BHARATHIDASAN UNIVERSITY

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Programme: M.Sc., Botany

Course Title : CELL BIOLOGY AND BIOINSTRUMENTATION
Course Code : 22PGBOT104

Unit – V

SEPARATION TECHNIQUES

Topic: HPLC

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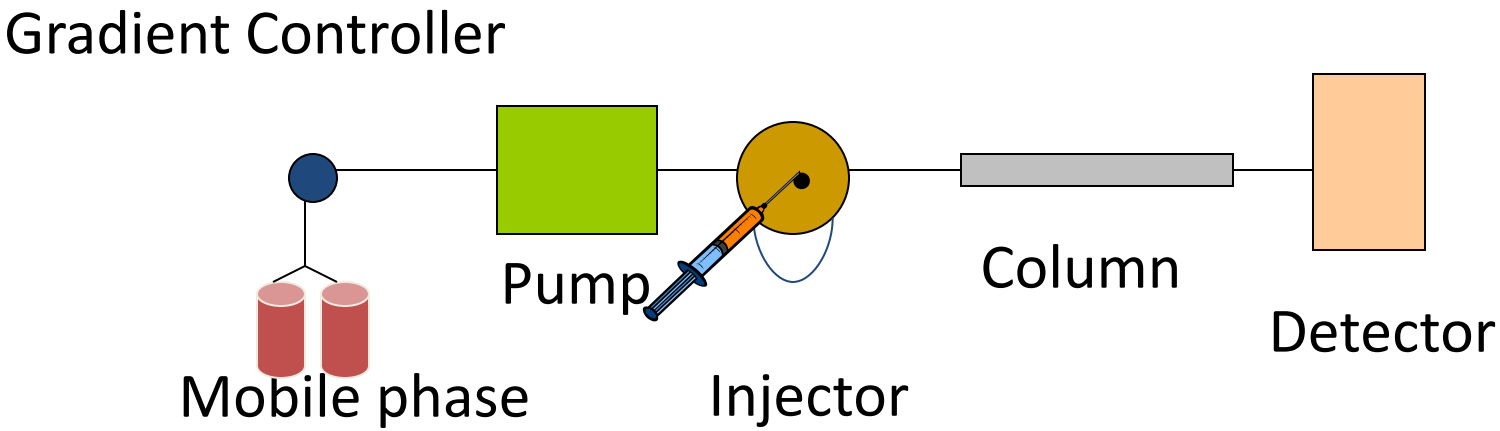
HPLC

- HPLC is characterized by the use of high pressure to push a *mobile phase* solution through column of *stationary phase* allowing separation of complex mixtures with high resolution.

