



*Chemical Analysis and Testing Task  
Laboratory Analytical  
Procedure*

**LAP-002**

**Procedure Title:** Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography

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# Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography

## Laboratory Analytical Procedure #002

### 1. Introduction

- 1.1 The carbohydrates making up a major portion of biomass samples are polysaccharides composed 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. The sample can then be quantified by ion-moderated partition HPLC.
- 1.2 This procedure has been adopted by ASTM as the Standard Test Method for Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography, E1758-95.

### 2. Scope

- 2.1 This method covers the determination of carbohydrates, expressed as the percent of each sugar present in a hydrolyzed biomass sample. The sample is taken through a primary 72% sulfuric acid hydrolysis, followed by a secondary dilute-acid hydrolysis.
- 2.2 Sample material 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), waste-paper (such as office waste, boxboard, and newsprint), washed acid- and alkaline-pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105°C oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.
- 2.3 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

### 3. References

- 3.1 Moore, W.E., and D.B. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products*. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.
- 3.2 Ethanol Project Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".
- 3.3 Ethanol Project Laboratory Analytical Procedure #003, "Determination of Acid-Insoluble Lignin in Biomass".
- 3.4 NREL Ethanol Project Laboratory Analytical Procedure #004, "Determination of Acid-Soluble Lignin in Biomass".

- 3.5 NREL Ethanol Project Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass".
- 3.6 TAPPI Test Method T264 om-88, "Preparation of Wood For Chemical Analysis." *In Tappi Test Methods*. Atlanta, GA: Technical Association of the Pulp and Paper Industry.
- 3.7 Vinzant, T.B., L. Ponfick, N.J. Nagle, C.I. Ehrman, J.B. Reynolds, and M.E. Himmel. 1994. "SSF Comparison of Selected Woods From Southern Sawmills." Appl. Biochem. Biotechnol., 45/46:611-626.

#### **4. Terminology**

- 4.1 Prepared Biomass - Biomass that has been prepared by lyophilization, oven drying, air drying, and in some instances by extraction, to reduce the moisture content of the sample so it is suitable for carbohydrate analysis.
- 4.2 Oven-Dried Weight - The moisture-free weight of a biomass sample as determined by LAP-001, "Standard Method for Determination of Total Solids in Biomass".

#### **5. Significance and Use**

- 5.1 The percent sugar content is used in conjunction with other assays to determine the total composition of biomass samples.

#### **6. Interferences**

- 6.1 Samples with high protein content may result in percent sugar values biased low, as a consequence of protein binding with some of the monosaccharides.
- 6.2 Test specimens not suitable for analysis by this procedure include acid- and alkaline-pretreated biomass samples that have not been washed. Unwashed pretreated biomass samples containing free acid or alkali may change visibly on heating.

#### **7. Apparatus**

- 7.1 Hewlett Packard Model 1090 HPLC, or equivalent, with refractive index detector.
- 7.2 HPLC columns, BioRad Aminex7 HPX-87C and/or Aminex7 HPX-87P (or equivalent).
- 7.3 Guard columns, cartridges appropriate for the column used.

Note: Deashing guard column cartridges from BioRad, of the ionic form  $H^+/CO_3^-$ , are an option when using an HPX-87P column. These cartridges have been found to be effective in eliminating baseline ramping.

- 7.4 Analytical balance readable to 0.1 mg.

- 7.5 Convection ovens with temperature control to  $45 \pm 3^{\circ}\text{C}$  and  $105 \pm 3^{\circ}\text{C}$ .
- 7.6 Autoclave capable of maintaining  $121 \pm 3^{\circ}\text{C}$ .
- 7.7 Water bath set at  $30 \pm 3^{\circ}\text{C}$ .
- 7.8 Desiccator containing anhydrous calcium sulfate.

