NREL
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Laboratory Analytical Procedure (LAP)

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A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker

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Technical Report
NREL/TP-510-42618
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NREL Laboratory Analytical Procedures for standard biomass analysis are available electronically at http://www.nrel.gov/biomass/analytical_procedures.html

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Procedure Title: Determination of Structural Carbohydrates and Lignin in Biomass

Laboratory Analytical Procedure

1. Introduction

1.1 Carbohydrates and lignin make up a major portion of biomass samples. These constituents must be measured as part of a comprehensive biomass analysis. Carbohydrates can be structural or non-structural. Structural carbohydrates are bound in the matrix of the biomass, while non-structural carbohydrates can be removed using extraction or washing steps. Lignin is a complex phenolic polymer.

1.2 Portions of this procedure are substantially similar to ASTM E1758-01 "Standard Method for the Determination of Carbohydrates by HPLC."

1.3 This procedure is suitable for samples that do not contain extractives. This procedure uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. The lignin fractionates into acid insoluble material and acid soluble material. The acid insoluble material may also include ash and protein, which must be accounted for during gravimetric analysis. The acid soluble lignin is measured by UV-Vis spectroscopy. During hydrolysis the polymeric carbohydrates are hydrolyzed into the monomeric forms, which are soluble in the hydrolysis liquid. They are then measured by HPLC. Protein may also partition into the liquid fraction. A measure of acetyl content is necessary for biomass containing hemicellulose with a xylan backbone, but not biomass containing a mannan backbone. Acetate is measured by HPLC.

2. Scope

2.1 This procedure is appropriate for extractives free biomass, which includes biomass that has been extracted using LAP "Determination of Extractives in Biomass," as well as process solids containing no extractives. Results are reported on an oven dry weight basis. Results may be reported on an as received biomass basis or an extractives free basis, depending on type of biomass used. LAP "Preparation of Samples for Biomass Compositional Analysis" should be used prior to this procedure.

2.2 This procedure is appropriate for biomass containing the components listed throughout the procedure. Any biomass containing other interfering components not listed must be further investigated.

2.3 A measure of acetyl content is necessary for biomass containing hemicellulose with a xylan backbone, but not biomass containing a mannan backbone.

2.4 All analyses should be performed in accordance with an appropriate laboratory specific Quality Assurance Plan (QAP).

3. Terminology

3.1 *Oven dry weight (ODW)*—the weight of biomass mathematically corrected for the amount of

moisture present in the sample at the time of weighing.

3.2 *Prepared biomass*—biomass prepared according to LAP "Preparation of Samples for Biomass Compositional Analysis."

3.3 *Extractives free biomass*—biomass after exhaustive water and ethanol extraction (refer to LAP "Determination of Extractives in Biomass").

3.4 *Acid insoluble lignin*—the residue remaining on a medium porosity filtering crucible after a two step hydrolysis, with correction for acid insoluble ash and acid insoluble protein, if necessary.

3.5 *Structural carbohydrates*—polymeric carbohydrates, namely cellulose and hemicellulose.

3.6 *Non-structural components*—non-chemically bound components of biomass that include but are not limited to sucrose, nitrate/nitrites, protein, ash, chlorophyll, and waxes.

4. Significance and Use

4.1 This procedure is used, possibly in conjunction with other procedures, to determine the amount of structural carbohydrates and lignin in a solid biomass sample.

5. Interferences

5.1 This procedure has been optimized for the particle size range specified in LAP "Preparation of Samples for Biomass Compositional Analysis." Deviation to a smaller particle size may result in a low bias in carbohydrate content (and consequent high lignin bias) due to excessive carbohydrate degradation. Deviation to a larger particle size may also result in a low bias in carbohydrate content (and consequent high lignin bias) due to incomplete hydrolysis of polymeric sugars to monomeric sugars.

5.2 Samples containing extractives are not suitable for this procedure. Extractives will partition irreproducibly, resulting in a high lignin bias.

5.3 Samples with an ash content above 10 wt % may not be suitable for this procedure, as the sample may contain soil or other minerals that will interfere with appropriate acid concentrations and may catalyze side reactions.

5.4 Samples with a moisture content above 10 wt % may not be suitable for this procedure, as the excess moisture will interfere with appropriate acid concentrations. Samples should be dried (air-dried or oven dried at less than 40°C) prior to this procedure.

