

# Major Analytical

## Method Development in GC

Following are the steps taken in the selection of method for GC.

- (1) Goal of analysis
- (2) Sample preparation
- (3) Choosing the detector
- (4) Selecting the column
- (5) Choosing the injection method

### (1) Goal of analysis:-

Following points are considered as goal of analysis -

- ⇒ What is required from analysis?
- ⇒ Is it qualitative identification?
- ⇒ Is it quantitative identification / Analysis of one or more components?
- ⇒ Do you need high precision?
- ⇒ How much analysis cost?
- ⇒ How much amount of analyte required?
- ⇒ Do you need high resolution.

### Sample preparation:-

The first step for successful chromatography of complex samples, it must be cleaned before its introduction into column. Different methods are used to isolate analyte from interfering species such as,

- Solid-phase microextraction

- Purge and trap
- Liquid extraction
- Super-Critical fluid extraction

- If you don't clean sample, chromatogram could contain a broad number of unresolved peaks.
- Moreover non-volatile substances will ruin the expensive chromatography column, so they should be removed.

### (3) Detector:-

The next step is to choose a detector. Each detector have specific features such as.

#### 1) Flame ionization Detector:-

It is probably most popular detector but it mainly respond to hydrocarbons. It is not sensitive as electron capture nitrogen phosphorus detector. The sample should contain  $> 10 \text{ ppm}$  of each analyte.

#### 2) Thermal Conductivity Detector:-

~~It respond to all classes of compounds but it is not very sensitive.~~

#### 3) Electron Capture detector:-

These are specific for halogen containing molecules, nitriles, nitro compounds and conjugated carbonyls.

The sample should contain  $> 100 \text{ ppb}$  of each analyte.

#### (4) Photo-ionization Detector:-

Specific for aromatic and unsaturated compounds.

#### (5) Nitrogen-phosphorus Detector:-

Respond to compounds having either of these 2 elements, but also respond to hydrocarbons.

→ A selective detector can be used to simplify the chromatogram by not responding to everything that is eluted.

The most general purpose of detector is mass spectrometer.

Mass spectrometry with selected reaction monitoring is excellent way to monitor one analyte of interest in a complex sample.

#### (4) Column Selection:-

~~Choices~~ The basic choices are stationary phase, column diameter, length and thickness of stationary phase. There are 3 kind of column according to these 3 choices.

- (i) Thin film narrow bore
- (ii) thick film narrow bore
- (iii) Thick film wide bore

## (1) Thin film narrow bore:-

Inner diameter: 0.1 - 0.32 mm  
Film thickness: ~ 0.2 μm

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### Advantages:-

- High resolution, trace analysis.
- Fast separation
- Low temperature
- Elute high Boiling point compounds.

### Disadvantages:-

- Low capacity
- Require high sensitivity
- Detector (not mass spectrometry).
- Surface activity of exposed silica.

## (2) Thick film narrow bore:-

Inner diameter: 0.25 - 0.32 mm

Film thickness: ~ 1-2 μm

### Advantages:-

- Good capacity
- Good resolution
- Easy to use
- Retain volatile compounds.
- Good for mass spectroscopy.

### Disadvantages:

- PUAC
- Moderate resolution
  - Long retention time for high Boiling point, compounds.

### (3) Thick film wide bore:-

Description :- 0.53 mm Film thickness  
Inner diameter :- ~2.5 um

Advantages:- High capacity. Good for thermal conductivity and IR detector, simple injection technique.

Disadvantages:- Low resolution, Long retention time for high Boiling point compounds.

⇒ To improve resolution, use a

- Longer column
- Narrower Column
- Different S. phase

→ An intermediate polarity S. phase will handle most separation that non-polar "compounds" column cannot.

→ For high polar compounds, a strongly polar column might be necessary.

### (5) Injection Method:-

The last major decision is how to inject the sample, There are three types of injection which given below

#### (1) Split Injection:-

⇒ Best for high conc. sample

- ⇒ High resolution
- ⇒ Can handle dirty sample as use packed liner.
- ⇒ Could cause thermal decomposition
- ⇒ poor quantitative analysis.

### (2) Split Less Injection:-

- ⇒ Required for dilute sample.
- ⇒ High resolution
- ⇒ Require Solvent trapping or cold trapping.
- ⇒ Moderate thermal stability
- ⇒ Poor quantitative analysis.

### (3) On-Column Injection:-

- ⇒ Best for quantitative analysis.
- ⇒ For thermally sensitive compounds
- ⇒ Low resolution
- ⇒ Can't handle dilute or concentration solution.

### Summary:-

In GC, a volatile liquid or gaseous solute is carried by mobile phase over stationary phase on the inside of a solid support. Each stationary phase must strongly retain solute according to "like dissolve like".

## ⇒ Resolution:-

If we divide the retention time by width. It give resolution.

$$\text{Resolution} = \frac{t_r}{\text{width}(W)}$$

"The ratio b/w the retention time and width is known as resolution."

→ It is dimensionless and unit less.

"It is degree of separation of two components."

The concentration of components is directly proportional to width and retention time. as concentration increase both width and retention time increase and vice versa

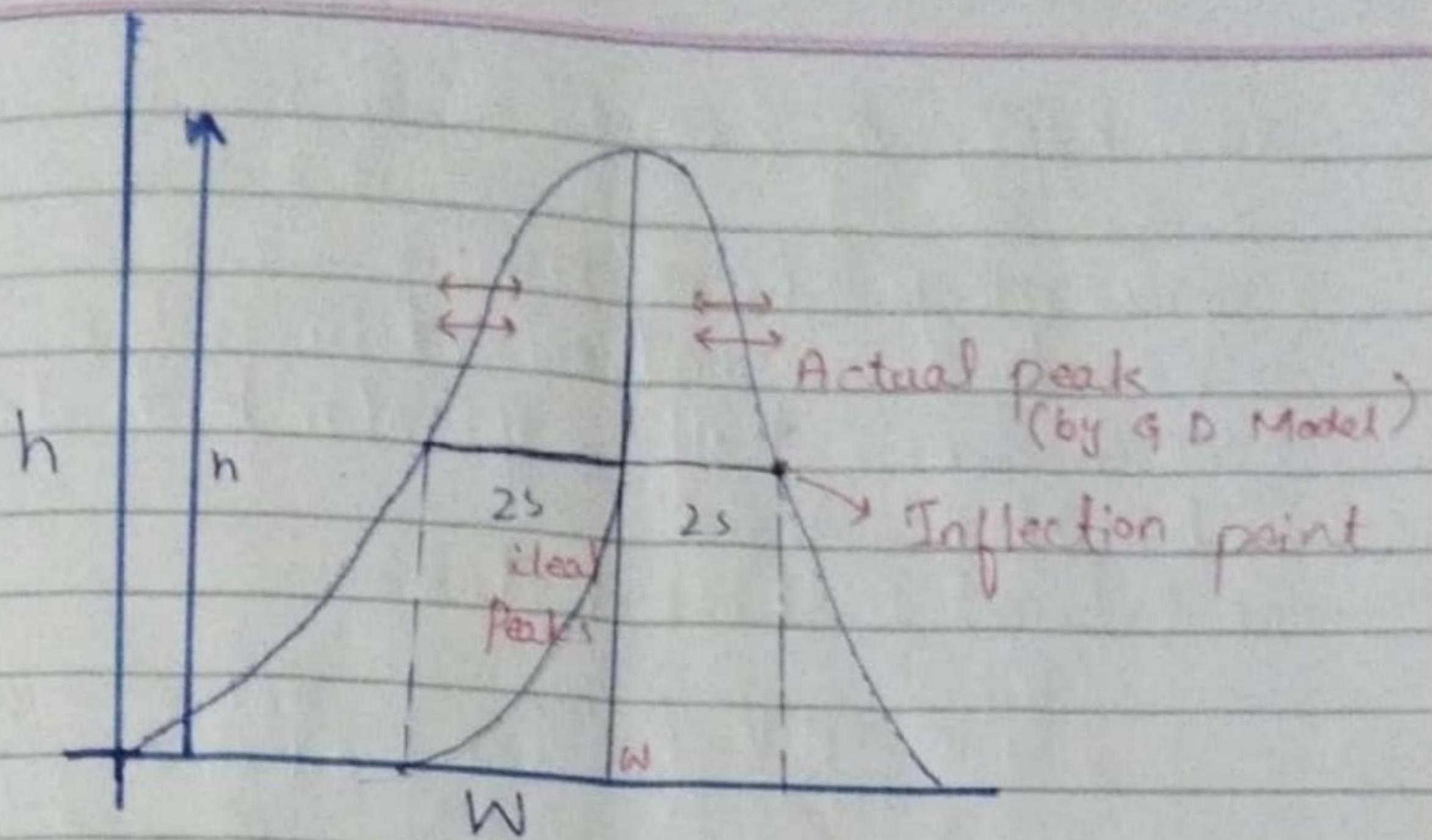
## ⇒ Model of Resolution:-

Gaussian- Distribution Model:-

According to this model.

At higher conc. peaks should be sharper.

It is an ideal condition, in actual practice at higher conc. of components peaks are broaden.



→ Standard Deviation :- ( $\sigma$ )

"Difference between actual peak and ideal peak is known as standard deviation."

$$\sigma/S = \sqrt{\frac{\sum x^2}{n} - \left(\frac{\sum x}{n}\right)^2}$$

→ Inflection Point:-

"Steepest part of any curve is known as inflection point."

"At steepest part slope is maximum."

$h \Rightarrow$  height of peaks

The half of height ( $\frac{1}{2}h$ ) = width of peak ( $\frac{1}{2}w$ )

The width of peak at inflection point is half of width at base point, this is an ideal condition.

→ Natural distribution of any material:-

When it is uniformly or freely thrown from height it form

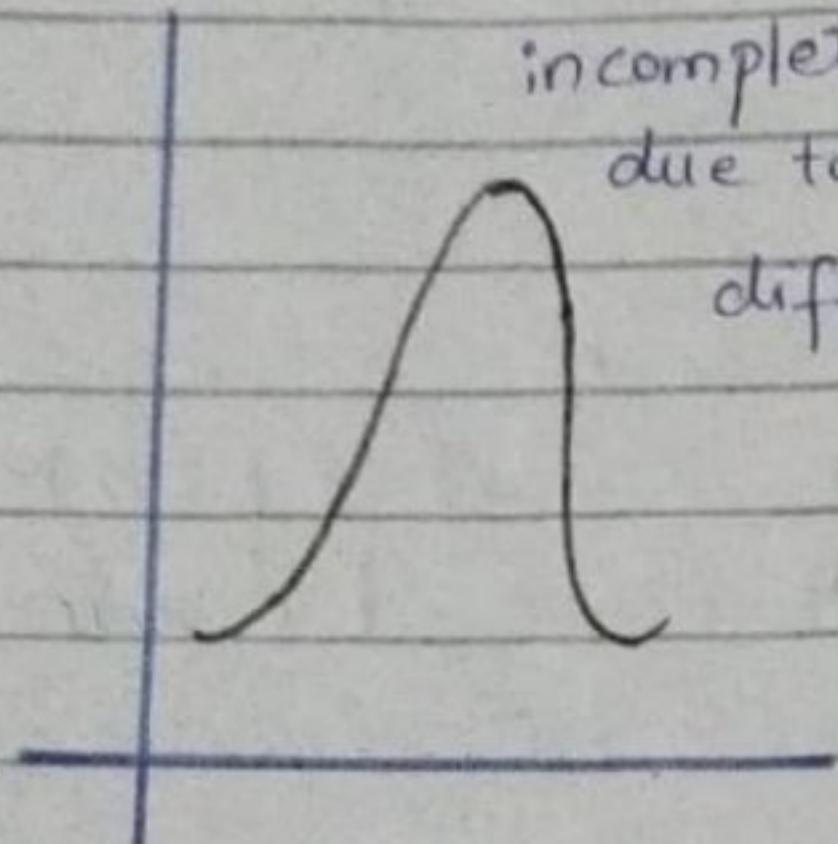
a shape of cone.

→ Gaussian Distribution:-

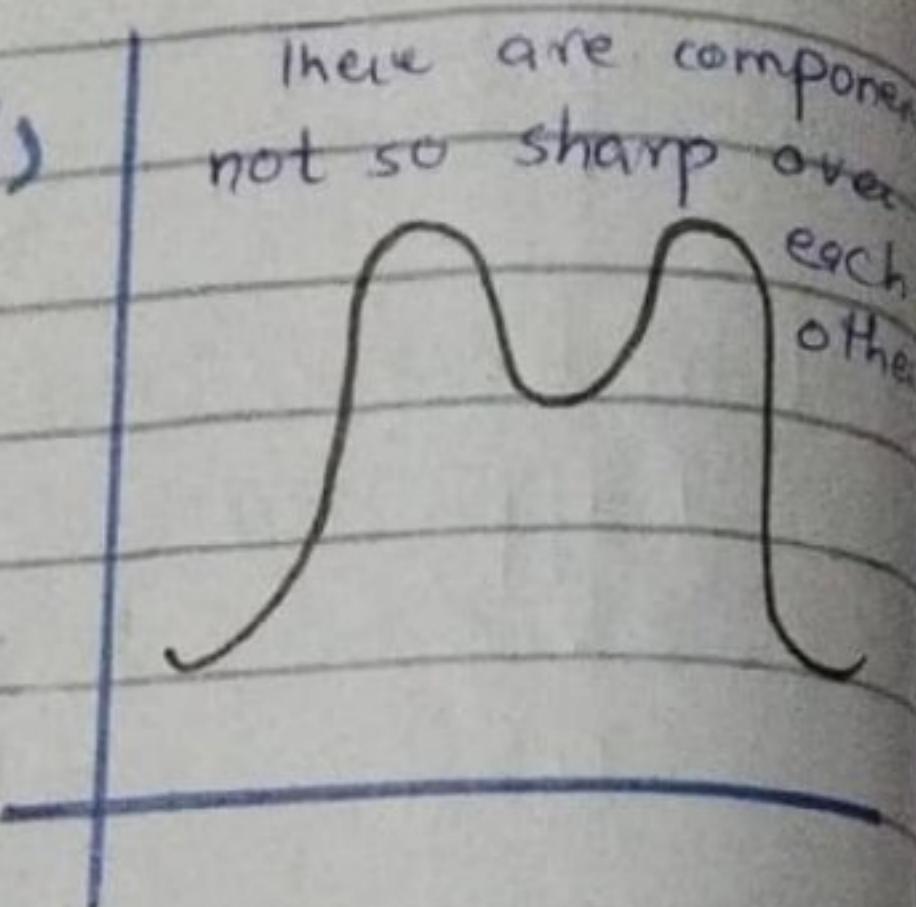
Standard deviation at inflection point in half of standard deviation at base point.

→ Natural Distributions:-

(i)

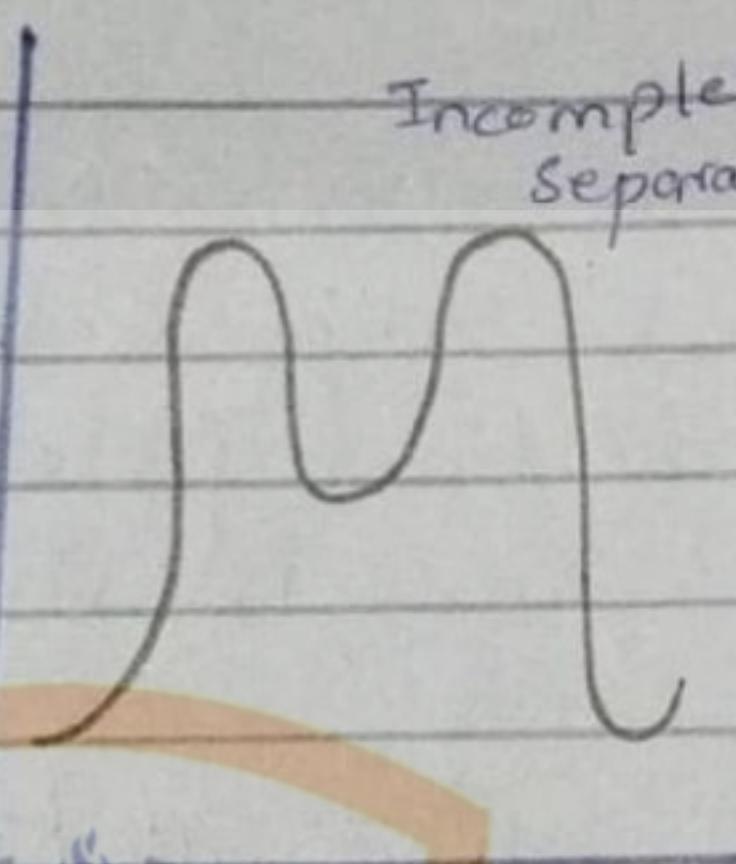


(ii)

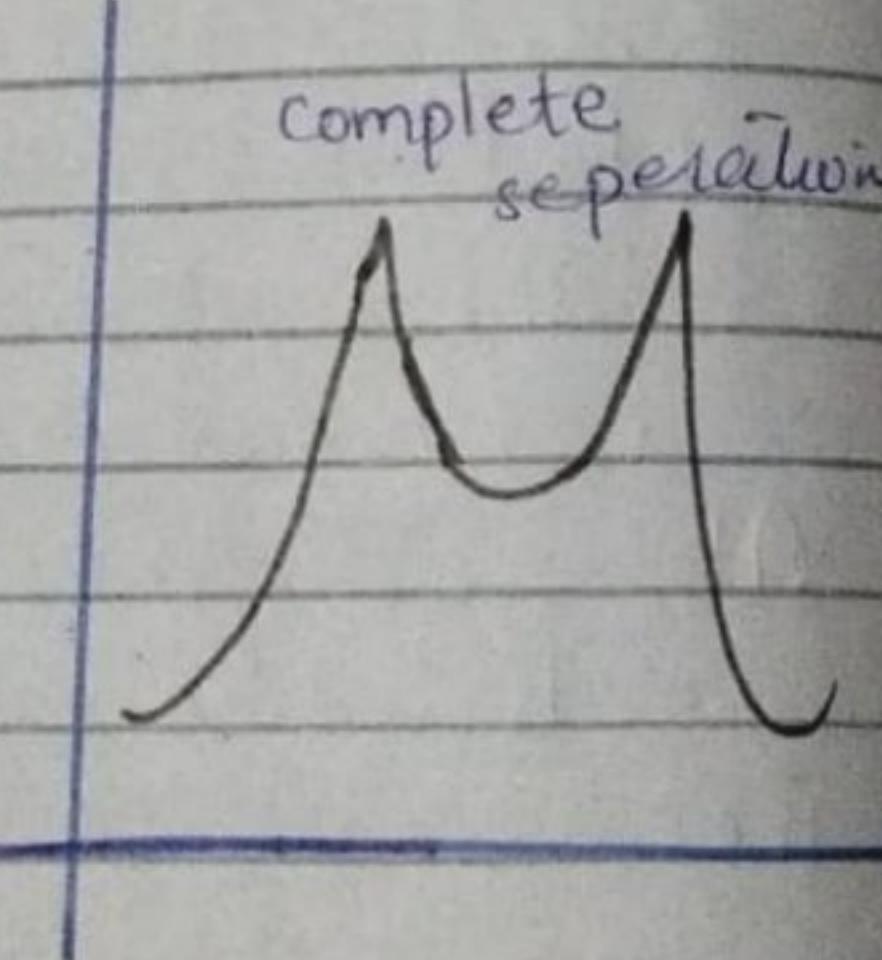


(i)

(iii)



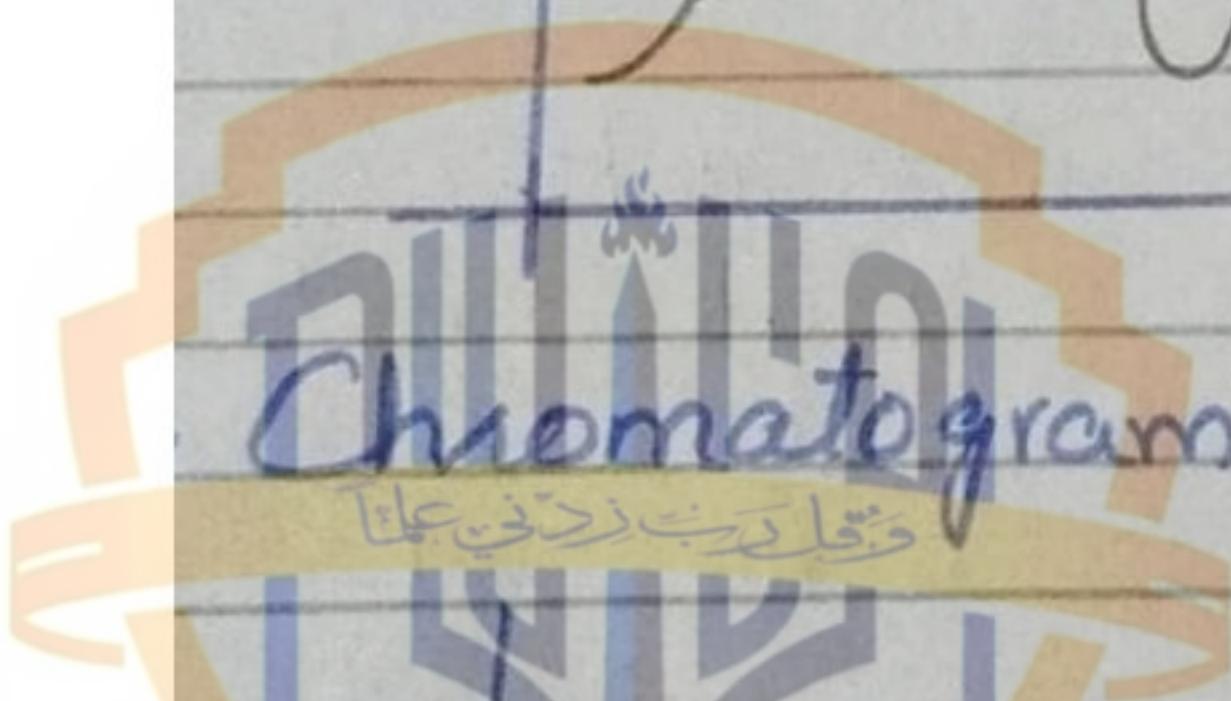
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(ii)