## 8. Reagents and Materials

### 8.1 Reagents

- 8.1.1 High purity sugars for standards (98%+) - two sets of glucose, xylose, galactose, arabinose, and mannose from different lots or manufacturers.
- 8.1.2 72% w/w  $\text{H}_2\text{SO}_4$  ( $12.00 \pm 0.02$  M or specific gravity 1.6389 at  $15.6^{\circ}\text{C}$  /  $15.6^{\circ}\text{C}$ ).
- 8.1.3 Calcium carbonate, ACS reagent grade.
- 8.1.4 Water, 18 megohm deionized.

### 8.2 Materials

- 8.2.1 Glass test tubes, 16x100 mm.
- 8.2.2 125 mL glass serum bottles, crimp top style, with rubber stoppers and aluminum seals to fit.
- 8.2.3 pH paper, suitable to cover the pH range of 4 to 7.
- 8.2.4 Disposable nylon syringe filters, 0.2  $\mu\text{m}$ .
- 8.2.5 Disposable syringes, 3 mL.
- 8.2.6 Autosampler vials, with crimp top seals to fit.
- 8.2.7 Erlenmeyer flasks, 50 mL.

## 9. ES&H Considerations and Hazards

- 9.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.
- 9.2 72%  $\text{H}_2\text{SO}_4$  is very corrosive and must be handled carefully.
- 9.3 Use caution when handling hot glass bottles after the autoclave step, as they may have become pressurized.

## 10. Sampling, Test Specimens and Test Units

- 10.1 Test specimens suitable for analysis by this procedure are as follows:
- biomass feedstocks, dried and reduced in particle size, if necessary.
  - pretreated biomass, washed free of any residual acid or alkali.
  - the solids fraction of fermentation residues.
- 10.2 The sample must not contain particles larger than 1 mm in diameter. If milling is required to reduce the particle size of the test specimen, a laboratory mill equipped with a 40 mesh (or smaller) screen should be used.
- 10.3 The total solids content of the "as received" test specimen (prior to any drying or extraction steps) must be determined by LAP-001 in parallel with the carbohydrate analysis. Record this value as %T<sub>as received</sub>.
- 10.4 Material with a total solids content less than 85%, on a 105°C dry weight basis, will require drying by lyophilization, oven drying, or air drying prior to milling or analysis. The amount of moisture lost as a result of the preparation procedure must be determined. This moisture content is used to calculate the total solids content of the sample based on its preparation and is recorded as %T<sub>prep</sub>. This value is used to correct the weight of the prepped material used in the carbohydrate analysis, as described in the calculations section. The prepared sample should be stored in a manner to ensure its moisture content does not change prior to analysis.

Note: Preparing samples for analysis by oven drying can produce hard chunks of material. This material must then be milled to reduce the size of the large pieces to less than 1 mm in diameter. The sample is then redried prior to testing.

- 10.5 Some samples may require extraction prior to analysis, to remove components that may interfere with the analysis. LAP-010, "Standard Method for the Determination of Extractives in Biomass", is used to prepare an extractives-free sample with a moisture content suitable for carbohydrate analysis. As part of this procedure, the percent extractives in the prepared sample, on a 105°C dry weight basis, is determined. This value, recorded as % extractives, can be used to convert the % sugar reported on a extractives-free basis to an as received (whole sample) basis.
- 10.6 The test specimen shall consist of approximately 0.3 g of sample. The test specimen shall be obtained in such a manner to ensure that it is representative of the entire lot of material being tested.

## 11. Procedure

- 11.1 This procedure is suitable for air-dried, lyophilized, and extracted biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. It is not suitable for samples that have been dried at a temperature exceeding 45°C.

Note: The total solids content of the original sample, %T<sub>as received</sub>, must be determined using LAP-001, prior to any preparatory steps. The total solids content of the sample based on its preparation, %T<sub>prep</sub>, must also be known.

- 11.2 Determine the total solids content of the prepared or extractives-free biomass sample by LAP-001 and record this value as %T<sub>final</sub>.

Note: Samples for total solids determination (LAP-001) must be weighed out at the same time as the samples for the carbohydrate determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere.

