STANDARD OPERATING PROCEDURE
STAN MAYFIELD BIOREFINERY PILOT PLANT

TITLE: Biomass Composition

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APPROVALS: Process Change Committee **DATE:**

A. Scope

This documentdescribes the procedure to perform a complete biomass composition analysis. This procedure is suitable for sugarcane bagasse samples that do not contain extractives. Other samples such as hardwoods, softwoods, or corn stover need to have the extractives removed prior to this procedure. It 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 contents of this SOP have been adapted from the National Renewable Energy Laboratory (NREL) Technical Reports NREL/TP-510-42618 (revised June 2010) and NREL/TP-510-42620 (revised August 2008).

B. Safety and Training Requirements

Refer to UF lab safety policies regarding equipment listed in section D below before starting any process work.

Review the location of fire extinguishers, fire blankets, safety showers, spill cleanup equipment and protective gear before beginning any process work.

During operations in the lab, the following safety gear will be utilized at all times:

- Safety Goggles
- Protective Gloves
- Lab Coat

CAUTION: Sulfuric acid is corrosive and should be handled with care. Use appropriate Personal Protective Equipment (PPE) when handling.

CAUTION: This procedure requires the handling of materials at high temperatures (575 °C). Make sure to wear appropriate PPE, including heat resistant gloves.

C. Related Documents and SOPs

1. NREL Technical Report: Determination of Structural Carbohydrates and Lignin in Biomass (NREL/TP-510-42618)
2. NREL Technical Report: Preparation of Samples for Compositional Analysis (NREL/TP-510-42620)
3. MSDS Binder
4. Moisture by Moisture Balance SOP-0503
5. Autoclave Operation SOP-0504

6. Sampling SOP-0511
7. Sugars, Organic Acids, and Inhibitors Concentration SOP-0505

D. Preparation/Materials/Equipment

1. Analytical balance, accurate to 0.1 mg
2. Convection drying oven, with temperature control of 105 ± 3 °C
3. Muffle furnace, equipped with a thermostat, set to 575 ± 25 °C
4. Water bath, set at 30 ± 3 °C
5. Autoclave
6. Filtration setup, equipped with a vacuum source and vacuum adaptors for crucibles
7. Desiccator containing desiccant
8. UV-Visible spectrophotometer
9. Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20°C)
10. Calcium carbonate, ACS reagent grade
11. Water, purified, 0.2 µm filtered
12. High purity standards : D-cellobiose, D(+)glucose, D(+)xylose, D(+)galactose, L(+)arabinose, and D(+)mannose
13. 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)
14. 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
15. Glass pressure tubes, minimum 90 mL capacity, with screw on Teflon caps and o-ring seals (Ace glass # 8648-30 tube with #5845-47 plug, or equivalent)
16. Teflon stir rods sized to fit in pressure tubes and approximately 5 cm longer than pressure tubes
17. Filtering crucibles, 25 mL, porcelain, medium porosity, Coors #60531 or equivalent
18. Bottles, wide mouth, 50 mL
19. Filtration flasks, 250 mL
20. Erlenmeyer flasks, 50 mL
21. Adjustable pipettors, covering ranges of 0.02 to 5.00 mL
22. Adjustable dispensers 50 - 100 mL
23. pH paper, range 4-9
24. Disposable syringes, 3 mL, fitted with 0.2 µm syringe filters

E. Detailed Procedure

1. Place an appropriate number of filtering crucibles in the muffle furnace at 575 ± 25 °C for a minimum of four hours.
2. Remove the crucibles from the furnace using a tweezers and heat resistant gloves directly into a desiccator and cool for one hour.
3. Weigh the crucibles to the nearest 0.1 mg and record this weight. Make sure the crucibles are marked with identifiers.
4. Place the crucibles 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.
5. Preparation of the sample.
    a. Air Drying
        i. This method is suitable for drying materials where ambient humidity allows the sample to air-dry to a moisture content below 10%.
        ii. Biomass samples must first be available as pieces with overall dimensions less than 5 by 5 by 0.6 cm (2 by 2 by 1/4 in.).
        iii. Spread out the biomass material on a suitable surface and allow the sample to air-dry prior to any milling. Do not pile the material deeper than 15 cm.
        iv. Determine the dry weight of the biomass sample once every 24 hours according to Moisture by Moisture Balance SOP-0503 until the moisture content is less than 10% and the change in weight is less than 1% in 24 h.
    b. Convection oven drying
        i. Select a container suitable for oven drying the biomass sample and dry this container at 45 ± 3°C for a minimum of 3 h.
        ii. Place the container in a desiccator and allow the container to cool to room temperature.
        iii. Weigh the container to the nearest 0.1 g and record this weight (W_t).
        iv. Place the biomass material into the dried container to a maximum depth of 1 cm.
        v. Weigh the container and biomass to the nearest 0.1 g and record this weight (W_{ws}).
        vi. Place the container and biomass in a drying oven maintaining the temperature at 45 ± 3°C.
        vii. Allow the material to dry for 24 to 48 h.
        viii. Remove the container and biomass from the drying oven, place in a desiccator and allow the sample to cool to room temperature.

