

Chapter 1

CARBOHYDRATES

1.1 Colour Reactions

Carbohydrates are widely prevalent in the plant kingdom, comprising the mono-, di-, oligo-, and polysaccharides. The common monosaccharides are glucose, fructose, galactose, ribose etc. The disaccharides, *i.e.*, the combination of two monosaccharides include sucrose, lactose and maltose. Starch and cellulose are polysaccharides consisting of many monosaccharide residues. Cellulose is the most abundant organic compound on this planet since it forms part of the cell wall in plants.

Aldehydes ($-\text{CHO}$) and ketones ($=\text{CO}$) are active groups in carbohydrates. Carbohydrates contain many hydroxyl groups as well. The number of hydroxyl groups varies with the number of carbon atoms. Monosaccharides contain the free aldehyde or ketone group. Some disaccharides have the free aldehyde group (maltose) and some do not have the free ones (sucrose). The polysaccharides, starch and cellulose, are polymers of monosaccharides linked through the active groups.

The chemical properties of saccharides vary depending upon the number of hydroxyl groups and the presence or absence of $-\text{CHO}/=\text{CO}$ groups. These variations are the basis in the development of colour reactions to identify the saccharides.

Some simple tests used to identify the presence/absence of certain saccharides are listed below:

REAGENTS

- Iodine solution*: Add a few crystals of iodine to 2% potassium iodide solution till the colour becomes deep yellow.
- Fehling's reagent A*: Dissolve 34.65 g copper sulphate in distilled water and make up to 500 mL.
- Fehling's reagent B*: Dissolve 125 g potassium hydroxide and 173 g Rochelle salt (potassium sodium tartrate) in distilled water and make up to 500 mL.

- *Benedict's qualitative reagent*: Dissolve 173 g sodium citrate and 100 g sodium carbonate in about 500 mL water. Heat to dissolve the salts and filter, if necessary. Dissolve 17.3 g copper sulphate in about 100 mL water and add it to the above solution with stirring and make up the volume to 1 L with water.
- *Barfoed's reagent*: Dissolve 24 g copper acetate in 450 mL boiling water. Immediately add 25 mL of 8.5% lactic acid to the hot solution. Mix well, Cool and dilute to 500 mL.
- *Seliwanoff's reagent*: Dissolve 0.05 g resorcinol in 100 mL dilute (1:2) hydrochloric acid.
- *Bial's reagent*: Dissolve 1.5 g orcinol in 500 mL of concentrated HCl and add 20 to 30 drops of 10% ferric chloride.

The reactions of carbohydrates are given in Table 1.1.

TABLE 1.1: Reactions of carbohydrates

<i>Experiment</i>	<i>Observation</i>	<i>Remarks</i>
<p>1. Molisch's Test</p> <p>Add two drops of Molisch's reagent (5% 1-naphthol in alcohol) to about 2 mL of test solution and mix well.</p> <p>Incline the tube and add about 1 mL of concentrated sulphuric acid along the sides of the tube.</p> <p>Observe the colour at the junction of the two liquids.</p>	<p>A red-cum-violet ring appears at the junction of the two liquids.</p>	<p>The colour formed is due to the reaction of alpha-naphthol with furfural and/or its derivatives formed by the dehydration of sugars by concentrated sulphuric acid. All carbohydrates react positively with this reagent.</p>
<p>2. Iodine Test</p> <p>Add a few drops of iodine solution to about 1 mL of the test solution.</p>	<p>Appearance of deep blue colour.</p>	<p>This indicates the presence of starch in the solution.</p> <p>The blue colour is due to the formation of starch-iodine complex.</p>
<p>3. Fehling's Test</p> <p>To 1 mL of Fehling's solution 'A', add 1 mL of Fehling's solution 'B' and a few drops of the test solution. Boil for a few minutes.</p>	<p>Formation of yellow or brownish-red precipitate.</p>	<p>The blue alkaline cupric hydroxide present in Fehling's solution, when heated in the presence of reducing sugars, gets reduced to yellow or red cuprous oxide and it gets precipitated. Hence, formation of the coloured precipitate indicates the presence of reducing sugars in the test solution.</p>

