A STUDY OF THE METHODS OF ESTIMATION OF CARBOHYDRATES, ESPECIALLY IN PLANT-EXTRACTS.

A New Method for the Estimation of Maltose in presence of other Sugars.

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During an investigation of the carbohydrates present in the mangold leaf, now in progress at the Rothamsted Experimental Station, we have made a special study of the methods of analysis applicable in such cases and have detected certain errors which are likely to occur in this class of work; although the investigation is still incomplete, it seems advisable to describe the methods which we have adopted to obviate these as far as possible.

The study of the accurate estimation of sugars in the complex mixtures occurring in plants may be said to date from the important memoir of Brown and Morris in 1893 entitled "A Contribution to the Chemistry and Physiology of Foliage Leaves[1]"; a few years later Brown, Morris and Millar published their tables[2] of the reducing power of pure dextrose, laevulose, maltose and invert sugar under certain defined conditions with varying concentrations of the sugars and determined the specific rotatory power of pure maltose. Quite recently Parkin[3] in studying the carbohydrates of the snowdrop leaf, which does not contain starch or maltose, tested certain points of analytical procedure necessary in dealing with plant-extracts.

Gravimetric Methods of Estimating Sugars.

In the estimation of mixed sugars such as occur in plant extracts undoubtedly the most satisfactory gravimetric method is to work under

[1] *Trans. Roy. Soc.*, 1893, **63**, 604. [2] *Trans. Roy. Soc.*, 1897, **71**, 72—123.
[3] *Biochem. J.*, 1912, **6**, 1.

the conditions laid down by Brown, Morris and Millar, using the tables already referred to. We have tested the accuracy of these tables by means of carefully purified specimens of dextrose, laevulose, cane sugar and maltose, dried *in vacuo* at 105—106° (except the laevulose which was heated at 75—80° only) under the conditions adopted by Brown, Morris and Millar[1], and have found a satisfactory agreement—that is to within 1 milligram on the weight of copper weighed, which Brown, Morris and Millar regard as the probable degree of accuracy of their method; when 0·20 to 0·35 grm. of copper is weighed the error is therefore within 0·5 per cent.

Possible Error in the Gravimetric Method due to the Action of Fehling Solution on Asbestos.

In our early experiments with purified sugars it was frequently found that the copper oxide weighed in duplicate experiments differed by very large amounts—often not by a mere milligram but by a centigram or more. It was at first thought that this was due to the use of a layer of asbestos in the Soxhlet or Gooch crucible which was insufficiently thick to retain the whole of the cuprous oxide during the filtration, although no oxide was visible in the filtered Fehling solution. On using a much thicker layer of asbestos ($\frac{1}{2}$" to 1"), however, as is usual in sugar-works

[1] Ost (*Chem. Zeit.*, 1897, **21**, 613) in reply to Brown, Morris and Millar's criticism of the value he had assigned to the specific rotatory power of maltose, based on a method in which the hydrated substance was weighed and the rotation for the anhydrous substance derived from this, threw doubt on their values, alleging that, when maltose is heated to a temperature above 95°, even *in vacuo* it begins to decompose, although without showing any external signs of change, the decomposition being indicated only by a falling off of rotatory power. According to Ost the values of the solution-densities and reducing powers given by Brown, Morris and Millar would therefore be only approximately correct ("können principiell nicht als exakt gelten") as slight change had probably occurred in the material used (dextrose, laevulose as well as maltose). This statement is reproduced in von Lippmann's *Chemie der Zuckerarten* (3rd edition, p. 1468), where preference is given to Ost's values of solution-densities.

We have not specially investigated this point but may point out that Ost makes use of a strange argument in support of his case, and refutes himself, when he maintains that the indication of decomposition having occurred in maltose is a *lowering* of the specific rotatory power and yet contends that Brown, Morris and Millar's *high* value for the specific rotation at 15·5° (137·93) as compared with his own (137·46) was due to this cause; had decomposition occurred a *lower* value would be expected. E. Schulze (*Chem. Zeit.*, 1902, **26**, 7) on the other hand maintains that maltose hydrate can be completely dehydrated at 100° in a current of air without any decomposition occurring, and Ling, Eynon and Lane (*7th International Congress App. Chem.*, 1910, **1**, 137) confirm Brown, Morris and Millar's tables of solution-densities. Our own results were also always in full accord with them.

(see Frühling, *Anleitung zur Zuckerindustrie*, 7th edition, 1911, p. 112; von Lippmann in *Chem. Tech. Untersuch.-Methoden*, 5th edition, III. p. 403), it was found that the differences were thereby considerably *increased*. An example will show the character of the results obtained. Using successive portions of 25 c.c. of the same invert sugar solutions (prepared from pure cane sugar) otherwise treated in exactly the same way:

Soxhlet A (previously used for two charges) gave 0·3453 grm. CuO. Soxhlet B (also used for two charges) gave 0·3410 grm. CuO, but Soxhlet C (freshly packed with $\frac{3}{4}''$ of asbestos and ignited) gave 0·3060 grm. CuO.

In this case the difference between the result C and results A and B is from 0·035 to 0·040 grm.

When thinner layers of asbestos were used in the Soxhlet tube smaller differences were observed, and it was found that by using approximately equal thicknesses of asbestos in different tubes results *differing in duplicate by not more than a milligram* could easily be obtained although these were far from being correct. An example may be given:

Taken 25 c.c. solution = 0·1356 grm. invert sugar.

Thin asbestos layer.

1. CuO = 0·3215 grm = 0·1343 invert sugar = 98·9 %
2. CuO = 0·3211 ,, = 0·1341 ,, ,, = 98·7 ,,
3. CuO = 0·3188 ,, = 0·1330 ,, ,, = 98·0 ,,

Thicker asbestos. 25 c.c. of same solution.

1. CuO = 0·3090 grm = 0·1287 invert sugar = 94·8 %
3. CuO = 0·3100 ,, = 0·1291 ,, ,, = 95·1 ,,

Herein lies probably the principal cause of the not infrequent disagreement between analysts dealing with sugar materials and the doubts which have been expressed as to the accuracy of gravimetric methods. The analyst, using approximately the same thickness of asbestos throughout his experiments, would obtain duplicates in close agreement although the actual result might be considerably at fault.

We became aware of this source of error by observing that, in the case of the particular variety of asbestos we were using (Kahlbaum's specially prepared long-fibre asbestos for Gooch crucibles), the loss of weight experienced with Fehling's solution was particularly pronounced. This asbestos, which when washed with 200 c.c. of boiling water containing 40 c.c. of concentrated nitric acid and subsequently with 300 c.c. of boiling water, showed practically no change in weight (not more than

0·0002 grm.), yet lost considerably on passing through it 50 c.c. of the hot Fehling solution as in an ordinary "Blank" made to determine the correction for self-reduction of the Fehling solution in Brown, Morris and Millar's method. Prior ignition of the asbestos did not alter this property. With thick layers of asbestos ($\frac{1}{2}$" to $\frac{3}{4}$") the loss of weight so caused amounted to several centigrams. When successive 50 c.c. quantities of Fehling solution were used, the loss of weight experienced with each successive charge rapidly diminished, and after about the third "Blank" there is the normal *gain* of 0·0015 to 0·0030 grm. CuO, corresponding with the correction necessary to introduce for self-reduction of the Fehling solution (Brown, Morris and Millar, *Trans.* 1897, **71**, 96). It is clear that there is present in the asbestos, as an impurity, some easily decomposable silicate which is gradually dissolved away by the strongly alkaline Fehling solution. The following numbers illustrate this; they were obtained with layers $\frac{1}{2}$" to $\frac{3}{4}$" thick of the asbestos.

		Soxhlet 1	Soxhlet 2	Soxhlet 3
1st.	50 c.c. Fehling	−0·0328	−0·0300	−0·0465
2nd.	,, ,,	—	−0·0020	−0·0026
3rd.	,, ,,	—	+0·0021	+0·0016

− indicates loss of weight, + gain of weight.

We find that by digesting the asbestos during 30 minutes with boiling 20 °/₀ sodium hydroxide, and then thoroughly washing with water, an asbestos is obtained which is quite suitable for use in a Gooch crucible or Soxhlet tube as it *undergoes no further perceptible loss when hot Fehling solution is passed through it.* Such asbestos gives the normal increase of 0·0015 to 0·0028 as the correction to be applied for the self-reduction of the Fehling solution, and on passing through it 100 c.c. of boiling 5 °/₀ sodium hydroxide the loss of weight is less than 0·0001 grm.

