Lignin Chromatography Lab:
HP-20 Separation of Lignin and Analysis by Size Exclusion HPLC and GC-MS

Introduction: Lignocellulose, i.e. non-food crops, is the most abundant form of biomass on Earth and annual production is estimated at 170 billion metric tons. Lignin comprises approximately 25% of lignocellulose and is considered the "by-product" of lignocellulosic biofuel as well as pulp and paper industries. Though it contains aromatic units, is highly oxidized, and therefore has a low heating value as compared to other carbon-based forms of energy, lignin is often burned to heat the mill's reactors.

Instead of burning lignin, it may be more sustainable to use lignin for bulk and commodity chemical applications – it is after all the largest natural source of aromatic carbon on Earth! Some examples of bulk applications include bio-dispersants, wood panel products, emulsifiers, polyurethane foams, automotive brakes, epoxy resins for printed circuit boards and thermoplastics[1]. A number of commodity chemicals and fuels such as phenols, benzene, toluene and xylene can also be derived from lignin[2].

Chemically speaking, lignin is a heterogeneous, polydisperse macromolecule derived from three monomeric phenylpropanoid units. The monomeric composition is feedstock dependent and is summarized in Figure 1. The depolymerization of lignin can be accomplished under a variety of techniques and has been extensively reviewed. [1,2] Solvent, catalyst, temperature, atmosphere, pressure, pH, etc. all determine the quality of resulting, depolymerized lignin.
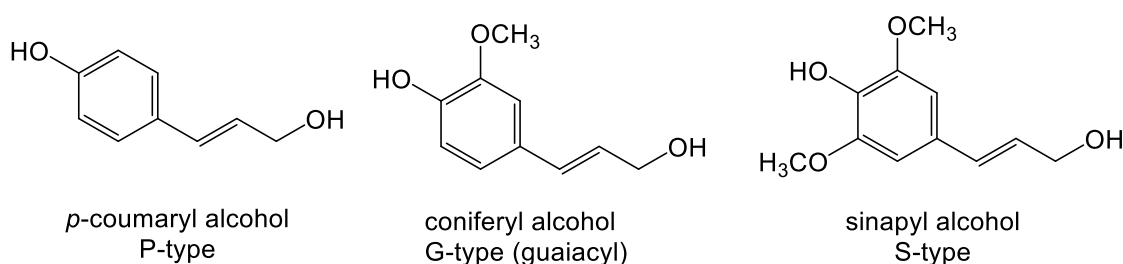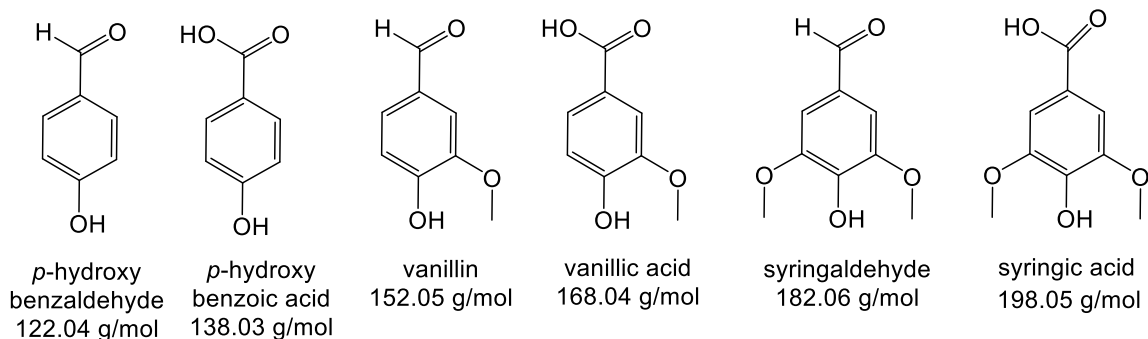

Lignin Monomer	Softwoods	Hardwoods	Grasses
p-coumaryl alcohol (P-type)	0.5-3.5%	Trace amounts	10-25%
coniferyl alcohol (G-type)	90-95%	25-50%	25-50%
sinapyl alcohol (S-type)	0-1%	50-75%	25-50%

Figure 1: *Top, the chemical structures of individual phenylpropanoid monomeric units. The distribution of monomeric units (P, G and S-type) in lignin obtained from the three feedstocks (softwoods, hardwoods and grasses).*

This experiment will focus on the lignin obtained from soda pulping reactions you conducted previously under oxidative (O_2) vs. inert (N_2) conditions. Because lignin can be oxidatively depolymerized to yield higher value compounds such as benzaldehydes and benzoic acids (Figure 2) we will compare the yields of selected benzaldehydes and benzoic acids using GC-MS. Note that oxidative lignin depolymerization is a free-radical process, and thus the lignin is subject to rearrangement to form more condensed versions of the lignin polymer. Because condensed lignin is often more chemically stable, condensation chemistry is unfavorable if the goal is to obtain low molecular weight products from lignin.

