PRACTICAL-I
(DBOTL01)
(MSC BOTONY)

ACHARYA NAGARJUNA UNIVERSITY

CENTRE FOR DISTANCE EDUCATION

NAGARJUNA NAGAR,

GUNTUR

ANDHRA PRADESH

M.Sc., Botany (Previous)
Practical- Plant Physiology
9.1 DEMONSTRATION OF OSMOSIS BY USING EGG MEMBRANE

Principle

If two solutions of different concentrations are separated by a semipermeable membrane (egg membrane), the membrane being selectively permeable will not allow to pass solute molecules whereas the solvent molecules will pass through it. According to the laws of diffusion, the movement of solvent molecules (water molecules) will be from the region of higher concentration to the region of their lower concentration or in other words from dilute solution to concentrated solution because the concentration of solvent molecules will be higher in the dilute solution and lower in the concentrated solution.

Materials and Reagents

Eggs-2
Beakers 500 ml – 2
Beakers 250 ml – 2
Graduated tubes – 2
Thread
Hydrochloric acid
Saturated sucrose solution or 1 Molarity sucrose solution

Procedure

1. Take an egg and remove its outer calcareous shell by carefully placing in a beaker containing 300 ml of 3:1 (3 parts HCl and 1 part water) HCl and water mixture.
2. Thoroughly stirr the contents by using glass rod to facilitate the uniform dissolution of the egg shell.
3. After the shell is completely dissolved, take out its inner contents by making a small hole on the surface of the egg membrane.
4. Wash the membrane well in running tap water and make into a sac by tying the membrane to one end of graduated tube having uniform bore.
5. Fill the sac completely with 1 M sucrose solution to demonstrate endosmosis or with pure water for exosmosis.
6. Fix the tube to a stand and keep the sac to be immersed in a beaker containing pure water in case of endosmosis or 1 M sucrose solution in case of exosmosis.
7. Keep the apparatus like that for sometime and note down the volume increase in the tube in case of endosmosis or decrease in case of exosmosis at different time intervals till the equilibrium condition arrives at.
8. Comment on your results.

9.6 ESTIMATION OF REDUCING SUGARS BY NELSON-SOMOGYI'S METHOD

Principle

Reducing sugars (mono or disaccharides by virtue of free aldehyde or ketonic group in their structure reduces cupric ions to cuprous ions ($Cu^{++} \leftrightarrow Cu^{+}$) in alkaline solutions at high temperatures. The alkali present in the Benedict's reagent enolises the reducing sugar to form enediols (forms of sugars) which are highly reactive and act as strong reducing agents.

Materials and Reagents

1. Banana fruits
2. Conical flasks
3. Burette.
4. Pipettes
5. Mortar & pestle
6. Measuring cylinders

Lead acetate

Sodium Oxalate

Quantitative Benedict's solution: Solution A: Dissolve 50 g sodium citrate, 50 g sodium carbonate and 31.25 g potassium thiocyanate – in distilled water and make the final volume to 200 ml.

Solution B: Dissolve 4.5 g $CuSO_4 . 5H_2O$ in distilled water and make the final volume to 25 ml.

Mix solution B drop by drop to solution A and stirr contents repeatedly.

Procedure

1. Prepare the plant extract by boiling 10 g banana fruit for about 15 minutes in 100 ml distilled water.
2. Grind the banana in mortar and pestle by using the same water in which it is boiled and filter it by using muslin cloth.
3. Add a pinch of lead acetate to the filtrate.
4. Filter it again.
5. Add a pinch of sodium oxalate to the filtrate and filter the contents.
6. Now take the filtrate as the sugar extract for estimating reducing sugars and make its final volume to 100 ml.
7. Take this extract in a clean burette.

8. Take 2 ml of Benedicts solution in a conical flask and boil by keeping it on electric heater or water bath.
9. Titrate the boiling Benedict's solution against the plant extract taken in a burette.
10. End point is blue to milk white.
11. Note down the volume of plant extract run down in the burette.
12. Continue the titration till to get 3 concurrent values.
13. Tabulate the results as follows.

S.No.	Volume of Benedict's solution	Burette readings		Volume of the extract run-down
		Initial	Final	
1				
2				
3				
Average				

Calculation: Amount of reducing sugars present in the material was calculated taking into account that 2 ml of Benedict's reagent requires 4 mg of sugar to be reduced.

