HPLC

B.V.Rao

Associate Professor

Chebrolu Hanumaiah

Institute of Pharmaceutical Sciences

Concept of Chromatography

- Chromatography is an analytical method that the compounds are physically separated prior to measurement
- The main purpose of chromatography is to **separate** and **quantify** the target sample in the matrix
- Column Chromatography was introduced by **Mikhail T Svet**
- HPLC was introduced by **Joseph J Kirkland**

HPLC

High Pressure Liquid Chromatography

- High pressure to be able to use small particle size to allow proper separation at reasonable flow rates

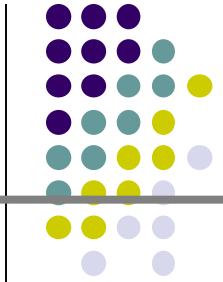
High Performance Liquid Chromatography

- High performance due to its reproducibility

Currently Refers to:

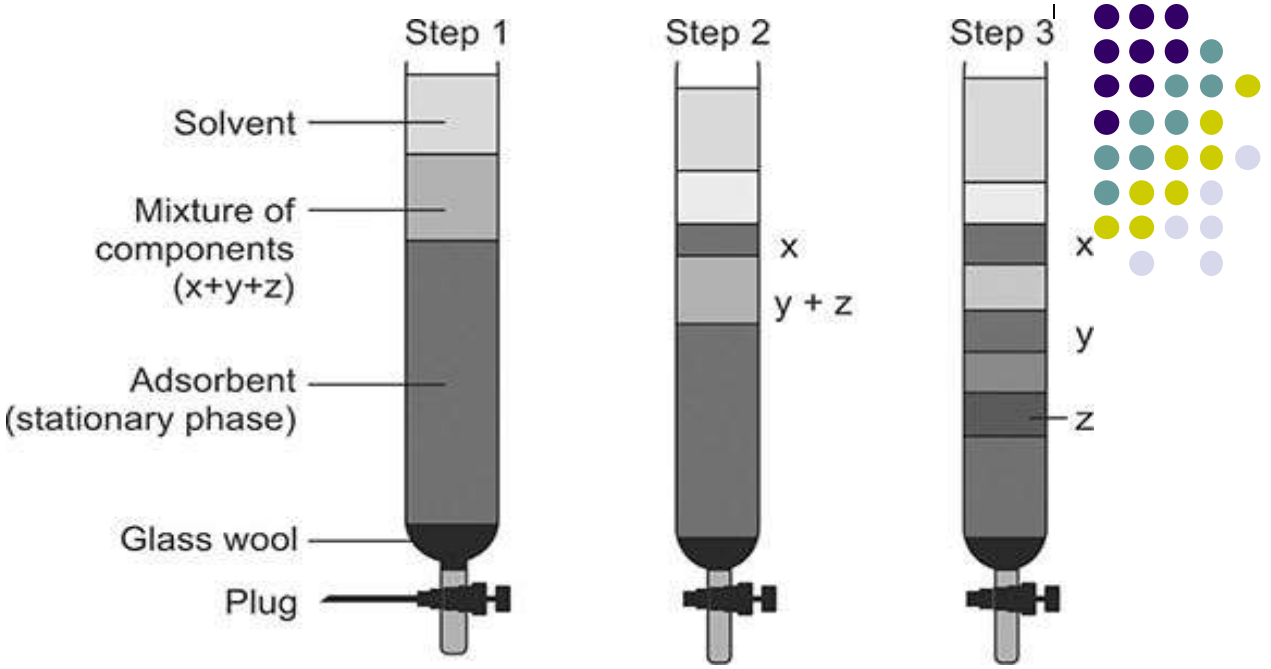
High Precision Liquid Chromatography

Why use HPLC?



- Simultaneous Analysis
- High Resolution
- High Sensitivity (ppm-ppb)
- Good Repeatability
- Small Sample Size
- Moderate Analysis Condition
- No Need to Vaporize the Sample Like GC
- Easy to Fractionate the Sample and Purify
- Non Destructive for Many Detectors

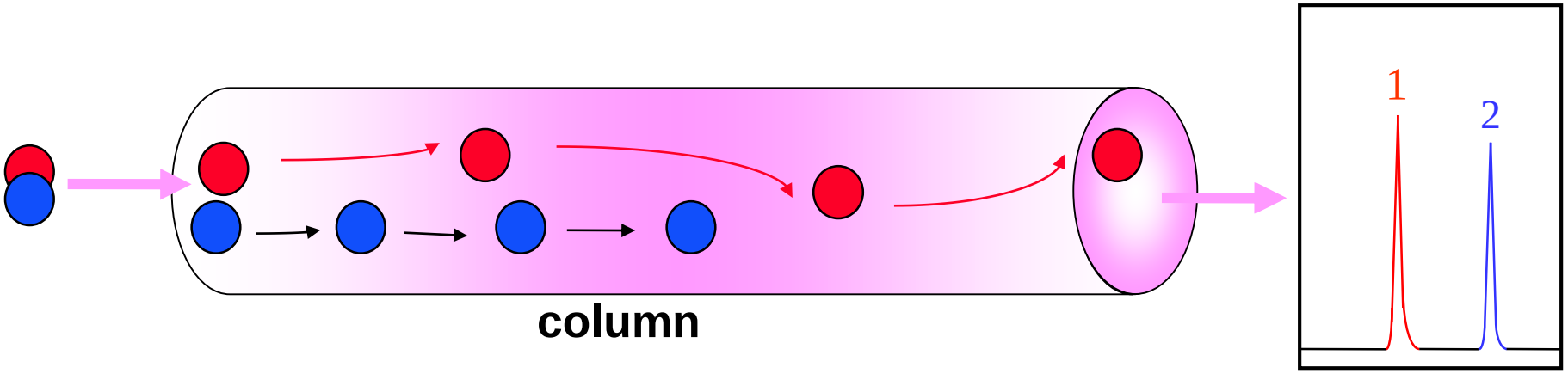
Introduction



- Compounds are separated by injecting a sample mixture onto the column. The different component in the mixture pass through the column at different rates due to differences in their partition/ adsorption behavior between the mobile phase and the stationary phase.

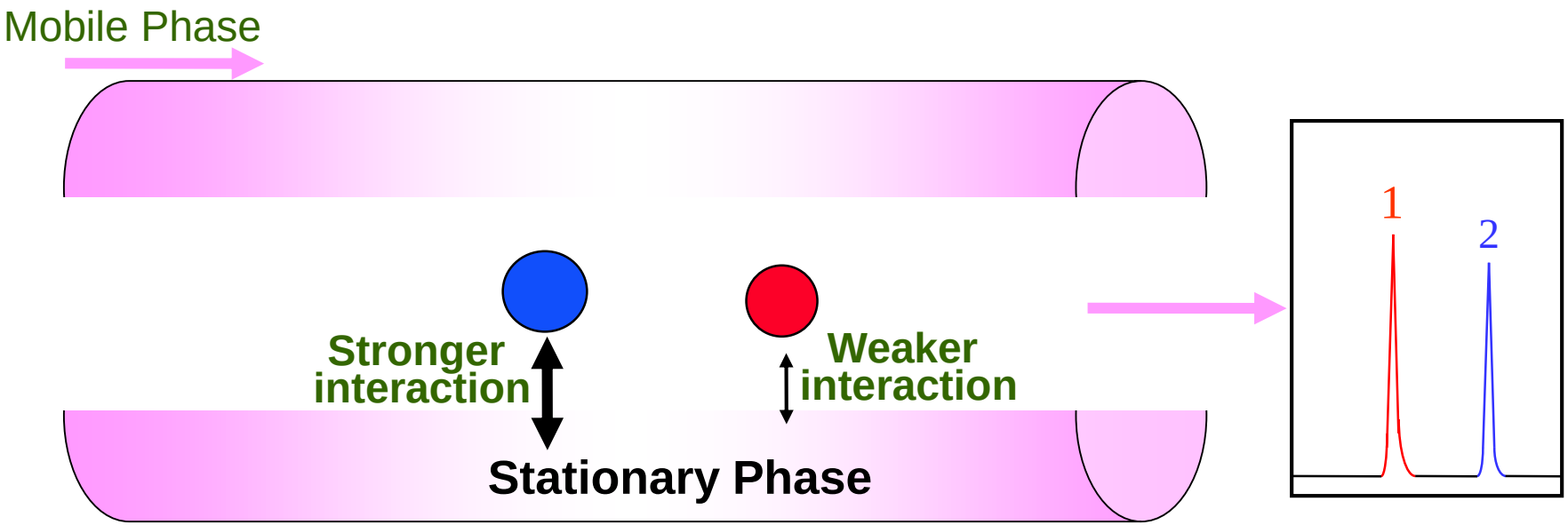
Separation Mechanism

Compounds are separated because the molecules move at different rates in the column.



