UGHN-110 FOOD ANALYSIS

BLOCK- I Introduction to Sampling and Analytical Techniques	
Unit I:	**Sample and Sampling Techniques**
Unit II:	**Analytical Techniques**
BLOCK- II Introduction to Photometric, Electrophoresis and Chromatography	
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U.P.Rajarshi Tandon Open University, Prayagraj

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FOOD ANALYSIS

BLOCK

1

BLOCK- I INTRODUCTION TO SAMPLING AND ANALYTICAL TECHNIQUES 01-46

U.P.Rajarshi Tandon Open University, Prayagraj

UGHN-110

FOOD ANALYSIS

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 UGHN-110 Food Analysis

BLOCK INTRODUCTION

In this block we have cover all the 4 units and as we know that collection of the correct sample for the analysis and process by which the researcher chooses the sample is a 'sampling techniques'. Essentially, there are two forms of sampling technique: 1) probability sampling which is focused on chance occurrences (such as random numbers, spinning a coin, etc.); and 2) non-probability sampling that based on preference of researcher, open & active population. Any of the approaches for nonprobability sampling include: purposeful sampling, convenient sampling, or quota sampling. Random sampling method is a type of probability sampling (such as simple random sample or stratified random sample). It is necessary to consider the various forms of sampling used in clinical trials, and to specifically discuss the approach. The researcher should not misrepresent the manuscript sampling method (e.g. using the term 'random sample' when the researcher has used convenience sample. The method of sampling will depend on the question of research.

The chemicals used in research are in the form of solutions that require purchasing or planning. The actual concentration value is not important for certain purposes but in some situations, the concentration of the solution and its preparation process need to be as precise as practicable. The comparison portion of the Flinn Laboratory Solution Preparation is planned for inexperienced as well as experienced solution maker. It offers useful details on the basic principles of preparing solutions and guidance in the high school science laboratory for preparing most of the solutions needed. The fundamental principles of solution preparation, solution from solid and liquid reagents, acid & base preparations, and sample and processing procedures are all explored in

unit 1.

Colorimetric is the method used to determine the colour species concentration. This method applies to dilute solutions and it is quantitative, it must come from a compound with definite characteristics of colour.

A spectrophotometer measures either the amount of light that a sample object reflects, or the amount of light that the sample object absorbs. The instrument operates by passing a light beam through a sample, and by measuring the light intensity that reaches a detector.

Atomic absorption is a process which involves the absorption of light by free atoms of an element at a given wavelength of that element. This is a method for determining the concentration of metals in their atomic state. In different phenomena of Atomic Spectroscopy (emission, absorption, and fluorescence), energy is given to the atoms in the form of thermal, radioactive, chemical, or electrical energy that is transformed to light energy through different atomic and electronic processes prior to measurement. Atomic absorption Spectrometry is useful not only for the identification of many elements present in samples but also for the quantitative determination. The methodology is precise and flexible so that significant numbers of individual items may be detected accurately in each sample. The theory, process, advantages and limitations of colorimetry, spectrophotometer and atomic absorption spectroscopy are given in unit 2.

Flame photometry is an analytical technique in which it calculates the radiation emitted by neutral atoms. Neutral atoms are formed by the injection of the sample into the fires, so the term is "Flame Photometry."

Spectrofluorimetry is a technique for the sample fluorescence identification and examination. Fluorescence is light absorption from a material (fluor) that reflects light or other electromagnetic radiation. Through this emission process, a light ray (usually UV radiation) excites the electron in a molecule that passes

from ground state to excited state with higher energy. When the electron returns to ground state, fluorescence is emitted.

Electrophoresis is a technique used to separate and sometimes purify macromolecules which differ in size, charge or conformation, particularly proteins and nucleic acids. When charged molecules are put in an electric field, they move, depending on their voltage, to either the positive or negative pole. Detail of flame photometry i.e. theory, process, advantages and disadvantage are given in unit 3.

Chromatography is a separation process in which the components to be separated are dispersed between two phases, one of which is called a stationary phase and the other is a mobile phase which moves in a definite direction on a stationary phase. The mixture component redistributes itself between two phases through a process that may include adsorption, partitioning, ion exchange or size exclusion.

Animal study frequently referred as animal behaviour is affected. Animal study and in vivo examination, involves the usage of non-human specimens in studies attempting to monitor the factors which influence the behaviour or biological mechanism being tested. See the principle, methods, types of chromatography and detail theory of animal assay given in unit 4.

Learning objectives are: -

Structure:

1.0 Standard solution

1.1 Introduction

1.2 Definition of standard solutions.

1.3 Different terms used in preparation on standard solution

1.4 Ideal procedure of calculating normality

1.5 Sample and sampling techniques

1.6 Introduction of sampling

1.7 Basic principles of sampling

1.8 Characteristics of sampling

1.9 Advantages of sampling

1.10 Disadvantages of sampling

1.11 Types of sampling

A. Probability sampling
B. Non-probability sampling

1.12 Types of error

1.13 Summary

STANDARD SOLUTION

1.1Introduction

Many of the reagents used in science are in the form of solutions which need to be purchased or prepared. For many purposes, the exact value of concentration is not critical; in other cases, the concentration of the solution and its method of preparation must be as accurate as possible. A solution is a homogeneous mixture created by dissolving one or more solutes in a solvent. The chemical present in a smaller amount, the solute, is soluble in the solvent (the chemical present in a larger amount). Solutions with accurately known concentrations can be

referred to as standard (stock) solutions.

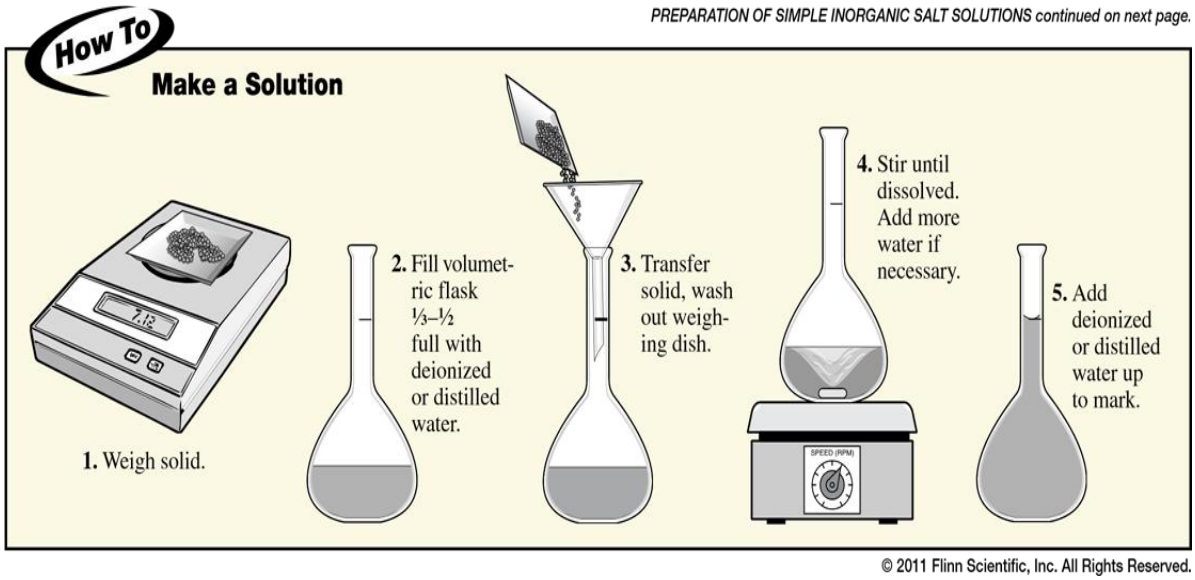


Figure 1:Preparation of standard solution

1. Definition of standard solutions

Figure 1 explain theknow the concentration terms of a solution once we know the relative amounts of the solute and the solvent making up the solution. Now let us describe a standard solution. A standard solution is a solution of which the concentration is accurately known. A standard solution is prepared by weighting a pure solute, dissolving it in a suitable solvent, usually water and making up the solution to a definite volume in a volumetric flask. The volumetric flask has a thin neck, which is marked with a line so it can be filled accurately to the correct capacity. The standard solution can then be used to find the concentration of a second solution with which it reacts. This is known as volumetric analysis or titration.

Different terms used in preparation on standard solution

A. Titration

A titration is a procedure used to identify the concentration of a solution by reacting it with a solution of known concentration and measuring the volume required for a complete reaction. The number of moles in the standard solution is calculated. Using a balanced equation for the reaction, the number of moles in the solution of unknown concentration can also be calculated. Once the number of moles for the solution is known, the concentration can be easily calculated.

B. Concentration

The concentration of a solution is a measure of how much solute is dissolved per unit of solvent.

concentration = amount of solute/volume of solvent

- Amount of solute is measured in moles
- Volume of solvent is measured in dm^3
- Concentration is measured in mol dm^{-3}
- Volumes are often expressed in cm^3, so a more useful equation includes a conversion from cm^3 to dm^3

Concentration = (no. moles × 1000) / volume mol dm^3=cm^3

C. Molarity

The most common unit of solution concentration is molarity (M). The molarity of a solution is defined as the number of moles of solute per one litre of solution. Note that the unit of volume for molarity is litres, not millilitres or some other unit. Also note that one litre of solution contains both the solute and the solvent. Molarity, therefore, is a ratio between moles of solute and litres of solution. To prepare laboratory solutions, usually a given volume and molarity are required. To determine molarity, the formula weight or molar mass of the solute is needed. The following examples illustrate the calculations for preparing solutions. If starting with a solid, use the following procedure: Calculate grams of solute (gs) required using equation 1.

gs = MMs x M x V.....Eq.1

Where,

MMs=molar mass (mass in grams of one mole of solute)

V=volume of solution required, in litres

M= molarity of solution required

Example: Prepare 800 mL of 2 M sodium chloride.

(MM NaCl = 58.45 g/mol)

gNaCl = 58.45 g/mol x 2 mol/L x 0.8 L

gNaCl = 93.52 g NaCl

Dissolve 93.52 g of NaCl in about 400 mL of distilled water, then add more water until final volume is 800 mL.If starting with a solution or liquid reagent: When diluting more

concentrated solutions, decide what volume (V_2) and molarity (M_2) the final solution should be. Volume can be expressed in litres or millilitres.

- **Determine molarity (M1:** -Calculate volume of starting solution (V_1) required using equation 2.

$$M_1V_1 = M_2V_2 \text{.....Eq. 2}$$

Note: V_1 must be in the same units as V_2.

Example: Prepare 100 mL of 1.0 M hydrochloric acid from concentrated (12.1 M) hydrochloric acid.

$M_1V_1 = M_2V_2$

(12.1 M) (V_1) = (1.0 M) (100 mL)

V_1 = 8.26 mL conc. HCl

Add 8.26 mL of concentrated HCl to about 50 mL of distilled water, stir, then add water up to 100 mL.

- **Percent Solutions:**-Mass percent solutions are defined based on the grams of solute per 100 grams of solution.

1. Volume percent solutions are defined as millilitres of solute per 100 mL of solution.

Example: v/v=10% v/v solution contains 10 ml of concentrate per 100 ml of solution (not the solvent).

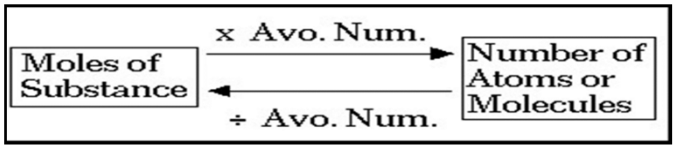
2. Mass-volume percent solutions are also very common. These solutions are indicated by w/v% and are defined as the grams of solute per 100 millilitres of solution.

Example: w/v=10% of w/v solution contains 10 g of solute in 100 ml of solution (not the solvent).

3. Mass-mass percent solutions are indicated by w/w% and are defined as the grams of solute per 100 milligrams.

Example: w/w=10% w/w solution contain 10 g of solute into 10 g of solvent

- **Use Avogadro's Number**: -Avogadro's Number is the number of atoms (or molecules) in 1 mole of substance.



Avogadro's no.=6.022 x 10^{23} per Mole.

Problem 1: 0.450 mole of Fe contains how many atoms?

•0.450 mol x 6.022 x 10^{23} mol^{-1}

Problem2: 0.200 mole of H_2O contains how many molecules?

•0.200 mol x 6.022 x 10^{23} mol^{-1}

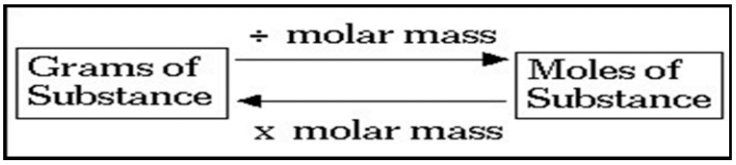
❖ **Use of molecular weight:** -Molecular weight is important, because it connects

The Macroscopic scale	The Microscopic scale
Where we all live	Where chemistry occurs
g of a substance	Number of molecules in mole

Use the molecular weight to connect between the two scales

Problem 3: Calculate the number of moles in 1.058 gram of H_2O. (MW of H_2O=18 g/mole).

Moles of substance=1.058□□×18□□/□□□□=19.044

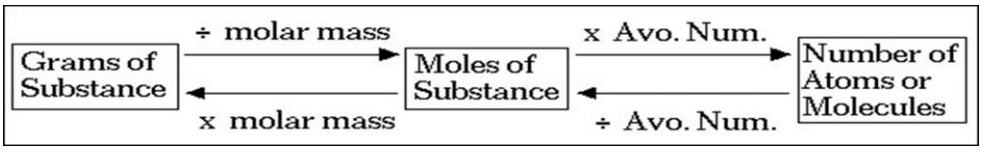


Moles of substance=1.058gm×18$gm/mole$

=19.044

Problem 4: Calculate the number of molecules in 1.058 gram of H_2O

No. of molecules=1.058gm×18$gm/mole$×6.022 x 10^{23} $mole^{-1}$



Determination of molecular weight (H_2SO_4)

Make a list of each element and the number of atoms of each element present in the substance (2H, 1S, 4O)

- Go to the periodic table and determine the atomic weight of each element.

H=1.00794

S=32.066

O=-15.9994

- Multiply each atomic mass by the number of atoms in the formula

H=1.00794 x 2 = 2.015

S=32.066 x 1 = 32.066

O=15.9994 x 4 = 63.998

Add up the results of above step

Molar mass of sulfuric acid =2.015+32.066+63.998 = 98.079

Problem 5: -Prepare 2M solution of NaCl (mw = 58)

Molecular weight = g/mole Na = 23, Cl = 35.5

1 mole = 58 g

2 moles = 116 grams of NaCl

116 grams of NaCl in 1litre solution.

Normality:-It is the number of gram equivalent of solute dissolved in 1 liter of Solutionand it is based on chemical reaction.

Example: - 1M solution of H_2SO_4

1 mole of H_2SO_4 in 1 liter of solution

But it gives 2 moles of acid. So, 1M of H_2SO_4 will be equivalent to 2 moles of acid. Normality represents the molar concentration 'only of the Acid Component (H^+ for Acid)' or 'only the base component (OH^- for base)' finally,

N = M x Number of H^+ or OH^- ions

2M H_2SO_4 = 4N

2M H_3PO_4 = 6N

2M HCl = 2N

2M NaOH = 2N

1.2Ideal procedure of calculating Normality

1. Preparing a Standard Solution from a Solid

Two methods exist for making a known-concentration solution from solids. With proper technique, either method will work in the General Chemistry Laboratory. Method 1: Transfer solid solute to weighing paper or small container, then directly into volumetric flask. A funnel can help transfer the solid into the vol. flask's thin neck. The vol. flask is then gently mixed with a small amount of solvent until the substance is completely dissolved. Add more solvent until the liquid surface reaches the calibration mark on the neck of the vol. flask (dilution to volume).Vol. The flask is then capped and repeatedly inverted to mix and dissolve the contents. Unwanted weighted solids may adhere to the original container,

weighing paper or funnel. Solids may also spill when transferred into the vol. flask's thin neck. The second method weighs the solid in a small beaker first. The beaker is stirred with a small amount of solvent until the solid dissolves. Then into the vol. flask. A funnel may need to be inserted into the vol. flask's thin neck.

Calculate compound's equivalent mass: - This is done by taking molecular weight of compound and dividing by the number of H^+ ions or OH^- ions

NaOH's equivalent mass = 40/1

H_2SO_4's equivalent mass = 98/2

For example: -Prepare 250 ml of 2N NaOH=2N x 40 x 0.250 = 20 g NaOH dissolve in 250 ml water.

For example: -Prepare 250 ml of 2N NaOH=2N x 40 x 0.250 = 20 g NaOH dissolve in 250 ml water.

2. <u>Preparing a Standard solution from a liquid</u>

Prepare 200 ml of 0.2 N H_2SO4 solution

MW = 98, number of H^+=2 so, Eq. wt=49

Vol. of solution=10 DX/eq. wt……eq. 3

Where, D=density of solution and X=% purity of solution

For 0.2 N of H_2SO_4 in 250 ml of water

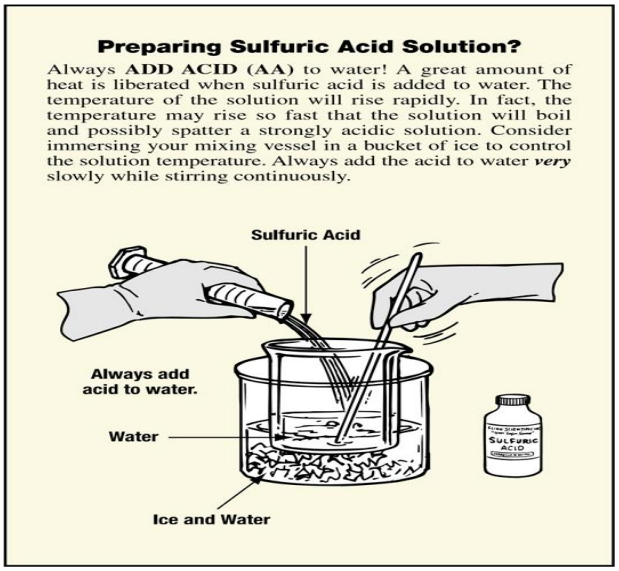
Vol. of solution (V_1) = (10 x 1.84 x 96)/49=18.4

After that

$N_1V_1=N_2V_2$

N1=0.2 x 200/18.4=0.277 ml

•**Caution –**Never add water into a large volume of concentrated acid it creates explosion risk because water into acid causes blast.



SAMPLE AND SAMPLING TECHNIQUES

1.3Introduction of sampling

In sampling, we choose units from a population of interest and then study them so that we can fairly generalise our findings back to the population from which they came. In Research Methodology, the practical formulation of the research is very important, so it should be done very carefully and with a lot of focus. It should also be done with a lot of good help. There are a lot of things to think about when you start to think about how to do this research on a more practical level, though. These problems are usually about how to figure out what the universe or the population looks like by looking at the characteristics of a specific part or a small part, which is called a sample. So now sampling can be described as the method or technique of choosing a small group of people to study, so that you can learn more about the rest of the world or the population.

Definition: -Choosing units (e.g., people, organisations) from a population of interest is called sampling. When we study the sample, we can fairly generalise our findings back to the population from which they were chosen. When it comes to Research Methodology, practical formulation of the research is very important. So, it should be done very carefully, with a lot of focus, and with the help of a lot of good people. There are a lot of things to think about when you start to think about how to do your research on a more practical level, though. Some of these issues are about knowing about the universe or the population by looking at the characteristics of a small part of it, which is called a sample. So now sampling can be

described as the method or technique of choosing a small group of people to study, so that you can learn more about the whole world or the population.

1.1.Population

A population is any complete group (i.e., people, sales territories, stores, etc.) sharing some common set of characteristics. It can be defined as including all people or items with the characteristic one wish to understand and draw inferences about them.

1.2.Population frame

A list, map, directory, or other source used to represent the population.

1.3.Census

A census is an investigation of all the individual elements making up the population a total listing rather than a sample.

1.4.Sample

A sample is a subset or some part of a larger population. It is "a smaller (but hopefully representative) collection of units from a population used to determine truths about that population" (Field, 2005). The sample has many advantages over a census or complete enumeration. When designed carefully, the sample may give results which are just accurate and sometimes more accurate than those of a census and is also considerably cheaper than the census. Hence a carefully designed sample may actually be better than a poorly planned and executed census (Rosander).

1.5.Sample design

A sample design is a definite plan for obtaining a sample from a given population (Kothari, 1998). It helps to decide the number of items to be selected in the sample i.e. the size of the sample. Purpose of sampling is to estimate an unknown characteristic of a population. It is all about selecting a random sample which is representative of the population under study. The idea is to compute a suitable value from the sample data relating to the test statistic by using the appropriate distribution. It constitutes a certain portion of the population or universe.

1.6.Sampling design

Sampling design refers to the technique or procedure, the researcher undergoes for selecting items as samples from the population or universe.

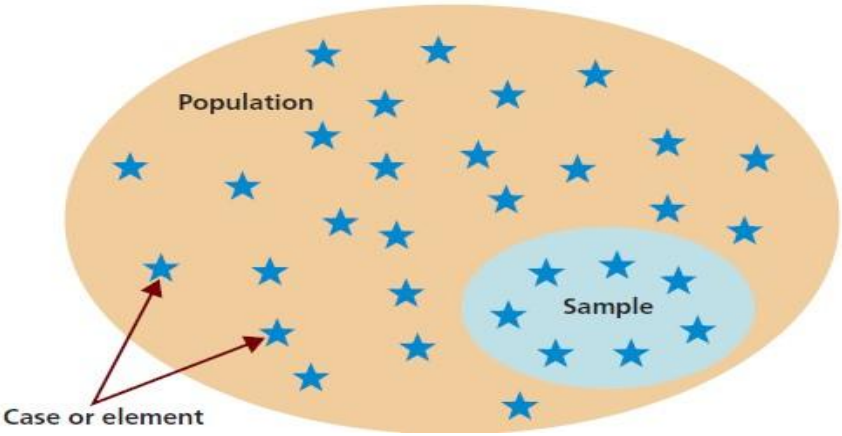


Figure 2: Diagrammatic representation of sampling process

2. Basic principles of sampling

Theory of sampling is based on the following laws: -

Law of Statistical Regularity – This law comes from the mathematical theory of probability. According to King," Law of Statistical Regularity says that a moderately large number of the items chosen at random from the large group are almost sure on the average to possess the features of the large group." According to this law the units of the sample must be selected at random.

Law of Inertia of Large Numbers – This law states that the other things being equal the larger the size of the sample; the more accurate the results are likely to be.

3. Characteristics of Sampling

Characteristics of the sampling technique

- Much cheaper.
- Saves time.
- Much reliable.
- Very suitable for carrying out different surveys.
- Scientific in nature.

4. Advantages of sampling

- Very accurate.
- Economical in nature.
- Very reliable.
- High suitability ratio towards the different surveys.
- Takes less time.

- In cases, when the universe is very large, then the sampling method is the only practicalmethod for collecting the data.

5. Disadvantages of sampling

- Inadequacy of the samples.
- Chances for bias.
- Problems of accuracy.
- Difficulty of getting the representative sample.
- Untrained manpower.
- Absence of the informants.
- Chances of committing the errors in sampling.

6. Types of sampling

Sampling methods are broadly categorized into two groups:

A. Probability sampling methods.
B. Non probability sampling methods

A. Probability sampling

If you use probability sampling, you need to know where the sample comes from. All the things in the universe have the same chance of being in the sample under this method. Lottery methods or picking a student from a box with your eyes closed or blindfolded are the best examples of random sampling. It is the best technique and the most fair method. It is the best way to choose samples that are representative. But the main problem is that for this method, we need the complete sampling frame, which is a list of all the items or population that we want to look at. This isn't always the case. There are five types of probability sampling:

1) Simple Random Sampling
2) Systematic Sampling
3) Stratified Random Sampling
    - Proportionate
    - Disproportionate
4) Cluster (or Area) Sampling
5) Multistage sampling

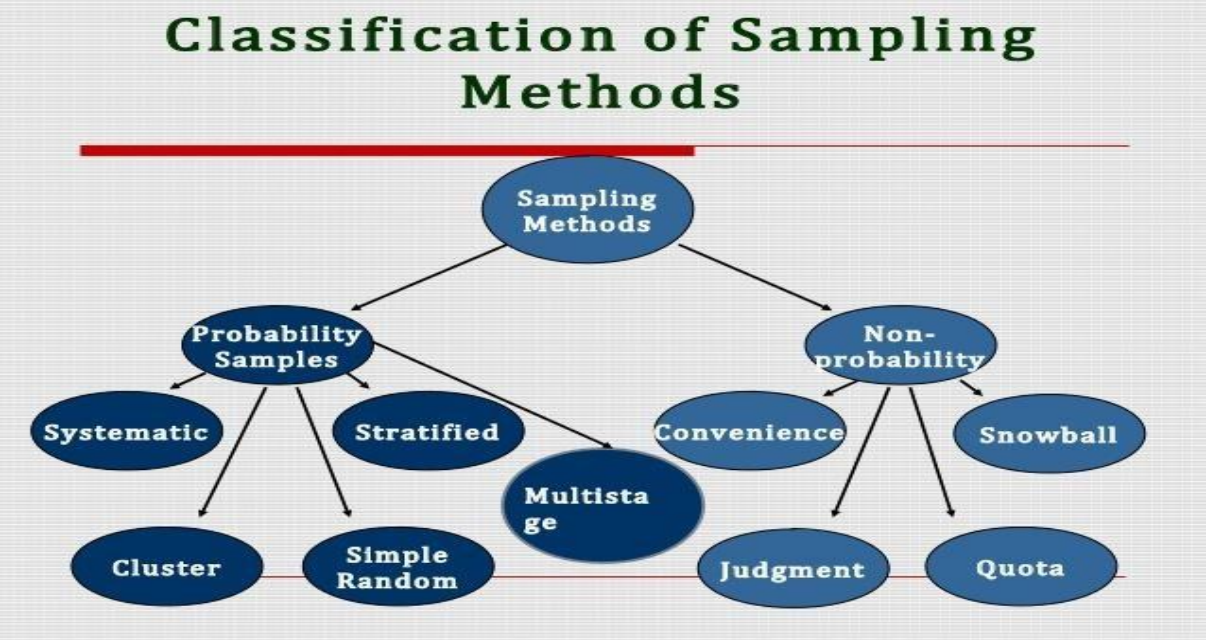


Figure 3: Classification of probability and non-probability sampling

1) Simple Random Sampling

It is a sampling procedure where each element in the population will have an equal chance of being selected in the sample. This process is simple because it requires only one stage of sample selection process. Here we number each frame unit from 1 to N. Then use a random number table or a random number generator to select *n* distinct numbers between 1 and *N*, inclusively. It is easier to perform for small populations but cumbersome for large populations.

Advantages

- Sample easy to select.
- Suitable sampling frame can be identified easily.
- Sample evenly spread over entire reference population.
- Cost effective.

Disadvantages

- Sample may be biased if hidden periodicity in population coincides with that of selection.
- Each element does not get equal chance.
- Ignorance of all element between two elements

2) Systematic Random Sampling

Systematic random sampling is convenient and relatively easy to measure. Here an initial starting point is selected by a random process; then every *n*th number on the list is selected. Thefirst sample element is selected randomly from the first k population elements (Figure 4).Thereafter, sample elements are selected at a constant interval, k from the ordered sequence frame.

$$k = N/n$$

where,

n= sample size, N=population size

k = size of selection interval

For example, someone wants to pick 50 out of a list of 10,000 purchase orders. This means that each purchase order from the previous fiscal year is numbered from 1 to 10,000 (N = 10,000). To do an audit, you need to look at a sample of 50 purchases orders (n = 50). There are k = 10,000/50 = 200. The first sample item was chosen at random from the first 200 purchase orders. Assume that the 45th purchase order was chosen.

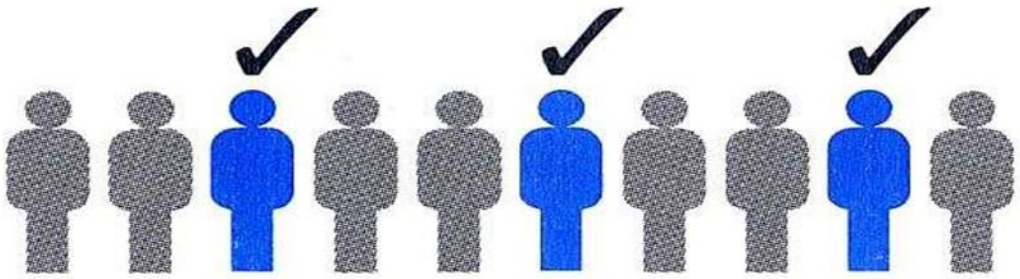


Figure 4: Diagrammatic representation of systematic random sampling

Advantages

- Sample easy to select.
- Suitable sampling frame can be identified easily.
- Sample evenly spread over entire reference population.
- Cost effective.

Disadvantages

- Sample may be biased if hidden periodicity in population coincides with that of selection.
- Each element does not get equal chance.

- Ignorance of all elements between two elements.

3) Stratified Random Sampling

In stratified random sampling, the population is first split into groups that are all the same. This could be based on a single criterion like male and female, or it could be based on a combination of more criteria like sex, caste, level of education, and so on. This method is usually used when there are a lot of different types of people in the population. People who are in the upper caste, middle caste, and backward caste are called O.B.C., S.C., and S.T. They are small farmers, big farmers, marginal farmers, landless farmers, and many other things as well. To get an accurate picture of a group of people's living standards, it's best to group them by caste, religion, or land ownership. Otherwise, some people might be under-represented or not be represented at all. It can be done in two ways:

a) Proportionate stratified random sampling

In the proportionate random sampling method, the researcher divides the population into groups based on known characteristics. Then, he or she draws a random sample from each group in the same proportion as its share of the population. That is, the population is split into several sub-populations based on some known characteristics. These sub-populations are called strata, and they are all the same. If there are 1000 people on a Gaon Panchayat, 60 percent of them are Hindus, 30 percent are Muslims, and 10 percent are scheduled tribes, how would the Panchayat run? Now, the investigator wants to draw a sample of 150 voters from the population, based on how many of them are in the general population at this point. There is a way to do this: You can multiply the number of people in the sample by their percentage. The sample size of Hindu voters will be 150 x 60% = 90; Muslims will be 150 x 30% = 45; and S.T. is 15% of the sample. So, the investigator needs to get the full list of voters for the G.P. and pick a random sample from each category as shown above. In this method, the sampling error is kept to a minimum, and the sample has all the characteristics of the population.

b) Disproportionate stratified random sampling

In this method, the number of people in each stratum isn't always the same as the number of people in that stratum. If the investigator wants to know how male and female Hindu, Muslim, and S.T. voters vote, he should take the same number of male and female voters

from each group. It's important to give each of them the same amount of attention. This is a type of sampling that isn't truly representative, but it can still be used in some situations.

Advantage

- Enhancement of representativeness to each sample.
- Higher statistical efficiency.
- Easy to carry out.

Disadvantage

- Classification error.
- Time consuming and expensive.
- Prior knowledge of composition and of distribution of population

4) Cluster Sampling

It is also called 'two-stage sampling'. In the first stage a sample of areas is chosen. In the second stage a sample of respondents within those areas is selected. Here population is divided into non overlapping clusters or areas of homogeneous units usually based on geographical dispersed population (Figure 5). Each cluster is a miniature, or microcosm, of the population. A subset of the clusters is selected randomly for the sample. If the number of elements in the subset of clusters is larger than the desired value of *n*, these clusters may be subdivided to form a new set of clusters and subjected to a random selection process.

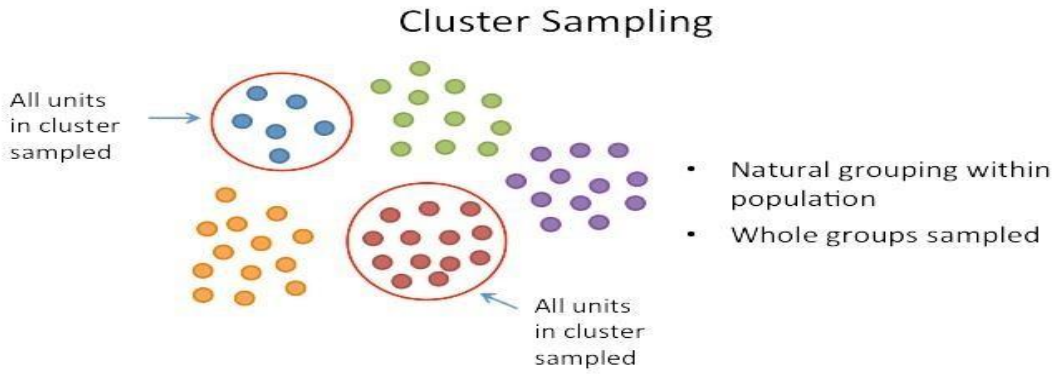


Figure 5: Diagrammatic representation of selection of cluster sampling from population

Advantages

- Can estimate characteristics of both cluster and population

Disadvantages

- The cost to reach an element to sample is very high.

- Each stage in cluster sampling introduces sampling error—the more stages there are, the more error there tends to be.

5) Multistage Sampling

Multi-stage sampling (also known as multi-stage cluster sampling) is a more complex form of cluster samplingwhich contains two or more stages in sample selection. In multi-stage sampling large clusters of population are divided into smaller clusters in several stages in order to make primary data collection more manageable in terms of cost effectiveness and time effectiveness. It is quite effective in primary data collection from geographically dispersed populations where face-to-face contact is required (e.g. semi-structured in-depth interviews).

Advantages

- More Accurate.
- More Effective.

Disadvantages

- Costly
- Each stage in sampling introduces sampling error—the more stages there are, the more error there tends to be.

B. Non probability sampling

Random sampling is a type of sampling that doesn't make sure that the items in the sample are chosen by the researcher. If you want to call it that, it's also called a purposeful sample or a judgement sample. In this example, if a researcher wants to make sure that the self-help groups in a state are making money and being self-sufficient in their chosen businesses, they might choose one or two districts with a lot of self-help groups and researchers who have been there for a long time. There are many mistakes in this type of sampling, which is why it's not a good idea to use this method. This type of sampling isn't used for very big or important things. Even though this may be done for research, it may not be done.

1) Some important techniques of non-probability sampling methods are –
2) Convenience sampling
3) Judgment sampling
4) Snowball sampling
5) Quota sampling

1) **Convenience Sampling**

A type of nonprobability sampling which involves the sample being drawn from that part of the population which is close to hand. That is, readily available and convenient (Figure 6). It is also termed as grab or opportunity sampling or accidental or haphazard sampling. Sample elements are selected for the convenience of the researcher. The researcher using such a sample cannot scientifically make generalizations about the total population from this sample because it would not be representative enough. This type of sampling is most useful for pilot testing.

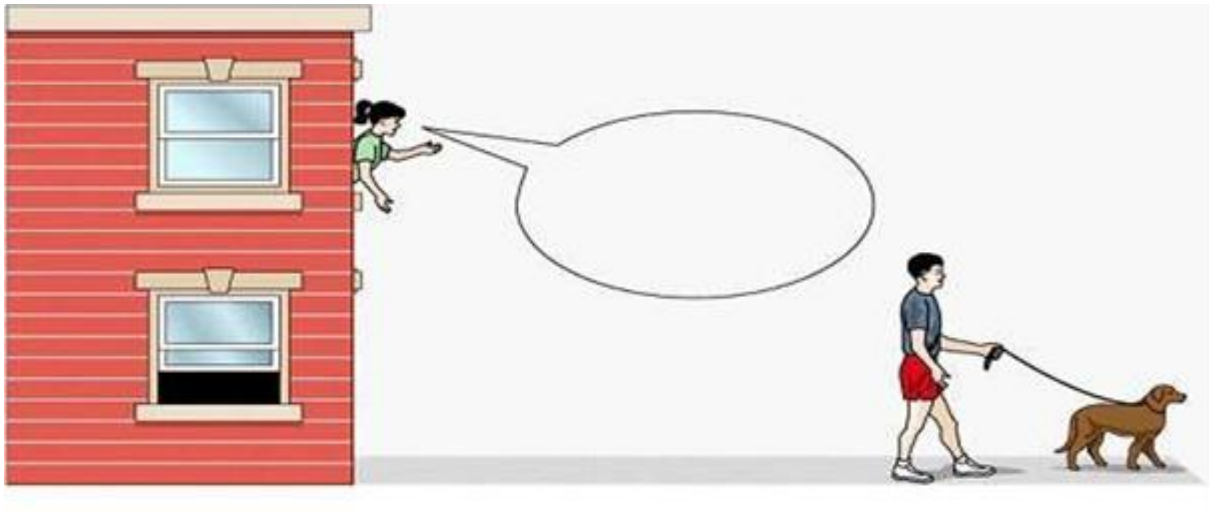


Figure 6: Diagrammatic representation of convenience sampling

Advantage

- Very low cost.
- A sample selected for ease of access, immediately known population group and good response rate.

Disadvantages

- Variability and bias cannot be measured or controlled.
- Projecting data beyond sample not justified.
- Restriction of Generalization.

2) **Judgment Sampling**

Here the sample elements are selected by the judgment of the researcher. The researcher chooses the sample based on who they think would be appropriate for the study. This is used primarily when there are a limited number of people that have expertise in the area being researched.

Advantages

- There is assurance of Quality response.
- Meet the specific objective.

Disadvantages

- Bias selection of sample may occur
- Time consuming process.

3) Quota Sampling

Here the population is first segmented into mutually exclusive subgroups, just as in stratified sampling. Then judgment is used to select subjects or units from each segment based on a specified proportion. In quota sampling the selection of the sample is non-random. For example, an interviewer may be told to sample 200 females and 300 males between the age of 45 and 60. He might be tempted to interview those who look most helpful.

Advantages

- Used when research budget is limited.
- Very extensively used/understood.
- No need for list of population elements.

Disadvantages

- Variability and bias cannot be measured/controlled.
- Time Consuming.
- Projecting data beyond sample not justified.

4) Snowball Sampling

This is a sociometric sampling technique generally used to study the small group. All the persons in a group identify their friends who in turn know their friends and colleagues, until the informal relationships converge into some type of a definite social pattern. It is just like the snowball going on increasing its size when rolling in an ice-field. In case of drug addict people it is difficult to find out who are the drug user but when one person is identified he can tell the names of his partner then each of his partner can tell another 2 or 3 whom he knows uses drug .This way the required number of element/people is identified and collects data. This method is suitable for diffusion of innovation, network analysis, and decision making.

Advantages

- Identifying small, hard-to reach uniquely defined target population.
- Useful in qualitative research.

- Access to difficult to reach populations (other methods may not yield any results).

Disadvantages

- Bias can be present.
- Not independent.
- Projecting data beyond sample not justified.

7. Types of Error

7.1.1. Sampling error

If researchers are not careful in planning and defining the sampling process, it can lead to faulty research findings. Sampling error is the error that occurs because of a representative samplefrom thepopulationrather than the entire population. In statistical terminology, it's the difference between thestatisticyou measure and theparameteryou would find if you took a census of the entire population. Sample error can't be eliminated, but it can be reduced. In general, it works like the larger the sample, the smaller the margin of error.

