GUIDE TO THE CARBOHYDRATE ANALYSIS OF FORAGE PLANTS

(Prepared by J. T. Sullivan, United States Regional Pasture Research Laboratory, State College, Pennsylvania, March 15, 1951)

This guide is for those conducting research in plant physiology or on the nutritive value of plants, particularly forage plants. It does not contain analytical directions, but refers to methods in published literature from which the research worker may make his own selection. The sources of these methods have been chosen on the bases of exposition and availability rather than on originality or priority and in many cases the method is described as a minor part of the research report. Only sources in English are quoted though the original may have appeared in some other language. The page numbers refer to the actual section or paragraph to be read rather than to the beginning of the article. All opinions and recommendations are personal ones of the writer of this guide and subject to modification.

In certain cases methods used at the Pasture Research Laboratory which have been found satisfactory and which may not occur elsewhere in the same form or detail are appended to this guide as Supplements. Suggestions as to inclusion or deletions are welcome. This guide will be revised as often as necessary.

Abbreviations to references are as follows:

Anal. Chem.	--- Up to Vol. 18(1946) this refers to Ind. Eng. Chem., Anal. Ed., beginning with Vol. 19(1947) to Anal. Chem.
Browne and Zerban	--- Physical and Chemical Methods of Sugar Analysis, 3rd Edition, John Wiley & Sons, Inc., 1941.
Cont. B. T. I.	--- Contributions of the Boyce Thompson Institute
J. A. C. S.	--- Jour. American Chemical Society.
J. A. O. A. C.	--- Jour. Assoc. Official Agricultural Chemists.
J. B. C.	--- Jour. Biological Chemistry.
Loomis and Shull	--- Methods in Plant Physiology, McGraw-Hill Book Co., 1937.
Methods	--- Methods of Analysis, Assoc. Official Agricultural Chemists, 6th Edition, 1945.
Norman	--- The Biochemistry of Cellulose, the Polyuronides, Lignin, etc. The Clarendon Press, Oxford, 1937.
P. P.	--- Plant Physiology (Journal).

1. SAMPLING

The research worker himself is best qualified to decide on the numbers and portions of plants to be sampled. Suggestions as to techniques of sampling various crops may be found in the following:

References:

General, committee report -- Tottingham et al, P. P. 2, 225(1927).

General -- Loomis and Shull, 251(1937).

Soybean plants -- Webster, P. P. 3, 31(1298).

Selecting uniform samples of leaves -- Vickery et al, P. P. 24, 335(1949).

2. PRESERVATION AND EXTRACTION

Because of the instability of many carbohydrates, plants must be treated to fix their composition until analysis can be completed.

(a) Drying.

Drying is the simplest procedure for plant preservation. Drying at low temperature will permit enzymatic changes. High temperatures in air will promote oxidation. Intermediate temperatures, 60-80°C., are probably safest. Attainment of the desired temperature and desiccation must be carried out as quickly as possible. Vacuum drying with moderate temperature and autoclaving before drying are variants. Freezing and drying while frozen are not recommended.

References:

General, committee report -- Tottingham et al. P. P. 1, 397(1926); also Link and Tottingham, J. A. C. S. 45, 439(1923).

(b) Alcoholic preservation and extraction.

Immersion of the fresh tissue, as quickly as possible after harvesting, in boiling alcohol of such quantity and strength as will give a final concentration of 70% or 80% is recommended. About 4 ml. of 95% alcohol for each gram of fresh plant tissue will give the proper concentration, with additional amounts of 70 or 80% alcohol, if necessary, to submerge the sample. An agent to neutralize plant acidity, as calcium carbonate, may be placed in the alcohol, if necessary. This is followed by successive washings with 80% alcohol, optionally in a Soxhlet extraction apparatus, to yield alcohol-soluble and alcohol-insoluble portions. When bulky coarse particles are present they should be removed from the alcohol after the first or second washing, air dried, ground, and returned for further extraction. The operations may be suspended and the samples placed in storage at any time after the tissue is killed.

