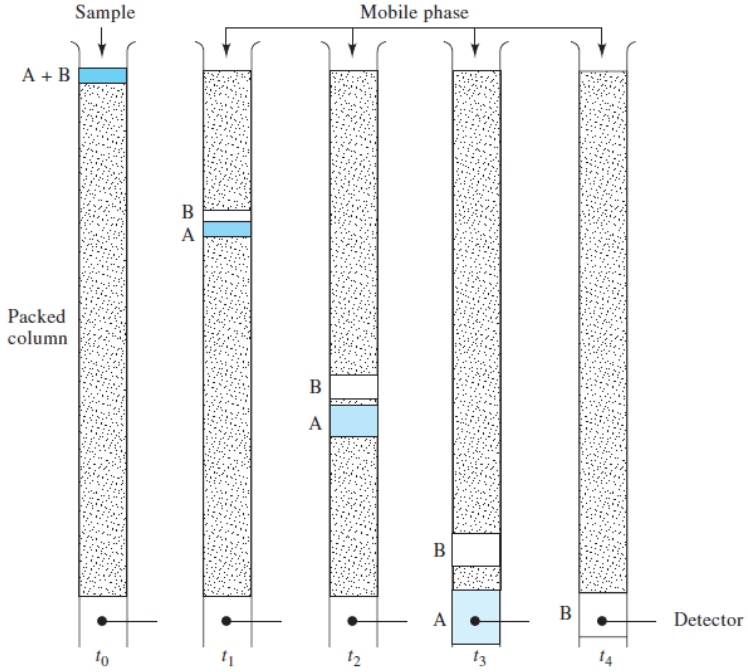
Chromatographic techniques

- Chromatography is a widely used method for the separation, identification, and determination of the chemical components in complex mixtures .
- **Chromatography** is a technique in which the components of a mixture are separated based on differences in the rates at which they are carried through a fixed or **stationary phase** by a gaseous or liquid **mobile phase**.
- **Elution** is a process in which solutes are washed through a stationary phase by the movement of a mobile phase.
- The mobile phase that exits the column is termed the **eluate**. An **eluent** is a solvent used to carry the components of a mixture through a stationary phase.

Column Chromatography

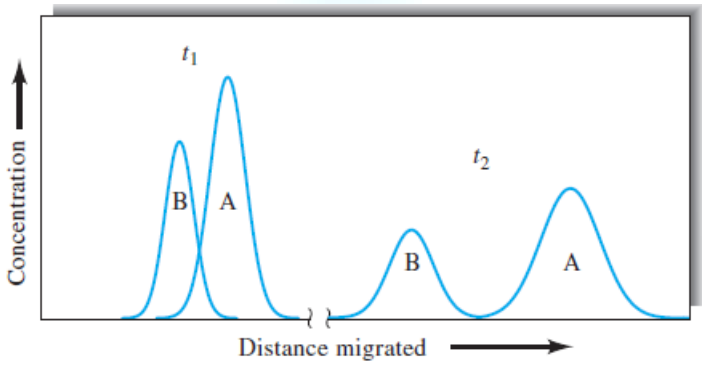
- The column consists of narrow-bore tubing that is packed with a finely divided inert solid that holds the stationary phase on its surface.
- The mobile phase occupies the open spaces between the particles of the packing.
- Initially, a solution of the sample containing a mixture of A and B in the mobile phase is introduced at the head of the column as a narrow plug at time t_0.
- The two components distribute themselves between the mobile phase and the stationary phase.
- Elution then occurs by forcing the sample components through the column by continuously adding fresh mobile phase.
- With the first introduction of fresh mobile phase, the **eluent**, the portion of the sample contained in the mobile phase moves down the column, where further partitioning between the mobile phase and the stationary phase occurs (time t_1).
- Partitioning between the fresh mobile phase and the stationary phase takes place simultaneously at the site of the original sample.
- Further additions of solvent carry solute molecules down the column in a continuous series of transfers between the two phases.
- Because solute movement can occur only in the mobile phase, the average *rate* at which a solute migrates *depends on the fraction of time it spends in that phase*.
- This fraction is small for solutes that are strongly retained by the stationary phase (component B for example) and large where retention in the mobile phase is more likely (component A).
- Ideally, the resulting differences in rates cause the components in a mixture to separate into **bands**, or **zones,** along the length of the column. Isolation of the separated species is then accomplished by passing a sufficient quantity of mobile phase through the column to cause the

individual bands to pass out the end (to be **eluted** from the from the column), where they can be collected or detected (times t_3 and t_4)



Chromatograms

- If a detector that responds to solute concentration is placed at the end of the column during elution and its signal is plotted as a function of time (or of volume of added mobile phase), a series of peaks is obtained.
- Such a plot, called a **chromatogram**, is useful for both qualitative and quantitative analysis.
- The positions of the peak maxima on the time axis can be used to identify the components of the sample. The peak areas provide a quantitative measure of the amount of each species.



Migration Rates of Solutes

The effectiveness of a chromatographic column in separating two solutes depends in part on the relative rates at which the two species are eluted. These rates in turn are determined by the ratios of the solute concentrations in each of the two phases.

Distribution Constants

All chromatographic separations are based on differences in the extent to which solutes are distributed between the mobile and the stationary phase. For the solute species A, the equilibrium is described by the equation

A(*mobile*) ⇋ A(*stationary*)

The equilibrium constant *K*c for this reaction is called a **distribution constant**, which is defined as

$$K_c = \frac{(a_A)_S}{(a_A)_M}$$

Where $(a_A)_S$ is the activity of solute A in the stationary phase and $(a_A)_M$ is the activity in the mobile phase. We often substitute c_S, the molar analytical concentrations of the solute in the stationary phase, for $(a_A)_S$ and c_M, the molar analytical concentration in the mobile phase, for $(a_A)_M$. Hence

$$K_c = \frac{c_S}{c_M}$$

Ideally, the distribution constant is constant over a wide range of solute concentrations, that is, c_S is directly proportional to c_M.

Retention Times

- The **dead time** (void time), t_M, is the time it takes for an unretained species to pass through a chromatographic column.
- All components spend at least this amount of time in the mobile phase.
- Separations are based on the different times, t_S, that components spend in the stationary phase.
- The **retention time**, t_R, is the time between injection of a sample and the appearance of a solute peak at the detector of a chromatographic column.

$$t_R = t_S + t_M$$

Migration Rates and Distribution Constants

To relate the rate of migration of a solute to its distribution constant, we express the rate as a fraction of the velocity of the mobile phase:

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

The Retention Factor, k

The **retention factor**, k_A, for solute A is related to the rate at which A migrates through a column. It is the amount of time a solute spends in the stationary phase relative to the time it spends in mobile phase.

The retention factor is an important experimental parameter that is widely used to compare the migration rates of solutes on columns. For solute A, the retention factor k_A is defined as

$$k_A = \frac{K_A V_S}{V_M}$$

The Selectivity Factor

The **selectivity factor**, a, for solutes A and B is defined as the ratio of the distribution constant of the more strongly retained solute (B) to the distribution constant for the less strongly held solute (A).

The **selectivity factor**, a, of a column for the two solutes A and B is defined as

$$\alpha = \frac{K_B}{K_A}$$

The selectivity factor for two analytes in a column provides a measure of how well the column will separate the two.

Column Efficiency

Two related terms are widely used as quantitative measures of chromatographic column efficiency: (1) **plate height**, *H*, and (2) **plate count** or **number of theoretical plates**, *N*. The two are related by the equation

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

where *L* is the length (usually in centimeters) of the column packing. The efficiency of chromatographic columns increases as the plate count *N* becomes greater and as the plate height *H* becomes smaller.

Enormous differences in efficiencies are encountered in columns as a result of differences in column type and in mobile and stationary phases. Efficiencies in terms of plate numbers can vary from a few hundred to several hundred thousand, while plate heights ranging from a few tenths to one thousandth of a centimeter or smaller are not uncommon.

As we know the breadth of a Gaussian curve is described by the standard deviation σ and the variance σ^2. Because chromatographic bands are often Gaussian and because the efficiency of a column is reflected in the breadth of chromatographic peaks, the variance per unit length of column is used by chromatographers as a measure of column efficiency. That is, the column efficiency H is defined as

$$H = \frac{\sigma^2}{L}$$

Thin-Layer Chromatography (TLC)

- Thin-layer chromatography (TLC) can be considered a form of liquid-solid chromatography in which the stationary phase is a thin layer on the surface of an appropriate plate.
- The mobile phase is drawn over the surface by capillary action.