Method of reducing sampling error

- Specific problem selection.
- Systematic documentation of related research.
- Effective enumeration.
- Effective pre testing.
- Controlling methodological bias.
- Selection of appropriate sampling techniques.

7.1.2. Non-Sampling error

This is due to poor data collection methods(like faulty instruments or inaccurate data recording, missing data, selection bias, non-response bias(where individuals don't want to or can't respond to a survey), poorly conceived concepts, vague definitions and defective questions. Increasing the sample size will not reduce these errors. Their key is to avoid making the errors in the first place with a well-planned design for the survey or experiment.

8. Summary

- A sample is a part of the whole population (group of individuals of interest to the researcher). It all comes down to the type of sample you choose. The more general the research results can be, the more important the sample is (external validity). Probability

samples are a good representation of the whole population. They can be used to make generalisations about the group from which they came. Non-probability sampling is based on the researcher's choice and isn't based on how many people there are.

- Sampling is the process by which some people (elements) in the population are chosen for a research study.

- The population is made up of all people who have a certain trait that is of interest to the researchers. Census: If we get data from all the people in the population, then we have one. Sample: If we only get data from a few people in the population, we have a small group.

- With probability sampling, a researcher can say how likely it is that an element (participant) will be in the sample. With nonprobability sampling, there is no way to figure out how likely it is that an element will be in a sample.

- Probability sampling is a more precise way to get a sample that is representative of the whole population, even though it can be more difficult and expensive.

- All the people in the population have an equal chance of being chosen to be in the sample when simple random sampling is used. The four steps of simple random sampling are: (1) defining the population, (2) making a list of all members, (3) drawing a sample, and (4) contacting the members of the sample after drawing the sample.

- Stratified random sampling is a type of probability sampling in which people are randomly chosen from certain groups (strata) of the population. To make sure the sample is more representative, this method can be used. It can also be used to make comparisons between people in different groups.

- Convenience sampling is fast and cheap because it only picks people who are available at the time of the study (such as introductory psychology students). The downside is that convenience samples aren't as representative as random samples, so results should be taken with a grain of salt.

- Quota sampling is when a certain percentage of people from certain groups of the population are chosen when there aren't lists of people in the population. Many polling groups use this method.

- The right sample size is based on a number of factors, including population variability, statistical issues, economic factors, and the availability of participants. In general, when you have more samples, you will have a smaller margin of error and be able to see smaller differences.

- The bigger the range of scores in the population, the bigger the sample size must be in order to be representative of the whole group of people.

- Systematic and random errors are both part of the sampling error. When the sample isn't drawn correctly, there is a "systematic error" (an error of the researcher). Random error is the amount to which the sample isn't completely representative of the rest of the people in the world. Even if you use the best sampling methods, there will still be some random error.

- We are seeing more and more information that is based on sample data. Understanding the principles of sampling, as well as the limitations of different methods, should help us be more critical of the information we get from them.

Self-AssessmentQuestions: -

1. How much sodium chloride is needed to make 1 litre of an aqueous 1M solution (MW=58.5 gm/mole)?
2. How much sodium chloride is needed to make 1 litre of an aqueous 2M solution (MW=58.5)?
3. How much sodium chloride is needed to make 1 litre of an aqueous 0.1M solution?
4. Make 1 litre of 1M aqueous solution of H2SO4 (MW=98.07, Sp. Gr. = 1.84, Purity=96%)?

5. Calculate the normality of a NaCl solution prepared by dissolving 2.9216 gm of NaCl in water and then topping it off with more water to a total volume of 500 ml (MW=58.44)?
6. Calculate the volume of concentrated aqueous sulphuric acid having Sp.Gr. 1.842 & containing 96% H_2SO_4, required to prepare 2 litre of 0.20 N H_2SO_4?
7. Calculate the volume of concentrated HCl, having a density of 1.188 gm/ml and containing 38% HCl by weight, needed to prepare 2 litre of 0.20 N HCl solution (MW=36.461)?
8. What is a cluster sampling?
9. What is stratified sampling?
10. What are the limitations and advantage of multistage sampling?
11. What specific steps would you have taken to obtain a representative sample?

Check your progress of unit 1 exercise

- Apply molarity and normality formula that are given in the eq. 1. 2 and 3.
- Check the answer of question no. 8, 9, 10, and 11 are given in the sample and sampling techniques part of unit.

Learning objectives are: -

Colorimeter:

2.0 Introductionof colorimeter

2.1 Principleof colorimeter

2.2 Parts of colorimeter

2.3 Working procedure of colorimeter

2.4 Applications of colorimeter

2.5 Advantages of colorimeter

2.6 Disadvantages of colorimeter

Spectrophotometer:

2.1.0 Introductionof spectrophotometer

2.1.1 Principleof spectrophotometer

2.1.2 Types of spectrophotometer

2.1.3 Instrumentationof spectrophotometer

2.1.4 Applications of spectrophotometer

2.1.5 Advantages of spectrophotometer

Atomic absorption spectrophotometer:

2.2.0 Introductionof atomic absorption spectrophotometer

2.2.1 Principle ofatomic absorption spectrophotometer

2.2.2 Methodologyof atomic absorption spectrophotometer

2.2.3 Instrumentation of atomic absorption spectrophotometer

2.2.4 Workingprocedureof atomic absorption spectrophotometer

2.2.5 Applications of atomic absorption spectrophotometer

2.2.6 Advantages of atomic absorption spectrophotometer

2.2.7 Disadvantages of atomic absorption spectrophotometer

COLORIMETRY

1. INTRODUCTION

A colorimeter is a device that is used to determine the absorbance of specific wavelengths of light by a solution. It measures absorbance and wavelength in the range of 400–700 nm (nanometer), i.e. in the visible spectrum of light. The Beer-Lambert law is most frequently used to determine the concentration of a known solute in a given solution using this device. When light strikes a colored solution is absorbed or transmitted. A colored solution absorbs the entire spectrum of white light and transmits only one color selectively. Louis Jules Duboscq invented colorimetry.

2. PRINCIPLE

A colorimeter is based on the photometric technique which states that when a beam of incident light of intensity I0 passes through a solution, a part of the incident light is reflected (I_r), a part is absorbed (I_a) and the rest of the light is transmitted (I_t)

Thus,

$$\mathbf{I_0 = I_r + I_a + I_t}$$

(I_r) is eliminated because of the measurement of (I_0) and it is sufficient to determine the (I_a). For this purpose, the amount of light reflected (I_r) is kept constant by using cells that have identical properties. The two fundamental laws of photometry on which the colorimeter is based demonstrate the mathematical relationship between the amount of light absorbed and the concentration of the substance.

Beer's Law: When monochromatic light passes through a colored solution, Beer's law states that the amount of light transmitted decreases exponentially as the concentration of the colored substance increases. According to this law, the amount of light absorbed is directly proportional to the solute concentration in the solution.

$$\mathbf{Log10\ I_0/It = AsC}$$

where,

A_s = Absorbency index

C = Concentration of Solution

Lambert's Law: According to Lambert's law the amount of light transmitted decreases

exponentially with an increase in thickness of the colored solution. Lambert's law states that the amount of light absorbed is directly proportional to the length and thickness of the solution under analysis.

$$\mathbf{A = \log 10\ I_0/It = A_s b}$$

Where,

A = Absorbance of test

A_s = Absorbance of standard

b = length / thickness of the solution

The mathematical representation of the combined form of Beer-Lambert's law is as follows:

$$\mathbf{Log10\ I_0 / It = A_s bc}$$

If b is kept constant by applying cuvette or standard cell then,

$$\mathbf{Log10\ I_0/It = A_s C}$$

The absorbency index is defined as = A/C l

Where,

C = concentration of the absorbing material (in gm/liter).

l = distance traveled by the light in solution (in cm).

In simplified form,

The colorimeter's operation is based on Beer-law, Lambert's which states that the amount of light absorbed by a color solution is proportional to its concentration and the length of a light path through it.

$$\mathbf{A \propto Cl}$$

Where,

A = Absorbance / Optical density of solution

C = Concentration of solution

l = Path length

$$\mathbf{A = \in C\ l}$$

$\in$ = Absorption coefficient

3. PARTS OF COLORIMETER:-Colorimeter will comprise the following parts:

1. Light source: -The most common source of light used in colorimeter is a tungsten filament.

2. Filter: -To select the particular wavelength filter or monochromators are used to split the light from the light source.

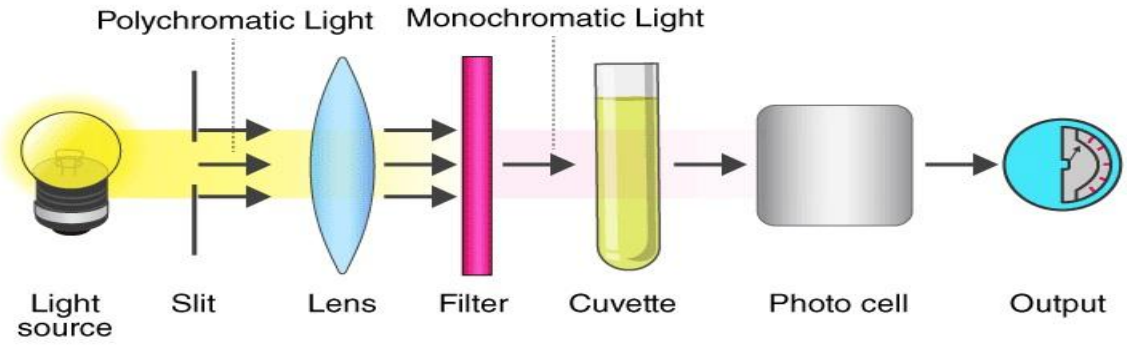


Figure 1: Diagrammatic representation of a process of colorimeter

3. Cuvette chamber: -The transmitted light passes through a compartment wherein the solution containing the colored solution is kept in a cuvette, made of glass or disposable plastic.The cuvette isa rectangular cell, square cell, or circular one. It is made up of optical glass for visible wavelength. The common cuvette is square and rectangular to avoid refraction artifacts, and the dimension of the cuvette is 1cm.

4. Detector: This is a photosensitive element that converts light into electrical signalsand the electric current is generated, this reflects the Galvanometer reading

5. Galvanometer: -The current from the detector is fed to the measuring device, theGalvanometer, which shows the meter reading that is directly proportional to the intensity of light.

4. WORKING PROCEDURE OF THE COLORIMETER

- A colorimeter must first be calibrated using standard solutions of the known concentration of the solute to be determined in the test solution. Fill the cuvettes with the standard solutions and place them in the colorimeter's cuvette holder.
- A light ray of a specific wavelength in the direction of the solution. Light passes through various lenses and filters. Lenses guide colored light and filters split a beam of light into different wavelengths, allowing only the required wavelength to pass through and reach the standard test solution cuvette.
- Once in the cuvette, monochromatic light (one wavelength) is partially reflected,

partially absorbed by the solution, and partially transmitted through the solution to the photodetector system. The photodetector system measures the intensity of transmitted light and sends it to the galvanometer.

- The galvanometer's electrical signals are displayed digitally.
- The absorption of the solution affects the galvanometer reading and corresponds to the concentration of the solute in the solution.
- The colorimeter measures the optical density or absorbance of a colored compound to determine its concentration.
- The rate of formation and disappearance of the light-absorbing compound in the visible spectrum of light can also be used to determine the reaction's progress.
- A colorimeter can identify a compound by measuring its absorption spectrum in the visible light spectrum.

5. ADVANTAGES OF COLORIMETER

- It is an inexpensive method, widely used in the quantitative analysis of colored samples, easy to carry, and transport.

6. DISADVANTAGES OF COLORIMETER

- Analysis of colorless compounds is not possible and does not work in IR and UV regions.

SPECTROPHOTOMETER

1. INTRODUCTION

Spectrophotometers are better than colorimeters. The principle, function, and use of both instruments are the same. Both of these are common tools for figuring out how much of a certain substance there is in biochemistry. Some examples are glucose, total protein in the blood, and a single bacteria cell. This is how you say it: This is how a spectrophotometer works: It looks at how much light comes back from a sample object or how much light is absorbed by the sample object. The instrument works by shining a beam of light through a sample and measuring how much light reaches a detector. It's called a chromophore when something or a molecule can take in light. They have unique absorption spectra that can be defined by a wavelength of maximum absorption, or max. This is because vibrational levels cause a wide absorption band. The absorption spectra can have a lot of different absorption peaks with different amplitudes. Biomolecules in the visible and ultraviolet (UV) ranges can absorb light from the sun. As the light passes through a solution with a chromophore, the intensity of the light goes down. Chromophore type, concentration, sample thickness, and other factors (like pH and solvent) all affect how much light a chromophore can absorb. These factors all affect how much light a chromophore can absorb. Assimilation is based on the Beer-Lambert law.

Beer-Lambert law: -According to Beer's law when monochromatic light passes through the colored solution, the amount of light transmitted decreases exponentially with an increase in the concentration of the colored substance.

Where I_0= Light Intensity entering a sample

I= Light Intensity exiting a sample

C= The concentration of analyte in the sample

L= The length of the light path in a glass sample cuvette

Ɛ= a constant for a particular solution and wavelength

2. PRINCIPLES OF SPECTROPHOTOMETER

The word is made up of the Greek words for "light" and "measure." Photometric working is based on the fact that the species of alkali metal and alkaline earth metals are broken apart by the heat from the flame source. Because of this thermal excitation, some of the atoms are moved to a higher energy level where they aren't going to be able to stay there. Direct absorption techniques can be used to measure how much radiation is absorbed by electrons that have been excited. Emission techniques can be used to measure how much radiation is emitted. The wavelength of the light that is emitted is unique to each element. Following the loss of energy, the exiting atoms will move to the low-energy ground state and emit some radiation, which can be seen in the visible range of the spectrum. The idea is that you can measure the spectrum of a sample that has atoms or molecules in it. Spectrum is a graph that shows how much radiation is absorbed or emitted by each sample as a function of frequency or wavelength (v or).

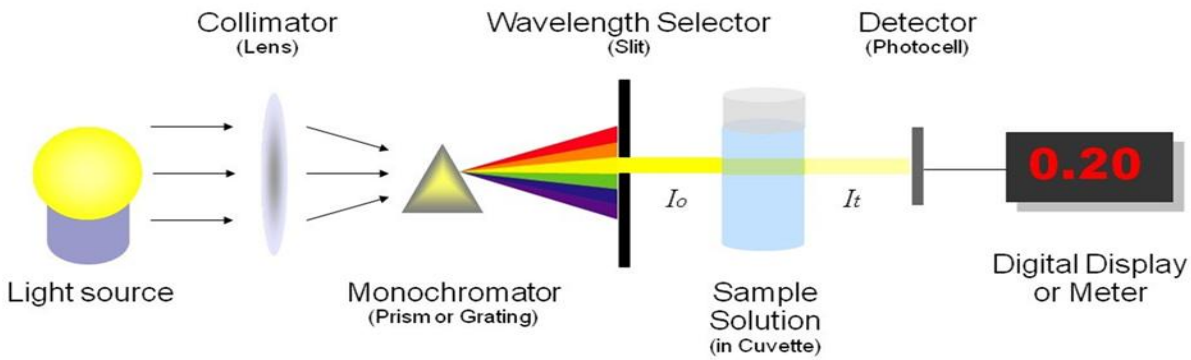


Figure 2: Instrumentation process of spectrophotometer

Spectrophotometers make monochromatic light and then accurately measure how much light there is. You need to have the light source, the monochromator, the sample stand, and the light detector (phototube) to use one. Tungsten lamps are used in most instruments to look at things in the visible range, and H2 or D2 lamps are used to look at things in the UV range. One way to make monochromatic light is to move a prism or use diffraction gradient filters. Monochromatic light shines through the sample and is then measured by a photomultiplier

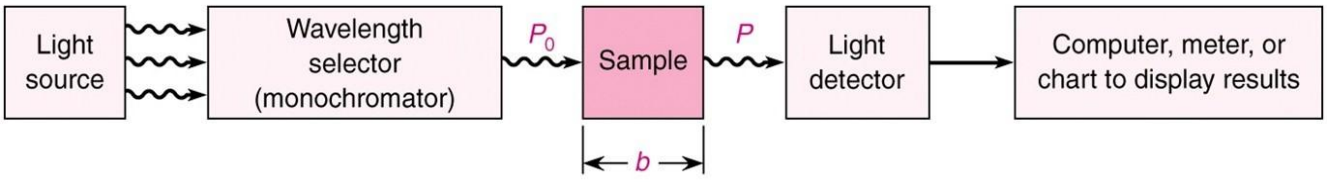
tube after it has passed through. A photomultiplier tube turns the energy of the light photons into electrons, which can then be used to do things like making electricity. The voltage caused by these electrons is measured by a meter and then turned into an absorbance value. This is how you figure out the Io (initial intensity): You calibrate your instrument with a "buffer blank." Because the blank and sample have different amounts of light shining on them, this difference is called "absorbance" (A). Spectrophotometers often come with extras like chart recorders or microprocessors that can help you analyze data. There is a different signal if there is more or less light being absorbed by the liquid at that time. It can be easy to figure out how much a substance in a solution is absorbed by light at the right wavelength to figure out how much that substance is in solution (Figure 2).

For example: -Hemoglobin appears red because the hemoglobin absorbs blue and green light rays muchmore effectively than red.The degree of absorbance of blue or green light is proportional to the concentration of hemoglobin.

3. TYPES OF SPECTROPHOTOMETER

There are two types of spectrophotometers: one that has one beam and one that has two beams. In this case, a double beam spectrophotometer compares the amount of light in two different places. One path has a reference sample on it. There was also a test sample for us to look at. A single beam spectrophotometer measures how much light is in the beam before and after a test sample is put in. For comparison, a double beam machine makes it easier and more stable for people to use. But a single beam machine can measure a wider range of light frequencies than a two-beam machine. When you use single beam machines that have simple optical systems, you can fit more into a small space.

Single beam spectrophotometer: -



Double beam spectrophotometer: -

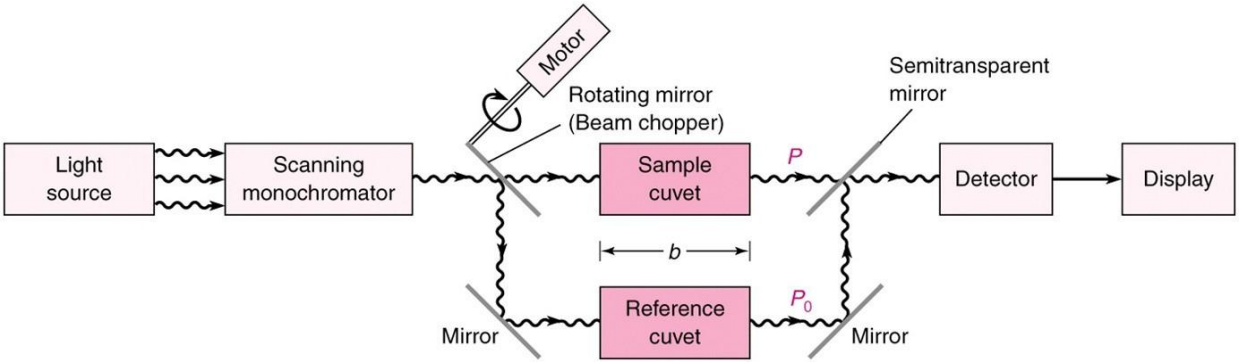


Figure 3: Types of spectrophotometer

4. **INSTRUMENTATION: -** There five basic optical instrument components are as follows:
   - **I.** Light source
   - **II.** Wavelength Selector for measurement (monochromators, prisms & filters).
   - **III.** Sample Container
   - **IV.** Detector/Photoelectric transducer
   - **V.** Signal Processor & Readout

I. Light sources: -Various UV radiation sources are as follows: -Deuterium lamp,hydrogen lamp**,** tungsten lamp**, a** xenon discharge lamp, and mercury arc lamp.Various visible radiation sources are as follows: -Tungsten lamp, mercury vapors lamp, and carbonone lamp.

II. Wavelength selectors: -Wavelength selectors output a limited, narrow, continuous group of wavelengths called a band. Two types of wavelength selectors: A) Filters and B) Monochromators.

a) Filters-Two types of filters: a) Interference Filters b) Absorption Filters
b) Monochromators-Wavelength selector that can continuously scan a broad range of wavelengths.Used in most scanning spectrometers including UV, visible, and IR

instruments. Prismsarerefractive type & reflective type and grating are diffraction type & transmission type.

III. **Sample compartment:** -Spectroscopy requires all materials in the beam path other than the analyte should be as transparent to the radiation as possible. The geometries of all components in the system should be such as to maximize the signal and minimize the scattered light.The material from which a sample cuvette is fabricated controls the optical window that can be used.Some typical materials are:

- Optical Glass: 335-2500 nm
- Special Optical Glass: 320-2500 nm
- Quartz (Infrared): 220-3800 nm
- Quartz (Far-UV): 170-2700 nm

IV. **Detectors:**-After the light has passed through the sample, we want to be able to detect and measure the resulting light.These types of detectors come in the form of transducers that can take energy from light and convert it into an electrical signal that can be recorded, and if necessary, amplified.Three common types of detectors are used i.e. barrier layer cells, photoemissive cell detector, and photomultiplier.

V. **Signal processor &readout:** -Amplifies or attenuates the transduced signal and sends it to a readout device as a meter, digital readout, chart recorder, computer, etc.

5. APPLICATION OF SPECTROPHOTOMETER: -

- ❖ Concentration measurement: -Prepare samples and a series of standard solutions with known concentrations, and then calibrate the spectrophotometer to its maximum light absorption setting. Determine the unknown's absorption and, using the standard plot, determine the corresponding concentration.

- ❖ Detection of Impurities: -UV absorption spectroscopy is a highly effective technique for determining impurities in organic molecules. Additional peaks can be observed as a result of sample impurities, which can be compared to the standard raw material. Additionally, impurities can be detected by measuring absorbance at a specific wavelength.

- Structure elucidation of organic compounds: -UV spectroscopy is useful for elucidating the structure of organic molecules, determining the presence or absence of unsaturation, and detecting the presence of heteroatoms. The location and combination of peaks can indicate whether the compound is saturated or unsaturated, whether heteroatoms are present or not, and so on.

- Quantitative analysis: -UV absorption spectroscopy can be used for the quantitative determination of compounds that absorb UV radiation. This determination is based on Beer's law which is as follows.

- Qualitative analysis: -UV absorption spectroscopy can characterize those types of compounds that absorb UV radiation.Identification is done by comparing the absorption spectrum with the spectra of known compounds.

- Chemical kinetics: -Kinetics of reaction can also be studied using UV spectroscopy.The UV radiation is passed through the reaction cell and the absorbance changes can be observed.

- Detection of Functional Groups: -This technique is used to detect the presence or absence of functional groups in the compound. The absence of a band at a particular wavelength is regarded as evidence for the absence of a particular group like benzene and toluene.

- Quantitative analysis of pharmaceutical substances: - Many drugs are either in the form of raw material or in the form of the formulation. They can be assayed by making a suitable solution of the drug in a solvent and measuring the absorbance at a specific wavelength.Diazepam tablets can be analyzed by 0.5% H_2SO_4 in methanol at the wavelength 284 nm.

- Examination of Polynuclear Hydrocarbons: -In the ultraviolet and visible regions,

benzene and polynuclear hydrocarbons exhibit distinctive spectra. Thus, polynuclear hydrocarbons can be identified by comparing their spectra to those of known polynuclear compounds. Polynuclear hydrocarbons are those that contain two or more closed rings; examples include naphthalene, C10H8, which contains two benzene rings adjacent to one another, and diphenyl, (C6H5)2, which contains two bond-connected benzene rings. Additionally referred to as polycyclic hydrocarbon.

- As HPLC detector: -A UV/Vis spectrophotometer may be used as a detector for HPLC.

- Enzyme activity: -In which the substrate and the product exhibit different λmax. Either the disappearance of substrate or the appearance of the product over time is measured.

6. ADVANTAGES OF SPECTROPHOTOMETRY

- It is often non-destructive (i.e., can measure and recover sample),
- It is selective (often a particular compound in a mixture can be measured without separation techniques)
- It has a short time interval of measurement (10-14 seconds).

ATOMIC ABSORPTION SPECTROPHOTOMETER(AAS)

1. INTRODUCTION

Atomic absorption is the process by which light is absorbed by free atoms of a particular element at a wavelength specific to that element. It is a technique for determining the concentration of metals in their atomic state. Atomic Spectroscopy's various phenomena (emission, absorption, and fluorescence) involve the supply of energy to the atoms in the form of thermal, electromagnetic, chemical, or electrical energy, which is then converted to light energy via various atomic and electronic processes prior to measurement. Atomic Absorption Spectrometry is advantageous for not only identifying but also quantifying a wide variety of elements present in samples. The technique is both specific and sensitive in that it can reliably identify individual elements in small amounts in each sample. Walsh and Alkemade coined the term atomic absorption spectroscopy to refer to this technique for analytical purposes. Walsh used hollow cathode lamps as a line source, lowering the required resolution for successful analysis significantly. Due to the widespread availability of photomultipliers, the complications associated with measuring absorption using a photographic plate were eliminated. Modulation enabled the detector to distinguish between the absorption and emission of atoms at the same wavelength. Additionally, he used the flame to atomize. It is found to be superior to other techniques because it is capable of determining 50-60 elements in quantities ranging from trace to large. These materials may be metallic or non-metallic.

2. PRINCIPLE

The principle is based on the Beer- Lambert's law. The absorption of radiation by the free atoms is proportional to their concentration.

$$\text{Absorbance} = \log_{10} \frac{I_0}{I_t} = KCL$$

Where,

I_o = Intensity of incident radiation

I_t = Intensity of transmitted radiation

C = Concentration of analyte

K = Constant

L = Path length

3. METHODOLOGYOF AAS

Atomization is the process by which a sample solution is aspirated into a flame or heated in a tube to convert it to atoms. The atoms then absorb light of a particular wavelength, promoting the electrons from the ground state to the excited state. Each element absorbs a specific wavelength of radiation, which serves as the basis for qualitative analysis. The amount of light absorbed also varies according to the number of atoms in the light path. A quantitative determination of the analyte can be made by detecting this amount of light. Each atom is composed of a positively charged nucleus surrounded by a cloud of electrons in rapid motion. Each electron in an atom can occupy a discrete set of energy levels. Each electron in an atom has a unique spacing between its energy levels, but electrons in similar atoms have identical spacing. The energy levels are usually labeled E0, the ground state, through E1, E2, etc. to E∞. For an unexcited atom, each electron is in the ground state. To excite the atom, one or more electrons can be raised to the first or higher energy levels by the absorption of energy. This energy can be supplied by photons or by collisions due to heat. Those electrons furthest from the nucleus require the least energy to go from the ground state E0 to the first energy level E1.

$$E = E_1 - E_0$$

The energy required for this transition can be supplied by a photon of light with energy given by:

$$E = h \nu$$

Where h is the Planck's constant and ν the frequency.

This corresponds to a wavelength (λ) of:

$$\lambda = h\, c/ E$$

Where c is the speed of light in a vacuum.

4. INSTRUMENTATION OF ATOMIC ABSORPTION SPECTROPHOTOMETER: -

an atomic absorptionspectrophotometer apparatus consists of:

4.1. Radiation source: The sample solution is aspirated into the flame or heated in a tube to convert it to atoms. The atoms then absorb radiation of a particular wavelength, promoting the electrons from their ground state energy level to their excited state energy level. Each element absorbs a specific wavelength of light, which serves as the basis for qualitative analysis. The amount of light absorbed also varies with the number of atoms in the light path. By detecting this amount of light, it is possible to make a quantitative determination of the analyte. Each atom contains a positively charged nucleus that is surrounded by a cloud of electrons in rapid motion. Each electron in an atom has a discrete set of possible energy levels. Each electron in the atom has a unique spacing between its energy levels, but electrons in similar atoms have identical spacing.

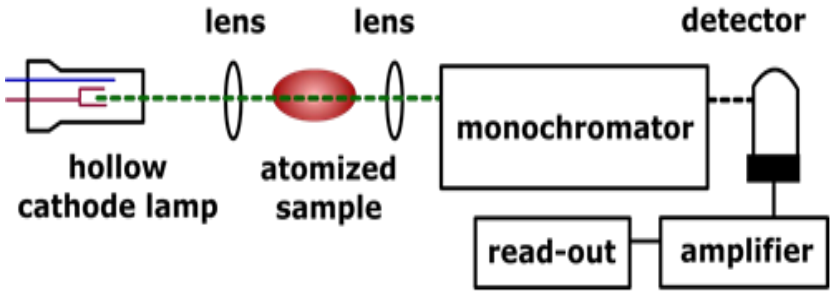


Figure 4: Instrumentation process of Atomic absorption spectrophotometer

In a process called sputtering, these gaseous ions bombard the cathode and eject metal atoms from it. As some sputtered atoms decay to their ground state, they emit radiation characteristic of the metal. These emitted rays serve as incident rays for the element being analyzed.

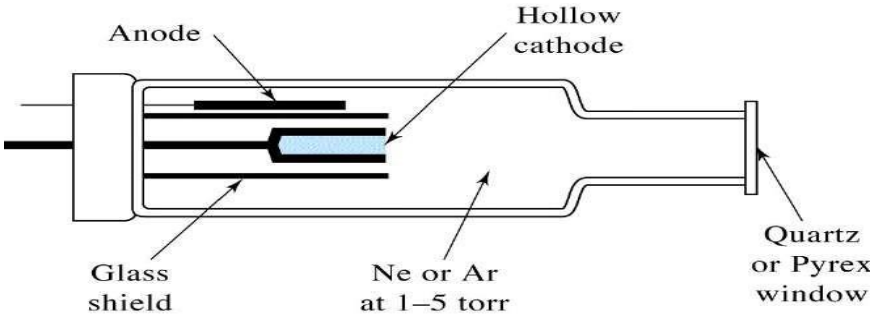


Figure 5: Diagrammatic representation of hollow cathode lamp

Another type of discharge lamp is the electrodeless discharge lamp. Within a quartz bulb, a trace amount of the metal or salt of the element for which the source is to be used is sealed. This bulb is contained within a small, self-contained radio frequency generator, or "driver." When the driver is powered, an RF field is created. Coupled energy vaporises and excites the atoms inside the bulb, causing them to emit their distinctive spectrum. They are frequently significantly more intense and, in some cases, more sensitive than comparable HCL. As a result, when an analysis is intensity limited, the precision and detection limits are increased. EDL are available for a wide variety of elements, including Sb, As, Bi, Cd, Cs, Ge, Pb, Hg, P, K, Rb, Se, Te, Th, Sn and Zn.

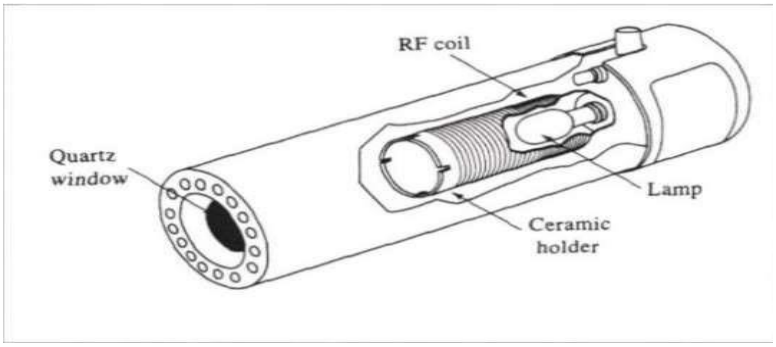


Figure 6: Diagrammatic representation of electrodeless discharge lamp

4.2. Atomizer:

Nebulization is the process by which the sample solution is introduced into the flame as a fine spray. Nebulization is the process by which a liquid is dispersed into particles using a rapidly moving gas, liquid stream, or mechanical means. This is immediately followed by atomization, a process in which high energy, such as that produced by a flame, converts molecules to atoms.

4.2.1. Flame Atomization:

The sample is atomized using a flame. When a sample is heated, it is decomposed into its constituent atoms. Excitation occurs when the flame reaches a high temperature. By absorbing a certain amount of energy, the atomized sample's electrons are promoted to higher orbitals.

4.2.2. Graphite furnace atomization: Graphite furnace atomic absorption spectrometry (GFAAS) (alternatively called electrothermal atomic absorption spectrometry (ETAAS)) is a type of spectrometry in which the sample is vaporized in a graphite-coated furnace. Rather than using the high temperature of a flame to generate atoms from the sample, non-flame methods involving electrically heated graphite tubes or rods are used.

4.2.3. Oxidants and fuel: Hydrogen, propane, butane, acetylene, and natural gas are all frequently used fuels. As oxidants, oxygen and nitrous oxide are used. Typically, an oxygen-acetylene flame is used. Its temperature is sufficient to produce sufficient atomization for the majority of elements detectable by AAS but not so high that ionization interference becomes significant. By altering the composition of the gas mixture, the analytical conditions can be optimized.

Table 1: Upper temperature limits for some gas mixtures

Combustible mixture	Maximum temperature (K)
Butane – Air	2200
Acetylene – Air	2600
Acetylene - Nitrous Oxide	3100
Acetylene - Oxygen	3400

4.3. Monochromator:

The instrument must be capable of providing a narrow bandwidth to separate the line chosen for determination from other undesirable lines. Usually used devices are gratings or prisms.

4.4. Detector:

The intensity of the light is fairly low, so a photomultiplier tube (PMT) is used to boost the signal intensity. A detector (a special type of transducer) is used to generate a voltage from the impingement of electrons generated by the photomultiplier tube.

5. WORKING PROCEDURE OF AAS:

Aqueous samples should be acidified (typically with nitric acid, HNO3) to a pH of 2.0 or less. Discoloration in a sample may indicate that metals are present in the sample. For example, a greenish color may indicate a high nickel content, or a bluish color may indicate a high copper content. A good rule to follow is to analyze clear (relatively dilute) samples first, and then analyze colored (relatively concentrated) samples. It may be necessary to dilute highly colored samples before they are analyzed.After the instrument has warmed up and been calibrated, a small aliquot (usually less than 100 microliters (µL) and typically 20 µL) is placed, either manually or through an automated sampler, into the opening in the graphite tube.The graphite furnace is an electrothermal atomizer system that can produce temperatures as high as 3,000°C. The heated graphite furnace provides the thermal energy to break chemical bonds within the sample and produce free ground-state atoms. Ground-state atoms then are capable of absorbing energy, in the form of light, and are elevated to an excited state.

6. APPLICATIONS OF ATOMIC ABSORPTION SPECTROPHOTOMETER

- Atomic absorption spectrometry has many uses in different areas of chemistry.
- The different methods of Atomic absorption spectrometry are very powerful for analyzing elements in a solution.
- The instruments are simple and easy to operate.
- They are useful when a few elements have to be determined in a large number of samples, as is the case in clinical or food analysis.
- Atomic absorption spectroscopy methods are of great importance compared with other methods of elemental analysis.
- In clinical analysis, it is used for analyzing metals in biological fluids such as blood and urine.

- An environmental analysis is utilized for monitoring the levels of various elements in rivers, seawater, drinking water, air, etc., Presence of contaminants can be monitored in foodstuffs like fruit juices and wines.

- AAS can trace the presence of pesticide residues in fruits and vegetables. In pharmaceuticals industries, it is used for the assay of drugs.

- The purity of the sample can be checked for minute quantities of a catalyst used in the manufacturing process (usually a metal) sometimes present in the final product.

- Levels of the toxic substances present in the products can be verified and reduced by this technique.

- By using Atomic absorption spectrometry in mining industries, the number of metals such as gold in rocks can be determined to see whether it is worth mining the rocks to extract the gold.

7. ADVANTAGES OFATOMIC ABSORPTION SPECTROPHOTOMETER

- High selectivity and sensitivity.
- Fast and simple working.
- Does not need metals separation.
- Specific because the atom of a particular element can only absorb radiation of theircharacteristic wavelength.

8. DISADVANTAGES OFATOMIC ABSORPTION SPECTROPHOTOMETER

- The analysis doesnot simultaneous.
- Cannot use for elements that give rise to oxides in flames.
- Limit types of cathode lamps (expensive).

Questions: -

1. What is the principle of calorimetry?
2. Define the types of spectrophotometer?
3. Describe the Lambert beer law with equation?
4. Which type of detector is commonly used in spectrophotometer?
5. Which type of radiation source that used in AAS?
6. What are the most common oxidants used in atomizer?

Check your progress of unit 2 exercise

- Check the answer of all question are given in the unit 2 read carefully.

BLOCK

2

BLOCK- II Introduction to Photometric, Electrophoresis and Chromatography

Unit III Photometric Methods and Electrophoresis Principle

Unit IV Chromatography Principle

UNIT III :PHOTOMETRIC METHODS AND ELECTROPHORESIS PRINCIPLE

Learning objectives are: -

3.0 **Spectrophotometer fluorimetry**

3.1 Introduction of spectrofluorimetry

3.2 Classification of spectrofluorimetry

3.3 Factor affecting the fluorescence

3.4 Instrumentation design of spectrofluorimetry

3.5 Applications of spectrofluorimetry

3.6 Advantage of spectrofluorimetry

3.7 Disadvantageof spectrofluorimetry

3.1 Flame photometer

3.1.1 Introduction of flame photometer

3.1.2 Principle of flame photometer

3.1.3 Working procedure of flame photometer

3.1.4 Applicationsof flame photometer

3.1.5 Advantage of flame photometer

3.1.6 Disadvantageof flame photometer

3.0Electrophoresis:

3.2.1 Introduction of electrophoresis.

3.2.2 Principle and methodology of electrophoresis.

3.2.3 Classification of electrophoresis-

3.2.4 Zone Electrophoresis

3.2.5 Moving boundary electrophoresis

3.2.6 Common medium used in electrophoresis

3.2.7 Factors influencing electrophoresis

3.2.8 Applications of electrophoresis

3.0 SPECTROPHOTOMETER FLUORIMETRY

3.1.INTRODUCTION

Fluorescence spectroscopy, fluorimetry, or spectrofluorimetry is a way to look for and look at fluorescence in a sample. A substance (fluor) that has taken in light or other types of electromagnetic radiation emits light. In this type of emission, a beam of light (usually UV light) moves an electron in a molecule from its ground state to a higher energy excited state, where it stays for a while. In this case, the electron goes back to the ground state and emits a glow. Fluorescence spectroscopy focuses mostly on electronic states (the ground state and the excited state) and vibrational states (the vibrational state). For any molecule, it requires EMR for excitation that why initially both spectrophotometry and spectrofluorimetry works on the same principle (see in previous unit 2) i.e. absorption till both reacts with sample and later on both works on different principles (photometry on absorption and fluorimetry on emission).Every molecule try to stabilize by giving its extra amount of energy to another molecule, thus in spectrophotometry, the extra amount of energy (provided by EMR) is transferred to neighboring molecules (i.e. solvent) in heat as a relaxation process while in spectrofluorimetry, the extra amount of energy (provided by EMR) is transferred to neighbouring molecules at longer wavelengths(in terms of Fluorescence).

1. CLASSIFICATION:

Based on the wavelength of emitted radiation when compared to absorbed radiation

1) **Stokes fluorescence:** emitting radiation has a longer wavelength than the radiation that is taken in.

2) **Anti-stokes fluorescence:** In this case, the wave length of emitted radiation is shorter than the wave length of absorbed radiation.

3) **Resonance fluorescence:** emitting and absorption waves are both same length.

