Laboratory Manual of Biochemistry

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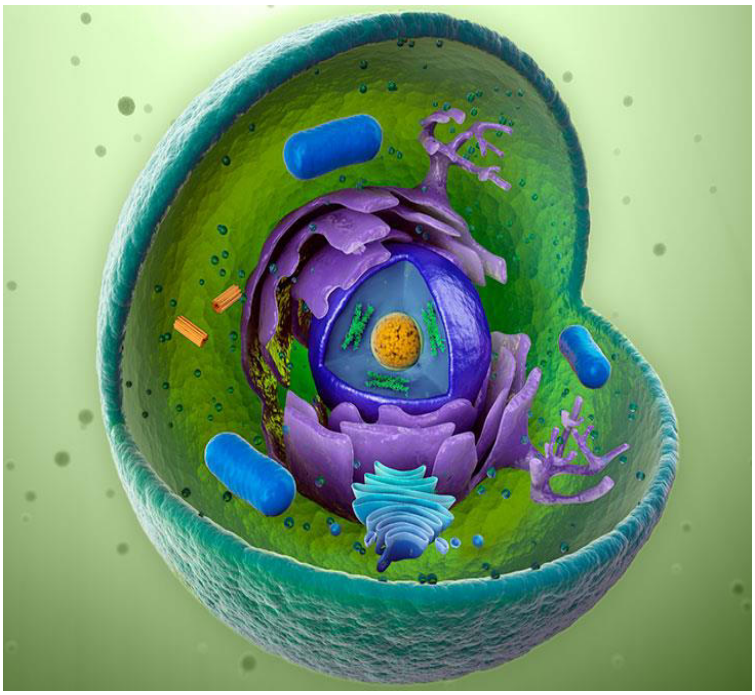

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A. Biochemistry: An Introduction

1. Introduction and Scope of Biochemistry

Biochemistry is the branch of life science which deals with the study of chemical reactions occurring in living cells and organisms. The term 'Biochemistry' was first introduced by the German Chemist Carl Neuberg in 1903. It takes into account the studies related to the nature of the chemical constituents of living matter, their transformations in biological systems and the energy changes associated with these transformations. Biochemistry may thus be treated as a discipline in which biological phenomena are analyzed in terms of chemistry. The branch of Biochemistry for the same reason, it has been variously named as 'Biological Chemistry' or 'Chemical Biology'. Modern biochemistry has two branches, descriptive biochemistry and dynamic biochemistry. Descriptive biochemistry deals with the qualitative and quantitative characterization of the various cell components and the dynamic biochemistry deals with the elucidation of the nature and the mechanism of the reactions involving these cell components. Many newer disciplines have been emerged from biochemistry such as enzymology (study of enzymes), endocrinology (study of hormones), clinical biochemistry (study of diseases), molecular biochemistry (study of biomolecules and their functions) etc. Along with these branches certain other specialties have also come up such as agricultural biochemistry, pharmacological biochemistry etc. Biochemistry is related to almost all the life sciences and without biochemistry background and knowledge, a thorough understanding of health and well-being is not possible. Those who acquire a sound knowledge of biochemistry can tackle the two central concerns of the biomedical sciences (1) the understanding and maintenance of health, and (2) the understanding and treatment of diseases.

1.1. Objectives of Biochemistry

The major objective of biochemistry is the complete understanding of all the chemical processes associated with living cells at the molecular level. To achieve this objective, biochemists have attempted to isolate numerous molecules found in cells, to determine their structures and to analyze how they function. Biochemical studies have illuminated many aspects of disease and the study of certain diseases has opened up new therapeutic approaches. In brief the objectives can be listed as follows:

- Isolation, structural elucidation and the determination of mode of action of biomolecules
- Identification of disease mechanisms
- Study of inborn errors of metabolism
- Study of oncogenes in cancer cells
- The relationship of biochemistry with genetics, physiology, immunology, pharmacology, toxicology etc.

Biochemistry is the study and application of substances, reactions and processes in animals, plants, bacteria and viruses. Biochemistry has vast scope. Biochemists work in hospitals, industry, drug design and development, environmental sciences, forestry, agriculture, dietetics, hormone production, vaccine research, virology, immunology, microbiology, toxicology, food science, plant science and associated areas and in areas from marine biology to entomology not just to carry out R&D work and develop new products but also to monitor the production, quality and safety of the product. Biochemists provide diagnostic service, carrying out tests on blood, urine and other body fluids, while researching the underlying causes of disease and methods of treatment. They find jobs in pharmaceutical and

agrochemical companies, food brewing and biotechnology industry. The postgraduate degree in biochemistry also provides opportunity for advanced teaching in universities, colleges, medical, dental and veterinary schools and consulting or allied work. Observational, organizational, computational skills, planning and team work are important for biochemists.

1.2. Scope and importance of Biochemistry in agriculture

- To evaluate nutritive value of cereals, pulses, poultry and cattle feed
- Development and exploitation of better genotypes
- Removal and inactivation of toxic or anti nutritional factors present in food grains in general and grain legumes in particular by breeding and chemical treatments E.g. BOAA in Lakhodi dal (*Lathyrus sativus*), trypsin inhibitors of soybean, aflatoxins of groundnut
- Food preservation and processing technology and post-harvest physiology of fruit crops and vegetables and their nutritional quality
- Biochemistry of disease and pest resistance
- Biochemistry of drought resistance: Proline and hydroxyproline imparts drought resistance to Jowar
- Formulation of balanced diet
- Use of non-conventional sources of protein foods viz., single cell proteins, fish protein concentrates, mushrooms and leaf proteins
- Developments in the field of intermediary metabolism i.e. synthesis and degradation of constituents of living tissues

B. General Laboratory Principles

2. Laboratory Safety Rules, Requirements and Regulations

Biochemistry laboratory provides controlled conditions in which scientific or technological research, experiments, and measurement may be performed. Laboratories used for scientific research take many forms because of the differing requirements of specialists in the various fields of science and engineering.

Fig.1. A Biochemistry Laboratory

2.1. Safety in the Laboratory

Safety in the biochemistry laboratory involves a cautious attitude and an awareness of potential hazards. Usually potential accidents can be anticipated and prevented. If safety precautions are followed, fewer accidents will occur. The number of laboratory accidents can be reduced if every student follows all of the directions given for the experiment and by the instructor. Special note should be taken of specific instructions that are given in an experiment to eliminate recognized potential hazards. Total awareness of hazards and dangers and what to do in case of an accident is the responsibility of the student and the instructor. Work in the biochemistry laboratory involves the use of inflammable solvents, some corrosive and toxic chemicals, and apparatus which, if used improperly, can cause minor to severe injury. All work with solvents and chemicals must be performed in the fume hoods not on the bench top. Safety glasses and shoes must be worn at all times while in the laboratory.

2.1.1. Solvents

- Never heat inflammable solvents, even small amounts, with or near a flame. As for refluxing or distillation, never place solvents in an open beaker. Pouring solvents in the vicinity of a flame is extremely hazardous. Use an oil bath, steam bath, water bath, heating mantle, or hot plate as a heat source whenever possible.
- Ethyl ether and Petroleum ether (bp 30-60°) are especially dangerous. Never heat them on a hot plate; always use a water or steam bath, and collect the distillate in an ice-cooled flask. In the case of Ethyl ether, the receiver should be a filtering or distilling flask connected to the condenser with a cork and with a piece of rubber tubing leading from side tube on the flask to the floor. This allows the heavy Ether vapours to spread along the floor instead of the desktop where they may be ignited by burners. Carbon disulphide is extremely hazardous. It has been known to ignite from hot steam pipes or electrical sparks, as from the thermostat on a hot plate, or the motor on a stirrer.
- If an inflammable solvent is spilled, have all workers at the desk turn off their burners and clean it up immediately using a cloth. Wring (squeeze)the solvent from the cloth into the solvent waste can and then rinse the cloth in the sink with much water. Use gloves.

- If Acetone is used to aid in drying glassware, use it sparingly and not near a flame.
- Inflammable solvents which you may have contact with are: Ether, Ligroin (Petroleum ether), Cyclohexane, Toluene, Xylene, Alcohols, Ethyl acetate, Carbon disulphide, Acetone, Dioxane etc. If in doubt about the inflammability of a solvent, assume that it is hazardous.
- Benzene and chlorinated solvents are toxic. In some cases, the toxic effect is cumulative. Avoid contact with the skin and inhalation of solvent vapours.
- Many organic solvents freely permeate latex gloves commonly used in laboratories, and are therefore inadequate protection of the skin from solvent vapours. Thicker neoprene or butyl rubber gloves are recommended.

2.1.2. Chemicals

- Especially corrosive substances which give off noxious fumes (e.g., Bromine, Acetyl chloride, Benzyl chloride, Phosphorus trichloride, Acetic anhydride, fuming Nitric and Sulphuric acids, Chlorosulphonic acid, Benzene sulfonyl chloride, etc.) should be handled in the hoods. Use proper gloves. Do not spill these chemicals on yourself or on the desktops. They will cause very painful burns. Do not put any of these in organic waste cans.
- Over the last several years a number of organic compounds have been confirmed as carcinogens and the list is steadily growing. It is best to assume that all chemicals are toxic, and possibly carcinogenic.
- Sodium and Potassium metals react explosively with water. They are rapidly corroded by the atmosphere and should be stored in kerosene or oil. These metals should not be allowed to come into contact with the skin. They may be handled with dry filter paper or tweezers. Unused pieces of metal may be destroyed by dropping into 95% Ethyl alcohol, or they may be returned to the bottle. Avoid all contact between chlorinated solvents and Sodium or Potassium.
- Concentrated acids and alkalis are corrosive to the desktops, clothing and skin. If there is a spill, first dilute with large volume of water. If an acid, neutralize with solid Sodium bicarbonate; and if a base, with 3% Acetic acid. Sulfuric acid is troublesome, since drops adhering to the tops of bottles tend to absorb moisture and run down the outside of the bottle.
- Mercury and its vapour are poisonous. Avoid spilling; the Institute should have special facilities for cleaning up mercury spills.

2.1.3. Apparatus

- Approved safety glasses, goggles, or a face shield must be worn at all times when in the lab. Normal prescription lenses are insufficient due to the possibility of explosion.
- When inserting tubing or thermometers into bored stoppers, it is wise to take some simple precautions. The tubing and stopper should be held by a towel, so that if the tubing breaks the towel will reduce the impact of the jagged edge. If the tubing does not enter the hole in the stopper easily, the hole may be made larger with a file (if a cork) or lubricated with water, alcohol, or Glycerine. Hold the tubing close to the stopper. In removing tubing from stoppers, follow the same technique. *Serious cuts have resulted from carelessness in inserting tubes in stoppers.*
- Closed systems are liable to explode if heated. Never carry out an atmospheric pressure distillation in a closed system.

- Do not support apparatus on books, boxes, pencils, etc. Use large, strong wooden blocks, rings, or lab jacks. Assemblies with a high centre of gravity (as when a reagent is added through the top of a condenser) should be assembled and operated with much care.
- Use glass stirring rods with care for breaking up solids. They are liable to break.
- Do not evacuate Erlenmeyer flasks larger than 50 ml (except filtering flasks). They may collapse.
- Oil baths and melting point baths can cause severe burns if spilled. Make sure they are well supported. Be especially careful not to get water into oil baths. Use electric heating mantles in preference to oil baths when possible.
- Dewar flasks and vacuum desiccators, implode easily when tipped over or dropped. Make sure the ones you use are wound on the outside with friction tape or are contained in protective shields, so they will not shower glass around the laboratory if broken.

2.1.4. Accidents

2.1.4.1. Fire

Personal safety is most important. If a person's clothing catches fire, he/she needs immediate help. Prevent him/her from running. If he/she is close enough, put him/her under the safety shower because it is more effective than a blanket. If not, make him/her lie down and smother (obstruct) the flames by rolling, wrapping with lab coats, blankets, towels, etc. Never turn a Carbon dioxide extinguisher on a person. If a fire breaks out, turn off all burners and remove solvents if time allows. Carbon dioxide extinguishers are must in the laboratory, their positions and operation should be known. Point the extinguisher at the base of the flames. Very small fires can be put out with a damp towel by smothering. The priority should be the safety of all than the matter of extinguishing the fire is considered. A few seconds delay can result in very serious injury, every person in the laboratory should plan in advance what he/she will do in case of such an emergency.

2.1.4.2. Chemicals

If corrosive chemicals are spilled on the clothing, immediate showering (with clothing on) is the best remedy. Safety showers are located by each door. If chemicals are spilled on the skin, wash them off with large volumes of water. Bromine should be washed off with water and the skin then massaged with Ethanol or Glycerine. Do not apply a burn ointment. If the chemical is spilled in the eye, it should immediately be washed out thoroughly with water using the eyewash sprayer in the sinks. If acid was involved, a weak solution of Sodium bicarbonate in an eyecup should then be used. If a base, Boric acid is effective. If corrosive chemicals are spilled on the desk, dilute them with a large volume of water and then neutralize with Sodium bicarbonate if an acid, or dilute Acetic acid if a base.

2.2. First Aid in the Laboratory

Accidents do not often happen in well-equipped laboratories if one of them understands safe laboratory procedures and are careful in following them. When an occasional accident does occur, it is likely to be a minor one. The following information will be helpful to you if an accident occurs.

- In accidental swallowing of acids or alkali, the mouth must be thoroughly rinsed with water. In the case of acid; rinse the mouth with dilute Sodium carbonate and in case of alkali; with dilute

Citric acid. If proper swallowing has occurred, the person should be made to drink water followed by milk in case of acids and lemon juice in case of alkali.

- Skin burns should be washed under running water or ice water and then apply Petroleum jelly or burn ointment and cover with sterile gauge.
- Inhalation injury by toxic fumes is best treated by shifting the person to an open fresh air atmosphere. Irritation to throat can be soothened with hot water vapour inhalation or a warm drink.
- Chemical injury to eyes must be treated by thorough washing with water and then applying 2% Sodium carbonate drops till referred to a specialist.

2.2.1. Hazards in the Lab - Important Terms

- **Severe Toxicity-** adverse effects of a substance that result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours)
- **Irritant-** causes redness, inflammation
- **Corrosive-** "eats away" tissue gradually
- **Carcinogenic-** causes cancer
- **Flammable-** easily set on fire
- **Bio hazard-** substances that pose a threat to the health of living organisms, primarily that of humans.

Fig.2. Hazards in the Lab

2.2.2. Personal Protective Equipment (PPE)

Personal Protective Equipment or PPE (Fig.3) refers collectively to equipment such as safety glasses, goggles, aprons, lab coats, gloves, protective shoes, respiratory protective equipment, ear defenders and similar equipment used to protect the person during their work.

Fig.3. Personal Protective Equipment (PPE)

2.3. Laboratory Requirements

The basic requirements of a biochemistry laboratory include: good computer attached with a printer, temperature control or cold room, dark room, sinks, and instruments such as autoclave, microscope, water distillation units, hot air ovens, titration assembly, chemical fume hoods, laminar flow work stations, incubators, deep freezer, refrigerators, water baths, refrigerated centrifuges, micro centrifuge, pH meter, trans-illuminator equipped with a camera or electronic imaging system, photometric assembly, electrophoresis units, chromatography assembly, PCR , blotting apparatus, microwave oven, balances, and many other miscellaneous things.

The laboratory needs a variety of glassware including reagent bottles, beakers, measuring cylinders, conical flasks, standard flasks, test tubes, burettes, pipettes, watch glass, glass slides, cover slips, glass rods, petridishes, and other culture vessels. Other requirements include syringes, needles, forceps, scalpels, membrane filters (to sterilize heat labile liquids), magnetic stirrers, orbital shakers, inoculation loops, stop watches, nitro cellulose membranes, parafilm, saran wrap, aluminium foil, marker pens, filter paper, ice buckets, latex gloves, plastic boxes, plastic bags, UV goggles, besides plastic bottles and

containers etc. A good supply of chemicals is a very essential aspect of equipping a laboratory as are uninterrupted power and water supply.

Fig.4. Laboratory Instruments

Fig.5. Laboratory Glasswares

2.4. Regulations in the Laboratory

- Safety glasses must be worn at all times by students and instructors. Visitors to the lab must be appropriately warned and safety glasses should be made available to them.
- Fume hoods must be used while working with chemicals that produce hazardous fumes.
- The location of fire extinguishers, safety showers, and eyewash stations must be known.
- There must be no unsupervised or unauthorized work going on in the laboratory.
- A laboratory is never a place for practical jokes or pranks.
- The toxicity of all the chemicals you will be working with must be known. Consult the instructor to aware on material safety data sheets (MSDSs), safety charts, and container labels for safety information about specific chemicals. Recently, many common organic chemicals, such as Benzene, Carbon tetra chloride, and Chloroform, have been deemed unsafe.
- Eating, drinking, or smoking in the laboratory is never allowed. Never use laboratory containers (beakers or flasks) to drink beverages.
- Shoes (not open-toed) must always be worn; hazardous chemicals may be spilled on the floor or feet.
- Long hair should always be tied back.
- Mouth pipetting is never allowed.
- Cuts and burns must be immediately treated. Use ice on new burns and consult a doctor for serious cuts.
- In the event of acid spilling on one's body, flush thoroughly with water immediately. Be aware that acid–water mixtures will produce heat. Removing clothing from the affected area while water flushing may be important, so as to not trap hot acid–water mixtures against the skin.

Acids or acid–water mixtures can cause very serious burns if left in contact with skin, even if only for a very short period of time.

- Weak acids (such as Citric acid) should be used to neutralize base spills, and weak bases (such as Sodium carbonate) should be used to neutralize acid spills. Solutions of these should be readily available in the lab in case of emergency.
- Dispose all waste chemicals from the experiments according to your instructor's directions.
- In the event of an accident, report immediately to your instructor, regardless of how minor you perceive it to be.
- Always be watchful and considerate of others working in the laboratory. It is important not to jeopardize their safety or yours.
- Always use equipment that is in good condition. Any piece of glassware that is cracked or chipped should be discarded and replaced.

It is impossible to foresee all possible hazards that may manifest themselves in a laboratory. Therefore, it is very important for all students to listen closely to their instructor and obey the rules of their particular laboratory in order to avoid injury.

2.5. Keeping Records and Communicating Experimental Results

2.5.1. The Laboratory Notebook

All students need to maintain a laboratory notebook. The notebook should be used for the recording of laboratory data and calculations, and is critically important for writing your lab reports. The purpose of a laboratory notebook is to allow anyone with some biochemical knowledge to understand exactly what you did. You need to record the information in sufficient detail so as to be able to repeat it, and you must be able to understand exactly what your results were. You will need good notes to be able to write your lab reports; in addition, as your understanding of biochemistry improves, your notebook may allow you to figure out why some parts of your experiments did not work as expected.

A lab notebook should contain many things:

- A Table of Contents to aid navigation of the notebook
- A date on each page
- A written introduction / explanation to yourself of the importance of the experiment
- Procedural notes (if following a published procedure, there is no need to copy the procedure. Just reference it, and note changes)
- Values collected (i.e., if a protocol called for using 5.0g of NaCl, how much did you actually use? 4.998 g?)
- All results collected, along with observations (did the tube turn pink, and the protocol didn't mention that? That's an observation!)
- Analysis of the data – legible tables, graphs, and calculations
- Brief conclusions
- Answers to analysis and comprehension questions for the future.

Lab reports are a more formal presentation of your results. Instead, they focus on clearly explaining the significance of the experiment, and give a careful, well-reasoned and clearly worded analysis of the results, leading the reader to the conclusion.

2.5.2. Experimental Write-up

Below is an outline that may be used as a guideline to write a complete report on an experiment.

Pre lab

I. Introduction
(a) Objective or purpose
(b) Theory
II. Experimental
(a) Table of materials and reagents
(b) List of equipment
(c) Flowchart
(d) Record of procedure
III. Data and Calculations
(a) Record of all raw data including printouts
(b) Method of calculation with statistical analysis
(c) Present final data in tables, graphs, or figures when appropriate
IV. Results and Discussion
(a) Conclusions
(b) Compare results with known values
(c) Discuss the significance of the data
(d) Was the original objective
(e) Literature references

Fig.6. Experimental Write-up

2.5.3. Communicating Results from Biochemistry Research

A scientific project is not complete until its discoveries have been communicated to colleagues around the world. The three most important methods or tools for communication are: the scientific paper, the oral presentation, and the poster. Although there are many differences in how to prepare for these three common methods of introducing new biochemical information, they all have one thing in common - the sharing of experimental results and conclusions. The distinct rules and traditions of each of the methods will be described and compared here.

2.5.3.1. The Scientific Paper

A paper published in a biochemical journal is a formal way to report research results to colleagues in the international biochemical community. Before writing such a document, one must first determine the journal to which the article will be submitted. The best advice is to submit the manuscript to the most prestigious journal that has a large audience interested in his or her specialized topic. You write up the results from an experiment in the form of a journal article, so it is important to understand the conventions used in preparing a manuscript for publication. Most biochemical journal articles have the same basic organization: Title, Abstract, Introduction, Experimental Methods, Results, Discussion, and References. The specific requirements for each of these sections vary among the many journals, so it is important to review several articles in different journals to get a flavour of what is expected.

2.5.3.2. The Oral Presentation

The purpose and mechanics of an oral presentation are quite different from preparing and publishing a paper. In an oral presentation, you have a fleeting moment to present data and attempt to convince your audience of the importance of your work. One advantage of the oral presentation, however, is that it provides an opportunity for more direct contact with your audience than does a paper; thus the opportunity exists for immediate questions and feedback. Presentations usually range from 15 to 60 minutes. Shorter presentations cover a much smaller unit of a research project, whereas 60-minutes talks (often called seminars) can give a broader exposure to the research area. Scientific presentations involve mixed media - oral and visual. The important verbal points are reinforced with the use of a visual aid such as a figure, graph, or other element. Scientific presenters today most often use power point, computer software that projects electronic slides onto a screen, although overhead transparencies are also acceptable and efficient. In visual aid, the slides must be carefully constructed with special concern for the total number of slides and the amount of information on each. The organization of a talk is similar to that of a paper-introduction, experimental methods, results, discussion, conclusions, and questions/comments.

2.5.3.3. The scientific poster

Scientific poster is a communication method that may be considered a hybrid, as it combines elements of the oral presentation (verbal expression and visual aids) with elements of a paper (printed text and figures). The poster has become the primary medium by which new scientific information is exchanged at all professional conferences, including local, regional, national, and international meetings. The poster, however, may be available to readers for long periods of time in the absence of the presenter.

2.6. Using Biochemical Reagents and Solutions

2.6.1. Water Purity

Water is the most common and widely-used substance in the biochemistry laboratory. Both the quality and quantity of water required must be considered for each lab application.

Applications of water usage include:

(1) Solvent for preparing most buffer and reagent solutions;
(2) Column chromatography;
(3) High-performance liquid chromatography;
(4) Tissue culture; and
(5) Washing glassware.

Ordinary tap water contains a variety of impurities including particulate matter, dissolved organics, in organics, and gases; and microorganisms and the natural degradation of microorganisms leads to the presence of byproducts called pyrogens. For most laboratory procedures, it is recommended that some form of purified water be used. The purity of water is usually measured in terms of resistivity. Unit for resistivity is Megohms- cm. There are five basic water purification technologies distillation, ion exchange, activated carbon adsorption, reverse osmosis, and membrane filtration. For most procedures

carried out in the biochemistry lab, water purified by ion-exchange, reverse osmosis, or distillation is usually acceptable. Of these three processes, distillation is the slowest, least energy efficient, least pure and most high maintenance especially in areas with hard water (needs regular de-scaling). Distilled water must also be stored to prevent contamination by microbes. For special procedures such as buffer standardization, liquid chromatography, and tissue culture, ultra-pure water, which is usually bottled and available commercially, should be used. Water that is purified only by ion-exchange will be low in metal-ion concentration, but may contain certain organics that are washed from the ion-exchange resin. These contaminants will increase the UV-absorbance properties of water. If sensitive UV-spectroscopic measurements are to be made, distilled water is better than de-ionized. If large volumes of high-purity water are required reverse osmosis should be the choice.

2.6.2. Cleaning Laboratory Glassware

The results of your experimental work will depend, to a great extent, on the cleanliness of your equipment, especially glassware used for preparing and transferring solutions. There are at least two important reasons for this: (1) many of the chemicals and biochemicals will be used in milligram, microgram, or even nanogram amounts. Any contamination, whether on the inner walls of a beaker, in a pipette, or in a glass cuvette, could be a significant percentage of the total experimental sample; (2) many biochemicals and biochemical processes are sensitive to one or more of the following common contaminants: metal ions, detergents, and organic residues. The preferred method for cleaning glassware is to begin with hot tap water. Rinse the glassware at least 10 times with this; then rinse 4–6 times with distilled or de-ionized water. Occasionally it is necessary to use a detergent for cleaning. Use a dilute detergent solution (0.5% in water) followed by 5–10 water rinses with distilled or de-ionized water. When you needed dry glassware in the organic laboratory, you probably rinsed the glassware with Acetone, which rapidly evaporated, leaving a dry surface. But, this technique coats the surface with an organic residue consisting of nonvolatile contaminants found in the Acetone. Because this residue could interfere with your experiments, it is best to refrain from acetone washing. Never clean cuvettes or any optically polished glassware with ethanolic KOH or other strong base, as this will cause etching. All glass cuvettes should be cleaned carefully with hot tap water or 0.5% detergent solution, in a sonicator bath or in a cuvette washer, followed by thorough rinsing with purified water.

2.6.3. Solutions: Concentrations

The concentrations for solutions used in the biochemistry laboratory may be expressed in several different units. The most common units are:

- ***Molarity (M):*** concentration based on the number of moles of solute per litre of solution.
- ***Percent by weight (% wt/wt):*** concentration based on the number of grams of solute per 100 g of solution.
- ***Percent by volume (% wt/vol):*** concentration based on the number of grams of solute per 100 ml of solution.
- ***Weight per volume (wt/vol):*** concentration based on the number of grams, milligrams, or micrograms of solute per unit volume.

2.6.4. Preparing and Storing Solutions

In general, solid solutes should be weighed on weighing paper or plastic weighing boats, with the use of an electronic analytical or top-loading balance. Liquids are more conveniently dispensed by volumetric techniques; however, this assumes that the density is known. If a small amount of a liquid is to be weighed, it should be added to a tared flask by means of a disposable Pasteur pipette with a latex bulb. The storage conditions of reagents and solutions in the biochemistry lab are especially critical. It is good practice to store all solutions in a closed container. Often it is necessary to store some solutions in a refrigerator at 4^{o}C. This inhibits bacterial growth and slows decomposition of the reagents. Some solutions may require storage below 0^{o}C. Stored solutions must always have a label containing the name and concentration of the solution, the date prepared, and the name of the preparer. All stored containers, whether at room temperature, or below freezing, must be properly sealed. This reduces contamination by bacteria and vapours in the laboratory air (Carbon dioxide, Ammonia, HCl, etc.). Volumetric flasks, of course, have glass stoppers, but test tubes, Erlenmeyer flasks, bottles, and other containers should be sealed with screw caps, corks, or hydrocarbon foil (Parafilm). Remember that hydrocarbon foil, a wax, is dissolved by solutions containing non-polar organic solvents like Chloroform, Diethyl ether, and Acetone. Bottles of pure chemicals and reagents should also be properly stored.

2.7. Quantitative Transfer of Liquids

2.7.1. Pipettes Fillers

The use of any pipette requires some means of drawing reagent into the pipette. Liquids should never be drawn into a pipette by mouth suction on the end of the pipette! Small latex bulbs are available for use with disposable pipettes. For volumetric and graduated pipettes, two types of bulbs are available. The features are special conical fitting that accommodates common sizes of pipettes.

Fig.7. Pipette Fillers

To use these, first place the pipette tip below the surface of the liquid. Squeeze the bulb with the left hand and then hold it tightly to the end of the pipette. Slowly release the pressure on the bulb to allow liquid to rise to 2 or 3 cm above the top graduated mark. Then, remove the bulb and quickly grasp the pipette with your index finger over the top end of the pipette. The level of solution in the pipette will fall slightly, but should not fall below the top graduated mark. If it does fall too low, use the bulb to refill.

2.7.1.1. Safety Pipette Fillers

Mechanical pipette fillers (made of silicone and sometimes called safety pipette fillers, propipettes, or pi-fillers) are more convenient than latex bulbs. These fillers are equipped with a system of hand-operated valves and can be used for the complete transfer of a liquid.

The three valves (A valve, S valve and E valve) release air, draw liquid in to the pipette, and accurately release liquid.

Never allow any solvent or solution to enter the pipette bulb! To avoid this, two things must be kept in mind:

1. Always maintain careful control while using valve S to fill the pipette.
2. Never use valve S unless the pipette tip is below the surface of the liquid. If the tip moves above the surface of the liquid, air will be sucked into the pipette and solution will be flushed into the bulb.

Fig.8. Safety Pipette Fillers

2.7.2. Pipettes

2.7.2.1. Disposable Pasteur Pipettes

Often it is necessary to perform a semi-quantitative transfer of a small volume (1–10 ml) of liquid from one vessel to another. Because pouring is not efficient, a Pasteur pipette with a small latex bulb may be used. Pasteur pipettes are available in two lengths (15 and 23 cm) and hold about 2 ml of solution. Used disposable pipettes should be discarded in special containers for broken glass.

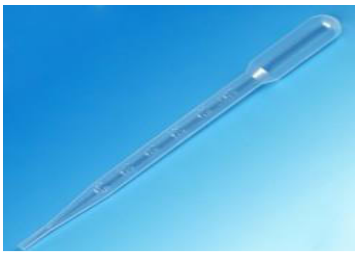


Fig.9. Disposable Pasteur Pipettes

2.7.2.2. Calibrated Pipettes

When a quantitative transfer of a specific and accurate volume of liquid is required, some form of calibrated pipette must be used.

2.7.2.2.1. Volumetric Pipettes

Used for the delivery of liquids required in whole-millilitre amounts (1, 2, 5, 10, 15, 20, 25, 50, and 100 ml). To use these pipettes, draw liquid with a latex bulb or mechanical pipette filler to a level 2–3 cm above the fill line. Release liquid from the pipette until the bottom of the meniscus is directly on the fill line. Touch the tip of the pipette to the inside of the glass wall of the container from which it was filled.

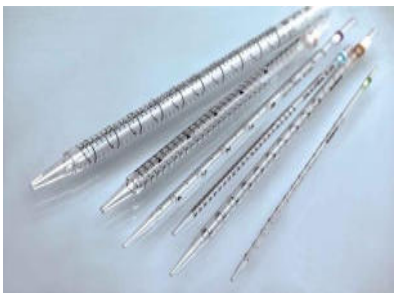


Fig.10. Volumetric Pipettes

Transfer the pipette to the inside of the second container and release the liquid. Hold the pipette vertically; allow the solution to drain until the flow stops, and then wait an additional 5–10 seconds. Touch the tip of the pipette to the inside of the container to release the last drop from the outside of

the tip. Remove the pipette from the container. Some liquid may still remain in the tip. Most volumetric pipettes are calibrated as "TD" (To Deliver), which means the intended volume is transferred without final blow-out; that is; the pipette delivers the correct volume.

Fractional volumes of liquid are transferred with graduated pipettes, which are available in two types:

2.7.2.2.2. Mohr Pipettes

Mohr pipettes are available in long- or short-tip styles. All Mohr pipettes are TD, and they are available in many sizes (0.1 to 10 ml). The use of a Mohr pipette is similar to that of a volumetric pipette.

Fig.11. Mohr Pipettes

2.7.2.2.3. Serological Pipettes

Serological pipettes are similar to Mohr pipettes, except that they are graduated downward to the very tip and are designed for blowout. Their use is identical to that of a Mohr pipette except that the last bit of solution remaining in the tip must be forced out into the receiving container with a rubber bulb. This final blow-out should be done after 15–20 seconds of draining.

Fig.12. Serological Pipettes

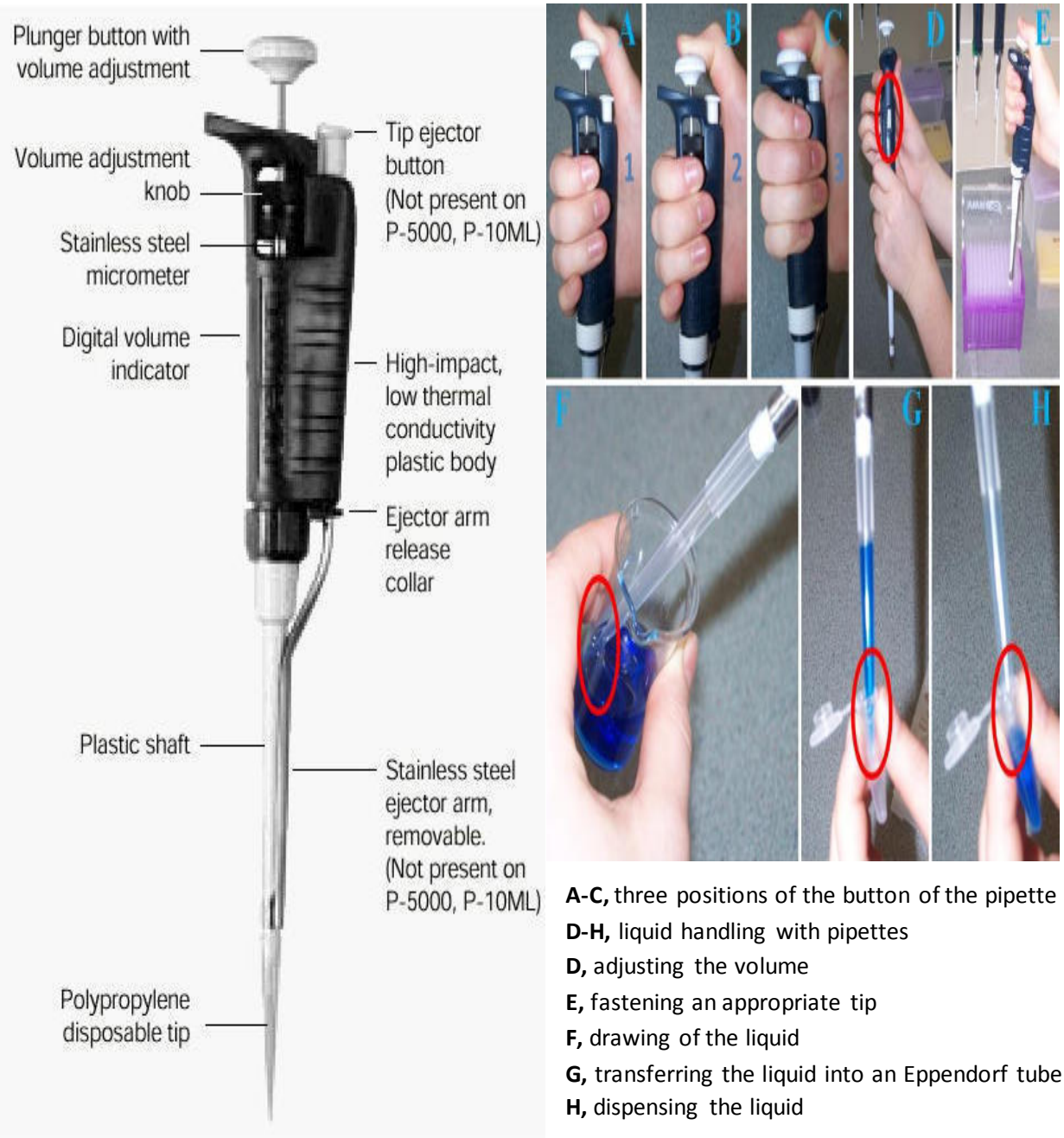
2.7.3. Cleaning and Drying Pipettes

Special procedures are required for cleaning glass pipettes. Immediately after use, every pipette should be placed, tip up, in a vertical cylinder containing warm tap water or a dilute detergent solution (less than 0.5%). The pipette must be completely covered with solution. This ensures that any reagent remaining in the pipette is forced out through the tip. If reagent solutions are allowed to dry inside a pipette, the tips can easily become clogged and are very difficult to open. After several pipettes have accumulated in the water or detergent solution, the pipettes should be transferred to a pipette rinser. Pipette rinsers continually cycle fresh water through the pipettes. Immediately after detergent wash, tap water may be used to rinse the pipettes, but distilled water should be used for the final rinse. Pipettes may then be dried in an oven.

2.7.4. Automatic Pipetting Devices / Micropipettes

For most quantitative transfers, including many repeated small-volume transfers, a mechanical microlitre pipettor (i.e., Eppendorf type, Pipetteman) is ideal. This allows accurate, precise, and rapid dispensing of fixed volumes from 1 to 10,000 µl (0.001 to 10 ml). The pipette's push-button system can be operated with one hand, and it is fitted with detachable polypropylene tips. The advantage of polypropylene tips is that the amount of reagent film remaining in the pipette after delivery is much less than for glass tips.

How to use an adjustable pipetting device?



A-C, three positions of the button of the pipette
D-H, liquid handling with pipettes
D, adjusting the volume
E, fastening an appropriate tip
F, drawing of the liquid
G, transferring the liquid into an Eppendorf tube
H, dispensing the liquid

Fig.13. Automatic Pipetting Device / Micropipette

Set the desired volume with the digital micrometer or plunger button. Attach a new disposable tip to the shaft of the pipette. Press tip on firmly with a slight twisting motion. Depress the plunger to the first positive stop, immerse the disposable tip into the sample liquid to a depth of 2–4 mm, and allow the pushbutton to return slowly to the up position and wait 1–2 seconds. To dispense sample, place the tip end against the side wall of the receiving vessel and depress the plunge slowly to the first stop. Wait 2–3 seconds, and then depress the plunger to the second stop to achieve final blow-out. Withdraw the device from the vessel carefully with the tip sliding along the inside wall of the vessel. Allow the plunger to return to the up position. Discard the tip by depressing the tip ejector button.

2.8. Statistical Analysis of Experimental Data

2.8.1. Statistical Analysis

An error in an experimental measurement is defined as a deviation of an observed value from the true value. There are two types of errors, determinate and indeterminate. Determinate errors are those that can be controlled by the experimenter and are associated with malfunctioning equipment, improperly designed experiments, and variations in experimental conditions. These are sometimes called human errors because they can be corrected or at least partially alleviated by careful design and performance of the experiment. Indeterminate errors are those that are random and cannot be controlled by the experimenter.

Two statistical terms involving error analysis that are often used and misused are *accuracy* and *precision.* Precision refers to the extent of agreement among repeated measurements of an experimental value. Accuracy is defined as the difference between the experimental value and the true value for the quantity. Because the true value is seldom known, accuracy is better defined as the difference between the experimental value and the accepted true value. Several experimental measurements may be precise (that is, in close agreement with each other) without being accurate. If an infinite number of identical, quantitative measurements could be made on a biosystem, this series of numerical values would constitute a *statistical population.* The average of all of these numbers would be the *true value* of the measurement. The alternative is to obtain a relatively small *sample of data,* which is a subset of the infinite population data. The significance and precision of these data are then determined by statistical analysis.

2.8.2. The Mean, Sample Deviation, and Standard Deviation

The arithmetic average, or mean, of the numbers is calculated by totaling all the experimental values observed for a sample (the counting rates, the velocity of the reaction, or protein concentration) and dividing the total by the number of times the measurement was made. *Sample deviation* is defined as the difference between the value for an observation and the mean value. A more useful statistical term for error analysis is *standard deviation,* a measure of the spread of the observed values.

2.8.3. Spreadsheet Statistics

A spreadsheet is an interactive computer application program for organization, analysis and storage of data in tabular form. It is common practice today to use computer spreadsheet programs for statistical analysis of biochemical data. The graphing abilities of spreadsheets make it easy to inspect data for errors and outliers, look for nonlinear relationships and non-normal distributions, and display the final results. Even to use something like SPSS, SAAS and SyStat, there will be many times when it's easier to enter the data in to a spreadsheet first, inspect it for errors, sort and arrange it, then export it in to a format suitable for statistics package.

C. Biochemistry Lab Techniques

3. Basic Instrumentation Practices

3.1. Mortar and Pestle

General Description

A mortar and pestle can be used to prepare ingredients or substances by crushing and grinding them in to a fine paste or powder. The mortar is a bowl, typically made of hard wood, ceramic or stone. The pestle is a heavy and blunt club shaped object, the end of which is used for crushing and grinding. The substance to be ground is placed in the mortar and ground, crushed or mixed using the pestle.

Fig.14. Mortar and Pestle

3.2. Desiccator

General Description

Desiccators are sealable enclosures containing desiccants used for preserving moisture sensitive items. A common use for desiccators is to protect chemicals which are hygroscopic or which react with water from humidity. Desiccators are sometimes used to remove traces of water from an almost dry sample. To prevent adsorption of moisture from the surrounding air, glassware is cooled in desiccators.

Fig.15. Vacuum desiccators (left) & Desiccators (right)

Where, desiccators alone are unsatisfactory, the sample may be dried at elevated temperature using Abderhalden's drying pistol.

Parts of a Desiccator

In laboratory use, the most common desiccators are circular and made of heavy glass. There is usually a removable platform on which the items to be stored are placed. The desiccant, usually an otherwise inert solid such as Silica gel, freshly calcined quicklime or Anhydrous Calcium chloride to absorb water, fills the space under the platform. The ground glass rim of the desiccator lid must be greased with a thin layer of Petroleum jelly or other lubricant to ensure an airtight seal. A stopcock may be included to permit the desiccators to be evacuated. Such models are usually known as vacuum desiccators. When a vacuum is to be applied, it is a common practice to criss cross the vacuum desiccators with tape, or to place it behind a screen to minimize damage or injury caused by an implosion. To maintain a good seal, vacuum grease is usually applied to the flanges.

3.3. Bunsen burner

General Description

A Bunsen burner, named after Robert Bunsen, is a common piece of laboratory equipment that produces a single open glass flame, which is used for heating, sterilization and combustion. The gas can be natural gas which is mainly Methane or a liquefied petroleum gas, such as Propane, Butane or a mixture of both.

Fig.16. Bunsen burner

Parts of a Bunsen burner

A Bunsen burner is made entirely of metal. In order to function properly, Bunsen burners must have:

(A) **Barrel**, that's approximately five inches long

(B) **Collar** with (C) **air holes**

(D)**Gas intake**

(E)**Gas valve**

(F)**Stand**, to keep all of the pieces from making contact with a work surface.

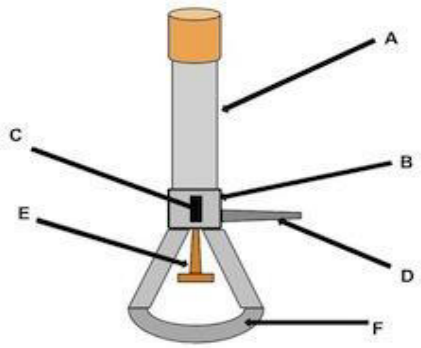


Fig.16.1. Parts of a Bunsen burner

Principle

The amount of air mixed with the gas stream affects the completeness of the combustion reaction. Less air yields an incomplete and thus cooler reaction, while a gas stream well mixed with air provides oxygen in an equimolar amount and thus a complete and hotter reaction. The air flow can be controlled by opening or closing the slot openings at the base of the barrel.

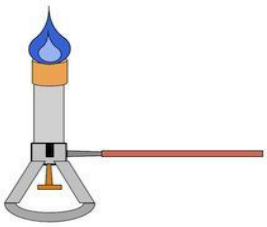


Fig.16.2. Burner Flame

This Fig.3.3.2 shows the burner producing two sets of blue flames. The inner flame is a smaller and a brighter blue, while the other flame is larger and a darker blue. The flame is hottest between the tip of the smaller flame and the tip of the larger flame.

Working

Once connected to a source of fuel, usually Methane, the Bunsen burner can be ignited with a spark. Incoming gas reacts with oxygen in a one-to-three ratio to produce a blue flame that comes out of the top of the barrel.

Adjusting the gas valve and on the Bunsen burner then changes the volume of gas flow; the more gas entering the burner, the larger the flame. The collar at the base of the barrel contains air holes to control the amount of oxygen reacting with the gas. The collar can be rotated to adjust oxygen intake. In general, the more oxygen present, the more intense and blue the flame will be. Less oxygen lends to a weaker yellow flame. The more oxygenated blue flame is both hotter and more controlled than the oxygen-deprived flame; scientists generally prefer a controlled blue flame.

1 **2** **3** **4**

1. Air hole closed (safety flame used for lighting or default)
2. Air Hole slightly open
3. Air hole half open
4. Air hole fully opens (Roaring blue flame)

Fig.16.3. Bunsen burner flames depend on air flow in the throat holes

3.4. Micro Centrifuge

Fig.17. Micro Centrifuge

General Description

A micro centrifuge, also called a microfuge, is an important piece of lab equipment; it is used to spin small (2 ml or less) liquid samples at high speeds (generally tens of thousands times g-force).

Working

- Choose a flat area on your table
- Put the line chord into a suitable 220 V 50 Hz power plug
- Timer can be set in pulse mode. Timed cycles from 1-15 minutes in increment of 1 minute and continuous mode
- The RESET- OFF key is to be pressed whenever time or rpm setting is to be changed.
- An audible beep sounds at the beginning and on completion of the cycle

Points to Remember

- After centrifugation wipe the inner chamber and keep open to be dried.
- Clean the rotor after use. There are chances for any spill of liquids used.
- Notice if any voltage fluctuation occurs.
- It shows drive fault if any error occurs. In that case switch off and then enter the program.
- Always make sure the required temperature is attained before the rotor starts.

3.5. Vortex mixer

General Description

Fig.18. Vortex mixer

A vortex mixer, or vortexer, is a simple device used commonly in laboratories to mix small vials of liquid. The vortex mixer was invented by the Kraft brothers (Jack A. Kraft and Harold D. Kraft). In a biochemical or analytical laboratory they may be used to mix the reagents of an assay or to mix an experimental sample and a dilutant.

Parts of a Vortex mixer

It consists of an electric motor with the drive shaft oriented vertically and attached to a cupped rubber piece mounted slightly off-center. As the motor runs the rubber piece oscillates rapidly in a circular motion.

Working

When a test tube or other appropriate container is pressed into the rubber cup (or touched to its edge) the motion is transmitted to the liquid inside and a vortex is created. Most vortex mixers have variable speed settings and can be set to run continuously, or to run only when downward pressure is applied to the rubber piece.

3.6. Rotary Shaker

Fig.19. Rotary Shaker

General Description

Rotary Shakers are ideal for mixing and development of cultures, chemicals, solvents, assays, etc. and for production of basic chemicals, pathological work, and various other applications. The shaker is also widely used for shaking solutions in Erlenmeyer flasks.

Rotary Shaker extracting, dissolving slow-reacting samples; cultivation of cells; extraction of mineral oil from soil, tissue culture for analytical diagnostics; de-aeration of tested biodegradable materials and samples; rotating closed containers for dialysis in a circular fashion.

Components of a Rotary Shaker

A typical shaker has a table board that oscillates horizontally, powered by an electric motor. The liquids to be stirred are held in beakers, jars, or Erlenmeyer flasks that are placed over the table; or, sometimes, in test tubes or vials that are nested into holes in the plate. Orbital shakers also exist, that shake the vessel in a circular fashion.

3.7. Homogenizer

Fig.20. Homogenizer

General Description

A homogenizer is a piece of laboratory or industrial equipment used for the homogenization of various types of material, such as tissue, plant, food, soil, and many others. Homogenization is a very common sample preparation step prior to the analysis of nucleic acids, proteins, cells, metabolism, pathogens, and many other targets.

Principle

High speed mechanical and hydraulic shear forces are real key to the success of this instrument. Rotor and stator generates a shearing action which insures that materials being processed are subjected to thousands of shearing actions each minute.

3.8. Fume hood

Fig.21. Fume hood

General Description

A fume hood or fume cupboard is a type of local ventilation device that is designed to limit exposure to hazardous or toxic fumes, vapours or dusts. A fume hood is typically a large piece of equipment enclosing five sides of a work area, the bottom of which is most commonly located at a standing work height. Two main types exist, ducted and recirculating (ductless).

Principle

The speed of the air moving through the hood opening is known as face velocity. Air is drawn in from the front (open) side of the cabinet, and either expelled outside the building or made safe through filtration and fed back into the room.

Instrumentation

A fume hood structure is basically a cabinet, with an open side (or sides) for access to the interior of the hood. A transparent, movable sash, allows the user to restrict or enlarge the fume hood opening. The hood is connected, via ductwork, to an exhaust fan, usually located on the roof of the building in which the hood is located. The exhaust fan draws air from the room in which the hood is in through the hood opening and out through the ductwork.

3.9. Laminar Air Flow Cabinet

General Description

A laminar flow cabinet or laminar flow closet or tissue culture hood is a carefully enclosed bench designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive materials.

