# Characterization of Non-Derivatized Plant Cell Walls Using High-Resolution Solution-State NMR Spectroscopy

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# Outline

- Objectives
- Approach
- What is Solution-State NMR
- Evolution of Methodology
- Characterizing the Whole Cell Wall
- Applying the 2D NMR Technique
- Conclusions
- Current and Future Work

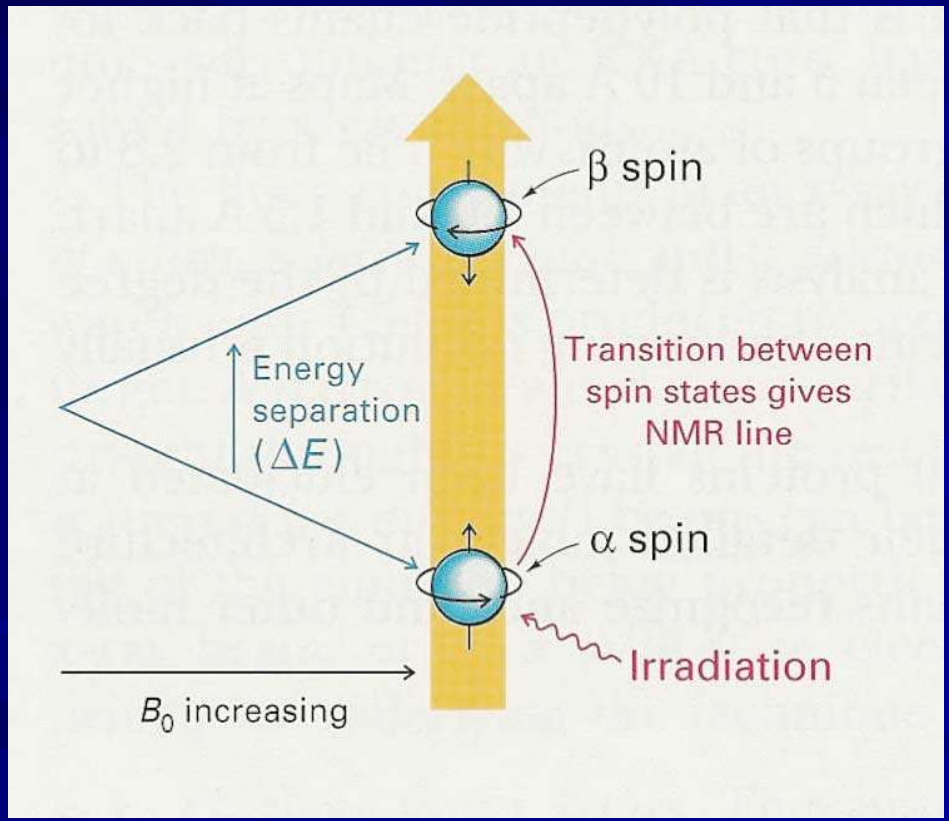
# Objectives

- Establish methodologies to retain the ‘native’ chemistry of wood cell wall components using wood dissolution techniques
- Characterize the whole cell wall using solution-state NMR experiments
- Explore applications of this technique, including:
  - adhesive bonding interactions
  - wood-decay mechanisms
  - others…

# Approach

- Leave the wood cell wall material in a minimally altered state so as to characterize all components without extensive extraction or isolation techniques
- Synthesize perdeuterated imidazole compound for dissolution of wood
- Use 2D solution-state NMR $^{1}H$-$^{13}C$ correlation experiments, which allow for increased S/N and enhanced peak dispersion as compared to other spectroscopies

# How NMR spectroscopy works

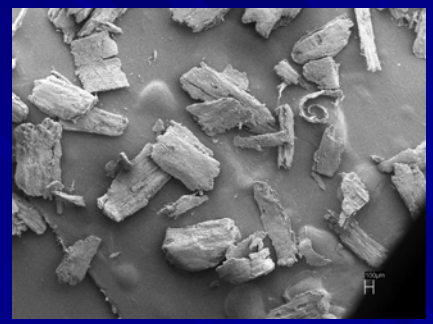


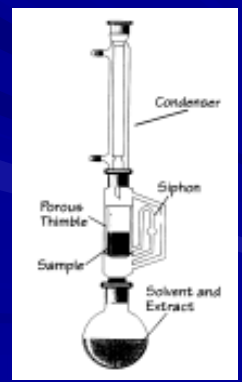

- Nuclear Magnetic Resonance is the phenomenon which occurs when radio frequency (rf) electromagnetic radiation interact with the nuclei of stable isotopes (e.g., $^{1}$**H,** $^{13}$**C,** $^{15}$**N**)
- Nuclei are immersed in a static magnetic field $\boldsymbol{B_o}$ while simultaneously exposed to a second applied magnetic field
- Chemical shift (absorption of energy changes with electron density)
- Determine chemical structure of molecules (covalent bonds between atoms)

# Non-degradative dissolution, I

Solid wood species

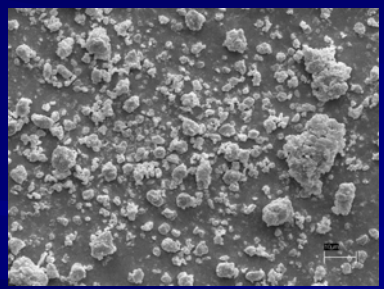
Wiley-Milled






Water
MeOH

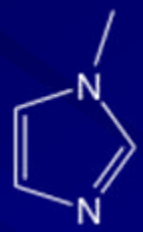
Acetone
$CHCl_3$



Ball-milled wood without extractives

# Non-degradative dissolution, II

10mL, DMSO + 5mL, N-methylimidazole



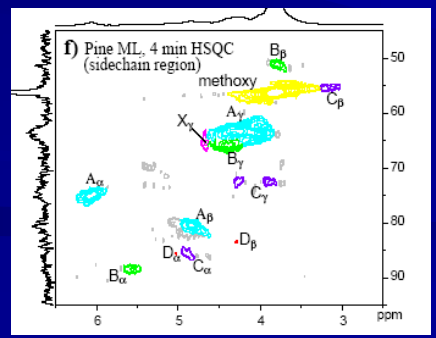
**500mg wood added and solution becomes clear in ~3 hrs.**

**Acetic anhydride, excess**

**EDTA wash**

**Dissolve Ac-Wood in $CDCl_3$**

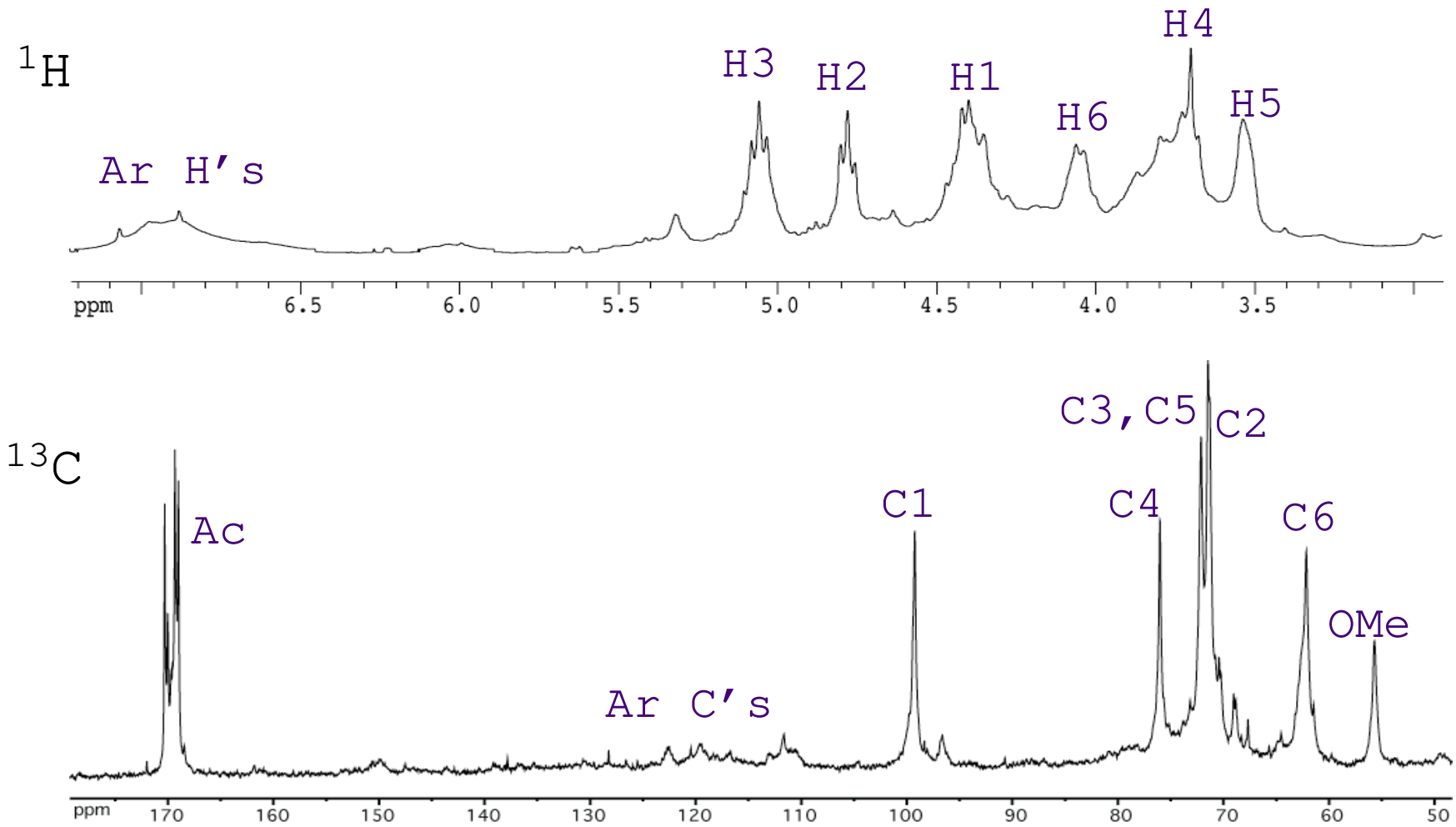
**60 - 100 mg Ac-Wood Into a 5 mm NMR tube**