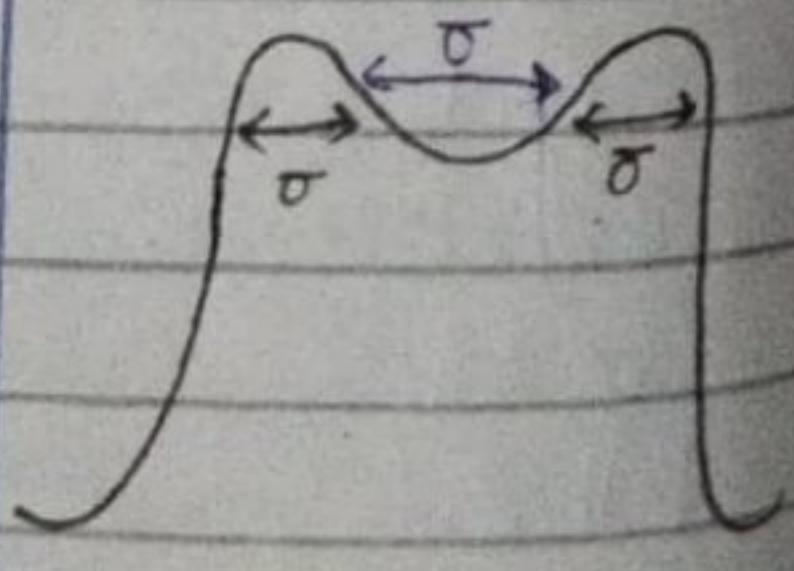
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(iv)



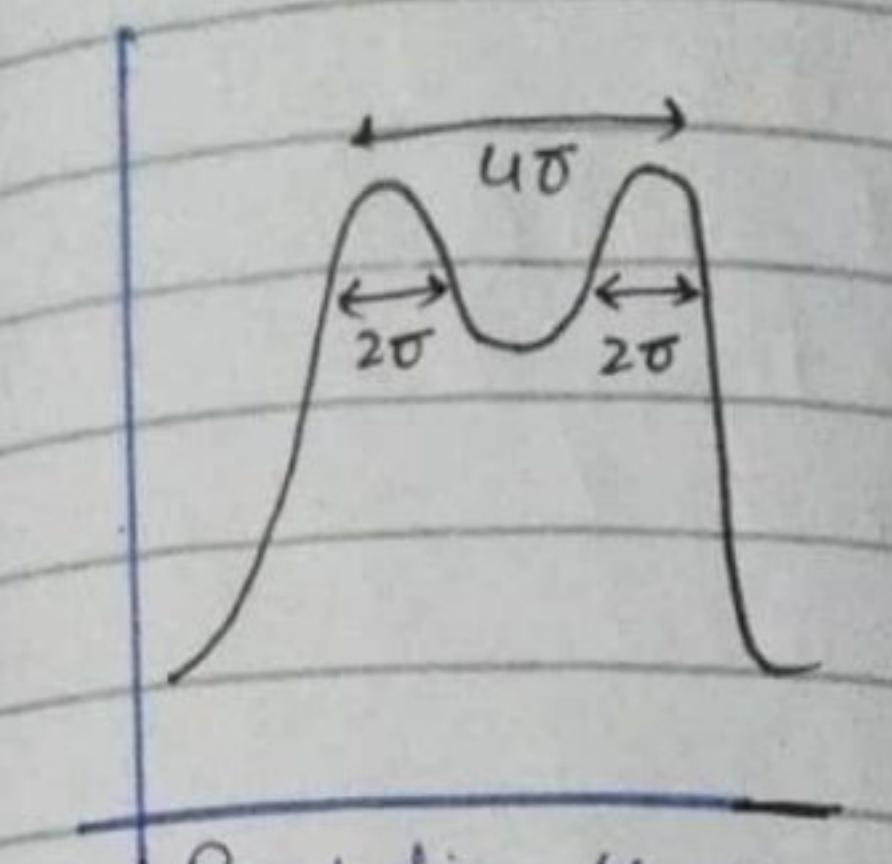
Chromatogram Explanations:-

(iii)



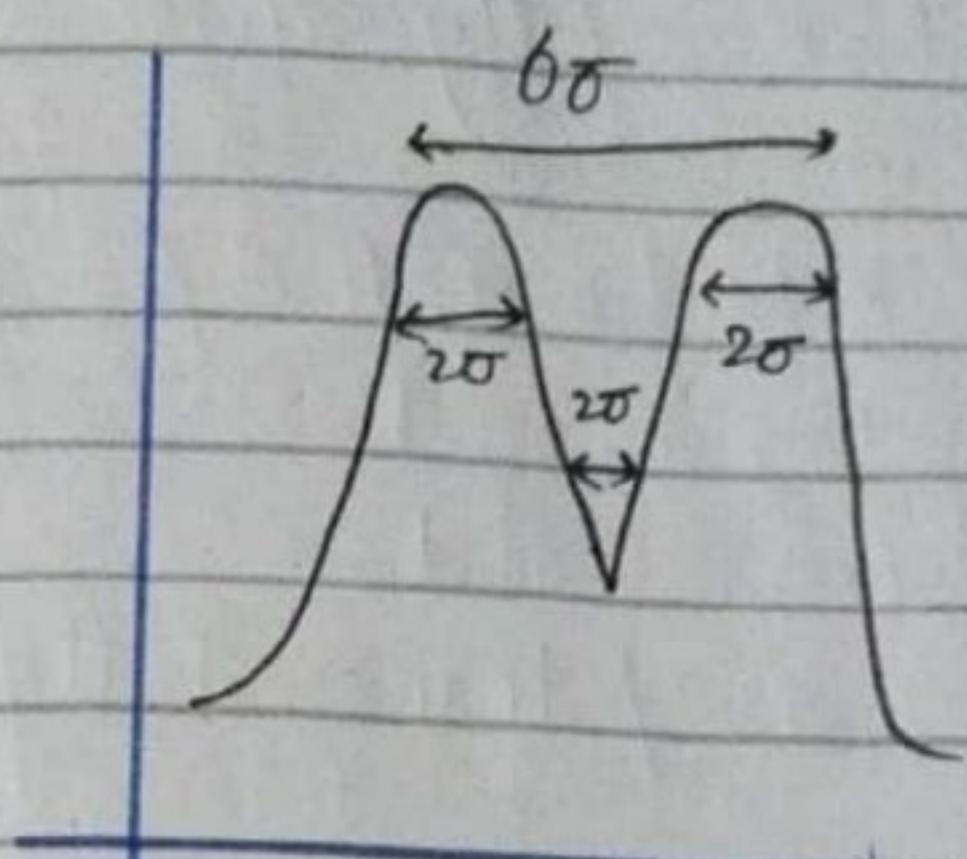
$$\text{Resolution} = \frac{\Delta}{\sigma} = 0.5$$

$$\text{Resolution} = \frac{\Delta}{\sigma} = 0.75$$



$$\text{Resolution } \frac{\Delta}{w} = 1$$

Perfect resolution occurs at 1.5



$$\text{Resolution} = \frac{6}{w} = 1.5$$

## Factors responsible for band broadening:-

i) No. of molecules:-

No. of molecules  $\uparrow \rightarrow$  Peak area  $\uparrow$

$\uparrow$  Broader Peak  $\leftarrow$  Peak length  $\uparrow$

ii) Diffusion:-

Lighter particles move rapidly through diffusion hence bands are broad.

iii) Sample volume:-

large volume of sample will result in band broadening.

iv) Physical state of sample:-

Sample may be solids, liquid, or gas.

Sample  $\rightarrow$  Gas  $\rightarrow$  Molecules move more rapidly due to lighter weight so bands are broader.

Sample  $\rightarrow$  liquid  $\rightarrow$  Molecules move slowly as compared to gas it gives sharper peaks.

Sample  $\rightarrow$  Solid  $\rightarrow$  It does not move so peaks whether broad or sharp.

# Applications of HPLC:-

The main purpose of the HPLC technique is to identify, quantify and purify particular analyte or compound.

HPLC is considered a crucial tool in a variety of pharmaceutical applications, including

- evaluating formulations
- checking purity
- monitoring changes due to process adjustments.

The results can be used to analyse finished drug products and their ingredients quantitatively and qualitatively during the manufacturing process.

HPLC is also used for the quantitative analysis of the vitamins due to instability of various vitamins, in which decomposition regularly occurs during sample preparation.

The HPLC is used in the quantitative determination of plasma levels of drugs and their metabolites.

It is most frequently used technique in forensic toxicology.

It has particular advantages in

cases of non-volatile, thermally sensitive and high-molecular weight substances that can be analyzed under mild conditions and without derivatization.

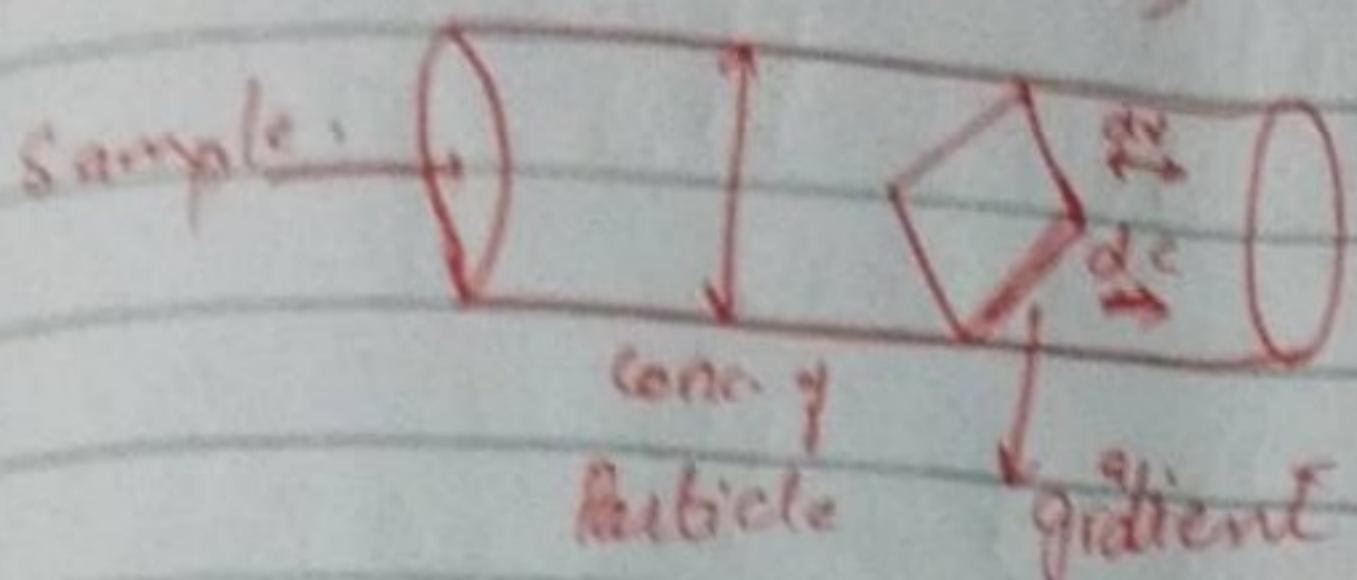
- One of its most common uses is to determine materials used in explosives.
- It helps forensic scientists to analyze substances such as gun powder, residue, fibers and toxins.

→ Furthermore, HPLC can be used in the following applications.

- Water purification.
- Detection of impurities in pharmaceutical industries.
- Pre-concentration of trace components.
- Ligand exchange chromatography.
- Ion-exchange chromatography of proteins.
- High-pH anion-exchange chromatography of carbohydrates and Oligosaccharides.

## Concentration Gradient:-

$$J = D \left( \frac{dx}{dc} \right)$$



$dx \Rightarrow$  change in distance

$D \Rightarrow$  Diffusion co-efficient  
it determine how many particles pass through gradient.

$$J = \text{flux}$$

Flux is a no. of moles of analyte passing through area (through gradient) of one meter square in one second is called flux.

Unit:

$$J = \text{mol/m}^2/\text{s} \Rightarrow \text{mol/m}^2$$

$$\bar{J} = \text{mol/m}^2\text{s}$$

## Size of Particles:-

Large size of particles  $\rightarrow$  Broad Peak

Small size of particles  $\rightarrow$  sharp Peaks

Very small size of  $\rightarrow$  sharper peaks.

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## → GAS CHROMATOGRAPHY

- Mobile Phase is mostly gaseous phase in gas chromatography stationary phase is non-volatile liquid and sometime solid.
- The analyte is gaseous which is transported through gaseous phase in a column with the help of carrier gas.
- Carrier gas introduced into the column in injection port which have a rubber septum and an oven.
- The carrier gas may be different mostly He, but may be  $N_2$  and  $H_2$ .
- The carrier gas taken depend upon the efficiency / quality and timing of gas separation.
- There are different types of chromatography on the basis of stationary phase.

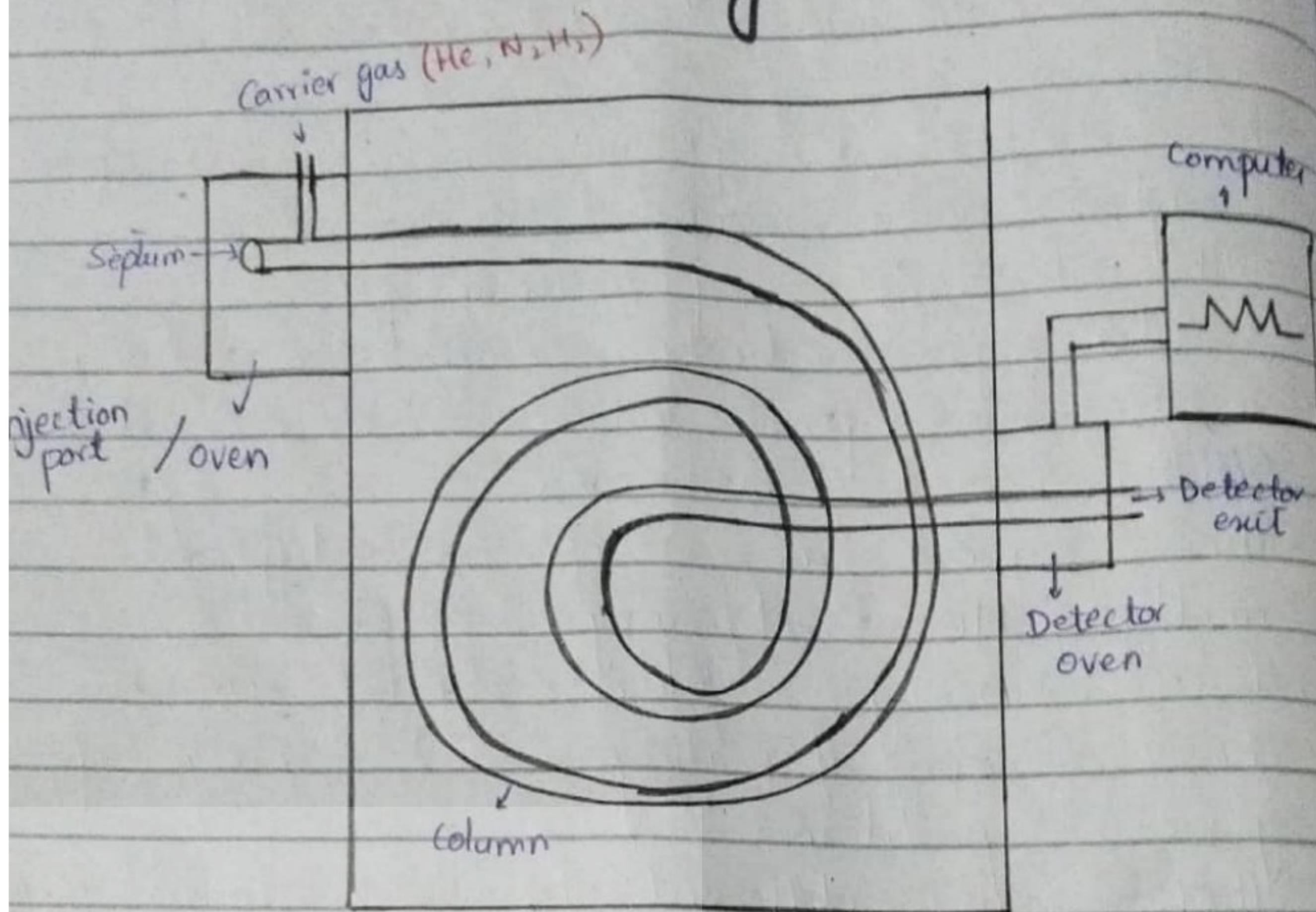
## → Types

(1) "The stationary phase is non-volatile liquid supported on a solid known as Gas Liquid Partition Chromatography".

(2) "If gas is directly absorbed on solid stationary phase is known as Gas Solid absorption chromatography".

- If sample is liquid it must be volatile and phase is non-volatile

## Schematic Diagram:-



## Process:-

- Sample introduced into column, when it reach oven which is preheated, it vaporize the sample.
- The carrier gas introduced from above flow vapours of sample across the column.
- The Sample go to detector which is connected with computer which give is (septum) spectrum.

# Types of Column:-

1. Open Tubular Column
2. Closed / Packed Column

Open Tubular Column is explained as follows.

## (1) Open Tubular Column:-

- The S. phase coated inside of the column which may be  $0.1-5\text{ }\mu\text{m}$ .
- The length of column is  $5-100\text{ m}$ . The most common column have  $30\text{ m}$  length.
- The intermediate distance of walls or diameter of column is  $0.1-0.53\text{ mm}$ .
- More narrow the column, more batter the separation of sample and less the time of ejection.
- But narrow column have less holding capacity of sample. Hence it is better when diameter is less.

→ If open Tubular Column is compared with packed Column, it have following points.

- Better Resolution
- Short analysis of time
- Greater Sensitivity.

→ But it has one disadvantage too.

- Low Sample holding capacity.

$5-100\text{ m length (30 m common)}$

$0.1-0.53\text{ mm}$   
diameter

$(0.1-5\text{ }\mu\text{m}) \downarrow \text{s. phase}$

## → Types of Open Tubular:-

- (i) WCOT
- (ii) SCOT
- (iii) PLOT

### (1) WOT:-

Stationary phase is liquid coated inside wall known as wall coated open tubular column.

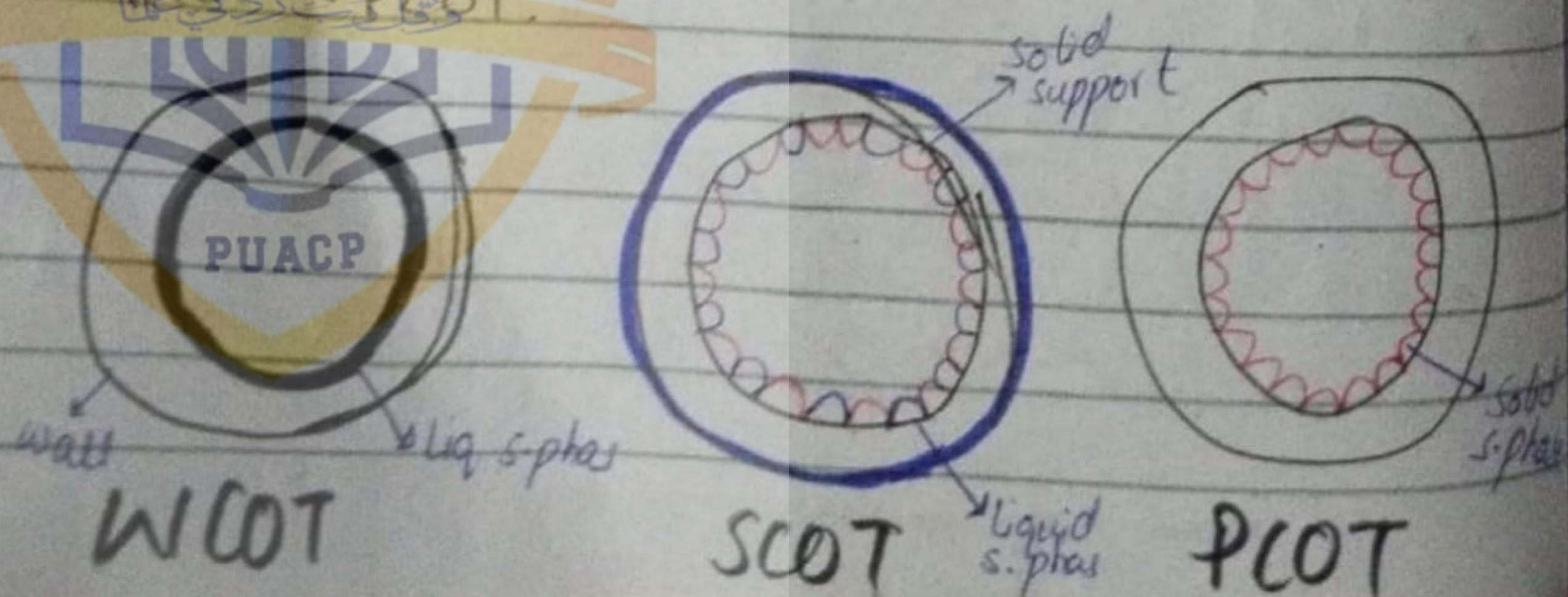
### (2) SCOT:-

The S. phase is liquid but supported by solid particle inside the walls of column known as support coated open tubular column.