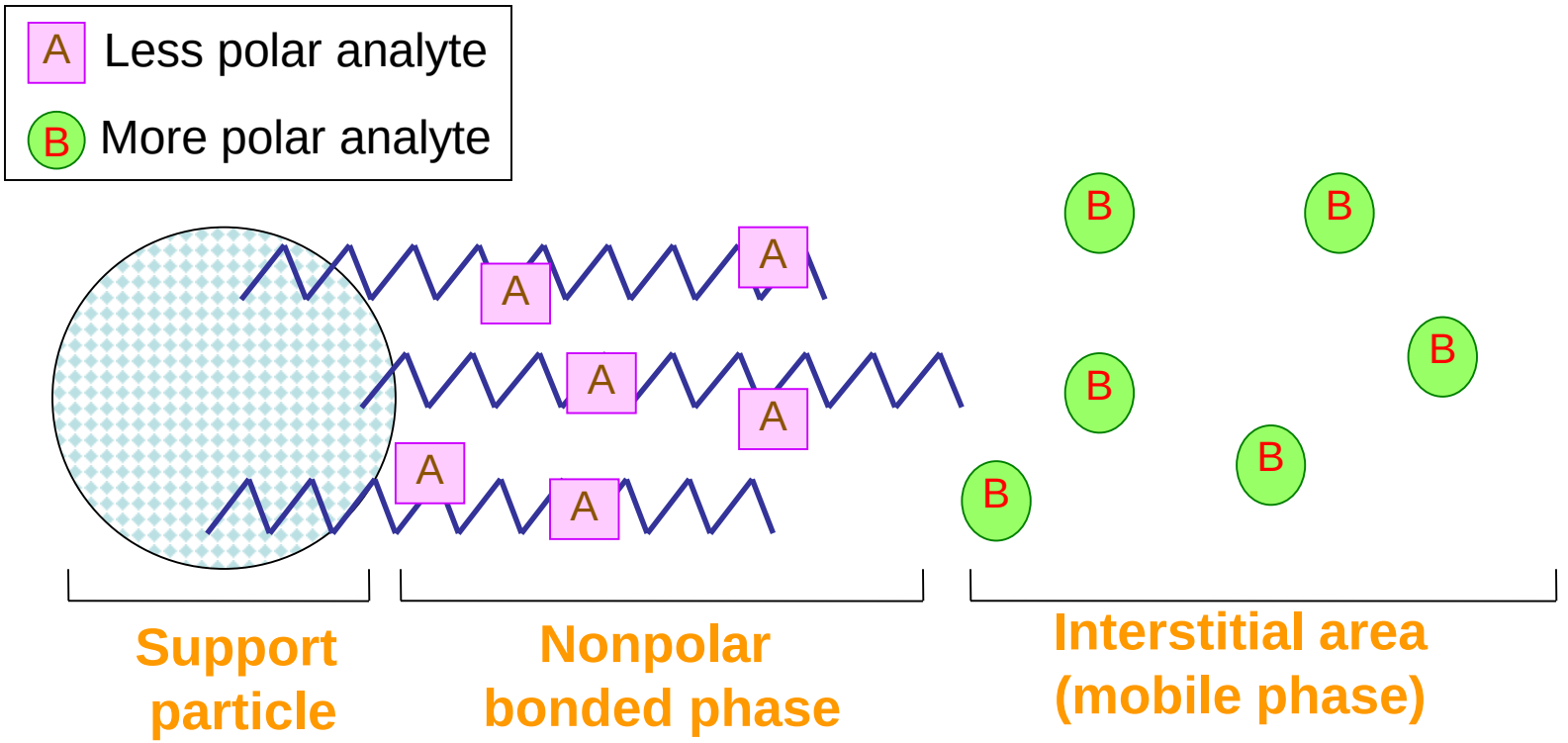
Separation Mechanism

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



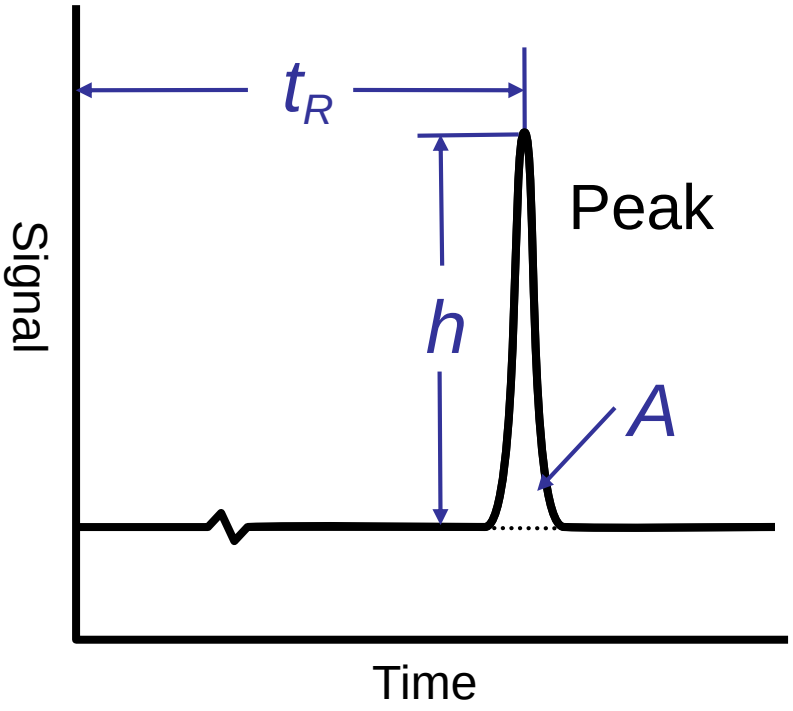
What Is the Interaction?

Hydrophobic Interaction



Less polar (more hydrophobic) analytes are more attracted and spend more time associated with the hydrophobic bonded phase, therefore, they are eluted last.

Chromatogram



t_R : Retention time

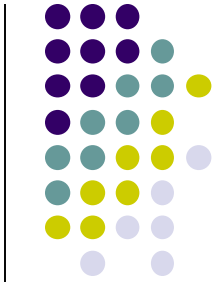
A : Area

h : Height

Some Important Terms

- **Chromatogram:** A plot of detector signal output versus time or elution volume.
- **Mobile phase:** The liquid that moves the solute through the column.
- **Stationary phase:** The packing material of the column, which is the immobile phase involved in the chromatographic process.
- **Peak:** The visual representation on the chromatogram based on the detector's electrical response due to the presence of a sample component inside the flow cell.
- **Retention time:** The time taken by the analyte peak to reach the detector after sample injection.
- **Qualitation:** An analysis process which is designed to identify the components of a substance or mixture.
- **Quantitation:** An analysis process which is designed to determine the amounts or proportion of the components of a substance.

Types of HPLC Techniques



Based on modes of Chromatography

- Normal Phase mode
- Reverse Phase mode

Based on principle of separation

- Adsorption – solid-liquid, Silica, Alumina
- Partition – Liquid-liquid, PEG coated on silica

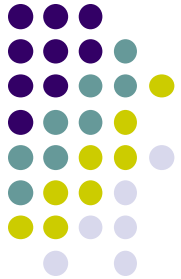
Based on elution technique

- Isocratic separation – Same Composition
- Gradient Separation – Different Composition

Dis-adv : **Time taking for reconditioning for next run**
Base line disturbance because of different solvents

