

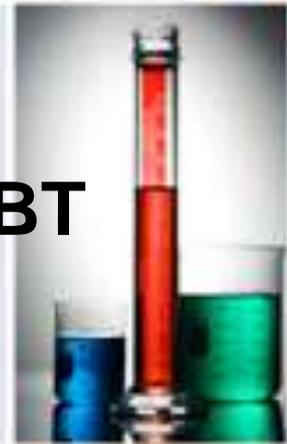
HPLC

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY



DIVYA B.

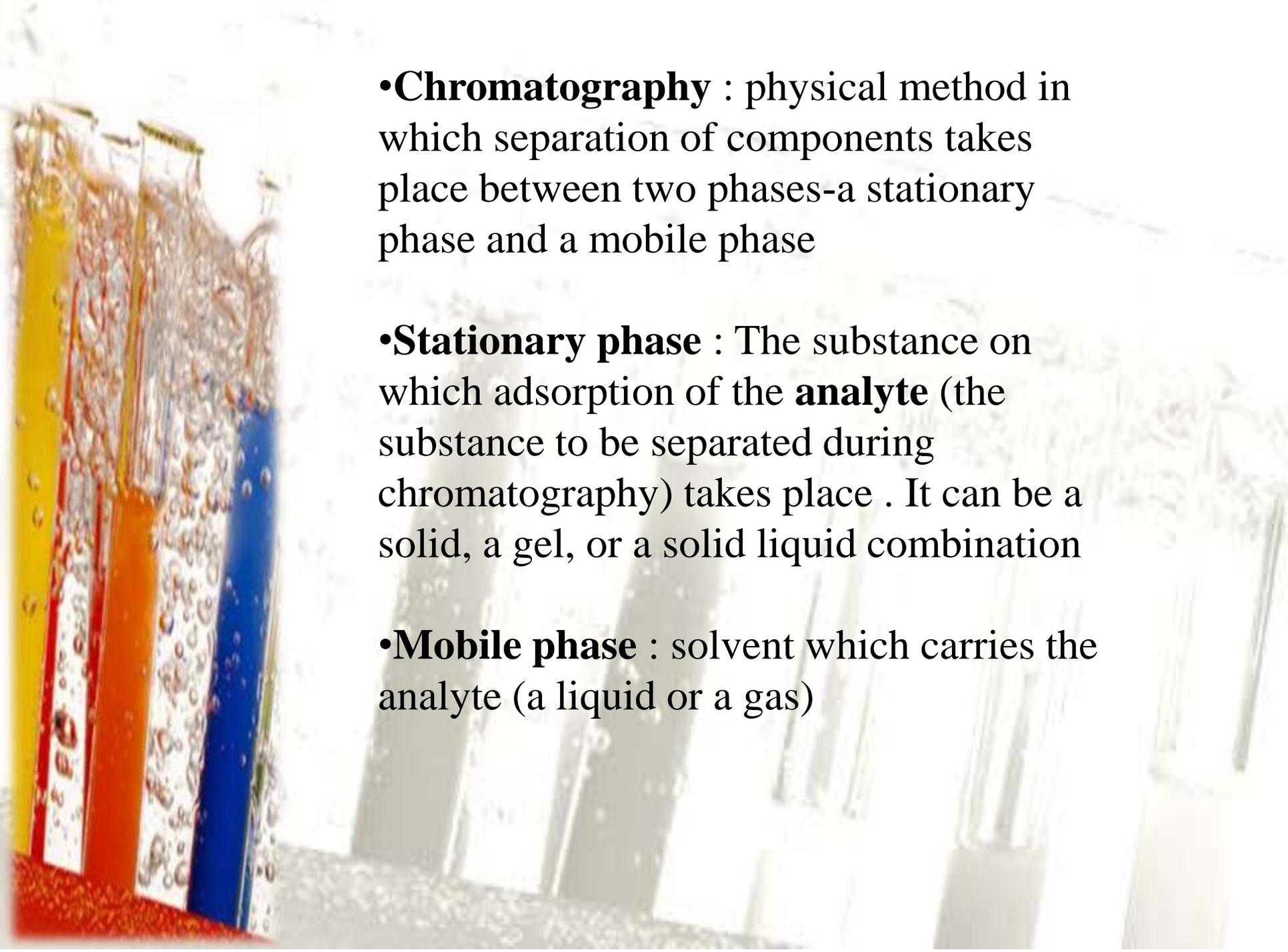
GRIET kukatpally
BACHELOR OF TECHNOLOGY –BT
Divya B.



INTRODUCTION

- HPLC stands for “High-performance liquid chromatography”(sometimes referred to as High-pressure liquid chromatography).
- High performance liquid chromatography is a powerful tool in analysis, it yields high performance and high speed compared to traditional columns chromatography because of the forcibly pumped mobile phase.
- HPLC is a chromatographic technique that can separate a mixture of compounds
- It is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of a mixture.





• **Chromatography** : physical method in which separation of components takes place between two phases-a stationary phase and a mobile phase

• **Stationary phase** : The substance on which adsorption of the **analyte** (the substance to be separated during chromatography) takes place . It can be a solid, a gel, or a solid liquid combination

• **Mobile phase** : solvent which carries the analyte (a liquid or a gas)

Chromatographic techniques are divided into different types based on :

The type of chromatographic bed used
i.e. column chromatography (gas chromatography) and planar chromatography (paper and thin layer)

The physical state of mobile phase
i.e. gas chromatography and liquid chromatography

The separation mechanism
i.e. ion-exchange and size exclusion

HPLC is a type of **liquid chromatography** where the sample is forced through a **column** that is packed with a stationary phase composed of irregularly or spherically shaped particles, a porous monolithic layer, or a porous membrane by a liquid (mobile phase) at high pressure.



COLUMN CHROMATOGRAPHY

Column chromatography involves the following:

1. Adsorption/retention of substance on stationary phase
2. Separation of adsorbed substance using mobile phase
3. Recovery of individual components by continuous flow of mobile phase
4. Quantitative and qualitative analysis of solute and the components which are recovered

- When distribution of solute is equal between two phases , then effective distribution $K_d = 1$
- Individual zones adjacent to each other in the column where there is sufficient space for equal distribution of solutes in between stationary and mobile phase are called theoretical plates
- Length of theoretical plate in the column is called plate height
- More the theoretical plates , more efficient the separation
- 2000 or more theoretical plates are ideal for a column
- Components which have more affinity towards mobile phase elute first

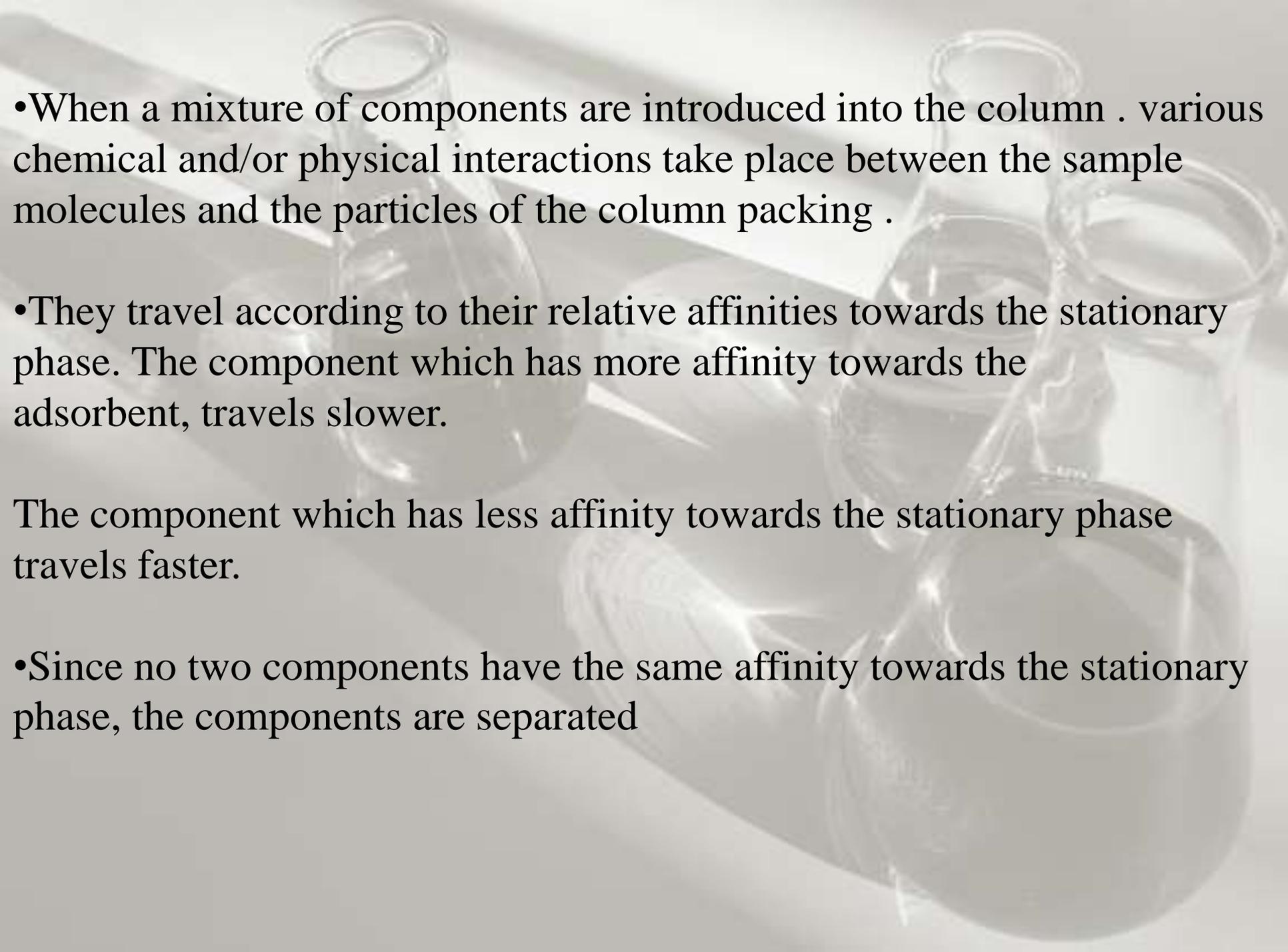
PRINCIPLE

To understand the principle of HPLC , we must first look at the principle behind liquid chromatography

Liquid chromatography is a separation technique that involves:

- the placement (injection) of a small volume of liquid sample
- into a tube packed with porous particles (stationary phase)
- where individual components of the sample are transported along the packed tube (column) by a liquid moved by gravity.

The main principle of separation is **adsorption** .

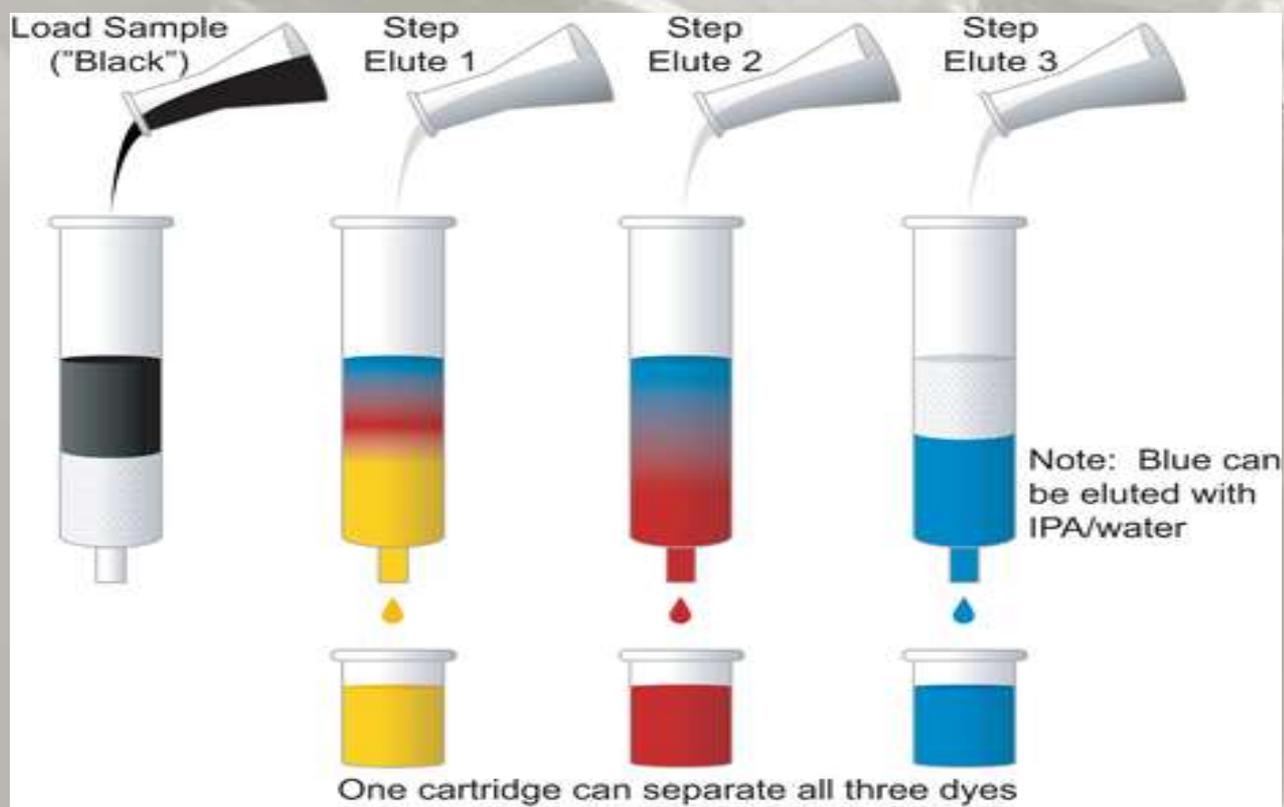
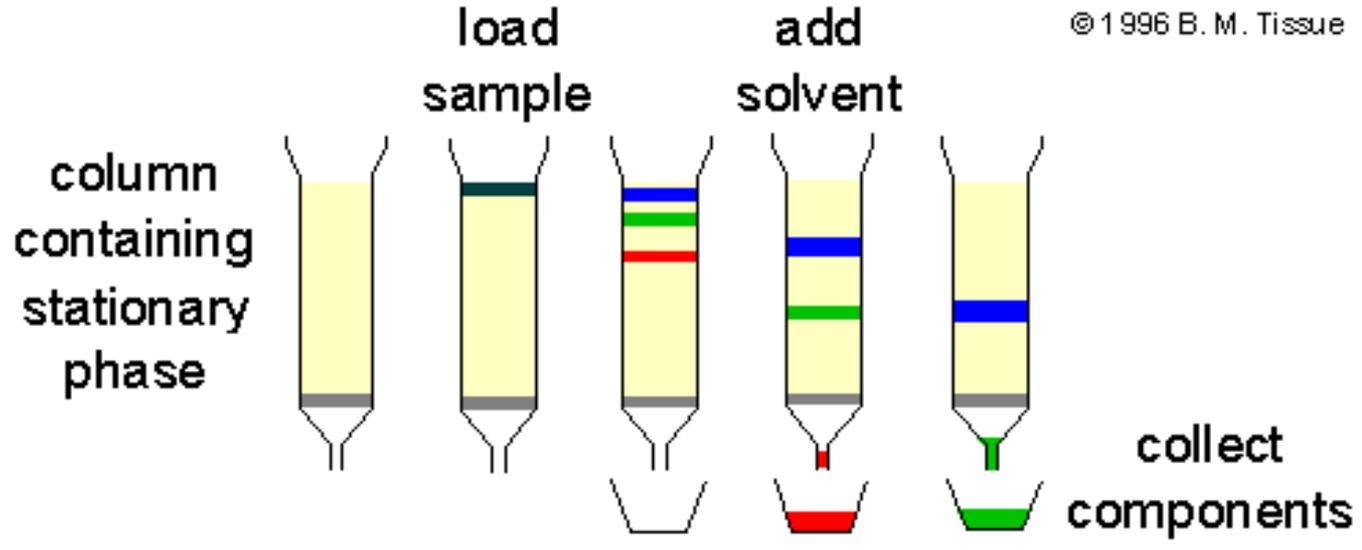


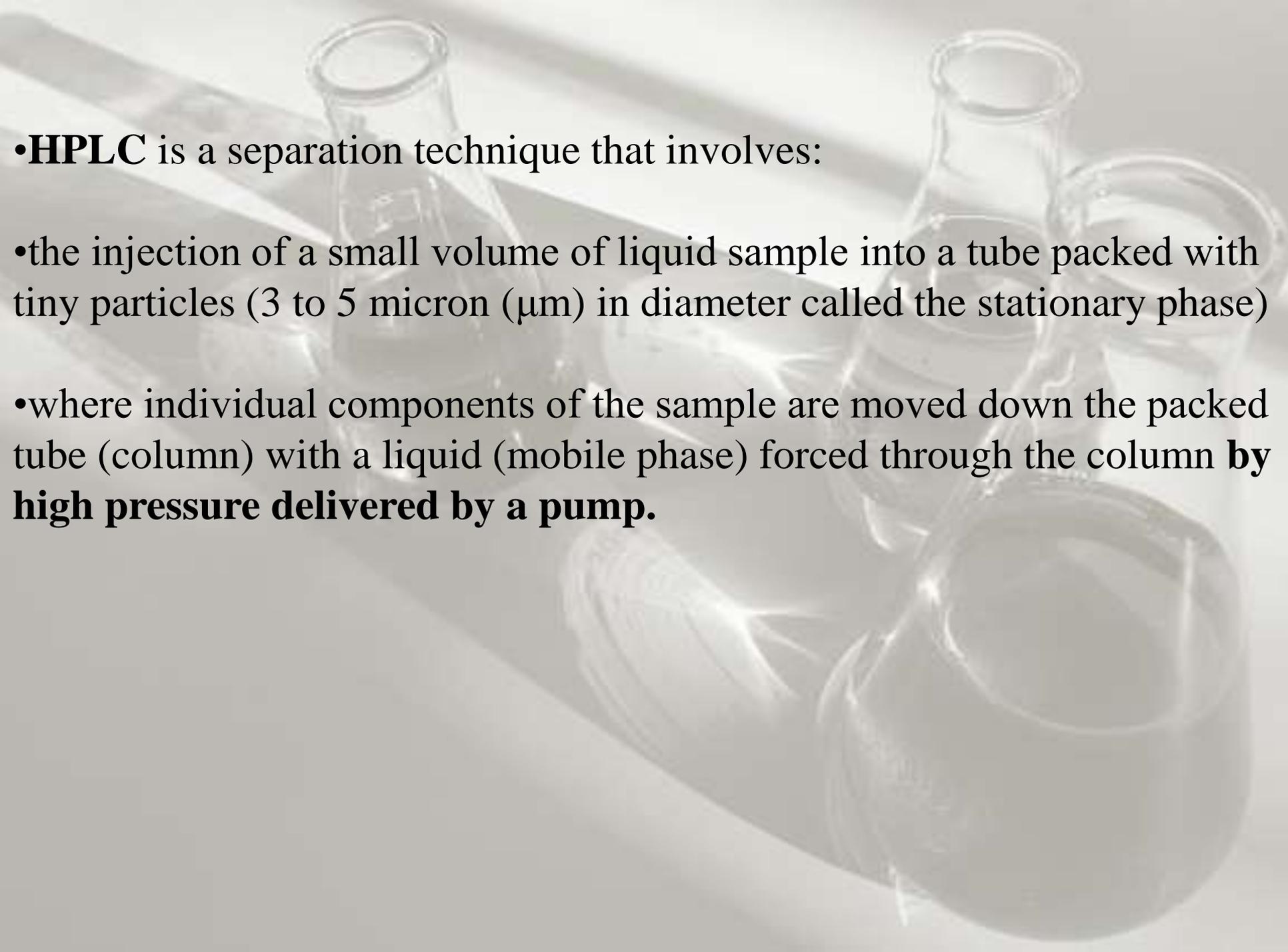
- When a mixture of components are introduced into the column . various chemical and/or physical interactions take place between the sample molecules and the particles of the column packing .

- They travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent, travels slower.

The component which has less affinity towards the stationary phase travels faster.

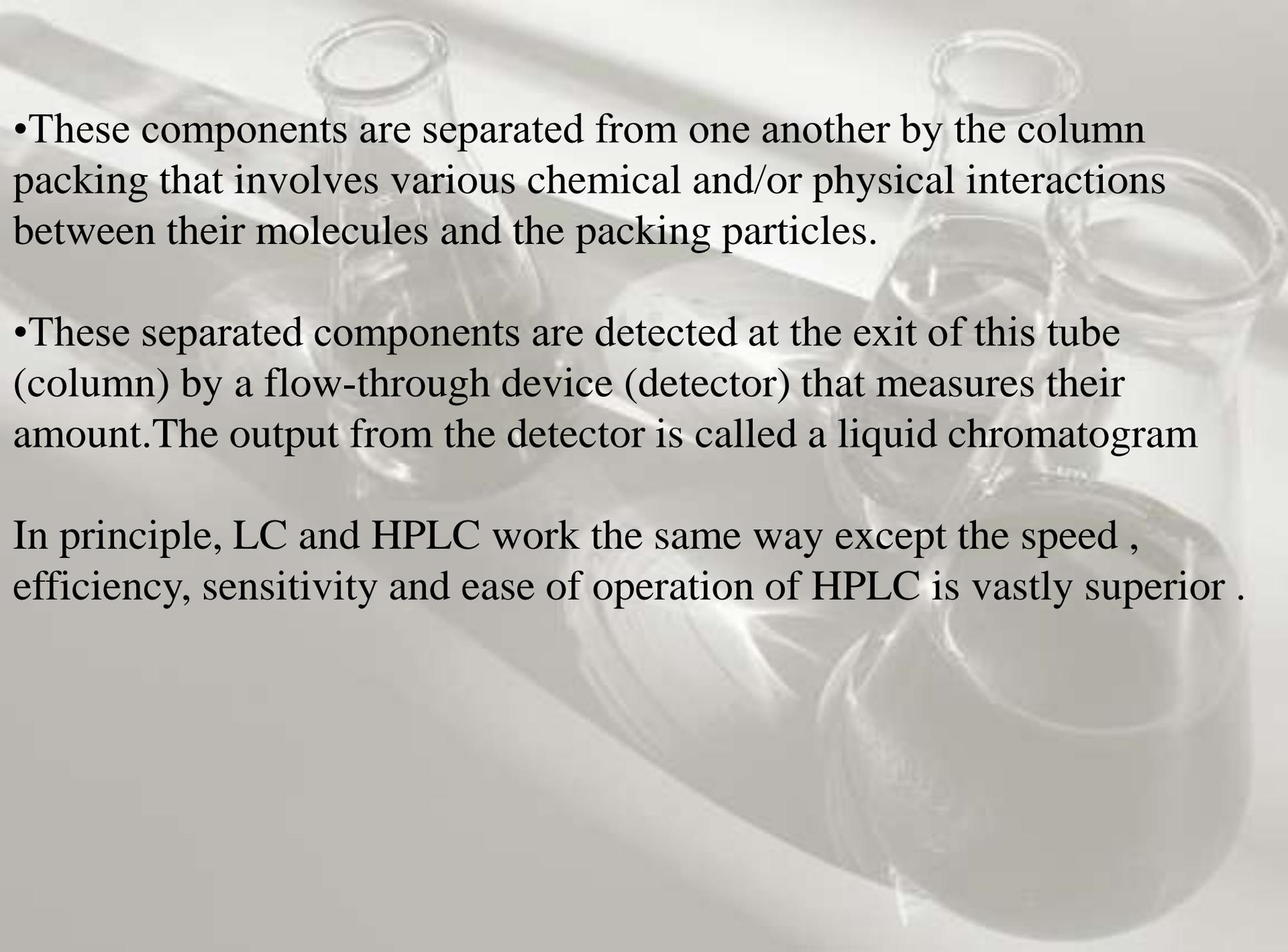
- Since no two components have the same affinity towards the stationary phase, the components are separated





• **HPLC** is a separation technique that involves:

- the injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (μm) in diameter called the stationary phase)
- where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column **by high pressure delivered by a pump.**



- These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.

- These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. The output from the detector is called a liquid chromatogram

In principle, LC and HPLC work the same way except the speed, efficiency, sensitivity and ease of operation of HPLC is vastly superior.

TYPES OF HPLC

I. BASED ON MODE OF SEPERATION

1. Normal phase chromatography - stationary phase is polar (hydrophilic) and mobile face is non-polar (hydrophobic).

2. Reverse phase chromatography- stationary face is non-polar (hydrophobic) and mobile face is Polar (hydrophilic).

- Polar-Polar bonds and Non Polar-Non Polar bonds have more affinity than Polar-Non Polar bonds.

