Analyzing Changes in Lignin Chemistry Due to Biofuel Production Processes

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NARA Supply Chain

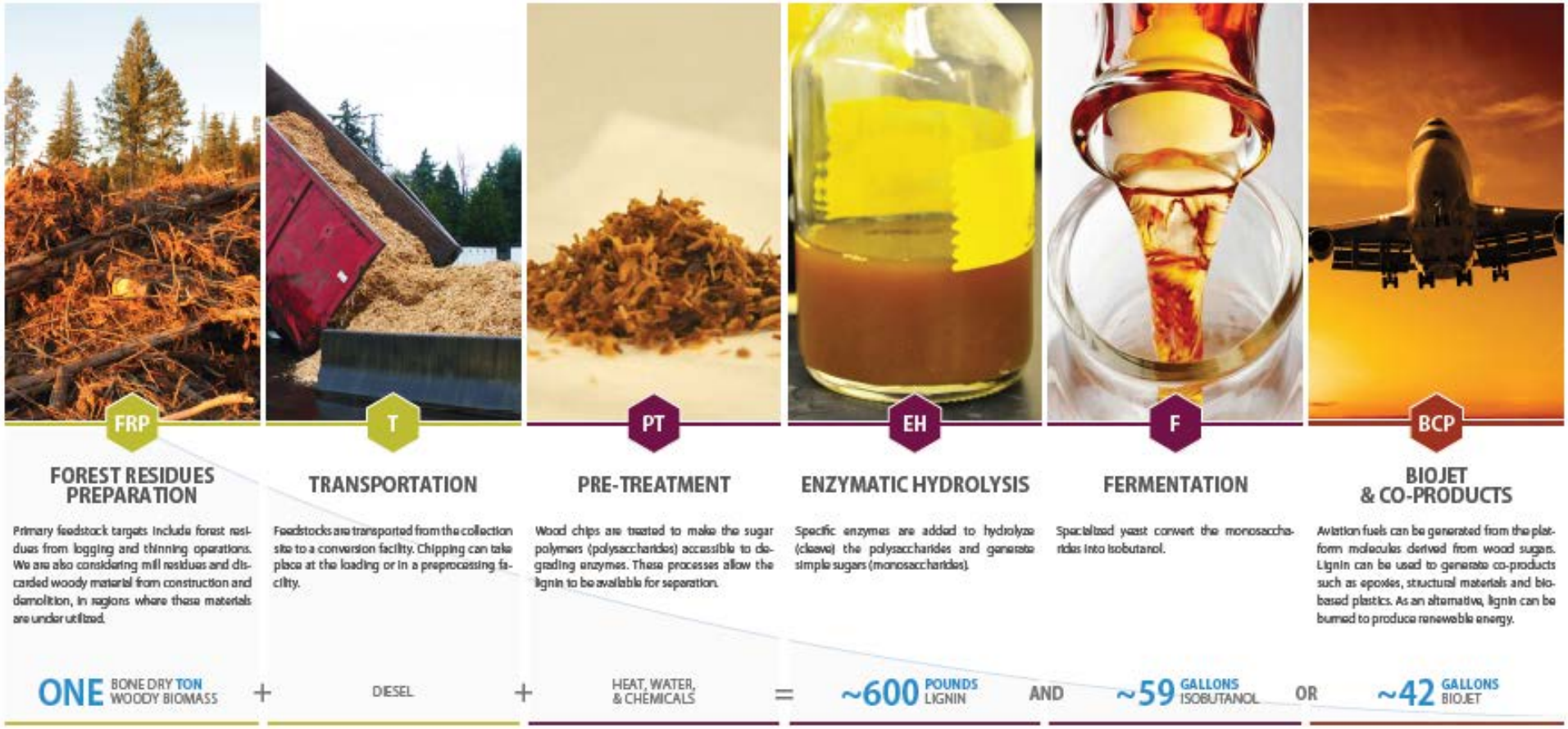


Image credit: NARA