Principles of Thin-Layer chromatography

- Typical thin-layer separations are performed on a glass plate that is coated with a stationary phase, which consists of a thin and adherent layer of finely divided particles.
- The particles are similar to those described in the discussion of adsorption, normal- and reversed-phase partition, ion-exchange, and size-exclusion column chromatography.
- Mobile phases are also similar to those used in high-performance liquid chromatography.

Preparation of Thin-Layer Plates

- A thin-layer plate is prepared by spreading an aqueous slurry of the finely ground solid onto the clean surface of a glass or plastic plate or microscope slide.
- Often a binder is incorporated into the slurry to enhance adhesion of the solid particles to the glass and to one another.
- The plate is then allowed to stand until the layer has set and adheres tightly to the surface; for some purposes, it may be heated in an oven for several hours.
- Several chemical supply houses offer precoated plates of various kinds.
- Costs are a few dollars per plate. The common plate dimensions in centimetres are 5x 20, 10 x 20, and 20 x 20.
- Commercial plates can be conventional and high-performance plates.
- Conventional plates have thicker layers (200 to 250 mm) of particles with particles sizes of 20 mm or greater.

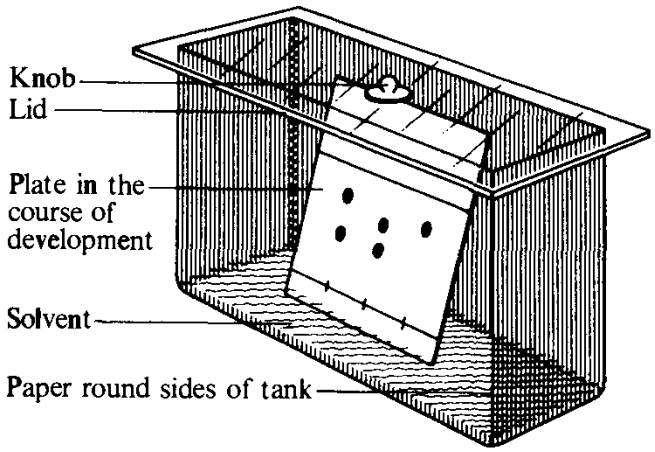
- High-performance plates usually have film thicknesses of 100 mm and particle diameters of 5 mm or less.

Sample Application

- Sample application is perhaps the most critical aspect of thin-layer chromatography.
- Usually the sample is applied as a spot 1 to 2 cm from the edge of the plate.
- Manual application of samples is performed by touching a capillary tube containing the sample to the plate or by use of a syringe.
- Mechanical dispensers, which increase the precision and accuracy of sample application, are available commercially.

Plate Development

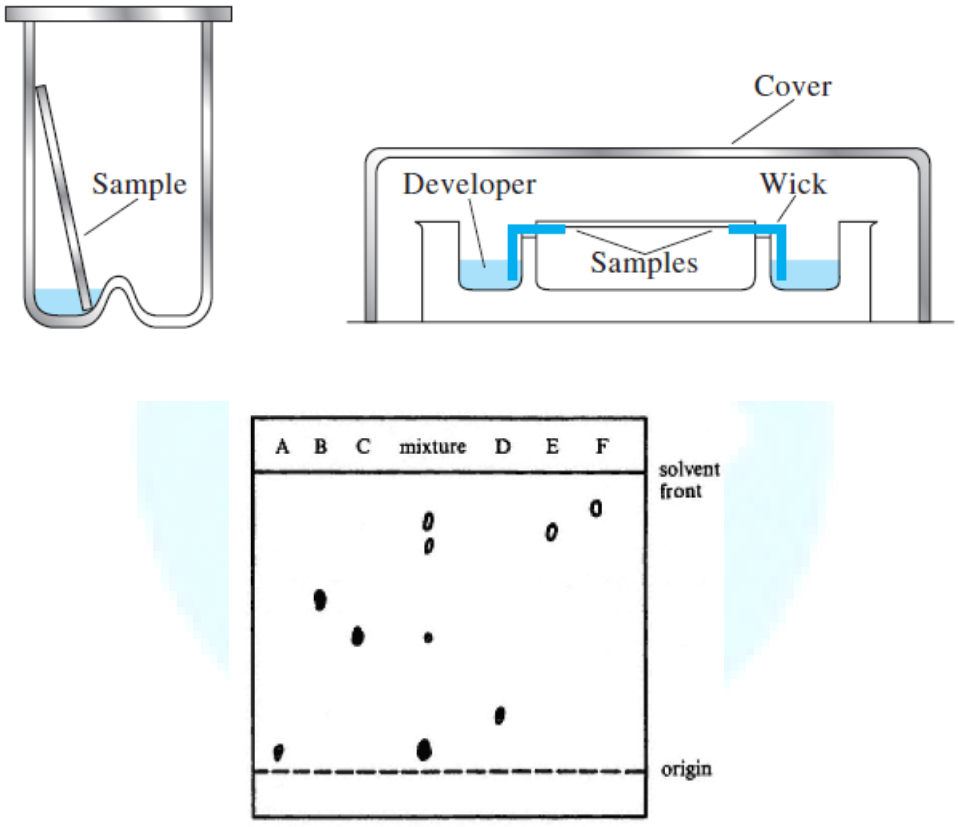
- **Plate development** is the process by which a sample is carried through the stationary phase by a mobile phase.
- It is analogous to elution in liquid chromatography.
- After applying a spot and evaporating the solvent, the plate is placed in a closed container saturated with vapors of the developing solvent.
- One end of the plate is immersed in the developing solvent, with care being taken to avoid direct contact between the sample and the developer.
- After the developer has traversed one half or two thirds of the length of the plate, the plate is removed from the container and dried.
- The positions of the components are then determined in any of several ways.



Locating Analytes on the Plate

- Several methods are used to locate sample components after separation.

- Two common methods that can be applied to most organic mixtures involve spraying with a solution of iodine or sulfuric acid. both of these reagents react with organic compounds to yield dark products.
- Several specific reagents (such as ninhydrin) are also useful for locating separated species.
- Another method of detection is based on incorporating a fluorescent material into the stationary phase.
- After development, the plate is examined under ultraviolet light.
- The sample components quench the fluorescence of the material so that all of the plate fluoresces except where the nonfluorescing sample components are located.



Thin-layer chromatogram.

The Retardation Factor

The retardation factor for this solute is given by

$$R_f = \frac{d_R}{d_M}$$

where d_R and d_M are linear distances measured from the origin line. Values for R_F can vary from 1 for solutes that are not retarded to a value that approaches 0.

Stationary Phase

Nature and Function of Thin -layer Materials

- Any of the materials used in column, ion-exchange and exclusion chromatography can be used for TLC provided that they can be obtained in the form of a homogeneous powder of fine particle size (1–50μm).
- Silica gel or silicic acid, has found the most widespread use in TLC.
- It functions primarily as a surface adsorbent if dried above 100°C, otherwise the adsorbed water acts as the stationary phase for a partition system.
- Plates coated with silica gel often contain about 10% w/w calcium sulphate (plaster of Paris) as a binder to improve adherence to the plate, although this is not essential if a very finely powdered material is used. Indicators which fluoresce under a UV lamp can be incorporated into the layer when it is prepared, e.g. the sodium salt of fluorescein.
- Alumina and kieselguhr (diatomaceous earth) are sometimes used as alternatives to silica gel but offer no particular advantages.
- For relatively polar compounds, octadecyl (C18 or ODS) modified plates are particularly useful.
- The production of plates with layers of very small and uniform particles, which result in more compact sample spots and improved resolution, has given rise to the term 'high performance' TLC or HPTLC.
- Cellulose powder is used for partition TLC where it acts largely as a solid support as in paper chromatography.
- More compact spots are obtained than with paper chromatography and development times are faster because of the fine particle size.
- A number of ion exchange cellulose powders are available for separations of ionic species.
- The mobile phase is drawn through the thin layer by capillary action, but the rate of movement is relatively fast because of the uniform and small particle size.
- TLC separations on a 20 × 20 cm plate take only 20–40 min compared with two hours or more for a comparable-sized paper chromatogram.