For ascertaining the total dry weight of the sample, on which percentages of carbohydrates may be based, add the weight of the alcohol-insoluble portion to the weight of total solids in the alcohol-soluble portion. Obtain the latter figure by evaporating an aliquot of the alcoholic extract to dryness in a tared weighing bottle. When removing any aliquot of the extract, have the extract at the same temperature at which it was made to volume. The moisture content of the original fresh sample is the difference between the fresh weight and the calculated total dry weight. This total dry weight may also be taken as the dry weight yield of some experimental unit material.

References:

General, committee report -- Tottingham et al, P. P. 1, 399(1926); also Loomis and Shull, 254(1937).

Directions for preserving plants in 80% alcohol -- Methods, Section 12.2 (b)(1945); also Browne and Zerban, 886(1941).

The use of $CaCO_3$ may be omitted -- Denny, Con. B. T. I. 5, 103(1933).

(c) Extraction of dry samples.

Dried and ground samples may be extracted by alcohol without the Soxhlet procedure and with a minimum of labor when sugars only are to be determined. Since the alcohol-insoluble portion is not washed free of sugars this method, without modification, is not recommended when polysaccharides are to be determined.

References:

Method used to extract sugars from grain and stock feed -- Methods, Section 27.31 (1945).

A similar procedure with plant material -- Hasselbring, P. P. 2, 233(1927).

(d) Extraction in the Waring blendor

The Waring blendor may be used to disintegrate and extract with alcohol either fresh tissue or tissue dried as in 2a, and after filtration and washing to obtain alcohol-soluble and alcohol-insoluble portions. Treatment in the blendor of fresh tissue with cold alcohol is not recommended if the several sugars are to be distinguished from one another as sucrose is hydrolyzed to some extent before the killing is complete. With tissue killed in hot alcohol, as in 2b, the disintegration and extraction may be completed with the blendor instead of by the Soxhlet procedure. The combination of killing the tissue in hot 80% alcohol, decanting after cooling, and then extracting in the blendor with fresh cold 60% alcohol, is recommended for rapid and complete extraction; concentrations of alcohol less than 60% must not be used with grasses because of the solubility of fructosan.

References:

Extraction of forage plants with cold alcohol, recommended for total sugar

but not for individual sugars unless the tissue has been previously killed--Thomas et al, Anal. Chem. 21, 1363(1949).

See Supplement A.

3. PREPARATION OF EXTRACTS FOR SUGAR DETERMINATIONS

The alcohol-soluble portion obtained in 2b, 2c, or 2d is used for sugar determination. Prepare an aqueous extract for sugar determination by evaporating the alcohol, adding water, and clarifying. The safest reagent for clarifying is neutral lead acetate, and the excess lead, after filtering, is removed by precipitation as an insoluble salt, i.e., oxalate or sulfate. Clarifying may be omitted if unnecessary, that is, if the reducing powers of clarified and unclarified solutions are the same. The entire alcohol-soluble portion need not be clarified, the sugar concentration being the deciding factor in the aliquots used and the amount of dilution.

References:

General, committee report -- Loomis et al, P. P. 2, 196(1927); also P. P. 10, 387(1935); also Loomis, P. P. 1, 179(1926); also Loomis and Shull, 277(1937).

Directions for clarifying -- Methods, Section 12-49(b), (1945).

4. DETERMINATION OF SUGARS

Aliquots of the aqueous solution obtained in Section 3 are used for the determination of sugars.

(a) Reducing sugars.

With few exceptions this determination is for the sum of the glucose and fructose, both occurring free. There are numerous methods for this determination. All, when properly standardized, give recovery with pure sugars, but all do not give the same results with plant extracts containing non-sugar substances. If it is wished to make a comparison of two or more methods with the plant material at hand, select that method as more nearly accurate which gives the lowest apparent sugar content. Also, choose a method with a sugar range of the same order as that in the sugar solutions to be analyzed, or else adjust the latter solutions by dilution or alter the size of the sample or of the aliquot. Methods employing copper salts are preferred over those using other oxidizing agents. Methods employing other than copper reagents are often more rapid but select one of such methods only if convinced by comparative trials of its reliability.

