HPLC Basics

Fundamentals of Liquid Chromatography (HPLC)

Courtesy of Agilent Technologies, Inc.

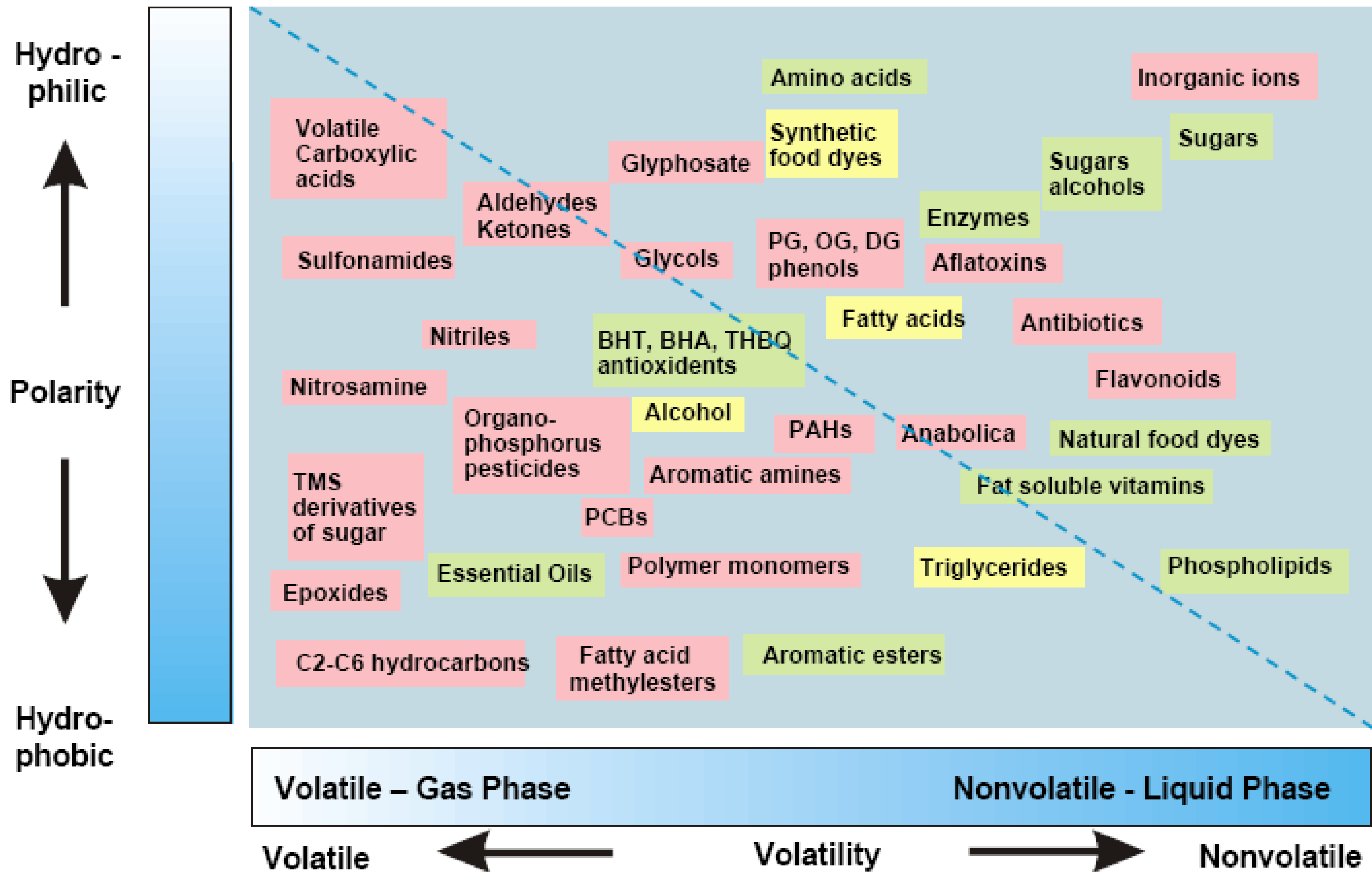
Fundamentals of High Performance Liquid Chromatography (HPLC)

This course will enable you to:

- Explain the general principles of HPLC analyses
- Know the major application areas of HPLC
- Identify the major components of an HPLC system and explain their principles of operation

Chromatographic Separation Techniques

Which separation technique for which compound?



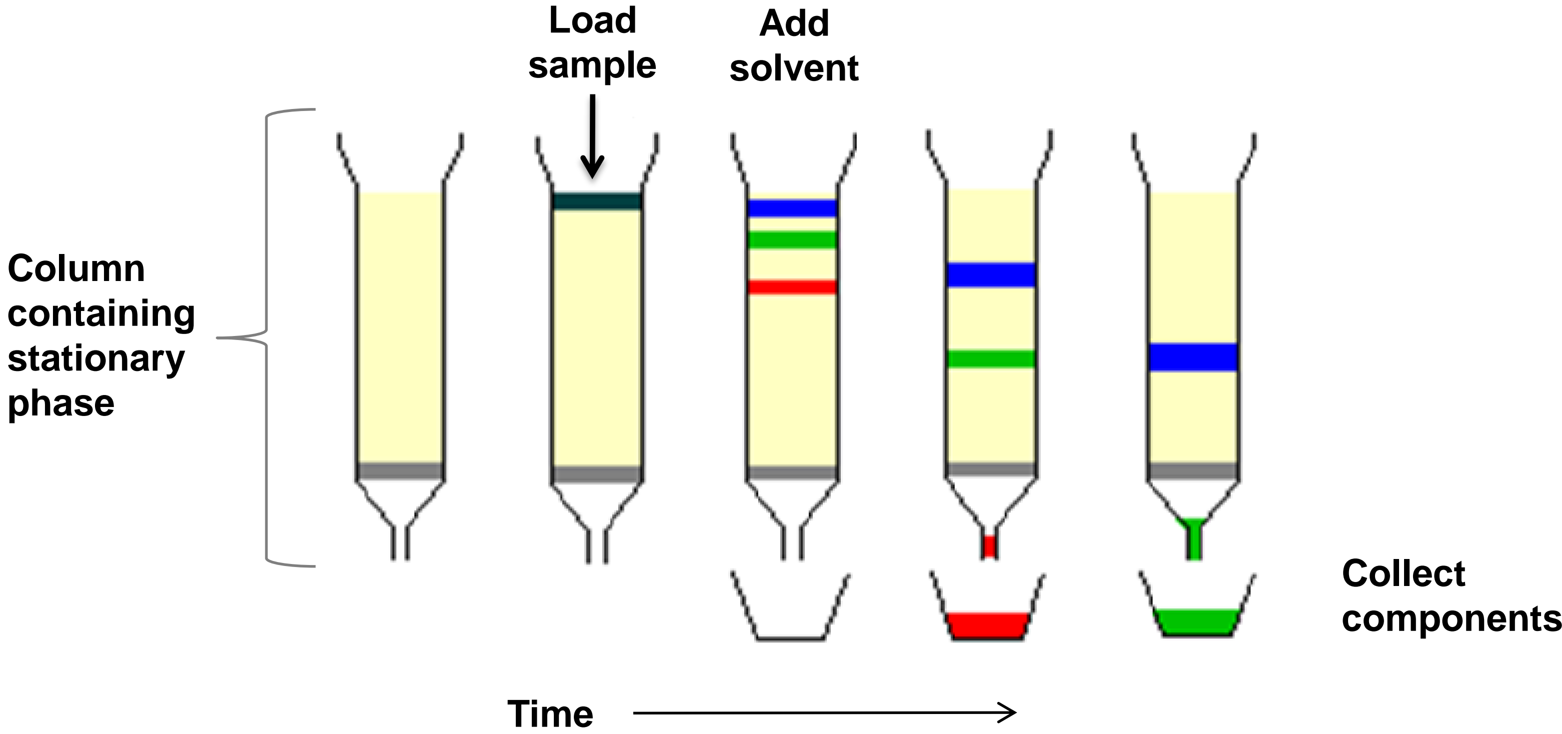
First, What is Liquid Chromatography?

