GPAT Online Class for B.Pharm Students

Ananthapuramu Local Branch

Pharmaceutical Analysis – Part 4
(4th July 2020)

By

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Pharmaceutical Analysis – Part 4
Chromatography : Types & Principles
HPLC Instrumentation

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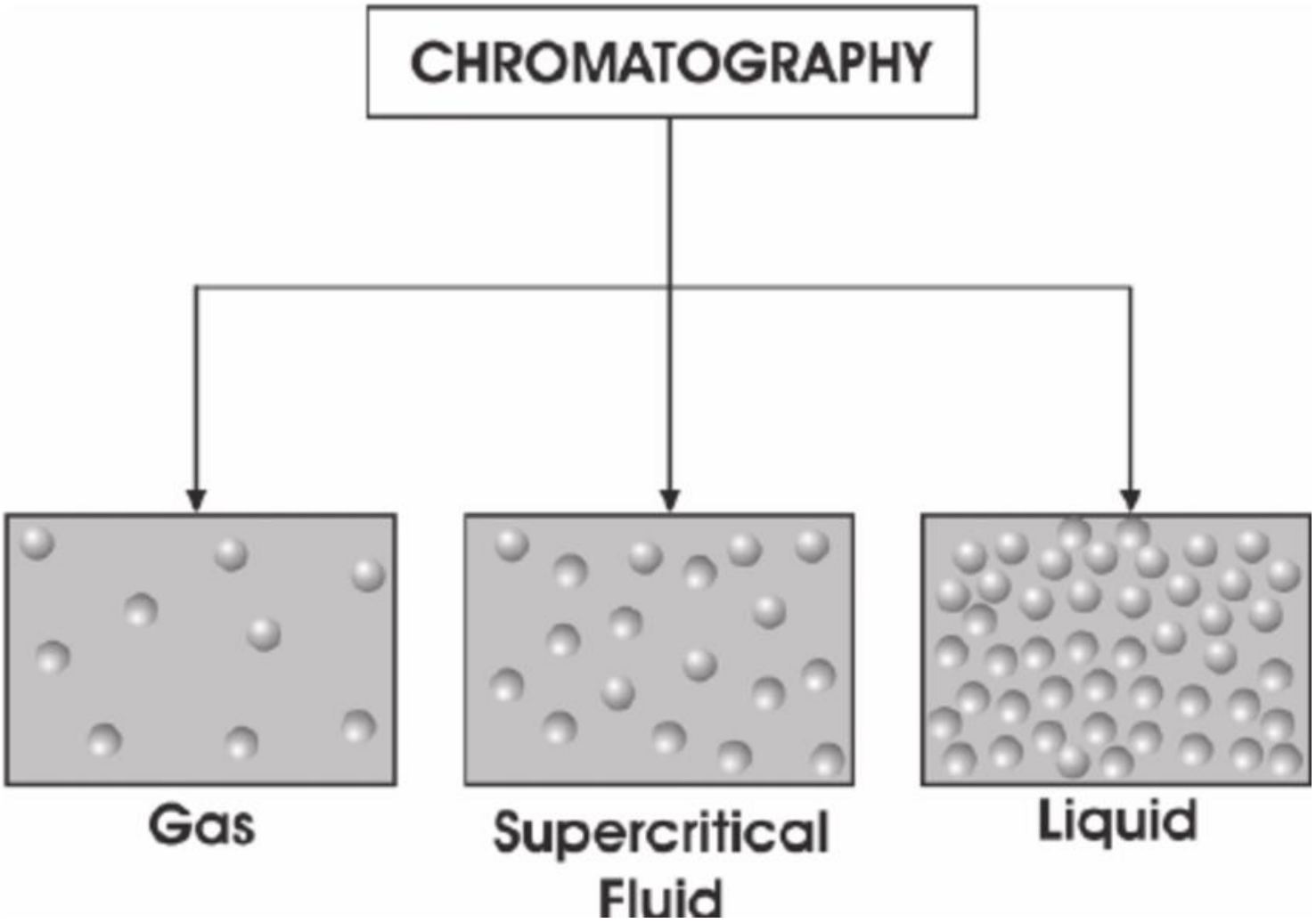
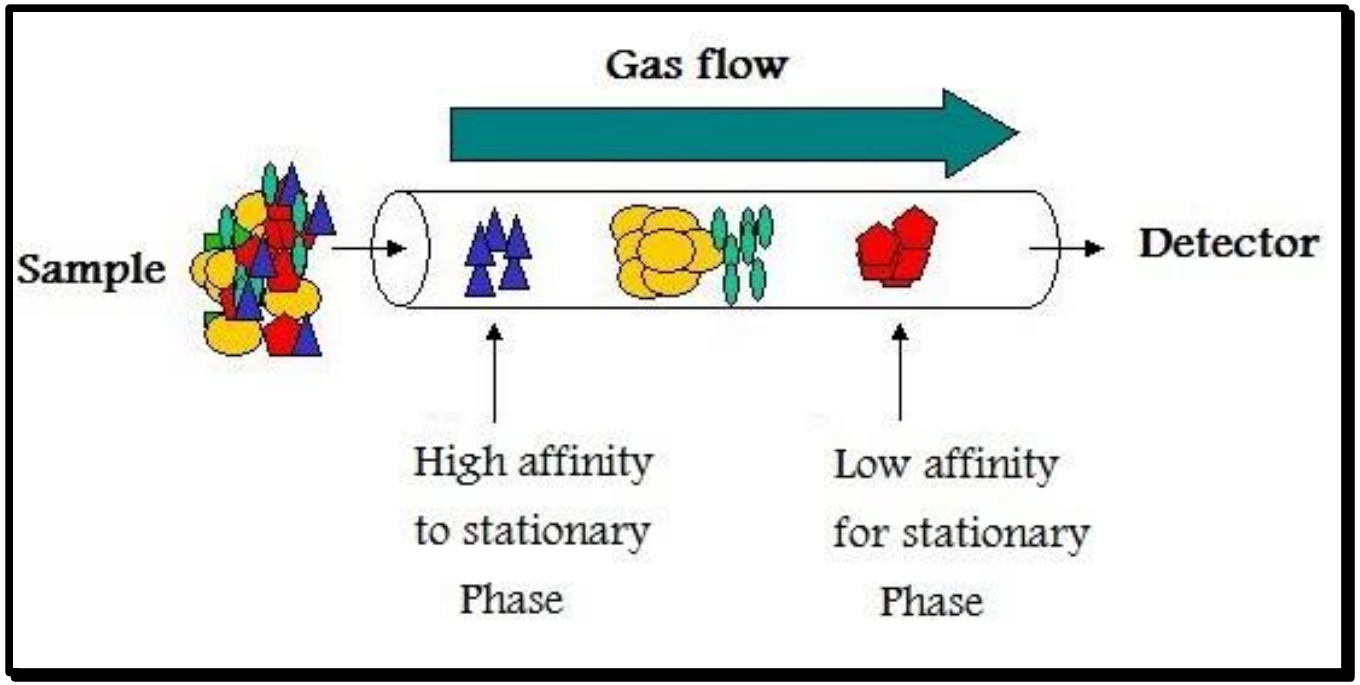

CHROMATOGRAPHY
Gas
Supercritical Fluid
Liquid

INTRODUCTORY PRINCIPLES

Chromatography is a combination of two words;

* **Chromo** – Meaning color

***Graphy** – representation of something on paper



HISTORY OF CHROMATOGRAPHY

Chromatography, literally "color writing", was first employed by Russian scientist Mikhail Tswett in 1903/1906. He continued to work with chromatography in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll , carotenes, and xanthophylls . Since these components have dif ferent colors (green, orange, and yellow,respectively) they gave the technique its name.

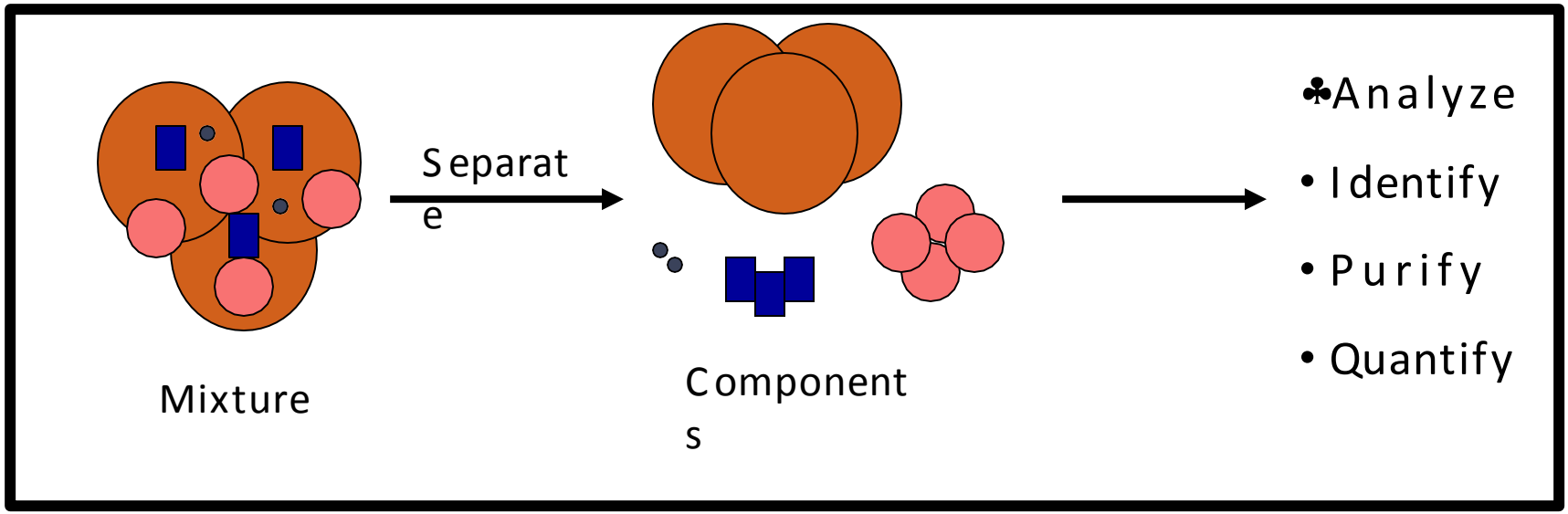
DEFINITION OF CHROMATOGRAPHY

IUPAC definition (International Union of pure and applied Chemistry) (1993):

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction.

The stationary phase may be a solid, or a liquid supported on a solid or gel, the mobile phase may be either a gas or a liquid.

CHROMATOGRAPHY



Glossary...

- **Chromatograph:** Instrument employed for a chromatography.
- **Eluent:** Fluid entering a column.
- **Eluate:** Fluid exiting the column.
- **Elution:** The process of passing the mobile phase through the column.
- **Flow rate:** How much mobile phase passed / minute (ml/min).
- **Linear velocity:** Distance passed by mobile phase per I min in the column (cm/min).

Components of Chromatography

Mobile Phase – gas or liquid that carries the mixture of components through the stationary phase.

Stationary Phase – the part of the apparatus that holds the components as they move through it, separating them.

Uses for Chromatography

Chromatography is used by scientists to:

- Analyze – examine a mixture, its components, and their relations to one another
- Identify – determine the identity of a mixture or components based on known components
- Purify – separate components in order to isolate one of interest for further study
- Quantify – determine the amount of the a mixture and/or the components present in the sample

Uses for Chromatography

Real-life examples of uses for chromatography:

- Pharmaceutical Company
- Hospital
- Law Enforcement
- Environmental Agency
- Manufacturing Plant

CHROMATOGRAPHY TERMS

Chromatogram:

It is the visual output of the chromatograph.

Chromatograph:

It is equipment that enables a sophisticated Separation.

Stationary phase (bounded phase):

It is a phase that is covalently bonded to the support particles or to the inside wall of the column tubing.

CHROMATOGRAPHY TERMS

Mobile phase:

It is the phase which moves in a definite direction.

Analyte (Sample):

It is the substance to be separated during chromatography.

Eluate:

It is the mobile phase leaving the column.

CHROMATOGRAPHY TERMS

Retention time:

It is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.

Eluent:

It is the solvent that will carry the analyte.

CHROMATOGRAPHY TERMS

Retardation factor (R):

Fraction of an analyte in the mobile phase of a chromatographic system.

$$R = \frac{\textit{Quantity of substance in mobile phase}}{\textit{Total quantity of substance in the system}}$$

TYPES OF CHROMATOGRAPHY

- **Liquid Chromatography** – separates liquid samples with a liquid solvent (mobile phase) and a column composed of solid beads (stationary phase)

- **Gas Chromatography** – separates vaporized samples with a carrier gas (mobile phase) and a column composed of a liquid or of solid beads (stationary phase)

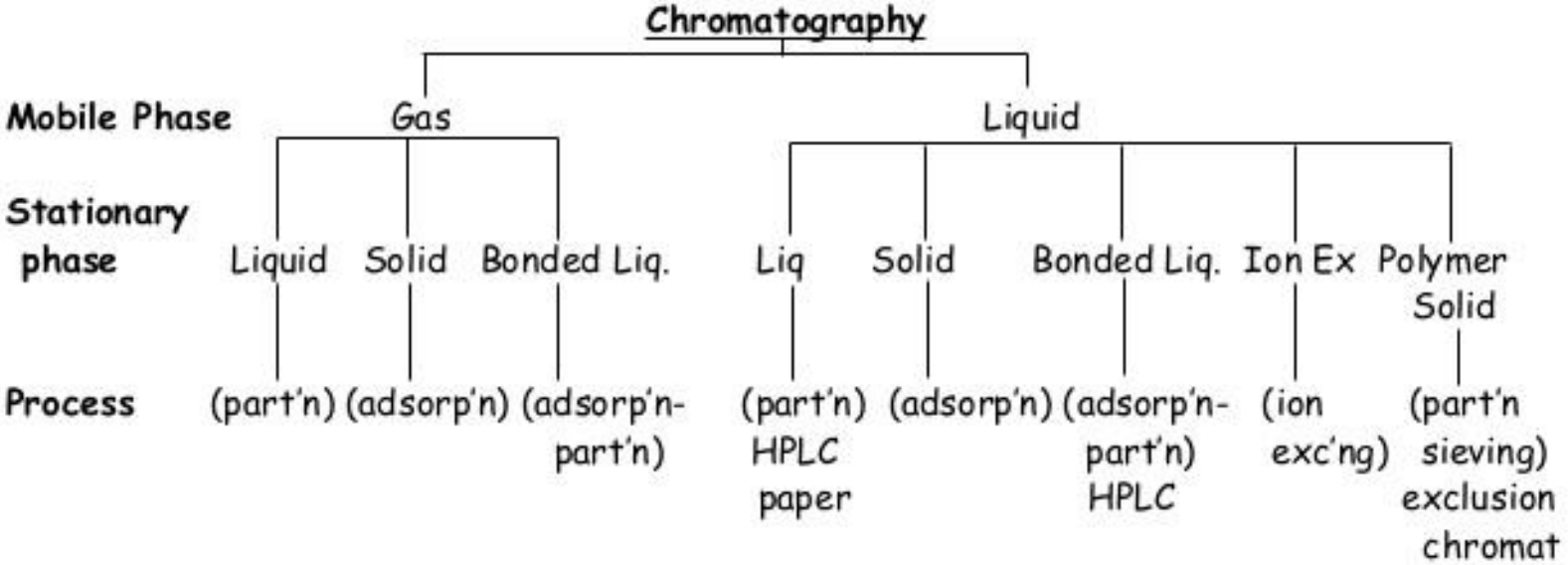
- **Paper Chromatography** – separates dried liquid samples with a liquid solvent (mobile phase) and a paper strip (stationary phase)

- **Thin- Layer Chromatography** – separates dried liquid samples with a liquid solvent (mobile phase) and a glass plate covered with a thin layer of alumina or silica gel (stationary phase)

Chromatography

Definition of chromatography: The differential migration of sample components dissolved in a mobile phase produced by selective retention by a stationary phase

Classification of chromatographic methods



Conditions associated with each kind of chromatography

- **Gas-liquid partition chromatography** involves establishing an equilibrium between components in the gaseous mobile phase and the liquid stationary phase

A. **Types of Chromatography**

- Planer Chromatography
  - Paper Chromatography
  - Thin layer Chromatography (TLC)
- Column Chromatography
  - Size exclusion Chromatography
  - Ion Exchange Chromatography
  - Affinity Chromatography
  - Reverse phase chromatography
  - Hydrophobic Interaction Chromatography
  - High performance liquid Chromatography (HPLC)
  - Gas Chromatography

B. **Classification of chromatographic method**

- Based on the shape of the Chromatographic bed
  - Planar Chromatography
  - Column Chromatography
- Based on the physical state of the mobile & stationary phases
  - Gas Chromatography
  - Liquid Chromatography
- Based on the mechanism of separation
  - Partition
  - Absorption
  - Size exclusion
  - Affinity
  - Ion exchange

HPLC

- HPLC- It was originally referred to as High Pressure Liquid Chromatography since high pressure is applied using a pumping system to the column.
- This pressure works by forcing the mobile phase through, at much higher rate increasing the resolution power.
- Due to its high efficiency and performance High Pressure Liquid Chromatography is referred to as High Performance Liquid Chromatography.

WHY USE HPLC?

- Simultaneous analysis
- High resolution
- High sensitivity
- Good repeatability
- Moderate analysis condition
- Easy to fractionate and purify
- Not destructive

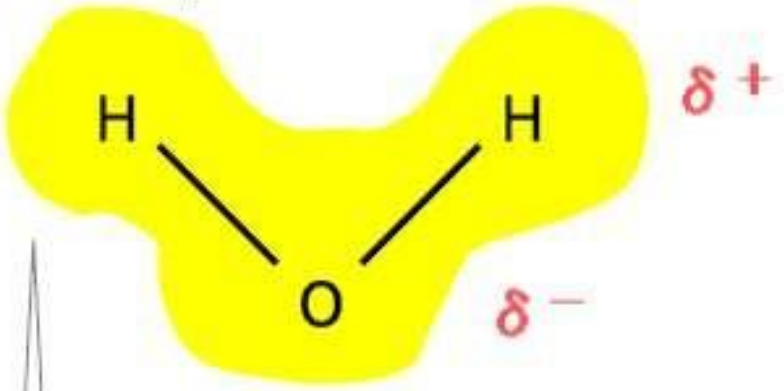
TYPES OF LIQUID CHROMATOGRAPHY

LC mode	Packing materials	Mobile phase	Interaction
Normal phase chromatography	Silica gel	n-Hexane/IPE	Adsorption
Reversed phase chromatography	Silica-C18(ODS)	MeOH/Water	Hydrophobic
Size exclusion chromatography	Porous polymer	THF	Gel permeation
Ion exchange chromatography	Ion exchange gel	Buffer sol.	Ion exchange
Affinity chromatography	Packings with ligand	Buffer sol.	Affinity

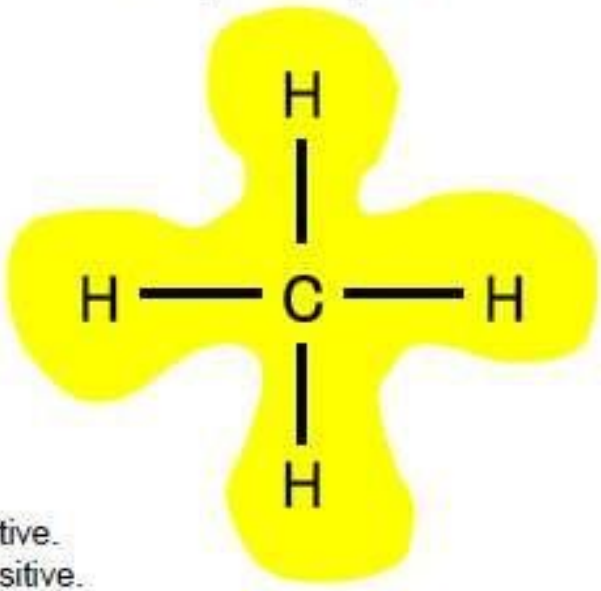
3. Separation mode

Polar compounds

Polar compound



Non-polar compound



Bonding electrons are not shared evenly.
The end of the bond with electrons becomes partially negative.
The end of the bondwithout electrons becomes partially positive.