Different samples of asbestos differ widely in their behaviour with boiling Fehling solution or sodium hydroxide; we have not met with an asbestos which is completely unaffected by these solutions, sample C given below being the best we have as yet obtained. In most cases, the loss is very considerable. In the following examples the asbestos had previously been boiled with hydrochloric and nitric acids, and showed no loss under this treatment. The numbers show the percentage loss of weight on boiling during 30 minutes with 10 °/₀ sodium hydroxide.

A.	White, long fibre	loss 6·94 °/₀
B.	White, long fibre	,, 5·85 ,,
C.	White	,, 0·18 ,,
D.	Blue	,, 0·75 ,,

Although the error that may arise in this way is often very considerable, none of the standard works of analysis we have consulted refers to the necessity of a preliminary treatment of the asbestos with alkali, although von Lippmann (*Chem. Tech. Untersuch.-Methoden*, III. 403) quotes a reference to a paper by Casamajor (*Zeitsch. anal. Chem.*, **22**, 552) in which the ordinary treatment of asbestos with *acids* before use is recommended. In Lippmann's *Chemie der Zuckerarten* (3rd edition, p. 594) the necessity of using asbestos of "guter, reiner und langfaserigen Qualität" is stated and reference made to Maercker (*Oester. Ung. Zeit. für Zuckerind. und Landw.*, **7**, 699; *Zeitsch. Ver. Deutsch. Zuckerind.*, **28**, 797), Killing (*Zeit. angew. Chem.*, 1894, 431) and Elion (*Rec. Trav. Chem.*, 1896, **15**, 116), who had previously pointed out the necessity of using "pure" asbestos. Killing in 1894 went so far as to state that suitable asbestos bids fair to become very scarce or perhaps altogether to disappear from the market and recommended a return to the old method of collecting the cuprous oxide on filter paper.

Weighing the Copper Precipitate from the Fehling Solution.

Numerous papers have been published (cp. Lippmann, *Chemie der Zuckerarten*, 3rd Aufl. 596—598) in which the necessity of reducing the cuprous oxide to metallic copper and weighing as such have been emphasised; the simpler operation of oxidation has been frequently stated to give erroneous results owing to the difficulty of ensuring complete oxidation, the possibility of reduction owing to the action of the flame gases on the cupric oxide, and other causes[1]. Elion for instance (*Zeit. angew. Chem.*, 1890, 325) states that whereas by weighing as copper in sugar estimations he obtained close agreement in four experiments (0·2734, 0·2730, 0·2730, 0·2730), on weighing as cupric oxide he obtained widely discordant results (0·3328, 0·3068, 0·2962, 0·2854).

We have found, on the contrary, that the conversion of cuprous into cupric oxide is practically complete when the following precautions are observed.

1. The precipitate of cuprous oxide is collected in a porcelain Gooch crucible (with sufficient thickness of asbestos), finally washing

[1] In Moissan's *Traité de Chimie Minérale* (Vol. v. p. 459) the oxidation of cuprous oxide on heating in air is said to be incomplete on the authority of Grunhüt (*Chem. Zeit.*, 1894, **18**, 447), Nihoul (*Chem. Zeit.*, 1894, **18**, 831) and Killing (*Zeit. angew. Chem.*, 1894, 431). As we shall show, this is erroneous, unless the blow-pipe flame is used.

with alcohol and ether in the ordinary way, dried at 100°, and after placing in a No. 1 porcelain crucible to act as a container and prevent direct contact of the flame, heated in a fairly powerful flame from a ½" Teclu burner or Fletcher Argand, until the weight is constant; generally, we simply keep the crucible over the flame during half an hour, allow to cool in the desiccator at least one hour, weigh and again heat another 30 minutes. The increase in weight in the second heating seldom exceeds 0·0005 grm.

2. *The blow-pipe should not be used*, as even when the Gooch is shielded by an outer crucible low results are obtained, probably owing to slight dissociation of the cupric oxide at the high temperature (compare Debray and Joannis, *Compt. Rend.*, 1884, **99**, 383 and 688). Its use, too, is more tedious than that of a Teclu burner.

We append a few examples illustrating this. We have never observed the hygroscopic tendency which is sometimes attributed to cupric oxide.

	Wt. Cu_2O	Wt. CuO	Ratio	Remarks
On blowpipe	1. 0·3730	0·4135	1·109	Heated on blow-pipe till constant in weight
	2. 0·3735	0·4140	1·109	
	3. 0·3864	0·4270	1·105	
	4. 0·2587	0·2857	1·105	
On Fletcher or Teclu burner	1. 0·3864	0·4297	1·112	
	2. 0·3767	0·4188	1·112	
	3. 0·3849	0·4271	1·110	
	4. 0·2410	0·2680	1·112	

The theoretical ratio $\frac{2CuO}{Cu_2O} = 1{\cdot}112$ (Cu = 63·57).

A similar ratio $\frac{CuO}{Cu_2O}$, viz. 1·110 to 1·112, is always obtained when dealing with purified sugar solutions, but when the ordinary solutions obtained from plant extracts (previously treated with basic lead acetate and then deprived of the excess of lead) are used, the ratio is considerably lower (1·095—1·106); whilst it is lowest in estimating the reduction of solutions which have been treated with invertase or yeast, or starch solutions treated with malt extract (see below).

The recommendation is frequently made to weigh the cuprous oxide as such, after drying at 100°, and this method is prescribed for example in the Official and Provisional Methods of Analysis of the Association of Official Agric. Chemists (*U.S. Dept. Agric., Bull.* 107). (Compare Allen's

Commercial Organic Analysis, Vol. I. p. 325.) Whilst this course is safe in the case of solutions of pure sugars, it involves large error when dealing with solutions derived from plant or animal extracts, or when inversion or hydrolysis has been effected by an enzyme, or after fermentation by yeasts, even though alumina cream has been subsequently used to clear the solutions. In such cases the cuprous oxide invariably contains some organic matter, which burns away when it is oxidised, so that the ratio $\frac{CuO}{Cu_2O}$ is thereby diminished[1]; it is probable that, in dealing with yeasts, invertase, etc., the cuprous oxide precipitated contains traces of copper salts of amino-acids as well as the colloidal organic matter carried down by adsorption. In such cases the cupric oxide weighed would be slightly higher than that actually due to reduction only; but the numerous experiments we have made with yeasts, invertase preparations, diastase, etc., would lead us to think that this error is relatively small, and negligible in comparison with other errors of sampling, etc., in this class of work.

We have made it a rule in our work to weigh both the cuprous and cupric oxide precipitate and calculate in each case the ratio $\frac{CuO}{Cu_2O}$. This throws an interesting light on the character of the solution dealt with, and on the purity of the cuprous oxide weighed. *But all calculations for sugars are based only on the weight of cupric oxide actually obtained.* We usually collect many successive charges in the same Gooch crucible one after the other; the same Gooch can be used for 10—20 charges without cleaning, the cuprous oxide from a fresh experiment being collected on top of the previous charge of cupric oxide when the latter is constant in weight.

Description of Heating Bath used.

In order to facilitate the analyses we have made use of the form of water bath shown in the sketch; it consists of a 10″ enamelled iron saucepan, 4½″ deep, into which a false bottom of copper plate is placed, so as to afford a convenient support for the beaker flasks used. The cover of the bath is made of copper and consists of two halves, each

[1] Using invertase in the form of autolysed yeast the ratio of $\frac{CuO}{Cu_2O}$ weighed varies between 1·060 to 1·090 according to the quantity used; with prepared diastase in starch transformations it is slightly higher (1·105—1·106) and with malt extract it again gives low figures similar to those obtained with autolysed yeast.

perforated with two $2\frac{1}{2}''$ holes, the edge of the plate being turned down so as to fit over the bath. Each half of the cover can be lifted off separately so as to admit the beaker flask containing the Fehling solution. We find that 250 c.c. conical beaker flasks, with a top diameter $2\frac{1}{4}''$ and bottom diameter $2\frac{7}{8}''$, give results in close agreement with the Brown, Morris and Millar tables, and are much more convenient for manipulation and heating than ordinary beakers.

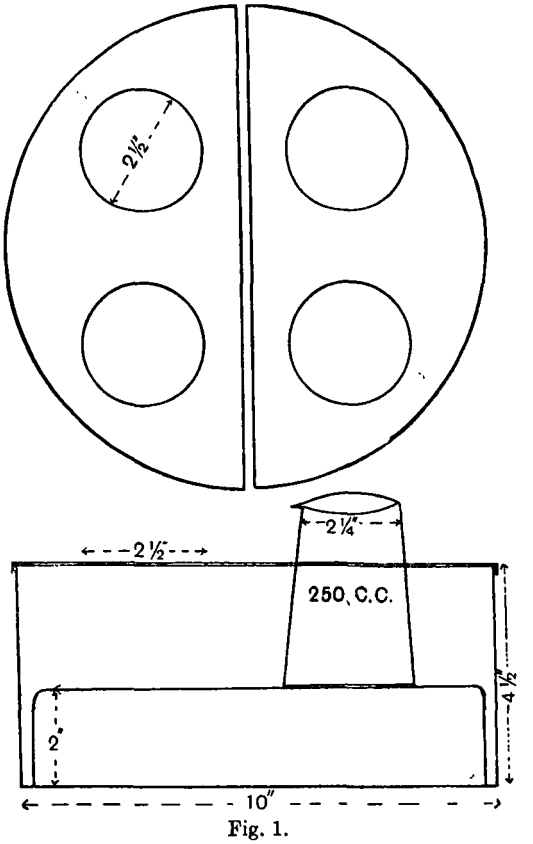

Fig. 1.