2 ml of Benedict's reagent is reduced by 4 mg of sugar

$\therefore$ X ml of plant extract is equal to 4 mg of sugar

$$100 \text{ ml of plant extract contains} = \frac{4}{X} \times 100 = Y \text{ mg}$$

10 g of banana contains Y mg of sugar

100 g of banana contains 100 x Y = Z mg

X = ml plant extract run down in the burette

Y = mg of sugar present in 100 ml plant extract

Z = % sugar

*****

9.7 DETERMINATION OF WATER POTENTIAL OF PLANT TISSUE BY GRAVIMETRIC METHOD

Principle

The gravimetric method involves the placement of pre-weighed plant tissue (potato tuber cylinders) into a graded series of sucrose solutions of known concentrations. After an incubation of certain time, the tissue is removed and weighed. The weight gain or loss is plotted against concentrations of sucrose solutions. When the points are connected, the intercept at the abscissa (through zero) represents the concentration of sucrose solution at which no gain or loss in weight occurs. This concentration will be taken for the calculation of water potential of plant tissue.

Materials and Reagents

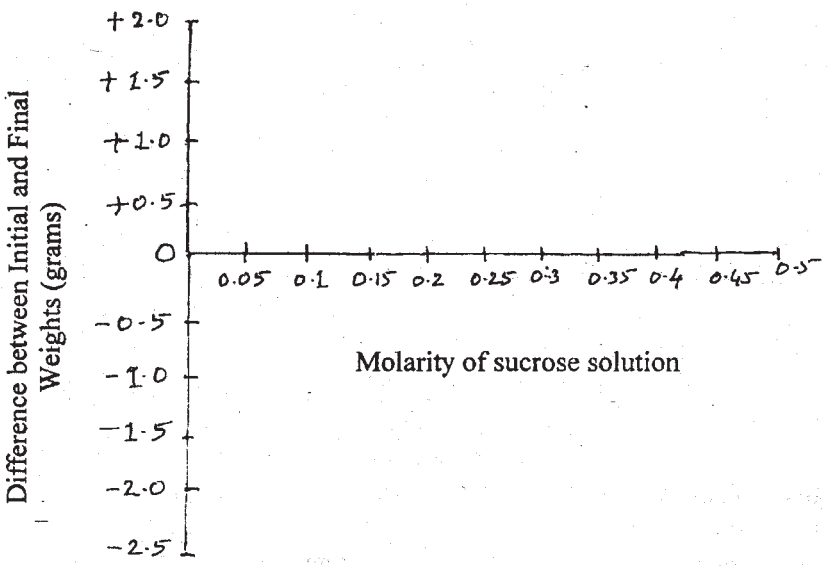
Tubers of white potato or swept potato
Cork borer
Razor blade
Petriplates or 50 ml beakers (11 nos.)
balance and weights
measuring cylinder
aluminium foil
Sugar solutions of molarities ranging from 0.05 M to 0.5 M (0.05, 0.1, 0.15, 0.20, 0.25, 0.3, 0.35, 0.40, 0.405 and 0.5).

Procedure

1. Take out approximately 4.5 cm length and 1 cm diameter potato tuber cylinder by using a 1 cm bore cork borer.
2. Trim the two ends of the cylinder to remove the corky portion.
3. Take weight of cylinder approximately having 5.5 g and measure its length.
4. Cut it into 10 uniformly sized discs.
5. Wash the discs thoroughly to remove starch and other organic constituents.
6. Blot the discs lightly between two pieces of filter paper or blotting paper in order to remove water adhered to the tissue.
7. Adjust the weight of discs accurately to 5 g by trimming one out of 10 discs.
8. Immediately after weighing, place the discs in a beaker or petriplate containing 50 ml of one of the sucrose solutions given in the following table:

Sucrose solutions (Molarity)	Fresh weight of cylinders		Change in weight (g)
	Initial (g)	Final (g)	
0.05	5		
0.10	5		
0.15	5		
0.20	5		
0.25	5		
0.30	5		
0.35	5		
0.40	5		
0.45	5		
0.50	5		

9. Repeat the same process for other concentrations of sucrose solutions.
10. Cover the beakers or plates with aluminium foil and set aside for 1 hour at room temperature.
11. After the prescribed time, remove the discs at a time from the sucrose solutions.
12. Quickly blot the discs with filter paper and measure the final total fresh weight.
13. For each treatment, calculate the increase or decrease in the original weight and enter the results in table given above.
14. Using the graph provided, plot the change in weight (ordinate) against the appropriate concentration of sucrose (abscissa).



Weight change in cylinders of potato tuber tissue as a function of the molarity of the ambient sugar solution.

