Electronic Supplementary Information for

A Facile Spectroscopic Method for Measuring Lignin Content in Lignocellulosic Biomass

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1. Supplementary Methods

Materials

All chemicals and reagents are commercial products and used as supplied. l-cysteine, microcrystalline cellulose and xylan were purchased from Sigma-Aldrich. Sulfuric acid (72%, 12 M, SA) was prepared by careful dilution of concentrated (95-98%) sulfuric acid purchased from Sigma-Aldrich or purchased from Fisher Scientific.

The lignocellulose biomass materials were ground with a Wiley mill and sieved to collect the fraction between 40-80 mesh for analysis. To remove non-cell-wall extractives, the ground biomass was Soxhlet-extracted with benzene/alcohol (v/v 2:1) for 4 h, followed by 95% ethanol extraction for 12 h; toluene can replace benzene for safety reasons, and simply using 80% ethanol extraction alone is quite effective especially for woody biomass.[1] For monocot or grass biomass or non-woody dicots extraction with water under sonication is recommended first to remove proteins and other water-soluble components. Each solvent-extracted biomass sample was kept in a sealed glass bottle after drying in an oven at 50 °C for 48 h, and placed in a vacuum desiccator over P_2O_5.

Monolignols (coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol) were prepared according to the published methods.[2,3] Milled wood lignins (MWLs) was prepared from the corresponding biomass according to the procedure described by Bjorkman.[4]

Synthesis of model compounds

Lignin model compounds were synthesized according to published methods.

The guaiacyl-guaiacyl (GG) β-ether, herein abbreviated GG-β-O-4 ether but sometimes also abbreviated as GG-β-GE, was synthesized as previously.[5]

Syringaresinol was synthesized from sinapyl alcohol by free radical coupling reaction according to reference.[6,7]

Arylglycerols (G-glycerol, S-glycerol and H-glycerol) were synthesized according to reference.[8]

Alkyl hydroxycinnamates (ethyl ferulate, ethyl sinapate and methyl *p*-coumarate) were synthesized from the corresponding hydroxycinnamic acids and alcohols in dry HCl generated from acetyl chloride in absolute alcohol, as previously.[2]

Preparation of DHPs

Each DHP was synthesized from total 1 mmol of monolignol [i.e., G-DHP made from 1 mmol of coniferyl alcohol (CA), DHP-A made from 0.9 mmol sinapyl alcohol (SA) and 0.1 mmol CA). The given amount of monolignol (Table S1) was dissolved in a flask containing 100 mL of 10% dioxane aqueous solution. In another flask a 100 mL solution of hydrogen peroxide (1.0 mmol) in 10% dioxane aqueous solution was prepared. Both solutions were added simultaneously by a peristaltic pump in rate of 0.1 mL/min into a 250 mL flask containing 1 mg horseradish peroxidase in 20 mL of 10% dioxane aqueous solution over about 16 h period, followed by

addition of 0.5 mg peroxidase every 5 h. After addition, the reaction mixture was further stirred for another 5 h. Finally, the DHP suspension was concentrated at 40 °C under vacuum on a rotatory evaporator to a volume of 100 mL and then freeze-dried.

Table S1. Compositional molar ratios and monolignol weight used for the preparation of DHPs

	Molar ratios	Starting monolignols (mg)
G-DHP	100% G, 1 mmol	180 mg
DHP-A	70%S (0.7 mmol): 30% G (0.3 mmol)	147 mg SA; 54 mg CA
DHP-B	50%S (0.5 mmol): 50% G (0.5 mmol)	105 mg SA; 90 mg CA
DHP-C	30%S (0.3 mmol): 70% G (0.7 mmol)	63 mg SA; 126 mg CA
DHP-D	20%S (0.2 mmol) : 75%G (0.75 mmol):5% H (0.05 mmol)	42 mg SA; 135 mg CA; 7.5 mg HA

Note: In this case only, SA is used for sinapyl alcohol, not sulfuric acid!

Table S2. Absorptivity of lignin related model compounds dissolved in cysteine-SA

Unit type	Model compounds	Absorptivity at 283 nm ε , (g^{-1} L cm^{-1})
G	GG-β-O-4 ether	14.31 ± 0.17
	G-glycerol	14.80 ± 0.18
	Ethyl ferulate	13.75 ± 0.04
S	Syringaresinol	11.02 ±0.14
	S-glycerol	3.95 ± 0.07
	Ethyl sinapate	3.45 ± 0.17
H	H-glycerol	6.94 ± 0.11
	Methyl *p*-coumarate	6.54 ± 0.16

Table S3. Lignin contents measured by the Klason method and the CASA lignin method for various species of lignocellulosic biomass

Biomass	Acid insoluble Lignin, % (IL)	Acid Soluble Lignin, % (AL)	Total Lignin, % (IL+AL)	CASA Lignin, %
Loblolly pine[a]	27.5±0.3	---	---	29.2±0.4
White spruce[a]	27.0±0.5	---	---	28.4±0.5
Black pine[a]	32.9±0.4	---	---	33.8±0.1
Mason pine[b]	31.0±0.4	2.14±0.00	33.1±0.4	33.6±0.1
Spruce[b]	28.7±0.2	2.02±0.01	30.7±0.2	30.6±0.1
Simao pine[b]	28.0±0.1	1.48±0.01	29.5±0.1	27.9±0.2
Chinese cedar[b]	33.7±0.2	1.91±0.01	35.6±0.2	36.7±0.5
Beech[a]	21.7±0.7	---	---	21.9±0.3
Balsa[a]	23.3±1.6	---	---	23.1±0.1
Willow[a]	21.2±1.6	---	---	21.3±0.4
Poplar[a]	21.1±0.9	---	---	24.9±0.7
Maple[a]	21.9±0.8	---	---	23.1±0.9
Walnut[a]	23.2±0.3	---	---	25.9±0.9
Palm[a]	20.8±1.3	---	---	22.7±0.5
Wheat straw[b]	22.9±0.2	2.62±0.01	25.5±0.2	25.5±0.7
Corn stalk[b]	19.4±0.2	2.51±0.01	21.9±0.2	21.7±1.2
Bamboo[b]	27.3±0.1	1.86±0.02	29.2±0.1	29.4±0.2
Rice straw[b]	17.7±0.3	2.75±0.02	20.5±0.3	19.4±0.1
Sugarcane bagasse[b]	18.3±0.1	2.56±0.01	20.9±0.1	21.4±0.3

Notes: [a] Lignin analysis was performed at the GLBRC, University of Wisconsin-Madison, acid-soluble lignin content was not measured; [b] Lignin analysis was performed at the South China University of Technology.