Polar compounds are soluble in polar solvents.
Non-polar compounds are soluble in non-polar solvents.

1.NORMAL PHASE CHROMATOGRAPHY:

- Stationary Phase – Polar nature.

Eg: SiO_2,Al_2O_3

- Mobile Phase – Non-Polar nature.

Eg:heptane,hexane,cyclohexane,$CHCl_3$,CH_3OH

- Mechanism:

✓ Polar compounds travels slower & eluted slowly due to higher affinity tost.phase

✓ Non-polar compounds travels faster & eluted 1st due to lower affinity tost.phase.

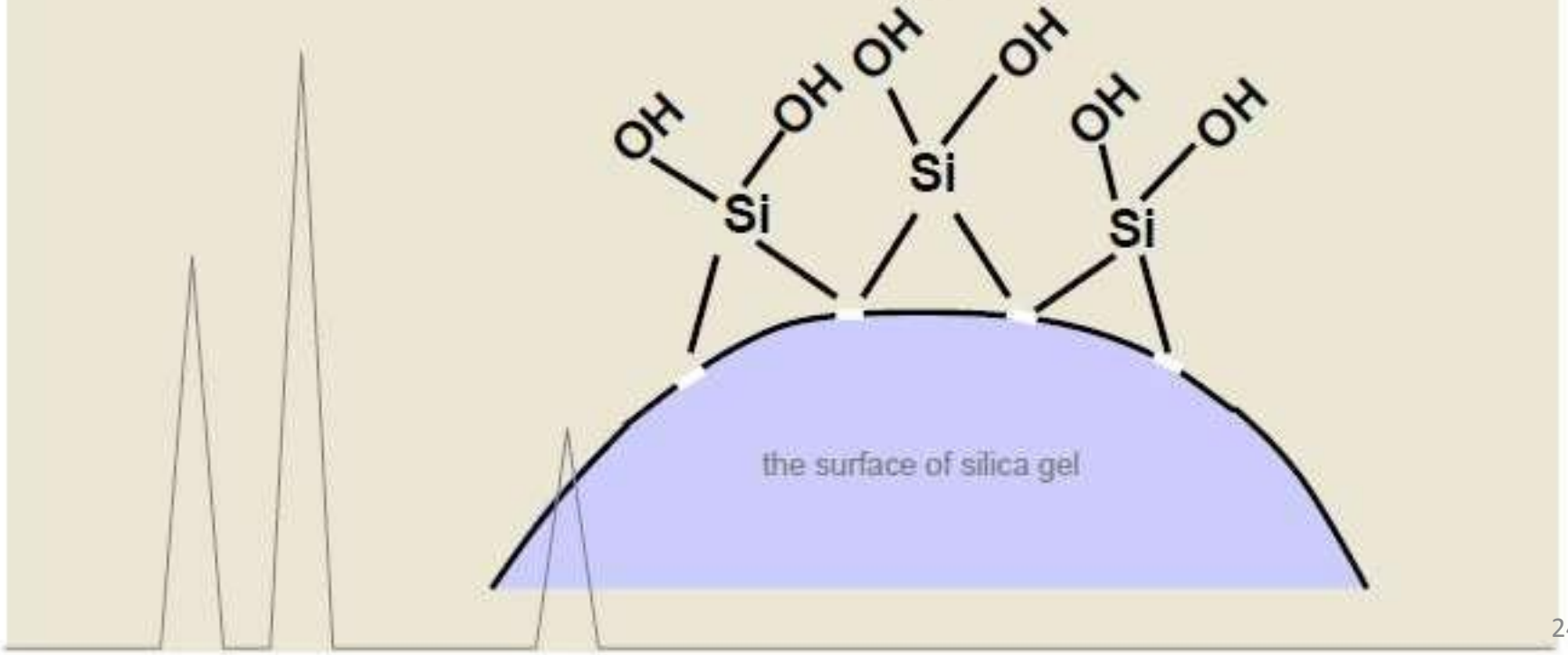
- This technique is not widely used in pharmaceutical separations.

3. Separation mode

Normal Phase Chromatography

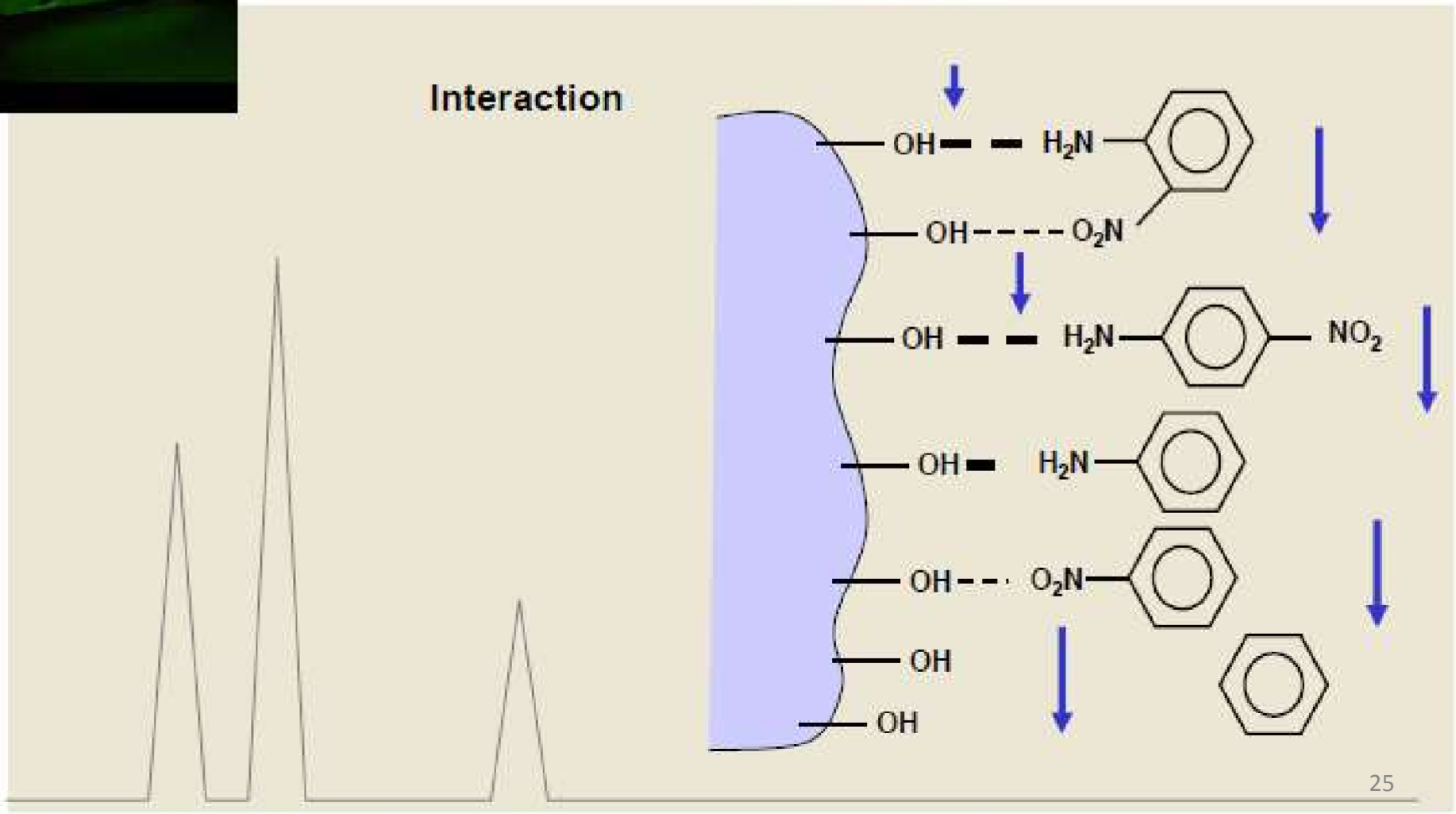
Packing material

The most popular packing material is silica gel.
It is believed that silanol radicals (-Si-OH) on the surface of silica gel act as the active site and the sample is separated.



3. Separation mode

Normal Phase Chromatography



Normal Phase Chromatography

Mobile phase solvents

n-Hexane	(n-Hex)
iso-Octane	(iso-Oct)
Chloroform	($CHCl_3$)
Dichloromethane	(CH_2Cl_2)
Ethylacetate	(AcOEt)
Isopropylalchol	(IPA)
Tetrahydrofran	(THF)
Dioxane	
Acetonitrile	(CH_3CN)
Ethanol	(EtOH)
Methanol	(MeOH)
Amines	
Acids	

2.REVERSE PHASE CHROMATOGRAPHY:

- Stationary Phase – Non-Polar nature.

  Eg: n-octadecyl, n-octyl, ethyl, phenyl diol, hydrophobic polymers.

- Mobile Phase – Polar nature.

  Eg: methanol or acetonitrile/water or buffer sometimes with additives of THF or dioxane.

- Mechanism:

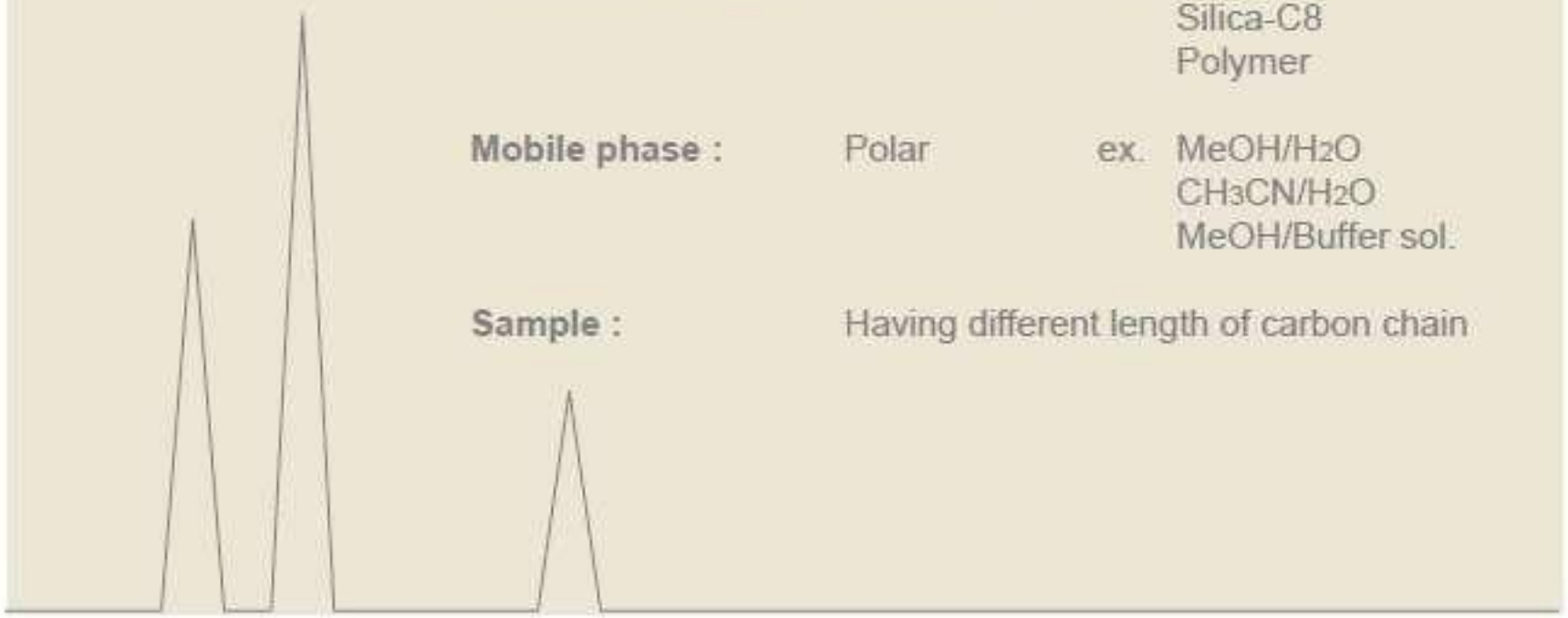
✓ Polar compounds travels faster & eluted 1st due to lesser affinity to st.phase

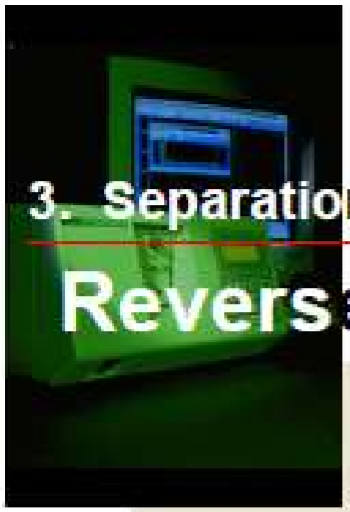
✓ Non-Polar compounds travels slower & eluted slowly due to higher affinity tost.phase

3. Separation mode

Reversed Phase Chromatography

Interaction :	Hydrophobic		
Packing materials :	Non-polar	ex.	Silica-C18 Silica-C8 Polymer
Mobile phase :	Polar	ex.	MeOH/H_2O CH_3CN/H_2O MeOH/Buffer sol.
Sample :	Having different length of carbon chain		

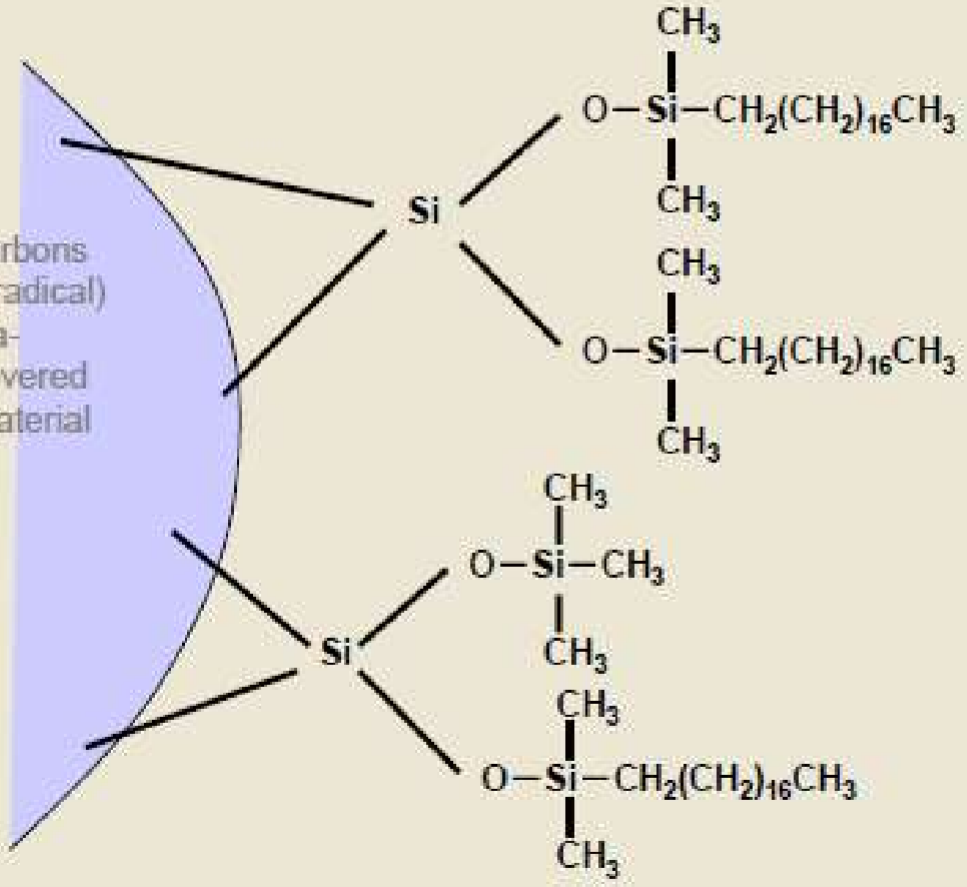




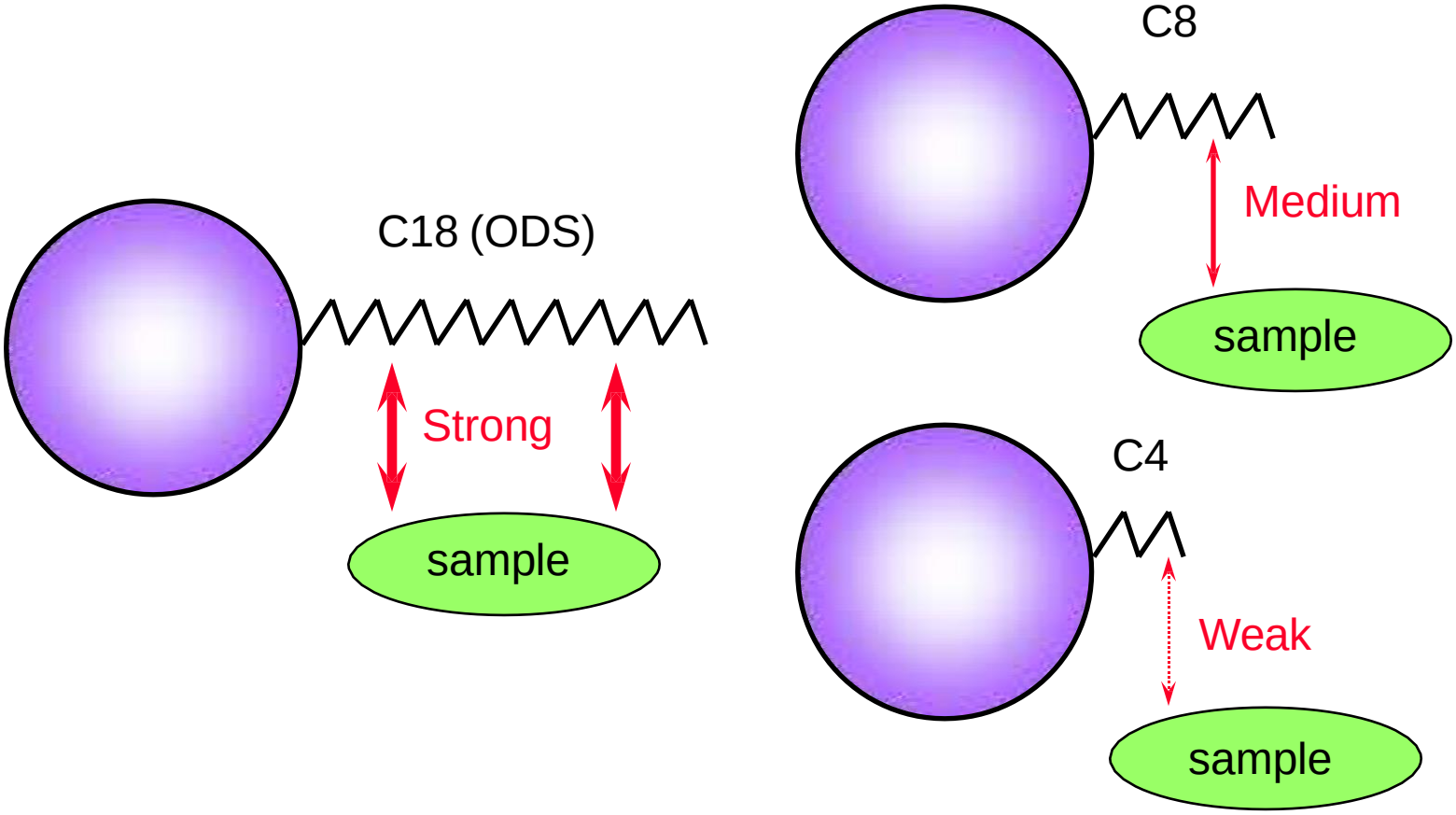
Reversed Phase Chromatography

Silica-C18 Packing materials

Commonly used packing materials are hydrocarbons having 18 carbon atoms (called the Octadecyl radical) which are chemically bonded to silica gel (Silica-ODS).Since the surface of the Silica-ODS is covered with hydrocarbon, the polarity of the packing material itself is very low.

