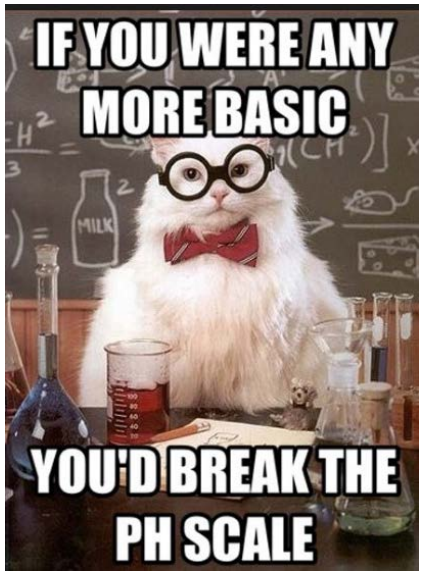
ASBC Annual Meeting
June 4–7 ■ Fort Myers, Florida
See what SCIENCE can brew for you

Basic Chromatography

Stacey Williams

06/06/2017



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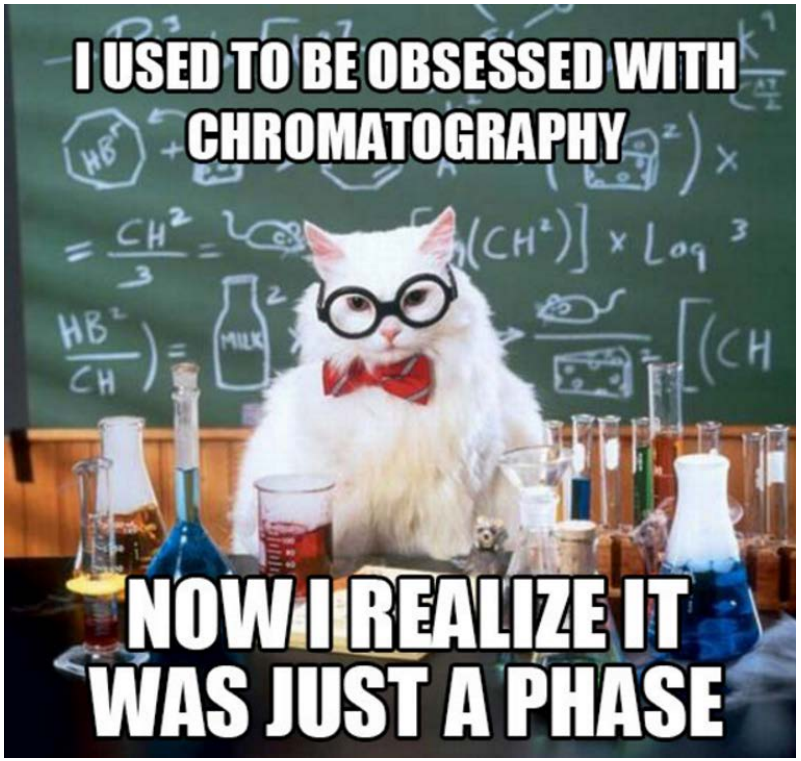


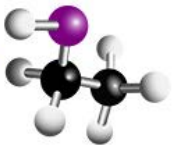
GC vs HPLC

GC

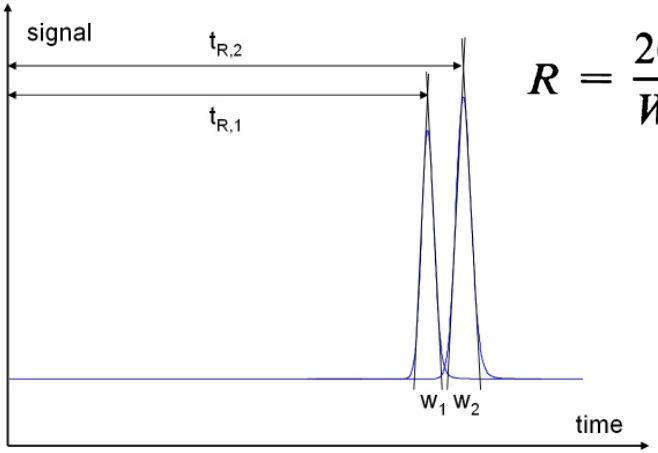
- Separation based on volatility/polarity
- Thermally stable
- Small molecules (MW<1000 Da)
- Mobile Phase: High purity gas
- Applications: Petroleum, flavors and fragrances, environmental, organic and inorganic molecules

HPLC, LC, UHPLC, UPLC

- Separation based on hydrophobicity, polarity, ion interactions, etc.
- Thermally stable/unstable
- Small and large molecules
- Mobile phase: Liquid comprised of different solvents
- Applications: Pharmaceuticals, foods, life sciences, polymers, sugars, acids, organic molecules, ions, and polymers



Fundamental Resolution Equation



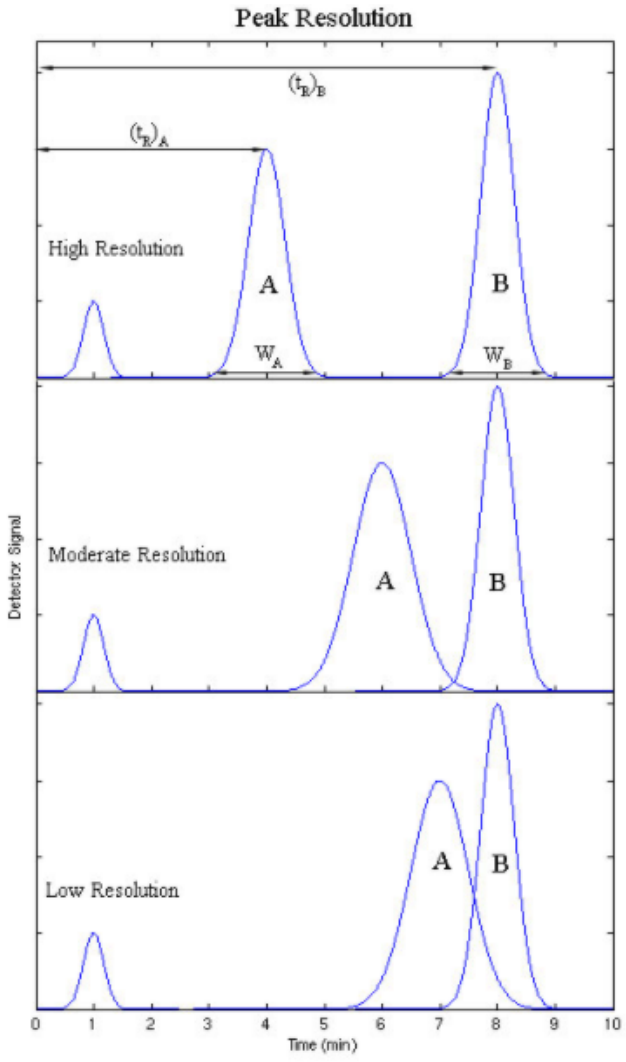
$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$$