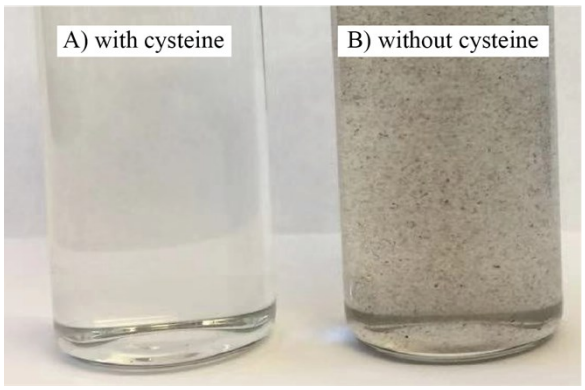
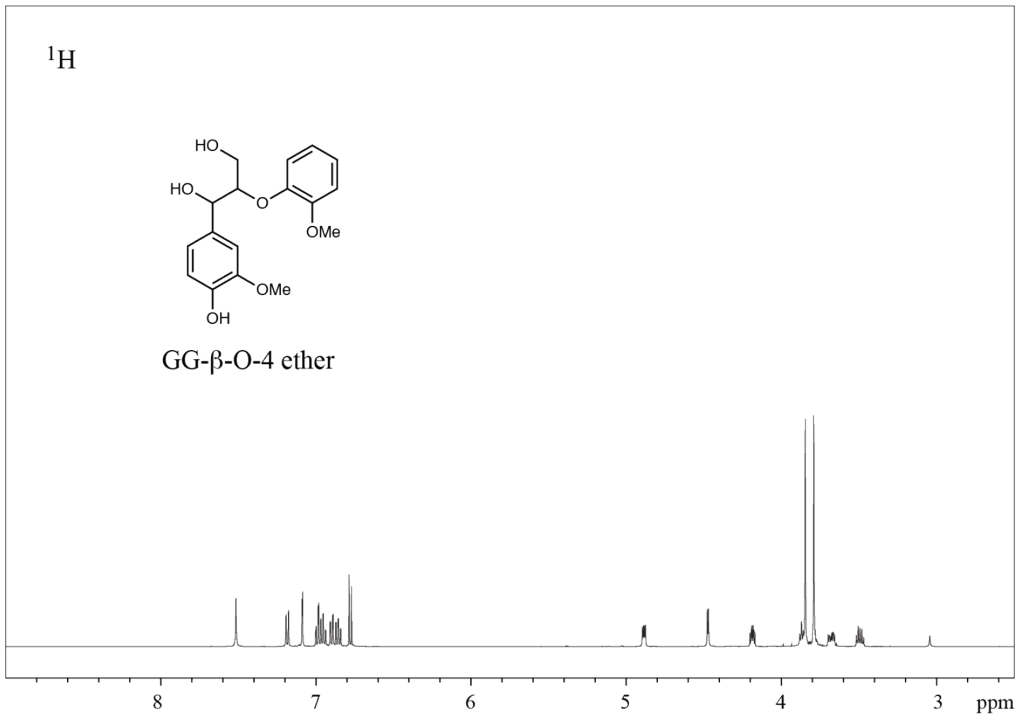
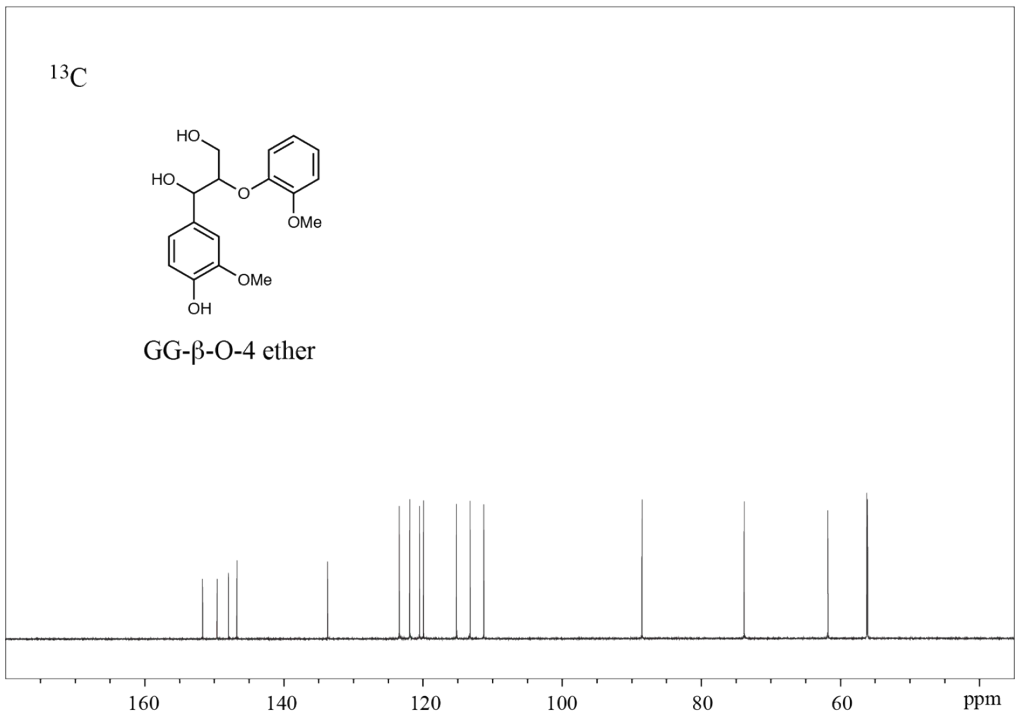
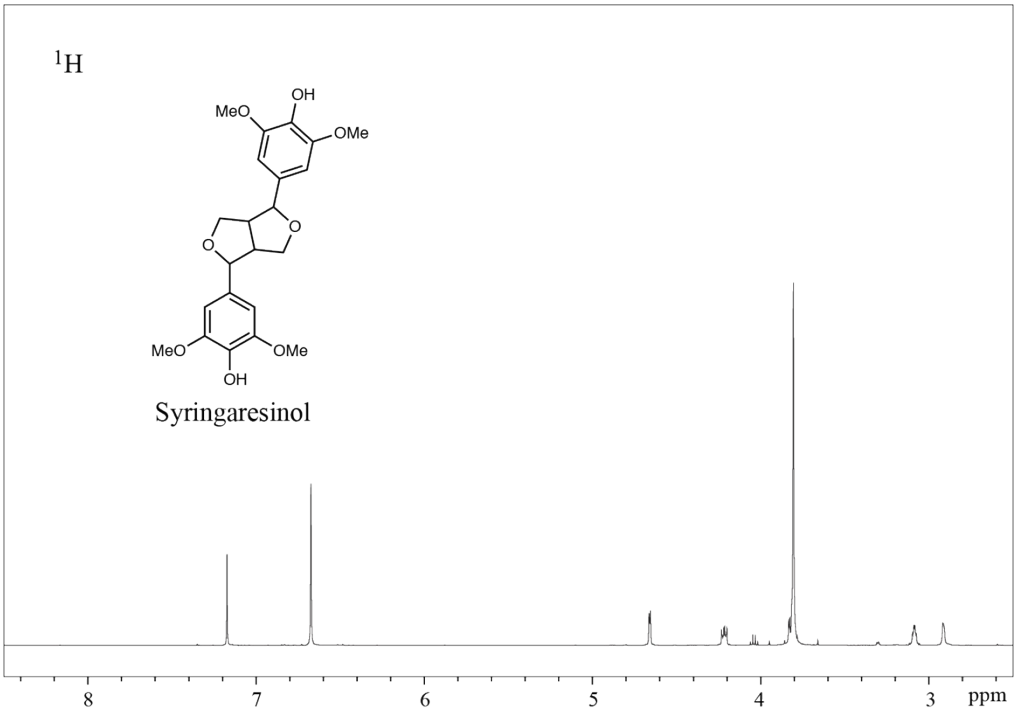