5.5 Samples containing protein will bias the acid insoluble lignin high unless the protein is accounted for in the gravimetric determination of acid insoluble material. An independent nitrogen analysis is required to estimate the protein content of the residue. The protein estimate is then subtracted from the acid insoluble residue measurement. Physical separation of the acid insoluble protein from the acid insoluble lignin is beyond the scope of this procedure.

5.6 This procedure is not suitable for samples containing added acid, base, or catalyst.

5.7 Certain guard columns for carbohydrate quantification may cause artifact peaks. Individual carbohydrates should be run on new columns and guard columns to verify to absence of artifact peaks.

6. Apparatus

6.1 Analytical balance, accurate to 0.1 mg.

6.2 Convection drying oven, with temperature control of 105 ± 3°C.

6.3 Muffle furnace, equipped with a thermostat, set to 575 ± 25 °C or equipped with optional ramping program.

6.4 Water bath, set at 30 ± 3 °C.

6.5 Autoclave, suitable for autoclaving liquids, set to 121 ± 3 °C.

6.6 Filtration setup, equipped with a vacuum source and vacuum adaptors for crucibles.

6.7 Desiccator containing desiccant.
6.8 HPLC system equipped with refractive index detector and the following columns:
6.8.1 Shodex sugar SP0810 or Biorad Aminex HPX-87P column (or equivalent) with ionic form H^+/CO_3^- deashing guard column.
6.8.2 Biorad Aminex HPX-87H column (or equivalent) equipped with an appropriate guard column.
6.9 UV-Visible spectrophotometer, diode array or single wavelength, with high purity quartz cuvettes of pathlength 1 cm.
6.10 Automatic burette, capable of dispensing 84.00 mL water, optional.

7. Reagents and Materials

7.1 Reagents
7.1.1 Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20°C) – (also commercially available as a reagent for the determination of fluorine, from Fluka #00647).
7.1.2 Calcium carbonate, ACS reagent grade.
7.1.3 Water, purified, 0.2 μm filtered.
7.1.4 High purity standards: D-cellobiose, D(+)glucose, D(+)xylose, D(+)galactose, L(+)arabinose, and D(+)mannose.
7.1.5 Second set of high purity standards, as listed above, from a different source (manufacturer or lot), to be used to prepare calibration verification standards (CVS).
7.2 Materials
7.2.1 QA standard, well characterized, such as a National Institute of Standards and Technology (NIST) biomass standard or another well characterized sample of similar composition to the samples being analyzed.
7.2.2 Pressure tubes, minimum 90 mL capacity, glass, with screw on Teflon caps and o-ring seals (Ace glass # 8648-30 tube with #5845-47 plug, or equivalent).
7.2.3 Teflon stir rods sized to fit in pressure tubes and approximately 5 cm longer than pressure tubes.
7.2.4 Filtering crucibles, 25 mL, porcelain, medium porosity, Coors #60531 or equivalent.
7.2.5 Bottles, wide mouth, 50 mL.
7.2.6 Filtration flasks, 250 mL.
7.2.7 Erlenmeyer flasks, 50 mL.
7.2.8 Adjustable pipettors, covering ranges of 0.02 to 5.00 mL and 84.00 mL.
7.2.9 pH paper, range 4–9.
7.2.10 Disposable syringes, 3 mL, fitted with 0.2 μm syringe filters.
7.2.11 Autosampler vials with crimp top seals to fit.

8. ES&H Considerations and Hazards

8.1 Sulfuric acid is corrosive and should be handled with care.
8.2 Use caution when handling hot pressure tubes after removal from the autoclave, as the pressurized tubes can cause an explosion hazard.
8.3 When placing crucibles in a furnace or removing them, use appropriate personal protective equipment, including heat resistant gloves.
8.4 Operate all equipment in accordance with the manual and NREL Safe Operating Procedures.
8.5 Follow all applicable NREL chemical handling procedures.

9. Sampling, Test Specimens, and Test Units

9.1 Care must be taken to ensure a representative sample is taken for analysis.
9.2 LAP "Preparation of Samples for Biomass Compositional Analysis" should be performed

prior to this analysis. Samples must have a minimum total solids content of 85%.

9.3 LAP "Determination of Extractives in Biomass" should be performed prior to this analysis if extractives are present in the sample.

9.4 LAP "Determination of Solids in Biomass" should be performed at the same time that samples for this analysis are weighed out.

