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Molecular weight determination of lignin via gel permeation chromatography

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We use this protocol and it's working

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Disclaimer

This protocol is for research purposes only.

Abstract

Lignin is a complex, aromatic biopolymer found in the cell walls of plants, where it imparts rigidity, hydrophobicity, and pathogen resistance. It is one of the three major components of lignocellulosic biomass, along with cellulose and hemicellulose.

The physicochemical properties of lignin depend on several factors including the plant species, the method of isolation, its chemical structure, and the abundance of functional groups. Among these characteristics, the molecular weight (MW) or molar mass distribution is important for lignin valorization to fuel, chemicals, and materials.

This protocol describes the most commonly used and versatile technique for quantifying the molecular weight of water-insoluble lignin using an organic gel permeation chromatography (GPC) system.[1-6]

Guidelines

This protocol utilizes a high-performance liquid chromatography system with diode array detection (HPLC-DAD) manufactured by Agilent Technologies as referenced in 'Materials'. A similar chromatography and detection system can be utilized; however, some parameter nomenclature may deviate depending on the manufacturer.

Materials

Standards:

- Polystyrene Calibration Kit (Mp 162-20,000 Da) **Agilent Technologies Catalog** #PL2010-0101
- Polystyrene Calibration Kit (Mp 580-3,000,000 Da) **Agilent Technologies Catalog** #PL2010-0100
- Toluene, HPLC plus **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #650579

Reagents:

- Acetic Anhydride **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #320120
- Pyridine anhydrous **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #270970
- Methanol (HPLC) **Fisher Scientific Catalog** #A452-1
- Tetrahydrofuran, suitable for HPLC, inhibitor-free **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #34865

Heating and stirring module for sample preparation :

Equipment	
Reacti-Therm	NAME
Triple block heating and stirring module	TYPE
Thermo Scientific	BRAND
PI18823	SKU
https://www.fishersci.com/shop/products/reacti-therm-dry-block-sample-incubation-system/PI18823	LINK

Consumables:

Syringes and 0.2 µm syringe filters for sample filtration to remove any undissolved particles

Equipment	
NORM-JECT® Luer slip 1 mL syringe	NAME
Lab grade polypropylene/polyethylene syringe	TYPE
B. Braun	BRAND
NJ-9166017-02	SKU
https://www.air-tite-shop.com/p-13-2-part-luer-slip-syringes.aspx?variantid=22&srsltid=AfmBOool1PzDGJM_S8-iJ8EYn2yNR7qMPKGilPhR6GpnJPGTh-g2jTbQ	LINK

Equipment	
0.2 µm PTFE syringe filters	NAME
syringe filter	TYPE
Agilent Technologies	BRAND
5191-5912	SKU
https://www.agilent.com/store/en_US/Prod-5191-5912/5191-5912?srsltid=AfmBOoqwi1hb5LUqNNFbQy6cGC0K51O7AEo0i2ttMDmLgp-IJECdG7Uo	LINK
13 mm	SPECIFICATIONS

GC vials and caps for sample preparation

Equipment	
Crimp top amber vials - 2 mL	NAME
GC vials	TYPE
Agilent Technologies	BRAND
5183-4496	SKU
https://www.agilent.com/store/productDetail.jsp?catalogId=5183-4496	LINK
certified, deactivated (silanized), 12 × 32 mm	SPECIFICATIONS

Equipment	
11 mm silver aluminum crimp caps	NAME
GC vial caps	TYPE
Agilent Technologies	BRAND
6183-4498	SKU
https://www.agilent.com/store/productDetail.jsp?catalogId=6183-4498	LINK
press-fit PTFE/natural red rubber bi-layer septum	SPECIFICATIONS

Equipment:

Pre-filter

Equipment	
Inline filter assembly and frits	NAME
stainless steel inline filter	TYPE
Agilent Technologies	BRAND
5067-1551 and 280959-904	SKU
https://www.agilent.com/store/productDetail.jsp?catalogId=5067-1551	LINK
2.1 mm, 0.2 µm, max 600 bar, 3 frits, 0.12 × 70 mm connecting capillary	SPECIFICATIONS

