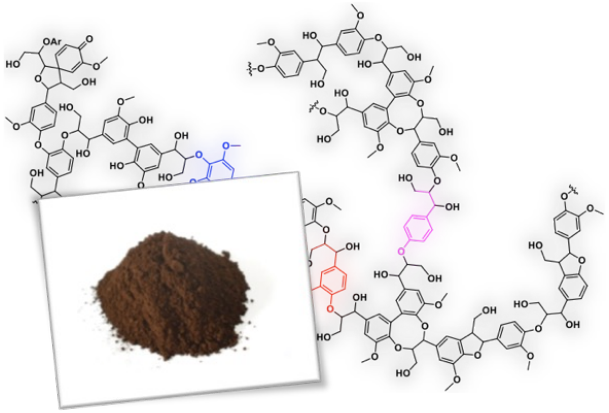
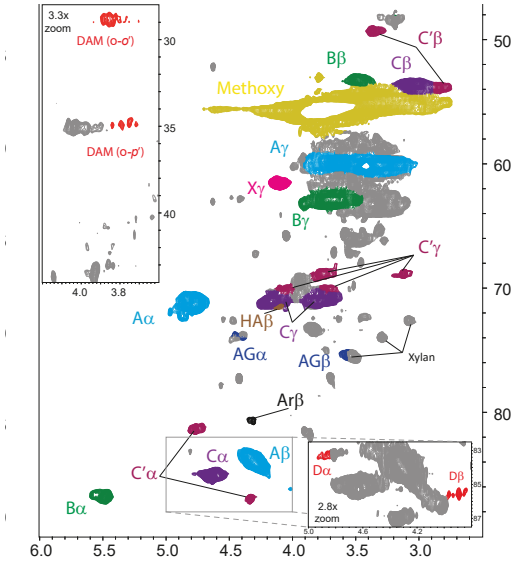
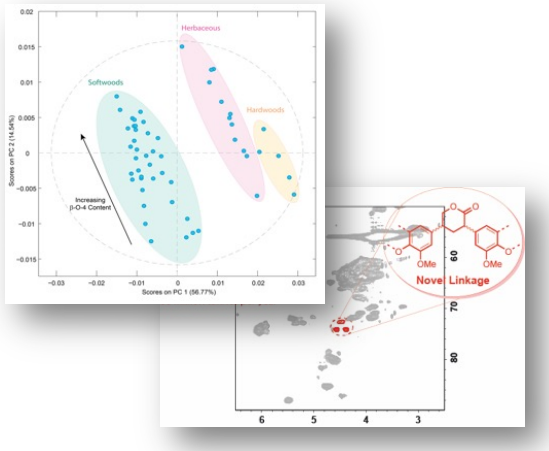
Lignin Molecular Structure Characterization by NMR







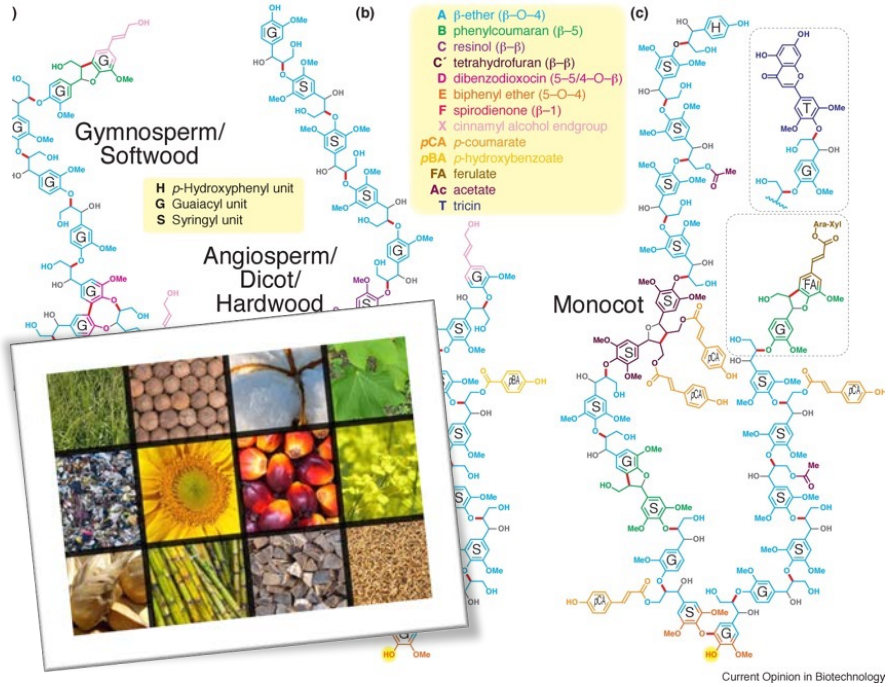
Universiteit Utrecht

Workshop
Lignocost Conference Wageningen 2022

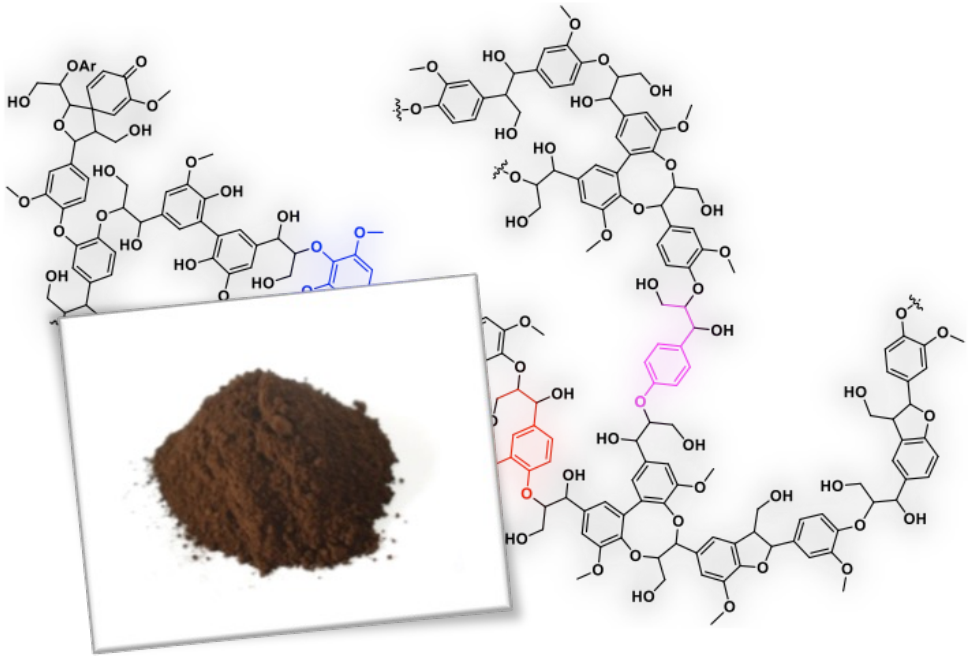
Pieter Bruijnincx

The Lignin Platform: Dealing With Structural Complexity

- There is **no such thing** as lignin



In planta lignins

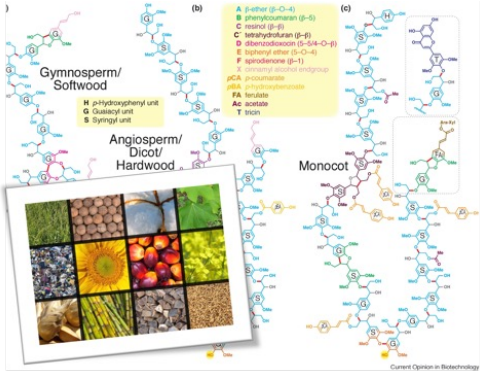


Technical lignins

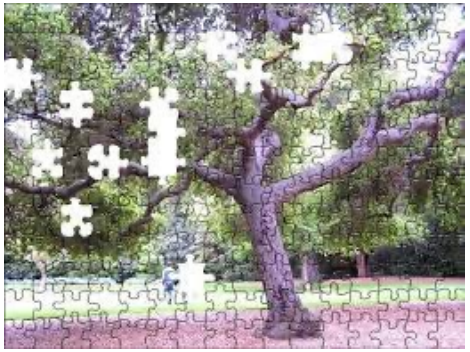
From Ralph, Lapierre, Boerjan *Curr. Opin. Biotechnol* 2019, 56, 240

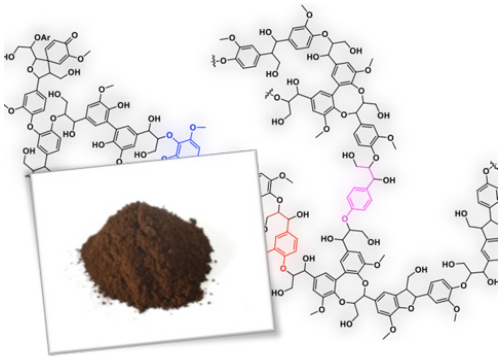
The Lignin Platform: Dealing With Structural Complexity

- What is it we seek to know?



- In planta lignin **structure elucidation**
- Species/mutation dependent **variation**
- **Valorization** opportunities

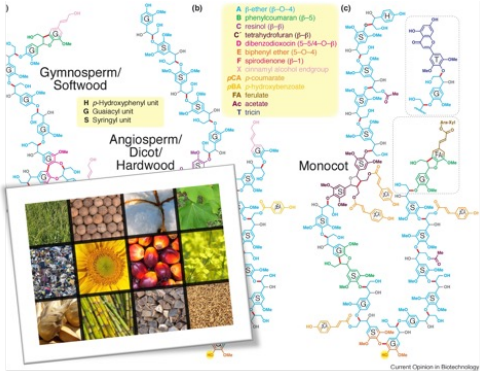


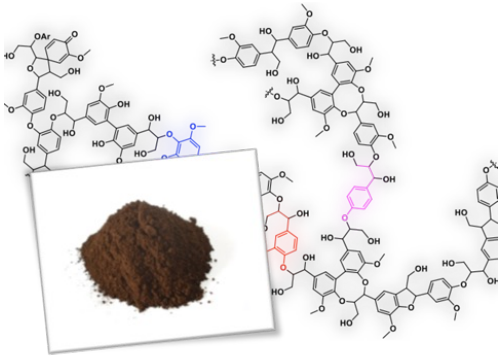


- Technical lignin **structure elucidation**
- Biorefinery operation dependent **variation**
- **Valorization** opportunities

The Lignin Platform: Dealing With Structural Complexity

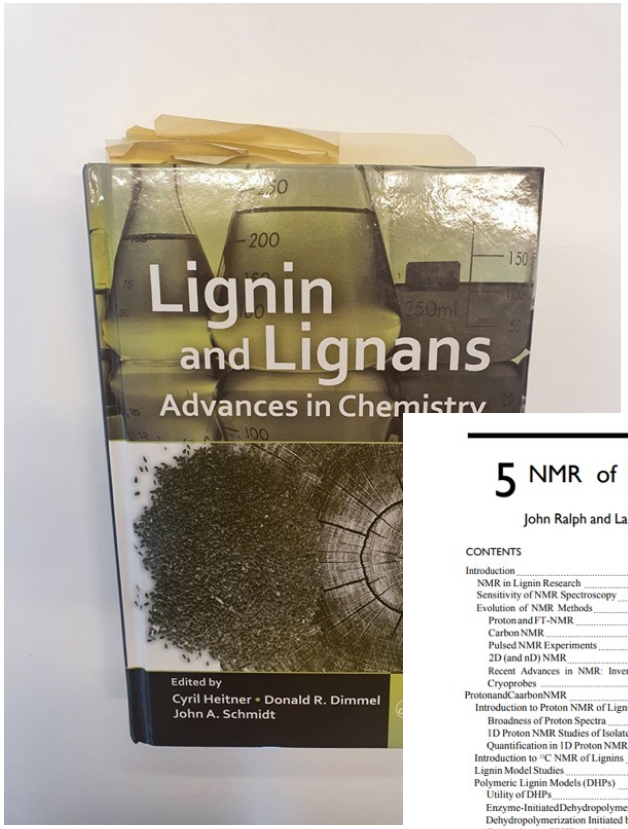
- What is it we seek to know?





- Monomer/pendant **composition**
- **Connectivity**: linkages within and to other components in the matrix
- **Functional group** type and density
- **Macromolecular properties**: shape/aggregation/molecular weight

- Comprehensive approach to lignin structure analysis needed
- No single technique suffices
- **NMR** is arguably the most **powerful** one though, when it comes to obtaining structural information



Lignin NMR

5 NMR of Lignins

John Ralph and Larry L. Landucci

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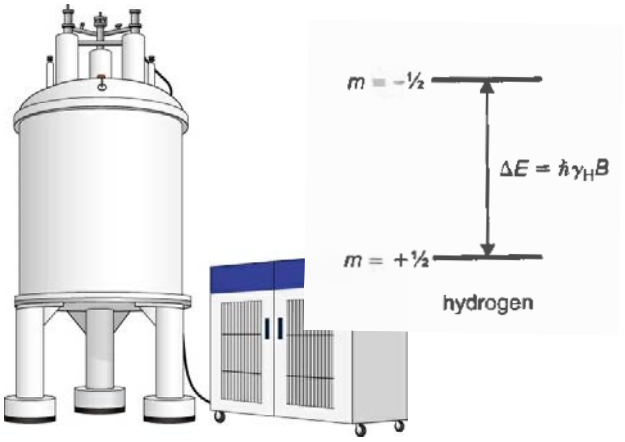
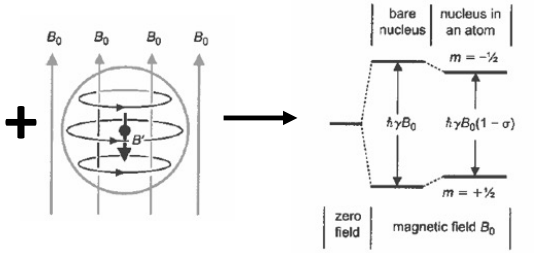
Several nuclei of interest suffer from low natural abundance: protons are essentially 100% ^{1}H (~0.015% ^{2}H); phosphorus is 100% ^{31}P; the major carbon isotope, ^{12}C, is not NMR-active, while the ^{13}C isotope is only present in 1.11% relative abundance. Consequently, ^{13}C NMR at natural abundance loses another two orders of magnitude in sensitivity. Also, the energy levels are even closer in ^{13}C than in ^{1}H (roughly a factor of $^{1}/_{4}$), resulting in an even smaller population difference between the levels and causing yet another decrease in sensitivity. Sample sizes typically in the milligram range are required to obtain good spectra in reasonable times. Despite this apparently abominable sensitivity, the information content of NMR spectra is significantly higher than for other spectral techniques. NMR alone can often fully identify compounds. Structure and bonding patterns are readily elucidated even in complex molecules.

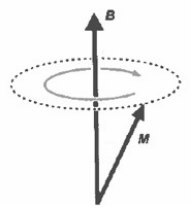
Considerable gains in sensitivity have been realized through other approaches. A 2-dimensional(2D) ^{13}C–^{1}H correlation spectrum, for example, can now be acquired much more quickly than a 1-dimensional (1D) ^{13}C spectrum [2]. Although a relatively insensitive spectroscopic method, NMR still provides an amazingly detailed picture of lignin structure from a combination of experiments, if sufficient sample (typically 10–100mg) is available.

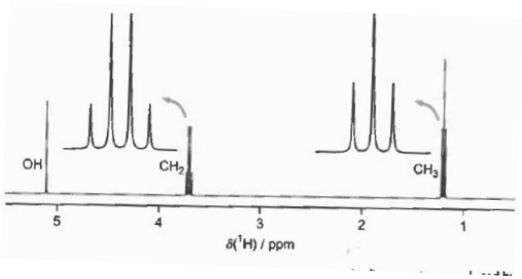
- NMR is arguably the most **powerful** one though, when it comes to obtaining structural information

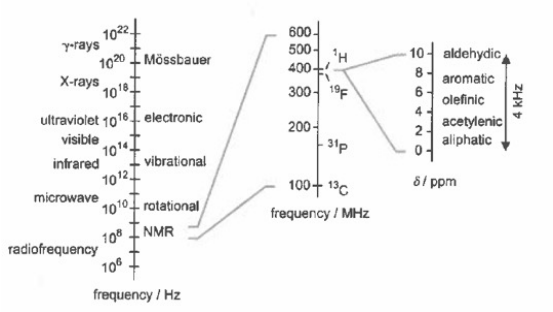
(Lignin) NMR











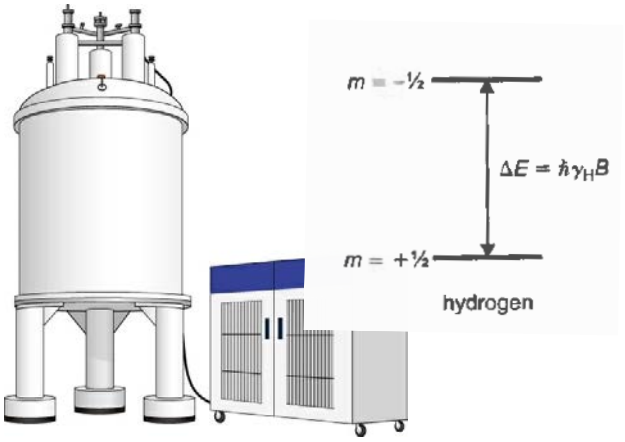
$$\frac{n_\beta}{n_\alpha} = \exp(-\Delta E/k_B T), \tag{1.11}$$

where $\Delta E = \hbar\gamma_H B$ (eqn 1.9) and k_B is Boltzmann's constant. Evaluating this expression at 300 K, one finds $\Delta E = 2.65 \times 10^{-25}$ J, $k_B T = 4.14 \times 10^{-21}$ J, and $\Delta E/k_B T = 6.4 \times 10^{-5}$. Thus, the energy required to reorient the spins is dwarfed by the thermal energy $k_B T$, so that there will be only a small excess of spins in the lower energy level. For such small values of $\Delta E/k_B T$, eqn 1.11 may be simplified using $\exp(-x) \approx 1 - x$, to obtain the relative population difference, or polarization, p

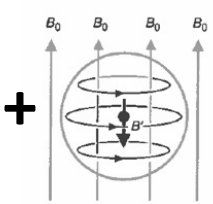
$$p = \frac{n_\alpha - n_\beta}{n_\alpha + n_\beta} \approx \frac{\Delta E}{2k_B T}. \tag{1.12}$$

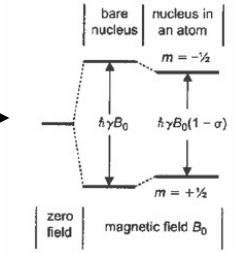
With the above numbers, eqn 1.12 gives $p \approx 3.200 \times 10^{-5}$. That is, for every 15,624 spins in the upper level there are 15,625 in the lower level. The polarization will be even smaller for ^{1}H nuclei in a weaker field, or for spins with smaller $|\gamma|$ (i.e. almost all other nuclei). This situation is in stark contrast to electronic spectroscopy at a frequency of, say, 10^{15} Hz. Here, ΔE is much larger than $k_B T$ at room temperature and almost all of the molecules are in the ground state, leaving the

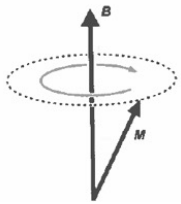
Lignin NMR

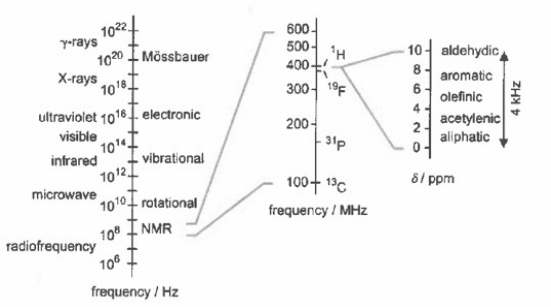


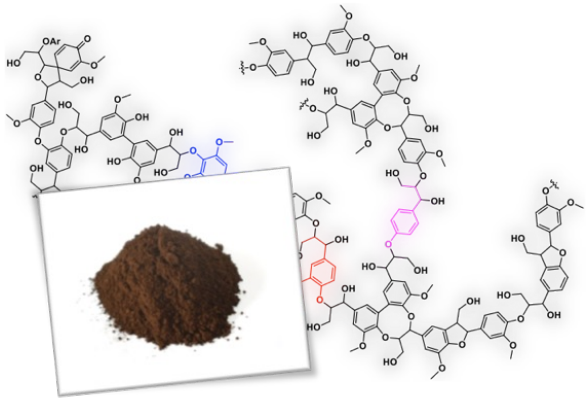
m = −½
ΔE = ħγ_H B
m = +½
hydrogen


B0 B0 B0 B0
B'


bare nucleus
nucleus in an atom
m = −½
ħγB0
ħγB0(1 − σ)
m = +½
zero field
magnetic field B0


B
M

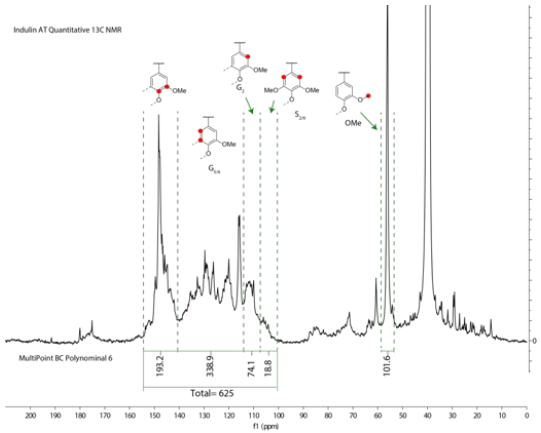

γ-rays
X-rays
ultraviolet
visible
infrared
microwave
radiofrequency
Mössbauer
electronic
vibrational
rotational
NMR
frequency / Hz
¹H
¹⁹F
³¹P
¹³C
frequency / MHz
aldehydic
aromatic
olefinic
acetylenic
aliphatic
4 kHz
δ / ppm