9.5 This procedure is suitable for samples that have been air dried or lyophilized. Samples dried at a temperature of 45°C or higher are not suitable for this procedure.

9.6 Steps 9.2 to 9.4 should be applied to the QA standard.

10. Procedure

10.1 Prepare the sample for analysis and hydrolyze.

10.1.1 Place an appropriate number of filtering crucibles in the muffle furnace at 575 ± 25 °C for a minimum of four hours. Remove the crucibles from the furnace directly into a desiccator and cool for a specific period of time, one hour is recommended. Weigh the crucibles to the nearest 0.1 mg and record this weight. It is important to keep the crucibles in a specified order, if they are not marked with identifiers. Permanent marking decals are available from Wale Apparatus. Do not mark the bottom of the filtering crucible with a porcelain marker, as this will impede filtration.

10.1.2 Place the crucible back into the muffle furnace at 575 ± 25 °C and ash to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible.

10.1.3 Weigh 300.0 ± 10.0 mg of the sample or QA standard into a tared pressure tube. Record the weight to the nearest 0.1 mg. Label the pressure tube with a permanent marker. LAP "Determination of Total Solids in Biomass" should be performed at the same time, to accurately measure the percent solids for correction. Each sample should be analyzed in duplicate, at minimum. The recommended batch size is three to six samples and a QA standard, all run in duplicate.

10.1.4 Add 3.00 ± 0.01 mL (or 4.92 ± 0.01 g) of 72% sulfuric acid to each pressure tube. Use a Teflon stir rod to mix for one minute, or until the sample is thoroughly mixed.

10.1.5 Place the pressure tube in a water bath set at 30 ± 3 °C and incubate the sample for 60 ± 5 minutes. Using the stir rod, stir the sample every 5 to 10 minutes without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.

10.1.6 Upon completion of the 60-minute hydrolysis, remove the tubes from the water bath. Dilute the acid to a 4% concentration by adding 84.00 ± 0.04 mL deionized water using an automatic burette. Dilution can also be done by adding 84.00 ± 0.04 g of purified water using a balance accurate to 0.01 g. Screw the Teflon caps on securely. Mix the sample by inverting the tube several times to eliminate phase separation between high and low concentration acid layers.

Note: The volume of the 4% solution will be 86.73 mL, as demonstrated in the following calculations.

Density 72% H_2SO_4= $d_{72\%\ H2SO4}$= 1.6338 g/mL

Density H_2O= d_{H2O}= 1.00 g/mL

Density 4% H_2SO_4 = $d_{4\%\ H2SO4}$ = 1.025 g/mL

1. The weight of 3.00 mL 72% H_2SO_4 is:

   3.00 mL 72% H_2SO_4 x $d_{72\%\ H2SO4}$= 4.90 g 72% H_2SO_4

2. The composition of 3 mL of 72% H_2SO_4 is:

4.90 g 72% H_2SO_4 x 72% (acid wt) = 3.53 g acid
4.90 g 72% H_2SO_4 x 28% (water wt) = 1.37 g water

3. The concentration of H_2SO_4 after dilution is:
   3.53 g acid / (84.00 g H_2O + 4.90 g 72% H_2SO_4) = 3.97 % H_2SO_4 (w/w)
4. The total volume of solution present after dilution is:
   (4.90 g H_2SO_4 + 84.00 g H_2O) x $(d_{4\%\ H2SO4})^{-1}$ = 86.73 mL

10.1.7 Prepare a set of sugar recovery standards (SRS) that will be taken through the remaining hydrolysis and used to correct for losses due to destruction of sugars during dilute acid hydrolysis. SRS should include D-(+)glucose, D-(+)xylose, D-(+)galactose, -L(+)arabinose, and D-(+)mannose. SRS sugar concentrations should be chosen to most closely resemble the concentrations of sugars in the test sample. Weigh out the required amounts of each sugar, to the nearest 0.1 mg, and add 10.0 mL deionized water. Add 348 µL of 72% sulfuric acid. Transfer the SRS to a pressure tube and cap tightly.

10.1.7.1 A fresh SRS is not required for every analysis. A large batch of sugar recovery standards may be produced, filtered through 0.2 µm filters, dispensed in 10.0 mL aliquots into sealed containers, and labeled. They may be stored in a freezer and removed when needed. Thaw and vortex the frozen SRS prior to use. If frozen SRS are used, the appropriate amount of acid must be added to the thawed sample and vortexed prior to transferring to a pressure tube.