Volumetric Methods.

We have spent some time in studying two of the volumetric methods which are generally regarded as the most accurate, viz. that due to Ling and Rendle (*Analyst*, 1905, **30**, 182; compare Ling and Jones, *Analyst*, 1908, **32**, 160), who make use of Fehling solution with ferrous thiocyanate as indicator; and the volumetric permanganate method as carried out by Bertrand (*Bull. Soc. Chim.*, 1906, [iii.], **35**, 1285).

Our sugar solutions were prepared with carefully purified dextrose (recrystallised several times from both methyl and ethyl alcohols) $[\alpha]_D^{15^\circ} = 52{\cdot}6^\circ$, maltose $[\alpha]_D^{15^\circ} = 138{\cdot}0^\circ$ and invert sugar, prepared from cane sugar; in some cases the solutions were made by drying a known weight of the sugar *in vacuo* at 106° (toluene bath) in a small glass flask fitted with a ground-in stopper, and connected with a flask containing phosphoric anhydride, and then dissolving the anhydrous sugar in water and making to a known volume at 15·0°. In others, the concentration was checked by density determinations, using Brown, Morris and Millar's tables[1].

Ling-Rendle-Jones Method.

Dextrose. 1. Taken 0·1975 grm. dextrose per 100 c.c. Found 0·1981 grm.

2. Taken 0·2303 grm. dextrose per 100 c.c. Found 0·2300 grm.

Cane Sugar. (Inverted according to Ling and Rendle.)
Taken 0·2105 grm. per 100 c.c. Found 0·2106 grm.

Maltose. Taken 0·2059 grm. per 100 c.c. Found 0·2056 grm.

These confirm the view generally held that this method is accurate to at least 1 part in 300, or about 0·3 %, and we regard it as the most nearly accurate volumetric method at present in use. It is in our opinion preferable to the Bertrand method, both on the ground of accuracy and rapidity.

Bertrand's Method. This method, which consists in dissolving the cuprous oxide in an acid solution of ferric sulphate and titrating the resulting solution with permanganate, had in principle already formed the subject of at least six papers and was provisionally adopted by the U.S. Dept. of Agriculture at least as far back as 1899 (*Bulletin* 46, Bureau of Chemistry), prior to Bertrand's publication of convenient tables, which led to its being widely used in biochemical work.

We have carried out a large number of experiments with this method of which the following are typical: the solutions used were made from carefully purified sugars, and had given good results with the Brown, Morris and Millar gravimetric method and the Ling volumetric method. Bertrand's details were followed precisely.

Dextrose. Taken 0·0658 grm. dextrose.

Found in five experiments average 0·0648 grm., that is an error of

[1] In all cases our numbers refer to true c.c. at 15° C.

about 1·5 per cent. It is noteworthy that Bertrand gives his $[\alpha]_D = 52{\cdot}0°$, whereas the more nearly correct value is probably 52·7°.

Cane sugar. (Invert sugar.)

We obtained, using 0·0961 grm. invert sugar, results which were from 3 to 5 % low. The cause for this probably lies in the fact that the conditions for inverting cane sugar employed by Bertrand, namely heating 4·75 grms. of cane sugar with 50 c.c. of 2 % hydrochloric acid for 10—15 minutes at 100°, invariably leads to the decomposition of laevulose, which is shown by the production of a pronounced yellow colour in the solution; this is visible after 3 to 4 minutes heating. It is not safe to heat cane sugar with 2 % hydrochloric acid above 70°. In inverting cane sugar according to Bertrand's conditions, we invariably observed a decided yellow colour in our solution, whether simply boiled or heated in a boiling-water bath. That decomposition occurs at 100° has been generally recognised since the work of Herzfeld (*Zeit. Ver. Zuck.-Ind.*, 1898, 699 and 742).

Maltose. When working with 0·0824 grm. maltose we obtained results 0·0812 to 0·0826. Here the agreement is better, but the range of probable error is still large—nearly 1 %. It is noteworthy that Bertrand made use of hydrated maltose dried in a desiccator over sulphuric acid until constant in weight; to this the objection raised by Brown, Morris and Millar against Ost, that it contains slightly more than the theoretical 5 % of water of crystallisation, for $1H_2O$, can be applied. The $[\alpha]_D$ given by Bertrand, viz. 137·4° (calculated for the anhydrous substance), as compared with what is probably the more correct value, 138·0°, agrees with this view.

In all the above experiments special care was taken to wash the cuprous oxide precipitate very thoroughly with 300—400 c.c. of boiling water, so as to remove the last traces of Fehling solution before adding the acid ferric sulphate solution. In all cases, too, the latter part of the operation was carried out as quickly as possible, so as to avoid the possibility of oxidation which, however, we satisfied ourselves by several experiments with acid solutions of ferrous sulphate is not to be feared under these conditions.

In our hands, the concordance between duplicate experiments made with this method was not such as is desirable in an accurate quantitative process. Bertrand speaks of this method as "un des plus pratiques et des plus précis," an opinion which we cannot endorse; we regard it simply as a fairly rapid approximate method, which may perhaps in certain cases be useful when no high degree of accuracy is required.

In the case of cane sugar Bertrand's tables need revision. On the score of rapidity this method falls far short of the method advocated by Ling, Rendle and Jones.

METHODS OF INVERTING CANE SUGAR IN THE ESTIMATION OF SUGARS IN PLANT EXTRACTS.

In estimating cane sugar in plant extracts it is impossible to invert with hydrochloric acid at 70°, even under Herzfeld conditions as, if maltose is present, a considerable proportion also undergoes hydrolysis to dextrose (see p. 460) and there is also the danger of pentoses undergoing decomposition. It is therefore necessary to make use of invertase or a weak acid, such as citric acid or oxalic acid. We give the particulars of the invertase method later, and will first consider some of the difficulties which may arise in using citric acid.

Boiling 2 % citric acid[1] has been frequently used for inverting cane sugar and was employed by Campbell (*J. Agric. Sci.*, 1912, **4**, 248) in studying the carbohydrates of the mangold leaf. We can confirm the generally accepted view, that boiling during 10 minutes with 2 % citric acid completely inverts cane sugar when alone.

1. Taken 1 grm. cane sugar, 4 grms. citric acid, 200 c.c. water, boiled 10 minutes, neutralised to phenolphthalein by sodium hydroxide and made up to 500 c.c.

Solution = 0·2105 grm. invert sugar per 100 c.c.

Found (Ling's method) 0·2089 grm. = 99·3 % inversion.

2. A duplicate inversion.

Found (Ling's method) 0·2108 grm. invert sugar per 100 c.c. 100·1 % inversion.

In a series of experiments carried out on mangold leaf extracts, from which tannins, bases, amino-acids, etc. had been removed by basic lead acetate in the usual way, it was found on estimating cane sugar by means of 2 % citric acid that either cane sugar appeared to be entirely absent, or only a very small proportion seemed to be present. When, however, invertase was used with the same solution, the presence of a relatively large amount of this substance was disclosed. It was ultimately found that the cause of the difference in the two methods was due to the presence in the solution of a large proportion of sodium acetate, which almost entirely inhibits the inverting action of a 2 % solution of citric acid.

[1] Throughout the percentage of citric acid we give refers to the percentage of the ordinary crystalline acid, $C_6H_8O_7 + H_2O$.

The sodium acetate was produced owing to the necessity of using a very large quantity of basic lead acetate in the removal of the amino-acids, etc., of the leaf; the slight excess of lead was precipitated by sodium carbonate, a relatively large proportion of which however was necessary to neutralise the acetic acid liberated by the amino-acids, tannins, etc. In this way the sugar solutions had become so enriched with sodium acetate as entirely to prevent inversion by citric acid of the concentration used (2 °/₀).

That this was actually the case is shown by the following experiments.