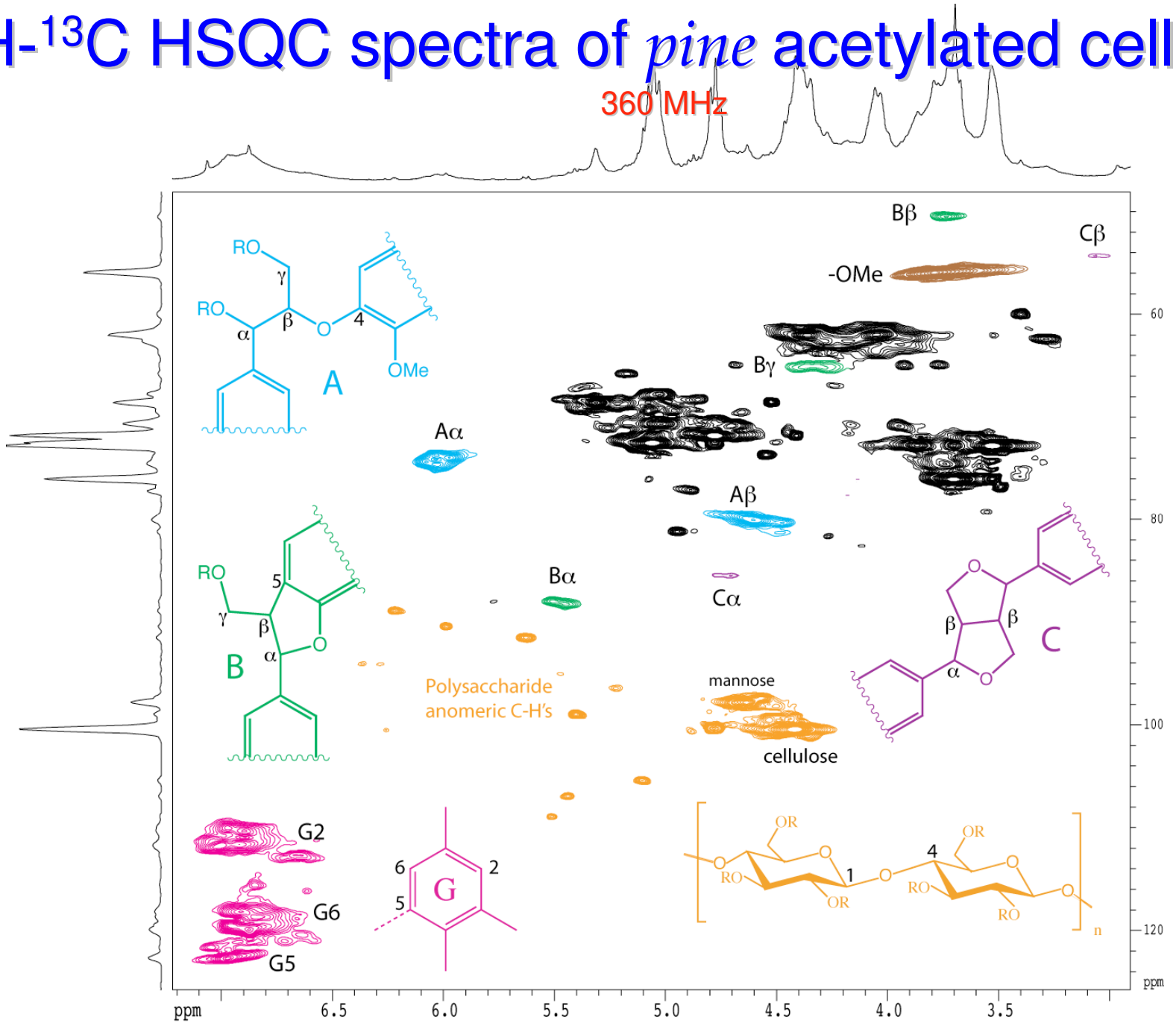

**NMR spectra**

Lu, F. and Ralph, J. (2003). *The Plant Journal*. 35(4) p. 535

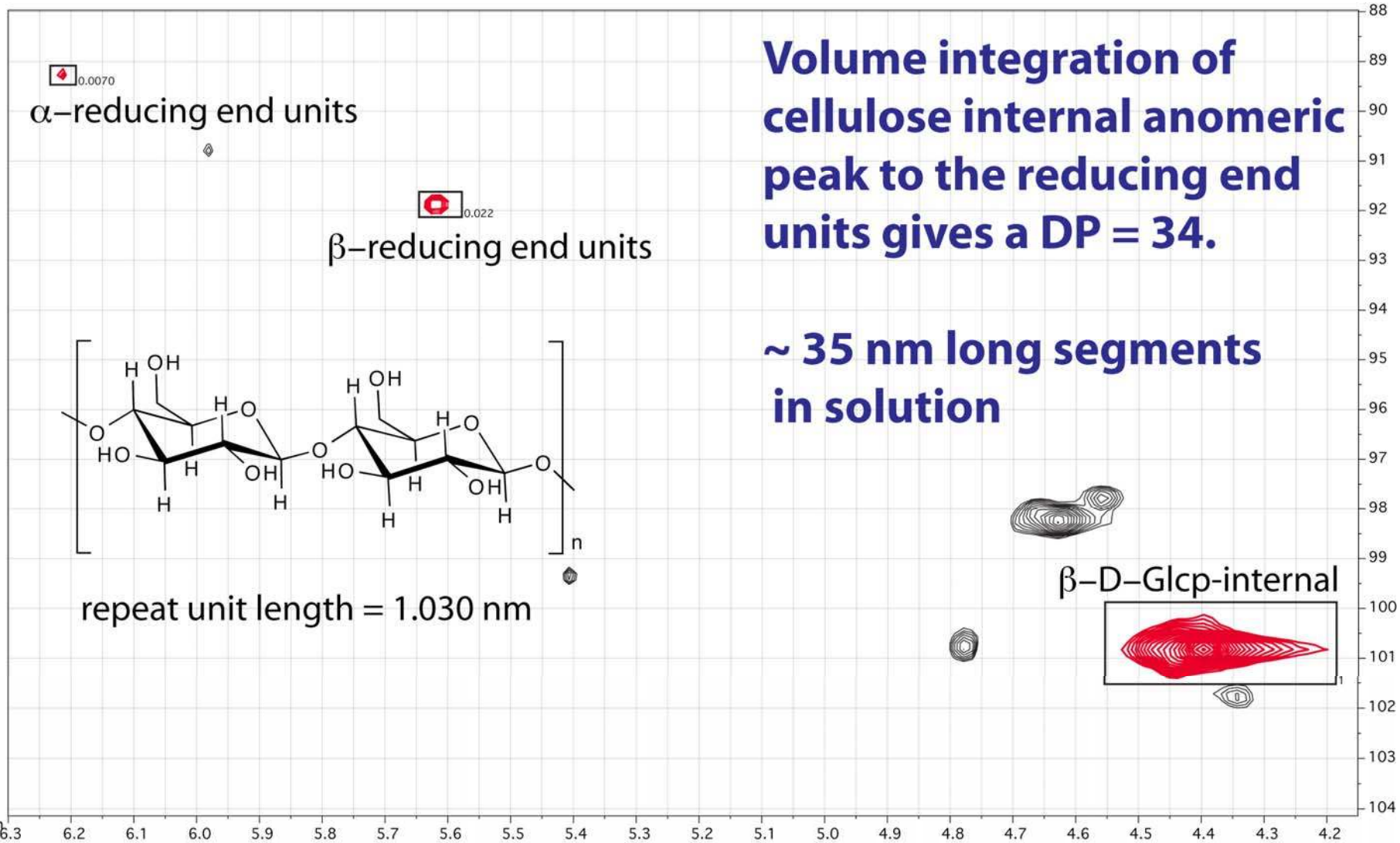
# 1D NMR spectra of *pine* acetylated cell walls