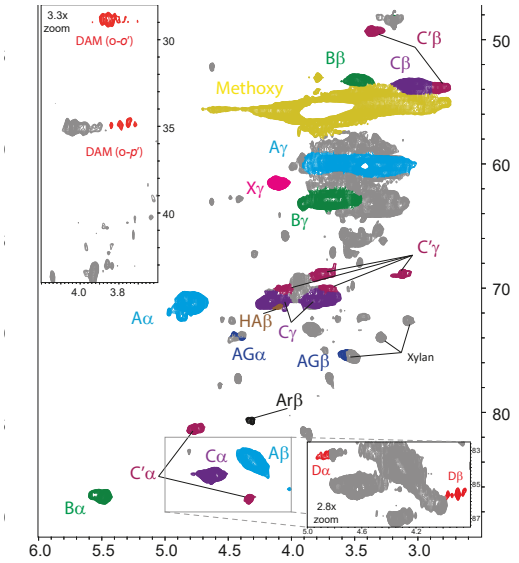


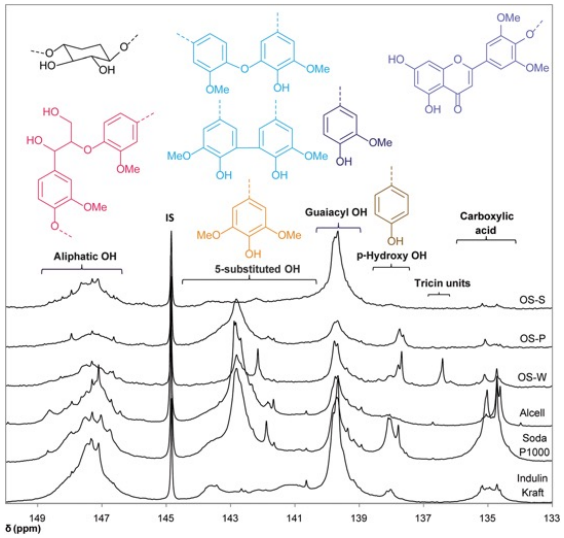
DMSO-d6
Aromatic region
Side-chain
Aliphatic region
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

Lignin NMR: Opportunities Abound

- **1D** ^{1}H and (quantitative) ^{13}C NMR
- **Multidimensional** NMR: TOCSY, HSQC$_{(0)}$, HMBC, HSQC-TOCSY, ….
- **Heteronuclear** NMR: ^{31}P, ^{19}F, …







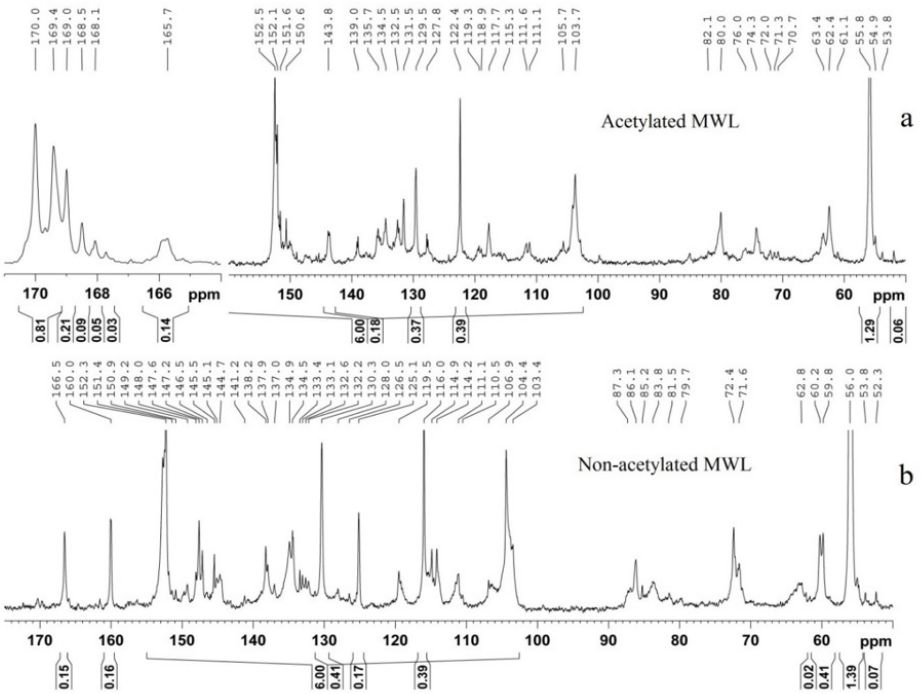
1D Lignin NMR: Limited Resolution Yet Quantitative

- (quantitative) ^{13}C NMR: powerful and historically important tool for lignin analysis

- **Quantitative** analysis of –OMe, -OH, -COOR, S/G, ArH, beta-O-4

- **Semi-quantitative** analysis of various linkages, degree of condensation, saturated aliphatics, etc.

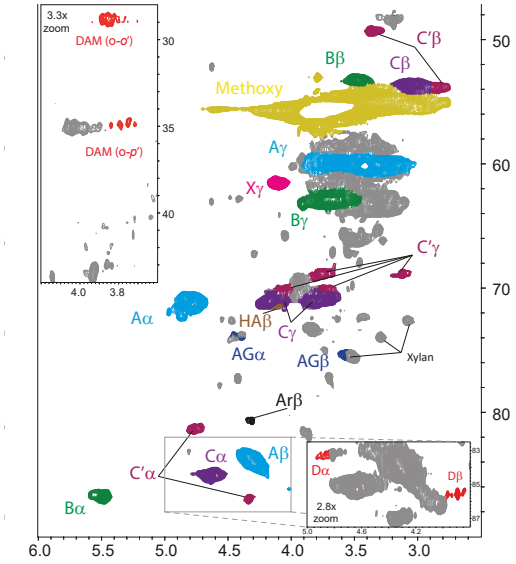
Figure 2. ^{13}C-NMR spectra of acetylated and non-acetylated bamboo lignin samples (Reprinted from [28]. Copyright 2013 De Gruyter).



- See e.g. Balakshin, Capanema RSC Adv., 2015, 5, 87187; Balakshin et al. Green Chem 2020, 22, 3985; Sun, Materials 2013, 13, 6

Multidimensional Lignin NMR: Opportunities Abound

- Multidimensional NMR: COSY, TOCSY, HSQC$_{(0)}$, HMBC, HSQC-TOCSY, etc, etc ….



- **HSQC** is the current go-to method for lignin structure analysis; **excellent for fingerprinting**
- **Combinations** of 2D NMR spectra and model compound spectra provide a **redundancy of information** and allow for **unambiguous** peak/structure assignments
- Spectra can be **multiplicity edited** (i.e. distinguish CH/CH3 and CH2 contours)
- Not limited to 2D: e.g. **3D HSQC-TOCSY**
- Note **quaternary carbons** cannot be identified by HSQC

Lignin NMR: HSQC

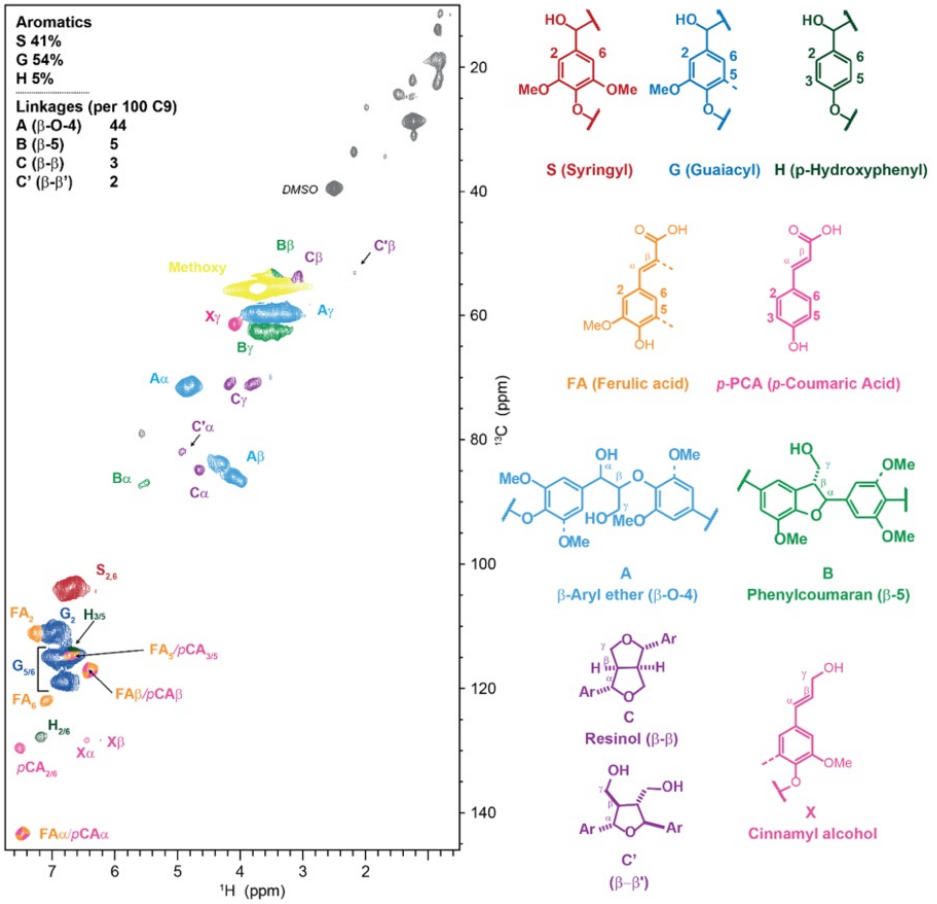


Fig. 3. HSQC analysis of the lignin PSS1 isolated after pilot scale soda pulping of *Miscanthus x giganteus*.

- Allows the **ratio of S/G/H** to be determined (be careful with H), using S2/6, G2 and H2/6
- Allows the **linkage abundance** to be semi-quantitatively determined (beware of quantification limitations)
- Allows **identification of end-units/pendants**; quantification of mobile pendants (e.g. p-coumarates, p-hydroxybenzoates) is overestimated due to differences in relaxation times
- Check your spectra **carefully** against the abundant literature

Lauwaert, Lancefield, Bruijnincx, Verberckmoes et al. Sep. Purif. Technol. 2019, 221, 226

Lignin NMR: HSQC

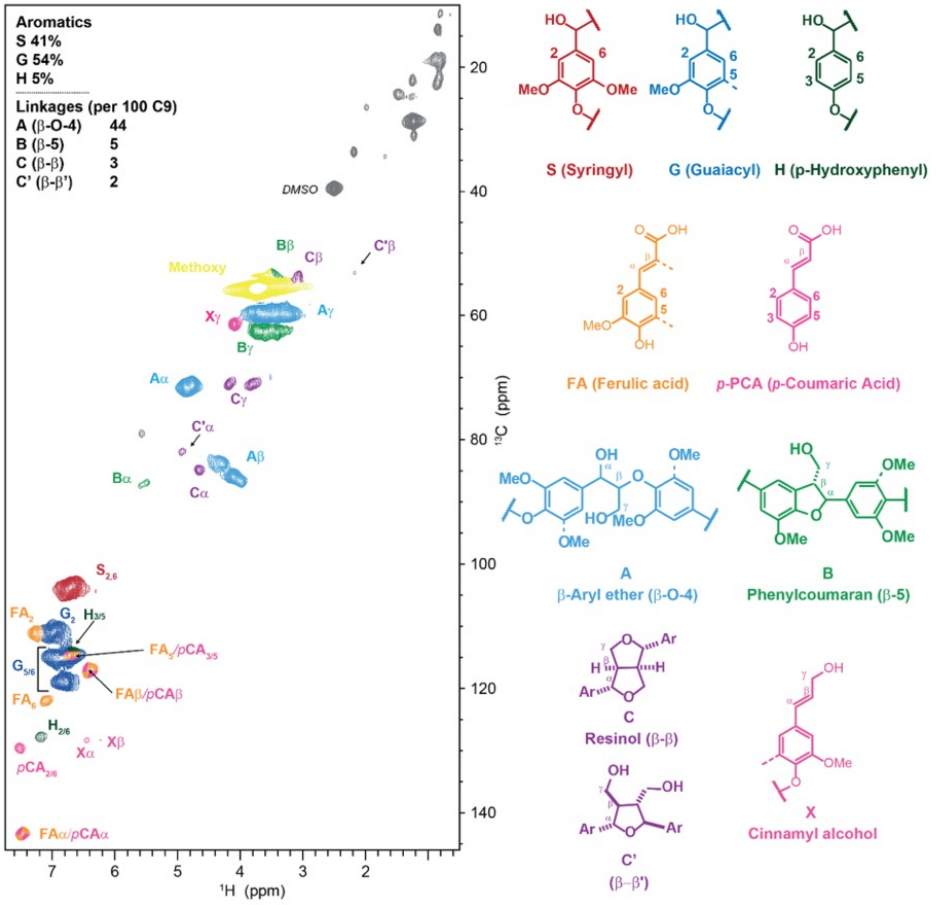


Fig. 3. HSQC analysis of the lignin PSS1 isolated after pilot scale soda pulping of *Miscanthus x giganteus*.

- Quantification is made **difficult** by the heterogeneous nature of lignin (resulting in difficulties in dealing with differences in T_2 relaxations, resonance offsets, coupling constant deviations, homonuclear couplings)

- **Methods for (semi-)quantification**:

  - Relative quantification based on HSQC without IS

    $I_X(\%) = I_X / (I_A + I_B + I_C + \ldots) \times 100\%$

  - **Semi-quantification based on HSQC with IS** (aromatics)

    $I_{C9} = 0.5 * I_{S2,6} + I_{G2} + 0.5 * I_{H2,6}$

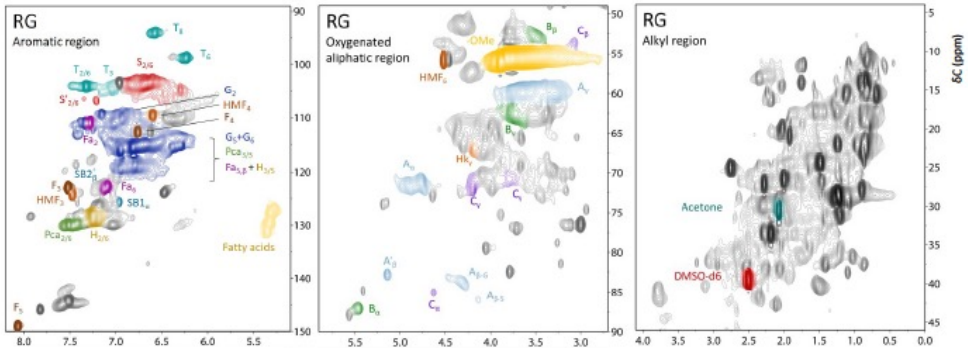
    $I_X(\%) = I_X / I_{C9} \times 100\%$

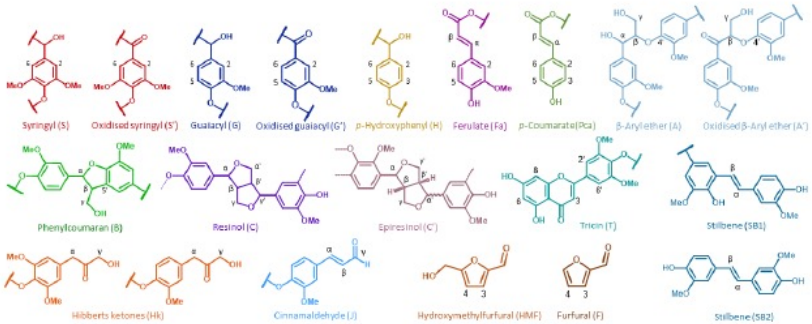
  - Quantification based on ^{13}C and HSQC

Lignin NMR: HSQC

Table S20 Assignments and correction factors for integral regions of HSQC-NMR spectra

Notation	Chemical Shift (δ / ppm)	Assignment	Correction Factor
$-OCH_3$	55.6/3.73	C–H in methoxyls	$\int x$
$S_{2,6}$	104.3/6.6	C_2–H_2 and C_6–H_6 in syringyl units	$\int x/2$
$S'_{2,6}$	106.5/7.3	C_2–H_2 and C_6–H_6 in syringyl units with α oxidization	$\int x/2$
G_2	110.8/6.9	C_2–H_2 in guaiacyl units	$\int x$
G'_2	111.6/7.5	C_2–H_2 in guaiacyl units with α oxidization	$\int x$
G_5	115.3/6.8	C_5–H_5 in guaiacyl units	$\int x$
G_6	119.0/6.9	C_6–H_6 in guaiacyl units	$\int x$
G'_6	123.5/7.6	C_6–H_6 in guaiacyl units with α oxidization	$\int x$
$H_{2,6}$	128.2/7.2	$C_{2,6}$–$H_{2,6}$ in *p*-hydroxyphenyl units	$\int x/2$
$T_{2,6}$	104.2/7.3	C_2–H_2 and C_6–H_6 in tricin units	$\int x/2$
T_5	104.5/7.1	C_5–H_5 in tricin units	$\int x$
T_6	98.9/6.3	C_6–H_6 in tricin units	$\int x$
T_8	94.2/6.6	C_8–H_8 in tricin units	$\int x$
Fa_2	111.2/7.3	C_2–H_2 in ferulates	$\int x$
Fa_6	122.9/7.1	C_6–H_6 in ferulates	$\int x$
$Pca_{2,6}$	130.1/7.5	C_2–H_2 and C_6–H_6 in *p*-coumarate	$\int x/2$
A_α	71.9/4.9	C_α–H_α in β-O-4′ substructures	$\int x$
A_β	84.8/4.3 (G) and 86.0/4.2 (S)	C_β–H_β in β-O-4′ substructures	$\int x$
A_γ	59.8/3.4	C_γ–H_γ in γ– hydroxylated β-O-4′ substructures	$\int x$
A'_β	83.1/5.3	C_β–H_β in α-oxidized β-O-4′ substructures	$\int x$
B_α	87.3/5.5	C_α–H_α in phenylcoumaran substructures	$\int x$
B_β	53.1/3.4	C_β–H_β in phenylcoumaran substructures	$\int x$
B_γ	62.6/3.7	C_γ–H_γ in phenylcoumaran substructures	$\int x$
C_α	85.2/4.7	C_α–H_α in β–β′ resinol substructures	$\int x/2$
C_β	53.6/3.1	C_β–H_β in β–β′ resinol substructures	$\int x/2$
C_γ	71.2/4.2 and 71.3/3.8	C_γ–H_γ in β–β′ resinol substructures	$\int x/2$
C'_α	86.9/4.4	C_α–H_α in β–β′ episinol substructures	$\int x$
C'_ω	81.4/4.8	C_ω–H_ω in β–β′ episinol substructures	$\int x$
C'_β	70.3/4.1 and 70.3/3.7	C_β–H_β in β–β′ episinol substructures	$\int x$
C'_γ	68.9/3.8 and 68.9/3.1	C_β–H_γ in β–β′ episinol substructures	$\int x$
C'_γ	53.9/2.8	C_γ–H_γ in β–β′ episinol substructures	$\int x$
I_α	128.2/6.4	C_α–H_α in cinnamyl alcohol end-groups	$\int x$
I_β	128.3/6.2	C_β–H_β in cinnamyl alcohol end-groups	$\int x$
I_γ	61.0/4.1	C_γ–H_γ in cinnamyl alcohol end-groups	$\int x$
J_α	153.4/7.61	C_α–H_α in cinnamaldehyde end-groups	$\int x$
J_β	126.0/6.8	C_β–H_β in cinnamaldehyde end-groups	$\int x$
Z-EE$_\alpha$	109.3/5.6	C_α–H_α in *Z*-enol ether	$\int x$
E-EE$_\alpha$	112.0/6.1	C_α–H_α in *E*-enol ether	$\int x$
$SB1_\alpha$	125.6/7.0	C_α–H_α in *trans*-stilbene substructures (β-1)	$\int x/2$
$SB2_\beta$	119.9/7.2	C_β–H_β in *trans*-stilbene substructures (β-5)	$\int x$
Hk_γ	67.2/4.2	C_γ–H_γ in Hibbert ketone structures	$\int x/2$
F_3	122.8/7.5	C_3– H_3 in furfural	$\int x$
F_4	112.6/6.8	C_4– H_4 in furfural	$\int x$
F_5	148.9/8.1	C_5– H_5 in furfural	$\int x$
HMF_3	124.3/7.5	C_3– H_3 in 5-hydroxymethylfurfural	$\int x$
HMF_4	109.6/6.6	C_4– H_4 in 5-hydroxymethylfurfural	$\int x$
HMF_6	55.8/4.5	C_6– H_6 in 5-hydroxymethylfurfural	$\int x/2$

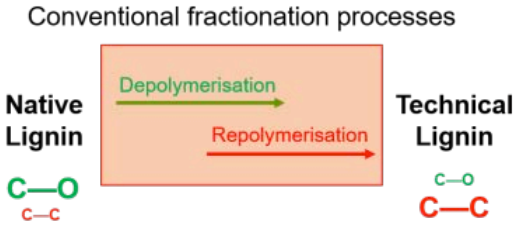




- Check your spectra **carefully** against the abundant literature

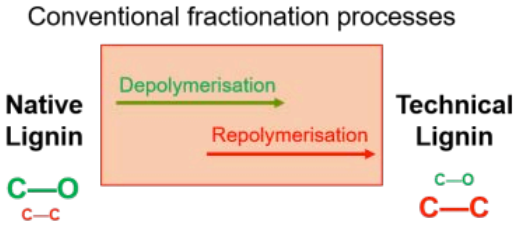
Smit, Riddell, Bruijnincx et al. ACS Sustainable Chem. Eng. 2022, 10, 6012

