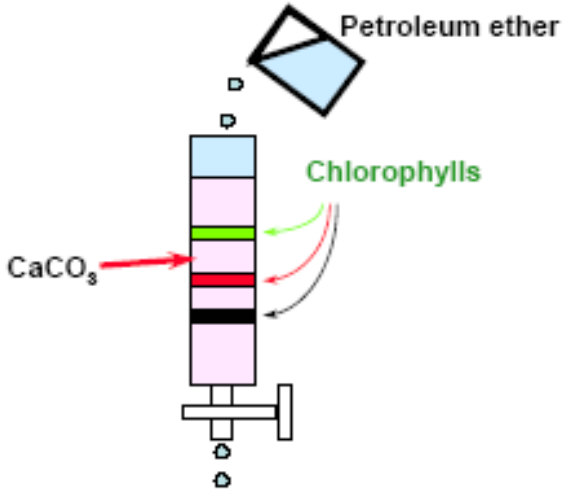
Chromatography -- what does it mean?

To write with colors -- literally translated from its Greek roots *chroma* and *graphein*, chromatography was first developed by the Russian botanist Mikhail Tswett in 1903 as he produced a colorful separation of plant pigments through a column of calcium carbonate. Chromatography has since developed into an invaluable laboratory tool for the separation and identification of compounds. Although color usually no longer plays a role in the process, the same principles of chromatography still apply.

Mikhail Tswett



First Chromatographic Separation

Chromatography -- what does it mean?

***Tswett stated*: Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system.**

Analytical Nomenclature of IUPAC:
Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction (IUPAC, 1993).

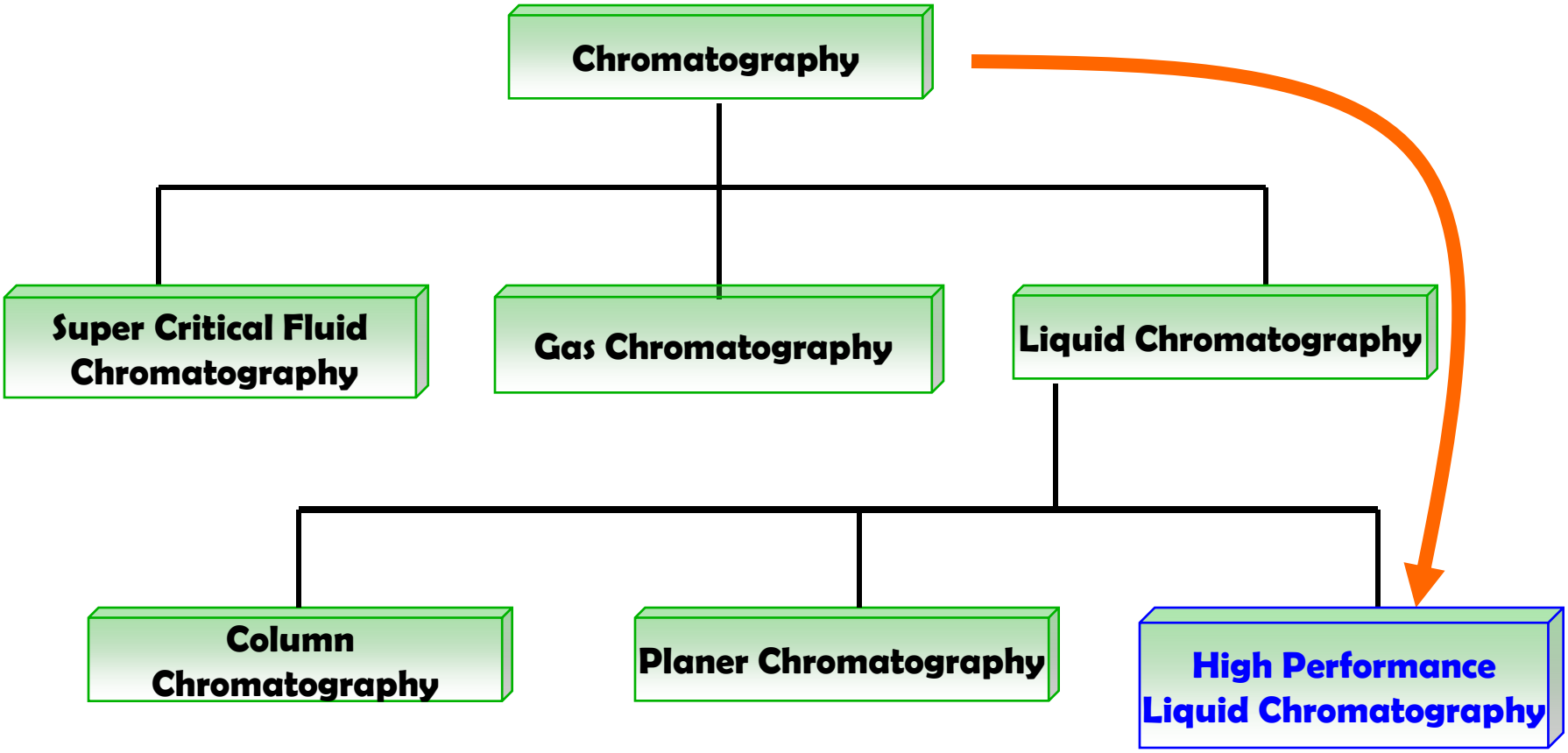
Chromatography: Method

Chromatograph: Machine

Chromatographer: Person

Chromatogram: Data

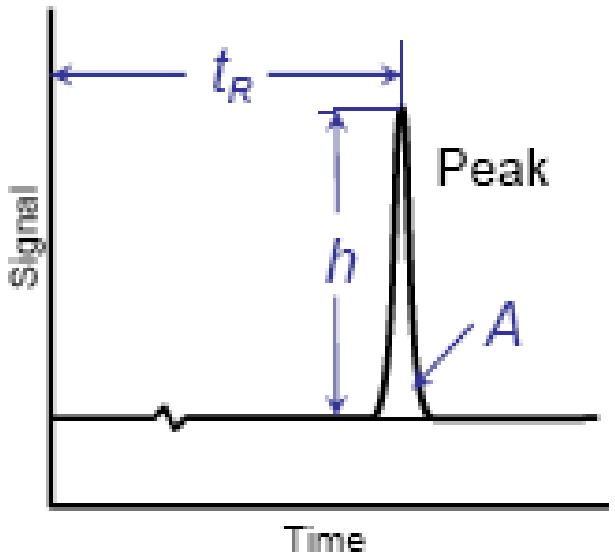
Chromatographic Tree



High Performance Liquid Chromatograph

Shimadzu Prominence

Chromatogram



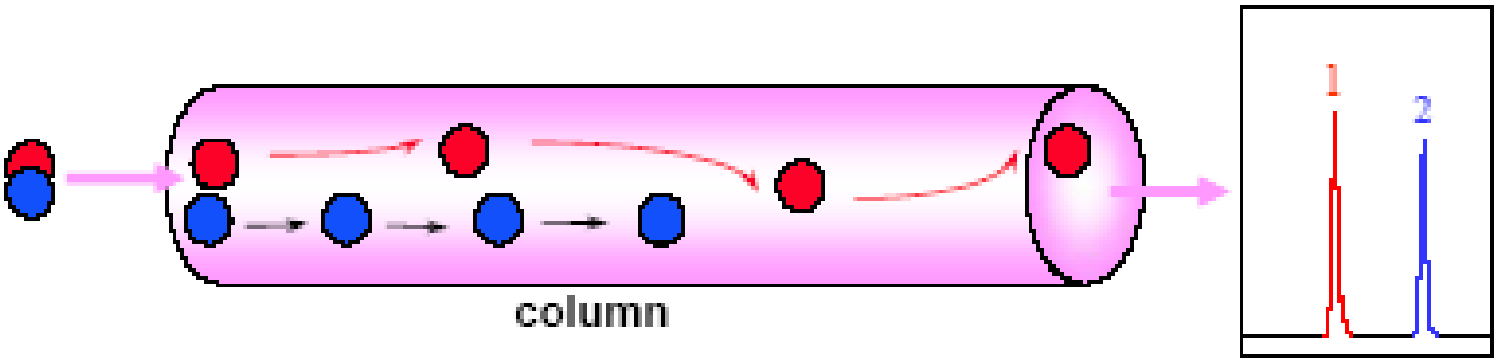
t_R : Retention time

A : Area

h : Height

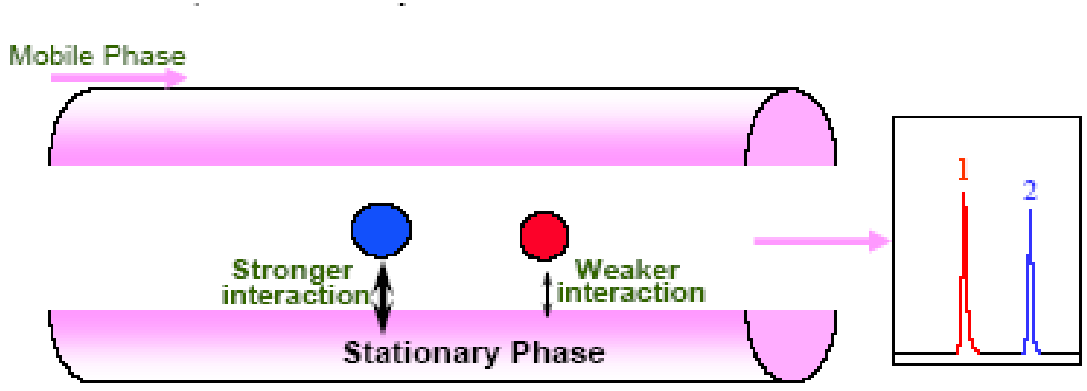
Separation Technique

- Compounds are separated due to the molecules moves at different rates in the column.

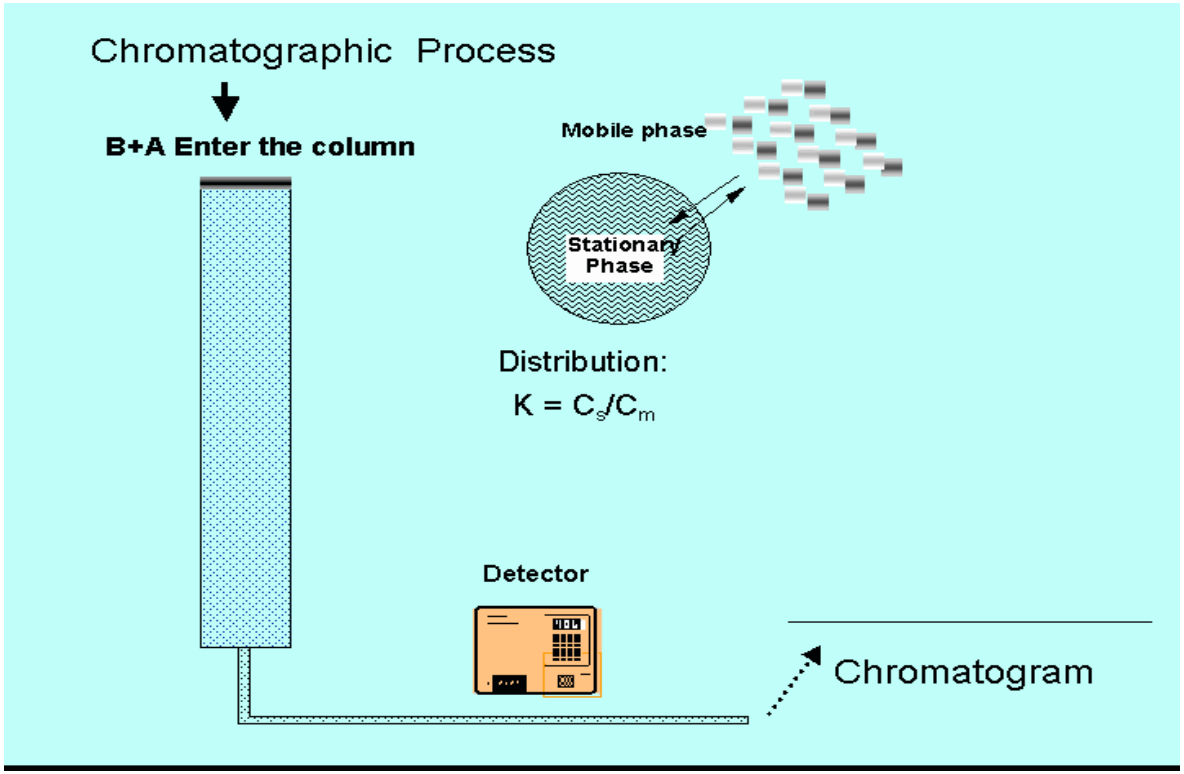


Separation Technique

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



Separation Technique



HPLC Phases

- ⇒**Normal Phase Mode**
- ⇒**Reverse Phase Mode**
- ⇒**Reverse Phase Ion Pairing Mode**
- ⇒**Ion Exchange Mode**
- ⇒**Chiral Separation Mode**