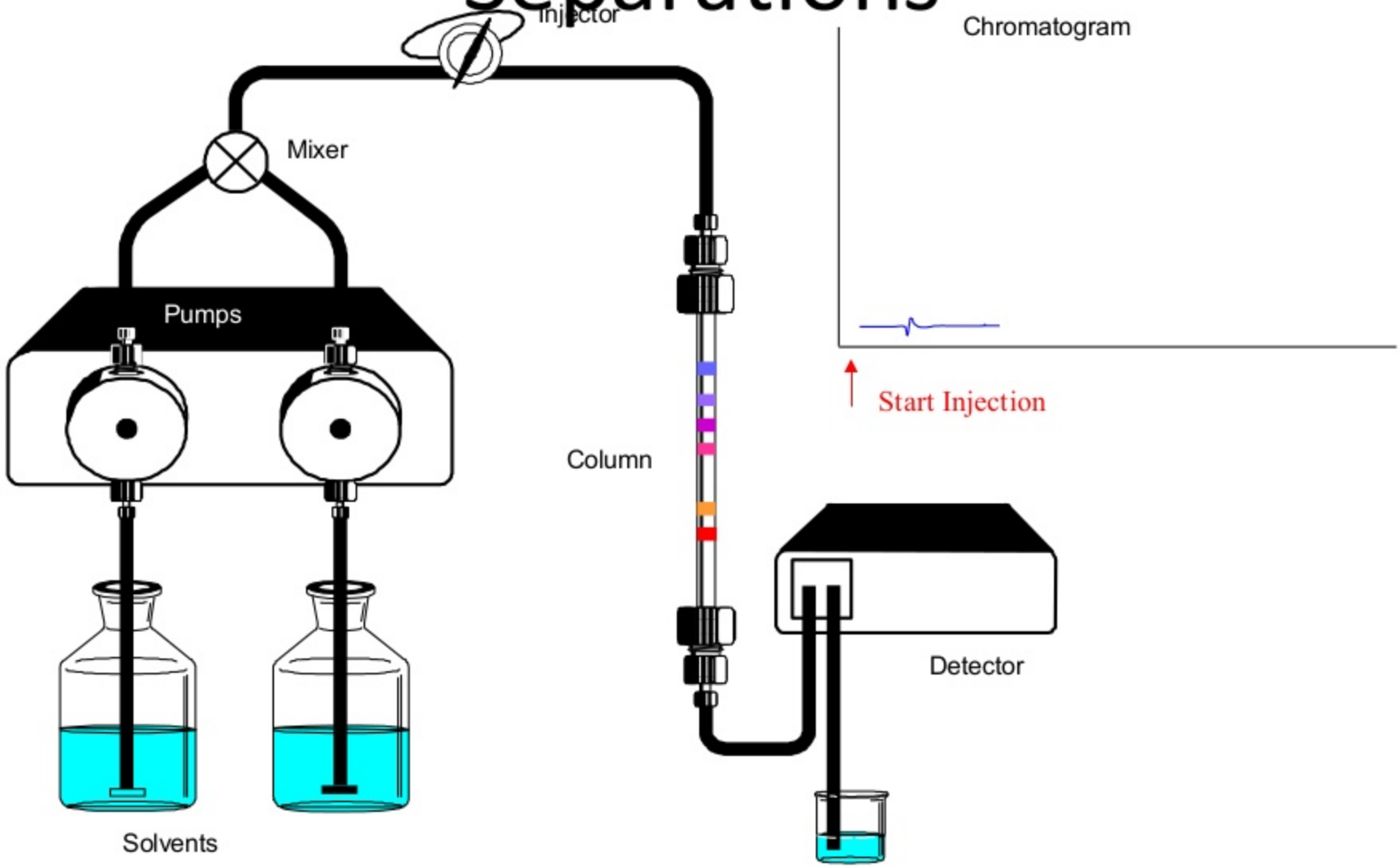
- HPLC is a form of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector.

- Compounds are separated by injecting a sample mixture onto the column. The different component in the mixture pass through the column at differentiates due to differences in their partition behavior between the mobile phase and the stationary phase. The mobile phase must be degassed to eliminate the formation of air bubbles.

Instrumentation



Separations



- A site in which a moving phase (mobile phase) and a non-moving phase (stationary phase) make contact via an interface.
- The affinity with the mobile phase and stationary phase varies with the solute. → Separation occurs due to differences in the speed of motion.

HPLC Separation

- Adsorption (liquid-solid) chromatography
- Partition (liquid-liquid) chromatography
  - Normal phase partition chromatography
  - Reversed phase partition chromatography (Stationary – nonpolar (hydrophobic), mobile- polar)
  - Attraction between nonpolar compound in the mixture and nonpolar adsorbant.
- Ion exchange chromatography
- Size exclusion chromatography

TYPES OF LIQUID CHROMATOGRAPHY

LC mode	Packing materials	Mobile phase	Interaction
Normal phase chromatography	Silica gel	n-Hexane/IPE	Adsorption
Reversed phase chromatography	Silica-C18(ODS)	MeOH/Water	Hydrophobic
Size exclusion chromatography	Porous polymer	THF	Gel permeation
Ion exchange chromatography	Ion exchange gel	Buffer sol.	Ion exchange
Affinity chromatography	Packings with ligand	Buffer sol.	Affinity

Adsorption

- A solid such as silica gel is used as the stationary phase, and differences, mainly in the degree of adsorption to its surface, are used to separate the solutes.
- Liquid-solid chromatography
- The retention strength increases with the hydrophilicity of the solute.