Biorefineries typically produce technical lignins



- Structure invariably **altered** by **biomass pretreatment**
- Structure is changed **considerably** and in different ways
- Know your lignin: **extensive analysis** is required

Biorefineries typically produce technical lignins



- HSQC is 'blind' to much of the structure

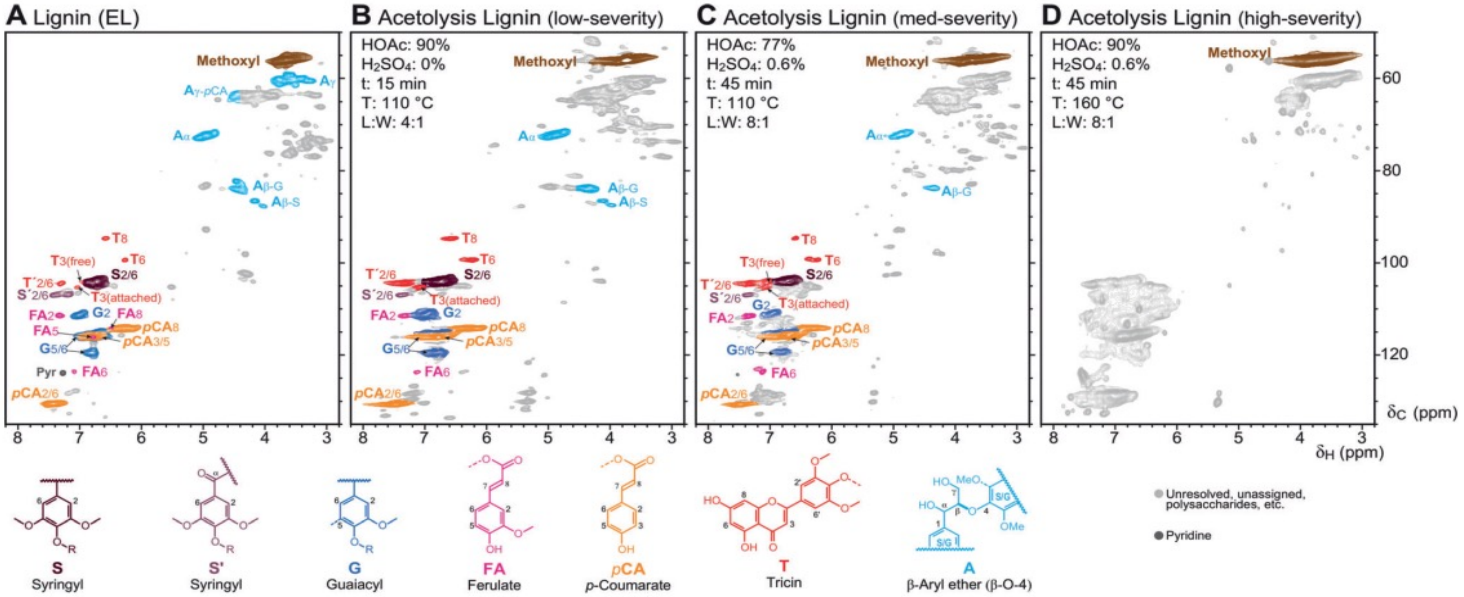


Figure 3. Influence of pretreatment severity on the nature of a processed lignin as revealed by HSQC NMR spectroscopy.

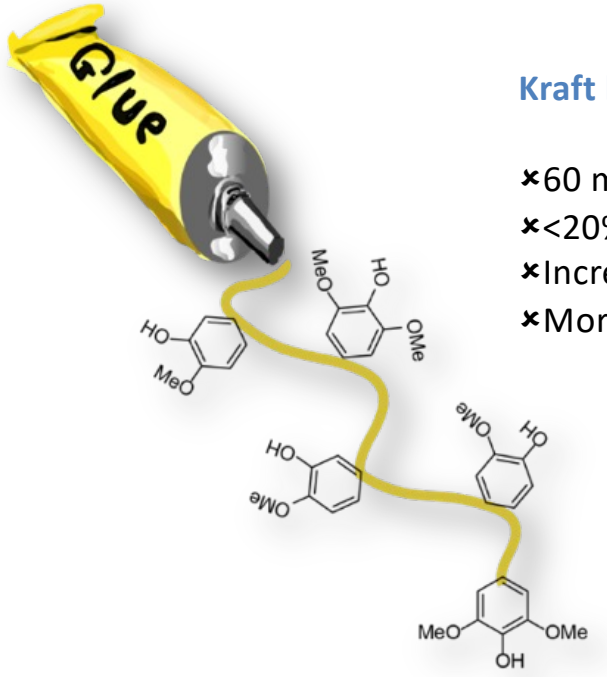
Rinaldi, Ralph, Bruijnincx, Weckhuysen et al. Angew. Chem. Int. Ed. 2016, 55, 8164

Kraft lignin: a case in point

Lancefield, Bruijnincx et al., Chem. Sci. 2018, 9, 6348

See also: Crestini , Argyropoulos Green Chem. 2017, 19, 4104

- The actual structure of the technical lignins is (in most cases) **largely unknown**

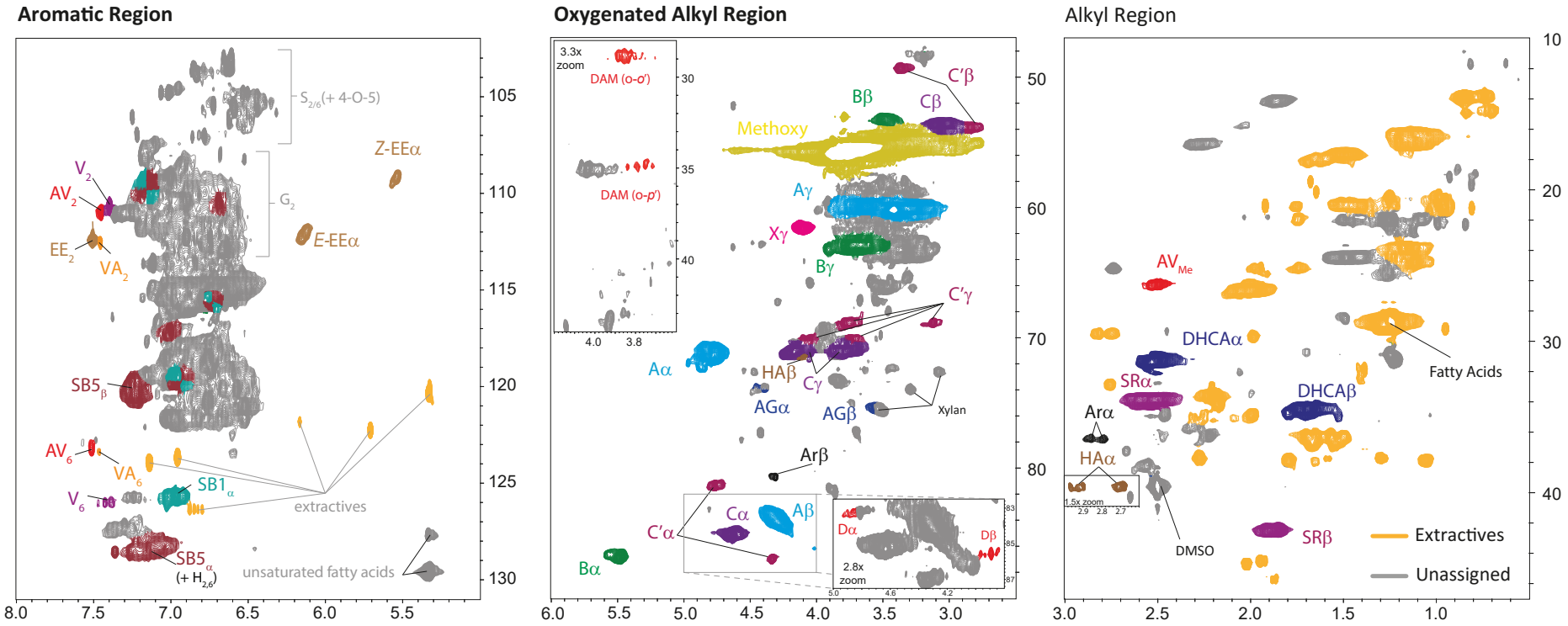


Kraft lignin

- ×60 mta produced per year
- ×<20% assigned by HSQC
- ×Increased complexity
- ×More C-C bonds and phenols

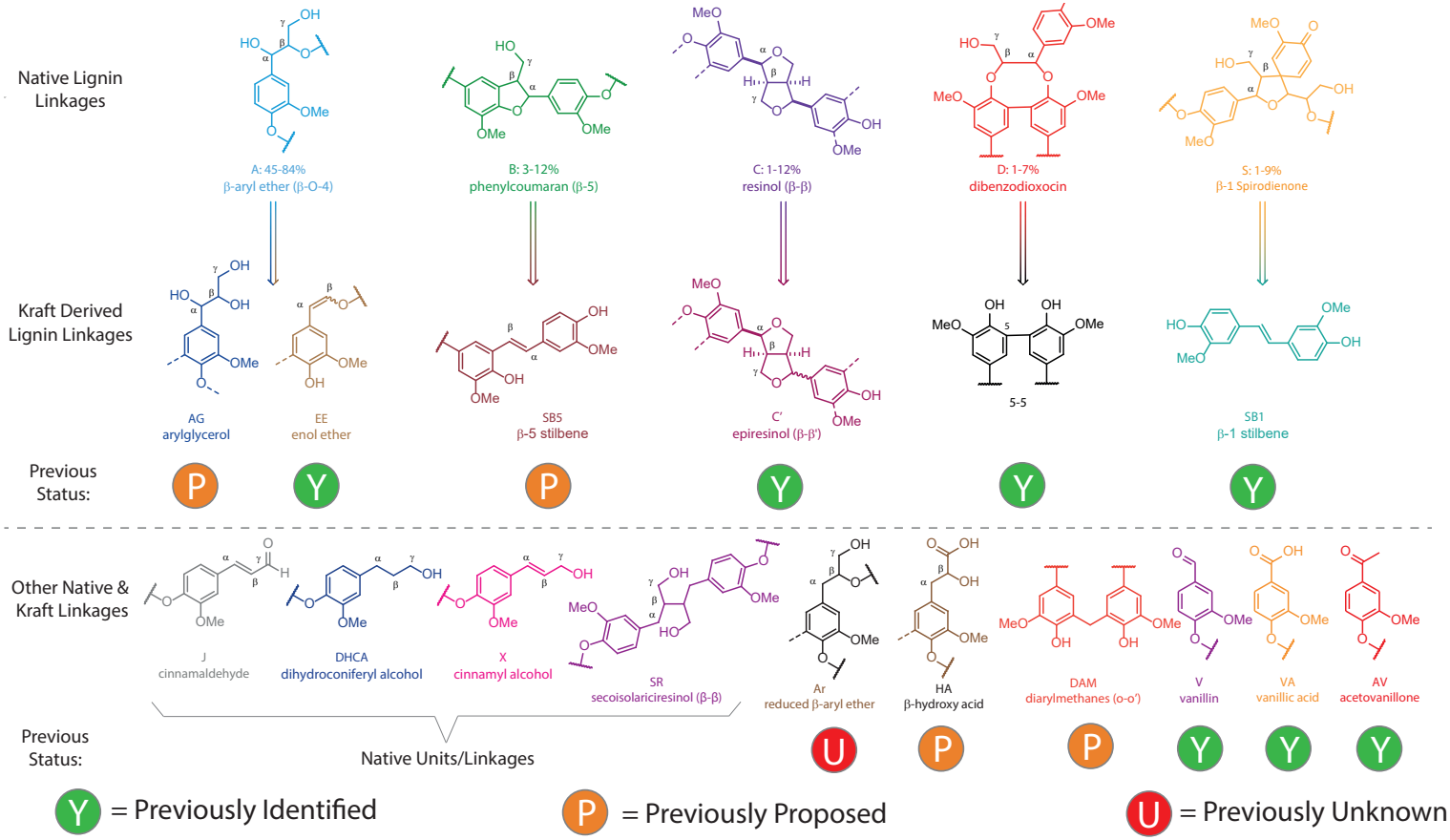
Kraft Lignin: Structure Elucidation by NMR

Indulin AT Lignin



- Quantification by HSQC accounts for up to ~**40% of the aromatic units** in kraft lignin

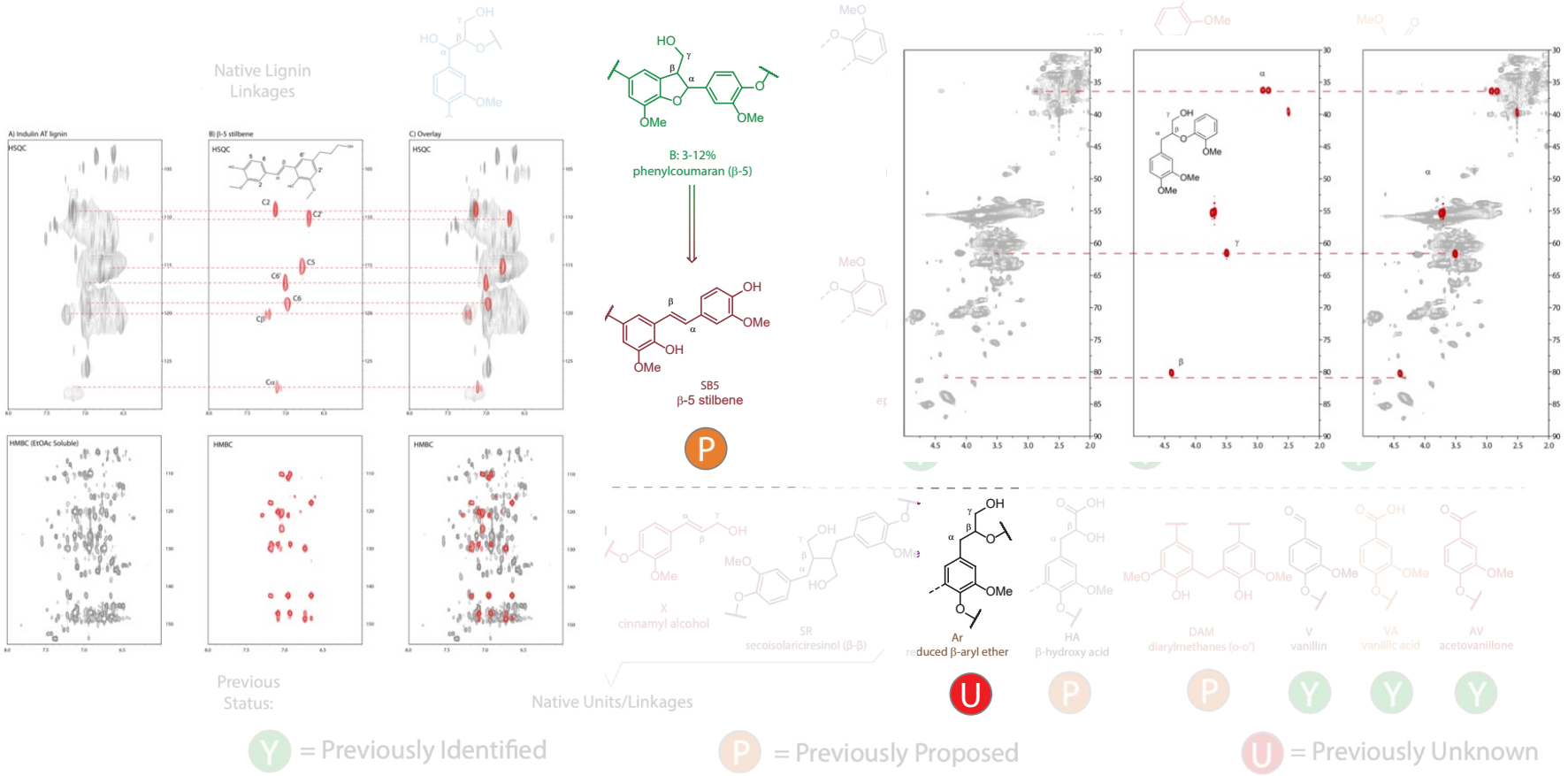
Kraft Lignin: Structure Elucidation by NMR



- Insight into the **chemistry of kraft pulping**

Lancefield, Bruijnincx et al., Chem. Sci. 2018, 9, 6348

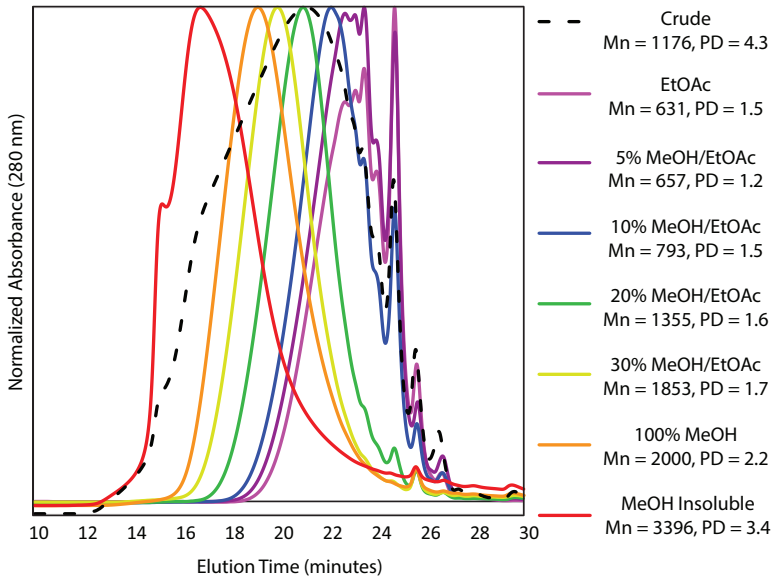
Authentic Model Compounds are Key

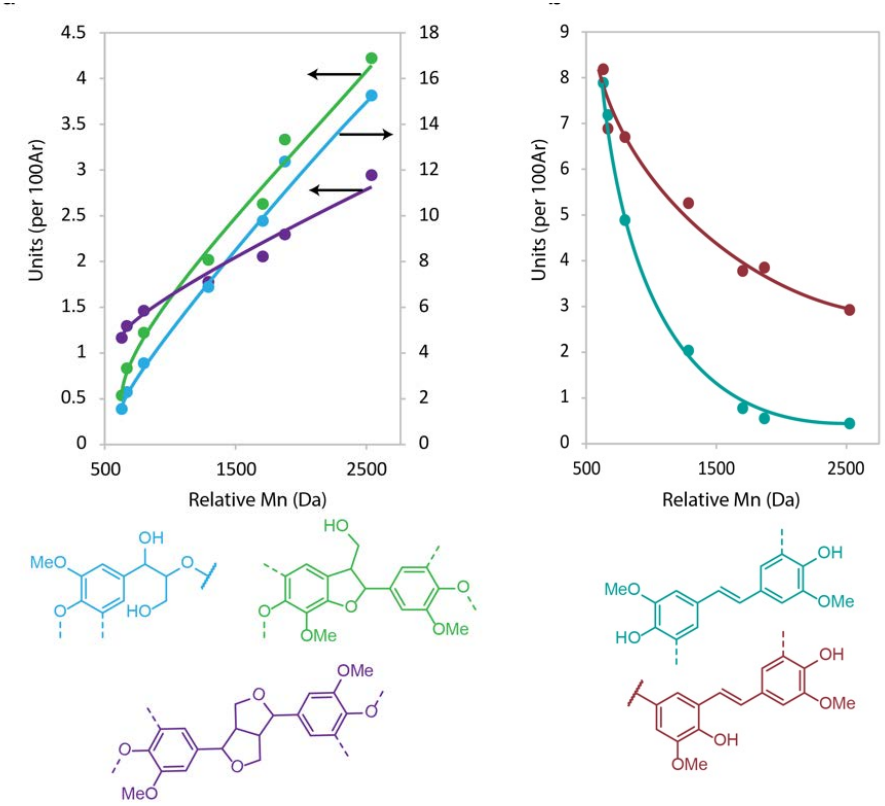


- Access to **high fidelity model compounds** have been key to understanding lignin structure

Deuss et al. Chemsuschem 2020, 13, 4238

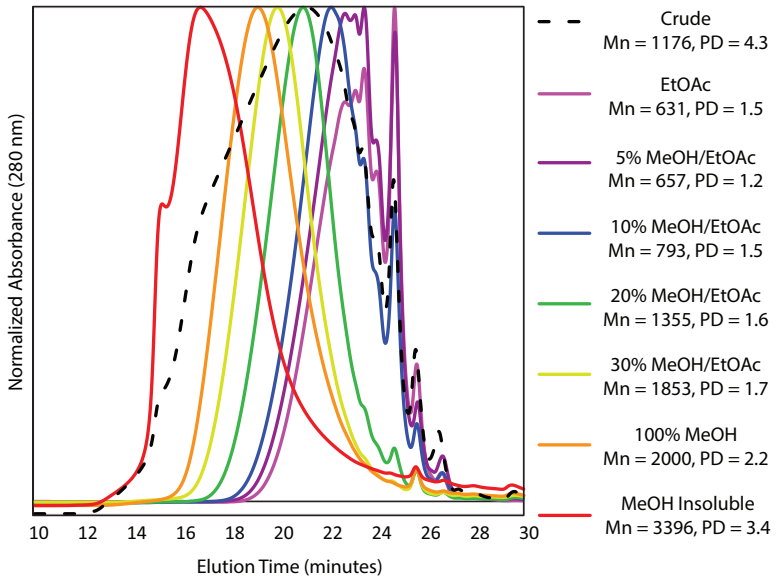
Fractionation Aids Quantification and Structure Elucidation