Source of Lignin in a Biorefinery

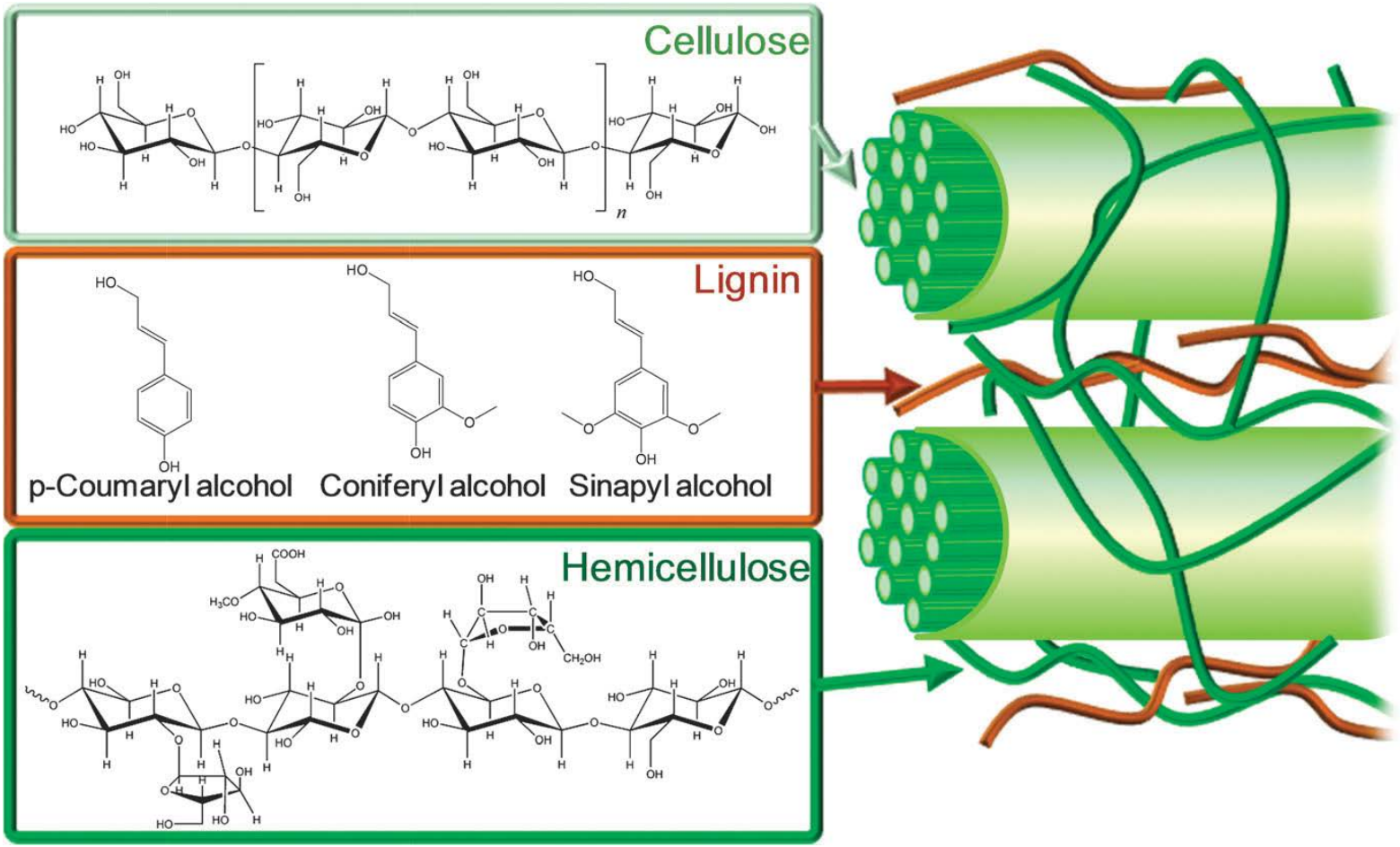
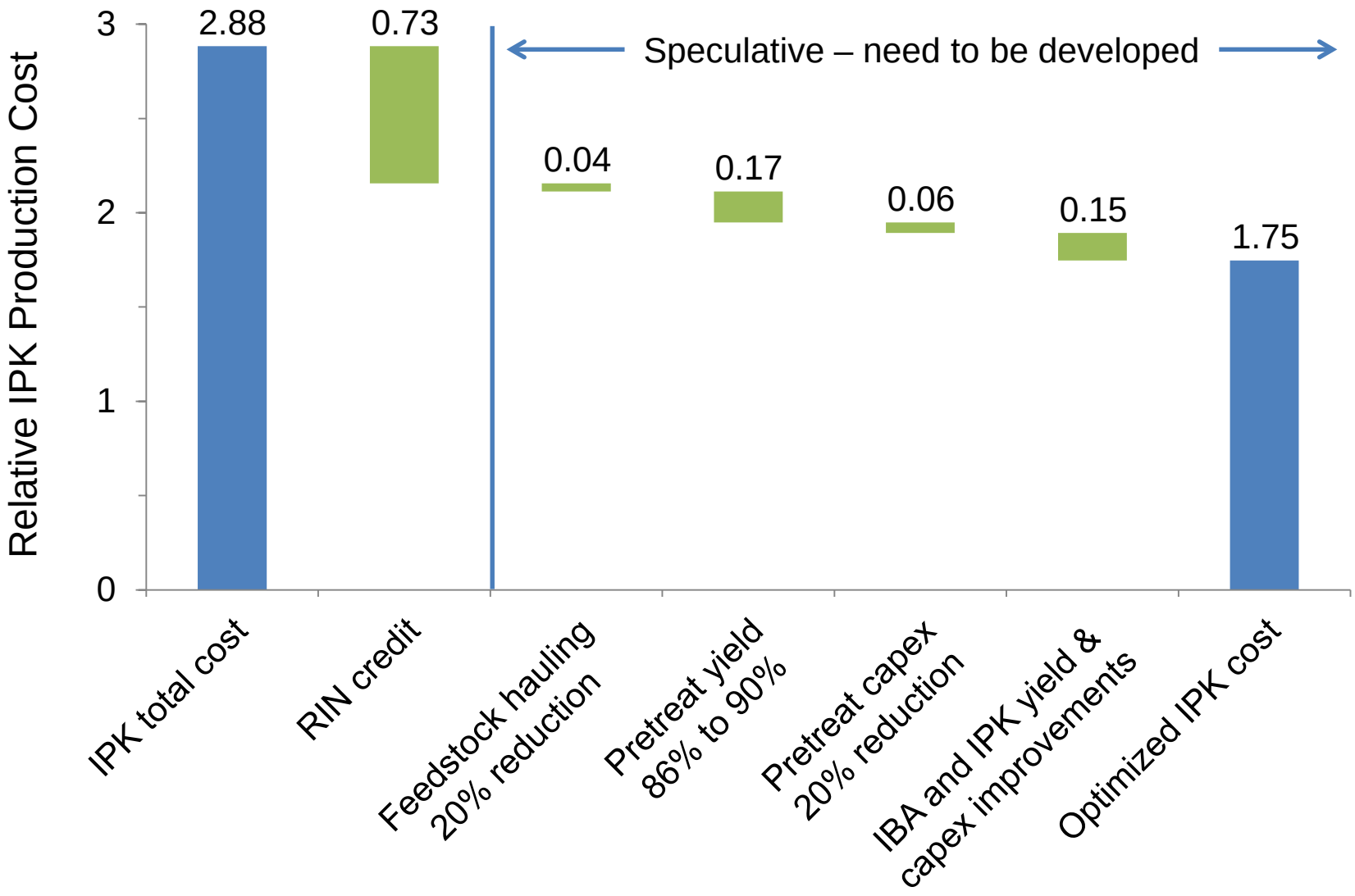


Image from: Alonso, D. M.; Wettstein, S. G.; Dumesic, J. A. *Chem. Soc. Rev.* **2012**, *41*, 8075-8098.