Baseline Separation ≥ 1.5

$$R_S = 1/4\sqrt{N} \times \frac{\alpha - 1}{\alpha} \times \frac{k}{1+k}$$

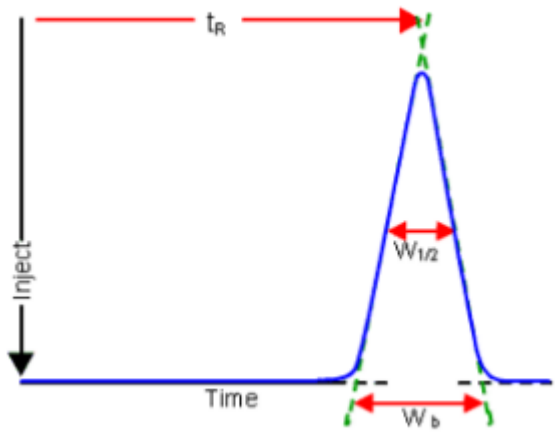
Efficiency Selectivity Retention

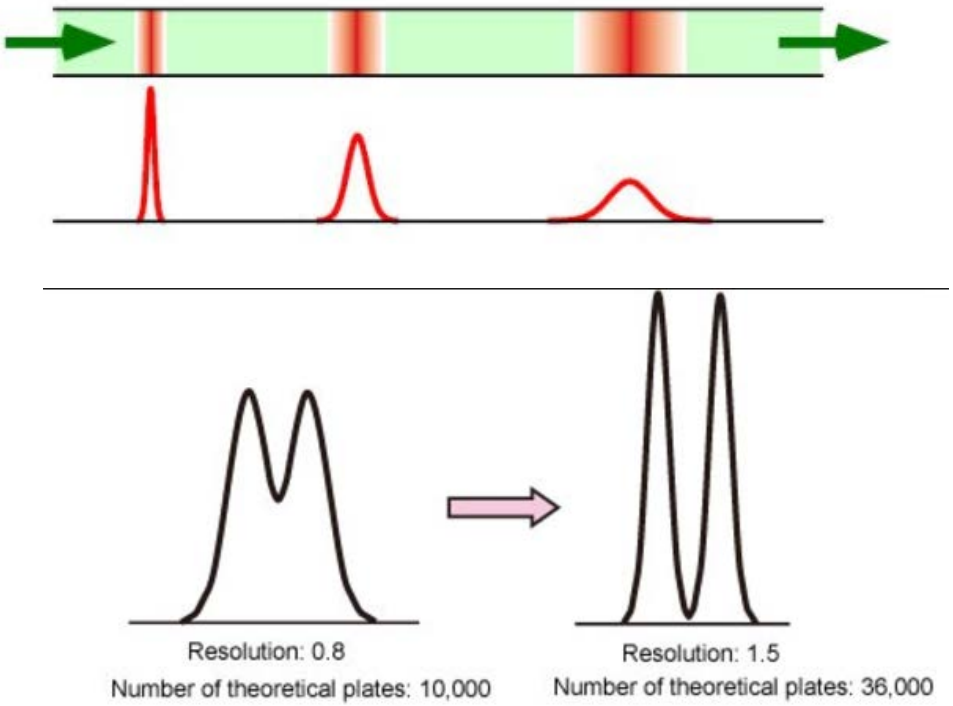
Improving separation can be accomplished by making changes to efficiency, selectivity and/or retention



Efficiency (Theoretical Plates)

$$N = 16\left(\frac{t}{W}\right)^2 \text{ or } N = 5.54\left(\frac{t}{W_{h/2}}\right)^2$$



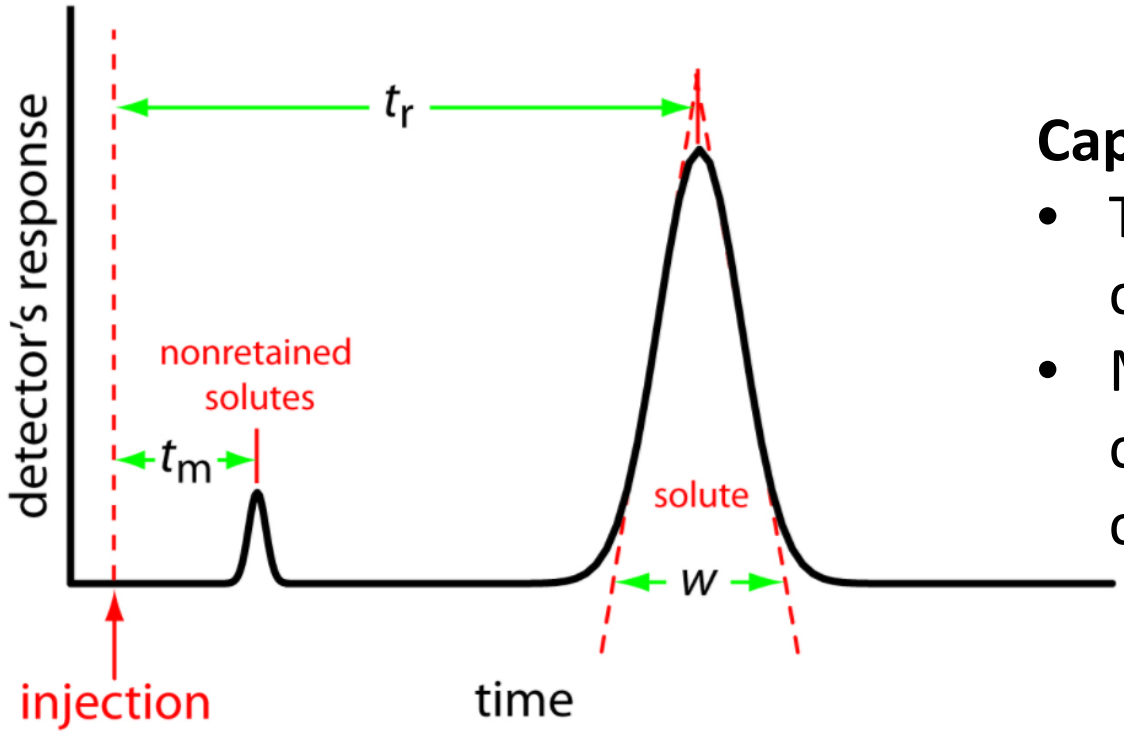


Efficiency affected by

GC or LC Column

Capacity Factor (Retention factor or K prime)