Partition

- A liquid is used as the stationary phase, and the solute is separated according to whether it dissolves more readily in the stationary or mobile phase.
- Liquid-liquid chromatography

Mode

- Normal phase:
- Polar stationary phase and non-polar solvent.

- Reverse Phase:
- - Non-polar stationary phase and a polar solvent.

- Normal Phase
  - Effective for separation of structural isomers
  - Offers separation selectivity not available with reversed phase
  - Stabilizes slowly and is prone to fluctuations in retention time
  - Eluents are expensive
- Reversed Phase
  - Wide range of applications
  - Effective for separation of homologs
  - Stationary phase has long service life
  - Stabilizes quickly
  - Eluents are inexpensive and easy to use

Common Reverse Phase Solvents

- Methanol
- Acetonitrile
- Tetrahydrafuran
- Water

Column

- Solid Support - Backbone for bonded phases.
  - Usually 10µ, 5µ or 3µ silica or polymeric particles.
- Bonded Phases - Functional groups firmly linked (chemically bound) to the solid support.
  - Extremely stable
  - Reproducible

Bonded Phases

- C-2 Ethyl Silyl (weak)
- C-8 Octyl Silyl (Medium)
- C-18 Octadecyl Silyl (Strong column)

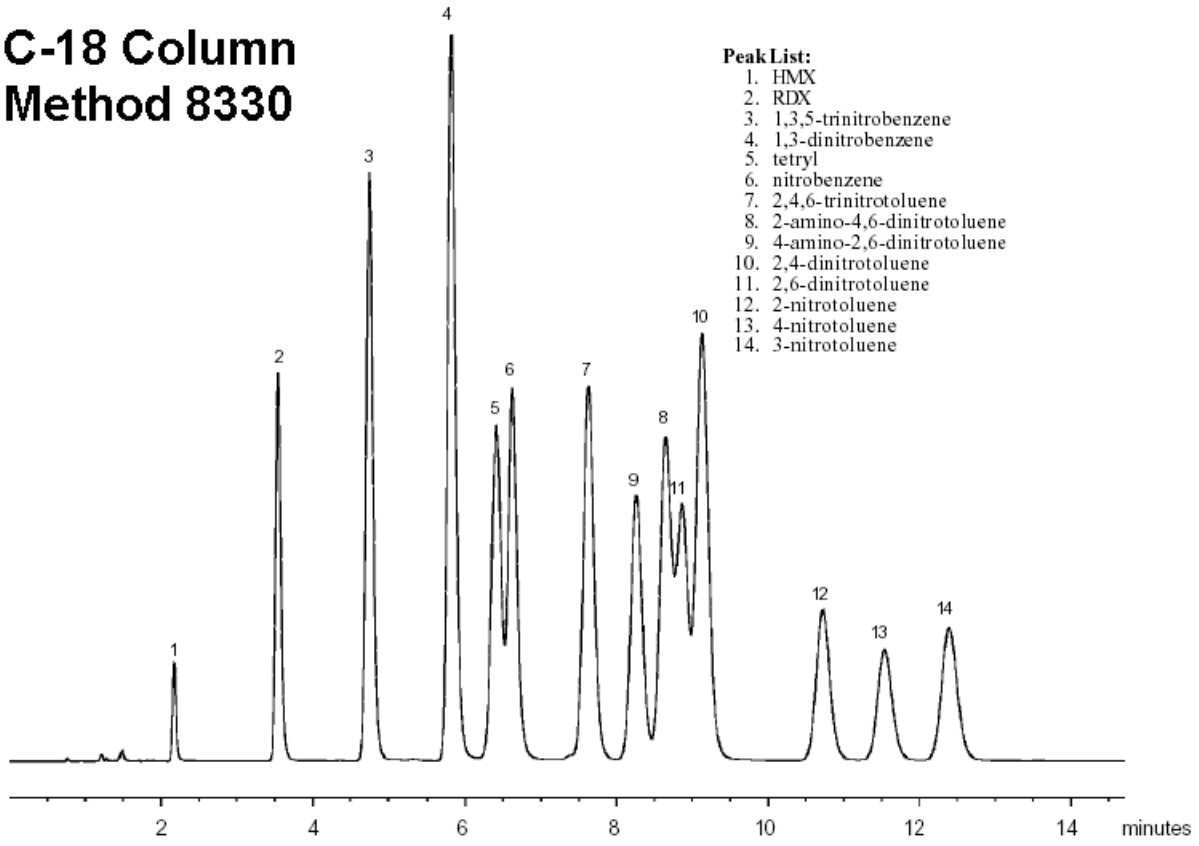
Gradient System

- Isocratic system
  - Constant eluent composition
  - Long analysis time and poor separation
- Gradient system
  - Varying eluent composition
    - HPGE (High Pressure Gradient)
    - LPGE (Low Pressure Gradient)
    - Good separation

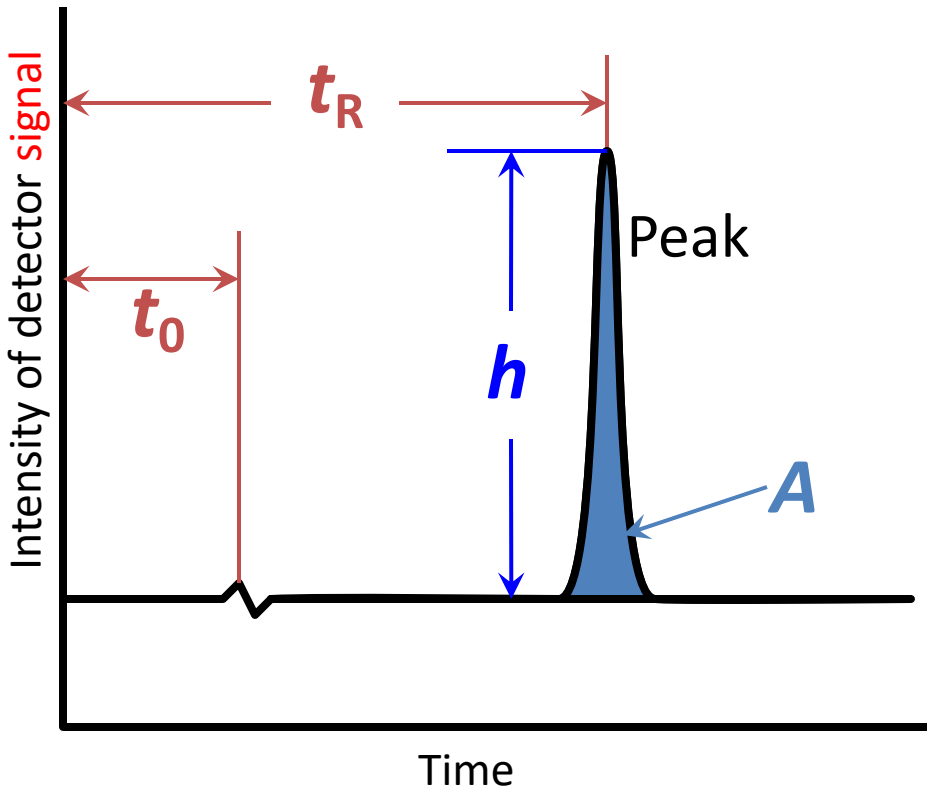
Detectors

- UV
  - Single wavelength (filter)
  - Variable wavelength (monochromator)
  - Multiple wavelengths (PDA)
- Fluorescence
- Electrochemical
- Mass Spectrometric

