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On the Determination of Reducing Sugars by Titration with the Sugar Solution

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The accuracy with which it is possible to carry out a quantitative determination of free aldoses and ketoses depends on the ratio between the free reducing groups and the glucoside bonds. The reason is that during the analysis itself there occurs a minimal rupture of glucoside bonds whereby new reducing groups are liberated.

The hydrolysis of the glucoside bonds is — other conditions being identical — dependent on the [OH^-]. This was already shown by Kjeldahl[1] with the disaccharides maltose and lactose. The inversion by OH^- of sucrose, which is strictly speaking a non reducing sugar, causes an increase of the »reduction power» with increasing pH, (Spengler, Tödt and Scheurer[2].) The same hydrolysis no doubt occurs to a greater or less extent with all oligo-and polysaccharides upon boiling in an alkaline solution.

The hydrolysis of glucoside bonds also depends on the oxidation-reduction-potential of the oxidizing agent. This was shown with partially broken down starch in our earlier paper »On the Determination of Reducing Sugars».[3]

Especially when the ratio between reducing groups and glucoside bonds is very small — as in the case of small amounts of invert sugar in cane sugar or in slightly broken down starch — the result of reductometric determinations may become problematic without proper corrections. It is possible to correct for the reducing effect of sucrose, but it is impossible to introduce corrections for the cleavage of glucoside bonds in a mixture of oligo- and polysaccharides such as they are formed by degradation of starch with α-amylases. The method involving an oxidizing agent with the lowest oxidation-reduction-potential in

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a medium with the lowest pH must in principle be preferable since it will give the most correct — although not the correct — result.

Of the oxidizing agents most generally employed in the determination of reducing sugars, the $Cu^{2+} \rightleftharpoons Cu^{+}$-system has by far the lowest oxidation-reduction-potential [3], and the use of copper methods must therefore, under otherwise identical conditions, cause the least oxidative cleavage of glucoside bonds. Of the different copper methods the preferable one must be the method in which the reaction mixture has the lowest pH and therefore causes the least hydrolytic cleavage of glucoside bonds.

Since copper methods for the determination of reducing sugars by direct titration with the sugar solution have gained a very widespread use, being so rapid, we have, bearing the mentioned points in mind, tried to improve the procedure. The reaction between reducing sugars and alkaline copper solutions is extremely slow at room temperature, and boiling is required to attain a reasonable velocity. But it is impossible to titrate a boiling sugar solution with alkaline copper solution. As there is a deficit of oxidizing agent, the alkali will alter the sugar before the oxidation has taken place. A direct determination is possible, however, by titrating the boiling copper solution with the sugar solution. In the determination of reducing sugars by direct titration a great advance was made by Lane and Eynon [4] when they discovered that methylene blue can be used as an indicator of the end point. Its use is based on the fact that it is reduced and decolorized by minute amounts of reducing sugars, but not so long as any cupric salt is present. Since, however, the reaction is accompanied by the precipitation of red Cu_2O, which remains suspended in the boiling medium, it may prove difficult, especially in poor light, to observe the decoloration of the methylene blue.

A method employing a reagent which contains complexly bound Cu^{2+} and $Fe(CN)_6^{4-}$ in a carbonate buffer solution has the following advantages over methods relying on Fehling's solution, which contains NaOH:

the hydrolytic cleavage of glucoside bonds in oligo- and polysaccharides becomes less,

the Cu^{+} is precipitated as almost white and very slightly soluble cuprous-ferrocyanide which does not interfere with the observation of the decoloration of methylene blue.

We have made no detailed investigation of the precipitate, cuprous-ferrocyanide, but probably it has the same composition as a potassium-cuprous-ferrocyanide investigated first by Messner [5], later by Bhaduri and Sarkkar [6] and lastly by Reihlen and Zimmermann [7], namely $K_2Cu_2Fe(CN)_6$, $7H_2O$.

