Determination of Reducing Sugar Content: Clinitest®, Benedict's Solution and the Rebelein Titration

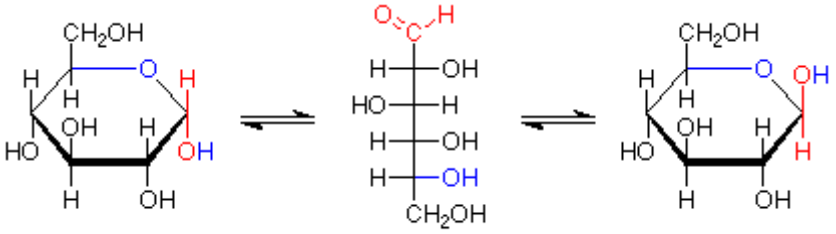
Chemical Concepts and Techniques:

The most important sugars present in wine and fruit juice are the hexoses - glucose and fructose. These are the sugars that yeast ferment to produce alcohol. They have the characteristic of being **reducing sugars**, as they contain functional groups capable of being oxidised and therefore causing reduction of other species under specific conditions.

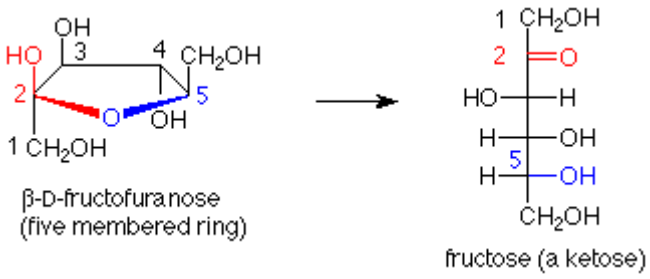
Structurally, reducing sugars must contain a free aldehyde or an alpha-hydroxy ketone capable of being oxidised. Hexose and pentose sugars in the free aldo- or keto- form or in equilibrium with these forms will fit in this category.

Hexoses:

Structures of **Glucose**:



Structures of **Fructose**:

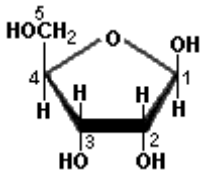


Pentoses:

The five carbon pentoses are also classified as reducing sugars and may contribute as much as 28% of the residual reducing sugar content of a dry table wine. Pentoses in

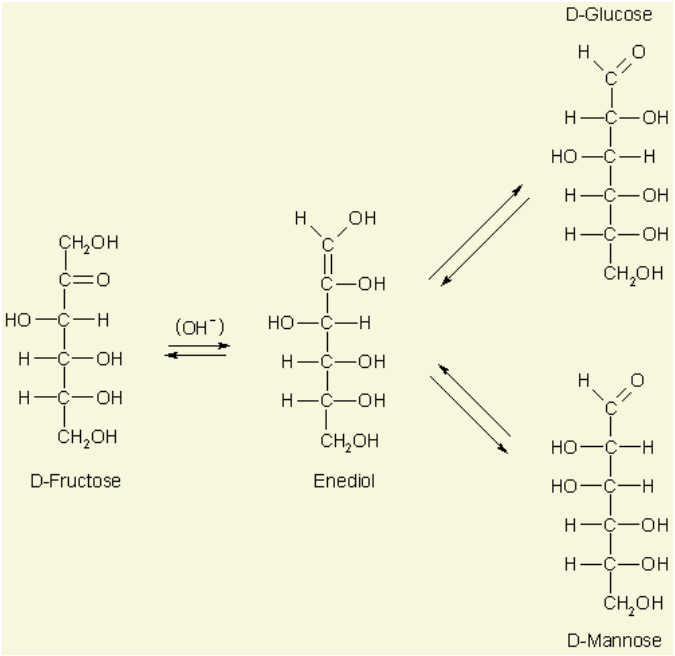
wine include ribose, arabinose and rhamnose. Most are not fermentable by yeast (although some can be utilised by bacteria).

