NMR Techniques for Biomass Characterization: Bridging Complex Structures and Renewable Energy Solutions

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A comprehensive review of the application of nuclear magnetic resonance (NMR) in the characterization of lignocellulosic biomass, a promising feedstock for chemical and fuel production, is presented. The diverse array of lignocellulosic materials, both naturally occurring and chemically or mechanically processed, and the various NMR techniques employed to elucidate their structural and chemical properties are explored. The review delves into the theoretical foundations of NMR and extends to detailed discussions on its use in analyzing whole biomass structures, cellulose, hemicellulose, lignin, lignin-carbohydrate complexes, and biomass derivatives such as platform chemicals, pyrolysis products, and digested substances. Additionally, it covers the application of NMR in monitoring reaction rates and studying biomass transformation processes *in situ*. Finally, the workflow of an NMR experiment and a model experiment of an unknown biomass sample are detailed to aid biomass researchers. This work aims to provide a comprehensive understanding of the current state of NMR research in biomass characterization and to inspire innovative approaches to address the complex chemical and structural challenges in this vital field of research.

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INTRODUCTION

Biomass is nearly universally accepted as an alternative fuel source that can partially replace or supplement petrochemical use. (Ben *et al*. 2019; Gokul *et al.* 2026; Mhetras *et al.* 2026). Therefore, a need to study and characterize biomass has emerged (Ge *et al*. 2016; Yoo *et al*. 2016; Araneda *et al*. 2022). Global trends are shifting towards using renewable resources such as biomass to alleviate dependence on petrochemicals due to heightened increases in net atmospheric CO_2 (Dufour *et al*. 2012; Elliot *et al*. 2019). Biomass as a whole can be divided into its three main bulk constituents: cellulose, hemicellulose, and lignin (Yoo *et al*. 2016; Chen *et al*. 2017). The composition of each component varies from species to species. Cellulose is the most abundant biopolymer on

Earth and is composed of repeating cellobiose units linked through β-(1,4) glycosidic bonds (Foston 2014; Synytsya and Novak 2014; Yoo *et al*. 2016). Hemicellulose is composed of various monosaccharides linked together as a copolymer with fewer structural units compared to cellulose (Shi *et al*. 2011; Yoo *et al*. 2016). Lignin is a heterogeneous, complex biopolymer that acts as structural support in plant species (Boerjan *et al*. 2003; Yoo *et al*. 2016; Jardim *et al*. 2020).

For the conversion of these biomass constituents into value-added products, specific processing steps are necessary (Ge *et al*. 2016; Elliot *et al*. 2019). Conversion steps include, but are not limited to, chemocatalytic carbohydrate conversion into platform chemicals, and conversion of technical lignins into products such as adhesives or bioplastics (Ge *et al*. 2016; Demuner *et al.* 2019; Elliot *et al*. 2019). To understand and utilize biomass as a resource, the products it produces, and the processes used to make these products, it is necessary to develop analytical methods to interrogate its structure at a molecular level (Ge *et al*. 2016; Yoo *et al*. 2016). Biomass is a complex entanglement of various chemical structures and polymers (Foston 2014; Yoo *et al*. 2016; Chen *et al*. 2017). Many analytical methods exist to determine properties associated with biomass components and chemical transformations (Ge *et al*. 2016; Yoo *et al*. 2016). No analysis is more powerful for chemical structural determination than nuclear magnetic resonance (NMR). In broad terms, NMR measures the spectroscopic effects of a nucleus in a magnetic field by analyzing the energy transition induced by a specific radio frequency to flip the spin of this nucleus (Feindel 2012; Reuhs and Simsek 2017). Nuclear magnetic resonance can probe chemical structures in the solid and solution-state, for multiple nuclei of interest, and in multiple dimensions of spectral analysis (Argyropoulos 1995; Taylor 2004; Pu *et al*. 2011; Wen *et al*. 2013; Adams 2016; Foston *et al*. 2016). Because many techniques exist for biomass chemical characterization *via* NMR methods, a holistic review describing NMR possibilities for biomass use is necessary.

Biomass characterization has been significantly amplified due to the NMR phenomena. Previously, the composition of whole, intact biomass could only be theorized. Various NMR studies have used solid-state NMR to determine not only the chemical environment but also the degree of crystallinity or morphology of the bulk sample, not obtainable through other analytical techniques (Foston 2014). Additionally, the development of solvent systems capable of dissolving intact plant cells has allowed NMR to provide further chemical characterization in multiple-dimensional NMR analysis in the solution-state (Ralph and Lu 2004; Kim and Ralph 2010; Yoo *et al*. 2016). For biomass-derived products such as platform chemicals or pyrolyzed biomass, NMR has provided an effective technique to verify the chemical composition of these chemically-altered materials (Prestes *et al*. 2007; Link *et al*. 2008; Yoshida *et al*. 2008; Falco *et al*. 2011; Melkior *et al*. 2012; Cao *et al*. 2013; Meng *et al*. 2013; Park *et al*. 2013; Wang *et al*. 2013; Singh *et al*. 2015; Ge *et al*. 2016; Hao *et al*. 2016; Mafu *et al*. 2016; Michailof *et al*. 2016; Ben *et al*. 2017; Carrier *et al*. 2017; Chen *et al*. 2017; Li *et al*. 2018; Lyu *et al*. 2018; Leng *et al*. 2019; Staš *et al*. 2020).

In the field of lignin, NMR has allowed for the broad quantification of its heterogeneous structure. Hydroxyl content, syringyl to guaiacyl (S/G) ratio, methoxyl content, inter-unit linkages, and lignin carbohydrate complex (LCC) content have all been quantified using various NMR techniques (Argyropoulos 1995; Granata and Argyropoulos 1995; Capanema *et al*. 2001, 2004; Liitiä *et al*. 2003; Balakshin *et al*. 2007; Hallac *et al*. 2009; Pu *et al*. 2011; Yuan *et al*. 2011; Balakshin and Capanema 2015a; Balakshin and Capanema 2015b; Zhu *et al*. 2015; Tarasov *et al*. 2018; Giummarella *et al*. 2019). Finally,

reaction progress and *in-situ* experiments can be detailed and may push the boundaries of what NMR has traditionally been capable of offering. (Dufour *et al*. 2012; Ko *et al*. 2014; Wang *et al*. 2016; Ben *et al*. 2019).

Herein, NMR studies on biomass are presented, and subsequent studies are outlined for specific biomass chemical units. This report is a brief guide to NMR as a technique for biomass research, an overview of NMR theory, a variety of biomass types and transformations studied using NMR, NMR techniques, both well-known and more innovative, that have been used to probe biomass, a guide for biomass researchers as to the workflow of a typical NMR experiment, and an in-house NMR experiment on an unknown biomass sample. Additionally, limitations such as the need for complex solvent systems to dissolve more intricate specimens, the time requirements of NMR experiments, and difficulties around discerning chemical structures that overlap in NMR spectra, among others, are also discussed. NMR is a powerful tool for biomass research, and this review should encourage more studies to generate improved approaches for biomass chemical characterization.

A holistic review is provided as a guideline to frame ideal studies for biomass types that have yet to be reported. The goal of the review is to avoid going into great technical details or to present specific peak assignments. Instead, it highlights how NMR has affected the biomass and biomass-derived products field and provides guidance for biomass researchers unfamiliar with NMR as an analytical technique. Reviews describing NMR studies generally provide details relating to one specific biomass material (Argyropoulos 1995; Pu *et al*. 2011; Kim *et al*. 2013; Foston 2014; Foston *et al*. 2016; Wang and Hong 2016; Ghassemi *et al*. 2022). However, a review of how NMR as a technique has advanced the field of biomass and biomass processing is absent, which this work intends to resolve.

NMR Theory: Basic Principles

NMR is a spectroscopic technique that immerses a sample of interest in a magnetic field (Feindel 2012; Reuhs and Simsek 2017). The compound is then subjected to a second, oscillating magnetic field that will flip the spin of certain nuclei at a specific radio frequency (Feindel 2012; Reuhs and Simsek 2017). These two magnetic fields are known as B_0 and B_1 respectively (Feindel 2012; Reuhs and Simsek 2017). The purpose of an NMR experiment is to quantify or elucidate the atomic structure of the compound of interest. The NMR effect was discovered near the end of World War II, and various scientists have contributed to this complex field to create a helpful multidisciplinary technology (Gerothanassis *et al*. 2002; Marion 2013; Singh and Singh 2022). Nobel prizes have been awarded to a multitude of researchers for NMR breakthroughs. These include Isidor Isaac Rabi in 1944 and Edward Mills Purcell and Felix Bloch in 1952 for developing the initial methods of NMR spectroscopy, Richard R. Ernst in 1991 for developing the idea for 2D NMR, and Kurt Wüthrich in 2002 for developing the idea for 3D NMR for biological macromolecules (Boesch 2004). Only nuclei with an odd atomic number can undergo the nuclear precession NMR phenomenon. This is due to a phenomenon known as nuclear spin (Marion 2013; Singh and Singh 2022). Protons, neutrons, and electrons all have nuclear spin. If an atom has a nuclear spin with the multiple of ½ due to an unpaired proton or neutron, an NMR signal will appear (Gerothanassis *et al*. 2002; Marion 2013; Singh and Singh 2022). The spin property makes an atom behave as a magnet. In the absence of a magnetic field, nuclear spins will orient at random (Singh and Singh 2022). In the presence external magnetic field (B_0), a nucleus will either align with or against a magnetic field (Feindel 2012; Gerothanassis *et al*. 2002; Singh and Singh 2022). In NMR spectroscopy

experiments, the energy of the external magnet can be at very low frequencies all the way up to 1.2 GHz (Bruker 2023).

The main hardware components of NMR magnets include the large external magnet, a radiofrequency transmitter, a radiofrequency coil or probe, a receiver, and a computer system that controls the electronics and parameters involved in NMR experiments (Mundy *et al*. 1983; Conradi 2022). The entire NMR system is complex, and knowledge of chemistry, physics, and electronics is needed to comprehend its full extent. Conradi (2022) and Mundy *et al*. (1983) both provide detailed explanations of the intricacies of NMR magnets. Solid-state NMR and solutions state NMR require different hardware setups. Specifically, the probes used in solid-state and solution-state NMR analyses are the primary difference based on how each technique functions. For solid-state NMR, a CP/MAS (Cross Polarization/Magic Angle Spinning) probe allows for a sample to be rotated at high speeds at what is known as the 'magic angle' to generate a suitable spectrum (Taylor 2004). The 'magic angle' will be further defined in solid-state NMR experiments discussions. Solution-state probes include broadband decoupling inverse probe (BBI), broad band observe probe (BBO), cryoprobes, *etc*., and do not require spinning at high frequencies at the 'magic angle' due to chemical isotropy inherent in solution-state NMR experiments (Dais and Spyros 2012; Marion 2013; Reuhs and Simsek 2017; Poulhazan *et al*. 2020; Bruker 2026).

When an external magnetic field is applied, nuclei will precess about the Lamour frequency either with or against the magnetic field. The rate of the precession is based on the strength of the external magnetic field (Reuhs and Simsek 2017; Singh and Singh 2022). Equation 1 and Fig. 1 explain and show the behavior of nuclear spin when an external magnetic field is applied (Singh and Singh 2022). The difference in spin orientation can be expressed as an equation using Boltzmann statistics (Feindel 2012). Pulses of the B_1 (applied oscillating magnetic field) magnetic field or radio frequency (RF) pulses will change the spin state of nuclei, which gives off an NMR signal as the nuclei relaxes back to equilibrium (Feindel 2012; Reuhs and Simsek 2017; Singh and Singh 2022). The applied frequency should match the processional frequency of each nucleus, creating a state called resonance, which corresponds to the energy difference between a nuclei's spin states (Reuhs and Simsek 2017). The resonance for each NMR active nucleus is different, and therefore, in an NMR experiment, different frequencies are applied depending on the nucleus in question (Gerothanassis *et al*. 2002). By applying various combinations of RF pulses and delays, known as a pulse sequence, with different durations and power levels, the nuclear spins of atoms are manipulated, and the NMR can show different parameters such as relaxation times, chemical shifts, and coupling constants (Reuhs and Simsek 2017). All three of these last concepts will be defined later in the review. Pulse sequences built into NMR software are commonly applied by novice NMR users, and the parameters can be optimized to provide the required sensitivity and resolution for the sample being analyzed (Parella 2010).

In the most straightforward NMR experiment, an RF (Radio Frequency) pulse is applied, which rotates the magnetization of a nucleus down the transverse plane, which the NMR detects (Feindel 2012; Reuhs and Simsek 2017). When an RF pulse is applied, the population of spin states will rearrange to the high-energy state conformation (Feindel 2012). Relaxation occurs exponentially along a longitudinal (z) axis, along the transverse axis (xy), or along the Lamour frequency to realign the nuclear spin with the applied magnetic field (Feindel 2012; Adams 2016; Singh and Singh 2022). These two processes are known as the spin-lattice (T_1) and spin-spin (T_2) relaxation respectively (Adams 2016;

Reuhs and Simsek 2017; Singh and Singh 2022). The Free Induction Decay (FID) is detected in an NMR experiment when a pulse is applied, and the nucleus undergoes T_1 and T_2 relaxation (Reuhs and Simsek 2017). Fourier transform (FT) is a mathematical operation that converts the time domain (FID) signal into the frequency domain. A visual representation of converting a time domain (FID) signal to Fourier transform signal is presented in Fig. 2 (Dais and Spyros 2012). NMR experiments are broadly defined as 'high field' and 'low field' depending on the magnetic field strength. As the B_0 field increases in magnitude the resonance frequency of the NMR signal increases, and consequently the signal-to-noise ratio increases (Dais and Spyros 2012). The ratio between baseline or background level 'noise' and desired 'signal' collected from a sample defines signal-to-noise ratio. In the last 20 years, NMR technology has improved dramatically to include larger external magnetic fields, cryoprobes to reduce noise, development of new pulse sequences, and improved spectrometer electronics and computer processing methods (Dais and Spyros 2012; Marion 2013). Improvements in the field of NMR aim to optimize experimental time and spectral resolution. More recently, techniques such as hyperpolarization have allowed for signal detection for minute sample concentrations up to one-millionth of a 'normal' concentrated amount (Epasto *et al*. 2022).

$$\delta = (\nu - \nu_{REF}) \times 10^6 / \nu_{REF} \quad (1)$$

Equation 1 is called Lamour frequency equation, where ω is the Lamour frequency or the frequency of the precessing motion of a nucleus in units of MHz, γ is the gyromagnetic ratio or a ratio of the magnetic moment (μ) and the angular momentum of the nucleus in units of MHz/Tesla (T), and B_0 is the external magnetic ratio or the strength of the external magnetic field in units of T. The Lamour frequency Eq. 1 is useful in assessing the frequencies of NMR active nuclei as shown in Fig. 1.