15. Find out the concentration of sugar solution which does not cause an increase or decrease in cylinder weight.

16. Calculate the water potential of plant tissue by using the formula = $\Psi_w = \frac{22.4 \times M \times 273}{T}$

Ψ_w = Water potential

M = Molarity of the sucrose solution at which no gain or loss in cylinder weight occurs

T = degrees kelvin (273 + room temp.)

*****

9.8 SEPARATION OF CHLOROPLAST PIGMENTS INTO TWO AND FOUR GROUPS

Principle

Chlorophyll *a*, chlorophyll *b*, carotenes and Xanthophylls are chloroplast pigments. They are separated into two groups (chlorophyll *a* – plus carotenes and chlorophyll *b* plus xanthophylls) and four groups (chl *a*, chl *b*, carotenes and xanthophylls) on the basis of their differential solubility in specific solvents and partitioning coefficients in a solvent made alkaline with KOH.

Materials and Reagents

1. Fresh Tecoma or Spinach leaves
2. Separatory funnels - 2
3. Measuring cylinders – 2 (100 ml)
4. Buckner funnels
5. Specimen tubes, separating funnel stands, Muslin cloth
6. 80% acetone (v/v)
7. Quartz sand
8. Calcium carbonate
9. Petroleum ether
10. 92% Methanol
11. Diethyl ether
12. 30% Methanolic KOH (30g KOH in 100 ml methanol)

Procedure

1. Take 10 g Spinach or Tecoma leaves after removing mid-vein.
2. Grind the leaves in a mortar and pestle by adding a pinch of $CaCO_3$, quartz sand and little by little volumes of 80% acetone.
3. When the acetone mixture is coloured deep green, filter the extract with muslin cloth.
4. Make the volume to 40 ml by adding 80% acetone.
5. Take 50 ml of petroleum ether into a separatory funnel.
6. Add 40 ml acetone solution of the chloroplast pigments.
7. Gently rotate the funnel.
8. Add 70 ml of distilled water by allowing the water to flow down the side of the funnel.
9. Gently rotate the funnel until the upper layer is quite green.
10. Permit the layers to separate and then draw off the lower acetone layer and discard.
11. Wash the petroleum ether solution by adding 50 ml of distilled water and gentle rotation.
12. Draw off the lower water and discard it.
13. Repeat the washing twice or thrice with 50 ml of distilled water.
14. Add 50 ml of 92% methyl alcohol and mix by gently rotating the funnel until two layers are formed.

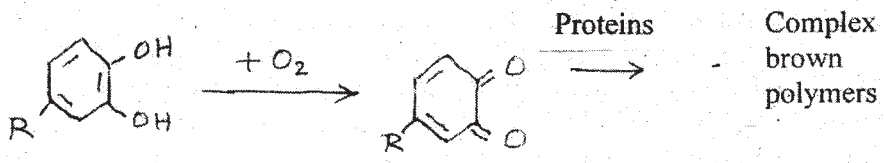
15. Upper petroleum ether layer now contains chlorophyll *a* and carotenes and lower methyl alcohol solution contains chlorophyll *b* and xanthophylls. This is the separation of chloroplast pigments into two groups.
16. Draw the methyl alcohol containing chlorophyll *b* and xanthophyll solution into a beaker and transfer it to a another separatory funnel.
17. To this, add 50 ml of diethyl ether and mix by rotating.
18. Now add distilled water, 5 ml at a time down the side of the funnel and rotate the funnel after each addition until two layers of liquid appear.
19. This usually requires 25 ml to 40 ml of distilled water.
20. Draw off the lower methyl alcohol solution and discard it.
21. Take 10 ml of ethyl ether solution into a one test tube and 10ml of petroleum ether solution into a another one.
22. Carefully pour 5 ml of freshly prepared 30% methanolic KOH down the wall of each test tube, shake and leave the tubes for 10 minutes.
23. Add 30 ml of distilled water to each tube, shake well, and allow them to settle for about few minutes.
24. The tube containing diethyl ether solution now gives the separation of its two pigments as chlorophyll *b* (bottom bluish green) and xanthophylls (upper yellow) and the tube containing petroleum ether shows chlorophyll *a* (bottom, dark green) and carotene (upper, orange-red).
25. Comment on your results.

