

STUDY MATERIAL

M.Sc., Chemistry (Non- Semester)

Major Paper VII – Analytical Chemistry



CENTRE FOR DISTANCE EDUCATION

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OCT- 2016

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TABLE OF CONTENTS

II- M.Sc CHEMISTRY, PAPER VII- ANALYTICAL CHEMISTRY

S.No.	TITLE	PAGE NO.
UNIT I	INSTRUMENTAL METHODS OF ANALYSIS	4-15
UNIT II	DATA AND ERROR ANALYSIS	16-46
UNIT III	CHROMATOGRAPHY	47-73
UNIT IV	THERMOANALYTICAL METHODS	74-89
UNIT V	ELECTROANALYTICAL TECHNIQUES AND FLURESCENCE SPECTROSCOPY	90-131

MAJOR PAPER VII-ANALYTICAL CHEMISTRY

UNIT I: INSTRUMENTAL METHODS OF ANALYSIS

COURSE OBJECTIVES

1. To know the principle and structure of EXAFS, FES and AAS spectroscopy
2. To learn the operation of various instruments
3. To learn the applications of EXAFS ,FES and AAS spectroscopy

EXTENDED X-RAY ABSORPTION FINE STRUCTURE (EXAFS)

Introduction

EXAFS spectroscopy provides structural information about a sample by way of the analysis of its X-ray absorption spectrum. It allows determining the chemical environment of a single element in terms of the number and type of its neighbours, inter-atomic distances and structural disorders. This determination is confined to a distance given by the mean free path of the photoelectron in the condensed matter, which is between 5 and 10 Å radius from the element.

These characteristics make EXAFS a powerful structural local probe, which does not require a long-range order. It is an important technique in several fields of natural sciences, from earth sciences to biochemistry. Since EXAFS is a technique selective for a particular element and sensible only for a short-range order, it is one of the most appropriate spectroscopic techniques to be applied in the following cases:

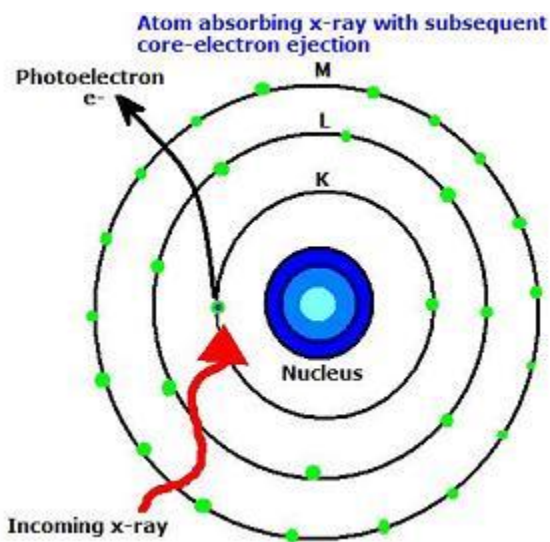
- amorphous solids, *e.g.*, ceramics
- liquids, *e.g.*, solutions of ionic compounds or gels which cannot be studied by X-ray diffraction,
- biomolecules, *e.g.*, solutions of metalloproteins,
- homogeneous and heterogeneous catalysts.

EXAFS does not require any particular experimental conditions, such as vacuum (at least in principle). There are several types of sample-holders that allow collecting experimental data under varying temperature and pressure, or while the sample is undergoing a chemical reaction. Measures taken under working conditions are of critical importance in the case of heterogeneous catalysts in order to understand their behavior during catalysis, and in studying temperature and pressure induced changes.

On the other hand, there are some problems associated with this spectroscopy. First, the necessity of synchrotron light as a source, which is expensive and not easily available. Second, the use of simulation and best-fit procedures to obtain structural parameters; these methods are time consuming and they sometimes give ambiguous or unreliable results.

Principle

However, if the system under study is properly chosen, EXAFS is able to supply useful and even essential information. The X-ray absorption coefficient for an atom, indicated as χ , is directly proportional to the probability of absorption of one photon and is a monotone decreasing function of energy. It shows several discontinuities known as absorption edges: they occur when the energy of the incident photons equals the binding energy of one electron of the atom and are classified with capital letters (K, L, M...) according to the principal quantum number of the electron in the ground state ($n = 1, 2, 3...$).



An incident photon is able to extract a core electron if its energy is equal to or greater than the edge energy. The ejected electron is called photoelectron and it has the characteristics of both a particle and a wave. Its kinetic energy is given by:

$$E = E_X - E_0$$

where E_X is the energy of the X-ray photon and E_0 the energy of the edge.

The structure of the XAS spectrum

When the X-ray photon energy reaches the ionisation energy of an inner shell atom, the absorption rapidly increases creating the ascending part of the absorption edge peak. After the maximum the decrease of the absorption is not smooth, but larger and smaller wave crests and troughs are super-positioned on the exponentially decreasing signal. This is the so-called XAFS (X-ray Absorption Fine Structure), which can give statistical information about the local

structure around the absorber atoms. The reason for the XAFS is the following. XAFS appear only if there are atoms near the absorbing atom, so in solid and liquid state and in molecular gasses. In case of free atoms as for example noble gasses no XAFS can be observed.

When the X-ray photon with $E > E^0$ is absorbed, its whole energy is transferred to an inner-shell electron, which will jump to unoccupied continuum level with symmetry properties defined by the dipole selection rules. If the configuration interaction is negligible, then the selection rule is $\Delta l = \pm 1$. The absorption edges are denoted according to the shell of the ejected core electron (K , L_I , L_{II} , L_{III}). The ejected photoelectron will have kinetic energy $E - E^0$. The outgoing photoelectron wave can be scattered by the neighbouring atoms, and the XAFS is caused by the interference of the outgoing and backscattered electron wave at the position of the absorber atom.

There is however a difference in the physical phenomenon taking place at low or higher kinetic energy. In the XANES region, where the kinetic energy of the photoelectron is low, multiple scattering of the photoelectron by the neighbouring atoms occurs. This is due to the fact, that the corresponding wave vector of the photoelectron is large enough to be comparable to the inter-atomic distances between the absorber and its neighbours, and the scattering amplitude is large. Due to the multiple-scattering process, XANES probes the 3 and more-body correlations and can give information not just about bond lengths (or inter-atomic distances), but about the bond angles, too.

In the EXAFS region the kinetic energy of the photoelectron is higher and consequently the wave length and the scattering amplitude is smaller, so mainly single scattering of the photoelectron by the neighbouring atoms takes place.

The scattering amplitude and phase shift caused by the backscatterer depends on of the neighbour's type, and the phase and the amplitude of the backscattered wave depends on the inter-atomic distance between the absorber and the backscatterer as well.

After the ejection of the photoelectron the absorber atom will be in an excited state due to the core hole. Relaxation can occur when an electron occupying a higher energy level jump down into the core hole. The energy difference between the levels is either emitted as a

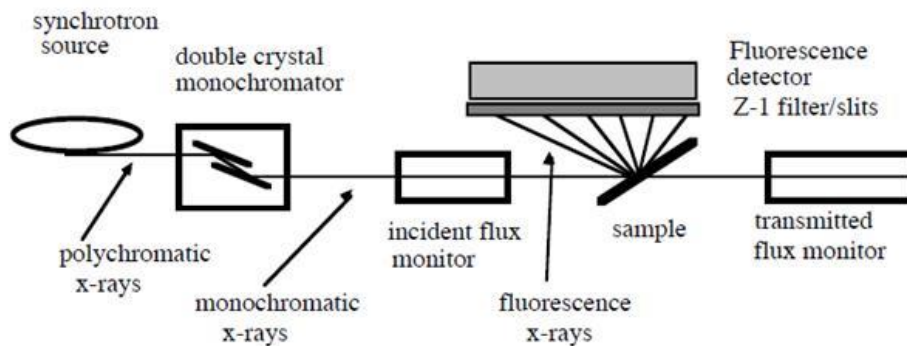
fluorescence photon (only rarely occur), or absorbed by another higher-level electron, which is emitted from the atom (Auger-electron).

The wave vector of the photoelectron (k) can be calculated from the X-ray photon energy, E and the ionization energy, E^0 by:

$$k = \sqrt{\frac{2m}{\hbar^2}(E - E^0)}$$

Variations of the XAFS spectroscopy are the following:

- **SEXAFS** (surface extended X-ray absorption fine structure) for the study of surfaces. It finds applications in the study of adsorption of atoms on surfaces, initial stages of epitaxial growth, catalysis, oxidation etc.
- **XMCD** (X-ray magnetic circular dichroism) for the study of magnetic materials. XMCD uses circularly polarized SR light and is based on the detection of differences in the absorption of right and left polarized light.
- **Quick EXAFS** for the study of dynamic systems. It requires the use of energy dispersive monochromators and CCD cameras which permit fast spectrum acquisition (in few msec).
- **μ -XAFS** which uses an SR beam of very small dimensions (nm to mm) and allows the study of inhomogeneous materials and acquisition of spectra from different positions of a sample with spatial compositional variations or distribution of different phases.



Surface-Extended X-ray Absorption Fine Structure (SEXAFS)

Surface-extended X-ray absorption fine structure (SEXAFS) is the surface-sensitive equivalent of the EXAFS technique. This technique involves the illumination of the sample by high-intensity X-ray beams from a synchrotron and monitoring their photo absorption by detecting in the intensity of Auger electrons as a function of the incident photon energy. Surface sensitivity is achieved by the interpretation of data depending on the intensity of the Auger electrons (which have an escape depth of $\sim 1\text{--}2$ nm) instead of looking at the relative absorption of the X-rays as in the parent method, EXAFS.

The photon energies are tuned through the characteristic energy for the onset of core level excitation for surface atoms. The core holes thus created can then be filled by non radiative decay of a higher-lying electron and communication of energy to yet another electron, which can then escape from the surface (Auger emission).

The photo absorption can therefore be monitored by direct detection of these Auger electrons to the total photoelectron yield. The absorption coefficient versus incident photon energy contains oscillations which are due to the interference of the backscattered Auger electrons with the outward propagating waves. The period of this oscillations depends on the type of the backscattering atom and its distance from the central atom. Thus, this technique enables the investigation of interatomic distances for adsorbates and their coordination chemistry.

Principle

The absorption of an X-ray photon by the atom excites a core level electron, thus generating a core hole. This generates a spherical electron wave with the excited atom as the center. The wave propagates outwards and get scattered off from the neighbouring atoms and is turned back towards the central ionized atom. The oscillatory component of the photoabsorption originates from the coupling of this reflected wave to the initial state . The Fourier transform of the oscillations gives the information about the spacing of the neighboring atoms and their chemical environment. This phase information is carried over to the oscillations in the Auger signal because the transition time in Auger emission is of the same order of magnitude as the

average time for a photoelectron in the energy range of interest. Thus, with a proper choice of the absorption edge and characteristic Auger transition, measurement of the variation of the intensity in a particular Auger line as a function of incident photon energy would be a measure of the photoabsorption cross section.

This excitation also triggers various decay mechanisms. These can be of radiative (fluorescence) or nonradiative (Auger and Coster–Kronig) nature. The intensity ratio between the Auger electron and X-ray emissions depends on the atomic number Z . The yield of the Auger electrons decreases with increasing Z .

ATOMIC ABSORPTION SPECTROSCOPY

Introduction and Principle

The first observation of an atomic absorption spectra was made by **Wollaston**, who in **1802**, described the dark lines in the solar system, but the technique was introduced for analytical purpose by **Walsh and by Alkemade and Milatz (1956)** under the designation **atomic absorption spectroscopy**.

Atomic absorption spectroscopy is an absorption method where radiation is absorbed by non-excited atoms in the vapour state. This technique involves the study of the absorption of radiation (usually in the ultraviolet visible region) by neutral atoms in the gaseous state. Thus in **atomic absorption spectroscopy**, the sample is first converted into an atomic vapour and then the absorption of atomic vapour is measured at a selected wavelength which is characteristic of each individual element. The measured absorbance is proportional to the concentration and analyses are made by comparing this absorbance with that given under the same experimental conditions by reference samples of known composition.

In other words, the procedure is based on flame absorption rather than flame emission and the basis of the method is the measurement of the light absorbed at the wavelength of a resonance line by the un excited atoms of the element.

Almost all analytical applications of the atomic absorption method at the present time involve spraying a solution of the sample into the flame, for this reason, the technique is also

known as **absorption flame photometry**. With the types of flame commonly used, the atomic absorption lines are very narrow (usually less than 0.05Å) and so it is extremely difficult to design a monochromator that is capable of isolating a sufficiently narrow band from the spectrum of a light source emitting a continuum. All commercially available atomic absorption spectrophotometers overcome this difficulty by using light sources which emit atomic spectral lines of the element to be determined under conditions which ensure that the lines in the spectrum are narrow, compared with the absorption line to be measured. With this arrangement peak absorptions can be measured and monochromator is only required to isolate the line to be measured from all other lines in the spectrum of the light source.

PROCEDURE

In an atomic absorption analysis the element being determined must be reduced to the element state, vapourised and imposed in the beam of radiation from the source. This process is frequently accomplished by drawing a solution of the sample as a fine mist, into a suitable flame. The flame thus serves as a function analogous to that of the cell and solution in conventional spectroscopy. **The absorption spectrum of an element in its gaseous atomic form consists of a series of well defined extremely narrow lines arising from electronic transition of the outer most electrons.**

The atomic absorption spectrophotometer is normally arranged so that the radiation from the **hollow cathode source** is focused by a lens to a slightly reduced image at the centre of a long (10cm) flame into which the solution to be measured is sprayed. A second lens then re-images the images into the entrance slit of the monochromator. **In the case of a double beam instrument**, the light from the source is split into two beams which are recombined before entering the monochromator. When an analysis is to be carried out, the source (hollow cathode lamp or other sharp line source) is placed in position and then warmed up for five to ten minutes. The choice of lamp current depends upon the following factors.

- (1) Age and quality of the source.
- (2) Sensitivity of the photomultiplier detector.
- (3) Slit width of the monochromator.
- (4) Inherent intensity and stability of the resonance line used.

In general, in order to obtain maximum sensitivity and a linear calibration curve, minimum hollow cathode current should be used consistent with adequate single intensity and stability.

In commercial instruments, provision is made for an **air-coal gas, air-propane, or air-acetylene flame**. The choice of suitable flame depends upon the following factors.

- (1) Metal to be determined.
- (2) The presence of the other materials.

The position and composition of the flame are adjusted to give maximum absorption, while a solution containing known concentration of the metal to be determined is sprayed on to the flame.

A complete analytical procedure is as follows- Allow the source lamp for the required element to warm up. Select the monochromator wavelength and slit width to resonance peak. With all light cut off from the detector adjust the read out to zero transmission, and with reference to zero concentration solution entering the flame, adjust to 100. Now run standard samples made up synthetically from a pure salt of the element being determined. Plot a graph between concentration and absorption, then run samples and read their analyses from the calibration graph.

The plot of concentration against absorbance is almost a straight line, but this line always curves slightly towards the concentration for various reasons,

Since the wavelengths of the particular element can be determined by exciting light, high selectivity is obtained. Moreover, since most of the atoms in the flame are present in the ground state and thus ready to interact with exciting beam, the technique is more sensitive than emission flame photometry.

Applications

Atomic absorption spectroscopy can be used to determine some sixty metals. The only limitation on the type of sample is that it must be capable of giving a solution of the metal concerned in either an aqueous or an organic solvent. W. Salvin and C.D.Manning (1963), C.L. Chakrabarti, G.R. Lyles and F.B. Dowling (1963) and V.A. Fassel and V.G. Mossotti (1963) have done preliminary work on metals forming oxides,

eg, Be, Al, Ti, V, Ta and the rare earths. They have shown that these metals required highly reducing oxyacetylene flame.

Analytical Applications

(i) Metallurgical and inorganic

Atomic absorption which is essentially a method of determining metals, has also been used widely in the fields of metallurgy and general inorganic analysis. Light alloys are almost completely analysed by this method. In aluminium, residual and alloying concentrations of magnesium, copper, zinc, lead, manganese, nickel are easily determined.

Atomic absorption is also used as an ideal method for the analysis of many ores and minerals.

Rowling, Amos and Greaves (1962) have analysed ores and concentrates for the determination of Ag, Cu, Co, Fe, Ni, Pb and Zn.

(ii) Bio Chemical analysis

A number of elements can also be determined in biological samples by using atomic absorption method. Willis, Cooke and Price have determined Na and K in biological fluids. They have shown that depressive effect of Phosphate and protein in samples of blood serum, cerebrospinal fluid and urine is overcome by adding a single diluents in each case.

(iii) Pollution Analysis

Atomic absorption technology is used in analyzing pollution samples. Council of British Archeology prepared a list of historic towns in England, Scotland and Wales for this purpose and found atmospheric lead contaminated by automobiles. Samples of air were passed through Whatmann. 1 filter paper to remove the particulate matter from the air. The filter paper was treated with 10% HNO₃, filtered to remove the insoluble and diluted. The concentration of lead was then determined by AAS using standards which were treated similarly. The results showed that heavily travelled roads maintained a much higher concentration of lead than the lesser travelled roads.

A large number of toxic metals present in water bodies as pollutants have been analysed by making use of AAS.

(iv) Miscellaneous Analyses

In agriculture, soils, extracts, plant materials and fertilizers have been analysed for the determination of Ca, Cu, Fe, K, Mg, Mn etc.

In oil analysis, crude oil, feedstocks and lubricating oils have been analysed for the determination of Ag, Ba, Ca, Cr and Pb.

Metallic impurities in petrol and refined oils can also be determined in petrol and oil industries. Coal ashes and boiler deposits have been analysed for the determination of Na, Mg, Sr, Ca, Fe and K. Electroplating solutions have also been analysed for Cd, Cu, Fe, Ni and Zn.

Advantages

- (1) The most important advantage of this technique is its high degree of freedom from interference from its environment, i.e. by the presence of other elements. Minute amount of an element can be determined in the presence of high concentration of other element.
- (2) It is more sensitive than emission flame photometry. More elements can be quantitatively determined by this technique.
- (3) This technique is quite independent of flame temperature.
- (4) It is rapid and requires only small amount of materials.
- (5) It is highly specific, because the atoms of a particular element can only absorb radiation of their own characteristic wavelength.

Disadvantages and Limitations

- (1) The main disadvantage of this method is the need of a separate lamp source for each element to be determined.
- (2) Elements such as Al, Ti, W, Mo etc. cannot be detected, when a flame is used to reduce the atomic state. This is due to the fact that these elements give rise to oxides in the flame.
- (3) The use of this technique is limited to metals only.

- (4) If aqueous solutions are used, the predominant anion affects the signal to a noticeable degree.

FLAME EMISSION SPECTROSCOPY

Introduction

The quantitative separation and determination of the alkali and alkaline earth metals by wet chemical tests has always been a difficult analytical problem. Emission spectroscopy, which deals with the examination of the energy emitted from a substance, is an obvious instrumental approach for the determination of these elements. It is a well known fact that a characteristic yellow light is emitted when a small amount of sodium is introduced into a flame of a Bunsen burner and the brightness of the flame varies with the amount of sodium or other metal introduced. Emission of such characteristic radiation by sodium or other metal and the correlation of emission intensity with the concentration of the element form the basis of **flame photometry**, which is actually a part of the **emission spectroscopy**.

The sample is dissolved in water or in an organic solvent, introduced into the flame by means of an atomizer under controlled conditions. The radiation from the flame enters a dispersing device in order to isolate the desired region of the spectrum. The intensity of isolated radiation can be measured by a photo tube and electronic amplifier. After carefully calibrating the photometer with solution of known composition and concentration, it is possible to correlate the intensity of a given spectral line of the unknown with the amount of an element present that emits the particular radiation. Atomization of solution permits the distribution of the sample or a portion is introduced in the flame. The most important applications of flame photometry have been to the analysis of sodium and potassium, particularly in biological fluids and tissues.

Principle

Flame photometry or Flame emission spectroscopy is a special area of emission spectroscopy in which a flame is used to excite the atoms. In this technique, excitation is brought about by spraying a solution of the sample into a hot flame. The basic principle of Flame emission spectroscopy rests on the fact that salts of metals when introduced under carefully controlled conditions into a suitable flame, are vapourised and excited to emit radiations that are

characteristic of each element. A series of processes occur when a solution containing an ion is nebulized through a flame.

- (a) The solvent gets vapourised leaving particles of salt.
- (b) The salt is subsequently vapourised by the flame.
- (c) Some of the atoms are vapourised by the flame.
- (d) The excited atoms emit radiation characteristic of their species.

Applications

- Flame photometry has been applied to the analysis of wide variety of materials, including biological fluids, soils, plant materials, cements, glasses and natural waters.
- The determination of lithium, Na, K, Cs, Rb, Ca, etc. have already been carried out by using flame photometry. These elements may be grouped as under,
 - 1. Elements commonly determined - Al, Ba, B, Ca, etc
 - 2. Elements easily determined but more or less neglected – Sb, Bi, Cd, Co etc.
 - 3. Elements with distinctive but less sensitive to flame – Be, Ge, Au, Mo, Hg, Nb etc.
 - 4. Elements determined by indirect means – Br, Cl, F, I, P etc.

Exercise

- 1. State the principle of EXAFS, AAS and FES.
- 2. Discuss the instrumentation of AAS.
- 3. List the applications of EXAFS.

UNIT-II

DATA AND ERROR ANALYSIS

OBJECTIVES

- 1. To identify the types of errors**
- 2. To know the different methods to minimize error**
- 3. To know the difference between precision and accuracy**
- 4. To have understanding of different statistical test.**
- 5. To learn the theoretical concepts of ANOVA**
- 6. To learn the applications of ANOVA**

Types of Errors in Experimental Data

Chemical analyses are affected by at least two types of errors. One type, Called indeterminate (or random) error, causes data to be scattered more or less symmetrically around a mean value. The precision of the data reflects the indeterminate errors in an analysis.

A second type of error, called determinate (or systematic) error, causes the mean of a set of data to differ from the accepted value.

A third type of error is gross error. Gross errors differ from indeterminate and determinate errors in that they usually occur only occasionally, are often large, and may cause a result to be either high or low. Gross errors lead to outliers-results that differ markedly from all other data in a set of replicate measurements.

DETERMINATE ERRORS

Determinate errors have a definite source that usually can be identified. They cause all the results from replicate measurements to be either high or low. Because they are unidirectional, determinate errors are also called systematic errors.

A probable source of this systematic error can be traced to the chemical nature of the sample, nicotinic acid. The analytical method used involves decomposition of the samples with hot concentrated sulfuric acid, which converts the nitrogen to ammonium sulfate. The amount of

ammonia in the ammonium sulfate is then determined in the measurement step. Experiments have shown that compounds containing a pyridine ring (such as nicotinic acid) are incompletely decomposed by sulfuric acid unless special precautions are taken. Without these precautions, low results are obtained.

SOURCES OF DETERMINATE ERRORS

Instrumental Errors

All measuring devices are sources of determinate errors. For example, pipettes, burettes, and volumetric flasks may have volumes slightly different from those indicated by their graduations. These differences may result from using glassware at a temperature that differs significantly from the calibration temperature, from distortions in container walls due to heating while drying, from errors in the original calibration, or from contaminants on the inner surfaces of the containers. Calibration eliminates most determinate errors of this type.

Method Errors

The nonideal chemical or physical behavior of the reagents and reactions upon which an analysis is based can introduce determinate method errors. Such sources of nonideality include the slowness of some reactions, the incompleteness of others, the instability of some species, the nonspecificity of most reagents, and the possible occurrence of side reactions that interfere with the measurement process.

Personal Errors

Many measurements require personal judgments. Examples include estimation the position of a pointer between two scale divisions, the color of a solution at the end point in a titration, or the level of a liquid with respect to a graduation in a pipet or burette. Judgments of this type are often subject to systematic, unidirectional errors. For example, one person may read a pointer consistently high, another may be slightly slow in activation a timer, and a third may be less sensitive to color changes. An analyst who is insensitive to color changes tends to use excess reagent in a volumetric analysis. Physical handicaps are often sources of personal determinate errors.

PRECISION

Precision describes the agreement between two or more measurements that have been made in exactly the same way. There are several ways to express precision.

Standard Deviation (s)

The standard deviation is a statistical term scientists and engineer use as a measure of precision. For small sets of data, we calculate the sample standard deviation s using the following equation:

$$S = \sqrt{\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N - 1}}$$

Where $x_i - \bar{x}$ is the deviation from the mean of the i th measurement.

Variance (S^2)

The variance is simply the squared of the standard deviation:

$$S^2 = \frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N - 1}$$

ACCURACY

Accuracy indicates the closeness of a measurement to its true or accepted value and is expressed by the error. Accuracy measurement agreement between a result and its true value. Precision describes the agreement among several results measured in the same way. Precision is determined by simply replicating a measurement. In contrast, accuracy can never be determined exactly because the true value of a quantity can never be known exactly. An accepted value is used instead.

THE SIGNIFICANT FIGURE

A simple way of indicating the probable uncertainty associated with an experimental measurement is to round the result so that it contains only significant figures. By definition, the significant figures in a number are all of the certain digits and the first uncertain digit. For example, when you read a 50-mL burette that has graduations every 0.1 mL, you can easily tell that the liquid level is greater than 30.2 mL and less than 30.3 mL.

A zero may or may not be significant, depending upon its location in a number. A zero that is surrounded by other digits is always significant (such as in 30.26 mL) because it is read directly and with certainty from a scale or instrument readout. On the other hand, zeros that are only located to the left of the decimal point are not significant. If we write 30.26 mL as 0.03026 L, the number of significant figures remains the same. The only function of the zero before the 3 is to locate the decimal point, so this zero is not significant. Terminal zeros may or may not be significant. For example, if the volume of a beaker is expressed as 2.0 L, the presence of the 0 tells us that the volume is known to a few tenths of a liter, and so, both the 2 and the 0 are significant figures. If this same volume is reported as 2000 mL, the situation becomes confused. The last two zeros are not significant if the uncertainty is still a few tenths of a liter or a few hundred milliliters. In order to follow the significant figure convention here, use scientific notation and report the volume as 2.0×10^3 mL.

THE POPULATION AND THE SAMPLE

In order to use statistics to treat our data, we must assume that a few replicate experimental results gathered in the laboratory are a tiny but representative fraction of an infinite number of results that could be collected if we had infinite time. Statisticians call this small set of data that sample and view it as a subset of a population, or a universe, of data that principle exists.

The laws of statistics apply strictly to a population of data only. To use these laws, we must assume that the handful of data that make up the typical sample truly represents the infinite population of results. Unfortunately, there is no guarantee that this assumption is valid. As a result, statistical estimates about the magnitude of indeterminate errors are themselves subject to uncertainty and therefore can only be made in terms of probabilities.

The Population Mean (μ) and the Sample Mean ($\bar{x}$)

We will find it useful to differentiate between the sample mean and the population mean. The sample mean is the mean of a limited sample drawn from a population of data.

