Research

Standard Operating Procedure for Determination of Lignin Structure with NMR Spectroscopy Within the Nanostructurome Methods Pipeline

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Abstract:
Standard operating procedure (SOP) for the characterization of Organosolv lignin from wood using nuclear magnetic resonance (NMR) spectroscopy is presented. The aim of this procedure within the Nanostructurome methods pipeline is to determine the structural composition of lignin, including the relative proportions of syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) units, as well as the distribution of interunit linkages such as β-O-4, β-β, and β-5 bonds. The extracted lignin was analyzed using 2D heteronuclear single quantum coherence (HSQC) NMR spectroscopy. This method enables precise structural elucidation of signals from aromatic and aliphatic regions. The mathematical model quantifies the abundance of aromatic units and interunit linkages, allowing for comparison of lignin fractions. Proper laboratory practices, including chemical handling, equipment maintenance, and data management, are outlined to ensure the reliability and reproducibility of results. Health and safety measures are emphasized, particularly regarding the disposal of byproducts such as black liquor and solvent waste. The SOP serves as a valuable guideline for lignin structural analysis, facilitating its application in biorefinery processes, material development, and bioengineering.

Keywords: Biomass; Lignin; NMR spectroscopy; 2D HSQC; Structure determination; Nanostructurome

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1. Definitions

NMR: nuclear magnetic resonance
HSQC: heteronuclear single quantum coherence spectroscopy
SOP: standard operating procedure
S-units: syringyl units
G-units: guaiacyl units
H-units: *p*-hydroxyphenyl units

2. Background

Due to the increasing awareness of environmental issues, significant attention is being directed towards obtaining substances from natural renewable sources, among which plant biomass is the only renewable source of organic carbon (Foong et al., (2020); Yoo et al., (2020); Lobato-Peralta et al., (2021)). The most common form of biomass is lignocellulosic biomass, which represents the most extensive and promising renewable carbon source on Earth, with an annual production of 181.5 billion tons (Dahmen et al., (2019)). The characterization of lignin is essential for determining its structure and properties. This, in turn, defines its final applicability and facilitates the development of general methods for its depolymerization and valorisation (Sun, (2020)).

Lignin is composed of cross-linked phenylpropane subunits: *p*-coumaryl (4-hydroxycinnamyl), coniferyl (3-methoxy-4-hydroxycinnamyl), and sinapyl alcohol (**Figure 1**) (Chio et al. (2019)). The proportion of individual monomeric subunits (H, G, and S) in lignocellulosic biomass depends on the type of plant biomass, its age, the plant part from which lignin is extracted, and the fractionation method used. Lignin with a high number of ether bonds, such as β-O-4, and fewer condensed C–C bonds, represents an excellent source for conversion into aromatic molecules. On the other hand, lignin with a high content of hydroxyl groups is suitable for polymer preparation (Sun, (2020)).

The most precise and useful method for identifying lignin structure is nuclear magnetic resonance spectroscopy (NMR), specifically two-dimensional heteronuclear single quantum coherence (2D HSQC) spectroscopy (**Figure 1**). This technique enables the rapid and straightforward determination of S, G, and H monomeric subunits and their linkages. Data from the aromatic region allow the determination of the ratio between the monomeric sub-

units of the entire lignin, while data from the aliphatic region provide insight into the proportion of C–C and C–O linkages. Using NMR spectroscopy, a quick structural analysis of lignin can be obtained, which is crucial for its further applications. Based on the ratio of S, G, and H monomeric subunits, the preferential conversion of a specific lignin into different products can be predicted (Wen et al., (2013); Lu et al., (2017); Mansfield et al., (2012)).

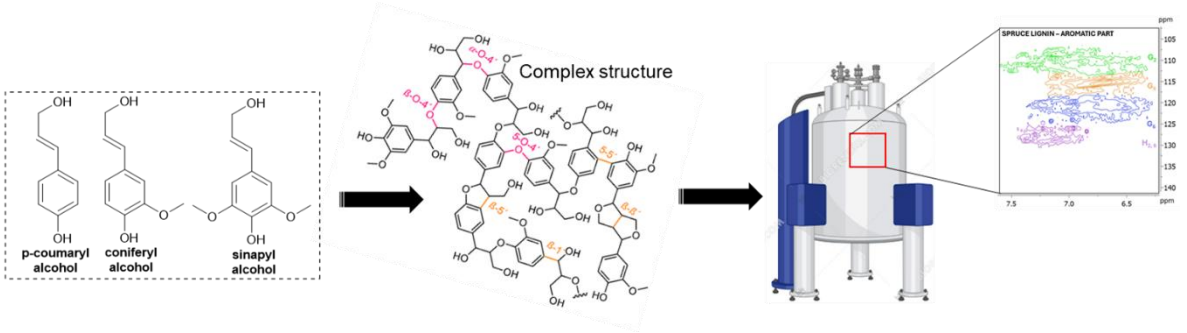

Figure 1. Left: simple propylene subunits that lignin structure is composed of after polymerization. Middle: complex structure of lignin macromolecule with specific bonds colored. Right: NMR instrument and 2D HSQC NMR spectrum of lignin. Different aromatic regions are in colors.

