A Practical Manual

Elementary Plant Biochemistry (HBB 111)

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PREFACE

Biochemistry is one of the fundamental subjects of life science and knowledge of its practical aspects is essential. It has always felt the necessity for a manual on orientation towards various aspects of practical biochemistry. The present manual is written in simple language covering the various practical aspects of course Elementary Plant Biochemistry and Biotechnology (HBB 111) of 2 (1+1) credits offered to the students of B.Sc. (Hons) Horticulture.

Students belonging to B.Sc. (Hons) Horticulture degree programmes taking the compulsory course of Elementary Plant Biochemistry will find it useful in learning various techniques by performing the experiments given in manual. Due to our experience in teaching various courses in concerned area and referring relevant published literature, the contents of the course manual has been designed. It covers all the biochemistry practicals mentioned in the syllabus of HBB 111 along with some introductory and necessary information regarding the subject for the orientation of the UG students.

We express our deep sense of gratitude to Dr. N. P. Singh, Hon'ble Vice Chancellor, BUAT, Banda, for providing valuable support and guidance in preparing the manual. We feel privileged to express our heartfelt gratitude to Dr. S. V. Dwivedi, Dean, College of Horticulture for his invaluable input, help, support, patience, and encouragement. Due to his time-to-time guidance, persuasion and motivation, we are able to bring this manual in the present shape and first time in the hands of our students.

We hope that the manual will meet the requirement of undergraduate students studying biochemistry and will be glad to accept constructive criticism and suggestions from the faculty, students and readers to make this manual a better one in future.

Date: June 2024

Authors

Syllabus as per ICAR 5th Deans Committee Report

Preparation of standard solutions and reagents; Carbohydrates: Qualitative reactions; Estimation of starch; Estimation of reducing and non-reducing sugars from fruits; Amino acids: Reactions of amino acids; Proteins: Estimation of proteins by Lowry's method; Fatty acids: Estimation of free fatty acids; Determination of iodine number of vegetable oils; Vitamins: Estimation of Ascorbic acid; Techniques: Paper chromatography, Thin layer chromatography; Electrophoresis of pigments extracted from flowers, Extraction of oil from oil seeds; Enzymes: Enzyme assay, Enzyme Immobilization.

Table of Contents

Do's for students

1. Maintain cleanliness of the working table.

2. Wear the lab coats while working in the laboratory.

3. Place the chemicals/reagent bottles on the rack in their respective positions.

4. Label the solutions/reagents/indicators with respect to concentration and date of preparation.

5. Clean and rinse with distilled water and dry the apparatus before use.

6. Handle the pipettes gently and put back to the stand after use so that the tips are not damaged.

7. Keep the burner/ heater away from the inflammable solvents such as diethyl ether, hexane etc.

8. Use only distilled/deionised water for analytical work.

9. For dilution of acids, add acid to distilled water in a flask but not vice versa.

10. Carefully pipette out acids/alkalies/corrosive reagents or use vacuupet

11. Evaporate the solvent extracts using water bath only or a stream of nitrogen wherever required.

12. Replace the caps of the solvents bottle immediately after use.

13. Ensure the closing of the taps, electrical points before leaving the lab at the end of the day.

14. Read the complete procedure and then start preparing solutions/reagents for the experiment.

15. After completion of the experiment, calculate the results and show these to the supervisor before leaving the laboratory.

16. Conduct all the experiments in duplicate/triplicate and submit records in time.

Exercise 1: General Laboratory Principles

1.1 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. 1.1 Biochemistry Laboratory

1.2 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 recognize d 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.

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.

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. Ether, Ligroin (Petroleum ether), Cyclohexane, Toluene, Xylene, Alcohols, Ethyl acetate, Carbon disulphide, Acetone, Dioxane etc are some of inflammable solvents which you might be dealing with in the Biochemistry laboratory. 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. 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. Approved safety glasses, goggles, or a face shield must be worn at all times when in the lab. Use glass stirring rods with care for breaking up solids. They are liable to break.

Personal safety is most important. If a person clothes catch 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. If a fire breaks out, turn off all burners and remove solvents if time allows. Fire extinguishers must be present in the laboratory.

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 Glycerin. If the chemical is spilled in the eye, it should immediately be washed out thoroughly with water.

1.3 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.

1.4 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, aluminum foil, marker pens, filter paper, ice buckets, latex gloves, plastic boxes, plastic bags, UV goggles, besides plastic bottles etc. A good supply of chemicals is a very essential aspect of equipping a laboratory as are uninterrupted power and water supply.

1.5. Keeping records and communicating experimental results

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
- Values collected
- 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.

Observations **Date**

i. What are the safety precautions to be kept in Biochemistry lab?

ii. Enlists different hazards in the laboratory.

iii. How do you maintain good record keeping in the laboratory?

Exercise 2: Identification of different laboratory equipment and glassware

2.1 Different instruments being used in Biochemistry lab

2.1.1 Mortar and Pestle 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.

2.1.2 Desiccator 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.

2.1.3 Bunsen burner 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.

2.1.4 Micro Centrifuge 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).

2.1.5 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.

2.1.6 Rotary Shaker 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 is used for 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.

2.1.7 Homogenizer 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.

2.1.8 Fume hood A fume hood or fume cupboard is a type of local ventilation device that is designed to limit exposure to hazardous or toxic fumes, vapors 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). 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.

2.1.9 Laminar Air Flow Cabinet 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. 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.

2.1.10 Electronic Weighing Balance 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. 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. Weight till the symbol "g" stabilizes.

2.1.11 Magnetic Stirrer Magnetic stirrer employs a rotating magnetic field to cause a stir bar 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. 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.

2.1.12 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.

2.1.13 pH Meter 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.

2.1.14 Microscope 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. 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.

2.1.15 Soxhlet Extractor 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.

2.1.16 Rotary Evaporator A rotary evaporator or rotovap is a chemical laboratory device used for the efficient and gentle removal of solvents from samples through evaporation. This technique involves applying heat to a rotating vessel at a reduced pressure, allowing solvents to boil at lower temperatures. The machine's lower pressure than atmospheric pressure increases the surface area, allowing evaporation to proceed more rapidly. Rotary evaporation is particularly useful for solvents with high boiling points, as atmospheric pressure requires high temperatures, which may cause side reactions like oxidation or decomposition. By lowering the pressure and boiling at a lower temperature, solvents with high boiling points are removed efficiently without unwanted side reactions.

2.1.17 Distillation Assembly In this equipment liquid is vaporized (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 evaporation 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.

2.1.18 Autoclave 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 (15 psi) 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.

2.1.19 BOD Incubator 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. 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.

2.1.20 Hot Air Oven A dry heat sterilization unit is a cost-effective and easy-to-install device that uses conduction to sterilize materials. The heat is absorbed by the equipment's outside surface and passed to the center, layer by layer, reaching the required sterilization temperature. Dry heat oxidizes molecules, destroying essential cell constituents and causing the organism to die. The temperature is maintained for almost an hour to kill resistant spores. Common time-temperature relationships for sterilization with hot air sterilizers include 1.170°C (340°F) for 60 minutes, 2.160°C (320°F) for 120 minutes, and 3.150°C (300°F) for 150 minutes or longer, depending on the volume.