1. 20 c.c. of a cane sugar solution containing 0·7504 grm. cane sugar was mixed with 50 c.c. of water and 5 c.c. of the ordinary basic lead acetate solution (Allen's *Commercial Organic Analysis*, 4th edition, Vol. I. p. 308) and solid sodium carbonate gradually added so as to precipitate the lead but using as little sodium carbonate in excess as possible; the solution was then diluted to 100 c.c., and 25 c.c. of the filtrate (= 0·1876 cane sugar) neutralised to phenolphthalein by adding a few drops of a citric acid solution. 0·5 grm. of solid citric acid was then added, so as to make a 2 °/₀ solution and the mixture boiled 10 minutes, after which it was cooled, neutralised with sodium hydroxide and heated with Fehling solution under Brown, Morris and Millar's conditions. *No weighable quantity of Cu_2O was obtained*, showing that under these conditions no inversion had occurred.

2. *Using Sodium Acetate only.*

It was calculated that 5 c.c. of the basic lead solution would give rise approximately to 1·13 grms. of sodium acetate, $C_2H_3O_2Na$, $3H_2O$; 40 c.c. of cane sugar solution (= 1·5008 grms.) and 2·26 grms. sodium acetate was diluted to 200 c.c. (Solution A), and 25 c.c. of this solution (= 0·1876 grm.) boiled during 10 minutes with 0·5 grm. citric acid. The solution was cooled, neutralised, and the reducing power estimated direct under Brown and Morris conditions:

0·1389 CuO = 0·0562 invert sugar = 0·0534 cane sugar
= 28·5 °/₀ inverted.

There had been some inversion, but nearly 75 °/₀ of the cane sugar had been left intact.

Some experiments were made to ascertain the concentration of citric acid necessary to invert cane sugar in presence of considerable quantities of sodium acetate.

It was found that more than 80 °/₀ of the cane sugar is inverted on boiling for 10 minutes with citric acid present to the extent of 2 °/₀ if

normal sulphuric acid is first added until the appearance of the first indication of change of colour with methyl orange.

3. 25 c.c. of Solution A in 2 (= 0·1876 grm. cane sugar) + 1·6 c.c. N-H_2SO_4 (first change of colour with methyl orange) + 0·532 grm. citric acid (= 2 %). Boiled 10 minutes, neutralised, and reducing power estimated.

CuO = 0·3973 grm. = 0·1701 invert sugar = 0·1616 cane sugar = 86·2 % inverted.

4. Similar experiments were made with the solution obtained in 1 by adding basic lead acetate to the cane sugar solution and subsequently precipitating with sodium carbonate.

25 c.c. (= 0·1876 grm. cane sugar) treated with N-H_2SO_4 (up to first indication of change of colour), solid citric acid added so as to give 2 % solution, boiled 10 minutes and neutralised.

(*a*) CuO = 0·3713 grm. = 0·1577 grm. invert sugar = 0·1498 cane sugar = 79·9 % inverted.

(*b*) CuO = 0·3910 grm. = 0·1670 grm. invert sugar = 0·1586 cane sugar = 84·6 % inverted.

The difference between the two experiments is probably due to a difference in the volume of sulphuric acid added, as the point of change with methyl orange is naturally very indistinct, owing to the sodium acetate present.

5. The same solution as in 4 was used. 25 c.c. (= 0·1876 grm. cane sugar) was neutralised to phenolphthalein by a concentrated citric acid solution, then an equal quantity of citric acid (to neutralise $NaHCO_3$), and finally 1 grm. of solid citric acid added so as to invert with 4 % of the latter; boiled 10 minutes and neutralised.

(*a*) CuO = 0·3142 = 0·1309 invert sugar = 0·1243 cane sugar = 66·3 % inversion.

(*b*) CuO = 0·3288 = 0·1376 invert sugar = 0·1302 cane sugar = 69·7 % inversion.

Here the amount of inversion is less than in 4.

6. *Adding N-H_2SO_4 to first change with methyl orange and then citric acid to make exactly 4 %.*

25 c.c. of solution 1 (deleaded by sodium carbonate) = 0·1876 grm. cane sugar + 2 c.c. N-H_2SO_4 + 1·08 grms. solid citric acid. Boiled 10 minutes and neutralised with sodium hydroxide.

0·4195 grm. CuO = 0·1811 grm. invert sugar = 0·1720 cane sugar = 91·76 % inversion.

7. Same treatment as 6, but citric acid exactly 5 °/₀ during inversion.

CuO = 0·4292 = 0·1858 grm. invert sugar = 0·1765 cane sugar = 94·1 °/₀.

Here inversion is still incomplete.

8. By adding sulphuric acid to first change, then citric acid to make a 10 °/₀ solution and boiling 10 minutes, inversion is complete.

CuO = 0·4500 = 0·1964 invert sugar = 0·1865 cane sugar = 99·5 °/₀[1].

Inversion with invertase in presence of salts.

It is remarkable that the action of invertase on cane sugar is not interfered with in the least by a proportion of sodium acetate which almost completely prevents inversion by 2 °/₀ citric acid.

1. To 25 c.c. of the above solution of cane sugar (= 0·1876 cane sugar) containing 1·13 °/₀ of sodium acetate, 1 c.c. of autolysed yeast was added and inverted for 2 hours at 40°. 3 c.c. of alumina cream was added, the solution filtered and the precipitate washed well, evaporating the washings so as finally to have 50 c.c. of the invert sugar solution for reduction under Brown, Morris and Millar conditions.

CuO = 0·4515 = 0·1873 grm. cane sugar inverted = 99·9 °/₀.

2. The same experiment was repeated, but making the cane sugar acid to litmus by adding one drop of *N*-sulphuric acid before adding the autolysed yeast.

CuO = 0·4542 grm. = 0·1885 grm. cane sugar = 100·6 °/₀.

A similar result was obtained by making the solution faintly acid to methyl orange with 1·6 c.c. of *N*-sulphuric acid before inversion; inversion was complete.

Invertase and boiling citric acid do not hydrolyse maltose under the conditions used in dealing with plant extracts.

It was important to make sure that in the inversion of cane sugar by either invertase or boiling citric acid, no maltose was hydrolysed to dextrose.

For this purpose carefully purified maltose was used (see p. 457), four times recrystallised from 80 °/₀ alcohol; the solution was standardised by preparing a concentrated solution and ascertaining the density,

[1] No loss of sugar is therefore caused by the use of basic lead acetate, as has sometimes been stated to be the case. This supposed loss has been probably due to incomplete inversion, brought about by the presence of sodium acetate. Parkin has also shown that no loss occurs by a series of special experiments.

using Brown, Morris and Millar's tables, the value so obtained being confirmed by ascertaining the reducing power.

Invertase.

25 c.c. of maltose solution representing 1·0032 grms. anhydrous maltose was digested with 1 c.c. autolysed yeast for 3 hours at 40°; 5 c.c. of alumina cream was added, the solution filtered and washed to 100 c.c. with boiling water. Taken 20 c.c. = 0·2006 grm. maltose.

1. CuO = 0·2700 = 0·1979 grm. maltose = 98·7 %.
2. CuO = 0·2716 = 0·1991 grm. maltose = 99·3 %.
3. CuO = 0·2700 = 0·1979 grm. maltose = 98·7 %.

The slight loss is probably due to retention of maltose by the alumina cream precipitate, in washing which only 70 c.c. of water was used. There has been no increase of reducing power such as would accompany hydrolysis of the maltose to dextrose.

Action of boiling 10 % citric acid on maltose.

A solution of maltose containing 1·941 grms. maltose per 100 c.c. at 15° was used. 10 c.c. of this reduces 0·2642 grm. CuO.

1. 50 c.c. of the maltose solution (= 0·9705 grm. maltose) was boiled 10 minutes with 5 grms. solid citric acid, cooled, neutralised with sodium hydroxide to phenolphthalein and made up to 100 c.c.; taken 20 c.c. = 0·1941 maltose.

CuO = 0·2768 = 0·1951 maltose = 100·6 %.

Very slight hydrolysis of maltose had occurred, representing an increase of reduction of 0·0036 grm. CuO.

2. 50 c.c. of the same solution was made faintly acid to methyl orange, by adding a trace of sulphuric acid, then 5 grms. solid citric acid were added, and the solution boiled 10 minutes and treated as in 1.

Expt. 1. CuO = 0·2677.

Expt. 2. CuO = 0·2715.

Average = 0·2696 = 0·1976 maltose = 101·8 %.

Slight hydrolysis of maltose occurred in these two separate experiments, representing an increase of 0·0035 grm. CuO in one case and 0·0073 in the other.

In another similar case a 1·0032 per cent. solution of maltose boiled with 10 % of citric acid showed in two experiments an average value of 101·6 % maltose. Slight hydrolysis had occurred.