The Need for Co-Products



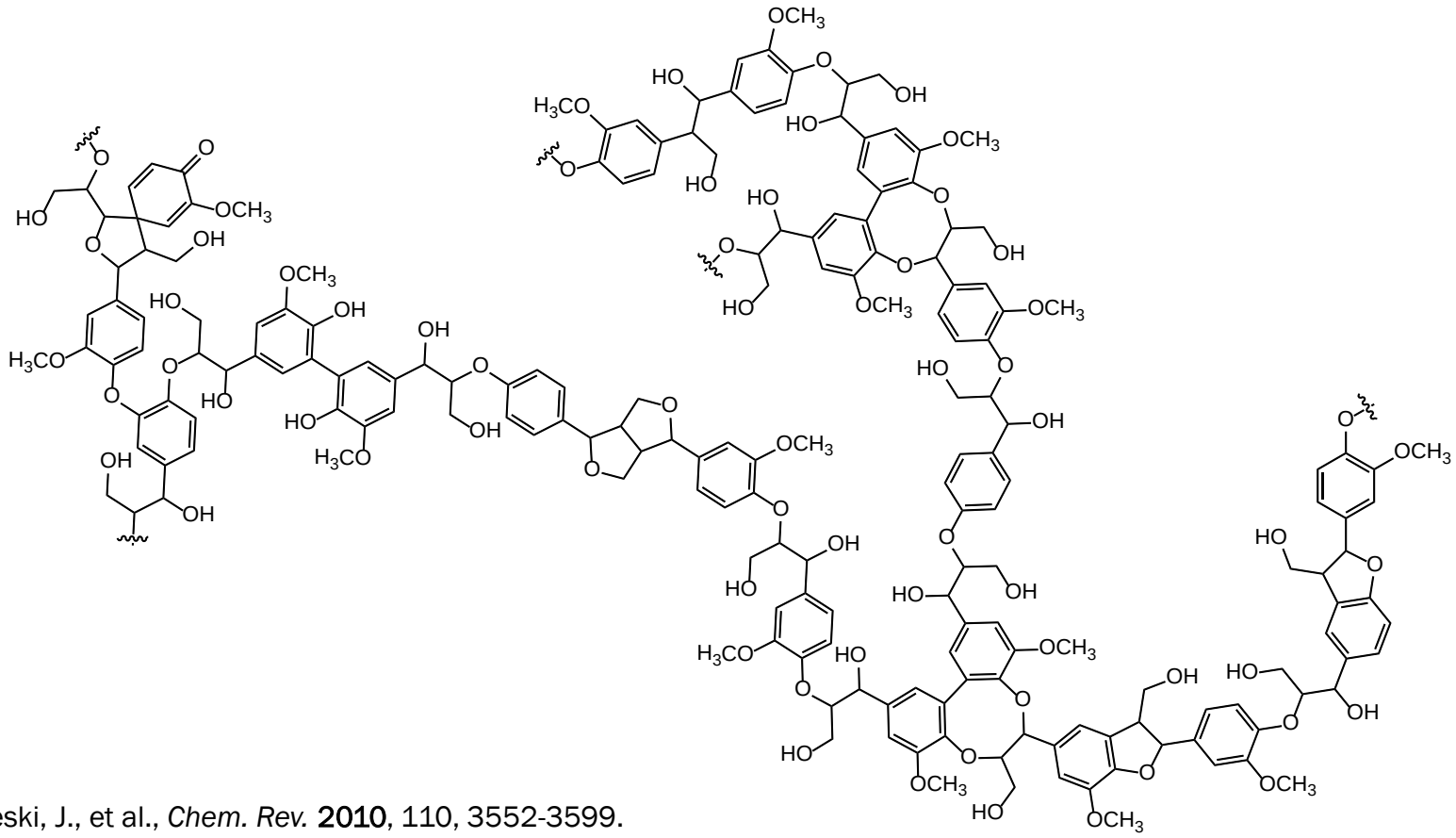
Credit: Gevan Marrs, NARA

Few Commercial Uses for Lignin

- **Annual lignin production:**
  - 40 to 50 million metric tons?
- **Annual commercial lignin consumption:**
  - ≈ 1.5 million metric tons
- **What is the problem? Why isn't lignin more widely used?**
- **Are the chances for success in lignin utilization better today?**

Lignin Complexity

- **Lignin is a complex molecule to begin with.**



Ref: Zakzeski, J., et al., *Chem. Rev.* **2010**, 110, 3552-3599.

Lignin Isolation Affects its Structure and Purity

- **Commercial**
  - Kraft pulping
  - Sulfite pulping

- **Non-commercial**
  - Organosolve pulping
  - Milled wood
  - Ionic liquid pretreatment
  - Mild acid hydrolysis

Photo credit: JBEI

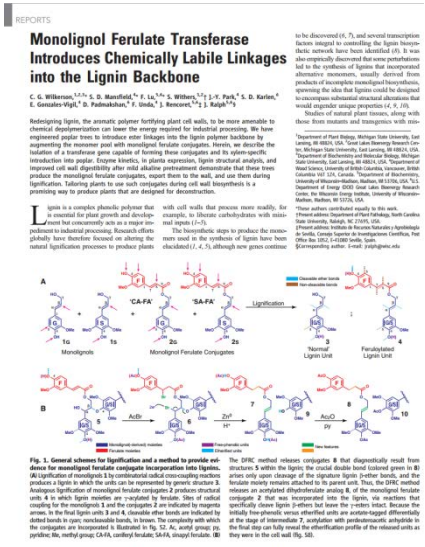
Drivers for Success

Near Term

- Cleaner commercial lignins
- Characterization methods are more powerful
- New market opportunities

Long Term

- Better methods to break specific lignin bonds
- Advances in biomass fractionation
- Ability to manipulate lignin biosynthesis



REPORTS

Monolignol Ferulate Transferase Introduces Chemically Labile Linkages into the Lignin Backbone

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Redesigning lignin, the aromatic polymer fortifying plant cell walls, to be more amenable to chemical depolymerization can lower the energy required for industrial processing. We have engineered poplar trees to introduce ester linkages into the lignin polymer backbone by augmenting the monomer pool with monolignol ferulate conjugates. Herein, we describe the isolation of a transferase gene capable of forming these conjugates and its xylem-specific introduction into poplar. Enzyme kinetics, in planta expression, lignin structural analysis, and improved cell wall digestibility after mild alkaline pretreatment demonstrate that these trees produce the monolignol ferulate conjugates, export them to the wall, and use them during lignification. Tailoring plants to use such conjugates during cell wall biosynthesis is a promising way to produce plants that are designed for deconstruction.

Lignin is a complex phenolic polymer that is essential for plant growth and development but concurrently acts as a major impediment to industrial processing. Research efforts globally have therefore focused on altering the natural lignification processes to produce plants with cell walls that process more readily, for example, to liberate carbohydrates with minimal inputs (*1*–*7*).

The biosynthetic steps to produce the monomers used in the synthesis of lignin have been elucidated (*1*, *4*, *5*), although new genes continue to be discovered (*6*, *7*), and several transcription factors integral to controlling the lignin biosynthetic network have been identified (*8*). It was also empirically discovered that some perturbations led to the synthesis of lignins that incorporated alternative monomers, usually derived from products of incomplete monolignol biosynthesis, spawning the idea that lignins could be designed to encompass substantial structural alterations that would engender unique properties (*4*, *9*, *10*).