$$k' = \frac{(t_R - t_M)}{t_M}$$

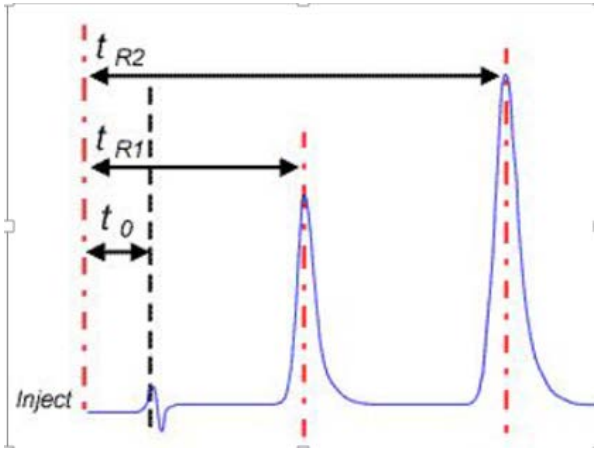


Capacity affected by

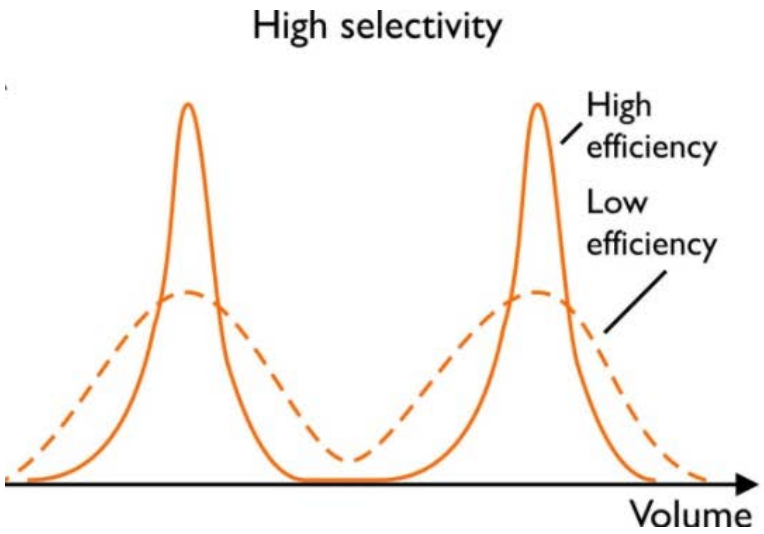
- Temperature and column specifics in GC
- Mobile phase composition and column specifics in LC

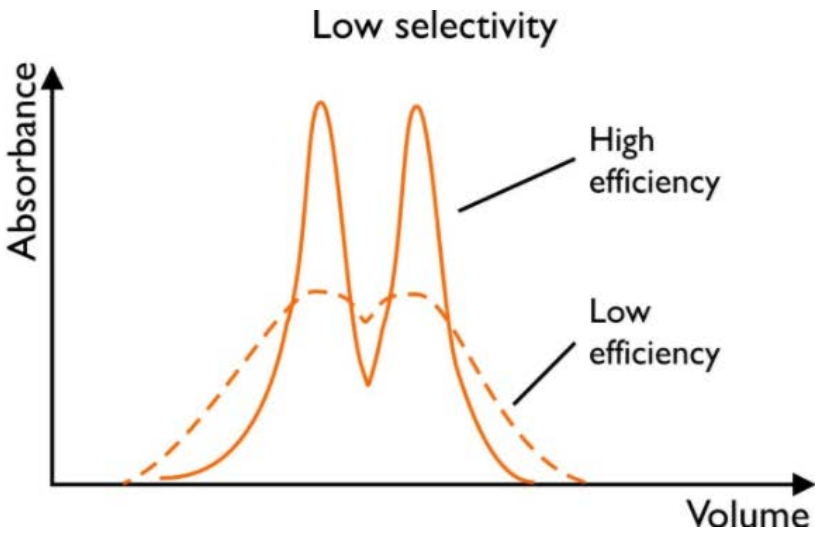
Selectivity (Separation Factor)

