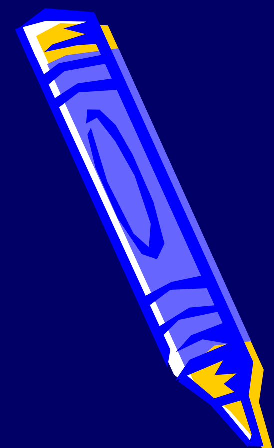
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY



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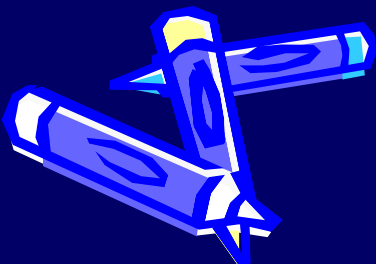
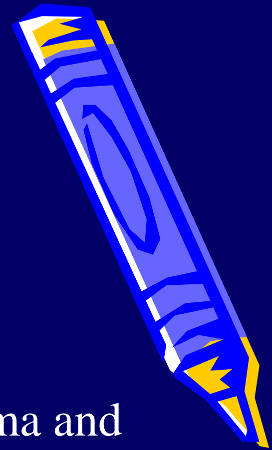


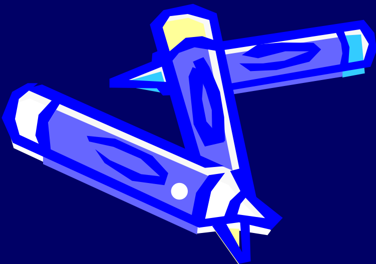
Chromatography: What does it mean?



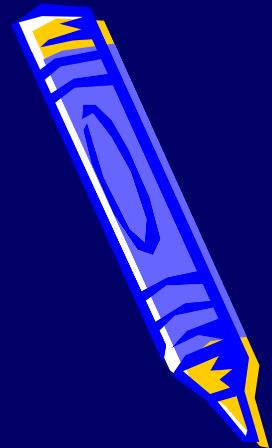
- To write with colors -- literally translated from its Greek roots chroma and graphein

Mikhail Tswett in 1903

- 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.
- First Chromatographic Separation



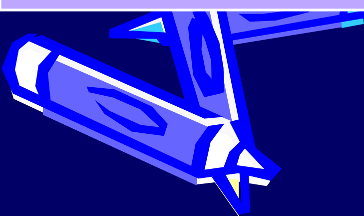
Chromatography -- what does it mean?



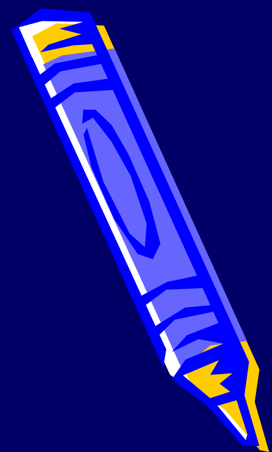
Tswett stated : Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system

Chromatography: Method
Chromatograph: Machine
Chromatographer: Person
Chromatogram: Data

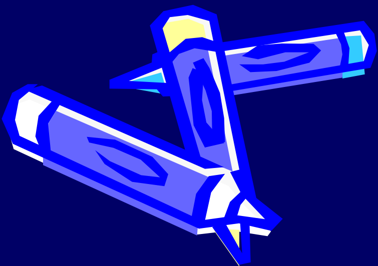
Analytical Nomenclature of IUPAC:
Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction (IUPAC, 1993).



Chromatographic Tree

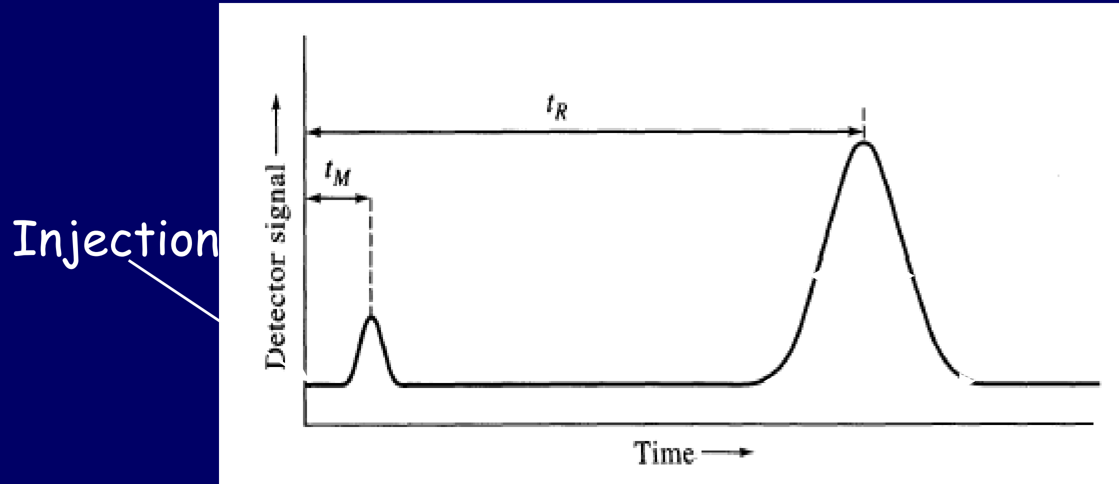


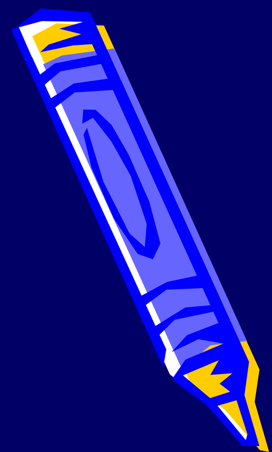
- Chromatography
  - Gas Chromatography
  - Liquid Chromatography
    - Planer Chromatography
    - Column Chromatography
      - HPLC

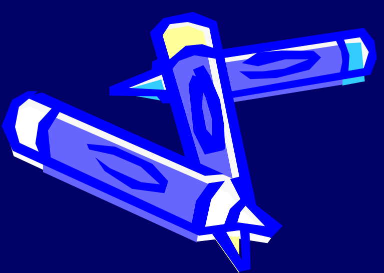


Chromatogram

Typical response obtained by chromatography



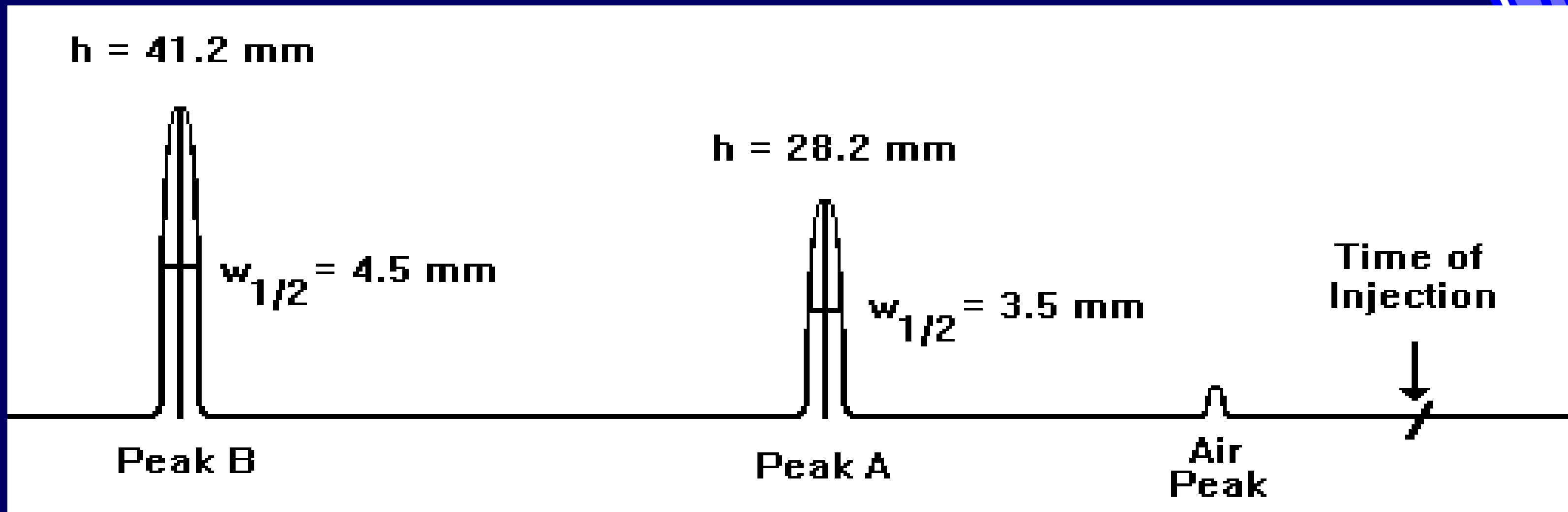




Where:

t_R = retention time
t_M = void time
W_b = baseline width of the peak
W_h = half-height width of the peak
h = Height of peak

Calculating Area



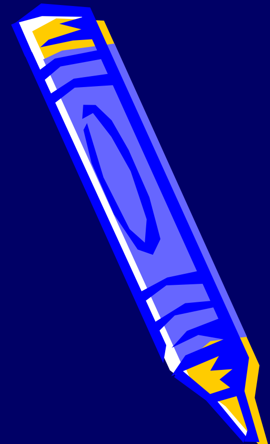
- For example, using a ruler, the Peak A was measured to have a height of 28.2 mm and a width at the half-height of 3.5 mm. Peak B has a height of 41.2 mm and a width at half-height of 4.5 mm. Therefore, the areas of the peaks can be calcuated a

$$\text{Area} = h * w_{1/2}$$

Peak A: $28.2 \text{ mm} * 3.5 \text{ mm} = 98.7 \text{ mm}^2$

Peak B: $41.2 \text{ mm} * 4.5 \text{ mm} = 185.4 \text{ mm}^2$

Using these areas, the percent of each compound in the sample can be calculated.