# $^{1}H$-$^{13}C$ HSQC spectra of *pine* acetylated cell walls



# What size is the wood particle?



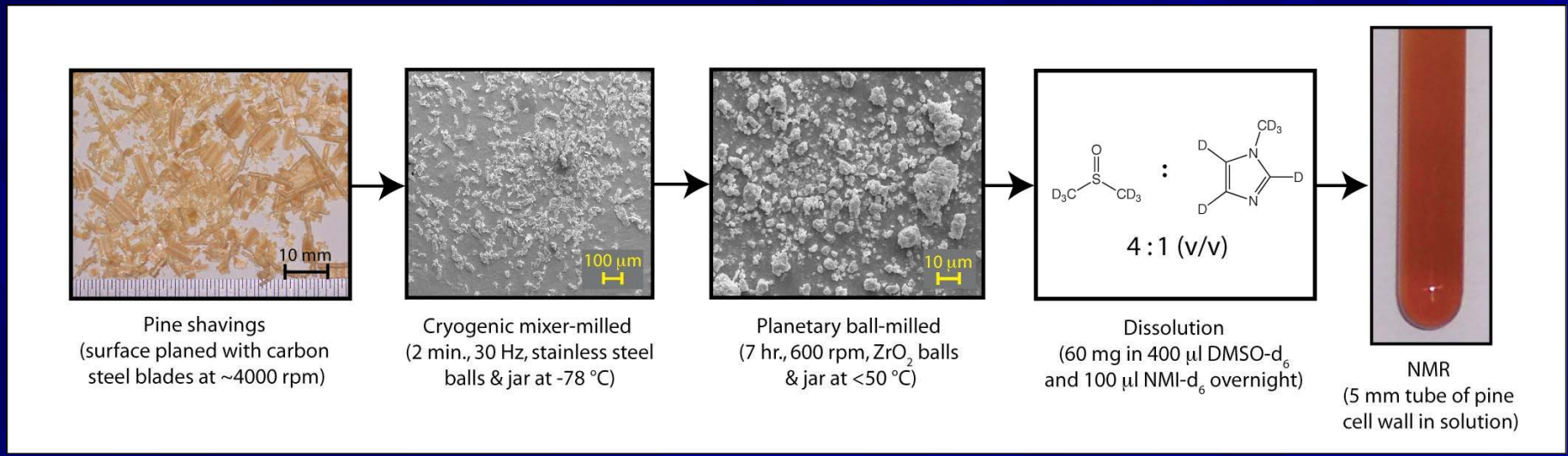
# ‘Native’-state of the cell wall

- Method of acetylating the whole cell wall allows for characterizing all wood components, but with *chemical modification*
- HSQC spectra reveal where and to what degree the chemical is reacting
- *How do we determine if a chemical reacts partially or not at all with wood?*
  - Remove acetylation and extraction steps
  - Use deuterated solvents

# DMSO-$d_6$ & NMI-$d_6$

- As you recall, mixing a specific ratio of dimethylsulfoxide and N-methylimidazole (v/v) can fully dissolve ball-milled wood
- Synthesis of NMI-$d_6$ is required
- At least 99.9 % D is needed to subdue the NMI proton signal in NMR
- Dissolution of non-extracted ball-milled wood directly in a 5mm NMR tube allows for rapid sample preparation

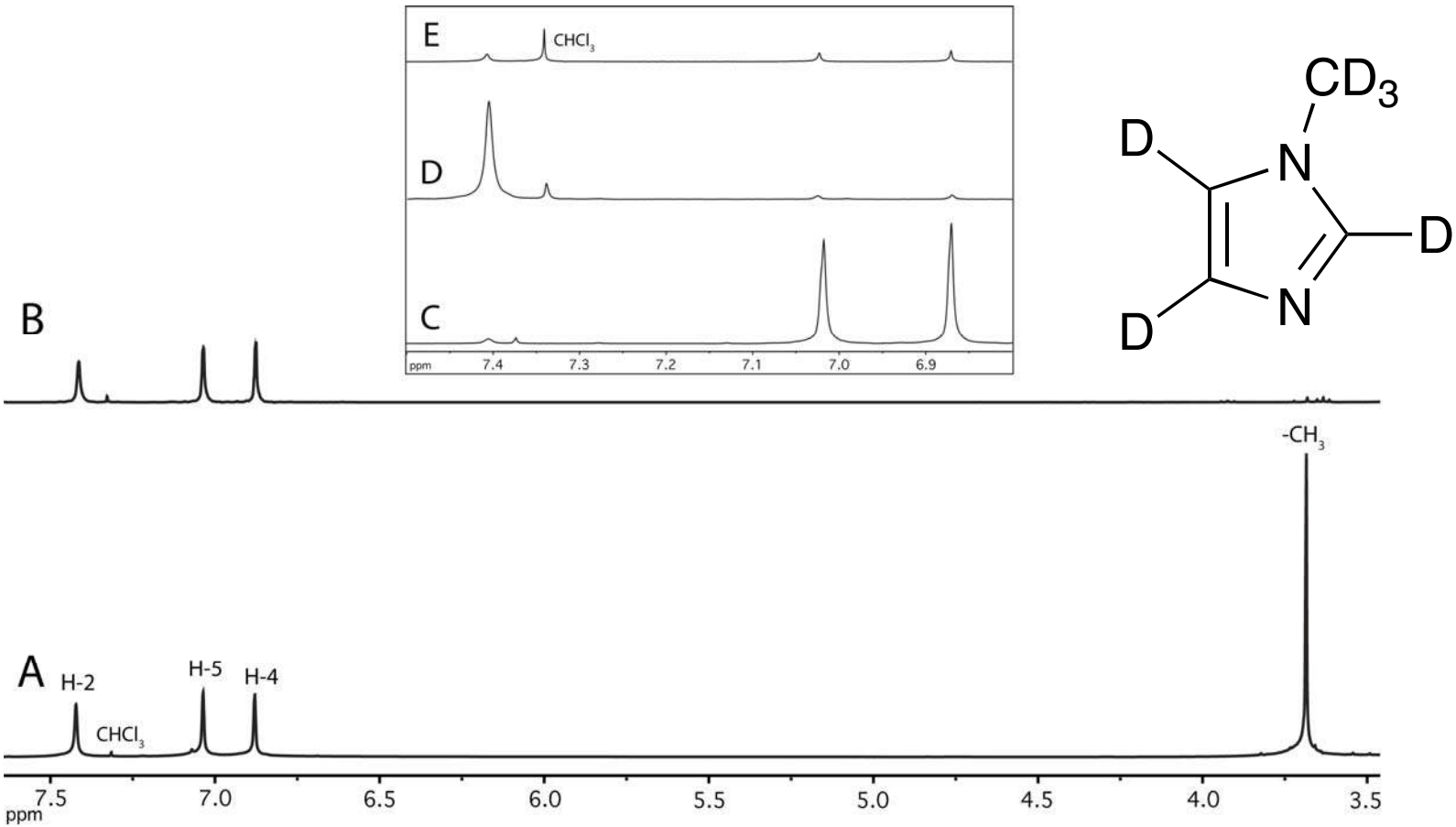
# Non-derivatized dissolution




- Acquiring quality NMR spectra requires removal of paramagnetic materials (e.g., Fe, $O_2$, Mn)
- Particle-size is important for dissolution
- *In situ* dissolution increases efficiency and dramatically decreases sample prep time

Yelle, D.J.; Ralph, J.; Frihart, C.R. (2008). *Mag. Res. Chem.* 46, 508-517

# Synthesis of NMI-$d_6$ , $^1$H spectra



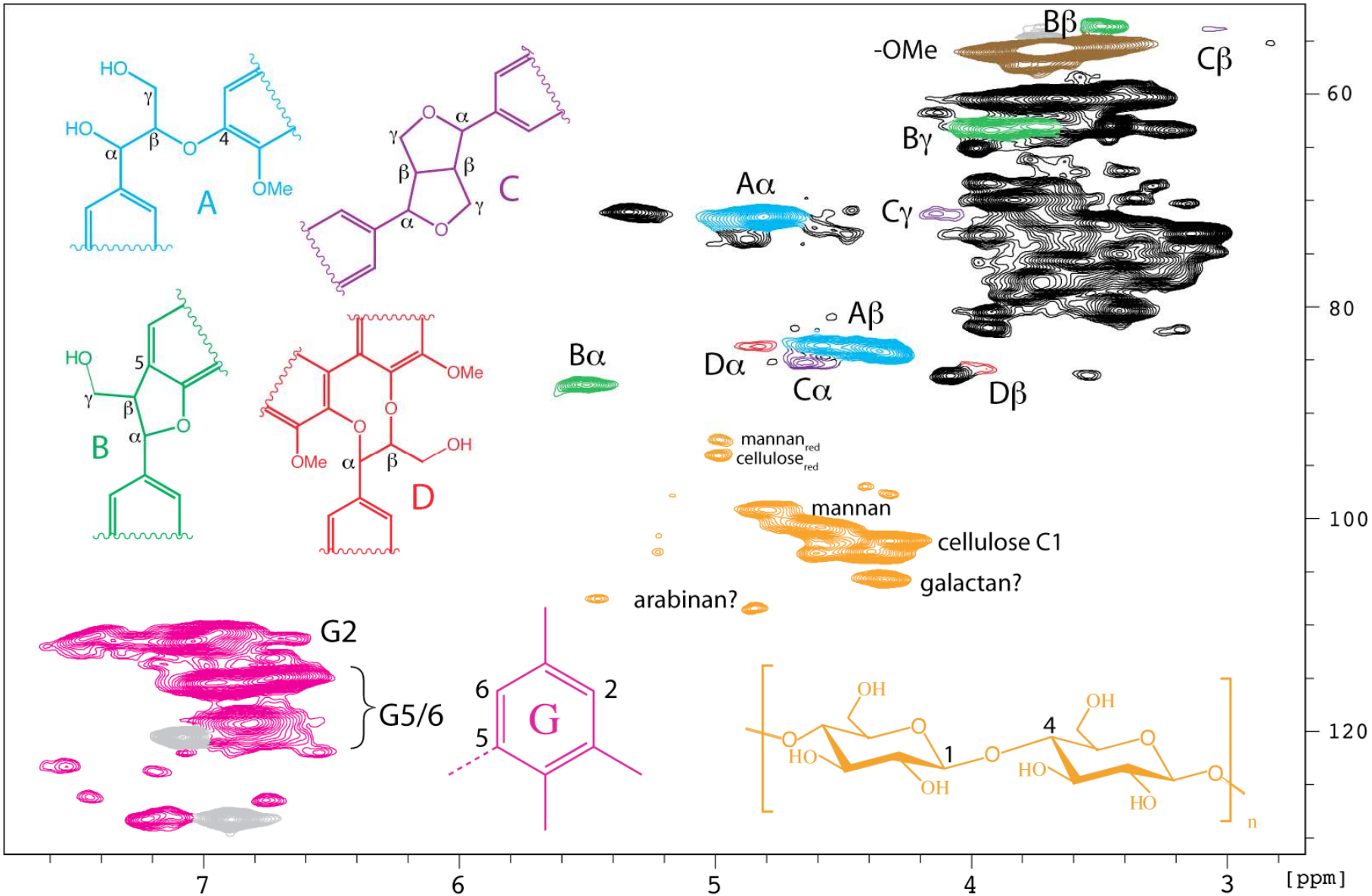




- Three steps: N-methylation ($CD_3OD$) & two aromatic ring deuterations ($D_2O$ with 10% Pd/C)