Figure S1. Comparison of photographs of two solutions: A) 10-fold diluted aqueous solution of the loblolly pine wood dissolved in cysteine-SA at 60 °C for 30 min; B) 10-fold diluted suspension of loblolly pine wood treated with 72% sulfuric acid (SA) at 60 °C for 30 min.

Figure S2. ^{1}H NMR and ^{13}C NMR spectra of lignin model compounds used in this study.



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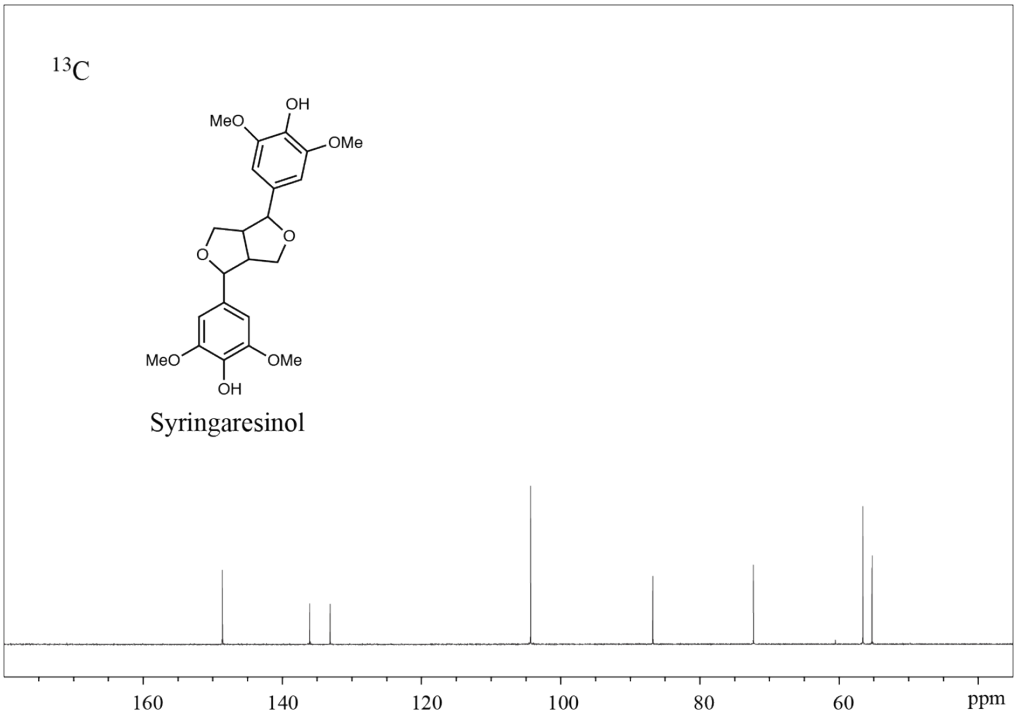
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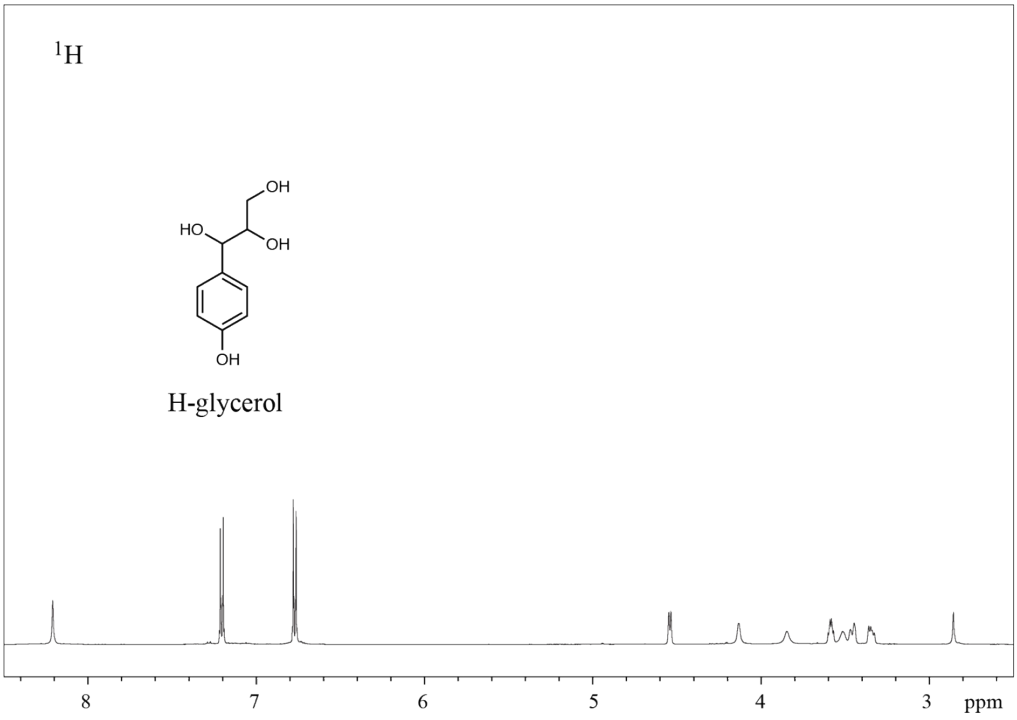