3. Although under the conditions of 1 and 2 maltose showed a slight but distinct hydrolysis, it was found that, under the conditions actually existing in the analysis of plant products, when large quantities of basic lead acetate have to be used, and the excess of lead is removed by sodium carbonate, the sodium acetate formed is sufficient to inhibit all hydrolysis of maltose by boiling 10 °/₀ citric acid; the increase of reduction brought about by citric acid therefore represents true cane sugar. This is, of course, *not* the case if the lead is removed by hydrogen sulphide, because then the solution becomes strongly acid with acetic acid, and unless the acidity is neutralised (to phenolphthalein) by sodium hydroxide prior to the addition of citric acid, an even greater hydrolysis of maltose than is given above would be experienced.

Maltose + sodium acetate and boiling 10 °/₀ *citric acid.*

(i) 50 c.c. of maltose solution (= 0·5016 grm. maltose), boiled 10 minutes with 5·0 grms. solid citric acid + 0·565 grm. sodium acetate; cooled, neutralised with sodium hydroxide to phenolphthalein and made up to 100 c.c. at 15°.

(*a*) 25 c.c. taken:

(0·1254 maltose) = 0·1724 CuO = 0·1259 maltose = 100·4 °/₀ maltose taken.

(*b*) 40 c.c. taken:

(0·2006 maltose) = 0·2724 CuO = 0·1996 maltose = 99·5 °/₀ maltose taken.

99·95 °/₀

Here no perceptible hydrolysis has occurred.

(ii) The same is true if to the solution of maltose containing sodium acetate, sulphuric acid is added so as to make the solution just change colour with methyl orange.

75 c.c. maltose solution (= 0·7524 grm. maltose) + 0·847 grm. sodium acetate + 4·25 c.c. N-H_2SO_4 + 3·17 grms. citric acid. Boiled 10 minutes, neutralised and made up to 100 c.c.

25 c.c. = 0·1881 grm. maltose taken.

Found 0·2579 CuO = 0·1889 maltose = 100·4 °/₀.

Practically no hydrolysis of maltose by 10 °/₀ citric acid is therefore to be feared. We have however invariably, in our analyses of plant materials, carried out the estimation of cane sugar both by invertase and 10 °/₀ citric acid. This as a general rule has given good agreement and a mutual check is thus obtained on the two methods: this procedure also ensures that the concentration of citric acid has been

sufficiently great to effect complete inversion. In certain cases, when very large quantities of basic lead solution have to be used, it might be necessary to employ citric acid of greater concentration than 10 °/₀, but we have as yet not met with such necessity.

We have found that considerable hydrolysis of maltose occurs on treatment with hydrochloric acid under either the Clerget or Herzfeld conditions usual in the estimation of cane sugar; we have therefore not employed this acid at all in dealing with plant products. (Compare below, p. 458.)

Estimation of Maltose.

It has been frequently proposed to estimate maltose by hydrolysis with dilute hydrochloric or sulphuric acid at 100°, noting the change of cupric reduction or specific rotatory power of the solution after allowing for the inversion of cane sugar present. Under carefully regulated conditions this method gives approximate results in the case of pure maltose or a mixture of maltose and dextrose (cf. Baker and Dick, *Analyst*, 1905, **30**, 79) but it is, as we shall show, inapplicable in all cases when cane sugar and laevulose or pentoses are present, as in the solutions prepared from plant extracts.

Brown and Morris in their classical paper of 1893 used hydrochloric acid under the conditions prescribed by Elion (*Zeit. angew. Chem.*, 1890, 291 and 321), 50 c.c. of the 1 °/₀ solution being heated with 3 c.c. of concentrated hydrochloric acid for 3 hours at 100° (boiling-water bath). They observed that the "fall of angle on inversion with acid was, for some unexplained reason, always somewhat less than it ought to be on the supposition that it was due only to the hydrolysis of maltose," and suggested that this "probably indicates the presence of a small quantity of a hydrolysable substance other than maltose and with a less optical activity." In our early analyses of extracts of mangold leaves, we invariably observed the same phenomenon; but as the solutions always contained a brown humus-like precipitate, and had thus undergone considerable decomposition, it appeared that a probable explanation of the increased rotation lay in the destruction of a laevorotatory substance in the solution. The relative instability of laevulose in presence of acids suggested that this was the constituent undergoing change. Experiments with cane sugar and laevulose fully confirmed this view. (Tables I and II.)

TABLE I. *Action of* 2·3 °/₀ *HCl on Cane Sugar* (2 *hrs. heating at* 100°).

Conditions. 50 c.c. cane sugar solution + 25 c.c. water + 5 c.c. conc. HCl. After heating, neutralised and made to 100·4 c.c. Taken 20 c.c.

Sugar per 100 c.c. during heating	HCl/100 c.c. during heating	CuO weighed ex. 20 c.c.	Sugar found after heating per 100 c.c.	°/₀ sugar accounted for	Actual weight of sugar destroyed grms.
1·125	2·30	0·4066	0·8321	83·21	0·1679
,,	,,	0·4075	0·8350	83·50	0·1650

TABLE II. *Action of* 2·44 °/₀ *HCl on Laevulose* (2 *hrs. at* 100°).

Conditions. 10 c.c. laevulose solution (0·6560 laevulose) + 60 c.c. water + 5 c.c. HCl (36·6 HCl/100 c.c.); after heating 2 hrs. at 100° neutralised with sodium hydroxide and made to 100 c.c. at 15° C.

Conc. of laevulose during heating	HCl/100 c.c. during heating	CuO ex. 25 c.c.	Laevulose found after heating	Laevulose °/₀ destroyed	Actual laevulose destroyed
0·8840 grms. per 100 c.c.	2·44	0·2625	0·4520	31·1	0·2040 grms.
,,	,,	0·2614	0·4500	31·4	0·2060 ,,

In both cases, although the concentrations of acid and sugar are not strictly the same, there has been destruction of, roughly, the same quantity of laevulose; in the case of the pure laevulose the proportion of acid present was somewhat higher, thus accounting for the somewhat greater destruction of the sugar. A considerable quantity of dark brown humus-like substance separated from both solutions, which also became much discoloured, as had been experienced in dealing with the actual plant extracts.

It is clear, therefore, that laevulose is destroyed very largely under the conditions recommended by Brown and Morris for hydrolysing maltose. In the above experiments the heating was only carried out for 2 hours, whereas for the hydrolysis of maltose heating for 3 hours was recommended. We have not made an actual determination of the result of heating during 3 hours with the above concentration of acid, but Tables III and IV show the result of heating for 3 hours with a slightly higher concentration.

TABLE III. *Action of* 4·71 °/₀ *HCl on Cane Sugar at* 100° (3 *hours*).

50 c.c. of 2 °/₀ cane sugar solution (=1·000 grm.)+15 c.c. water+10 c.c. conc. hydrochloric acid. Heated 3 hrs. in boiling water, neutralised and made to 100 c.c. at 15° C. Used 20 c.c. for reduction.

Conc. of cane sugar during heating, per 100 c.c.	HCl/100 c.c. during heating	CuO from 20 c.c.	Cane sugar accounted for after heating*	°/₀ cane sugar destroyed	Actual laevulose destroyed grms.
1·333	4·71	0·2691	0·5310	46·9	0·4690
,,	,,	0·2691	0·5310	46·9	0·4690
,,	,,	0·2713	0·5355	46·45	0·4655
,,	,,	0·2710	0·5350	46·50	0·4650
		Average ...	0·5331	46·65	0·4669

* Using dextrose reducing figure.

TABLE IV. *Action of* 4·58 °/₀ *HCl on Laevulose at* 100° (3 *hours*).

10 c.c. of laevulose solution (0·6560 grm. laevulose) +60 c.c. water +10 c.c. conc. hydrochloric acid. Heated 3 hrs. in boiling water, neutralised and made to 100 c.c. Used 25 c.c. for reduction.

Conc. of laevulose during heating	HCl/100 c.c. during heating	CuO from 25 c.c.	Laevulose left	Laevulose °/₀ destroyed	Actual laevulose destroyed
0·8288	4·58	0·0339	0·0586	91·07	0·5974 grms.
0·8288	4·58	0·0518	0·0880	86·6	0·5680 ,,
		Made to 101·7 c.c.			

With both cane sugar and laevulose a considerable decomposition was made evident by the production of much brown, humus-like material. Laevulose is thus largely destroyed by heating with dilute hydrochloric acid for such prolonged periods as 2 to 3 hours at 100°. On the other hand, the figures for cane sugar would suggest that the dextrose remains mainly unchanged even after the more prolonged heating with nearly 5 °/₀ HCl. This point was specially tested, and Tables V and VI give the results.

TABLE V. *Action of* 2·35 °/₀ *HCl on Dextrose at* 100°.

