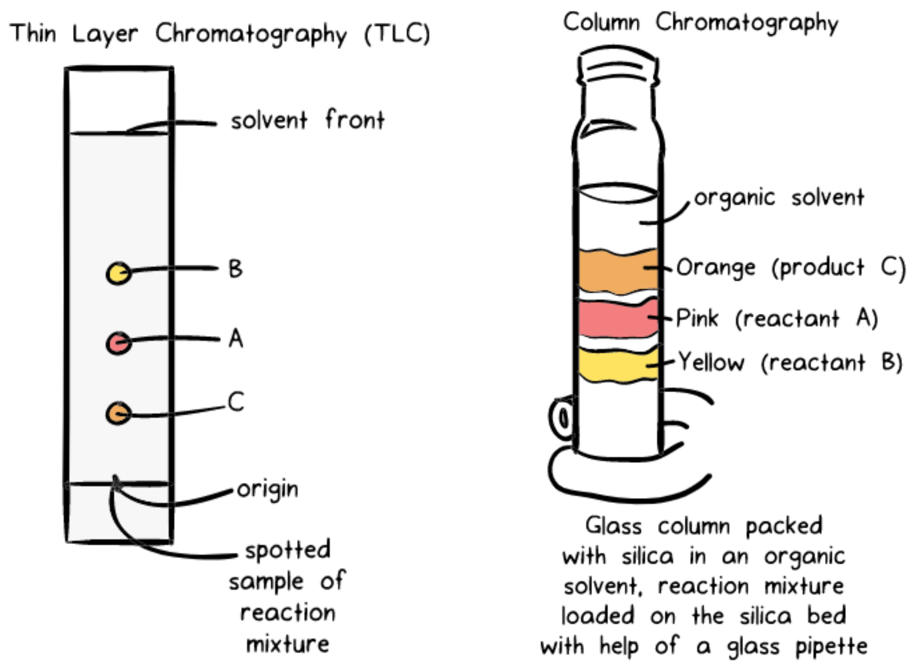
Experiment 8

Chromatography (HPLC)

INTRODUCTION

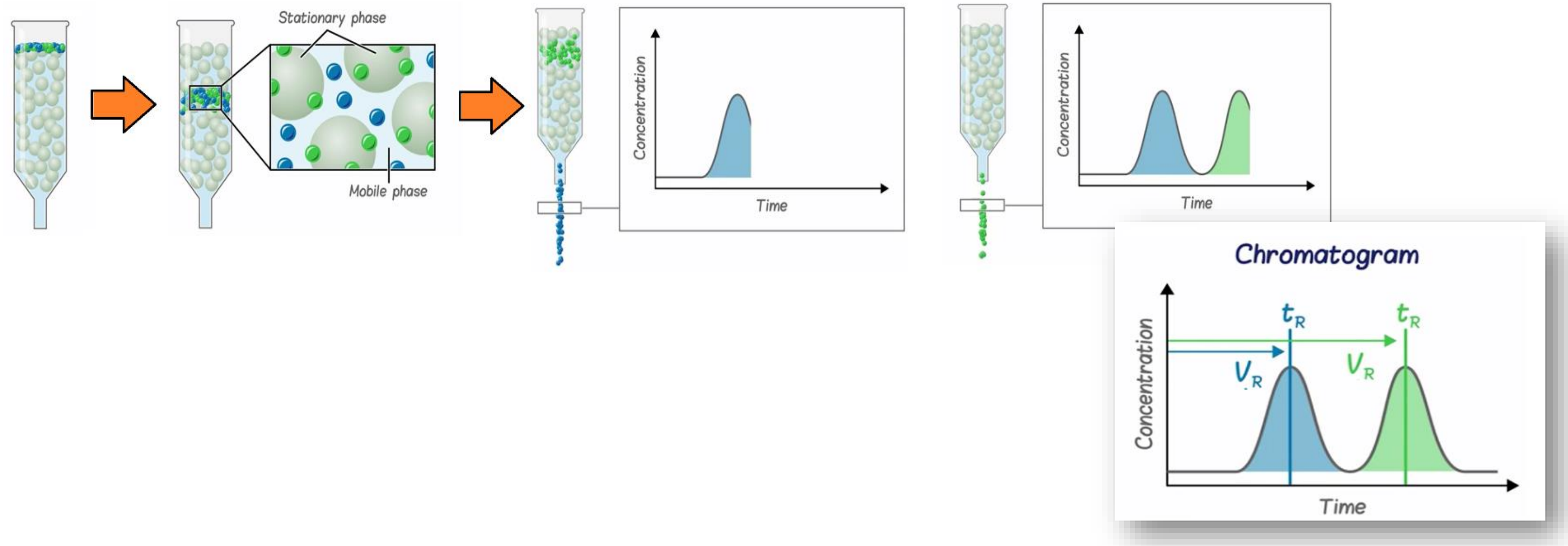
Chromatography is an analytical technique **for separation** of a complex **mixture** of chemical substances **into its individual components**, so that the individual components can be thoroughly analyzed.

- Its principle relies on the **differential affinities** of the individual substances for a gas or liquid **mobile medium** and for a solid or liquid **stationary medium** through which they pass. So, the components of the mixture will distribute unequally between the two immiscible phases.



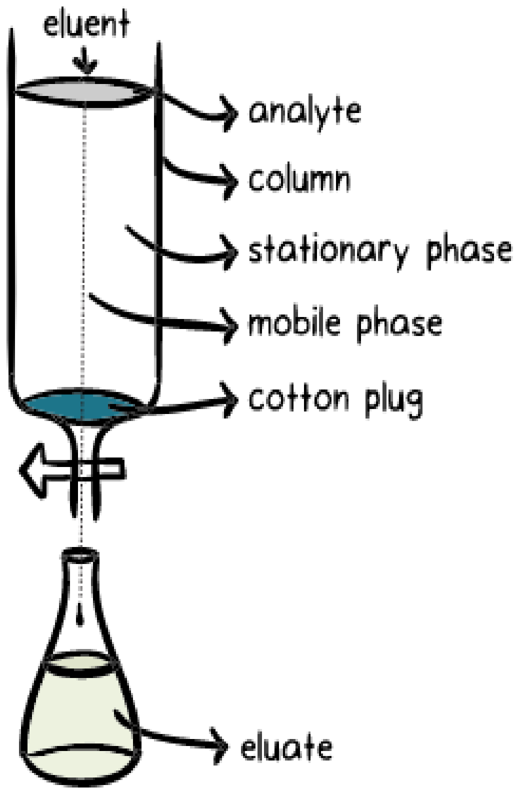
Purpose of Chromatography

- **Analytical:** determine chemical composition of a sample (qualitatively and then quantitively).
- **Preparative:** purify and collect one or more components of a sample.



TERMS RELATED TO CHROMATOGRAPHY

- **Analyte:** The mixture of substances that are to be separated and analyzed during chromatography.
- **Mobile Phase:** Liquid or gaseous solvent that flows over or through the stationary phase in a definite direction through the column.
- **Stationary Phase:** Solid or liquid or a mixture of solid/liquid medium which is immobilized (fixed) on the support particles or on the inner wall of the column tubing.
- **Eluent:** Fluid entering the column consist of mobile phase & analyte.
- **Effluent (Eluate):** Fluid leaving the column.



Applications for Chromatography

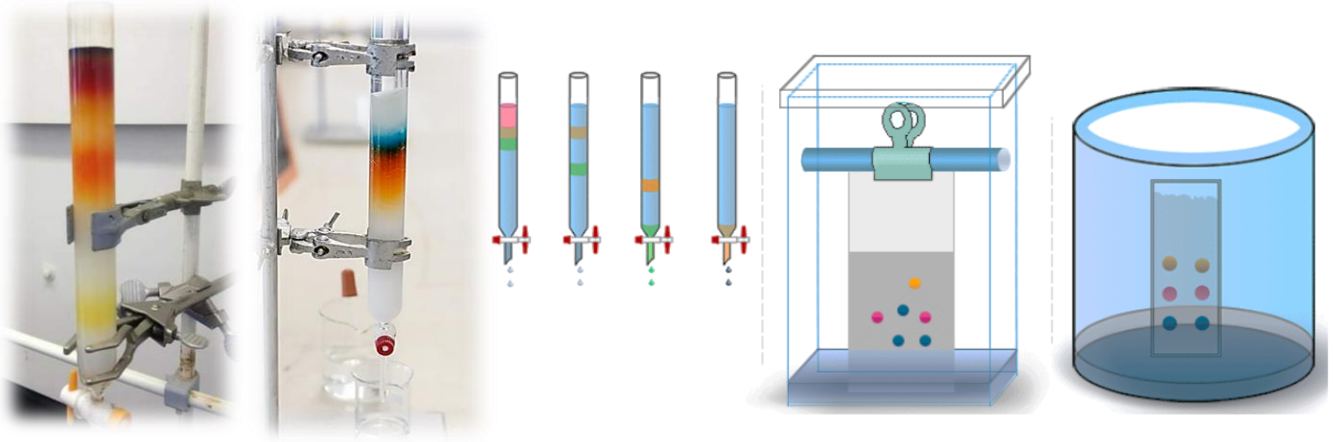
- **Pharmaceutical Company** – determine amount of each chemical found in new product
- **Hospital** – detect blood or alcohol levels in a patient's blood stream
- **Law Enforcement** – to compare a sample found at a crime scene to samples from suspects
- **Environmental Agency** – determine the level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

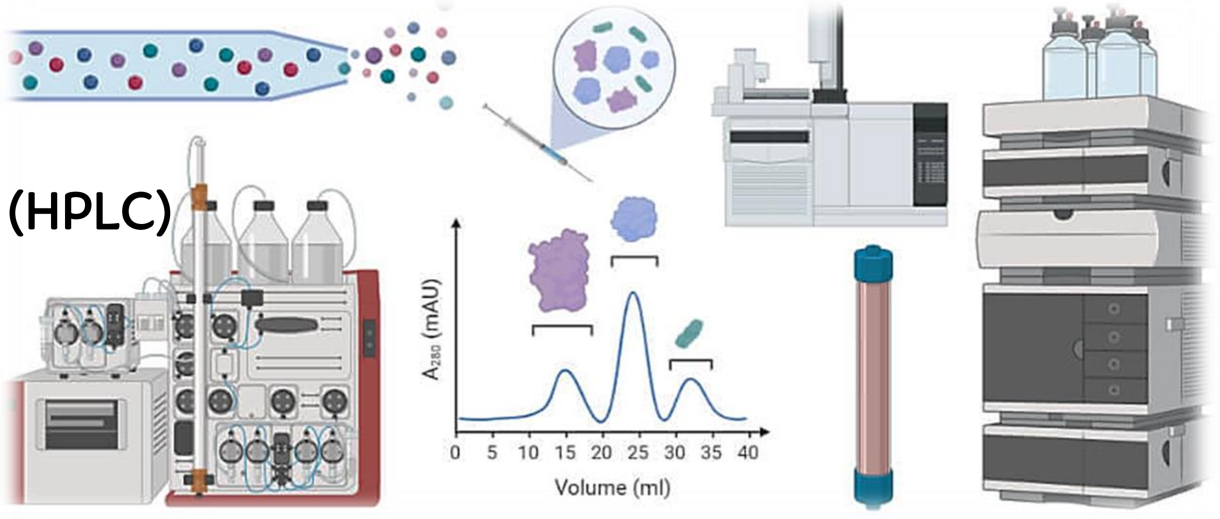
Classification of Chromatography

- **Separation Method** (Attractive Forces)
  - Adsorption Chromatography
  - Partition Chromatography
  - Exclusion Chromatography
  - Ion Exchange Chromatography
  - Affinity Chromatography
- **Mobile Phase**
  - Liquid Chromatography
  - Gas Chromatography
- **Stationary Phase**
  - Solid Chromatography
  - Liquid Chromatography