<p>4. <i>Benedict's Test</i></p> <p>To 2 mL of Benedict's reagent add five drops of the test solution. Boil for five minutes in a water bath. Cool the solution.</p>	<p>Formation of red, yellow or green colour/precipitate.</p>	<p>As in Fehling's test, the reducing sugars because of having potentially free aldehyde or keto group reduce cupric hydroxide in alkaline solution to red coloured cuprous oxide. Depending on the sugar concentration yellow to green colour is developed.</p>
<p>5. <i>Barfoed's Test</i></p> <p>To 1 mL of the test solution add about 2 mL of Barfoed's reagent. Boil it for one minute and allow to stand for a few minutes.</p>	<p>Formation of brick-red precipitate.</p>	<p>Only monosaccharides answer this test. Since Barfoed's reagent is weakly acidic, it is reduced only by monosaccharides.</p>
<p>6. <i>Seliwanoff's Test</i></p> <p>To 2 mL of Seliwanoff's reagent add two drops of test solution and heat the mixture to just boiling.</p>	<p>Appearance of deep red colour.</p>	<p>In concentrated HCl, ketoses undergo dehydration to yield furfural derivatives more rapidly than do aldoses. These derivatives form complexes with resorcinol to yield deep red colour.</p> <p>It is a timed colour reaction specific for ketoses.</p>
<p>7. <i>Bial's Test</i></p> <p>To 5 mL of Bial's reagent add 2–3 mL of solution and warm gently. When bubbles rise to the surface cool under the tap.</p>	<p>Appearance of green colour or precipitate.</p>	<p>It is specific for pentoses. They get converted to furfural. In the presence of ferric ion orcinol and furfural condense to yield a coloured product.</p>
<p>8. <i>Test for non-reducing sugars such as sucrose:</i></p> <p>(a) Do Benedict's test with the test solution.</p> <p>(b) Add 5 drops of concentrated HCl to 5 mL of test solution in another test tube. Heat for five minutes on a boiling water bath.</p>	<p>No characteristic colour formation.</p> <p>Appearance of red or yellow colour.</p>	<p>Indicates the absence of reducing sugars in the given solution.</p> <p>Indicates the formation of reducing sugars from non-reducing sugars after hydrolysis with acid.</p>

(Contd.)

<p>Add 10% sodium hydroxide solution to give a slightly alkaline solution (test with red litmus paper). Now perform Benedict's test with this hydrolysed solution.</p>		
<p>9. Mucic Acid Test Add a few drops of conc. HNO_3 to the concentrated test solution or substance directly and evaporate it over a boiling water bath till the acid fumes are expelled. Add a few drops of water and leave it overnight.</p>	<p>Formation of crystals.</p>	<p>The both end carbon groups are oxidized to carboxylic groups. The resultant saccharic acid of galactose is called mucic acid which is insoluble in water.</p>
<p>10. Osazone Test To 0.5 g of phenylhydrazine hydrochloride add 0.1 g of sodium acetate and 10 drops of glacial acetic acid. To this mixture add 5 mL of test solution and heat on a boiling water bath for about half an hour. Allow the tube to cool slowly and examine the crystals under a microscope.</p>	<p>Glucose, fructose and mannose produce needle-shaped yellow osazone crystals, whereas lactosazone is mushroom-shaped. Different osazones show crystals of different shapes. Maltose produces flower-shaped crystals.</p>	<p>The ketoses and aldoses react with phenylhydrazine to produce a phenylhydrazone which in turn reacts with another two molecules of phenylhydrazine to form the osazone.</p>

NOTES:

1. For osazone test, the reaction mixture should be between pH 5 and 6. Fructose takes 2 min to form the osazone whereas for glucose it is 5 min. The disaccharides take a longer time to form osazones. Disaccharides form crystals only on cooling.
2. When a mixture of carbohydrates is present in the test sample, chromatographic methods should be employed to identify the individual sugars.

READING

1. Sadasivam, S. and Theymoli Balasubramanian (1985). *Practical Manual (Undergraduate)*, Tamil Nadu Agricultural University, Coimbatore, p. 2.

1.2 Determination of Reducing Sugars by Nelson-Somogyi Method

Sugars with reducing property (arising out of the presence of a potential aldehyde or keto group) are called reducing sugars. Some of the reducing sugars are glucose, galactose, lactose

and maltose. The Nelson-Somogyi method is one of the classical and widely used methods for the quantitative determination of reducing sugars.

PRINCIPLE

The reducing sugars when heated with alkaline copper tartrate 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.

MATERIALS

Alkaline Copper Tartrate

- (i) Dissolve 2.5 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20 g anhydrous sodium sulphate in 80 mL water and make up to 100 mL.
- (ii) Dissolve 15 g copper sulphate in a small volume of distilled water. Add one drop of sulphuric acid and make up to 100 mL.

Mix 4 mL of B and 96 mL of solution A before use.

Arsenomolybdate reagent: Dissolve 2.5 g ammonium molybdate in 45 mL water. Add 2.5 mL sulphuric acid and mix well. Then add 0.3 g disodium hydrogen arsenate dissolved in 25 mL water. Mix well and incubate at 37°C for 24–48 hours.

Standard glucose solution: Stock: 100 mg in 100 mL distilled water.

Working standard: 10 mL of stock diluted to 100 mL with distilled water [100 µg/mL].

