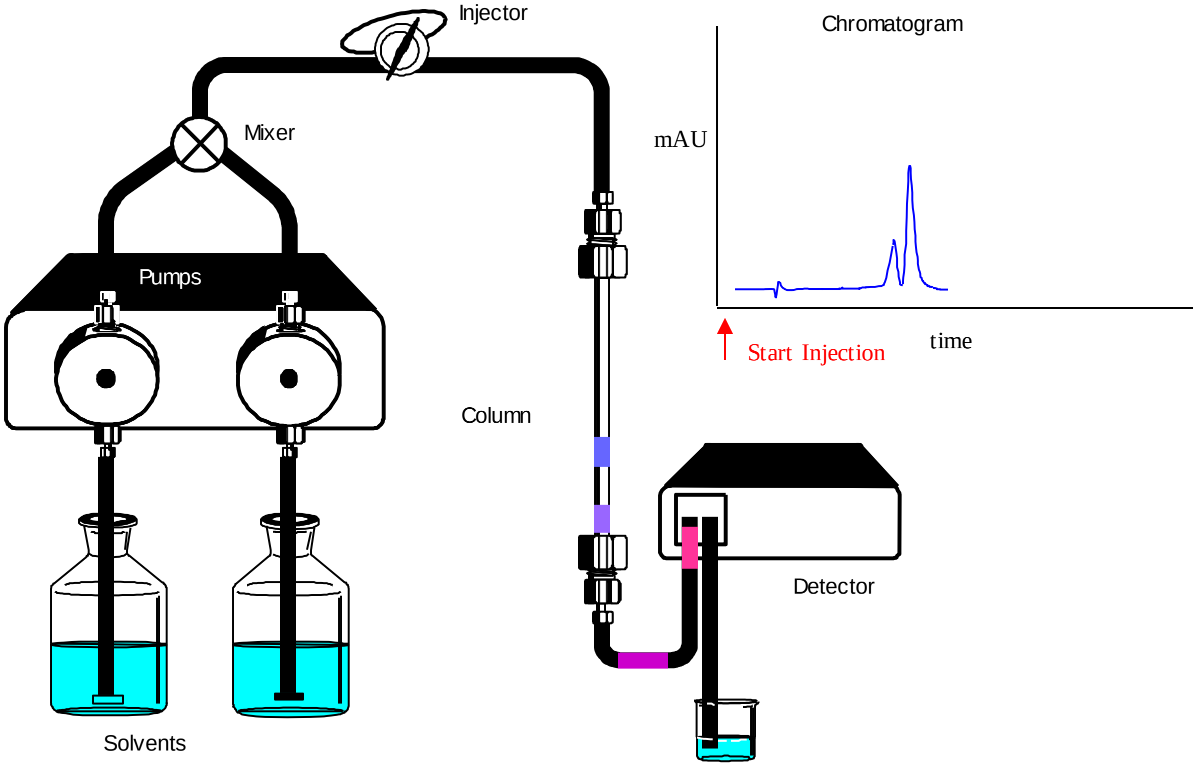

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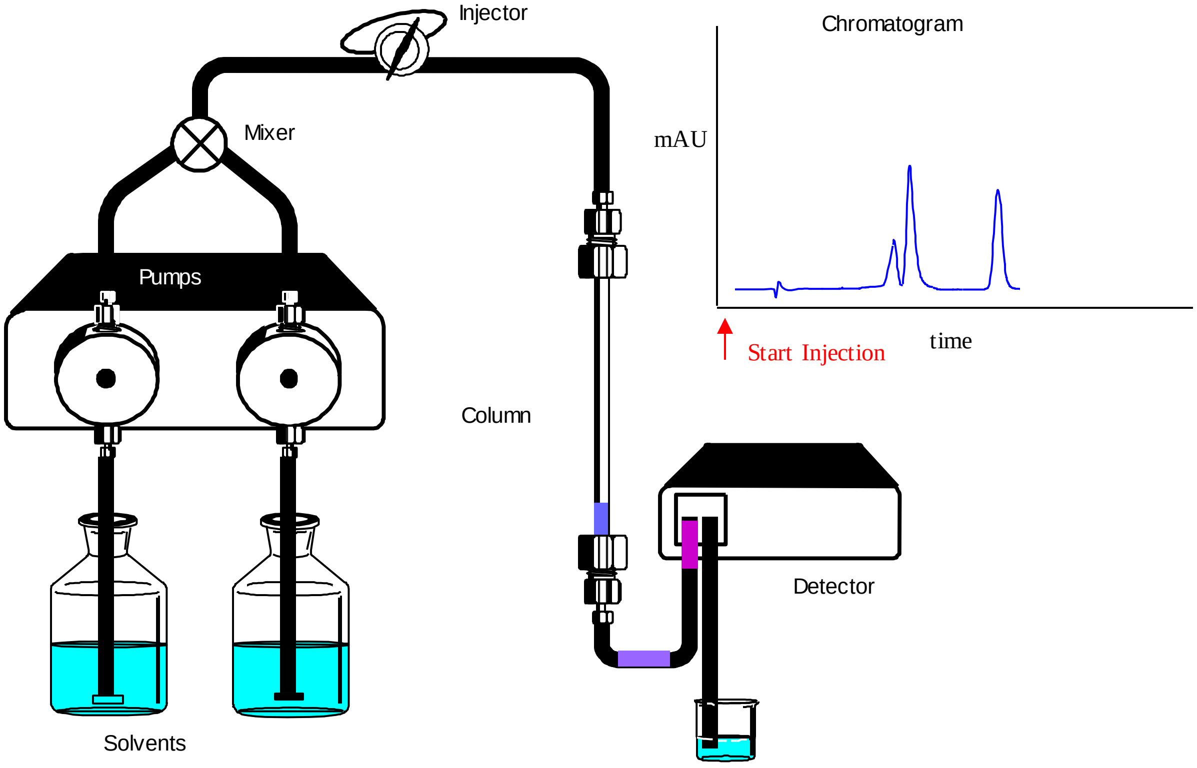

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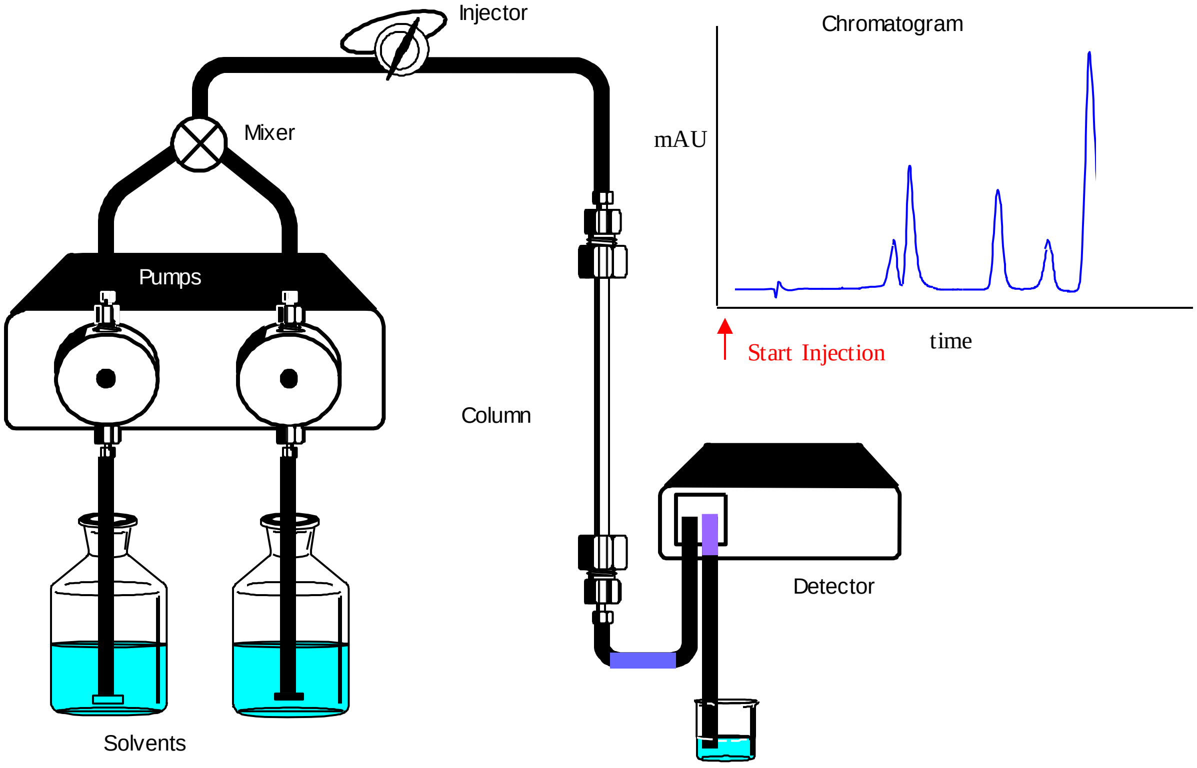