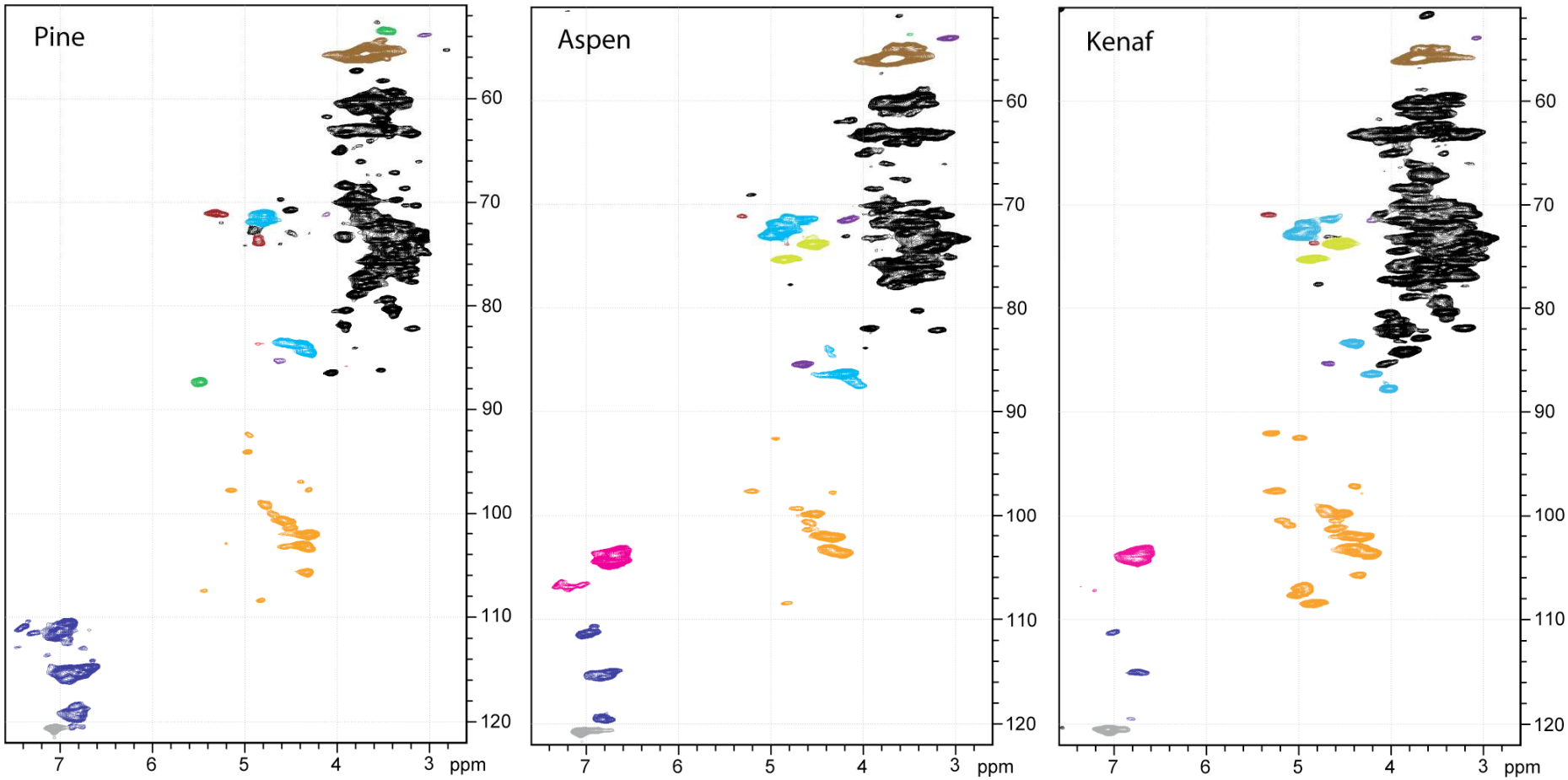
# $^{1}H$-$^{13}C$ HSQC spectra of *pine* 'native' cell walls

360 MHz



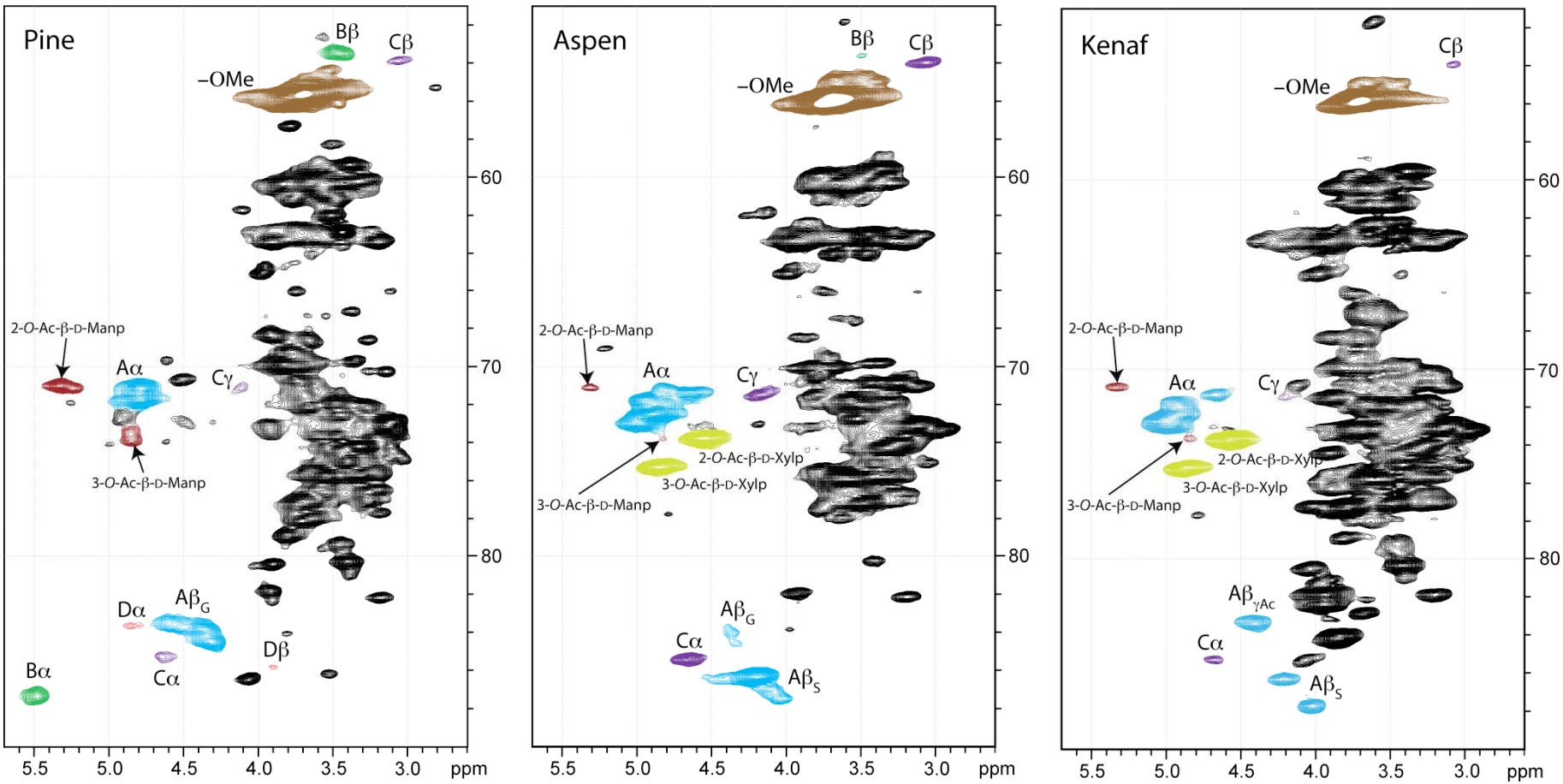
# $^{1}H$-$^{13}C$ HSQC of whole cell walls