Fig.22. Laminar Air Flow Cabinet

Principle

The principle behind laminar air flow is the passage of continuous air flow at uniform velocity. HEPA filters designed to create a particle free working environment and provide product protection. Air taken through the filtration system is then exhausted across the work surface. Commonly, the filtration system comprises of a prefilter and a HEPA filter. The laminar air flow cabinet is enclosed on the sides and constant positive air pressure is maintained to prevent the intrusion of contaminated room air.

Instrumentation

A laminar flow hood consists of a filter pad, a fan and a HEPA (High Efficiency Particulate Air) filter. The fan sucks the air through the filter pad where dust is trapped. After that the prefiltered air has to pass the HEPA filter where contaminating fungi, bacteria, dust etc. are removed. Now the sterile air flows into the working (flasking) area where you can do all your flasking work without risk of contamination. AUVC germicidal lamp to sterilize the interior and contents when not in use (It is important to switch this light off during use, to limit exposure to skin and eyes as stray ultraviolet light emissions can cause cancer and cataracts).

3.10. Electronic Weighing Balance

General Description

Balances are designed to meet the specific weighing requirement in the laboratory working environment. These balances come in precision designs and operating characteristics that allows making quick and accurate measurements.

Fig.23. Electronic Weighing Balance

Principle

Electronic weighing balance accurately measures the weight of chemicals. Calibrate the balance by internal calibration. Place the weighing boat and tare the weight. Wait till it becomes zero. Chemical

should be weighed slowly according to the need. Wait till the symbol “g” stabilizes next to the weight shown.

Note

Always have the knowledge about the maximum and minimum quantity which can be weighed using the balance. Never spill the chemicals on the weighing pan, if it happens wipe off with tissue. Switch off fan and close windows nearby when working with it as it may cause fluctuations in the value due to interaction with air density. Always use weigh boats or butter paper to weigh.

3.11. Magnetic Stirrer

General Description

Magnetic stirrer employs a rotating magnetic field to cause a stir bar (also called "flea") immersed in a liquid to spin very quickly, thus stirring it. The rotating field may be created either by a rotating magnet or a set of stationary electromagnets, placed beneath the vessel with the liquid.

Fig.24. Magnetic Stirrer

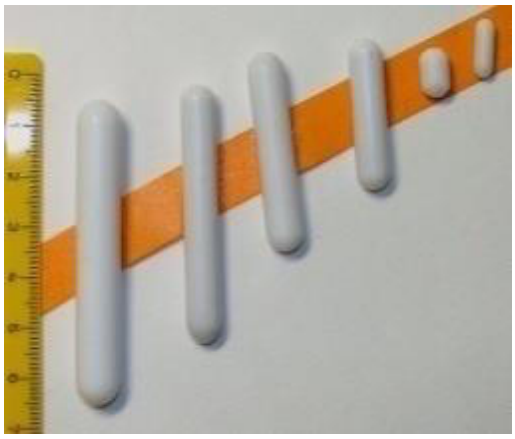


Fig.24.1. Stir bars

A stir bar is the magnetic bar placed within the liquid which provides the stirring action. The stir bar's motion is driven by another rotating magnet or assembly of electromagnets in the stirrer device, beneath the vessel containing the liquid. Stir bars are typically coated in teflon, or less often in glass.

Components of a Magnetic Stirrer

A stirrer magnet placed in the liquid and a magnetic drive located outside the vessel. Both, stirrer magnet and magnetic drive form a magnetic circuit. For trouble-free stirring in liquids with different viscosities the magnetic drive shall have a wide range of different speeds.

Principle

In principle, it is difficult to find the most effective magnetic stirring bar for a particular application, but important factors are the vessel shape and the viscosity of the stirring medium. The ideal configuration is where the magnet of the stirring bar and the magnet of the drive are of equal length and with a minimum distance between them.

3.12. Water Bath

General Description

Fig.25. Water Bath

A water bath is made from a container filled with heated water. It is used to incubate samples in water at a constant temperature over a long period of time. All water baths have a digital or an analogue interface to allow users to set a desired temperature.

Utilizations include warming of reagents, melting of substrates or incubation of cell cultures. It is also used to enable certain chemical reactions to occur at high temperature. Water bath is a preferred heat source for heating flammable chemicals instead of an open flame to prevent ignition. Different types of water baths are used depending on application. For all water baths, it can be used up to 99.9°C.

Principle

It is a system for the control of temperature in which a vessel containing the material to be heated is set into or over one containing water and receiving the heat directly.

Working

It has a double walled, outer body made of MS sheet, powder coated, inner body made of stainless steel, and 304 quality sheet glass wool insulation, fitted with 30^{o}C to 110^{o}C thermostat. By means of these, heat is transferred to the medium (water or oil) until reaching the temperature selected with a control device (thermostat or similar). It is provided with concentric rings with a diameter of 75 mm suitable to work on 220V AC supply. It is accompanied with a digital display temperature controller. In general they use water, but some baths use oil.

Before using the water bath, verify that it is clean and that accessories needed are installed. The steps normally followed are:

- Fill the water bath with fluid to keep the temperature constant (water or oil). Verify that once the containers to be heated are placed, the fluid level is between 4 and 5 cm from the top of the tank.
- Install the control instruments needed, such as thermometers and circulators. Use additional mounts provided for this purpose. Verify the position of the thermometer's bulb or thermal probe to ensure that the readings are correct.
- If water is used as the warming fluid, verify that it is clean. Some manufacturers recommend adding products which prevent the formation of fungus or algae.
- Put the main switch No.1 in the ON position (the numbers identifying the controls herein correspond to those shown in the diagram). Some manufacturers have incorporated controls with microprocessors which initiate auto-verification routines once the ON switch is activated.
- Select the operation temperature using the menu No.2 buttons and the buttons for adjusting the parameters.
- Select the cut-off temperature (in water baths with this control). This is a safety control which cuts off the supply of electricity if it exceeds the selected temperature. This is selected also by using the menu button and is controlled by the parameter adjustment buttons.

Avoid using the water bath with the substances indicated below:

- Bleach
- Liquids with high Chlorine content
- Weak saline solutions such as Sodium chloride, Calcium chloride or chromium compounds
- Strong concentrations of any acid
- Strong concentrations of any salt
- Weak concentrations of Hydrochloric, Hydrobromic, Hydroiodic, Sulphuric or Chromic acids
- Deionised water, as it causes corrosion and perforation in the stainless steel

Safety

- Avoid the use of the water bath in environments where there are flammable and combustible materials. The equipment has components (resistors generating very high temperatures) which could start an accidental fire or explosion.
- Always connect the equipment to an electrical outlet with a ground pole to protect the user and the equipment from electrical discharges.
- Use the water bath exclusively with non-corrosive or non-flammable liquids.
- When working with substances that generate vapours, place the water bath under a chemical hood or in a well-ventilated area.
- Remember that liquids incubated in the water bath tank can produce burns if hands are inadvertently placed inside it.
- Take into account that the water bath is designed for use with a liquid inside the tank. If the inside is dry, the temperature of the tank can become very high. Use the diffusing tray for placing the container inside of the filled tank of the water bath. This has been designed for distributing the temperature in a uniform way.
- Avoid using the water bath if any of its controls is not working, e.g. the temperature or limit controls.

Cleaning

Frequency: Monthly

- Turn off and disconnect the equipment. Wait until it cools to avoid the risk of burns and accidents.
- Remove the fluid used for heating. If it is water, it can be poured through a siphon. If it is oil; collect into a container with an adequate capacity.
- Remove the thermal diffusion grid located at the bottom of the tank.
- Disassemble the circulator and clean to remove scale and potential algae present.
- Clean the interior of the tank with a mild detergent. If there is any indication of corrosion, use substances for cleaning stainless steel. Rub lightly with synthetic sponges or equivalent. Avoid using steel wool to remove rust stains as these leave particles of steel which could accelerate corrosion.
- Avoid bending or striking the temperature control capillary tube generally located at the bottom of the tank.
- Clean the exterior and interior of the water bath with clean water.

3.13. pH Meter

General Description

pH meter used for potentiometrically measuring the pH, which is either the concentration or the activity of hydrogen ions, of an aqueous solution. It usually has a glass electrode plus a calomel reference electrode, or a combination electrode. pH meters are usually used to measure the pH of liquids, though special probes are sometimes used to measure the pH of semi-solid substances.

Fig.26.pH Meter

Components of a pH meter

Basic potentiometric pH meters simply measure the voltage between two electrodes and display the result converted into the corresponding pH value. They comprise a simple electronic amplifier and a pair of probes, or a combination probe, and some form of display calibrated in pH. The probe is the key part; it is a rod-like structure usually made of glass, with a bulb containing the sensor at the bottom. Frequent calibration with solutions of known pH, perhaps before each use, ensures the best accuracy. To measure the pH of a solution, the probe is dipped into it.

Principle

pH measurement is based on the use of a pH sensitive electrode (usually glass), a reference electrode, and a temperature element to provide a temperature signal to the pH analyzer. The pH electrode uses a specially formulated, pH sensitive glass in contact with the solution, which develops a potential (voltage) proportional to the pH of the solution. The reference electrode is designed to maintain a constant potential at any given temperature, and serves to complete the pH measuring circuit within the solution. It provides a known reference potential for the pH electrode. The difference in the potentials of the pH and reference electrodes provides a millivolt signal proportional to pH.

Working

- Turn on pH Meter. Lift up the electrode and clean the electrode tip by pressing with tissue paper.
- Calibrate using buffer 4±0.01 and buffer 7±0.01
- The buffers should come to the room temperature before calibration.
- Place the electrode in the solution to know the pH.
- If there is an increase in pH, stabilize it with adding 0.1N HCl which lowers the pH. If there is a decrease in pH, stabilize it with adding 0.1N NaOH which increases the pH.
- Add acid or alkali drop by drop and always stir well the solution with glass rod after each addition.

Note

- Read the pH when √A appears.
- Always keep the electrode dipped in 3 mol/l KCl solution, never leave the electrode dry.
- Be cautious about the electrode level displayed on the screen. It symbolizes the fitness of electrode. Any kind of unusual appearance can cause pH fluctuation.
- Buffer solutions should always be clear without any turbidity or mycelia growth. Same is the case if the buffer is used to protect electrode.

- Always prepare fresh buffer solutions every month for calibration.
- Always prepare buffer solutions in sterile water.
- Never mishandle the electrode like using it for stirring the solutions.
- Always use sterile water to clean the electrode after dipping it in solutions.
- Never place the electrode in solutions which are in extreme temperature conditions.
- No hurry should be there for reading the pH.
- Do not forget to make entry with appropriate comments in the log book.
- The equipment and its premises should be clean if there is any spill.

3.14. Microscope

General Description

A microscope is an instrument used to see objects that are too small for the naked eye. The science of investigating small objects using such an instrument is called microscopy.

Fig.27. Microscope

The most commonly used microscope for general purposes is the standard compound microscope. It magnifies the size of the object by a complex system of lens arrangement.

Principle

It has a series of two lenses; (i) the objective lens close to the object to be observed and (ii) the ocular lens or eyepiece, through which the image is viewed by eye. Light from a light source (mirror or electric lamp) passes through a thin transparent object. The objective lens produces a magnified 'real image' (first image) of the object. This image is again magnified by the ocular lens (eyepiece) to obtain a magnified 'virtual image' (final image), which can be seen by eye through the eyepiece. As light passes directly from the source to the eye through the two lenses, the field of vision is brightly illuminated. That is why; it is a bright-field microscope.

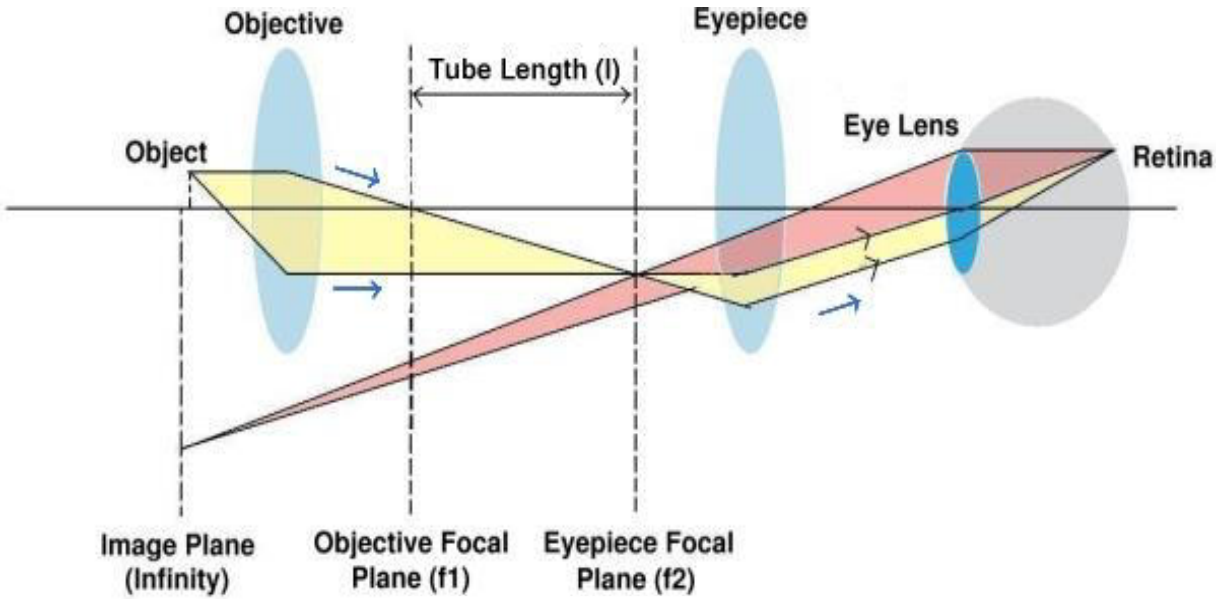


Fig.27.1. Working Principle of a Microscope

Parts of a Microscope

(I)Mechanical Parts: These are the parts, which support the optical parts and help in their adjustment for focusing the object.

- **Base or Metal Stand:** The whole microscope rests on this base. Mirror, if present, is fitted to it.
- **Pillars:** It is a pair of elevations on the base, by which the body of the microscope is held to the base.
- **Inclination joint:** It is a movable joint, through which the body of the microscope is held to the base by the pillars. The body can be bent at this joint into any inclined position, as desired by the observer, for easier observation.
- **Curved Arm:** It is a curved structure held by the pillars. It holds the stage, body tube, fine adjustment and coarse adjustment.
- **Body Tube:** It is usually a vertical tube holding the eyepiece at the top and the revolving nosepiece with the objectives at the bottom. The length of the draw tube is called 'mechanical tube length' and is usually 140-180 mm (mostly 160 mm).
- **Draw Tube:** It is the upper part of the body tube, slightly narrower, into which the eyepiece is slipped during observation.
- **Coarse Adjustment:** It is a knob with rack and pinion mechanism to move the body tube up and down for focusing the object in the visible field. As rotation of the knob through a small angle moves the body tube through a long distance relative to the object, it can perform coarse adjustment.
- **Fine Adjustment:** It is a relatively smaller knob. Its rotation through a large angle can move the body tube only through a small vertical distance. It is used for fine adjustment to get the final clear image.
- **Stage:** It is a horizontal platform projecting from the curved arm. It has a hole at the centre, upon which the object to be viewed is placed on a slide. Light comes from the light source below the stage passes through the object into the objective.
- **Mechanical Stage (Slide Mover):** Mechanical stage consists of two knobs with rack and pinion mechanism. The slide containing the object is clipped to it and moved on the stage in two dimensions by rotating the knobs, so as to focus the required portion of the object.
- **Revolving Nosepiece:** It is a rotatable disc at the bottom of the body tube with three or four objectives screwed to it. The objectives have different magnifying powers. Based on the required magnification, the nosepiece is rotated, so that only the objective specified for the required magnification remains in line with the light path.

(II) Optical Parts: These parts are involved in passing the light through the object and magnifying its size.

- **Light Source:** Modern microscopes have in-built electric light source in the base. The source is connected to the mains through a regulator, which controls the brightness of the field.
- **Diaphragm:** If light coming from the light source is brilliant and all the light is allowed to pass to the object through the condenser, the object gets brilliantly illuminated and cannot be visualized properly. Therefore, an iris diaphragm is fixed below the condenser to control the amount of light entering into the condenser.
- **Condenser:** The condenser or sub-stage condenser is located between the light source and the stage. It has a series of lenses to converge on the object, light rays coming from the light source. After passing through the object, the light rays enter into the objective.

The 'light condensing', 'light converging' or 'light gathering' capacity of a condenser is called 'numerical aperture of the condenser'. Similarly, the 'light gathering' capacity of an objective is called 'numerical aperture of the objective'. If the condenser converges light in a wide angle, its numerical aperture is greater and vice versa.

There are three types of condensers as follows:

(a) Abbe condenser (Numerical aperture=1.25): It is extensively used.

(b) Variable focus condenser (Numerical aperture =1.25)

(c) Achromatic condenser (Numerical aperture =1.40): It has been corrected for both spherical and chromatic aberration and is used in research microscopes and photomicrographs.

- **Objective:** It is the most important lens in a microscope. Usually three objectives with different magnifying powers are screwed to the revolving nosepiece.

The objectives are:

(a) Low power objective (X 10): It produces ten times magnification of the object.

(b) High dry objective (X 40): It gives a magnification of forty times.

(c) Oil-immersion objective (X100): It gives a magnification of hundred times, when immersion oil fills the space between the object and the objective.

The scanning objective (X4) is optional. The primary magnification (X4, X10, X40 or X100) provided by each objective is engraved on its barrel. The oil-immersion objective has a ring engraved on it towards the tip of the barrel.

3.15. Refractometer (Abbe's Hand Refractometer)

Fig.28. Refractometer

General Description

A refractometer is an optical instrument that is used to determine the refractive index of a substance, measuring how light is bent as it moves through the substance.

Principle

The instrument works on the critical angle principle, utilizing lenses and prisms to project a shadow line onto a small glass reticle inside the refractometer, which is then viewed by the inspector through a magnifying eyepiece.

The Fig.3.15.1 shows light enters from the left and passes through the liquid sample. When the light hits the prism at the bottom of the liquid, it suddenly is slowed more than in the liquid because the prism has a higher refractive index.

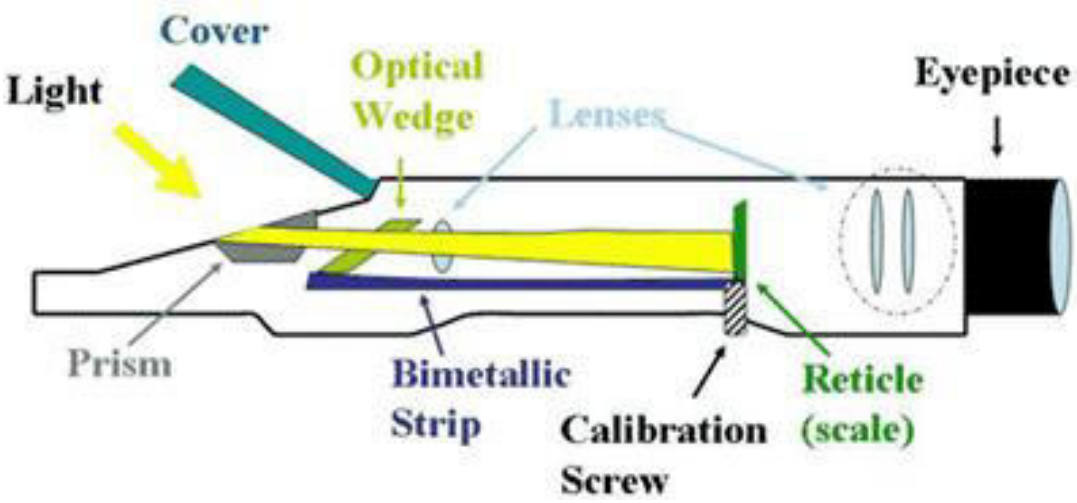


Fig.28.1 Working Principle of a Refractometer

In the case of a refractometer, the light bends in proportion to the liquid's refractive index. As the light then travels down the refractometer, it passes through lenses and lands on a scale. The bending of the light at the liquid/prism interface sends the light higher or lower in the scale's grid. Observer then looks through the viewfinder on the other end and read where the light is falling on the scale. Light covers a portion of the scale, and the remainder is dark. The dividing line between light and dark is the place to read the scale. Calibration is accomplished by turning the calibration screw, which raises or lowers the reticle (the scale) relative to the path of the light.

Working

Operation consists of placing 1 or 2 drops of the water sample on the prism, closing a glass plate over the sample, then looking through the eyepiece for the reading. The water sample is sandwiched between the measuring prism and the cover plate. Light traveling through the sample is either passed through or totally internally reflected. The net effect is that a shadow line is formed between the illuminated area and the dark area. It is at the point that this shadow line crosses the scale that a reading is taken.

3.16. Crude Fibre Apparatus

General Description

Crude fibre apparatus is used in the determination of crude fibre in feed, food and other agricultural products. The Apparatus is a reflux condenser designed to operate with speed and accuracy. The procedure involves subjecting a sample to the stimulated action of the digestion system. Samples are boiled in acid and washed and then boiled in alkali and washed again. Remaining solids are isolated and termed insoluble fibre or crude fibre - the indigestible parts of agricultural products such as cellulose and other materials.

Fig.29. Crude Fibre Apparatus

Principle

Crude fibre refers to the residue of a feed that is insoluble after successive boiling with dilute acid and alkali. In the Weende's method when the sample is subjected to acid and alkali digestion, we obtain a

residue comprising ash (mineral matter) of the feed and the resistant fraction of carbohydrate. When the residue is ignited the organic matter gets oxidized leaving the inorganic residue or ash. Thus the difference in weight of the residue before and after ashing gives the weight of crude fibre.

Instrumentation

- **Manifold Assembly-** The hood features a molded, 6" diameter exhaust connection sized to allow for a minimum of static pressure loss through the hood structure and one that still can provide a good transport velocity through the exhaust system.
- **Condenser Head System-** These cone shaped heads located above each heater provide an essential part of the digestion process. The vapours from the boiling digestion medium are condensed on the condenser heads and returned to the medium continually washing the sides of the beakers in the process.
- **Heater System-** Each 350 watt adjustable spring loaded heater provides an even distribution of heat through the use of coiled heater wires embedded in a ceramic material.
- **Variable Heat Control Switch-** Provides variable input from 20-100% of capacity to each heater.
- **Elevation Control Knob-** Adjusts beakers positions.
- **Main Power Switch-** Turn apparatus on and off without need of changing variable heat control switches.

3.17. Soxhlet Extractor

General Description

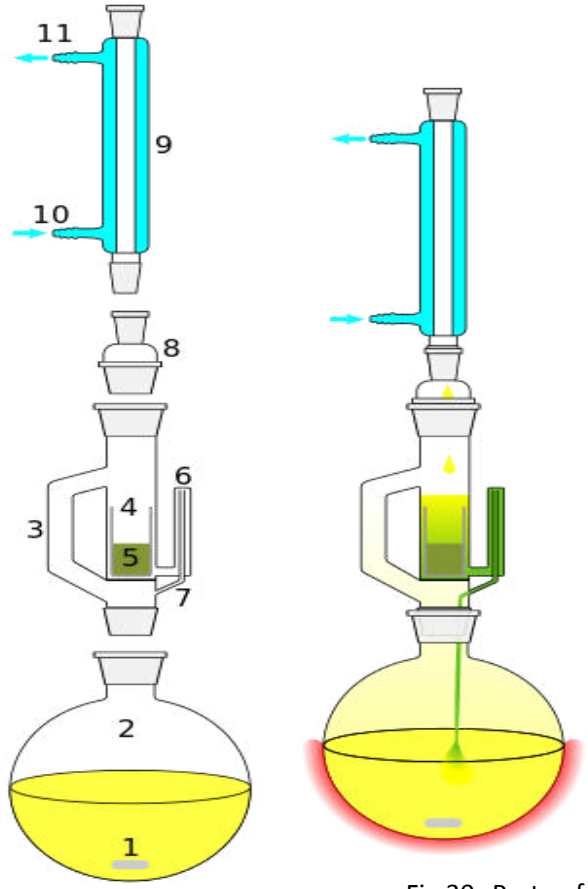
A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet. It was originally designed for the extraction of a lipid from a solid material. Typically, a Soxhlet extraction is used when the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. It allows for unmonitored and unmanaged operation while efficiently recycling a small amount of solvent to dissolve a larger amount of material.

Principle

The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound dissolves in the warm solvent. When the Soxhlet chamber is almost full, the chamber is emptied by the siphon. The solvent is returned to the distillation flask. The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask.

Instrumentation

A Soxhlet extractor has three main sections: A percolator (boiler and reflux) which circulates the solvent, a thimble (usually made of thick filter paper) which retains the solid to be laved, and a siphon mechanism, which periodically empties the thimble.



1: Stirrer bar
2: Still pot (the still pot should not be overfilled and the volume of solvent in the still pot should be 3 to 4 times the volume of the Soxhlet chamber)
3: Distillation path
4: Thimble
5: Solid
6: Siphon top
7: Siphon exit
8: Expansion adapter
9: Condenser
10: Cooling water in
11: Cooling water out

Fig.30. Parts of a Soxhlet Extractor

Working

Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by meansof a rotary evaporator, yielding the extracted compound. The insoluble portion of the extracted solid remains in the thimble, and is usually discarded.

3.18. Kjeldahl Apparatus

General Description

The Kjeldahl method was developed for determining the nitrogen contents in organic and inorganic substances. The Kjeldahl method was developed in 1883 by Johann Kjeldahl. The central basis used in this procedure is the oxidation of the organic compound using strong Sulphuric acid. As the organic material is oxidized the carbon it contains is converted to Carbon dioxide and the hydrogen is converted into water. The nitrogen, from the amine groups found in the peptide bonds of the polypeptide chains, is converted to ammonium ion, which dissolves in the oxidizing solution, and can later be converted to ammonia gas.

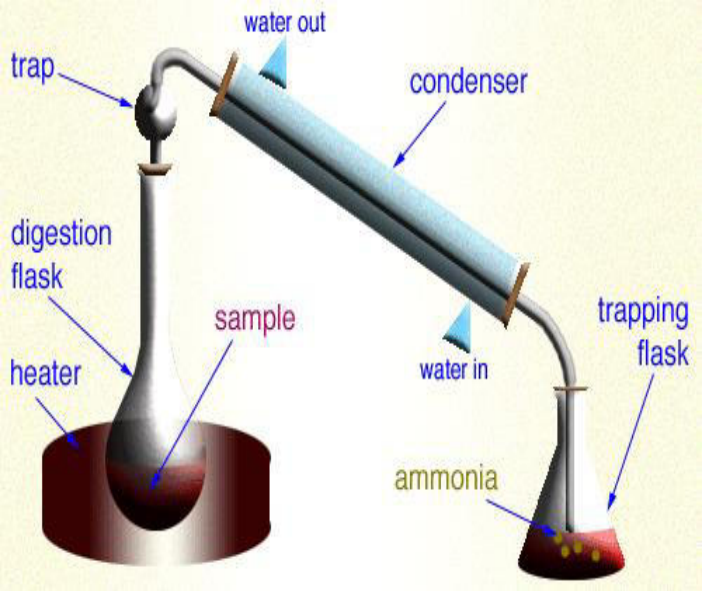


Fig.31. Kjeldahl Apparatus

Principle

A sample is digested with a strong acid so that it releases nitrogen which can be determined by a suitable titration technique. The amount of protein present is then calculated from the nitrogen concentration of the sample.

The method consists of heating a substance with Sulphuric acid, which decomposes the organic substance by oxidation to liberate the reduced nitrogen as Ammonium sulphate. In this step Potassium sulphate is added to increase the boiling point of the medium (from 337°F to 373°F / 169°C to 189°C). Chemical decomposition of the sample is complete when the medium has become clear and colourless (initially very dark). The solution is then distilled with Sodium hydroxide (added in small quantities) which converts the ammonium salt to ammonia. The amount of ammonia present (hence the amount of nitrogen present in the sample) is determined by back titration. The end of the condenser is dipped into a solution of Boric acid. The ammonia reacts with the acid and the remainder of the acid is then titrated with a Sodium carbonate solution with a Methyl orange pH indicator.

Procedure

Step 1: Digestion of the Sample

This is the most time-consuming step in the analysis. The purpose of this step is to break down the bonds that hold the polypeptides together, and convert them to simpler chemicals such as water, Carbon dioxide and, of course, ammonia.

Such reactions can be considerably speeded up by the presence of a catalyst and by a neutral substance, such as Potassium sulphate (K_2SO_4), which raises the boiling point of the digesting acid and thus the temperature of the reaction.

Catalysts are also used to help in the digestion process; many different ones have been tried including selenium, mercury, copper, or ions of mercury or copper.

Digestion is accomplished by:

- Weighing out approximately 1g of the sample containing protein, making a note of the weight, and placing the sample into a digestion flask, along with 12-15 ml of concentrated Sulphuric acid (H_2SO_4).
- Adding seven grams of Potassium sulphate and a catalyst, usually copper.
- Bringing the digestion tube/flask and mixture to a "rolling boil" (about 370°C to 400°C) using a heating block.
- Heating the mixture in the tube/flask until white fumes can be seen, and then continuing the heating for about 60-90 minutes.
- Cooling the tube/flask and cautiously adding 250 ml of water.

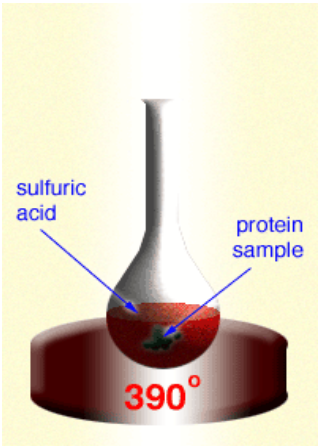


Fig.31.1. Step 1 Digestion

Step 2: Distillation

The purpose of the next step, distillation, is to separate the ammonia (that is, the nitrogen) from the digestion mixture. This is done by,

- Raising the pH of the mixture using Sodium hydroxide (45% NaOH solution). This has the effect of changing the ammonium ($NH4^+$) ions (which are dissolved in the liquid) to ammonia (NH_3), which is a gas.
- Separating the nitrogen away from the digestion mixture by distilling the ammonia (converting it to a volatile gas, by raising the temperature to boiling point) and then trapping the distilled vapours in a special trapping solution of about 15 ml HCl (Hydrochloric acid) in 70 ml of water.
- Removing the trapping flask and rinsing the condenser with water so as to make sure that all the ammonia has been dissolved.

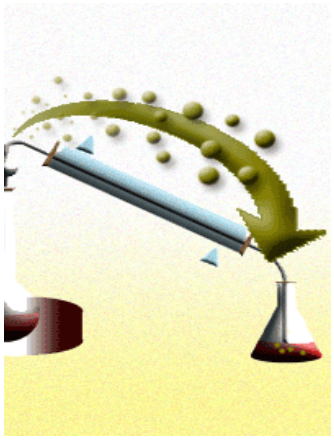


Fig.31.2. Step 2: Distillation

Step 3: Titration

As the ammonia dissolves in the acid trapping solution, it neutralizes some of the HCl it finds there. What acid is left can then be "back titrated", that is titrated with a standard, known solution of base (usually NaOH). In this way the amount of ammonia distilled off from the digestive solution can be calculated, and hence the amount of nitrogen in the sample determined.

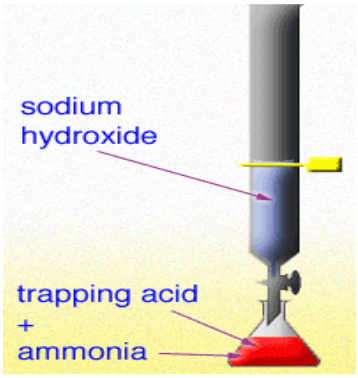


Fig.31.3. Step 3: Titration

The quantities of acid, and hence ammonia are determined by,

- Adding an indicator dye to the acid/ammonia trapping solution. This dye should turn a strong colour, indicating that a significant amount of the original trapping acid is still present.

- Putting a standard solution of NaOH (Sodium hydroxide) into the burette (a long tube with a tap at the end), very slowly, and adding small amounts of the Sodium hydroxide solution to the acid solution with the dye.
- Watching for the point at which the dye turns orange, indicating that the "endpoint" has been reached and that now all the acid has been neutralized by the base.
- Recording the volume of the neutralizing base (Sodium hydroxide solution) that was necessary to reach the endpoint.
- Performing a calculation to find out the amount of ammonia, and nitrogen came from the sample.

3.19. Muffle Furnace

General Description

A muffle furnace is a front-loading box-type oven or kiln for high-temperature applications such as fusing glass, creating enamel coatings, ceramics and soldering and brazing articles. They are used in order to determine what proportion of a sample is non-combustible and non-volatile that is called as ash.

Fig.32. Muffle Furnace

Principle

Muffle furnaces are usually heated to desired temperatures by conduction, convection, or blackbody radiation from electrical resistance heating elements. Therefore there is (usually) no combustion involved in the temperature control of the system, which allows for much greater control of temperature uniformity and assures isolation of the material being heated from the byproducts of fuel combustion.

Instrumentation

The furnace chamber is heated by electric resistance elements and is insulated with ceramic fibre insulation. The controller is located under the furnace chamber and is well insulated from the heat generated in the furnace chamber. A door safety switch removes power to the heating elements whenever the furnace door is opened. The temperature is controlled by one of three types of controllers.

3.20. Rotary Evaporator

General Description

A rotary evaporator or rotovap is a device used in chemical laboratories for the efficient and gentle removal of solvents from samples by evaporation.

Fig.33. Rotary Evaporator

Principle

Rotary evaporation is a technique which employs a rotary evaporator in order to remove excess solvents from samples by applying heat to a rotating vessel at a reduced pressure. An impotant concept that this technique applies is that liquids boil when the vapour pressure is equal to the external pressure or atmospheric pressure. The machine utilizes a lower pressure than atmospheric pressure which allows solvents to boil at lower temperatures. Furthermore, the rotation increases the surface area and

therefore evaporation proceeds more rapidly. Rotary evaporation is useful for evaporating solvents that have high boiling points. This is because evaporating these solvents at atmospheric pressure requires high temperatures which may cause side reactions such as oxidation or decomposition of the compound to occur. Therefore, by lowering the pressure and boiling at a lower temperature, solvents with high boiling points are removed efficiently without the occurrence of unwanted side reactions.

Components of a rotary evaporator

- A **motor unit** that rotates the evaporation flask or vial containing the sample.
- A **vapor duct** that is the axis for sample rotation, and is a vacuum-tight conduit for the vapour being drawn off the sample.
- A **vacuum system**, to substantially reduce the pressure within the evaporator system.
- A **heated fluid bath** (generally water) to heat the sample.
- A **condenser** with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and Acetone are placed.
- A **condensate-collecting flask** at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
- A **mechanical or motorized** mechanism to quickly lift the evaporation flask from the heating bath.

Procedure

- Ensure that both the aspirator pump and the recirculating water bath (5 gallon bucket) are filled with ice.
- Check that the power strip is turned on and plugged in.
- Verify that the bump trap is clean and dry.
- Add your sample to a round-bottomed flask. (Note: The RB flask should not be more than half full with liquid.)
- Attach the round-bottom flask to the ground glass joint of the bump trap at the end of the distillation tube. (Note: Some assemblies will have an adapter between the bump trap and round-bottom flask.)
- Ensure that all glassware is held securely in place with a plastic Keck clip and/or ring cap.
- Turn on the rotary evaporator motor (green switch).
- Adjust the dial to rotate the flask at medium speed.
- Turn the aspirator pump.
- Seal the vacuum by closing the valve at the top of the diagonal rotary evaporator condenser. (i.e. turn the knob until the arrow on it points straight down towards the attached tubing).
- If necessary, carefully lower the round-bottom flask into the water heating bath. (Note: The hot water should cover the liquid level in the flask. If the RB flask is more than half-way full, the water should touch the bottom of the RB flask and as the liquid evaporates, the RB flask can be further immersed in the water).
- Stop the rotovap when there is no more liquid dripping from the condenser coils for 30 seconds. (Note: For small volumes, dripping may not occur, wait 1-2 minutes and observe if there is any change.)

Shutdown (Essentially the reverse of setup)

- Lift the flask out of the water bath.
- Break the vacuum by opening the top valve at the top of the rotary evaporator condenser.
- Turn off the aspirator pump.
- Turn the flask rotation dial down to zero.
- Carefully remove the round-bottom flask.

3. 21. Titration Assembly

General Description

Titration, also known as titrimetry, is a common laboratory method of quantitative chemical analysis that is used to determine the unknown concentration of an identified analyte. Since volume measurements play a key role in titration, it is also known as volumetric analysis. A reagent, called the titrant or titrator is prepared as a standard solution. A known concentration and volume of titrant reacts with a solution of analyte or titrant to determine concentration. The volume of titrant reacted is called titration volume.

Principle

Titration is a process by which the concentration of an unknown substance in solution is determined by adding measured amounts of a standard solution that reacts with the unknown. Then the concentration of the unknown can be calculated using the stoichiometry of the reaction and the number of moles of standard solution needed to reach the so called end point.

The analyte: the solution of unknown concentration is known as the analyte. During titration the titrant is added to the analyte in order to achieve the equivalence point and determine the concentration of the analyte.

The standard solution: the solution of known concentration. An accurately measured amount of standard solution is added during titration to the solution of unknown concentration until the equivalence or endpoint is reached.

Components of a Titration Assembly

The technique uses a particular set of apparatus with which volumes of solutions can be measured to an accuracy of greater than 0.1cm^3.

Table.1. Titration Assembly

1. Burette	Used to deliver and measure the volume of a solution.
2. Pipette	Used to deliver an accurate volume (aliquot) of solution.
3.Volumetric flask	Used to prepare and store a standard solution of known concentration.
4. Conical Flask (Erlenmeyer Flask)	Used to contain the reaction mixture (the initial pipetted aliquot of solution plus the addition of solution from the burette).
5.Glass Funnel	Used to facilitate pouring of solution into the burette.
6. Beaker	Used to facilitate the acquisition of an aliquot of solution using the pipette, and, helps prevent possible contamination of stock solution.
4. Wash Bottle	Perfect for dispensing liquids or for cleaning glasswares.

Fig.34. Components of a Titration Assembly

In a titration the pipette is used to transfer 25cm^3 (usually to ±0.05 cm^3) of a solution in to a conical flask another solution reacts with the pipette solution in the conical flask is carefully added from a burette until it has all exactly reacted. This is called the end point of the titration (or equivalence point of the titration). There needs to be a way of knowing when the end point is reached. An indicator of some kind may be needed. For example, in the titration of strong acid and a strong base a few drops of Methyl orange or Phenolphthalein could be used.

Procedure

A typical titration begins with a beaker or Erlenmeyer flask containing a very precise volume of the analyte and a small amount of indicator placed underneath a calibrated burette or chemistry pipetting syringe containing the titrant. Small volumes of the titrant are then added to the analyte and indicator until the indicator changes colour in reaction to the titrant saturation threshold, reflecting arrival at the endpoint of the titration. Depending on the endpoint desired, single drops or less than a single drop of the titrant can make the difference between a permanent and temporary change in the indicator. When the endpoint of the reaction is reached, the volume of reactant consumed is measured and used to calculate the concentration of analyte by,

$$Ca = \frac{Ct \times Vt \times M}{Va}$$

Where, **Ca** is the concentration of the analyte, typically in molarity; **Ct** is the concentration of the titrant, typically in molarity; **Vt** is the volume of the titrant used, typically in liters; **M** is the mole ratio of the analyte and reactant from the balanced chemical equation; and **Va** is the volume of the analyte used, typically in liters.

Elements of Titration

- **The end point:** It indicates once the equivalence point has been reached. It is indicated by some form of indicator which varies depending on what type of titration being done. For example, if a colour indicator is used, the solution will change colour when the titration is at its end point. The equivalence point is the ideal point for the completion of titration. At the equivalence point the correct amount of standard solution must be added to fully react with the unknown concentration.
- **Equivalence point**: Is when the moles of a standard solution (titrant) equal the moles of a solution of unknown concentration (analyte).
- **The calibrated burette:** it is the main piece of equipment required for a titration method. Calibration is important because it is essential for the burette to be as accurate as possible in order to dispense very precise amounts of liquid into the sample. A burette is a long cylindrical piece of glass with an open top for pouring in the titrant. At the bottom there is a carefully formed tip for dispensing. Burettes usually have a plastic stopper that can easily be turned to deliver mere fractions of a drop of titrant, if needed. Burettes come in many sizes and are marked in millilitres and fractions of millilitres.
- **The Indicator:** the use of an indicator is in performing a successful titration reaction. The purpose of the indicator is to show when enough standard solution has been added to fully react with the unknown concentration. Indicators must only be added to the solution of unknown concentration when no visible reaction will occur. Depending on the solution being titrated, the choice of indicator can become key for the success of the titration.

3.22. Distillation Assembly

General Description

In this equipment liquid is vapourized (turned to steam), re-condensed (turned back into a liquid) and collected in a container. Distillation is a process of separating the component substances from a liquid mixture by selective evapouration and condensation. Distillation may result in essentially complete separation (nearly pure components), or it may be a partial separation that increases the concentration of selected components of the mixture. In either case the process exploits differences in the volatility of mixtures components.

Fig.35. Distillation Assembly

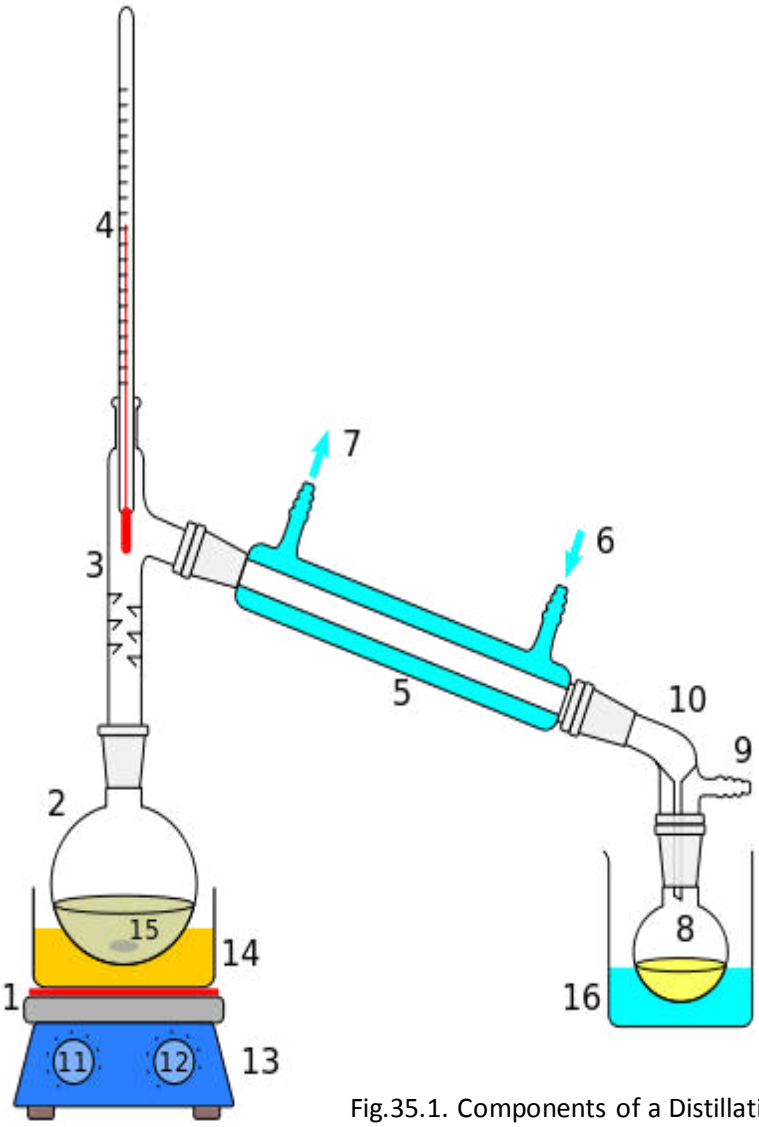
Laboratory scale distillations are almost exclusively run as batch distillations. The device used in distillation, sometimes referred to as a still, consists at a minimum of a reboiler or pot in which the source material is heated, a condenser in which the heated vapour is cooled back to the liquid state, and a receiver in which the concentrated or purified liquid, called the distillate, is collected.

Batch distillation refers to the use of distillation in batches, meaning that a mixture is distilled to separate it into its component fractions before the distillation still is again charged with more mixture and the process is repeated.

Principle

The separation of components from a liquid mixture via distillation depends on the differences in boiling points of the individual components. Also, depending on the concentrations of the components present, the liquid mixture will have different boiling point characteristics. Therefore, distillation processes depend on the vapour pressure characteristics of liquid mixtures. A liquid boils when its vapour pressure equalizes with the surroundings.

Components of a Distillation Assembly



1: A source of heat
2: Still pot
3: Still head
4: Thermometer/
Boiling point temperature
5: Condenser
6: Cooling water in
7: Cooling water out
8: Distillate/receiving flask
9: Vacuum/gas inlet
10: Still receiver
11: Heat control
12: Stirrer speed control
13: Stirrer/heat plate
14: Heating (Oil/sand) bath
15: Stirring means e.g. boiling chips or mechanical stirrer
16: Cooling bath.

Fig.35.1. Components of a Distillation Assembly

3.23. Mixer Grinder

General Description

In a laboratory, most materials required for sampling are, in practice, non-homogenous mixtures. The best method of obtaining a small representative sample of the non-uniform whole is to take a quantity of the material large enough to be compositionally representative and reduce it to a fine homogenous powder. For this purpose a grinder is usually used.

Fig.36. Mixer Grinder

Principle

In grinder samples grinding them between opposite cutter plates and then separated by centrifugal force. Most of the devices are powered electrically and in some cases are operated manually.

3.24. Microwave Oven

General Description

Fig.37. Microwave Oven

Microwave ovens are used for heating and defrosting in laboratories. Microwave heating, which uses electromagnetic energy in the frequency range 300- 3000 MHz, can be used successfully to heat many dielectric materials.

However, improper use of a microwave can pose a number of hazards including:

- Ignition of flammable vapours
- Electrical shock from ungrounded or faulty units
- Ignition of materials being heated
- Pressure build-up in sealed containers
- Integrity of containers holding materials
- Sudden boiling of liquid in an open container following removal

Principle

A microwave oven heats samples by passing microwave radiation through it. Microwaves are radio waves. In the case of microwave ovens, the commonly used radio wave frequency is roughly 2,500 Megahertz (2.5 gigahertz). Radio waves in this frequency range have an interesting property: they are absorbed by water, fats and sugars. When they are absorbed they are converted directly into atomic motion and motion is converted into heat. Microwaves in this frequency range have another interesting property: they are not absorbed by most plastics, glass or ceramics. And metal reflects microwaves, which is why metals cause spark in a microwave oven. The reason that metal reflects microwaves is that no electronic waves resident in inside of conductor because conductors conductivity is infinity as we studied in our course. The property is possible because the frequency 2,500 megahertz is resonance frequency of water. Molecules of all food are consist of a dipole and have positive charge in one side and have negative charge in another side. If we put electromagnetic fields in this, all molecules are rearranged: positive charge is to negative.

Pole and charge is to positive pole. In this process molecules heat is produced by Friction. The frequency of microwave oven is 2,500 megahertz as we saw before. Then Microwave of this frequency change the direction of electromagnetic fields 2,500,000,000 times in 1 second. Consequently the heat efficiency of a microwave oven is high. In a conventional oven, the heat has to migrate (by conduction) from the outside of the food toward the middle. In microwave cooking, the radio waves penetrate the food and excite water and fat molecules pretty much evenly throughout the food. There is no “heat having to migrate toward the interior by conduction”. There is heat everywhere all at once because the molecules are all excited together. There are limits of course. Radio waves penetrate unevenly in thick pieces of food (they don’t make it all the way to the middle), and there are also “hot spots” caused by wave interference. The whole heating process is different. In a microwave oven, the air in the oven is at room

temperature, so there is no way to form a crust. That is because it heats up foods by 'microwaves' instead of 'heat conduction'.

Steps to Working Safely with Microwaves

- Never attempt to heat, flammable liquids or solids, hazardous substances or radioactive materials in any type of microwave oven.
- Do not place metal items inside the microwave, including aluminum foil and plastic coated magnetic stirrer bars.
- Do not modify the microwave in any way, including the removal of the grounding pin or change of the plug.
- Never use a laboratory microwave for food preparation (or kitchen microwave for laboratory materials).
- Do not heat sealed containers in a microwave. Even a loosened cap or lid poses a significant risk since microwaves can heat material so quickly that the container explodes either in the oven or shortly after removal.
- Take care to avoid overheating liquids. It is possible to raise water to a temperature greater than normal boiling point; when this occurs, any disturbance to the liquid can trigger violent boiling that could result in severe burns.

