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Standard Test Method for Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography[1]

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INTRODUCTION

The carbohydrates making up a major portion of biomass samples are polysaccharides constructed primarily of glucose, xylose, arabinose, galactose, and mannose subunits. The polysaccharides present in a biomass sample can be hydrolyzed to their component sugar monomers by sulfuric acid in a two-stage hydrolysis process. These monosaccharides can then be quantified by ion-moderated partition HPLC.

1. Scope

1.1 This test method covers the determination of carbohydrates present in a biomass sample, expressed as the percent, by mass, of each sugar on a 105°C dried mass basis.

NOTE 1—The percent sugar must be corrected for the water of hydrolysis before calculating the actual mass percent of the polysaccharide in the original biomass sample.

1.2 Sample materials suitable for this procedure include hard and soft woods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), wastepaper (such as office waste, boxboard, and newsprint), acid or alkaline-pretreated biomass (washed free of any residual acid or alkali), and the solid fraction of fermentation residues. All results are reported relative to the 105°C oven-dried mass of the sample.

1.3 The options for the types of samples to be analyzed in this test method are as follows:

1.3.1 *Prepared Biomass Samples:*

1.3.1.1 *Air Dried ($\%T_{ad}$)*—The percent, by mass, of total solids of the air-dried sample.

1.3.1.2 *45°C Dried ($\%T_{45}$)*—The percent, by mass, of total solids of the 45°C dried sample.

1.3.1.3 *Freeze Dried ($\%T_{fd}$)*—The percent, by mass, of total solids of the freeze dried sample.

1.3.2 *Extractives-Free Sample ($\%T_{ext}$)*—The percent, by mass, of total solids of the extracted sample determined at 105°C.

1.4 The values stated in SI units are to be regarded as the standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Note 3 *and Note 4.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

E1690 Test Method for Determination of Ethanol Extractives in Biomass

E1721 Test Method for Determination of Acid-Insoluble Residue in Biomass

E1756 Test Method for Determination of Total Solids in Biomass

E1757 Practice for Preparation of Biomass for Compositional Analysis

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *as received biomass*—the biomass material as it is received in its field or process collected state.

3.1.2 *oven-dried mass*—the moisture-free mass of a biomass sample dried at 105°C as described in Test Method E1756.

[1] This test method is under the jurisdiction of ASTM Committee E48 on Bioenergy and Industrial Chemicals from Biomass and is the direct responsibility of Subcommittee E48.05 on Biomass Conversion.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

3.1.3 *prepared biomass*—material that has been treated according to Practice E1757 in order to raise the total solids content above 85 %, by mass, based on an oven-dried solids mass.

3.2 *Abbreviations*—Abbreviations of standards used in the procedure, and definitions of terms used in the calculations are as follows:

3.2.1 C_1—known concentration of sugar recovery standard before hydrolysis, in mg/mL.

3.2.2 C_2—concentration of sugar recovery standard detected by HPLC after hydrolysis, in mg/mL.

3.2.3 C_{corr}— concentration of sugar in hydrolyzed sample corrected for hydrolysis, in mg/mL.

3.2.4 C_{spl}— concentration of sugar in hydrolyzed sample detected by HPLC, in mg/mL.

3.2.5 *CVS (calibration verification standard)*— standards used in determining the quality of the calibration curve as well as the quality of the standard reagents used in preparing the calibration standards.

3.2.6 m_1—initial mass of sample, in mg.

3.2.7 *% extractives*—the percentage, by mass, of extractives in the prepared biomass sample as described in Test Method E1690.

3.2.8 $\%R_{srs}$— percent recovery of sugar recovery standard, as determined in 13.2.

3.2.9 $\%sugar_{extractives\text{-}free}$—the percentage, by mass, of sugar on an extractives-free 105°C dry weight basis, as determined in 13.6.1.

3.2.10 $\%\ sugar_{whole\ sample}$—the corrected mass percent sugar value on an extractives-free basis corrected to an as received (whole sample) 105°C dry mass basis.

3.2.11 $\%T_{45}$—percentage, by mass, of total solids of the sample prepared by drying at 45°C, as described by Practice E1757.

3.2.12 $\%T_{105}$—percentage, by mass, of total solids in the sample, dried at 105°C, as determined by Test Method E1756.

3.2.13 $\%T_{ad}$— percentage, by mass, of total solids of the air-dried sample determined at 105°C as described by Test Method E1756.

