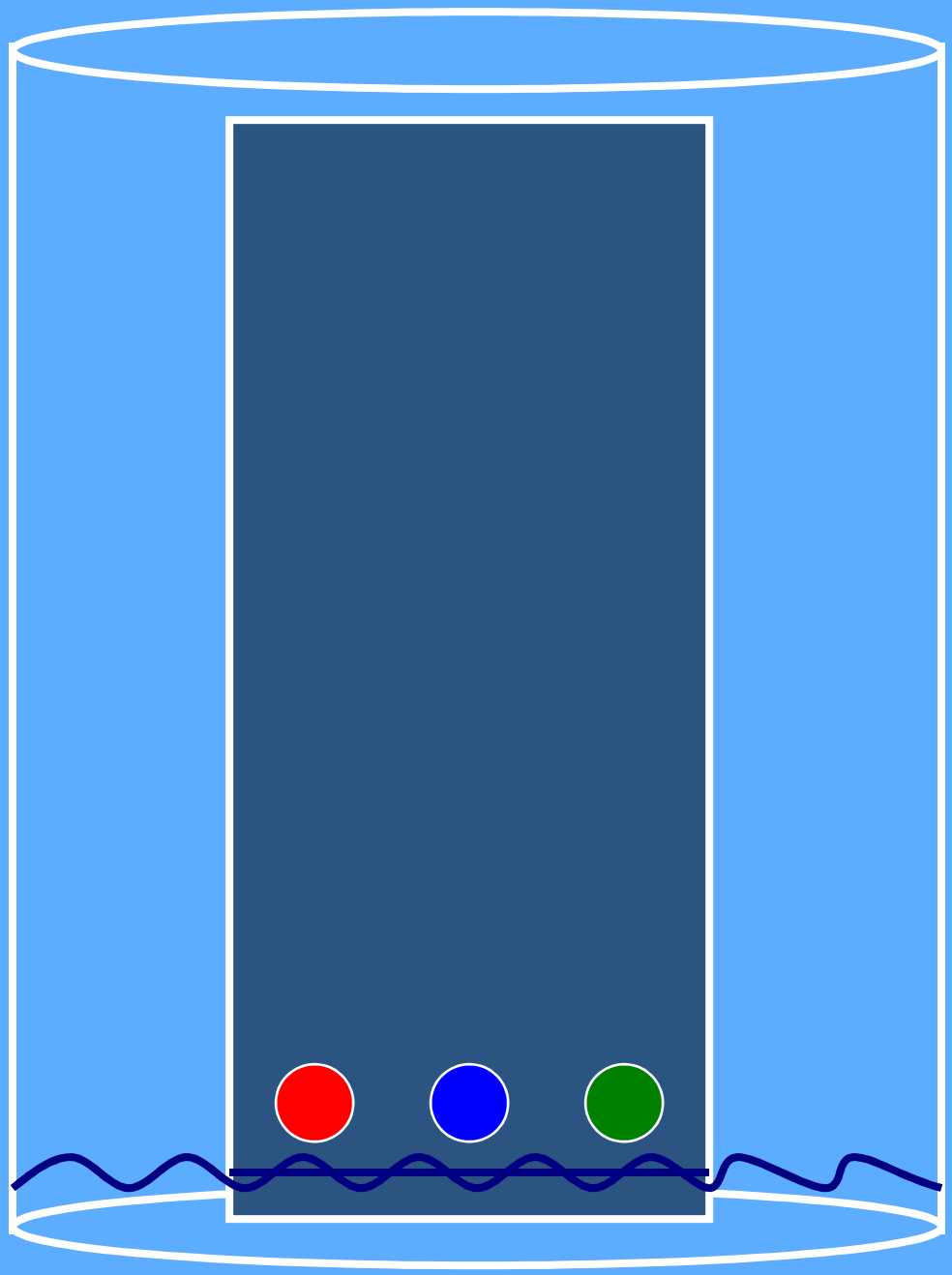
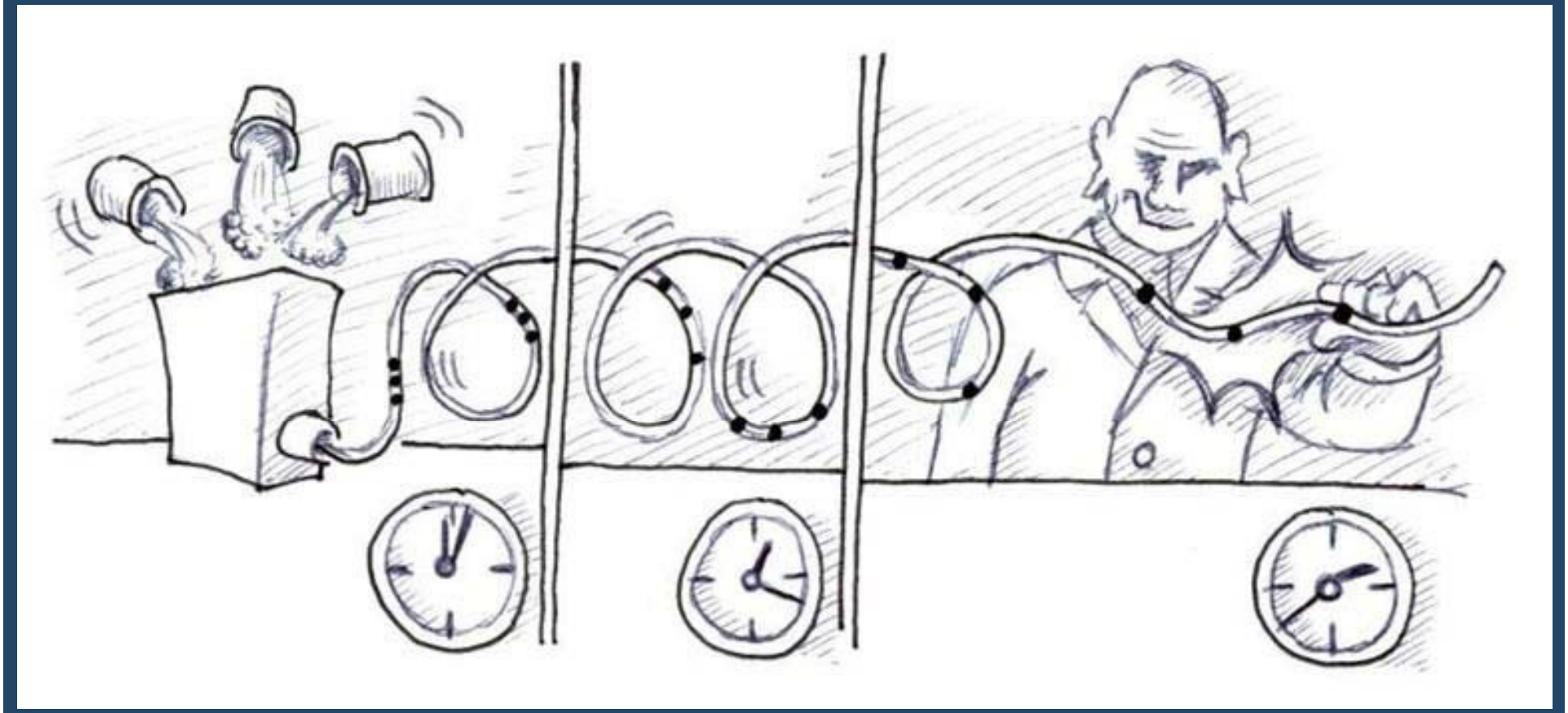
# HPLC Workshop

16 June 2009





What does this do ?

# Chromatography Theory Review

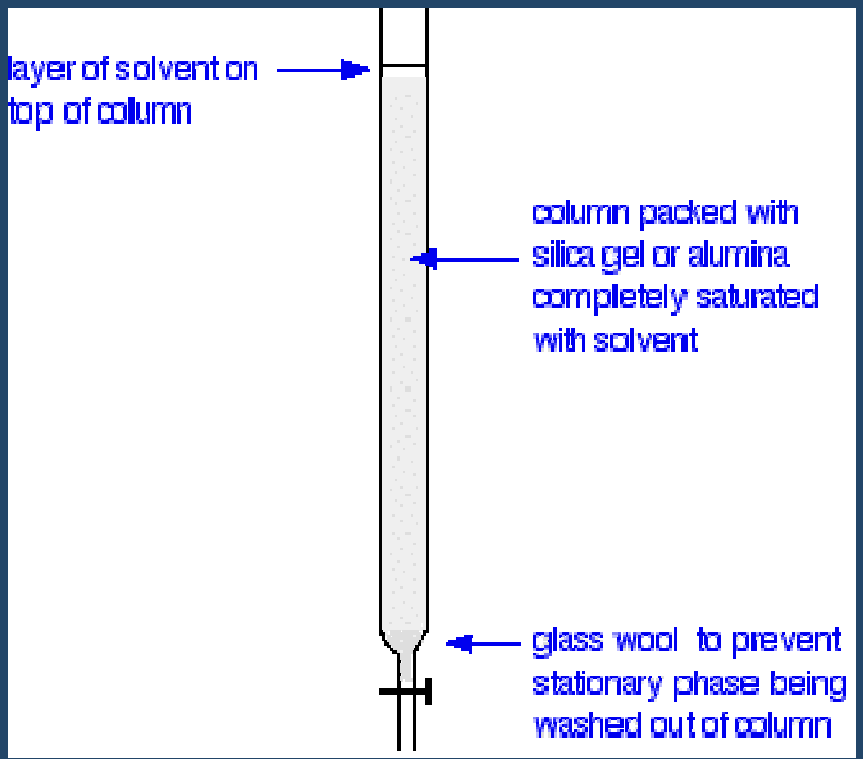
- Several chromatographic techniques
- Even though each method utilizes different techniques to separate compounds, the principles are the same.
- Common to all:
  - Stationary phase-
    - a solid or a liquid supported on a solid
  - Mobile phase-
    - A liquid or gas

# Chromatography Theory Review

- As the mobile phases passes through the stationary phase, it carries the components of the sample mixture with it.
  - The components of the sample will be attracted to the stationary phase, but there will also be a competing attraction for the mobile phase.
  - Each component will have its own characteristic balance of attraction to the mobile/stationary phase.
    - So the components will not move at the same speed and are separated.