- 11.3 Weigh  $0.3 \pm 0.01$  g of the prepared or extractives-free sample to the nearest 0.1 mg and place in a 16x100 mm test tube. Record as  $W_1$ , the initial sample weight in grams. Each sample must be run in duplicate, at minimum.
- 11.4 Add  $3.00 \pm 0.01$  mL ( $4.92 \pm 0.01$  g) of 72% H<sub>2</sub>SO<sub>4</sub> and use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.
- 11.5 Place the test tube in the water bath set at  $30 \pm 1^\circ\text{C}$  and hydrolyze for 2 hours.
- 11.6 Stir the sample every 15 minutes to assure complete mixing and wetting.
- 11.7 Weigh out  $0.3 \pm 0.01$  g of each high purity sugar (predried at  $45^\circ\text{C}$ ) to the nearest 0.1 mg, and place each in its own 16x100 mm glass test tube. Add acid, hydrolyze, and stir these sugars as described in the previous three steps. These sugar recovery standards (SRS) will be taken through the remaining steps in the procedure in parallel with the samples. The calculated recovery of the SRSs will be used to correct for losses due to the destruction of sugars during the hydrolysis process. It may be useful to run selected SRSs in duplicate, particularly if specific sugars are deemed critical.
- 11.8 Prepare a method verification standard (MVS) by weighing out  $0.3 \pm 0.01$  g of a well characterized standard material suitable for analysis. Add acid, hydrolyze, and stir the MVS as was done with the samples and SRSs (see 11.4-11.6 above). This MVS will be taken through the remaining steps in the procedure in parallel with the samples and the SRSs, and is used to test the reproducibility of the method as a whole.

Note: A suitable method verification standard, *Populus deltoides*, may be obtained from NIST (research material #8492).

- 11.9 Upon completion of the two hour hydrolysis step, transfer each hydrolyzate to its own serum bottle and dilute to a 4% acid concentration by adding  $84.00 \pm 0.04$  mL deionized water. Be careful to transfer all residual solids along with the hydrolysis liquor. The total weight added to the tared bottle is 89.22 g (0.3 g sample, 4.92 g 72% H<sub>2</sub>SO<sub>4</sub>, and 84.00 g deionized water). Since the specific gravity of the 4% acid solution is 1.0250 g/mL, the total volume of solution,  $V_F$ , is 87.0 mL.
- 11.10 Stopper each of the bottles and crimp aluminum seals into place.

- 11.11 Set the autoclave to a liquid cycle to prevent loss of sample from the bottle in the event of a loose crimp seal. Autoclave the samples in their sealed bottles for 1 hour at  $121 \pm 3^{\circ}\text{C}$ .
- 11.12 After completion of the autoclave cycle, allow the samples to cool for about 20 minutes at room temperature before removing the seals and stoppers.
- 11.13 These autoclaved solutions may also be used for the determination of acid-insoluble residue and/or acid-soluble lignin, in parallel with this carbohydrate determination.

Note: If acid-insoluble lignin and/or acid-soluble lignin 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 prior to proceeding with the carbohydrate determination. Refer to LAP-003, "Determination of Acid-Insoluble Lignin in Biomass", 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 hours, preferably within 6 hours of hydrolysis. Refer to the procedure "Determination of Acid-Soluble Lignin in Biomass" (LAP-004) for details.

- 11.14 Transfer 20 mL aliquots of each hydrolyzate, or filtrate, to 50 mL Erlenmeyer flasks.
- 11.15 Neutralize with calcium carbonate to a pH between 5 and 6. Do not over-neutralize. Add the calcium carbonate slowly with frequent swirling to avoid problems with foaming. Monitor the pH of the solution with pH paper to avoid over-neutralization.
- 11.16 Filter the neutralized hydrolyzate using a 3 mL syringe with a 0.2  $\mu\text{m}$  filter attached. One portion of the hydrolyzate should be filtered directly into a sealable test tube for storage. A second portion should be filtered directly into an autosampler vial if the hydrolyzate is to be analyzed without dilution. If the concentration of any of the analytes is expected to exceed the validated linear range of the analysis, dilute the hydrolyzate as required and filter into an autosampler vial for analysis.