4) **Photo Luminescence.** It is called light that doesn't have heat or cold light. There are two main types:

a) Fluorescence: part of energy is lost due to vibrational transitions and remaining energy is emitted as uv/visible radiation of longer wavelength than incident light.

b) Phosphorescence: When things are going well, excited singlet states move to a triplet state. When going from a triplet state to a ground state, radiation is released.

- This is called a "non-radiative transition." In molecular species, energy transitions can happen in a variety of vibrational levels of a single excited state. Because the vibrational level of an excited state matches the vibrational level of the ground state, some energy is lost as heat until it reaches the lowest vibrational level of the excited state. Electrons lose some energy in a non-radiative transition when they reach the lowest vibrational level of the excited state. Then, they follow a radiative transition. Radiative transition happens when electrons in a molecule fall back down from a high-energy excited state to a lower-energy ground state inside the molecule. Then, the energy emitted as light is measured. Most of the time, the emitted light has a longer wavelength (lower energy) than the radiation that was absorbed (higher energy). It is possible for the electrons or molecules to fall into one of several vibrational levels in the ground state during a radiative transition; this will make them emit different frequencies of light. It is a type of luminescence when the light that is emitted is in the range that we can see When fluorescence happens, the absorbed radiation is in the ultraviolet range of the

electromagnetic spectrum, which isn't visible to the human eye. The light that comes out is in the visible range.

Stokes Shift: Stokes shift is named after Irish physicist George G. It is the difference between the wavelength of absorption maxima and the emission maxima.

- Wavelength of absorbed radiation (having low wavelength units and higher energy) is denoted by a.
- Wavelength of emitted (fluorescence) radiation (having higher wavelength unit values and lower energy is denoted by b .

Stokes shift = b-a

A lot of good things happen when you use the compounds that have a bigger Stokes shift. In general, the more Stokes shift there is, the less interference there will be, because the excitation and emission spectra don't match up.

Fluors and fluorophores are two types of chromophores that show fluorescence. Fluorophores are organic molecules that have 20 to 100 Daltons. Fluorescent molecules take in electromagnetic radiation in the visible range and then give off radiation at a different wavelength in the visible. A good example is ethidium bromide (493 nm/620 nm). Most of the time, fluorescent molecules take in electromagnetic radiation in the UV range and give off light in the visible range. Green fluorescent protein has a wavelength of 360 nm and a wavelength of 508 nm, like this:

- Intrinsic fluors: In the case of proteins, aromatic groups are found in the side chains of amino acids, like tyrosine, tryptophan, and phenylalanine. Cofactors like FMN, FAD, and NAD also glow.
- Extrinsic fluors: Non-fluorescent compounds can be found by attaching a fluorescent probe to them (or fluor). 1- Anilino-8-naphthalene sulfonate, fluorescein (for protein), ethidium bromide, and acridine orange are some of them (for DNA).

Fluor has a unique emission spectrum (fluorescence), as well as a unique absorbance spectrum that changes depending on its structure and the chemicals in its environment. There

will be a lot of electrons at room temperature that are in the ground state and the lowest vibrational level (S0V0). In this case, the electromagnetic radiation gives electrons high-energy states that they can reach by taking in photons (Figure 1). A lot of people get excited in less than 10 to 15 seconds. From 0.5 to 8 ns (0.5 to 8 x 10-9 s), the life of the excited state is very short. In some cases, it can last for up to 2 sec (this situation can arise as a consequence of a phenomenon associated with electrons called magnetic spin). There is a lot of heat lost quickly because electrons don't move through space. Because of the collision degradation, the lowest vibrational energy is in the lowest excited state because it has the least energy (S1V0). Fluorescence is the energy that is released when electrons reach the lowest vibrational level of the excited state. They return to the ground state in less than 10-8 s, and the energy is called fluorescence. In fluorescence emission measurement, the excitation wavelength is always the same, but the detection wavelength can be different as well. Detection is always the same, but the emission wavelength changes.

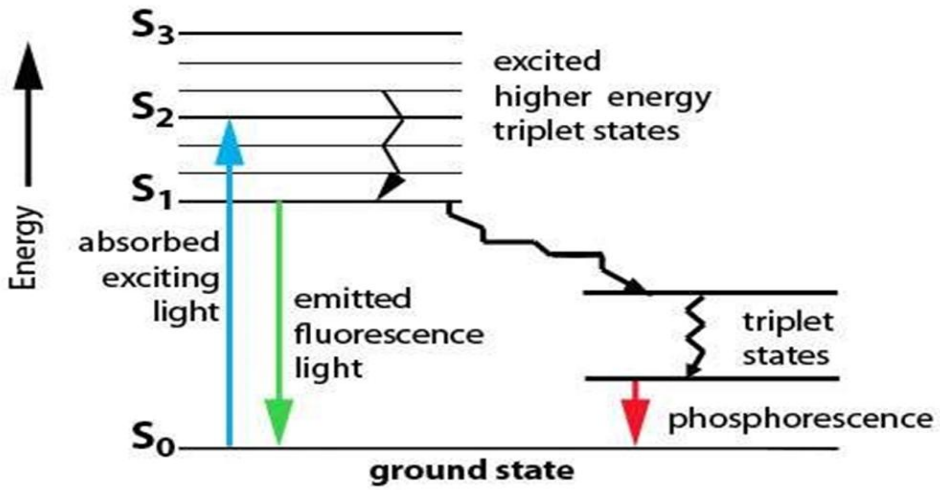


Figure 1: Energy level of fluorescence and phosphorescence

Fluorescent indicators:

- Intensity and colour of the fluorescence of many substances depend upon the pH of solutions. These are called fluorescent indicators and are generally used in acid base titrations.

Example

- Eosin – pH 3.0-4.0 – colourless to green.
- Fluorescein – pH 4.0-6.0 – colourless to green.

2. **FACTOR AFFECTING THE FLUORESCENCE**

i. Nature of molecules

ii. Nature of substituent
iii. Effect of concentration
iv. Adsorption
v. Electron
vi. Light
vii. Oxygen
viii. pH
ix. Temperature & viscosity
x. Intensity of incident light
xi. Path length: -
xii. Quenching:

3. INSTRUMENTATION

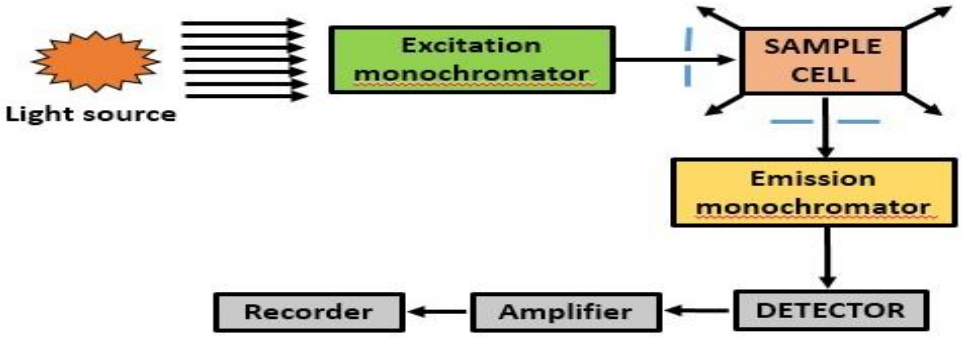


Figure 2: Systematic process chart of fluorimetry spectrophotometer

a) Light source: Light from a mercury lamp comes out at its best. The Xenon arc has a continuous emission spectrum with the same intensity in the 300-800 nm range. It can also be used above 200 nm, but it isn't very bright.

b) Monochromators: Monochromators that use a diffraction grating are the most common. It is used two monochromators to get the colour There is only one monochromator (excitation monochromator) that is used to choose the excitation wavelength from the beam that is coming in. If something is fluorescent, it will show the fluorescence in all directions. One monochromator (emission monochromator) and another monochromator are used to figure out the spectrum of the light emitted by a plant. The excitation

wavelengths that are chosen most often are in the ultraviolet range, and the emission wavelength is in the visible range.

c) **Detector:**It can be a single-channel detector that only picks up one wavelength at a time or a multichannel detector that picks up all the wavelengths. Both have their advantages and disadvantages. The detector is a very sensitive photocell (eg: red sensitive photomultiplier for wavelengths greater than 500nm).

There are two setups for the illumination of the sample:

1. **90° illumination:** When radiation is absorbed before it reaches the fluorescent molecule, pre-filter effects happen. When the molecule emits less fluorescence before it can get out of the cuvette, this is called post-filter effects. These effects get bigger as the concentration of the sample goes up. The use of microcuvettes helps to some extent with this (Figure 3).

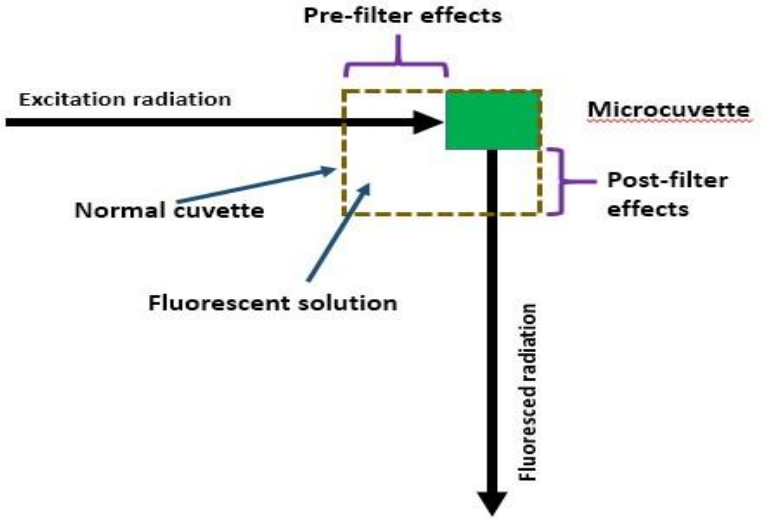


Figure 3: Diagram showing pre and post filter effect

2. **Front face illumination:**This type of lighting removes the effects of pre- and post-filters. When you use front face illumination, you use a cuvette that only has one optical face. Both the excitation and the emission happen on the same face. Setup: This one isn't as sensitive as a 90° light source (Figure 4).

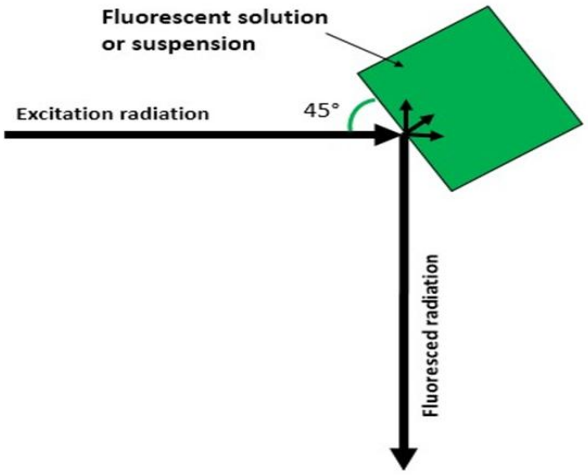


Figure 4: Diagram showing front face illumination

Instrument design: It is designed into two types-

1) **Single beam Fluorimeter**

- Tungsten lamp as source of light.
- The primary filter absorbs visible radiation and transmits uv radiation.
- Emitted radiation measured at 90o by secondary filter.
- Secondary filter absorbs UV radiation and transmits visible radiation.

Advantages

- Simple in construction.
- Easy to use.
- Economical

Disadvantages

- It is not possible to use a reference solution & sample solution at a time.
- Rapid scanning to obtain Excitation & emission spectrum of the compound is not possible.

2) **Double beam fluorimeter**

- Similar to a single beam instrument.
- Two incident beams from light source pass through primary filters separately and fall on either sample or reference solution.
- The emitted radiation from sample or reference passes separately through a secondary filter.

Advantage

- Sample & reference solution can be analysed simultaneously.

Disadvantage

- Rapid scanning is not possible due to use of filters.

4. APPLICATION OF FLUORIMETRY

- Fluorescent probes: It helps in the detection of biological compounds which is present in very low concentration in a mixture. They are applied to characterize folding intermediates and surface hydrophobicity.

- Protein and peptide structure: The intrinsic fluorescence such tryptophan, tyrosine and phenylalanine present in the protein are responsible for the fluorescence exhibited by the proteins. Proteins are generally excited at 280 nm and fluorescence is measured at 295 nm.

- Tyrosine emits fluorescence less than the tryptophan and its fluorescence is quenched by the tryptophan present in its vicinity. Phenylalanine gives weak fluorescence and its fluorescence is only observed when both tyrosine and tryptophan are absent. Any conformational change in the protein therefore changes the absorbance. Cofactors such as FMN, FAD, NAD exhibit fluorescence and are also applied in protein structural studies.

- Membrane Structure: The intensity fluorescence of a fluorescently labelled molecule is dependent upon the solvent/environment in which it is present. Changes in the pH or solvent polarity affects the conformation and therefore structure changes can be monitored by the changes in the fluorescence.

- Fluorescence recovery after photobleaching (FRAP): Fluorescent probes are used to measure how quickly molecules move across layers or thin membranes. This technique is called FRAP. Fluorescence is measured in the sample under study. An optical microscope with a time line camera is used to look at the sample and take pictures. FRAP can also be used to look at the protein binding in the cell membrane, the cell surface, and the free energy in phospholipid layers, among other things.

- Fluorescence resonance energy transfer (FRET): FRET, electronic energy transfer, or dipole-dipole coupling can transfer energy from donor to acceptor flour. To achieve this, the distance between the donor and acceptor must be small, and the donor and acceptor fluorescence spectrums must overlap. The donor fluorescence alone will fluoresce.

- Fluorescence immunoassay (FIA): FIA is a sophisticated technique that uses fluorescent probes to label either antigen or antibody interactions. Primary antibodies detect antigen by binding. Washing removes excess primary antibody. The secondary antibody labelled with fluor detects the antigen-antibody complex. Washing removes excess secondary antibody. Spectrofluorimetry detects fluorescence at a specific wavelength. The technique's main flaw is high background fluorescence. To reduce background fluorescence and increase sensitivity, two methods are used. First, large Stokes shift fluorescence (europium chelates) should be preferred. A good fluorimetry design delays detection of emitted light while the background fluorescence decreases.

- Fluorescence activated cell sorter (FACS): It is called flow cytometry. FACS is a type of flow cytometry. Fluorescence and light scattering are used to separate or sort cells from a mixture of cells (cell suspension) into different compartments. It is a way to physically separate or sort cells from a mixture of cells (cell suspension). Cells in a suspension are

allowed to pass through a small nozzle in a stream of water. This fast-moving stream of liquid is broken into droplets, each with a single cell inside. This is done by vibrating.

- Micro-spectrofluorimetry: A spectrofluorometer has a microscope that lets you see how an antibody binds to a single bacterial cell or subcellular organelle, and it also helps you tell cancerous cells from healthy ones because they have different proteins that make them glow. It's because some malignant cells have more nucleic acid content than normal cells. This means that these malignant cells take up more acridine orange dye that binds to the DNA and makes them easier to tell apart from the normal cell.

5. ADVANTAGES OF SPECTROFLUORIMETER

- More sensitive when compared to other absorption techniques. Concentrations as low as μg/ml or ng/ml can be determined.
- Precision up to 1% can be achieved easily.
- As both excitation & emission wavelengths are characteristic it is more specific than absorption methods.

6. DISADVANTAGE OF SPECTROFLUORIMETER

- Rapid scanning is not possible due to use of filters.

FLAME PHOTOMETER

1. INTRODUCTION

Flame photometry is one of the branches of atomic absorption spectroscopy. It is also known as flame emission spectroscopy. Currently, it has become a necessary tool in the field of analytical chemistry. Flame photometer can be used to determine the concentration of certain metal ions like sodium, potassium, lithium, calcium and cesium etc. In flame photometer

spectra the metal ions are used in the form of atoms. The International Union of Pure and Applied Chemistry (IUPAC) Committee on Spectroscopic Nomenclature has named this technique as flame atomic emission spectrometry (FAES). It is a device that is used in inorganic chemical analysis for the determination of certain metal ions such as potassium, sodium, calcium, and lithium. The photoelectric flame photometry is based on determining the intensity of emitted light produced when a metal is introduced into a flame. During 1980s Bowling Barnes, David Richardson, John Berry and Robert Hood developed an instrument to measure the low concentrations of sodium and potassium in a solution. They named this instrument as Flame photometer. The principle of flame photometer is based on the measurement of the emitted light intensity when a metal is introduced into the flame. The wavelength of the colour gives information about the element and the colour of the flame gives information about the amount of the element present in the sample.

2. PRINCIPLE OF FLAME PHOTOMETER

The compounds of the alkali and alkaline earth metals (Group II) dissociate into atoms when introduced into the flame. Some of these atoms further get excited to even higher levels. But these atoms are not stable at higher levels (Figure. 5).

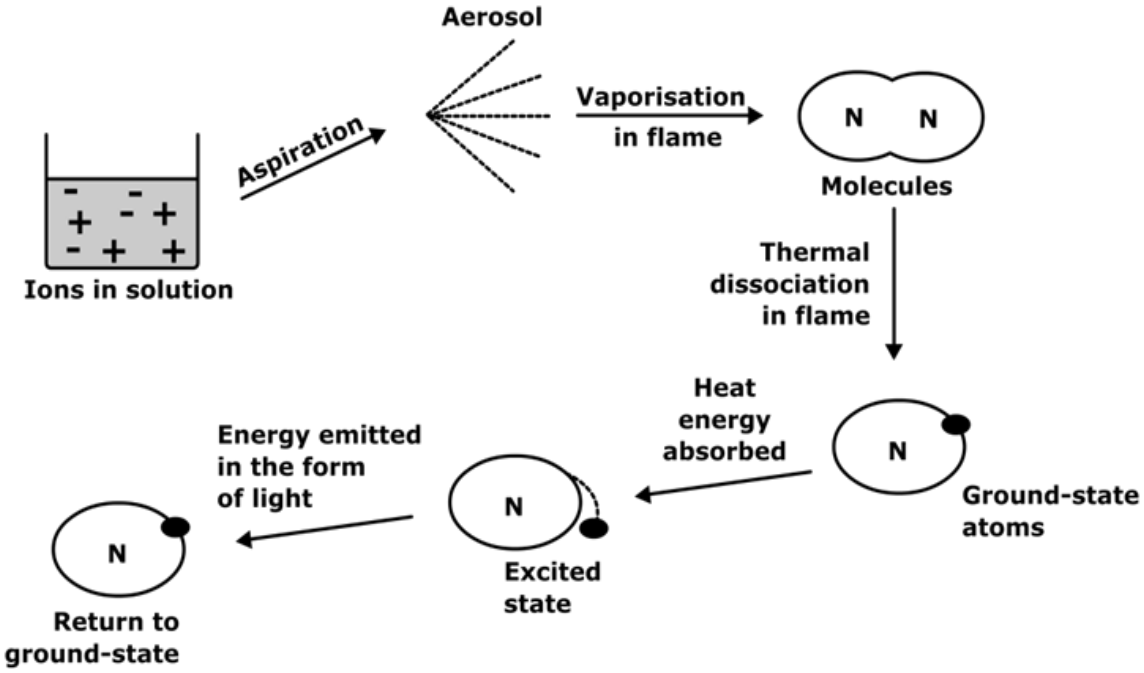


Figure 5: Principle of flame photometer

The liquid is sprayed with a non-shiny flame in the form of a fine mist that is colored according to the characteristic emissions of the elements, e.g. Sodium (Na), Potassium (K), Calcium (Ca) and Lithium (Li).

Table 1. List of emission of element with their specific flame color

Element	Emitted wavelength	Flame color
Sodium	589 nm	Yellow
Potassium	766 nm	Violet
Barium	554 nm	Lime green
Calcium	622 nm	Orange
Lithium	670 nm	Red

Before beginning a working procedure of flame photometry, we must recognize the various components essential to perform the process. The flame photometer consists of four major components such as a source of flame, nebulizer, optical system, photodetector and recorder.

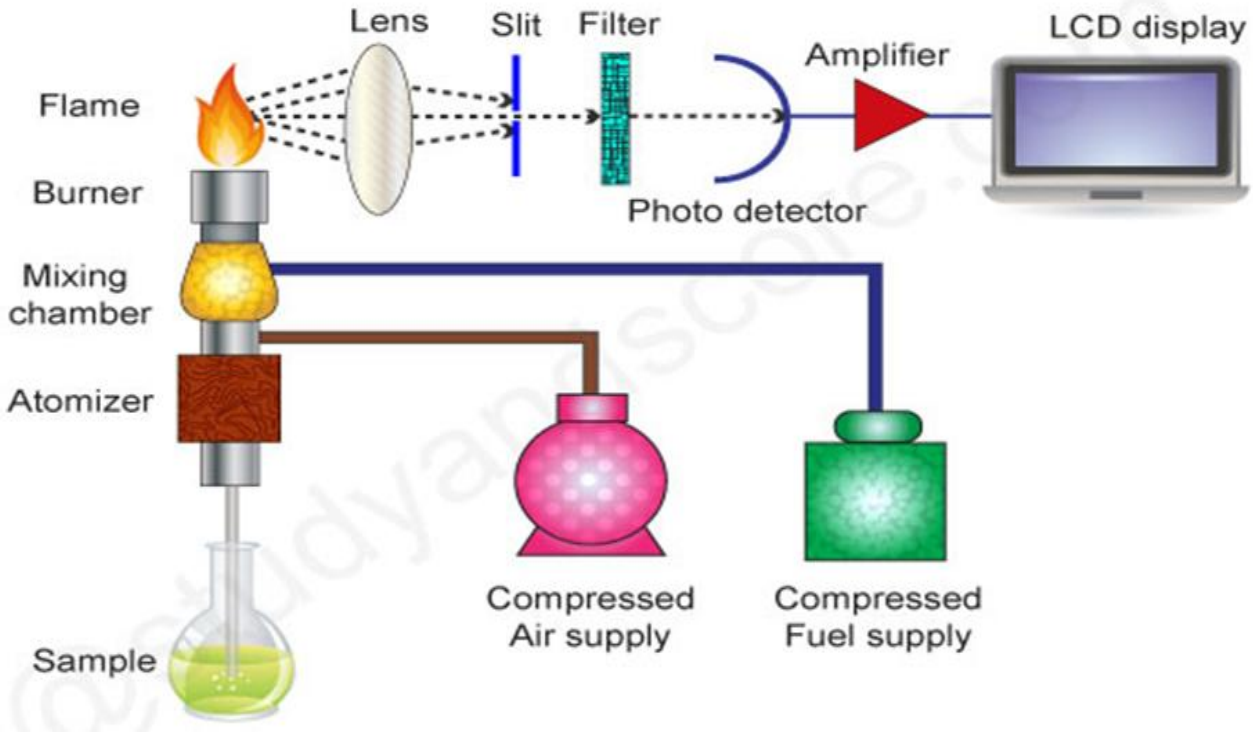


Figure 6: Diagrammatic representation of instrumentation of flame photometer

I. **Source of flame:** A Burner in the flame photometer is the source of flame, which converts the sample into excited atoms by spraying the sample into the combination of fuel and oxidant. Fuel and oxidants are essential for production of flame. It can be

maintained in a stable form and at a constant temperature. The temperature of the flame is one of the critical factors in flame photometry and it is important because converting the sample into neutral atoms which get excited by heat, because at high temperature ions will be formed instead of neutral atoms and at low temperature the atoms will not get excited. Various type of burner is used such as Mecke burner, Laminar flow burner and total consumption burner. The temperature plays an important role in converting the sample into neutral atoms which get excited by heat, because at high temperature ions will be formed instead of neutral atoms and at low temperature the atoms will not get excited.

II. **Nebulizer:** This helps to move the sample solution of the molecules into a flame at a constant rate. In mixing chamber fuel and oxidants are mixed together and then transported to the flame. Fuel-oxidant ratio is very important in flame, it is responsible for maintaining the flame temperature. Following is the description of events going on in flame-

Desolvation → Vaporization → Atomization → Excitation → Emission process

- Desolvation: Desolvation involves drying a sample in a solution. The metal particles in the solvent are dehydrated by the flame and thus solvent is evaporated.
- Vaporization: The metal particles in the sample are also dehydrated. This also led to the evaporation of the solvent.
- Atomization: Atomization is the separation of all atoms in a chemical substance. The metal ions in the sample are reduced to metal atoms by the flame.
- Excitation: The electrostatic force of attraction between the electrons and nucleus of the atom helps them to absorb a particular amount of energy. The atoms then jump to the higher energy state when excited.
- Emission: Since the higher energy state is unstable the atoms jump back to the ground state or low energy state to gain stability. This jumping of atoms emits radiation with characteristic wavelength. The radiation is measured by the photo detector.

III. **Optical system:** The optical system consists of three elements such as lens, filter, and a convex mirror. This separates the wavelength of any other extraneous emissions to be measured. The convex mirror transmits the light emitted from the atoms. Convex mirror also helps to focus the emissions to the lens. The lens helps to focus the light on a point or slit.

IV. **Photodetector:** The intensity of radiation emitted by the flame is measured by photo detector. The radiation emitted by atoms are in visible region (400nm-700nm). Flame photometric detector (FPD) is famous for its hydrogen rich flame because it is much cooler than oxygen rich flame which is used in Flame ionization detector (FID). Chemiluminescent reaction is used by flame photometric detector in the hydrogen-air flame. Flame photometric detector is sensitive for Sulphur and phosphorous comprising molecules. Flame photometric detector works by passing the effluent through the low temperature hydrogen-air flame, then the Sulphur and phosphorus are converted into emitting species. The wavelength maximum for the emitting species of S compounds is at about 394 nm, the Sulphur compounds are excited to S_2. The wavelength maximum for the emitting species of P-compounds is at about 512-526 nm, the phosphorus compounds are excited to HPO.

V. **Recorder:** Recorder records the data obtained from the detector and the display unit read-out this data.

3. WORKING PROCEDURE

Both the standard stock solution and a sample solution are made with distilled water that is very clean and clear.

A photodetector looks at the flame through a very narrow band optical filter that only lets in the wavelength (nm) that the chosen element emits. The output of the photodetector is sent to electronic modules that give digital readings of the concentration of the element you want to measure. There must be known concentrations of a solution in order for the method to be calibrated before it can test an unknown liquid sample.

With the help of a compressor, compressed air is sent to an atomizer in a measuring system so that air can be drawn into the atomizer tip and into the mixing chamber. This air is then sucked into the mixing chamber. A controlled amount of liquid petroleum gas (LPG) is also used in the mixing chamber. The atomized sample and gas are then delivered to the burner and set on fire. When the flame is stable for about 5 minutes, it is turned off and the process starts again. Now that the instrument is turned on, the lids of the filter chamber can be opened

so that the correct colour filters can be put in. It is done by spraying distilled water into a flame to make the galvanometer's readings go back to 0. When you spray the most concentrated standard working solution into the flame, you can change how sensitive it is. Full-scale movement of the galvanometer has been recorded now, so we can see how it moves. Again, distilled water is sprayed into the flame to get the same readings from the galvanometer. The galvanometer is then set back to zero. Afterward, each of the standard working solutions is sprayed in three times into the on-shiny flame to see what the galvanometer read. After each time you spray, you need to wash the sprayer thoroughly. Use the galvanometer to figure out the average of all of the readings on it. Use the graph of concentration to figure out how much of the element there is in the sample.

4. APPLICATIONS OF FLAME PHOTOMETER

- Flame photometer can be applied both for quantitative and qualitative analysis of elements. The radiations emitted by the flame photometer are characteristic to particular metal. Hence with the help of Flame photometer we can detect the presence of any specific element in the given sample.

- The presence of some group II elements is critical for soil health. We can determine the presence of various alkali and alkaline earth metals in soil sample by conducting flame test and then the soil can be supplied with specific fertilizer.

- The concentrations of Na^+ and K^+ ions are very important in the human body for conducting various metabolic functions. Their concentrations can be determined by diluting and aspirating blood serum sample into the flame.

- Soft drinks, fruit juices and alcoholic beverages can also be analyzed by using flame photometry to determine the concentrations of various metals and elements.

5. ADVANTAGES OF FLAME PHOTOMETER:

- It is used to look for sodium, calcium, potassium, and lithium ions. The intensity of the light that is emitted affects how many ions are found.
- This is a quick, appropriate, selective, and responsive analysis. This is how it should be done.
- It's a simple and cheap way to do research.
- It is used for both qualitative and quantitative tests of metal ions.
- The things that aren't usually looked at in this way can be found.
- Metal ions can also be found in very small amounts in the sample.

6. DISADVANTAGES OF FLAME PHOTOMETER:

- This method can only be used to look at liquids.
- In some cases, it takes longer to make the sample.
- You can't learn about the molecular structure of the metal ion that is in the sample with this method because it doesn't look at it.
- Each and every metal atom can't be found out by looking at flames.
- This method can't measure the concentration of the metal ion very well.

ELECTROPHORESIS

1. INTRODUCTION

“Electrophoresis” literally means running in the electric field. It moves to the electrodes with the opposite charge, but the electric field is cut off before it gets there. The movement of charged species in an electric field gives the sample different mobility based on its charge, which helps to separate them. The movement of the charged particle is slowed down by adding a polymeric gel. This gives enough time to separate the sample. The polymeric gel is inert, does not charge, and does not slow down the process by binding the molecule. Instead, it makes pores of different sizes (based on the concentration of polymer). Samples pass through these pores, and as a result, their electrophoretic mobility is slowed down because of this (Figure 7).

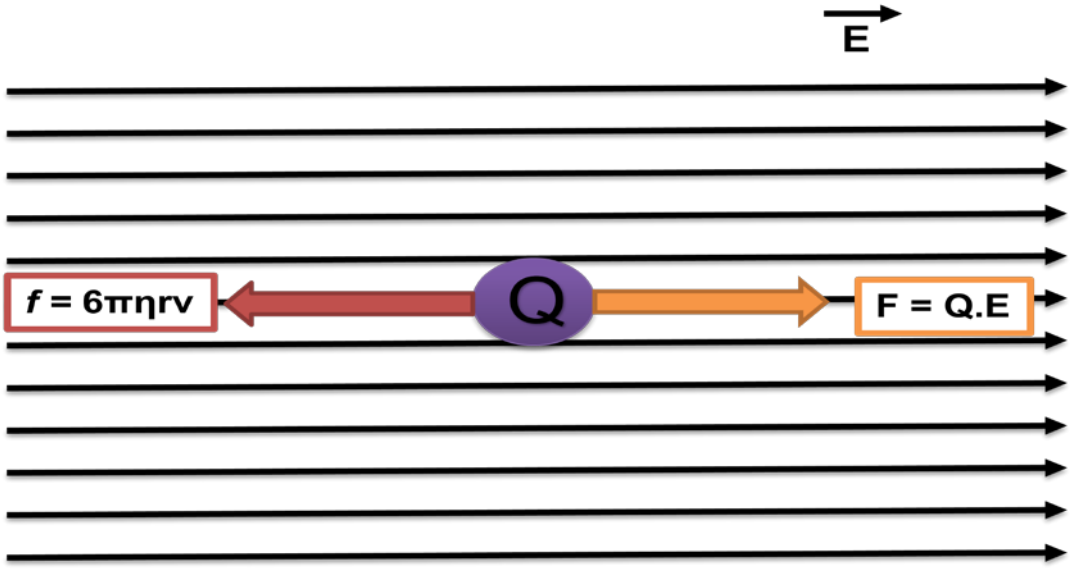


Figure 7: Movement of the charged particle in an external field.

Suppose a charged particle has net charge Q and the external electric field is E, then the force F responsible for giving electrophoretic mobility,

F= Q.E…… Eq (1)

The friction forces F which is opposing the movement of the charged particle is as follows

F= *f*. v……Eq (2),

Here *f* is the friction coefficient and the v is the velocity of the electrophoretic mobility. The movement of a spherical through a liquid medium (gel) of the viscosity η, the friction coefficient *f* is given by:

***f* = 6Πηrv……Eq (3)**

The place where, F=F or QE=6Πηrv

The electrophoretic mobility v is given by: **v =Q /6Πηr**

As Q=ze, where z is the valency and e are the electronic charge, the electrophoretic mobility can be expressed as:

$$v = \frac{ze}{6\Pi\eta r}$$

If you want to move faster through an electrophoretic medium, you need more charge and less viscosity, as well as a bigger molecule. In the case of relative mobility, the charge or radius of the molecule plays a big role. It is important to know how big a globular protein is

because it has a radius (r) that is related to the size of the macromolecule. The relative mobility, v', looks like this:

$$v' = \textbf{Charge/ mass} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\textbf{Eq (4)}$$

2. PRINCIPLE AND METHODOLOGY

All types of electrophoresis are directed by the single set of general principles illustrated by Equation:

$$\textbf{Mobility of a Molecule} = \frac{\textbf{(Applied Voltage) (Net charge on the Molecule)}}{\textbf{Friction of the Molecule}}$$

The speed at which a molecule moves, or how quickly it moves, increases with more voltage and more net charge on the molecule. The mobility of a molecule, on the other hand, decreases when there is more molecular friction, or resistance to movement through a viscous medium, because of the size and shape of the molecule and how it moves through the medium. Overall, the rate at which molecules move increases with time. This is because movement is defined as the rate of migration.

There is no molecular sieving in a buffer solution that is not a free buffer solution. The speed of a particle is related to the strength of the field E, multiplied by its electrophoretic mobility μ:

$$\mathbf{v = \mu E}$$

The velocity (v) and Field strength (E) both are vectors, while the mobility (μ) is scalar, being positive for cations and negative for anions.

When molecules move, the speed at which they do so is influenced by factors like the strength and size and shape of the molecules as well as their net charge and ionic strength. The viscosity, temperature, and viscosity of the medium in which they are moving also play a role. Rapid, simple, and very sensitive: This is a good way to look at things. It is also used as a diagnostic tool to look at the properties of a single charged species. It is the foundation for a lot of analytical methods that help you separate molecules by their charge, size, or affinity for each other. During an electric field, charged molecules move in the direction of electrodes that are oppositely charged to each other. Positively charged molecules move toward the

cathode, and negatively charged molecules move toward the anode. This is how it works: This movement is caused by the charge on the molecules and the electricity that is applied through the electrodes. The specimen is placed at one end of the electrodes. They move to their respective electrodes when electricity is put into them. But the molecular weight of the molecule has an effect on how the molecule moves around. When electrophoresis paper or gel is used to separate a mixture, different bands can be seen along the paper after the process is done. This is because molecules move at different speeds based on their weight.

3. CLASSIFICATION OF ELECTROPHORESIS TECHNIQUES:

Electrophoresis is usually broken down into two types based on whether or not there is a solid medium or matrix that helps the charged molecules move through the electrophoretic system. When there is no solid support medium, solution electrophoresis systems use water as buffers. Such systems can mix up samples because of the diffusion of the charged molecules, which can make them less clear during sample application, separation, and removal. This can cause a loss of resolution. So, solution electrophoresis systems need to use special tools to keep the aqueous solutions in the electrophoresis cell stable. Different types of electrophoresis techniques are made depending on whether or not it is done with or without a support medium.

Electrophoresis can be classified into two main types:

1) **Zone electrophoresis (ZE)**
2) **Moving-boundary electrophoresis (MBE)**

I. <u>ZONE ELECTROPHORESIS-</u>

In this method, an inert polymeric supporting media is used between the electrodes to separate and analyses the sample. The supporting media used in zone electrophoresis are absorbent paper, gel of starch, agar and polyacrylamide. The presence of supporting media minimizes mixing of the sample and that makes the analysis and purification of the molecule from the gel much easier than the moving boundary electrophoresis. The gel electrophoresis is the best example of zone electrophoresis. Zone Electrophoresis is classified into following types:

a) Paper Electrophoresis

b) Gel Electrophoresis

c) Thin Layer Electrophoresis

d) Cellulose acetate Electrophoresis

Advantages:

- Useful in biochemical investigations.
- Small quantities of samples can be analyzed.
- Cost is low and easy maintenance.

Disadvantages:

- Unsuitable for accurate mobility and isoelectric point determination.
- Due to the presence of supporting medium, technical complications such as capillary
- flow, electro osmosis, adsorption and molecular sieving are introduced.
-

a) PAPER CHROMATOGRAPHY

The technique of paper electrophoresis is simple and inexpensive and requires only micro quantities of plasma for separation.

- The support medium is a filter paper.
- The electrophoresis apparatus in its simplest form consists of two troughs to contain a buffer solution, through which electric current is passed.
- Frequently used in isolating proteins, amino acids and oligopeptides.

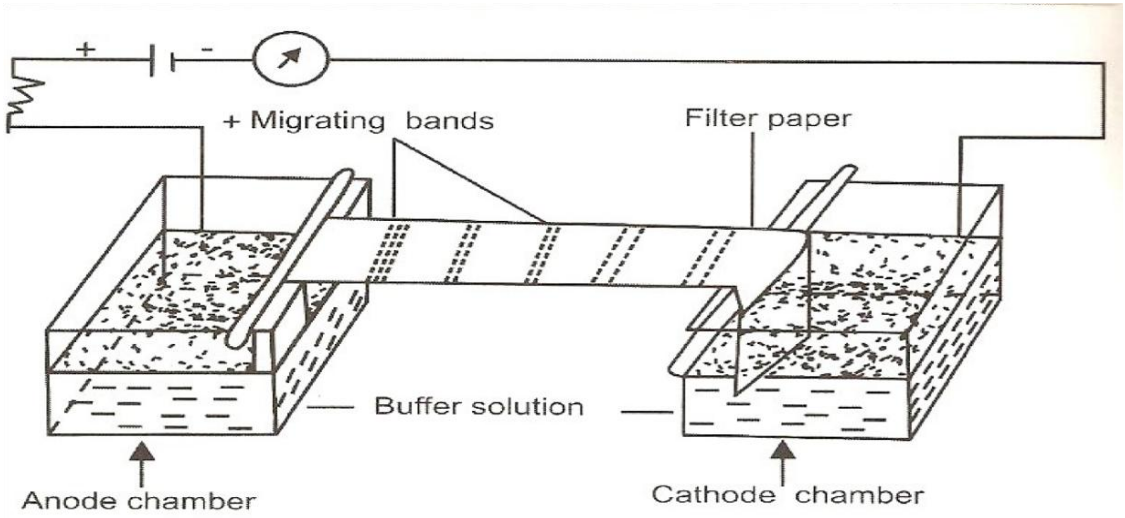


Figure 8: Structure of paper chromatography

Procedure

- In Figure 8, a long strip of filter paper is moistened with a suitable buffer solution of the desired pH and the sample is applied transversely across the central part of the strip.
- Ends are fixed to dip in buffer solutions in two troughs fitted with electrodes.
- Electric field of about 20 volts/cm is established.
- The charged particles of the sample migrate along the strip towards respective electrodes of opposite polarity, according to net charges, sizes and interactions with the solid matrix.
- Homogeneous group of particles migrate as a separate band.
- The electrophoresis is carried out for 16-18 hours.
- Separated Proteins are fixed to a solid support using a fixative such as Acetone or Methanol.
- Proteins are stained (bromophenol blue) to make them visible.
- The separated proteins appear as distinct bands

Advantages:

- It is economical.
- Easy to use.