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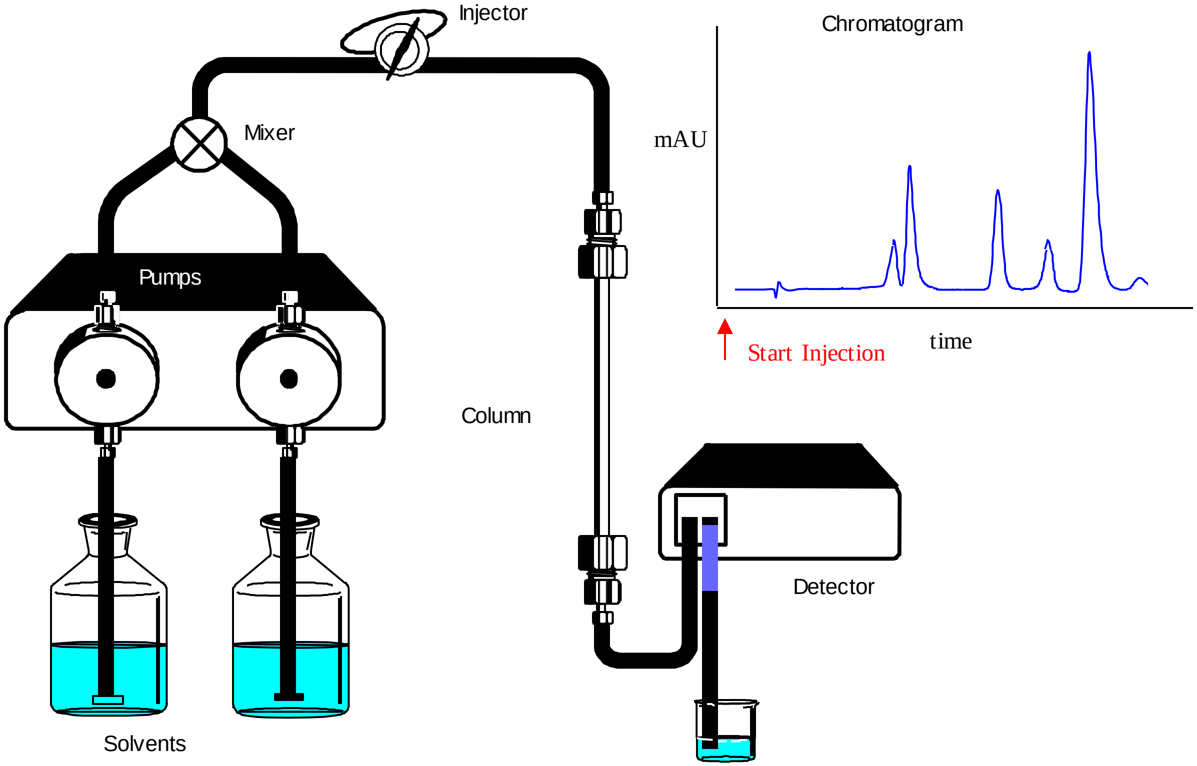

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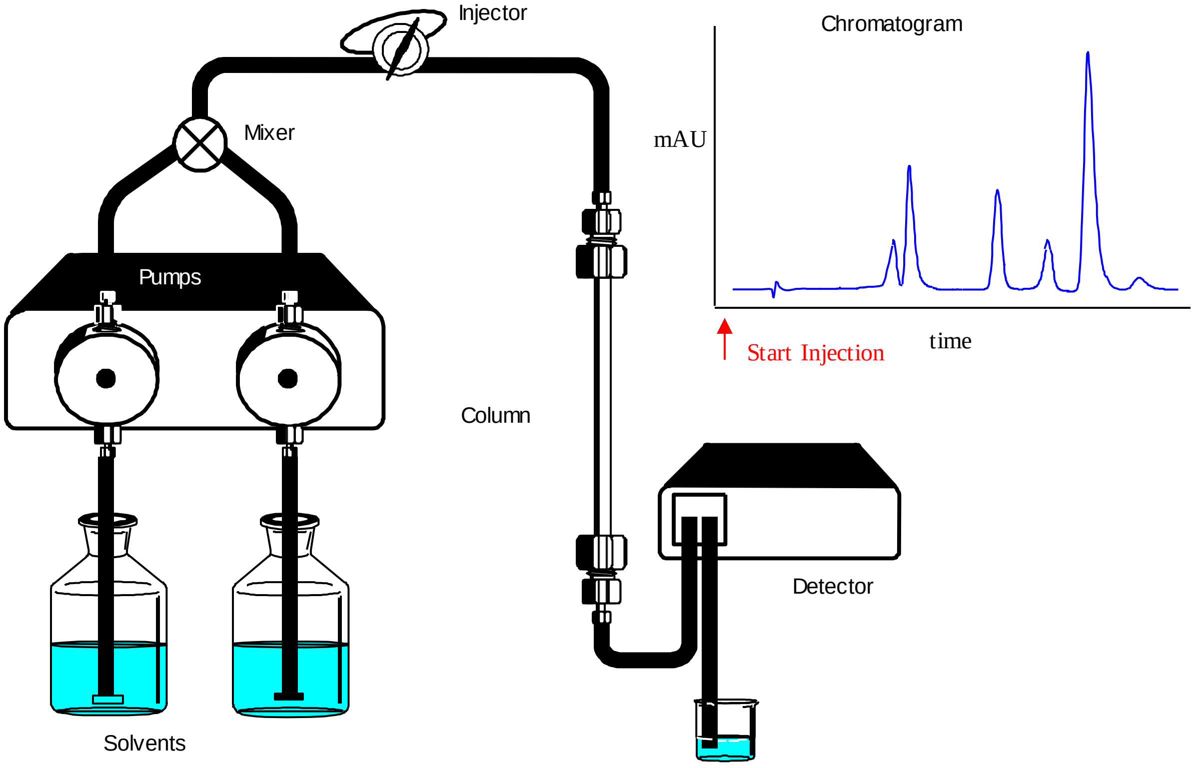
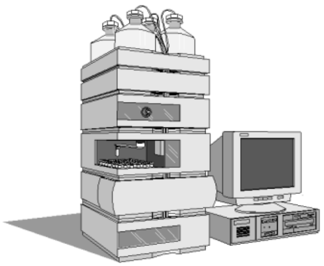
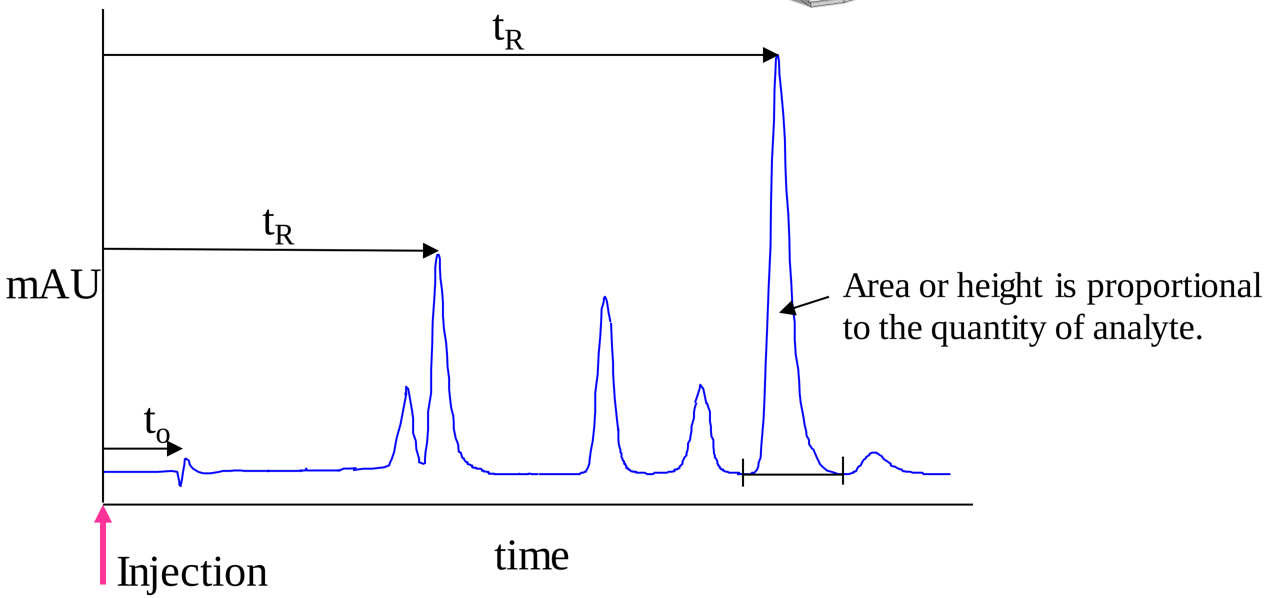