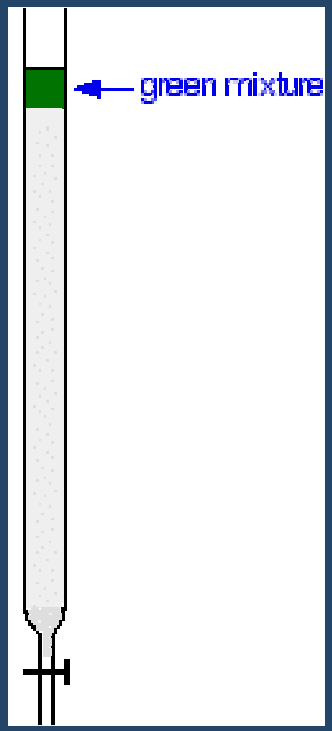
# Column Chromatography

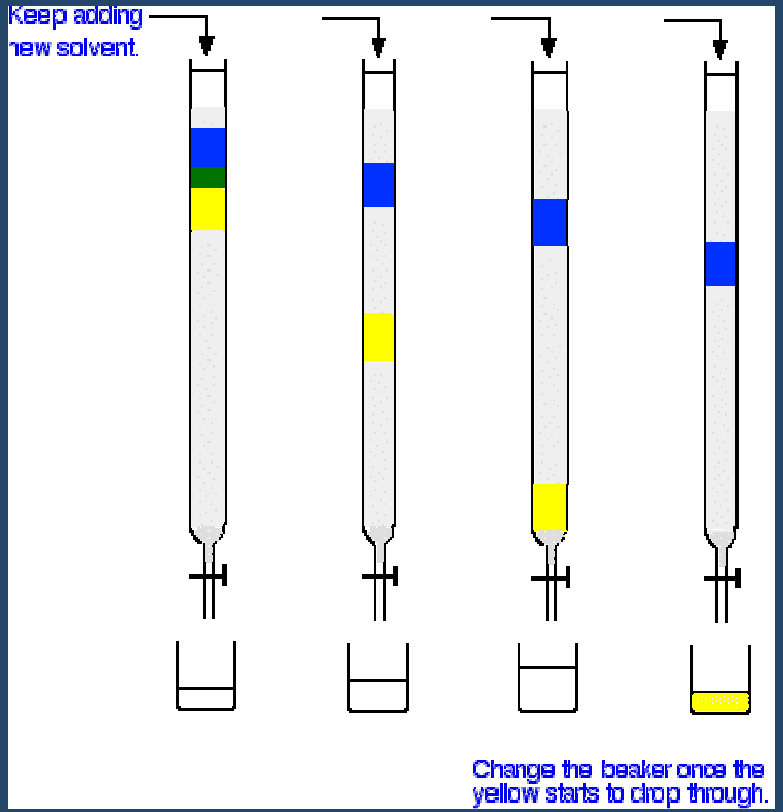
- Similar to thin layer chromatography
  - Stationary phase = silica gel on support
  - Mobile phase = liquid solvent
- In column chromatography, this stationary phase is packed into a vertical glass column.
- Mobile phase moves down the column as a result of gravity.



# Column Chromatography

- Example of column chromatography separation:
  - Blue compound = more polar
    - Adsorb more to the silica gel
    - Elutes slower
  - Yellow compound = less polar
    - Spends much of its time in the mobile phase
    - Elutes faster



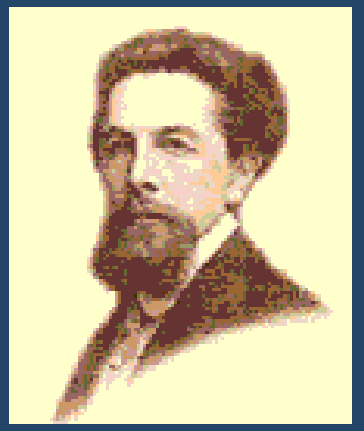
# HPLC Introduction:

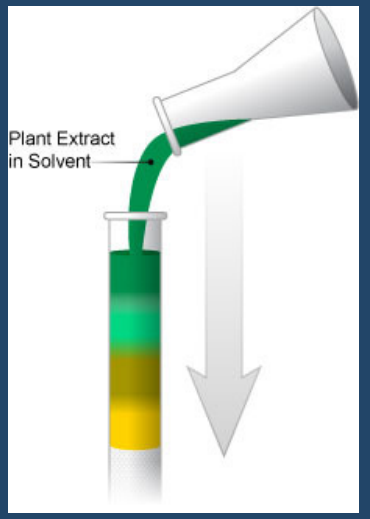
- HPLC = improved form of column chromatography
- Instead of the mobile phase moving through the column as a result of gravity, it is forced through the column under high pressure.
  - Typical operating pressures: 500-6000psi
- To get improved separation – smaller sized packing material is required (<10µm).
  - Smaller packing = greater resistance to flow
    - Low flow rate = solute diffusion
    - Higher pressures needed to generate the needed solvent flow
    - Gravity is too slow- high pressure greatly speeds up the procedure.

# HPLC History

## 1903: Russian botanist Mikhail Tswett

- Separated plant pigments through column adsorption chromatography
  - Packed open glass column with particles
    - Calcium carbonate and alumina
  - Poured sample into column, along with pure solvent
  - As the sample moved down the vertical column, different colored bands could be seen.
    - Bands correlated to the sample components.
- Coined the term chromatography from the Latin word meaning "colour writing".





# HPLC History

Early 1950s: First appearance of GC

- Almost immediately became popular.
- Work began on improving LC

1964: J. Calvin Giddings

- Published a paper entitled "Comparison of the Theoretical Limit of Separating Ability in Gas and Liquid Chromatography" in the journal *Analytical Chemistry.*
  - Outlined ways to improve LC: smaller packing size, increased pressure
  - In theory, he demonstrated how LC could actually be more efficient than GC.
    - Increased number of theoretical plates

# HPLC History

1966: Horváth

- Built the first HPLC instrument and gave it its name
  - HPLC = High Pressure Liquid Chromatography.

1970's: HPLC became popular with an increase in technology

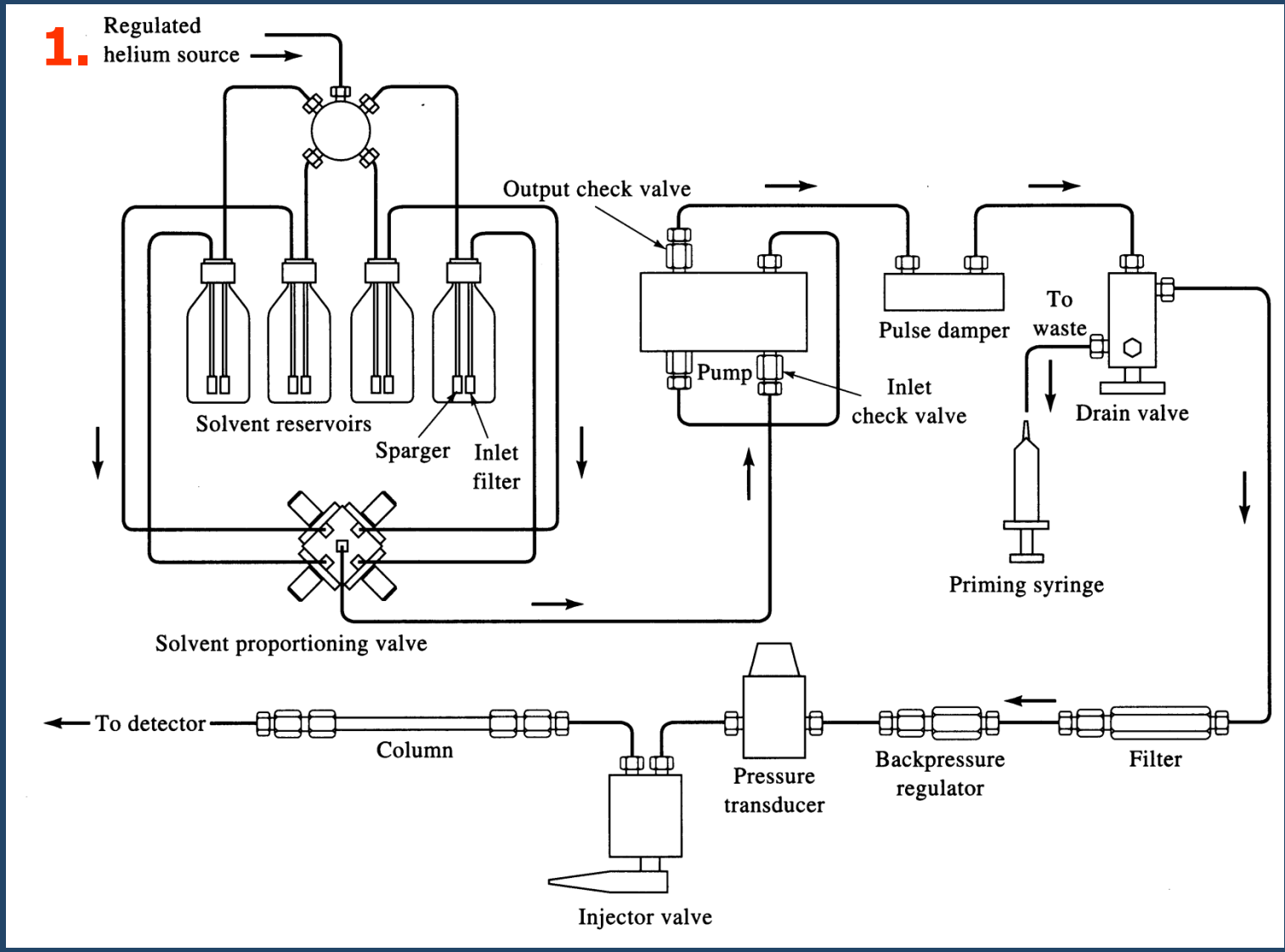
- Improved columns and detectors
- Production of small silica packing material
  - By 1972 particle sizes less than 10μm were introduced
- This allowed for more precise and rapid separations.
- As new technology continued to develop, HPLC became more efficient.
  - HPLC = High Performance Liquid Chromatography