Types of Chromatography Methods

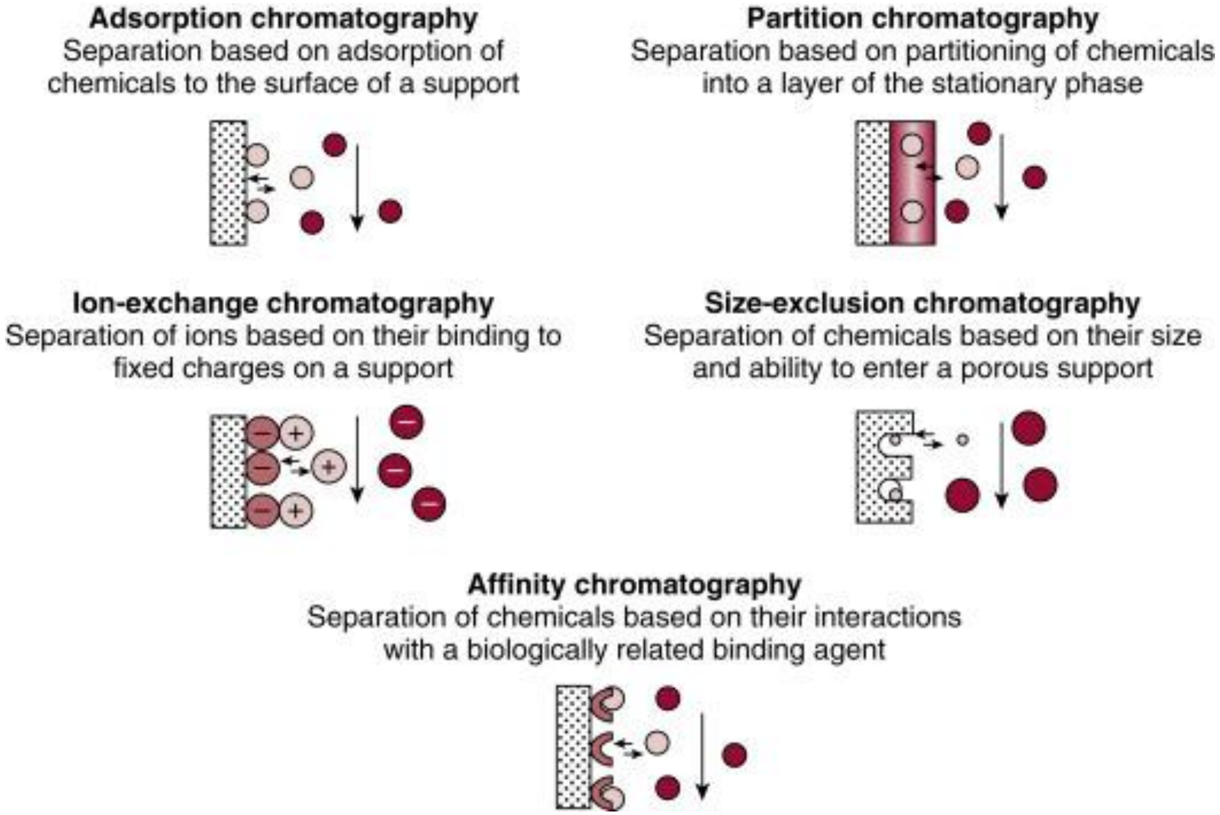
- Paper Chromatography
- Thin Layer Chromatography (TLC)
- Gas Chromatography (GC)
- Column Chromatography
- Liquid Chromatography (LC)
- High Performance Liquid Chromatography (HPLC)



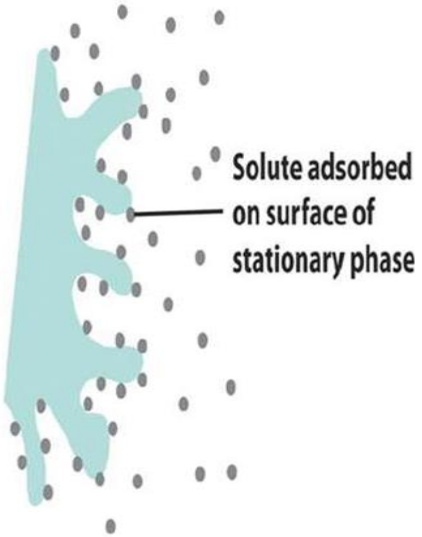


Classification based on Attractive Forces

1. Adsorption chromatography
2. Ion Exchange chromatography
3. Partition chromatography
4. Size Exclusion chromatography
5. Affinity chromatography



1. Adsorption Chromatography

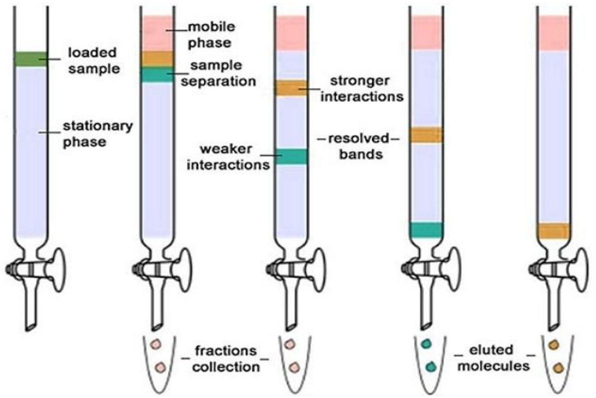


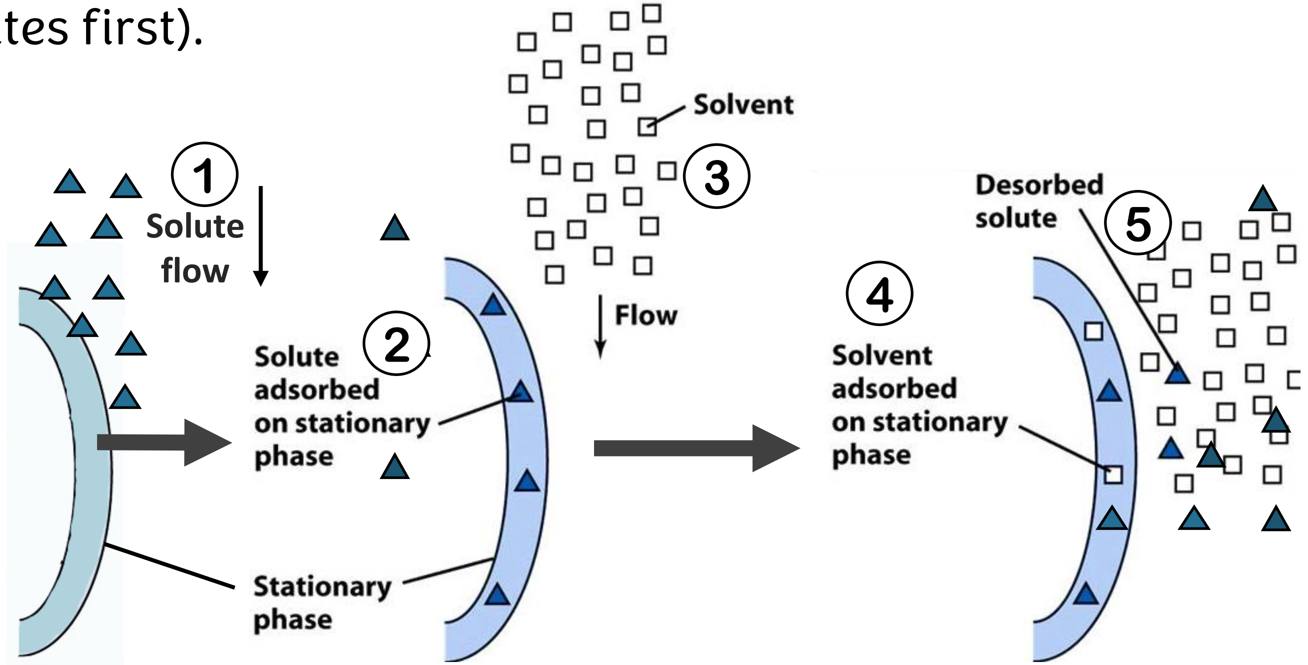
Separation based on their adsorption onto the surface of solid (stationary phase). Based on different non-covalent interactions (H-bond, ionic, hydrophobic, vander waal…).

- **Normal phase-separation: Polar analytes** → adsorbs strongly → Polar stationary phase (ex: Silica or Alumina) → Nonpolar mobile phase
- **Reverse phase-separation: Non-polar analytes**→ adsorbs strongly → Non-polar stationary phase (carbon chain bonded to silica) → Polar mobile phase

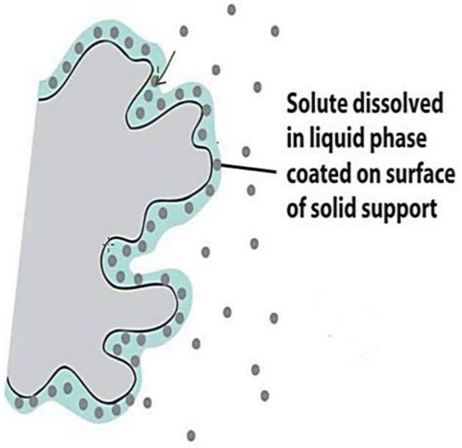
Degree of adsorption (less tightly bound → elutes first).

Ex: Column chromatography CC, TLC, HPLC





2. Partition Chromatography

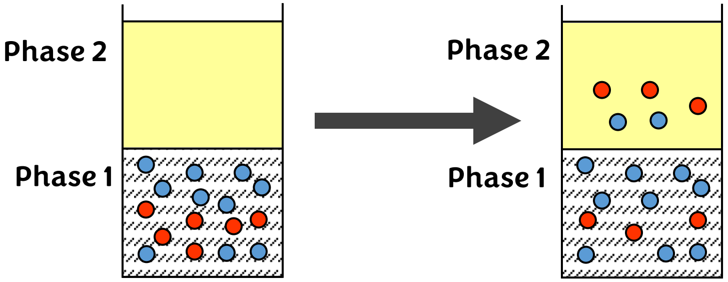


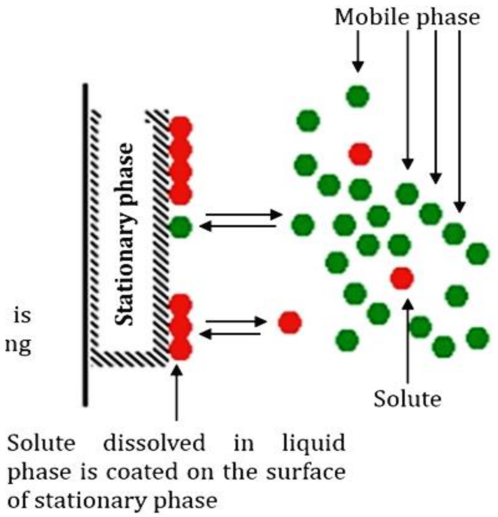
Solute are separated based on their partition (K_d) between a liquid mobile phase and a liquid stationary phase coated on a solid support.

$K_d \rightarrow$ ratio of concentration of solute in two phases (Solubility differences).