The Chromatogram



t_o - elution time of unretained peak

t_R- retention time - determines sample identity



Applications

- Monitoring materials that may jeopardize occupational safety or health
- Monitoring pesticide levels in the environment.
- To survey food and drug products,
- To identify confiscated narcotics
- To determine the amount of such chemical compounds found in new drugs in pharmaceutics

Summary

- The modern day technique is greatly enhanced in terms of selectivity, resolution, through miniaturization and the use of very elaborate stationary phases.
- Therefore HPLC is widely used for separation of molecules in biological, pharmaceutical, food, environmental and industrial process

advantages of HPLC

- Small diameter, reusable stainless steel columns
- Column packing with very small particles
- Control flow of mobile phase
- Precise sample introduction
- Good pumping system
- Special continuous flow detectors- can handle small flow rates and detect very small amounts
- Rapid analysis
- High resolution

disadvantages of HPLC

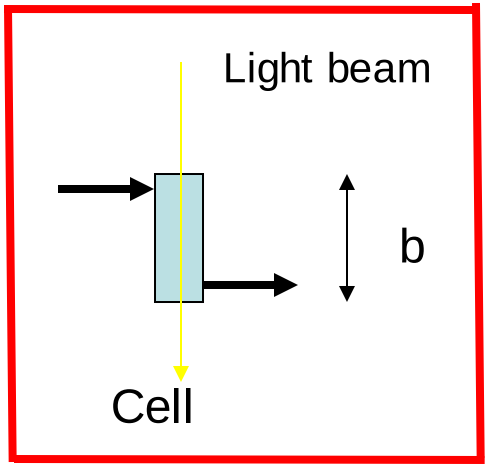
- Cost
- Complexity
- Low sensitivity for some compounds
- Irreversibly adsorbed compounds not detected
- Co-elution difficult to detect

Detectors used in HPLC

Type	Principle	Detection limit	Comments
Spectro-photometer	Measure absorbance of light	<1 ng	Analyte must absorb UV or visible light
Fluorometers	Measures fluorescence	pg to ng	Analyte must fluoresce
Electro-chemical detectors	Electrochemically measures oxidized/ reduce analyte	pg to ng	Useful for catecholamines
Mass spectrometer	Detects ions after separation by mass-to-charge ration	fg to ng	Analyte must be converted to ionized form
Refractometer	Measure change in refractive index	1 μg	Detection of most compounds but relatively low sensitivity

• UV Absorption Detectors

- The most common type of detector
- Principle: absorption of ultraviolet (or visible) light
- Follows Beer's Law: $A = -\log(I/I_o) = \varepsilon bC$
  - I = intensity of light (I_o for blank)
  - ε = molar absorptivity (constant)
  - b = path length
  - C = concentration



- Best results for $0.001 < A < 1$
- Fast response – sensitivity trade off in path length (can select cell volumes)

• UV Absorption Detectors

- Sensitivity to Compounds (ε values)
  - Best for compounds with conjugated double bonds, aromatic groups or strongly absorbing functional groups (e.g. R-NO_2, R-I, R-Br)
  - Poor response for compounds with few or weakly absorbing functional groups (worst for R-CN, R-NH_2, R-F; poor for R-OR', R-OH, R-COOH, R-COOR')
- Solvents:
  - Requires use of solvents that absorb poorly in UV

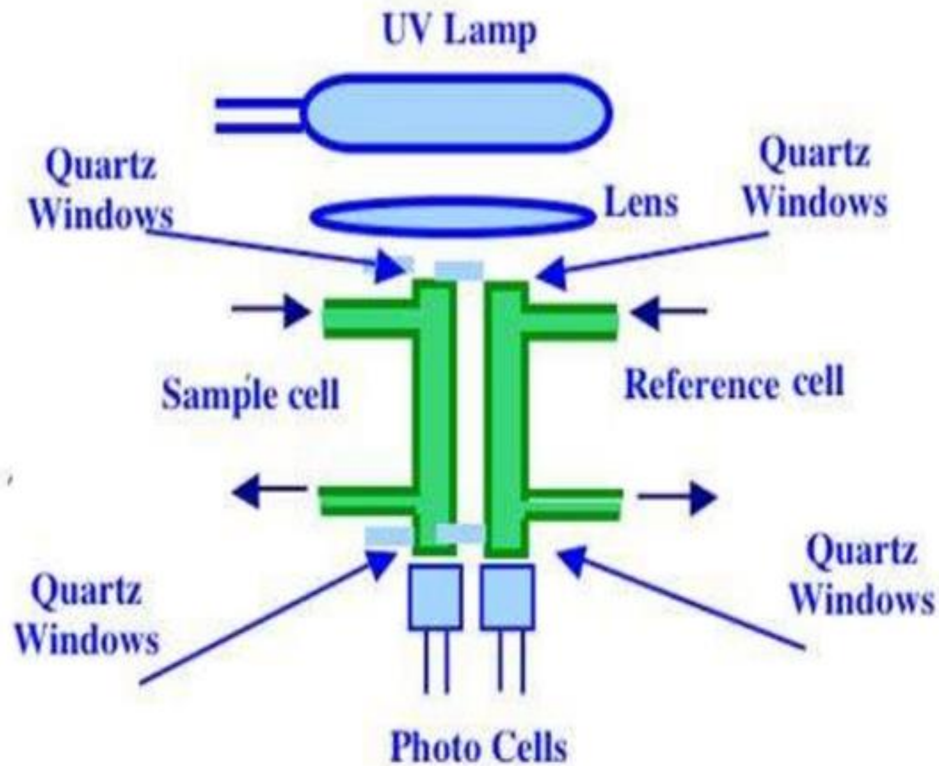
Absorbance detectors

UV/Visible detectors

- Solute property detector
- Three types
  - Fixed wavelength detector
  - Variable wavelength detector
  - Diode array detector

Fixed Wavelength detector

- 254nm
- Higher detection capacity.
- Hg vapour lamp(discharge lamp)
- Focus of light through two absorption cells.
- Volume of cell is kept constant.

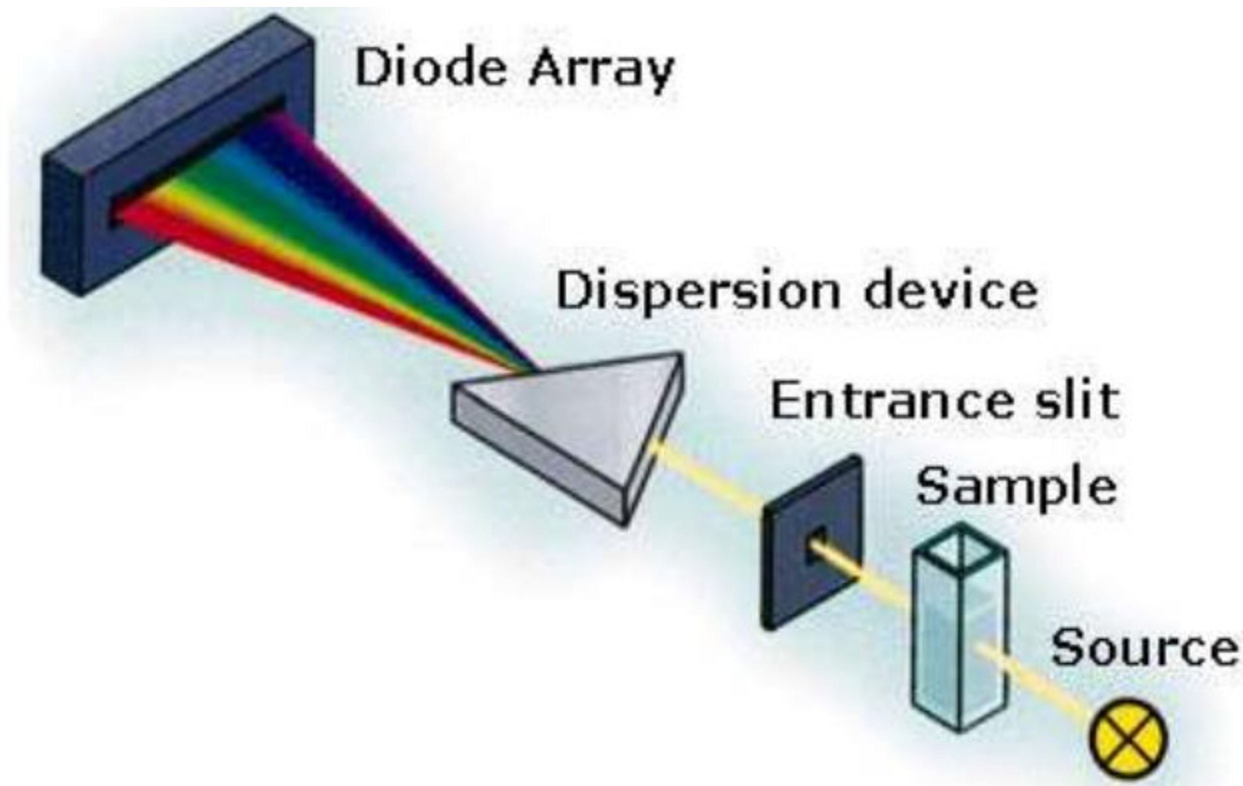


Variable Wavelength Detectors

- Relatively wide band pass UV-Visible spectrophotometer coupled to a chromatographic system.
- Offers a wide selection of UV & Visible wavelengths with increased cost.
- For complete spectrum, eluent flow must be stopped to trap the component of interest in the detector cell.