3.2.14 $\%T_{ext}$— percentage, by mass, of total solids of the extracted sample determined at 105°C as described by Test Method E1756.

3.2.15 $\%T_{fd}$— percentage, by mass, of total solids of the sample prepared by freeze drying, as described by Test Method E1756.

3.2.16 $\%T_{prep}$— percentage, by mass, of total solids of the sample prepared by freeze drying, $\%\ T_{fd}$, or by drying at 45°C, $\%\ T_{45}$, as determined by Practice E1757.

3.2.17 *SRS (sugar recovery standards)*—standards used to determine sugar recovery after hydrolysis.

3.2.18 V_F—volume of filtrate, 87.0 mL.

4. Significance and Use

4.1 The percentage, by mass, of sugar content is used in conjunction with other assays to determine the total composition of biomass samples.

5. Interferences

5.1 Samples with high protein content may result in the percentage, by mass, of sugar values being biased low, as a consequence of protein binding with some monosaccharides.

5.2 Test specimens not suitable for analysis by this procedure include alkaline and acid-pretreated biomass samples that have not been washed. Unwashed pretreated biomass samples containing free acid or alkali may change visibly on heating.

6. Apparatus

6.1 *Analytical Balance,* readable to 0.1 mg.

6.2 *Autoclave,* capable of maintaining 121 ± 3°C.

6.3 *Convection Ovens,* temperature control to 45 ± 3 and 105 ± 3°C.

6.4 *Desiccator,* using anhydrous calcium sulfate.

6.5 *Guard Columns,* cartridges appropriate for the column used.

NOTE 2—Deashing guard column cartridges from BioRad,[3] of the ionic form H^+/CO_3^-, are an option when using an HPX-87P[3] column, or equivalent. These cartridges are effective in eliminating baseline ramping.

6.6 *Hewlett Packard*[4] Model 1090 HPLC, or equivalent, with refractive index detector.

6.7 *HPLC Columns, BioRad* HPX-87C[3] or HPX-87P,[3] or both, or equivalent.

6.8 *Water Bath,* set at 30 ± 1°C.

7. Reagents and Materials

7.1 *Chemicals:*

7.1.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

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[3] The sole source of supply of the apparatus known to the committee at this time is BioRad Aminex®, HPX-87C and Aminex® HPX-87P, available from BioRad, Main Office, 3300 Regatta Boulevard, Richmond, CA 94804. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[4] Available from Hewlett-Packard, HP Analytical Direct, 2850 Centerville Road, Wilmington, DE 19808.

[5] *Reagent Chemicals, American Chemical Society Specifications* , American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

7.1.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type 1 of Specification D1193.

7.1.3 *Calcium Carbonate.*

7.1.4 *High-Purity Sugars (98 % +, By Mass)*—Two sets of glucose, xylose, galactose, arabinose, and mannose, meeting the requirements described above, dried at 45°C. The sugars are used in preparing calibration standards, calibration verification standards (CVS), and sugar recovery standards (SRS). The sugars used in preparing the calibration standards should be from a source (manufacturer or lot) other than that used in preparing the CVS. Either set of sugars may be used for preparing the SRS solutions used in determining sugar recoveries after hydrolysis.

7.1.5 *Sulfuric Acid Solution (72 % w/w or 12.00 ± 0.02 M)*—Slowly add 665 mL of concentrated sulfuric acid (H_2SO_4) to 300 mL of water while cooling in an ice bath and stirring. Allow to come to room temperature. Adjust the relative density to 1.6389 ± 0.0012 at 15.6°C/15.6°C.

7.2 *Materials:*

7.2.1 *Autosampler Vials,* with crimp top seals to fit.

7.2.2 *Disposable Syringes,* 3 mL.

7.2.3 *Disposable Syringe Filters,* nylon, 0.2 µm.

7.2.4 *Glass Serum Bottles,* crimp top style, 125 mL, with rubber stoppers and aluminum seals to fit.