The Sample Standard Deviation (s) and the Population Standard Deviation (σ)

We must also differentiate between the sample standard deviation and the population standard deviation. The sample standard deviation as was defined in Equation 2-2; that is,

$$s = \sqrt{\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N - 1}}$$

Sample mean = $\bar{x}$, where

$$\bar{x} = \frac{\sum_{i=1}^N x_i}{N}$$

when N is small.

Population mean = μ , where $\mu = \bar{x}$ when $N \rightarrow \infty$.

$$\mu = \frac{\sum_{i=1}^N x_i}{N}$$

CONFIDENCE LIMITS

The exact value of the mean μ for a population of data can never be determined exactly because such a determination requires an infinite number of measurements. Statistical theory allows us to set limits around an experimentally determined mean ($\bar{x}$), however, and the true mean μ lies within these limits with a given degree of probability. These limits are called confidence limits, and the interval they define is known as the confidence interval.

The size of the confidence interval, which is derived from the sample standard deviation, depends on the certainty with which s is known. If there is reason to believe that s is a good approximation of σ , then the confidence interval can be significantly narrower than if the estimate of s is based upon only two or three measurements.

The size of the confidence interval, which is derived from the sample standard deviation, depends on the certainty with which s is known. If there is reason to believe that s is a good approximation of σ , then the confidence interval can be significantly narrower than if the estimate of s is based upon only two or three measurements.

REJECTION OF OUTLIERS

When a set of data contains an outlying result that appears to differ excessively from the average, a decision must be made whether to retain or reject the result. The choice of criterion for the rejection of a suspected result has its perils. If we set a stringent standard that makes the rejection of a questionable measurement difficult, we run the risk of retaining results that are spurious and have an inordinate effect on the average of the data. If we set lenient limits on precision and thereby make the rejection of a result easy, we are likely to discard measurements that rightfully belong in the set and thus introduce a bias to the data. It is an unfortunate fact that no universal rule can be invoked to settle the question of retention or rejection.

Statistical Tests

Several statistical procedures such as the Q test and T_n test have been developed to provide criteria for rejection or retention of outliers. Such tests assume that the distribution of the population data is normal, or Gaussian. Unfortunately, this condition cannot be proved or disproved for samples consisting of many fewer than 50 results. Consequently, statistical rules, which are perfectly reliable for normal distributions of data, should be used with extreme caution when applied to samples containing only a few data.

The Q Test

The Q test is a simple, widely used statistical test. In this test the absolute value of the difference between the questionable result x_q and its nearest neighbor x_n is divided by the spread w of the entire set to give the quantity Q_{exp} :

$$Q_{\text{exp}} = \left| \frac{x_q - x_n}{w} \right|$$

This ratio is then compared with rejection values Q_{crit} found in Standard Table . If Q_{exp} is greater than Q_{crit} , the questionable result can be rejected with the indicated degree of

$$\frac{|x_q - \bar{x}|}{s}$$

confidence.

The Tn Test, the American Society for Testing Materials (ASTM) T_n test, the quantity T_n serves as the rejection criterion.

Here, x_q is the questionable result, and $\bar{x}$ and s are the mean and standard deviations of the entire set including the questionable result.

STATISTICAL AIDS TO HYPOTHESIS TESTING

Much of scientific and engineering endeavor is based upon hypothesis testing. Thus, in order to explain an observation, a hypothetical model is advanced and tested experimentally to determine its validity. If the results from these experiments do not support the model, we reject it and seek a new hypothesis. If agreement is found, the hypothetical model serves as the basis for further experiments. When the hypothesis is supported by sufficient experimental data, it becomes recognized as a useful theory until such time as data are obtained that refute it.

Experimental result seldom agree exactly with those predicted from a theoretical mode. Consequently, scientists and engineers frequently must judge whether a numerical difference is a manifestation of the indeterminate errors inevitable in all measurements. Certain statistical tests are useful in sharpening these judgments.

Tests of this kind make use of a null hypothesis, which assumes that the numerical quantities being compared are, in fact, the same. The probability of the observed differences

appearing as a result of indeterminate error is then computed from statistical theory. Usually, if the observed difference is greater than or equal to the difference that would occur 5 times in 100 (the 5% probability level), the null hypothesis is considered questionable and the difference is judged to be significant. Other probability levels, such as 1 in 100 or 10 in 100, may also be adopted, depending upon certainty desired in the judgment.

The kinds of testing that chemists use most often include the comparison of (1) the mean from an analysis x with what is believed to be the true value ; (2) the means x_1 and x_2 from two sets of analyses; (3) the standard deviations s_1 and s_2 or σ_1 and σ_2 from two sets of measurement; and (4) the standard deviation s of a small set of data with the standard deviation of the methods for making these comparison.

Hypothesis Testing - Analysis of Variance (ANOVA)

Introduction

This module will continue the discussion of hypothesis testing, where a specific statement or hypothesis is generated about a population parameter, and sample statistics are used to assess the likelihood that the hypothesis is true. The hypothesis is based on available information and the investigator's belief about the population parameters. The specific test considered here is called analysis of variance (ANOVA) and is a test of hypothesis that is appropriate to compare means of a continuous variable in two or more independent comparison groups. For example, in some clinical trials there are more than two comparison groups. In a clinical trial to evaluate a new medication for asthma, investigators might compare an experimental medication to a placebo and to a standard treatment (i.e., a medication currently being used). In an observational study such as the Framingham Heart Study, it might be of interest to compare mean blood pressure or mean cholesterol levels in persons who are underweight, normal weight, overweight and obese.

The technique to test for a difference in more than two independent means is an extension of the two independent samples procedure discussed previously which applies when there are exactly two independent comparison groups. The ANOVA technique applies when there are two or more than two independent groups. The ANOVA procedure is used to compare the means of

the comparison groups and is conducted using the same five step approach used in the scenarios discussed in previous sections. Because there are more than two groups, however, the computation of the test statistic is more involved. The test statistic must take into account the sample sizes, sample means and sample standard deviations in each of the comparison groups.

If one is examining the means observed among, say three groups, it might be tempting to perform three separate group to group comparisons, but this approach is incorrect because each of these comparisons fails to take into account the total data, and it increases the likelihood of incorrectly concluding that there are statistically significant differences, since each comparison adds to the probability of a type I error. Analysis of variance avoids these problems by asking a more global question, i.e., whether there are significant differences among the groups, without addressing differences between any two groups in particular (although there are additional tests that can do this if the analysis of variance indicates that there are differences among the groups).

The fundamental strategy of ANOVA is to systematically examine variability within groups being compared and also examine variability among the groups being compared.

Learning Objectives

1. Perform analysis of variance by hand
2. Appropriately interpret results of analysis of variance tests
3. Distinguish between one and two factor analysis of variance tests
4. Identify the appropriate hypothesis testing procedure based on type of outcome variable and number of samples

The ANOVA Approach

Consider an example with four independent groups and a continuous outcome measure. The independent groups might be defined by a particular characteristic of the participants such as BMI (e.g., underweight, normal weight, overweight, obese) or by the investigator (e.g., randomizing participants to one of four competing treatments, call them A, B, C and D). Suppose that the outcome is systolic blood pressure, and we wish to test whether there

is a statistically significant difference in mean systolic blood pressures among the four groups. The sample data are organized as follows:

	Group 1	Group 2	Group 3	Group 4
Sample Size	n_1	n_2	n_3	n_4
Sample Mean	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$	$\bar{X}_4$
Sample Standard Deviation	s_1	s_2	s_3	s_4

The hypotheses of interest in an ANOVA are as follows:

- $H_0: \mu_1 = \mu_2 = \mu_3 \dots = \mu_k$
- H_1 : Means are not all equal.

where k = the number of independent comparison groups.

In this example, the hypotheses are:

- $H_0: \mu_1 = \mu_2 = \mu_3 = \mu_4$
- H_1 : The means are not all equal.

The null hypothesis in ANOVA is always that there is no difference in means. The research or alternative hypothesis is always that the means are not all equal and is usually written in words rather than in mathematical symbols. The research hypothesis captures any difference in means and includes, for example, the situation where all four means are unequal, where one is different from the other three, where two are different, and so on. The alternative hypothesis, as

shown above, capture all possible situations other than equality of all means specified in the null hypothesis.

Test Statistic for ANOVA

The test statistic for testing $H_0: \mu_1 = \mu_2 = \dots = \mu_k$ is:

$$F = \frac{\sum n_j (\bar{X}_j - \bar{X})^2 / (k-1)}{\sum \sum (X - \bar{X}_j)^2 / (N-k)}$$

and the critical value is found in a table of probability values for the F distribution with (degrees of freedom) $df_1 = k-1$, $df_2 = N-k$. The table can be found in "Other Resources" on the left side of the pages.

In the test statistic, n_j = the sample size in the j^{th} group (e.g., $j = 1, 2, 3$, and 4 when there are 4 comparison groups), $\bar{X}_j$ is the sample mean in the j^{th} group, and $\bar{X}$ is the overall mean. k represents the number of independent groups (in this example, $k=4$), and N represents the total number of observations in the analysis. Note that N does not refer to a population size, but instead to the total sample size in the analysis (the sum of the sample sizes in the comparison groups, e.g., $N=n_1+n_2+n_3+n_4$). The test statistic is complicated because it incorporates all of the sample data. While it is not easy to see the extension, the F statistic shown above is a generalization of the test statistic used for testing the equality of exactly two means.

NOTE: The test statistic F assumes equal variability in the k populations (i.e., the population variances are equal, or $s_1^2 = s_2^2 = \dots = s_k^2$). This means that the outcome is equally variable in each of the comparison populations. This assumption is the same as that assumed for appropriate use of the test statistic to test equality of two independent means. It is possible to assess the likelihood that the assumption of equal variances is true and the test can be conducted in most statistical computing packages. If the variability in the k comparison groups is not similar, then alternative techniques must be used.

The F statistic is computed by taking the ratio of what is called the "between treatment" variability to the "residual or error" variability. This is where the name of the procedure

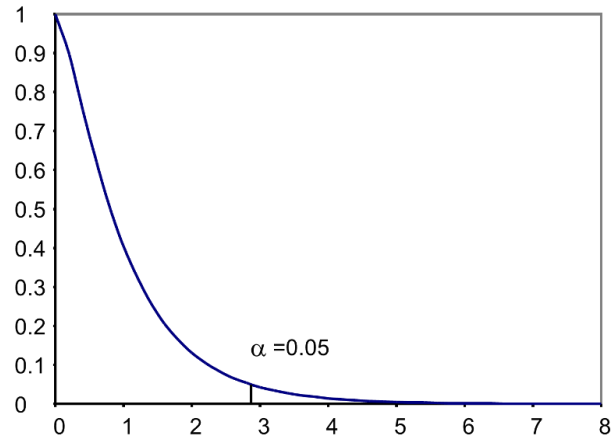
originates. In analysis of variance we are testing for a difference in means (H_0 : means are all equal versus H_1 : means are not all equal) by evaluating variability in the data. The numerator captures between treatment variability (i.e., differences among the sample means) and the denominator contains an estimate of the variability in the outcome. The test statistic is a measure that allows us to assess whether the differences among the sample means (numerator) are more than would be expected by chance if the null hypothesis is true. Recall in the two independent sample test, the test statistic was computed by taking the ratio of the difference in sample means (numerator) to the variability in the outcome (estimated by S_p).

The decision rule for the F test in ANOVA is set up in a similar way to decision rules we established for t tests. The decision rule again depends on the level of significance and the degrees of freedom. The F statistic has two degrees of freedom. These are denoted df_1 and df_2 , and called the numerator and denominator degrees of freedom, respectively. The degrees of freedom are defined as follows:

$$df_1 = k-1 \text{ and } df_2 = N-k,$$

where k is the number of comparison groups and N is the total number of observations in the analysis. If the null hypothesis is true, the between treatment variation (numerator) will not exceed the residual or error variation (denominator) and the F statistic will be small. If the null hypothesis is false, then the F statistic will be large. The rejection region for the F test is always in the upper (right-hand) tail of the distribution as shown below.

Rejection Region for F Test with $\alpha = 0.05$, $df_1 = 3$ and $df_2 = 36$ ($k = 4$, $N = 40$)



For the scenario depicted here, the decision rule is: Reject H_0 if $F \geq 2.87$.

The ANOVA Procedure

We will next illustrate the ANOVA procedure using the five step approach. Because the computation of the test statistic is involved, the computations are often organized in an ANOVA table. The ANOVA table breaks down the components of variation in the data into variation between treatments and error or residual variation. Statistical computing packages also produce ANOVA tables as part of their standard output for ANOVA, and the ANOVA table is set up as follows:

Source of Variation	Sums of Squares (SS)	Degrees of Freedom (df)	Mean Squares (MS)	F
Between Treatments	$SSB = \sum n_j (\bar{X}_j - \bar{X})^2$	$k-1$	$MSB = \frac{SSB}{k-1}$	$F = \frac{MSB}{MSE}$
Error (or Residual)	$SSE = \sum \sum (X - \bar{X}_j)^2$	$N-k$	$MSE = \frac{SSE}{N-k}$	
Total	$SST = \sum \sum (X - \bar{X})^2$	$N-1$		

where

- X = individual observation,
- $\bar{X}_j$ = sample mean of the j^{th} treatment (or group),
- $\bar{X}$ = overall sample mean,
- k = the number of treatments or independent comparison groups, and
- N = total number of observations or total sample size.

The ANOVA table above is organized as follows.

- The first column is entitled "**Source of Variation**" and delineates the between treatment and error or residual variation. The total variation is the sum of the between treatment and error variation.
- The second column is entitled "**Sums of Squares (SS)**". The between treatment sums of squares is

$$SSB = \sum n_j (\bar{X}_j - \bar{X})^2$$

and is computed by summing the squared differences between each treatment (or group) mean and the overall mean. The squared differences are weighted by the sample sizes per group (n_j). The error sums of squares is:

$$SSE = \sum \sum (X - \bar{X}_j)^2$$

and is computed by summing the squared differences between each observation and its group mean (i.e., the squared differences between each observation in group 1 and the group 1 mean, the squared differences between each observation in group 2 and the group 2 mean, and so on). The double summation (SS) indicates summation of the squared differences within each treatment and then summation of these totals across treatments to produce a single value. (This will be illustrated in the following examples). The total sums of squares is:

$$SST = \sum \sum (X - \bar{X})^2$$

and is computed by summing the squared differences between each observation and the overall sample mean. In an ANOVA, data are organized by comparison or treatment groups. If all of the data were pooled into a single sample, SST would reflect the numerator of the sample variance computed on the pooled or total sample. SST does not figure into the F statistic directly. However, $SST = SSB + SSE$, thus if two sums of squares are known, the third can be computed from the other two.

- The third column contains **degrees of freedom**. The between treatment degrees of freedom is $df_1 = k-1$. The error degrees of freedom is $df_2 = N - k$. The total degrees of freedom is $N-1$ (and it is also true that $(k-1) + (N-k) = N-1$).
- The fourth column contains **"Mean Squares (MS)"** which are computed by dividing sums of squares (SS) by degrees of freedom (df), row by row. Specifically, $MSB = SSB/(k-1)$ and $MSE = SSE/(N-k)$. Dividing $SST/(N-1)$ produces the variance of the total sample. The F statistic is in the rightmost column of the ANOVA table and is computed by taking the ratio of MSB/MSE .

Example:

A clinical trial is run to compare weight loss programs and participants are randomly assigned to one of the comparison programs and are counseled on the details of the assigned program. Participants follow the assigned program for 8 weeks. The outcome of interest is weight loss, defined as the difference in weight measured at the start of the study (baseline) and weight measured at the end of the study (8 weeks), measured in pounds.

Three popular weight loss programs are considered. The first is a low calorie diet. The second is a low fat diet and the third is a low carbohydrate diet. For comparison purposes, a fourth group is considered as a control group. Participants in the fourth group are told that they are participating in a study of healthy behaviors with weight loss only one component of interest. The control group is included here to assess the placebo effect (i.e., weight loss due to simply

participating in the study). A total of twenty patients agree to participate in the study and are randomly assigned to one of the four diet groups. Weights are measured at baseline and patients are counseled on the proper implementation of the assigned diet (with the exception of the control group). After 8 weeks, each patient's weight is again measured and the difference in weights is computed by subtracting the 8 week weight from the baseline weight. Positive differences indicate weight losses and negative differences indicate weight gains. For interpretation purposes, we refer to the differences in weights as weight losses and the observed weight losses are shown below.

Low Calorie	Low Fat	Low Carbohydrate	Control
8	2	3	2
9	4	5	2
6	3	4	-1
7	5	2	0
3	1	3	3

Is there a statistically significant difference in the mean weight loss among the four diets? We will run the ANOVA using the five-step approach.

- **Step 1.** Set up hypotheses and determine level of significance

$$H_0: \mu_1 = \mu_2 = \mu_3 = \mu_4 \quad H_1: \text{Means are not all equal} \quad \alpha=0.05$$

- **Step 2.** Select the appropriate test statistic.

The test statistic is the F statistic for ANOVA, $F=MSB/MSE$.

- **Step 3.** Set up decision rule.

The appropriate critical value can be found in a table of probabilities for the F distribution. In order to determine the critical value of F we need degrees of freedom, $df_1=k-1$ and $df_2=N-k$. In

this example, $df_1=k-1=4-1=3$ and $df_2=N-k=20-4=16$. The critical value is 3.24 and the decision rule is as follows: Reject H_0 if $F \geq 3.24$.

- **Step 4.** Compute the test statistic.

To organize our computations we complete the ANOVA table. In order to compute the sums of squares we must first compute the sample means for each group and the overall mean based on the total sample.

	Low Calorie	Low Fat	Low Carbohydrate	Control
n	5	5	5	5
Group mean	6.6	3.0	3.4	1.2

If we pool all $N=20$ observations, the overall mean is $\bar{X} = 3.6$.

We can now compute

$$SSB = \sum n_j (\bar{X}_j - \bar{X})^2$$

So, in this case:

$$SSB = 5 (6.6 - 3.6)^2 + 5 (3.0 - 3.6)^2 + 5 (2.4 - 3.6)^2 + 5 (1.2 - 3.6)^2$$

$$SSB = 45.0 + 0.2 + 28.8 + 1.8 = 75.8$$

Next we compute,

$$SSE = \sum \sum (X - \bar{X}_j)^2$$

SSE requires computing the squared differences between each observation and its group mean.

We will compute SSE in parts. For the participants in the low calorie diet:

Low Calorie	(X - 6.6)	(X - 6.6)²
8	1.4	2.0
9	2.4	5.8
6	-0.6	0.4
7	0.4	0.2
3	-3.6	13.0
Totals	0	21.4

Thus, $\Sigma(X - \bar{X}_1)^2 = 21.4$

For the participants in the low fat diet:

Low Fat	(X - 3.0)	(X - 3.0)²
2	-1.0	1.0
4	1.0	1.0
3	0.0	0.0
5	2.0	4.0
1	-2.0	4.0
Totals	0	10.0

Thus, $\Sigma(X - \bar{X}_2)^2 = 10.0$

For the participants in the low carbohydrate diet:

Low Carbohydrate	(X - 3.4)	(X - 3.4)²
3	-0.4	0.2

5	1.6	2.6
4	0.6	0.4
2	-1.4	2.0
3	-0.4	0.2
Totals	0	5.4

Thus, $\Sigma(X - \bar{X}_3)^2 = 5.4$

For the participants in the control group:

Control	(X - 1.2)	(X - 1.2)²
2	0.8	0.6
2	0.8	0.6
-1	-2.2	4.8
0	-1.2	1.4
3	1.8	3.2
Totals	0	10.6

Thus, $\Sigma(X - \bar{X}_4)^2 = 10.6$

Therefore, $SSE = \Sigma \Sigma(X - \bar{X}_j)^2 = 21.4 + 10.0 + 5.4 + 10.6 = 47.4$

We can now construct the **ANOVA table**.

Source of Variation	Sums of Squares (SS)	Degrees of Freedom (df)	Means Squares (MS)	F
Between Treatment	75.8	4-1=3	75.8/3=25.3	25.3/3.0=8.43
Error (or Residual)	47.4	20-4=16	47.4/16=3.0	
Total	123.2	20-1=19		

- **Step 5.** Conclusion.

We reject H_0 because $8.43 \geq 3.24$. We have statistically significant evidence at $\alpha=0.05$ to show that there is a difference in mean weight loss among the four diets.

ANOVA is a test that provides a global assessment of a statistical difference in more than two independent means. In this example, we find that there is a statistically significant difference in mean weight loss among the four diets considered. In addition to reporting the results of the statistical test of hypothesis (i.e., that there is a statistically significant difference in mean weight losses at $\alpha=0.05$), investigators should also report the observed sample means to facilitate interpretation of the results. In this example, participants in the low calorie diet lost an average of 6.6 pounds over 8 weeks, as compared to 3.0 and 3.4 pounds in the low fat and low carbohydrate groups, respectively. Participants in the control group lost an average of 1.2 pounds which could be called the placebo effect because these participants were not participating in an active arm of the trial specifically targeted for weight loss. Are the observed weight losses clinically meaningful?

Exercise:1

Calcium is an essential mineral that regulates the heart, is important for blood clotting and for building healthy bones. The National Osteoporosis Foundation recommends a daily calcium intake of 1000-1200 mg/day for adult men and women. While calcium is contained in some foods, most adults do not get enough calcium in their diets and take supplements. Unfortunately some of the supplements have side effects such as gastric distress, making them difficult for some patients to take on a regular basis.

A study is designed to test whether there is a difference in mean daily calcium intake in adults with normal bone density, adults with osteopenia (a low bone density which may lead to osteoporosis) and adults with osteoporosis. Adults 60 years of age with normal bone density, osteopenia and osteoporosis are selected at random from hospital records and invited to participate in the study. Each participant's daily calcium intake is measured based on reported food intake and supplements. The data are shown below.

Normal Density	Bone Osteopenia	Osteoporosis
1200	1000	890
1000	1100	650
980	700	1100
900	800	900
750	500	400
800	700	350

Is there a statistically significant difference in mean calcium intake in patients with normal bone density as compared to patients with osteopenia and osteoporosis? We will run the ANOVA using the five-step approach.

- **Step 1.** Set up hypotheses and determine level of significance

$H_0: \mu_1 = \mu_2 = \mu_3$ H_1 : Means are not all equal

$\alpha=0.05$

- **Step 2.** Select the appropriate test statistic.

The test statistic is the F statistic for ANOVA, $F = MSB/MSE$.

- **Step 3.** Set up decision rule.

In order to determine the critical value of F we need degrees of freedom, $df_1 = k - 1$ and $df_2 = N - k$. In this example, $df_1 = k - 1 = 3 - 1 = 2$ and $df_2 = N - k = 18 - 3 = 15$. The critical value is 3.68 and the decision rule is as follows: Reject H_0 if $F \geq 3.68$.

- **Step 4.** Compute the test statistic.

To organize our computations we will complete the ANOVA table. In order to compute the sums of squares we must first compute the sample means for each group and the overall mean.

Normal Density	Bone Density	Osteopenia	Osteoporosis
$n_1 = 6$	$n_2 = 6$	$n_3 = 6$	
$\bar{X}_1 = 938.3$	$\bar{X}_2 = 715.0$	$\bar{X}_3 = 800.0$	

If we pool all $N = 18$ observations, the overall mean is 817.7.

We can now compute:

$$SSB = \sum n_j (\bar{X}_j - \bar{X})^2$$

Substituting:

$$SSB = 6(938.3 - 817.7)^2 + 6(715.0 - 817.7)^2 + 6(800.0 - 817.7)^2$$

Finally,

$$SSB = 87,266.2 + 63,283.7 + 1,879.7 = 152,429.6$$

Next,

$$SSE = \sum \sum (X - \bar{X}_j)^2$$

SSE requires computing the squared differences between each observation and its group mean.

We will compute SSE in parts. For the participants with normal bone density:

Normal Bone Density	(X - 938.3)	(X - 938.3) ²
1200	261.7	68,486.9
1000	61.7	3,806.9
980	41.7	1,738.9
900	-38.3	1,466.9
750	-188.3	35,456.9
800	-138.3	19,126.9
Total	0	130,083.4

Thus, $\sum (X - \bar{X}_1)^2 = 130,083.4$

For participants with osteopenia:

Osteopenia	(X - 715.0)	(X - 715.0)²
1000	285.0	81,225.0
1100	385.0	148,225.0
700	-15	225.0
800	85.0	7,225.0
500	-215.0	46,225.0
700	-15	225.0
Total	0	283,350.0

Thus, $\Sigma(X - \bar{X}_2)^2 = 283,350.0$

For participants with osteoporosis:

Osteoporosis	(X - 715.0)	(X - 715.0)²
890	90	8,100.0
650	-150	22,500.0
1100	300	90,000.0
900	100	10,000.0
400	-400	160,000.0
350	-450	202,500.0
Total	0	493,100.0

Thus, $\Sigma(X - \bar{X}_3)^2 = 493,100.0$

$$SSE = \Sigma(X - \bar{X}_j)^2 = 130,083.4 + 283,350.0 + 493,100.0 = 906,533.4$$

We can now construct the **ANOVA Table**.

Source of Variation	Sums of Squares (SS)	Degrees of freedom (df)	Mean Squares (MS)	F
Between Treatments	152,429.6	2	76,214.8	1.26
Error or Residual	906,533.4	15	60,435.6	
Total	1,058,963.0	17		

- **Step 5.** Conclusion.

We do not reject H_0 because $1.26 < 3.68$. We do not have statistically significant evidence at $\alpha = 0.05$ to show that there is a difference in mean calcium intake in patients with normal bone density as compared to osteopenia and osteoporosis. Are the differences in mean calcium intake clinically meaningful? If so, what might account for the lack of statistical significance?

Two-Factor ANOVA

The ANOVA tests described above are called one-factor ANOVAs. There is one treatment or grouping factor with $k \geq 2$ levels and we wish to compare the means across the different categories of this factor. The factor might represent different diets, different classifications of risk for disease (e.g., osteoporosis), different medical treatments, different age groups, or different racial/ethnic groups. There are situations where it may be of interest to compare means of a continuous outcome across two or more factors. For example, suppose a clinical trial is designed to compare five different treatments for joint pain in patients with osteoarthritis. Investigators might also hypothesize that there are differences in the outcome by sex. This is an example of a two-factor ANOVA where the factors are treatment (with 5 levels) and sex (with 2 levels). In the two-factor ANOVA, investigators can assess whether there are differences in means due to the treatment, by sex or whether there is a difference in outcomes by the combination or interaction of treatment and sex. Higher order ANOVAs are conducted in the same way as one-factor ANOVAs presented here and the computations are again organized in

ANOVA tables with more rows to distinguish the different sources of variation (e.g., between treatments, between men and women). The following example illustrates the approach.

Exercise: 2

Consider the clinical trial outlined above in which three competing treatments for joint pain are compared in terms of their mean time to pain relief in patients with osteoarthritis. Because investigators hypothesize that there may be a difference in time to pain relief in men versus women, they randomly assign 15 participating men to one of the three competing treatments and randomly assign 15 participating women to one of the three competing treatments (i.e., stratified randomization). Participating men and women do not know to which treatment they are assigned. They are instructed to take the assigned medication when they experience joint pain and to record the time, in minutes, until the pain subsides. The data (times to pain relief) are shown below and are organized by the assigned treatment and sex of the participant.

