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Laboratory Analytical Procedure (LAP)

Issue Date: 06/01/2007

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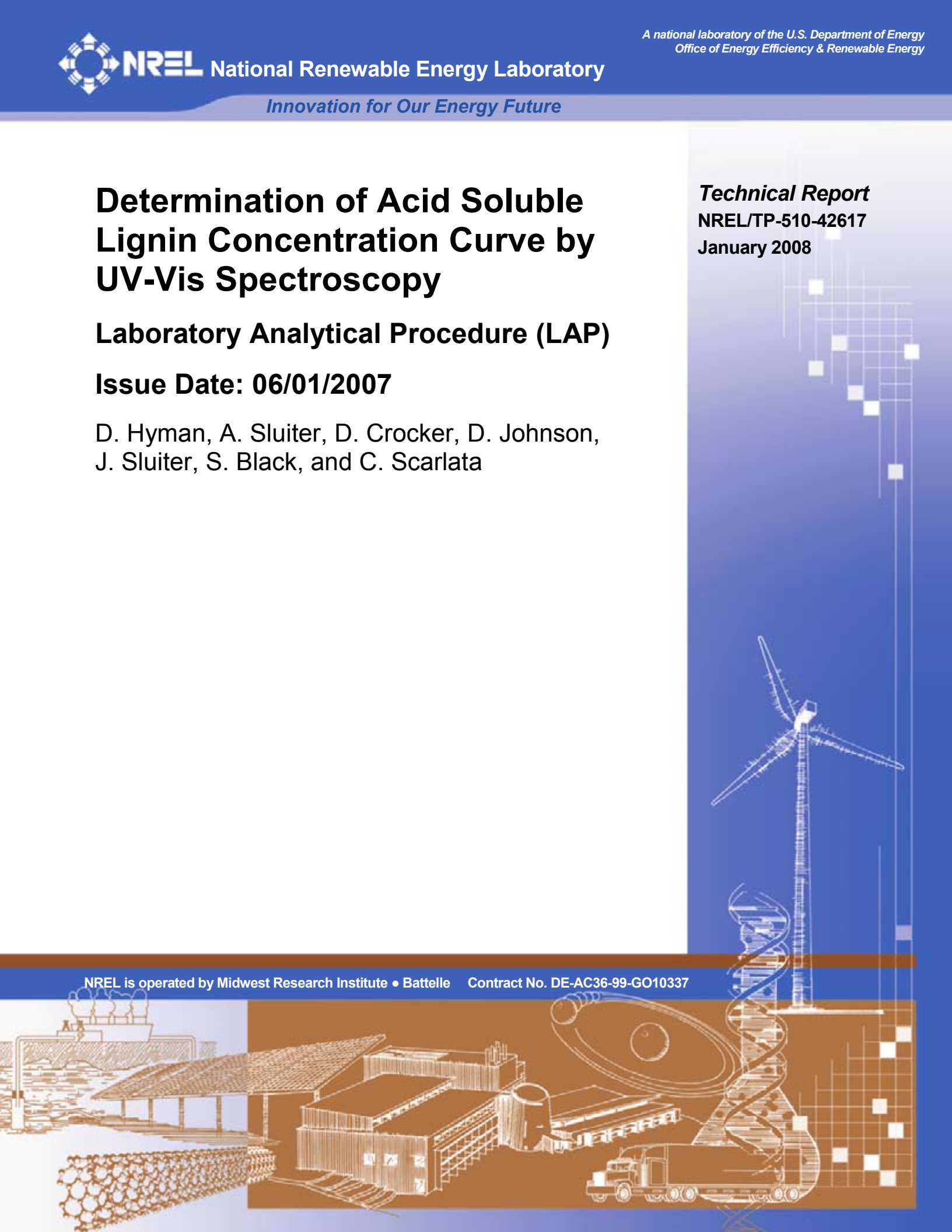
Technical Report
NREL/TP-510-42617
January 2008