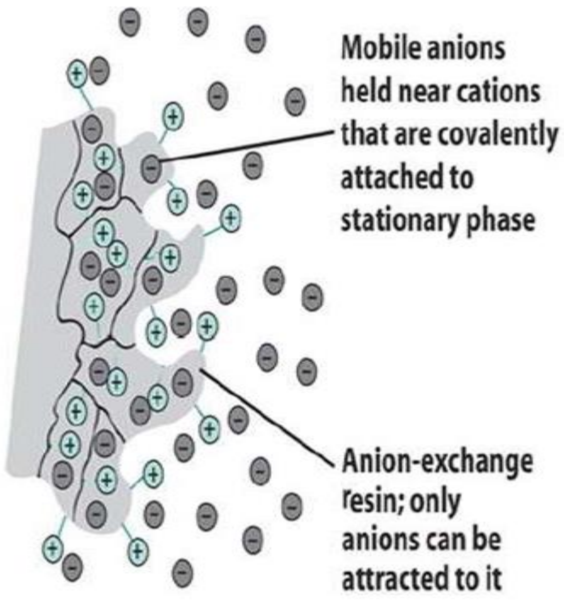
- **Normal** – analyte is nonpolar organic; stationary phase MORE polar than the mobile phase.
  Ex: TLC, Paper Chromatography

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





3. Ion Exchange Chromatography

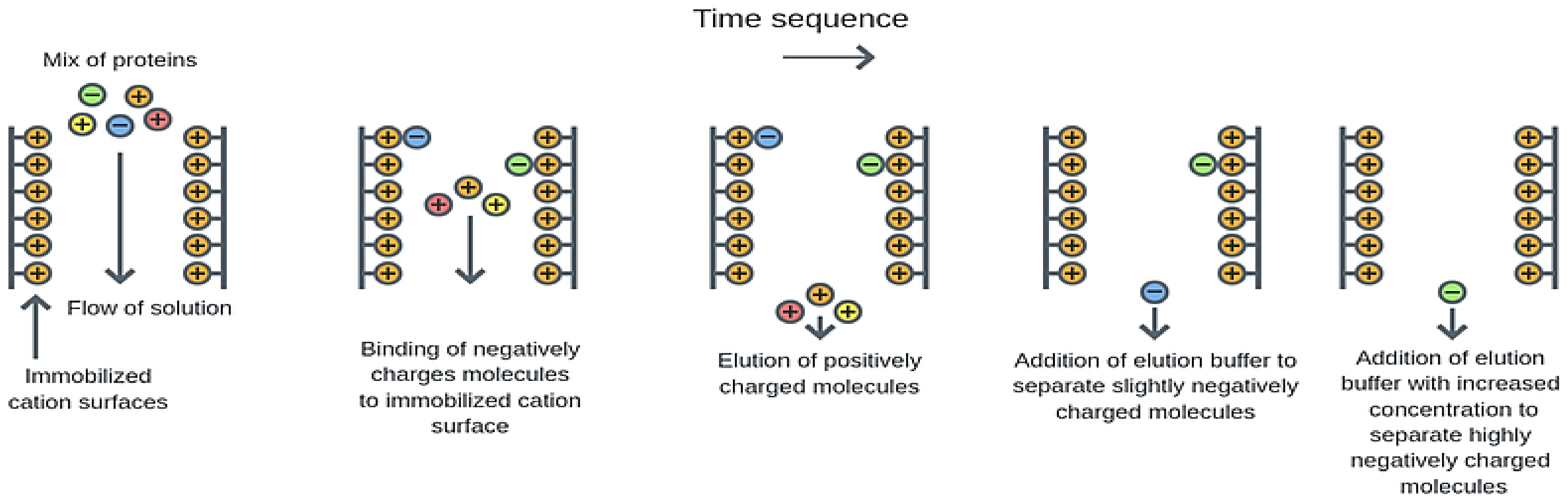


Use ionic stationary phase (Ionic exchanger)

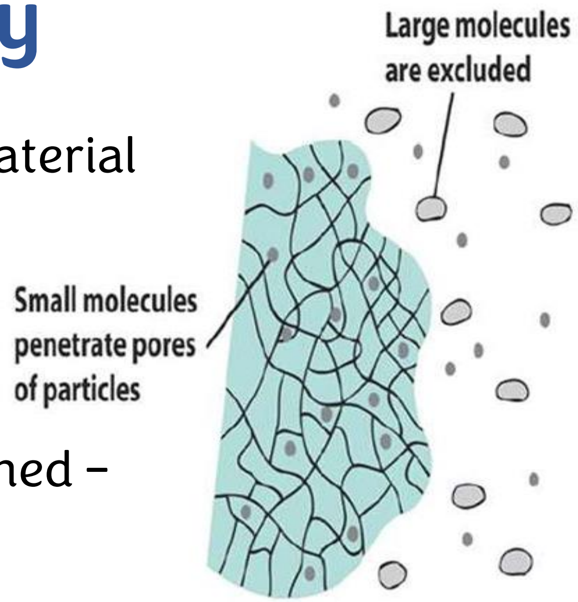
- ➢ ions separated based on their tendency to displace counter ions adsorbed on stationary phase (Depends on charge, hydration, "solubility").
- ➢ Used for analysis of proteins, nucleic acid, amino acids and its base pair.

- ○ Anionic stationary phases: used for cation separation (**attracts anions**).
- ○ Cationic stationary phases: for anion separation for ionic compounds (**attracts cations**)

Ex: GC, HPLC



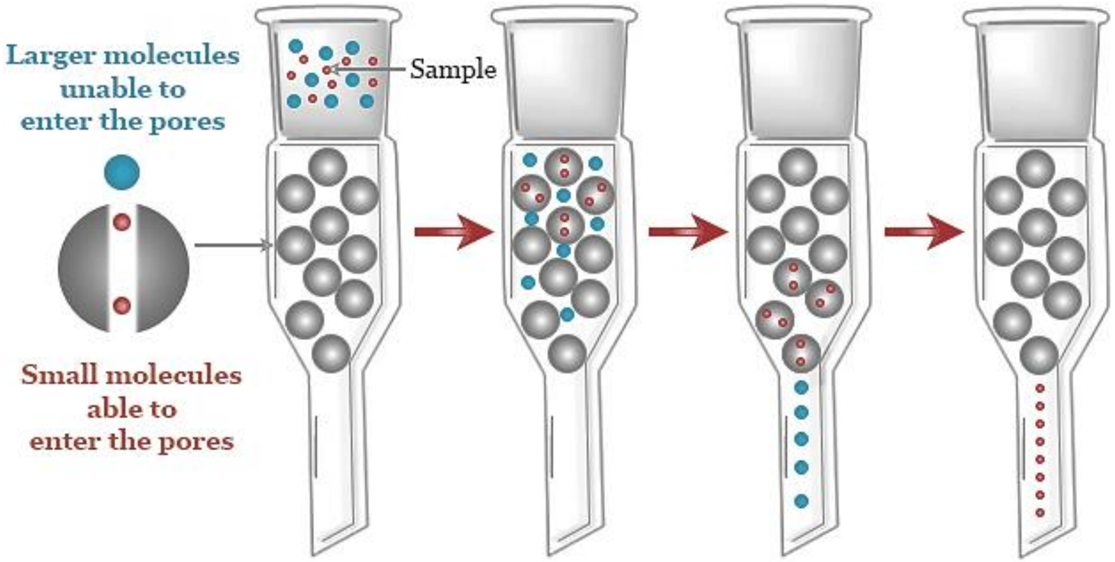
4. Size (Molecular)Exclusion Chromatography



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

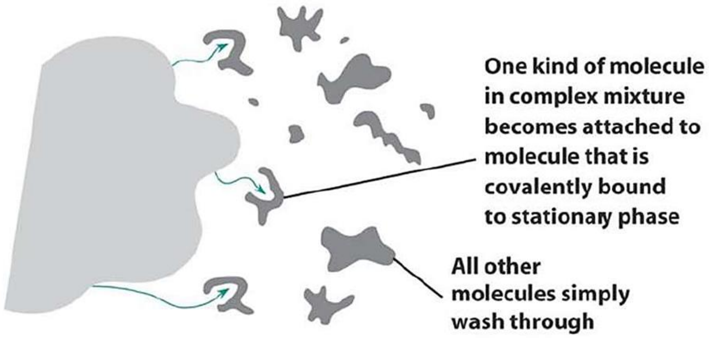
- Stationary phase is a porous matrix
- Very large molecules can't get into the pores – unretained
- Very small molecules get hung up into pores for a long time – most retained – longest retention time

Ex: GC, HPLC

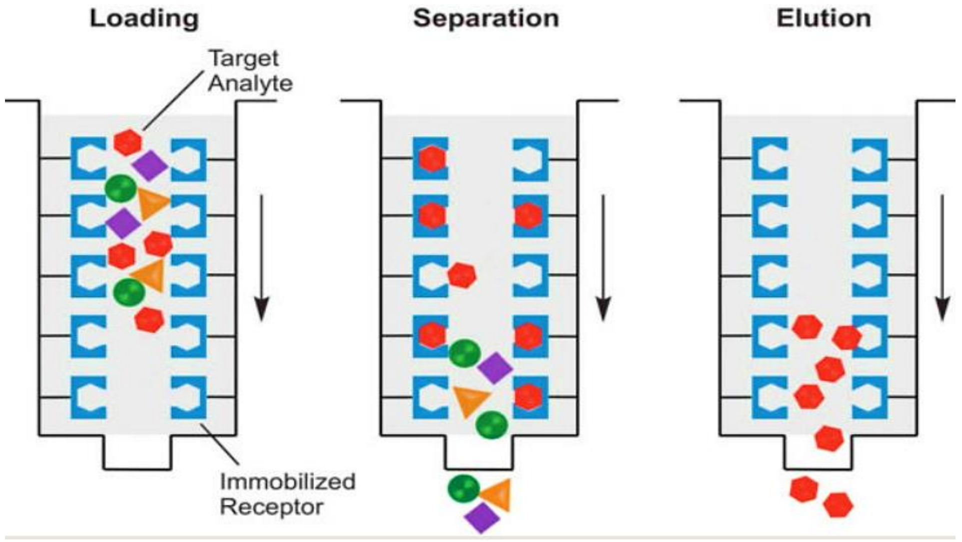


5. Affinity chromatography

The mixture are separated when the element having an affinity towards the stationary phase binds to the stationary phase. In contrast, other components are eluted with the mobile phase.