- **Fraction analysis** improved HSQC linkage quantification
- large MW range and high polydispersity: **Relaxation characteristics** of polymers means quantitative analysis is biased toward low MW components (even with Q-HSQC)

Fractionation Aids Quantification and Structure Elucidation



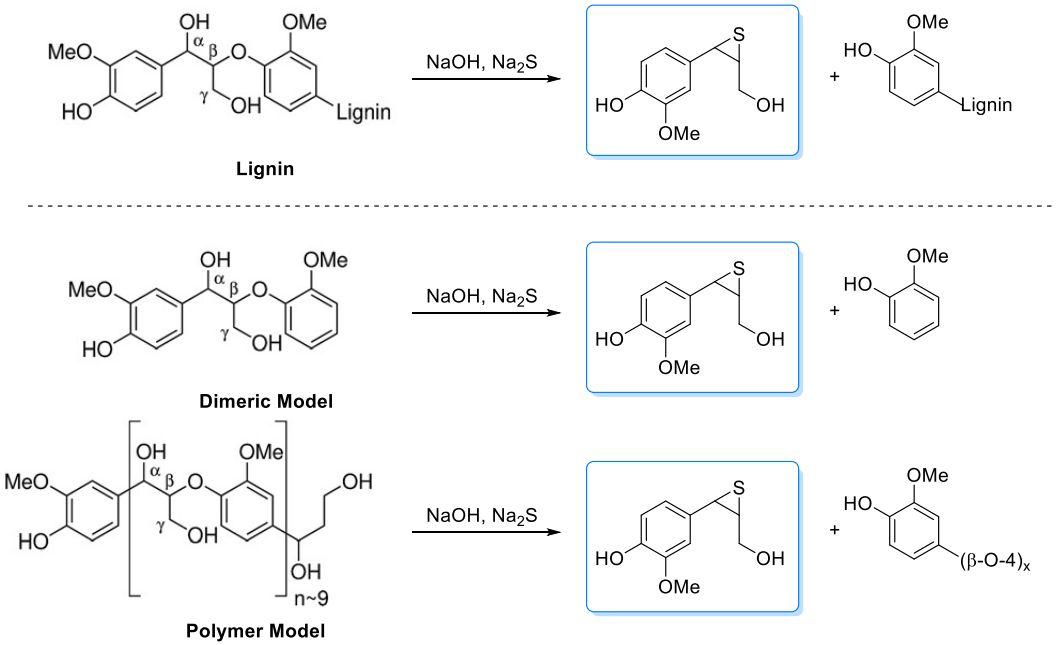
Unit[a]	Spruce CEL	Indulin AT kraft lignin		
		Crude	MW adjusted[d]	MW adjusted + $HSQC_0$[d]
A	38.5	7.0	9.8	10.3
B	13.0	1.6	2.8	2.6
C	3.0	1.6	2.1	1.8
D	2.4	0.2	—	—
X	5.8	1.8	1.7	1.6
SR	1.0	2.1	2.0	1.8
DHCA	5.2	4.7	4.6	4.1
J	3.7	0.0	0.0	0.0
S	2.9	0.0	0.0	0.0
C′	0.0	0.5	0.6	0.6
Z-EE	0.0	1.1	1.0	1.0
E-EE	0.0	2.8	2.5	3.1
SB1	0.0	2.7	1.5	1.9
SB5	0.0	6.5	3.8	—
AG	1.4	2.2[b]	3.8[b]	—
V	1.4	0.8	—	—
AV	0.0	0.7	—	—
VA	2.0	0.4	—	—
HA	0.0	0.8	—	—
Ar	0.0	0.5	—	—
Total[c]	84.3	45.3	38.6	35.2

- Bias in HSQC NMR experiments **can be significant**, but does not affect overall balance of assignment ~40%.
- Native overestimated, end groups underestimated, kraft derived underestimated

NMR of Model Compounds: New Insights from Synthetic Kraft Lignin

- What about the **other 60%** of kraft lignin?

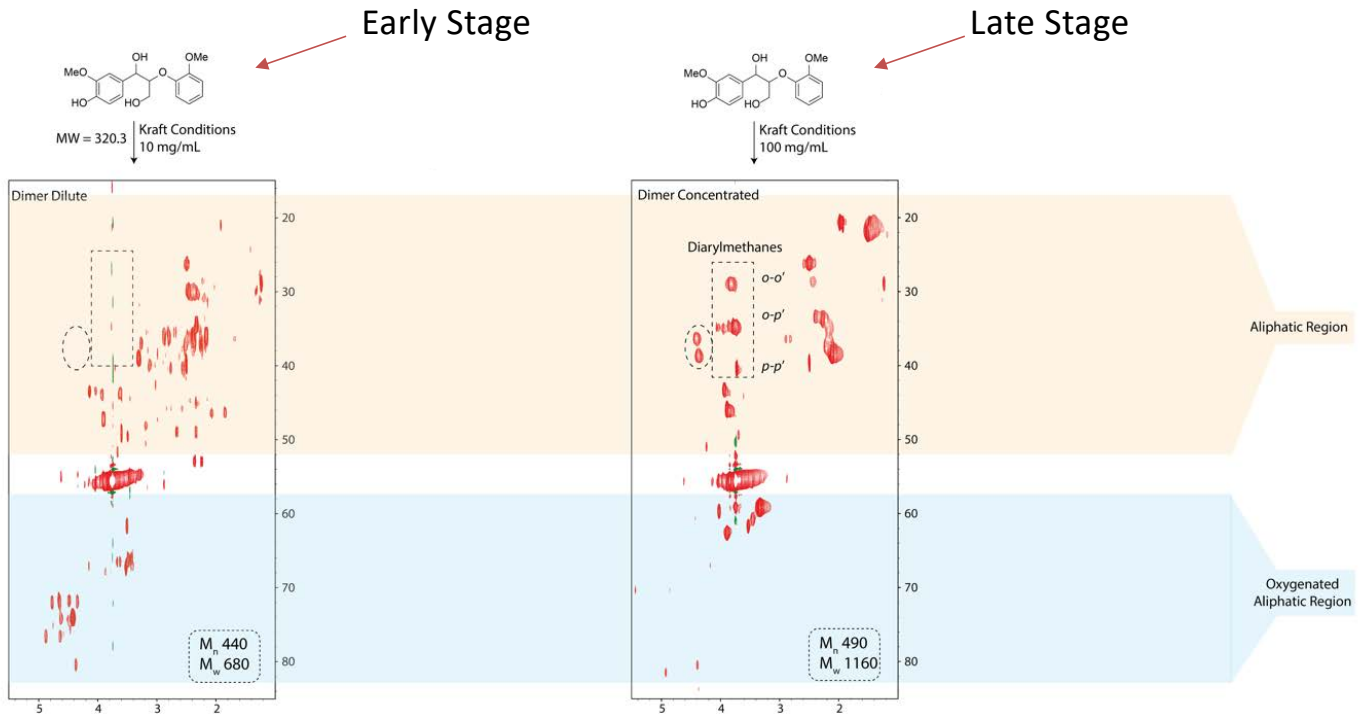
- Only ~15% β-O-4 linkages or derived products, i.e. ~45% still missing.



Phenol Content ~20%
No Guaiacol Released
High Molecular Weight

- Identical **reactive intermediates** from lignin and lignin model compounds

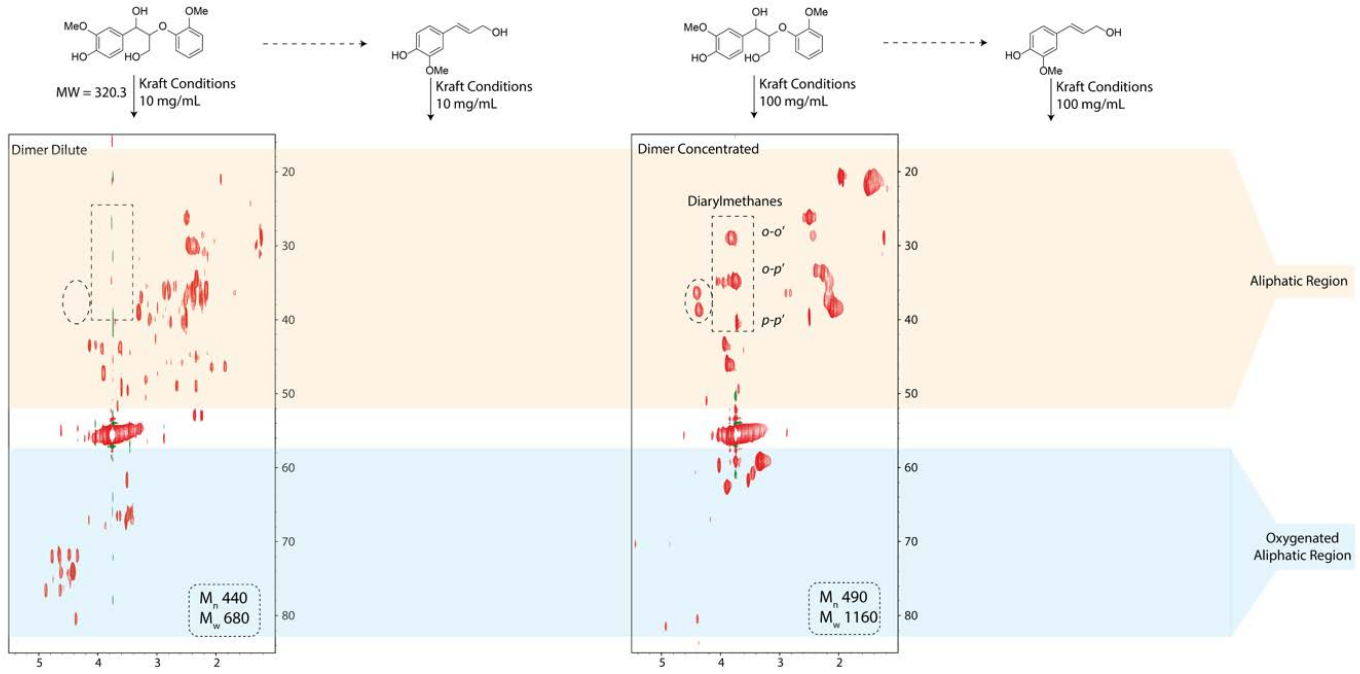
New Insights from Synthetic Kraft Lignin: Dimers



Kraft conditions = 0.25 M Na_2S, 0.12 M NaOH, H_2O, 170 ° C, 2 hours.

- Dimers and polymers mimic kraft chemistry remarkably well

New Insights from Synthetic Kraft Lignin: Dimers



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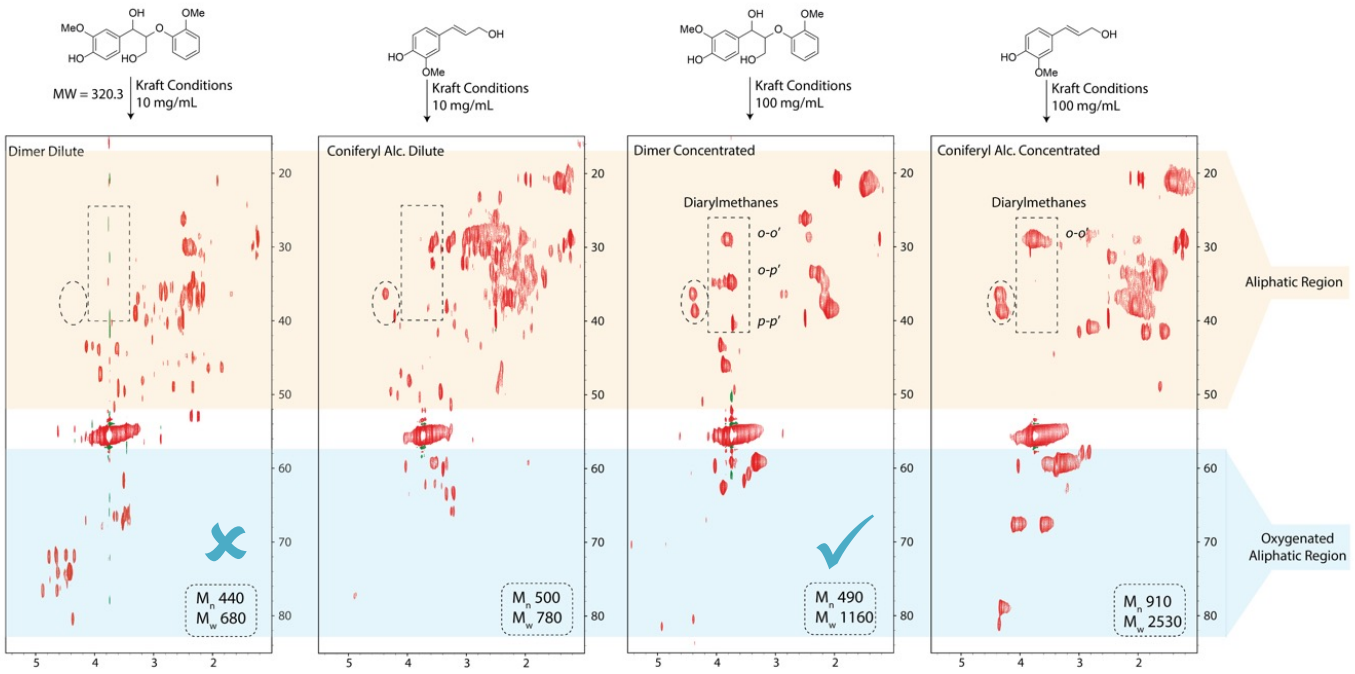
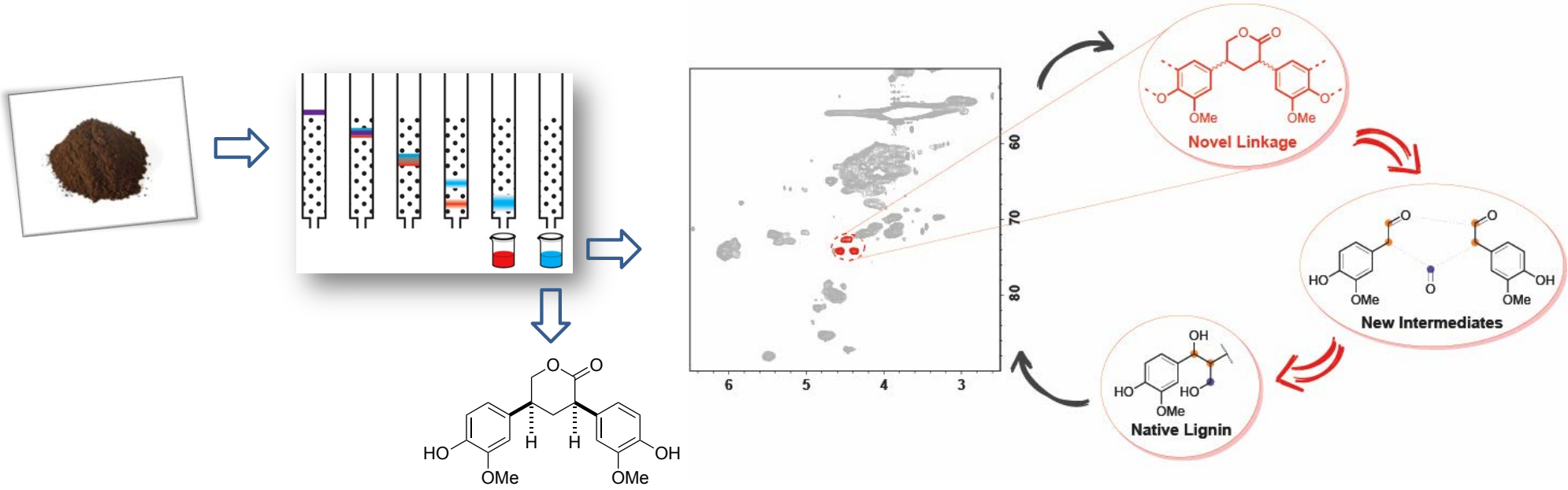
New Insights from Synthetic Kraft Lignin: Dimers



Kraft conditions = 0.25 M Na_2S, 0.12 M NaOH, H_2O, 170 ° C, 2 hours.

- Dimers and polymers **mimic Kraft chemistry** remarkably well
- Coniferyl alcohol cannot account for all condensation

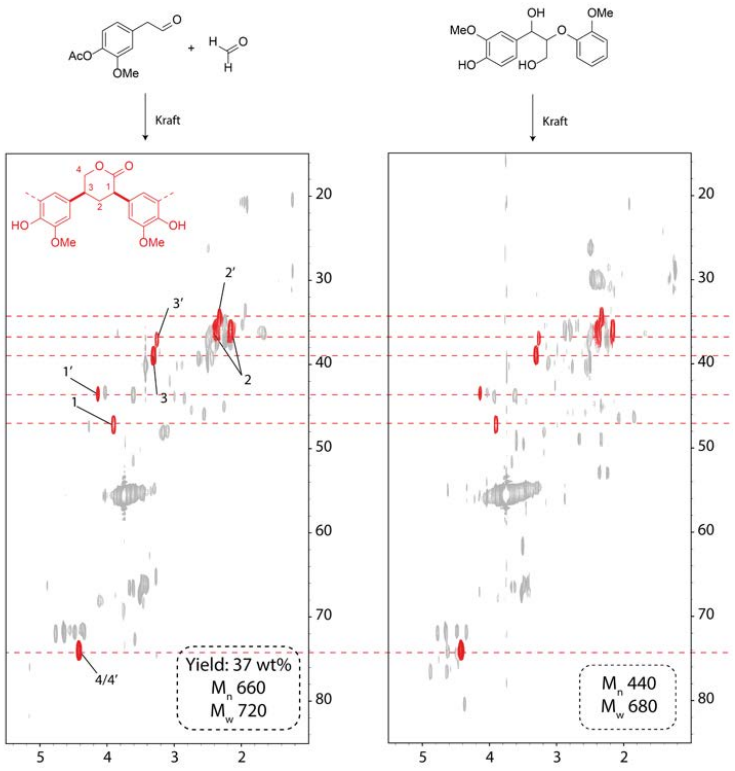
Kraft Lignin: A Synthetic Chemist's Approach

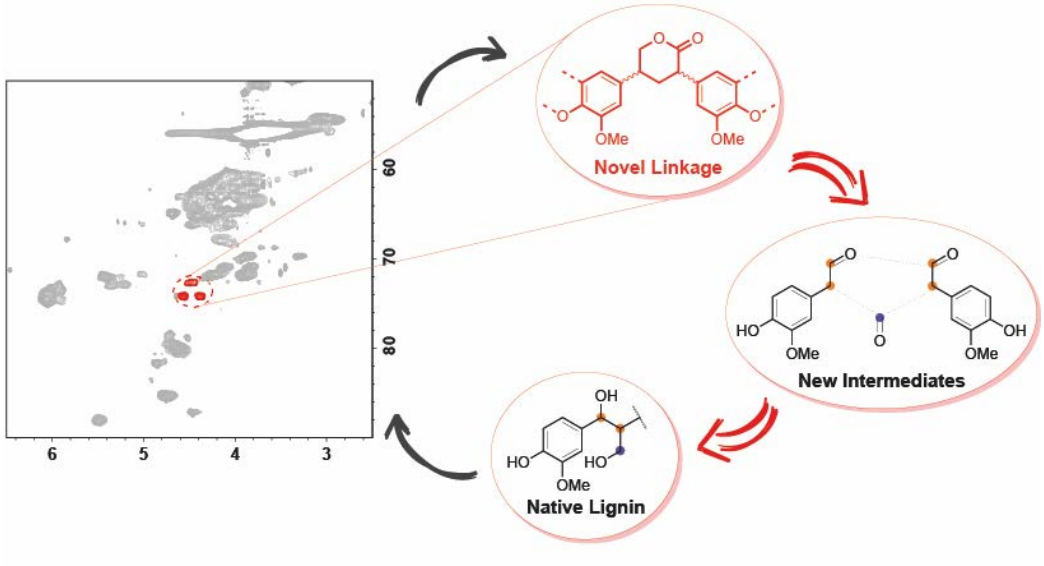


- Structural insight from smallest Mw fraction: **new lactone** isolated
- Lactone revealed a **new mechanism of condensation**