C-18 Column
Method 8330



Chromatogram



t_R : Retention time

t_0 : Non-retention time

A : Peak area

h : Peak height

- Higher degree of separation!
  → Refinement of packing material (3 to 10 µm)
- Reduction of analysis time!
  → Delivery of eluent by pump
  → Demand for special equipment that can withstand high pressures
- volatile and non –volatile
- based on retention time

Advantages of High Performance Liquid Chromatography

- High separation capacity, enabling the batch analysis of multiple components
- Superior quantitative capability and reproducibility
- Moderate analytical conditions
  - Unlike GC, the sample does not need to be vaporized.
- Generally high sensitivity
- Low sample consumption
- Easy preparative separation and purification of samples

Fields in Which High Performance Liquid Chromatography Is Used

- Biogenic substances
  - Sugars, lipids, nucleic acids, amino acids, proteins, peptides, steroids, amines, etc.
- Medical products
  - Drugs, antibiotics, etc.
- Food products
  - Vitamins, food additives, sugars, organic acids, amino acids, etc.
- Environmental samples
  - Inorganic ions
  - Hazardous organic substances, etc.
- Organic industrial products
  - Synthetic polymers, additives, surfactants, etc.

GC and GC-MS

- GC- Separation
- MS – molecular mass Identification

GC- Desired characters:

The compound must be volatile , should posses thermal stability (250-300°C).

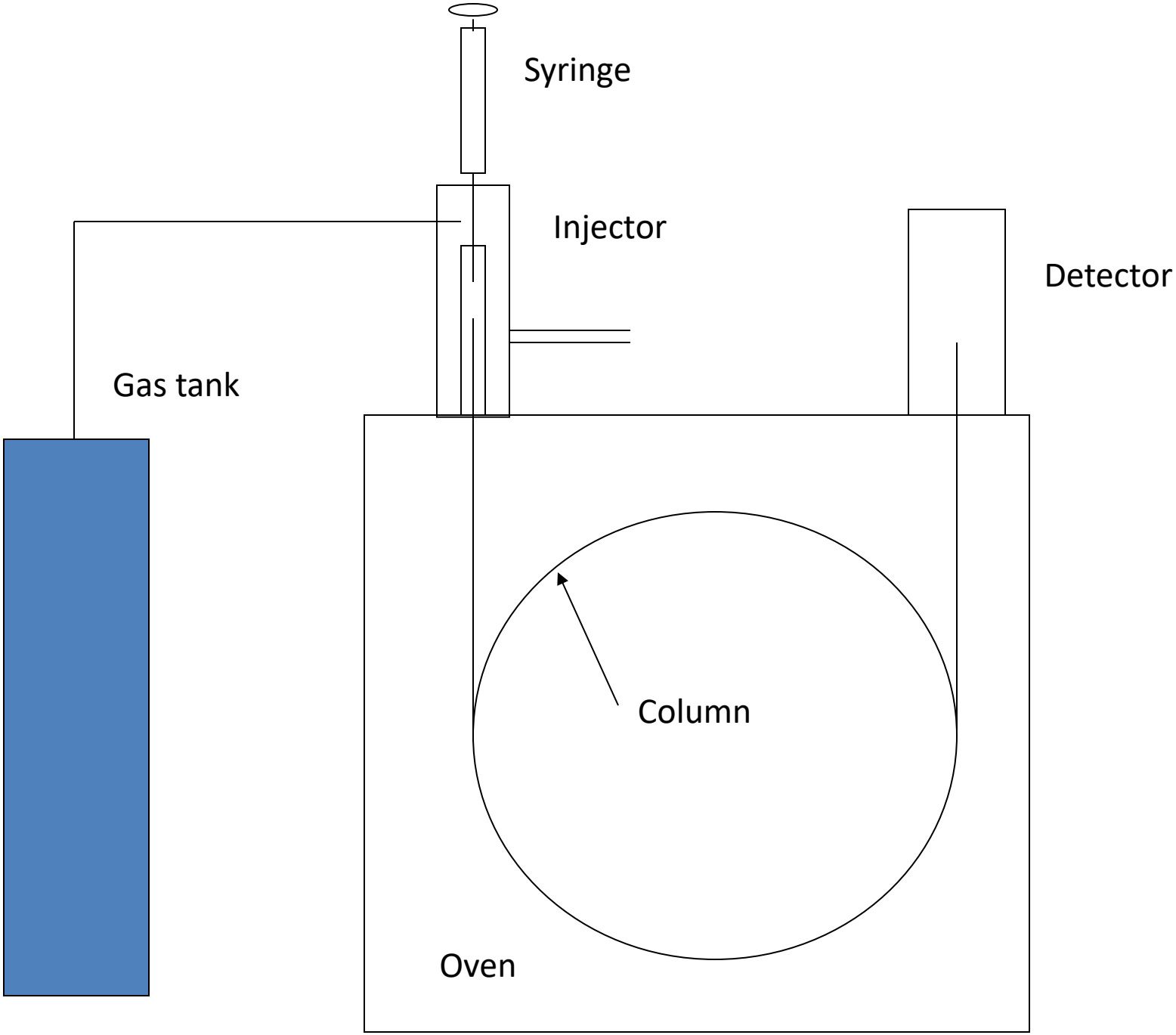
- Separation of volatile organic compounds
- Volatile – when heated, VOCs undergo a phase transition into intact gas-phase species
- Separation occurs as a result of unique equilibria established between the solutes and the stationary phase (the GC column)
- An inert carrier gas carries the solutes through the column