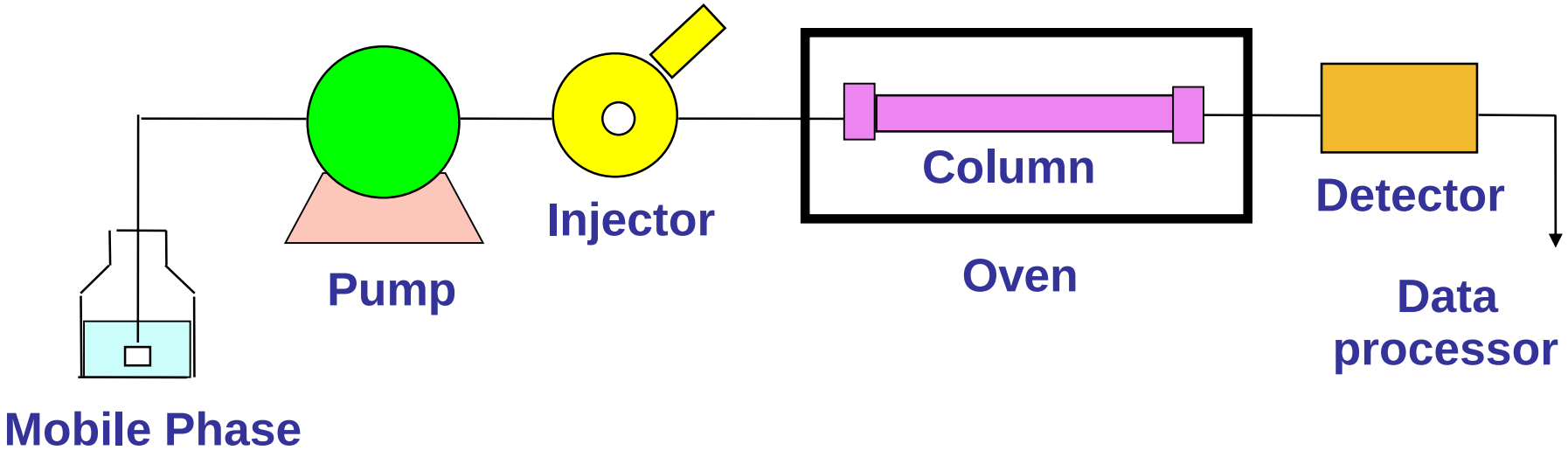
Based on Scale of operation

- Analytical HPLC
- Preparative HPLC

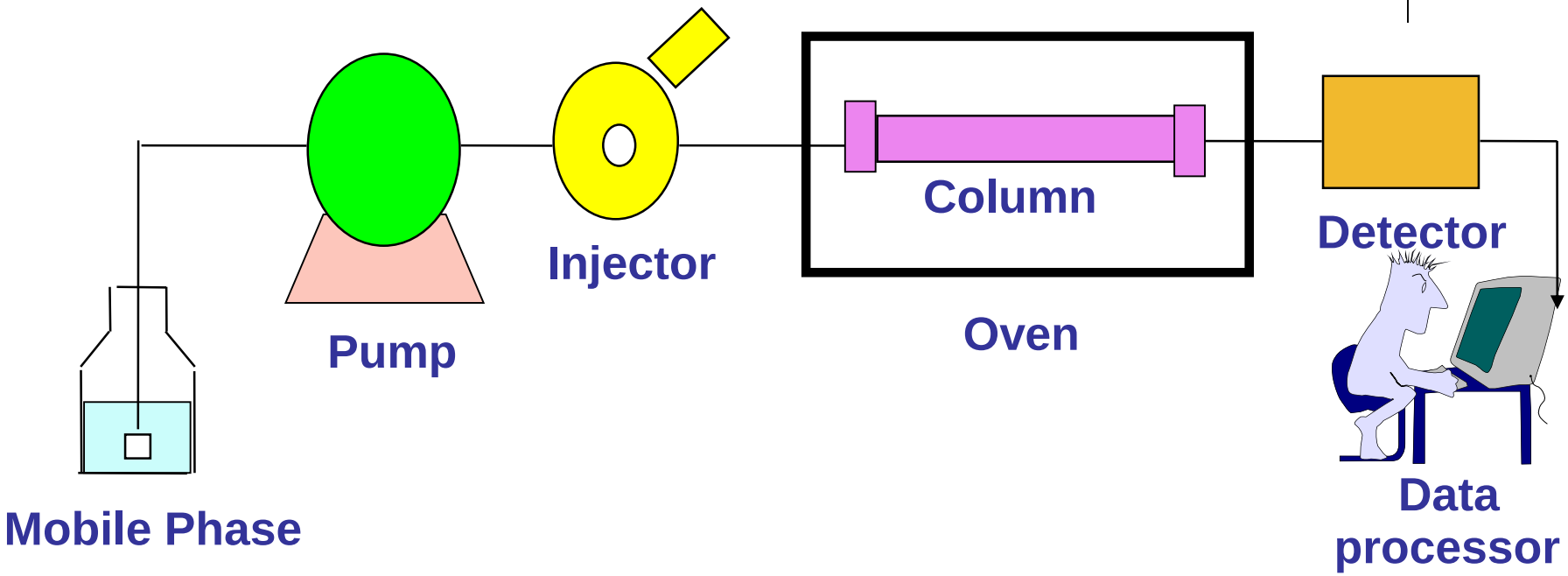


INSTRUMENTATION

Flow Diagram of HPLC



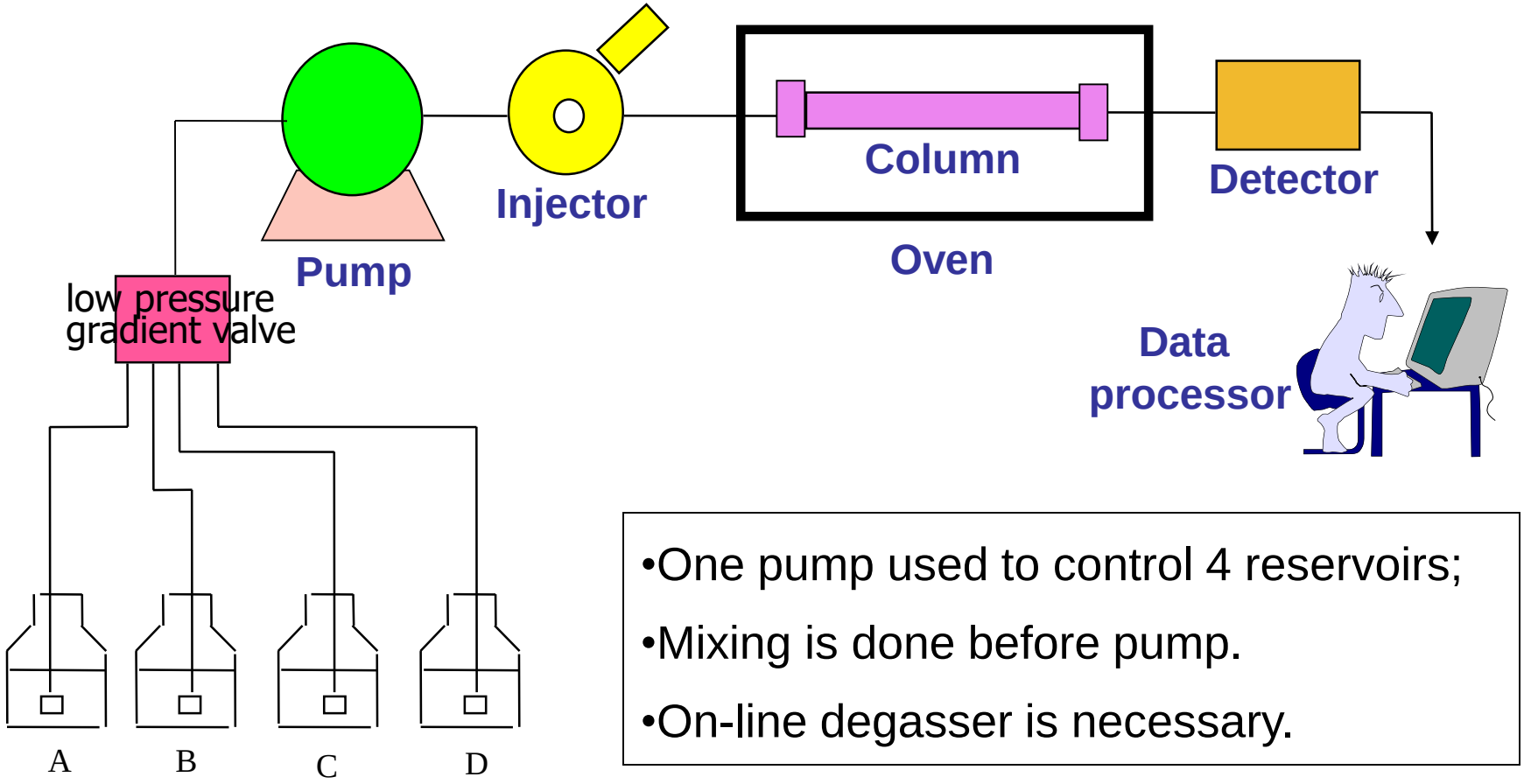
Isocratic System



Simple system with one pump and one solvent reservoir.

