Electronic Supporting Information (ESI)

A facile and fast method for quantitating lignin in lignocellulosic biomass using acidic lithium bromide trihydrate (ALBTH)

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Table S1. Formation of pseudo lignin fraction from extractives of herbaceous biomass by ALBTH method

Extractives[a]	IL[b] (%)	SL[c] (%)	Arabinose (%)	Galactose (%)	Glucose (%)	Xylose (%)	Mannose (%)
Corn stover	11.7	6.2	0.6	n.d.	0.9	0.3	n.d.
Switchgrass	14.9	4.5	0.6	0.5	5.9	0.4	n.d.

Note: a. Extractives were isolated from hot-water extraction from corn stover and switchgrass;
b and c denoted the pseudo insoluble lignin and soluble lignin from the extractives, respectively;
n.d. denoted not detected.
% was calculated based on the dry weight of the isolated extractives.

Table S2. Formation of humins from cellulose in ALBTH assay at 110 °C

	40 mM HCl		150 mM HCl	
	30 min	120 min	30 min	60 min
Humins yield (wt%)	0.35	1.35	4.14	6.78

Table S3. Residual carbohydrates in insoluble lignin of various biomass by ALBTH method

Method	ALBTH-lignin residue				
	Arabinan (%)	Galactan (%)	Glucan (%)	Xylan (%)	Mannan (%)
Poplar-30 min	n.d.	n.d.	0.05	0.11	n.d.
Poplar-60 min	n.d.	n.d.	0.04	0.08	n.d.
Poplar-120 min	n.d.	n.d.	0.04	0.06	n.d.
Switchgrass-30 min	0.34	n.d.	0.04	0.06	n.d.
Switchgrass-60 min	0.29	n.d.	0.04	0.04	n.d.
Switchgrass-120 min	0.27	n.d.	0.04	0.03	n.d.
Corn stover-30 min	0.18	n.d.	0.26	0.06	n.d.
Corn stover-60 min	0.14	n.d.	0.08	0.04	n.d.
Corn stover-120 min	0.13	n.d.	0.04	0.02	n.d.
Aspen-30min	n.d.	0.01	0.04	0.07	n.d.
Douglas fir-30 min	0.01	0.02	0.04	0.02	0.02
Eucalyptus-30 min	n.d.	0.02	0.05	0.08	n.d.

Note: * percentage of residual carbohydrates, based on initial biomass feedstocks