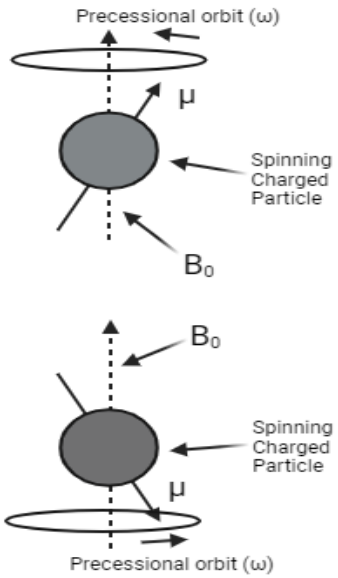


Fig. 1. Visual representation of nuclear spin behavior in the presence of an external applied magnetic field where ω is the Lamour frequency (Reuhs and Simsek 2017).

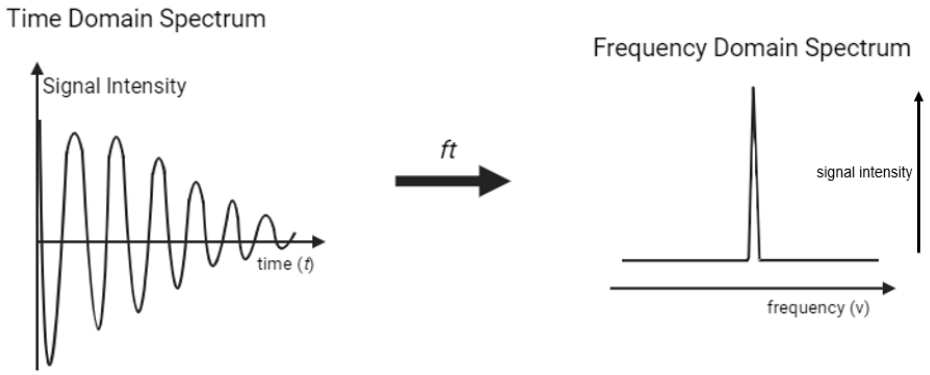


Fig. 2. The leftmost spectrum is a time domain spectrum known as Free Induction Decay (FID), and the right-most spectrum is a frequency domain spectrum that results from the Fourier Transformation (FT) of the FID (Dais and Spyros 2012).

NMR Techniques for Biomass Research

Various techniques exist in NMR experiments to elucidate the composition of a compound, polymer, or system of interest. NMR, broadly, is very commonly used in the fields of medicine and chemistry to perform MRIs or assess the purity of chemical reactions (Feindel 2012; Toman *et al.* 2016). Solution-state NMR experiments are the most commonly used in biomass research. As the name suggests, solution-state NMR requires the biomass in question to be dissolved in an NMR inactive solution (nuclei that are spin inactive). Deuterated dimethyl sulfoxide (DMSO-d_6) is the most used NMR solvent for biomaterials; however, alternative solvents have been used to dissolve complex milled biomass. Specifically, studies have been conducted using pyridine d_5/DMSO d_6, DMSO d_6, hexamethylphosphoramide d_{18} (HMPA-d_{18}), DMSO–N-methylimidazole (DMSO/NMI), DMSO tetrabutylammonium fluoride (DMSO/TBAF), and ionic liquids (Ralph and Lu 2004; Kim and Ralph 2010; Wen *et al*. 2013; Yuan *et al*. 2013; Foston *et al*. 2016; Yoo *et al*. 2016). Deuterated solvents are used for direct dissolution of biomass for NMR analysis, whereas non-deuterated solvents are used to dissolve biomass initially, followed by evaporation and the subsequent dissolution in a deuterated solvent for NMR analysis. While deuterated solvents do give off an NMR signal, the signal of deuterated solvents is much less intense compared to non-deuterated solvents.

Solid-state NMR is comparatively advantageous compared to solution-state NMR when studying complex structures that consist of multiple structural components as found in whole intact woody biomass or pyrolytic chars (Foston 2014). Additionally, if the solvent system used to dissolve a biomass sample is ineffective or expensive, solid-state NMR can be more effective. Solid-state NMR techniques also do not require purification, which can change the chemical structure of the original material (Foston 2014; Ghassemi *et al*. 2022). A disadvantage of solid-state NMR, however, is that molecules in the solid-state are not oriented isotopically with the external magnetic field, which results in broader lines and worse resolution in the resulting spectrum. (Marion 2013; Foston 2014; Ghassemi *et al*. 2022). Various techniques are employed in solid-state NMR to increase the resolution of generated spectra: namely, magic angle spinning (MAS), cross-polarization (CP), relaxation, dipolar coupling, spin diffusion, and multiple combinations of these techniques (Blümich 1988; Foston 2014; Reuhs and Simsek 2017; Ghassemi *et al*. 2022). In most solid-state NMR studies, a ^{13}C CP/MAS is a preferred technique to elucidate biomass structures (Atalla 1984; Atalla and Vanderhart 1999; Liitia *et al*. 2000; Taylor 2004; Link

et al. 2008; Ben *et al*. 2017; Reuhs and Simsek 2017; Walker *et al*. 2020). Using this method, magic angle spinning spins the sample at the inclined 'magic angle' of 54.74º with respect to the external magnetic field (B_0). This allows for the chemical shift anisotropy and spin-spin interactions between sample nuclei to average and results in better resolution at this angle (Foston 2014; Adams 2016; Wang and Hong 2016; Reuhs and Simsek 2017; Ghassemi *et al*. 2022; Rodrigues *et al*. 2022). In solid-state NMR, CP increases the sensitivity of lower gyromagnetic nuclei such as ^{13}C. This is done by transferring polarization from more abundant excited ^{1}H nuclei to less abundant ^{13}C nuclei to enhance the signal and resolution of the resulting spectrum (Liitia *et al*. 2000; Taylor 2004; Foston 2014; Adams 2016; Reuhs and Simsek 2017; Rosnow *et al*. 2017; Walker *et al*. 2020; Ghassemi *et al*. 2022; Rodrigues *et al*. 2022). As the spinning speed of a magic angle experiment increases to speeds up to 100 kHz, the spectral resolution improves in tandem. More recent advances in solid-state NMR rotor design mean smaller-sized rotors can be implemented allowing for higher spinning speeds (Sternberg *et al*. 2018). This means that NMR signals from intact samples can be more easily distinguished as spinning speed increases which provides increasing possibilities for biomass solid-state NMR studies.

Because only certain nuclei are NMR active (have spin properties), only a few nuclei are typically analyzed (Reuhs and Simsek 2017). A one-dimensional (1D) NMR study detects one isotope at a time; however, the spectrum can be modified by other nuclei interacting with the isotope being detected. A representation of a 1D NMR experiment for kraft pine lignin is presented in Fig. 3. A simple 1D experiment proceeds by exciting the nucleus of interest with an RF pulse wherein the FID is generated, as the molecule relaxes. When the FID is Fourier transformed, the 1D spectrum is made (Reuhs and Simsek 2017). The most common and most sensitive nucleus to analyze is hydrogen (^{1}H) also known as proton NMR. The most popular nuclei used in biomass research are ^{1}H, ^{13}C, and ^{31}P. A list of NMR active nuclei used in biomass research is presented in Table 1. ^{13}C and ^{31}P experiments have been used in biomass experiments in order to quantify specific functional groups (Capanema *et al*. 2004; Balakshin and Capanema 2015a).

In order to implement and analyze 1D NMR experiments, the concepts of chemical shift and coupling need to be understood. The NMR spectrum will identify individual atoms depending on their location within the molecule and the shielding effect from surrounding electrons (Marion 2013; Reuhs and Simsek 2017). The difference between resonance frequencies of nuclei in an experimental compound and a standard compound is known as chemical shift (Marion 2013; Reuhs and Simsek 2017). The chemical shift can be expressed *via* Eq. 1 and is independent of the magnetic strength used (Marion 2013). Atoms with similar chemical shifts can produce overlapping peaks in the generated spectra (Giummarella *et al*. 2019). In addition to chemical shift, the interactions of nearby nuclei, called coupling, is essential when considering setting up 1D NMR experiments. If two NMR detectable nuclei are either three bonds or less apart, as in the case of hydrogen and carbon, or up to five or six bonds apart, as in the case of phosphorus and fluorine their spins will often interact, and the resulting NMR signal will split into multiple peaks (Reuhs and Simsek 2017). This phenomenon is also known as scalar coupling or J-coupling (Marion 2013). Dipolar coupling is relevant in solid-state NMR and occurs due to dipole-dipole interactions between two nuclear spins (Taylor 2004). Coupling can be present between the same isotope (homonuclear coupling) and different isotopes (heteronuclear coupling). The signal of each coupled nucleus will split based on the number of neighboring nuclei capable of generating an NMR signal. Each coupled nucleus is separated by a distinct value in Hz known as a coupling constant. Ethanol ^{1}H NMR spectra are commonly used to depict

coupling constants, and a good example is illustrated by Gerothanassis *et al*. (2002). Coupling constants in a 1D experiment give information about the molecular structure because connectivity can be inferred based on each coupling constant (Marion 2013). To alleviate coupling effects in non-^{1}H nuclei (usually ^{13}C) 1D NMR experiment, a secondary radio frequency pulse on the proton channel is applied which is called decoupling (Marion 2013). Decoupling is most important in ^{13}C NMR due to the heteronuclear coupling effects of protons in the sample. Decoupling experiments have three modes: one which applies a decoupling pulse throughout the experiment (decoupling with the Nuclear Overhauser Effect (NOE)), another is to decouple only during the acquisition period (inverse gated or decouple without NOE), and finally, during the relaxation period (gated) (Hui 2019). The NOE effect arises when one population of spin-active nuclei is excited by an RF pulse, creating magnetization, which is transferred to another set of nuclei connected via dipolar interactions. This is common for proton magnetization being transferred to carbon nuclei, which increases the carbon signal (Marion 2013; Kumar Anil and Christy 2017; Hui 2019). When doing an inverse gated coupling experiment, the NOE effect is nullified, meaning, in the case of ^{13}C NMR, will decrease the signal in the resulting spectrum (Hui 2019). To do a ^{13}C experiment that is quantitative, the inverse gated experiment should be used. An NOE experiment can also provide information about dipolar or short-range through-space spin interactions between nuclei (Marion 2013).

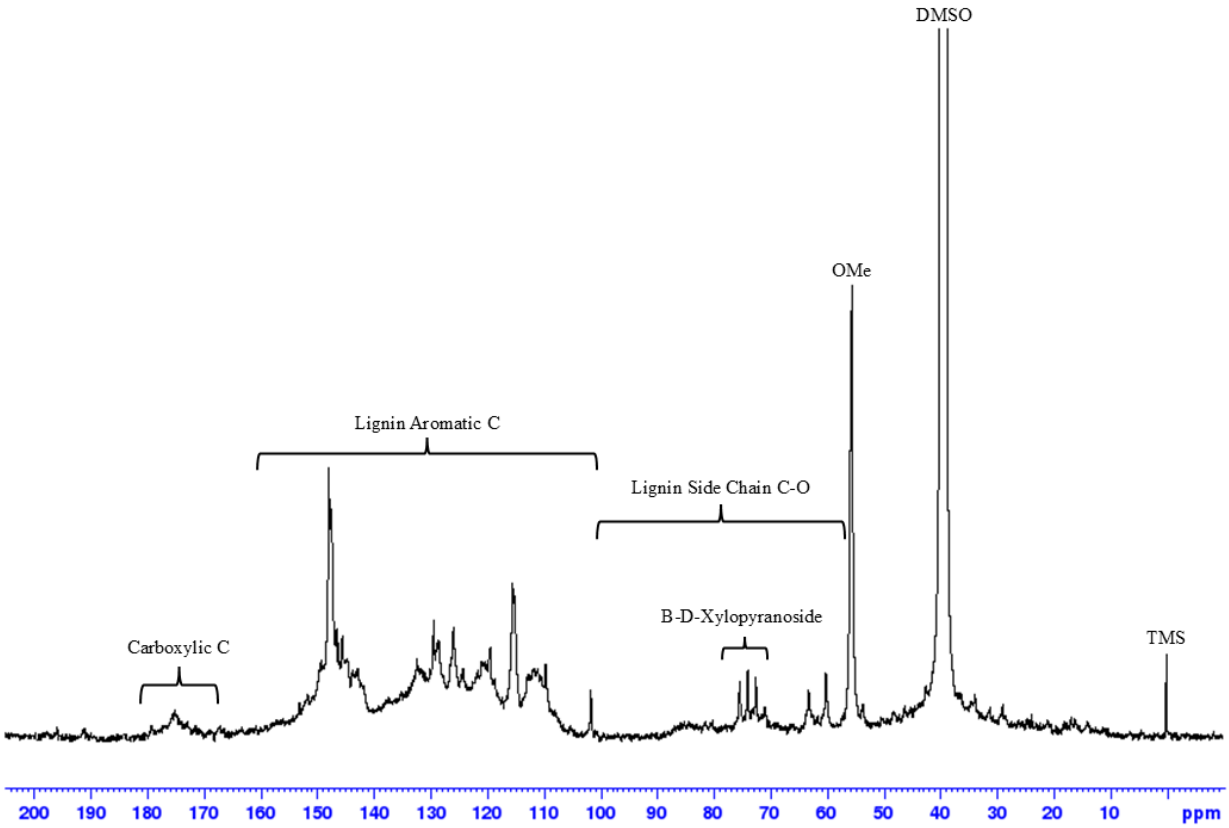


Fig. 3. ^{13}C NMR spectrum of pine kraft lignin. Kraft lignin was isolated by adjusting the pH of alkaline kraft black liquor samples to pH 2.5. Pulping was carried with an active alkali and sulfidity percentage of 19 and 25 respectively, a liquor to wood ratio of 4:1 and a final H-factor of 1400. Chemical cluster assignments are bracketed or labeled. OMe, DMSO, and TMS stand for methoxyl, dimethyl sulfoxide, and tetramethylsilane, respectively.

Table 1. Natural Abundance and Sensitivity Relative to ^{1}H of Commonly-used NMR Active Nuclei. Adapted from (Bottomley and Griffiths 2014)

Nucleus	Natural Abundance (%)	Relative Sensitivity
^{1}H	99.98	1.0
^{13}C	1.11	0.004
^{15}N	0.36	0.0004
^{19}F	100.00	0.83
^{31}P	100.00	0.5

Multiple dimension NMR is a technique that can alleviate signal overlap concerns by correlating multiple nuclei scans in one spectrum (Giummarella *et al*. 2019). Atoms with similar chemical shifts in one dimension are separated in the second dimension (often ^{13}C) by the nuclear chemical shift (Marion 2013; Giummarella *et al*. 2019). 2D NMR was invented by Richard R. Ernst by introducing an additional frequency axis to an NMR experiment, which leads to a rectangular matrix of correlated signals between two nuclei (Aue *et al*. 1976; Marion 2013). Many different 2D NMR experiments exist in both solid and solution-states, and depending on the research objectives, the chemistry of the compound, polymer, or system in question can be elucidated (Liitiä *et al*. 2003; Ralph and Lu 2004; Balakshin *et al*. 2007; Kim and Ralph 2010; Balakshin *et al*. 2011; Yuan *et al*. 2011; Le Brech *et al*. 2015; Wang and Hong 2016; Yoo *et al*. 2016; Elliot *et al*. 2019).