3. Purpose, Scope and Applicability

There are many examples of lignin valorization; however, characterization of lignin is urgent and important research prior to lignin valorization. When considering the utilization of lignin as a promising feedstock, there are some key aspects regarding the characterization of lignin to bear in mind. The under-utilization of industrial lignins is mainly due to their complicated, heterogeneous, destructive, and condensed structures. Simultaneously, different biorefinery processes also produce increasing lignin streams with various structural characteristics and properties. Various fractionation processes cause condensation of lignin, and this poses many difficulties for lignin valorization. Particularly depolymerization of condensed lignin leads to low yields of monomers. Therefore, other than developing viable valorization methods for different sources of lignin, structural characterization of lignin fractions could open the way towards new fractionation and valorization methods. In other words, the different sources of lignin complicate the development and optimization of new processes for their value-added applications (Sun, (2020); Orella et al., (2019)).

The purpose of this SOP is to focus on characterization of kraft lignin and determination of its structure

The scope of this SOP is to determine the structure of lignin derived from spruce wood and produced with craft method.

The applicability of this SOP is broad across various scientific fields, as lignin structural characterization plays a crucial role in multiple applications. In bioengineering, understanding lignin's composition and monomeric ratios enables genetic modifications to improve plant digestibility and industrial processing efficiency. In materials science, lignin with a high content of hydroxyl groups is essential for developing lignin-based polymers, while lignin rich in β-O-4 linkages is valuable for catalytic depolymerization into aromatic chemicals. In environmental and energy research, structural insights aid in optimizing biorefinery processes for sustainable biofuel production. Furthermore, advancements in lignin analysis support the emerging field of lignin nanoparticles, expanding applications in nanotechnology and biomaterials.

4. Health and Safety Warning

Lignin extraction and purification processes pose several health and environmental risks that require careful management. The disposal of black liquor, a byproduct of lignin extraction, generates high concentrations of persistent organic pollutants (POPs) as effluent, which harm aquatic flora and fauna. Adverse effects can include respiratory stress, mixed oxygenase activity, toxicity and mutagenicity, liver damage, or genotoxicity. They can also

cause health hazards such as diarrhea, vomiting, headaches, nausea, and eye irritation in children and employees (Mandal et al., (2023)).
Additionally, certain extraction methods, like the sulfite process, yield lignin products with high sulfur content and co-extract hemicellulose, complicating purification efforts. The lack of selectivity in these processes can lead to lignin with high ash and carbohydrate content (Saadan et al., (2024)).
Moreover, the use of strong acids in some extraction methods poses risks of unwanted side reactions and alterations in the biopolymer structure, which can affect the quality and functionality of the extracted lignin (Karlsson and Lawoko, (2023)).
Therefore, it is crucial to carefully consider and mitigate these risks when selecting and optimizing lignin extraction and purification methods to ensure safety and maintain the integrity of the lignin product.

5. Cautions

All chemicals used in the lignin extraction and purification process should be handled in accordance with applicable safety regulations and good laboratory practices. Proper storage conditions, including temperature control and segregation of incompatible substances, must be maintained to prevent hazardous reactions. Chemical waste should be disposed of following institutional guidelines and environmental regulations. The NMR spectrometer should be operated following manufacturer guidelines and institutional safety protocols. Regular maintenance, including cryogen refilling and probe calibration, must be performed to ensure optimal performance.

6. Personnel Qualifications / Responsibilities

Handling with chemicals should be performed by trained professionals. Operation with delicate NMR equipment should be performed by trained professionals.

7. Materials, Equipment and Supplies

Materials: Spruce biomass was milled to 0.25 mm particles. The following chemicals were used: ethyl acetate (Sigma-Aldrich), hydrochloric acid (Sigma-Aldrich), acetone (Sigma-Aldrich), and deuterated dimethyl sulfoxide (DMSO-d6, Eurisotop).
Devices: Retsch ZM200 mill, Bruker Avance Ultrashield 600 Plus NMR spectrometer.
Other equipment: NMR tubes for preparing lignin solutions in d6-DMSO.