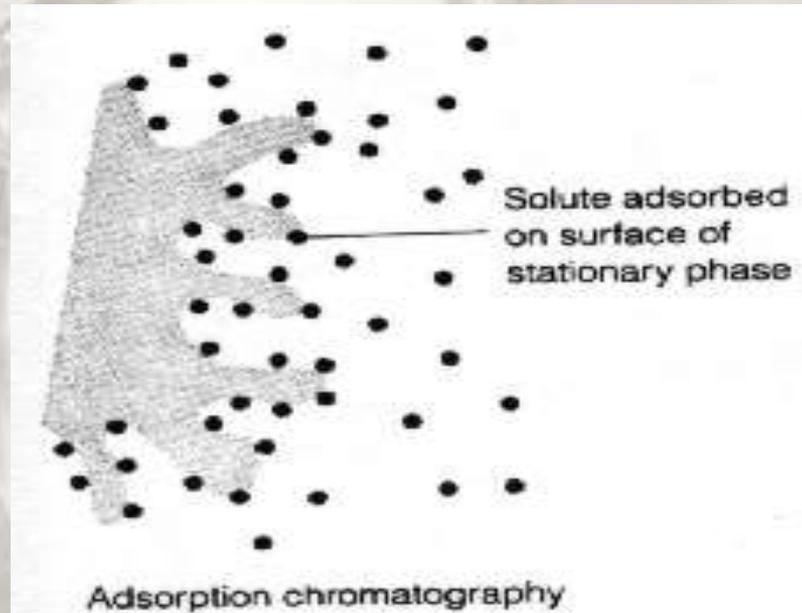
- Reverse phase chromatography is more commonly used as drugs are usually hydrophilic

II. BASED ON PRINCIPLE OF SEPERATION

1. Absorption Chromatography

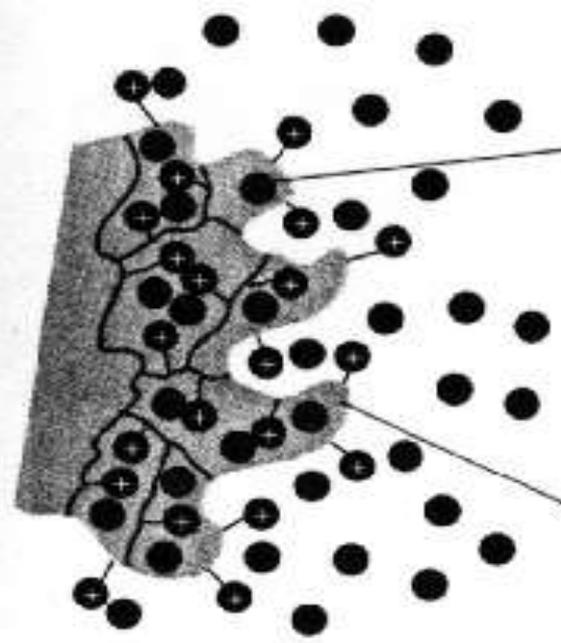
- In the Absorption Chromatography solute molecules bond directly to the surface of the stationary phase
- the component which has more affinity towards mobile phase elutes first & the component which has less affinity towards stationary phase elutes later.

No two components have same affinity towards mobile phase & stationary phase.



2. Ion-exchange chromatography

- Ion exchange chromatography is a process that allows the separation of ions and polar molecules based on their charge.
- It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids.
- Retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded.
- The use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.



Mobile anions held near cations that are covalently attached to stationary phase

Anion-exchange resin; only anions can be attracted to it

Ion-exchange chromatography

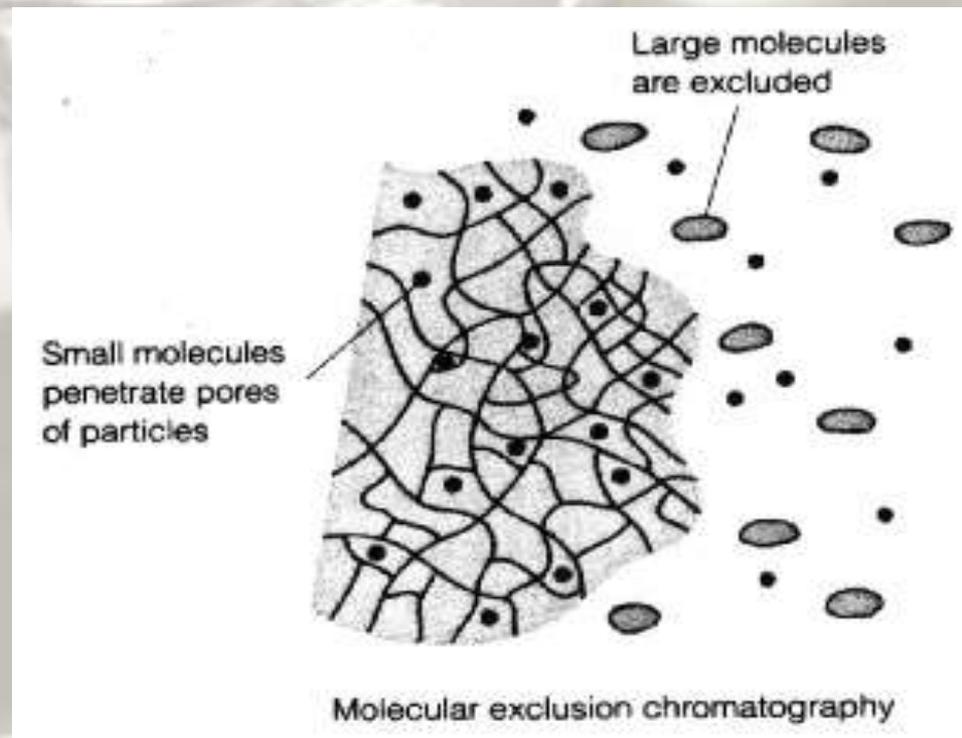
3. Ion-pair chromatography

- It is a form of chromatography in which ions in solution can be "paired" or neutralized and separated as an ion pair on a reversed-phase column.
- Ion-pairing agents are usually ionic compounds that contain a hydrocarbon chain that imparts a certain hydrophobicity so that the ion pair can be retained on a reversed-phase column.

4. gel permeation chromatography

- This type of chromatography lacks an attractive interaction between the stationary phase and solute.
- The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size.

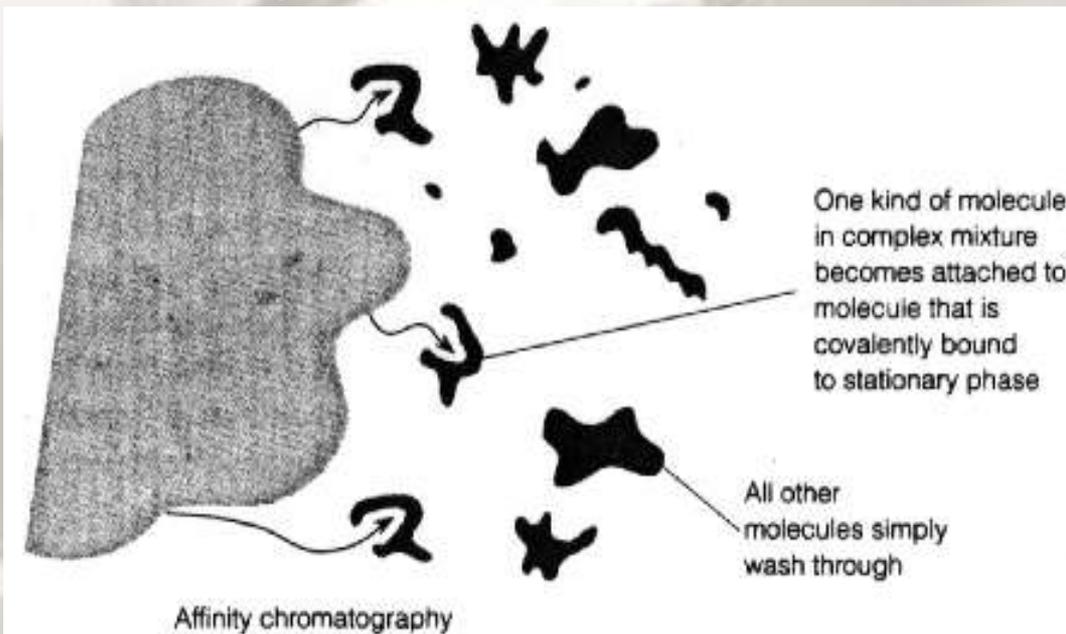
•The pores are normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.



5. Affinity Chromatography

- This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase.

- For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of proteins are passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or pH.



6. *Chiral chromatography*

• It involves the separation of stereoisomers. In the case of enantiomers, these have no chemical or physical differences apart from being three-dimensional mirror images. Conventional chromatography or other separation processes are incapable of separating them. To enable chiral separations to take place, either the mobile phase or the stationary phase must themselves be made chiral, giving differing affinities between the analytes.

III. BASED ON ELUTION TECHNIQUE

1. Isocratic elution

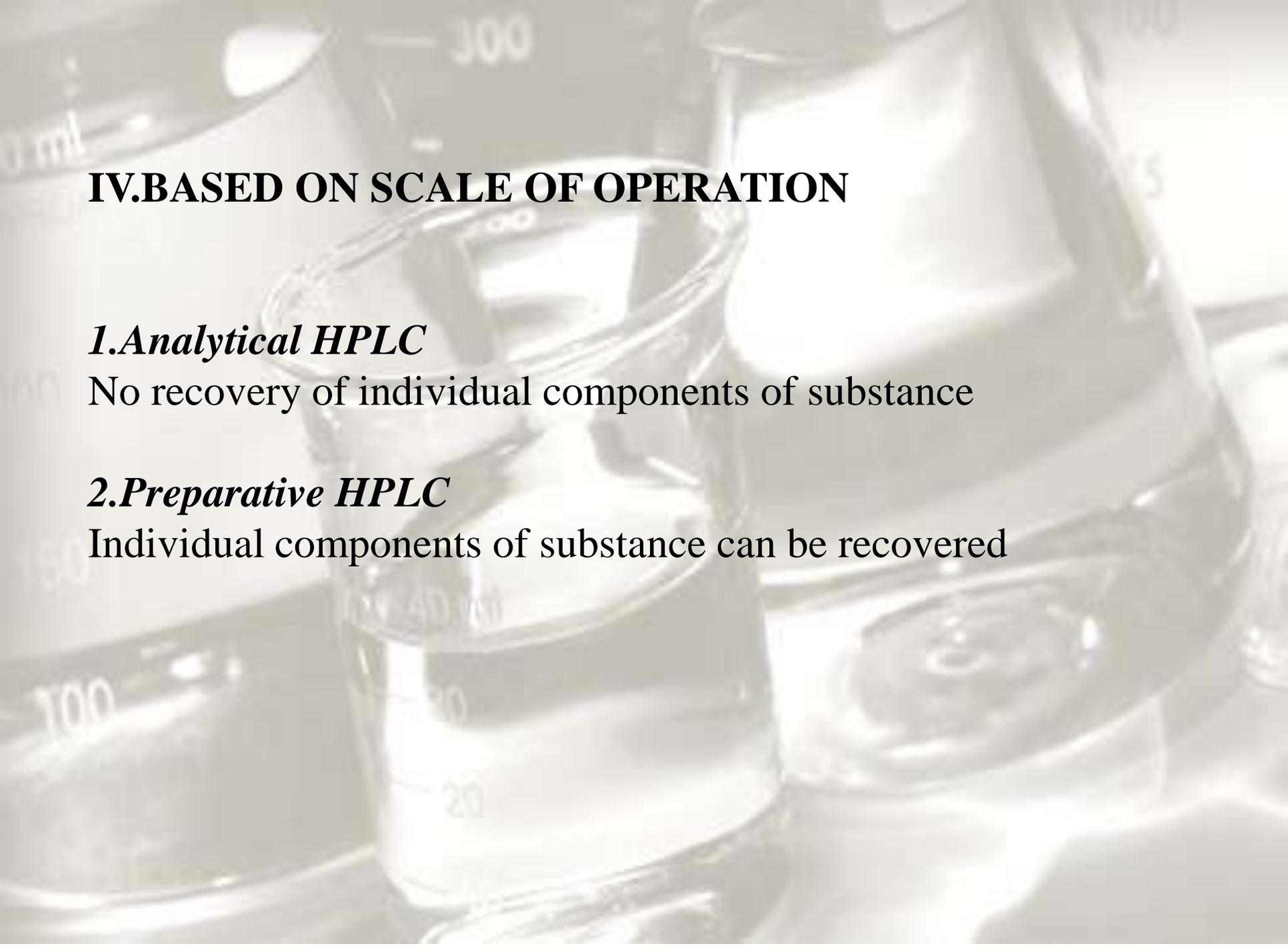
- A separation in which the mobile phase composition remains constant throughout the procedure is termed **isocratic elution**

- In isocratic elution, peak width increases with retention time linearly with the number of theoretical plates. This leads to the disadvantage that late-eluting peaks get very flat and broad.

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

2. *Gradient elution*

- A separation in which the mobile phase composition is changed during the separation process is described as a **gradient elution**
- Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower peaks . This also improves the peak shape and the peak height
 - Best for the analysis of complex samples
 - Often used in method development for unknown mixtures
 - Linear gradients are most popular



IV. BASED ON SCALE OF OPERATION

1. Analytical HPLC

No recovery of individual components of substance

2. Preparative HPLC

Individual components of substance can be recovered

V.BASED ON TYPE OF ANALYSIS

1. Qualitative analysis

Analysis of a substance in order to ascertain the nature of its chemical constituents

We can separate individual components but cannot assess the quantity in this analysis

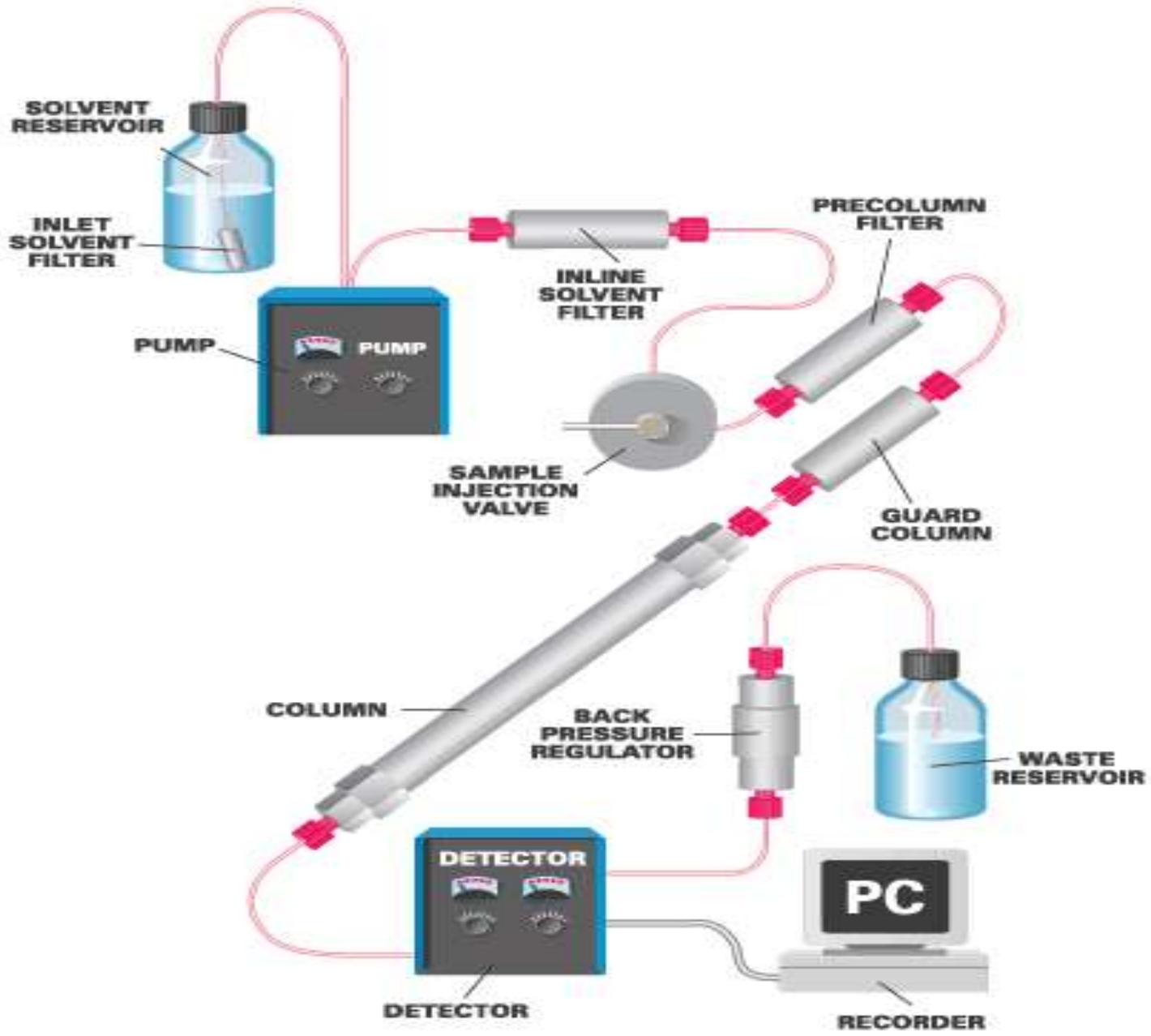
2. Quantitative analysis

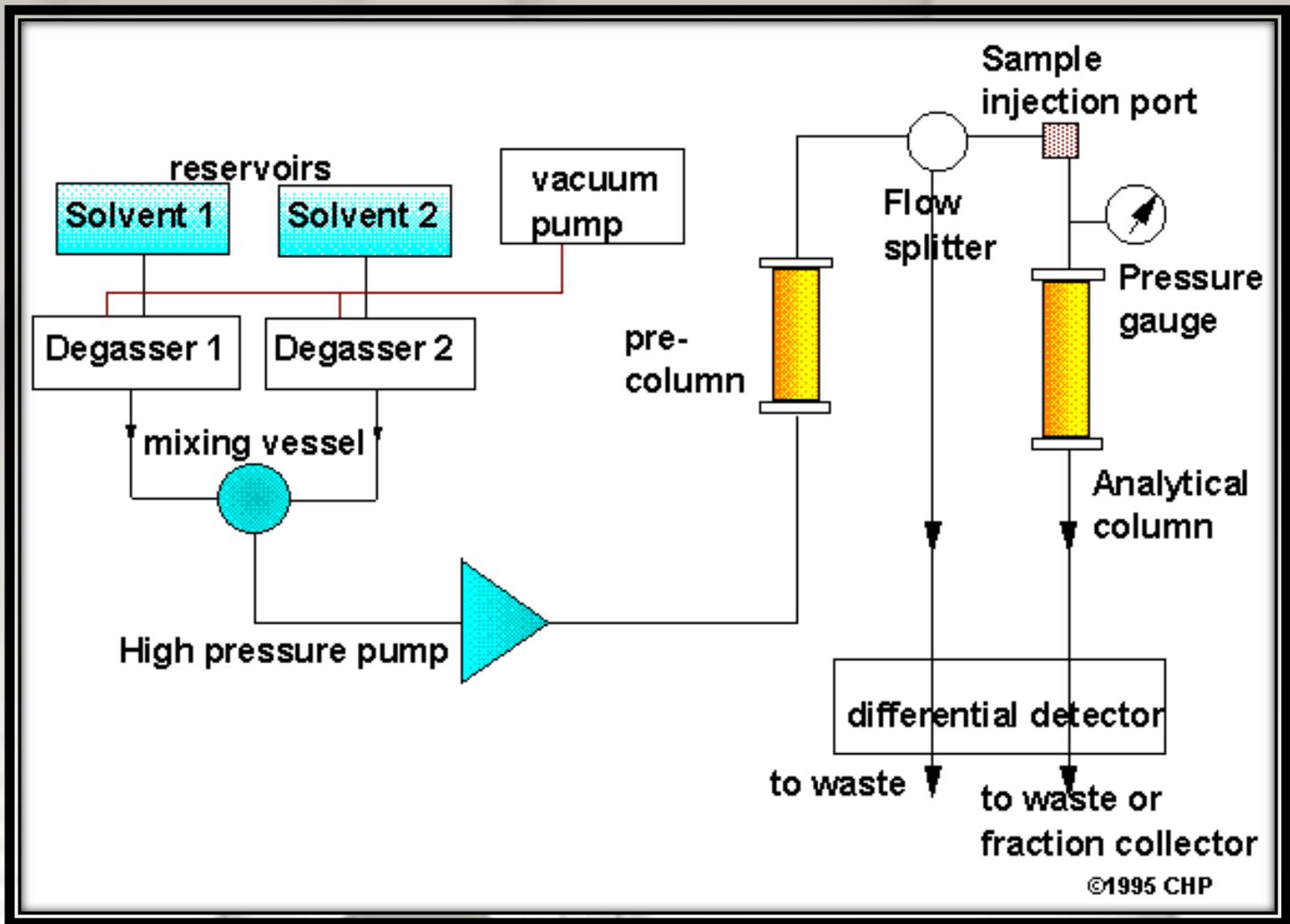
Determining the amounts and proportions of its chemical constituents .