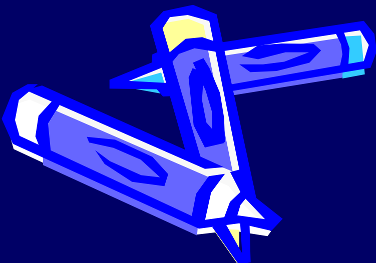
The Aim of Separation

- Qualitative Analysis:

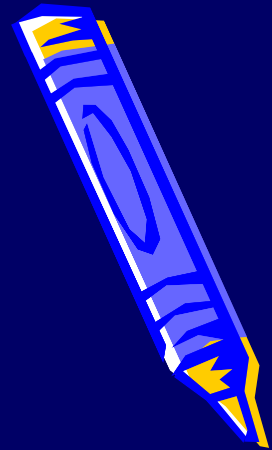
  What Chemical Compounds are included in the sample

- Quantitative Analysis:

  What amount of the compounds are included in the sample



Qualitative Analysis by HPLC



Sample (Unknown)

Detector Response

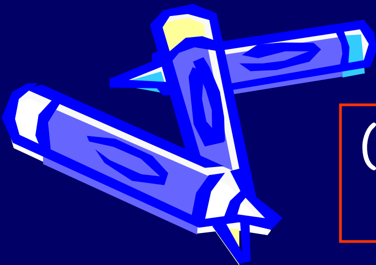
Standard (Known)

Component A

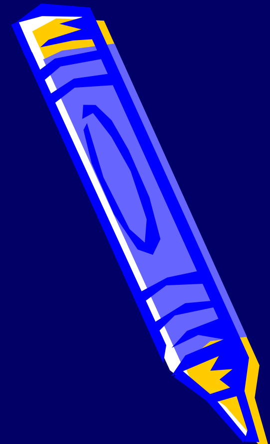
Component B

Retention time (min)

(Sample components elute at the same retantion time if the analytical conditions are same)



Quantitative Analysis by HPLC



$Au/As = Cu/Cs$

Component A

Component B

Sample (Unknown) 20 µL

Peak Area 700

Peak Area 1000

Detector Response

Standard (Known) 20 µL

Component A

100 ppm

Peak Area 1000

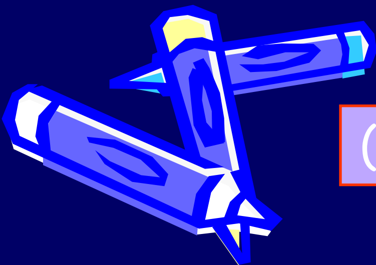
Component B

50 ppm

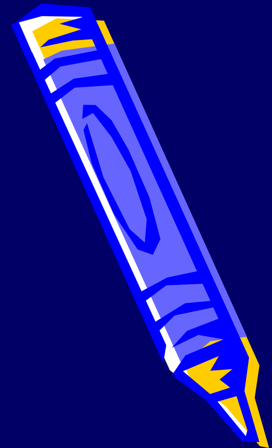
Peak Area 500

Rt_1 Rt_2

Retention time (min)



(Peak Area is proportional to the amount of analyte)



Quantitative Analysis HPLC

- $Y = mx + c^2$

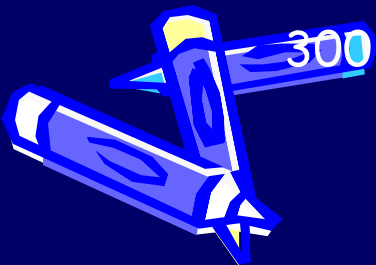
Calibration Curve

1500
1300
Component B in Sample
1000
Component A in sample
700
500
300

Peak Area

25 50 75 100 150

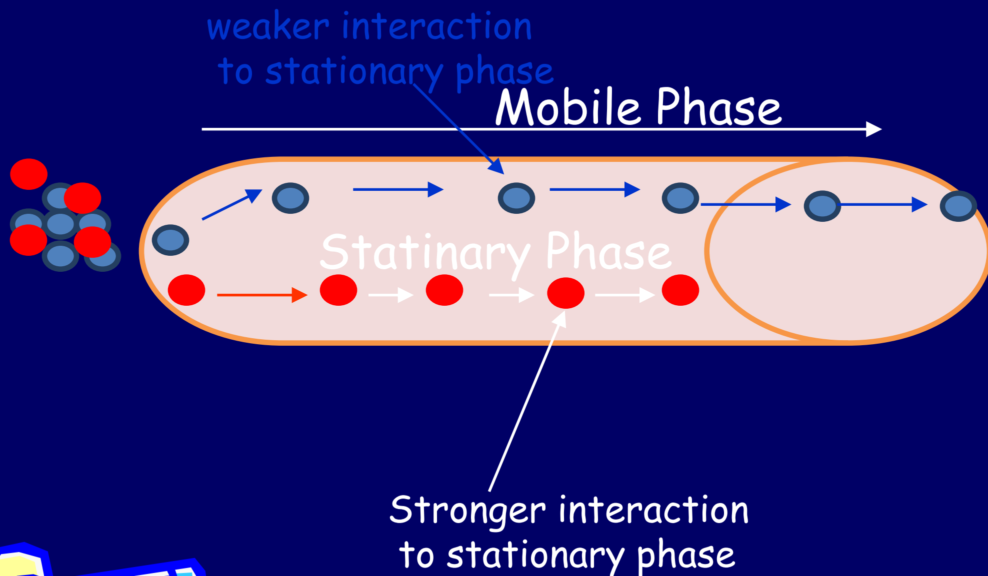
Concentration (ppm)

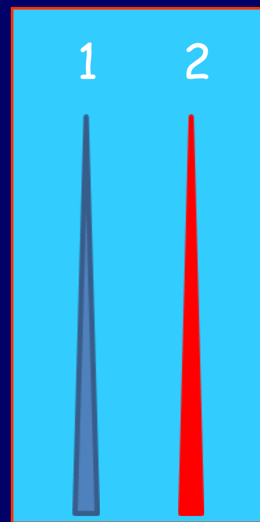


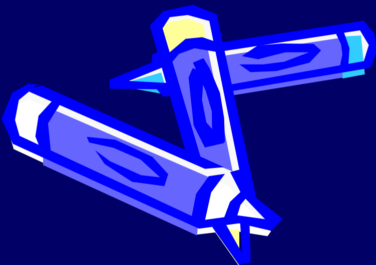
Separation Principle

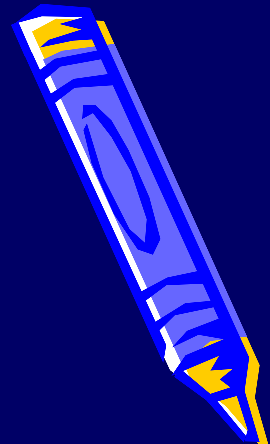
(compounds have different interactions)

- Separation Technique Compounds are separated due to the molecules moves at different rates in the column.
- Due to different interaction between stationary phase and different sample, the molecules move at different rate, therefore separation can be done.