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

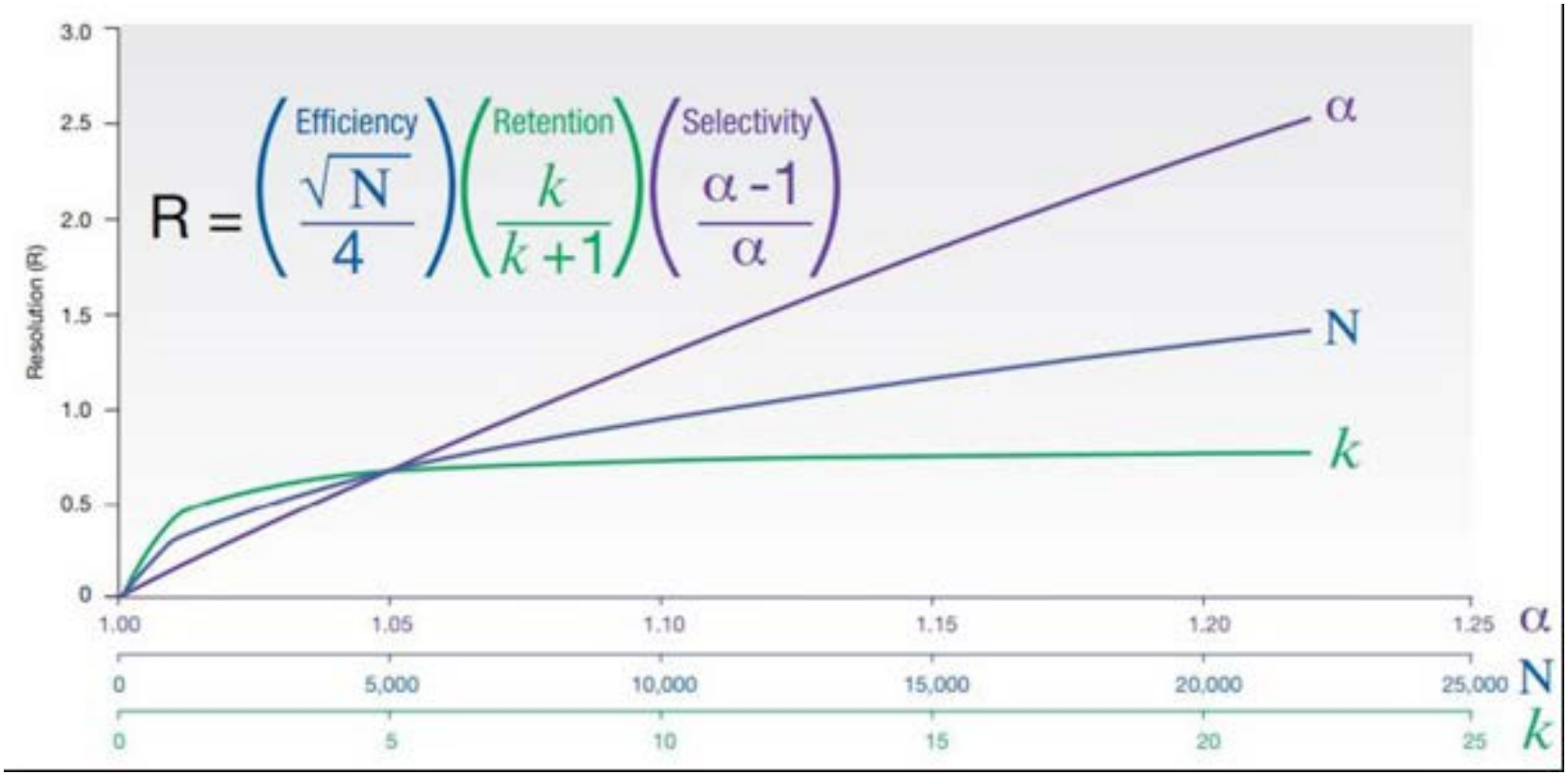


Selectivity affected by
Column stationary phase

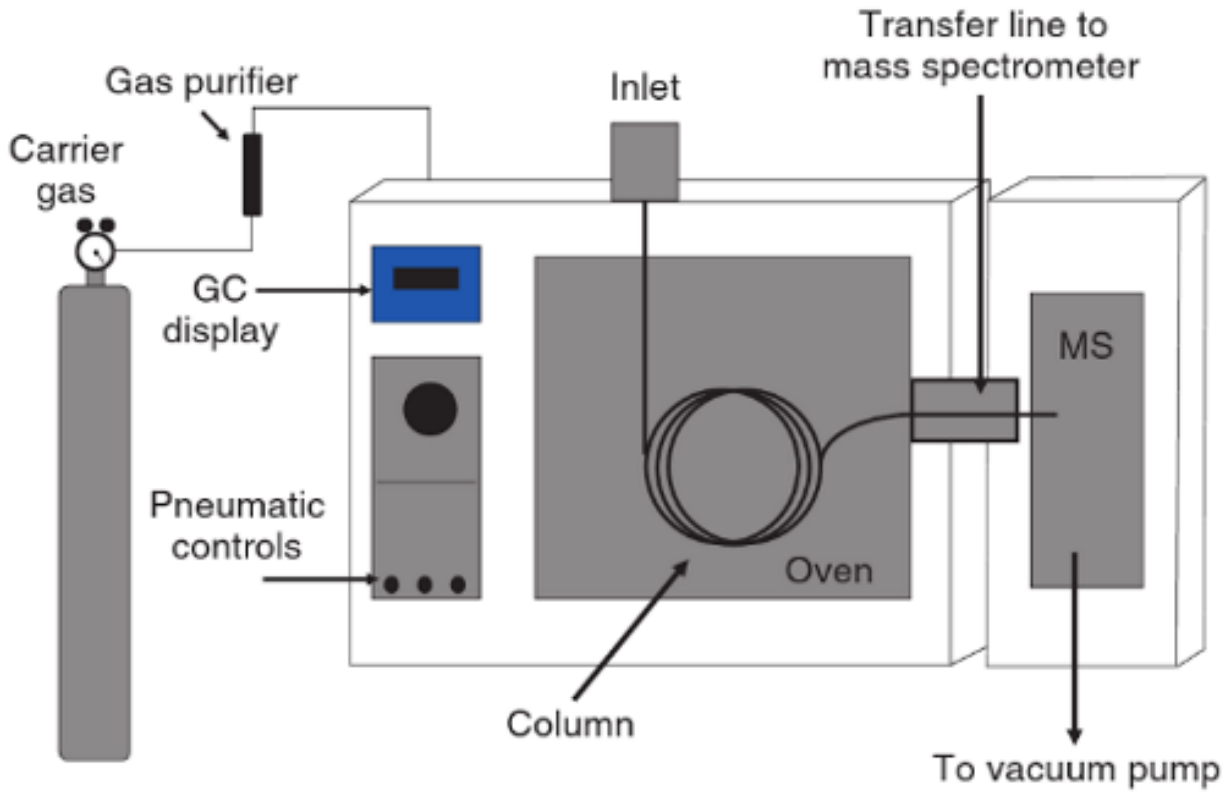




Resolution Equation

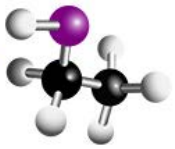


Gas Chromatography (GC)



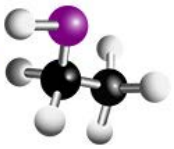
GC Components

Component	Purpose	Types
Autosampler	Introduces sample into the inlets	• SPME and SBSE (fiber and stir bar) • Headspace (dynamic and static) • Liquid (syringe) • Purge and Trap
Inlet	Volatilizes sample and introduces it into the carrier gas and onto the column	• Split/Splitless • On column • Cryogenic Trap
Oven	Temperature control of the column	• Isothermal • Gradient
Detector	Detects individual components as they elute off the column	• Destructive • Non-Destructive



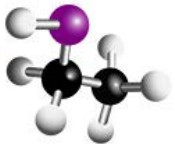
Autosamplers

Type	Benefits	Special considerations
SPME (solid phase microextraction)	Solvent free - highly sensitive extraction technique for organic compounds	Multiple phases available for a wide range of applications
SBSE (Stir Bar Sorptive Extraction)	Efficient extraction of organic compounds from aqueous matrices. Up to 1000x more sensitive than SPME	Available in 2 phases: PDMS and PDMS/EG
Headspace (static or dynamic)	Most suited for the analysis of the very light volatiles in samples	Higher boiling volatiles and semi-volatiles are hard to detect. Sensitivity is limited
Liquid (syringe)	The entire sample is introduced into the inlet	Choose an appropriate liner to accommodate the vaporized sample's volume and to avoid flashback
Purge and Trap	Effective for extracting volatile organic compounds from a sample matrix	Increased sensitivity when measuring environmental pollutants in water and volatiles in beverages

