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Summative Mass Closure

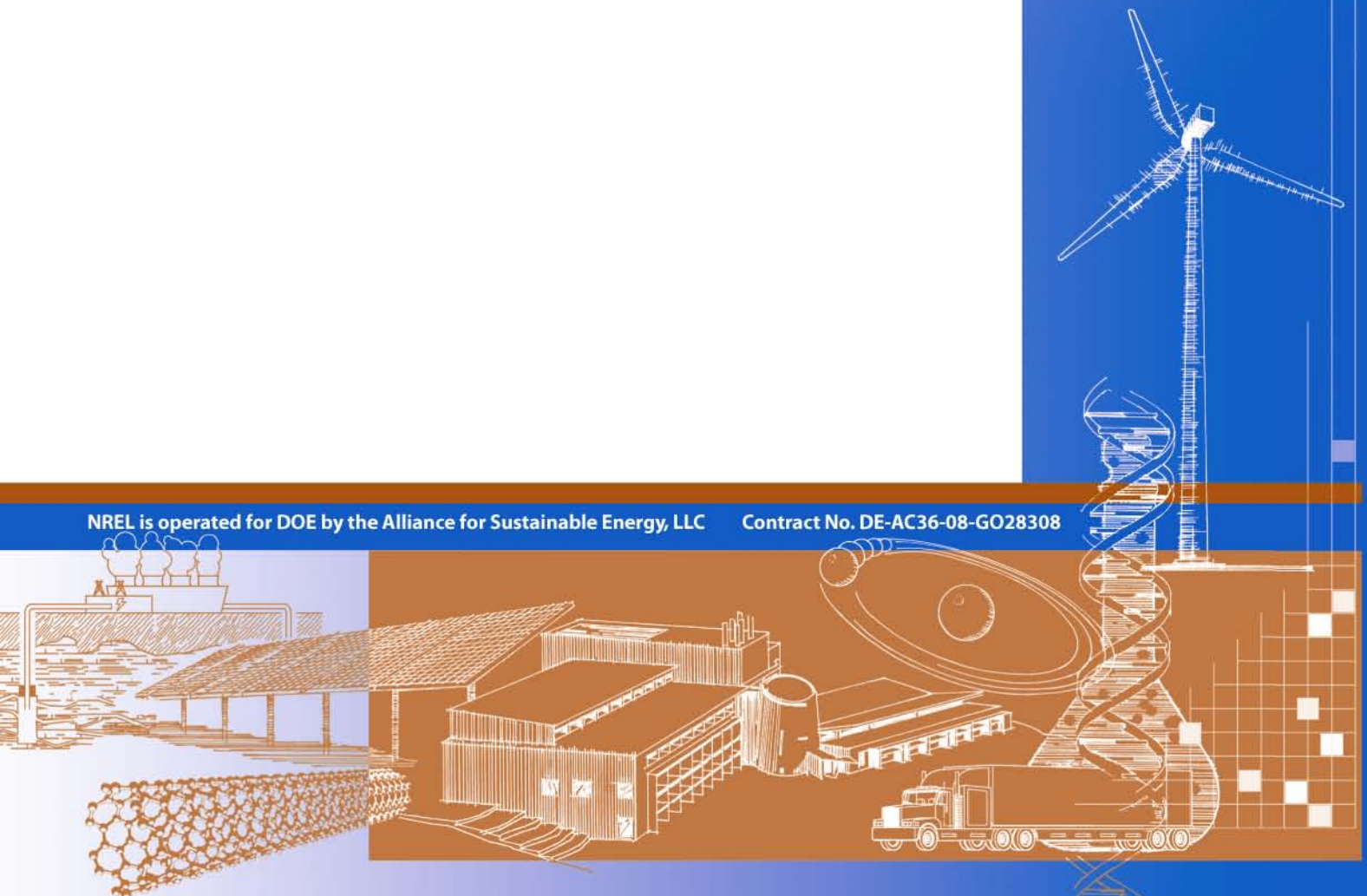
Laboratory Analytical Procedure (LAP) Review and Integration

Issue Date: April 2010

Revision Date: July 2011
(Version 07-08-2011)

J. Sluiter and A. Sluiter

Technical Report
NREL/TP-510-48087
Revised July 2011



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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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Summative Mass Closure – LAP Review and Integration: Feedstocks