Note: It is advisable to determine the initial glucose concentration of the sample using an alternative technique, such as a YSI glucose analyzer, in order to predict whether or not the glucose in the sample will fall within the linear range of the analysis.

- 11.17 The portion of the neutralized hydrolyzate filtered into the test tube should be securely sealed, labeled, placed in the refrigerator, and reserved in case a repeat analysis is required. The sample should be stored for no longer than two weeks.
- 11.18 Prepare a series of sugar calibration standards in deionized water at concentrations appropriate

for creating a calibration curve for each sugar of interest. A suggested scheme for the HPX-87C column is to prepare a set of multi-component standards containing glucose, xylose, and arabinose in the range of 0.2 -12.0 mg/mL. For the HPX-87P column, galactose, and mannose should be included as additional components in the standards. Extending the range of the calibration curves beyond 12.0 mg/mL will require validation.

- 11.19 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using sugars obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each sugar contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.
- 11.20 Analyze the calibration standards, the CVS, the samples, the SRSs, and the MVS by HPLC using a Biorad Aminex7 HPX-87C or HPX-87P column for glucose, xylose, and arabinose. If mannose and galactose are also to be determined, a Biorad Aminex7 HPX-87P column must be used instead. For many analyses, it is useful to run the same samples on both columns and compare the results. The following instrumental conditions are used for both the HPX-87C and the HPX-87P columns:

Sample volume: 50 µL.

Eluant: 0.2 µm filtered and degassed, deionized water.

Flow rate: 0.6 mL/min.

Column temperature: 85°C.

Detector: refractive index.

Run time: 20 minutes data collection plus a 15 minute post-run.

## 12. Calculations

- 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 from each SRS taken through the two-stage hydrolysis. This will give an estimate of the amount of each individual sugar destroyed during the hydrolysis procedure.

$$\% R_{srs} = \frac{C_2}{C_1} \times 100\%$$

Where: %R<sub>srs</sub> = % recovery of sugar recovery standard

C<sub>1</sub> = known concentration of sugar recovery standard before hydrolysis, in mg/mL

C<sub>2</sub> = concentration of sugar recovery standard detected by HPLC after hydrolysis, in mg/mL

- 12.3 Use the percent recovery of the appropriate sugar recovery standard to correct sugar concentration values (in mg/mL) obtained from HPLC for each sugar detected in the hydrolyzed sample.

$$C_{corr} = C_{spl} \div \frac{\%R_{srs}}{100\%}$$

Where:

C<sub>corr</sub> = concentration of sugar in hydrolyzed sample corrected for hydrolysis, in mg/mL

C<sub>spl</sub> = concentration of sugar detected in the hydrolyzed sample by HPLC, in mg/mL

%R<sub>srs</sub> = % recovery of sugar recovery of standard, as determined in the previous step

- 12.4 For lyophilized, air dried, or oven dried samples, or for samples requiring no preparation, calculate the percentage of each sugar present in the sample on an as received 105°C dry weight basis as follows:

$$\% \text{ Sugar} = \frac{C_{corr} \times \frac{1 \text{ g}}{1000 \text{ mg}} \times V_F}{W_1 \times \frac{\%T_{as \text{ received}}}{\%T_{prep}}} \times 100\% = \frac{C_{corr} \times \frac{1 \text{ g}}{1000 \text{ mg}} \times V_F}{W_1 \times \frac{\%T_{final}}{100\%}} \times 100\%$$

Where: W<sub>1</sub> = initial weight of sample, in grams

V<sub>F</sub> = volume of filtrate, 87.0 mL

C<sub>corr</sub> = concentration of sugar in hydrolyzed sample corrected for loss on hydrolysis, is determined in previous step, in mg/mL

%T<sub>as received</sub> = % total solids content of the original sample (prior to any preparation steps) on a 105°C dry weight basis, as determined by the LAP-001

%T<sub>prep</sub> = % total solids content of the sample as determined during the preparation of the sample for analysis (by lyophilization, oven-drying, or air drying)

%T<sub>final</sub> = % total solids content of the prepared sample used in this carbohydrate analysis, on a 105°C dry weight basis, as determined by the LAP-001

Note: If the sample used in the analysis required no preparation (analyzed as received), then %T<sub>prep</sub> = 100% and %T<sub>final</sub> = %T<sub>as received</sub>.