10.1.8 Place the tubes in an autoclave safe rack, and place the rack in the autoclave. Autoclave the sealed samples and sugar recovery standards for one hour at 121°C, usually the liquids setting. After completion of the autoclave cycle, allow the hydrolyzates to slowly cool to near room temperature before removing the caps. (If step 10.2 is not performed, draw a 10 mL aliquot of the liquor for use in step 10.5.)

10.2 Analyze the sample for acid insoluble lignin as follows.

10.2.1 Vacuum filter the autoclaved hydrolysis solution through one of the previously weighed filtering crucibles. Capture the filtrate in a filtering flask.

10.2.2 Transfer an aliquot, approximately 50 mL, into a sample storage bottle. This sample will be used to determine acid soluble lignin as well as carbohydrates, and acetyl if necessary. Acid soluble lignin determination must be done within 6 hours of hydrolysis. If the hydrolysis liquor must be stored, it should be stored in a refrigerator for a maximum of two weeks. It is important to collect the liquor aliquot before proceeding to step 10.2.3.

10.2.3 Use deionized water to quantitatively transfer all remaining solids out of the pressure tube into the filtering crucible. Rinse the solids with a minimum of 50 mL fresh deionized water. Hot deionized water may be used in place of room temperature water to decrease the filtration time.

10.2.4 Dry the crucible and acid insoluble residue at 105 ± 3 °C until a constant weight is achieved, usually a minimum of four hours.

10.2.5 Remove the samples from the oven and cool in a desiccator. Record the weight of the crucible and dry residue to the nearest 0.1 mg.

10.2.6 Place the crucibles and residue in the muffle furnace at 575 ± 25 °C for 24 ± 6 hours.

10.2.6.1 A furnace with temperature ramping may also be used.

Furnace Temperature Ramp Program:
Ramp from room temperature to 105 °C
Hold at 105 °C for 12 minutes

Ramp to 250 °C at 10 °C / minute
Hold at 250 °C for 30 minutes
Ramp to 575 °C at 20 °C / minute
Hold at 575 °C for 180 minutes
Allow temperature to drop to 105 °C
Hold at 105 °C until samples are removed

10.2.7 Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight. Place the crucibles back in the furnace and ash to a constant weight. (The amount of acid insoluble ash is not equal to the total amount of ash in the biomass sample. Refer to LAP "Determination of Ash in Biomass" if total ash is to be determined.)

10.3 Analyze the sample for acid soluble lignin as follows.

10.3.1 On a UV-Visible spectrophotometer, run a background of deionized water or 4% sulfuric acid.

10.3.2 Using the hydrolysis liquor aliquot obtained in step 10.2.2, measure the absorbance of the sample at an appropriate wavelength on a UV-Visible spectrophotometer. Refer to section 11.3 for suggested wavelength values. Dilute the sample as necessary to bring the absorbance into the range of 0.7–1.0, recording the dilution. Deionized water or 4% sulfuric acid may be used to dilute the sample, but the same solvent should be used as a blank. Record the absorbance to three decimal places. Reproducibility should be ± 0.05 absorbance units. Analyze each sample in duplicate, at minimum. (This step must be done within six hours of hydrolysis.)

10.3.3 Calculate the amount of acid soluble lignin present using calculation 11.3.

10.4 Analyze the sample for structural carbohydrates.

10.4.1 Prepare a series of calibration standards containing the compounds that are to be quantified, referring to Table 1 for suggested concentration range. Use a four point calibration. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.

10.4.1.1 Table 1. Suggested concentration ranges for 10.4.1 calibration standards

Component	Suggested concentration range (mg/ml)
D-cellobiose	0.1–4.0
D(+)glucose	0.1–4.0
D(+)xylose	0.1–4.0
D(+)galactose	0.1–4.0
L(+)arabinose	0.1–4.0
D(+)mannose	0.1–4.0
CVS	Middle of linear range, concentration not equal to a calibration point (2.5 suggested)

10.4.1.2 A fresh set of standards is not required for every analysis. A large batch of standards may be produced, filtered through 0.2 μm filters into autosampler

vials, sealed and labeled. The standards and CVS samples may be stored in a freezer and removed when needed. Thaw and vortex frozen standards prior to use. During every use, standards and CVS samples should be observed for unusual concentration behavior. Unusual concentrations may mean that the samples are compromised or volatile components have been lost. Assuming sufficient volume, standards and CVS samples should not have more than 12 injections drawn from a single vial. In a chilled autosampler chamber, the lifetime of standards and CVS samples is approximately three to four days.