8. Hazards

8.1 Handle the sulfuric acid carefully to avoid contact with skin or clothing, as it is corrosive.

8.2 The glass bottles are hot and may be pressurized after the autoclave step. Use caution when handling.

9. Sampling, Test Specimens, and Test Units

9.1 Test specimens suitable for analysis by this procedure are:

9.1.1 Prepared biomass prepared according to Practice E1757, and

9.1.2 Extractives-free material prepared according to Test Method E1690.

10. Calibration and Standardization

10.1 Prepare a series of three to six sugar standards in deionized water at concentrations appropriate for preparing calibration curves to quantitfy each sugar of interest. An HPX-87C[3] column, or equivalent, is used to analyze glucose, xylose, and arabinose. If mannose and galactose are also to be quantified, an HPX-87P[3] column, or equivalent, must be used instead. Typically, the concentrations of these sugar standards cover the range starting at the detection limit of the instrument and extending up to 4.0 mg/mL.

10.2 Prepare an independent CVS, as described in 8.1.2, for each set of calibration standards, using sugars obtained from a source other than that used in preparing the calibration standards. The CVS will contain precisely known amounts of each sugar contained in the calibration standards, at a concentration in the middle of the validated range of the calibration curve. The CVS will be analyzed after each calibration curve and at regular intervals in the HPLC sequence, as dictated by good laboratory practice. The CVS is used in confirming the quality of the calibration curve(s) and the standard reagents used in preparing the calibration standards. An additional benefit is obtained by bracketing groups of samples in the sequence with the CVS, assuring the analyst of the quality of the calibration curve throughout the run.

11. Procedure

11.1 An overview of the overall analytical sequence is as follows:

11.1.1 Hydrolysis of sample with 72 % sulfuric acid,

11.1.2 Hydrolyzate dilution and autoclaving,

11.1.3 Filtration of insolubles if separate analysis is desired,

11.1.4 Neutralization of hydrolyzate,

11.1.5 Filtration of sample prior to HPLC analysis,

11.1.6 HPLC analysis of sugar standards, CVS, SRS, and hydrolyzate samples, and

11.1.7 Calculation of sugar contents.

11.2 For prepared biomass samples, determine the total solids by Test Method E1756 and record the total solids value as $\%T_{105}$. This prepared sample should be stored in a manner to ensure its moisture content does not change before the analysis begins.

11.2.1 If Test Method A of this practice is used (air drying), determine the total solids content of this prepared sample by Test Method E1756 and record the total solids value as $\%T_{ad}$.

11.2.2 If Test Method B of this practice is used (drying at 45°C), record the total solids calculated in this practice, $\%T_{45}$, as $\%T_{prep}$.

11.2.3 If Test Method C of this practice is used (freeze drying), record the total solids calculated in this practice, $\%T_{fd}$, as $\%T_{prep}$.

11.3 If extractives-free material is used, determine the total solids content of the extractive-free material by Test Method E1756 and record this value as $\%T_{ext}$.

11.4 Weigh 300 ± 10 mg of the prepared or extractives-free sample to the nearest 0.1 mg and place in 16x 100 mm glass test tube. Record as m_1, the initial mass of sample in grams.

NOTE 3—**Warning:** 72 % w/w sulfuric acid is very corrosive and should be handled by trained personnel only.

11.5 Add 3.00 ± 0.01 mL (4.92 ± 0.01 g) of 72 % w/w H_2SO_4 to the test tube containing the sample and stir for 1 min or until thoroughly mixed.

11.6 Place the test tube containing the sample into the water bath controlled to 30 ± 1°C and hydrolyze for 1h. Stir approximately every 15 min to ensure the sample is completely mixed and wet.

11.7 Weigh out 300 ± 10 mg of each high purity sugar standard (dried at 45°C), described in 8.1.4, to the nearest 0.1 mg and place each in its own individual 16x 100 mm glass test tube. Add acid and hydrolyze these sugars as described in the previous two steps. These SRS's will be taken through the remaining steps in the procedure in parallel with the samples.

The calculated recovery of the SRS will be used to correct for losses caused by the destruction of sugars during the hydrolysis process.

11.8 Transfer each hydrolyzate to a glass bottle and dilute to 4 % w/w acid concentration by adding 84.00 ± 0.04 mL water. Be careful to transfer all the residual solids along with the hydrolysis liquor. The total mass added to the tared bottle is 89.22 g (0.3 g sample, 4.92 g 72 % w/w H_2SO_4, and 84.00 g deionized water). Because the relative density of the 4 % w/w acid solution is 1.0250, the total volume of solution, V_F, is 87.0 mL.