Para-hydroxybenzaldehyde, vanillin and syringaldehyde are examples of value-added products derived from lignin (Figure 2). While these compounds can also be synthesized from petrochemicals, they are often much more valuable if obtained from natural sources.[4] For example, vanillin is used in the flavoring industry and costs approximately $1500/kg when derived from natural sources as compared to $15/kg when produced from petrochemicals[5].

| *p*-hydroxy benzaldehyde 122.04 g/mol | *p*-hydroxy benzoic acid 138.03 g/mol | vanillin 152.05 g/mol | vanillic acid 168.04 g/mol | syringaldehyde 182.06 g/mol | syringic acid 198.05 g/mol |

Figure 2: Benzoic acids and benzaldehydes derived from various types of lignin.

In this lab, we will perform a hydrophobic chromatography separation of the depolymerized lignin using HP-20 resin. The fractions obtained by the chromatography will then be subjected to HPLC and GC analysis to see if there is a difference in the lignin's molecular size ranges and composition. Prior to chromatography, it is necessary to adjust the pH of the reaction mixture to ensure that lignin fragments are not in ionic (i.e. phenolate) form. The neutral charge of the resulting (acidic pH) material will ensure binding to the hydrophobic HP-20 resin.
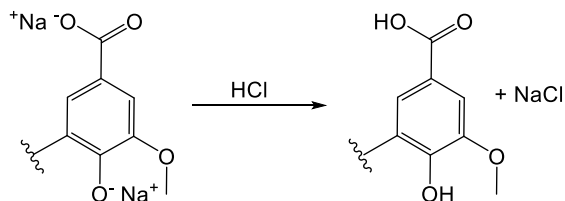
Figure 3: *Acidification of lignin fragment with HCl. Protonation of phenolate to phenol ensures a neutral species which can be absorbed to hydrophobic resin.*

Prelab Questions:

1) What structures from Figure 2 are derived primarily from grass lignin? If you were to use brewery waste as your feedstock for lignin oxidation, what structures would you expect to find as compared to pine? What is the advantage to using pine as a feedstock?

2) What is the result of "over-oxidation" of lignin? If you subjected vanillin to excessive oxidation, which of the structures in Figure 2 would you expect to produce? What is the "over-oxidation" product of syringaldehyde?

3) Using Scheme 1 below, propose a reasonable reaction mechanism for the loss of water in the final step.
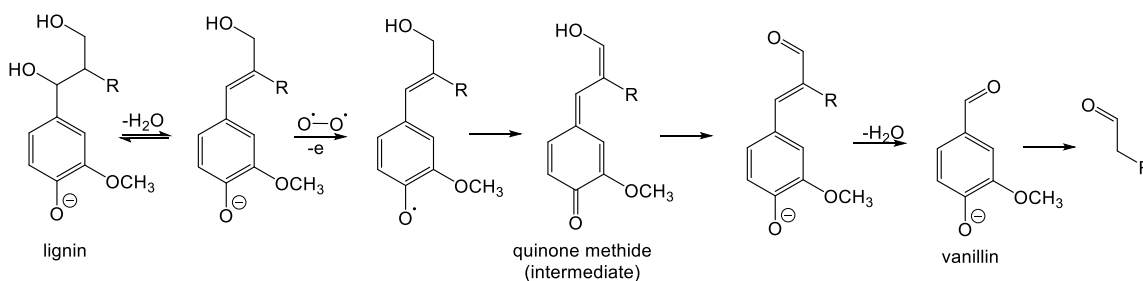
Scheme 1: *Oxidation and hydrolysis of lignin to vanillin.*

4) Is HP-20 chromatography an example of a *reversed phase* or a *normal phase* separation technique?

5) Why is it important to protonate the lignin with HCl prior to chromatography? What solvent will be used to *wash* the resin of unwanted polar compounds? What solvent will be used to *elute* the desired non-polar compounds?

6) What is the theory behind *size exclusion chromatography (SEC)*? Why do we use SEC to assess our lignin depolymerization chemistry? What are some requirements for solvents used for SEC of lignin?

Procedure:

1) Complete Table 1 below using Parr *Spec View* software from the reactions performed in the Pulping Experiment, your lab notebooks and experimental methods.