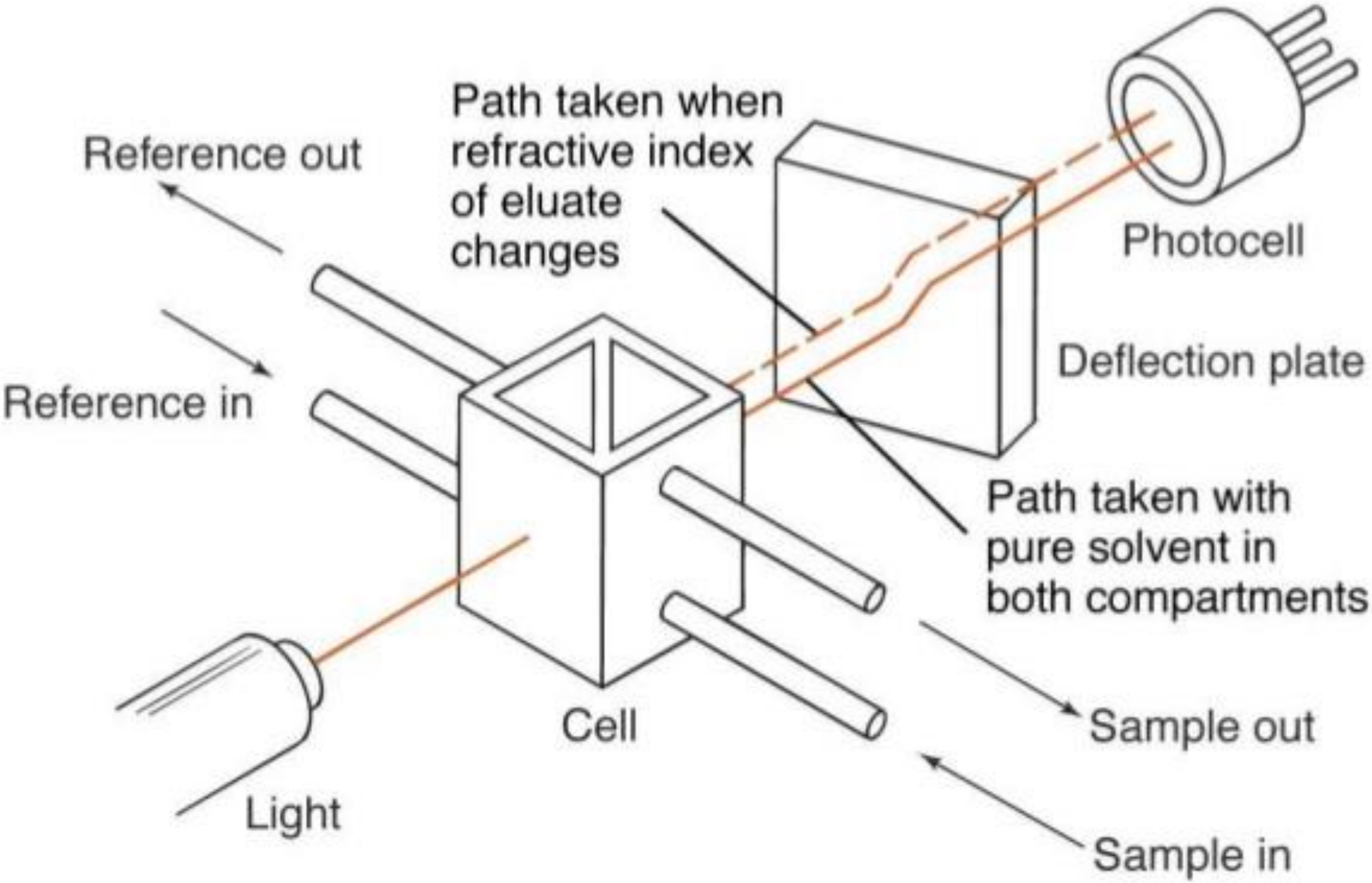
Diode Array Detector

- Scanning Wavelength Detector
- Required to obtain a real time spectrum of each solute as it elutes
- Work in parallel, monitoring all wavelength
- Xenon lamp
- Complete development of chromatogram



• Refractive Index Detectors

- Principle:
  - liquids with different refractive index will diffract light differently
  - Composition will determine refractive index
  - Any compound with a refractive index different than the solvent's is detectable
- Advantage:
  - Most universal detector (can detect weakly absorbing compounds)
- Disadvantages:
  - Gradients are not possible
  - Requires thermal stability
  - Generally not very sensitive


Path taken when
refractive index
of eluate
changes
Reference out
Reference in
Photocell
Deflection plate
Path taken with
pure solvent in
both compartments
Cell
Light
Sample out
Sample in

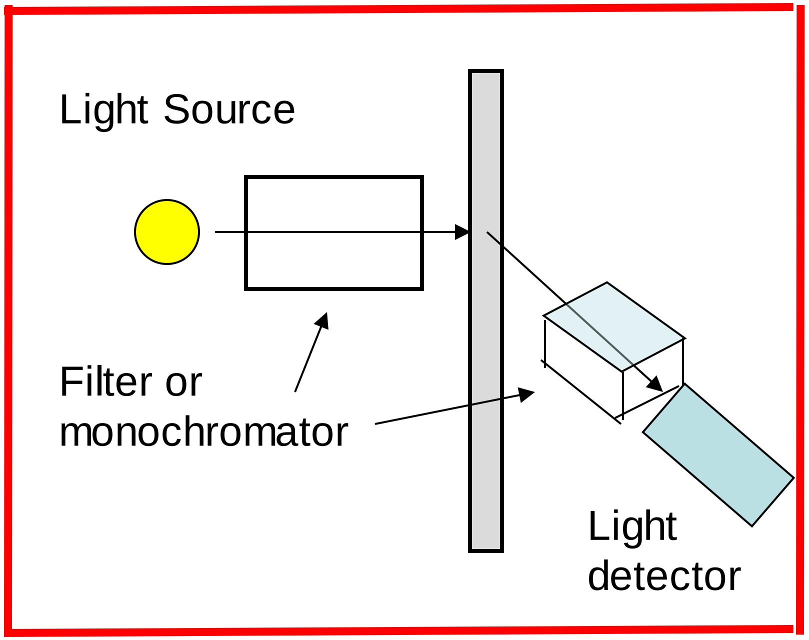
• Fluorescence Detectors

$$M + h\nu \rightarrow M^*$$

$$M^* \rightarrow M^{*\prime} \text{ (lower vibrational level)}$$

$$M^{*\prime} \rightarrow M + h\nu'$$

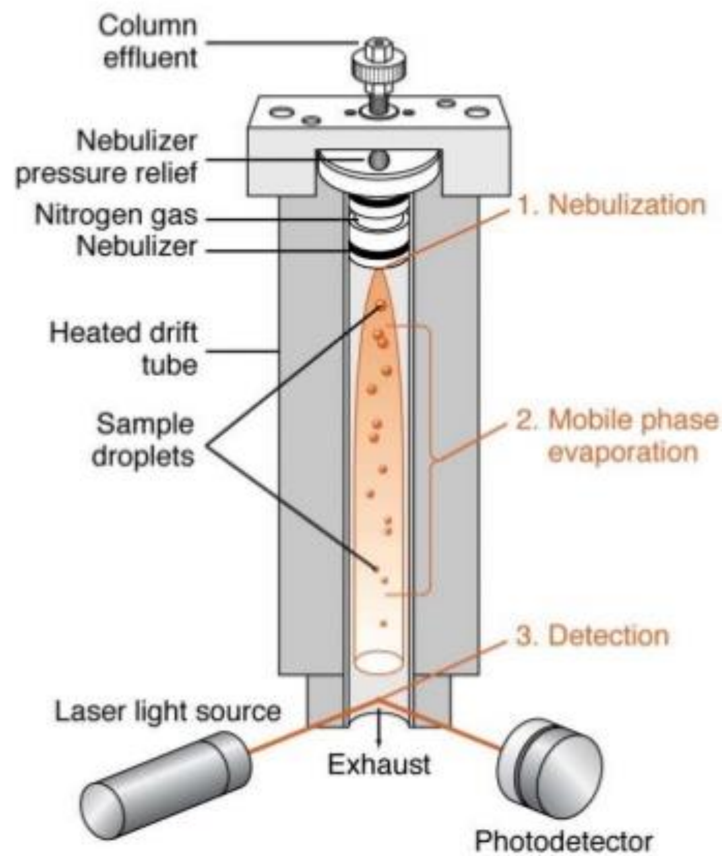
- Detection Principle:
  - Light promotes molecules to excited electronic state
  - Excited molecules transition from lowest excited state back to the ground state and emit light in the process
- Equipment:
  - High intensity light source
  - Filters or monochromators to select wavelengths (before and after cell)
  - Sensitive light detector