- The sample is effectively vaporized into the gas phase and separated into its various components using a capillary column coated with a stationary (liquid or solid) phase. The compounds are propelled by an inert carrier gas such as helium, hydrogen or nitrogen. As components of the mixture are separated, each compound elutes from the column at a different time based on its boiling point and polarity. The time of elution is referred to as a compound's retention time

- once the components leave the GC column, they are ionized and fragmented by the mass spectrometer using electron or chemical ionization sources. Ionized molecules and fragments are then accelerated through the instrument's mass analyzer
- ions are separated based on their different mass-to-charge (*m/z*) ratios

Components

- Carrier Gas, N_2 or He, 1-2 mL/min
- Injector
- Oven
- Column
- Detector


Syringe
Injector
Detector
Gas tank
Column
Oven

- A GC syringe penetrates a septum to inject sample into the vaporization camber
- Instant vaporization of the sample, 280 °C
- Carrier gas transports the sample into the head of the column
- Purge valve controls the fraction of sample that enters the column

Principle

- is partition
- Gas is used as a mobile phase and liquid coated solid support is used as a stationary support.
- The component which is more soluble in stationary phase travels slower and eluted later.
- Hence components are separated according to their partition coefficient.

- Injection port:
- Sample (>15microliter liquid form) should be injected in to the GC and the sample is carried by the inert gas, usually helium.
- The injection port is heated to 300° C to cause the chemicals become gases.

Detectors

- Flame Ionization Detectors (FID)
- Electron Capture Detectors (ECD)
- Electron impact/chemical ionization (EI/CI) Mass spectrometry

Flame Ionization Detector

- Effluent exits column and enters an air/hydrogen flame
- The gas-phase solute to form electrons and ions
- These ions collected at an electrode held above the flame
- The current reaching the electrode is amplified to give the signal

Electron Capture Detector

- Ultra-sensitive detection of halogen-containing species
- Pesticide analysis

- . G

- The samples should be ionized and converted to charged particles by ion source. This is done by either chemical ionization or electron ionization.
- Then the sample is separated according to their mass by charge ratio (m/Z).

What kind of info can mass spec give you?

- Molecular weight
- Elemental composition (low MW with high resolution instrument)
- Structural info (hard ionization or CID)

GC/MS

- GC works on the principle that a mixture will separate into individual substances when heated.
- Sample introduced in to GC vaporized at 250° C and swept in to the column by the carrier gas
- Sample components emerge from column flowing into the capillary column interface connecting the GC and MS (He should be removed).
- Identification of compound based on its mass spectrum , that every compound has a unique fragmentation.

- Thank You