Disadvantages:

- Certain compounds such as proteins, hydrophilic molecules cannot be resolved due to the adsorptive and ionogenic properties of paper which results in tailing and distortion of component bands.
- Electroosmosis
-

b) GEL ELECTROPHORESIS

Separation is brought about through molecular sieving technique, based on the molecular size of the substances. Gel material acts as a "molecular sieve". Gel is a colloid in a solid form (99% is water). It is important that the support media is electrically neutral. Different types of

gels which can be used are; Agar and Agarose gel, Starch, Sephadex, Polyacrylamide gels. A porous gel acts as a sieve by retarding or, in some cases, by completely obstructing the movement of macromolecules while allowing smaller molecules to migrate freely (Figure 9).

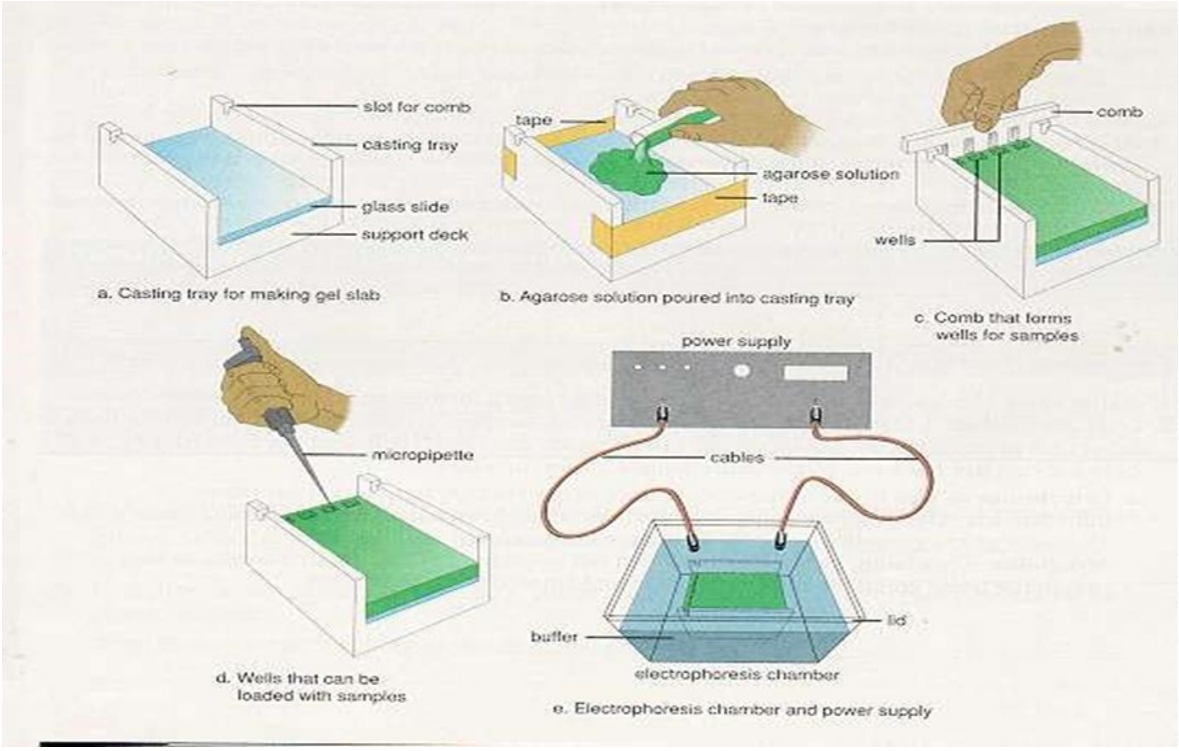


Figure 9: Structure of gel electrophoresis

Gel Electrophoresis is carried out in two methods:

I. Vertical Gel Electrophoresis: The electrophoresis in this system is performed in a discontinuous way with a buffer in the upper and lower tank connected by the gel slab. It has multiple modifications in the running condition to answer multiple analytical questions.

II. Horizontal Gel Electrophoresis: The electrophoresis in this system is performed in a continuous way and the electrophoresis is performed in the horizontal direction.

Polyacrylamide gel electrophoresis (PAGE)

- When acrylamide monomers are put together with methylene-bis-acrylamide, the monomers are linked together. In this picture, the structure of acrylamide (CH_2=CH-CO-NH_2)and polyacrylamide gel is shown. It is held together by covalent cross-links. This gel is more durable than agarose gels. It is thermostable, clear, strong, and a little bit chemically inert. Gels are not charged and come in a variety of pore sizes. They are separated by their charge to mass ratio and molecular size, which is called "Molecular sieving" (Figure 10).

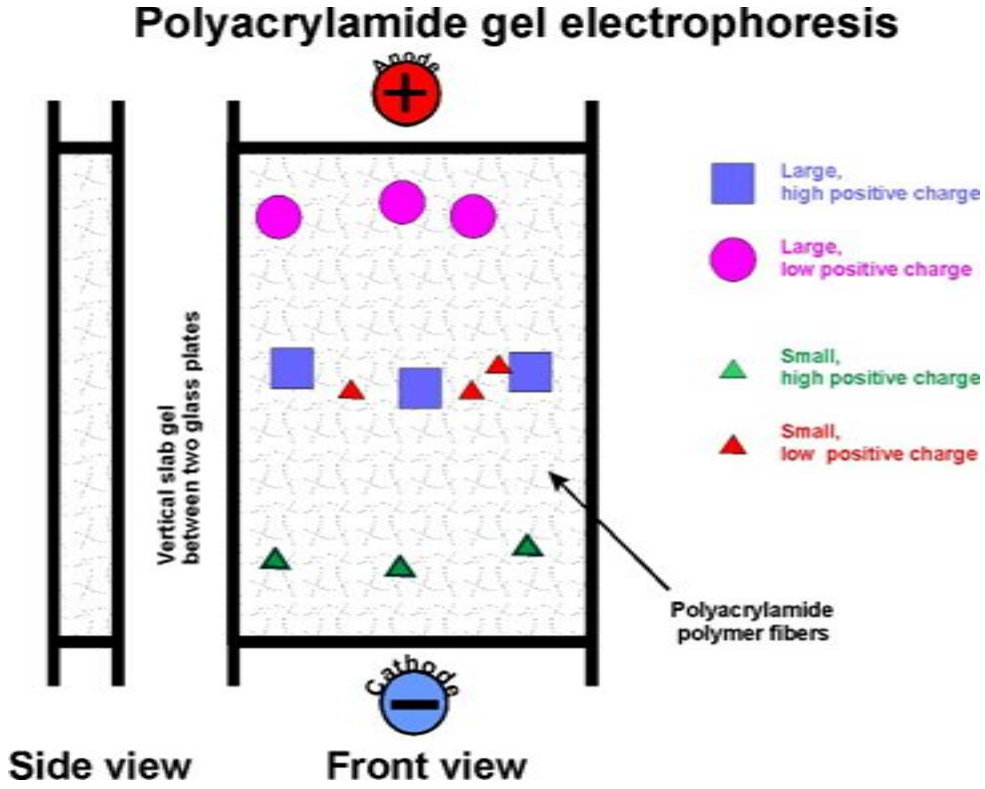


Figure: 10 Structure of polyacrylamide gel electrophoresis

Advantages:

- Gels can be used at a wide range of pH and temperature, and they stay the same.
- Gels with different pore sizes can be made. This is how it works:
- Compared to other things, it's easy to understand and the separation speed is good for its size.

Types of page

PAGE can be classified according the separation conditions into:

Native-PAGE:

- So that the analyte stays in its natural shape, native gels are run in non-denaturing conditions.
- People separate things based on how charged and big and round macromolecules are, as well as how they look and how they move.
- Useful for separating or removing proteins from a mixed group.
- This was the first way to do electrophoresis.

Denatured-PAGE OR SDS-PAGE:

- This means that proteins are separated based on how big they are in terms of molecular weight.
- The most common way to figure out the MW of proteins.
- It is very useful for checking the purity of protein samples with this tool.

c) THIN LAYER ELECTROPHORESIS: Electrophoretic migrations (separations) through a thin layer of inert material, such as cellulose, supported on a glass or plastic plate.

- Studies can be carried out in a thin layer of silica, kieselguhr, alumina.
- The studies with these materials offer advantages of speed and resolution when compared with paper.
- They have greatest application in combined electrophoretic-chromatography studies in two- dimensional study of proteins and nucleic acid hydrolysates.

Advantages:

- Less time consuming and good resolution.

Application:

- Widely used in combined electrophoretic-chromatography studies in two-dimensional study of proteins and nucleic acid hydrolysates.

d) CELLULOSE ACETATE ELECTROPHORESIS

It contains 2-3 acetyl groups per glucose unit and its adsorption capacity is less than that of paper and it gives sharper bands. It provides a good background for staining glycoproteins.

Advantage:

- No tailing of proteins or hydrophilic materials.
- Available in a wide range of particle sizes and layer thicknesses.
- Give sharp bands and offer good resolution.
- High voltage can be applied which will enhance the resolution.

Disadvantage:

- Expensive.
- Presence of sulphonic and carboxylic residue causes induced electroosmosis during electrophoresis.

Application:

- Widely used in analysis of clinical and biological protein samples (albumin and globulins).
- Alternative to paper electrophoresis.

II. MOVING BOUNDARY ELECTROPHORESIS

In this method, electrophoresis is done in a liquid, without any kind of support. The sample is dissolved, the buffer and molecules move to their opposite-charged electrodes, and they all end up in the same place. It is done in a U-shaped tube with platinum electrodes at the ends of both arms. This is how moving boundary electrophoresis is done: (Figure 11). Tube: At each end, there is a refractometer. This instrument is used to measure how the refractive index of the buffer changes during electrophoresis because there are molecules in the buffer. Samples are put in the middle of the U tube, and then the apparatus is connected to an outside power source. Charged molecules move to the other electrode as they pass through the refractometer, and this change can be seen on the refractometer. Whenever a good molecule comes through, samples can be taken out of the apparatus along with the buffer.

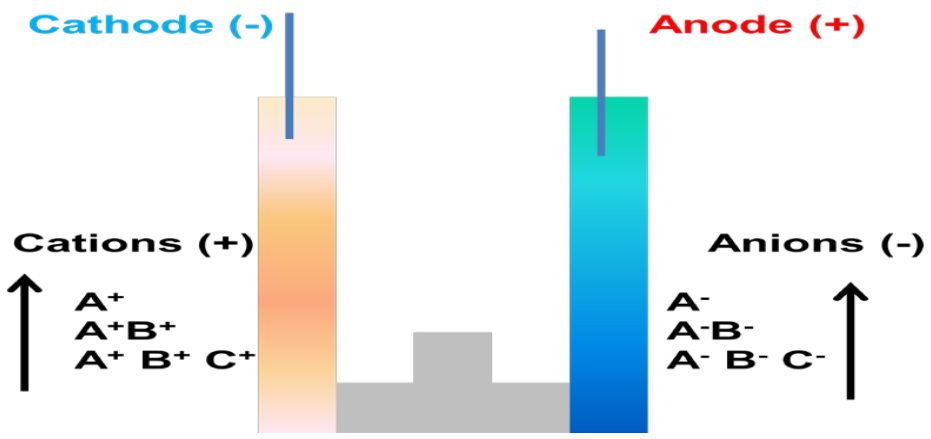


Figure 11: Movement of the charged particle in a moving boundary electrophoresis.

Advantages:

- It doesn't need to be done with denaturing agents to get biologically active fractions out of the water.
- A standard way to measure electrophoretic mobility.
- The optical system can detect very small amounts of the sample (0.05mg/ml by Interferometric optical system).

Disadvantages:

- Resolution is very low because the sample is mixed together and parts of the sample overlap each other, which makes it hard to see.
- When you use electrophoresis, you can't use it to separate and analyse a very complicated biological sample. Instead, you can use it to look at how the molecule behaves in an electric field.

It is divided into following types:

a) Capillary Electrophoresis
b) Electroosmotic Flow
c) Isotachophoresis
d) Immuno-electrophoresis
e) Isoelectric Focusing

a) CAPILLARY ELECTROPHORESIS

Using electrophoresis, charged molecules move toward the opposite pole and separate from one another based on how they look and move. Capillary electrophoresis has grown into a group of different separation techniques that use high voltages to separate things in buffer-filled capillaries. This is called capillary electrophoresis. It is a way to do electrophoresis in capillaries that are filled with buffer and have a small internal diameter, like 25 to 100 mm (ID). A high voltage, usually between 10 and 30 kV, is used. There are usually capillaries with an inner diameter of about 50 m. They are usually about 0.5 to 1 m long. Because of electroosmotic flow, all of the sample components move toward the negative electrode. This also stops the electroosmotic flow in the capillary. Gel can also be used to fill the capillary. Separation is done the same way as in gel electrophoresis, but the capillary allows for better resolution, more sensitivity, and on-line detection. Electrite solution fills the capillary, which lets electricity flow through it and make its way inside of the capillary. There are reservoirs filled with electrolyte where the ends of the capillary are dipped so that they can be cleaned. Electrodes (platinum) are inserted into the electrolyte reservoirs to complete the circuit and make electricity flow (Figure 12).

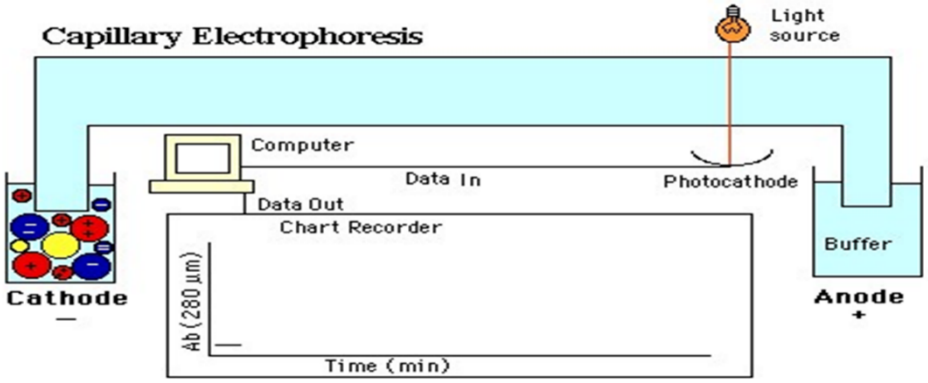


Figure: 12 Process of capillary electrophoresis

Sample application is done by either

I. High voltage injection-potential is applied causing the sample to enter capillary by combination of ionic attraction and electroosmotic flow.

II. Pressure injection-pressure difference is used to drive the sample into capillary by applying vacuum.

- When PD is used, the net migration goes in the direction of the cathode.

- People call this process Electro Osmotic Flow. Even substances that have a net negative charge move toward the cathode because of this process.
- When the neutral molecule moves at the same speed as the end of the sentence, they both move together. This means that positively charged species move faster. The speed is the sum of EOF and Electrophoretic mobility. Those molecules that aren't positive have to wait.

b) ELECTROOSMOTIC FLOW

The surface of the silicate glass capillary has functional groups that are negatively charged. They attract positively charged counterions. There are positive-charged ions that move toward the negative electrode, and solvent molecules move in the same direction. Electroosmotic flow is the name for this overall movement of solvent. In a separation, the electroosmotic flow moves at the same speed as the uncharged molecules move during the separation (with very little separation). Ions with positive and negative charges move faster, and ions with positive and negative charges move more slowly (Figure 13).

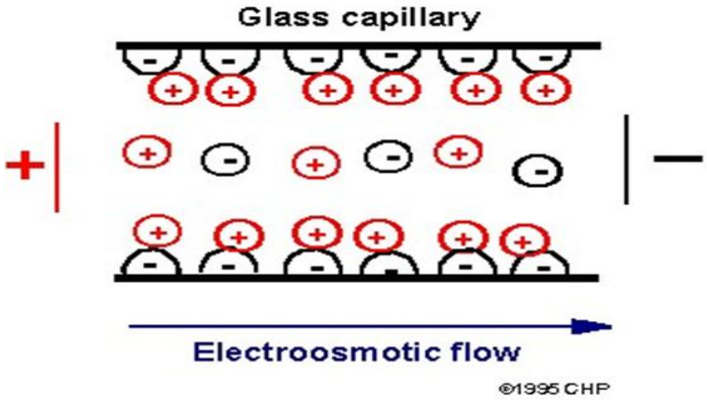


Figure: 13 Process of electroosmotic flow

A small volume of sample is moved into one end of the capillary. The capillary passes through a detector, usually a UV absorbance detector, at the opposite end of the capillary.

- Sample ions move toward their correct electrode when a voltage is applied. This usually happens through the detector.

- In this case, an electropherogram is made. It shows how the response of a detector changes over time.

c) ISOTACHOPHORESIS: The technique of isotachophoresis depends on the development of potential gradients.

Principle:

- Using moving boundary electrophoresis.

- The analytes are followed by a leading electrolyte (e.g. chloride) and a trailing electrolyte (e.g. glycinate).

- The separation solution is normally an aqueous medium containing sucrose to increase density.

- Where isoelectric focusing requires a pH gradient in the system. Isotachophoresis relies on a potential gradient.

- The ionic components of the sample are separated by stacking them in order of their mobilities, resulting in high resolution.

- When the voltage is applied, the analytes migrate in order of decreasing mobility.
- Once the potential gradient is established, all analytes move at the same speed.
- Individual zones border but do not overlap.
- No background electrolyte (buffer) is used in isotachophoresis, so only charged sample ions carry current.

- A faster moving component completely separates from a slower moving component, creating a depleted charge region that increases resistance and thus local voltage.
- Its increased conductivity causes the slower component to migrate faster and close the gap, concentrating it.
- In the end, all ions migrate at the same rate in zones of varying thickness, depending on their initial concentrations.

Application:

- Isotachophoresis that has been used for the separation of proteins as well as inorganic substances.

d) IMMUNOELECTROPHORESIS

- The immune system produces antibodies to foreign macromolecules. Each antibody binds to a single epitope on a macromolecule (antigen). So antibodies can detect and quantify specific proteins in complex mixtures.

- Immunoelectrophoresis is the study of antigen-antibody reactions using electrical potential. Antigens diffuse towards each other, forming precipitating arcs where antigen antibody complexes form. This process is known as immunoelectrophoresis.

- Antibody is placed in a trough parallel to the electrophoresis direction.

- Run the electrophoresis to precipitate arcs from the Ab-Ag complex.

- The antigens will be distributed in separate spots along a line passing through the well and parallel to the direction of current flow.

Method:

- It is usually carried out in 2% agar gel medium.

- The antigen mixture is applied into a small circular well cut out of agar and the initial electrophoretic separation is carried out depending on their charge and molecular weight.

- After the initial separation; the antibody mixture is then introduced into a narrow slot in the gel about 0.5 to 1.0 cm from the separated antigens.

- During this period, the antigen components diffuse radially outwards, towards the diffusing antibody and precipitation takes place in elliptical arcs as related antigens and antibodies diffuse into one another.

Advantages:

- Because of the applied field and the pH gradient, high resolution can be achieved.

Disadvantages:

- Carrier ampholytes are used in high concentrations, requiring high voltage power sources (up to 2000V) and power levels ranging from 2 to 50 W. The electrophoretic matrix must thus be cooled.

Application:

- Used to separate proteins and peptides.
- Used in enzymology, membrane biochemistry, microbiology, and immunology research to separate and identify serum protein.

e) ISOELECTRIC FOCUSING

Principle:

It is the pH of all proteins. So long as the current flows, proteins with a net charge will migrate towards the opposite electrode in electrophoresis. Each protein migrates to a specific pH area when the supporting medium has a pH gradient. The protein's pH matches the gradient's, resulting in distinct protein bands. A method for determining protein isoelectric points Thus, a mixture of proteins can be electrophoresed in a solution with a stable pH gradient from anode to cathode, with each protein migrating to its isoelectric point. Isoelectric focusing Protein migrates to isoelectric pH 0 where its net charge is zero. Protein migrates towards the cathode when its pH is below its PI. If the protein is negatively charged in solution, it will migrate to the anode. With no net charge, they will be in Zwitterion form and cannot move. An oligomer with aliphatic amino and carboxylic acid groups with a low molecular mass (600-900D) and a wide isoelectric point range. Ampholytes help maintain pH gradients in high-voltage systems. Use immobilized pH gradient gels (acrylamide derivatives covalently linked to ampholytes).
Ampholytes polyacrylamide

a. Cathode (-) electrode solution

b. Anode (+) electrode solution

Method:

- Ampholytes are added to the gel to create a pH gradient from anode to cathode.
- A protein mixture is put in a gel well.
- Proteins enter the gel and migrate until they reach their pH equivalent (PI).
- Proteins are thus focused into a narrow band around their PI.
- The column's anode connects to a phosphoric acid reservoir, while the cathode connects to a sodium hydroxide reservoir.
- Both solutions diffuse into the column from their respective reservoir valves, creating a PH gradient between the acidic anode and alkaline cathode.
- To migrate to PH regions with no net charge, the carrier ampholytes must close the valves and turn on the current. They will then remain stationary.

Advantages:

- High resolution is possible due to the applied field and pH gradient reducing band spreading.
- Proteins separated by 0.001 pH units can be separated.

Disadvantages:

- Given the high concentration of carrier ampholytes, high voltage (up to 2000v) is required.
- So the electrophoretic matrix must be cooled, which can be difficult.

Applications:

- For separating proteins and peptides.
- For research in enzymology, immunology, cytology and taxonomy.

4. COMMON MEDIUM USED IN ELECTROPHORESIS:

- **Cellulose Acetate:** Each hexose ring of the polysaccharide chain has two hydroxyl groups esterified to acetate (in general, in the C-3 and C-6 positions). Cellulose acetate is widely used in clinical chemistry because it is a quick-to-equilibrate support that separates proteins from biological fluids. This system has become the standard for combined electrophoresis and robotics. Large serum proteins can migrate in cellulose acetate. Despite the fact that focusing and two-dimensional techniques on cellulose acetate membranes have been described, cellulose acetate is only used in clinical electrophoresis.

- **Agarose Gels:**Agarose is a seaweed polysaccharide. Agarose is a purified linear galactan hydrocolloid obtained from agar or agar-bearing marine algae (Rhodophyta) directly. Agarose gels are simple to make and nontoxic. Agarose gels have a wide separation range but poor resolving power.
- **Polyacrylamide Gels:** The most resourceful of all matrices are the polyacrylamide gels. Their popularity is because of several fundamental properties:
  Transparency in ultraviolet (280 nm)

Electrical neutrality (no charged groups)
Wide range of pore sizes.

People who make polyacrylamide use acrylamide in a way that makes it cross-linked. Polyacrylamide gels are much more difficult to make than agarose gels. These gels have a very small range of separation, but they are very good at getting things to show up. These gels are used to look at and separate protein mixtures. Acrylamide is known to be a powerful neurotoxin.

5. FACTORS INFLUENCING ELECTROPHORESIS:

Proteins move in different ways depending on a lot of things. Molecules have to move through the gel as they move from one pole to the other to get there. They can weave in and out of the gel matrix more easily than bigger molecules. It moves faster if it has more net charge, has a shape like a ball and is smaller.

a. The buffer pH

- It will change the direction and speed of protein migration.

- There are many things that affect how proteins move, and one of them is how charged the proteins are. Proteins are made up of a sequence of amino acids that can be ionised depending on how acid or basic they are. To figure out how charged a protein is, add up all the electricity on its surface that is caused by the environment.

- It will depend on how strong their net surface charges are: If a protein has a lot of positive charges, it will move toward the cathode at a faster rate. On the other hand, the protein that has more negative charges will move towards the anode at a faster speed. It can be done this way because proteins can be separated based on their electric charges.
- Proteins in a sample will have different charges based on the pH of the buffer that they are mixed with, so this is how it works. P.I. (isoelectric point): At this point, the specific protein has no net charge and doesn't move in an electric field. At a pH above the pI, the protein has a net negative charge, so it moves toward the anode and away from the other

side. It gets a positive charge on its surface at a pH below the pI, so it moves toward the cathode.

b. The buffer ionic strength

- It affects how much of the current the proteins carry.

- At low ionic strength, the proteins will carry a lot of the current, which means they will move faster than other things. At high ionic strength, the buffer ions will carry most of the current, so the proteins will move very slowly. It might help to think of this effect of ionic strength in terms of an analogy. There are two counters in a bank: one for deposits (the anode) and one for withdrawals (the cathode). Electrons are the money in this bank. The ions can be thought of as customers who are waiting to be served at either of the two counters in the bank. They can be thought of as being at opposite ends of the bank.

- In electrophoresis, then, a low ionic strength is better because it speeds up the movement of proteins. A low ionic strength is also better because it doesn't make as much heat. If the ionic strength is increased, the electrical resistance will go down, but the current will go up. A high ionic strength buffer, on the other hand, will cause more heat, so a low ionic strength buffer is better.

c. The voltage gradient

- The rate of migration will depend on the voltage gradient: There is more voltage gradient in the electric field, protein will move towards the anode (or the cathode) at a faster rate.

d. Electro-osmosis

- Electro-osmosis is the name for when a liquid moves next to a solid medium in an electric field. In the applied electric field, electro-osmosis distorts the sample stream and limits

the separation. For example, Paper electrophoresis has poor resolution because of electro osmosis. The surface of paper has -e, so the buffer has +e derived from hydrogen ions because of electrostatic induction.

- Then +e drives a buffer to cathode in an electric field, these flows distort the electrophoretic migration of samples by causing a varying residence time. Thus, samples will move more or less than normal.

6. APPLICATIONS OF ELECTROPHORESIS

- DNA Sequencing.
- Medical Research.
- Protein research/purification.
- Agricultural testing.
- Separation of organic acid, alkaloids, carbohydrates, amino acids, alcohols, phenols, nucleic acids, insulin.
- In the food industry.
- Electrophoresis is also used for separation of carbohydrates and vitamins.
- Quantitative separation of all fractions of cellular entities, antibiotics, RBC, Enzymes etc. is possible.

Questions: -

1. What is the full form of PAGE?
2. Define the meaning of fluorescence?
3. What is the difference between spectrophotometer and spectrofluorimeter?
4. Describe the intrinsic and extrinsic fluorescence?
5. What is the main purpose to use nebulizer?
6. Zone electrophoresis is classified into how many types?

Check your progress of unit 3 exercise

- Carefully read the unit 3 and check your answer that all are given in this unit.

UNIT4: CHROMATOGRAPHY PRINCIPLE

Learning objectives are:

1. Chromatography

A. Introduction of Chromatography
B. History of Chromatography
C. Principle of Chromatography
D. Types of Chromatography
    1. Paper Chromatography
    2. Thin layer chromatography
    3. Gas chromatography
    4. High performance liquid chromatography

2. ANIMAL ASSAY

A. Introduction
B. Definition
C. Three Rs principle (in animal experimentation)
D. Choice of animal species for experimentation
E. Sources of experimental animals
F. Physical laboratory facilities
G. Diets and feeding
H. Transport of laboratory animals
I. An aesthesia and euthanasia
J. Disposal of animal carcasses
K. Laboratory animal ethics

CHROMATOGRAPHY

A. INTRODUCTION OF CHROMATOGRAPHY

The term chromatography (chroma = a colour; graphein = to write) is the collective term for a set of laboratory techniques for the separation of mixtures. Chromatography involves a sample (or sample extract) being dissolved in a mobile phase (which may be a gas, a liquid or asupercritical fluid).The mobile phase then has to go through a stationary phase that isn't moving or moving at all. The phases are chosen so that different parts of the sample can be dissolved in each one. People who want to move through the stationary phase will have to move slower than people who don't want the stationary phase to move at all but the mobile phase moves quickly. Different sample parts will become separated from each other as they move through a stationary phase because of their different mobility rates. High-performance liquid chromatography (H.P.L.C.) and gas chromatography use columns, which are small tubes filled with stationary phase. The mobile phase is then forced through the columns, which are filled with stationary phase. The sample moves through the column by adding more and more mobile phase. This process is called elution.

B. HISTORY

The subject of chromatography was introduced into scientific world in a very modest way by M. Tswett in 1906. He used a method to separate different types of pigments, like chlorophylls and xanthophylls, from each other. To do this, he poured a solution of these compounds into a glass column that was filled with finely divided calcium carbonate. After the last, Thompson and Way knew that soils could exchange ions. Most of a century after Adams and Holmes saw how ion exchange worked in broken records, they looked at it again in 1935. Seeing this opened up a new field for making ion exchange resins. In 1941, Martin and Synge came up with the idea of Gas-Liquid Chromatography, which is how it works. They also helped to make Liquid-Liquid chromatography better. In 1944, from the Martin

lab, it was reported that paper chromatography could be used to separate amino acids. In 1952, both Synge and Martin were given the Nobel Prize for their work on chromatography. It was discovered in 1959 that gel filtration chromatography could be used to separate low molecular weight substances from high molecular weight substances. This method is called gel-filtration chromatography. In 1960, liquid chromatography got even better. This led to the development of High-Performance Liquid Chromatography. During the 1970s, a new type of adsorption chromatography, called affinity chromatography, came out. This type of chromatography was mostly based on biological interactions. A new field was born, called supercritical fluid chromatography, and it was very new.

C. PRINCIPLES OF CHROMATOGRAPHY

The molecules that are found in a biological system or in synthetic chemistry are made through a series of reactions that use intermediates. A product that has a lot of small things in it is called "impurities," and these small things need to be separated from the main product for biotechnology applications. When some molecules have similar physical and chemical properties, this can be used to tell them apart. Properties of the physical and chemical world that can be used to separate molecules are-

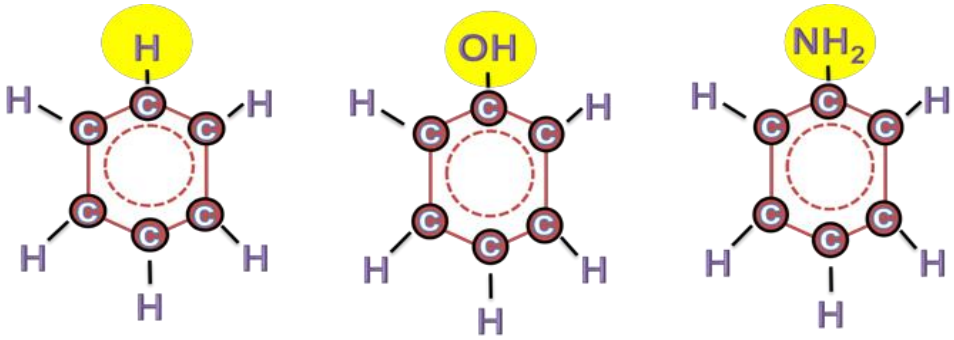
Physical Properties

1. Molecular weight
2. Boiling point (in case both are liquid, as in this case)
3. Freezing point
4. Crystallization
5. Solubility
6. Density

Chemical Properties

1. Functional Group, for example, phenol has –OH whereas aniline has NH_2.
2. Reactivity towards another reagent to form complex

Example: - Figure 1 show that if you have a mixture of compound 1 (benzene) and compound 3 (Aniline) and you would like to purify benzene rather than aniline. In this situation, you can take the physical and chemical properties of benzene into the account and isolate it from the mixture.



Name	Benzene	Phenol	Aniline
Molecular formula	C_6H_6	C_6H_6O	$C_6H_5NH_2$
Molar mass (g mol^{-1})	78.11	94.11	93.13
Density	0.8765 g cm^{-3}	1.07 g cm^{-3}	1.0217 g ml^{-1}
Melting point (°C)	5.5	40.5	-6.3
Boiling point (°C)	80.1	181.7	184.13

Figure 1: Chemical Structure and physical Properties of benzene, phenol and aniline.

The mixture of compound 1 and compound 3 is shown in Figure 2. If we are looking for them based on their boiling point, we can see them in Figure 2. This mixture will become two different things when we heat it up: one liquid phase and one gas phase. There will be a lot of compound 1 and 3 molecules in both liquid and gaseous phases. As the temperature nears the boiling point of compound 1, more of 1 will be in the vapour phase than the liquid phase. Whereas a lot more compounds 3 will be in the liquid phase than the other way around. As this process goes on, at some point, two molecules will separate from each other. To describe the distribution of compound 1 between two different phases A and B, the distribution coefficient (Kd) is shown in the following way:

$$Kd = \frac{\text{Concentration in Phase A}}{\text{Concentration in Phase B}}$$

In the same way, one can also take advantage of other physical and chemical factors. With each and every physical and chemical parameter, the molecules in the mixture will spread out based on how they behave in that parameter.

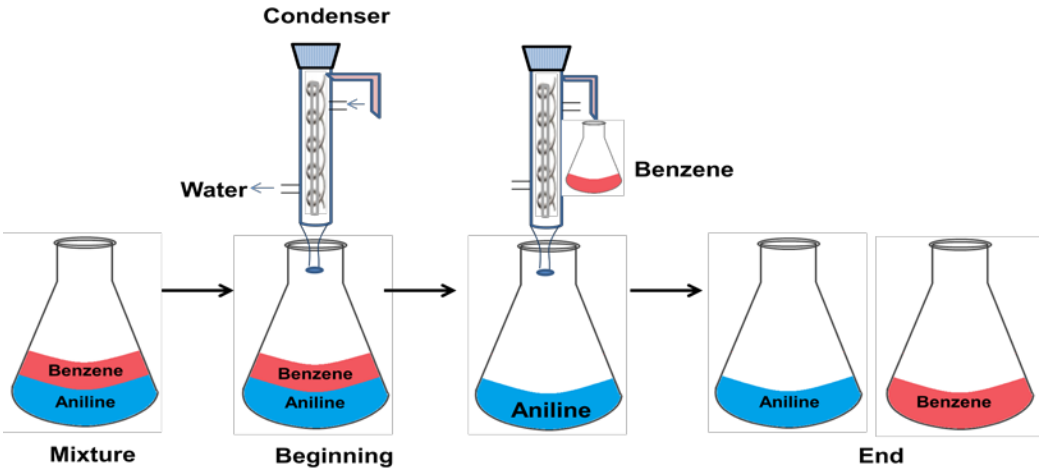


Figure 2: Distribution of molecules during distillation

D. TYPES OF CHROMATOGRAPHY

- ❖ **On the basis of chromatographic bed shape (two dimensional)**

1. Paper Chromatography
2. Thin Layer Chromatography

- ❖ **On the basis of physical state of mobile phase**

1. Liquid Chromatography
2. Gas Chromatography

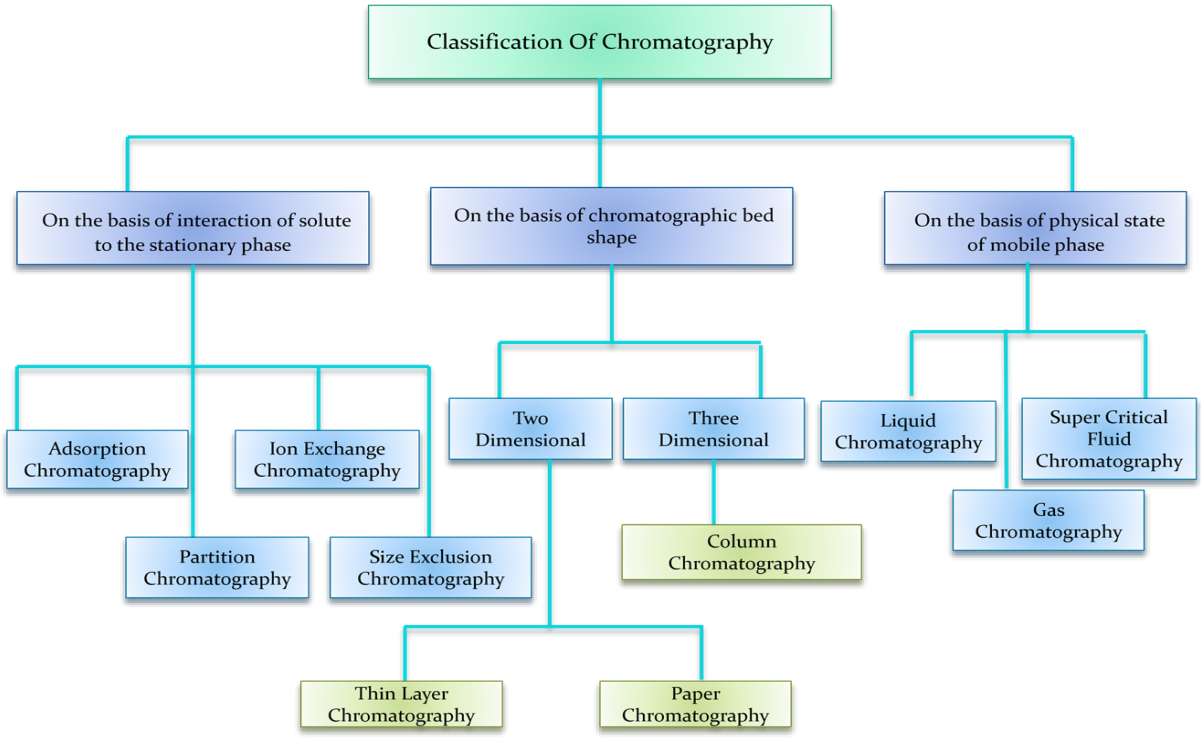


Figure 2: Classification of chromatography

1. Paper Chromatography

1.1.Introduction

This analytical method is called "Paper chromatography." It is used to separate coloured chemicals or substances that are also called pigments from each other. If you want to try out different colours of paint, this can also be used with secondary or primary colours. This method has been mostly replaced by thin layer chromatography, but it is still a good way to teach. Double-way paper chromatography, also known as two-dimensional chromatography, is when two solvents are used and the paper is rotated 90° in between. This is good for separating complex mixtures of compounds that have the same polarity, like amino acids. It should be made of good paper if filter paper is used, so it should be thick. Paper chromatography is thought to be the easiest and most common way to use chromatography because it can be used to isolate, identify, and measure organic and inorganic compounds. It was first thought up by a German scientist named Christian Friedrich Schonbein (1865).

1.2.Principle of paper chromatography

The principle behind partition chromatography is that the substances are spread out or partitioned between two liquids. One phase is the water that is in the pores of the filter paper, and the other phase is the liquid that moves over the paper. Water in the stationary phase and solvents in the mobile phase don't mix well with each other, so the compounds in the mixture get separated as the mobile phase moves. It can also be called adsorption chromatography, where the solid surface of the paper is the stationary phase and the liquid phase that moves is the moving phase. But most of the time, paper chromatography is used for applications that use partition chromatography, which means that two liquids are separated by paper.

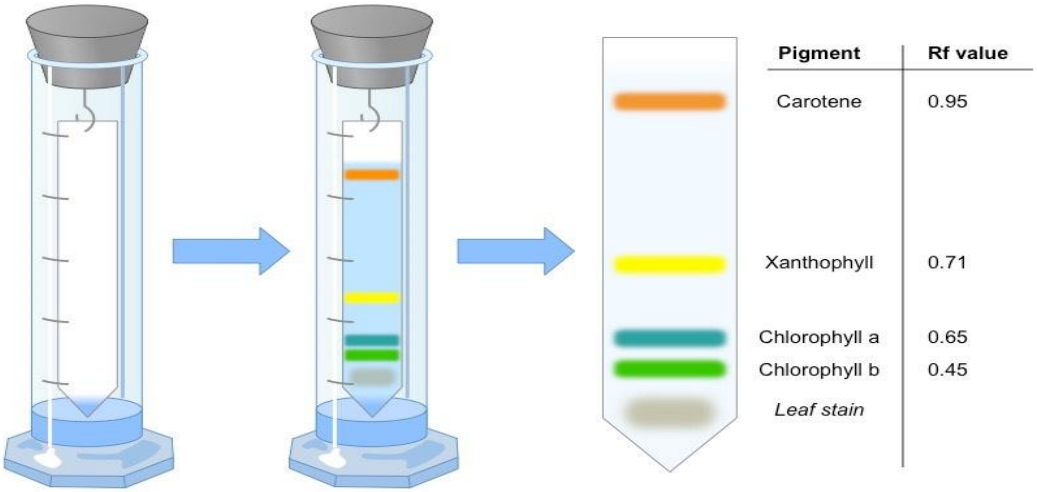


Figure 3: Process of paper chromatography

1.3.Types of paper chromatography

1.3.1. Paper Adsorption Chromatography

It's called an adsorbent when it's made of paper that's been coated with silica or alumina. The solvent is called the "mobile phase." .