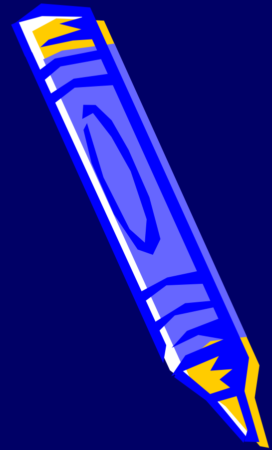






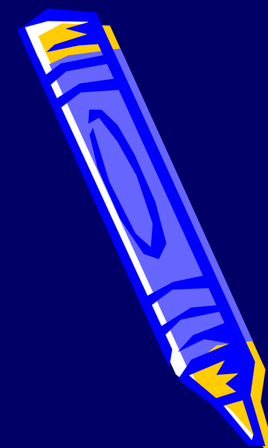


Types of HPLC Techniques



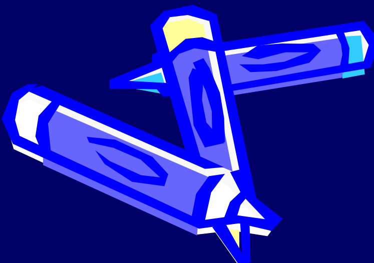
- Based on Modes of chromatography
  - Normal Phase HPLC
    Stationary Phase: Polar
    Mobile Phase: Non Polar
  - Reverse Phase HPLC
    Stationary Phase: Non Polar
    Mobile Phase: Polar
- Based on scale of operation
  - Analytical HPLC
  - Preparative HPLC

Types of HPLC



Based on principle of separation

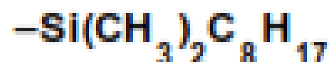
- Adsorption Chromatography
- Ion Exchange Chromatography
- Size Exclusion Chromatography
- Affinity Chromatography
- Chiral Chromatography



Stationary Phase Ligands

Stationary phase — **Functionality**

- C_{18} —

- C_8 — 

- tC_2 —

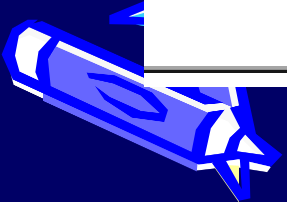
- Aminopropyl —

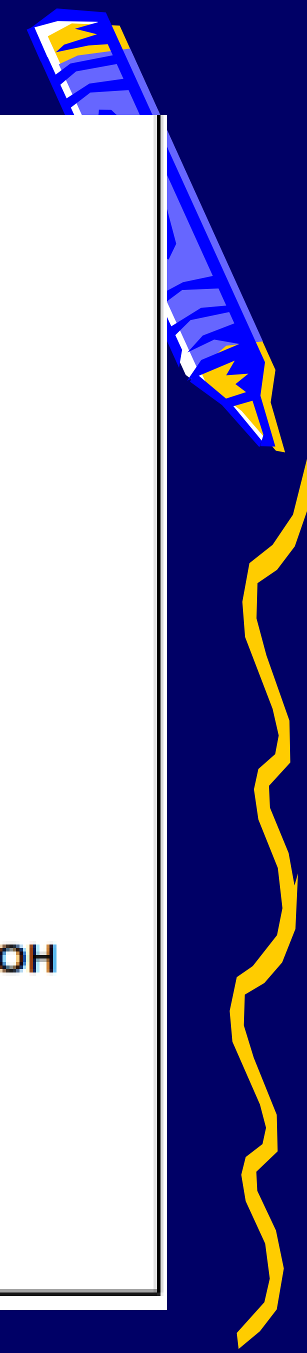
- Cyanopropyl —

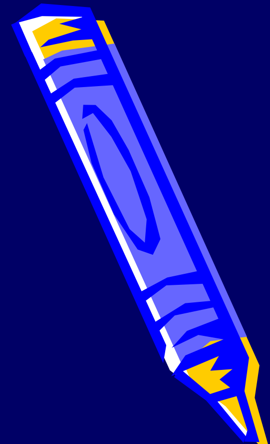
- Diol —

Retention time

Chain length CN Phenyl NH2 C4 C8 C18

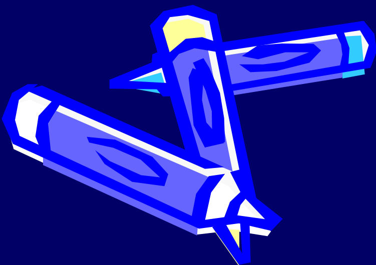


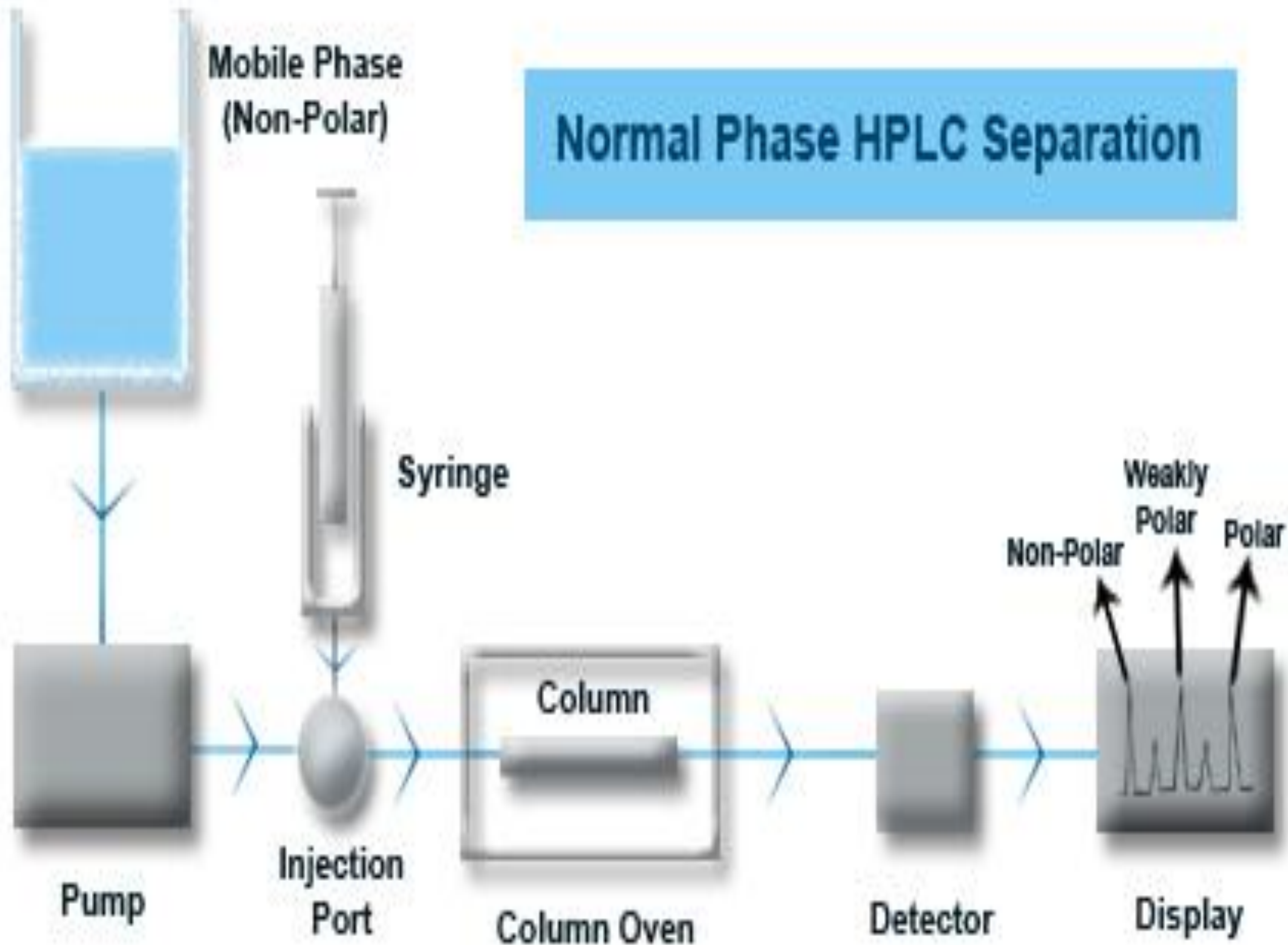




Normal Phase HPLC

- In this mode, the column packing is polar (e.g. silica
- gel, cyanopropyl-bonded, amino-bonded, etc.) and
- the mobile phase is non-polar (e.g. hexane, iso-
- octane, methylene chloride, ethyl acetate)
- Normal phase separations are performed less than 10% of the time.
- The technique is useful for:
- water-sensitive compounds

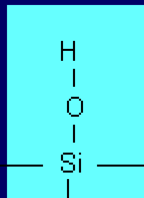
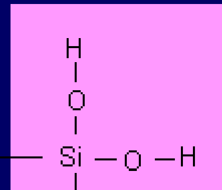
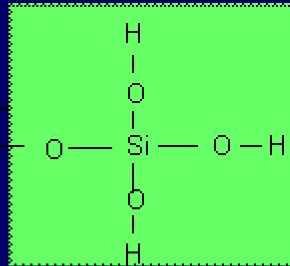


