National Renewable Energy Laboratory
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Summative Mass Closure

Laboratory Analytical Procedure (LAP) Review and Integration: Pretreated Slurries

Issue Date: August 2010

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(Version 07-08-2011)

J. Sluiter and A. Sluiter

Technical Report
NREL/TP-510-48825
Revised July 2011

NREL is operated for DOE by the Alliance for Sustainable Energy, LLC Contract No. DE-AC36-08-GO28308

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Prepared under Task No. BB07.2230

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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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Operated by the Alliance for Sustainable Energy, LLC

Contract No. DE-AC36-08-GO28308

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Summative Mass Closure – LAP Review and Integration: Pretreated Slurries

Laboratory analytical procedures (LAPs) developed at the National Renewable Energy Laboratory (NREL) have been optimized to provide compositional analysis for biomass feedstocks as well as for intermediary products of dilute acid pretreatment. The chemical pretreatment of biomass is a common process in the bioethanol industry. A wide array of pretreatment chemistries and reactions allows for the tuning of components released from the biomass.

These NREL LAPS are optimized for woody or herbaceous species that have been pretreated with dilute acid at elevated temperatures. They have been successfully applied with no issues to biomass pretreated with steam or hot water at neutral pH. Biomass pretreated under basic conditions may require substantial method adjustment to obtain accurate compositional analysis information. Biomass that has been mildly treated or degraded, for example, during ensiling, is not suitable for this type of analysis and may be better considered a feedstock for analysis purposes.

Dilute acid pretreatment produces a slurry composed of undigested biomass solids and an aqueous phase. The aqueous phase, or "liquor," typically will contain large portions of the hemicellulose-derived products, some of the lignin, and any degradation products from the acid pretreatment. The cellulose typically remains in the solid fraction of the slurry along with whatever lignin did not dissolve during pretreatment. Minor constituents, such as ash and protein, are usually split between the two phases.

A discussion of each LAP that is necessary to obtain complete compositional analysis of dilute acid-pretreated biomass slurries is presented below.

Sampling Pretreated Slurry

Pretreated slurries are very heterogeneous and sampling problems can dramatically affect the ability to accurately characterize the material. It is recommended that the slurry be thoroughly mixed immediately before and also during sampling. Failure to immediately sample will not change the composition of the washed solids or the liquors but can significantly bias any attempts to determine fraction allocations (i.e., total and insoluble solids measurements).

LAP: Determination of Insoluble Solids in Pretreated Biomass Material

Due to the variety of components present in the slurry, this LAP recommends separating the solid and aqueous phases for parallel analyses. This separation is described in the LAP "Determination of Insoluble Solids in Pretreated Biomass Material." This LAP is used to determine the fraction of insoluble solids (FIS) that is present in the pretreated slurry. It describes the detailed measurement of solids and liquids that is necessary to relate measured component concentration back to the slurry as a whole. The wash water from the rinsing process must be kept if FIS is to be determined. This process may also be used to isolate liquor or washed solids samples. In this case, many of the measurements and calculations can be eliminated.

Centrifugation and filtration methods are discussed for isolation of liquors from solids in this LAP. Both methods separate a pure sample of liquor from the slurry for analysis, prior to any

washing of the solids, and both methods detail steps for washing the solids free of soluble materials by repeated rinsing with water. An alternative for recovering liquor from the slurry is by pressing with a pneumatic or hydraulic press.

Centrifugation is the recommended method for most biomass samples. A large capacity centrifuge is required to process enough pretreated biomass for compositional analysis. Analysts should retain as much of the fine solids during the washing procedures as is practical, as these materials may have significantly different chemical makeup.

Filtration can be faster than centrifugation for samples that are lightly pretreated or for samples with a more liquid consistency. When using the filtration method, do not expose the liquors to excessive vacuum, as evaporation of the water will cause concentration of the solubilized components.