ix. Weigh the container and biomass to the nearest 0.1 g and record this weight.
x. Return the sample to the drying oven for minimum of 4 h, maintaining the temperature at 45 ± 3°C.
xi. Remove the container and biomass from the drying oven, place in a desiccator and allow the sample to cool to room temperature.
xii. Weigh biomass to the nearest 0.1 mg and record this weight.
xiii. Return the samples to the drying oven at 45°C for 1 h.
xiv. Remove the container and biomass from the drying oven, place in a desiccator and allow the sample to cool to room temperature.
xv. Weigh biomass to the nearest 0.1 mg and record this weight.
xvi. Repeat steps E.1.b.xiv through E.1.b.xvi until the change in the mass of the biomass is less than 1% in one hour. Record this final weight (W_{ds}).
xvii. Calculate the dry weight (DW) of the biomass using the following formula:

$$DW = \frac{W_{ds}}{W_{ws}}$$

6. 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.
7. Label the pressure tube with a permanent marker. 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.
8. Add 3.00 ±0.01 mL (or 4.92 ± 0.01 g) of 72% sulfuric acid to each pressure tube.

CAUTION: Sulfuric acid is corrosive and should be handled with care. Use appropriate Personal Protective Equipment (PPE) when handling.

9. Use a Teflon stir rod to mix for one minute, or until the sample is thoroughly mixed.
10. Place the pressure tube in a water bath set at 30 ± 3 °C and incubate the sample for 60 ± 5 minutes.
    a. Using the stir rod, stir the sample every five to ten minutes without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.
11. Upon completion of the 60-minute hydrolysis, remove the tubes from the water bath.
12. Dilute the acid to a 4% concentration by adding 84.00 + 0.04 mL deionized water. Dilution can also be done by adding 84.00 ± 0.04 g of purified water using a balance accurate to 0.01 g.

13. Screw the Teflon caps on securely and 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.

14. 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.
    a. SRS should include D-(+)glucose, D-(+)xylose, D-(+)galactose, -L(+)arabinose, and D-(+)mannose.
    b. Weigh out the required amounts of each sugar, to the nearest 0.1 mg, and add 10.0 mL deionized water.
    c. Add 348 µL of 72% sulfuric acid.
    d. Transfer the SRS to a pressure tube and cap tightly.

       Note: 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.

15. 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 using the liquids setting according to the Autoclave Operation SOP-0504.

    CAUTION: Use caution when handling hot pressure tubes after removal from the autoclave, as the pressurized tubes can cause an explosion hazard.

16. After completion of the autoclave cycle, allow the hydrolysates to slowly cool to near room temperature before removing the caps.
17. Vacuum filter the autoclaved hydrolysis solution through one of the previously weighed filtering crucibles. Capture the filtrate in a filtering flask.
18. 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.

NOTE: Acid soluble lignin determination must be done within six hours of hydrolysis. If the hydrolysis liquor must be stored, it should be stored in a refrigerator for a maximum of two weeks.

19. Acid Insoluble Lignin:
    a. Use deionized water to quantatively transfer all remaining solids out of the pressure tube into the filtering crucible.
    b. 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.
    c. Dry the crucible and acid insoluble residue in the convection drying oven at 105 ± 3 °C until a constant weight is achieved, usually a minimum of four hours.
    d. Remove the samples from the convection drying oven and cool in a desiccator.
    e. Record the weight of the crucible and dry acid insoluble residue (AIR) to the nearest 0.1 mg and calculate % AIR.

$$\% \, AIR = 100 * \frac{Weight_{crucible+AIR} - Weight_{crucible}}{DW_{sample}}$$

    f. Place the crucibles and residue in the muffle furnace at 575 ± 25 °C for 24 ± 6 hours.
    g. Carefully remove the crucible from the furnace using a tweezers and heat resistant gloves directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles.
    h. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight.
    i. Place the crucibles back in the furnace and ash to a constant weight.
    j. Calculate the % acid insoluble ash (AIA) in the sample.

$$\%AIA = 100 * \frac{Weight_{crucible+ash} - Weight_{crucible}}{DW_{sample}}$$

    k. Calculate the % acid insoluble lignin (AIL).

$$\%AIL = \%AIR - \%AIA$$

NOTE: For biomass containing high amounts of protein, the amount of protein needs to be subtracted from the AIR (along with the AIA) in order to obtain the AIL (see the NREL Technical Report NREL/TP-510-42618).