PROCEDURE

1. Weigh 100 mg of the sample and extract the sugars with hot 80% ethanol twice (5 mL each time).
2. Collect the supernatant and evaporate it by keeping it on a water bath at 80°C.
3. Add 10 mL water and dissolve the sugars.
4. Pipette out aliquots of 0.1 or 0.2 mL to separate test tubes.
5. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard solution into a series of test tubes.
6. Make up the volume in both sample and standard tubes to 2 mL with distilled water.
7. Pipette out 2 mL distilled water in a separate tube to set a blank.
8. Add 1 mL of alkaline copper tartrate reagent to each tube.
9. Place the tubes in a boiling water for 10 minutes.
10. Cool the tubes and add 1 mL of arsenomolybdic acid reagent to all the tubes.
11. Make up the volume in each tube to 10 mL with water.
12. Read the absorbance of blue colour at 620 nm after 10 min.
13. From the graph drawn, calculate the amount of reducing sugars present in the sample.

CALCULATION

Absorbance corresponds to 0.1 mL of test = x mg of glucose

$$\begin{aligned} 10 \text{ mL contains} &= \frac{x}{0.1} \times 10 \text{ mg of glucose} \\ &= \% \text{ of reducing sugars} \end{aligned}$$

READINGS

1. Somogyi, M. (1952). *J. Biol. Chem.*, 200, 245.
2. Krishnaveni, S.; Theymoli Balasubramanian and Sadasivam, S. (1984). *Food Chem.*, 15, 229.

1.3 Estimation of Reducing Sugar by Dinitrosalicylic Acid Method

For sugar estimation an alternative to Nelson-Somogyi method is the dinitrosalicylic acid method—simple, sensitive and adoptable during handling of a large number of samples at a time.

MATERIALS

- *Dinitrosalicylic Acid Reagent (DNS Reagent)*
Dissolve by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 mL 1% NaOH. Store at 4°C. Since the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite may be added at the time of use.
- 40% Rochelle salt solution (Potassium sodium tartrate).

PROCEDURE

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

CALCULATION

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

READING

1. Miller, G.L. (1972). *Anal. Chem.*, 31, p. 426.

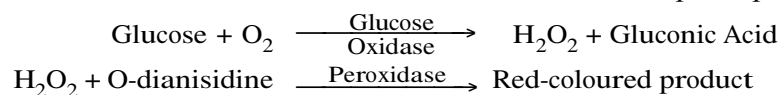
1.4 Determination of Glucose by Glucose Oxidase Method

Glucose is a widely distributed simple sugar with an active aldehyde group. Estimation of glucose by glucose oxidase gives the true glucose concentration eliminating the interference by other reducing sugars.

PRINCIPLE

Glucose oxidase catalyses the oxidation of alpha-D-glucose to D-glucono-1, 5 lactone (gluconic

acid) with the formation of hydrogen peroxide. The oxygen liberated from hydrogen peroxide by peroxidase reacts with the O-dianisidine and oxidises it to a red chromophore product.



MATERIALS

☐ *Glucose Oxidase Peroxidase Reagent*

Dissolve 25 mg O-dianisidine completely in 1 mL of methanol. Add 49 mL of 0.1 M phosphate buffer (pH 6.5). Then add 5 mg of peroxidase and 5 mg of glucose oxidase to the above prepared O-dianisidine solution.

☐ *Standard*: Dissolve 100 mg glucose in 100 mL water. Dilute 10 mL of this stock to 100 mL to obtain the working standard.

PROCEDURE

1. To 0.5 mL of deproteinised plant extract (deproteinization is not necessary in samples with very low protein content) add 0.5 mL distilled water and 1 mL glucose oxidase-peroxidase reagent.
2. Into a series of test tubes pipette out 0 (blank), 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard glucose solution and make up the volume to 1.0 mL with distilled water. Then add 1 mL of glucose oxidase-peroxidase reagent.
3. Incubate all the tubes at 35°C for 40 minutes.
4. Terminate the reaction by the addition of 2 mL of 6 N-HCl.
5. Read the colour intensity at 540 nm.

CALCULATION

From the standard graph, calculate the amount of glucose present in the sample preparation.

READINGS

1. Malik, C.P. and Singh, M.B. (1980). *Plant Enzymology and Histoenzymology*, Kalyani Publishers, New Delhi, p. 278.
2. Krishnaveni, S.; Theymoli Balasubramanian and Sadasivam, S. (1984). *Food Chem.*, 15, 229.

1.5 Determination of Total Carbohydrate by Anthrone Method

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharides which cannot be split by hydrolysis into more simpler sugars. The carbohydrate content can be measured by hydrolysing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

PRINCIPLE

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

MATERIALS

- 2.5 N HCl
- Anthrone reagent*: Dissolve 200 mg anthrone in 100 mL of ice-cold 95% H₂SO₄. Prepare fresh before use.
- Standard glucose*: Stock—Dissolve 100 mg in 100 mL water. Working standard—10 mL of stock diluted to 100 mL with distilled water. Store refrigerated after adding a few drops of toluene.

PROCEDURE

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

CALCULATION

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

NOTE:

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

READING

1. Hedge, J.E. and Hofreiter, B.T. (1962). In: *Carbohydrate Chemistry*, 17 (Eds. Whistler R.L. and Be Miller, J.N.), Academic Press, New York.