Table of Time to Pain Relief by Treatment and Sex

Treatment	Male	Female
A	12	21
	15	19
	16	18
	17	24
	14	25
B	14	21
	17	20
	19	23
	20	27
	17	25
C	25	37

	27	34
	29	36
	24	26
	22	29

The analysis in two-factor ANOVA is similar to that illustrated above for one-factor ANOVA. The computations are again organized in an ANOVA table, but the total variation is partitioned into that due to the main effect of treatment, the main effect of sex and the interaction effect. The results of the analysis are shown below (and were generated with a statistical computing package - here we focus on interpretation).

ANOVA Table for Two-Factor ANOVA

Source of Variation	Sums of Squares (SS)	Degrees of freedom (df)	Mean Squares (MS)	F	P-Value
Model	967.0	5	193.4	20.7	0.0001
Treatment	651.5	2	325.7	34.8	0.0001
Sex	313.6	1	313.6	33.5	0.0001
Treatment * Sex	1.9	2	0.9	0.1	0.9054
Error or Residual	224.4	24	9.4		
Total	1191.4	29			

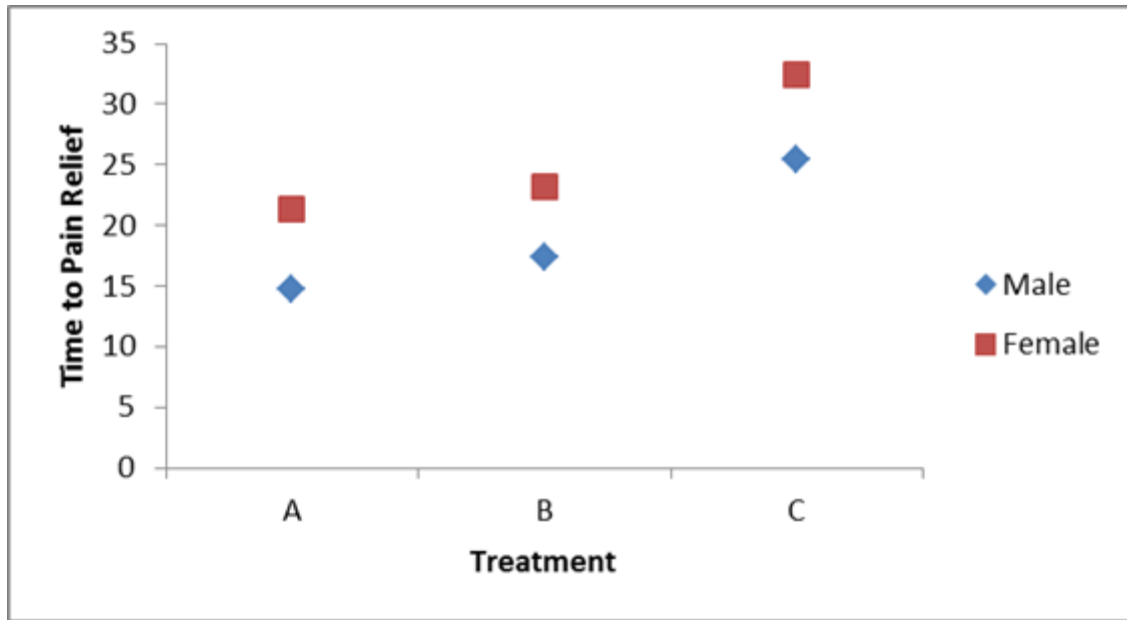
There are 4 statistical tests in the ANOVA table above. The first test is an overall test to assess whether there is a difference among the 6 cell means (cells are defined by treatment and sex). The F statistic is 20.7 and is highly statistically significant with $p=0.0001$. When the overall

test is significant, focus then turns to the factors that may be driving the significance (in this example, treatment, sex or the interaction between the two). The next three statistical tests assess the significance of the main effect of treatment, the main effect of sex and the interaction effect. In this example, there is a highly significant main effect of treatment ($p=0.0001$) and a highly significant main effect of sex ($p=0.0001$). The interaction between the two does not reach statistical significance ($p=0.91$). The table below contains the mean times to pain relief in each of the treatments for men and women (Note that each sample mean is computed on the 5 observations measured under that experimental condition).

Mean Time to Pain Relief by Treatment and Gender

Treatment	Male	Female
A	14.8	21.4
B	17.4	23.2
C	25.4	32.4

Treatment A appears to be the most efficacious treatment for both men and women. The mean times to relief are lower in Treatment A for both men and women and highest in Treatment C for both men and women. Across all treatments, women report longer times to pain relief (See below).



Notice that there is the same pattern of time to pain relief across treatments in both men and women (treatment effect). There is also a sex effect - specifically, time to pain relief is longer in women in every treatment.

Suppose that the same clinical trial is replicated in a second clinical site and the following data are observed.

Table - Time to Pain Relief by Treatment and Sex - Clinical Site 2

Treatment	Male	Female
A	22	21
	25	19
	26	18
	27	24
	24	25
B	14	21
	17	20
	19	23

	20	27
	17	25
C	15	37
	17	34
	19	36
	14	26
	12	29

The ANOVA table for the data measured in clinical site 2 is shown below.

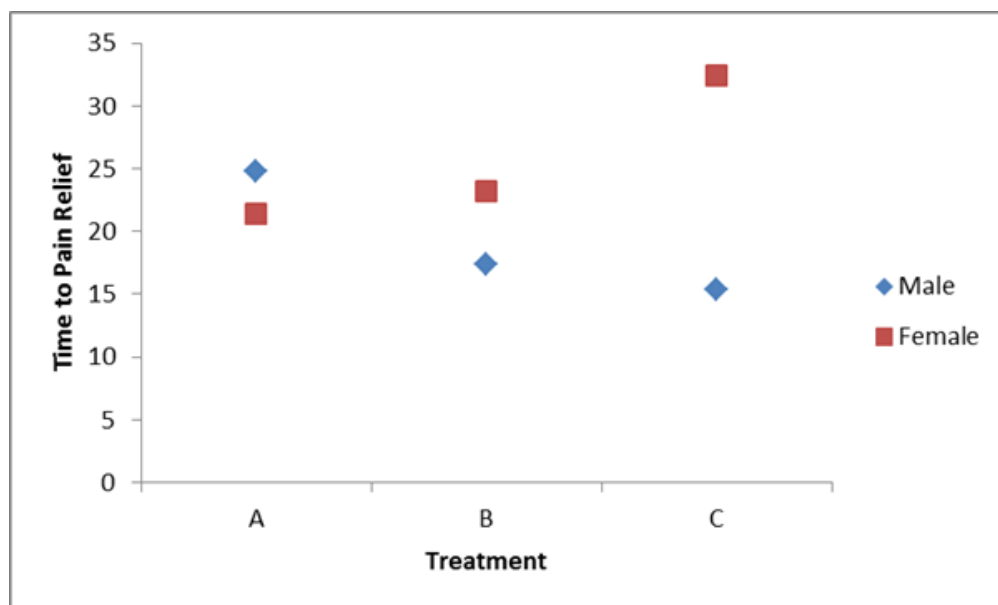
Table - Summary of Two-Factor ANOVA - Clinical Site 2

Source of Variation	Sums of Squares (SS)	Degrees of freedom (df)	Mean Squares (MS)	F	P-Value
Model	907.0	5	181.4	19.4	0.0001
Treatment	71.5	2	35.7	3.8	0.0362
Sex	313.6	1	313.6	33.5	0.0001
Treatment * Sex	521.9	2	260.9	27.9	0.0001
Error or Residual	224.4	24	9.4		
Total	1131.4	29			

Table - Mean Time to Pain Relief by Treatment and Gender - Clinical Site 2

Treatment	Male	Female
A	24.8	21.4
B	17.4	23.2
C	15.4	32.4

Notice that now the differences in mean time to pain relief among the treatments depend on sex. Among men, the mean time to pain relief is highest in Treatment A and lowest in Treatment C. Among women, the reverse is true. This is an interaction effect (see below).



Notice above that the treatment effect varies depending on sex. Thus, we cannot summarize an overall treatment effect (in men, treatment C is best, in women, treatment A is best).

When interaction effects are present, some investigators do not examine main effects (i.e., do not test for treatment effect because the effect of treatment depends on sex). This issue is complex and is discussed in more detail in a later module.

UNIT-III

Chromatography

OBJECTIVES

- To understand the basics of various separation techniques.
- To learn the methods of chromatographic techniques.
- To know the various applications of separation techniques.

Chromatography

Chromatography is a non-destructive procedure for resolving a complex mixture into its individual fractions or compounds.

- Its name is derived from two words: "chromo" meaning colour, and "graphy" meaning writing. In other words, colour bands are formed in the procedure, which are then measured or analysed. These bands are due to separation of individual compounds at different lengths on the column, as seen in column chromatography and on paper in paper chromatography.
- It is a separation procedure, and the separated entities are identified by other analytical techniques like UV-visible, Infrared, NMR (nuclear magnetic resonance), Mass spectroscopy and so forth.
- For a quantitative analysis, measurements of the area under the curve in the chromatogram are taken.
- However, in modern methods like HPLC or gas chromatograph, colour bands can't be seen.

The basic principle of chromatography has advanced a lot to cater to the growing needs of the industry and for research purposes.

Definition

The term 'Chromatography' is defined as the process of separation of the individual components of a mixture based on their relative affinities towards stationary and mobile phases.

Principle

The samples are subjected to flow by mobile liquid onto or through the stable stationary phase. The sample components are separated into fractions based on their relative affinity towards the two phases during their travel.

The component with a greater affinity to the stationary layer travels slower and at a shorter distance, while that with a lesser affinity travels faster and longer.

TYPES

I. Based on the technique employed in separation of components, chromatography is broadly classified as

(i) Adsorption - based

In this, the stationary layer is a solid while the mobile phase is liquid. The compounds travel on the solid surface under the influence of mobile liquid. The separation depends on the extent of physical adsorption to the solid surface.

(ii) Partition - based

In this mode, both the stationary and mobile phase are liquids. So the compounds have an affinity based on their partition coefficients into the individual liquid layer. The one with the greater partition to mobile liquid has a higher affinity to it, so it travels faster, and vice versa.

II. Based on the type of stationary material used for separation, there are two types:

(i) Normal phase: Here, the stationary material is polar in nature and hence the compounds with a higher polarity elute out last while non-polar compounds come out first.

(ii) Reverse phase: Here, the stationary material is non-polar in nature and hence the compounds with a lower polarity elute out last, and vice versa.

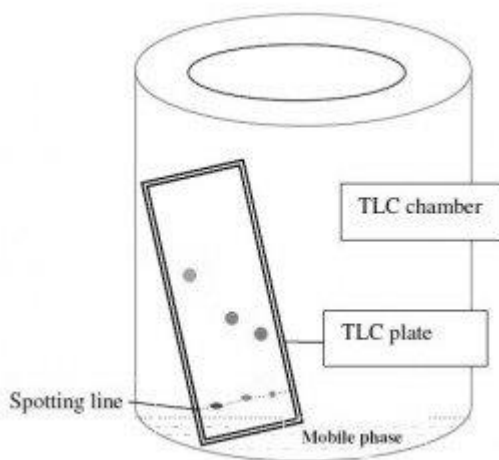
In most HPLC analyses, the type used is the reverse one, as many of the biological, phytochemical compounds, and drugs estimated by HPLC are polar in nature.

III. There are many developments which have occurred over years based on the requirements and also technology employed in evaluation of mixtures.

These techniques can be broadly divided into two. They are **planar** and **columnar** techniques.

(i) Planar technique:

In this type, the stationary phase is a plane surface (two dimensions surface where only length and breadth are taken as area), on which chromatograms are formed.



This method is adopted in techniques like (1) using **paper**, (2) Thin layer chromatography and (3) High performance thin layer chromatography (**HPTLC**).

(ii) Columnar technique:

In this type, there is use of a column wherein on its walls lies a stationary phase, while the mobile phase is flushed through the column.

The techniques which employ this method are: Gas chromatography, **HPLC**, Size exclusion and Ion exchange.

Pros and Cons of Both Techniques:

Both techniques have their pros and their cons.

- Planar techniques have the advantages of faster separation, visualization of formed chromatograms, or spots, and they are less expensive. However they are not useful for preparative purposes.

- Columnar techniques have the advantages of having better or more effective separations of even complex mixtures — possibility to yield to a large amount of compounds by separation of mixtures, i.e in preparative mode. However they have the disadvantage of being expensive, time consuming, and cumbersome (heavy).

PAPER CHROMATOGRAPHY

Paper chromatography is one of the types of chromatography procedures which run on a piece of specialized paper. It is a planar chromatography system wherein a cellulose filter paper acts as a stationary phase on which the separation of compounds occurs.

Principle:

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.

Applications of Paper Chromatography

Paper chromatography is specially used

- for the separation of a mixture having polar and non-polar compounds.
- for separation of amino acids.
- to determine organic compounds, biochemicals in urine, etc.
- for the determination of hormones, drugs, etc. in the pharma sector.

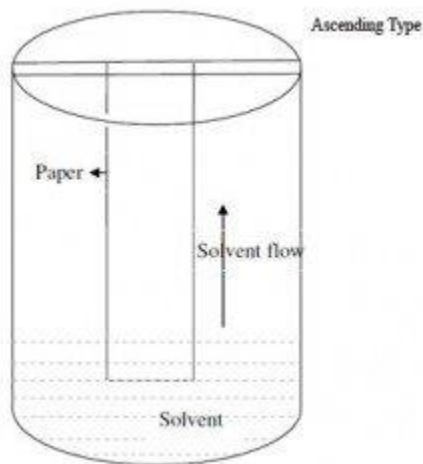
- for evaluation of inorganic compounds like salts and complexes.

Types or Modes of Paper Chromatography

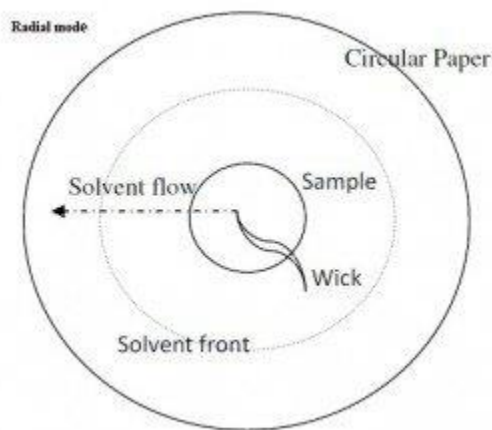
Based on the way the development of chromatogram on paper is done in procedures, we have, broadly, five types of chromatography.

1. Ascending chromatography: As the name indicates, the chromatogram ascends. Here, the development of paper occurs due the solvent movement or upward travel on the paper.

The solvent reservoir is at the bottom of the beaker. The paper tip with sample spots just dips into the solvent at the bottom so that spots remain well above the solvent.



Ascending Paper chromatography



Radial paper chromatography

2. Descending chromatography: Here the development of paper occurs due to solvent travel downwards on the paper. The solvent reservoir is at the top. The movement of solvent is assisted by gravity besides the capillary action.

3. Ascending- descending mode: Here solvent first travels upwards and then downwards on the paper.

4. Radial mode: Here the solvent travels from center (mid-point) towards the periphery of circular chromatography paper. The entire system is kept in a covered petridish for the development of the chromatogram.

The wick at the center of paper dips into mobile phase in a petri dish, by which the solvent drains on to the paper and moves the sample radially to form the sample spots of different compounds as concentric rings.

5. Two-dimensional chromatography: Here the chromatogram development occurs in two directions at right angles. In this mode, the samples are spotted to one corner of rectangular paper and allowed for first development. Then the paper is again immersed in mobile phase at a right angle to the previous development for second chromatogram.

Experimental Procedure:

The experimental method involves the following steps:

a) Selection of suitable type of development: This depends on the complexity of the mixture, solvent, paper, etc. But in general ascending type or radial type chromatography are used as they are easy to perform, handle, less time-consuming and also give chromatogram faster.

b) Selection of suitable filter paper: Filter paper is selected based on pore size, the quality of the sample to be separated, and also mode of development.

c) Preparation of sample: Preparation of sample involves dissolution of sample in suitable solvent used in making mobile phase. *The solvent used should be inert with the sample* under analysis.

d) Spotting of sample on the paper: Samples are to be spotted at proper position on the paper preferably using a capillary tube.

e) Development of chromatogram: Sample spotted paper is subjected to development by immersing it in the mobile phase. The mobile phase moves over the sample on the paper under the capillary action of paper.

f) Drying of the paper and detection of the compounds: Once the development of chromatogram is over, the paper is held carefully at the borders so as to avoid touching the sample spots and dried using an air drier. Sometimes the detecting solution is sprayed in the developed paper and dried to identify the sample chromatogram spots.

THIN LAYER CHROMATOGRAPHY (TLC):

TLC is a type of planar chromatography.

- It is routinely used by researchers in the field of phyto-chemicals, biochemistry and so forth, to identify the components in a compound mixture, like alkaloids, phospholipids, and amino acids.
- It is a semi quantitative method consisting of analysis.
- High performance thin layer chromatography (HPTLC) is the more sophisticated or more precise quantitative version.

Principle

Similar to other chromatographic methods, thin layer chromatography is also based on the principle of separation.

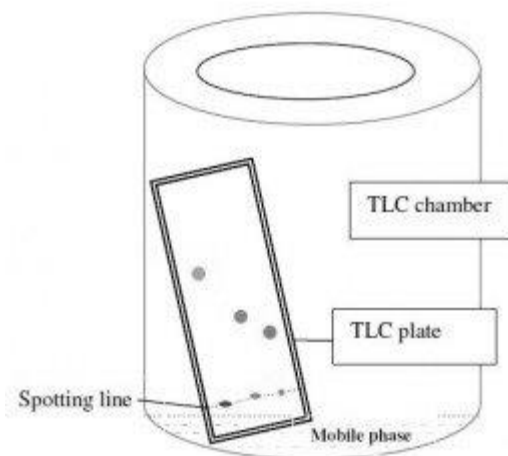
1. The separation depends on the relative affinity of compounds towards stationary and the mobile phase.
2. The compounds under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved.

3. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character are identified by means of suitable detection techniques.

System Components

TLC system components consists of

1. **TLC plates:** Preferably ready made with a stationary phase and these are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
2. **TLC chamber:** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
3. **Mobile phase:** This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.
4. **A filter paper:** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.



Experimental Procedure:

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are preferred.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect this way).
4. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
5. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent — as shown in the picture) for development.
6. Allow sufficient time for the development of spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber, or any other methods as recommended for the said sample.

Advantages

- It is a simple process with a short development time.
- It helps with the visualization of separated compound spots easily.
- The method helps to identify the individual compounds.
- It helps in isolating of most of the compounds.
- The separation process is faster and the selectivity for compounds is higher (even small differences in chemistry is enough for clear separation).
- The purity standards of the given sample can be assessed easily.
- It is a cheaper chromatographic technique.

Applications

1. To check the purity of given samples.
2. Identification of compounds like acids, alcohols, proteins, alkaloids, amines, antibiotics, and more.
3. To evaluate the reaction process by assessment of intermediates, reaction course, and so forth.
4. To purify samples, i.e for the purification process.
5. To keep a check on the performance of other separation processes.

Being a semi quantitative technique, TLC is used more for rapid qualitative measurements than for quantitative purposes. But due its rapidity of results, easy handling and inexpensive procedure, it finds its application as one of the most widely used chromatography techniques.

COLUMN CHROMATOGRAPHY

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid - liquid technique in which the stationary phase is a solid & mobile phase is a liquid. The principle of column chromatography is based on differential adsorption of substance by the adsorbent.

- 1.The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc.
- 2.The selection of solvent is based on the nature of both the solvent and the adsorbent.
- 3.The rate at which the components of a mixture are separated depends on the activity of the adsorbent and polarity of the solvent. If the activity of the adsorbent is very high and polarity of the solvent is very low, then the separation is very slow but gives a good separation. On the other hand, if the activity of adsorbent is low and polarity of the solvent is high the separation is rapid but gives only a poor separation i.e., the components separated are not 100% pure.

4. The adsorbent is made into slurry with a suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc.
5. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and is allowed to pass through the column.
6. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption.
7. The component with greater adsorption power will be adsorbed at the top and the other will be adsorbed at the bottom.
8. The different components can be desorbed and collected separately by adding more solvent at the top and this process is known as *elution*. That is, the process of dissolving out of the components from the adsorbent is called elution and the solvent is called **Eluent**.
9. The weakly adsorbed component will be eluted more rapidly than the other.
10. The different fractions are collected separately. Distillation or evaporation of the solvent from the different fractions gives the pure components.
11. Intermolecular forces, which vary in strength according to their type, make organic molecules to bind to the stationary phase. The stronger the intermolecular force, the stronger the binding to the stationary phase, therefore the longer the compound takes to go through the column.

Intra-molecular hydrogen bonding is present in o-nitrophenol. This is due to the polar nature of the O-H bonds which can result in the formation of hydrogen bonds within the same molecule. But in para-nitro phenol, inter molecular hydrogen bonding (between H and O atoms of two different para-nitro phenol molecules) is possible. As result of inter molecular hydrogen bonding para-nitro phenol undergo association that increases the molecular weight, whereby decreasing volatility.

Procedure:

1. Preparation of the Column:

1. Place the column in a ring stand in a vertical position.
2. A plug of glass wool is pushed down to the bottom of the column.
3. Prepare slurry of silica gel with a suitable solvent & pour gently into the column.
4. Open the stop cock & allow some solvent to drain out. The layer of solvent should always
5. cover the adsorbent; otherwise cracks will develop in the column.

2. Adding the Sample to the Column:

1. Dissolve the sample mixture in a minimum amount of solvent (petroleum ether).
2. Remove the solvent by placing the mixture in a rotary evaporator at a low temperature.
3. Place the dry powder on a piece of weighing paper and transfer it to the top of the column through the funnel.

3. Developing the Chromatogram:

1. Attach a dropping funnel filled with petroleum ether on to the column.
2. Add petroleum ether continuously from the funnel to the top of the column.
3. Open the stopcock carefully.
4. The components of the mixture run down the column forming two separate yellow bands.

4. Recovering the Constituents:

1. Continue running the petroleum ether till both the bands are eluted out separately.
2. Collect the constituents in two different R.B flasks. (o-nitrophenol is obtained first, followed by p-nitro phenol.).
3. Evaporate the solvent by placing the mixture in a rotary evaporator.

Retardation factor

In chromatography, the **retardation factor (R_f)** is the fraction of an analyte in the mobile phase of a chromatographic system. In planar chromatography in particular, the retardation factor **R_f** is defined as the ratio of the distance traveled by the center of a spot to the distance

traveled by the solvent front. Ideally, the values for R_F are equivalent to the R values used in column chromatography.

Although the term **retention factor** is sometimes used synonymously with retardation factor in regard to planar chromatography the term is not defined in this context. However, in column chromatography, the retention factor or **capacity factor (k)** is defined as the ratio of time an analyte is retained in the stationary phase to the time it is retained in the mobile phase, which is inversely proportional to the retardation factor.

$$R_f = \frac{\text{migration distance of the distance}}{\text{migration distance of the solvent front}}$$

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - HPLC

High performance liquid chromatography is a powerful tool in analysis. It is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster.

It also allows us to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

The column and the solvent

In HPLC, there are two variants in use depending on the relative polarity of the solvent and the stationary phase.

(i) Normal phase HPLC

This is essentially just the same as you will already have read about in thin layer chromatography or column chromatography. Although it is described as "normal", it isn't the most commonly

used form of HPLC. The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm. Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.

(ii) Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol.

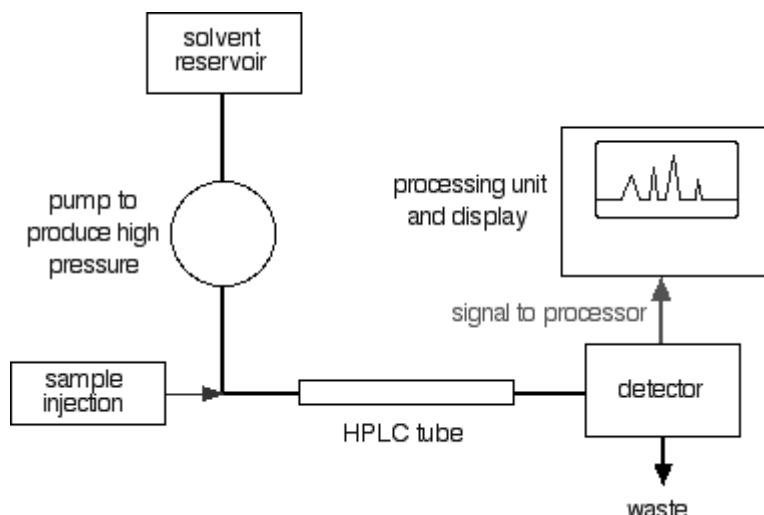
In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column.

That means that now it is the polar molecules that will travel through the column more quickly.

Reversed phase HPLC is the most commonly used form of HPLC.

A flow scheme for HPLC



Injection of the sample

Injection of the sample is entirely automated, and you wouldn't be expected to know how this is done at this introductory level. Because of the pressures involved, it is not the same as in gas chromatography.

Retention time

The time taken for a particular compound to travel through the column to the detector is known as its retention time. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

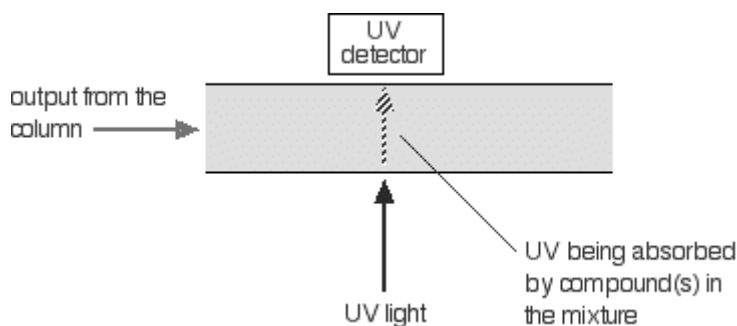
Different compounds have different retention times. For a particular compound, the retention time will vary depending on: the pressure used (because that affects the flow rate of the solvent), the nature of the stationary phase (not only what material it is made of, but also particle size), the exact composition of the solvent, the temperature of the column. That means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds.

The detector

There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses ultra-violet absorption.

Many organic compounds absorb UV light of various wavelengths. If you have a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, you can get a direct reading of how much of the light is absorbed.

The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.



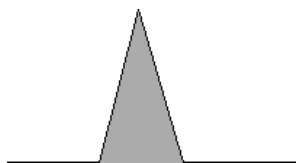
Interpreting the output from the detector

The output will be recorded as a series of peaks - each one representing a compound in the mixture passing through the detector and absorbing UV light. As long as you were careful to control the conditions on the column, you could use the retention times to help to identify the compounds present - provided, of course, that you (or somebody else) had already measured them for pure samples of the various compounds under those identical conditions.