# Overview of the HPLC Process

- Mobile phase pumped through column at high pressure.
- Sample is injected into the system.
- Separation occurs as the mobile phase and sample are pumped through the column.
- Each sample component will elute from the column, one at a time, and will be detected by one of several possible detector types.
- The response of the detector to each component eluted will be displayed on a chart or computer screen.
  - Known as a chromatogram.
    - Each compound eluted will show up as a peak on this chromatogram.
- Data processing equipment are used to analyze the data generated.

http://www.studyhplc.com/chromatographyanimation.php

# Diagram of HPLC Apparatus:



# Design & Operation of an HPLC Instrument

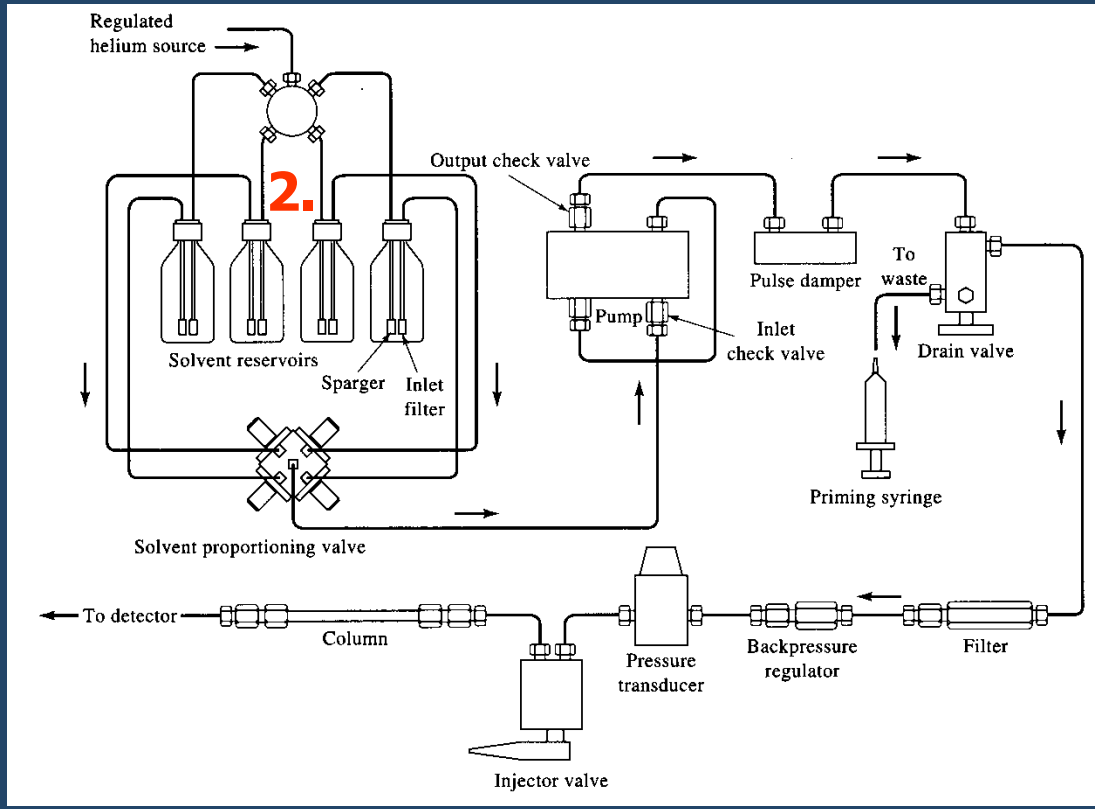
## 1) Mobile phase degassing:

- Dissolved gases in the mobile phase can come out of solution and form bubbles as the pressure changes from the column entrance to the exit.
  - May block flow through the system
- Sparging is used to remove any dissolved gas from the mobile phase.
  - An inert and virtually insoluble gas, such as helium, is forced into the mobile phase solution and drives out any dissolved gas.
- Degassing may also be achieved by filtering the mobile phase under a vacuum.

# Design & Operation of an HPLC Instrument

## 2) Solvent reservoirs:

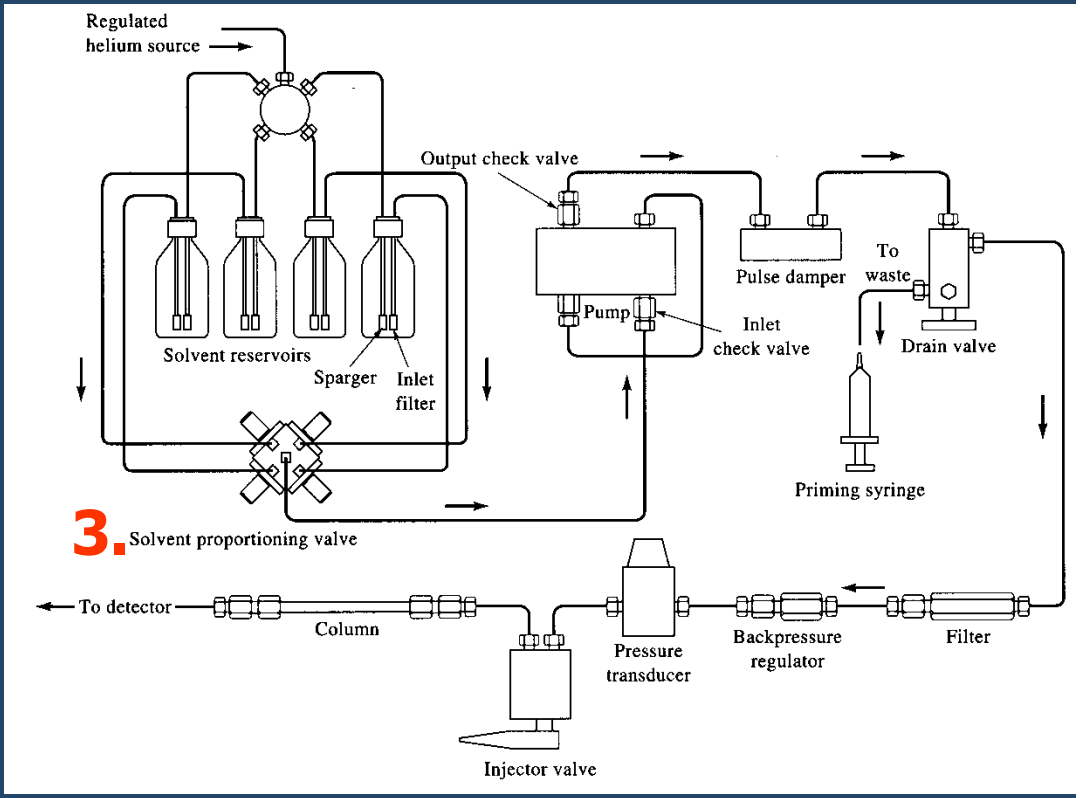
- Individual reservoirs store the mobile phase components until they are mixed and used.
- May also manually prepare the mobile phase mixture and store in a single reservoir.



# Design & Operation of an HPLC Instrument

## 3) Mobile phase mixing:

- Solvent proportioning valve can be programmed to mix specific amounts of solvent from the various reservoirs to produce the desired mobile phase composition.