20 c.c. dextrose solution (0·8216 grm.) + 50 c.c. water + 5 c.c. HCl conc. After heating neutralised and made to 100 c.c. at 15°.

Time of heating	Grms. dextrose per 100 c.c. during heating	Grms. HCl per 100 c.c. during heating	CuO ex. 20 c.c.	Dextrose found	Dextrose °/₀ found	Dextrose actually destroyed
2 hrs. at 100°	1·095	2·35	0·3948			
,,	,,	,,	0·3955			
,,	,,	,,	0·3950			
,,	,,	,,	0·3948			
		Average...	0·3950	0·8140	99·0	0·0076 grms.

Dextrose in 1 °/₀ solution is only slightly decomposed by 2 hours' heating with 2·35 °/₀ HCl at 100°, although even here the dextrose actually destroyed is 7·6 mgrm., but when heated 4 hours in 4·38 °/₀ solution, with a greater concentration of acid (4·15 °/₀) the amount of decomposition is considerable, the actual dextrose destroyed amounting to as much as 0·2400 grm. (Table VI).

TABLE VI. *Action of* 4·15 °/₀ *HCl on Dextrose at* 100°.

75 c.c. of dextrose solution (3·726 grms.) + 10 c.c. conc. HCl. Heated and made to 100 c.c. 20 c.c. diluted to 100 c.c. and 25 c.c. taken for reduction.

Time of heating	Grms. dextrose per 100 c.c. during heating	Grms. HCl per 100 c.c. during heating	CuO ex. 25 c.c.	Dextrose found	Dextrose °/₀ found	Dextrose actually destroyed
4 hrs. at 100°	4·38	4·15	0·4180			
,,	,,	,,	0·4195			
		Average...	0·4188	3·486	93·6	0·2400

It was hoped that it would be possible to arrange the conditions for the hydrolysis of maltose by dilute acid so as at the same time to leave the laevulose intact, but the following experiments showed that it was only possible to obtain anything like complete hydrolysis of maltose under conditions which bring about considerable destruction of laevulose.

TABLE VII. *Hydrolysis of Maltose* by Hydrochloric Acid at* 100°.

20 c.c. maltose solution (=0·8226 grm.) + 5 c.c. conc. HCl + 50 c.c. water. After heating, neutralised and made to 100 c.c.

Time of heating	Grms. maltose per 100 c.c. during heating	Grms. HCl per 100 c.c. during heating	CuO ex. 20 c.c.	Maltose found per 100 c.c.	Maltose % calculated from dextrose formed
1 hour	1·097	2·35	0·4060		
,,	,,	,,	0·4057		
		Average...	0·4058	0·7983	97·05†
2 hours	1·097	2·35	0·4096		
,,	,,	,,	0·4077		
		Average...	0·4087	0·8051	97·88
3 hours	1·097	2·35	0·4080		
,,	,,	,,	0·4091		
		Average...	0·4086	0·8045	97·82

* For the purpose of our experiments Kahlbaum's maltose was recrystallised several times from 80 % alcohol; the commercial material contains generally from 10 to 15 % of dextrin and great care is required in recrystallisation to remove this. We dissolve the maltose in 80 % alcohol, and leave to cool, when the dextrin separates as an oily layer from which the solution of the purer sugar is decanted and the process repeated. Our finally purified material had almost identically the same physical properties and the same reducing power as given by Brown, Morris and Millar (*Trans.*, 1897), within the error of 0·5 %.

25 c.c. of a solution containing 0·8226 grm. anhydrous maltose (dried *in vacuo* at 105°) per 100 c.c. gave

(1) 0·2809 CuO = 0·2059 maltose = 100·1 %
(2) 0·2821 CuO = 0·2067 ,, = 100·5 ,,

† Baker and Dick after 90 minutes' heating found with 1 % maltose + 20 c.c. water + 1 c.c. conc. HCl (sp. gr. 1·16) a hydrolysis of 96·5 %.

The maltose was heated with hydrochloric acid for 1, 2 and 3 hours in a long-necked flask fitted with a reflux and heated in boiling water; before hydrolysis the maltose was approximately 1 % and the acid 2·35 %.

Hydrolysis is not complete after 1 hour's heating at 100°, as is shown by the slight increase in reduction after 2 hours' heating; the increase in hydrolysis is however balanced by the increasing destruction of dextrose, which after 2 hours, according to p. 456, becomes distinctly noticeable.

With slightly stronger acid and longer periods of heating, *lower* results are obtained for maltose, as might be anticipated from the destruction of dextrose which occurs (compare Table VI, p. 456); the following results illustrate this:

TABLE VIII. *Hydrolysis of Maltose by* 4·15 °/₀ *HCl at* 100°.

75 c.c. dilute maltose solution (=0·6171 grm.) + 10 c.c. conc. HCl; after heating at 100° neutralised and made to 100 c.c.

Time of heating	Grms. maltose per 100 c.c. during heating	Grms. HCl per 100 c.c. during heating	CuO ex. 20 c.c.	Maltose found per 100 c.c.	Maltose °/₀ calculated from dextrose formed
3 hours	0·7261	4·15	0·3105		
,,	,,	,,	0·3117		
,,	,,	,,	0·3119		
		Average...	0·3114	0·5905	95·70
4 hours	0·7261	4·15	0·3068		
,,	,,	,,	0·3064		
,,	,,	,,	0·3061		
		Average...	0·3064	0·5805	94·06

Hydrolysis of Maltose at 70°.

Hydrolysis of maltose in 1 °/₀ solution by 2·44 °/₀ hydrochloric acid at 70°, is very slow, and even after 24 hours' heating only 94 °/₀ is converted into dextrose; it is possible that slight decomposition of the dextrose formed may take place during this prolonged period, but this point was not specially pursued, as it was found that laevulose certainly underwent considerable decomposition under these conditions and to an extent rendering it impossible accurately to estimate maltose in presence of cane sugar and laevulose by hydrolysis at this temperature.

The solutions remained colourless throughout the whole time of heating. In calculating the percentage of maltose converted in the above hydrolyses it must be remembered that the reducing power represented by CuO is due to a mixture of dextrose and unconverted maltose. In the last three experiments the original concentration of maltose is slightly different from that of the first three experiments, but the concentration of hydrochloric acid is maintained the same.

TABLE IX. *Action of 2·44 °/₀ Hydrochloric Acid on Maltose at 70°*

20 c.c. of maltose solution (0·7620 grm.) + 50 c.c. water + 5 c.c. conc. HCl; after heating neutralised and made to 100°.

Time of heating	Grms. maltose per 100 c.c. during heating	Grms. HCl per 100 c.c. during heating	CuO ex. 25 c.c.	°/₀ maltose converted
2 hrs. at 70°	1·016	2·44	0·3198	
,,	,,	,,	0·3193	
		Average...	0·3195	28·85
3 hrs. at 70°	1·016	2·44	0·3320	
,,	,,	,,	0·3328	
		Average...	0·3324	34·3
6 hrs. at 70°	1·016	2·44	0·3705	
,,	,,	,,	0·3693	
		Average...	0·3700	52·4
20·69 of another maltose solution (0·7186 grm.) + 49·3 c.c. water + 5 c.c. HCl conc., as above.				
12 hrs. at 70°	1·042	2·44	0·4449	
,,	,,	,,	0·4447	
		Average...	0·4448	83·3
18 hrs. at 70°	1·042	2·44	0·4635	
,,	,,	,,	0·4635	
		Average...	0·4635	92·1
24 hrs. at 70°	1·042	2·44	0·4673	
,,	,,	,,	0·4672	
		Average...	0·4673	93·9

TABLE X. *Action of 4·58 % Hydrochloric Acid on Maltose at 70°.*

20 c.c. of maltose solution (=0·9150 grm. anhydrous maltose) + 50 c.c. water + 10 c.c. conc. HCl was heated 3 and 6 hours at 70°. The solution was neutralised with sodium hydroxide and made to 100 c.c. at 15°. The reducing power was estimated in 20 c.c.

Time of heating	Conc. of maltose during heating grms. per 100 c.c.	HCl per 100 c.c. during heating	CuO ex. 20 c.c.	% Maltose converted
3 hours	1·144	4·58	0·3955	
,,	,,	,,	0·3916	
		Average...	0·3935	69·9
6 hours	1·144	4·58	0·4300	
,,	,,	,,	0·4300	
		Average...	0·4300	87·6

The curves summarise the above results.