NREL has developed a suite of Laboratory Analytical Procedures (LAPs) for the analysis of biomass. Many of these methods build on years of research in the biomass analytical field. Choosing the appropriate combinations of LAPs allows for the summative mass closure of biomass feedstocks and process intermediates. By combining the appropriate LAPs, the goal is to break the biomass sample down into constituents that sum to 100% by weight. Some of these constituents are individual components, such as individual carbohydrates, and some are groups of compounds, such as extractable material. However, the goal of these analyses is to characterize all of the material in the sample. It is imperative to follow the LAPs as they are written, as small deviations can have a large detrimental impact on the constituent values. This document covers the summative mass closure for biomass feedstocks only.

There are many subtle differences in the analytical suites for differing feedstocks. This LAP is designed to help in the decision making process. It will assist in the selection of appropriate LAPs and help organize the flow from one LAP to the next in the analysis sequence. It is not meant to replace the specific LAPs in any way, but to aid in the development of a comprehensive analysis specific to the feedstock. The LAPs have been optimized for corn stover and generally work well on woody feedstocks and herbaceous materials such as switchgrass, sorghum, and miscanthus, although minor adjustments may be necessary. Unusual feedstocks will typically require some method development to capture constituents not included in the LAP suite.

There are several important points within the compositional analysis suite where decisions must be made to optimize the analysis. Some of these decisions are based on the type of biomass present, and some decisions must be made to obtain complete summative mass closure of all constituents. Discussions to aid these decisions are included with LAP discussions below. Following the LAP discussions is a flowchart of analyses and a walkthrough of the flowchart using an example feedstock, including some troubleshooting.

Preparation of Samples for Compositional Analysis

Biomass samples typically arrive from the field in an intact or semi-intact state that includes soil or other debris and significant moisture content. Proper sample preparation will minimize interferences in subsequent compositional analyses. Sample drying, particle size reduction, and potential sieving are discussed in this LAP.

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. 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, most suitable for low ambient humidity. 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^{o}C, most suitable for samples likely to be unstable at ambient conditions or where ambient humidity does not allow for air-drying, is covered as well. Oven drying at 105^{o}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 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 moisture requirements discussed above. Milling wet samples can result in the degradation of the sample during milling. 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.

Sieving of a sample is sometimes necessary to accurately analyze a biomass feedstock but may interfere with representative sampling. The acid hydrolysis steps have been optimized for a -20/+80 particle size, and deviation to a larger particle size distribution will cause structural carbohydrates to be incompletely dissolved into solution. Such deviation will result in higher acid insoluble lignin values and lower overall structural carbohydrates, especially cellulose. Deviation from the recommended particle size to a smaller particle size may result in over hydrolysis of the structural carbohydrates, contributing to an overproduction of sugar degradation products, which can complicate the acid soluble lignin measurement.

Sieving was originally developed for the analysis of very homogeneous materials, such as wood samples. However, in herbaceous feedstocks, fines (-80 mesh) often contain a disproportionately large percentage of inorganic materials as compared to the bulk sample. Removal of the fines will cause an overall change in composition. In such cases sieving is not recommended, to ensure the integrity of the sample. Alternately, sieving may be performed, but the constituent values of the fines should be mathematically added back into the whole value before reporting; this calculation is not discussed. Sieving can be performed to remove a portion of the higher ash content fraction. This is only the case when the ash content of the extracted biomass is high enough to interfere with hydrolysis. Further discussion of ash interference is included with the hydrolysis discussions.

Determination of Total Solids in Biomass

Due to the high variability of moisture content in biomass feedstocks, 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^{o}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.