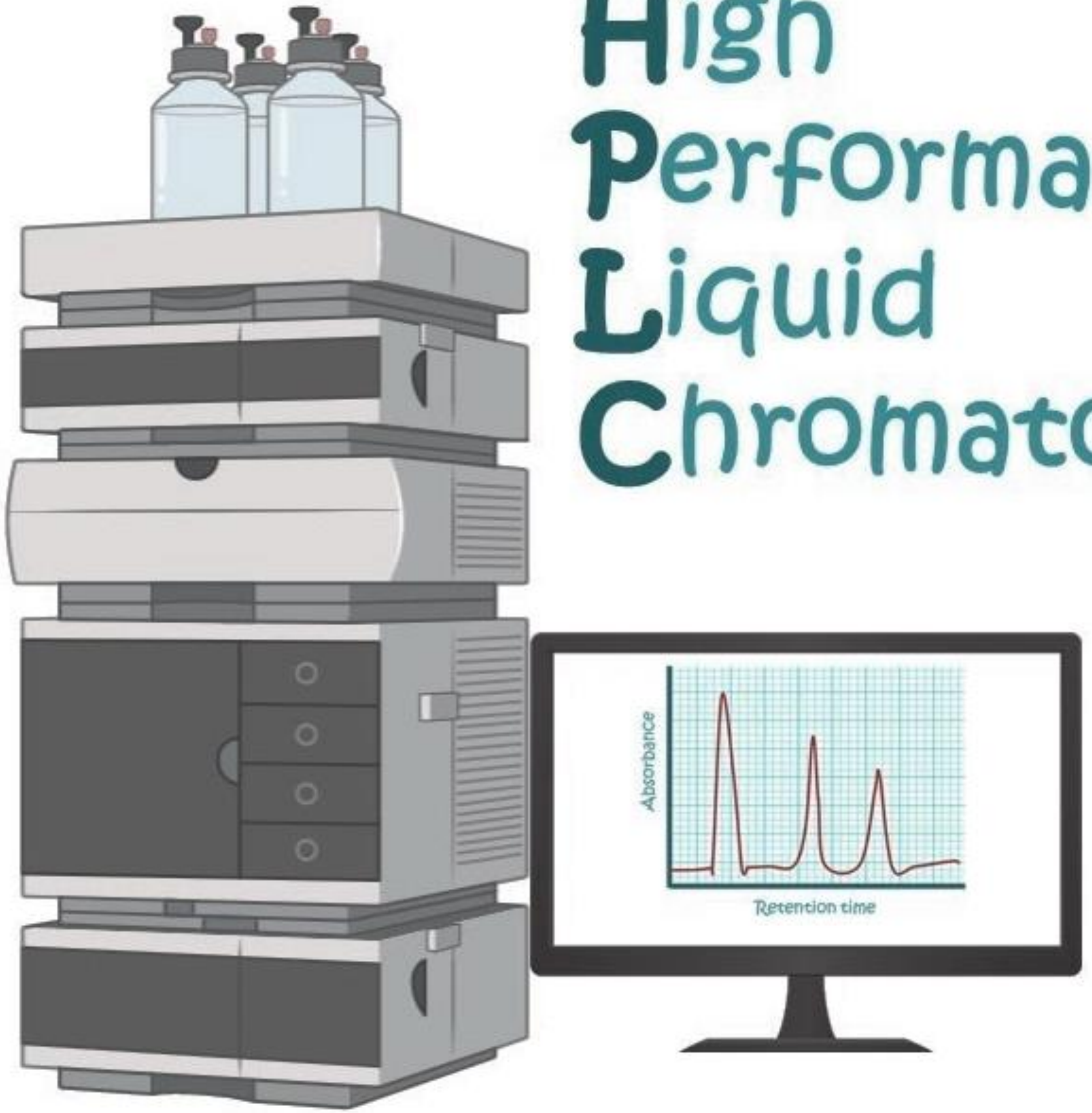


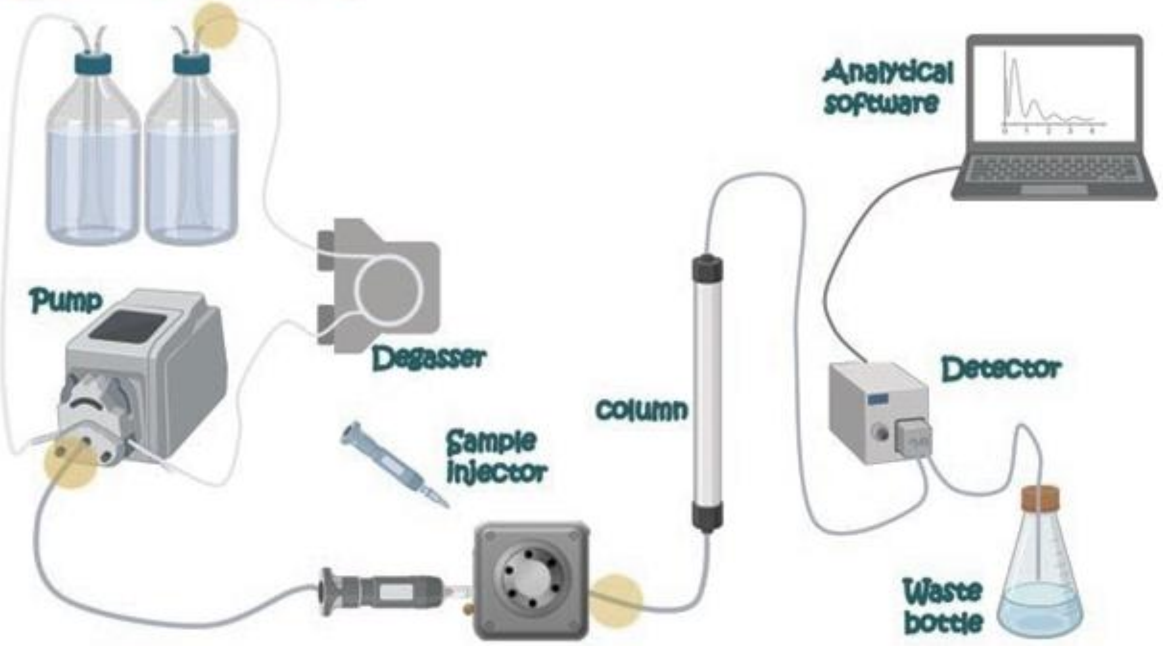
- The substrate/ ligand is bound to the stationary phase so that the reactive sites for the binding of components are exposed.
- Now, the mixture is passed through the mobile phase where the components with binding sites for the substrate bind to the substrate on the stationary phase while the rest of the components are eluted out with the mobile phase.
- The components attached to the stationary phase are then eluted by changing the pH, ionic strength, or other conditions.



High **P**erformance **L**iquid **C**hromatography

- High Performance → High resolution power
- Liquid → Mobile Phase is liquid





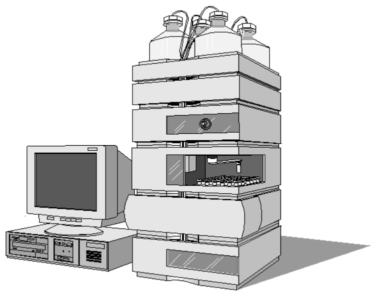
High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a modified form of column chromatography where the components of a mixture are separated based on their affinity with the stationary phase. It is an extension of conventional LC.

- ❖ Very powerful technique (High resolution & High Pressure).
- ❖ The stationary phase is a granular material with very small porous are tightly particles packed in a separation column.

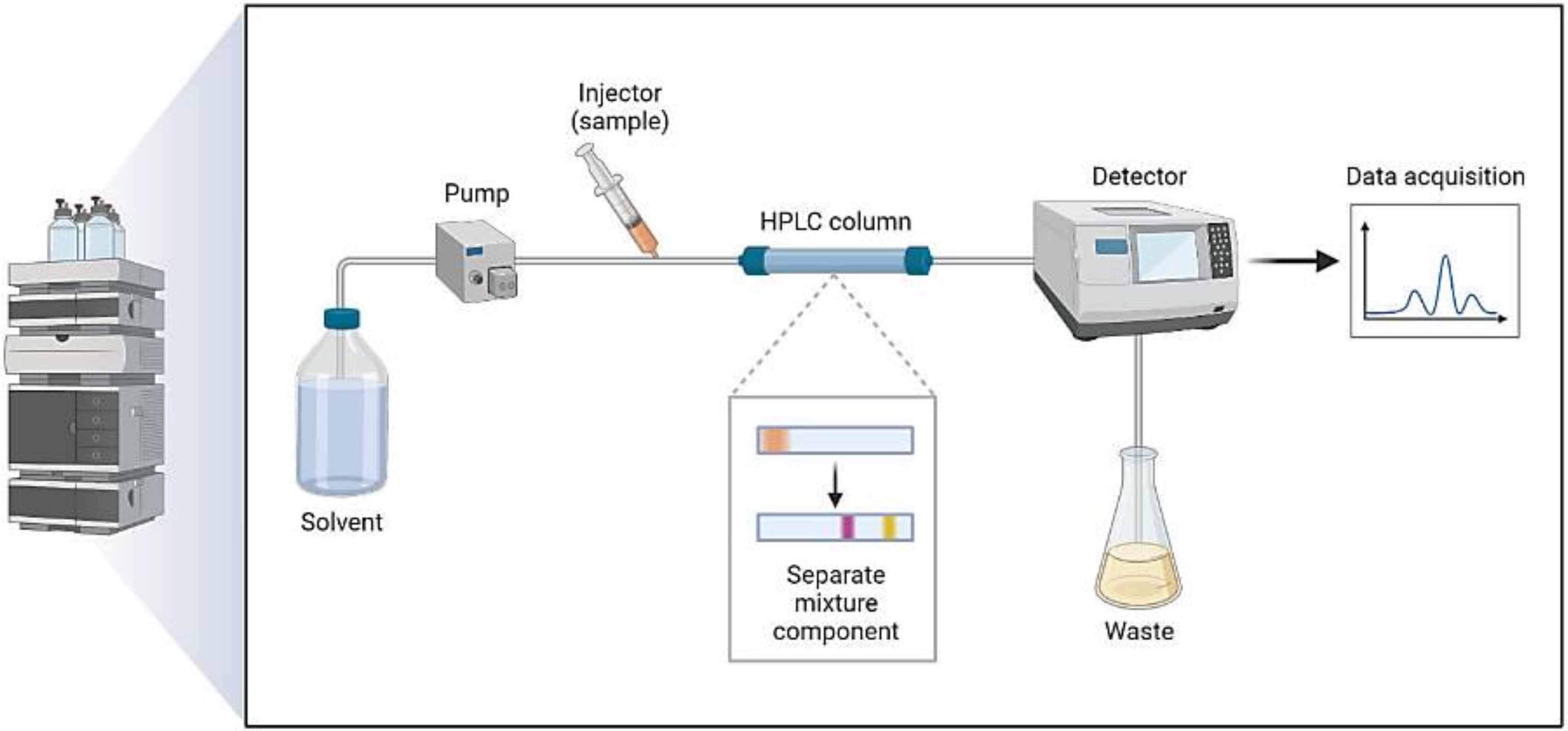
  Particles size is very small → larger surface area → higher interaction → Better separation of the molecules flowing through it.

- ❖ The Eluent (liquid mobile phase) is forced through the column with extremely high pressure (up to 400 atm or 6,000 psi) by a pump
- ❖ separation time is highly reduced to few minutes rather than hours and days.



HPLC Instrumentation Components

1. Solvent reservoir 2. Pump 3. Injector 4.Column 5.Detector 6.Recorder or Data system.



1. **Solvent reservoir:** Solvent reservoir is also known as mobile phase reservoir. The high viscous solvent is discouraged to use as it takes much more time to travel through column, and high pressure is required for the viscous solvent. These leads to peak broadening, and hence better not to use such solvent.

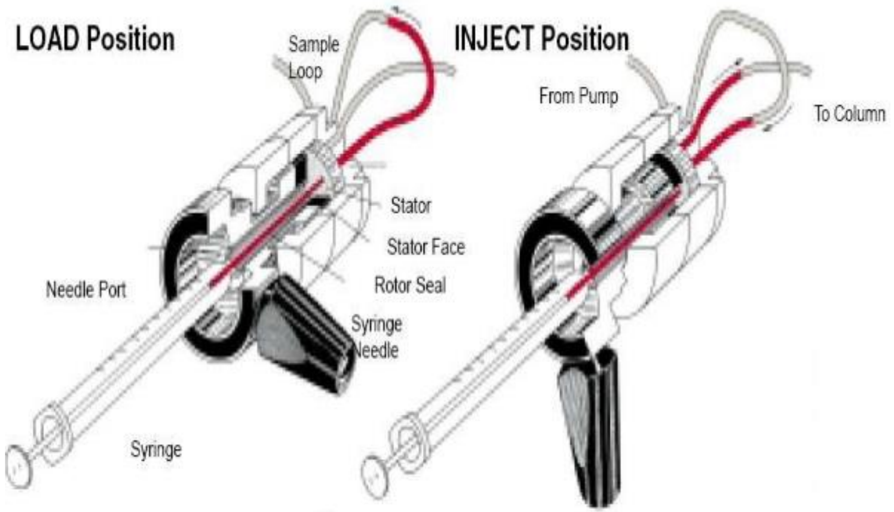
- The choice of solvent depends on the nature of sample and the sensitivity of the detector.

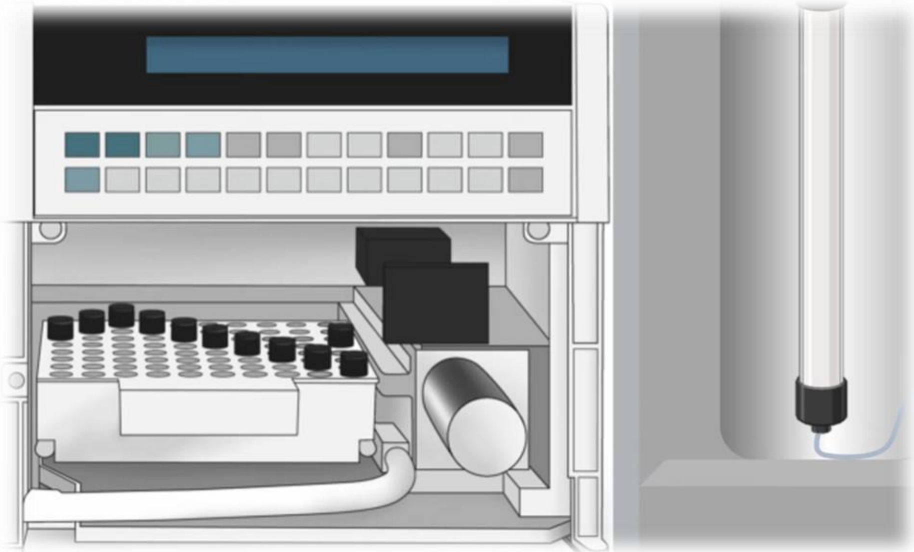
2. **Pump:** The pump's function is to propel the liquid mobile phase through the column at a set flow rate, which is measured in (mL/min). Which forces the mobile phase at a much a higher velocity than the gravity-flow columns.

- A pump can deliver a constant mobile phase composition (isocratic) or a rising mobile phase composition (gradient) during the chromatographic experiment.