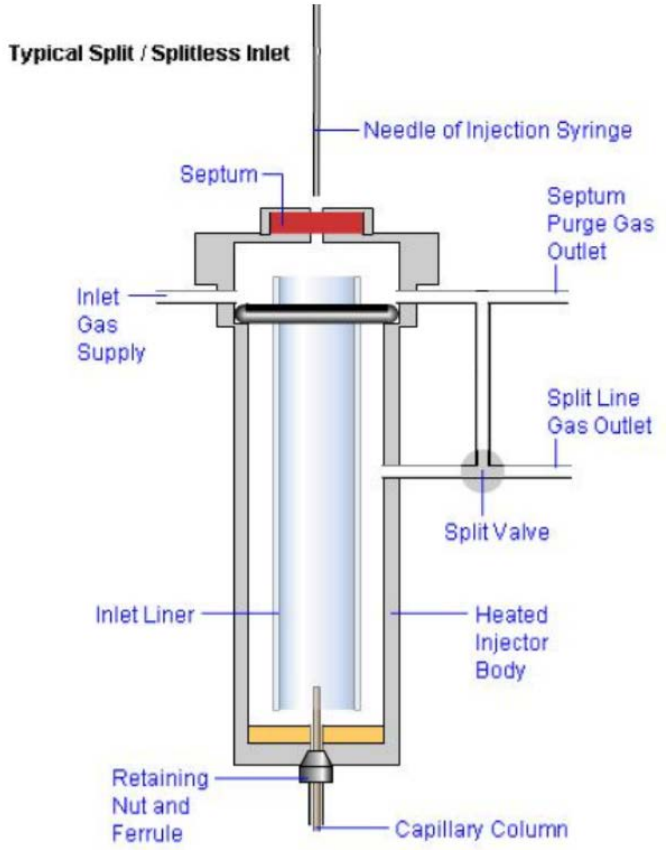


Inlets

Type	Definition	Uses
Vaporizing (Split/Splitless)	Most commonly used with capillary analysis	Split - general injections Splitless - trace analysis.
Cool-on-column or On-column	No exposure to high inlet temps during injection	Samples with wide boiling point ranges, thermally sensitive, trace analysis
Programmed thermal vaporizing injector (PVT)	Similar to cool-on-column – can operated in different modes	Ultra-Trace analysis of difficult sample types
Cryogenic Trap	Volatized analytes are focused on the head of the column by forced cooling to sub-ambient temperatures	On-column trace enrichment, Air and Gas sampling, highly volatile samples.
Thermal Desorption	Used to analyze volatile and semi volatile sample components from a solid matrix	Hazardous gas monitoring and environmental air analysis
Pyrolysis	Used to thermally cleave nonvolatile samples into volatile fragments	Polymers, fibers, microorganisms, geological samples



Split Injections

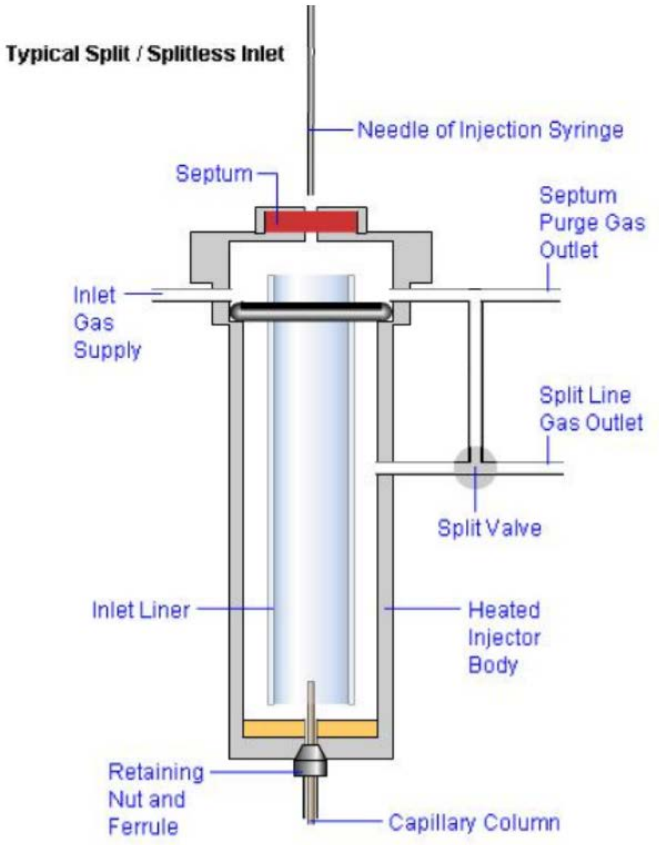


- Concentrated analytes
- Split valve is open the entire injection
- Sample is vaporized, mixed with carrier gas and then split in the inlet
- Split ratios from 1:1 to 1:500

$$\textbf{Split Ratio} = \frac{\textbf{Split vent flow}}{\textbf{Column flow}}$$

$$\textbf{Total Flow} = \textbf{Split Vent Flow} + \textbf{Purge flow} + \textbf{Column Flow}$$