# Design & Operation of an HPLC Instrument

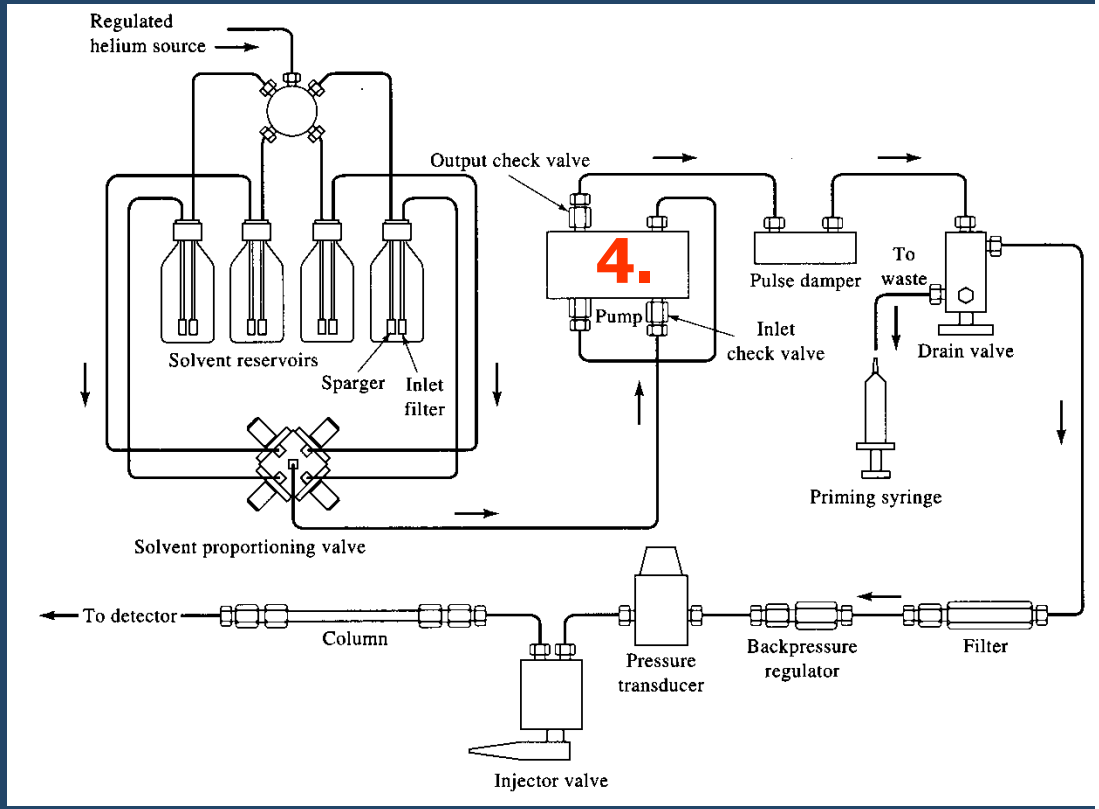
## 3) Mobile phase mixing:

- Isocratic elution:
  - operate at a single, constant mobile phase composition
- Gradient elution:
  - Vary the mobile phase composition with time
  - If there is a wide polarity range of components to be eluted.
  - Allows for faster runs.
  - Ex: mobile phase composition can be programmed to vary from 75% water: 25% acetonitrile at time zero to 25% water: 75% acetonitrile at the end of the run.
    - More polar components will tend to elute first.
    - More non-polar components will elute later in the gradient.

# Design & Operation of an HPLC Instrument

## 4) HPLC pump:

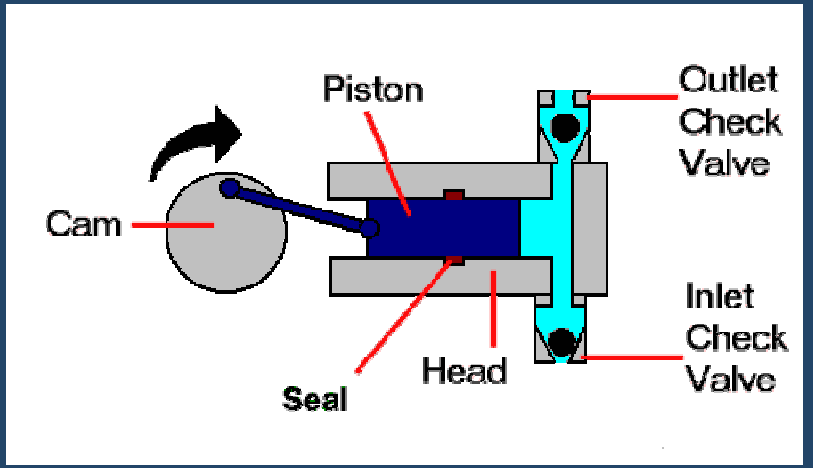
- Fill stroke: mobile phase is pulled from the solvent side
- Exhaust stroke: the mobile phase is pushed from the injector to the column head.
  - This is where the high pressure is generated



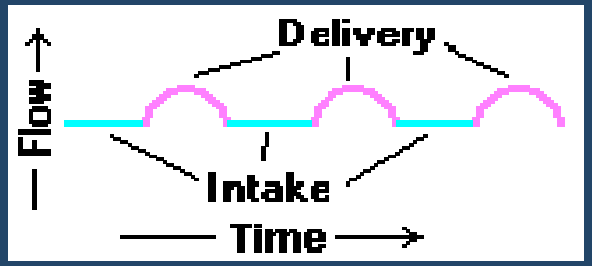
# Design & Operation of an HPLC Instrument

## 4) HPLC pump:

- Most common = reciprocating piston type
- Flow rates change during pumping cycle
  - Want to minimize flow surges
    - Pulse dampener
    - Dual pistons
      - While one piston fills, the other delivers



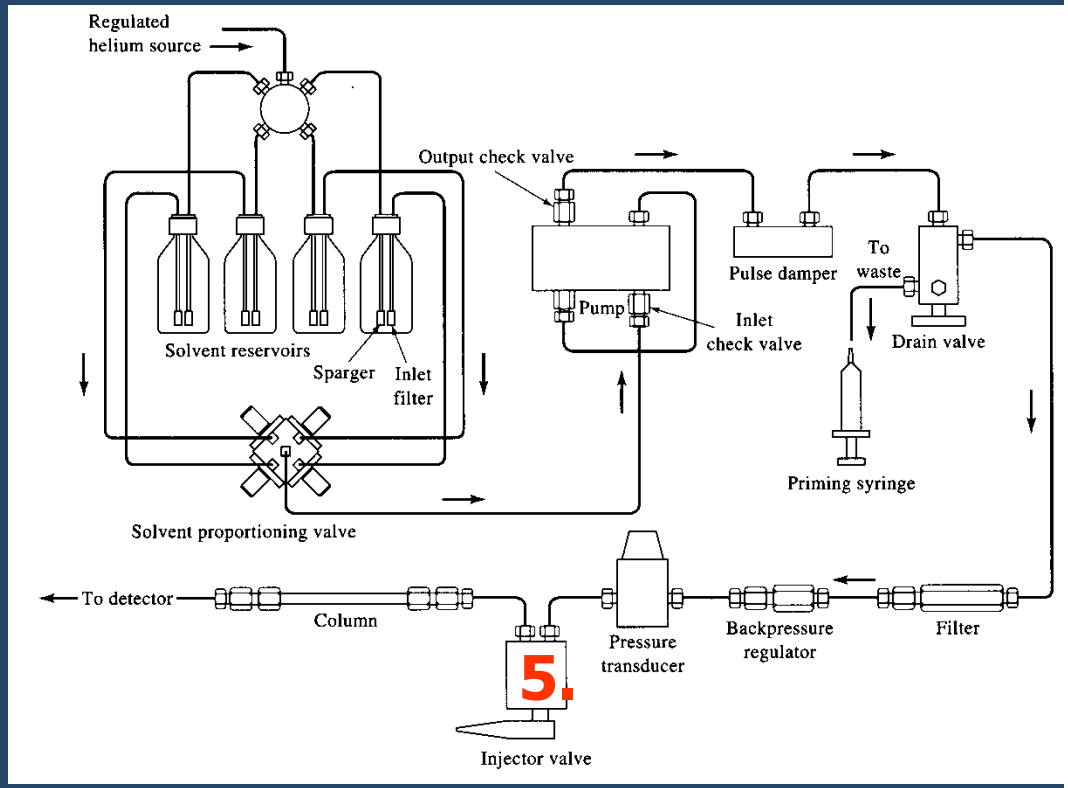

http://www.lcresources.com/resources/getstart/2b01.htm



# Design & Operation of an HPLC Instrument

## 5) Injector:

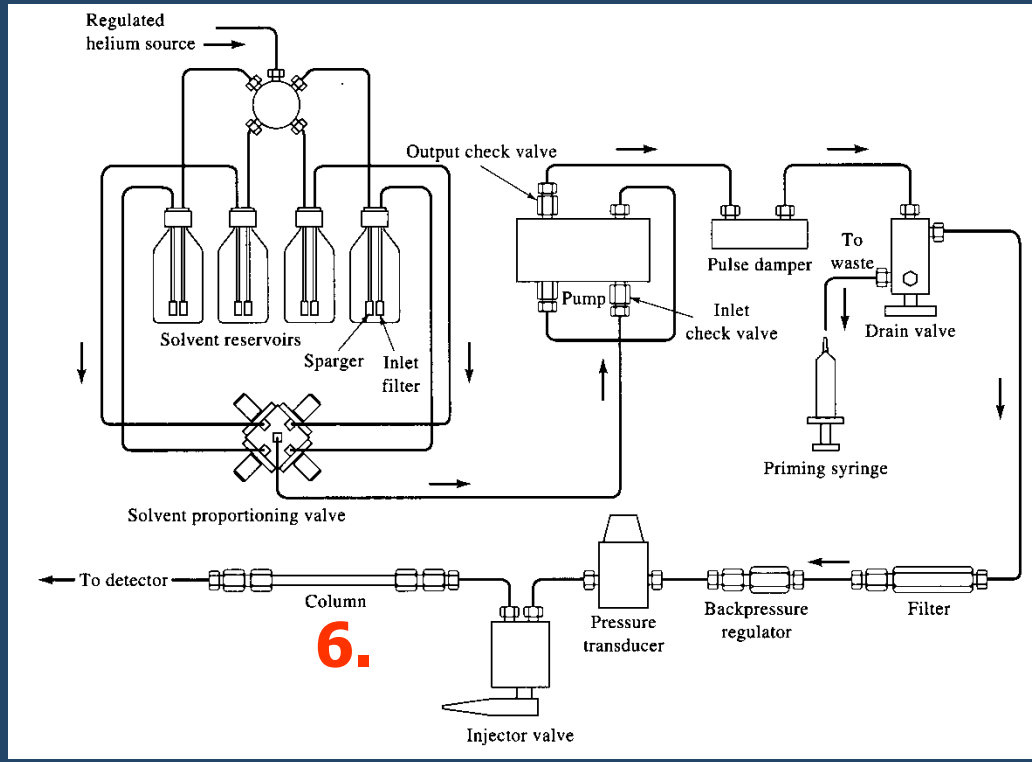
- Introduces the sample into the mobile phase stream to be carried into the column.
- Syringe = impractical for use in highly pressurized systems.
- Rotary injection valve is used.