3. Injector: The injector is used to insert the liquid sample into the mobile phase’s flow stream.

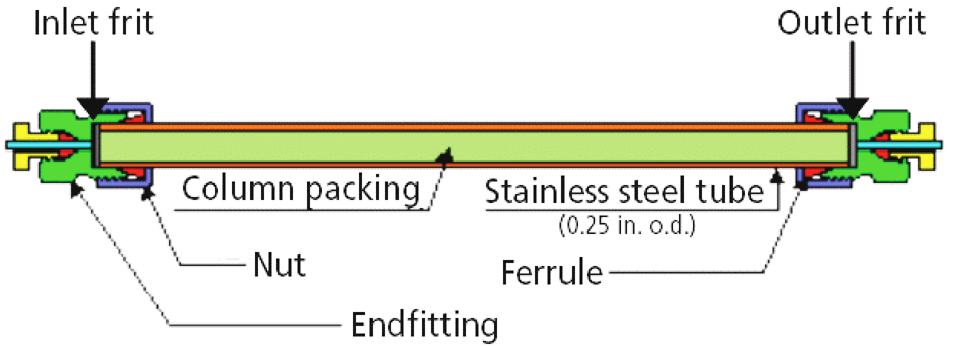
- ➢ Sample quantities range from 0.1 to 100 microliters (L) at high pressure.
- ➢ It is either done using a syringe or The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.





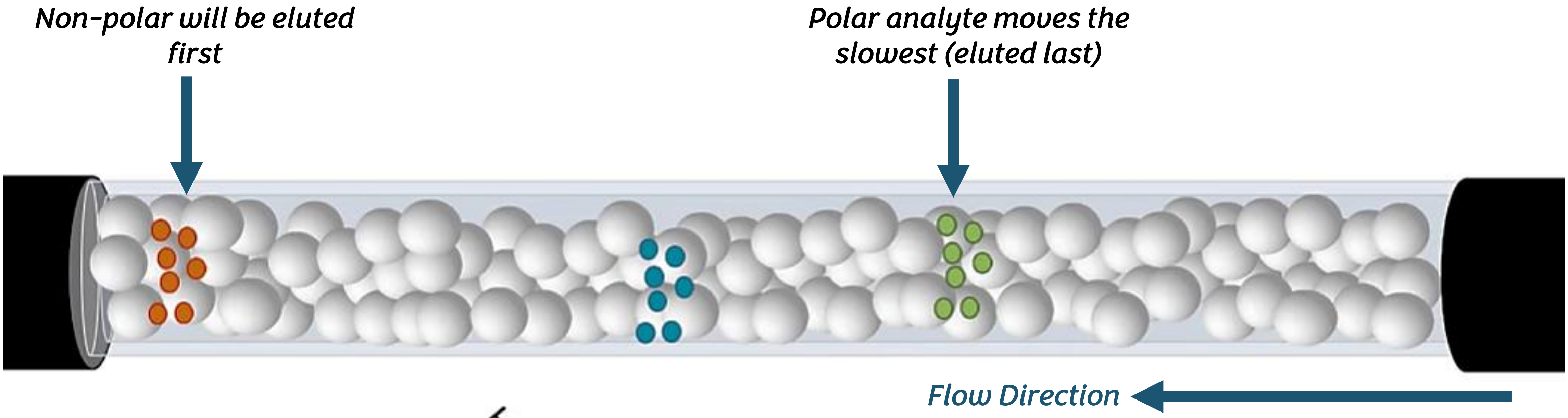
4. **HPLC Column:** It is known as the heart of the chromatograph. Mostly, stainless steel is used as materials for the construction of the tubing (High pressure).

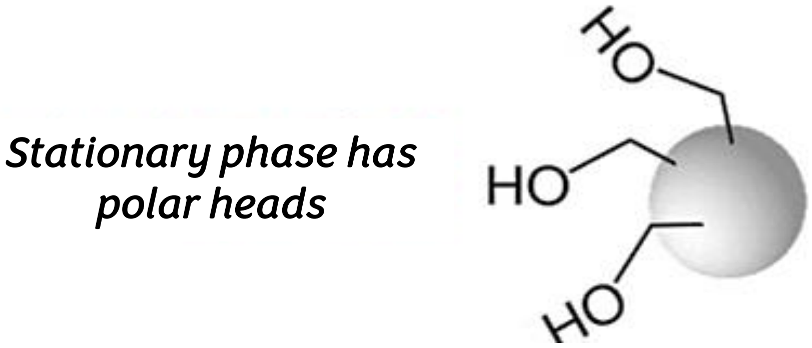
- The column length generally varies from 5 cm to 30 cm, and its diameter ranges from 2-50 mm.
- Silica and alumina particle is used as packing materials.
- The mobile phase is aspirated from the solvent reservoir and forced through the system's column and detector by a pump.



Normal Phase HPLC

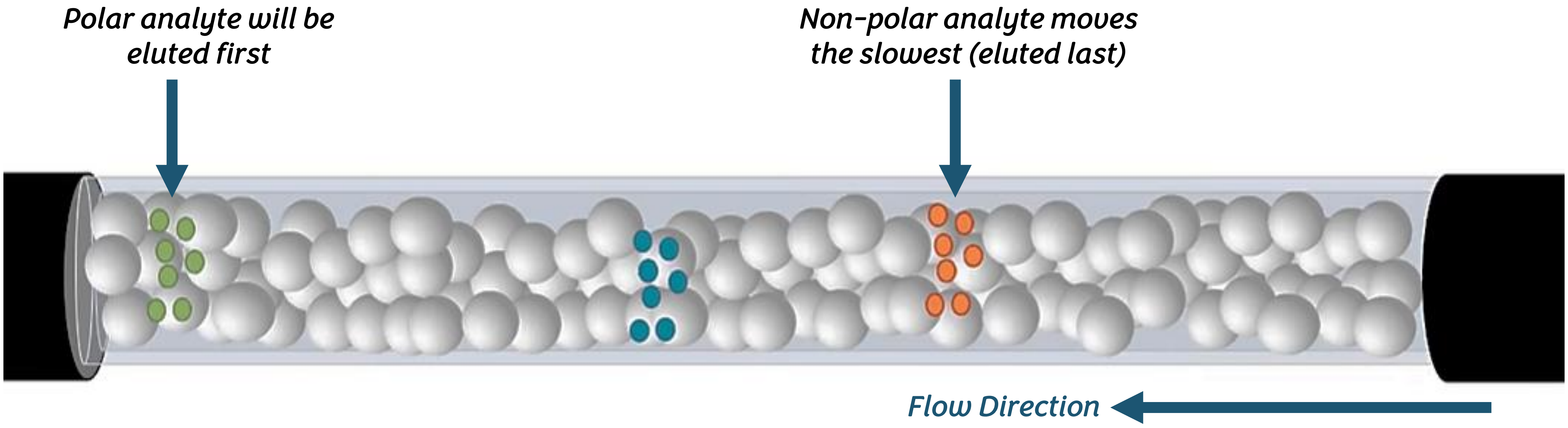
The stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase).

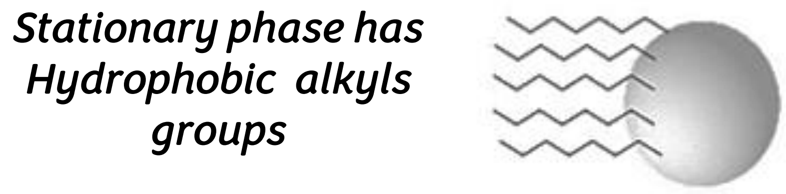




Reverse Phase HPLC

The stationary phase is less polar than the mobile phase (e.g., water-methanol mixture as the mobile phase, C18= octadecylsilyl (ODS) as the stationary phase).

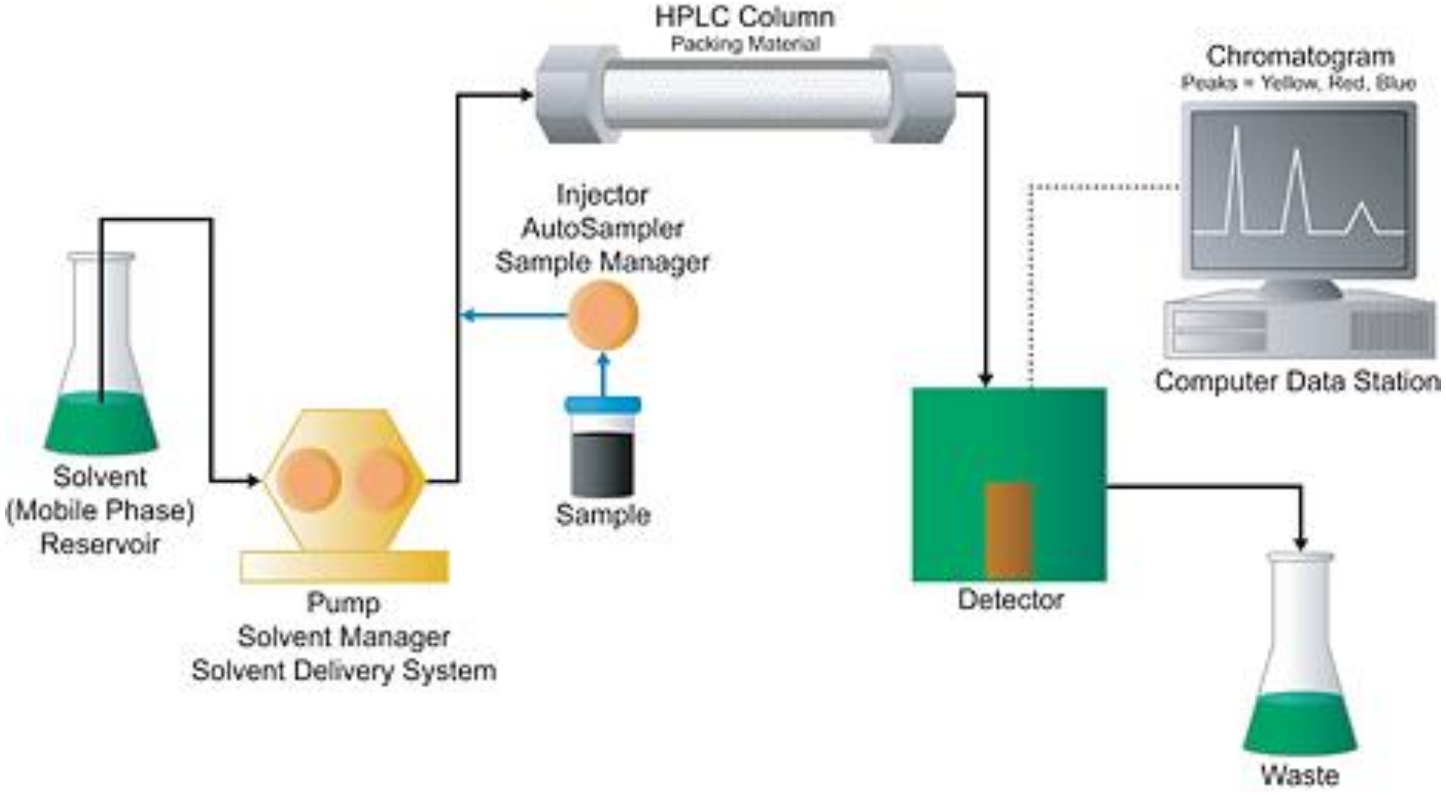




Elution Modes in HPLC:

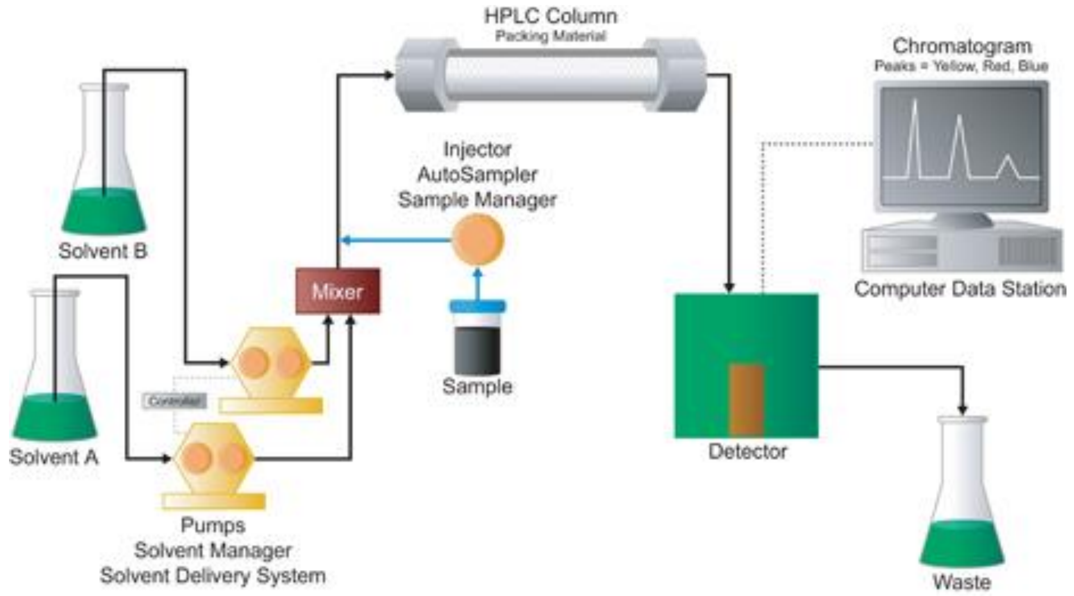
A. Isocratic

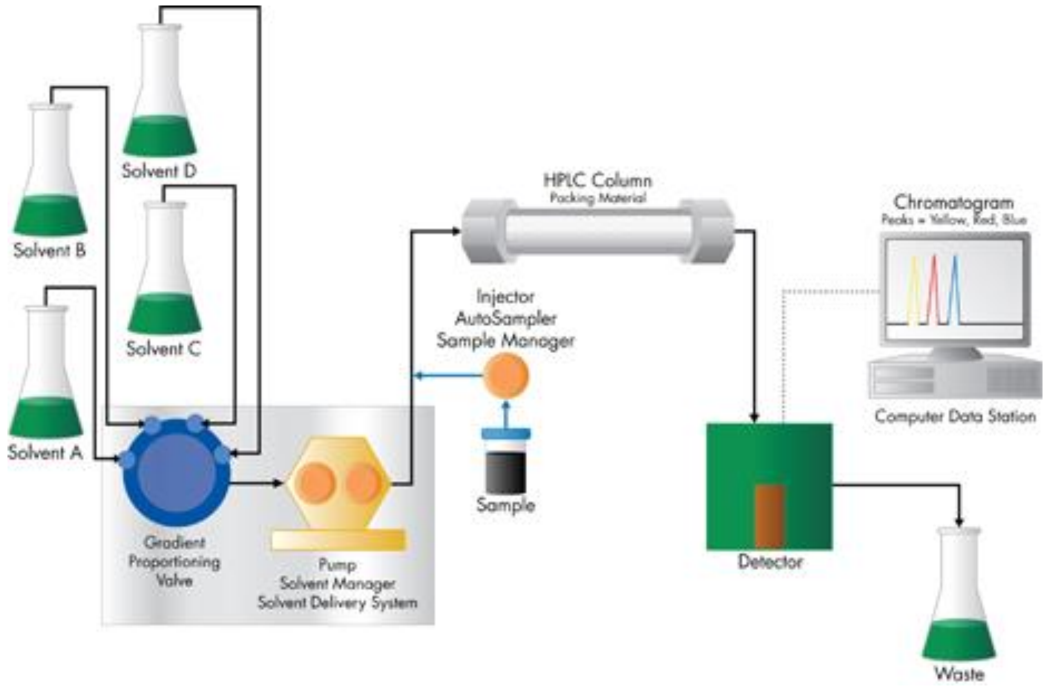
The mobile phase is either a pure solvent or a mixture *remains the same throughout the run.*



A. Gradient

The *mobile phase composition changes during the separation.*



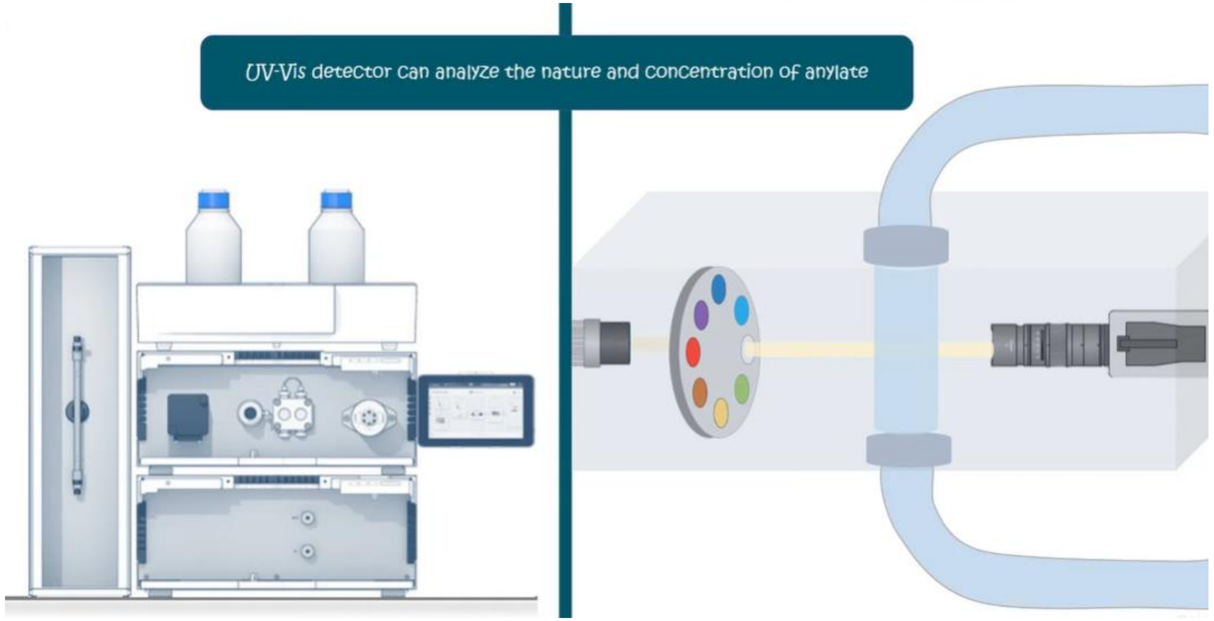


5. **Detector:** It is used to observe the obtained separated elute as it leaves the column. The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.

- This difference is monitored as a form of an electronic signal. This signal is proportional to the concentration of each separated component
- Crucial in trace elements (high sensitivity).
- Simplifies quantification
- Insensitive to changes in type of solvent, flow rate and temp.
- There are different types of detectors available

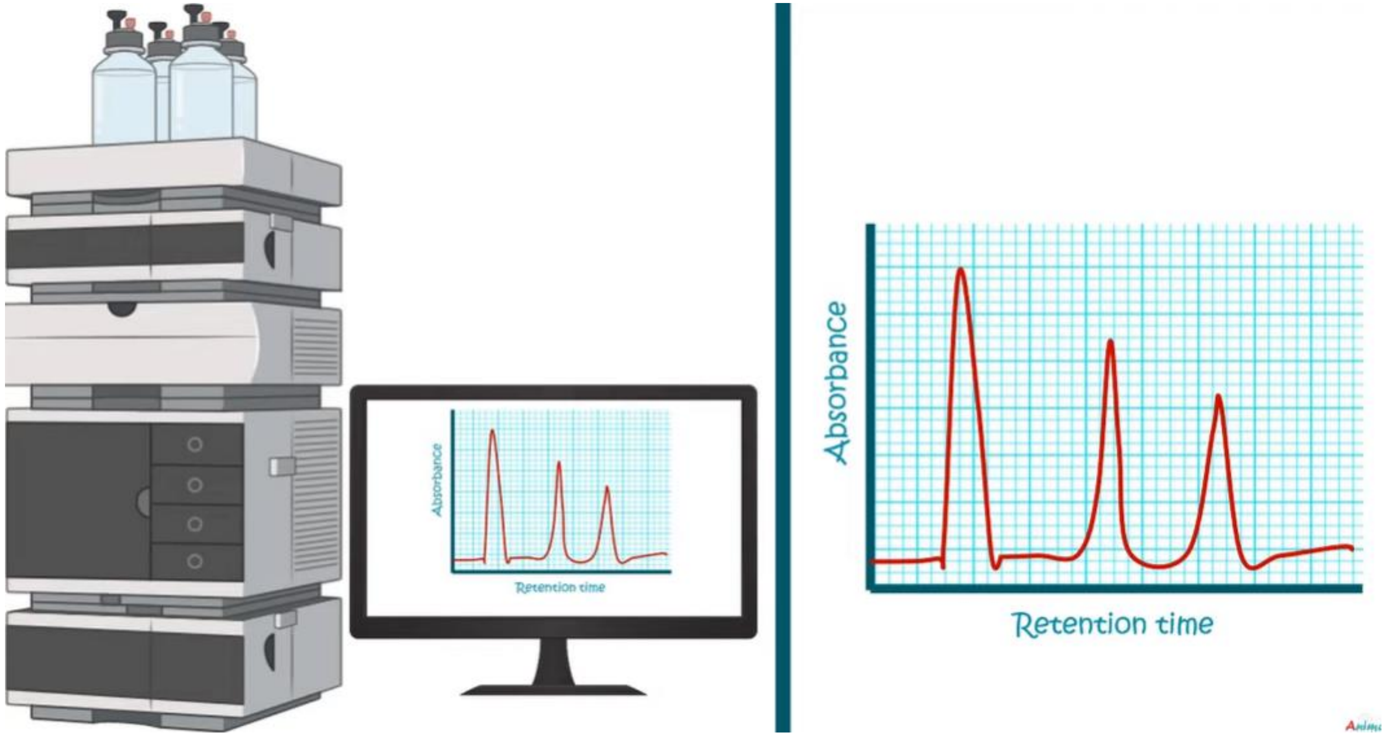
The most widely used detection methods

- Spectrophotometers
- Fluorometers
- Electrochemical detectors
- Mass spectrometer
- Refractive index detector



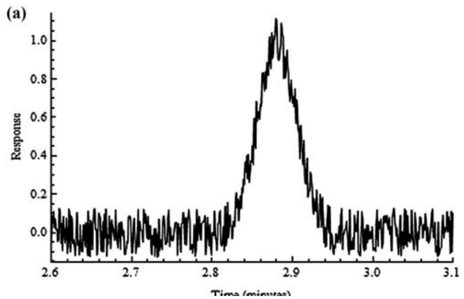
6. **Recorder:** The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes.

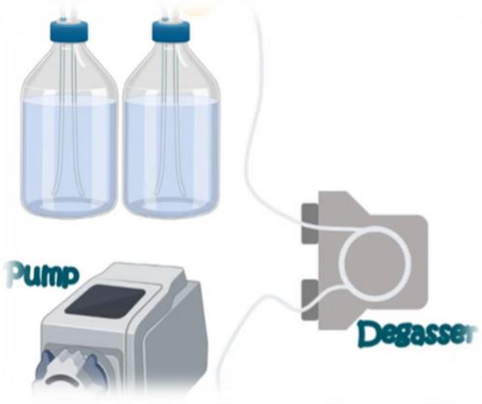
- A computer-based data processor (integrator) is used to process various types of data.
- It takes the signal from the detector and makes use of it to decide the time of elution (retention time) of the sample components (qualitative analysis) and the quantity of pattern (quantitative analysis).