Analytical columns

Equipment	
PLgel 10^4 Å column	NAME
polystyrene-divinylbenzene copolymer filled column	TYPE
Agilent Technologies	BRAND
PL1110-6140	SKU
https://www.agilent.com/en/product/gpc-sec-columns-standards/organic-gpc-sec-columns/plgel	LINK
inner diameter 7.5 mm, length 300 mm, particle size 10 µm, MW range 10-450 kDa	SPECIFICATIONS

Equipment	
PLgel 10^3 Å column	NAME
polystyrene-divinylbenzene copolymer filled column	TYPE
Agilent Technologies	BRAND
PL1110-6130	SKU
https://www.agilent.com/en/product/gpc-sec-columns-standards/organic-gpc-sec-columns/plgel	LINK
inner diameter 7.5 mm, length 300 mm, particle size 10 µm, MW range 0.2-60 kDa	SPECIFICATIONS

Equipment	
PLgel 50 Å column	NAME
polystyrene-divinylbenzene copolymer filled column	TYPE
Agilent Technologies	BRAND
PL1110-6115	SKU
https://www.agilent.com/en/product/gpc-sec-columns-standards/organic-gpc-sec-columns/plgel	LINK
inner diameter 7.5 mm, length 300 mm, particle size 10 µm, MW range 0.1-1.5 kDa	SPECIFICATIONS

HPLC System

Equipment

1260 Infinity HPLC System	NAME
High performance liquid chromatography system	TYPE
Agilent Technologies	BRAND
G1311C Quat Pump, G1329B Autosampler, G1316A TCC, G4212B DAD	SPECIFICATIONS

Safety warnings

All chemicals used for this procedure are hazardous. Read the Safety Data Sheet (SDS) for each chemical listed and follow all applicable chemical handling and waste disposal procedures. Manufacturer specific SDS information can be found by following the catalog numbers of compounds in 'Materials' list.

Before start

All solvents and chemicals used are listed in the 'Materials' section. These are excluded from in-line references to maintain clarity and keep the steps concise.

Preparation of standards

1 **Standards**

1. Prepare polystyrene (PS) stock solutions by grouping the eighteen PS standards with varying molecular weights as shown in Table 1.
2. Dissolve 5 mg of each standard in the group into 50 mL of tetrahydrofuran (THF) along with 1 mL of toluene.
3. Transfer 1 mL of each stock solution to a 2 mL amber GC vial. These will be used to create the calibration curve.

Note

Stock solutions should be kept in a refrigerator capable of maintaining a temperature of 4 °C.

1.1 **PS standard stock solutions' molecular weights**

Table 1. Polystyrene standard mixtures including toluene (Mw 92.0 Da)

Sample	#	Retention Time[a] (min)	Mp (Da)	Log Mp
PS1	1	13.74	677,500	5.83
	2	16.20	68,000	4.83
	3	20.42	5,050	3.70
	4	23.40	950	2.98
	5	28.88	92	1.96
PS2	1	14.08	465,000	5.67
	2	17.23	34,500	4.54
	3	20.99	3,600	3.56
	4	24.09	580	2.76
	5	28.87	92	1.96
PS3	1	14.32	369,500	5.57
	2	17.89	22,000	4.34
	3	21.17	3,250	3.51
	4	28.88	92	1.96
PS4	1	14.63	271,800	5.43
	2	18.89	11,600	4.06
	3	22.40	1,700	3.23
	4	28.88	92	1.96
PS5	1	13.57	980,000	5.99
	2	15.04	170,000	5.23
	3	19.27	9,200	3.96
	4	22.91	1,250	3.10
	5	28.88	92	1.96

[a] Retention times may varying depending on the HPLC system used.

Preparation of samples

2 **Acetylation procedure**

1. Dissolve 20 mg of air-dried lignocellulosic material in a mixture of 0.5 mL pyridine and 0.5 mL acetic anhydride in 5 mL conical vials.

2. Heat vials at 40 °C in a heating and stirring module, as described in 'Materials', for 24 h with stirring.
3. Terminate the reaction with an addition of 0.2 mL of methanol.
4. Subsequently, add 1 mL aliquots of methanol to each sample and dry under nitrogen.
5. Repeat methanol additions 7-10 times.
6. Further dry samples in a vacuum oven at 40 °C overnight.
7. Suspend the dried, acetylated samples in THF to achieve a final dilution of 2 mg of lignin residue to 1 mL of THF.
8. Filter the samples through a 0.2 µm PTFE filter into a GC vial.