### (3) PLOT:-

The S. phase is porous solid (carbon) coated on the inner wall known as Porous Layer Open Tubular Column.

SCOT have most sample holding capacity due to increased surface area because of liquid on solid. But efficiency is intermediate b/w WCOT & PLOT.



## → Chromatography Comparison:-

- Different chromatograph of two different column diameter are drawn.
- The separation of 0.25mm diameter is better than 0.32mm diameter.  
Hence narrow diameter is preferred.

## Composition of Column:-

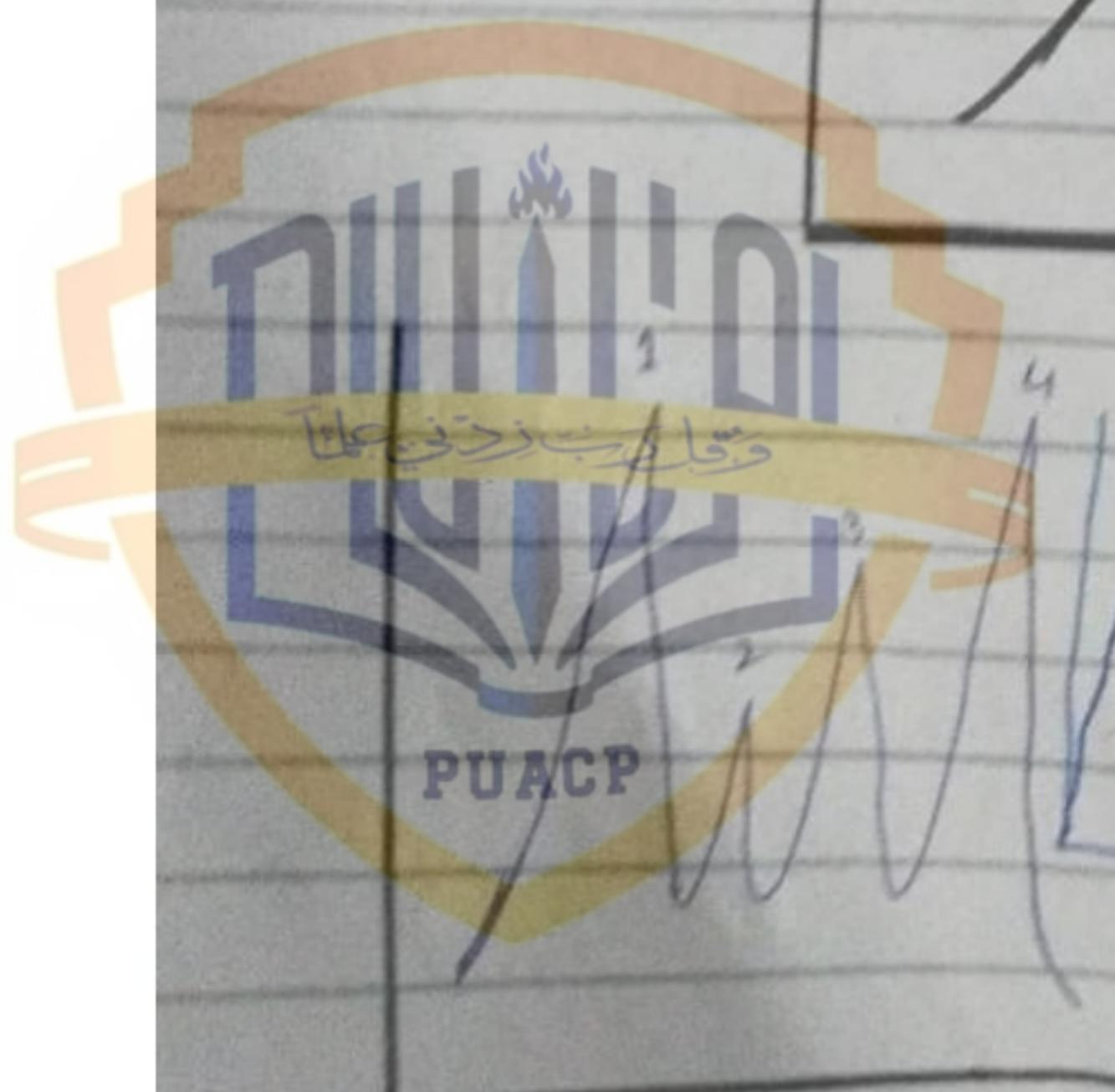
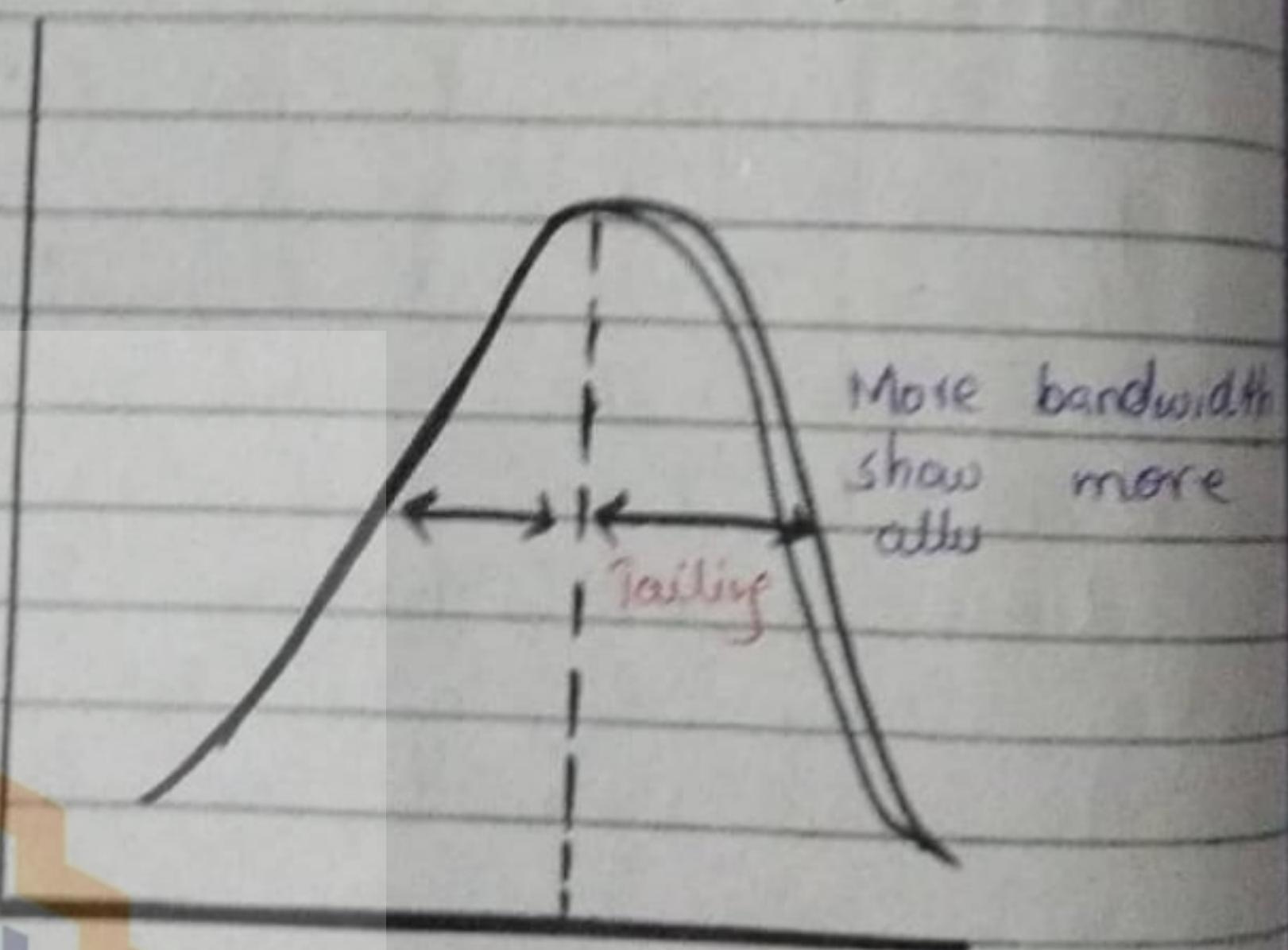
- The column is made of silicon coated with polyamide which bear temperature upto  $350^{\circ}\text{C}$ .
  - In some cases, s. phase adhere some particle of analyte more as compare to other particles of solute / analyte. This give rise to "Tailing".
  - In which the allution speed is decreased. As some part of solute adhere more strongly to s. phase as compared to other.
  - As time passed, the thickness of coating decreases as a result silicon gel exposed. Hence few other particles also allute with analyte.
  - As a result signal to noise produced.
- Ratio b/w desired and undesired signal is signal to Noise Ratio.
- gives rise to background signal.

## → How to check Column?

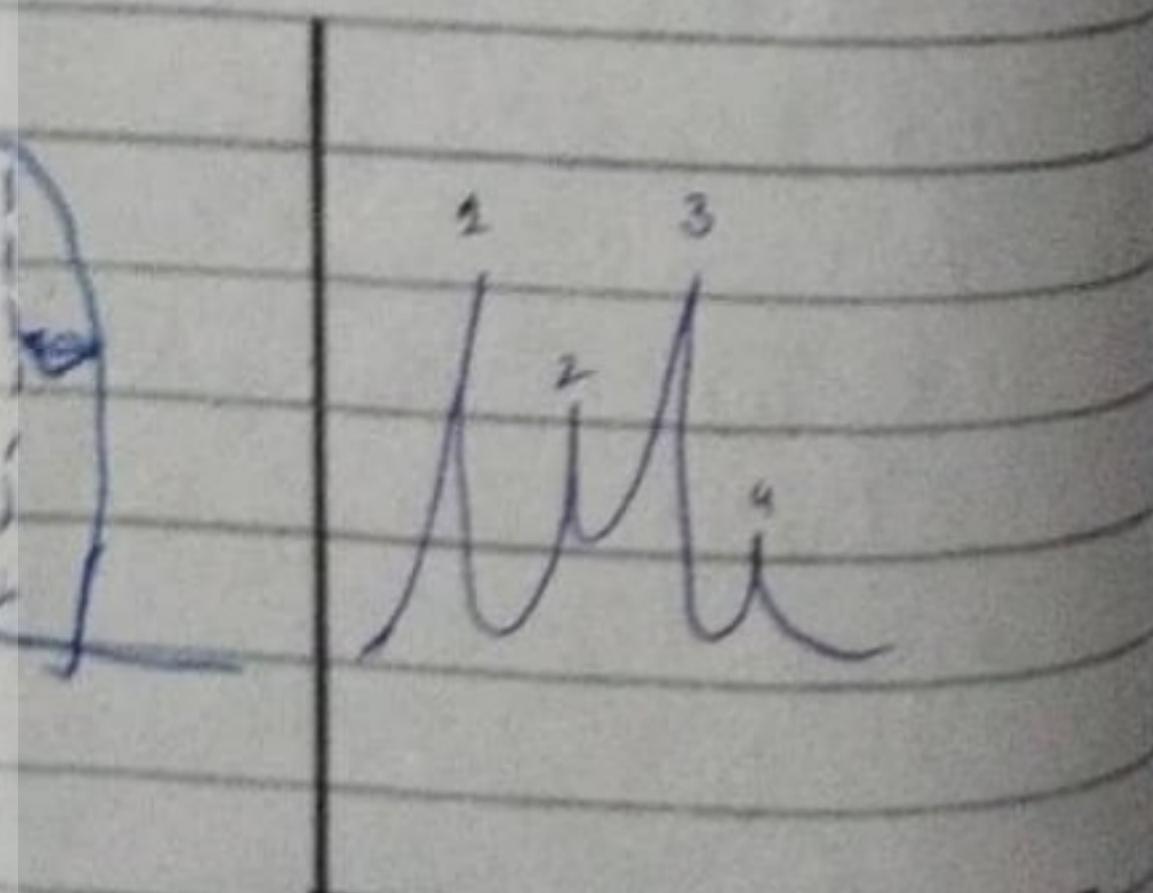
- A standard with known retention time taken and passed through column if retention time is similar to original, it means column is correct if not then there's need to change the column.
- The choice of Liquid phase is based on "Like dissolve Like". Non-polar column are best for non-polar solute.
- Columns of intermediate polarity are best for intermediate polarity solut, and similar for strong.

B

- A
- B
- C
- D
- E
- F
- G
- H
- I
- J



0.25 mm  
diameter



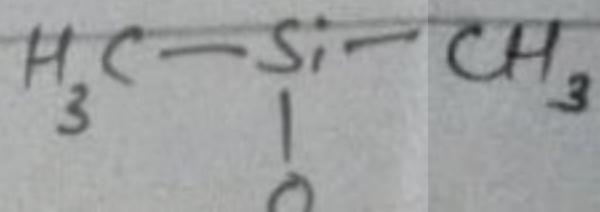
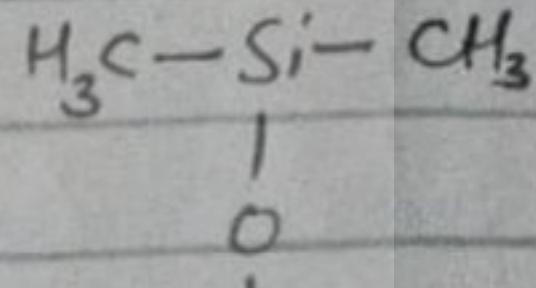
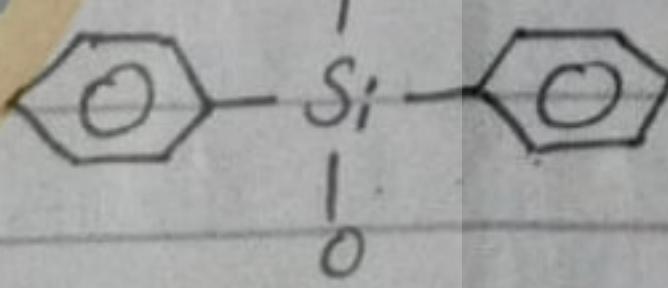
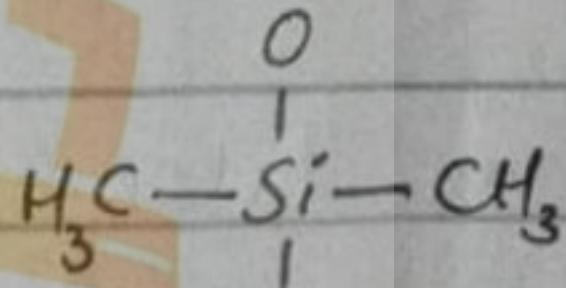
0.32 mm  
diameter

## Background Signal:-

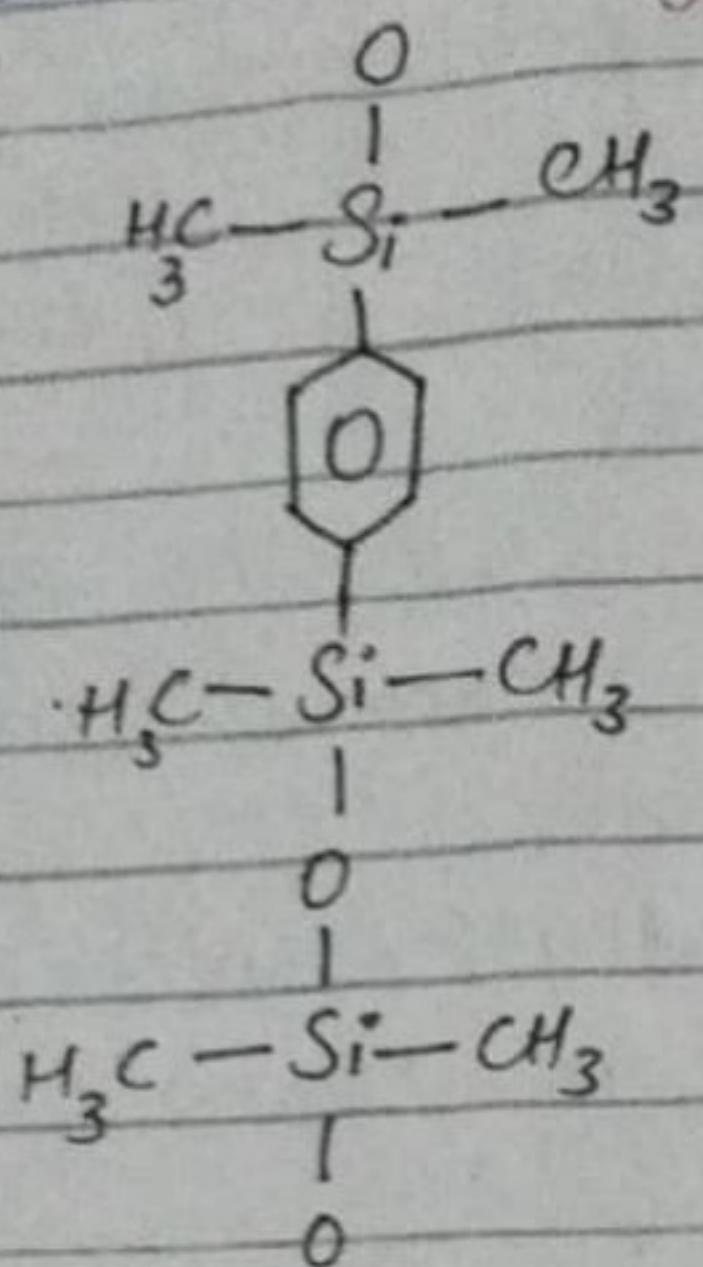
- At high temperature (more than its range) the s. phase start decompose.
- When s. phase broke, the silica exposed, the decomposition of s. phase is known as Bleed.
- As a result of bleeding, few products are formed by combination of Silica and stationary phase.
- These products allute with the analyte and Background radiations are generated in detector.  
"Background signals are extraneous signals produced along with the phenomenon to be observed."
- These background signals reduce signal to Noise ratio. The ratio b/w desired and undesired signal measured in Decible (DB) units.
- Methyl and aryl group attached to silicon at alternate position. known as Dimethyl diaryl polysiloxane. This is used as s. phase.

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- In other S. phase aryl group is present at the centre | in blw silicon group known as Arylene Polysiloxane



- Arylene Polysiloxane is more temperature tolerant and give less bleeding. Hence less background signal produced, so there's no effect or signal to noise ratio.
- That's why Arylene polysiloxane is preferred for coating as S. phase.

## ⇒ How to Reduce Interference :-

Few precautions are taken to reduce

The following

- Thinnest layer of S. phase is coated on column walls.
- Narrow column is used
- Short length Column / Size.
- If column S. phase got O<sub>2</sub> it bleed

- S-phase hence the carrier gas shouldn't be  $O_2$ . To avoid  $O_2$  Sucraber are used which absorb  $O_2$ .
- 1ppb  $O_2$  is enough to bleed S-phase  $H_2O$  hydrolyze S-phase and bleed the S-phase.
- Makers know that silanol group of S-phase exposed and initiate bleeding So manufacturers eliminates the silanol group.
- Ionic group Liquid are also used as S-phase in newly, coated columns.
- They are less volatile hence decrease bleeding.