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

Determination of Ash in Biomass

Inorganic materials are present in both whole and structural, or extracted, biomass samples. In addition to contributing significantly to total mass closure, inorganic material may interfere with acid hydrolysis. LAP "Determination of Ash in Biomass" describes two methods for the determination of %Ash in biomass. The LAP provides instructions for ash determination in a muffle furnace set to 575°C with prior preignition and describes the use of a ramping muffle furnace with no preignition. The calculation for determining %Ash is below.

$$\%Ash = \frac{(Weight_{crucible\ plus\ ash} - Weight_{crucible})}{ODW_{sample}} \times 100$$

Determination of Protein in Biomass

Herbaceous feedstocks can contain a significant amount of protein in the stalks and leaves, which will interfere with lignin measurements in subsequent analyses. Quantification of the protein will allow this interference to be mathematically minimized. Measurement of protein in biomass is performed indirectly by measurement of nitrogen content and use of a nitrogen-to-protein conversion multiplier. The typically used nitrogen-to-protein conversion value of 6.25 is not generally applicable to biomass proteins. Instead, calculation of the conversion factor is done by measurement of individual amino acids in the feedstock of interest.

Because a portion of the protein is often removed during the extraction process, protein analysis is performed on both whole and extractives-free materials.

Determination of Extractives in Biomass

Nonstructural materials in biomass often contribute significantly to the mass closure and will interfere with the characterization of carbohydrates and lignin. LAP "Determination of Extractives in Biomass" describes extraction processes to both remove and quantify the extractable portion of a biomass feedstock using successive ethanol and water extractions.

Extraction with ethanol is required for all biomass types to ensure the removal of waxy materials that co-precipitate during filtration of the acid hydrolysate. When analyzing woody feedstocks, ethanol extraction alone is generally sufficient to remove interfering extractable material, including sap and resins. Herbaceous feedstocks require a water extraction prior to the ethanol extraction to allow for additional quantification of components more commonly found in herbaceous feedstocks. Nonstructural water soluble components commonly include inorganic material in the form of soil or fertilizers, proteins that are easily washed from the biomass, and a diverse array of carbohydrates, especially sucrose. Sucrose, a dimer of glucose and fructose, is of particular interest to fermentation and can be abundant in an herbaceous plant, but it is easily lost during the hydrolysis stages. Early removal and analysis of sucrose allows for better quantification of the structural glucose present in a feedstock as well. The LAP describes the sampling of the water extractable material to determine sucrose concentration.

Once a sample has been extracted, subsequent analytical values must be corrected for that removal to bring all values to a whole biomass basis. That calculation is included in the calculation list below.

Note that herbaceous feedstocks are typically higher in inorganic materials (commonly soil or fertilizer) and protein than woody feedstocks are. The water extraction process will remove some of these materials; therefore ash and protein measurements are recommended both before and after extraction.

The formula below should be used in calculating %Extractives content for both water and ethanol extractives.

$$\%Extractives = \frac{Weight_{flask\ plus\ extractives} - Weight_{flask}}{ODW_{sample}} \times 100$$

The formula below is used when a portion of the extractives are removed for sucrose analysis.

$$\%Extractives = \frac{Weight_{flask\ plus\ extractives} - Weight_{flask}}{ODW_{sample}} \times \frac{Volume_{total\ solution}}{Volume_{solution\ minus\ sample\ removed}} \times 100$$

The calculation to correct from an extractives free basis to a whole biomass basis for any component measured on the extracted biomass is the following:

$$Component_{whole\ biomass} = Component_{extractives\ free} \times \frac{100 - \%Extractives}{100}$$

$$\%Sucrose = \frac{Concentration_{sucrose} \times Volume_{total\ solution}}{ODW_{sample}} + \frac{Concentration_{glucose} \times 1.9 \times Volume_{total\ solution}}{ODW_{sample}} \times 100$$

Determination of Structural Carbohydrates and Lignin in Biomass

Structural carbohydrates and lignin make up the bulk of most feedstocks and often represent the most interesting portions. 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. The 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 polymers indicates incomplete hydrolysis, and those carbohydrates will not be captured. During hydrolysis, the conversion of polymers to monomers in the carbohydrates results in the addition of a hydrogen 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 monomers. 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 ash determination of 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. Some herbaceous feedstocks may need to have the acid insoluble residue corrected for protein as well, as a significant portion of the protein from the feedstock can condense into that fraction. The specific amount of protein that will co-condense can vary between feedstocks. Individual feedstocks need to be evaluated for protein condensation into the acid insoluble residue. This evaluation is not included in the method.

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 represent an important portion 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.

Unextracted biomass feedstock 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, 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 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

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

ε = 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).