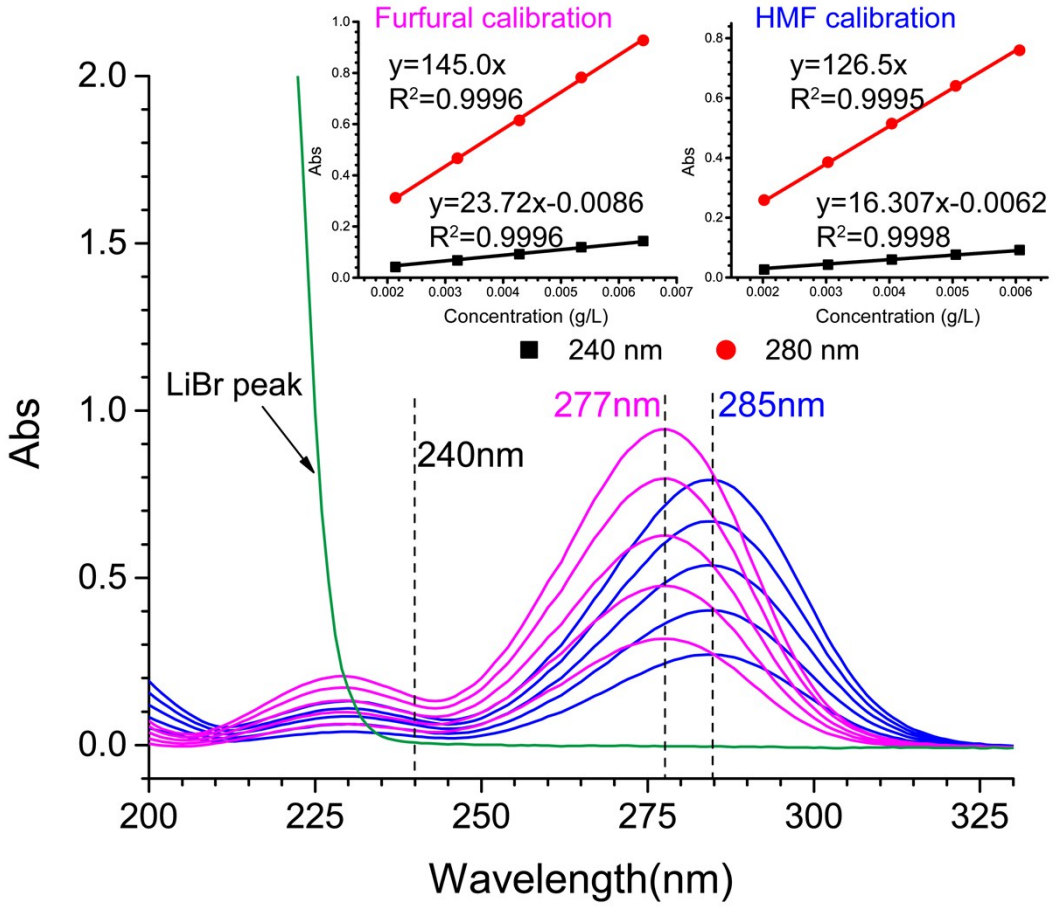

Figure S1. UV spectra of HMF (blue, 2.02 to 6.06 mg/L) and furfural (magenta, 2.14 to 6.42 mg/L) and acidic lithium bromide solution (green, 220 mg/L LiBr).

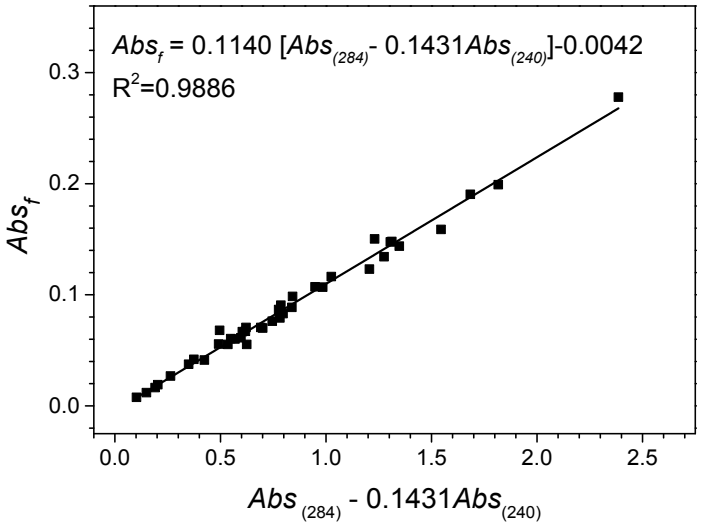

Figure S2. Linear correlation of absorption deduction ($^{Abs_{(284)} - 0.1431Abs_{(240)}}$) with furan absorption correction (^{Abs_f}) based on the multiple linear regression results.