But you can also use the peaks as a way of measuring the quantities of the compounds present. Let's suppose that you are interested in a particular compound, X.

If you injected a solution containing a known amount of pure X into the machine, not only could you record its retention time, but you could also relate the amount of X to the peak that was formed.

The area under the peak is proportional to the amount of X which has passed the detector, and this area can be calculated automatically by the computer linked to the display. The area it would measure is shown in green in the (very simplified) diagram.



If the solution of X was less concentrated, the area under the peak would be less - although the retention time will still be the same. For example:



This means that it is possible to calibrate the machine so that it can be used to find how much of a substance is present - even in very small quantities.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC).

High performance thin layer chromatography (HPTLC) is an enhanced form of thin layer chromatography (TLC). A number of enhancements can be made to the basic method of thin layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements.

Automation is useful to overcome the uncertainty in droplet size and position when the sample is applied to the TLC plate by hand. One recent approach to automation has been the use of piezoelectric devices and inkjet printers for applying the sample.

The spot capacity (analogous to peak capacity in HPLC) can be increased by developing the plate with two different solvents, using two-dimensional chromatography. The procedure begins with development of sample loaded plate with first solvent. After removing it, the plate is rotated 90° and developed with a second solvent.

Solvent extraction

Solvent extraction, also known as **Liquid–liquid extraction** or **partitioning**, is a method to separate a compound based on the solubility of its parts. This is done using two liquids that don't mix, for example water and an organic solvent.

Solvent extraction is used in the processing of perfumes, vegetable oil, or biodiesel. It is also used to recover plutonium from irradiated nuclear fuel, a process which is usually called nuclear reprocessing. The recovered plutonium can then be re-used as nuclear fuel.

In this process one of the components of a mixture dissolves in a particular liquid and the other component is separated as a residue by filtration.

ION EXCHANGE CHROMATOGRAPHY

Ion chromatography (or ion-exchange chromatography) is a chromatography process that separates ions and polar molecules based on their affinity to the ion exchanger.

It works on almost any kind of charged molecule—including large proteins, small nucleotides, and amino acids.

It is often used in protein purification, water analysis, and quality control. The water soluble and charged molecules such as proteins, amino acids, and peptides bind to moieties which are oppositely charged by forming ionic bonds to the insoluble stationary phase.

The equilibrated stationary phase consists of an ionizable functional group where the targeted molecules of a mixture to be separated and quantified can bind while passing through the column. This method applies the idea of the interaction between molecules and the stationary phase which are charged oppositely to each other.

Cation exchange chromatography is used when the desired molecules to separate are cations.

Anion exchange chromatography is used to separate anions, meaning that the beads in the column contain positively charged functional groups to attract the anions.

The bound molecules then can be eluted and collected using an eluent which contains anions and cations by running higher concentration of ions through the column or changing pH of the column.

One of the primary advantages for the use of ion chromatography is only one interaction involved during the separation as opposed to other separation techniques; therefore, ion chromatography may have higher matrix tolerance.

However, there are also disadvantages involved when performing ion-exchange chromatography, such as constant evolution with the technique which leads to the inconsistency from column to column.

Principle:

Ion-exchange chromatography separates molecules based on their respective charged groups. Ion-exchange chromatography retains the analyte molecules on the column based on coulombic (ionic) interactions. Essentially, molecules undergo electrostatic interactions with opposite charges on the stationary phase matrix.

The stationary phase consists of an immobile matrix that contains charged ionizable functional groups or ligands. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. To achieve electro-neutrality, these inert charges couple with exchangeable counter ions in the solution. Ionizable molecules that are to be purified compete with these exchangeable counter ions for binding to the immobilized charges on the stationary phase. These ionizable molecules are retained or eluted based on their charge. Initially, molecules that do not bind or bind weakly to the stationary phase are first to wash away. Altered conditions are needed for the elution of the molecules that bind to the stationary phase.

The concentration of the exchangeable counter ions, which competes with the molecules for binding, can be increased or the pH can be changed. A change in pH changes the charge on the particular molecules and, therefore, alters binding. The molecules then start eluting out based on the changes in their charges from the adjustments. Further such adjustments can be used to release the protein of interest.

Additionally, concentration of counter ions can be gradually varied to separate ionized molecules. This type of elution is called gradient elution. On the other hand, step elution can be used in which the concentration of counter ions are varied in one step. This type of chromatography is further subdivided into cation exchange chromatography and anion-exchange

chromatography. Positively charged molecules bind to anion exchange resins while negatively charged molecules bind to cation exchange resins.

The ionic compound consisting of the cationic species M^+ and the anionic species B^- can be retained by the stationary phase. Cation exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group:

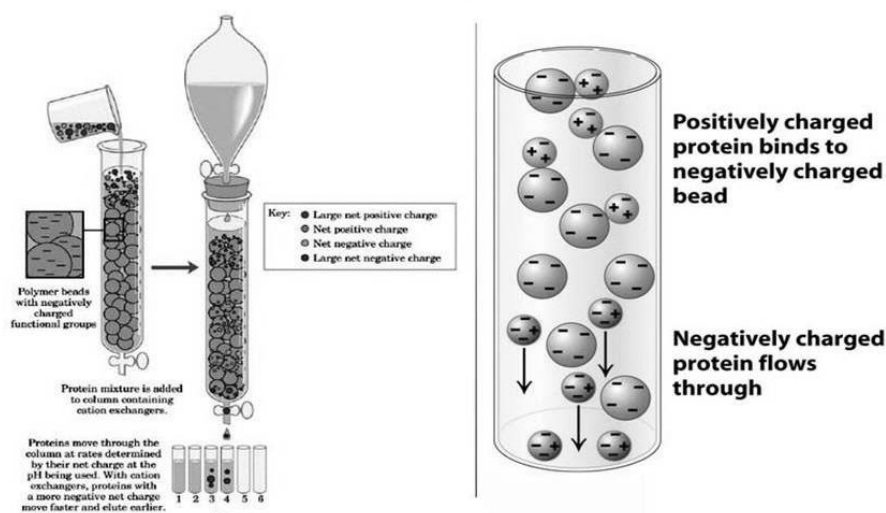


Anion exchange chromatography retains anions using positively charged functional group:



Note that the ion strength of either C^+ or A^- in the mobile phase can be adjusted to shift the equilibrium position, thus retention time. The ion chromatogram shows a typical chromatogram obtained with an anion exchange column.

Procedure :



It is possible to perform ion exchange chromatography in bulk, on thin layers of medium such as glass or plastic plates coated with a layer of the desired stationary phase, or in chromatography columns.

Thin layer chromatography or column chromatography share similarities in that they both act within the same governing principles; there is constant and frequent exchange of molecules as the mobile phase travels along the stationary phase. It is not imperative to add the sample in

minute volumes as the predetermined conditions for the exchange column have been chosen so that there will be strong interaction between the mobile and stationary phases.

Furthermore, the mechanism of the elution process will cause a compartmentalization of the differing molecules based on their respective chemical characteristics. This phenomenon is due to an increase in salt concentrations at or near the top of the column, thereby displacing the molecules at that position, while molecules bound lower are released at a later point when the higher salt concentration reaches that area. These principles are the reasons that ion exchange chromatography is an excellent candidate for initial chromatography steps in a complex purification procedure as it can quickly yield small volumes of target molecules regardless of a greater starting volume.

Chamber (left) contains high salt concentration. Stirred chamber (right) contains low salt concentration. Gradual stirring causes the formation of a salt gradient as salt travel from high to low concentrations. Comparatively simple devices are often used to apply counter ions of increasing gradient to a chromatography column. Counter ions such as Copper (II) are chosen most often for effectively separating peptides and amino acids through complex formation.

A simple device can be used to create a salt gradient. Elution buffer is consistently being drawn from the chamber into the mixing chamber, thereby altering its buffer concentration. Generally, the buffer placed into the chamber is usually of high initial concentration, whereas the buffer placed into the stirred chamber is usually of low concentration. As the high concentration buffer from the left chamber is mixed and drawn into the column, the buffer concentration of the stirred column gradually increases. Altering the shapes of the stirred chamber, as well as of the limit buffer, allows for the production of concave, linear, or convex gradients of counter ion. A multitude of different mediums are used for the stationary phase. Among the most common immobilized charged groups used are trimethylaminoethyl (TAM), triethylaminoethyl (TEAE), diethyl-2- hydroxypropylaminoethyl (QAE), aminoethyl (AE), diethylaminoethyl (DEAE), sulpho (S), sulphomethyl (SM), sulphopropyl (SP), carboxy (C), and carboxymethyl (CM). Successful column packing is an important aspect of ion chromatography. Stability and efficiency of a final column depends on packing methods, solvent used, and factors that affect mechanical properties of the column. In contract to early inefficient dry- packing methods, wet

slurry packing, in which particles that are suspended in an appropriate solvent are delivered into a column under pressure, shows significant improvement.

Three different approaches can be employed in performing wet slurry packing: the balanced density method (solvent's density is about that of porous silica particles), the high viscosity method (a solvent of high viscosity is used), and the low viscosity slurry method (performed with low viscosity solvents).

Polystyrene is used as a medium for ion-exchange. It is made from the polymerization of styrene with the use of divinylbenzene and benzoyl peroxide. Such exchangers form hydrophobic interactions with proteins which can be irreversible. Due to this property, polystyrene ion exchangers are not suitable for protein separation. They are used on the other hand for the separation of small molecules in amino acid separation and removal of salt from water.

Polystyrene ion exchangers with large pores can be used for the separation of protein but must be coated with a hydrophilic substance. Cellulose based medium can be used for the separation of large molecules as they contain large pores. Protein binding in this medium is high and has low hydrophobic character.

DEAE is an anion exchange matrix that is produced from a positive side group of diethylaminoethyl bound to cellulose or Sephadex. Agarose gel based medium contain large pores as well but their substitution ability is lower in comparison to dextrans. The ability of the medium to swell in liquid is based on the cross-linking of these substances, the pH and the ion concentrations of the buffers used. Incorporation of high temperature and pressure allows a significant increase in the efficiency of ion chromatography, along with a decrease in time.

Temperature has an influence of selectivity due to its effects on retention properties. The retention factor ($k = (tR_g - tMg) / (tMg - t_{ext})$) increases with temperature for small ions, and the opposite trend is observed for larger ions. Despite ion selectivity in different mediums, further research is being done to perform ion exchange chromatography through the range of 40-175°C. An appropriate solvent can be chosen based on observations of how column particles behave in a solvent. Using an optical microscope, one can easily distinguish a desirable dispersed state of slurry from aggregated particles.

GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample.

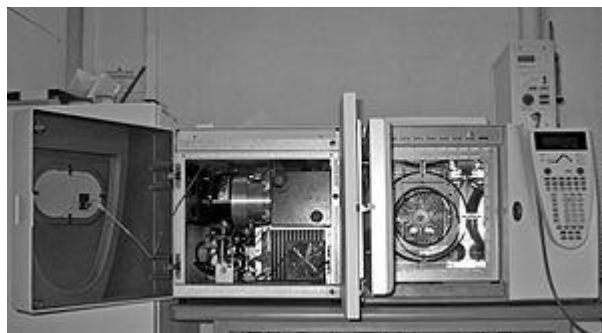
Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples, including that of material samples obtained from planet Mars during probe missions as early as the 1970s.

GC-MS can also be used in airport security to detect substances in luggage or on human beings.

Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification. Like liquid chromatography–mass spectrometry, it allows analysis and detection even of tiny amounts of a substance.

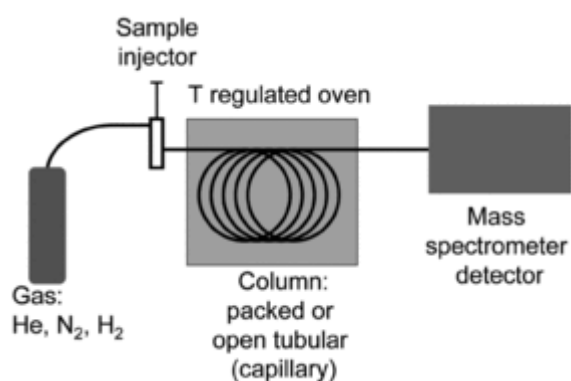
GC-MS has been widely heralded as a "gold standard" for forensic substance identification because it is used to perform a 100% specific test, which positively identifies the presence of a particular substance. A nonspecific test merely indicates that any of several in a category of substances is present. Although a nonspecific test could statistically suggest the identity of the substance, this could lead to false positive identification.

Instrumentation



**The insides of the GC-MS, with the column of the
Gas chromatograph in the oven on the right**

The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-charge ratio.



Schematic diagram of GC-MS

These two components, used together, allow a much finer degree of substance identification than either unit used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography using a traditional detector (e.g. Flame ionization detector) cannot differentiate between multiple molecules that happen to take the same amount of time to travel through the column (*i.e.* have the same retention time), which results in two or more molecules that co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Therefore, when an identifying mass spectrum appears at a

characteristic retention time in a GC-MS analysis, it typically increases certainty that the analyte of interest is in the sample.

Purge and trap GC-MS

For the analysis of volatile compounds, a purge and trap (P&T) concentrator system may be used to introduce samples. The target analytes are extracted and mixed with water and introduced into an airtight chamber. An inert gas such as Nitrogen (N₂) is bubbled through the water; this is known as purging. The volatile compounds move into the headspace above the water and are drawn along a pressure gradient (caused by the introduction of the purge gas) out of the chamber. The volatile compounds are drawn along a heated line onto a 'trap'. The trap is a column of adsorbent material at ambient temperature that holds the compounds by returning them to the liquid phase. The trap is then heated and the sample compounds are introduced to the GC-MS column via a volatiles interface, which is a split inlet system. P&T GC-MS is particularly suited to volatile organic compounds (VOCs) and BTEX compounds (aromatic compounds associated with petroleum).^[8]

Applications

- **Environmental monitoring and cleanup**
- **Criminal forensics**
- **Law enforcement**
- **Sports anti-doping analysis**
- **Chemical warfare agent detection**
- **Chemical Engineering**
- **Food, beverage and perfume analysis**
- **Astrochemistry**
- **Medicine**

McReynolds Constants

McReynolds values provide a systematic approach to ranking GC stationary phases by polarity and simplify the task of cross-comparing phases to determine whether they are equivalent. They also are useful for predicting elution orders among related analytes and resolution of analytes from differing chemical families. Values are obtained by measuring the degree to which a phase retains several probe compounds, relative to their retention by a nonpolar phase (squalane). An extensive table of McReynolds values in this bulletin enables you to use the McReynolds system without expending the time to derive the values.

The selectivity of a stationary phase can be accurately estimated by determining the degree to which polar compounds are retained by the phase, relative to their retention by a nonpolar phase (squalane). The retention index system used for many years by gas chromatographers is a systematic method for expressing and comparing such data. In this system, the retention index for any normal paraffin is defined as 100 times the number of carbon atoms in the molecule, regardless of the stationary phase, column dimensions, or analytical conditions used. For example, the retention indices for n-hexane, n-heptane, and n-octane are 600, 700, and 800, respectively. For all compounds except the n-paraffins, the column dimensions and operating conditions must be specified. Retention indices for compounds other than n-paraffins are determined as follows, using benzene as an example.

1. Inject a sample containing benzene, n-hexane, n-heptane, and n-octane onto the column and determine the retention times.
2. Correct for dead volume by subtracting the retention time for an unretained compound from each time measured in step 1.
3. For each of the three n-paraffins, plot the logarithm of the adjusted retention time (t'_R) against the retention index. For example, on 20% squalane at 120°C, n-hexane has a t'_R of 15 minutes, n-heptane has a t'_R of 19 minutes, and n-octane has a t'_R of 25 minutes (Figure A). The intersection of log15 min on the t'_R axis and 600 on the retention index axis gives the point for hexane; log19 min and 700 and log25 min and 800 give the other two points.
4. Connect the three points. The plot thus obtained is the basis for determining the retention index for benzene on this phase and under these conditions. The retention index for benzene is read at the point on the plot at log17 min (t'_R for benzene).

$$I = 100Z + 100[\log t'_R(i) - \log t'_R(z)] / \log t'_R(z+1) - \log t'_R(z)$$

Where, $t'_R(i)$ is the corrected retention time required to elute the compound of interest from the column $t'_R(z)$ is the corrected retention time for the n-alkane eluted prior to the compound of interest $t'_R(z+1)$ is the corrected retention time for the n-alkane eluted after the compound of interest Z is the carbon number of the n-alkane of retention $t'_R(z)$

EXERCISE:

Answer the following Questions

1. Write down principles of TLC, column and ion exchange chromatographic techniques?
2. In what way TLC differs from paper chromatography?
3. Explain the GC-MS techniques with applications.
4. Solvent extraction-Explain.

Unit IV

Thermo Analytical Techniques

OBJECTIVES

- **To understand the basics of various thermo analytical techniques.**
- **To learn the Instrumentation of thermo analytical techniques.**
- **To know the various applications of thermo analytical techniques.**

Introduction

Thermal analysis is the analysis of a change in a property of a sample, due to temperature. The sample is usually in the solid state and the changes that occur on heating include melting, phase transition, sublimation, and decomposition.

4.1. Thermo Gravimetric Analysis Technique

Principle

TGA measures the amount of weight change of a material, either as a function of increasing temperature, or as a function of time (temperature of the sample is increased), in an atmosphere of nitrogen, helium, air, other gas, or in vacuum. A plot of mass or mass percent as a function of time is called a thermogram, or a thermal decomposition curve.

Instrumentation

Requirements:

- (i) **Sensitive analytical balance:**

The most common type of balance is capable of weighing the sample range from 5 to 20 mg.

- (ii) **Furnace :**

The sample holder of the balance must be placed inside the furnace. The temperature range for most furnaces is zero to 1500°C.

(iii) **A purge gas system providing an inert atmosphere . Nitrogen or Argon are usually used to purge the furnace and prevent oxidation of the sample.**

(iv) **A microcomputer /microprocessor for instrument control and data acquitstion and display.**

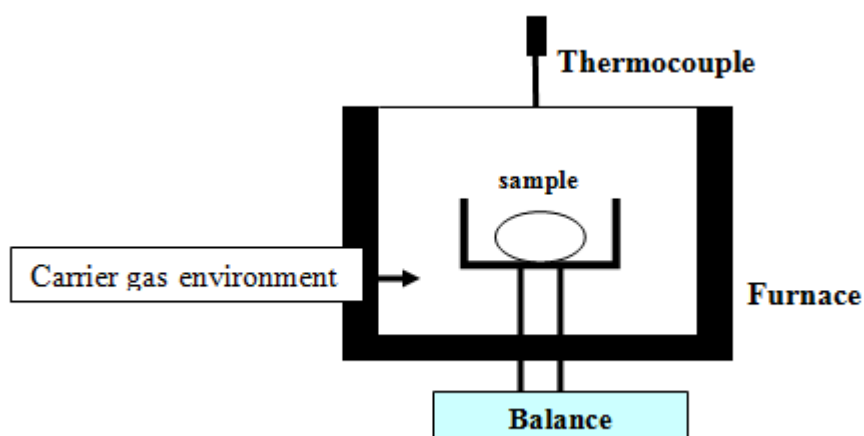


Fig.4. 1.1 Schematic diagram for Thermo Gravimetric Analytical Technique

In this analysis, the sample is placed in a sample holder (which is made up of platinum) of a microbalance. The entire arrangement is placed within a small oven, temperature of which is controlled and monitored. This temperature is measured by immersing a small thermocouple close to the sample container. The recorded temperatures then lead or lag the actual sample temperature.

Modern thermobalances use a computerized temperature control unit which finds the difference between the temperature of the thermocouple and the temperature specified in read only memory (ROM) to adjust the voltage to the heater. Analysis is carried out by raising the temperature of the sample gradually in a flow of air or inert gas such as N_2 , Ar or He and the sample weight (or percentage) is plotted against temperature.

The parameters affecting the TGA are: heating rate, amount of sample, carrier gas flow, nature of carrier gas etc.

Applications

1. Thermograms provide information about decomposition mechanisms for various polymeric preparations. Decomposition patterns are characteristics for each kind of polymer and in some cases can be used for identification of various polymers.

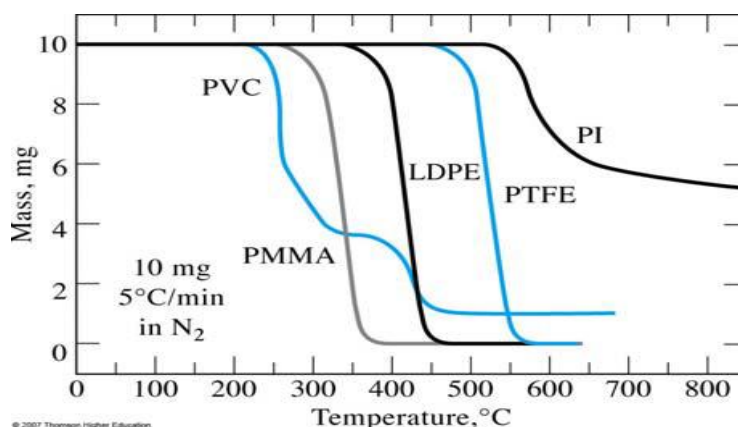


Fig. 4.1.2. Thermograms for some Common Polymeric Materials

(PVC = polyvinyl chloride; PMMA = polymethyl-methacrylate;
LDPE = low-density polyethylene; PTFE = polytetrafluoroethylene;
PI = aromatic polyimide.)

2. Following Fig. illustrates how a thermogram is used for quantitative analysis for a polymeric material.

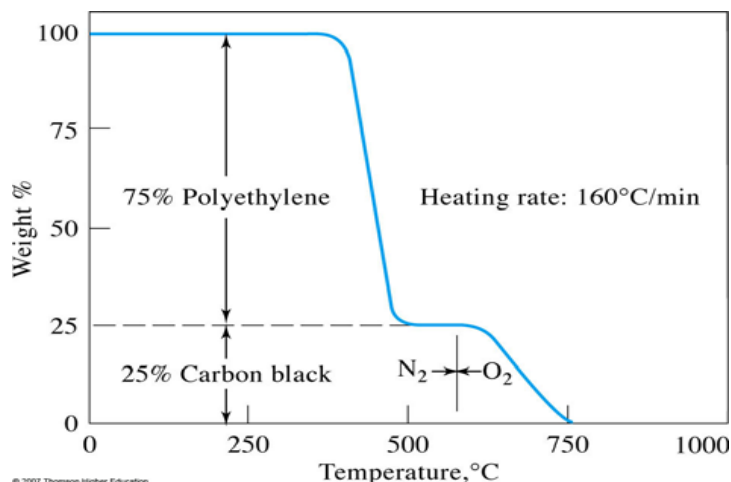


Fig. 4.1.3. Thermogravimetric determination of Carbon Black in Polyethylene.

The sample is polyethylene that has been formulated with fine carbon –black particles to inhibit degradation from exposure to sunlight.

3. Thermogravimetric analysis of Calcium oxalate monohydrate. Thermogram of this compound is shown below.

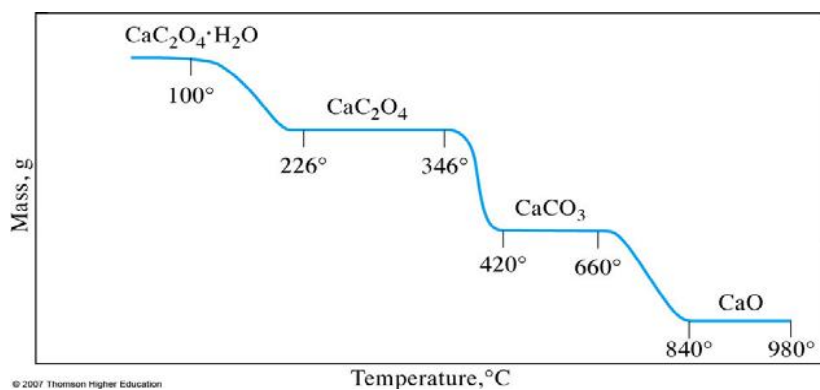
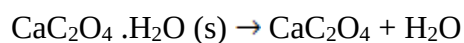
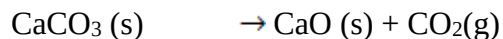
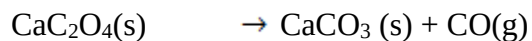


Fig.4.1.4. Thermogram of Calcium Oxalate Monohydrate

Successive plateaus correspond to the formation of anhydrous salt, calcium carbonate and calcium oxide.



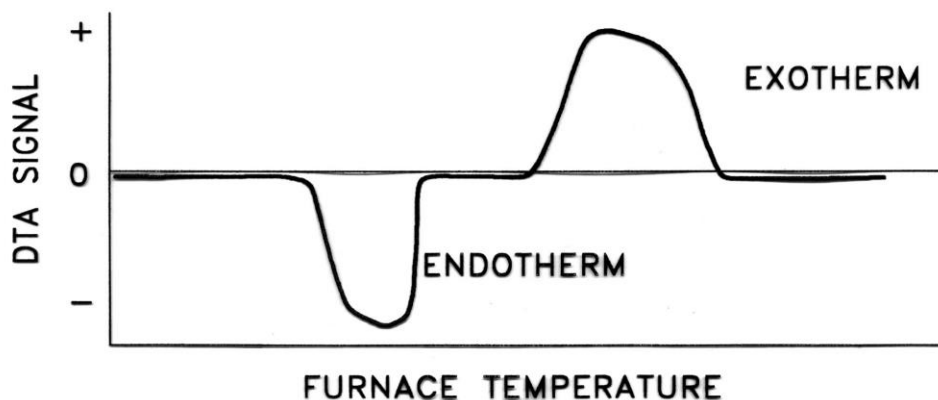


Thermogram indicates that loss of water begins at 100⁰C, loss of CO at 400⁰ C and loss of CO₂ at 600⁰C.

4.2 Differential Thermal Analysis (DTA)

Principle

In DTA, the difference in temperature is measured between a sample and an inert reference material, which are subjected to a controlled temperature programme. The difference in temperature (ΔT) is plotted against sample temperature to give a differential thermogram .



DTA peaks result from:

1) Physical changes

- * Endothermic: fusion, vaporization, sublimation, absorption, desorption.
- * Exothermic: adsorption, crystallization.

2) Chemical reactions

- * Endothermic: dehydration, reduction, decomposition.
- * Exothermic: oxidation in air or O₂, polymerization, catalytic reactions.