Mobile Phase

- The choice of mobile phase is largely empirical but general rules can be formulated.
- A mixture of an organic solvent and water with the addition of acid, base or complexing agent to optimize the solubility of the components of a mixture can be used.
- For example, good separations of polar or ionic solutes can be achieved with a mixture of water and *n*-butanol.
- Addition of acetic acid to the mixture allows more water to be incorporated and increases the solubility of basic materials, whilst the addition of ammonia increases the solubility of acidic materials.
- If the stationary phase is hydrophobic, various mixtures of benzene, cyclohexane and chloroform provide satisfactory mobile phases.
- It should be emphasized that a large degree of trial and error is involved in their selection.

- For TLC on silica gel, a mobile phase with as low a polarity as possible should be used consistent with achieving a satisfactory separation.
- Polar solvents can themselves become strongly adsorbed thereby producing a partition system, a situation which may not be as desirable.

Qualitative Analysis

- Components are identified by comparison of their R_f values with those of standards run under identical conditions, or by removing the materials from the chromatogram and subjecting them to further qualitative tests, e.g. spot-tests, mass spectrometry, infrared spectrometry.
- Chromatographic materials and conditions are usually so variable that it is advisable to run standards with samples to ensure that comparisons are valid.

Quantitative Analysis

- Thin-layer chromatography does not provide quantitative information of the highest precision and accuracy.
- Linear relationships between the mass of a substance and the logarithm or square-root of the spot area can sometimes be established under very closely controlled conditions.
- The optical absorbance of a spot determined by reflectance measurements can be similarly related to mass, or the substances can be scraped from the plate and dissolved in a suitable solvent for a spectrometric determination.
- The main difficulties with area and density measurements lie in defining the boundaries of spots and controlling chromogenic reactions in a reproducible manner.
- Relative precision can be as good as 1–2% but is more usually 5–10%.

Applications of Thin -layer Chromatography

- Thin-layer chromatography is very widely used, mainly for qualitative purposes; almost any mixture can be at least partially resolved.
- Inorganic applications, such as the separation of metals in alloys, soil and geological samples, and polar organic systems, such as mixtures of amino acids or sugars in urine, are particularly suited to cellulose TLC.
- The versatility of TLC has resulted in a rapid spread in its use in all fields especially for the separation of organic materials.
- It is ideally suited to following the course of complex reactions, quality control, purity checks, clinical diagnosis and forensic tests.

PAPER CHROMATOGRAPHY

- In paper chromatography, the sample mixture is applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action.
- Components of the mixture are carried along with the solvent up the paper to varying degrees, depending on the compound's preference to be adsorbed onto the paper versus being carried along with the solvent.
- The paper is composed of cellulose to which polar water molecules are adsorbed, while the solvent is less polar, usually consisting of a mixture of water and an organic liquid.
- The paper is called the stationary phase while the solvent is referred to as the mobile phase.
- Performing a chromatographic experiment is basically a three-step process:

  1) application of the sample
  2) "developing" the chromatogram by allowing the mobile phase to move up the paper, and 3) calculating R_f values and making conclusions.

- In order to obtain a measure of the extent of movement of a component in a paper chromatography experiment, we can calculate an "R_f value" for each separated component in the developed chromatogram.
- An R_f value is a number that is defined as:

$$R_f = \frac{\text{distance traveled by component from application point}}{\text{distance traveled by solvent from application point}}$$

PRINCEPLE OF PAPER CHROMATOGRAPHY

- The principle involved is partition chromatography wherein the substances are distributed or partitioned between liquid phases.
- One phase is the water, which is held in the pores of the filter paper used; and other is the mobile phase which moves over the paper.
- The compounds in the mixture get separated due to differences in their affinity towards water (in stationary phase) and mobile phase solvents during the movement of mobile phase under the capillary action of pores in the paper.
- The principle can also be adsorption chromatography between solid and liquid phases, wherein the stationary phase is the solid surface of paper and the liquid phase is of mobile phase.
- But most of the applications of paper chromatography work on the principle of partition chromatography, i.e. partitioned between to liquid phases.
- The distance between the point of application of the sample and the band (separation zone), is referred to as R_f value.
- Each chemical components has a definite R_f value.
- Hence it is used as an identification value.
- Those chemical components with lower affinity and adsorption toward the stationary phase move or travel faster, while those with higher affinity adsorption towards the stationary phase move or travel slower.

Stationary phase:

- It is liquid that is, the water trapped in the molecular structure of the paper and is invisible.
- Supporting material for the stationary phases is the matrix of cellulose fibers of chromatography paper.
- Chromatography papers are available in three running characteristics: slow, medium, and fast.
- Most frequently used chromatographic paper is Whatmann No.1 or its equivalent.

Mobile phase:

- It is generally a liquid e.g. for a pure paper system, the eluent may be a single solvent or a mixture of solvents that can move through the paper.
- Among mixed solvent systems, a water-organic mixture is frequently used, e.g. n-butanol, acetic acid: water (4:1:5, top layer) for flavonoid, glycosides, acetic acid: conc.HCl: water (30:3:10) (Forrestal system for flavonoid aglycones), toluene:acetic acid: water (4:1:5, upper phase for flavonoid aglycones).

- The solvents used are selected from eluotropic series which is a list of solvents arranged in order of increasing polarity.
- Petroleum ether < n-hexane< carbon tetrachloride< toluene < benzene < chloroform < dichloromethane < diethyl ether < n-butanol < isopropanol < acetone < ethanol < methanol < water
- Generally, higher the solubility of a solute in a solvent, the greater the solute mobility in that solvent.
- If a solute dissolve more readily in the mobile phase, then it will travel with the solvent, hence the partition occurs between two phases.
- Different solutes travel at different rates up the paper, it is a result of their different solubility in two phases.

GAS CHROMATOGRAPHY

- In gas chromatography, the components of a vaporized sample are separated by being distributed between a mobile gaseous phase and a liquid or a solid stationary phase held in a column.
- In performing a gas chromatographic separation, the sample is vaporized and injected onto the head of a chromatographic column.
- Elution is brought about by the flow of an inert gaseous mobile phase. In contrast to most other types of chromatography, the mobile phase does not interact with molecules of the analyte.
- The only function of the mobile phase is to transport the analyte through the column.

Two types of gas chromatography are encountered:

- Gas-liquid chromatography (GLC)
- Gas-solid chromatography (GSC)

Basic instrumental setup

Carrier Gas System

- The mobile phase gas in gas chromatography is called the **carrier gas** and must be chemically inert.
- Helium is the most common mobile phase, although argon, nitrogen, and hydrogen are also used.
- These gases are available in pressurized tanks.
- Pressure regulators, gauges, and flow meters are required to control the flow rate of the gas.
- Classically, flow rates in gas chromatographs were regulated by controlling the gas inlet pressure.
- A two-stage pressure regulator at the gas cylinder and some sort of pressure regulator or flow regulator mounted in the chromatograph were used.
- Inlet pressures usually range from 10 to 50 psi (lb/in2) above room pressure, yielding flow rates of 25 to 150 mL/min with packed columns and 1 to 25 mL/min for open tubular capillary columns.
- With pressure-controlled devices, it is assumed that flow rates are constant if the inlet pressure remains constant.
- Newer chromatographs use electronic pressure controllers both for packed and for capillary columns.
- With any chromatograph, it is desirable to measure the flow through the column.
- A soap film is formed in the path of the gas when a rubber bulb containing an aqueous solution of soap or detergent is squeezed; the time required for this film to move between two graduations on the burette is measured and converted to volumetric flow rate.
- Usually, the flow meter is located at the end of the column.
- The use of electronic flow meters has become increasingly common.
- Digital flow meters are available that measure mass flow, volume flow, or both.
- Volumetric flow measurements are independent of the gas composition.
- Mass flow meters are calibrated for specific gas compositions, but, unlike volumetric meters, they are independent of temperature and pressure.