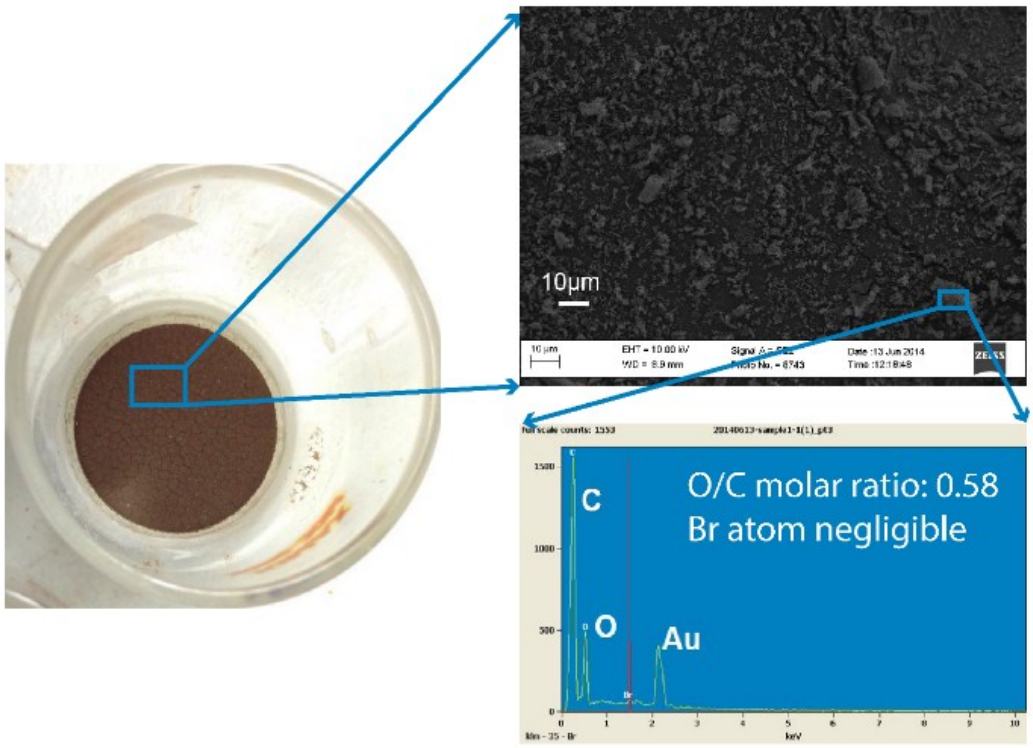

Figure S3. SEM-EDS analysis of ALBTH lignin residue from extractives-free aspen.

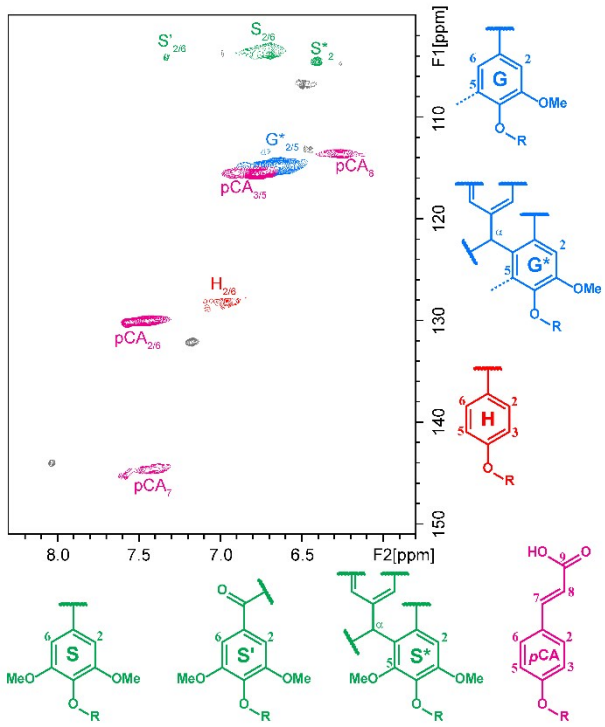

Figure S4. The aromatic region of 2D ^{1}H-^{13}C correlation (HSQC) spectrum from corn stover lignin residue by ALBTH method dissolved in DMSO-d6. Correlation signals were categorized and color coded by the type of aromatic units (S: syringyl, G: guaiacyl, H: *p*-hydroxyphenyl, *p*CA: *p*-coumarate).