Washing of the solids is critical, as the residual liquor present in the solids contains acid that will concentrate as the material dries, potentially hydrolyzing some of the solids. Additionally, the sugars dissolved in the liquor can contribute significantly to the measured component concentrations. To ensure complete removal of soluble sugars, the concentration of glucose is monitored in the rinse water. Xylose is the most concentrated sugar in the liquor, but glucose is an easier sugar to monitor. If a fast xylose measurement is available, it may be used in place of the glucose measurement.

Isolation of samples for saccharification and fermentation

The procedure detailed above is specifically designed for the isolation of materials for compositional analysis. If materials are to be isolated for enzymatic hydrolysis or fermentation, there are additional considerations.

- Washed pretreated solids should not be dried before enzymatic hydrolysis or fermentation, as this can cause cell wall collapse. Cell wall collapse is thought to limit the availability of the surfaces to enzymes.

- Unwashed pretreated solids should not be squeezed at excessive pressures as this may lead to cell wall collapse. Tests have been performed at up to ~600 psig of direct pressure that show no detrimental effect on enzymatic hydrolysis. Further testing should be done before exceeding this pressure.

Liquor Analysis

Liquor analysis is detailed in the LAP "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples."

Washed Pretreated Solids Analysis

The procedure for chemical compositional analysis of washed pretreated solids is very similar to that for an extracted feedstock sample. Washed pretreated solids do not require extraction, as the extractable material is removed by the pretreatment process and the washing of the slurry. Additionally, chemical changes in the biomass will result in extraction methods removing more material than anticipated, some of which cannot be characterized. The discussion below is substantially similar to the LAP "Summative Mass Closure – LAP Review and Integration:

Feedstocks." Sections that are not used in analysis of washed pretreated solids have been removed, and the most important points are detailed below.

The differences between the procedures for compositional analysis of extracted feedstock and washed pretreated solids are discussed here.

- In addition to the drying methods available for feedstocks, washed pretreated solids may be dried in a vacuum oven set to 40°C. Vacuum levels of 15–20 in. Hg are sufficient to remove water from the washed pretreated solids. In a well sealed vacuum oven, it may be necessary to provide a small bleed of airflow to prevent the oven's atmosphere from becoming saturated with moisture.

- Milling of *feedstock* samples is carefully controlled to prevent over-hydrolysis of small particles. Washed pretreated solids are much harder to mill to a specific particle size because there is usually significant degradation of the structure of the biomass. Nevertheless, it is still necessary to mill the solids, but fines are not removed by sieving. Generally, a smaller particle size is acceptable for pretreated material.

- Protein analysis is performed on the washed pretreated solids, but it is difficult to determine a conversion factor for nitrogen to protein due to the degradation of the proteins during acid pretreatment. The protein content is still reported, but it is important to realize that this is best viewed as a tracking number.

- The extinction coefficient used for the feedstock should also be used for the pretreated materials when determining the acid soluble lignin content.

Flow Chart of Analysis

The flowchart of analysis in Figure 1 provides an example of a complete pretreated slurry preparation. It illustrates the process of washing a slurry sample and the isolation of fractions for compositional analysis. The flow chart refers to NREL procedures, not detailed here, that are required in conjunction with this LAP. The flow chart is not designed to incorporate FIS determination, as several additional steps are necessary for that analysis.