Quantity of the impurity and individual components can be assessed

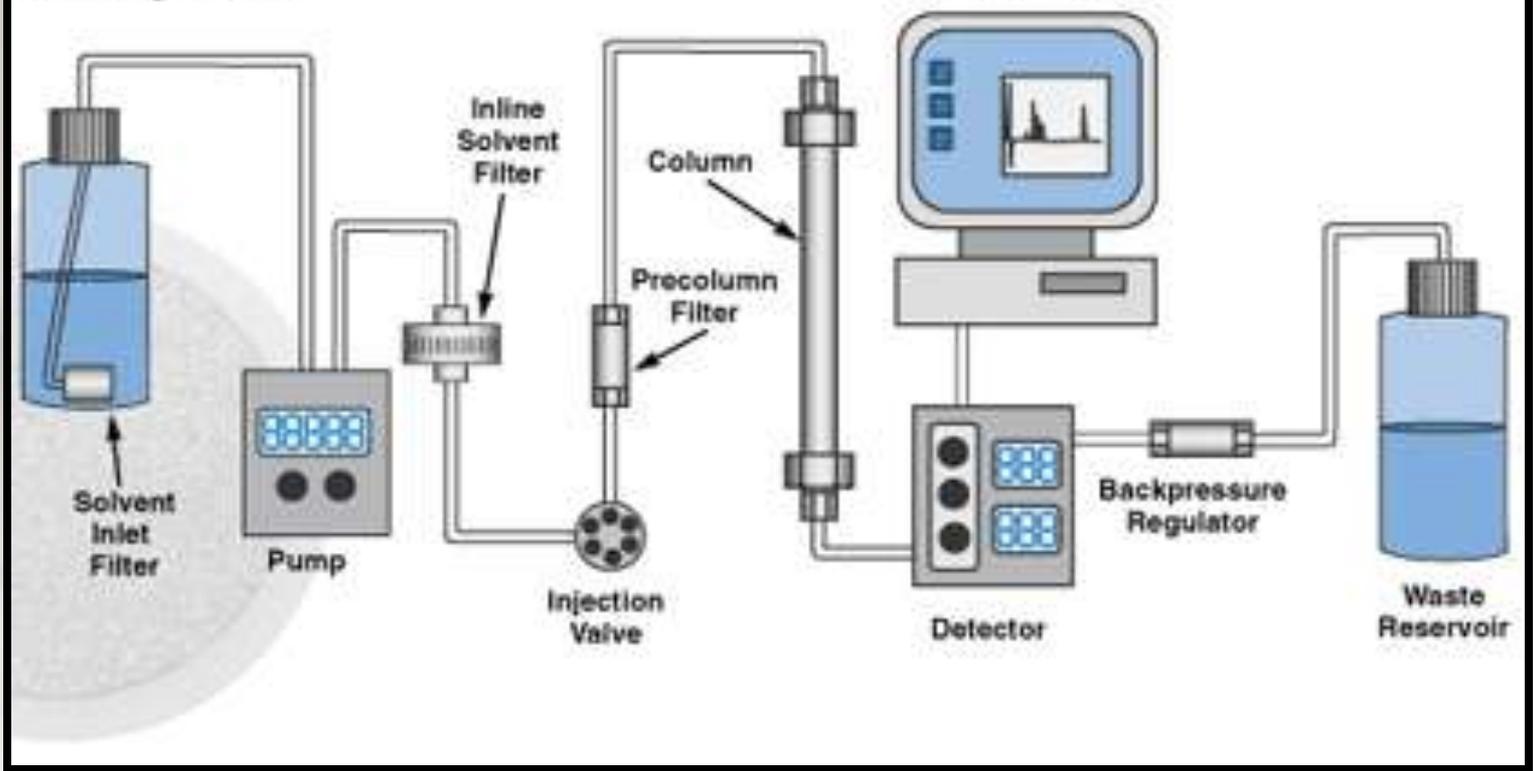
INSTRUMENTATION







HPLC System



1000ml

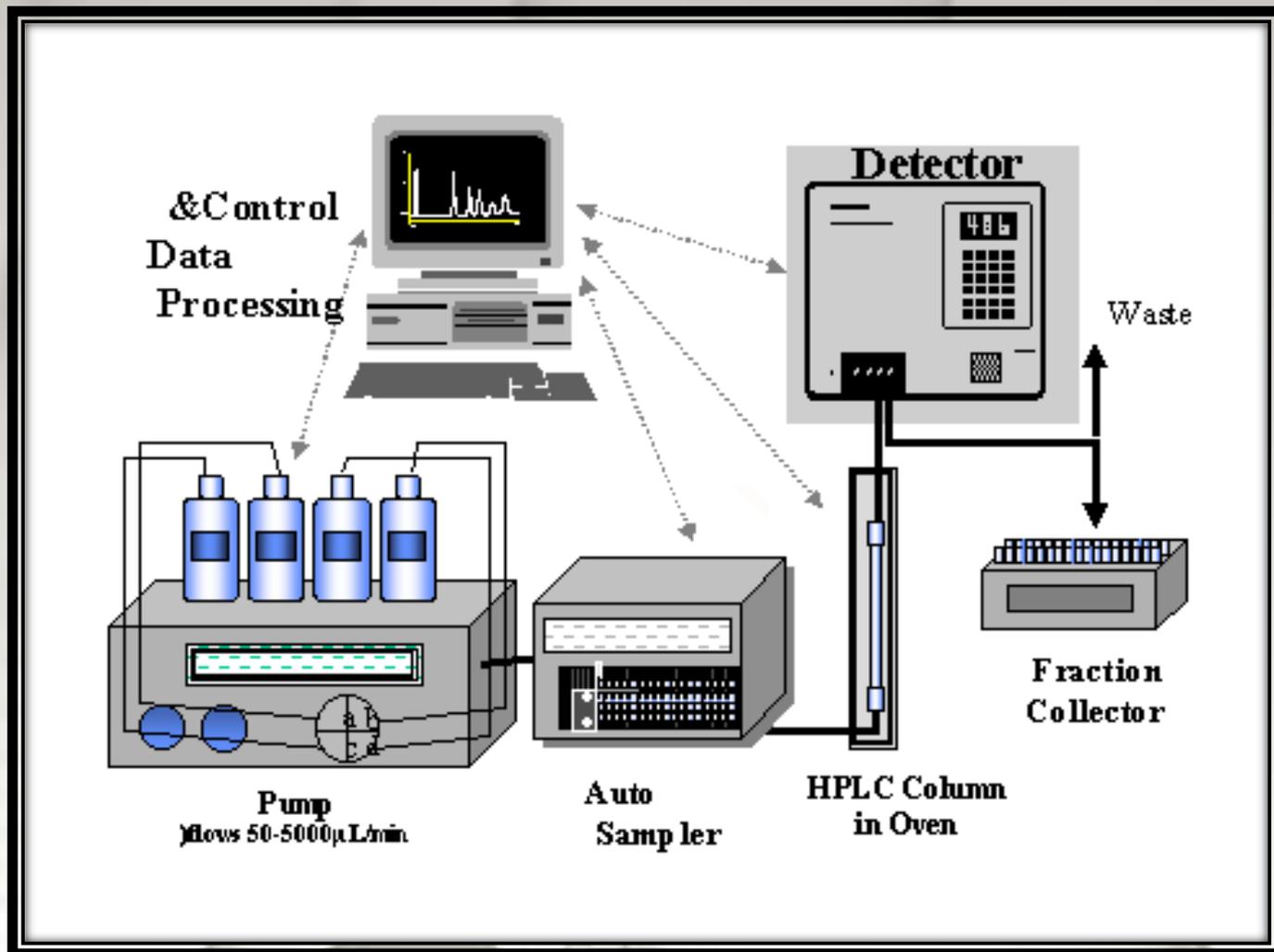
USA

APPROXIMATE VOLUME

800

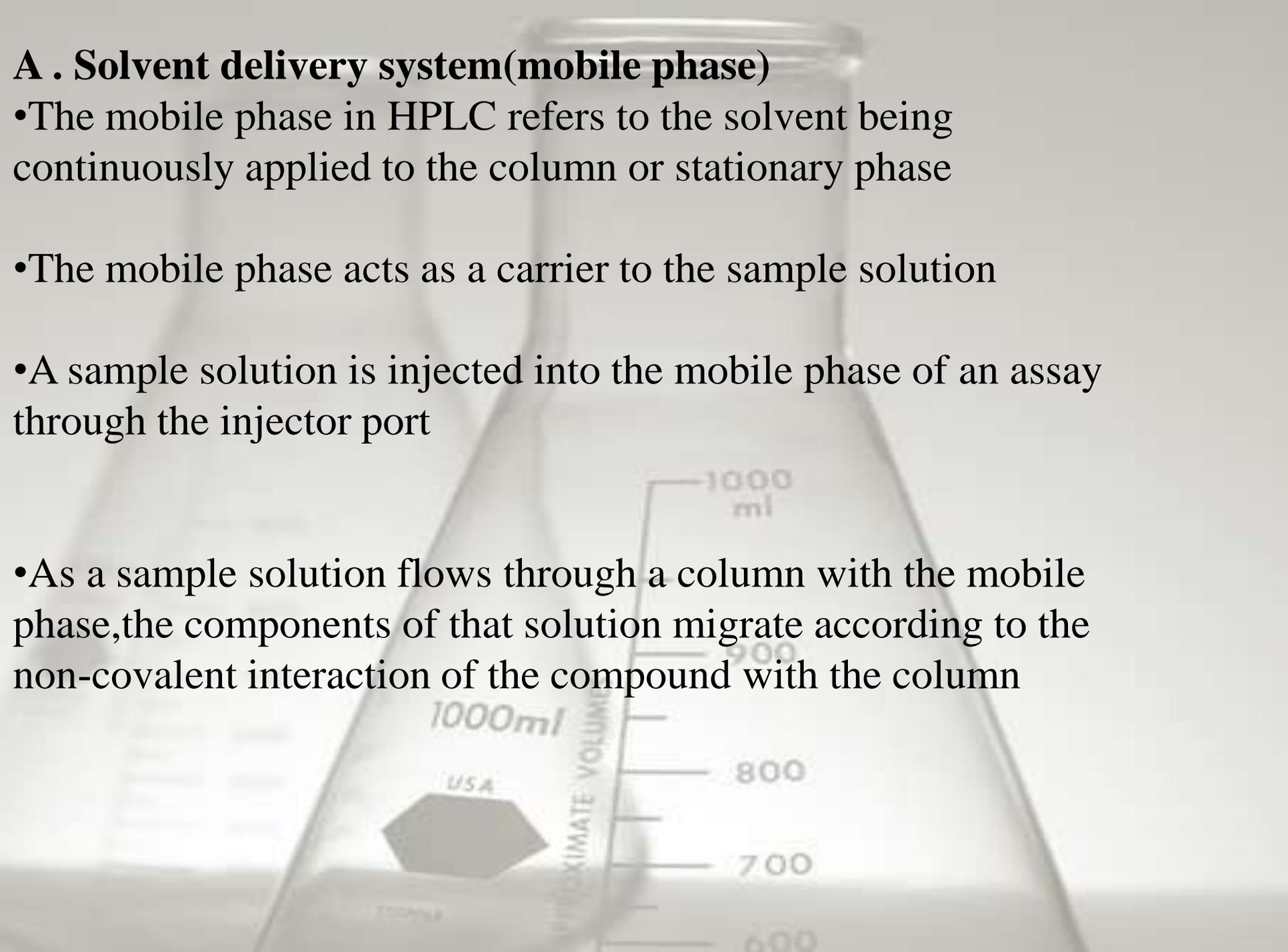
700

600

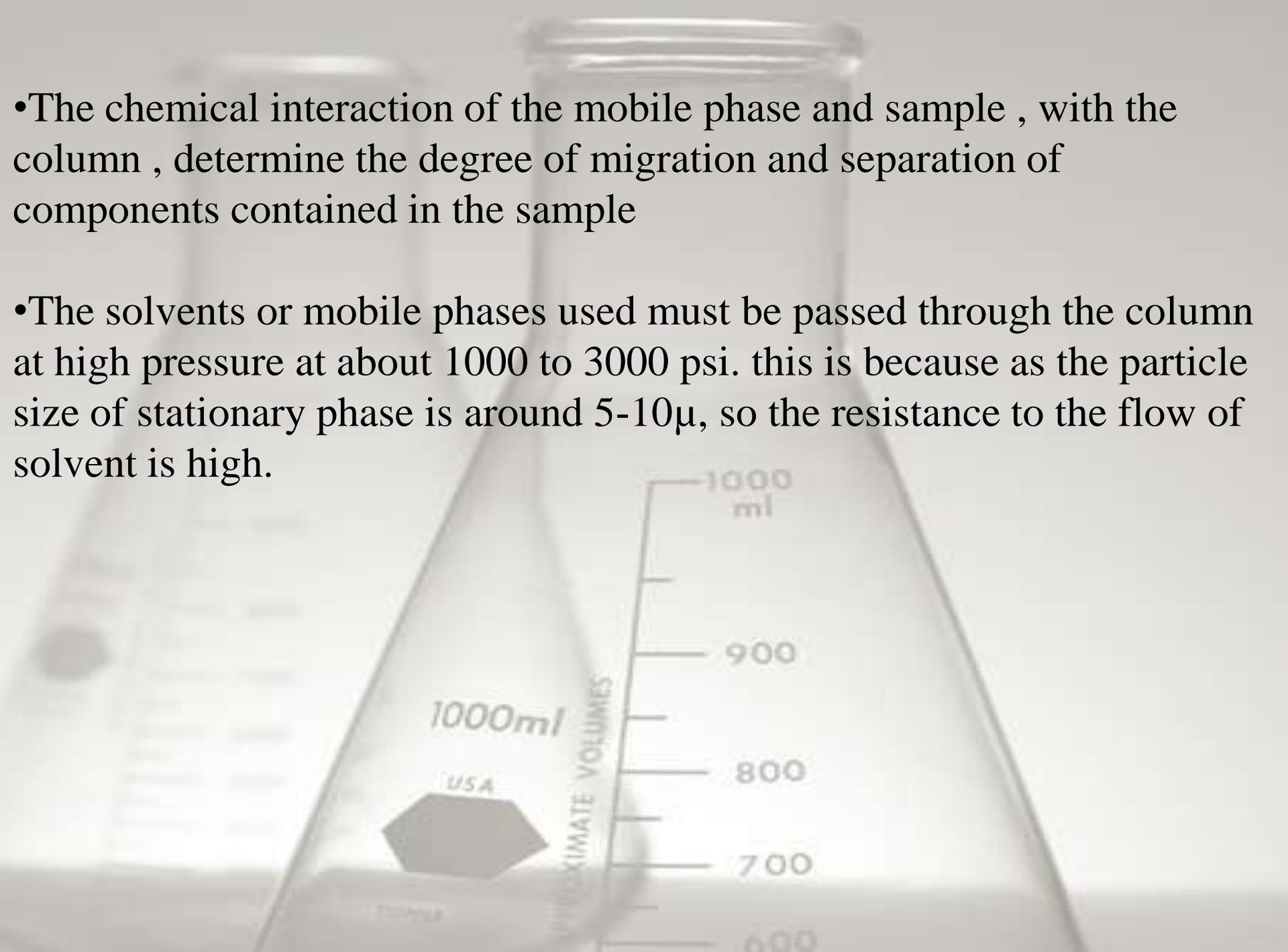


A . Solvent delivery system(mobile phase)

- The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase
- The mobile phase acts as a carrier to the sample solution
- A sample solution is injected into the mobile phase of an assay through the injector port
- As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interaction of the compound with the column



- The chemical interaction of the mobile phase and sample , with the column , determine the degree of migration and separation of components contained in the sample
- The solvents or mobile phases used must be passed through the column at high pressure at about 1000 to 3000 psi. this is because as the particle size of stationary phase is around 5-10 μ , so the resistance to the flow of solvent is high.



B.Pumps

- The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min).
- Normal flow rates in HPLC are in the 1-to 2-mL/min range.
- Typical pumps can reach pressures in the range of 6000-9000 psi (400- to 600-bar).
- During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient).

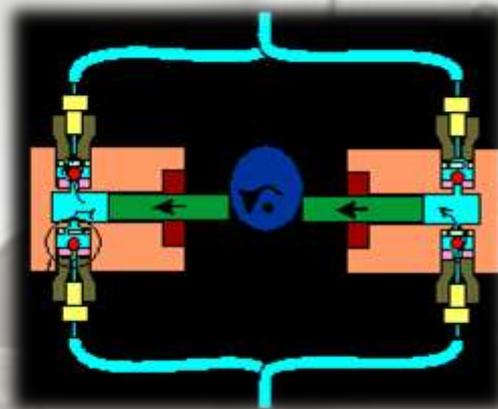
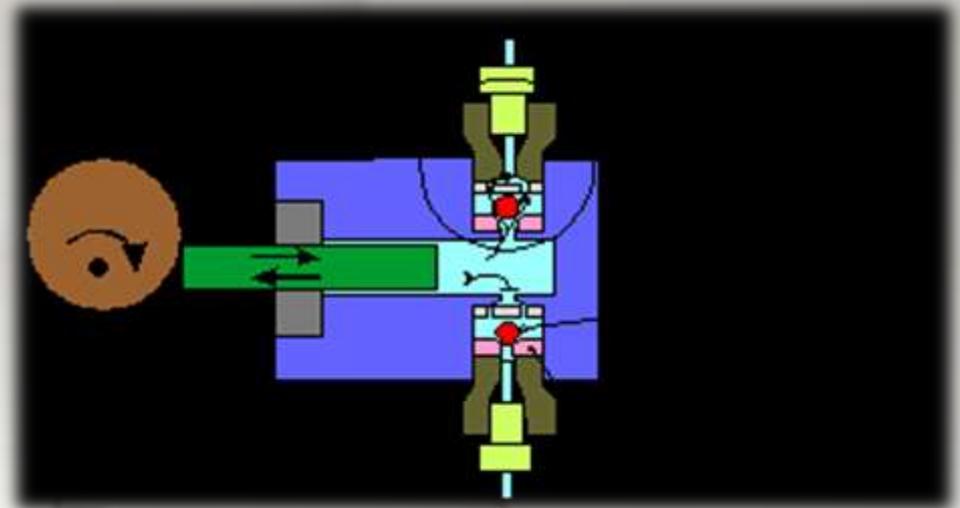
Types of HPLC pumps

There are several types of pumps used for HPLC analysis, most commonly used are reciprocating piston pump, syringe pump and constant pressure pump

1. Reciprocating piston pumps:

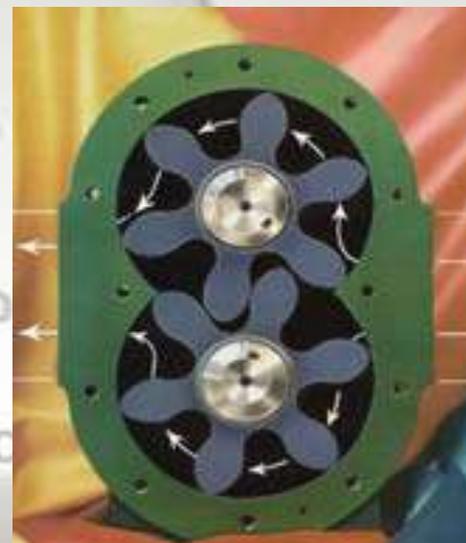
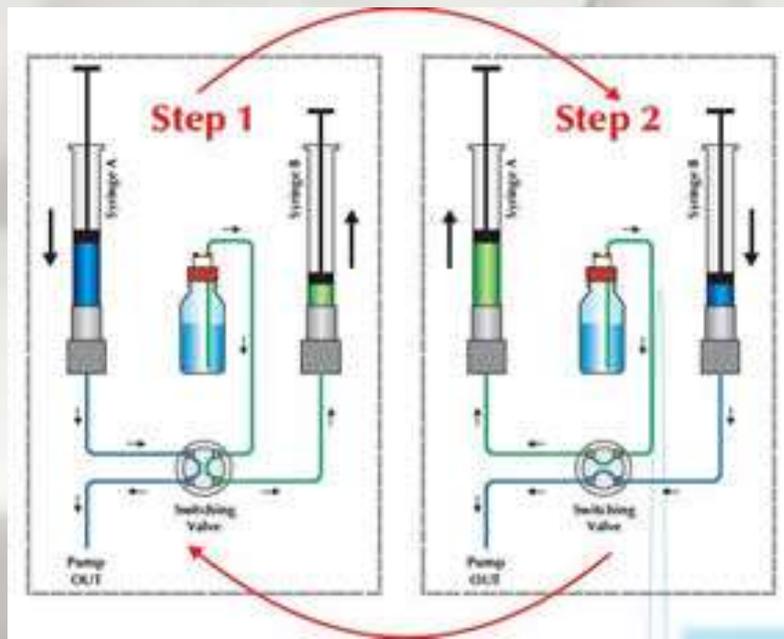
- Consists of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 μ L in volume
- On the back stroke, the separation column valve is closed, and the piston pulls in solvent from the mobile phase reservoir
- On the forward stroke, the pump pushes solvent out of the column from the reservoir
- A wide range of flow rates can be attained by altering the piston stroke volume during each cycle, or by altering the stroke frequency.

- Dual and triple head pump consists of identical piston chamber units which operate at 180 or 120 degrees out of phase(this system is significantly smoother because one pump is filling while the other is in the delivery cycle.



2. Syringe type pump

- These are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500mL
- The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor.



3. Constant pressure pump

- In these types of pumps, the mobile phase is driven through the column with the use of pressure from the gas cylinder
- A low-pressure gas source is needed to generate high liquid pressures
- The valving arrangement allows the rapid refill of the solvent chamber whose capacity is about 70mL
- This provides continuous phase flow rates

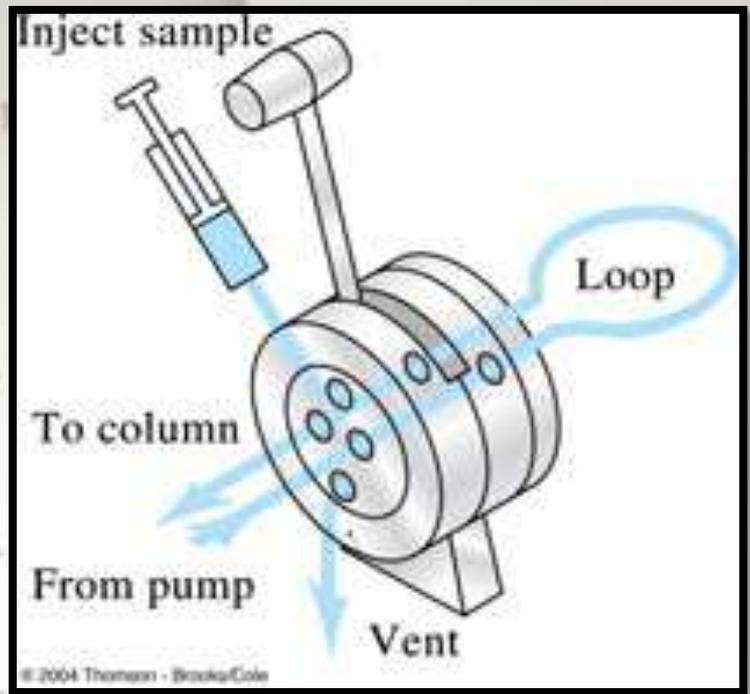
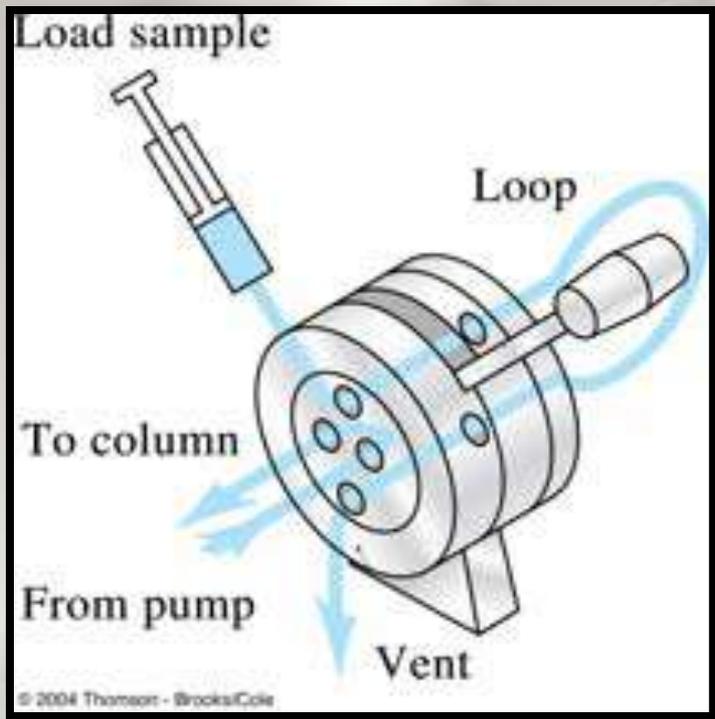


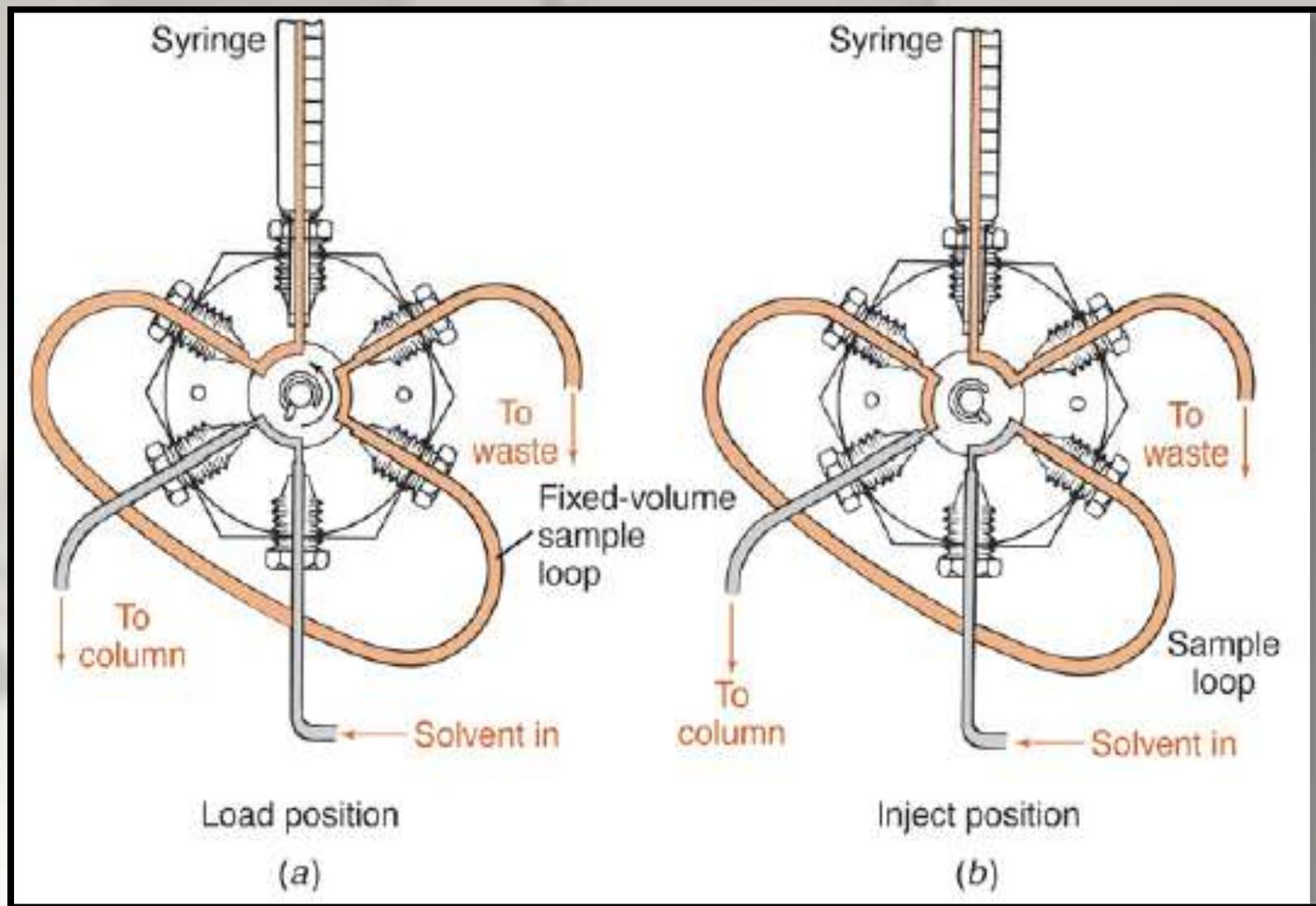
C. Injector:

- The injector serves to introduce the liquid sample into the flow stream of the mobile phase for analysis.
- It is equipped with six port valves so that a sample can be injected into the flow path at continuous pressure
- For a manual injector, the knob is manually operated to deliver the sample to the column
- The knob is set to **LOAD** position for sample injection using a syringe , the sample is injected into the sample loop , which is separated from the flow path
- The knob is turned to **INJECT** position and the eluent travels through the loop from the pump and delivers the sample to the column

- Typical sample volumes for manual injector are 5-to 20-microliters (μL).
- The injector must also be able to withstand the high pressures of the liquid system.
- An autosampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical. It can continuously inject variable volume a of $1 \mu\text{L} - 1 \text{ mL}$