Example of pentose structure (**Ribose**):



Hexoses and pentoses share the quality of existing in aqueous solution in two or more different forms (cyclic and non-cyclic) in equilibrium. The non-cyclic is the least common but it is present in small amounts in the wine or juice matrix. In alkaline solution sugars undergo decyclisation to yield the corresponding non-cyclic aldo- and keto- forms. This form has an aldehydic group at the end of the chain and is the group that is oxidised.

Technically, fructose should not behave as a reducing sugar under normal conditions However, fructose gives a positive reducing sugar test also because **fructose is converted to glucose and mannose under alkaline conditions**. The conversion can be explained by the keto-enol tautomerism – see below
The reduction of copper using fructose is not only to be attributed to the fact that the ketose is isomerised into an aldose. The treatment of fructose with alkali may cause decomposition of the carbon chain. More products with reducing capability are formed. Fructose is generally oxidised more rapidly and at lower temperatures than glucose.



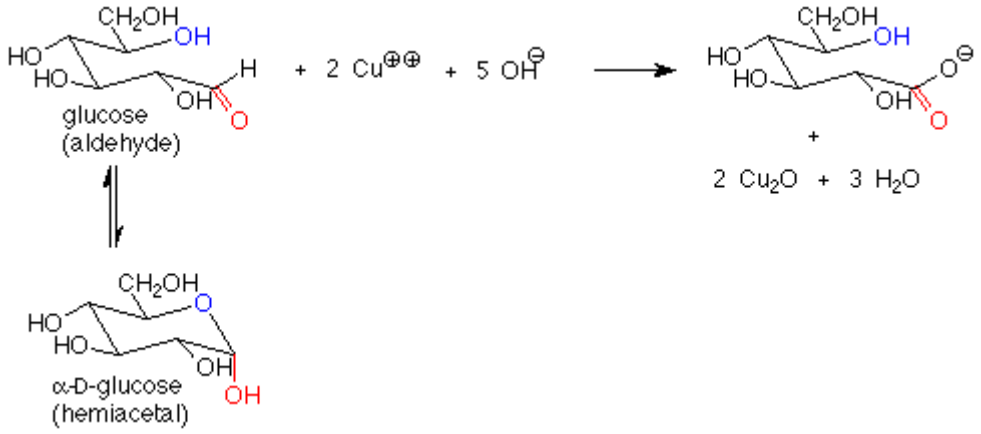
Keto-enol tautomerism - Fructose

Chemical methods for determining reducing sugars generally involve reaction with copper (II) under alkaline conditions. The sugars are oxidised as they reduce the copper from copper (II) to copper (I).

The reactions occurring are:

$$\mathbf{2Cu^{2+} + Reducing\ Sugars \rightarrow Cu^{+} + Oxidised\ Products}$$
$$\mathbf{2Cu^{+} + 2OH^{-} \rightarrow 2CuOH + Cu_2O + H_2O}$$

For example, with glucose as the reducing sugar the reaction is



The reduced forms of copper show a characteristic colour change from the blue of Cu(II) to yellow/orange/red of Cu(I), depending on the copper(I) salt formed.
The colour changes through this reaction may be complicated by the oxidation and subsequent degradation of the sugar compounds. Dependent variables for this reaction include the type and concentration of sugars present, the concentration of base and the temperature and time of reaction. Low temperatures and high alkali concentration favour the formation of red copper oxide precipitate.

Sodium potassium tartrate (Clinitest®/Rebelein) or **sodium citrate** (Benedict's Solution) are included in the reagent mix as it assists separation of the copper oxide precipitate while holding sugar oxidation products and unreacted Cu (II) in solution. The ligand (tartrate or citrate) forms a complex with the copper(II) ions and stops the copper(II) ion from precipitating with the hydroxide ions as copper(II) hydroxide. This would not be a very good oxidising agent. The trick is that the **copper(II) ions have to be alkaline to be oxidising but that they must be in solution not in a precipitate**
Cu (I) does not form a tartrate complex and precipitates readily from solution.