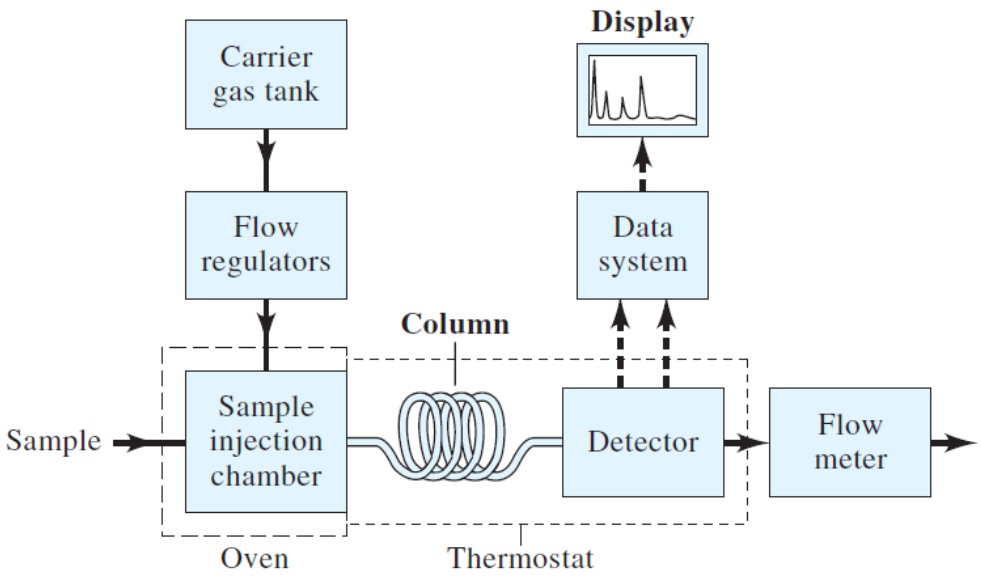
Sample Injection system

- For high column efficiency, a suitably sized sample should be introduced as a “plug” of vapor.
- Slow injection or oversized samples cause band spreading and poor resolution.
- Calibrated microsyringes, are used to inject liquid samples through a rubber or silicone diaphragm, or septum, into a heated sample port located at the head of the column.
- The sample port is usually kept at about 50^0C greater than the boiling point of the least volatile component of the sample.
- For ordinary packed analytical columns, sample sizes range from a few tenths of a microliter to 20 mL.
- Capillary columns require samples that are smaller by a factor of 100 or more.
- For these columns, a sample splitter is often needed to deliver a small known fraction (1:100 to 1:500) of the injected sample, with the remainder going to waste.

- Commercial gas chromatographs intended for use with capillary columns incorporate such splitters, and they also allow for splitless injection when packed columns are used.
- For the most reproducible sample injection, newer gas chromatographs use autoinjectors and autosamplers.
- With such autoinjectors, syringes are filled, and the sample injected into the chromatograph automatically.
- In the autosampler, samples are contained in vials on a sample turntable.
- The autoinjector syringe picks up the sample through a septum on the vial and Injects the sample through a septum on the chromatograph.
- For introducing gases, a sample valve is often used instead of a syringe.
- With such devices, sample sizes can be reproduced to better than 0.5% relative.
- Liquid samples can also be introduced through a sampling valve.
- Solid samples are introduced as solutions or alternatively are sealed into thin-walled vials that can be inserted at the head of the column and punctured or crushed from the outside.

Column Configurations and Column Ovens

- The columns in gas chromatography are of two general types: **packed columns** or **capillary columns**.
- Chromatographic columns vary in length from less than 2 m to 60 m or more.
- They are constructed of stainless steel, glass, fused silica, or Teflon.
- In order to fit into an oven for thermosetting, they are usually formed as coils having diameters of 10 to 30 cm.
- Column temperature is an important variable that must be controlled to a few tenths of a degree for precise work.
- Thus, the column is normally housed in a thermostated oven.
- The optimum column temperature depends on the boiling point of the sample and the degree of separation required.
- Roughly, a temperature equal to or slightly above the average boiling point of a sample results in a reasonable elution time (2 to 30 min).
- For samples with a broad boiling range, it is often desirable to use **temperature programming** whereby the column temperature is increased either continuously or in steps as the separation proceeds.
- In general, optimum resolution is associated with minimal temperature.
- The cost of lowered temperature, however, is an increase in elution time and, therefore, the time required to complete an analysis.
- Analytes of limited volatility can sometimes be determined by forming derivatives that are more volatile.
- Likewise, derivatization is used at times to enhance detection or improve chromatographic performance.



Chromatographic Detectors

Characteristics of the Ideal Detector

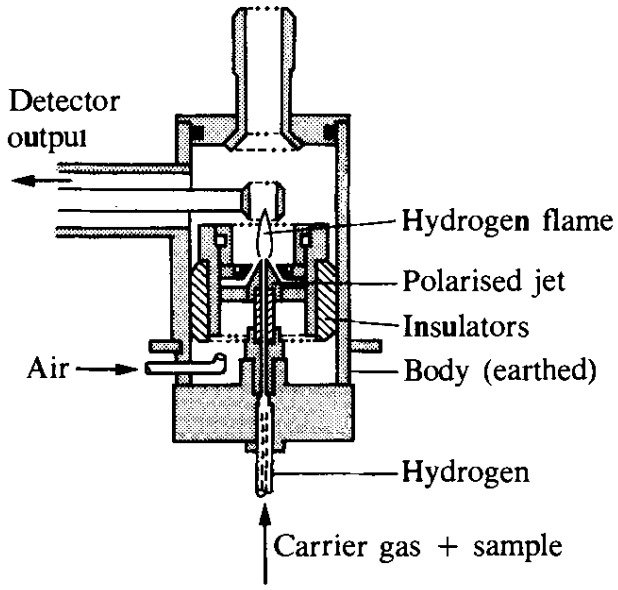
The ideal detector for gas chromatography has the following characteristics:

1. Adequate sensitivity. In general, the sensitivities of present-day detectors lie in the range of 10^{-8} to 10^{-15} g solute/s.
2. Good stability and reproducibility.
3. A linear response to solutes that extends over several orders of magnitude.
4. A temperature range from room temperature to at least 400^0C.
5. A short response time that is independent of flow rate.
6. High reliability and ease of use. To the greatest extent possible, the detector should be foolproof in the hands of inexperienced operators.
7. Similarity in response toward all solutes or, alternatively, a highly predictable and selective response toward one or more classes of solutes.
8. Nondestructive of sample.

Flame Ionization Detectors

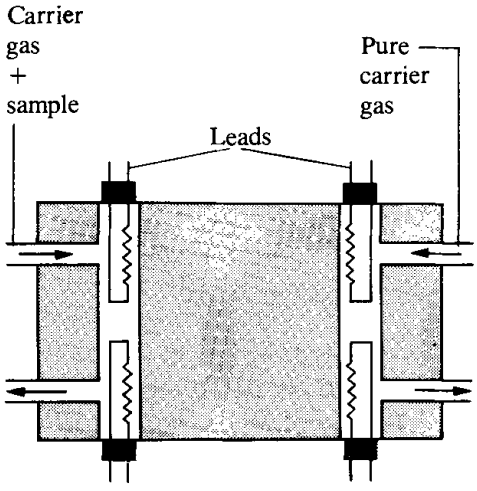
- The **flame ionization detector** (FID) is the most widely used and generally applicable detector for gas chromatography.
- With a FID, effluent from the column is directed into a small air/hydrogen flame.
- Most organic compounds produce ions and electrons when pyrolyzed at the temperature of an air/hydrogen flame.

- These compounds are detected by monitoring the current produced by collecting the ions and electrons.
- A few hundred volts applied between the burner tip and a collector electrode located above the flame serves to collect the ions and electrons.
- The resulting current (10^{-12} A) is then measured with a sensitive picoammeter.
- The ionization of carbon compounds in a flame is a poorly understood process, although it is observed that the number of ions produced is roughly proportional to the number of *reduced* carbon atoms in the flame.
- Because the flame ionization detector responds to the number of carbon atoms entering the detector per unit of time, it is a *mass sensitive* rather than a *concentration-sensitive* device.
- As such, this detector has the advantage that changes in flow rate of the mobile phase have little effect on detector response.
- Functional groups, such as carbonyl, alcohol, halogen, and amine, yield fewer ions or none at all in a flame.
- In addition, the detector is insensitive toward noncombustible gases, such as H_2O, CO_2, SO_2, and NO_x.
- These properties make the flame ionization detector a most useful general detector for the analysis of most organic samples including those that are contaminated with water and the oxides of nitrogen and sulfur.
- The FID exhibits a high sensitivity (,10^{-13} g/s), large linear response range (10^7), and low noise. It is generally rugged and easy to use.
- Disadvantages of the flame ionization detector are that it destroys the sample during the combustion step and requires additional gases and controllers.