*****

9.9 DEMONSTRATION OF THE ACTIVITY OF THE ENZYME POLYPHENOL OXIDASE

Principle

The brownish or blackish discoloration that develops in certain tissues shortly after injury by cutting, mining or bruising is the result of the activity of a copper containing oxidase known as polyphenol oxidase. It is a terminal oxidase and acts on substrates that are O-dihydroxy phenols like pyrocatechol. Oxidation of an O-dihydroxyphenol (pyrocatechol) by O_2 results in the production of the corresponding O-quinone. These quinones are very reactive and condense with amino acids and proteins present in plant tissues to yield complex brown polymers.



Pyrocatechol Orthoquinone

Appearance of brown colour indicates the activity of polyphenol oxidase.

Materials and Reagents

1. Potato tubers
2. Test tubes
3. Beakers
4. Muslin cloth
5. Pipettes
6. Measuring cylinders
7. **pH 6 buffer: Solution A:** Dissolve 23.89 g sodium hydrogen phosphate in distilled water and make the final volume to 1 litre. **Solution B:** Dissolve 9.08 g potassium dihydorgen phosphate in distilled water and make the final volume to 1 litre. Mix solution A and B in the ratio of 12:88.
8. 0.02% pyrocatechol: Dissolve 20 mg pyrocatechol in 100 ml distilled water.

Procedure

1. Take 10 g potato tuber bits in a muslin cloth and dip it in a beaker containing 100 ml of distilled water and shake well for about 15 minutes.
2. Polyphenol oxidase is a water soluble enzyme and when potato bits are shaken in water, the enzymes dissolves in water. It serves as an enzyme extract.
3. Take five clean test tubes.
4. Add 5 ml of enzyme extract to each test tube.
5. Now add buffer, pyrocatechol and water to each test tube as given below:

Test Tube No.	Enzyme extract (ml)	Buffer (ml)	Pyrocatechol (ml)	Distilled water (ml)
1	5	2	0.5	--
2	5	--	0.5	2
3	5	2	--	0.5
4	5	--	--	2.5
5	5 (boiled for two minutes)	2	0.5	--

6. Shake well and keep all the test tubes for 15 minutes.
7. Observe the colour changes and comment upon your results.

*****

9.10 ESTIMATION OF THE ACTIVITY OF THE ENZYME CATALASE

Principle

H_2O_2 produced in several reactions in plant cells is highly toxic and must be scavenged promptly to avoid injury to metabolic machinery of the tissue. The enzyme catalase decomposes H_2O_2 as:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The estimation of catalase activity is based on the fact that it dicomposes H_2O_2 and the remaining H_2O_2 in the reaction mixture is estimated volumetrically by using potassium permanganate as an oxidizing agent.

Materials and Reagents

1. Germinating seeds of mungbean or wheat.
2. 0.045 M H_2O_2 : (Take 2.5 ml of H_2O_2 and make upto 100 ml with distilled water)
3. 0.05 N $KMnO_4^+$
4. 12% H_2SO_4.
5. **pH7 phosphate buffer: Solution A:** Dissolve 11.86 g disodium hydrogen phosphate in distilled water and make the volume to 1 litre. **Solution B:** Dissolve 9.07 g potassium dihydrogen phosphate in distilled water and bring the volume to 1 litre.
   Mix solution A and B in the ratio of 61 : 39
6. Mortar and Pestle
7. Test tubes
8. Pipettes
9. Burette
10. Measuring Jar .
11. Conical flasks
12. Muslin cloth
13. Funnel
14. Volumetric flask (25 ml)

Procedure

1. Take 1 g germinating seeds after removing seed coat and moisture.
2. Grind the seeds in a mortar and pestle by adding little by little of pH 7 buffer.
3. Filter the extract by using muslin cloth.
4. Make the total volume of extract to 25 ml by repeating grinding and washing of debris left out with the same buffer.
5. Take 2 ml of extract in a conical flask.

9.2 ESTIMATION OF CHLORIDE

Principle

Chloride is one of the essential plant nutrient. It is required for various metabolic activities. In plants, it is present in higher amounts than the surroundings, i.e., accumulated in plants against concentration gradients. This element can be estimated titrimentrically with $AgNO_3$ as a reactant. $AgNO_3$ by reacting with chloride forms Ag Cl.

$$Ag\ NO_3 + NaCl \rightarrow Ag\ Cl + NaNO_3$$

Materials and Reagents

Hydrilla twigs
0.02 N Silver Nitrate
0.02 N Sodium chloride
5% Potassium chromate
Pipette 10 ml
Burette
Conical flasks
Volumetric flasks
Pestle & Mortar