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

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

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

Determination of Starch in Biomass

Starch, a non-crystalline glucose polymer, is often found in biomass feedstock that contains grain. LAP "Determination of Starch in Biomass" is a procedure that is based heavily on the

Megazyme Total Starch Assay (amyloglucosidase/α-amylase method). The major difference in the NREL adaptation is the analysis for quantification of glucose. The LAP changes from a derivatization for colorimetric detection to HPLC glucose analysis. Because the quantification of glucose is nonspecific to starch, extraction of the biomass is recommended prior to the starch assay to remove any nonstructural free glucose. Failure to remove free glucose will artificially elevate the starch content of the biomass sample. If this procedure is performed in conjunction with carbohydrate (cellulose and hemicelluloses) determination, the contribution of glucose from starch will be included in the total glucose value.

$$\%Starch = \frac{Glucose\ Conc_{HPLC} \times \frac{Volume_{Solution}}{ODW}}{1.11 \times \%Recovery_{starch\ standard}} \times 100$$

Example of Flowchart Use and Decisions

The flow chart of analysis (Figure 1) provides an example of a complete biomass feedstock analysis. The paragraphs below will step through an analysis of a whole feedstock sample, detailing the decisions necessary at each step.

For the purposes of demonstrating the flow chart and decision making steps, a hypothetical herbaceous feedstock will be used as an example. This feedstock is a potential energy crop that is harvested off of the ground. The plant is known to produce grain late in life, but this particular sample should not contain grain, according to the sample source. Before being shipped for analysis the sample was dried and milled through a 2-mm screen and sealed in a plastic bag.

As this sample has already been milled to an appropriate particle size distribution, the first decision to be considered for this sample is whether sieving is necessary. Initial ash measurements indicate an ash content of 12%, which is greater than the recommended 10%, and may interfere with the acid hydrolysis steps. Sieving could be tested to determine if some of the ash could be partitioned into the fines (-80 mesh), but as the plant was harvested off of the ground, the high ash content is likely soil and can be reduced by water extraction. Since the sample is herbaceous, water extraction is already part of the analysis suite.

Extraction is the next major consideration for the sample. As discussed above, all herbaceous materials are extracted with water as well as with ethanol. Prior to extraction with water, samples must have protein and total solids measurements performed, in addition to the ash measurement, which has already been done. Water extraction is required to quantify sucrose levels for herbaceous materials, but it also proves to be efficient at reducing the ash content to 5%. If it had not, sieving would need to be reconsidered to reduce the ash content to less than 10% before hydrolysis. Ethanol extraction follows the water extraction to ensure no complications regarding acid insoluble lignin measurements. The sample is now extractives-free and ready for hydrolysis.

Prior to hydrolysis the sample must again have total solids, protein, and ash measurements performed. Total solids will be used to convert values to a dry weight basis. Protein and ash measurements are used to determine the amount of these constituents removed during the extraction process.

Hydrolysis is run as described in the LAP, but the sample seems to have difficulty filtering while removing the acid insoluble lignin. Carbohydrate analysis, acetyl analysis, and acid soluble lignin measurement proceed as expected.

Once the data is compiled, the glucan measurement seems disproportionately high and the mass closure is significantly below 100%, while the other constituent values are what can be expected in similar feedstocks. Additionally, the acid soluble lignin results seem to have higher than expected errors. These problems may stem from one issue or a combination of problems. Two potential areas of interference will be examined.

If the plant did contain grain that was not detected in the field, the sample may have starch that was not accounted for. Since starch is measured as glucan in the analysis, starch content would explain the high glucan values, but not the low mass closure.

The unusually slow filtration of the acid insoluble lignin and the high acid insoluble lignin errors are commonly related. It is likely that there is something in the hydrolysate solution that the LAPs have not been optimized for, and an additional step is needed. In this case, further solvent extractions would be a good consideration to remove additional fractions of extractable material.

Closing

The LAPs described above cover the summative mass closure for biomass feedstocks only, and some are not appropriate for chemically or thermally altered materials. The goal is to break the biomass sample down into constituents that sum to 100% by weight. If the constituents do not sum to 100%, the LAP flow chart should be revisited to determine missing or incorrectly quantified components. There are many subtleties in the analytical suites for differing feedstocks, and this LAP is not meant to replace the specific LAPs in any way.