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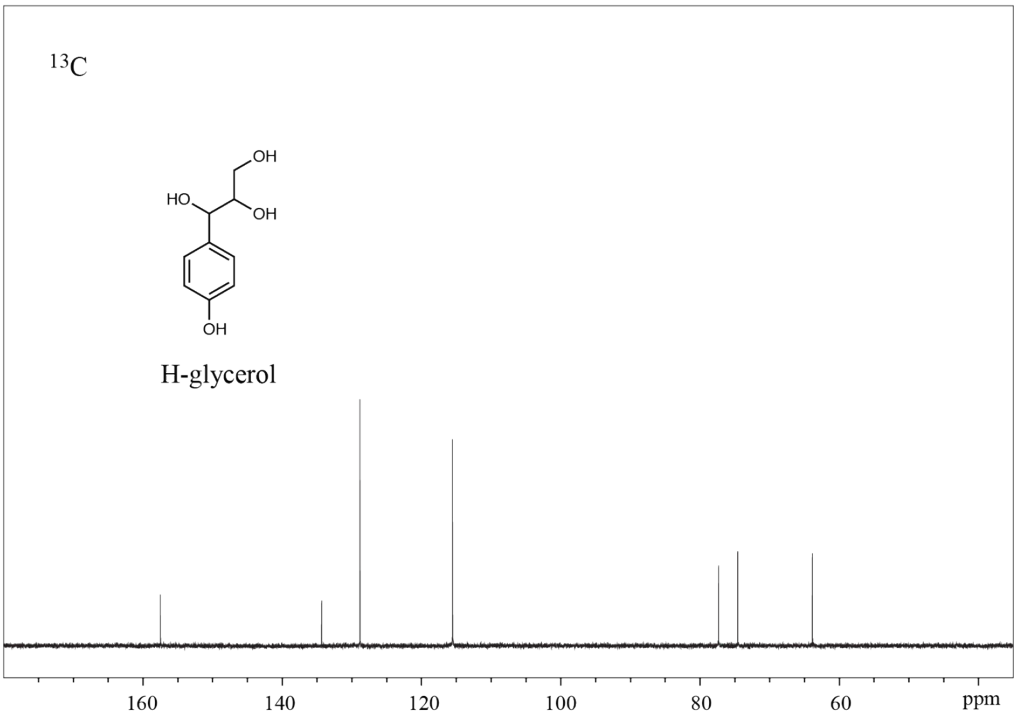
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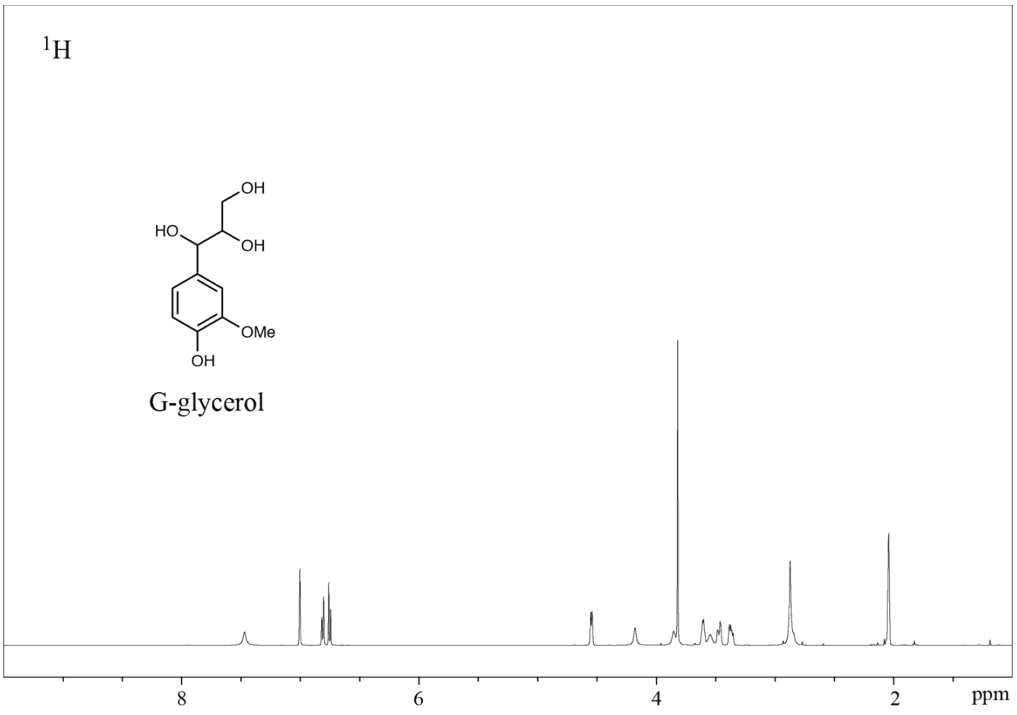
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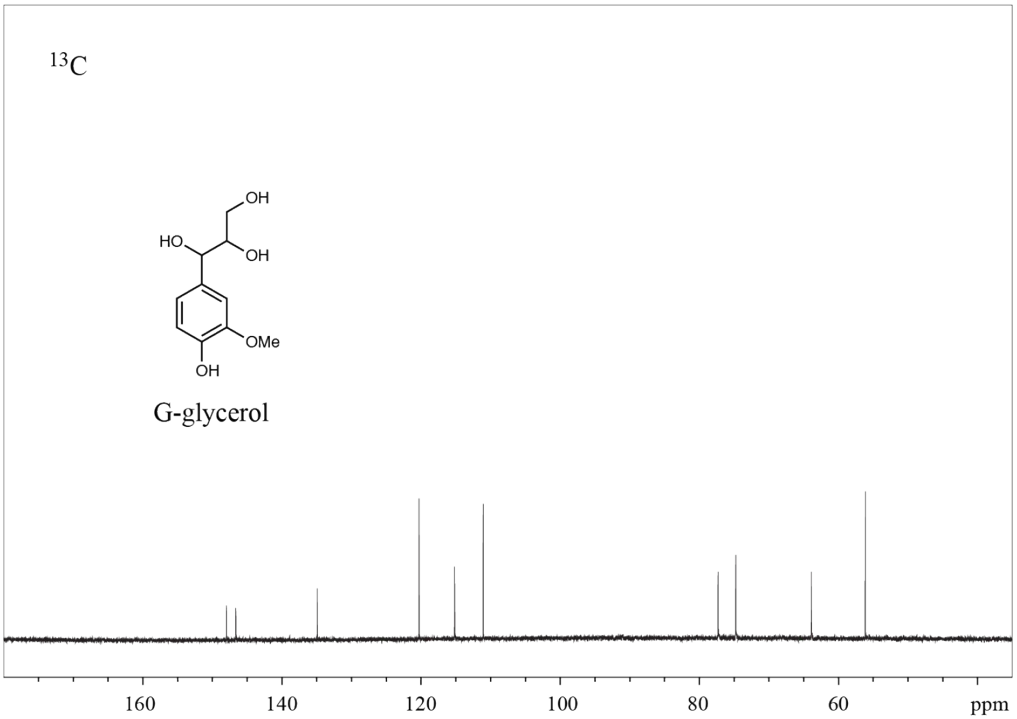
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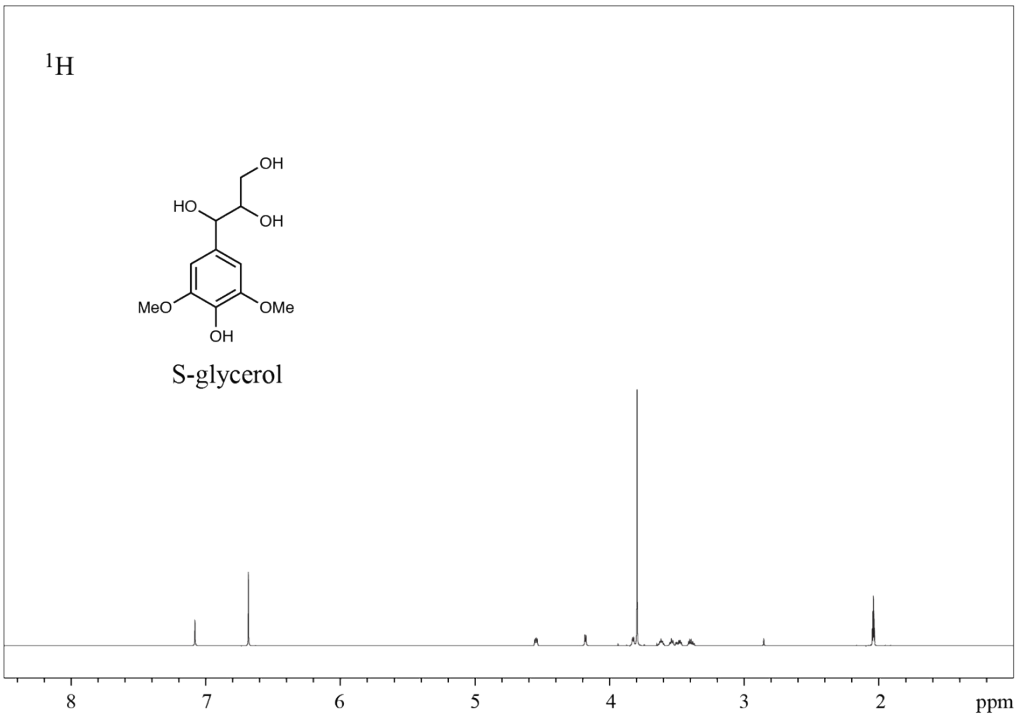
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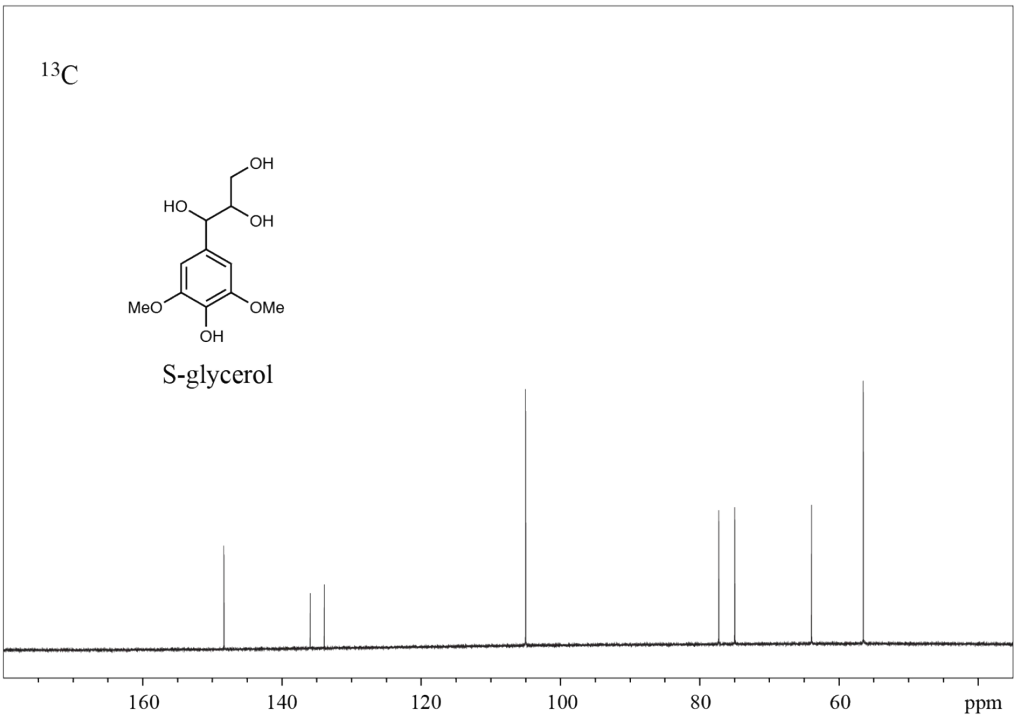
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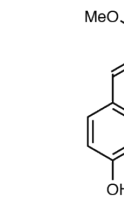
^{1}H