Additional safety measures must be considered when using a microwave to melt agar

- Large amounts (e.g. 250 ml) of solidified agar should not be warmed in a microwave unless first chopped up with a sterile spatula. Not doing this can cause explosive vapourization in solid agar where vapour cannot escape.
- Use loose fitting sterile foam plugs or loose 'Kim wipe" plugs, rather than just relying on loosely placed cap.
- Ensure a good amount of headspace is available in the container above the material being heated.
- Set the power and timings correctly. Do not overheat.
- Thermal gloves and a face shield must be worn when removing a container from the microwave.
- Care should be taken when placing the container on the bench as not to cause unnecessary disturbance of the agar, which may cause the agar to boil over.

3.25. Refrigerator

General Description

Laboratory refrigerators are used to cool samples or specimens for preservation. They differ from standard refrigerators used in homes or restaurant because they need to be totally hygienic and completely reliable.

Fig.38. Refrigerator

Principle

The refrigerating effect in refrigeration systems is based on the use and control of the phase transition processes of evapouration. As the refrigerant evapourates it absorbs energy (heat) from its surroundings and by placing an object in thermal contact with the evapourating refrigerant it can be cooled to low temperature.

Different substances have different melting and boiling points. Gold for example melts at 1064°C, chocolate at 26°C and most refrigerants melt at temperatures around -100°C.

For a substance that is present in two of its phases at the same time or undergoing a phase change pressure and temperature become dependent. If the two phases exist in a closed container and the two phases are in thermal equilibrium the condition is said to be saturated. If the temperature of the two phase mixture is increased the pressure in the container will also increase. The relationship between pressure and temperature for saturated conditions (liquid and vapour) is typically called the vapour pressure curve. Using the vapour pressure curve one can determine what the pressure will be for an evapourating or condensing process. The evapouration process in a refrigeration system is one of the processes where the term superheat is used. Superheat is a very important term in the terminology of refrigeration but it is unfortunately used in different ways. It can be used to describe a process where refrigerant vapour is heated from its saturated condition to a condition at higher temperature. The term superheat can also be used to describe or quantify the end condition of the before mentioned process. Superheat can be quantified as a temperature difference between the temperature measured with a thermometer and the saturation temperature of the refrigerant measured with a pressure gauge. Superheat mined from a single measurement of temperature alone a measurement of pressure or saturation temperature is also needed. When superheat is quantified it should be quantified as a temperature difference and, consequently, be associated with the unit [K]. If quantified in [°C] it can be the cause of mistakes where the measured temperature is taken for the superheat or vice versa.

3.26. Deep Freezer

General Description

Deep freezers are elegantly designed with the best quality refrigeration system to meet the elevated standards of performance and controlled temperature storage needs of the pharmacy, medical, industry, biotech and clinical applications.

Fig.39. Deep Freezer

Principle

The basic principle behind a freezer is evapouration. When a liquid evapourates, it causes the surrounding area to cool. Water can't be used in freezer though, because it evapourates at too high a temperature. But some liquids evapourate at very low temperatures. For example, Isobutene (becoming more common in domestic freezers) evapourates at very low temperatures. This ability to evapourate at very low temperatures means that it cools surfaces which are already very cold.

Instrumentation

- **Evapourato**r: Evapouration is affected by air pressure. The higher the air pressure, the less a liquid will evapourate.
- The **compressor** takes in the refrigerant (as gas); raise the air pressure which converts the refrigerant gas to liquid.
- As the refrigerant liquid flows from the compressor to the **expansion valve** the high air pressure stops it evapourating and instead it gives off heat and becomes cooler. The refrigerant liquid flows through the expansion valve where the air pressure is much lower .This cause the refrigerant liquid to evaporate which cause the pipe to become very cold inside the freezer.
- One key component of the freezer is the **thermostat**. The thermostat senses the temperature inside the freezer and when it drops below a certain temperature it turns off the motor so the flow of the refrigerant liquid stops. When the temperature rises above a certain level the thermostat turns on the motor and the refrigeration process restarts.

- **Refrigerant Liquids:** Different freezers have different refrigerant liquids. Which liquid is used is important for two main reasons. Firstly different liquids are more or less efficient for use in a freezer. Less efficient liquids will use more electricity and therefore cost you more to run your freezer. Secondly, some refrigerant liquids contribute significantly more than others to global warming and destruction of the ozone layer. Pre-1990 many freezers used CFC which caused significant environmental damage to the atmosphere. This was then replaced with MFC which does not destroy the ozone layer but still does contribute to global warming. Currently HC and Isobutene are used as refrigerant liquids in most domestic freezers. Both are a good with Isobutene being the best.

3.27. BOD Incubator

General Description

BOD incubators often called low temperature incubators, which is one of most important lab equipment in many research centres, hospitals and other pharmaceutical labs. Bacteriological incubators are known as exceptional lab incubators specially designed for a variety of incubation and testing applications. The series of BOD incubator is perfect for BOD testing and other related jobs of research.

Fig.40. BOD Incubator

Principle

The major principle behind the BOD incubator is to maintain constant environment condition for any particular kind of study such as cell cultures, microbiological etc.

3.28. Hot Air Oven

General Description

It is a dry heat sterilization unit. A dry heat cabinet is easy to install and has relatively low operating costs; it is nontoxic and does not harm the environment and it is noncorrosive for metal and sharp instruments.

Fig.41. Hot Air Oven

Principle

Sterilization by dry heat is accomplished through conduction. The heat is absorbed by the outside surface of the equipment, and then passes towards the centre of it, layer by layer. The entire system will eventually reach the temperature required for sterilization. Dry heat does most of the damage by oxidizing molecules. The essential cell constituents are destroyed and the organism dies. The temperature is maintained for almost an hour to kill the most difficult of the resistant spores. The most common time-temperature relationships for sterilization with hot air sterilizers are:

1. 170°C (340°F) for 60 minutes,

2. 160°C (320°F) for 120 minutes,

3. 150°C (300°F) for 150 minutes or longer depending upon the volume

Working

Working principle of hot air oven is the forced circulation of hot air inside the chamber of oven. As it is a universal scientific fact that in any chamber hot air rises above, so by utilizing this principle when the hot air reaches the top of chamber it is circulated back to bottom by a fan installed inside the chamber and hence optimum amount of heat is achieved gradually inside the hot air oven.

There are two types of dry-heat sterilizers: one is the static-air type and the other is forced-air type. The static-air type is referred to as the oven-type sterilizer as heating coils in the bottom of the unit cause the hot air to rise inside the chamber via gravity convection. This type of dry-heat sterilizer is much slower in heating, requires longer time to reach sterilizing temperature, and is less uniform in temperature control throughout the chamber than is the forced-air type.

Note

Dry heat sterilization technique requires longer exposure time (1.5 to 3 hours) and higher temperatures than moist heat sterilization. Dry heat ovens are used to sterilize items that might be damaged by moist heat or that are impenetrable to moist heat (e.g., powders, petroleum products, sharp instruments).

The hot air oven is mounted on four rubber foots to prevent slipping and this protects the bench surface. The control panel houses a main ON/OFF switch indicator lamp and temperature setting knob. The scale is calibrated in 5°C steps.

3.29. Autoclave

General Description

An autoclave is a large pressure cooker. It is a moist sterilization unit. It is a pressure chamber used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 121°C (249°F) for around 15–20 minutes depending on the size of the load and the contents. It was invented by Charles Chamberland in 1879.

Fig.42. Autoclave

Parts of an Autoclave

Autoclaves have four basic parts

- **Water Intake-** An autoclave needs water to make the steam used for sterilization. A water intake hookup or hose, it allowing the user to pump water directly into the machine.
- **Chamber-** The chamber is the space where the user places items to sterilize. In the chamber are wire racks, which will hold various items upright or lying down and allow for steam penetration from all angles.
- **Control Panel-** The control panel allows the user to customize the autoclaving process. Some materials can withstand higher temperatures, while some must be autoclaved at lower temperatures for longer time.
- **Machinery-** Autoclaves must have an air pump system to remove the oxygen in the chamber and create a vacuum which then fills with pressurized steam created from the water in the

reservoir. The water becomes heated either via a heating element inside the water reservoir or a heat-generating mechanism that completely surrounds the reservoir.

Principle

It operates under the principle of steam under pressure as the sterilizing agent. High pressures enable steam to reach high temperatures, thus increasing its heat content and killing power. Most of the heating power of steam comes from its latent heat of vapourization (the amount of heat required to convert boiling water to steam).

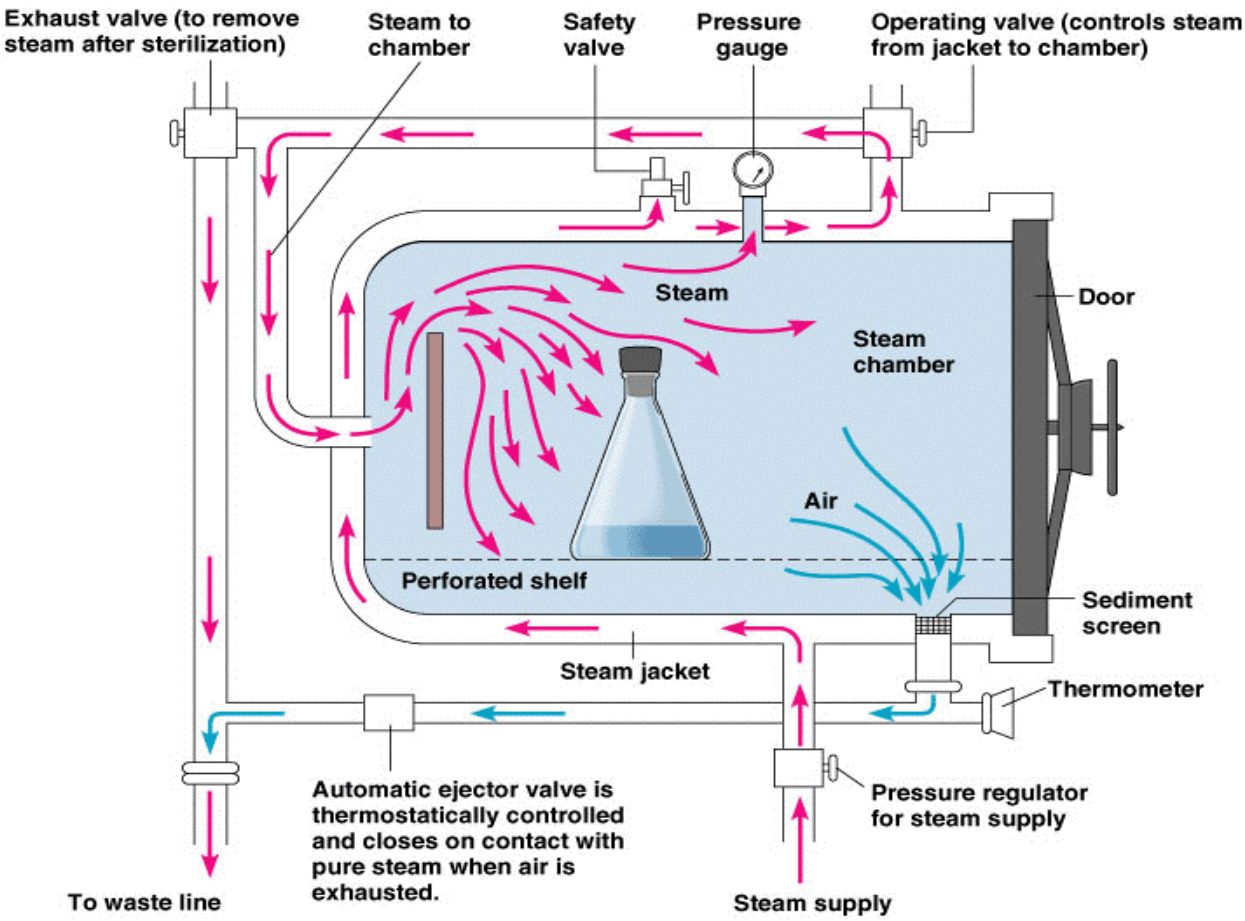


Fig.42.1. Principle of Autoclave

Steam is able to penetrate objects with cooler temperatures because once the steam contacts a cooler surface; it immediately condenses to water, producing a concomitant 1,870 fold decreases in steam volume. This creates negative pressure at the point of condensation and draws more steam to the area. A condensation continues so long as the temperature of the condensing surface is less than that of steam; once temperatures equilibrate, a saturated steam environment is formed. Achieving high and even moisture content in the steam-air environment is important for effective autoclaving. The ability of air to carry heat is directly related to the amount of moisture present in the air. The more moisture present, the more heat can be carried, so steam is one of the most effective carriers of heat. Steam therefore also results in the efficient killing of cells and the coagulation of proteins.

Moist heat is thought to kill microorganisms by causing coagulation of essential proteins. Another way to explain this is that when heat is used as a sterilizing agent, the vibratory motion of every molecule of a microorganism is increased to levels that induce the cleavage of intramolecular hydrogen bonds

between proteins. Death is therefore caused by an accumulation of irreversible damage to all metabolic functions of the organism. Death rate is directly proportional to the concentration of microorganisms at any given time. The time required to kill a known population of microorganisms in a specific suspension at a particular temperature is referred to as thermal death time (TDT). All autoclaves operate on a time/temperature relationship; increasing the temperature decreases TDT, and lowering the temperature increases TDT.

Standard temperatures/pressures employed are 115¡C/10 psi 121¡C/15 psi and 132¡C/27 psi. (Psi=pounds per square inch).

Working

Steam enters the chamber jacket, passes through an operating valve and enters the rear of the chamber behind a baffle plate. It flows forward and down through the chamber and the load, exiting at the front bottom. A pressure regulator maintains jacket and chamber pressure at a minimum of 15 psi, the pressure required for steam to reach 121^{o}C (250^{o}F).Overpressure protection is provided by a safety valve. The conditions inside are thermostatically controlled so that heat (more steam) is applied until 121^{o}C is achieved, at which time the timer starts, and the temperature is maintained for the selected time.

Note

Please note that after loading and starting the autoclave, the processing time is measured after the autoclave reaches normal operating conditions of 121¡C (250¡F) and 15 psi pressure, not simply from the time you push the "on" button. Due to the fact that autoclaves utilize steam, heat and pressure the risk of personal exposure and potential harm is great. Personnel should wear proper personal protective equipment, i.e. heat resistant gloves, eye protection and a lab coat, particularly when unloading the autoclave. Regularly inspect the autoclave for proper operation. Do not assume that the temperature and pressure is down before opening the chamber. Look at the gauges. Even if the pressure gauge shows "0", open the chamber carefully; crack the door to allow steam to dissipate (don’t fling the door open, as steam might come out and burn you). After opening the door, let items sit for 5 minutes before handling. This will reduce the chance of boil-over and burns. Never place sealed containers in an autoclave they might explode. This allows for expansion during the cycle. Caps must be slightly loose so that pressure created during the cycle does not cause the vessel to break. For screw-cap containers, you can make the lid hand tight and then loosen the lid by one-half turn. Always leave a few inches of "head room" in your containers. That way, if the item boils, it won’t spray out into your face. Liquids to be autoclaved must be in an autoclavable vessel that is at least twice as large as the volume to be autoclaved (i.e. If you are autoclaving 1 litre of media, you need to put it in a flask that hold at least 2 litres) . Agar will clog the drain in the autoclave and break it.

Do not autoclave items containing solvents, volatile or corrosive chemicals (Phenol, Trichloro acetic acid, Ether, Chloroform, etc.) or any radioactive materials.

3.30. Gel Documentation System

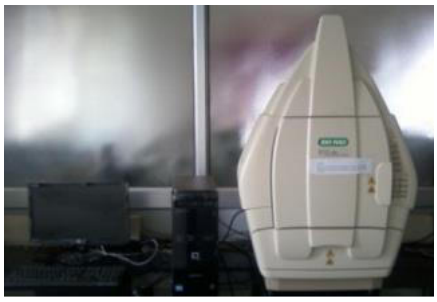


Fig.43. Gel Documentation System

General Description

A gel doc, also known as a gel documentation system, gel image system or gel imager, is equipment widely used in molecular biology laboratories for the imaging and documentation of nucleic acid and protein suspended within polyacrylamide or agarose gels. These gels are typically stained with Ethidium bromide or other fluorophores such as SYBR Green.

Principle

Principle of fluorescence with fluorescent staining of nucleic acids, a fluorescent substance that has bound to nucleic acids is excited by ultraviolet irradiation and emits fluorescent light. The fluorescent substance Ethidium bromide binds specifically to nucleic acid and the amount of binding depends on the molecular weight and concentration of the nucleic acid. In other words, a band for a large molecular weight or large amount will shine brighter; conversely, fluorescence will be weaker for a band for a small molecular weight or small amount.

Instrumentation

- **Source of irradiation:** UV Transilluminator 20 x 20 cm, 312 nm (254 nm selectable)
- **Base Plate:** A sample tray, a gel viewer
- A set of **filters**
- **Imaging device:** A camera (CCD with high resolution) unit integrated with darkroom, camera controller, video monitor and printer.
- **Readout system:** Computerized system

3.31. Centrifuge

Fig.44. Centrifuge

General Description

A centrifuge is the equipment generally driven by an electric motor that puts an object to rotate around fixed axis, and a perpendicular force is applied to axis. The particles get separated according to their size, shape, density, viscosity of the medium and rotor speed.

Principle

The centrifuge involves principle of sedimentation, where the acceleration at centripetal force causes denser substance to separate out along the radial direction at the bottom of the tube. In centrifugation, the lysate is rotated at a certain speed (expressed as rotations per minute (RPM)). This rotation imposes a force on the particles perpendicular to the axis of rotation. The force is called a relative centrifugal force (RCF), expressed as a multiple of the force of Earth's gravitational force (x g). When a particle is subjected to centrifugal force, it will migrate away from the axis of rotation at a rate dependent on the particle's size and density.

Instrumentation

- That part of the centrifuge that holds the centrifugation tubes is called the centrifuge rotor.
- Centrifuges are designed so that a number of different rotors can be used by the instrument. There are three types of centrifuge rotors: fixed-angle rotors, swinging-bucket rotors, and vertical rotors.
- Fixed-angle and swinging-bucket rotors are the most commonly used. In a fixed-angle rotor, the centrifuge tubes are spun at a fixed angle, which is usually approximately 35 degrees. They are most commonly used for pelleting cells and subcellular components.
- With swinging-bucket rotors, the tubes are free to swing out perpendicular to the axis of rotation as the rotor rotates. This rotor is particularly useful in density-gradient centrifugation schemes.

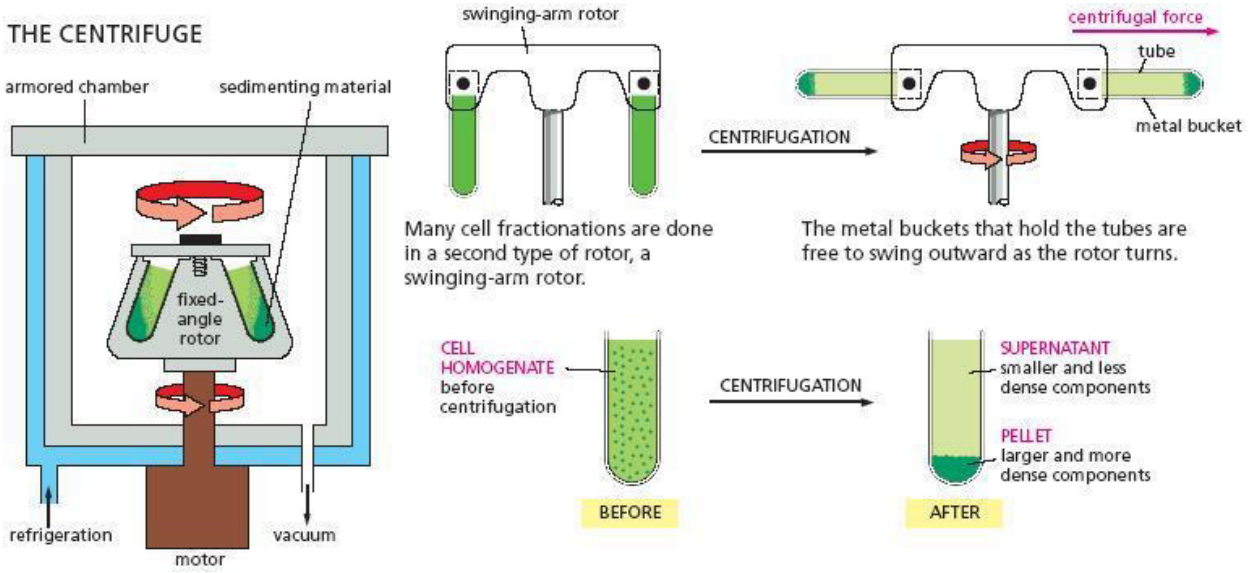


Fig.44.1. Rotors and Centrifugation

Differential Centrifugation

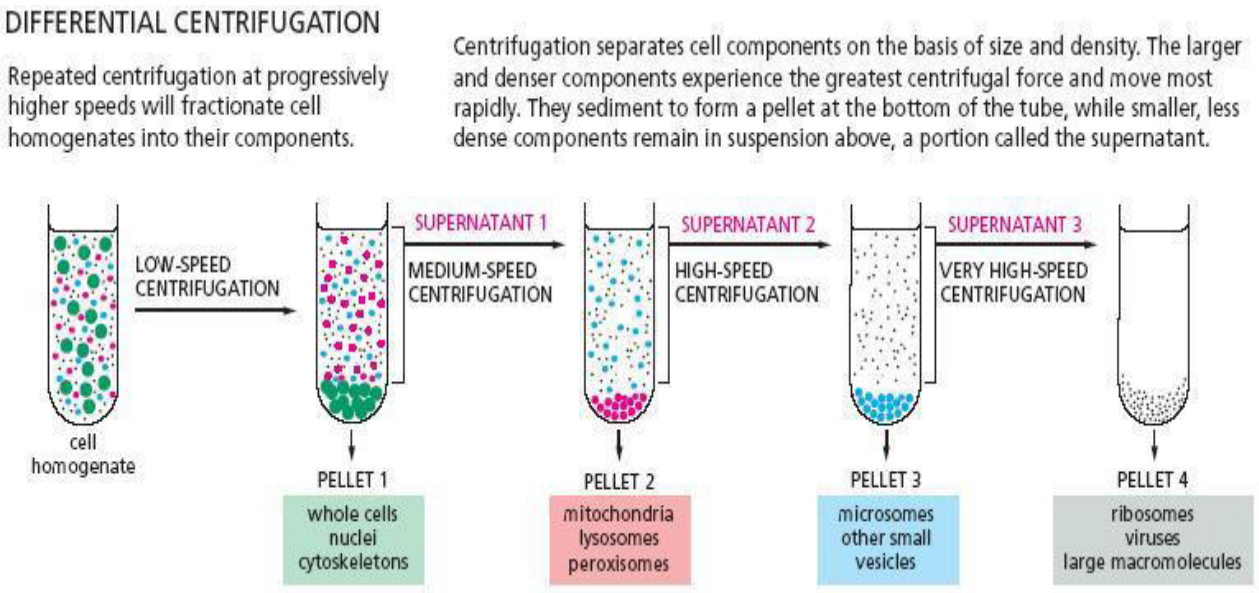


Fig.44.2. Differential Centrifugation

Differential centrifugation is one of two major types of centrifugation schemes. Differential centrifugation is the sequential centrifugation of a cell lysate at progressively increasing centrifugation force, isolating cellular components of decreasing size and density. The separation of the cellular components is based solely on their sedimentation rate through the centrifugation medium, which, in turn, is dependent on the size and shape of the cellular components. In differential centrifugation, each centrifugation step results in the production of a pellet, usually containing a mixture of cellular components of the same size and/or density. The fluid resting above the pellet, the supernatant can be removed and subjected to additional centrifugations to generate pellets containing other cellular components of lesser size and / or density.

3.32. Refrigerated Centrifuge

Fig.45. Refrigerated Centrifuge

General Description

It is extensively used in chemistry, biology, and biochemistry for isolating and separating suspensions. It additionally provides the cooling mechanism to maintain the uniform temperature throughout the operation of the sample.

Principle

Refrigerated centrifuge works on the concept of sedimentation principle by holding up the sample tubes with a capacity of 2ml, 10ml and 50ml in rotation around a fixed axis. In this, the centripetal force causes the denser substances to separate out along the radial direction in the bottom of the centrifuge tube. The rate of the centrifugation is calculated by the acceleration applied to the sample and it is typically measured in revolution per minute (RPM) or relative centrifugal force (RCF). The particles settling velocity during centrifugation depends on the function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity.

Working

- Switch on the mains. The power indicator will get illuminated.
- Setting the Program Number: Press the PROG button. The required program number can be set by using the UP or DOWN.
- Setting the timer: Press the PROG button again. The display panel will show Zero and the timer setting is initialized. The required time setting can be made by pressing the UP arrow for the increment and DOWN arrow for decreasing the time. Set the time required for the program and release the button.
- Setting the temperature: Press the PROG again. Now the system is ready for temperature setting. Set the required temperature by pressing the UP or DOWN arrow till the required temperature is set. Release the button.
- Selection of RPM: Press the PROG button again. The option to select the display mode to read normal display in RPM\RCF can be selected by using the UP or DOWN
  Setting the rotor speed: Press the PROG button again. Use the UP or DOWN arrow to set the required speed. Release the button
- Setting the acceleration time: Press the PROG button again. Use UP or DOWN arrow to set the acceleration time within the range of 60-240 seconds. Release the button.

- Setting the deceleration time: Press the PROG button again. The system is ready to set the deceleration time. Use UP or DOWN arrow to set the deceleration time within the range of 45-240 seconds. Release the button.
- Rotor Selection: Press the PROG button again. The system is ready to set the rotor selection. The rotor heads are numbered and select the specific rotor head number which you plan to use in this program. Use arrow UP or DOWN select the specific number of the rotor head you plan to use. Release the button, saving the setting and locking the program.
- Now you have completed the setting of the parameters for the first program and the same is automatically registered and saved as parameters of the set program number as program 1. Press the RETURN key to come back to Normal Display mode.

3.33. Thermal Cycler (PCR machine)

General Description

The thermal cycler (also known as a thermo cycler, PCR machine or DNA amplifier) is a laboratory apparatus used to amplify segments of DNA via the Polymerase Chain Reaction (PCR). The device has a thermal block with holes where tubes holding the PCR reaction mixtures can be inserted. The cycler then raises and lowers the temperature of the block in discrete, preprogrammed steps.

Fig.46. Thermal Cycler (PCR machine)

Polymerase Chain Reaction (PCR)

PCR stands for the Polymerase Chain Reaction and was developed in 1987 by Kary Mullis (which won him a Nobel Prize) and associates. With this technique it is possible to make virtually unlimited copies of a single DNA molecule even though it is initially present in a mixture containing many different DNA molecules. It is used to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule. The polymerase chain reaction can be used to amplify both double and single stranded DNA.

Principle

The cycling reactions: There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

1. Denaturation: This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

2. Annealing: The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should only bind to a perfectly complementary part of the template. If the temperature is too low, the primer could bind imperfectly. If it is too high, the primer might not bind. Typically the annealing temperature is about 3-5°C below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

3. Extension: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. At each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

4.Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

5. Final hold: This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

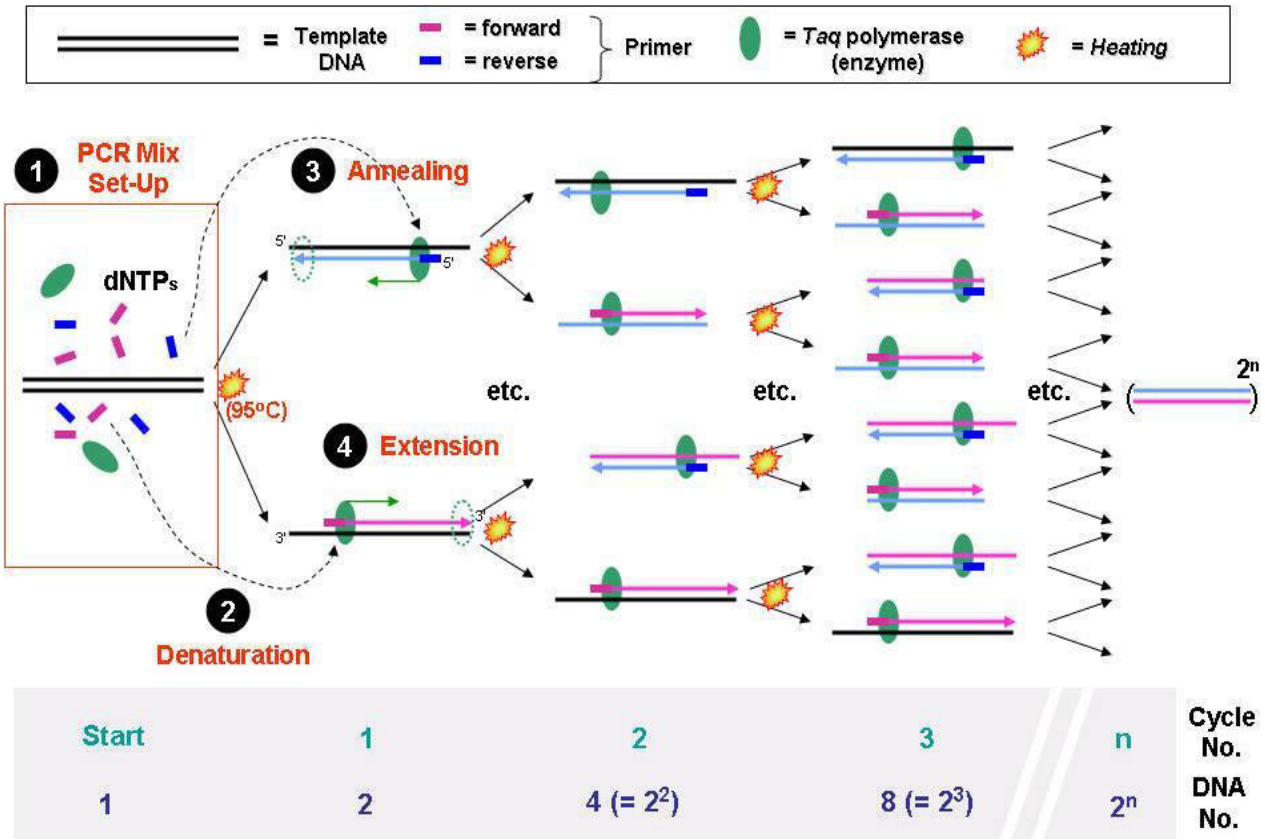


Fig.46.1. Principle of Polymerase Chain Reaction

Requirements

- **DNA template:** That contains the DNA region (target) to be amplified.
- **Two primers (Forward primer & Backward primer):** Which are complementary to the DNA regions at the 5' (five prime) or 3' (three prime).
- A **thermostable DNA polymerase**: Such as Taq polymerase.
- **Deoxynucleoside triphosphates (dNTPs):** The building blocks from which the DNA polymerases synthesizes a new DNA strand.
- **Buffer solution:** Providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- **Divalent cations:** Magnesium or manganese ions; generally Mg2+ is used.
- **Nuclease free water**

Procedure

1. The DNA molecule carrying a target sequence is denatured by heat at 90-95^{o}C for 20 seconds. The two strands separate due to breakage of the hydrogen bonds holding them together. Oligonucleotide primers are added.

2. A reaction mixture containing all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) and a thermostable DNA polymerase is added. A DNA polymerase (Taq) that is not denatured by the high temperature needed to separate the DNA strands is used. It is usually sourced from *Thermus aquaticus*, a bacterium isolated from hot springs.

3. The mixture is allowed to cool to a lower temperature (50-65^{o}C). Each strand of DNA molecule becomes annealed with an oligonucleotide primer complementary to either end of the target sequence. Primer annealing takes 20 seconds.

4. The temperature is raised to 60-75^{o}C and primers are extended by the action of DNA polymerase for 30 seconds. The polymerase synthesizes complementary sequence the 5' to 3' direction away from each of the primers. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain. Polymerization continues until each newly synthesized strand has proceeded far enough to contain the site recognized by the other primer. At this point there would be exactly two copies of the target DNA sequence.

5. The mixture is heated again at 90-95^{o}C to denature the molecules and separate the strands and the cycle repeated. Each new strand then acts as a template for the next cycle of synthesis. Thus amplification proceeds at an exponential (logarithmic) rate, i.e. amount of DNA produced doubles at each cycle. The amplified product at the end of PCR is called amplicon.

3.34. Blotting Techniques

General Description

Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support generally nylon or nitrocellulose membranes. Blotting of nucleic acid is the central technique for hybridization studies. Nucleic acid labeling and hybridization on membranes have formed the basis for a range of experimental techniques involving understanding of gene expression, organization, etc. Blotting techniques are used to identify unique proteins and nucleic acid sequences. They have been developed to be highly specific and sensitive and have become important tools in both molecular biology and clinical research.

Principle

The blotting methods are fairly simple and usually consist of four separate steps: electrophoretic separation of protein or of nucleic acid fragments in the sample; transfer to and immobilization on paper support; binding of analytical probe to target molecule on paper; and visualization of bound probe. Molecules in a sample are first separated by electrophoresis and then transferred on to an easily handled support medium or membrane. This immobilizes the protein or DNA fragments, provides a faithful replica of the original separation, and facilitates subsequent biochemical analysis. After being transferred to the support medium the immobilized protein or nucleic acid fragment is localized by the use of probes, such as antibodies or DNA, that specifically bind to the molecule of interest. Finally, the position of the probe that is bound to the immobilized target molecule is visualized usually by

autoradiography. Three main blotting techniques have been developed and are commonly called Southern, Northern and Western blotting.

3.34.1. Southern blot

Southern blot is a method used to check for the presence of a DNA sequence in a DNA sample. The method is named after its inventor, the British biologist Edwin Southern.

Procedure

- Restriction endonucleases are used to cut high molecular weight DNA strands into smaller fragments, which are then electrophoresed on an agarose gel to separate them by size.
- If the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl, which depurinates the DNA fragments, breaking the DNA into smaller pieces, thus allowing more efficient transfer from the gel to membrane.
- If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (containing NaOH) to denature the double-stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe and destroys any residual RNA that may still be present in the DNA.
- A sheet of nitrocellulose (or nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is used to move the DNA from the gel on to the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
- The membrane is then baked in a vacuum or regular oven at 80°C for 2 hours or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane. The membrane is then exposed to a hybridization probe a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.
- After hybridization, excess probe is washed from the membrane and the pattern of hybridization is visualized on x-ray film by autoradiography in the case of a radioactive or fluorescent probe or by development of colour on the membrane if a chromogenic detection method is used.

Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome. A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication). Modification of the hybridization conditions (i.e., increasing the hybridization

temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.

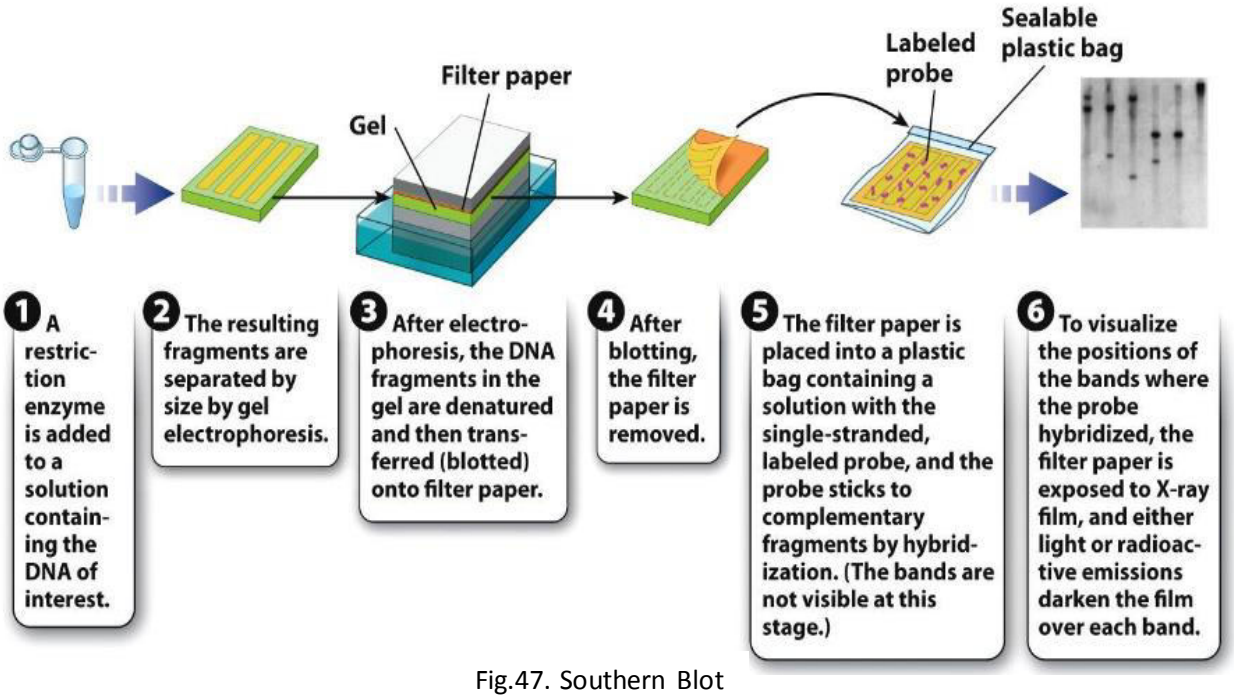


Fig.47. Southern Blot

3.34.2. Northern blot

The Northern blot technique is used to study gene expression by detection of RNA (or isolated mRNA) in a sample. With Northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. This technique was developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot. The major difference is that RNA, rather than DNA, is analyzed in the Northern blot.

Procedure

The blotting procedure starts with extraction of total RNA from a homogenized tissue sample. The mRNA can then be isolated through the use of oligo (DT) cellulose chromatography to maintain only those RNAs with a poly (A) tail. RNA samples are then separated by gel electrophoresis. A nylon membrane with a positive charge is the most effective for use in Northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction preventing RNA degradation by high temperatures. Once the RNA has been transferred to the membrane it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. The membrane is washed to ensure that the probe has bound specifically. The hybrid signals are then detected by x-ray film and can be quantified by densitometry.

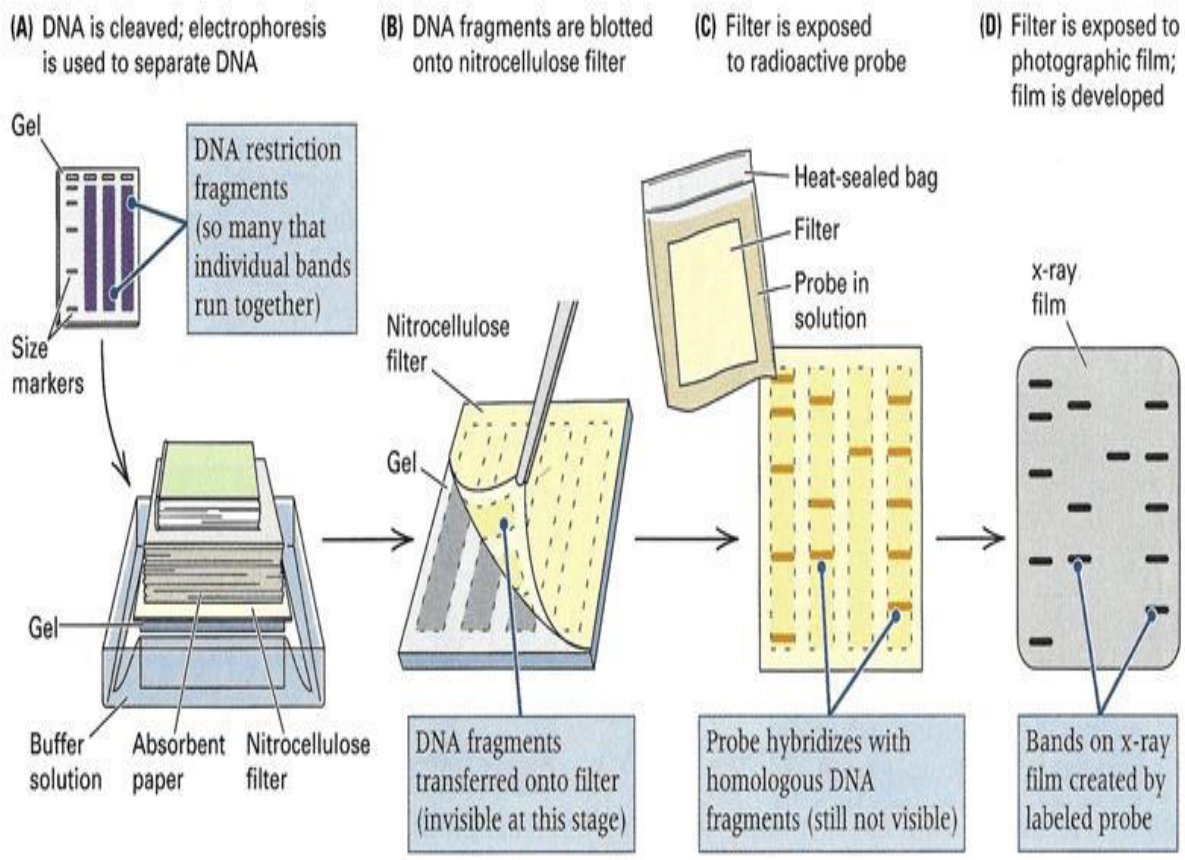


Fig.48. Northern Blot

3.34.3. Western blot

The Western blot (alternatively, immunoblot) is used to detect specific proteins in a given sample of tissue homogenate or extract. The method originated from the laboratory of George Stark at Stanford. The name Western blot was given to the technique by W. Neal Burnette.

Procedure

The first step in a Western blot is to prepare the protein sample by mixing it with a detergent called Sodium dodecyl sulfate, which makes the proteins unfold into linear chains and coats then with a negative charge. Next, the protein molecules are separated according to their sizes using a method called gel electrophoresis. Following separation, the proteins are transferred from the gel onto a blotting membrane. Although this step is what gives the technique the name "Western blotting," the term is typically used to describe the entire procedure.

Once the transfer is complete, the membrane carries all of the protein bands originally on the gel. Next, the membrane goes through a treatment called blocking, which prevents any nonspecific reactions from occurring. The membrane is then incubated with an antibody called the primary antibody, which specifically binds to the protein of interest. Following incubation, any unbound primary antibody is washed away, and the membrane is incubated yet again, but this time with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is linked to a reporter enzyme that produces colour or light, which allows it to be easily detected and imaged. These steps permit a specific protein to be detected from among a mixture of proteins.

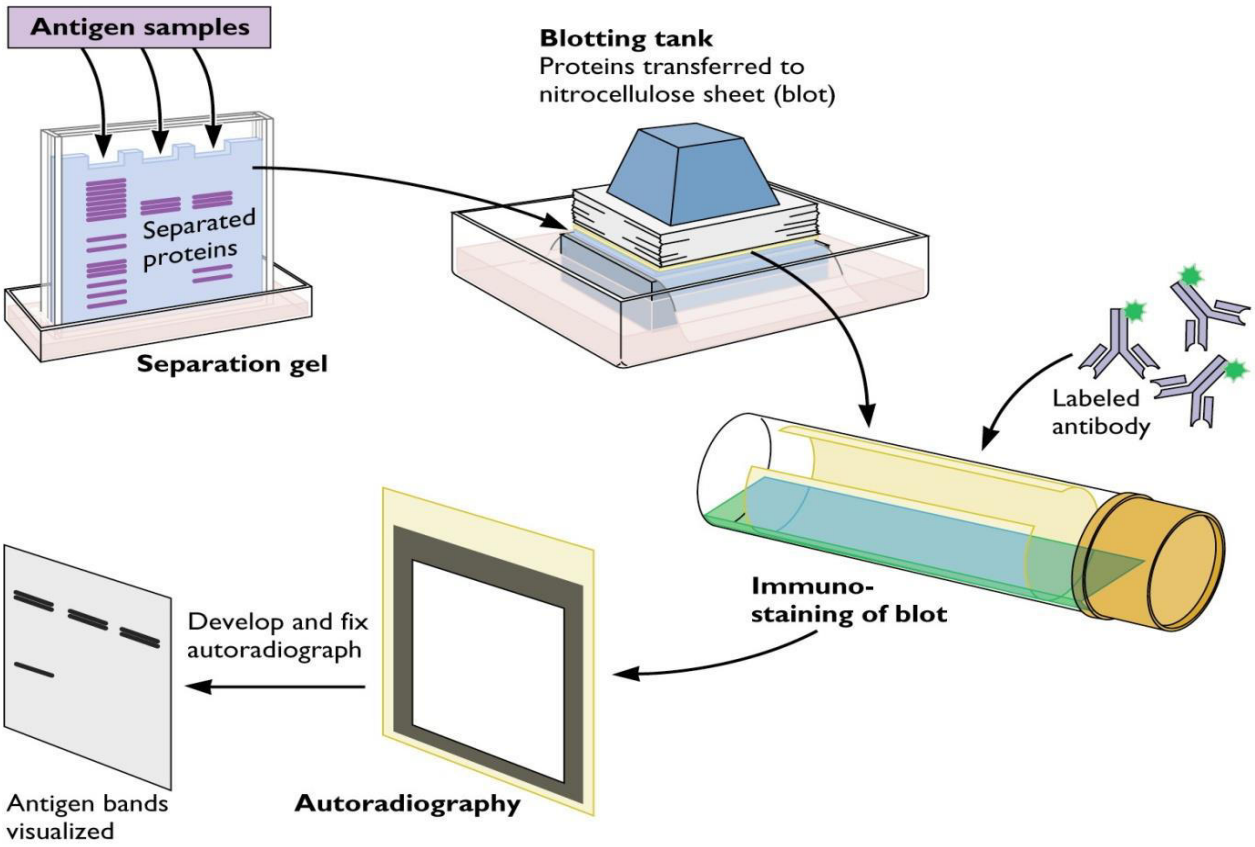


Fig. 49.Western blot

3.35. Electrophoresis

General Description

The term electrophoresis means Electro= electric field + Phoresis= migration. So as the name indicates, electrophoresis is a method of separation of charged molecules in an electric field.

The charged molecules under the influence of electric field migrate towards oppositely charged electrodes. The molecules with positive charge move towards cathode and molecules with negative charge move towards Anode. The migration is due to charge on the molecules and potential applied across the electrodes.

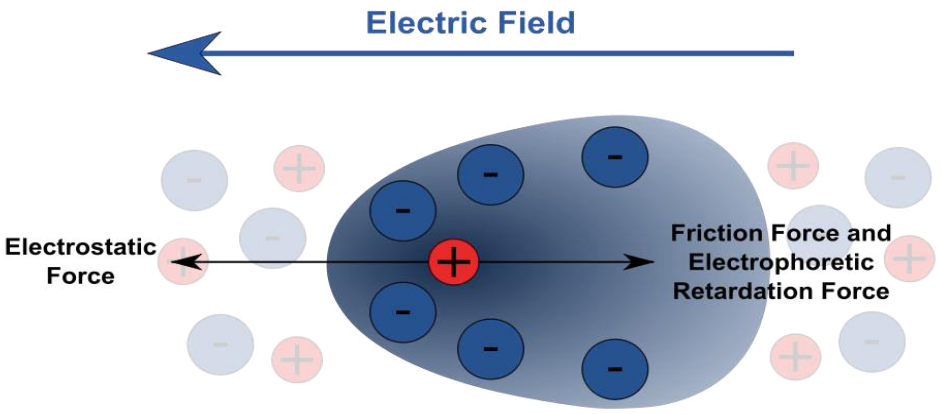


Fig.50. Movement of charged particles in an electric field

Principle

The charged molecule moves to their counter charge electrodes but electric field is removed before it reaches the electrode. Movement of the charged species in an electric field gives differential mobility to the sample based on the charge and consequently resolves them. Movement of the charged particle is retarded with the addition a polymeric gel so that a sufficient time is available for resolving the sample. The polymeric gel is inert, uncharged and does not cause retardation by binding the molecule. Instead it, forms pores of different size (depending on the concentration of polymer) and sample pass through this pore and as a result their electrophoretic mobility is reduced.

Electrophoretic techniques: Different types of electrophoresis techniques are designed depending upon whether it carried out in the presence or absence of a supporting media.

3.35.1. Moving boundary electrophoresis

In this method, the electrophoresis is carried in solution, without a supporting media. The sample is dissolved the buffer and molecules move to their respective counter charge electrodes. Moving boundary electrophoresis is carried out in a U shape tube with platinum electrodes attached to the end of both arms.