1.6 Phenol Sulphuric Acid Method for Total Carbohydrate

The phenol sulphuric acid method to estimate total carbohydrates is described below.

PRINCIPLE

In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green coloured product with phenol and has absorption maximum at 490 nm.

MATERIALS

- *Phenol 5%*: Redistilled (reagent grade) phenol (50 g) dissolved in water and diluted to one litre.
- Sulphuric acid 96% reagent grade.
- *Standard glucose*: Stock—100 mg in 100 mL of water. Working standard—10 mL of stock diluted to 100 mL with distilled water.

PROCEDURE

1. Follow the steps 1 to 4 as given in anthrone method for sample preparation.
2. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard into a series of test tubes.
3. Pipette out 0.1 and 0.2 mL of the sample solution in two separate test tubes. Make up the volume in each tube to 1 mL with water.
4. Set a blank with 1 mL of water.
5. Add 1 mL of phenol solution to each tube.
6. Add 5 mL of 96% sulphuric acid to each tube and shake well.
7. After 10 min shake the contents in the tubes and place in a water bath at 25–30°C for 20 min.
8. Read the colour at 490 nm.
9. Calculate the amount of total carbohydrate present in the sample solution using the standard graph.

CALCULATION

Absorbance corresponds to 0.1 mL of the test = x mg of glucose

$$\begin{aligned} 100 \text{ mL of the sample solution contains} &= \frac{x}{0.1} \times 100 \text{ mg of glucose} \\ &= \% \text{ of total carbohydrate present.} \end{aligned}$$

READINGS

1. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A. and Smith, F. (1956). *Anal. Chem.*, 26, p. 350.
2. Krishnaveni, S.; Theymoli Balasubramanian and Sadasivam, S. (1984). *Food Chem.*, 15, p. 229.

1.7 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 amylose and amylopectin. Starch is hydrolysed into 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 hydrolysed to glucose and dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone.

MATERIALS

- Anthrone*: Dissolve 200 mg anthrone in 100 mL of ice-cold 95% sulphuric acid.
- 80% ethanol.
- 52% perchloric acid.
- Standard glucose*: Stock—100 mg in 100 mL water. Working standard—10 mL of stock diluted to 100 mL with water.

PROCEDURE

1. Homogenize 0.1–0.5 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.
2. To the residue add 5.0 mL of water and 6.5 mL of 52% perchloric acid.
3. Extract at 0°C for 20 min. Centrifuge and save the supernatant.
4. Repeat the extraction using fresh perchloric acid. Centrifuge and pool the supernatants and make up to 100 mL.
5. Pipette out 0.1 or 0.2 mL of the supernatant and make up the volume to 1 mL with water.
6. 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 water.
7. Add 4 mL of anthrone reagent to each tube.
8. Heat for eight minutes in a boiling water bath.
9. 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.

READINGS

1. Hodge, J.E. and Hofreiter, B.T. (1962). In: *Methods in Carbohydrate Chemistry*, (Eds. Whistler, R.L. and Be Miller, J.N.), Academic Press, New York.
2. Thayumanavan, B. and Sadasivam, S. (1984). *Qual. Plant Foods Hum. Nutr.*, 34, p. 253.

1.8 Determination of Amylose

Starch is composed of two components, namely amylose and amylopectin. Amylose is a linear or non-branched polymer of glucose. The glucose units are joined by α -1-4 glucosidic linkages. Amylose exists in coiled form and each coil contains six glucose residues.

PRINCIPLE

The iodine is adsorbed within the helical coils of amylose to produce a blue-coloured complex which is measured colorimetrically.

MATERIALS

- Distilled ethanol.

- 1 N NaOH.
- 0.1% phenolphthalein.
- Iodine reagent*: Dissolve 1 g iodine and 10 g KI in water and make up to 500 mL.
- Standard*: Dissolve 100 mg amylose in 10 mL 1 N NaOH; make up to 100 mL with water.

PROCEDURE

1. Weigh 100 mg of the powdered sample, and add 1 mL of distilled ethanol. Then add 10 mL of 1 N NaOH and leave it overnight.
2. Make up the volume to 100 mL.
3. Take 2.5 mL of the extract, add about 20 mL distilled water and then three drops of phenolphthalein.
4. Add 0.1 N HCl drop by drop until the pink colour just disappears.
5. Add 1 mL of iodine reagent and make up the volume to 50 mL and read the colour at 590 nm.
6. Take 0.2, 0.4, 0.6, 0.8 and 1 mL of the standard amylose solution and develop the colour as in the case of sample.
7. Calculate the amount of amylose present in the sample using the standard graph.
8. Dilute 1 mL of iodine reagent to 50 mL with distilled water for a blank.