8. Computer Hardware and Software

Hardware: Office PC.
Software: Microsoft Excel, Bruker TopSpin 4.1.1, MestReNova.
Saving and sharing: Cloud and/or Drive documents.

9. Mathematical Model of Lignin 2D HSQC NMR Calculations

In this SOP we follow the general course from previously published articles (Zijlstra et al., (2019)).
The amount of **all aromatic units (T.A.)** in lignin is defined as

$$T.A. = S_{unit} + G_{unit} + H_{unit}, \quad (1)$$

where S_{unit} is the sum of the integrals in the 2D HSQC NMR spectrum for the S and S' units, G_{unit} is the sum of the integrals in the 2D HSQC NMR spectrum for the G_2 and G_5 and G_6 units, H_{unit} is the sum of the integrals in the 2D HSQC NMR spectrum for the H and H' units (**Figure 2**).

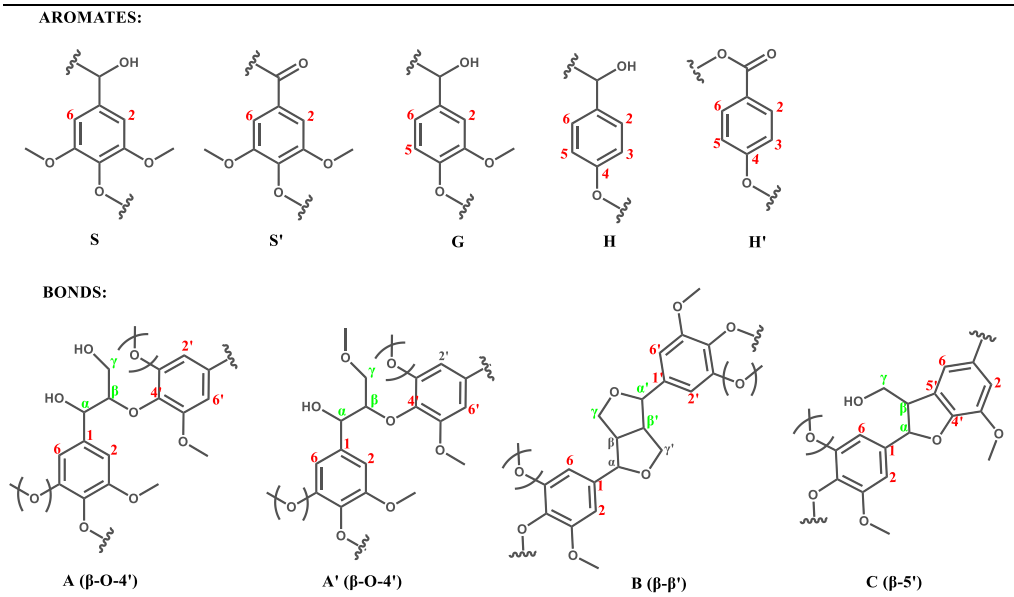

Figure 2: Schematic representation of the aromatic units and the bonds between them in the structure of lignin. H atoms used in integration are in red, bonds in green.

Since there are two S-unit regions in the 2D HSQC NMR spectrum, the following applies

$$S_{unit} = (S_{2/6} + S'_{2/6}) / 2, \quad (2)$$

where $S_{2/6}$ is the area integral of the $S_{2/6}$ unit in the 2D HSQC NMR spectrum and $S'_{2/6}$ is the area integral of the $S'_{2/6}$ unit in the 2D HSQC NMR spectrum.

Since there are two H-unit regions in the 2D HSQC NMR spectrum, the following applies

$$H_{unit} = (H_{2/6} + H'_{2/6}) / 2, \quad (3)$$

where $H_{2/6}$ is the area integral of the $H_{2/6}$ unit in the 2D HSQC NMR spectrum and $H'_{2/6}$ is the area integral of the $H'_{2/6}$ unit in the 2D HSQC NMR spectrum.

Since there are three G-unit regions in the 2D HSQC NMR spectrum, the following applies

$$G_{unit} = (G_2 + G_5 + G_6) / 3, \quad (4)$$

where G_2 is the area integral of the G_2 unit in the 2D HSQC NMR spectrum, G_5 is the area integral of the G_5 unit in the 2D HSQC NMR spectrum, G_6 is the area integral of the G_6 unit in the 2D HSQC NMR spectrum.