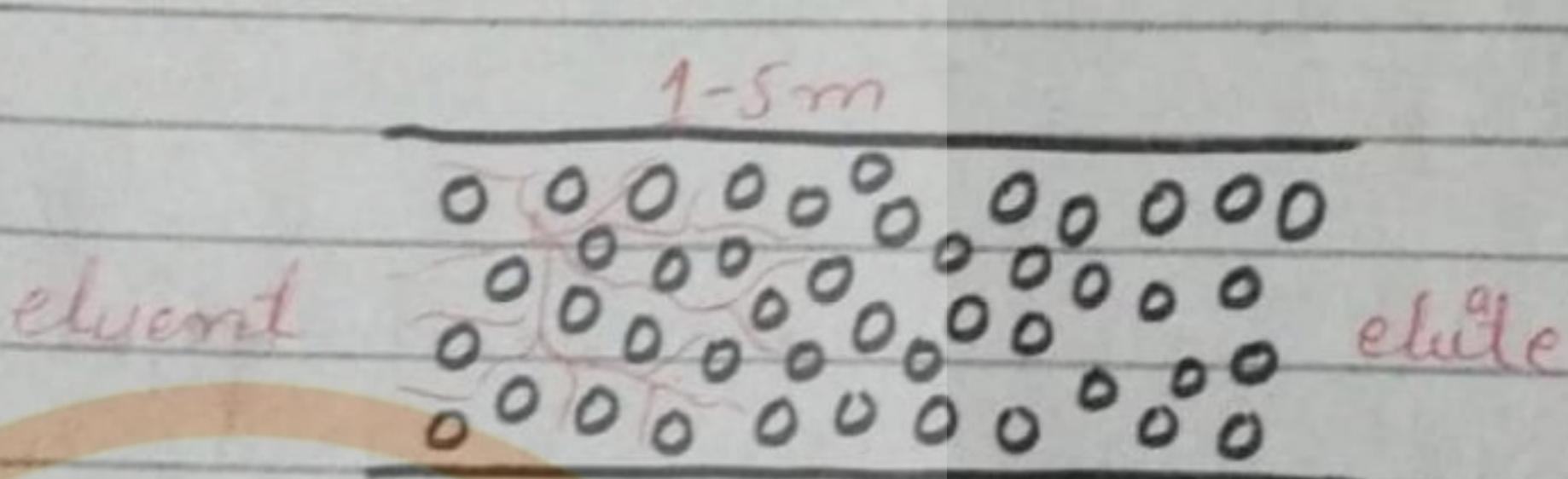
- In PLOT, The solid should be porous like carbon, alumina, styrene, benzene.
- They have n Molecular sieves. which separate the different compounds.

### → Molecular Sieves:-

are inorganic or organic materials with cavities into which small molecules enter and are partially retained. Molecules such as  $H_2$ ,  $O_2$ ,  $N_2$ ,  $CO_2$  and  $CH_4$  can be separated from one another. Gases can be dried by dried by passage through traps containing molecular sieves because water is strongly retained. Inorganic sieves can be regenerated (dried) by heating to  $300^\circ C$  in vacuum or under flowing  $N_2$ .

## (2) Packed Columns

- The internal diameter is 3-6mm
- The length of column is 1-5m.
- Usually solid particle are used in packed column in the lumen of column.
- In few column, filling is of liquid supported by solid.
- Sample introduced into the column and it have the multiple paths to allow sample.
- That's why it take more time so analysis time is more for packed column and Resolution is less.
- But sample holding capacity is more as compared to open tubular column.



- Solid particle used to pack column are mostly ~~silica~~ silica or Teflon.
- If solute is ~~Tenaciously~~ <sup>Tenaciously</sup> soluble, mean form strong bond with s. phase then solid particle taken are Teflon

## Van Deemter Equation:

$$H \approx A + \frac{B}{u_e} + C u_e$$

where

$A \Rightarrow$  Multiple path  
 $\frac{B}{u_e} \Rightarrow$  Longitudinal diffusion

$C u_e \Rightarrow$  Equilibrium time

$H \Rightarrow$  plate Height

- If particle used to fill have small size and uniform in size the factor A reduce.
- As small particle have very small gap between them making less path for sample
- If A reduce, plate height also reduce and also time to equilibrium reduce as now there are less path for sample
- So sample pass earlier from column
- Hence Efficiency of column increased
- The size of particle is measured in two factors

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- Mesh number is no. of openings in one linear inch of screen.
- If a screen have 100 opening then have 100 mesh no.

## → Retention Index :-

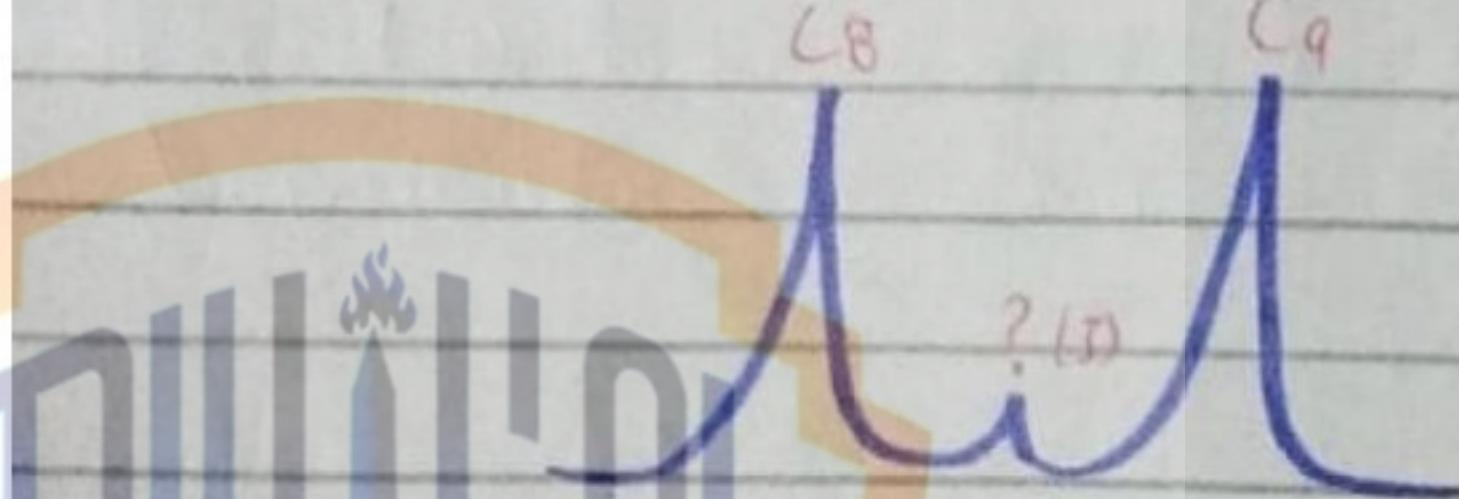
"Retention index relate retention time of solute to the retention time of linear alkane."

E.g:-

A linear alkane like octane have 8C. The retention index is 800 for 8C.

- Similarly for Nonane  $I = 900$ .
- It means 1C have 100 retention (time) Index.
- If a substance is separating in b/w alkane of two known retention index, then to find out I of such substance, following formula is used.

$$I = 100 \left( n + (N-n) \frac{\log t_r'(\text{unknown}) - \log t_r'(n)}{\log t_r'(N) - \log t_r'(n)} \right)$$



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$n \Rightarrow$  No. of C of smaller alkane (8C)

$N \Rightarrow$  No. of C of larger alkane (9C)

$t_{r'(\text{unknown})}$  = given

$t_r' =$  Adjusted retention time

$$t_r' = t_r - t_m$$

$t_r \Rightarrow$  Retention time

$t_m \Rightarrow$  Time of unretained solute.

⇒ For Example:-

$$t_r(\text{CH}_4) = 0.5 \text{ min}$$

$$t_r(\text{octane}) = 14.3 \text{ min}$$

$$t_r(\text{Untknown}) = 15.7 \text{ min}$$

$$t_r(\text{Nonane}) = 18.5 \text{ min}$$

$$I = ?$$

$$t_m = \text{CH}_4 \Rightarrow \text{unretained.}$$

$$t_{r'} = t_r - t_m$$

(Unknown)

$$t_{r'(\text{u})} = 15.7 - 0.5 \\ = 15.2$$

$$t_{r'(\text{n})} = 14.3 - 0.5 \\ = 13.8$$

$$t_{r'(\text{N})} = 18.5 - 0.5 \\ = 18$$

Put values in formula:-

$$I = 100 \left( n + (N-n) \frac{\log t_{r'(\text{u})} - \log t_{r'(\text{n})}}{\log t_{r'(\text{N})} - \log t_{r'(\text{n})}} \right)$$

$$= 100 \left( 8 + (9-8) \frac{\log 15.2 - \log 13.8}{\log 18 - \log 13.8} \right)$$

$$\boxed{I = 836}$$

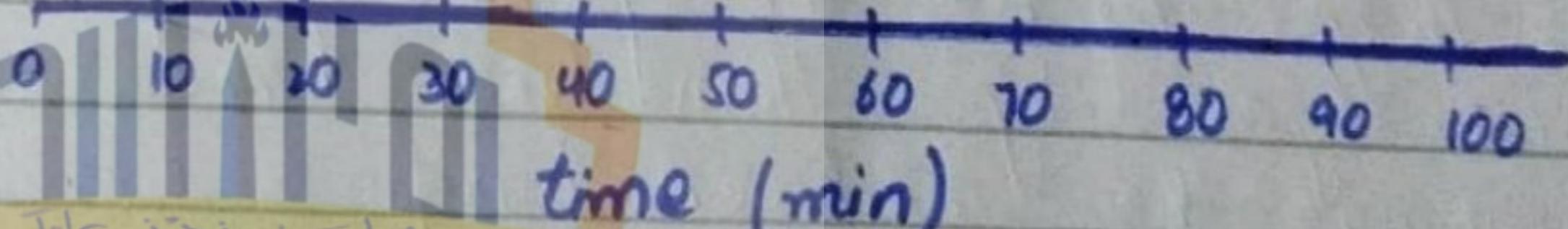
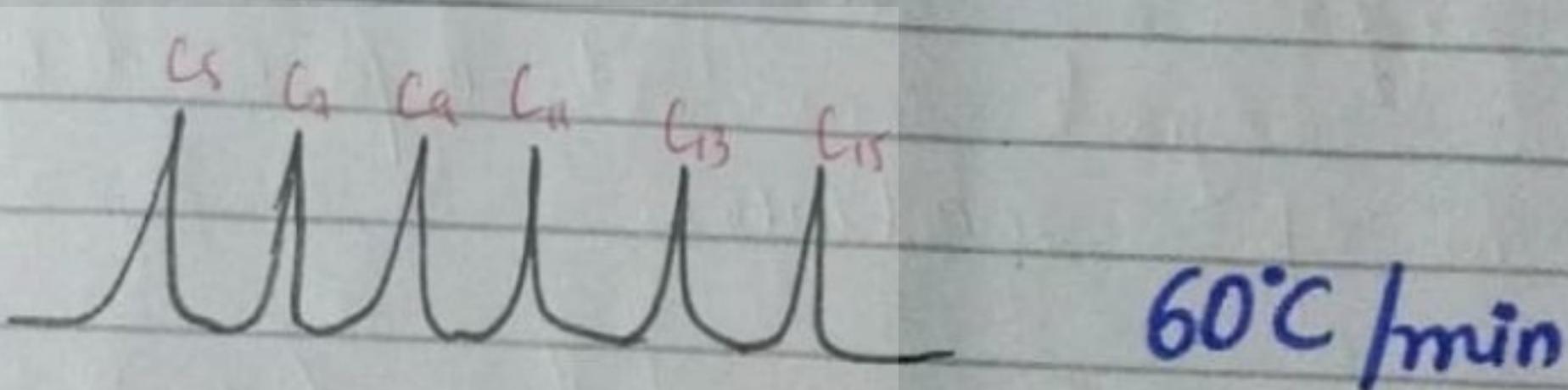
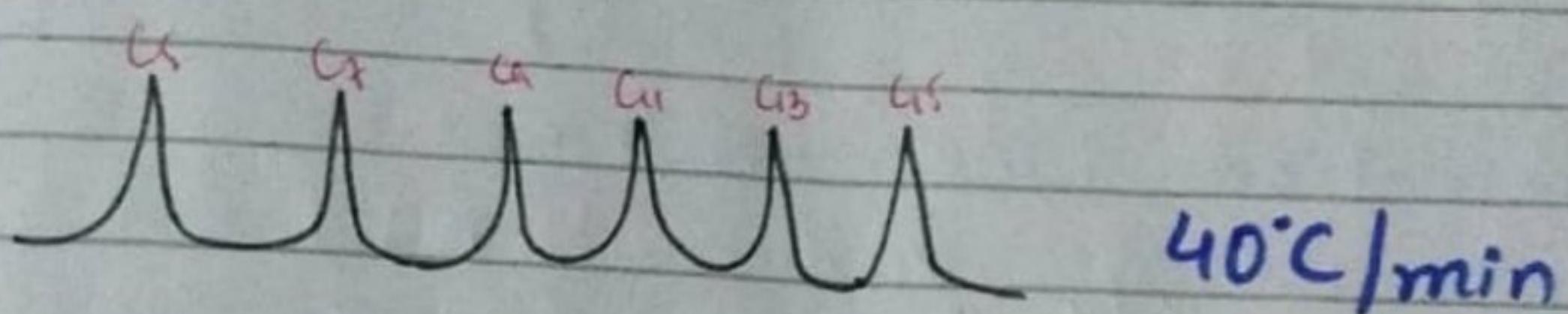
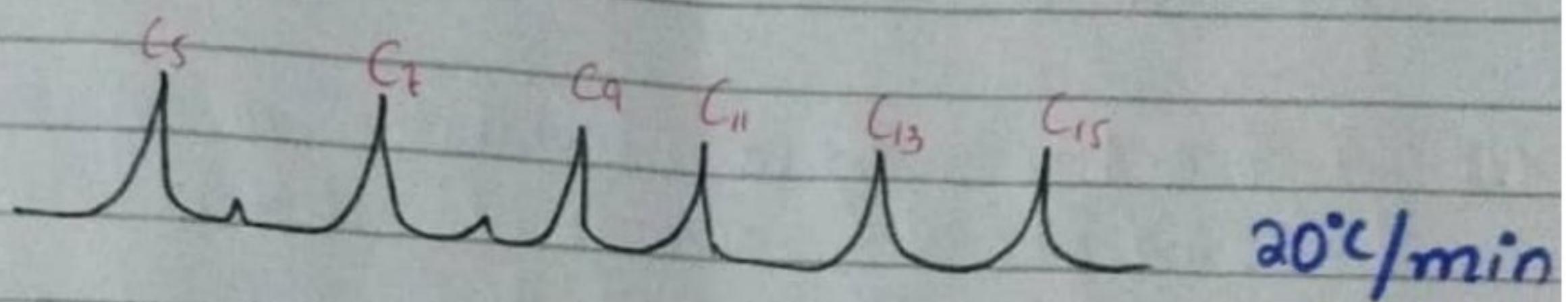
# Temperature And Pressure Programming:-

- In chromatography column is temperature programmed, a label is attached which have two temperature values, lower and higher. Assume it is  $50-300^\circ\text{C}$ .
- This label means  $50^\circ\text{C}$  is lowest temperature given to this column and at this temp column have long life span.
- While  $300^\circ\text{C}$  is highest temperature can be given to this column and this known as **programmed Temperature**.
- At that temperature, elution of the sample is very fast, but life span of column is decreased, as at this Temperature S-phase decomposes and show **Bleeding** process.
- All chromatographic column are temp. programmed and few are both temp. and pressure programmed.  
The separation of  $C_5-C_{15}$  at different temperatures is done. Such as first spectrum is drawn at  $20^\circ\text{C}/\text{min}$ .  
In next step temperature is increased upto  $40^\circ\text{C}/\text{min}$ .  
The (increased) heavier alkane first elutes at  $100\text{ min}$  but at high

Temp. it allute after 70min. Further temp is increased upto  $60^{\circ}\text{C}/\text{min}$ . Now heavier alkane allute after 80min.

→ This show that,

- Increase in temperature, decrease the allution time.
- Peaks are sharp, less broad.
- Resolution increase.



The pressure broadening required for substances which can't bear higher Temp and decompose before allution. Such substances are alluted by pressure programming.

⇒ The substance which allute earlier such as C<sub>5</sub> is alluting at same time even at different Temperature. These substances are first alluted at constant flow Temp. Once they are alluted then temperature programming is done.

⇒ The signs which show that column is degraded,

- (1) If baseline signal number increase it indicate column is degraded.
- (2) Peaks are broad.
- (3) Tailing
- (4) Retention time increase.

Baseline Signal:-

Signal recorded before allusion of substances.

Some spectrophotograph are electronically built up, they increase the pressure of carrier gas by themselves hence allusion time.

# Applications of Gas Chromatography:-

Gas chromatography was discovered by **Mikhail Semyonovich** in early 1900's.

The separation technique is used to first split the chemical components of mixture and determine the presence or absence of each component as well as to measure the level of each detected compound.

## → Food Analysis:-

The Food industry relies on the technique of G.C for several applications, including qualitative and quantitative analysis of food.

- The analysis of food additives
- Components of flavor and aroma.
- detection and analysis of ~~the~~ components / contaminants such as environmental pollutants, pesticides, fumigants and naturally occurring toxins.

## Quality Control:-

The pharmaceutical industry uses

gas chromatography to help produce pure products in large quantities. The method is used to ensure the purity of the produced material, eliminated inconsistencies in pharmaceutical products.

The industry also uses Gas-Chromatography to analyze compounds to check for trace contaminants.

Currently, there is also a growing use of the method within the pharmaceutical industry to separate chiral compounds.

Interiors of new automobiles release a significant amount of volatile organic compounds (VOCs).

For this reason, gas chromatography has been adopted by the automotive industry.

To identify and measure the chemicals that are released into air inside the car from its

Carpets

door linings

Pedals

seat covers.

Research :-

Gas chromatography is fundamental

to many research areas.

- For analysis of meteorites
- For analysis of natural products
- To analyze composition of meteorites that fall to the earth.
- Provide information regarding the nature of life outside of earth.
- To determine presence of ribose in meteorites, the building block of RNA.

## Forensics :-

It is used to determine the circumstances of a person's death such as:

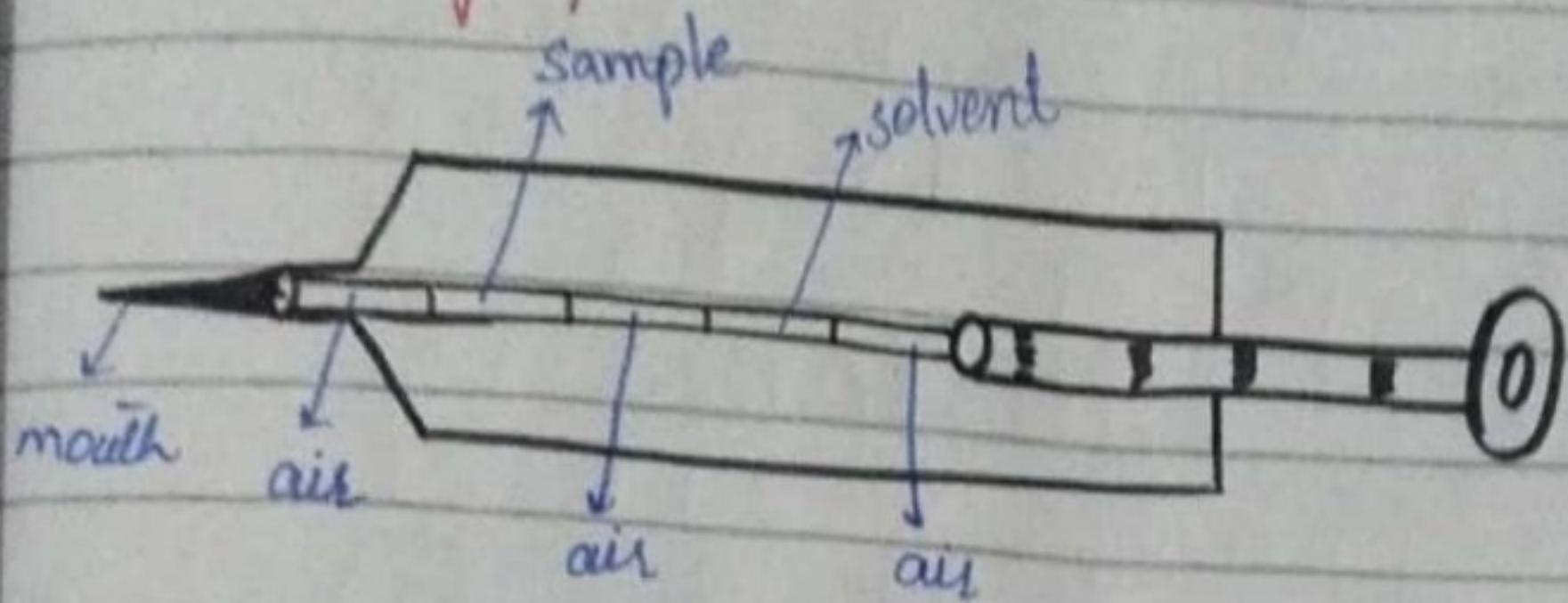
- Whether they ingest poison.
- Consumed drugs
- Alcohol in the hours prior.

Scientists take samples of blood and fibers from the crime scene and analyze them using gas chromatography to help investigators piece together the facts.

PUACP

## → Sample Injection:-

How sample is injected into gas chromatograph.



(Sandwich Injection)

Syringe is used for injection. Before use, it is washed with solvent for several times.

Before filling sample, air is filled into syringe and then filled with solvent then air and then sample.

## → Rubber Septum:-

Sample is introduced into septum, which is pre-heated.

When syringe is injected into septum, the air goes into the septum. If air is not filled, the sample / solvent first enter the septum and volatile part of solvent evaporated.

→ In 1<sup>st</sup> 2<sup>nd</sup> filling of air is done,  
2<sup>nd</sup> Air filling is done for  
two reason.

- To completely propel the solvent
- To separate the sample and  
solvent.

## Types of Injection:-

- (1) Split Injection
- (2) Splitless Injection
- (3) On-Column Injection

### (1) Split Injection:-

It is used to inject small  
sample into chromatograph  
column.