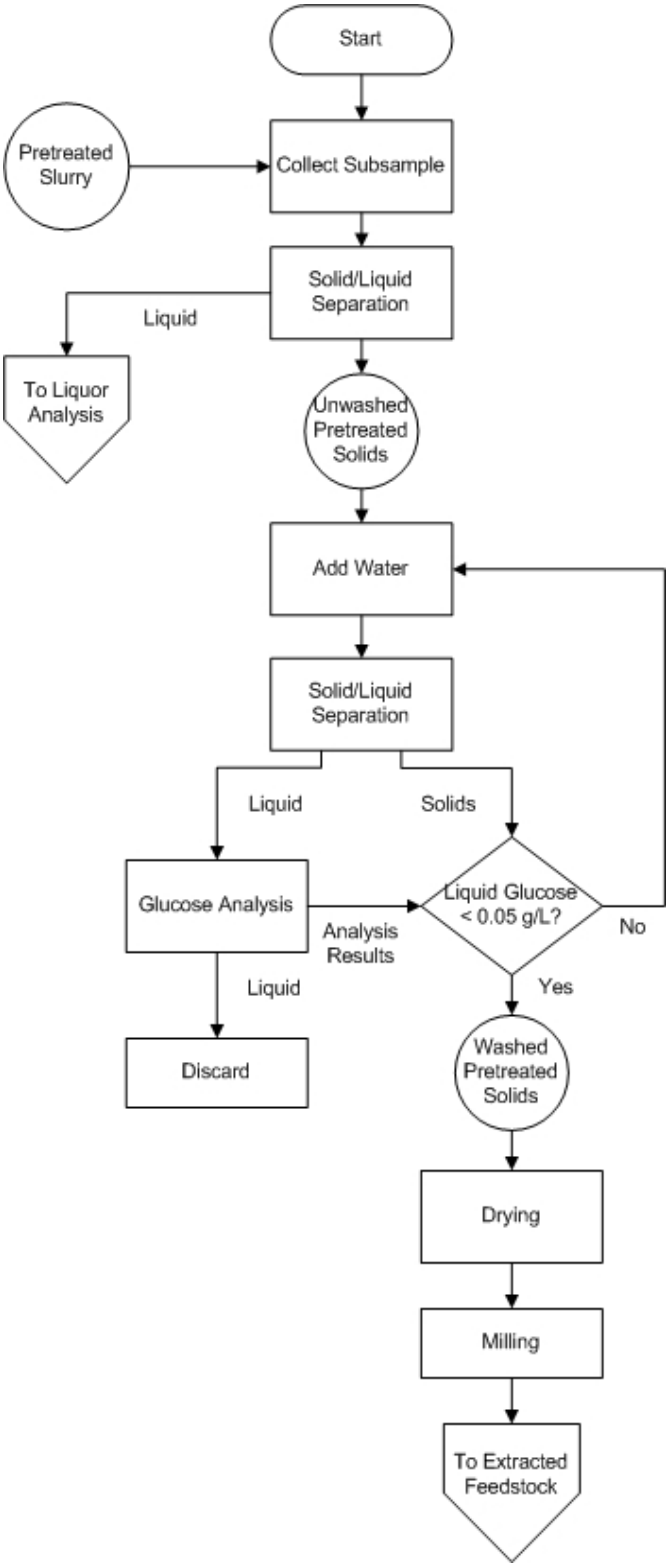

Figure 1. Flowchart of analysis

LAP: Preparation of Samples for Compositional Analysis

The first consideration for sample preparation is drying. If a sample contains greater than 10% moisture by weight, it must be dried to ensure compatibility with subsequent analyses steps. LAP "Preparation of Samples for Compositional Analysis" describes the appropriate drying procedures for the compositional analysis suite. Three options are presented within the LAP. One option is air-drying, which is most suitable for locations with low ambient humidity conditions. When ambient humidity is too high to permit this technique, samples must be monitored for degradation and microbial growth until the moisture content is less than 10% by weight. Convection oven drying at 45°C, most suitable for samples likely to be unstable at ambient conditions or when ambient humidity does not allow for air-drying, is covered as well. Oven drying at 105°C should be used with care, as many biomass types chemically change when exposed to prolonged heat. The last technique covered is lyophilization drying, or freeze drying, which is suitable for samples that are at risk for degradation at elevated temperature and therefore are not suitable for convection oven drying or air-drying. Lyophilization is an unsuitable technique for large or bulk samples and those with large pieces of biomass.