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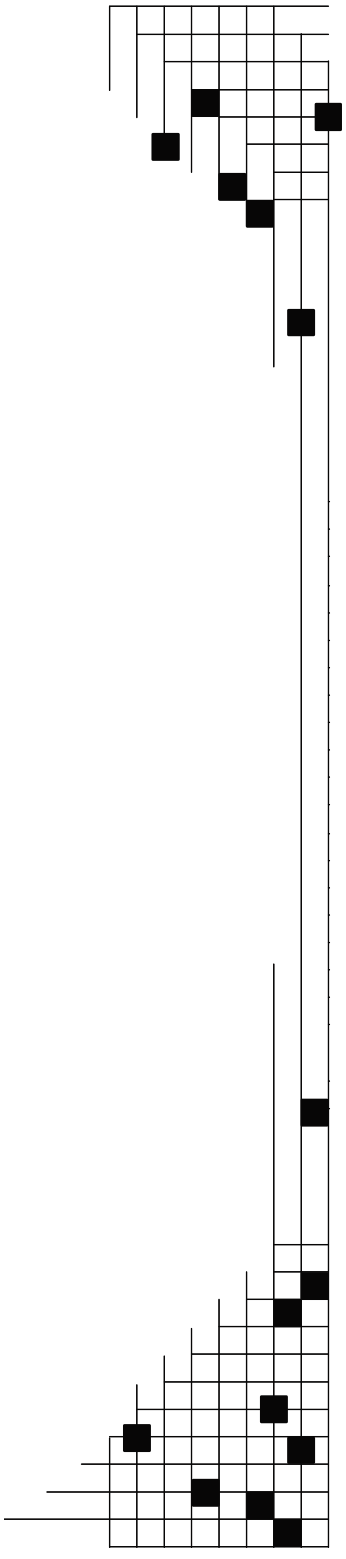

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Procedure Title: Determination of Acid Soluble Lignin Concentration Curve by UV-Vis Spectroscopy

Laboratory Analytical Procedure

1. Introduction

1.1 Lignin is a complex phenolic polymer found in biomass feedstocks and biomass derived products. Lignin contributes a significant mass to the composition of biomass. A fraction of the lignin content can be solubilized during certain analytical or industrial procedures. Accurately measuring the lignin value is critical to mass closure. This procedure describes the isolation of lignin from an acidic solution, and measurement of a maximum peak and absorptivity constant specific to that sample or biomass type. These values can usually be applied to other samples of the same matrix. Ultraviolet-Visible spectroscopy (UV-Vis) is employed in this technique, and measurements are made at a peak maxima that minimizes the confounding influence of other analytes. Reference appendix 15.1 for further information on confounding analytes.

1.2 The solubilized lignin fraction is extracted from a calibration liquor or other matrix using methyl isobutyl ketone (MIBK). The solvent is removed, and the fraction is dried. The extracted material is dissolved in water to produce a set of lignin concentration standards. A wavelength maximum is identified that minimizes interferences. A response curve is generated using a UV-Vis spectrometer. To analyze an unknown liquor sample, the sample is diluted until it is within the range of the response curve. The lignin concentration of the unknown liquor sample is determined using Beer's Law and a dilution factor relative to the intact sample.

2. Scope

This procedure is appropriate for acidic hydrolyzate liquors from biomass.
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.
All analyses should be performed in accordance with an appropriate laboratory specific Quality Assurance Plan (QAP).

3. Terminology

3.1 *HMF*—hydroxymethyl furfural
3.2 *MIBK*—Methyl Isobutyl Ketone
3.3 *Acid Soluble Lignin*- The fraction of lignin solubilized during certain analytical procedures, such as LAP "Determination of Structural Carbohydrates and Lignin in Biomass", or during certain industrial or pretreatment procedures
3.4 *Absorptivity constant*- Reference section 11.1, also known as molar extinction coefficient

4. Significance and Use

This procedure is used, possibly in conjunction with other procedures, to determine the amount of lignin in a solid biomass sample or hydrolyzate liquor.

5. Interferences

5.1 Methyl Isobutyl Ketone (MIBK) forms emulsions easily with water. Care must be taken not to shake such a mixture too vigorously.
5.2 A neutralization step must be done after the MIBK extraction. This prevents the concentration of trace acid during solvent removal, which may cause sample degradation. This neutralization must be performed after the MIBK extraction to ensure that all of the lignin is extracted. (Lignin has a higher affinity for MIBK than it does for acidic solutions.)
5.3 Sodium Hydrogen Carbonate (Sodium Bicarbonate) loses buffering capacity when exposed to oxygen for extended periods. Buffers for neutralization should be created each time an extraction is performed to keep the reagent fresh.
5.4 Biomass samples tend to degrade when exposed to temperatures above 40°C. When removing solvent avoid raising the materials above this temperature.
5.5 This method has been optimized to reduce interference from HMF and furfural that are likely to be present in the matrix by specifying an extended wavelength, such as 320 nm.
5.6 MIBK can be difficult to evaporate. Adding small amounts of water to a solution of MIBK creates an azeotrope with a lower boiling point than MIBK, thus aiding evaporation.