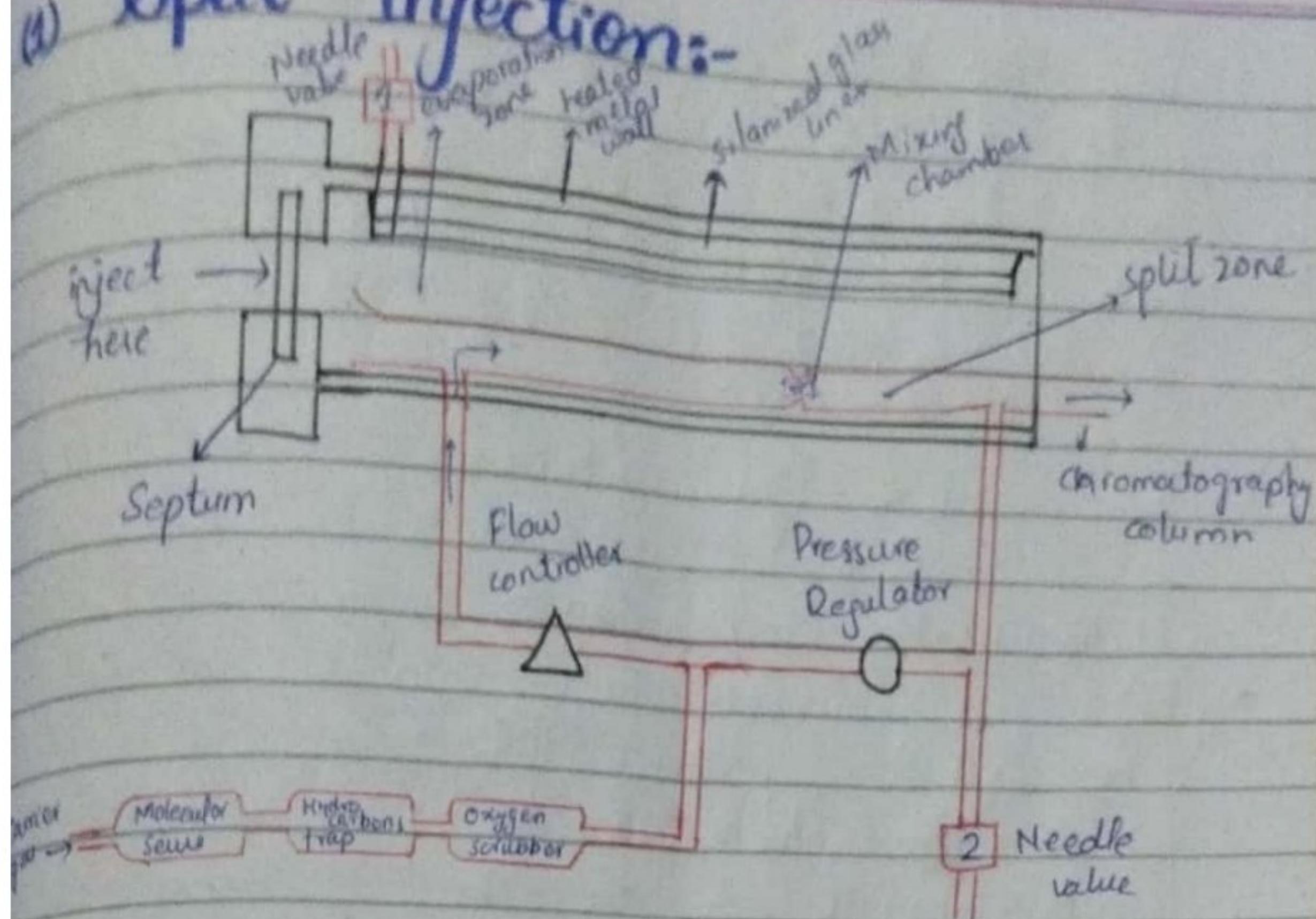
### (2) Splitless Injection:-

It is used to inject trace amount  
of sample. The B.P of solute  
and B.P of solvent  
is low.

### (3) On-Column Injection:-

When sample is Thermally unstable  
on-column Injection is used. Solute  
is unstable means solvent have high B.P.

## (a) Split Injection:-



→ If concentration of analyte is  $> 0.1\%$  of the sample, then split injection method is used.

→ To get more accurate separation small conc. of sample (nearly  $1\mu\text{L}$ ) should be used. As only small portion of sample will reach column, other is wasted ( $0.2 - 2\%$ )

## Working:-

- Sample is injected into septum. If injection is manual, after 20 should be changed. For PUM auto injection it changed after 100 injection.
- The septum zone is pre-heated nearly  $350^\circ$ . The sample vaporize after

- coming in this region (evaporation zone) If Temperature is ~~to~~ not this much then low B.P element is vaporized first and high B.P after (later) Sample injected is less than one second.
- The carrier gas introduced from other opening after passing molecular sieves, hydrocarbons trap and oxygen scrubber. The gas mixed with sample in mixing zone. And further split zone is present in which sample split and a small amount go to the column and large amount went off. Went off is controlled by the pressure control.
- The sample leave injection through needle valve 2.
- The injection is sealed with saturated glass lining which is completely sealed so that carrier gas can't enter these lining.

→ The proportion of sample that not reaches the chromatography column is known as split ratio. It is 50:1 to 600:1

- After sample is flushed ~~from~~ injection port, needle valve 2 is closed and carrier gas flow is reduced.

Quantitative analysis through this injection are not accurate because split ratio is not reproducible from run to run.

1uL liquid injection creates 0.5mL of gas volume.

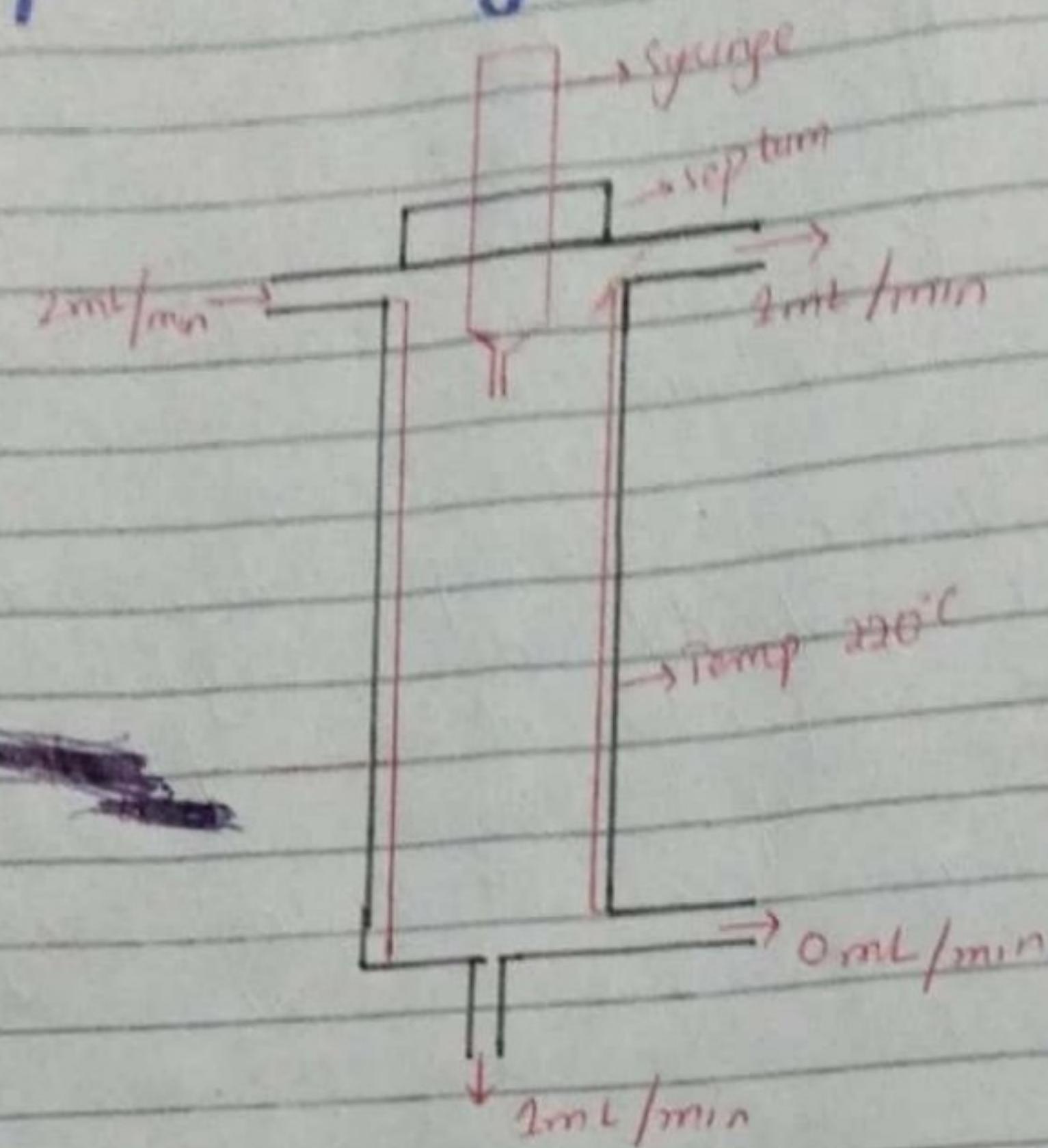
- Some vapors escape backward. So temperature should be high enough to minimize fractionation of sample.
- However, if Temp is too high, decomposition occurs during injection.

Septum purge gas is introduced through needle valve 1 at rate of 1 mL/min to remove excess vapour and gas that bleed from hot rubber septum.

- For dirty samples, These injection are used and packing material can be placed inside liner to absorb undesirable component of sample.

- Glass wool may be placed at near end of liner. So liquid on outside of syringe needle get wiped off by wool before needle is withdrawn.

## (2) Splitless Injection:-



- It is straight empty tube without mixing chamber.

- It is used for trace analysis of analyte  $< 0.01\%$ .

- There is not much difference b/w split and splitless injection, only of glass liner.

- Split have narrow glass liner and have mixing chamber for mixing of carrier gas and sample.

- While have hollow tube which don't have any mixing chamber.

Moreover Temp of split was  $350^\circ\text{C}$  while splitless have  $< 220^\circ\text{C}$

Sample introduced through rubber less

than 1 sec in split but in splitless sample injection time is increased upto 2 sec.

- It is briefly for split and for splitless it is slowly
  - The residence time is increased (1 min) for splitless that's why Temp is decreased, so low B.P. component don't evaporate fastly.
  - The amount of sample reaching chromatography column was little for split, while for splitless it is about 80%.
- Solvent The initial column Temp is set  $40^{\circ}\text{C}$  below B.P. of solvent. So solvent condense at spot of initial part of column.
- When solute pass through this part, it is trapped by solvent known as **solvent trapping**.

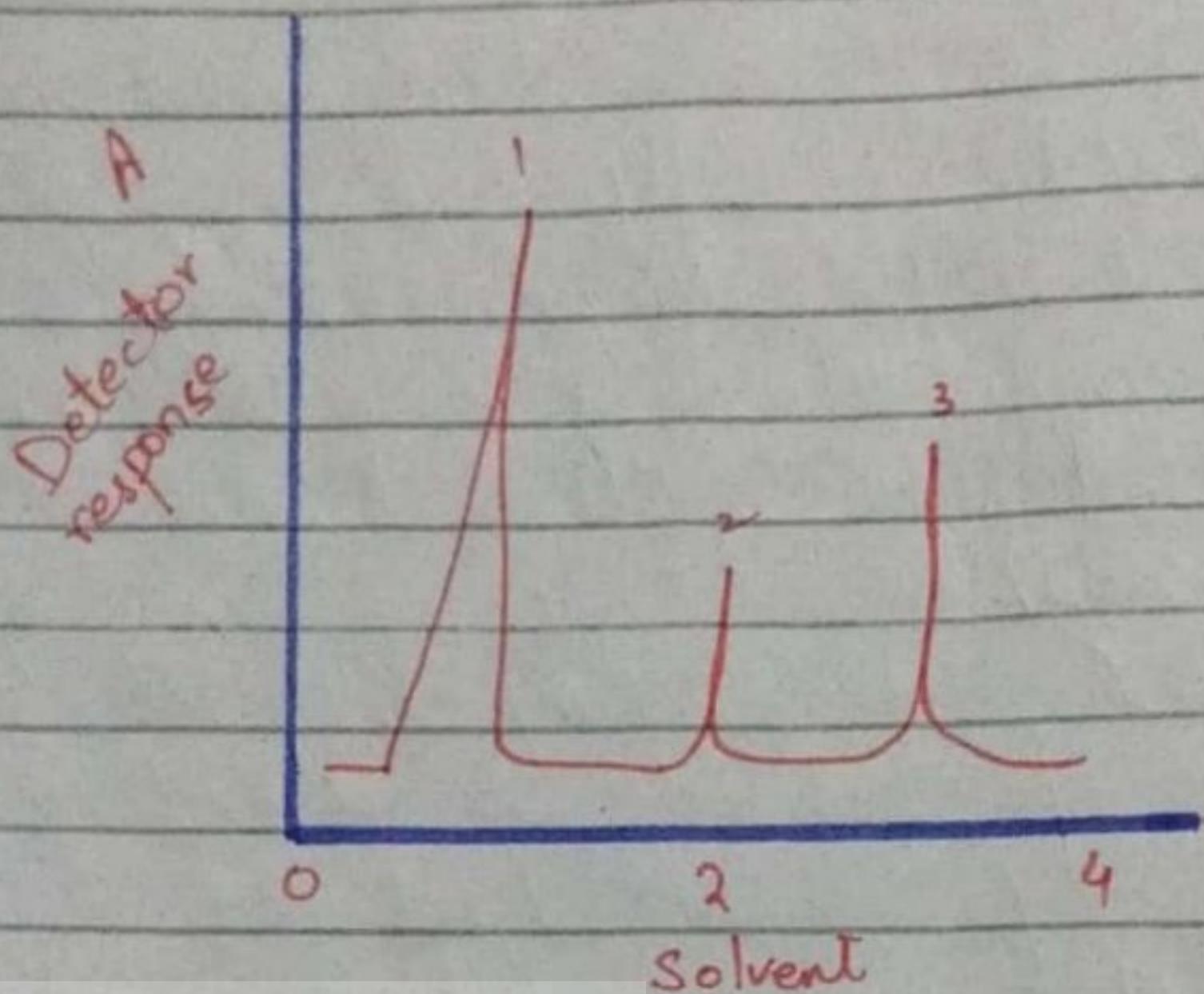
→ The advantage of solvent trap is that its sharp the peaks without trapping. Peaks are broad.

### → Cold Trapping:

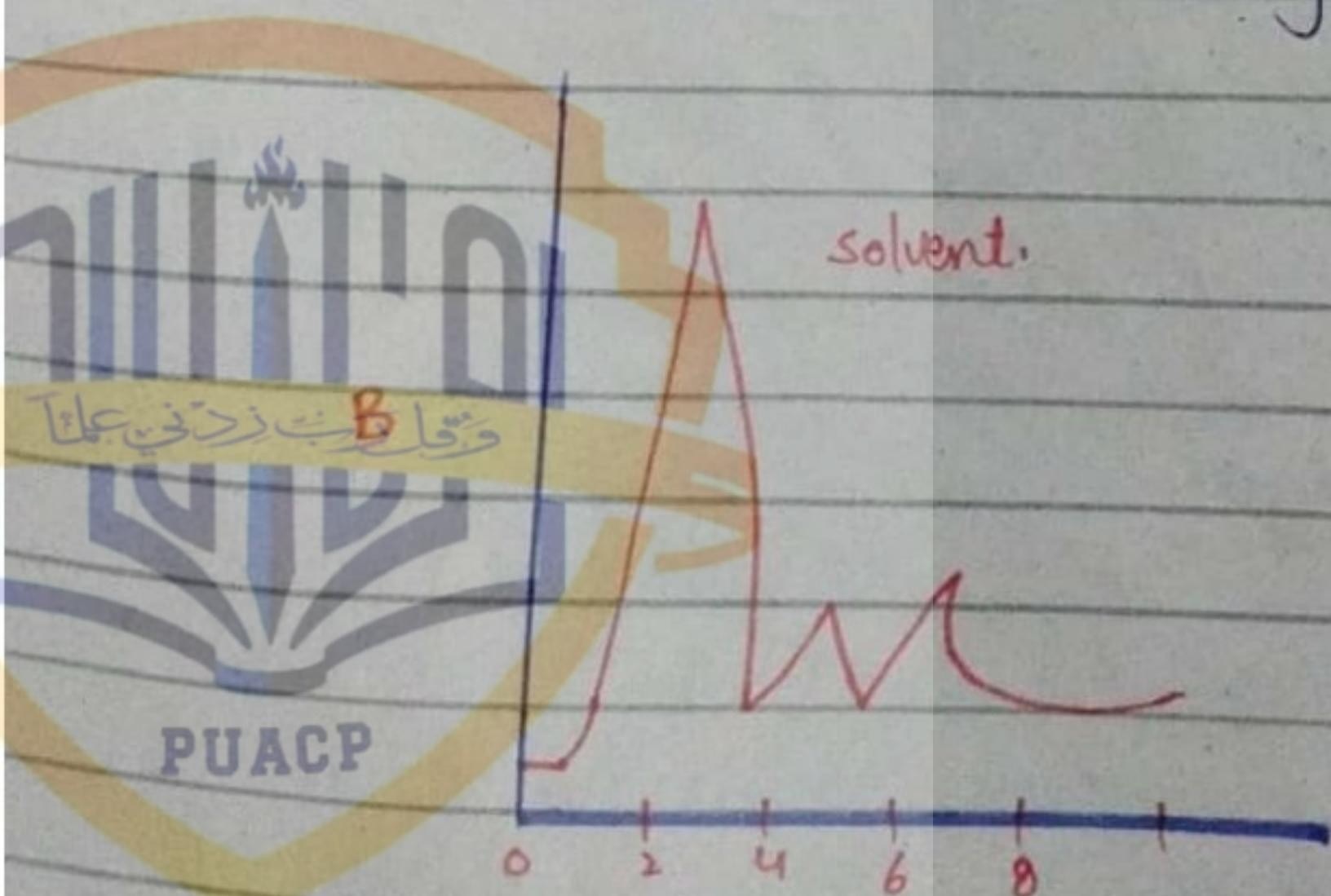
The initial column Temp is adjusted below  $150^{\circ}\text{C}$  B.P. of <sup>PUACP</sup> the component which have low B.P. condense here. Then column is rapidly warmed for high B.P.

For low boiling solutes, cryogenic focusing required.

In graph A, the peaks are sharp for split injection, because sample flows readily

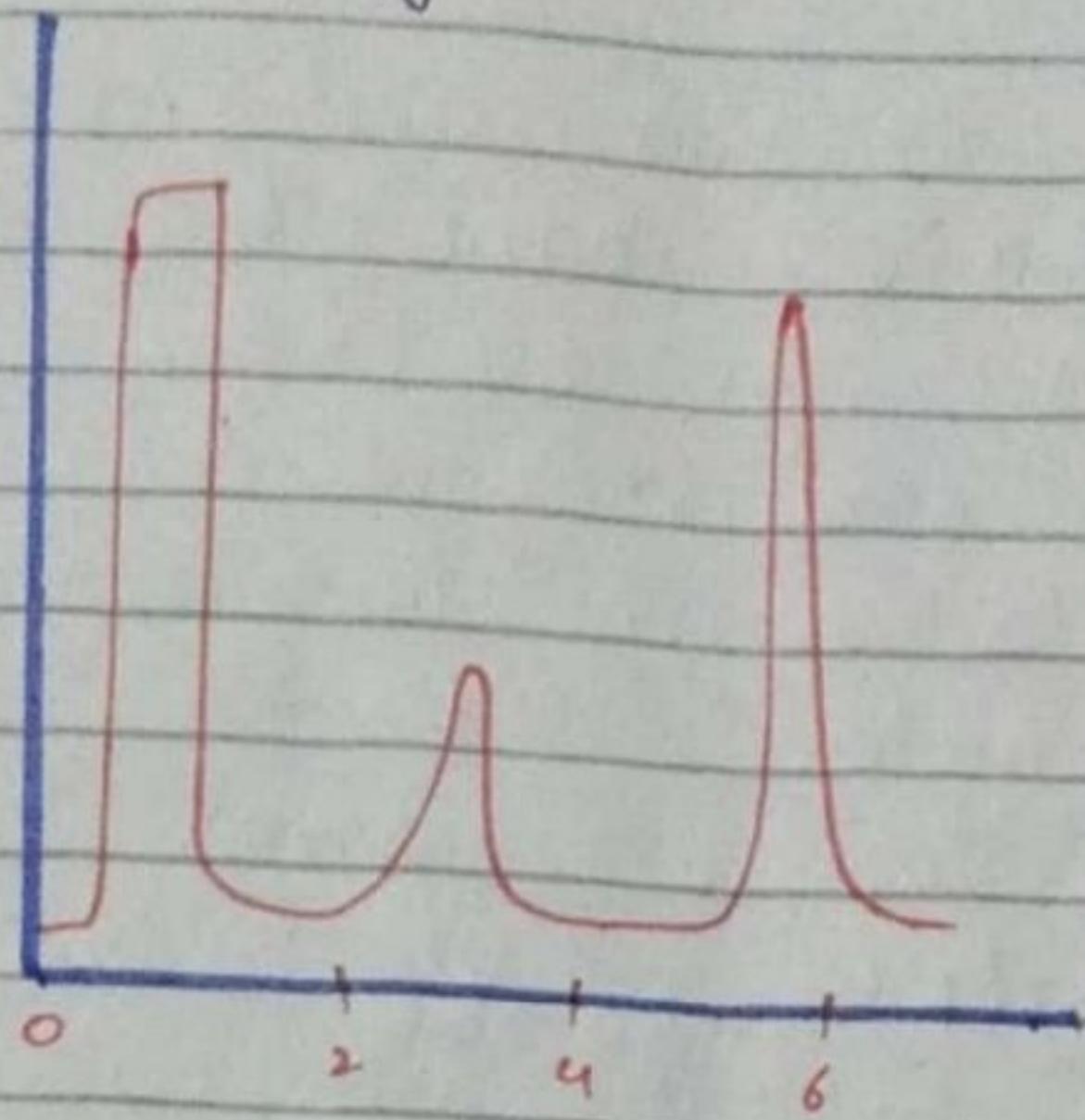


In graph B, split vent closed carries gas purge sample readily, peaks are broadened and tailing occurs.