- Liquid chromatography is a separation technique that involves:
  - the placement (injection) of a small volume of liquid sample
  - into a tube packed with porous particles (stationary phase)
  - where individual components of the sample are transported along the packed tube (column) by a liquid moved by gravity.
- The components of the sample are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.
- The separated components are collected at the exit of this column and identified by an external measurement technique, such as a spectrophotometer that measures the intensity of the color, or by another device that can measure their amount.

☞ Note: The modern form of liquid chromatography is now referred to as “flash chromatography”

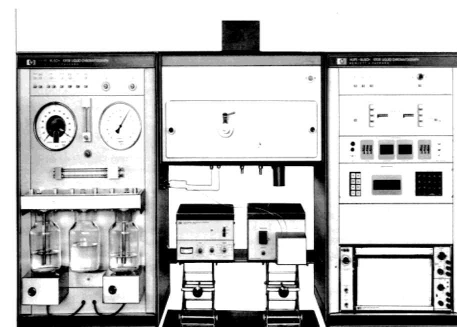
Note: Look for the comparison with HPLC on page 7

Principles of Liquid Chromatography



Then, What is HPLC?

- **HPLC** is an abbreviation for
  High Performance Liquid Chromatography
  (It has also been referred to as High Pressure LC)
- HPLC has been around for about 35 years and is the largest separations technique used
- The history of HPLC:
  - Beginning of the 60's: start of HPLC as High Pressure Liquid Chromatography
  - End of the 70's improvements of column material and instrumentation – High Performance Liquid Chromatography
  - Since beginning of the 80's: "boom" in HPLC started
  - Since 2006 new terms popped up like UPLC, RRLC, UFLC, RSLC, …..



HPLC in 1973

HPLC in 2009

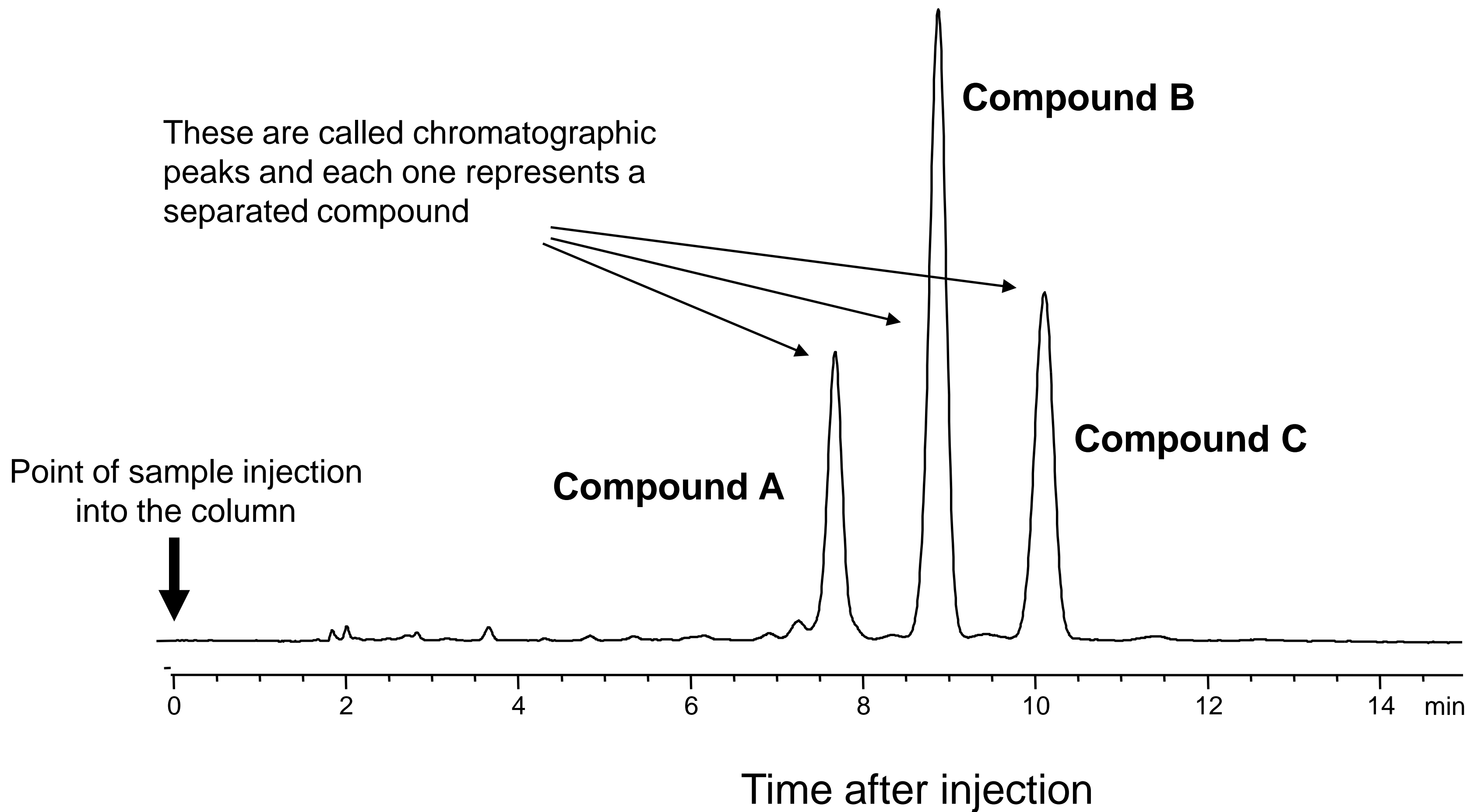
What is HPLC?

- **HPLC** is a **separation technique** that involves:
  - the injection of a small volume of liquid sample
  - into a tube packed with tiny particles (3 to 5 micron (µm) in diameter called the **stationary phase**)
  - where individual components of the sample are moved down the packed tube (**column**) with a liquid (**mobile phase**) forced through the column by high pressure delivered by a pump.
- These **components are separated** from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.
- These separated components are detected at the exit of this tube (**column**) by a flow-through device (**detector**) that measures their amount. An output from this detector is called a "**liquid chromatogram**".

☞ **In principle, LC and HPLC work the same way except the speed, efficiency, sensitivity and ease of operation of HPLC is vastly superior.**

Note: Compare this description to that on page 4 about "Liquid Chromatography"

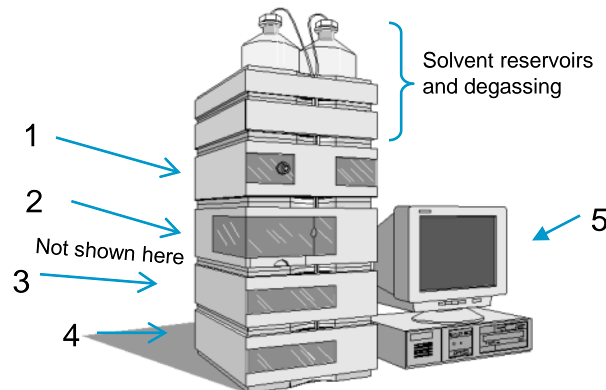
What Does a Liquid Chromatogram Look Like?



This is the chromatogram resulting from the injection of a small volume of liquid extracted from a vitamin E capsule that was dissolved in an organic solvent. Modern HPLC separations usually require 10- to 30-minutes each.

What does a high pressure LC look like?