- Advantages:
  - Greater sensitivity possible (for molecules with high fluorescence efficiencies) because easy to detect small signal against zero background (see below)
  - Much greater selectivity because few molecules fluoresce, particularly at selected wavelengths
- Disadvantages:
  - Limited to relatively few molecules (although derivatization is also possible)

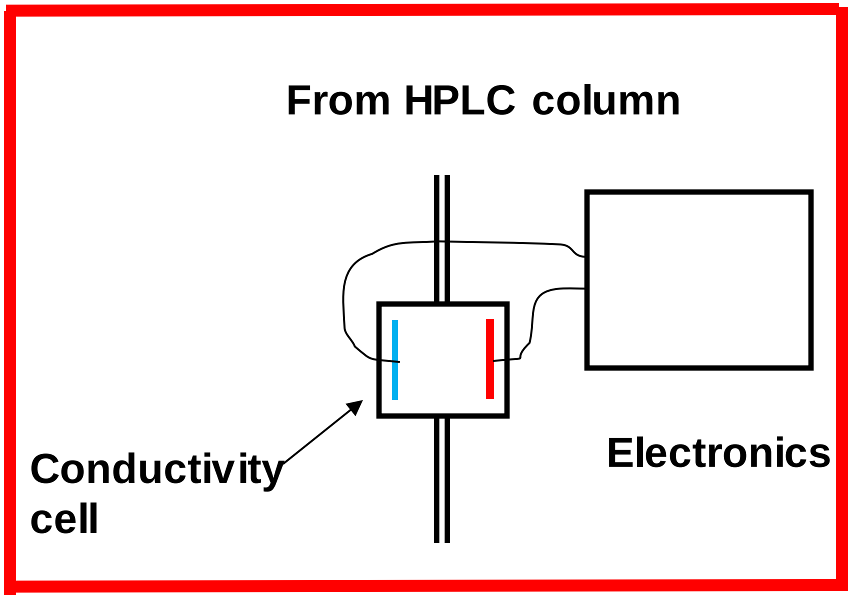
- ELSD (Evaporative Light Scattering Detector)

- Universal, destructive
- Useful for large molecules and wide linear range.
- Analytes are de-solvated in the detector.
- Molecules pass through a large cuvette for a UV-VIS instrument.
- The reduction in light intensity detected (due to scattering by the analytes) is measured.



• Ion Exchange Chromatography

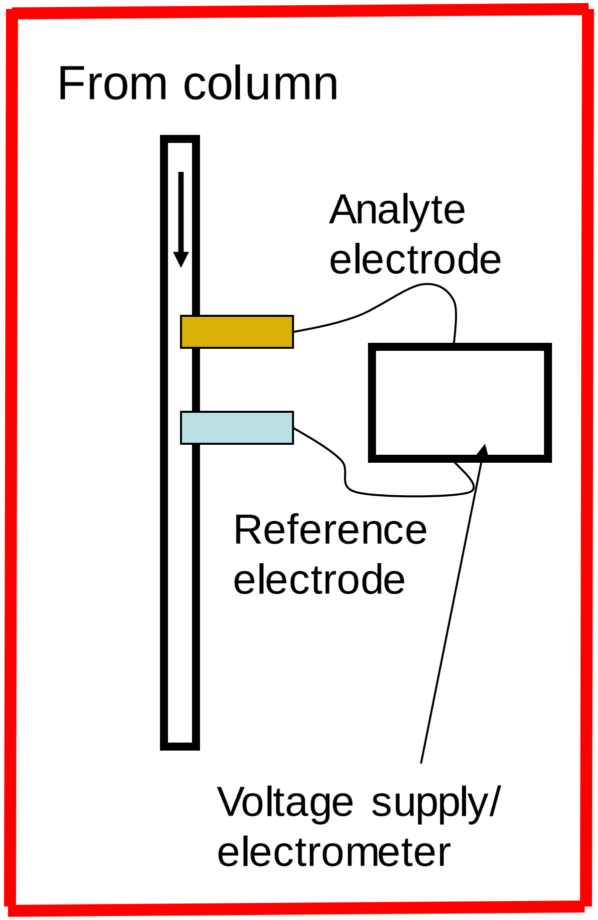
- Types of Instruments:
  - Single column
  - With analytical plus suppressor columns
- Detection in Single Column Instruments
  - Other detection methods (fairly common)
  - Conductivity detection
- Conductivity Detector
  - Resistance measured (AC circuit)
  - Conductivity = 1/(resistance)
  - Ions in solution create conductance
  - Conductivity depends on ion concentration and size



• Electrochemical Detectors

– Principle:

- Redox reactions occur at electrodes following column
- Potential cycle used to periodically oxidize/reduce analytes at electrode
- Current depends on concentration of analyte being reduced or oxidized (similar to A in UV detector)
- Electrode potential determines classes of compounds that are detectable (similar to λ in UV detector)



• Mass Spectrometry

Bonds in **cation radicals** begin to break (fragment)

$M^{+\cdot} \longrightarrow M^{+} + M^{\cdot}$

Cation radical — fragment

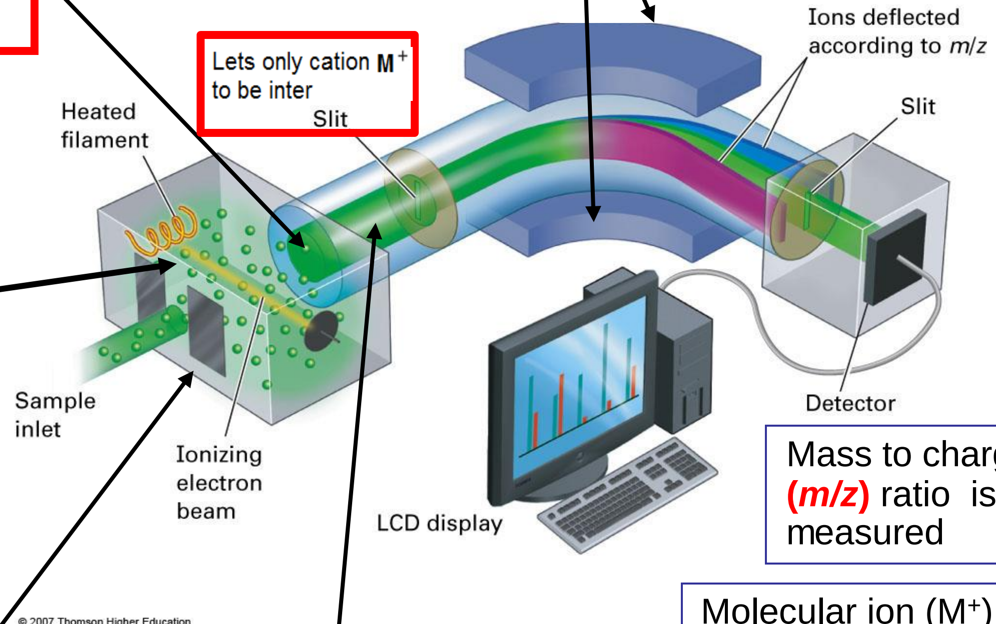
Magnet

Lets only cation M^{+} to be inter

Sample vaporized and subjected to bombardment by electrons that remove an electron creating a cation radical