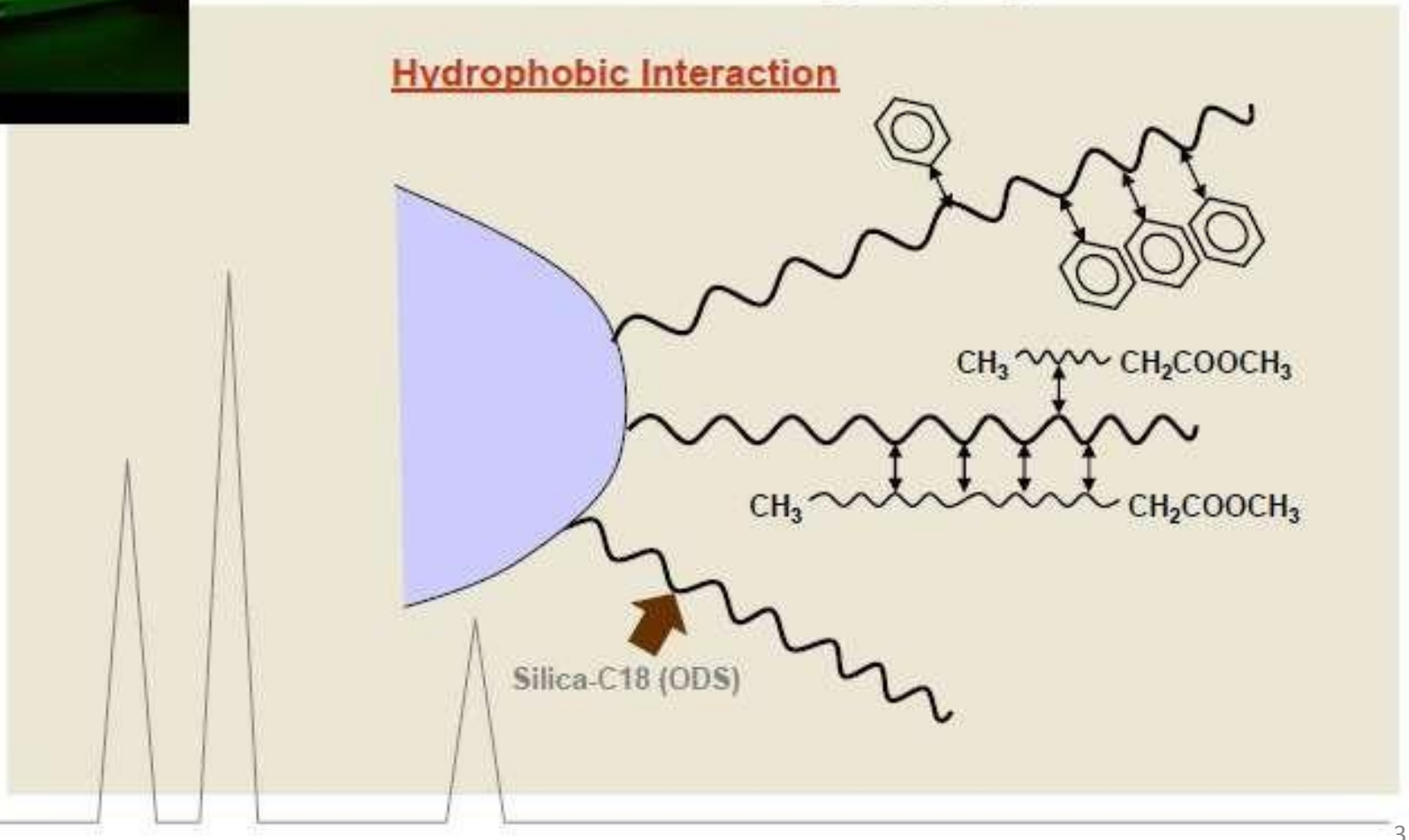


Effect of Stationary Phase



3. Separation mode

Reversed Phase Chromatography



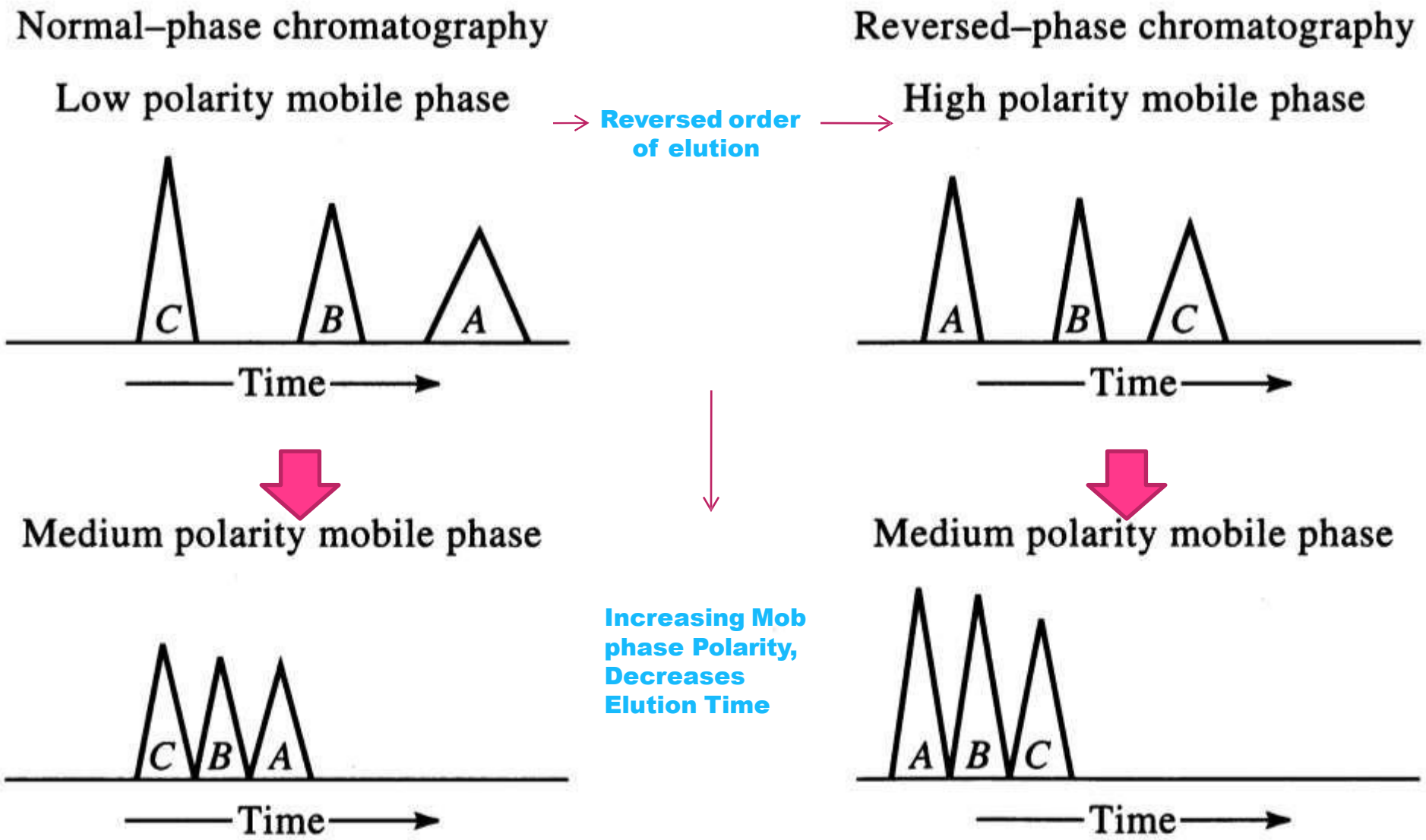
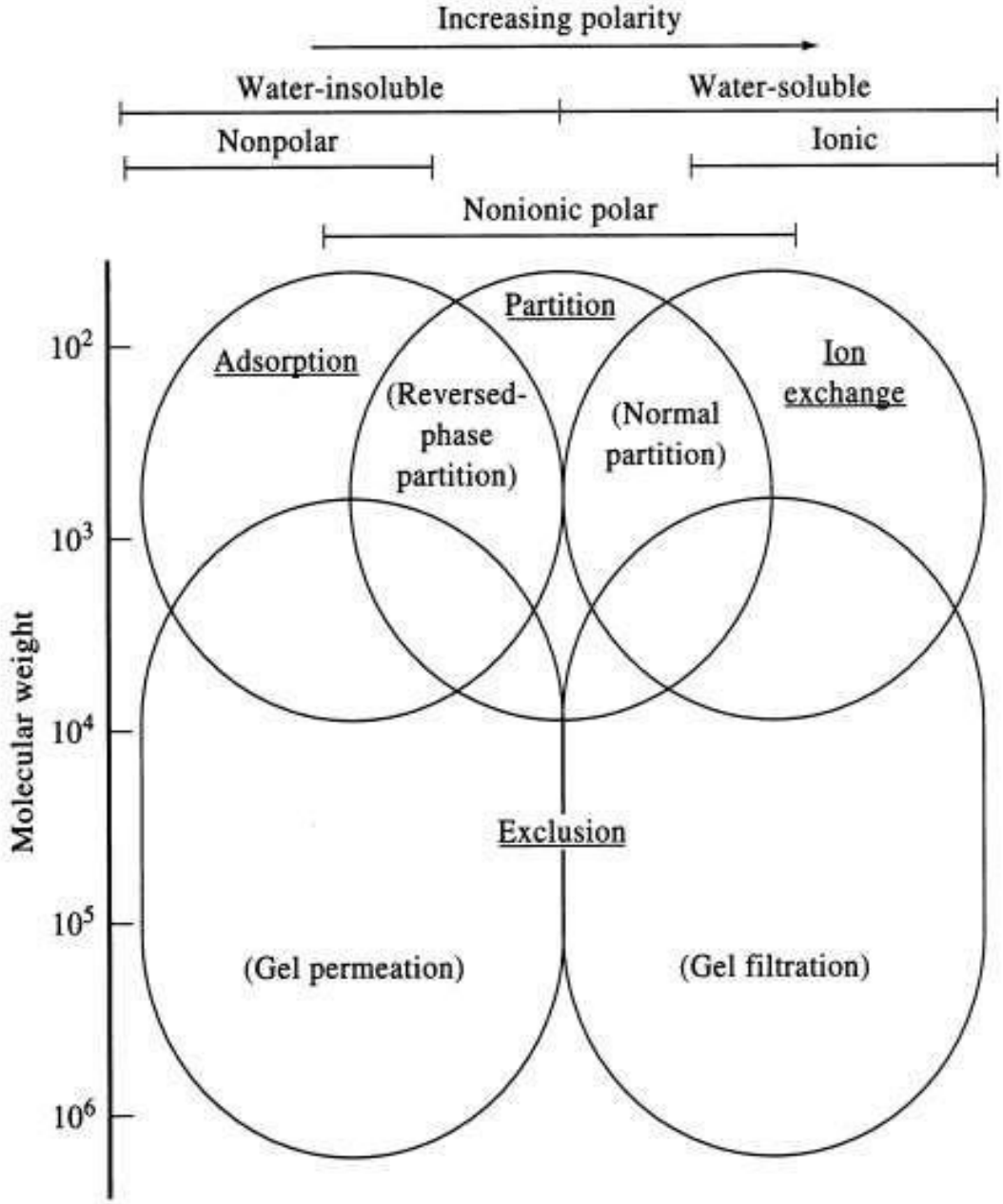


Figure 28-14 The relationship between polarity and elution times for normal-phase and reversed-phase chromatography.

TYPES OF HPLC DEPENDS ON:



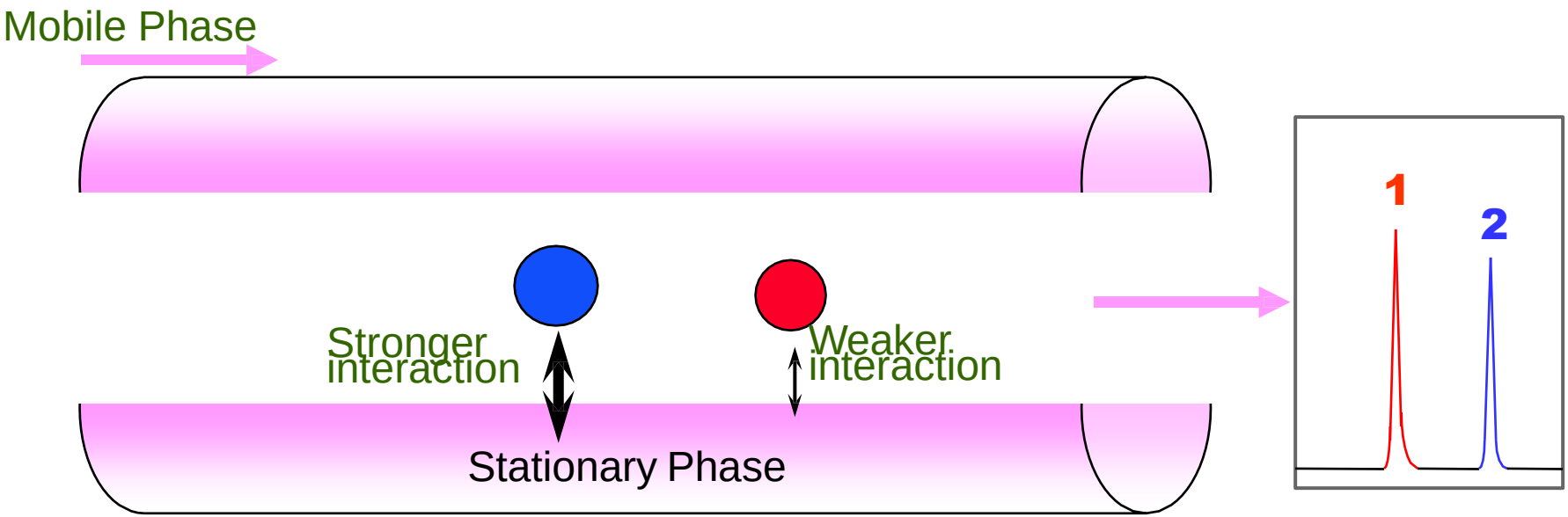
- Molecular weightof solute
- Water solubility ofsolute
- Polarity of solute
- Ionic and non-ionic character of solute

ADSORPTION CHROMATOGRAPHY:

- The principle of separation is adsorption.
- Separation of compounds takes place based on the difference in the affinity of the compunds towards stationary phase as in the normal and reverse phase.
- The lesser the affinity of the sample particles towards the stationary phase the faster the time of elution of the sample.

Separation Mechanism

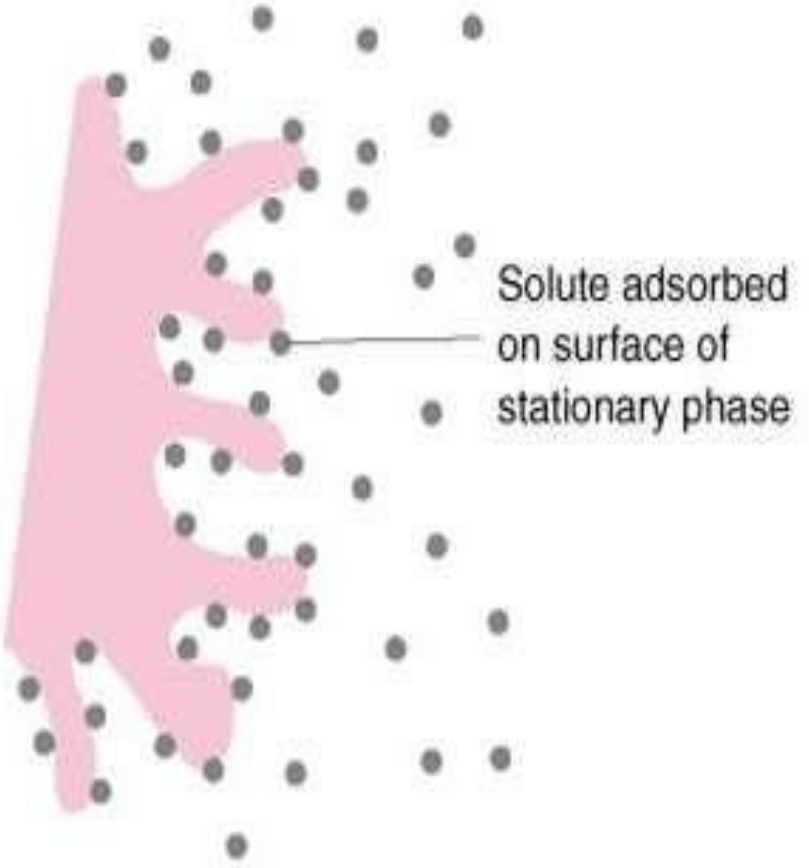
Due to different interaction between stationary phase and different sample, the molecules move at different rate, therefore separation can be done.



PARTITION CHROMATOGRAPHY:

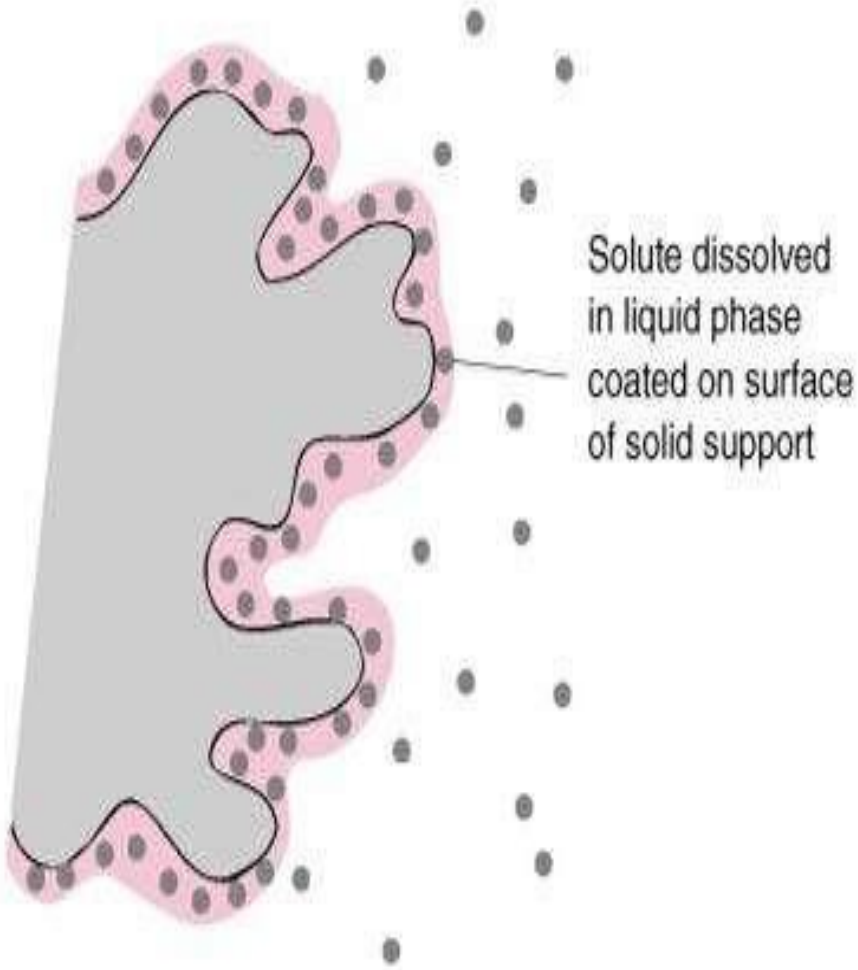
- In this the stationary phase is a liquid which is coated on the solid support on the column.
- The mobile phase is also a liquid.
- When solute along with the mobile phase is passed over the stationary phase it gets dissolved to the surface of the liquid coated to the solid support.
- The compounds which have more partition co-efficient are eluted slowly when compared to the compounds with low partition co-efficient.