Syringe

Syringe

To waste

To waste

Fixed-volume sample loop

Sample loop

To column

To column

Solvent in

Solvent in

Load position

Inject position

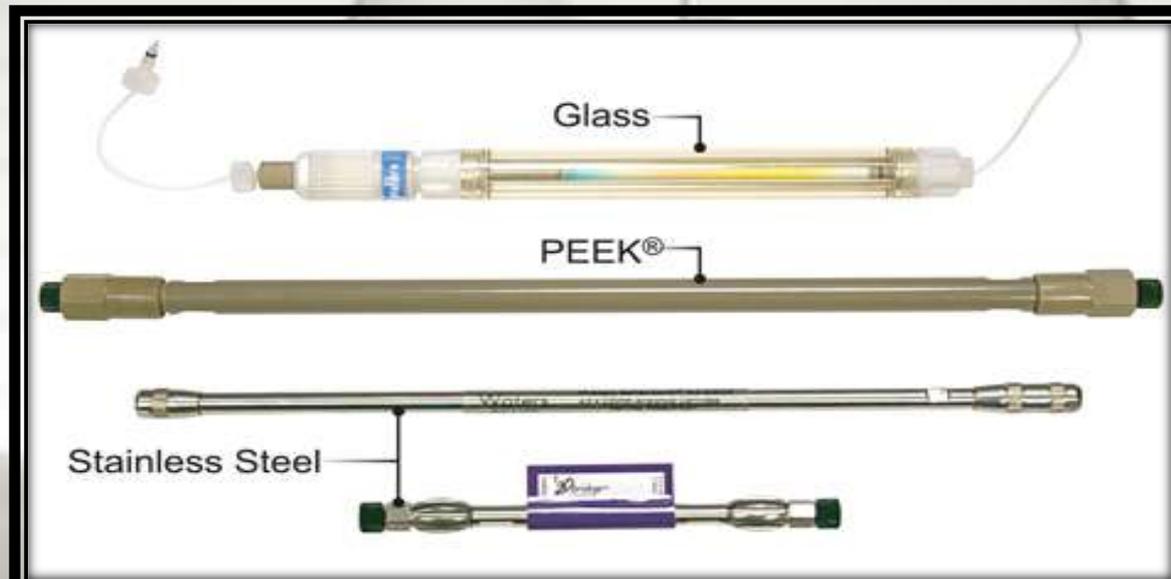
(a)

(b)

D.Column

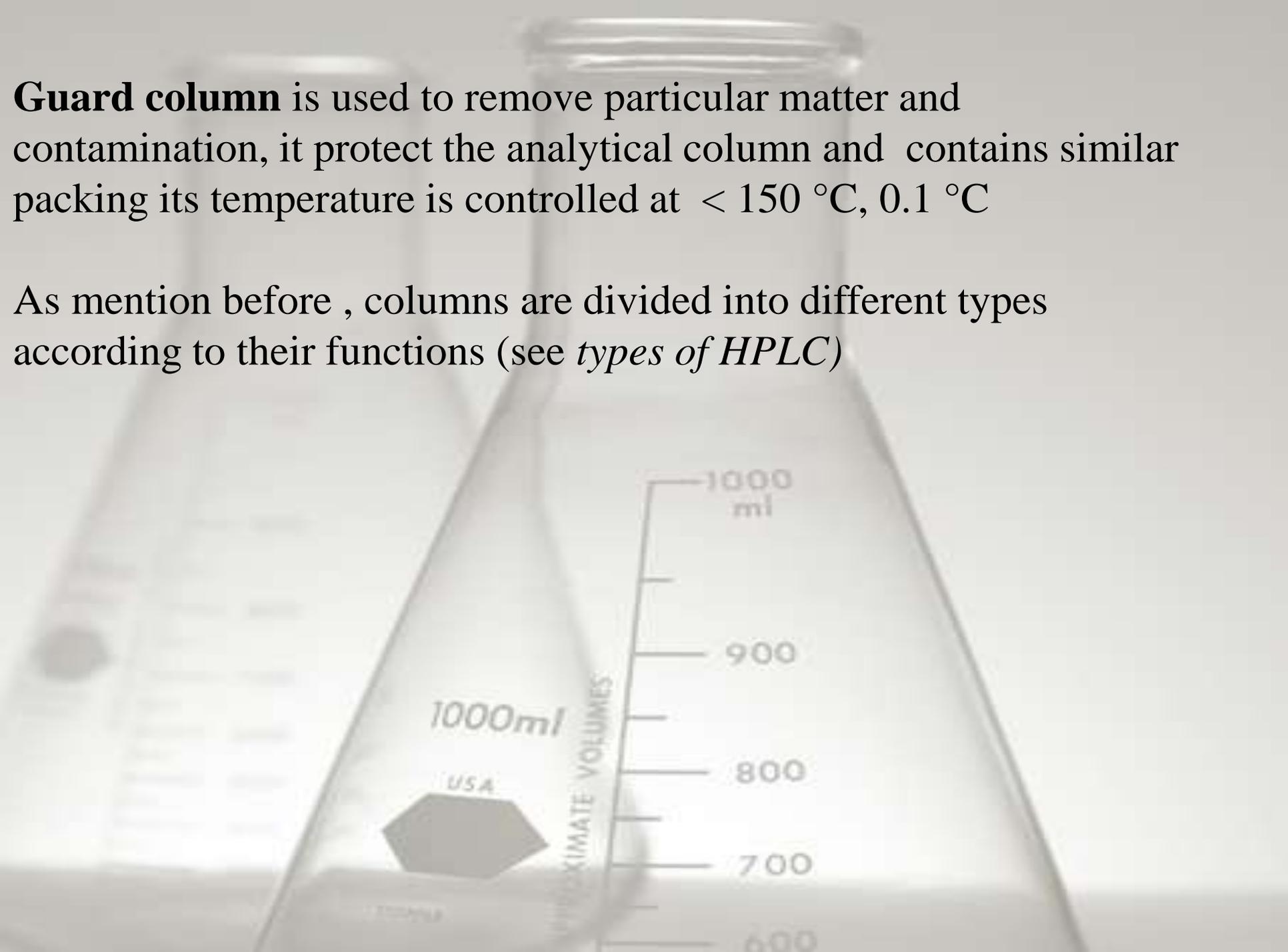
- Considered the “heart of the chromatograph” the column’s stationary phase separates the sample components of interest using various physical and chemical parameters.
- It is usually made of stainless steel to withstand high pressure caused by the pump to move the mobile phase through the column packing other material include PEEK and glass
- The small particles inside the column are called the “packing” what cause the high back pressure at normal flow rates.
- Column packing is usually silica gel because of its particle shape , surface properties , and pore structure give us a good separation

- Other material used include alumina, a polystyrene-divinyl benzene synthetic or an ion-exchange resin
 - Pellicular particle: original, Spherical, nonporous beads, proteins and large biomolecules separation (dp: 5 μm)
 - Porous particle: common used, dp: 3 ~ 10 μm . Narrow size distribution, porous microparticle coated with thin organic film
- The dimensions of the analytical column are usually
 - straight, Length(5 ~ 25 cm), diameter of column(3 ~ 5 mm), diameter of particle(35 μm). Number (40 k ~ 70 k plates/m)



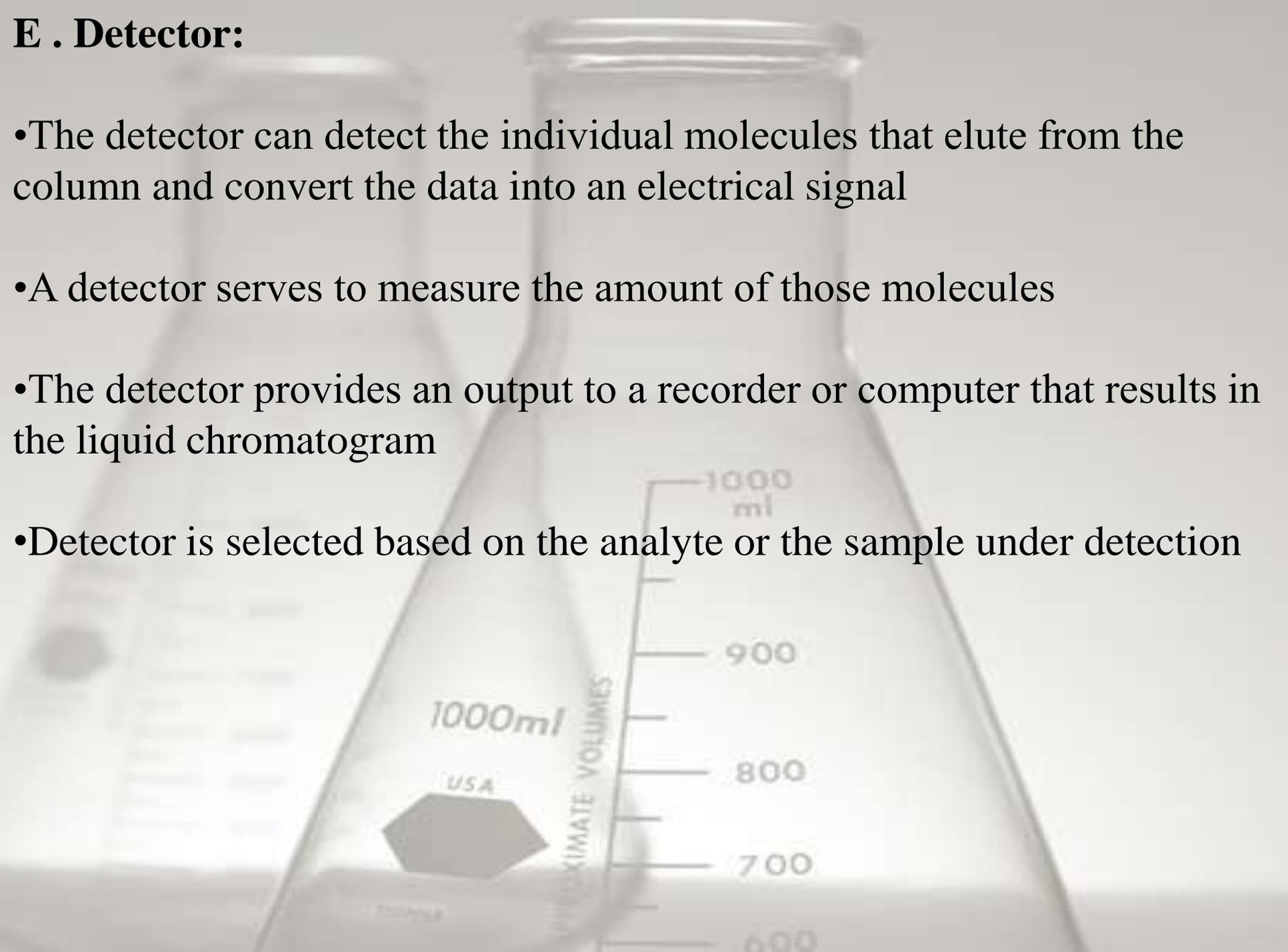
Guard column is used to remove particular matter and contamination, it protect the analytical column and contains similar packing its temperature is controlled at $< 150\text{ }^{\circ}\text{C}$, $0.1\text{ }^{\circ}\text{C}$

As mention before , columns are divided into different types according to their functions (see *types of HPLC*)



E . Detector:

- The detector can detect the individual molecules that elute from the column and convert the data into an electrical signal
- A detector serves to measure the amount of those molecules
- The detector provides an output to a recorder or computer that results in the liquid chromatogram
- Detector is selected based on the analyte or the sample under detection



Commonly used detectors in HPLC

Ultraviolet (UV)

- This type of detector responds to substances that absorb light.
- The UV detector is mainly to separate and identify the principal active components of a mixture.
- UV detectors are the most versatile, having the best sensitivity and linearity.
- UV detectors cannot be used for testing substances that are low in chromophores (colorless or virtually colorless) as they cannot absorb light at low range.
- They are cost-effective and popular and are widely used in industry

Fluorescence

- This is a specific detector that senses only those substances that emit light. This detector is popular for trace analysis in environmental science.
- As it is very sensitive, its response is only linear over a relatively limited concentration range. As there are not many elements that fluoresce, samples must be synthesized to make them detectable.

Mass Spectrometry

- The mass spectrometry detector coupled with HPLC is called HPLC-MS. HPLC-MS is the most powerful detector, widely used in pharmaceutical laboratories and research and development.
- The principal benefit of HPLC-MS is that it is capable of analyzing and providing molecular identity of a wide range of components.

Refractive Index (RI) Detection

The refractive index (RI) detector uses a monochromator and is one of the least sensitive LC detectors.

- This detector is extremely useful for detecting those compounds that are non-ionic, do not absorb ultraviolet light and do not fluoresce.
- e.g. sugar, alcohol, fatty acid and polymers.

F . Data processing unit (Computer)

- Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis).
- The concentration of each detected component is calculated from the area or height of the corresponding peak and reported.

OPERATION

Switch on instrument



Check system set up



Prime the pump



Prepare the column



```
graph TD; A[Set-up software(for system flushing)] --> B[Software set up(for sample run)]; B --> C[Sample injection]; C --> D[Chromatograph data acquisition];
```

Set-up software(for system flushing)

Software set up(for sample run)

Sample injection

Chromatograph data acquisition

STANDARD TEST PROCEDURE

Based on the STP the method of operation is determined

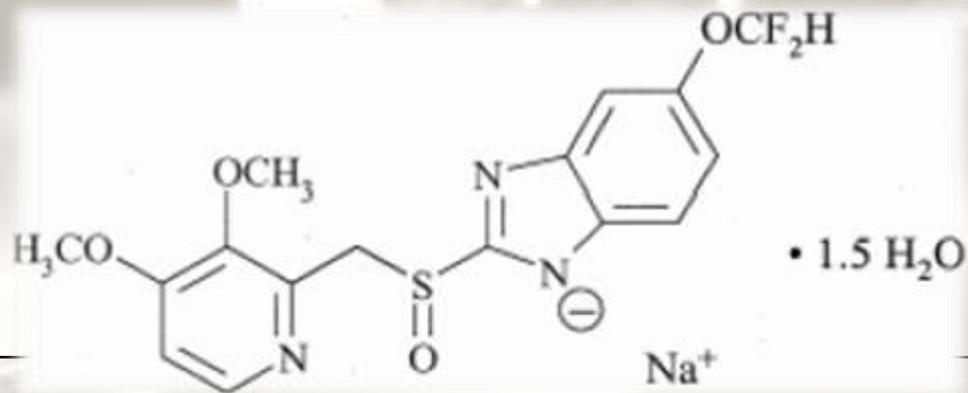
EXAMPLE:

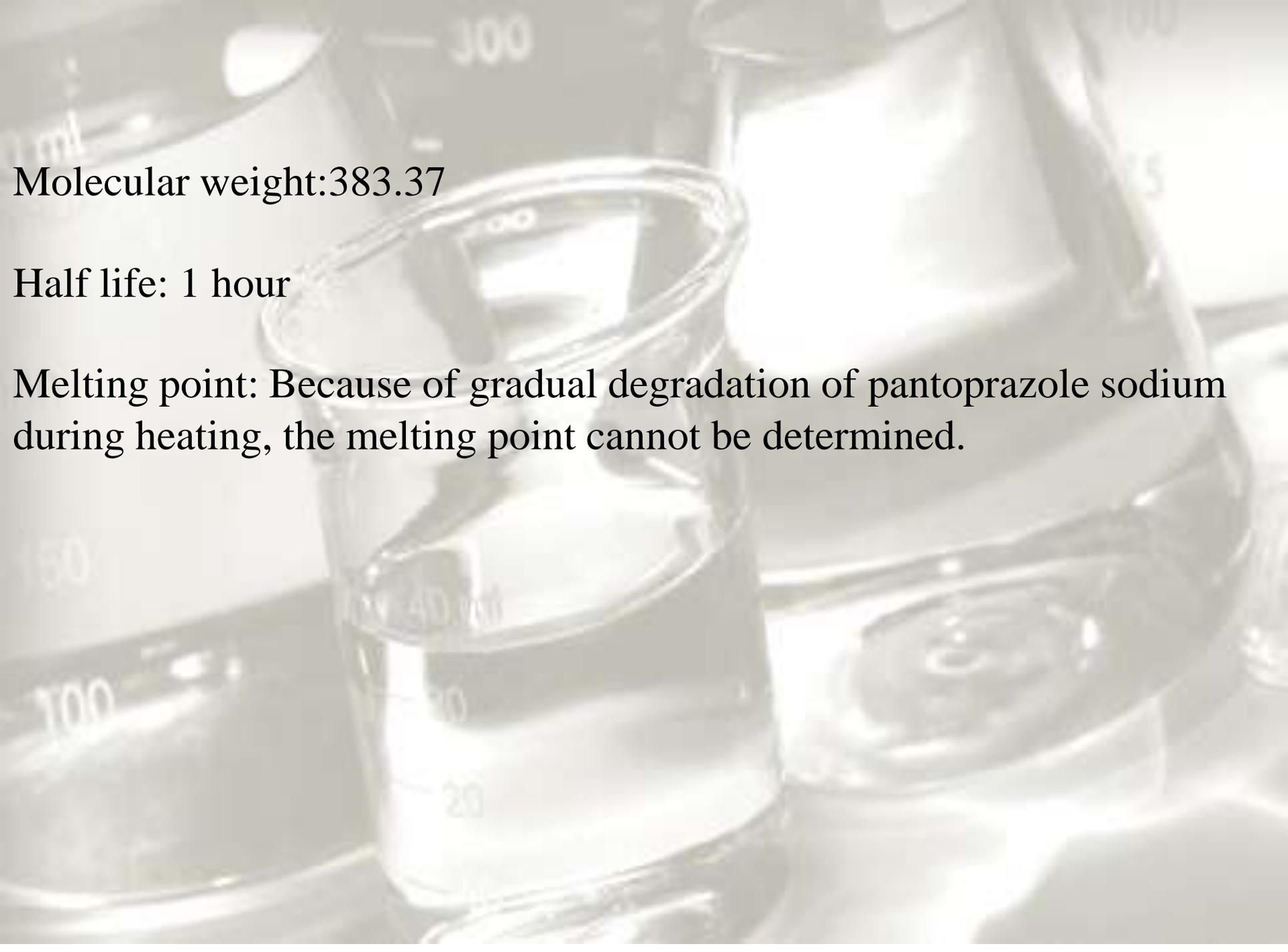
Drug name: Pantoprazole(USP)

Description : white to off-white crystalline powder

Solubility: freely soluble in water, very slightly soluble in phosphate buffer at pH 7.4, and practically insoluble in n-hexane

Molecular formula: $C_{16}H_{15}F_2N_3O_4S$



A background image of laboratory glassware, including a beaker and an Erlenmeyer flask, both containing clear liquids. The glassware has faint markings and numbers, such as '300', '100', and '50'.

Molecular weight:383.37

Half life: 1 hour

Melting point: Because of gradual degradation of pantoprazole sodium during heating, the melting point cannot be determined.

ASSAY

Assay is done to determine the main component in the drug

range of assay is 99%-101%

For a powder solution

Assay = $\frac{\text{sample area}}{\text{standard area}} \times \text{Standard Wt. in mg/dilution} \times$

$\frac{\text{Dilution/sample Wt.}}{\text{in Mg}} \times \% \text{ purity of standard}$

For a tablet

Assay = $\frac{\text{sample area}}{\text{standard area}} \times \text{Standard Wt. in mg/dilution} \times$

$\frac{\text{Dilution/sample Wt.}}{\text{in Mg}} \times \text{Avg wt./label vol. claim} \times \% \text{ purity of standard}$

Chromatographic Conditions

- Mobile Phase → Methanol: buffer in a 50:50 ratio

Instrumentation Conditions

- Column- C_{18} , 12.3x 4.0mm, 5 μ m (silica gel particle size).
- Injector Volume- 20 μ m
- Flow rate- 1.5ml/min
- Column Oven Temperature- Room Temperature
- Detector Wave Length- 260nm
- Run Time- 10 minutes

1. Preparation of solutions:

1. Preparation of buffer : dissolve 1.32gm of diammonium hydrogen orthophosphate in 500ml of distilled water and adjust the pH to 3 with orthophosphoric acid (OPA)

2. Preparation of mobile phase:

Add 500ml of prepared buffer solution to 500ml of MeOH and mix properly.

Filter through 0.45 μ filter paper

Sonicate for degassing

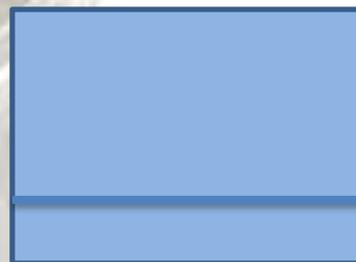
3. Preparation of standard solution

Add Pantoprazole sesquihydrate to a 20ml flask and add methanol

Operation:

- Equilibrate the column with mobile phase till the base line is stabilized
- Inject 20 μ l of mobile phase into the system and record the chromatogram(blank)

voltage



time

- Inject 20 μ l of standard solution into the system and record the chromatogram
- Inject the 20 μ l of sample solution into the system and record the chromatogram
- Wash the column with distilled water for 15 minutes and with MeOH for 15 minutes

Washing

- Methanol (any solvent) for 10-30 minutes
- H₂O (10-30 minutes)

Washing

- Mobile Phase
- Standard
- Sample

Analysis

- H₂O
- MeOH

Wash again

Preparation of standard and & samples is done by ***PPM calculations***
(Parts per million)

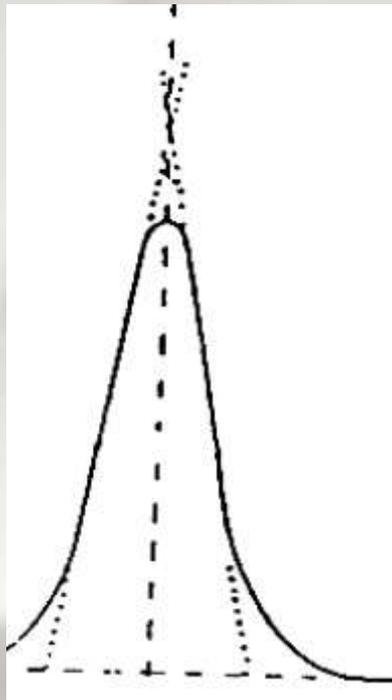
PPM= wt. in mg / Vol. in ml. 1000 (1 PPM is 1 mg in 1 lt solution)

PARAMETERS

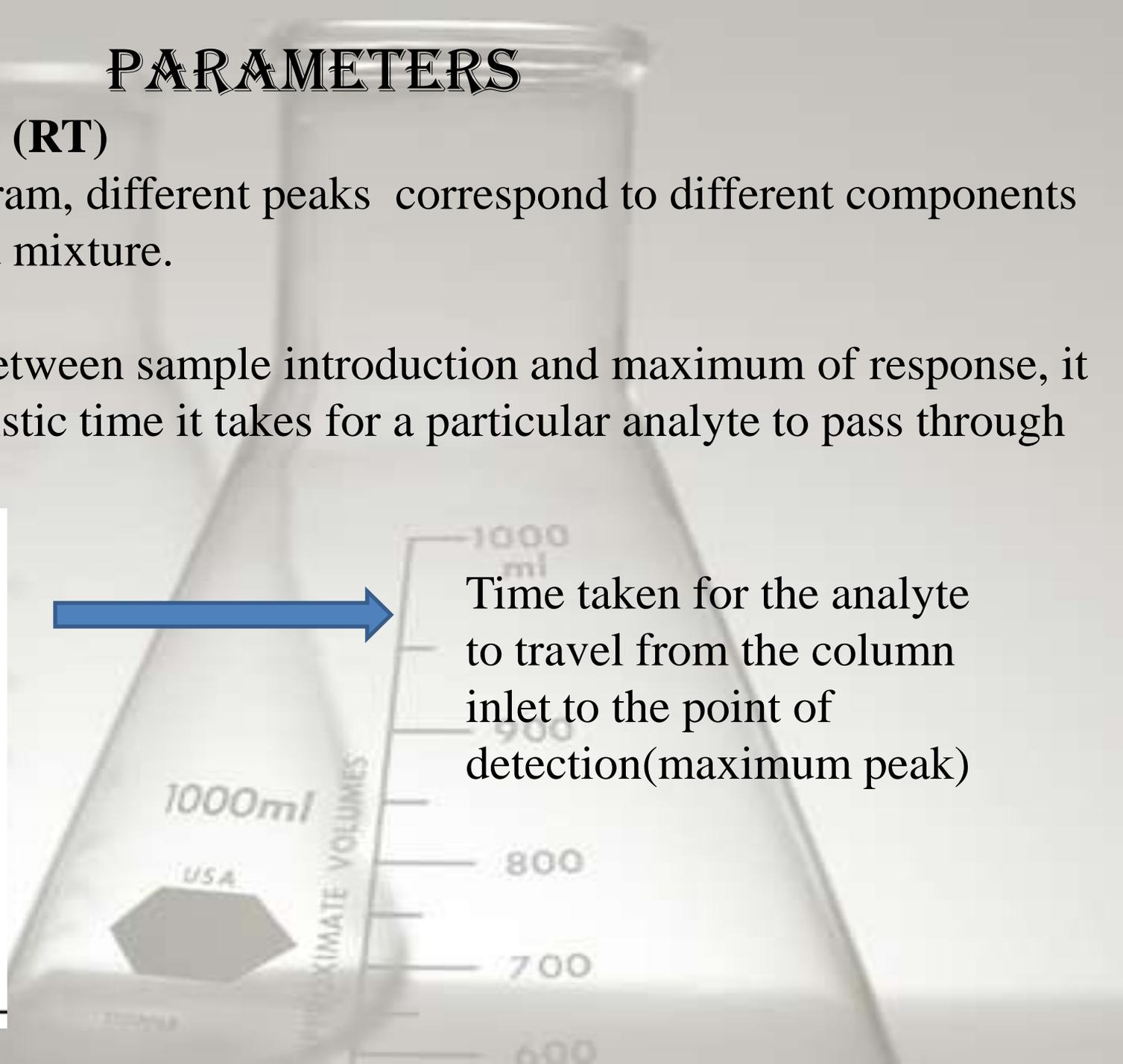
Retention time (RT)