There are two distinct steps, (1) the reduction of the copper, and (2) the determination of the amount of copper reduced. Numerous methods have been proposed for each step and in general any combination of (1) and (2) will work. The reduced copper obtained in (1) is insoluble and

it is recommended that it be separated from the reduction mixture before carrying out (2). Its titration in the presence of the reduction mixture is possible but is not recommended indiscriminately for plant extracts. Some published methods are accompanied by tables or formulae for translating copper values to sugar. These tables are based on (1), regardless of the procedure used for (2). The analyst may make his own standardization with pure sugars. Pure glucose (dextrose) and sucrose may be obtained for this purpose from the National Bureau of Standards and are furnished without charge to educational institutions.

References:

General, committee report -- Loomis et al, P. P. 2, 197(1927).

The Quisumbing and Thomas reduction method, macro, with optional method for copper determination, with tables, requires constant temperature bath, highly recommended -- Methods, Section 12.51 (1945); also Browne and Zerban, 802 (1941); also Quisumbing and Thomas, J.A.C.S. 43, 1503 (1921).

The Munson and Walker reduction method, macro, with optional method for copper determination, with tables, requires simple equipment but more careful attention to heating conditions -- Methods, Section 34.39(1945); also Loomis and Shull, 269(1937); also Browne and Zerban, 800 (1941); also Munson and Walker, J.A.C.S. 28, 663(1906).

The reduced copper obtained in either of the above two methods may be determined by permanganate titration -- Methods, Section 34.44(1945), or by weighing -- Methods, Section 34.40(1945).

A semimicro method, with reduced copper separated by filtration and then determined by permanganate titration, with equations for glucose, fructose, and invert sugar (as sucrose) -- Phillips, J. A. O. A. C. 24, 181 (1941).

For a modification of the above, see Supplement B.

(b) Fructose (levulose)

If fructose is the only reducing sugar present it may be determined by any reducing sugar method, as in 4a, which has been properly standardized against pure fructose. However, in most cases glucose is also present. Determine the reducing power of the fructose after destroying that of the glucose by oxidation with iodine under alkaline conditions. This iodine treatment does not affect fructose but in determining the reducing power of the fructose a method must be used which takes into account that iodide salts have been added to the solution.

References:

See Supplement C.

That part of a method for fructosan which follows the hydrolysis of

fructosan and concerns the determination of fructose, a method involving the iodine oxidation of glucose -- Norman et al, Iowa State College Jour. Sci. 15, 302 (1941).

Fructose may also be determined by a colorimetric method using the Seliwanoff reaction -- Hubbard and Loomis, J.B.C. 145, 643 (1942).

Other colorimetric methods have been proposed for fructose but have not been sufficiently tested with plant extracts to be recommended.

(c) Sucrose

Use another aliquot (usually a smaller one) of the aqueous solution obtained in Section 3 for the determination of sucrose. First hydrolyze the sucrose to invert sugar by (1) invertase or (2) acid, and then determine its reducing power, as in 4(a). Invertase is more specific for the hydrolysis of sucrose than is acid and the procedure contains fewer steps but in some plant extracts, especially in grasses, the completion of hydrolysis by invertase is attained very slowly. For this reason invertase is not recommended for grasses nor when the results are desired quickly.

References:

For the use of invertase read -- Loomis and Shull, 275 (1937); also Loomis P. P. 10, 388 (1935).

Specific directions in the use of invertase -- Methods, Section 12.53 (1945).

Hydrolysis of sucrose by hydrochloric acid at room temperature, Methods, Section 34.24(c) (1945); also Loomis and Shull, 274-5 (1937).

Hydrolysis of sucrose by hydrochloric acid at 70°, a more rapid procedure, using that part of the Clerget procedure concerned with the hydrolysis only (not the polarization) -- Browne and Zerban, 407 (1941); also Methods, Section 34.24(b)(1945).

See Supplement D.

5. POLYSACCHARIDES

For the determination of polysaccharides use the alcohol-insoluble portion prepared in 2(b), ground to 40 mesh or finer if required. Treat some of this portion so that the polysaccharides are hydrolyzed to simple sugars and determine their reducing power by methods described in 4. Clarify the hydrolyzate as in 3 before the reduction, if necessary. If an alcoholic extraction had not been carried out on the material, the presence of sugars not derived from the polysaccharides should be taken into account.