The most straightforward 2D experiment shows correlations between protons on adjacent carbons and involves pulsing multiple 1D sequences with varying delay times, creating a two-dimensional matrix of data that can be Fourier transformed from the time domain into the frequency domain in two dimensions (Dais and Spyros 2012; Marion 2013). This is known as correlation spectroscopy (COSY). Spins of two NMR active nuclei evolve during an evolution delay (t_1), exchange magnetization through either J-coupling or NOE, and are detected during the detection period (t_2) (Marion 2013). This creates a two-dimensional spectrum with both axes being identical (Marion 2013). The correlation between protons can be extended by a technique known as total correlation spectroscopy, commonly referred to as TOCSY. This technique shows homonuclear bond correlation for protons attached to carbons in a continuous chain from two, three, and sometimes four bonds away. A complementary 2D technique called Nuclear Overhauser spectroscopy (NOESY) shows correlations based on proximity rather than bonding, as in COSY and TOCSY. The COSY, TOCSY, and NOESY experiments are all known as homonuclear NMR experiments.

Heteronuclear experiments such as heteronuclear single quantum coherence (HSQC) display a ^{13}C spectrum on the y-axis and a ^{1}H spectrum on the x-axis (Dais and Spyros 2012). This spectrum produces signals for carbon and hydrogen atoms that are directly bonded to one another. (Dais and Spyros 2012; Giummarella *et al*. 2019). An example of a 2D HSQC spectrum of kraft pine lignin is presented in Fig. 4. To visualize atoms multiple bond lengths away, heteronuclear multiple bond correlation (HMBC) shows connections between carbon and hydrogen atoms two, three, and, at times, four bonds away (Giummarella *et al*. 2019). HMBC NMR is valuable for visualizing quaternary and carbonyl carbons and therefore connecting spin systems for carboniferous biomass samples. 2D NMR, specifically to detect two heterogeneous nuclei, is seen as semi-

quantitative due to different coupling constants in excited nuclei, off-resonance effects (pulse not applied at Lamour frequency), relaxation effects of both T_1 and T_2 and the effect of proton coupling (Foston *et al*. 2016). Using internal standards similar to the compounds of interest makes 2D NMR a quantitative study (Heikkinen *et al*. 2003).

A third dimension can be added to alleviate further signal overlapping in the second dimension. A third-dimensional (3D) NMR experiment will separate spin systems from one nucleus in the second dimension with a second nucleus which cross sections this 2D spectrum in the third dimension (Schirra 1996; Liitiä *et al*. 2003; Ralph and Lu 2004). For instance, the most common 3D NMR study in biomass studies is a 3D HSQC-TOCSY (Total Correlation Spectroscopy) method. The ^{1}H-^{1}H (TOCSY) (xy-axes) spin systems are separated by a ^{13}C (z-axis) spectrum to create HSQC spectra (Schirra 1996; Liitiä *et al*. 2003; Ralph and Lu 2004). This allows individual analysis of specific spectra sections to be analyzed for enhanced characterizability (Schirra 1996; Liitiä *et al*. 2003); however, 3D NMR studies are time-consuming and require a detailed experimental plan. While the multi-dimensional techniques mentioned are for solution-state NMR studies, a variety of techniques also exist for solid-state NMR studies. 2D ^{13}C Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE) (^{13}C-^{13}C) and 2D (^{1}H-^{13}C) heteronuclear correlation (HETCOR) have been used in biomass studies (Foston 2014; Le Brech *et al*. 2015; Wang and Hong 2016). 2D HETCOR NMR was used as a high-field technique for intact biomass and biochar to provide high-resolution spectra (Le Brech *et al*. 2015). Throughout this article, the pulse sequences HSQC and HETCOR will be used repeatedly. Both are 2D techniques that show peaks for protons attached to carbons, with HSQC generally being the more sensitive technique, but for some systems the NMR hardware limitations may require that HETCOR be the method of choice.

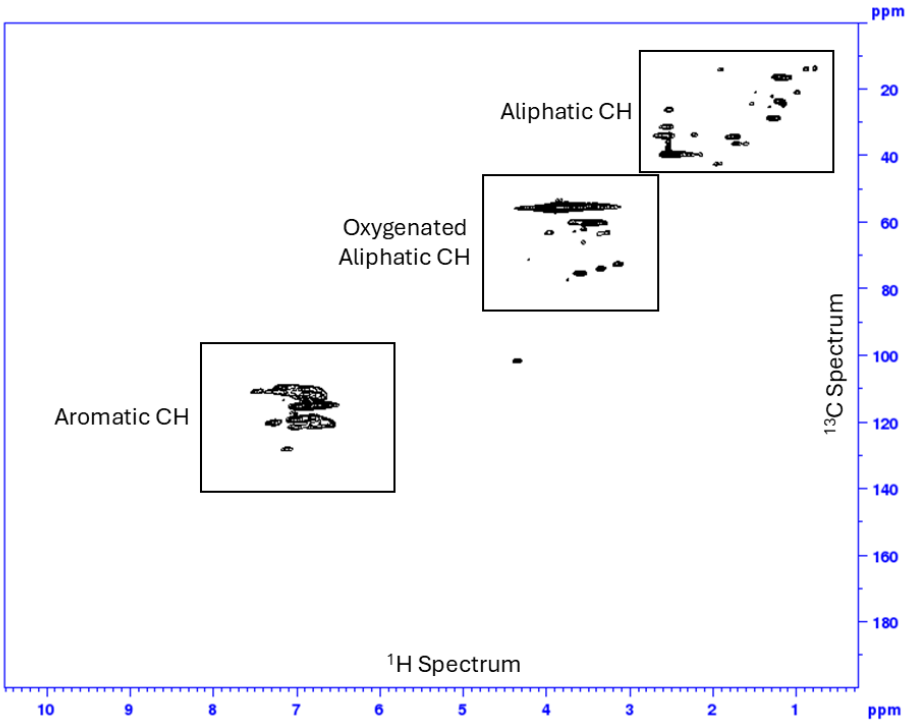


Fig. 4. 2D HSQC spectrum of pine kraft lignin. The x-axis is a 1D ^{1}H spectrum and the y-axis is a 1D ^{13}C spectrum. This allows for deconvolution of signals that overlap at the same chemical shift in 1D spectra by the addition of the second dimension. The boxed-in regions represent clusters of the lignin chemical structure.

While the above solution and solid-state NMR techniques are typically used in biomass research, many creative NMR methods exist and have been used for more specialized questions. For example, a technique known as pure shift NMR will alleviate homo-nuclear couplings in a proton NMR spectrum and deconvolute the spectrum into single peaks. This technique has been used previously to investigate the hydrogenation of biomass-derived platform chemicals (Zhao *et al*. 2021). Time domain NMR has also been used to report solids content in kraft black liquor (Nikolskaya *et al*. 2019) and evaluate biomass pretreatments for enzyme saccharification (Bianquini *et al*. 2020). Time domain NMR works because the T_1 and T_2 relaxation times, calculated when a sample is pumped through a magnetic field, are correlated with the viscosity, molecular mass, or rigidness of the sample depending on the sample state (Dais and Spyros 2012; Nikolskaya *et al*. 2019; Bianquini *et al*. 2020). A diagram of a time-domain NMR system used to analyze solids content in spent pulping liquor is displayed in Fig. 5 (Nikolskaya *et al*. 2019). A commonly used pulse sequence in time-domain NMR is a Carr-Purcell-Meiboom-Gill (CPMG) sequence, which can accurately measure T_2 relaxation time. Fast-field cycling (FFC) NMR is another relaxometry NMR technique. FFC NMR relies on spin-lattice relaxation times (T_1) and external applied magnetic field variations to study complex biomass systems. A three-step pulse sequence consisting of polarization (intense, longitudinal magnetization is induced for a fixed period), relaxation (magnetization energy relaxes to equilibrium over a specific amount of time), and acquisition (^{1}H pulse used to make the magnetization energy observable and generates an FID signal) is used to measure variations in molecular relaxation and reveal information on molecular dynamics (Conte and Alonzo 2013).

Soil porosity, rock permeability, biomass transformations, and natural organic matter dynamics have been studied using FFC NMR, and the technique has the potential to measure the fate of chemical contaminants in polluted soils (Conte and Alonzo 2013). A diffusion-ordered spectroscopy (DOSY) experiment separates complex samples by their diffusion coefficients, closely related to the molecular weight. DOSY is a pseudo-2D NMR experiment that uses a compound's diffusion coefficient to separate compounds in a mixture on the y-axis of the spectrum, while any overlapping diffusion coefficients are separated with either a ^{1}H or TOCSY spectrum on the x-axis. Chemically modified biomass mixtures of platform chemicals provide an ideal sample for DOSY experiments because they can be quantified using a ^{1}H spectrum and separated *via* their diffusion rates to avoid signal overlapping that would occur if the mixture was just analyzed using ^{1}H NMR (Ge *et al*. 2016; Lyu *et al*. 2018). A representation of a DOSY NMR spectrum is shown in a subsequent section for a coniferyl alcohol experiment (Fig. 9). Examples of unique NMR techniques will continue to be utilized as biomass research evolves.

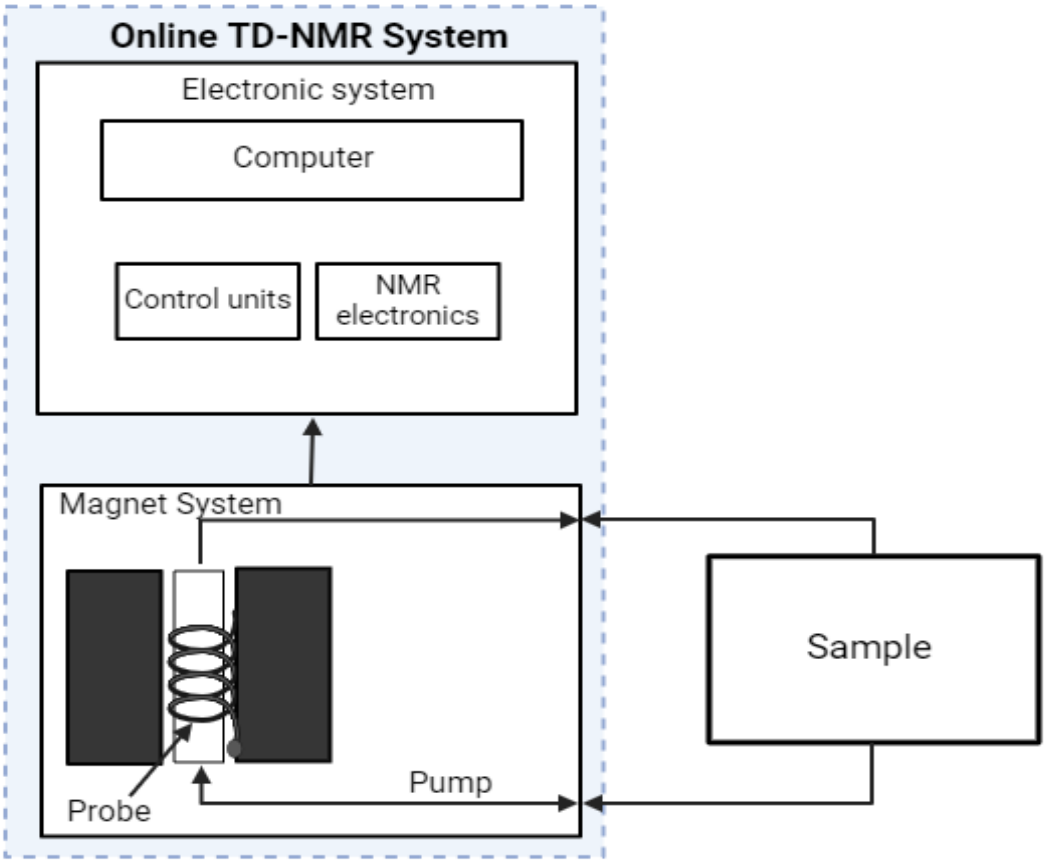


Fig. 5. Time domain NMR system adapted from Nikolskaya *et al*. (2019).

There are a multitude of NMR techniques that are used for biomass research, and some of their applications, advantages, and limitations are listed in Table 2.