Thermal Conductivity Detectors

- The **thermal conductivity detector** (TCD), which was one of the earliest detectors for gas chromatography, still finds wide application.
- This device consists of an electrically heated source whose temperature at constant electric power depends on the thermal conductivity of the surrounding gas.
- The heated element may be a fine platinum, gold, or tungsten wire or, alternatively, a small thermistor.
- The electrical resistance of this element depends on the thermal conductivity of the gas.
- Four thermally sensitive resistive elements are often used.
- A *reference pair* is located ahead of the sample injection chamber and a *sample pair* immediately beyond the column.
- Alternatively, the gas stream can be split. The detectors are incorporated in two arms of a simple bridge circuit, such that the thermal conductivity of the carrier gas is cancelled.
- In addition, the effects of variations in temperature, pressure, and electric power are minimized.
- Modulated single-filament TCDs are also available.
- The thermal conductivities of helium and hydrogen are roughly six to ten times greater than those of most organic compounds.
- Thus, even small amounts of organic species cause relatively large decreases in the thermal conductivity of the column effluent, resulting in a marked rise in the temperature of the detector.
- Detection by thermal conductivity is less satisfactory with carrier gases whose conductivities closely resemble those of most sample components.
- The advantages of the TCD are its simplicity, its large linear dynamic range (about five orders of magnitude), its general response to both organic and inorganic species, and its nondestructive character, which permits collection of solutes after detection.
- The chief limitation of this detector is its relatively low sensitivity.
- Other detectors exceed this sensitivity by factors of 10^4 to 10^7. The low sensitivities of TCDs often precludes their use with capillary columns where sample amounts are very small.



Electron Capture Detectors

- The **electron capture detector** (ECD) has become one of the most widely used detectors for environmental samples because this detector selectively responds to halogen-containing organic compounds, such as pesticides and polychlorinated biphenyls.
- In this detector, the sample eluate from a column is passed over a radioactive b emitter, usually nickel-63.
- An electron from the emitter causes ionization of the carrier gas (often nitrogen) and the production of a burst of electrons. In the absence of organic species, a constant standing current between a pair of electrodes results from this ionization process.
- The current decreases markedly, however, in the presence of organic molecules containing electronegative functional groups that tend to capture electrons.
- Compounds, such as halogens, peroxides, quinones, and nitro groups, are detected with high sensitivity.
- The detector is insensitive to functional groups such as amines, alcohols, and hydrocarbons.
- Electron capture detectors are highly sensitive and have the advantage of not altering the sample significantly (in contrast to the flame ionization detector, which consumes the sample).
- The linear response of the detector, however, is limited to about two orders of magnitude.

Other GC Detectors

- Other important GC detectors include the thermionic detector, the electrolytic conductivity or Hall detector, and the photoionization detector.
- The thermionic detector is similar in construction to the FID.
- With the thermionic detector, nitrogen- and phosphorous-containing compounds produce increased currents in a flame in which an alkali metal salt is vaporized.
- The thermionic detector is widely used for organophosphorus pesticides and pharmaceutical compounds.
- With the electrolytic conductivity detector, compounds containing halogens, sulfur, or nitrogen are mixed with a reaction gas in a small reactor tube.
- The products are then dissolved in a liquid that produces a conductive solution.
- The change in conductivity as a result of the presence of the active compound is then measured.
- In the photoionization detector, molecules are photoionized by UV radiation.
- The ions and electrons produced are then collected with a pair of biased electrodes, and the resulting current is measured.
- The detector is often used for aromatic and other molecules that are easily photoionized.
- Gas chromatography is often coupled with the selective techniques of spectroscopy and electrochemistry.

GAS-SOLID CHROMATOGRAPHY

- Gas-solid chromatography is based on adsorption of gaseous substances on solid surfaces.
- Distribution coefficients are generally much larger than those for gas-liquid chromatography.

- This property renders gas-solid chromatography useful for separating species that are not retained by gas-liquid columns, such as the components of air, hydrogen sulfide, carbon disulfide, nitrogen oxides, carbon monoxide, carbon dioxide, and the rare gases.
- Gas-solid chromatography is performed with both packed and open tubular columns.
- For the latter, a thin layer of the adsorbent is affixed to the inner walls of the capillary.
- Such columns are sometimes called **porous layer open tubular columns**, or PLOT columns.

High-Performance Liquid Chromatography

- High-performance liquid chromatography (HPLC) is the most versatile and widely used type of elution chromatography.
- The technique is used by scientists for separating and determining species in a variety of organic, inorganic, and biological materials.
- In liquid chromatography, the mobile phase is a liquid solvent containing the sample as a mixture of solutes.
- The types of high-performance liquid chromatography are often classified by the separation mechanism or by the type of stationary phase.

These include
(1) partition, or liquid-liquid, chromatography
(2) adsorption, or liquid-solid, chromatography
(3) ion-exchange, or ion, chromatography
(4) size-exclusion chromatography
(5) affinity chromatography
(6) chiral chromatography

Principles

- Separation of mixtures in microgram to gram quantities by passage of the sample through a column containing a stationary solid by means of a pressurized flow of a liquid mobile phase; components migrate through the column at different rates due to different relative affinities for the stationary and mobile phases base on adsorption, size or charge.

EQUIPMENT FOR HPLC

Solvent Delivery Systems

- These include solvent reservoirs and inlet filters, solvent degassing facilities and one or more pumps with associated pressure and flow controls.
- Most systems are microprocessor or computer controlled enabling parameters to be selected and monitored during operation using simple keypad dialogues.
- A single solvent may be used as the mobile phase for *isocratic* elution or mixtures of two to four solvents (*binary*, *ternary* and *quaternary*) blended together under microprocessor or computer control for *gradient* elution, i.e. where the composition of the mobile phase is altered during the chromatographic run so as to optimize the separation.
- Pumps for HPLC should be capable of delivering a constant, reproducible and pulse-free supply of mobile phase to the column at flowrates between 0.1 and at least 5 $cm^3 min^{-1}$ and at operating pressures up to about 3000 psi (200 bar).
- They should be chemically inert to the various solvents that may be used and preferably have a very small hold-up volume to facilitate rapid changes of mobile phase and for gradient elution.

Sample Injection System

- Sample injection in HPLC is a more critical operation than in GC.
- Samples may be injected either by *syringe* or with a *valve injector* although the former is now rarely used.
- Valves, which can be used at pressures up to about 7000 psi (500 bar), give very reproducible results for replicate injections (< 0.2% relative precision) and are therefore ideal for quantitative work.
- They consist of a stainless steel body and rotating central block into which are cut grooves to channel the mobile phase from the pump to the column.
- The sample is loaded into a stainless steel loop incorporated into the valve body or attached externally whilst the mobile phase is passed directly to the column.
- By rotating the central block, the flow can be diverted through the loop thereby flushing the sample onto the column.
- Returning the block to its original position enables the next sample to be loaded ready for injection.
- Although the sample injected is generally a fixed volume as determined by the size of the loop, these are interchangeable and range from 2 µl to over 100 µl. *Multiport valves* which can accommodate several loops of different sizes are available, and some loops can be used partially filled.
- Automated injection systems that allow a series of samples and standards to be injected over a period of time whilst the instrument is unattended and under variable chromatographic conditions are frequently used in industrial laboratories.

The Column

- Columns are made from straight lengths of precision-bore stainless-steel tubing with a smooth internal finish.
- Typically they are 10–25 cm long and 4–5 mm i.d. Microbore columns, 20–50 cm long and with an i.d. of 1–2 mm, are sometimes used where sample size is limited and to minimize solvent consumption because the volumetric flow rate through them is less than a quarter of that through conventional columns.
- The stationary phase or packing is retained at each end by thin stainless-steel frits or mesh disks of 2 µm porosity or less.
- Columns are packed by a *slurry method* which involves suspending the particles of packing in a suitable solvent and 'slamming' it into the column rapidly and at pressures in excess of 3000 psi (200 bar).

Stationary Phase (Column Packing)

- Unmodified or chemically modified microparticulate silicas (3, 5 or 10 µm) are preferred for nearly all HPLC applications.
- The particles, which are totally porous, may be spherical or irregular in shape but it is essential that the size range is as narrow as possible to ensure high column efficiency and permeability.