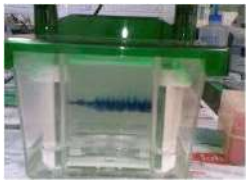
2.1.21 Centrifuge A centrifuge is the equipment generally driven by an electric motor that puts an object to rotate around fixed axis, and a perpendicular force is applied to axis. The particles get separated according to their size, shape, density, viscosity of the medium and rotor speed.

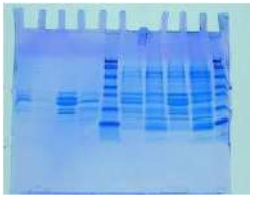
2.1.22 Refrigerated Centrifuge It is extensively used in chemistry, biology, and biochemistry for isolating and separating suspensions. It additionally provides the cooling mechanism to maintain the uniform temperature throughout the operation of the sample.

2.1.23 Thermal Cycler (PCR machine) 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.

2.1.24 Gel Documentation System 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 50 other fluorophores such as SYBR Green.

2.1.25 Polyacrylamide gel electrophoresis (PAGE) is a widely used analytical technique for separating and characterizing proteins. It involves polymerizing a solution of Acryl amide and Bis-acrylamide, with the pore size determined by the ratio and concentration of Acryl amide. High ratios and concentrations cause low electrophoretic mobility. Ammonium per sulphate (APS) is used to induce polymerization, which is stabilized by TEMED. Sodium dodecyl sulfate (SDS) is an amphipathic detergent that binds non-covalently to proteins, causing them to denature and disassociate from each other, excluding covalent cross-linking. SDS also confers a negative charge.





2.1.26 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. 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.

2.1.27 UV Visible spectrophotometer 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. Spectrophotometer works with the principle of Beer-Lambert Law.

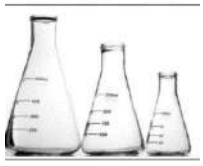
2.1.28 Atomic absorption Spectrophotometry (AAS) is widely used for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. In AAS two main types of atomization sources used, flame and graphite furnace.

2.2 Different glassware being used in Biochemistry lab

Test tube A test tube, also known as a sample tube, is a common piece of laboratory glassware consisting of a finger-like length of glass or clear plastic tubing, open at the top and closed at the bottom.

Beakers are used as containers. They are available in a variety of sizes. Although they often possess volume markings, these are only rough estimates of the liquid volume. The markings are not necessarily accurate.

Erlenmeyer flask are often used as reaction vessels, particularly in titrations. As with beakers, the volume markings should not be considered accurate.



Volumetric flask are used to measure and store solutions with a high degree of accuracy. These flasks generally possess a marking near the top that indicates the level at which the volume of the liquid is equal to the volume written on the outside of the flask. These devices are often used when solutions containing dissolved solids of known concentration are needed.

Graduated cylinder Graduated cylinders are used to transfer liquids with a moderate degree of accuracy.

Pipette are used for transferring liquids with a fixed volume and quantity of liquid must be known to a high degree of accuracy

Graduated pipette These Pipettes are calibrated in the factory to release the desired quantity of liquid.

Burette are devices used typically in analytical, quantitative chemistry applications for measuring liquid solution. Differing from a pipette since the sample quantity delivered is changeable, graduated Burettes are used heavily in titration experiments. In Burette and Pipettes it is common to measure the volume delivered. This means that two volume measurements will be made. The volume delivered is the difference in these two volumes, Vfinal - Vinitial.

Burette clamp Burette clamp is scientific equipment which used specifically to hold and secure a burette on a stand, so that a burette is fixed and more convenient for the experiment. Burette clamp can be made by many materials such as plastic and cast iron. However, iron clamp with rubber knob to hold burette are likely to be more durable. Usually Burette clamp comes in double, which means it can hold two burettes.

Funnel A tube or pipe that is wide at the top and narrow at the bottom, used for guiding liquid or powder into a small opening.

Test tube stand Test tube racks are laboratory equipment used to hold upright multiple test tubes at the same time.

Test tube holder A test tube holder is used to hold test tubes. It is used for holding a test tube in place when the tube is hot or should not be touched.

Petri dish A petri dish is a small dish shaped like a cylinder.

Glass rod A glass stirring rod, glass rod, stirring rod or stir rod is a piece of laboratory equipment used to mix chemicals and liquids for laboratory purposes. They are usually made of solid glass, about the thickness and slightly longer than a drinking straw, with rounded ends.

Wash Bottle A wash bottle is a squeeze bottle with a nozzle, used to rinse various pieces of laboratory glassware, such as test tubes and round bottom flasks. Wash bottles are sealed with a screw-top lid.

Spatula In laboratories, spatulas and microspatulas are small stainless-steel utensils, used for scraping, transferring, or applying powders and paste like chemicals or treatments. Many spatula brands are also resistant to acids, bases, heat, and solvents, which make them ideal for use with a wide range of compound.

Round-bottom flasks (also called round-bottomed flasks or RB flasks) are types of flasks having spherical bottoms used as laboratory glassware, mostly for chemical or biochemical work.

Separatory funnel A separatory funnel, also known as a separation funnel, separating funnel, or colloquially sep funnel, is a piece of laboratory glassware used in liquid-liquid extractions to separate (partition) the components of a mixture into two immiscible solvent phases of different densities.

Crucible A crucible is a ceramic or metal container in which metals or other substances may be melted or subjected to very high temperatures.

Observations **Date**

i. **Enlist any 10 major equipment being used in biochemistry laboratory with their principle and use.**

ii. Enlist and draw any 10 major glassware being used in biochemistry laboratory with their principle and use.

Exercise 3: Preparation of standard solutions and reagents

3.1 Common terms

- **Buffer:** A solution which tends to maintain a constant pH when excess acid or base is added.
- **Concentrated:** For some commonly used acids and bases, the maximum solubility (at room temperature) in an aqueous solution or as a pure liquid.
- **Concentration:** The relative amount of solute and solvent in a solution.
- **Hydrates:** Compounds containing water chemically combined in a definite ratio. Computations using formula weight must take the water molecules into account.
- **Miscible:** The ability of two liquids to be completely soluble in one another.
- **Molality:** A concentration unit (m); defined as the number of moles of solute divided by the number of kilograms of solvent.
- **Molar Mass:** The mass of a mole of any element or compound.
- **Molarity:** A concentration unit (M); defined as the number of moles of solute divided by liters of solution.
- **Normality:** A concentration unit (N); defined as the number of equivalents of solute per liter of solution. (e.g., 1 M H_2SO_4 = 2 N H_2SO_4)
- **Saturated Solution:** A solution that contains the maximum amount of a particular solute that will dissolve at that temperature.
- **Solute:** The substance which is dissolved, or has gone into solution (typically a solid).
- **Solution:** A uniform homogeneous mixture of two or more substances. The individual substances may be present in varying amounts.
- **Solvent:** The substance which does the dissolving (typically a liquid, such as water or alcohol). Must be greater than 50% of the solution.
- **Standard Solution:** A very precise solution, usually to 3–4 significant figures, used in quantitative analysis or an analytical procedure.
- **Supersaturated Solution:** A solution that contains more solute than equilibrium conditions allow; it is unstable and the solute may precipitate upon slight agitation or addition of a single crystal.

