Qualitative Analysis of Carbohydrates

1. Solubility: The monosaccharides and oligosaccharides are readily soluble in water due to polar hydroxyl groups, which forms H-bonds with water. The polysaccharides owing to their large molecular weight, however, make translucent colloidal solutions.

2. Qualitative tests for Carbohydrates: While analyzing a sample containing a mixture of carbohydrates, particularly the sugars, several difficulties are encountered in their qualitative as well as quantitative analysis. These difficulties are attributed to their structural and chemical similarity and also with respect to their stereoisomerism. Therefore, during biochemical investigation it becomes necessary to establish whether a given sample contains carbohydrates or not. Several rapid tests are available to establish the presence or absence of a sugar or a carbohydrate in a sample. These tests are based on specific colour reactions typical for their group. In the laboratory, it is advisable to perform these tests with the individual rather than mixture of sugars. The sensitivity of these tests can be confirmed by using sugar solutions of different concentrations (0.1-1%).

A. General tests for carbohydrates: The most commonly used tests to detect the presence of carbohydrates in a solution are:

a) Molisch’s test: It is a group test for all carbohydrates, whether free or in combined form. Despite its limitations, it is routinely used to detect the presence of carbohydrates.

Principle: The reaction is based on the fact that concentrated H₂SO₄ catalyses the dehydration of sugars to form furfural (from pentoses) or hydroxymethyl furfural (from hexoses). These furfurals then condense with sulfonated alpha-naphthol to give a purple or violet coloured product. Polysaccharides and glycoproteins also give a +ve reaction. In the event of the carbohydrate being a poly- or disaccharide, the acid first hydrolyses it into component monosaccharides, which then get dehydrated to form furfural or its derivatives.

Reagents: i) Conc.H₂SO₄
          ii) Molisch’s reagent: Alpha-naphthol 5%(w/v) in 95% ethanol.

Procedure: Take 1-2 mL of unknown solution and add 2-3 drops of Molisch’s reagent and mix the contents. Incline the tube and carefully pour 1-2 mL of conc.H₂SO₄ down the side of
tube so that the acid forms a layer beneath the aqueous solution. The formation of a purple or violet ring or zone at the junction of two layers indicates the presence of carbohydrates.

**Precautions:**

i) Alpha-naphthol solution is unstable and should be prepared fresh.

ii) The conc.\(\text{H}_2\text{SO}_4\) should be added carefully along the sides of the test tube causing minimal disturbance to the contents of the tube.

**Limitations:**

- In addition to carbohydrates, furfurals as such, some organic acids, aldehydes and ketones also give this test. Secondly, a concentrated sugar solution may give a red colour instead of purple owing to charring action of acid.

b) ** Anthrone test:**

**Principle:** Anthrone reaction is another general test for carbohydrates. Its principle is same as that for Molisch’s test except that the furfurals and hydroxy-methyl furfurals give condensation products with anthrone that are bluish green in colour.

![Anthrone reaction](image)

Reagents: i) Anthrone reagent: 0.2\%(w/v) solution in conc.\(\text{H}_2\text{SO}_4\).

Procedure: Add about 2 mL of Anthrone reagent to about 0.5-1mL of the test solution in a test tube and mix thoroughly. Observe whether the colour changes to bluish green. If not, then examine the tubes again after keeping them in boiling water bath for ten minutes. A blue green colour indicates positive test.

B. **Specific tests for carbohydrates:**

a) **Iodine test for polysaccharides:** This test is performed to distinguish polysaccharides from mono- and disaccharides.

**Principle:** Iodine forms coloured adsorption complexes with different polysaccharides. These complexes are formed due to the adsorption of iodine on the polysaccharide chains. The intensity of the colour depends on the length of the unbranched or linear chain available for the complex formation. Thus, amyllose, the unbranched helical component of starch gives a deep blue colour and amylopectin, the branched component gives red colour because the chains do not coil effectively. Glycogen, which is also highly branched, gives red colour with iodine. This test is conducted in acidic or neutral solutions.
Reagents: i) Iodine solution: Prepare 2%(w/v) solution of KI in water to which add a few crystals of iodine until the solution assume a deep yellow colour.
   ii) Starch solution: Dissolve 1g starch in about 10-20mL boiling water and make the volume to 100mL with saturated sodium chloride solution.

Procedure: Take 2-3 mL of the test solution in a test tube and add 1-2 drops of dil.HCl. Mix and then add 1-2 drops of iodine solution. Mix and observe the colour change. Heat the tube and observe the colour again. Blue colour disappears on heating and reappears on cooling.

b) Tests based on reducing property of carbohydrates: Sugars possessing a free, or potentially free, aldehyde or ketone group act as reducing agents and this fact becomes the basis of the tests performed for distinguishing them from the non-reducing sugars. Such sugars have the property of readily reducing alkaline solutions of the metals like copper, bismuth, mercury, iron and silver. The aldo sugars are oxidized to the corresponding aldonic acids whereas the keto sugars give rise to shorter chain acids. If the alkaline copper solution is heated in the absence of reducing sugar, it forms black precipitate of cupric oxide:

\[
\text{Heat} \quad \text{Cu} (\text{OH})_2 \rightarrow \text{CuO} + \text{H}_2\text{O}
\]