10.4.2 Prepare an independent calibration verification standard (CVS) for each set of calibration standards. Use reagents from a source or lot other than that used in preparing the calibration standards. Prepare the CVS at a concentration that falls in the middle of the validated range of the calibration curve. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.

10.4.3 Using the hydrolysis liquor obtained in step 10.2.2, transfer an approximately 20 mL aliquot of each liquor to a 50 mL Erlenmeyer flask.

10.4.4 Use calcium carbonate to neutralize each sample to pH 5–6. Avoid neutralizing to a pH greater than 6 by monitoring with pH paper. Add the calcium carbonate slowly after reaching a pH of 4. Swirl the sample frequently. After reaching pH 5–6, stop calcium carbonate addition, allow the sample to settle, and decant off the supernatant. The pH of the liquid after settling will be approximately 7. (Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.)

10.4.5 Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.2 μm filter into an autosampler vial. Seal and label the vial. Prepare each sample in duplicate, reserving one of the duplicates for analysis later if necessary. If necessary, neutralized samples may be stored in the refrigerator for three or four days. After this time, the samples should be considered compromised due to potential microbial growth. After cold storage, check the samples for the presence of a precipitate. Samples containing a precipitate should be refiltered, while still cold, through a 0.2 μm filter.

10.4.6 Analyze the calibration standards, CVS, and samples by HPLC using a Shodex sugar SP0810 or Biorad Aminex HPX-87P column equipped with the appropriate guard column.

HPLC conditions:
Injection volume: 10–50 μL, dependent on concentration and detector limits
Mobile phase: HPLC grade water, 0.2 μm filtered and degassed
Flow rate: 0.6 mL/minute
Column temperature: 80–85 °C
Detector temperature: as close to column temperature as possible
Detector: refractive index
Run time: 35 minutes
Note: The deashing guard column should be placed outside of the heating unit and kept at ambient temperature. This will prevent artifact peaks in the chromatogram.

10.4.7 Check test sample chromatograms for presence of cellobiose and oligomeric sugars. Levels of cellobiose greater than 3 mg/mL indicate incomplete hydrolysis. Fresh samples should be hydrolyzed and analyzed.

10.4.8 Check test sample chromatograms for the presence of peaks eluting before cellobiose (retention time of 4–5 minutes using recommended conditions). These peaks may indicate high levels of sugar degradation products in the previous sample, which is indicative of over hydrolysis. All samples from batches showing evidence of over-hydrolysis should have fresh samples hydrolyzed and analyzed.

10.5 Analyze the sample for acetyl content if necessary.

10.5.1 Prepare 0.005 M (0.01 N) sulfuric acid for use as a HPLC mobile phase. In a 2 L volumetric flask, add 2.00 mL of standardized 10 N sulfuric acid and bring to volume with HPLC grade water. Filter through a 0.2 μm filter and degas before use. If 10N sulfuric acid is not available, concentrated sulfuric acid may also be used. 278 μL concentrated sulfuric acid brought to volume in a 1 L volumetric flask with HPLC grade water will also produce 0.005 M sulfuric acid.

10.5.2 Prepare a series of calibration standards containing the compounds that are to be quantified. Acetic acid is recommended, formic acid and levulinic acid are optional. A range of 0.02 to 0.5 mg/mL is suggested. An evenly spaced four point calibration is suggested. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.

10.5.3 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using components obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each compound contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.

10.5.4 Prepare the sample for HPLC analysis by passing a small aliquot of the liquor collected in step 10.2.2 through a 0.2 μm filter into an autosampler vial. Seal and label the vial. If it is suspected that the sample concentrations may exceed the calibration range, dilute the samples as needed, recording the dilution. The concentrations should be corrected for dilution after running.

10.5.5 Analyze the calibration standards, CVS, and samples by HPLC using a Biorad Aminex HPX-87H column equipped with the appropriate guard column.