Samples must be milled prior to compositional analysis if the particle size is greater than 2 mm. Since the subsequent methods are optimized for a 2-mm or less particle size, milling the sample will likely be necessary. Prior to milling, the sample must meet the moisture requirements discussed above. Milling a wet sample can result in the degradation of the sample. The mill must be monitored to ensure that it is operating at optimal temperature. An overheated mill can cause extractable material to separate from the biomass and deposit on the heated metal portions of the mill. Milling with dry ice is not recommended, as the oils in dry ice leave a residue on the biomass.

LAP: Determination of Total Solids in Biomass

Due to the high variability of moisture content in biomass, all biomass compositional analysis results are reported on dry weight basis. This allows for comparison of biomass samples on a consistent baseline. At several points in the compositional analysis suite, a %Total Solids determination is required. The LAP "Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples" details appropriate procedures for such determinations. The LAP describes drying by convection oven at 105°C and drying by infrared moisture balance.

This determination should be done at the same time as the corresponding procedure that calls for a %Total Solids determination, as the moisture content of a biomass sample can change quickly. The values obtained during this analysis are used to mathematically correct the sample back to a dry weight basis. The sample aliquot used for %Total Solids analysis has been exposed to elevated temperatures and should not be used in further analyses, with the exception of determining ash content on an extremely limited sample quantity.

Calculation of %Total Solids is shown below.

$$\%Total\ Solids = \frac{(Weight_{dry\ pan\ plus\ dry\ solids} - Weight_{dry\ pan})}{Weight_{sample\ as\ received}} \times 100$$

Calculation of the dry weight of a sample or its Oven Dried Weight (ODW) is described in the following formula.

$$ODW_{sample} = \frac{(Weight_{air\ dried\ sample} \times \%Total\ Solids)}{100}$$

LAP: Determination of Structural Carbohydrates and Lignin in Biomass

Structural carbohydrates and lignin make up the bulk of most feedstocks and pretreated materials and often represent the most interesting portions. The LAP "Determination of Structural Carbohydrates and Lignin in Biomass" describes the acid hydrolysis and subsequent analyses of acid soluble and acid insoluble portions. It describes the preparation and two-stage sulfuric acid hydrolysis of the sample and includes the use of sugar recovery standards, which are used to correct for loss of carbohydrates during hydrolysis. This LAP also describes carbohydrate analysis, including preparation of standards, hydrolysate neutralization, HPLC method setup, and acetyl analysis.

The determination of carbohydrates using this method requires that all carbohydrates be in monomeric form. The presence of sugar oligomers indicates incomplete hydrolysis, and those carbohydrates will not be captured. During hydrolysis, the conversion of carbohydrate polymers to monomers results in the addition of a hydrogen atom and a hydroxyl group to each monomer. An anhydro correction is used to mathematically convert the monomeric values back to a structural polymeric value.

Sugar recovery standards (SRSs) are used to account for sample sugar degradation during the dilute sulfuric acid step. SRSs are used to mimic the behavior and degradation of sample monomeric sugars. Since these values can fluctuate depending on a variety of factors, SRSs are included with every sample analysis. They are independent from the sample but are run in parallel. Because carbohydrate concentration will affect degradation levels, it is imperative to mimic the sample carbohydrate concentrations as closely as possible in the SRSs. Since this correction is critical, duplicate or triplicate SRSs are recommended.

The LAP details the steps necessary to determine acid insoluble residue, including filtration of the hydrolysate and determination of ash in the residue. Acid insoluble residue, once corrected for ash and protein content if necessary, is considered high molecular weight lignin. This definition of lignin is considered a behavior-based lignin definition, as opposed to a chemical based definition, which would include further characterization of the material. Acid insoluble residue must be corrected for ash, as a significant portion of the ash in the whole biomass is acid insoluble. For samples containing protein, the contribution of protein should be mathematically removed. Generally, all of the protein in the whole pretreated sample is assumed to condense into the insoluble solids. The specific amount of protein that will co-condense can vary between biomass types, but this number is exceptionally difficult to determine since the protein from the whole sample is degraded.

