8. Mass Spectrometry/ Gas Chromatography

There are many important analytical techniques, which are not spectroscopic in nature.

a) Mass Spectrometry
Mass spectrometry is the most precise physical method of determining molecular mass. It separates ions based on their mass-to-charge ratios, \( m/z \).

The method relies on the effects of magnetic and electric fields on a beam of ions. Therefore, the molecules of interest must first be vapourized and ionized.
There are a variety of different types of mass spectrometry which vary in the method of vapourisation and ionization:

- Spark source mass spectrometry (SSMS)
  Ion source = radiofrequency electric spark

- Inductively coupled plasma mass spectrometry (ICPMS)
  Ion source = high-temperature argon plasma

- Matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS)
  Ion source = UV laser pulse
  The sample is first implanted into a solid matrix containing a UV-absorbing chromophore, e.g. 2,5-hydroxybenzoic acid. The excited chromophore acts as a source of $\text{H}^+$, thus ionizing the sample.

- Electrospray ionization mass spectrometry (ESI-MS)
  Ion source = electrically charged needle
  The sample (in a suitable solvent) is sprayed from a small needle, to which a high voltage (4,000 V) is applied. This yields charged droplets, from which the solvent is evaporated.
SSMS and ICPMS rely on heat to vapourize the molecules of interest. They are suitable for low molecular weight molecules.

The temperatures necessary to vapourize biological macromolecules are, however, so high that the molecules would be completely degraded. MALDI and ESI-MS are most appropriate for studying biological molecules.

*Mass analyzer*

As well as different methods of ionization there are also different methods of analyzing the masses of the ions produced. We shall discuss two popular techniques:

- **Double-focussing mass analyzer**
  Separates the ions based on their deflection in a magnetic field.

- **Time-of-flight (TOF) mass analyzer**
  Analyzes the time it takes an ion to traverse a constant distance after acceleration in an electric field.
Double-focussing mass analyzer

The ions first pass through a slit, $S_1$, and enter a region of crossed electric and magnetic fields (velocity selector). Those ions which are undeflected pass through a second slit, $S_2$, into a region where there is only a magnetic field. In the second region the ions follow a circular path, whose radius of curvature, $r$, is determined by the ions mass-to-charge ratio.
Principle of the double-focussing mass analyzer

In the first region of crossed electric and magnetic fields, the electric field causes an upward deflection of the ions. The magnetic field causes a downward deflection. Only ions which are undeflected will pass through $S_2$. Therefore, for the ions passing through $S_2$ the forces of from the electric and magnetic fields must be equal.

The force, $F_1$, from an electric field is:

$$F_1 = qE$$  

(8.1)

The force, $F_2$, from the magnetic field is:

$$F_2 = qvB$$  

(8.2)

$q =$ charge on the ion (C)
$E =$ electric field strength (V m$^{-1}$)
$B =$ magnetic field strength (T)
$v =$ velocity of the ions (m s$^{-1}$)

For the ions passing through $S_2$, $F_1 = F_2$, so that

$$v = \frac{E}{B}$$  

(8.3)

The first region, therefore, selects ions whose velocity is given by eq. (8.3).
In the second region, the magnetic field causes the ions to follow a curved path.

The force on the ions from the magnetic field, \( B' \), is given by
\[
F = q v B' \quad (8.4)
\]

This force causes a centripetal acceleration, \( a \), of the ions, which is given by
\[
a = \frac{v^2}{r} \quad (8.5)
\]

\( r = \) radius of curvature (m)

The force on the ions can also be expressed using Newton's second law of motion, \( F = ma \), as
\[
F = \frac{m v^2}{r} \quad (8.6)
\]

\( m = \) mass of the ions (kg)

Equating eqs. (8.4) and (8.6) yields
\[
\frac{m}{q} = \frac{B' r}{v} \quad (8.7)
\]
The velocity of the ions can now be substituted into eq. (8.7) from eq. (8.3), which yields

$$\frac{m}{q} = \frac{BB'r}{E}$$  \hspace{1cm} (8.8)

The charge in Coulombs, \(q\), can be substituted by \(ze_0\), where \(z\) is the valence of the ion (i.e. +1, +2, etc) and \(e_0\) is the charge on an electron. This leads to the final equation for the mass-to-charge ratio, \(m/z\):

$$\frac{m}{z} = \frac{BB're_0}{E}$$  \hspace{1cm} (8.10)

\(e_0 = 1.6 \times 10^{-19} \text{ C}\)

Since \(B\), \(B'\) and \(E\) are known and \(e_0\) is a constant, the mass-to-charge ratio can be calculated directly from \(r\).
Time-of-flight (TOF) mass analyser

In the source region the ions are accelerated by an electrical potential difference, $V$. In the drift region they move at a constant velocity towards the detector. The time it takes them to reach the detector is determined by their mass-to-charge ratio.