Table 2. NMR Techniques that have been Used in Biomass Research and their Advantages and Limitations

NMR Technique	Application in Biomass Research	Advantages	Limitations
Solution-State 1D (^{1}H)	Provides chemical structural information of a compound of interest	-Quick experimental time -Highest sensitivity NMR analysis (Dais and Spyros 2012; Singh and Singh 2022)	-Significant signal overlap difficulties specifically in complex polymers or molecules (Rinaldi 2004; Ge *et al*. 2016; Hao *et al*. 2016; Giummarella *et al*. 2019)
Solution-State 1D (non-proton)	Provides chemical structural information of a compound of interest	-Small amount of time needed for an experiment depending on nucleus abundance (Rinaldi 2004; Singh and Singh 2022) -High sensitivity compared to multidimensional NMR analyses (Rinaldi 2004; Dais and Spyros 2012; Singh and Singh 2022)	-Signal overlap difficulties specifically in complex polymers or molecules (Rinaldi 2004; Hao *et al*. 2016; Ge *et al*. 2016; Giummarella *et al*. 2019;) -Higher experimental time compared to ^{1}H experiments
Solution-State 2D (COSY, HSQC, HMBC, *etc*.)	Provides chemical structural information of a compound of interest	-Provides significant resolution needed for biomass characterization (Rinaldi 2004; Balakshin *et al*. 2007; Dais and Spyros 2012; Hao *et al*. 2016; Giummarella *et al*. 2019) -Can tailor to the nuclei of interest (Rinaldi 2004)	-High NMR field needed for optimal spectral resolution (Giummarella *et al*. 2019) -Time-consuming measurements (Rinaldi 2004) -Lower sensitivity compared to 1D NMR (Rinaldi 2004; Marion 2013)
3D (HSQC-TOCSY)	Provides chemical structural information of a compound of interest	-Best resolution of all solution-state NMR techniques (Liitiä *et al*. 2003; Ralph and Lu 2004) -Identification of minute structural detail (Liitiä *et al*. 2003; Ralph and Lu 2004; Rinaldi 2004)	-Time-consuming measurements (more than 2D NMR) (Rinaldi 2004) -Lower sensitivity compared to 1D and 2D NMR (Rinaldi 2004)
Solid-State 1D (^{13}C CP/MAS)	Provides chemical structural information of a compound of interest	-Structural information in the native state (Foston 2014; Poulhazan *et al*. 2020)	-Complex spectra to interpret with wide peak widths (Dais and Spyros 2012; Reuhs and Simsek 2017; Poulhazan *et al*. 2020)
Solid-State 2D (Hetcor, J-inadequate, *etc*.)	Provides chemical structural information of a compound of interest	-Structural information in the native state (Foston 2014; Poulhazan *et al*. 2020)	-Significant signal overlap (Poulhazan *et al*. 2020)
Diffusion Ordered Spectroscopy (DOSY)	Separates chemicals in a mixture via their diffusion coefficient, and quantifies each specific chemical structure in said mixture (Ge *et al*. 2016)	-Able to quantify components in a mixture (Ge *et al*. 2016; Lyu *et al*. 2018; Harvey *et al*. 2023) -Allows for molecular size and shape information (Ge *et al*. 2016; Lyu *et al*. 2018)	-Overlapping signals in components with similar diffusion coefficients (Lyu *et al*. 2018; Harvey *et al*. 2023) -Limited applications (used for mixtures) (Ge *et al*. 2016; Lyu *et al*. 2018; Harvey *et al*. 2023)
Relaxometry Techniques (Time-domain, FFC, *etc*.)	Provides insights into relaxation behavior of a sample's nuclei and therefore can infer specific physical and chemical properties of the sample (Dais and Spyros 2012; Conte and Alonzo 2013; Nikolskaya *et al*. 2019)	-Ability to correlate the spectra to physical properties of a sample (Dais and Spyros 2012; Nikolskaya *et al*. 2019) -Able to gain useful molecular dynamics information (Conte and Alonzo 2013; Foston 2014; Nikolskaya *et al*. 2019)	-Not able to quantify chemical structure (Nikolskaya *et al*. 2019) -Need to set up an NMR system/study (Nikolskaya *et al*. 2019)

Comparison of NMR to Other Analytical Techniques

Biomass research has been evolving alongside NMR. As biomass researchers have breakthroughs in their research, NMR can effectively characterize new compounds and polymers of interest. Other analytical techniques exist to chemically characterize biomass samples, however. These techniques and their inherent advantages and disadvantages are listed in Table 3.

Table 3. Comparison between Commonly Used Analytical Techniques for Chemical Characterization in Biomass Research

	NMR	Gas or Liquid Chromatography Mass Spectrometry	Infrared (FT-IR)
Advantages	-Can provide information about the entire sample. -Non-destructive -Inherently quantitative: signal intensity is proportional to the number of nuclei, so no analyte-specific calibration curve is needed (with proper acquisition parameters, e.g., adequate relaxation delay) -Many 1D, 2D, and 3D techniques -Automated instruments -Can measure kinetics and diffusion -Can interrogate local environments -Can show the stereochemistry of functional groups -Shows connectivity -Compatible with solid and liquid samples	-Highly sensitive -Quantitative with very low detection limits; requires calibration with standards for each analyte -Automated instruments -Less expensive than NMR -Short experimental time	-Short experimental time -Functional group determination -Inexpensive -Semi-quantitative when calibrated (e.g., chemometric/PLS models) -Less upkeep -Spectra easier to interpret than NMR -Compatible with solid and liquid samples -Automated instruments -Non-destructive -Easy sample preparation
Disadvantages	-Expensive -Complexity: expertise is needed for choosing the proper experiment and interpreting the results -Extensive sample preparation -Low sensitivity -Long experimental time -Costly upkeep: need someone knowledgeable to maintain and calibrate the instrument to work properly -Quantitation depends on the experiment (^{1}H and quantitative ^{13}C are reliable; standard 2D HSQC is only semi-quantitative)	-Expensive -For Gas Chromatography, the sample needs to be volatile -Sample destruction -Cannot determine connectivity, local environments, stereochemistry, and functional groups -Cannot measure kinetics or diffusion -Extensive sample preparation -Response factors and matrix effects complicate quantification; thermally labile compounds may degrade (GC)	-Limited structural information -Spectral overlap -Limited techniques available -Cannot determine connectivity, local environments, or stereochemistry -Quantification requires calibration against a primary method (often NMR or wet chemistry); absorptivities differ among functional groups -Will not provide details on all the sample depending on how much sample is being analyzed

Workflow of an NMR Experiment

When designing an NMR experiment, sample preparation, desired results, and knowledge of which NMR techniques will provide those desired results are important to consider. For a solution-state experiment, the solvent and concentration are determined by the strength of the external magnet, the probe type, and the nucleus of interest. As the strength of the external magnet increases, the strength of NMR signals improves, and, as a

result, less sample is needed (Reuhs and Simsek 2017). The same is true for the probe type. For example, if a cryoprobe is used, thermal noise is reduced, and less sample is required for analysis (Ralph and Lu 2004). Probe selection in an NMR experiment is important depending on the nucleus of interest, the concentration of the sample, and whether solid-state or solution-state NMR is used. Finally, certain nuclei are more abundant than others. For example, hydrogen has a natural abundance of 99.9%, whereas ^{13}C has a natural abundance of 1% (Gerothanassis *et al*. 2002). Samples need to be more concentrated when doing ^{13}C NMR to account for low natural abundance and improve the sensitivity of NMR signals. The solvent used in NMR biomass experiments should be deuterated to minimize the solvent peak in the NMR spectrum. Dissolution of biomass (specifically cellulose) can be challenging due to inter- and intra-molecular hydrogen bonding (Foston *et al*. 2016). Gelation is known to occur even when using solvent systems capable of dissolving lignocellulose (Foston *et al*. 2016). Because biomass samples do not have an inherent concentration for NMR experiments, experiments in this review will have details on the amount of sample and the solvent used in dissolution whenever possible. Finally, a proton NMR spectrum is recommended before running complex NMR experiments. This is to check the shimming and tuning and ensure the expected NMR signals are present. After all the NMR experiments have concluded, it is often useful to run a final proton NMR experiment to ensure that the NMR instrument performance did not change and that the sample has not changed or precipitated during the run. Since many experiments can be easily run on automated instruments it is important that the experiments are well understood so the data isn't misinterpreted. Consulting with an NMR expert is always a wise decision before setting up a study to make sure all the parameters are optimized for your sample. Additionally, selecting experiments that will give the desired results is important and will dictate how a sample is prepared, the amount of time needed for experimentation, and the amount of work needed to interpret the spectra. This review acts as a guide for biomass research to make informed decisions when constructing an NMR study. All the recommendations outlined here come from the authors' institutional knowledge and personal experiences.

Spectroscopic Analysis of Coniferyl Alcohol

To illustrate how NMR can be used to chemically characterize an unknown biomass substance, impure coniferyl alcohol, which had been stored on a shelf for an unknown amount of time, and had undergone unknown chemical reactions, was dissolved in DMSO at ~ 2.0 mg/mL, and several NMR experiments were conducted. The sample had undergone a color change during its time in storage from white to brown-colored solid. Therefore, it was theorized that the substance had self-polymerized in an unknown manner. The changes from a pure coniferyl alcohol substance to the unknown sample could be visualized using NMR. It was hypothesized that coniferyl alcohol reacted to form dimers, trimers, and oligomers, similarly to how lignin naturally polymerizes. For this study, 1D ^{1}H and ^{13}C NMR experiments, 2D HSQC and HMBC experiments, and a DOSY experiment were used as initial techniques to visualize the reactions that occurred to the pure coniferyl alcohol when it was stored. Figures 6, 7, 8, and 9 show these spectra of impure coniferyl alcohol and relevant assignments. Assignments were possible due to the work from Ralph *et al*. (2024), as they provide a useful database for ^{13}C and ^{1}H assignments for specific compounds found in lignin NMR spectra.

Assignments are comparable to those of fully polymerized lignin when analyzing the 1D NMR spectra. An example of the similarities can be visualized when comparing

kraft lignin ^{13}C spectra (shown in Fig. 3) to the impure coniferyl alcohol sample (Fig. 6). Specifically, both spectra contain assignments for methoxy functional groups, an aliphatic carbon spectral region, and an aromatic carbon spectral region can be identified in both the coniferyl alcohol sample and the kraft lignin sample. However, a few discrepancies were present when comparing the spectra to a kraft lignin spectrum or even native lignin spectrum. Specifically, the assignments between δ_H 9.5-10.5 ppm in the ^1H spectrum, and δ_C 200-190 ppm in the ^{13}C spectrum correspond with aldehydes. Native lignin polymers do not react in a way that leads to ketone or aldehyde groups; therefore, an unexpected reaction that deviated from native lignin polymerization occurred. Additionally, pure coniferyl alcohol is a $C_{10}H_{12}O_3$ structure, meaning there should be assignments for ten carbons in the ^{13}C spectrum, and 12 hydrogens in the ^1H structure, although more peaks (doublets, triplets, *etc*.) will be visible on a single hydrogen due to peak splitting by spin-spin coupling from non-equivalent neighboring hydrogen atoms. However, the impure coniferyl alcohol ^{13}C spectrum has over 113 carbon atoms, meaning a significant number of reactions occurred in this sample. Aromatic and aliphatic groups can be roughly described based on certain regions of the 1D spectra. Methoxy groups are easy to identify, specifically in the ^{13}C spectrum, as there is a large peak at δ_C 58 ppm. However, a more detailed analysis is difficult given that the nature of the polymer was unknown. Without any knowledge of the reactions and subsequent products of a complex polymer, it can be extraordinarily difficult to deduce exactly what product was produced. Therefore, NMR does come with limitations if the nature of the system being analyzed is not well understood.

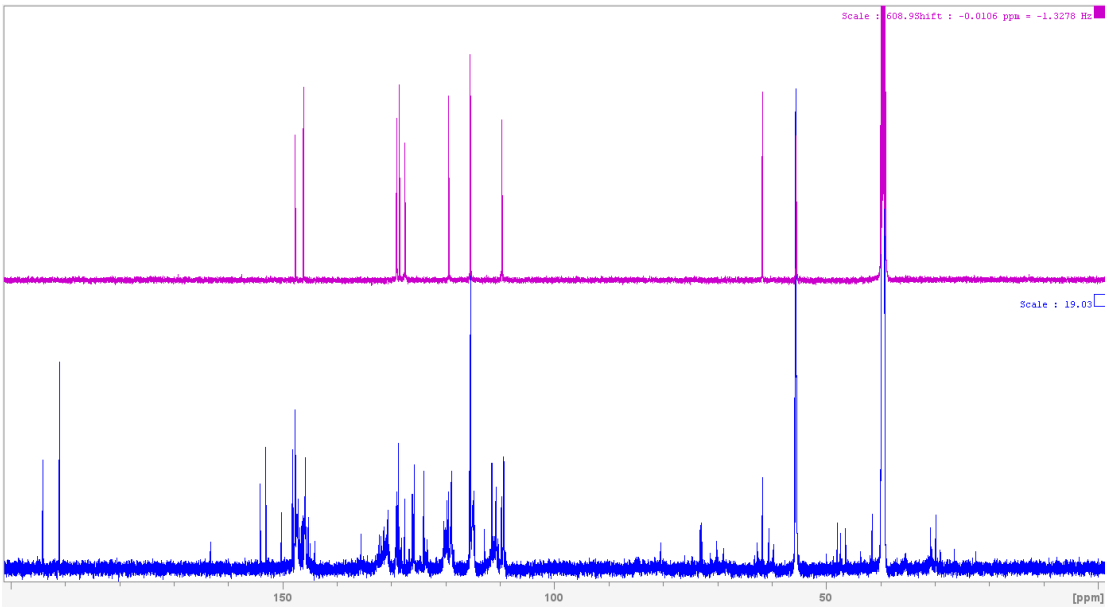


Fig. 6. ^{13}C NMR spectra for pure coniferyl alcohol (shown in purple) compared to the unknown biomass structure (shown in blue). The 10 peaks shown in the pure coniferyl alcohol spectrum represent the 10 carbons present in a coniferyl alcohol compound. Over 100 singular peaks were assigned in the unknown biomass sample. The large peak near 40 ppm in both spectra is from the DMSO solvent. Pure coniferyl alcohol spectrum was accessed from the *bmrb.io* website.

The NMR techniques 2D HSQC and HMBC show chemical connectivity in the unknown biomass structures, and when combined, the chemical structure can begin to be characterized to a further extent. Exact carbon-hydrogen assignments would need to be compared to other literature sources of similar compounds or polymers. The most comparable spectrum in this work is Fig. 7, a 2D multiplicity edited HSQC spectrum of softwood kraft lignin. The aromatic, oxygenated aliphatic, and unoxygenated aliphatic regions of both spectra have similar chemical shifts. The aromatic regions of both spectra are present between δ_H ~8-5.5 ppm and δ_C ~100-140 ppm. Oxygenated aliphatic groups were present between δ_H ~5.5-2.5 ppm and δ_C ~90-50 ppm. Finally, unoxygenated aliphatic groups were contained between δ_H ~3.5-0.5 ppm and δ_C ~50-10 ppm. Unlike softwood kraft lignin, the impure coniferyl alcohol sample chemical shift assignments were present during the literature review process. Therefore, only some specific signals can be definitively assigned when attempting to assign them through other studies in literature. Specifically, the C2, C5, C6 carbons, the alpha carbon on a 5-5 linkage, and methoxy functional groups are part of the aromatic and oxygenated aliphatic regions of the spectra. The aldehyde group is also visible in the 2D HSQC spectrum between δ_H 10 and δ_C 20-30 ppm, signifying that this signal is most likely from a proton on the aldehyde functional group. This signal would appear between δ_C 191-194 ppm if the spectrum were run with a wider carbon range, and instead, folded over to show up between δ_C 20-30 ppm. The HMBC spectrum (Fig. 8), when overlaid with the HSQC spectrum, shows correlations between signals from the HSQC spectrum to long-range proton-carbon bonds *via* the HMBC signals. Therefore, it is possible to deduce the connectivity of the biomass system. While a full picture of the system can be theoretically mapped out, a knowledge of the initial sample would allow for cross-reference to the literature to fully understand the specimen. For this specific sample, it was difficult to cross-reference peaks in these spectra to spectra of other biomass samples, besides certain peaks correlating with regional connectivity. For example, it is possible to distinguish that the aldehyde C- has long-range correlations with aromatic C-H signals. However, to parse out the entire system without knowledge of the products of this system would be a futile effort. This is a downside to analyzing unknown biomass samples using NMR; however, regional chemical assignments can still be undertaken for similar samples.