11.9 Stopper the bottles and crimp the aluminum seals into place in preparation for the next step.

11.10 Set the autoclave to a liquid vent cycle to prevent loss of sample from the bottle in the event of a loose crimp seal. Autoclave the sample in the sealed bottle for 1 h at 121 ± 3°C.

NOTE 4—**Warning:** Handle the sealed bottle with caution after the autoclave step, as it may have become pressurized.

11.11 After completing the autoclave cycle, allow the bottles to cool for about 20 min at room temperature before removing the seals and stoppers.

11.12 These autoclaved solutions may also be used for determining acid-insoluble residue or acid-soluble lignin, or both, in parallel with this carbohydrate determination.

NOTE 5—If acid-insoluble residue or acid-soluble lignin, or both, determinations are to be conducted on a sample, the residual solids must be collected by filtering the hydrolyzate through an ashed and weighed filtering crucible before proceeding with the carbohydrate determination. Refer to Test Method E1721 for details. If an acid-soluble lignin determination is to be conducted, a portion of the filtrate must be reserved for analysis. Acid-soluble lignin should be analyzed within 24 h, preferably within 6 h of hydrolysis.

11.13 Transfer a 20 mL aliquot of each hydrolyzate, or filtrate, to 50 mL Erlenmeyer flasks.

11.14 Neutralize with calcium carbonate to a pH between 5 and 6. Do not over-neutralize. Add the calcium carbonate slowly to avoid problems with foaming.

11.15 Filter each neutralized hydrolyzate directly into a capped test tube using a 3 mL syringe with a 0.2 µm filter attached and place in ice bath. If the hydrolyzate is to be analyzed without dilution, filter an additional portion directly into an autosampler vial. If the solution requires dilution, withdraw the necessary amount, dilute as required, then filter the diluted sample into an autosampler vial.

NOTE 6—The initial glucose concentrations of the samples could be determined using an alternative technique, such as a glucose analyzer,[6] to predict whether or not the sugars in the samples will fall within the linear range of the analysis.

11.16 Place the remainder of each filtered sample into the refrigerator as soon as possible, and reserve in case a repeat analysis is required. The samples should be stored for no longer than two weeks.

[6] A YSI, Model 2700 Select, available from Yellow Springs Instrument Co., Inc., Yellow Springs, OH 45387, has been found to be satisfactory for this purpose.

11.17 Analyze the calibration sugar standards, the CVS's, the hydrolyzed SRS's, and the hydrolyzed samples by HPLC using either the HPX-87C[3] or HPX-87P[3] column, or their equivalents, as described in 11.1. For many analyses, it is useful to run the same samples on both columns and compare the results.

11.18 The following instrumental conditions are used for both the HPX-87C[3] and the HPX-87P[3] columns, or their equivalents:

11.18.1 *Sample Volume*—50 µL,

11.18.2 *Eluant*—0.2 µm filtered, 18 megohm deionized water (de-gassed with helium or vacuum),

11.18.3 *Flow Rate*—0.6 mL/min,

11.18.4 *Column Temperature*—85°C,

11.18.5 *Detector*—refractive index, and

11.18.6 *Run Time*—20 min data collection, 15 min post-run.

12. Calculation

12.1 Create a calibration curve by linear regression analysis for each sugar to be quantified. From these curves, determine the concentration in mg/mL of the sugars present in each solution analyzed by HPLC.

12.2 Calculate the amount of sugar recovered, in percent, from each sugar recovery standard taken through the two-stage hydrolysis. This will estimate the amount of each individual sugar destroyed during the hydrolysis procedure:

$$\%\ recovery_{srs} = \frac{C_2}{C_1} \times 100\,\% \tag{1}$$

where:

C_1 = known concentration of sugar recovery standard before hydrolysis, in mg/mL, and

C_2 = concentration of sugar recovery standard detected by HPLC after hydrolysis, in mg/mL.

12.3 Use the percentage recovery of the sugar recovery standard to correct sugar concentration values (in mg/mL) obtained from HPLC for each hydrolyzed sample:

$$C_{corr} = \frac{C_{spl}}{\frac{\%R_{srs}}{100\,\%}} \tag{2}$$

where:

$\%R_{srs}$ = percent recovery of sugar recovery standard, as determined in 12.2,

C_{corr} = concentration of sugar in hydrolyzed sample corrected for hydrolysis, in mg/mL, and

C_{spl} = concentration of sugar in hydrolyzed sample detected by HPLC, in mg/mL.

