CHROMATOGRAPHY

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Program Objectives

- The principles of chromatography
- Fundamental concepts in Gas and liquid Chromatography (GC and LC)
- Equipment used
- GC and LC in analytical chemistry

History of Chromatography

- Mikhail Tswett (1906)- Russian botanist separated plant pigments on $CaCO_3$ column with petroleum solvent. The technique was given the name Chromatography (literally *writing in colour*).
- In 1941 Martin and Synge separated amino acids in paper (Nobel Prize in 1952).
- Explosive growth in applications since then.

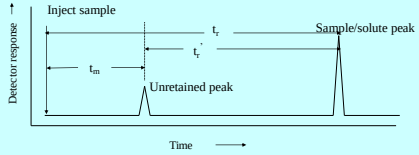
Principles of Chromatography

- Widely used for the *separation, identification and determination* of the chemical components in a *complex mixture*.
- Have in common the use of a *stationary phase* and a *mobile phase.*
- Chromatographic methods of two types:
  - ***column chromatography*** (GC, LC)
  - ***planar chromatography*** (TLC)

Classification	Specific Method	Stationary Phase	Type of equilibrium
Liquid chromatography (LC) (Mobile phase a liquid)	Liquid-liquid or partition	Liquid adsorbed onto solid	Partition between immiscible liquids
	Liquid-bonded phase	Organic species bonded to solid surface	Partition between liquid and bonded surface
	Liquid-solid, or adsorption	Solid	Adsorption
	Ion exchange	Ion-exchange resin	Ion exchange
	Size exclusion	Interstices of polymeric solid	Partition/sieving
Gas chromatography (GC) (Mobile phase a gas)	Gas-liquid	Liquid adsorbed onto solid surface	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to solid surface	Partition between gas and bonded liquid
	Gas-solid	Solid	Adsorption
Supercritical-fluid chromatog. (SFC) (mobile phase a supercritical fluid)		Organic species bonded to solid surface	Partition between supercritical fluid and bonded surface

Theory of Chromatography

- The Chromatogram
  - Plot of detector response against time.



Relationship between Void Volume and t_m

- Void volume (V_m) is the volume of mobile phase contained in the void space of the column.
- $V_m = t_m \times u_v$
  Where u_v = volume flow rate (ml/min)
- V_m is a constant for a given column, but t_m changes with the flow rate.
- Retention volume (V_r) is the volume of mobile phase required to elute a solute from the column.
- $V_r = t_r \times u_v$
- V_r is independent of the flow rate unlike t_r.

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Capacity factor: $k' = \frac{t_r - t_m}{t_m} = \frac{t_r'}{t_m}$

Since time is propotional to volume, $k' = \frac{V_r - V_m}{V_m}$

For two compounds:

Selectivity coefficient: $\alpha = \frac{t_{r_2}'}{t_{r_1}'} = \frac{k'_2}{k'_1}$

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Column Efficiency

- Two factors which contribute to the separation of two compounds by chromatography are:
  - differences in retention times;
  - broadness of peaks.
- Column efficiency is concerned with band broadening.
- Concentration of solute emerging from a column is Gaussian in shape.
  - Characterized by σ, the standard deviation.
  - Related to peak's width at different positions, commonly at half height and at base.
  - Peak width obviously related to band broadening.

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- "band broadening" is related to the goodness of the column [or column efficiency, N, (the number of theoretical plates)]
  - It can be shown:

$$N = \left(\frac{t_r}{\sigma}\right)^2 = 16\left(\frac{t_r}{w_b}\right)^2 = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2$$

- N depends on column length.
- HETP (Height Equivalent of a Theoretical Plate)

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

- HETP (H) is a term which describes column efficiency irrespective of column length:

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Relative Retention and Resolution

- Consider two peaks on a chromatogram:
  - Relative position and separation defined by:
    - Relative retention
    - Peak resolution.
- Peak resolution, R $= \frac{t_{r2} - t_{r1}}{\frac{w_{b1} + w_{b2}}{2}}$

Note that peaks are essentially base- line resolved when resolution = 1.5.