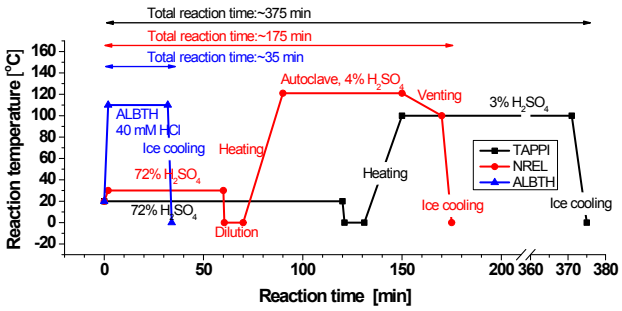

Figure S5. Comparison of ALBTH, NREL and TAPPI reaction duration for lignin quantitation.

The determination of Abs_f in equation (3) by direct HPLC quantitative analysis and pseudo double-wavelength spectrophotometric method

To diminish the interference of HMF and furfural from sugar dehydration on soluble lignin quantitation, we started with directly subtracting the absorbance of furfural and HMF. Specifically, first, standard calibration curves of absorbance vs. concentration were created for furfural and HMF using a UV spectrophotometer. As shown in Fig. S1, the absorbance of both furfural and HMF followed the Beer-Lambert law at 240 nm (Eqn. (S1) for furfural and Eqn. (S2) for HMF, respectively), where Abs_{240} is the absorbance of the furans at 240 nm, and c (g/L) is the concentration of the furan standards. Second, the concentrations of furfural and HMF in the hydrolysate from the ALBTH method after n times dilution (n, dilution factor for SL quantitation of the hydrolysate) were determined by HPLC analysis as $c_{furfural}$ and c_{HMF} (g/L), respectively. Then, the contributions of furfural and HMF to the absorbance of the hydrolysate at 240 nm (Abs_f) could be calculated from Eqn. (S3).

$$Abs_{240} = 23.72c - 0.0086 \quad \text{(S1)}$$

$$Abs_{240} = 16.31c - 0.0062 \quad \text{(S2)}$$

$$Abs_f = 23.72c_{furfural} + 16.31c_{HMF} - 0.0148 \quad \text{(S3)}$$

Another alternative is to use the pseudo double-wavelength spectrophotometric method, in which the portion of absorption from furfural and HMF at 240 nm (Abs_f) was estimated from the absorbance of the hydrolysate at two wavelengths, 284 nm (the isosbestic wavelength of furfural and HMF in water) and 240 nm (the characteristic wavelength of SL), according to an empirical equation regressed from the experimental data, and therefore no HPLC analysis was required to quantitate furfural and HMF. In detail, the Abs_f calculated from HPLC analysis and corresponding absorbance at 240 and 284 nm ($Abs_{(240)}$ and $Abs_{(284)}$) of the hydrolysates from 40 sets of experimental data from different biomass under different conditions were regressed using a multiple linear regression model in Origin Lab 9.1. As shown in Fig. S2, a linear correlation between Abs_f and $[Abs_{(284)} - 0.1431Abs_{(240)}]$ was found with $R^2 = 0.9886$. Therefore, instead of using HPLC to quantitate furan compounds and then determining the absorbance correction (Abs_f) at 240 nm from furfural and HMF with Eqn. (3) above, Abs_f can be directly estimated using empirical Eqn. (S4) after reading the absorbance ($Abs_{(240)}$ and $Abs_{(284)}$) of the hydrolysate at 240 nm and 284 nm, respectively. In summary, Because of the elimination of the HPLC analysis of

furfural and HMF, the pseudo double-wavelength method is recommended for the ALBTH method to quantitate SL of woody biomass. As mentioned above, no correction is needed for herbaceous biomass because of the negligible interference of HMF and furfural absorptions at 320 nm.

$$Abs_f = 0.1140\left[Abs_{(284)} - 0.1431 Abs_{(240)} \right] - 0.0042 \quad \text{(S4)}$$

Recommended ALBTH Protocol for Lignin Quantitation in Lignocellulosic Biomass

1. Scope

This procedure is for the fast quantitation of lignin content of lignocellulosic biomass.