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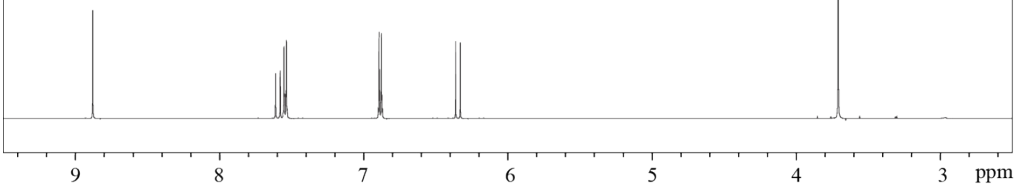

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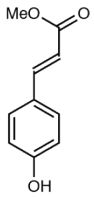

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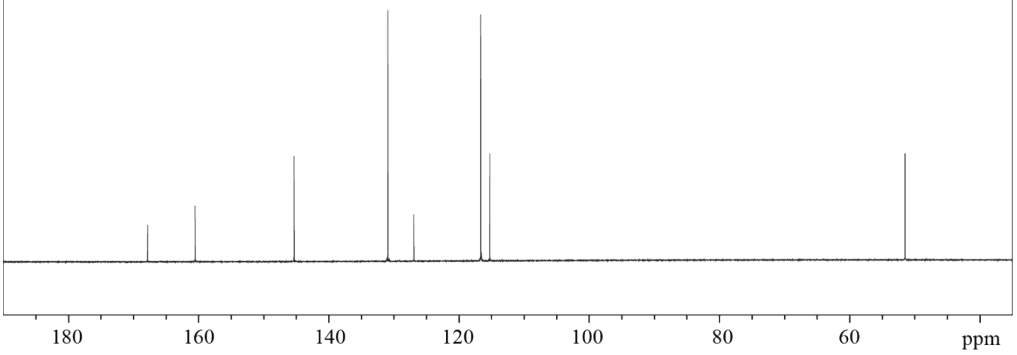