(1) Describing the 5 major HPLC components and their functions …



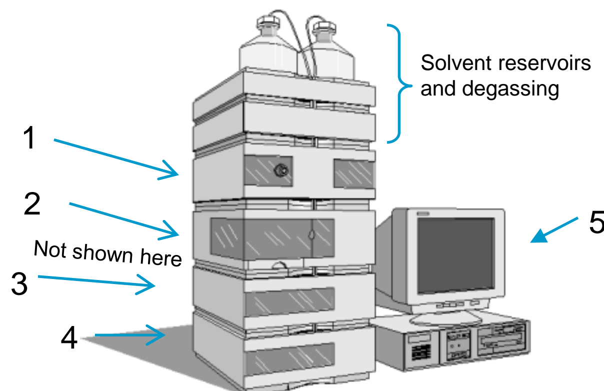
1. Pump:

- 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).

2. 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).
    - The injector must also be able to withstand the high pressures of the liquid system.
- An autosampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical.

(2) Describing the 5 major HPLC components and their functions …



3. Column:

- Considered the "heart of the chromatograph" the column's stationary phase separates the sample components of interest using various physical and chemical parameters.
  - The small particles inside the column are what cause the high backpressure at normal flow rates.
  - The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph.

4. Detector:

- The detector can see (detect) the individual molecules that come out (elute) from the column.
  - A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyze the sample components.
  - The detector provides an output to a recorder or computer that results in the liquid chromatogram (i.e., the graph of the detector response).

5. Computer:

- Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis).

What is HPLC used for?

Separation and analysis of non-volatile or thermally-unstable compounds

HPLC is optimum for the separation of chemical and biological compounds that are non-volatile

☞ NOTE: If a compound is volatile (i.e. a gas, fragrance, hydrocarbon in gasoline, etc.), gas chromatography is a better separation technique.

Typical non-volatile compounds are:

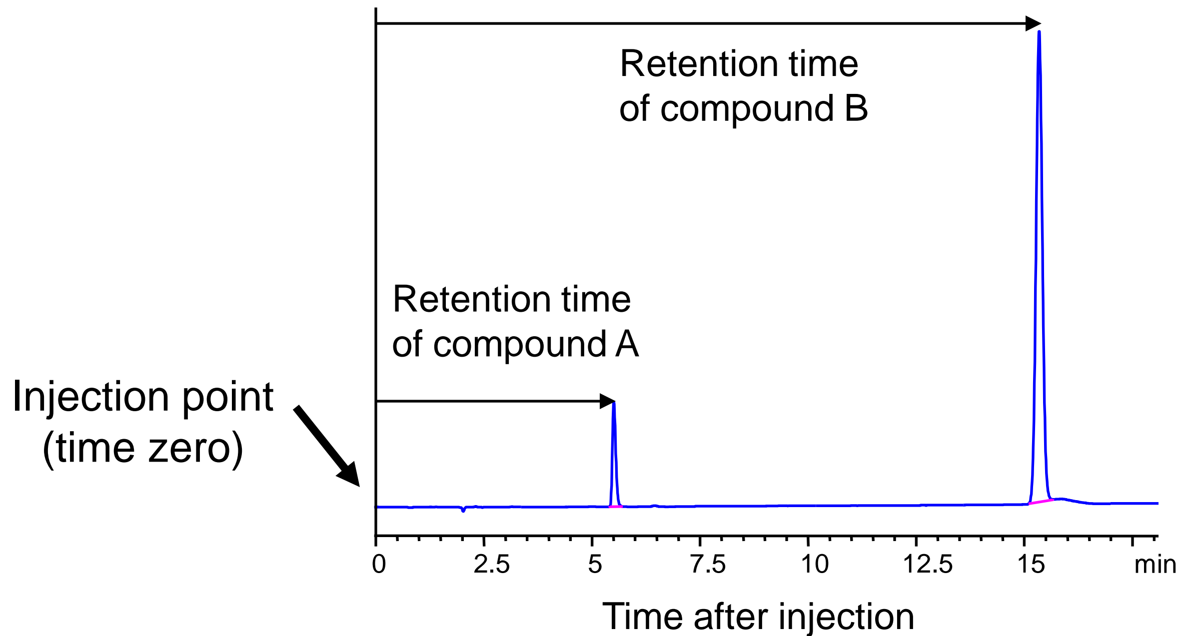
- Pharmaceuticals like aspirin, ibuprofen, or acetaminophen (Tylenol)
- Salts like sodium chloride and potassium phosphate
- Proteins like egg white or blood protein
- Organic chemicals like polymers (e.g. polystyrene, polyethylene)
- Heavy hydrocarbons like asphalt or motor oil
- Many natural products such as ginseng, herbal medicines, plant extracts
- Thermally unstable compounds such as trinitrotoluene (TNT), enzymes

What is HPLC used for?

Qualitative analysis

The identification (ID) of individual compounds in the sample;

- the most common parameter for compound ID is its retention time (the time it takes for that specific compound to elute from the column after injection);
- depending on the detector used, compound ID is also based on the chemical structure, molecular weight or some other molecular parameter.



What is HPLC used for?

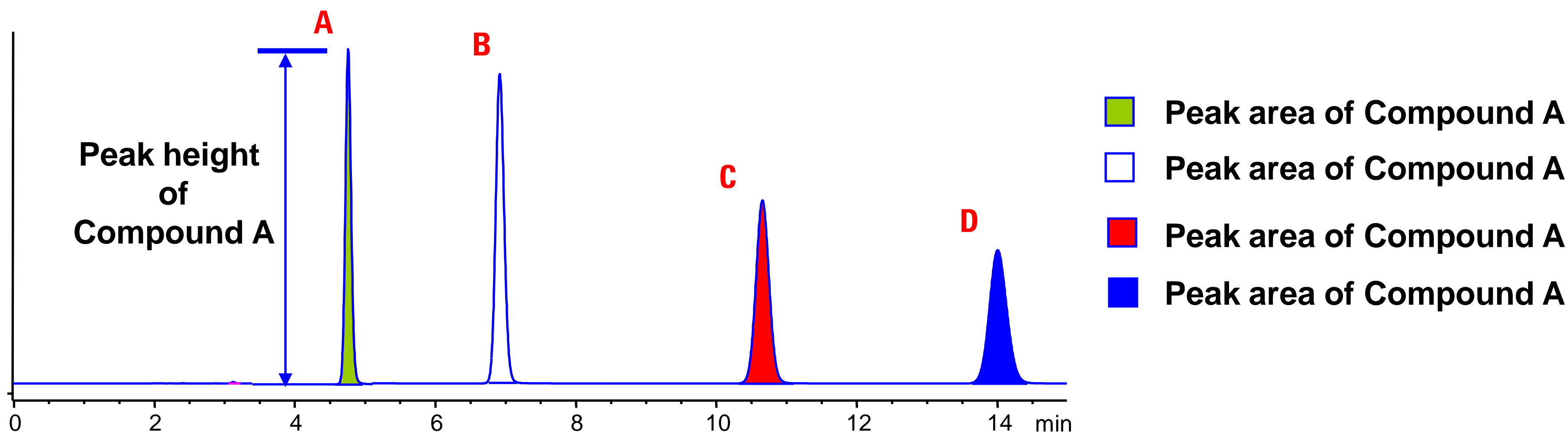
Quantitative Analysis

The measurement of the amount of a compound in a sample (concentration); meaning, how much is there?;

There are two main ways to interpret a chromatogram (i.e. perform quantification):

1. determination of the peak height of a chromatographic peak as measured from the baseline;
2. determination of the peak area (see figure below);

In order to make a quantitative assessment of the compound, a sample with a known amount of the compound of interest is injected and its peak height or peak area is measured. In many cases, there is a linear relationship between the height or area and the amount of sample.