Lancefield, Bruijnincx et al., Chem. Sci. 2018, 9, 6348

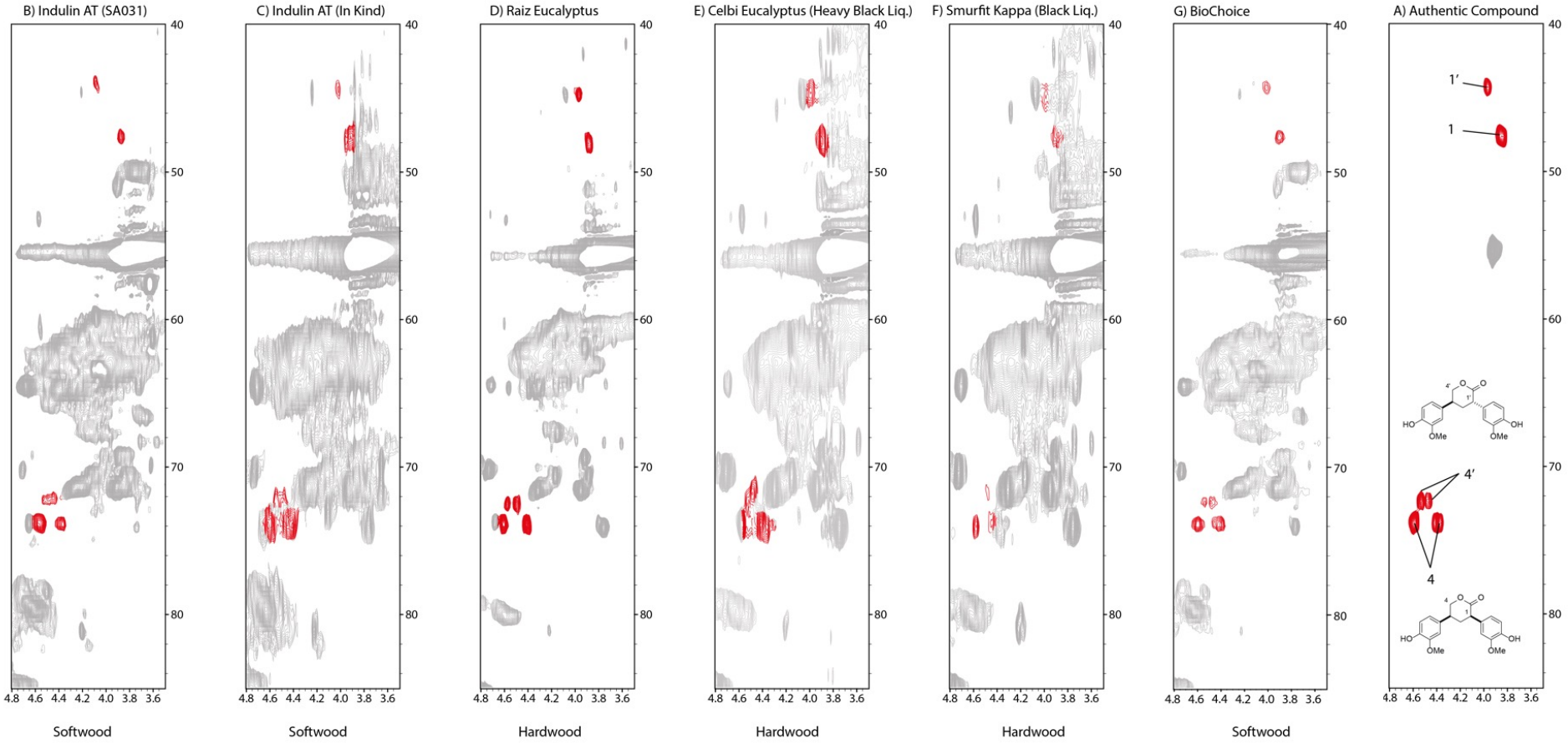
Kraft Lignin: A Highly Diagnostic New Linkage





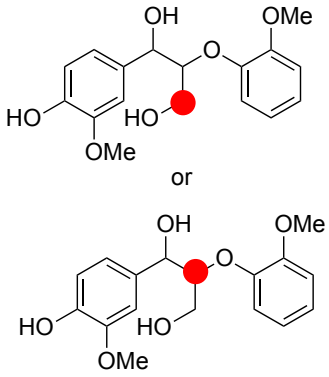
- A minor, new lactone linkage is highly diagnostic of a **new homovanillin condensation** pathway

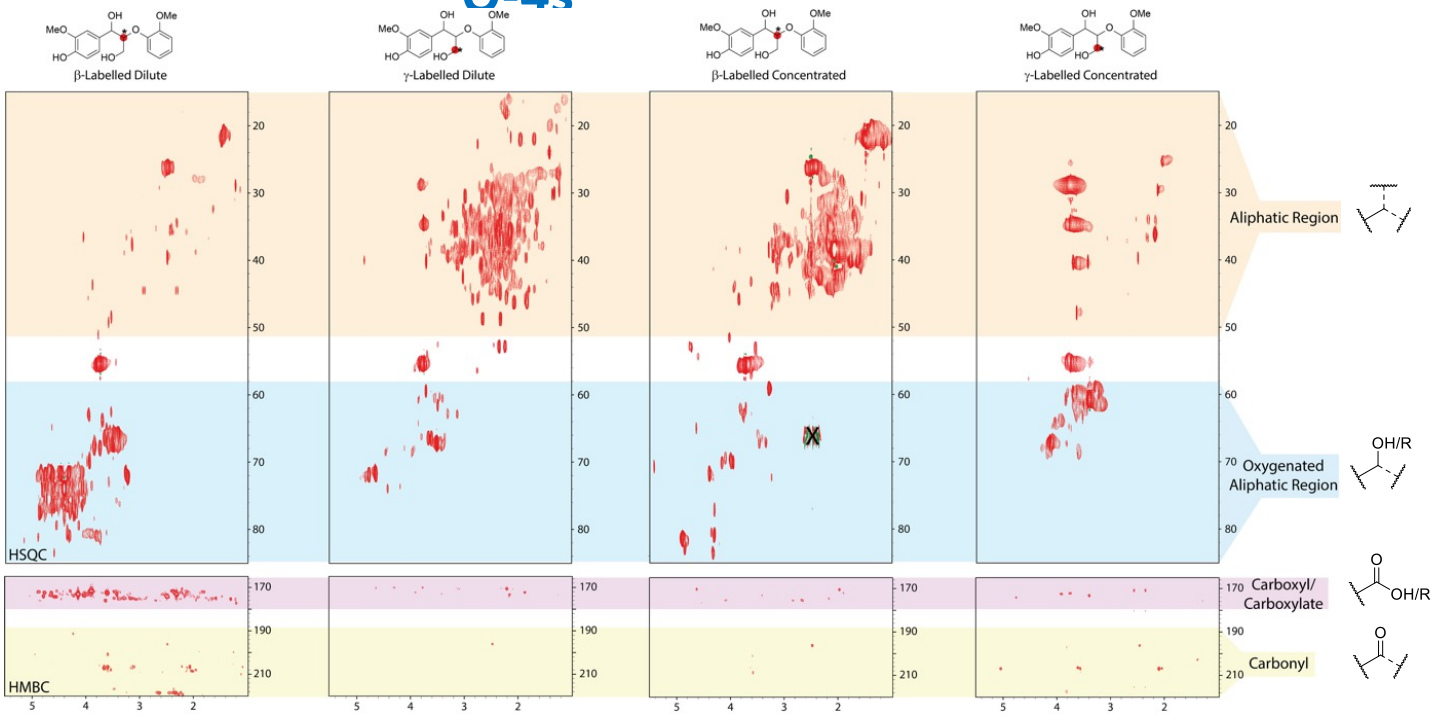
Kraft Lignin: A Highly Diagnostic New Linkage



- This lactone is detected in **all Kraft lignins analyzed**; mechanism is generally applicable

Labeled Model Compounds: The Fate of the Other β-O-4s



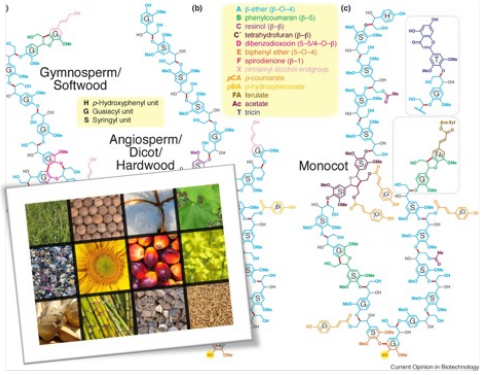


- β- and γ-carbon labels end up in **>150 unique new chemical environments**
- Fate of the β–O-4 seems **intractible**: another way in which Kraft lignin is **recalcitrant**

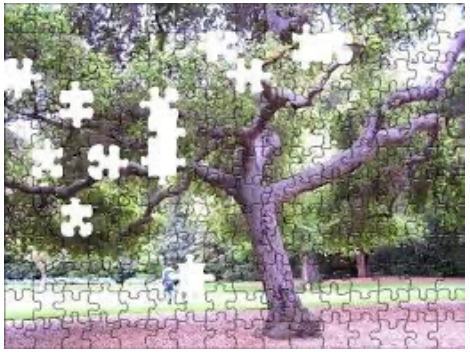
Lancefield, Bruijnincx et al., Chem. Sci. 2018, 9, 6348

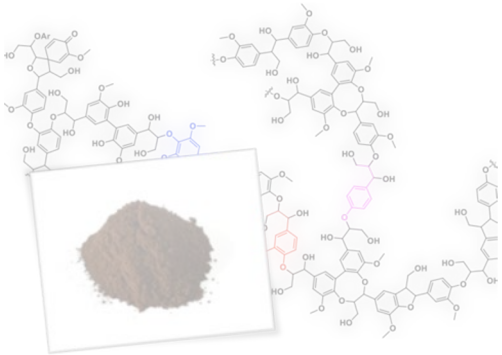
The Lignin Platform: Dealing With Structural Complexity

- What is it we seek to know?



- In planta lignin **structure elucidation**
- Species/mutation dependent **variation**
- **Valorization** opportunities





- Technical lignin **structure elucidation**
- Biorefinery operation dependent **variation**
- **Valorization** opportunities

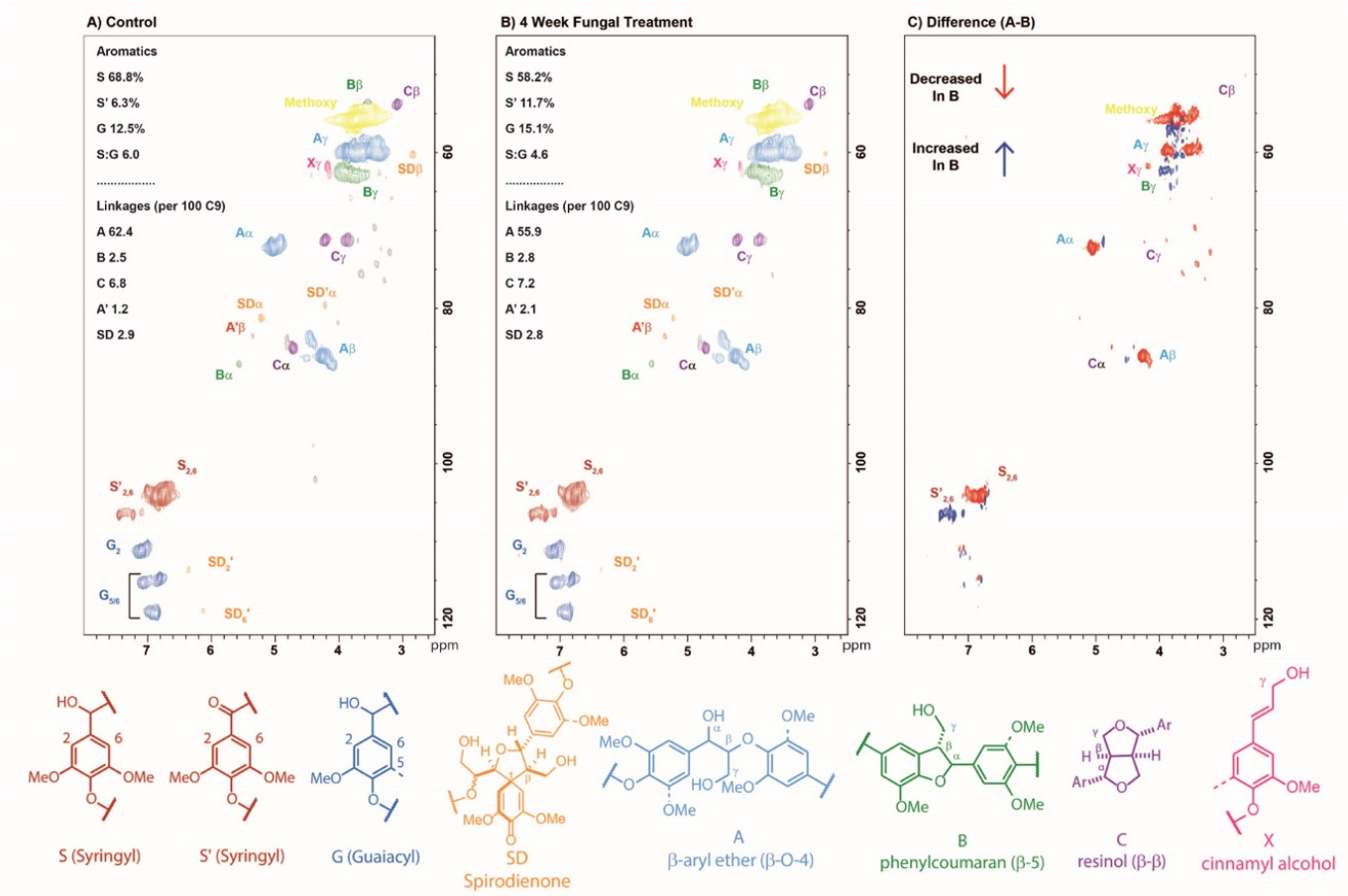
Lignin NMR: HSQC of whole biomass

- Wood degradation by ***D. Squalens*** (white rot fungi)

Daly, Lancefield, Bruijnincx, de Vries et al. Environ Microbiol 2018, 20, 4141

Lignin NMR: HSQC of whole biomass

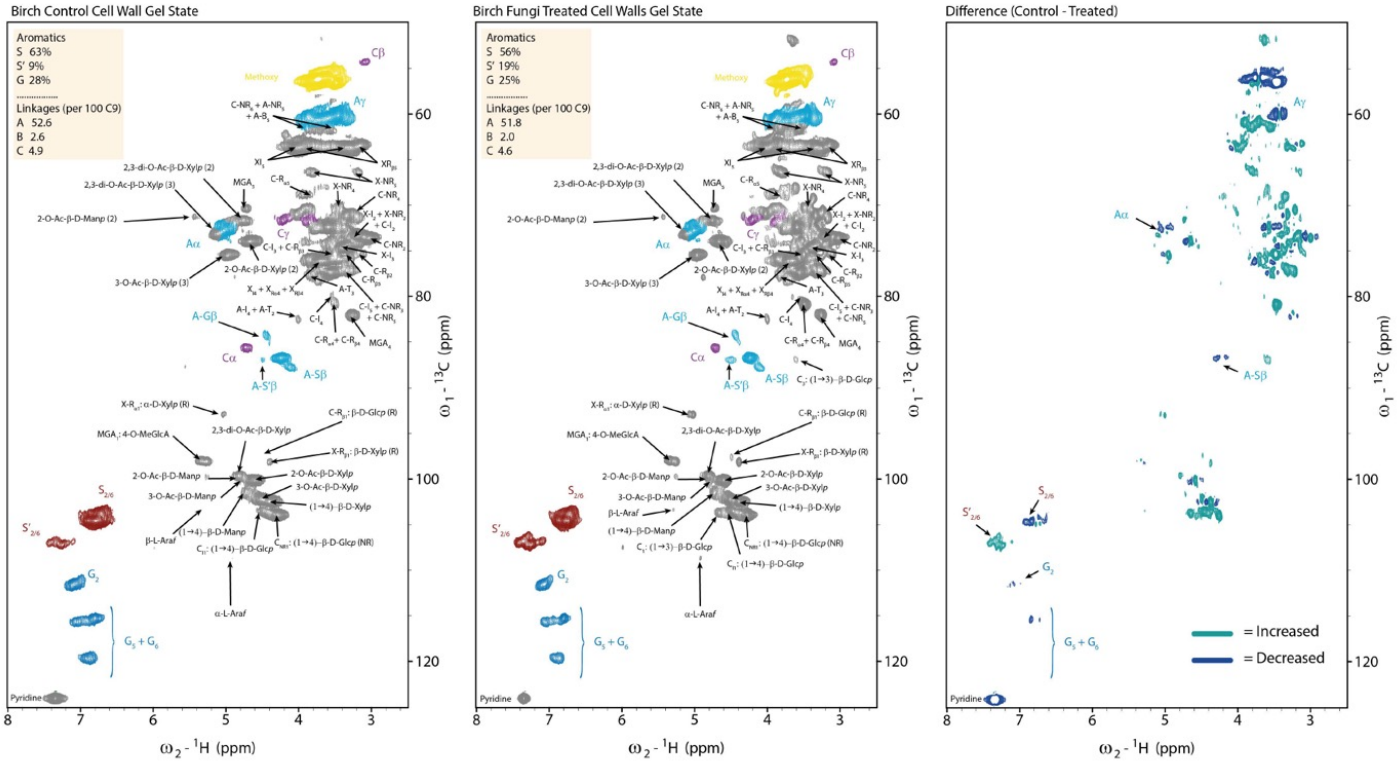
- **Isolate the lignin** by enzymatic treatment using cellulase/hemicellulase cocktail
- Measure **solution HSQC** NMR
- **Difference spectra** provide insight into the complexity



Daly, Lancefield, Bruijnincx, de Vries et al. Environ Microbiol 2018, 20, 4141

Lignin NMR: HSQC of whole biomass

- Directly measure the **whole biomass sample** by **gel-state whole cell wall** HSQC NMR



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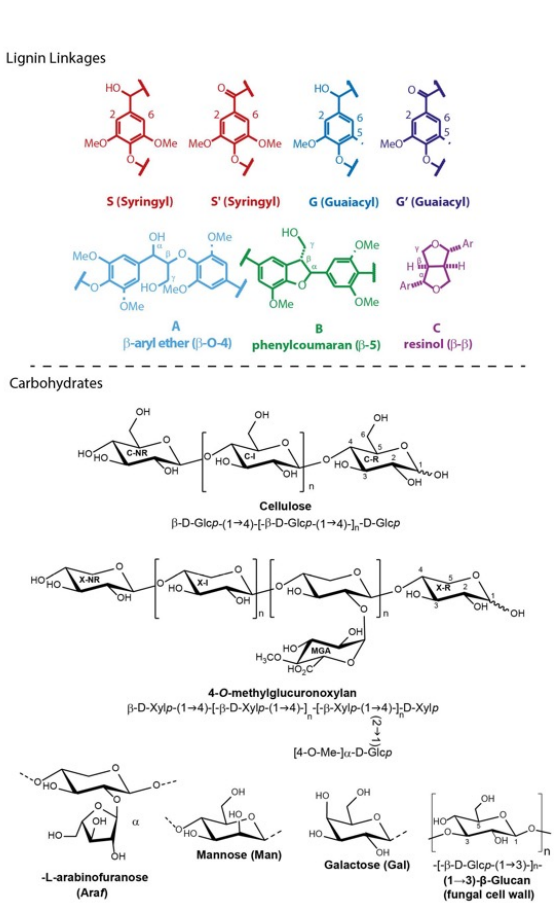
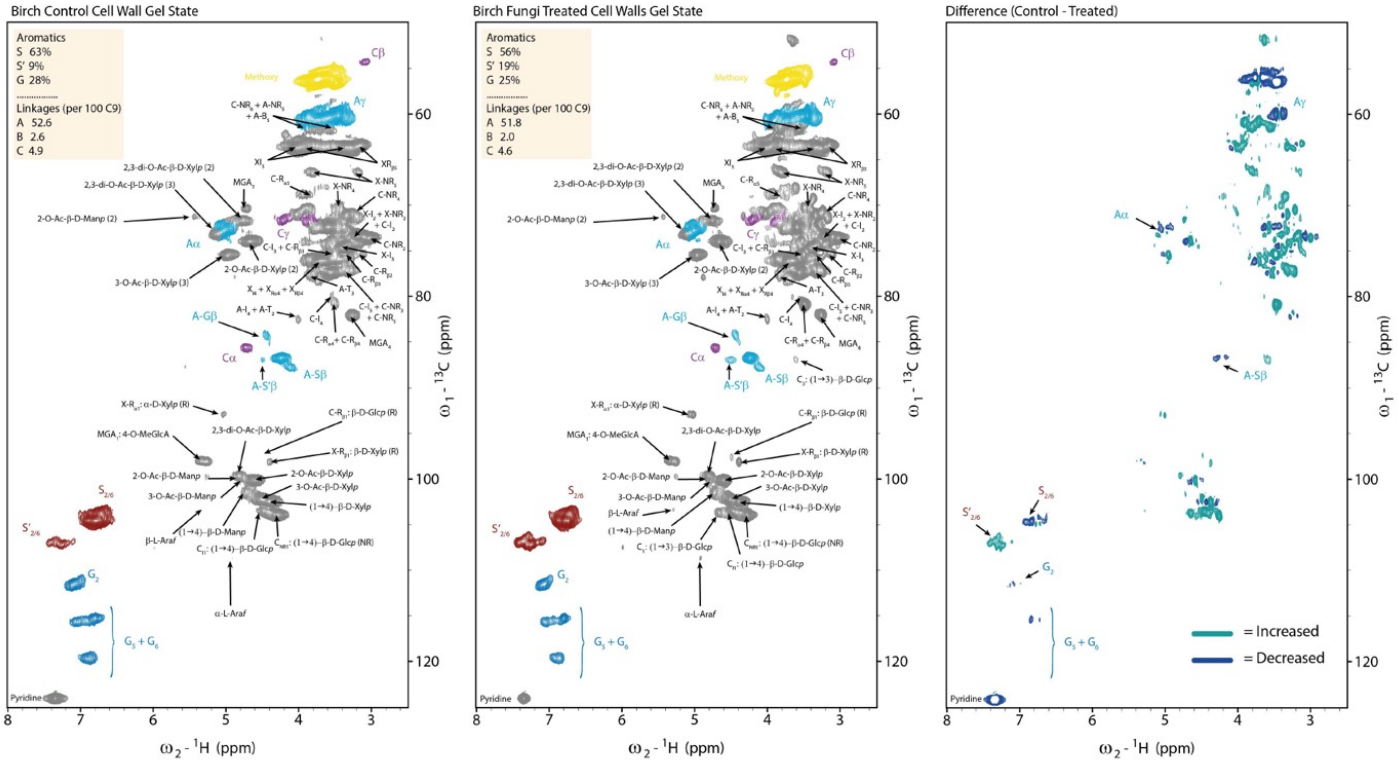
Lignin NMR: HSQC of whole biomass