1.3.2. Paper Partition Chromatography

Water or moisture in the pores of cellulose fibers that make up filter paper is what makes it a stationary phase. A solvent is then used to move the solvent around the paper. In general, paper chromatography mostly refers to paper partition chromatography, but there are other types of paper chromatography as well.

1.4.Instrumentation of paper chromatography

1. Stationary phase & papers used
2. Mobile phase
3. Developing Chamber
4. Detecting or Visualizing agents

1.4.1. Stationary phase and papers: - Whatman filter papers of different grades like No.1, No.2, No.3, No.4, No.20, No.40, No.42 etc. In general, the paper contains 98-99% of α-cellulose, 0.3-1% β -cellulose. Other modified papers are-

- Acid or base washed filter paper.

- Glass fiber type paper.
- Hydrophilic Papers-Papers modified with methanol, formamide, glycol, glycerol etc.
- Hydrophobic papers: Acetylation of OH groups lead to hydrophobic nature, hence can be used for reverse phase chromatography.
- Impregnation of silica, alumna, or ion exchange resins can also be made.

1.4.2. Mobile phase: -Pure solvents, buffer solutions or mixture of solvents can be used.Examples-

- Hydrophilic mobile phase.

Isopropanol: Ammonia:water- 9:1:2

Methanol: water-4:1

N-butanol: glacial acetic acid: water-4:1:5

- Hydrophobic mobile phases

dimethyl ether: cyclohexane kerosene: 70% isopropanol

There are two types of solvents that are used most often: polar solvents and nonpolar solvents. The choice depends on the type of substance being separated. If pure solvents don't work well, a mixture of solvents with the right polarity can be used.

1.4.3. Chromatographic chamber**:** -There are two types of solvents that are used most often: polar solvents and nonpolar solvents. The choice depends on the type of substance being separated. If pure solvents don't work well, a mixture of solvents with the right polarity can be used.

1.4.4. Visualizing agent: **-**Colorless analytes can be found by staining them with reagents like iodine vapour, ninhydrin, and so on. Radiolabeled and fluorescently labelled analytes can be found by measuring radioactivity and florescence.

1.5. Steps in Paper Chromatography: -In paper chromatography, the sample mixtureis applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action. The basic steps include:

Step 1: Selection of Solid Support: Fine quality cellulose paper with defined porosity, high resolution, negligible diffusion of sample and favouring good rate of movement of solvent.

Step 2: Selection of Mobile Phase: Different combinations of organic and inorganic solvents may be used depending on the analyte.

Example-Butanol: Acetic acid: Water (12:3:5) is suitable solvent for separating amino-acids.

Step 3: Saturation of Tank: The inner wall of the tank is wrapped with the filter paper before

solvent is placed in the tank to achieve better resolution.

Step 4: Sample Preparation and Loading: If solid sample is used, it is dissolved in a suitable solvent. Sample (2-20ul) is added on the base line as a spot using a micropipette and air dried to prevent the diffusion.

Step 5: Development of the Chromatogram: Sample loaded filter paper is dipped carefully into the solvent not more than a height of 1 cm and waited until the solvent front reaches near the edge of the paper.

Step 6: Different types of development techniques can be used:

- Type with ascending development: The solvent moves against gravity, just like in traditional type. It takes a lot of paper to get rid of the spots. The spots are kept in a chamber that has a solvent at the bottom.
- People do this kind of thing in a special room with the solvent holder on top. Soap and water are used to clean up a spot on a paper. There are two ways to do this: one is called descending chromatography, which means the solvent moves from top to bottom.
- Ascending–descending chromatography: A mix of the two techniques above is called ascending–descending chromatography. Only the distance between them got longer. First, they went up, then they went down.
- Circle development: The spot is kept in the middle of a circle paper. All around, the solvent moves through a wick at the centre.
- After the development, the solvent front is marked and the left to dry in a dry cabinet or oven.
- Detection: Colorless analytes are found by staining them with reagents like iodine vapour, ninhydrin, and so on.
- Rf values: Some compounds in a mixture travel almost as far as the solvent does; others stay closer to the base line than the rest of the mixture. In this case, the distance that a compound moves away from the solvent is the same as long as other factors, such as how the paper is made and what kind of solvent it is, stay the same. The value of the Rf value is how far the substance has moved away from the sample (Figure 4).

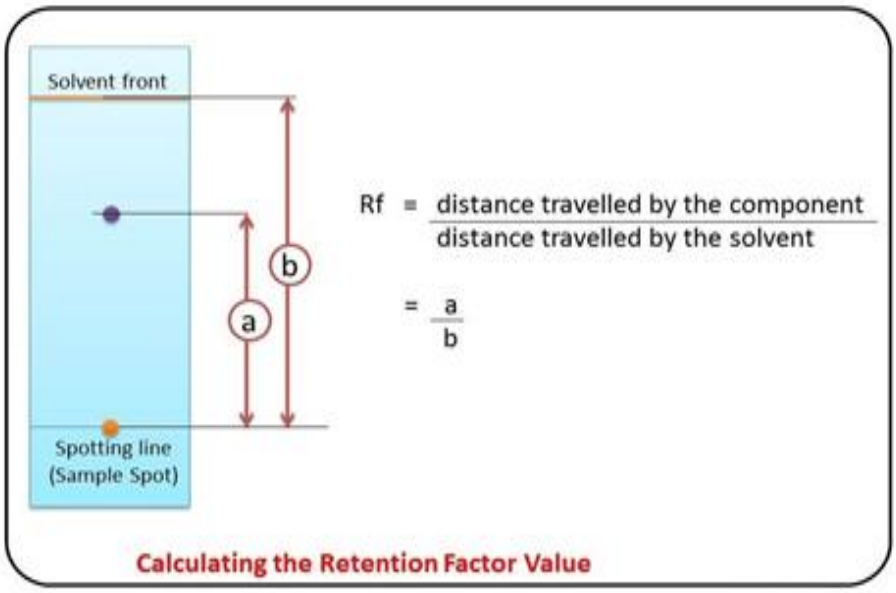


Figure 4: Formula derived for the calculation of Rf value

Development: The solvent moves against gravity, just like in traditional type. Spots are kept at the bottom of a piece of paper and kept in a chamber with a solvent at the bottom.

People do this kind of thing in a special room with the solvent holder on top, and they do it there. The spot is kept at the top, and the solvent moves down the paper as it dries. There are two ways to do this: one is called "descending chromatography," which means the solvent moves from top to bottom.

It's called ascending–descending chromatography because it's a mix of the two techniques above. Only the length of separation has changed. First, the person rises, then the person descends.

At the centre of the paper, there's a small spot where you can write. The solvent moves through a wick in the middle and spreads out in all directions at the same rate.

After the development, the solvent front is marked, and the left to dry in a dry cabinet or oven.

Analytes that aren't colored can be found by staining with reagents like iodine vapour, ninhydrin, and so on.

Rf values: Some compounds in a mixture travel almost as far as the solvent does; others stay closer to the base line than the rest of the mix. In this case, the distance that a compound moves away from the solvent is the same as long as other factors, such as how the paper is made and what type of solvent it is, stay the same. Distance from the solvent, or Rf value, is

how far you've gone (Figure 4).

1.6.Application of paper chromatography

- To check the control of purity of pharmaceuticals.
- For detection of adulterants.
- Detect the contaminants in foods and drinks.
- In the study of ripening and fermentation.
- For the detection of drugs and dopes in animals & humans.
- In analysis of cosmetics.
- Analysis of the reaction mixtures in biochemical labs.

1.7.Advantages of paper Chromatography

- Simple
- Rapid
- Paper Chromatography requires very less quantitative material.
- Paper Chromatography is cheaper compared to other chromatography methods.
- Both unknown inorganic as well as organic compounds can be identified by paper chromatography method.
- Paper chromatography does not occupy much space compared to other analytical methods or equipment.
- Excellent resolving power

1.8.Limitations of paper Chromatography

- Large quantity of sample cannot be applied on paper chromatography.
- In quantitative analysis paper chromatography is not effective.
- Complex mixture cannot be separated by paper chromatography.
- Less Accurate compared to HPLC.

2.Thin Layer Chromatography

2.1.Introduction

To separate and analyse complex biological or non-biological samples into their parts, an analytical chromatography called thin layer chromatography is used to do this. There are two phases in this method of separation: one that stays the same, and the other that moves in a certain direction. If you choose the right stationary phase and mobile phase, they can be of different types.

2.2.Principle

TLC, like other chromatographic methods, works by separating. Compatibility of compounds with stationary and mobile phases determines separation. Compounds in the mobile phase (capillary action) cross the stationary phase surface. Affinity compounds to stationary phase travel slowly while others travel quickly. Thus, the mixture's components are separated. Individual components are visualised as spots at their respective travel levels on the plate. Detection techniques identify their nature or character. After coating a glass or aluminium foil with the stationary phase, the sample is allowed to run in the presence of a mobile phase (solvent). Unlike other chromatography techniques, the mobile phase diffuses from bottom to top (in most of the chromatography techniques, mobile phase runs from top to bottom by gravity or pump). The mobile phase distributes the sample into the solvent and stationary phases. The stationary phase slows the molecule's movement while the mobile phase exerts an effective force on the sample. Suppose the force caused by mobile phase is $\mathbf{F_m}$ and the retardation force by stationary phase is $\mathbf{F_s}$, then effective force on the molecule will be $\mathbf{(F_m-F_s)}$ through which it will move (Figure 5). The molecule immobilizes on the silica gel (where, $F_m=F_s$) and the position will be controlled by multiple factors:

- Nature or functional group present on the molecule or analyte.
- Nature or composition of the mobile phase
- Thickness of the stationary phase.
- Functional group present on stationary phase.
- If the distance travelled by a molecule on TLC plate is $\mathbf{D_m}$ whereas the distance travelled by the solvent is $\mathbf{D_s}$, then the retardation factor (R_f) of molecule is given by:

$$\mathbf{Rf} = \frac{\mathbf{Distancetravelledbysubstance\ (Dm)}}{}$$

Distancetravelledbysolvent (Ds)

R_f value is characteristic to the molecule as long as the solvent system and TLC plate remains unchanged. It can be used to identify the substance in a crude mixture.

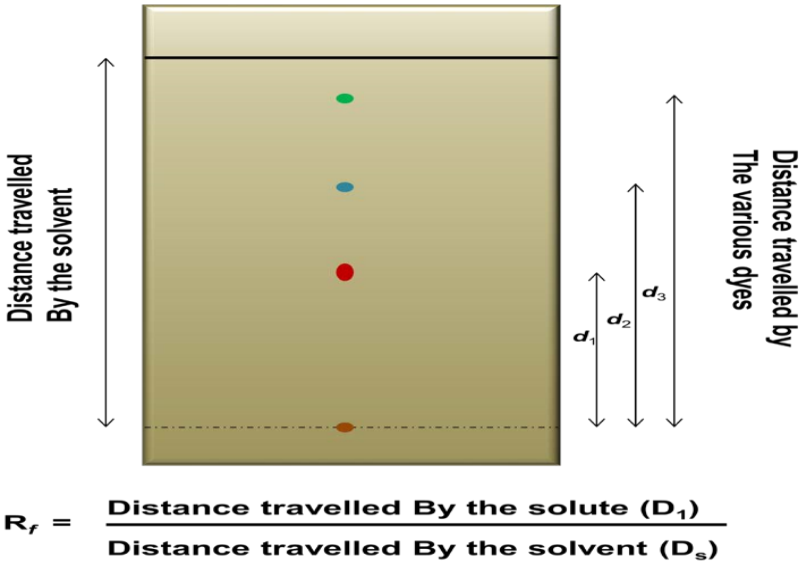


Figure 5: Principle of thin layer chromatography

2.3. Operation techniques of thin layer chromatography-Several steps are required to perform a thin layer chromatography to analyse a complex sample. These preparatory and operational steps are as follows:

- **Thin Layer Chromatography Chamber-**There are two types of thin layer chromatography chambers: rectangular and cylindrical. They are both made of transparent non-reactive materials, mostly glass (Figure 6). It is covered from top to bottom with a thick glass sheet, and the joints are sealed with a high vacuum grease to keep solvent vapour from getting out of the machine. Each side of the chamber is covered with a Whatman filter paper to make sure it's the same temperature on all sides of it. The chamber is filled with a solvent system, and it is allowed to humidify the chamber with the solvent vapour that comes from the solvent system. It's important for the solvent front to move in a straight line during TLC so that it looks even.

- **Preparation of TLC plate**-A silica slurry is made in water and spread on the glass or alumina sheet as a thin layer. Then, the sheet is left to dry. When it's done baking in a hot air oven, the plate is ready for some TLC. The layer is very thin (0.1-0.25 mm) for analytical purposes and very thick (0.4-0.21 mm) for preparative or bioassay purposes.

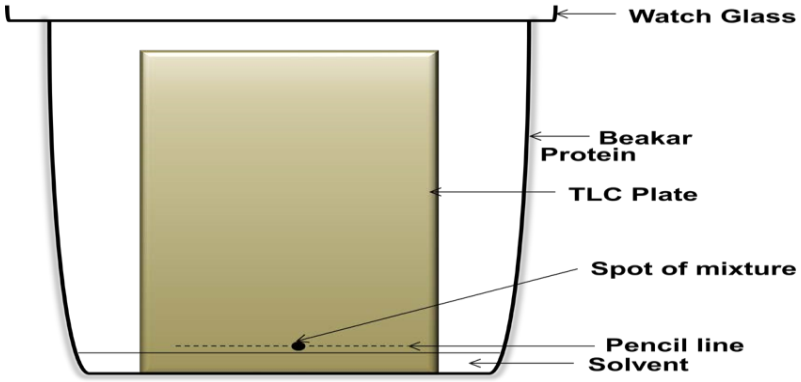


Figure 6: Thin Layer chromatography chamber

- **Spotting:** Figure 7 shows the steps that need to be taken to spot. A pencil is used to draw a line a little away from the bottom. Samples are put into the capillary tube or into a pipette to be taken. The capillary is put on the silica plate and the sample is allowed to flow out. It's important to know how thick the layer is so that the right amount of liquid can be used to apply it. A hair dryer can be used instead of letting the spot dry out in the air.

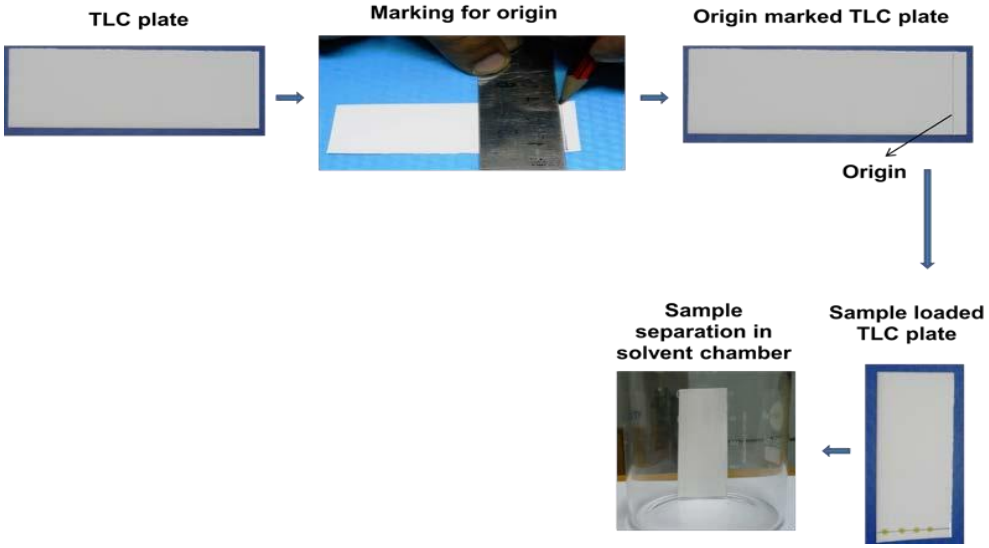


Figure 7: Events in spotting during thin layer chromatography

- **Running of the TLC:** It is put in the TLC chamber after the spot has dried. The spot should not be below the solvent level at all. Allow the solvent front to move all the way to the end of the piece of paper.

- **Analysis of the chromatography plate-**When the plate is done inside, it's taken out and dried outside. If the compound is coloured, it will make a spot. For these substances, there is no need for extra staining. There are two ways to make a chromatogram.

- **Staining procedure-** In the staining procedure, TLC plate is sprayed with the staining reagent to stain the functional group present in the compound. For example: - Ninhydrin is used to stain amino acids.

- **Non-staining procedure-** In non-staining procedure spot can be identify by following methods-

- **Autoradiography-** A TLC plate can be placed along with the X-ray film for 48-72 hrs (exposure time depends on type and concentration of radioactivity) and then X-ray film is processed.

- **Fluorescence-** Several heterocyclic compounds give fluorescence in UV due to presence of conjugate double bond system. TLC plate can be visualized in an UV chamber (Figure 8) to identify the spots on TLC plate.

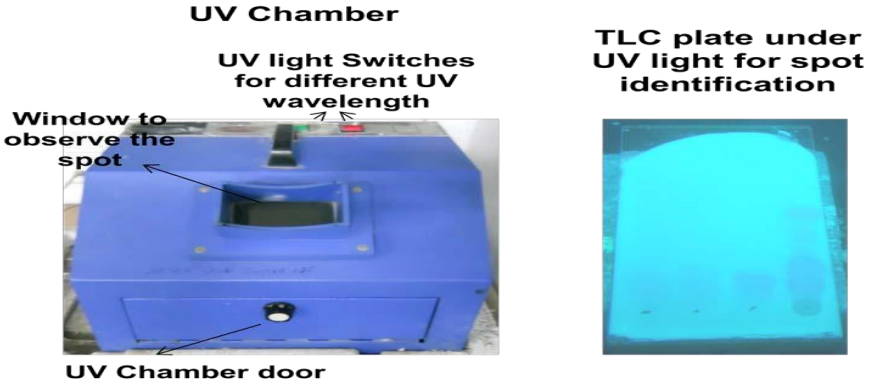


Figure 8: UV-Chamber and UV illuminated TLC plate.

2.4.Technical troubles with thin layer chromatography-

- **Tailing effect-**In general sample forms round circular spot on the TLC plate. It is due to the uniform movement of the solvent front throughout the plate. But in few cases instead of forming a spot, a compound forms a spot with long trail or rocket shape spot (Figure 9). it is due to few reasons as given below:

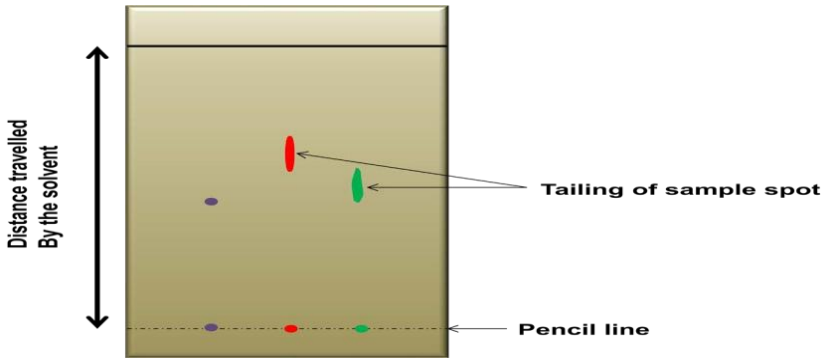


Figure 9: Tailing effect in thin layer chromatography.

- **Over-loading-** if the sample is loaded much more than the loading capacity of the TLC plate, it appears as spot with trail or rocket shape spot. A diluted sample can be tested to avoid this.

- **Fluctuation in temp or opening of chamber-** If there will be fluctuation in temperature or solvent saturation in the chamber (due to opening of the chamber during running), it disturbs the flow of solvent front and consequently cause spot with trail. it can be avoided by maintaining a uniform temperature and the opening of the chamber should be minimized especially during running.

- **No movement of sample-** In few cases, a sample doesn't move from the spot after the run is completed. These problems are common with high molecular weight substances such as protein or chemicals with large number of functional groups. In this case, a change in polarity or pH of solvent system can be explored to bring the compound into the solvent front so that it run on silica plate to get resolved.

- **Movement is too fast-**In few cases, the movement of a compound is too fast and does not give time to interact with the matrix to resolve into individual compounds. In this case, a change in polarity of solvent system can be explored to retard the running of the sample.

2.5.Applications of Thin layer Chromatography

- Composition analysis of biomolecules
- Quality testing of molecules
- Identification of impurities in a sample
- Progress of chemical reaction
- Estimation of biomolecules

2.6.Advantages of Thin layer Chromatography

- It is a simple with a short development time.
- It is a cheaper chromatographic technique.
- The purity standards of the given sample can be assessed easily.
- It helps with the visualization of separated compound spots easily.
- 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).

2.7.Limitations of Thin layer Chromatography

- It cannot tell the difference between enantiomers and some isomers.
- In order to identify specific compounds, the Rf values for the compounds of interest must be known beforehand.

- TLC plates do not have long stationary phases. Therefore, the length of separation is limited compared to other chromatographic techniques.

3. Gas Chromatography

3.1.Introduction

When you use gas chromatography, the mobile phase is a gas, and each component is separated into vapours. This makes it different from other types of analysis. There are many small molecules in the gas phase, so it is used to separate and find them. An either a gas or a liquid is vaporised in the port. Helium is used as the "carrier gas" in gas chromatography because it has a low molecular weight and isn't a good conductor of electricity. The pressure is put on, and the mobile phase moves the analyte through the column with the help of the pressure. The separation is done by coating a column with a stationary phase.

3.2.Principle of Gas chromatography

The equilibrium for gas chromatography is partitioning, which means that the components of the sample will spread out between the stationary phase and the moving phase. People who are more attracted to the stationary phase spend more time in the column than people who are more attracted to the mobile phase. This means that they elute later and have a longer retention time (Rt) than people who are more attracted to the mobile phase. People are attracted to the stationary phase because they have a lot of intermolecular interactions. The polarity of the stationary phase can be chosen to maximise these interactions, which will lead to more separation. Ideal peaks have Gaussian distributions and are symmetrical because the analytes interact with the column in a way that isn't always predictable.

3.3.Types of gas chromatography

Based on mobile phase and stationary phase used, Gas Chromatography can be broadly

classified into two:

A. Gas-Liquid Chromatography (GLC), the name of the technique refers to the mobile phase (gas) and stationary phase (liquid) respectively. In gas chromatography, the partition co-efficient is used to separate the components of a mixture. GC, HPLC, SCFC are chromatographic methods. In a thin layer or along the inner wall of capillary tubing, the non-volatile liquid is coated on inert solid powder (solid support). This liquid film aids in the separation of stationary and mobile phases of the sample (carrier gas). The inert support increases the liquid film's surface area for better interaction with the sample. For GLC, the solid support can be anything from glass powder to diatomaceous earths to crushed firebricks to carbon black. The liquid layer applied on top of the solid support should have low volatility and high decomposition temperatures. A thin liquid coating or an inert solid support bearing liquid film is applied to the walls of open tubular columns. Its internal diameter is 0.1-0.53 mm and its length is 30 to 100 metres. Capillary columns outperform packed columns in terms of resolution and runtime.

B. Gas-Solid Chromatography (GSC), the name of the technique refers to the mobile phase (gas) and stationary phase (solid) respectively. The sample components are retained by physical adsorption. The basic principle of gas solid chromatography is adsorption. The solid stationary phase is a powdered active adsorbent. The column length is up to 10 m with internal diameters ranging from 2-4 mm. The columns are filled with porous materials like activated carbon, silica, and alumina. The stationary phase selectively adsorbs and desorbs volatile components. Fritz Prior invented solid-state gas chromatography in 1947. It is used to separate geometric isomers as well as low molecular gases such H_2, CO_2, CO, oxides of N etc.,

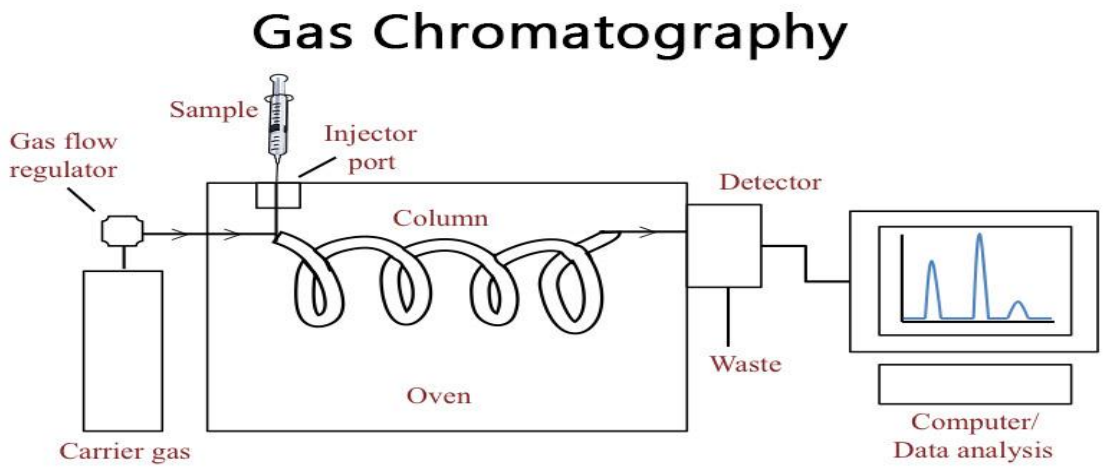


Figure 10: Parts of gas chromatography

3.4.Parts of Gas chromatography

Gas chromatography is mainly composed of the following parts:

- **Carrier gas in a high-pressure cylinder with attendant pressure regulators and flow meters**: Helium, N_2, H, Argon is used as carrier gases. Helium has a higher thermal conductivity than most organic vapours, making it ideal for thermal conductivity detectors. When using a lot of carrier gas, N_2 is preferred. Toggle valve, flow metre, capillary restrictors, and pressure gauge (1-4 atm). The flow rate is controlled by capillary restrictors and a needle valve mounted on the flow meter's base. The efficiency of a gas chromatograph depends on maintaining a constant gas flow.

- **Sample injection system**: A micro syringe injects liquid samples into a heated metal block via a self-scaling silicon-rubber septum. By-pass loops and valves or a gas-tight syringe inject gas samples. Sample volumes are typically 0.1-0.2 ml.

- **The separation column**: The column is made of metals bent into a U shape or coiled into an open spiral or a flat pancake shape. Copper is good to 250^0. Swage lock fittings help insert columns. Various column sizes are used based on requirements.

- **Liquid phases**: The only limitations are volatility, thermal stability, and ability to wet the support. There is no one phase that works for all temperatures.
  Non-Polar-Paraffin, squalene, silicone greases, apiezon L, silicone gum rubber. These materials separate the components in order of their boiling points.

  Intermediate Polarity-Their non-polar structure allows them to dissolve both polar and nonpolar solutes. Like this. High boiling alcohols are separated using diethyl hexyl phthalate.

  Polar-Car bowaxes: Liquid phases with a large proportion of polar groups. Separation of polar and non-polar substances.

  Hydrogen bonding-Polar liquid phases with high hydrogen bonding e.g. Glycol.
  Specific purpose phases – Relying on a chemical reaction with solute to achieve separations. e.g $AgNO_3$ in glycol separates unsaturated hydrocarbons.

- **Supports**: The support material's structure and surface characteristics affect the support's efficiency and degree of separation. Inert support capable of immobilising large volumes of liquid phase as thin film on its surface. Large surface area ensures rapid equilibrium between stationary and mobile phases. The support should be strong enough to withstand handling and pack into a uniform bed. Particles of diatomaceous earth kieselguhr fusion into coarse aggregates when heated with Na_2CO_3. Low-surface-area, low-porosity glass beads can coat up to 3% stationary phases. Porous polymer beads with varying degrees of styrene cross-linking with alkyl-vinyl benzene are also used.

- **Detector**: Detectors sense the arrival of the separated components and provide a signal. These are either concentration-dependent or mass dependant. The detector should be close to the column exit and the correct temperature to prevent decomposition.

- **Recorder**: The recorder should be generally 10 mv (full scale) fitted with a fast

response pen (1 sec or less). The recorder should relate to a series of good quality resistances connected across the input to attenuate the large signals. An integrator may be a good addition.

3.5. The procedure of Gas Chromatography

Step 1: Sample injection and vaporization

1. A small amount of liquid sample to be analysed is drawn up into a syringe.
2. The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.
3. The injection of the sample is a "point" in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
4. The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporize.
5. The vaporized components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.

Step 2: Separation in the column

- Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will, therefore, have the longest retention time (Rt). It will emerge from the gas chromatograph last.
- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.

- If we consider a 2-component mixture in which component A is more polar than component B then:

1. component A will have a **longer retention time** in a polar column than component B
2. component A will have a **shorter retention time** in a non-polar column than component B

Step 3: Detecting and recording results

1. The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.
2. The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
3. The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last.

3.6.Advantages of Gas Liquid Chromatography over Gas Solid Chromatography

- Shorter analysis time and better resolution between the peaks.
- GLC can be used for both, quantitative and qualitative analysis.
- Large concentration range of samples can be evaluated in GLC.
- Wide range of stationary phase i.e. liquid coating is available for large number of separations.

3.7.Limitations of Gas Chromatography

The primary limitation of gas chromatography is that the substance being analysed must be volatile, with a defined fraction in the gaseous phase. Organic substances with molecular weights over 500 have insufficient volatilities. While high temperatures (up to 300°C) increase volatility, they can also cause decomposition.

3.8.Applications of Gas Chromatography

- Since the 1950s, gas chromatography has evolved. Its high sensitivity makes it useful in industrial, pharmaceutical, chemical, and biotechnology laboratories.
- Due to its wide applicability, GC is used to analyse many compounds both

qualitatively and quantitatively.

- Its high sensitivity allows it to separate and analyse compound mixtures at very low concentrations. GC can be used to test the purity of a substance, prepare pure compounds from a mixture, or identify a compound.
- It also works for compounds with boiling points below 300°C. GC is used to analyse complex mixtures due to its high sensitivity and resolution speed. GC is a standard analytical method in R&D.
- Many industries such as petrochemical, food, and drug use GC in quality control to check for purity and contaminants. GC is now being used in the food, fragrance, and environmental sectors.

4. High performance liquid chromatography

4.1.Introduction

The acronym HPLC was coined by late Prof. Csaba Horvath in 1970 to Pittcon paper because high pressure was used to create flow required for liquid chromatography in packed columns. Initially, pumps could handle 500 psi. Hence the name High-Pressure Liquid Chromatography. Then came the early 1970s technological leap. These new HPLC instruments used improved injectors, detectors, and columns, and could generate up to 6,000 psi of pressure. Due to technological advancements, the name was changed to high performance liquid chromatography (HPLC), but the acronym remained. It's also called High-Resolution Liquid Chromatography. LC (High Performance Liquid Chromatography) As in other types of column chromatography, the sample mixture is pumped into the solvent (the mobile phase) and then passed through the packing material (stationary phase).

4.2.Scope of HPLC

Ionic species, macromolecules, labile products, and other low molecular weight compounds are best separated by HPLC. On exchange, absorption, partition, and size exclusion are all HPLC separation modes. The greater variety of stationary phases also expands the utility of liquid chromatography. Recycling, pre or post column derivatization, solvent and gradient programming, column switching devices, specific detectors, and ancillary (tandem)

techniques are all examples of HPLC applications.

4.3.Principle of High-Performance Liquid Chromatography (HPLC)

Purification takes place in a stationary-mobile phase separation column. An ultrafine granular stationary phase is used in a separation column. The mobile phase is a solvent or solvent mixture forced through the separation column under high pressure. A syringe injects the sample into the mobile phase flow from the pump to the separation column via a valve with a connected sample loop. Following this, the sample components migrate at varying rates through the column due to interactions with the stationary phase. After leaving the column, the substances are detected by a suitable detector and sent to the computer's HPLC software. After this operation/run, a chromatogram is obtained in HPLC software on the computer. The chromatogram identifies and quantifies substances. High-Performance Liquid Chromatography Equipment (HPLC).

4.4. Instrumentation of HPLC

- **Pump:** The pump system evolved from HPLC. The pump draws eluent from the solvent reservoir into the liquid chromatography system. Pumps must be able to generate high pressures, as well as a controllable and reproducible flow rate. Most current LC pumps work by moving a motor-driven piston back and forth (reciprocating pumps). This piston motion creates “pulses”.

- **Injector:** Next to the pump is an injector. Using a syringe, the sample is introduced into the eluent flow. The most common injection method uses sampling loops. The auto sampler (auto-injector) system allows repeated injections at predetermined times.

- **Column:** The separation occurs within the column. Instead of glass columns, modern columns are often prepared in stainless steel. Compared to calcium carbonate, silica or polymer gels are commonly used. LC eluents range from acidic to basic. Most column housing is stainless steel, which is resistant to many solvents.

- **Detector:** Analytes are separated inside a column, and the separation is observed by a

detector. When no analyte is present, the eluent composition is stable. The presence of analyte alters the eluent composition. Detector measures these differences. This difference is monitored electronically. Various detectors are available.

- **Recorder:** The change in eluent detected by a detector is an electronic signal, not visible to the human eye. Historically, the pen-chart recorder was widely used. An electronic data processor (integrator) is now more common. They range from simple systems with a built-in printer and word processor to those with software specifically designed for an LC system and features like peak-fitting, baseline correction, automatic concentration calculation and molecular weight determination.

- **Degasser**: The eluent used in LC analysis may contain invisible gases like oxygen. Gas in the eluent is detected as noise, causing an unstable baseline. A degasser removes gases with polymer membrane tubing. The numerous tiny pores on the polymer tube's surface allow air through but not liquid.

- **Column Heater:** The LC separation is often largely influenced by the column temperature. In order to obtain repeatable results, it is important to keep consistent temperature conditions. Also, for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C). Thus, columns are generally kept inside the column oven (column heater).

4.5.Types of High-Performance Liquid Chromatography (HPLC)

1. **Normal phase:**

Column packing is polar (e.g silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers, and chiral compounds.

2. **Reverse phase:**

The column packing is non-polar (e.g C18), the mobile phase is water and miscible solvent (e.g methanol). It can be used for polar, non-polar, ionisable and ionic samples.

3. **Ion exchange:**

Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cautions.

4. **Size exclusion:**

Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

4.6. Application of HPLC

HPLC is used to separate and analyse thermally-unstable or non-volatile compounds.
Common non-volatile compounds are:

- Pharmaceutical products such as acetaminophen (Tylenol), aspirin or
- ibuprofen etc
- Sodium chloride and potassium phosphate (salts)
- Blood protein / egg white (protein components)
- Polystyrene, polyethylene (polymers)
- Asphalt / motor oil (hydrocarbons)
- Herbal medicines, plant extracts (natural products)
- TNT, enzymes (thermally unstable compounds)
- HPLC can be enforced to any of the fields as environmental, pharmaceuticals, food, forensic, nutraceuticals, industrial and cosmetics,

4.7. Advantages of HPLC

- High sensitivity: HPLC can analyse nanogram and picogram samples.
- Find molecules with high precision and resolution.
- Highest accuracy: Separation of components in complex mixtures.
- Extremely quick and efficient: The process takes between 10 and 30 minutes to complete.
- Extremely reproducible.
- Automated: Basic HPLC requires little training.
- Security, data management, report and instrument validation.
- Strong and adaptable.

- Manages all aspects of analysis and increases productivity from sample to instrument to result reporting.
- Monitoring of column effluent
- Ion exchange and exclusion column separations are done quickly.
- Both aqueous and non-aqueous samples require no pre-treatment.

4.8. Disadvantages of HPLC

- HPLC provides high selectivity for specific analysis with a variety of solvents and column packing's.
- An analysis can determine multiple components.
- In some ways, HPLC outperforms GLC. HPLC is not limited to volatile or thermally stable solutes and offers a wide range of mobile and stationary phases.
- HPLC costs a lot of money because it uses expensive organics.
- Existing HPLC methods are simple to use, but establishing new methods or troubleshooting problems can be difficult due to the variety of columns, modules, and mobile phases.
- HPLC cannot detect compounds that are irreversibly adsorbed. Thus, HPLC is insensitive to such compounds.
- For example, gas chromatography is better for volatile separation.

ANIMAL ASSAY

A. INTRODUCTION OF ANIMAL ASSAY:

Animal experimentation is the use of non-human animals in experiments. The research takes place in universities, medical schools, pharmaceutical companies, farms, defense facilities, and commercial animal testing facilities. Research on genetics, developmental biology, and

behavior is pure research. Applied research includes biomedical research, xeno transplantation, drug testing, and cosmetics testing. Animals are used for research, education, and breeding. Biomedical scientists generally work to unravel the complex processes of life and provide new measures for human, animal, and environmental health. So they need some freedom and adequate facilities to use animals as needed. Clearly, certain life processes require the involvement of the entire animal system. In-vitro tests can only provide limited data. They can't completely replace animals in tests. That's why using animals is still required by law. Nevertheless, scientists must ensure that animal experiments are justified and unavoidable, that no unnecessary pain or injury is caused to the animals, and that they are kept in the best possible conditions.

B. DEFINITION

A non-human animal is used in an experiment. The research takes place in universities, medical schools, pharmaceutical companies, farms, defense establishments, and commercial animal testing facilities. Research in genetics, developmental biology, and behavior is pure research. Applied research includes biomedical research, xeno transplantation, drug testing, and cosmetics testing. Animals are used for research, breeding, and education. They study the complex processes of life and devise new ways to improve the health and welfare of people, animals, and the planet. They need to be free to use animals as needed and have adequate facilities to do so. A whole animal system is required to study certain life processes. In vitro tests can only provide limited information. In experiments, they cannot completely replace animals. Because of this, the use of animals remains mandatory. To ensure that animals are not subjected to unnecessary pain or injury while being kept in the best possible conditions, scientists must ensure that the experiments they conduct are justified and unavoidable.

THREE Rs PRINCIPLE (in animal experimentation)

The Three Rs (3Rs) are guiding principles for more ethical use of animals in testing. These were first described by W.M.S. Russell and R.L. Burch in 1959.

The 3Rs are:

1. Replacement which refers to the preferred use of non-animal methods over animal methods whenever it is possible to achieve the same scientific aims. These methods

include computer modeling.

2. Reduction which refers to methods that enable researchers to obtain comparable levels of information from fewer animals, or to obtain more information from the same number of animals.
3. Refinement which refers to methods that alleviate or minimize potential pain, suffering or distress, and enhance animal welfare for the animals used. These methods include non-invasive techniques.