Studies of natural plant tissues, along with those from mutants and transgenics with mis-

Fig. 1. General schemes for lignification and a method to provide evidence for monolignol ferulate conjugate incorporation into lignins. (**A**) Lignification of monolignols **1** by combinatorial radical cross-coupling reactions produces a lignin in which the units can be represented by generic structure **3**. Analogous lignification of monolignol ferulate conjugates **2** produces structural units **4** in which lignin moieties are γ-acylated by ferulate. Sites of radical coupling for the monolignols **1** and the conjugates **2** are indicated by magenta arrows. In the final lignin units **3** and **4**, cleavable ether bonds are indicated by dotted bonds in cyan; noncleavable bonds, in brown. The complexity with which the conjugates are incorporated is illustrated in fig. S2. Ac, acetyl group; py, pyridine; Me, methyl group; CA-FA, caffeyl ferulate; SA-FA, sinapyl ferulate. (**B**) The DFRC method releases conjugates **8** that diagnostically result from structures **5** within the lignin; the crucial double bond (colored green in **B**) arises only upon cleavage of the signature lignin β-ether bonds, and the ferulate moiety remains attached to its parent unit. Thus, the DFRC method releases an acetylated dihydroferulate analog **8**, of the monolignol ferulate conjugate **2** that was incorporated into the lignin, via reactions that specifically cleave lignin β-ethers but leave the γ-esters intact. Because the initially free-phenolic versus etherified units are acetate-tagged differentially at the stage of intermediate **7**, acetylation with perdeuteroacetic anhydride in the final step can fully reveal the etherification profile of the released units as they were in the cell wall (fig. S8).

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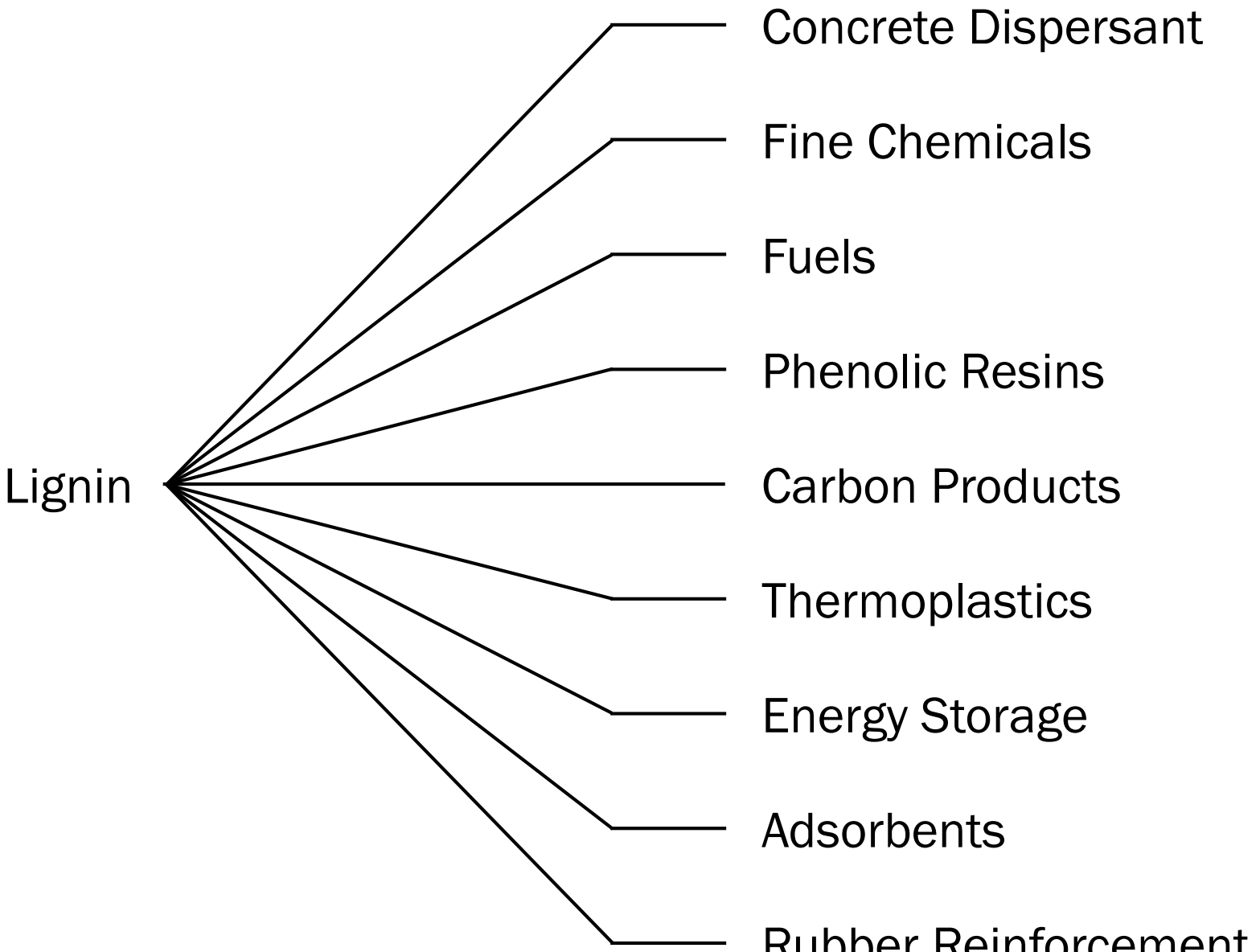
Many Possibilities from Lignin