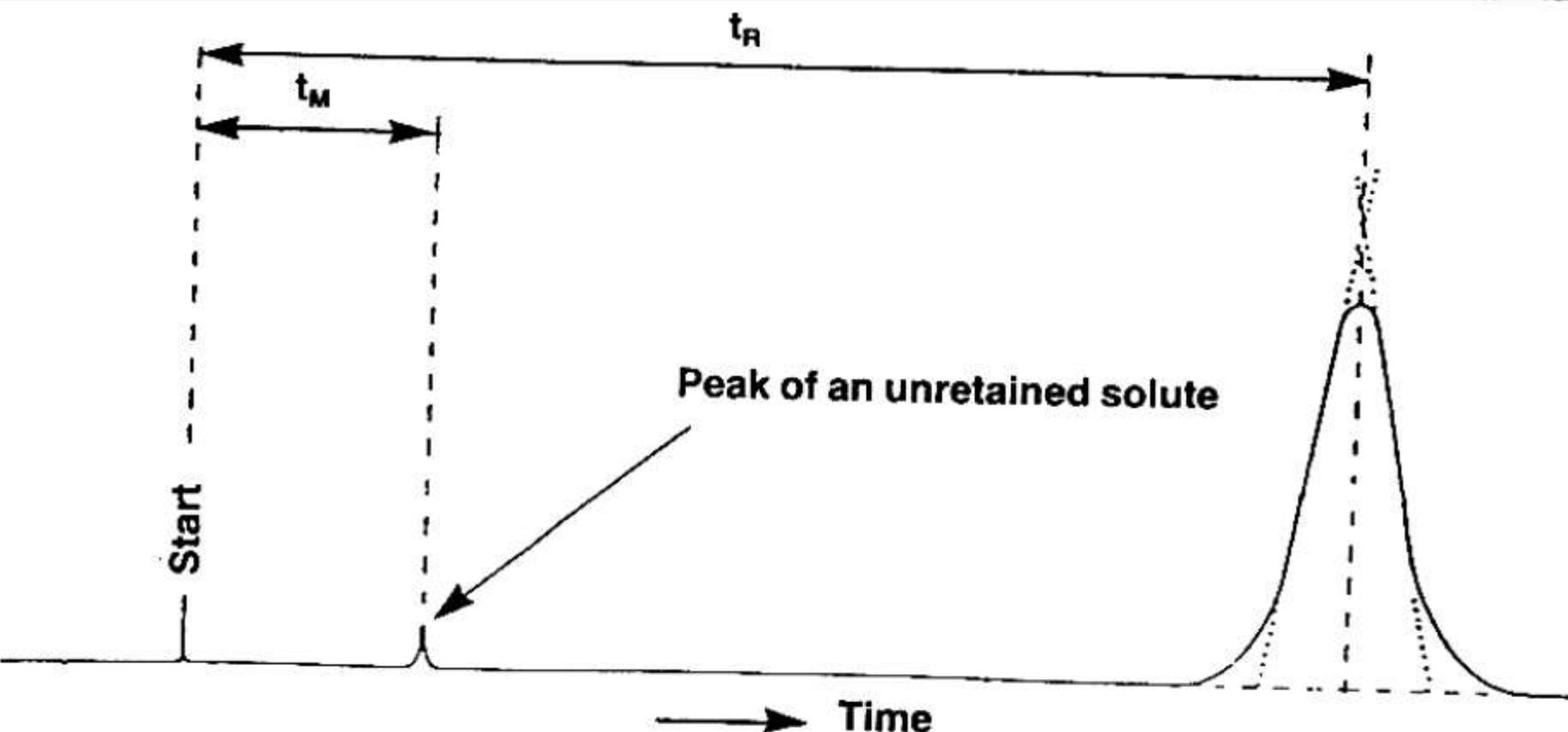
In a chromatogram, different peaks correspond to different components of the separated mixture.

Time elapsed between sample introduction and maximum of response, it is the characteristic time it takes for a particular analyte to pass through the system



Time taken for the analyte to travel from the column inlet to the point of detection (maximum peak)





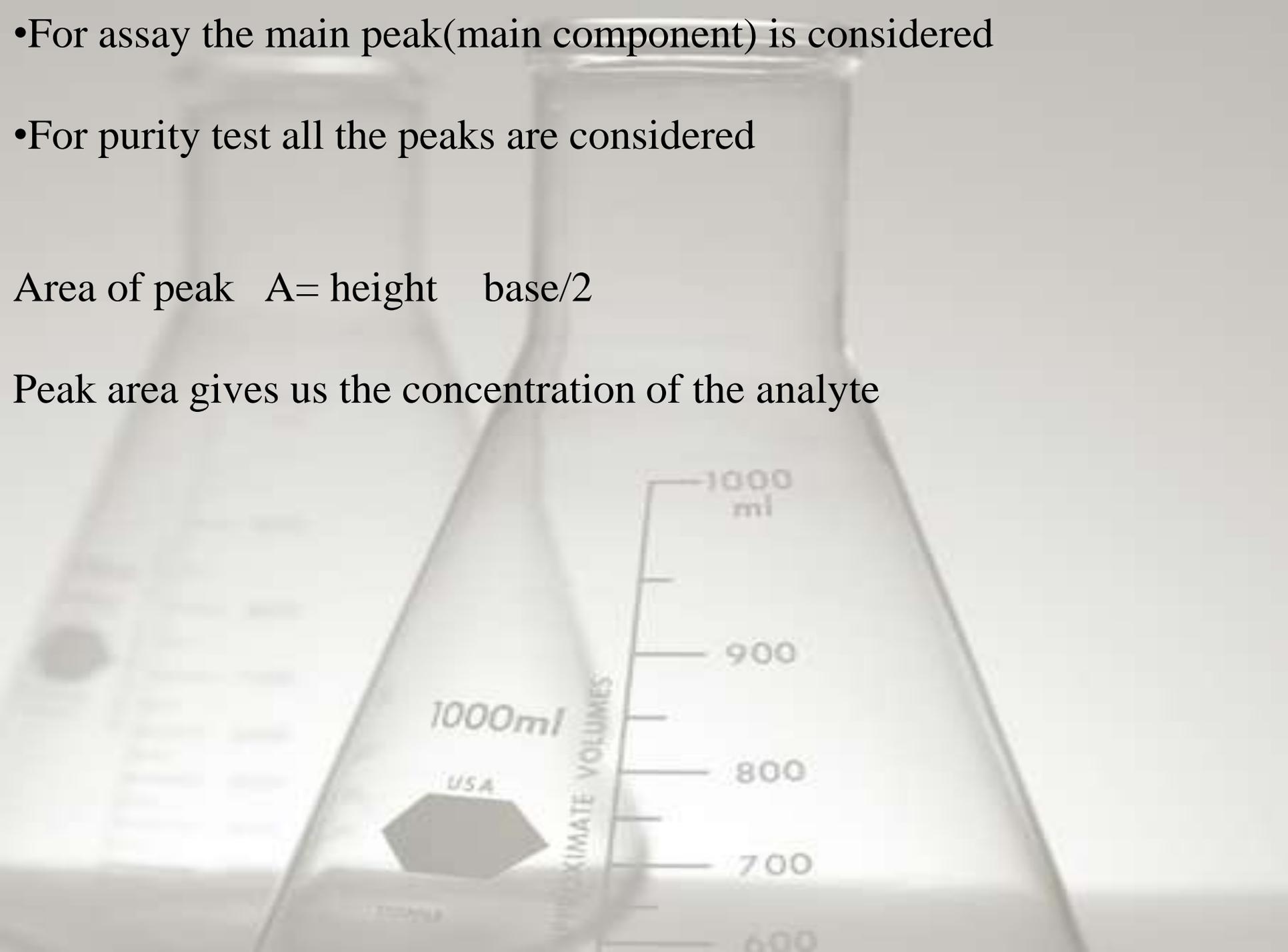
$$k' = \frac{t_R - t_m}{t_m}$$

t_m = unretained peak's retention time
 t_R = retention time of the peak of interest

- For assay the main peak(main component) is considered
- For purity test all the peaks are considered

Area of peak $A = \text{height} \times \text{base}/2$

Peak area gives us the concentration of the analyte



2.Retention volume:

Retention volume is the volume of carrier gas required to elute 50% of the component from the column. It is the product of retention time and flow rate.

$$\text{Retention volume} = \text{Retention time} \times \text{flow rate}$$

3.Seperation factor:

Separation factor is the ratio of partition coefficient of the two components to be separated.

$$S = K_a / K_b = (t_b - t_0) / (t_a - t_0)$$

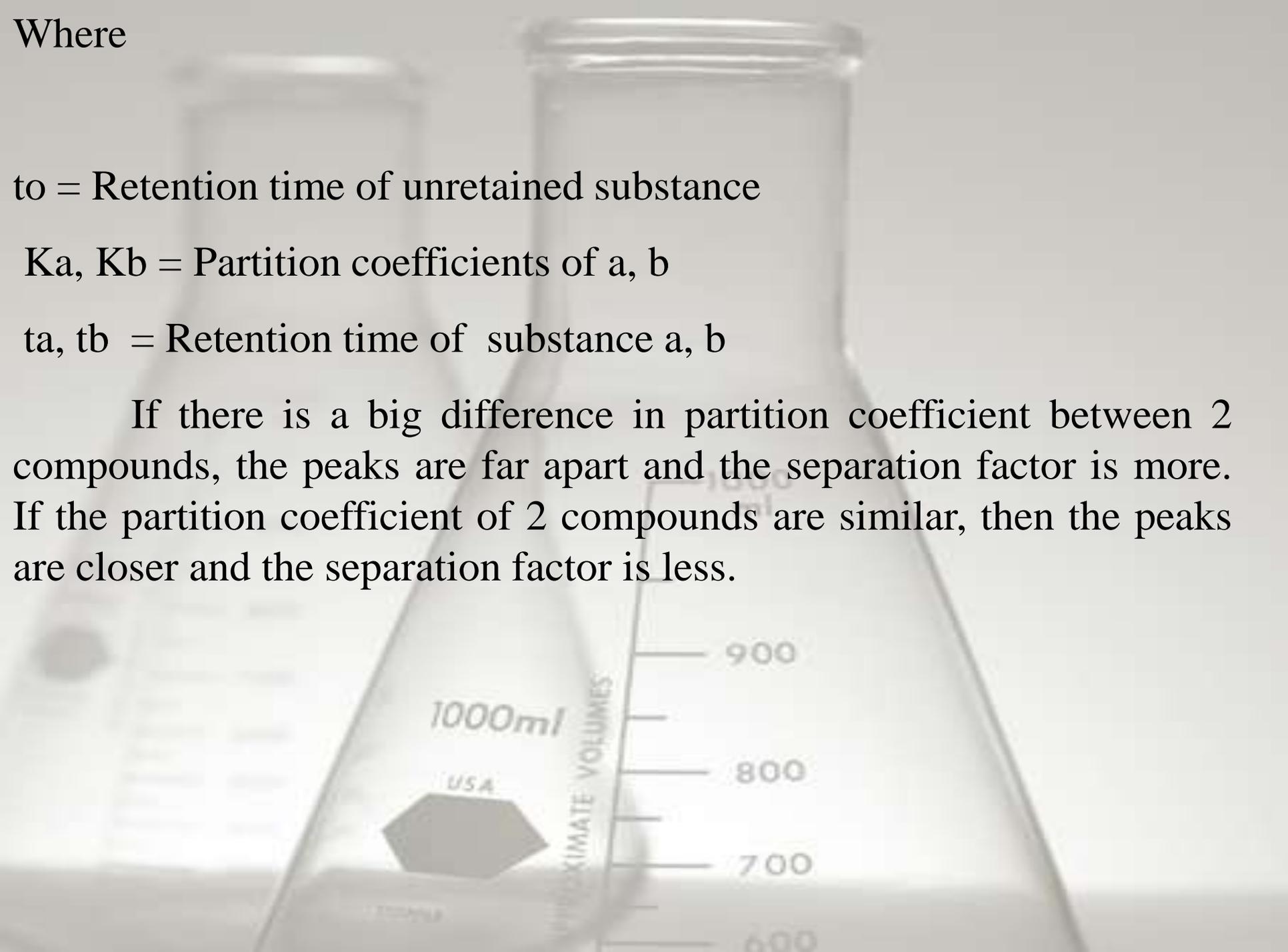
Where

t_0 = Retention time of unretained substance

K_a, K_b = Partition coefficients of a, b

t_a, t_b = Retention time of substance a, b

If there is a big difference in partition coefficient between 2 compounds, the peaks are far apart and the separation factor is more. If the partition coefficient of 2 compounds are similar, then the peaks are closer and the separation factor is less.



4. Resolution:

Resolution is the measure of extent of separation of 2 components and the base line separation achieved.

$$R_s = 2 (R_{t_1} - R_{t_2}) / w_1 + w_2$$

5. Height Equivalent to a Theoretical Plate (HETP):

A theoretical plate is an imaginary or hypothetical unit of a column where distribution of solute between stationary phase and mobile phase has attained equilibrium. It can also be called as a functional unit of the column.

A theoretical plate can be of any height, which describes the efficiency of separation. If HETP is less, the column is more efficient. If HETP is more, the column is less efficient.

HETP = length of the column/ no. of theoretical plates

HETP is given by Van deemter equation

$$\text{HETP} = A + B/u + Cu$$

Where A = Eddy diffusion term or multiple path diffusion which arises due to the packing of the column. This can be minimized by uniformity of packing.

B = Longitudinal diffusion term or molecular diffusion.

C = Effect of mass transfer.

u = flow rate or velocity of the mobile phase.

6. Efficiency:

Efficiency of a column is expressed by the theoretical plates.

$$n = 16 R_t^2 / w^2$$

Where n = no of theoretical plates

R_t = retention time

w = peak width at base

R_t and w are measured in common units (cm or mm , min or sec). No of theoretical plates

Is high, the column is said to be highly efficient. For GLC, a value of 600/metre is sufficient. But in HPLC, high values like 40,000 to 70,000/meter are recommended.

7. Asymmetry factor:

A chromatographic peak should be symmetrical about its centre and said to follow Gaussian distribution. But in practice due to some factors, the peak is not symmetrical and shows tailing or fronting.

Fronting is due to saturation of stationary phase and can be avoided by using less quantity of sample. Tailing is due to more active adsorption sites and can be eliminated by support pretreatment.

Asymmetry factor (0.95 to 1.05) can be calculated by $AF = b/a$ (b, a calculated by 5% or 10% of the peak height).

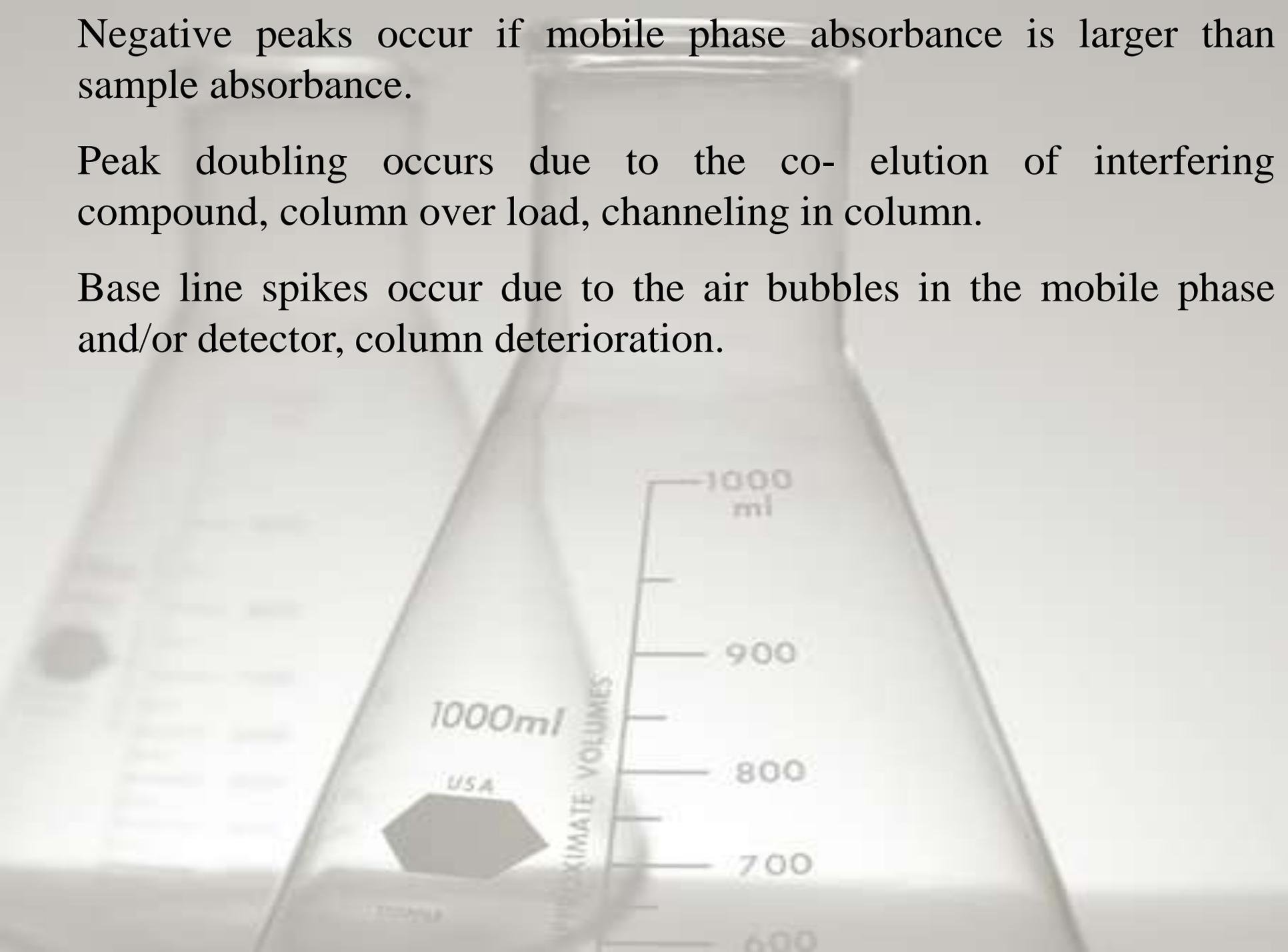
Broad peaks occur due to the more conc. of sample, large injection volume, column deterioration.

Ghost peaks occur due to the contamination of the column, compound from earlier injections.

Negative peaks occur if mobile phase absorbance is larger than sample absorbance.

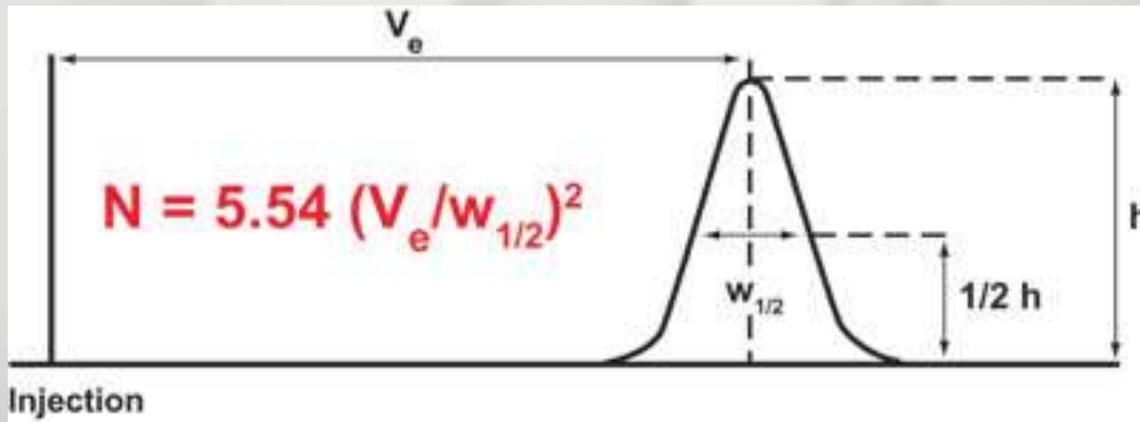
Peak doubling occurs due to the co-elution of interfering compound, column over load, channeling in column.

Base line spikes occur due to the air bubbles in the mobile phase and/or detector, column deterioration.



SYSTEM SUITABILITY

1. Calculation of the number of Theoretical Plates (half-height method)



Where:

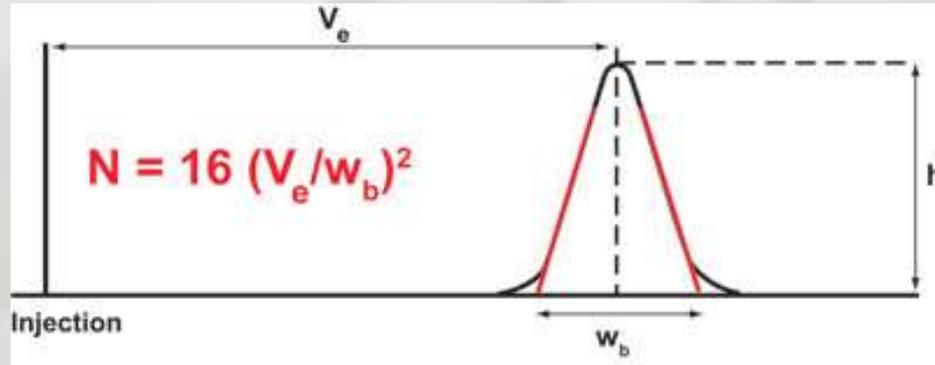
N = Number of theoretical plates

V_e = elution volume or retention time (mL, sec, or cm)

h = peak height

$w_{1/2}$ = width of the peak at half peak height (mL, sec, or cm)

2. Calculation of the number of Theoretical Plates (USP method)



Where:

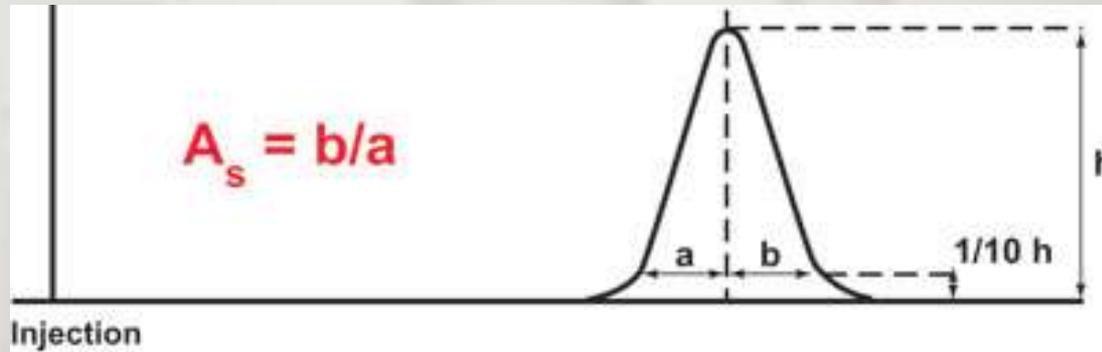
N = Number of theoretical plates

V_e = elution volume, retention time or retention distance (mL, sec, or cm)

h = peak height

w_b = width of the peak at the base line (mL, sec, or cm)

3. Calculation of peak Asymmetry Factor (used by Tosoh)



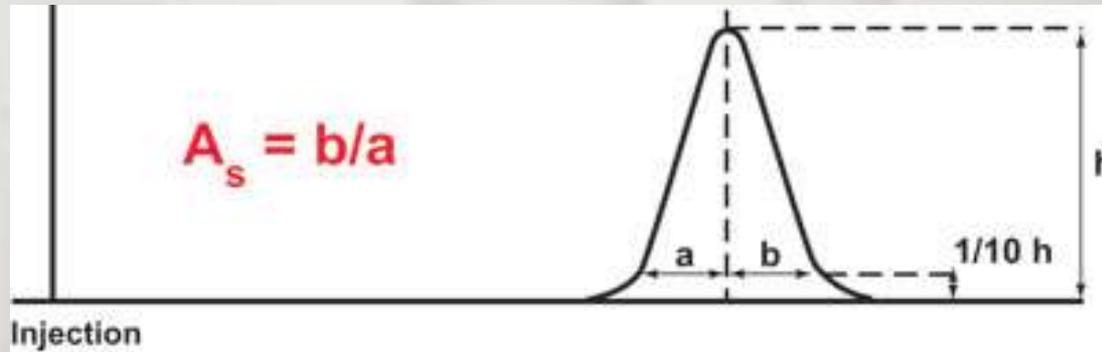
Where:

A_s = peak asymmetry factor

b = distance from the point at peak midpoint to the trailing edge (measured at 10% of peak height)

a = distance from the leading edge of of peak to the midpoint (measured at 10% of peak height)

3. Calculation of peak Asymmetry Factor (used by Tosoh)



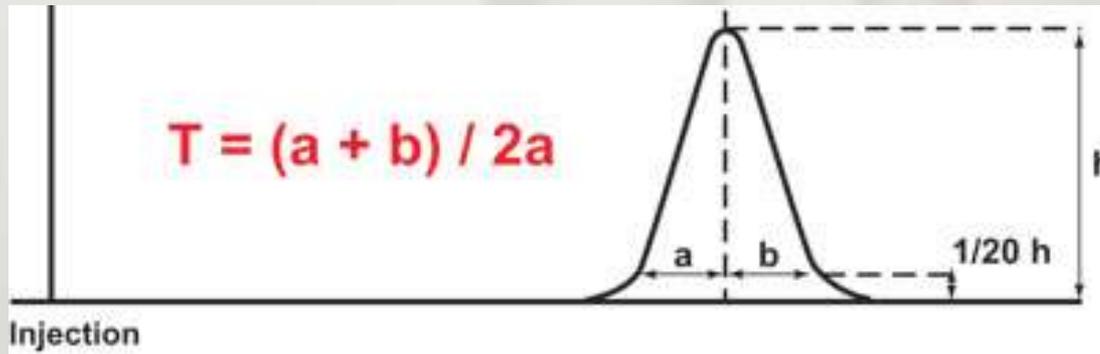
Where:

A_s = peak asymmetry factor

b = distance from the point at peak midpoint to the trailing edge (measured at 10% of peak height)

a = distance from the leading edge of of peak to the midpoint (measured at 10% of peak height)

4. Calculation of Tailing Factor (USP method)



Where:

T = tailing factor (measured at 5% of peak height)

b = distance from the point at peak midpoint to the trailing edge

a = distance from the leading edge of the peak to the midpoint

5. Calculation of the Height Equivalent to a Theoretical Plate (HETP)

$$H = L / N$$

Where:

H = Height equivalent of a theoretical plate

L = Length of the column

N = Number of theoretical plates

Dimensions: when using HPLC or UHPLC columns, H is usually expressed in μm .