ADSORPTION CHRT:



Adsorption chr omatography

PARTITION CHRT:

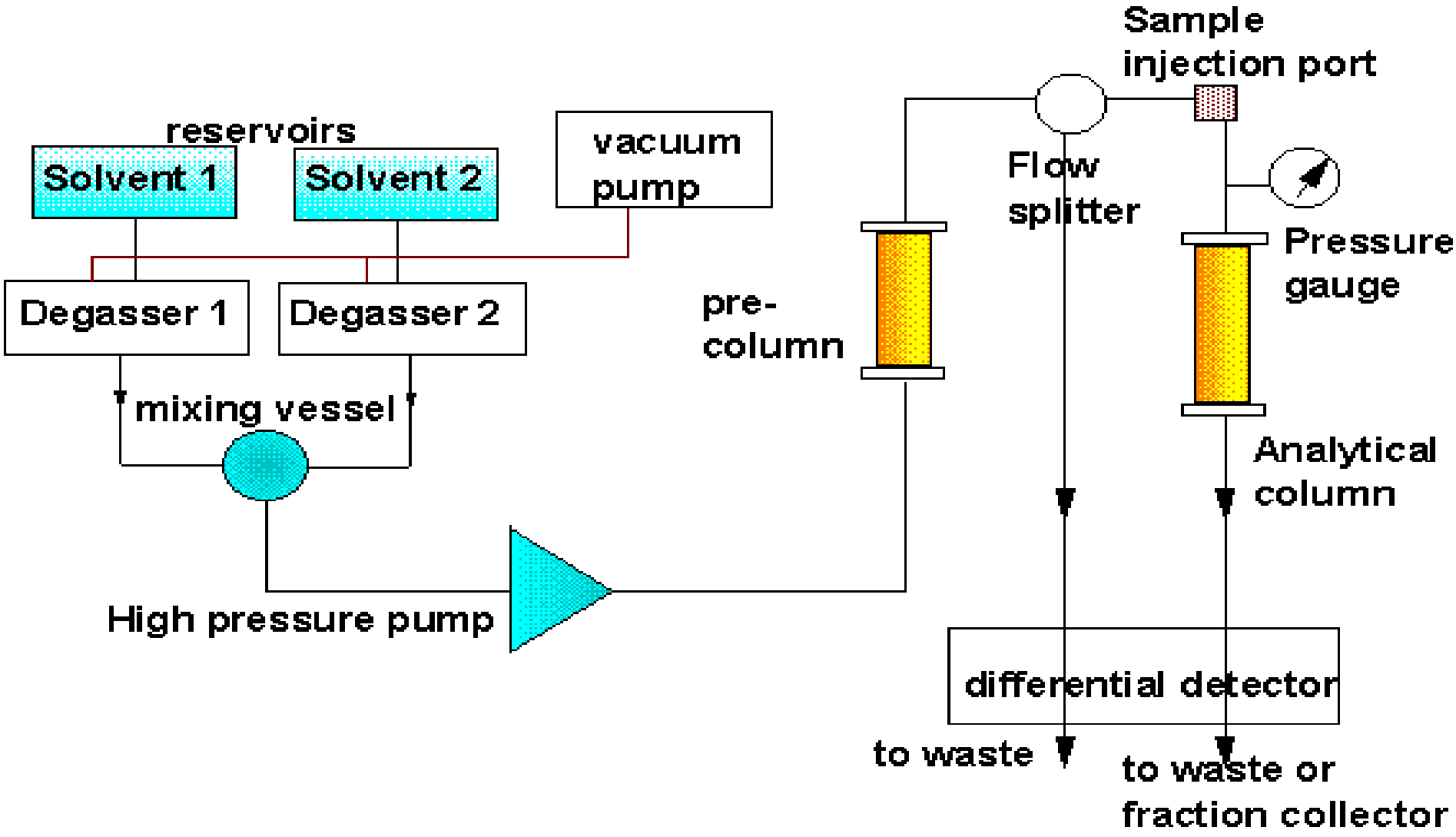


Partition chromatography

INSTRUMENTATION OF HPLC

- Solvent storage bottle
- Gradient controller and mixing unit
- De-gassing of solvents
- Pump
- Pressure gauge
- Pre-column
- Sample introduction system
- Column
- Detector
- Recorder

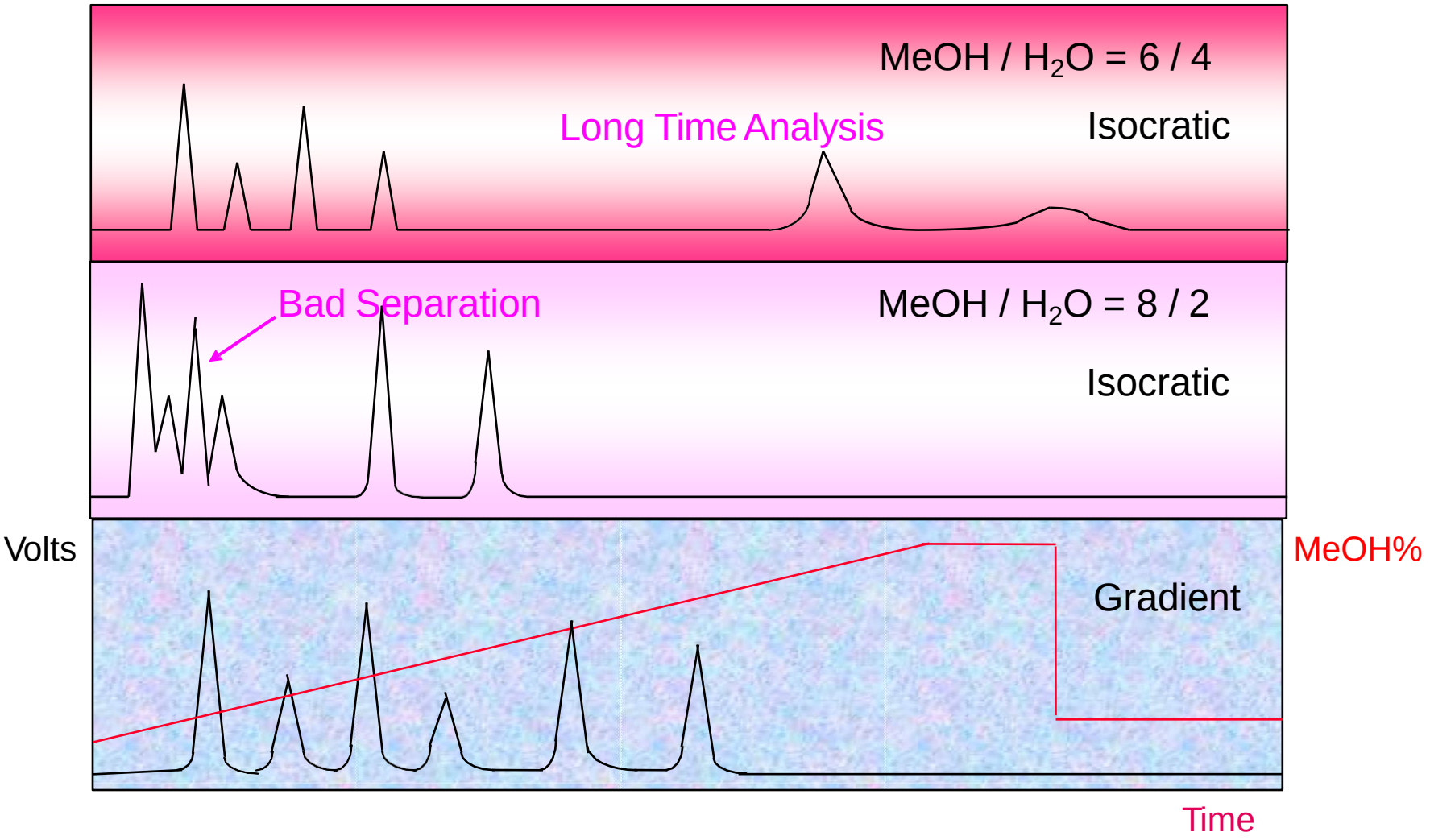
FLOW DIAGRAM OF HPLC INSTRUMENT



❖GRADIENT CONTROLLER

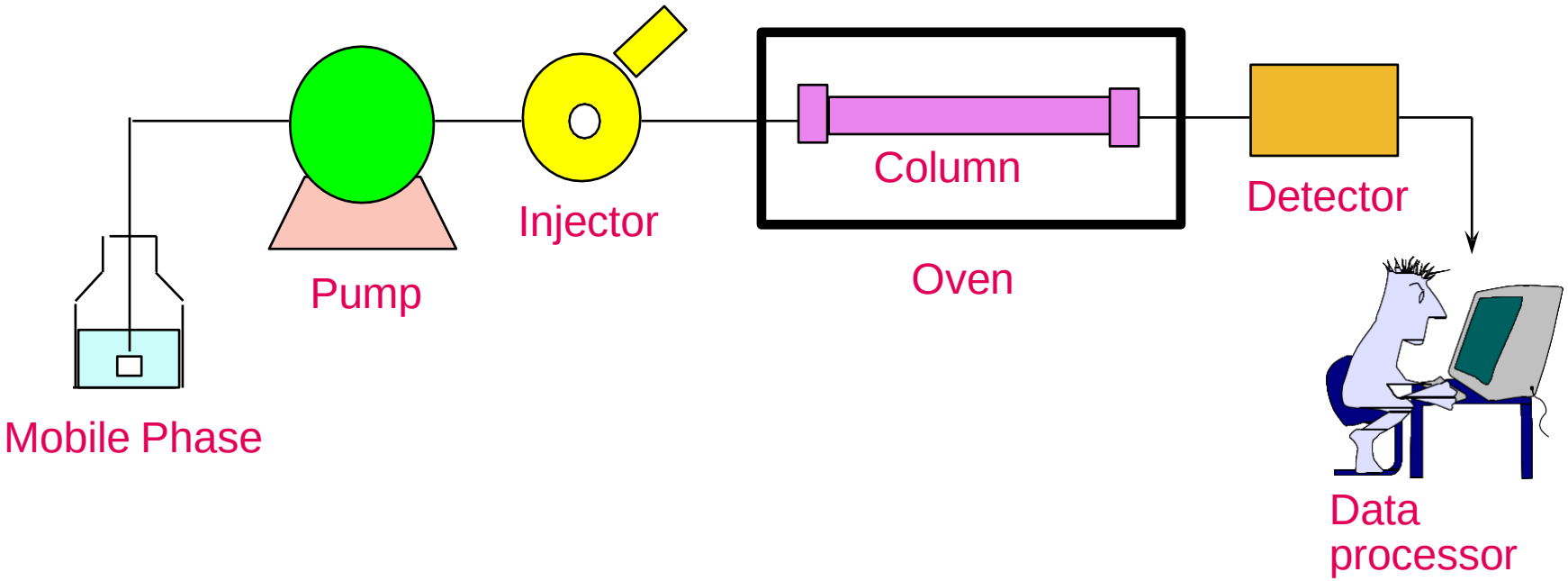
- Isocratic solvents- mobile phase is prepared by using pure solvent or mixture of solvents which has same eluting power or polarity.
- Gradient solvents- in this the polarity of the solvent is gradually increased & hence the solventcomposition has to be changed.
- Hence this gradient controller is used when two or more solvent pumps are used for such separations.

Elution Modes



(Column : ODS)

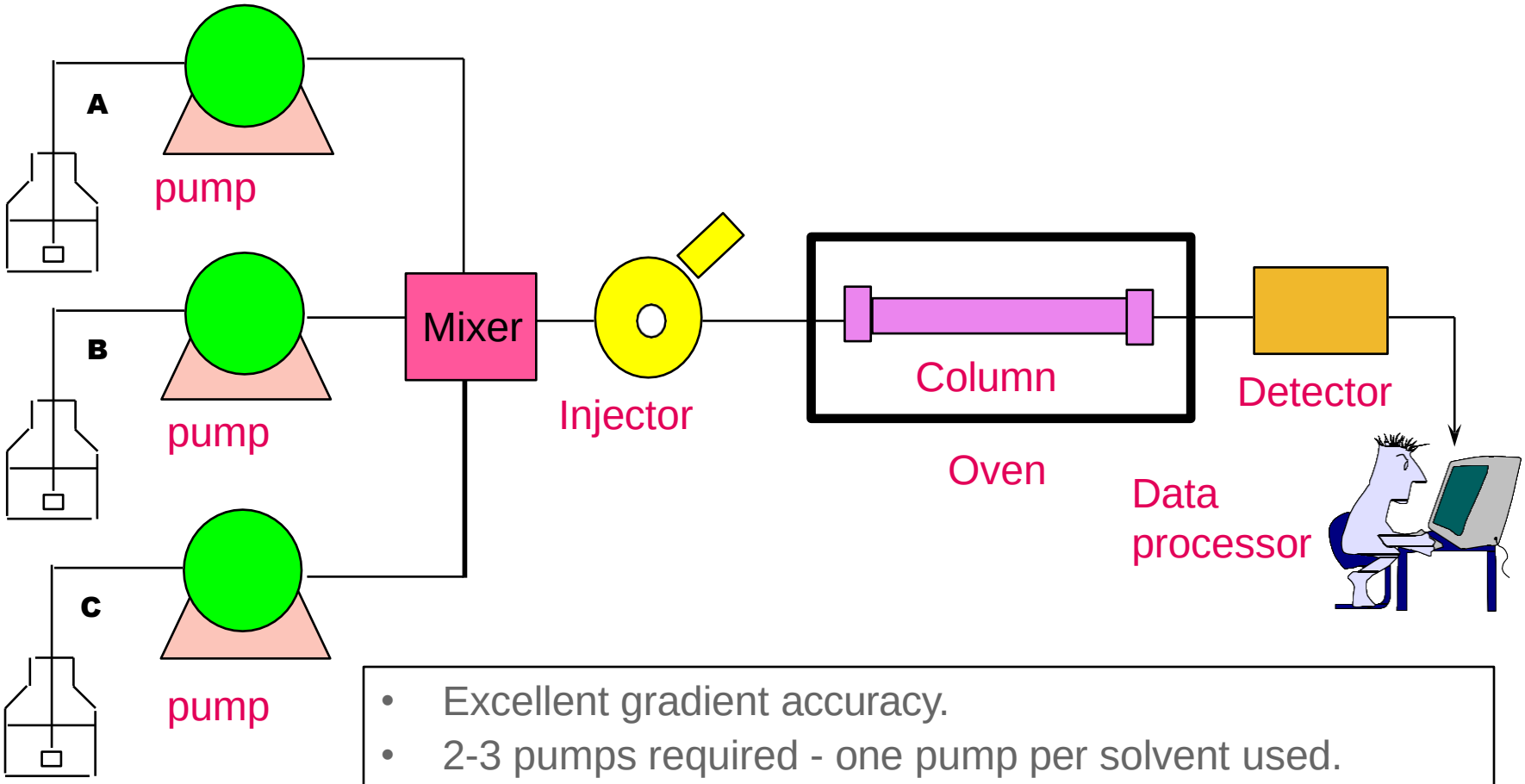
Isocratic System



Simple system with one pump and one solvent reservoir.

If more than one solvent is used, solvents should be premixed.

High-pressure Gradient System



- Excellent gradient accuracy.
- 2-3 pumps required - one pump per solvent used.
- On-line degassing may not be critical.

❖MIXING UNITS:

Mixing units

- Low pressure-mixing chamber which uses Helium for de-gassing
- High pressure-mixing chamber does not require Helium for de-gassing

Mixing of solvents done by

- Static mixer-which is packed with beads
- Dynamic mixer-with magnetic stirrer & operates under high pressure

❖DEGASSING OF SOLVENTS:

- Several gases are soluble in organic solvents, when high pressure is pumped, the formation of gas bubbles increases which interferes with theseparation process, steady baseline & shape of the peak.
- Hence de-gassing is very important and it can be done by various ways.

(I) VACUUM FILTRATION:

- ✓ De-gassing is accomplanished by applying a partial vacuum to the solvent container.
- ✓ But it is not always reliable & complete.

(II) HELIUM PURGING:

- ✓ Done by passing Helium through the solvent.
- ✓ This is very effective but Helium is expensive.

(III) ULTRASONICATION:

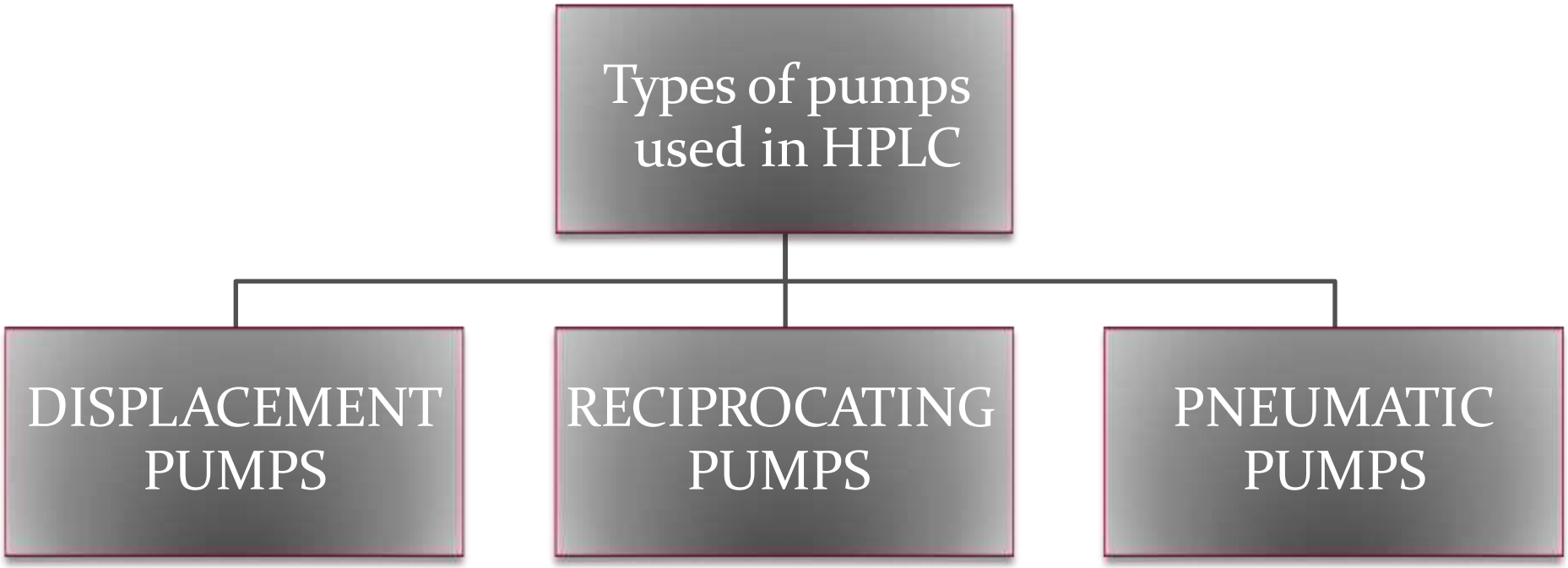
- ✓ Done by using ultrasonicator which converts ultra high frequency to mechanical vibrations.