References:

General, committee report -- Willian et al, P. P. 2, 91 (1927).

(a) Fructosans (lovulosans), in grasses

Fructosans are found in grasses only, are water-soluble though present in the alcohol-insoluble portion, are hydrolyzed easily with acids of low concentration or with weak acids, and yield mainly fructose with some minor quantities of glucose. The conditions of hydrolysis should not be made more severe than necessary or else other polysaccharides may be attacked or the fructose may be destroyed. After hydrolysis determine either total reducing power by a method as in 4(a), or determine fructose only by a method as in 4(b).

References:

Fructosan dissolved with hot water, filtered from residue, hydrolyzed with oxalic acid, fructose determined in hydrolyzate by a reduction method - Norman et al. Iowa State Coll. Jour. Sci. 15, 302 (1941).

Hydrolysis of fructosan with oxalic acid without previous solution and separation from the residue, see Supplement E.

Hydrolysis of fructosan and determination of fructose colorimetrically in one operation by a method proposed for inulin - Hubbard and Loomis, J.B.C. 145. 643(1942).

A similar procedure, designed for the guayule plant - McRary and Slattery J. B. C. 157, 162 (1945).

(b) Water-soluble carbohydrates.

Extract a sample dried as in 2(a) with water to remove all water-soluble carbohydrates. This mixture, if from grasses, contains reducing sugars, sucrose, and fructosan. Convert the last two into reducing sugars by a method of hydrolysis as in 5(a), clarifying as in 3, and determining the reducing power as in 4(a). This mixture if from legumes contains no appreciable amount of carbohydrates other than sugars.

References:

A cold water or a hot water extraction, a method proposed for the guayule plant. Hot water extraction should be avoided in legumes or other plants containing starch. -- Traub and Slattery, Botan. Gaz. 108, 295(1946).

Water-soluble carbohydrates in grasses may be considered identical with "total available carbohydrates", see 5(d).

(c) Starch.

Starch is usually absent in grasses of the temperate zone and its determination is confined to legumes. Use the alcohol-insoluble portion obtained in 2(b) or a portion freed from sugars by any suitable method of extraction with alcohol or cold water. Hydrolyze the starch to glucose by the use of diastase and determine the reducing power of the glucose as in 4(a). Nine parts of starch yield ten parts of glucose. No method for starch is highly recommended at present.

References:

General, committee report -- Willaman et al, P. P. 2, 91(1927); also Sullivan, Anal. Chem. 7, 311(1935); also Thurber, J.A.O.A.C. 23, 128(1940).

Hydrolysis with takadiastase followed by hydrolysis with HCl to insure the conversion of all starch hydrolytic products to glucose -- Johnston and Doro, P. P. 4, 39(1929).

Takadiastase (undiluted) with a low reducing blank may be obtained from Parke-Davis and Co.

Hydrolysis of starch with takadiastase under conditions of time, acidity, and concentration which convert all the starch to glucose -- Denny, Contrib. B.T.I. 6, 129(1934); also Browne and Zerban, 862(1941).

A procedure similar to the above is given in Supplement F.

Hydrolysis of starch with salivary diastase, followed by hydrolysis with dilute HCl to convert all starch hydrolytic products to glucose -- Loonis and Shull, 143 (1937).

Hydrolysis with salivary diastase followed by hydrolysis with dilute H_2SO_4 --Clements, P. P. 3, 445(1928).

Hydrolysis of starch with malt diastase, followed by hydrolysis with dilute HCl to convert maltose to glucose -- Browne and Zerban, 858 (1941); also Methods, Section 27.35 (1945).

Solubilization of starch with alcoholic HCl, followed by hydrolysis with salivary diastase to maltose, and the reducing power determined by a method standardized with maltose -- Hassid et al, Anal. Chem. 12, 142 (1940).