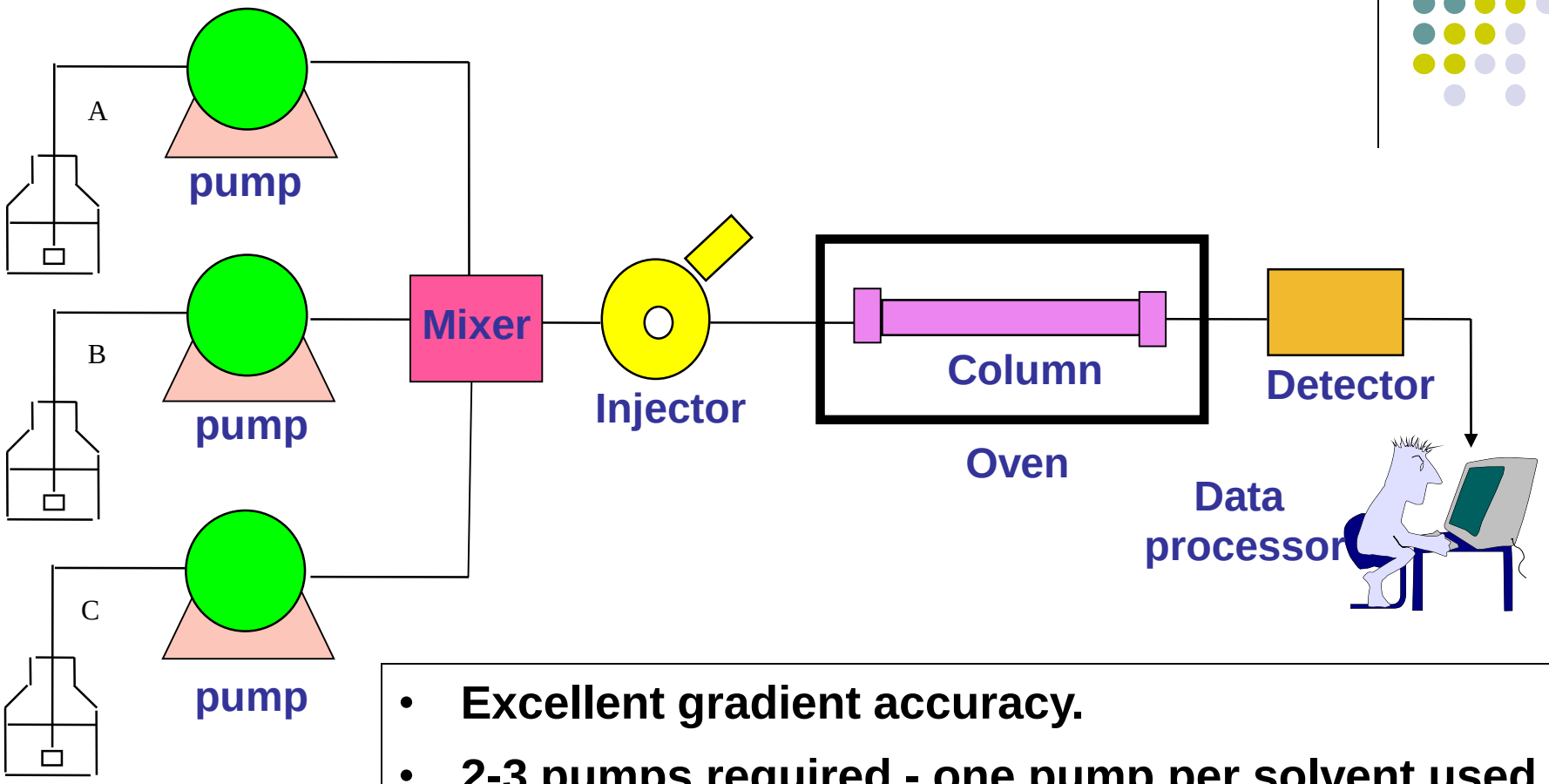
If more than one solvent is used, solvents should be premixed.

Low-pressure Gradient System



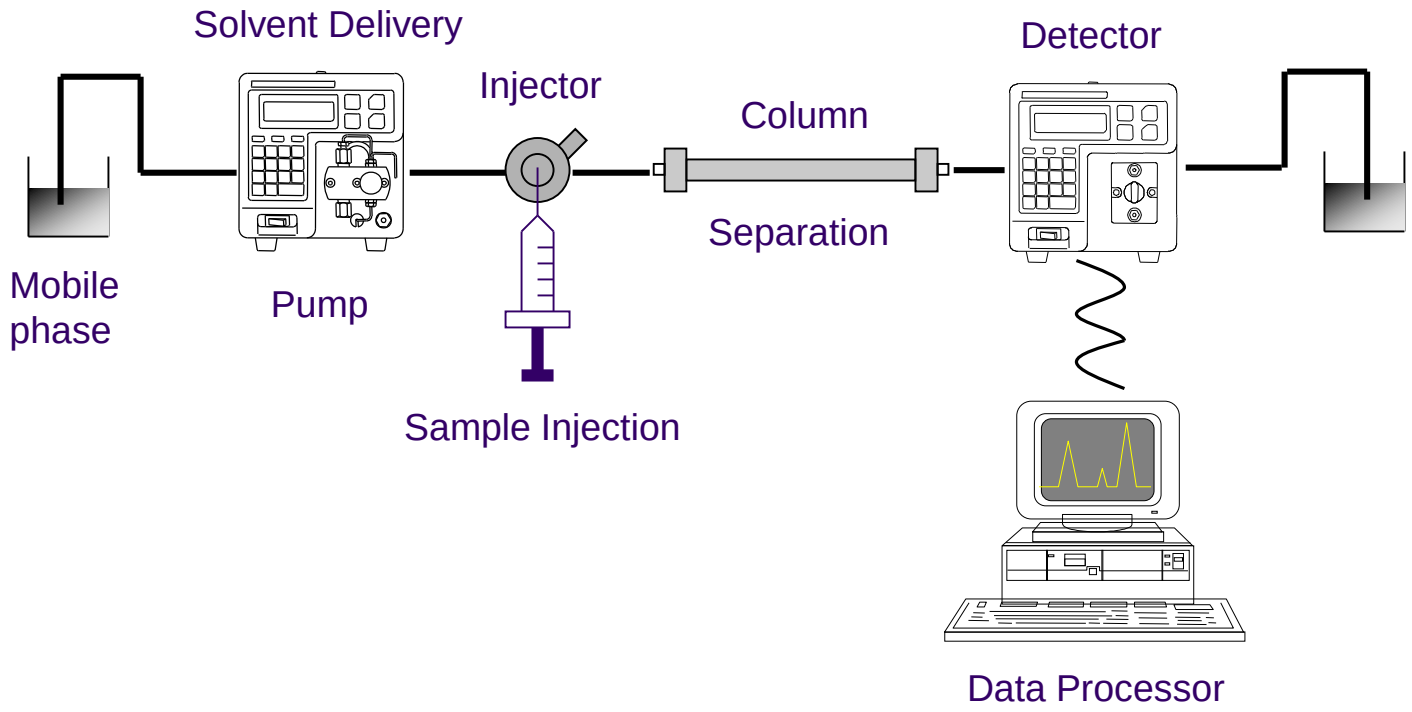
- One pump used to control 4 reservoirs;
- Mixing is done before pump.
- On-line degasser is necessary.

High-pressure Gradient System



- **Excellent gradient accuracy.**
- **2-3 pumps required - one pump per solvent used.**
- **On-line degassing may not be critical.**
- **The sum of the flow rates delivered by each pump is maintained constant**

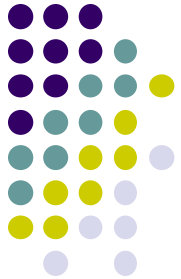
HPLC Basic Instrumentation



Components of A Liquid Chromatography System

- Mobile Phase / Solvent Reservoir
- Degasser
- Solvent Delivery System (Pump)
- Injector
- Precolumn
- Column
- Temperature Control
- Detectors
- Recorder (Data Collection)

The Mobile Phase in HPLC



- **Must do the following:**
  - **solvate the analyte molecules and the solvent they are in**
  - **be suitable for the analyte to transfer "back and forth" between during the separation process**

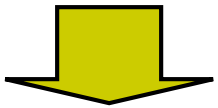
- **Must be:**
  - **Compatible with the instrument (pumps, seals, fittings, detector, etc)**
  - **Compatible with the stationary phase**
  - **Readily available (often use liters/day)**
  - **Adequate purity**
  - **Free of gases (which cause compressability problems)**
  - **Low viscous – methanol than ethanol**

Mobile Phase for Reversed Phase HPLC

- Water / buffer + Organic solvent
  - **Organic solvents:**
  - Methanol
  - Acetonitrile
  - THF
  - **Buffer:**
  - Phosphate buffer
  - Acetate buffer
  - Ammonia buffer
- Ratio of aqueous and organic solvents is important

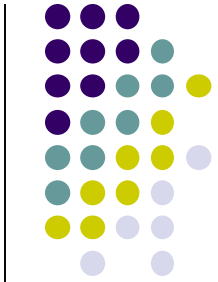
Degasser

- **Problems caused by dissolved air(O_2, N_2)in mobile phase (Deaerated mobile phase)**
  - Unstable delivery in pump
  - Bigger noise and large baseline-drift in detector cell



- **In order to avoid causing the problems, mobile phase should be degassed.**
  - vacuum pumping systems
  - distillation system
  - a system for heating and stirring the solvents
  - sparging system – Passing an inert gas of low solubility through the solvent

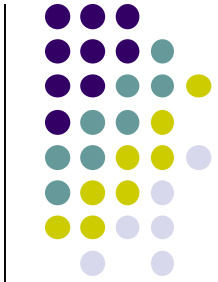
PUMPS



Four basic types of LC Pumps are:

- Pneumatic pumps – Preparative purpose only
- Motor driven syringe type pumps
- Reciprocating pumps
- Hydraulic amplifier pumps – not in use

Motor driven syringe type pumps



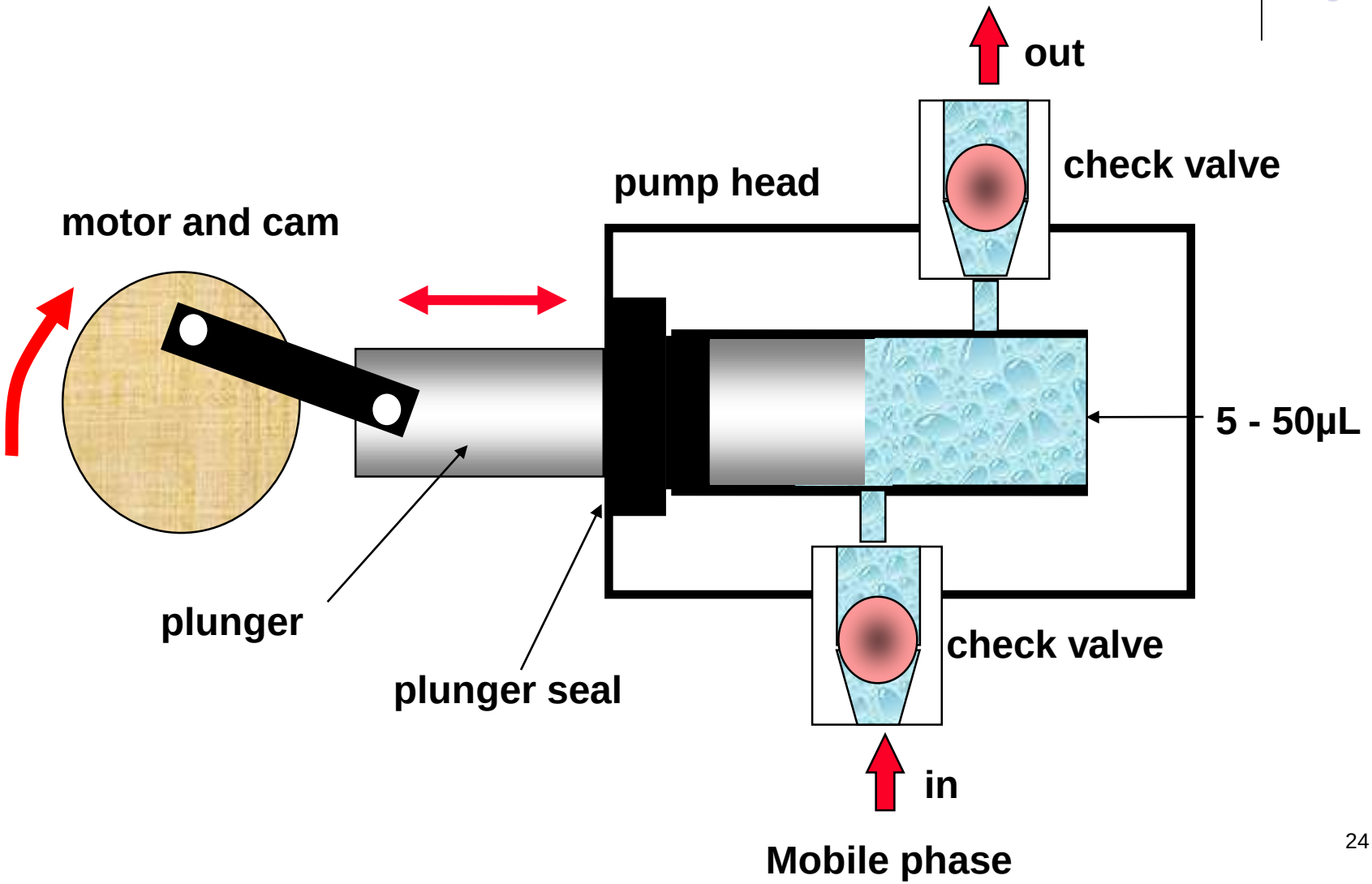
Works on the principle of positive solvent displacement
Double syringe pumps are available:

one for column

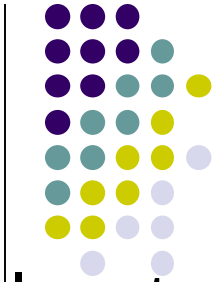
one from reservoir

- **Advantages:**
  - Simple
  - Inexpensive
  - Pulse free
  - Stable flow rate
  - Lowest dead volume
- **Disadvantages**
  - Limited capacity
  - Not suitable for gradient elution

Plunger Reciprocating Pump



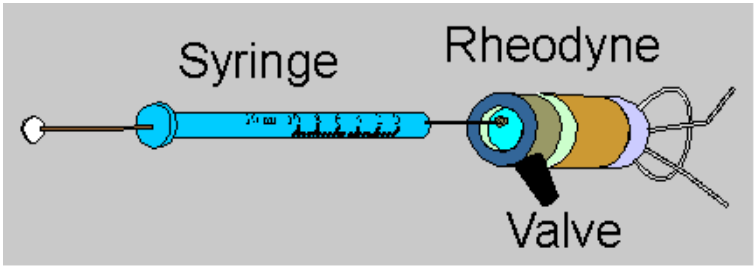
Plunger Reciprocating Pump



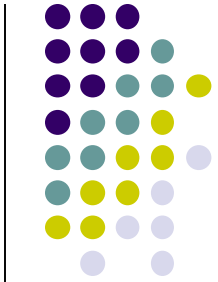
- Consists of a small chamber in which the solvent is pumped by the back and forth motion of a motor-driven piston
- Advantage
  - Low pressure fluctuation
  - Very easy to replace other solvent
- Disadvantage
  - Change the plunger seal
  - Flow variation
  - Small volume of solvent delivery is possible

Sample Injection Systems

- Convenient to use
- For injecting the solvent through the column
- Minimize possible flow disturbances
- Volumes must be small
- .1-500 μL
- Sampling loops
  - interchangeable loops (5-500 μL at pressures up to 7000 psi)
  - Chemically inert
  - Reproducible



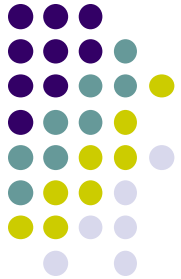
Different ways of sample injection



- **Fixed volume valve injection**
  - First HPLC sample injection system
  - Valve loop is filled with sample
  - Reproducible sample amounts can be injected
- **Variable injection valve injection**
  - Flow restrictors are used b/w pump-column
- **On column injection**
  - Injected by means of syringe through septum
  - Simple method of injection
  - Dis-adv :Leaching effect of the mobile phase in contact with septum , leads to a ghost peak


1.5
1.0
0.5ml
1.5
1.0
0.5ml
1.5
1.0
0.5ml

Peak broadening depends on



- Type of injection system used
- Connection between injector and column
- Injection volume and time taken for injection

- Commonly used Injectors

Syringe injection - Trouble is more

- Peak broadening

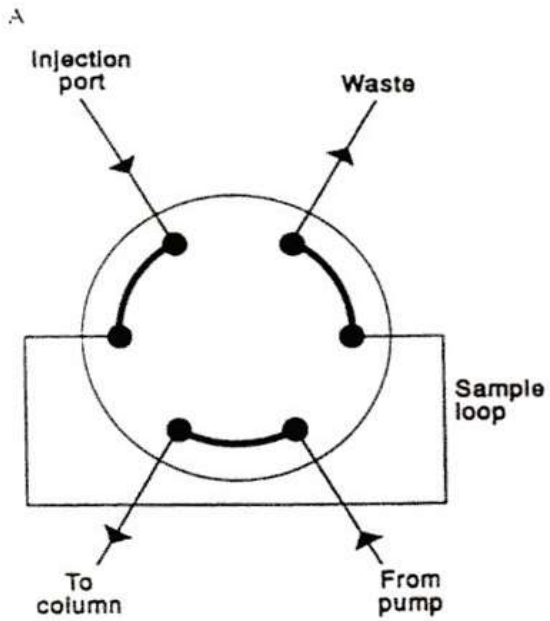
Valve injection – Automation is possible