Procedure

1. Take 10 ml of 0.2 N Sodium chloride solution in a conical flask.
2. Add 3 drops of 5% potassium chromate.
3. Titrate it against 0.02 N Ag NO_3.
4. Appearance of permanent reddish brown colour is the end point.
5. Note down the volume of $AgNO_3$ run down in the burette.
6. Continue the titration till three concurrent values obtained.
7. Weigh 2.5 g fresh Hydrilla twigs.
8. Macerate it in a mortar and pestle by adding quartz sand and distilled water.
9. Squeeze the contents through muslin cloth.
10. Make volume of the extract to 100 ml.
11. Take 10 ml of extract in a conical flask.
12. Add 3 drops of 5% $K_2Cr_2O_7$.
13. Titrate it against 0.02 N $AgNO_3$.
14. Note down the burette readings.
15. Similarly continue the experiment with pond water collected from where the Hydrilla twigs are taken.

6. Add 1 ml of 0.045 M H_2O_2.
7. Incubate the reaction mixture at 25°C for 5 minutes.
8. Stop the reaction by adding 10 ml of 12% H_2SO_4.
9. Titrate reaction mixture against 0.05 N $KMnO_4$.
10. Appearance of a faint purple colour that persists for at least 15 sec. denotes end point.
11. Repeat the titrations till three concurrent values were obtained.
12. Similarly, maintain a control on which the enzyme activity has been stopped by adding H_2SO_4 prior to the addition of H_2O_2.
13. Express the activity of enzyme as amount H_2O_2 decomposed in one minute time as per the following formula:

1 ml of 0.05 N $KMnO_4$ reacts with 0.85 mg of H_2O_2

$$\text{Amount of } H_2O_2 \text{ destroyed by catalase in 5 minutes} = \frac{25}{2} \times 0.85 \times \frac{V}{W}$$

V = Volume of difference on $KMnO_4$ consumed in blank and sample titrations

W = Weight of the plant material

*****

9.11 PHYSIOLOGICAL ANATOMY OF C_3 AND C_4 PLANTS

Principle

Plants which fix the CO_2 through Calvin's cycle are called C_3 type of plants and those that fix CO_2 through Hatch and Slack pathway are called C_4 type. These two groups of plants not only differ in their mode of fixation of CO_2 but also in their anatomical features. The C_4 plants possess a special type of leaf anatomy, the **Kranz Anatomy** due to which they show high photosynthetic rates and high efficiency.

Materials and Reagents

Both C_3 and C_4 dicot and monocot plants
Watch glasses
Coverslips
Slides
Microscope
Needle and brush
Iodine stain solution (Dissolve 2 g potassium iodide in 5 ml of distilled water and add 1 g idonine. Make a volume to 300 ml with distilled water).

Procedure

1. Take fresh leaves of C_3 and C_4 plants such as *Sorghum, Maize, Saccharum, Euphorbia hirta, Tribulus, Eleusine, Cleome gynandra. Alternanthera* and wash them thoroughly in running tap water.
2. Take free hand sections of leaves with a blade.
3. Place them on a glass slide and stain with iodine solution.
4. Mount the slides with coverslips.
5. Observe the mounted slides under microscope.
6. Find out the anatomical differences i.e. presence of chloroplast containing bundle sheath around the vascular bundles and neatly draw the diagrams of observed species.

9.12 DETERMINATION OF AEROBIC RESPIRATION

Principle

During the process of respiration CO_2 is released. This CO_2 is absorbed by the barrium hydroxide to form barrium carbonate. the remaining barrium hydroxide is titrated against 0.1 N HCl. The difference between blank and sample titre value can be taken to estimate the rate of respiration by the repairing organs.

Materials and Reagents

Germinating seeds or Calotropis flower buds
Conical flasks, 250 ml 4
Glass bent tubes
Rubber tube
Suction pump
10% Sodium hydroxide
0.1 N Barrium hydroxide
0.1 N HCl
Phenolphthalein indicator solution