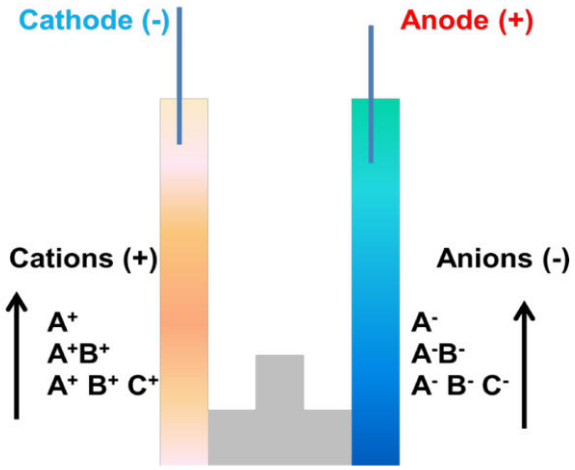


Fig.51. Moving boundary electrophoresis

At the respective ends, tube has refractometer to measure the change in refractive index of the buffer during electrophoresis due to presence of molecule. Sample is loaded in the middle of the U tube and then the apparatus is connected to the external power supply. Charged molecule moves to the opposite electrode as they passes through the refractometer, a change can be measured. As the desirable molecule passes, sample can be taken out from the apparatus along with the buffer.

3.35.2. Zone electrophoresis

In this method, an inert polymeric supporting media is used between the electrodes to separate and analyze 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. This is of two types:

3.35.2.1. Paper electrophoresis

Is a technique which employs a Whatman filter paper No.1 which is moistened by a buffer and then connected at two ends to two opposite charged electrodes.

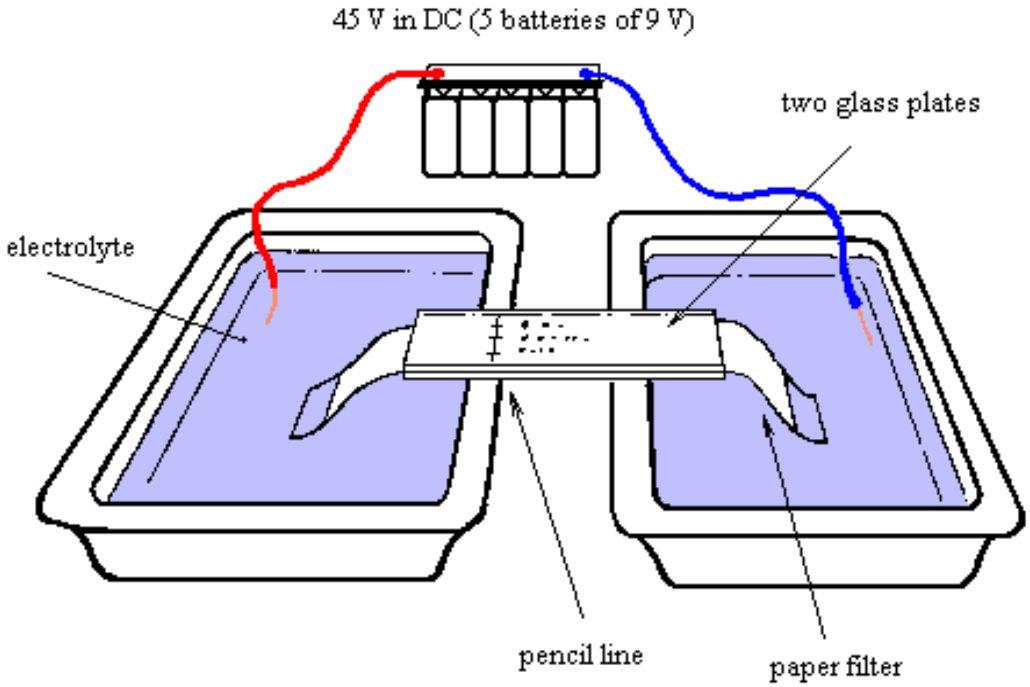


Fig.52.Paper electrophoresis

Then sample is applied on to one end and let for separation of components under electric field. After separation, the paper is dried and stained to get coloured bands.

3.35.2. 2. Gel electrophoresis

3.35.2.2.1. Vertical Gel Electrophoresis

The electrophoresis in this system performed in a discontinuous way with buffer in the upper and lower tank connected by the gel slab.

3.35.2.2.1. 1. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

PAGE: Polyacrylamide gel electrophoresis (PAGE) is probably the most common analytical technique used to separate and characterize proteins. A solution of Acryl amide and Bis-acryl amide is polymerized. Acrylamide alone forms linear polymers. The Bis-acrylamide introduces cross links between polyacrylamide chains. The 'pore size' is determined by the ratio of Acrylamide to Bis-acrylamide, and by the concentration of Acryl amide. A high ratio of Bis-acryl amide to Acrylamide and a high Acrylamide concentration cause low electrophoretic mobility. Polymerization of Acrylamide and Bis-acrylamide monomers is induced by Ammonium per sulphate (APS), which spontaneously decomposes to form free radicals. TEMED, a free radical stabilizer, is generally included to promote polymerization.

SDSPAGE: Sodium dodecyl sulfate (SDS) is an amphipathic detergent. It has an anionic head group and a lipophilic tail. It binds non-covalently to proteins, with a stoichiometry of around one SDS molecule per two amino acids. SDS causes proteins to denature and disassociate from each other (excluding covalent cross-linking). It also confers negative charge. In the presence of SDS, the intrinsic charge of a protein is

masked. During SDS PAGE, all proteins migrate toward the anode (the positively charged electrode). SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by molecular weight.

Instrument

It has two buffer chambers, upper chamber and a lower chamber. Both chambers are fitted with the platinum electrodes connected to the external power supply from a power pack which supplies a direct current or DC voltage. The upper and lower tank filled with the running buffer is connected by the electrophoresis gel casted in between two glass plates (rectangular and notched). There are additional accessories needed for casting the Polyacrylamide gel such as comb (to prepare different well), spacer, gel caster etc.

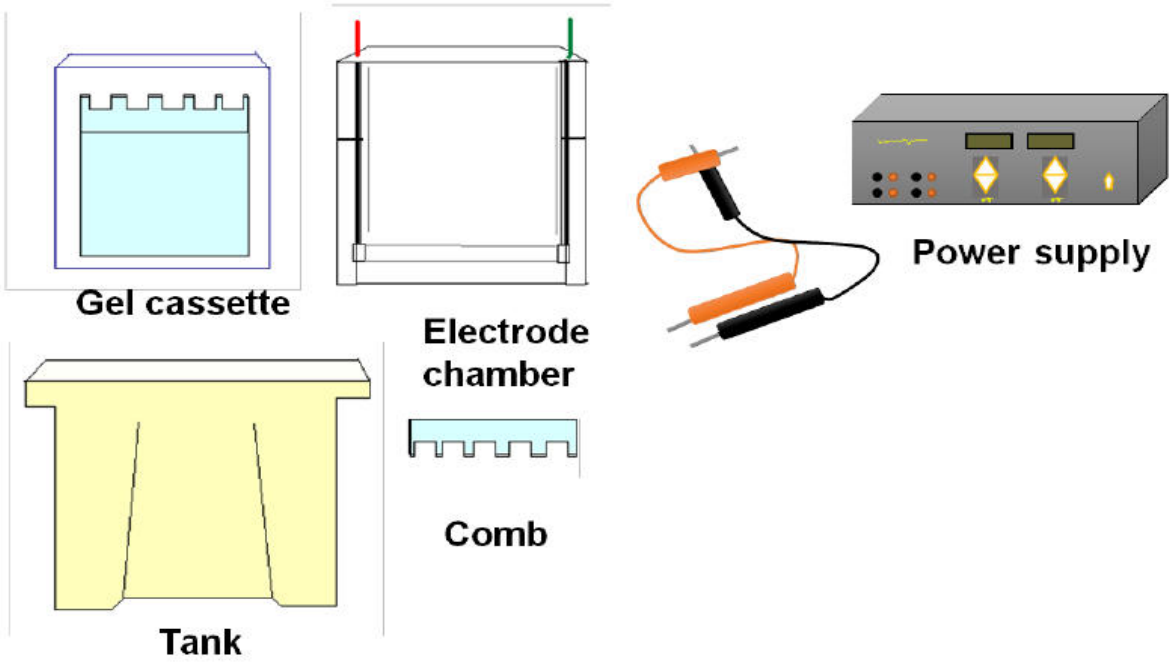


Fig.53. SDS-PAGE Instrument

Buffer and reagent for electrophoresis

- **N, N, N', N'-tetramethylethylenediamine (TEMED) -** it catalyzes the Acrylamide polymerization.
- **Ammonium persulphate (APS)-**it is an initiator for the Acrylamide polymerization.
- **Tris-HCl-** it is the component of running and gel casting buffer.
- **Glycine-** it is the component of running buffer.
- **Bromophenol blue**- it is the tracking dye to monitor the progress of gel electrophoresis.
- **Coomassie brilliant blue R250-** it is used to stain the Polyacrylamide gel.
- **Sodium dodecyl sulphate-** it is used to denature and provide negative charge to the protein.
- **Acryl amide (a potent neurotoxin)-**monomeric unit used to prepare the gel.
- **Bis-acrylamide-** cross linker for polymerization of Acrylamide monomer to form gel.

Casting of the gel

The Acrylamide solution (a mixture of monomeric Acrylamide and a bifunctional cross linker Bis-acrylamide) is mixed with the TEMED and APS and poured immediately in between the glass plate fitted into the gel caster. Ammonium persulphate in the presence of TEMED forms oxygen free radicals and induces the polymerization of Acryl amide monomer to form a linear polymer. These linear monomers are interconnected by the cross linking with Bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of Acryl amide and amount of Bis-acrylamide in the gel.

In a vertical gel electrophoresis system, we cast two types of gels, stacking gel and resolving gel. First the resolving gel solution is prepared and poured into the gel cassette for polymerization. A thin layer of organic solvent (such as butanol or isopropanol) is layered to stop the entry of oxygen (oxygen neutralizes the free radical and slow down the polymerization) and make the top layer smooth. After polymerization of the resolving gel, a stacking gel is poured and comb is fitted into the gel for construction of different lanes for the samples.

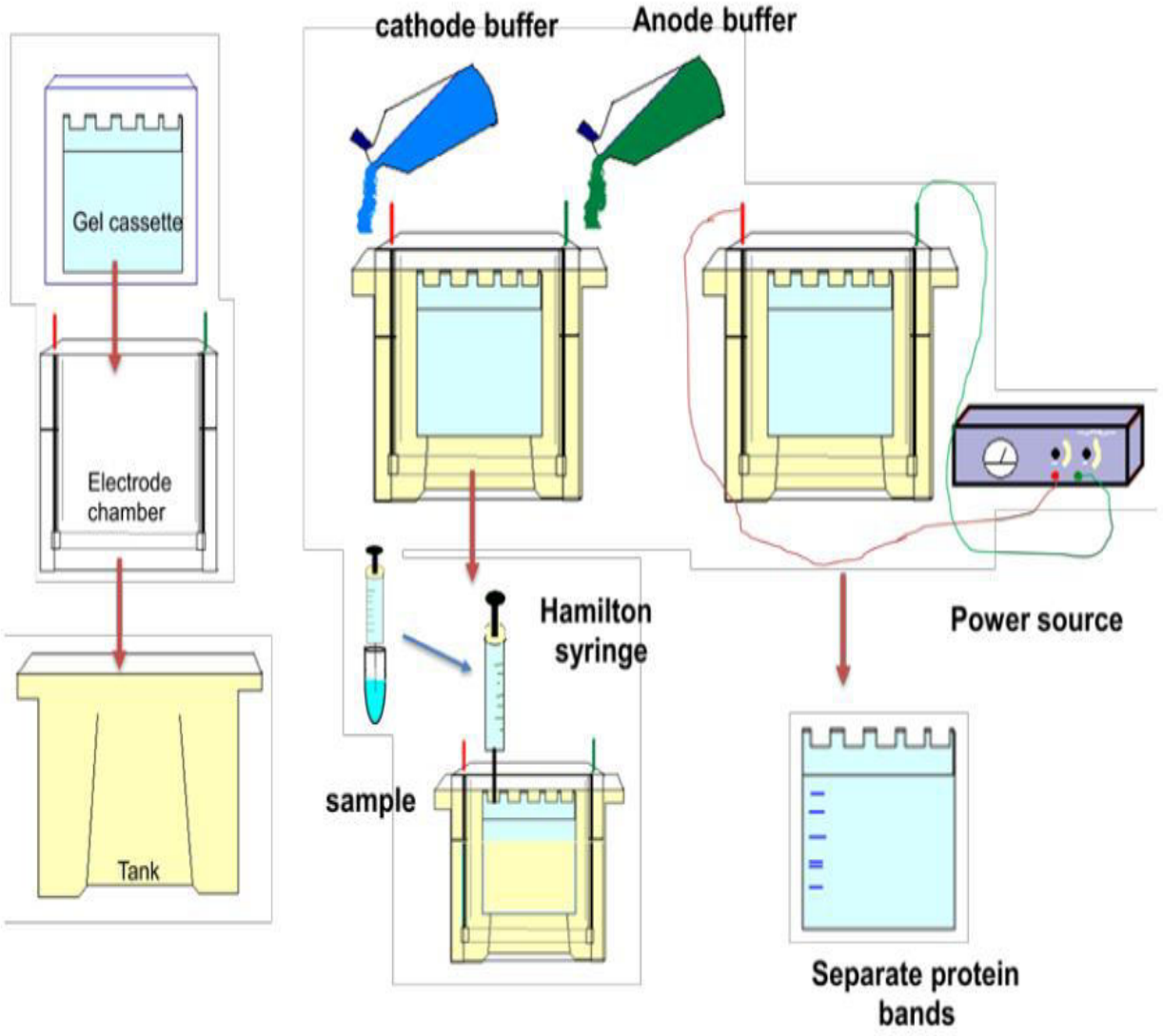


Fig.53.1 Casting of the Gel

Running of the gel

The sample is prepared in the loading dye containing SDS, β- mercaptoethanol in glycerol to denature the sample and presence of glycerol facilitates the loading of sample in the well. As the samples are filled vertically there is a distance drift between the molecules at the top Vs at the bottom in a lane. This problem is taken care once the sample runs through the stacking gel. The pH of the stacking gel is 6.8 and at this pH, glycine is moving slowly in the front whereas Tris- HCl is moving fast. As a result, the sample gets sandwiched between Glycine-Tris and get stacked in the form of thin band. As the sample enters into the resolving gel with a pH 8.8, the glycine is now charged, it moves fast and now sample runs as per their molecular weight (due to SDS they have equal negative charge). After tracking dye reaches to the bottom of the gel, gel is taken out from the glass plate with the help of a spatula and it is stained with Coomassie brilliant blue R250 dye. The dye stains protein present on the gel.

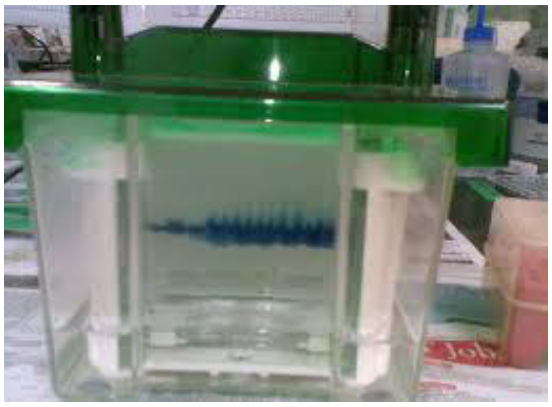


Fig.53.2.Running of the Gel

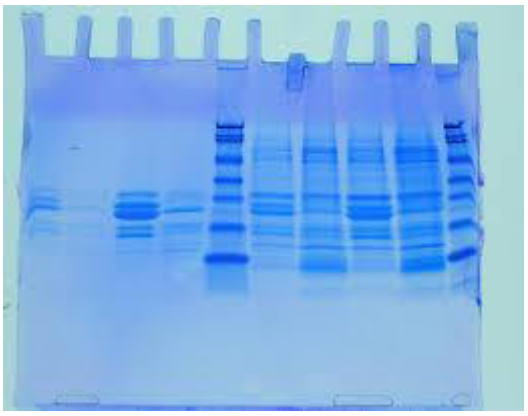


Fig.53.3. Stained Gel

3.35.2.2.2. Horizontal Gel Electrophoresis

The electrophoresis in this system is performed in a continuous way and the electrophoresis is performed in the horizontal direction.

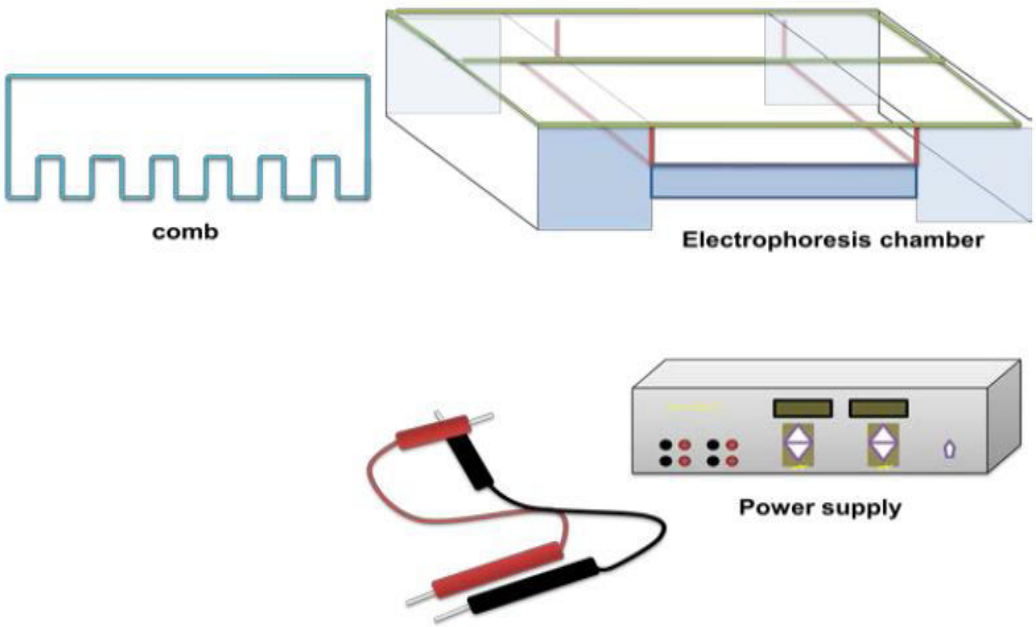


Fig.54. Horizontal Gel Electrophoresis

The electrophoresis in this gel system is performed in a continuous fashion with both electrodes and gel cassette submersed within the buffer. The electrophoresis chamber has two platinum electrodes placed on the both ends are connected to the external power supply from a power pack which supplies a direct current or DC voltage. The tank filled with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories needed for casting the agarose gel such as comb (to prepare different well), spacer, gel caster etc.

Buffer and reagent for electrophoresis

1. Agarose-polymeric sugar used to prepare horizontal gel for DNA analysis.

2. Ethidium bromide (a potent carcinogen)-for staining of the agarose gel to visualize the DNA.

3. Sucrose- For preparation of loading dye for horizontal gel.

4. Tris-HCl- The component of the running buffer.

5. Bromophenol blue-Tracking dye to monitor the progress of the electrophoresis.

Casting of the agarose gel

The Agarose powder is dissolved in a buffer (TAE or TBE) and heated to melt the Agarose. Hot Agarose is poured into the gel cassette and allowed it to set. A comb can be inserted into the hot Agarose to cast the well for loading the sample. In few cases, we can add Ethidium bromide within the gel so that it stains the DNA while electrophoresis.

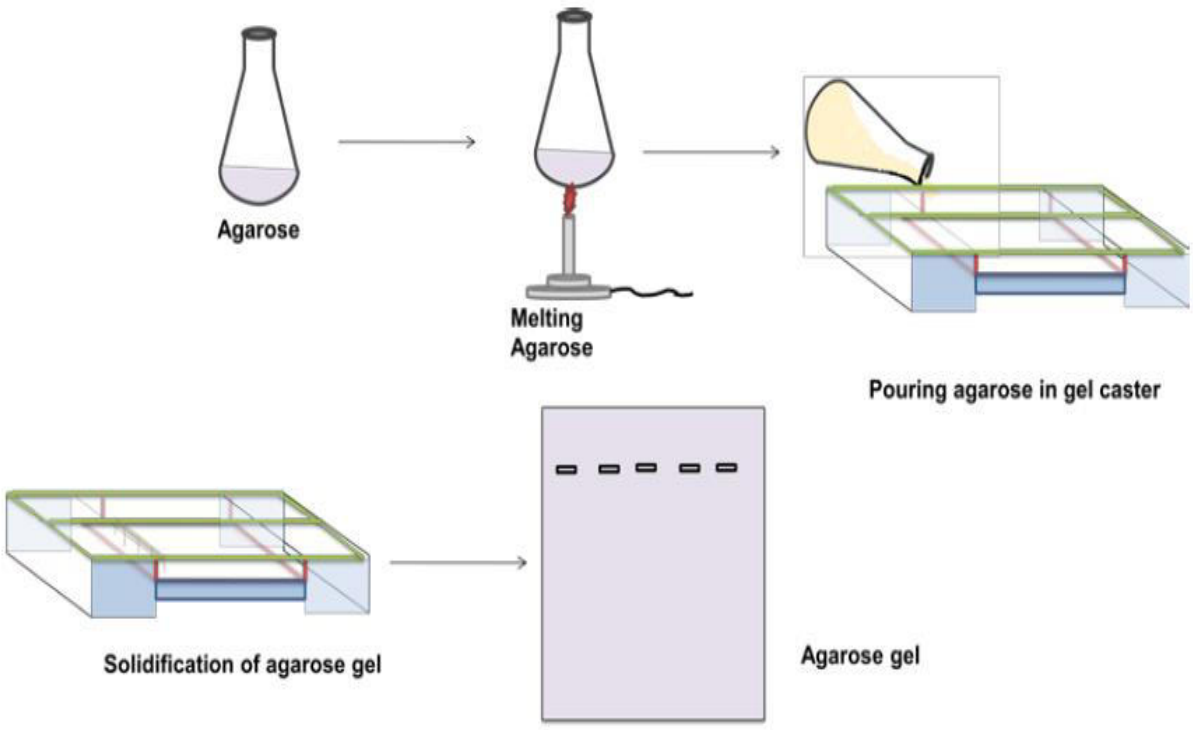


Fig.54.1. Casting of the Agarose Gel

Running and staining

The gel cassette is placed in the electrophoresis tank submerged completely and DNA loaded into the well with the help of pipetman and run with a constant voltage. DNA runs from negative to positive end and Ethidium bromide (EtBr) present in the gel stain the DNA. Observing the agarose gel in a UV-chamber shows the DNA stained with EtBr as orange coloured fluorescence.

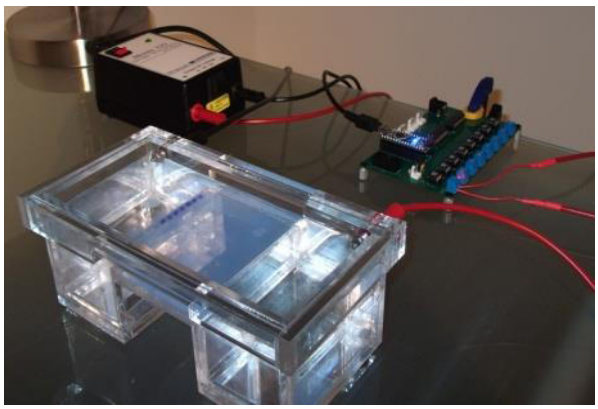


Fig.54.2.Running of the Gel

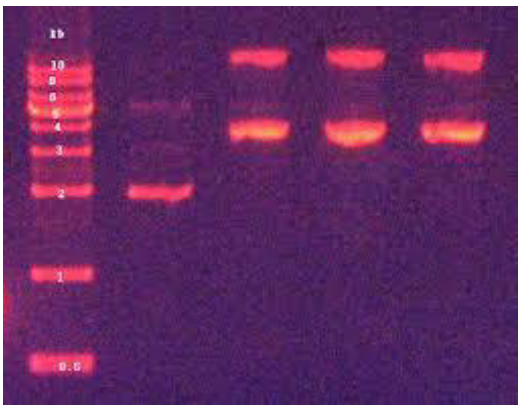


Fig.54.3. Visualization of the Gel

3.36. Chromatography

General Description

Chromatography is a physicochemical method for separation of complex mixtures.

Principle

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase: a separation is achieved.

Types of Chromatography

3.36.1. On the basis of interaction of solute to stationary

3.36.1.1. Adsorption Chromatography

Adsorption chromatography utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

Here the molecules or components of the mixture travel with different rates due to differences in their affinity towards stationary phase.

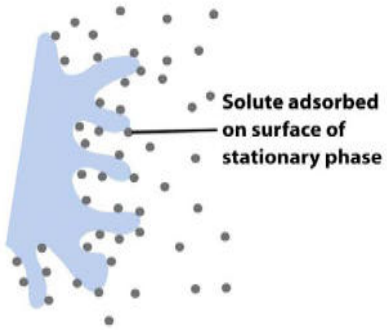


Fig.55. Adsorption Chromatography

Adsorption means a physical attachment between the compound and the particles of stationary phase. Based on the nature, polar compounds adsorb with stronger or greater intensity to the polar stationary phase while non-polar compounds adsorb better to the non-polar stationary phase than polar components. Hence during separation of components, when we use a polar stationary phase, polar components elute out late due to greater adsorption and non-polar components get out of the column or elute out first. This is exactly reverse on using a non-polar stationary phase.

3.36.1.2. Partition Chromatography

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.

Here the molecules get preferential separation in between two phases. i.e. both stationary phase and mobile phase are liquid in nature. So molecules get dispersed into either phase preferentially.

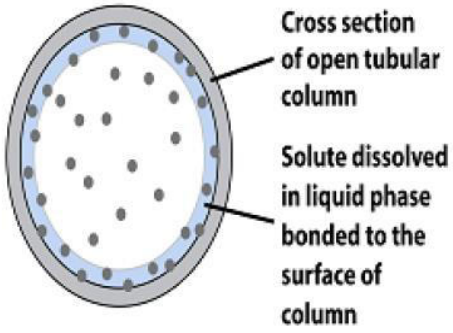


Fig.56. Partition Chromatography

Polar molecules get partitioned into polar phase and vice-verse. This mode of partition chromatography applies to liquid-liquid, liquid-gas chromatography and not to solid-gas chromatography. Because partition is the phenomenon in between a liquid and liquid or liquid and gas or gas and gas. But not in solid involvement.

3.36.1.3. Ion Exchange Chromatography

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces. Ion exchange chromatography is commonly used to separate charged biological molecules such as proteins, peptides, amino acids or nucleotides.

The amino acids that make proteins are zwitterionic compounds that contain both positively and negatively charged chemical groups. Depending on the pH of the environment, proteins may carry a net positive charge, a net negative charge, or no charge. The pH at which a molecule has no net charge is called its isoelectric point or PI.

In a buffer with a pH greater than the PI of the protein of the interest, the protein will carry a net negative charge; therefore, a positively charged anion exchange resin is chosen to capture this protein.

In a buffer with a pH greater than the PI of the protein of interest, the protein will carry a net negative charge; therefore, a positively charged anion exchange resin is chosen to capture this protein.

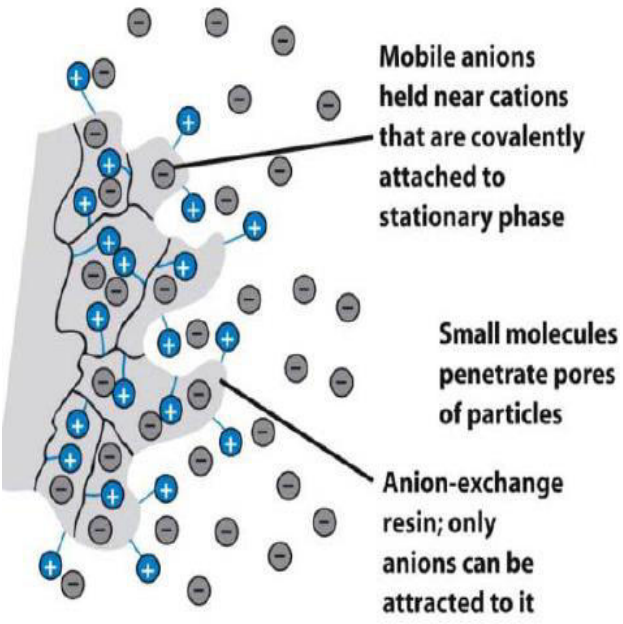


Fig.57. Ion Exchange Chromatography

In a buffer with a pH lower than the pI of the protein of interest, the protein will carry a net positive charge; therefore, a negatively charged cation exchange resin is chosen.

When an ion exchange chromatography column is loaded with a sample at a particular pH, all proteins that are appropriately charged will bind to the resin.

3.36.1.4. Molecular Exclusion Chromatography

Also known as gel permeation or gel filtration, this between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

Molecular exclusion chromatography separates molecules based on their size by filtration through gel. The gel consists of spherical beads containing pores of a specific size distribution.

Separation occurs when molecules of different sizes are included or excluded from the pores within the matrix. Small molecules diffuse in to the pores and their flow through the column is retarded according to their size, while large molecules do not enter the pores and are eluted in the columns void volume. Consequently, molecules separate based on their size as they pass through the column and are eluted in order of decreasing molecular weight.

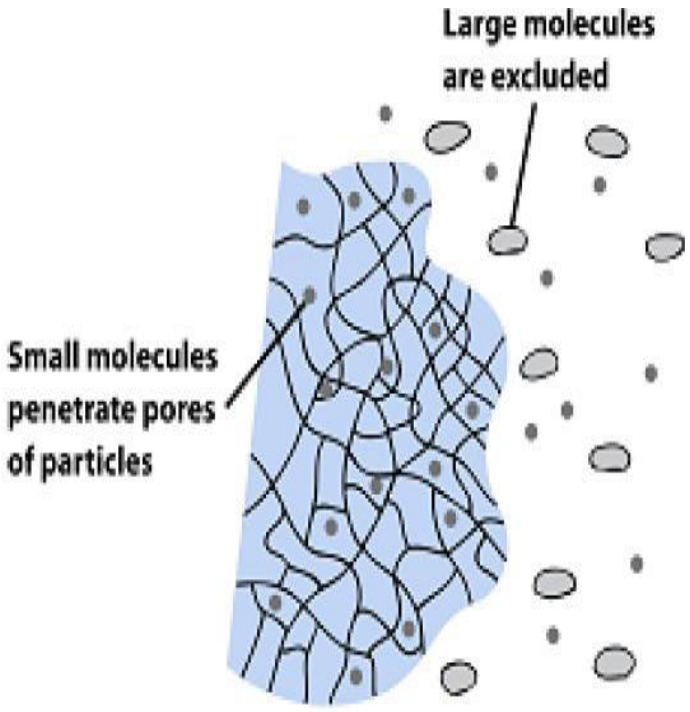


Fig.58. Molecular Exclusion Chromatography

3.36.2. On the basis of chromatographic bed

3.36.2.1. Column Chromatography

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample.

In Column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column. The mobile phase, a liquid, is added to the top and flows down through the column by either gravity or external pressure. Column chromatography is generally used as a purification technique: it isolates desired compound from a mixture.

The mixture to be analyzed by column chromatography is placed inside the top of the column. The liquid solvent (or eluent) is passed through the column by the application of air pressure. An equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column.

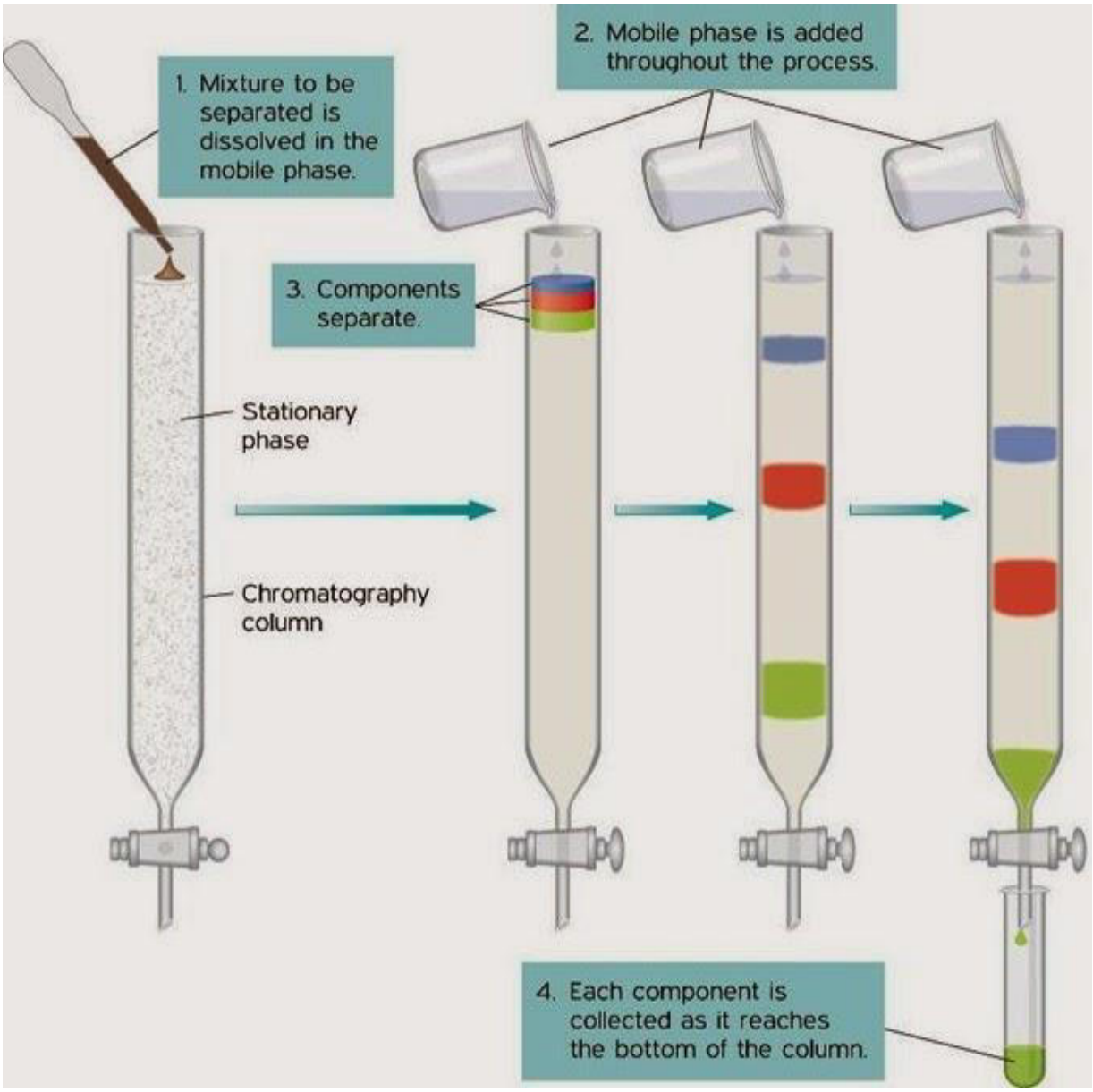


Fig.59. Column Chromatography

3.36.2.2. Planar Chromatography

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific Retention factor (R_f) of each chemical can be used to aid in the identification of an unknown substance.

3.36.2.2.1. Paper Chromatography

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of Cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

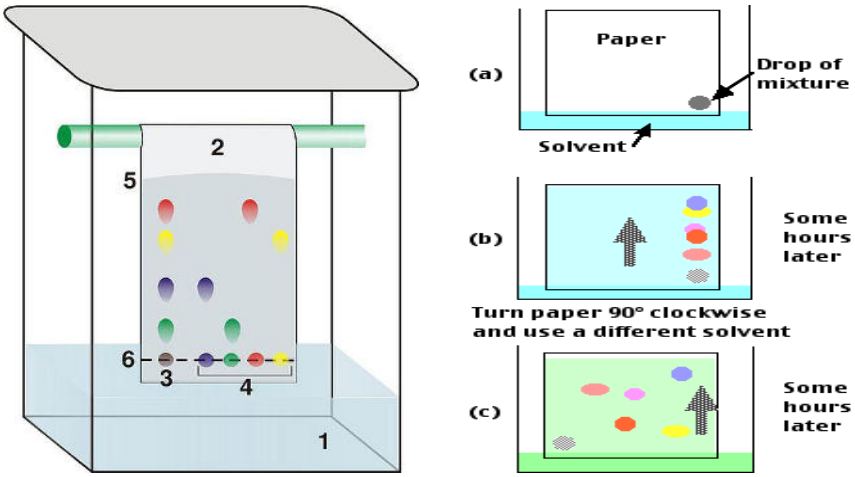


Fig.60. Paper Chromatography

3.36.2.2.2. Thin Layer Chromatography

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like Silica gel, Alumina, or Cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, high-performance TLC can be used.

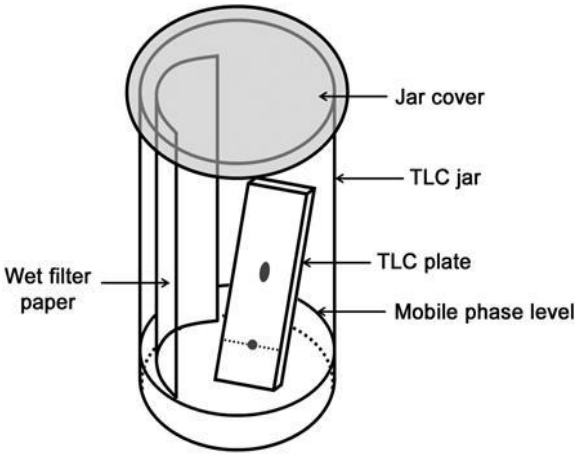


Fig.61.Thin Layer Chromatography

3.36.2.3. Techniques by physical state of mobile

3.36.2.3.1. Gas Chromatography

Gas chromatography (GC) is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically "packed" or "capillary". Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium).

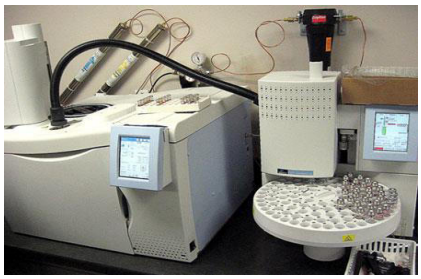


Fig.62. Gas Chromatography

The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical, environmental monitoring, and industrial chemical fields. It is also used extensively in chemistry research.

3.36.2.3.2. Liquid Chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred as high performance liquid chromatography (HPLC).

Fig.63. Liquid Chromatography

3.36.2.3.3. Affinity Chromatography

Affinity chromatography is based on selective non-covalent interaction between an analyte and specific molecules. Examples include antibody/antigen, enzyme/substrate, and enzyme/substrate interactions. It is very specific, but not very robust. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained. Affinity chromatography often utilizes a biomolecules affinity for a metal (Zn, Cu, Fe, etc.).

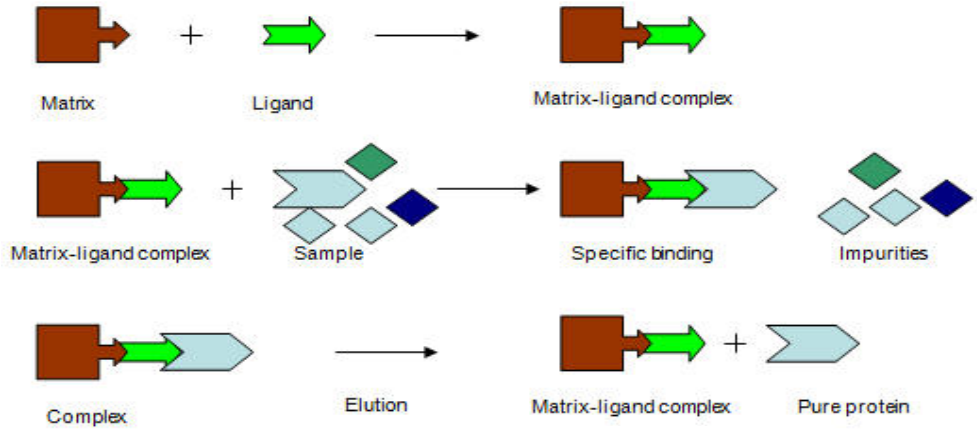


Fig.64. Affinity Chromatography

3.36.2.3.4. High Performance Liquid Chromatography (HPLC)

HPLC instruments could develop up to 6,000 psi of pressure, and incorporated improved injectors, detectors, and columns. With continued advances in performance during this time smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography. High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid.

Fig.65. HPLC

Instrumentation

- A **reservoir** (Solvent Delivery) holds the solvent (called the mobile phase, because it moves).
- A **high-pressure pump** solvent manager is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.
- An **injector** (sample manager or auto sampler) is able to introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column.
- The **column** contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware.
- A **detector** is needed to see the separated compound bands as they elute from the HPLC column (most compounds have no colour, so we cannot see them with our eyes). The mobile phase exits the detector and can be sent to waste, or collected, as desired.
- When the **mobile phase** contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is called preparative chromatography.
- The **high-pressure tubing and fittings** are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.
- The detector is wired to the **computer data** station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents.

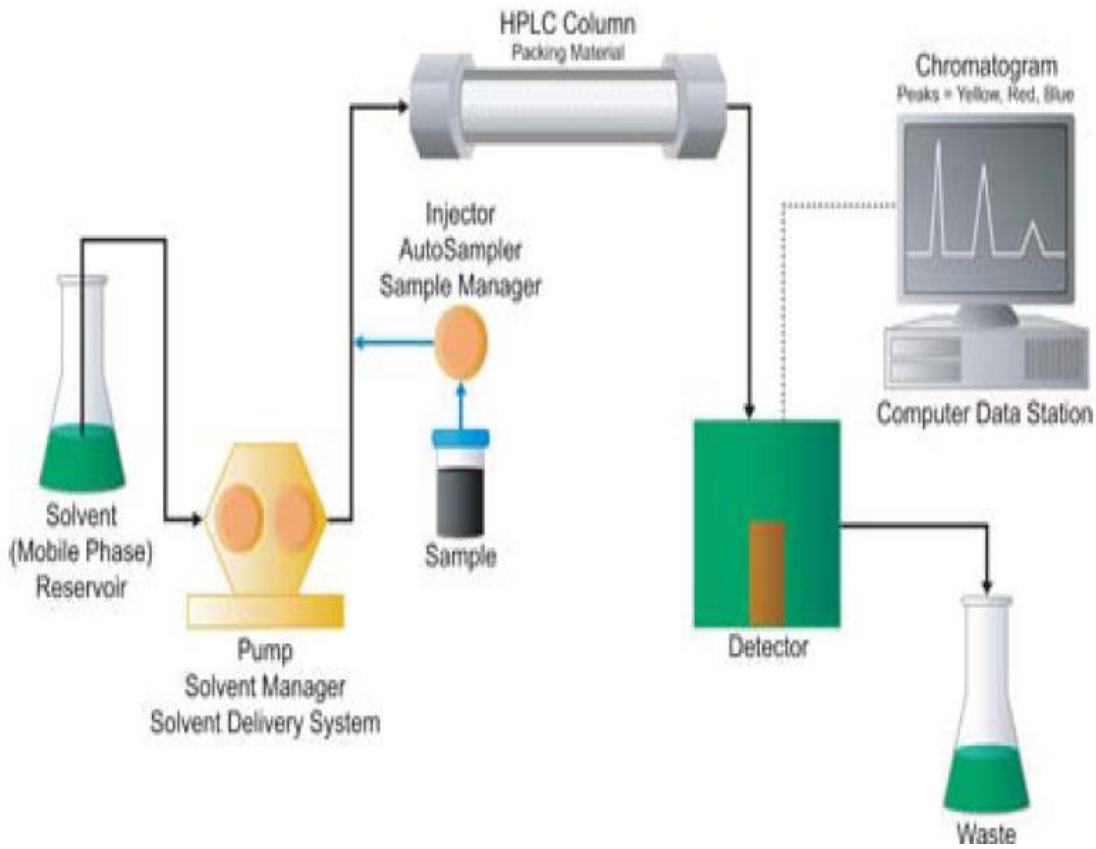


Fig.65.1. HPLC Instrumentation

Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an evaporative-light-scattering detector (ELSD). The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a mass spectrometer (MS) to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

3.37. Photometry and Spectrophotometry

Photometry is the measurement of the luminous intensity of light or the amount of luminous light falling on a surface from such a source. Spectrophotometry is the measurement of the intensity of light at selected wavelengths. The term photometric measurement was defined originally as the process used to measure light intensity independent of wavelength. Modern instruments isolate a narrow wavelength range of the spectrum for measurements. Those that use filters for this purpose are referred to as filter photometers, where as those that use prisms or gratings are called spectrophotometers.

Principle

Light can be described as a wave. This wave has an electric component and a magnetic component which are perpendicular to each other. Electromagnetic radiation exhibits a direction of propagation and wave-like properties (i.e., oscillations).

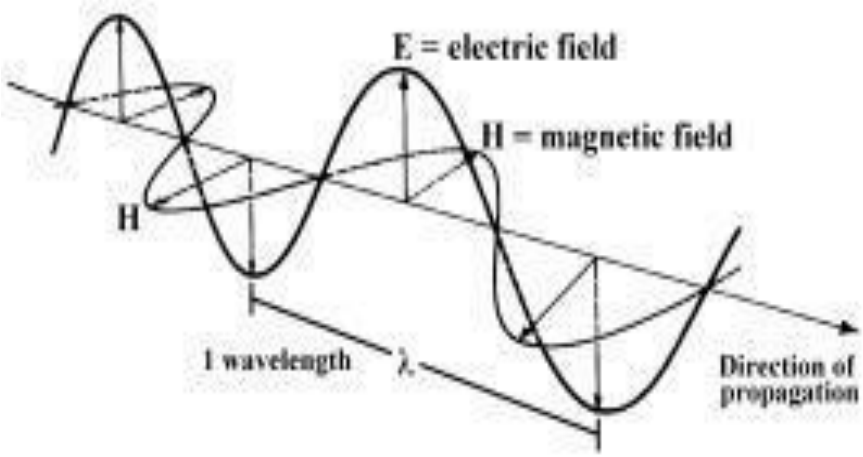


Fig.66. Definition of Light

The energy of electromagnetic radiation is defined as:

$$E = hc/\lambda = h\upsilon$$

Where, E = energy, h = Planck's constant, c = the speed of light, λ = the wave length, and υ= frequency

Light behaves both as a wave and as a particle. The conceptual particle of light is called a photon and is represented by hυ. Electromagnetic radiation exhibits a wide spectrum and specific ranges of wavelengths have names. The energy of electromagnetic radiation is inversely proportional to its wavelength.

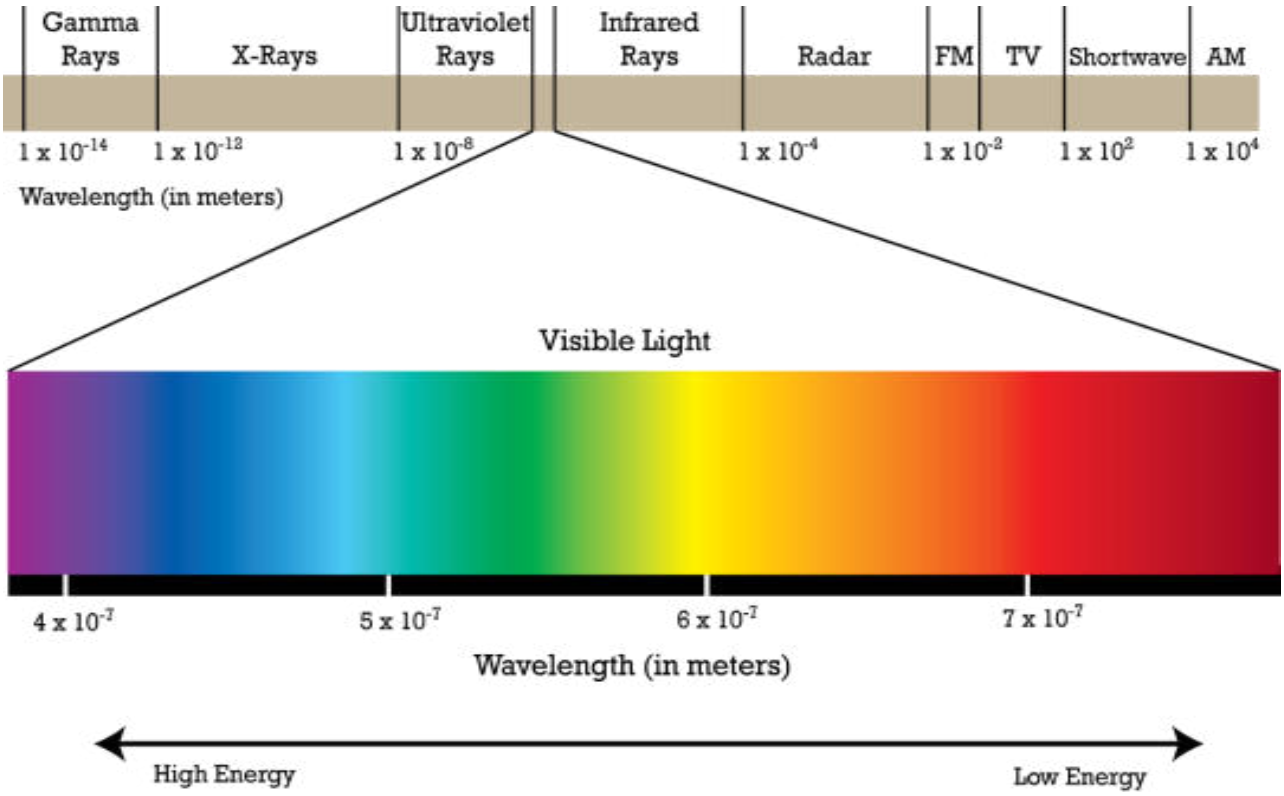


Fig.67. Electro Magnetic Spectrum

When a light wave encounters a particle, or molecule, it can be either scattered (i.e., direction changed) or absorbed (energy transferred). Molecules can only absorb discreet packets of energy, or quanta. Absorption occurs when the energy of the photon corresponds to differences between energy levels in that particular molecule. Absorption of the energy from the photon elevates the molecule to an excited state.