Instrumentation

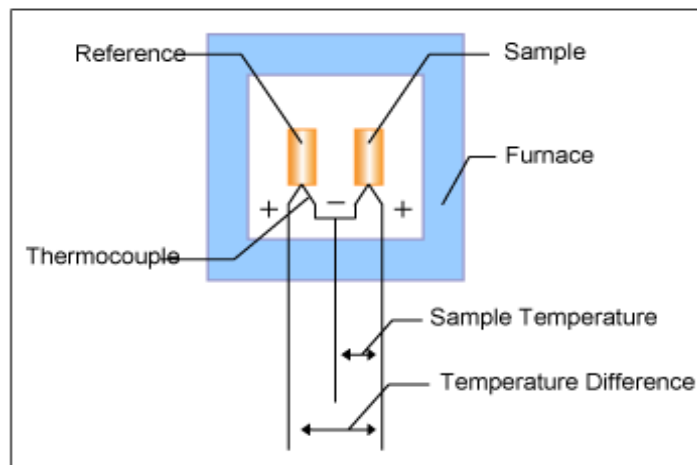


Fig. 4.2. 1. Block diagram of DTA

The sample (few mg) and an inert reference are placed in small aluminium dishes that are located above sample and reference thermocouples in an electrically heated furnace. Reference material is an inert substance such as alumina, silicon carbide or glass beads. The furnace is controlled under a temperature program and the temperature of the sample and the reference are changed. During this process, a differential thermocouple is set up to detect the temperature difference (ΔT) between the sample and the reference. Also, the sample temperature (T_s) is detected from the thermocouple on the sample side.

In a differential thermogram, (T_s) is served as abscissa (X-axis) and (ΔT) is served as ordinate (Y-axis).

The temperature should be the same until thermal event occurs, such as melting, decomposition or change in the crystal structure. In an endothermic event takes place within the sample, the temperature of the sample will lag behind that of the reference and a minimum will be observed on the curve. On the contrary, if an exothermic event takes place, then the temperature of the sample will exceed that of the reference and a maximum will be observed on the curve. The area under the endotherm or exotherm is related to the enthalpy of the thermal event, ΔH .

Differential thermogram for heating a polymer is shown below.

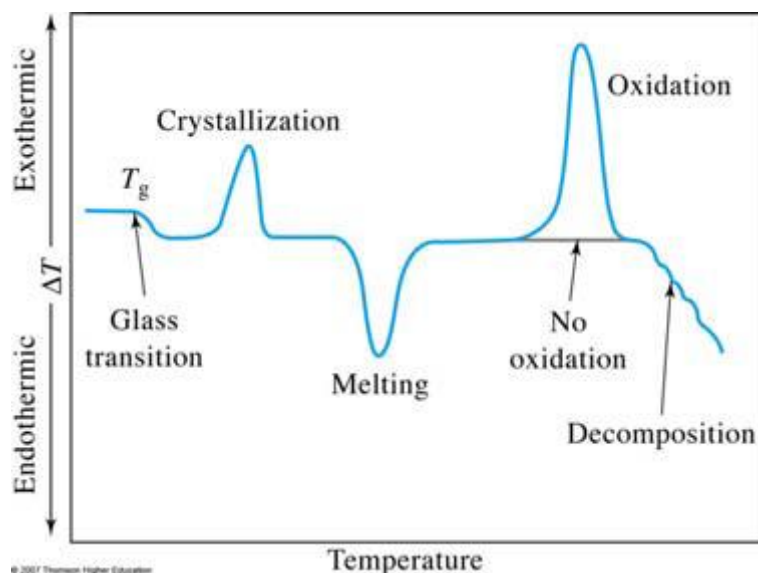


Fig. 4. 2. 2. Differential Thermogram of Polymer

The initial decrease in (ΔT) is due to the glass transition, (T_g) at which glassy amorphous polymer becomes flexible or rubber like nature. During this process heat will neither be absorbed nor be released.i.e ($\Delta H = 0$).Two maxima are the result of exothermic process (heat evolved from the analyte) and two minima are the result of endothermic process (heat absorbed by the analyte).The first exothermic peak is due to the crystal formation. The second exothermic peak is possible when the heating is performed in the presence of oxygen or air.

The first endothermic peak arises due to the melting of the microcrystals which are formed in the previous exothermic process.The final negative change in (ΔT) results from the endothermic decomposition of the polymer to produce a variety of products.

Applications

- 1.To determine the thermal behaviour and composition of naturally occurring and manufactured polymers.
- 2.To determine the thermal behaviour of pure Inorganic compounds.
- 3.To study the phase transitions.

Ex: Differential thermogram for sulfur.

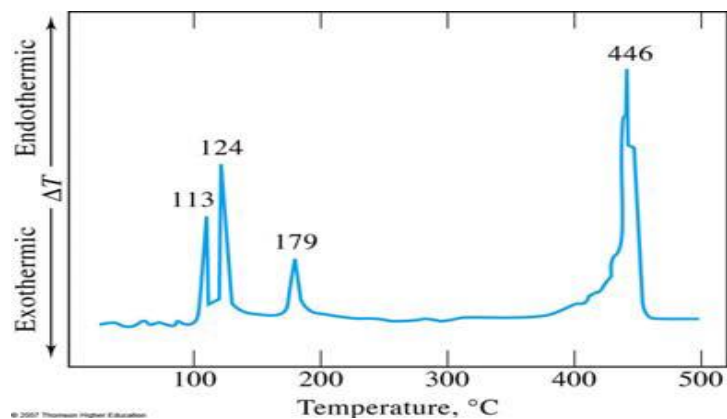


Fig. 4. 2. 3. Differential Thermogram of Sulphur

The peak at 113⁰C corresponds to the change from the rhombic to the monoclinic.

The peak at 124⁰C corresponds to the melting point of the element.

The peak at 179⁰C corresponds to the liquid phase transition of sulphur.

The peak at 446⁰C corresponds to the boiling point of the sulphur.

4. To determine the melting ,boiling and decomposition points of organic compounds.

Ex: Differential thermogram for benzoic acid.

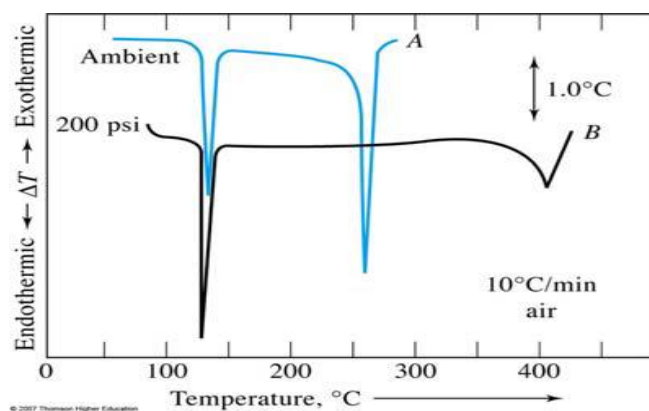


Fig. 4.2. 4. Differential thermogram of Benzoic acid

The first peak corresponds to the melting point and the second to the boiling point of the acid.

4.3 Differential Scanning Calorimetry (DSC)

Principle

Differential scanning calorimetry or DSC is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature while the two are subjected to a controlled temperature programme. In DSC, the differences in energy are measured.

Instrumentation

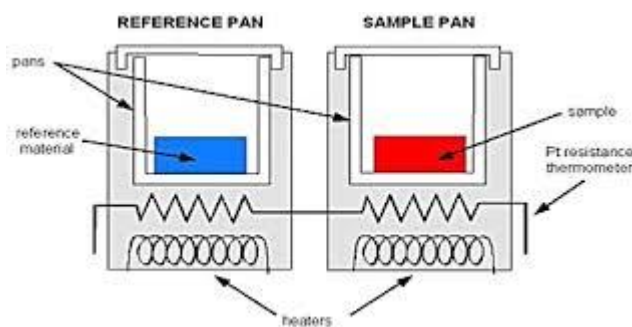


Fig. 4. 3. 1. Schematic diagram of Power Compensated DSC

Power Compensated DSC

Power Compensated calorimeter has two independent furnaces, one for heating the sample and the other for heating the reference. The furnaces are small and imbedded in a large temperature controlled heat sink. Above the furnaces are the sample and reference holders, which have platinum resistance thermometers imbedded in them to monitor the temperatures of the two materials continuously. Two control circuits are employed, one for average temperature control and one for differential temperature control.

Average temperature control is used to maintain the same temperature of the sample and the reference. Differential temperature control is used to find the difference in the amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature .

The average temperature of the sample and the reference is taken as the abscissa (X-axis), difference in the amount of heat (i.e) heat flow is taken as ordinate (Y-axis) in the thermogram.

Heat flux DSC

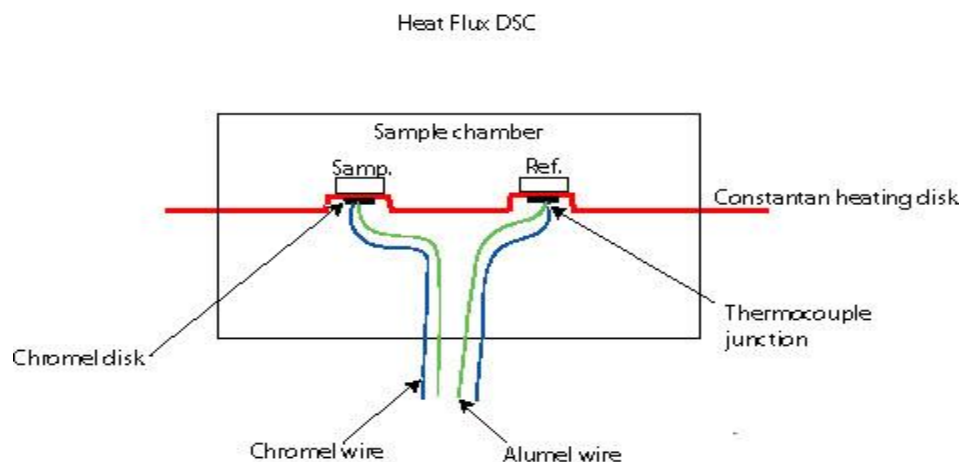


Fig. 4.3. 2. Schematic diagram of Heat flux DSC

Heat flows in to sample and reference material via an electrically heated constantan (constantan is an alloy with 60% copper and 40% nickel.) thermoelectric flask. A platinum pan is placed on constantan disc. Heat is transferred through the discs and upto the sample and reference via pan. Sample holder and the reference holder are connected by a low resistance heat path. The differential heat flow to the sample and the reference is monitored by constantan /chromel (chromel is an alloy made of 95%Ni,1%Si ,2%Mn and 2%Al)thermocouple. Thermocouple is a junction between two metal that produces voltage due to a temperature difference. The differential heat flow into the two pans directly proportional to the difference in output of the two thermocouple junction. The sample temperature is measured by means of chromel /alumel junction under the disc.

Temperature of the sample is taken as the abscissa (X-axis), difference in the amount of heat (i.e) heat flow is taken as ordinate (Y-axis) in the thermogram.

A typical DSC thermogram is shown below.

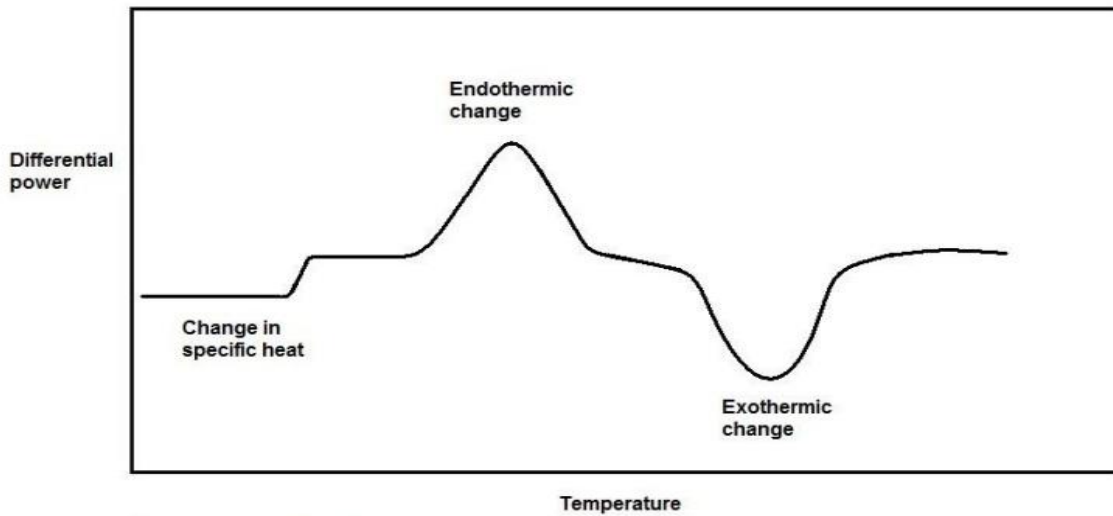
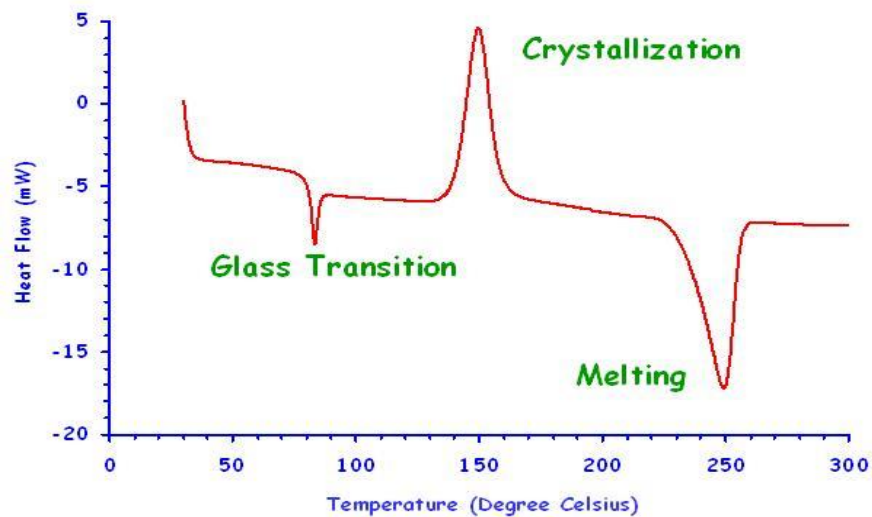


Figure 2. Typical DSC thermogram

Applications

1. To determine the thermal behaviour of polymers

The DSC curve for an amorphous sample of polyethylene Terephthalate is shown



below.

Fig. 4. 3. 3. DSC Curve for Polyethylene Terephthalate

DSC curves shows peak for glass transition, crystallization and melting. It does not show peak for oxidation, because the experiment was carried out in an atmosphere of nitrogen

2. It is used in the pharmaceutical industry to check the purity of drug samples.

4.4 Thermometric Titrations

In the thermometric titration, titrant is added at a known constant rate to a titrand until the completion of the reaction is indicated by a change in temperature. The endpoint in a thermometric titration is indicated by sharp break in the temperature versus volume curve.

Conditions for thermometric titration

1. Thermometric titrations are carried out in adiabatic conditions.
2. The reaction should be accompanied by sufficient heat change that can be measured.
3. Heat of dilution of the product should be negligible.
4. The reaction should be simple, one step change.

Instrumentation

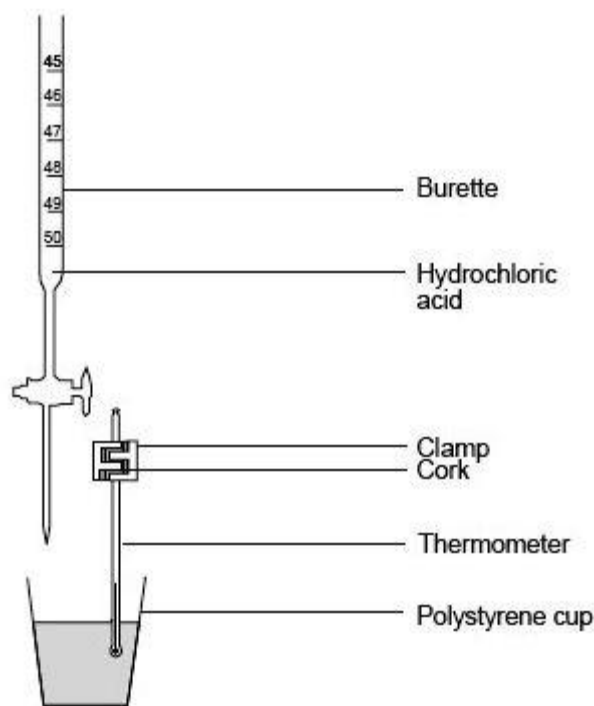


Fig. 4. 4. 1. Thermometric Titration Instrument

Titration Instrument:

A simple schematic representation of thermometric titration unit is shown above. It consists of motor-driven thermostated burette, thermally insulated adiabatic titration vessel [Dewar flask]. A known volume of the solution to be estimated is taken in the Dewar Flask. The tip of the burette is dipped into the solution in the vessel. The vessel is covered by a thick, insulating material containing holes for inserting the thermistor, the burette and the stirrer. The titrant is added at a constant rate which the solution in the Dewar flask is constantly stirred. The thermistor assembly is connected to one arm of a wheatstone bridge and its resistance is recorded directly on a recorder. The temperature measuring thermistor unit should be capable of measuring temperature change of 0.01°C . The titrant should be 10-100 times more concentrated than the solution in the Dewar flask, so that the titration can be done by the addition of only a small quantity of the titrant.

Titration of Hydrochloric Acid with Sodium Hydroxide:

A known volume of hydrochloric acid to be estimated is taken in the Dewar flask. The titrant NaOH solution is added from the burette. Temperature readings are recorded for different volume of the titrant added. The thermogram is obtained by plotting temperature versus titrant volume [fig.4.4.2].

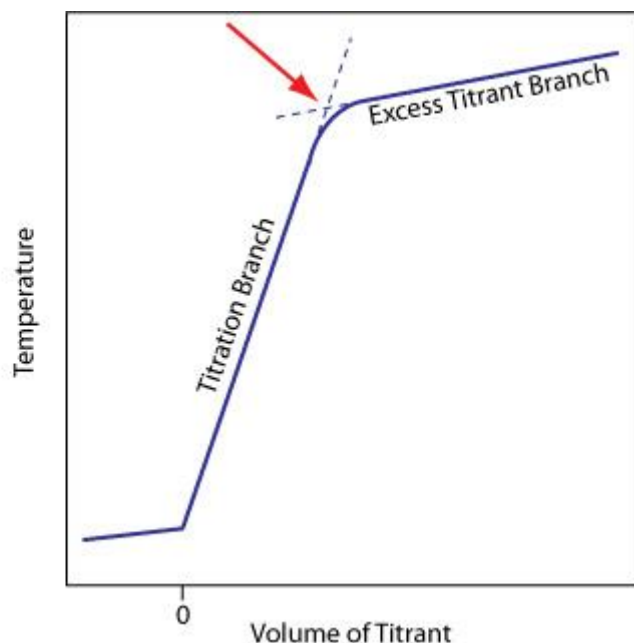


Fig. 4.4.2. Thermogram for the Titration of Hydrochloric Acid with Sodium Hydroxide

The neutralization point is indicated by a sharp, inflexion point C. Thermometric Titrimetry is one of the few methods of titration not based solely on considering of the free energy change ΔG and hence on the equilibrium constant of the reaction. ΔH and ΔG , not ΔG is measured in this method. Hence this titration may give useful results even when ΔG is zero or positive.

Complexometric titrations:

Consider, for example, the titration of calcium and magnesium with EDTA. The stability constants of the chelates differ by less than two orders of magnitudes, so that titration based on an indicator can give only the sum of the concentrations of the two ions. However, for the reaction of Mg^{2+} with EDTA the enthalpy is +5.5 kcal/mole whereas that for the Ca^{2+} reaction is

-5.7kcal/mole. This distinct difference in ΔH values makes it possible for the adoption of thermometric titration for estimating these two ions.

Application of Thermometric Titrations:

Several types of titrations have been followed successfully by the thermometric method. These include neutralizations of both strong and weak acids, precipitations, redox reactions and complex formations. These titrations can be carried out in aqueous or non-aqueous medium. Besides in water, titration have been reported in acetic acid, carbon tetrachloride, benzene and nitrobenzene. The precision of a thermometric titration is usually + or - 1%.

Exercises

1. With a diagram , explain the experimental setup used inTGA.
2. Explain how DTA differs from TGA.
3. Give an account of the applications of TGA.
4. Discuss the various applications of DTA.
5. Outline briefly the Instrumentation part of DSC along with its application.
6. Explain the principle of thermometric titrations.

UNIT V

ELECTROANALYTICAL TECHNIQUES AND FLUORESCENCE SPECTROSCOPY

OBJECTIVES

- To gain the basic information about the various types of electrochemical sensors.
- To know about the various types of electrodes, which are used in electrochemical techniques.
- To learn the Instrumentation and applications of voltammetric techniques.

ELECTROCHEMISTRY

Electrochemistry deals with the transfer of electrons from one substance to another. This transfer creates a current, the magnitude of which can give us clues about the analyte.

5.1.1 ELECTROCHEMICAL SENSOR

A chemical sensor can be defined as a device that provides continuous information about a specific chemical species in the environment. All chemical sensors consist of a transducer, which transforms the response into a detectable signal on modern instrumentation, and a chemically selective layer, which isolates the response of the analyte from its immediate environment. Electrochemical sensors are designed to detect and respond to an analyte in the gaseous, liquid or solid state. Electrochemical sensors are especially attractive because of their remarkable detectability, experimental simplicity and low cost. There are three main types of electrochemical sensors: potentiometric, amperometric and conductometric.

POTENTIOMETRIC: For potentiometric sensors, a local equilibrium is established at the sensor interface, where either the electrode or membrane potential is measured, and information

about the composition of a sample is obtained from the potential difference between two electrodes.

There are three basic types of potentiometric devices: ion- selective electrodes (IES), coated wire electrodes (CWES) and field effect transistors (FETS).

5.1.2. ION-SELECTIVE ELECTRODE

Ion-selective electrode also known as a **specific ion electrode (SIE)**, is a transducer (or sensor) that converts the activity of a specific ion dissolved in a solution into an electrical potential. The voltage is theoretically dependent on the logarithm of the ionic activity, according to the Nernst equation. Ion-selective electrodes are used in analytical chemistry and biochemical/biophysical research, where measurements of ionic concentration in an aqueous solution are required.

Ion-selective electrodes possess a high degree of selectivity. The selectivity of the ISE is determined by the composition of the membrane. Ideally the membrane allows the uptake of only one specific ion into it. The analyte ion may be a cation or an anion. The three main components of making a measurement at an ISE are an inner reference, or standard, solution and an outer analyte, or sample, solution separated by a thin membrane. These components are shown in Figure 5.1.2.1

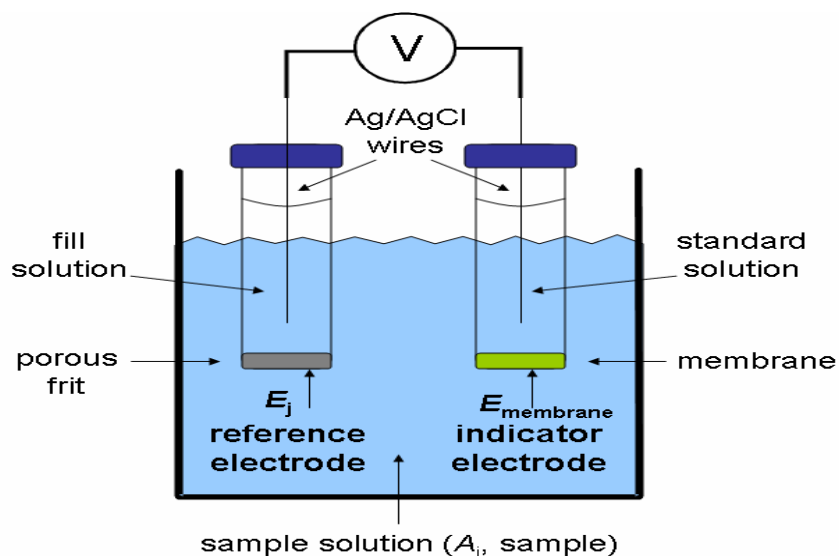


Figure 5.1.2.1 Ion-selective electrode

Different strategies for producing an electrode that is selective to one species are based primarily on the nature and composition of the membrane material. Depending on the nature of the membrane, ISEs can be divided into three groups: glass, liquid or solid electrodes.

Properties of Ion –Selective Membranes

a. Minimal solubility

A necessary property of an ion selective medium is that its solubility in analyte solutions approaches zero.

b. Electrical conductivity

A membrane must exhibit electrical conductivity, generally this conduction takes the form of migration of singly charged ions within the membrane.

c. Selective reactivity with the analyte.

A species within the membrane must be capable of selectively binding the analyte ion.

5.1.2.1 Glass membrane electrodes:

The most famous glass electrode determines H^+ activity or pH. The membrane is composed of a silicate glass. Glass electrodes can also be constructed that are sensitive to other cations such as sodium.

pH electrode

The internal filling solution is HCl; H^+ is the ion that binds to the membrane; Cl^- is the ion that governs the potential of the reference electrode AgCl/Ag. (Fig 5.2.1.1) shows an older type with separate pH and reference electrodes.

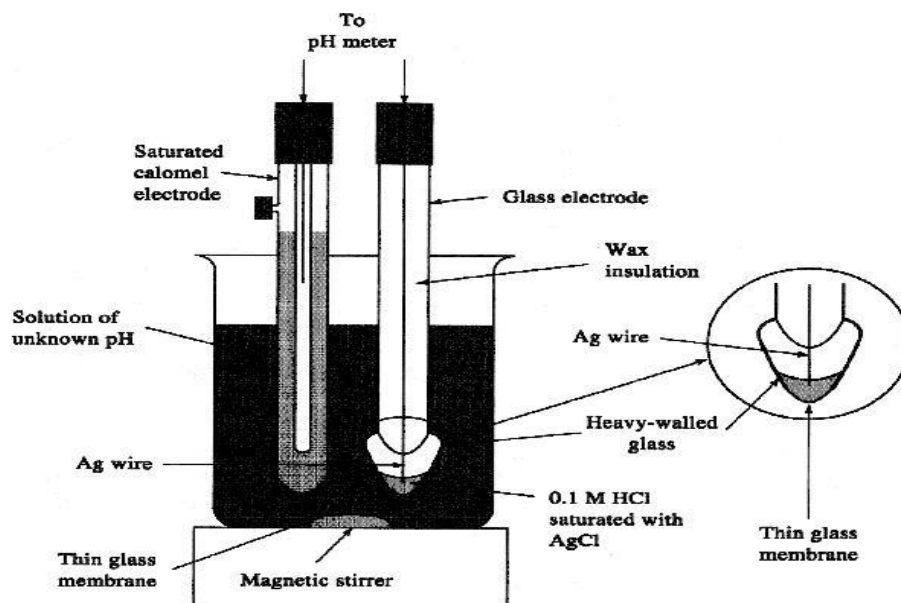


Fig 5.1.2.1.1 Typical electrode system for measuring pH

5.1.3 Crystalline / solid state membrane electrodes:

The membrane is composed of an insoluble inorganic salt. An ion-exchange process leads to the formation of a potential at the membrane. An example for ion-exchange process is shown below.