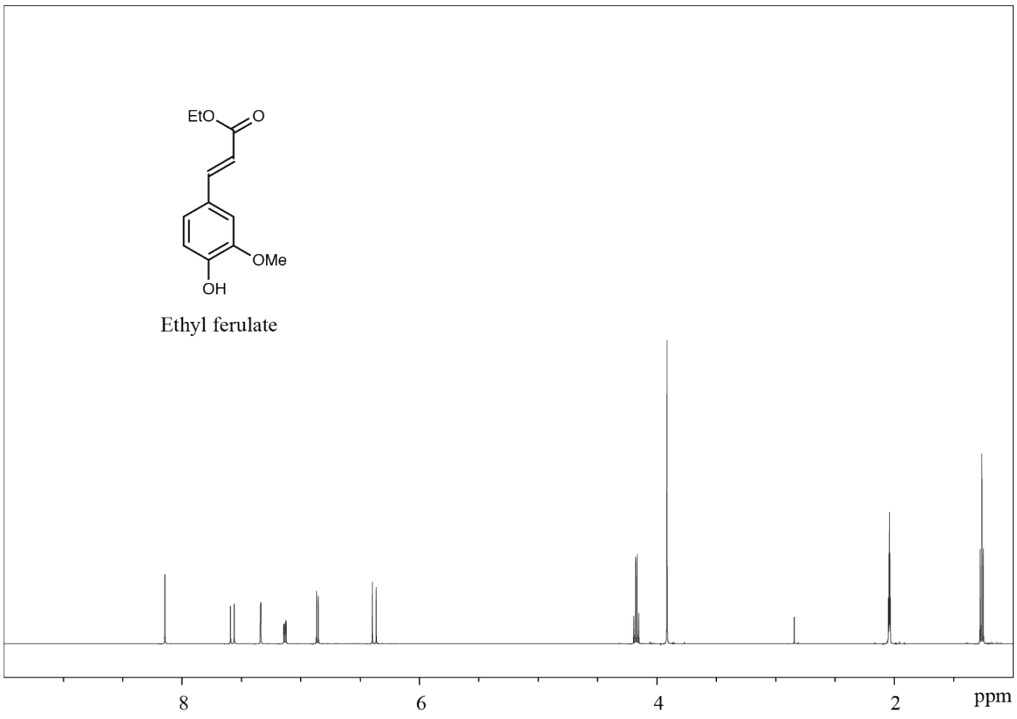
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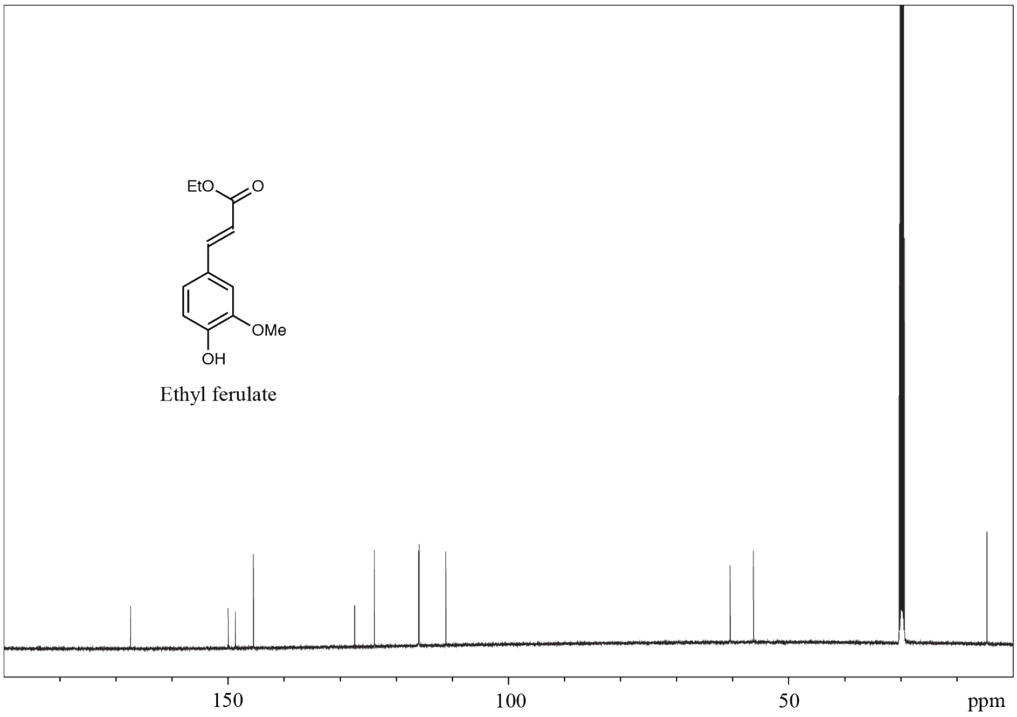
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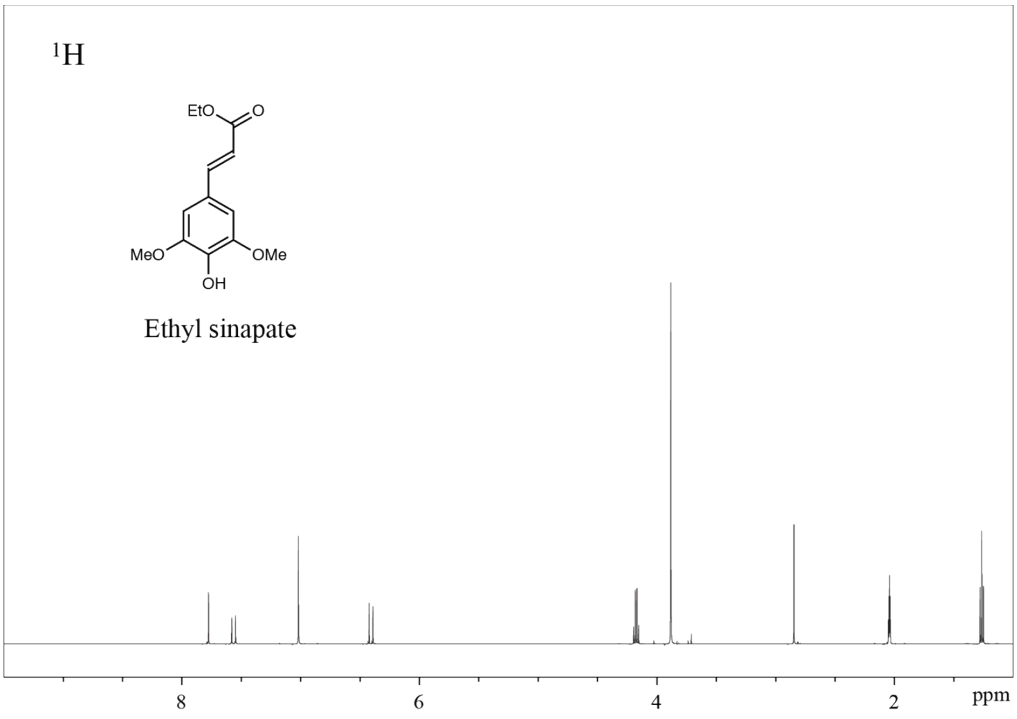
