Biomass Analysis Technology Team

Laboratory Analytical Procedure LAP-002CS

Procedure Title: **Determination of Structural Carbohydrate Content in Corn Stover Feedstocks by High Performance Liquid Chromatography**

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1. Introduction

1.1 Corn Stover is composed largely of three biopolymers; cellulose, a polymer of glucose; hemicellulose, an acetylated arabinoxylan with minor amounts of galactose and mannose; and lignin, a complex phenolic polymer. This procedure requires a two-stage hydrolysis process to separate the complex, polymeric biomass matrix into forms that can be more easily measured and quantified. During hydrolysis, the polysaccharides present in a corn stover sample are hydrolyzed to their component sugars. The monomeric sugars and associated by-products are then quantified by ion-moderated partition HPLC.

1.2 This procedure is similar to portions of ASTM E1758, Standard Test Method for the Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography.

2. Scope

2.1 This procedure covers methods for quantifying the carbohydrate content of a biomass sample. The results are, reported as anhydro-sugars expressed as a weight percent of the original biomass sample. Results are reported relative to the 105°C oven-dried weight of a specified sample. Specified samples include as-received, structural, and prepared stover. Since the measurements are made on the extractives-free material, the results may also be reported on an extractives-free basis.

2.2 This procedure has been optimized for the analysis of carbohydrate content in extractives-free corn stover.

2.3 This procedure quantifies the structural carbohydrate content of corn stover feedstocsk. Some corn stover samples contain significant amounts of non-structural sugars that will be removed in the water extraction step in LAP 10 "Determination of Extractives in Corn Stover". The total carbohydrate content for corn stover should include both values.

2.3 All analyses shall be performed according to the guidelines established in the Biofuels Program Experimental Data Quality Assurance Plan (QAP).

3. Terminology

3.1 Prepared Stover - Biomass that has been prepared according to LAP-000.

3.2 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".

3.2 Structural Carbohydrates

3.3 As-received Stover

3.4 Structural Stover – Agricultural residue such as whole corn plants (excluding grain kernels), or parts of the corn plant, such as leaves, culm, pith etc..

3.5 Summative Analysis

3.6 High Protein

3.7 High Ash

4. Significance and Use

4.1 This procedure is used in conjunction with other assays to determine the total composition of corn stover samples.

5. Interferences

5.1 Samples with high protein content may result in percent sugar values biased low, as a consequence of association between protein and carbohydrates.

5.3 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.

5.3 Samples with ash contents above 10% may contain soil, which may result in percent sugar values biased low as a consequence of side reactions to products not measured in this procedure.

5.4 Failure to remove extractable materials such as starch and non-structural sugars may result in a high bias in percent sugar values.

6. Apparatus

6.1 Hewlett Packard Model 1090 HPLC, or equivalent, with refractive index detector. HPLC column, BioRad Aminex® HPX-87P (or equivalent).

6.2 Guard columns, cartridges appropriate for the column used.

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

6.3 Analytical balance readable to 0.1 mg.

7. Reagents and Materials

7.1 Reagents

7.1.1 Test samples of biomass after processing through LAP 19 "Hydrolysis of Corn Stover for Compositional Analysis"

7.1.2 Test samples of the QA biomass standard after processing through LAP 19 "Hydrolysis of Corn Stover for Compositional Analysis"

7.1.3 Test Samples of the High purity sugars standards and Calibration verification standards after processing through LAP 19 "Hydrolysis of Corn Stover for Compositional Analysis"

7.1.4 Calcium carbonate, ACS reagent grade.

7.2 Materials

7.2.1 pH paper, suitable to cover the pH range of 4 to 7.

7.2.2 Disposable nylon syringe filters, 0.2 μm.

7.2.3 Disposable syringes, 3 mL.

7.2.4 Autosampler vials, with crimp top seals to fit.

7.2.5 Erlenmeyer flasks, 50 mL.

8. ES&H Considerations and Hazards

8.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8.2 Operate all equipment in accordance with NREL Safe Operating Procedures.

8.3 H_2SO_4 is very corrosive and must be handled carefully

8.4 Use caution when handling hot glass bottles after the autoclave step, as they may have become pressurized, creating an explosion hazard.

8.5 Use caution when removing crimp-top seals after the autoclave step, to avoid sharp edges on the aluminum seals and glass bottles.

9. Sample Preparation and Pre-Analysis Requirements

9.1 Prior to analysis using this procedure, corn stover samples should be processed according to LAP-021 Preparation of Corn Stover for Compositional Analysis, LAP-010 Determination of Extractives in Corn Stover and LAP 019 Hydrolysis of Biomass for Compositional Analysis.

9.2 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.