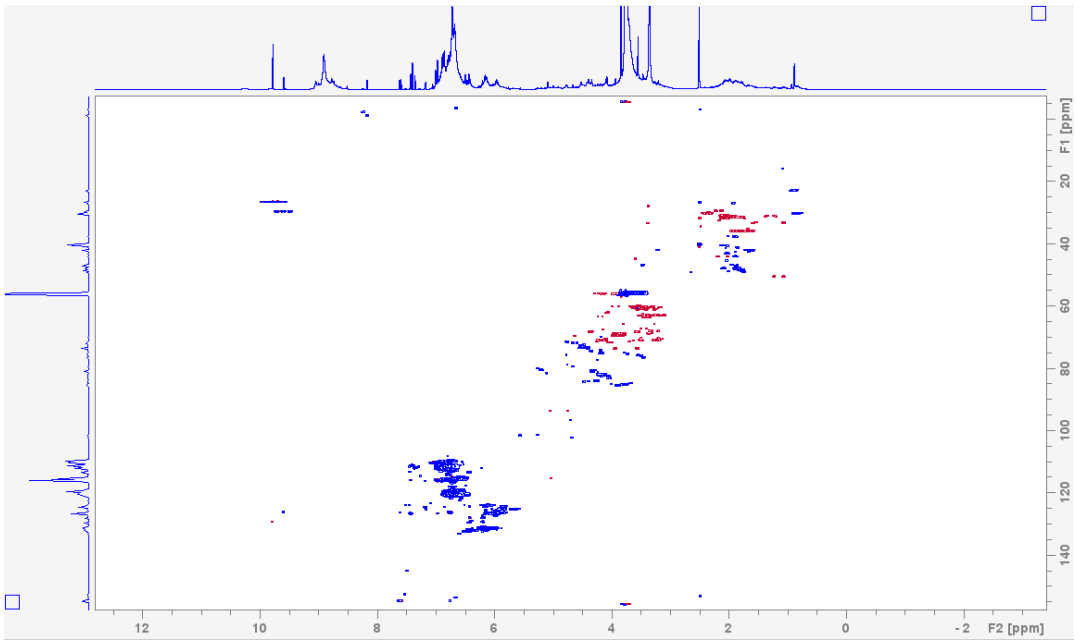


Fig. 7. 2D multiplicity edited HSQC NMR spectrum of the unknown coniferyl alcohol substance used in these experiments. The red signal indicates CH_2 groups, while the blue signal equates to CH and CH_3 groups. The spectrum along the top is the trace of the ^{1}H spectrum, while the vertical spectrum is a trace of the ^{13}C spectrum. Each signal represents a carbon attached to a proton. The spectrum can be divided into three major sections: unoxygenated aliphatic groups (δ_H ~3.5-0.5 and δ_C ~50-10 ppm), oxygenated aliphatic groups (δ_H ~5.5-2.5 and δ_C ~90-50 ppm), and aromatic groups (δ_H ~8-5.5 and δ_C ~100-140 ppm). The number of signals reveals the complex nature of the polymerized coniferyl alcohol sample and provides a simplified model of lignin.

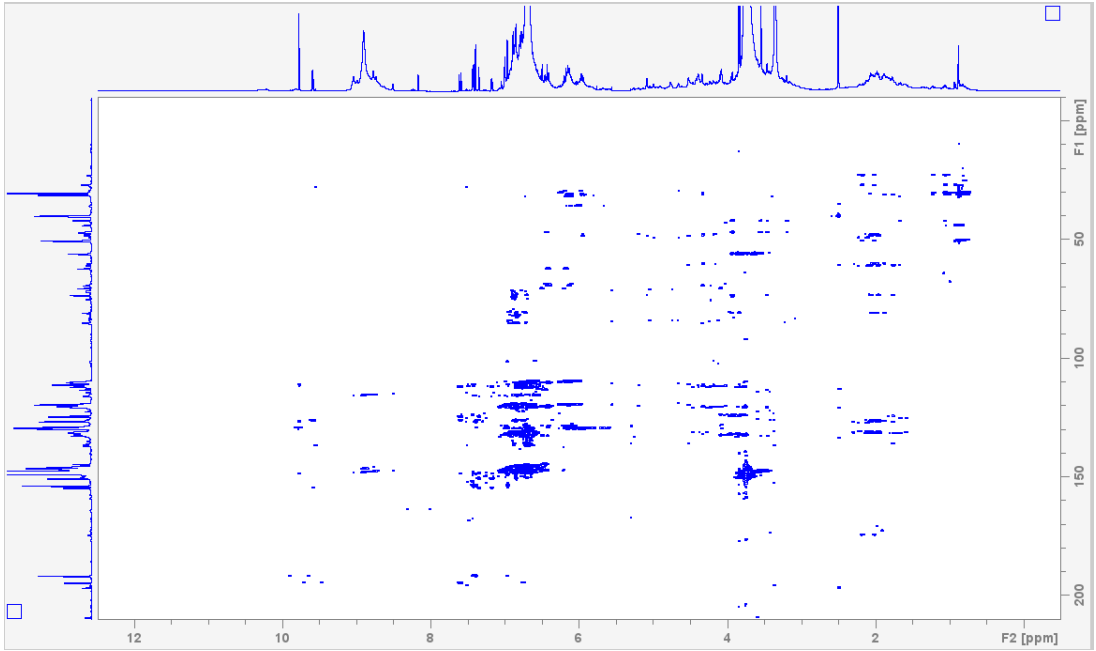


Fig. 8. 2D HMBC NMR spectrum of the unknown coniferyl alcohol substance used in these experiments. The spectrum along the top is the trace of the ^{1}H spectrum, while the vertical spectrum is a trace of the ^{13}C spectrum. The 2D HMBC NMR spectrum shows long-range correlation between ^{1}H and ^{13}C spectra, which are separated by two, three, and at times, four bonds away.

The hypothesis for what happened to this unknown sample was that it polymerized into a lignin-like substance, forming shorter-chain oligomers, a DOSY NMR technique was employed to separate larger polymers from shorter monomers, dimers, and trimers based on their respective diffusion coefficients. The results are detailed in Fig. 9. This spectrum is included to demonstrate the complexity of the unknown sample.

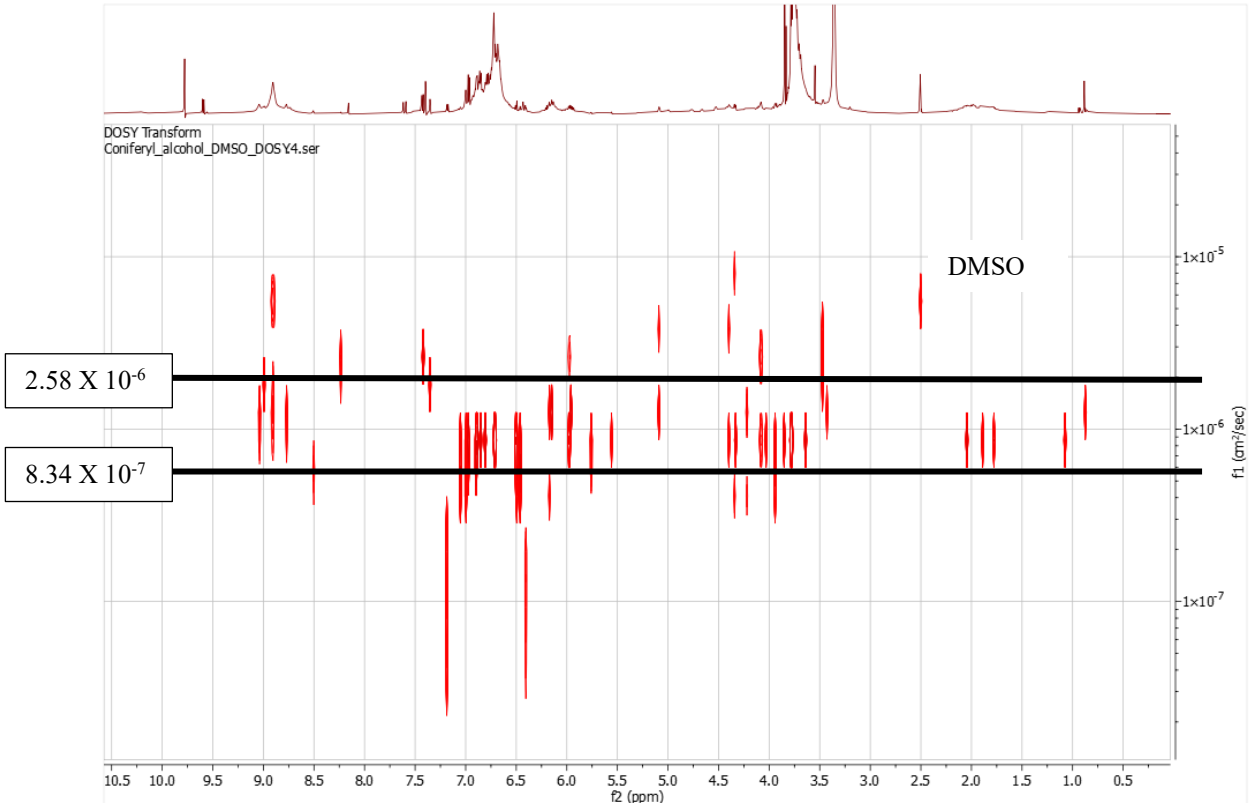


Fig. 9. DOSY spectrum of the unknown coniferyl alcohol sample. The DOSY spectrum consists of the ^{1}H spectrum on the x-axis and each component's diffusion coefficient on the y-axis. Two diffusion coefficients that correspond to a number of peaks are defined on the spectrum. DMSO is used as a reference compound with an estimated molecular weight of 78 g/mol.

The diffusion coefficient in the DOSY spectrum correlates directly with the molecular weight of each species in this spectrum. In fact, a DOSY spectrum can be used as an alternative to gel permeation chromatography (GPC) to estimate the molar mass and polydispersity of biomass, such as lignin, and, in this case, an unknown polymerized coniferyl alcohol sample. A signal that is lower on the diffusion coefficient axis (y-axis) corresponds with higher molecular weight compounds and polymers. As can be seen, a variety of molecular weight compounds and polymers are present. Any compound with a similar diffusion coefficient corresponds to a specific polymer or compound. Therefore, it is possible to identify the chemical composition of each species given the alignment in the diffusion spectrum, which is separated into the ^{1}H dimension.

Various limitations exist when analyzing this spectrum. For analytical accuracy, DOSY requires molecules and polymers to be spherical, as any other shape can yield abnormal diffusion characteristics that require a correction factor to obtain a more accurate diffusion coefficient. While this works for compounds such as DMSO, DOSY accuracy for lignin-like polymers can be affected by their non-spherical shape and tendency to aggregate in solution. Additionally, unlike some other NMR techniques, DOSY requires calibration

with standard polymers, such as polystyrene, to establish a Mark-Houwink-Sakurada (MHS) relationship, an equation that relates the intrinsic viscosity of a polymer to its molecular weight to characterize its branching and rigidity.

While the unknown coniferyl alcohol sample was difficult to analyze due to its unknown nature, NMR can provide valuable insight into what occurred while this sample sat on a laboratory benchtop. Specifically, it was theorized that, because of the sample's brown hue, some degree of polymerization occurred. The severity of this polymerization can be visualized in the ^{13}C spectrum, as evidenced by the total peak count compared to a pure coniferyl alcohol sample. Additionally, the exact chemical structures of the polymerized coniferyl alcohol were investigated using 2D HSQC and HMBC spectra. Finally, the extent of polymerization and the chemical structures of the various polymers can be visualized using DOSY spectra. This highlights the potential of NMR for use with other biomass systems through a realistic experiment.

Previous Biomass NMR Studies: Whole Biomass Composition and NMR

Biomass can be studied holistically, as isolated components, or as transformed value-added products *via* NMR spectroscopy. Whole biomass cell walls have been extensively studied using NMR in solid and solution states. Atalla and Vanderhart (1999) pioneered a novel method for using solid-state NMR to quantify cellulose in biomass (Atalla 1984). Hallac *et al*. (2009) used solid-state ^{13}C CP/MAS NMR to study *Buddleja davidii* (summer lilac) biomass to determine cellulose crystallinity, S/G ratio in its lignin, and degree of lignin condensation. This study used a Bruker Avance-400 spectrometer operating at 100.59 MHz for ^{13}C NMR. Their sample was packed into a 4 mm ZrO_2 rotor, spun at 8 kHz, and scanned 8129 times. More recently, Foston (2014) reviewed progress in solid-state NMR for whole biomass systems. The author discussed structures that can be elucidated and strategies for analysis acquisition, showing how NMR can be a powerful tool for understanding the chemical structure of intact biomass and how it can be dissolved in solvent systems and analyzed for its chemical composition. Fu *et al*. (2015) isolated lignin from lignocellulosic biomass using ionic liquid and quantified the cellulose/hemicellulose component from the lignin component in biomass using ^{13}C CP/MAS NMR. The parameters for their NMR experiments include using a 600 MHz Bruker Advance III spectrometer equipped with a 4 mm HXY solid-state MAS probe. A spin rate of 12.5 KHz, acquisition time of 0.02 seconds, a temperature of 278 K, and 4096 scans were used to acquire their spectra. Multi-dimensional (2D and 3D) solid-state NMR techniques (HETCOR and MAS) provide more resolution to cell wall polymers with non-destructive methods (Le Brech *et al*. 2015; Wang and Hong 2016). Yoo *et al*. (2016) used a combination of DMSO-d_6 and HMPA-d_{18} (4:1, v/v) to dissolve biomass directly without a time-consuming isolation procedure. This procedure provided a feasible route for low-field NMR quantification using a room temperature probe instead of a cryoprobe and did not discuss biomass concentration in the solvent system. Ralph and Lu (2004) used 3D NMR of solubilized ball-milled pine cell walls in DMSO/NMI and DMSO/TBAF to reveal details of the lignin structure. This study used a 500 MHz Bruker DMX-500 equipped with an inverse gradient $^{1}H/^{13}C/^{15}N$ cryoprobe. Finally, Kim and Ralph (2010) dissolved cell wall gels in a mixed DMSO-d_6 and pyridine-d_5 solvent system for 2D HSQC NMR spectroscopy. A cryoprobe was used in order to obtain good spectral resolution in less experimental time. However, this method was unsuitable for long-range experiments, like HMBC, for example, due to the rapid relaxation of nuclei, which loses the magnetization required for long-range experiments. The consideration when studying whole biomass in

the solution-state is that the solvent system implemented needs to maintain the structural integrity of the plant cell walls. This requires a solvent system capable of dissolving cellulose, hemicellulose, and lignin. This task can be challenging and requires a substantial amount of mechanical treatment (for example, ball-milling) to completely dissolve the biomass into the solvent system (Kim and Ralph 2010). Whole biomass used in solid-state NMR still needs to be milled, although not to the same extent (Hallac *et al*. 2009).