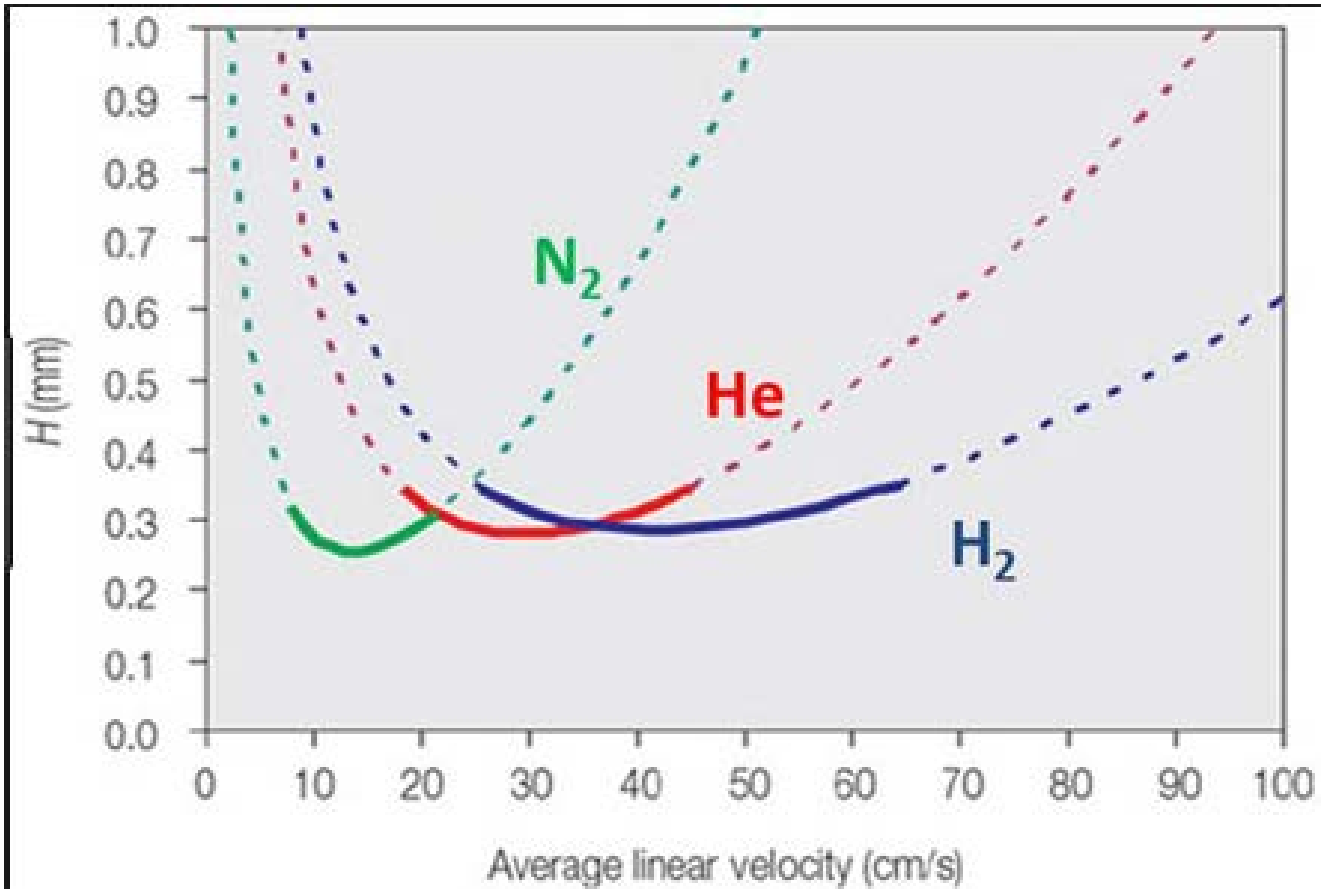
Splitless Injections



- Trace analysis
- Split valve is closed the entire injection
- Sample is vaporized, mixed with carrier gas and then entire sample enters column
- After a predetermined time the split valve is opened to purge inlet
- Initial oven temperature set at 20ºC below solvent boiling point

$$\textit{Total Flow} = \textit{Column Flow} + \textit{Purge flow}$$

Choosing an appropriate carrier gas



$$HETP = \frac{L}{N}$$

L = Column Length (mm)
N = Theoretical plates

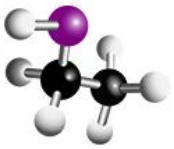
Column Selection

- Internal Diameter (0.1 – 0.53 mm)

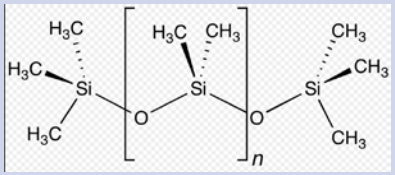
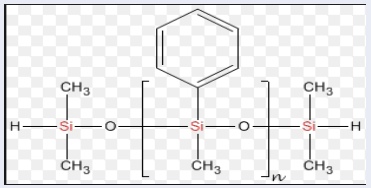
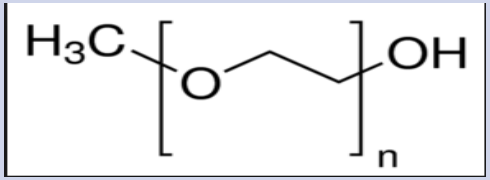
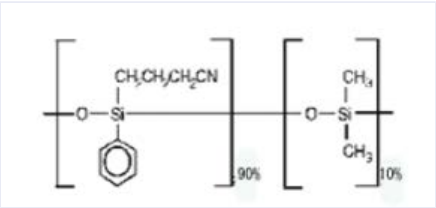
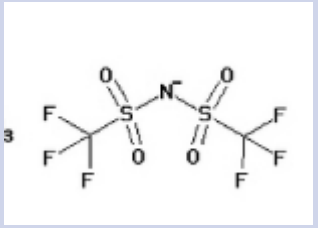
Parameter	Effect	Example
Column Efficiency (N)	Inversely Proportional	Column ID = 0.1 mm, N = 12,500
		Column ID = 0.53mm, N = 2240
Column Capacity (ng each analyte)	Directly Proportional (also depends on film thickness)	Column ID = 0.25 mm, Capacity = 50-100 ng
		Column ID = 0.53 mm, Capacity = 1000-2000 ng

- Column Length (5 m to 100 m)
  - Efficiency (N) and Retention (K) is directly proportional to length
  - Resolution (R) is proportional to the square root of column length
- Film Thickness (0.10 – 5.0 µm)

Parameter	Result
Retention (K)	Thicker films increase K for volatile analytes
	Thinner films decrease K for highly retained analytes

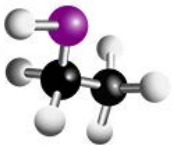


Stationary Phase

Stationary Phase	Polarity	Common Applications	Structure
Dimethyl Polysiloxane/5 % Phenyl Methyl Polysiloxane	Non-Polar	Hydrocarbons, aromatics, PCB containing samples	
35 - 50% Phenyl Methyl Polysiloxane	Intermediate Polar	Pesticides, PCB's herbicides, aromatic hydrocarbon isomers	
Polyethylene Glycol	Polar	FAMES, alcohols, essential oils, glycols, food, flavor and fragrance compounds	
Biscyanopropyl	Highly Polar	Geometric isomers of FAMES	
1,5-Di(2,3-dimethylimidazolium)pentane bis(trifluoromethanesulfonyl)imide	Extremely Polar	Polarizable analytes, benzene, oxygenates in petroleum products	

Detectors

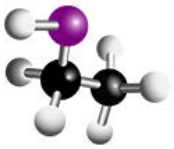
Name	Type of response (selectivity)	Destructive	Type of Gas	Response Characteristic
Flame Ionization Detector (FID)	Responds to C-H bonds	Yes	H_2, Air, He and N_2	Mass
Thermal Conductivity Detector (TCD)	Universal	No	H_2, Ar, He and N_2	Concentration
Electron Capture Detector (ECD)	Electronegative groups (halogens, nitrates and conjugated carbonyls)	No	H_2, He and N_2	Concentration
Flame Photometric Detector (FPD)	Phosphorous or Sulfur	Yes	H_2, Air, and N_2	Mass
Chemiluminescence Detector (SCD, NCD)	Sulfur and Nitrogen	Yes	H_2, Air, O_2	Mass
Nitrogen/Phosphorus Detector, Thermionic Detector (NPD)	Nitrogen or Phosphorous	Yes	H_2, Air, He, N_2	Mass
Mass Spectrometer (MS)	Ionized molecular fragments	Yes	H_2, N_2, He	Mass