9.3 The total solids content of the original sample, $\%T_{as\ received}$, must be determined using LAP-001, prior to any preparatory steps. The total solids content of the sample based on its preparation, $\%T_{C\text{-}L}$, must also be known.

9.4 The air-dried weight, W_1, and the total solids content of the extractives-free stover , *%T final*, as recorded in LAP 019 Hydrolysis of Biomass for Compositional Analysis will be used to calculate percent composition.

9.5 The sugar recovery standards (SRS) were taken through LAP 019 "Hydrolysis of Biomass for Compositional Analysis" with the biomass samples. The calculated recovery of the SRSs will be used to correct for losses due to the destruction of sugars during the hydrolysis process.

9.6 The QA standard was taken through LAP 019 "Hydrolysis of Biomass for Compositional Analysis" with the biomass samples. The QA standard is a well-characterized material used as a quality control check to test the accuracy of the analysis.

9.7 This procedure is performed after LAP 19 "Hydrolysis of Biomass for Compositional Analysis" and begins with the autoclaved solutions of hydrolyzed biomass.

9.8 This procedure uses the same bulk test materials as the following LAPS. If these tests will be performed, care must be taken not to consume the entire sample in this analysis.

LAP 03CS	Determination of Acid-Insoluble lignin in corn Stover
LAP 04CS	Determination Acid Soluble Lignin in Corn Stover
LAP 17CS	Determination of O-Acyl Groups in Biomass by HPLC
LAP TBD	Determination of Uronic Acids in Corn Stover (in development)

10. Procedure

10.1 Using the high temperature markers, individually label the crucibles needed for lignin analysis and ignite them in the muffle furnace at 575 ±25°C to achieve a constant weight of ±0.3 mg. Store the ignited crucibles in a desiccator until needed. Record the weight of the ignited crucible, W_2 to the nearest 0.1 mg. To improve tare accuracy, avoid direct contact with skin when handling crucibles.

10.2 Vacuum filter the autoclaved hydrolysis solution through one of the previously ignited filter crucibles into a vacuum flask.

10.3 For carbohydrate analysis and/or an acid-soluble lignin analysis transfer the undiluted filtrate into a sample storage bottle. If the hydrolysis liquor is not used immediately for further analysis, store in refrigerator at 4°C. The acidic hydrolysis liquor can be stored in the refrigerator for a maximum of 2 weeks.

> Note: Acid-soluble lignin should be analyzed within 6 hours to prevent loss due to precipitation of soluble lignin.
> Caution: Undiluted filtrate must be collected before proceeding to the washing step.

10.4 Transfer a 20 mL aliquot of each hydrolysis liquor (filtrate), to a 50 mL Erlenmeyer flask.

10.5 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.

Note 1: Over neutralizing to a pH greater than 10 will result in loss of sugars. Over-neutralized samples should be discarded.

Note 2: Over neutralizing the sample to a pH greater than 6 can affect the chromatography by introducing salts that cause baseline disruption.

10.6 Filter the neutralized hydrolyzate using a 3 mL syringe with a 0.2 µm filter attached. One portion of the hydrolyzate should be filtered directly into a sealable container for storage. A second portion should be filtered directly into an HPLC auto sampler vial.

10.7 The portion of the neutralized hydrolysis liquor that was filtered into the storage container should be securely sealed, labeled, placed in a refrigerator, and reserved in case a repeat analysis is required. The sample should be stored for no longer than two weeks. If the sample cannot be analyzed in two weeks, the sample should be stored frozen.

Note 1: After cold storage check the samples for the presence of a precipitate. Samples with a precipitate should be re-filtered through 0.2 µm filter before proceeding .

Note 2: After storage, check the samples for the presence of mold. Samples with mold should be discarded.

10.8 Analyze the calibration standards, the CVS, the test samples, the SRSs, and the QA standards by HPLC. Bracket groups of six samples with CVS standards. The following instrumental conditions are recommended:

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.

10.9 Before reporting HPLC data perform the following data QA checks.

10.9.1 Check that the CVS concentrations fall within ± 3% (relative) of known value. Accept data for test samples between acceptable CVSs. Reject data for samples that are not bracketed by two acceptable CVSs. Re-run rejected samples.

10.9.2 Check for abnormal chromatography, including, baseline disruptions, broad or asymmetric peaks, or spikes indicative of a bubble. Reject data for samples with unacceptable chromatography. Re-run rejected samples.

10.9.3 Check the calibration response factors for consistency within the HPLC run. Compare response factors to historical values. Flag unusual data for future reference.

10.9.4 Check test sample chromatograms for presence of cellobiose and oligomerics sugars. Levels of cellobiose greater than 1% indicate incomplete hydrolysis. Samples should be re-run.