2. Apparatus

2.1 Wiley mill

2.2 Soxhlet extraction setup

2.3 Analytical balance with 0.1 mg readability

2.4 Drying oven for moisture determination at temperature of 105 ± 2 °C

2.5 Muffle furnace for ash determination at temperature of 575 ± 25 °C

2.6 Oil bath heated by hot plate with magnetic stirrer and temperature controller

2.7 Desiccator with fresh desiccant

2.8 Magnetic stirrer

2.9 Vacuum filtration set with a vacuum pump/water aspirator and filtration flask

2.10 Glass filtrating crucibles (30 mL, low form with medium pore size)

2.11 UV-Visible spectrophotometer with quartz cuvettes (1cm path-length)

3. Procedure

3.1 Sample preparation

3.1.1 Biomass grounding: Grind chipped or chopped air-dry biomass using a Wiley mill to pass a 2-mm screen. Sieve the ground biomass and collect the fraction between 20 and 100 mesh for analysis.

3.1.2 Removal and determination of extractives: Extract woody biomass with 95% ethanol for 16 h or herbaceous biomass with hot-water for 8 h followed by 95% ethanol for 16 h using Soxhlet extractor according to NREL/TP-510-42619 “Determination of Extractives in Biomass”.

3.1.3 Determine moisture content of the extractives-free biomass

3.2 ALBTH hydrolysis of the biomass sample

3.2.1 Dry filtering crucibles in a drying oven at 105 ± 2 °C for at least 6 hours (or until constant weight). Transfer the crucibles from the oven to a desiccator and cool down for 30 ± 5 min. Weigh the crucibles and record the values to the nearest 0.1 mg.

3.2.2 Weigh 300.0 ± 5.0 mg of sample into a 40 mL glass vial with screw top.

3.2.3 Add 4.50 ± 0.10 mL of 60 % LiBr solution with 40 mM HCl to the vial. Mix the solution and the biomass with magnetic stirring at room temperature for two minutes. Ensure the solid sample evenly soaked in the solution. Seal the vial with an open top cap with Teflon lined septa.

Note: To prepare 100 mL 60% LiBr solution (1.71 g/mL, 20 °C) with 40 mM HCl, weigh 102.6 g of anhydrous LiBr, dissolve in 68.1 g of water. Cool down the solution to room temperature and add 0.394 g of 37% HCl. Vortex the solution and store it in a bottle with a cap.

3.2.4 Put the vial in an oil bath at 110 ± 2 °C, turn on the magnetic stirring at 400 ± 50 rpm, and hydrolyze the sample for 30 ± 3 min.

3.2.5 Upon the end of the hydrolysis, take the vial from the oil bath to an ice water bath. Transfer the hydrolyzed sample to a 50-mL volumetric flask, rinse the vial with water and transfer all solids completely into the flask, and fill the flask with deionized water to the mark.

3.3 Gravimetrical quantitation of insoluble lignin

3.3.1 Filter the hydrolysate in the volumetric flask under vacuum through a pre-weighed filtering crucible. Collect 20 mL the filtrate (before washing and dilution) and store in a sample storage bottle for the determination of soluble lignin and furan compounds.

3.3.2 Transfer all the remaining solids out of the volumetric flask into the filtering crucible by deionized water. Wash thoroughly the insoluble residue on the crucible with deionized water to remove residual LiBr and HCl.

3.3.3 Place the crucible together with the insoluble residue in a drying oven at 105 ± 2 °C for a minimum of 12 h or until a constant weight is achieved.

3.3.4 Move the crucible into a desiccator to cool down for 30 ± 5 min and record the weight of the crucible with the insoluble residue to the nearest 0.1 mg.