3.2 Preparation of molar solutions

The most common unit of solution concentration is molarity (M). The molarity of a solution is defined as the number of moles of solute per one liter of solution. Note that the unit of volume for molarity is liters, not milliliters or some other unit. Also note that one liter of solution contains both the solute and the solvent. Molarity, therefore, is a ratio between moles of solute and liters of solution. To prepare laboratory solutions, usually a given volume and molarity are required. To determine molarity, the formula weight or molar mass of the solute is needed. The following examples illustrate the calculations for preparing solutions.

3.2.1 If starting with a solid, use the following procedure:

- Determine the mass in grams of one mole of solute, the molar mass, MM_s.
- Decide volume of solution required, in liters, V.
- Decide molarity of solution required, M.
- Calculate grams of solute (g_s) required using equation 1.

  Equation 1 is $g_s = MM_s \times M \times V$

Example: Prepare 800 ml of 2 M sodium chloride.

(MM_{NaCl} = 58.45 g/mol)

g_{NaCl} = 58.45 g/mol x 2 mol/L x 0.8 L

g_{NaCl} = 93.52 g NaCl

Dissolve 93.52 g of NaCl in about 400 ml of distilled water and then add more water until final volume is 800 ml.

3.2.2 If starting with a solution or liquid reagent:

When diluting more concentrated solutions, decide what volume (V_2) and molarity (M_2) the final solution should be. Volume can be expressed in liters or milliliters.

- Determine molarity (M_1) of starting, more concentrated solution.
- Calculate volume of starting solution (V_1) required using equation 2. Note: V_1 must be in the same units as V_2.

Equation 2 is $M_1V_1 = M_2V_2$

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

$M_1V_1 = M_2V_2$

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

V_1 = 8.26 ml conc. HCl

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

3.3 Preparation of Percent Solutions

3.3.1 Mass percent solutions are defined based on the grams of solute per 100 grams of solution.

Example: 20 g of sodium chloride in 100 g of solution is a 20% by mass solution.

3.3.2 Volume percent solutions are defined as milliliters of solute per 100 ml of solution.

Example: 10 ml of ethyl alcohol plus 90 ml of H_2O (making approx. 100 ml of solution) is a 10% by volume solution.

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

Example: 1 g of phenolphthalein in 100 ml of 95% ethyl alcohol is a 1 w/v% solution.

3.4 Conversion between Percent Solutions

You may wish to convert mass percent to volume percent or vice versa. If so, follow this procedure:

A 10% by mass solution of ethyl alcohol in water contains 10 g of ethyl alcohol and 90 g of water.

- The formula for determining the volume of the component (ethyl alcohol in our example) is:

$$\text{Volume} = \frac{\text{mass of ethyl alcohol}}{\text{density of ethyl alcohol}}$$

For example: Mass of ethyl alcohol = 10 g (given)

Density of ethyl alcohol = 0.794 g/ml

$$\text{Volume} = \frac{10\,\text{g}}{0.794\,\text{g/mL}} = 12.6\ \text{ml}$$

- Determine the volume of the total solution by dividing the mass of the solution by the density of the solution.
- Determine the percent by volume by dividing the volume of the component by the volume of the solution.

3.5 Calculating molarity from Percent Solutions

To determine the molarity of a mass percent solution, the density of the solution is required. Use the following procedure:

1. Determine the mass of solution by multiplying the volume of the solution by the density of the solution.

Mass = volume x density

2. Determine concentration in percent by mass of the solute in solution. *Change to the decimal equivalent.*

3. Calculate the molar mass of the compound, MM.

4. Multiply mass (step 1) by mass % (step 2) and divide by molecular mass (step 3) to find the number of moles present in the whole solution.

5. Divide the number of moles (step 4) by the volume in liters of the solution to find the molarity of the solution.

Example: Determine molarity of 37.2% hydrochloric acid (density 1.19 g/ml).

1. Mass of solution = 1,000 ml x 1.19 g/ml = 1,190 g

2. Mass % = 37.2 % = 0.372

3. Molar mass of hydrochloric acid = 36.4 g/mol

4. $$\frac{\text{mass x mass \%}}{MM_{HCl}} = \frac{1{,}190\text{ g x } 0.372}{36.4\text{ g/mol}} = 12.1\text{ moles}$$

5. Molarity = moles/liters = 12.1 moles/1 liter = 12.1 M

3.6 Stock solutions

A stock solution is a concentrated solution that will be diluted to some lower concentrated for actual use. Stock solutions are used to save preparation time, conserve materials, reduce storage space, and improve the accuracy with which working lower concentration solutions are prepared.

3.7 Preparation of commonly used Buffers

3.7.1 Acetic acid- sodium acetate buffer:

Reagents required:

- Acetic acid 0.2 M: 1.5 ml of glacial acetic acid is made upto 100 ml with distilled water.
- Sodium acetate solution: 0.64 g of sodium acetate or 2.72 g of sodium acetate trihydrate is dissolved in 100ml distilled water.

Procedure: Pipette out exactly 36.2 ml of sodium acetate solution into 100 ml of standard flask and add 14.8 ml of glacial acetic acid, make the volume 100 ml using distilled water using distilled water. This gives 0.2 M of acetic acid and sodium acetate buffer. The pH is measured with pH meter. The pH meter is first standardized with pH buffer. Wash electrode with distilled

water and introduce into 0.2 M acetic acid-sodium acetate buffer prepared, the pH of solution is 4.6.

3.7.2 Barbitone buffer:

Reagents required:

- Diethyl barbituric acid.
- Sodium diethyl barbititrate

Procedure: Dissolve 2.85 g of diethyl barbituric acid and 14.2 g of sodium diethyl barbititrate in distilled water and upto 1 liter. This gives the barbitone buffer. The pH meter is first standardized with pH buffer. Wash electrode with distilled water and introduce into barbitone buffer prepared, the pH of solution is 6.8.

3.7.3 Citrate buffer:

Reagents required:

- Citric acid: dissolve 2.101 g of citric acid in 100 ml distilled water.
- Sodium citrate solution 0.1 M: dissolve 2.941g of sodium citrate in 100 ml distilled water.

Procedure: Mix 46.5 ml of citric acid with 3.5 ml of sodium citrate solution and make volume upto 100 ml with distilled water. It corresponds to 0.1 M citrate buffer and standardized with pH meter and measures the pH of the prepared solution. This gives citrate buffer at pH 2.5.

3.7.4 Phosphate buffer:

Reagents required:

- Monobasic: dissolve 2.78 g of sodium/potassium dihydrogen phosphate in 100 ml of distilled water.
- Dibasic sodium/potassium phosphate (0.2 M): dissolve 5.3 g of disodium/dipotassium hydrogen phosphate or 7.17 g sodium /potassium hydrogen phosphate in 100 ml distilled water.