10.9.5 Check test sample chromatograms for the presence peaks eluting before cellobiose (RT of 4-5 minutes using recommended conditions). These peaks may indicate high levels of sugar degradations products in the previous sample. High levels of sugar degradation products indicate over-hydrolysis. All samples from batches showing evidence of over-hydrolysis should be re-run.

10.9.6 An additional check for over-hydrolysis can be made using LAP 17 "Determination of O-Acyl groups in biomass by HPLC" Evidence of elevated amounts of Formic and levulinic acids may indicate over-hydrolysis.

11. Calculations

11.1 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.

11.2 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:

$\%R_{srs}$ = % recovery of sugar recovery standard

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

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

9.4 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_{corr} = concentration of sugar in hydrolyzed sample corrected for sugar loss during hydrolysis, in mg/mL

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

$\%R_{srs}$ = % recovery of sugar recovery of standard, as determined in the previous step

12.4 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.

$$\% \; Sugar_{extractives-free} = \frac{C_{corr} \; x \; \frac{1 \; g}{1000 \; mg} \; x \; V_F}{W_1 \; x \; \frac{\% T_{final}}{100\%}} \; x \; 100\%$$

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

Where:

C_{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_1 = initial weight of extractives-free biomass sample, in grams
$\%T_{final}$ = % 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 LAP-001"Determination of Total solids Content in Biomass"

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

$$\%\ Sugar_{whole\,sample} = \%\ Sugar_{extractives\text{-}free}\ x\ \frac{(100\% - \%\ extractives)}{100\%}$$

Where:
$\%\ Sugar_{extractives\text{-}free}$ = % sugar on an extractives-free 105°C dry weight basis, as determined in the previous step
% extractives = % extractives in the extracted sample as determined using LAP 10CS " Determination of Extractives in Corn Stover" calculate the percentage of each sugar present on an extractives-free 105EC dry weight basis and then correct this value to an as received (whole sample) 105°C dry weight basis.

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

$$\%\ Sugar_{extractives\text{-}free} = \frac{C_{corr}\ x\ \frac{1\ g}{1000\ mg}\ x\ V_F}{W_1\ x\ \frac{\%T_{final}}{100\%}}\ x\ 100\%$$

Where: C_{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_1 = initial weight of extracted sample, in grams

$\%T_{final}$ = % 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

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

$$\%\ Sugar_{whole\,sample} = \%\ Sugar_{extractives\text{-}free}\ x\ \frac{(100\% - \%\ extractives)}{100\%}$$

Where:

$\%\ Sugar_{extractives\text{-}free}$ = % sugar on an extractives-free 105°C dry weight basis, as determined in the previous step

% 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 no more than 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.

10.5 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%.

11.6 Data obtained by replicate testing of an extractives-free hybrid poplar sample in five different laboratories gave a standard deviation in glucose content of 1.90% and a CV of 4.0%.

14.3 Data obtained by replicate testing of extractives-free NIST SRM 8491 sugar cane bagasse in nine different laboratories gave a standard deviation in glucose content of 1.90% and a CV of 4.1%.

15. Quality Control

14.6 *Reported significant figures:* Report the percentage of each sugar present in the hydrolyzed sample to no more than 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.

12.7 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.

14.8 *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.

14.9 *Relative percent difference criteria:* The relative percent difference must be less thatn 6%. If the difference is too large, the sample must be re-run.

14.10 *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 8491 (*baggasse*) may be used as the MVS in carbohydrate analysis of grasses.

14.11 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in section 11.19 of this procedure.

14.12 *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 data should be noted.

14.13 *Sample storage:* Samples should be stored in an airtight container and refrigerated.

14.14 *Standard storage:* Standards should be kept frozen in airtight vials or test tubes. Vortex mix the standards vigorously upon thawing to ensure thorough mixing.

14.15 *Standard preparation:* Standards are prepared according to section 11.18 of this procedure.

14.16 *Definition of a batch*: Any number of samples that are analyzed and recorded together. The maximum size of a batch is limited by the equipment constraints. A batch cannot be larger than what is practical for the equipment used.

14.17 *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.

15. Appendixes None

16. References

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16.3 NREL Biofuels Program Laboratory Analytical Procedure #003CS, "Determination of Acid-Insoluble Residue in Corn Stover".

16.4 NREL Biofuels Program Laboratory Analytical Procedure #004CS, "Determination of Acid-Soluble Lignin in Corn Stover".

16.5 NREL Biofuels Program Laboratory Analytical Procedure #010CS, "Standard Method for the Determination of Extractives in Corn Stover".

16.6 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.

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16.8 NREL Biofuels Program Laboratory Analytical Procedure #015CS, "HPLC Analysis of the Liquid Fractions of Process Samples for Organic Acids, Glycerol, HMF, and Furfural".

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