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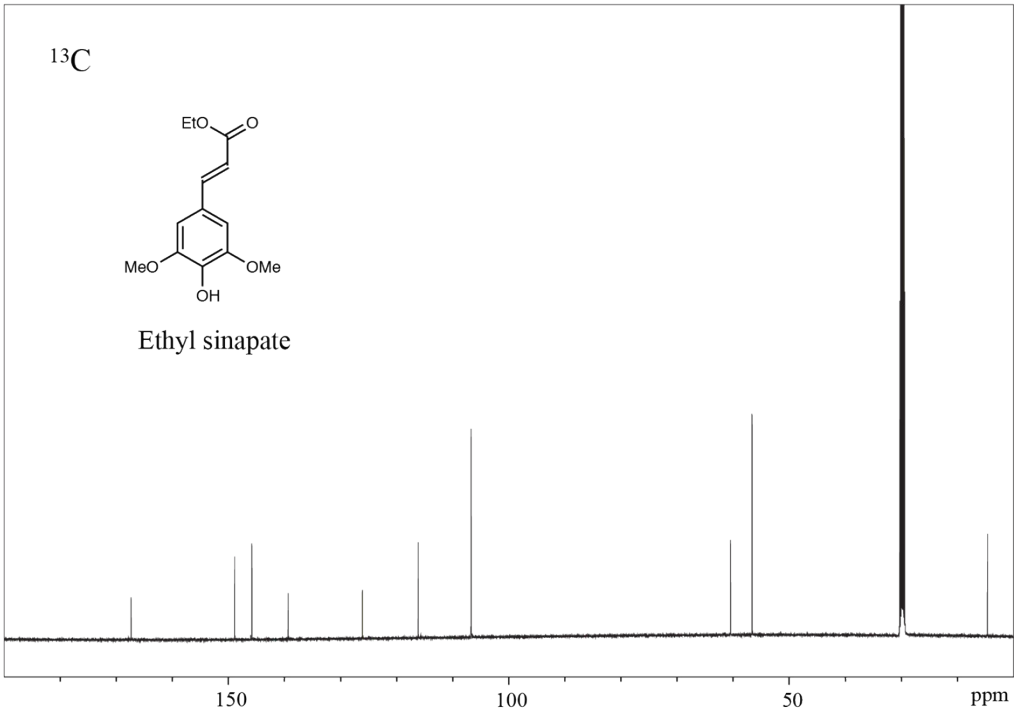
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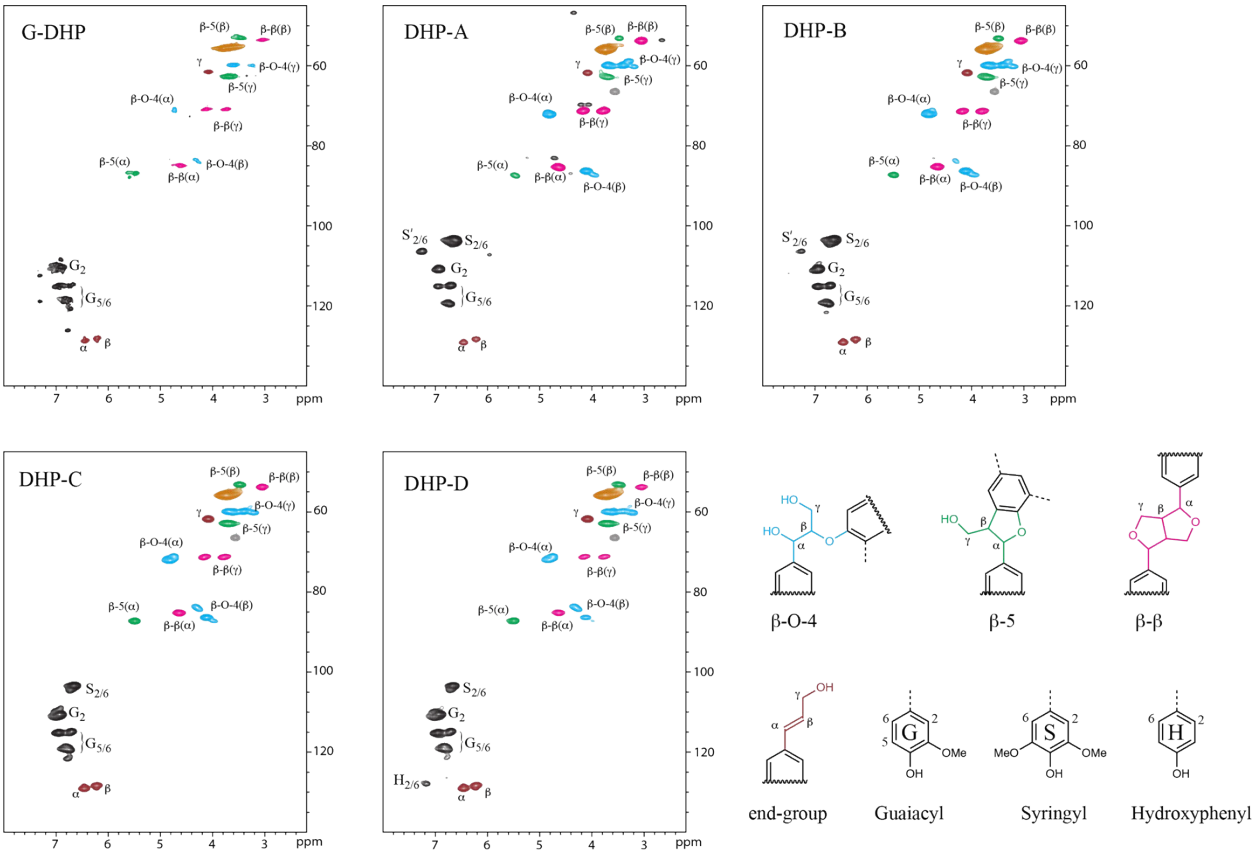
Figure S3. HSQC NMR spectra of DHPs used in this study