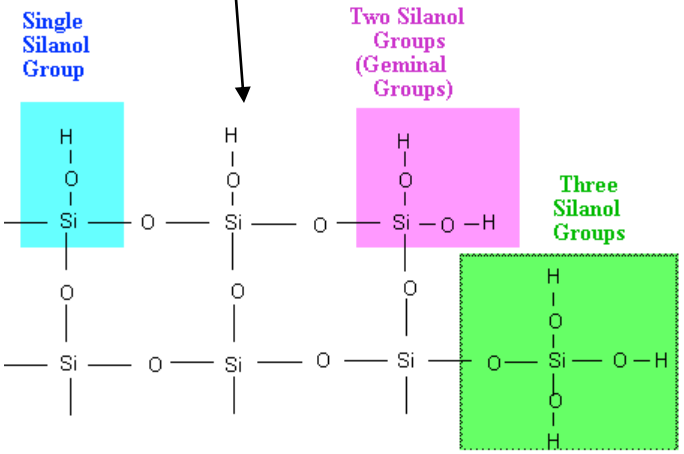
Normal Phase

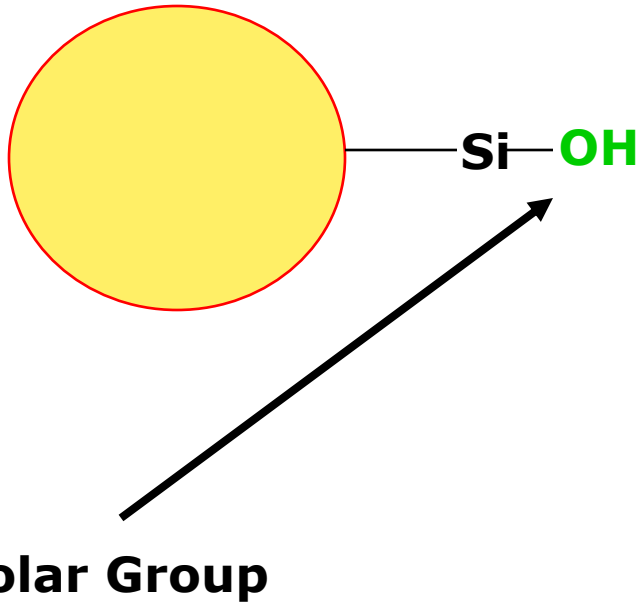
- ✓Stationary Phase is Polar
- ✓Mobile Phase is Non Polar

Base material for Stationary Phase

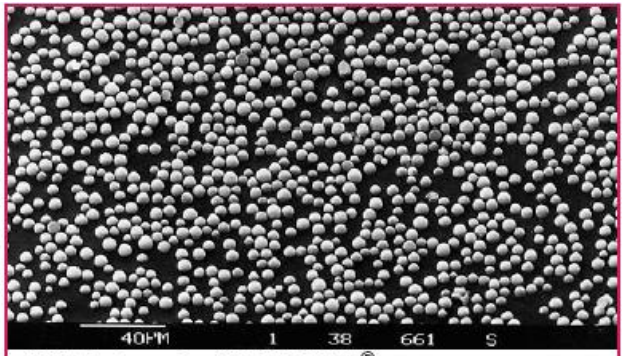
SiO_2

O=Si=O

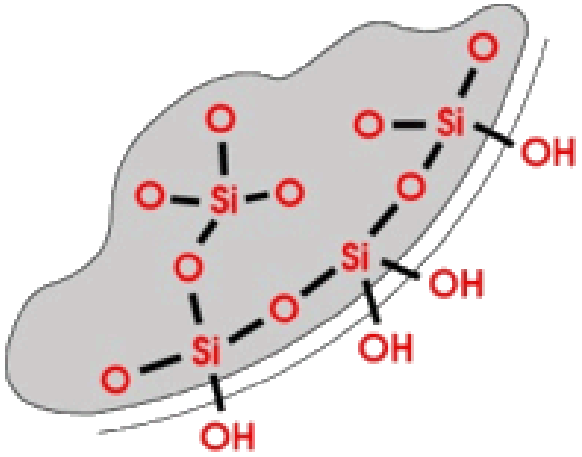


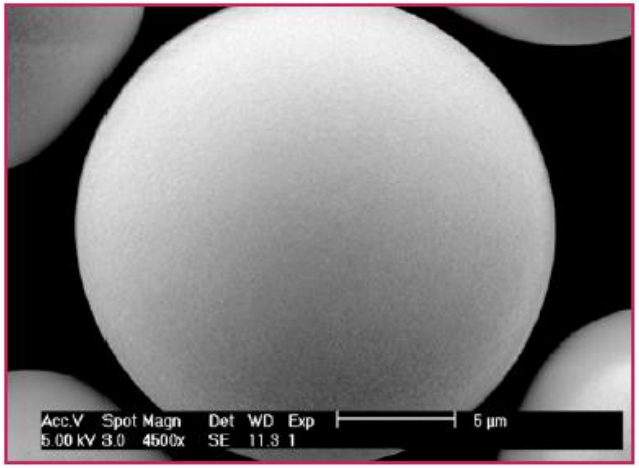


Silica Gel

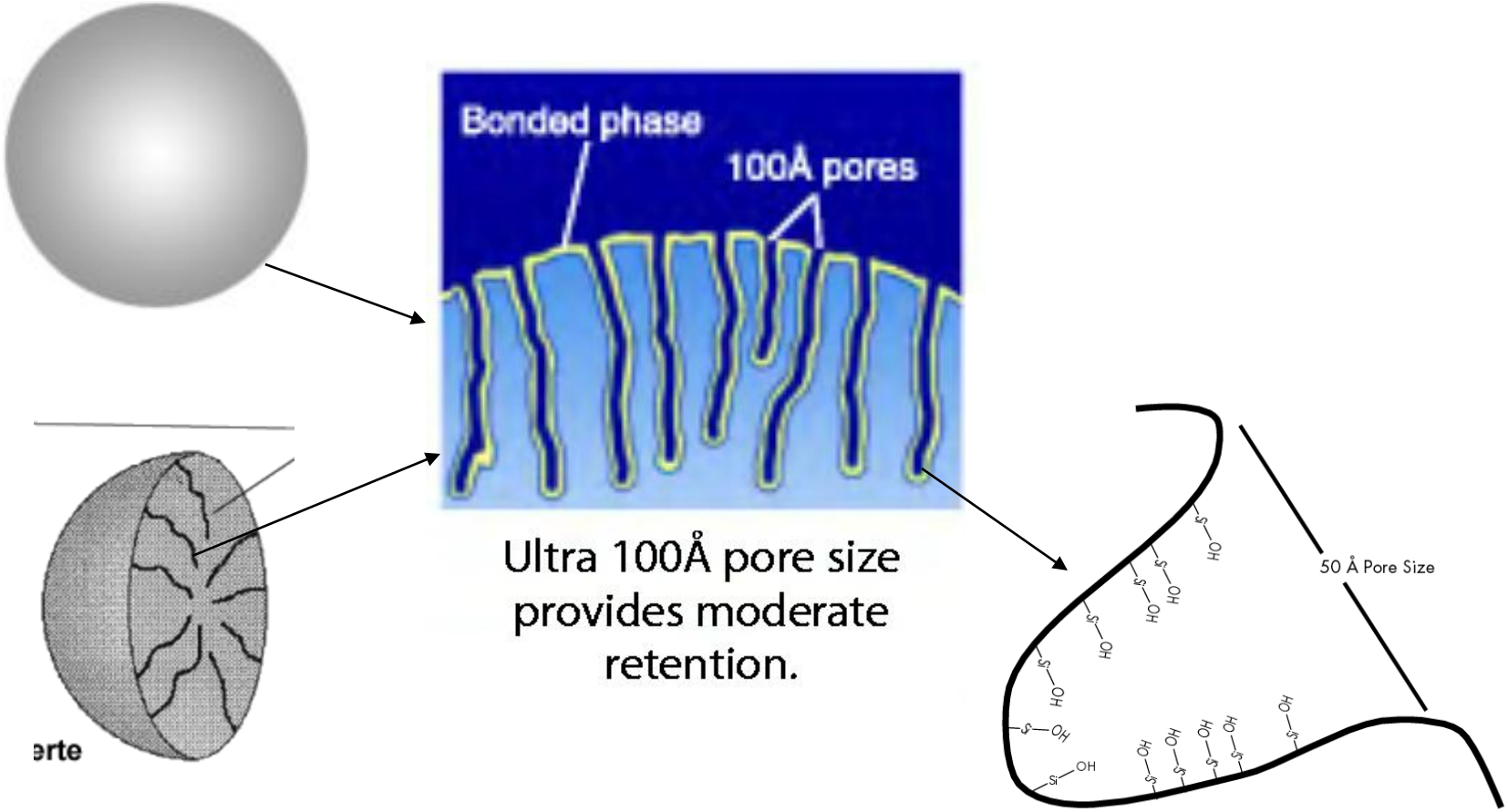


SEM photograph of NUCLEOSIL®
(magnification see scale, 1 cm ≈ 40 µm)

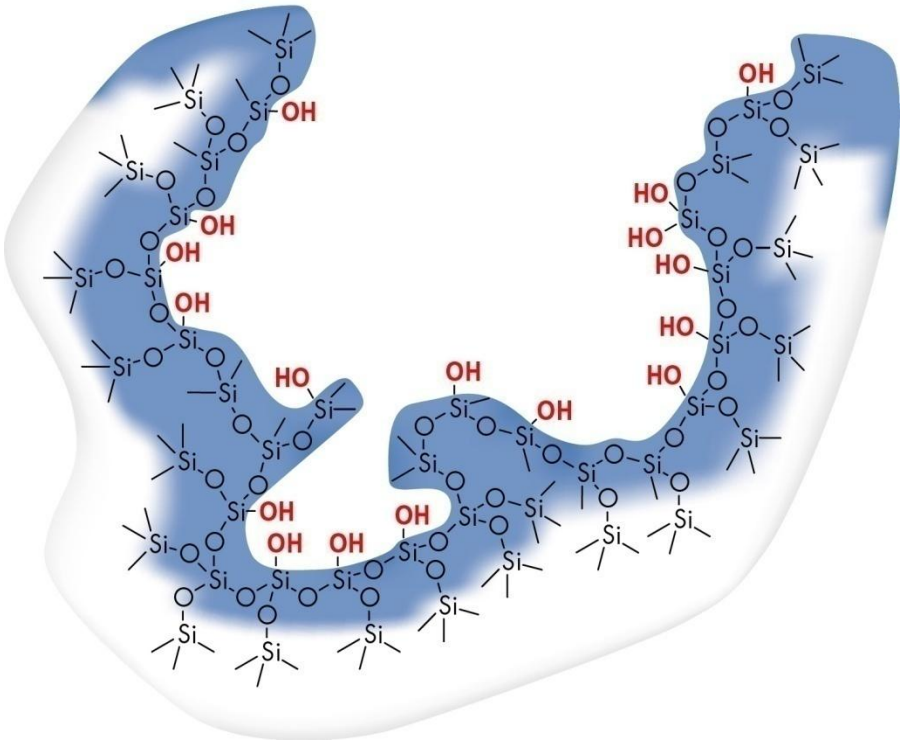




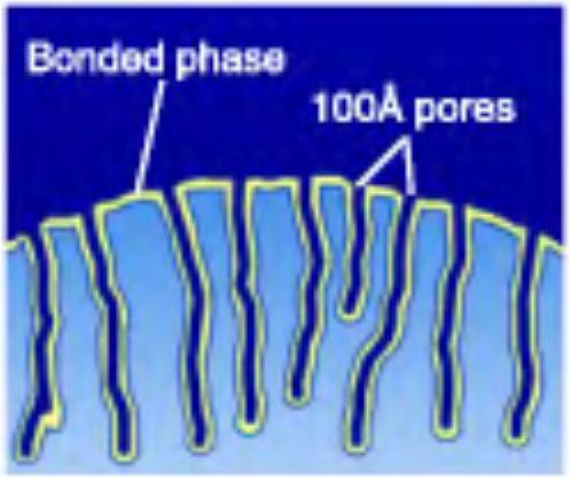
Silica gel


Bonded phase
100Å pores
Ultra 100Å pore size provides moderate retention.
50 Å Pore Size

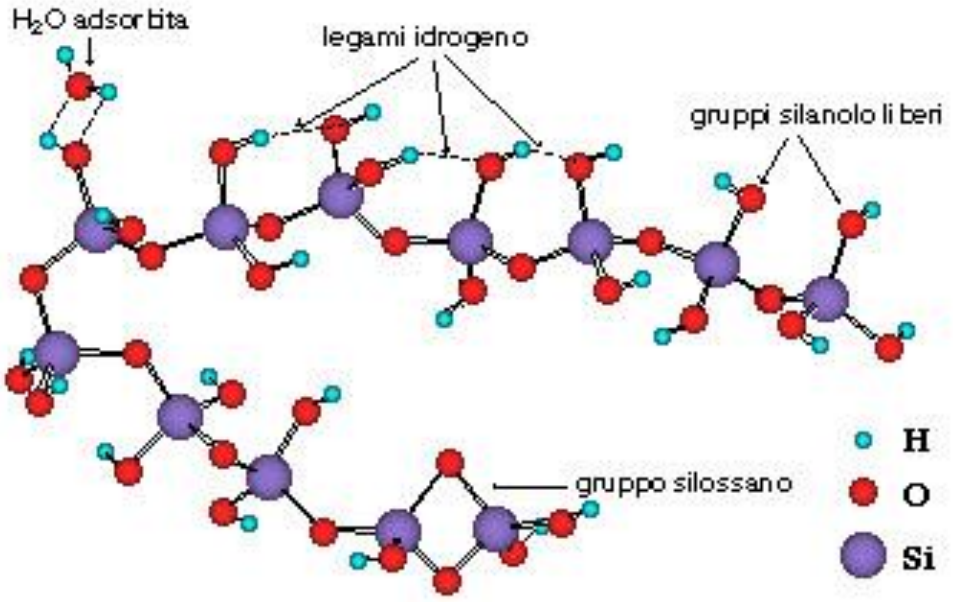
Silica gel



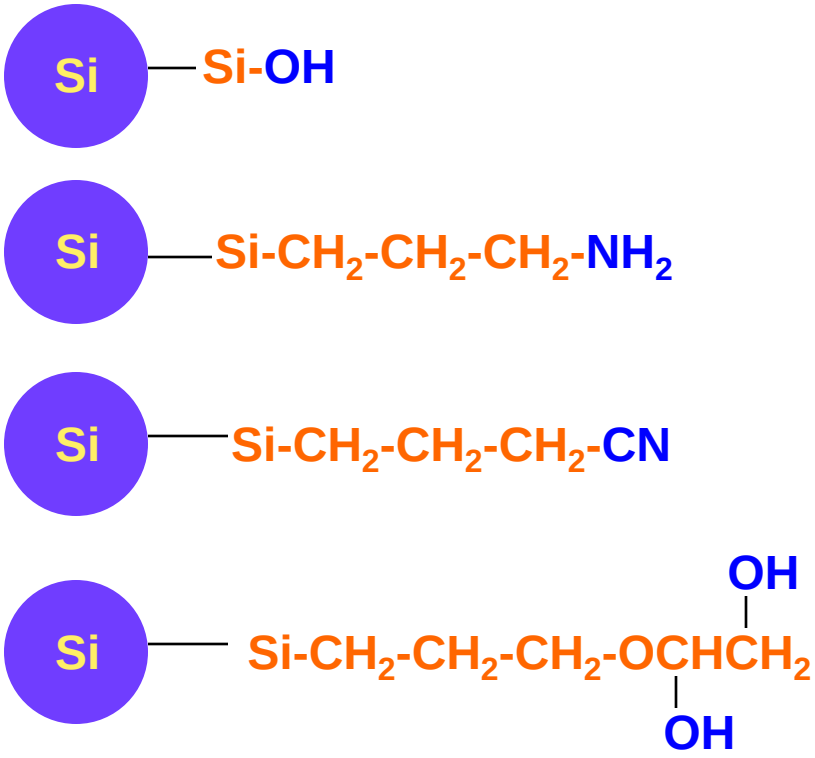
Silica gel



Ultra 100Å pore size provides moderate retention.