$M \xrightarrow{e^{-}} M^{+\cdot} + e^{-}$

Organic molecule — Cation radical



Mass to charge (***m/z***) ratio is measured

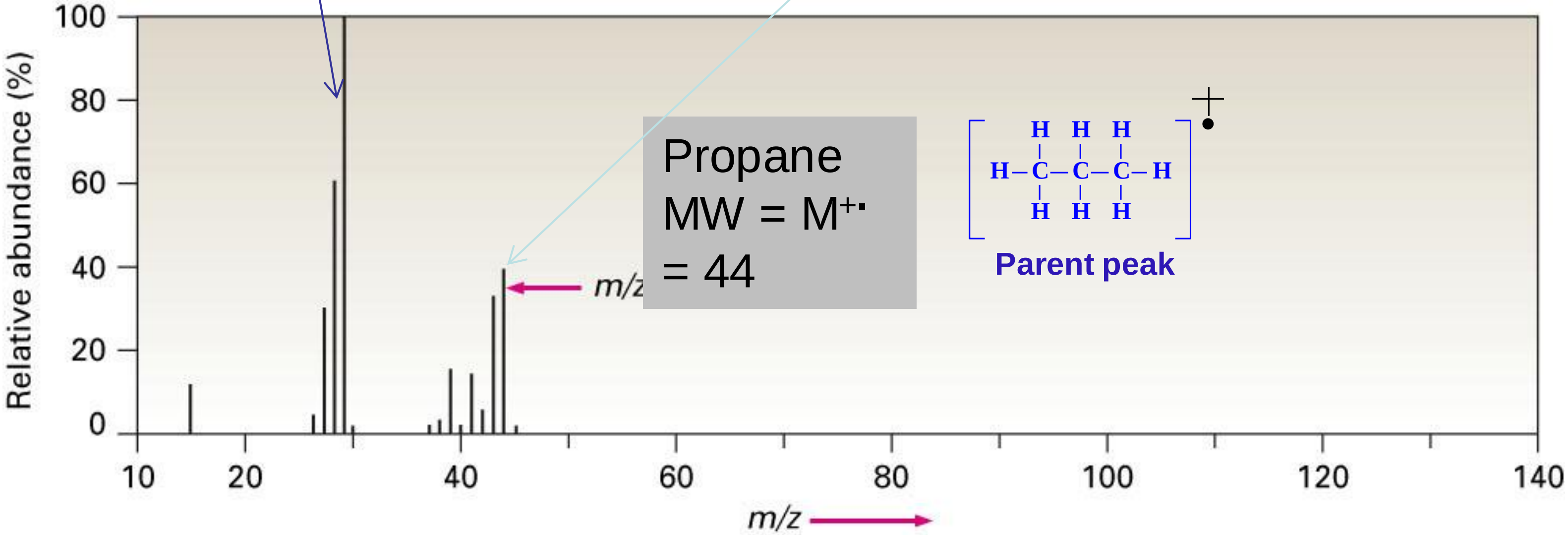
Molecular ion (M^{+}) shows **molecular weight**

Acceleration

Ionization unit

The Mass Spectrum

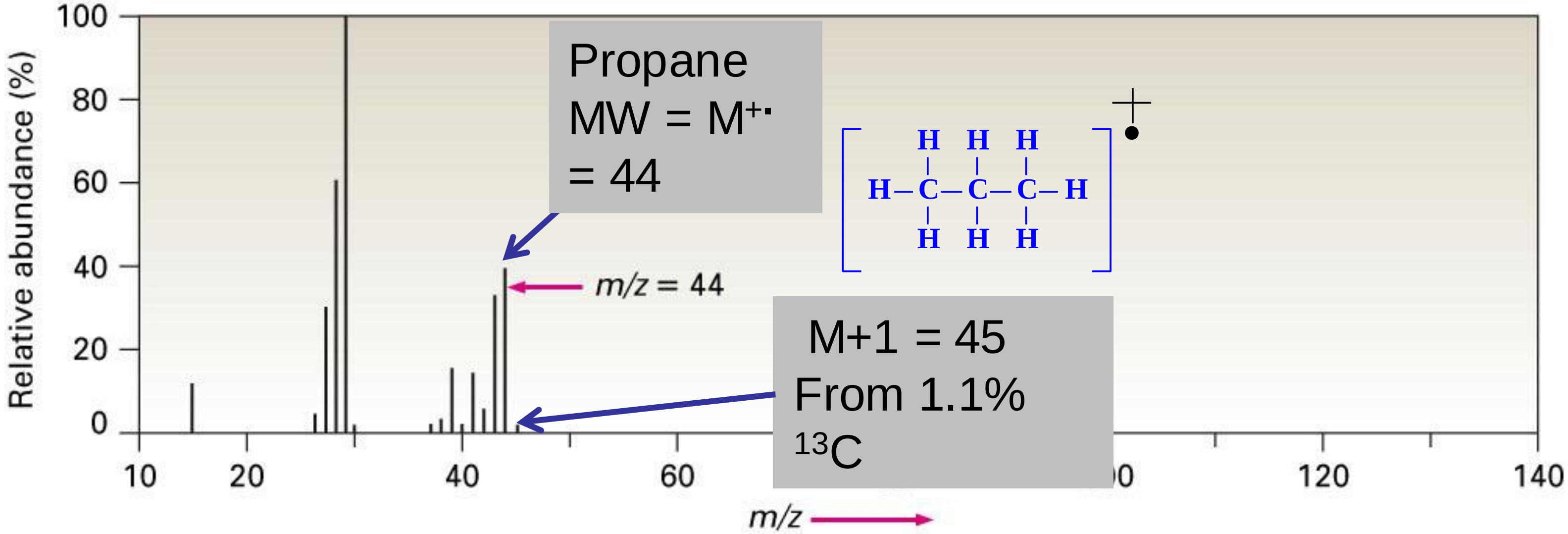
- Plot mass of ions **(m/z) (x-axis)** versus the intensity of the signal (roughly corresponding to the **number of ions) (y-axis)**
- Tallest peak is **base peak** (100%)
  - Other peaks listed as the % of that peak
- Peak that corresponds to the unfragmented **radical cation $[M]^{+\cdot}$ is parent peak**



Determining the molecular formula
M and M+n peak :

Peaks above the molecular weight appear as a result of naturally occurring heavier isotopes in the sample

- M ^{12}C (98.9%) and (M+1) from (1.1%) of ^{13}C in nature
- (M and M+2) in (75.8%) /(24.2%) ratio = ^{35}Cl and ^{37}Cl
- (M and M+2) in (50.7%) /(49.3%) ratio = ^{79}Br and ^{81}Br



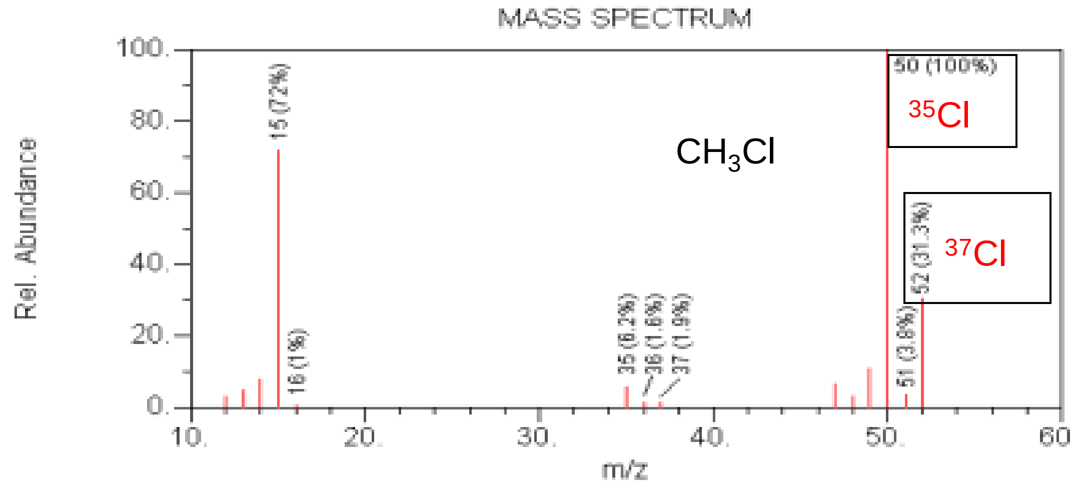
Natural abundances of Isotopes of some common elements

Element	Major Isotope	RA	M + 1 Isotope	RA	M + 2 Isotope	RA
Hydrogen	^{1}H	100				
Carbon	^{12}C	98.9	^{13}C	1.1		
Nitrogen	^{14}N	99.6	^{15}N	0.4		
Oxygen	^{16}O	99.8			^{18}O	0.2
Fluorine	^{19}F	100				
Sulfur	^{32}S	94.8	^{33}S	0.8	^{34}S	4.4
Chlorine	^{35}Cl	75.8			^{37}Cl	24.2
Bromine	^{79}Br	50.7			^{81}Br	49.3
Iodine	^{127}I	100				

