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PROCEDURES FOR ANALYSIS OF SOLIDS AND LIQUORS

FROM

CELLULOSIC SOURCES

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Presented herein are the procedures we currently use to analyze cellulosic materials from all sources as well as the liquors and solids produced from these materials through our own treatments and hydrolyses.

GENERAL SCHEME

As shown in Fig. 1, solid materials are first ground or milled to a suitable size. Then they are analyzed for ash, determined as the residue after ignition at 600°C; for extractives, defined as the material removed by extraction with the benzene/ethanol azeotrope; for lignin, the organic residue after a 2-stage hydrolysis in 72 w/w% and 4 w/w% H_2SO_4; and for carbohydrates.

The carbohydrate content is determined as the monomer sugars generated by exhaustive hydrolysis of the solid, as measured by the glucose oxidase-peroxidase and gas chromatographic methods. The content of each monomer is then converted to the weight of the sugar in the polymerized form.

Liquors, such as those generated by the acid or base treatments of cellulosic materials or enzymatic hydrolyses of same are analyzed as follows: the monomer sugar content is measured by the glucose oxidase-peroxidase and gas chromatography methods. Each liquor is then exhaustively hydrolyzed in 4% H_2SO_4. By this procedure, all polymerized sugar is hydrolyzed to monomer form. The resultant increase in monomer sugar content is labelled as polymerized sugar.

Procedures for Solids [1]

1. Milling and Sieving

If 100 gm or more of air dry material is available, it is ground in a Wiley mill through a 2 mm screen. The ground material is sieved through 0.25mm

and 0.35 mm opening screens to collect the 0.25 mm to 0.35 mm fraction, which is used for subsequent analysis. With smaller amounts of material, grinding with a Waring blender suffices.

The material is dried in an 105°C oven for at least 4 hours or until no weight change occurs with further drying. This dry material is used for subsequent analysis. Once a material is dried it must be stored in desiccator or sealed container to avoid moisture uptake. Alternatively, a sample of the air dry material can be assayed for moisture content by the change in weight after drying at 105°C.

2. Extraction

An accurately weighed portion of the dry material is placed in a tared glass extraction crucible equipped with a coarse (40-60 μ pore size) or extracoarse (170-220 μ pore size) fritted glass filter. The material is extracted in a soxhlet apparatus with benzene/ethanol (2:1) for at least 6 hours or until no change in weight occurs after additional extraction. The material is sucked reasonably dry and placed in a 105°C oven to dry. The change in weight of the material during extraction is taken as the extractives content, i.e.,

$$\% \text{ Extractives} = \frac{(\text{Gross wt. pre-extraction}) - (\text{Gross wt. post extraction})}{(\text{Gross wt. pre-extraction}) - (\text{Tare wt.})} \times 100.$$

$$\text{Extraction Factor} = \frac{100 - (\% \text{ extractives})}{100}$$

The extraction factor above is used to correct values of the assays performed on extracted material, subsequently.

3. Exhaustive Hydrolysis

The dry extracted material is exhaustively hydrolyzed to yield a sugar solution and acid insoluble residue as follows: 72 w/w% H_2SO_4 is made by

carefully adding 665 ml of concentrated H_2SO_4 to 300 ml H_2O in a one-liter volumetric flask placed in an ice bath. After cooling the solution is brought up to one liter with H_2O. The acid strength may have to be adjusted at this point due to the irregularities in acid strength of concentrated H_2SO_4. A portion of solution can be diluted and titrated with standard base to determine its normality. 72 w/w% H_2SO_4 is equivalent to 24.1 N.

A convenient amount of dry extracted material is accurately weighed and placed in a vial equipped with a teflon-lined screw top. 0.5 gm of material and a 9.5 dram vial are convenient sizes. One ml of 72 w/w% H_2SO_4 is added per 0.1 gm of material. The slurry is mixed on a vortex mixer, allowed to stand 3 minutes and mixed again. The vials are placed in a 30°C bath for one hour during which time they are removed and mixed at least three times. The slurry is quantitatively transferred to a 250 ml erlenmyer flask using 28 ml H_2O per ml of 72 w/w% H_2SO_4 used. This dilution produces 4 w/w% H_2SO_4 solution for the secondary hydrolysis. The flasks are capped with watch glasses and placed in a preheated autoclave (we use the horizontal electrical type exclusively). The flasks are heated for one hour at which time the autoclave is slowly allowed to depressurize to prevent the samples from boiling over.