Stationary Phase for Normal Phase



Unmodified Silica (USP-L3)

Amino (USP-L11)

Cyano (USP-L10)

Diol (USP-L)

Mobile Phase

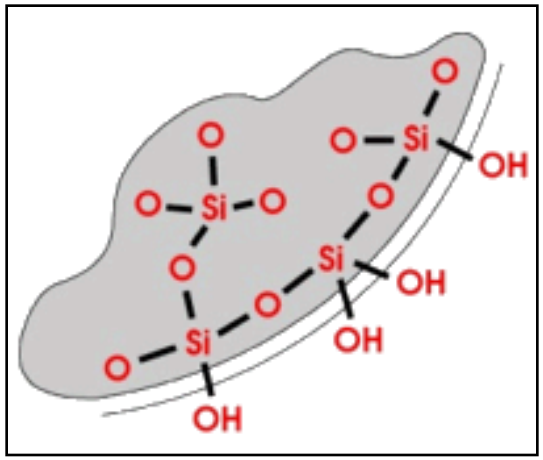
Primary solvents(non-polar)

- -Hydrocarbons (Pentane, Hexane, Heptane, Octane)
- -Aromatic Hydrocarbons (Benzene, Toluene, Xylene)
- -Methylene chloride
- -Chloroform
- -Carbon tetrachloride

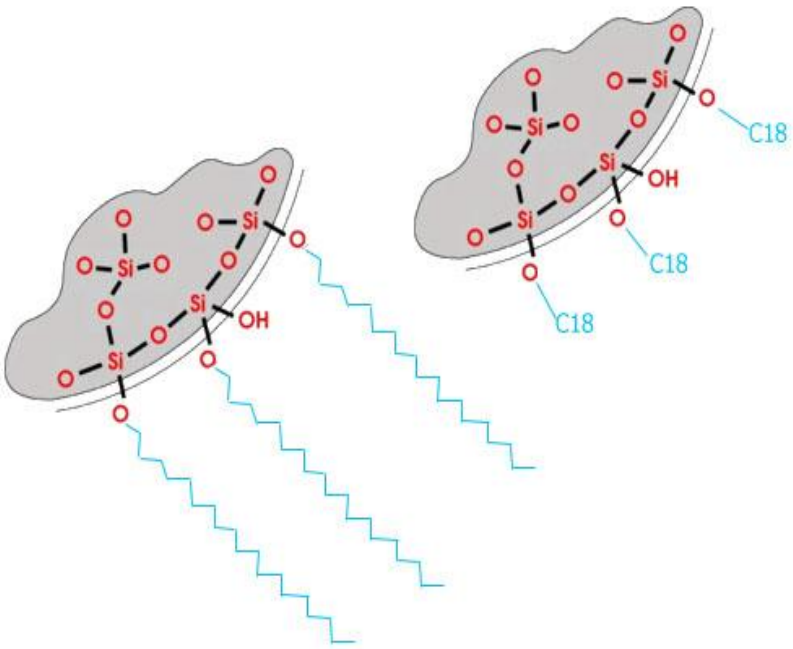
Secondary solvents

- -Methyl-t-butyl ether (MTBE), Diethyl ether, THF, Dioxane, Pyridine, Ethyl acetate, Acetonitrile, Acetone, 2-propaol, ethanol, methanol
- A primary solvent is used as mobile phase. Addition of secondary solvents is to adjust retention time.

Reverse Phase

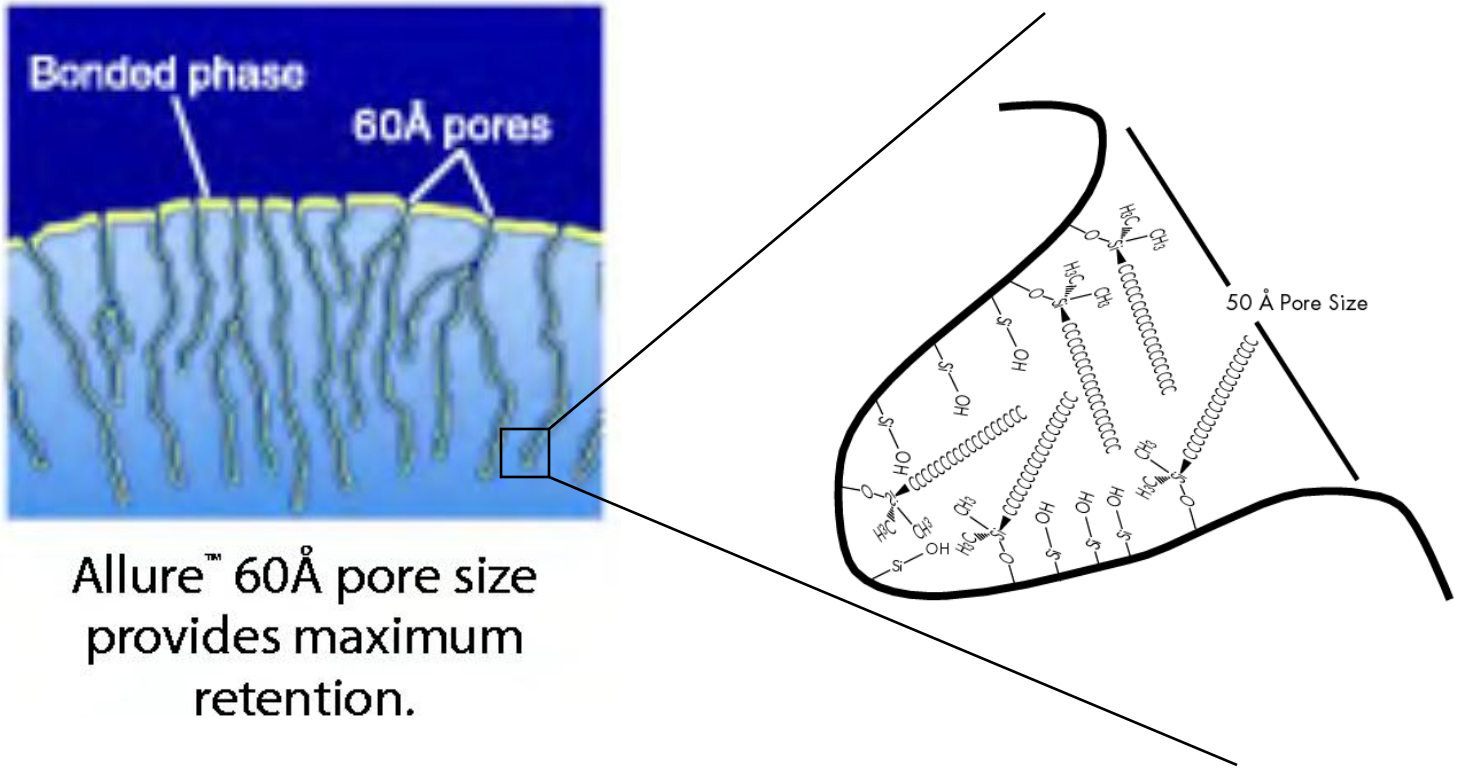


Normal Phase



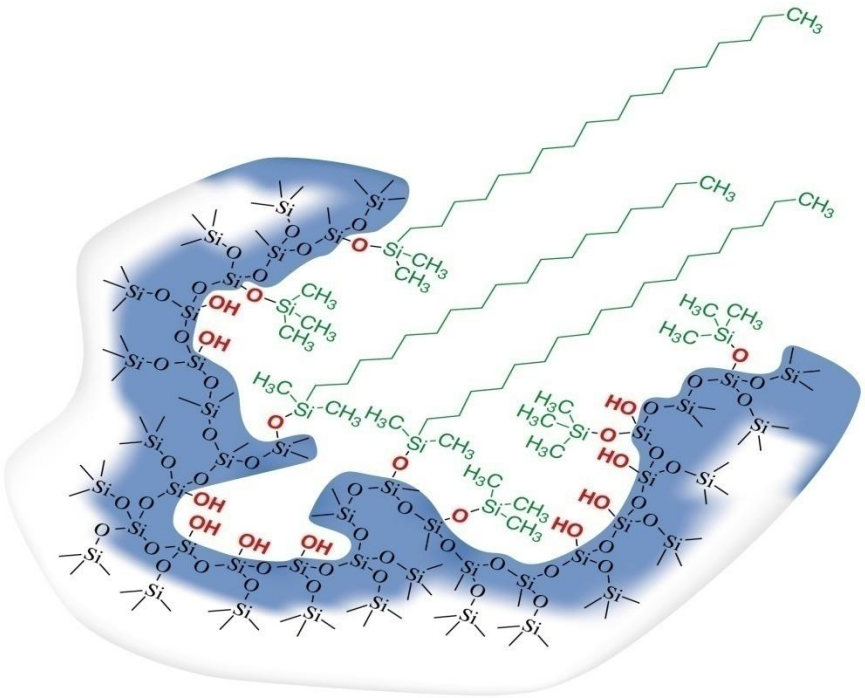
Reverse Phase

Reverse Phase

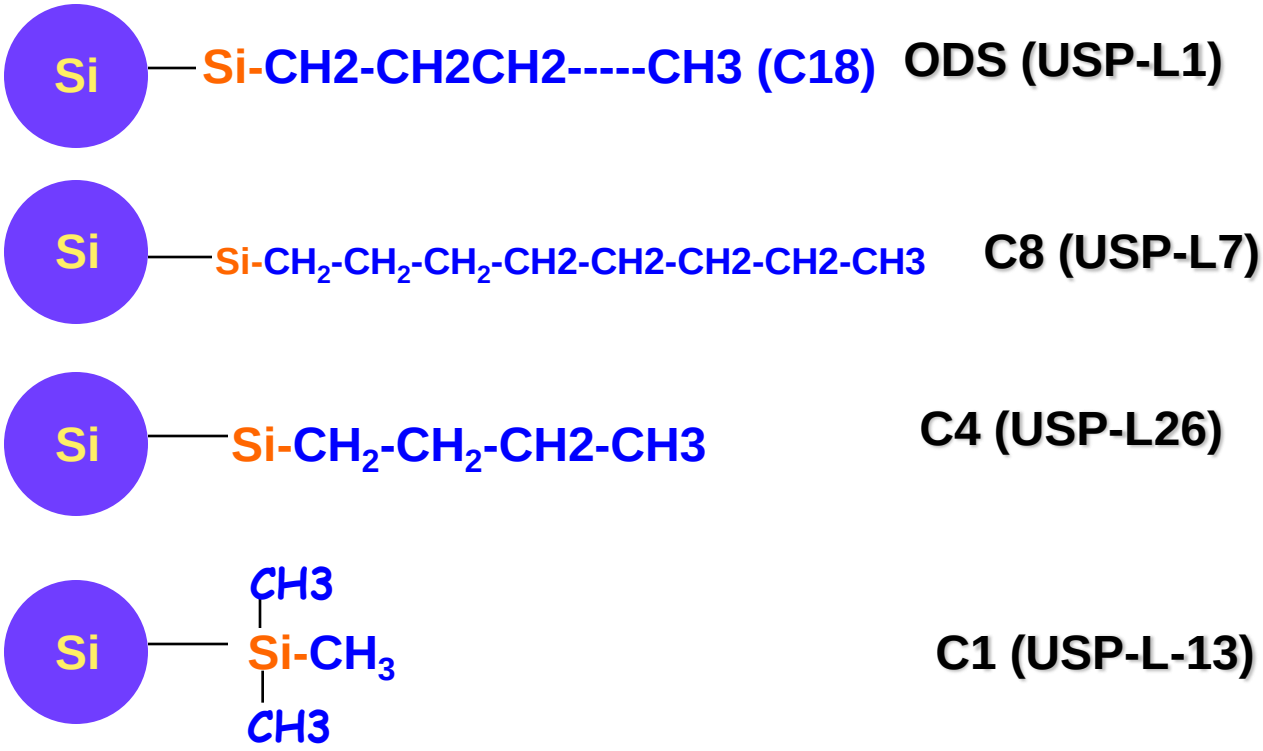


Allure™ 60Å pore size provides maximum retention.