Solubilization of starch with alcoholic HNO_3, extraction with 20% alcohol, hydrolysis to glucose with HCl -- Niemann et al, P. P. 10, 579(1935).

The acid hydrolysis method, Methods, Section 27.33(1945), is not recommended for plants. But the hydrolyzate obtained by this method from legumes may be neutralized and treated with yeast, as in 5(e). The loss in reducing power as a result of fermentation may be assumed to be that of glucose and the glucose may be assumed to have been derived chiefly from starch.

(d) Total available carbohydrates.

This term includes all carbohydrates considered available to plants from a physiological standpoint and also perhaps the most readily digestible from a nutritional standpoint. They include all sugars, fructosan, starch, and perhaps glucosides if present. Analysis for this total without obtaining any information on the individual compounds is sometimes resorted to. Use unextracted material dried as in 2(a).

References:

Hydrolysis with takadiastase, followed by hydrolysis with .3N HCl on the boiling water bath. Includes sugars, fructosan and starch. With grasses

only, and with no or little starch present, and with the analyses made at locations near sea level, the HCl concentration may be cut to 0.1 N. -- Meinann, P. P. 22, 280(1947).

Clarase may be used instead of takadiastase, and other modifications of the above are suggested by Lindahl et al., P. P. 24, 286(1949).

With grasses only, and if starch is absent, total available carbohydrates may be considered identical with water-soluble carbohydrates and may be determined as in 5(b).

(c) Hemicelluloses, acid-hydrolyzable carbohydrates, pentosans, etc. These substances are usually determined on the alcohol-insoluble portion, free from sugars, as obtained in 2(b) and from which fructosan or starch has been removed. The starting material would be therefore the residues remaining after the fructosan had been extracted as in 5(a) or 5(b), or after starch had been removed as in 5(c) or 5(d). Some water-soluble hemicelluloses would have been removed along with the fructosan or starch but they may be a small part of the total hemicellulose. An alternative would be to use as starting material the alcohol-insoluble portion obtained as in 2(b), to hydrolyze the hemicellulose to reducing sugars and to correct for the starch or fructosan which has been hydrolyzed along with the hemicellulose. The correction for starch may be made by deducting the percent starch (previously determined on another portion) from the percentage of the total of hemicellulose plus starch. The correction for fructosan cannot be made in that way, as fructose is partly destroyed by the acid hydrolysis and the correction must be for that part only of the fructose remaining undestroyed in the hydrolyzate and which must be measured in a separate fructose determination.

The conditions of hydrolysis vary and are somewhat empirical and the products are mixtures of sugars. The total reducing power may be expressed as glucose, or distinctions may be made between hexoses and pentoses, or between fermentable and non-fermentable sugars. Neutralization and clarification must follow hydrolysis and precede any measurement of reducing power.

References:

General, committee report -- Millaman et al, P. P. 2, 93(1927).

General -- Norman, 63(1937); also M. Phillips, J.A.O.A.C. 23, 124(1940); also Loonis and Shull, 290(1937).

Hydrolysis by boiling with 2.5% HCl for 2.5 hours - Murneek, P. P. 1,10(1926).

Hydrolysis by boiling with about 1% HCl for 2.5 hours -- Johnston and Dore, P. P. 4, 39(1929).

Hydrolysis in a boiling water bath with 0.7 N HCl for 3 hours - Hasselbring, P. P. 2, 235(1927).

Hydrolysis in a boiling water bath with 2.5% H_2SO_4 for 2.5 hours -- Clements, P. P. 3, 446(1928).

Hydrolysis on a boiling water bath with 1.1% HCl for 4.25 hours, at a high altitude -- Weinmann, P. P. 15, 472(1940). This time may be shortened at low altitudes.

Nonfermentable sugars (galactose, pentoses) may be distinguished from fermentable sugars (glucose, mannose) in the hydrolyzate by treating the hydrolyzate with yeast. The reducing power after fermentation is a measure of the non-fermentable sugars; the difference in reducing power between yeast-treated and untreated aliquots represents the fermentable sugars. For the procedure of fermentation -- Phillips and Smith, New Hampshire Tech. Bull. 81,6(1943); also Gawadi, P. P. 22, 438(1947).