The TOF method is generally used in conjunction with the MALDI and ESI ionization methods, because both allow synchronized formation of the ions.

Typical flight times = 1-30 microseconds
Principle of the time-of-flight mass analyzer

In the source region the ions are accelerated by the potential difference, $V$. The kinetic energy, $E_K$, they have at the end of the source region is given by

$$E_K = qV \quad (8.11)$$

However, the kinetic energy is also related to the mass and the velocity of the ions by

$$E_K = \frac{1}{2}mv^2 \quad (8.12)$$

Equating eqs. (8.11) and (8.12), the velocity of the ions as they enter the drift region is given by

$$v = \sqrt{\frac{2qV}{m}} \quad (8.13)$$

Since no further potential is applied in the drift region, the velocity of the ions will remain constant and it is, therefore, related to the time, $t$, that it takes to traverse the drift region by

$$v = \frac{D}{t} \quad (8.14)$$
Now equating eqs. (8.13) and (8.14), and rearranging,

$$\frac{m}{q} = 2V\left(\frac{t}{D}\right)^2 \quad (8.15)$$

Substituting $ze_0$ for $q$ gives the mass-to-charge ratio:

$$\frac{m}{z} = 2Ve_0\left(\frac{t}{D}\right)^2 \quad (8.16)$$

Since the applied voltage, $V$, and the length of the drift region, $D$, are known, the mass-to-charge ratio can be calculated directly from $t$.

Applications:
Determination of molecular weight.
Identification of unknown molecules.

- Organic molecules
  In a spark source mass spectrometer the ionization spark also provides sufficient energy to fragment the molecule. Each molecule has a characteristic fragmentation pattern, which can be used for identification. e.g. ketones
• Biological molecules

MALDI-TOF-MS or ESI-MS are generally used. Because of relatively low energy used for vapourisation and ionization, fragmentation doesn't generally occur within the instrument. Fragmentation can be carried out by enzymic digestion, e.g. protein digestion by trypsin. Each protein has a characteristic digestion pattern.
b) Chromatography

Chromatographic techniques are used to separate closely related components of complex mixtures and to quantify the concentrations of the individual components. All chromatographic techniques have some common principles:

- **Mobile phase**
  The sample is transported in a mobile phase, which may be a gas or a liquid.

- **Stationary phase**
  The mobile phase is forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface.

The two phases are chosen so that the components of the sample distribute themselves to varying degrees between the mobile and the stationary phases. If a component interacts strongly with the stationary phase, it will move slowly with the mobile phase. If a component interacts weakly with the stationary phase, it will move more quickly with the mobile phase.
Differences in mobility ⇒ components separate into bands or zones

\[ t_2 > t_1 \]

In the various different chromatographic techniques the separation of the components can come about because of their different sizes, masses, charge or chemical composition.
General Principles of Chromatography

Consider a solute species, A, which distributes between the mobile and stationary phases according the equilibrium,

\[ A_{\text{mobile}} \leftrightarrow A_{\text{stationary}} \]

The equilibrium constant for this reaction is called the *distribution constant* or *partition coefficient*,

\[ K = \frac{c_s}{c_m} \quad (8.17) \]

where \( c_s \) is the concentration in the stationary phase and \( c_m \) is the concentration in the mobile phase.

The time it takes an analyte to pass down the column and reach the detector is called the *retention time*, \( t_r \). The time for an unretained species to reach the detector is called the dead time, \( t_d \). The velocity of analyte migration is,

\[ v = \frac{L}{t_r} \quad (8.18) \]

where \( L \) is the length of the column.
The velocity of the molecules of the mobile phase, \( u \), is

\[
u = \frac{L}{t_m}
\]  \hspace{1cm} (8.19)

The velocity of the solute is related to that of the mobile phase by

\[
v = u \times \text{fraction of time solute spends in mobile phase}
\]

This fraction of time is, however, also equal to the moles of solute in the mobile phase divided by the total number of moles of solute in the column:

\[
v = u \times \frac{\text{moles of solute in mobile phase}}{\text{total moles of solute}}
\]
The number of moles of solute can be expressed in terms of the concentrations in the mobile and stationary phases and their volumes as:

\[
v = u \times \frac{1}{1 + c_s V_s / c_m V_m} \quad (8.20)
\]

Substituting \( K \) for \( c_s / c_m \) from eq. (8.17) yields,

\[
v = u \times \frac{1}{1 + KV_s / V_m} \quad (8.21)
\]

Now substituting this expression into eq. (8.18) and rearranging yields the following expression

\[
t_r = \frac{L(1 + KV_s / V_m)}{u} \quad (8.22)
\]

Relatively long retention time and thus a good separation are enhanced by a long column (large \( L \)), strong interaction with the stationary phase (large \( K \)) and a high relative ratio of the stationary-to-mobile phases (high \( V_s / V_m \)).