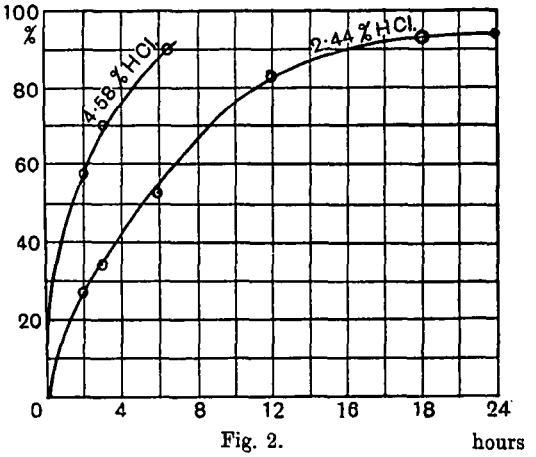

Fig. 2.

Action of Hydrochloric Acid on Dextrose at 70°.

The following results show that dextrose is only very slightly changed by prolonged heating (36 hours) with 2·44 hydrochloric acid at 70°; but stronger acid (4·88 %) brings about noticeable decomposition in 24 hours.

TABLE XI. *Action of* 2·44 °/₀ *HCl on Dextrose at* 70°.

20 c.c. dextrose solution (0·7640 grm. dextrose) + 50 c.c. water + 5 c.c. HCl (36·6 HCl/100 c.c.). Heated 36 hrs. at 70° C.; neutralised with sodium hydroxide and made to 100 c.c. at 15° C. Conc. of dextrose during heating = 1·0187 grm. per 100 c.c.

HCl/100 c.c. during heating	CuO ex. 25 c.c.	Dextrose found after heating	Dextrose °/₀ accounted for	Actual dextrose destroyed
2·44	0·4380	0·7608	99·6	0·0032 grms.

TABLE XII. *Action of* 4·88 °/₀ *HCl on Dextrose at* 70°.

20 c.c. dextrose solution (0·7640 grm. dextrose) + 45 c.c. water + 10 c.c. HCl (36·6 HCl/100 c.c.). Heated 24 hrs. at 70° C.; neutralised with sodium hydroxide and made to 100 c.c. at 15° C. Conc. of dextrose during heating = 1·0187 grm. per 100 c.c.

HCl/100 c.c. during heating	CuO ex. 25 c.c.	Dextrose found after heating	Dextrose °/₀ accounted for	Actual dextrose destroyed
4·88	0·4341	0·7532	98·6	0·0108 grms.

TABLE XIII. *Action of* 2·44 °/₀ *Hydrochloric Acid on Laevulose at* 70°.

10 c.c. laevulose solution (0·6560 grm. laevulose) + 60 c.c. water + 5 c.c. conc. HCl. Heated under reflux for 2, 18 and 24 hours at 70°. After heating, neutralised with sodium hydroxide and made to 100 c.c. at 15°. Used 25 c.c. for reduction.

Time of heating	Grms. laevulose per 100 c.c. during heating	Grms. HCl/100 c.c. during heating	CuO ex. 25 c.c.	Laevulose found	Laevulose °/₀ left	Laevulose destroyed grms.
2 hours	0·8840	2·44	0·3687	0·6524	99·50	0·0036
,,	,,	,,	0·3696	0·6540	99·73	0·0020
		Average...	0·3691	0·6532	99·62	0·0028
18 hours	0·8840	2·44	0·3605	0·6368	97·1	0·0192
,,	,,	,,	0·3602	0·6360	97·0	0·0200
		Average...	0·3603	0·6364	97·05	0·0196
24 hours	0·8840	2·44	0·3530	0·6224	94·9	0·0336
,,	,,	,,	0·3504	0·6160	93·9	0·0400
		Average...	0·3517	0·6192	94·5	0·0368

In 0·8840 °/₀ solution laevulose is only very slightly destroyed by 2 hours' heating with 2·44 °/₀ hydrochloric acid at 70°/₀; but longer

periods of heating bring about considerable destruction, so that it is impossible to hydrolyse maltose even at 70° in presence of laevulose, without destruction of the latter.

TABLE XIV. *Action of* 4·88 °/₀ *Hydrochloric Acid on Laevulose at* 70°.

10 c.c. laevulose solution (0·6560 grm.) + 55 c.c. water + 10 c.c. conc. HCl. Heated 6 hours at 70°. Neutralised and made to 100 c.c. at 15°; taken 25 c.c. for reduction.

Time of heating	Grms. laevulose per 100 c.c. during heating	Grms. HCl per 100 c.c.	CuO ex. 25 c.c.	Laevulose found	Laevulose °/₀ left	Actual laevulose destroyed
6 hours	0·8840	4·88	0·3520	0·6200	94·5	0·0360
,,	,,	,,	0·3552	0·6272	95·6	0·0288
		Average...	0·3536	0·6236	95·1	0·0324

In this case the destruction of laevulose in 6 hours is almost as great as with the 2·44 °/₀ acid in 24 hours.

Estimation of Maltose in presence of other Sugars by means of Maltase-free yeasts.

It has been known for some years that certain species of yeast do not contain maltase and hence are incapable of fermenting maltose; owing to the impossibility of estimating this substance in presence of the other sugars likely to be present in plant extracts by means of the ordinary methods we decided to ascertain whether these yeasts are suitable for purposes of quantitative estimation of this sugar[1].

For this purpose we fermented a solution of maltose both alone and mixed with cane sugar, with pure cultures of *S. exiguus*, *S. anomalus* and *S. marxianus*, which Dr H. B. Hutchinson was good enough to prepare for us. To the solution of sugar, 5 c.c. of yeast water was added and the mixture, after being sterilised by 10 minutes' heating in the autoclave, was inoculated with a trace of the pure yeast from an agar-yeast-water tube-culture.

[1] Baker and Dick (*Analyst*, 1905, **30**, 79) have suggested the use of *S. marxianus* for *detecting* maltose in presence of dextrose, by the increase of specific rotation and drop in reducing power which occur in fermenting the mixed sugars with this yeast; they fermented, however, only for a relatively short time and did not completely remove the dextrose as we have done, so as to make the method a quantitative one.

The flask containing the liquid is stoppered with cotton wool in the usual manner and incubated at 25° for three to four weeks. When the fermentation is complete, 5 c.c. of alumina cream is added and the solution boiled to expel alcohol: it is then filtered, and the precipitate washed until the filtrate has a volume of 100 c.c.

An aliquot portion (50 c.c.) can then be used for the cupric reduction.

TABLE XV.

	Yeast	Time	CuO from 50 c.c.	Maltose found	% Maltose found
1st Series					
Maltose (0·2006) + Cane Sugar (0·3751)	*S. exiguus*	17 days	0·1525	0·2224	110·8
	S. anomalus	23 ,,	0·1355	0·1974	98·4
	S. marxianus	21 ,,	0·1348	0·1962	97·4
Maltose only	*S. anomalus*	21 ,,	0·1360	0·1980	98·7
	S. marxianus	21 ,,	0·1340	0·1952	97·3
2nd Series					
Maltose (0·3704) + Cane Sugar (0·2000)	*S. exiguus*	31 ,,	0·2535	0·3712	100·2
	S. marxianus	31 ,,	0·2548	0·3736	100·8
	S. marxianus	31 ,,	0·2547	0·3734	100·7

In the first series the high result with *S. exiguus* is undoubtedly due to incomplete fermentation, the time being insufficient. The slightly low results in the other experiments in this series are due either to the maltose used being slightly contaminated with dextrose, or, more probably, to experimental error on the relatively small quantity of maltose taken. In the second series a very carefully purified maltose was used and a larger quantity taken so as to minimise the proportional error. In this case it will be seen that in spite of a very vigorous growth of yeast the maltose is quantitatively recovered, whilst the cane sugar is completely fermented away.

By the use of these special maltase-free yeasts it is therefore possible accurately to estimate maltose in presence of other sugars (dextrose, laevulose and cane sugar) which are completely fermented by them. We have applied this method now for some considerable time to the analysis of plant extracts and find that it is generally necessary also to introduce a correction for the presence of reducing

substances such as pentoses[1] which remain after fermentation by the yeast is complete. This correction is obtained by carrying out fermentation with a pure culture of ordinary distillery or baker's yeast, which ferments away the maltose as well as the other sugars, but leaves a slight residual reduction due to pentoses, etc.; on subtracting this value from the reducing value obtained by using the maltase-free yeasts, the cupric reduction due to maltose alone is obtained.

Estimation of Maltose in Plant-extracts.

For this purpose the plant extract, from which amino-acids, tannins, etc., have been removed by means of basic lead acetate, has to be entirely freed from lead before the yeasts will grow satisfactorily. For this purpose two methods may be used:

1. Solid sodium carbonate is added little by little until no further precipitate is produced. The filtrate, which still contains traces of lead, is made slightly acid with hydrochloric acid and treated with hydrogen sulphide as in 2.

2. The excess of lead is removed directly by hydrogen sulphide. In this case the solution becomes strongly acid owing to the presence of free acetic acid and must be partly neutralised by adding dilute sodium carbonate solution until the reaction is faintly but distinctly acid to litmus paper (see p. 467).