- For separations based on adsorption, an unmodified silica, which has a polar surface due to the presence of silanol (Si–OH) groups, is used.
- Appropriate chemical modification of the surface by treatment with chloro- or alkoxy-silanes, e.g. $R(CH_3)_2SiCl$, produces *bonded-phase* packings which are resistant to hydrolysis by virtue of forming siloxane (Si—O—Si—C) bonds.
- Materials with different polarities and chromatographic characteristics can be prepared.
- The most extensively used are those with a non-polar hydrocarbon-like surface, the modifying groups, R, being octadecyl (C_{18} or ODS), octyl or aryl.
- More polar bonded phases, e.g. amino-propyl, cyanopropyl (nitrile) and diol, and cation and anion exchange materials are also available.

Mobile Phase

- Unlike GC, in HPLC appropriate selection of the mobile phase composition is crucial in optimizing chromatographic performance.
- The eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components.
- For 'normal phase' separations (polar stationary phase/non-polar mobile phase) eluting power *increases* with increasing solvent polarity whereas for 'reverse phase' separations (non-polar stationary phase/polar mobile phase) eluting power *decreases* with increasing solvent polarity.
- Many solvents for HPLC require purification before use as the impurities may either be strongly UV absorbing, e.g. aromatic or alkene impurities in *n*-alkanes, or they may be of much higher polarity than the solvent itself, e.g. traces of water or acids, or ethanol in chloroform, etc.
- All mobile phases should be filtered and degassed before pumping through the column, the former to prevent contamination and clogging of the top of the column and the latter to prevent noise in the detector from the formation of air bubbles due to the pressure dropping to atmospheric at the column exit.

Detectors

- The ideal HPLC detector should have the same characteristics as those required for GC detectors, i.e. rapid and reproducible response to solutes, a wide range of linear response, high sensitivity and stability of operation.
- No truly universal HPLC detector has yet been developed but the two most widely applicable types are those based on the absorption of UV or visible radiation by the solute species and those which monitor refractive index differences between solutes dissolved in the mobile phase and the pure mobile phase.
- Other detectors which are more selective in their response rely on such solute properties as fluorescence, electrical conductivity, diffusion currents (amperometric) and radioactivity.

UV/Visible Photometers and Dispersive Spectrophotometers

- These detectors respond to UV/visible absorbing species in the range 190–800 nm and their response is linear with concentration, obeying the Beer-Lambert law.

- They are not appreciably flow or temperature sensitive, have a wide linear range and good but variable sensitivity.
- *Photometers* are designed to operate at one or more fixed wavelengths only, e.g. 220, 254, 436 and 546 nm, whereas *spectrophotometers* facilitate monitoring at any wavelength within the operating range of the instrument.
- Both types of detector employ low-volume (10 µl or less) flow-through cells fitted with quartz windows.

Diode Array Spectrophotometers

- These can provide more spectral information than photometers or conventional dispersive spectrophotometers but are much more expensive and generally less sensitive.
- However, they enable sets of complete UV or UV and visible spectra of all the sample components to be recorded as they elute from the column.

Fluorescence Detectors

- These are highly selective and among the most sensitive of detectors.
- They are based on filter fluorimeters or spectrofluorimeters but are usually purpose-designed for HPLC or capillary electrophoresis.
- Fluorescence detectors are relatively insensitive both to pulsations in the mobile phase flow and to temperature fluctuations.

Refractive Index (RI) Monitors

- There are several types of RI detector, all of which monitor the difference between a reference stream of mobile phase and the column effluent.
- Any solute whose presence alters the refractive index of the pure solvent will be detected, but sensitivity is directly proportional to the difference between the refractive index of the solute and that of the solvent.
- At best they are two orders of magnitude less sensitive than UV/visible detectors.
- All RI detectors are highly temperature-sensitive, and some designs incorporate heat exchangers between column and detector to optimize performance.

Electrochemical Detectors

- *Conductance monitors* can be used where the sample components are ionic and providing that the conductivity of the mobile phase is very low.
- They are used extensively in *ion chromatography* for the detection of inorganic anions, some inorganic cations and ionized organic acids.
- *Amperometric detectors*, which are based on the principle of polarography, rely on measuring the current generated in an electrochemical cell at a fixed applied potential by the facile oxidation or reduction of an eluted compound at the surface of a glassy carbon, gold or platinum micro-electrode.

Ion-exchange Chromatography

Principles

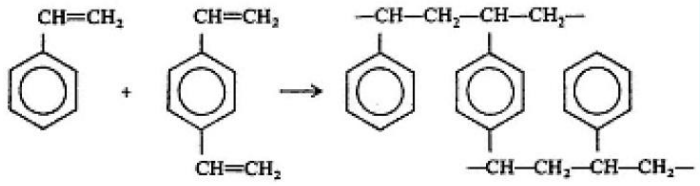
- Separation of ionic materials in microgram to gram quantities by passage of a solution through a column or across a surface consisting of a porous polymeric resin incorporating exchangeable ions.

Apparatus and Instrumentation

- Glass columns for separation by gravity flow; glass, metal or nylon tubing for pressurized systems; fraction collector, detector and recorder.

Structure of Ion-exchange Resins

- The most widely used type of resin is a copolymer of styrene and divinyl benzene produced in bead form by suspension polymerization in an aqueous medium.



- Cation or anion-exchanging properties are introduced into the resin by chemical modification after polymerization.
- *Cation-exchangers* can be subdivided into *strong-acid* types containing —SO_3H groups and *weak-acid* types containing —COOH groups.
- *Anion-exchangers* comprise *strong-base* types incorporating quaternary ammonium groups (—N^+R_3) and *weak-base* types incorporating primary, secondary or tertiary amines.

Non-resinous Ion-exchange Materials

- Cellulose, modified by the introduction of ionic groups, is available in paper form or as a powder for use in TLC and is particularly useful for the separation of macromolecules and biological materials.
- Cation exchangers are produced by introducing acidic groups, e.g. —OCH_2SO_3H (sulphomethyl, SM), —OCH_2COOH (carboxymethyl, CM), which are bonded to the cellulose structure via ether or ester linkages. Anion exchangers are formed by reacting the cellulose with epichlorhydrin and an amine, e.g. —$OCH_2CH_2N^+(C_2H_5)_3$ (triethylaminoethyl, TEAE).

Selectivity

- The affinity between a resin and an exchangeable ion is a function both of the resin and the ion.

- Ion exchange is an equilibrium process which for a cationic resin can be represented by the equation

$$n\mathrm{R^-H^+} + \mathrm{M}^{n+} = (\mathrm{R^-})_n\mathrm{M}^{n+} + n\mathrm{H^+}$$

Where R represents the resin matrix.

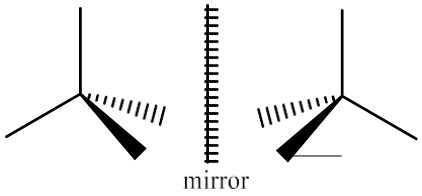
- The equilibrium constant, also known as the *selectivity coefficient*, is given by

$$K = \frac{[\mathrm{M}^{n+}]_\mathrm{R}[\mathrm{H^+}]^n}{[\mathrm{M}^{n+}][\mathrm{H^+}]^n_\mathrm{R}}$$

Ion-exchange Separations

- Most separations are performed using columns of resin and an elution procedure.
- The sample is introduced as a small band at the top of the column from where the various components are moved down the column at a rate depending on their selectivity coefficients.
- Sorption isotherms are approximately linear in dilute solutions so that elution peaks are symmetrical.
- Tailing can be expected at high concentrations as the isotherms curve towards the mobile phase concentration axis.
- The mobile phase contains an ion of low resin affinity, and the separated components collected at the bottom of the column are thus accompanied by a relatively high concentration of this ion.
- Procedures often adopted in ion-exchange chromatography are *gradient elution*, involving continuous variation of the composition of the eluting agent, *stepwise elution*, in which the composition is altered at specific points during the separation, and *complexing elution* where a reagent which forms complexes of varying stability with the sample components is included in the solution.
- Acids, bases and buffers are the most widely used eluting agents.
- Separated components emerging in the column effluent can be monitored by means of a physical measurement, e.g. UV or visible absorbance, refractive index, conductivity or radioactivity.
- Alternatively, separate fractions can be collected automatically and subjected to further analysis.