Pretreatment Number (Notebook page/Date)	#1	#2
Feedstock (*Genus, species*)		
Mass (g)		
0.5 M NaOH Volume (mL)		
Feedstock Moisture Content (%)		
Atmosphere (N_2 or O_2)		
Temperature (^{o}C)		
Reaction time (min)		
Charge pressure (psi)		
Highest pressure reached		
Mass of cellulose obtained		

Table 1: *Comparison of biomass pretreatment conditions*

2) With separate lignin experiments (N_2 and O_2) follow the procedure below.

3) Using a pH meter and 3M HCl, drop the pH of your lignin to 3. This will ensure protonation of the phenolates. Some lignin will precipitate.

4) Set aside 10 mL of each type (N_2 and O_2) of well mixed lignin for SEC measurements. You want to vortex before sampling to ensure sampling from all size ranges (i.e. precipitated and non-precipitated lignin).

5) With remaining lignin make slurries of your separate lignin experiments (N_2 and O_2), each with 50g of HP-20 resin in a 200 mL beaker.

6) Create 5-10 mL fractions (100% water, 90% water in MeOH, 80% water in MeOH, etc. -> -> 100% MeOH) by column chromatography (or vacuum filtration using a Buchner funnel) of the slurry.   Rinse resin with approximately 100 mL of 100% acetone and store for reuse.

7) Dry the samples on the rotary evaporator to complete your mass balance (optional).

8) Syringe filter (0.2 micron) the fractions into a GC vial. They should be perfectly translucent. You may also dilute the fractions as needed for analysis.

SEC HPLC and GC-MS Analysis:

Prepare polystyrene standards for SEC. From the 25 mg/mL stock, prepare standards at 500 µg/mL in N-methyl-pyrrolidinone (NMP). Prepare a 500 µg /mL vanillin sample from vanillin. Run SEC HPLC using the column/guard column and conditions provided by your instructor. Complete the Table 2 below, distinguishing the retention time of the standards to their molecular weights. You will also have to dissolve/sonicate "native" lignin into NMP to use as a standard. Be sure to properly filter the sample prior to HPLC.

Polystyrene Standard	Molecular Weight	Retention Time (min.sec)	UV Absorbance at 254 and 280 nm
1			
2			
3			
4			
5			
6			

Table 2: *SEC Polystyrene Standard Results*

Prepare GC-MS Standards and a Calibration Curve for Vanillin and Syringaldehyde. Complete Table 3 below.

Standard	Concentration µM	Retention Time (min.sec)	Peak Area
Vanillin	50		
Vanillin	150		
Vanillin	450		
Vanillic acid	50		
Vanillic acid	150		
Vanillic acid	450		
Syringaldehyde	50		
Syringaldehyde	150		
Syringaldehyde	450		

Table 3: *Calibration data for selected GC-MS analytes of interest.*

Conclusions:

Using the data in Table 2, estimate the molecular weights of the different lignin that you analyzed. Did you see a reduction in lignin molecular weight in the pretreated lignin as compared to native lignin?

Using the peak area data in Table 3, plot the calibration curves for your benzaldehydes. Be sure to include axis labels, title, legend, R^2 value and equation on the chart. Based on your understanding of the chemistry and the GC, do you think it is OK to assume the retention time and estimate the yield of syringic acid using the data from vanillin/vanillin acid and syringaldehyde?

Using point-slope-form, quantify the percent yield of the individual benzaldehydes and benzoic acids from original biomass sample for your two pretreatment reactions and complete Table 4 below. This will require several accurate back calculations. Be sure to account for all dilutions and partitioning of original biomass/lignin fractions.

Reaction Conditions	Vanillin Concentration μM	Syringaldehyde Concentration μM	Vanillic Acid Concentration μM	Syringic Acid Concentration μM
N$_2$				
O$_2$				

Table 4: Percent yield of selected benzaldehydes and benzoic acids under various reaction conditions

What are your conclusions about the yields of benzaldehydes and benzoic acids from various pressure reaction conditions?

Were other compounds observed in amounts greater than those listed in Table 3? What are their identities by GC-MS analysis? Are they of industrial value?

How can the reaction conditions be optimized to favor one product over the other (e.g. benzaldehyde vs. benzoic acid)?

References:

1. Li, C. et al. Catalytic transformation of lignin for the production of chemicals and fuels. *Chem. Rev.* **2015**, 115, 21, 11559-11624.
2. Zakzeski, et al. The Catalytic Valorization of Lignin for the Production of Renewable Chemicals *Chem. Rev.* **2010**, 110, 6, 3552-3596.
3. George, A. et al. The effect of ionic liquid cation and anion combinations on the macromolecular structure of lignins. *Green Chem*. **2011**, 13, 2237-3385.
4. Fache, M. et al. Vanillin production from lignin and its use as a renewable chemical. *ACS Sust. Chem. Eng.* **2016**, 4, 35-46.
5. Evolva Company Website: http://www.evolva.com/vanillin/