# Design & Operation of an HPLC Instrument

## 6) Column:

- Usually constructed of stainless steel
  - glass or Tygon may be used for lower pressure applications (<600 psi).
- Length: 5-100cm
  - 10 to 20cm common
- Diameter:
  - Typical: 2.1, 3.2, or 4.5mm
  - Up to 30mm for preparative applications



# Design & Operation of an HPLC Instrument

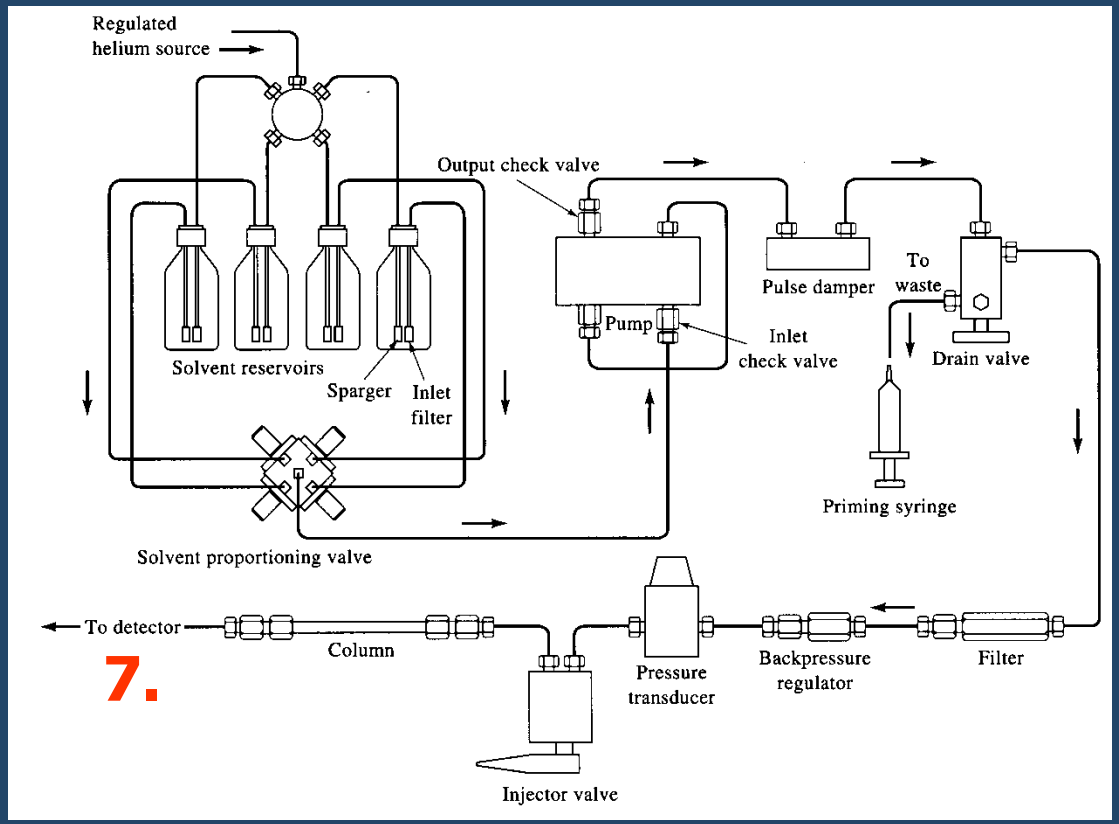
## 6) Column:

- Guard column: Protects the analytical column
  - Particles
  - Interferences
  - Prolongs the life of the analytical column
- Analytical column: Performs the separation

# Design & Operation of an HPLC Instrument

## 7) Detector:

- The component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram.
- A wide variety of detectors exist.
- Must have high sensitivity- small sample sizes are used with most HPLC columns

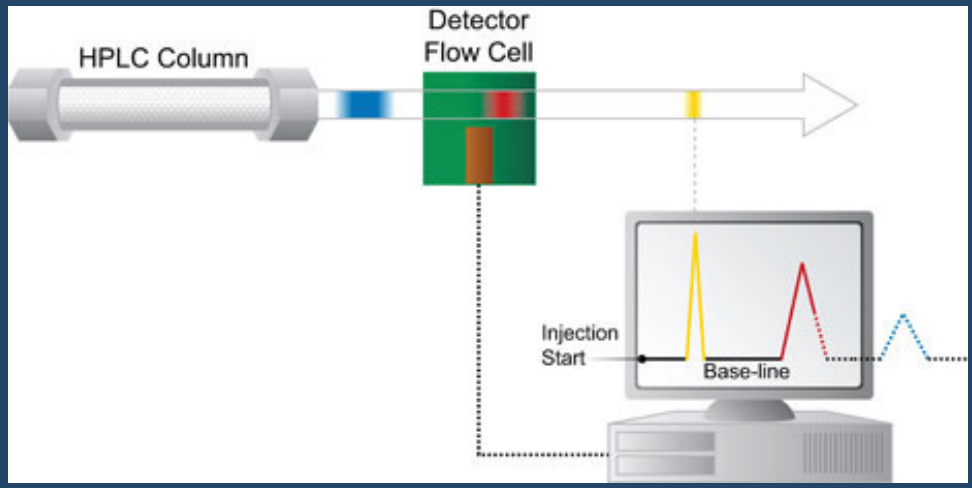


# Detection in HPLC

*There are six major HPLC detectors:

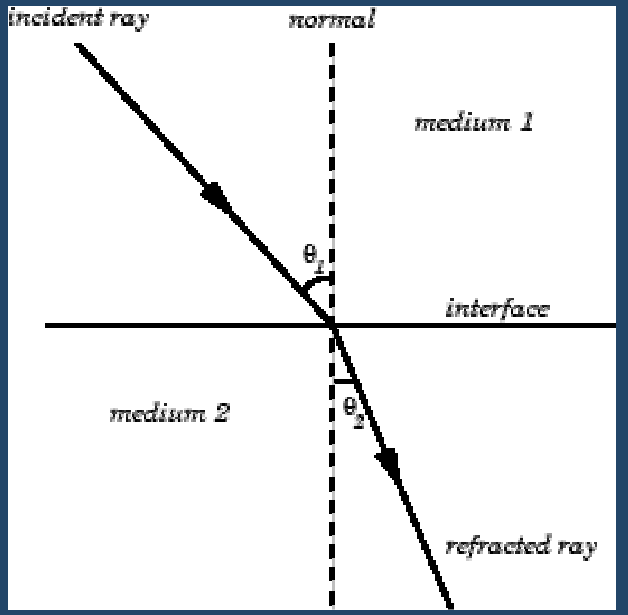
- Refractive Index (RI) Detector
- Evaporative Light Scattering Detector (ELSD)
- UV/VIS Absorption Detectors
- The Fluorescence Detector
- Electrochemical Detectors (ECDs)
- Conductivity Detector

* The type of detector utilised depends on the characteristics of the analyte of interest.



# Refractive Index Detector

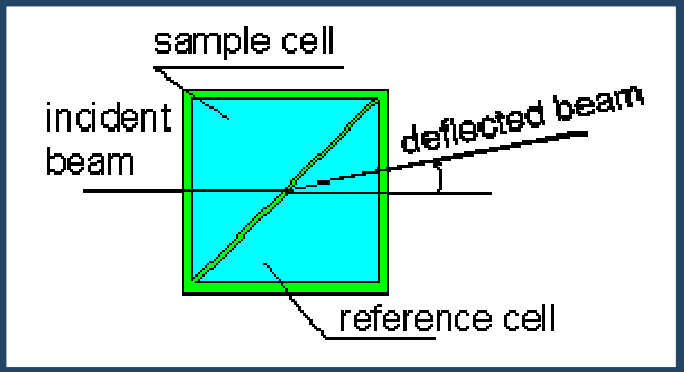
- Based on the principle that every transparent substance will slow the speed of light passing through it.
  - Results in the bending of light as it passes to another material of different density.
  - Refractive index = how much the light is bent
- The presence of analyte molecules in the mobile phase will generally change its RI by an amount almost linearly proportional to its concentrations.




http://farside.ph.utexas.edu/teaching/302l/lectures/img1154.png

# Refractive Index Detector

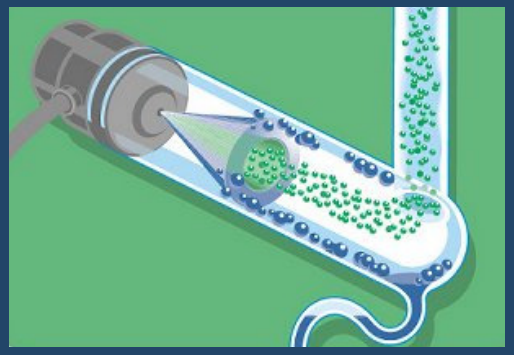
- Affected by slight changes in mobile phase composition and temperature.
- Universal-based on a property of the mobile phase
- It is used for analytes which give no response with other more sensitive and selective detectors.
  - RI = general
    - responds to the presence of all solutes in the mobile phase.
- Reference= mobile phase
- Sample= column effluent
- Detector measures the differences between the RI of the reference and the sample.