NARA Supply Chain

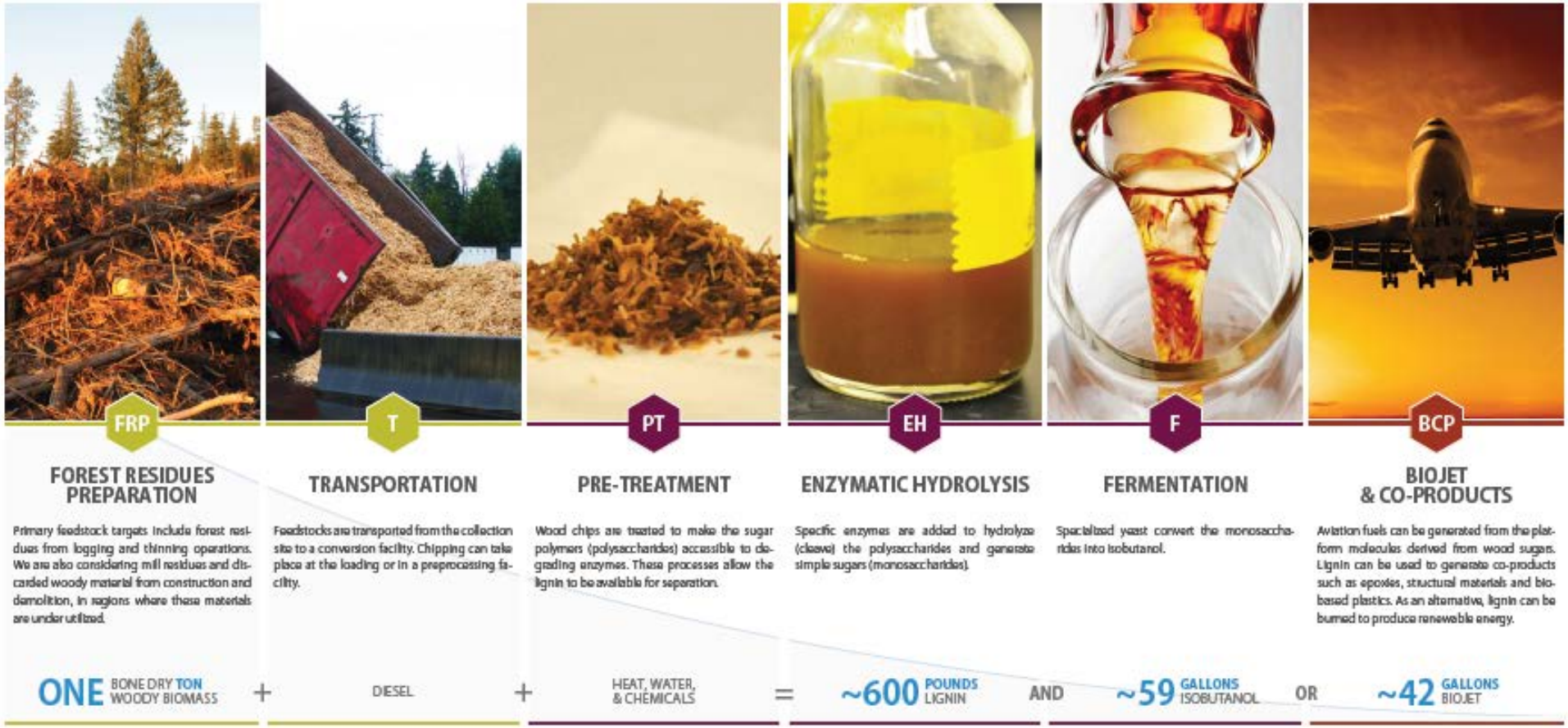
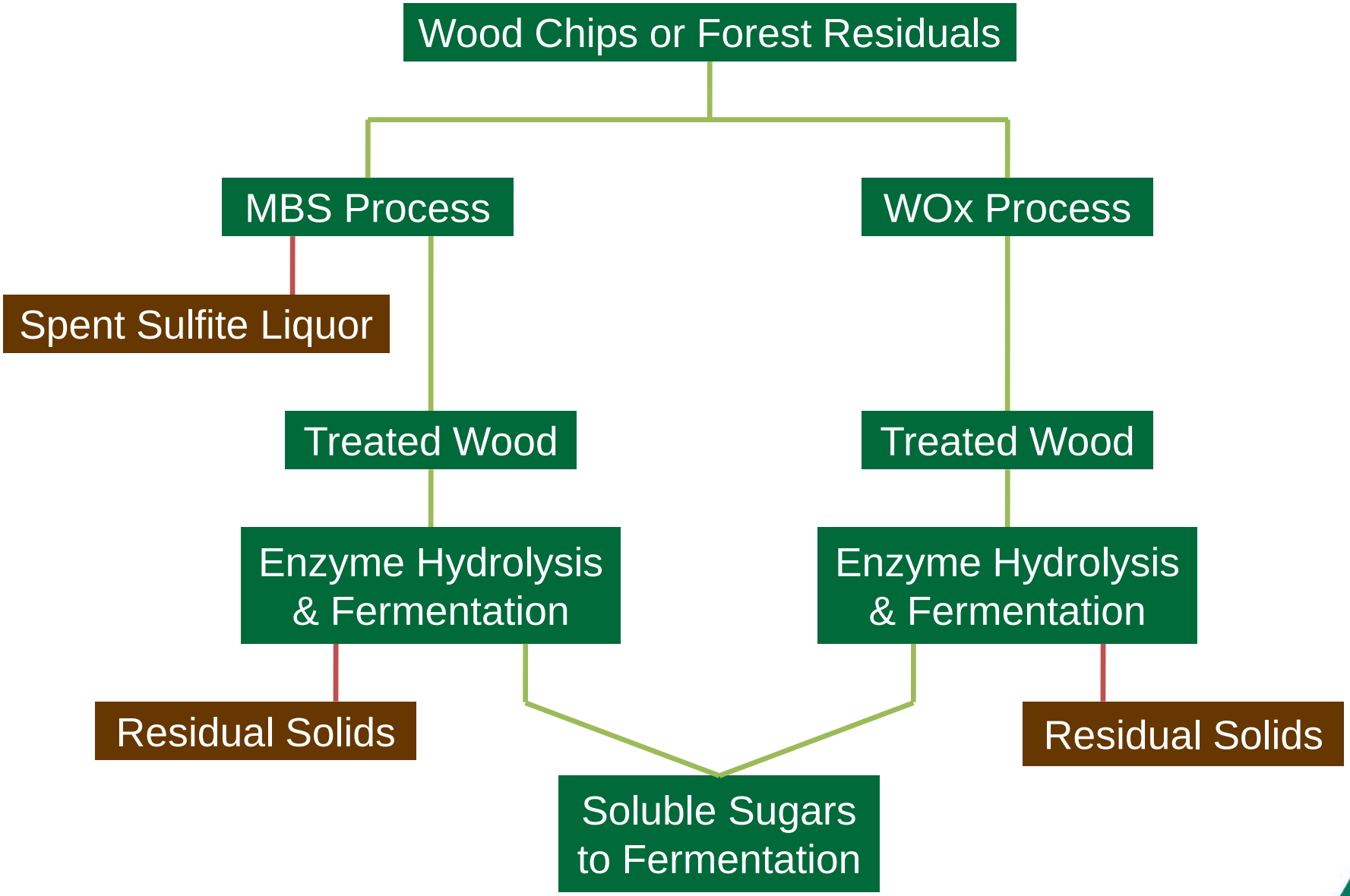
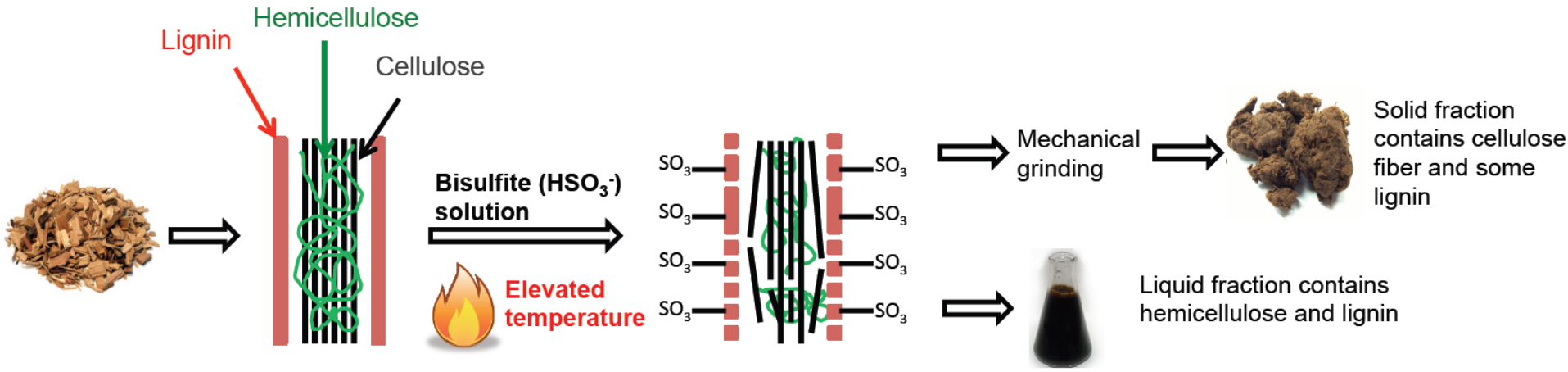