Table 1. Reagents.

	g/l	moles/l	ml/ analysis	mmoles/ analysis
I $CuSO_4$, $5H_2O$	24.97	0.1	10.00	1.00
II K-Na-tartrate, $4H_2O$	141	0.5	10.0	5.0
II K_2CO_3	138	1.0	10.0	10.0
III $K_4Fe(CN)_6$, $3H_2O$	51	0.12	5.0	0.6
IV Methylene blue in water	2.0	—	—	—

Owing to the acid action of the Cu^{2+}ion, the mixing of the reagents in the prescribed quantities is accompanied by a conversion of 1/5 of the K_2CO_3 into $KHCO_3$, so that the ratio K_2CO_3:$KHCO_3$ becomes 4 : 1.

$$Cu^{2+} + 10\ CO_3^{2-} + 2\ H_2O \rightleftharpoons Cu(OH)_2 + 8\ CO_3^{2-} + 2\ HCO_3^-$$

The pH of such a buffer solution is about 10.4. The $CuSO_4$ and the tartrate solutions must be carefully mixed in order to prevent any precipitation of cupric-ferrocyanide on adding the ferrocyanide solution. If the prescribed ratio $Fe(CN)_6^{4-}$:$Cu^{2+} = 0.6 : 1$ is substantially reduced, Cu^+ will partly be precipitated as Cu_2O; if the ratio is increased no advantage is gained. In Fehlings solution the ratio Cu^{2+}:tartrate is 1 : 4.4. The ratio 1 : 5 is found to be very suitable for our purpose. K_2CO_3 is found to be preferable to Na_2CO_3. To replace copper tartrate with potassium-copper-carbonate in order to eliminate any organic substance [3] proves impossible, since Cu^{2+} is so loosely bound in the potassium-copper-carbonate that cupric-ferrocyanide is immediately precipitated. Cupric-ferrocyanide does not precipitate directly upon mixing of the reagents I, II and III since Cu^{2+} is complexly bound by tartrate. But the complex is not stable enough to prevent a slow precipitation. It is therefore preferable, before starting a series of analysis to mix I and II and first to add III immediately before heating. Any cupric-ferrocyanide precipitated will however dissolve during the heating.

STANDARDIZATION AND METHOD OF TITRATION

For accurate work one should standardize against the same pure sugar under exactly the same conditions as those under which one is working. In the case of glucose one should either use glucose which has been purified by recrystallization from absolute ethanol or anhydrous glucose C. P. All preparations must be dried at 100° C for 1—2 hours. Standard invert sugar solution is prepared by acid hydrolysis of sucrose C. P.[8, 9]. Since it undoubtedly will be impossible for many analysts to prepare maltose of the necessary purity, and

since even the best commercial preparations are not of the desired purity we have in the following table listed relative values for maltose, glucose being put at 100.

The standard solutions shall contain 1.200 g anhydrous glucose or invert sugar, or 2.000 g maltose hydrate per 1000 ml. The standard solution is poured into a 50 ml burette the tip of which is bent twice at right angles, to prevent too much heating of the burette. 10.00 ml of I (automatic pipette!) and 10 ml of II are pipetted into a 100 ml flat bottom flask. After shaking, 5 ml of III are added. Only the $CuSO_4$ solution has to be pipetted accurately. The flask is placed on a wire gauze over a Bunsen burner. The burette is arranged so that the tip of the stopcock extends a few mm into the neck of the flask. 24 ml of glucose solution are added. After shaking, the contents are heated to boiling. Glass bead! When the solution boils, a stopwatch is started. In order to exclude atmospheric oxygen the reaction mixture must at no time cease boiling. After boiling for 2 minutes, 2—3 drops of methylene blue are added, and at intervals of about 15 seconds 3—4 drops of the sugar solution. When all Cu^{2+} has been reduced to Cu^{+}, the methylene blue is reduced whereby the blue colour disappears. The less the overtitration, the slower the colour change will take place. The decoloration is so distinct that the titration can be repeated with but a few drops' deviation. The titration must be completed within a boiling time of 3 minutes $\pm$ 15 seconds. The determination is repeated another 3 times, adding immediately 0.2 ml less sugar solution than the total consumption. The number of milligrams corresponding to 10.00 ml 0.1 *M* $CuSO_4$ is calculated from the mean of the last three determinations. The corresponding values for other sugars may be obtained by multiplying with the factors given in the following table. (The sugars used are the same as described in our previous paper [3]). If great accuracy is desired, the standardization must, as already mentioned, be made against the same sugar as the one which it is desired to determine, and must be repeated each time one of the components of the reagent is renewed. Ordinarily, though, it will only be necessary to standardize against glucose when the $CuSO_4$ solution is renewed.