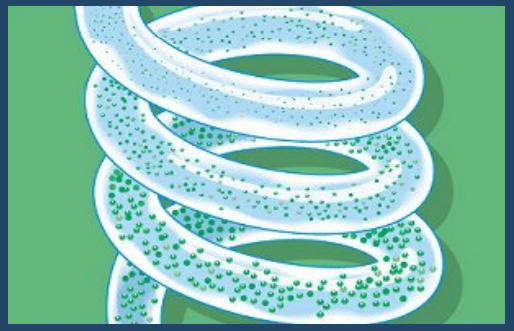


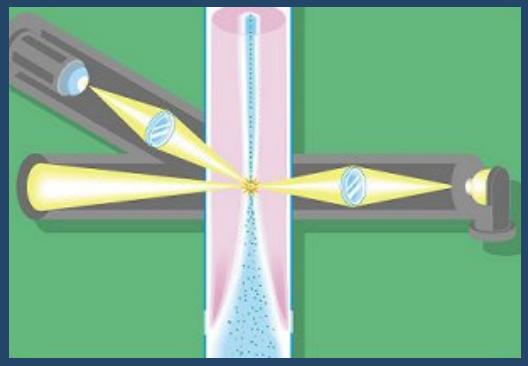

http://hplc.chem.shu.edu/HPLC/index.html

# Evaporative Light Scattering Detector (ELSD)

- Analyte particles don't scatter light when dissolved in a liquid mobile phase.
- Three steps:
  1) Nebulize the mobile phase effuent into droplets.
     - Passes through a needle and mixes with hydrogen gas.
  2) Evaporate each of these droplets.
     - Leaves behind a small particle of nonvolatile analyte
  3) Light scattering
     - Sample particles pass through a cell and scatter light from a laser beam which is detected and generates a signal.



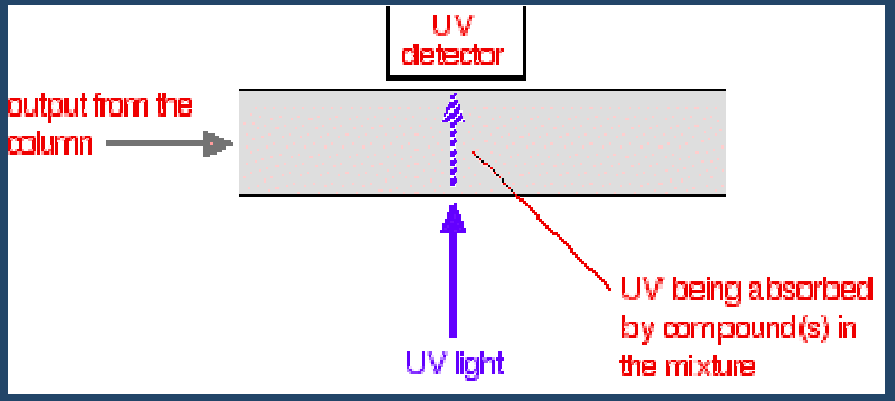




http://www.sedere.com/WLD/whatis.html

# UV/VIS Absorption Detectors

- Different compounds will absorb different amounts of light in the UV and visible regions.
- A beam of UV light is shined through the analyte after it is eluted from the column.
- A detector is positioned on the opposite side which can measure how much light is absorbed and transmitted.
  - The amount of light absorbed will depend on the amount of the compound that is passing through the beam.

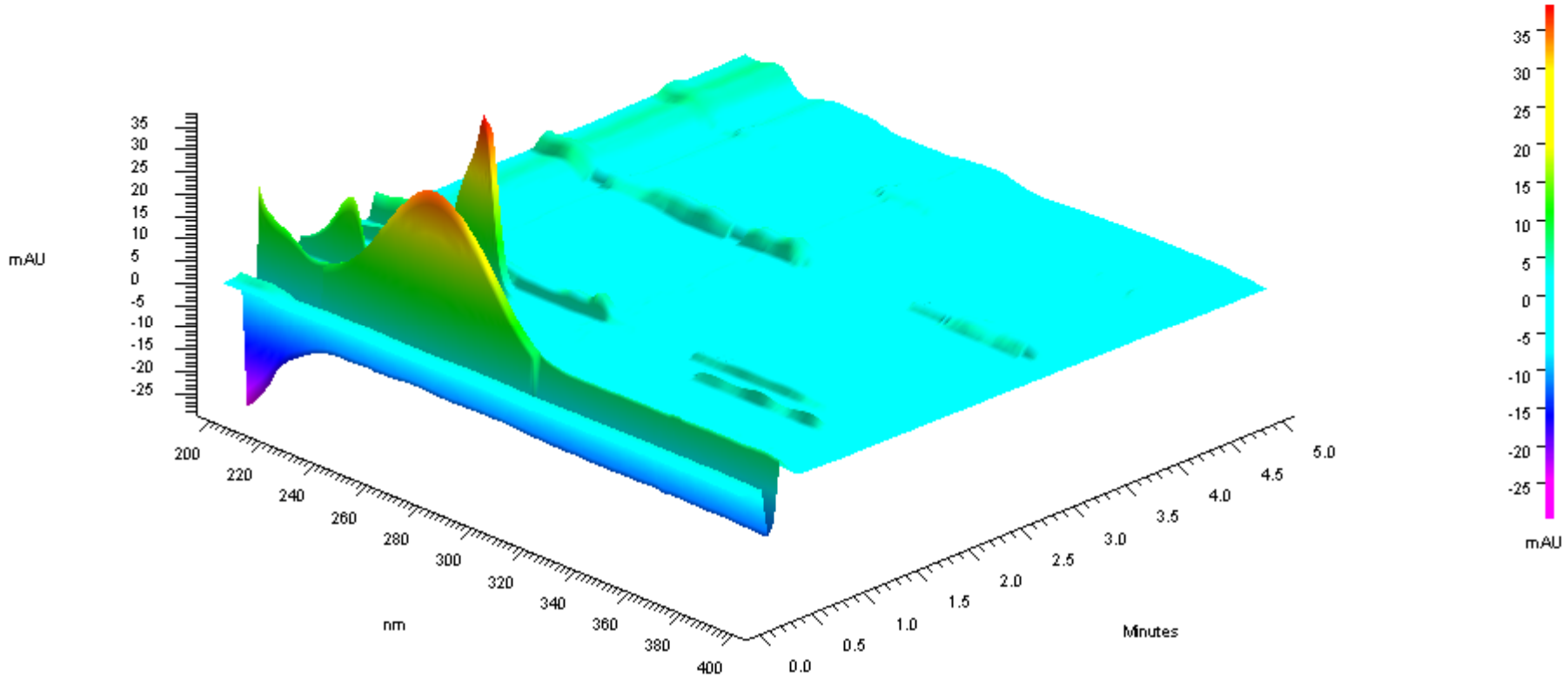



http://www.chemguide.co.uk/analysis/chromatography/hplc.html

# UV/VIS Absorption Detectors

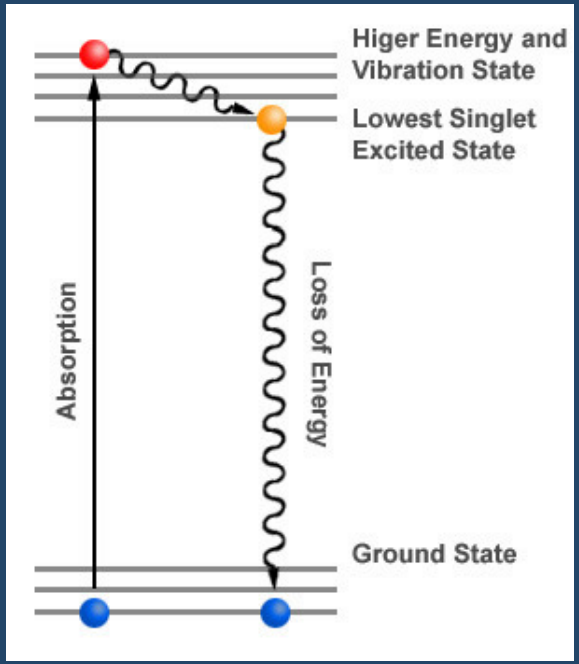
- Beer-Lambert law: $A=\varepsilon bc$
  - absorbance is proportional to the compound concentration.
- Fixed Wavelength: measures at one wavelength, usually 254 nm
- Variable Wavelength: measures at one wavelength at a time, but can detect over a wide range of wavelengths
- Diode Array Detector (DAD): measures a spectrum of wavelengths simultaneously

# 3D View – absorbance vs time vs wavelength



# The Fluorescence Detector

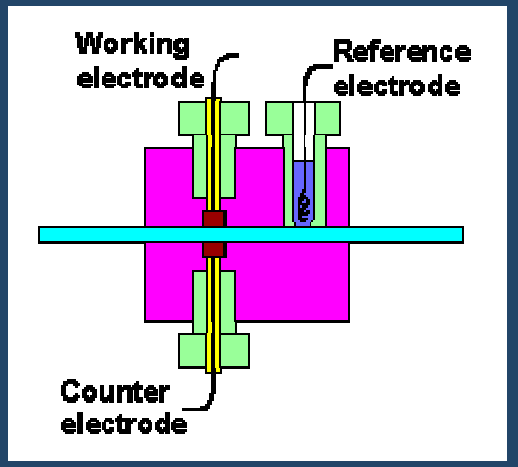
- Measure the ability of a compound to absorb then re-emit light at given wavelengths
- Some compounds will absorb specific wavelengths of light which, raising it to a higher energy state.
- When the compound returns to its ground state, it will release a specific wavelength of light which can be detected.
- Not all compounds can fluoresce / more selective than UV/VIS detection.