The 3Rs aim to improve animal welfare and scientific quality where animal testing is unavoidable. These 3Rs are now widely used in testing facilities worldwide and incorporated into legislation and regulations.

B. CHOICE OF ANIMAL SPECIES FOR EXPERIMENTATION

a) Factors influencing the selection: - Several factors influence the choice of animal species used in laboratory experiments.

i. The presence or absence of physiological systems and response like those in man, e.g. The dog does not acetylate aromatic amines and the monkey and the guinea pig require exogenous ascorbic acid while the rodents do not.

ii. The ease of handling, housing, breeding and maintenance in healthy and normal environment and cost of production, e.g. the rabbit is easy to handle and administer intravenous injections.

iii. Experience in terms of past use in other laboratories and by other investigators. To give few examples, the rabbit skin is relatively sensitive to irritants so that great deals of experimentation on skin irritants have been conducted in rabbit. The monkey is useful in behavioral work. Nerve demyelization is best investigated in the chicken. Sensitization studies in the animals are best conducted in the guinea pig.

iv. The ease of experimentation in a short duration. To cite a few instances for long term studies species having a relatively shorter life span allows one to examine the effect of a chemical or exposure to irradiation etc. through several generation or entire life span.

Mouse and rate have been the most standardized and popular of all laboratory animals. Rabbits, guinea pigs and dogs perhaps would be classified next.

b) Factors affecting the experimentation

The use of animals in various investigations demands rigorous investigational discipline. Temperature, humidity, ventilation, nutrition, housing conditions, cleanliness, disease control and personnel care are best a few of the more obvious factors requiring the attention of the investigator.

C. SOURCES OF EXPERIMENTAL ANIMALS

Scientists should obtain animals for experiments from reputable sources. Wild animals like monkeys, feral dogs and cats are used in research because they are readily available and cheaper than colony bred animals. Before being used in experiments, wild and feral animals are generally quarantined and stabilized. Because these animals' health and genetic status are unknown, careful screening during quarantine is required. Purchase of wild and feral animals should be approved by institutional animal ethics committees and certified suppliers. The only legitimate source for research animals should be recognized scientific animal facilities with known genetic and health status animal colonies. Only such animals can deliver results. Scientists should demand defined animals from organized colonies, avoiding unscrupulous traders who not only supply poor stock but also keep animals in unethical and unhygienic conditions.

E. PHYSICAL LABORATORY FACILITIES

(i) Housing and Environment

- The living circumstances of laboratory animals are critical. They should be kept in a structure far away from human habitations, free from dust, smoke, noise, wild

rodents, insects, and birds. The building, cages, and environment of animal rooms directly affect animal quality.

- The space in an animal facility should be well-divided for diverse purposes. Storage (8-10%), washing (8-10%), office and personnel (8-10%), machine rooms (4-5%), quarantine and corridors should fill the remaining area (12-15%).

- Metal (stainless steel, galvanized iron sheet/rods) or plastic (polypropylene/polycarbonate) cages are recommended. They should be the right size for the animals and have adequate food and watering facilities. These surfaces must be free of cracks and sharp edges for cleaning and safety. A good quality bedding should be sterilized before usage. Common Indian bedding materials include rice husk, sawdust, dried grass, and crushed corn cobs.

- The environment of the animal room (Macro-environment) and cage (Micro-environment) influences the animal's productivity and experimental efficiency. Because animals are sensitive to changes in temperature, humidity, light, sound, and ventilation, sudden changes should be avoided.

- Variation in room temperature affects food and drink consumption. A 4°C temperature change can affect biological reactions tenfold. Temperature impacts ovulation and lactation. Temperature rises promote ammonia buildup in high humidity. Insufficient ventilation promotes respiratory discomfort in both animals and humans, reducing resistance to infection. Animal rooms must have sufficient ventilation with 10-12 air changes per hour of 100% fresh air.

- Other elements include light and sound. Light intensity, wave length, and photo cycle affect animal health and behavior. Sounds in the animal rooms can cause ear damage, hypertension, cannibalism, etc.

(ii) Hygiene and Disease Control

- The building for housing the animals should be provided with barriers to control the entry of contamination into the building through men, material and wild animals. Strict barriers should be provided to avoid the entry of wild rodents, birds, insects and pests. Visitors and service staff should be allowed entry with care and when necessary.

- On the exit side an efficient monitoring service should be established to monitor the prevalence of any infection in the colony. A regular medical checkup of the staff, postmortem of dead and sacrificed animals and screening of waste material of the rooms are essential.

- (iii) Personal and Training

- The management of an animal facility relies heavily on the selection of staff, especially those working in animal rooms or transportation.

- If the crew is working in an animal room, they must be given with all necessary protective gear. Personal hygiene should be maintained through lockers, wash basins, toilets, and bathrooms. It is also vital that the workers be regularly checked for zoonotic infections and are not acting as a source of illness transmission to the animals. He should guarantee that anybody working in the animal home is properly vaccinated, especially against tetanus and other zoo noses.

- All employees must receive initial in-house training. Several weeks must be spent training new employees in animal handling, cage cleaning, and the necessity of hygiene, disinfection, and sterilization. They should also be familiar with the activities of healthy and sick animals so they can identify sick animals during their daily cage checks.

- The National Centre's shall offer appropriate training programmes for workers working in animal breeding and holding units. Orientation training programmes for investigators working in diverse fields should be developed. This course should cover the following topics: -

a) biology and husbandry of laboratory animals

b) genetic make-up

c) microbiology and diseases

d) health-hazards in the animal house

e) anesthesia, analysis and experimental procedures

f) alternatives to animal use

g) ethical aspects and legislation

The training courses and workshops may also be organized for the senior level biological scientists to evoke awareness among them about the use of animals in research, alternatives available and the ethical and legal provisions in regard to use of animals. This is particularly important care and management for the supervisory staff and veterinarians exists in the country.

Table 1: The national level training programmed of following type are essentially required:

S. N.	Training level	Qualification	Duration	Course contents
1	Technician level	Matriculate	6-12weeks	Basics
2	Supervisory level	. Graduate	12-24weeks	Comprehensive
3	Scientist level	Veterinary/medical Graduate/Post Graduate in Natural Sciences	8-12 weeks	Specialized

iv) Records and Evaluation

- Disease-free animals are of high quality. A genetically anesthetized animal should have all the traits of a strain. Regular monitoring of genetic purity indicators must be meticulously recorded.
- An animal facility's records must be kept properly. Simple yet complete forms, preferably computer compatible. Excessive and unneeded recording is not useful. Records of breeding and experimenting are required, as are records of all experimental animals.
- Food and other supplies should be noted. Incinerator, boiler, and air-conditioning

plant log books should be kept. Monthly and annual activity reports should be provided for evaluation and future planning.

v) Experimentation and Veterinary Care

- Qualified investigators should normally oversee the experimental animal units. Experiment and post-operative care must be adequately housed and equipped. The experimental unit's equipment should be suitable for the experiments. No technique should be utilised that causes unnecessary pain to animals. Following surgery, animals should be housed in comfortable holding cages, under the supervision of a competent scientist or veterinarian.
- Animal facility managers should be veterinarians or skilled in laboratory animal science. If an animal is sick or injured, a veterinarian must be on hand to care for it. A veterinarian could also aid with animal anesthesia and surgery research.

F. DIETS AND FEEDING

(a) Nutritional Requirements

- The presence of nutritional deficits and imbalances may affect the outcome of an experiment. Therefore, laboratory animals must be fed a balanced diet depending on their species' nutritional needs. Dietary factors, food ingredients, and feeding procedures require special attention. Protein, carbohydrate and fat should be provided in the proper proportions for each animal species.

- Dietary items should be free of dust, mould, fungi, and other pollutants. Each animal must receive the proper amount of feed for upkeep and production.
- The feed must be tasty for the animals to eat it in sufficient quantities. The animals suffer from nutritional deficiencies due to any unpleasant odor.
- No drugs, hormones, or antibiotics should be given to the meal as these may disrupt the animals' regular metabolism and generate skewed results.
- Ingredients and prepared feed must be handled with care to avoid contamination. To avoid waste, the food must be supplied to the animals in the suitable hoppers. In other circumstances, the diet is split between 2-3 meals.
- Commercially available palliated diets for many species are currently available. They are cheap and straightforward to use. However, feed quality varies from batch to

batch. Manufacturers should be required to label each bag with the food type, date of manufacturing, batch number, ingredients, and chemical makeup. Random chemical analysis for important nutrients is required to periodically monitor food quality.

- Ad labitum access to clean chlorinated water

(b) Classification of Diets: -Diets for laboratory animals are classified according to the degree of refinement of the ingredients:

- Naturally processed whole grains like wheat, corn, and millet, as well as elements refined to a minimum like fish meal, soy bean meal, and wheat bran are called natural intergradient diets. These include the inability to completely control nutrient concentration, difficulty in modifying composition to study specific nutrients, and the risk of pesticide residues, heavy metals, or other agents altering the response to experimental treatments.
- Purified diets contain refined ingredients such as casein (protein), sugar or starch (carbohydrate), vegetable oil or lard (fat), and cellulose (fibre). Inorganic salts and vitamins are added. The capacity to duplicate nutrient concentration or change it to induce nutritional deficits or excesses is a benefit of utilizing pure diets. Chemical contamination of these diets is unlikely. Sadly, not all species readily consume them.
- Amino acids, carbohydrates, triglycerides, essential fatty acids, minerals, and vitamins are employed to make these diets. These are valuable in investigations where careful nutrient concentration control is required, but are too costly for widespread use.

(c) Formulation of Diets

- Nutritional composition, availability, and palatability of ingredients utilized in research animal diets must be known. When creating natural ingredient diets, more than one component should be utilized for each nutritional class. This improves diet quality by reducing variance in nutritional composition and increasing palatability. It is important to compensate for nutritional losses that occur during feed processing procedures such as sterilization or nutrient interactions.
- The materials must be finely ground, mixed in the amounts stated in the formula, and stored in containers until used.
- Large quantities of ingredients are added directly, whereas minor quantities, such vitamins and minerals, are supplied via premixes.

- Vitamin and mineral premixes are separated to reduce vitamin oxidation by minerals. That way, the nutrient concentrations are dispersed evenly throughout the diet. As a carrier, one of the main ingredients should be used to ensure that at least one percent of the premix is consumed.
- Diets for laboratory animals can be supplied as meal or wash (wet or dry), flaked or baked, pellets, semi-moist, or gel.

Note: Diets should be stored under low temperature and humidity. Areas where diets are stored or processed should be kept clean and enclosed to prevent entry of wild rodents and other pests. Almost care must be taken to avoid any contamination by any rodenticides, insecticides, hormones, antibiotics into the diet.

(d) Feeding Practices

There are different methods for feeding animals.

i) Ad libitum feeding: Feed and water are always made available for the animal and it can consume as much as it needs.

ii) Pair-feeding: This method of feeding is practiced in nutritional experiments. For a paired feeding trial, the animals should be grouped in pairs, with each number of a pair being as similar as possible in body weight, of the same sex and preferably from the same litter. One animal of each pair is assigned to the experimental group, the other to the control.

iii) Restricted Feeding: In studies where, maximum longevity is vital (cancer research, gerontology etc.) the animals are fed on nutritionally adequate diets but slightly less than the daily normal ration.

G. TRANSPORT OF LABORATORY ANIMALS

- Transporting animals is highly important and must be done with care. The key factors for animal transportation include modality, containers, animal density in cages, food and water, and protection from illnesses, injuries, and stress.
- The means of transport for animals is determined by distance, season, climate, and animal species. Considering the foregoing, animals can be transported via road, rail, or air. In any case, shipping stress should be avoided, and containers should be large

enough to allow for comfortable mobility and protection from probable accidents. Food and water should be provided in proper containers or forms to ensure they obtain enough food and water while travelling. To avoid congestion and infighting, transport containers (cages or crates) should be of adequate size.

H. ANESTHESIA AND EUTHANASIA

The scientists should ensure that painful treatments are performed under adequate anesthetic for each animal type. During the experiment, the animal must not be conscious of pain and must be anaesthetized throughout. If the scientist feels he must stop the experiment or has caused irreparable injury, the animal should be sacrificed by anesthetic overdose. Using neuromuscular blocking drugs requires proper general anesthesia. If an animal is to be sacrificed after an experiment, the investigator must guarantee that the animal is clinically dead before sending it for disposal.

(a) Anesthesia

- Sedatives, analgesics, and anesthetics should be used to control pain or distress in experiments unless the results are compromised. Anesthesia affects the circulatory, respiratory, and thermoregulatory systems as well as the brain.
- Pre-anesthetics, which block parasympathetic activation of the cardio-pulmonary system and diminish salivary secretion, are used to prepare the animal for anesthesia. The most common anti-cholinergic is atropine. Depending on the surgery, local or general anesthesia may be employed.
- Local Anesthesia: Local anesthetics block the nerve supply to a specific location and are only used for minor procedures. This is done under specialist supervision for surgical infiltration, nerve blocks, and epidural and spinal anesthesia.
- General Anesthesia: Several substances are inhaled. Barbiturates and other general anesthetics are injected intravenously or intramuscularly. When employing an anesthetic, keep in mind the species' differences. Excessive salivation, convulsions, excitation, and disorientation should be avoided. After anesthesia and post-operative stress, the animal should be kept in veterinarian care.

(b) Euthanasia

Euthanasia is used when an animal must be slaughtered to end an experiment or for other

ethical reasons. The treatment should be short and painless, with no fear or worry. For euthanasia to be considered compassionate, it must first depress the central nervous system, causing pain insensitivity. The method chosen will depend on the study's purpose, the animal's species, and the number of sacrifices. The approach should always meet the following criteria:

a. Death, without causing anxiety, pain or distress with minimum time lag phase.
b. Minimum physiological and psychological disturbances.
c. Compatibility with the purpose of study and minimum emotional effect on the observer and operator.
d. Location should be separate from animal rooms and free from environmental contaminations.
e. Method should be reliable, reproducible and safe to the personnel involved.
f. Simple and economical.

I. DISPOSAL OF ANIMAL CARCASSES

- All animal carcasses, whether healthy, infected, or radioactive, must be bagged before disposal. All healthy or contagious animals can be buried deep in the ground or incinerated. Radioactive animals should be placed in double polythene bags and thrown in a suitable pit. Contact the Bhabha Atomic Research Centre in Mumbai for details. Personnel handling infectious or radioactive materials should be given strict instructions not to bring them out in open containers for disposal.
- An infectious or radioactive agent must be carefully disposed of so that infection does not spread to other animals or personnel handling such animals during the experiment or during disposal. Staff handling such animals for disposal must be informed of the hazards and equipped with protective clothes, gloves, and masks.

J. LABORATORY ANIMAL ETHICS

- All scientists working with laboratory animals must have a deep ethical consideration for the animals they are dealing with. From the ethical point of view, it is important that such considerations are taken care at the individual level, at institutional level and

finally at the national level.

- Individually each investigator has an obligation to abide by all the ethical guidelines laid down in this regard at institutional level. The Head of the Institution, maintaining animals for scientific experiments, should constitute an Animal Ethics Committee for experimentation to ensure that all experiments conducted on animals are rational, do not cause undue pain or suffering to the animals and only minimum number of animals are used. The constitution and terms of reference of the Animal Ethics Committee should be well defined.
- An Animal Ethics Committee should include: a senior biological scientist of the Institute, two scientists from different biological disciplines, a veterinarian involved in care of animals, the scientist in charge of animal facility, a scientist from outside the institute, a non-scientific socially aware member and a member or nominee of appropriate regulatory authority of Govt. of India. A specialist may be co-opted while reviewing special projects using hazardous agents such as radioactive substance and deadly micro-organisms etc. The investigator may also be called in for any clarification, if required.
- The Animal Ethics Committee has to examine all projects involving use of animals before implementation, to ensure that minimum number of animals is used in the project and the ethical guidelines are strictly adhered to. It will also examine that the scientists and technicians handling animals possess adequate skill to perform the experiment. All animals will be maintained under standard living conditions and experiments will be conducted with care. All invasive experiments will be conducted under proper anesthesia and on termination of an experiment, the animal will be humanely sacrificed under anesthesia. Before disposal it must be ensured that the animal is clinically dead.

a) Ethical Guidelines for Use of Animals in Scientific Research

3. Animal experiments should be undertaken only after due consideration of their relevance for human or animal health and the advancement of knowledge.
4. The animals selected for an experiment should be of an appropriate species and quality, and minimum number should be used to obtain scientifically and statistically valid results.
5. Investigators and other personnel should treat animals with kindness and should take

proper care by avoiding or minimizing discomfort, distress or pain.

6. Investigators should assume that all procedures which would cause pain in human beings may cause pain in other vertebrate species also (although more needs to be known about the perception of pain in animals).
7. Procedures that may cause more than momentary pain or distress should be performed with appropriate sedation, analgesia or anesthesia in accordance with accepted veterinary practice. Surgical or other painful procedures should not be performed on anaesthetized animals.
8. At the end of, or when appropriate during an experiment, the animal that would otherwise suffer severe or chronic pain, distress, discomfort, or disablement that cannot be relieved or repaired should be painlessly killed under anesthesia.
9. The best possible living condition should be provided to animals used for research purpose. Normally the care of animals should be under the supervision of a veterinarian or a person having adequate experience in laboratory animal care.
10. It is the responsibility of the investigator to ensure that personnel conducting experiment on animals possess appropriate qualifications or experience for conducting the required procedures. Adequate opportunities have to be provided by the institution for in service training for scientific and technical staff in this respect.
11. In-vitro systems to replace or reduce the number of animals should be used wherever possible.

b) In-vitro Systems to Replace Animals

Several in vitro systems can be used to reduce/replace animals in experimentation. These systems could be the living or the non-living systems. The living systems are tissue and organ culture, lower animals and microorganisms and human volunteers in restricted cases. The non-living systems could also be used in place of animals in certain areas and these include chemicals, mechanical models, mathematical models, computer simulation, DNA recombinant technology and synthetic substances.

Questions: -

1. What is the principle of liquid chromatography?
2. Define the instrumentation of paper chromatography?
3. What is the Rf value?
4. How to prepare TLC plate?
5. What is the difference between the GLC and GSC?
6. HPLC stand for?
7. What is the 3Rs principal in animal experiment?

Check your progress of unit 4 exercise-

- Read all the facts of unit 4 carefully after that you will be able to answer the given questions.

BLOCK

3

BLOCK- III ANALYTICAL INSTRUMENTS

Unit V:Electromagetic radiation based analytical instrumentation

Unit VI:Analytical balance, pH meter and refractometer

S

UNIT-V: ELECTROMANGETIC RADIATION BASED ANALYTICAL INSTRUMENTATION

Course structure:

5.0 OBJECTIVE

After going through this unit you will be able to-

- Understand the basic concept of Spectroscopy and Electromagnetic spectroscopy.
- Differentiate the Absorption and Emission. The principles of spectroscopy.
- Know various types Spectroscopy their basic arrangement, single and double spectroscopy.

- Understand basic components of spectroscopy and various detectors such as Photovoltaic cells, Photo Tubes (Photoemission Cells), and Photomultiplier Tubes.
- Know the details of UV- Visible Spectroscopy, UV- Visible range of Spectrum, Colorimetry and their applications.
- Understand basic concept of colorimetry its working principles and various components of colorimeter.
- Understand the Vibrational (Infra Red) Spectroscopy, Spectral Range, Applications of IR Spectroscopy.
- Follow the concept of Rotational (Microwave) Spectroscopy, Microwave Range and its application in analysis of various constituents.
- Know the details of Atomic Absorption Spectroscopy (AAS), principle on which it works various components.
- Understand the basic concept of Nuclear Magnetic Resonance (NMR), difference of NMR, P-NMR (H-NMR) and FT- NMR. Application of this Non destructive technique of Analysis.

5.1: INTRODUCTION

Electromagnetic Radiation based analytical instruments are widely used in modern times to evaluate the quality of food and its constituents including adulterations in food items. Electromagnetic radiation based analytical technique; spectroscopy utilizes the interaction between electromagnetic radiation and matter to provide information about food properties such as molecular composition, structure, dynamics and interactions. The different techniques like UV- Visible, Colorimetric, fluorescence, Infra Red, Microwave and atomic Absorption Spectroscopy and Nuclear Magnetic Resonance (NMR) are extensively used as analytical technique. Electromagnetic Radiation when interact with matter as a particle of energy (Photon) can absorb and move to excited state is absorption spectroscopy where as in emission spectroscopy atom or molecule when return to its ground state emits energy in form of radiation. Different types of energetic transition that can occur in atoms and molecules like electronic, rotational, vibrational translational and nuclear transition spectra are

applied in modern analytical techniques. Absorption, Emission and Raman Spectroscopy are applied in quality evaluation of foods. Ultra Violet-Visible spectroscopy and colorimetry use Ultra Violet and Visible range of EM spectra and absorption of monochromatic light by matter is measured which is proportional to the concentration of analyte in solution. The colorimetric method is based on principle of Beer- Lambert's Law which utilizes absorption of spectra in solution to determine concentration of analyte. Different types of detectors like phototubes (Photoemission cell), photomultiplier tube are used. Infra red spectroscopy is based on vibrational spectroscopy. Rotational spectroscopy or Microwave spectroscopy measures transition between rotational and energy levels in gaseous molecules. Atomic absorption spectroscopy is very effectively used for quantitative determination of chemical elements using absorption of optical radiation by free atoms in the gaseous state. AAS gives quick result. Nuclear Magnetic Resonance (NMR) is a non destructive technique which uses radio frequency waves that induces transition between magnetic energy levels of nuclei of a molecule.

5.2: SPECTROSCOPY:

The spectroscopy is analytical technique which utilizes the interactions between electromagnetic radiation and matter to provide information about food properties such as molecular composition, structure, dynamics and interactions. The instruments commonly used to analyze food material are based on spectroscopy (e.g. UV- Visible, Fluorescence, Atomic, Infrared and Nuclear Magnetic Resonance Spectroscopy). These instruments are based on principle of **"interactions between Electromagnetic radiation energy and matter to provide information about food properties"**. Operating principles of these instruments depends on understanding the distribution of energy with in atoms and molecules, the characteristics of electromagnetic (EM) radiation, and the interaction of EM radiations.

ELECTROMAGNETIC RADIATION:

Electromagnetic radiation is characterized by its wavelength (or**λ)**, Frequency **ν**and energy, **E.**

$$E = h\nu = hc / \lambda \qquad \text{and speed of light, } c = \nu\lambda$$

Where, **h** = Planck's constant &**c** = speed of light in a vacuum.

Light waves and other types of energy that radiate (travel out) from where theyproduced are called **Electromagnetic Radiation**. Together, they make up the **electromagnetic spectrum (Figure-1)**. Our eyes can see only a limited part of the electromagnetic spectrumi.e.the colorful rainbow we see on sunny-rainy days, which is an *incredibly tiny* part of all the electromagnetic radiation that zaps through our world. We call the energy as we can seethe **visible light,** radio waves, microwaves, and all the rest, all made up of **electromagnetic waves**. These are up-and-down, wave-shaped patterns ofelectricity and magnetism that race along at right angles to one another, at the speed of light (300,000 km per second or 186,000 miles per second, which is fast enough to go 400 times round the world in a minute). The light we can see visible light, stretches in a spectrum from red (the lowest frequency and longest wavelength of light our eyes can register) through orange, yellow, green, blue, and indigo to violet (the highest frequency and shortest wavelength we can see)**Figure-1**.

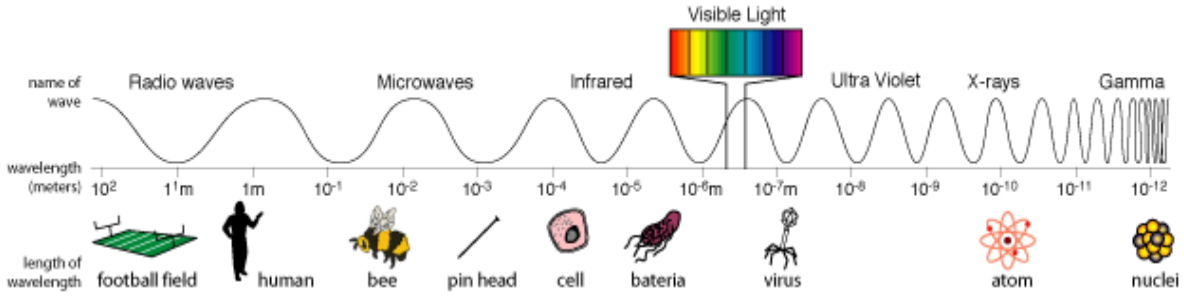


Figure 1: Electromagnetic Waves, wave lengths and measurement limits (Radio Waves- Microwaves- Infrared-Visible Light-Ultraviolet-X-Rays-Gamma Rays)

Electromagnetic Radiation travels in waves as shown in **Figure-2** below. Electromagnetic waves differ from each other in wave length. Wave length is distance between "**one wave crest to the next**" is called **wave length or lambda (λ)**. It follows the unique property that longer the wave lengths have lower energy and shorter wave lengths have higher energy.

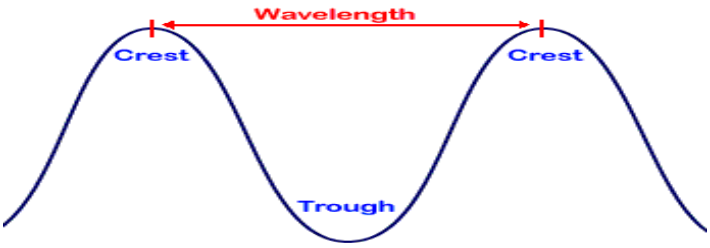


Figure-2: Diagrammatic Representations of Electromagnetic Waves

Each molecular species has a unique set of energy levels that depends on its unique atomic structure (electrons, protons, neutrons) and atomic arrangements in a molecule (type and arrangements of atoms and connecting bonds). The lowest of energy level is called ground state, while higher levels are called exited state. The potential energy of a molecule is contributions from a number of different sources, like electronic, vibrational, rotational, translation and nuclear. When electromagnetic wave is passed through a matter, as a particle of energy (photon) can absorb energy and electron move to an excited state (**Absorption**). If the wave have energies that are equal to the difference between two energy levels we have, ($dE = h\upsilon$). The excited atom or molecule while returning back to ground state emits energy in the form of radiation like waves (**Emission**). The emitted energies are exactly equal to the difference between two energy levels ($dE = h\upsilon$). The energy of the photon in different regions of the electromagnetic spectrum correspond to different types of energetic transitions that can occur in atom and molecules, e.g. electronic, rotational, vibrational, translation, nuclear transitions. A monochromatic electromagnetic wave that propagates through a vacuum can be described completely by its frequency (υ), wave length (λ) and amplitude (A). The period (T), intensity (I), velocity (c), energy (E) have an expression,

$\mathbf{E = h\upsilon = h/T = hc / \lambda =}$ **hc**; {Where, h = Planks constant (6.6262×10^{-34} JS)}.

This relationship indicates that monochromatic radiation (i.e. radiation of a single frequency) contains photons that all have the same energy. The EM spectrum consists of radiation that ranges in wave length form 10^{-12} m (high energy) to 10^{-4} m (low energy). Different regions are called as- Cosmic rays, Gamma rays, X-rays, Ultra Violet, Visible, Infrared, Micro waves, Radio ways.

Absorption: When Electromagnetic (EM) wave energy falls on atom or molecule and cause electron to move an excited state is called **absorption**. At room temperature the ground state of atoms and molecules are highly populated and transition usually occur from the ground state to higher energy levels. But at higher temperature more energy

levels are occupied the transition occur between higher energy levels and may also become important. If radiation of different wavelengths (energies) falls on atoms or molecules, it will absorb photon at that wavelength which corresponds to exact difference between two different energy levels within the material. A plot of the fraction of photon absorbed at particular wavelength versus the energy of photon is called **absorption spectrum**.

Emission: Emission of radiation is reverse of absorption when atom or molecule releases energy in form of photon of radiation. After absorption of energy by a molecule, rose to excited state and will exist for a very short time, than relaxed back to ground state by dissipating its energy. In spectroscopy EM waves are measured due to absorption, emission, transmission and reflection. Determination of concentration of certain compounds in food is done by measuring the EM waves due to interaction between EM waves and the matter. Simple absorption is measured by expression-

A = α b c; [Where, α = constant absorptivity; b = path length; c = concentration of sample].

Spectra can be classified in two categories-

(a) Atomic Spectra: Spectra arise from transition of an electron between the atomic energy levels (Involves only electronic transitions).

(b) Molecular Spectra: Spectra arise from transition on an electron between the molecular energy levels (Involve electronic, rotational and vibrational transitions).

5.3 TYPES OF SPECTROSCOPY:

Spectroscopy is of **three** types-

(i) Absorption,

(ii) Emission and

(iii) Raman Spectroscopy.

When radiations are passed through a sample, certain characteristic wave lengths are removed by the process of absorption. The decrease in the intensity of radiation due to excitation of electrons to high energy level is measured and it becomes the basis of colorimetric or Spectrophotometric. Spectrophotometer is of **two** types, (i) Single Beam and (ii) Double Beam spectrophotometer **(See Figure -3).**

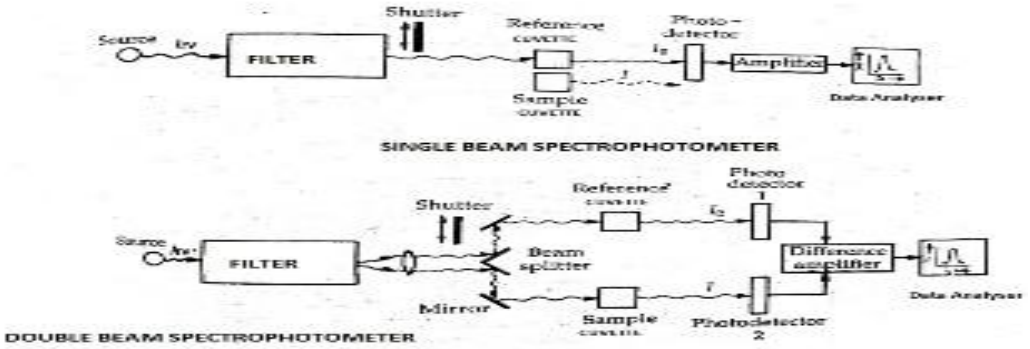


Figure- 3: Line diagram of Components of Single & Double Beam Spectrophotometer

5.4 COMPONENTS OF SPECTROPHOTOMETER:

Spectrophotometer consists of a light source, lens, filter (slit) or monochromator, cell for keeping sample and a detector as shown diagrammatically in **Figure-4&5 below-**

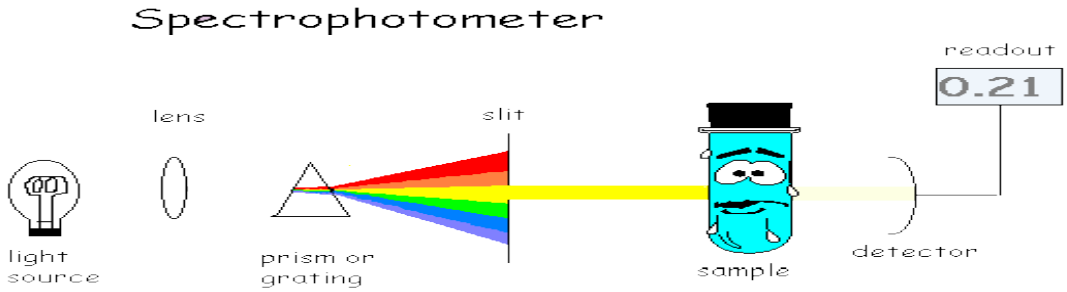


Figure-4: Working of Spectrophotometer and its Components

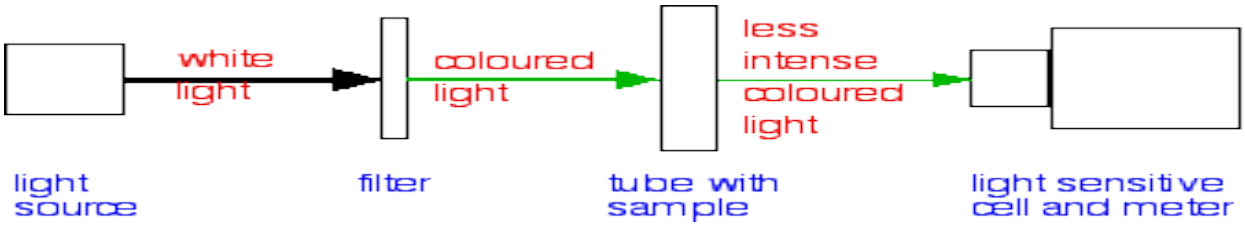


Figure-5: Line diagram of Basic Components of Spectrophotometers

All spectrometers, spectrophotometers have the following basic components-

1. Light Source: A continuous light source of radiant energy covering the region of spectrum in which the instrument is designed to work. The tungsten filament lamp is the most common source of visible radiation (wave length 400 nm to 750 nm) which is widely used. The tungsten lamp must emit constant over long period of time.

Tungsten is most satisfactory material for lamp filaments but the carbon arc is used when a more intense source of visible light is required. In UV or IR region spectrometry, Hydrogen or a Nernst glower are used.

2. **Filter or Monochromator:**

Both filter and Monochromator allow the light of required wavelength to pass through but absorb the light of other wavelengths. A device is required to select a narrow band from wavelength of the continuous spectra, therefore, filters or monochromators or both are used. A suitable filter can select a desired wavelength band, which means a particular filter or inter changeable filter may be used for a specific analysis. Filters are of two types, *Absorption filter* and *interference filter*. An absorption filter is a solid sheet of glass that has been colored by a pigment, which is dissolved or dispersed in the glass. Dyed gelatin or similar material can also be used as absorption filter. Interference filter is a semitransparent metal film deposited on a glass plate. Then it is coated a thin layer of some dielectric material followed by protective coating.

Monochromator successfully isolates band of wavelengths usually much more than a narrower filter. Narrower filter contains an entrance slit, a dispersing prism or grating and an exit slit. A grating consists of a large number of parallel lines (grooves) ruled on a highly polished surface such as alumina.

3. **Slits:**

There are two slits, entrance slit and exit slit. The main function of the entrance slit is to provide a narrow source of light (width) so that there is no overlapping of monochromatic images. From this the exit slit select a narrow band of dispersed spectrum for observation by the detector. Both the slits are so adjusted that it will just be passed by the exit slit having equal width.

4. **Cells:**

The cell holds the sample usually having rectangular shape with a one centimeter internal path length. Cell material may be of high quality Pyrex glass, silica, quartz and plastic called *cuvette*. Pyrex glass cuvette transmits 320-2500 nm range and silica cuvette transmits 170-2500 nm. In infrared spectrum KBr and other salt material are used.

5. **Detectors:**

Three types photosensitive devices are used to detect radiations, Photovoltaic cells (barrier layer or photonic cells), Phototubes (photoemission tubes) and photomultiplier tubes.

(a) Photovoltaic cells:

Photovoltaic cells are also called as barrier layer or *photonic cells* which consist of a metal based plate like iron or aluminium, this acts as one electrode. On its surface, a thin layer of a semiconductor metal like selenium is deposited. Then the surface of selenium is covered by a very thin layer of silver or gold which acts as a second collector electrode. When radiation is incident upon the surface of selenium, electrons are generated at the selenium silver interface. These electrons are collected by silver and create an electric voltage. Difference between silver surface and base of the cell causing photocurrent is measured which is directly proportional to the intensity of the radiant beam. This cell is connected to a galvanometer and amplifier for measurement. It is less sensitive to low radiation.

(b) Phototubes:

Photo tubes are also called as *photoemission cells*. A phototube consists of an evacuated glass bulb containing light sensitive cathode in the form of half cylinder of metal. The inner surface of cathode is coated with light sensitive layer such as cesium or potassium oxide and sliver oxide. A metal ring inserted near the center of the bulb act as anode. When radiation is incident upon the cathode, photoelectrons are emitted. These are attracted and collected by anode. Due to the flow of these electrons there occurs a IR drop across the resistance (R) that is proportional to the current which is amplified and measured.

(c) Photomultiplier tube:

A *photomultiplier tube* consists of an electrode covered with a photo emission material. This tube also contains a large number of plates, known as *dynodes*. Each dynode is covered with a material which emits several electrons (2-5) for each electron striking on its surface. Also each dynode is charged at a successively higher potential. When light radiation incident upon the cathode surface of dynode where secondary electrons in greater number fall on another dynodes again multiplying the electrons ultimately fall on collector anode. Most of the photomultiplier tubes have about10 dynodes. Each maintained 75-100 V more positive than proceeding dynode, overall amplification factor of about 10^6 can be achieved.

6. Power Supply: The power supply serves a triple functions. (i) It decreases the line voltage to operating level. (ii) It converts alternating current to direct current with a rectifier. (iii) It supplies smooth and constant voltage.

5.5 ELECTRONIC (UV-VISIBLE) SPECTROSCOPY:

As discussed earlier theSpectrometry refers to the determination technique that is based upon the production or interaction of electromagnetic radiation with matter as given in following table-1.

Table-1:Spectrum of Electromagnetic Radiations and Quantum Changes:

Wave length (λ)	<100 Pm	0.1-10 nm	100-380nm	380-780nm	0.78-100µm	100µm-1cm	1-100cm
Radiation	γ- ray	X-ray	UV	Visible	IR	Micro Wave	Radio waves
Quantum change	Change in Nuclear Configuration	Change of electron distribution	Change of electron distribution		Change of configuration	Change of Orientation	Change of Spin

Spectroscopy involves transition of electrons within a molecule or an ion from lower to higher electronic energy levels or *vice-versa* by absorption or emission of radiation falling in the UV- Visible range of spectrum. As the electronic energy levels are quantized, it appears as bands of electronic spectra of sample molecule in gaseous state. Properties of Electromagnetic Radiation is given in table-2 below-

Table-2: Properties of Electromagnetic Spectrum-

Type of Radiation	**Frequency Range (Hz)**	**Wavelength Range**	**Type of Transition**
Gamma-rays	1020-1024	Less than (<) 1 pm	Nuclear Transition
X-rays	10^{17} -10^{20}	1 nm-1 pm	Inner Electron
Ultraviolet	10^{15} -10^{17}	400 nm-1 nm	Outer Electron
Visible	**4-7.5 X 10^{14}**	**750 nm-400 nm**	**Outer Electron**
Near-Infrared	1 X 10^{14} -4 X 10^{14}	2.5 µm-750 nm	Outer Electron, MolecularVibrations
Infrared	10^{13} -10^{14}	25 µm-2.5 µm	Molecular Vibrations
Microwaves	3 X 10^{11} -10^{13}	1 mm-25 µm	Molecular Rotations,

			Electron Spin Flips
Radio waves	Less than (<) 3 X 10^{11}	More than (>) 1 mm	Nuclear Spin Flips

2.6 COLORIMETRY

Colorimetry is a group of photometric methods of quantitative analysis based on **"the determination of the concentration of substances in a colored solution by measuring the amount of light absorbed by the solution."** The most widely used method for determining the concentration of biochemical compounds is **Colorimetry**, which makes use of the property that when white light (Visible Range) passes through a colored solution, some wavelength are absorbed more than others as shown in **Figure-6** below.