Image credit: NARA

NARA Co-Product Streams



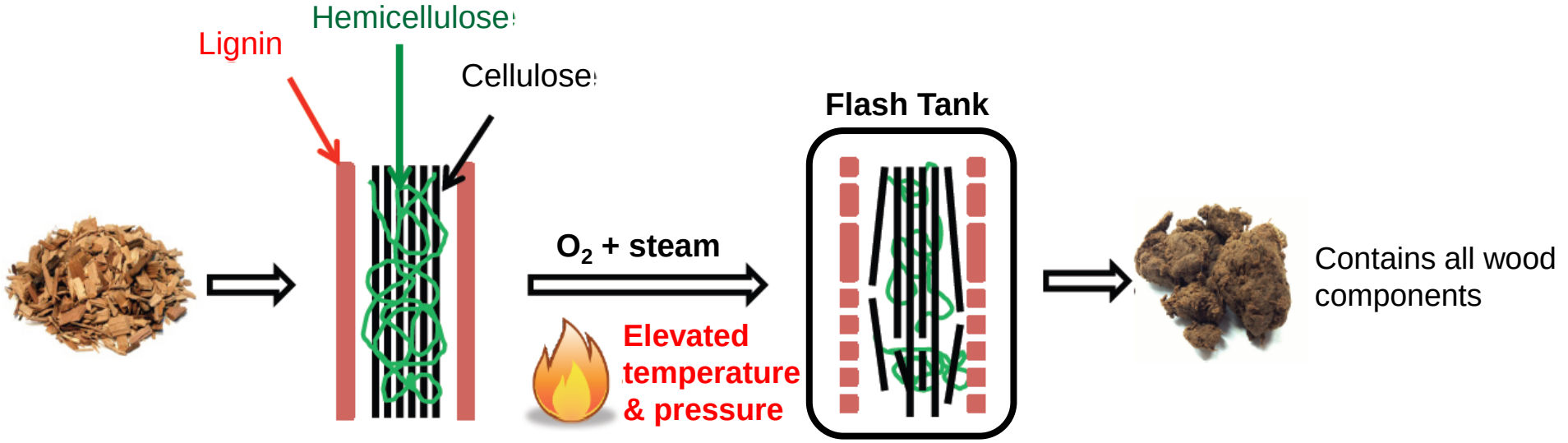
Mild Bisulfite Pretreatment (MBS)



Process development by Johnway Gao and Dwight Anderson at Catchlight Energy and J.Y. Zhu at the Forest Products Laboratory

Refs: Zhang, C., et al. *BioEnergy Research.* **2012**, *5*, 978-988.
Gao, J., et al. *Biotechnology for Biofuels* **2013**, *6*, 10.

Wet Oxidation Pretreatment



Process development by Dr. Brigitte Ahring at Washington State University's Bioproducts, Sciences & Engineering Laboratory

Ref: Rana, D., et al. *Bioresour. Technol.* **2012,** *121*, 61-67.

Composition of Fermentation Residual Solids (FRS)

Sample	Total Klason Lignin	Total Polysaccharide	Ash	Sulfur
Feedstock	34.9%	54.4%	0.2%	0.0%
MBS FRS as rec'd	70.4%	18.9%	8.9%	2.4%
MBS FRS washed	69.4%	23.9%	4.1%	1.8%
Wet Ox FRS as rec'd	53.0%	43.9%	2.0%	0.1%
Wet Ox FRS washed	53.4%	47.3%	0.5%	0.0%