When galatose is known to be absent the nonfermentable reducing power is a measure of pentoses released by acid hydrolysis. It is probable that this figure also represents total pentose.

Pentosans may also be determined by distillation of the original plant material with 12% HCl and precipitation of the furfural with phloroglucinol - Methods, Section 27, 38(1945).

6. STRUCTURAL CONSTITUENTS

The structural constituents are the celluloses and lignin (For hemicelluloses see 5). Each is determined as the residual material after the removal of substances less resistant to various reagents. Use the original dried and unextracted material as in 2(a) or use the residue from any of the preceding treatments.

(a) Cellulose.

Cellulose may be determined as "crude" or "natural" celluloso and as "true" celluloso, the difference being that the former contains the associated cellulosan.

References:

General -- Norman,17 (1937).

A method for "natural" cellulose, with the removal of lignin and hemicellulose by alternate treatment with hypochlorite and sulfite solutions; the method of Norman and Jenkins; very tedious and probably approximate -- Matrone et al, J. Animal Sci. 5, 307(1946); also Ellis, J.A.O.A.C. 32, 289(1949).

A method for "true" cellulose with the removal of carbohydrates and lignin by a mixture of nitric and acetic acids; a more rapid method; the product intended to be free, but probably not entirely so, from cellulosans; the method of Kuerschner and Hanak -- Crampton and Maynard, J. Nutrition 15, 391 (1938).

A modification of the above, omitting the use of the centrifuge -- Phillips and Smith, New Hampshire Tech. Bull. 81, 4(1943).

A variation of the above,recommended as a rapid method for crude fiber, but essentially a method for "true" cellulose -- Whitehouse et al, J.A.O.A.C. 28, 1948(1945).

(b) Lignin

Lignin is separated from cellulose by solution of the latter in 72% H_2SO_4. The residue is lignin. Different methods vary as to the preliminary steps to remove lipoids, protein, and hydrolyzable carbohydrates before treatment with the 72% acid, and in other respects. All methods are tedious and capable of improvement in speed and accuracy.

References:

General -- Norman, 167(1937); also M. Phillips, J.A.O.A.C. 23, 110(1940).

A method using pepsin and 5% H_2SO_4 in turn to remove protein; convenient filtration with fritted glass filter tubes; a two-hour contact with 72% H_2SO_4 at 20°. -- Ellis et al, J. Animal Sci. 5, 290(1946); also Ellis, J. A. O.A.C. 32, 288(1949).

A method using pepsin to remove protein; a 15 minute period of contact with 72% H SO in the presence of formaldehyde.--Crampton and Maynard, J. Nutrition 15, 390(1938).

Supplements to "Guide to the Carbohydrate Analysis of Forage Plants"

Supplement A

Method for the preservation and extraction of plant material with the use of the Waring Blendor.

Cut the material in small conveniently sized pieces and drop into boiling 95% alcohol, using about 4 ml. alcohol per gram of fresh sample. Cover with glass and boil gently on the steam bath for about 15 minutes. Cool, decant the supernatant liquid into a volumetric flask, transfer the solid pieces to the jar of a Waring blendor, using cold 60% alcohol to cover the pieces, and grind for 7 minutes. Pour into a Buchner funnel fitted with a Whatman #2 filter paper and wash with 60% alcohol. Make one final washing with 95% alcohol. Add the filtrate and washings to the volumetric flask and dilute to the mark with 60% alcohol. Remove the insoluble portion from the paper before it is completely dry.

Supplement B

Method for the Determination of Reducing Sugars. This is a modification of the method of Phillips, J.A.O.A.C. 24, 181-2(1941).

I. Reagents:

(a) Oxidizing solutions - (1) Dissolve exactly 20 g. $CuSO_4 \cdot 5H_2O$ in water containing 2 drops conc. H_2SO , and dilute to 1 liter. (2) Dissolve 50 g. Rochelle salts, 50 g. Na_2CO_3 (anhyd.) and 40 g. $NaHCO_3$ in water and dilute to 1 liter.