3.3.5 Place the crucible together with insoluble residue in Muffle furnace at 575 ± 25 °C for a

minimum of 12 h or until a constant weight is achieved.

3.3.6 Cool down the crucible with remaining ash in a desiccator for 30 ± 5 min and record the weight of the crucible with ash to the nearest 0.1 mg.

3.4 Spectrophotometric evaluation of soluble lignin

3.4.1 Acquire a background spectrum of deionized water on a UV-Visible spectrophotometer.

3.4.2 Scan the hydrolysate aliquot collected in step 3.3.1 from 200 nm to 350 nm wavelength on the UV-Visible spectrophotometer. Dilute the samples with deionized water, if necessary, so that the absorbance at the interested wavelength (240 nm for woody biomass and 320 nm for herbage) falls into the range of 0.2-0.8. Read and record the absorbance at 240 nm and 284 nm for woody biomass and the absorbance at 320 nm for herbaceous biomass.

4. Calculation for lignin quantitation

4.1 Calculate the insoluble lignin (*IL*, wt%) of the biomass sample

$$IL\ (\%) = \frac{(m_{c+r} - m_c) - (m_{c+a} - m_c)}{m_{ods}} \times \frac{(100 - \sigma)}{100} \times 100$$

$$m_{ods} = m_s \times \frac{x}{100}$$

Where:

m_{ods}, (g), oven dry weight of extractives-free biomass sample;

x, (wt%), weight percentage dryness of the extractives-free biomass sample, x = 100 – moisture (%);

m_s, (g), air dry weight of extractives-free biomass sample;

m_c, (g), oven dry weight of the net filtering crucible after reaching constant weight;

m_{c+r}, (g), oven dry weight of the filtering crucible plus ALBTH insoluble residue;

m_{c+a}, (g), dry weight of the filtering crucible plus ALBTH ash after furnace ignition;

σ, (wt%), weight percentage of the extractives in the oven dry biomass sample.

4.2 Calculate the soluble lignin (*SL*, wt%) of the biomass sample

$$SL(\%) = \frac{Abs' \times V_f \times \delta}{\varepsilon \times m_{ods} \times 1} \times \frac{(100 - \sigma)}{100} \times 100$$

$$Abs' = Abs_T - Abs_f$$

$$Abs_f = 0.1140\left[Abs_{(284)} - 0.1431 Abs_{(240)}\right] - 0.0042$$

Where:

V_f (L), total volume of the filtrate (50 mL);

δ, dilution factor;

ε, absorptivity of biomass lignin at the appropriate wavelength, 25 $g^{-1} \cdot L \cdot cm^{-1}$ for woody biomass at 240 nm and 30 $g^{-1} \cdot L \cdot cm^{-1}$ for herbaceous biomass at 320 nm, respectively, adopted from the NREL method;

Abs', corrected UV absorbance of the diluted filtrate after subtracting UV absorbance of hydroxymethylfurfural (HMF) and furfural at 240 nm for woody biomass (No correction is necessary for herbaceous biomass hydrolysate because of the negligible absorbance of HMF and furfural absorptions at 320 nm. For herbaceous biomass, $Abs' = Abs_{T=}$ absorbance at 320 nm);

Abs_T, total UV absorbance of the diluted hydrolysate at appropriate wavelength (240 nm for woody biomass and 320 nm for herbaceous biomass);

Abs_f, UV absorbance of the furan fractions (HMF and furfural) in the diluted hydrolysate at 240 nm wavelength for woody biomass, Abs_f approximately equals zero for herbaceous biomass at 320 nm;

$Abs_{(284)}$, total UV absorbance of the diluted hydrolysate at 284 nm wavelength;

$Abs_{(240)}$, total UV absorbance of the diluted hydrolysate at 240 nm wavelength;

σ, (wt%), weight percentage of the extractives in the oven dry biomass sample.