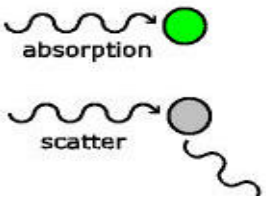


Fig.68. A light Wave

A molecule or substance that absorbs light is known as a chromophore. Chromophore exhibit unique absorption spectra and can be defined by a wavelength of maximum absorption, or λmax. A large number of biological molecules absorb light in the visible and ultraviolet (UV) range.

The net effect of absorption is that the intensity of the light decreases as it passes through a solution containing a chromophore. The amount of light absorbed depends on the nature of the chromophore, the concentration of the chromophore, the thickness of the sample, and the conditions (e.g.: pH, solvent, etc.)

Absorption is governed by the Beer-Lambert Law:

$$I = Io10^{-\varepsilon dc} \text{ or } \log(I/Io) = -\varepsilon dc$$

Where,

Io = initial light intensity, I = final light intensity, ε = molar extinction coefficient, d = thickness, and c = molar concentration.

Absorption (A) will be defined by: $A = -\log(I/Io) = \varepsilon dc$

3.37.1.1. Colorimeter

General Description

A colorimeter is a light sensitive device used for measuring the transmittance and absorbance of light passing through a liquid sample. The device measures the intensity or concentration of the colour that develops upon introducing a specific reagent into a solution.

Fig.69. Colorimeter

Instrumentation

The three main components of a colorimeter are a light source, a cuvette containing the sample solution, and a photocell for detecting the light passed through the solution.

The instrument is also equipped with either coloured filters or specific LEDs to generate colour. The output from a colorimeter may be displayed by an analog or digital meter in terms of transmittance or absorbance. In addition, a colorimeter may contain a voltage regulator for protecting the instrument from fluctuations in mains voltage. Some colorimeters are portable and useful for onsite tests, while others are larger, bench-top instruments useful for laboratory testing.

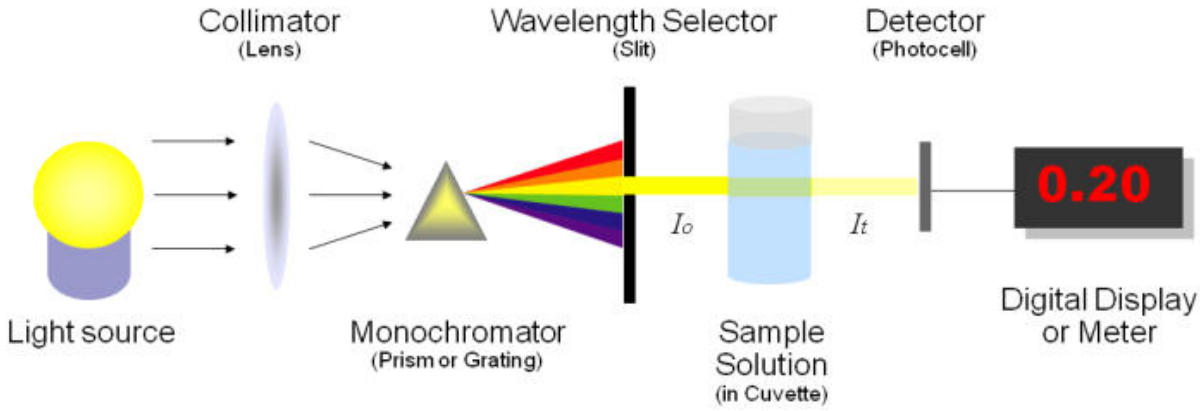


Fig.69.1. Instrumentation

Principle

The colorimeter is based on Beer-Lambert's law, according to which the absorption of light transmitted through the medium is directly proportional to the medium concentration.
According to Beer's law when monochromatic light passes through the coloured solution, the amount of light transmitted decreases exponentially with increase in concentration of the coloured substance.

$$I_t = I_o^{e\text{-}KC}$$

According to Lambert's law the amount of light transmitted decreases exponentially with increase in thickness of the coloured solution.

$$I_t = I_o^{e\text{-}kt}$$

Therefore, together Beer-Lambert's law is:

$$I_E/I_o = e^{-KCT}$$

Where,

I_E = intensity of emerging light, I_o = intensity of incident light, e = base of neutral logarithm, K = a constant, T = thickness of the solution and C = concentration

3.37.1.2. Spectrophotometer

Fig.69.2. Spectrophotometer

General Description

The spectrophotometer is an instrument which measures the amount of light of a specified wavelength which passes through a medium. They produce monochromatic light and then accurately measure the light intensity.

Principle

Spectrophotometer works with the principle of Beer-Lambert Law.

Instrumentation

The major components of a spectrophotometer are the light source, a monochromator, sample holder, a light detector (phototube), and a meter.

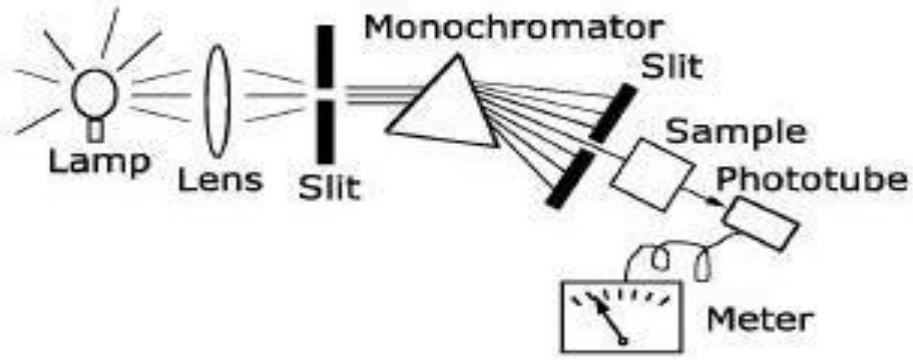


Fig.69.3. Instrumentation

In most instruments a tungsten lamp is used for the visible range and either high pressure H_2 or D_2 lamps are used for UV range. Monochromatic light is generated by either

1) A movable prism,

2) A diffraction gradient, or

3) Filters.

Working

1. White light from a bulb (source) is focused into a narrow beam by passing it through a thin slit.
2. A prism is used to split the beam of white light into its component colours, in the same way that water droplets can split sunlight into its component colours to make a rainbow. Different colours of light have a different wavelength: the distance between the peaks of the light waves, measured in nanometres (where 1 nanometre is 10^{-9}metres).
3. A second thin slit, just after the prism, can be moved from side to side to select just one colour of light to pass through to the sample.
4. The light passes through a container with the liquid sample inside (usually the light passes through 1 cm thickness of the liquid).

5. A light detector measures how much light is transmitted through the sample, and compares this with how much light was emitted by the source. The difference between these values gives a measure of how much light was absorbed by the sample: *i.e.,* the absorbance (A), often also called the optical density(OD). The absorbance varies with wavelength, so measurements of this type always specify the wavelength of light that was shone through the sample.

3.37.1.3. Flame Photometer

Fig.70. Flame Photometer

General Description

Flame photometry is a branch of atomic spectroscopy is used for inorganic chemical analysis for determining the concentration of certain metal ions such as sodium, potassium, lithium, calcium, cesium etc. In flame photometry the species (metal ions) used in the spectrum are in the form of atoms.

Principle

The basis of flame photometric working is that, the species of alkali metals (Group I) and alkaline earth metals (Group II) are dissociated due to the energy provided by the flame source. Atoms of many metallic elements, when given sufficient energy such as that supplied by a hot flame, emit this energy at wavelengths characteristic for the element. A specific amount or quantum of thermal energy is absorbed by an orbital electron. The electrons being unstable in this high energy (excited) state, release their excess energy as photons of a particular wavelength as they change from the excited state to their previous or ground state. If the energy is dissipated as light, the light may consist of one or more than one energy level and therefore of different wavelengths. These line spectra are characteristic for each element. Under constant and controlled conditions, the light intensity of the characteristic wavelength produced by each of the atoms is directly proportional to the number of atoms that are emitting energy, which in turns directly proportional to the concentration of the substance of interest in the sample.

Table.2. Elements, Emitted wavelength and Characteristic colour on Flame photometry

Name of the element	Emitted wavelength range (nm)	Observed colour of the flame
Potassium (K)	766	Violet
Calcium (Ca)	622	Orange
Sodium (Na)	589	Yellow
Magnesium (Mg)	285	Blue

Instrumentation

- **A cylinder of compressed gas:** Various combinations of gases include acetylene and oxygen for the hottest flame and natural gas acetylene and propane in combination with either O_2 or compressed air.

- **Two stage pressure regulator:** High pressure tubing must be used to lead the gases to the flame.
- **Source of flame**: A burner that provides flame and can be maintained in a constant form and at a constant temperature.
- **Nebulizer and mixing chamber:** Helps to transport the homogeneous solution of the substance into the flame at a steady rate.
- **Optical system (optical filter):** The optical system comprises three parts: convex mirror, lens and filter. The convex mirror helps to transmit light emitted from the atoms and focus the emissions to the lens. The convex lens help to focus the light on a point called slit. The reflections from the mirror pass through the slit and reach the filters. This will isolate the wavelength to be measured from that of any other extraneous emissions. Hence it acts as interference type colour filters.
- **Photo detector:** Detect the emitted light and measure the intensity of radiation emitted by the flame. That is, the emitted radiation is converted to an electrical signal with the help of photo detector. The produced electrical signals are directly proportional to the intensity of light.

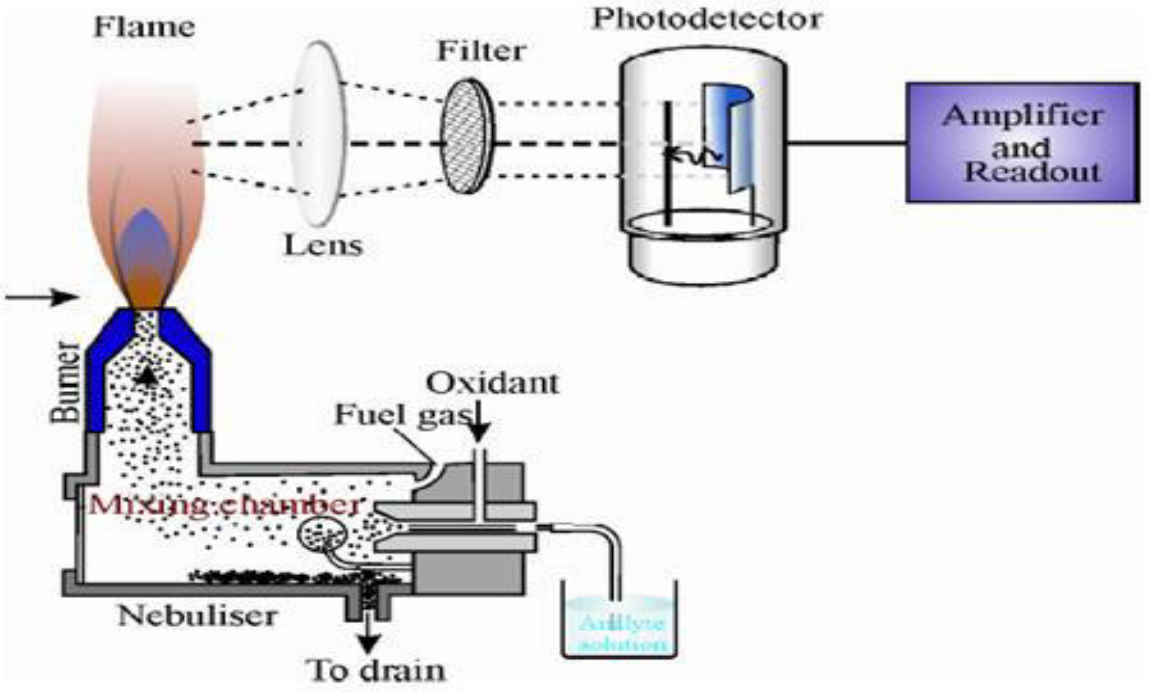


Fig.70.1. Instrumentation

Working

Nebulisation: The solution of the substance to be analyzed is first aspirated into the burner, which is then dispersed into the flame as fine spray particles.

Overview of the Process

- The solvent is first evaporated leaving fine divided solid particles.
- This solid particles move towards the flame, where the gaseous atoms and ions are produced.
- The ions absorb the energy from the flame and excited to high energy levels.
- When the atoms return to the ground state radiation of the characteristic element is emitted.
- The intensity of emitted light is related to the concentration of the element.

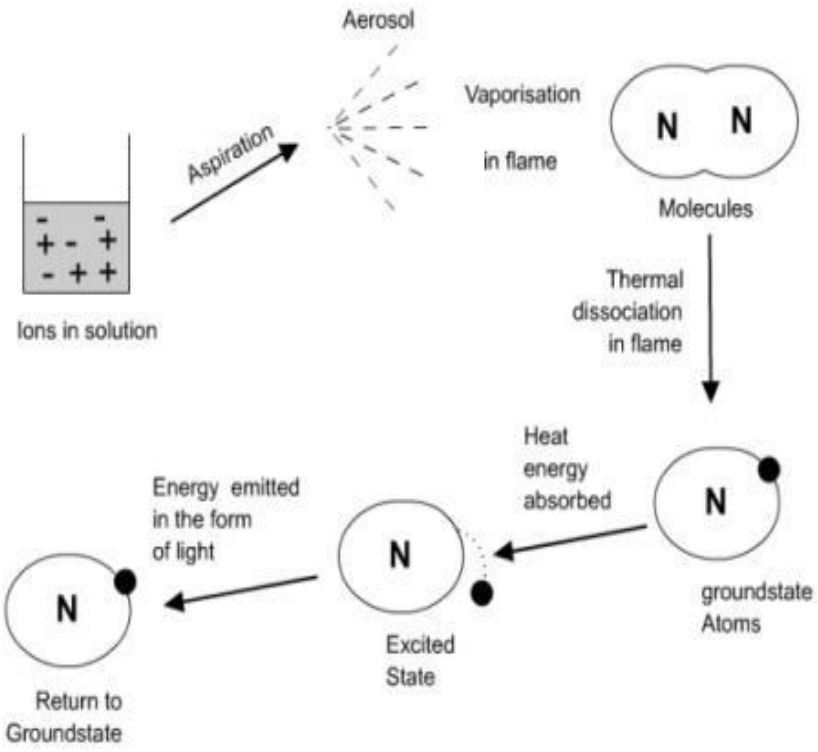


Fig.70.2. Overall Process occurs in a Flame Photometer

3.37.1.4. Atomic Absorption Spectrophotometer

General Description

Atomic absorption Spectrophotometry (AAS) is widely used for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. In AAS two main types of atomization sources used, flame and graphite furnace.

Fig.71. Atomic Absorption Spectrophotometer

Principle

Atomic Absorption (AA) occurs when a ground state atom absorbs energy in the form of light of a specific wavelength and is elevated to an excited state. The amount of light energy absorbed at this wavelength will increase as the number of atoms of the selected element in the light path increases. The relationship between the amount of light absorbed and the concentration of analytes present in known standards can be used to determine unknown sample concentrations by measuring the amount of light they absorb.

Instrumentation

Atomic absorption spectrophotometry requires a primary light source, an atom source. The light source normally used is a hollow cathode lamp (HCL) or an electrode less discharge lamp (EDL). In general, a different lamp is used for each element to be determined, although in some cases, a few elements may be combined in a multi-element lamp. The light from the lamp is directed through a flame onto a monochromator, which selects the preferred analytical wavelength. The light from the monochromator is detected by a photo multiplier tube and converted to an electrical signal.

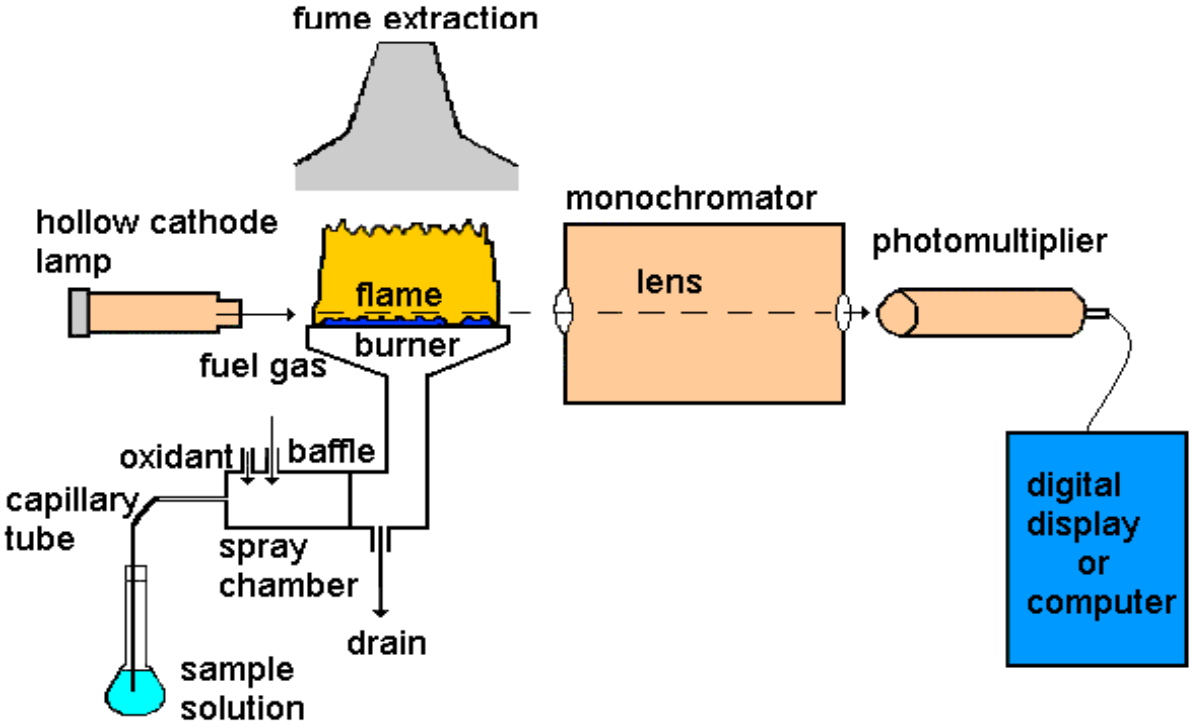


Fig.71.1. Instrumentation

Working

In general, after choosing the proper hollow cathode lamp for the analysis, the lamp should be allowed to warm up for a minimum of 15 minutes unless operated in a double beam mode. During this period, align the instrument, position the monochromator at the correct wavelength, select the proper monochromator slit width, and adjust the hollow cathode current according to the manufacturer's recommendation. Subsequently, light the flame and regulate the flow of fuel and oxidant, adjust the burner and nebulizer flow rate for maximum percent absorption and stability, and balance the photometer. Run a series of standards of the element under analysis and construct a calibration curve by plotting the concentrations of the standards against the absorbance. For those instruments which read directly in concentration set the curve corrector to read out the proper concentration. Aspirated the samples and determined the concentrations either directly or from the calibration curve. Standards must be run each time a sample or series of samples are run.

3.37.1.4.1. Graphite furnace AAS

Graphite Furnace Atomic Absorption Spectrometry (GFAAS) (also known as Electro thermal Atomic Absorption Spectrometry (ETAAS)) is a type of spectrometry that uses a graphite-coated furnace to vaporize the sample. In GFAAS, samples are deposited in a small graphite or pyrolytic carbon coated graphite tube, which can then be heated to vapourize and atomize the analyte.

Fig.72. Graphite furnace AAS

Principle

Graphite furnace AAS (GFAAS) involves electro thermal atomization inside a graphite tube furnace instead of flame atomization.

Instrumentation

- **A source of light** (lamp) that emits resonance line radiation.
- **An atomization chamber** (graphite tube) in which the sample is vapourized.
- **A monochromator** for selecting only one of the characteristic wavelengths (visible or ultraviolet) of the element of interest.
- **A detector,** generally a photomultiplier tube.
- **A signal processor,** computer system.

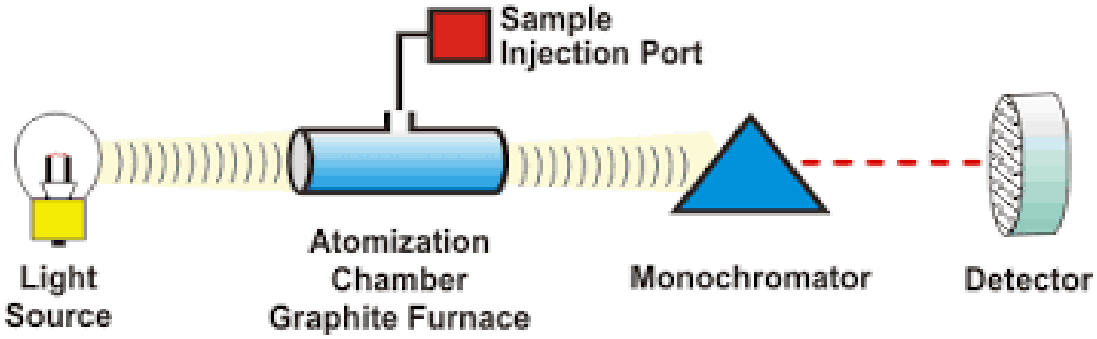


Fig.72.1. Instrumentation

Working

With Graphite Furnace Atomic Absorption (GFAA), the sample is introduced directly into a graphite tube, which is then heated in a programmed series of steps to remove the solvent and major matrix components and to atomize the remaining sample. All of the analyte is atomized, and the atoms are retained within the tube (and the light path, which passes through the tube) for an extended period of time.

Due to the small volume of the tube (9 x 50 mm), small volumes (1-100µl) of sample can be analyzed and the technique is more sensitive than flame AAS. Detection limits for most metals are 100-1000 lower with GFAAS than with flame AAS. GFAAS may be used to analyze those samples which contain metal concentrations below the detection limit of the flame AAS.

3.37.1.5. Mass spectrometer

Mass spectrometry is a powerful technique that is used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities (as little as 10^{-12} to 10^{-15} molecules for a compound of mass 1000 Daltons). The compounds can be identified at very low concentrations in chemical complex mixtures.

Fig.73. Mass spectrometer

Principle

A Mass spectrometer is an instrument that measures the masses of individual molecules that have been converted into ions, i.e., molecules that have been electrically charged.

Components of a Mass Spectrometer

Components of the mass spectrometer are:

- Inlet system
- Ion source
- Mass analyzer and
- Detector

Samples can be introduced to the mass spectrometer directly *via.* Solids probe, or in the case of mixtures, by the intermediary of chromatography devices (e.g. Gas chromatography, Liquid chromatography). Once in the source, sample molecules are subjected to ionization. Ions formed in the source acquire some kinetic energy and leave the source. A calibrated analyzer then analyzes the passing ions as function of their mass to charge ratios. Different kind of analyzer can be used viz. Magnetic, Quadrupole, Ion trap, Fourier Transform, Time of Flight etc. The ion beam exiting the analyzer assembly is then detected and the signal is registered.

The analyzer and detector of the mass spectrometer and often the ionization source too, are maintained under high vacuum to give the ions a reasonable chance of travelling from one end of the instrument to the other without any hindrance process also, is under complete data system control on modern mass spectrometers.

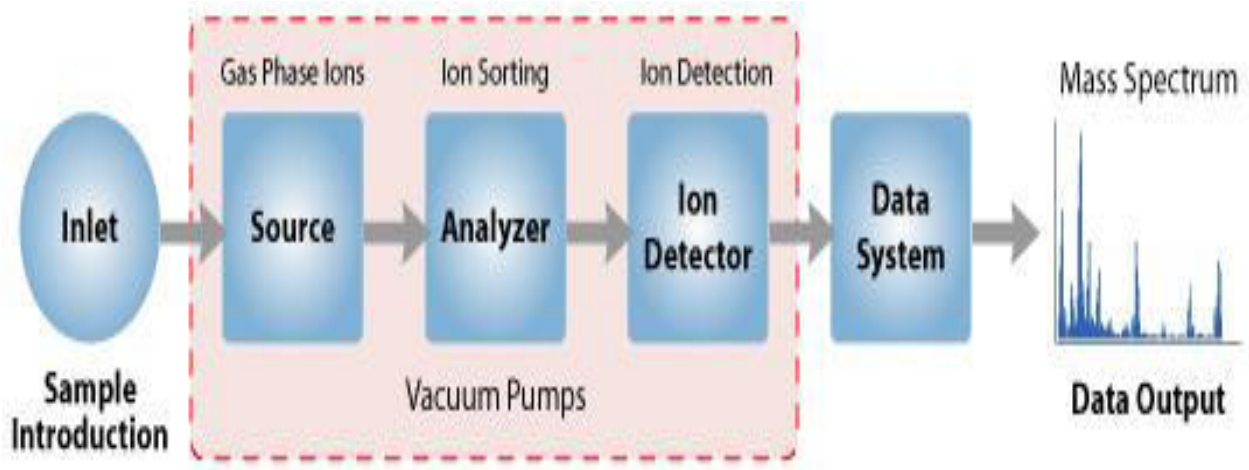


Fig.73.1. Instrumentation

Methods of Sample Ionization

❖ **Electron Ionization and Chemical Ionization**

Electron Ionization (EI) is widely used in mass spectrometry for relatively volatile samples that are insensitive to heat and have relatively low molecular weight. The spectra, usually containing many fragmentation peaks, are useful for structural characterization and identification. Small impurities in the sample are easy to detect. Chemical Ionization (CI) is used to enhance the abundance of the molecular ion. For both ionization methods, the molecular weight range is 50 to 800 Da.

❖ **Fast Atom Bombardment Ionization**

Fast atom bombardment ionization is a softer ionization method than EI. The spectrum often contains peaks from the matrix, which is necessary for ionization, a few fragments and a peak for a protonated or deprotonated sample molecules. FAB is used to obtain the molecular weight of sensitive, non-volatile compounds.

❖ **MALDI-TOF**

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules such as polymers and other macromolecules, which tend to be fragile and fragment when ionized by more conventional ionization methods. It is similar in character to electro spray ionization (ESI) in that both techniques are relatively soft ways of obtaining large ions in the gas phase, though MALDI produces far fewer multiply charged ions.

❖ **Electron spray Ionization (ESI)**

Electron spray Ionization allows production of molecular ions directly from samples in solution. It can be used for small and large molecular-weight biopolymers (proteins, peptides, carbohydrates and DNA fragments), and lipids. ESI is different than other atmospheric pressure ionization processes (e.g. MALDI) since it may produce multiply charged ions, effectively extending the mass range of the analyzer to accommodate the kDa-MDa orders of magnitude observed in proteins and their associated polypeptide fragments.

ESI uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. Ionic species in solution can thus be analyzed by ESI-MS with increased sensitivity. Neutral compounds can also be converted to ionic form in solution or in gaseous phase by protonation or cationisation (e.g. metal cationisation), and hence can be studied by ESI-MS.

The transfer of ionic species from solution into the gas phase by ESI involves three steps:

(1) Dispersal of a fine spray of charge droplets, followed by

(2) Solvent evaporation and

(3) Ion ejection from the highly charged droplets.

Tube, which is maintained at a high voltage (e.g. 2.5 – 6.0 KV) relative to the wall of the surrounding chamber. A mist of highly charged droplets with the same polarity as the capillary voltage is generated. The application of a nebulising gas (e.g. nitrogen), which shears around the eluted sample solution, enhances a higher sample flow rate. The charged droplets, generated at the exit of the electro spray tip, pass down a pressure gradient and potential gradient toward the analyzer region of the mass spectrometer. With the aid of an elevated ESI-source temperature and/or another stream of nitrogen drying gas, the charged droplets are continuously reduced in size by evaporation of the solvent, leading to an increase of surface charge density and a decrease of the droplet radius. Finally, the electric field strength within the charged droplet reaches a critical point at which it is kinetically and energetically possible for ions at the surface of the droplets to be ejected into the gaseous phase. The emitted ions

are sampled by a sampling skimmer cone and are then accelerated into the mass analyzer for subsequent analysis of molecular mass and measurement of ion intensity.

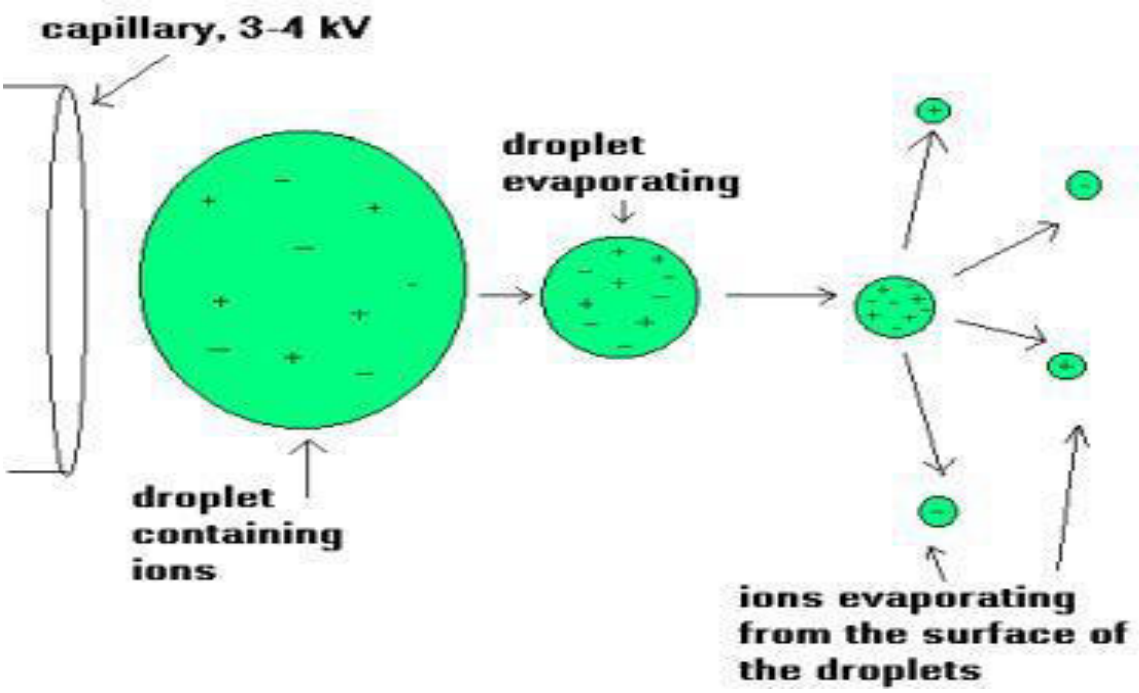


Fig.73.2. Electron spray Ionization

D. Fruit Analysis

4. Pineapple

Pineapple, *Ananas comosus*, belongs to the *Bromeliaceae* family. It originated in America and later spread in European countries. Now it is widely available in India. It is the most amazing fruit packed with nutrients and health benefits. Pineapples are actually not just one fruit but a composite of many flowers whose individual fruitlets fuse together around a central core. Each fruit let can be identified by an "eye," the rough spiny marking on the pineapples surface. Pineapples have a wide cylindrical shape, a scaly green, brown or yellow skin and a regal crown of spiny, blue-green leaves. The fibrous flesh of pineapple is yellow in colour and has a vibrant tropical flavour that balances the tastes of sweet and tart. The area closer to the base of the fruit has more sugar content and therefore a sweeter taste and more tender texture.

Fig.74. Pineapple *(Ananas comosus)*

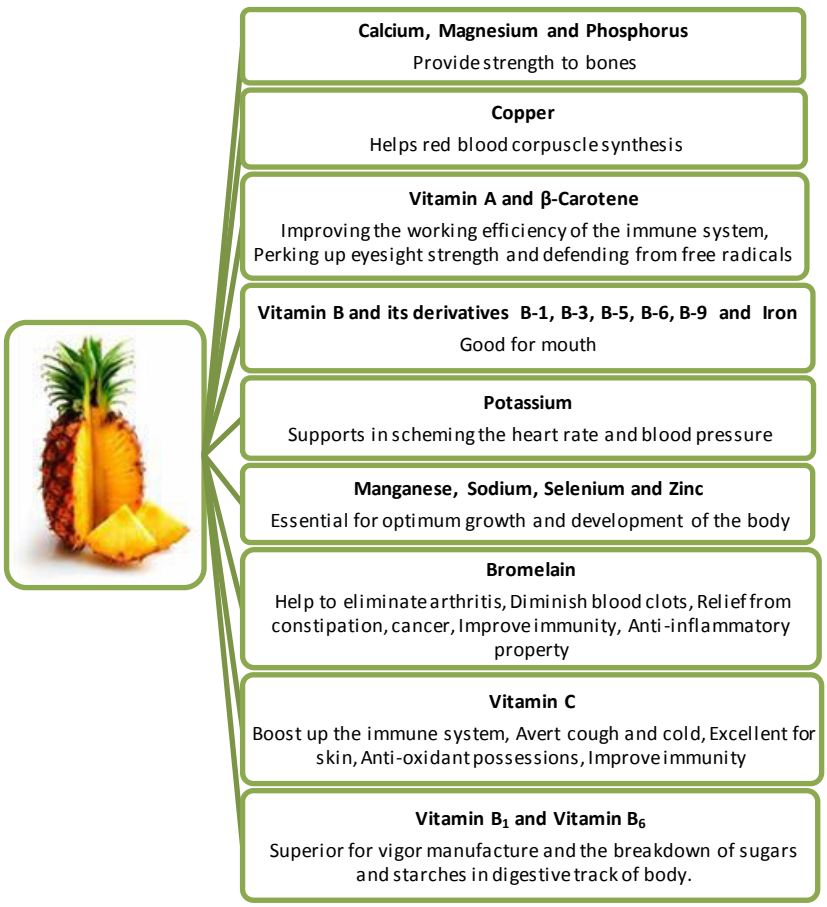


Fig.75. Health Benefits of Pineapple

Table.3. Pineapple Nutritive Value per 100 g

Principle	Nutrient Value	Percentage of RDA (%)
Energy	50 Kcal	2.5
Carbohydrates	13.52 g	10
Protein	0.54 g	1
Total Fat	0.12 g	<1
Cholesterol	0 mg	0
Dietary Fiber	1.40 g	4
Vitamins		
Folates	18 µg	4.5
Niacin	0.500 mg	4
Pyridoxine	0.112 mg	9
Riboflavin	0.018 mg	1.5
Thiamin	0.079 mg	6.5
Vitamin A	58 IU	2
Vitamin C	47.8 mg	80
Vitamin E	0.02 mg	<1
Vitamin K	0.07 µg	0.5
Minerals		
Sodium	1 mg	0
Potassium	109 mg	2.5
Calcium	13 mg	1.3
Copper	0.110 mg	12
Iron	0.29 mg	3.5
Magnesium	12 mg	3
Manganese	0.927 mg	40
Phosphorus	8 mg	1
Selenium	0.1 µg	<1
Zinc	0.12 mg	1
Phyto Nutrients		
Carotene-β	35 µg	--
Crypto-xanthin-β	0 mcg	--

4.1. Basic Fruit Analysis of Pineapple

4.1.1. Collection of Pineapple fruit Samples

4.1.1.1. Selection of Fruits

All the fruits are required for the physical or chemical studies. Selection is randomized or individual fruits are selected for studies. Select a pineapple that is plump and fresh-looking. The leaves in the crown should be fresh and green, and the body of the pineapple firm. The first method is to look at the scales on the side of the pineapple. These are called eyes. If a pineapple has eyes of a uniform size all the way to the top, that's a good sign the pineapple is ripe.

Fig.76. Selection of Pineapple Fruits

Avoid the ones where the eyes near the top are significantly smaller than the ones at the base. The second is to smell the bottom of the pineapple where the cut stem is located. It should have a faint pineapple scent, but should not smell too strong or fermented. Too strong a pineapple smell means that the pineapple is overripe and might be mushy. Picking the small leaves on the top of the pineapple near the centre of the rosette can also tell you if it is ready, as long as the leaves are not wilted.

4.1.1.2. Method of Plucking

Plucking of pineapple fruit is actually done with a sharp cutting instrument, can be a cutter or a knife which gives a clean and smooth cut on the stock. Try to get a uniform stock length. Proper documentation and labeling were done prior and after harvesting.

Fig.77. Plucking of Pineapple Fruit

4.1.1.3. Sorting

Fruits for analysis are sorted. The fruits showing any symptom of infection or damage on surface should be scrupulously rejected and only healthy are sorted for conducting study.

4.1.1.4. Surface Cleaning

After cutting off the stalks or other foliage parts which remain attached to the fruits after harvest, they should be meticulously cleaned before use. Surface cleaning can be done by using clean dry/ moistened cloth piece.

4.1.1.5. Bringing to analytical laboratory

Fruits of pineapple are brought to the laboratory as soon as possible after they are plucked and stored in the same atmosphere conditions to maintain the minimal change of the physio-chemical conditions.

4.1.2. Determination of Constituents by Physical Methods

There may in fact, be a large number of physical characteristics of fruits that are worthy to test. Those are as follows

4.1.2.1. Weight

Weight of a fruit is considered to be an important factor in judging its compactness, maturity, juice content, carbohydrate and other chemical constituents. It is done by physical balance. Balance should be properly set, placed and leveled, accuracy ensured before use, weighing done accurately and the reading noted carefully.

Fig.78. Balance

4.1.2.2. Volume

Volume that is the size of a fruit is another important factor. In market consumers prefer large-sized ones for many fruits. Volume of the fruit can be determined by measuring the volume of a liquid that is water which is actually displaced by it.

4.1.2.3. Overall Length

The length of a fruit is referred to by many as the space, that is, straight line distance between its stalk-end and the stylar end. It appears to be more appropriate to consider the total length of a fruit, which may be termed as its overall length. This can be done by slide calipers, L-shaped sets etc.

Fig.79. Slide caliper

4.1.2.4. Maximum width

To refer width, the diameter of a fruit in its centre is emphasized. It can be done by measuring the distance from the extreme points at two sides using slide calipers, L-shaped sets etc.

4.1.2.5. Shape

Fruits belonged to a species or a variety of it has some characteristic shape of their own, although variation within some limit is not considered to be an uncommon feature.

4.1.2.6. Firmness/ Pulp firmness

Fig.80. Penetrometer

Firmness of a healthy fruit is linked to the degree of its physiological maturity. With progress of development, maturation and ripening either in the pre-harvest or in the post-harvest condition, the fruits undergo gradual softening to a greater or a lesser extent depending on species, varieties, environment and the use of agro-inputs. Enzymatic conversion of pectic compounds may cause this. This can be measured by penetrometer, by hand-feeling, and by pin drop method.

4.1.2.7. Peel colour

The colour of the fruit surface is an important factor in determining an appeal to the consumers. The change in colour is due to accumulation of one or more forms of pigments in different combinations. This can be measured by, the use of colour-dictionary, eye-estimation etc.

Fig.81. Peel colour

4.1.2.8. Peel smoothness

The peels of fruits get smoothened with advancement of their development, maturation and ripening. In pineapple, the eyes become less raised. It can be judged by hand feeling of a group of testers.

Fig.82. Peel smoothness

4.1.2.9. Peel wax

Plant wax is an ester of a higher fatty acid with long chain alcohol other that glycerol. It can be done by simply rubbing the surface with fingers or using a tissue paper.

4.1.2.10. Peel thickness

The thickness of the peel is considered to be a character of importance of many fruits. To measure the peel thickness, it has to be excoriated from the fruit. It should be carefully cut out with a knife in order to separate from the inner part of the fruit. Slide calipers are used to measure the thickness.

Fig.83. Peel thickness

4.1.2.11. Colour of the edible part

The colour of the inside part undergoes change with progress of their development. The color changes are due to various combinations of chlorophylls, carotene, xanthophylls, anthocyanin, anthoxanthin etc. So, by estimating the relative intensity of the colour may be done by chemical analysis of pigments or by several group eye estimations. Juice colour can be determined by optical density value, which is done in colorimeter. Absorbance is obtained against distilled water.

Fig.84. Colour of the edible part

4.1.2.12. Edible matter content

Although in fruit culture, the quantitative productivity of a fruit crop is conventionally determined in considering the yield of the whole fruits or the number of them that are harvested from a given number of trees or an unit area of land, it is more apposite to determine how much matter that is consumable to the human beings has actually been obtained from the same trees or plants or the same area of land. In pineapple outer rachis, bract, perianth and pericarp all fused together. In it usually we get approximately 68% of human consumable matter. It can be measured by,

Fig.85. Edible matter content

Weight of consumable matter (g)/weight of whole intact fruits (g) x 100

or

Volume of juice (ml)/ weight of the whole intact fruit (g) x 100

4.1.2.13. Flavour

Flavour is charm to a fruit. Flavour is due to the existence of adverse type of volatile compounds e.g., alcohols, esters, aldehydes, ketones, ethers, halides, hydrocarbons and others in different proportions. It will change with development. It can be measured by ultra-sophisticated chemical procedures or by smelling power of expert persons.

4.1.2.14. Seed content

Presence of seeds in a fruit is considered a demerit or a merit. Consumers always prefer seedless fruits. But seeds are required for the production of seedlings. Seed content can be measured by conventional way, cutting and removed by rubbing and washing. Records to keep usually include the number of bold and less bold seeds per fruit and weight of one hundred seeds. Viability of seeds also sometimes considered.

Fig.86. Seed content

4.3. Sample Preparation for Chemical Analysis

Pineapple is belonging to the pulpy fruits. The pulpy fruit consists of those, fleshy pulp of which serves as the edible part.

Firstly, the fleshy edible part of a pulpy fruit should be scrupulously taken out from the given sample of fruit. The non-edible parts of them have no use and hence, are discarded. Then the pulpy fruit is cut into very small pieces after which, the pieces should thoroughly mixed together and piled at a place. The pile is then disrupted to make several lots from it at random basis. From each lot, some pieces are picked up randomly and these are mixed together and heaped again while the remaining ones are discarded. This heap of pieces is broken and several lots are made in the same way as before. This process is repeated several times and lastly, the representative sample for analysis is obtained.

5. Passion fruit

Passion fruit is a member of Passiflora and grows on a climbing plant. Passion fruit is native from Brazil. It is a woody, perennial vine that bears a delicious fruit .There is mainly three types of passion fruit under cultivation. They are the yellow passion fruit **(***Passiflora edulis forma flavicarpa***)** which is suited to tropical conditions or the plains, the purple passion fruit (*Passiflora edulis forma edulis*) which grows best under sub-tropical conditions or high altitudes and the giant granadilla (*Passiflora quadrangularis*).

The plants have a weak taproot and extensive ivory-coloured lateral roots. The stem is usually solitary, up to 7 cm in basal diameter, extends 5 to 10 m or more into the crowns of trees, and is covered by a thin, flaky, light brown bark. The stem-wood is light and brittle. The twigs are yellow-green, turning brown, and support themselves on vegetation by means of tendrils that arise at the leaf axils. The leaves are alternate, green to yellow-green, three-lobed (on mature plants) with serrate edges. The petioles are 3 to 6 cm long and the blades are 5 to 11 cm long by 4 to 10 cm broad. Solitary flowers arise at the leaf axils. The flowers measure 5 to 7 cm across with five greenish-white sepals and five white petals topped with a fringe-like corona of straight purple and white rays. There are five stamens with large anthers and a triple-branched style. The fruit is globose or ovoid, purple or yellow and 4 to 7 cm in diameter. Inside a thick rind are many dark-brown to black seeds enveloped in small sacs filled with aromatic yellow or orange juice.

Giant granadilla (***Passiflora quadrangularis***)

The giant granadilla, *Passiflora quadrangularis*, produces the largest fruit any species within the genus Passiflora. The fruit appears to be in round or ovoid shape and measuring about 20 to 30 centimetres in length and 18 centimetres in diameter. The fruit has a tough peel with a waxy nature; the peel ranges in shade from dark purple to light yellow or pumpkin colour which is sweet and acidulous.

Fig.87. Giant granadilla (*Passiflora quadrangularis*)

Health Benefits of Giant Granadilla

- Use as an antiscorbutic (prevention or relief of scurvy) and stomachic.
- The flesh is set as a sedative to get relieve from the nervous headache, asthma, diarrhea, dysentery, neurasthenia and insomnia.
- The leaf decoction is a vermifuge (a medicine that expels intestinal worms) and is used for bathing skin afflictions (Pain or suffering).
- Leaf poultices are applied for liver complaints.
- Powdered root when mixed with oil is used as a soothing poultice.
- The leaf extraction is used as a medicine to treat intestinal writhes cured.

Table.4. Giant granadilla Nutritive Value per 100 g

Principle	Thick Flesh	Arils and Seeds
Moisture	94.4 g	78.4 g
Protein	0.112 g	0.299 g
Fat	0.15g	1.29 g
Crude Fiber	0.7 g	3.6 g
Ash	0.41 g	0.80 g
Minerals		
Calcium	13.8 mg	9.2 g
Phosphorus	17.1 mg	39.3 g
Iron	0.80 mg	2.93 mg
Vitamins		
Thiamine	0.004 mg	0.019 mg
Riboflavin	-	0.003 mg
Niacin	0.033 mg	0.120 mg
Ascorbic Acid	0.378 mg	15.3 mg
Phyto Nutrients		
Carotene	14.3 mg	-

Yellow Passion fruit (*Passiflora edulis forma flavicarpa*)

Yellow passion fruit is more energetic in comparison to the purple variety. These fruits are spherical to oblong and have a smooth yellow surface. Their pericarp is tougher and they are larger with a length of 6 to 12 centimetres and diameter of 4 to 7 centimetres. They are comparatively less savory and a little more acidic.

Fig.88. Yellow passion fruit (*Passiflora edulis forma flavicarpa*)

Purple Passion fruit (*Passiflora edulis forma edulis*)

This is one of the most common varieties which feature tiny globular or ovoid fruits, about 4 to 9 centimetres in length and 4 to 7 centimetres in diameter. They have a breakable pericarp and a darkish yellow pulp which occupies 35 to 50% of the fruit's weight.