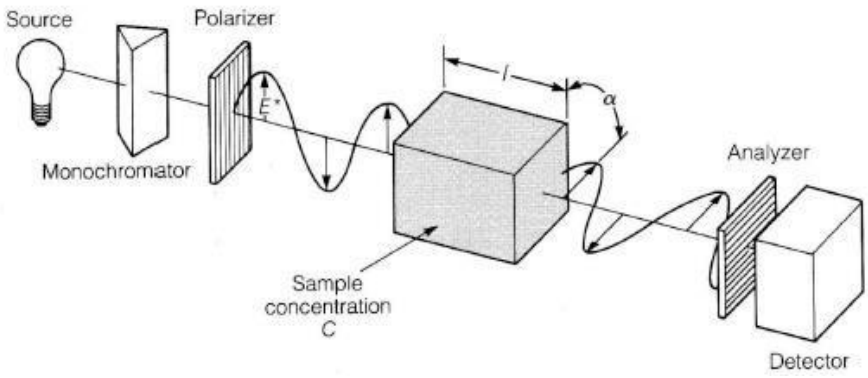
ORD & CD: Fundamental theory of ORD and CD depends on the chirality. Chirality: Mirror images but not superimposable

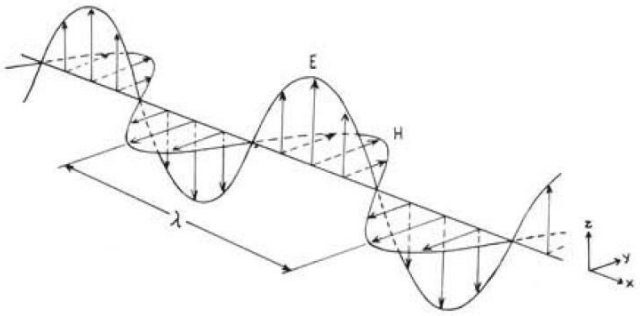


Chiral molecules exist as pair of isomers, they lacks plane of symmetry, and they are optically active.

Enantiomers: Same chemical formula/stereoisomers. Mirror images not super imposable to each other. They have identical physical properties *e.g.*, density, mp, bp, vp etc. only one difference (exception) they reacts to light differently. Even, NMR spectroscopy cannot distinguish enantiomers.

Polarized light: Ordinary light consists of many components which vary in wavelength and amplitude. Light which oscillates in a single plane is called plane-polarized or linearly polarized light. Electric vectoris randomly oriented/distributed around the z-axis in x-y plane.





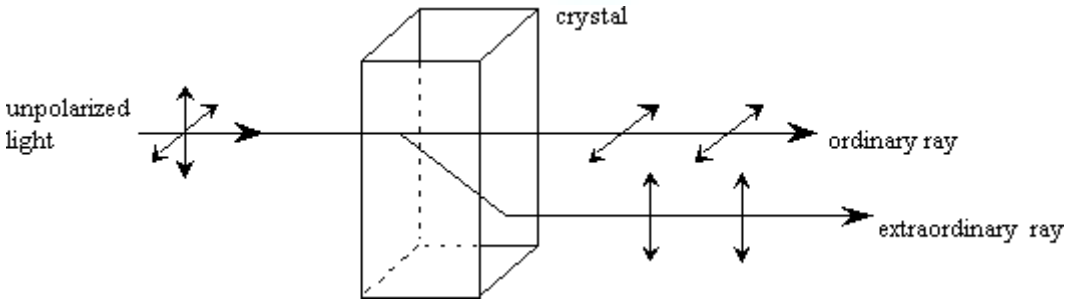
Types of polarized light

- Plane polarized light consists of two circularly polarized components of equal intensity.
- Two circularly polarized components are like left- and right-handed springs/helices.
- As observed by looking at the source, right-handed circularly polarized light rotates clockwise.

Optical activity: interactions between enantiomers & polarized light and its consequences.It has two phenomena:

1. Birefringence
2. Dichroism

Birefringence: its and optical properties that involves direction dependences of refractive index, double refraction (Birefringence)

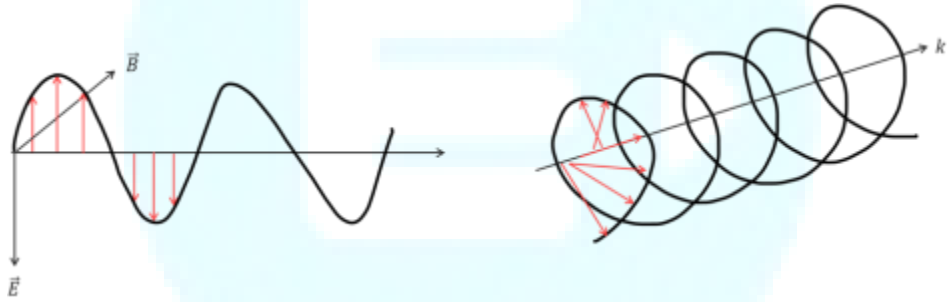


Since Birefringence refers to the direction-dependent index of Refraction. Hence Asymmetric

crystals andplastics under mechanical stress can give birefringence. It is of two types – Linear and Circular

Linear Birefringence: it's an optical property that involves unequal refraction/speed of linear polarized light in two orthogonal planes.

Circular Birefringence: It is an optical property that involves unequal refraction/speed of left circularly polarized light (LCPL) and right circularly polarized light (RCPL). Shorter wavelength rotates more (clockwise – dextrorotary & anti clock wise – laevorotatory). Since the speed of light in a medium is manifested in the refractive index of the medium, the essential property of an optically active substance is that it has different refractive indexes for the left and the right circularly polarized light, nL and nR, respectively. Linearly (left) and circularly (right) polarized light are shown below.



ORD Spectroscopy: It is observed by dependence of optical rotation on wavelength. Optical Rotatory Dispersion method measures the ability of optically active compound to rotate plane polarized light, as a function of the wavelength. ORD based on index of refraction.

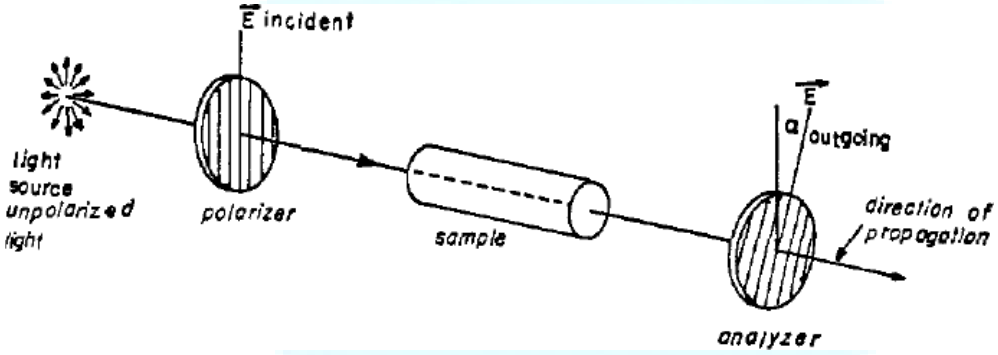
- If the refractive indices of the sample for the left and right handed polarized light are different, whenthe components are recombined, the plane-polarized radiation will be rotated through an angle α
- nl, nr are the indices of the refraction for left-handed and right handed polarized light
- α is in radians per unit length [$\alpha = nl\text{-}nr/\lambda$].

- ORD curve is a plot of molar rotation [α] or M vs λ
- Clockwise rotation is plotted positively; counter clockwise rotation is plotted negatively
- ORD is based solely on the index of refraction
- plain curve is the ORD for a chiral compound that lacks a chromophore
- Chiral compounds containing a chromophore can give anomalous, or Cotton effect, curves

Specific rotation (α)**:** The specific rotation of a chemical compound is defined as the observed angle of optical rotation, when plane-polarized light is passed through a sample with a path length of 1 dm and a sample concentration of 1g/ml

Specific rotation (α) = 100 x $\alpha_O/l.c$

Where, α_O is observed rotation, l is path length (dm) and c is concentration (g/100 ml).