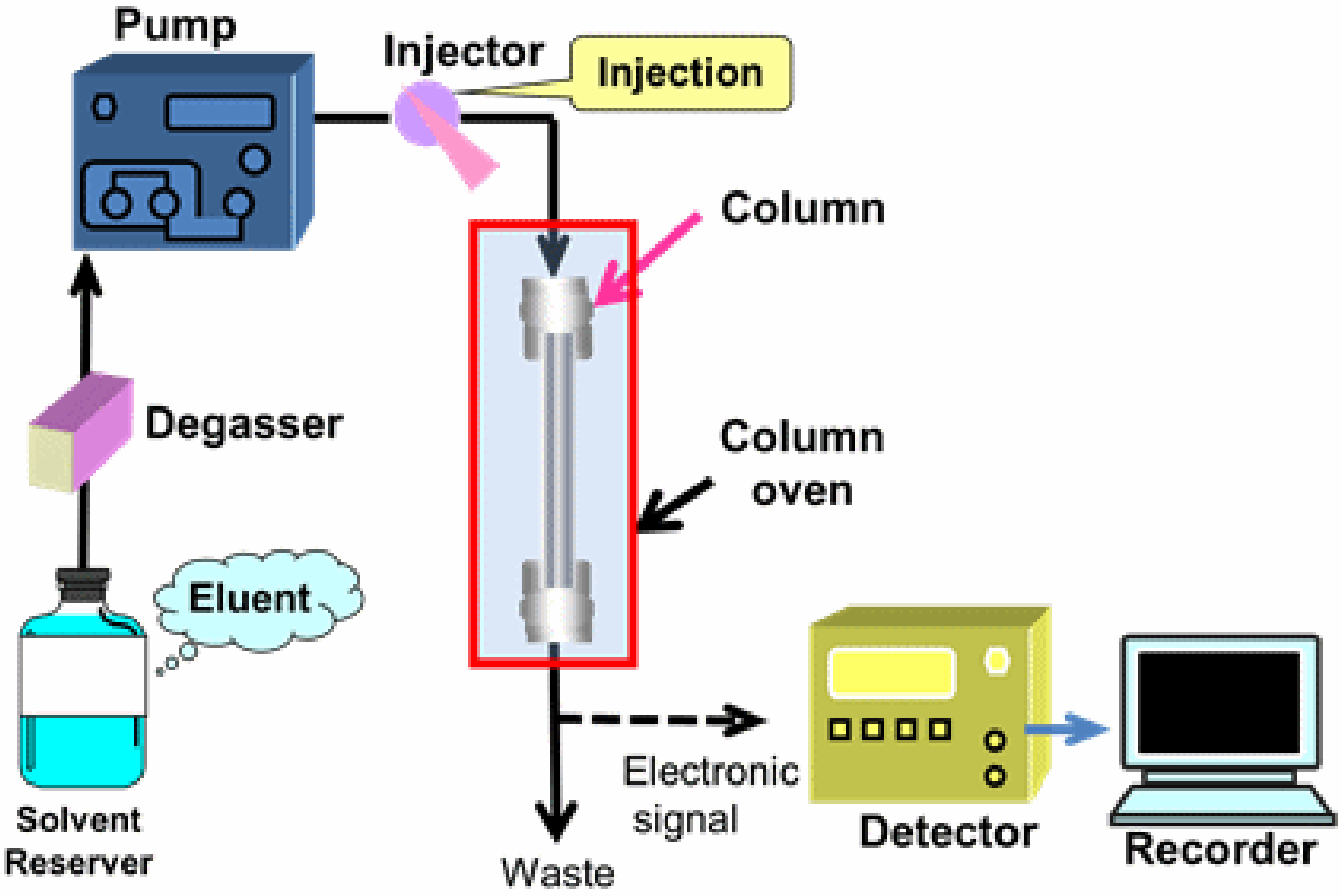
Types of Analysis

- **Quantitative Analysis**
  - Linear dynamic range of detector
  - Limit of detection
  - Calibrations
    - External Calibration
    - Internal Standards
    - Standard Addition

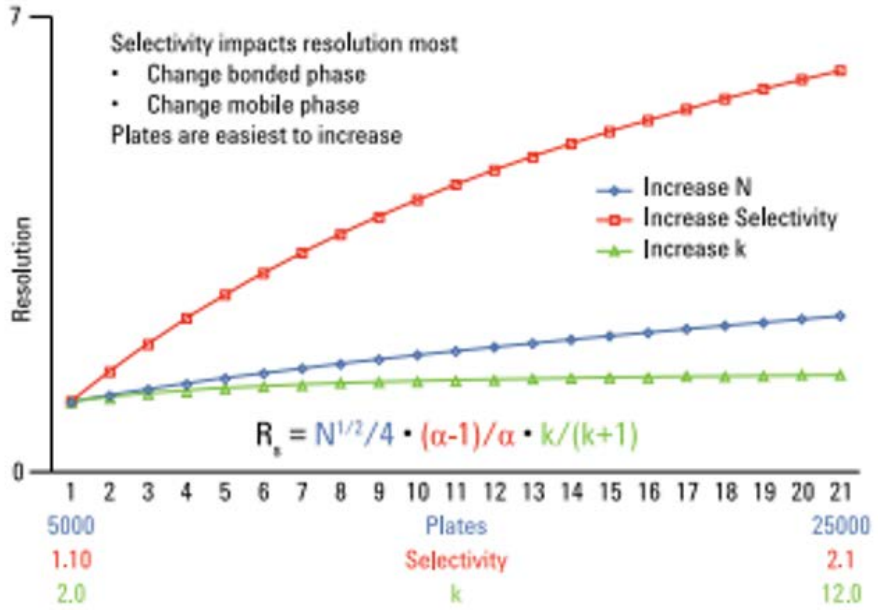
- **Qualitative Analysis**
  - Limit of detection
  - Always use the same GC/Column/Analysis Method
  - Non-targeted analysis
  - Identification of unknowns based on
    - Mass (NIST ID)
    - Spiking studies
    - Retention times
    - Relative Retention times
    - Retention Index (I.E. Kovats)

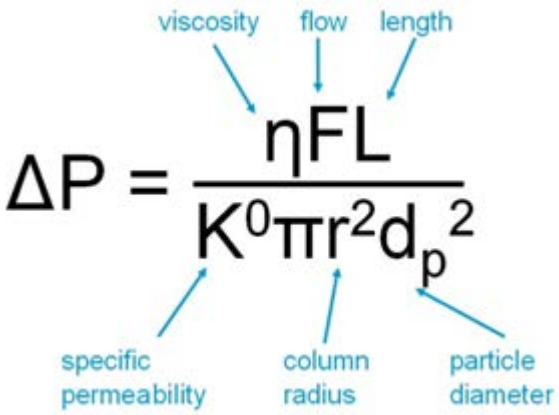


High Performance Liquid Chromatography (HPLC)



HPLC - Fundamentals of Performance



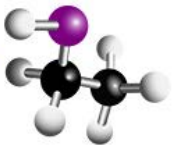


Pressure – (P)