Procedure

1. Take four conical flasks with two holed rubber corks fitted with bent glass tubes.
2. Keep all the four flasks on a serial order and number them from left to right (as 1, 2, 3 and 4).
3. Take 200 ml of 10% NaOH in the first flask and 200 ml of 0.1 N Barrium hydroxide (BaOH) in the second flask, 20 g of flower buds or germinating seeds in the third flask and 200 ml of 0.1 N $Ba(OH)_2$ in the fourth flask.
4. Keep all inlets of the glass tubes to be immersed in respective solutions and outlets above the solutions.
5. Connect all flasks with rubber tubing.
6. Connect the fourth flask to a suction pump.
7. Keep the whole apparatus air tight by applying wax.
8. Maintain the suction for an hour.
9. Take 20 ml of barrium hydroxide from fourth flask into a conical flask and titrate it against 0.1 N HCl using phenolphthalein as indicator.
10. Similarly do the titration for blank.
11. Calculate the rate of respiration according to the following formula:

$$\text{Rate of respiration (mg } CO_2.h^{-1} g^{-1}) = \frac{\text{titre value (Blank-Experiment)} \times \text{Normality of HCl} \times (\text{½ gram molecular weight of } CO_2) \times 200}{\text{Sample weight} \times 20}$$

9.13 ESTIMATION OF TITRATABLE ACIDITY

Principle

In CAM plants carboxylation occurs during night time and decarboxylation during day time, which results in accumulation of malic acid in vacuoles during night time. The leaf material of dark exposed CAM plant therefore, contains high titratable acidity than do light exposed ones.

Materials and Reagents

Bryophyllum leaves (both dark and light exposed)
Beakers
Conical flasks
Burette
Measuring jars
Mortar and pestle
Pipettes
0.1 N Sodium hydroxide
Phenolphthalein indicator

Procedure:

1. Take 10 g Bryophyllum leaves in a beaker containing distilled water.
2. Boil the contents for about 15 minutes.
3. Then paste the leaves in mortar by adding same distilled water in which the leaves were boiled.
4. Filter the extract and make a volume upto 100 ml.
5. Take 10 ml of the extract in a conical flask and add 2 drops of phenolpthalein.
6. Titrate the contents with 0.1 N NaOH taken in a burette.
7. End point is the appearance of pink colour.
8. Note down the volume of NaOH run down.
9. Continue the titration till three concurrent values obtained.
10. Calculate the titratable acidity in terms of normality by using the following formula:

$$N_1V_1 = N_2V_2$$

V_1 = Volume of the extract = 10 ml
N_i = Normality of the extract - ?
V_2 = Volume of NaOH run down = X ml
N_2 = Normality of NaOH = 0.1 N

$$N_1 = \frac{V_2 \times N_2}{V_1}$$

11. Titratable acid number (TAN) is the number of 0.1 N NaOH required to titrate the acid conte present in free state for 100 g fresh material and is calculated as follows:

10 ml of extract requires = X ml of NaOH

$$100 \text{ ml of extract requires} = \frac{X \times 100}{10} \text{ ml of NaOH}$$

$$\text{i.e. 10 g leaf material contains acid equivalent to} = \frac{X \times 100}{10} \text{ ml of NaOH}$$

$$100 \text{ g leaf material contains acid equivalent to} = \frac{X}{10} \times 100 \times 100 \text{ ml NaOH}$$

$$TAN = \frac{X \times 100 \times 100}{10}$$

*****

16. Calculation:

1 ml of 0.02 N NaCl = 0.71 mg of chloride

Volume of 0.02 N Ag NO_3 used to neutralize the chloride present in 10 ml of 0.02 N

NaCl (7.1 mg chloride) = x ml

$\therefore$ 1 ml of Ag NO_3 = 7.1 x $^1/_X$ mg of chloride

By using this standardized value calculate the amount of chloride present in the 100 g plant material and pond water.

17. Comment on your results.

*****

9.3 ESTIMATION OF NITROGEN BY MICRO-KJELDAHL'S METHOD

Principle

Any nitrogen containing substance on digestion with concentrated sulphuric acid is converted into ammonium sulphate ($(NH)_2SO_4$). On distillation of the digested sample with NaOH, NH_3 is liberated and trapped in boric acid containing methyl red as an indicator. Ammonia reacts with boric acid to form ammonium borate which is then determined volumetrically by titrating against standardized 0.02 N H_2SO_4 and the amount of nitrogen determined. Since proteins contain about 16% nitrogen, the protein content of the sample is calculated by multiplying its nitrogen content by 6.25. The value obtained is referred to as crude protein content since this procedure estimates total organic nitrogen rather than true proteins. This method is commonly used for determination of crude protein content of grains, forage and animal feeds.