Procedure: 39 ml of dihydrogen sodium/potassium phosphate is mixed with 61 ml of disodium/dipotassium hydrogen phosphate this made up to 200 ml with distilled water. This gives phosphate $(PO_4)_2$ buffer of 0.2 M. Wash electrode with distilled water and introduce it into phosphate buffer prepared. The pH of the solution is 6.8.

Observations Date

i. Prepare 100 ml volume of 1% solution of NaCl

ii. Prepare a 0.01 M NaCl solution with 500 ml volume

iii. Prepare a 100 ml volume of 0.5 M phosphate buffer of 6.5 pH

iv. How will you prepare a 100 ml volume of 0.1 M working solution from 10 M stock solution?

Exercise 4: To perform qualitative tests on carbohydrates

Theory: Carbohydrates are defined as polyhydroxy aldehydes or ketones, hence, called aldoses or ketoses. The basic unit of carbohydrate is monosaccharide, which cannot be hydrolyzed again. Most of the tests for carbohydrates are based on two facts:

(i) Reducing reaction due to the presence of free reducing group, which may be aldehyde or ketone in nature. Due to this property carbohydrates give some tests like Fehling test, Benedict's test, Barfoed's test etc.

(ii) The conversion of sugar into furfural or its derivatives in presence of strong acids. These derivatives condense with phenol (α-napthol, resorcinol, orcinol) to give coloured derivatives. The relative rate of dehydration of different sugars also depends on the nature and strength of the acidity. Due to this fact they give some tests e.g., Molisch test, Seliwanoff's test and Bial's test.

1. Molisch Test

Principle: Concentrated H_2SO_4 hydrolyses glycosidic bond to give the monosaccharides, the latter on further reaction with acid dehydrates to form furfural or fufural derivatives. These compounds combine with sulfonated α-naphthol to give a Purple coloured complex. All carbohydrates give this fest.

Requirements: Conc. H_2SO_4, α-naphthol, alcohol and test solution.

Procedure: Add 2 drops of Molisch reagent to two ml of test solution. Then carefully pour about 1ml concentrated H_2SO_4 down the side of the tube to form two layers. A reddish violet zone or ring is formed at the junction of two liquids.

2. Benedict's Test

Principle: Carbohydrates with a free aldehyde or ketone group have reducing property in alkaline solution. Alkaline copper reagents when react with a reducing sugar give rise cuprous oxide, which depending on concentration imparts green, yellow, orange or red colour.

Requirements: Benedict reagent, Test solution

Procedure: Add 5 drops of the test solution to 2 ml of Benedict's reagent and heat to boiling. Cool the tube. If a reducing sugar is present a colour will form which may be red, orange, yellow or green depending upon the amount of sugar present. This test is positive for all reducing sugars.

3. Seliwanoff's Test

Principle: Ketoses are dehydrated more rapidly than the aldoses, to give furfural derivatives, which then condense with orcinol to form a red complex. Prolonged heating must be avoided.

Requirements: Seliwanoff‟s reagent, Test solution

Procedure: Add 2 drops of test solution to 2ml of Seliwanoff‟s reagent. Heat to boiling for 1 min. If a ketose is present a red colour will appear as in fructose. Sucrose also gives as positive test due to hydrolysis into glucose and fructose.

4. Bial's Test

Principle: When pentoses are heated with concentrated HCI, furfural is formed which condenses with orcinol in presence of ferric ions to give a blue green colour. The colour is specific for

pentoses, glucuronate and their polymers. Prolonged heating of some hexoses also yield hydroxy methyl furfural, which also reacts with orcinol to give coloured complex.

Requirements: Bial's reagent

Procedure: Add about 1ml of test solution to 2.5 ml of the reagent in a test tube and heat to boiling. A blue green colour on cooling indicates the presence of pentose sugar.

5. Barfoed's Test

Principle: Barfoed's reagent is weakly acidic and is only reduced by monosaccharides. Prolonged heating may also hydrolyze disaccharides to give a positive result. The precipitation of cuprous oxide is less dense than with the Benedict's solution and is best to leave tube to stand to allow the precipitate to settle. The colour of the cuprous oxide is also different being a more brick red rather than orange brown as obtained in Benedict‟s test.

Requirements: Barfoed's reagent, test solution

Procedure: Add 4 ml. of reagent to 1ml. of the test solution. Put the tube in water bath and note the time for the formation of red spot at the bottom of the tube. Monosaccharides take lesser time than the disaccharides.

6. Iodine Test

Principle: Iodine test is performed to distinguish polysaccharide from mono and disaccharides. Iodine forms coloured adsorption complexes with polysaccharides. Adsorption is a surface phenomenon and it decreases with temperature and vice versa. That's why polysaccharide-iodine complexes lose and gain colour on heating and cooling respectively.

Procedure: Take 3 ml of test solution in a test tube and add single drop of dil HCI. Mix and add one or two drops of lodine solution. Mix again and observe the colour change. A blue colour indicates the presence of starch, a reddish blue or purple for dextrin and wine red colour for glycogen.

Observations Date

i. Perform the different qualitative tests for carbohydrates as per the table and record your observations:

Sugar	Benedict test	Barfoed's Test	Seliwanoff's Test	Molisch Test	Iodine Test
Glucose					
Fructose					
Sucrose					
Lactose					
Starch					

Indicate your results as "+" or "-" for different test

Exercise 5: Estimation of total sugars by Dubois method

Principle

Carbohydrates (simple sugars, oligosaccharides, polysaccharides, and their derivatives) react in the presence of strong acid and heat to generate furan derivatives that condense with phenol to form stable yellow-gold compounds that can be measured spectrophotometrically at 490 nm.

Reagents

- 95% Sulphuric acid
- 5% Phenol (w/v, redistilled)
- Stock standard glucose solution: 100 milligrams of glucose mixed with 50ml double-distilled water. Make up to 100 ml.
- Working standard glucose solution: Take 10 ml of the stock solution and build the total to 100 ml using double-distilled water.

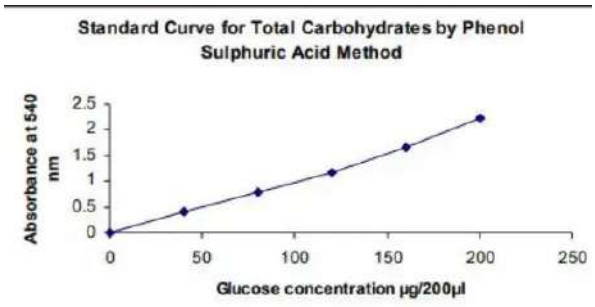
Procedure

Extraction

Powdered dry seeds (200 mg) were extracted with 10 ml of 80% ethanol at 80°C for 30 min. The supernatant was collected by decantation. This procedure was repeated once with 80% ethanol and twice with 70% ethanol. From the pooled supernatant, ethanol was evaporated at 50°C and final volume was made to 25 ml with water for estimation of free sugars. The sugar free residue was dried at 60°C for estimation of starch.