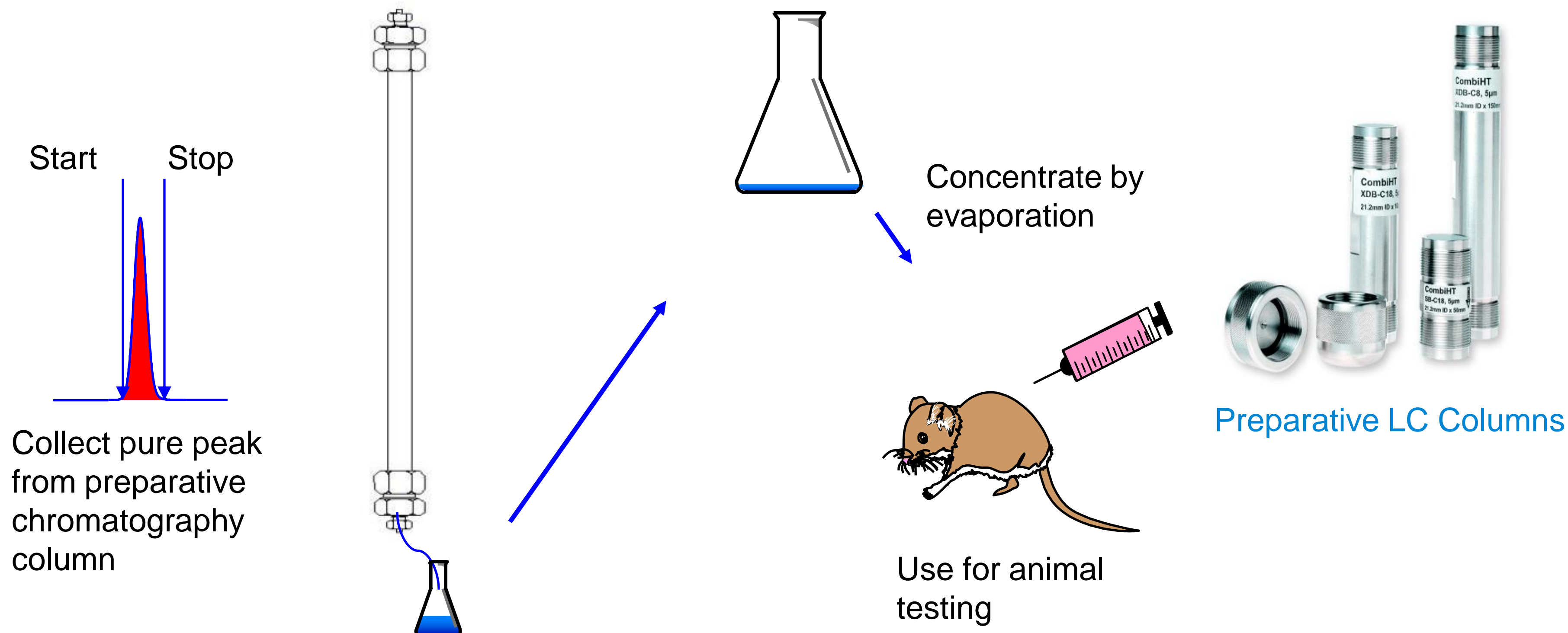


What is HPLC used for?

Preparation of Pure Compound(s)

- By collecting the chromatographic peaks at the exit of the detector,
- and concentrating the compound (analyte) by removing/evaporating the solvent,
- a pure substance can be prepared for later use (e.g. organic synthesis, clinical studies, toxicology studies, etc.).

This methodology is called preparative chromatography.

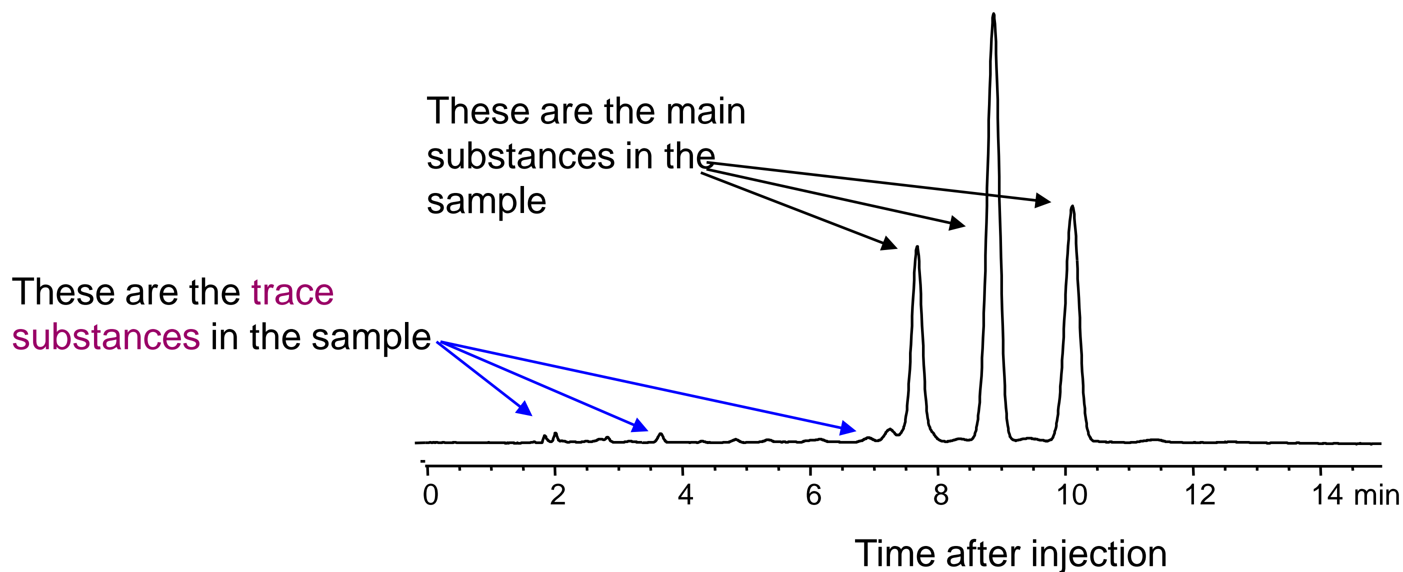


What is HPLC used for?

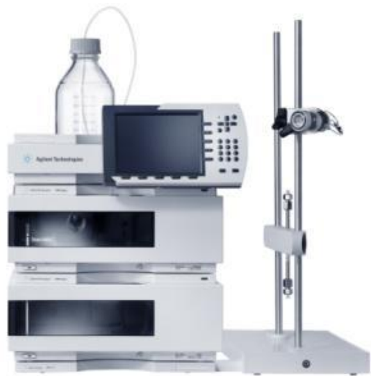
Trace analysis

A trace compound is a compound that is of interest to the analyst but it's concentration is very low, usually less than 1% by weight, often parts per million (ppm) or lower;

- the determination of trace compounds is very important in pharmaceutical, biological, toxicology, and environmental studies since even a trace substance can be harmful or poisonous;
- in a chromatogram trace substances can be difficult to separate or detect;
- high resolution separations and very sensitive detectors are required.



Examples of Different Instruments and Configurations



Modular HPLC System – basic configuration with isocratic pump, manual injector, variable wavelength detector, and hand-held controller

Modular HPLC System – high-end configuration with quaternary pump, autosampler, column thermostat, diode array detector, and computer with control and data analysis SW

Integrated HPLC System "all parts in one box" – different configurations possible, here with gradient pump, autosampler, column oven, VWD, and computer with control and data analysis SW (not shown on picture)

Let’s Look at Individual Modules…

Pump

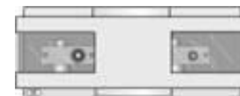
Pump Module – types:

- Isocratic pump - delivers constant mobile phase composition;
  - solvent must be pre-mixed;
  - lowest cost pump
- Gradient pump - delivers variable mobile phase composition;
  - can be used to mix and deliver an isocratic mobile phase or a gradient mobile phase