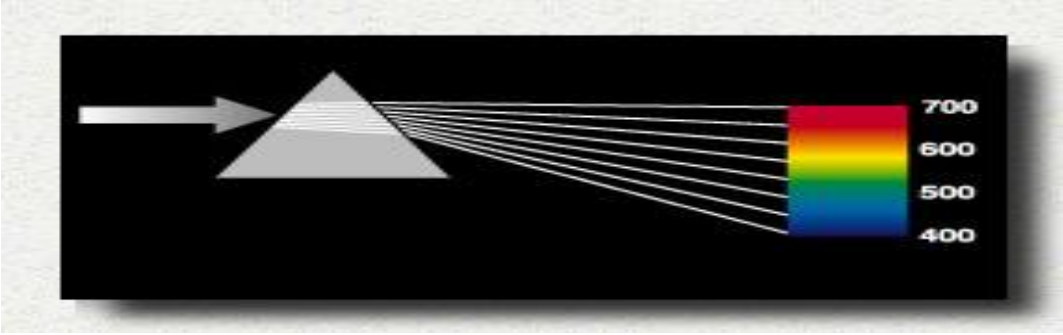


Figure-6:Visible Range of Electromagnetic Spectrum (VIBGYOR or ROYGBIV, The mnemonic used to remember the colors of visible spectrum.)

Colorimeter is an instrument that measures the absorbance of a solution at a particular frequency (color) of visual light to be determined. Colorimeters hence make it possible to determine the concentration of a known solute, since it is proportional to the absorbance.

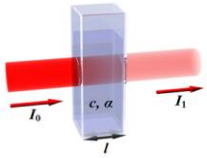
The term Colorimetry originates from the times when measurements were done by comparing the color of a component under investigation with a standard color by the eye. While this principle is still the basis of modern techniques but measurement with eye has been replaced by a photon detector (From this the term **photometry** came about).

The concerned matter may **produce** or **absorb** electromagnetic radiation from which the term **emission** and **absorption** spectrometry originates. Further **spectroscopic** techniques are characterized according to the type of radiation involved, which may range from X-ray to Radiofrequency or in other words: which part of the spectrum is used. Now a day's spectrometric methods are most applied analytical

techniques in the world. Particularly the Radiation in UV (180-380 nm), Visible (380-780 nm) and Infrared (780-40,000 nm) is most commonly used.

Theory of Colorimetry and Spectrophotometry

When light passes from a homogeneous medium, a part of incident light is reflected, a part is absorbed by medium and the remainder is allowed to transmit as such**(Figure-7)**.



$$I_0 = I_a + I_t + I_r$$

Figure-7: Light passing through a medium in cell

If a comparison cell is used, the value of **Ir** is very small (<4%) hence can be eliminated for air-glass interfaces then we have,

$$I_0 = Ia + I_t$$

Bouguer actually investigated the range of absorption of light with the thickness of medium. But credit was enjoyed by **Lambert**, who gave the law as-

Lambert's Law:-

"When a beam of light is allowed to pass through a transparent medium, the rate of decrease of intensity with the thickness of medium and is directly proportional to the intensity of light."

$d_I / d_t \propto I$ where, I = Intensity of incident light, t= thickness of medium

Law shows that there is logarithm relationship between transmittance and length of path.

$A = \log I_0 / I_t$ The ratio I_o / I_t is **Transmittance** and the ratio I_t / I_0 is called **Absorbance**

Beer's Law: - "When monochromatic beam is passed through a medium, the intensity of beam decreases exponentially with the increase in concentration of the absorbing substance arithmetically."

$$I_t = I_0 e^{-k'c}$$

$I_t = I_0.10^{-act}$ (Where, c is concentration)

Or $\log I_0/I_t = act$ **(Beer-Lambert law)**

In equation value of 'a' depends on unit of concentration, '**c**'. If c is expressed in mole dm^{-1} and t in cm, then 'a' is replaced by symbol '**ε**', which is **absorption coefficient** or **molar absorptivity**.

There exists a relationship between absorption, **A**, the transmittance **T** and the molar absorption coefficient, '**ε**';

$A = \varepsilon ct = \log I_0 / It = \log 1/T = -\log T$

$\varepsilon = A/ct$, if A = 1 mole dm^{-3} and t = 1cm , then we have,

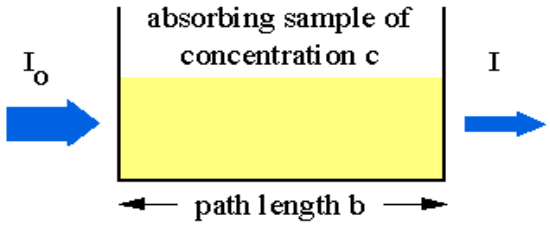
$\varepsilon = A$

Transmittance is given by the equation:

$T = I/I_o$

Where, **I**is the intensity of the light after it has gone through the sample &**I_o** is the initial light intensity.

Absorbance is related to the %T**(Figure-8)**:



Absorbance, $A = -\log T = -\log (I/I_o)$

Figure-8: Absorbance of light in sample medium

Nature of Molar Absorptivity and Absorbance:

Absorptivity reflects the sensitivity of the procedure. ***Absorbance*** is extensive property of a substance, where as***absorptivity***is its intensive property.

A = εct, (where, A= absorbance,**ε** = absorptivity, c = concentration and t = path length)

Deviations of Beer's Law-

From the Beer's law it is clear that if we plot absorbance 'A' against concentration, a straight line passing through the origin should be obtained. Deviations from law are reported as positive or negative due to following factors.

1. If a colored solution having foreign substances, whose ion do not react chemically with the colored component may affect light absorption and may after the value of the extinction coefficient.
2. If colored substance ionizes, dissociates or associates in solution may cause deviation in the curve.
3. Presence of impurities that fluoresce or absorb radiation may cause error in measurement of absorbance.

4. Deviation may occur if monochromatic light is not used.
5. Undesirable radiation which falls on detector may cause deviation in measurement.
6. If solution species undergoes polymerization or polymeric forms cause deviation in measurement.
7. Laws cannot be applied to suspensions, the homogeneity affects the measurements.

Interaction of Electro Magnetic Radiation with matter:-

Table-3: Visible colors of different wave length regions in absorption and transmission of light-

Wave length (λ) in nm	**Absorbed colors**	**Transmitted colors**
380-450	Violet	Yellow-green
450-495	Blue	Yellow
495-570	Green	Violet
570-590	Yellow	Blue
590-620	Orange	Green -Blue
620-750	Red	Blue-Green

Types of Absorption Spectrophotometer

1. **Photometer-** The instrument which measures the ratio, or some function of the two of radiant power of two electromagnetic beams.

The instrument consists of a filter to isolate a narrow wavelength and a photocell or phototube to measures the intensity of radiation.

2. **Colorimeter:** The instrument is used for measuring absorption in the visible region is called colorimeter.
3. **Spectrophotometer:** The instrument that measures the ratio or a function of the two of the radiant power of two electromagnetic beams over a large wavelength region**(Figure-9)**. The instrument consists of a scanner or Monochromator and sensitive detectors like phototubes or photomultiplier.

COMPONENTS OF INSTRUMENT-

All photometers, colorimeters and spectrophotometers have the following basic components.

1. Source-A continuous source of radiant energy covering the region of spectrum in which the instrument is designed to work. The **Tungsten filament** lamp is the most common source of visible radiation (Wave length 4000 Å to 7500 Å) which is widely used. The tungsten lamp must emit constant over long periods of time. Tungsten is the most satisfactory material for lamp filaments but the **carbon arc** is used when a more intense source of visible light is required. If colorimetric is carried out in the UV or IR region, the source commonly used is **hydrogen lamp** or a **Nernst Glower** respectively.

2. Filter or Monochromator-Both filter or Monochromator allow the light of the specific (required) wavelength to pass through medium that absorb the light of other wavelengths. A device is required to select a narrow band from wavelengths of the continuous spectra, therefore, filters or Monochromator or both are used (Figure-4). A suitable filter can select a desired Wavelength band, which means a particular filter may be used for a specific analysis. If analysis is carried out for several species a large number of filters have to be used and interchanged.

Filters are of **two** types- **absorptionfilters** and **interference filters**. An absorption filter is a solid sheet of glass that has been colored by a pigment which is dissolved or dispersed in the glass. Dyed gelatin or similar materials can also be used as absorption filters. Absorption filters are classed as either **cut off** or **band pass filters.**

Figure-4: Monochromator or Filters and Approximate Range of Spectrum Band-

Colors of filters	**Approximate range of absorption band(in nm)**
Yellow	450
Orange	500
Red	575
Purple	450-650

Blue	480
Green	575-700

Interference filter functions on interference phenomena at desired wavelength, thus permitting rejection of unwanted radiation by selective reflection.

In interference filter, a semitransparent metal film is deposited on a plate of glass, then it is coated a thin layer of some dielectric material (Mg F_2) followed by coating of a film for mechanical protection.

When a ray of light is incident upon an interference filter, a part of light is reflected back whereas remaining is transmitted. Interference filters have a bad pass of 100-150 Å and peak transmittance of 40-60 %. The ideal filter yields maximum transmittance over a narrow range of wavelengths as possible. Both of these criteria are better met with interference filters than with the absorption type.

Monochromator successfully isolates band of wavelengths usually much more than a narrower filter. The essential elements of a Monochromator are an entrance slit, a dispersion **prism** of **grating** and an **exit slit**. A grating consists of a large number of parallel lines (grooves) ruled on a highly polished surface as alumina. Generally 15,000-30,000 lines per square inch are drawn for UV and Visible regions.

3. **Slits**: There are two slits, **entrance slit** and **exit slit**. The main function of the entrance slit is to provide a narrow source of light so that there is no overlapping of monochromatic images. From this the exit slit selects a narrow band of dispersed spectrum for observation by the detector. Both the slits are so adjusted that it will just be passed by the exit slit having equal width.

4. Cells: The cell holding the sample usually having rectangular shape with a 1cm. internal path length. Circular cells, similar to test tubes are also available in matched sets. Cell material may be of high quality Pyrex glass, silica quartz and plastic. Pyrex glass transmits 320-2500nm range and silica 170-2500nm. In infra-red spectrum KBr and other salt material are used.

5. Detectors: three types of photosensitive devices are used to detect radiations, **photovoltaic cells** (barrier layer or photonic cells), phototubes (photoemission tubes) and photomultiplier tubes.

A. Photovoltaic cells: also called as barrier layer or photonic cells consists of a metal base plate like iron or aluminum which acts as one electrode, on its surface, a thin layer of a semiconductor metal like selenium is deposited. Then the surface of selenium is covered by a very thin layer of silver or gold which acts as a second collector electrode.

When radiation is incident upon the surface of selenium, electrons are generated at the selenium silver interface. These electrons are collected by silver and create an electric voltage difference between silver surface and base of the cell causing photocurrent, directly proportional to the intensity of the radiant beam. This cell is connected to a galvanometer and amplifier for measurement. Photovoltaic cells are sensitive over the whole visible region but less sensitive in blue region. Due to low internal resistance it can be amplified by conventional electronic circuit. It show fatigue effect after a few minutes which can be less sensitive to low radiation.

A. Phototubes: also called as **photoemission cells**. Phototube consists of an evacuated glass bulb containing light sensitive cathode in the form of half cylinder of metal. The inner surface of cathode with light sensitive layer such as cesium or potassium oxide and silver oxide. A metal ring inserted near the center of the bulb acts as anode.

When radiation is incident upon the cathode, photoelectrons are emitted. These are attracted and collected by anode. Due to the flow of these electrons there occurs a IR drop across the resistance (R) which is proportional to the current. This current may be amplified and measured.

B. Photomultiplier tube: A photomultiplier tube consists of an electrode covered with a photoemission material; this tube also contains a large number

of plates, known as **dynodes.** Each dynode is covered with a material which emits several electrons (2-5) for each electrons striking on its surface. Also each dynode is charged at a successively higher potential.

When light radiation incident upon the cathode surface of dynodes where secondary electrons in greater number fall on another dynodes again multiplying the electrons ultimately fall on collector anode. Most of the photomultiplier tubes have about 10 dynodes. Each maintained 75-100 v more positive than proceeding dynodes, overall amplification factor of about 10^6can be achieved.

6. Power Supply: The power supply servers a triple function.

a) It decreases the line voltage to operating level.

b) It converts alternating current to direct current with a rectifier.

c) It supplies smooth and constant voltage.

Photoelectric colorimeters: Photoelectriccolorimetersare of two types-

1. Single beam instrument- The essential parts of single beam instruments are-

a) A source of light with a concave reflector

b) An adjustable diaphragm

c) A colored glass filter (Monochromator)

d) A cuvette for holding the absorbing medium.

d) A single photocell to receive the radiation.

e) A directly connected galvanometer.

In order to determine the absorbance of a solution, the cuvette is filled up with pure solvent. Then the diaphragm is adjusted so that the meter reads full scale (100%). Now the solvent is replaced by solution without disturbing the diaphragm. Then the meter will read the percent transmittance from which the absorbance of solution can be calculated.

2. Double Beam-Electro Photometer

In double beam instrument a Potentiometer, null- balance measuring system is employed. In order to operate null balance galvanometer is adjusted to bring the needle at midscale. The blank solution is kept in both light beams. A potentiometer dial $\mathbf{R_2}$in adjusted to read 100 percent transmittance and then slide wire contact $\mathbf{R_1}$is adjusted to null the galvanometer. Standard and unknown solutions are introduced

into the measurement beam and slide wire contact $\mathbf{R_2}$is adjusted to re-null the meter. The transmittance (linear) or absorbance (non-linear) can then be read off from the potentiometer dial for each sample.

Applications of electronic Spectroscopy:

1. Characterization of aromatic compounds.
2. Detection of impurities.
3. Control of purification (Detection of Impurities).
4. Determination of unknown concentrations (Quantitative Measurements).
5. Determination of Molecular Weights (Absorption at different wave lengths).
6. Study of kinetics of chemical reactions.

Figure-9: Spectrophotometers (Visible and UV-Visible)

5.7 PROGRESS EXERCISE-1

A. Answer the following questions-

1. What is meant by Spectroscopy?

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2. What is the difference between absorption and emission spectroscopy?

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3. What are the components of spectrophotometer?

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4. What are the types of radiations in electromagnetic spectrum?

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5. What is Colorimetry?

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B. Fill in the blanks with appropriate words to complete the sentences-

1. Electromagnetic Radiation travels in ……with specific energy levels.
2. Each molecular species has a …… set of energy levels.
3. When electromagnetic wave energy falls on atoms or molecules can cause electron to move to……..state called absorption.
4. After absorption of energy by a molecule rose to excited state and then relaxed back to ground state by …….. of its energy.

5. A device is required to select a narrow band from wavelength of a continuous spectra by ……..
6. Detectors are …….. devices that are used to detect radiation.
7. Colorimetry is based on principle of …….. law.

5.8 VIBRATIONAL (INFRA RED) SPECTROSCOPY:

Vibrational spectroscopy involves the transition between vibrational energy levels of molecules on absorption of radiation falling in spectral range of 500-4000 cm $^{-1}$ (Infra red region). A single vibrational energy change is accompanied by a large number of rotational energy changes. Thus, the vibrational spectra appear as vibrational-rotational bands.

During the vibration of a molecule, if there is a change in dipole movement, it will lead to the generation of an oscillating in electric field. This will lead to the generation of absorption IR-spectra or Emission IR-spectra. The molecular vibration may be due to (i) Stretching (Symmetrical or asymmetrical) or due to (ii) Bending (in-plane or out of the plane).

Application of Infra red Spectroscopy:

1. Determination of force constant of vibrational spectrum.
2. Determination of Purity of molecule.
3. Identification of unknown compounds (matching of Molecules).
4. Identification of Functional groups in organic molecules.
5. Determination of intra, inter molecular hydrogen bonding.
6. Elucidation of structure as it gives valuable information's (like molecular symmetry, dipole moment, bond length, bond strength, etc).

5.9: ROTATIONAL (MICROWAVE) SPECTROSCOPY:

Rotational (Microwave) Spectroscopy involves the transition between rotational energy levels of a gaseous molecule having permanent dipole moment on absorption of radiation (in range of 1-100 cm^{-1}), microwave region.

The molecule with permanent dipole moment generates the oscillating electric field on rotation, hence can interact with the electric field of microwave radiation. Such a molecule exhibits rotational spectrum and known as microwave active molecule (e.g. HCl, CN, CO, etc). If molecule having no permanent dipole moment are known microwave inactive molecules (e.g. O_2, N_2, H_2 or O=C=S, H-=C-H, etc.).

Application or Rotational Spectroscopy:

1. Determination of bond length of a polar molecule.
2. Determination of symmetry in a molecule.
3. Determination of dipole moment of gaseous molecule.

5.10 ATOMIC ABSORPTION SPECTROSCOPY (AAS)

Atomic Absorption Spectroscopy (AAS) is a spectral analytical method for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 differentelements in solution or directly in solid samples used in pharmacologyand research**(Figure-11)**. Atomic absorption spectroscopy was first used as an analytical technique, and the underlying principles were established in the second half of the 19th century by Robert Wilhelm Bunsen and Gustav Robert Kirchhoff, both professors at the University of Heidelberg, Germany. The modern form of AAS was largely developed during the 1950s by a team of Australian chemists. They were led by Sir Alan Walsh at the Commonwealth Scientific and Industrial Research Organization (CSIRO), Division of Chemical Physics, in Melbourne, Australia.

Atomic absorption spectrometry has many uses in different areas of chemistry such as clinical analysis of metals in biological fluids and tissues such as whole blood, plasma, urine, saliva, brain tissue, liver, muscle tissue, semen, in some pharmaceutical manufacturing processes, minute quantities of a catalyst that remain in the final drug product, and analyzing water for its metal content.

Principle:

The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires standards with known analyte content to establish the relation

between the measured absorbance and the analyte concentration and relies therefore on the **Beer-Lambert Law.**In short, the electrons of the atoms in the atomizer can be promoted to higher orbital (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an absorption line is only of the order of a few Pico meters (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert Law.

Components of Instrument:

In order to analyze a sample for its atomic constituents, it has to be atomized. The atomizers most commonly used nowadays are flames and electro thermal (graphite tube) atomizers. The atoms should then be irradiated by optical radiation, and the radiation source could be an element-specific line radiation source or a continuum radiation source. The radiation then passes through a monochromator in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector**(Figure-12)**.

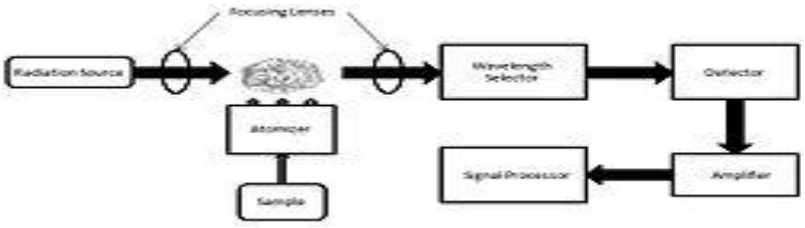

Figure-12: Atomic Absorption Spectrometer Block Diagram

1. **Atomizers**

The atomizers most commonly used nowadays are (spectroscopic) flames and electro thermal (graphite tube) atomizers. Other atomizers, such as glow-discharge atomization, hydride atomization, or cold-vapor atomization might be used for special purposes.

2. **Flame Atomizers**

The oldest and most commonly used atomizers in AAS are flames, principally the air-acetylene flame with a temperature of about 2300 °C and the nitrous oxide system (N_2O)-acetylene flame with a temperature of about 2700 °C. The latter flame, in addition, offers a

more reducing environment, being ideally suited for analyte with high affinity to oxygen.A laboratory flame photometer that uses propane operated flame atomizer

Figure-13: Atomic Absorption Spectroscopy

(Source: https://en.wikipedia.org/wiki/Atomic_absorption_spectroscopy)

Liquid or dissolved samples are typically used with flame atomizers. The sample solution is aspirated by a pneumatic analytical nebulizer, transformed into an aerosol, which is introduced into a spray chamber, where it is mixed with the flame gases and conditioned in a way that only the finest aerosol droplets (less than 10 μm) enter the flame. This conditioning process is responsible that only about 5% of the aspirated sample solution reaches the flame, but it also guarantees a relatively high freedom from interference.

On top of the spray chamber is a burner head that produces a flame that is laterally long (usually 5–10 cm) and only a few mm deep. The radiation beam passes through this flame at its longest axis, and the flame gas flow-rates may be adjusted to produce the highest concentration of free atoms. The burner height may also be adjusted, so that the radiation beam passes through the zone of highest atom cloud density in the flame, resulting in the highest sensitivity.The processes in a flame include the stages of desolvation (drying)in which the solvent is evaporated and the dry sample nano-particles remain, vaporization (transfer to the gaseous phase) in which the solid particles are converted into gaseous molecule, atomization in which the molecules are dissociated into free atoms, and ionization where (depending on the ionization potential of the analyte atoms and the energy available in a particular flame) atoms may be in part converted to gaseous ions.Each of these stages includes the risk of interference in case the degree of phase transfer is different for the analyte in the calibration standard and in the sample. Ionization is generally undesirable, as it reduces the number of atoms that are available for measurement, i.e., the sensitivity.In flame AAS a steady-state signal is generated during the time period when the

sample is aspirated. This technique is typically used for determinations in the mg L^{-1} range, and may be extended down to a few μg L^{-1} for some elements.

We have to distinguish between line source AAS (LS AAS) and continuum source AAS (CS AAS). In classical LS AAS, as it has been proposed by Alan Walsh, the high spectral resolution required for AAS measurements is provided by the radiation source itself that emits the spectrum of the analyte in the form of lines that are narrower than the absorption lines. Continuum sources, such as deuterium lamps, are only used for background correction purposes. The advantage of this technique is that only a medium-resolution monochromator is necessary for measuring AAS; however, it has the disadvantage that usually a separate lamp is required for each element that has to be determined. In CS AAS, in contrast, a single lamp, emitting a continuum spectrum over the entire spectral range of interest is used for all elements. Obviously, a high-resolution monochromator is required for this technique, as will be discussed later.

3. Hollow Cathode Lamps (HCL):

Hollow Cathode Lamps are the most common radiation source in LS-AAS. Inside the sealed lamp, filled with argon or neon gas at low pressure, in a cylindrical method cathode containing the element of interest and an anode. A high voltage is applied across the anode and cathode, resulting in an ionization of filled gas. The gas ions are accelerated towards the cathode and, upon impact on the cathode, sputter cathode material that is excited in the glow discharge to emit the radiation of sputtered material, i.e. the element of interest. Most lamps will handle a handful of elements i.e. 5-8. A typical machine will have two lamps, one will take care of five elements and the other will handle four elements for a total of nine elements analyzed.

4. Electrodeless Discharge Lamps (EDL)

Electrodeless Discharge Lamps (EDL) contain a small quantity of the analyte as a metal or a salt in a quartz bulb together with an inert gas, typically argon gas, at low pressure. The bulb is inserted into a coil that is generating an electromagnetic radio frequency field, resulting in a low-pressure inductively coupled discharge in the lamp. The emission from an EDL is higher than that from an HCL, and the line width is generally narrower, but EDLs need a separate power supply and might need a longer time to stabilize.

5. Deuterium lamps

Deuterium HCL or even hydrogen HCL and deuterium discharge lamps are used in LS AAS for background correction purposes. The radiation intensity emitted by these lamps decreases significantly with increasing wavelength, so that they can be only used in the wavelength range between 190 and about 320 nm.

Figure-14:Xenon lamp as a continuous radiation source

6. **Continuum sources**

When a continuum radiation source is used for AAS, it is necessary to use a high-resolution monochromator, as will be discussed later. In addition, it is necessary that the lamp emits radiation of intensity at least an order of magnitude above that of a typical HCL over the entire wavelength range from 190 nm to 900 nm. A special high-pressure xenon short arc lamp, operating in a hot-spot mode has been developed to fulfill these requirements**(Figure-14)**.

- **Spectrometer**

As already pointed out above, there is a difference between medium-resolution spectrometers that are used for LS AAS and high-resolution spectrometers that are designed for CS AAS. The spectrometer includes the spectral sorting device (monochromator) and the detector.

- **Spectrometers for LS AAS**

In LS AAS the high resolution that is required for the measurement of atomic absorption is provided by the narrow line emission of the radiation source, and the monochromator simply has to resolve the analytical line from other radiation emitted by the lamp.This can usually be accomplished with a band pass between 0.2 and 2 nm, i.e., a medium-resolution monochromator. Simple monochromator of the Czerny-Turner designed are typically used for LS AAS. Photomultiplier tubes are the most frequently used detectors in LS AAS, although solid state detectors might be preferred because of their better signal-to-noise ratio.

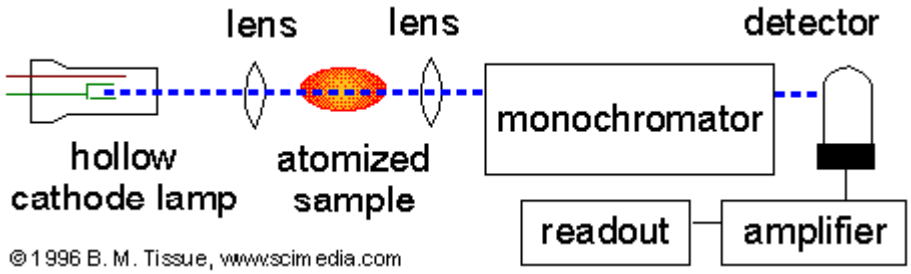

Figure-15: Schematic diagram of an Atomic-Absorption experiment.

Atomic-Absorption (AA) spectroscopy uses the absorption of light to measure the concentration of gas-phase atoms. Since samples are usually liquids or solids, the analyte atoms or ions must be vaporized in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The analyte concentration is determined from the amount of absorption. Applying the Beer-Lambert law directly in AA spectroscopy is difficult due to variations in the atomization efficiency from the sample matrix, and no uniformity of concentration and path length of analyte atoms (in graphite furnace AA). Concentration measurements are usually determined from a working curve after calibrating the instrument with standards of known concentration.

Components of Instrument:

1. Light source

The light source is usually a hollow-cathode lamp of the element that is being measured. Lasers are also used in research instruments. Since lasers are intense enough to excite atoms to higher energy levels, they allow Atomic Absorption and atomic fluorescence measurements in a single instrument. The disadvantage of these narrow-band light sources is that only one element is measurable at a time.

2. Atomizer

Atomic Absorption spectroscopy requires that the analyte atoms be in the gas phase. Ions or atoms in a sample must undergo desolvation and vaporization in a high-temperature source such as a flame or graphite furnace. Flame Absorption can only analyze solutions, while graphite furnace AA can accept solutions, slurries, or solid samples.Flame AA uses a slot type burner to increase the path length, and therefore to increase the total absorbance (Beer-Lambert law). Sample solutions are usually aspirated with the gas flow into a nebulizing/mixing chamber to form small droplets before entering the flame.

The graphite furnace has several advantages over a flame. It is a much more efficient atomizer than a flame and it can directly accept very small absolute quantities of sample. It also provides a reducing environment for easily oxidized elements. Samples are placed directly in the graphite furnace and the furnace is electrically heated in several steps to dry the sample, ash organic matter, and vaporize the analyte atoms.

3.Light separation and detection

AA spectrometers use monochromators and detectors for UV and visible light. The main purpose of the monochromator is to isolate the absorption line from background light due to interferences. Simple dedicated AA instruments often replace the monochromator with a band pass interference filter. Photomultiplier tubes are the most common detectors for AA Spectroscopy.

Figure-16: Picture of a Flame Atomic-Absorption Spectrometer:

(Source: http://elchem.kaist.ac.kr/vt/chem-ed/spec/atomic/aa.htm)

Atomic Emission Spectroscopy (**AES**):

Atomic Emission Spectroscopy (AES) is a method of chemical analysis that uses the intensity of light emitted from a flame, plasma, arc, or spark at a particular wavelength to determine the quantity of an element in a sample. The wavelength of the atomic spectral line gives the identity of the element while the intensity of the emitted light is proportional to the number of atoms of the element.

Flame Emission Spectroscopy (FES):

Flame Emission Spectroscopy (FES) is a technique where sample of a material (analyte) is brought into the flame as either a gas, sprayed solution, or directly inserted into the flame by use of a small loop of wire, usually platinum. The heat from the flame evaporates the solvent and breaks chemical bonds to create free atoms. The thermal energy also excites the atoms into excited electronic states that subsequently

emit light when they return to the ground electronic state. Each element emits light at a characteristic wavelength, which is dispersed by a grating or prism and detected in the spectrometer.A frequent application of the emission measurement with the flame is the regulation of alkali metals for pharmaceutical analytics.

Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES):

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) uses an inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element.

Advantages of ICP-AES are excellent limit of detection and linear dynamic range, multi-element capability, low chemical interference and a stable and reproducible signal. Disadvantages are spectral interferences (many emission lines), cost and operating expense and the fact that samples typically must be in a liquid solution.

Spark and Arc Atomic Emission Spectroscopy

Spark or arc atomic emission spectroscopy is used for the analysis of metallic elements in solid samples. For non-conductive materials, the sample is ground with graphite powder to make it conductive. In traditional arc spectroscopy methods, a sample of the solid was commonly ground up and destroyed during analysis. An electric arc or spark is passed through the sample, heating it to a high temperature to excite the atoms within it. The excited analyte atoms emit light at characteristic wavelengths that can be dispersed with a monochromator and detected. In the past, the spark or arc conditions were typically not well controlled, the analysis for the elements in the sample was qualitative. However, modern spark sources with controlled discharges can be considered quantitative. Both qualitative and quantitative spark analysis are widely used for production quality control in foundry and metal casting facilities.

2.11 NUCLEAR MAGNETIC RESONANCE (NMR):

Nuclear Magnetic Resonance (NMR) is a branch of spectroscopy in which radio frequency waves induce transition between magnetic energy levels of nuclei of a molecule. Keeping the nuclei of molecule in magnetic field the magnetic energy level is created. The nuclei with spin quantum number are greater than zero which can exhibit the NMR phenomenon. If proton has spin quantum number ½ so it can exhibit NMR and known as Proton Magnetic Resonance (**PMR**).The principle behind NMR is that "**Nuclei**

have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap) and recorded in form of NMR spectrum".Proton is a spinning magnet since it has both electric charge and mechanical spin. Hence it will process around the axis of an applied magnetic field. After absorption of energy, proton will pass into higher energy state and starts processing in opposite orientation. Absorption energy is recorded in the form of NMR spectrum. NMR is used to study the properties of molecules containing magnetic nuclei by means of the application of a magnetic field and the orientation of the frequency at which they come into resonance with a radio frequency of electromagnetic fields. This technique is known as **Proton-NMR** (**H-NMR**) when applied to proton spin. The NMR basically consists of a magnet, a radio frequency source and a detecting system.

NMR is a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation. This energy is at a specific resonance frequency which depends on the strength of the magnetic field and the magnetic properties of the isotope of the atoms; in practical applications, the frequency is similar to very high frequency (VHF) andultra high frequency (UHF) television broadcasts (60–1000 MHz). NMR allows the observation of specific quantum mechanical magnetic properties of the atomic nucleus. Many scientific techniques exploit NMR phenomena to study molecular physics, crystals, and non-crystalline materials through nuclear magnetic resonance spectroscopy. NMR is also routinely used in advanced medical imaging techniques, such as in Magnetic Resonance Imaging (MRI).

- **Magnetic Resonance Imaging (MRI)**

MRIis a test that uses a **magnetic** field and pulses of radio wave energy to make pictures of organs and structures inside the body. In many cases, MRI gives different information about structures in the body than can be seen with an X-ray, ultrasound, or computed tomography (CT) scan.

Nuclear Magnetic Resonance (NMR) Spectroscopy is a non-destructive analytical technique that is used to probe the nature and characteristics of molecular structure. A **simple** NMR experiment produces information in the form of a spectrum, which is able to provide details about: The types of atoms present in the sample.

NMR spectroscopy is one of the principal techniques used to obtain physical, chemical, electronic and structural information about molecules due to either the chemical

shift, Zeeman effect, or the Knight shift effect, or a combination of both, on the resonant frequencies of the nuclei present in the sample. It is a powerful technique that can provide detailed information on the topology, dynamics and three-dimensional structure of molecules in solution and the solid state. Thus, structural and dynamic information is obtainable (with or without "magic angle" spinning (MAS)) from NMR studies of quadrupolar nuclei (that is, those nuclei with spin $S > 1/2$) even in the presence of magnetic "dipole-dipole" interaction broadening (or simply, dipolar broadening) which is always much smaller than the quadrupolar interaction strength because it is a magnetic vs. an electric interaction effect.

Additional structural and chemical information may be obtained by performing double-quantum NMR experiments for quadrupolar nuclei such as 2H. Also, nuclear magnetic resonance is one of the techniques that have been used to design quantum automata, and also build elementary quantum computers.

- **Non-Destructive Testing:**

Nuclear magnetic resonance is extremely useful for analyzing samples non-destructively. Radio waves and static magnetic fields easily penetrate many types of matter and anything that is not inherently ferromagnetic. For example, various expensive biological samples, such as nucleic acids, including RNA and DNA, or proteins, can be studied using nuclear magnetic resonance for weeks or months before using destructive biochemical experiments. This also makes nuclear magnetic resonance a good choice for analyzing dangerous samples.

- **Application Chemistry**

By studying the peaks of nuclear magnetic resonance spectra, chemists can determine the structure of many compounds. It can be a very selective technique, distinguishing among many atoms within a molecule or collection of molecules of the same type but which differ only in terms of their local chemical environment. NMR spectroscopy is used to unambiguously identify known and novel compounds, and as such, is usually required by scientific journals for identity confirmation of synthesized new compounds.

By studying T_2 information, a chemist can determine the identity of a compound by comparing the observed nuclear precession frequencies to known frequencies. Further structural data can be elucidated by observing *spin-spin* coupling, a process by which the precession frequency of a nucleus can be influenced by the magnetization transfer from nearby chemically bound nuclei. Spin-spin coupling is observed in NMR of Hydrogen-1

(1 NMR), since its natural abundance is nearly 100%; isotope enrichment is required for most other elements.Because the nuclear magnetic resonance *timescale* is rather slow, compared to other spectroscopic methods, changing the temperature of a T_2 experiment can also give information about fast reactions, such as the Cope rearrangement or about structural dynamics, such as ring-flipping in cyclohexane. At low enough temperatures, a distinction can be made between the axial and equatorial hydrogen in cyclohexane.

An example of nuclear magnetic resonance being used in the determination of a structure is that called "Bucky balls", composition C_{60}. This now famous form of carbon has 60 carbon atoms forming a sphere. The carbon atoms are all in identical environments and so should see the same internal **H** field. Unfortunately, buckminsterfullerene contains no hydrogen and so ^{13}C, nuclear magnetic resonance has to be used. ^{13}C spectra require longer acquisition times since carbon-13 is not the common isotope of carbon (unlike hydrogen, where 1H is the common isotope).Modification of this technique is Fourier Transform NMR (FT-NMR). The sample is held in a strong pulse of radio frequency energy. The radiation changes the orientations of the nuclear spins in a controlled way. When they return to equilibrium, they emit radio frequency radiation which in monitored and analyzed mathematically.

2.12 SUMMARY

Electromagnetic Radiation based analytic are widely used in modern times to evaluate the quality of food and its constituents including adulterations in food. Electromagnetic radiation based analytical techniques of spectroscopy which utilize the interaction between electromagnetic radiation and matter to provide information about food properties such as molecular composition, structure, dynamics and interactions. These different techniques are UV- Visible, Colorimetric, fluorescence, Infra Red, Microwave and atomic Absorption Spectroscopy and Nuclear Magnetic Resonance (NMR). Electromagnetic Radiation travels in form of waves. When it passes through matter as a particle of energy (Photon) can absorb and move to excited state, called absorption spectroscopy. The excited atom or molecule when return to its ground state emits energy in form of radiation called Emission Spectroscopy. Different types of energetic transition that can occur in atom and molecules like electronic, rotational, vibrational translational and nuclear transitional spectra are applied in spectroscopy. Spectroscopy is divided in

three types' absorption, emission and Raman Spectroscopy. The components of spectrophotometer are light source, lens, monochromator or filter, slit, cell or cuvette and detector. UV- Visible spectroscopy and colorimetry use UV and Visible range of EM spectra and absorption of monochromatic light is measured. The colorimeter is based on principle of Beer- Lambert's Law which utilizes absorption of spectra in solution to determine concentration of analyte. Different types of detectors like phototubes (Photoemission cell), photomultiplier tube are used. Infra red spectroscopy is based on vibrational spectroscopy. Rotational spectroscopy is known as Microwave spectroscopy (1-100 cm^{-1}) which measures transition between rotational and energy levels of gaseous molecules having dipole movement. Atomic absorption spectroscopy is used for quantitative determination of chemical elements using absorption of optical radiation by free atoms in the gaseous state. AAS constitute atomizer, flame atomizer, light source, lamps (EDL and deuterium), monochromator, amplifier and detector. Nuclear Magnetic Resonance (NMR) is a branch of spectroscopy which uses radio frequency waves induces transition between magnetic energy levels of nuclei of a molecule. If proton spin quantum is used then it is called Proton Magnetic Resonance (PMR or H-NMR). NMR is non destructive technique of analysis; it is used in MRI, Chemical research.

2.13 KEY WORDS

- **Electromagnetic Radiations:** Is a form of energy that is transmitted through space at enormous velocities like UV, Visible, IR, X-ray. γ-ray
- **Light:**Light is called visible radiation of electromagnetic Radiation.
- **Photon of quanta:** discrete packets of electromagnetic radiation energy or particle called photons or quanta. [**E= hv = hc =hc_υ = λ]**Where, h= plank's constant 6.63×10^{-34}js, c= speed of light $3\times10^{10}cms^{-1}$
- **Wavelength (λ):** Wave length is the linear distance between successive maxima or minima of a wave (i.e. Distance between one crest to another in wave).
- **Velocity (v):**The wave front through a medium depends on both the medium and frequency (frequency × Wave length).
- **Frequency (υ):**It the number of oscillations of the electric field vector per unit time (in seconds) and is equal to 1/P or Number of cycles passing a fixed point per unit time.

- **Electromagnetic Spectrum:** Range of EM spectrum from x-ray to radio- frequency.

- **Wave number(v):** The number of waves per cm (unit length) and is equal to $1/\lambda$ Cm^{-1}
- **The Radiant Power (P):**Is the energy of a beam that reaches a given area per unit time, It is express in watts (w).
- **Intensity:**Is the radiant power per unit solid angle.
- **Speed of light (c):** In vacuum light travels its maximum velocity, [In air velocity is only 0.03% ;i.e. **c** = $2.99792 \times 10^{8} ms^{-1}$ or $3 \times 10^{10} cm\ S^{-1}$]
- **Amplitude:** Is a vector quantity that provides a measure of the electric or magnetic field strength at a maximum point in the wave.
- **Period:** Period of an electromagnetic wave is the time in seconds that is takes for successive maxima or minima to pass a point in space.
- **Monochromatic Radiation:** Term refers to radiation of a single colori.e. single wavelength or frequency.
- **Polychromatic light:** Multicolored light is light of many wavelengths.
- **Spectrometry:** The techniques that are based upon the production or interaction of electromagnetic radiation.
- **Absorption (A):** Is the fraction of incident radiation that is absorbed by the solution.
- **Transmittance (T):** Is the fraction of incident radiation that is passed through (transmitted) a medium.
- **Colorimetry:** Measurement of the intensity of visible radiation transmitted through a solution or transparent solid often called as Spectrophotometry.
- **Photocell:**A device used to measure the amount of light transmitted through a solution/ medium.