Assay

To 1 ml of appropriately diluted sugar extract, 1 ml of 5% phenol was added followed by addition of 5 ml of concentrated sulphuric acid. The sulphuric acid was poured directly in the center of test tube to ensure proper mixing of the solution. After 30 min, the absorbance was measured at 490 nm against the reagent blank. The concentration of total sugars (as glucose) was calculated from glucose standards (10-100 µg) run simultaneously.



Test Sample = Concentration of unknown "x" in µg/200 µl = ……. x 5 µg/ml

Observations **Date**

i. Estimate total sugars in different fruit crops.

Exercise 6: 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.

Reagents

- 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

(A) 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°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.

Observations **Date**

i. Estimate the starch from different tuber crops.

Exercise 7: Estimation of reducing sugars by Nelson 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 H2SO4 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 H2SO4 and mix well. Then add 0.3 g Disodium hydrogen arsenate dissolved in 25 ml distilled water. Mix well and incubate at 370 C 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 (%) =

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

Observations Date

i. Estimate reducing sugars in different fruit crops as per Nelson method.

Exercise 8: To perform qualitative tests on amino acids and proteins

Amino acids are organic compounds that have amino and carboxylic groups due to which they have both acidic and basic properties. Amino acids are the building blocks of proteins and are joined together in the protein molecule by peptide bond. Hence, tests performed by the amino acids are also performed by the proteins but proteins impart some additional reactions due to peptide bonds.

1. **Ninhydrin Test**:

Principle: Ninhydrin is a powerful oxidizing agent, which reacts with all α-amino acids to give a purple coloured complex. The reaction is also given by primary amines and ammonia, but there is no liberation of CO_2. Proline and hydroxy proline react with ninhydrin but in this case yellow colour is obtained instead of purple colour.

Reagents: Test solution, Ninhydrin (0.2%) in acetone

Procedure: Place 1ml of test solution in a test tube and add 5 drops of ninhydrin solution and boil for 2 minutes. A purple colour indicates the presence of amino acids. This test is given by all α- amino acids and proteins. Many primary amines may also give a positive ninhydrin test.

2. **Hopkins-Cole test**:

Principle: This test is given by those amino acids, which contain indole group, such as tryptophan. The indole group of amino acid reacts with glyoxylic acid in the presence of concentrated H_2SO_4 to give a purple colour (glacial acetic acid, which has been exposed to light, contains glyoxylic acid).

Reagents: Test solution, glacial acetic acid, which has been exposed, to light and sulphuric acid.

Procedure: Add 2ml of glacial acetic acid to 2ml of the test solution then pour about 2ml of concentrated H2SO4 carefully down the side of a sloping test tube to form two layers. A violet ring is formed at the junction of two liquids, which indicates the presence of indole group.

3. **Sakaguchi Test:**

Principle: This test is specific for only those compounds, which contain guanidine group, such as arginine. Guanidine reacts with α- naphthol in presence of an oxidizing agent, e.g., bromine water to give a red colour.

Materials: Test solution, sodium hydroxie 40%, α-naphthol, bromine water

Procedure: Mix 1 ml of 40% strong alkali with 2 ml of amino acid solution and add 1-2 drops of α-naphthol. Mix thoroughly and add 5 drops of bromine water. A red colour indicates the presence of arginine.

4. **Xanthoproteic test**:

Principle: Amino acids containing aromatic nucleus when react with concentrated HNO_3 form yellow coloured nitro derivatives. The salts of these derivatives are orange in alkaline condition. Phenols also impart a positive test.

Reagents: Test solution, conc. HNO_3, 40% NaOH solution

Procedure: Take 0.5 ml test solution in a test tube and add equal amount of concentrated HNO_3. On cooling yellow colour forms. Now to this solution add 40% NaOH solution to make it alkaline, a bright orange colour confirms the amino acid containing aromatic ring.

5. **Millon's Test:**

Principle: Compounds containing hydroxybenzene radical react with Millon's reagent to form red complexes. The only amino acid having hydroxybenzene ring is tyrosine. Thus, this test is specific for the amino acid tyrosine and the protein containing this amino acid. Tyrosine when reacted with acidified mercuric sulphate solution gives yellow precipitate of mercury-amino acid complex. On addition of sodioum nitrate solution and heating, the yellow complex of mercury-amino acid complex converts to mercury phenolate which is in red color.

Reagents: Millon's reagent, Test solution, 1 % sodium nitrite

Procedure: Take 1ml test solution in dry test tube and add 1ml of Millon's reagent and mix well. Boil gently for 1 minute. Cool under tap water. Now add 5 drops of 1 % sodium nitrite. Heat the solution slightly. Look for the development of brick red precipitate.

Positive Millon's test: Brick red color (Tyrosine and phenol solution)

Negative Millon's test: no red color (arginine)

6. **Biuret Test**:

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 540 nm. 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:

- 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
- 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

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 37ºC 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%.

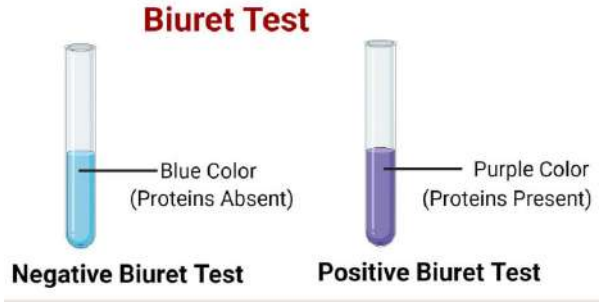
Calculation

$$\text{Concentration of the Protein (mg \%)} = \frac{\text{O.D. (Test)}}{\text{O.D. (Std)}} \times \frac{\text{Conc. (Std)}}{\text{Aliquot (test)}} \times 100$$

Result and Interpretation of Biuret Test

Positive Biuret Test: Formation of purple color after the addition of Biuret reagent. (Tube with albumin solution will turn purple.)

Negative Biuret Test: No formation of violet/purple color (or formation of blue color) solution after the addition of Biuret reagent. (Water will turn to blue color.)



Observations Date

i. Perform different qualitative tests on amino acids in various horticultural crops

Exercise 9: Estimation of total free amino acids by ninhydrin method

The ninhydrin test is a chemical test which is used to check whether a given analyte contains amines or α-amino acids. In this test, ninhydrin is added to a test solution of the analyte. The development of a deep blue colour indicates the presence of ammonia, primary/secondary amines, or amino acids in the analyte.

Principle

The amino group belonging to a free amino acid undergoes a chemical reaction with ninhydrin, which behaves as an oxidizing agent. When exposed to ninhydrin, the amino acid undergoes oxidative deamination, resulting in the liberation of CO_2, NH_3, and an aldehyde along with hydrindantin (which is a reduced form of ninhydrin). Now, the ammonia goes on to react with another ninhydrin molecule to form diketohydrin (which is also known as Ruhemann's complex). This complex is responsible for the deep blue colour. When the analyte contains Imino-acids like proline, a yellow-coloured complex is formed. When asparagine is used, the colour of the resulting complex is brown.