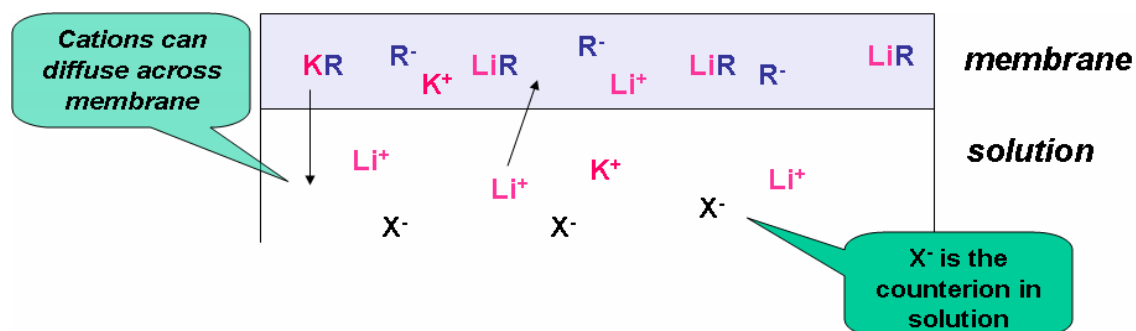


Figure 5.1.3.1 Ion-exchange process

F⁻ electrode

Single crystal LaF₃ is widely used to determine F⁻. The crystal is usually doped with europium to improve the conductivity. The membrane is a thin slice of crystalline LaF₃, which has very low solubility in water

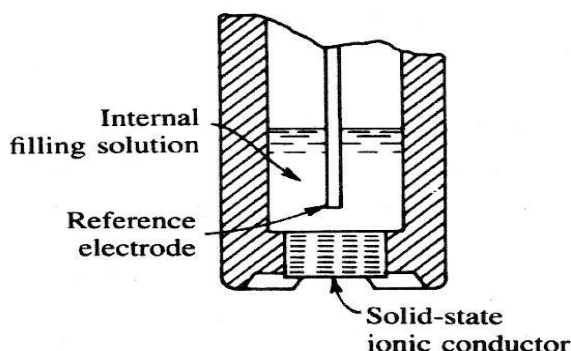


Fig 5.1.3.2 Typical crystalline membrane electrode

The chemistry at the each membrane solution interface is: $\text{LaF}_3(\text{s}) \rightleftharpoons \text{LaF}_2^+(\text{s}) + \text{F}^-(\text{aq})$
The formation of LaF₂⁺ creates a charge at the surface. The equilibrium will be shifted to the right for the solution with a smaller F⁻ concentration, and the potential will become more positive relative to the other side of the membrane. It is this potential difference across the LaF₃ crystal membrane that is measured and related to F⁻ concentration. The fluoride electrode is extremely selective for F⁻ but can experience interference from OH⁻ above pH 8. The internal filling solution is NaCl + NaF; F⁻ is the ion that binds to the membrane; Cl⁻ is the ion that governs the potential of the reference electrode AgCl/Ag. Na⁺ is merely a spectator ion.

Silver salt electrodes:

Depend on the property that Ag_2S is conducting and can be pressed into disks of moderate mechanical strength, with or without a second ionic solid. Design is similar to the LaF_3 electrode.

Ag_2S membrane alone: senses either Ag^+ or S^{2-}

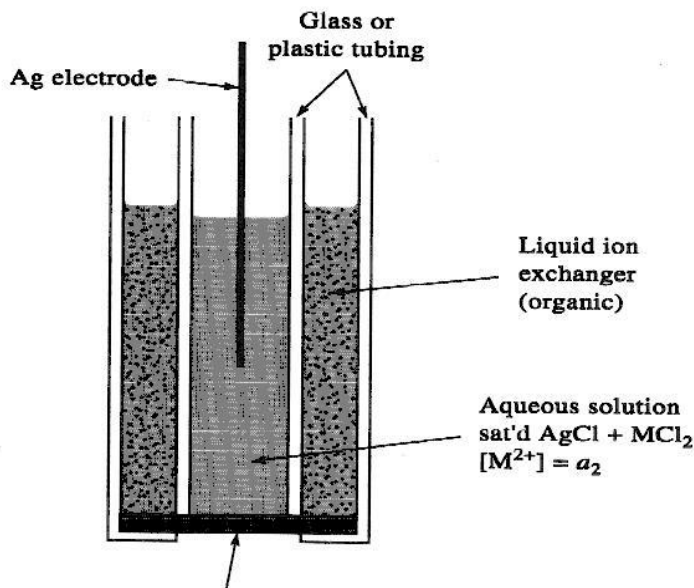
Ag_2S with other silver salt AgX senses X^-

Ag_2S with other metal sulfide, MS senses M^{2+} (Cu, Cd, Pb)

Silver salt electrodes are irreversibly damaged by exposure to highly acidic solutions, and by the presence of certain interferences (metals whose sulfides are more insoluble than the analyte of interest). Thus Hg^{2+} is incompatible with CuS ; Hg^{2+} and Cu^{2+} are incompatible with PbS , etc.

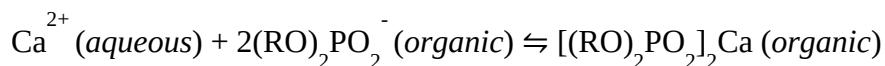
5.1.4 Liquid membrane electrodes:

An ion-exchanger or ionophore (neutral macrocyclic ion carrier) is dissolved in a viscous organic liquid membrane. Without the exchanger or ionophore the ion of interest is unable to penetrate the membrane.



5.1.4.1. Schematic diagram of Liquid membrane electrodes

With the exchanger or ionophore present, the processes described in Figures 5.3.1 and 5.4.1 transport the ion into the membrane. One of the most famous liquid membrane electrodes has been used for calcium determination. Initially, researchers attempted to use glass membrane electrodes (which had been successful for monovalent cations such as H⁺ and Na⁺) to detect divalent cations, such as Ca²⁺. When this was determined to be unfeasible, liquid membranes were developed. This electrode works by an ion-exchange process. The cation-exchanger is an aliphatic diester of phosphoric acid, (RO)₂PO₂⁻, where each R group is an aliphatic hydrocarbon chain containing between 8 and 16 carbons. The phosphate group can be protonated, but has a strong affinity for Ca²⁺. The cation exchanger is dissolved in an organic solvent and held in a porous compartment between the analyte solution and internal reference calcium chloride solution. The ion-exchanger uptakes Ca²⁺ into the membrane by the following mechanism,



Calcium ISEs are commonly used to measure calcium ion activity in biological fluids, as calcium ion is important in many physiological processes, such as bone formation.

5.1.5. Ion-selective field effect transistors

The ion-sensitive field-effect transistor (ISFET) is an electrochemical sensor that produces electrical response in accordance to ionic concentration due to the ionic activity at the exposed gate window. ISFETs are potentiometric sensors that produce response by virtue of potential reaction at the electrolyte/membrane interface similar to the more frequently used ion-selective electrode .

The membrane at the gate window acts as the receptor and the basic structure of a metal-oxide field-effect transistor functions as a transducer. The construction of ISFET requires the fabrication of metal-oxide field-effect (MOSFET) transistor thus giving ISFET the advantage of being solid-state silicon based and compatible with standard MOSFET fabrication technology. Which enhances chemical sensing nature of the ISFET . The gate of the ISFET is covered with an insulating layer of silicon Nitride.

Sharing the same silicon platform is an added convenience for integrating sensing and computational modules in smart sensor systems that are portable.

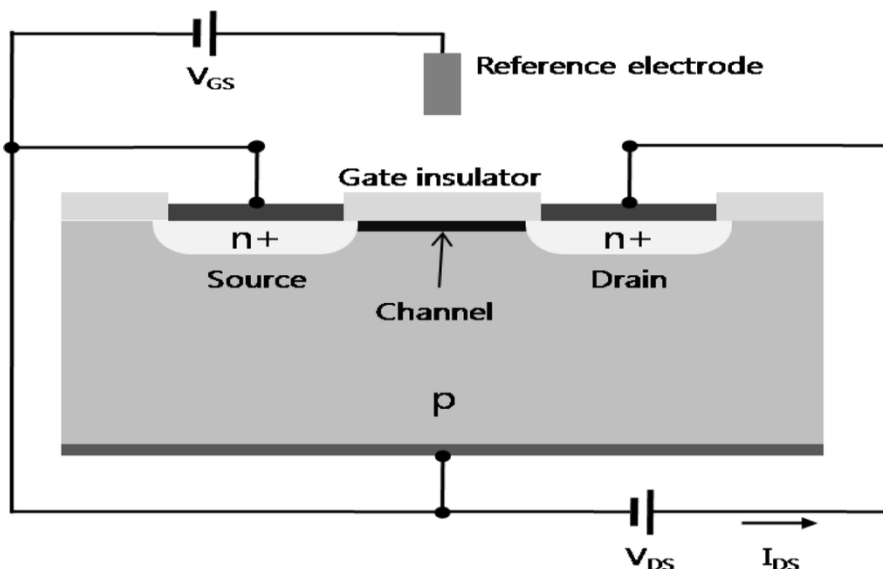


Fig.5.1.5. 1 Schematic diagram for Ion-selective field effect transistors

The analyte solution, containing hydronium ion in this example, is in contact with this insulating layer and with a reference electrode. The surface of the gate insulator functions very much like the surface of a glass electrode. Protons from the hydronium ions in the test solution are adsorbed by available microscopic sites on the silicon nitride. Any change in the hydronium ion concentration of the solution results in a change in the concentration of adsorbed protons. The change in concentration of adsorbed protons then gives rise to a changing electrochemical potential between the gate and source, which in turn changes the conductivity of the channel of the ISFET. The conductivity of the channel can be monitored electronically to provide a signal that is proportional to the logarithm of the concentration of hydronium ion in the solution.

Applications:

Each ISFET may be coated with a different polymer so that measurements of several different species may be made. Their small size (approximately 1 to 2 mm²), rapid response time relative to glass electrodes, and ruggedness suggest that ISFETs may be the ion detectors of the future for many applications.

This makes ISFET an appealing technology for environmental, agricultural and clinical applications. One of the challenges for the ISFET sensor is the need to demonstrate high selectivity. Selectivity is the ability to respond to primarily only one ion species in the presence of other species. In the presence of mixed ions of equal charge number and similar ionic radii to the main ion of interest, ISFETs exhibit response towards the interfering ionic activity. The sensor signal in mixed-ion solution would then represent combined activity rather than information from a single ion type.

5.1.6 SENSORS FOR THE ANALYSIS OF GASES IN SOLUTION

5.1.6.1 AMPEROMETRIC : Amperometric sensors exploit the use of a potential applied between a reference and a working electrode, to cause the oxidation or reduction of an electroactive species; the resultant current is measured.

PRINCIPLES

Most gas sensors are amperometrically based where the concentrations of gas are monitored by current measurements with the latter arising from electrolysis (oxidation or reduction) of a target gas at a working electrode.

APPARATUS TECHNIQUES

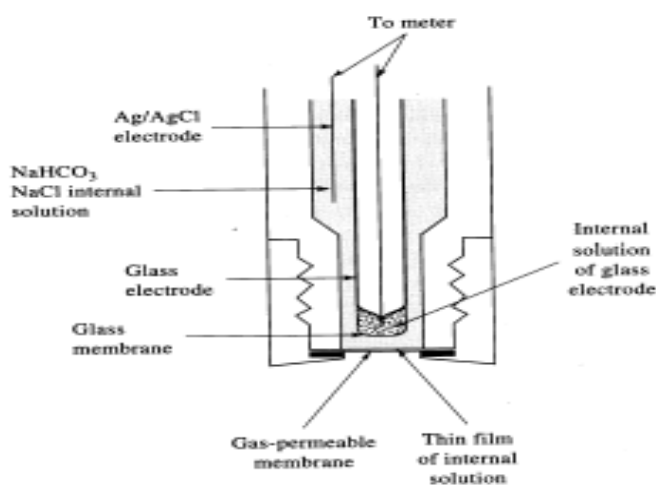


Fig.5.1. 6.1 Schematic diagram for Gas-sensing probes

Gas-sensing probes:

Gas-sensing probes are remarkably selective and sensitive devices for determining dissolved gases or ions that can be converted to dissolved gases by pH adjustment. The heart of the probe is a thin, porous membrane, which is easily replaceable. This membrane separates the analyte solution from an internal solution containing sodium bicarbonate and sodium chloride. A pH-sensitive glass electrode having a flat membrane is fixed in position so that a very thin film of the internal solution is sandwiched between it and the gas-permeable membrane. A

silver/silver chloride reference electrode is also located in the internal solution. It is the pH of the film of liquid adjacent to the glass electrode that provides a measure of the carbondioxide content of the analyte solution on the other side of the membrane.

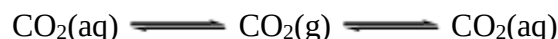
Gas-Permeable Membranes:

Two types of membrane material are encountered, microporous and homogeneous. Microporous materials are manufactured from hydrophobic polymers such as polytetraflouroethylene or polypropylene, which have a porosity (void volume) of about 70% and a pore size of less than 1 μ m. Because of the water-repellent properties of the film, water molecules and electrolyte ions are excluded from the pores; gaseous molecules, on the other hand, are free to move in and out of the pores by effusion and thus across this barrier. Typically, microporous membranes are about 0.1mm in thickness.

Homogeneous films, in contrast, are solid polymeric substances through which the analyte gas passes by dissolving in the membrane, diffusing and then solvating into the internal solution,. Silicone rubber is the most widely used material for construction. Homogeneous films are generally thinner than microporous ones (0.01 to 0.03mm) in order to hasten the transfer of gas and thus the rate of response of the system.

Mechanism of Response:

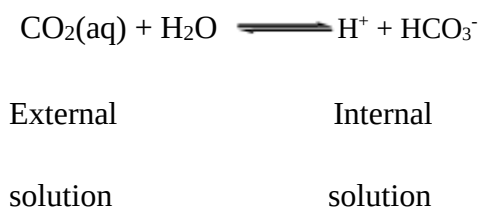
When a solution containing dissolved carbon dioxide is brought into contact with the microporous membrane show in **Fig.5.1.6.1. 1**, the gas effuses through the membrane, as described by the reactions.



Because the pores are numerous and small, equilibrium is rapidly established (in a few secs to a few minutes) between the external solution and the thin film of internal solution adjacent to the membrane. In the internal solution, another equilibrium is established that causes the pH of the internal surface film to change; that is,



A glass electrode immersed in the film of internal solution then detects the pH change. The overall reaction for the processes just described is given by



APPLICATIONS

There are various gas sensing electrodes are available to detect different gases.

They are shown in the following table :

SERIAL .NO	Gas	Equilibrium in Internal solution	Sensing electrode
1	NH ₃	$\text{NH}_3 + \text{H}_2\text{O} \rightleftharpoons \text{NH}_4^+ + \text{OH}^-$	Glass,pH
2	CO ₂	$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$	Glass,pH
3	HCN	$\text{HCN} \rightleftharpoons \text{H}^+ + \text{CN}^-$	Ag ₂ S,pCN
4	HF	$\text{HF} \rightleftharpoons \text{H}^+ + \text{F}^-$	LaF ₃ ,pF
5	H ₂ S	$\text{H}_2\text{S} \rightleftharpoons 2\text{H}^+ + \text{S}^{2-}$	Ag ₂ S,pS
6	SO ₂	$\text{SO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HSO}_3^- + \text{H}^+$	Glass,pH
7	NO ₂	$2\text{NO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+$	Immobilized ionexchange,pNO ₃

5.1.7.CONDUCTOMETRIC: conductometric sensors are involved with the measurement of conductivity at a series of frequencies. Given the impressive progress in the electrochemical sensor area, and their growing impact on analytical chemistry

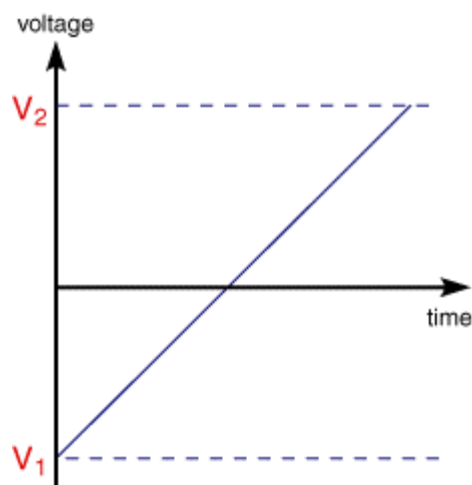
5.1.8.Linear Sweep Voltammetry

Introduction

Linear sweep voltammetry is a general term applied to any *voltammetric method* in which the potential applied to the working electrode is varied linearly in time. These methods would include polarography, cyclic voltammetry, and rotating disk voltammetry.

Instrumentation

In linear sweep voltammetry (LSV) a fixed potential range is employed much like potential step measurements. However in LSV the voltage is scanned from a lower limit to an upper limit as shown below.



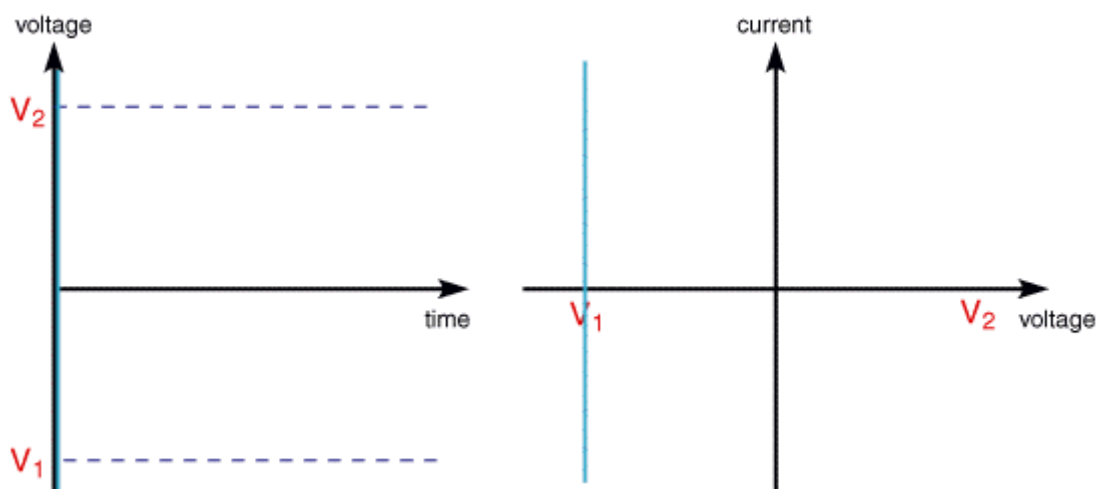
The voltage scan rate (v) is calculated from the slope of the line. Clearly by changing the time taken to sweep the range we alter the scan rate. The characteristics of the linear sweep voltammogram recorded depend on a number of factors including:

- The rate of the electron transfer reaction(s)
- The chemical reactivity of the electroactive species
- The voltage scan rate

In LSV measurements the current response is plotted as a function of voltage rather than time, unlike potential step measurements. For example if we return to the $\text{Fe}^{3+}/\text{Fe}^{2+}$ system



then the following voltammogram would be seen for a single voltage scan using an electrolyte solution containing only Fe^{3+} resulting from a voltage sweep

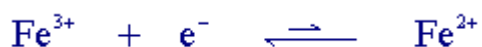


The scan begins from the left hand side of the current/voltage plot where no current flows. As the voltage is swept further to the right (to more reductive values) a current begins to flow and eventually reaches a peak before dropping. To rationalise this behaviour we need to consider the influence of voltage on the equilibrium established at the electrode surface. If we consider the electrochemical reduction of Fe^{3+} to Fe^{2+} , the rate of electron transfer is fast in comparison to the voltage sweep rate. Therefore at the electrode surface an equilibrium is established identical to that predicted by thermodynamics. You may recall from equilibrium electrochemistry that the Nernst equation:

$$E = E^{\ominus} + \frac{RT}{nF} \ln \frac{[\text{Fe}^{3+}]}{[\text{Fe}^{2+}]}$$

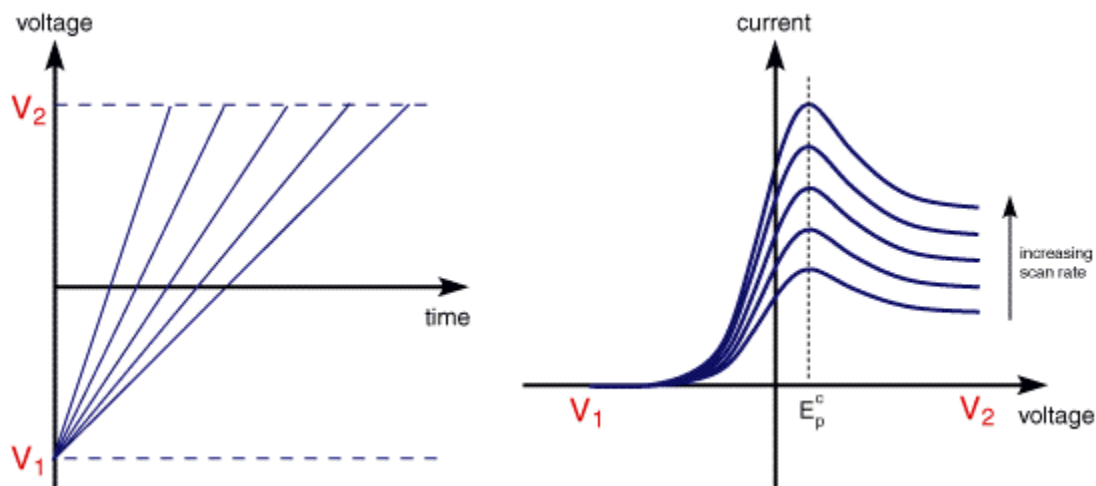
predicts the relationship between concentration and voltage (potential difference), where E is the applied potential difference and $E^{\ominus}$ is the standard electrode potential. So as the voltage is swept from V_1 to V_2 the equilibrium position shifts from no conversion at V_1 to full conversion at V_2 of the reactant at the electrode surface. The exact form of the voltammogram can be rationalised by

considering the voltage and mass transport effects. As the voltage is initially swept from V_1 the equilibrium at the surface begins to alter and the current begins to flow:



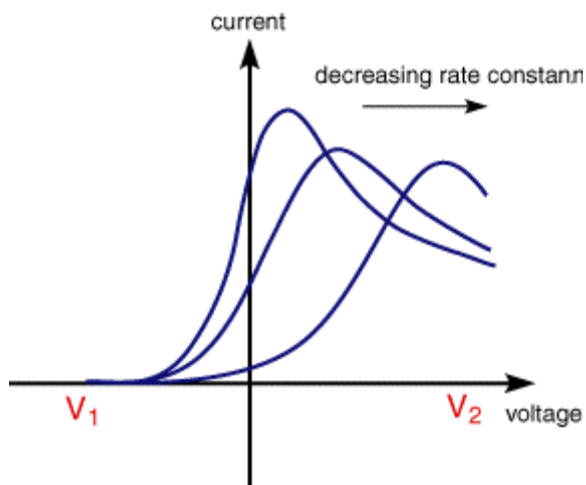
The current rises as the voltage is swept further from its initial value as the equilibrium position is shifted further to the right hand side, thus converting more reactant. The peak occurs, since at some point the diffusion layer has grown sufficiently above the electrode so that the flux of reactant to the electrode is not fast enough to satisfy that required by the Nernst equation. In this situation the current begins to drop just as it did in the potential step measurements. In fact the drop in current follows the same behaviour as that predicted by the Cottrell equation.

The above voltammogram was recorded at a single scan rate. If the scan rate is altered the current response also changes. The figure below shows a series of linear sweep voltammograms recorded at different scan rates for an electrolyte solution containing only Fe^{3+}



Each curve has the same form but it is apparent that the total current increases with increasing scan rate. This again can be rationalised by considering the size of the diffusion layer and the time taken to record the scan. Clearly the linear sweep voltammogram will take longer to record as the scan rate is decreased. Therefore the size of the diffusion layer above the electrode surface will be different depending upon the voltage scan rate used. In a slow voltage scan the diffusion layer will grow much further from the electrode in comparison to a fast scan. Consequently the flux to the electrode surface is considerably smaller at slow scan rates than it is at faster rates. As the current is proportional to the flux towards the electrode the magnitude of the current will be lower at slow scan rates and higher at high rates. This highlights an important point when examining LSV (and cyclic voltammograms), although there is no time axis on the graph the voltage scan rate (and therefore the time taken to record the voltammogram) do strongly effect the behaviour seen. A final point to note from the figure is the position of the current maximum, it is clear that the peak occurs at the same voltage and this is a characteristic of electrode reactions which have rapid electron transfer kinetics. These rapid processes are often referred to as reversible electron transfer reactions.

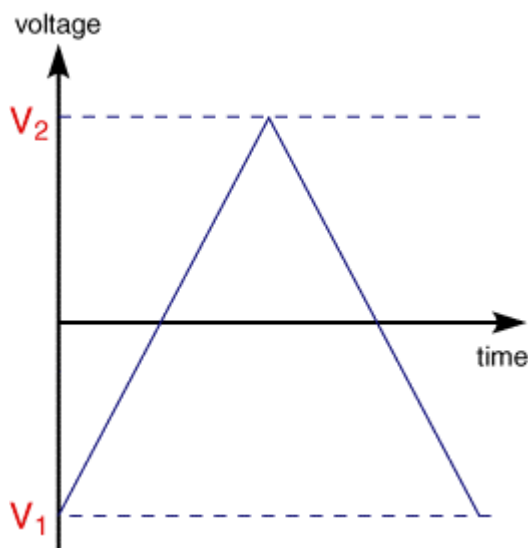
This leaves the question as to what would happen if the electron transfer processes were 'slow' (relative to the voltage scan rate). For these cases the reactions are referred to as quasi-reversible or irreversible electron transfer reactions. The figure below shows a series of voltammograms recorded at a single voltage sweep rate for different values of the reduction rate constant (k_{red})



In this situation the voltage applied will not result in the generation of the concentrations at the electrode surface predicted by the Nernst equation. This happens because the kinetics of the reaction are 'slow' and thus the equilibria are not established rapidly (in comparison to the voltage scan rate). In this situation the overall form of the voltammogram recorded is similar to that above, but unlike the reversible reaction now the position of the current maximum shifts depending upon the reduction rate constant (and also the voltage scan rate). This occurs because the current takes more time to respond to the applied voltage than the reversible case.

5.1.9 Cyclic Voltammetry

Cyclic voltammetry (CV) is very similar to LSV. In this case the voltage is swept between two values (see below) at a fixed rate, however now when the voltage reaches V_2 the scan is reversed and the voltage is swept back to V_1



Theory of Cyclic Voltammetry

Voltammetry is the electrochemical technique in which the current at an electrode is measured as a function of the potential, applied to the electrode. The potential is varied in some systematic manner and the resulting current-potential plot is called a voltammogram.