2.14 PROGRESS EXERCISE-2

A. **Answer the following questions-**

1. What is Monochromator?

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2. Explain Vibrational Spectroscopy.

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3. What is rotational (Microwave) Spectroscopy?

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4. Explain Atomic Absorption Spectroscopy?

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5. On which principle NMR is used?

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A. Write full form of following Abbreviations-

1. NMR……
2. AAS……
3. UV……..

4. IR……

5. EMR……

6. LS……..

7. CS…….

8. HCL…….

9. EDL…….

10. AES……

11. FES……

12. ICP-AES…….

13. PMR……

14. HNMR…….

15. MRI…….

16. CT……

17. FT…….

2.15 SOME RECOMMENDED BOOKS:

1. Instrumental Methods of Chemical Analysis, Gurdeep Raj Chatwal and Sham K. Anand, Himalaya Publishing House, 2015, ISBN- 9789351420880.

2. Handbook of Analytical Instrument, R. S. Khandpur, Tata McGraw Hills Education (2006), ISBN-10: 0070604606; ISBN-13: 978- 0070604605.

3. Basic Clinical Biochemistry and Instrumentation, Aruna Singh and Poonam Bachheti, Vayu Education of India, First Edition 2015, ISBN-10: 938507718X, ISBN-13: 978 9385077180.

4. Principle of Instrumental Analysis, Skoog, Douglas, Canada Thomas Brooks/cole, ISBN-0: 495-01201-7.

5. Atomic Absorption, fluorescence and flame Emission Spectroscopy-a practical approach, R. J. Reynold and K. C. Thompson (1978), New York, Wiley, ISBN0-47026478-0.

6. Nuclear Magnetic Resonance, Application to organic chemistry, John D. Roberts, McGraw Hills Book Company, ISBN 9781258811662.

7. Methods in Food Analysis; Rui M. S. Crutz, IorKhmelinskii, Margarida Vieira, June 2014, CRC Press; ISBN 9781482231953.

8. Physical Properties of Foods by Ignacio Arana, 2012, CRC Press, ISBN No 9781439835364.

9. Handbook of Food Analysis Instruments; Ed. Smith Otles, CRC Press, ISBN-9781420045666.

10. Handbook of Analysis and quality control for Fruit and Vegetable Products, Ranganna, Tata McGraw- Hill Publication Company, Ltd., New Delhi.

11. Principles of Instrumental Analysis, D. A. Skoog and J. J. Leary (1992); Saunders College Publishing, Florida.

12. Instrumental Methods of Food analysis, A. J. Mac Leod; Elek Science. London.

2.16 ANSWER KEY TO THE PROGRESS EXERCISES:

ANSWER KEY OF EXERCISE-1 (2.7)

A. Your answer should include following points-

1. Spectroscopy is analytical technique which utilizes the interactions between electromagnetic radiation energy and matter to provide information about molecular composition.
2. When electromagnetic wave is passed through a matter the particle of energy (Photon) is absorbed energy and electron move to an excited state called absorption spectroscopy, whereas the excited electrons in atoms or molecules return back to the ground state emits energy in form of radiation like waves, is called emission spectroscopy.
3. The spectrophotometer is consists of light source, filter or monochromator, slits, cells, detectors and power supply.
4. The electromagnetic radiations consist of 7 wave's i.e. γ -Rays, X-rays, Ultraviolet, Visible, near Infra Red&Infra Red, Microwaves and Radio waves.
5. The colorimetry is photometric method in which quantitative determination of concentration in a solution is measured by absorption of monochromatic light.
6. **Correct words for fill in the blanks-**
   1. Waves
   2. Peculiar
   3. Excited
   4. Emission
   5. Monochromator
   6. Photosensitive
   7. Beer- Lambert's

ANSWER KEY OF EXERCISE-2 (2.14)

A. Your answer should include following points-

1. Monochromator allows the light wave of specific wave length is allowed to pass through.
2. Vibrational spectroscopy involves the transition between vibrational energy levels of molecules on absorption of radiation in spectral region of Infra Red (500 to 4000cm^{-1}).
3. Rotational or microwave spectroscopy involves the transitional between rotational energy levels of a gaseous molecule having permanent dipole movement on absorption of radiation in range of 1 to 100 cm^{-1}(Microwave region).
4. The atomic absorption spectroscopy is a spectral analytical method for the quantitative determination of chemical elements using absorption of optical radiation by free atoms in the gaseous state.
5. Nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level and recorded in form of NMR spectrum.

B. Full form of given Abbreviations-

1. Nuclear Magnetic Resonance
2. Atomic Absorption Spectroscopy
3. Ultra Violet
4. Infra Red
5. Electro Magnetic Radiation
6. Line source
7. Continuum source
8. Hollow Cathode Lamps
9. Electrodeless Discharge Lamps
10. Atomic Emission Spectroscopy
11. Flame Emission Spectroscopy
12. Inductively Coupled Plasma – Atomic Emission Spectroscopy
13. Proton Magnetic Resonance
14. Proton (H^+) - NMR
15. Magnetic Resonance Imaging
16. Coupled Tomography
17. Fourier Transform

UNIT-VI: ANALYTICAL BALANCE, pH METER AND REFRACTOMETER

Course Structure-

6.0 OBJECTIVES:

After reading this unit you will understand-

- The principle of balances used in laboratory for precise measurements.
- Different types and handling of analytical balances.
- Working of Analytical Balances.
- Types and Working of Electronic Balances.
- What is pH and scale of Acidity?
- Measurement of pH of solutions.
- Different types of Electrodes used in pH measurement.
- Different types of Refractometer used in Laboratory.
- Principle of Refractometer.
- Measurement of Brix (Sugar content in fruit juices).
- Determination of Total soluble solids (TSS).

6.1 INTRODUCTION:

Food quality testing and evaluation commonly require specific instrumentation such as weight measurement of food product by balance, acidity measurement by pH meter and sugar or Brix measurement by refractometer. There is wide range of balances used for specific food products. Food products are sold in the market with proper weight measurements. Balances compare the weight of substance with the weight of set standard mass (standard weights). Most commonly used balances are beam balance, spring balances; top loading balances, scale balances, analytical balances, micro or precise balances, etc. These are properly handled and proper methodology is used to get proper result.

The acidity of food product determines the quality of food. There is certain specific standards for acidity in food products beyond the range food products are not acceptable by consumers or various food quality regulation standards. The acidity is usually determined accurately by pH meter using specific methodology. Various pH meters are used in food quality measurements. Refractometers are used to measure the soluble sugar

content of food products and beverages. It is based on principle of refractive index of juice, which determines the sugar content of Brix of juice.

6.2 ANALYTICAL BALANCES:

Principle:

Weight (W) and mass (M) of food material is important measure often used synonymous but are quite different. The **Mass** of substance "is a definite property which can be used as a measure of quantity" where the Weight of a substance 'is the force which is the result of interaction of the gravitational force on the substance".

This relationship is expressed by formula as (**$W = M \times g$**)

Where, W = weight (determined by balance), M = mass and g = gravitational force or acceleration.

Balance compares the weight of a substance with the weight of set standard mass. Since gravity becomes common equally between the weight of standard and object indicate equality to mass. In practice 'weight' is used in place of 'mass' conventionally. Balances are used to measure weight.

Types of Balances:

A balance compares the mass of two sets of objects, while a scale determines the mass of an object or set of objects. The most common types in use today are Pan Balances, Beam Balances, Spring Balances, Top-loading Balances, Analytical Balances, Precision Scales Balances, Electronic Balances and Moisture Analyzing Balances.

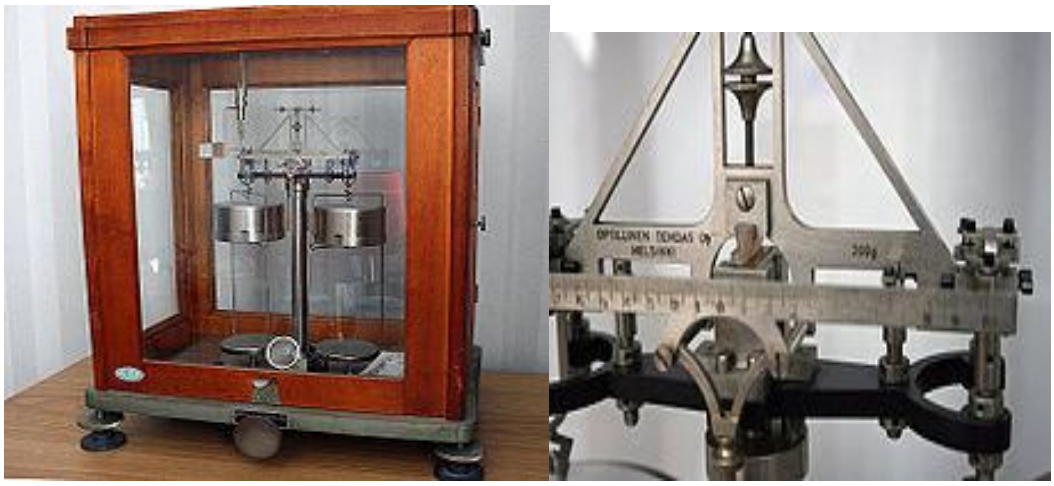
Analytical Balance:

An analytical balance (also called as Laboratory Balances) is a class of balance basically designed to measure small mass in the sub-milligram range. The measuring pan of an analytical balance (0.1 mg or below) is inside a transparent enclosure with doors so that dust does not collect and so any air currents in the room do not affect the balancer's operation (**Figure 1 A**). This enclosure is often called a draft shield. The use of a mechanically vented balance safety enclosure, which has uniquely designed acrylic airfoils, allows a smooth turbulence-free airflow that prevents balance fluctuation and the measure of mass down to 1 µg without fluctuations or loss of product. Also, the sample must be at room temperature to prevent natural convection from forming air currents inside the enclosure from causing an error in reading. Single pan mechanical substitution balance maintains consistent response

throughout the useful capacity is achieved by maintaining a constant load on the balance beam, thus the fulcrum, by subtracting mass on the same side of the beam to which the sample is added.

Analytical balance is one of the most important tools very sensitive used for quantitative measurements and designed to weigh the substance to a sufficiently high degree of precision. It consists of a beam mounted at its center upon a prism- form, knife edged agate or synthetic sapphire (corundum) which rests upon an agate plate attached to the central beam support **(Figure 1B)**. Two terminal agate knife edges, fixed at equal distance from which the pans are hung, contact being made upon agate planes fixed to the stirrups. A long pointer is attached to the centre to the beams which moves over a scale at the foot of pillar and serves to indicate the deflection of the beam when the beam is in operation. The three prisms like knife edged agate attached to the beam and working on agate planes the friction to give precise weighing. To reduce wear, the balance beam can be raised by arrest knob. While taking out or adding weights to the pan or when not in use the balance should be arrested. The balance is enclosed in a glass cabinet which protects the balance from dust, air, operator's breath, etc. The balance is adjusted to a horizontal position indicated by spirit level or plumb bob that is provided in balance.

The beam has a scale with graduation zero to 10. In most of balances zero mark remains at center of beam and mark 10 on the beam right over the pan. Each division is further divided into 5-10 parts. Rider is used on this scale which is a bend light aluminum wire of 10 mg, when kept on pan or 10^{th} position on beam. Each further division gives 1 mg weight of rider. This 10 division in 10 parts each gives 0.1 mg weight of rider.



(Source: https://en.wikipedia.org/wiki/Analytical_balance)

Figure -1 A (Balance in cabinet) and B (Beam mounted on knife edged agate)

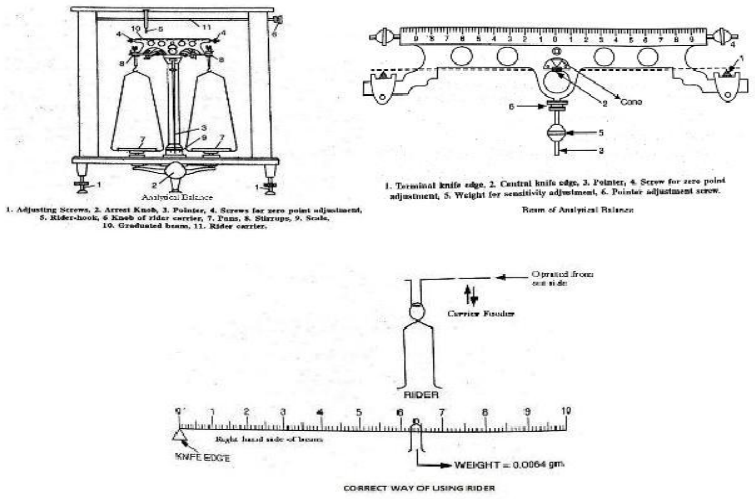


Figure-2: Line diagram of Analytical Balance

(Source: Practical Manual of Agricultural Chemistry by A. K. Gupta, Kalyani Publisher-ISBN 81-272-3475-3)

Handling:

Before weighing observe the beam and stirrups in proper place; check the plumb line, adjust with screws to make balance horizontal. Clean the balance pans with soft brush or soft cloth. Place the rider at zero '0' position with the help of rider carrier. Cover the front door; slowly release the beam with arrest knob so that pointer swings on the scale equally either side. If pointer is not swinging equally, then adjust zero point with screws provided on both ends of beam. After zero adjustment, place the object to be weighed on left hand side of pan. Add weights with the help of forceps on right hand pan and slowly release the arrest knob and observe the movement of pointer. Add weights, fractional weights from weight box and move rider, after arresting the knob repeatedly till pointer moves equidistance from center.

Electronic analytical scales measure the force needed to counter the mass being measured rather than using actual masses. As such they must have calibration adjustments made to compensate for gravitational differences. They use an electromagnet to generate a force to counter the sample being measured and out puts the result by measuring the force needed to achieve balance. Such measurement device is called electromagnetic force restoration.

Different Types of Analytical Balances Commonly Found In Laboratories:

Balances can be found in nearly every laboratory setting throughout the world. Their ability to accurately measure weights and provide detailed data makes them indispensable tools. This piece will take a look at some of the different types of balances commonly found in laboratories throughout the world, all of which are available in the market.

- **Micro balances**: Microbalances are generally designed to measure, weigh and provide data on the tiniest of samples. Most models can effectively provide data for samples weighing between 0.1 and 0.0001 milligrams **(Figure-3)**. These types of balances are generally used to weigh highly valuable substances in minute quantities. Manufacturers also stock an extensive range of ultra micro balances, which are designed to weigh and provide data on even smaller samples. These units typically come standard with draft shields so that dust and other foreign particles do not make their way into the dish and corrupt data and materials being worked on.

(Source: https://en.wikipedia.org/wiki) (Source: https://www.alibaba.com/product-detail)

Figure-3: Electronic Balance **Figure-4: Carat Balance**

- **Gold and carat balances:**

  Gold and carat balances, as the name implies, are designed to weigh gold as well as give carat values **(Figure-4)**. These are more commonly found in jewellary design workshops and retail jeweleroutlets, however, they are used in some laboratory exercises too. These units are not limited to weighing gold; they are also commonly used to measure and provide carat values for both precious and semiprecious stones and metals.

- **Scale Balances:**

  Scale balances are designed to measure weight quickly. Various types of scale balances are available like double and triple scale balances. These balances are not used for accurate/ minute measurements. These balances consist of pan, shaft and

scale on which movable weights are attached to the scale for determining the weight. These balances need to be calibrated again and again with standard weights.

(Source: https://en.wikipedia.org/wiki/Triple_beam_balance)

Figure-5: Scale Balance

- **Analytical balances:**

  Analytical balances are found throughout most laboratories. They are mostly used to weigh substances and samples between 0.01 to 500 milligrams (**Figure-1 above**). These units' measuring pans are usually encased in a glass box so as to prevent any dust particles settling in the pan. The particles can often disrupt efficient testing and provide incorrect data if they should settle in the pan. Analytical balances are accurate and precise instruments to measure weights. They require a draft-free location on a solid bench that is free of vibrations. Modern balances have built-in calibration weights to maintain accuracy. Handled objects with tongs, gloves, or weighing paper to prevent fingerprints. When the weights on the plates of this balance are equal, the needle mid-rod points straight up. Very precise measurements are achieved by ensuring that the fulcrum of the beam is friction-free (a knife edge is the traditional solution), by attaching a pointer to the beam which amplifies any deviation from a balance position and finally by using the lever principle, which allows fractional weights to be applied by movement of a small weight along the measuring arm of the beam, as described above. For greatest accuracy, there needs to be an allowance for the buoyancy in air, which effect depends on the densities of the weights and the sample. While the word "weigh" or "weight" is often used, any balance scale measures *mass*, which is independent of the force of gravity. The moments of force on eitherside balance, and the acceleration of gravity on each side cancels out, so a change in the strength

of the local gravitational field will not change the measured weight. Mass is properly measured in grams, kilograms, pounds, ounces, or slugs.

The original form of a **weighing scale** consisted of a beam with a fulcrum at its center. For highest accuracy, the fulcrum would consist of a sharp V-shaped pivot seated in a shallower V-shaped bearing. To determine the mass of the object, a combination of reference weights was hung on one end of the beam while the object of unknown mass was hung on the other end. For high precision work, the center beam balance is still one of the most accurate technologies available, and is commonly used for calibrating test weights. To reduce the need for large reference weights, an off-center beam can be used. A scale with an off-center beam can be almost as accurate as a scale with a center beam, but the off-center beam requires special reference weights and cannot be intrinsically checked for accuracy by simply swapping the contents of the pans as a center-beam balance can. To reduce the need for small graduated reference weights, a sliding weight, called a poise, can be installed so that it can be positioned along a calibrated scale. Poise adds further intricacies to the calibration procedure, since the exact mass of the poise must be adjusted to the exact lever ratio of the beam. For greater convenience in placing large and awkward loads, a platform can be "floated" on a cantilever beam system which brings the proportional force to a "nose iron" bearing; this pulls on a "steelyard rod" to transmit the reduced force to a conveniently sized beam. This design

Analytical balances are instruments used for precise determining mass of matter. Analytical balances are sensitive and expensive instruments, and upon their accuracy and precision the accuracy of analysis result depends. The most widely used type of analytical balance is wide range of balances with a capacity of 100 g and a sensitivity of 0.1 mg. Not one quantitative chemical analysis is possible without usage of balances, because, regardless of which analytical method is being used, there is always a need for weighing a sample for analysis and the necessary quantity of reagents for solution preparation.

The working part of the balance is enclosed in a glass-fitted case. The baseplate is usually of black glass or black slate. The beam has agate knife-edges at its extremes, supporting stirrups from which balance pans are suspended. Another agate or steel knife-edge is fixed exactly in the middle of the beam on its bottom

side. This knife-edge faces downwards and supports the beam. When not in use and during loading or unloading of the pans, the balance should be arrested

Use of weighing bottle:

The weighing bottle is tipped above the container to receive the sample and a small amount is allowed to fall out of the weighing bottle. The weighing bottle is tipped back up and tapped gently to make sure all of the substance falls back in the bottle and doesn't remain on the bottle rim. The cap is replaced and the bottle weighed once again. The difference between the first and second weighing represents the amount transferred. If your sample has a tendency to absorb water and thus to gain weight when exposed to the moisture of the air, this method must be used to minimize exposure to the atmosphere. Still, the method is not foolproof and has its own perils: (1) several transfers may be necessary until an amount close to that needed is added to the receiving container. (2) Too much may be transferred the first time, forcing one to discard the entire sample. (3) Sample which remains on the weighing bottle rim may be lost and produce a weighing error. This peril is repeated each time a transfer is attempted. .

- **Electronic Balance:**

Electronic Balance is electro-magnetic device designed for easy to operate and quick method of weighing. Proper standardization and calibration is needed to measure the weight accurately **(Figure-6, 7).** The pan of balance is situated on hollow metal cylinder, surrounded by a coil and fitted over the inner hole of cylindrical permanent magnet. When object is placed on the pan the indicator arm moves downward with increasing the light causing striking of the photocell of the null detector. The current falling photocell is amplified in to the coil which creates magnetic field and returns the pan into original null position. The current which keeps the pan and object in null position is directly proportion to the weight of the object. Taring is needed before placing the object on pan.

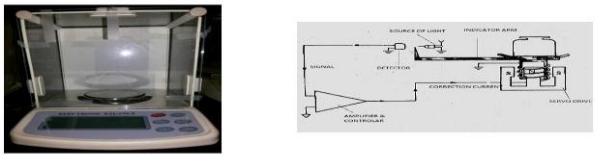


Figure - 8: Electronic Balance

Figure-6 Electronic Balance

Figure-7: Digital Electronic Balance

6.3 Measurement of pH: (The Scale of Acidity):

Acidity of Food products are measured by specific instrument called pH meter which is based on the quantitative measurement of free Hydrogen ion that cause acidity in solution. Acid is a substance which has tendency to donate proton in the solution. The free proton in solution when increases than 1 X 10^{-7} gram ion / liter, results the solution acidic. Sorenson in 1909 gave mathematical measurement of such minute amount is "the negative logarithm of Hydrogen ion concentration" expressed as pH. The negative logarithm of Hydrogen ion in the solution becomes whole number 7. Less than 7.0 shows acidic and morethan7.0 becomes alkaline. This gives quick and most accurate measurement of acidity. The instrument is based on following principle-

Principle:

Acidity and Alkalinity is measured in terms of pH values. pH is an expression of amount of hydrogen ions [H+] in aqueous medium, mathematically expressed as "Negative logarithm of Hydrogen ion).

$pH = - \log [H^+]$ or $\log 1 / [H^+]$

Acid- base neutralization reactions are basically reaction between Hydrogen ($H^{+)}$ and Hydroxyl (OH^-) ions to form water. Pure water dissociates feebly to-

$2H_2O = [H_3O^+] + [OH^-]$, Appling law of mass action we have,

$[H_3O^+] \times [OH^-] = Kw$ (Ionization constant)

Since concentration of H_2O is extremely great as compared to that of $[H_3O^+]$ and $[OH^-]$ and regarded as constant, 1 then,

$[H_3O^+]$ x $[OH^-]$ = Kw

Kw is constant of pure water which is $1x10^{-14}$. Hence $[H_3O^+] = [OH^-] = 1 \times 10^{-7}$ g ion/l

Now it is the relative concentration of H_3O^+ and OH^- which decides whether an aqueous solution is acidic or alkaline. In neutral water the hydrogen and hydroxyl ion concentration will be equal i.e. 1 X 10^{-7} gram ion /liter. Thus if H^+ concentration of solution is increased 10 times (i.e.10^{-6} gion/L) then it is acidic. The H^+ ion concentration will obviously be decreased 10 times i.e. 10^{-8} g ion/liter.

It is cumbersome to express the concentration of H^+ or OH^- ions in extremely small numerical value (Smallest being 10^{-14}, which is 0.000, 000, 000, 000, 01 g). Sorenson therefore, suggested the use of negative exponent (logarithms) with the negative sign removal so that it is expressed by whole number 7, 14. Thus pH can be expressed in numerical values ranging from 0 to 14, the neutral point being 7.0. More than 7.0 is alkaline and less than 7.0 is acidic nature of solution.

pH meter:

A **pH Meter** is a scientific instrument that measures the hydrogen-ion concentration (or pH) in a solution, indicating its acidity or alkalinity (Figure-8). The pH meter measures the difference in electrical potential between a pH electrode and a reference electrode due to hydrogen ions in a solution. It usually has a glass (Hydrogen) electrode plus a calomel (Mercury) reference electrode, or a combination electrode. In addition to measuring the pH of liquids, a special probe is sometimes used to measure the pH of semi-solid substances. pH meter is basically a potentiometer used for measuring the potential or voltage. It has similarly two electrodes like potentiometer, an indicator electrode and reference electrode (known or standard electrode of known potential). The potential of indicator electrode is combination of reference electrode is generated by ionic activity of solution and is measured b potentiometer. This potentiometer is calibrated to measure the ionic activity due to activity of hydrogen ion hence called pH meter. Generally hydrogen electrode, calomel electrode and silver electrodes are used as reference electrodes and ion-selective glass membrane electrode is used as indicator or working electrode. pH meter generally has combination of standard calomel and glass membrane ion selective electrode. The calomel electrode contains a platinum plate connected with metallic mercury (calomel), surrounded by saturated $HgCl_2$ and connected by N- KCl solution.

(Source: https://en.wikipedia.org/wiki/pHmeter) (Source: milwaukeeinnst.com)

Figure-8: pH meters

pH meters range from simple and inexpensive pen-like devices to complex and expensive laboratory instruments with computer interfaces and several inputs for indicator and temperature measurements to be entered to adjust for the variation in pH caused by temperature. Specialty meters and probes are available for use in special applications, harsh environments, etc. There are also holographic pH sensors, which allow pH measurement colorimetrically. The Glass electrode is used in measurement of pH which is a glass membrane consists of a porous bulb of sensitive glass membrane which acts a semi permeable membrane. The bulb is filled with saturated silver chloride connected with silver plate and wire. A silver wire in this solution acts as silver reference electrode. Due to porous semi permeable ion sensitive reference electrode it is called glass electrode (Figure-9).

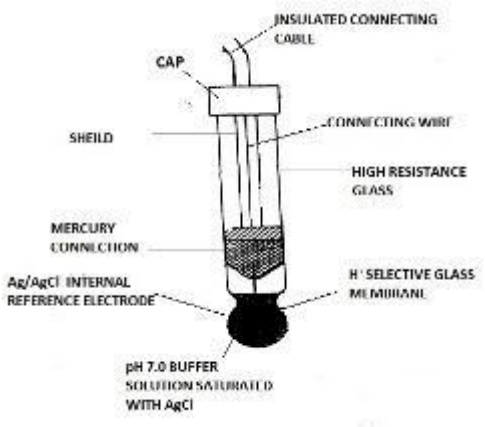


Figure – 9: Glass Electrode

Calibration of pH meter:

The pH meter should be calibrated before each measurement in precise work. For normal use calibration should be performed at the beginning of each day. The reason for this is that the

glass electrode does not give a reproducible e. m. f. over longer periods of time Calibration should be performed with at least two standard buffer solutions that span the range of pH values to be measured. For general purposes, buffers at pH 4.00 and pH 10.00 are acceptable. The pH meter has one control (calibrate) to set the meter reading equal to the value of the first standard buffer and a second control which is used to adjust the meter reading to the value of the second buffer. A third control allows the temperature to be set. Standard buffer sachets, which can be obtained from a variety of suppliers, usually state how the buffer value changes with temperature. For more precise measurements, a three buffer solution calibration is preferred. As pH 7.0 is essentially, a "zero point" calibration (taking to zero or taring a scale), calibrating at pH 7.0 first, calibrating at the pH closest to the point of interest (e.g. either pH 4.0 or 10.0) second and checking the third point will provide a more linear accuracy to what is essentially a non-linear problem. Some meters will allow a three-point calibration and that is the preferred scheme for the most accurate work. Higher quality meters will have a provision to account for temperature coefficient correction, and high-end pH probes have temperature probes built in. The calibration process correlates the voltage produced by the probe (approximately 0.06 volts per pH unit) with the pH scale. After each single measurement, the probe is rinsed with distilled water or deionized water to remove any traces of the solution being measured, blotted with a scientific wipe to absorb any remaining water which could dilute the sample and thus alter the reading, and then quickly immersed in a solution suitable for storage of the particular probe type.

Method of Measurement:

In a clean beaker distilled water is taken and dipped the electrodes and adjusted zero. Prepared the standard buffer solution and electrodes are dipped in solution after drying the surface of electrode with soft clean cloth or blotting/tissue paper. Temperature is recorded and adjusted with separate knob and then switched on the instrument. Adjusted the pH of solution as prepared (pH of known buffer), with the knob provided for adjustment. Taken out the electrode from solution, washed the electrode and dried with blotting paper gently. Precaution is taken not to touch the bulb with bare fingers or rub the electrode. Sample solution is taken in beaker and dipped the electrodes in solution and measured the pH and recorded. Taken out the electrodes and process is repeated several times to get the constant value. The instrument is calibrated that gives direct result and does not need any calculation.

6.4 REFRACTOMETER:

Refractometer is an instrument used to determine total soluble solids (TSS) in solution, juice, etc. It is based on principle of Refractive index. Refractive Index is a useful physical property of pure liquids and often identified with refractive index. The refractive index is defined as-

"Refractive Indexis the ratio of the velocity of light in vacuum to that in the medium".

When a beam of light is passed from one medium to other (air to liquid), it suffers refraction that is "a change of direction". If beam of light passes from less dense to more dense medium (air to liquid), the beam of light is refracted towards the normal so that the angle of refraction is less than the angle of incidence Q (theta).

Nr = sin Q / Sin O == V (air) / V (liquid)

This diversion is due to difference in velocities of light in condensed phase (liquid, solid) than in air. This physical property is used to determine the concentration of solute in solution. The refractive index of a solvent generally changes if a solute is dissolved in it. The solute increased the density of solvent (becomes more dense medium) hence refractive index increases with, the concentration of solute.

dnr /dc = n (solution) – n (solvent) / Concentration of solution.

dnr / dc = specific refractive index increment that is, the change in refractive index of a solvent with change of solute concentration.

Types of Refractometer: There are four main types of Refractometer, traditional handheld Refractometer, digital handheld Refractometer, laboratory or Abbe Refractometer, and inline process Refractometer. Refractive index is a material constant, dependent on the chemical composition of a substance. Due to the dependence of the refractive index on the wavelength of the light used (*i.e.* dispersion), the measurement is normally taken at the wavelength of the sodium line D-line (Na_D) of ~589 nm. This is either filtered out from daylight or generated with a monochromatic light-emitting diode (LED). The two different refractive indexes are classified using a polarization filter.

A wine grape grower with Refractometer

Digital Brix Refractometer

Hand held Refractometer

Abbe Refractometer

(Source:https://upload.wikimedia.org/wikipedia/commons/5/58/WinzerMitRefraktometer.jpg)

Figure-10: Different Refractometers

Measurement of Brix (Sugar Content) in fruits:

Refractometer is instruments that measure the refractive index of grape using the phenomenon of light refraction or of total internal reflection of light. The sugar content of grapes is determined by measuring the refractive index of their juice. The refractometer reading can then be converted to the required units using a standard curve (calibration) based on the concentration of sucrose in sucrose-water solutions. Like hydrometers, refractometer can also be purchased with a variety of scales. The measurement of refractive index is subject to temperature effects and therefore must be corrected for temperature. Most bench-type Refractometer, and some hand-held digital types, have automatic temperature compensation. It is also very important that sufficient time be allowed for temperature equilibration of the sample, a common cause of error in many laboratories.

The individual concentration of the different sugars (fructose, sucrose, etc) does not matter as much as the total sugar concentration for the flavor of the fruit and degrees Brix (°Bx) measure the total amount of sugar in a standard amount of water. Simple instrument, Refractometer is used to measure the nutritional content of fruits and vegetables. It works on the principle of light bending when it passes from air into water. Due to dissolved sugars,

minerals and other nutrients in fruit and vegetable juices, they are denser than water and bend light more. The amount of light bends by juice is measured in **Degree "Brix" (^{0}Bx)**. There are values of degrees Brix for each fruit and vegetable ranging from poor to excellent. The excellent level generally gives the plant immunity to disease and insects, and creates health in animals and people.

Refractometer is easy to use, even for inexperienced operators. To make a reading, place 2 to 3 drops of the liquid sample on the prism surface, close cover & point toward any light source. Focus the eyepiece by turning to the right or left. Locate the point on the graduated scale where the light & dark fields meet. Read the % sucrose (solids content) on the scale. The chart represents values for juices of mature crops. For reference, pure (distilled) water has a reading of "0" degrees Brix. Within a given species of plant, the crop with a higher refractive index will have higher sugar content, higher mineral content, higher protein content and a greater specific gravity or density. This adds up to a sweeter tasting, more minerally nutritious food (maximum nutritional value) with a lower nitrate and water content and better storage characteristics. Crops with higher refractive index will produce more alcohol from fermented sugars and be more resistant to insects, thus resulting in decreased insecticide usage. For resistance to insects, maintain a Brix of 12 or higher in the juice of the leaves of any plant. Crops with higher sugar content will have a lower freezing point and therefore be less prone to frost damage.

Determination of Total Soluble Sugar (TSS) or Brix by Refractometer:

Refractometer contains two prisms of equal size and thickness placed on one another (Figure-11). The surface is ground to serve as diffusing screen providing rays in every direction. There is small space between the lower prism P and the upper prism R, where small portion of liquid under examination is kept. The lower prism is closed so as to have fine film of liquid is formed between both the prisms. Observe with eye piece, kept the telescope straight against light.

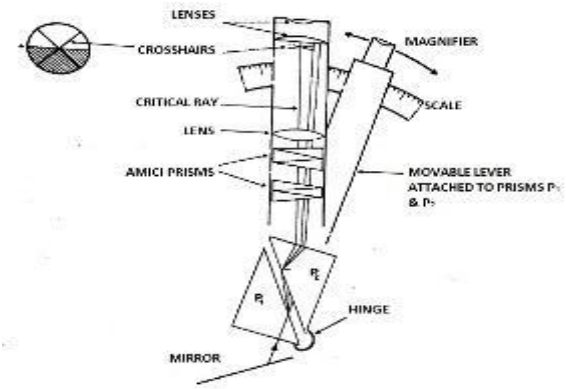


Figure – 11: Line diagram of Refractometer

The ray after passing through the diffused surface AB, enter into the liquid medium at different angles of incidence. A particular ray going along the grazing incidence (i.e. at an angle slightly less than 90 ^{0}) will pass through prism R at an angle O (*theta)* which is equal to the critical angle. According to the critical angle phenomenon,

Sin O (*Sine theta*) = nr /Nr

Where, nr is the refractive index of the liquid and Nr is that of prism R. The ray emerging from A C at an angle α is viewed by telescope T. The telescope is fixed and prism box is rotated such a way to get the incidence of the critical ray with the cross- wire of the eye-piece. Abbe Refractometer is ideal instrument for the measurement of refractive index (RI) of solutions.

A revised hand Refractometer is used frequently containing prism box, telescope in a line and natural light is used. This is calibrated in such a way that it gives direct measurement of total dissolve solids or total soluble sugar in unknown solutions.

The Refractometer is checked before use. The prisms are cleaned by moist tissue paper or soft linen cloth. Placed few drops of water on the prism (specimen chamber) closed and looked through eye piece with the projection inlet facing towards light. The point on the scale is noted when boundary line of the shaded area intersects with the unshaded area. If necessary, eye piece is rotated to either side for clear vision to zero. The specimen chamber is cleaned and dried with muslin cloth or tissue paper. For determination of TSS, a drop of sample (juice, solution, syrup, etc.) is placed on the prism (specimen chamber) closed and viewed through eye piece against light and the percentage is read directly. The temperature is set at 20 ^{0}C or temperature correction is done.

6.5 SUMMARY

The balance is most important instrument used to determine the weight or mass of food products. Marketing of food products are done on the weight which is measured by various types of balances like Pan Balance, Scale Balance, Spring Balances, Top loading Balances. While Analytical Balances, Micro Balances, Electronic Balances are used for quality testing and evaluation of products and precise weighing. Different balances have specific handling processes to determine the greater accuracy of products. The acidity of food products are quickly and accurately measured by specific instrument called pH meter which is based on measurement of electrode potential of solution (potentiometer). The ph Meter measures the difference in electrical potential between pH electrode and a reference electrode. Various pH meters are available for specific purposes. Calibration of pH meter is required before measurement of pH with known buffers. Soluble Sugar content in food products and beverages are measured by specific instrument called Refractometer, which measures the refraction of plane of light by the solution. This is based on the physical property of pure liquids and determines refractive index of test solution. There are several types of refractometer such as Table top refractometer, Hand- held Refractometer, Digital refractometer and Abbe refractometer. Soluble sugar content in food products and beverages are quickly determined and quality control is done by Refractometers.

6.6 KEY WORDS

- **Weight**- Is the force which is the result of interaction of the gravitational force on the subject.
- **Mass**- Is a definite property which can be used as a measure of quantity.
- **Balance**- Instrument which determines the mass of an object or set of objects.
- **Analytical Balance**- Is a class of Balance design to measure small mass in sub milligram range (0.1 mg or below).
- **Micro Balance**- Is designed to measure, weigh the object in micro range (0.0001 mg).
- **Scale Balance**- A balance designed to have Scale on which weight moves to adjust the mass of heavy or light objects quickly.

- **Electronic Balance**- Balance work on electromagnetic principle used to weigh the mass accurately and quickly.
- **pH meter**- Is a potentiometer designed to measure the hydrogen ion concentration (acidity) in a solution by measuring the difference of electrical potential produced by hydrogen between electrodes.
- **Calibration**- Calibration of instrument is done with standard value to get reproducible results.
- **Refractometer**- An optical instrument used to measure refraction of a polarized light (Refractive Index) by thin layer of solution for determining the soluble sugar in the solution/juice.
- **Hand Held Refractometer**- A portable refractometer design to measure the soluble sugar in solution or Brix of juice at the site of collection.

6.7 PROGRESS EXERCISE

A. Answer the following Questions-

1. What is difference between weight and mass of a substance?

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2. What are the different types of balances?

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3. What is pH of a solution?

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4. What is pH meter?

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5. What is Refractometer?

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B. Fill in the blanks with appropriate words.

1. Mass is a definite property which can be used as measure of ……of substance.
2. Balance compares the weight of substance with set of ……. Mass.
3. Analytical Balance consists of a beam mounted at it center upon prism form ……which can rest agate plate.
4. Electronic Balance is …….device designed to weight accurate and easy to operate.
5. pH is negative ……. of hydrogen ion concentration in a solution.
6. Refractive Index is the ratio of ……in Vacuum to that of medium.
7. The amount of light bends by juice is measured in …… of juice.
8. Buffer solution used for calibration of pH meter has ……pH.
9. Concentration of hydrogen and hydroxyl ions in neutral water is ……../liter.
10. pH meter is ……. which measures potential difference produced by hydrogen ion on electrodes.

6.8 SOME RECOMMENDED BOOKS

6.9 ANSWER KEY TO THE PROGRESS EXERCISE

A. Your answer should include the following points-

1. Weight is the force which is result of interaction of gravitational force on the substance whereas mass is a definite property which can measure the quantity of substance.
2. The types of Balances are Pan balance, Scale balance, Analytical balance, electronic balance, digital balance and microbalances.
3. pH measures the acidity/alkalinity of a solution. pH is negative logarithm of hydrogen ion concentration (Acidity) in the solution.
4. pH meter is a potentiometer design to measure the hydrogen ion concentration (Acidity) in a solution by measuring the difference of electrical potential produced by hydrogen ion between electrodes.
5. Refractometer is an optical instrument used to measure the angle refraction of a polarized light by thin layer of solution between two prisms for determining the soluble sugar in solution.

B. Appropriate words for fill in the blanks.

1. Quantity
2. Standard
3. Knife aged agate
4. Electromagnetic
5. Logarithm
6. Velocity of light
7. Degree Brix
8. Known/Standard
9. 1 X 10^{-7}gram ion /lit.
10. Potentiometer