  – Binary gradient pump – delivers two solvents

  – Quaternary gradient pump – four solvents

Gradient vs. Isocratic Conditions



Pump

Isocratic

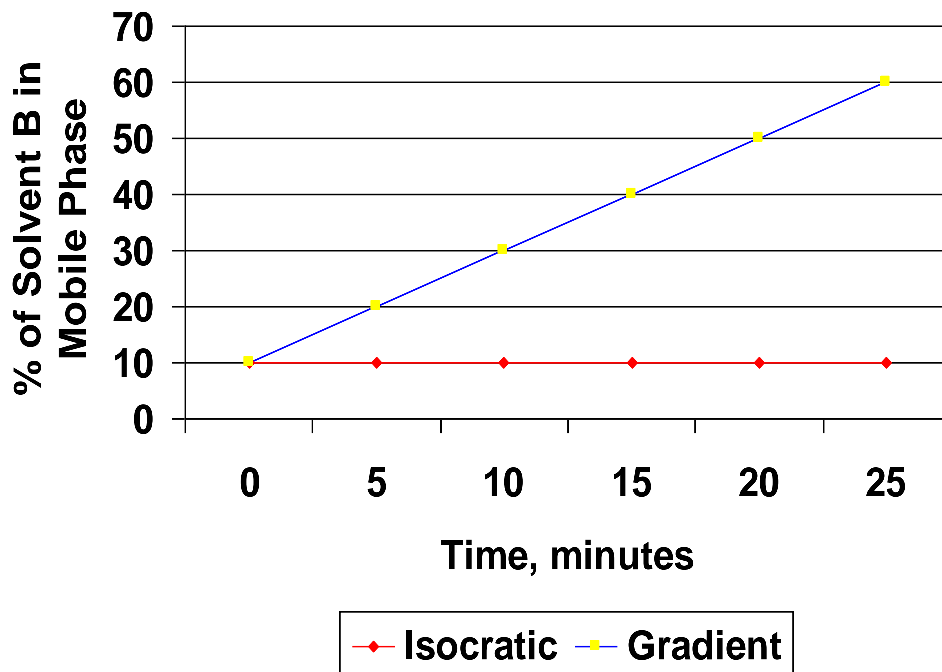
mobile phase solvent composition remains constant with time

- Best for simple separations
- Often used in quality control applications that support and are in close proximity to a manufacturing process

Gradient

mobile phase solvent ("B") composition increases with time

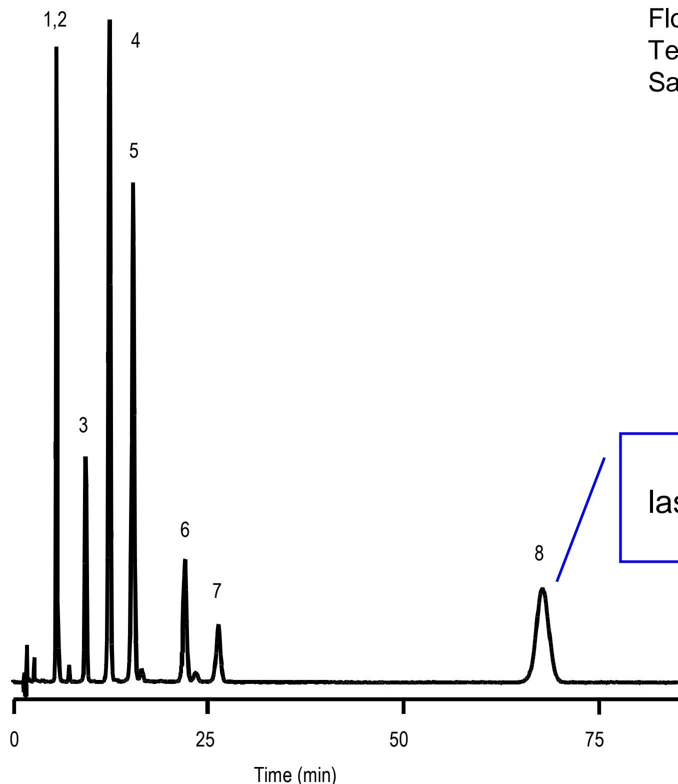
- Best for the analysis of complex samples
- Often used in method development for unknown mixtures
- Linear gradients are most popular (for example, the "gradient" shown at right)



Why Are Mobile Phase Gradients Used in HPLC?

Separation of Herbicides on ZORBAX StableBond-C18

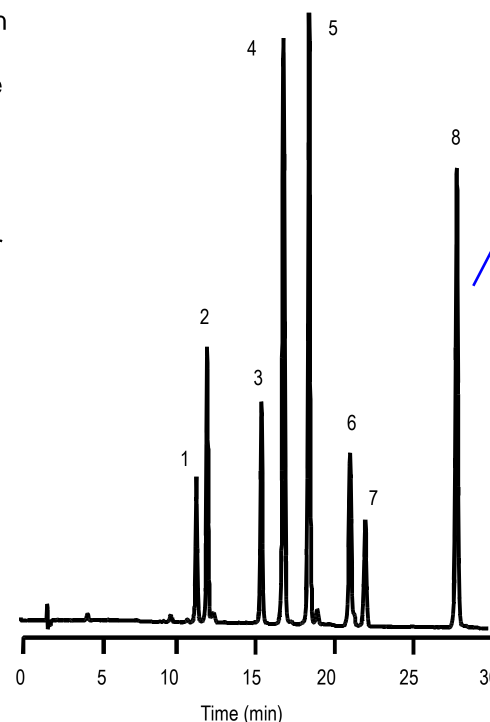
Isocratic Elution
70% water/30% Acetonitrile



Note:
last peak eluted in ~70 minutes

Column:	**ZORBAX SB-C18** **4.6 x 150 mm, 5 µm**
Mobile Phase:	A: H_2O with 0.1% TFA, pH 2 B: Acetonitrile
Flow Rate:	1.0 mL/min
Temperature:	35°C
Sample:	1. Tebuthiuron 2. Prometon 3. Prometryne 4. Atrazine 5. Bentazon 6. Propazine 7. Propanil 8. Metolachlor

Gradient Elution
20 – 60% Acetonitrile in water in 30 min.



Note:
last peak eluted in ~ 28 minutes and it is sharper

Sample Injection

… how is a sample actually put into an LC system

Manual Injector:

1. User manually loads sample into the injector using a syringe
2. and then turns the handle to inject sample into the flowing mobile phase

   … which transports the sample into the beginning (head) of the column, which is at high pressure

Autosampler:

1. User loads vials filled with sample solution into the autosampler tray (100 samples)
2. and the autosampler automatically
   1. measures the appropriate sample volume,
   2. injects the sample,
   3. then flushes the injector to be ready for the next sample, etc., until all sample vials are processed …

   … for unattended automatic operation

HPLC Columns

LC Columns

☞ **Within the Column is where separation occurs.**

☞☞ **Key Point – Proper choice of column is critical for success in HPLC**

Types of columns in HPLC:

- Analytical [internal diameter (i.d.) 1.0 - 4.6-mm; lengths 15 – 250 mm]
- Preparative (i.d. > 4.6 mm; lengths 50 – 250 mm)
- Capillary (i.d. 0.1 - 1.0 mm; various lengths)
- Nano (i.d. < 0.1 mm, or sometimes stated as < 100 µm)

LC Columns - analytical

Materials of construction for the tubing

- Stainless steel (the most popular; gives high pressure capabilities)
- Glass (mostly for biomolecules)
- PEEK polymer (biocompatible and chemically inert to most solvents)

HPLC Columns Packing Materials

LC Columns

- Columns are packed with small diameter porous particles.
  - The most popular sizes are: 5-µm, 3.5- µm and 1.8-µm
- Columns are packed using high-pressure to ensure that they are stable during use
  - most users purchase pre-packed columns to use in their liquid chromatographs
- These porous particles in the column usually have a chemically bonded phase on their surface which interacts with the sample components to separate them from one another
  - for example, C18 is a popular bonded phase
- The process of retention of the sample components (often called analytes) is determined by the choice of column packing and the selection of the mobile phase to push the analytes through the packed column.

LC column packing material

Typical Mechanism of an HPLC Separation

Analyte molecules are attracted to the chemically bonded phase

Chemically bonded phase

Silica
Base Packing

Separation Modes of HPLC

LC Columns

☞ Key Point to Remember:

The correct selection of the column packing and the mobile phase are the most important factors in successful HPLC.