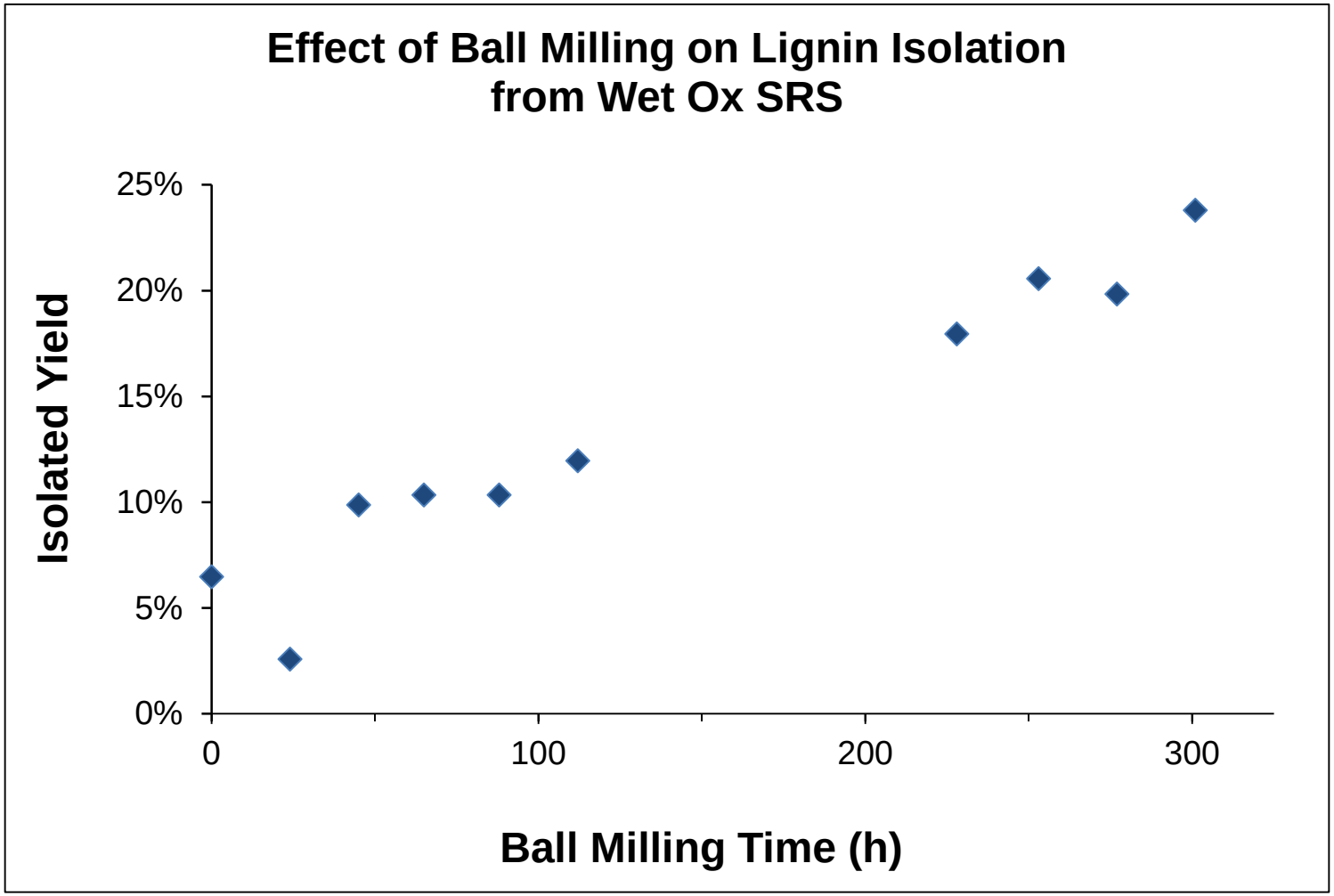
% oven dry

How to Determine FRS Lignin Chemistry?

- **Saccharification and fermentation residual solids are insoluble in all attempted solvents.**
- **Need to extract high purity, unmodified lignin from SRS and FRS samples.**
- **Can we simply extract lignin using dioxane/water mixture, similar to milled wood lignin?**
- **Not really.**

Sample	Yield
MBS SRS	2.8%
Wet Ox SRS	8.3%

Ball Milling Increases Extracted Lignin Yield



Enzymatic Mild Acidolysis Lignin (EMAL)

Procedure developed by the Argyropoulos research group at NC State. (Ref: Guerra, A., et al. *J. Agric. Food. Chem.* 2006, 54, 5939-5947.)

Ball Milling

21 days

Enzymatic Hydrolysis

48 hours

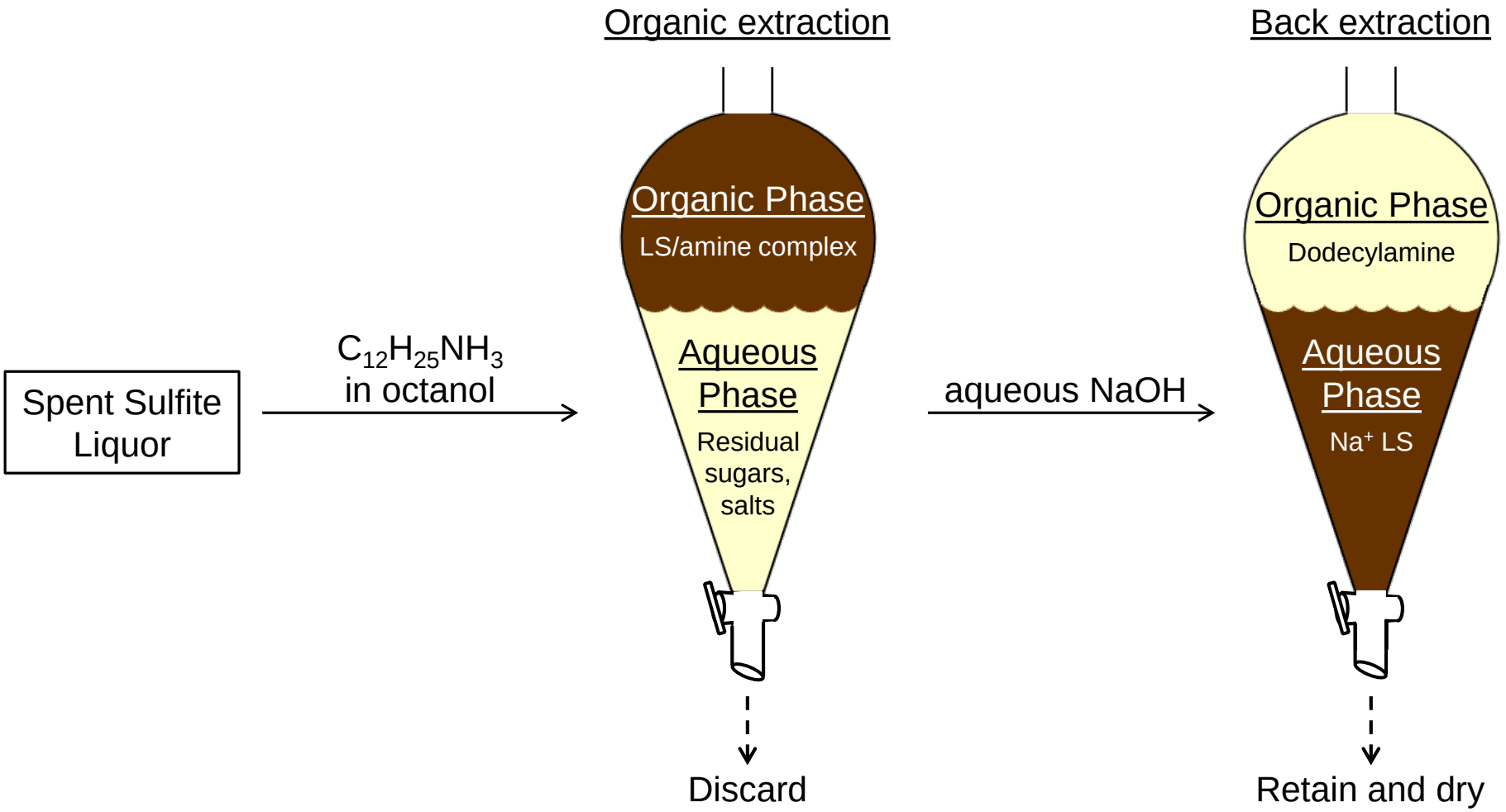
Solvent Extraction

2 hours

Lignin Isolation Yields

Sample	Enzymatic Hydrolysis Yield	Lignin Extraction Yield	Extracted Lignin Purity
Forest Residuals	42.8%	52.4%	81.0%
Mild Bisulfite FRS	64.7%	52.5%	95.4%
Wet Oxidation FRS	86.4%	44.9%	94.2%