❖ PUMP:

- The solvents or mobile phase must be passed through a column at high pressures at up to 6000 psi(lb/in^2) or 414 bar.
- As the particle size of stationary phase is smaller (5 to 10µ) the resistance to the flow of solvent will be high.
- That is, smaller the particle size of the stationary phase the greater is the resistance to the flow of solvents.
- Hence high pressure is recommended.

➢REQUIREMENTS FOR PUMPS:

- Generation of pressure of about 5000 psi.
- Pulse free output & all materials in the pump should be chemically resistant to solvents.
- Flow rates ranging from 0.1 to 10 mL/min
- Pumps should be capable of taking the solvent from a single reservoir or more than one reservoir containing different solvents simultaneously.

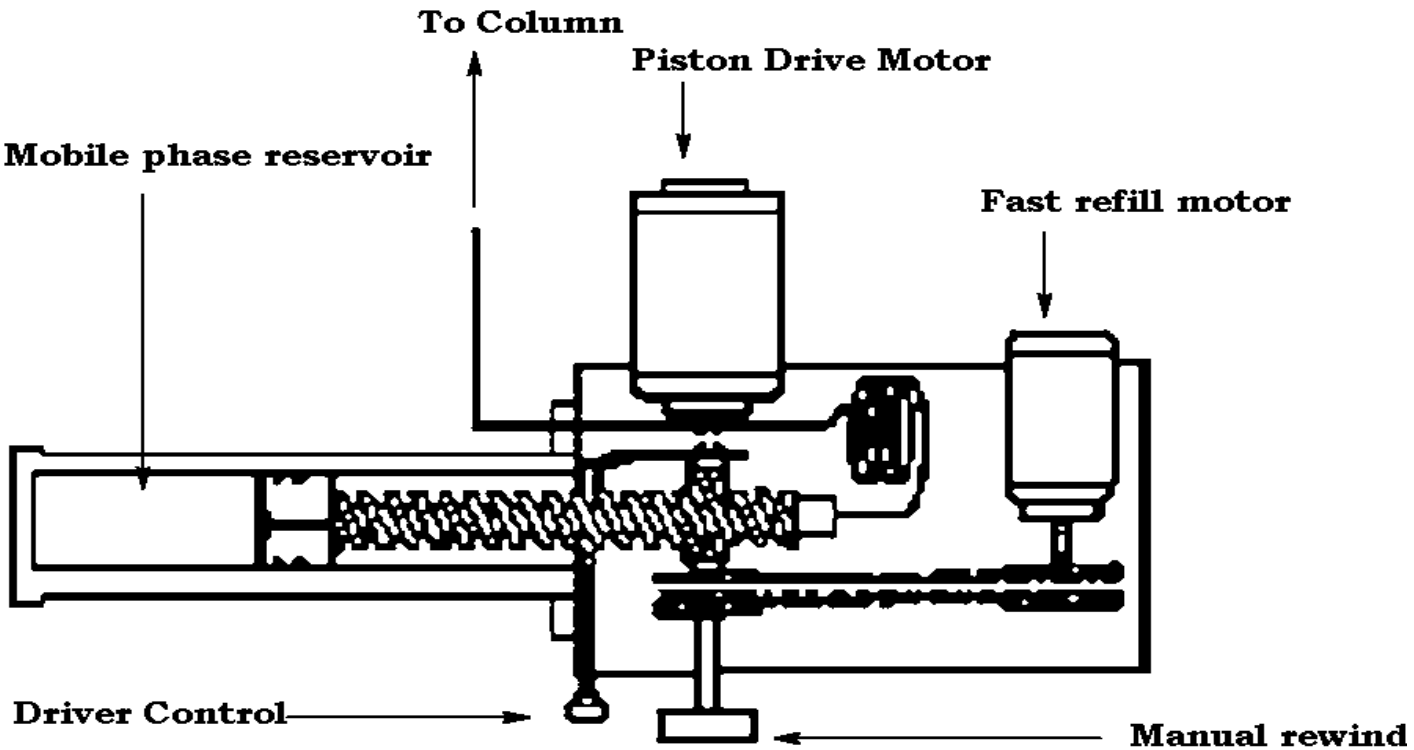

Types of pumps used in HPLC
DISPLACEMENT PUMPS
RECIPROCATING PUMPS
PNEUMATIC PUMPS

PUMP A

PUMP B

➢DISPLACEMENT PUMPS

- It consists of large, syringe like chambers equipped with a plunger activated by a screw driven mechanism powered by a stepping motor.
- So it is also called as Screw Driven Syringe Type Pump.
- Advantages:- It produces a flow that tends to be independent of viscosity & back pressure.
- Disadvantages:- It has a limited solvent capacity(~250) & considerably inconvenient when solvents must bechanged.



DISPLACEMENT PUMP

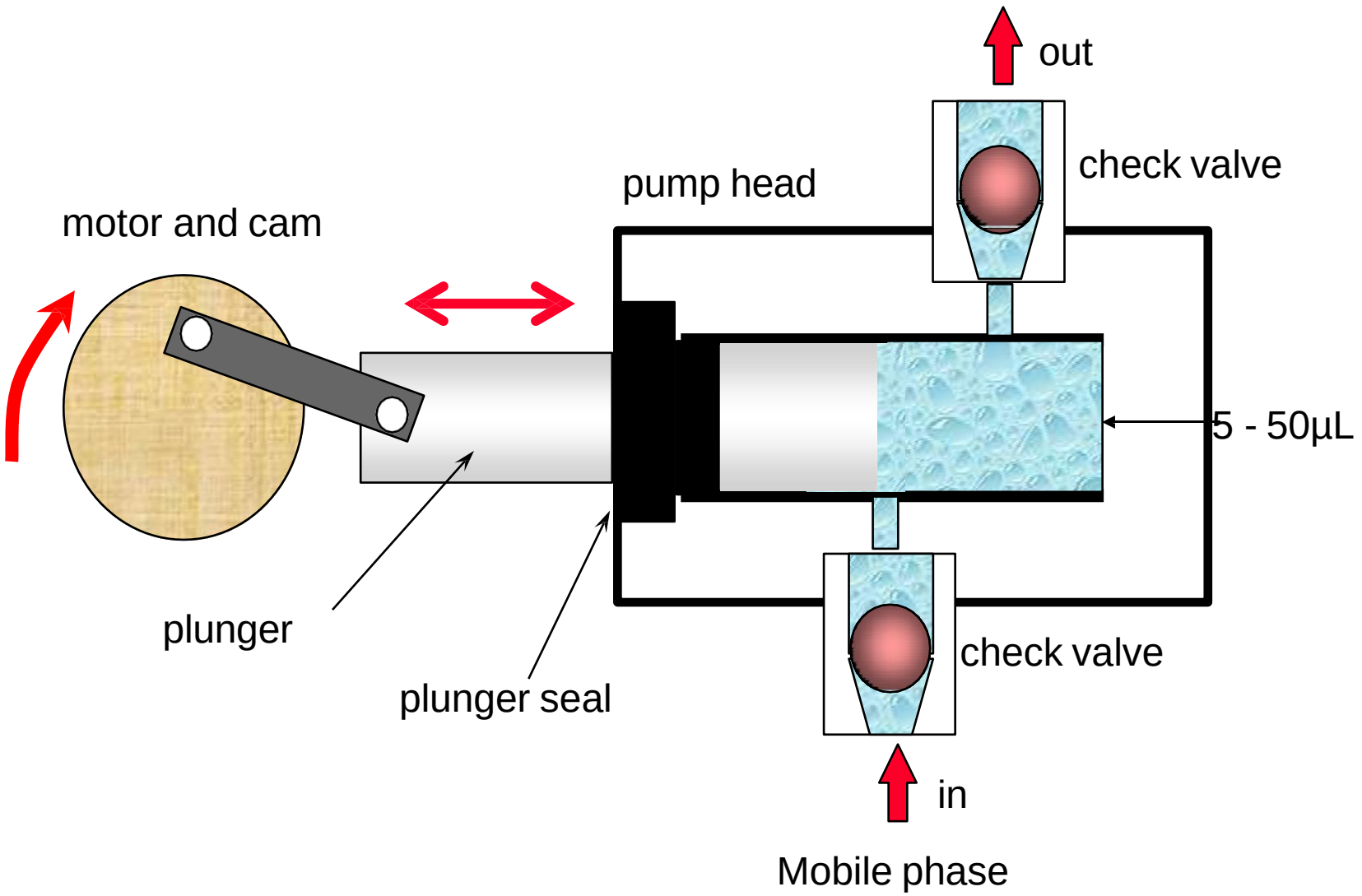
➢RECIPROCATING PUMPS:

- This pump transmits alternative pressure to the solvent via a flexible diaphragm ,which in turn is hydraulically pumped by a reciprocating pump.
- **DISADVANTAGES**:-

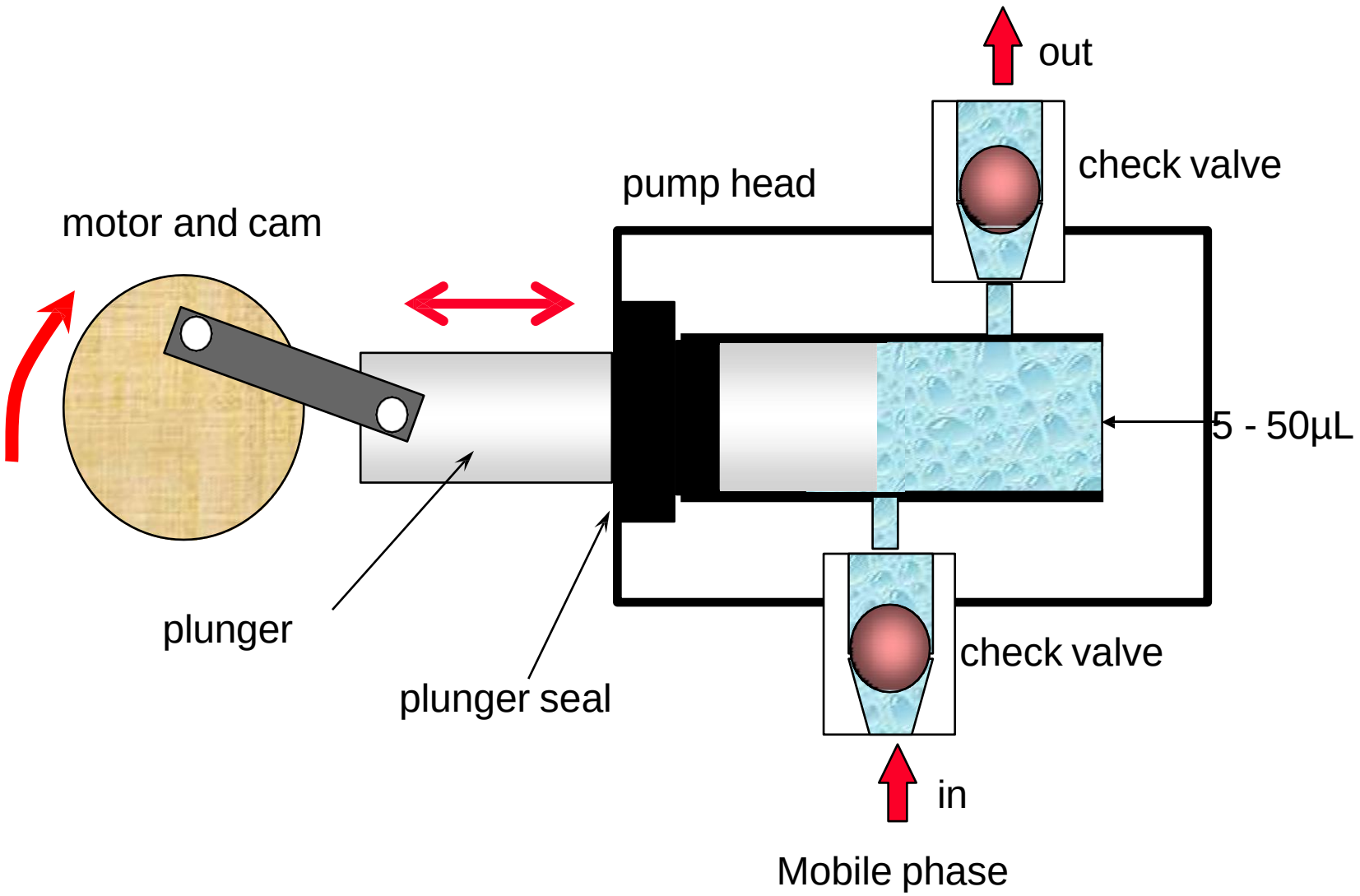
  Produces a pulsed flow which is damped because pulses appear as baseline noise on the chromatograph.

  This can be overcome by use of dual pump heads or elliptical cams to minimize such pulsations.

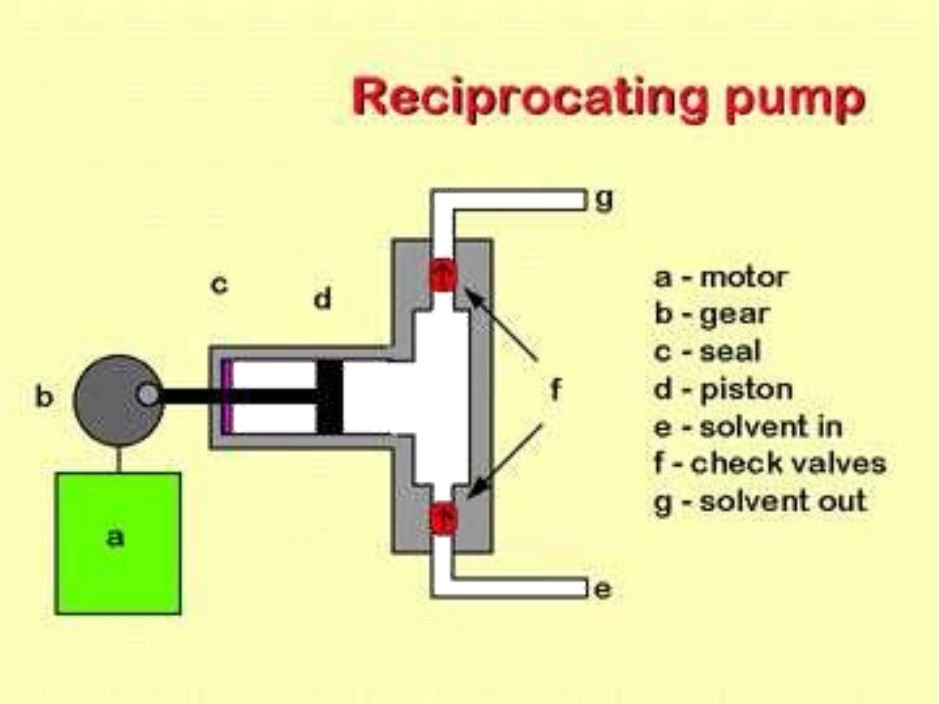
Plunger Reciprocating Pump



Plunger Reciprocating Pump



- Solvent is pumped back and forth by a motor driven piston
- Two ball check valves which open & close which controls the flow
- The piston is in direct contact with the solvent
- Small internal volume 35-400µL
- High output pressure upto 10,000 psi
- Ready adaptability togradient elution and constant flow rate

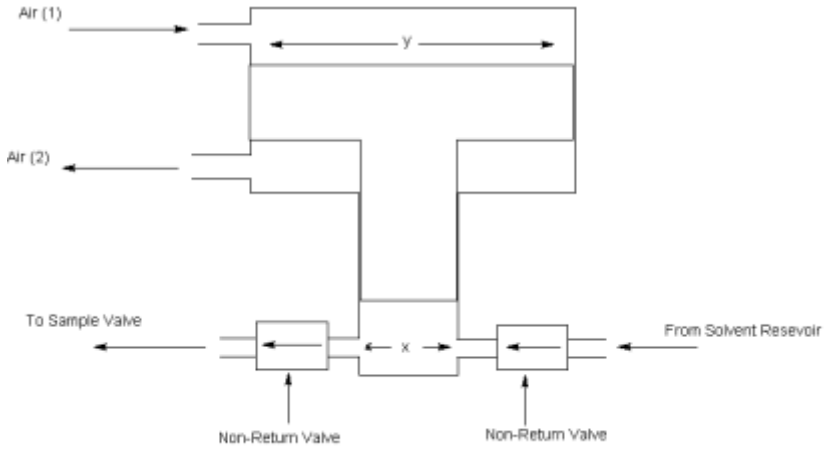


ADVANTAGES:

- Have small internal volume of 35-400µL
- Higher output pressures up to 10,000 psi.
- Adaptability to gradient elution.
- Large solvent capacities & constant flow rates.
- Largely independentof column back pressure & solvent viscosity.