6. Calculation of Reduced Plate Height (h)

Giddings introduced dimensionless parameters for H and also for the linear velocity. Dimensionless parameters allow the direct comparison of the efficiency of two or more columns packed with different particle size packing materials. According to the theory, a well packed column should have a reduced plate height (h) in the range of 2-3 at a reduced velocity (v) of about 3. Here we only provide the formula for h.

$$h = H / d_p$$

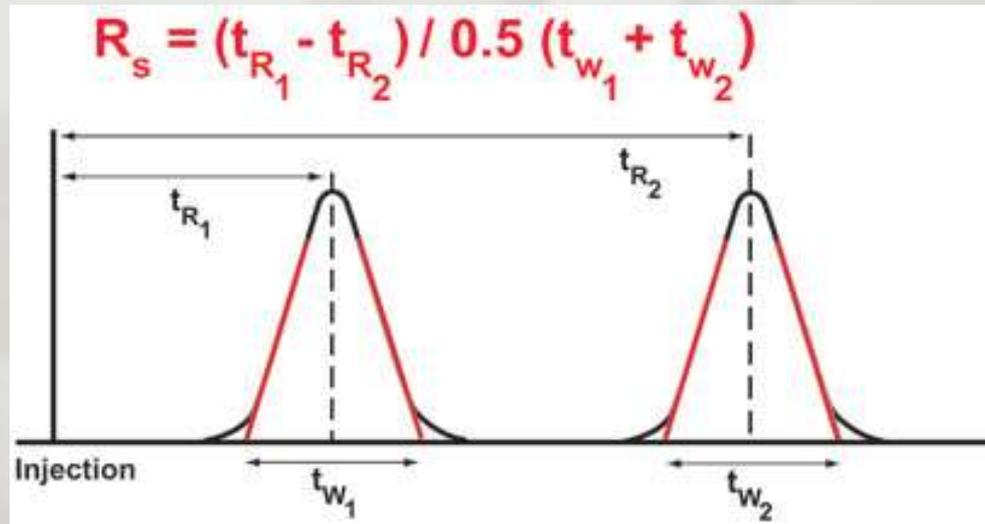
Where:

h = reduced plate height (sometimes referred to as the number of band widths)

H = height equivalent of a theoretical plate (μm)

d_p = mean particle size (μm)

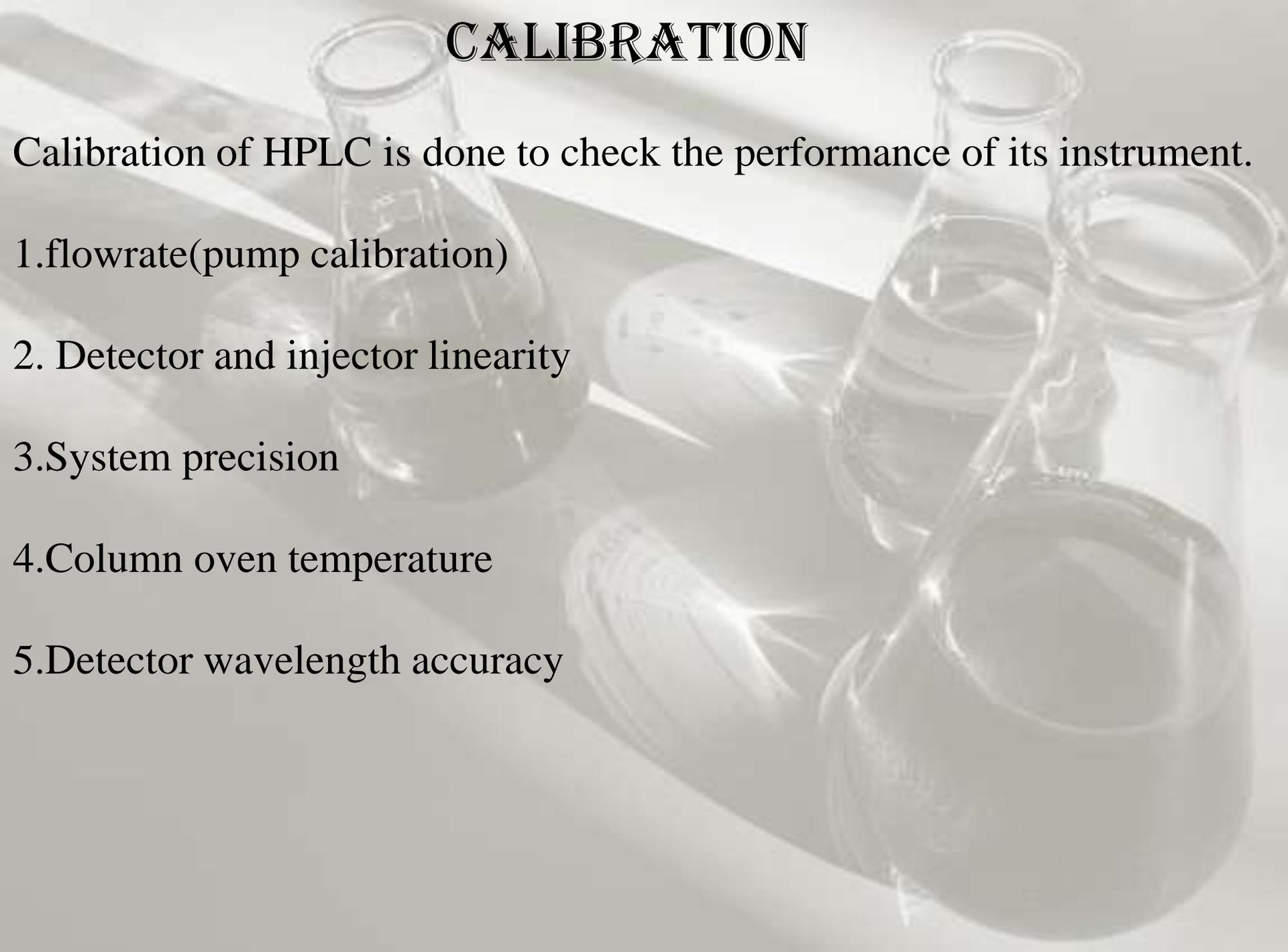
7. Calculation of chromatographic Resolution



Note: t_{w_1} and t_{w_2} are obtained from the intersection of the tangents with the baseline.

For a symmetrical Gaussian peak the tangents are drawn at 0.6 times the peak height.

CALIBRATION



Calibration of HPLC is done to check the performance of its instrument.

1. flowrate (pump calibration)
2. Detector and injector linearity
3. System precision
4. Column oven temperature
5. Detector wavelength accuracy

1. Pump calibration

- Disconnect the column and connect the inlet and outlet tubing's with a union.
- Prime all the lines at 5 ml/min flow rate with water and ensure that flow line is free from air bubbles.
- Set the flow rate at 1ml / min and collect the mobile phase (water) in a dry preweighed beaker and collect the mobile phase for 10 min. weigh the beaker to get the weight of mobile phase.
- Calculate the flow rate by dividing the weight obtained with weight per ml and 10 (run time).
- Calculate the corresponding flow rate. Carry out the experiment in duplicate.

Acceptance criteria

0.05ml for small quantities

0.1ml for larger quantities

2. Detector and Injector linearity

Column : ODS or C18

Mobile phase : milli Q water and acetonitrile (80:20)

Flow rate: 1ml/min

Temperature: 40 Centigrade

Detector wavelength: 272 nm

Runtime: 10min

Preparation of stock solution:

- Take 50mg of caffeine and transfer into 50ml of flask and make it up to the mark with the diluent
- *Caffeine is used as it gives a multiwavelength response and is stable*
- Prepare solutions of different PPM (100-600)
- Inject them and calculate the area
- Correlation coefficient r is used to check the detector linearity and cannot be less than .999
- $r = \frac{N\sum XY - \sum X\sum Y}{\sqrt{[N\sum X^2 - (\sum X)^2][N\sum Y^2 - (\sum Y)^2]}}$
- The graph obtained between concentration and area is linear

3. System precision

- From the stock solution pipette out 1ml of stock solution and transfer into 10ml flask and make it upto the mark with diluent for 100PPM conc.
- Calculate the percentage of RSD of areas of 5 different injections
- % RSD = $\frac{\text{standard deviation}}{\text{average value of above 5 injection areas}} \times 100$
- Where $SD = \sqrt{\frac{\sum(X-M)^2}{n-1}}$

N= # of injections

M=average area

X=area

For system precision % of RSD is not more than 1

4. Column oven temperature

1. Set the column oven temperature to 30 and leave it for 30 minutes
2. Open the door of the column oven and keep the thermometer and leave it for 30 min
3. now note down the reading in the thermometer
4. Similarly change the column oven temperature to 40 C and 50 C and repeat the above procedure

Acceptance criteria

2 c

5. Detector wavelength accuracy

Column : C18

Mobile phase: HPLC grade methanol

Flow rate: 1 ml/min

Retention time: 5 minutes

Wavelength: 272 nm

1. Inject the methanol and record blank
2. Inject 20 ml of standard solution at 268 nm
3. Similarly inject the standard solution by increasing the nm upto 278 nm (increasing it 1 at a time)

Acceptance criteria

273 nm 1

MAINTAINANCE

1.RESERVOIR

Possible Cause

1. Blocked inlet frit

2. Gas bubbles

Preventive Maintenance

1. a. Replace (3–6 months)

b. Filter mobile phase with
0.4 - 0.5 μm filter

2. Degas mobile phase,
sonification

2. PUMP

Possible Cause

1. Air bubbles
2. Pump seal failure
3. Check valve failure
4. Improper cleaning

Preventive Maintenance

1. Degas mobile phase , do not change mobile phase during run
2. Replace (3 months), clean with 1 N acid
3. Filter mobile phase; use inlet-line frit ; keep spare
4. Clean with Isopropyl alcohol , mobile phase container must be cleaned with mobile phase and other sections with solvent

3.INJECTOR

Possible Cause

- 1.Washing
2. Rotor seal wear
- 3.Syringe

Preventive Maintenance

- 1.wash before and after use
2.
 - a. Do not overtighten
 - b. Filter samples
- 3.Sterilize when fresh sample is used

4. Column

Possible Cause

1. Number of injections
2. Blocked frit
3. Void at head of column

Preventive Maintenance

1. 2000 or less
- 2.. a. Filter mobile phase
b. Filter samples
c. Use in-line filter and/or guard column
- 3.. a. Avoid mobile phase pH >8
b. Use guard column
c. Use precolumn (saturator column)

5. Detector

Possible Cause

1. Lamp failure;
decreased detector
2. Bubbles in cell

Preventive Maintenance

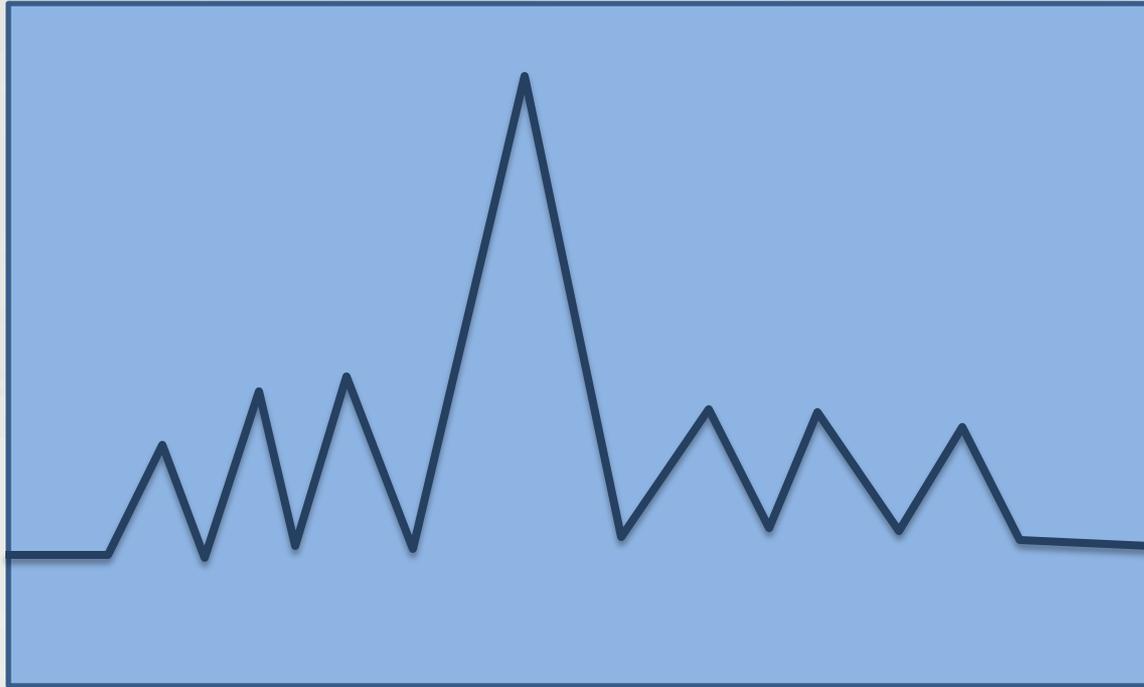
1. Replace (6 months) or
keep spare lamp
response; increased
detector noise
2. a. Keep cell clean
b. Use restrictor after cell
c. Degas mobile phase

6. Software

Update frequently , around 6-12 months

TROUBLESHOOTING

Identifying problem using a chromatogram

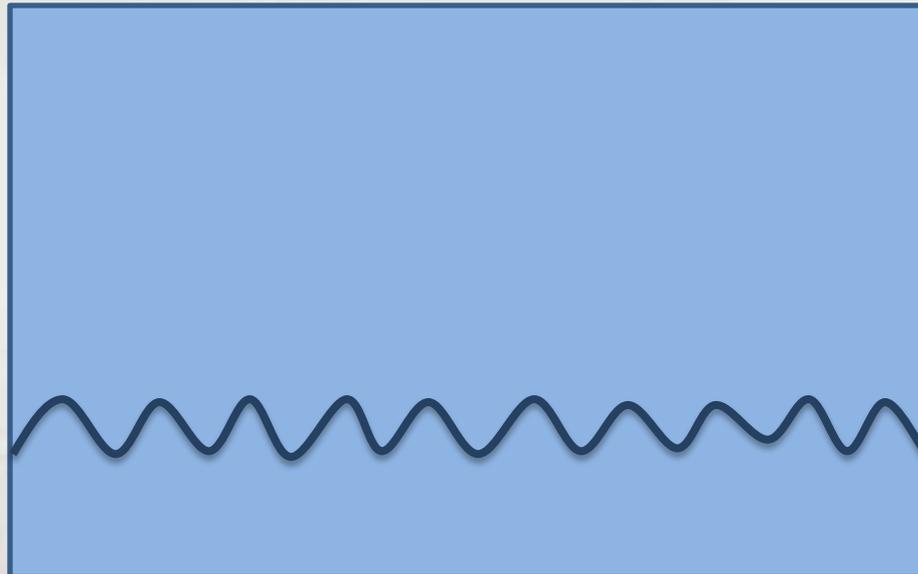


1. The above pattern occurs when the mobile phase is not suitable

2. Baseline noise



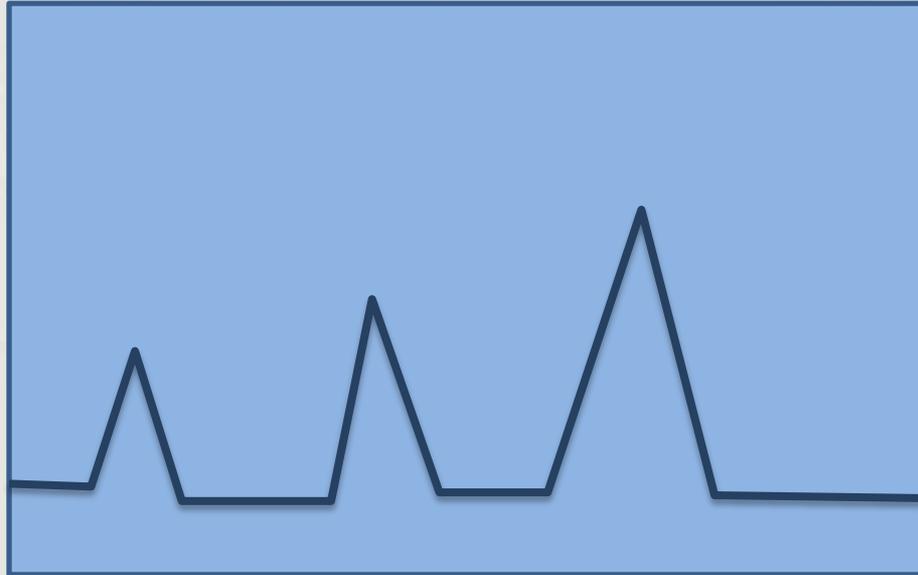
I. Blank base(normal)



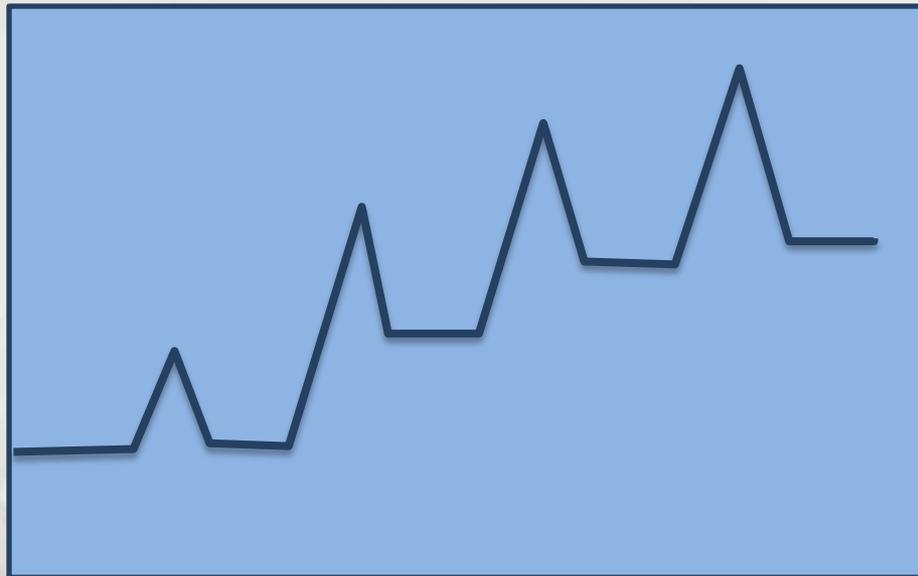
II. Noisy :

- occurs when there is contamination of mobile phase
- improper cleaning

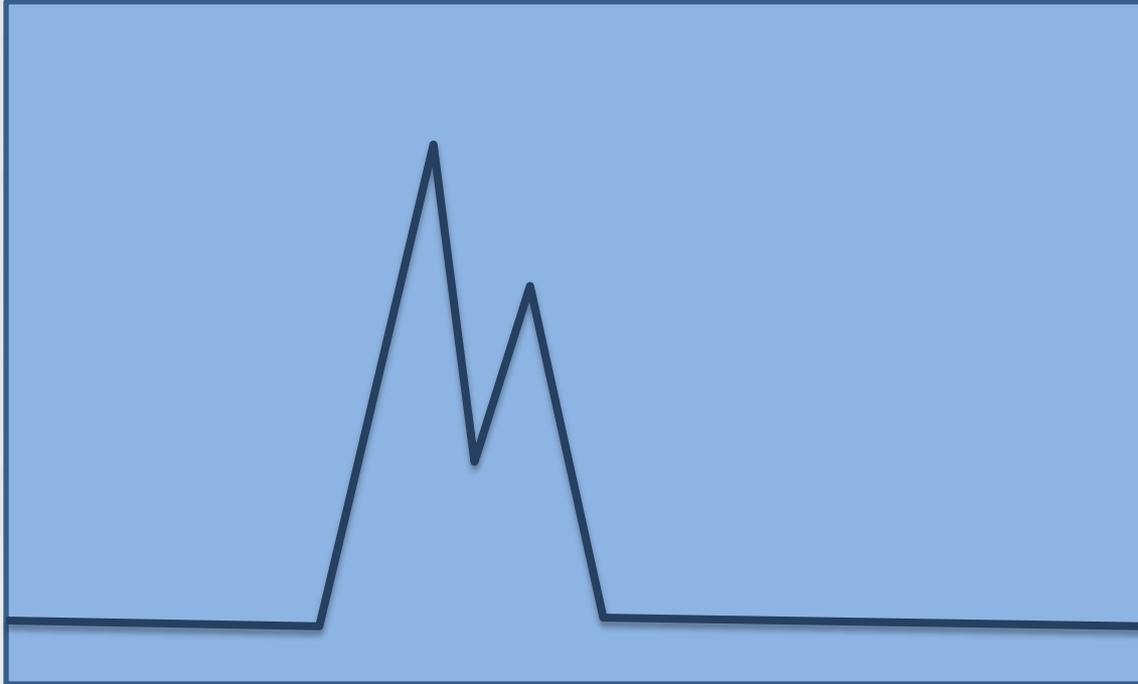
3.Drift



- Temperature is unstable
- Contamination of column



4. Peak tailing



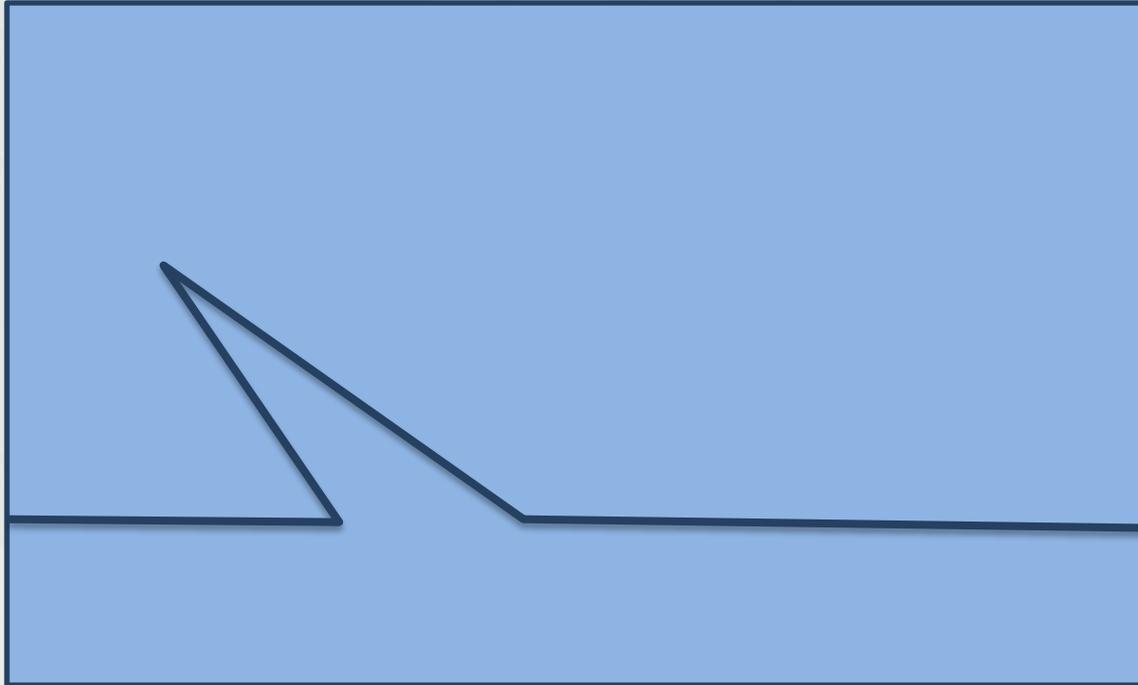
- Improper mixing of mobile phase (90% of the time)
- Problems with column (30% of the time)