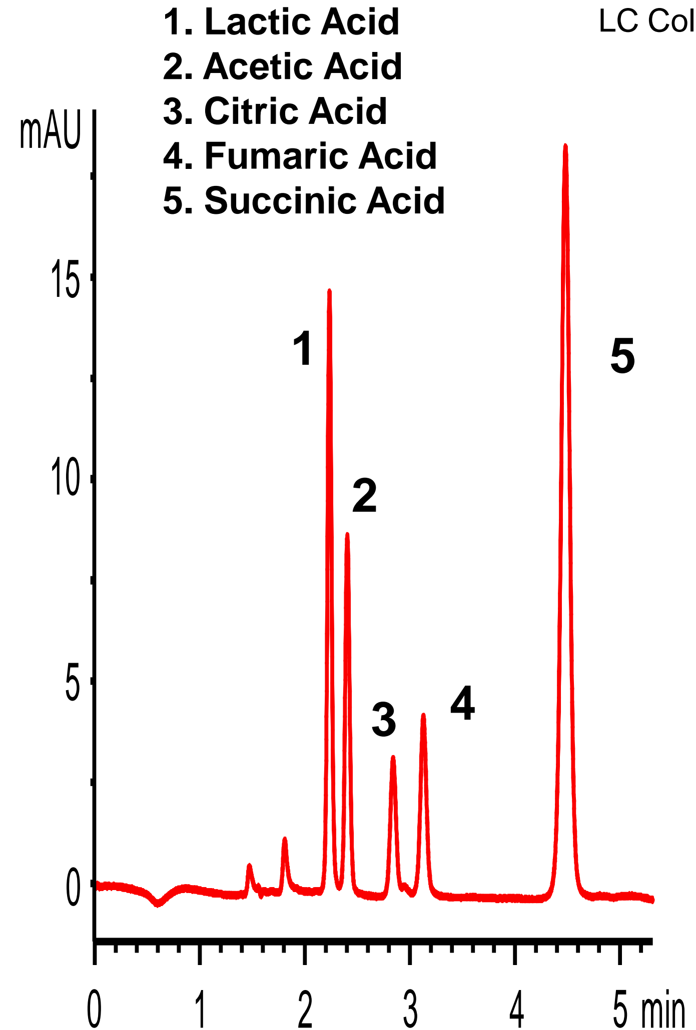
There are four major separation modes that are used to separate most compounds:

1. **Reversed-phase** chromatography
2. **Normal-phase and adsorption** chromatography
3. **Ion exchange** chromatography
4. **Size exclusion** chromatography

….Let's briefly look at each mode

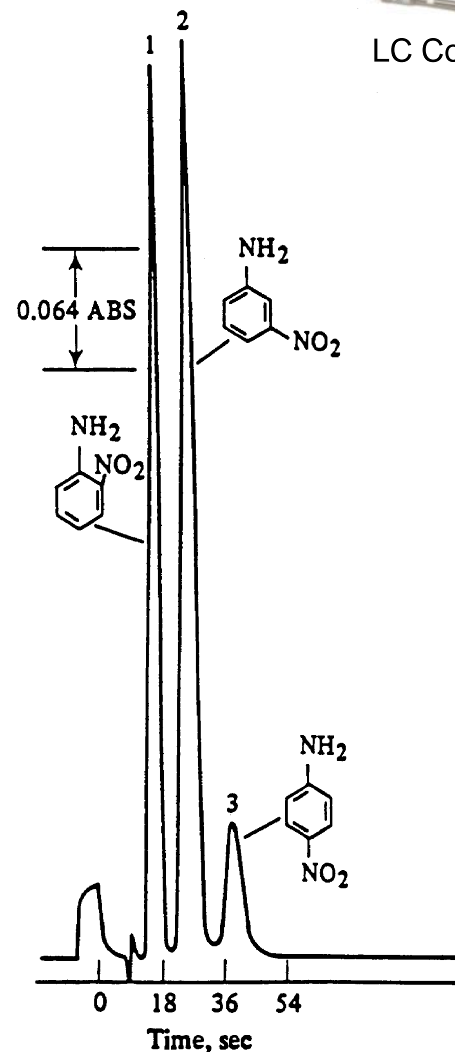
(1) Reversed-Phase Chromatography (RPC)

- 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
- For samples containing a wide range of compounds, gradient elution is often used …
  - One begins with a predominantly water-based mobile phase and then adds organic solvent as a function of time.
  - The organic solvent increases the solvent strength and elutes compounds that are very strongly retained on the RPC packing



(2) Normal Phase or Adsorption Chromatography

- 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
  - geometric isomers
  - cis-trans isomers
  - class separations
  - and chiral compounds.



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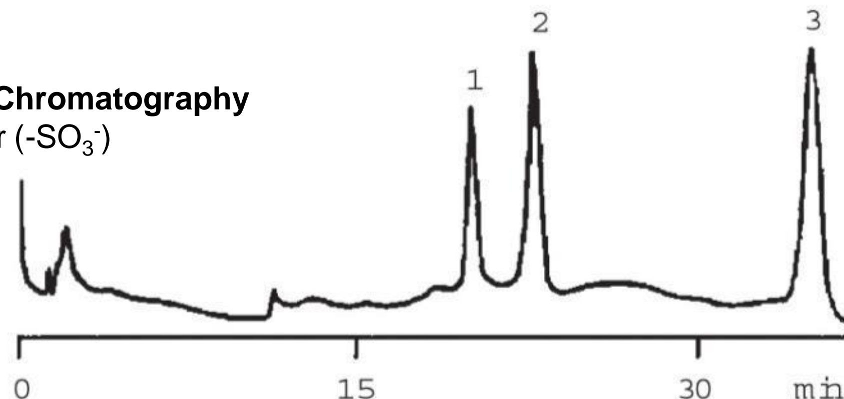
(3) Ion Exchange Chromatography

LC Columns

- In ion exchange, the column packing contains ionic groups (e.g. sulfonic, tetraalkylammonium) and the mobile phase is an aqueous buffer (e.g. phosphate, formate, etc.).
- Ion exchange is used by about 20% of the liquid chromatographers
- The technique is well suited for:
  - the separation of inorganic and organic anions and cations in aqueous solution.
  - Ionic dyes, amino acids, and proteins can be separated by ion exchange because such compounds are salt in brine water,

Application Example of Ion Exchange Chromatography
Basic proteins on strong cation exchanger ($-SO_3^-$)
1. RNA polymerase
2. Chymotrypsinogen
3. Lysozyme

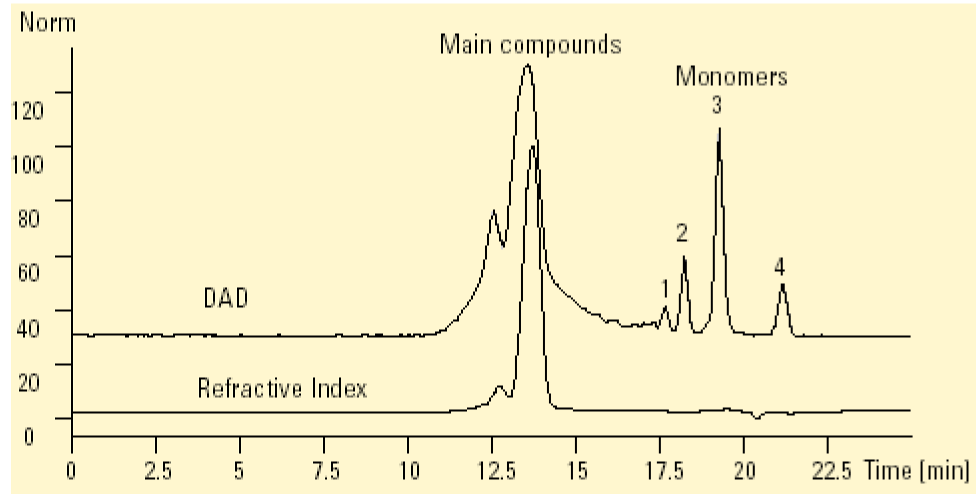


(4) Size Exclusion Chromatography (SEC)