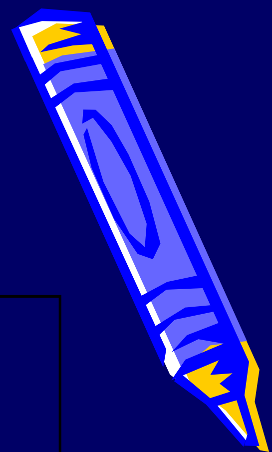
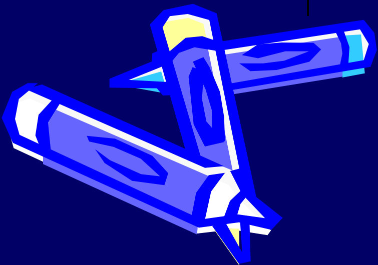


Normal Phase HPLC Separation
Mobile Phase
(Non-Polar)
Syringe
Weakly
Polar
Non-Polar
Polar
Column
Pump
Injection
Port
Column Oven
Detector
Display

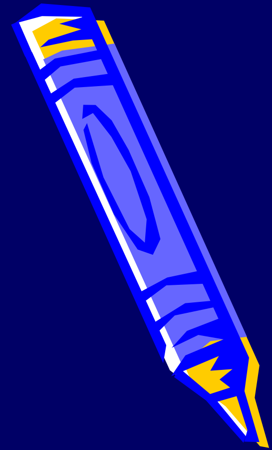
Stationary Phase

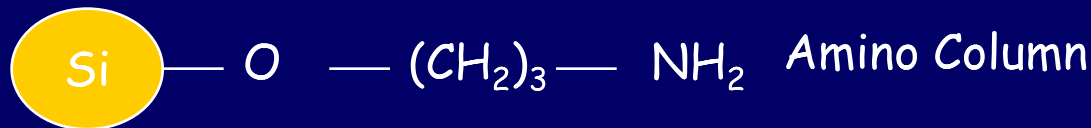
- SiO_2
- O===Si===O + H_2O → Silica gel