## 500 MHz cryoprobe



# $^{1}H$-$^{13}C$ HSQC of aliphatic region

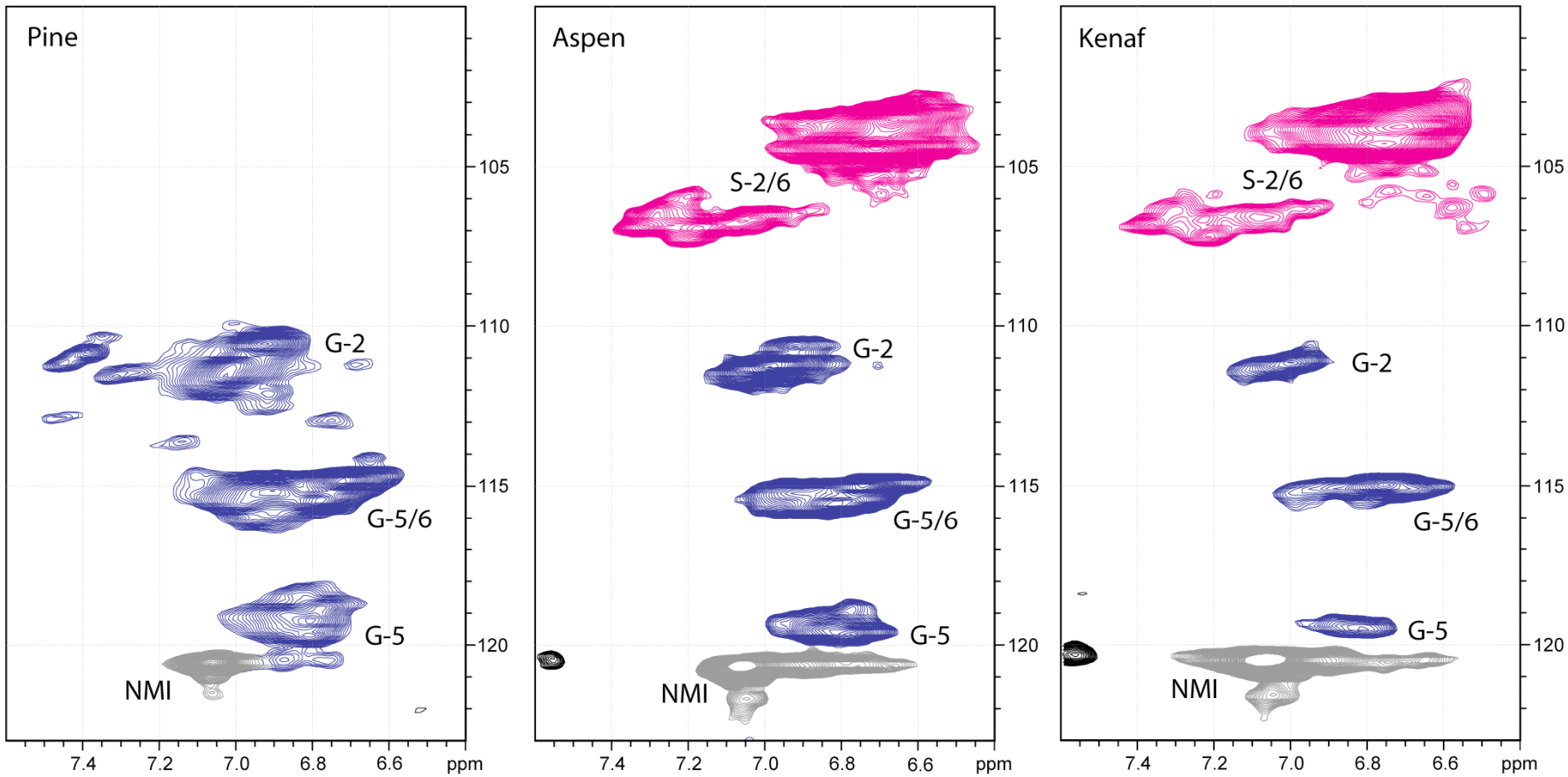
## 500 MHz cryoprobe




~ 60% βaryl ether units are acetylated

# $^{1}H$-$^{13}C$ HSQC of aromatic region

## 500 MHz cryoprobe

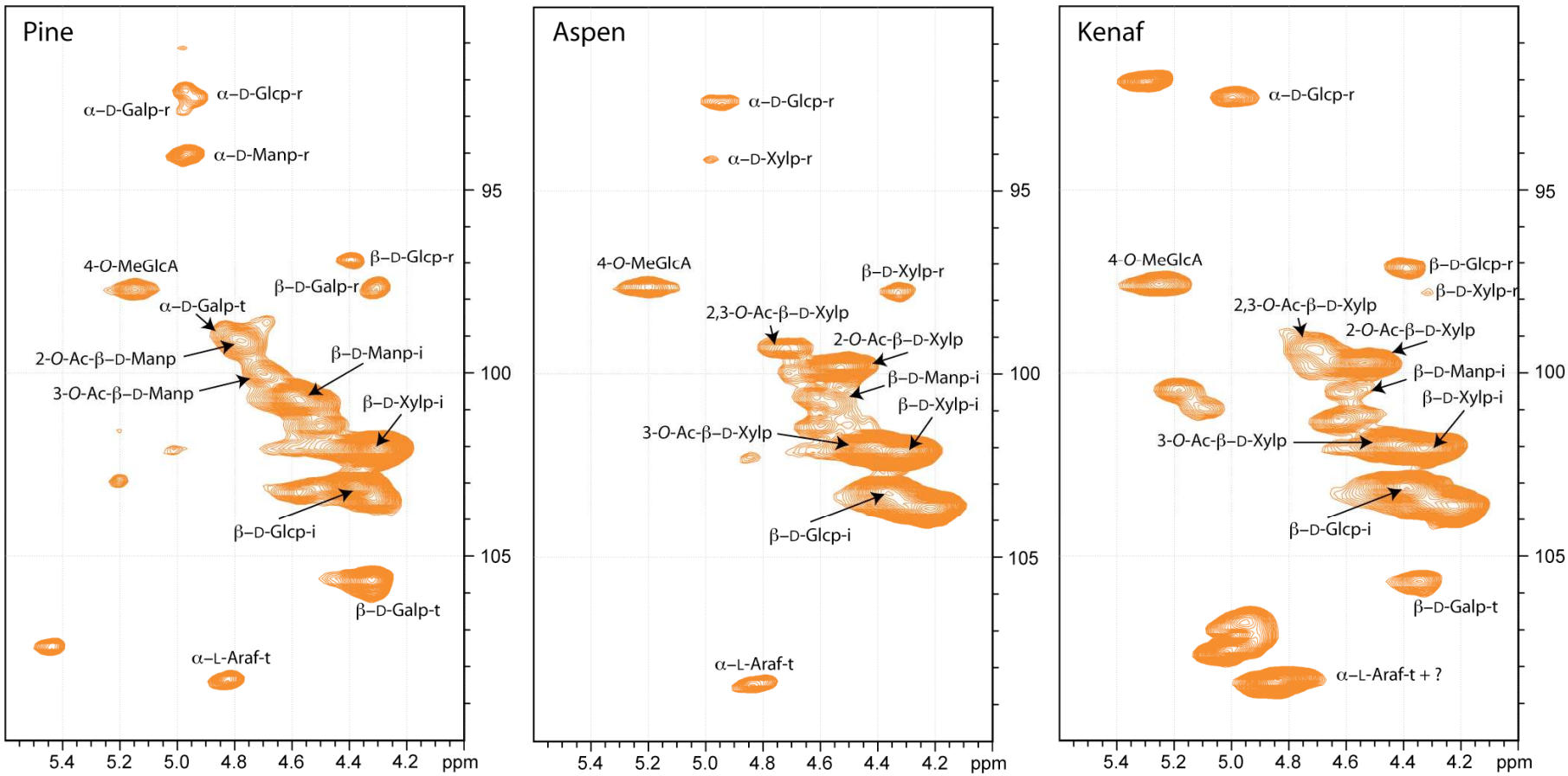



S:G = 2.0

S:G = 4.6

# $^{1}H$-$^{13}C$ HSQC of anomeric region

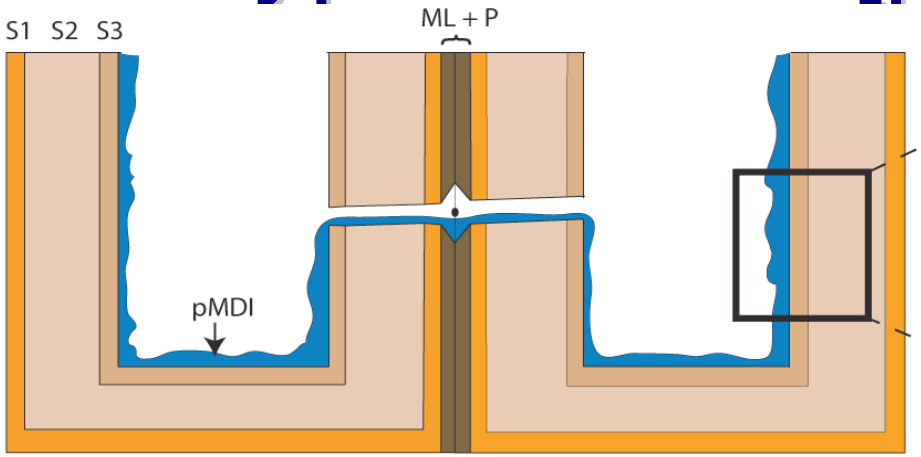
## 500 MHz cryoprobe

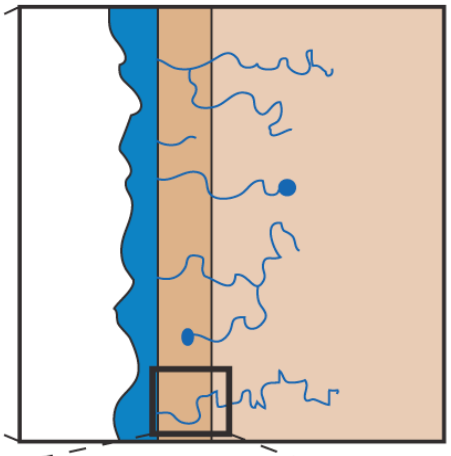


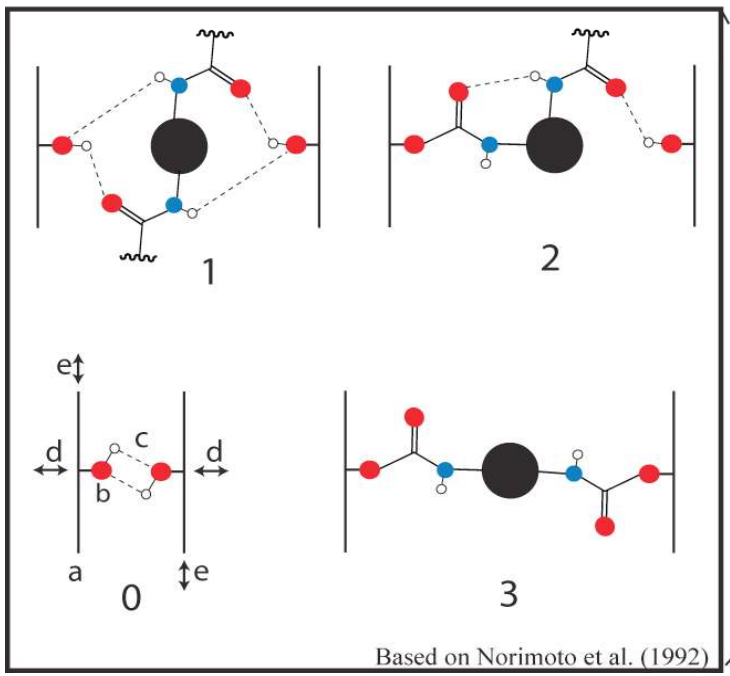