- Cyclic voltammetry is generally used to study the electrochemical properties of an analyte in solution

- Voltammetry can be used to analyze any chemical species that is electroactive, i.e., that can be made to oxidize or reduce.
- The potential of the electrode is the controlled parameter that causes the chemical species to be oxidized or reduced.
- The potential can be thought of as “electron pressure” which either forces a species in solution to gain an electron (reduction) or lose an electron (oxidation). As the potential of the electrode becomes more negative, it becomes more strongly reducing. Conversely, as the potential becomes more positive, it becomes more strongly oxidizing (Figure 5.1.9.1). Therefore, the redox reaction taking place on the electrode can be controlled by controlling the electrode potential.
- The current, on the other hand, is simply a measure of electron flow. The current is due to electron transfer that takes place when an oxidation or reduction occurs on the electrode surface. This type of current is termed **Faradaic**. In voltammetry, the Faradaic current is proportional to concentration.
- The current due to reduction (cathodic current) is, by convention, assigned a positive sign. The current due to oxidation (anodic current) is assigned a negative sign.

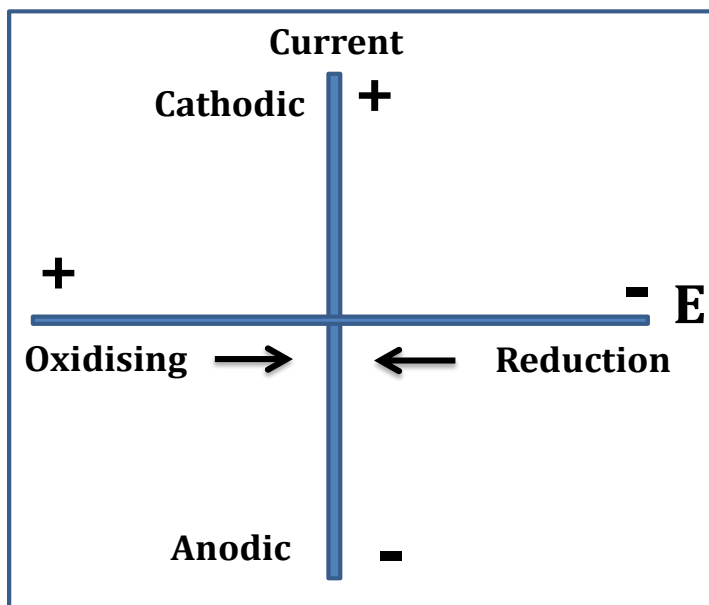
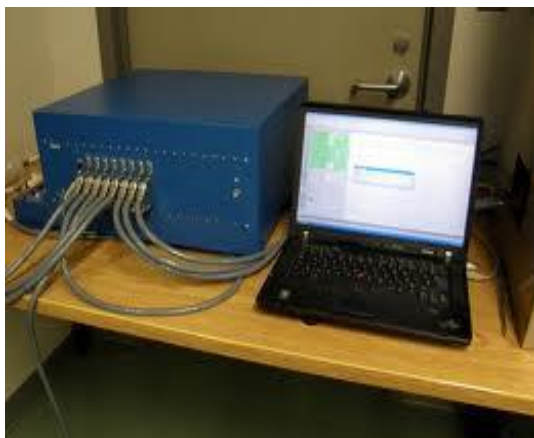


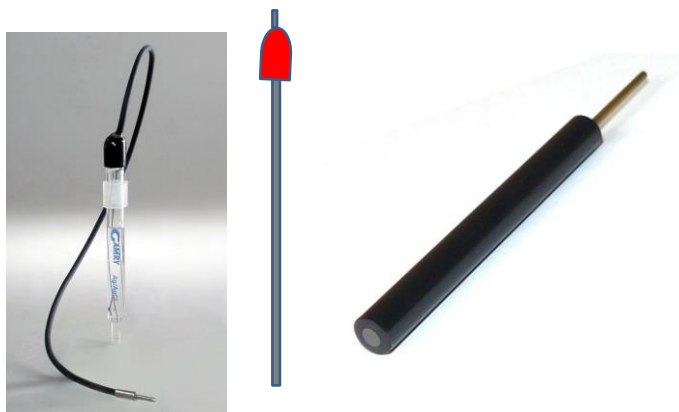
Figure 5.1.9.1: Convention for plotting voltammograms

Instrumentation

- ✓ A CV system consists of an electrolysis cell, a potentiostat, a current-to-voltage converter, and a data acquisition system.

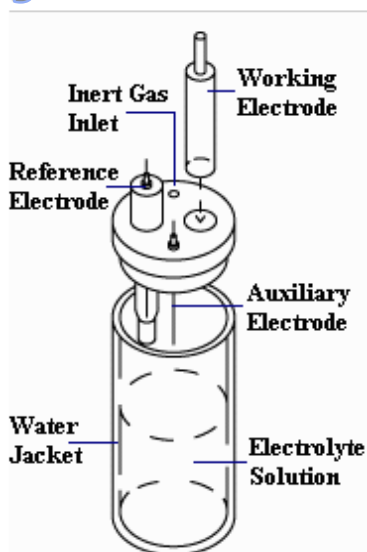


- ✓ The electrolysis cell consists of a working electrode, counter electrode, reference electrode, and electrolytic solution.



- ✓ The working electrode's potential is varied linearly with time, while the reference electrode maintains a constant potential.
- ✓ The counter electrode conducts electricity from the signal source to the working electrode. The purpose of the electrolytic solution is to provide ions to the electrodes during oxidation and reduction.
- ✓ A potentiostat is an electronic device which uses a dc power source to produce a potential which can be maintained and accurately determined, while allowing small currents to be drawn into the system without changing the voltage.
- ✓ The current-to-voltage converter measures the resulting current, and the data acquisition system produces the resulting voltammogram.

Cyclic voltammetry



Working electrode is the electrode at which the analyte is oxidized or reduced

The potential is controlled between the working and **reference electrode**

Electrolysis current passes between the working electrode and a **counter electrode**

An excess of a nonreactive electrolyte called a **supporting electrolyte** which carries the current

Migration of ions may be due to

- (i) **Diffusion**
- (ii) **Convection (unstirred solution, no convection)**
- (iii) **Transference (reduced by using supporting electrolyte)**

Convection may be eliminated but not transference.

If electrolysis is carried out in excess of indifferent electrolyte whose ions are not discharged at applied potential, then the entire amount of current will be carried by the ions of indifferent electrolytes. Such electrolytes are **supporting electrolytes**.

Therefore using supporting electrolyte, transference (t) becomes unity.

Electrodes used in CV

Electrodes are static and sit in unstirred solutions during cyclic voltammetry. This "still" solution method results in cyclic voltammetry's characteristic diffusion controlled peaks. Stirring the solution between cyclic voltammetry traces is important as to supply the electrode surface with fresh analyte for each new experiment. Since cyclic voltammetry usually alters the charge of the analyte, it is common for reduced or oxidized analyte to precipitate out onto the electrode. It is often necessary to clean electrodes between scans.

Common materials for working electrodes include glassy carbon, platinum, and gold. These electrodes are generally encased in a rod of inert insulator with a disk exposed at one end. A regular working electrode has a radius within an order of magnitude of 1 mm. Having a controlled surface area with a defined shape is important for interpreting cyclic voltammetry results.

To run cyclic voltammetry experiments at high scan rates, a regular working electrode is insufficient. High scan rates create peaks with large currents and increased resistances which result in distortions. Ultramicroelectrodes can be used to minimize the current and resistance.

The counter electrode, also known as the auxiliary or second electrode, can be any material which conducts easily and won't react with the bulk solution.

The reference electrode provides a stable potential with which the potential of the working electrode is compared. The most common types of reference electrodes are the saturated calomel electrode and the silver/silver chloride electrode.

Nitrogen purging

The cell should incorporate a mechanism for deaeration of the solution by purging with nitrogen. Molecular oxygen is a reducible species and will therefore contribute to the background current if allowed to remain in solution. A purge time of 3-5 minutes is generally adequate. The nitrogen should be pre-saturated with the supporting electrolyte prior to purging to avoid evaporative losses and temperature changes. The cell should be blanketed with nitrogen while the polarogram is being run to avoid oxygen intrusion.

Addition of Supporting Electrolyte:

In most cases, an electrolyte is added to the sample before analysis. This is done to insure the conductive media which polarography requires (minimum 10^{-3} M).

For Aqueous Solutions – KCl (supporting electrolyte)

For Non-Aqueous Solutions – TBAP Tetrabutylammoniumperchlorate (Supporting electrolyte)

Cyclic Voltammogram:

The potential of the working electrode is measured against a reference electrode which maintains a constant potential, and the resulting applied potential produces an excitation signal such as that of figure 5.1.9.2.

- ❖ In the forward scan of figure 5.1.9.2, the potential first scans negatively, starting from a greater potential (a) and ending at a lower potential (d).
- ❖ The potential extrema (d) is called the **switching potential**, and is the point where the voltage is sufficient enough to have caused an oxidation or reduction of an analyte.
- ❖ The reverse scan occurs from (d) to (g), and is where the potential scans positively.
- ❖ Figure 5.1.9.2 shows a typical reduction occurring from (a) to (d) and an oxidation occurring from (d) to (g).
- ❖ It is important to note that some analytes undergo oxidation first, in which case the potential would first scan positively. This cycle can be repeated, and the scan rate can be varied. The slope of the excitation signal gives the scan rate used.

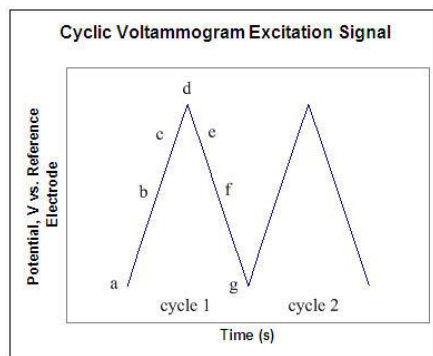


Figure 5.1.9.2: CV Excitation Signal

- In cyclic voltammetry, the electrode potential ramps linearly versus time as shown. This ramping is known as the **experiment's scan rate** (V/s).
- As the waveform shows, the forward scan produces a current peak for any analytes that can be reduced (or oxidized depending on the initial scan direction) through the range of the potential scanned.

- The current will increase as the potential reaches the reduction potential of the analyte, but then falls off as the concentration of the analyte is depleted close to the electrode surface.
- If the redox couple is reversible, then when the applied potential is reversed, it will reach the potential that will reoxidize the product formed in the first reduction reaction, and produce a current of reverse polarity from the forward scan. This oxidation peak will usually have a similar shape to the reduction peak.
- As a result, information about the redox potential and electrochemical reaction rates of the compounds are obtained.

For instance if the electronic transfer at the surface is fast and the current is limited by the diffusion of species to the electrode surface, then the current peak will be proportional to the square root of the scan rate. This relationship is described by the Cottrell equation.

$$i = \frac{nFAc_j^0\sqrt{D_j}}{\sqrt{\pi t}}$$

where,

i = current, in unit A

n = number of electrons (to reduce/oxidize one molecule of analyte j , for example)

F = Faraday constant, 96,485 C/mol

A = area of the (planar) electrode in cm^2

c_j^0 = initial concentration of the reducible analyte j in mol/cm^3 ;

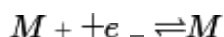
D_j = diffusion coefficient for species j in cm^2/s

t = time in s.

Important Parameters of Cyclic Voltammogram

A cyclic voltammogram is obtained by measuring the current at the working electrode during the potential scans. **Figure 5.1.9.3** shows a cyclic voltammogram resulting from a single electron reduction and oxidation.

Consider the following reversible reaction:



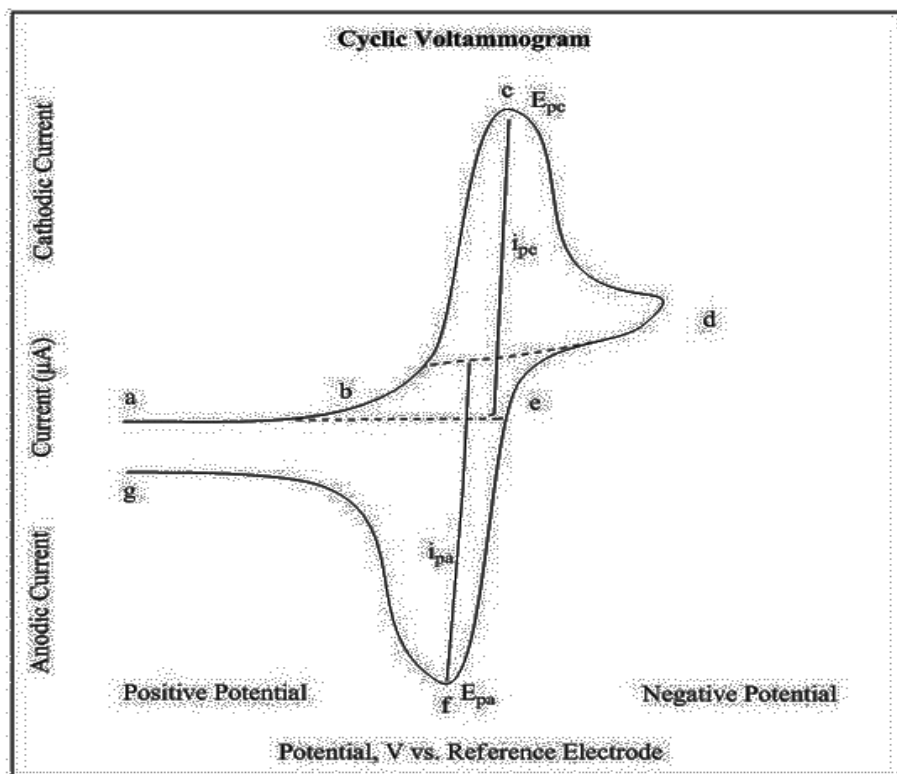


Figure 5.1.9.3: Voltammogram of a Single electron oxidation-reduction

In Figure 5.1.9.3, the reduction process occurs from (a) the initial potential to (d) the switching potential. In this region the potential is scanned negatively to cause a reduction. The resulting current is called cathodic current (i_{pc}). The corresponding peak potential occurs at (c), and is called the cathodic peak potential (E_{pc}). The E_{pc} is reached when all of the substrate at the surface of the electrode has been reduced.

After the switching potential has been reached (d), the potential scans positively from (d) to (g). This results in anodic current (i_{pa}) and oxidation to occur. The peak potential at (f) is called the anodic peak potential (E_{pa}), and is reached when all of the substrate at the surface of the electrode has been oxidized.

- 1. The Important parameters of a cyclic voltammogram** are the magnitudes of the peak currents, i_{pa} and i_{pc} and the potentials at which the peaks occur, E_{pa} and E_{pc} .

2. One method for measuring i_p involves extrapolation of **base current**. The establishment of correct baseline is essential for the accurate measurement of peak currents.

3. Reversible system

- (i) A redox couple in which both species rapidly exchange electrons with the working electrode is termed an electrochemically reversible couple. Such a couple can be identified from a cyclic voltammogram by measurement of the potential difference between the two peak potentials. The equation that applies to a system that is both electrochemically and chemically reversible:

$$\Delta E_p = E_{pa} - E_{pc} \approx 0.058/n$$

Where n is the number of electron transferred and E_{pa} and E_{pc} are the cathodic and anodic peak potentials respectively, in volts.

(Or)

$$|E_{pc} - E_{pa}| = 57\text{mv}/n \text{ for an } n \text{ electron process.}$$

This $0.058/n$ separation of peak potentials is **independent of scan rate for a reversible couple**, but is slightly dependent on switching potential and cycle number.

- (ii) When the scan rate v is increased, i_{pa} and i_{pc} both increase in proportion to $v^{1/2}$. Plots of **i_{pa} and i_{pc} versus $v^{1/2}$ should be linear** with intercepts at the origin **for a reversible couple**.
- (iii) The values of i_{pa} and i_{pc} are **similar in magnitude for a reversible couple** with no kinetic applications. That is,

$$i_{pa}/i_{pc} \approx 1$$

4. Formal Reduction Potential

The potential midway between the two peak potentials is the formal reduction potential (corrected for the reference electrode being used) of the couple:

$$E^0 = (E_{pa} + E_{pc})/2$$

5. Irreversible System

Electrochemical irreversibility is caused by slow electron exchange of the redox

species with the working electrode. **Electrochemical irreversibility** is characterised by a separation of peak potentials that is greater than $0.059/n$ V and that is dependent on the scan rate.

6. Thin Layer Voltammetry

Thin layer voltammetry (TLV) is a very slow technique, and is used in quantitative studies of electrode reaction with small heterogeneous rate constants. It is applicable to examination of metal deposition and adsorption. In combination with optical monitoring of products, it is proved helpful in mechanism studies of redox controlled solution chemistry.

A plot of I vs E defines the ideal TLV

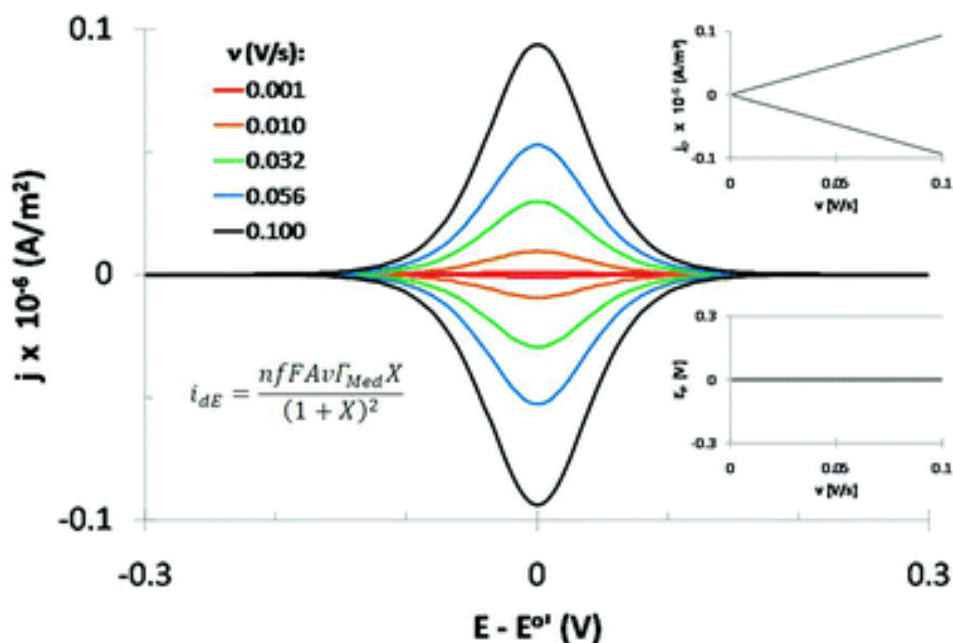


Figure 5.1.9.4. Thin Layer Voltammetry for a reversible one- electron process.

The peak is symmetrical about E° and that the current drops to zero after the potential passes through E_p and I_p is directly proportional to scan rate. The number of coulombs required to oxidise a substance within a thin layer cell is given by Faraday's law

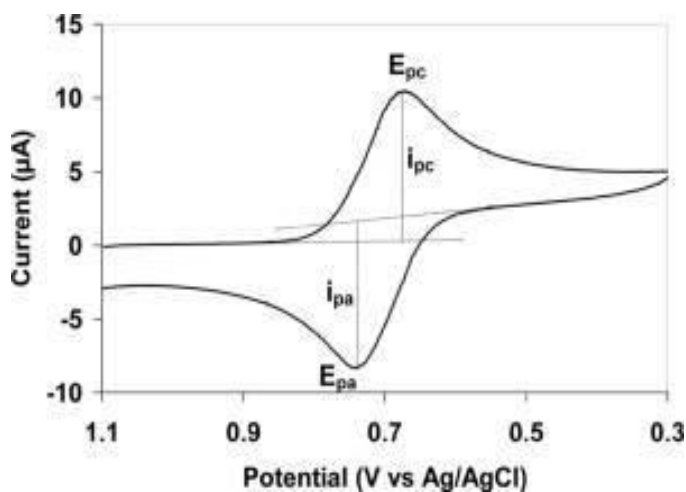
$$Q = nFVC_R$$

V = Volume of thin layer cavity.

C = Initial concentration of R

If we reverse the scan direction, a reduction wave is obtained for the reverse reaction which is an exact mirror image of the oxidation wave. This result is obtained only for a reversible electrode reaction.

Characterization

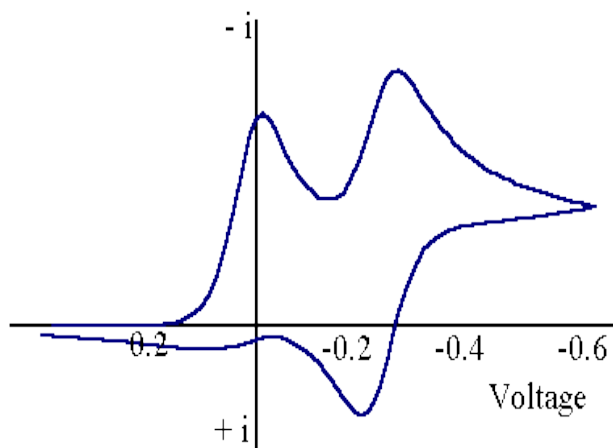


For reversible reactions: i_{pc}/i_{pa} is unity

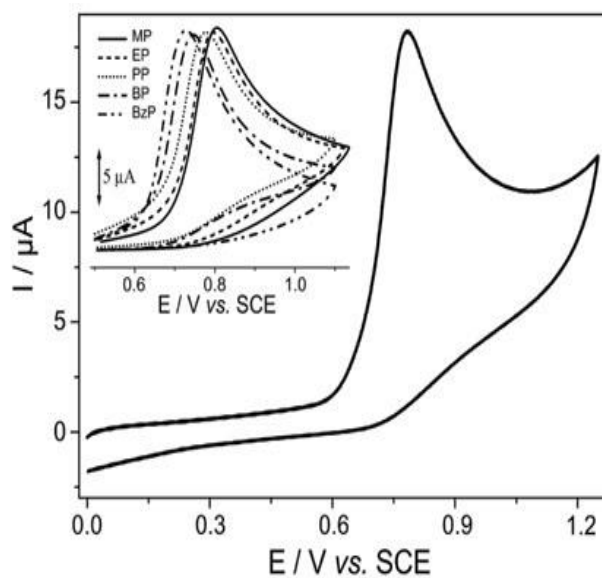
E_p remains constant with respect to v

i_{pc}/i_{pa} vs $v^{1/2}$ should be linear

$\Delta E_p = (E_{pa} + E_{pc})/2$; for 1 electron reaction $\Delta E = 59 \text{ mV}$

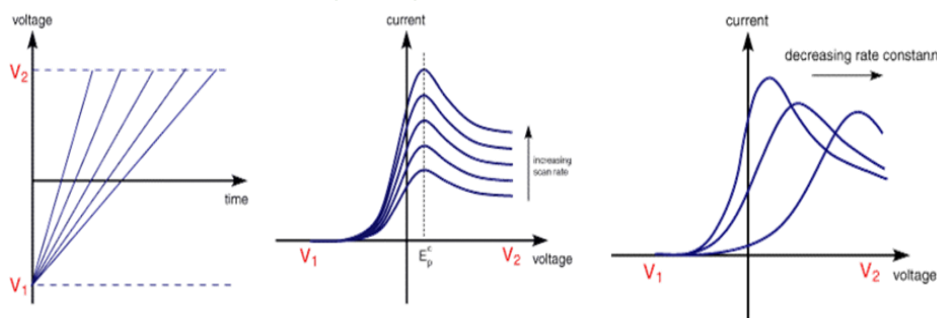


For Quasireversible system



For Irreversible System

What happens to i_p and E_p when scan Rate (v) is increased ?



For quasi or irreversible reactions

i_{pc}/i_{pa} is not unity

E_p shifts positive

Based on ΔE standard heterogeneous rate constant (k_s) can be calculated using Nicholson theory

- ❖ When such reversible peaks are observed, thermodynamic information in the form of half cell potential $E^0_{1/2}$ can be determined.
- ❖ When waves are semi-reversible such as when i_{pa}/i_{pc} is less than or greater than 1, it can be possible to determine even more information especially kinetic processes.
- ❖ When waves are non-reversible, it is impossible to determine what their thermodynamic $E^0_{1/2}$ is with cyclic voltammetry. This $E^0_{1/2}$ can be determined, however it often requires equal quantities of the analyte in both oxidation states.
- ❖ When a wave is non-reversible cyclic voltammetry can not determine if the wave is at its thermodynamic potential or shifted to a more extreme potential by some form of overpotential. The couple could be irreversible because of a chemical process, a common example for transition metals is a shift in the geometry of the coordination sphere. If this is the case, then higher scan rates may show a reversible wave. It is also possible that the wave is irreversible due to a physical process most commonly some form of precipitation .

VARIATIONS

In some experiments, **an electroactive species is fixed to the surface of the electrode, for instance in microparticle voltammetry.** Potentiodynamic techniques also exist that add low-

amplitude ac perturbation to a potential ramp and measure variable response in a single frequency (ac voltammetry) or in many frequencies simultaneously (**Potentiodynamic Electrochemical Impedance Spectroscopy**). The response in alternating current is two-dimensional – it is characterised by amplitude and phase. The amplitude and phase depend differently on frequency for constituents of ac response attributed to **different processes (charge transfer, diffusion, double layer charging, etc.)**. Frequency response analysis enables simultaneous monitoring of the various processes that contribute to the potentiodynamic ac response of electrochemical system.

DISTINCTIONS

Cyclic voltammetry is not a hydrodynamic technique. In a hydrodynamic technique flow is achieved at the electrode surface by stirring the solution, pumping the solution, or rotating the electrode as is the case with rotating disk electrodes and rotating ring-disk electrodes. These techniques target steady state conditions which appear the same scanned from the positive or the negative, thus limiting them to linear sweep voltammetry.

If a voltammetric measurement is made using a dropping mercury electrode, the technique is termed polarography.¹ The dropping mercury electrode (DME), which consists of mercury flowing through a capillary and emerging from the orifice as a continual series of mercury drops, is deserving of this special consideration because it is the most useful electrode for analytical purposes. This is because the mercury drips expose a clean, reproducible surface of constant area to the solution.

5.1.10. CHRONOAMPEROMETRY

Chronoamperometry is an electrochemical technique in which the potential of the working electrode is stepped and the resulting current from faradaic processes occurring at the electrode (caused by the potential step) is monitored as a function of time.

When a potential step large enough to cause an electrochemical reaction is applied to an electrode, the current changes with time. The study of this current response as a function of time is called Chronoamperometry (CA).

A solid electrode is immersed in a solution containing the oxidized form (Ox) of a redox couple at some known initial concentration (C_0), say 1.0 mM. Initially the electrode is poised at a potential (E_i) well positive of the E_0' for the Red/Ox couple, assuring that only Ox is present in

the solution. The solution also contains an excess of some inert electrolyte, to assure that flux to the electrode is strictly diffusion controlled.

At a time designated t_0 , the potential is stepped to a value significantly more negative than the E_0' for the redox couple (Es), and Ox in the vicinity of the electrode is immediately converted (assuming reversible kinetics) to Red.