(b) Prepare a dilute suspension of $Al(OH)_3$ by diluting ordinary alumina cream without supernatant liquid with 19 parts water. Shake before using.

(c) Dissolve 100 g. cryst. ferric ammonium sulfate in 1 liter water.

(d) Dissolve about 300 ml. conc. H_2SO_4 and about 60 ml. 85% H_3PO_4 in water and dilute to 1 liter.

(e) Dissolve 0.75 g. orthophenanthroline monohydrate and 0.25 g. ferrous sulfate in water and dilute to 1 liter.

(f) Dissolve 3.32 g. ferrous sulfate in water and dilute to 1 liter.

(g) Prepare a 0.01 N solution of ceric sulfate as follows: weigh out sufficient $Ce(SO_4)_2$ or $Ce(HSO_4)_4$, depending on the purity of the salt, to contain 1.403 g. Ce. Dissolve with heat in 600 ml. water containing 50 ml. conc. H_2SO_4. Cool. Dilute to 1 liter with water.

To standardize weigh out accurately about 0.33 g. pure sodium oxalate (Bureau of Standards sample), dissolve in water containing 10 ml. conc. HCl, and dilute to ½ liter with water. Into a flask pipette a 50 ml. aliquot of the oxalate solution, and add from a burette a slight excess (not over 1.0 ml. excess) of the ceric sulfate, or until the yellow color persists after warming to 40 degrees. Add 1 ml. indicator (e) and back titrate with the ferrous solution (f) to the appearance of the orange color. Deduct the back titration from the volume of ceric sulfate and deduct a blank on the indicator. Calculate the normality of the ceric sulfate. Normality equals g. oxalate/(ml. ceric sulfate x 0.67). Dilute if necessary to 0.01 N with 5% H_2SO_4.

II. Reduction procedure:

In a 50 ml. Pyrex round bottom centrifuge tube place 5 ml. of each of the oxidizing solutions (a). Add 10 ml. of sugar solution containing 0.25 to 4.0 mg. reducing sugars (If a smaller volume is sufficient, add water to make up the difference). Mix, cover with a loose glass stopper, and immerse for exactly 15 minutes in a boiling water bath at such a depth that the level of the water in the bath is 1 inch above the bottom of the tube and the top of the tube protrudes 1 inch above the cover of the bath through a close fitting but not tight hole. (Eight determinations are made at a time in a circular bath of 8 inch diameter with an 8-hole plywood cover). Remove and cool in running water. Add about 10 ml. of $Al(OH)_3$ suspension (b) and centrifuge 5 minutes at 2000 R.P.M. Decant and discard the supernatant liquid. Wash the copper precipitate by adding about 25 ml. water, shaking gently, centrifuging, and decanting. Wash twice, if the sugar solution contained iodide or oxalate salts; otherwise once is sufficient.

III. Titration of reduced copper:

To the washed copper oxide add about 10 ml. ferric ammonium sulfate (c), transfer to a small flask, rinse the tube once with about 5 ml. acid solution (d) and a few times with water, adding the rinsings to the flask. Add 1 ml. indicator solution (e) and titrate with ceric sulfate (g) in a burette reading to divisions of 0.02 ml. or less. Deduct a blank determination (about 0.3 ml.). Calculate reducing sugar either from a curve prepared by the use of glucose or invert sugar or from an equation similarly prepared by the method of least squares.

The method of least squares is as follows:
Let $y = nx + k$, when y = mg. sugar and x = mg. Cu.
The best values for n and k are obtained by solving the equations:

$Sy = n \cdot Sx + N \cdot k$

$Sxy = n \cdot Sx^2 + Sx \cdot k$, when N is the number of pairs of values of x and y obtained experimentally.

Supplement C

Method for the determination of fructose in the presence of aldose.

Supplement C (cont'd)

I. Reagents:

(h) Dissolve 25.44 g. iodine and 34.0 g. potassium iodide in water and dilute to 1 liter.

(i) Prepare immediately before using a 0.3 N solution of sodium sulfite (1.9g/100 ml.) in water, and from this, by dilution, solutions of 0.03 N and 0.01 N.