6. Apparatus

6.1 Analytical balance, accurate to 0.1 mg
6.2 Diode array UV-Vis spectrometer
6.3 Vacuum solvent evaporation system, such as a Bucchi Rotavapor

7. Reagents and materials

7.1 Reagents
- 7.1.1 Methyl Isobutyl Ketone (MIBK) a.k.a. 4-Methyl-2-pentanone, HPLC grade
- 7.1.2 Nanopure water
- 7.1.3 Sodium Hydrogen Carbonate ($NaHCO_3$) a.k.a. Sodium Bicarbonate
- 7.1.4 Acetone, spectroscopic grade
- 7.1.5 Ethyl alcohol, HPLC grade
- 7.1.6 Sodium Bicarbonate Buffer
  - 7.1.6.1 Add approximately 2 grams of Sodium Bicarbonate to 100 mL water.
  - 7.1.6.2 Stir until dissolved.
  - 7.1.6.3 Prepare fresh buffer each day.

7.2 Materials
- 7.2.1 Graduated cylinder, 100 mL
- 7.2.2 Beakers, 250 mL, or sized to accommodate sample volume
- 7.2.3 pH paper, range 2-10
- 7.2.4 Round bottom flasks, 50 mL, or sized to accomodate sample volume
- 7.2.5 Separation funnel, 250 mL, or sized to accommodate sample volume
- 7.2.6 Erlenmeyer flasks, 250 mL
- 7.2.7 Volumetric flask, sized to accommodate sample amount, refer to step 10.3.5
- 7.2.8 Adjustable pipettors, covering appropriate ranges needed for dilutions

8. ES&H Considerations and Hazards

8.1 Acidic compounds can be corrosive and should be handled with care.

8.2 MIBK has moderate health risks and is extremely flammable. Refer to MSDS for more information.
8.3 Operate all equipment with the appropriate manual and NREL Safe Operating Procedures.
8.4 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.

10. Procedure

10.1 Separate the lignin fraction
- 10.1.1 Add the hydrolyzate sample (or liquor) to an appropriately sized separation funnel.
- 10.1.2 Add 25 mL MIBK to separation funnel for every 100 mL of sample.
- 10.1.3 Agitate gently, at about one inversion per second. Avoid forming an emulsion by mixing too vigorously. Vent the separation funnel during each inversion.
- 10.1.4 Allow mixture to separate for at least 30 minutes.
- 10.1.5 If an emulsion is still present then add several drops of high purity acetone and repeat step 10.1.4, or let the mixture sit for a longer period of time.
- 10.1.6 Separate the fractions into labeled beakers.
- 10.1.7 Repeat steps 10.1.1-10.1.6 at least two more times with aqueous phase. The MIBK phases from each separation can be combined.

10.2 Neutralize the MIBK fraction
- 10.2.1 Place the MIBK fraction in clean separation funnel. This fraction now contains the soluble lignin.
- 10.2.2 Wash the MIBK fraction by adding a 10 mL aliquot of the Sodium Bicarbonate solution to the separation funnel and inverting several times. Vent the separation funnel during each inversion.
- 10.2.3 Separate the fractions into labeled beakers. Using pH paper, check the pH of the aqueous phase as it drips from the separation funnel. Collect the wash water, but do NOT mix it with the original hydrolyzate liquor.
- 10.2.4 Repeat steps 10.2.1-10.2.3 until the pH of the aqueous phase is greater than 6.5.

10.3 Dry the MIBK fraction and prepare the stock solution
- 10.3.1 Weigh an appropriately sized oven dry round bottom flask and place the MIBK extract in the flask.
- 10.3.2 Remove the solvent from the MIBK fraction using a Rotavapor solvent removal system or similar technique. The water bath heating the round bottom flask should be set at 30-35 °C. MIBK can be difficult to evaporate. When only several milliliters of solvent are left, add small amounts of water (a few milliliters at a time) to the MIBK to create an azeotrope with a lower boiling point than MIBK, until all solvent is gone.
- 10.3.3 Place the round bottom flask and residue from step 10.3.2 in a vacuum oven at 40°C for a minimum of 12 hours. Repeat until there is no further weight loss.
- 10.3.4 Weigh the flask to determine the total mass of lignin isolated in the MIBK fraction. Quantitatively transfer the lignin from the dried round bottom into a pre-weighed volumetric flask. The size of the flask depends on the amount of lignin present. (A resulting solution of at least 1 mg/mL is desirable.) Use just enough ethanol to dissolve the lignin in the round bottom flask. It may be necessary to mix this for a

prolonged amount of time to ensure complete dissolution. Quantitatively transfer the ethanol/lignin solution to the volumetric flask. Evaporate off the ethanol under a stream of nitrogen until dry. Dry in a vacuum oven at 40°C for at least 12 hours.