Materials and Reagents

1. Long necked Micro-Kjeldahl flasks (50 ml capacity).
2. Digestion rack
3. Micro-Kjeldahl apparatus
4. Burette (50 ml capacity), conical flasks (50 ml), measuring cylinder (50 ml), balance and weights.
5. Conc. H_2SO_4.
6. **Catalyst:** Selenium dioxide powder or a mixture of $CuSO_4.5H_2O$ and K_2SO_4 in the ratio of 1:4.
7. 40% NaOH. Take 400 g of NaOH, dissolve it in water and make the volume to 1 litre.
8. **2% boric acid:** Dissolve 4 g of reagent grade boric acid in water and make the volume to 100 ml.
9. 0.02 N HCl.

Procedure

1. Take 100 mg of finely powdered grain sample on a long necked digestion flask. Add 3 ml of Conc. H_2SO_4 and a pinch of catalyst mixture.
2. Digest the sample on heater or Kjeldahl heating unit till the solution becomes clear.
3. Take 10 ml of 4% boric acid on 50 ml conical flask and to it add 3 drops of methyl red indicator. Place this receiving flask in such a way that outlet of the condenser of micro-Kjeldahl's distillation apparatus dips into the boric acid solution.
4. Transfer the acid digested sample to the distillation jacket of micro-Kjeldahl's apparatus. Add about 15 to 20 ml of 40% NaOH to the aliquot of the digested sample. Wash the flask with distilled water and pour the contents into jacket through a funnel. Immediately close the stopcock.
5. Allow the steam to pass through the distillation jacket to distill ammonia till the volume of the solution in the conical flask becomes approximately double of the initial volume.

6. Remove the receiving flask, rinse the condenser outlet tip into the receiving flask with water.
7. Titrate the contents of flask against 0.02 N H_2SO_4 till the yellowish green colour changes to pink.
8. Run a blank preparation which has been identically prepared except that it does not contain the sample.

Calculations:

1 ml of 0.02 N H_2SO_4 = 0.00028 g nitrogen.

Since an average nitrogen content of most proteins is 16%

$\therefore$ 1 g of nitrogen = $^{100}/_{16}$ = 6.25g protein

Volume of 0.02 N H_2SO_4 used for blank = x ml

Volume of 0.02 N H_2SO_4 used for sample = y ml

Titre volume of sample = (y-x) ml

Nitrogen present in 0.1 g or 100 mg of sample = (y-x) x 0.00028

$$\text{Amount of nitrogen present in 100g sample} = \frac{(y-x)\ 0.00038 \times 100}{0.1}$$

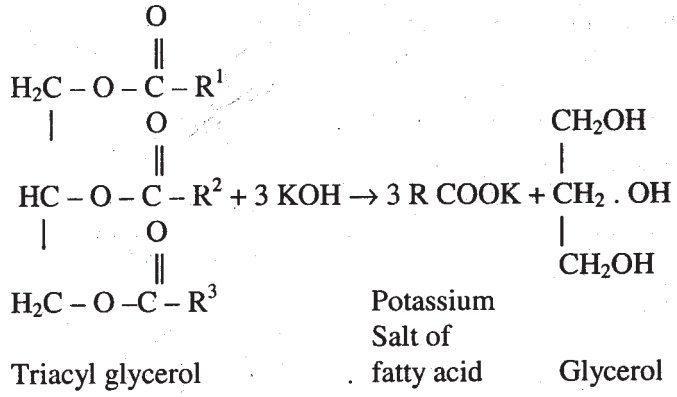
$$\text{\% Protein content in sample} = \frac{6.25 \times (y-x) \times 0.00028 \times 100}{0.1}$$

*****

9.4 DETERMINATION OF SAPONIFICATION VALUE OF FATS AND OILS

Principle

Hydrolysis of fat with an alkali results in the formation of salts of fatty acids (also called soap) and glycerol. This process is called saponification. From the amount of potassium hydroxide utilized during hydrolysis, the saponification value of a given fat sample can be calculated. The saponification value is defined as milligrams of potassium hydroxide required to saponify one gram of the given fat.



From the above reaction, it may be noted that 3 molecules of KOH are consumed for saponification of each molecule of triacylglycerol irrespective of chain length of fatty acid. Evidently, each gram of a triacyl-glycerol with shorter chain fatty acids will contain larger number of molecules of the tri acyl glycerol and will thus require much more KOH. The saponification value is thus an indication of an average molecular weight of the fatty acids on an acyl glyceride.

The procedure involves refluxing of known amount of fat or oil with a fixed but an excess of alcoholic KOH. The amount of KOH remaining after hydrolysis is determined by back titrating with standardized 0.5 N HCl and the amount of KOH utilized for saponification can thus be calculated.