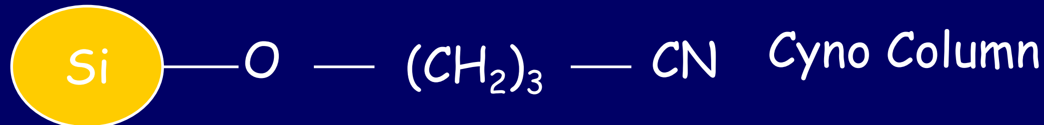




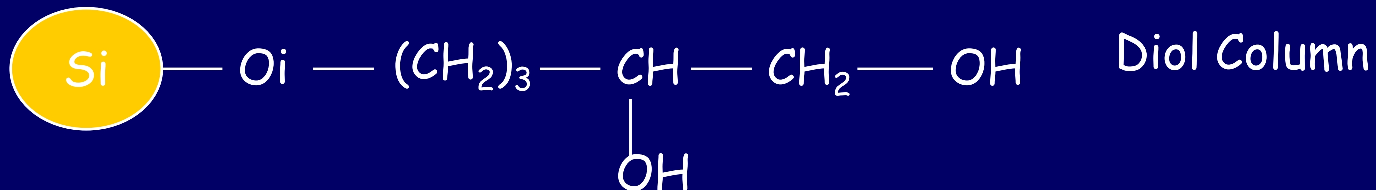



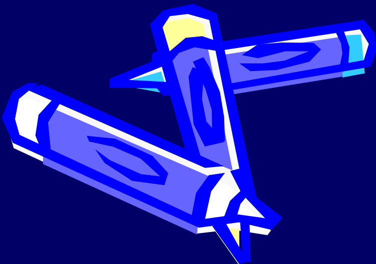
Stationary Phase for Normal phase



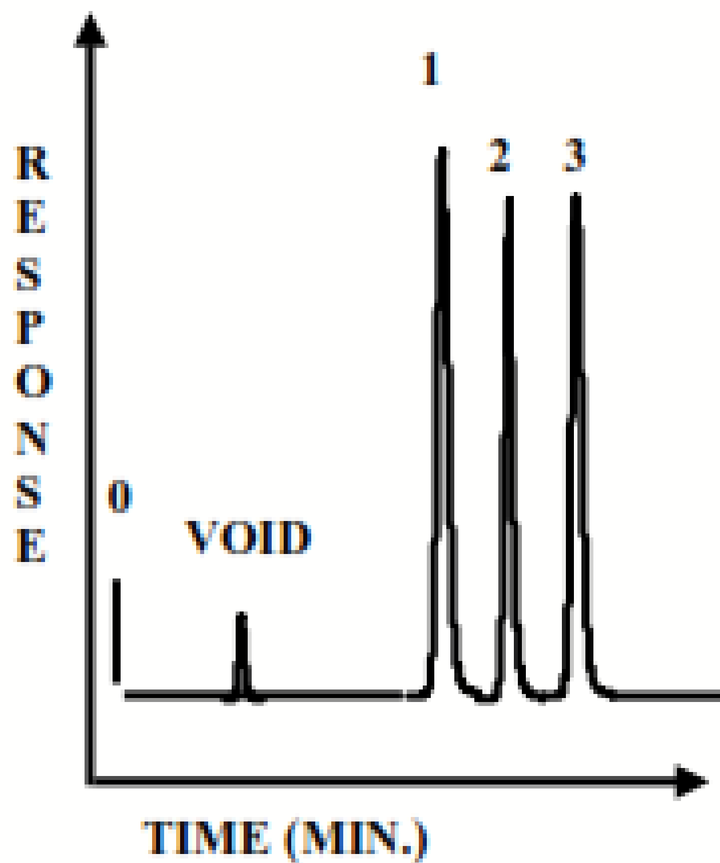


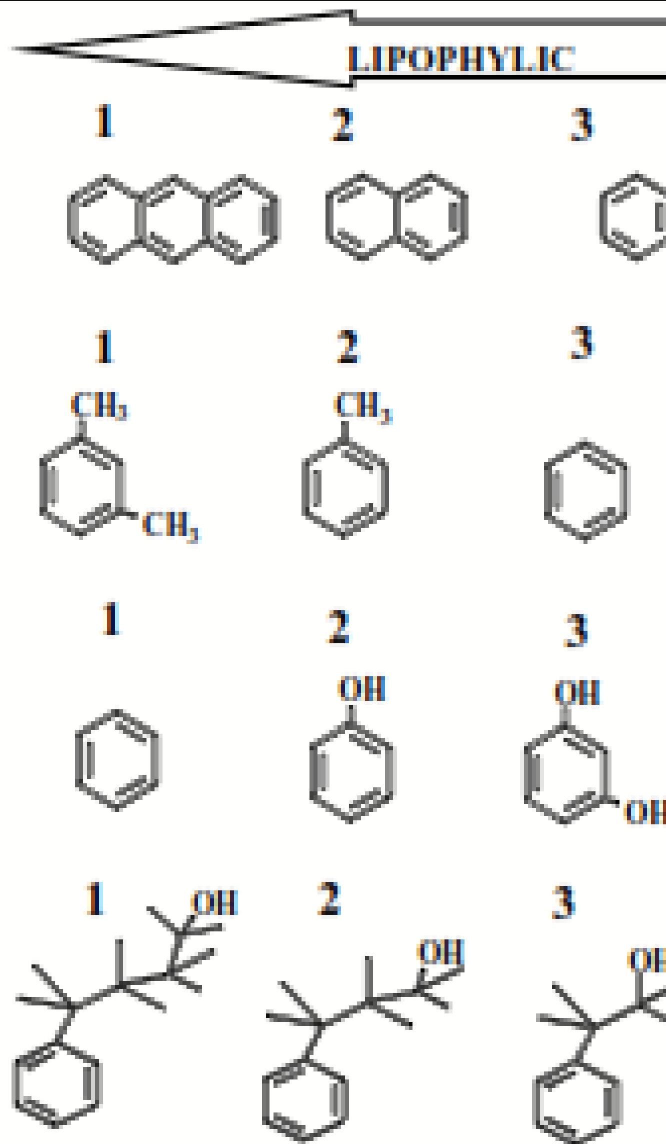




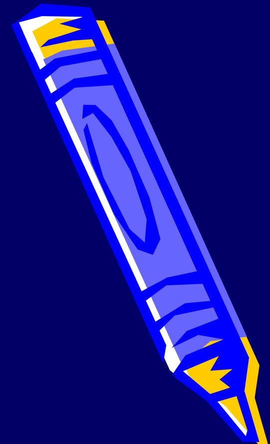


ELUTION ORDER IN NORMAL PHASE

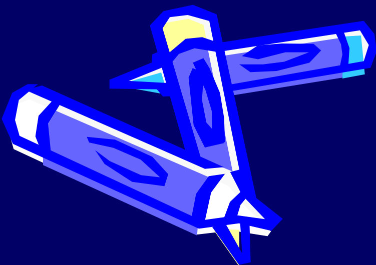


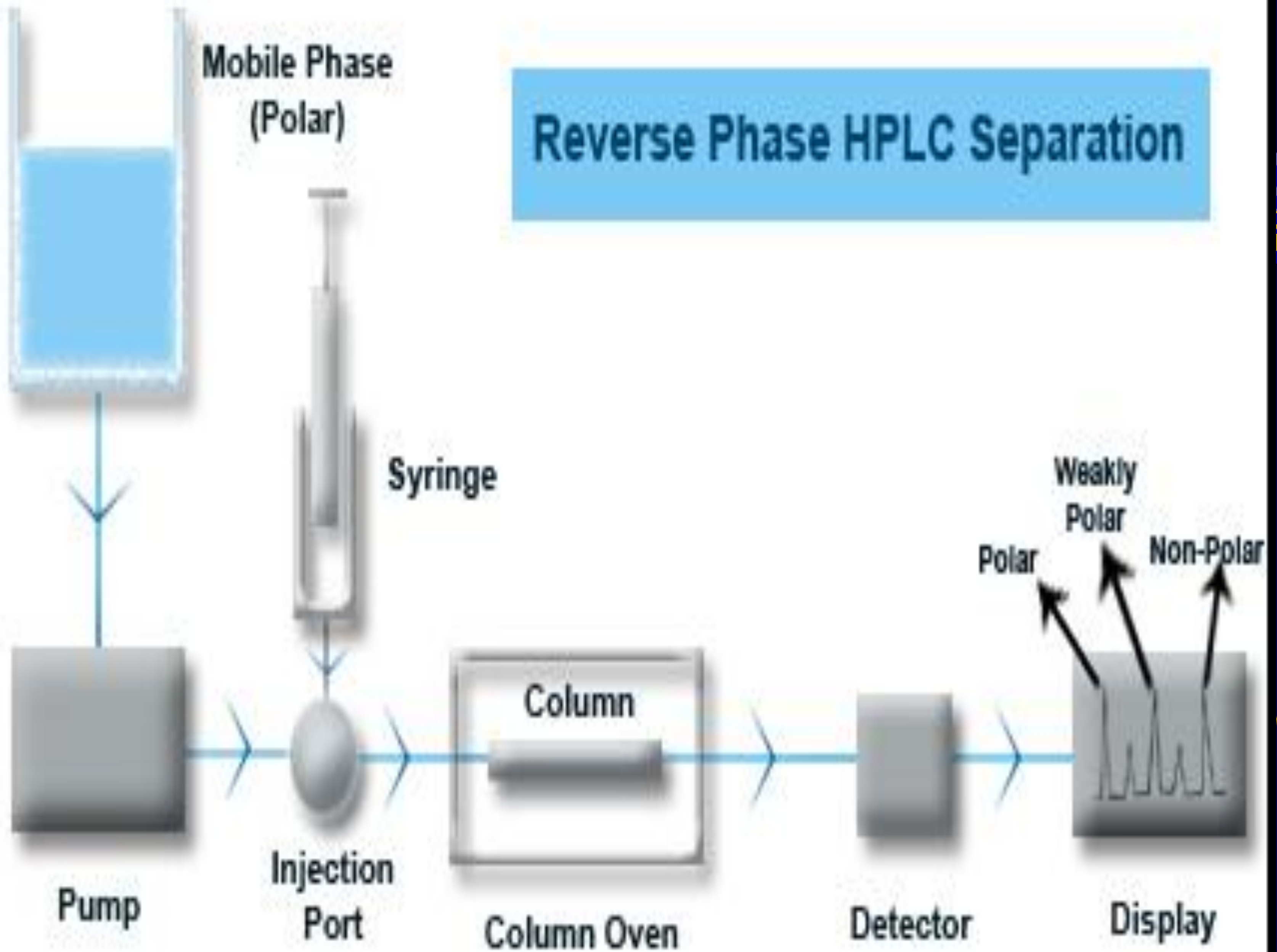


Reverse Phase HPLC

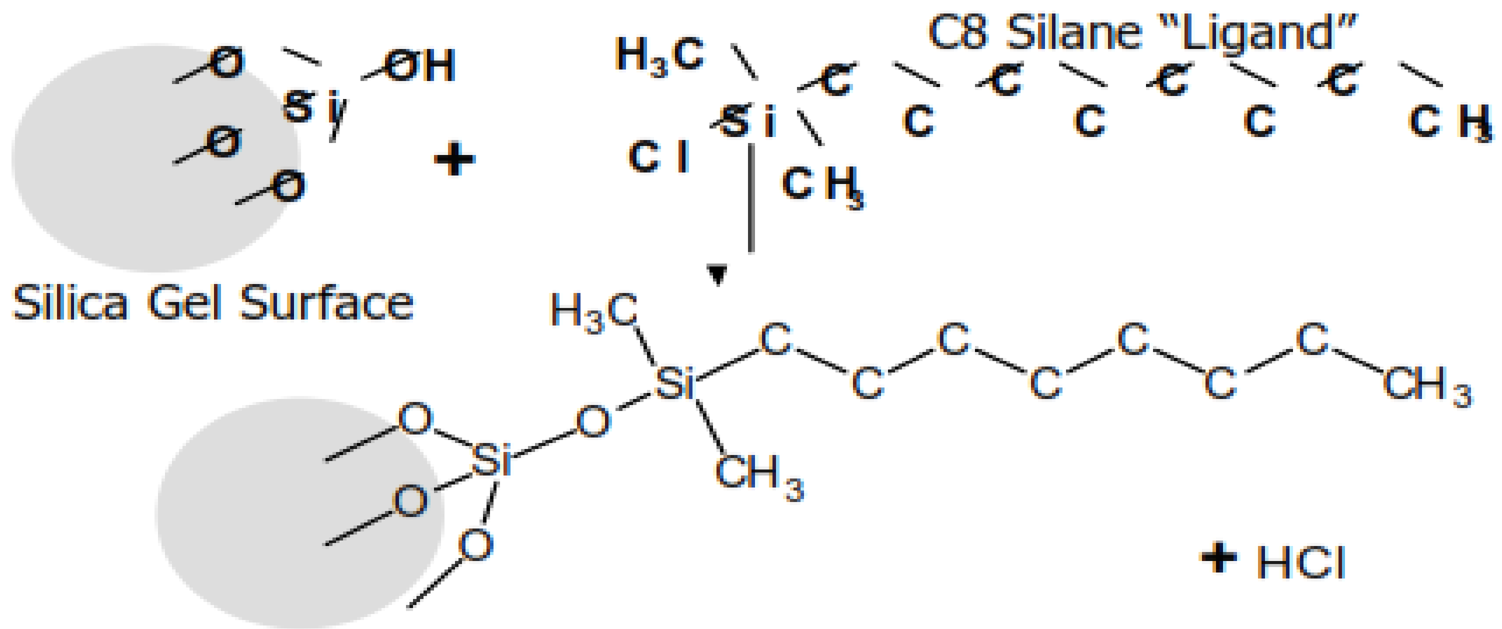


- The column packing is non-polar (e.g. C18, C8,
- C3, phenyl, etc.) and the mobile phase is water
- (buffer) + water-miscible organic solvent
- (e.g. methanol, acetonitrile)
- • RPC is, by far, the most popular mode …
- • over 90% of chromatographers use this mode
- • The technique can be used for non-polar, polar,
- ionizable and ionic molecules …
- • making RPC very versatile