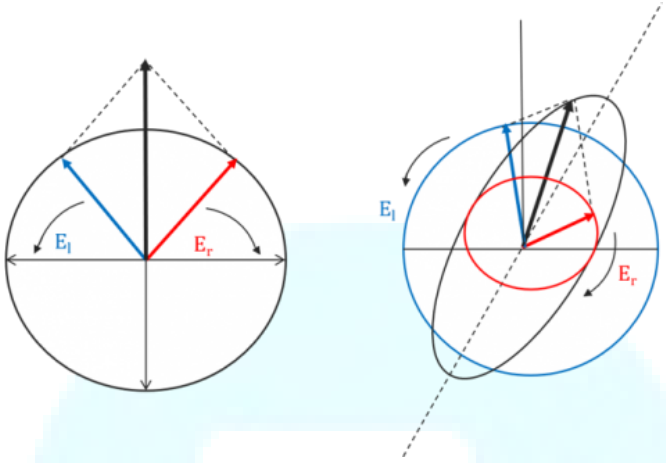
Dichroism: Dichroism – direction depended light absorption. It is of two types – Linear Dichroism andCircular Dichroism.

Linear Dichroism: A differential absorption in parallel and perpendicular directions. Linear Dichroism (LD) is a spectroscopic technique that can be used with systems that are either intrinsically oriented, or can be oriented during an experiment by external forces. It gives information about conformation and orientation of structures within molecules.

To measure *LD,* the sample is oriented then the difference in absorption of light linearly polarized paralleland perpendicular to the orientation axis is measured

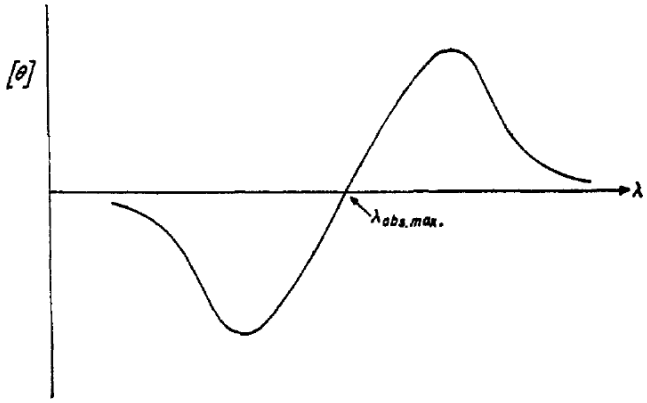
$$LD = A_{||} - A_{\perp}$$

Circular Dichroism (CD): Some materials possess special properties of absorption of the left circularly polarized light to different extent that the right circularly polarized light. Thisphenomenon is called circular dichroism. CD is the differential absorption of LCPL andRCPL. Elliptically polarized light shown below.



The difference between the absorption of left and right handed circularly-polarised light and is measured as a function of wavelength. CD is measured as a quantity called mean residue ellipticity, whose units are *degrees-cm^2/dmol*. So emerging light is elliptically polarized. CD Spectrum is difference of $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$

Plot of molar ellipticity (θ) as a function of wavelength. CD – exhibited because of dextrorotatory & laevorotatory compounds.



- Measurement of how an optically active compound *absorbs* right- and left-handed

circularly polarized light

- All optically active compounds exhibit CD in the region of the absorption band
- For CD, the resulting transmitted radiation is not plane-polarized but ellipticallypolarized
- Optically active absorbing chromophores present different extinction coefficients for R and L circularly polarized waves
- The technique is good at estimating alpha helical content, and at studyingdynamic changes is secondary structure

Applications

- α – helix of properties has its distinct spectral signatures. Nucleic acid double helix (αhelix & β sheet). Comparison of the UV absorbance (left) and the circular dichroism(right) of poly-L-lysine in different secondary structure conformations as a function ofpH.
- Proteins – CD spectra sensitive to secondary structure change – α- β- polyproline,irregular
- Aromatic amino acids – CD spectra sensitive to tertiary structure changePhenylamine
- Tryptophan, tyrosine etc.
- Proteins 2^{o} structure determination
- pH, heat or solvent induced structural changes
- proteins folding unfolding
- ligand or ion induced structural changes
- nucleic acid/peptide/hormones structural behavior

CD- differential absorption of light and right circularly polarized light

Region	Application
Near UV CD >250 nm	Tertiary structure of protein
UV CD	secondary structure of protein

UV/Vis CD	Charge transfer transitions
Near IR CD	Geometric & electronic structure by probing metal d-dtransitions
Vibrational CD	Structural studies of proteins and DNA

CD & ORD comparison

In the absence of magnetic field – only chiral molecule show CD and ORDIn the presence of magnetic field- all molecules show CD & ORD

ORD – differential speed of LCDL and RLPL $C_L \neq C_R$

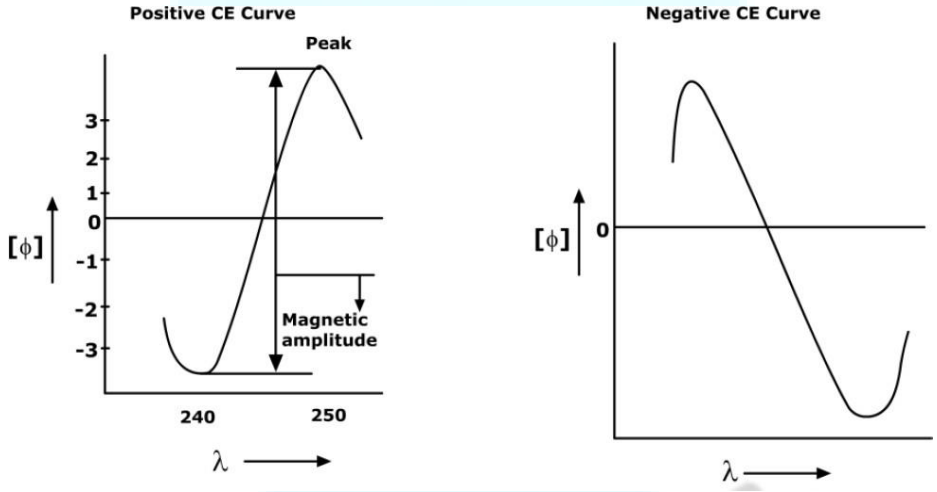
CD – differential absorption of LCDL & RLPL $\varepsilon_L \neq \varepsilon_R$

ORD	CD
• ORD is the refractive indices of thesample for the left and right handed polarized light are different, when the components are recombined, the plane polarized radiation will be rotatedthrough angle α (differential speed of LCDL and RLPL $C_L \neq C_R$)	• CD is a differential absorption of left and right handed circularly polarized light (differential absorption of LCDL & RLPL $\varepsilon L \neq \varepsilon R$)
• ORD spectra are dispersive	• CD spectra are absorptive
• In ORD the circular polarized light is usedis not converted to elliptical light	• In CD the circular polarized light is usedand its converted to elliptical light
• ORD grades are obtained by plotting specific rotation vs wavelength	• CD graphs are obtained by plottingmolar ellipticity vs wavelength

Cotton Effect: The characteristics change in ORD &/or CD in the vicinity of an absorbband. The combination of both (circular birefringence and circular dichroism) effect inthe region in which optically active absorption bands are observed gives rise to a phenomenon called cotton effect.

Cotton effect curves: It has the following points:

- These curves will show the high peak ns through which depends on the absorbinggroups.
- These curves will obtain for the compounds which are having asymmetric carbon &chromophore which absorbs near UV region.
- A chromophore with +ve cotton effect cause a right rotation at low frequency.
- A chromophore with negative cotton effect cause left rotation at low frequency.
- It is of two types: Single cotton effect curves and multiple cotton effect curves.



Advantage of ORD & CD:

- Simple and quick experiments
- No extensive preparation
- Relatively low concentration/amounts of sample required for experiment
- Any size of macromolecules can be observed
- Better resolution, better sensitivity and Easier to assign.

ORD – differential speed of LCDL and RLPL CL

≠ CRCD – differential absorption of LCDL & RLPL

εL ≠ εR

ORD	CD
• ORD is the refractive indices of thesample for the left and right handed polarized light are different, when the components are recombined, the planepolarized radiation will be rotatedthrough angle α (differential speed of LCDL and RLPL CL ≠ CR)	• CD is a differential absorption of left and right handed circularly polarized light (differential absorption of LCDL & RLPL εL ≠ εR)
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