Algae

Algae represent a critical natural carbon sink, with the potential to be used as a biofuel source as it can be readily grown in a bioreactor and has a relatively high energy content (Akhter *et al*. 2016). Its chemical and physical composition is necessary for its downfield utilization, and therefore, its characterization is paramount. Additionally, the plethora of algae species with different properties means that certain algal compositions are targeted for optimal biofuel production. A large collection of plant tissues and microbes, including algae, was detailed by Bonanomi *et al*. (2024). Specifically, solid-state ^{13}C CP/MAS NMR broadly characterized specific carbon-containing functional groups, which allowed for the comparison of chemical compositions between species. Microalgal membranes are comprised of lipids, proteins, carbohydrates, phytosterols, and pigments such as chlorophyll (Poulhazan *et al*. 2020). Algal biofuel production is correlated with the lipid and carbohydrate content and growth rate (Akhter *et al*. 2016; Ning *et al*. 2020).

These two factors will be parameters in genetic modification studies, and specifically, lipid content can be monitored with analytical techniques such as NMR. Ning *et al*. (2020) utilized a technique known as Comprehensive Multiphase (CMP)-NMR to study subcritical water extraction of two algae (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*) and two cyanobacteria species (*Synechocystis* and *Spirulina*) with varying reaction temperatures and with and without a basic catalyst. CMP-NMR allows the NMR user to study the components, phases, and interfaces in an unaltered solid-state sample.

CMP-NMR was used in this study to evaluate three components of these cyanobacteria species after a subcritical water extraction was applied: the liquid extract, the desired swollen components, and solid components that could not be extracted. About 10 mg of the extraction products and 30 μL of D_2O were added to the dry sample in the rotor. A 500 MHz Bruker Avance III spectrometer with a 4 mm C-H-N-D CMP probe spun at 6666 Hz was used in this study. Results provide a clear picture of the percentage and composition of components extracted in each species. Of the two algae species analyzed, *Chlamydomonas* showed better overall extractability, but the *Chlorella vulgaris* NMR spectrum indicated higher carbohydrate extractability compared to the former algae species, which could be more desirable for bioethanol production (Ning *et al*. 2020). Solution and solid-state NMR techniques have been used to study various algae species' chemical compositions (Akhter *et al*. 2016; Poulhazan *et al*. 2020). Solution-state NMR studies utilizing ^{13}C labeling have the capability of building a metabolic profile of various algae species as well as analyzing the protons by combining water suppression with 1D and 2D NMR techniques (Akhter *et al*. 2016).

Solid-state NMR techniques focus on the architecture of whole cells and have probed ^{13}C and ^{15}N nuclei. Similar to solution-state algae NMR studies, ^{13}C and ^{15}N labelling is necessary for spectral resolution. Multiple dimensions may be used in isotopically labeled algae solid-state NMR studies and have been successfully done so for studying biological membranes *in situ* or in whole cells with enhanced spectral resolution (Poulhazan *et al*. 2020). As algae becomes a predominant biofuel feedstock and more

species are bred or cultivated, NMR will be able to quantify its composition so we can predict how it will perform.

Studies of Thermochemical Conversion

The creation of value-added products from biomass is critical if society is to create a sustainable bioeconomy and combat global climate change. Two major pathways exist for biomass conservation to value-added chemicals: enzymatic and thermochemical conversion. At the time of writing, the thermochemical route is the most used method.

Platform chemicals

Biomass can transform into platform chemicals and various end products depending on the feedstock, chemistry applied, and isolated biomass components. Platform chemicals can generally be defined as an array of simple chemical constituents capable of being used as a building block for industrially relevant secondary chemical products. Platform chemical studies use NMR as a technique to detect desirable products and the purity of the chemical being isolated (Yoshida *et al*. 2008; Wang *et al*. 2013; Singh *et al*. 2015; Ge *et al*. 2016; Li *et al*. 2018; Lyu *et al*. 2018). Pretreatment techniques may also be evaluated, and the effectiveness of certain parameters (ionic liquid treatment, temperature, *etc*.) on platform chemical yield in these systems can be quantified using NMR techniques (Sievers *et al*. 2009; Hossain *et al*. 2019). Singh *et al*. (2015) used ball-milled corn stover as a feedstock to test various pretreatment techniques and analyze the chemical composition using 2D HSQC NMR. Around 50 mg of ball-milled samples of corn stover were dissolved in 600 µL of DMSO-d_6 and analyzed using a Bruker Avance 600 MHz instrument equipped with a 5 mm inverse-gradient $^{1}H/^{13}C$ cryoprobe. DMSO was used as an internal standard reference peak. Carbohydrate and lignin moieties were identified and compared in each pretreated sample, and the lignin S/G ratio was obtained. Li *et al*. (2018) studied 54 commonly associated compounds in biomass pretreatment using ^{31}P NMR. The authors used 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) to phosphitylate components of interest, and the determined chemical shifts were assigned and displayed. Each compound was added at 0.02 mmol into 0.5 mL of solvent. Each phosphitylated compound could be dissolved in a pyridine/$CDCl_3$ solvent system. Meng *et al*. (2013) evaluated cellulose pore size and accessibility by pretreatment methods (acid hydrolysis and steam explosion) and determined the molecular mobility using various NMR relaxometry techniques such as NMR cryoporometry, NMR relaxometry, and NMR diffusion experiments.

These techniques analyze the pore size distribution in a porous medium and take advantage of melting point differences between liquid in pores compared to in the bulk, monitoring the nuclear relaxation of adsorbed surface water and the diffusion coefficient, respectively. For these techniques, low-field 300 and 400 MHz spectrometers were used. Ge *et al*. (2016) utilized DOSY NMR spectra to separate and quantify lignocellulosic biomass into cellulose, hemicellulose, and lignin constituents. Structures associated with cellulose, hemicellulose, and lignin were all assigned *via* this technique. These studies provide valuable information on the pretreatment process that can convert biomass into platform chemicals in an efficient manner. A Bruker AV-III 400 MHz NMR spectrometer equipped with a 5 mm PABBO BB/^{19}F-^{1}H/D probe was used, and a 1% w/w concentration for each compound was prepared in 400 µL of deuterated solvents. Chen *et al*. (2017) prepared bioplastics using esterified lignocellulosic biomass. ^{1}H NMR and 2D HSQC analyses using a 600 MHz Bruker Advance III spectrometer determined the reactivity of

polysaccharides towards homogeneous transesterification. These combined techniques confirmed a carbene transesterification reaction mechanism between cellulose and vinyl esters.

Pyrolysis

Biomass pyrolysis subjects a substance to a non-oxygenated environment at high temperatures. NMR has been used to quantify pyrolyzed structures. Torrefaction is a soft-pyrolysis technique that uses relatively low pyrolysis temperatures (200-300 ºC) to produce a combustible biofuel. Melkior *et al*. (2012), Park *et al*. (2013), and Mafu *et al*. (2016) each performed solid-state NMR analyses of torrefied biomass. Each study used ^{13}C CP-MAS solid-state NMR experiments to elucidate the aromaticity of treated biomass as torrefaction progressed to enhanced temperatures and times. At higher temperatures and shorter residence times, the pyrolysis process can thermochemically produce solid (biochar), liquid (bio-oil), and gaseous (syngas) products, which can be used in the production of biofuels (Michailof *et al*. 2016). NMR is useful in determining the chemical structures of solid and liquid fast pyrolysis products.

Solid-state NMR is useful for characterizing unmodified biochar chemical structures. The chemical composition of biochar is an aromatic carbon structure that includes amorphous aromatic rings and crystalline condensed polycyclic hydrocarbon layers (Leng *et al*. 2019). In biochar made using temperatures between 300-700 ºC, the condensed benzene structure analyzed by solid-state NMR increased from 8 carbons to 76 carbons (Cao *et al*. 2013; Leng *et al*. 2019). Ben *et al*. (2017) studied the biochar of six different biomass products using solid-state ^{13}C CP/MAS NMR. A Bruker DSX 300 MHz NMR spectrometer was used with samples ground, packed into 7 mm ZrO_2 rotors, and spun at 4 kHz. Additionally, this solid-state technique was employed by Lv *et al*. (2024) to analyze coal samples with different volatile matter content on a dry and ash-free basis (V_{daf}). The authors were able to obtain an aromaticity percentage value, a key component in predicting the calorific value of coal, using ^{13}C CP/MAS NMR, which allows for the ability to characterize key temperature increments for pyrolysis, characteristic index, and tar quality. Results for all biomass types indicated more condensed aromatic structures in biochar produced at higher temperatures.

Biochar composition also depended on the composition of the original feedstock used to produce it. For example, biochar produced from pine bark had higher C-O aromatic and aliphatic groups and aliphatic C-C bonds, which the authors posited was due to higher lignin and tannin content in the pine bark. Link *et al*. (2008) pyrolyzed biochar of various non-wood and softwood species at various heating rates up to 800 ºC and characterized them *via* solid-state ^{13}C CP/MAS and ^{23}Na MAS NMR techniques with a Bruker Avance 300 MHz spectrometer. The heating rate did not affect carbon structure, and the aromaticity was similar to previously reported aromaticity values for biochar. Complications using ^{23}Na MAS NMR were due to increased sodium concentration, broad spectrum width, and relaxation behavior of the nucleus. Additionally, sodium content was analyzed because its presence can act as a catalyst in the pyrolysis and gasification process (Link *et al.* 2008). Le Brech *et al*. (2015) used high field 2D HETCOR solid-state NMR to chemically characterize biochar at a low pyrolysis temperature. The study effectively identified the mechanisms of aromatization of each biomass component during the pyrolysis process.

Typically, in 2D solid-state NMR experiments, it is advantageous to isotopically label samples with ^{13}C. However, the authors claimed that a high-field magn*et* alleviated the need for isotopic labeling. Falco *et al*. (2011) compared pretreated hydrothermal

carbons to pyrolytic carbons to evaluate the relative furan-to-arene ratio from equivalent feedstocks. Using a combination of solid-state ^{13}C NMR techniques such as CPMAS, Non-quaternary suppression (NQS), and single quantum double-quantum (SQ-DQ), the furan-to-arene ratio and relative composition of complex carboniferous structures were observed. NQS quantifies quaternary carbon atoms, and SQ-DQ allows crucial information to be extracted in complex systems with many interactive spin systems. Additionally, a Bruker Avance 300 MHz spectrometer equipped with 4mm zirconia rotors spun at 14 kHz was used in this study (Falco *et al.* 2011).

Bio-oil is a primary product from thermochemical transformation of biomass fast pyrolysis. Feedstocks used for fast pyrolysis are lignocellulose, sugars, and triglycerides (Staš *et al*. 2020). Compared to petroleum, bio-oil is comprised of more heteroatoms, such as nitrogen and oxygen (Croce *et al*. 2017). Studies using solution-state NMR have been used to quantify the chemical composition of bio-oil. 1D and 2D techniques have been used to quantify bio-oil composition with various nuclei and to optimize the pyrolysis process with optimal bio-oil composition for its utilization (Hao *et al*. 2016; Staš *et al*. 2020). ^{13}C, ^{19}F, and ^{31}P NMR were used to probe carbonyl, carboxylic acid, and phenol functional groups in the acquired bio-oils (Staš *et al*. 2020). ^{19}F NMR experiments specifically using treatment from 4-(trifluoromethyl)phenylhydrazine are excellent for analyzing carbonyl functional groups because the technique can determine aldehyde and ketone contents separately (Hao *et al*. 2016).

Bio-oil derived from lignin or cellulose yields different chemical compositions when it undergoes fast pyrolysis (Carrier *et al*. 2017; Staš *et al*. 2020). Carrier *et al*. (2017) used solution-state ^{13}C NMR to provide a quantitative distribution of functional groups in bio-oil derived from various ^{13}C-enriched feedstocks. Samples were dissolved in DMSO-d_6, and a hexamethyldisiloxane internal standard was added. Around 15-20 mg of their sample was added to 600 μL of the solvent and internal standard at different ratios depending on their sample. Chromium (III) acetylacetonate was used as a relaxation agent. Functional group content was related to the chemical composition of the feedstock, with methoxyl and hydroxyl carbon atoms being attributed to cellulose-derived bio-oil and secondary alkyl carbon atoms being prevalent in lignin and hemicellulose bio-oil derivatives. Other bio-oil-related NMR studies include bio-oil water content and aging effects (Michailof *et al*. 2016; Staš *et al*. 2020). Prestes *et al*. (2007) developed a low-field rapid detection method using ^{1}H NMR to determine the fatty acid profile of oilseeds.

Oils used in biodiesel production require a balance of saturated and unsaturated fatty acid moieties to control biodiesel's viscosity and stability, respectively. The authors noted that T_2 relaxation time has an inverse relationship with viscosity, and viscosity increases with carbon chain length in the triglycerides analyzed. Fatty acid content needs to meet a specification for biodiesel utilization; thus, this procedure offers a quick method to meet specific specifications. Specifically, in the European Union, biodiesel requires a maximum degree of unsaturation, maximum linolenic acid (three double bonds) content of 12%, and maximum polyunsaturated fatty acid (at least four double bonds) content of 1% (Prestes *et al*. 2007). Overall, NMR provides the blueprint for chemical quantification and qualification of biomass transformations.

Aerobic and anaerobic digestions

Aerobic (with access to oxygen) and anaerobic (without access to oxygen) digestions are biomass decomposition techniques that occur during municipal solid waste (MSW) processing. NMR can quantify individual components of the feedstock and the

subsequent chemical changes during aerobic and anaerobic digestions. De La Cruz *et al*. (2014) studied chemical changes in hardwood, softwood, and newspaper during anaerobic decomposition under multiple temperature conditions (mesophilic and thermophilic). Using 2D HSQC NMR, a 700 MHz Bruker Avance III spectrometer equipped with a QNP cryoprobe, and an internal standard DMSO-d_6 peak, chemical differences between anaerobically decomposed hardwood and softwood samples can be quantified. For example, glucose and xylose contents in hardwoods decreased in both temperature conditions, while no discernible change in sugar content occurred in the softwood sample (De La Cruz *et al*. 2014). No lignin depolymerization was observed, but a decrease in overall β-O-4, resinol, and phenylcoumaran content was discerned. The chemical composition of wheat straw during its decomposition was studied in a 12-month aerobic and anaerobic decomposition study by Gao *et al*. (2016). Differences in functional group composition were discerned in anaerobic and aerobic conditions at different timescales. Solid-state NMR analysis was performed using a 400 MHz Bruker Avance III spectrometer using ^{13}C CP-MAS, 4 mm sample rotors, and spun at 14 kHz. In ^{13}C cross-polarization/total sideband suppression (CP/TOSS) experiments, 5 kHz was used as a spinning speed. The CP/TOSS technique was employed to improve the sensitivity (CP) and spectral resolution from unwanted sidebands when the RF pulse interacts with neighboring spins (TOSS). In their spectra, aromatic C-O and C-C and COO/N-C=O functional group content increased in each condition, while O-alkyl C content decreased. Anaerobic and aerobic conditions accounted for differences in NCH and Alkyl C-content, with anaerobic conditions allowing for a larger percentage of these functional groups to be retained or recorded (Gao *et al*. 2016). Tambone *et al*. (2010) used a large sample quantity to study 23 samples of varying organic composition, including eight ingestates (initial substrate in anaerobic decomposition), eight digestates (solid-liquid by-product), three composts (aerobic decomposition), and four digested sludges (anaerobic digestion). The primary motivation of this study was to evaluate the fertilization ability and quality of organic matter. Solid-state CP MAS ^{13}C NMR, a 600 MHz Bruker AMX spectrometer, a 4 mm CP MAS probe, and a spinning speed of 10 kHz were used to reveal differences in organic matter chemical composition between digestates and ingestates, as well as differences concerning compost and digested sludges. Digestates also exhibited a high nutrient content (available nitrogen, phosphorus, and potassium), making them a good candidate for use as fertilizer (Tambone *et al*. 2010).