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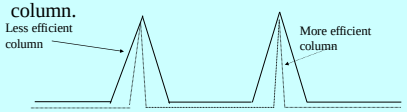
- However, R depends on the efficiency of the column.



Resolution of peaks with the same relative retention (α)

- Efficient columns (large N) give narrow peaks.

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Resolution and Column Efficiency

- Resolution achievable on a given column can differ depending on column efficiency(N), selectivity coefficient(α) and capacity factor(k')

- Expressed by the following relationship:

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{k_{av}'+1}\right)$$

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- What this equation tells us:

  resolution is proportional to the square root of *N*, α and k'

  - ↑ N by
  - – ↑column length
  - – ↓ the size of packing material (H is smaller for smaller particle size, thus ↑ N)
  - – using the optimum flow rate (Smallest or optimum H obtained at the optimum flow rate)

  - ↑ α by changing stationary &/or mobile phase
  - ↑ k' by changing mobile phase composition

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GAS CHROMATOGRAPHY

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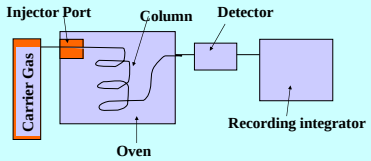
Gas Chromatographic Systems

- Essential components of a GC system are:



- Injector block, column oven and detector block are all heated.

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- Sample injected into a *heated block* where it is *vaporised*

- Vapour carried by carrier gas through column

- *Interaction* with stationary phase is *different for different molecules* - Separation results.

- Sample molecules *are carried through the column (eluted)by the carrier gas.*

- *Solutes are detected by the detector as they elute from the column*

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- Carrier gas must be pure, inert and dry; He, N_2 and H_2.

- Retention times directly proportional to flow rate

- Good flow control required.

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- Samples introduced using:
  - liquid syringes
  - gas-tight syringes
- Sample size determined by type of column and detector

Sample size	Column	Detector
0.001-0.01uL	Capillary 1/16 inch packed	FID
0.2-1.0uL	1/8 inch packed	FID

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Capillary GC Injectors

- Objectives:
  - Sample to occupy shortest possible column length
  - Sample to be identical before and after injection.
- To achieve these objectives you need to consider: "backflash", injector temperature and inlet discrimination.

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- Backflash.
  - Except with 'on-column' injectors, all injectors utilise vaporisation to introduce sample
  - When vaporised, sample may expand beyond capacity of the injector liner and this leads to "backflash".
  - Sample now occupies a large volume and takes a longer time to be swept onto the column.
  - Results in peak tailing and "ghosting".
  - Minimise using :
    - Small injection volumes
    - Large volume liners
    - Optimal injector temp.

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- Injector Temp.
  - Sufficiently hot to ensure "instant" vaporisation of sample.
  - Too low leads to incomplete vaporisation and broad peaks.
  - Too high leads to degradation of analyte.
  - 200 - 250^{o}C usually satisfactory.
- Inlet Discrimination
  - Less volatile components vaporised more slowly than the more volatile.
  - Greater proportion of more volatile component introduced onto the column.

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Injection Techniques

- Split Injection.
  - Simple and most commonly encountered capillary injection technique.
  - Low sample capacity of capillary columns requires low sample amounts
    - not possible directly with microlitre syringes
  - Amount of sample introduced on to the column controlled by split flow. Typically 10:1 to 100:1

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- Splitless Injection
  - Used for trace analysis where component amounts are < 200ng.
  - With split valve off, sample vaporised and carried onto column.
  - Sample introduced into column as abroad band and needs to be "re-focussed" to obtain narrow bands.
  - Initial column oven temp. is maintained ~ 40^{0}C below B.Pt. of sample solvent. Solvent condenses at front of the column forming a narrow (re-focussed) band of solutes in it.