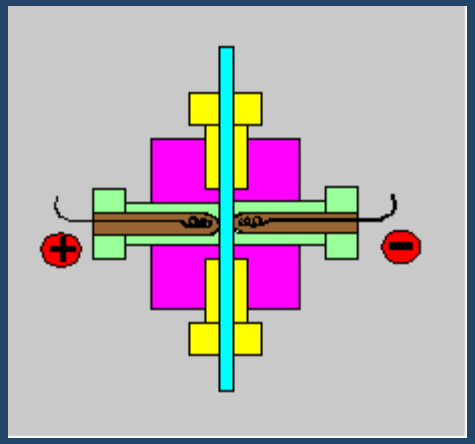

http://mekentosj.com/science/fret/images/fluorescence.jpg

# Electrochemical Detectors (ECDs):

- Electrochemical Detectors (ECDs):
  - Used for compounds that undergo oxidation/reduction reactions.
  - Detector measures the current resulting from an oxidation/reduction reaction of the analyte at a suitable electrode.
  - Current level is directly proportional to the concentration of analyte present.
- Conductivity Detector:
  - Records how the mobile phase conductivity changes as different sample components are eluted from the column.



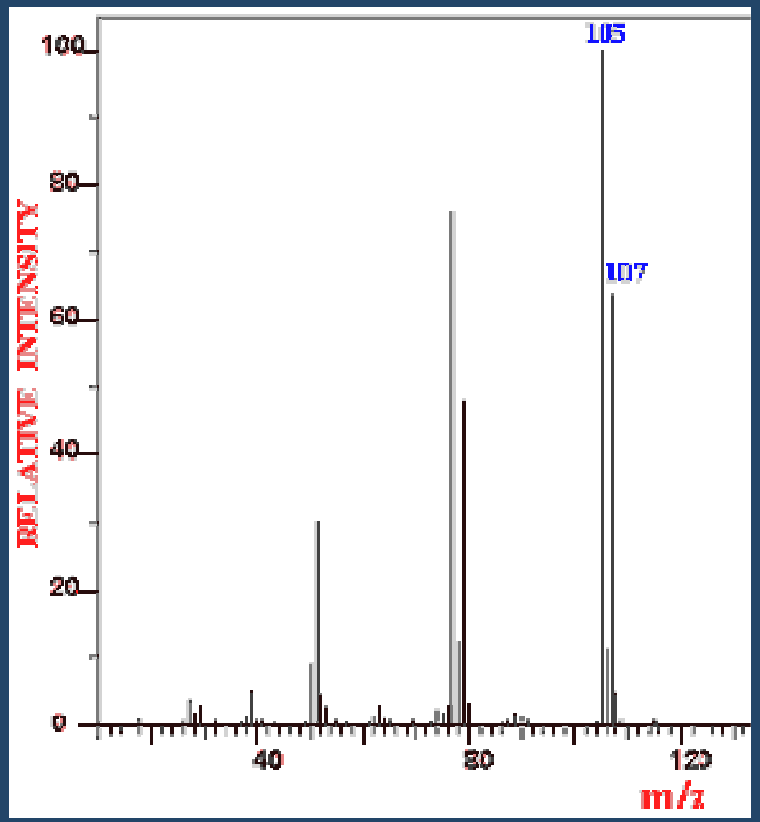

http://hplc.chem.shu.edu/HPLC/index.html



http://hplc.chem.shu.edu/HPLC/index.html

# Interfacing HPLC to Mass Spectrometry

- Mass Spectrometry = an analytical tool used to measure the molecular mass of a sample.
  - Measures the mass to charge ratio
- Allows for the definitive identification of each sample component.
- Most selective HPLC detector, but also the most expensive.




http://www.chem.queensu.ca/FACILITIES/NMR/nmr/mass-spec/index.htm

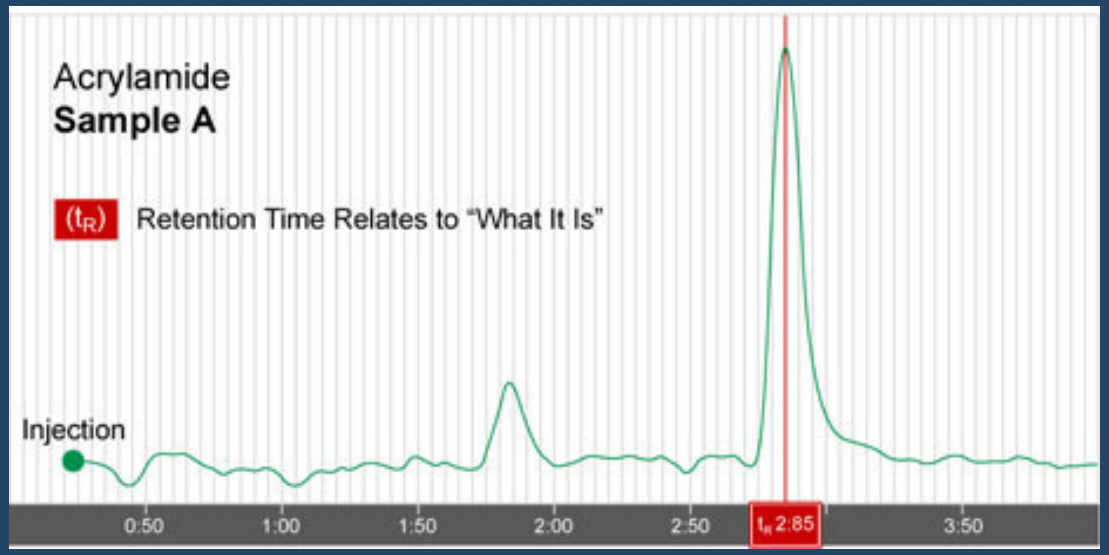
# Picture of a Typical HPLC System

# Retention Time- $t_R$

- The elapsed time between the time of analyte injection and the time which the maximum peak height for that compound is detected.
- Different compounds will have different retention times.
  - Each compound will have its own characteristic balance of attraction to the mobile/stationary phase.
    - So they will not move at the same speed.
- Running conditions can also effect $t_R$:
  - Pressure used, nature of the stationary phase, mobile phase composition, temperature of the column