HPLC conditions:
Sample volume: 50 μL
Mobile phase: 0.005 M sulfuric acid, 0.2 μm filtered and degassed
Flow rate: 0.6 mL/minute
Column temperature: 55–65 °C
Detector temperature: as close to column temperature as possible
Detector: refractive index
Run time: 50 minutes

11. Calculations

11.1 Calculate the oven dry weight (ODW) of the extractives free sample, using the average total solids content as determined by the LAP "Standard Method for the Determination of Total Solids in Biomass."

$$ODW = \frac{Weight_{\ air\ dry\ sample}\ x\ \%\ Total\ solids}{100}$$

11.2 Calculate and record the weight percent acid insoluble residue (AIR) and acid insoluble lignin (AIL) on an extractives free basis.

$$\%\ AIR = \frac{Weight_{crucible\ plus\ AIR} - Weight_{\ crucible}}{ODW_{sample}}\ x\ 100$$

$$\%\ AIL = \frac{\left(Weight_{crucible\ plus\ AIR} - Weight_{\ crucible}\right) - \left(Weight_{crucible\ plus\ ash} - Weight_{\ crucible}\right) - Weight_{\ protein}}{ODW_{sample}}\ x\ 100$$

Where:
$Weight_{protein}$ = Amount of protein present in the acid insoluble residue, as determined in LAP "Determination of Protein Content in Biomass." This measurement is only necessary for biomass containing high amounts of protein. Our studies have shown that, for a corn stover feedstock, only a minor fraction of the protein condenses into the residue. For a pretreated corn stover, a significant amount of the nitrogenous material condenses into the AIR. The amount of nitrogen in the residue can be determined. If a nitrogen value is determined, the user should be aware that the nitrogen-to-protein factor will no longer be valid, as the nitrogenous material will likely have degraded.

11.3 Calculate the amount of acid soluble lignin (ASL) on an extractives free basis.

$$\%\ ASL = \frac{UVabs\ \ x\ Volume_{\ filtrate}\ x\ Dilution}{\varepsilon\ x\ ODW_{sample} \times Pathlength}\ x\ 100$$

Where:
UVabs = average UV-Vis absorbance for the sample at appropriate wavelength (see table below)
$Volume_{hydrolysis\ liquor}$ = volume of filtrate, 86.73 mL

$$\text{Dilution} = \frac{Volume_{sample} + Volume_{diluting\ solvent}}{Volume_{sample}}$$

ε = Absorptivity of biomass at specific wavelength (see table below)
ODW_{sample} = weight of sample in milligrams
Pathlength = pathlength of UV-Vis cell in cm

Absorptivity constants for acid soluble lignin measurement for select biomass types

Biomass type	Lambda max (nm)	Absorptivity at lambda max (L/g cm)	Recommended wavelength (nm)	Absorptivity at recommended wavelength (L/g cm)
Pinus Radiata- NIST SRM 8493	198	25	240	12
Bagasse- NIST SRM 8491	198	40	240	25
Corn Stover- NREL supplied feedstock	198	55	320	30
Populus deltiodes- NIST SRM 8492	197	60	240	25

Note: Lambda max values often contain interfering peaks from carbohydrate degradation products. Recommended wavelength values have been chosen to minimize these interferences.

11.4 Calculate the total amount of lignin on an extractives free basis.

$$\%\ Lignin_{ext\ free} = \%\ AIL + \%\ ASL$$

11.5 Calculate the total lignin value to an as received basis, if necessary

$$\%\ Lignin_{as\ received} = \left(\%Lignin_{ext\ free}\right) \times \frac{\left(100 - \%\ Extractives\right)}{100}$$

Where:
% Extractives = percent extractives in the prepared biomass sample, as determined in LAP "Determination of Extractives in Biomass"

11.6 Create a calibration curve for each analyte to be quantified using linear regression. From these curves, determine the concentration in mg/mL of each component present in the samples analyzed by HPLC, correcting for dilution if required.