-- Highly précised one

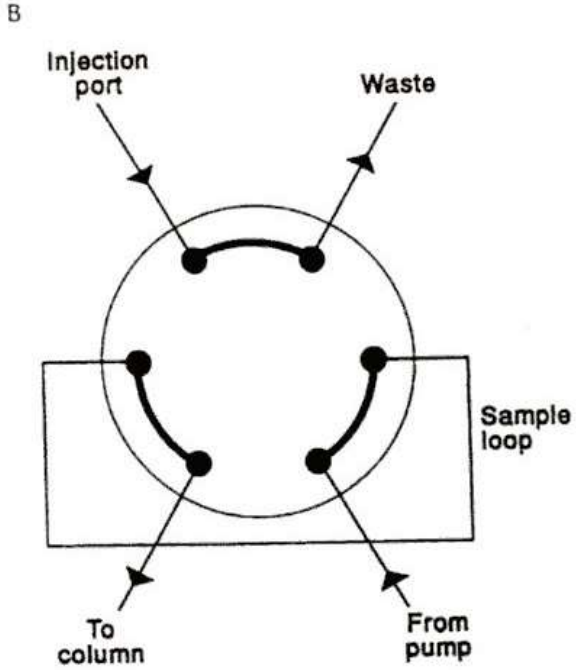
-- Constant pressure is maintained

Manual injector

- **Valve injectors**

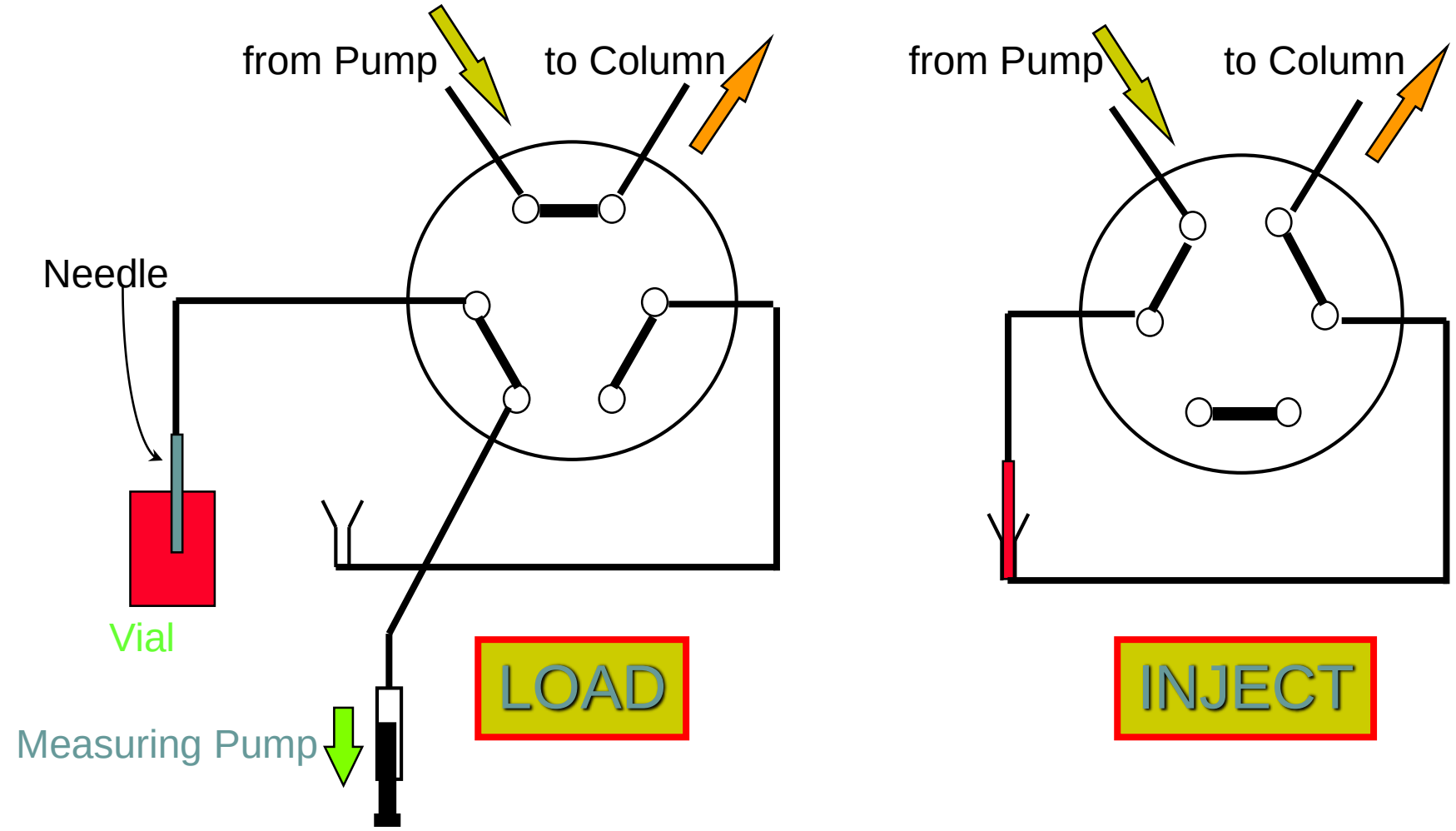


(a) Isolated from the pump eluent Stream (LOAD position)



(b) Positioned in it (INJECT position)

Direct Injection Auto Sampler



Guard column

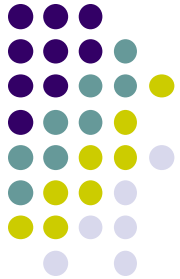
- Protection device, often included just prior to the analytical column to chemically remove components of the sample that would foul the main column
- Guard column filled with removable protective cartridge

Troubleshooting: if pressure in system high check the cartridge in the guard column

Guard - Protects the analytical column.

- Particles
- Interferences
- Prolongs the life of the analytical column

Columns and Stationary Phases.

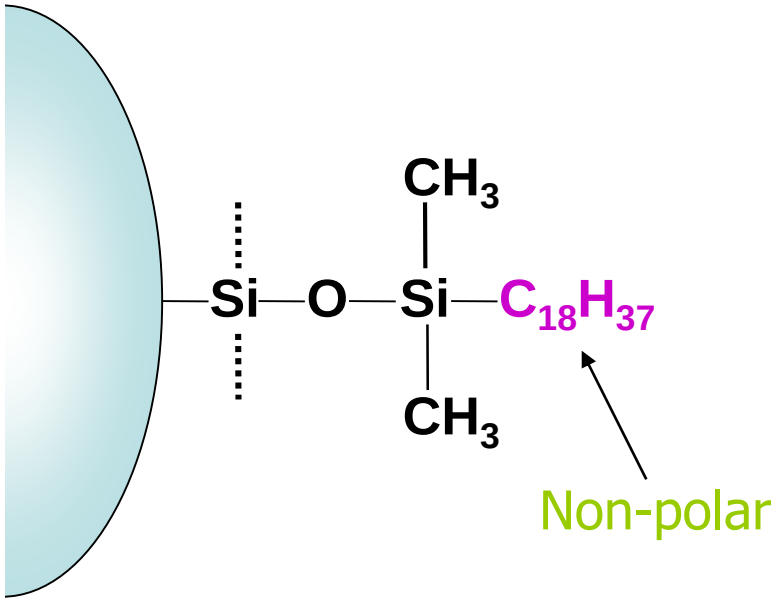


- **HPLC is largely the domain of packed columns some research into microbore/capillary columns is going on.**

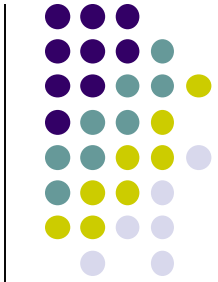
- **Stationary phases are particles which are usually about 1 to 20 μm in average diameter (often irregularly shaped)**
- **In Adsorption chromatography, there is no additional phase on the stationary phase particles (silica, alumina, Fluorosil).**
- **In Partition chromatography, the stationary phase is coated on to (often bonded) a solid support (silica, alumina, divinyl benzene resin)**

Stationary Phase in Reversed Phase Column

- C18 (ODS) type
- C8 (octyl) type
- C4 (butyl) type
- Phenyl type
- TMS type
- Cyano type