Infinitely long retention times are, however, impractical, so that a compromise must always be made.
The relative positions of peaks due to different components of a mixture are defined by their *retention factor*, $k'$:

$$k' = \frac{t_r - t_m}{t_m} \quad (8.23)$$

In the case of TLC, rather than use the time within the column, an analogous expression involving the distance travelled along the plate is used.

Examples:
- **Gas chromatography**
  Mobile phase = gas
  Stationary phase = polymeric liquid film adsorbed onto a solid, i.e. glass beads, packed into a column.
  Basis of separation: chemical interaction, i.e. polar compounds interact strongly with polar stationary phases, non-polar compounds interact more strongly with non-polar stationary phases.
• Gel chromatography (molecular sieve chromatography)
  Mobile phase = liquid
  Stationary phase = liquid in pores of a polymeric solid (gel)
  Basis of separation: molecular size, i.e. small molecules enter the pores of the gel and pass more slowly through the column.

Types of gel: dextran (Sephadex), polyacrylamide, agarose (Sepharose)

The fractionation range depends on the size of the pores in the gel,
e.g. Sephadex G-100 separates proteins in the molecular weight range 4,000-150,000 g mol\(^{-1}\).
• Thin-Layer Chromatography (TLC)
  Mobile phase = liquid
  Stationary phase = layer (~ 0.5 mm) of sorbent (e.g. silica gel, cellulose) spread over the surface of a glass or plastic plate.
  Basis of separation: adsorption to the stationary phase.

The solvent moves up the plate via capillarity, taking the components of the sample with it.

The method is often used for separating mixtures of small organic molecules.
• Ion-exchange Chromatography
  Mobile phase = water
  Stationary phase = ion-exchange resin, i.e. a polymeric solid with chemically bound charged groups.
  Basis of separation: charge and charge density

  e.g. a typical group used for a cation exchange resin is $\text{SO}_3^-$.  

  ![Diagram of ion-exchange resin structure]

  First the substances to be separated are bound to the exchange (i.e. at the top of a column). Secondly, the column is eluted using a solution containing ions which exchange for those bound.

  Cations can be eluted from a cation exchange column by adding a solution of HCl, so that $\text{H}^+$ exchanges for the bound cations. Ions with a small charge or a small charge density (i.e. large ions) elute more rapidly than those with a high charge or high charge density.
• Affinity Chromatography
Mobile phase = liquid
Stationary phase = insoluble matrix to which a binding molecule is covalently coupled (e.g. the ligand of an enzyme)
Basis of separation: specific chemical interaction.

Application: purification and isolation of specific biological molecules,
e.g. glutathione immobilized on sepharose is used for the purification of glutathione transferase.
• High-Performance Liquid Chromatography (HPLC)
  Mobile phase = liquid
  Stationary phase = spherical nonporous glass or polymer beads (diameter 30-40 µm) coated with a thin porous layer of silica, alumina or ion-exchange resin.
  Basis of separation: adsorption to the stationary phase.

The separation achieved via chromatography depends on the length of the column and the surface area available for adsorption.
In HPLC very small beads are used to pack the column, thus giving a superior resolution

e.g. separation of ketoglutaric acid (1), citric acid (2) malic acid (3), fumaric acid (4) and succinic acid (5) via ion-exchange chromatography (A) and HPLC (B).

Because of the small bead size the samples must be pumped at high pressure through the column.
Chromatographic procedures are sometimes combined with other analytical techniques to provide improved separation or identification. Here just two examples:

a) Gas Chromatography/Mass Spectrometry (GC/MS)

After separation of a mixture of organic molecules in a gas chromatograph, the chemical compositions of the individual components are identified from their mass spectra.
b) Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In the method of electrophoresis the sample is subjected to an electric field by placing two electrodes at either end of a capillary, column or plate, in which the sample is contained. Components of the sample which are positively charged ions (cations) migrate towards the cathode and negatively charged ions (anions) migrate towards the anode.

The speed at which an ion moves (e.g. a charged protein molecule) depends on its electrical surface potential, which is determined by its surface charge.
In the method of SDS-PAGE, proteins are pretreated with the detergent SDS. Under these conditions most proteins undergo denaturation. They adopt a random-coil conformation and bind dodecyl sulphate (DS) ions, gaining a negative surface charge. The amount of DS bound per unit weight of protein is constant (1.4 g of DS/g of protein), so that all proteins have the same charge-to-mass ratio and the same surface potential. Therefore, their separation is not based on charge but on their molecular mass.

Small proteins pass through the crosslinked chains of the polyacrylamide gel more easily.

Distance travelled $\propto$ molecular mass
Example:

Protein separation (gels stained with coomassie blue).