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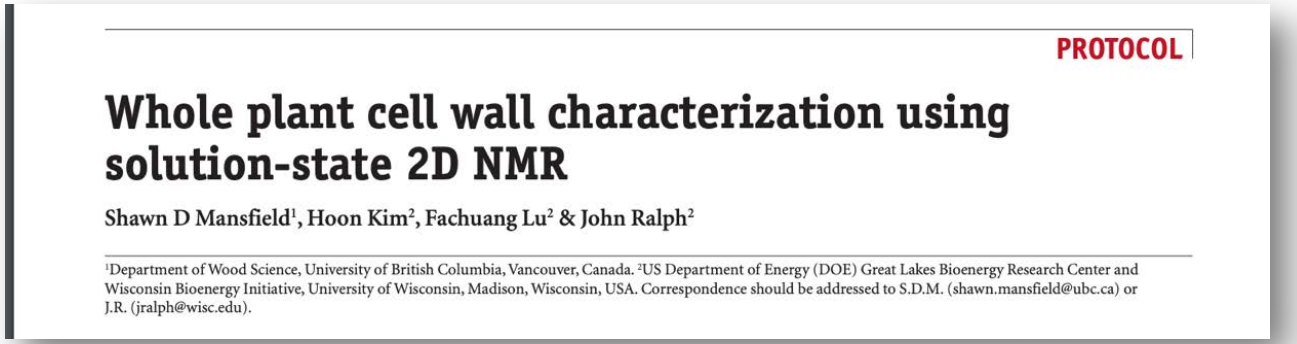
Lignin NMR: HSQC of whole biomass

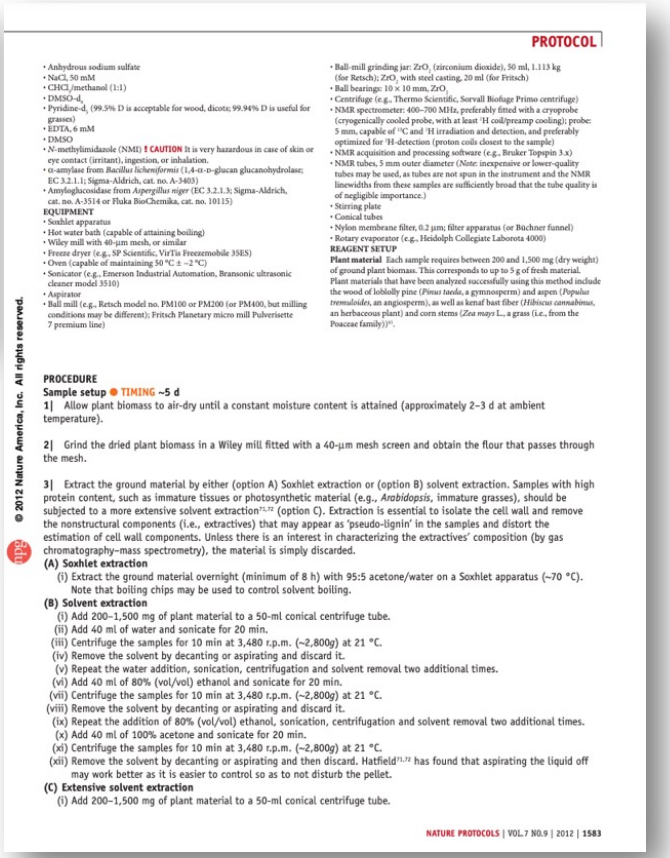


PROTOCOL

Whole plant cell wall characterization using solution-state 2D NMR

Shawn D Mansfield[1], Hoon Kim[2], Fachuang Lu[2] & John Ralph[2]

[1]Department of Wood Science, University of British Columbia, Vancouver, Canada. [2]US Department of Energy (DOE) Great Lakes Bioenergy Research Center and Wisconsin Bioenergy Initiative, University of Wisconsin, Madison, Wisconsin, USA. Correspondence should be addressed to S.D.M. (shawn.mansfield@ubc.ca) or J.R. (jralph@wisc.edu).



PROTOCOL

- Anhydrous sodium sulfate
- NaCl, 50 mM
- $CHCl_3$/methanol (1:1)
- DMSO-d_6
- Pyridine-d_5 (99.5% D is acceptable for wood, dicots; 99.94% D is useful for grasses)
- EDTA, 6 mM
- DMSO
- *N*-methylimidazole (NMI) ! CAUTION It is very hazardous in case of skin or eye contact (irritant), ingestion, or inhalation.
- α-amylase from *Bacillus licheniformis* (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1; Sigma-Aldrich, cat. no. A-3403)
- Amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3; Sigma-Aldrich, cat. no. A-3514 or Fluka BioChemika, cat. no. 10115)

EQUIPMENT

- Soxhlet apparatus
- Hot water bath (capable of attaining boiling)
- Wiley mill with 40-μm mesh, or similar
- Freeze dryer (e.g., SP Scientific, VirTis Freezemobile 35ES)
- Oven (capable of maintaining 50 °C ± −2 °C)
- Sonicator (e.g., Emerson Industrial Automation, Bransonic ultrasonic cleaner model 3510)
- Aspirator
- Ball mill (e.g., Retsch model no. PM100 or PM200 (or PM400, but milling conditions may be different); Fritsch Planetary micro mill Pulverisette 7 premium line)
- Ball-mill grinding jar: ZrO_2 (zirconium dioxide), 50 ml, 1.113 kg (for Retsch); ZrO_2 with steel casting, 20 ml (for Fritsch)
- Ball bearings: 10 × 10 mm, ZrO_2
- Centrifuge (e.g., Thermo Scientific, Sorvall Biofuge Primo centrifuge)
- NMR spectrometer: 400–700 MHz, preferably fitted with a cryoprobe (cryogenically cooled probe, with at least ^1H coil/preamp cooling); probe: 5 mm, capable of ^{13}C and ^1H irradiation and detection, and preferably optimized for ^1H-detection (proton coils closest to the sample)
- NMR acquisition and processing software (e.g., Bruker Topspin 3.x)
- NMR tubes, 5 mm outer diameter (*Note:* inexpensive or lower-quality tubes may be used, as tubes are not spun in the instrument and the NMR linewidths from these samples are sufficiently broad that the tube quality is of negligible importance.)
- Stirring plate
- Conical tubes
- Nylon membrane filter, 0.2 μm; filter apparatus (or Büchner funnel)
- Rotary evaporator (e.g., Heidolph Collegiate Laborota 4000)

REAGENT SETUP

Plant material Each sample requires between 200 and 1,500 mg (dry weight) of ground plant biomass. This corresponds to up to 5 g of fresh material. Plant materials that have been analyzed successfully using this method include the wood of loblolly pine (*Pinus taeda*, a gymnosperm) and aspen (*Populus tremuloides*, an angiosperm), as well as kenaf bast fiber (*Hibiscus cannabinus*, an herbaceous plant) and corn stems (*Zea mays* L., a grass (i.e., from the Poaceae family))[17].

PROCEDURE

Sample setup ● TIMING ~5 d

1| Allow plant biomass to air-dry until a constant moisture content is attained (approximately 2–3 d at ambient temperature).

2| Grind the dried plant biomass in a Wiley mill fitted with a 40-μm mesh screen and obtain the flour that passes through the mesh.

3| Extract the ground material by either (option A) Soxhlet extraction or (option B) solvent extraction. Samples with high protein content, such as immature tissues or photosynthetic material (e.g., *Arabidopsis*, immature grasses), should be subjected to a more extensive solvent extraction[71,72] (option C). Extraction is essential to isolate the cell wall and remove the nonstructural components (i.e., extractives) that may appear as 'pseudo-lignin' in the samples and distort the estimation of cell wall components. Unless there is an interest in characterizing the extractives' composition (by gas chromatography–mass spectrometry), the material is simply discarded.

(A) Soxhlet extraction

(i) Extract the ground material overnight (minimum of 8 h) with 95:5 acetone/water on a Soxhlet apparatus (~70 °C). Note that boiling chips may be used to control solvent boiling.

(B) Solvent extraction

(i) Add 200–1,500 mg of plant material to a 50-ml conical centrifuge tube.
(ii) Add 40 ml of water and sonicate for 20 min.
(iii) Centrifuge the samples for 10 min at 3,480 r.p.m. (~2,800*g*) at 21 °C.
(iv) Remove the solvent by decanting or aspirating and discard it.
(v) Repeat the water addition, sonication, centrifugation and solvent removal two additional times.
(vi) Add 40 ml of 80% (vol/vol) ethanol and sonicate for 20 min.
(vii) Centrifuge the samples for 10 min at 3,480 r.p.m. (~2,800*g*) at 21 °C.
(viii) Remove the solvent by decanting or aspirating and discard it.
(ix) Repeat the addition of 80% (vol/vol) ethanol, sonication, centrifugation and solvent removal two additional times.
(x) Add 40 ml of 100% acetone and sonicate for 20 min.
(xi) Centrifuge the samples for 10 min at 3,480 r.p.m. (~2,800*g*) at 21 °C.
(xii) Remove the solvent by decanting or aspirating and then discard. Hatfield[71,72] has found that aspirating the liquid off may work better as it is easier to control so as to not disturb the pellet.

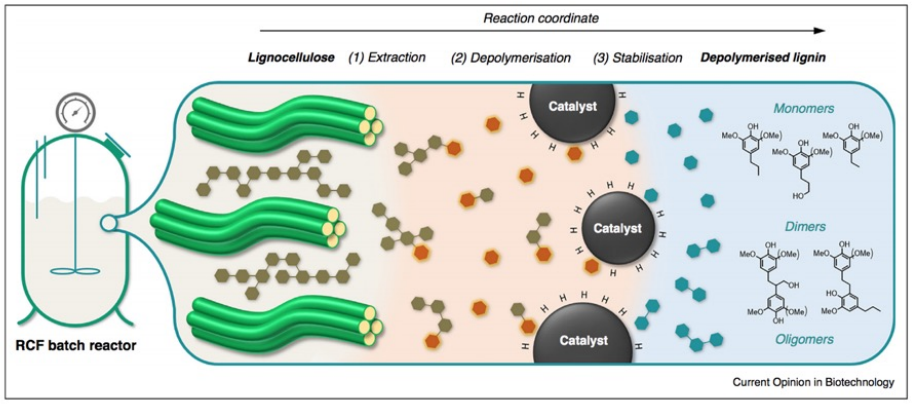
(C) Extensive solvent extraction

(i) Add 200–1,500 mg of plant material to a 50-ml conical centrifuge tube.

- Collect biomass sample, remove extractives by solvent wash; dry
- Ball mill
- Dissolve milled cell wall material in d6-DMSO/d5-py (4:1) to give a uniform gel for NMR analysis
- Run your HSQC

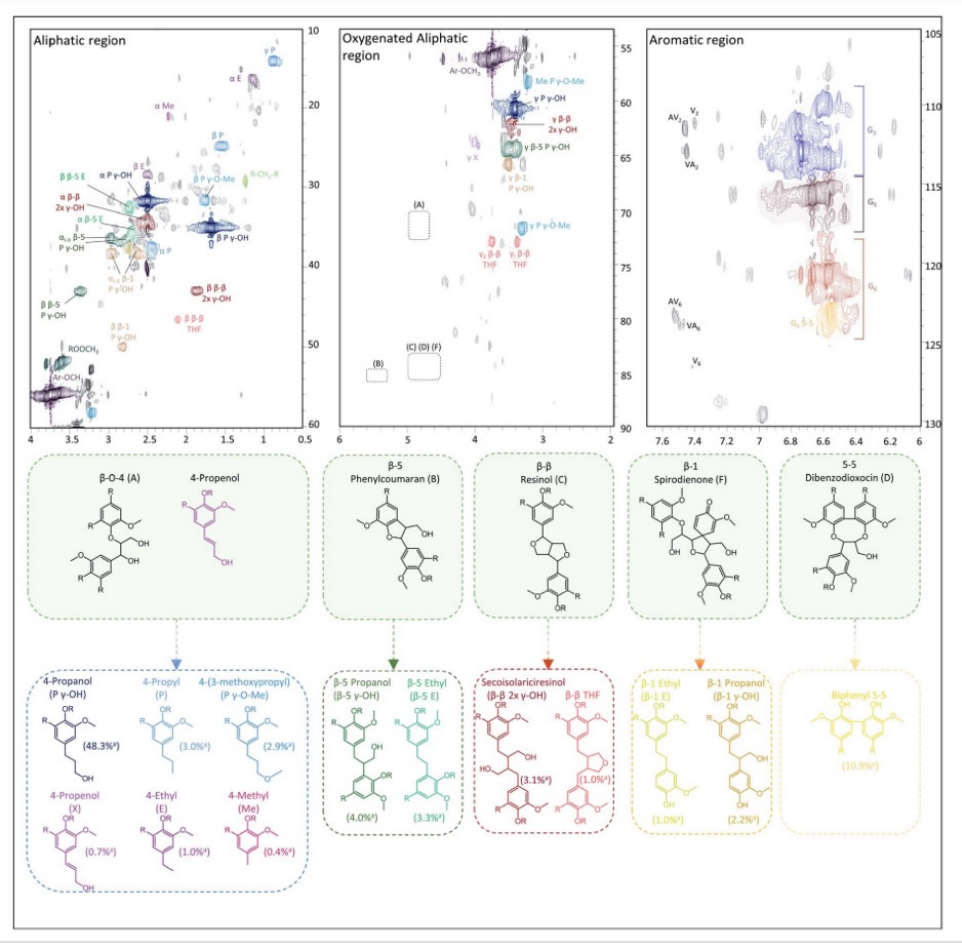
Mansfield, Ralph, Nat Prot 2012, 7, 1579

Lignin NMR: HSQC of lignin-derived product mixtures



Sels et al. Curr. Opin. Biotechnol. 2019, 56, 193

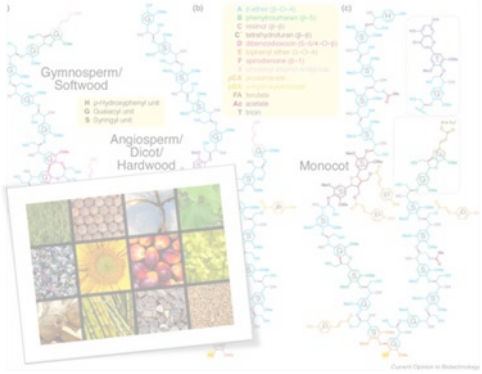
- Lignin derived products (e.g. From pyrolysis, RCF, liquifaction, etc) are **complex**
- Extensive characterization is again required



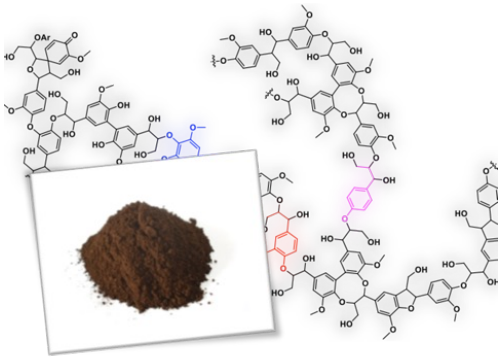
Sels et al. Chem. Sci. 2020, 11, 11498

The Lignin Platform: Dealing With Structural Complexity

- What is it we seek to know?



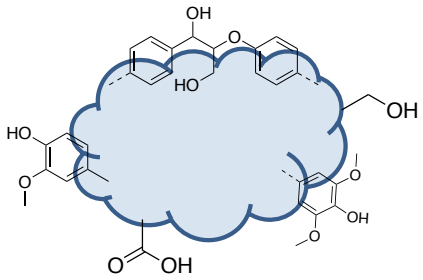
- In planta lignin **structure elucidation**
- Species/mutation dependent **variation**
- **Valorization** opportunities



- Technical lignin **structure elucidation**
- Biorefinery operation dependent **variation**
- **Valorization** opportunities

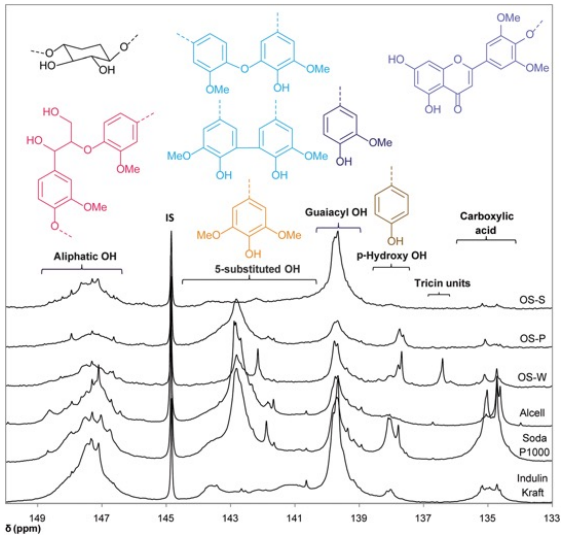
Lignin NMR: Opportunities Abound

- Structure analysis: **functional groups**



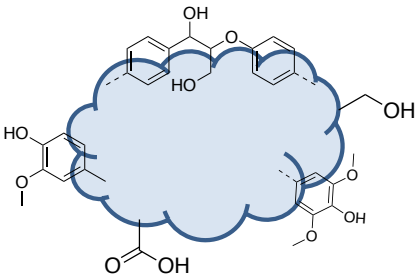
- (Mw-dependent) insight into functional group **density and type** is essential for (mechanistic) understanding of lignin formation and properties and to **guide further valorization**

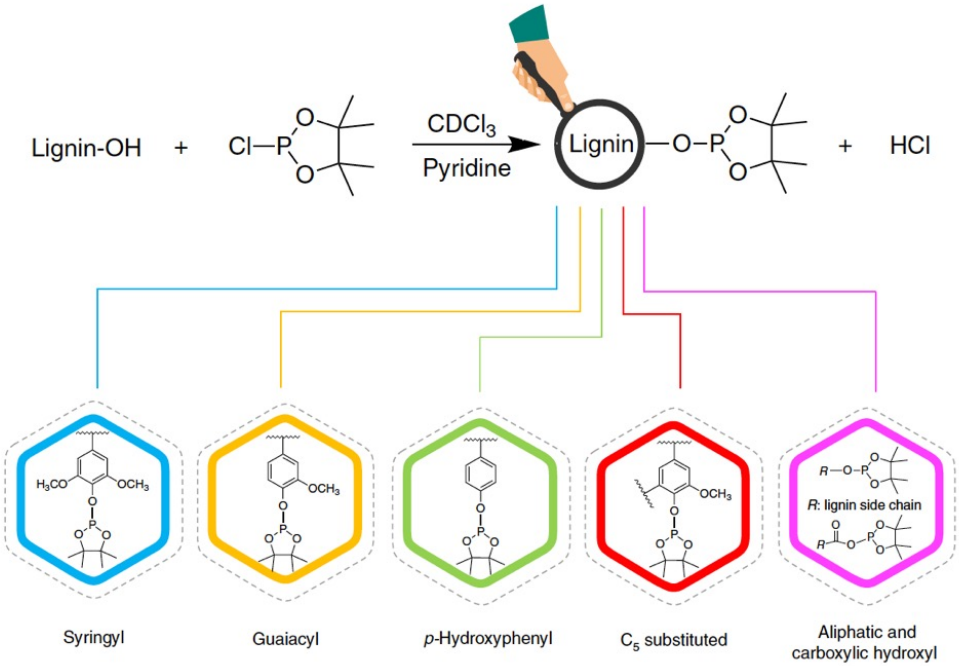
- **Heteronuclear** NMR: ^{31}P, ^{19}F, …



(Lignin) NMR: ^{31}P NMR

- Structure analysis: functional groups





nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-019-0191-1

Determination of hydroxyl groups in biorefinery resources via quantitative ^{31}P NMR spectroscopy

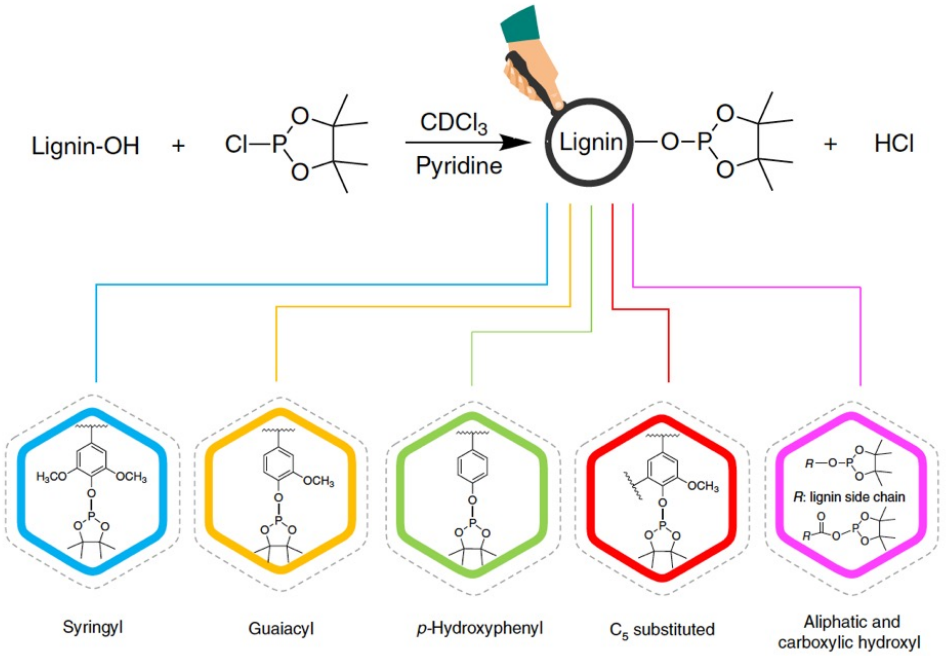
Xianzhi Meng[1], Claudia Crestini[2*], Haoxi Ben[3], Naijia Hao[1], Yunqiao Pu[4], Arthur J. Ragauskas[1,4,5*] and Dimitris S. Argyropoulos[6*]