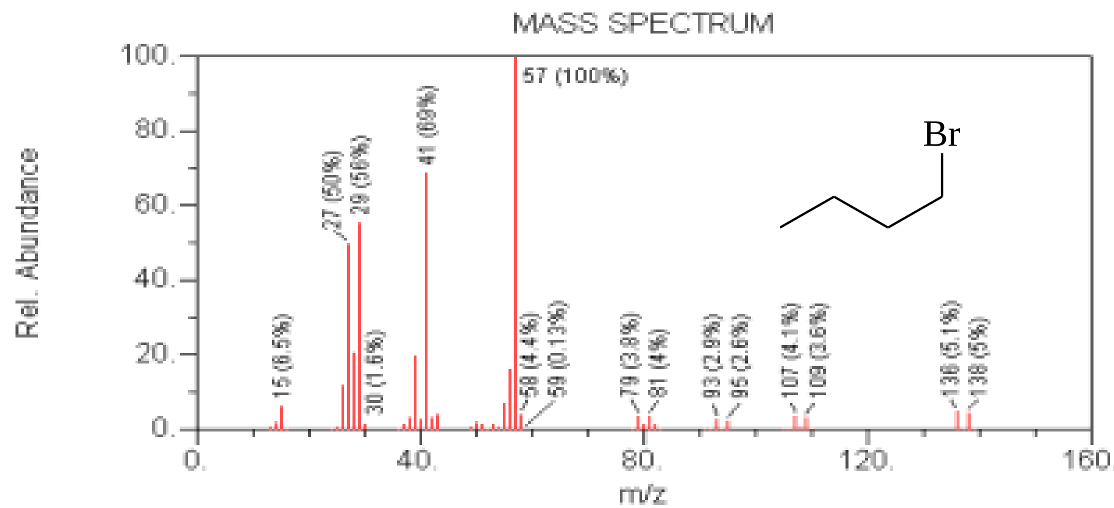
The relative abundance (RA) of the most abundant isotope is listed as 100, and the abundances of the other isotopes are listed relative to that number. The M + 1 isotope is the one that is responsible for the peak at *m/z* one unit higher than the peak for $M^{+\cdot}$.

M^+ peak: Halides

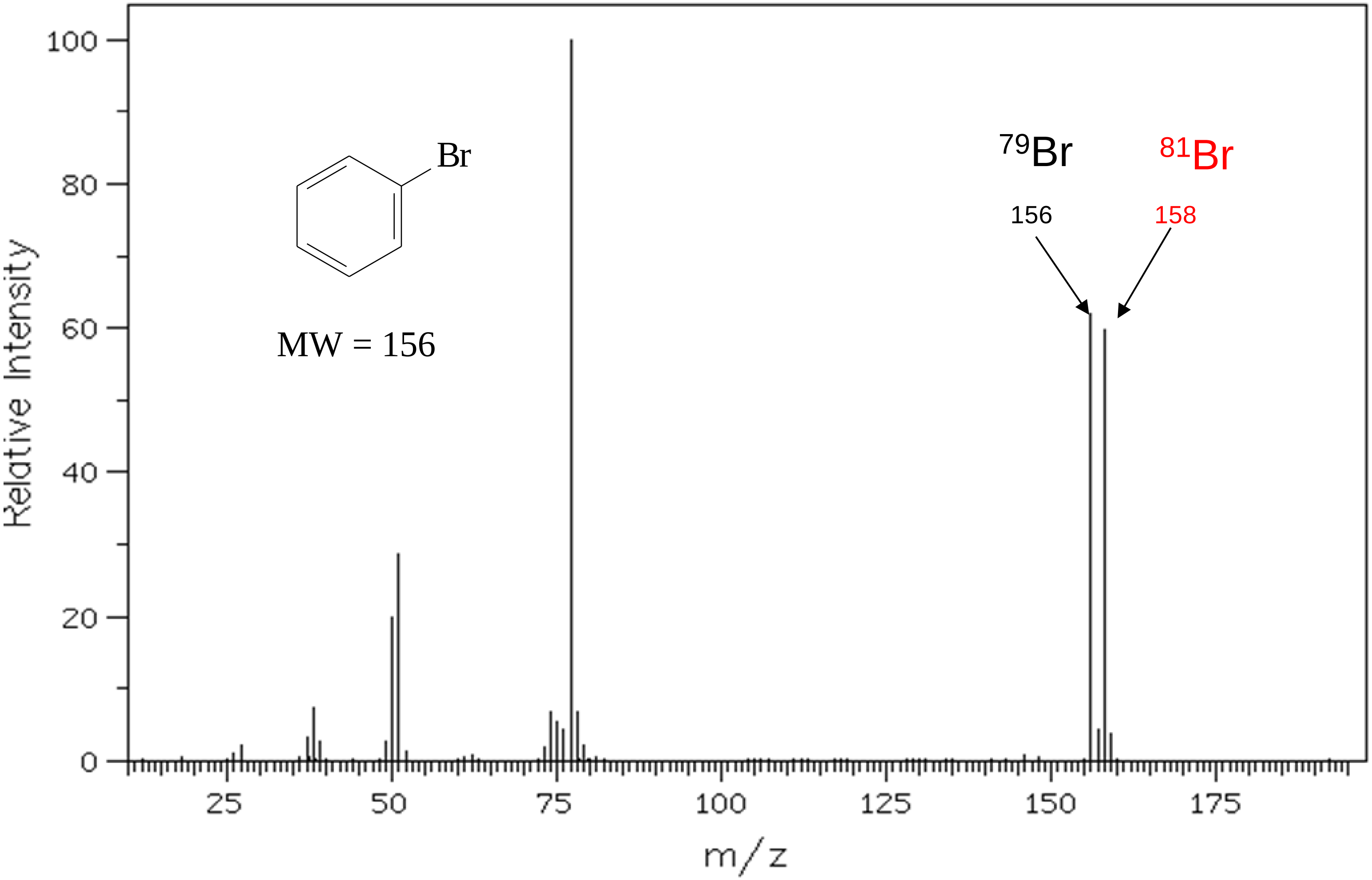
M^+ and M+2 in 75.8%:
24.2% (~ 3:1) ratio
= ^{35}Cl and ^{37}Cl



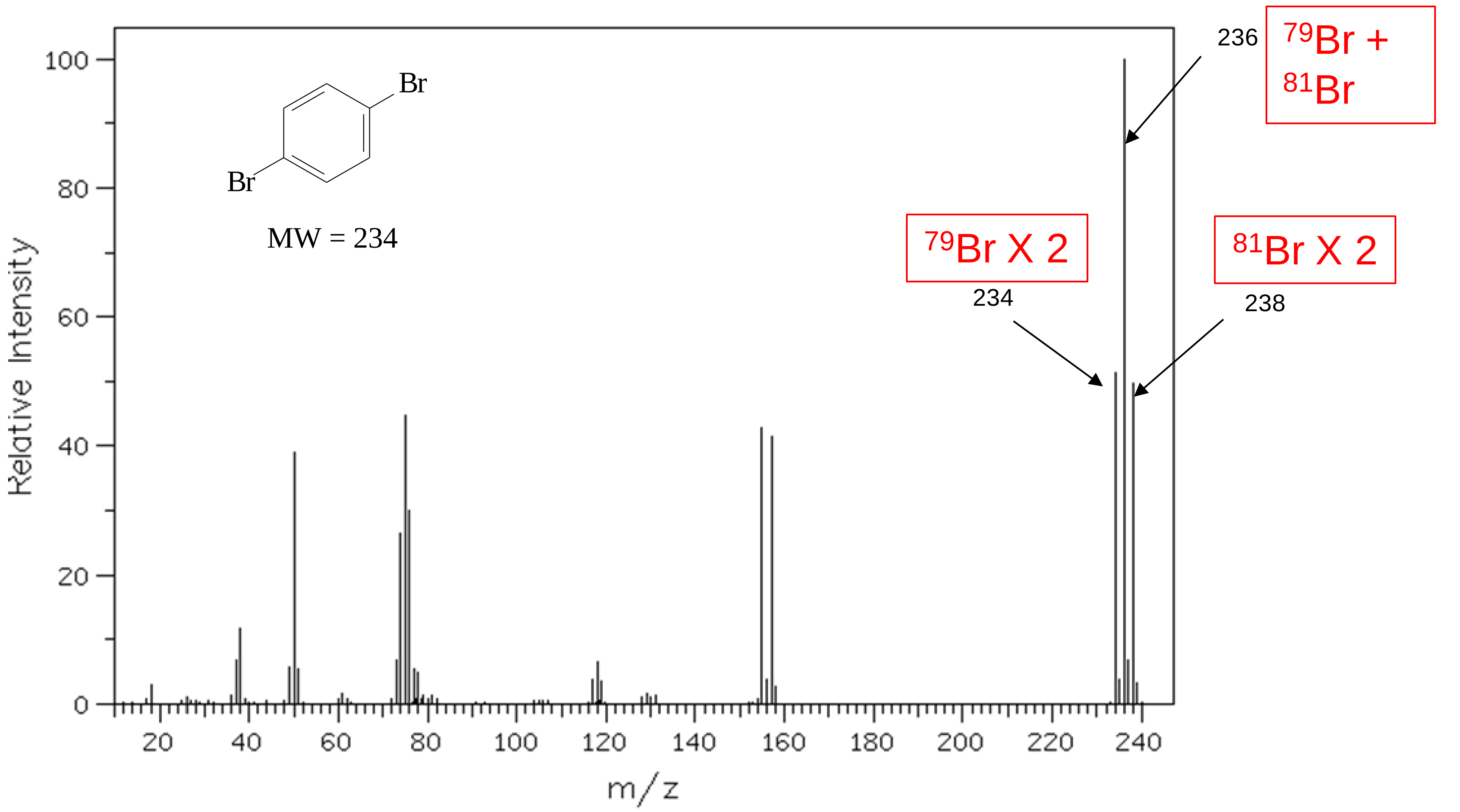
M^+ and M+2 in 50.7%:
49.3% (~ 1:1) ratio
= ^{79}Br and ^{81}Br