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- On-Column Injection.
  - Used for samples that decompose above their boiling point.
  - Samples are injected directly on to the column using a special syringe.
  - The initial column temperature is low enough to condense solutes in a narrow zone.
  - Subsequent warming of the column initiates the chromatographic separation.
  - As there is no or minimum sample loss, this is best suited for quantitative analysis.

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Columns

- "Heart"of GC.
- Two types
  - Packed and Open Tubular (capillary).
  - Advantages of capillary columns are:
    - little resistance to gas flow.
    - Absence of multiple path effect
- Capillary columns have the stationary phase cross linked and chemically bonded to the inner surface of a fused silica capillary

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- Tubing with different internal diameters are available.
  - Commonly 0.25, 0.32, 0.53 mm ID.
- Variety of types and thicknesses of stationary phases available.
- Most common stationary phases based on silicone polymers

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Selecting Stationary Phases

- Stationary phase polarity.
  - Greatest effect on resolution of a mixture of analytes.
  - Determines manner in which analyte molecules interact with stationary phase
  - "Like dissolves like" is applicable
  - Polar phases rely almost entirely on the interaction between the functional groups in the analyte molecules and those in the stationary phase

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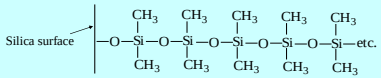
Selecting Stationary Phases

- Use least polar phase that provides satisfactory separation.
- Examples of common phases include:
  - Polydimethyl siloxane (non-polar)



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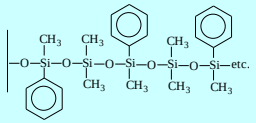
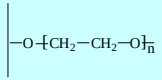
  - Polyphenyl methylsiloxane (intermediate polarity)



  - Carbowax (polar)



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Column Oven Temperature

- Columns housed in an oven with fine temperature control
- Because vapour pressure increases with increasing temperature, retention times decrease
- Resolution of two components also decreases with increasing temperature and increases with decreasing temperature.
- Can make use of both of these.

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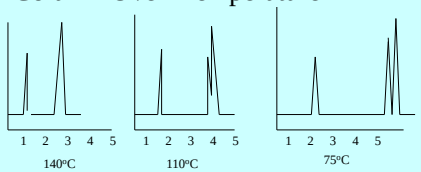
Column Oven Temperature



- Generally a 30°C change in oven temperature halves (or doubles) retention time

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Temperature Programming

- Too high a temperature can cause poor resolution
- Too low a temperature causes ill-defined peaks and excessive retention times
- Thus could have a problem in the analysis of a mixture with a wide range of affinities to the stationary phase (eg. Separating a mixture of hydrocarbons ranging from C_5H_{12} to $C_{16}H_{34}$)
- Change temperature with time (eg. 10^0C increase per min.)

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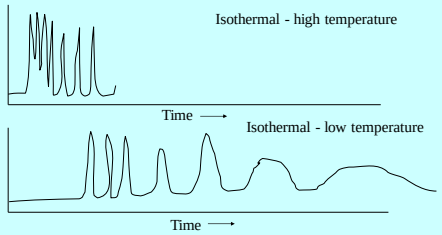

- Problem can be overcome with temperature programming - i.e. changing column oven temperature with time

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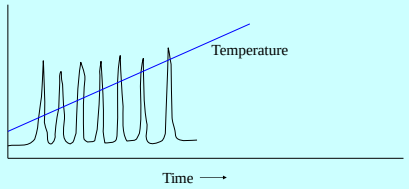

- Temperature programming:
  - allows analysis of mixtures containing a wide range of solutes
  - gives overall better shaped peaks
  - gives better detection limits

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Detectors

- Thermal conductivity detector (TCD)
  - Based on the principle that the conductivity of a W filament changes with the composition of the gas that comes into contact with it.
  - Concentration is proportional to the change in conductivity
  - Universal response
  - Good stability
  - Good linear range
  - Minimum detectable quantity ~10 ppm