10.3.5 Weigh the volumetric flask with lignin. This step is necessary because not all of the lignin transfers from the round bottom flask to the volumetric flask.

10.3.6 Re-dissolve the lignin in ethanol in the volumetric flask, bringing the solution to volume. This is the stock solution that will be used to make dilutions for the standard curve. A stock solution of at least 1 mg/mL is desirable to perform serial dilutions.

10.4 Build A Standard Curve

10.4.1 Create a set of aqueous standards using the stock solution made in step 10.3.7. Formulate standards at concentrations that give a linear response versus absorbance, usually within an absorbance range of 0.1 to 2.5 absorbance units, maximum. See Table 1 for example dilutions and concentrations.

10.4.2 Measure the absorbance of each standard at an appropriate wavelength on a UV-Visible spectrophotometer. See appendix A for an example scenario of appropriate wavelength determination. Record the absorbance to three decimal places. Reproducibility should be $\pm$ 0.05 absorbance units. Analyze each sample in duplicate, at minimum.

10.4.3 Use good analytical techniques to create a curve of concentration versus UV-VIS absorption at an appropriate wavelength, e.g. 240, 280, or 320 nm. Determine the extinction coefficient (ε) for lignin by taking the slope of the curve.

Table 1

Table 1 example is based on the extraction of ASL from a solids analysis hydrolysis liquor
% ASL is calculated for the stock solution using the equation:
(Conc. of stock soln. from step 10.3.7)*((Volume hydrolyzate)/(Dry weight solid sample))*100)
Assuming that the stock solution is 100.0 mg lignin in 100 mL water,
%ASL = 1.000 mg/mL *(87.00 mL / 290.0 mg) *100 = 30.0%

Standard #	% ASL stock	Dilution factor		mL of stock in 25 mL water	% ASL with dilution	Final Conc. of standard (mg/ml)
1	30.0	10		2.5	3.00	0.100
2	30.0	20		1.25	1.50	0.050
3	30.0	50		0.5	0.60	0.020
4	30.0	65		0.385	0.46	0.015
5	30.0	80		0.313	0.38	0.013
6	30.0	100		0.25	0.30	0.010
7	30.0	120		0.208	0.25	0.008
8	30.0	160		0.156	0.19	0.006
9	30.0	250		0.1	0.12	0.004
10	30.0	500		0.05	0.06	0.002
11	30.0	1000		0.025	0.03	0.001
12	30.0	2000		0.0125	0.02	0.0005

10.5 Measure An Unknown

10.5.1 Dilute the sample so that the UV-VIS absorbance at the wavelength selected in step 10.4.2 is within the calibration range. Record the absorbance to three decimal places. Reproducibility should be $\pm$ 0.05 absorbance units. Analyze each sample in duplicate, at minimum.

10.5.2 Calculate the amount of acid soluble lignin present using calculation 11.1 for process liquors, and 11.2 for liquors from solids analyses.

11. Calculations

11.1 Beer's law can be expressed as

$$A_\lambda = \varepsilon bc,$$

Where:

A_λ = average UV-Vis absorbance at a specific wavelength
ε = absorptivity constant at a specific wavelength in L/g-cm
b is the path length through the sample in cm
c is the concentration of a single analyte in mg/ml

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

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

where:
UVabs = average UV-Vis absorbance for the sample at specified wavelength
$Volume_{hydrolysis\ liquor}$ = volume of filtrate, 87 mL

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

ε = absorptivity constant of biomass at specific wavelength in L/g-cm

11.3 To 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

11.4 To 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 wavelength used as well as the absorptivity constant.
12.2 Report the acid soluble lignin on a mg/mL basis, or a dry weight percent if using LAP "Determination of Structural Carbohydrates and Lignin in Biomass".
12.3 For replicate analyses, report the average and relative percent difference.