# Determining isocyanate reactivity

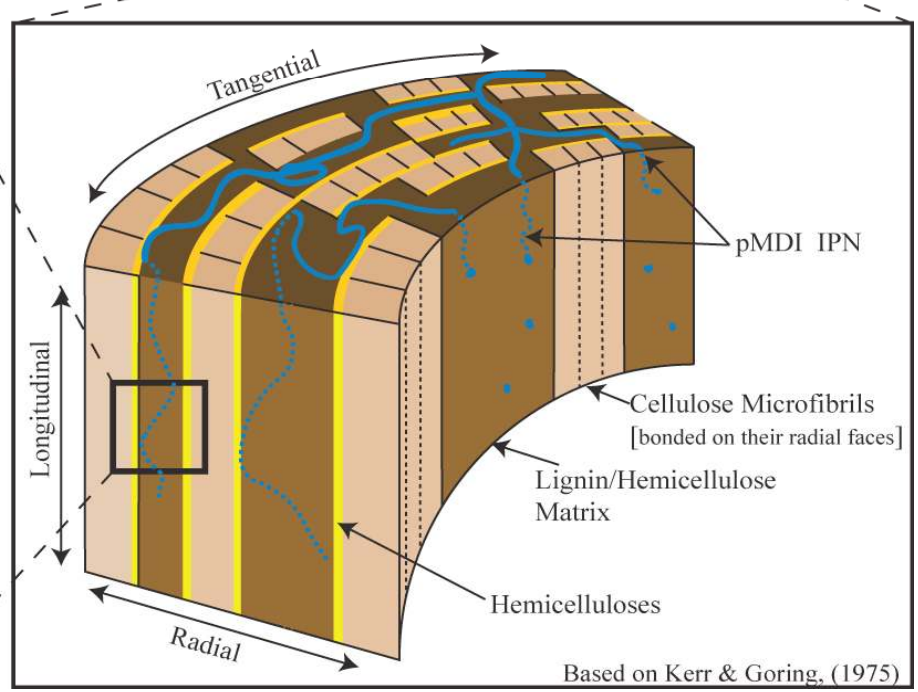
- Use solution-state NMR to assign peaks in HSQC spectra of the reaction products between model pMDI and model wood components
- Characterize changes in wood component chemistry as model pMDI reacts with wood hydroxyls
  - Detect $^{1}H$-$^{13}C$ correlations in wood

# Hypotheses [pMDI example]



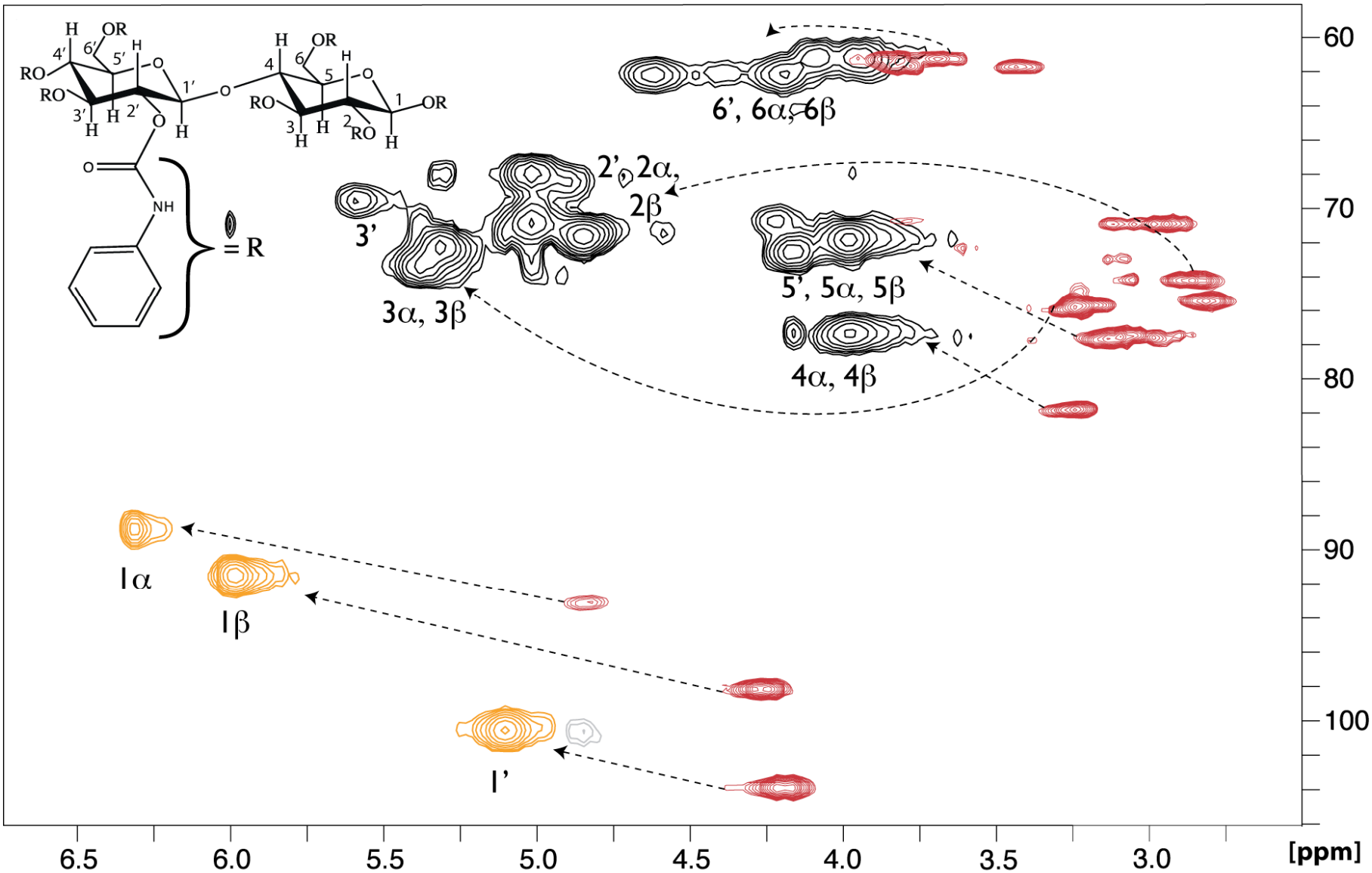





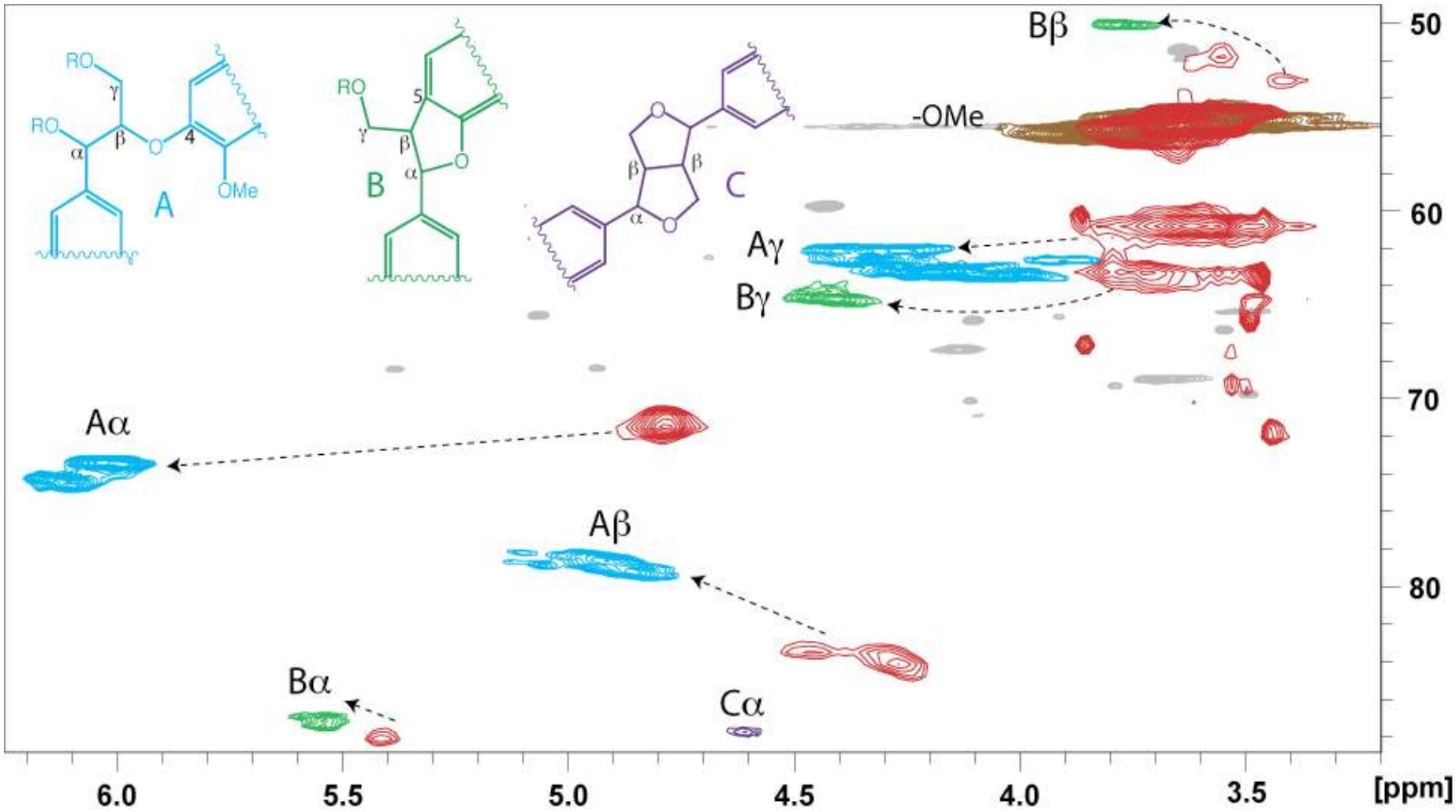
# 2D HSQC spectra of Ph-NCO reacted *cellobiose*