CALCULATION

$$\begin{aligned} \text{Absorbance corresponds to 2.5 mL of the test solution} \\ &= x \text{ mg amylose 100 mL contains} \\ &= \frac{x}{2.5} \times 100 \text{ mg amylose} = \% \text{ amylose.} \end{aligned}$$

NOTES:

1. The sample suspension may be heated for 10 min in a boiling water-bath instead of overnight dissolution.
2. The amount of amylopectin is obtained by subtracting the amylose content from that of starch.

READINGS

1. McCready, R.M.; Guggolz, J.; Siliviera, V. and Owens, H.S. (1950). *Anal. Chem.*, 22, p. 1156.
2. Juliano, B.O. (1971). *Cereal Sci. Today*, 16, 334.
3. Thayumanavan, B. and Sadasivam, S. (1984). *Plant Foods Hum. Nutr.*, 34, p. 253.

1.9 Estimation of Cellulose

Cellulose, a major structural polysaccharide in plants, is the most abundant organic compound in nature, and is composed of glucose units joined together in the form of the repeating units of the disaccharide cellobiose with numerous cross linkages. It is also a major component in many of the farm wastes.

PRINCIPLE

Cellulose undergoes acetolysis with acetic/nitric reagent forming acetylated cellodextrins which get dissolved and hydrolyzed to form glucose molecules on treatment with 67% H₂SO₄. This

glucose molecule is dehydrated to form hydroxymethyl furfural which forms green coloured product with anthrone and the colour intensity is measured at 630 nm.

MATERIALS

- *Acetic/Nitric reagent*: Mix 150 mL of 80% acetic acid and 15 mL of concentrated nitric acid.
- *Anthrone reagent*: Dissolve 200 mg anthrone in 100 mL concentrated sulphuric acid. Prepare fresh and chill for 2 h before use.
- 67% sulphuric acid.

PROCEDURE

1. Add 3 mL acetic/nitric reagent to a known amount (0.5 g or 1 g) of the sample in a test tube and mix in a vortex mixer.
2. Place the tube in a water-bath at 100°C for 30 min.
3. Cool and then centrifuge the contents for 15–20 min.
4. Discard the supernatant.
5. Wash the residue with distilled water.
6. Add 10 mL of 67% sulphuric acid and allow it to stand for 1 h.
7. Dilute 1 mL of the above solution to 100 mL.
8. To 1 mL of this diluted solution, add 10 mL of anthrone reagent and mix well.
9. Heat the tubes in a boiling water-bath for 10 min.
10. Cool and measure the colour at 630 nm.
11. Set a blank with anthrone reagent and distilled water.
12. Take 100 mg cellulose in a test tube and proceed from Step No. 6 for standard. Instead of just taking 1 mL of the diluted solution (Step 7) take a series of volumes (say 0.4–2 mL corresponding to 40–200 µg of cellulose) and develop the colour.

CALCULATION

Draw the standard graph and calculate the amount of cellulose in the sample.

READING

1. Updegroff, D.M. (1969). *Anal. Biochem.*, 32, p. 420.

1.10 Estimation of Hemicellulose

Hemicelluloses are non-cellulosic, non-pectic cell wall polysaccharides. They are regarded as being composed of xylans, mannans, glucomannans, galactans and arabinogalactans. Hemicelluloses are categorized under 'unavailable carbohydrates' since they are not split by the digestive enzymes of the human system.

PRINCIPLE

Refluxing the sample material with neutral detergent solution removes the water-solubles and materials other than the fibrous component. The left out material is weighed after filtration and expressed as Neutral Detergent Fibre (NDF).

MATERIALS *Neutral Detergent Solution*

Weigh 18.61 g disodium ethylenediamine tetraacetate and 6.81 g sodium borate decahydrate. Transfer to a beaker. Dissolve in about 200 mL of distilled water by heating and to this, add a solution (about 100–200 mL) containing 30 g of sodium lauryl sulphate and 10 mL of 2-ethoxy ethanol. To this add a solution (about 100 mL) containing 4.5 g of disodium hydrogen phosphate. Make up the volume to one litre and adjust the pH to 7.0.

 Decahydronaphthalene. Sodium sulphite. Acetone.**PROCEDURE**

1. To 1 g of the powdered sample in a refluxing flask add 10 mL of cold neutral detergent solution.
2. Add 2 mL of decahydronaphthalene and 0.5 g sodium sulphite.
3. Heat to boiling and reflux for 60 min.
4. Filter the contents through sintered glass crucible (G-2) by suction and wash with hot water.
5. Finally give two washings with acetone.
6. Transfer the residue to a crucible, dry at 100°C for 8 h.
7. Cool the crucible in a desiccator and weigh.

CALCULATION

Hemicellulose = Neutral detergent fibre (NDF) – Acid detergent fibre (ADF)

NOTE:

See Lignin for determining acid detergent fibre.

READING

Goering, H.D. and Vansoest, P.J. (1975). *Forage Fibre Analysis*, U.S. Deptt. of Agriculture, Agricultural Research Service, Washington.