➢ PNEUMATIC PUMPS:

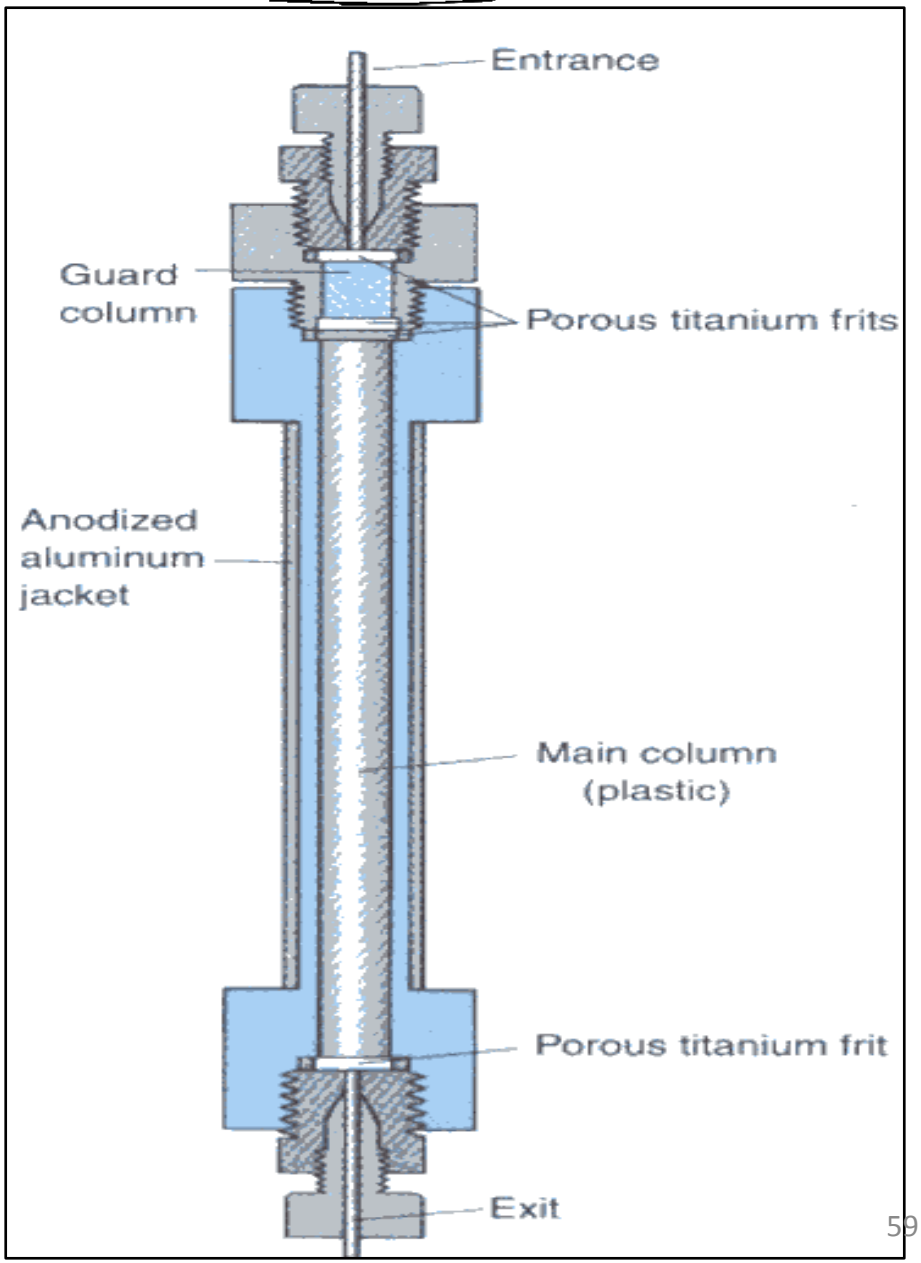
- In this pumps, the mobile phase is driven through the columnwith the use of pressure produced from a gas cylinder.
- It has limited capacityof solvent



❖ COLUMN:

- There are various columns that can be used in HPLC method.
- They are as follows:

- Guard Column
- Derivatizing Column
- Capillary Column
- Fast column
- Analytical Column
- Preparatory Column



➢GUARD COLUMN:

- Guard columns are placed anterior to the separating column.
- This protects and prolongs the life & usefulness of the separating column.
- They are dependable columns designed to filter or remove:-

✓ particles that clog the separating column,

✓ compounds and ions that could ultimately cause 'baseline drift', decreased resolution, decreased sensitivity and create false peaks.

- ✓ Compounds that may cause precipitation upon contact with the stationary or mobile phase.
- ✓ Compounds that may co-elute and cause extraneous peaks & interfere with the detection and quantification.
- ✓ These columns must be changed on a regular basis in order to optimize theirprotectiveness.

➢ DERIVATIZING COLUMN

- Derivatization involves a chemical reaction between an analyte and a reagent to change the chemical and physical properties of an analyte.
- The four main uses of derivatization in HPLC are:

✓ Improve detectability,

✓ Change the molecular structure or polarity of analyte for better chromatography,

✓ Change the matrix for better separation,

✓ Stabilize a sensitive analyte.

- Pre or post primary column derivatization can be done.
- Derivatization techniques includes –acetylation, silylation, acid hydrolysis.
- **DISADVANTAGES**: It becomes a complexprocedure and so it acts as a source of error to analysis and increases the total analysistime.
- **ADVANTAGES**: Although derivatization has drawbacks, it may still be required to solve a specific separation or detectionproblem.

➢CAPILLARY COLUMNS:

- HPLC led to smaller analytical columns called as micro-columns, capillary columns which have diameter less than a millimeter.
- Sample used – is in nanolitre volumes, decreased flow rate, decreased solvent volume usage which leads to cost effectiveness.
- Disadvantage:- since it is miniatured flow rate is difficult to produce & gradient elution is not efficient.

- MICROBORE and SMALLBORE columns are also used for analytical and small volumes assay.
- Diameter of small-borecolumns is 1-2mm.
- The instrument must also be modified to accommodate these smaller capacitycolumns.

➢ FAST COLUMNS:

- This column also have the same internal diameter but much shorter length than most other columns & packed with particles of 3µm in diameter.
- Increased sensitivity, decreased analysis time, decreased mobile phase usage & increased reproducibility.

➢ ANALYTICAL COLUMN:

- This is the most important part of HPLC which decides the efficiency of separation
- Length- 5 to 25 cm ,Internal Diameter 3 to 5mm.
- Particle size of packing material is 3 to 5µm.
- LC columns achieve separation by different intermolecular forces b/w the solute & the stationary phase and those b/w the solute & mobile phase.

➢PREPARATORY COLUMN:

- Length – 10 to 15 cm, Int. diameter – 4.6mm
- Packed with particles having 5µm asdiameter.
- Columns of this time generate 10,000 plates per column.
- It consists of back pressure regulator and fraction collector.
- This back pressure regulator is placed posterior to the HPLC detector.

❖SAMPLE INJECTOR SYSTEM:

- Several injector devices are available either for manual orauto injection of the sample.

(i) Septum Injector

(ii)Stop Flow Injector

(iii)Rheodyne Injector

Rheodyne Manual injector

(I)SEPTUM INJECTOR:

- These are used for injecting the sample through a rubber septum.
- This kind of injectors cannot be commonly used , since the septum has to withstand high pressures.

(II)STOP FLOW(ON LINE):

- In this type the flow of mobile phase is stopped for a while & the sample is injected through a valve.

(III)RHEODYNE INJECTOR:

- It is the most popular injector and is widely used.
- This has a fixed volume of loop, for holding sample until its injected into the column, like 20µL, 50µL or more.
- Through an injector the sample is introduced into the column.
- The injector is positioned just before the inlet of the column.

SELECTION VALVE:
By using the selection valve we can select whether the purpose is for analytical purpose or preparative purpose.

LOAD POSITION:
In this position the sample is loaded into the sample loop.

INJECT POSITION: In this position the loaded sample is injected into the column by the forcful flow of the solvent into the sample loop by which the sample is introduced into the column.

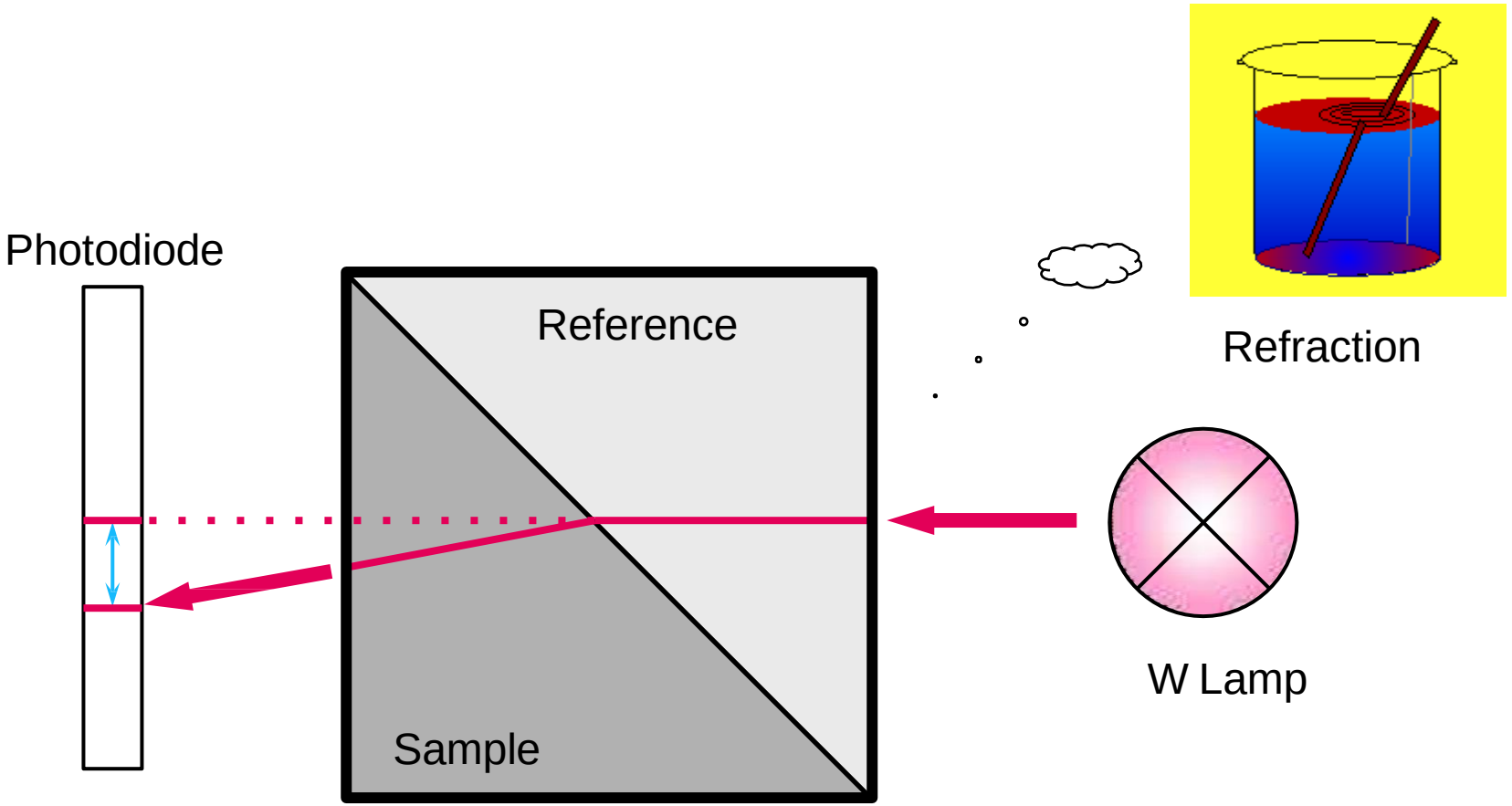
❖ DETECTORS:

- Absorbance (UV/Vis or PDA)
- Refractive index (detects the change in turbidity)
- Fluorescence (if the analyte is fluorescent)
- Electrochemical (measures current flowing through a pair of electrodes, on which a potential difference is imposed, due to oxidation or reduction of solute)
- Conductivity (for ions)
- Light scattering
- Mass spectrometry (HPLC-MS)

❖ ABSORBANCE DETECTORS:

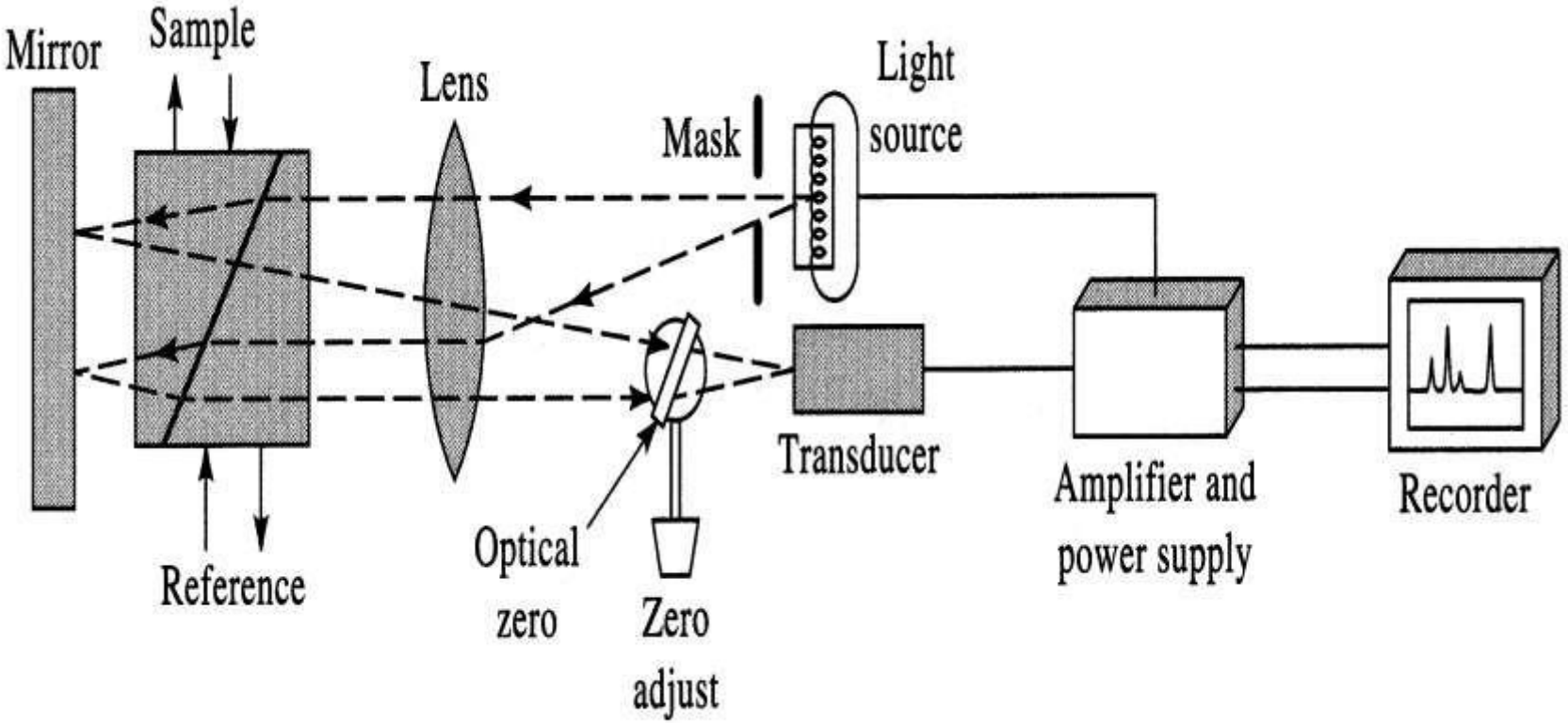
- The UV/Vis source usually comes from a monochromatorso the wavelength can be selected, or scanned.
- If wavelength scanning is desired, the flow is stopped long enough for the scan to take place.
- Fixed wavelength-measures at single wavelength usually 254nm.
- Variable wavelength-measures at single wavelength at a time but can detect over a wide range of wavelengths simultaneously.

Refractive Index Detector



REFRACTIVE INDEX (RI) DETECTOR:

- Nearly universal but poor detectionlimit.



- Detection occurs when the light is bent due to samples eluting from the columns, and this isread as a disparity b/w the two channels.
- It is not much used for analytical applications because of low sensitivity & specificity.
- When a solute is in the sample compartment, refractive index changes will shift the light beam from the detector.

➢FLUORIMETRIC DETECTORS:

- It is based on the fluorescent radiation emitted by some compounds.
- The excitation source passes through the flow cell to a photodetector while a monochromator measures the emission wavelengths.
- More sensitive and specific.
- The disadvantge is that most compounds are not fluorescent in nature.

Fluorescence of Compounds

Fluorescence is a type of luminescence in which the light energy is released in the form of a photon in nanoseconds to microseconds

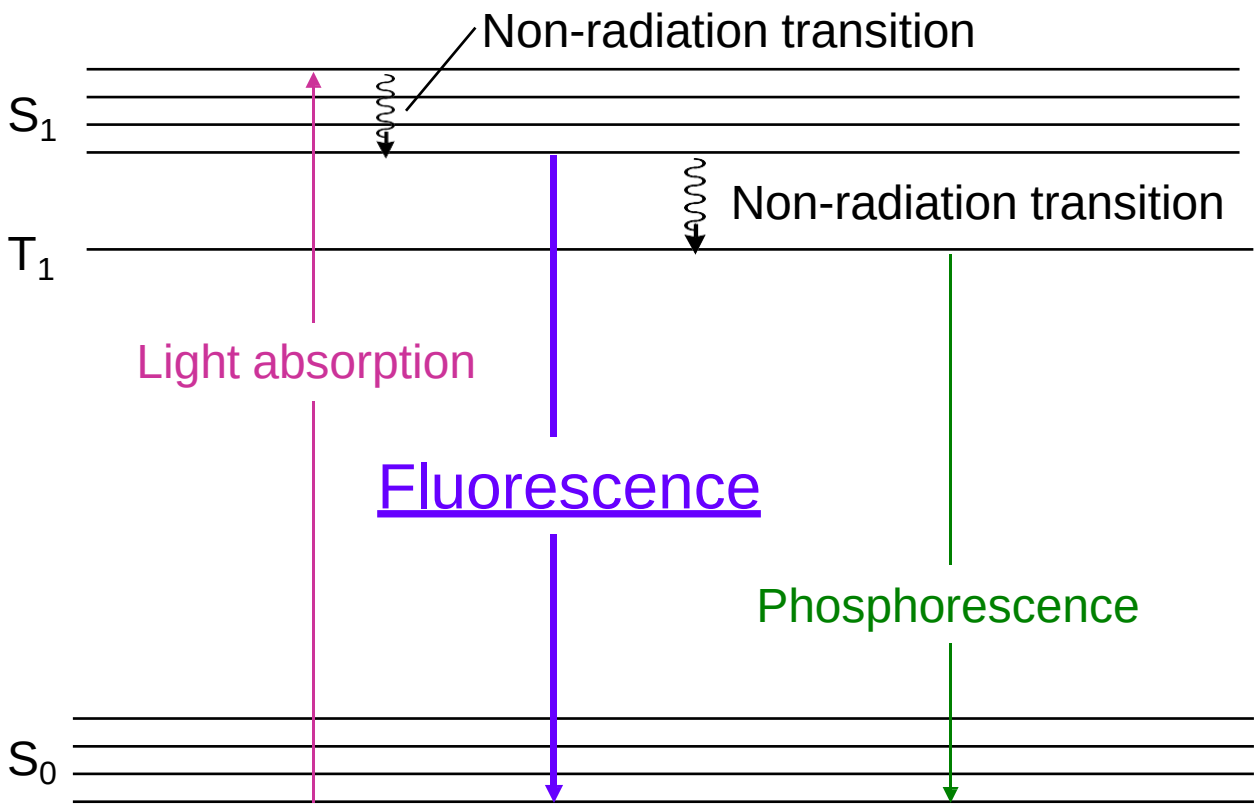
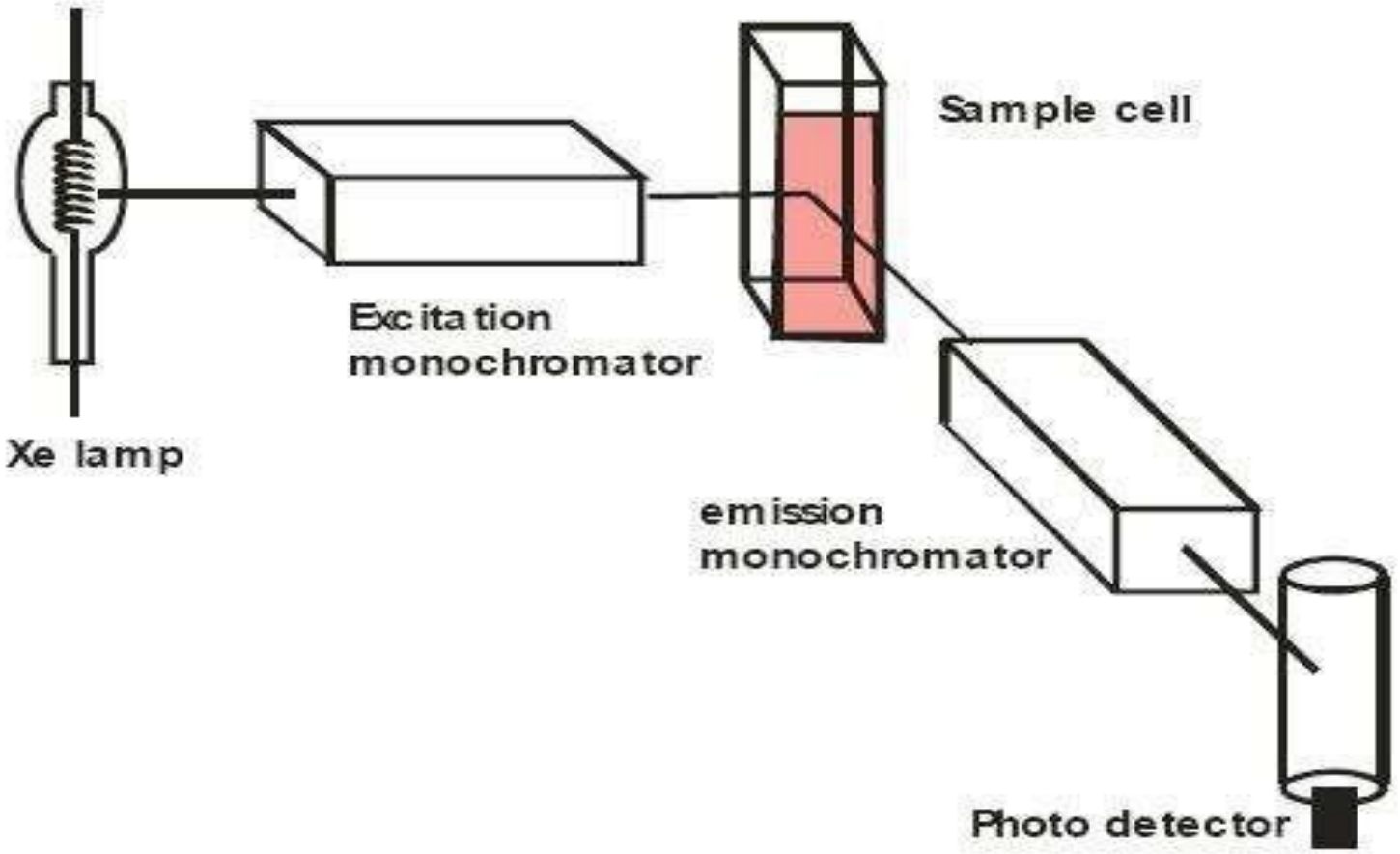


DIAGRAM OF FLUORESCENCE DETECTORS



Fluorescence Detector

Advantage

- Sensitivity is higher than UV-Vis detector
- Selectivity is high because relatively few compounds fluorescence
- Compatible with gradient elution

Disadvantage

- Difficult to predict fluorescence
- Greatly affected by environment
  - Solvent
  - pH
  - Temperature
  - Viscosity
  - Ionic strength
  - Dissolved gas

➢ AMPEROMETRIC DETECTOR:

- Amperometric detectors works based on the reducing and oxidizing property of the sample when a potential is applied.
- The diffusion current recorded is directly proportional to the concentration of the compound recorded.
- DISADVANTAGE: This detector is applicable only when the functional groups present in the sample can be either oxidized or reduced.
- ADVANTAGE: Highly sensitive detector.