Fig.89. Purple passion fruit (*Passiflora edulis forma edulis*)

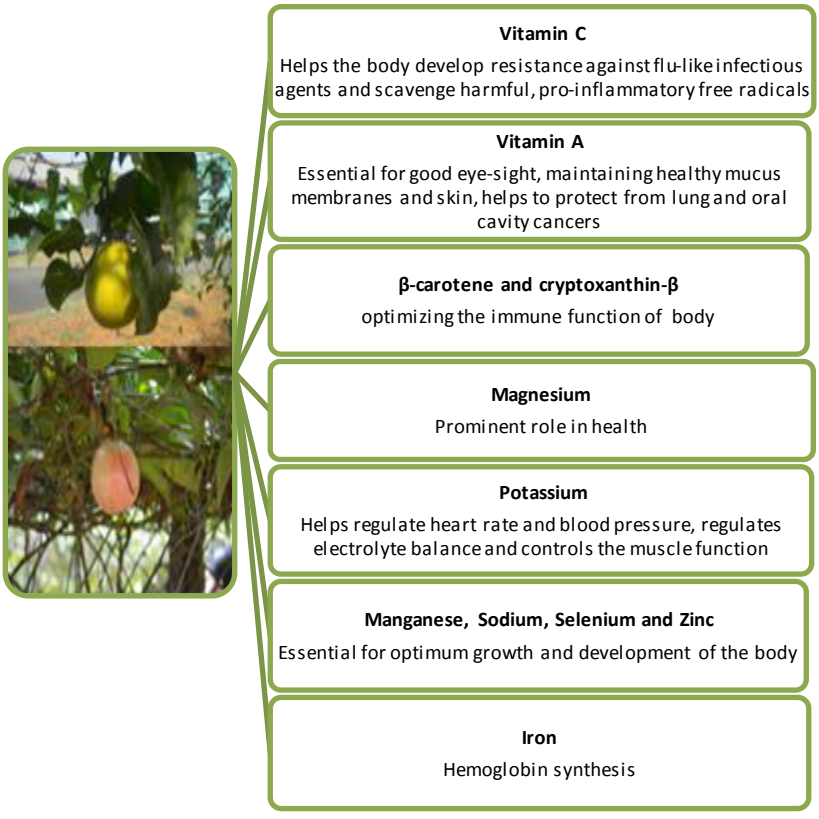


Fig.90. Health Benefits of Purple passion fruit and Yellow passion fruit

Table.5. Purple passion fruit and Yellow passion fruit Nutritive Value per 100 g

Principle	Nutrient Value	Percentage of RDA
Energy	97 Kcal	5%
Carbohydrates	23.38 g	18%
Protein	2.20 g	4%
Total Fat	0.70 g	3%
Cholesterol	0 mg	0%
Dietary Fiber	10.40 g	27%
Vitamins		
Folates	14 µg	3%
Niacin	1.500 mg	9%
Pyridoxine	0.100 mg	8%
Riboflavin	0.130 mg	10%
Thiamin	0.00 mg	0%
Vitamin A	1274 IU	43%
Vitamin C	30 mg	50%
Vitamin E	0.02 µg	<1%
Vitamin K	0.7 mg	0.5%
Minerals		
Sodium	0 mg	0%
Potassium	348 mg	7%
Calcium	12 mg	1.2%
Copper	0.086 mg	9.5%
Iron	1.60 mg	20%
Magnesium	29 mg	7%
Phosphorus	68 mg	10%
Selenium	0.6 µg	1%
Zinc	0.10 mg	1%
Phyto Nutrients		
β -Carotene	743 µg	--
Cryptoxanthin-β	41 µg	--
Lycopene	0 µg	--

5.1. Basic Fruit Analysis of Passion Fruit

5.1.1. Collection of Passion fruit Samples

5.1.1.1. Selection of Fruits

All the fruits are required for the physical or chemical studies. Selection is randomized or individual fruits are selected for studies. Select a passion fruit looking wrinkled skin, indicating it is ripe.

Fig.91. Selection of Fruits

5.1.1.2. Method of Plucking

Plucking of passion fruit is actually done with a sharp cutting instrument which gives a clean and smooth cut on the part and hence, cutting with a knife or pulling a fruit by hand is not considered to be a correct procedure.

5.1.1.3. Sorting

The fruits for analysis are collected at random basis, not only the healthy ones but those infected with insect pests or diseases, or mechanically damaged may also be plucked. At the first step, the fruits showing any symptom of infection or damage on surface should be rejected such that only those which at least outwardly look healthy are only sorted out for conducting study.

Fig.92. Sorting

5.1.1.4. Surface Cleaning

The fruits are surface cleaned by rubbing it with a piece of dry clean cloth, or rubbing it with moistened and then with a dry cloth-piece. If this procedure is not enough to clean it properly, washing with clean and preferably running water.

5.1.1.5. Bringing to analytical laboratory

Passion fruits are brought to the laboratory as soon as possible after they are plucked and stored in the same atmosphere conditions to maintain the minimal change of the physio-chemical conditions.

5.1.2. Determination of Constituents by Physical Methods

There may in fact, be a large number of physical characteristics of fruits that are worthy to test. Those are as follows

5.1.2.1. Weight

Fig.93. Balance

Weight of a fruit is considered to be an important factor in judging its compactness, maturity, juice content, carbohydrate and other chemical constituents. It is done by physical balance. Balance should be properly set, placed and leveled, accuracy ensured before use, weighing done accurately and the reading noted carefully.

5.1.2.2. Volume

Volume that is the size of a fruit is another important factor. In market consumers prefer large-sized ones for many fruits. Volume of the fruit can be determined by measuring the volume of a liquid that is water which is actually displaced by it.

5.1.2.3. Overall Length

Fig.94. Slide caliper

The length of a fruit is referred to by many as the space, that is, straight line distance between its stalk-end and the stylar end. It appears to be more appropriate to consider the total length of a fruit, which may be termed as its overall length. This can be done by slide calipers, L-shaped sets etc.

5.1.2.4. Maximum Width

To refer width, the diameter of a fruit in its centre is emphasized. It can be done by measuring the distance from the extreme points at two sides using slide calipers, L-shaped sets etc.

The practice lies in testing whether the fruit passes or not through a ring of desired diameter. If the fruit passes out, it is considered not to have fully grown, till then for which it is left to grow further *in-situ* instead of harvesting at that stage. On the other hand, if it fails to pass through the ring, it is considered to have grown to desired degree and hence, it is harvested then.

5.1.2.5. Shape

Fruits belonged to a species or a variety of it has some characteristic shape of their own, although variation within some limit is not considered to be an uncommon feature.

5.1.2.6. Firmness/ Pulp firmness

Firmness of a healthy fruit is linked to the degree of its physiological maturity. With progress of development, maturation and ripening either in the pre-harvest or in the post-harvest condition, the fruits undergo gradual softening to a greater or a lesser extent depending on species, varieties, environment and the use of agro-inputs. Enzymatic conversion of pectic compounds may cause this. This can be measured by penetrometer, by hand-feeling, and by pin drop method.

Fig.95. Penetrometer

5.1.2.7. Peel colour

The colour of the fruit surface is an important factor in determining an appeal to the consumers. The change in colour is due to accumulation of one or more forms of pigments in different combinations. This can be measured by, the use of colour-dictionary, eye estimation etc.

5.1.2.8. Peel smoothness

The peels of fruits get smoothened with advancement of their development, maturation and ripening.

5.1.2.9. Peel wax

Plant wax is an ester of a higher fatty acid with long chain alcohol other that glycerol. It can be done by simply rubbing the surface with fingers or using a tissue paper.

5.1.2.10. Peel thickness

The thickness of the peel is considered to be a character of importance of many fruits. To measure the peel thickness, it has to be excoriated from the fruit. It should be carefully cut out with a knife in order to separate from the inner part of the fruit. Slide – calipers is used to measure the thickness.

Fig.96. Peel thickness

5.1.2.11. Colour of the edible part

The colour of the inside part undergoes change with progress of their development. The colour changes are due to various combinations of chlorophylls, carotene, xanthophylls, anthocyanin, anthoxanthin etc. So, by estimating the relative intensity of the colour may be done by chemical analysis of pigments or by several group eye estimations. Juice colour can be determined by optical density value, which is done in colorimeter. Absorbance is obtained against distilled water.

Fig.97. Colour of the edible part

5.1.2.12. Edible matter content

Although in fruit culture, the quantitative productivity of a fruit crop is conventionally determined in considering the yield of the whole fruits or the number of them that are harvested from a given number of trees or an unit area of land, it is more apposite to determine how much matter that is consumable to the human beings has actually been obtained from the same trees or plants or the same area of land. In passion fruit usually we get approximately 43-56% of human consumable matter. It can be measured by,

Weight of consumable matter (g)/weight of whole intact fruits (g) x 100

or

Volume of juice (ml)/ weight of the whole intact fruit (g) x 100

Fig.98. Edible matter content

5.1.2.13. Flavour

Flavour is charm to a fruit. Flavour is due to the existence of adverse type of volatile compounds e.g., alcohols, esters, aldehydes, ketones, ethers, halides, hydrocarbons and others in different proportions. It will change with development. It can be measured by ultra-sophisticated chemical procedures or by smelling power of expert persons.

5.1.2.14. Seed content

Presence of seeds in a fruit is considered a demerit or a merit. Consumers always prefer seedless fruits. But seeds are required for the production of seedlings. Seed content can be measured by conventional way, cutting and removed by rubbing and washing. Records to keep usually include the number of bold and less bold seeds per fruit and weight of one hundred seeds. Viability of seeds also sometimes considered.

Fig.99. Seed content

5.3. Sample Preparation for Chemical Analysis

Passion fruit is belonging to the Juicy fruits. The juicy fruits comprise those, in which, only the juice of the fruits forms the consumable part.

In the case of juicy fruits, extraction of juice is needed to be done at first. A commonly available fruit juice extractor having perforations at the bottom and lower part of it is convenient to squeeze out juice from fruits. The fruits are to be taken in the cup of the extractor to its capacity and it's covered with the lid. Then pressure is given on the handle, due to which the juice will be squeezed and will come outside through the perforations which are collected below.

After squeezing, the lid should be opened and the fruits should be stirred to turn the upper ones to the lower and the lower ones to the upper layers. Then again it is covered with lid and pressed to squeeze out the juice. The process is repeated several times till the recovery of juice is complete. The extracted juice need to be made free from pulp-pieces from which, it should be filtered to get the clear juice having no opacity.

E. Quality Indices of Fruits

Quality in fruits, as in other products, is that combination of characteristics which makes them desirable to the buyer or user. Fruits are ordinarily chosen by appearance, because other methods for determination of quality are rarely available to the casual purchaser. Thus, to the vendor of fruits, whether at the field, the wholesale display, or in the retail store, such factors as size, shape, colour and freedom from external defects, are of compelling importance.

6. Sensory Attributes of Fruit

Sensory analysis is a scientific discipline used to evoke measure, analyze and interpret reactions to those characteristics of foods as they are perceived by the senses of sight, smell, taste, touch and hearing. The consumer acceptance of fruits most often relies upon the inherent flavour and textural quality of the product. Incorporating sensory evaluation will help in the selling a consumer friendly product with increased acceptance. When done properly, sensory information can provide reliable and useful information about fruits and vegetables which no instrument can measure their perceptual characteristics.

The sensory properties of foods are related to three major attributes:

Appearance - colour, size, shape

Flavour - odour, taste and

Texture - mouth feel, viscosity and hearing

The consumer integrates all of those sensory inputs such as appearance, aroma, flavour, hand-feel, mouth-feel and chewing sounds into a final judgment of the acceptability of that fruit.

6.1. Appearance and Colour of Fruit

Present emphasis is on the matter of eye appeal. Unless a commodity has eye appeal it is difficult to market, even though flavour and nutritional characteristics of the product or variety may be quite superior. Size is another factor that affects market acceptance of the fruit that is produced. Standard sizes have become rather generally adopted, and fruit not within those limits, usually has to be marketed as culls or processing in to some by product other than fresh product.

An important factor in eye appeal is good colour. External amount and intensity of surface colour on a fruit of any type or cultivar is of prime importance to its appeal in the market place. Colour is the one characteristic that make fruit distinctive and attracts the buyer's eye. Colour is such important aspects of fruit quality that grade standards almost universally specify the amount and shade of characteristic colour required to meet a designated grade. The determination of internal colour in an intact fruit enables periodic measurement of chlorophyll as related to its decrease during ripening, or changes in internal plant pigments such as carotenes or anthocyanins.

6.2. Texture/Mouth feel

Texture is an important component of fruit quality. Flavour may be affected by texture because release of taste compounds in the mouth is related to tissue structure. Texture is related to those attributes of quality associated with the sense of feel, as experienced by the fingers, the hand or in the mouth.

Included in texture are such sensations as hardness, softness, crispness, meatiness, juiciness and toughness.

Sounds produced by a food during mastication (chewing) or physical handling. It includes pitch (frequency of sound), loudness (intensity of the sound) and persistence (endurance of sound over time).

Viscosity of the fruits related to the temperature and the nature of the compounds present.

6.3. Flavour of Fruit

Fruit flavour is difficult to determine by chemical means, primarily because a complex group of volatile compounds is combined in most to provide the typical flavour. Often the same compounds are present in genetically unrelated fruits, with the proportion of each, or the presence or absence of a few, resulting in vastly different flavours.

Flavour, is the combined impression perceived via the chemical senses from a product in the mouth, i.e., it does not include appearance and texture. It includes aromatics, taste and chemical feeling factors.

Odour of a product is detected when food volatiles enter the nasal passage and are perceived by the olfactory system.

7. 9 Point Hedonic Scale

The 9-point hedonic scale, originally developed by Peryam and Girardot (1952), is the most commonly used scale for assessing liking and preferences of foods. It is a balanced scale around a neutral point with category labels that have been determined by Jones and Thurstone (1955) to be fairly evenly spaced psychologically.The adjectives used to label the nine points also aid in interpretation of panel means. Typically, the hedonic scale is used with untrained panelists who frequently use the product of interest, and it is considered the gold standard for assessing degree of liking.

Table.6. 9 Point Hedonic Scale

Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

F. General Biochemical Parameters

8. Proximate Analysis

This refers to the determination of the major constituents of feed and it is used to assess if a feed is within its normal compositional parameters or somehow been adulterated. This method partitioned nutrients in feed into: total protein, crude fat, carbohydrate, ash, and moisture reported as the percentage composition of the product, which are expressed as the content (%) in the feed respectively.

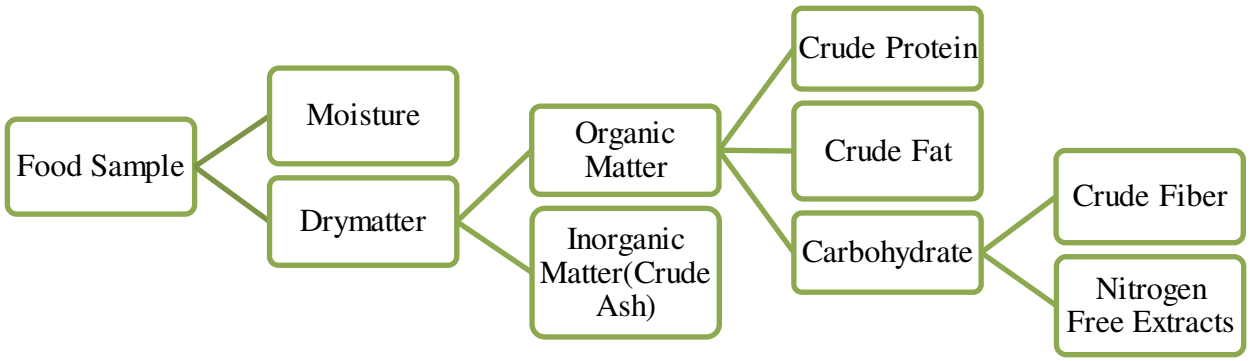


Fig.100. Proximate Composition

- **Moisture** (Water, Volatile substances)

The active ingredients from the view of feed nutrition are present in the part of dry matter (solid matter); therefore the level of moisture content is an important factor in both economy and storage. As the assay for moisture in the feed measures loss on drying by heating at normal pressure as moisture, the result includes most of volatile substances other than H_2O. Therefore, it may be more appropriate to be referred to as volatile matter rather than moisture for accuracy. Organic acids such as acetic acid and butyric acid in silage as well as ammonia and flavour components in feed materials are also vapourized and thus measured as moisture. When the feed is spread on sheet and left at rest, moisture absorption or release proceeds, and under a constant relative humidity comes to equilibrium at the moisture content corresponding to the constant relative humidity (RH) (equilibrium moisture content), which indicates comparatively stable water content of the feed in the air-dry state.

- **Total Ash** (soluble & insoluble minerals including macro & micro forms)

A sample is incinerated by heating to be crude ash. When a sample is incinerated without special treatment, there always is contamination with charred organic matter, resulting in a blackish colour. Therefore, it cannot be considered as pure ash (inorganic salts), and is referred to as crude ash. Ash content of a material represents inorganic residue remaining after destruction of organic matter, which is generally considered equivalent to the mineral content.

- **Crude Protein** (True Protein & Non Protein Nitrogen)

Crude protein is actually a chemical analysis of the food where by the amount of nitrogen present is used to estimate the amount of protein in the food. It is defined as the value obtained by quantitating nitrogen in a sample by the Kjeldahl method (in which nitrogen compounds in the sample is degraded by Sulphuric acid to become Ammonia, Sodium hydroxide is added, steam distillation is conducted under

the alkaline conditions, distilled ammonia is absorbed in acid and measured by titration) and multiplying the result by the factor 6.25.

Note: Generally the nitrogen content of protein is 16 % on average; thus the inverse number of this (100/16 = 6.25) is used as the factor.

- **Crude Fat** (Lipids, pigments)

Crude fat is the term used to refer the crude mixture of fat soluble material present in a sample. It is also known as the ether extract or the free lipid content and is the traditional measure of fat in food products. The common approach for total crude fat determination is based on the solubility of lipids in non-polar organic solvents such as Hexane, Petroleum ether or supercritical liquid Carbon dioxide with or without a solvent modifier.
A sample is extracted with ether using the Soxhlet extractor, to obtain the extract as ether extract. Ether extract contains, in addition to fat, oil-soluble dyes (such as chlorophyll and carotenoids), wax, free fatty acids, lecithin, cholesterin, and phospholipids, etc. Generally, ether extract of oil meal is around 1 % and the major component is oil and fat, therefore the energy value is high. When feeds are stored for a long period, a phenomenon is observed in that moisture does not change while ether extracts decreases gradually. This is because unsaturated fatty acids contained in feeds are oxidatively polymerized absorbing oxygen in the air and becomes insoluble in ether.

- **Crude Fibre** (cellulose, fiber with nitrogen attached & alkali insoluble lignin)

A sample is boiled sequentially with dilute acid and then with dilute alkali, and then sequentially washed with Ethanol and Diethyl ether, and the residue is subtracted by its ash, and the result is defined as crude fibre. Crude fibre is primarily measured to comprehend indigestible parts in feeds, and is consisted mainly of a part of lignin, pentosan, chitin, etc., in addition to cellulose. These compounds are collectively called as fibre.
Crude fibre consists largely of cellulose and lignin (97%) plus some mineral matter. It represents only 60% to 80% of the cellulose and 4% to 6% of the lignin.

- **Total Carbohydrate**

Carbohydrate is important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The basic unit of carbohydrates is the monosaccharides which cannot be split by hydrolysis in to simpler sugars. The carbohydrate content can be measured by hydrolyzing the polysaccharides in to simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

- **Nitrogen Free Extract** (sugars, organic acids, pectin, hemicelluloses and alkali soluble lignin)

Nitrogen free extract consisting of carbohydrates, sugars, starches, and a major portion of materials classed as hemicelluloses in feeds. When crude protein, fat, water, ash and fibre are added and the sum is subtracted from 100, the difference is NFE.

8.1. Determination of Moisture Content by Hot Air Oven Method

Principle

The water content of dried food stuffs is kept very low in order to extent shelf life.

Procedure

- The sample is thoroughly homogenize in a domestic mixer
- Weigh 2-10 g (±1 mg) of homogenize material in a clean dried petri-dish pre-dried at 98°C for 60 minutes
- Dry the sample by heating for a period ranging from 2 to 3 hours to overnight in a hot air oven at 100±1°C
- Weigh the sample periodically until it reaches a constant weight
- The percent moisture content can be calculated from the difference between the initial sample weight (W_I) and the final sample weight after drying (W_D)

Calculation

$$\% \text{ Moisture} = \frac{W_I - W_D}{W_I} \times 100$$

Where, W_I - Initial sample weight
W_D- Final sample weight

8.2. Determination of Crude Ash Content Using Muffle Furnace

Principle

Ash is the residue obtained after incineration of the dry material at high temperatures and appears as grey-white coloured powder.

Procedure

- Heat a platinum crucible to 600°C in a muffle furnace for 1 hour, cool in a desiccator and weigh (W_1)
- Weigh accurately 2 g of the dried sample (W_2) in to a crucible and heat at low flame by keeping on a clay triangle to char the organic matter
- Keep the charred material inside the previously set muffle furnace and heat for 6 to 8 hours to greyish white ash
- Cool the crucible in a desiccator and weigh (W_3)
- Heat the crucible again for further 30 minutes to confirm completion of ashing, cool and weigh

Calculation

$$\% \text{ of ash content (g /100 g)} = \frac{(W_3 - W_1) \times 100}{(W_2 - W_1)}$$

Where, W_1- Weight of crucible

W_2- Weight of dry matter with crucible taken for ashing

W_3- Weight of crucible with ash

8.3. Determination of Total Protein

8.3.1. Determination of Crude Protein by Kjeldahl Method

Principle

The nitrogen compounds in the sample are converted in Ammonium sulphate on treated with concentrated Sulphuric acid. Upon distillation with excess alkali, the ammonia is liberated which is absorbed in 2% Boric acid and is estimated by titration with standard excess N/50 Sulphuric acid.

Procedure

- Weigh 1 – 2 g of homogenized wet sample in to a Kjeldahl flask of 100 ml capacity
- Add a few glass beads and a pinch of digestion mixture (a mixture of $CuSO_4$ and K_2SO_4in the ratio of 1:8) and 10 ml of concentrated Sulphuric acid
- Digest over a burner till solution turns colourless
- To the digested solution in digestion flask add distilled water in small quantities with shaking and cooling till the addition of water does not generate heat
- Transfer quantitatively in to a 100 ml standard flask and make up the volume
- Pipette 5 ml of the makeup solution and transfer to the reaction chamber of the micro Kjeldahl distillation apparatus
- Rinse down with distilled water
- Add 2 drops of Phenolphthalein indicator and 40% Sodium hydroxide till the indicator changes to pink
- Distilled for 4 minutes and absorbed the ammonia liberated in 2% Boric acid containing a drop of Tashiro's indicator and determined the amount of ammonia by treating with N/50 Sulphuric acid.

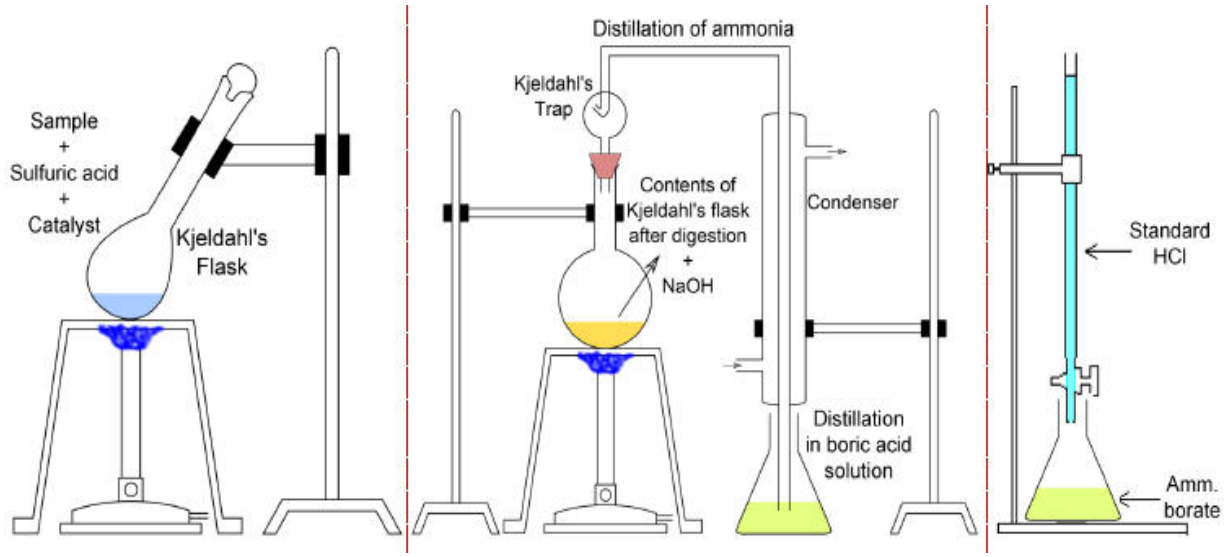


Fig.101. Kjeldahl Method 1. Digestion, 2. Neutralization and Distillation, 3. Titration

Calculation

$$\% \text{ of Crude protein} = \frac{\frac{14}{50} \times \text{vol}\frac{N}{50} \times H_2SO_4 \times 100 \times 100 \times 6.25}{5 \times \text{weight of sample} \times 1000}$$

or

$$\frac{N \times 14 \times 6.25}{\text{Wt. of sample}}$$

8.3.2. Determination of Total Protein by Biuret method

Principle

Under alkaline conditions copper ions present in Biuret reagent interact with –CO-NH-groups present in adjacent dipeptide and form a purple coloured complex. This can measure spectrophotometrically at 540nm. The method is fairly specific and there is little interference with other compound containing –CO-NH-groups like urea and other like reducing sugar can interact with cupric ions.

Reagents

- Standard protein solution (Bovine Serum Albumin (BSA) 5 mg/ml): Dissolve 500 mg of BSA and make up the final volume 100 ml with distilled water
- Biuret reagent: Dissolve 3 g of $CuSO_4$ and 9 g Sodium potassium tartarate in 500 ml of 0.2M NaOH. Add 5 g of KI and make up the final volume 1 litre with 0.2 M NaOH
- Distilled water
- Test sample

Procedure

- Pipette 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes
- Pipette 0.5 ml and 1 ml of the sample extract in two other test tubes
- Make up the final volume 2 ml with distilled water along with the blank tubes
- Add 3 ml Biuret reagent in all tubes. Mix all the reagents properly and incubated at 37^{o}C for 15 minutes
- Measure the colour complex spectrophotometrically at 520 nm
- Draw the standard curve of concentration of BSA vs. Optical Density (O.D.)
- Put the OD of the unknown solution and calculate the concentration of the protein present in given sample from standard graph and formula express result in mg%

Calculation

$$\text{Concentration of the Protein (mg \%)} = \frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Aliquot (test)}} \times 100$$

8.3.3. Determination of Total Protein by Lowry's Method

Principle

The blue colour developed by the reduction of the Phosphomolybdic-Phophotungstic components in the Folin-Ciocalteau reagent by the amino acids Tyrosine and Tryptophan present in the protein plus the colour developed by the Biuret reaction of the protein with the Alkaline cupric tartarate are measured in the Lowry's method.

Reagents

- 2% Sodium carbonate in 0.1 N Sodium hydroxide (Reagent A)
- 0.5% Copper sulphate ($CuSO_4.5H_2O$) and 1% Potassium sodium tartarate in 100 ml distilled water (Reagent B)
- Alkaline copper solution: Mix 50 ml of A and 1 ml of B prior to use (Reagent C)
- Folin-Ciocalteau Reagent (Reagent D)- Reflux gently for 10 hours a mixture consisting of 100 g Sodium tungstate ($Na_2WoO_4.2H_2O$), 25 g Sodium molybdate ($Na_2MoO_4.2H_2O$), 700 ml distilled water, 50 ml of 85% Phosphoric acid, and 100 ml of concentrated Hydrochloric acid in a 1.5 litre flask. Add 150 g Lithium sulphate, 50 ml distilled water and a few drops of Bromine water. Boil the mixture for 15 minutes without condenser to remove excess bromine. Cool, dilute to 1 litre and filter. The reagent should have no greenish tint (Determine the acid concentration of the reagent by titration with 1 N NaOH to a Phenolphthalein end-point)
- Protein Solution (Stock Standard) - Weigh accurately 50 mg of bovine serum albumin and dissolve in distilled water and make up to 50 ml in a standard flask
- Working Standard- Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. 1 ml of this solution contains 200 µg protein

Procedure

- Extraction of Protein from sample: Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500 mg of the sample and grinded well with a pestle and mortar in 5-10 ml of the buffer. Centrifuged and used the supernatant for protein estimation
- Pipette 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes
- Pipette 0.1 ml and 0.2 ml of the sample extract in two other test tubes
- Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank
- Add 5 ml of reagent C to each tube including the blank. Mix well and allow standing for 10 minutes
- Then add 0.5 ml of reagent D, mix well and incubated at room temperature in the dark for 30 minutes. Blue colour is developed
- Take the readings at 660 nm
- Draw a standard graph and calculate the amount of protein in the sample and express the amount of protein in mg/g or 100 g sample

Calculation

$$\text{Concentration of the Protein (mg \%)} = \frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Aliquot (test)}} \times 100$$

8.3.4. Determination of Total Protein by Bradford Method

Principle

The assay is based on the ability of proteins to bind Coomassie brilliant blueG250 and form a complex whose extinction coefficient is much greater than the free dye.

Reagents

- Standard Protein Solution (BSA) 20 µg/ml: Prepare stock solution (1 mg/ml) and dilute with distilled water to make concentration 20 µg/ml.
- Bradford Reagent: Dissolve 100 mg of Coomassie brilliant blueG250 in 50 ml of 95% Ethanol. Cover it tightly and incubate for an hour. Slowly add 100 ml of 85% concentrated Ortho-Phosphoric acid and make the volume to 1 litre with distilled water. Filter the reagent through Whatman filter paper No.1 and store in amber bottle. Reagent is stable only for 2-3 weeks
- 0.2 M Phosphate Buffer (pH – 7.5): Prepare 0.2 M Monobasic Sodium phosphate and 0.2 M Dibasic Sodium phosphate and mix until the pH becomes 7.5

Procedure

- Pipette 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes
- Pipette 0.5 ml and 1 ml of the sample extract in two other test tubes. A tube with 2 ml of phosphate buffer and 2 ml of Bradford reagent serves as the blank
- Make up the final volume 2 ml with phosphate buffer and added 2 ml Bradford reagent in all tubes
- Mix all the reagents properly and incubate at 37^{o}C for 15 minutes
- Measure the colour complex spectrophotometrically at 595 nm
- Draw the standard curve of concentration of BSA vs. Optical Density (O.D)
- Put the OD of the unknown solution and calculate the concentration of the protein present in given sample from standard graph and formula express result in mg%

Calculation

$$\text{Concentration of the Protein (mg \%)} = \frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Aliquot (test)}} \times 100$$

8.4. Determination of Crude Fat by Soxhlet Method

Principle

Fat soluble in organic solvents can be extracted from moisture free samples by using solvents like Petroleum ether, Ethyl ether etc. The solvent is evaporated and fat is estimated gravimetrically.

Procedure

- Weigh, accurately 5-10 g (W_1) of dry sample into a thimble and keep a cotton plug on top of it
- Place the thimble in a Soxhlet apparatus and add ½ volumes of Ether into a pre-weighed flat-bottom flask (W_2) and distilled for 16 hours (Cool the apparatus and filter the solvent into a pre-weighed conical flask (W_2)

- Rinse the flask of the apparatus with small quantities of Ether and then added washings to the above flask)
- Remove Ether by evapouration and dried the flask with fat at 80-100°C, cool in a desiccator and weigh (W_3)

Calculation

$$\text{Fat content (g/ 100\%)} = \frac{(W_3\text{-}W_2) \times 100}{W1} = X$$

$$\text{Fat content (g / 100\%)} = \frac{\text{Weight of fat} \times 100}{\text{Weight of sample}} = X$$

Where, W1- Weight of dry matter taken for extraction

W2- Weight of flat bottom flask

W3- Weight of flask with fat

Conversion of dry weight to wet weight basis

$$\text{Wet Weight} = \frac{X \times (100 - \text{moisture})}{100}$$

8.5. Determination of Total Carbohydrate

8.5.1. Determination of Total carbohydrate by Furfural Colorimetric Method

Principle

Carbohydrate is determined by the furfural colorimetric method after treatment with concentrated Sulphuric acid. The intensity of the pink colour is measured at 520 nm.

Procedure

- About 30-50 mg of material is weigh out into a 20 ml centrifuge tube and heat in a boiling water bath for 30 minutes with 4 ml 10% Tri Chloro acetic acid (TCA) and about 30 mg of Ag_2SO_4
- After centrifuging, the clear supernatant and the subsequent washings of the residue with the TCA solution were transferred to a 25 ml graduated flask and make up to the volume
- 2 ml aliquots were taken in duplicates and carefully layered over a 6 ml of concentrated H_2SO_4 taken in a boiling tube
- The tubes were quickly agitate to mix the contents thoroughly and heated for 6.5 minutes in a vigorously boiling water bath
- After rapid cooling to room temperature the optical density is measure at 520 nm
- Blanks were run with each batch of analysis and Glucose is use to observe the standard curve

8.5.2. Determination of Total carbohydrate by Anthrone Reagent

Principle

Carbohydrates are first hydrolyzed in to simple sugars using dilute Hydrochloric acid. In hot acidic medium Glucose is dehydrated to Hydroxyl methyl furfural. This compound forms with Anthrone a green coloured product with an absorption maximum at 630 nm.

Reagents

- 2.5N HCl
- Anthrone reagent: Dissolve 200 mg anthrone in 100 ml of ice-cold 95% Sulphuric acid. Prepare fresh before use
- Standard Glucose (stock): Dissolve 100 mg in 100 ml distilled water
- Working standard: 10 ml of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of Toluene

Procedure

- Weigh 100 mg of the sample in to a boiling tube
- Hydrolyze by keeping it in a boiling water bath for 3 hours with 5 ml of 2.5 N HCl and cool to room temperature
- Neutralize with solid Sodium carbonate until the effervescence ceases
- Make up the volume to 100 ml and centrifuge
- Collect the supernatant and take 0.5 and 1 ml aliquots for analysis
- Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard, '0' serves as blank
- Make up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water
- Then add 4 ml of Anthrone reagent
- Heat for 8 minutes in a boiling water bath
- Cool rapidly and read the green to dark green colour at 630 nm
- Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis
- From the graph calculate the amount of carbohydrate present in the sample tube

Calculation

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

Note

Cool the contents of all the tubes on ice before adding ice cold anthrone reagent.

8.5.3. Determination of Total Carbohydrate by Phenol Sulphuric Acid Method

Principle

In hot acidic medium Glucose is dehydrated to Hydroxyl methyl furfural. This forms a green coloured product with Phenol and has absorption maximum at 490 nm.

Reagents

- Phenol 5%: Redistilled (Reagent grade) phenol (50 g) dissolved in water and diluted to 1 litre
- Sulphuric acid 96% reagent grade
- Standard glucose Stock: Dissolve 100 mg in 100 ml of water
- Working standard: 10 ml of stock diluted to 100 ml with distilled water

Procedure

- Follow the steps 1 to 4 as given in anthrone method for sample preparation
- Pipette 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard in to a series of test tubes
- Pipette 0.1 and 0.2 ml of the sample solution in two separate test tubes. Make up the volume in each tube to 1 ml with water
- Set a blank with 1 ml of water
- Add 1 ml of phenol solution to each tube
- Add 5 ml of 96% Sulphuric acid to each tube and shake well
- After 10 minutes shake the contents in the tubes and place in a water bath at 25 to 30^{o}C for 20 minutes
- Read the colour at 490 nm
- Calculate the amount of total carbohydrate present in the sample solution using the standard graph

Calculation

Absorbance corresponds to 0.1 ml of the test = X mg of glucose

$$100 \text{ ml of the sample solution contains} = \frac{X}{0.1} \times 100 \text{ mg of glucose} = \% \text{ of total carbohydrate present}$$

8.6. Determination of Crude Fibre

The crude fibre content in sample was determined by sequential acid and alkali hydrolysis method.

Principle

During the acid and subsequent alkali treatment, oxidative hydrolytic degradation of the native cellulose and considerable degradation of lignin occur. The residue obtained after final filtration is weighed, incinerated. Cooled and weighed again. The loss in weight gives the crude fibre content.

Reagents

Sulphuric acid solution (0.255 ± 0.005 N): 1.25 g concentrated Sulphuric acid diluted to 100 ml (Concentration must be checked by titration)

Sodium hydroxide solution (0.313 ± 0.005 N): 1.25 g Sodium hydroxide in 100 ml distilled water (Concentration must be checked by titration with standard acid)

Procedure

- Extract 2 g of ground material with Ether or Petroleum ether to remove fat (Initial boiling temperature 35-38^{o}C and final temperature 52^{o}C). If fat content were below 1%, extraction may be omitted
- After extraction with Ether boil 2 g of dried material with 200 ml of Sulphuric acid for 30 minutes with bumping chips
- Filter through muslin and washed with boiling water until washings are no longer acidic
- Boil with 200 ml of Sodium hydroxide solution for 30 minutes
- Filter through muslin cloth again and wash with 25 ml of boiling 1.25% H_2SO_4, three 50 ml portions of water and 25 ml alcohol
- Remove the residue and transfer to ashing dish (pre weighed dish W_1)
- Dry the residue for 2 hours at 130 ± 2^{o}C. Cool the dish in a desiccator and weigh (W_2)
- Ignite for 30 minutes at 600 ± 15^{o}C
- Cool in a desiccator and reweighed (W_3)

Calculation

$$\% \text{ Crude fibre in ground sample} = \frac{\text{Loss in weight on ignition } (W_2 - W_1) - (W_3 - W_1)}{\text{Weight of the sample}} \times 100$$

G. Determination of Chemical Constituents

9. Estimation of Titrable Acidity

The acid content of foods directly affects their flavour. Practically all foods contain an acid or a mixture of acids. These acids may occur naturally, may be produced by action of microorganisms, or may be added in such products as ketch up or chili sauce during their manufacture. Total acidity determinations are useful as a measure of this tartness. In some cases a high concentration of acid may be an indication of spoilage and rancidity while in other cases high acid content is necessary for preservation.

Total acid is usually determined by titrating an aliquot of sample with a base of known strength using a suitable indicator to determine the end point.

9.1 Titrimetric Method

Reagents

- Sodium hydroxide: 0.1 N
- Phenolphthalein indicator: Dissolve 1 g of indicator in 60 ml of 95 per cent Ethyl alcohol and dilute to 100 ml with distilled water

Procedure

- **Preparation of sample**
  Juices: All fruit juices should be thoroughly mixed by shaking to ensure uniformity of sampling. Filter the juice through filter paper to remove the coarse particles which can result in an inaccurate measured sample
  Fresh fruits: In order to secure a sample of the water soluble acids, the fresh fruits must be crushed. This can be accomplished by placing the fruit in a small hand operated press type juice extractor or a layer of cheese cloth and squeezing it tightly until the pulp is fairly dry. If the juice sample contains fibres and particles, the liquid should be centrifuged, or filtered until the juice is clear
- Weigh 25 g of macerated sample or pipette a 25 ml sample and transfer to a 250 ml volumetric flask
- Dilute to 100 ml with distilled water. Shake and filter through Whatman No. 4 filter paper
- Pipette 50 ml of the filtrate into a 250 ml Erlenmeyer flask and added 50 ml of distilled water
- Add 0.3 ml of 1% Phenolphthalein solution and titrate with 0.1 N Sodium hydroxide solution until the pink colour develops
- Record the volume of the Sodium hydroxide used and calculate the acidity

Calculations

Total acid may be expressed on three different bases

1. As volume (ml) of 0.1 N NaOH per 100 ml of sample

Let x ml of 0.1 N NaOH is used for 50 ml of the solution

$$\text{The amount of 0.1 N NaOH used / 100 ml Juice} = \frac{X}{50} \times 100$$

2. As grams of acid

To calculate results in terms of grams of acid, all acid present are calculated in terms of one acid. In general, the predominant acid present in the juice or product is used for calculation purposes using the "theoretical" rather than the actual equivalent weight.

Calculate grams of acid in the aliquot titrated

$$\text{Gram acid in aliquot} = \frac{\text{ml of 0.1 N NaOH} \times \text{equivalent wt. of acid}}{10000}$$

3. As percent of acid (titrating with 0.1 N NaOH)

$$\% \text{ acid} = \frac{\text{ml 0.1 N NaOH} \times \text{factor}}{\text{Volume of sample in ml}} \times 100$$

Table.7. Factor for calculating acidity

Acid	Formula weight	Equivalent weight	Factor
Acetic	60.05	60.05	0.0060
Butyric	88.10	88.10	0.0088
Citric	192.12	64.04	0.0064
Lactic	90.08	90.08	0.0090
Malic	134.09	67.05	0.0067
Oleic	282.46	282.46	0.282
Oxalic	90.04	45.02	0.0045
Succinic	118.09	59.05	0.0059
Stearic	284.47	284.47	0.0284
Tartaric	150.08	75.04	0.0075

Note: For liquid sample, if the specific gravity of the sample is approximately 1.0 (same as water) it is not necessary to weigh out the sample for titration as the volume and weight of the sample will be the same. So volume in ml will be equivalent to weight in gram. However, for samples of specific gravity greater or less than 1.0, the aliquot should be weighed.

9.2. pH Meter Method

The point of neutrality i.e. the end point of titration may also be determined using a pH meter.

Checking the pH meter

- Make sure the pH meter has warmed up before use - allow about 30 minutes
- Remove the electrode from the distilled water in the storage beaker and dry

- Place the electrode into the beaker containing a buffer solution of pH 7 and calibrate the meter to the same figure
- Whenever readings are taken, ensure that the electrode is not in contact with the sides or base of the beaker
- Remove the electrode and after rinsing in distilled water, place in the solution to be tested; the electrode should not have any contact with the glass

Procedure

- Ensure the tap on the burette is shut and using a funnel pour the 0.1 M solution of NaOH into the burette until it reaches the zero mark. Do not spill the solution onto the skin
- Slowly titrate the NaOH into the juice/water solution. Care must be taken that the NaOH is dropped directly into the solution and does not adhere to the glass; otherwise the reading may be false
- While titrating care must be taken to continually swirl the solution in the beaker to keep it thoroughly mixed. This is essential, particularly when the solution nears neutrality. It is important to determine the point of neutrality or the end point of titration very exactly. The end point can easily be missed, which will give an inaccurate reading for the test. It is important therefore that towards the end of the titration the NaOH is added a drop at a time
- Using a pH meter, while titrating the digital readout will be seen to climb from around 4 or 5. When the reading reaches 7 proceed carefully. The point of neutrality or the end point of titration is reached at pH 8.1. If this figure is exceeded the test is not acceptable and must be repeated
- When the pH meter reads 8.1 read off the amount of NaOH used on the burette and record
- Remove the electrode and rinse it in distilled water ready for the next test. Do not allow it to become contaminated
- Refill the burette for each subsequent test
- Clean the equipment thoroughly and rinse with distilled water. Detergents must not be used

Note: When testing very acidic juices e.g. lemons and limes a larger amount of NaOH is required. Therefore, when the NaOH reaches the 25 ml mark on the scale the burette tube should be recharged as described above. When the end point is reached the various readings are added together and recorded to produce a figure of NaOH used for each titration.

10. Estimation of Total Soluble Solids

Total soluble solids (TSS) of a given sample of fruit juice representing various chemical substances present in it in soluble form. It indicates a measure of sugars present in the sample. The amount of TSS present in the juice of a fruit is also considered to be a reliable index in judging its maturity. In accordance with the harvest – maturity of many fruits is assessed in considering the TSS of their juices.

Principle

The TSS of a given fruit juice sample is determined in a quicker way with the help of the refractometer. The instrument works on the principle of refractive index of the sample and gives the refractive index as $^{\circ}$Brix.

Procedure

- The lid that is covering plate of the refractometer which rest over the prism-plate and is attached with it at the base end with a hinge which can be unfolded backward. By doing so, both the lid and the prism plate are exposed
- The lid and the prism plate are then carefully wash with a jet of clean water to ensure that they have no stain on its surface
- Water adhered on the prism plate and the lid as well as the surrounding parts of them, if any is completely wipe off with blotting paper or absorbent cotton
- The lid of the prism plate is then wash with distilled water and the water adhered on them is blotted out. The cleaning is best done by rubbing the lid and the prism plate gently and carefully with absorbent cotton, soaked with rectified spirit
- Then, with the help of a previously cleaned dropper or a glass rod, a drop of distilled water is carefully dropped on the surface of the prism plate. The lid is folded forward and placed over the prism plate to cover it. At this position, the lid and the prism plate are firmly held together with fingers to avoid unfolding of the lid
- The refractometer is held to point towards light. The eyepiece of it is brought close to any eye of the observer who should look into the eyepiece to have a view of the image of the scale. The scale focusing knob should be conveniently rotated to adjust it at such a position where the scale is most clearly visible. The shaded part would be seen to insert the unshaded part at zero position of the scale which indicates no reading with respect to distilled water. If it is not there then the reading should be brought to zero by rotating the scale calibrating screw
- The lid is then folded backward. The distilled water that remains adhered over the lid and the prism plate is completely blotted out and dried in air for a few minutes
- A clear sample of fruit juice, TSS of which is to be determined is taken in the dropper, or a drop may be taken with the glass rod. A drop of juice is then carefully placed on the prism-plate
- Reading of the juice sample as ^{o}Brix is obtained and amount of TSS is expressed accordingly

11. Estimation of Total Phenolic Compounds

Phenols, the aromatic compounds with hydroxyl groups, are widespread in plant kingdom. They include an array of compounds like tannin; flavanols etc. and occur in all parts of the plants where they offer resistance to diseases and pests. Estimate the phenolic compounds using the same extract prepared for soluble sugar estimation.

11.1. Estimation of Total Phenols

Phenols react with Phospho molybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Reagents

- Folin-ciocalteau reagent: Dilute the Folin-ciocalteau reagent (2 N) with distilled water in 1:1 (v/v) ratio before use
- Saturated Sodium carbonate solution: Dissolve the Anhydrous Sodium carbonate (35.0 g) in 100 ml of distilled water by heating on a water bath at 70-80°C. Cool the contents for overnight and use the supernatant

Procedure

- Take 1 ml of the extract in a test tube and dilute with distilled water (7.5 ml)
- Mix well the contents. Add to this, 0.5 ml of dilute Folin- ciocalteau reagent
- Shake the tubes thoroughly and after 3 minutes, add 1 ml of saturated Sodium carbonate and make total volume to 10 ml with distilled water
- Allow the tubes to stand for 1 hour and read the absorbance at 725 nm using spectrophotometer
- Use distilled water in place of sample extract as reagent blank and rest of procedure for blank is same
- Determine the amount of total phenols in the sample from the standard curve prepare simultaneously by taking tannic acid as the standard phenol and express the data as mg g^{-1} dry weight

11.2. Estimation of O-dihydroxy Phenols

Reagents

- Arnow's reagent

(a) 10 g Sodium nitrite

(b) Sodium molybdate - 10 g Dissolve these in distilled water separately, mix and make volume to 100 ml with distilled water.

- 0.5 N HCl
- 0.5 N NaOH

Procedure

- Take 2 ml of the sample extract in a test tube
- To this, add 2 ml of 0.5 N HCl, 1 ml of Arnow's reagent and 4 ml of distilled water in succession
- Add 2 ml of 0.5 N NaOH, pink colour will appear. Now, shake the solution
- Make volume to 15 ml with distilled water and measure the intensity of the colour at 515 nm
- Run blank simultaneously without the addition of Arnow's reagent and calculate the amount of O-dihydric phenols by taking Chlorogenic acid as standard and express the data as mg g^{-1} dry weight

11.3. Estimation of Total Flavanoid Content

Reagents

- HCl
- CH_3OH
- Cinnamaldehyde
- Catechin

Procedure

- Make up the sample volume to 1 ml
- Add 5 ml of Chromogen reagent (HCl + CH_3OH + Cinnamaldehyde in 1:3:0.004 ratio) and take the absorbance at 640 nm
- Use Catechin as a standard and express the result as microgram Catechin equivalents per milligram (µg CE/mg) sample

11.4. Estimation of Flavanols

Reagents

- Aluminium chloride (0.1 M): Dissolve 24.143 g of Aluminium chloride in 1 litre of distilled water

Procedure

- Take 1 ml of the extract in a test tube and evapourate to dryness on a water bath maintain at 60-70°C
- Dissolve the residue in 10 ml of 0.1 M Aluminium chloride solution and thereafter, read the absorbance of the solution at 420 nm using spectrophotometer
- Prepare a blank simultaneously and calculate the amount of flavanols by taking rutin as standard and express the data as mg g^{-1} dry weight

12. Estimation of Total Antioxidant Activity (TAA)

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk of chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables.

The antioxidant activity of a sample is checked on the basis of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity. The reaction between an antioxidant and DPPH can be presented as follows.

When an antioxidant reduces DPPH° to DPPH-H, decrease in absorbance occurs. Thus, the degree of discolouration shows the scavenging potential of an antioxidant in terms of hydrogen donating ability.

Procedure

- Mix DPPH (500 µl, 0.5 mM in methanol) solution with sample extract and make up the volume to 3.5 ml with methanol
- Incubate the mixture in dark for 45 minutes at room temperature and record the absorbance at 515 nm in a spectrophotometer
- Use Butylated hydroxy anisol (BHA) as a standard antioxidant compound
- Calculate the DPPH radical scavenging activity by using the formula

Calculation

$$\text{DPPH° scavenging activity (\%)} = [(Ac-As)/Ac] \times 100$$

Where, Ac is the absorbance of positive control solution and

As is the absorbance of test solution

13. Estimation of Reducing Sugar

In fruits both reducing and non-reducing sugars are present in varying amount. Reducing sugars are those hexose ($C_6H_{12}O_6$) sugars, which can reduce compounds such as alkaline (ammoniacal) silver nitrate solution, cupric salt solution etc., because they themselves are oxidized.