Note

If insoluble material remains, collect the solids before filtering the liquids through a 0.2 µm filter. Dry the collected solids and weigh the amount to determine what ratio of sample is not represented in the GPC analysis.

GPC analysis

3 **Method specifications**

Analysis is performed using an Agilent 1260 series HPLC system with a DAD. Complete method parameters are in the tables below. Use the analytical columns listed in 'Materials'. Total run time for each sample is 40 minutes.

Quaternary pump configuration	
Flow rate	1 mL/min
Maximum pressure	100 bar
Mobile phase	100.0% THF (v/v)

Note

To eliminate air in the HPLC system, purge the solvent lines by bypassing the columns and increasing the flow rate of THF from 1.0 mL/min to 2.5 mL/min in 0.5 mL increments. Once this has been completed, reduce the flow rate to 0.2 mL/min, return the columns to the flow path and increase the flow rate by 0.2 mL/min increments to a final flow rate of 1.0 mL/min.

Column compartment parameters	
Temperature	25 °C

Autosampler parameters	
Injection volume	25 µL
Draw speed	200 µL/min
Eject speed	200 µL/min
Wash needle after injection	yes

Diode array configuration	
Wavelength (bandwidth)	220.0 nm (10 nm)
	260.0 nm (80 nm)
	270.0 nm (10 nm)
Reference wavelength (bandwidth)	550.0 nm (100 nm)
Peakwidth	>0.10 min (2.5 Hz)
Spectrum	Store all
	190-400 nm
	2.0 nm step

Note

Lignin components are analyzed using the 260 nm wavelength (bandwidth 80 nm).

3.1 **Vials and experimental run set up**

1. Fill the first and second vials in the autosampler with THF (the first vial will be used to clean the injection needle after each injection and the second will be used to run 3-5 blanks at the beginning of the run).
2. Place the five PS standard solutions (as prepared in Step 1) after the THF vials, going from PS1 to PS5.
3. Run them in order with two runs of PS1 at the beginning.
4. Place samples after the standards.
5. After every ten samples, run a PS5 and again at the end of the run. This ensures instrument drift remains within the determined acceptance criteria.
6. Finish with one THF blank to clear any residues from the system, then shutdown the system.

Note

If at the end of the run the peak retention times of the five standards from PS5 are +/-0.1 minute out of range of each other, then re-run the samples that occurred after the deviation.