Reagents

- 80% ethanol
- 0.2M citrate buffer (pH 5.0)
- Ninhydrin reagent (Dissolve 0.8 g of stannous chloride in 500 mL of 0.2 M citrate buffer, pH 5. Add this solution to 20 g ninhydrin in 500 mL of methyl cellosolve), prepare fresh and store in brown bottle.
- Standard leucine solution (10mg/100ml water). Standard graph is different for different amino acids. So it is advisable to prepare a composite mixture of alanine, aspartic acid, tryptophan, proline and cysteine in equal amounts.
- Diluent solvent 50% ethanol or 50% propanol

Procedure

Sample preparation

Grind a known quantity (500mg) of sample in a pestle and mortar with small quantity of acid-washed sand. Add 5-10 ml of 80% ethanol (Boiling 80% ethanol may also be used if the tissue is tough). Filter or centrifuge. Repeat the extraction and pool the supernatant. The volume of the sample can be reduced by evaporation. This extract is used for estimation of total amino acids.

Assay

- Pipette out 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of aliquot from the standard solution in different test tubes.
- Make up the volume to 1.0 ml with water. Add 1 ml of Ninhydrin reagent and mix. Heat in a boiling water bath for 20 minutes
- Add 5ml of the diluent while still on the water bath and mix.
- Keep the tube for 10 minutes and read the O.D. at 570nm.
- In the same way, the unknown sample can be processed.

- Draw a standard curve and determine the concentration of unknown solution using standard curve. Calculate the concentration of amino acid as mg/100 ml

Observations Date

i. Estimate total free amino acids in different crop samples.

Exercise 10: Determination of soluble protein content by Lowry method

Principle

The method is based on the principle that the phenolic group of tyrosine and trytophan residues (amino acid) in a protein produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate, molybdate and phosphate. The method is sensitive down to about 10 μg/ml and is probably the most widely used protein assay.

Reagents

- Reagent A - 2% Na_2CO_3 in 0.1 N NaOH
- Reagent B - 1% solution of $CuSO_4$ $5H_2O$ in distilled water
- Reagent C - 2% sodium potassium tartarate
- Reagent D - Prepare fresh before use by mixing reagents B and C in 1:1 ratio
- Reagent E - Prepare by adding 1 ml of reagent D to 50 ml of reagent A
- Reagent F - 1 N Folin-Ciocalteau reagent (Mix Folin-Ciocalteau reagent 2N and distilled water in 1:1 ratio)
- Reagent G - 20% Trichloroacetic acid

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

Concentration of the Protein (mg %) = $\frac{\text{O.D. (Test)}}{\text{O.D. (Std)}} \times \frac{\text{Conc. (Std)}}{\text{Aliquot (test)}} \times 100$

Exercise 11: Estimation of free fatty acids

A small quantity of free fatty acids (FFA) is usually present in oils along with the triglycerides. The free fatty acid content is known as acid number/acid value. It increases during storage. The amount of free fatty acids present or acid value of fat is a useful parameter which gives an indication about the age and quality of the fat.

Principle

The free fatty acid in oil can be determined volumetrically by titrating the sample with potassium hydroxide in the presence of phenolphthalein indicator. The acid number is defined as the mg KOH requires to neutralize the free fatty acids present in 1g of sample. However, the free fatty acid content is expressed as oleic acid equivalents.

Materials:

a. 1% phenolphthalein in 95% ethanol.
b. Neutral alcohol – to be neutralized with KOH to a faint but permanent pink colour just before using.
c. 0.1N KOH solution: 5.6g of potassium hydroxide 1L of distilled water. The solution is standardized by titrating it with 0.1N oxalic acid using phenolphthalein as an indicator till permanent pink colour appears.

Method

a. Take 1-10g of oil or melted fat in a clean and dry conical flask.
b. Add 25 ml of hot neutral alcohol followed by few drops of phenolphthalein indicator.
c. Titrate the content with 0.1N KOH with vigorous shaking to the first permanent pink color which persists for fifteen seconds is obtained.

Calculation

Acid value = FFA (%) x 1.99

Observations Date

i. Estimate free fatty acids in different oil samples.

Exercise 12: Determination of iodine number of vegetable oils

The glycerides of the unsaturated fatty acids unite with a definite amount of iodine and the iodine value is therefore a measure of the degree of unsaturation.

Principle

The material is treated, in carbon tetrachloride medium, with a known excess of iodine monochloride solution in glacial acetic acid (Wijs solution). The excess of iodine monochloride is treated with potassium iodide and the liberated iodine estimated by titration with sodium thiosulphate solution.

Materials:

Reagents

a. Potassium Dichromate.
b. Concentrated Hydrochloric Acid.
c. Potassium Iodide Solution - Prepare a fresh solution by dissolving 10 g of KI free from potassium iodate, in 90 ml of water.
d. Starch Solution - Triturate 5 g of starch and 0.01 g of mercuric iodide with 30 ml of cold water and slowly pour it with stirring into one litre of boiling water. Boil for three minutes. Allow to cool and decant off the supernatant clear liquid.
e. Standard Sodium Thiosulphate Solution (0.1N).
f. Glacial acetic Acid.
g. Iodine Monochloride (ICl) - 98 %.
h. Wijs Iodine Monochloride Solution: Dissolve 10 ml of iodine monochloride in about 1800 ml of glacial acetic acid (chemically pure) and shake vigorously. Pipette 5 ml of this, add 10 ml of KI solution and titrate with 0.1 N standard Na2 S2 O3 solution, using starch solution as indicator. Adjust the volume of the solution till it is approximately 0.2 N.
i. Carbon Tetrachloride or Chloroform - inert to Wijs solution.

Method

a. Melt the sample if not already melted and then filter it through a filter paper to eliminate any impurities and residual moisture. Ensure both the sample and the glass apparatus are impeccably clean and dry.
b. Weigh an accurate amount of the oil or fat using the difference method, and place it into a clean, dry 500 ml iodine flask or a well-ground glass-stoppered bottle containing 25 ml of carbon tetrachloride. Agitate to dissolve the contents thoroughly. Then, add 25 ml of Wijs solution and replace the glass stopper after wetting it with KI solution. Swirl the mixture for thorough mixing and let it stand in darkness for 30 minutes for non-drying and semi-

drying oils, and for 1 hour for drying oils. Simultaneously, conduct a blank test under similar experimental conditions.

c. After the designated time, add 15 ml of KI solution and 100 ml of water, ensuring to rinse the stopper as well. Titrate the liberated iodine with standard $Na_2S_2O_3$ solution, continuously swirling the bottle contents to prevent any local excess until the solution turns straw yellow in color. Finally, add 1 ml of starch solution and continue the titration until the blue color formed disappears after thorough shaking with the stopper on.