11.7 Calculate and record the amount of each calibration verification standard (CVS) recovered following HPLC analysis.

$$\%\,CVS\ recovery = \frac{conc.\,detected\ by\ HPLC, mg/mL}{known\ conc.\,of\ \ standard, mg/mL}\ x\ 100$$

11.8 For the sugar recovery standards (SRS), calculate the amount of each component sugar recovered after dilute acid hydrolysis, accounting for any dilution made prior to HPLC analysis. Average any replicate ($\%R_{sugar}$) values obtained for each individual sugar and report $\%R_{avg,\ sugar}$.

$$\%\ R_{sugar} = \frac{conc.\,detected\ by\ HPLC, mg/mL}{known\ conc.\,of\ \ sugar\ before\ hydrolysis, mg/mL}\ x\ 100$$

11.9 Use the percent hydrolyzed sugar recovery values calculated in step 11.8 to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples ($C_{cor.\ sample}$), accounting for any dilution made prior to HPLC analysis.

$$C_x = \frac{C_{HPLC}\ x\ dilution\ \ factor}{\%\ R_{ave.\,sugar}\ /\ 100}$$

Where: C_{HPLC} = conc. of a sugar as determined by HPLC, mg/mL
$\%\ R_{ave.\ sugar}$ = average recovery of a specific SRS component
$C_x = C_{cor.\ sample}$, concentration in mg/mL of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis

11.10 Calculate the concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or162/180) for C-6 sugars (glucose, galactose, and mannose).

$$C_{anhydro} = C_{corr} \times Anhydro\ correction$$

Note: To be completely correct, the hydrolysis of the hemicellulose branches should be accounted for in the xylan values, since the loss of functional groups adds either a proton or a hydroxide to the xylan. Two examples of branch compounds that are quantified in this LAP are acetate and minor sugars such as galactan, arabinan, and mannan. However, for the typical concentrations of acetate and minor sugars seen in biomass samples, the resulting changes in xylan values are negligible, and so this correction is not performed. If desired, the correction can be applied per the equations below. However, this correction actually increases the uncertainty of the xylan value, since the uncertainties of the measured concentration values of the minor sugars are much larger than for xylose. It also makes the assumption that each of the minor sugars and acetate groups branch directly from the xylan backbone, which is likely an erroneous assumption.

$$C_{anhydro\_xylan} = (Xylose_{corr} \times (1 - Xylose_{AAcorrection} - Xylose_{Minorsugarcorrection})) \times 132/150$$

Where: $Xylose_{AAcorrection} = \frac{Acetic\_acid\_concentration}{Xylose\_concentration} \times \frac{17(OH^{-}MW)}{132(xyloseMW)}$

$$Xylose_{Minorsugarcorrection} = \frac{Minor\_sugar\_concentration}{Xylose\_concentration} \times \frac{1(H^{+}MW)}{132(xyloseMW)}$$

11.11Calculate the percentage of each sugar on an extractives free basis.

$$\%\ Sugar_{ext\ free} = \frac{C_{anhydro} \times V_{filtrate} \times \frac{1g}{1000mg}}{ODW_{sample}}\ x\ 100$$

Where: $V_{filtrate}$ = volume of filtrate, 86.73 mL

11.12Calculate the percentage of each sugar on an as received basis, if necessary.

$$\%\ Sugar_{as\ received} = \left(\%Sugar_{ext\ free}\right) \times \frac{\left(100 - \%\ Extractives\right)}{100}$$

Where:
% Extractives = percent extractives in the prepared biomass sample, as determined in LAP "Determination of Extractives in Biomass"

11.13Calculate the acetate percentage on an extractives free basis.

$$\%\ Acetate_{ext\ free} = \frac{C_{AA,HPLC}\ x\ Volume_{filtrate} x\ Conversion\ factor}{ODW_{sample}}\ x\ 100$$

Where:
$C_{AA,HPLC}$ = concentration in mg/mL of acetic acid as determined by HPLC
$Volume_{hydrolysis\ liquor}$ = volume of filtrate, generally 86.73 mL
Conversion factor = (59/60) = 0.983, the conversion from acetic acid to acetate in biomass

Note (July 8, 2011): The conversion factor has been updated from previous versions of the LAP. The previous value was (43/60) = 0.717.