2017 ASBC Meeting

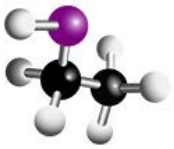
HPLC Components

Component	Purpose	Types
Pump	• Delivers mobile phase though the column at high pressures • Degasses mobile phase	• Quaternary • Binary
Autosampler	• Introduces sample onto the column	• Fixed loop • Flow through
Thermostat	• Temperature control of the column	• Block Heater • Air Bath
Detector	• Detects individual components as they elute off the column	• Destructive • Non-Destructive



Mobile Phase

- **Use good lab practices**
  - HPLC grade solvents and reagents (LC/MS grade is better)
  - Filter the aqueous component (especially buffers)
  - Know the pH ranges of different salts
  - Adjust pH of buffer prior to adding organic
  - Measure components one at a time.
- **Know your compound**
  - What are you trying to separate (acid/base, non-polar/polar/neutral/isomers/size of compound)
- **Know your mobile phase components**
  - Compatible with the column, analyte, and detection
- **Know your column**
  - Reverse phase vs Normal phase



Column Selection

❖ **Pore size**

Size of molecule	Pore Size
MW < 2000 (small molecule)	80 – 120 Å
MW > 2000 (large molecules)	300 Å

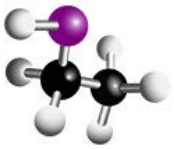
❖ **Particle size and column length**

- HPLC – 3.5 - 5 µm, UHPLC - sub 2-3 µm
- Resolution is proportional to the square root of column length

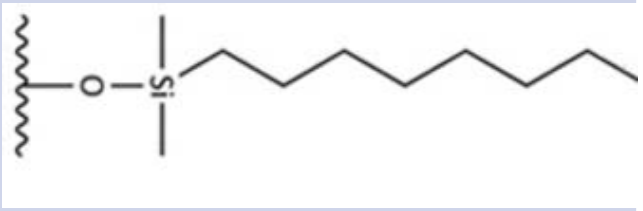
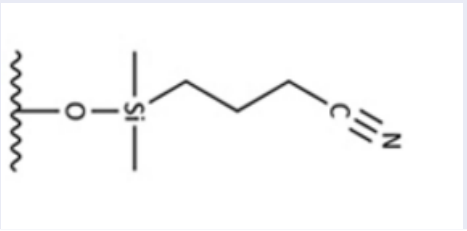
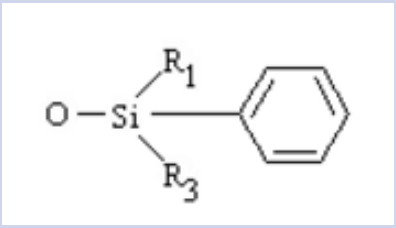
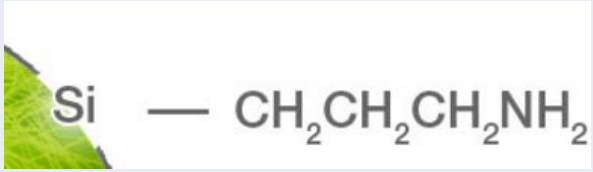
Parameter	Effect	Pressure
Column Efficiency	Directly proportional to column length	Directly proportional to column length
	Doubles when particle size decreases by 1/2	Increases 4 times when particle size decreases by 1/2
Retention	Directly proportional to column length	
	Decreases when particle size decreases	

❖ **Column dimensions**

- Column diameter based on application
  ex. 4.6mm for standard separations, 2.1mm for MS detection

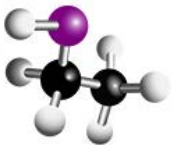


Common RP Stationary Phases

Stationary Phase	Interactions	Molecule types	Structure
Alkyl bonded phase (C18, C8, C4)	Dispersive (Hydrophobic)	Neutral and non- polar compounds, weak acids and bases, proteins and peptides	
CN (cyano)	Electrostatic/ dipole/Ionic	Polar and basic compounds (carboxyl, carbonyl and amine containing compounds)	
Phenyl	π-π interactions	Molecules varying polarity and aromaticity	
Amino	Ion- exchange (anions)	Simple and complex sugars, sugar alcohols, other hydrogen-bonding compounds	

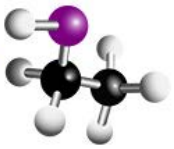
Types of Chromatography

Type	Mobile phase/Stationary phase	Molecule Types
Reverse Phase	Polar mobile phase, Non polar stationary phase	Non-polar, polar, ionizable and ionic molecules, small molecules, peptides, nucleotides, proteins
Normal Phase	Non polar mobile phase (not miscible with water) Polar stationary phase	Isomers, compounds are too hydrophobic or hydrophilic for RP
HILIC	Non polar mobile phase (miscible with water) Polar stationary phase	Polar compounds that are not retained or poorly retained by RP
Ion-Chromatography	Mobile phase is an aqueous buffer, Stationary phase contains ionic groups	Ionic and ionizable compounds



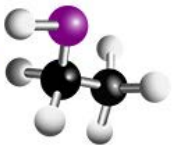
Detectors

Type	Definition	Destructive?
UV/VIS	Compounds absorb UV/visible light in the 190 – 600nm range	No
Fluorescence	Compounds having specific functional groups are excited by shorter wavelength energy and emit higher wavelength radiation called fluorescence	No
Refractive Index	Measures refractive index of an analyte relative to the solvent	No
ELSD	A photomultiplier tube measures the scattering of light caused by nebulized compounds	Yes
MS	Ionizes chemical species and sorts the ions based on their mass/charge ratio	Yes



Quantitative vs Qualitative

- **Quantitative Analysis**
  - Linear dynamic range of instrument
  - Limit of detection
  - Area Percent
  - Calibrations
    - External Calibration
    - Internal Standards
    - Standard Addition
- **Qualitative Analysis**
  - Limit of detection
  - Always use the same HPLC/Column/Analysis Method
  - Identification of unknowns based on
    - Retention times
    - Spiking studies
    - Spectral peak Identification (DAD, MS, MS/MS, SRM)
    - Peak Purity (DAD)
    - Relative Retention Time (RRT)



Useful References

- Troubleshooting guides
  - https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/Bulletin/4497.pdf
  - https://www.agilent.com/cs/library/eseminars/Public/HPLC%20Column%20and%20System%20Troubleshooting.pdf
  - http://www.waters.com/waters/library.htm?cid=511436&lid=1528445&locale=en_US
  - http://www.restek.com/pdfs/GNWC1723-UNV.pdf
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  - https://az621941.vo.msecnd.net/documents/f5d3c182-7a88-49d6-8d3e-a0c959f1d9e0.pdf
- Books
  - “Basic Gas Chromatography” – Harold McNair and James Miller
  - “Practical Problem Solving in HPLC” – Stavros Kromidas
  - “Practical HPLC Method Development” – Lloyd R. Snyder