List of Revisions

Version 07-08-2011:

- Page 1: Drying temperature in a convection oven in sample preparation section has been corrected from 105^{o}C to 45^{o}C.
- Page 6: %ASL equation has been corrected.
- Page 7: Acetic acid to acetate modifier has been corrected.

Original version (April 2010).

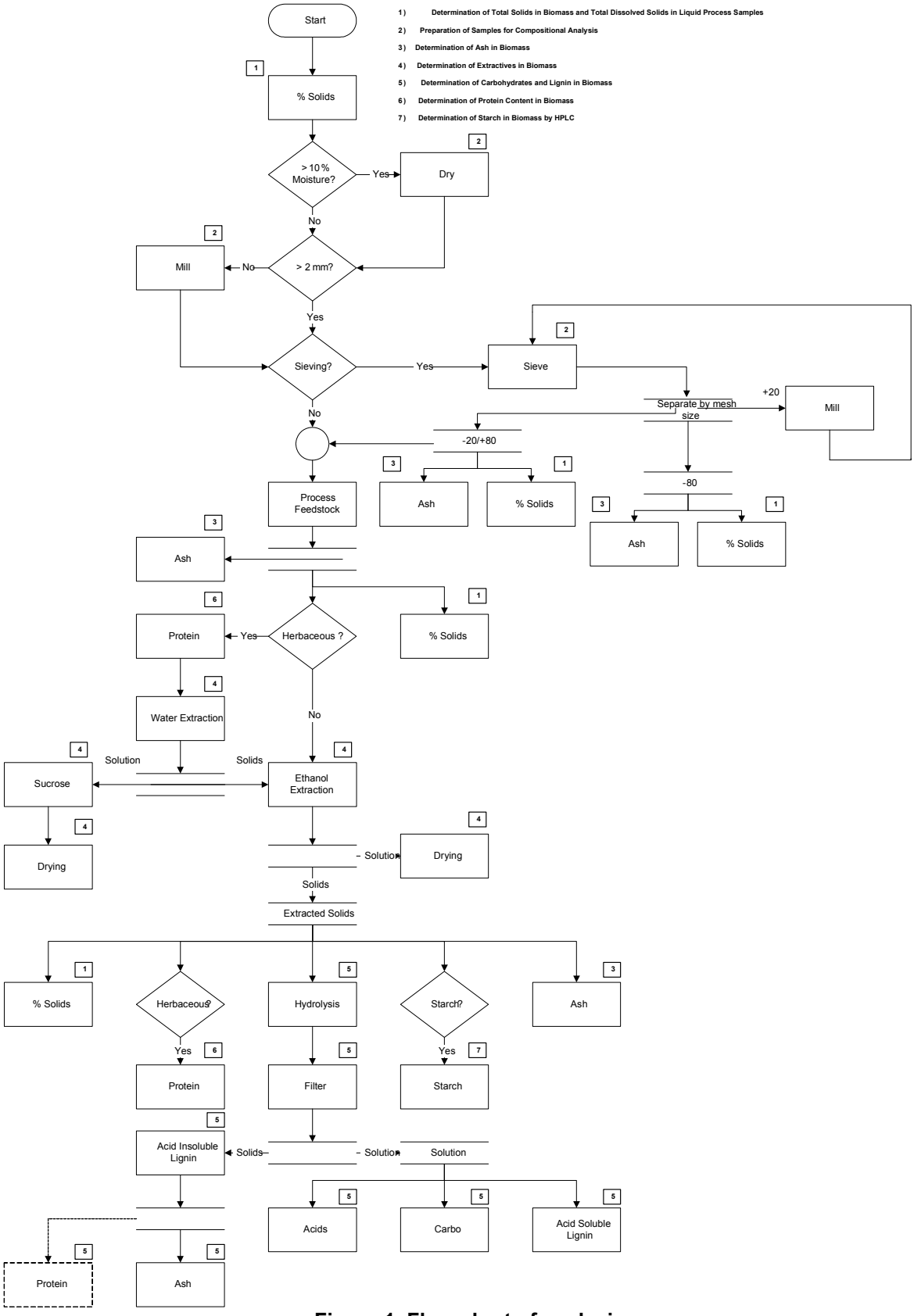

Figure 1. Flow chart of analysis