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Flame ionisation detector (FID)

– Organic compounds in the eluting gas is burnt in a mixture of H_2 and air. Resulting ion current is measured.
– Concentration is proportional to the ion current produced
– Sample is destroyed
– Most widely used detector
– Selective to organic compounds
– More sensitive (x100) than thermal conductivity detector

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•Integration

- Electronic integrators
- Computer-aided integration and instrument control

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Qualitative Analysis

- What is present?
- Information from:
  - retention times
  - selective detector response
- t_R, t_R' and α
- Retention Index
  - Most useful system is that attributed to **Kovats**
  - Takes advantage of linear relationship between log t_R' of members of a homologous series and the number of carbon atoms in the molecules

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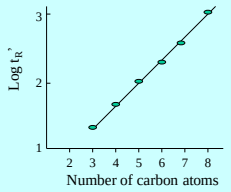

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- The Kovat's index of the analyte (I) is calculated based on the retention times of the unknown analyte and two n-alkanes which elute in front and behind it (all run on the same column).
- *I* for any alkane is 100 x number of carbon atoms in the molecule, regardless of the stationary phase and the temperature.
- I of <u>all other solutes</u> vary slightly with temperature and markedly with stationary phase. The I values for solutes on common stationary phases are given in tables, and used for identification of the unknown solute.

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Equation method-

$$I = 100 \times \left[n + (N - n) \frac{\log t_{R'(unknown)} - \log t_{R'(n)}}{\log t_{R'(N)} - \log t_{R'(n)}} \right]$$

- Where I is the Kovat's index for the unknown solute.
- n and N are the number of carbon atoms in the smaller alkane and that in the larger alkane respectively.

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Problem

- If the retention time of the unretained peak is 0.5 min and that of an unknown is 15.7 min, calculate the Kovat's retention index for the unknown. (Octane and Nonane elute from the same column at 14.3 min and 18.5 min respectively)

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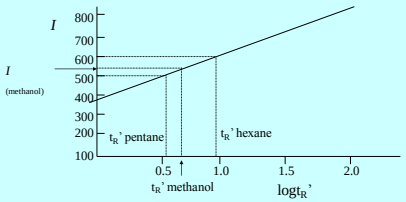
Graphical method-

- Eg. Methanol elutes between pentane and hexane.



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Quantitative Analysis

- How much is present?
- Steps involve:
  - sampling and sampling techniques
  - chromatography
  - integration
  - calibration
  - calculation
- Need to minimise error in each step to minimise overall error

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External Standardisation

- Sample and standard solutions are prepared in the same solvent
- Equal volumes of sample and standard solutions are injected and chromatographed
- Concentration of the analyte in the sample is determined based on the relative peak areas of analyte in the standards and sample (using single point or multi-point calibration)
- **peak area of analyte α concentration of analyte**

– Relies on consistent and reproducible injection of small volumes.

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Internal Standardisation

- Equal amount of an internal standard is added to all sample and standard solutions at the preparation step
- Approximately same volumes of sample and standard solutions are injected and chromatographed
- Concentration of the analyte in the sample is determined based on the peak areas of analyte and the internal standard in standards and the sample.
- **peak area ratio of analyte/int.std α concentration ratio of analyte/int.std**
  – overcomes the problem of having to inject consistent and reproducible small volumes.

eg. In gas chromatography

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- A suitable internal standard:
  - must not be present in the sample being analysed
  - must elute with a retention time separate from any component in the sample (and preferably should not add to analysis time)
  - should preferably be similar in chemical structure to the analyte(s) of interest in the sample

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Internal Standardisation- Multilevel Calibration

- In the analysis of ethanol in a blood sample, a series of standard aqueous solutions of ethanol and the blood sample were all treated alike. An aliquot portion (0.1mL) of each was diluted with a standard aqueous solution of 1-propanol(0.1mL; concentration 0.1mgcm^{-3}). Each sample was then analysed by gas chromatography with the following results:

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Sample	Area Ethanol	Area Propanol
Ethanol Standards:		
0.5 mgcm^{-3}	5518	12754
0.75 mgcm^{-3}	7563	11893
1.0 mgcm^{-3}	10350	12084
1.25 mgcm^{-3}	13935	12870
1.5 mgcm^{-3}	15628	12314
Blood Sample:	9862	12604

- Firstly, construct a calibration curve.