The hot solutions are individually filtered through dry procelain filtering crucibles. The residues are washed repeatedly with hot H_2O. The crucibles are dried in the 105°C oven, cooled in a desiccator and weighed. They are then placed in a muffle furnace (600°C) and ashed for at least 6 hours or until no weight change occurs after additional heating. The ashed crucibles are cooled in a desiccator and weighed. The change in weight after ashing is taken as the total acid insoluble content of the extracted material. This value is multiplied by the extraction factor to determine the content in the unextracted material, i.e.,

% Total Acid Insolubles =

$$\frac{\text{(Gross wt. residue \& crucible) - (Gross wt. after ashing)}}{\text{(wt. starting sample)}} \times$$

(extraction factor) x 100.

The supernates and collected washings are neutralized to pH 5-6 by addition of $CaCO_3$ (1.4 gm of $CaCO_3$ per 1 ml of 72% H_2SO_4 used). The $CaCO_3$ must be added slowly since foaming is a big problem. Addition of a drop antifoam (10% solution GE Antifoam 60) helps here. The neutralized solution is filtered free of $CaSO_4$ precipitate through a medium or coarse porosity fritted glass funnel. The precipitate is washed several times with H_2O. The total volume is accurately measured by dilution to a fixed volume in a volumetric flask. A portion of the supernate is then centrifuged to remove any residual solid and saved for further analysis. It can be stored by freezing or addition of a preservative (e.g., 0.01% merthiolate) and refrigerating.

4. Carbohydrate Analysis

Liquors are analyzed by the glucoseoxidase-peroxidase (GOP) and gas-liquid chromatography (GLC) methods. The GOP method is a highly specific assay for monomer glucose. Many variations of the method have been used.[2,3] For our assay the reagent is made as follows: 1200 units of purified glucose oxidase (1 ml of Sigma Chemical Co., type V) and 10 mg peroxidase (Sigma Chemical Co., type II, 125-200 purpurogallin units per 1 mg) is added to 500 ml of 0.1M citrate buffer, pH 5.6. To this add 10 ml of a 1% solution of ortho-dianisidine dihydrochloride (Sigma Chemical Co.) in H_2O. Care must be taken when handling o-dianisidine, for it is a known carcinogen. 8 ml of this reagent is added to 2 ml of sample containing between 10 and 200 µg of glucose in a test tube. The tubes are incubated in a 37°C bath for one hour. The reaction is stopped by addition of 2 drops of concentrated HCl. The

tubes are allowed to stand at least for 5 minutes before reading their absorbance at 420 nm against a reagent blank. The color is stable for several hours. It is a good idea to do color blanks of each solution since there may be absorption at 420 nm by the solution alone. Also when working with new materials one should "spike" these solutions with known amounts of standard glucose to see if the sensitivity changes, an indication that interference is occurring.

A standard curve with at least a few points should be made with each run. The standards should be made from pure glucose and made at least a day in advance so that mutarotation can be accomplished (GOP is specific for B-D - glucose).

Once the glucose content of an hydrolysis liquor is known the value is converted to give the weight of the sugar in the starting solid. This is done by multiplying the concentration of glucose in the supernate by the total volume of the supernate, by a factor converting the weight of glucose as a monomer to its weight as a polymer, and by dividing by a factor to correct for loss of sugar during exhaustive hydrolysis. This gives the glucan content in the extracted material. Multiplying by the extractive factor gives the content in the original material, i.e.,

% Glucan (polymerized glucose) = (glucose, g/l) x (.9, polymerization factor) x (volume supernate, ℓ) ÷ (weight of material assayed, gm) x extraction factor ÷ recovery factor x 100.

Gas-liquid chromatography (GLC) allows quantification of each monomer sugar. Currently we assay for arabinose, xylose, mannose, galactose, and glucose. In order for sugars to be analyzed by GLC they must be freed of H_2O and made volatile by derivatization. There are many types of derivatives that can be formed and methods for doing it.[4-7] Probably the most common type and the one

we use are the trimethylsilyl derivatives (TMS). The procedure we use is as follows.

A volume of sugar solution containing from 1 to 10 mg of sugar is freeze dried to remove all H_2O. The dry residue is brought up in 1 ml of dimethylsulfoxide solvent, containing about 5 g/l 2-OH-pyridine and 2 g/l myo-inositol, in a 1 dram vial with a teflon lined screw top. The vial is placed in a 40°C oven and allowed to equilibrate at least 6 hours, preferably overnight. After equilibration, 1 ml of hexamethyldisilazane and 1/2 ml of chlorotrimethylsilane are added to the vial which is mixed and allowed to stand for about 1 hour. Two phases are formed. The lower phase is discarded. To the upper phase is added an equal volume of H_2O. After mixing the two phases are allowed to separate and the lower phase is discarded. The upper is dried by addition of anhydrous Na_2SO_4. The derivatized sugars are now ready for analysis.