Lignosulfonate Isolation Procedure



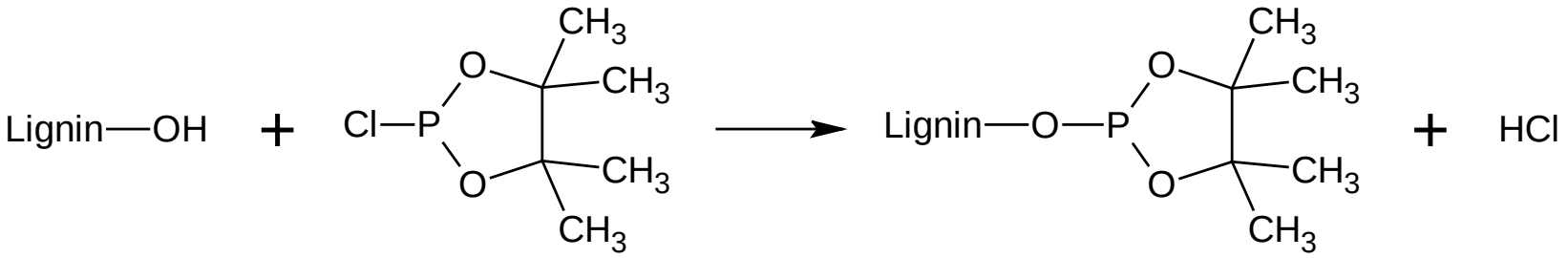
Ref: Ringena, O., et al. *Holzforschung* **2005,** 59, 405-412.

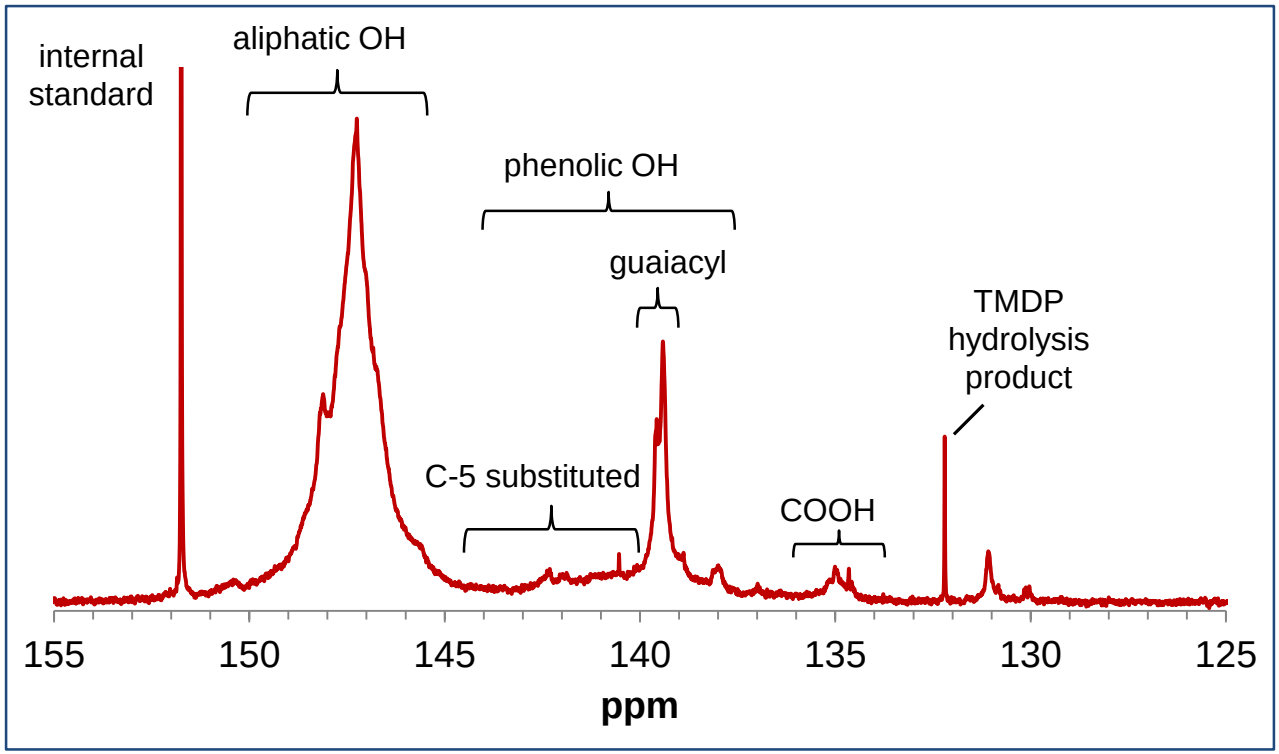
Elemental Analysis of Isolated Lignins

Sample	Sulfur	Carbon	Hydrogen	Nitrogen	Oxygen*
Forest Residual Lignin	0.032	61.0	6.0	0.07	32.9
Wet Oxidation Lignin	0.096	64.9	5.3	0.96	28.7
Mild Bisulfite Lignin	1.13	63.3	5.7	1.01	28.9
MBS Lignosulfonic acid	8.10	52.1	4.8	0.36	34.7

* Oxygen determined by difference
All values % oven dry

^{31}P NMR Analysis of EMALs





Quant. ^{31}P NMR spectrum of forest harvest residual EMAL

Hydroxyl Group Comparison

Sample	Aliphatic OH	Aromatic OH	C-5 Substituted OH	COOH
Forest Residuals Lignin	4.31	1.11	1.45	0.03
Wet Oxidation Lignin	1.93	2.86	1.32	0.21
Mild Bisulfite Lignin	1.38	2.71	1.66	0.22
MBS Lignosulfonic acid	2.42	3.96	1.73	0.10

Molecular Weight Analysis

Sample	Mn	Mw	PDI
Forest Residuals Lignin	26.8 ±	54.6	1.94
Wet Oxidation Lignin	34.1 ±	45.7	1.34
Mild Bisulfite Lignin	47.5	56.4	1.19
MBS Lignosulfonic acid	4.7	28.0	5.92

Summary

- Developing value-added products from lignin-rich process residuals will be critical to large scale biorefinery commercial success.
- Different pretreatment technologies produce residuals with different properties.
- The residues from Mild Bisulfite and Wet Oxidation pretreatments contain a lot more than just lignin.
- Processes to upgrade lignin must be robust enough to handle significant impurity content and variability in the feedstock.

Summary

- **High purity lignin was isolated from the Mild Bisulfite and Wet Oxidation residuals and characterized with respect to elemental content, hydroxyl group content, and molecular weight**

Acknowledgements

- Colleagues Dave Fish and Ian Dallmeyer
- NARA Summer undergraduate intern Steve Cline

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