Cellulose and Hemicellulose Analysis Using NMR

Cellulose and hemicellulose have comparatively simple chemical structures compared to lignin; however, NMR can provide helpful analysis of these structures. Cellulose is the most abundant biopolymer on earth and is composed of repeating linear β-(1,4) D-glucosaspyranosyl units (Foston 2014). Hemicellulose is another biopolymer found in plants, comprised of C6 or C5 inter-linked sugar units (Foston 2014). Quantification of hemicellulose and cellulose in intact biomass has been discussed, but solution-state NMR can distinguish specific properties, such as crystallinity, moisture content, and differences in chemical structures, of biomass oligomers and polysaccharides. Large degree of polymerization (DP) makes cellulose polymers insoluble in most solvents, so solvent systems are needed for smaller DP cellulose oligomers (Hallac *et al*. 2009; Foston 2014; Sasaki *et al*. 2024; De Carvalho *et al*. 2025). To solubilize cellulose for solution-state NMR chemical quantification, ionic liquids (IL) and a deuterated solvent (usually DMSO) are typically used. For example, Fliri *et al*. (2023) used a

tetralkylphosphonium ionic liquid butylphosphonium acetate, diluted with deuterated DMSO, and Sasaki *et al*. (2024) used lithium chloride and deuterated DMSO to chemically dissolve cellulose. While butylphosphonium acetate and DMSO were able to dissolve larger cellulose polymers, LiCl and DMSO were only able to dissolve cellulose oligomers. Additional NMR techniques have been used to study cellulose and hemicellulose, too. Relaxometry NMR (probes differences in specific nuclei relaxation rates in T_1 and T_2) determined the moisture content in cellulose biomass (Foston 2014). A 2D wide-line separation (WISE) experiment, which separates protons' relaxation by transferring magnetization to adjacent carbon atoms, was performed on onion cellulose. This experiment determined that hydrated cellulose was solely on the surface of microfibrils (Foston 2014). Cellulose degradation methods have also been investigated. Walker *et al*. (2020) investigated biomass pretreatment techniques to allow cellulose accessibility for cellulase enzymes using solid-state ^{13}C CP/MAS NMR, a 500 MHz Bruker Avance III spectrometer, at a rotor speed of 4 kHz.

The authors additionally noted that 200 mg of the sample was packed into 4 mm rotors. NMR has also been used to distinguish between polysaccharide chemical structures for different biomass types. De Carvalho *et al*. (2025) compared β-glucans isolated from yeast and mushroom species. Polysaccharide samples were isolated, methylated, and ultimately dissolved in DMSO-d_6. 1D and 2D NMR were used initially to distinguish between polysaccharide linkage patterns and to predict the homogeneous nature of the cellulose structures, and DOSY served as a complementary technique capable of distinguishing differences in mixed β-glucan samples where branched glucan units were attached. Proton spin relaxation editing was used to differentiate similar chemical shifts between proton rotations in crystalline (cellulose) and amorphous (hemicellulose and lignin) regions of biomass. This technique proved viable for predicting sugar yields of enzymatically hydrolyzed sugars in residual cellulose. Hallac *et al*. (2009) used solid-state ^{13}C CP/MAS NMR to determine the crystallinity of *Buddleja davidii* cellulose, although other analytical methods, such as x-ray diffraction (XRD), can accomplish this as well (Kim *et al*. 2013).

Differences in pyrolysis products made from cellulose and hemicellulose feedstocks were analyzed *via* various NMR procedures (Melkior *et al*. 2012; Mafu *et al*. 2016; Michailof *et al*. 2016; Ben *et al*. 2017, 2019; Rosnow *et al*. 2017). Monosaccharide composition in *Eucalyptus globulus* black liquor sampled at different pulping times was quantified using 1D proton, 2D TOCSY, and HSQC NMR techniques. Polysaccharides with different degrees of polymerization were isolated using acid precipitation. 2D NMR allowed for a multitude of xylan derivatives and amylopectin, which was first reported in this study (Lisboa *et al*. 2005). Due to alternative methods to characterize cellulose and hemicellulose and the simplicity of their structural chemistry, NMR studies on specifically hemicellulose and cellulose are scarce and uninformative.

Lignin Chemical Composition Analysis using NMR

Of the components in biomass, lignin is the most complex and heterogeneous; therefore, creative ways to characterize lignin are needed to fully understand, quantify, and ultimately use lignin as a value-added product. Each lignin sample has a different chemical composition and is a prime candidate for analysis using solution or solid-state NMR. Lignin biosynthesis requires three lignin precursor compounds: *p*-coumaryl, coniferyl alcohol, and synapyl alcohol (Boerjan *et al*. 2003; Wen *et al*. 2013). Through the action of enzymes, these compounds are synthesized into *p*-hydroxyphenyl (H), guaiacyl (G), and

syringyl (S) units (Boerjan *et al*. 2003; Wen *et al*. 2013). These monolignol units are differentiated by the amount of methoxyl groups on the aromatic ring. Softwood lignin is comprised of only G units, hardwood lignin is comprised of S and G units, and non-wood species are comprised of all three lignin units (Boerjan *et al*. 2003; Lu *et al*. 2017). Lignin can be isolated using various feedstocks and methods. Lignin can be isolated using milling, enzymatic hydrolysis, precipitation from pulping liquor, organosolv, acidolysis and thioacidolysis, ionic liquid degradation, and as a pyrolysis byproduct (Zhu *et al*. 2015; Lu *et al*. 2017; Jardim *et al*. 2020). Chemical characterization of lignin isolated from these various methods has benefited from the analytical power of NMR.

NMR is able to quantify information regarding lignin functional groups and characteristics, including but not limited to hydroxyl content (Argyropoulos 1995; Granata and Argyropoulos 1995; Capanema *et al*. 2004; Pu *et al*. 2011; Balakshin and Capanema 2015a; Balakshin and Capanema 2015b; Hu *et al*. 2016; Li *et al*. 2018; Meng *et al*. 2019; Popova *et al*. 2020; Gracia-Vitoria *et al*. 2022; Sun Mun *et al*. 2022; Jiang *et al*. 2023), inter-unit linkages (Capanema *et al*. 2001, 2004; Liitiä *et al*. 2003; Balakshin and Capanema 2015b; Hu *et al*. 2016; Sun Mun *et al*. 2022; Jiang *et al*. 2023), methoxy content (Capanema *et al*. 2004; Zhu *et al*. 2014; Balakshin and Capanema 2015b; Hu *et al*. 2016; Sun Mun *et al*. 2022), and degree of condensation (Capanema *et al*. 2004; Balakshin and Capanema 2015b; Hu *et al*. 2016). Lignin hydroxyl content is an indicator of lignin reactivity due to how it polymerizes, and free phenoxy or aliphatic hydroxyl groups mean that cleavage of the lignin biopolymer has occurred (Pu *et al*. 2011). Granata and Argyropoulos (1995) proposed a novel approach using ^{31}P NMR by selectively tagging hydroxyl groups in polysaccharides and phenolic hydroxyl and carboxylic acid groups in lignin with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP). The "tagging" procedure is a chemical reaction detailed in Fig. 10. This method can distinguish between aliphatic, benzylic, and phenolic hydroxyls due to chemical shifts in phosphitylated hydroxyls. Additionally, Pu *et al*. (2011) reviewed ^{31}P NMR to analyze hydroxyl group content in lignin and lignin-derived pyrolysis products. The basic principle is that lignin or lignin-derived bioproducts can be tagged using TMDP, which bonds to aliphatic, phenolic, and carboxylic hydroxyl groups in the presence of an organic base.

For ^{31}P analysis of lignin, an anhydrous pyridine, and deuterated chloroform solvent system (~1.6:1, v/v), a relaxation agent (usually chromium (III) acetylacetonate), and internal standard are used to generate optimal spectra. Generally, around 20-40 mg of lignin is dissolved in 0.5 mL of solvent. Around 0.05-0.1 mL of TDMP is added and stirred into this solution, which is then transferred into an NMR tube. A representation of selective TMDP tagging is presented in Fig. 10 (Pu *et al*. 2011). Lignin acetylation allows for quantitative ^{13}C NMR analysis of phenolic and aliphatic hydroxyl groups, similar to the ^{31}P procedure. To compare the two analyses, an internal standard, normally 1,3,5-trioxane allows for lignin quantification with the same units (mmol/g).

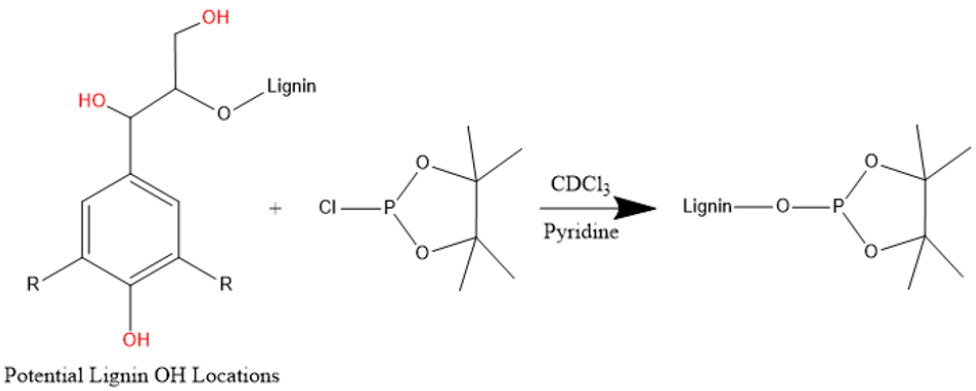


Fig. 10. Phosphitylation mechanism of TDMP and lignin adapted from Pu *et al*. (2011).

Lignin methoxy content and degree of condensation are quantifiable using ^{13}C and multi-dimensional NMR techniques. Quantitative ^{13}C NMR can be undertaken using an internal standard or *via* integration of the chemical shift assigned to the aromatic and vinylic carbons in the lignin structure (Xia *et al*. 2001; Capanema *et al*. 2004; Balakshin and Capanema 2015b; Balakshin *et al*. 2016; Hu *et al*. 2016). Quantitative information can be gained without an internal standard by integrating the region assigned to aromatic carbons in lignin (δ_C 163-100 ppm) and dividing the resulting integral by 6.12. This assumes that signals from six aromatic carbons and signals from 0.12 vinylic carbons are equivalent to one aromatic ring (Capanema *et al*. 2004). Detailed procedure and ^{13}C signal assignments have been described by Balakshin and Capanema (2015b) and Capanema *et al*. (2004). Lignin samples used in these studies were milled wood lignin (MWL), a lignin isolated from wood or other biomass, and technical lignins precipitated from various pulping liquors, respectively.

Therefore, quantitative ^{13}C NMR is viable for a variety of lignins. However, different integrations and results must be expected, given the inherent structural changes that lignin undergoes during the pulping process (Balakshin and Capanema 2015b). General assignments or clusters in a typical quantitative ^{13}C NMR lignin spectrum are divided between the aromatic region (δ_C 163-100 ppm) and oxygenated aliphatic region (δ_C 90-58 ppm). Each cluster can be further divided to distinguish specific carbon assignments in various lignin structures. Methoxyl groups present a single peak between δ_C 58-54 ppm and can be easily integrated, although a small amount of other lignin moieties exist in MWL at this chemical shift. Degree of condensation calculations have different equations based on the lignin sample being analyzed (Capanema *et al*. 2004; Balakshin and Capanema 2015b). ^{13}C spectra can be unreliable if factors such as baseline correction, probe type, signal-to-noise ratio, and lignin concentration in the sample. A more detailed discussion of the essential factors that go into a quantitative ^{13}C experiment is presented in the literature (Balakshin and Capanema 2015b).

While 1D NMR techniques can provide a great deal of quantitative information regarding lignin structure and functional groups, 2D and 3D NMR techniques provide both lignin structure and functional group detail as well as lignin inter-unit and lignin-carbohydrate linkage data. As such, multi-dimensional analysis of lignin samples has been extensively studied in the literature (Capanema *et al*. 2001, 2004; Marita *et al*. 2001; Liitiä *et al*. 2003; Lisboa *et al*. 2005; Balakshin *et al*. 2007; Yuan *et al*. 2011, 2013; Wen *et al*. 2013; Min *et al*. 2014; Lupoi *et al*. 2015; Constant *et al*. 2016; Hu *et al*. 2016; Lu *et al*.

2017; Tarasov *et al*. 2018; Bergs *et al*. 2021; Suota *et al*. 2021; Jiang *et al*. 2023). In lignin NMR, HSQC is the most common technique, although HMQC, HMBC, and 3D HSQC-TOCSY are useful in this field. Compared to 1D experiments, 2D HSQC requires longer experiment times, but provides enhanced resolution which justifies its usage. Additionally, lignin inter-unit linkages, specifically β-O-4, β-5, and β-β can be quantified using this technique (Liitiä *et al*. 2003). An HSQC spectrum separates carbon atoms in the proton dimension, and therefore, analysis of each signal is more straightforward to integrate and provides enhanced accuracy in quantification compared to ^{13}C NMR (Giummarella *et al*. 2019). It is, however, essential to mention that heteronuclear multiple dimension NMR techniques are considered semi-quantitative unless specific pulse sequences are developed (Giummarella *et al*. 2019). Despite this, multi-dimensional NMR analysis is crucial for thoroughly understanding lignin chemical structure.