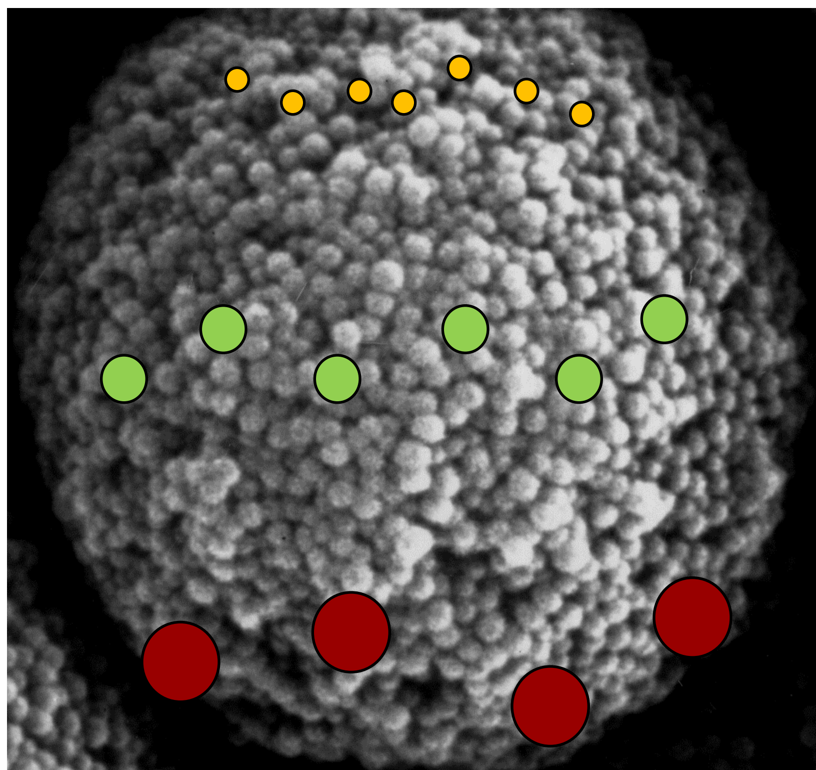
LC Columns

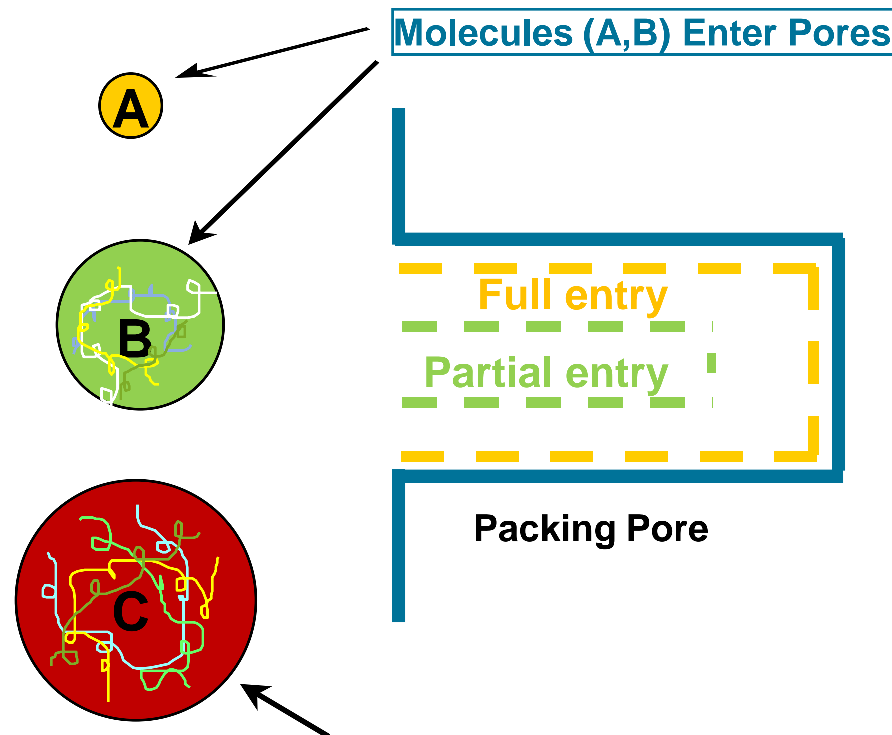
- In SEC, there is no interaction between the sample compounds and the column packing material. Instead, molecules diffuse into pores of a porous medium. Depending on their size relative to the pore size, molecules are separated. Molecules larger than the pore opening do not diffuse into the particles, while molecules smaller than the pore opening enter the particle and are separated. Large molecules elute first. Smaller molecules elute later
- The SEC technique is used by 10-15% of chromatographers, mainly for polymer characterization and for proteins.
- There are two modes:
  - non-aqueous SEC [sometimes termed Gel Permeation Chromatography (GPC)] and
  - aqueous SEC [sometimes referred to an Gel Filtration Chromatography (GFC)].

Gel Permeation Chromatogram of Polybutadiene polymer on non-aqueous SEC (GPC) column;
The monomers elute after the polymer;
column: PLgel mixed-D gel



Mechanism of SEC





Molecule (C) Will be Excluded from Pores

Molecules must freely enter and exit pores to be separated. Largest molecules elute first, followed by intermediate size molecules and finally the smallest molecules elute last.

C B A

Signal

Time

Temperature Control in HPLC

Why is it needed?

Reproducibility

- Retention in HPLC is temperature-dependent
- If temperature varies, then it is difficult to assign "peaks" to specific compounds in the chromatogram and the peak areas/heights may vary

Solubility

- Certain chemical compounds may have low solubility in the HPLC mobile phase
- If they are injected into the flow stream they may precipitate or other difficulties may arise

Stability

- Certain chemical compounds, especially biological compounds such as enzymes or proteins, may not be stable at room temperature or higher
- The temperature needs to be much lower down to 4°C

How is Temperature Control Achieved?

Three (3) ways the temperature of a column could be controlled, use:

1. Oven
2. Heater Block
3. Water bath

Heater block

Column placed in
Heater block

Agilent 1200 Series Column Compartment
(temperature range: 10 above ambient to 100°C)

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Detection in HPLC

There are many detection principles used to detect the compounds eluting from an HPLC column.

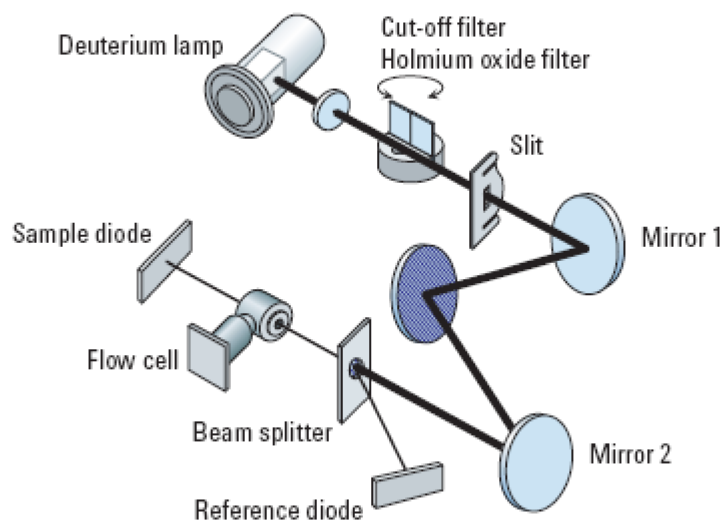
The most common are:

- **Spectroscopic** Detection
- **Refractive Index** Detection
- **Fluorescence** Detection