13. Precision and Bias

13.1 None to report

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 independent duplicates from two different dilutions, at minimum.
14.3 Blank: nanopure water
14.4 Relative percent difference criteria: Determined by data quality objectives and laboratory specific Quality Assurance Plan.
14.5 Calibration verification standard: none
14.6 Sample size: Minimum volume of sample sufficient to yield enough lignin to create a calibration curve that falls above the lower detection limit of the UV-Vis instrument
14.7 Sample storage: Acidic hydrolysis liquors may be stored in a refrigerator for up to two weeks. Lignin can precipitate and adhere to containers during storage.
14.8 Standard storage: Acidic hydrolysis liquors may be stored in a refrigerator for up to two weeks. Neutral lignin solutions may be stored in a refrigerator for up to four days. Lignin can precipitate and adhere to containers during storage.
14.9 Standard preparation: Refer to section 10.
14.10 Definition of a batch: Any number of samples that are analyzed and recorded together.
14.11 Control charts: All values may be control charted if desired.

15. Appendices

15.1

Influence of Carbohydrate Degradation Products on the Measurement of Acid Soluble Lignin

David K. Johnson

Furfural and hydroxymethyl furfural (HMF) are the main acid catalyzed degradation products of pentoses and hexoses. Hydrolyzates produced in the analysis of biomass samples always contain small amounts of these furans. Both furans are very strongly absorbing in the UV spectral range from 190 to 300 nm. The analysis of biomass also results in the dissolution of a small fraction of the lignin in biomass.

The current method for determining the quantity of lignin dissolved in an acid hydrolyzate is to measure the UV absorbance of the hydrolyzate at 205nm and apply an absorbtivity of 110 L/g-cm to calculate the concentration of acid soluble lignin in the sample. The wavelength, 205nm, is chosen because it corresponds to a minimum in the UV spectrum of furfural and HMF, and is in a region where lignin components absorb strongly. However, the absorption of the furans is still significant at 205 nm (see Table 1). Recently new methods have been explored using different wavelengths and absorbtivities determined for specific biomass samples. The absorbtivity of acid soluble lignins from pine, sugarcane bagasse, corn stover, poplar, and other feedstocks have been determined at their λ_{max} (197 – 202 nm) and at 240 nm. The latter wavelength corresponds to another minimum in the spectra of the furans, but again there is still a significant absorbance by the furans at these wavelengths (Table 1). Table 1 shows the λ_{max} and the relative absorbtivities of furfural, HMF and some lignin-derived compounds found in acid hydrolyzates by reversed-phase HPLC. The relative absorbtivity in the table is the absorbtivity of a substance at a given wavelength compared to its absorbtivity at its λ_{max}. A comparison of the spectra of the furfurals and lignin-derived compounds found in biomass hydrolyzates is shown in Figure 1.

Table 2. λ_{max} and Relative Absorbtivities of Furfurals and Lignin-derived Components Found in Biomass Hydrolyzates

		Relative Absorbtivies at Wavelengths Shown			
	λ_{max} (nm)	200 nm	205 nm	240 nm	320 nm
Furfural	278	11%	6%	16%	1%
HMF	284	26%	13%	12%	3%
p-Coumaric acid	308	35%	39%	19%	83%
Ferulic Acid	322	58%	53%	60%	99%
Vanillin	230	90%	98%	45%	55%
Hydrolyzate	190	77%	61%	28%	11.2%
Hydrolyzate-Furfural	190	64%	52%	18%	10.5%

Because of the high level of absorbance of the lignin-derived compounds at high wavelengths (300 nm and above) at the end of the absorption of the furans, a high wavelength absorbance should also be considered for the measurement of acid-soluble lignin.

Figure 2 shows a reversed-phase HPLC chromatogram for a biomass hydrolyzate from a dilute acid pretreatment of corn stover. The hydrolyzate contains a large peak for furfural from degradation of pentoses in the corn stover and a HMF peak from degradation of the hexoses. The lignin-derived components appear later in the chromatogram and contain verifiable peaks for vanillin, p-coumaric acid and ferulic acid. p-Coumaric acid is normally the largest peak in biomass hydrolyzates. Figure 2 also contains the chromatogram for the hydrolyzate after it was azeotropically rotovapped with n-octane to remove furfural (water was also removed). After rotovapping the sample was brought back to its original weight by adding water to return the retained components back to their original concentrations. The chromatogram indicates that while 96% of the furfural was removed all the other components remained in the solution including the HMF.