360 MHz



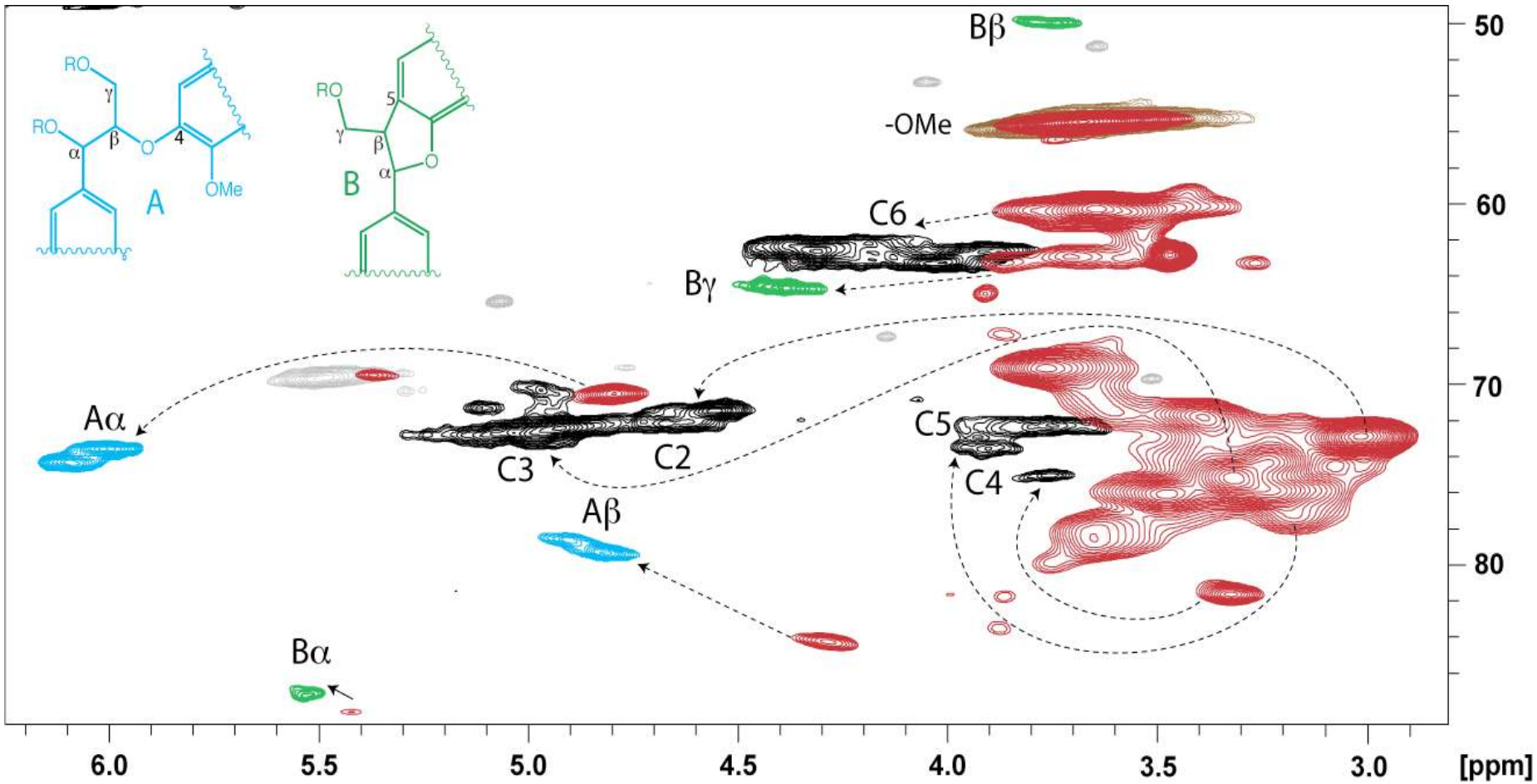
# 2D HSQC spectra of Ph-NCO reacted *MWL*

500 MHz cryoprobe



# $^{1}H$-$^{13}C$ HSQC spectra of Ph-NCO reacted *pine* ball-milled cell walls

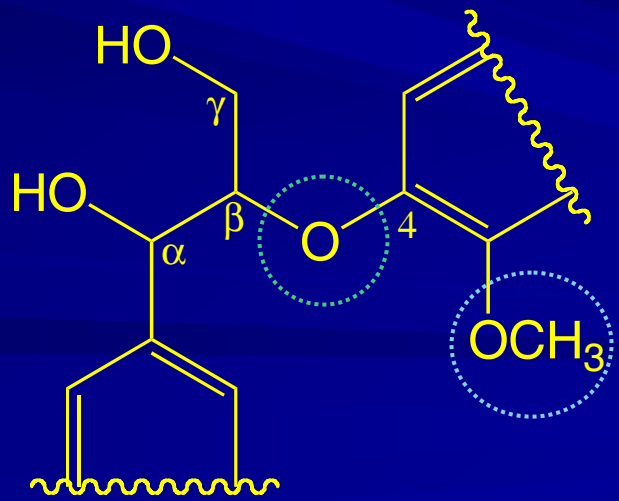
500 MHz cryoprobe



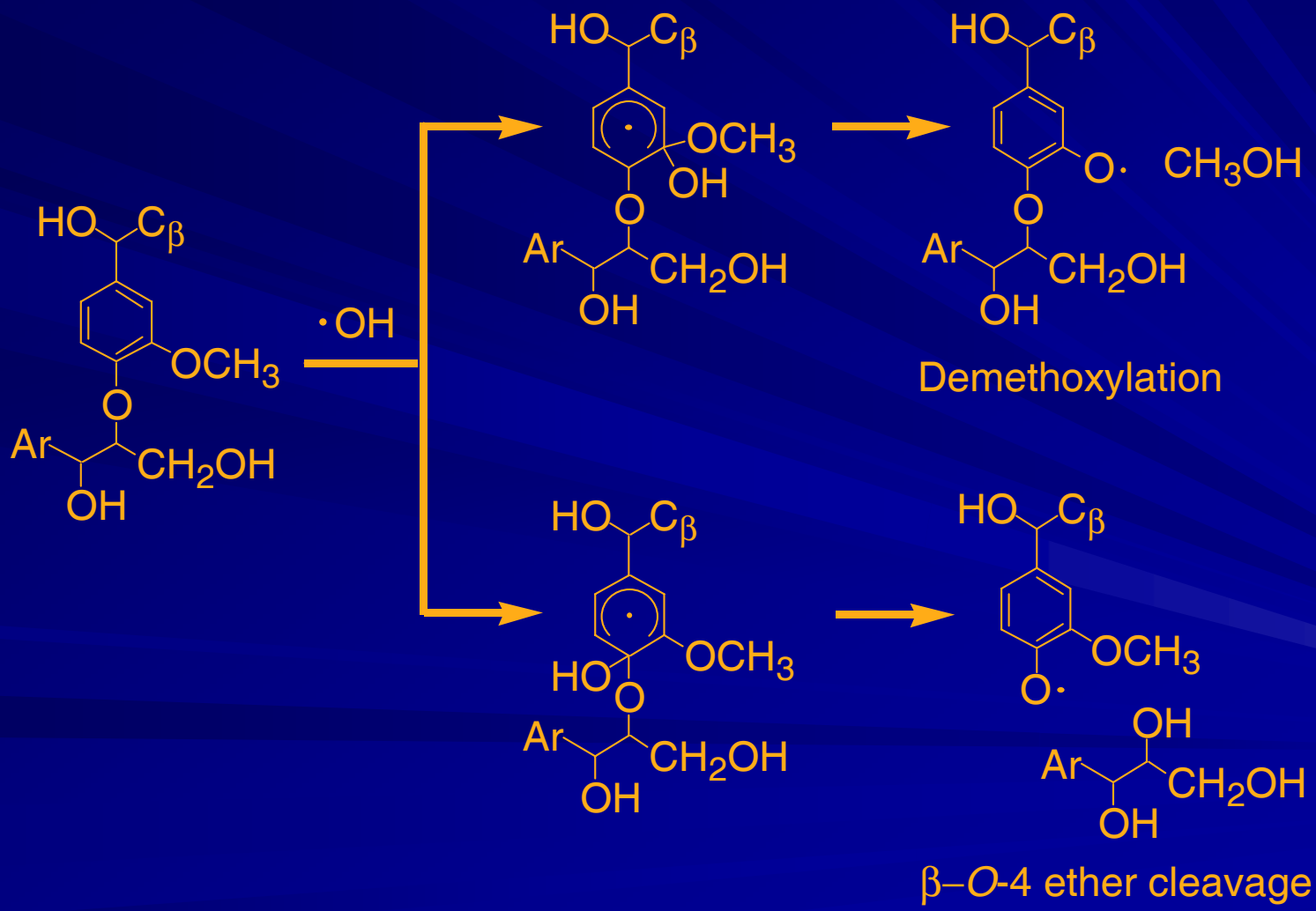
# Using 2D NMR to Study Brown-Rot Decay

# Strategy

- Measure extent of demethoxylation
- Measure extent of β-*O*-4 ether cleavage, if any
  - GC/MS
  - Integrate β-*O*-4 ether peaks in 2D NMR spectra to quantify cleavage
- No apparent reason for a •OH to attack one type of aryl ether over the other