1.11 Determination of Fructose and Inulin

Fructose, a keto-hexose (called as fruit sugar), is usually accompanied by sucrose in fruits like apple. Honey is a rich source of fructose.

PRINCIPLE

The hydroxymethyl furfural formed from fructose in acid medium reacts with resorcinol to give a red colour product.

MATERIALS

-
- Resorcinol reagent:*
- Dissolve 1 g resorcinol and 0.25 g thiourea in 100 mL glacial acetic acid. This solution is indefinitely stable in the dark.

- *Dilute HCl*: Mix five parts of conc. HCl with one part of distilled water.
- *Standard fructose solution*: Dissolve 50 mg of fructose in 50 mL water. Dilute 5 mL of this stock to 50 mL for a working standard.

PROCEDURE

1. To 2 mL of the solution containing 20–80 µg of fructose add 1 mL of resorcinol reagent.
2. Then add 7 mL of dilute hydrochloric acid.
3. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard and make up the volume to 2 mL with water. Add 1 mL of resorcinol reagent and 7 mL of dilute HCl as above.
4. Set a blank along with the working standard.
5. Heat all the tubes in a water-bath at 80°C for exactly 10 min.
6. Remove and cool the tubes by immersing in tap water for 5 min.
7. Read the colour at 520 nm within 30 min.
8. Draw the standard graph and calculate the amount of fructose present in the sample using the standard graph.

Inulin

Inulin is a polymer made of fructose units with β -2-1 linkage. It is found in onion, garlic and in many other plant parts.

Sample Extraction

Grind the sample and extract in 80% ethanol for six hours to remove free sugars. Dry the sample and take 500 mg in a 100 mL conical flask. Add 20 mL of water and heat it in a water bath at 90°C for 10 min. Collect the extract and then add 70 mL of water. Replace the flask for another 30 min with occasional shaking to dissolve the fructosan, then remove and cool it at room temperature. Combine the extracts and filter the solution if it is not clear and make up to 100 mL in a standard flask.

To estimate the inulin content in the extract follow the procedure given for fructose estimation. The amount of inulin is expressed in terms of fructose concentration.

READING

Ashwell, G. (1957). In: *Methods in Enzymol.* 3 (Eds. Colowick, S.J. and Kaplan, N.O.), Academic Press, New York, p. 75.

1.12 Estimation of Pectic Substances

Pectic substances abundantly exist in the middle lamella of the plant cells. There are three types of pectic substances—pectic acids, pectin and protopectin. Pectic acid is an unbranched molecule made up of about 100 units of D-galacturonic acid residues. The monomers are linked through 1–4 linkages. Pectin is an extensively esterified pectic acid. Several carboxyl groups exist as methyl esters. Pectic acid is water soluble whereas pectin forms a colloidal solution. Protopectin is a larger molecule than pectic acid and pectin. During ripening of fruits, conversion of protopectin into pectic acid and pectin takes place. The pectins in fruits vary in their methoxyl content and in jellying power.

Two methods are described below for the estimation of pectin: one gravimetric and the other, colorimetric.

I. Gravimetric Method

PRINCIPLE

Pectin is extracted from plant material and saponified. It is precipitated as calcium pectate by the addition of calcium chloride to an acid solution. After thoroughly washing to eliminate chloride ions, the precipitate is dried and weighed.

MATERIALS

- 1 N Acetic acid (Dilute 30 mL of glacial acetic acid to 500 mL with water).
- 1 N Calcium chloride solution: Dissolve 27.5 g anhydrous CaCl_2 in water and dilute to 500 mL.
- 1% Silver nitrate: Dissolve 1 g AgNO_3 in 100 mL water.
- 0.01 N HCl
- 0.05 N HCl
- 0.3 N HCl

PROCEDURE

1. Weigh 50 g of blended sample into a 1 L beaker and add 300 mL 0.01 N HCl. Boil for 30 min and filter under suction. Wash the residue with hot water and collect the filtrate.
2. To the residue add 100 mL 0.05 N HCl, boil for 20 min filter, wash and collect the filtrate.
3. To the residue now add 100 mL 0.3 N HCl, boil for 10 min, filter, wash and collect the filtrate.
4. Pool the filtrates. Cool and make to volume (500 mL).
5. Pipette out 100–200 mL aliquots into 1 L beakers.
6. Add 250 mL water and neutralize the acid with 1 N NaOH using phenolphthalein indicator. Add an excess of 10 mL of 1 N NaOH with constant stirring and allow it to stand overnight.
7. Add 50 mL 1 N acetic acid and after 5 min, add 25 mL 1 N calcium chloride solution with stirring. Allow it to stand for 1 h.
8. Boil for 1 to 2 min.
9. Filter through a pre-weighed Whatman No. 1 filter paper (see note 1).
10. Wash the precipitate with almost boiling water until the filtrate is free from chloride.
11. Test the filtrate with silver nitrate for chloride.
12. Transfer the filter paper with the calcium pectate, dry overnight at 100°C in a weighing dish, cool in a desiccator and weigh.