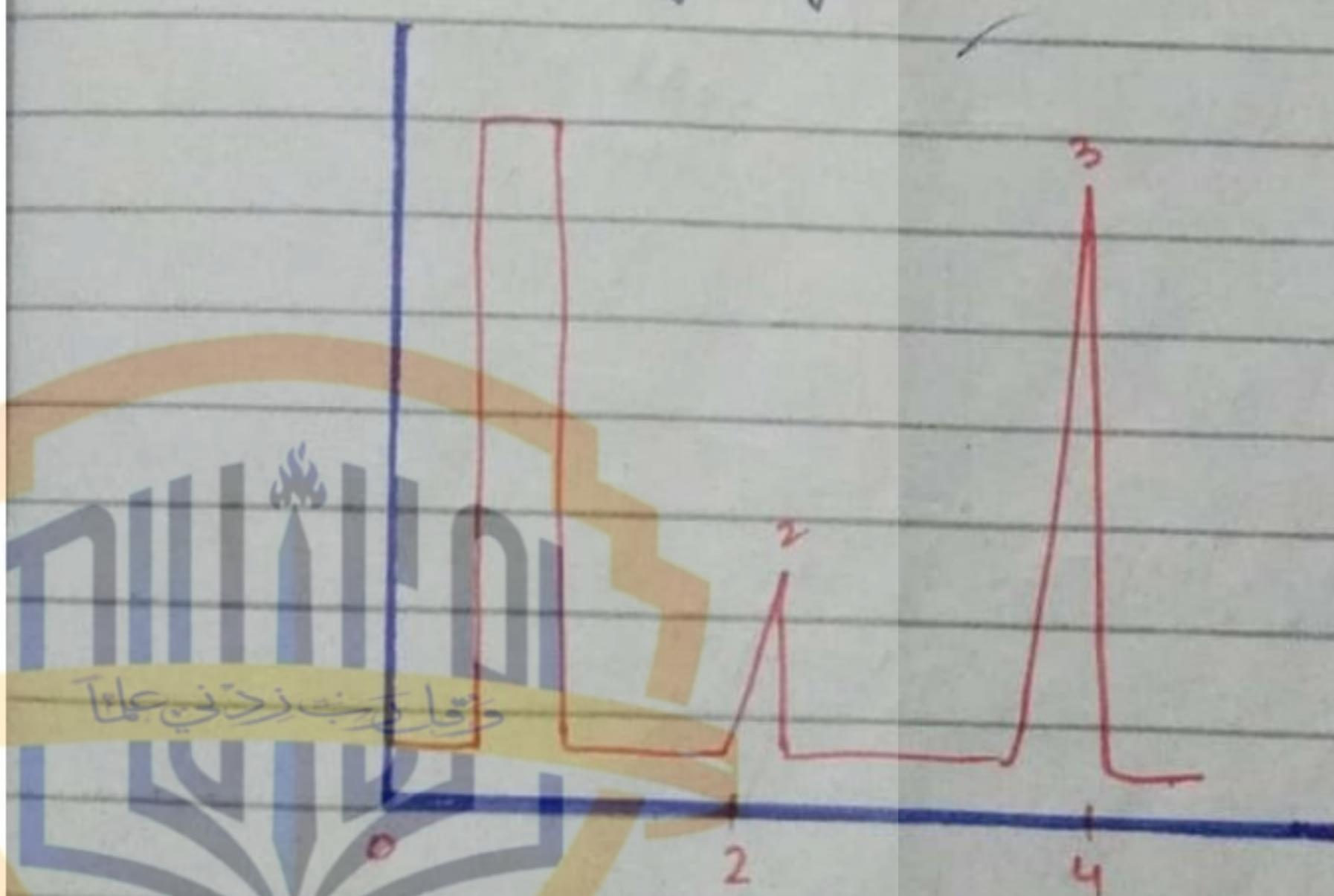


In graph C it is same as B but split vent opened after 30sec

sample fastly flow through column.  
and ↑ less tailing occur.



For graph D solvent trapping only for splitless.  
Initial T is less and solute peaks are sharp. Column is initially cooled to 25°C to trap solvent and solutes at the begining in column.

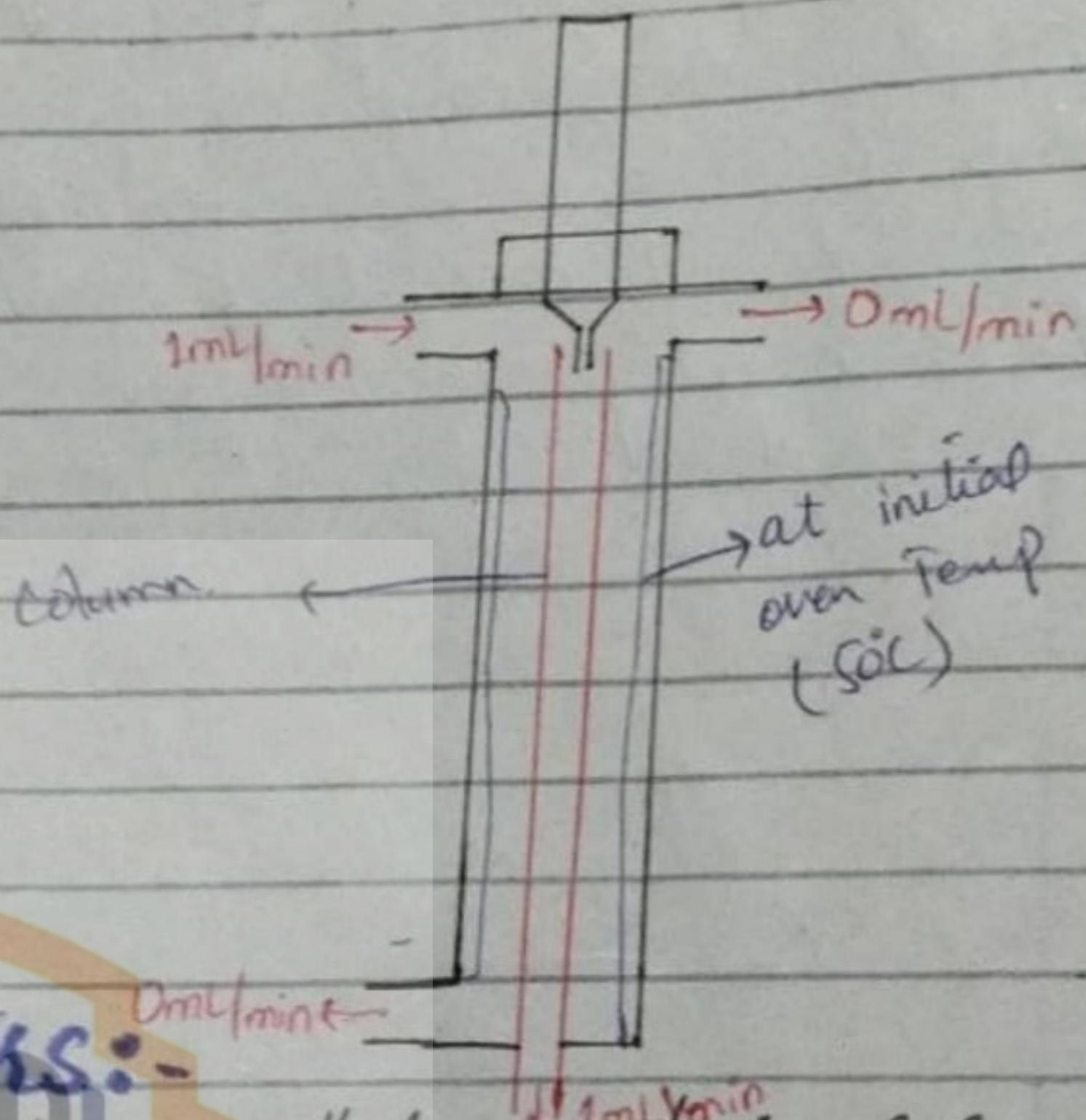


Actual ACP peaks areas in D are greater than those in A because most of the sample is applied to the column in D, but small fraction in A.

## On Column Injection:-

On column injection used for compound that decompose above their B.P. and is preferred for quantitative analysis.

- On column injection port is used. Sample go to column through port, but in this, sample is directly passed through injection to column.
- The initial temperature is very low to condense solute in a narrow zone.
- Needle is coated with silica, any other needle do not give good result.



## Detectors:-

The devices that used in G.C and measure the component of mixture being eluted from column.

## Thermal conductivity Detector:-

- These are most used in past due to properties that.

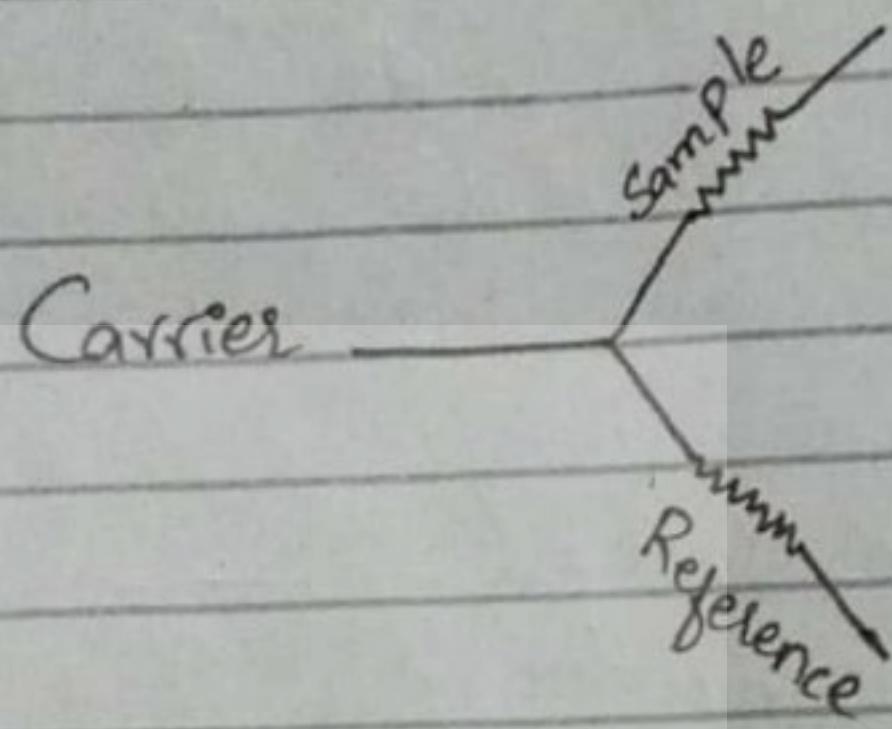
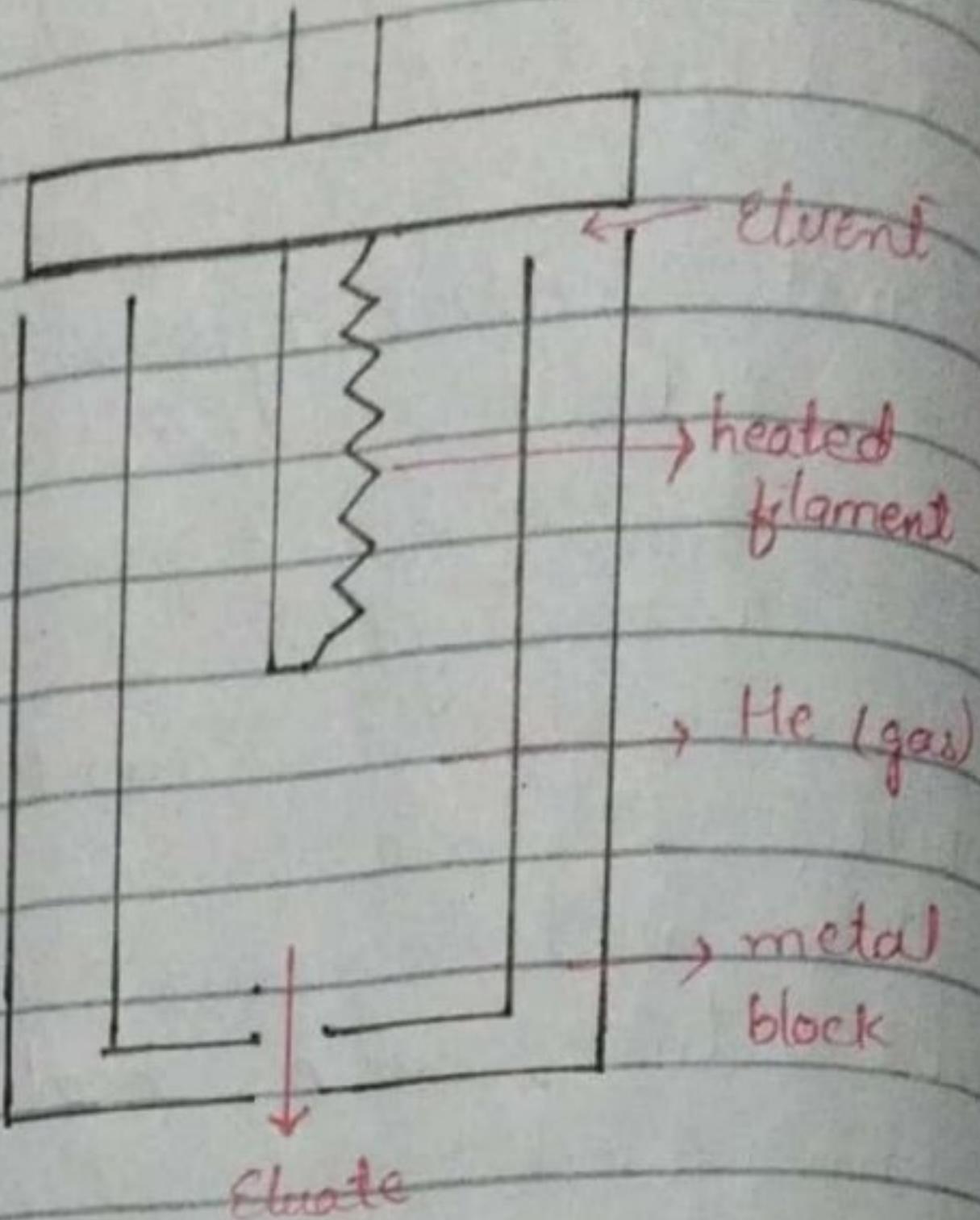
- They are simple
- Universal
- Used for packed column
- Respond to all analyte

- Thermal conductivity is the ability of a substance to transfer heat from a hot region to cold region.
- Diagram:- A carrier gas  $\text{He}$  is filled in this column.  $\text{H}_2$  is most conductive gas while 2<sup>nd</sup>  $\text{He}$  is most conductive.
- When allute come outside the column, it strike with heated tungsten filament, before eluting due to which conductivity of  $\text{He}$  is reduced.
- Three factors are controlling the sensitivity of detectors.

- (i) Flow rate:- Sensitivity  $\propto \frac{1}{\text{Flow rate}}$   
Sensitivity is inversely related to flow rate. It is more sensitive at lower flow rate.
- (2) Difference of Temperature between block and Filament:- Sensitivity  $\propto \frac{1}{\text{Diff}}$   
It is inversely related to the sensitivity. So block is maintained at low Temp to allow solute remain gaseous.
- (3) Filament's Temperature:-  $\propto$  sensitivity.  
It is directly related to the sensitivity.
- The filament get hottes, its electrical resistance increase and voltage

across filament change. Detect or measure change in voltage.

Diagram:-



- The carrier gas going toward sample, have two resistance filament one is sample and other is reference filament.
- Carrier gas divided into two routes now the measure of give the voltage change in resistance measurement.
- The resistance of sample filament is measured w.r.t reference filament.
- The reference filament minimize flow difference when Temp is changed. Sensitivity increase with square of filament current.

→ However to avoid burning out filament, maximum current shouldn't exceed. So recommended current shouldn't be exceeded when carrier gas is not flowing.

## Flame Ionization Detector:-

- The eluate enters flame ionization detector.
- Mixture of gases such as  $H_2$  added into it. The eluate along with  $H_2$  when flow, it is again mixed with air.
- If eluate have carbon atom the carbon is converted into CH radical.
- Except carbonyl and carboxyl carbon, all convert into CH radical.
- The CH radical mixed with air and when reach to flame it convert into  $CHO^+$  (formyl cation). along which  $e^-$  are produced.



- If  $10^5 C$  enter the column only 1C atom ionize and produce  $e^-$ .
- The process of ionization depend upon no. of susceptible C atom entering the flame as if C atom not entered in flame, they will not ionize.

### Dark Current:-

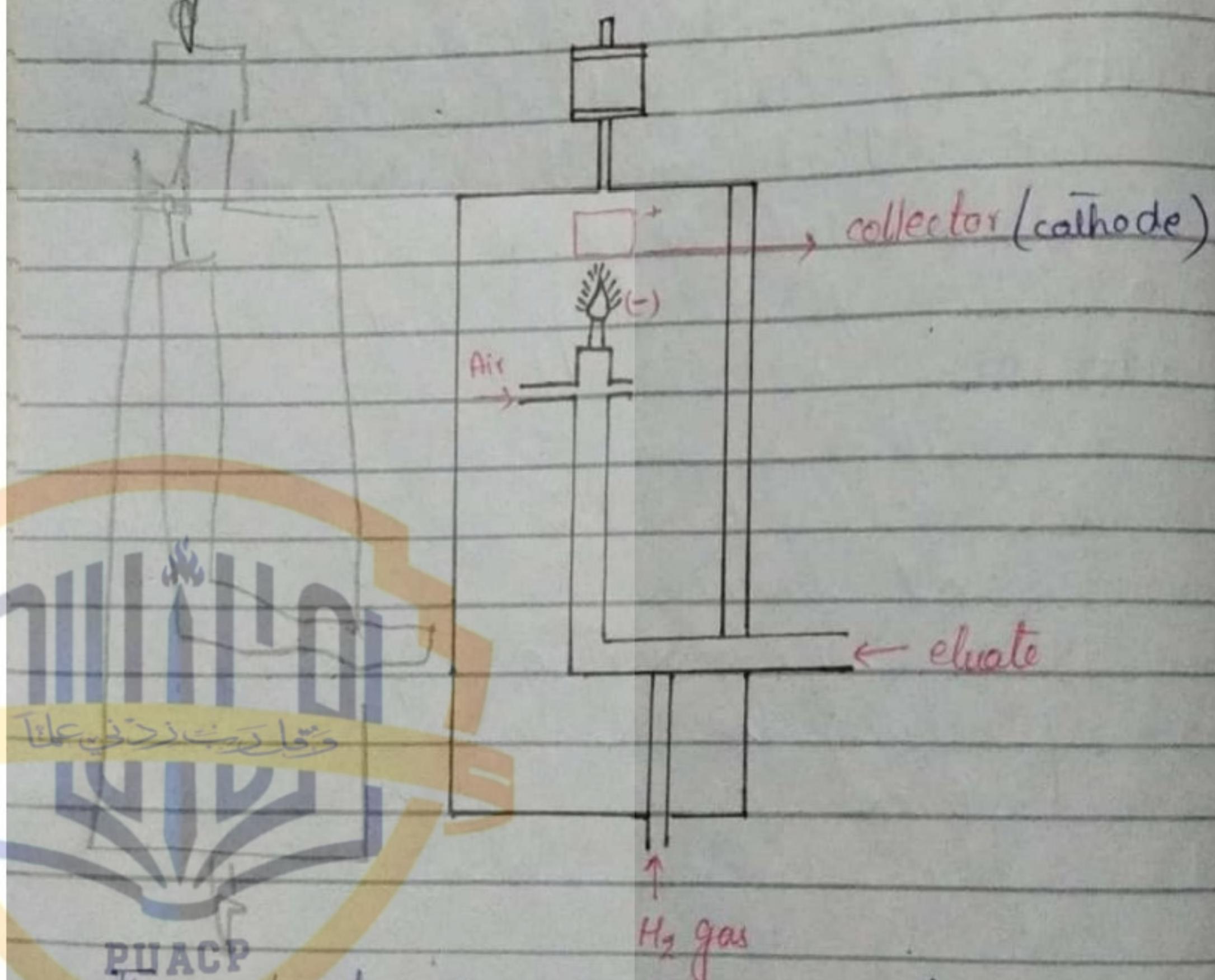
- If C atom not entering flame, nearly  $10^{-14} A$ , blue flame tip and cathode.
- A collector is present at tip of flame, which is a cathode and flame is

negative, so current flow between them.