Benedict's solution contains sodium carbonate rather than sodium hydroxide as the alkali agent, which reduces the risk of hexose degradation due to the action of alkali.

However, Benedict's solution does not produce sufficient heat of reaction to oxidise all reducing sugars present and so the reaction vessel must be heated.
Clinitest® tablets contain sufficient sodium hydroxide, and the reaction mixture is sufficiently concentrated to generate their own heat of reaction.

For more accurate quantitative results the **Rebelein titration** is used. Copper is present in excess, and the residual copper (II) remaining after the reaction of sugar is reduced with **iodine** followed by a back titration against **sodium thiosulphate**. The reactions are as follows:

$$2Cu^{2+} + 2I^{-} \rightarrow 2Cu^{+} + I_2$$

and the back titration:

$$I_2 + 2S_2O_3^{2-} \rightarrow 2I^{-} + S_4O_6^{2-}$$

Equipment Required:

For Clinitest® or Benedict's Solution tests
Test tubes 20 x 150mm disposable glass acceptable
Pasteur pipettes or plastic transfer pipettes
Laboratory boiling water bath or
Large beaker and hot plate for hot water bath

For titration method
125mL or 150mL Erlenmeyer flasks
2mL bulb pipette
Heat source – hot plate or Bunsen burner
5mL bulb pipette
10mL bulb pipettes
50mL burette

Reagent Preparation:

Clinitest Tablets: *Commercial preparation from Bayer Healthcare. Available at some chemist or from Bayer Healthcare direct (cheaper).* ***Do not buy Clinistix strips instead*** *– they are specific to glucose and do not measure fructose levels.*
Distilled or deionised water

Benedict's Solution: *Dissolve 173g sodium citrate and 100g anhydrous sodium carbonate in 800 ml water, with warming if necessary. Dilute this solution to 850 ml with distilled water. Dissolve 17.3g of copper (II) sulphate pentahydrate in 100 ml water and add to the carbonate-citrate solution. Dilute the mixed solutions to 1.0L with distilled water.*
Glucose standards: *Do not use table sugar to prepare standards as sucrose is not a reducing sugar.*
100g/L stock solution*: Weigh 100g of glucose, dissolve in distilled water and dilute to volume. Solution should be stable for a few weeks if refrigerated. Prepare diluted standards on the day of use.*
10g/L: *Pipette 10mL of the stock, dilute to 100.0mL*
8g/L: *Pipette 8mL of the stock, dilute to 100.0mL*

6g/L: *Pipette 6mL of the stock, dilute to 100.0mL*
4g/L: *Pipette 4mL of the stock, dilute to 100.0mL*
2g/L: *Pipette 2mL of the stock, dilute to 100.0mL*

Rebelein Solution Z1: Add 1.0mL of concentrated sulphuric acid to 600mL of distilled water and mix thoroughly. Weigh 41.92g accurately of copper sulphate Pentahydrate CuSO4.5H2O. Dissolve the copper sulphate in some of the acid solution and quantitatively transfer to a 1.0L volumetric flask, using the acid solution as transfer rinse. Top up with the remaining acid solution, and dilute to volume with distilled water. Mix well.
Rebelein Solution Z2: Dissolve 250g (weighed accurately) sodium potassium tartrate in about 600mL distilled water. Carefully add 80g of sodium hydroxide to this solution. (Caution: heat generated!). Allow the mixture to cool. Quantitatively transfer the cooled mixture to a 1.0L volumetric flask and make to volume with distilled water and mix well. Store in a plastic bottle.
Rebelein Solution Z3: carefully add 100mL of a 1M sodium hydroxide solution to about 600mL of distilled water. Mix well. Weigh accurately 300g of potassium iodide. Dissolve the potassium iodide in a portion of the alkaline solution, transfer quantitatively to a 1.0L volumetric flask. Dilute to volume with distilled water. Mix well. Store in a plastic container.
Rebelein Solution Z4: Slowly and carefully add 175mL of concentrated sulphuric acid to around 825mL of cold distilled water. (Caution: heat evolved!) Mix well and allow to cool. Store in a sealed glass container.
Rebelein Solution Z5: Weigh 20g of potassium iodide and 10g starch in separate beakers. Add 10mL of 1M sodium hydroxide to approximately 500mL of distilled water and mix well. Use the hydroxide solution to dissolve the potassium iodide and starch in separate beakers. Transfer each solution quantitatively to the same 1.0L volumetric flask. Add any remaining sodium hydroxide solution and dilute to volume with distilled water. Mix well. Store in a plastic container.
Rebelein Solution Z6: Accurately weigh 13.78g of sodium thiosulphate Na2S2O3.5H2O and dissolve in distilled water. Quantitatively transfer to a 1.0L volumetric flask. Add 50mL of 1M sodium hydroxide. Dilute to volume with distilled water. Mix well. Store in a plastic container.
1M Sodium Hydroxide: Weigh 40g of sodium hydroxide. Add carefully and slowly with stirring to about 800mL of distilled water. (Caution: heat evolved!) After all the sodium hydroxide is dissolved, allow to cool and transfer to a 1.0L volumetric flask. Dilute to volume with distilled water. Mix well. Store in plastic.
Boiling granules