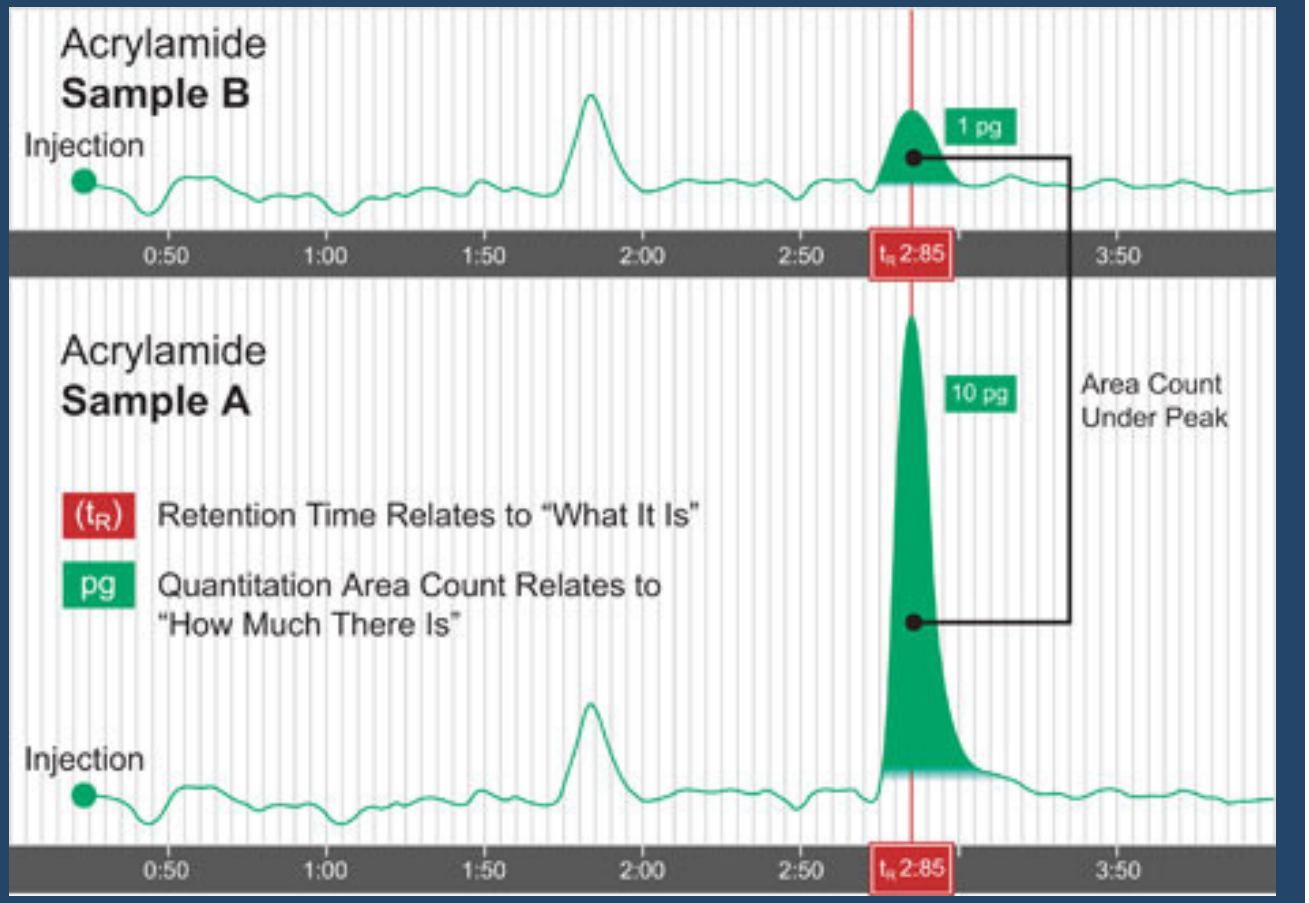
# Retention Time- $t_R$

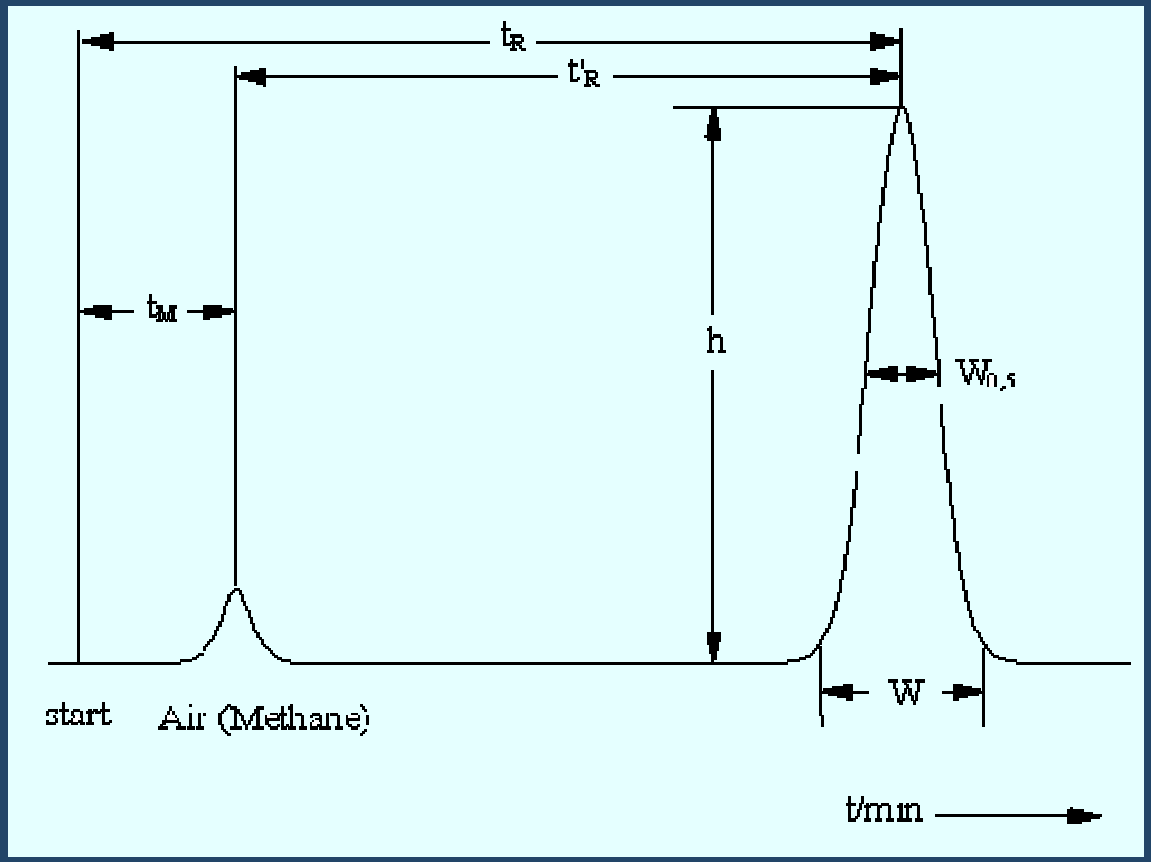
- If you are careful to keep the conditions constant, you may use $t_R$ to help you identify compounds present.
  - Must have measured $t_R$ for the pure compounds under identical conditions.



# Determining Concentration

- In most cases, sample peaks on the chromatogram can be used to estimate the amount of a compound present.
- The more concentrated, the stronger the signal, the larger the peak.






- $t_R$: Retention time
- $t'_R$: Adjusted retention time = ($t_R$- $T_m$)
- $T_m$: Dead time
- $W_{0,5}$: Peak width at half height
- h: Height of signal

# Types of HPLC

- There are numerous types of HPLC which vary in their separation chemistry.
  - All chromatographic modes are possible:
    - Ion-exchange
    - Size exclusion
- Also can vary the stationary & mobile phases:
  - Normal phase HPLC
  - Reverse phase HPLC

# Chromatographic Modes of HPLC

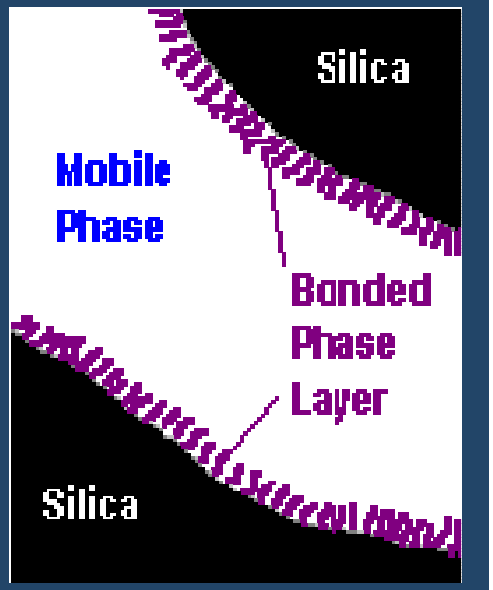
- Ion exchange:
  - Used with ionic or ionizable samples.
  - Stationary phase has a charged surface.
    - opposite charge to the sample ions
  - The mobile phase = aqueous buffer
  - The stronger the charge on the analyte, the more it will be attracted to the stationary phase, the slower it will elute.
- Size exclusion:
  - Sample separated based on size.
  - Stationary phase has specific pore sizes.
  - Larger molecules elute quickly.
  - Smaller molecules penetrate inside the pores of the stationary phase and elute later.

# Normal Phase HPLC

- Stationary phase: polar, silica particles
- Mobile phase: non-polar solvent or mixture of solvents
- Polar compounds:
  - Will have a higher affinity for the polar, stationary phase
  - Will elute slower
- Non-polar compounds:
  - Will have a higher affinity for the non-polar, mobile phase
  - Will elute faster

# Reverse Phase HPLC

- Stationary phase: non-polar
  - Non-polar organic groups are covalently attached to the silica stationary particles.
    - Most common attachment is a long-chain n-C18 hydrocarbon
      - Octadecyl silyl group, ODS
- Mobile phase: polar liquid or mixture of liquids
- Polar analytes will spend more time in the polar mobile phase.
  - Will elute quicker than non-polar analytes
- Most common type of HPLC used today.




http://www.lcresources.com/resources/getstart/3a01.htm

# HPLC Applications

- Can be used to isolated and purify compounds for further use.
- Can be used to identify the presence of specific compounds in a sample.
- Can be used to determine the concentration of a specific compound in a sample.
- Can be used to perform chemical separations
  - Enantiomers
  - Biomolecules

# HPLC Applications

*HPLC has an vast amount of current & future applications*

- Some uses include:
  - Forensics: analysis of explosives, drugs, fibers, etc.
  - Proteomics: can be used to separate and purify protein samples
    - Can separate & purify other biomolecules such as: carbohydrates, lipids, nucleic acids, pigments, proteins, steroids
  - Study of disease: can be used to measure the presence & abundance of specific biomolecules correlating to disease manifestation.
  - Pharmaceutical Research: all areas including early identification of clinically relevant molecules to large-scale processing and purification.

# FPLC- A Modification of HPLC

- In 1982 Pharmacia introduced a new chromatographic method called FPLC.
  - FPLC = Fast Protein Liquid Chromatography
- FPLC is basically a "protein friendly" HPLC system.
  - Stainless steel components replaced with glass and plastic.
    - Stainless steel was thought to denature proteins
    - Also many ion-exchange separations of proteins involve salt gradients; thought that these conditions could results in attack of stainless steel systems.
  - FPLC can also be used to separate other biologically active molecules, such as nucleic acid.

# FPLC- A Modification of HPLC

- FPLC is an intermediate between classical column chromatography and HPLC.
  - FPLC pump delivers a solvent flow rate in the range of 1-499ml/hr
    - HPLC pump= 0.010-10ml/min
  - FPLC operating pressure: 0-40 bar
    - HPLC= 1-400bar
    - classic chromatography= atmospheric pressure
    - Since lower pressures are used in FPLC than in HPLC, a wider range of column supports are possible.

# The Impact of HPLC

- HPLC has such widespread application it is impossible to convey its extensive impact.
  - Has many advantages in situations were a nonvolatile or thermally unstable sample must be separated.
    - As with many biochemical samples
  - Great speed and resolution
    - Resolution = how well solutes are separated
  - Columns don't have to be repacked
  - Adaptable to large-scale, preparative procedures.