→ When eluate pass between them  $\sim 10^{-12} \text{ A}$   
Current is produced which is converted into voltage and amplified and filtered to reduce high resonance frequency and (resonance frequency) converted into signal.

$10^{-12} \text{ A} \rightarrow \text{voltage} \rightarrow \text{Amplify} \rightarrow \text{filter}$   
Signal ←

Diagrams:-



- The limit is 100 times smaller than detection limit of the thermal conductivity detector.  
→ It means it can detect even at a lower level.

If  $N_2$  is taken as mixing gas, its detection limit reduce further to 50% level.

### Detection of limit Detection power

In open tubular Column,  $N_2$  gas is taken as a make up gas. If it is very narrow OTC, (open tubular column) it can even detect them. It is most sensitive to hydrocarbons and show no sensitivity to non-hydrocarbon.

### Electron Capturing Detector:-

This detector is sensitive for some classes of compounds such as,

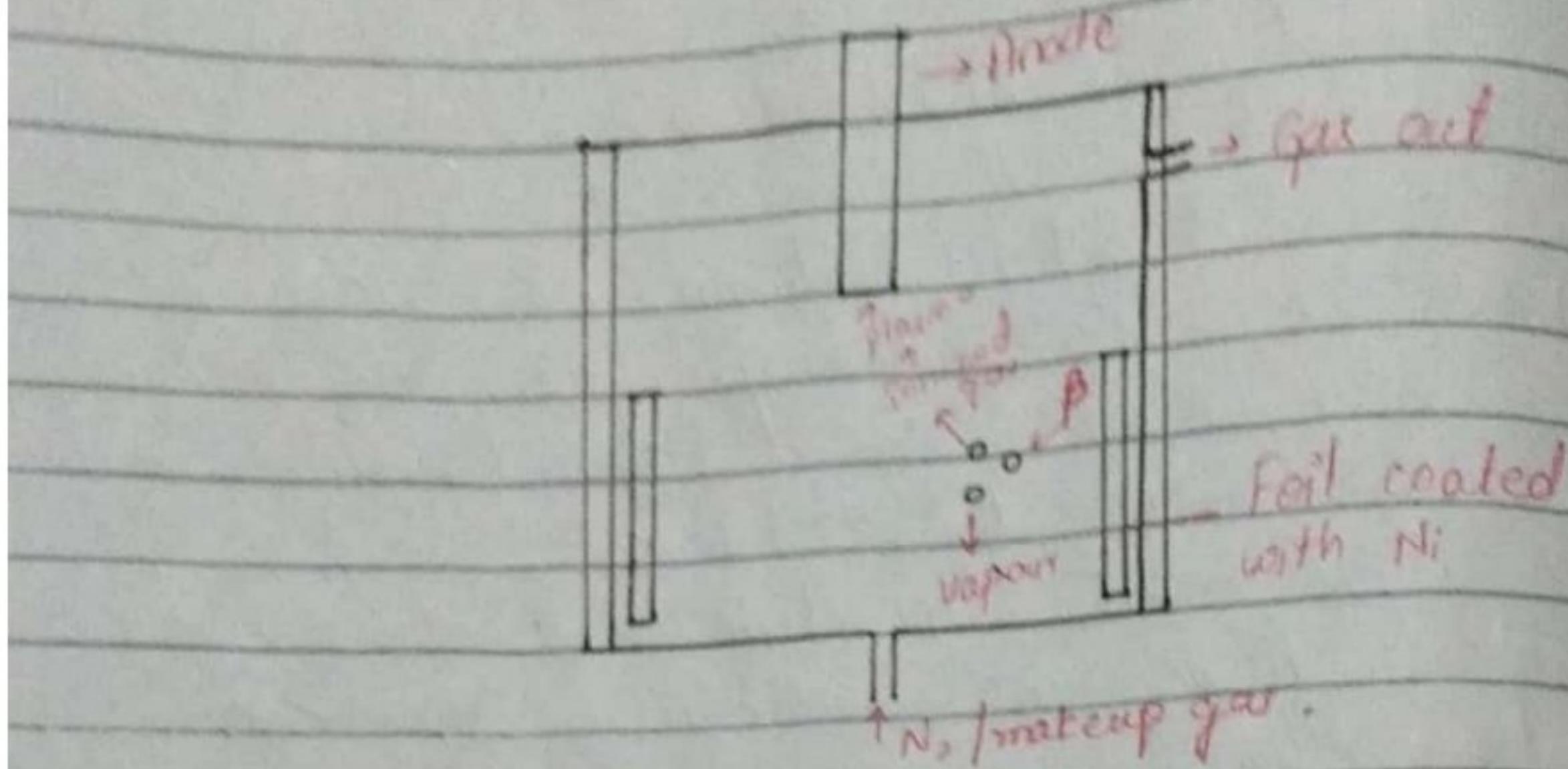
- Halogen containing molecules
- Nitro compounds
- Nitrile compounds
- Conjugated carbonyl compounds
- Organometallic Compounds.

There are few classes, for which they are totally insensitive, such as

- Hydrocarbons
- Alcohol
- Ketones

→ Carrier gas.

Mixtly N<sub>2</sub> gas is used as carrier gas. Sometimes 3% CH<sub>4</sub> in Argon is also used as a carrier gas.



→ The carrier gas/make-up gas is entered into detector.

→ A foil coated with Ni present in detector. Ni is radioactive element emitting radiations which may be  $\beta$  or  $\gamma$  rays.

→ The  $\beta$  or  $\gamma$  rays strike with the gas and gas is ionized.

→ The gas vapours along with the  $e^-$  and ions is known as plasma.

→ Plasma is attracted toward anode, as a result a small amount of current generated which is maintained at constant level with the help of pulses of frequency.

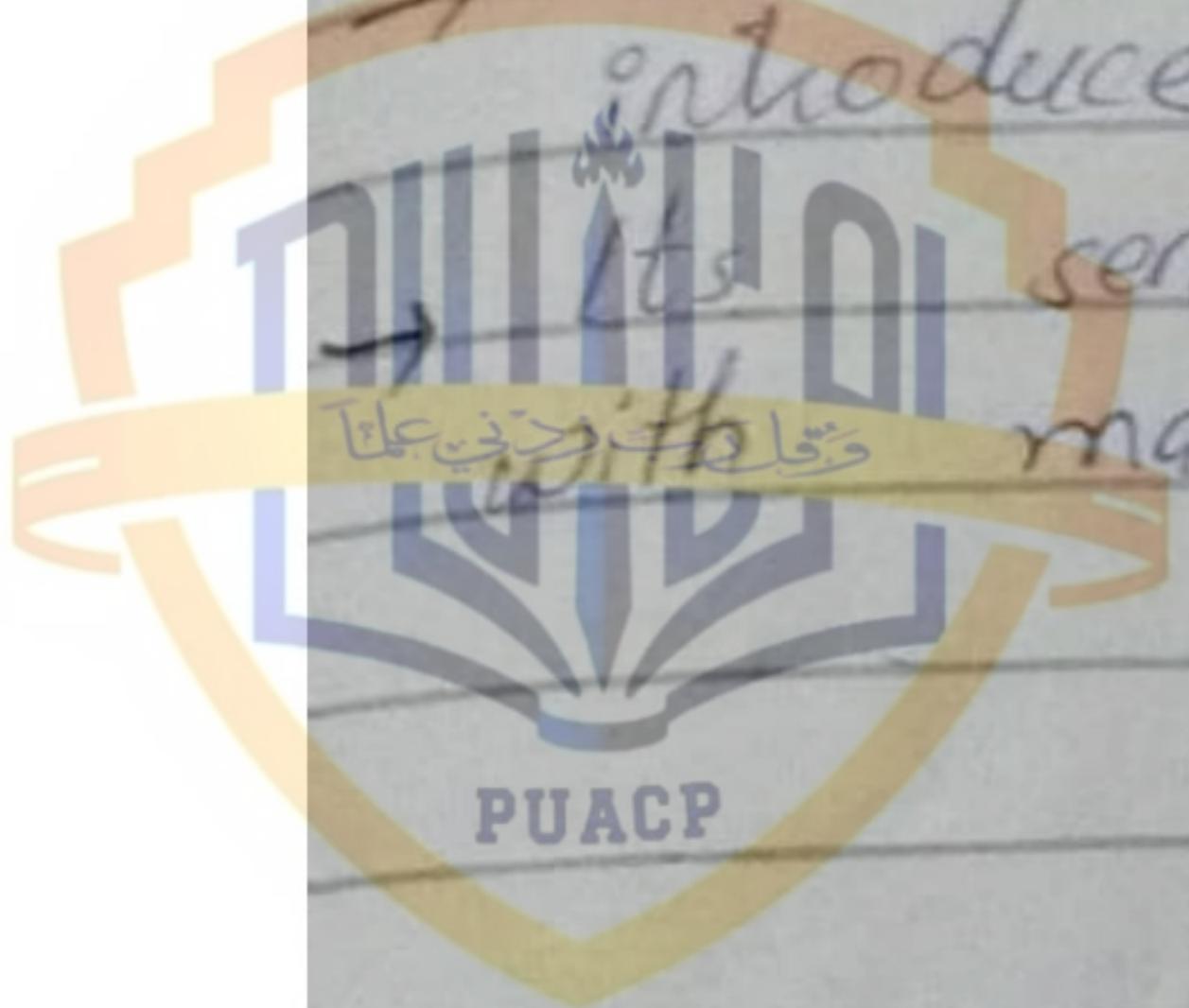
→ Non-analyte is passed through detector, few analyte have ability to capture  $e^-$ . As a result,  $e^-$  moving toward anode decrease in no. so amount of current

generating is reduced.

→ The current is maintained by frequency pulses. If no. of pulses decrease or increase, it is the signal of detector.

→ This is signal of the analyte, being introduced into detector.

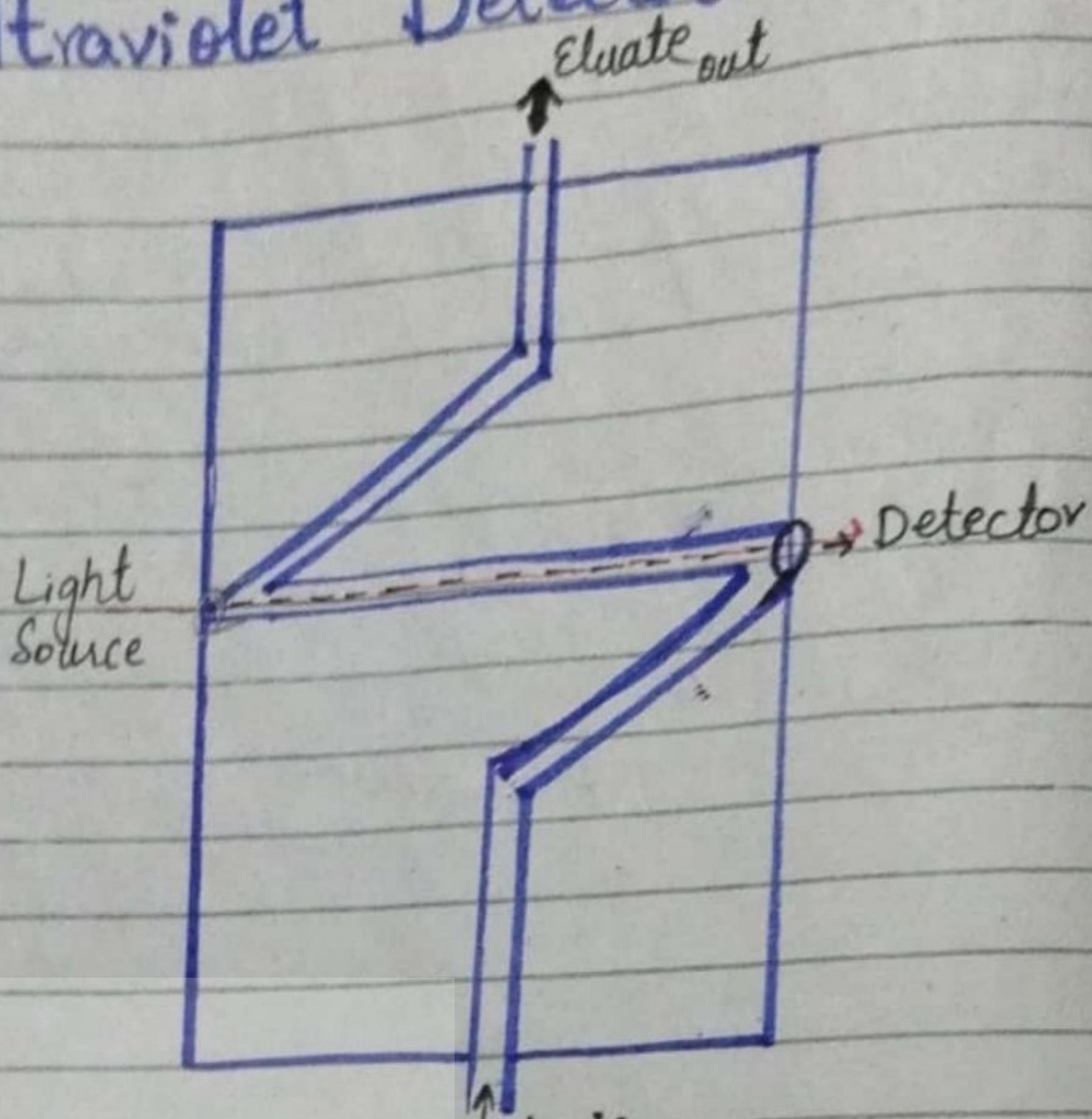
→ Its sensitivity can be compared with mass - spectrometer detector.



# HPLC:-

## Detectors:-

### (1) Ultraviolet Detector:-



→ It uses a flow <sup>eluate in</sup> cell and is most common detector used in HPLC, because many solutes absorb UV light and at the base of their absorbance components of solute and solvent measured. → Solutes introduced as it flows in flow cell (eluates in) beam of intense beam of <sup>emission of</sup> 254nm Mercury vapour lamp. or 261 nm

More versatile instruments have broad band deuterium, Xenon or tungsten lamps and a

monochromator, so you can choose an optimum wavelength for your analyte.

### Types of Sources:-

- At fixed wavelength, specific wavelength of sample on which light detected. if the absorbance is known. (Aspirine)
- At variable wavelength at which a sample give maximum absorbed and detected. (several elements).
- It is better than fixed wavelength.
- More versatile version of UV detector. Photo-diode array detector is ~~record~~ replaced that <sup>record the</sup> spectrum of each solute as it is eluted.
- Noise level is just 1% in UV detector.
- Iso-chritic elution (single solvent) elution done by
- Gradient elution (more than one solvent).
- UV detector is good for gradient elution with non-absorbing solvent

### Fluorescence Detector:-

- Excite the eluate or analyte with ~~the same~~ laser and measure fluorescence. (Parallel excited and deexcited state)
- Detectors are very sensitive but respond only to few analytes that show fluorescence.
- The substance which don't fluoresce, they are derivitized like epinephrine.

noradrenaline and dopamine.  
→ To increase the utility of fluorescence or electrochemical detectors.  
Fluorescent or electroactive groups can be covalently attached to analyte.

that substance is added in it which attach and cause fluorescence.  
Tb(EDTA) is added to the hormone (epi...) from a chromat column.  
change the sample = Derivitization

⇒ Derivitized sample before introducing in column. Sometimes, Derivitization is done after eluate out of sample, then chemical is added and this type of derivitization is called Post-column derivitization.

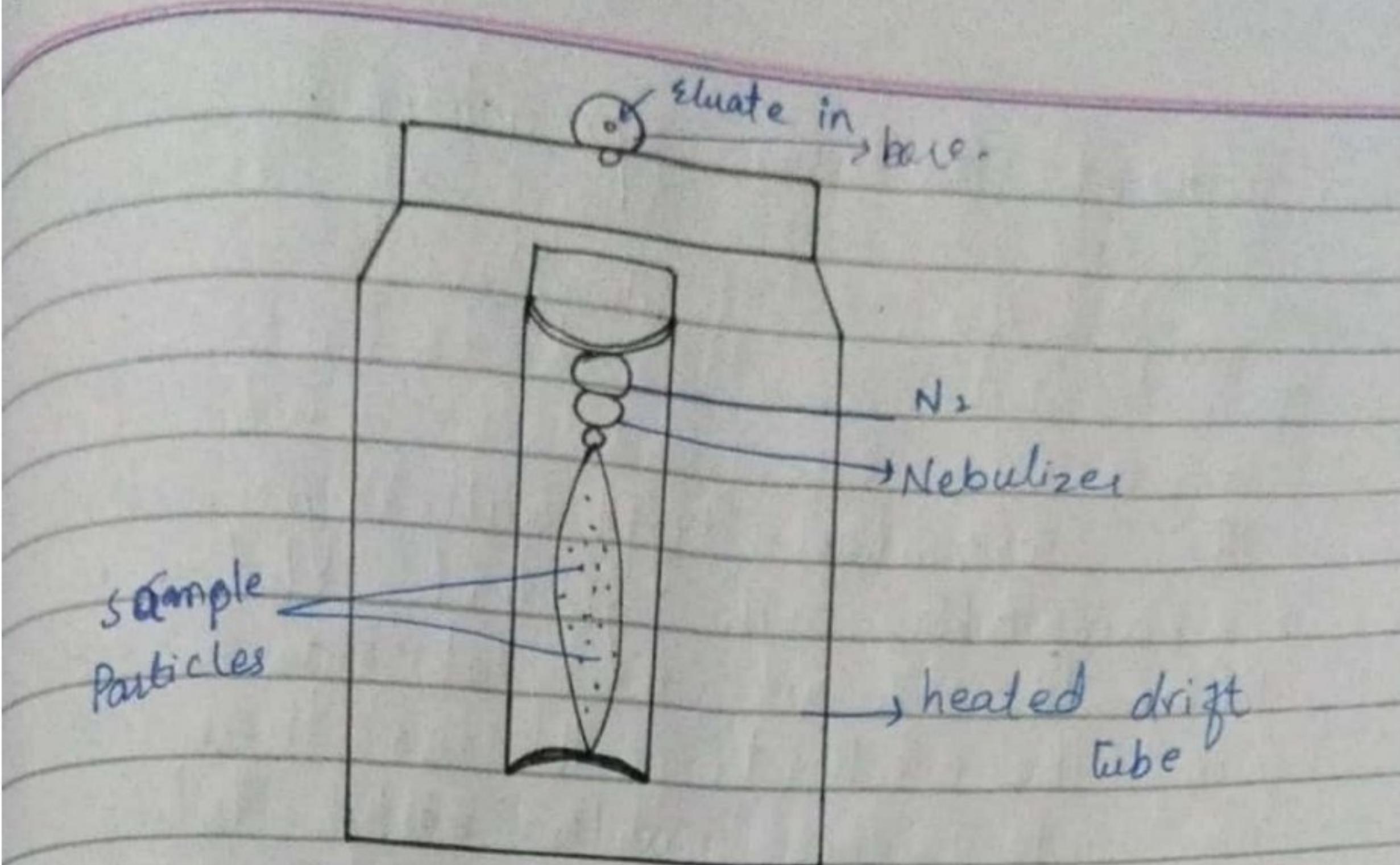
Response of UV detectors show minor response for lipids and carbohydrates.

⇒ Compounds can be excited near 300nm and emit strongly at 500-600nm.

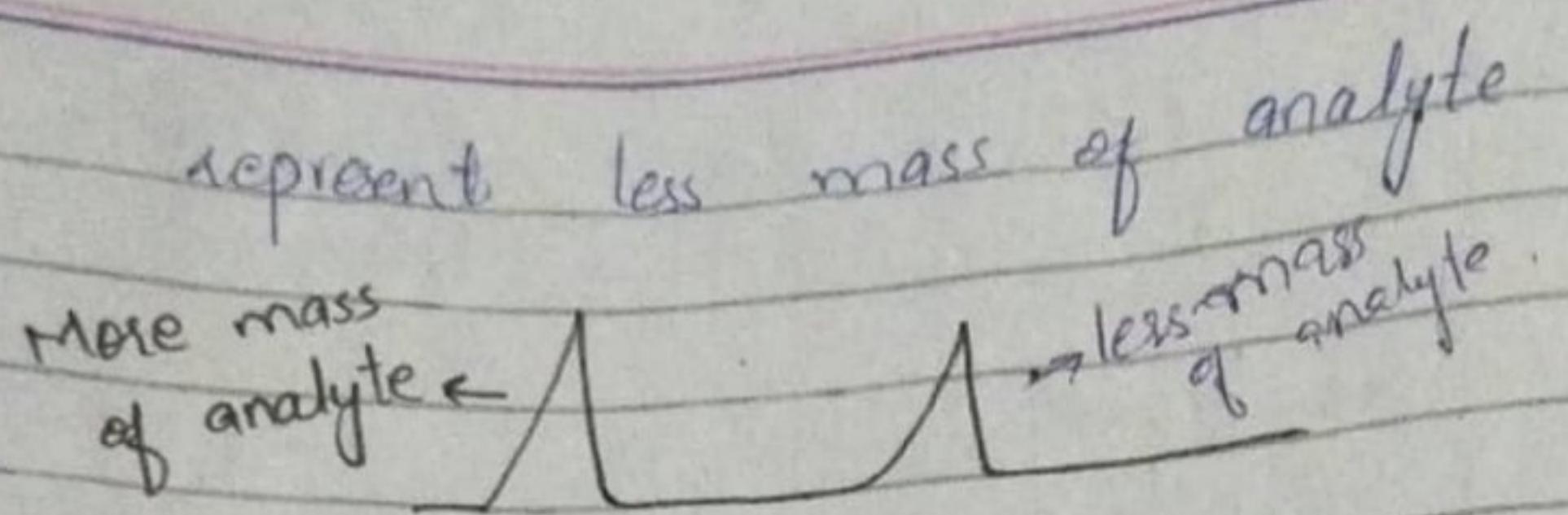
⇒ The detection limit is 10 to 100nM with Tb fluorescence.