AMPEROMETRIC DETECTOR:

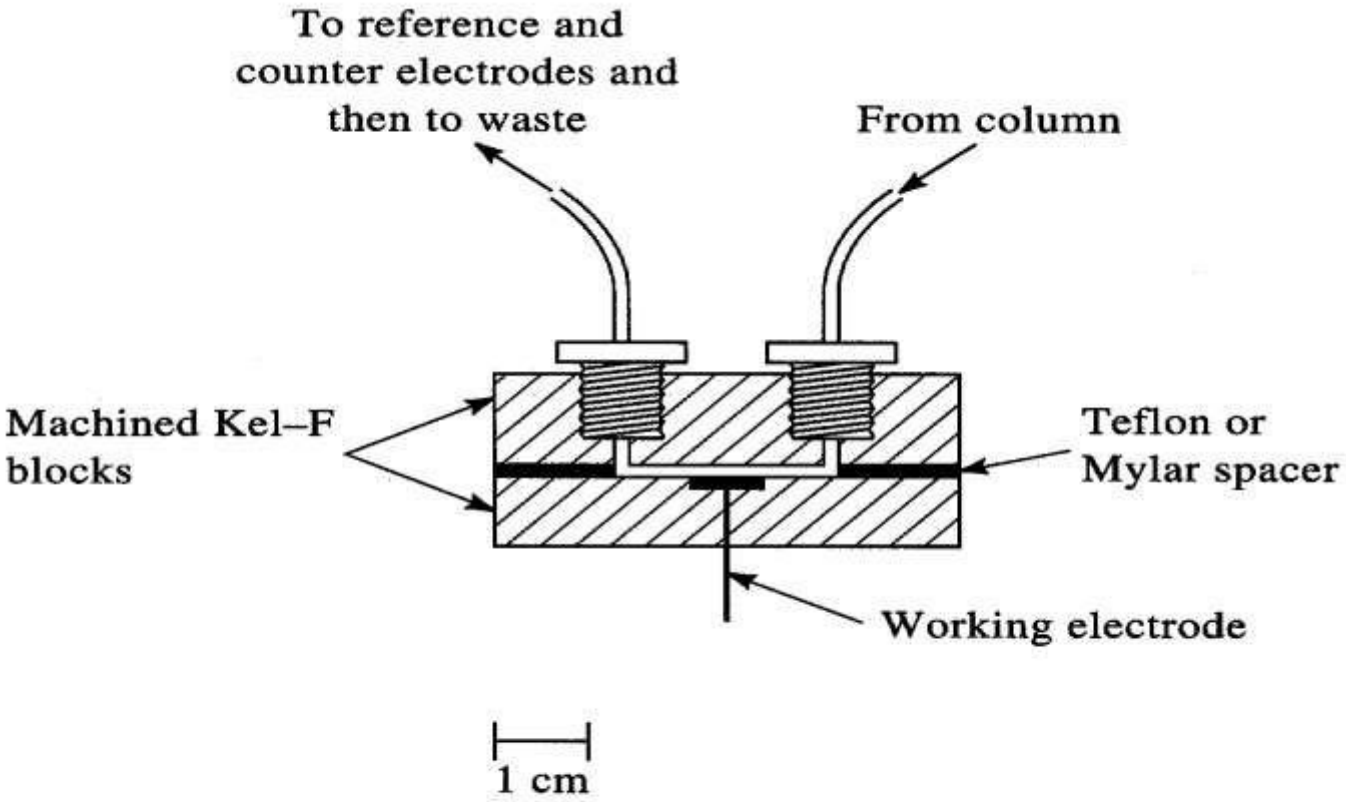
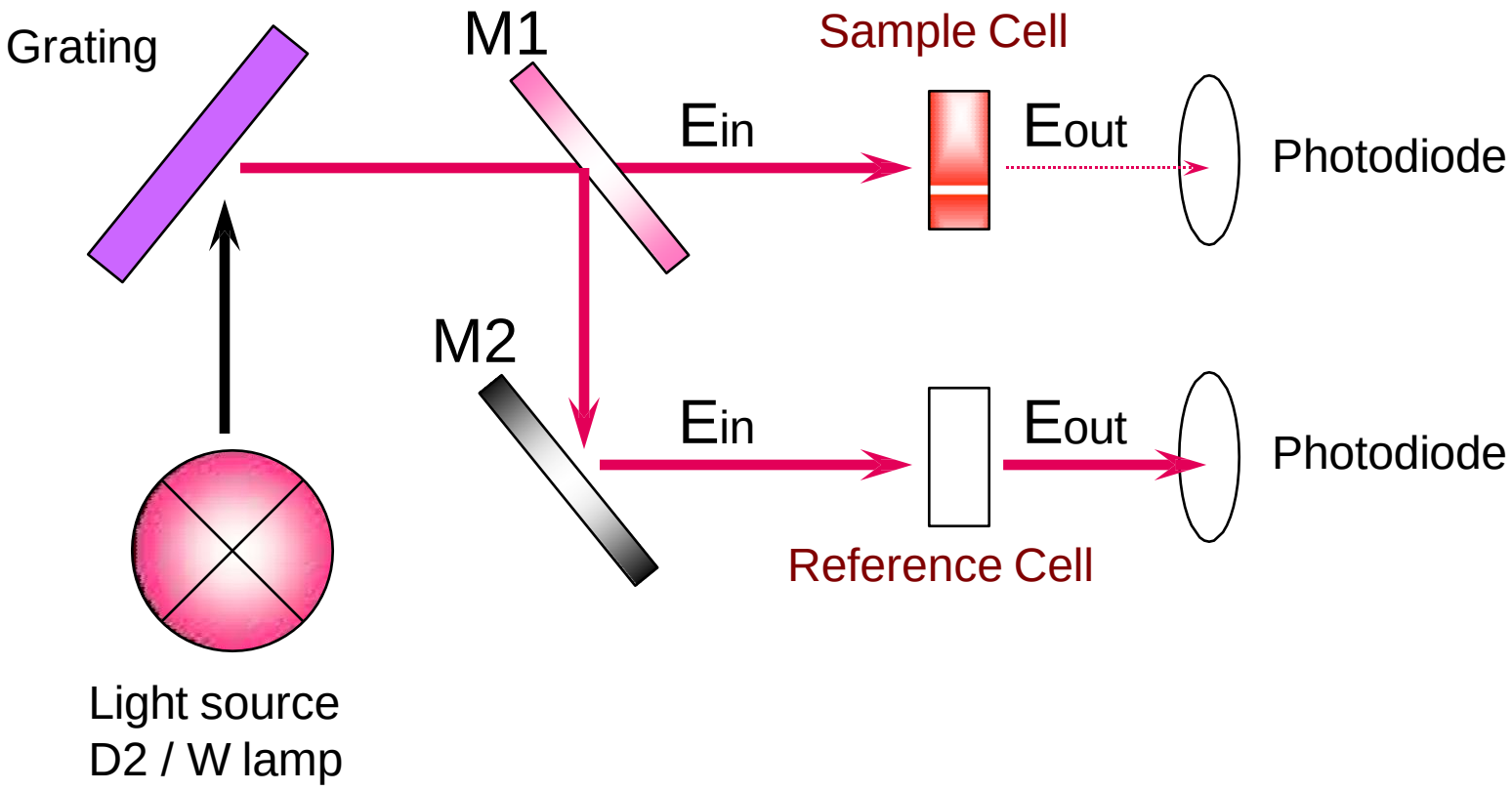


Figure 28-13 Amperometric thin-layer detector cell for HPLC.

Instrumentation of UV-Vis Detector



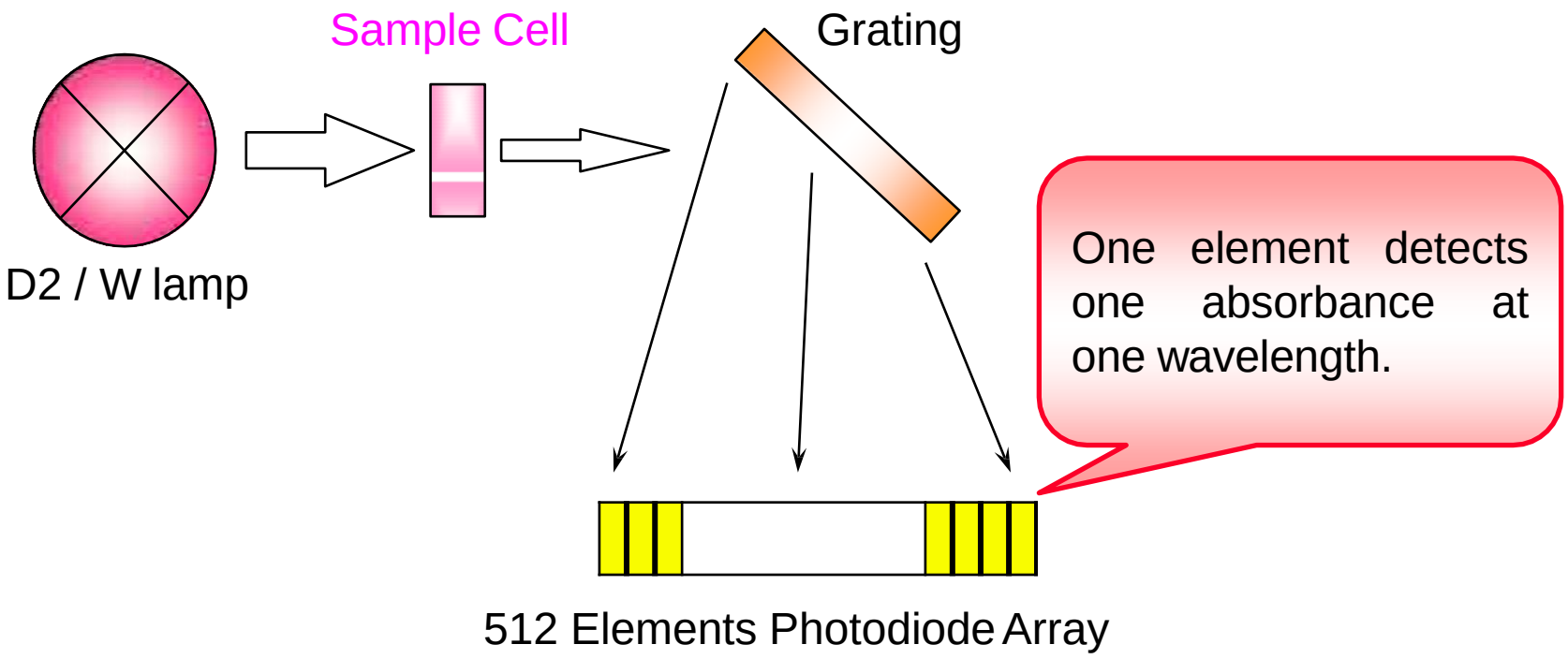
Ultraviolet / Visible Detector

- Advantage:
  - Sensitivity is high
  - Relative robust to temperature and flow rate change
  - Compatible with gradient elution
- Disadvantage:
  - Only compounds with UV or visible absorption could be detected.
- Additional Functions
  - Dual Wavelength mode
  - Wavelength Time Program mode
  - Wavelength Scan mode

➢PHOTODIODE ARRAY DETECTORS:

- This is a recent detector which is similar to UV detector which operates from 190-600nm.
- Radiations of all wavelength fall on the detector simultaneously.
- The resulting spectra is a three dimensional plot of Response Vs Time Vs Wavelength.
- ADVANTAGE: The wavelength need not be selected but detector detects the responses of all compounds.

➢PHOTODIODE ARRAY DETECTORS:

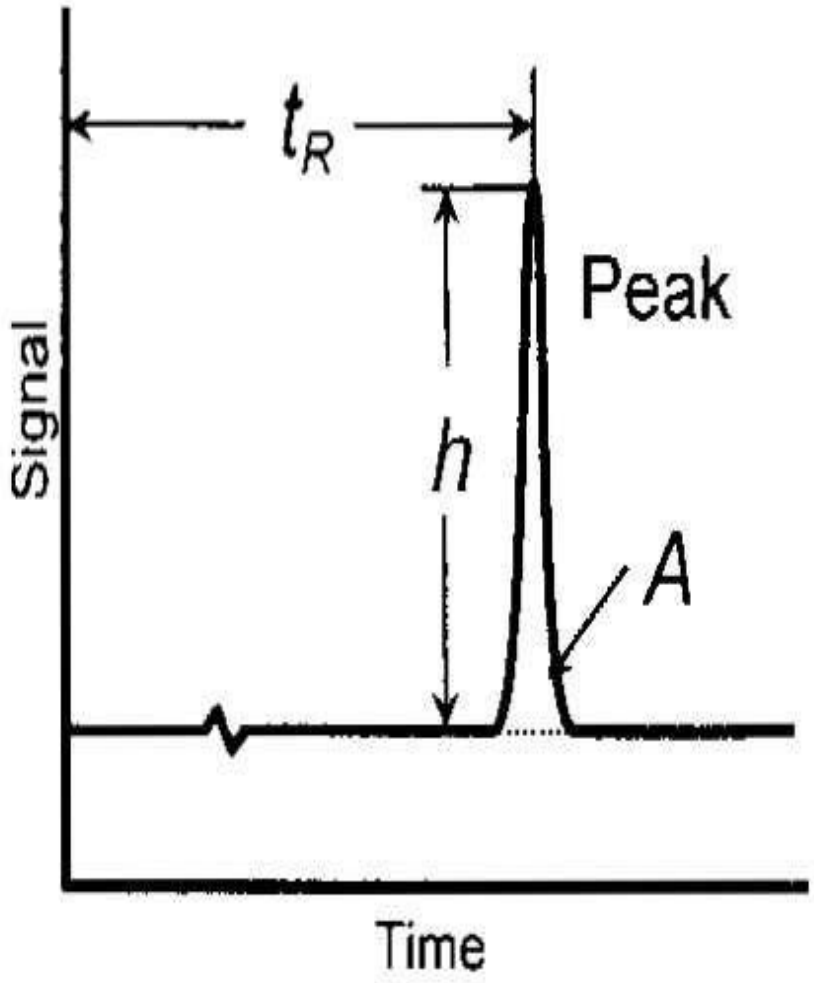


❖RECORDERS AND INTEGRATORS:

- Recorders are used to record responses obtained from the detectors after amplification,if necessary.
- They record the baseline & all the peaks obtained with respect tot ime.
- Retention time can be found out from this recordings,but area under curve cannot be determined.

❖ INTEGRATORS:

- These are improved versions of recorders with some data processing capabilities.
- They can record the individual peaks with retention time,height,width of peaks,peak area,percentage area,etc.
- Integrators provides more information on peaks than recorders.
- In recent days computers and printers are used for recording and processing the obtained data & for controlling several operations.