CALCULATION

The pectin content is expressed as % calcium pectate

$$\% \text{ calcium pectate} = \frac{\text{Wt. of calcium pectate} \times 500 \times 100}{\text{mL of filtrate taken} \times \text{Wt. of sample for estimation}}$$

NOTES:

The filter paper for Step No. 9 should be prepared as described below:

1. Wet the filter paper in hot water, dry in oven at 102°C for 2 h. Cool in a desiccator and weigh in a covered dish.
2. The theoretical yield of calcium pectate from pure galacturonic anhydride is 110.6%.

II. Colorimetric Method**PRINCIPLE**

Galacturonic acid is reacted with carbazole in the presence of H_2SO_4 and the colour developed is measured at 520 nm.

MATERIALS

- 60% Ethyl alcohol (Mix 500 mL 95% alcohol and 300 mL water).
- 95% Ethyl alcohol.
- Purified ethyl alcohol (Reflux 1 L of 95% ethyl alcohol with 4 g zinc dust and 2 mL conc. H_2SO_4 for 15 h and distill in all glass distillation apparatus. Redistill with 4 g zinc dust and 4 g KOH).
- 1 N and 0.05 N Sodium hydroxide.
- H_2SO_4 (Analytical grade).
- 0.1% Carbazole reagent:* Weigh 100 mg recrystallized carbazole, dissolve and dilute to 100 mL with purified alcohol.

PROCEDURE

1. Weigh 100 mg pectin (see notes section for the preparation of pectin) and dissolve in 100 mL of 0.05 N NaOH.
2. Allow it to stand for 30 min to deesterify the pectin.
3. Take 2 mL of this solution and make up to 100 mL with water.
4. Pipette out 2 mL of deesterified pectin solution and add 1 mL carbazole reagent. A white precipitate will be formed.
5. Add 12 mL conc. H_2SO_4 with constant stirring.
6. Close the tubes with rubber stopper and allow to stand for 10 min to develop the colour.
7. To set a blank add 1 mL of purified ethyl alcohol in the place of carbazole reagent.
8. Read the colour at 525 nm against blank, exactly 15 min after the addition of acid.

STANDARD

Weigh 120.5 mg galacturonic acid monohydrate (from a sample vacuum dried for 5 h at 30°C) and transfer to a 1 L volumetric flask. Add 10 mL 0.05 N NaOH and dilute to volume with water. After mixing, allow it to stand overnight. Dilute 10, 20, 40, 50, 60 and 80 mL of this standard solution to 100 mL with water. Take 2 mL of these solutions for colour developing and proceed as in the case of the sample. Draw a standard curve—the absorbance *versus* concentration.

CALCULATION

Read the concentration of the anhydrogalacturonic acid corresponding to the reading of the sample, and calculate as follows:

$$\% \text{ anhydrogalacturonic acid} = \frac{\mu\text{g of anhydrogalacturonic acid in the aliquot} \times \text{Dilution} \times 100}{\text{mL taken for estimation} \times \text{Wt. of pectin sample} \times 1,000,000}$$

NOTES:

1. Carbazole is recrystallized from toluene.
2. An alternate procedure adopted for colour development is as follows:
Take 12 mL of conc. H_2SO_4 in a test tube, cool in an ice-bath, and add 2 mL of the deesterified pectin solution and again cool. Heat the contents in a boiling water-bath for 10 min, cool to 20°C and add 1 mL of 0.15% carbazole reagent in purified ethyl alcohol. Allow it to stand for 25 ± 5 min at room temperature to develop the colour. Read the absorbance at 520 nm. Standards should also be treated similarly.

III. Extraction and Purification of Pectin

1. Blend the fresh sample. If the material is dry grind.
2. Transfer 100 g macerated sample (10 g dry tissue) to a pre-weighed 1 L beaker containing 400 mL water.
3. Add 1.2 g freshly ground sodium hexametaphosphate and adjust to pH 4.5.
4. Heat with stirring at $90\text{--}95^\circ\text{C}$ for 1 h. Check the pH in every 15 min and maintain at pH 4.5 with citric acid or NaOH. Replace water lost by evaporation at intervals. However, do not add water at the last 20 min.
5. Add 4 g filter aid and 4 g ground paper pulp. Filter rapidly through a fast filter paper coated with 3 g moistened fast filter aid.
6. Collect at least 200 mL of the filtrate in a preweighed container. Cool as rapidly as possible. Now, note the weight of the filtrate.
7. If the filtrate contains less than 0.2% pectin, concentrate the filtrate under vacuum to attain this concentration.
8. To three volumes of ethanol, isopropanol or acetone containing 0.5 N HCl, pour the cooled, weighed filtrate. The slurry should be at pH 0.7–1. Stir for 30 min.
9. Centrifuge or filter. Wash the precipitate with the same solvent containing HCl. Then, wash repeatedly with 70% alcohol or acetone until the precipitate is essentially chloride-free or the pH is above 4.
10. Dehydrate the precipitate further in 400 mL acetone. Dry overnight *in vacuo* with a slow stream of dry air passing through the oven.
11. Weigh the precipitate and use this pectin for analysis.
12. The dried pectin should be free from ammonia for which a small sample of the pectin is heated with 1 mL of 0.1 N NaOH and ammoniacal odour can be noticed or tested with a moistened litmus paper. If ammonium ions are present wash with acidified 6% alcohol, followed by neutral alcohol to remove the acid and dry.