In the presence of a reducing sugar, however, the alkaline solution of copper is reduced to insoluble yellow or red cuprous oxide:

\[
\text{Heat} \quad \text{Sugar} + 2 \text{Cu(OH)}_2 \rightarrow \text{Aldonic acid} + \text{Cu}_2\text{O} + 2 \text{H}_2\text{O}
\]

i) Fehling’s test: Rochelle salt acts as a chelating agent in this reaction:

\[
\text{Heat} \quad \text{CuSO}_4 + 2\text{KOH} \rightarrow \text{Cu(OH)}_2 + \text{K}_2\text{SO}_4
\]

\[
2\text{Cu(OH)}_2 + \text{Reducing Sugar} \rightarrow 2\text{Cu}_2\text{O} + \text{Aldonic acid}
\]

Reagents: i) Fehling’s solution A: Dissolve 69.38 g of Copper Sulfate in DW and make the volume to 1 L.
   ii) Fehling’s solution B: Dissolve 250 g NaOH in DW, add 346 g of Sodium Potassium Tartrate and make the volume up to 1 L.

Mix equal volumes of A & B solutions just before use because mixing causes deterioration with time.

Procedure: Add 1mL of Fehling’s reagent to 1mL of the test solution. Mix thoroughly and place the test tubes in boiling water bath. Formation of yellow or red precipitates of Cuprous Oxide indicates the presence of reducing sugar.

Note: i) In case of mild reduction, leave the solution to stand for 10-15 minutes, then decant the supernatant. A small amount of red or yellow precipitates may then be seen adhering to the inner side of the tube.
   ii) Fehling’s test is performed only alkaline solution.
   iii) Cuprous Oxide is dissolved by ammonia. Hence it is not possible to detect small quantities of reducing sugars in fluids saturated with ammonium salts e.g. urine.

ii) Benedict’s Test: Benedict modified the Fehling’s solution to produce an improved single reagent which is quite stable. Sodium Citrate functions as a chelating agent. It is very sensitive and even small quantities of reducing sugars(0.1%) yield enough precipitates.
Reaction:

\[
\begin{align*}
Na_2CO_3 + 2H_2O & \rightarrow 2NaOH + H_2CO_3 \\
2NaOH + CuSO_4 & \rightarrow Cu(OH)_2 + Na_2SO_4 \\
Cu(OH)_2 & \rightarrow CuO + H_2O \\
D-Glucose + 2 CuO & \rightarrow D-gluconic acid + Cu_2O \\
& \text{(Red ppt)}
\end{align*}
\]

**Reagents:** i) Benedict’s qualitative reagent: Dissolve 173g Sod. Citrate and 100g anhydrous Sod. Carbonate in about 800mL water by gently heating the contents. Then in a separate beaker dissolve 17.3g Copper Sulfate in about 100mL DW. Pour this solution slowly, with constant stirring into the Carbonate-Citrate mixture and make upto 1 L with DW.

Procedure: Add 0.5-1mL of the test solution to about 2mL of Benedict’s reagent. Keep the test tubes in boiling water bath. Observe the formation of green, orange, yellow or red precipitates which indicates the presence of reducing sugar in the given solution.

**Note:**

i) This test is especially suitable for the detection of reducing sugar in urine because it is more specific than Fehling’s test which is also positive for non-reducing substances such as urates present in urine.

ii) This is a semi-quantitative test.

iii) Barfoed’s Test: This test is performed to distinguish between a reducing mono- and disaccharide. Monosaccharides are more reactive reducing agents than disaccharides and thus react in about 1-2 min while the reducing disaccharides take 7-12 min to get hydrolysed in the acidic solution and then react. Thus, the difference in reducing property can be detected.

Reagents: i) Barfoed’s reagent: Dissolve 66.5 g of Cupric Acetate in about 900 mL DW. Boil and add 9 mL of Glacial Acetic Acid. Cool and make the volume to 1 L with DW and filter if necessary.

Procedure: Take 2-3 mL of Barfoed’s reagent in a test tube and add 1 mL of the given test solution. Keep the test tubes in boiling water bath for 1-2 min only. Then allow the tubes to cool down for a while. Thin red precipitates, at the bottom or sides of the tube indicates the presence of a reducing monosaccharide.
Note: i) The boiling should not be prolonged beyond 1-2 min, otherwise reducing disaccharides also respond to this test.
   ii) This test does not work in the detection of reducing sugar in urine owing to the presence of chloride ions.

iv) Picric Acid Test: It is another test for the detection of reducing sugars. The reducing sugars react with Picric Acid to form a red coloured Picramic Acid.