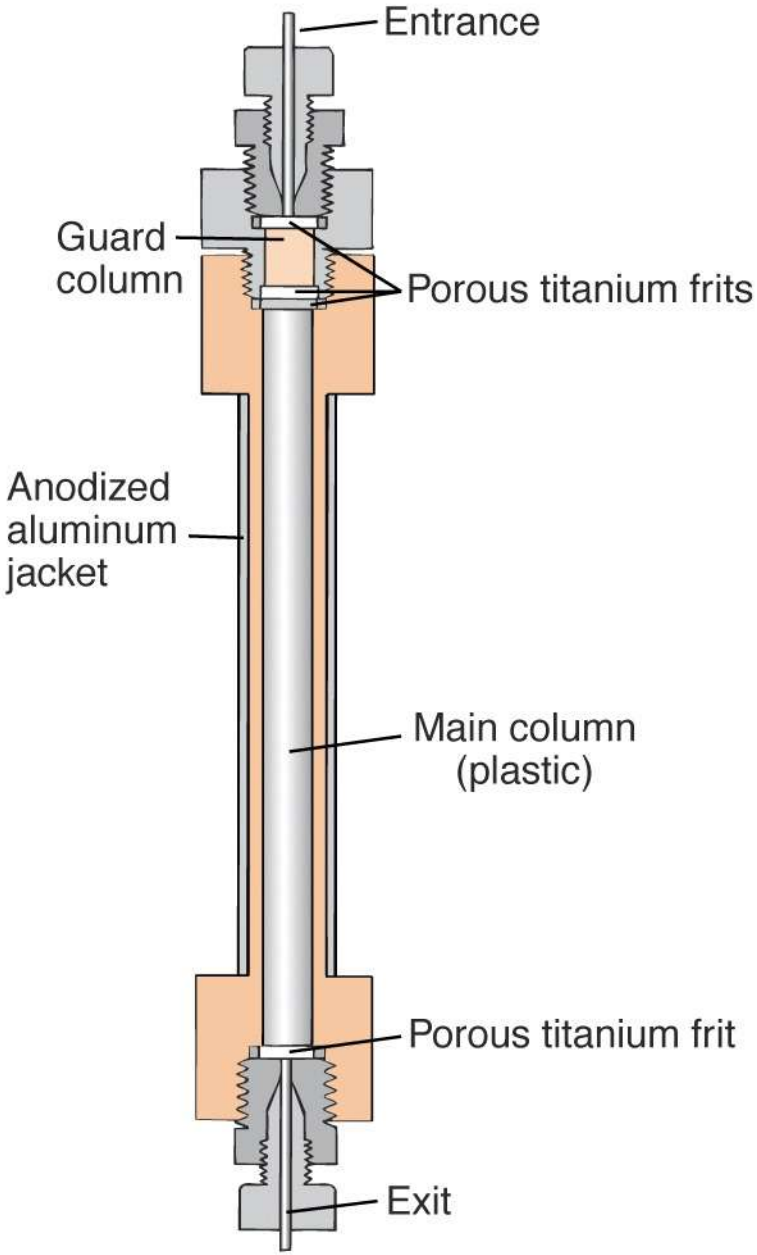
Stationary Phases



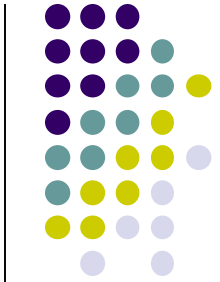
- **Polar (“Normal” Phase):**
  - **Silica, alumina**
  - **Cyano, amino or diol terminations on the bonded phase**

- **Non-Polar (“Reversed Phase”)**
  - **C18 to about C8 terminations on the bonded phase**
  - **Phenyl and cyano terminations on the bonded phase**

- **Mixtures of functional groups can be used!!**

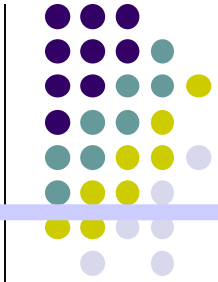

Entrance
Guard column
Porous titanium frits
Anodized aluminum jacket
Main column (plastic)
Porous titanium frit
Exit

Properties of Detector



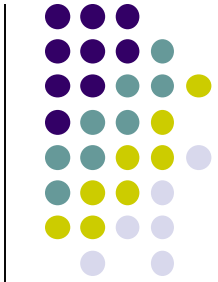
- Adequate sensitivity
- Stability and reproducibility
- Short response time
- Minimum volume for reducing zone broadening
- High reliability and ease of use
- Similarity in response toward all analytes
- Non-destructive

Detectors for HPLC



- UV-VIS Ultraviolet / Visible detector
- PDA Photodiode Array detector
- RF Fluorescence detector
- CDD Conductivity detector
- RID Refractive Index detector
- ECD Electrochemical detector
- ELSD Evaporative light scattering detector
- MS Mass spectrophotometer detector

Ultraviolet / Visible Detector



Advantage:

- **Sensitivity is high**
- **Relative robust to temperature and flow rate change**
- **Compatible with gradient elution**

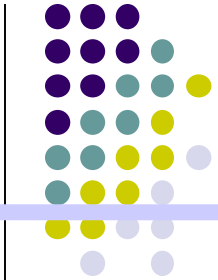
Disadvantage:

- **Only compounds with UV or visible absorption could be detected.**

Additional Functions

- **Dual Wavelength mode**
- **Wavelength Time Program mode**
- **Wavelength Scan mode**

PDA Detector



Advantages:

- **PDA Detector could analyze a sample simultaneously at many different wavelengths.**
- **UV Visible spectra are useful for compound identification, checking peak purity, as well as finding the optimum absorbance for the compounds.**
- **UV Visible spectra of many compounds could be stored in the spectrum libraries, which are useful for compound identification.**
- **Relatively robust to temperature and flow rate fluctuations**
- **Compatible with gradient elution.**

Disadvantages:

- **Slightly less sensitive than UV-Visible detector.**

Refractive Index Detector

Advantage

Responds to nearly all solutes

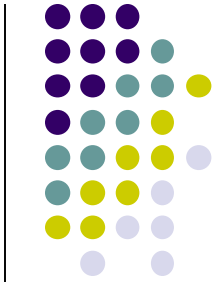
Unaffected by flow rate

Disadvantage

Not as sensitive as most other types of detectors

Could not be used with gradient elution

Selection of Detectors



Detectors	Type of compounds can be detected
UV-Vis & PDA	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
RF	Fluorescent compounds, usually with fused rings or highly conjugated planar system.
CDD	Charged compounds, such as inorganic ions and organic acid.
ECD	For easily oxidized compounds like quinones or amines.
RID & ELSD	For compounds that do not show characteristics usable by the other detectors, eg. polymers, sacharides.

Parameters used in HPLC

CAPACITY FACTOR
RESOLUTION
ASYMMETRY FACTOR (TAILING FACTOR)
EFFICIENCY

Retention : When a component in a sample interacts with the stationary phase in the column and a delay in elution occurs.

Column efficiency : Goodness of a column

Parameters used in HPLC

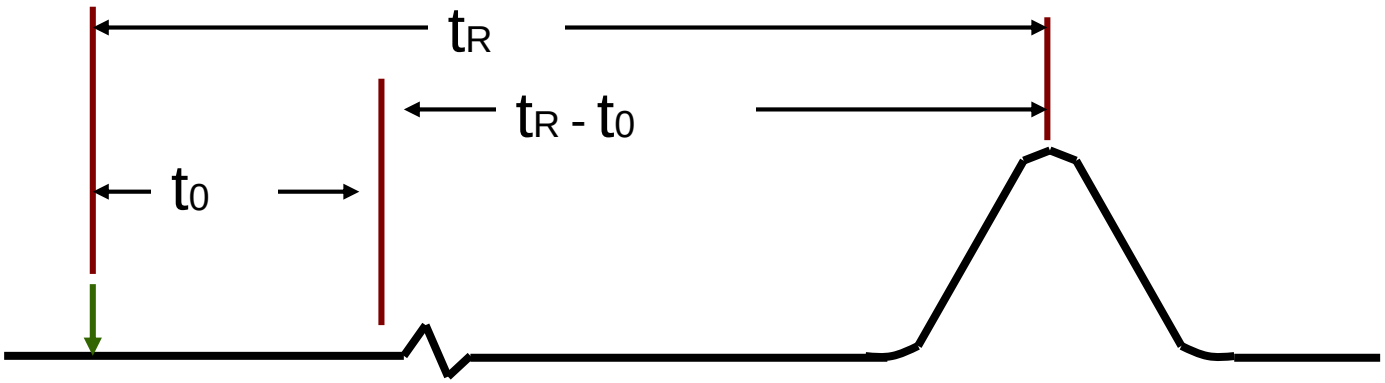
Retention parameters

Rt : Retention Time (the time between the injection point and the maximum detector response for correspondent compound)

vR: retention volume (tR x eluent flow rate)

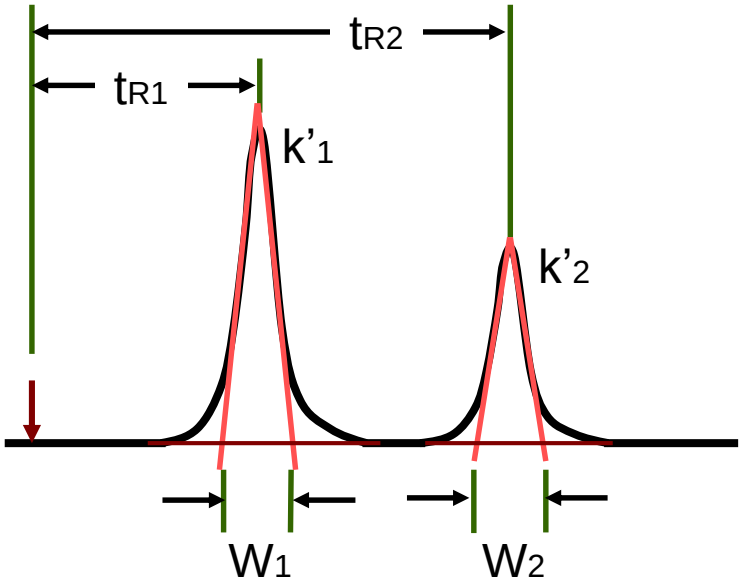
k’ : capacity factor

t0 : the time required for the component not retained by the column to pass through the column



Resolution

The resolution of two bands is a function of both their relative Retentions and peak width.