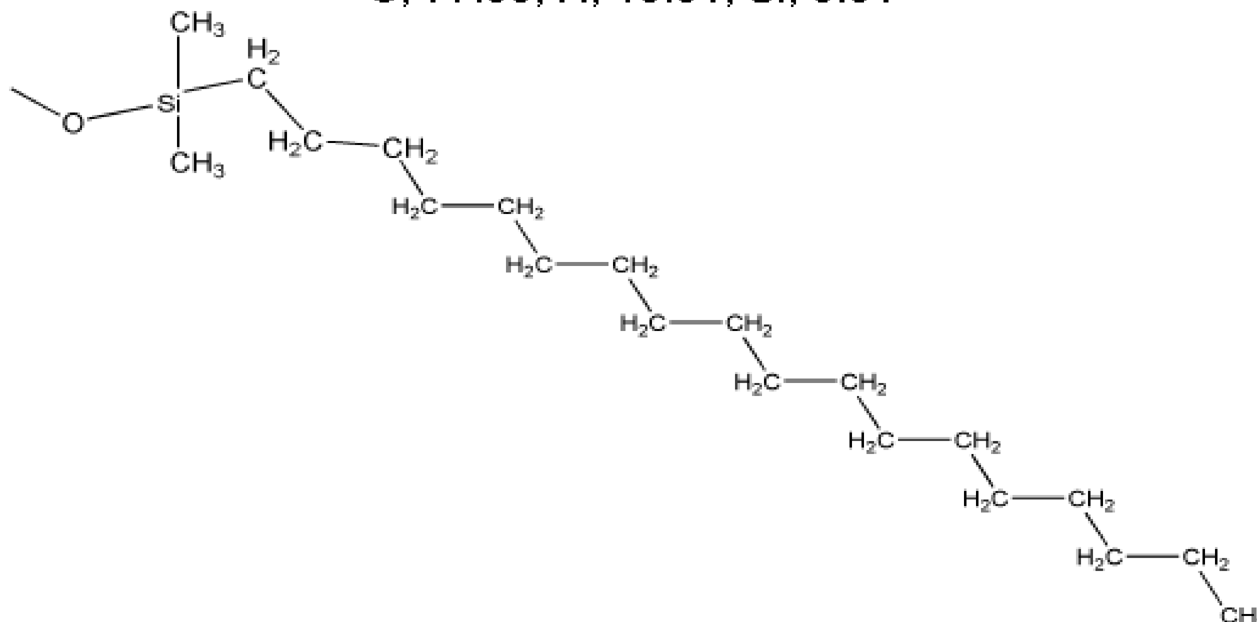



Reverse Phase HPLC Separation
Mobile Phase
(Polar)
Syringe
Weakly
Polar
Polar
Non-Polar
Column
Pump
Injection
Port
Column Oven
Detector
Display

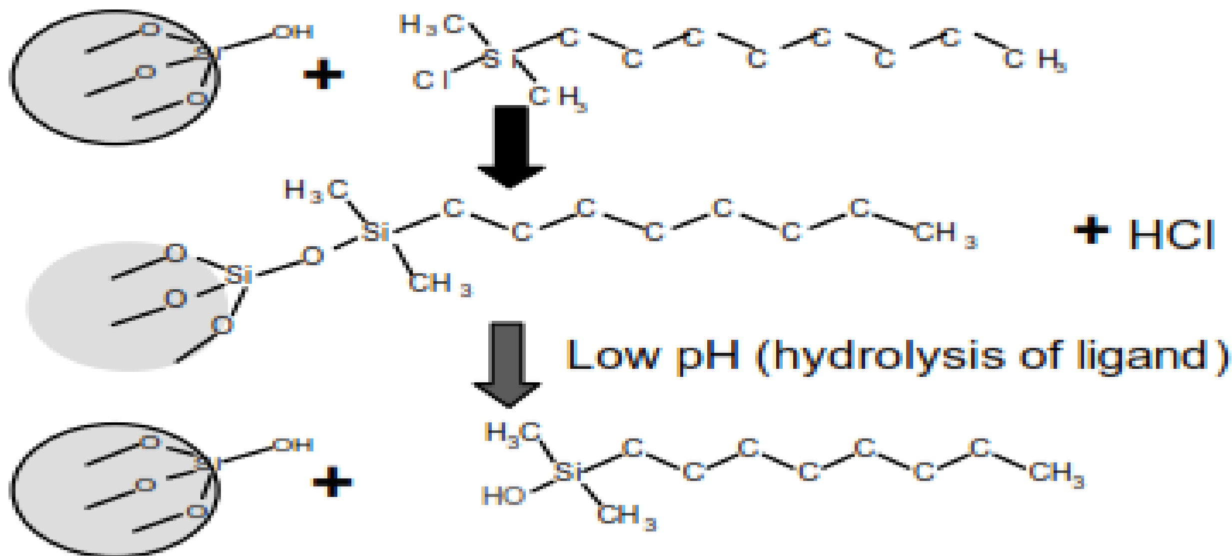
Making a Bonded Phase Material: Monofunctional Synthesis