Nat Prot 2019, 14, 2627

(Lignin) NMR: ^{31}P NMR

Very versatile method; examples of analysis of whole biomass, pyrolysis oils, tannins, LCCs

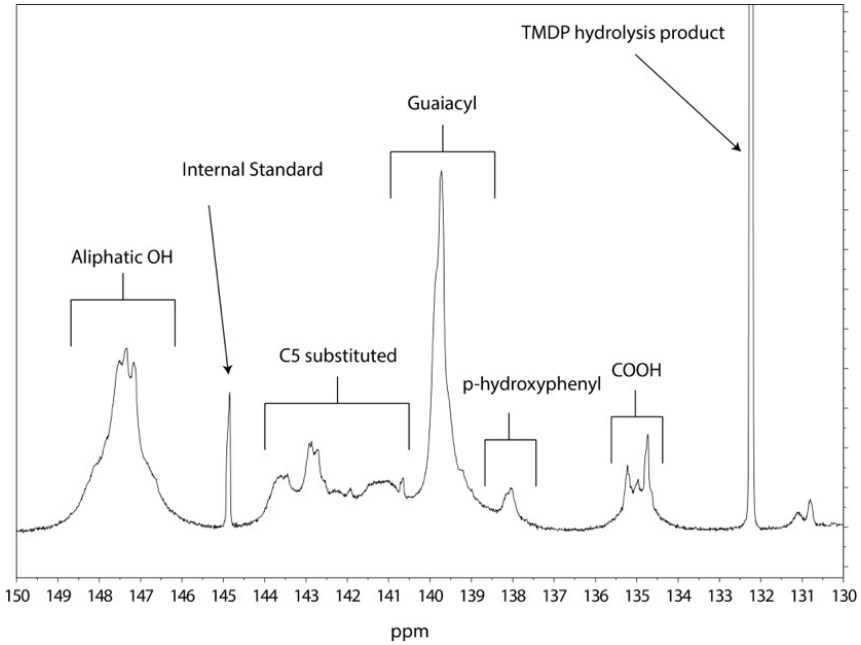
- Pyridine/$CDCl_3$ (1.6/1)
- TMDP
- $Cr(acac)_3$ as relaxation agent
- IS: NHND, cholesterol, cyclohexanol, …
- **Little sample** is needed and acquisition is **relatively fast**



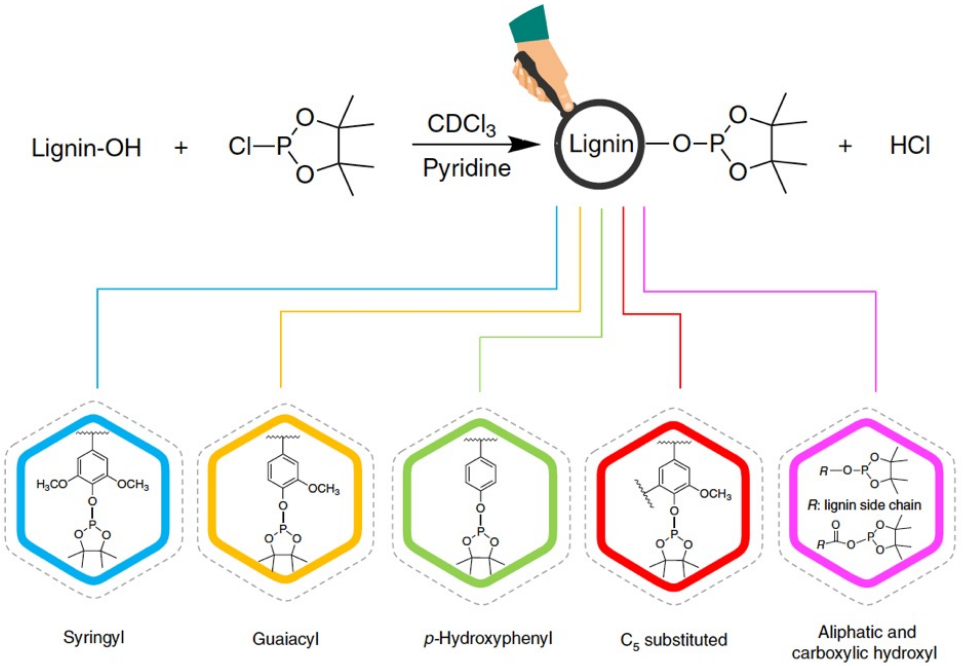
Crestini, Ragauskas, Argyropoulos et al. Nat Prot 2019, 14, 2627

(Lignin) NMR: ^{31}P NMR

- Structure analysis: **functional groups**



Indulin AT (Lancefield, Bruijnincx et al. Chem Sci 2018)



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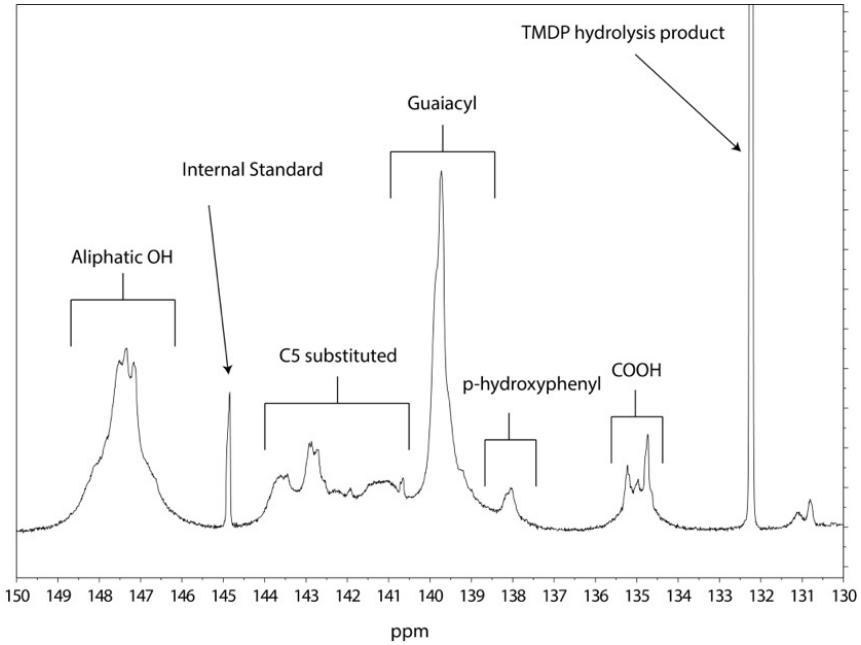
(Lignin) NMR: ^{31}P NMR

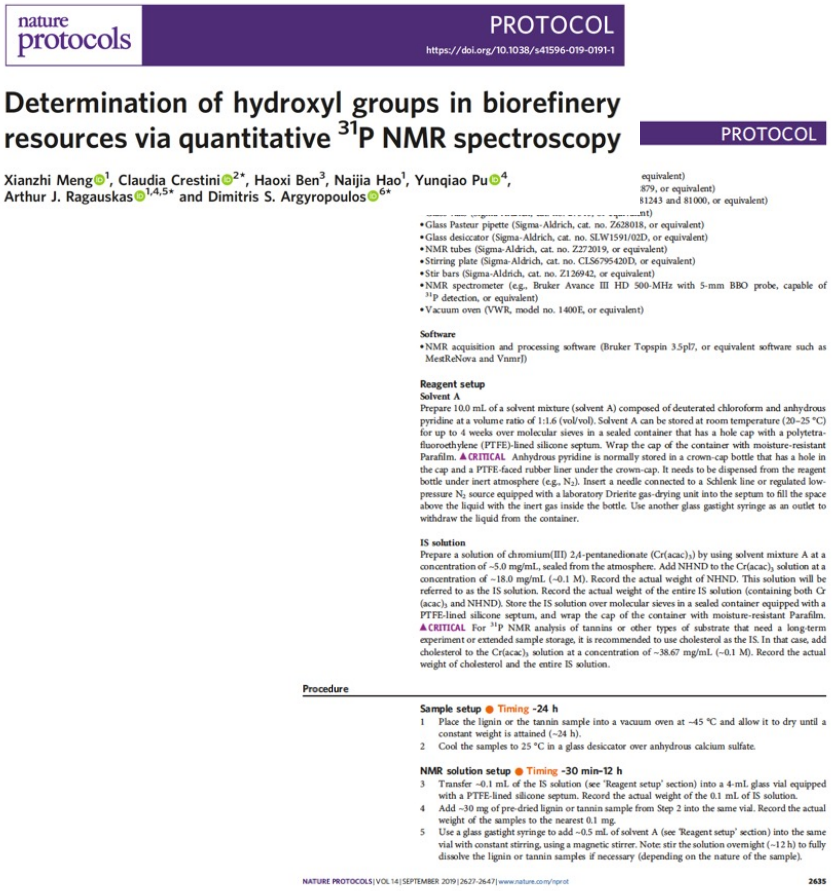
- Structure analysis: **functional groups**



Indulin AT (Lancefield, Bruijnincx et al. Chem Sci 2018)

- Reproducibility needs careful attention (error bars vary from person to person and lab to lab
- TMDP is expensive (and sometimes not available)
- Samples need to be **relatively pure** (free of ash, aldehydes, sugars, sulfur species, …)
- S and G condensed cannot be separated

(Lignin) NMR: ^{31}P NMR



nature protocols

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PROTOCOL

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equivalent)
879, or equivalent)
81243 and 81000, or equivalent)

- Glass Pasteur pipette (Sigma-Aldrich, cat. no. Z628018, or equivalent)
- Glass desiccator (Sigma-Aldrich, cat. no. SLW1591/02D, or equivalent)
- NMR tubes (Sigma-Aldrich, cat. no. Z272019, or equivalent)
- Stirring plate (Sigma-Aldrich, cat. no. CLS6795420D, or equivalent)
- Stir bars (Sigma-Aldrich, cat. no. Z126942, or equivalent)
- NMR spectrometer (e.g., Bruker Avance III HD 500-MHz with 5-mm BBO probe, capable of ^{31}P detection, or equivalent)
- Vacuum oven (VWR, model no. 1400E, or equivalent)

Software

- NMR acquisition and processing software (Bruker Topspin 3.5pl7, or equivalent software such as MestReNova and Vnmrj)

Reagent setup

Solvent A

Prepare 10.0 mL of a solvent mixture (solvent A) composed of deuterated chloroform and anhydrous pyridine at a volume ratio of 1:1.6 (vol/vol). Solvent A can be stored at room temperature (20–25 °C) for up to 4 weeks over molecular sieves in a sealed container that has a hole cap with a polytetrafluoroethylene (PTFE)-lined silicone septum. Wrap the cap of the container with moisture-resistant Parafilm. ▲ CRITICAL Anhydrous pyridine is normally stored in a crown-cap bottle that has a hole in the cap and a PTFE-faced rubber liner under the crown-cap. It needs to be dispensed from the reagent bottle under inert atmosphere (e.g., N_2). Insert a needle connected to a Schlenk line or regulated low-pressure N_2 source equipped with a laboratory Drierite gas-drying unit into the septum to fill the space above the liquid with the inert gas inside the bottle. Use another glass gastight syringe as an outlet to withdraw the liquid from the container.

IS solution

Prepare a solution of chromium(III) 2,4-pentanedionate ($Cr(acac)_3$) by using solvent mixture A at a concentration of ~5.0 mg/mL, sealed from the atmosphere. Add NHND to the $Cr(acac)_3$ solution at a concentration of ~18.0 mg/mL (~0.1 M). Record the actual weight of NHND. This solution will be referred to as the IS solution. Record the actual weight of the entire IS solution (containing both $Cr(acac)_3$ and NHND). Store the IS solution over molecular sieves in a sealed container equipped with a PTFE-lined silicone septum, and wrap the cap of the container with moisture-resistant Parafilm. ▲ CRITICAL For ^{31}P NMR analysis of tannins or other types of substrate that need a long-term experiment or extended sample storage, it is recommended to use cholesterol as the IS. In that case, add cholesterol to the $Cr(acac)_3$ solution at a concentration of ~38.67 mg/mL (~0.1 M). Record the actual weight of cholesterol and the entire IS solution.

Procedure

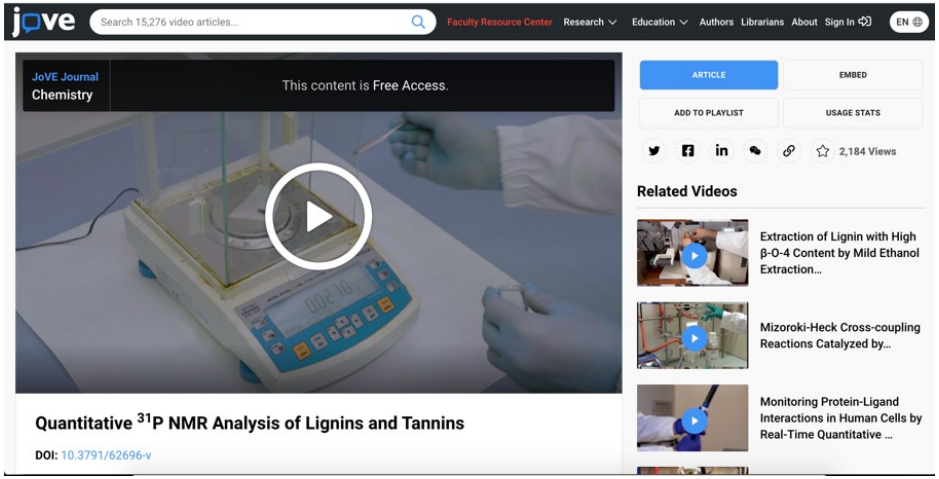
Sample setup ● Timing ~24 h

1 Place the lignin or the tannin sample into a vacuum oven at ~45 °C and allow it to dry until a constant weight is attained (~24 h).
2 Cool the samples to 25 °C in a glass desiccator over anhydrous calcium sulfate.

NMR solution setup ● Timing ~30 min–12 h

3 Transfer ~0.1 mL of the IS solution (see 'Reagent setup' section) into a 4-mL glass vial equipped with a PTFE-lined silicone septum. Record the actual weight of the 0.1 mL of IS solution.
4 Add ~30 mg of pre-dried lignin or tannin sample from Step 2 into the same vial. Record the actual weight of the samples to the nearest 0.1 mg.
5 Use a glass gastight syringe to add ~0.5 mL of solvent A (see 'Reagent setup' section) into the same vial with constant stirring, using a magnetic stirrer. Note: stir the solution overnight (~12 h) to fully dissolve the lignin or tannin samples if necessary (depending on the nature of the sample).

Nat Prot 2019, 14, 2627



jove

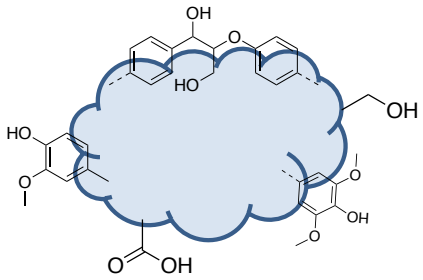
Quantitative ^{31}P NMR Analysis of Lignins and Tannins

Dimitris S. Argyropoulos[2], Nicolò Pajer[1], Claudia Crestini[1]

J. Vis. Exp. (174), e62696

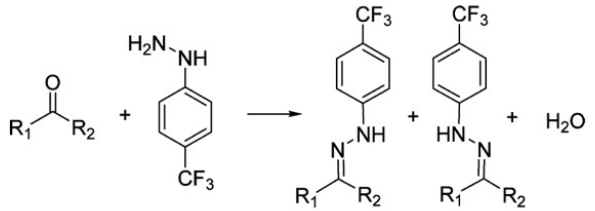
(Lignin) NMR: ^{19}F NMR

- Structure analysis: **functional groups**



- (Mw-dependent) insight into functional group **density and type** is essential for (mechanistic) understanding of lignin formation and properties and to **guide further valorization**

Scheme 1. Hydrazone Formation with 4-(Trifluoromethyl)phenylhydrazine[a]



[a]For nonsymmetrical ketones, both the *E* and *Z* isomers are typically observed.

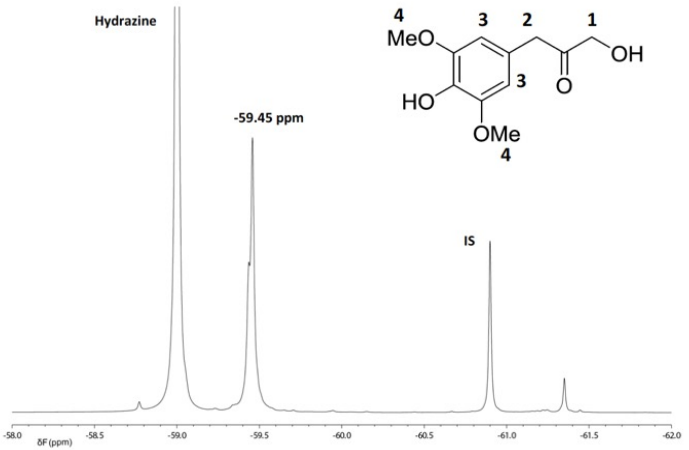
Constant, Weckhuysen, Bruijnincx et al. ACS Sustainable Chem. Eng. 2017, 5, 965; see also Lachenal et al. Holzforschung 2001, 55, 286

(Lignin) NMR: ^{19}F NMR

- d6-DMSO
- 4-(trifluoromethyl)phenylhydrazine
- Cr(acac)3 as relaxation agent
- IS: 1-methyl-4-(trifluoromethyl)benzene
- **Little sample** is needed and **acquisition is relatively fast**

But:

- Reproducibility needs careful attention
- Reactions are **not as clean** as with ^{31}P reagent



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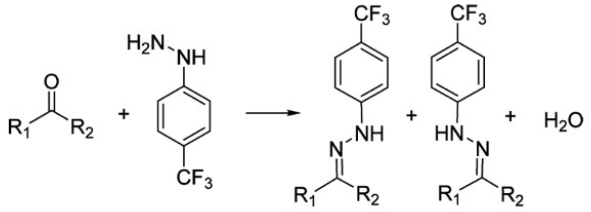

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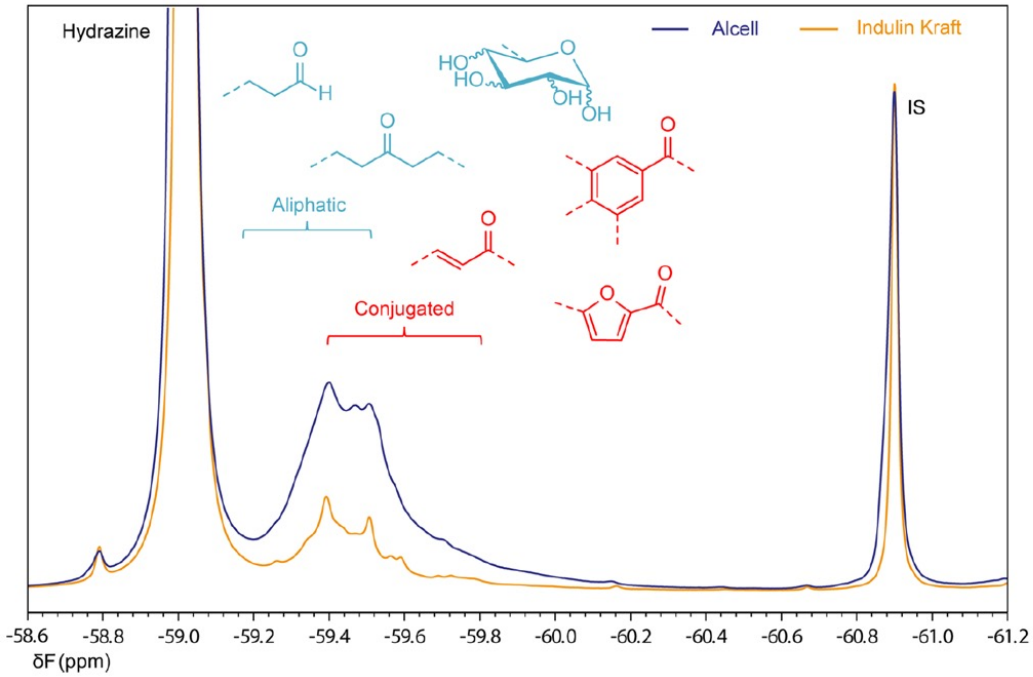


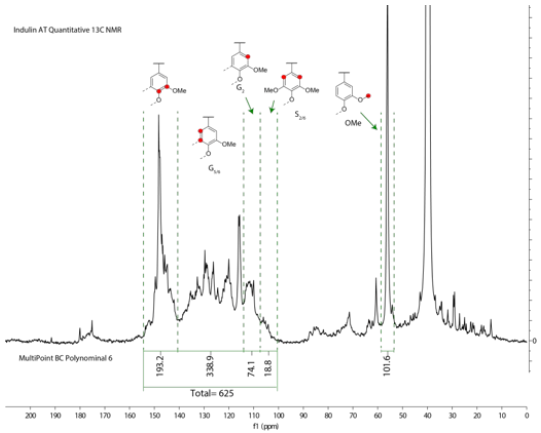
Table 2. Carbonyl Group Quantification in Humin and Lignins by ^{19}F NMR[a]