Materials and Reagents

1. Reflux condenser
2. Water bath
3. Burette
4. Test compounds (tristearin, coconut oil, corn oil, butter).
5. **Fat solvent:** A mixture of 95% ethanol and ether (1:1, v/v)
6. **0.5 N alcoholic KOH:** Prepare 0.5 N solution of KOH by dissolving 28.05 g of KOH pellets in 20 ml of water and making the volume to 1 litre with 95% ethanol.
7. 1% phenolphthalein solution in 95% alcohol

8. 0.5 N HCl.

Procedure

1. Weigh accurately 1 g of the fat sample in a conical flask and dissolve it in about 3 ml of the fat solvent (Reagent No. 5).
2. Add 25 ml of 0.5 N alcoholic KOH, attach a reflux condenser to it and reflux the contents on boiling water bath for 30 minutes.
3. Cool it to room temperature and add a few drops of phenolphthalein into the flask.
4. Titrate the contents of the flask with 0.5 N HCl till the pink colour disappears.
5. Similarly, run a blank by refluxing 25 ml of 0.5 N alcoholic KOH without any fat sample.

Calculations

0.5 N alcoholic KOH in blank = x ml

0.5 N alcoholic KOH in test sample = y ml

Titre value for sample = (x-y) ml

$$\text{Saponification value} = \frac{28.05 \times \text{titre value}}{\text{Weight of sample (g)}}$$

The multiplication factor 28.05 in the above equation is included since 1 ml of 0.5 N KOH contains 28.05 mg of KOH.

Precautions

1. Electric heater should be used during refluxing as alcohol is highly imflammable.
2. During refluxing, effective cooling of condenser is required so that alcohol does not get evaporated during saponification.

*****

9.5 ESTIMATION OF CHLOROPHYLL

Principle

Chlorophylls are present as green pigments within the chloroplasts of plants. Chlorophyll *a*, chlorophyll *b*, and carotenoids are chloroplast contained pigments. They play an important role in the photosynthetic process. The quantity of chlorophylls not only denotes about the efficiency of photosynthesis and it varies among C_3 and C_4 plants.

The pigments of the chloroplast are readily soluble in acetone, which provides an excellent extraction medium for their isolation. Absorption maxima of isolated chlorophyll *a* and chlorophyll *b* are then determined with spectrophotometer for their estimation.

Reagents and materials

Acetone 80% (v/v)
Calcium carbonate, Quartz sand
Fresh leaves (spinach or other chlorophyll containing tissue)
Mortar and pestle
Muslin cloth or Whatman No.42 filter paper
Volumetric flasks, 50 ml, 100 ml
Buchner funnel
Centrifuge
Pipette, 5 ml, 10 ml
Spectrophotometer

Extraction of Chlorophyll

1. Place 1 g (fresh weight) of small cut pieces of spinach leaves (or other fresh green leaves) into a clean mortar.
2. Add a pinch of $CaCO_3$, quartz sand and 40 ml of 80% acetone.
3. Grind the tissue to a fine pulp.
4. Carefully transfer the resulting green liquid to Buchner funnel containing a pad of Whatman No.1 filter paper or musclin cloth.
5. Repeat the grinding of the pulp with a fresh 30 ml aliquot of 80% acetone and then filter the slurry into the flask containing the first extract. After the second extraction, the tissue should be devoid of chlorophyll.
6. If filtering is carried out by using musclin cloth, the resulting filterate should be centrifuged at 3000 rpm for about 5 minutes.
7. Adjust the final volume of filtrate to 100 ml by adding sufficient 80% acetone.
8. Transfer a small quantity of filtrate to the cuvettes.
9. Measure the absorbance at 645 for chlorophyll *b* and 663 for chlorophyll *a* with a spectrophotometer. Be sure to read against an 80% acetone solvent blank.

10. Calculate the amount of chlorophyll present on the extract on the basis of milligrams of chlorophyll per gram of leaf tissue extracted according the following equations:

$$\text{mg of chlorophyll a/g fresh weight} = \frac{[12.7\ (OD_{663}) - 2.69\ (OD_{645})\ ] \times V}{a \times 1000 \times W}$$

$$\text{mg of chlorophyll b/g tissue} = \frac{[22.9\ (OD_{645}) - 4.68\ (OD_{663})\ ] \times V}{a \times 1000 \times W}$$

Where a = length of path of light in the cell (usually 1 cm)

V = Volume of the extract in ml, and

W = Fresh weight of the sample in g

OD = Optical density reading of the chlorophyll extract at specific indicated wavelength.

*****