READING

Ranganna, S. (1979). *Manual of Analysis of Fruit and Vegetable Products*, Tata McGraw-Hill Publ. Co. Ltd., New Delhi, p. 634.

1.13 Estimation of Crude Fibre

Crude fibre consists largely of cellulose and lignin (97%) plus some mineral matter. It represents only 60–80% of the cellulose and 4–6% of the lignin. The crude fibre content is commonly used as a measure of the nutritive value of poultry and livestock feeds and also in the analysis of various foods and food products to detect adulteration, quality and quantity.

PRINCIPLE

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

MATERIALS

- *Sulphuric acid solution* (0.255 ± 0.005 N): 1.25 g concentrated sulphuric acid diluted to 100 mL (concentration must be checked by titration).
- *Sodium hydroxide solution* (0.313 ± 0.005 N): 1.25 g sodium hydroxide in 100 mL distilled water (concentration must be checked by titration with standard acid).

PROCEDURE

1. Extract 2 g of ground material with ether or petroleum ether to remove fat (Initial boiling temperature 35–38°C and final temperature 52°C). If fat content is below 1%, extraction may be omitted.
2. After extraction with ether boil 2 g of dried material with 200 mL of sulphuric acid for 30 min with bumping chips.
3. Filter through muslin and wash with boiling water until washings are no longer acidic.
4. Boil with 200 mL of sodium hydroxide solution for 30 min.
5. Filter through muslin cloth again and wash with 25 mL of boiling 1.25% H₂SO₄, three 50 mL portions of water and 25 mL alcohol.
6. Remove the residue and transfer to ashing dish (preweighed dish W₁).
7. Dry the residue for 2 h at 130 ± 2°C. Cool the dish in a desiccator and weigh (W₂).
8. Ignite for 30 min at 600 ± 15°C.
9. Cool in a desiccator and reweigh (W₃).

CALCULATION

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

READING

Maynard, A.J. (Ed.) (1970). *Methods in Food Analysis*, Academic Press, New York, p. 176.

1.14 Estimation of Pyruvic Acid

Pyruvic acid or pyruvate is an important metabolic intermediate. It is greatly produced in the terminal step of glycolysis and funnels to TCA cycle for further oxidation for releasing the chemical energy. It can be determined following the procedure given below:

PRINCIPLE

The DNPH (2,4-dinitrophenyl hydrazine) reacts with pyruvate after the addition NaOH giving a brown colored hydrazone product which can be estimated colorimetrically at 510 nm.

MATERIALS

- *Phosphate buffer pH 9.4*
A: 0.2 M solution of monobasic sodium phosphate $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (27.8 g in 1000 mL).
B: 0.2 M solution of dibasic sodium phosphate (53.65 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L or 17.7 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1 L).
19 mL of A and 81 mL of B, diluted to a total of 200 mL.
Store in refrigerator.
- *Pyruvate, Standard*
Dissolve 22 mg sodium pyruvate in 100 mL water in a standard flask.
- *2, 4-Dinitrophenyl hydrazine (DNPH)*
Dissolve 19.8 mg of DNPH in 10 mL of conc. HCl and make to 100 mL with water.
Store it in an amber bottle at room temperature.
- *Sodium hydroxide 0.8 N*
Dissolve 16 g sodium hydroxide in one litre water.
- *Plant extract*
Grind 6 g of plant material in 15 mL of phosphate buffer. Centrifuge at 25,000 g for 15 min. Use the supernatant as plant extract.

PROCEDURE

- 1 Pipette out 50 μL , 75 μL , 100 μL , 150 μL , 200 μL of pyruvate standard solution and 0.5 mL, 1.0 mL, 1.5 mL, and 2.0 mL of sample extract into test tubes and make up the volume to 2.0 mL with phosphate buffer (pH 7.4).
2. Set a blank with no pyruvate solution.
3. Add 0.5 mL of DNPH solution to each tube.
4. Incubate at 37°C for 20–30 min.
5. Add 5 mL of NaOH solution to each tube, mix well and incubate for 10 min at room temperature.
6. Record the absorbance at 610 nm.
7. Draw the standard graph and calculate the amount of pyruvic acid present in the sample using the graph.