Calculation

$$\text{Iodine value} = \frac{12.69\ (\text{B} - \text{S}) \times \text{N}}{W}$$

Where,

B = Volume, in ml, of $Na_2S_2O_3$ solution required for the blank,

S = volume, in ml, of $Na_2S_2O_3$ solution required for the sample,

N = normality of $Na_2S_2O_3$ solution, and

W = weight, in g, of the material taken for the test.

Observations Date

i. Estimate iodine number of the following vegetable oils

Type of Oil	Iodine Value
Coconut oil	
Groundnut oil	
Mustard oil	
Sesame oil	
Sunflower oil	
Soybean oil	
Palm oil	

Exercise 13: Estimation of ascorbic acid

Ascorbic acid is a good antioxidant when present in reduced form and is widely distributed in fresh fruits and leafy vegetables such as ber, guava, mango, papaya, cabbage and spinach. Ascorbic acid content is estimated according to the method of Roe (1964) which is based on the reduction of 2, 6-dichlorophenol indophenol (2, 6-DCPIP) by ascorbic acid.

Principle

Ascorbic acid reduces the 2, 6-dichlorophenol indophenol dye to a colorless leuco-base. 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 colour in acidic medium. Oxalic acid is used as the titrating medium.

Reagents

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

Procedure

1. Pipette out 5ml of the working standard solution into a 100ml of conical flask.
2. Add 10ml of 4% oxalic acid and titrate against the dye (V_1 ml). End point is the appearance of pink colour which persists for a few minutes. The amount of dye consumed is equivalent to the amount of ascorbic acid.
3. Extract the sample (0.5-5g depending on the sample) in 4% oxalic acid and make up to a known volume (100ml) and centrifuge.
4. Pipette out 5ml of this supernatant, add 10ml of 4% oxalic acid and titrate against the dye (V_2 ml).

Calculations

Amount of ascorbic acid mg/100ml sample

$$\frac{0.5\ mg}{V1\ ml} \times \frac{V2\ ml}{5\ ml} \times \frac{100\ ml}{Wt.of\ the\ sample} \times 100$$

Observations Date

i. Estimate ascorbic acid content in the citrus fruits using DCPIP method.

Exercise 14: To perform paper electrophoresis

Principle:

Paper Chromatography is used for separating chemicals based on their different properties such as solubility, size, mass, etc. and thus, allows scientists to distinguish various organic and inorganic materials. This technique is commonly used in crime scenes or in laboratories to identify an unknown compound by comparing it to known compounds. Paper Chromatography is divided into two phases: Stationary Phase and Mobile Phase. Stationary Phase describes the paper before the solvents start to move up and Mobile Phase is when the solvents migrate up the chromatography paper while carrying the solute (e.g. pigments).

In this experiment, we will observe black ink consists of other pigments and how different pigments in black ink separate based on their solubility. Capillary action allows solvent to travel up the paper and different pigments in black ink will separate based on their solubility. Pigment that is more soluble in polar solvent will travel up the paper along with the solvent, but pigments that are less soluble in polar solvent will travel more slowly because of their interaction with the chromatography paper.

Requirements:

i. Chromatography Paper (White Paper Towels can be used as a substitute)
ii. Solvents: must be polar (Ex: distilled water & rubbing alcohol)
iii. Ruler
iv. Pencil
v. Black Ink Pens/ Markers
vi. Cup
vii. Tape

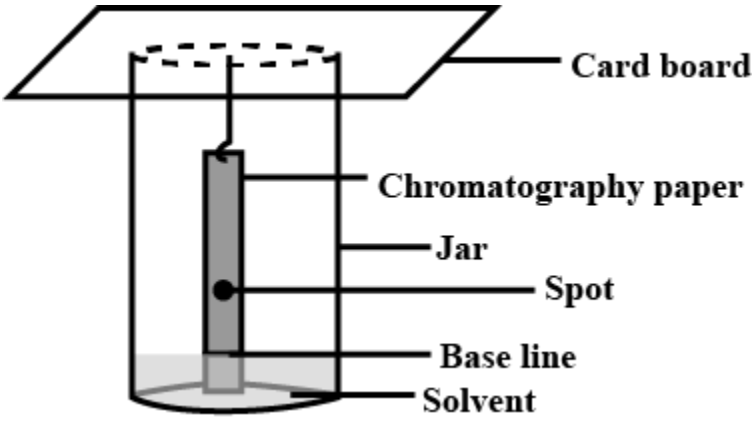
Procedure:

i. Preparing chromatography paper: Cut the paper towel: 2 cm by 15 cm. Then, measure 4 cm from one end and draw a line using a pencil and also a midline dividing the 4 cm line you just drew (as shown in the picture below). This is the baseline. It's important to use pencil because lead will not dissolve in water, preventing experimental error in chemical separation.
ii. Using black ink pen or marker, draw a small dot at the cross section of two lines you have drawn previously.
iii. Preparing Solvent: Mix equal amounts of water and rubbing alcohol in the cup (height ~ 2 cm). Rubbing alcohol by itself is also fine. Make sure the height of the solvent is less than 4cm. (You don't need large amounts of solvent, just a little bit as depicted in the picture below).
iv. Place the chromatography paper (aka. Paper towel) inside the cup and make sure that the solvent Does Not reach where the ink is (there should be a gap between ink and solvent).

Also, make sure that the chromatography paper doesn't touch the bottom of the cup. Tape the other end of chromatography paper to a pencil and place it on the cup.

v. Wait for about 15 minutes. Then, take out the chromatography paper.

vi. Using the same set up, repeat the experiment with different types of black ink pen/ marker.



Calculation of Rf value:

$$Rf = \frac{\text{distance travelled by solute (ink) from baseline}}{\text{distance travelled by solvent from baseline}}$$

Observations Date

i. Perform paper chromatography for the identification of unknown compound.

Exercise 15: Assay of enzyme activity and specific activity of acid phosphatase enzyme

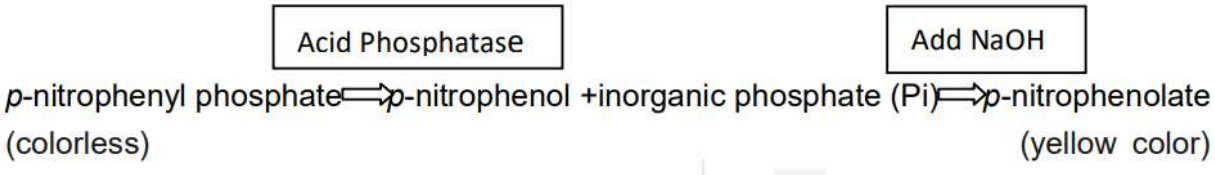
Enzymes that many biochemical reactions occur in living organisms and these are catalyzed by specific biocatalysts called enzymes. They are mostly protein in nature and found in all the cells. Every cell contains thousands of different enzymes. The term enzyme (meaning in yeast) was coined by F.W. Kuhne (1878). Enzymes play a pivotal role in the metabolism and different cellular functions. They change the rate of a chemical reaction without affecting the final equilibrium. However, these are not consumed in the biochemical reaction. Only small quantities of enzyme are required to bring about the transformation of a large number of substrate molecules. Enzymes exhibit high degree of specificity for their substrates. They work optimally only under certain well defined surrounding conditions of pH, temperature, substrate concentration etc. In this practical you will learn how to assay the activity of an enzyme taking the example of acid phosphatase. Phosphatases are ubiquitous and abundant enzymes. They catalyze removal of phosphate from a molecule. Acid phosphatase acts to liberate phosphate under acidic conditions and is made in the liver, spleen, bone marrow and prostate gland.