❖ Degasser

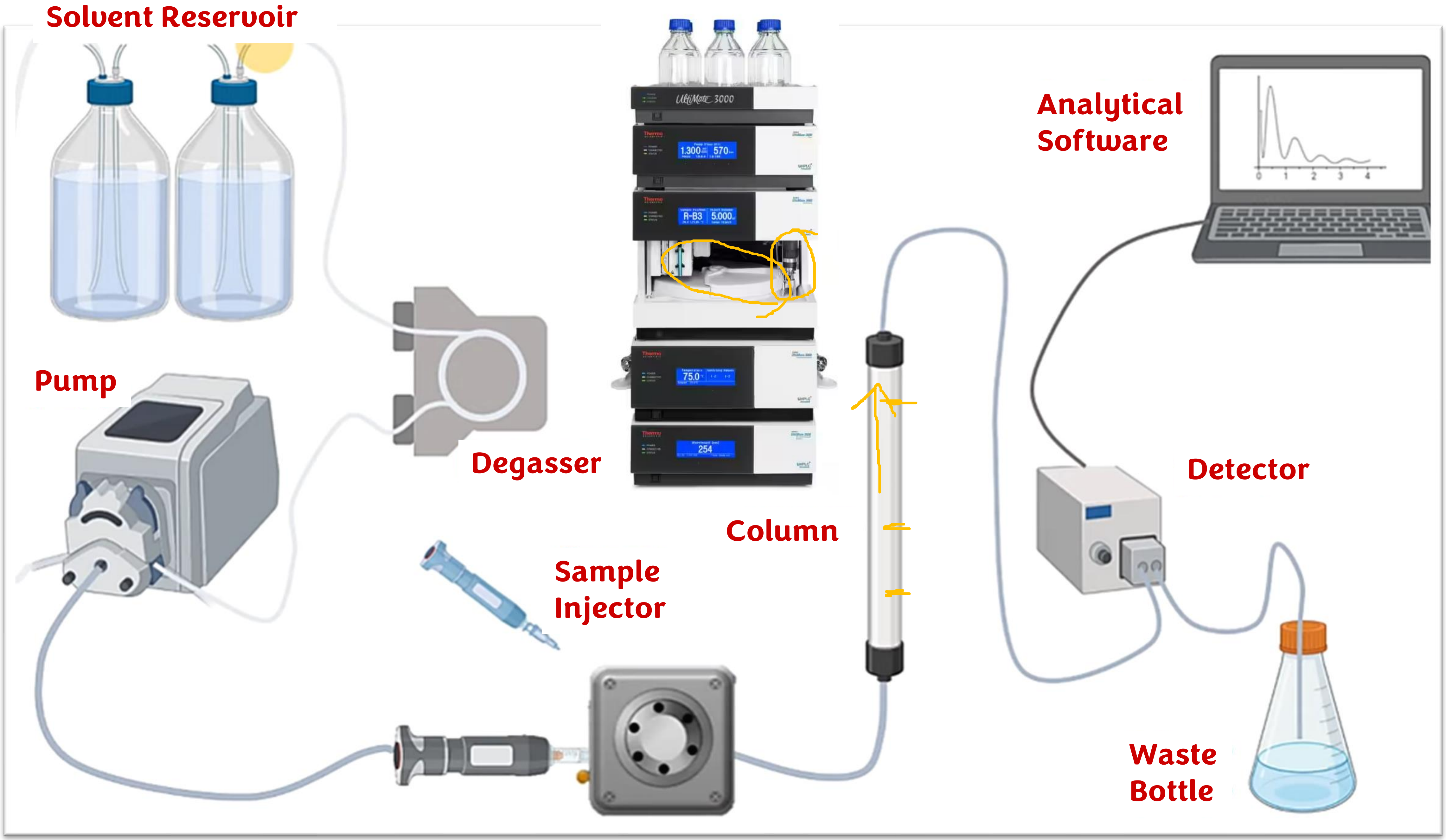
The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as noise and causes an unstable baseline. Degasser uses special polymer membrane tubing to remove gases.



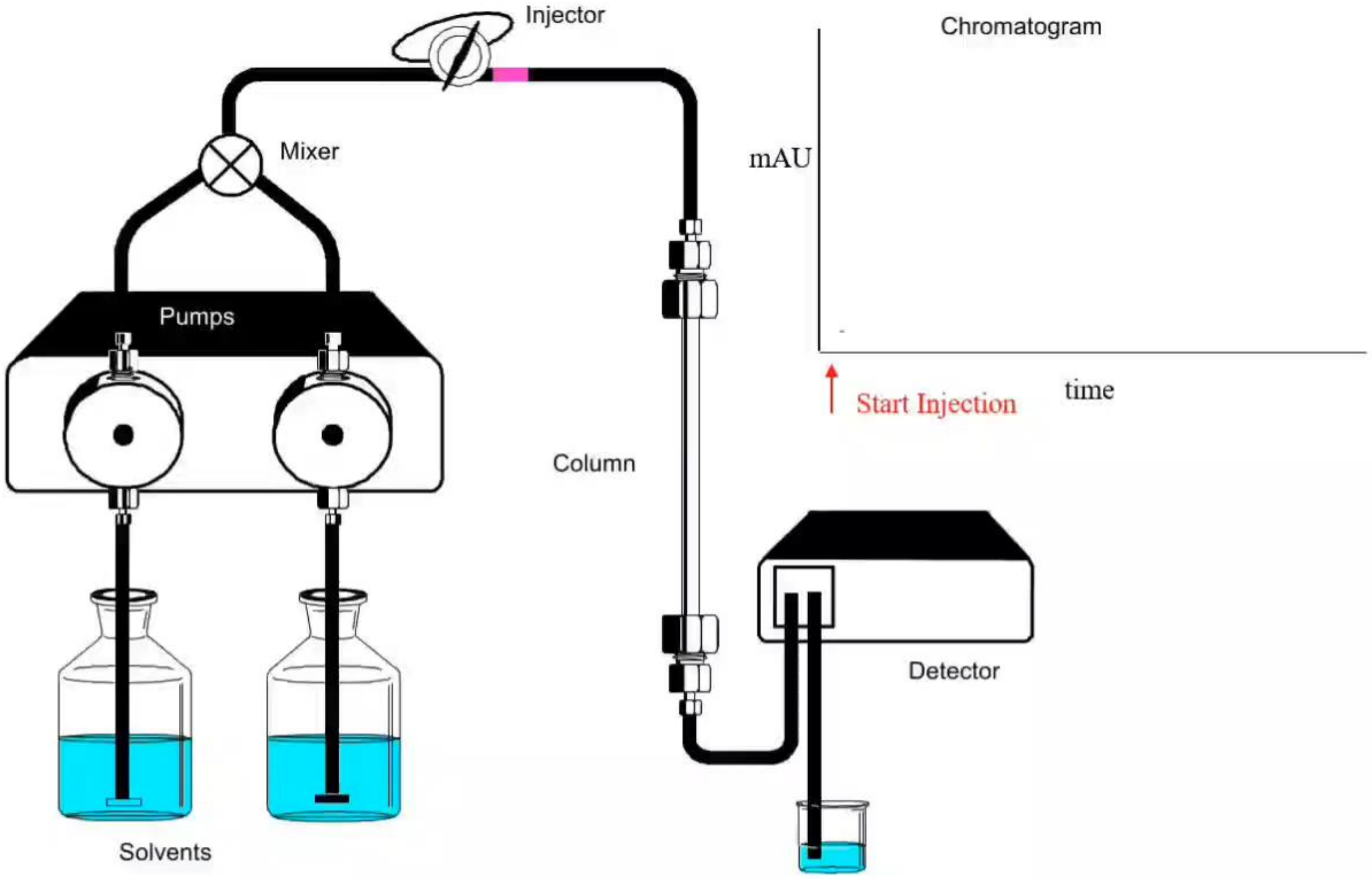


❖ Column Heater

The LC separation is often largely influenced by the column temperature. In order to obtain repeatable results, it is important to keep consistent temperature conditions. Also, for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C). Thus, columns are generally kept inside the column oven (column heater).


Solvent Reservoir
UltiMate 3000
Analytical Software
Pump
Degasser
Detector
Column
Sample Injector
Waste Bottle

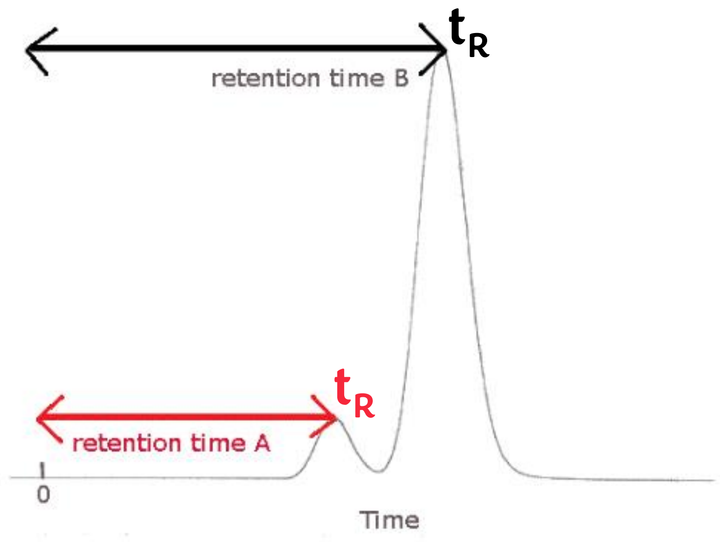
Separation in HPLC

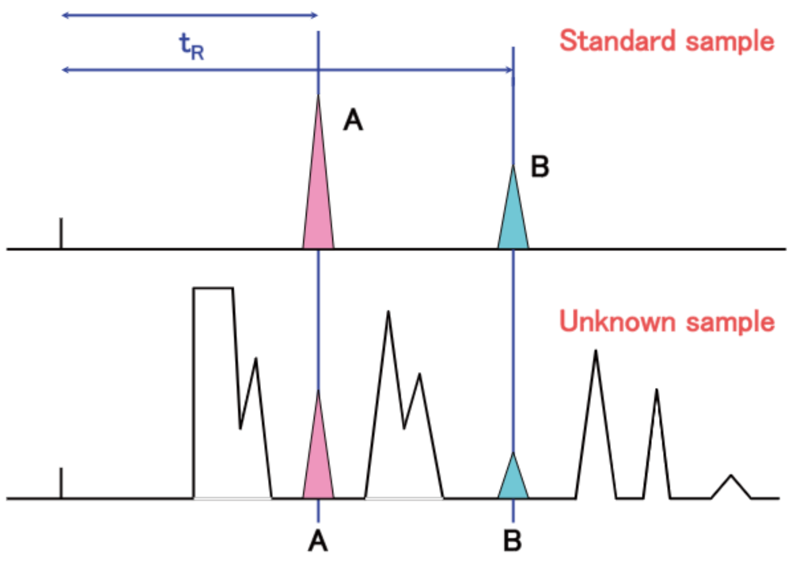


The Retention Time (t_R)

- The time taken for a particular compound to travel through the column to the detector