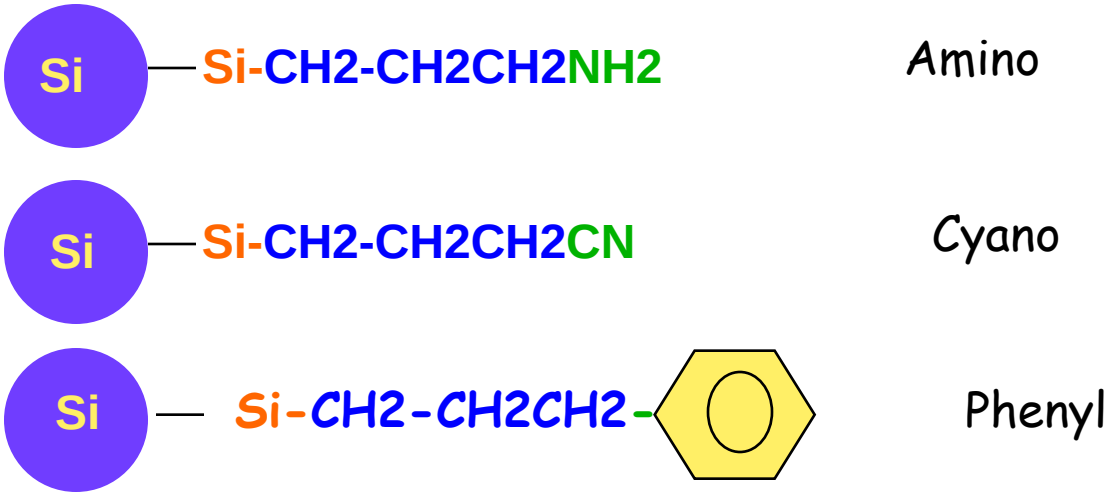
Reverse Phase



Column for Reverse Phase



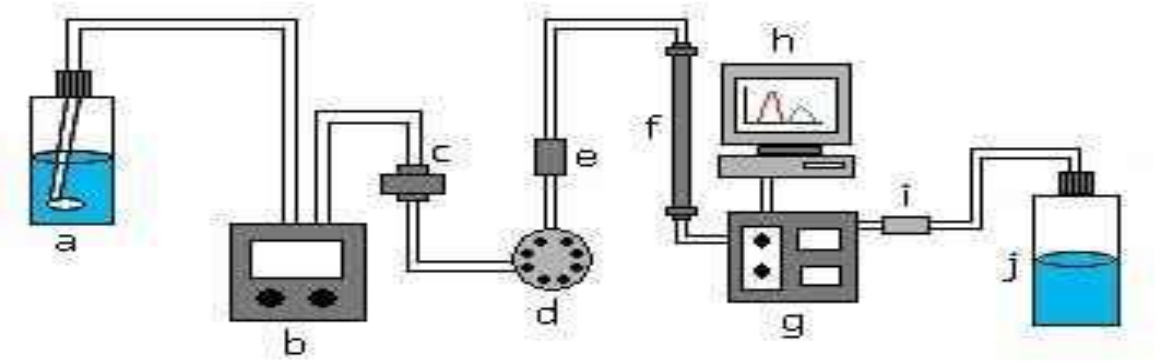
Column for Reverse Phase



HPLC Instrumentation

- 1. Pump
- 2. Column
- 3. Injector
- 4. Detector
- 5. Data Processor

HPLC Instrumentation



A basic LC system consists of (a) a solvent inlet filter, (b) pump, (c) inline solvent filter, (d) injection valve, (e) precolumn filter, (f) column, (g) detector, (h) recorder, (i) backpressure regulator, and a (j) waste reservoir.

Some common definitions

- **ELUTION:** This term describes the transport of a species through the stationary phase by the continuous flow (addition) of mobile phase.
- **ELUANT:** Mobile phase that carries the sample through the column.
- **ELUATE or EFFLUENT:** Mobile phase with separated components after they emerge from the column.
- **ISOCRATIC ELUTION:** A separation in which the mobile phase composition remains unaltered. The mobile phase may comprise of a single solvent or a pre-mixed mixture of solvents.
- **GRADIENT ELUTION:** HPLC is frequently used for the separation of mixtures that contain compounds with a wide range of polarities. In such situations, isocratic conditions may not provide an acceptable separation (i.e., it is not possible to obtain sufficient resolution or the separation takes an unacceptably long period of time). To solve these problems, the composition of the mobile phase is changed during the separation. Two or three solvents that differ in polarity are employed. After sample introduction, the ratio of these solvents is programmed to vary either continuously or in steps, resulting in enhanced separation efficiency.
- The terms 'binary gradient', ternary gradient', and quaternary gradient' refer to the use of 2, 3, and 4 solvents, respectively, to make up the mobile phase composition in a gradient elution method.

- **DISTRIBUTION CONSTANT**: It describes the equilibrium involving the transfer of an analyte between the mobile and stationary phases. This constant, also called partition ratio or partition coefficient, is defined as K= Cs/Cm, a ratio of the analyte molar concentration in the stationary phase to that in the mobile phase.
- **RETENTION TIME, t**: The time taken by the analyte peak to reach the detector after sample introduction is called the retention time. A more accurate measure of the retention time of an analyte is obtained by subtracting from this value the time taken for an unretained solute to emerge from the column (i.e., the dead time, t0), resulting in the adjusted retention time, t'. The retention time is the most important parameter for component identification under set experimental conditions.
- **CAPACITY FACTOR** (or **Retention Factor**) **k'**: A measure of the retention volume (or time) of a particular component of the sample with a given combination of stationary phase and mobile phase. It is defined for species A as k' A = (tA-t0)/t0, where t0 is the retention time for an unretained compound and t'A = adjusted retention time of species A.

Isocratic Vs Gradient

- **Isocratic elution has a constant mobile phase composition**
  - **Can often use one pump!**
  - **Mix solvents together ahead of time!**
  - **Simpler, no mixing chamber required**
  - **Limited flexibility, not used much in research**
    - **mostly process chemistry or routine analysis.**

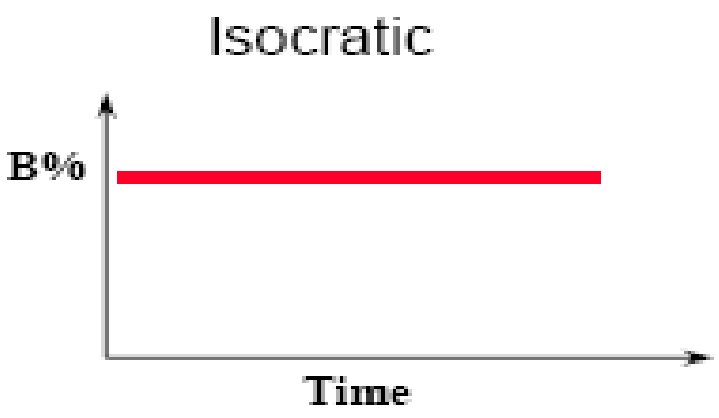
- **Gradient elution has a varying mobile phase composition**
  - **Uses multiple pumps whose output is mixed together**
    - **often 2-4 pumps (binary to quarternary systems)**
  - **Changing mobile phase components changes the polarity index**
    - **can be used to subsequently elute compounds that were previously (intentionally) "stuck" on the column**
    - **Some additional wear on the stationary phase**
    - **Column has to re-equilibrate to original conditions after each run (takes additional time).**