# Possible Reactions of •OH with Lignin

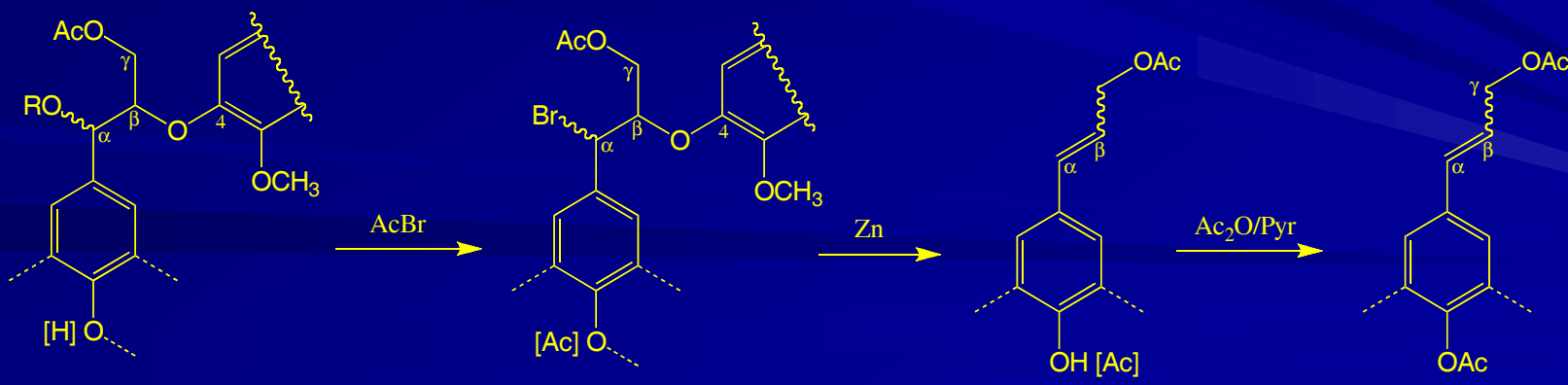


# Material

- ***Picea glauca*** was innoculated with ***Gloeophyllum trabeum*** to give a 50-70% wt. loss after 16 weeks.
- Decayed wood and control wood were ground in a coffee grinder + dry ice and ball-milled into **~30 nm particle size**.
- Dissolved wood using DMSO-NMI system for 2D solution-state NMR spectroscopy

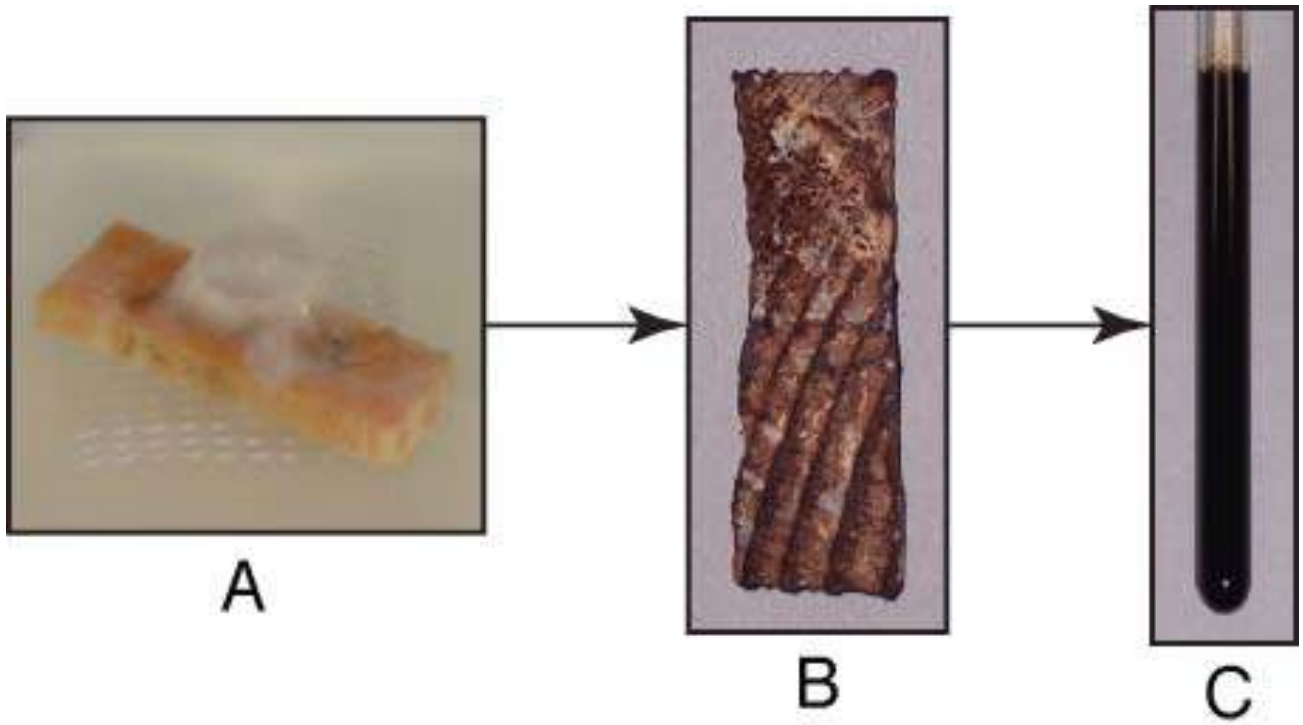
# Wet Chemistry

- Determine -**$OCH_3$** content
  - Zeisel-Vieböck-Schwappach approach
    - Titration of **$I_2$**
- Determine β-*O*-4 content of lignin
  - Derivatization Followed by Reductive Cleavage (DFRC) method
    - GC/MS to quantify cleaved monomers




R = H or Aryl

# Solution-State 2D NMR




A. One week innoculation
B. Appearance of a typical spruce wafer 16 weeks after innoculation
C. The decayed sample after milling and dissolution

# Methoxyl and β-*O*-4 Content Resuts

| Sample | Methoxyls | Arylglycerol-aryl ethers by DFRC analysis | |
|---|---|---|---|
| | g/g lignin[a] | g/g lignin[a] | g/g methoxyls |
| Sound wood | 0.168 | 0.111 | 0.661 |
| Decayed wood | 0.107 | 0.020 | 0.187 |

[a]Klason lignin plus acid-soluble lignin

-Biodegraded wood was 36% deficient in methoxyl groups

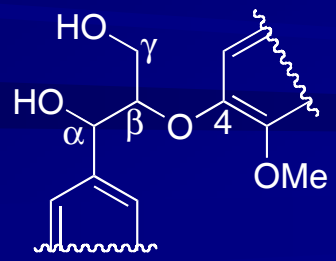
-Assayable arylglycerol-β-aryl ether was 82% deficient in on a total lignin basis and 72% deficient on a methoxyl basis

# Solution-State NMR Results

Relative integrals of contours in HSQC spectra of solubilzed spruce

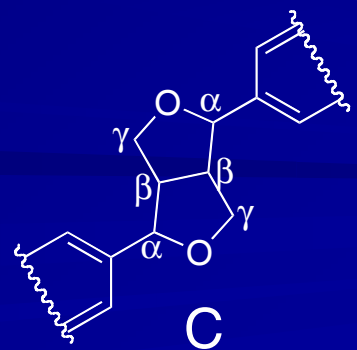
| Lignin Structure | Sound Wood Integral | Decayed Wood Integral |
|---|---|---|
| Methoxyl | 1.00 | 1.00 |
| Aα | **0.079** (69) | **0.023** (70) |
| Bα | 0.024 (21) | 0.007 (21) |
| Cα | 0.011 (10) | 0.003 (9) |

Numbers in parentheses show what percent of the sum of the integrals for structures Aα, Bα and Cα is accounted for by the integral for each individual structure.



A

B



C

Yelle, D.J.; Ralph, J.; Lu, F.; Hammel, K.E. (2008). *Environ. Microbiol.* In press (online)

# Conclusions

- Non-degradative whole cell wall dissolution is a powerful tool for analysing wood chemistry
- This improved wood dissolution method allows characterization of all cell wall polymers in their essentially native-states using NMR
- Model compound NMR spectra can be used to directly compare to NMR spectra of chemically reacted wood
- Decayed wood chemistry can be investigated with solution-state NMR to show substantial differences in lignin side-chain unit composition
- Continuously searching for new applications for this 2D NMR technique

# Acknowledgements

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University of Wisconsin, Madison, NMRFAM