- 12.5 If the biomass was prepared according to the "Standard Method for the Determination of Ethanol Extractives in Biomass" (LAP-010), first calculate the percentage of each sugar present on an extractives-free 105°C dry weight basis and then correct this value to an as received (whole sample) 105°C dry weight basis.

12.5.1

Calculate the percentage of each sugar on an extractives-free basis as follows:

$$\% \text{ Sugar}_{\text{extractives-free}} = \frac{C_{\text{corr}} \times \frac{1000 \text{ mg}}{1 \text{ g}} \times V_F}{W_I \times \frac{\% T_{\text{final}}}{100\%}} \times 100\%$$

Where:  $C_{\text{corr}}$  = concentration of sugar in hydrolyzed sample corrected for loss on hydrolysis, as determined above, in mg/mL  
 $V_F$  = volume of filtrate, 87.0 mL  
 $W_I$  = initial weight of extracted sample, in grams  
 $\%T_{\text{final}}$  = % total solids content of the prepared sample used in this carbohydrate analysis (in this case, extracted sample), on a 105°C dry weight basis, as determined by the LAP-001

- 12.5.2 Correct the % sugar value on an extractives-free basis, calculated above, to an as received (whole sample) 105°C dry weight basis as follows:

$$\% \text{ Sugar}_{\text{whole sample}} = \% \text{ Sugar}_{\text{extractives-free}} \times \frac{(100\% - \% \text{ extractives})}{100\%}$$

Where:

$\% \text{ Sugar}_{\text{extractives-free}}$  = % sugar on an extractives-free 105°C dry weight basis, as determined in the previous step  
 $\% \text{ extractives}$  = % extractives in the extracted sample as described in the Standard Method for the Determination of Extractives in Biomass

### 13. Report

- 13.1 Report the percent sugar present in the sample, to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. Cite the basis used in the report.
- 13.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

## 14. Precision and Bias

- 14.1 Data obtained by replicate testing of a hybrid poplar in one laboratory, using a HPX-87P column, gave a standard deviation in glucose content of 1.90% and a CV of 3.95%.
- 14.2 Data obtained by replicate testing of an extractives-free hybrid poplar sample in five different laboratories gave a standard deviation of 1.90% and a CV of 4.0%.

## 15. Quality Control

- 15.1 *Reported significant figures:* Report the percentage of each sugar present in the hydrolyzed sample to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. Cite the basis used in the calculation.
- 15.2 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.
- 15.3 *Blank:* The only requirement is a reagent blank, which starts out as an empty 16x100 mm test tube (ie, no sample) which is taken through all the procedural steps.
- 15.4 *Relative percent difference criteria:* The RPD for glucose must be less than 6.1%. If the RPD is too large, the sample must rerun.
- 15.5 *Method verification standard:* A method verification standard must be run in duplicate with every batch. This method utilizes a well characterized standard material suitable for analysis. For example, NIST 8492 (*Populus deltoides*) is used as the MVS in carbohydrate analysis of hardwoods.
- 15.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in section 11.19 of this procedure.
- 15.7 *Sample size:* A minimum of 0.6 grams of sample (on a dry weight basis) are required for duplicate analyses. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.
- 15.8 *Sample storage:* Samples shall be stored in an airtight container and refrigerated.
- 15.9 *Standard storage:* Standards should be kept frozen in airtight vials or test tubes. Vortex the standards vigorously upon thawing to ensure thorough mixing.
- 15.10 *Standard preparation:* Standards are prepared according to section 11.18 of this procedure.
- 15.11 *Definition of a batch:* Any number of samples which are analyzed and recorded together. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.
- 15.12 *Control charts:* The result of each replicate analysis of the method verification standard is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standards is to be control charted.