After adjusting PH with buffer

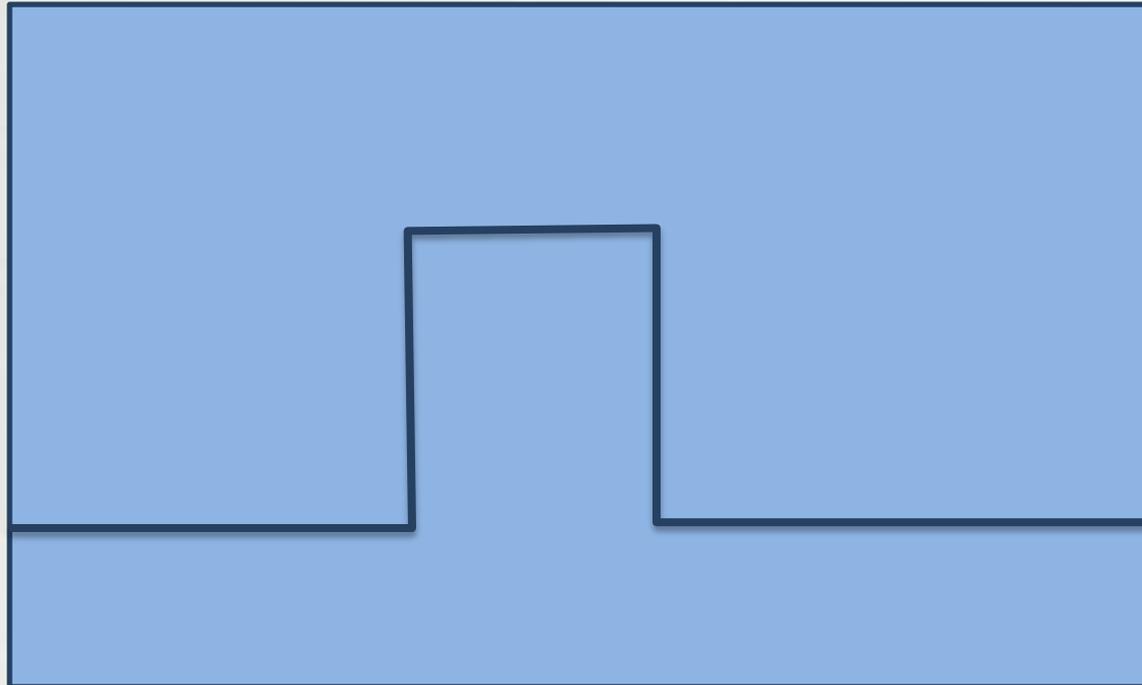


Good resolution

5. Peak fronting



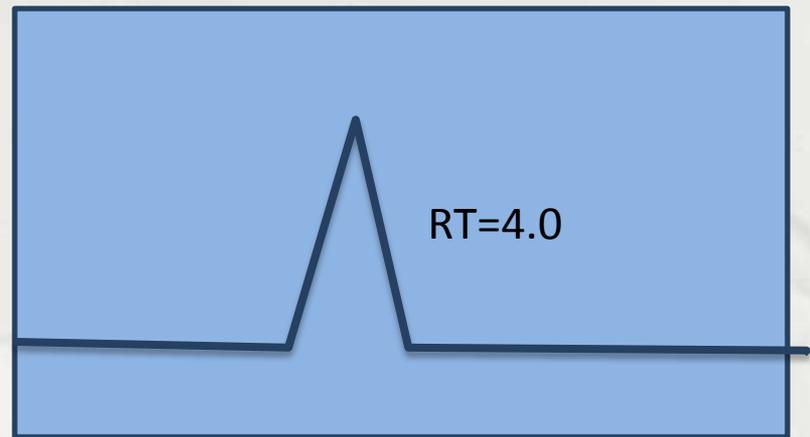
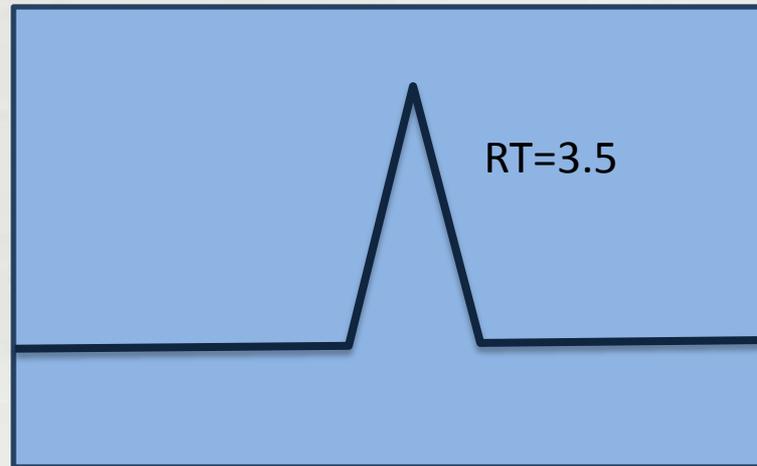
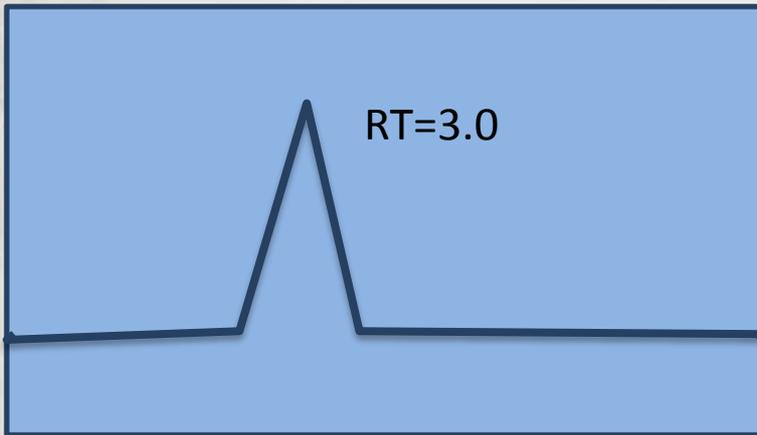
- Low temperature
- Overloading of sample
- Flow rate
- Overfilling of column
- Insufficient mixing of mobile phase



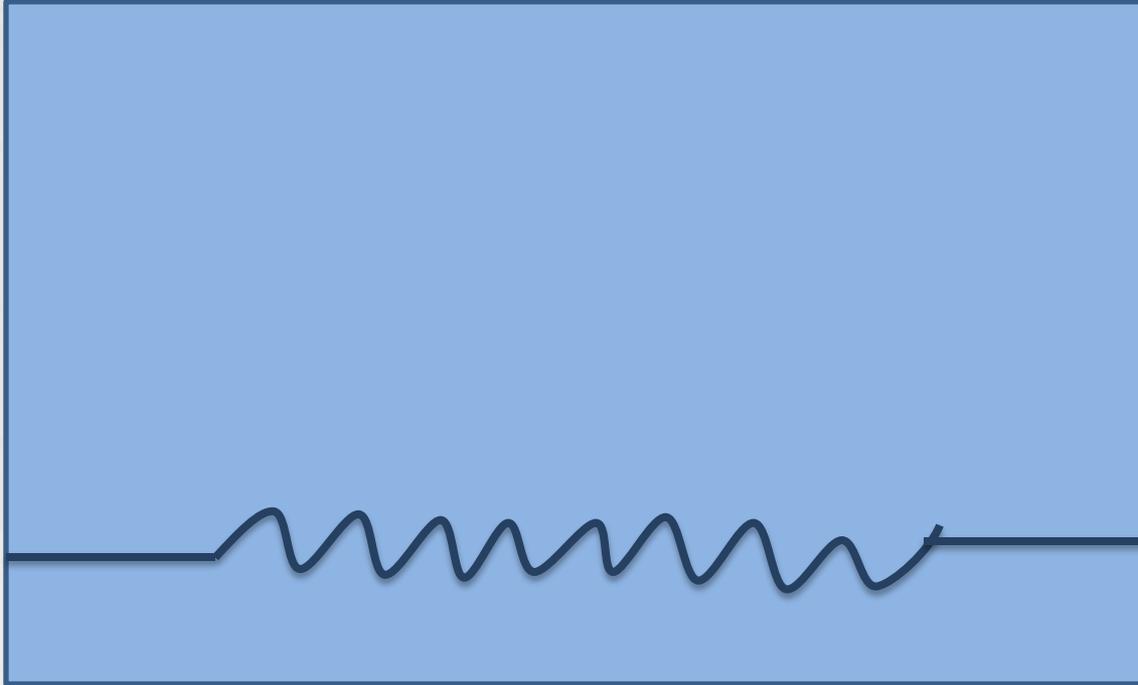
6.High concentration of sample

7.Retention time variation

Occurs due to problems with mobile phase

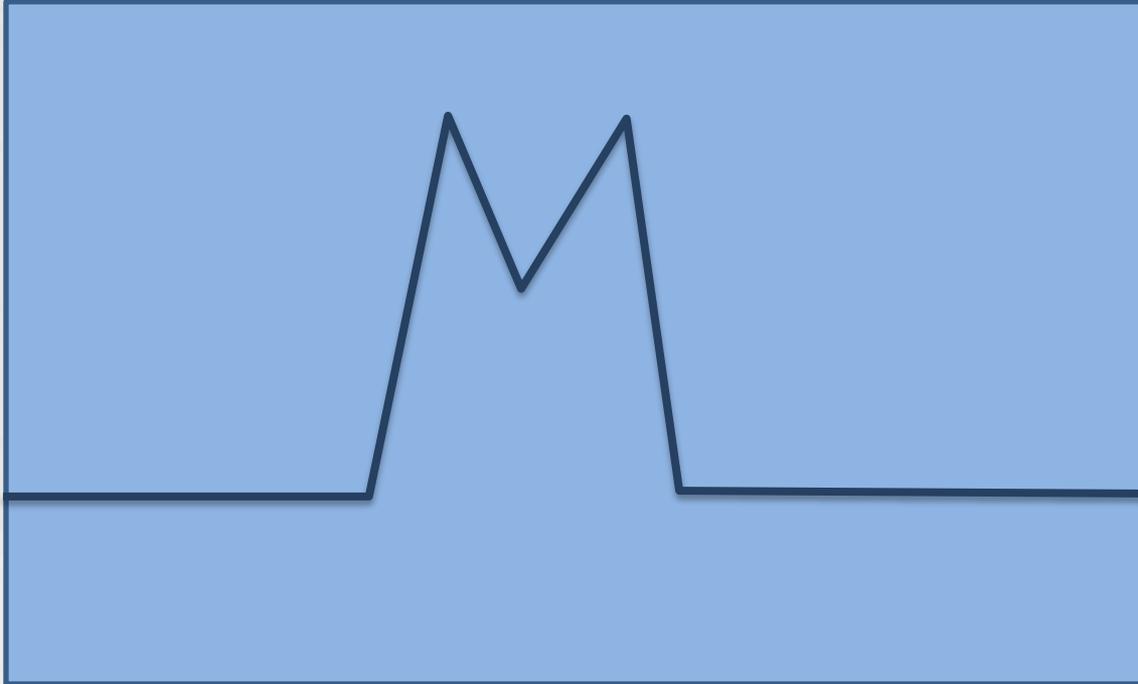


8. Communication error



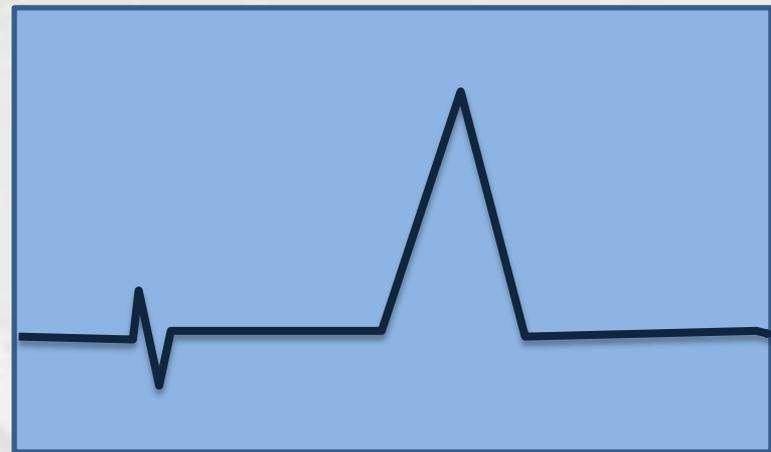
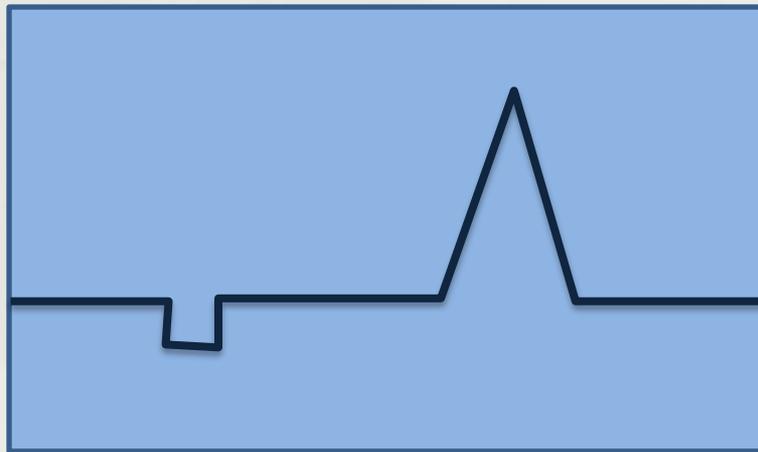
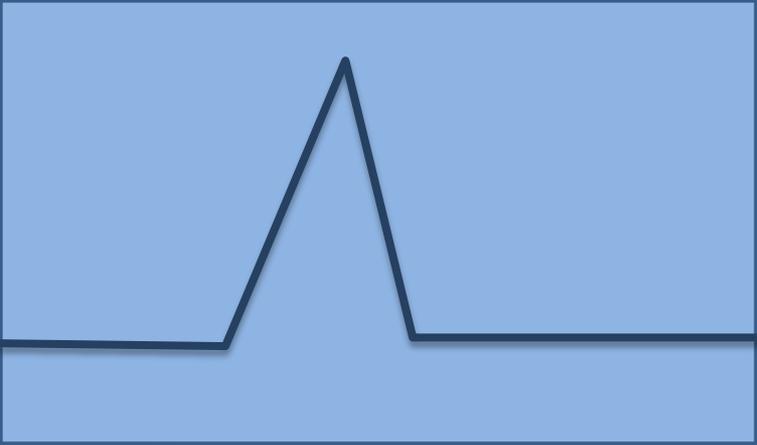
Due to problem in communication between detector and the software

9. Peak splitting



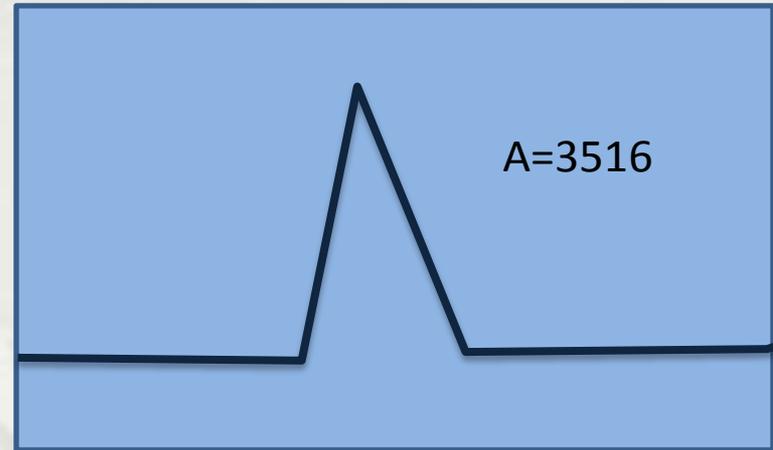
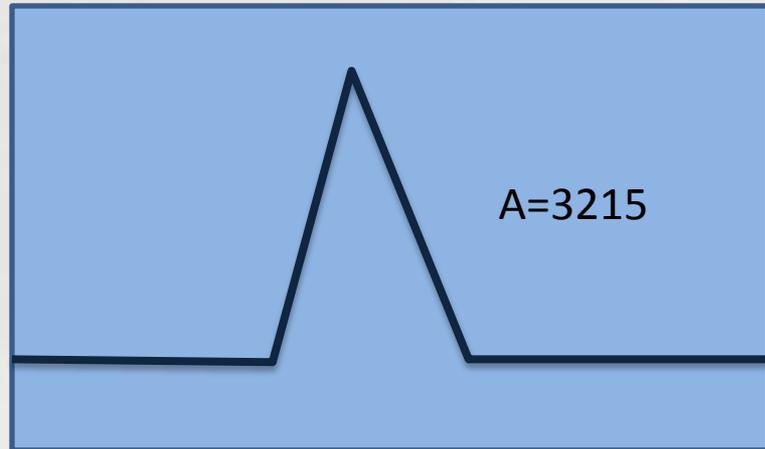
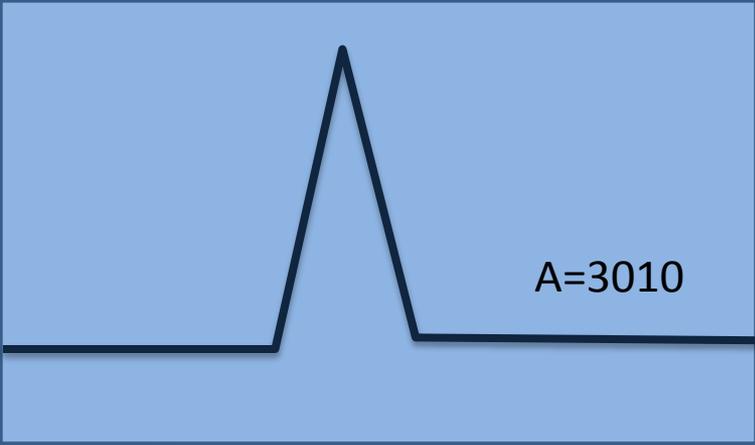
Occurs when column lifetime is diminished

10. Power fluctuation



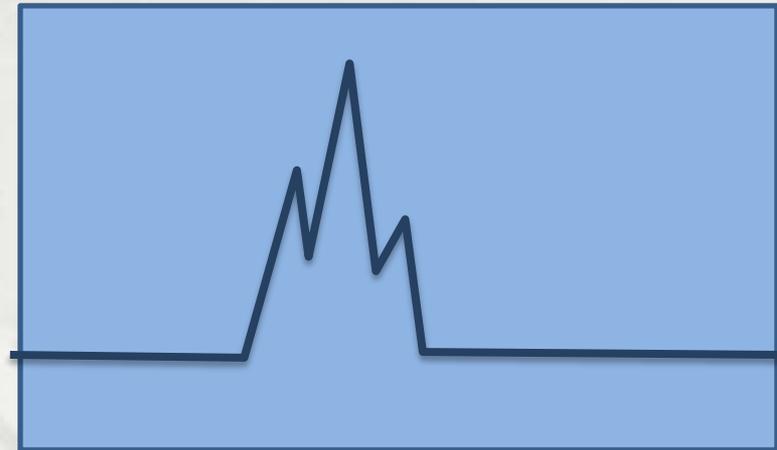
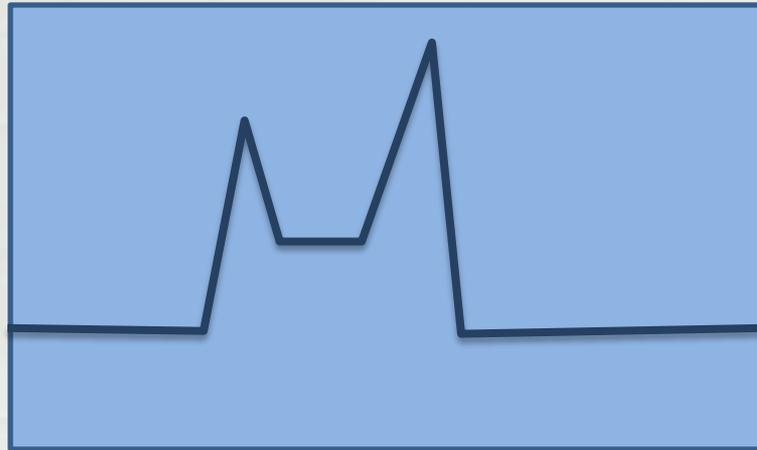
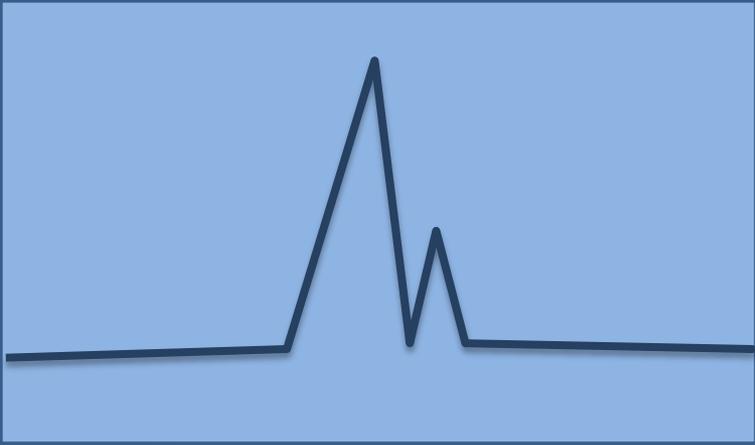
11. Variation in area