Preparation of the column for GLC has been covered at length by Malcolm Evett (8) and others (9). Suffice it to say that a column from 1-3 ft. in length containing a liquid phase of low to moderate polarity (e.g. SE30, OV-17, SF96) on a diatomacrous earth support (e.g. Chromasorb G or W) which has been acid washed and silylated should be used. We have had success with a low liquid coatings (1-3%). Using a Aerograph 1520 gas chromatograph, equipped with a temperature programmer we have found that an initial temperature of 120°C to 150°C followed by a programmed temperature rise of 8°C per minute to be a good combination to give a short analysis time with good resolution. Carrier gas flows of 20-50 cc/min., air flow of 300-500 cc/min., and H_2 flows of 20-30 cc/min. were used.

Provided that a good flat baseline can be achieved the recorder peaks can be measured with a disc integrator. We use a Leeds and Northrup Speedomax G Model S Recorder equipped with a Disc Chart Integrator-Model 203. The

sugar derivative peaks are quantified in terms of the area of the internal standard peak (myo-inositol). For each run the sugar peak areas are divided by the area of the internal standard peak, i.e.,

$$\text{Sugar peak ratio units} = \frac{\text{Area sugar peak x attenuation}}{\text{Area internal standard peak x attenuation}}$$

This ratio is then compared to a standard curve made by analyzing standard sugar solutions by the above method and plotting standard sugar ratio units vs. weight of sugar.

Once the quantity of a sugar is determined by GLC it can be converted back to its weight in the starting material in much the same way as described for glucose in the GOP method, i.e.,

% Polymerized sugar in solid = (sugar in solution, g/l) x (polymerization factor) x (volume supernate, l) ÷ (volume freeze dried, ml/1 ml DMSO solvent) ÷ (recovery factor) ÷ (weight of material assayed, gm) x extraction factor x 100.

A listing of the polymerization and recovery factors for each sugar is shown in Figures 3 and 4.

5. H_2O Wash

Material which has been extracted with benzene/ethanol is now washed with hot H_2O to remove acid insoluble material other than lignin. This is done by placing a known weight of material in a tared glass extraction crucible into a soxhlet apparatus. Boiling chips and antifoam should be added to the H_2O in the distillation flask to prevent bumping or foaming. The material should be washed overnight. After drying in the 105°C oven the washed material is weighed, and an H_2O extraction factor is determined as follows:

$$H_2O \text{ Wash Factor} = \frac{\text{(Gross wt. post wash) - (Tare wt.)}}{\text{(Gross wt. pre wash) - (Tare wt.)}}$$

6. Exhaustive Hydrolysis

An exhaustive acid hydrolysis is preformed on the dry H_2O washed material. The procedure is exactly as before, the only exception being that the liquor generated by the hydrolysis is neither neutralized or saved. The residue collected in the dry procelain crucible is washed, dried, weighed, ashed, and weighed again. The change in weight after ashing is taken as the lignin content of the extracted, H_2O washed material. Multiplication by the extraction and H_2O wash factors gives the lignin content of the starting material, i.e.,

$$\% \text{ Lignin} = \frac{(\text{Gross wt. dry crucible \& lignin}) - (\text{Gross wt. ashed crucible})}{(\text{wt. of dry starting material})} \times (\text{extraction factor}) \times (H_2O \text{ wash factor}) \times 100.$$

The difference between the total acid insolubles content and the lignin content gives the acid insolubles content of the starting material, i.e.,

% Acid Insolubles = (% total acid insolubles) - (% lignin)

PROCEDURES FOR LIQUORS

Liquors are analyzed by the GOP and GLC methods as described previously. These analyses give the monomer sugar content of the liquors. To determine the total sugar content of the liquor it is exhaustively hydrolyzed as follows: a 4% H_2SO_4 solution is made by adding 1 ml of 72% H_2SO_4 to 28 ml of liquor or combined liquor and H_2O. The acid solution is heated in an autoclave (we use the horizontal electrical kind) for 1 hour and then cooled slowly. Anti-foam and 1.4 gm $CaCO_3$ are added per 1 ml 72% H_2SO_4 used to neutralize the solution. The $CaSO_4$ solid is filtered off with a coarse or medium porosity fritted glass filter funnel. The total volume is measured using a volumetric flask. A sample is further cleared by centrifugation if necessary and stored.