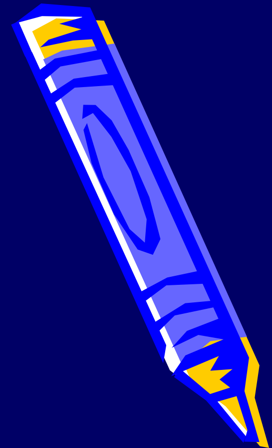
RP-18 Stationary Phase

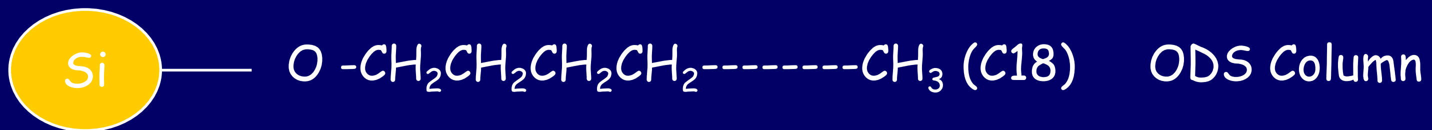


pH affect on Silica based column

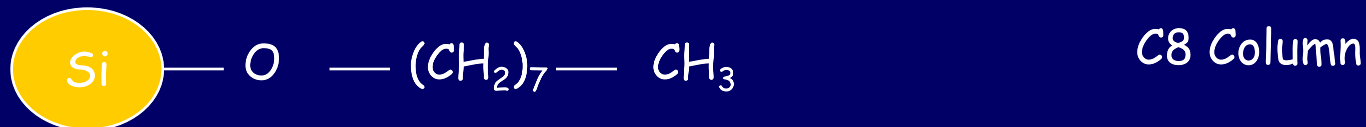


Stationary Phase for Reverse phase

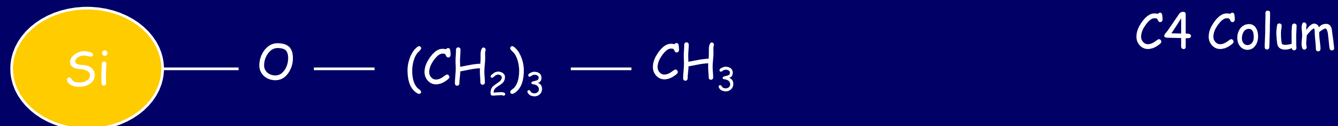




ODS Column

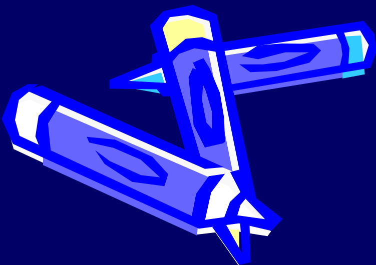


C8 Column

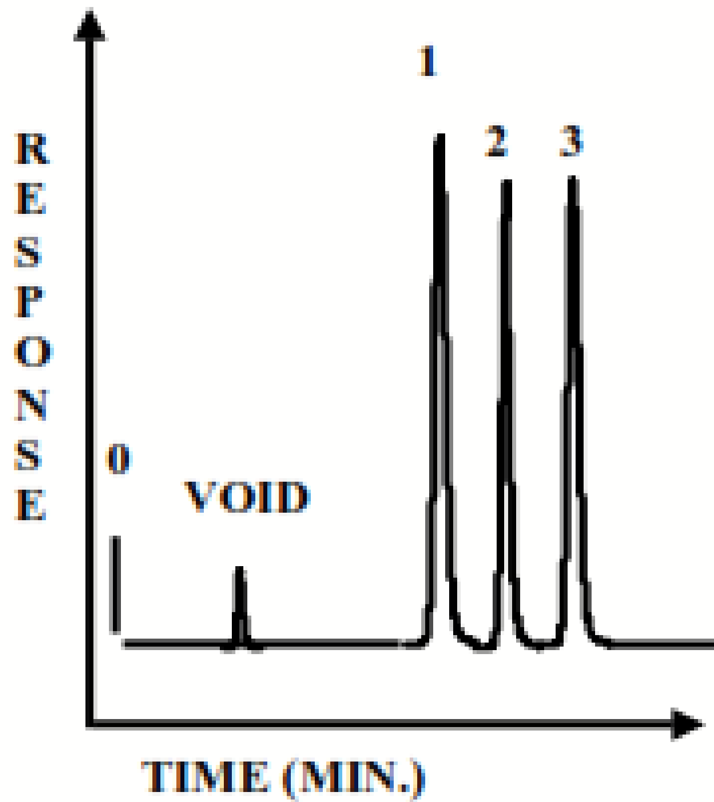


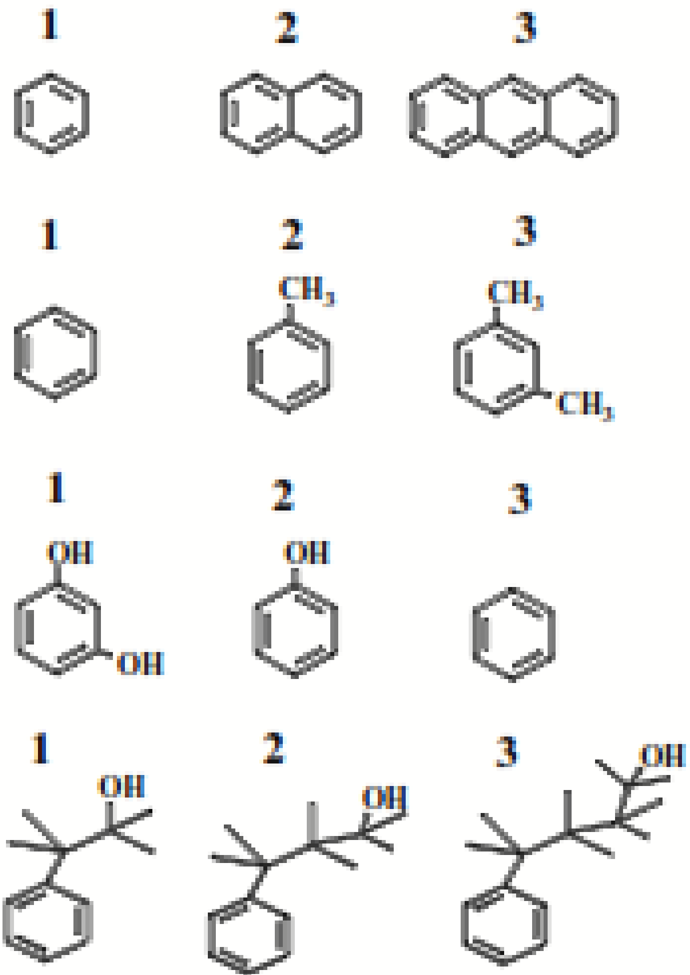
C4 Colum

Phenyl Column



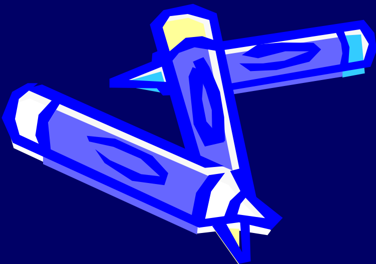
ELUTION ORDER IN REVERSED PHASE

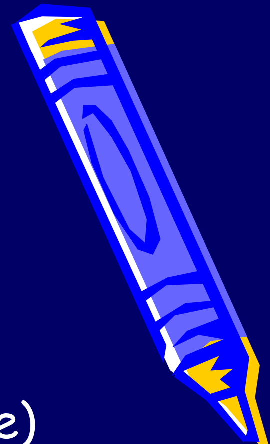




HPLC instrumentation

1. M.P reservoirs
2. Eluent degas module
3. Solvent delivery pumps
4. Manual / Auto injector
5. Analytical column
6. Detector
7. Data processor

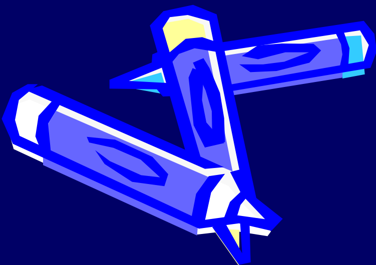


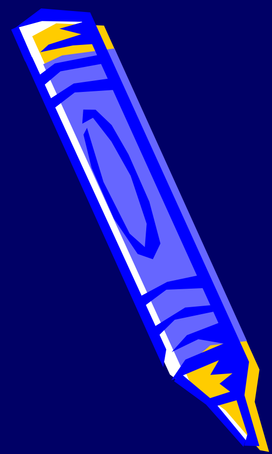


PUMP

- 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).

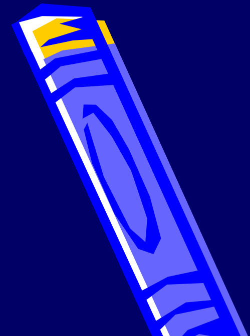


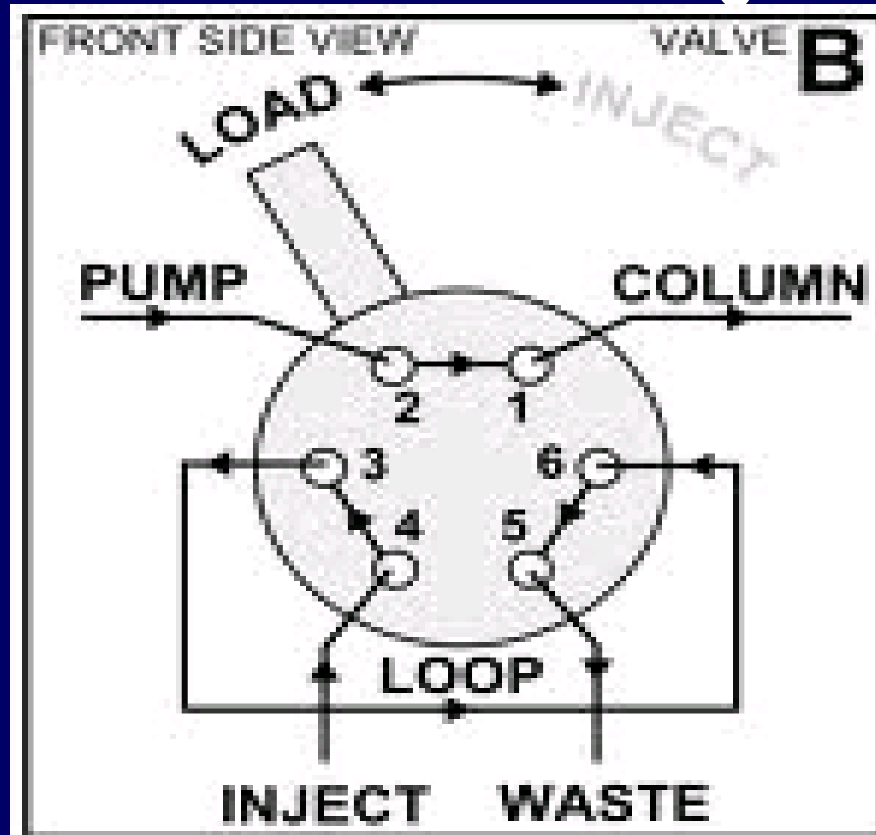


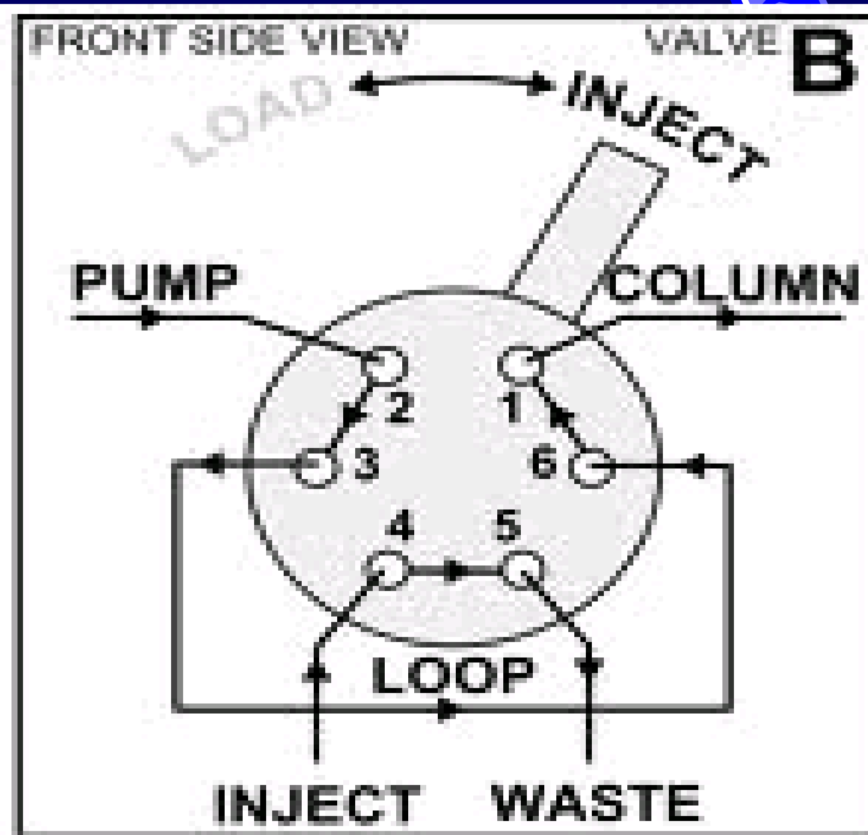
INJECTOR

- The injector serves to introduce the liquid sample into the flow stream of the mobile phase.
- Typical sample volumes are 5- to 20-microliters (µL).
- An autosampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical.