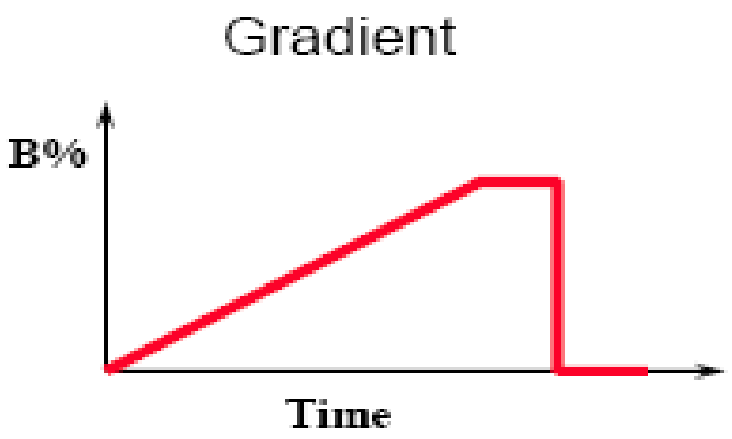
HPLC configuration by eluting mode

Isocratic system

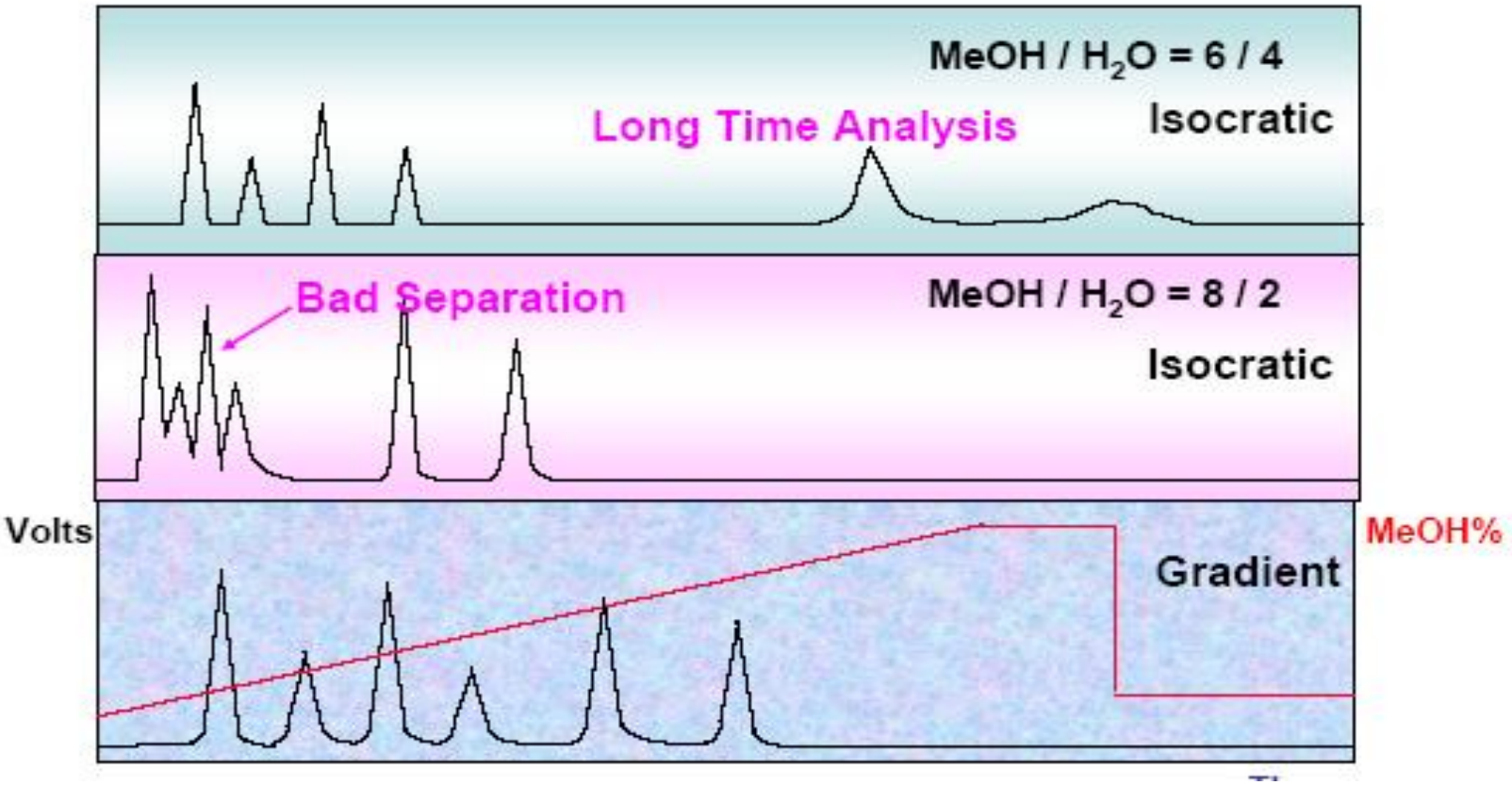
Low-pressure gradient system

High-pressure gradient system

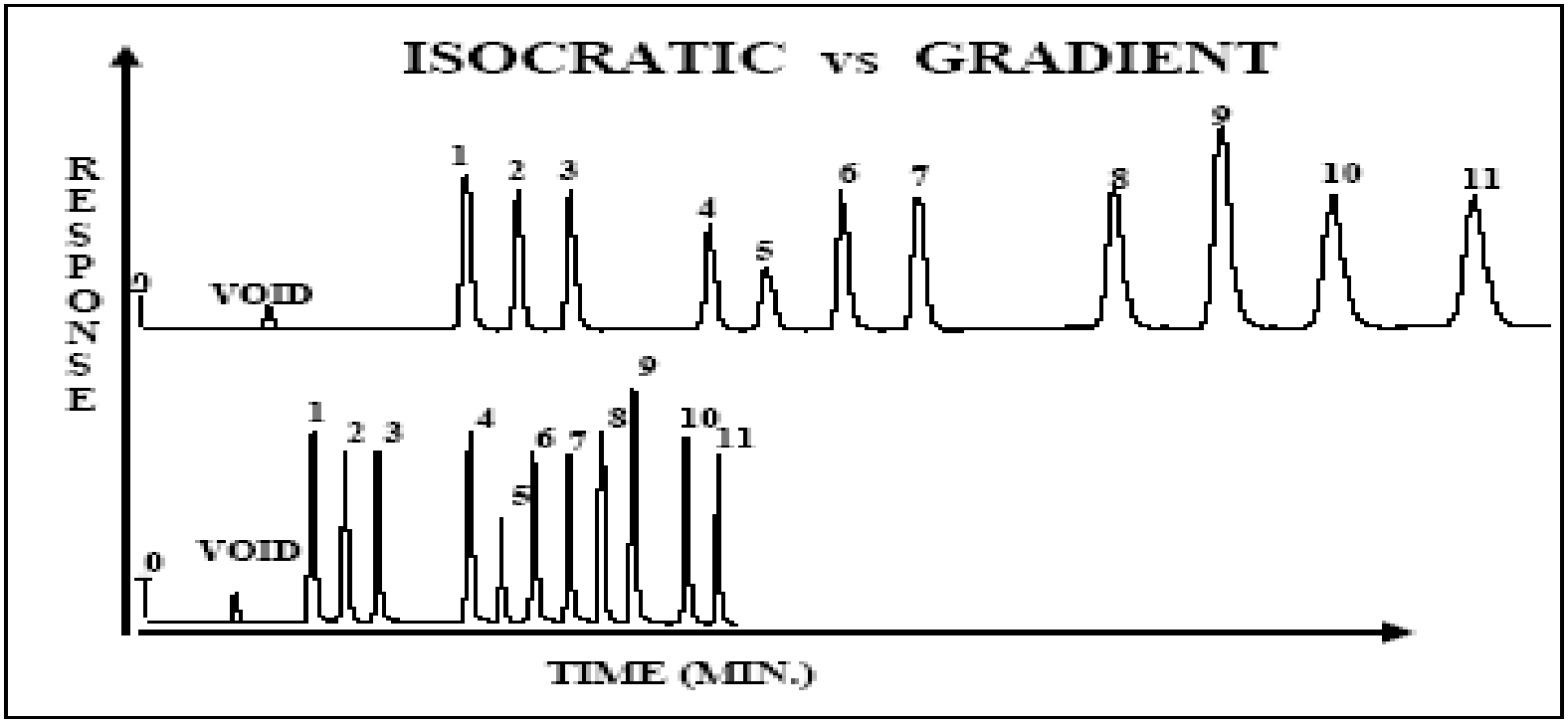




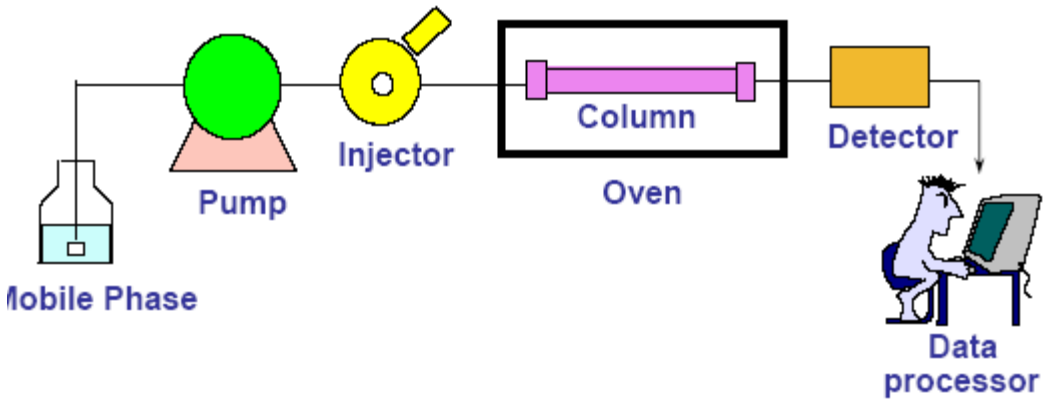
Gradient VS Isocratic



Isocratic VS Gradient



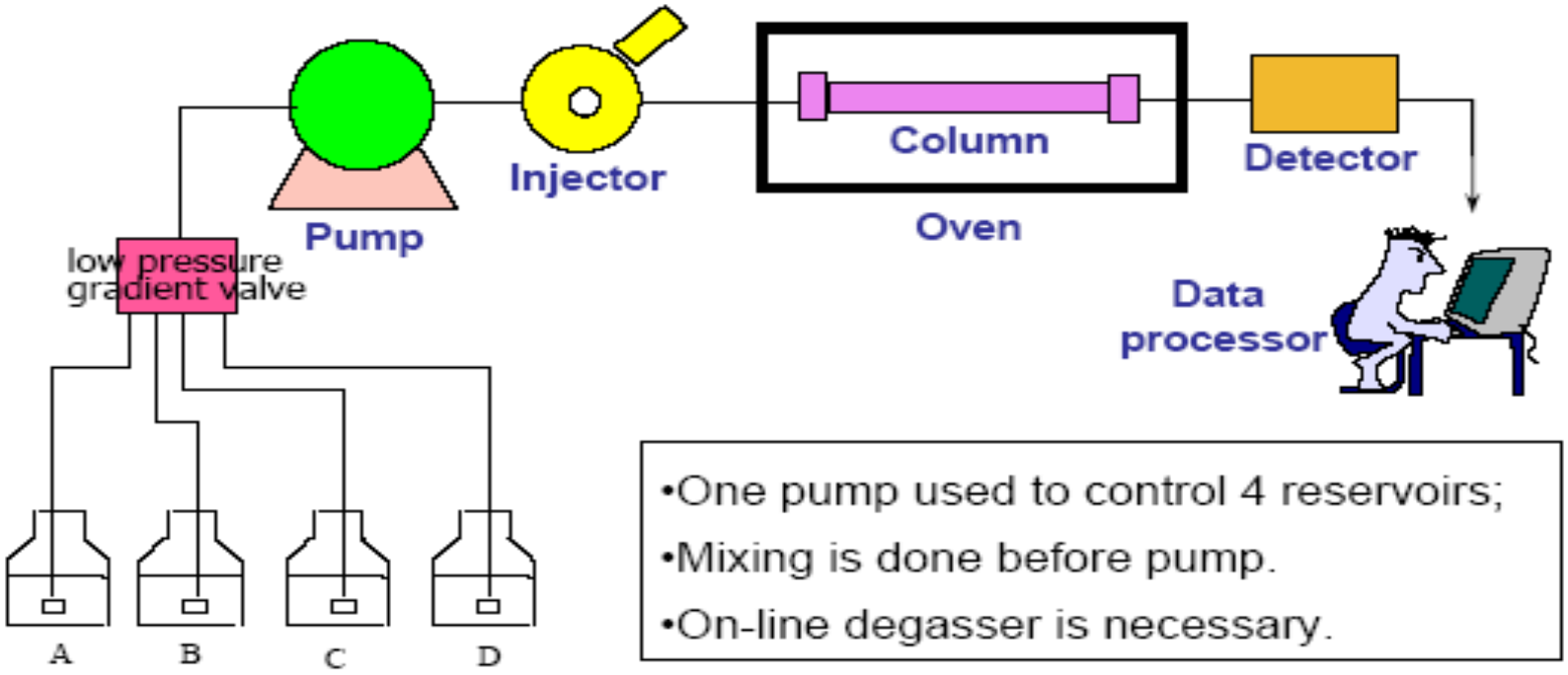
Isocratic



Simple system with one pump and one solvent reservoir.

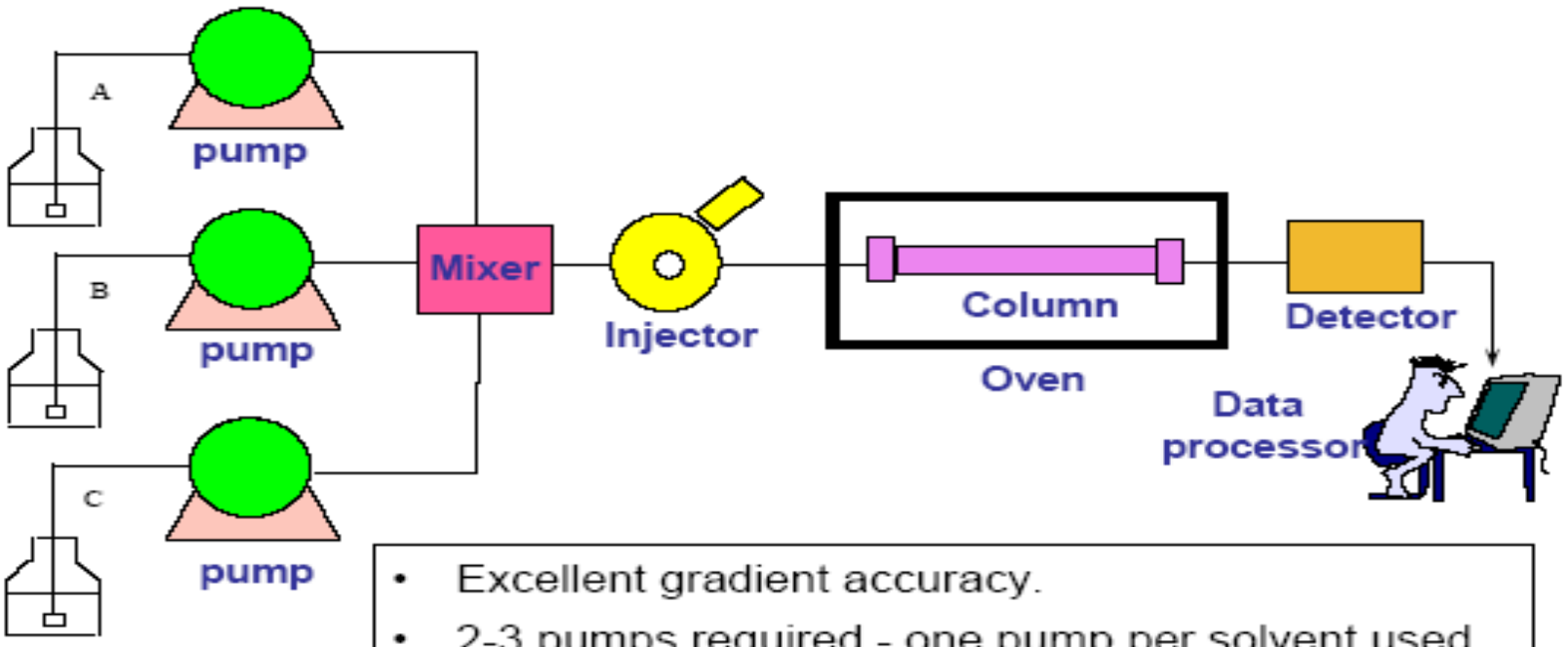
If more than one solvent is used, solvents should be premixed.

Low Pressure Gradient



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

High Pressure Gradient



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

Injector

- Manual Injector
- Auto Injector (Sampler)
- Advantage of Auto Injector (Sampler)

Benefits of Auto injector

--Wide variability in injection volume (0.1 mL-2mL)

--Precision is better or equal to manual injection

--High sample throughput and less labor intensive

Detectors

- 1. UV-Vis /Photodiode Array Detector
- 2. Refractive Index Detector
- 3. Fluorescence Detector
- 4. Conductivity Detector
- 5. Electro Chemical Detector
- 5. Evaporate Light Scattering Detector
- 6. Mass Detector

Data Processor

- Software or Integrator or Recorder

Column Particle Physical Characteristics

- ***Column Dimensions***
- • Length and internal diameter of packing bed

- ***Particle Shape***
- • Spherical or irregular

- ***Particle Size***
- • The average particle diameter, typically 3-20µm

- ***Surface Area***
- • Sum of particle outer surface and interior pore surface, in m2/gram

Column Particle Physical Characteristics

- ***Pore Size***
- • Average size of pores or cavities in particles, ranging from 60-10,000Å
- ***Bonding Type***
- • Monomeric - single-point attachment of bonded phase molecule
- • Polymeric - multi-point attachment of bonded phase molecule
- ***Carbon Load***
- • Amount of bonded phase attached to base material, expressed as %C

Column length

- **Effect on chromatography**

- **Short** (30-50mm) - short run times, low backpressure
- **Long** (250-300mm) - higher resolution, long run times

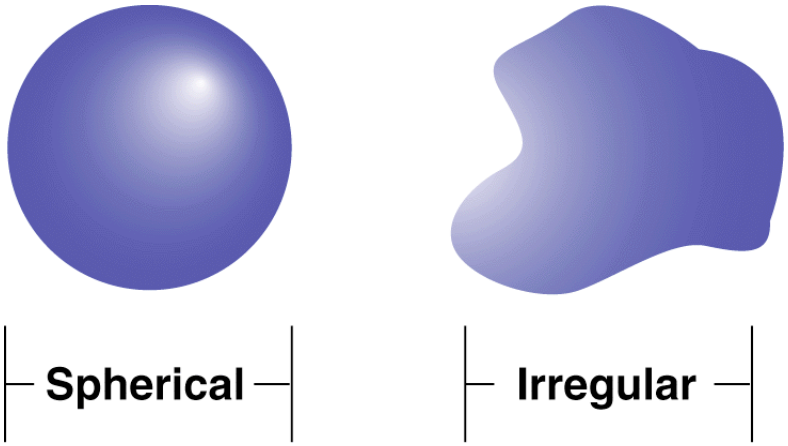
Column ID

- **Narrow** (≤ 2.1mm) - higher detector sensitivity, Sharp peak
- **Popular ID** – 4.6 mm
- **Wide** (10-22mm) - high sample loading

Base Material

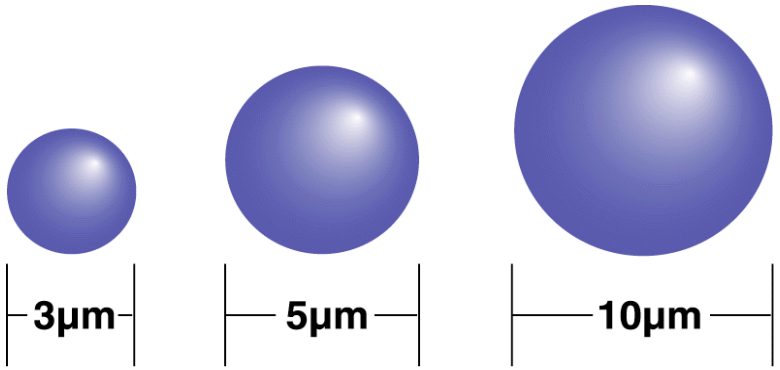
Particle Shape

- **Effect on chromatography**
- Spherical particles offer reduced back pressures and longer column life when using viscous mobile phases like 50:50 MeOH:H2O.
- Nice peak Shape



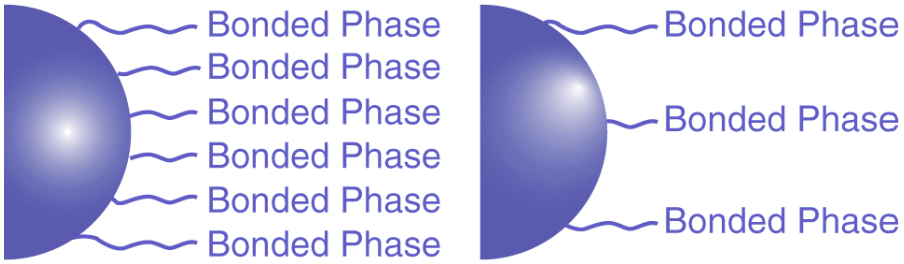
Particle Size

- **Effect on chromatography**
- Smaller particles offer higher efficiency, but also cause higher backpressure. Choose 3µm particles for resolving complex, multi-component samples. Otherwise, choose 5 or 10µm packings.