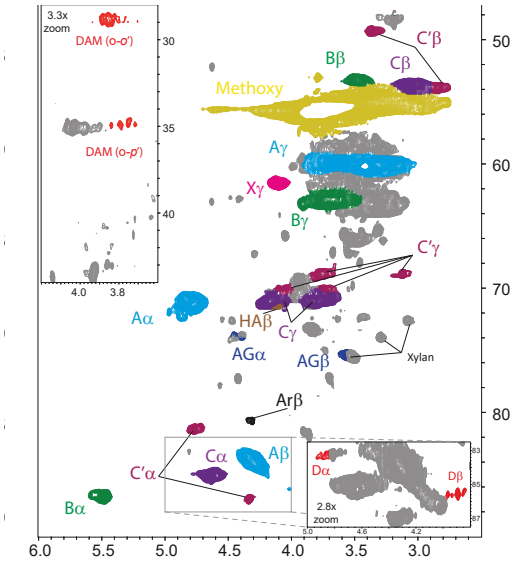
polymer	mmol function/g of polymer	wt %	CO per C_{900}[b]
humin	2.35	6.6	
Indulin Kraft lignin	0.60	1.7	10.8
Alcell lignin	1.17	3.3	20.9

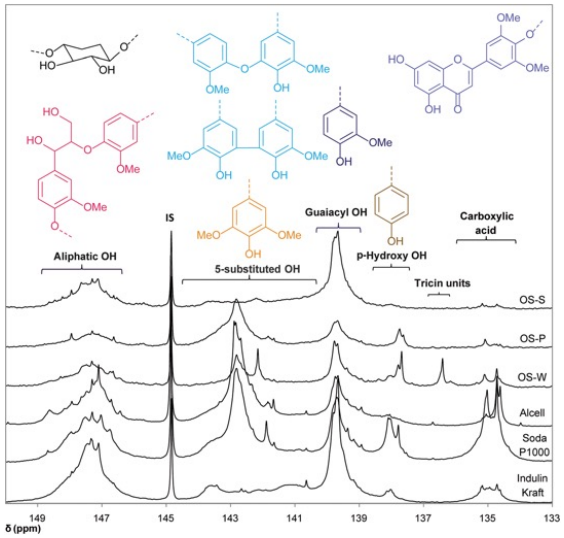
Constant, Weckhuysen, Bruijnincx et al. ACS Sustainable Chem. Eng. 2017, 5, 965

Lignin NMR: Opportunities Abound

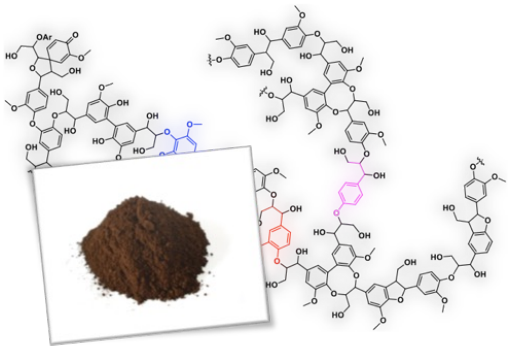
- **1D** ^{1}H and (quantitative) ^{13}C NMR
- **Multidimensional** NMR: TOCSY, HSQC$_{(0)}$, HMBC, HSQC-TOCSY, ….
- **Heteronuclear** NMR: ^{31}P, ^{19}F, …



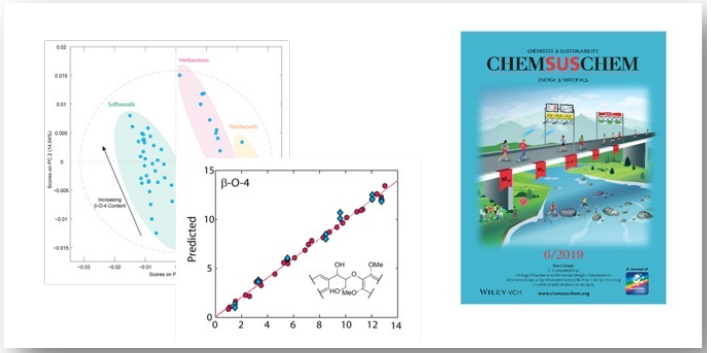




Lignin: The Analytical Challenge

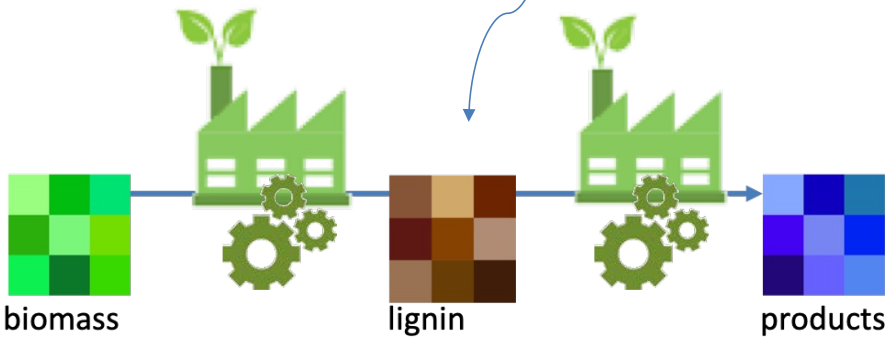


Rapid lignin analysis by ATR-IR



Analytical challenges in lignin valorization are manifold:

- **Structure elucidation**: what is (a technical) lignin?
- **Structure variation**: crop, seasonal, process-dependent variations in key lignin parameters
- **Structure dynamics:** how do we get online/real time information on, e.g., depolymerization?



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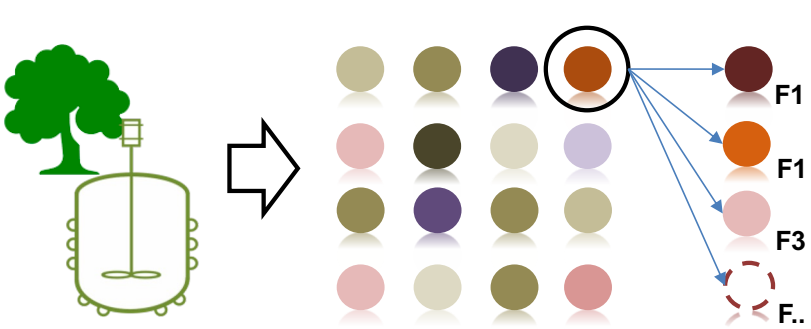
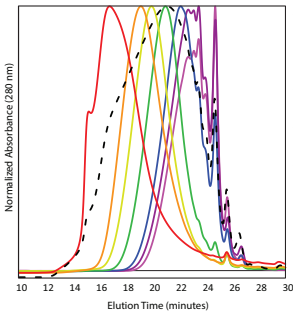
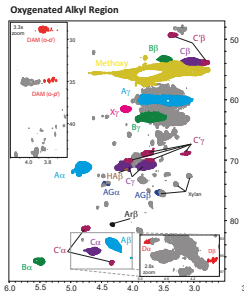


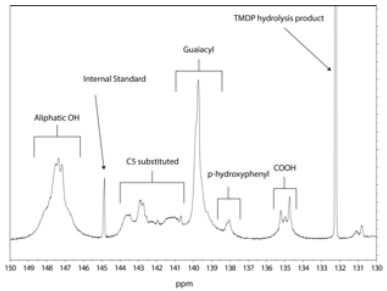
GPC



HSQC NMR

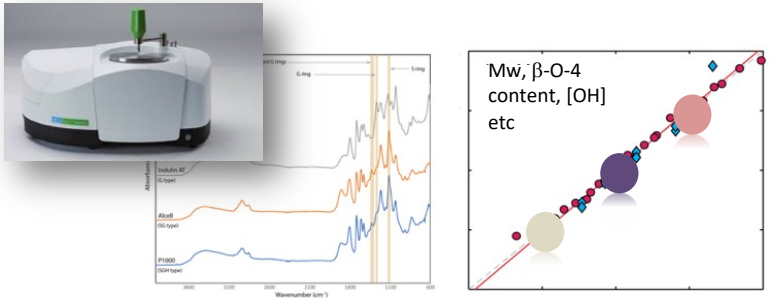


^{31}P NMR



- Different botanical origin and refining technology give…

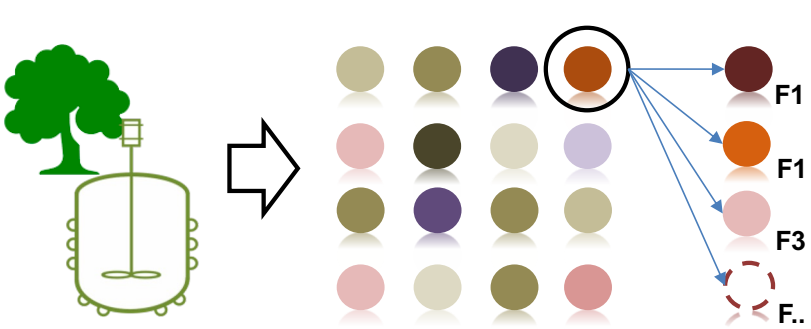
… structurally different technical lignins and lignin fractions



- **Sample load** becomes an issue
- **Rapid analysis** by ATR-IR with chemometric analysis can **reduce** that heavy burden

Lancefield, Bruijnincx et al. *ChemSusChem*, 2019, 12, 1139

Lignin: The Analytical Challenge



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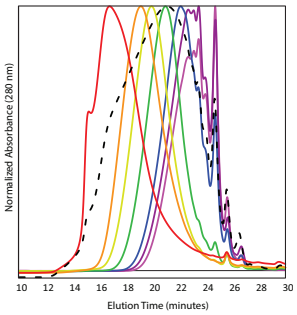

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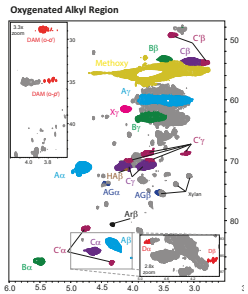

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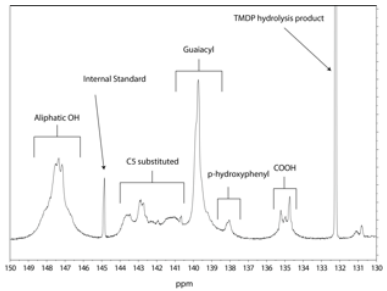

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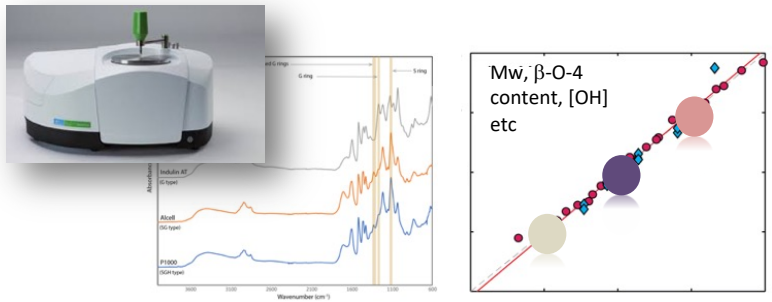


- Previously: e.g., hydroxyl group content, S/G ratios,… of different technical lignins

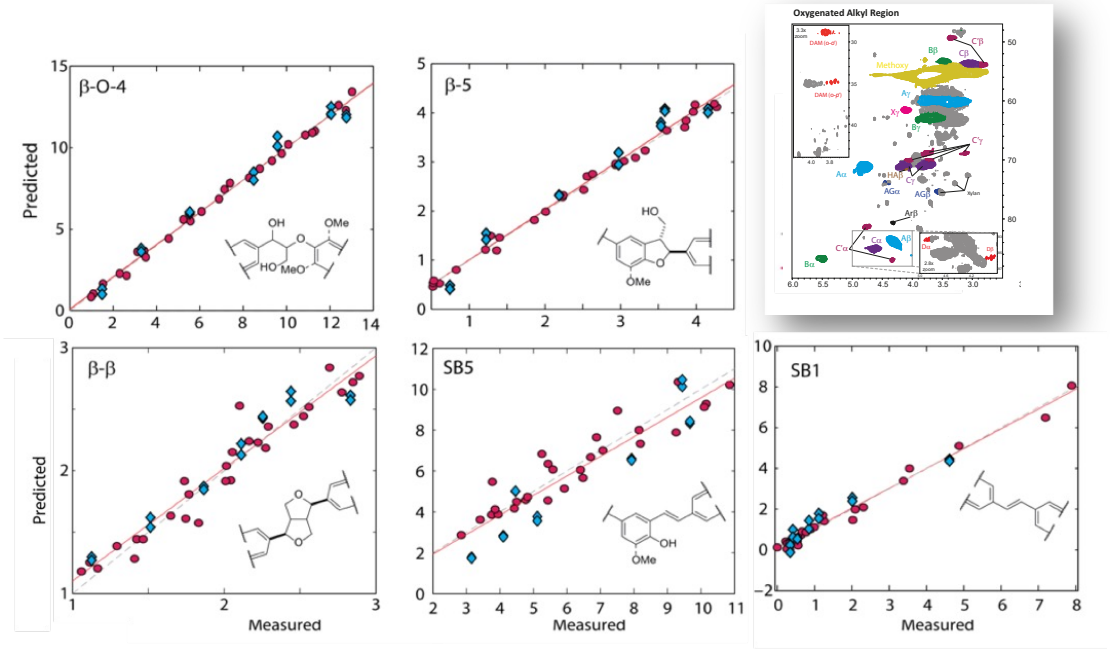
  e.g., Boeriu, Gosselink, Ind. Crops Prod. 2004, 20, 205

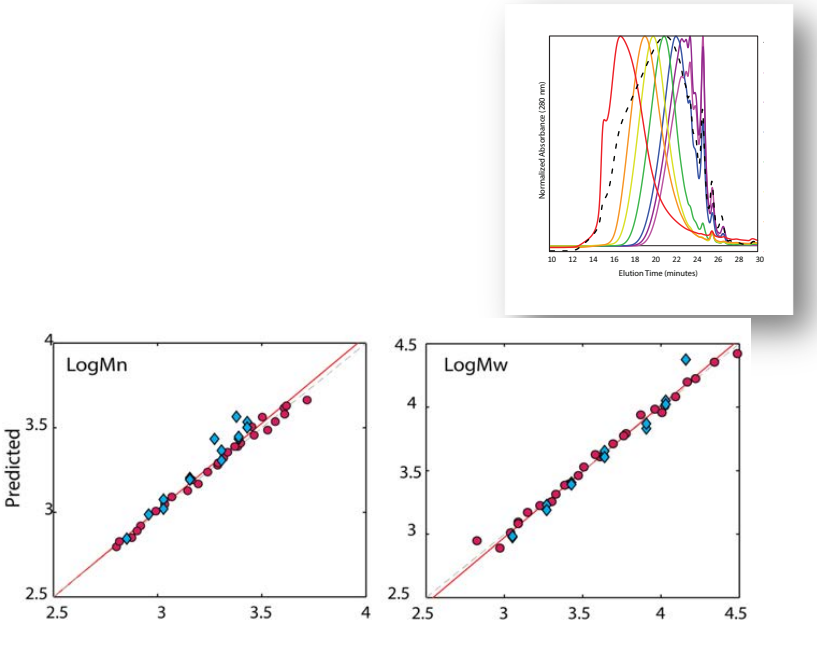
- Now expanded to **linkage abundance** and **molecular weight** information

Kraft Lignins as Showcase Sample Set: Linkage Abundance

Excellent prediction of:

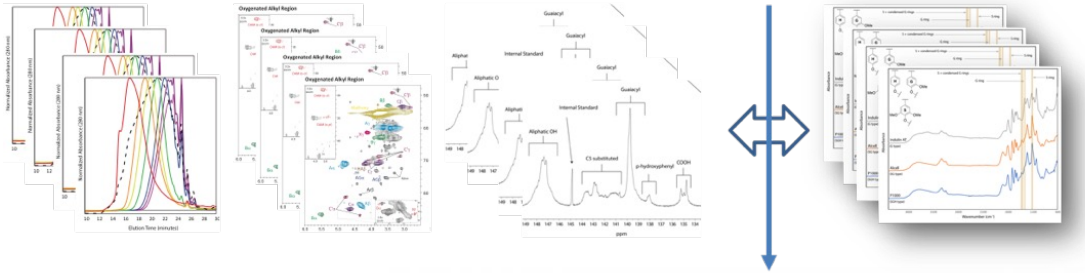
- **linkage abundance**
- **molecular weight** (Mn and Mw)



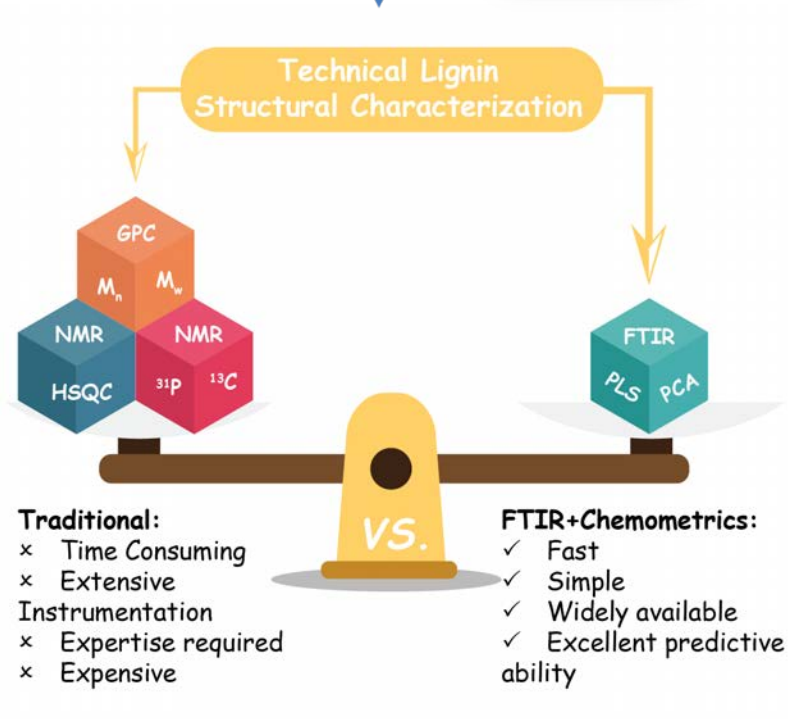


Lancefield, Bruijnincx et al., ChemSusChem 2019, 12, 1139

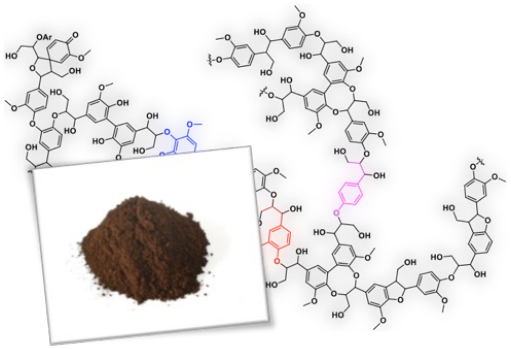
Rapid and Complete Lignin Analysis by ATR-IR



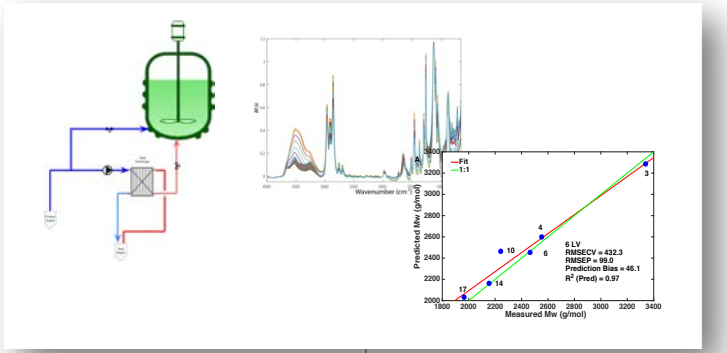
- Quantification is **comparable to standard GPC and HSQC NMR**
- When calibration set is in place, analysis of new samples is rapid and free of operator-bias



Lignin: The Analytical Challenge

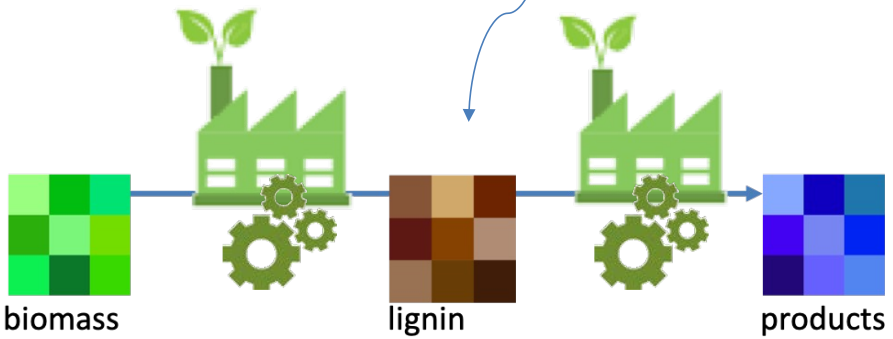


Rapid reaction analysis by *operando* ATR-IR



Analytical challenges in lignin valorization are manifold:

- **Structure elucidation**: what is (a technical) lignin?
- **Structure variation**: crop, seasonal, process-dependent variations in key lignin parameters
- **Structure dynamics:** how do we get online/real time information on, e.g., depolymerization?



Khalili et al., *Chem. Methods* 2021, doi.org/10.1002/cmtd.202100041; *ChemSusChem* 2021, accepted

Acknowledgments

Universiteit Utrecht

Lignin

SuBiCat A new Generation

NWO

سابک sabic

CatchBio

TNO innovation for life

BASF The Chemical Company

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