Since the G units in the 2D HSQC NMR spectrum partially overlap with the H units, the latter have to be subtracted from the G units to obtain

$$G_{unit} = (G_2 + G_5 + G_6 - H_{2/6} - H'_{2/6}) / 3, \quad (5)$$

When the Eq.(2), Eq.(3) and Eq.(5) are included in Eq.(1), we get

$$T.A. = (S_{2/6} + S'_{2/6}) / 2 + (G_2 + G_5 + G_6 - H_{2/6} - H'_{2/6}) / 3 + (H_{2/6} + H'_{2/6}) / 2 \quad (6)$$

The number of all bonds between monomeric units in lignin structure is defined as

$$\sum bonds = (A_\alpha(bonds) + B_\alpha(bonds) + C_\alpha(bonds)) * 100 / T.A., \quad (7)$$

where A_α(bonds) is the sum of the integrals in the 2D HSQC NMR spectrum from A_α and A'_α bonds, B_α(bonds) is the integral in the 2D HSQC NMR spectrum from the B_α bonds, C_α(bonds) is the integral in the 2D HSQC NMR spectrum from the C_α bonds.

Since there are two A_α(bonds) regions in the 2D HSQC NMR spectrum, the following applies

$$A_\alpha(bonds) = (A_\alpha + A'_\alpha) / 2, \quad (8)$$

where A_α is the integral in the 2D HSQC NMR spectrum for A_α bonds and A'_α is the integral in the 2D HSQC NMR spectrum for A'_α bonds.

When the Eq.(8) is included in Eq.(7), we get

$$\sum bonds = ((A_\alpha + A'_\alpha) / 2 + B_\alpha(bonds) + C_\alpha(bonds)) * 100 / T.A.$$

10. Step by Step Procedure

10.1. Isolation of lignin

Lignin is isolated using ACE high-pressure tubes at 120 °C. Ground spruce biomass (5 g) is placed into the tube, followed by the addition of 50 mL of ethyl acetate and 1 mL of 37% hydrochloric acid. The mixture is stirred at 120 °C for 1 hour.
After the reaction, the residual biomass is filtered off, and the solvent is evaporated under reduced pressure using a rotary evaporator. The resulting concentrated residue is dissolved in 10 mL of acetone, and 100 mL of water is slowly added to induce lignin precipitation. The precipitated lignin is collected by filtration, washed with water, and air-dried overnight.

10.2. Sample preparation for NMR analysis

All lignin samples are prepared following the same procedure. A total of 40 mg of dry, pre-isolated lignin is dissolved in 0.5 mL of deuterated DMSO (d-DMSO). The solution is then filtered and transferred into an NMR tube for analysis.

10.3. NMR analysis

2D HSQC NMR spectra are recorded at 25 °C using a Bruker Avance III 500 spectrometer. The following acquisition parameters are used: F2 range = 10 to 0 ppm, F1 range = 158 to -8 ppm, number of scans (ns) = 24, dummy scans (ds) = 16, number of increments (ni) = 256, relaxation delay (d1) = 1.47 s, CNST[2] = 145, and pulse program = hsqcetgpsi2. The spectra are processed using Bruker TopSpin 4.1.1 and MestReNova software.

10.4. Determination of lignin structure

Integrate the signals in the aromatic and aliphatic region that correspond to the three different aromatic units and bonds between them (**Table 1**).
The aromatic region is used as a reference standard, and the fraction of bonds between aromatic subunits is expressed as the number of occurrences per 100 aromatic units.

Percentage of aromatic units:
T.A. (total aromatics) is calculated by using Eq.(6).
S_{unit} is calculated by using Eq.(2).
H_{unit} is calculated by using Eq.(3).
G_{unit} is calculated by using Eq.(5).

Percentage of S-units = S_{unit} / T.A. × 100%
Percentage of G-units = G_{unit} / T.A. × 100%
Percentage of H-units = H_{unit} /T.A. × 100%

Percentage of bonds between monomers:
T.A. (total aromatics) is calculated by using Eq.(6).
A_{α}(bonds) is calculated by using Eq.(8).

Percentage of β-O-4′ bonds = A_{α}(bonds) / T. A. × 100%
Percentage of β-β′ bonds = B_{α}(bonds) / T. A. × 100%
Percentage of β-5′ bonds = C_{α}(bonds) / T. A. × 100%

10.5. Data acquisition

Table 1. The area of each sub-unit (S, G, H, I and -OCH_3) and the linkages between (A, B, C) in the 2D HSQC NMR spectrum (Yuan et al., (2011)).