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Internal Standardisation- Multilevel Calibration

Sample	Area Ethanol	Area Propanol	Area Ethanol/ Area Propanol
Ethanol Standards:			
0.5 mgcm^{-3}	5518	12754	0.433
0.75 mgcm^{-3}	7563	11893	0.634
1.0 mgcm^{-3}	10350	12084	0.857
1.25 mgcm^{-3}	13935	12870	1.08
1.5 mgcm^{-3}	15628	12314	1.27

- A plot of A_e/A_{is} against C_e should be a straight line

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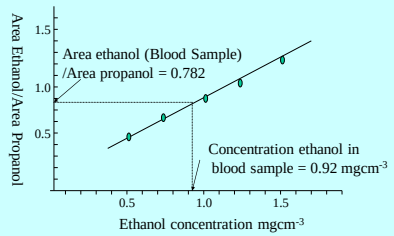
Internal Standardisation- Multilevel Calibration



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High Performance Liquid Chromatography (HPLC)

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Introduction

- Mobile phase is a liquid.
- Particle size of the stationary phase is very small in HPLC (0.3μm - 10μm).
- Pressures required to enable flow rates of 1 - 5 mL per minute are several hundred atmospheres
- Columns are usually stainless steel, 25 cm in length and 2- 5mm in diameter.

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Modes of HPLC

- Mode of HPLC defined by the stationary phase:
- **Normal phase HPLC**
  - Plain Silica or Alumina (adsorption chromatography)
  - Polar phase chemically bonded to silica surface
    - Cyano, amino
- **Reversed Phase HPLC**
  - Non-polar phase chemically bonded to silica surface
    - C-18, C-8, C-3

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- Mobile phase types:

Normal phase HPLC

- Non polar or less polar solvents as mobile phase (eg. Hexane, diethyl ether)

* more polar solvent has higher eluent strength

- **Reversed Phase HPLC**
  - Polar solvents as mobile phase (eg. Methanol, acetonitrile, water)

* less polar solvent has higher eluent strength

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Modes of Elution

- **Isocratic elution**

– Single solvent or a solvent mixture used for elution

eg. 100% methanol or 80% methanol(20% water)

- **Gradient elution**

– Increasing amounts of stronger solvent added to the weaker solvent during elution

eg. 20% methanol to 80% methanol within 15 minutes for a reversed phase separation

– Used for mixtures of solutes with widely differing polarities

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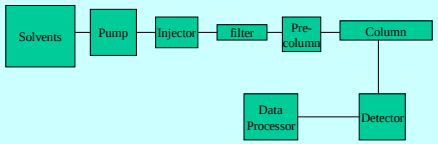
HPLC system



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- **Solvent requirements:**
  - Pure
  - Particle free
  - Degassed
- **Pump requirements:**
  - Pressures up to 6000 psi
  - Pulse free
  - Flow rates of 0.1mL - 10mL/min
  - Flow reproducibility of 0.5% relative or better
  - Resistance to corrosion by a variety of solvents

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- **Sample Injection:**
  - Most commonly by loop valve
- **Detectors:**
  - Absorbance (UV/Vis - variable λ or PDA)
  - Refractive index (detects the change in turbidity)
  - Fluorescence (if the analyte is fluorescent)
  - Electrochemical (measures current flowing through a pair of electrodes, on which a potential difference is imposed, due to oxidation or reduction of solute)
  - Mass spectrometry (HPLC-MS)
  - Conductivity (for ions)
  - Light scattering

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