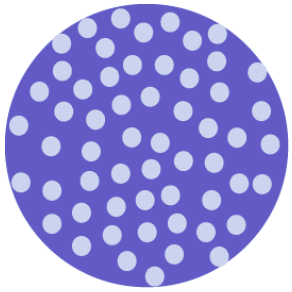
Carbon Load

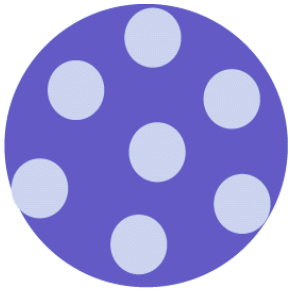
- **Effect on chromatography**
- Higher carbon loads generally offer greater resolution and longer run times. Low carbon loads shorten run times.

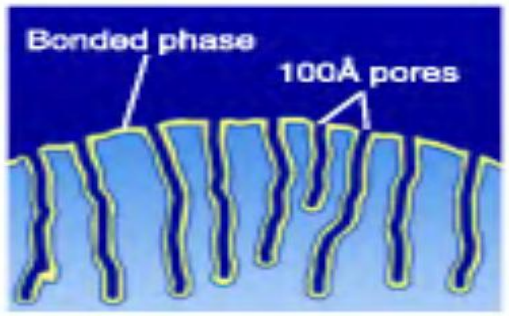


Surface Area

- **Effect on chromatography**
- High surface area generally provides greater retention, capacity and resolution for separating complex, multi-component samples. Low surface area packings generally equilibrate quickly, especially important in gradient analyses.



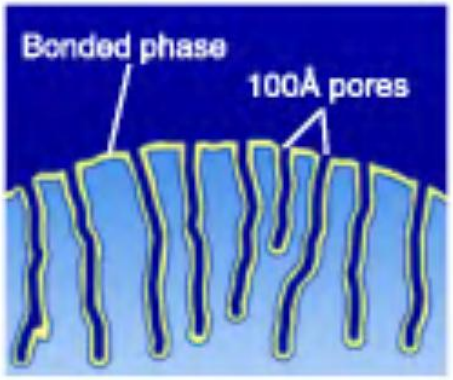




Ultra 100Å pore size provides moderate retention.

Pore Size

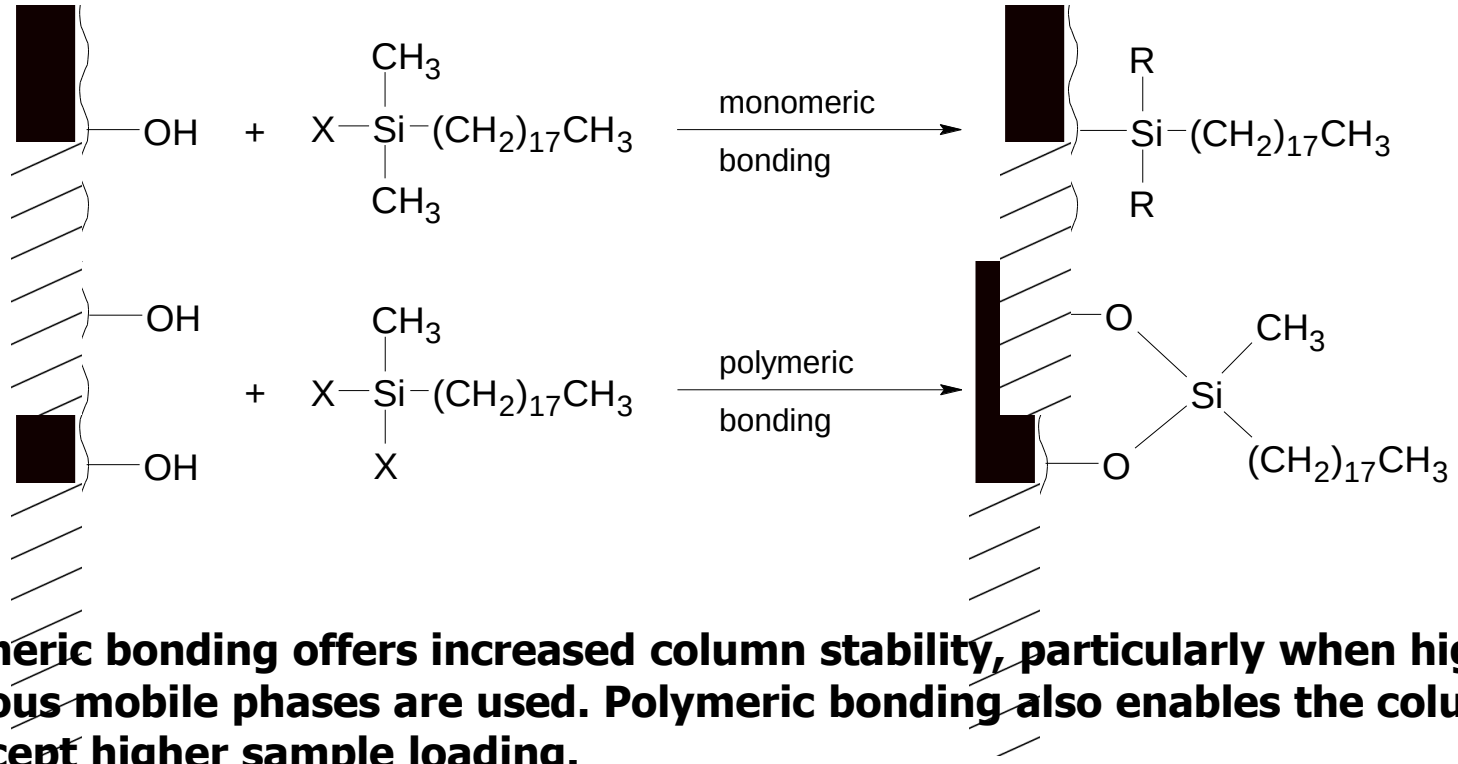
- **Effect on chromatography**
- Larger pores allow larger solute molecules to be retained longer through maximum exposure to the surface area of the particles. Choose a pore size of 150Å or less for sample MW ≤ 2000. Choose a pore size of 300Å or greater for sample MW > 2000.



Ultra 100Å pore size provides moderate retention.

Bonding Type

- **Effect on chromatography**
- Monomeric bonding offers increased mass transfer rates, higher column efficiency, and faster column equilibration.



Polymeric bonding offers increased column stability, particularly when highly aqueous mobile phases are used. Polymeric bonding also enables the column to accept higher sample loading.

System Suitability

- ✓Repeatability,%RSD (CV)
- ✓Capacity factor, k’
- ✓Selectivity/Relative Retention Time
- ✓Theoretical Plates, N (Efficiency)
- ✓ Resolution
- ✓Asymmetry/Tailing Factor

Repeatability, CV (%RSD)

$$S_R\,(\%) = \frac{100}{\bar{X}}\left[\frac{\sum_{i=1}^{N}(X_i - \bar{X})^2}{N-1}\right]^{\frac{1}{2}},$$

X= *(X1+X2……..Xn-1+Xn)/n*

N= *: Number of Analysis*

X1..Xn *: Retention time (or area or heights)*

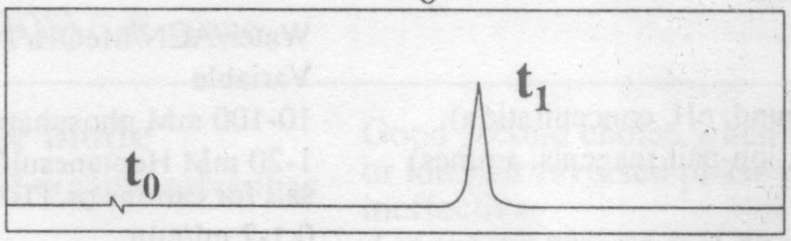
X *: Average*

C.V *: Coefficient of Variation*

Capacity Factor, K'

Capacity factor is relative retention of a sample to the elution of non-retain sample

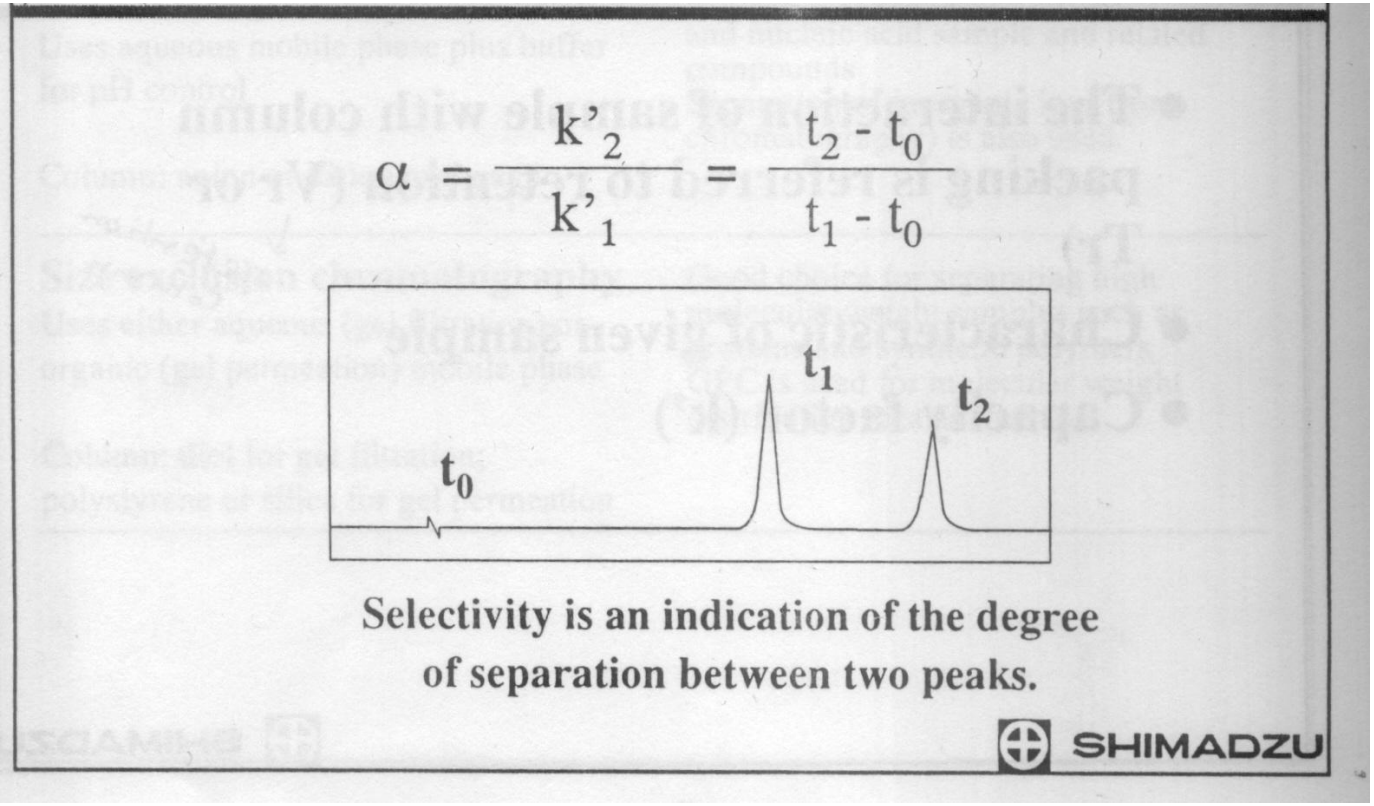
$$k' = \frac{t_1 - t_0}{t_0}$$



t_1 = retention time of a solute peak
t_0 = column void time solvent peak (non retained peak)

SHIMADZU

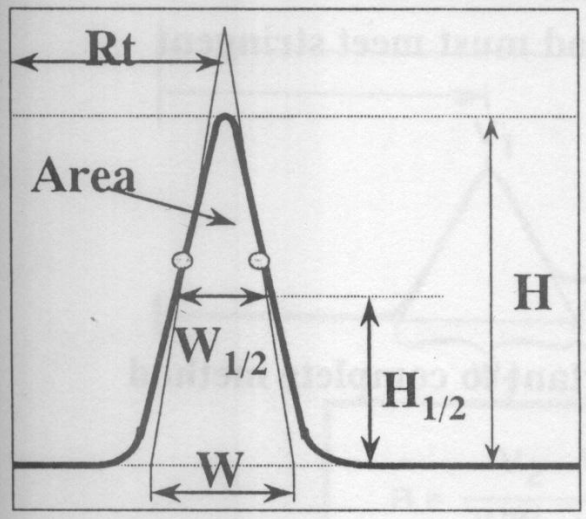
Selectivity/Relative Retention Time, α



RRT = Capacity factor of slower analyte /that of faster analyte.
It should be more than one.