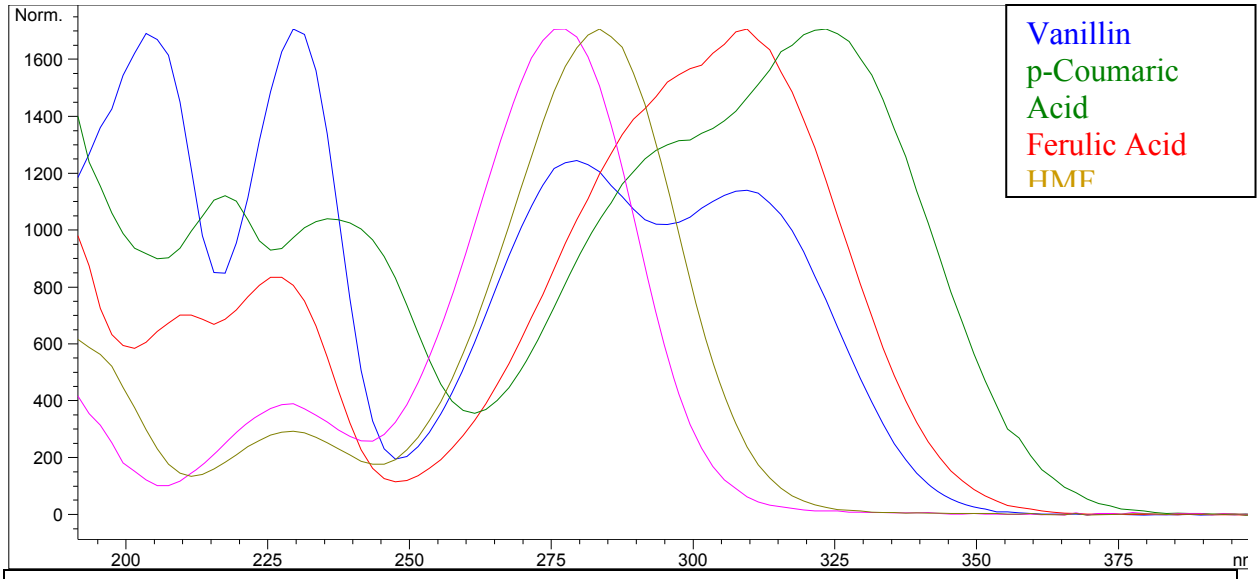

Figure 1. UV Spectra of Biomass Hydrolyzate Components

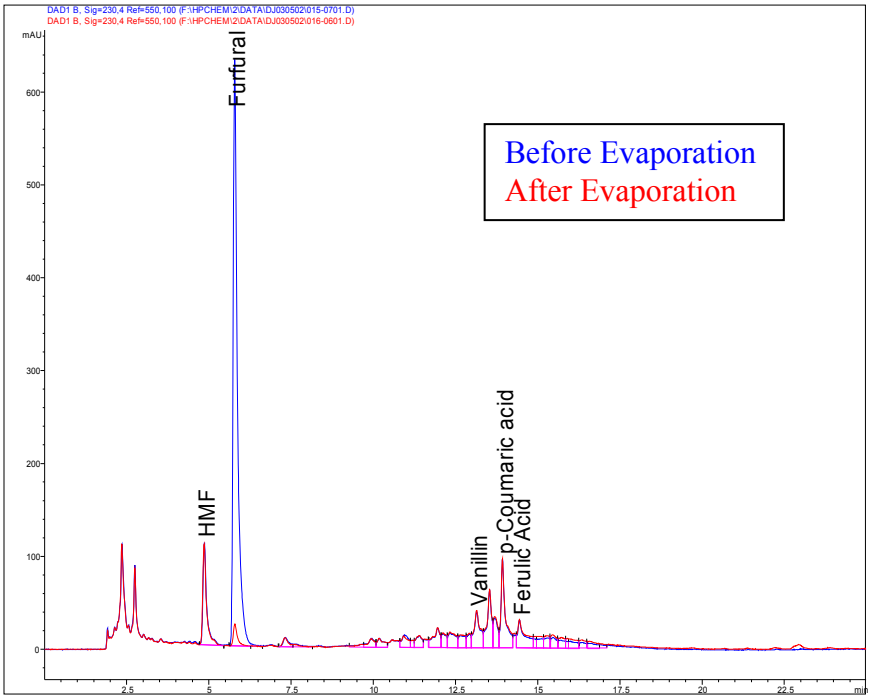

Figure 2. Reversed-phase HPLC of Biomass Hydrolyzate Before and After Removal of Furfural by Evaporation