11.14Calculate the acetyl percentage on an as received basis, if necessary.

$$\%\ Acetyl_{as\ received} = \left(\%Acetyl_{ext\ free}\right) \times \frac{\left(100 - \%\ Extractives\right)}{100}$$

11.15To report or calculate the relative percent difference (RPD) between two samples, use the following calculation.

$$RPD = \left(\frac{(X_1 - X_2)}{X_{mean}} \right) \times 100$$

Where:

X_1 and X_2 = measured values
X_{mean} = the mean of X_1 and X_2

11.16To report or calculate the root mean square deviation (RMS deviation) or the standard deviation (st dev) of the samples, use the following calculations.
First find the root mean square (RMS) of the sample using:

$$RMS = x_m = mean = \sqrt{\left(\frac{\sum_1^n x}{n} \right)^2}$$

Then find the root mean square deviation, or standard deviation, using:

$$RMSdeviation = \sigma = stdev = \sqrt{\frac{\sum_1^n (x_i - x_m)^2}{n}}$$

Where:

x_m = the root mean square of all x values in the set
n = number of samples in set
x_i = a measured value from the set

12. Report Format

12.1 Report the weight percent of lignin, each sugar, and acetate. Report on an as received basis, correcting for extractives if necessary.
12.2 For replicate analyses, report the average and relative percent difference.

13. Precision and Bias

13.1 *Round robin testing* – For a report documenting an international round robin test of biomass analysis methods, including this procedure, see Milne et al., 1992.

14. Quality Control

14.1 Reported significant figures or decimal places: Determined by data quality objectives and laboratory specific Quality Assurance Plan.

14.2 Replicates: Run all samples in duplicate.
14.3 Blank: None.
14.4 Relative percent difference criteria: Determined by data quality objectives and laboratory specific Quality Assurance Plan.
14.5 Calibration verification standard: Calibration verification standards should be independently prepared and analyzed as appropriate, per the procedure.
14.6 Sample size: 4g, minimum, of sample, extracted if necessary (including amount necessary for percent solids determination).
14.7 Sample storage: Hydrolysis liquors may be stored in a refrigerator for up to two weeks. Neutralized hydrolysis liquors may be stored in a refrigerator for up to four days.
14.8 Standard storage: HPLC standards should be stored in a freezer and removed when needed. Thaw and vortex samples prior to use.
14.9 Standard preparation: Standards should be prepared as described in the procedure, including a QA standard.
14.10 Definition of a batch: Any number of samples that are analyzed and recorded together. The recommended size is three to six samples with a QA standard, all run in duplicate.
14.11 Control charts: All CVS, SRS recoveries, and NIST or QA standards should be control charted.

15. Appendices

15.1 None

16. References

16.1 NREL BAT Team Laboratory Analytical Procedure "Determination of Total Solids in Biomass."
16.2 NREL BAT Team Laboratory Analytical Procedure "Determination of Ash in Biomass."
16.3 NREL BAT Team Laboratory Analytical Procedure "Determination of Extractives in Biomass."
16.4 NREL CAT Task Laboratory Analytical Procedure#002 "Determination of Structural Carbohydrate Content in Biomass by High Performance Liquid Chromatography."
16.5 NREL CAT Task Laboratory Analytical Procedure#003 "Determination of Acid Insoluble Lignin in Biomass."
16.6 NREL CAT Task Laboratory Analytical Procedure#017 "Determination of o-Acetyl Groups in Biomass by High Performance Liquid Chromatography."
16.7 NREL CAT Task Laboratory Analytical Procedure#019 "Hydrolysis of Corn Stover for Compositional Analysis."
16.8 Moore, W., and D. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products*. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.
16.9 ASTM E1758-01 "Standard method for the Determination of Carbohydrates by HPLC." In *2003 Annual Book of ASTM Standards, Volume 11.05.* Philadelphia, PA: American Society for Testing and Materials, International
16.10 Milne, T. A.; Chum, H. L.; Agblevor, F. A.; Johnson, D. K. (1992). "Standardized Analytical Methods." Biomass & Bioenergy. Proceedings of International Energy Agency Bioenergy Agreement Seminar, 2-3 April 1992, Edinburgh, U.K.. Vol. 2(1-6), 1992; pp. 341-366

17. List of Revisions

Version 08-03-2012:

- Page 11: Section 11.10 includes notes on the correction of the xylan number for minor sugars and acetate, as well as has a corrected $C_{anhydro\_xylan}$ equation.

Version 07-08-2011:

- Page 9: Section 11.2 includes notes on nitrogenous material in AIR.
- Page 9-10: Section 11.3 includes pathlength in the ASL equation, which has always been assumed to be 1 cm, but may differ.
- Page 11-12: Section 11.10 includes explanations and equations to correct xylan for branched hemicelluloses components.
- Page 12: Section 11.13 corrects the acetic acid to acetate conversion factor.

Previous version (July 2010).