- Problem with injector
- Fluctuation in flow rate
- Error in detection

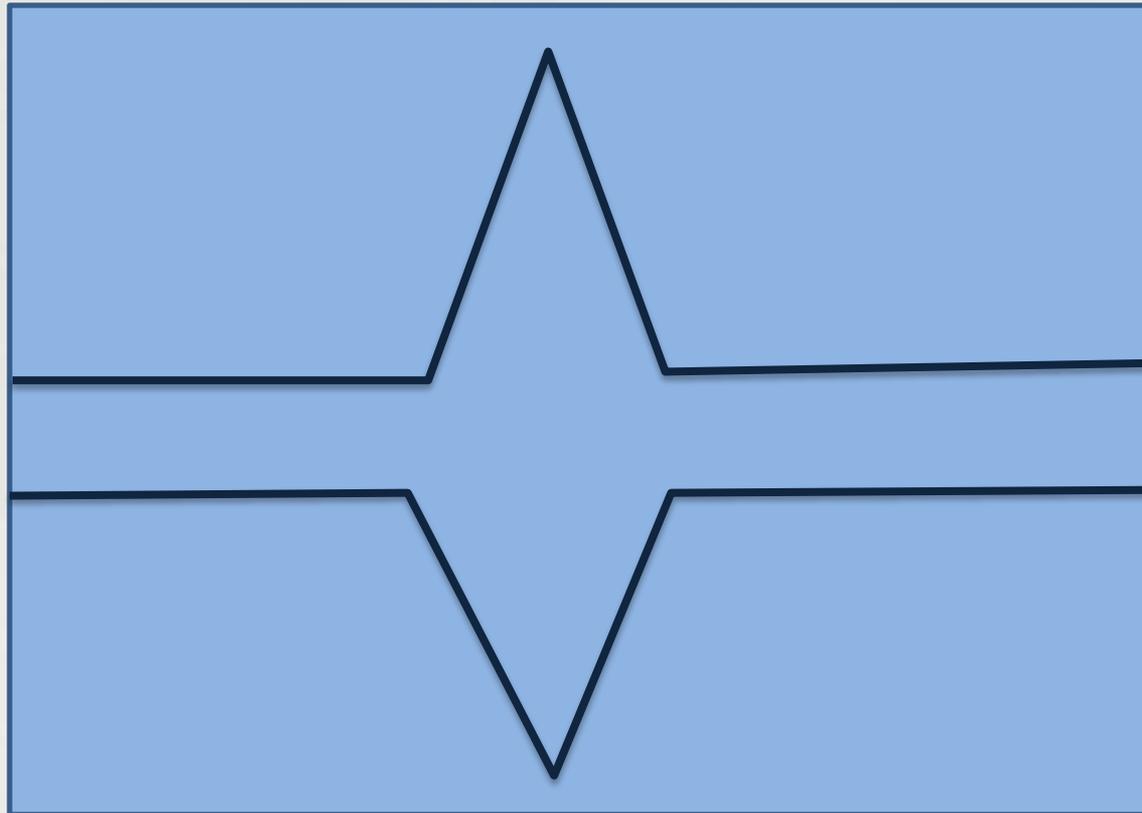


12. Ghost peaks

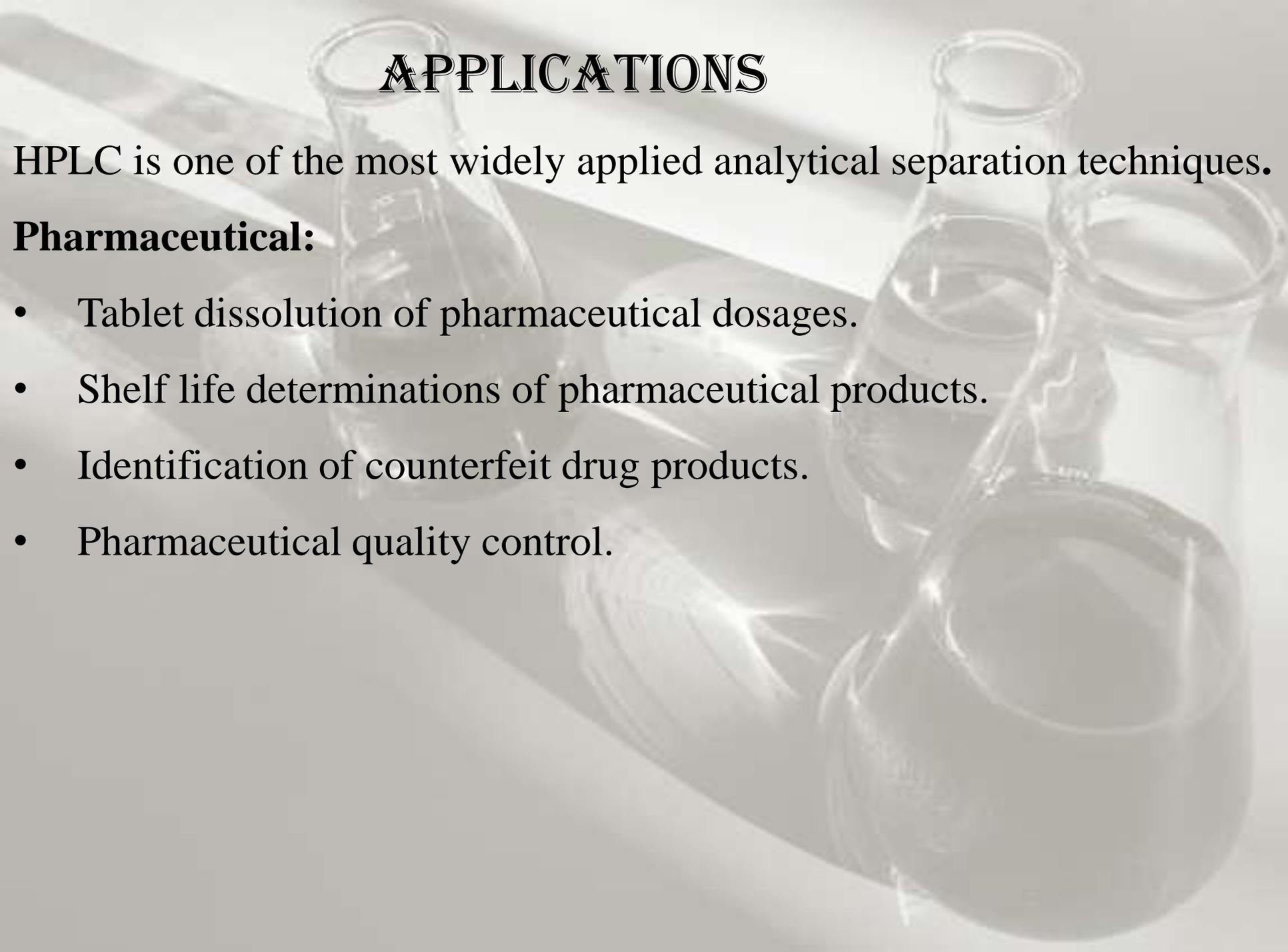
- Contamination of mobile phase
- Upset equilibrium
- Production not finished



13. Detector lifetime diminished



APPLICATIONS

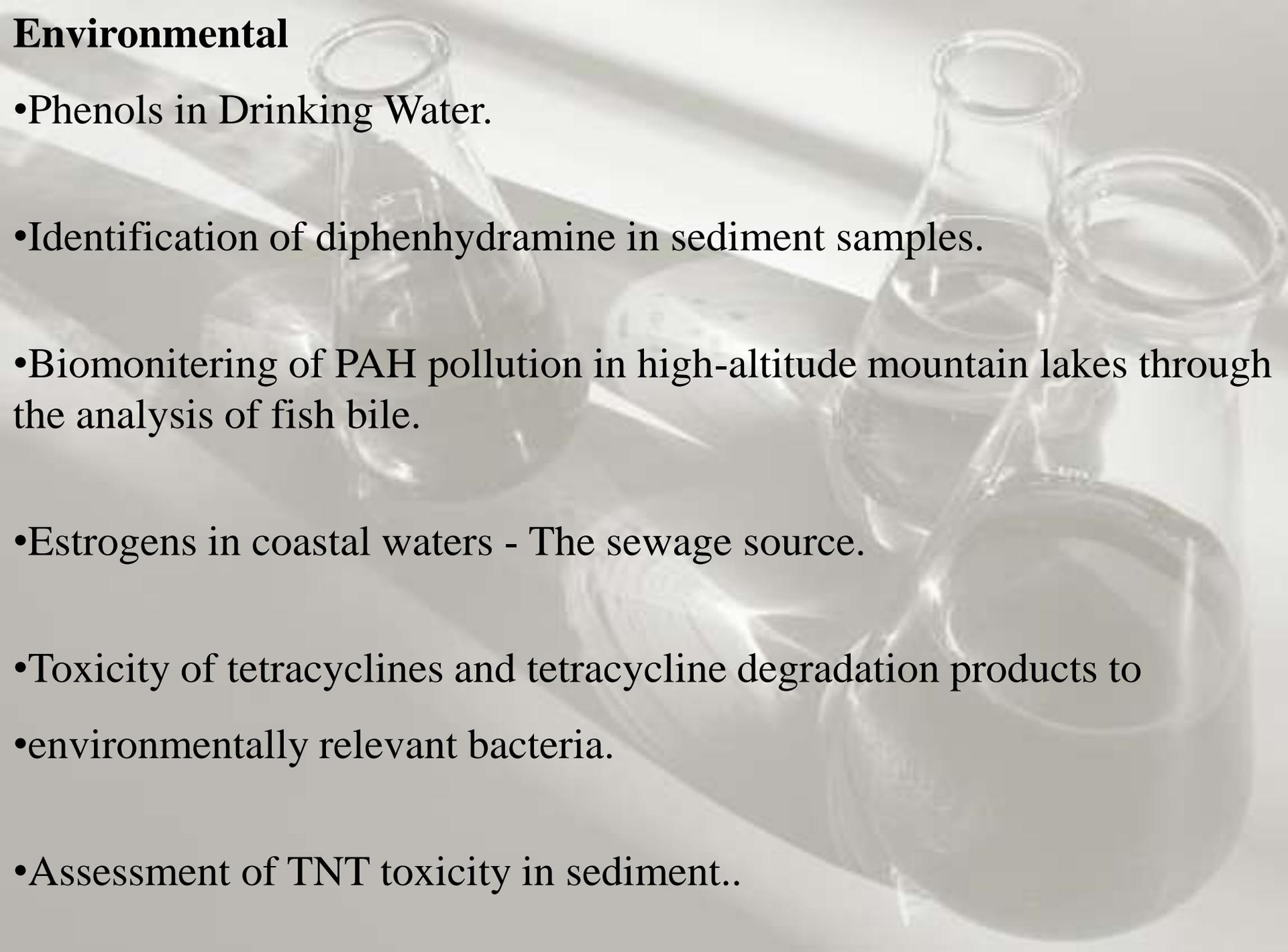


HPLC is one of the most widely applied analytical separation techniques.

Pharmaceutical:

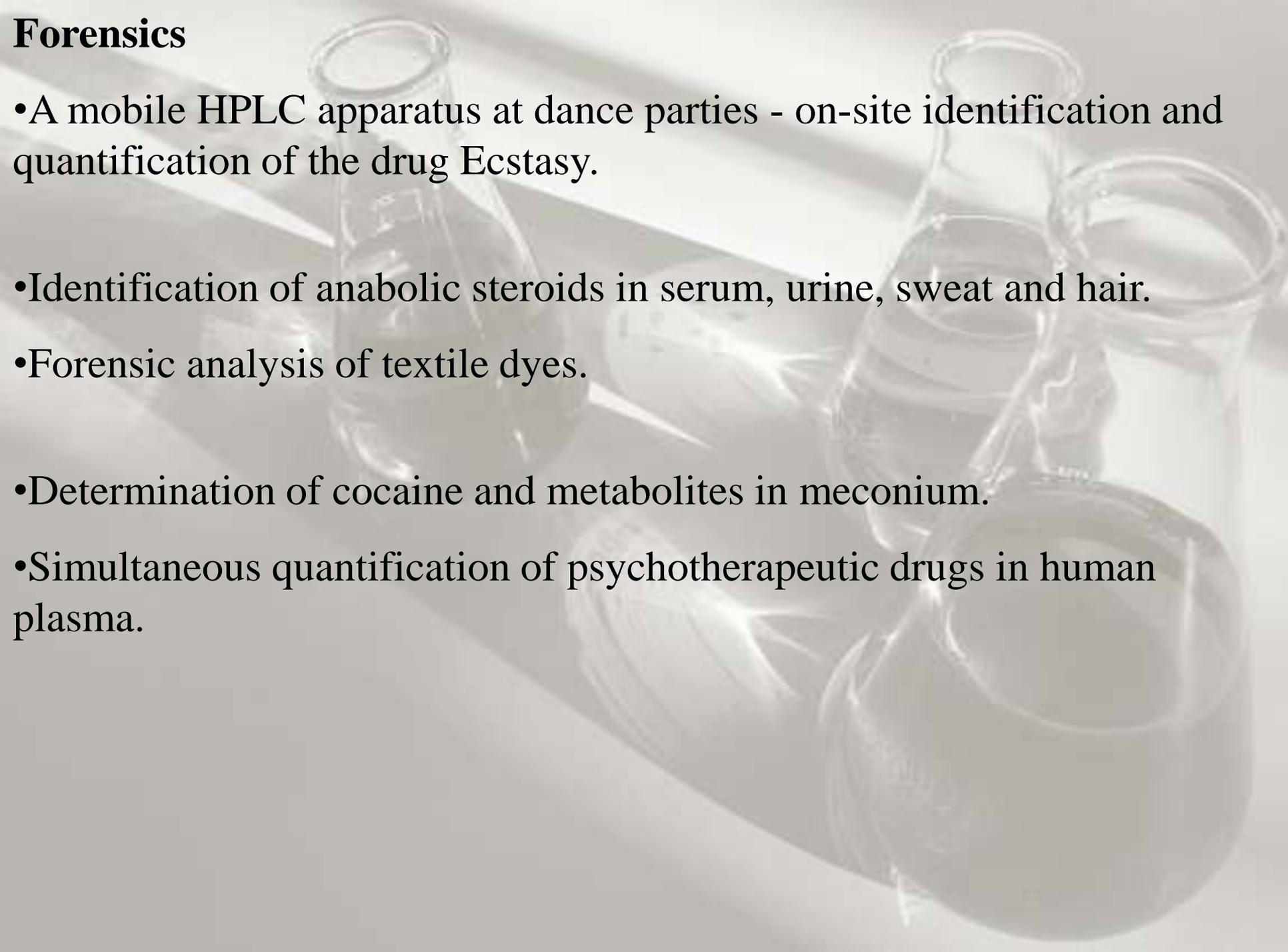
- Tablet dissolution of pharmaceutical dosages.
- Shelf life determinations of pharmaceutical products.
- Identification of counterfeit drug products.
- Pharmaceutical quality control.

Environmental



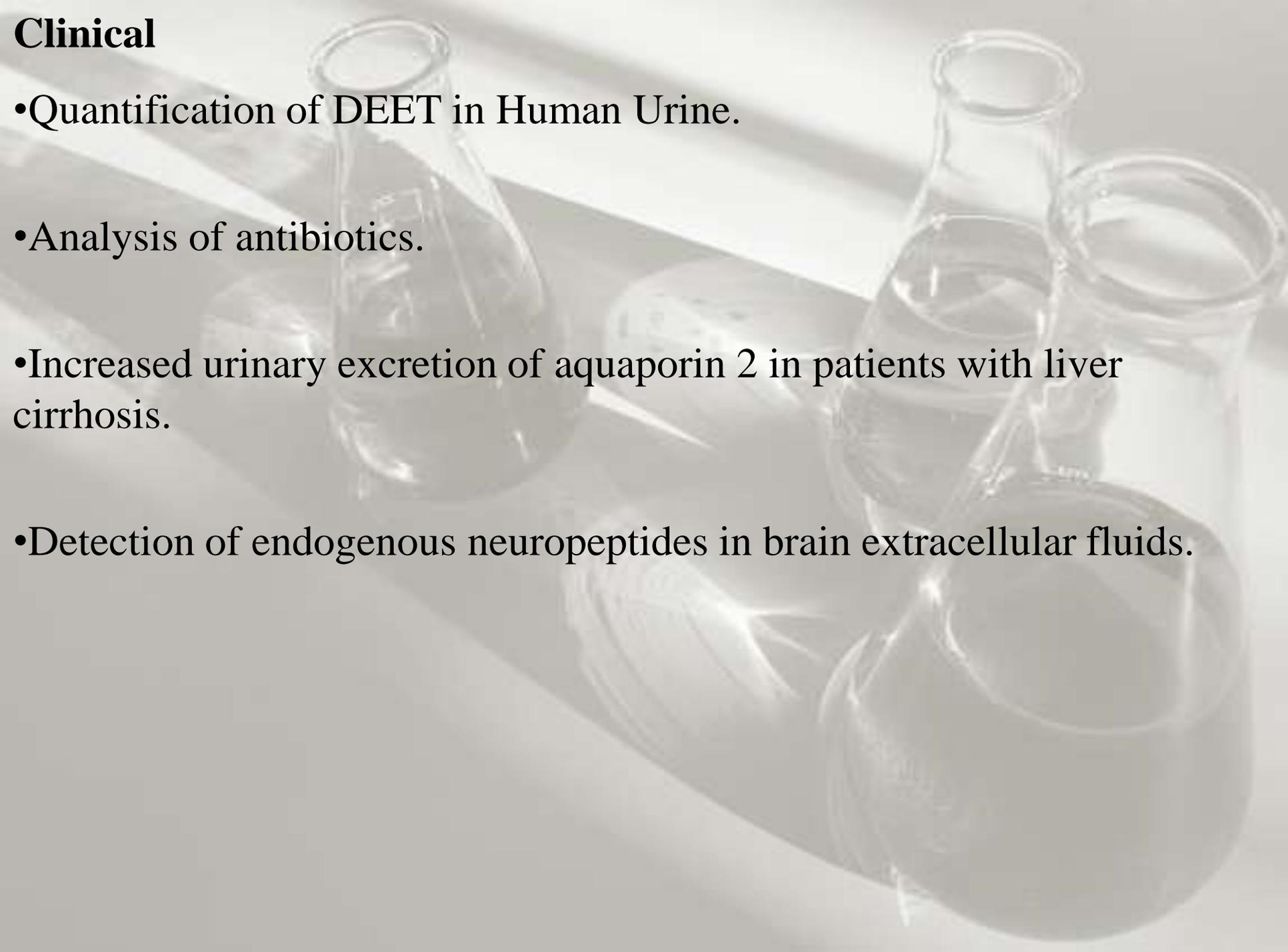
- Phenols in Drinking Water.
- Identification of diphenhydramine in sediment samples.
- Biomonitoring of PAH pollution in high-altitude mountain lakes through the analysis of fish bile.
- Estrogens in coastal waters - The sewage source.
- Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria.
- Assessment of TNT toxicity in sediment..

Forensics



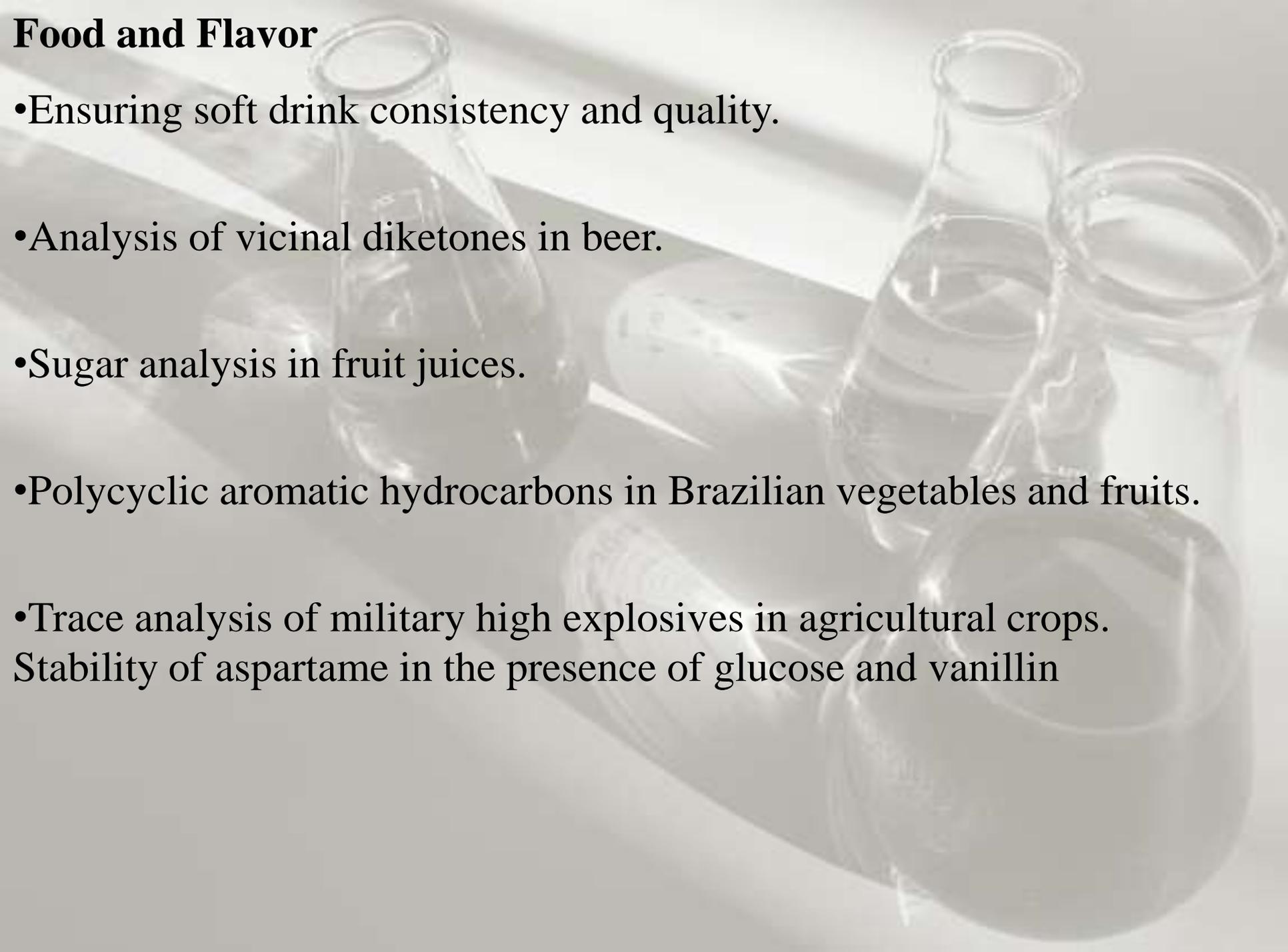
- A mobile HPLC apparatus at dance parties - on-site identification and quantification of the drug Ecstasy.
- Identification of anabolic steroids in serum, urine, sweat and hair.
- Forensic analysis of textile dyes.
- Determination of cocaine and metabolites in meconium.
- Simultaneous quantification of psychotherapeutic drugs in human plasma.

Clinical



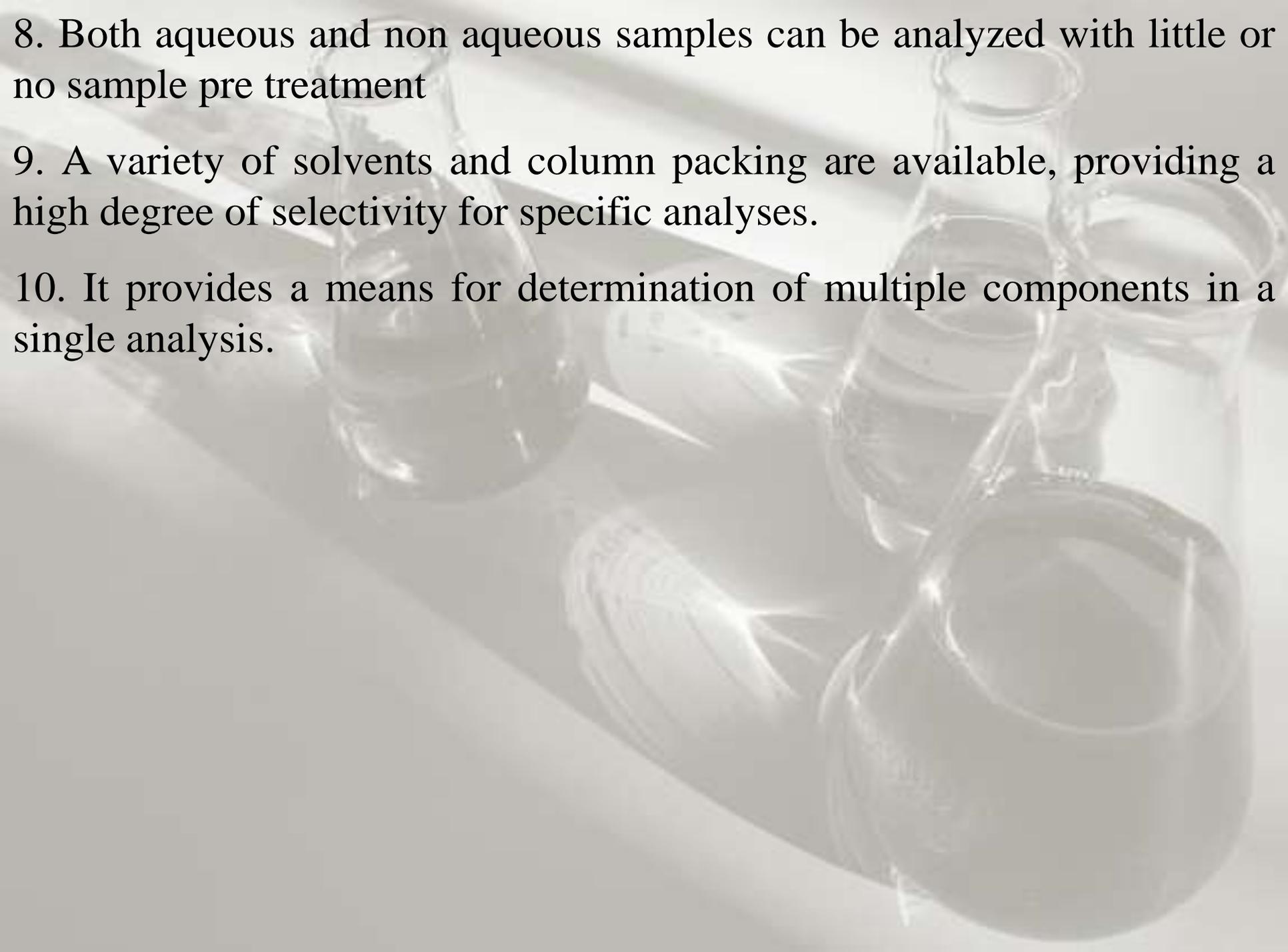
- Quantification of DEET in Human Urine.
- Analysis of antibiotics.
- Increased urinary excretion of aquaporin 2 in patients with liver cirrhosis.
- Detection of endogenous neuropeptides in brain extracellular fluids.

Food and Flavor

- Ensuring soft drink consistency and quality.
 - Analysis of vicinal diketones in beer.
 - Sugar analysis in fruit juices.
 - Polycyclic aromatic hydrocarbons in Brazilian vegetables and fruits.
 - Trace analysis of military high explosives in agricultural crops.
- Stability of aspartame in the presence of glucose and vanillin
- 

ADVANTAGES OF HPLC:

1. Separations fast and efficient (high resolution power)
2. Continuous monitoring of the column effluent
3. It can be applied to the separation and analysis of very complex mixtures
4. Accurate quantitative measurements.
5. Repetitive and reproducible analysis using the same column.
6. Adsorption, partition, ion exchange and exclusion column separations are excellently made.
7. HPLC is more versatile than GLC in some respects, because it has the advantage of not being restricted to volatile and thermally stable solute and the choice of mobile and stationary phases is much wider in HPLC

- 
8. Both aqueous and non aqueous samples can be analyzed with little or no sample pre treatment
9. A variety of solvents and column packing are available, providing a high degree of selectivity for specific analyses.
10. It provides a means for determination of multiple components in a single analysis.

THANK YOU!