12.4 If the biomass was prepared according to Part A of Practice E1757, calculate the percent, by mass, of each sugar present in the as-received sample, on a 105°C dried mass basis as follows:

$$\%\ sugar = \frac{C_{corr} \times V_F}{m_1 \times \frac{\%T_{ad}}{100\,\%}} \times 100\,\% \tag{3}$$

where:

m_1 = initial mass of sample, in mg,
V_F = volume of filtrate, 87.0 mL,
C_{corr} = concentration of sugar in hydrolyzed sample corrected for hydrolysis, as determined in 13.3, in mg/mL, and
$\%T_{ad}$ = percent, by mass, of total solids of the air-dried sample determined at 105°C as described by Test Method E1756.

12.5 If the biomass was prepared according to Part B or C of Practice E1757 calculate the percent, by mass, of each sugar present in the as-received hydrolyzed sample, on a 105°C dried mass basis as follows:

$$\%\ sugar = \frac{C_{corr} \times V_F}{m_1 \times \frac{\%T_{105}}{\%T_{prep}}} \times 100\,\% \quad (4)$$

where:

C_{corr} = corrected sugar concentration of the hydrolyzed sample, in mg/mL,
V_F = volume of filtrate, 87.0 mL,
m_1 = initial mass of prepared sample, in mg,
$\%T_{105}$ = percent, by mass, of total solids in the sample, dried at 105°C, as determined by Test Method E1756, and
$\%T_{prep}$ = percent, by mass, of total solids of the sample prepared by freeze drying, $\%T_{fd}$, or by drying at 45°C, $\%T_{45}$, as determined by Practice E1757.

12.6 If the biomass was prepared according to Test Method E1690, first calculate the percent, by mass, of each sugar present on an extractives-free 105°C dried mass basis and then correct this value to an as received (whole sample) 105°C dried mass basis.

12.6.1 Calculate the percent, by mass, of sugar present on an extractives-free basis as follows:

$$\%\ sugar_{extractives-free} = \frac{C_{corr} \times V_F}{m_1 \times \frac{\%T_{ext}}{100\,\%}} \times 100\,\% \quad (5)$$

where:

C_{corr} = corrected sugar concentration of the hydrolyzed sample, in mg/mL,
V_F = volume of filtrate, 87.0 mL,
m_1 = initial mass of extracted sample, in mg, and
$\%T_{ext}$ = percent, by mass, of total solids of the extracted sample determined at 105°C as described by Test Method E1756.

12.6.2 Correct the percent, by mass, of sugar value on an extractives-free basis, calculated above, to an as received (whole sample) 105°C dried mass basis as follows:

$$\%\ sugar_{whole\ sample} = \%\ sugar_{extractives-free} \times \frac{(100\,\% - \%\ extractives)}{100\,\%} \quad (6)$$

where:

$\%\ sugar_{extractives\text{-}free}$ = the percent, by mass, of sugar on an extractives-free 105°C dried mass basis, as determined in 12.6.1, and
$\%\ extractives$ = the percent, by mass, of extractives in the extracted sample as described in Test Method E1690.

13. Report

13.1 Report the percent, by mass, of sugar present in the sample, to two decimal places, on a 105°C dried mass basis.

14. Precision and Bias

14.1 Data obtained by replicate testing of glucose in a hybrid poplar in one laboratory, using a HPX-87P[3] column, gave a standard deviation of 1.90 %, and a coefficient of variation percent (CV) of 3.95 %.[7,8]

14.2 Data obtained by replicate testing of glucose in an extractives-free hybrid poplar in five laboratories, using a HPX-87P[3] column, gave a standard deviation of 1.90 %, by mass, and a CV, by mass, of 4.0 %.[7,8]

15. Keywords

15.1 agricultural residue; biomass; carbohydrates; fermentation residue; herbaceous; wastepaper; wood

[7] Ehrman, C. I., and Himmel, M. E., "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure," *Biotechnology Techniques*, 8(2), 1994, pp. 99–104.

[8] Vinzant, T. B., Ponfick, L., Nagle, N. J., Ehrman, C. I., Reynolds, J. B., and Himmel, M. E., "SSF Comparison of Selected Woods from Southern Sawmills" *Applied Biochemical Biotechnology*, 45/46, 1994, pp. 611–626.