- From the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

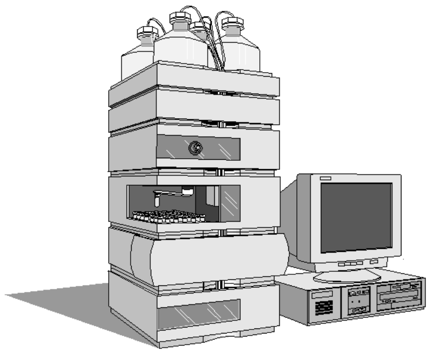


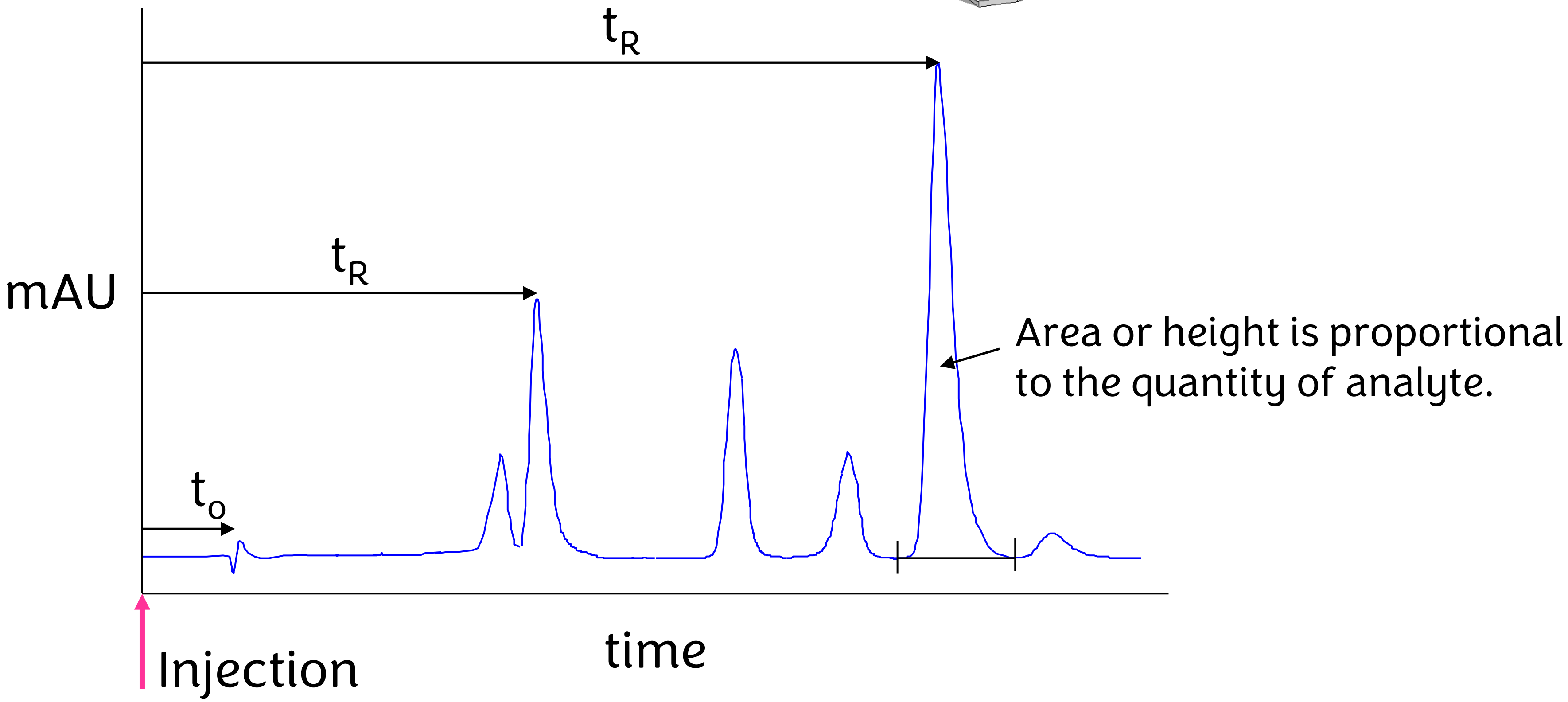


The Chromatogram

t_o – elution time of unretained peak

t_R – retention time – determines sample identity

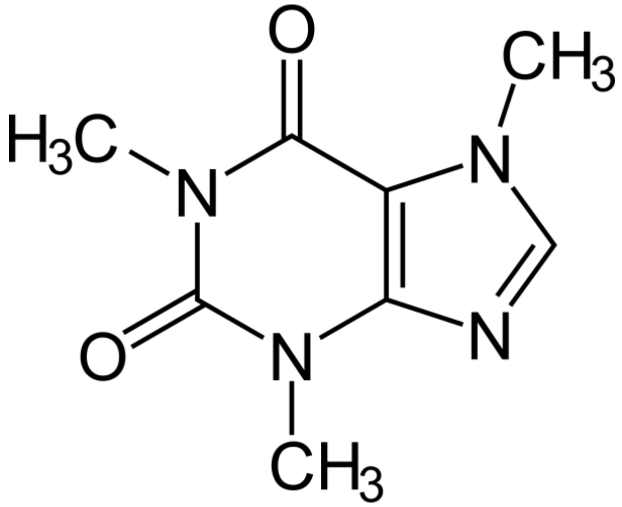


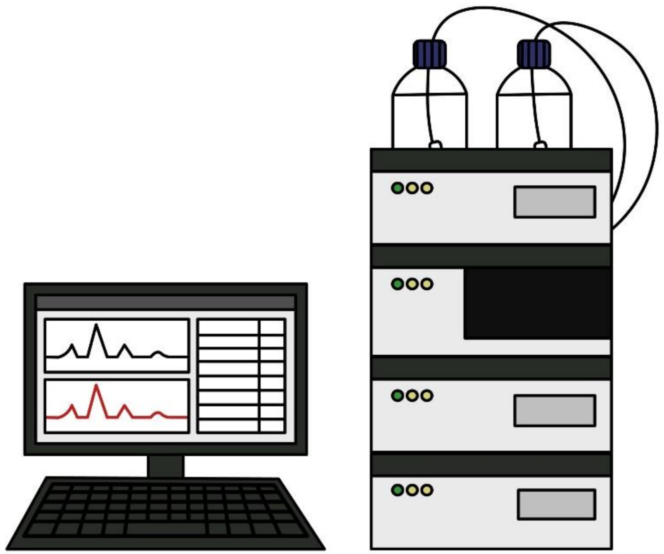


Practical Part

Objective

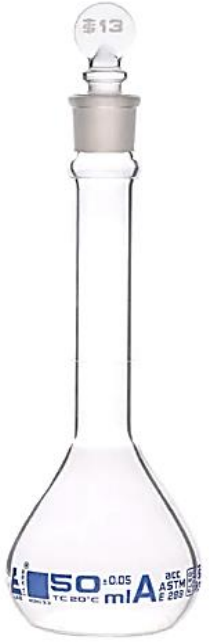
- ❖ Determination of **Caffeine sample assay (purity)** using HPLC.
- ❖ Get familiar with the HPLC instrument and its usage.





Glassware

Each group will be supplied by the following glassware:



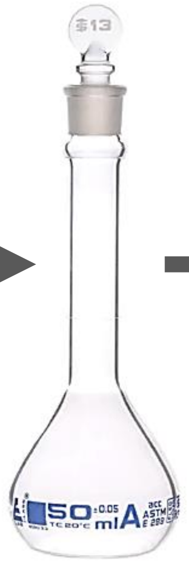
1 flask

1 flask

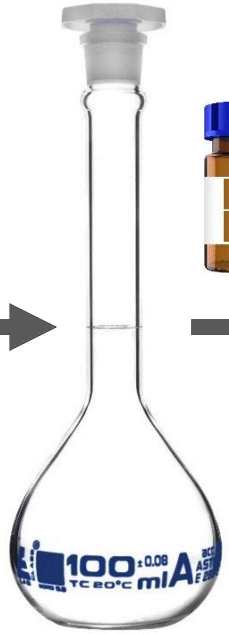
Procedure

Preparation of the Caffeine sample solution by dissolving the 25mg of the unknown powder in 50ml diluent. Then carrying a dilution of 5ml to 100ml diluent.

Weigh 25 mg of the caffeine unknown sample then dissolve in 50 ml diluent



Dilute 5 ml to 100 ml diluent



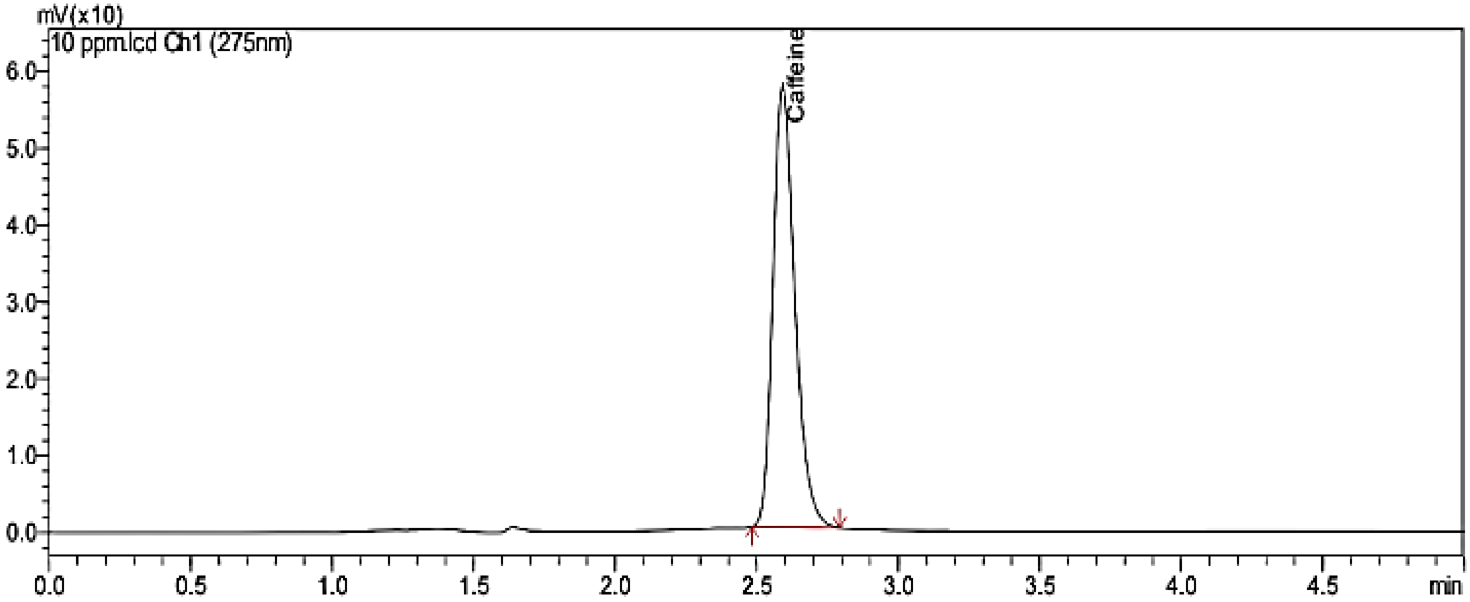
Instrumentation Method

Mobile Phase	Water: Methanol (60:40) both are HPLC grade
Column	Zorbax Eclipse Plus 18C Column Pore size: 5µ Internal diameter: 4.6nm Length: 150 mm. Reverse phase: ODS
Flow rate	1 ml/min
UV detection (λ)	275 nm
Sample injection volume	10 µl
Column Temperature	40°C
Run time	5 min

Data Interpretation

- ✓ *From the HPLC analysis the data will be obtained as an* ***area under the curve*** *(AUC), measured with (mV) unit.*

- ✓ *Students will be provided with a calibration curve data to make the needed calculations.*

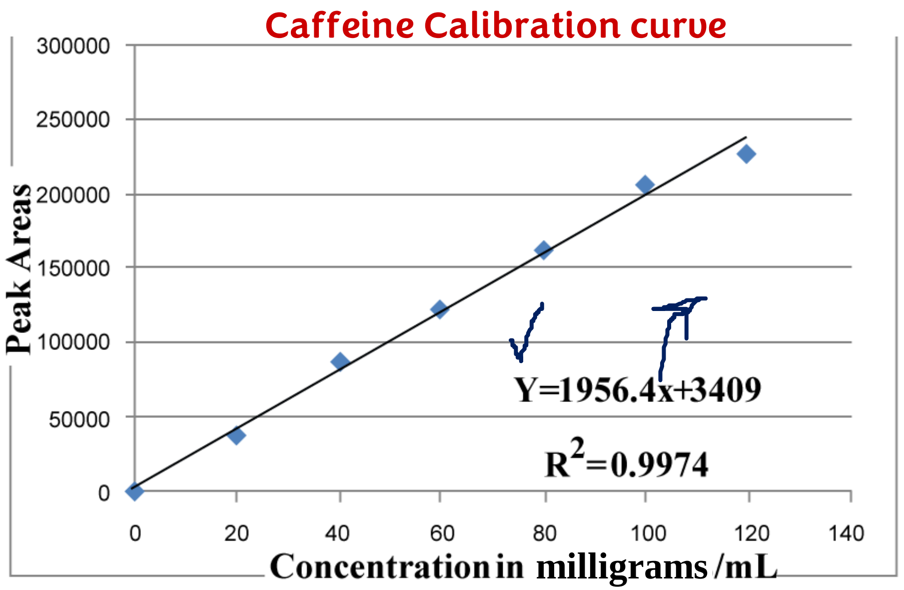


Chromatogram of caffeine , Detector 275nm

- ✓ *After finishing measurements, students will obtain their* ***unknown sample area*** *from the device → then using the calibration curve you can calculate the concentration for your caffeine in the sample(D.f).*

- ✓ *From the calibration curve → find the concentration of your unknown caffeine sample → Multiplied by the volume used and D.f → Amount in (mg)*

mg



- ✓ ***Then determine the Caffeine sample purity as follows:***

$$\% \text{ Purity} = \frac{\text{Actual Amount}}{\text{Theoretical Amount}} \times 100\%$$

MSc. Farah Hudaib