Acid soluble lignin is low molecular weight lignin that is solubilized in the acidic hydrolysis solution. Inclusion of acid soluble lignin concentration in the total lignin value is necessary, as acid soluble lignin can be a significant fraction of the lignin. The LAP describes the

measurement of acid soluble lignin but does not detail the determination of the proper extinction coefficient for feedstocks. A short list of common extinction coefficients is included in the LAP.

This LAP contains several notable interferences, such as high moisture or ash content in the sample. High moisture content, above 10% by weight, dilutes the acid concentration beyond the specifications of the LAP, possibly resulting in incomplete hydrolysis. Similarly, ash content above 10% by weight may buffer the acid, causing an effective reduction in acid concentration. However, not all inorganic material in biomass has this buffering effect. The buffering effect of excessive inorganic material should be determined prior to analysis if this problem is suspected.

Extractives remaining in very mildly pretreated feedstocks will interfere with this method. Extractives can deposit on the filter during separation of the acid soluble and acid insoluble fractions, resulting in excessive filtration time and potential concentration of the liquid fraction. Extractives can also partition irreproducibly between the acid soluble and acid insoluble fractions, thereby compromising the lignin values.

The calculations for %Acid Insoluble Lignin (%AIL) and %Acid Soluble Lignin (%ASL) are included below along with necessary carbohydrate calculations.

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

Where $Weight_{protein}$ = amount of protein present in the acid insoluble residue, as determined in the LAP "Determination of Protein Content in Biomass." This measurement is only necessary for biomass containing high amounts of protein.

$$\%ASL = \frac{UVabs \times Volume_{hydrolysis\ liquor} \times Dilution}{\varepsilon \times ODW_{sample\ (mg)} \times Pathlength} \times 100$$

Where:

UVabs = average UV-Vis absorbance for the sample at the appropriate wavelength

$Volume_{hydrolysis\ liquor}$ = volume of filtrate, 87mL

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

$\varepsilon =$ Absorptivity of biomass at specific wavelength (see table below).

Table 1. 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 radiate	198	25	240	12
Sugarcane bagasse	198	40	240	25
Corn stover	197	55	320	30
Populus deltoids	197	60	240	25

Calculate the total amount of lignin on an extractive free basis as below:

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

For the determination of carbohydrates, first correct the sugar concentrations for degradation during hydrolysis:

$$\%R_{sugar} = \frac{conc.of\ sugar\ after\ hydrolysis\ by\ HPLC, mg/mL}{conc.of\ sugar\ before\ hydrolsis\ by\ HPLC, mg/mL} \times 100$$

$$Conc_{sugar} = \frac{Conc_{HPLC} \times dilution\ factor}{\%R_{sugar}/100}$$

Where:

$Conc_{HPLC}$ = concentration of an individual sugar as determined by HPLC after hydrolysis, mg/mL.

$\%R_{ave.sugar}$ = average recovery of a specific SRS component ($\%R_{sugar}$)

$Conc_{sugar}$ = $Conc_{corr.sample}$, concentration in mg/mL of a sugar in the hydrolysate sample after correction for loss on 4% hydrolysis.

Next, 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).

$$Conc_{anhydro} = Conc_{Corr.sample} \times Anhydro\ correction$$

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

$$\%Acetyl_{ext\ free} = \frac{Conc_{acetic\ acid,HPLC} \times Vol_{filtrate} \times 0.983}{ODW_{sample}}$$

List of Revisions

Version 07-08-2011:

- Page 5: Drying temperature in a convection oven in sample preparation section has been corrected from 105°C to 45°C.
- Page 7: %ASL equation has been corrected.
- Page 9: Acetic acid to acetate modifier had been corrected.

Original version (August 2010).