(j) Prepare 0.25 N NaOH, 1.0 N NaOH, and 0.33 N H_2SO_4.

(k) Prepare solution of 0.1 g. alpha-napthoflavone in 100 ml. alcohol.

II. Determination of fructose:

Place 5 ml. of solution containing 0.5 to 4.0 mg. fructose in a centrifuge tube weighed to 0.05 g. Cool in refrigerator. If not neutral, adjust to neutrality or faint alkalinity. Add 1.0 ml. iodine solution (h) and 1.0 ml. 0.25 N NaOH. Stopper and place in refrigerator (about 8 degrees C) for 2 hours. Add 0.85 ml. 0.33 N H_2SO_4 . Decolorize the iodine with the sulfite solutions (i), using 0.3 N for most of it, finishing with 0.03 N and finally with 0.01 N sulfite, adding about 3 drops indicator (k) before obtaining the final end-point and avoiding an excess of sulfite of more than 1 drop of 0.01 N. Adjust to neutrality with 1.0 N NaOH and 0.33 N H_2SO_4 with methyl red. Add water to bring the contents of the tube to 10.1 g. Add 5 ml. of each of the oxidizing solutions (a) and continue the determination of the reducing power as in Supplement A. Standardize the procedure with fructose in the presence of iodine of the same concentration as above.

Supplement D

Method for the determination of total sugars and of sucrose

Place 5 ml. of solution containing 0.25 to 4.0 mg. total sugars in a centrifuge tube weighed to 0.05 g. Add 6 drops conc. HCl and place in a water bath at 70 degrees C temperature for 7 minutes. Cool, neutralize with 1.0 N NaOH and adjust with 0.33 N H_2SO_4 to methyl red. Add water to bring the contents of the tube to 10.1 g. Add 5 ml. of each of the oxidizing solutions (a) and continue the determination of the reducing power as in Supplement B. Calculate either as sucrose or as invert sugar. This is total sugars. To obtain sucrose deduct the reducing sugars determined in another aliquot carried out without hydrolysis. If the results are expressed as invert sugar, multiply by 0.95 to express as sucrose.

Supplement E

Determination of fructosan

I. Place a weighed quantity of alcohol-insoluble portion of a grass sample containing 8-80 mg. fructosan in a small Erlenmeyer flask and add 50 ml. 0.25% oxalic acid. Cover loosely and place in a water bath at 80 degrees C for 1 hour. Cool, neutralize with 0.1 N NaOH and 0.33% H_2SO_4, leaving acid to phenolphthalein. Transfer to a 100 ml. volumetric flask, add 1 ml. saturated neutral lead acetate, dilute to the mark with water, shake, allow to settle and filter. To the filtrate add sufficient solid anhydrous sodium sulfate to precipitate all the excess lead. Filter. Determine fructose in a 5 ml. aliquot of the clear filtrate. Correct, if necessary, for the volume of the insoluble residue in the volumetric flask.

II. An optional procedure is to hydrolyze fructosan on the bath at 80 degrees as above. While still warm add about 1 g. powdered calcium carbonate and stir. Cool, neutralize to phenolphthalein with a saturated solution of barium hydroxide, make slightly acid with dilute sulfuric acid, filter into a volumetric flask, washing the residue and precipitate with warm water. Cool the filtrate, dilute to the mark with water. If necessary, filter again, and use 5 ml. of clear filtrate for fructose determination, as in Supplement C.

Supplement F

Determination of starch

Place a weighed quantity of the alcohol-insoluble portion of legume plants containing at least 3.0, preferably about 50 mg, starch in a small flask, add 50 ml. water, and heat in a boiling water bath under reflux 1/2 hour. Cool, add 10 ml. acetate buffer of pH 4.5, 0.1 g. undiluted takadiastase, and a few drops of toluene. Close with a cork stopper, and incubate for 5 days, at least 2 of which are at 38 degrees C. Remove toluene by warming the open flasks on the steambath, transfer to a 100 ml. volumetric flask, cool, and clarify, meanwhile diluting to the mark. Determine reducing power as glucose on the cleared filtrate and convert to starch by multiplying by 0.9.