The activity of an enzyme is most frequently expressed in terms of units (U) which is defined as: One unit is the amount of enzyme that catalyses the conversion of 1 micromole of substrate per minute under defined conditions. In certain cases, the unit is too large and the activity can be more conveniently expressed in terms of nmol/min or pmol/min. The purity of an enzyme is expressed in terms of the specific activity, which is the number of enzyme units (U) per milligram of protein.

Acid Phosphatase Activity: One enzyme unit hydrolyses 1 µmole of *p*-nitrophenyl phosphate per minute, at pH 4.8 at 37°C.

Principle

The enzyme acid phosphatase catalyzes the conversion of the substrate, *p*-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate (Pi). The enzyme activity is measured by recording the absorbance due to the formation of *p*-nitrophenol, spectrophotometrically at 410 nm. The concentration of *p*-nitrophenol is calculated from the standard curve.



By measuring the quantity of p-nitrophenolate, we are measuring the quantity of *p*-nitrophenol (the product) which was present when the NaOH was added to the assay mixture to stop the reaction. The NaOH added has two effects:

a) it converts p-nitrophenol to p-nitrophenolate (yellow); and b) it stops the activity of the phosphatase enzyme.

Reagents

- Buffer: 0.1 M Sodium citrate buffer (pH 4.8) Solution A: 0.1 M citric acid monohydrate ($C_6H_8O_7.H_2O$ MW 210.14)
- Solution B: 0.1 M trisodium citrate dihydrate ($C_6H_5O_7Na_3.2H_2O$ MW 294.12)
  Take 40.0 mL citric acid monohydrate (Solution A) in a volumetric flask (100 mL) and make up to mark with trisodium citrate dihydrate (solution B).
- Substrate solution: p-Nitrophenyl phosphate, 15 mM dissolved in above mentioned 0.1 M citrate buffer (pH 4.8)
- 0.2N Sodium hydroxide solution Dissolve 8 gm sodium hydroxide pellets in 1000 mL distilled water. Standardize with 0.1N oxalic acid using phenolphthalein as indicator).
- 2.0 mM p-nitrophenol in citrate buffer (pH 4.8)

Procedure

- **Extraction of enzyme**: Take 10 g of fresh washed potato tubers and peel the skin. Cut these into small pieces with a sharp stainless knife. Weigh 5 g of the cut potatoes and transfer into a mechanical blender. Add 10 mL of cold 0.1M citrate buffer (pH 4.8) and homogenize the contents for ~5 minutes. Squeeze the homogenate through six layers of cheese cloth and centrifuge in cold at 10,000 g for 30 minutes to obtain the clear supernatant. Decant the supernatant (designated the crude extract) into a clean 15 mL conical flask and store in refrigerator. Use this fraction as a source of enzyme.
- **Assay of enzyme:** Take clean test tubes and pipette out the following reagents along with blank.

Name of the reagent	Blank tube (ml)	Test sample (ml)
0.1M Citrate Buffer (pH 4.8)	0.4	0.4
p-Nitrophenyl phosphate	0.5	0.5
Mix well and equilibrate at 37°C at 10 min		
Enzyme extract	-	0.2
Mix well and incubate at 37°C at 10 min		
0.2 NaOH	4.0	4.0
Enzyme extract	0.1	-

Record the absorbance of test and blank at 410 nm, using a spectrophotometer.

Calculation

You may also calculate the acid phosphatase activity (U/mL) in the enzyme extract by using the following equation:

$$\text{Enzyme activity (units/mL extract)} = \frac{(A_{410} \text{ of Test} - A_{410} \text{ of Blank}) \times 5 \times df}{10 \times 18.3 \times 0.1}$$

where,

5.0 = total assay volume
10 = incubation time
18.3 = Millimolar extinction coefficient of p-Nitrophenol at 410nm (ε)
0.2 = volume of enzyme extract
df = dilution factor

Determine the concentration of protein in enzyme extract by Bradford/Lowry's method which you have already performed in your exercise no. 10.

$$\text{Specific activity} = \frac{\text{Units/mL enzyme extract}}{\text{mg protein/ mL enzyme extract}}$$

Observations Date

i. Estimate the activity and specific activity of acid phosphatase enzyme in potato samples.

References

AOAC (1990). Official Methods of Analysis. Association of Official Analytical Chemists, Washington, D.C.

AOAC (2000). Official method - Acids (volatile) in oils and fats. 17th edition (925.41).

AOAC (2000). Official method – Iodine absorption number of oils and fats. 17th edition (920. 159).

AOAC (2000). Official methods of analysis 16th Ed. Association of Official Analytical Chemists, Washington, D. C. USA

Clegg, K. M. (1956). The application of anthrone reagent to the estimation of starch in cereals. J. Sci. Food Chem. Agric. 7: 40-44.

Dubois, M., Gilles, K.A., Hamilton, J.J., Rubress, P.A. and Smith, F. (1956). Colorimetric method for determination of sugar and related substances. Anal. Chem. 28:350-356.

FSSAI (2012) Manual of methods of analysis of foods oils and fats. Food safety and standards authority of India Ministry of health and family welfare Government of India New Delhi.

Joy, P. P., Surya, S. and Aswathy, C. (2015) Laboratory Manual of Biochemistry. Kerala agricultural university, pineapple research station, Vazhakulam, Muvattupuzha, Ernakulam, Kerala.

Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, J.L. (1951). Protein measurement with Folin-phenol reagent. Biol. Chem. 193:265-275.

Luddy, E.F., Barford, R.A. and Riemenschneider, R.W. (1960). Direct conversion of lipid components to their fatty acid methyl esters. J. Amer. Oil Chem. Soc. 37:447-451.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for determination of glucose. J. Biol. Chem. 153: 375-380.

Pal, A., Jain V.,Madan, S., Chugh, L. K., Singal, H. R., Tokkas, J., Kumari, N., Mandhania, S., Wadhwa, N. and Joshi, U. N. (2012) Basic and Applied Biochemistry: A Practical Manual, Department of Biochemistry, College of Basic Sciences & Humanities, CCS Haryana Agricultural University Hisar.

Roe, J.H. (1964). Chemical determination of ascorbic dehydroascorbic and diketogluconic acids. In: Met. Biochem. Anal. I., (Ed.D. Glick) Interscience, New York. 115-139.

Somogyi, M. (1952). Notes on sugar determination. J. Biol. Chem. 195: 19-23.

Williams, P. G., Kazima, F. D. and Hlynka, I. (1970). A rapid colorimetric procedure for estimating amylose content of starches. Cereal Chem. 47: 411-420.