Resolution : $R_s = 2 \times \frac{t_{R2} - t_{R1}}{W_1 + W_2}$

Separation factor : $\alpha = \frac{k'_2}{k'_1}$

Number of theoretical plates (N): **compare efficiencies of a system for solutes that have different retention times**

$$N = (t_R/\sigma)^2$$

or for a Gaussian shaped peak

$$N = 16\,(t_R/W_b)^2$$

$$N = 5.54\,(t_R/W_h)^2$$

The larger the value of N is for a column, the better the column will be able to separate two compounds.

- the better the ability to resolve solutes that have small differences in retention

- N is independent of solute retention

- N is dependent on the length of the column

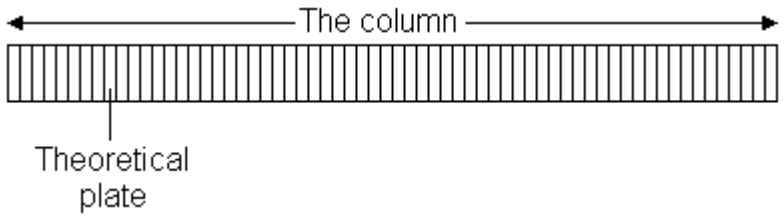


Plate height or height equivalent of a theoretical plate (H or HETP): compare efficiencies of columns with different lengths:

$$H = L/N$$

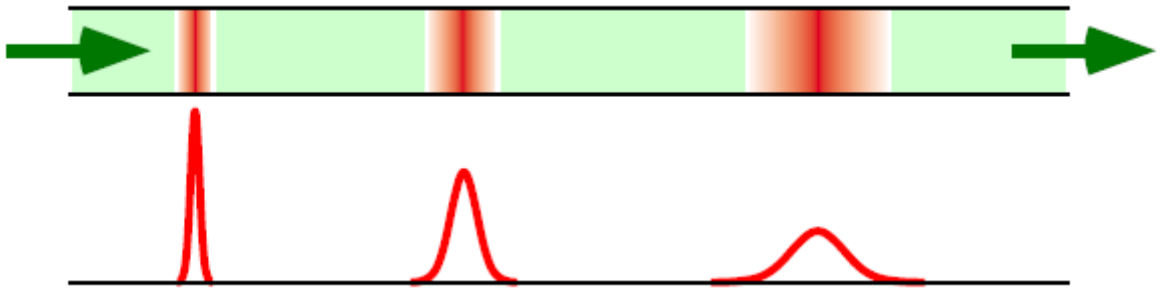
where: L = column length
N = number of theoretical plates for the column

Note: H simply gives the length of the column that corresponds to one theoretical plate

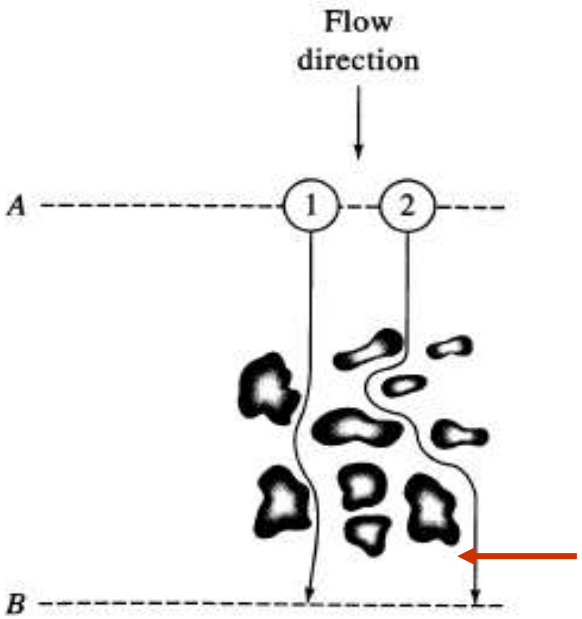
H can be also used to relate various chromatographic parameters (e.g., flow rate, particle size, etc.) to the kinetic processes that give rise to peak broadening:

Why Do Bands Spread?

a. Eddy diffusion
b. Mobile phase mass transfer
c. Stagnant mobile phase mass transfer
d. Stationary phase mass transfer
e. Longitudinal diffusion

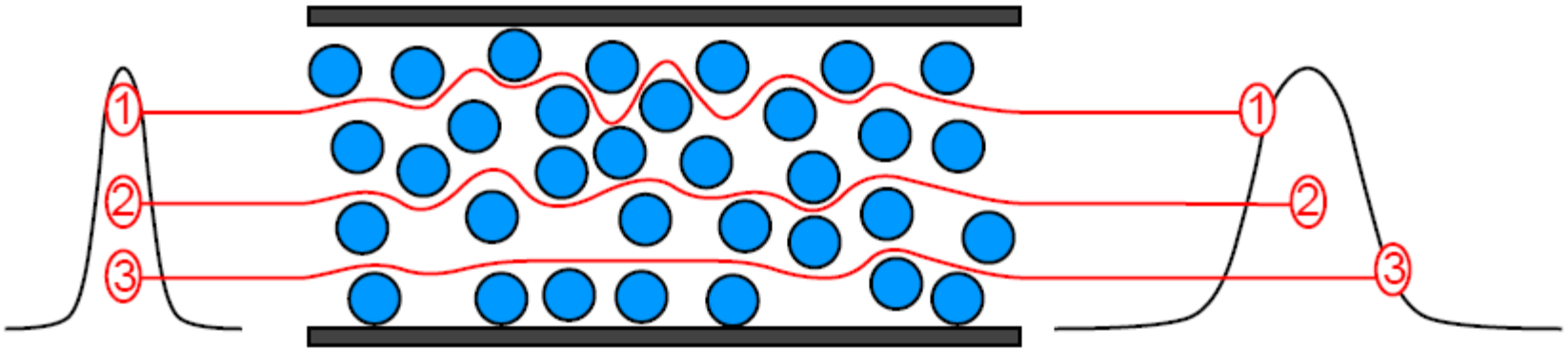


a.) *Eddy diffusion* – a process that leads to peak (band) broadening due to the presence of multiple flow paths through a <u>packed</u> column.

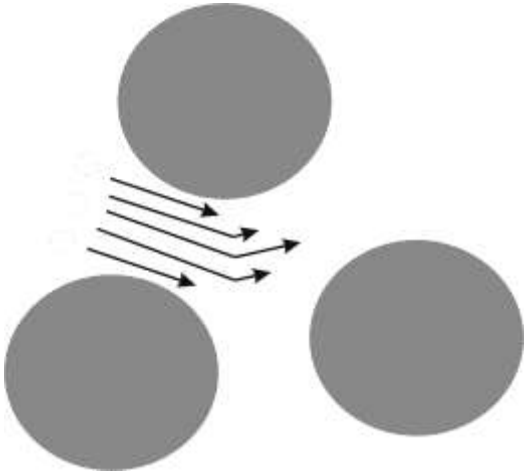


As solute molecules travel through the column, some arrive at the end sooner then others simply due to the different path traveled around the support particles in the column that result in different travel distances.

Longer path arrives at end of column after (1).



b.) Mobile phase mass transfer – a process of peak broadening caused by the presence of different flow profile within channels or between particles of the support in the column.



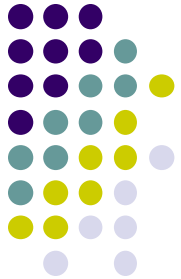
A solute in the center of the channel moves more quickly than solute at the edges, it will tend to reach the end of the channel first leading to band-broadening

The degree of band-broadening due to eddy diffusion and mobile phase mass transfer depends mainly on:

1) the size of the packing material
2) the diffusion rate of the solute

Applications of HPLC

Field	Typical mixtures
Pharmaceuticals	Antibiotics, sedatives, steroids, Amino analgesics, crude drugs, cosmetics
Biochemical	acids, proteins, peptides, carbohydrates, lipids, enzymes, medicines, hormone
Food products	Mycotoxins, additives, saccharides, amino acids, vitamins, fatty acid, coloring agents, antibacterials
Industrial chemicals	Condensed aromatics, surfactants, propellants, dyes, polymers, plasticizers
Forensic chemistry	Drugs, poisons, blood alcohol, narcotics
Environmental field	Inorganic ions, organic acids, agricultural chemicals, pesticides, herbicides, phenols,
Clinical medicine	Bile acids, drug metabolites, urine extracts, estrogens



THANK YOU