Sample solution loading and injection







Analytical COLUMN of HPLC

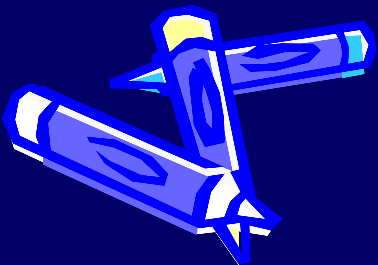
- Column: is a heart of chromatography system
- It is a place where separation of components take place
- It is usually made up of stainless steel, with ¼ inch external diameter and 4.6 mm internal diameter and up to 25 cm long. These may also be available in other dimensions
- Has stainless steel gauze/frit at the end of the column to retain packing

Guard Column

small column placed between injector and column
It extends column life by preventing entry of materials from sample or solvent into column.

It should have the same packing as of analytical column.

Ratio of guard column: analytical column (1:15 or 1:25)

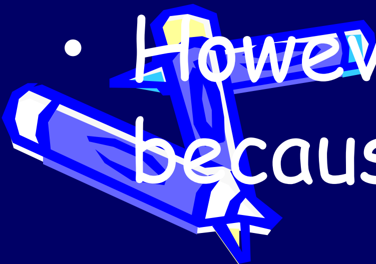


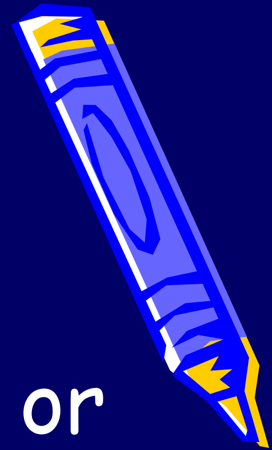
Column packing

- Three types of packing are used in HPLC columns

1-Fully porous

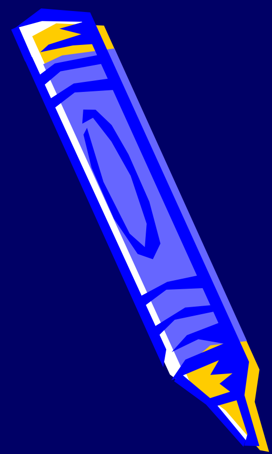
- Originally used silica or alumina
- Has porous channels through the packing
- Give low efficiency because solute takes a long time to diffuse from porous structure
- No longer used in analytical HPLC
- However, still used in preparative columns because of high sample capacity





2-Superficially porous layer beads

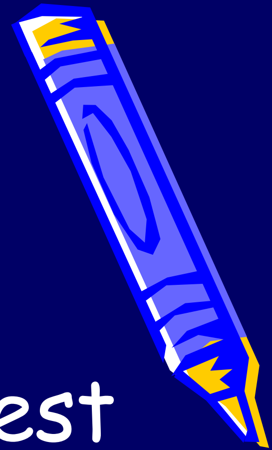
- Rough surface
- Consists of inert solid core of glass or plastic within a thin outer coating of silica or modified silica
- Fast mass transfer
- High efficiency
- Rapid re-equilibribriation
- For analytical separation
- More efficient than porous packing



3-Micro-particulate

- Small diameter 3, 5, 10 μm
- Fully porous
- Spherical or irregular
- Analytical or preparative
- Combines the best features of fully porous and superficially porous beads

Sample preparation



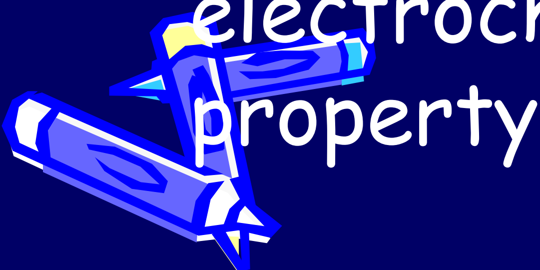
- Goal of sample preparation: obtain a sample with the components of interest free from interfering constituents of the matrix at a suitable concentration for detection and measurement in a suitable solvent
- Sample preparation: liquid-liquid extraction and solid phase extraction
- Sample should be dissolved in same solvent or mixture like mobile phase wherever possible

Detectors

- Monitor mobile phase emerging from the column
- Its output is an electrical signal which is proportional to some property of the mobile phase/solute or both e.g.

1-Refractive index- property of solutes and mobile phase (bulk property)

2- Absorbance (UV/Vis, fluorescence) and electrochemical activity- solute property



- Required characteristics of detectors

1- Adequate sensitivity

2-Linearity (Wide linear dynamic range)

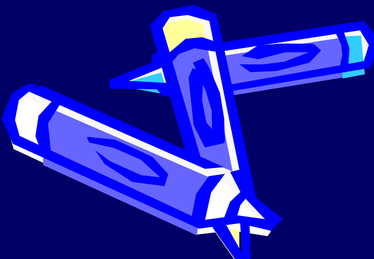
3-Universal or selective response

4-Predictable response, unaffected by changes in conditions

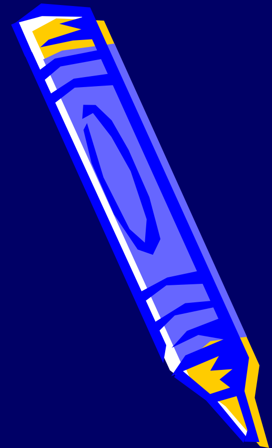
5- Short response time

6- Non-destructive

7- Cheap, reliable and easy to use

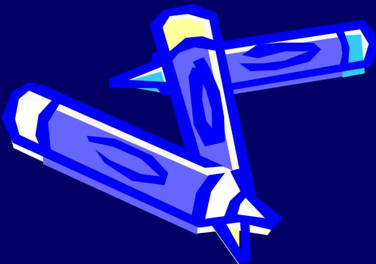


Types of detectors



- <u>Bulk property detectors</u>

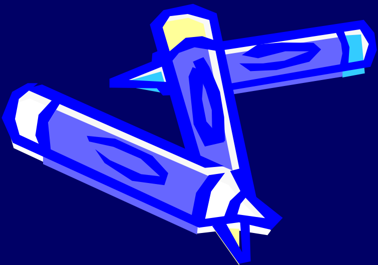
- 1- Principle: Sense the difference in refractive index between column eluent and reference beam of pure mobile phase
- Universal, not sensitive (limit of detection 1 μg)
- Difficult to do gradient elution work
- Must have good control of temperature of the instrument and composition of mobile phase

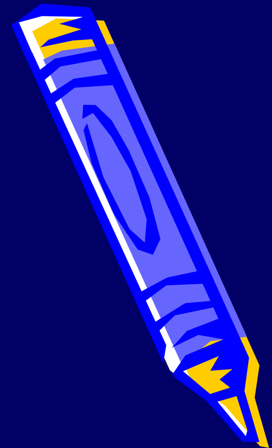


- <u>Solute property Detector</u>

1-UV/Vis spectrophotometer

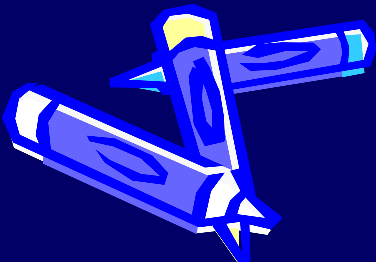
- Most popular
- Only detects solute that absorb UV/Vis radiation
- Mobile phase should not absorb radiation
- Absorption of radiation by solute is a function of concentration according to Beer Lambert's law
- Limit of detection is sub ng





Absorbance detectors UV/Visible detectors

- Three types
  - Fixed wavelength detector
  - Variable wavelength detector
  - Diode array detector



- **Spectrofluorometric detectors**

1-Absorb UV radiation and subsequently emit radiation of longer wavelength, either instantly (fluorescence) or after a time of delay (phosphorescence)

2-For compounds that are inherently fluorescent, otherwise compound has to be made fluorescent by derivatization using suitable reagent

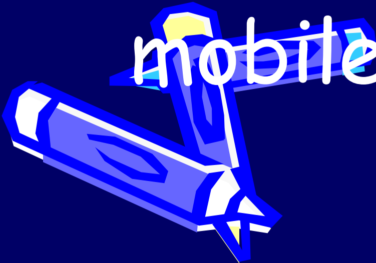
3- Limit of detection 1pg

- <u>Electrochemical detectors</u>

1-Measure conductance of eluent (analyte must be ionic or redox system)

2-Measures current associated with oxidation and reduction of solutes, and may be coulometric or amperometric

1- Amperometric detector-most commonly used- a known potential is applied across a set of electrodes typically glassy carbon as a working electrode. It requires conducting mobile phase. Limit of detection pg



Thank You