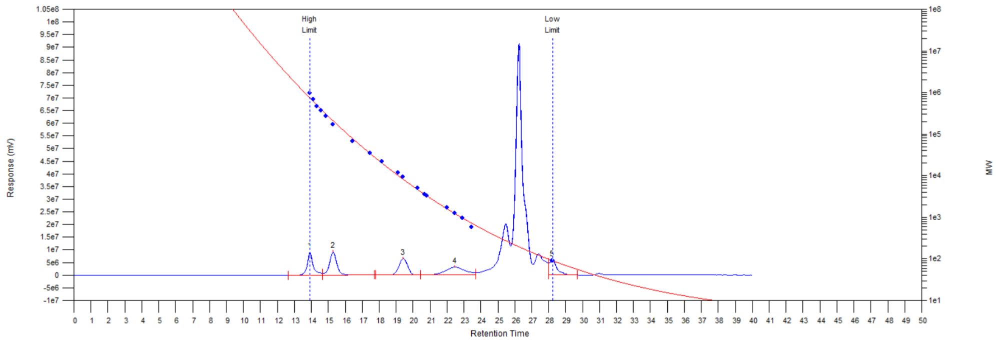
Data analysis

4 **Data analysis:**

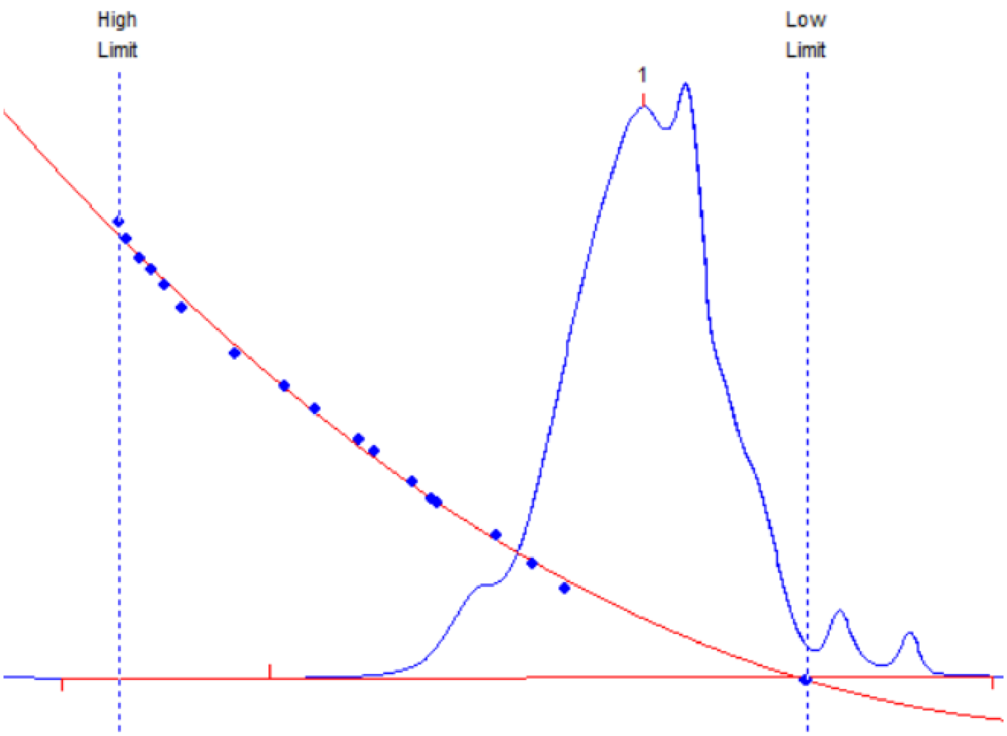
Data analysis is completed using Agilent Cirrus GPC Offline GPC/SEC software version 3.4.1 and Agilent OpenLab CDS, ChemStation Edition version 1.167.13.0. Other GPC analysis software can be utilized; however the depiction of the data and results provided may vary from the examples presented.

Example chromatograms

5



- Figure 1. Example of a calibration curve with PS5 standard in the background as viewed in Agilent Cirrus GPC Offline GPC/SEC software version 3.4.1.



- Figure 2. Example of a sample chromatogram as viewed in Agilent Cirrus GPC Offline GPC/SEC software version 3.4.1.

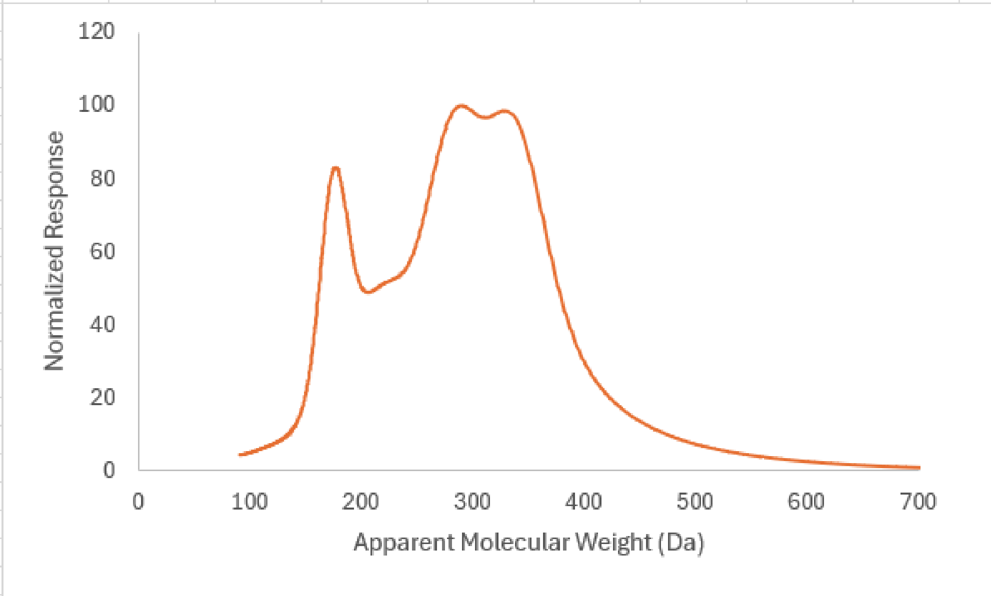


Figure 3. Example of a final molecular weight plot a viewed in Microsoft Excel software version 2025.

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