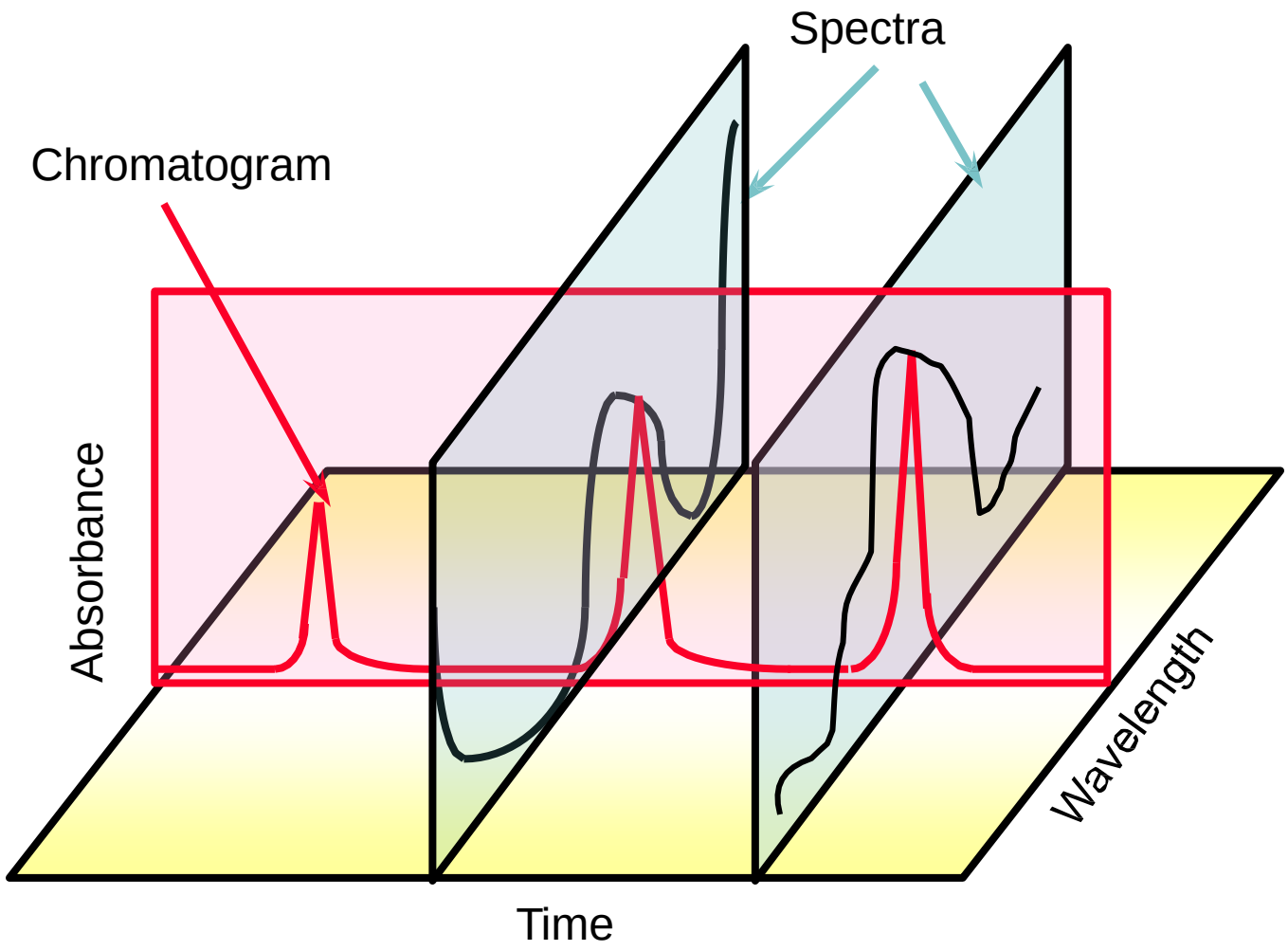


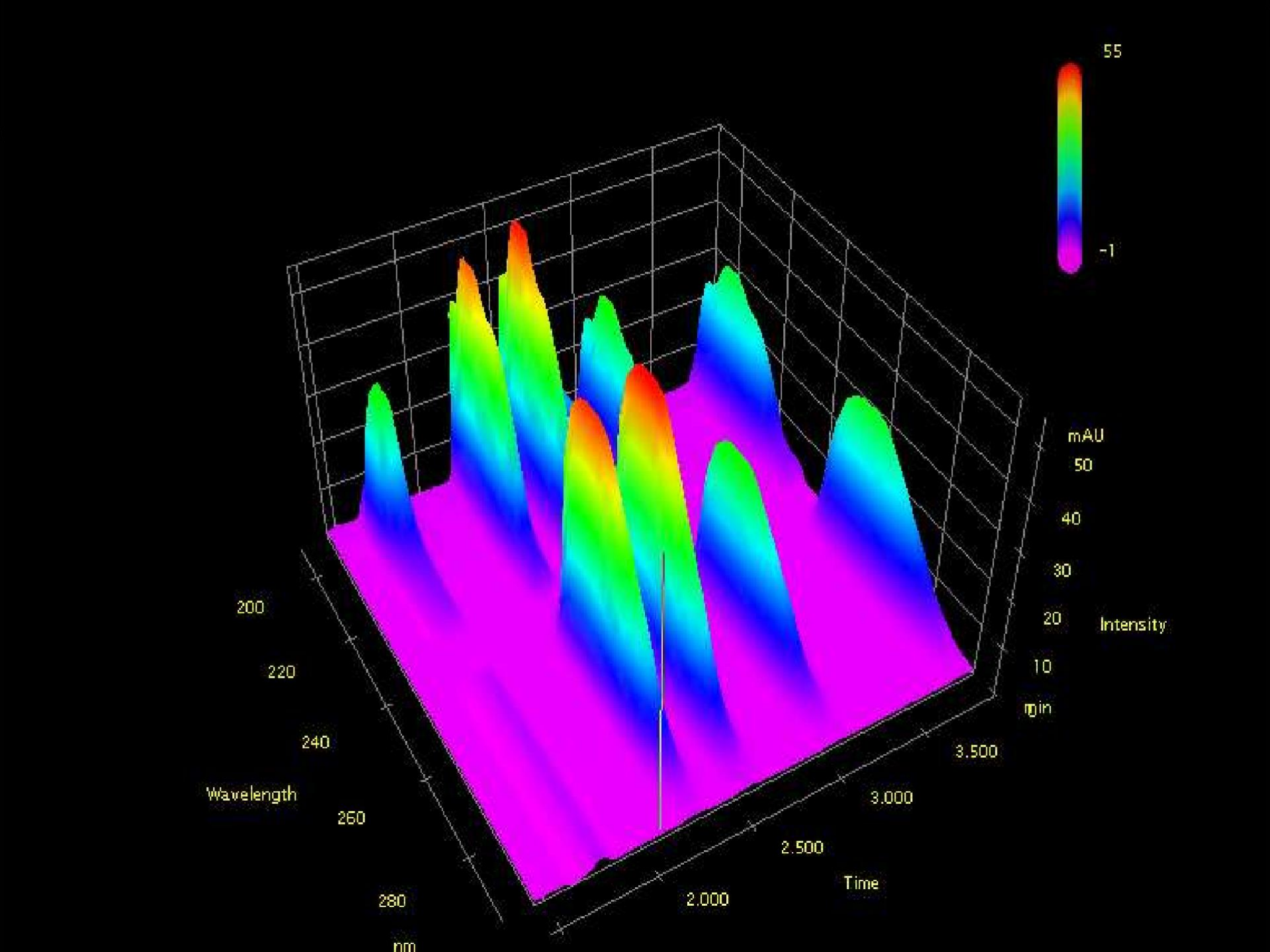
t_R : Retention time

A : Area

h : Height

Photodiode Array Detector (3-D Data)




55
-1
mAU
50
40
30
20
10
Intensity
min
3.500
3.000
2.500
2.000
Time
200
220
240
260
280
Wavelength

❖PARAMETERS:

- Retention time(R_t)
- Retention volume(Vr)
- Separation factor(S)
- Resolution
- Theoritical plates
- Column efficiency
- Assymetry factor

Column efficiency
in
Column Chromatography

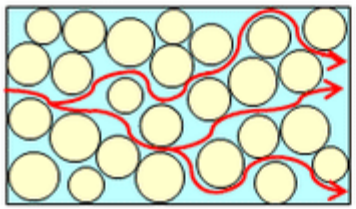
Van Deemter Equation

$$H = A + \frac{B}{u} + Cu$$

- H = height of a theoretical plate
- u = average linear velocity of the mobile phase
- *A* = eddy diffusion term
- *B* = longitudinal or ordinary diffusion term
- *C* = nonequilibrium or resistance to mass transfer term

$$H = A + B/u + Cu$$ (Van Deemter equation)

Eddy diffusion

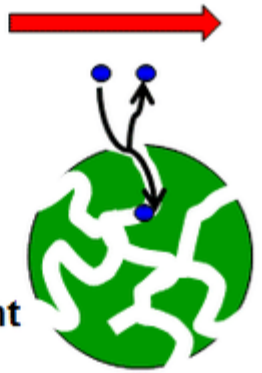


Molecular diffusion in longitudinal direction in a column.

diffusion

Mass transfer between mobile and stationary phase.

Mobile phase



$C \propto d_p^2/D$

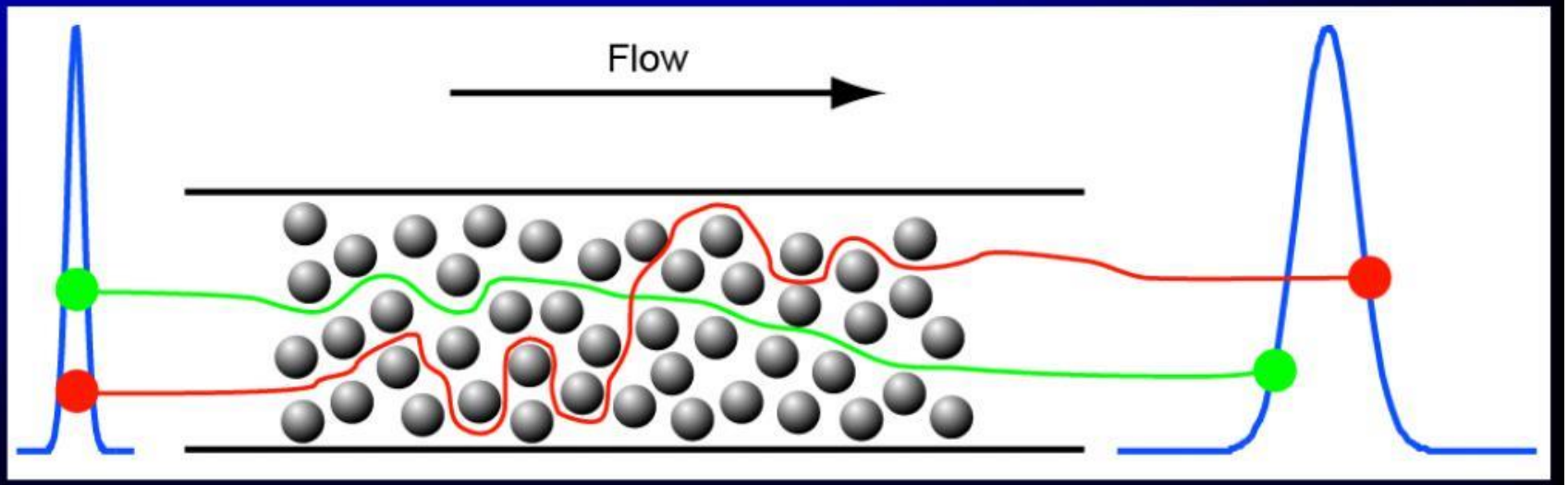
D = Diffusion coefficient

d_p = particle diameter

Porous particle

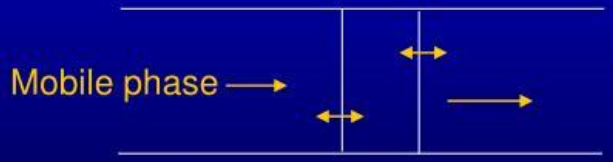
Van Deemter "A" Term

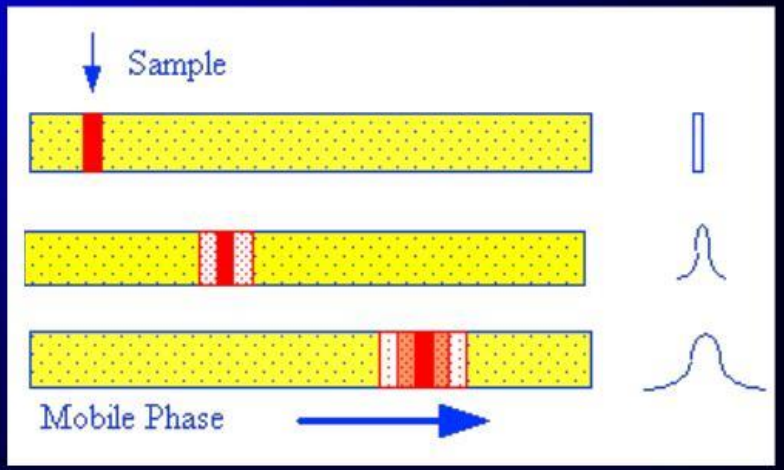
- The "A" Term: Eddy diffusion
  - molecules may travel unequal distances in a packed column bed
  - particles (if present) cause eddies and turbulence
  - "A" depends on size of stationary particles (small is best) and their packing "quality" (uniform is best)



Van Deemter "B" Term

- The "B" Term: Longitudinal diffusion
  - The concentration of analyte is less at the edges of the band than at the center.
  - The analyte diffuses out from the center to the edges.
  - If *u* is high or the diffusion constant of the analyte is low, the "B" term has less of a detrimental effect

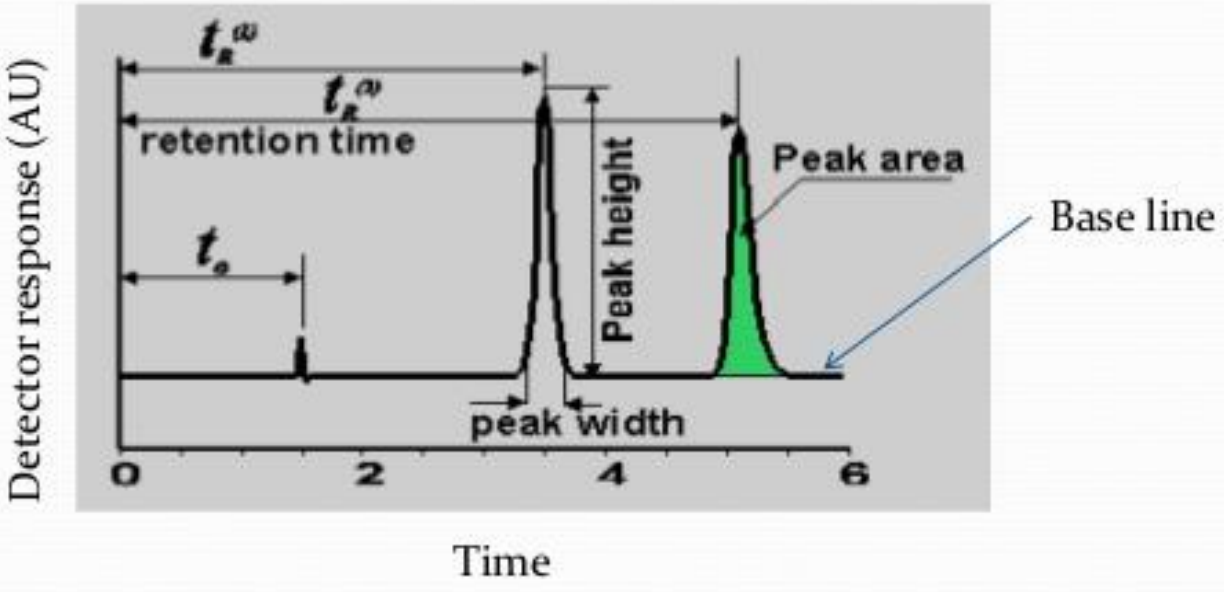




Note: The functional form of the term is B/u

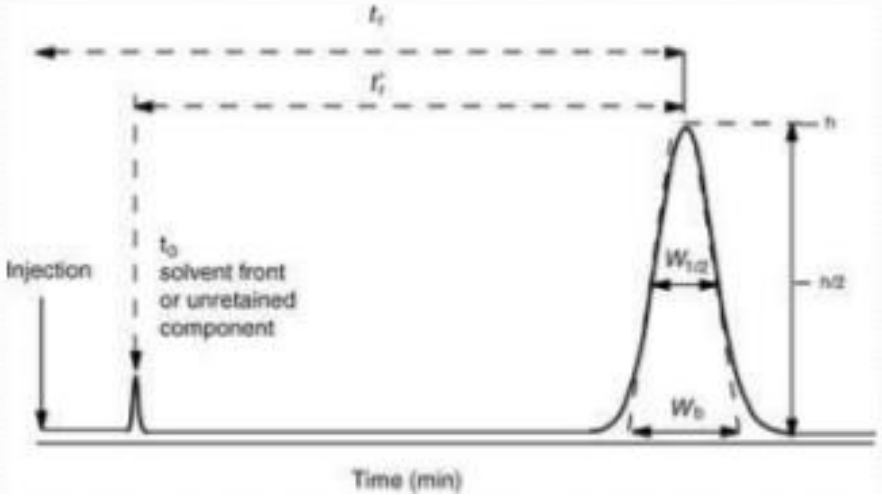
Chromatogram

- A chromatogram is a pictorial record of the detector response as a function of elution volume or retention time.
- It consists of a series of peaks, ideally symmetrical in shape, representing the elution of individual analytes.
- It is a two-dimensional plot with the ordinate axis giving concentration in terms of detector response (AU) and the abscissa represents the time (t).
- The base line represents any time period during which only mobile phase is passing through the detector



Quantitative analysis

- The objective of this approach is to confirm the presence of a specific analyte in a test sample and to quantify its amount.
- **Quantification** is achieved on the basis of **peak area** coupled with an appropriate calibration graph.
- The **area of each peak** in a chromatogram can be shown to be **proportional** to the **amount of the analyte** producing the peak.
- The **area** of the peak is determined by the **product** of the **height of the peak (hP)** and its **width at half the height (wh).**



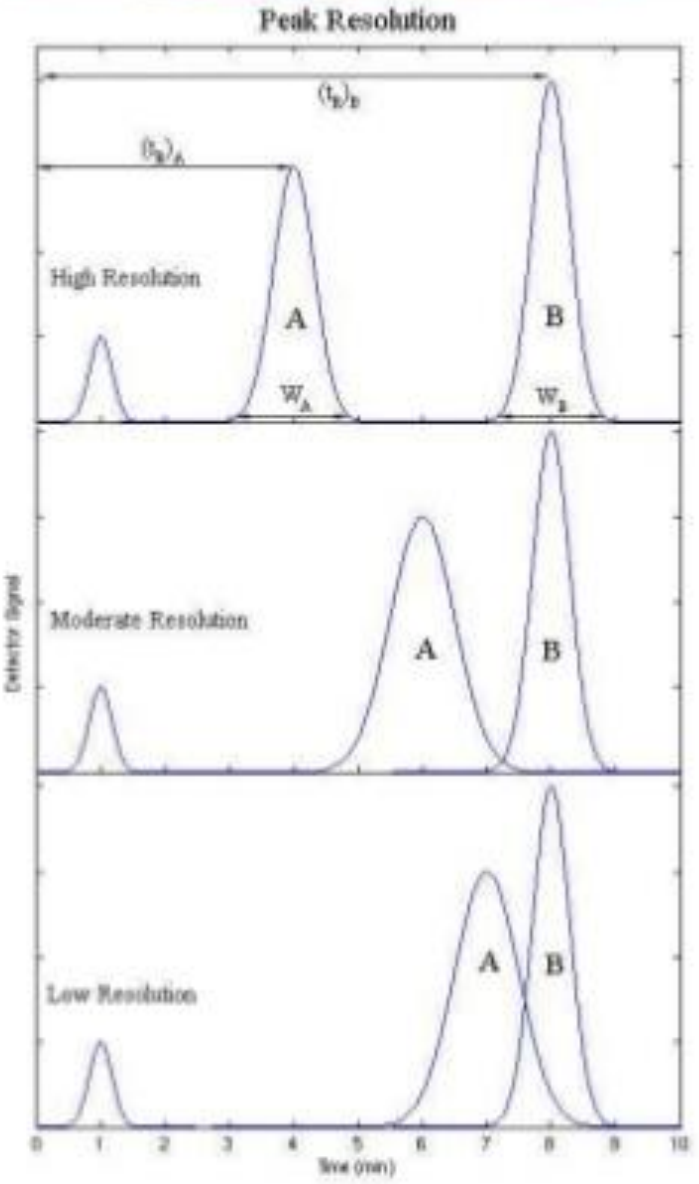
Resolution

- The most important thing in HPLC is to obtain the **optimum resolution in the minimum time**.
- A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well separated to a degree at which the area or height of each peak may be accurately measured.
- Resolution depends on-

  1.Selectivity (Separation factor)

  2. Efficiency

  3. Retention (Capacity factor)



Efficiency

- The efficiency of a chromatographic peak is a measure of the dispersion of the analyte band as it travels through the HPLC system and column.
- In an ideal world, chromatographic peaks would be pencil thin lines, however, due to dispersion effects the peaks take on their familiar 'Guassian' shape.
- Efficiency can be increased by increasing the column length , reducing the column internal diameter .