Recommended protocol for lignin quantitation in lignocellulosic biomass
by the CASA Lignin Method

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 0.01 mg readability

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

2.7 Desiccator with fresh desiccant

2.8 Magnetic stirrer

2.8 Glass vial (4 mL) with Teflon-lined cap

2.8 Volumetric flask (50 mL, 100 mL)

2.9 Micropipette (0.1–1.0 mL)

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

3. Procedure

3.1 Sample preparation

3.1.1 Biomass grinding: Grind chipped or chopped air-dry biomass using a Wiley mill to pass a 1-mm screen. Sieve the ground biomass and collect the fraction between 40 and 80 mesh for analysis.

3.1.2 Removal of extractives: Extract woody biomass with benzene/alcohol (v/v 2:1) using Soxhlet extractor for 4 h, followed by extraction with 95% ethanol for 12 h. [Toluene can replace benzene for safety reasons, and simply using 80% ethanol extraction alone is quite effective especially for woody biomass.[1]] For monocot or grass biomass or non-woody dicots extraction with water under sonication is recommended first to remove proteins and other water-soluble components.

3.2 Dissolution of the biomass sample

3.2.1 Prepare cysteine (0.1 g/mL) stock solution (cysteine-SA): weigh 10 g of L-cysteine and add into 100 mL 72% sulfuric acid (SA) in a glass bottle, stir the mixture with a magnetic stir-bar at room temperature until a homogenous solution is formed.

ES & H considerations: Sulfuric acid is corrosive and should be handled with care.

3.2.2 Weigh 5.0–10.0 mg of sample into a 4 mL glass vial with screw-top.

3.2.3 Add 1.0 mL of cysteine-SA solution and the mixture is sealed with a Teflon-lined cap and stirred with

magnetic stir-bar at room temperature for 60 min.

3.2.4 Transfer the biomass solution with a glass pipet into a volumetric flask containing about 70 mL of DI water (for 100 mL flask), rinse the vial with DI water 4 times (3 mL for each) to completely transfer all content in the vial into the volume flask. Fill the volume flask to the volume marker with DI to exactly 100 mL.

3.3 Spectrophotometric evaluation of CASA-lignin content

3.3.1 Prepare a blank solution by diluting 1 mL of the cysteine stock solution with DI water to volume as the final volume in step 3.2.4.

3.3.1 Acquire a baseline (background spectrum) using the blank solution made in step 3.3.1 on a UV-Visible spectrophotometer.

3.3.2 Scan the biomass solution made in step 3.2.4 from 230 nm to 400 nm wavelength on the UV-Visible spectrophotometer or measure the absorbance at 283 nm.

4. Calculation for lignin content (CASA_L, %) of the biomass sample

$$CASA\_L,(\%) = \frac{Abs. \times V}{\varepsilon \times m_s \times L} \times 100 \qquad (1)$$

where $A_{bs.}$ is the UV absorbance of the diluted solution at λ = 283 nm; V is the total volume of the diluted solution in liters (L); m_s (g) is the dry mass weight of the solvent-extracted lignocellulosic sample; L is the light path length, 1 cm for here; ε ($g^{-1}.L.cm^{-1}$) is the UV-absorption coefficient (absorptivity) of lignin at λ = 283 nm, 17.25 for softwood lignin and 11.23 for hardwood and monocot lignins.

References

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