Method:

Estimation of reducing sugars using Clinitest® tablets:

1. Using a Pasteur pipette or plastic transfer pipette, add 10 drops of distilled water to a test tube.
2. Add 5 drops of the solution to be tested
3. Add 1 Clinitest® tablet and allow to react. (Caution: the reaction generates heat, do not touch the bottom of the test tube)
4. When bubbling has ceased, swirl the test tube to mix.

5. Compare the colour after 15 seconds with the supplied colour chart. Where the test colour is in between two colour plates, estimate the sugar content.

Estimation of reducing sugars using Benedict's Solution:

1. Set up a rack of 7 test tubes.
2. Using a hotplate and large beaker, prepare a boiling water bath (or use a laboratory boiling water bath if available)
3. Add 1mL of Benedict's solution to each test tube.
4. Add 5 drops of distilled water to the first test tube, and 5 drops of each of the glucose standard to the next 5 test tubes.
5. Add 5 drops of the sample to be tested to the final test tube.
6. Lightly stopper the test tubes and place in the boiling water bath for 5 minutes.
7. Remove and allow to cool.
8. Compare the sample solution colour to the standards and estimate the sugar content.

Determination of reducing sugars by Rebelein Titration:

1. Pipette 10.0mL of Z1 solution and 5.0mL of Z2 solution into an Erlenmeyer flask. Add a few boiling granules.
2. Pipette 2.0mL of wine sample into the flask.
3. Heat until boiling, allow to boil for 30 seconds. Remove the flask from the heat source and allow to cool to room temperature.
4. Add 10.0mL of each of Z3, Z4, Z5 solutions in that order.
5. Fill the burette with Z6 (standard thiosulphate solution)
6. Record the initial burette reading.
7. Titrate the mixture in the Erlenmeyer flask, shaking the flask well to mix throughout the titration.
8. The endpoint is cream. The solution will fade from yellow-brown (free iodine) to a blue-grey from the starch, before turning cream.
9. Record the final burette reading.
10. Carry out a reagent blank using 2.0mL of distilled water instead of wine, at the same time as the test sample. Treat the blank identically (steps 1-9).
11. Calculate the net titres for both the sample and the distilled water blank. The blank titre should be in the range 29-31mL. The sample titre will be less.
12. Calculate the reducing sugar content using the formula below. If no sample dilution was performed, the dilution factor = 1:

Reducing sugars (g/L)
= Dilution factor x [Blank titre(mL) – Sample titre(mL)]

Points to consider:

References:

Benedict, S. R. "The detection and estimation of reducing sugars", *J. of Bio. Chem.*, **5**: 485-487 (Mar 1908)

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Iland, Ewart, Sitters, Markides & Bruer, *Techniques for chemical analysis and quality monitoring during winemaking*, Patrick Iland Wine Promotions, 2000

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