Hexose sugars are divided in to two main groups, which are aldo hexose and keto hexose. Aldo hexose or aldose contains aldehydes group and keto hexose or ketose contains ketone group. Aldehydes are strong reducing agents. Hexose which contain aldehyde groups e.g., glucose, galactose, mannose etc. are reducing sugars. Ketones are however more resistant to oxidation than aldehydes, because it involves the breaking of a relatively stable C-C bond. Hence they do not ordinarily reduce Alkaline silver nitrate or Cupric salt solution. But that fructose contains ketone, it is able to reduce readily as easily oxidizable CO-CH_2OH group is present in it and it act as reducing sugar.

Non reducing sugar e.g., Sucrose is a disaccharide and cannot reduce Alkaline silver nitrate or Cupric salt solution.

The reducing sugars will be often expressed in terms of Glucose, since Glucose is the most predominant reducing sugar present in fruits.

13.1. Estimation of Reducing Sugar by Titration Method

Principle

The reducing sugar present in the sample reduces the alkaline solution of cupric salt (Fehling's solution) to red cuprous oxide. Methylene blue, a redox indicator is employed to detect the end point of the titration. The red colour of the cuprous oxide formed during the reaction will be the intense blue colour of the indicator. But when all the cupric ions of the Fehling's solution are reduced to cuprous by the sugar solution, then the next few drops of sugar solution added reduce and thus decolourize. The indicator so that the red colour of the cuprous oxide become visible which is taken as the end point of the titration.

Reagents

- Fehling solution A: Dissolve 69.28 g $CUSO_4.5H_2O$ in water and make up to 1 litre
- Fehling solution B: Dissolve 346 g of Rochelle salt (Potassium sodium tartarate, $KNaC_4H_4O_6.4H_20$) and 100 g of NaOH in water and make up to 1 litre
- Methylene blue indicator solution, 1% in distilled water
- Neutral Lead acetate ($C_2H_3O_{22}pb$, $3H_2O$) solution, 45% in distilled water
- Potassium oxalate solution, 22% in distilled water
- Standard Glucose solution: 5 mg/ml

Procedure

- Pulp the fruit in a blender and filter through Whatman No.4 filter paper
- Weigh and transfer about 25 g of the filtered juice in to a 250 ml volumetric flask
- Add about 100 ml of water and neutralize with 1 N NaOH
- Add 2 ml of Lead acetate solution, shake, and allow standing for 10 minutes
- Add 22 ml of Potassium oxalate solution to remove the excess of lead make up the volume with water and filter

- Pipette 5 ml each of Fehling's solutions A and B in to a 250 ml conical flask
- Add about 50 ml of distilled water and 2 or 3 glass beads
- Boil the content vigorously and while boiling add the clarified fruit juice taken in a burette, till the blue colour just disappears
- Add 0.5 ml of Methylene blue indicator and allow it to boil for 1 minute
- While boiling, complete the titration as quickly as possible by adding 2-3 drops of sugar solution at 5-10 seconds intervals, until the indicator is completely decolourize and the brick red colour of cuprous oxide becomes dominant
- Calculate the content of reducing sugars as gram of Glucose per 100 g of the juice (percentage)

Calculation

Weight of the fruit juice taken for analysis = W (25 g)

Volume made up to = 250 ml

Volume of the clarified juice reacted with 10 ml of Fehling's solution (A+B) = T.V (ml)

10ml of Fehling's solution (A+B) = 0.05 g of glucose

Percentage of reducing sugars in the juice (g of glucose per 100 g of juice) = $0.05 \times \frac{250}{T.V} \times \frac{100}{W}$

13.2. Estimation of Reducing Sugars by Nelson-Somogyi's Method

Principle

The reducing sugars when heated with Alkaline copper tartarate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with Arsenomolybdic acid, the reduction of Molybdic acid to molybdenum blue takes place. The blue colour developed is compared with a set of standards in a colorimeter at 620 nm.

Reagents

- Alkaline copper tartarate: dissolve 2.5 g Anhydrous Sodium carbonate, 2 g Sodium bicarbonate, 2.5 g Potassium sodium tartarate and 20 g Anhydrous Sodium sulphate in 80 ml water and make up to 10 ml (solution A). Dissolve 15 g Copper sulphate in a small volume of distilled water. Add 1 drop of H_2SO_4 and make up to 100 ml (solution B) before use
- Arsenomolybdate reagent: dissolve 2.5 g Ammonium molybdate in 45 ml distilled water. Add 2.5 ml H_2SO_4 and mix well. Then add 0.3 g Disodium hydrogen arsenate dissolved in 25 ml distilled water. Mix well and incubate at 37^0C for 24 to 48 hours
- Standard stock Glucose solution: 100 ml distilled water
- Working standard: dilute 10 ml of stock solution to 100 ml with distilled water (100 µg/ml)

Procedure

- Weigh 100 mg of the sample and extract the sugars with hot 80% alcohol twice (5 ml each time)
- Collect the supernatant and evaporate on water bath
- Add 10 ml of water and dissolve the sugars
- Pipette aliquots of 0.1 or 0.2 ml of alcohol free extract to separate tubes
- Pipette 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution into a series of test tubes
- Make up the volume in both sample and standard tubes to 2 ml; with distilled water

- Pipette 2 ml distilled water into a separate tube to serve as a blank
- Add 1 ml of Alkaline copper tartarate reagent to each time
- Place the tubes in boiling water for 10 minutes
- Cool the tubes and add 1 ml of Arsenomolybdic acid reagent to all the tubes
- Make up the volume in each tube to 10 ml with water
- Read the absorbance of blue color at 620 nm after 10 minutes
- From the graph drawn, calculate the amount of reducing sugars present

Calculation

Reducing sugars in sample (%) =

$$\frac{\text{Sugar value from graph (g)}}{\text{Aliquot sample used (0.1 or 0.2 ml)}} \times \frac{\text{Total volume of alcohol free extracts (10 ml)}}{\text{Weight of sample}} \times \frac{1}{100}$$

Note: Although the colour is stable, determine the absorbance at a fixed time after the addition of Arsenomolybdate reagent.

13.3. Estimation of Reducing Sugar by Dinitro Salicylic Method

For sugar estimation an alternative to Nelson-Somogyi's method in the Di nitro salicylic acid method- simple, sensitive and adoptable during handling of a large number of samples at a time.

Materials

- Dinitro Salicylic acid reagent (DNS reagent): Dissolve by stirring 1 g Dinitro salicylic acid ,200 mg crystalline Phenol and 50 mg Sodium sulphite in 100 ml 1% NaOH. Store at 4°C. Since the reagent deteriorates due to Sodium sulphite, if long storage is required, Sodium sulphite may be added at time of use
- 40% Rochelle Salt Solution (Potassium Sodium Tartarate)

Procedure

- Follow, steps 1 to 3 as in Nelson- Somogyi's method to extract the reducing sugars from the test material
- Pipette 0.5 to 3 ml of the extract in test tubes and equalize the volume to 3 ml with water in all the test tubes
- Add 3 ml of DNS reagent
- Heat the contents in a boiling water bath for 5 minutes
- When the contents of the tubes are still warm, add 1ml of 40% Rochelle salt solution
- Cool and read the intensity of dark red colour at 510 nm
- Run a series of standards using glucose (0 to 500 µg) and plot a graph

Calculation

Calculate the amount of reducing sugars present in the sample using the standard graph.

14. Estimation of Total sugar

The non-reducing sugars which are not titratable are first hydrolyzed to reducing sugars. Thus after hydrolysis, the non-reducing sugars are converted to reducing sugars while the reducing sugars that are already present in the sample remain unchanged. Accordingly, all the sugars that are present after hydrolysis remain as reducing sugars. This is conveniently termed as total sugars.

Principle

The non-reducing sugar of the sample (sucrose) is hydrolyzed to reducing monosaccharides by treatment with dilute acid. The total reducing sugars are then estimated by titration with Fehling's solution.

Reagents

- Fehling solution A: Dissolve 69.28 g $CUSO_4.5H_2O$ in water and make up to 1 litre
- Fehling solution B: Dissolve 346 g of Rochelle salt (Potassium sodium tartarate, $KNaC_4H_4O_6.4H_20$) and 100 g of NaOH in distilled water and make up to 1litre
- Methylene blue indicator solution, 1% in distilled water
- Citric acid, solid
- NaOH solution, 1 N

Procedure

- Pipette 50 ml of the clarified juice solution (prepared for the estimation of reducing sugars) in to a 250 ml conical flask
- Add 5 g of Citric acid and 50 ml of distilled water
- Boil gently for 10 minutes to complete the inversion of sucrose and then cool
- Transfer the content to a 250 ml volumetric flask and neutralize with 1 N and make up the volume
- Titrate the made up solution with 10 ml of Fehling's solution as detailed in the estimation of reducing sugars
- Express the total sugars as g of glucose per 100 g of the original juice

Calculation

Weight of the fruit juice taken for analysis = W (g)

Volume made up to = 250 ml

Volume of the clarified juice used for the inversion = 50 ml

Percentage of total sugars in the juice (g of glucose per 100 g of juice) = $0.05 \times \frac{250}{T.V} \times \frac{250}{50} \times \frac{100}{W}$

15. Estimation of Non-Reducing Sugar

- The difference between the total sugars and the reducing sugars will represent the non-reducing sugar
- The non-reducing sugar will be expressed as gram of sucrose per 100 g of the juice

Calculation

Percentage of Non-reducing sugars in the juice (g of glucose per 100 g of juice)

= Percentage of Total sugars – Percentage of Reducing sugars

16. Estimation of Starch by Anthrone Reagent

Starch is an important polysaccharide. It is the storage form of carbohydrate in plants abundantly found in roots, tubers, stems, fruits and cereals. Starch, which is composed of several glucose molecules, is a mixture of two types of components namely amylase and amylopectin. Starch is hydrolyzed in to simple sugars by dilute acids and the quantity of simple sugars is measured colorimetrically.

Principle

The sample is treated with 80% alcohol to remove sugars and then starch is extracted with Perchloric acid. In hot acidic medium starch is hydrolyzed to Glucose and dehydrated to hydroxy methyl furfural. This compound forms a green coloured product with Anthrone.

Requirements

- Anthrone reagent: Dissolve 200 mg Anthrone in 100 ml of ice cold 95% Sulphuric acid. Prepare fresh before use
- 80% Ethanol
- 52% Perchloric acid
- Standard Glucose (stock): Dissolve 100 mg in 100 ml distilled water
- Working standard: 10 ml of stock diluted to 100 ml with distilled water

Procedure

- Homogenize 0.1 to 0.5 g of the sample in hot 80% Ethanol to remove sugars. Centrifuge and retain the residue. Wash the residue repeatedly with hot 80% Ethanol till the washings do not give colour with Anthrone reagent. Dry the residue well over a water bath
- To the residue add 5.0 ml of water and 6.5 ml of 52% Perchloric acid
- Extract at 0^{o}C for 20 minutes. Centrifuge and save the supernatant
- Repeat the extraction using fresh Perchloric acid. Centrifuge and pool the supernatants and make up to 100 ml.
- Pipette 0.1 or 0.2 ml of the supernatant and make up the volume to 1 ml with distilled water
- Prepare the standards by taking 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard and make up the volume to 1 ml in each tube with distilled water
- Add 4 ml of Anthrone reagent to each tube
- Heat for 8 minutes in a boiling water bath
- Cool rapidly and read the intensity of green to dark green colour at 630 nm

Calculation

Find out the glucose content in the sample using the standard graph. Multiply the value by a factor 0.9 to arrive at the starch content.

17. Determination of Amylases

(α-1, 4 glucan 4-glucanohydrolase *EC 3.2.1.1* and α-1, 4 glucan maltohydrolase*EC 3.2.1.2*)

Starch degrading enzymes - universally distributed - act on glycogen and related polysaccharides. α-amylase cause endo cleavage of substrates and hydrolyses α- 1, 4 linkages in a random manner. It has the ability to bypass α- 1, 6 branch points. The viscosity reduction of the substrate is fast but the production of reducing sugars is slow. β- Amylase hydrolyses alternate bonds from the non-reducing end of the substrate. The enzyme degrades amylase, amylopectin or glycogen in an exo or stepwise fashion by hydrolyzing alternative glycosidic bonds. The end product is β- maltose. β- Amylase is incapable of bypassing branch points i.e., 1, 6- glycosidic linkages amylopectin and glycogen. This results in about 55% conversion of amylopectin to maltose. The other product is a large limit dextrin. The viscosity reduction of the substrate due to β- amylase action is slow but the production of reducing sugars is fast.

Principle

The reducing sugars produced by the action of α-and/or β- amylase react with Dinitro salicylic acid and reduce it to a brown coloured product, Nitro amino salicylic acid.

Reagents

- Sodium acetate buffer, 0.1 M pH 4.7
- Starch, 1% solution: Prepare a fresh solution by dissolving 1 g starch in 100 ml Acetate buffer. Slightly warm, if necessary
- Dinitro Salicylic Acid reagent (DNS reagent): Dissolve by stirring 1 g Dinitro salicylic acid, 200 mg crystalline Phenol and 50 mg Sodium sulphite in 100 ml 1% NaOH. Store at 4^{o}C. Since the reagent deteriorates due to Sodium sulphite, if long storage is required, Sodium sulphite may be added at the time of use
- 40% Rochelle Salt Solution (Potassium sodium tartarate)
- Maltose solution: Dissolve 50 mg Maltose in 50 ml distilled water in a standard flask and store it in a refrigerator
- Extraction of amylases: Extract 1 g of sample material with 5-10 volumes of ice cold 10 mM Calcium chloride solutions overnight at 4^{o}C or for 3 hours at room temperature. Centrifuge the extract at 54,000 g at 4^{o}C for 20 minutes. The supernatant is used as enzyme source
- Extraction of β- Amylases (Free and Bound): The free β- amylase is extracted from Acetone defatted sample material in 66 mM Phosphate buffer (pH 7.0) containing 0.5 M NaCl. The extract is centrifuged at 20,000 rpm for 15 minutes. The supernatant is used as a source free β-amylase. The pellet is then extracted with Phosphate buffer containing 0.5% 2-mercaptoethanol. The clear extract is used as source of bound β- amylase. All operations are carried out at 4^{o}C

Procedure

- Pipette 1 ml of starch solution and 1 ml of properly diluted enzyme in a test tube
- Incubate it at 27^{o}C for 15 minutes
- Stop the reaction by the addition of 2 ml of Dinitro salicylic acid reagent
- Heat the solution in a boiling water bath for 5 minutes
- While the tubes are warm, add 1 ml Potassium sodium tartarate solution
- Then cool it in running tap water
- Make up the volume to 10 ml by addition of 6 ml water
- Read the absorbance at 560 nm
- Terminate the reaction at zero time in the control tubes
- Prepare a standard graph with 0-100 µg Maltose

Calculation

A unit of α- or β- Amylase is expressed as mg of maltose produced during 5 minutes incubation with 1% starch.

18. Extraction of Bromelain

Bromelain is a general name for a family of sulpha hydril proteolytic enzymes that are found in tissues of plant family Bromeliaceae, of which pineapple, *Ananas comosus*, is best known.

Procedure

Note: All the steps of bromelain extraction should be maintained at 4^{o}Cto prevent protein denaturation during the process.

- Peel pineapple, remove its eyes, and wash with distilled water
- Pineapple and its stem part are cut in to small pieces. Weigh 50 g of each part (Remaining steps have to be separately done for both fruit and stem)
- Add chilled aquadest (4^{o}C) in the ratio of 1:1 (50 g fruit part : 50 ml aquadest)
- Homogenize the samples in a blender
- Filter both the homogenates (crude extract) through muslin cloth to remove the solid parts
- 40 ml of each sample is taken for centrifugation
- Centrifuge each sample at 5000 rpm for 10 minutes to obtain sediment and supernatant
- Collect the supernatant and centrifuge at 10000 rpm for 10 minutes, dispose the supernatant
- Mixed the sediments from both the centrifugation process and dilute with 25 ml cold aquadest
- Transfer the sediment mixture in to a beaker and stirr using magnetic stirrer maintained in an ice bath (The process is done to homogenize all of the sediment)
- The mixture of sediment is subject to further centrifugation at 10000 rpm for 10 minutes at 4^{o}C. The supernatant obtained is the crude enzyme and it is stored at 4^{o}C till further procedure

Purification of Bromelain

The purification of bromelain by size exclusion chromatography is carried out at cold room 4^{o}C.

Procedure

- 1 g of Sephadex G-50 superfine is weigh in a clean beaker and mix with 10 ml of buffer solution pH of 7.4
- The matrix suspension is transfer in to glass column; the column is set vertically with the bottom valve close. Let the matrix settle for 15 minutes until the Sephadex is precipitated
- The buffer is drain by opening the bottom valve and the valve is closed afterward. 15 ml more of buffer is add to the clean Sephadex that trap to the column and then drain the buffer
- 10 ml extracted enzyme is carefully add to the column. In addition 30 ml of PBS (Phosphate Buffer Solution) is also added. Allow the system to settle for 5 minutes
- 10 tubes are prepared and place under the column. The valve is open and the enzyme sample is allowed to be filter through the Sephadex according to the molecular weight. Each tube is filled with 4 ml eluate
- The valve is close. 10 tubes containing eluate are collect and test for the protein content and enzyme activity

Enzyme Activity Determination

Procedure

- In to Eppendorf tube,250 µl of 1% casein and is mix with 250 µl of 0.05 M buffer pH 7.4 and 250 µl enzyme sample – 0.04 mM $CaCl_2$ mixture (1:1) is mixed
- The mixture is incubating at 37^{o}C for 20 minutes
- The reaction is stop with 750 µl 5% TCA (Trichloro acetic acid)
- The mixture is centrifuge at 8000 rpm for 10 minutes at 4^{o}C
- In two clean Eppendorf tube add 300 µl supernatant, 1000 µl 0.5 M Na_2CO_3 and 200 µl Folin–Ciocalteau reagent, which is diluted by miliq water (Folin–Ciocalteau: Miliq water = 1:2) and mix well
- The absorbance is measure with spectrophotometrically at 578 nm
- As standard, tyrosine which concentration of 0.625, 1.25, 2.5 and 5 mM is use instead of enzyme

19. Estimation of β-carotene

β-Carotene is a red-orange pigment abundant in fruits, vegetables and cereals. It is precursor of vitamin A.

Reagent

- Water saturated n-butanol: Mix n-butanol and water in ratio of 6:2 (v/v) and shake vigorously. Then allow to stand till it separates into two phases; the upper clear layer is water saturated n-butanol

Procedure

- Disperse 10 g of sample in 50 ml water saturated n-butanol to make a homogenous suspension
- Shake gently and allow to stand overnight (16 hours) at room temperature in dark
- Shake the suspension again and filter through Whatman filter paper no. 14. Make the volume of filtrate to 100 ml

- Measure the absorbance (A) of the clear filtrate at 440 nm in spectrophotometer using saturated n-butanol as a blank

Calculation

$$\beta\text{-carotene content (ppm)} = 0.0105 + 23.5366 \times A$$

20. Estimation of Ascorbic Acid

Ascorbic acid otherwise known as Vitamin C is an antiscorbutic. It is a good antioxidant when present in reduced form and is widely distributed in fresh fruits. It is a water soluble and heat labile vitamin.

20.1 Volumetric Method

Principle

Ascorbic acid reduces the 2,6-dichlorophenol indophenol dye to a colourless leucobase. The ascorbic acid gets oxidized to dehydroascorbic acid. Though the dye is a blue coloured compound, the end point is the appearance of pink colour. The dye is pink coloured in acid medium. Oxalic acid is used as the titrating medium.

Reagents

- Oxalic acid 4%
- Dye solution: Weigh 42 mg Sodium bicarbonate in to a small volume of distilled water. Dissolve 52 mg 2,6-dichloro indophenols in it and make up to 200 ml with distilled water
- Stock standard solution: Dissolve 100 mg ascorbic acid in 100 ml of 4% oxalic acid solution in a standard flask (1 mg/ml)
- Working standard: Dilute 10 ml of the stock solution to 100 ml with 4% oxalic acid. The concentration of the working standard is 100 µg/ml

Procedure

- Pipette 5 ml of the working standard solution in to a 100 ml conical flask
- Add 10 ml of 4% oxalic acid and titrate against the dye (V_1ml). End point is the appearance of pink colour, which is persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid
- Extract the sample (0.5 – 5 g depending on the sample) in 4% Oxalic acid and make up to a known volume (100 ml) and centrifuge
- Pipette 5 ml of this supernatant, add 10 ml of 4% Oxalic acid and titrate against the dye (V_2ml)

Calculation

$$\text{Amount of Ascorbic acid mg / 100 g sample} = \frac{V_1 \text{ ml}}{0.05} \times \frac{5 \text{ ml}}{V_2} \times \frac{\text{Weight of the sample}}{100 \text{ ml}} \times 100$$

Note

Acetic- metaphosphoric acid mixture may also be used instead of 4% Oxalic acid.

20.2. Colorimetric Method

Ascorbic acid is also determined colorimetrically. The dehydroascorbic acid alone reacts quantitatively and not the other reducing substances present in the sample extract. Thus this method given an accurate analysis of ascorbic acid content than the dye method.

Principle

Ascorbic acid is first dehydrogenated by bromination. The dehydroascorbic acid then react with 2,4-di nitro phenyl hydrazine to form osazone and dissolved in Sulphuric acid to give an orange-red colour solution which is measured at 540 nm.

Materials

- 4% Oxalic acid solution
- 0.5% Sulphuric acid
- 2% 2,4-di nitro phenyl hydrazine (DNPH) reagent – Dissolve by heating 2 g DNPH in 100 ml 0.5 N H_2SO_4. Filter and use
- 10% Thiourea solution
- 80% Sulphuric acid
- Bromine water – Dissolve 1-2 drops of liquor bromine in approximately 100 ml cool water
- Ascorbic acid stock solution – See previous procedure

Extraction

Grind 0.5-5 g of sample material either mechanically or using a pestle and mortar in 25-50 ml 4% oxalic acid solution. Centrifuge or filter and collect the liquid

Transfer an aliquot (10 ml) to a conical flask and add Bromine water drop wise with constant mixing. The enolic hydrogen atoms in ascorbic acid are removed by Bromine. When the extract turns orange yellow due to excess bromine, expel it by blowing in air. Make up to a known volume (25 or 50 ml) with 4% Oxalic acid solution.

Similarly, convert 10 ml of stock ascorbic acid solution in to dehydro form by bromination

Procedure

- Pipette 10-100 µg standard dehydro ascorbic acid solution in to a series of test tubes
- Similarly pipette different aliquots (0.1 ml-2 ml) of brominated sample extract
- Make up the volume in each tube to 3 ml by adding distilled water
- Add 1 ml of DNPH reagent followed by 1-2 drops of thiourea to each tube
- Set a blank as above but with distilled water in place of ascorbic acid solution
- Mix the contents of the tubes thoroughly and incubate at 37^{o}C for 3 hours
- After incubation dissolve the orange-red osazone crystals formed by adding 7 ml of 80% Sulphuric acid.
- Measure absorbance at 540 nm
- Plot a graph ascorbic acid concentration versus absorbance and calculate the ascorbic acid content in the sample

21. Determination of Dietary Fibre

Dietary fibre part of plant material in the diet which is resistant to enzymatic digestion which includes cellulose, non-cellulosic polysaccharides such as hemicelluloses, pectin substances, gums, mucilage's and a non-carbohydrate component lignin. The diets rich in fibre such as cereals, nuts, fruits and vegetables have a positive effect on health since their consumption has been related to decreased incidence of several diseases. Dietary fibre can be determined by different methods; Enzymic gravimetric and Enzymic-chemical method.

Principle

The sample is defatted; starch is gelatinized with α-amylase, and then removed, along with the protein by enzymatic digestion using protease and Amyloglucosidase. The fibre is precipitated with Ethanol and filtered to produce a residue. Total dietary fibre is then determined gravimetrically with a correction for protein and ash.

Total dietary fibre= weight (residue) - weight (ash + protein)

Reagents

- Petroleum ether
- Ethyl alcohol
- Acetone
- Sodium phosphate
- Dibasic anhydrous Sodium phosphate; Monobasic anhydrous Sodium phosphate
- Sodium hydroxide
- 1.0 N, Hydrochloric acid
- 1.0 M HCl

Preparation of Reagents

Use distilled or deionized water to prepare solutions.

- **78% Ethanol:** Place 207 ml of distilled water into 1 liter volumetric flask. Dilute to volume with 95% Ethanol. Mix and bring to volume again with 95% Ethanol if necessary. Mix
- **Phosphate Buffer, 0.08 M, pH 6.0:** Dissolve 1.4 g of Na_2HPO_4 and 8.4 g of NaH_2PO_4, anhydrous in approximately 700 ml of distilled water. Dilute to 1 litre with distilled water. Check pH and adjust if necessary with either NaOH or H_3PO_4. Store in tightly capped container at room temperature
- **Sodium Hydroxide Solution, 0.275 N:** Dilute 275 ml of 1.0 N NaOH solution to 1 litre with distilled water in a volumetric flask. Store in a tightly capped container at room temperature
- **Hydrochloric Acid Solution, 0.325 M:** Dilute 325 ml of 1.0 M HCl solution to 1 litre with distilled water in a volumetric flask. Store in a tightly capped container at room temperature

Enzymic-gravimetric Method

Procedure

- **Sample Preparation:** Fresh fruits are clean with water and external moisture wipe with a dry cloth. The edible portion of the individual fruits is separate, weigh and homogenize thoroughly in a mixer and a known weight of the sample is quantitatively transfer into 500 ml round bottom flasks (glass) and lyophilized. Freeze dried sample is extract with Diethyl ether to remove the fat content. The total soluble free sugars present in the sample are extracted with 80% hot Ethanol. The sample is rinse with Acetone to remove any traces of moisture from the sample and finally, dry in a hot air oven at 50°C for 1 hour. The dried samples are then powdered in to fine powder
- Weigh four 1 g samples of each material to be tested into tall form beakers. Sample weights should not differ by more than 20 mg. Record weights to 0.1 mg
- Add 50 ml of pH 6.0 Phosphate buffer to each beaker
- Add 0.10 ml α-Amylase to each beaker and mix well
- Cover each beaker with aluminum foil and place in a boiling water bath. Agitate beakers gently at 5 minutes intervals. Incubate for 15 minutes after the internal temperature of the beakers reaches 95°C
- Allow solutions to cool to room temperature
- Adjust the pH of the solutions to 7.5 ± 0.2 by adding 10 ml of 0.275 N NaOH to each beaker. Check pH; adjust if necessary with either NaOH or HCl
- Immediately before use, make a 50 mg/ml solution of protease in phosphate buffer. Pipette 0.1 ml (5 mg Protease) into each beaker
- Cover each beaker with aluminum foil and place in 60°C water bath. With continuous agitation, Incubate for 30 minutes after the internal temperature of the beakers reaches 60°C
- Allow solutions to cool to room temperature
- Adjust the pH of the solutions to between pH 4.0 and 4.6 by adding 10 ml of 0.325 M HCl to each beaker. Check pH; adjust if necessary with either NaOH or HCl
- Add 0.1 ml of Amyloglucosidase to each beaker
- Cover each beaker with aluminum foil and place in 60°C water bath. With continuous agitation, incubate for 30 minutes after the internal temperature of the beakers reaches 60°C
- Add 4 volumes of 95% Ethanol to each beaker
- Let solutions set overnight at room temperature to allow complete precipitation
- **Filtration:** Wet and redistribute the bed of Celite in each crucible using 78% Ethanol. Apply gentle suction to draw Celite onto frit as an even mat. Maintain gentle suction and quantitatively transfer the precipitate and suspension from each beaker to its respective crucible. Wash the residue with three 20 ml portions of 78% Ethanol, two 10 ml portions of 95% Ethanol, and two 10 ml portions of Acetone. A gum may form with some samples, trapping liquid. Breaking the surface film with a spatula will improve the rate of filtration. Be sure to rinse any material adhering to the spatula into the crucible. The time for filtration and washing will vary from 0.1 to 6 hours per crucible, averaging about 0.5 hour per crucible
- Dry crucibles containing residues overnight in a 105°C air oven or 70°C vacuum oven
- Cool all crucibles in a desiccator, weigh to nearest 0.1 mg, and record this weight as “Residue + Celite+ Crucible Weight" or W_2

- Analyze the residues from two samples and two blanks for protein by Kjeldahl nitrogen analysis. Use 6.25 as the factor to convert ammonia determined in the analysis to protein except where nitrogen content in the protein sample is known
- Ash the residue in the crucibles from two samples and two blanks for 5 hours at 525°C. Cool in desiccator, weigh to nearest 0.1 mg and record this weight as "Ash + Celite + Crucible Weight" or W_3.

Calculation

Residue Weight = W_2-W_1
Ash Weight = W_3-W_1

Where, W_1- Celite + Crucible Weight,
W_2- Residue + Celite+ Crucible Weight,
W_3- Ash + Celite + Crucible Weight

$$B = R\ Blank - P\ Blank - A\ Blank$$

$$\%\ TDF = [(R_{Sample} - P_{Sample} - A_{Sample} - B)/SW] \times 100$$

Where, TDF = Total Dietary Fiber
R = Average Residue Weight (mg)
P = Average Protein Weight (mg)
A = Average Ash Weight (mg)
SW = Average Sample Weight (mg)

22. Estimation of Mineral Content

Minerals help the body to grow, develop and stay healthy and perform many different functions from building strong bones to transmitting nerve impulses. Some minerals are also used to make hormones or maintain a normal heart-beat. Minerals are also important components of various enzymes, hemoglobin, chlorophyll in plants and electron transport system.

Reagents

- Diacid mixture – Mix Nitric acid and Perchloric acid in 4:1 ratio just before use
- Hydrochloric acid (1%) – Add 1 ml of concentrated HCl in 50 ml double distilled water and make volume 100 ml with double distilled water

Procedure

- Digest 1 g powdered sample with 15 ml of diacid mixture (4 HNO_3 : 1 $HClO_4$) in a conical flask by heating on hot plate in open space till clear white precipitates settle down at the bottom of the conical flask
- Dissolve the precipitates in 1% HCl prepared in double distilled water, filter and make volume of the filtrate to 50 ml with double distilled water

A. Micronutrients

The contents of iron (Fe), zinc (Zn) may be estimated, from the extract prepared,

B. Macronutrients

The amount of phosphorus, potassium, sodium, calcium and magnesium may be estimated from the same extract by the procedures mentioned below

22.1. Determination of Phosphorus by Colorimetric Method

Reagents

- Ascorbic acid (10%): Prepare by dissolving 10 g Ascorbic acid to 100 ml of distilled water
- Ammonium molybdate 2.5%: Prepare by dissolving 2.5 g Ammonium molybdate to 100 ml of distilled water
- Reagent C: Prepare by mixing 6 N Sulphuric acid, distilled water, 2.5% Ammonium molybdate and 10% Ascorbic acid in the ratio of 1:2:1:1 (v/v) at the time of use

Procedure

- Take 1 ml of the diluted extract (1 ml extract + 9 ml distilled water), in a test tube and make volume to 4 ml with distilled water
- Then, add 4 ml of reagent C to it and mix well
- Incubate the contents at 37^{o}C in a water bath for 90 minutes and cool at room temperature
- Read the absorbance at 820 nm against a suitable blank and calculate the phosphorus content with the help of a calibration curve of Mono potassium dihydrogen orthophosphate and express as mg g-1 dry weight

22.2. Determination of Sodium and Potassium Using Flame Photometry

Principle

In flame Photometry, sample is sprayed under constant and controlled condition the light intensity of the characteristic wavelength is produced by each of the atom is directly proportional to the number of atoms that are emitting energy, which in turn is directly proportional to the concentration of the substance of interest in the sample.

Reagents

- Double distilled deionized water- The method is very sensitive; the water must be free of metal ions
- Stock Standard Sodium Solution – 1000 ppm
- Stock standard potassium solution – 1000 ppm
- Test sample

Procedure

- Prepare 20, 15, 10 and 5 ppm sodium and potassium standards by dilution of the standard solutions using deionised water. Deionised water is used as the blank solution
- To 10 ml of the fruit juice, add 50 ml of deionized water

- Filter the fruit juice solution through an ashless filter paper (e.g. Whatman 540) into a litre volumetric flask. Ensure that the solid particles retained by the paper are washed thoroughly, collecting the washings into the same 1 litre flask. Dilute to the mark with deionized water and mix by inversion
- Switch on the instrument and allow it to warm up for 4-5 minutes
- Turn on the knob for the air supply and regulate the air pressure
- Turn the gas supply on and light with and obtain a non-luminous flame
- Spray in to the flame by dipping the tubing for the spray in take in water
- Calibrate the instrument using standards
- Then inject the diluted sample in to the flame and note the readings and calculate the concentration in milli equivalence

22.3. Determination of Calcium Using Flame Photometry

Reagents

- Standard Calcium solution 100 mg/l (containing 50 ml 4 M Perchloric acid per litre)
- 1% Ammonium oxalate solution
- 25% Ammonia solution
- 4 M Perchloric acid

Procedure

- Pipette 5 ml fruit juice into a 10 ml graduated stoppered centrifuge tube
- Add 5 ml of 1% Ammonium oxalate and 3 drops ammonia solution
- Shake and allow standing for 30 minutes
- Centrifuge at 300 rpm for 2 minutes
- Decant the supernatant and allow the tube to drain inverted for 30 seconds
- Add 0.5 ml 4 M Perchloric acid and shake
- Heat for 1 minute in a boiling water bath
- Cool and dilute to the 10 ml mark with distilled water
- Calibrate the flame photometer using a 100 mg/l calcium standard solution containing 50 ml 4 M Perchloric acid
- Aspirate the sample directly into the flame photometer
- The calcium concentration is calculated in milli equivalence

22.4. Determination of Zinc by Colorimetric Dithizone Method

Principle

The sample is wet or dry ashed. Zinc is extracted are Zinc dithizonate with CCl_4 for colour measurement.

Reagents

- **Copper sulphate solution 2 mg Cu/ml:** Dissolve 8 g of $CuSO_4.5H_2O$ in water and diluted to 1000 ml
- **Ammonium citrate solution:** Dissolve 225 g of $(NH_4)2HC_6H_5O_7$ in water and make alkaline to phenol red with NH_4OH (pH 7.5), and add 75 ml in excess. Dilute to 2000 ml. Extract this solution immediately before use as follows. Add slight excess of dithizone and extract with CCl_4 until solvent layer is clear bright green. Remove excess dithizone by repeated extraction with $CHCl_3$, and finally with CCl_4. Excess dithizone must be entirely removed

- **Dimethyl glyoxime solution:** Dissolve 2 g of reagent in 10 ml of NH_4OH and 200-300 ml water, filter, and dilute to 1000 ml with water
- **α-nitroso- β-naphthol solution:** Dissolve 0.25 g in $CHCl_3$ and dilute to 500 ml
- **Chloroform**
- **Di phenyl thio carbazone solution:** Dissolve 0.05 of dithizone in 2 ml of NH_4OH and 100 ml of water and extract repeatedly with CCl_4 until solvent layer is clear bright green. Discard solvent layer and filter aqueous portion through washed ashless filter paper
- **Carbon tetrachloride**
- **Dilute hydrochloric acid (0.04 N):** Dilute required amount of HCl with water
- Zinc standard solution:
  - Stock solution (500 µg Zn/ml): Dissolve 0.0500 g pure Zinc in slight excess of dilute HCl and dilute to 100 ml
  - Working solution (5 µg/ml): Dilute 10 ml of stock solution to 1000 ml with 0.04 N HCl

Procedure

Preparation of sample

Sample preparation is done by following anyone of the two procedures given below.

- **Wet ashing:** Accurately, weigh into 300 or 500 ml Kjeldahl flask, representative sample of about 25 g. Evapourate the liquid sample to small volume. Add Concentrated HNO_3 and heat cautiously until first vigorous reaction subsides and then add 2 to 5 ml Concentrated H_2SO_4. Continue heating, adding more HNO_3 in small portions and need to prevent charring, until solution is clear and almost colourless. Continue heating until dense fumes of H_2SO_4 evolve and all HNO_3 has been remove. Cool, dilute with approximately 25 ml of water filter, if necessary through prewashed fast filter paper and dilute the filtrate through 100 ml with water.
- **Dry ashing:** Accurately, weigh into a clean platinum or silica dish, a representative portion of sample (about 25 g). Char the sample and ash at temperatures not exceeding 500°C. raise the temperature of the muffle furnace slowly to avoid ignition. When ash is carbon free, dissolve ash under watch glass in, minimum volume of HCl. Add about 20 ml of water and evapourate to near dryness on team bath. Add 20 ml 0.1 N HCl and continue heating for 5 minutes. Filter through pre washed fast filter paper into 100 ml volumetric flask. Wash dish and filter with 5 to 10 ml portions of 0.1 N HCl. Cool and dilute to volume with 0.1 N HCl.

Isolation and determination

To a suitable aliquot of ash solution of sample, add 2 drops of Methyl red indicator and 1 ml of $CuSO_4$ solution and neutralize with NH_4OH. Add enough HCl to make solution about 0.15 N with respect to HCl. Adjust the pH of this solution, as measured with glass electrode, to 1.9 to 2.1. Pass stream of H_2S in solution until precipitation is complete. Filter through fine paper. Receive the filtrate in 250 ml beaker, wash flask and filter with 3 or 4 small portions of water. Gently boil filtrate until odour of H_2S can no longer be detected. Add 5 ml of saturated-bromine water and continue boiling until bromine free. Cool, neutralize to Phenol red with NH_4OH and make slightly acid with HCl. Dilute resultant solution to definite volume. At this stage, for optimum conditions of measurement, the solution should contain 0.2 to 1 µg of Zn/ml.

To 20 ml aliquot of this prepared solution, in a 125 ml separator, add 5 ml of Ammonium citrate solution, 2 ml Dimethyl glyoxime solution and 10 ml of α-nitroso- β-naphthol solution and shake for 2 minutes. Discard solvent layer and extract with 10 ml of $CHCl_3$. Discard solvent layer.

The aqueous phase following removal of cobalt and nickel, which at this point has pH 8.0 to 8.2, add 2 ml of dithizone solution and 10 ml of CCl_4 and shake for 2 minutes. Let phases separate and remove aqueous layer as completely as possible, withdrawing liquid with pipette attached to vacuum line. Wash down sides of separator with about 25 ml of water and without shaking again draw of aqueous layer. Add 25 ml of 0.04 N HCl and shake 1 minute. Drain and discard solvent, being careful to dislodge and remove drops of solvent that floats on surface. To acid solution, add 5.0 ml of Ammonium citrate solution and 10.0 ml of CCl_4 (pH of solution at this point should be 8.8 to 9.0). Determine the volume of dithizone solution and shake for 2 minutes. Pipette exactly 5.0 ml of solvent layer into clean, dry test tube, dilute with 10.0 ml of CCl_4 and mix well and determine absorbance' A' at 540 nm.

Convert absorbance 'A' to µg of zinc from standard curve and calculate Zn content of sample.

Determination of volume of Dithizone to be added

To separator containing Zinc standard solution equivalent to 20 µg Zinc and diluted to 25 ml with 0.04 N HCl, add 5.0 ml citrate solution, 10.0 ml of CCl_4 and add dithizone reagent in 0.1 ml increments , shaking briefly after each addition until faint yellow in aqueous phase indicates bare excess of reagent.

Multiply the volume of dithizone solution required by 1.5 and adds this volume (to nearest 0.05 ml) to all samples.

Preparation of standard curve

Prepare series of separators containing 0, 5, 10, 15 and 20 µg of Zinc diluted to 25 ml with 0.04 N HCl. Add to each separator, 5.0 ml of citrate solution, 10 ml of CCl_4 and determined volume of dithizone solution and shake for 2 minutes. Pipette exactly 5.0 ml of solvent layer into clean, dry test tube. Dilute with 10.0 ml of CCl_4. Mix well and read absorbance at 540 nm

Plot 'A' against concentration and draw smooth curve through points.

22.5. Determination of Iron by Colorimetric Method

Principle

Organic matter in the sample is destroyed by ashing, and the resulting ash is dissolved in Hydrochloric acid and diluted to a known volume with water.

Whole of the Iron present in the aliquot of ash solution is reduced with Hydroxylamine hydrochloride and Fe (II) is determined spectrophotometrically as its coloured complex with α-α-dipyridyl, the solution being buffered with acetate buffer solution. Absorption of resulting complex is read at 510 nm.

Reagents

- **Magnesium nitrate solution (50% w/v):** Dissolve 50 g of $Mg(NO_3)_2.6\ H_2O$ in distilled water and dilute to 100 ml with distilled water
- **Concentrated Hydrochloric acid**

- **Hydroxylamine hydrochloride solution (10% w/v**): Dissolve 10 g H_2NOH. HCl in distilled water and dilute to 100 ml
- **Acetate buffer solution:** Dissolve 8.3 g Anhydrous NaOAC (previously dried at 100°C) in distilled water, add 12 ml of Glacial acetic acid and dilute to 100 ml
- **α-α-dipyridyl solution (0.1% w/v):** Dissolve 0.1 g of α-α-dipyridyl in distilled water and dilute to 100 ml. Keep this reagent in cool and dark place.
- **Iron standard solution(0.01mg Fe/ml):**
  - Dissolve 0.3412 g $Fe(NH_4)_2(SO_4)_2.6\ H_2O$ in distilled water, add 2 drops of concentrated HCl and dilute to 100 ml
  - Dilute 5 ml of solution to 250 ml
- **Ortho phenanthroline solution (0.1% w/v):** Dissolve 0.1 g of O-phenanthroline in 80 ml of distilled water at 80°C, cool and dilute to 100 ml with distilled water. Keep in cool and dark place.

Preparation of sample

Weigh accurately, a suitable quantity of well homogenized sample, into a clean and tared silica dish. If sample contains more water, dry on a water bath. Char the sample (in the dish) on low flame of a burner till all the volatile matter escapes and smoking ceases. Transfer the dish to a cold muffle furnace and raise the temperature slowly to 450°C. Continue ashing at 450°C till practically carbon-free ash is obtained. (If carbon is present in ash even after 4 to 5 hour of ashing, remove the dish from furnace, cool and moisten the ash with 1 ml of Magnesium nitrate solution, dry on water bath and ash in furnace at 450°C. After the ash is carbon free remove the dish from furnace and cool.

Add 5 ml of concentrated HCl letting acid rinse the upper portion of the dish and evapourate to dryness on a water bath. Dissolve residue by adding exactly 2.0 ml of concentrated HCl, heat for 5 minutes on steam bath with watch glass covering the dish. Rinse watch glass with water, filter into a 100 ml volumetric flask, cool and dilute to volume.

Determination of Iron

Pipette 10 ml aliquot of ash solution into 25 ml volumetric flask, and add 1 ml hydroxylamine hydrochloride solution. After 5 minutes, add 5 ml buffer solution and 1 ml of O-phenanthroline solution or 2 ml of Dipyridyl solution and dilute to volume. Determine absorbance of solution at 510 nm. From absorbance reading, determine Fe content present in aliquot of ash solution taken by referring to standard curve.

Preparation of Standard curve

Pipette 0, 0.5, 1, 1.5, 2.0, 3.0 and 4.0 ml of Fe standard solution into a series of 25 ml volumetric flasks and add to each of them exactly 0.2 ml of concentrated HCl dilute each of them to exactly 10 ml with water, and then add reagents in the same way as for the sample, plot the quantity of Fe (in mg) against the absorbance.

Calculation

Iron content of sample (mg Fe/ 100 gm sample)

$$= \frac{\text{Quantity of Fe in aliquot of ash solution}}{\text{Aliquot of ash solution taken for determination}} \times \frac{\text{Total volume of ash solution}}{\text{Weight of the sample taken for ashing}} \times 100$$

H. Appendices

23. Appendix I - Abbreviations

A	Absorbance
AAS	Atomic Absorption Spectrometry
AOAC	Association of Official Analytical Chemists
APS	Ammonium Per Sulphate
BHA	Butylated Hydroxy Anisol
BOAA	β- N- oxalyl- amino- L- alanine
BOD	Biological Oxygen Demand
bp	Boiling Point
BSA	Bovine serum albumin
CFC	Chlorofluro Carbon
CI	Chemical Ionization
DNPH	2,4 – di nitro Phenyl Hydrazine
DNS	Di Nitro Salicylate
dNTP's	Deoxy Nucleoside Tri Phosphates
DPPH	1,1- di phenyl -2- picrylhydrazyl
EDL	Electrode less Discharge Lamp
EDTA	Ethylene Diamine Tetra Acetic acid
EI	Electron Ionization
ELSD	Evapourative Light Scattering Detector
Eq. wt	Equivalent weight
ESI	Electron Spray Ionization
ETAAS	Electro Thermal Atomic Absorption Spectrometry
EtBr	Ethidium Bromide
FAAS	Flame Atomic Absorption Spectrometry
FAB	Fast Atom Bombardment
FCR	Folin-Ciocalteau reagent
GC	Gas Chromatography
GFAAS	Graphite Furnace-AAS
GLP	Good Laboratory Practice
HC	Hydro Carbon
HCL	Hollow Cathode Lamp
HEPA	High Efficiency Particulate Air
HPLC	High-performance liquid chromatography
M	Molarity
MALDI	Matrix Assisted Laser Desorption / Ionization
MFC	Mixed Fluid Cascade
MS	Mass spectrometry
MSDS	Material safety data sheet
NaOAc	Sodium Acetate
NFE	Nitrogen Free Extract
nm	Nanometre(s) = 10^{-9} metre
OD	Optical Density

PA	Poly Acrylamide
PBS	Phosphate buffered saline triphosphate
PC	Paper chromatography
PCR	Polymerase chain reaction
PMT	Photomultiplier Tube
PPE	Personal Protective Equipment
RCF	Relative Centrifugal Force
R&D	Research and Development
RDA	Recommended Dietary Allowance
Rf	Relative mobility in chromatography
RH	Relative Humidity
rpm	Revolution Per Minute
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SI	Systeme International d' Unites
TCA	Tri Chloro Acetic acid
TD	To Deliver
TDF	Total Dietary Fibre
TDT	Thermal Death Time
TEMED N,N,N^1,N^1	Tetramethylethylenediamine
TLC	Thin layer chromatography
T_M	Melting Temperature
TSS	Total Soluble Solids
UV	Ultraviolet
VIS	Visible

24. Appendix II - Glossary

Absorbance (A): A measure of the amount of light absorbed by a substance suspended in a matrix. The matrix may be gaseous, liquid, or solid in nature. Most biologically active compounds (e.g., proteins) absorb light in the Ultra Violet (UV) or visible light portion of the spectrum. Absorbance is used to quantitate the concentration of the substance in question.

Absorption: From the Latin ab, away, and sorbere, to suck into. Absorption is the taking up of nutrients, water etc. by assimilation.

Acid: A substance that contains hydrogen atoms in its molecular structure, with a pH in the range from 0-6, which will react with a base to form a salt. Acids normally taste sour and feel slippery.

Affinity chromatography: A technique for separating a protein from a mixture on the basis of a property specific to that particular protein. For example, one substrate for lactate dehydrogenase is NADH; a column with NADH or a structurally related molecule may bind lactate dehydrogenase (and probably other dehydrogenases) with high affinity, while not binding the vast majority of other proteins.

Alkali solutions: When preparing concentrated alkali solutions (e.g., 40% NaOH) dissolve the alkali in distilled water under cooling.

Amylase: A term that is used to refer to a category of enzymes that catalyzes the chemical reaction in which amylose (starch) molecules are hydrolytically cleaved to molecular pieces (e.g., the polysaccharides maltose, maltotriose, α-dextrin etc.).

Angstrom (Å): A unit used for measuring lengths shorter than 100 **Å** such as those of atoms and molecules (nanometer, nm is used for longer dimensions);

1 **Å** = 10^{-10} metre or 10^{-8} centimetre or 0.1 nanometre

Anion: Negatively-charged particle or ion

Aquadest: Distilled water

Assay: A test that measures a response to a test substance or the efficacy of the test substance.

Ascorbic Acid: Ascorbic acid is a naturally occurring organic compound with antioxidant properties. It is white solid and soluble in water. Ascorbic acid is one form of vitamin C. It was originally called L-hexuronic acid, but, when it was found to have vitamin C activity in animals.

Band: A chromatographic zone that is a region where the separated substance is concentrated.

Biochemistry: The study of chemical processes that comprise living things (systems); the chemistry of life and living matter. It takes into account the studies related to the nature of the chemical constituents of living matter, their transformations in biological systems and the energy changes associated with these transformations. Biochemistry may thus be treated as a discipline in which biological phenomena are analyzed in terms of chemistry.