Reagents: i) Saturated picric acid: Dissolve 13 g Picric Acid in 100 mL DW, boil and cool.
   ii) Sodium Carbonate (10% solution).
Procedure: Add 1 mL of the above reagent to 1 mL of the test solution followed by 0.5 mL of 10% Sod. Carbonate solution. Heat the test tube in a boiling water bath. Appearance of red colour indicates the presence of reducing sugar in the solution.

c) Seliwanoff’s test for keto sugars

Principle: This test is a timed colour reaction specific for keto hexoses. Thus it is used to distinguish aldohexoses from ketohexoses. In the presence of HCl ketohexoses undergo dehydration to yield 4-hydroxy methyl furfural more rapidly than aldohexoses. Further these furfural derivatives condense with resorcinol to form a red coloured complex.

Reagents: i) Seliwanoff’s reagent: Dissolve 50 mg resorcinol in 100 mL dilute HCl (1:2).
Procedure: To about 2 mL of Seliwanoff’s reagent add 1 mL of the test solution and warm in a boiling water bath for 1 min. Appearance of a red colour indicates the presence of ketohexose (fructose).

Note: i) Aldohexoses e.g. glucose also react if boiling is prolonged because it is transformed into fructose by the catalytic action of acid.
   ii) Sucrose and inulin also give this test because these are hydrolysed by acid to give fructose.

d) Bial’s test for pentoses
Principle: This test is specific for pentoses and the compounds containing pentoses and thus useful for the determination of pentose sugars. Reaction is due to the formation of furfural in the acid medium which condenses with orcinol in the presence of ferric ions to give a blue green coloured complex.
Reagents: i) Bial’s reagent: Dissolve 1.5 g of orcinol in 100 mL of conc. HCl and add 20-30 drops of 10% Ferric Chloride solution to it. Prepare fresh.
Procedure: To about 2 mL of Bial’s reagent add 4-5 drops of test solution. Heat in a boiling water bath until bubbles of gas rise to the surface. Formation of green solution and precipitate indicates the presence of a pentose sugar.

**e) Test for sucrose**: This test is performed only when there is no precipitation in Barfoed’s test.

Principle: Sucrose present in the unknown solution is hydrolysed by acid to glucose and fructose. The resulting fructose formed in the solution is then tested by Seliwanoff’s reagent

Reagents: i) Conc. HCl
  ii) Seliwanoff’s reagent
  iii) Sodium Carbonate
Procedure: To about 2-3 mL of the test solution add 1-2 drops of conc. HCl and boil in a water bath for about 8-10 minutes. Then add about 5 mL of Seliwanoff’s reagent and again keep it in the water bath for 1 minute. Appearance of red colour indicates the presence of fructose which is the hydrolytic product of sucrose.
Note: Acid hydrolyzed sample after cooling and then neutralizing with Sodium Carbonate can be tested by Benedict’s reagent for reducing sugars.

**f) Mucic acid test for galactose**

Principle: This test is highly specific for galactose which is either independently present in solutions or obtained by the hydrolysis of lactose. Galactose is converted to Saccharic acid on heating with HNO₃ (a strong oxidizing agent). Mucic acid (galactaric acid) which is formed from galactose due to the oxidation of both aldehyde & primary alcoholic group at C1&C6. It is the only Saccharic acid which is insoluble in cold water and thus helps in the identification of galactose.

Reagents: i) Conc. HNO₃
Procedure: Take about 50 mg galactose and 50 mg glucose separately in test tubes. Add 1 mL DW and 1 mL conc. HNO₃ to each tube. Heat the tubes in a boiling water bath for about 1 hr. Add 5 mL DW and let the tubes to stand and cool slowly. Colourless needle like crystals will indicate the presence of galactose.
Note: Lactose will also give this test.

**g) Phenylhydrazine test / Osazone Test**

This test is used to differentiate the maltose and lactose

**Principle:**
An organic compound phenylhydrazine reacts with carbonyl carbon of sugar to form the osazones. These osazone crystals have yellow colour characteristics shapes and melting point, time of formation and solubility. The characteristics features of osazone are given in the following table:-
<table>
<thead>
<tr>
<th>Carbohydrate (Osazone)</th>
<th>Time of formation (Minutes)</th>
<th>Solubility in boiling water</th>
<th>Crystalline structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructosazone</td>
<td>2</td>
<td>Insoluble</td>
<td>Needle shape</td>
</tr>
<tr>
<td>Glucosazone</td>
<td>5</td>
<td>Insoluble</td>
<td>Needle shape</td>
</tr>
<tr>
<td>Galactosazone</td>
<td>20</td>
<td>Insoluble</td>
<td>Thorny ball shape</td>
</tr>
<tr>
<td>Maltosazone</td>
<td>30-45</td>
<td>soluble</td>
<td>Sunflower/Star shape</td>
</tr>
<tr>
<td>Lactosazone</td>
<td>30-45</td>
<td>soluble</td>
<td>Cotton ball/Powder puff shape</td>
</tr>
</tbody>
</table>

**Procedure:**
Take 7-8 ml of carbohydrate solution in a test tube and to this add a pinch of phenylhydrazine and double the quantity of sodium acetate and 10 drops of acetic acid. Dissolve by shaking and allow cooling slowly. Observe the shape of crystal under low power of microscope (10x).

**Observations and inference:**
The lactose forms powder puff shape crystals, maltose forms sunflower shaped or star shaped crystals, while the glucose and fructose form identical needle shaped crystals.