As a consequence of the conversion, the concentration of Ox directly adjacent to the electrode is reduced to zero from its initial, bulk concentration of 1.0 mM. This leads to the formation of a concentration gradient for Ox, through which Ox from the bulk of solution must diffuse to the electrode surface, where it is immediately reduced to Red. The longer the electrode remains at this reducing potential, the further away from the electrode the region depleted of Ox, called the diffusion layer, extends. A similar, but opposite gradient exists for Red, which after formation is free to diffuse away from the electrode surface, reaching further into the bulk as time passes. This situation is summarized in Figure.5.1.10.1

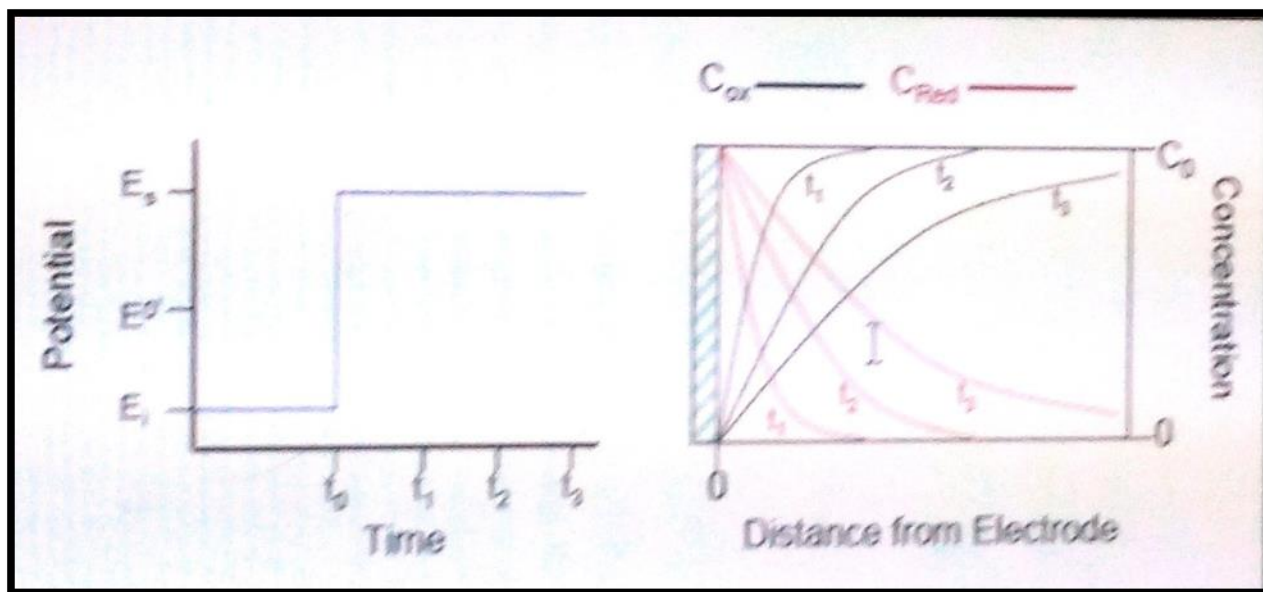


Figure.5.1.10.1

the faradaic current under diffusion controlled conditions is related directly to the concentration gradient, $\partial C_i / \partial x$, evaluated at $x = 0$. Thus, as the slope of the concentration profile for Ox

decreases with time following the potential step, so the current will be observed. In the example above, the concentration of Ox at the surface was driven immediately to zero for a step to a potential well negative of the E_0' for the couple. In general for a reversible system (reduction), the ratio of [Red] to [Ox] at the surface is given by the Nernst equation at any potential, with the ratio reaching 100:1 at a value 118 mV more negative than the E_0

Chronoamperometry experiments are most commonly either *single potential step*, in which only the current resulting from the forward step as described above is recorded, or *double potential step*, in which the potential is returned to a final value (E_p) following a time period, usually designated as τ , at the step potential (E_s).

The most useful equation in chronoamperometry is the Cottrell equation, which describes the observed current (planar electrode) at any time following a large forward potential step in a reversible redox reaction (or to large overpotential) as a function of $t^{-1/2}$.

$$i_t = \frac{nFA C_0 D_0^{1/2}}{\pi^{1/2} t^{1/2}}$$

where n = stoichiometric number of electrons involved in the reaction; F = Faraday's constant (96,485 C/equivalent), A = electrode area (cm^2), C_0 = concentration of electroactive species (mol/cm^3), and D_0 = diffusion constant for electroactive species (cm^2/s).

APPLICATION

The CA technique is also useful for determining the rate constant of the slow step in a reaction. An example is the ECE mechanism, a reaction in which the first step is an electrochemical reaction (E), the intermediate step is a chemical reaction (C) that forms an electrochemically active species and the final step is the electrochemical reaction (E) of that species. Alberts and Shain¹² have shown that for the ECE mechanism, the apparent value of n can be calculated from the Cottrell equation:

$$n = \frac{i_t \pi^{1/2} t^{1/2}}{FAC_0 D_0^{1/2}}$$

and that from a simple working curve, values of the rate constant k can be calculated. Many researchers have used this approach to help determine reaction mechanisms and kinetic constants

5.1.11 Chronocoulometry

In a CC experiment, the charge used in an oxidation or reduction is measured and then plotted with respect to time. The excitation waveform is the same as that used for CA, but the resulting curve shows an increase of charge with time, rather than the decrease of current with time seen in CA.

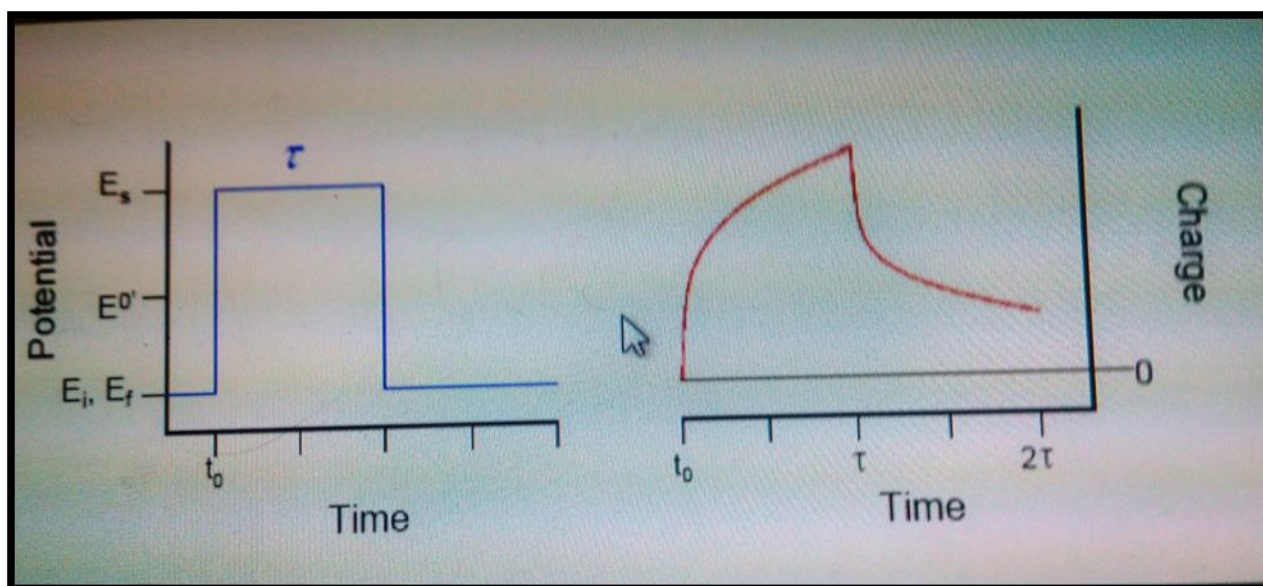


Figure 5.1.11.1

In a chronocoulometry experiment, the total charge (Q) that passes during the time following a potential step is measured as a function of time. Q is obtained by integrating the current, i , during the potential step. For a well behaved system (diffusion only), the charge observed following a potential step to a point significantly more negative (for a reduction) of the E^0 , is described by the integrated Cottrell equation, known as the Anson equation

$$Q_d = \frac{2nFACoDo^{1/2}t^{1/2}}{\pi^{1/2}}$$

Potential step programs in chronocoulometry are identical to those used in chronoamperometry. The most obvious advantage of recording charge rather than current is that Q increases with time following a potential step, reaching a maximum at the end of the step, where the most meaningful experimental data generally is observed.

Measuring Charge Since CC is based on the same assumptions as CA, the charge at any time can be calculated by simply integrating the Cottrell equation and adding corrections for the charge due to the double layer and any interfacial interactions (for example, adsorption). The resulting equation is:

$$Q = \frac{2nFACoD\tau^{1/2}}{\pi^{1/2}} + Q_{dl} + Q_i$$

Q = charge n = number of electrons in the reaction F = Faraday's constant A = electrode area Co = bulk concentration D = diffusion coefficient Q_{dl} = charge due to double layer Q_i = charge due to interfacial interaction. If Q is plotted versus $\tau^{1/2}$ the result is the Anson plot shown in Figure 16. If we assume that no adsorption occurs, the slope of the line is:

$$Q = \frac{2nFACoD\tau^{1/2}}{\pi^{1/2}}$$

and the intercept is the double layer charge. If adsorption does occur, the line in Figure 5.1.11.2 would be offset by the charge due to this interfacial phenomenon. It is easy to differentiate between the charge due to the double layer and that due to adsorption. You merely run the same experiment on an electrolyte blank. The blank will not exhibit the adsorption effect but will exhibit the double layer charging current (see 5.1.11.2).

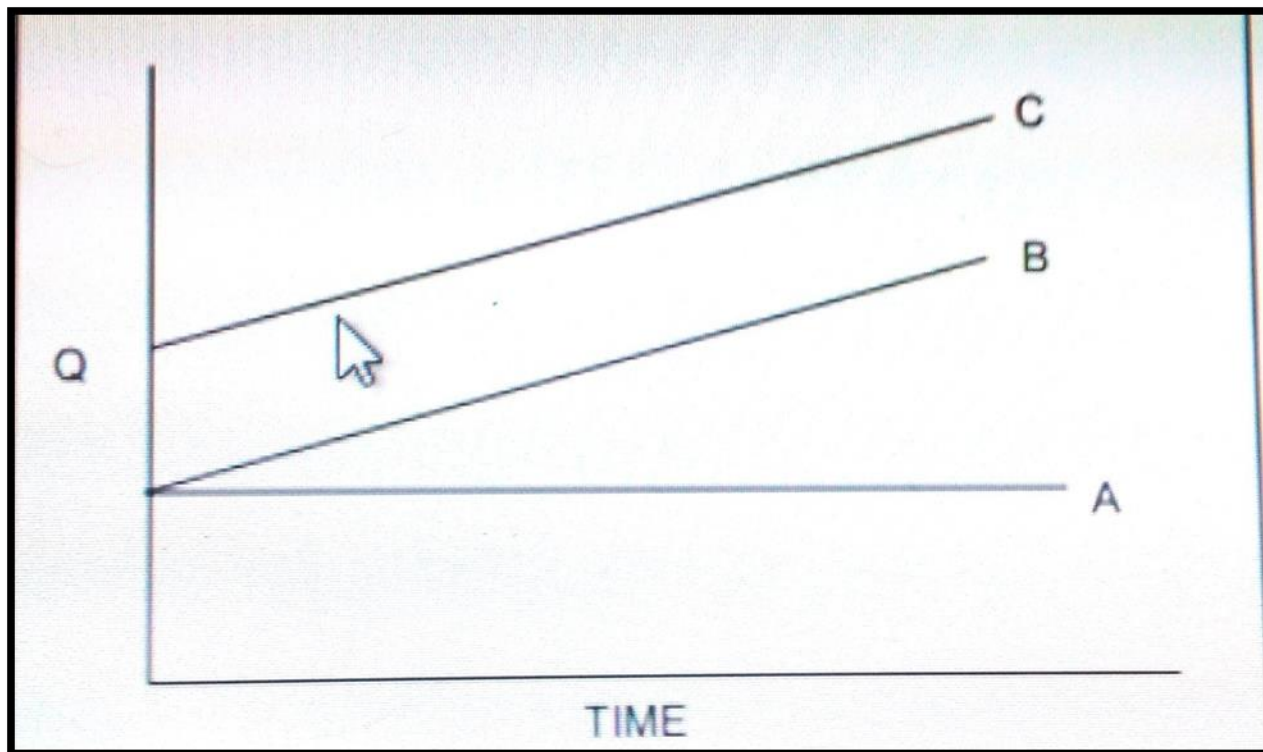


Figure 5.1.11.2

5.1.12.CHRONOPOTENTIOMETRY (CP)

Chronopotentiometry (CP) is the most basic constant current experiment. Chronopotentiometry (CP) is a technique in which a constant current, or a current step, is applied to the electrode and the resulting potential change is plotted versus time. Data Interpretation A typical chronopotentiogram is shown in Figure 5.1.12

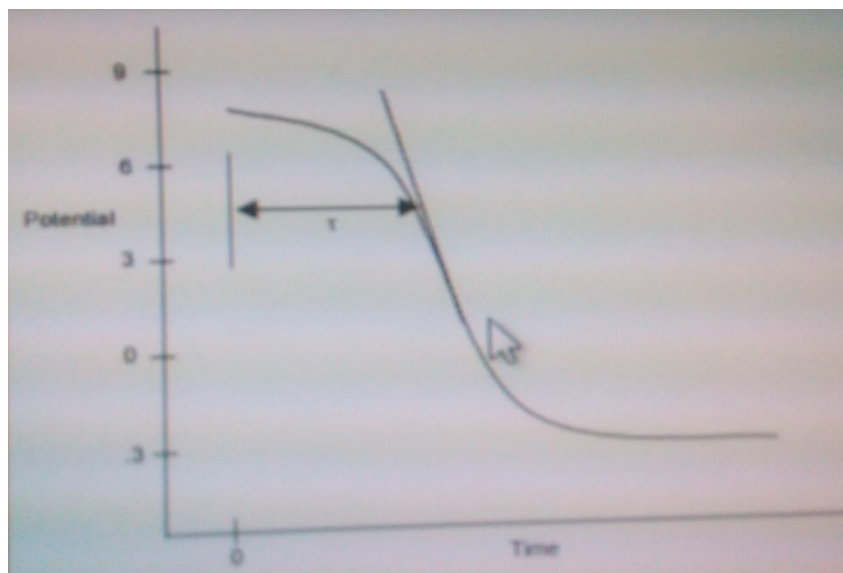


Figure 5.1.12.1

A typical chronopotentiogram is shown in Figure 5.1.12. You can understand the curve by considering the events at the electrode surface. In order for current to flow at the electrode, a reaction must take place. The electrode potential is the minimum required to drive a reaction that will support the constant current. If a species is present which is oxidized or reduced more easily than the solvent, the electrode will be driven to the potential appropriate for that reaction. As the concentration of that species at the electrode surface moves toward zero, the potential of the electrode moves toward the next stable potential of the system. The chronopotentiogram in Figure 17 is for a single species in an acid solution. The electrode assumes an initial potential required to reduce metal ion M^{++} to metal ion M^+ . As the concentration of M^{++} at the electrode surface decreases, the potential of the electrode gradually moves more negative until the metal ion concentration is depleted at the electrode surface. As soon as no more metal ion exists at the surface, the electrode potential shifts to the potential needed to support a reaction that will give rise to the desired current. In this case, that is the reduction of H^+ to H_2 and a potential of -0.3 V versus SCE. rise to the desired current. In this case, that is the reduction of H^+ to H_2 and a potential of -0.3 V versus SCE. Transition time is the time between cell turn on and the rapid change in potential. This quantity is

related by the Sand equation to the diffusion constant, the areas of the electrode, the bulk concentration and the number of electrons involved in the reaction:

$$\tau^{1/2} = \frac{\pi^{1/2} n F A D^{1/2}}{2i}$$

i = current τ = transition time F = Faraday's constant A = area D = diffusion constant of species
 C_b = concentration of species Examination of the Sand equation shows that the transition time is proportional to the concentration since all other values are constant for a given experiment.

Multiple CPs In a series of CP experiments, you might vary either the concentration of the species or the applied constant current. If you vary the current, you might want to plot $\tau^{1/2}$ versus i . For a Nernstian system under diffusion control, this plot should be a horizontal line (see Figure 5.1.12.2). If the system is non-Nernstian, the Sand equation does not apply and you will see deviations from the horizontal line.

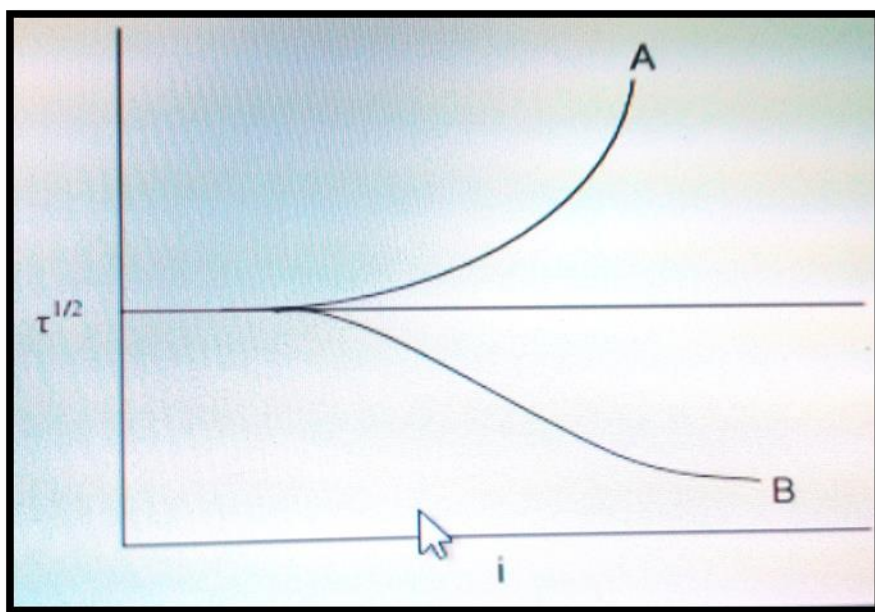


Figure 5.1.12.2

A positive (upward) deviation is usually due to adsorption effects. The transition time is longer than under diffusion control because of the adsorbed species. As the current is increased, the transition time does not decrease proportionally. It is always larger due to the

adsorbed species. At very large currents the adsorbed species makes a more pronounced contribution to the transition time (see Figure 5.1.12.2). A negative (downward) deviation is usually due to a preceding reaction. This is because the transition time value will be smaller if a preceding reaction limits the concentration of the species at the electrode. The reaction is kinetically controlled and not diffusion controlled. If the concentration of the species is lower it will take less time than the Sand equation predicts for the transition to a different potential. At higher currents, the deviation becomes greater as the difference in time required to produce the electroactive species remains the same.

5.1.13 Theory of Polarography

It is an electro analytical method of studying the composition of dilute electrolytic solution by plotting current voltage curves (Polarogram).

It is based on the electrolysis of a solution using a cell which consists of a polarizable electrode (working electrode or microelectrode) such as dropping mercury electrode and a non polarizable electrode (reference electrode) such as calomel, Ag|AgCl, a mercury pool.

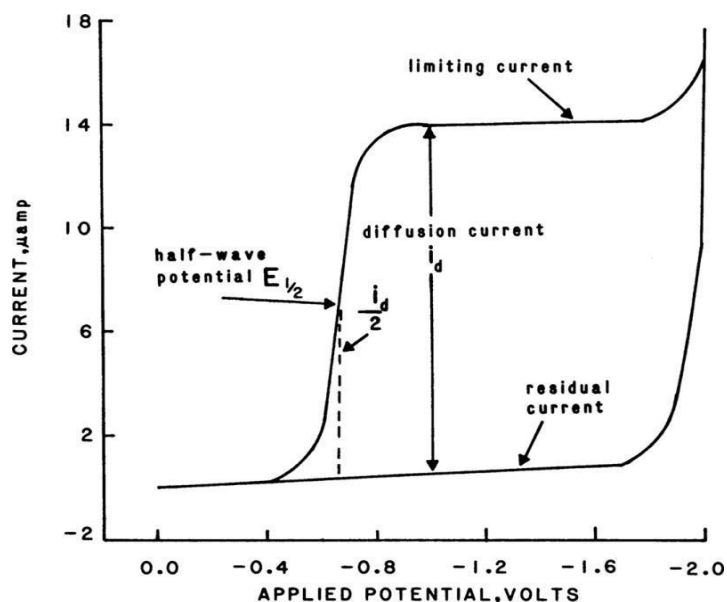


Figure 5.1.13.1: Polarogram

The electrodes are connected to a variable voltage source (0-2V). A very sensitive ammeter or galvanometer is included in the circuit. The resulting changes in current through the solution is noted. The potential at the working electrode is measured by a voltmeter relative to reference electrode.

The rate determining step in discharge of ions is **diffusion** (i.e)

- Depends upon **concentration polarization** produced at the microelectrodes.
- The **increase or decrease of electrode potential from the reversible value as a result of change in concentration in the vicinity of electrode**
- **Slow diffusion** of ions towards or away from the electrode.

There will be no concentration polarization if change in concentration of ions is compensated instantaneously by quick migration of ions either by agitation of solution or by raising temperature.

Diffusion Current and half-Wave potential

The current density at which rapid increase of potential takes place is known as i_d or limiting current density,

i_d = limiting rate at which particular ionic species can be discharged.

$$i_d = (DnF/\delta)C^*$$

The electrolysis current obtained as i_d is referred to as **diffusion current**.

The limiting current = height of curve = concentration of electroactive species.

If the test solution contains a single electroactive species, current voltage curve (polarogram) will have the shape.

The potential at centre of rising part of the wave = half wave potential ($E_{1/2}$) which is characteristic of the species being discharged and is independent of concentration

From Ilkovic equation

$$E = E_{1/2} - (0.0591/n) \log (i/i_d - i)$$

5.2.1 Synchronous fluorescence spectroscopy (SFS)

Synchronous fluorescence spectroscopy (SFS) plays an important role in the simultaneous analysis of multi component samples due to the remarkable advantages of spectral simplification, light scattering reduction, and selectivity improvement over conventional fluorescence spectroscopy.

In conventional fluorescence spectroscopy, two types of spectra are generally measured. When a sample is excited at a fixed wavelength λ_{ex} , an emission spectrum is produced by recording the emission intensity as a function of the emission observation is conducted at the fixed λ_{em} . The broad nature and spectral overlap of conventional fluorescence spectra can be overcome, and enhanced selectivity can be obtained using synchronous fluorescence spectroscopy (SFS).

In SFS, the λ_{ex} and λ_{em} are scanned simultaneously. Depending on the scan rate three basic types of SFS technique are possible.. An excitation spectrum may be obtained when λ_{ex} is scanned while the (i) Constant-wavelength SFS is very simple technique as the scan rate is constant for both monochromators and, therefore, a constant wavelength interval, $\Delta\lambda$, is kept between λ_{em} and λ_{ex} . (ii) Variable-angle SFS is known as the second technique. The excitation and emission wavelengths may be varied simultaneously but at different rates. (iii) The third technique, constant-energy SFS, has not been used much. SFS is often considered as a convenient technique for the analysis of multi-component samples without resorting to tedious separation procedures.

5.2.2 Spectral hole burning

It is the frequency selective bleaching of the absorption spectrum of a material, which leads to an increased transmission (a "spectral hole") at the selected frequency.

Two basic requirements, that must be met for the observation of this phenomenon, are:

1. The spectrum is in homogeneously broadened
2. The material undergoes, subsequent to light absorption, a modification which changes its absorption spectrum. Typical materials include dye molecules dissolved in suitable host matrices and the frequency selective irradiation is usually realized by a narrow band laser.

5.2.3 Flow cytometry

In biotechnology, **flow cytometry** is a laser- or impedance-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus.

5.2.4. Fluorimeters

Fluorescence is the emission of visible light by a substance that has absorbed light of a different wavelength. The emitted photon has a longer wavelength.

fluorometer or fluorimeter is a device used to measure parameters of fluorescence: its intensity and wavelength distribution of emission spectrum after excitation by a certain spectrum of light. These parameters are used to identify the presence and the amount of specific molecules in a medium. Modern fluorometers are capable of detecting fluorescent molecule concentrations as low as 1 part per trillion.

INSTRUMENTATION

The essential components of an instrument used to measure fluorescence of the sample are:

- Excitation light sources –Lamps –low pressure mercury vapour lamp, Xenon arc lamp and various sources of lasers.
- Filters or Monochromators –Used for wavelength selection of both the excitation beam and the resulting fluorescence radiation.
- Transducers-The typical luminescence signal is of low intensity ; large amplifier gains are thus required for its measurement. Photomultiplier tubes are the most common transducers in sensitive fluorescence measurements.
- Sample holder –Both cylindrical and rectangular cells fabricated of glass or silica employed for fluorescence measurements.
- Detector
- Readout device

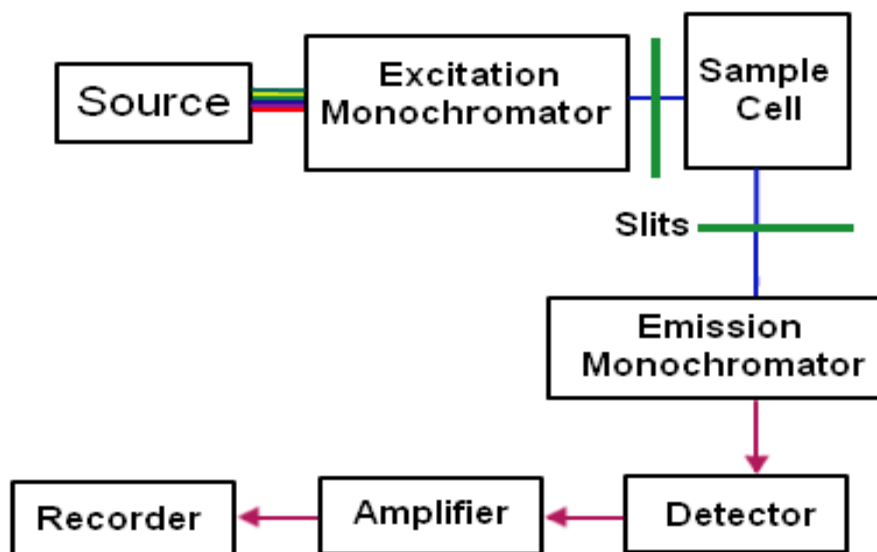


Figure 5.2.4.1.Components of a fluorometer

Exercises

1. Match the following

1.Coulometry

2.Ion selective electrode

3.Polarography

4.amperometry

a.dropping mercury electrode

b.current efficiency

c. dead stop endpoint

d.membrane potential

e.condurometer

Ans : 1.b; 2.d; 3.a;4.c

2. List out the various types of electrodes used in potentiometric sensors.

3. Describe briefly the amperometric sensors.

4. Briefly explain the theory of cyclic voltammetry.

5. What are the different wave forms used in voltammetry?
6. Explain why an excess of supporting electrolyte is added to electroactive species in polarographic analysis?
7. Outline briefly the chronoamperometry electrochemical technique.
8. Give an account of chronocoulometry technique.
9. With a chronopotentiogram ,explain the process involved in it.
10. write short notes on the following
 - a. Synchronous fluorescence spectroscopy
 - b. Spectral hole burning
 - c. Flow cytometry
 - d. Fluorimeter

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