Theoretical Plate

The ability of a column to minimize peak spreading is reffered to column efficiency



Equation :

$$N = 16 \times (Rt / W)^2$$

Modified equation for actual measurement :

$$N = 5.54 \times (Rt / W_{1/2})^2$$

Modified equation for integrator :

$$N = 6.28 \times (Rt \times H / Area)^2$$

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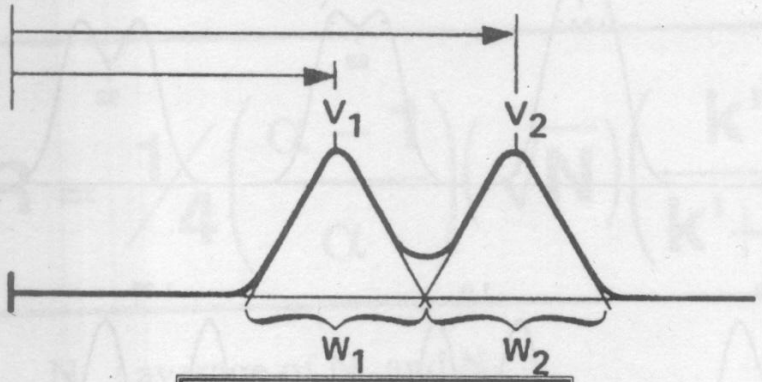
Resolution

Resolution (R or Rs) value for complete separation of two neighboring peaks should be >1.5.



$$R = \frac{V_2 - V_1}{\frac{1}{2}(W_1 + W_2)}$$

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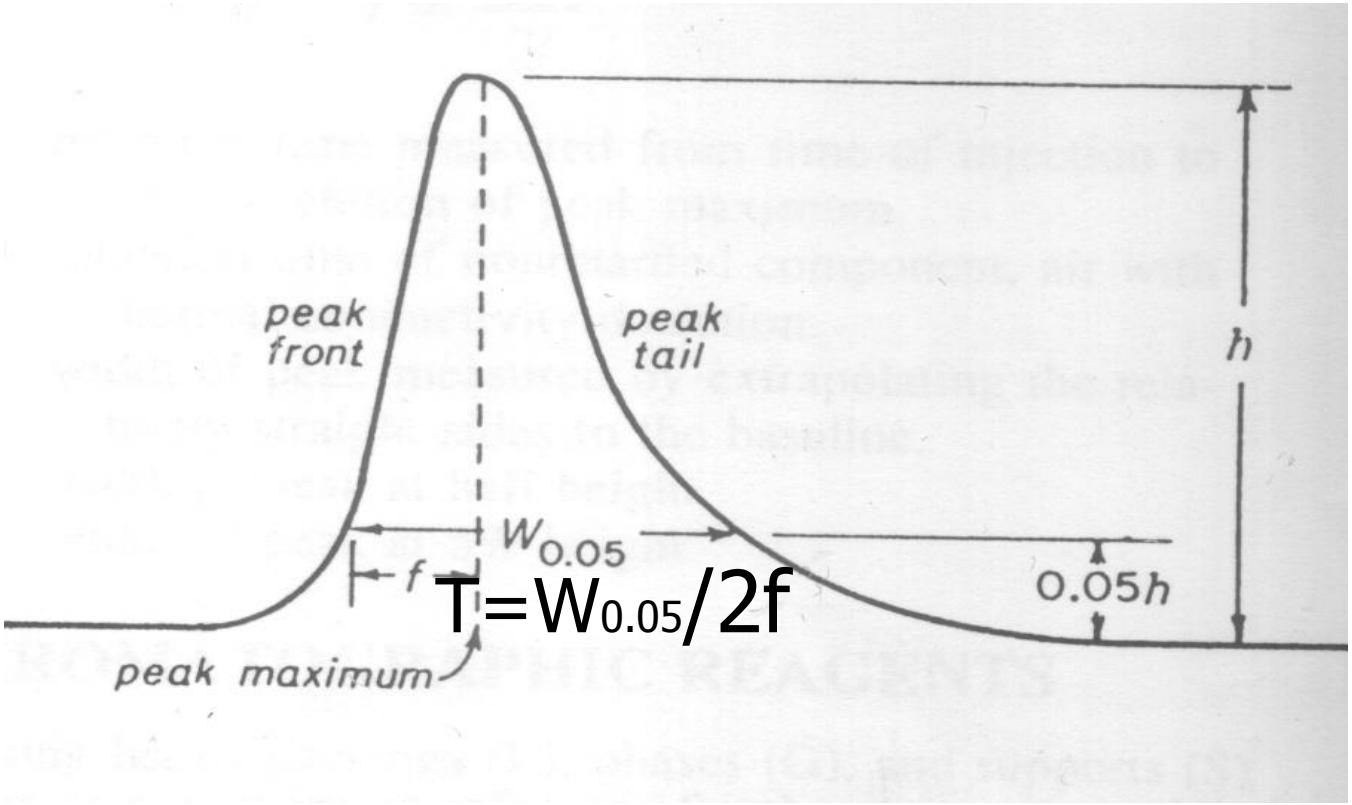
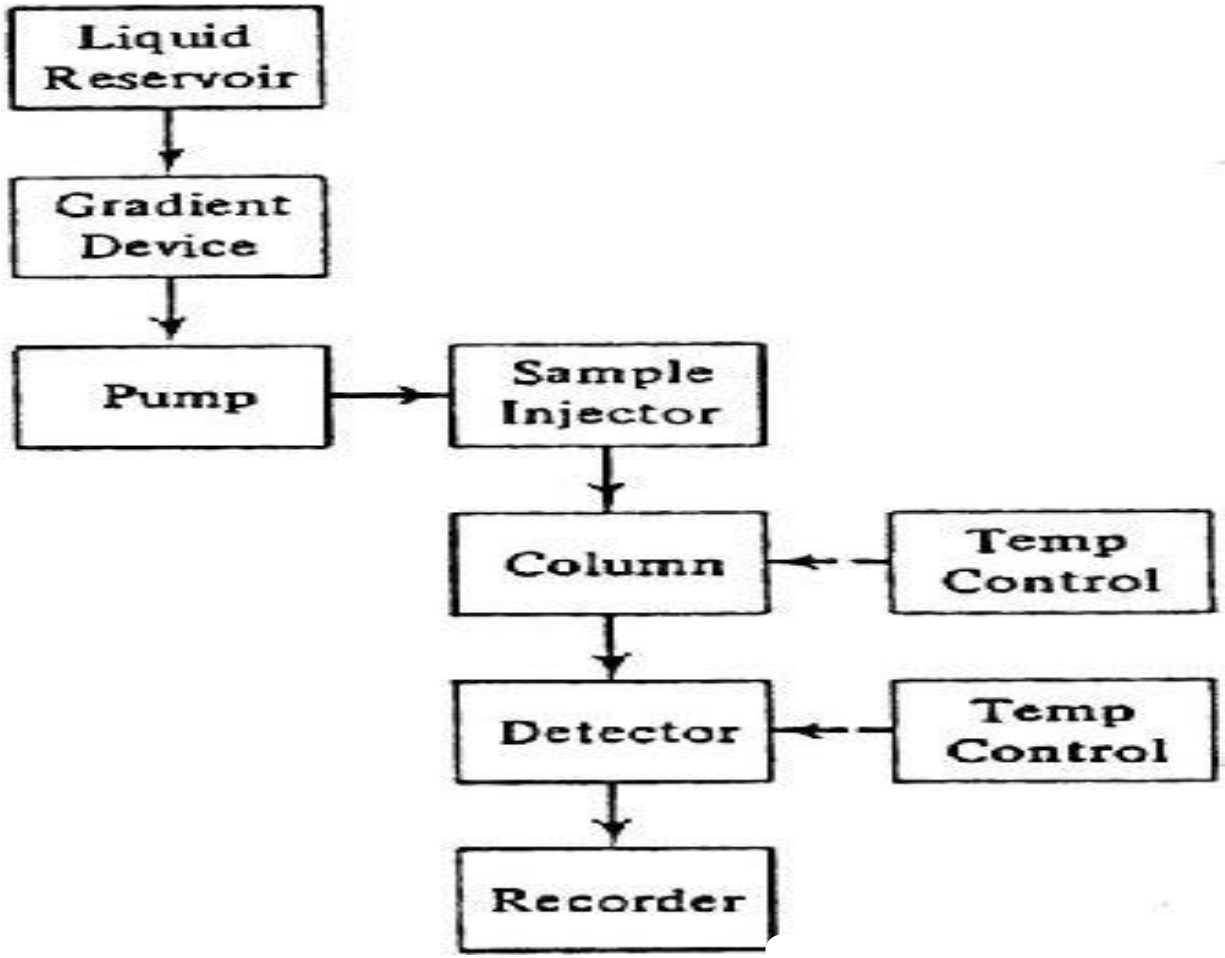
Tailing Factor,T



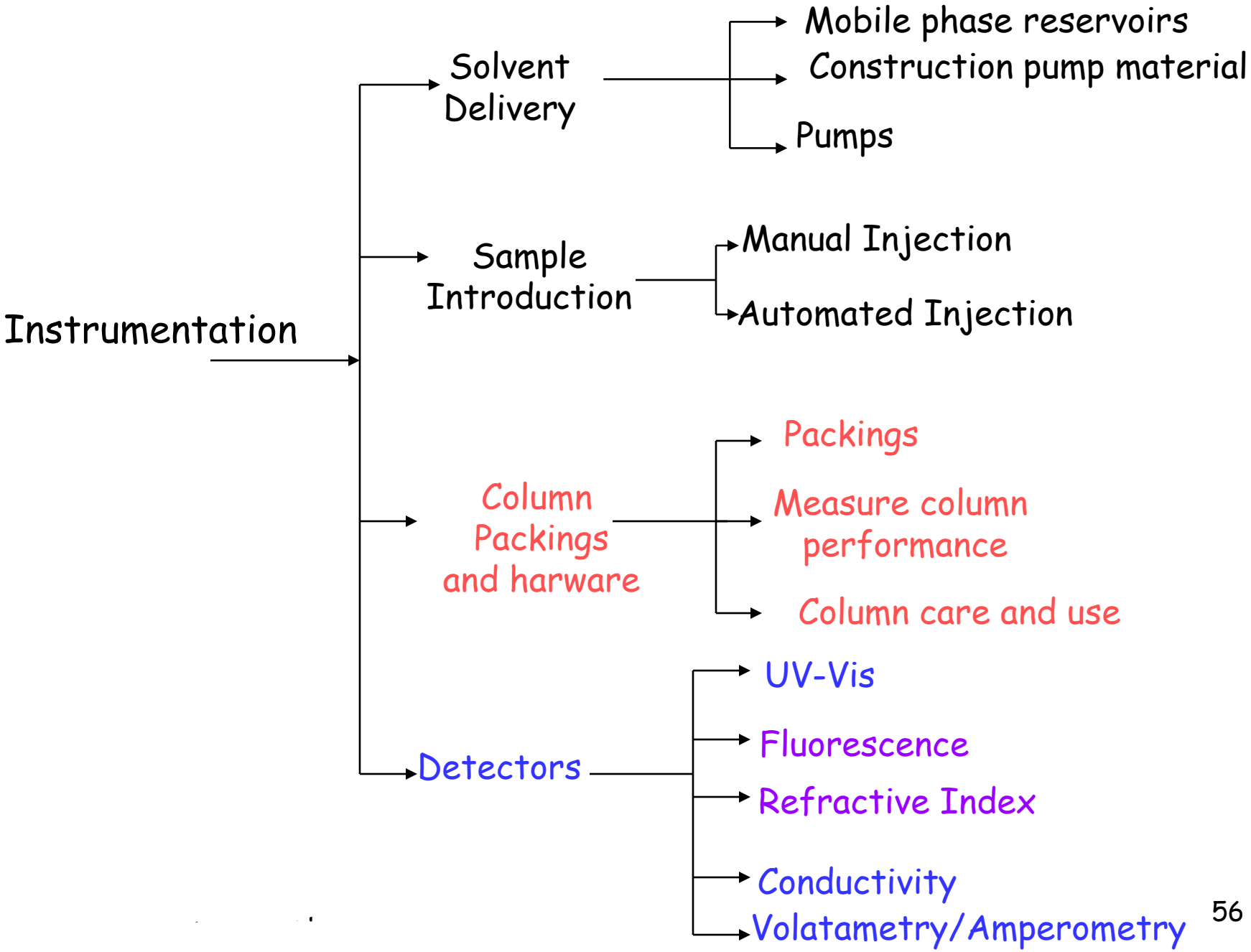
$T = W_{0.05}/2f$

HPLC

Instrumentation: Block diagram of a liquid Chromatography



HPLC Instrumentation and Techniques



Q. What is the purpose of a sinker frit or push filter?

-- Push filter (10 mm) prevent any dust or particulate matter to enter the pump.
--This is because particulate matter can interfere with pumping action, damage valves and seals. In addition, it may also damage the column (by collecting at the top of the column).

Q. Why He degassing of the liquid mobile phase is required?

DEGASSING: the practice of removing air from the mobile phase; degassing can be achieved by bubbling He gas into the M.P

-- He degassing removes dissolve oxygen from the M.P
-- The presence of oxygen in mobile phase causes bubble formation resulting in air in the flow system and pump pressure will change causing error in the chromatogram (due to air bubble formation in the detector cell)

C8 and C18 column

Difference-

C18: octyl decyl chain linked to silica

C8: Only octyl chain linked to silica

C18 is widely used, the polymeric C18 format incorporates a tri-functional silylation procedure whereby the octadecyl group is bonded to 2 or 3 silica atoms on the silica gel backbone. This increased silylation results in far greater column stability particularly in acidic mobile phase conditions. Stereo recognition capability is also greater than that of the monofunctional silylation type of C18.