It has been our custom to carry out five fermentations with each plant extract to be analysed, viz. one each with *S. anomalus*, *S. exiguus* and *S. marxianus*, and two with distillery yeast. The agreement between the results with the different special yeasts has generally been entirely satisfactory. Certain differences however in the behaviour of the yeasts may here be noted.

S. anomalus grows rapidly and gives a very bulky mass of yeast; it is apparently less efficient as a sugar remover than *S. marxianus* or *S. exiguus*, that is, it is slower in its action, and a greater yeast growth accompanies the removal of a certain weight of sugar. It shows a decided tendency to grow at the surface as a film. The cuprous oxide obtained in the subsequent reductions often filters very slowly.

S. marxianus is more sensitive to slight excess of acid than the

[1] The pentoses present in plant extracts are apparently not fermented by either baker's yeast or the special maltase-free yeasts we have used. Experiments on this point are still in progress.

other two yeasts, and refuses to grow in acid solutions in which the others readily multiply. *S. exiguus* is probably the most convenient for general use.

Pentoses.

Pentoses are generally present to some considerable extent in the solutions obtained by the extraction of foliage leaves, and, after the treatment with basic lead acetate and subsequent removal of excess of lead, exercise a reducing action on Fehling solution. In view of recent work, especially that of Levene and Jacobs (*Ber.*, 1909, **42**, 2469, 2474, 2703; *Biochem. Zeit.*, 1910, **28**, 127), it is probable that pentoses play an extremely important part in the leaf's activity, especially *d*-ribose, which is an essential constituent of the nucleus of both plant and animal cells; the pentoses present in the aqueous alcoholic extract of leaf tissue are very possibly largely derived from nucleic acids. There is, however, also the possibility of the presence of arabinose and xylose as well as methylpentoses. In calculating the proportions of dextrose and laevulose we therefore have to make allowance, after subtracting the reduction due to the maltose (when this is actually present), for the pentoses; here we are faced with the difficulty that we do not know in any particular case what pentoses actually are present or what is their reducing power under the special conditions of the actual analysis.

In the present state of our knowledge we must be content with approximations, but the nature of these will affect the accuracy of the values for dextrose and laevulose, which are calculated from the primary reducing power of the plant solutions.

We have in our experiments estimated the total pentoses by distilling a quantity of the solution of which the "direct" reducing power of the sugars is determined, with hydrochloric acid according to the ordinary A.O.A.C. method (see Allen's *Commercial Organic Analysis*, I. 401; Bulletin 107, U.S.A. Dept. of Agriculture), weighing the furfural formed as phloroglucide. We intend to introduce a correction for the pentoses by ascertaining their reducing power under the conditions in which we have made our analyses.

Analysis of Plant Extracts.

The scheme of the analysis of a plant extract, such as that of foliage leaves, which we have adopted, may be outlined as follows:

Extract evaporated *in vacuo* (700 to 740 mm.) to small volume. Made to a definite volume, say 500 c.c.

2 portions of 20 c.c. each evaporated to dryness and dried *in vacuo*, 18 hours at 100°. This gives *total dry matter* in extract. (20 c.c. kept as reserve in case of accident.)

440 c.c. treated with basic lead acetate, filtered under pressure on Büchner funnel and washed to known volume (2 litres) = Solution A.

300 c.c. of Solution A is deleaded by solid Na_2CO_3 and made up to 500 c.c. = Solution B.

1. 25 c.c. of B is used for *direct reduction* and *polarised*[1]; the reduction is due to *dextrose, laevulose, maltose, pentoses.*

2. *For cane sugar.* 50 c.c. of B is inverted:

(*a*) *By invertase.* Make faintly acid to *methyl orange* by a few drops of concentrated sulphuric acid, and add 1—2 c.c. autolysed yeast and two or three drops of toluene and leave 24 hours at 38—40°C. After this period, add 5 to 10 c.c. alumina cream, filter and wash to 100 c.c. Take the reducing power of 50 c.c. (= 25 c.c. B) and polarise.

(*b*) *By* 10 °/$_0$ *citric acid.* Make faintly acid to methyl orange by a few drops of conc. sulphuric acid and add a weighed quantity of citric acid crystals so as to have 10 °/$_0$ of the crystalline acid ($C_6H_8O_7 + H_2O$) present. Boil 10 minutes, cool, neutralise (to phenolphthalein) with sodium hydroxide, make to 100 c.c. and determine *reducing power* of 50 c.c. (= 25 c.c. B). Polarise.

Cane sugar is calculated from the increase of reducing power or change of rotation caused by inversion. The values obtained by the two methods *a* and *b* should agree closely.

3. *For maltose.* Another 300 c.c. of Solution A is deleaded by means of hydrogen sulphide and filtered, the precipitated sulphide being washed until the total volume of filtrate and washings is about

[1] The polarisation of these dilute solutions is usually small and it is therefore necessary to take the reading with a long tube (at least 200 mm. in length), with an instrument reading accurately to $\frac{1}{100}°$, the temperature being maintained constant at 20° C. within $\frac{1}{10}°$. It is an easy matter, using a Lowry thermo-regulator, and circulating the water by means of a small pump, to keep the temperature constant to $\frac{1}{100}°$; but we find that differences of temperature less than $\frac{1}{10}°$ hardly make a perceptible difference in the readings with such dilute solutions as we have worked with.

450 c.c. Air is then sucked through this for about 1½ hours to expel hydrogen sulphide, a very little ferric hydroxide is added to remove the last traces of the latter, and the solution is made to 500 c.c. It is filtered and

50 c.c. fermented (*a*) with *S. marxianus*
„ „ (*b*) „ *S. anomalus*
„ „ (*c*) „ *S. exiguus*

and two lots *d* and *e* of 50 c.c. are fermented with baker's yeast. It is generally necessary, in order to ensure good growth of the yeast, to reduce the acidity by adding 2 to 5 c.c. of *N*-sodium carbonate to the 50 c.c. to be fermented; 5 c.c. of sterilised yeast water is also added, the mixture is sterilised in the usual way and inoculated in the inoculating chamber with the pure culture of yeast. It is then stoppered with cotton wool and the yeast allowed to incubate for 21 to 28 days at 25°.

After completion of fermentation, 5 c.c. alumina cream is added, the solution made to 100 c.c. at 15°, filtered and 50 c.c. used for reduction. The *difference* between the *average* reduction with *a*, *b*, *c* and the average of *d* and *e* gives the reduction due to *maltose*.

4. *Pentoses.* These are approximately determined in 50 c.c. of A by distilling with hydrochloric acid according to the A.O.A.C. method, weighing as phloroglucide.

5. When the reduction in 1 due to pentose and maltose has been allowed for, the remaining direct reduction is due to dextrose and laevulose; the actual proportions of these two sugars are calculated from the reducing power combined with the corrected specific rotation as suggested by Brown and Morris in their 1893 paper.

In conclusion, we wish to express our best thanks to Dr H. B. Hutchinson for his kindness in preparing pure cultures of the yeasts employed and assistance in their manipulation and to Mr G. C. Sawyer for help in the analyses.

Summary.

1. Certain sources of error encountered in the estimation of sugars in plant extracts are dealt with. Large errors in the gravimetric method may be obtained unless special care is taken in purifying the asbestos by boiling for at least 30 minutes with 20 % sodium hydroxide. Weighing the reduced copper as cuprous oxide is likely to give rise to

large error, and a process of weighing as cupric oxide, with certain precautions, is recommended.

2. The volumetric methods of Ling and of Bertrand have been studied; the former is preferable in all respects to the latter, which we regard as only roughly approximate.

3. In dealing with plant extracts, owing to the accumulation of sodium acetate in the solutions analysed, inversion by citric acid of lower concentration than 10 °/₀ is generally incomplete. Inversion by invertase is, however, not interfered with by this salt. To estimate cane sugar inversion both by invertase and 10 °/₀ citric acid is recommended. No loss of sugars occurs owing to the use of basic lead acetate as has been sometimes stated; the supposed loss is probably due to incomplete inversion caused by the presence of sodium acetate.

4. It is shown by a detailed study of the action of dilute hydrochloric acid on different sugars that it is impossible completely to hydrolyse maltose at either 70° or 100° without simultaneously destroying large quantities of laevulose or dextrose.

5. The only available method for the accurate estimation of maltose consists in the employment of special maltase-free yeasts, such as *S. exiguus*, *S. marxianus* or *S. anomalus*, introducing a correction (for pentoses, etc.) obtained by a special fermentation with baker's or brewer's yeast.

6. A scheme for the quantitative estimation of sugars in plant material is given.