Mark	δ_H/δ_C [ppm]	Assignation
$S_{2,6}$	[6.3-7.0/101.5-108.0]	C_2-H_2 and C_6-H_6 in syringyl units (S)
$S'_{2,6}$	[7.2-7.4/105.0-109.0]	C_2-H_2 and C_6-H_6 in oxidised (C=O) syringyl units (S')
G_2	[6.7-7.2/108.5-113.0]	C_2-H_2 in guaiacyl units (G)
G_5	[6.38-7.15/113.2-117.5]	C_5-H_5 in guaiacyl units (G)
G_6	[6.5-7.0/117.5-123.0]	C_6-H_6 in guaiacyl units (G)
$H_{2,6}$	[7.1-7.29/126.5-131.0]	C_2-H_2 and C_6-H_6 in *p*-hydroxyphenyl units (H)
$H'_{2,6}$	[7.5-7.75/127.3-131.0]	C_2-H_2 and C_6-H_6 in *p*-hydroxybenzoate units (H)
I_{α}	[6.44-6.54/128.1-128.5]	C_{α}-H_{α} in *p*-hydroxycinnamyl alcohol
$I_{ß}$	[6.25-6.45/128.1-128.5]	$C_{ß}$-$H_{ß}$ in *p*-hydroxycinnamyl alcohol
I_{γ}	[4.0-4.10/61.4]	C_{γ}-H_{γ} in *p*-hydroxycinnamyl alcohol
-OCH_3	3.7/55.6	C-H in methoxy groups
A_{α}	[4.6-5.0/70.0-74.0]	C_{α}-H_{α} in ß-0-4' units (A)
A'_{α}	[4.50-4.85/79.0-83.0]	C_{α}-H_{α} in ß-0-4' units (A)
$A_{ß}$, $A'_{ß}$	[4.0-4.45/80.0-86.0]	$C_{ß}$-$H_{ß}$ in ß-0-4' units (A)
A_{γ}, A'_{γ}	[3.1-4.1/83.5-87.0]	C_{γ}-H_{γ} in ß-0-4' units (A)
B_{α}	[4.5-4.8/83.5-87.0]	C_{α}—H_{α} in ß-ß' resinol units (B)
$B_{ß}$	[2.96-3.2/52.0-55.5]	$C_{ß}$-$H_{ß}$ in ß-ß' resinol units (B)
B_{γ}	[3.7-3.94/69.5-73.5]; [4.1-4.2/69.5-72.5]	C_{γ}-H_{γ} in ß-ß' resinol units (B)
C_{α}	[5.35-5.65/85.5-89.0]	C_{α}—H_{α} in phenylcoumaran units (C)
$C_{ß}$	[3.36-3.58/52.0-53.7]	$C_{ß}$-$H_{ß}$ in phenylcoumaran units (C)
C_{γ}	[3.5-3.9/61.5-64.5]	C_{γ}-H_{γ} in phenylcoumaran units (C)

11. Data and Records Management

All the experimental details are recorded within the lab journal carefully. All raw as well as treated data is stored in electronic form with physical backup for a minimum of 10 years after data generation.

12. Waste Management

In this SOP, ethyl acetate is collected during the vacuum evaporation process and is either recycled or disposed of as a waste organic solvent. The first filtration process generates a

solid residue composed of cellulose and hemicellulose, which is repurposed for further reactions. In the second filtration step, where lignin is obtained as the solid residue, the remaining aqueous phase is classified and collected as waste aqueous solvents.

13. Related Protocols or SOPS

There are many reported protocols for determination of lignin structure (reviewed for example by Lupoi et al., (2014), Jiang et al., (2018), Karlsson et al., (2023), Lui et al., (2024)).

14. Quality Control and Quality Assurance Section

14.1. Instrument Calibration

Calibrating a Nuclear Magnetic Resonance (NMR) spectrometer is essential to ensure accurate and reproducible results. The calibration process involves several key steps, including frequency calibration, field homogeneity adjustments, and shimming optimization (Andris et al., 2021).

14.2. Critical Processes Parameters and Checkpoints

Number of Scans (NS) and Relaxation Delays (D1) are critical parameters. The former has a major impact on the signal-to-noise ratio, while the latter must be suitable for quantitative analysis, which is what lignin structure determination is.

15. Conclusions

NMR spectroscopy is a powerful analytical tool that enables precise structural elucidation, quantitative analysis, and dynamic studies of molecular systems. The implementation of standardized methodologies, rigorous experimental protocols, and continuous technological advancements enhances the accuracy, reproducibility, and efficiency of NMR-based investigations. To ensure optimal performance and reliability, best practices and standard operating procedures should be regularly reviewed and updated in alignment with scientific progress and evolving research demands. Moreover, integrating NMR spectroscopy within a comprehensive quality management system tailored to scientific exploration can further improve data integrity while preserving the flexibility necessary for innovative research.

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Data Availability Statement: Data available at request.

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Conflicts of Interest: The authors declare no conflict of interest.

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