Table 2. The reduction equivalent (RE) of various sugars.

	RE	Glucose = 100
Glucose, anhydrous	6.0	100
Invert sugar	5.9	101
Galactose	5.2	117
Maltose, anhydrous	7.3	159
Lactose, hydrate	7.9	152

DETERMINATION

To avoid any appreciable change of pH in the reaction mixture, it is absolutely necessary to neutralize acid or alcaline sugar solutions. If the approximate concentration of the sugar in the sample is unknown, one should proceed by the incremental method of titration. 10 ml of the sugar solution are added immediately and the mixture is heated to boiling. Glass bead! After boiling for 1 minute, the sugar solution is added in portions of several milliliters at intervals of 15 seconds. When the blue colour of the copper has almost disappeared, 2—3 drops of methylene blue are added, and the addition of sugar solution continues drop by drop until the blue colour of the indicator disappears. The actual analysis is now arranged on the basis of one or several such preliminary experiments. Since the oxidation of the sugar — though to a slight degree — is dependent on the reaction volume (see later), the final volume must be the same as in the standardization. For higher precision the sugar solution in question must either be suitably diluted or one must add enough water before boiling to make the final volume of the reaction mixture total 50 ml. Finally, care must be taken to have the definitive determination completed within a total boiling time of 3 minutes $\pm$ 15 seconds, and otherwise the analysis has in all details to follow the same procedure as that of the standardization.

The standard error of the titration is about 0.2 ml, corresponding to a relative error of about 0.8 %.

DISCUSSION

The effect of the reaction volume has only been investigated in the case of glucose, and is evident from the following figures:

Total reaction volume in ml	Titration with ml	RE
45	20	6.08
50	25	6.05
55	30	6.03

The reduction equivalent, RE, decreases with increasing reaction volume. If the total reaction volume is kept between 45 and 55 ml, one may, without committing too great errors, reckon with a constant RE. If particular accuracy is required, the final volume should be as close to 50 ml as possible.

The oxidation of the sugars does not proceed stoichiometrically. As in other methods employing Cu^{2+} or $Fe(CN)_6^{3-}$, galactose is oxidized less than glucose. Glucose and invert sugar reduce 6 equivalents Cu^{2+} per molecule.

This corresponds, theoretically, to a break-down to a pentonic acid. The disaccharides maltose and lactose show a larger consumption of Cu^{2+}, owing to hydrolysis of the glucoside bonds.

During the last year the method has been employed in hundreds of routine analyses at the Tuborg Laboratory and at the Laboratory of Løvens kemiske Fabrik, Copenhagen.

SUMMARY

A rapid method of determination of reducing sugars by titration with the sugar solution is described. A reaction mixture is chosen which gives the lowest hydrolytic and oxidative cleavage of glucoside bonds in oligo- and polysaccharides. The reaction mixture contains Cu^{2+}, tartrate and $Fe(CN)_6^{4-}$ in a carbonate buffer; pH about 10.4. Cu^{+}, formed by reaction of Cu^{2+} with the sugar, is under these conditions precipitated as almost white potassium-cupro-ferrocyanide, which does not interfere with the observation of the decoloration of methylene blue indicator.

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