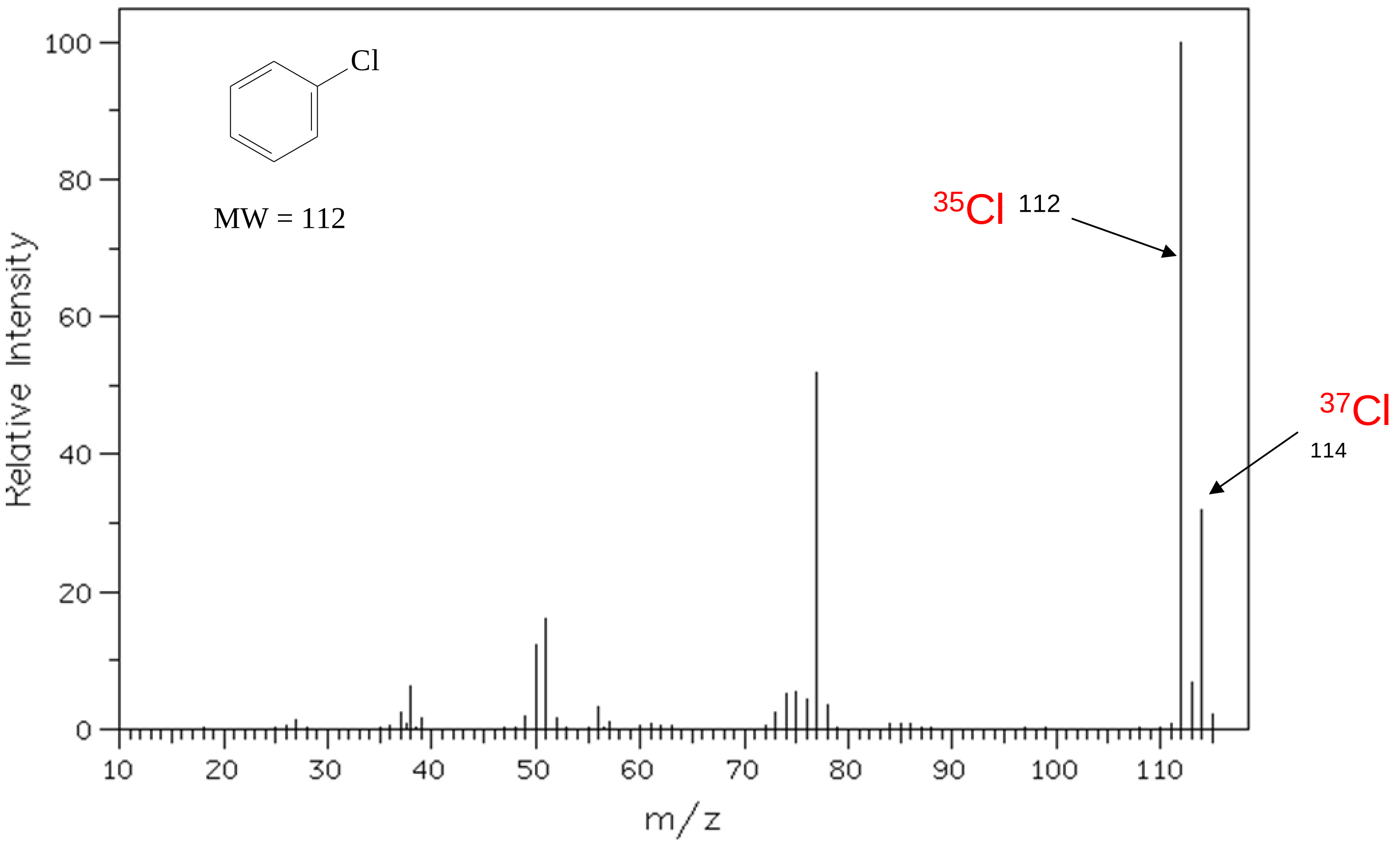
Determining the molecular formula



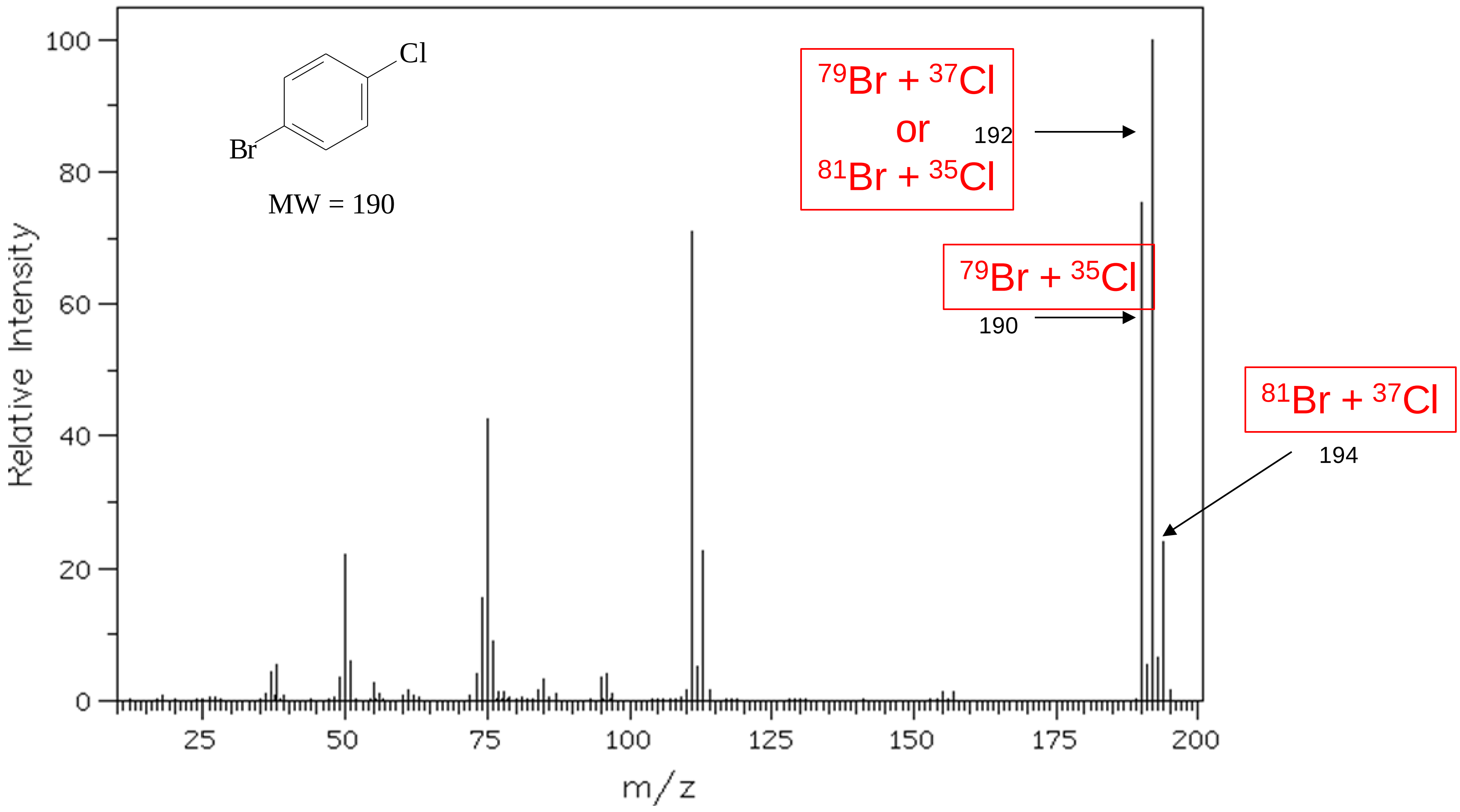
Determining the molecu



Determining the molecular formula



Determining the molecular formula



Determining the molecular formula