The liquor generated by this hydrolysis is analyzed by GOP and GLC. The

total sugar content of the original liquor is found by multiplying by the dilution and by dividing by the recovery factor (Fig. 4), i.e.,

$$\text{Total Sugar Content} = (\text{Sugar in hydrolyzed liquor, g/}\ell) \times \frac{\text{final volume}}{\text{initial volume}} \div (\text{recovery factor}).$$

The difference between the total sugar content and the monomer sugar content gives the content of sugar which was polymerized in the starting liquor. Multiplying by the polymerization factors (Fig. 3) gives the weight of this sugar in the starting liquor, i.e.,

$$\text{Polymerized sugar} = \{(\text{Total sugar content}) - (\text{Monomer sugar content})\} \times \text{polymerization factor}$$

CONCLUSION

The percentages of the solids will total to less than 100% since not all materials are assayed for, including protein and sugar derivatives. Also the ash content measurement can be inaccurate since heating the residue to 600°C causes loss of volatiles and oxidation to occur.

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FIGURE 1 - SCHEME OF SOLIDS ANALYSIS

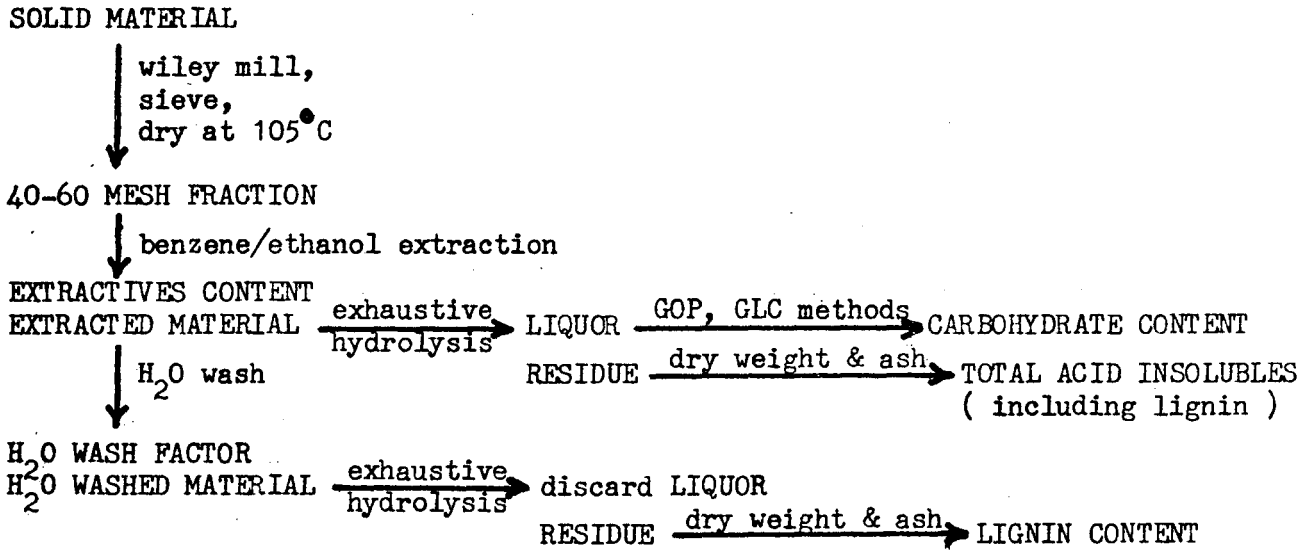

FIGURE 2 - SCHEME OF LIQUORS ANALYSIS

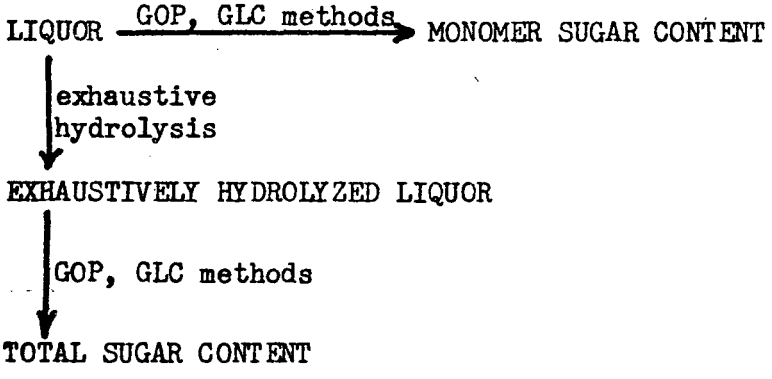

FIGURE 3 - POLYMERIZATION FACTORS (1)

SUGAR	POLYMERIZATION FACTOR
Arabinose	0.88
Galactose	0.9
Glucose	0.9
Mannose	0.9
Xylose	0.88

FIGURE 4 - RECOVERY FACTORS (1)

SUGAR	RECOVERY FACTOR
Arabinose	0.924
Galactose	0.957
Glucose	0.950
Mannose	0.916
Xylose	0.866

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