Advantages of Silica Packing in HPLC

a) Can withstand high pressure generated when 10-30 cm columns with 3-10 mm particles are used.

b) Silica is abundant, inexpensive and available in a variety of shapes, sizes and degree of porosity.

c) Functional group can be readily bonded to silanol and the chemistry of bonding reactions are well understood.

Limitations of Silica Packing in HPLC

--Major limitation is its instability at high and low pH (i.e. above pH 8 or below pH 2)

Advantage of resin-based packing over silica based packing:

---Stability over a wide pH range (1-13)

Disdvantages are:

(a) Unstable in presence of organic solvents

(b) Polymers swell in the organic solvent

Column Care and Use

a. The manufacturer's recommendation regarding M.P pH, flow rates and organic modifier content, temperature as well as maximum operating pressure etc should be followed
b. Use of HPLC grade water and HPLC grade organic solvents, analytical reagent grade chemicals (buffers) must be used to prepare solvents and standards.
c. M.P should always be filtered through an appropriate solvents compatible filter (e.g 0.45 or 0.2 µm filter) and vacuum degassing before use
d. M.P flow rate should be altered in small increments (0.2mL/min -> 1mL/min to avoid column back pressure particularly for softer S.P packings
e. When not in use store column according to manufacture's recommendation

   - For short term storage i.e overnight, columns can be stored in the eluent used in last analysis

   - For middle term storage i.e 2 days or over be weekend, columns should be flushed with pure water to prevent microbial growth.

   - For long term storage, silica based columns should be stored in a suitable solvent. The water content should not be higher than 50%. The best storing solvent is Acetonitrile.

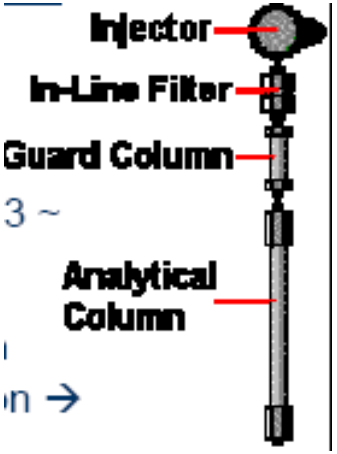
Use of Guard Column in HPLC

Guard column is used at two points in the HPLC flow system

(A) Between pump and the injector -------→ Remove particulates and contaminants from mobile phase

Under very harsh mobile phase conditions (above pH 7 and 40°C, and with a buffer salt concen-tration > 50 mM), a silica saturator column should also be used. As the mobile phase passes through this guard column, it dissolves enough silica to saturate (or nearly so) the mobile phase. Then when the mobile phase reaches the analytical column, no additional packing can dissolve. Therefore, column life is extended at high pH. In addition, some HPLC columns are designed for operation at higher pH.

(B) Between Injector and analytical column --→ Remove contaminants From sample before it enters the column



--Guard columns are very short columns (0.5-3.0cm), which are packed with the same S.P as the analytical column except that the particles are generally large to minimize pressure drops

Guard columns are used primarily to protect the analytical column from strongly adsorbed sample and matrix. Over time the packing dissolves, resulting in increased backpressure, column voids and broad peaks.

When to replace guard columns?

A guard column is most effective if it is replaced before the chromatography of the analytical column deteriorates. This can be hard to determine, so many chromatographers select a specific time interval (every week) or sample interval (every 100 samples) for changing the guard column. This is probably the most effective way to minimize contamination of the analytical column. Alternatively, peak shape deterioration and backpressure increases (no more than 10%)can serve as reminder.

System Suitability Check (Column Performance)

This is to test the suitability of the instrument according to the requirements of prescribed in the individual monograph. By using specified chemical reference substances, adjust the following parameters to comply with the requirements specified in the individual monograph i.e to match-

1. Number of theoretical plates of the column (n)
2. The repeatability
3. Resolution Factor ® of the column
4. Tailing Factor (T) of the column

Procedure:

Preparation of the sample: Powder the sample e.g CMM (Carminomycin) and sieve before analysis. Quantity of the sample powder should be of at least 5 times as much as those needed for analysis.

General requirement for the apparatus:

a. Stationary and mobile phase as per individual monograph
b. ODS (Octadesylsilane) column
c. UV detector
d. Room temperature

1. Theoretical Plate Count (n)

It is a measure of column efficiency. It should not be less than the value specified in the monograph. It is calculated by using the following equation-

$$n = 5.54 \left(\frac{t_R}{W_{h/2}}\right)2$$

Where, t_R = the retention time of the marker peak in the standard solution or analyte peak in the test solution

$W_{h/2}$ = the peak width of the marker peak in the standard solution or analyte peak in the test solution

2. Repeatability

It is expressed as an estimated RSD of at least 5 to 10 replicate injections of the standard solution.

RSD of the peak area and Retention time should comply with the requirement specified in the monograph. Details will be later...

3. Resolution Factor ®

To ensure the accuracy of quantitative analysis, the R value of the analyte peak must be larger than 1.5, unless other wise specified. It is calculated by the following equation-

$$R = \frac{2(t_{R2} - t_{R1})}{W_1 + W_2}$$

Where, t_{R1} and t_{R2} = the retention times of the two adjacent peaks 1 and 2 respectively

W_1 and W_2 = the widths of two adjacent peaks 1 and 2 respectively

4. Tailing Factor (T)

It is necessary to inspect the T value of the peak, especially when using the peak height method. It should also be complied with the requirement specified in the individual monograph. It is calculated by using following equation-

$$T = \frac{W_{0.05h}}{2d_1}$$

Where, $W_{0.05h}$ = the peak width at 0.05 of the peak height

d_1 = the distance between the perpendicular line passing through the peak maximum and the leading edge of the peak at 0.05 of the peak height.

How to assess the precision of Quantitative Measurement

Procedure:

1. Select a suitable substance and make repeated injections of a solution using the HPLC chromatographic conditions suggested for that material
2. Atleast 5 replicate injections should be made
3. Measure the peak height of each peak and if an integrator is available, the peak areas.
4. Calculate the relative **standard deviation (RSD)** (coefficient of variation) of the result

$$RSD = \frac{\sqrt{\frac{\Sigma(x - \bar{x})^2}{(n-1)}}}{\bar{x}} \times 100$$

where, x is the individual peak height (or areas)

$\bar{x}$ is the mean peak height (or the area)

N is the number of replicate injection

Values of RSD below 1% indicate precision is satisfactory. If it is excess of 2% indicates that individual measurement of peak height (or the area) may be subject to considerable error.

UV Detector

UV-visible detectors were among the first detectors utilized for liquid chromatography and remain by far the most popular. Three popular types-

a. Fixed-wavelength detectors which operate from a discrete source.

b. Variable-wavelength detectors which utilize a continuum source and a monochromator.

c. Rapid-scanning detectors which are generally based on a linear photodiode-array light detector.

A fixed-wavelength detector will generally offer lower limits of detection than a variable-wavelength detector operated at the same wavelength.

Variable-Wavelength detector with the use of a silicon photodiode light detector gives a very inexpensive detector with high signal-to-noise ratio. The inherent sensitivity of this detector makes it useful for virtually all organic compounds with any degree of conjugation or other chromophore. This detector can also be used at other wavelengths by filtering the emission source to give other lines, or even using phosphor screens to give lines not available from mercury. These other wavelengths will have significantly lower signal-to-noise ratios.

- **The popularity of variable-wavelength detectors has increased greatly, both due to improved sensitivity from operating at the wavelength of maximum absorbance of the solute of interest, and also due to the ability to operate at wavelengths where other solutes will *not* absorb. Perhaps the greatest operating difficulty with these detectors is the somewhat limited lamp lifetime, especially when compared to the low-pressure mercury lamp used in the fixed-wavelength 254nm detector.**

Refractive Index Detector

RI detectors are an excellent example of the problems facing universal detectors. They respond to changes in the refractive index of the mobile phase, and this changes not only with solute concentration, but also with temperature, pressure, dissolved gases, and changes in mobile-phase conditions. Therefore, they are generally applicable only for isocratic separations, and to be operated at maximum sensitivity they need careful temperature and flow control.

Problems associated with HPLC technique

A. Baseline:- 1. drift, 2. noise, irregular, 3. noise regular
B. Column back pressure: 1. higher than usual, 2. lower than usual
C. Ghost Peak
D. Peak shapes, incorrect: 1. broad, 2. fronting, 3.rounded, 4. split, 5. tailing
E. Peaks: 1. height change, 2. missing, 3.negative, 4. no peaks, 5. unresolved
F. Retention times, variable
G. Selectivity Change

Some problems are discussed below:-

1. <u>No peaks/Very small peaks</u>:

<u>Probable Cause</u>: **a**. Detector lamp off **b**. Loose/broken wire between detector and integrator or recorder **c**. No mobile phase flow **d**. No sample/deteriorated sample/wrong sample **e**. Settings too high on detector or recorder

<u>Remedy/Comment</u>: **a**. Turn lamp on **b.** Check electrical connections and cables **c**. No flow (see problem no.2) **d.** Be sure automatic sampler vials have sufficient liquid and no air bubbles in the sample. Evaluate system performance with fresh standard to confirm sample as source of problem **e**. Check attenuation or gain settings. Check lamp status. Auto zero if necessary.

2. No flow

Probable causes:- **a**. Pump off **b**. Flow interrupted/obstructed **c**. Leak **d**. Air trapped in pump head (Revealed by pressure fluctuations)

Remedy/Comments: **a**. Start pump **b**. Check mobile phase level in reservoir. Check flow through out the system. Examine sample loop for obstruction or air lock. Make sure mobile phase components are miscible and mobile phase is properly degassed. **c**. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary **d.** Disconnect tubing at guard column (if necessary) or analytical column inlet. Check for flow. Purge pump at high flow rate (e.g 5 to 10 ml/min), prime system if necessary. (Prime each head separately). If system has check valve, loosen valve to allow air to escape. If problem persists, flush system with 100% methanol or isopropanol. If problem still persists, contact system manufacturer.

3. Tailing peaks

Probable cause: a. Guard or analytical column contaminated/worn out **b**. Mobile phase contaminated/deteriorated **c.** Interfering components in sample

Remedy:- **a**. Remove guard column (if present) and attempt analysis. Replace guard column if necessary. If analytical column is source of problem, use appropriate restoration procedure. If problem persists, replace column. **b**. Check make up of mobile phase **c.** Check column performance with standard

4. Ghost Peak

Probable Causes: **a**. Contamination in Injector or column **b.** Late eluting peak (usually broad) present in sample

Remedy: **a**. Flush injector between analyses (a good routine practice). If necessary, run strong solvent through column to remove late eluters. Include final wash step in gradient analyses, to remove strong retained compounds **b**. (i). Check sample preparation (ii) Include (step) gradient to quickly elute components.

5. Negative Peak(s):

Probable Cause: **a**. Recorder leads reversed **b**. Refractive index of solute less than that of mobile phase (RI Detector) **c**. Sample solvent and mobile phase differ greatly in composition **d.** Mobile phase more absorptive than sample components to UV wavelength.

Remedy: **a**. Check polarity **b**. Use mobile phase with lower Refractive Index or reverse recorder leads **c**. Adjust or change sample solvent. Dilute sample in mobile phase whenever possible **d.** (i) Change polarity when using indirect UV detection. Or (ii) Change UV wavelength or use mobile phase that does not adsorb chosen wavelength

6. No Pressure/Pressure Lower than Usual

Probable cause: **a**. Leak, **b.** M.P. flow interrupted/obstructed, **c.** Air trapped in pump head (revealed by pressure fluctuation), **d**. Leak at column inlet end fitting, **e.** Air trapped elsewhere in system, **f**. Worn pump seal causing leaks around, **g.** Faulty check valve, **h**. Faulty pump seal

Remedy : **a.** Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary. **b.** Check mobile phase level at reservoir. Check flow throughout system. Examine sample loop for obstruction or air lock. Make sure mobile phase components are miscible and properly degassed. **c.** Disconnect tubing at guard column (if present) or analytical column inlet. Check for flow. Purge pump at high flow rate (e.g 10ml/min.), prime system if necessary. Prime each pump head separately. If system has check valve, loosen valve to allow air to escape. **d.** Reconnect column and pump solvent at double the flow rate. If pressure is still low, check for leaks at inlet fitting or column end fitting. **e**. Disconnect guard and analytical column and purge system. Reconnect columns. If problem persists, flush system with 100% methanol or isopropanol. **f.** Replace seal. If problem persists, replace piston and seal. **g.** Rebuild or replace valve. **h.** Replace seal

7. Pressure Higher Than Usual

Probable Cause: **a**. Problem in pump. Injector. In-line filter, or tubing **b**. Obstructed guard column or analytical column

Remedy : a. Remove guard column and analytical column from system. Replace with union and 0.010" I.D. or larger tubing to reconnect injector to detector. Run pump at 2-5 ml/min. **If pressure is minimal, follow cause and remedy b.** If not, isolate cause by systematically eliminating system components, starting with detector, then in-line filter, and working back to pump. Replace filter in pump if present. **b**. Remove guard column (if present) and check pressure. Replace guard column if necessary. If analytical column is obstructed, reverse and flush the column, while disconnected from the detector. If problem persists, column may be clogged with strongly retained contaminants. Use appropriate restoration procedure. If problem still persists, change inlet frit or replace column.

8. Loss of Resolution :

Probable Cause : **a**. Mobile phase contaminated /deteriorated (causing retention times and or selectivity to change). **b.** Obstructed guard or analytical column.

Remedy : **a**. Prepare fresh mobile phase **b**. Remove guard column and attempt analysis. Replace guard column (if necessary). If analytical column is obstructed, reverse and flush. If problem persists, column may be clogged with strongly retained contaminants. Use appropriate restoration procedure. If problem still persists, change inlet frit or replace column.

Thank You