Lignin-Carbohydrate Complex Study using NMR Techniques

Hemicellulose and lignin, and to a lesser extent, cellulose and lignin, have been theorized to be linked together chemically to form lignin-carbohydrate complexes (LCCs) (Tarasov *et al*. 2018; Giummarella *et al*. 2019). Lignin-carbohydrate linkages are primarily formed *via* three reaction pathways: benzyl ether, benzyl or gamma esters, and phenyl glycoside (Tarasov *et al*. 2018; Giummarella *et al*. 2019). Linkages between carbohydrates and lignin have been modeled to be thermodynamically favorable during the biosynthesis of lignin (Beck *et al*. 2022). A quinone methide intermediate in lignin is attacked at the alpha position by a primary hydroxyl group on a polysaccharide (Beck *et al*. 2022). Due to the complexity of this structure and the infrequency of lignin-carbohydrate linkages, isolation/fractionization and multidimensional NMR are needed for their detection (Giummarella *et al*. 2019). A combination of ^{13}C and 2D HSQC is most commonly used for LCC detection after it has been isolated from biomass. Balakshin *et al*. (2007, 2011), in multiple studies, compared different isolation techniques and used 2D and 3D NMR methods. Milled wood lignin (MWL), LCC extracted from acetic acid (LCC-AcOH), and cellulase enzyme lignin (CEL) were all dissolved in DMSO-d_6 and analyzed for LCC linkages *via* ^{13}C, 2D HSQC, and 2D HMBC. Chromium (III) acetylacetonate (0.01 M) was added as a relaxation agent. Benzyl ether, gamma ester, and phenyl glycoside linkages were identified in varying amounts in the isolated lignin and quantified using Eq. 2 (Balakshin *et al*. 2011).

Benzyl ester linkages were not identified in any of the samples tested. The authors discussed the best lignin isolation method for quantifying each linkage and concluded that each linkage has a different method of isolation that will show more linkages. Therefore, one should prepare their samples with the research goals in mind. For optimal spectral resolution, a 950 MHz spectrometer equipped with a cryoprobe was used for the 2D spectra (Balakshin *et al*. 2011). Phenyl glycoside typically shows up around δ_C 102.8-99.2/δ_H 4.72-4.28 ppm, benzyl ether linkages around δ_C 82.5-80.0/δ_H 4.7-4.3 ppm, and benzyl and gamma ester linkages around δ_C 75-77/δ_H 6.0-6.2 and δ_C 62-65/4.0-4.5 respectively (Balakshin *et al*. 2007). During a typical LCC experiment, the 2D spectrum generated can give valuable information on the lignin structure alongside any lignin-carbohydrate linkages. Yuan *et al*. (2011) was able to compare LCC and lignin structural content in MWL and mild acidolysis lignin (MAL) using a 400 MHz Bruker AVIII spectrometer. In this study, 125 mg of lignin was dissolved in 0.5 mL of DMSO-d_6 with 0.01 M chromium (III) acetylacetonate used as a relaxation agent. Not only did benzyl ether and gamma ester content differ between the isolation methods, but the authors found

differences in *p*-hydroxycinnamyl alcohol end groups and percentage of gamma acetylation, with MWL having a larger number and percentage of each. LCCs remain challenging to identify even with high-field multiple-dimension NMR techniques.

$$BE = 2D_{BE}/2D_{90\text{-}78/5.7\text{-}3.0} * {}^{13}C_{90\text{-}78}/{}^{13}C_{163\text{-}103} * 600 \quad (3)$$

$$PhGlc = 2D_{PhGlc}/2D_{103\text{-}96/5.5\text{-}3.8} * {}^{13}C_{103\text{-}96}/{}^{13}C_{163\text{-}103} * 600 \quad (4)$$

$$GEst = 2D_{GEst}/2D_{65\text{-}58/5.0\text{-}3.8} * {}^{13}C_{65\text{-}58}/{}^{13}C_{163\text{-}103} * 600 \quad (5)$$

Equations 3, 4, and 5 were used for the calculations of the number of lignin-carbohydrate linkages per 100Ar. $2D_{BE}$, $2D_{PhGlc}$, and $2D_{Gest}$ are the resonance frequency of benzyl ether, phenyl glycoside, and gamma ester linkages, respectively in a 2D spectrum divided by the total resonance frequencies in the chemical shift region associated with each LCC. This is multiplied by the resonance frequencies of clusters in the carbon spectrum. 600 is the number of aromatic carbons in 100Ar. These equations are adapted from Balakshin *et al*. (2011).

A significant component of municipal solid waste (MSW) can be classified as biomass or derivatives of biomass products. Increasing attention has been put into studying MSW, specifically for its utilization and decomposition. MSW is a broad term used for various waste materials, and many have been studied. Specifically, solid and pretreated MSW with varying compositions (obtained from a landfill), coffee and vegetable residues, wood, pine needle, garden trimmings, pig slurry, sewage waste, and activated sludges have been characterized using various solution-state ^{1}H, ^{13}C, ^{31}P, and solid-state ^{13}C and ^{15}N NMR techniques (Chefetz *et al*. 1996; Pichler *et al*. 2000, 2001; Gigliotti *et al*. 2002; Tambone *et al*. 2009; Amir *et al*. 2010; Caricasole *et al*. 2010, 2011). In Europe specifically, pretreatment of MSW *via* composting or anaerobic digestion is used to reduce adverse ecological effects (Pichler *et al*. 2001).

To characterize pretreated MSW, ^{13}C CPMAS solid-state NMR is predominately used (Chefetz *et al*. 1996; Pichler *et al*. 2000, 2001; Caricasole *et al*. 2010, 2011), although Pichler *et al*. (2000) quantified nitrogen-containing functional groups using solid-state ^{15}N NMR (Pichler *et al*. 2000). Components in pretreated municipal solid have been assigned to specific regions in a solid-state ^{13}C spectrum. Lipids, proteins, and plastics and their carbon-containing functional groups will appear between δ_C 0-50 ppm. Carbohydrates in MSW are in the O-alkyl C region between δ_C 50-110 ppm and generally dominate the ^{13}C spectrum. Aromatic units belonging to lignin and polystyrene in MSW will be between δ_C 110-140 ppm. Finally, carbonyl, carboxylic, ketone, aldehyde, (poly)amide, and ester groups will appear between δ_C 160 and 220 ppm (Pichler *et al*. 2000, 2001). The general trend in aerobic, anaerobic, and composting studies is that carbohydrate content in MSW decreases as a function of time while signals from alkyl, functional groups between δ_C 160-220 ppm, and aromatic carbon regions increase (Chefetz *et al*. 1996; Pichler *et al*. 2000, 2001; Chen 2003; Tambone *et al*. 2009; Caricasole *et al*. 2010, 2011). Additionally, plastic content does not degrade, and therefore, NMR signals will relatively increase (Pichler *et al*. 2001).

Contaminated MSWs present a danger to the surrounding environment. The fate of ^{13}C-labeled contaminants such as phenol, bisphenol A, pentachlorophenol, and 2,4,6-trichlorophenol was obtained by evaluating ^{13}C NMR spectra of anaerobically incubated MSW samples taken at different times and conditions (thermophilic and mesophilic) which

were dissolved in deuterated water (D_2O) (Limam *et al*. 2013, 2016). Chemical fate was determined by the mineralization of these phenolic-based compounds into carbon dioxide and methane, which appear on the ^{13}C spectrum (Limam *et al*. 2013, 2016). Microorganisms in a composting environment use organic matter as a carbon and nitrogen source, and the end product of this utilization, as well as dead microorganism cell walls, are classified as humic substances (Chefetz *et al*. 1998). This process is important for net primary production and can be an indicator of overall composting efficiency (Chefetz *et al*. 1998). As is true in ^{13}C CPMAS NMR studies in MSW, ^{13}C CPMAS NMR studies of humic substances in compost observed that as composting commences, polysaccharide structures bound to humic structures are degraded, whereas humic structures are enriched with aromatic structures (Chefetz *et al*. 1998; Castaldi *et al*. 2005).

Pyrolysis reactions to produce biochar and bio-oils utilizing MSW as a feedstock have been undertaken in previous studies and quantified using solution-state ^{1}H and ^{13}C and solid-state ^{13}C CP/MAS NMR (Ateş *et al*. 2013; Tursunov *et al*. 2020; Hilber *et al*. 2024). $CDCl_3$ was used as the solvent for the solution-state samples. Solid-state NMR has been used to study biochar produced from plastic-contaminated biowaste when co-pyrolyzed with biomass to assess its suitability for agricultural use (Hilber *et al*. 2024). Additionally, early studies involving pyrolysis and compost have been reviewed (Chen 2003). As the world searches for techniques to utilize the growing amount of produced MSW, NMR can quantify important chemical structures in MSW derivatives and determine the fate of harmful contaminants.

Kinetic and *in-situ* Studies

NMR provides a unique opportunity in biomass studies to probe reaction kinetics and changes in chemical composition during a chemical reaction. The progress of a reaction can be monitored in an NMR study *via* chemical shift changes, which can be probed *in situ*, through the use of variable-temperature or pressure-rated NMR tubes over various time intervals, investigating molecular dynamics, and characterizing reaction intermediates and products to determine specific reaction pathways. Additionally, aliquots at various points of a reaction can be taken and analyzed at specific timed intervals via NMR to monitor a targeted species in a chemical reaction. Therefore, monitored chemical reactions do not have to adhere to a specific time limit. Ben *et al*. (2019) used two model compounds (methylcyclopentane and cyclohexane) representing abundant pyrolysis oil compounds to investigate ring-opening reactions using various catalysts and temperatures for efficient pyrolysis oil value-added product upgrading. 100 μL of model compounds were mixed with 0.5 μL of tetramethylsilane-d_{12} as an internal standard, as well as 2 mg of catalyst. A high-pressure NMR tube was needed for this study.

A pressurized deuterium isotope tracing ^{2}H NMR system was utilized in this study to probe the H/D exchange rate between reaction products. The reaction rate was dependent upon the position of the ring, with the unhindered side expected to be the most efficient. Rubidium was found to be the most efficient catalyst, and the higher temperature value yielded faster reaction rates (Ben *et al*. 2019). A ^{1}H *in situ* NMR process has been used to investigate the mobile or liquid intermediate phase present during the pyrolysis process. This was done at a specific pyrolysis temperature using a ^{1}H NMR pulse sequence able to distinguish between the peak shape of protons in a solid structure (Gaussian-like distribution) and protons in a mobile or liquid structure (Lorentzian-like distribution) (Dufour *et al*. 2012; Ben *et al*. 2019).

Solid-state ^{13}C CPMAS NMR has also been used to monitor specific functional groups in torrefied softwood biomass pyrolyzed at differing temperatures (Wang *et al*. 2016). Chemical shift differences between cellulose, hemicellulose, and lignin in torrefied biomass allowed researchers to conclude the fate of functional groups in each predominant wood component. Specifically, as torrefaction temperature increased, hemicellulose moieties deacetylated and depolymerized, cellulose crystalline structure was destroyed, and lignin aryl ether linkages and propyl side branches were cleaved (Wang *et al*. 2016). Through their experimental design, an activation energy model for the parallel reaction in pyrolysis was applied to analyze pyrolysis kinetics: degradation of hemicellulose and lignin pyrolysis, parallel degradation in cellulose pyrolysis, and parallel condensation during hemicellulose and lignin pyrolysis. Variance in their model was due to changes in structural composition, and the most significant amount of variance was determined to be between 25 °C to 200 °C and 250 °C to 300 °C (Wang *et al*. 2016). Co-pyrolysis between polyethylene terephthalate (PET) and macadamia nut shells was analyzed using ^{1}H and ^{13}C CPMAS solid-state NMR with Total Suppression of Spinning Sidebands (TOSS) to investigate thermal degradation rate at differing blending ratios and heating rates (Ko *et al*. 2014). Results indicated a symbiotic relationship between the two feedstocks during pyrolysis, which enhanced the total carbon yield due to the macadamia nutshell catalyzing the degradation of PET-yielding polycyclic aromatic hydrocarbons (PAHs) through a cross-linking reaction (Ko *et al*. 2014).

Reaction pathways of hydrothermal pretreated D-glucose were studied using *in situ* ^{13}C NMR spectroscopy (Kimura *et al*. 2011). The reaction rate of D-glucose isomers to D-fructose isomers and, subsequently, the reaction rate for dehydration of D-fructose into 5-hydroxymethylfurfural (HMF) was quantified using this NMR technique. The most prominent HMF peak in the NMR spectra came from the sample with the longest reaction time and at the highest temperature (Kimura *et al*. 2011). NMR is a useful technique for biomass kinetic studies but has been underutilized to this point. Further research is necessary to answer important questions about the biomass chemical reaction mechanism and optimize biomass transformation reactions.

CONCLUSIONS AND FUTURE PERSPECTIVES

Anthropogenic climate change has driven biomass research to be a solution for alternative energy resources. Due to the complexity of biomass structures, complex analysis is needed to grasp a complete chemical characterization. NMR can decipher between different functional groups and structures, and with various NMR techniques, biomass chemical structure can be probed to a significant level of accuracy. The application of larger NMR magnets and the use of a variety of 2D and 3D NMR techniques will allow for both enhanced clarity and ways to quantify different research questions with new biomaterials as the field advances. We can resolve signal overlaps easily through the application of higher-field magnets, which now have reached 1 GHz. A review of the NMR phenomenon, specific solid and solution-state NMR techniques, a section for a biomass NMR study workflow, an in-house unknown biomass substance NMR experiment, and a list of biomass and biomass-derived materials characterized using NMR analysis were presented. This work aims to highlight what has been done to probe biomass chemical structure using NMR techniques and promote the use of this technology to answer complex problems inherent in biomass research.

1. Many of the NMR techniques discussed here are innovative and can be implemented in the field of biomass. Specifically, only a few kinetic NMR studies were found. Given the multitude of new biomass-derived products, a more holistic picture of their manufacturing and any reaction intermediates can be derived.
2. Isotopic labeling is also novel and has the potential to generate more accurate and complete carbon-derived 1-, 2-, and 3-D solid-state NMR spectra. This has the potential to alleviate signal overlap issues in multi-dimensional solid-state NMR, as more ^{13}C atoms produce a stronger NMR signal and therefore allow for more resolved spectra.
3. As external NMR magnets get larger and, therefore, more powerful, higher resolution NMR spectra can be generated. Using NMR to structurally characterize plant cell walls has only recently been realized. Creative approaches to determining plant cell structure can still be implemented.
4. Additionally, only a few 3D NMR studies exist. This could be due to how long a 3D spectrum takes to generate and the fact that 2D NMR techniques can resolve most structures of interest. 3D NMR is typically used in protein studies.
5. As new biomass types and treatments emerge, NMR can probe their structure and identify their composition. Finally, complex NMR techniques such as DOSY, time-domain NMR, and *in-situ* NMR studies have the potential to be applied to biomass research questions, and each method remains underutilized. Research into new strategies to use the NMR phenomenon will continue to come to light, and biomass research and NMR technology can symbiotically evolve together.
6. AI tools such as NMRmind and NMRformer have been developed for faster NMR data interpretation for both complex and simple NMR spectra. Additionally, tools to denoise complex NMR spectra will allow for peaks to appear near the noise floor, which can be missed in manual interpretation. These phenomena could allow for easier automation of NMR spectral interpretation (Tian et al. 2026).

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