Figure 3 contains the UV spectra of the hydrolyzate before and after evaporation. As can be seen from Figure 3 removal of furfural had a significant affect on the spectrum throughout the wavelength range from 190 nm to about 320 nm. From 320 nm, however, the spectrum was virtually unchanged. Table 1 lists the relative absorbtivities of the corn stover hydrolyzates. The relative absorbtivities for the hydrolyzate-furfural sample were calculated using the absorbtivity at the λ_{max} of the hydrolyzate before furfural was removed to show the influence that furfural had on what would have been the measurements taken for assessing the acid-soluble lignin content. It is clear that furfural had a significant affect on the absorbance at 200 and 205 nm, and the largest affect at 240 nm, however, the affect at 320 nm was minimal.

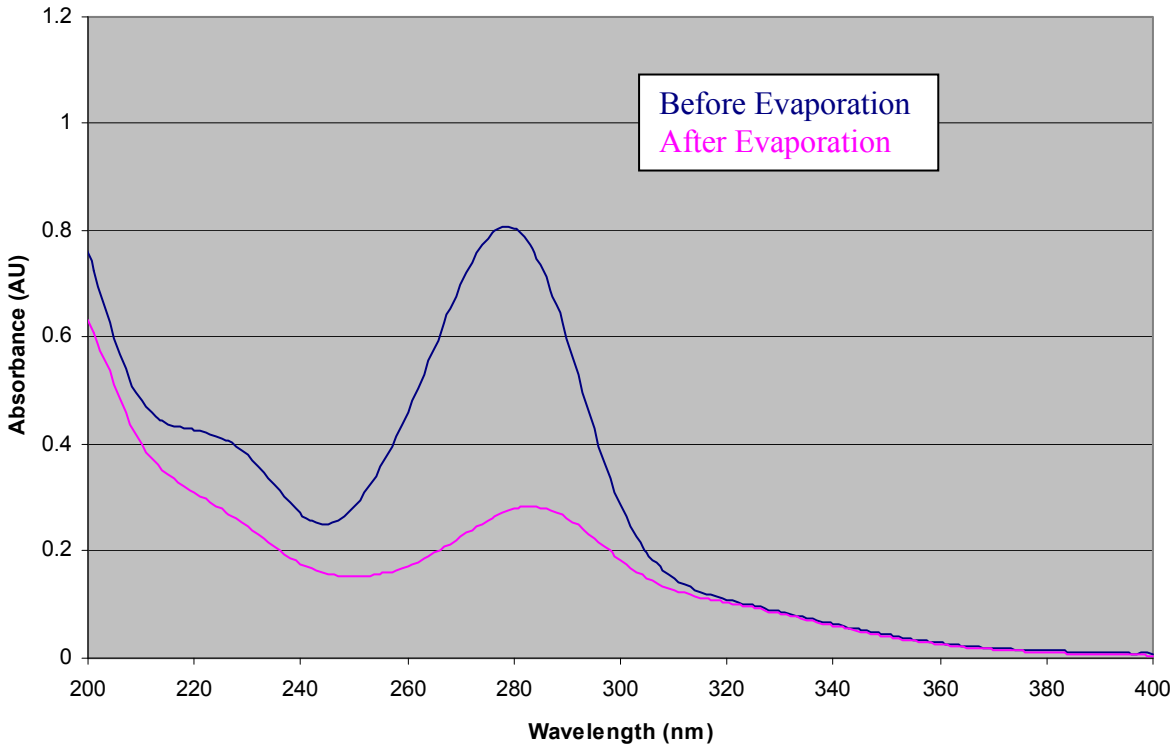

Figure 3. UV Spectra of a Corn Stover Prehydrolyzate Before and After Evaporation to Remove Furfural

I would like to recommend that a higher wavelength, such as 320 nm, be assessed for the measurement of acid-soluble lignin, especially for feedstocks such as corn stover that have the potential for generating larger amounts of furfural and HMF than is seen in some other feedstocks such as wood.

Internal NREL publication, 2005.

16. References

16.1. Johnson, David K., Influence of Carbohydrate Degradation Products on the Measurement of Acid-Soluble Lignin, internal publication, 2005.