20. Acid Soluble Lignin;
    a. On a UV-Visible spectrophotometer, run a background of deionized water or 4% sulfuric acid (240 nm for sugarcane bagasse).

b. Using the hydrolysis liquor aliquot obtained in step E.18., measure the absorbance (UV_{abs}) of the sample at 240 nm (for sugarcane bagasse; for other biomass see NREL Technical Report NREL/TP-510-42618) on an UV-Visible spectrophotometer.
   i. 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.
   ii. Record the absorbance to three decimal places. Reproducibility should be + 0.05 absorbance units.
   iii. Analyze each sample in duplicate, at minimum.

c. Calculate the % acid soluble lignin (ASL)

$$\%ASL = \frac{UV_{abs} * V_{filtrate} * Dilution}{\varepsilon * DW * Pathlenght} * 100$$

   i. The ε refers to the absorptivity of the biomass at the wavelength used (for sugarcane bagasse at 240 nm it is 25 L/g cm); Pathlength refers to the pathlength of the cell in cm (for different biomass refer to NREL Technical Report NREL/TP-510-42618); $V_{filtrate}$ = 86.73 mL.

21. Calculate the total amount of lignin.

$$\%Lignin = \%AIL + \%ASL$$

22. Analyze the sample for structural carbohydrates.
    a. Prepare a series of calibration standards containing the compounds that are to be quantified. Use a four point calibration. Suggested concentration ranges for calibration standards (mg/mL);
       i. D-cellobiose, 0.1-4.0
       ii. D(+)glucose, 0.1-4.0
       iii. D(+)xylose, 0.1-4.0
       iv. D(+)galactose, 0.1-4.0
       v. L(+)arabinose, 0.1-4.0
       vi. D(+)mannose, 0.1-4.0

       NOTE: A fresh set of standards is not required for every analysis. A large batch of 6 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.

b. Prepare an independent Calibration Verification Standard (CVS) for each set of calibration standards.
   i. Use reagents from a source or lot other than that used in preparing the calibration standards.
   ii. Prepare the CVS at a concentration that falls in the middle of the validated range of the calibration curve (2.5 suggested).
   iii. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples.
   iv. The CVS is used to verify the quality and stability of the calibration curve throughout the run. Calculate the recovered sugars for each CVS.

$$\%CVS\ recovery = \frac{[Conc\ detected\ by\ HPLC]}{[Known\ conc\ standard]} * 100$$

c. Using the hydrolysis liquor obtained in step E.18., transfer an approximately 20 mL aliquot of each liquor to a 50 mL Erlenmeyer flask.
d. Use calcium carbonate to neutralize each sample to pH 5 – 6.
   i. Avoid neutralizing to a pH greater than 6 by monitoring with pH paper.
   ii. Add the calcium carbonate slowly after reaching a pH of 4.
   iii. Swirl the sample frequently.
   iv. After reaching pH 5 – 6, stop calcium carbonate addition, allow the sample to settle, and decant off the supernatant.
   v. The pH of the liquid after settling will be approximately 7.
   vi. Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.
e. Analyze the samples for sugars according to the Sugars, Organic Acids, and Inhibitors Concentration SOP-0506.
   i. 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.
   ii. 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 degradations 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.

iii. For the sugar recovery standards (SRS), calculate the amount of each component sugar recovered ($\%R_{sugar}$) after dilute acid hydrolysis, accounting for any dilution made prior to HPLC analysis.

$$\%R_{sugar} = \frac{[Conc\ detected\ by\ HPLC]}{[Known\ conc\ before\ hydrolysis]} * 100$$

iv. Use the $\%R_{sugar}$values obtained in the previous step 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.

$$C_{cor.sample} = \frac{[Conc\ detected\ by\ HPLC] * Dilution}{\%R_{sugar}/100}$$

f. 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)

$$C_{anhydro} = C_{cor.sample} * Anhydro\ correction$$

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 as detailed in the NREL Technical Report NREL/TP-510-42618 (p. 11-12). 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.

23. Analyze the sample for acetyl content (if necessary);
    a. Prepare a series of calibration standards containing acetic acid, formic acid, and levulinic acid. A range of 0.02 to 0.5 mg/mL is suggested, along with a four point calibration.
    b. Prepare an independent calibration verification standard (CVS) for each set of calibration standards
        i. Use components obtained from a source other than that used in preparing the calibration standards.

ii. 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.
iii. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples.
iv. The CVS is used to verify the quality and stability of the calibration curve throughout the run.

c. Prepare the sample for HPLC analysis according to Sugars, Organic Acids, and Inhibitors Concentration SOP-0506.

24. Calculate the percentage of each sugar.

$$\%Sugar = \frac{C_{anhydro} * V_{filtrate}}{DW} * 100$$

a. $V_{filtrate}$ = 86.73 mL

25. Calculate the acetate percentage on an extractives free basis.

$$\%Acetate = \frac{C_{AA,HPLC} * V_{filtrate} * Conversion\ factor}{DW} * 100$$

a. $C_{AA,HPLC}$ refers to the concentration of acetic acid as determined by HPLC; $V_{filtrate}$ = 86.73 mL; Conversion factor = 59/60 = 0.983, the conversion from acetic acid to acetate in biomass.