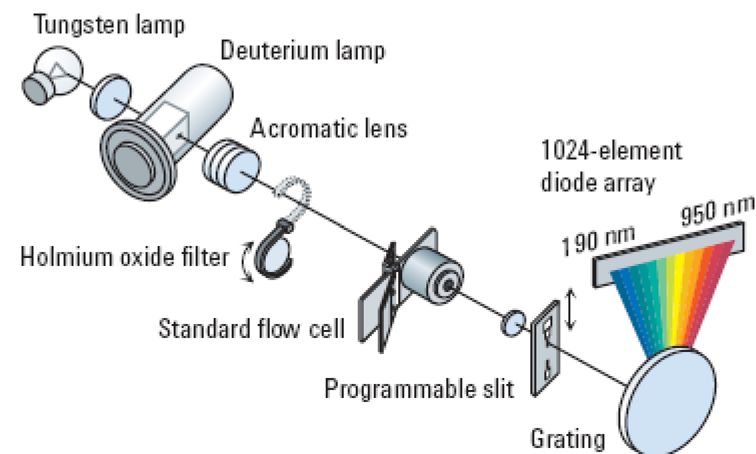
Spectroscopic Detection

Ultraviolet (UV) Absorption

- An ultraviolet light beam is directed through a flow cell and a sensor measures the light passing through the cell.
- If a compound elutes from the column that absorbs this light energy, it will change the amount of light energy falling on the sensor.
- The resulting change in this electrical signal is amplified and directed to a recorder or data system.
- A UV spectrum is sometimes also obtained which may aid in the identification of a compound or series of compounds.



Variable Wavelength Detector



Diode Array Detector

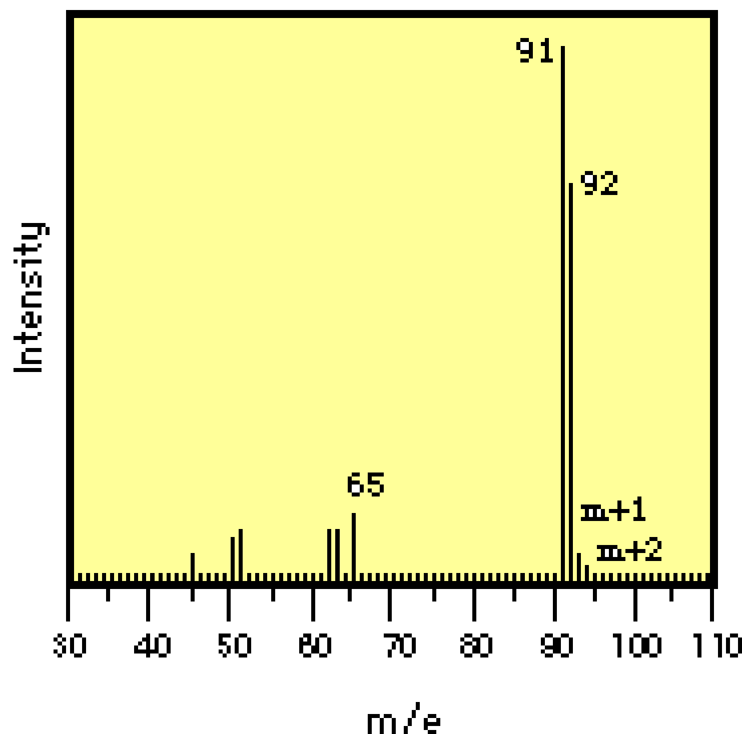
Spectroscopic Detection

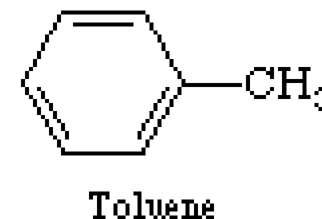
Mass Spectroscopy (MS)

- An MS detector senses a compound eluting from the HPLC column first by ionizing it then by measuring it's mass and/or fragmenting the molecule into smaller pieces that are unique to the compound.
- The MS detector can sometimes identify the compound directly since its mass spectrum is like a fingerprint and is quite unique to that compound.

Here is a mass spectrum of a simple chemical compound, **toluene**.

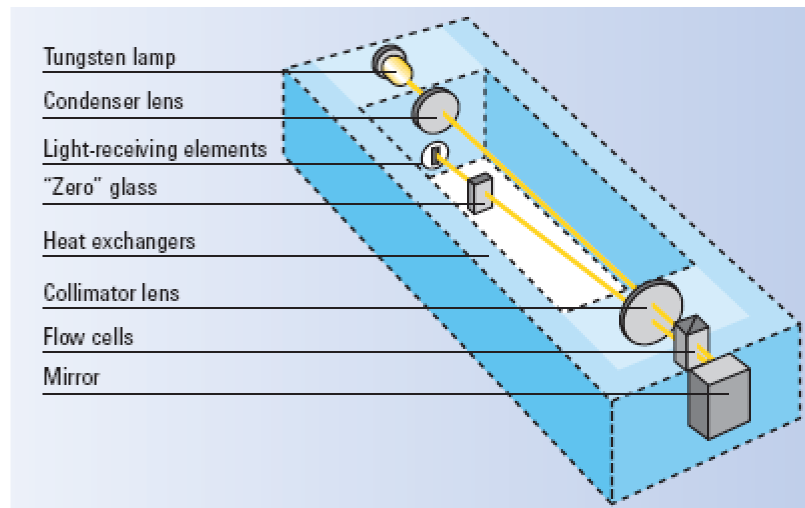
The pattern of lines is very unique to this compound. The largest peak in the spectrum occurs at a mass of 91, which is a fragment ion generated by loss of a hydrogen atom.





Refractive Index (RI) Detection

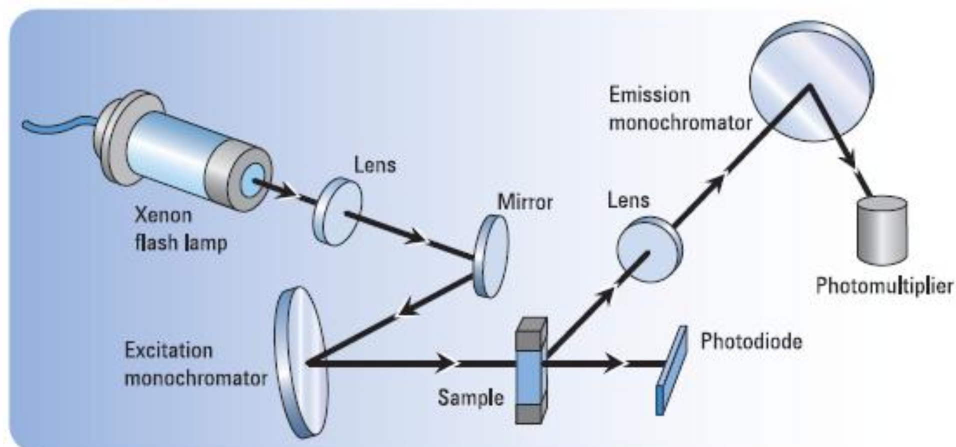
- **The ability of a compound or solvent to deflect light provides a way to detect it.**
- The **RI** is a measure of molecule's ability to deflect light in a flowing mobile phase in a flow cell relative to a static mobile phase contained in a reference flow cell.
- The amount of deflection is proportional to concentration.
- The RI detector is considered to be a universal detector but it is not very sensitive.



Schematic of a Deflection Type of RI Detector

Fluorescence Detection

- Compared to UV-Vis detectors fluorescence detectors offer a higher sensitivity and selectivity that allows to quantify and identify compounds and impurities in complex matrices at extremely low concentration levels (trace level analysis).
- Fluorescence detectors sense only those substances that fluoresce



Summary HPLC Basics

In this course, you were introduced to the:

General principles of HPLC and application uses of HPLC

Components of HPLC instrument configurations

Major separation modes in HPLC

Overview of HPLC columns