## (a) Evaporative Light-Scattering Detector:-

PUACP  
This detector is used for samples, all analytes which have less volatile than mobile phase



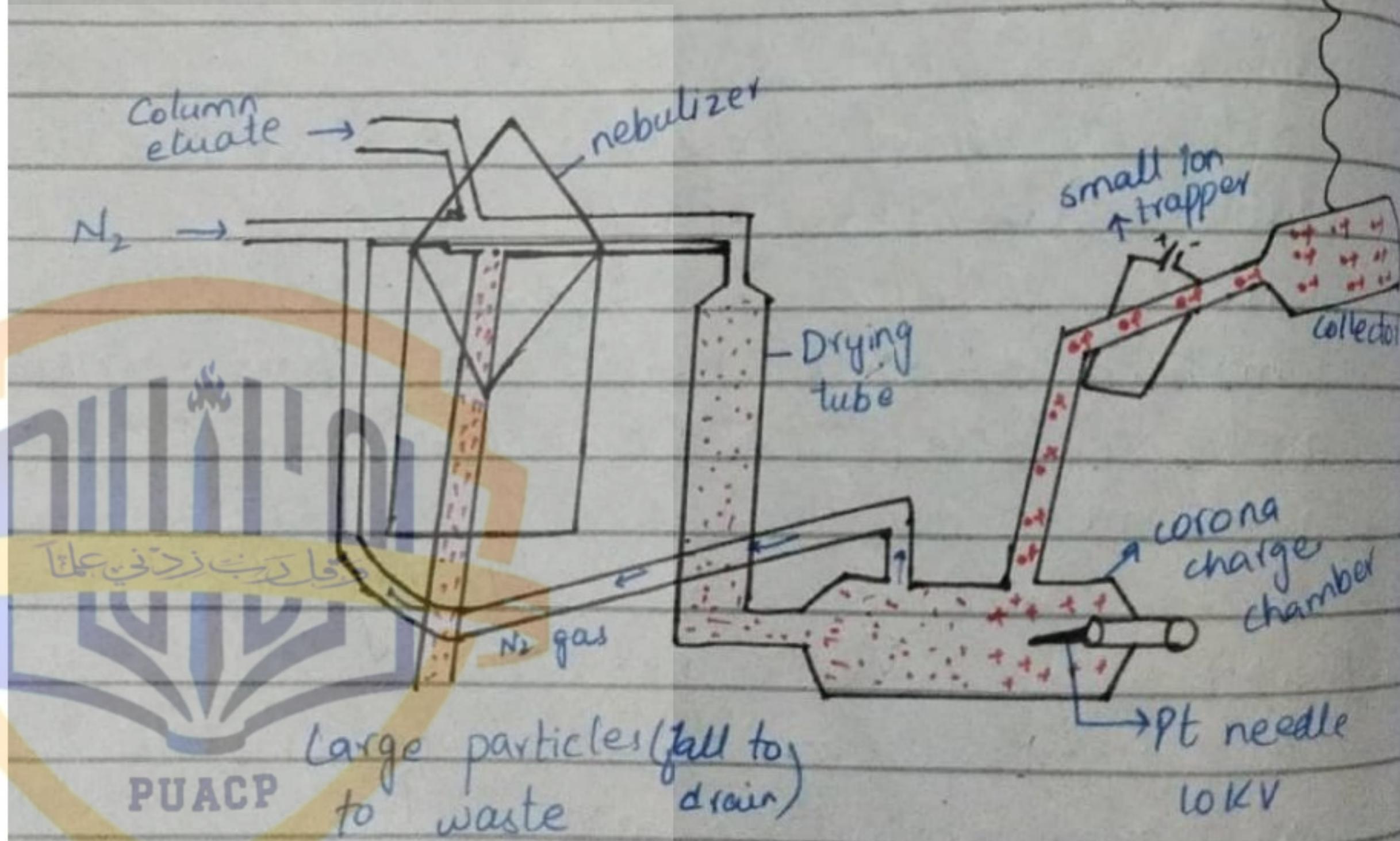
- Eluate from column in into detector from top and  $N_2$  gas mixed which is inert and don't react.
- ⇒ Now this mixture is based through a very tiny hole (base) and nebulized (from a mixture of fine particles).
- ⇒ Solvent from mixture evaporate due to heated drift tube.
- ⇒ Fine particles of samples are remained. Light fall on these particles and scattered light don't fall at base some point.
- ⇒ By scattering we identify how much particles present.
- ⇒ It P.D.F depends upon mass of analyte not on molecular mass.
- ⇒ Given peaks, larger peak represent more mass of analyte while smaller



The evaporative light-scattering detector is compatible with gradient elution. Also there are no peaks associated with the solvent front, so there is no interference with early eluting peaks.

$$\log A = \alpha + \beta \log M + \text{const} \frac{\text{mass of analyte}}{\text{area of signal}}$$

### 3) Charged Aerosol Detector (CAD)



"Sensitive and almost universal detector with nearly equal response to equal masses of non-volatile analyte."

- From the top of nebulizer, column eluate is introduced,  $N_2$  gas is also introduced in nebulizer.
- Gas mixed with column eluate and forms aerosol (fine particles) that fine particles move into a tube which is known as Drying Tube.
- Solvent evaporates in drying tube, and fine particles  $\Rightarrow$  Mist produced (Aerosol) leaving behind aerosol particles which is 1% of Large particles waste from analyte.
- the solvent passes through a tube.
- Drying tube extends toward a chamber which is known as corona charge chamber.
- In this chamber a needle is attached which is made up of Platinum.
- Pt needle is maintained at 10KV as compared to outer case of corona chamber.
- $N_2$  gas reaches to this chamber and passes over a Pt needle and converted into  $N_2^+$ , due to high voltage of platinum.
- $$PUACN_2 \rightarrow N_2^+ + e^-$$
- And particles gain positive charge. these are called charged aerosol particles.

- Charge particles passes through small ion traps and the trap plates of the <sup>mobile</sup> ions.
- ⇒ All  $N_2$  molecules don't ionize & remaining  $N_2$  molecules go back to that tube from which  $N_2$  was passing.
- ⇒ Aerosol charged particles are massive and can't traped through trap and a **collector** is attach to trap which collects the particle.
- ⇒ An **Electrometer** which detect charged particles and <sup>represent in the form of</sup> spectrum  $\phi$  is attached to collector.
- Equal masses give equal responses.

**Peak generated & Mass of analyte**  
 mostly used in pharmaceutical industries.

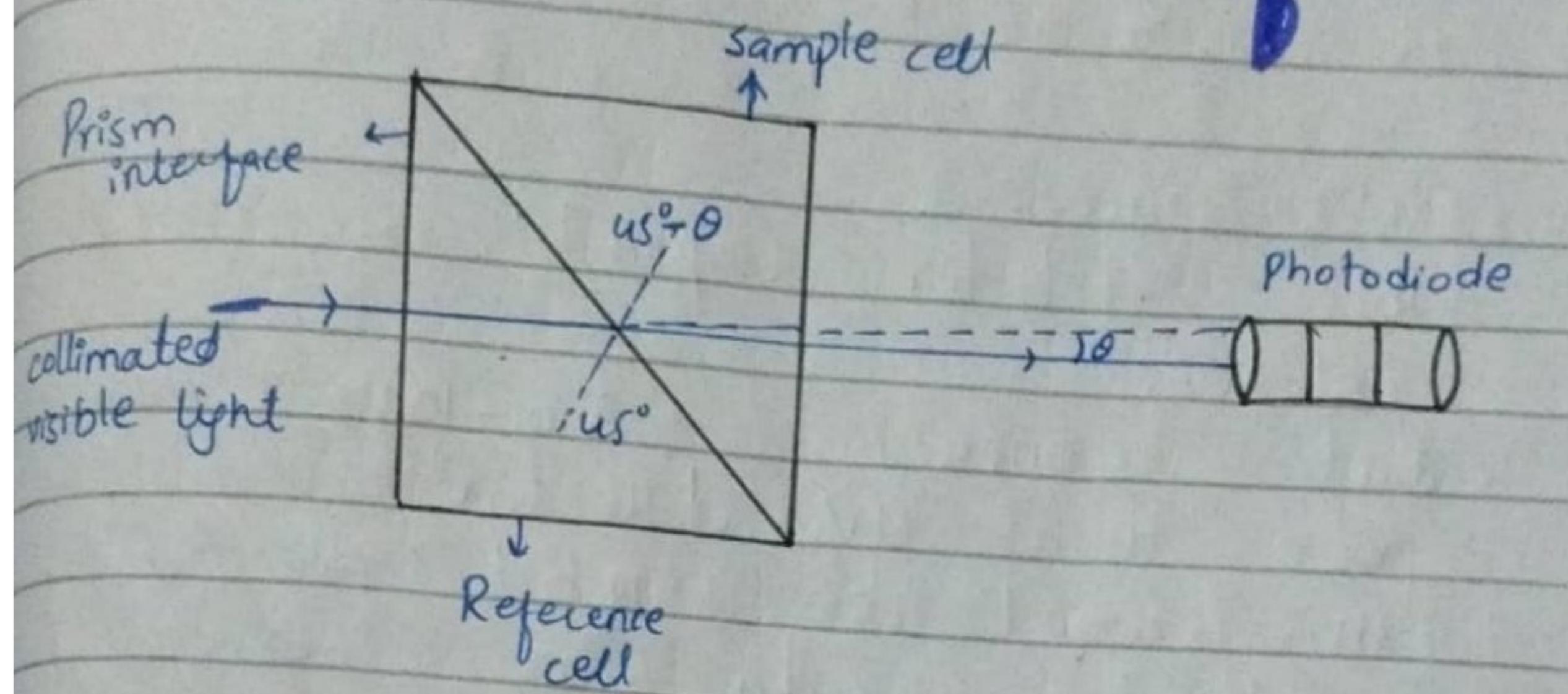
→ Comparison with UV detector:-  
 It show same response for Lipids and carbohydrates as for other compounds. For detection of Lipid and carbohydrate UV detector give minor response while charge aerosol detector show response atoms.

### ⇒ Characteristics:-

Response depends on solvent composition with higher response for higher % age of volatile organic solvent and lower response for water. As organic composition of a

gradient increases, so does the response. However the detector can still be used with gradient elution.

## 4) Refractive Index Detector



triangular compartments

Two prism are combined having prism interface. Two cells are formed.

- Reference Cell
- Prism Sample Cell

Both compartment containing capacity of 5-10 uL. Collimated visible (parallel) light passed through these compartments.

Both cell contain a solvent. photodiode array is attached to cell.

After passing both cells direct array. The light do not reflect and angle is 45°.

- When solvent is replaced with sample it shows deflection and angle is  $\theta$ .
- Now light fall on some other point at photodiode array. The angle of deflection is equal to  $45^\circ - \theta$ .

### Advantage:-

This detector respond to all

solutes.

### Shortcomings:-

- But response is 100-1000 times poor than UV detector.
- This can't be used for the gradient elusion, as we can't match sample and reference cell as solvent is changing.
- It is very sensitive for temp and pressure change. Even show change on  $0.01^\circ\text{C}$  Temp.
- Due to its low sensitivity, it can't be used for trace analysis.
- The collimated light filtered before passing to remove IR radiation as they heat up the sample.

# Applications of HPLC

Since arriving on the scene in **1967**, High performance Liquid chromatography (HPLC) has become a staple technique for separating, identifying and quantifying different components in a mixture.

## 1) Pharmaceutical Development:-

- One of the most common uses of HPLC is in the manufacturing process of pharmaceutical products.
- HPLC is a reliable and precise way to check product purity.
- As a result, it can help pharmaceutical manufacturers develop the purest products.
- However, due to its expensive nature on a large scales, it is not typically the primary method when drugs go on to be produced in bulk.

## 2) Medical Diagnose:-

- Because it can be used to separate components from mixtures, HPLC also lends itself to the analysis of nutrients in blood and other medical samples. Again while it's expensive compared to

alternatives. HPLC can deliver much more precise results when measuring for things like vitamin D deficiency. In this case, rather than measuring vitamin D directly, HPLC can be used to measure to concentration of 5-hydroxyvitamin D [25(OH)D] - a metabolite associated with the product of vitamin D.

### (3) Forensic

Similarly to the above, HPLC is also used for the detection of drug traces in urine. Usually used in tandem with mass spectrometry, the technique can be applied to detect everything from doping agents, metabolites and conjugates to opioids, cocaine, LSD, cannabis and ketamine.

When drug traces are detected, the HPLC findings can be used as evidence in police charges as well as sporting disqualifications. Because this can be used to check for metabolites produced by a reaction to the substance - the method is useful for modern drugs which are designed specifically to disappear within the body.

#### (4) Medical Research:-

As well as identifying nutrient levels for a direct diagnosis, HPLC is often used to analyse biological samples from people with existing diagnoses.

By identifying specific metabolites in patients with **Parkinson's** or heart disease, for example, researchers can use them as biomarkers to assist with early diagnosis for future patients.

#### (5) Food Production:-

Over the past few decades, use of chemicals in agriculture and food production has become far more common. In many cases, this can be problematic, with chemical residues remaining on products and posing health risks to consumers.

HPLC can also be used to identify and quantify pesticides along with preservatives and artificial flavourings and colourants.

#### (6) Improving HPLC Technology:-

As with any method, HPLC comes with its own limitations.

One of these is portability, with

samples needing to be transferred to a laboratory before analysis can take place. The article

## Experience and Applications of a New Portable HPLC Machine

discusses how a portable HPLC instrument can address those challenges and assist with analyses outside the laboratory.

### → PLATE THEORY:-

The variance for diffusive band spreading is

$$\sigma = \sqrt{2Dt} \quad \text{ii}$$

If the solute has travelled a distance  $x$  at a linear flow rate  $u_x$ , then the time it has been on the column is  $t$ .

Distance covered by solute =  $x$

Flow rate of solute to =  $u_x$

The  $\gg$  covers this distance

Time taken by solute =  $t = \frac{x}{u_x}$

Taking HACP equation (i)

$$\sigma = \sqrt{2Dt}$$

Taking square on both side.

$$\sigma^2 = 2Dt$$

$$\sigma^2 = \frac{2D}{u_x} x$$

Since  $t = \frac{x}{u_x}$

$$\sigma^2 = \left( \frac{2D}{u_x} \right) x$$

$$\sigma^2 = H x$$

$$\therefore H = \frac{2D}{u_x}$$

$$H = \frac{\sigma^2}{x}$$

$$\sigma \propto x$$

" $H$  is the proportionality constant between the square of variance and distance traveled by solute is called plate height."

→ The name came from the theory of distillation in which separation could be performed in discrete stages called plates.

→ Plate height is also called the height equivalent to a theoretical plate.

→ It is approximately the length of column required for one equilibration of solute between mobile and stationary phases.

→ The smaller the plate height, the narrower the bandwidth and better the resolution.

→ Plate height is different for different solutes as every solute have different affinity and have different diffusion co-efficients.

For • Gas Chromatography

0.1 - 1 mm

• HPLC

~ 104 m

• Capillary electrophoresis

< 14 m

For solute emerging from a column of length L, the number of plates N in the entire column can be find out by:

$$N = \frac{L}{H}$$

where  $H = \frac{\sigma^2}{x}$

So,

$$N = \frac{L}{\sigma_x^2}$$

$$N = \frac{Lx}{\sigma^2}$$

$$N = \frac{L^2}{\sigma^2}$$

As x and L both are length.

where  $\sigma = \frac{W}{4}$

W = width of band in begining.

$$N = \frac{L^2}{(W/4)^2}$$

$$N = \frac{L^2}{W^2/16}$$

$$N = \frac{16L^2}{W^2}$$

→ Both length and width are the same unit "meter."

→ Number of plates will be dimension less.

If retention time is replaced by length

$$N = \frac{16t_r^2}{W^2}$$

$$W = 40^\circ$$

$$N = \frac{16t_r^2}{160^2}$$

$$W^2 = 160^2$$

$$N = \frac{t_r^2}{\sigma^2}$$

If we use the width at half-height also called the half width instead of the width at the base of column we get.

$$N = \frac{5.55t_r^2}{W_{1/2}^2}$$

## Plate Height Equations:-

Plate height  $H$ , is proportional to The variance of a chromatographic band, the smaller the plate height, the narrower the band.

The van Deemter equation tells us how the column and flow rate affect plate height:

$$H = A + \frac{B}{U_x} + C U_x^2$$

where

$H \Rightarrow$  Plate Height

$A \Rightarrow$  multiple path

$B/u_x \Rightarrow$  Longitudinal Diffusion.

$Cu_x \Rightarrow$  Equilibration time.

$u_x \Rightarrow$  Linear flow rate

$A, B, C \Rightarrow$  Constants.

The value of constant depends upon

- Stationary phase
- Column

- Packed Column:-

The constants  $A, B, C$  are participates and their value does not equal to zero, means these all involve in separation. Multiple paths available.

$A, B, C \neq 0$

- Open Column:-

Solute don't have multiple path, there is only one way for solute to flow.

(And) Therefore multiple path factor reduce narrow band.

$A = 0$

PUACP  
Capillary Electrophoresis:-

Both factor  $A$  and  $C$  is equal to zero

$A, C = 0$

→ Plate height decrease and separation will be better.

## Longitudinal Diffusion:- $(\frac{B}{U_x})$

We apply a thin, disk-shaped band of solute to the center of a column, the band would slowly broaden as molecules diffuse from the high concentration within the band to regions of lower concentration.

→ Diffusional broadening of a band called Longitudinal diffusion because it takes place along the axis of the column.

The term  $(\frac{B}{U_x})$  arises from longitudinal diffusion.

Time spent by solven  $\alpha$  Separation

Flow rate of solute  $\frac{1}{\text{separation}}$

Diffused  $\leftarrow$  [solute]

column

The variance resulting from diffusion

$$\sigma^2 = 2D_m t$$

where

$D_m \Rightarrow$  Diffusion co-efficient

$t \Rightarrow$  Time spent in the column

$\sigma^2 \Rightarrow$  Diffusion band broadening.

Since  $t = \frac{L}{U_x}$

$$\sigma^2 = \frac{2D_m L}{U_x}$$

Plate height  $H_D = \frac{\sigma^2}{L}$

Putting the value of  $\sigma^2$  in above eq.

$$H_D = \frac{2D_m K}{U_x K}$$

$$H_D = \frac{2D_m}{U_x}$$

$$\therefore 2D_m = B$$

$$H_D = \frac{B}{U_x}$$

## Finite Equilibration Time

b/w Phases:-

Van Deemter Equation.

$$H = A + \frac{B}{U_x} + C U_x$$

$C U_x \rightarrow$

Finite equilibration time of  
Solute between stationary  
phases and mobile phase.

It is also known as mass transfer plate height due to mass transfer

$$H_{\text{mass transfer}} = C_{11x} \\ = (C_s + C_m) \cdot 11x$$

where

$C_s \Rightarrow$  mass transfer in stationary phase  
 $C_m \Rightarrow$  mass transfer in mobile phase.

→ Mass transfer in Stationary phase:-

$$C_s = \frac{2K}{3(K+1)^2} \cdot \frac{d^2}{D_s}$$

where

$K \Rightarrow$  retention factor

$d \Rightarrow$  thickness of stationary phase.

$D_s \Rightarrow$  Diffusion co-efficient of solute in stationary phase.

If thickness "d" is smaller the plate height will also be less and separation will be better.

→ Mass transfer in Mobile phase:-

$$C_m = \frac{1 + 6k + 11k^2}{24(K+1)^2} \cdot \frac{r^2}{D_m}$$

where PUACP

$r \Rightarrow$  Column radius.

$D_m \Rightarrow$  Diffusion co-efficient of solute in mobile phase.

As if column radius "r" decrease plate height will also decrease and separation will be better.

If temperature is increased mass transfer plate height is decreased which increases the diffusion coefficient of solute in the stationary phase and separation will be better.

## Multiple Flow Paths:-

The term A in the van Deemter equation arises from multiple effects / ~~for~~ multiple flow paths.

In a tubular packed column, the factor A involves in it. When solute is entered in column, suppose A, B and C are solutes at a time elution will not be at same time / different elution time.