Buffer: A solution containing both a weak acid and its conjugate weak base whose pH changes only slightly on the addition of acid or alkali.

Carbohydrates (saccharides): A large class of carbon-hydrogen-oxygen compounds. Monosaccharides are called simple sugars, of which the most abundant is D-glucose. It is both the major fuel for most organisms and constitutes the basic building block of the most abundant polysaccharides, such as starch and cellulose. While starch is a fuel source, cellulose is the primary structural material of plants. Carbohydrates are produced by photosynthesis in plants. Most, but not all, carbohydrates are represented chemically by the formula $C_x(H_2O)_n$, where x and n are variable numbers, e.g. glucose: $C_6H_{12}O_6$. On the basis of their chemical structures, carbohydrates are classified as polyhydroxy aldehydes, polyhydroxy ketones, and their derivatives.

Cation: Positively-charged particle or ion

Centrifuge: A machine that is used to separate heavier from lighter molecules and cellular components and structures.

Chromatogram: A series of separated bands or zones detected either visually, as in paper chromatographic or thin layer chromatographic separations or indirectly by a detection system.

Chromatography: A method, often used in laboratories, which enables the easy and efficient separation of mixtures of chemical compounds using the phenomenon of adsorption. The technique's strengths are especially manifested in the separation of isomers and natural materials.

Desiccation: The process by which a substance is dried out and the moisture removed; desiccation is often carried out in a desiccator, which contains a substance which will take up water.

EDTA: Ethylene diamine tetra acetic acid is an amino poly carboxylic acid and a colourless, water-soluble solid. Its conjugate base is named ethylene diamine tetra acetate. It is an organic molecule is able to chelate (bind) certain other molecules such as divalent metal cations due to chemical groups at their juxtaposition within that molecule.

Effluent: Mobile phase that has exited from the column

Electrophoresis: Electrophoresis is an analytical method frequently used in molecular biology and medicine. It is applied for the separation and characterization of proteins, nucleic acids and subcellular sized particles like viruses and small organelles. Its principle is that the charged particles of a sample migrate in an applied electrical field. If conducted in solution, samples are separated according to their surface net charge density. The most frequent applications, however, use gels (Polyacrylamide, Agarose) as a support medium. The presence of such a matrix adds a sieving effect so that particles can be characterized by both charge and size. Protein electrophoresis is often performed in the presence of a charged detergent like Sodium dodecyl sulphate (SDS) which usually equalizes the surface charge and, therefore, allows for the determination of protein sizes on a single gel. Additives are not necessary for nucleic acids which have a similar surface charge irrespective of their size.

Eluate: A compound or mixture that has been separated in and exited from the column.

Eluent: Mobile phase

Elution: Removal of solute from a stationary phase by passage of a suitable mobile phase.

Enzyme: Enzymes are proteins that have catalytic functions indispensable to maintenance and activity of life. All chemical reactions occurring in a living organism are dependent on the catalytic actions of enzymes, and this is why enzymes are called biotransformers. Each enzyme is highly specific with regard

to the type of chemical reaction that it catalyzes, and to the substances (called substrates) upon which it acts. All enzymes are proteins; containing additional non-protein components called coenzymes.

Experiment: The test of a hypothesis

Ion-Exchange Chromatography: Separation of ionic compounds (which include nucleic acids and proteins) in a chromatographic column containing a polymeric resin (i.e., the stationary phase) having fixed charge groups. The process works in that charges of the column (stationary phase) interact with the opposite charges of the material which has dissolved in the material that is flowing through the column (mobile phase). The charge interaction between the column material and, i.e., the protein has the effect of slowing down the rate of movement of protein through the column. The other molecule, meanwhile, which not interact with the column, flow right on-through. This constitutes the separation process.

Lakhodi dal (*Lathyrus sativus*): It is also known as 'khesari' it grown and consumed all over Maharashtra by the rural for production and sale.

Microscope: An optical instrument that uses a lens or a combination of lenses to produce magnified images of small objects, especially of objects too small to be seen by the unaided eye.

Miliqwater: Ultrapure laboratory grade water that has been filtered and purified by reverse osmosis.

Mobile phase: The mixture of solvents that is percolated through the column.

Mobility: The velocity of a particle or ion attains for a given voltage. A relative measure of how quickly an ion moves in an electric field.

Molecular sieving: Separation of molecules on the basis of their effective sizes.

Northern Blotting: A research test/methodology used to transfer RNA fragments from an agarose gel (e.g., following gel electrophoresis) to a filter paper without changing the relative positions of the RNA fragments (e.g., re electrophoresis separation grid).

Polyacrylamide Gel: A "sieving" gel, which is used in electrophoresis

Polymerase Chain Reaction (PCR): A reaction that uses the enzyme DNA polymerase to catalyze the formation of more DNA strands from an original one by the execution of repeated cycles of DNA synthesis. Functionally, this is accomplished by heating and melting double-stranded DNA (hydrogen bonded) into single-stranded DNA (non-hydrogen bonded) and producing an oligo nucleotide primer complementary to each DNA strand. The primers bind to DNA and mark it in such a way that the addition of DNA polymerase and deoxy nucleoside tri phosphates causes a new strand of DNA, which is complementary to the target section of DNA. The process described previously is repeated (trait, product etc.) again and again to produce millions of copies (amplicons) of the desired strand of DNA.

Resolution: The degree of separation between two components by chromatography.

Retention time: The time that has elapsed from the injection of sample into the chromatographic system to the recording of the peak maximum of the component in the chromatogram.

Reverse phase: A chromatographic mode in which the mobile phase is more polar than the stationary phase.

R_f: A ratio used in paper chromatography, and thin-layer chromatography that is the difference from the origin to the centre of the separated zone divided by the distance from the origin to the solvent front.

SDS: Sodium dodecyl sulphate, also known as Sodium lauryl sulphate (SLS) is a surfactant used in biochemical and biotechnological applications for the solubilization of membrane components and hard-to- solubilize molecules. The SDS/PA in water solution helps to separate out contaminants commonly present in samples from plant tissues. Because DNA molecules are much more soluble in SDS/PA solution than are those contaminant molecules. Above a critical concentration, SDS forms micelles in water are thought to be responsible for its solubilizing action.

Southern Blot Analysis: A test that is performed on biological samples such as plant DNA. Gel electrophoresis is used to separate the DNA fragments according to size, and then those fragments are transferred to a filter (blot). Radio labeled DNA probes or RNA probes are added, and the ones which are complementary to each of the fragments will hybridize to those respective DNA fragments. The location (on the blot) and "radioactive label" of those hybridized probes can then be utilized to determine the nature of the DNA that was in those plant cells.

Southern Blot: Is a kind of electro blot, in which the substances separated and transferred are nucleic acids.

Stationary Phase: The portion of separation system that is immobilized in the column.

Support: The particles on which the stationary phase is held.

Ultracentrifuge: A high-speed centrifuge that can attain revolving speeds up to 85,000 rpm and centrifugal fields up to 500,000 times gravity. The machine is used to sediment (i.e., cause to settle out) macromolecular structures in a mixture/solution. In general, a centrifuge is a machine that whirls test tubes around rapidly, like a merry-go-round, to force the heavier suspended materials to the bottoms of those test tubes before the lighter material.

Ultra filtration: A separation methodology that uses the ability of synthetic semi permeable membranes to discriminate between molecules in the mixture, primarily on the basis of size and shape of the molecule. Invented and developed by Dr. Roy J. Taylor in the 1950s and 1960s, ultra filtration is typically used for the separation of relatively high-molecular-weight solutes (e.g., proteins, gums, polymers and other complex organic molecules) and colloidally dispersed substances (e.g., minerals, microorganisms, etc.) from their solvents.

Units (U): A measure of biological activity of a substance, as defined by various standardized assays.

Western Blot: Is a kind of electro blot, in which the substances separated and transferred are proteins.

25. Appendix III - Definitions

1. **Atomic weight:** The average mass of atoms of an element, calculated using the relative abundance of isotopes in a naturally occurring element.
2. **Molecular weight:** The sum of the atomic weights of all the atoms in a molecule is its molecular weight.
3. **Equivalent weight:** Equivalent weight of a substance is the number of grams of the substance required to react with, replace one mole of H_2O^+ or OH^-.The equivalent weight of an acid is the weight that contains one atomic weight of acidic hydrogen. i.e., the hydrogen that reacts during neutralization of acid with base.
4. **Percent Solution (w/v):** One percent solution of a substance contains one gram of the substance in 100 ml of the solvent. If v/v is given, it means 1 ml in 100 ml of solvent.
5. **Molar solution (M):** One molar solution of a substance contains one mole or one molecular weight of the substance in one litre of solution.
6. **Normal solution (N):** One normal solution of a substance contains one equivalent or one gram equivalent weight of the substance in one litre solution.
7. **Buffer:** A solution containing both a weak acid and its conjugate weak base whose pH changes only slightly on the addition of acid or alkali.
8. **pH:** pH is a value taken to represent the acidity or alkalinity of an aqueous solution. It is defined as logarithm of the reciprocal of the hydrogen ion concentration of the solution.

   $$\text{i.e., } pH = \log \frac{1}{[H^+]}$$

9. **Acidic buffer solutions:** An acidic buffer solution is simply one which has a pH less than 7. Acidic buffer solutions are commonly made from a weak acid and one of its salts - often a sodium salt. A common example would be a mixture of Ethanoic acid and Sodium ethanoate in solution. Change the pH of the buffer solution by changing the ratio of acid to salt, or by choosing a different acid and one of its salts.
10. **Alkaline buffer solutions:** An alkaline buffer solution has a pH greater than 7. Alkaline buffer solutions are commonly made from a weak base and one of its salts. A frequently used example is a mixture of ammonia solution and Ammonium chloride solution.
11. **Dilute acids:** While preparing dilute acids, add slowly to water preferable under cooling.
12. **Alkali solutions:** When preparing concentrated alkali solutions (e.g., 40% NaOH) dissolve the alkali in distilled water under cooling.

Units of Length

1 micron= 1µ= 1µm= $1x10^{-6}$ m

1Å= 0.1nm= $1x10^{-4}$ µm=$1x10^{-10}$ m

1nm=10Å=$1x10^{-3}$ µm= $1x10^{-9}$m

Conversion Units of Temperature

Temp. in ^{0}F= (Temp. in ^{0}Cx 1.8) + 32

Temp. in ^{0}C= (Temp. in ^{0}F-32)x 5/9

Temp. in K = (Temp. in ^{0}F+459.67) x 5/9

26. Appendix IV - Major Chemicals and Reagents Used in Biochemistry

Acetic acid	An organic acid; is one of the simplest carboxylic acids
Acetone	An organic compound; simplest example of the ketones
Acetylene	A hydrocarbon and the simplest alkyne; widely used as a fuel and chemical building block
Ammonia	inorganic; the precursor to most nitrogen-containing compounds; used to make fertilizer
Ammonium hydroxide	Aqueous ammonia; used in traditional qualitative inorganic analysis
Baeyer's reagent	An alkaline solution of Potassium permanganate; used in organic chemistry as a qualitative test for the presence of unsaturation, such as double bonds;
N-Bromosuccinimide	Used in radical substitution and electrophilic addition reactions in organic chemistry
Butanone (methyl ethyl ketone)	Organic compound; similar solvent properties to Acetone but has a significantly slower evaporation rate
Carbon disulfide	A non-polar solvent; used frequently as a building block in organic chemistry
Carbon tetrachloride	Toxic, and its dissolving power is low; consequently, it has been largely superseded by deuterated solvents
Collins reagent	Used to selectively oxidize primary alcohols to an aldehyde
Copper(I) iodide	Useful in a variety of applications ranging from organic synthesis to cloud seeding
Diethyl ether	Organic compound; a common laboratory solvent
Dimethyl ether	The simplest ether; a useful precursor to other organic compounds and an aerosol propellant
Dimethylformamide	Organic compound; a common solvent for chemical reactions
Ethanol	A powerful psychoactive drug; used in alcoholic beverages, in thermometers, as a solvent, and as a fuel
Fehling's reagent	Used to differentiate between water-soluble aldehyde and ketone functional groups
Formaldehyde	The simplest aldehyde; an important precursor to many other chemical compounds, such as polymers and polyfunctional alcohols
Formic acid	The simplest carboxylic acid; often used as a source of the hydride ion
Grignard reagents	The most common application is for alkylation of aldehydes and ketones
Hydrochloric acid	A highly corrosive, strong mineral acid with many industrial uses
Hydrofluoric acid	Valued source of fluorine, precursor to numerous pharmaceuticals; highly corrosive
Hydrogen peroxide	An oxidizer commonly used as a bleach
Imidazole	An organic compound; this aromatic heterocyclic is a diazole and is classified as an alkaloid
Isopropyl alcohol	Simplest example of a secondary alcohol; dissolves a wide range of non-polar compounds
Manganese dioxide	Used as a pigment and as a precursor to other manganese compounds; used as a reagent in organic synthesis for the oxidation of allylic alcohols

Methyl tert-butyl ether	A gasoline additive; also used in organic chemistry as a relatively inexpensive solvent
Millon's reagent	An analytical reagent used to detect the presence of soluble proteins
Nitric acid	Highly corrosive and toxic strong acid; used for the production of fertilizers, production of explosives, and as a component of aqua regia
Palladium(II) acetate	A catalyst for many organic reactions by combining with many common classes of organic compounds to form reactive adduct
Perchloric acid	A powerful oxidizing agent; readily forms explosive mixtures; mainly used in the production of rocket fuel
Phosphoric acid	A mineral acid with many industrial uses; commonly used in the laboratory preparation of hydrogen halides
Phosphorus pentachloride	One of the most important phosphorus chlorides; a chlorinating reagent. Also used as a dehydrating agent for oximes which turn them into nitriles
Phosphorus tribromide	used for the conversion of alcohols to alkyl bromides
Phosphorus trichloride	Most important of the three phosphorus chlorides; used to manufacture organophosphorus compounds; used to convert primary and secondary alcohols into alkyl chlorides, or Carboxylic acids into acyl chlorides
Potassium dichromate	A common inorganic chemical reagent, most commonly used as an oxidizing agent in various laboratory and industrial applications
Potassium hydroxide	A strong base; precursor to most soft and liquid soaps as well as numerous potassium-containing chemicals
Potassium permanganate	A strong oxidizing agent; can be used to quantitatively determine the total oxidizable organic material in an aqueous sample; a reagent for the synthesis of organic compounds
Raney nickel	An alternative catalyst for the hydrogenation of vegetable oils; in organic synthesis, used for desulfurization
Silver oxide	Used to prepare other silver compounds; in organic chemistry, used as a mild oxidizing agent
Silver nitrate	Precursor to many other silver compounds; commonly used in inorganic chemistry to abstract halides
Sodium hydride	A strong base used in organic synthesis
Sodium hydroxide	Strong base with many industrial uses; in the laboratory, used with acids to produce the corresponding salt, also used as an electrolyte
Sodium nitrite	Used to convert amines into diazo compounds
Sulfuric acid	Strong mineral acid; major industrial use is the production of Phosphoric acid
Tetra hydro furan	One of the most polar ethers; a useful solvent; its main use is as a precursor to polymers

27. Appendix V - System International (SI) Units, Conversions and Derivations

The French **Systeme International d' Unites** (the SI system) is accepted conversion for all units of measurements.

Table.8. SI Units of Mass

Weight	Symbol	Multiple of gram	Lower equivalent
Megagram	M	10^6	1000 kilogram
Kilogram	K	10^3	1000 gram
Gram	G	1	1000 milligram
Milligram	M	10^{-3}	1000 microgram
Microgram	µ	10^{-6}	1000 nanogram
Nanogram	N	10^{-9}	1000 picogram
Picogram	P	10^{-12}	1000 femtogram

Table.9. SI units – Basic

Quantity	SI unit	Symbol (basic SI units)	Quantity	SI unit	Symbol (basic SI units)
Basic units			**Basic units**		
Length	Metre	M	Plane angle	Degree	°
Mass	Kilogram	Kg	Plane angle	Minute	’
Time	Second	S	Plane angle	Second	’’
Electric current	Ampere	A	Length	Foot	’
Temperature	Kelvin	K	Length	Inch	’’
Luminous intensity	Candela	Cd	Length	Angstrom	A
Amount of substance	Mole	Mol	Area	Barn	B
Time	Minute	Min	Volume	Litre	L
Time	Hour	H	Mass	Tonne	T
Time	Day	D	Pressure	Bar	Bar

Table.10. Constants of Acids and Bases

Acid or base	Formula	Molecular weight	Commercial concentrated reagent		
			Specific gravity	**% by weight**	**Molarity(M)**
Acetic acid	CH_3COOH	60.1	1.05	99.5	17.4
Ammonium hydroxide	NH_4OH	35.0	0.89	28	14.8
Formic acid	HCOOH	46.0	1.20	90	23.4
Hydrochloric acid	HCl	36.5	1.18	36	11.6
Nitric acid	HNO_3	63.0	1.42	71	16.0
Perchloric acid	$HClO_4$	100.5	1.67	70	11.6
Phosphoric acid	H_3PO_4	80.0	1.70	85	18.1
Sulphuric acid	H_2SO_4	98.1	1.84	96	18.0

Table.11. SI units – Derived

Quantity	**SI unit**	**Symbol (basic SI units)**	**Definition of SI unit**	**Equivalent in SI units**
Force	Newton	N	$kgms^{-2}$	Jm^{-1}
Energy, work, heat	Joule	J	kgm^2s^{-2}	Nm
Power, radiant flux	Watt	W	kgm^2s^{-2}	Js^{-1}
Electric charge, quantity	Coulomb	C	As	JV^{-1}
Electric potential difference	Volt	V	$kgm^2s^{-3}A^{-1}$	JC^{-1}
Electric resistance	Ohm	Ω	$kgm^2s^{-2}A^{-2}$	VA^{-1}
Pressure	Pascal	Pa	$kgm^{-1}S^{-2}$	Nm^{-2}
Frequency	Hertz	Hz	S^{-1}	
Magnetic flux density	Tesla	T	$kgS^{-2}A^{-1}$	Vsm^{-2}
Other units based on SI				
Area	Square metre	m^2		
Volume	Cubic metre	m^3		
Density	Kilogram per cubic metre	kgm^{-3}		
Concentration	Mole per cubic metre	mol m^{-3}		

Table.12. SI prefixes and multiplication factors

Multiplication factor		**Prefix**	**Symbol**
1 000 000 000 000 000 000 000 000	$=10^{24}$	yotta	Y
1 000 000 000 000 000 000 000	$=10^{21}$	zetta	Z
1 000 000 000 000 000 000	$=10^{18}$	exa	E
1 000 000 000 000 000	$=10^{15}$	peta	P
1 000 000 000 000	$=10^{12}$	tera	T
1 000 000 000	$=10^{9}$	giga	G
1 000 000	$=10^{6}$	mega	M
1 000	$=10^{3}$	kilo	K
100	$=10^{2}$	hecto	H
10	$=10^{1}$	deca	Da
0.1	$=10^{-1}$	deci	D
0.01	$=10^{-2}$	centi	C
0.001	$=10^{-3}$	milli	M
0.000 001	$=10^{-6}$	micro	μ
0.000 000 001	$=10^{-9}$	nano	N
0.000 000 000 001	$=10^{-12}$	pico	P
0.000 000 000 000 001	$=10^{-15}$	femto	F
0.000 000 000 000 000 001	$=10^{-18}$	atto	A
0.000 000 000 000 000 000 001	$=10^{-21}$	zepto	Z
0.000 000 000 000 000 000 000 001	$=10^{-24}$	yocto	Y

Table.13. SI Units and Conversion Table

SI Units		**Conversion Table**		
Length and area		**fps Units**	**SI Units**	**Reciprocal**
Micron (μm, μ)	=10^{-6} m	**Length**		
Angstrom (A)	=10^{-10} m	1 inch (in)	=2.54 x 10^{-2}m	39.370079
Fermi (fm)	=10^{-15} m	1 foot (ft)	=0.3048 m	3.280839
Are (a)	=100 m^2	1 yard (yd)	=0.9144 m	1.093613
Barn (b)	=10^{-28} m^2	1 fanthom	=1.8288 m	0.546806
Mass		1 chain	=20.1168 m	4.97097 x 10^{-2}
Tonne (t)	=10^6g=1000kg	1 furlong	=2.01168 x 10^2 m	4.97097 x 10^{-3}
fps Units		1 mile (mi)	1.609344 x 10^3 m	6.213712 x 10^{-4}
Length		**Area**		
12 inches	=1 foot (ft)	1 in^2	=6.4516 x $10^{-4}$$m^2$	1.550003 x 10^3
3 feet	=1 yard (yd)	1 ft^2	=9.290304 x $10^{-2}$$m^2$	10.763910
22 yards	=1 chain	1 yd^2	=0.836127 m^2	1.195990
10 chains	=1 furlong	1 mi^2	=2.589988 x 10^6 m^2	3.861022 x 10^{-7}
8 furlongs	=1 mile (mi)	1 acre	=4.046856 x 10^3 m^2	2.471054 x 10^{-4}
6 feet	=1 fanthom	**Volume**		
6080 feet	=1UK nautical mile	1 in^3	=1.638706 x 10^{-5} m^3	6.102374 x 10^4
Area		1 ft^3	=2.831685 x $10^{-2}$$m^3$	35.31467
4840 $yard^2$	=1 acre	1yd^3	=0.764555 m^3	1.307950
640 acres	=1 $mile^2$	1 fluid ounce (fl oz)	=2.841306 x $10^{-5}$$m^3$	3.519508 x 10^4
Mass		1 pint (pt)	5.682613 x $10^{-4}$$m^3$	1.759754 x 10^3
16 ounces (oz)	=1 pound (lb)	1 quart (qt)	=1.136523 x $10^{-3}$$m^3$	8.798770 x 10^2
14 pounds	=1 stone	1 gallon (gal)	=4.54609 x $10^{-3}$$m^3$	2.199692 x 10^2
28 pounds	=1 quarter	1 bushel (bu)	=0.036369 m^3	27.495944
4 quarters	=1 hundredweight	1 US gallon (=231in^3)	=3.785412 x 10^{-3} m^3	2.641721 x 10^2
20 hundredweight	=1 ton	**Mass**		
Volume		1 ounce (oz)	=2.834952 x 10^{-2} kg	35.273962
20 fluid ounces	=1 pint (pt)	1 pound (lb)	=0.45359237 kg	2.204623
2 pints	=1 quart (qt)	1 stone	=6.350293 kg	0.158473
4 quarts	=1 gallon	1 quarter	=12.700586 kg	7.873652 x 10^{-2}
		1 hundredweight	=50.802345 kg	1.968413 x 10^{-2}
		1 ton	=1.016047 x 10^3 kg	9.842065 x 10^{-4}

Table.14. Designation of large numbers

	USA	**UK**
10^6	Million	Million
10^9	billion	Milliard
10^{12}	trillion	Billion
10^{15}	quadrillion	Billiard
10^{18}	quintillion	Trillion

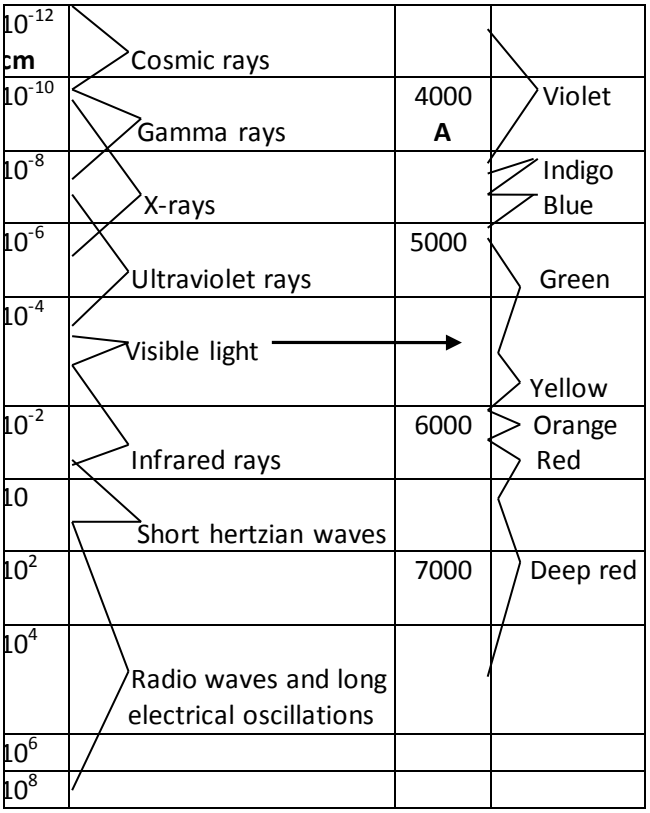
Table.15. Conversion Factors

Parameter	Conversion	Reciprocal
P_2O_5	=P x 2.29	0.43668
K_2O	=K x 1.2	0.83333
Urea	=N x 2.174	0.46
SSP	=P_2O_5 x 6.25	0.16
MOP	=K_2O x 1.67	0.275438
Organic matter %	=C x 1.74	0.5747

Table.16. Soil test rating

Parameter	Low	Medium	High
O.C. %	< 0.5	0.5-0.75	> 0.75
N kg/ha	< 280	280-560	> 560
P kg/ha	< 10	10-25	> 25
K kg/ha	< 110	110-280	> 280

Table.17. Electromagnetic and visible spectra



Useful data

1 atom = 764 mm Hg = 1036 cm water = 1013 mbar = pF 3 = 1.03329 kg/cm^2 = 1013231 dynes/cm^2
1 ha = 0.01 km^2 = 10000 m^2 = 2.471 ac = 0.003861 mile^2
1 ac = 100 cents = 0.4 ha = 4047m^2 = 43560ft^2,1 cent = 40.47m^2 = 436ft^2
1 mile = 8 furlong = 80chains = 1760yards = 5280ft
1 ton = 20 hundredweight (cwt) = 80 quarter = 2240 pounds = 35840 ounces
1 HP = 76.0404 kg.m/s = 745.7 watts = 550 ft.lb/s
Erosion: v velocity, v^2 erosive power, v^5 amount eroded, v^6 size of materials carried away.
When slope is increased 4 times: increase in velocity 2 times, erosivity 4 times, quantity 32 times, size 64 times.
Construct bunds every 3 ft vertical drop or 300 ft length whichever is less
Solar constant: 2cal/cm^2/minute. Average energy received on earth from sun.
Plants use only 0.4-0.5% energy. Algae use the maximum, 2.5%
Beer's law $I=I_o e^{-KL}$
Hopkins bioclimatic law: Crop phonological events are delayed by 1 day for every 1^o latitude, 5^o longitude and 400 ft altitude.
15 cm 1 ha furrow slice = 2.2 million kg
1 ha 1 mm water = 10 m^3
1 cusec (cubic foot/s) = ft^3/s = 28.3 l/s = 1 acre inch/hour
1 cumes (cubic metre/s) = m^3/s = 35.3 cusecs
$^oC = (^oF – 32)\ 5 \div 9$; $^oF = 1.8^oC + 32$
Balanced fertilizer application based on balanced ratio of NPK 4:16:1 in the economic part.
Protein contains 16% N, Protein % =N% x 6.25
Organic matter contains 58% carbon, Organic matter % =C % x 1.724
Soil water potential, $\psi = \psi_{m/p} + \psi_{o/s} + \psi_g + \psi_a$
Pan evaporation, E_o=4-6 mm/day, $Et_o = E_o$ x 0.6-0.8
Irrigation requirement: depth=3-8cm, interval=8-10days, IW/CPE=0.9 for sensitive crops, 0.6 for hardy crops
N x Eq. wt=g/l, N=Eq. wt/l, ppm=μg/ml=mg/l=me/l x Eq. wt.
mmhos/cm, EC x 640=TSS, ppm; EC x 0.064=TSS%; EC x 10=TSS, me/l; EC x –0.36=$\psi_{o/s}$ (+ osmotic pressure)
NPK recovery by crop: <40, <20, 80-90%
Cation adsorption to clay: Al>Fe>Si>H>Ca>Mg>K>Na
Anion adsorption to clay: SiO_4>PO_4>MO_4>SO_4>NO_3>Cl
(Cation and anion leaching in reverse order)
Anion toxicity to crops: HCO_3>CO_3>Cl>SO_4>NO_3
Anaerobic reduction during flooding: O>NO_3>Mn>Fe>S>C
Lyophilic series/Displacement capacity of anions: F>OH>HCO_3>PO_4>SiO_4
Plant mobile nutrients: N, P, K, Mg, Cl, S
Plant immobile elements: Ca, Fe, Mn, Zn, Cu, Mo, B
Particle size: solution < 10A colloid 1000A > suspension
Neutralising value (Ca equivalent): $CaCO_3$ 100, $MgCO_3$ 119, $Ca(OH)_2$ 136, CaO 179
Residual (equivalent) acidity: $CaNH_4NO_3$ zero, Urea 80, NH_4NO_3 60, $(NH_4)_2SO_4$ 110, $(NH_4)_3PO_4$ 86, NH_4Cl 128, anhydrous NH_3 148
Essential elements: C, H, O, N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, Mo, B, Cl, Co
Soils having >20% o.m. = organic; >70% sand = sandy; >40% clay = clayey; 27-52% silt = silty soil

28. Appendix VI - Solution Preparation

I. Buffers

A buffer, as defined by Van Slake is "a substance which by its presence in solution increases the amount of acid or alkali that must be added to cause unit change in pH". Buffers are thus very important components in experiments designed to study biological reactions by maintaining a constant concentration of hydrogen ions within the physiological range.

Measurement of pH

pH can be measured in many ways. An accurate and practical method for measuring pH involves the use of pH meter. It consists of a glass electrode with a glass bulb made of very thin glass that is permeable to hydrogen ions. Standardization against a buffer of known H^+ concentration is required since concentration of H^+ inside the bulb of glass electrode changes with time.

The pH scale usually runs from 0 to 14, with 7 representing neutrality and pH value above 7 characterize basic conditions and pH value below 7 represent acidic conditions.

The pH of the mobile phase (eluent) is adjusted to improve component separation and to extend the column life.

This pH adjustment should involve not simply dripping in an acid or alkali but using buffer solutions. A buffer solution is prepared as a combination of weak acids and their salts (sodium salts, etc.) or of weak alkalis and their salts. Common preparation methods include:

i. Dripping an acid (or alkali) into an aqueous solution of a salt while measuring the pH with a pH meter.
ii. Making an aqueous solution of acid with the same concentration as the salt and mixing while measuring the pH with a pH meter. However, if the buffer solution is used as an HPLC mobile phase, even small errors in pH can lead to problems with separation reproducibility. Therefore, it is important to diligently inspect and calibrate any pH meter that is used. This page introduces a method that does not rely on a pH meter. The method involves weighing theoretically calculated fixed quantities of a salt and acid (or alkali) as shown in the table below. Consider the important points below.

Denoting Buffer Solutions

A buffer solution denoted, "100 mM Phosphoric acid (sodium) buffer solution pH = 2.1," for example, contains Phosphoric acid as the acid, sodium as the counter ion, 100 mM total concentration of the Phosphoric acid group, and a guaranteed buffer solution pH of 2.1.

Maximum Buffer Action Close to the Acid (or Alkali) pKa

When an Acetic acid (sodium) buffer solution is prepared from 1:1 Acetic acid and Sodium acetate, for example, the buffer solution pH is approximately 4.7 (near the Acetic acid pKa), and this is where the maximum buffer action can be obtained.

Buffer Capacity Increases as Concentration Increases

The buffer capacity of an Acetic acid (sodium) buffer solution is larger at 100 mM concentration than at 10 mM, for example. However, precipitation occurs more readily at higher concentrations.

Beware of Salt Solubility and Precipitation

The salt solubility depends on the type of salt, such as potassium salt or sodium salt. Salts precipitate out more readily when an organic solvent is mixed in.
In addition, avoid using buffer solutions based on organic acids (carboxylic acid) as much as possible for highly sensitive analysis at short UV wavelengths.

Tabe.18. Technical Programme for the Preparation of Buffer Solutions

100 mM phosphoric acid (sodium) buffer solution (pH=2.1) Sodium di hydrogen phosphate di hydrate (M.W.=156.01)..50 mmol (7.8 g) Phosphoric acid (85 %, 14.7 mol/l).....50 mmol (3.4 ml) Add water to make up to 1 l
10 mM phosphoric acid (sodium) buffer solution (pH=2.6) Sodium di hydrogen phosphate di hydrate (M.W.=156.01)..5 mmol (0.78 g) Phosphoric acid (85 %, 14.7 mol/l).....5 mmol (0.34 ml) Add water to make up to 1 l (Alternatively, dilute 100 mM phosphoric acid (sodium) buffer solution (pH=2.1) ten times.)
50 mM phosphoric acid (sodium) buffer solution (pH=2.8) Sodium di hydrogen phosphate di hydrate (M.W.=156.01)..40 mmol (6.24 g) Phosphoric acid (85 %, 14.7 mol/l).....10 mmol (0.68 ml) Add water to make up to 1 l
100 mM phosphoric acid (sodium) buffer solution (pH=6.8) Sodium di hydrogen phosphate di hydrate (M.W.=156.01)..50 mmol (7.8 g) Sodium di hydrogen phosphate 12-hydrate (M.W.=358.14)..50 mmol (17.9 g) Add water to make up to 1 l
10 mM phosphoric acid (sodium) buffer solution (pH=6.9) Sodium di hydrogen phosphate di hydrate (M.W.=156.01)..5 mmol (0.78 g) Sodium di hydrogen phosphate 12-hydrate (M.W.=358.14)..5 mmol (1.79 g) Add water to make up to 1 l (Alternatively, dilute 100 mM phosphoric acid (sodium) buffer solution (pH=6.8) ten times.)
20 mM citric acid (sodium) buffer solution (pH=3.1) Citrate di hydrate (M.W.=210.14).....16.7 mmol (3.51 g) Sodium citrate di hydrate (M.W.=294.10)..3.3 mmol (0.97 g) Add water to make up to 1 l
20 mM citric acid (sodium) buffer solution (pH=4.6) Citrate di hydrate (M.W.=210.14).....10 mmol (2.1 g) Sodium citrate di hydrate (M.W.=294.10)..10 mmol (2.94 g) Add water to make up to 1 l
20mM (acetic acid) ethanolamine buffer solution pH=9.6 Monoethanolamine (M.W.=61.87, d=1.017)...20 mmol (1.22 ml)

Acetic acid (glacial acetic acid, 17.4 mol/l).....10 mmol (0.575 ml) Add water to make up to 1 l
100 mM acetic acid (sodium) buffer solution (pH=4.7) Acetic acid (glacial acetic acid) (99.5 %, 17.4 mol/l).....50 mmol (2.87 ml) Sodium acetate trihydrate (M.W.=136.08).....50 mmol (6.80 g) Add water to make up to 1 l
100 mM boric acid (potassium) buffer solution (pH=9.1) Boric acid (M.W.=61.83).....100 mmol (6.18 g) Potassium hydroxide (M.W.=56.11).....50 mmol (2.81 g) Add water to make up to 1 l
100 mM boric acid (sodium) buffer solution (pH=9.1) Boric acid (M.W.=61.83).....100 mmol (6.18 g) Sodium hydroxide (M.W.=40.00).....50 mmol (2.00 g) Add water to make up to 1 l

Glycine-NaOH Buffer

Stock Solutions

A: 0.2 M solution of glycine (15.01 g in 1 l)

B: 0.2 M NaOH

50 ml of A+ x ml of B, diluted to a total of 200 Ml

Table.19. Glycine-NaOH Buffer - Stock Solutions

X	pH
4.0	8.6
6.0	8.8
8.8	9.0
12.0	9.2
16.8	9.4
22.4	9.6
27.2	9.8
32.0	10.0
38.6	10.4
45.6	10.6

Phosphate Buffer

Stock solutions

A: 0.2 M solution of monobasic phosphate (27.8 g in 1000 ml)

B: 0.2 M solution of the dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml)

x ml of A, y ml of B, diluted to a total of 200 Ml

Table.20. Phosphate Buffer - Stock solutions

X	Y	pH	X	Y	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	89.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49	6.8	5.3	94.7	8.0

II. Preparation and Dilution of Solutions

a. Percent solution
   Solution in the exact concentration of the solute in 100 ml of liquid is known percentage solution. The concentration may be expressed as weight or volume.
   Percentage (v/v) = x ml in 100 ml of solution
   Percentage (w/v) = x g in 100 ml solution

III. Preparation of Concentrated Solutions

a. Molar solution (M) (mole): The SI unit of amount of substances, equal to the quantity containing as many elementary units as there are atoms in 0.012 kg of Carbon-12 or One gram molecular weight of the solute dissolved in 1000 ml of solvent
b. Normal solution (N): One gram equivalent weight of the solute dissolved in 1000 ml of distilled water
c. Molar (mol): containing one mole of solute per kilogram of solvent

IV. Preparation of Standard Solutions

1. Standard NaOH Solutions (0.1 M or 0.1 N)

Reagents

- Approximately 0.1 N NaOH solution: Prepare a saturated solution of sodium hydroxide as follows. Add in small portions about 100g of AR grade NaOH to 100 ml of water in a flask, stopper and allow to stand for a few days or until the Na_2CO_3 settles to the bottom. The clear solution which is carbonate-free contains about 50 g of NaOH/100 ml (approx. 50% solution or 12.5 M). From this solution, pipette out 5.3ml of clear supernatant liquid and transfer to a 1.0 litre rubber stoppered bottle and dilute up to the mark
- Standard HCl solution (0.1 N): Prepare and standardize as described before
- Phenolphthalein solution: Dissolve 0.5 g of phenolphthalein in 100 ml of 95% alcohol

Method

Using a pipette, transfer 20 ml of standard HCl solution into 150 ml beaker. Add 25 ml of water and one drop of phenolphthalein indicator. Titrate with the sodium hydroxide (approx. 0.1 N) until a faint pink colour is obtained. Repeat titration 2-3 times.

Calculation

The exact normality of NaOH using the relationship $N_1 V_1 = N_2 V_2$

2. Standard H_2SO_4 Solution (0.05 M/0.1 N)

Reagents

- Approximately 0.05 M/0.1 N H_2SO_4 Solution: Dilute 3.0 ml of pure concentrated H2SO4 solution (sp. Gr. 1.84, 96%) to 1.0 l with water in a volumetric flask and mix thoroughly)
- Anhydrous Na_2CO_3 solution (0.1 N): Prepare as described under standard HCl
- Methyl orange indicator: Prepare as described under standard HCl

Method

Fill a clean, dry burette with H_2SO_4 solution (0.05 M/0.1 N) and titrate against a known volume (10 ml) of anhydrous Na_2CO_3 solution using 2 drops of methyl orange as an indicator until an orange or faint pink color end point is obtained. Repeat titration 2-3 times.

Calculation

The exact normality of H_2SO_4 using the relationship $N_1 V_1 = N_2 V_2$

3. Standard HCl Solution (0.1 M or 0.1 N)

Reagents

- Approximately 0.1 M or 0.1 N HCl Solution: Dilute 9.0 ml (sp. Gr. 1.18, 35%) or 8.5 ml (sp. Gr. 1.19, 37%) of pure concentrated HCl to 1.0 litre with distilled water in a volumetric flask. Invert several times and transfer to a clean, dry bottle.
- Anhydrous Na_2CO_3 solution (0.1 N): Dry the pure anhydrous Na_2CO_3 on a watch glass in the oven at 110°C quantitatively to a beaker and dissolve in about 20 ml of water. Transfer to 100ml volumetric flask with washings, up to the mark and mix.
- Methyl orange indicator solution: Dissolve 0.5 g of Methyl orange in 1.0 litre of distilled water

Method

Fill a clean, dry burette with HCl solution (approx0.1M or 0.1N) and titrate against a known volume (10 ml) of Sodium carbonate (0.1 N) using 2 drops of Methyl orange as an indicator until an orange or faint pink colour end point is obtained. Note the titre value and repeat titration 2-3 times.

Calculation

Using$N_1 V_1 = N_2 V_2$ relationship, the exact normality of HCl can be calculated.

Where, N_1 – Normality of HCl to be calculated

N_2 - Normality of Na_2CO_3 = 0.1N

V_1 - Volume of HCl (titre value)

V_2 –Volume of Na_2CO_3 (10ml)

Note:

Methyl red also may be used in place of Methyl orange as an indicator provided the CO_2 in solution is expelled by boiling before the end point is reached.

4. Standard Potassium Permanganate Solution (0.1N)

Reagents

- Potassium Permanganate Solution (0.1 N approximate): Dissolve about 3.2g of pure crystals of Potassium permanganate in 200 ml of hot water and allow to stand for 60 minutes at about 100°C. The mouth of the container is well closed with a glass-stopper to disallow any addition of dust particles into the permanganate solution. The solution is then filtered after 2 days standing through either a sintered filter or a filter of glass wool but never filter through a filter paper because the latter reduces the strength of permanganate considerably. The solution is kept in dark because of its sensitivity to light
- Oxalic acid ($H_2C_2O_4$. $2H_2O$) or Sodium oxalate $(COONa)_2$(0.1N): Dissolve 6.3 g of pure crystals of oxalic acid in 200 ml of water and transfer to a litre flask. Make up with water or slowly heat 6.7 g of pure sodium oxalate salt in a platinum crucible until the flame of carbon monoxide formed disappears. Dissolve the contents of crucible with water and make up to 1.0 litre
- 4N H_2SO_4

Method

Dilute 10 ml of standard Oxalic acid or oxalate solution to 20 ml water and treat with 15 ml of 4 N H_2SO_4. Heat the solution to 70-80°C and titrate against permanganate solution until the solution becomes slightly pink. The colour is permanent. Calculate the normality of permanganate solution using the relationship

Calculation

UsingN_1 V_1 = N_2 V_2 relationship, normality of permanganate solution can be calculated.

5. Standardization of the Fehling's Solution

Reagents

- Fehling's solution A (Copper sulphate solution): Dissolve 34.639 g of $CuSO_4.5H_2O$ (pure recrystallized) in distilled water, dilute to 500 ml and filter
- Fehling's solution B (Alkaline tartarate solution): Dissolve 173 g of Rochelle salt (Sodium potassium tartarate) and 50 g of NaOH in distilled water dilute to 500 ml. Allow to stand for 2 days and filter. Mix equal volumes (5 ml each) of both immediately before use
- Standard glucose solution (0.5%): Dissolve accurately 0.5 g of pure Anhydrous Glucose powder in distilled water and dilute to 100 ml with distilled water
- 1.0% Methylene blue: Dissolve 1.0 g in 100 ml 95% alcohol

Method

Into a conical flask, pipette out 5.0 ml of each of Fehling's solutions A and B. Dilute with about 10 ml of water and add 3 glass beads to it. Keep the flask over a wire gauze or hot plate and bring to boil. Add from the burette the standard glucose solution slowly, 1ml at a time till the colour just turns from blue to red. Then add 3-4 drops of 1.0% Methylene blue indicator which gives a blue colour to the solution and continue to add the glucose solution drop-wise till a brick red colour end point of solution is obtained.

Again in another conical flask, take 5.0ml of each of the Fehling's solutions A and B, dilute with about 10 ml of water and add 3 glass beads into it. Boil the contents as above and add from the burette the standard glucose solution 2.0ml less than the quantity required in the first titration. Then add 3-4 drops of Methylene blue indicator and continue the titration till the brick red coloured end point is obtained. Repeat the whole process of titration 3-4 times and calculate the mean of the last three readings (excluding the first reading). From the mean titration reading, calculate the amount of anhydrous glucose corresponding to 10 ml of the mixed Fehling's solutions A and B.

Note:

The titration should be completed within total boiling time of about 3 minutes. Note down the first burette reading.

Calculation

Let 'a' ml be the mean titration reading.

10ml of Fehling's A&B together = 'a' ml of std. glucose solution.

Now, 100 ml of standard glucose solution = 0.5 g of glucose

$$\therefore \text{'a' ml of std. glucose solution} = 0.5 \times \frac{a}{100} \text{ g of glucose}$$

$\therefore$ 10 ml of Fehling's solutions = $0.5 \times \frac{a}{100}$ g of glucose

29. Appendix VII – Fruit Composition and Nutritive Value

Table.21. Fruit Composition and Sugar Contents (Grams per 100 g of Edible Portion)

Fruit	Water	Carbohydrates	Protein	Fat	Fibre	Fructose	Glucose	Sucrose	Maltose	Total sugar
Apple	86	12	0.3	Tr	2	5.6	1.8	2.6	-	10
Apricot	88	9.5	0.8	Tr	2.1	0.4	1.9	4.4	-	6.7
Avocado	79	5.9	1.5	12	1.8	0.1	0.1	-	-	0.2
Banana	75	20	1.2	0.3	3.4	2.9	2.4	5.9	-	11.3
Cherry	80	17	1.3	0.3	1.2	6.1	5.5	-	-	11.6
Grape	82	16.1	0.6	Tr	0.9	6.7	6	0	0	12.9
Guava	82	15.7	1.1	0.4	5.3					
Kiwi fruit	84	9.1	1	0.4	2.1					
Mango	84	15	0.6	0.2	1	3.8	0.6	8.2	-	12.7
Melon	92	6	0.1	Tr	1					
Orange	87	10.6	1	Tr	1.8	2	1.8	4.4	-	8.3
Papaya	89	9.8	0.6	0.1	1.8					
Passion fruit P										
Passion fruit Y										
Peach	89	9	0.6	Tr	1.4	4	4.5	0.2	-	8.7
Pear	86	11.5	0.3	Tr	2.1	5.3	4.2	1.2	-	10.7
Pineapple	84	12	1.2	Tr	1.2					
Plum	84	9.6	0.8	Tr	2.2	3.2	5.1	0.1	0.1	8.6
Raspberry	86	11.9	1.2	0.6	6.5					
Strawberry	91	5.1	0.7	0.3	2.2	2.3	2.6	1.3	-	6.2
Watermelon	93	8	1	Tr	0.6	2.7	0.6	2.8	-	6.2

Table.22. Vitamin and Mineral Content of Fruits (Value per 100 g of Edible Portion)

Fruit	Vitamin C (mg)	Vitamin E (mg) (a tocopherol)	Vitamin A (fg RAE)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Pyridoxine (mg)	Folate (fg)	Fe (mg)	Ca (mg)	P (mg)	Mg (mg)	K (mg)	Na (mg)	Zn (mg)	Cu (mg)	Se (fg)
Apple	4.6	0.18	3	0.017	0.026	0.091	0.041	3	0.12	6	11	5	107	1	0.04	0.027	0
Apricot	10	0.89	96	0.03	0.04	0.6	0.054	9	0.39	13	23	10	259	1	0.2	0.078	0.1
Avocado	10	2.07	7	0.067	0.13	1.738	0.257	58	0.55	12	52	29	485	7	0.64	0.19	0.4
Banana	8.7	0.1	3	0.031	0.073	0.665	0.367	20	0.26	5	22	27	358	1	0.15	0.078	1
Cherry	7	0.07	3	0.027	0.033	0.154	0.049	4	0.36	13	21	11	222	0	0.07	0.06	0
Grape	10.8	0.19	3	0.069	0.07	0.188	0.086	2	0.36	10	20	7	191	2	0.07	0.127	0.1
Guava	183.5	0.73	31	0.05	0.05	1.2	0.143	14	0.31	20	25	10	284	3	0.23	0.103	0.6
Kiwi fruit	75	-	9	0.02	0.05	0.5	-	-	0.41	26	40	30	332	5	-	-	-
Orange	53.2	0.18	11	0.087	0.04	0.282	0.06	30	0.1	40	14	10	181	0	0.07	0.045	0.5
Papaya	61.8	0.73	55	0.027	0.032	0.338	0.019	38	0.1	24	5	10	257	3	0.07	0.016	0.6
Passion fruit	30	0.02	64	0	0.13	1.5	0.1	14	1.6	12	68	29	348	28	0.1	0.086	0.6
Passion fruit P																	
Passion fruit Y																	
Peach	6.6	0.73	16	0.024	0.031	0.806	0.025	4	0.25	6	20	9	190	0	0.17	0.068	0.11
Pear	4.2	0.12	1	0.012	0.025	0.157	0.028	7	0.17	9	11	7	119	1	0.1	0.082	0.1
Pineapple	36.2	0.02	3	0.079	0.031	0.489	0.11	15	0.28	13	8	12	115	1	0.1	0.099	0.1
Plum	9.5	0.26	17	0.02	0.026	0.417	0.029	5	0.17	6	16	7	157	0	0.1	0.057	0
Raspberry	26.2	0.87	2	0.032	0.038	0.598	0.055	21	0.69	25	29	22	151	1	0.42	0.09	0.2
Strawberry	58.8	0.29	1	0.024	0.022	0.386	0.047	24	0.42	16	24	13	153	1	0.14	0.048	0.4