Lignin Microwave Protocol
Keil Lab

Updated Oct 2018

Adapted from Goni and Montgomery 2000

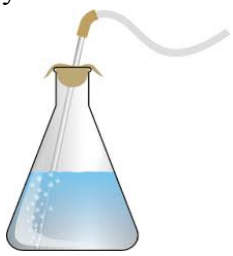
1.1 Preparation (do this the night before)

1) Prepare a 2N NaOH solution in an Erlenmeyer flask by dissolving 16 g NaOH(s) in 200 mL of milli-Q water. This is enough solution for six samples.
    a. Adjust this amount accordingly for however many vessels you will be running. If you are running 12 samples, you will need 350 mL of solution (28 g NaOH and 350 mL milli-Q water)

2N NaOH

Number of samples	Milli-Q Water (ml)	NaOH (g)
-	50	4
2	100	8
4	150	12
6	200	16
8	250	20
10	300	24
12	350	28

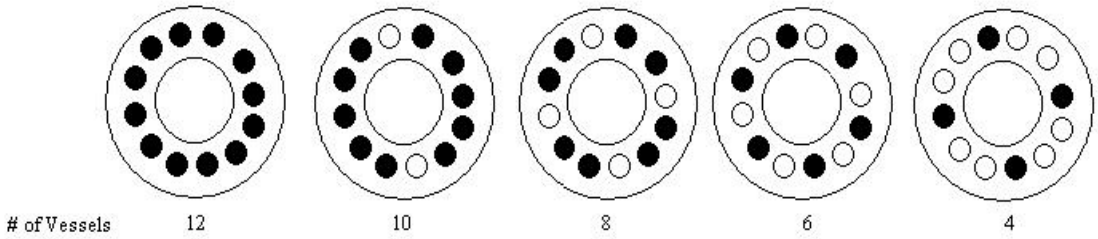
2) Cover flask with parafilm. Bubble this 2N NaOH solution overnight with Nitrogen gas to remove dissolved oxygen in the water. Attach a glass pipet to the nitrogen line in the fume hood and carefully poke the glass pipet through the parafilm and lower it into the solution. Slowly increase the nitrogen gas by adjusting the knob on the front of the hood. A gently bubble stream is all you need. Be sure to label the flask with the contents (2N NaOH), date and your initials.



1.2 Oxidation

The oxidation process is carried out using the MARS 5 system (CEM corp), during which up to twelve samples are simultaneously oxidized. The samples need to be very similar in percent organic carbon so that the amount of sediment in each vessel is nearly the same – this helps to prevent uneven heating of the samples. If uneven heating occurs, there is typically a significant amount of vapor transfer between vessels, which can potentially result in one or more vessels running dry – without a sufficient amount of liquid in the vessel, the CuO will quickly heat up and start sparking and burning – very bad!

1) Into each 120 mL PFA vessel, load ~500 mg fine CuO powder and ~75 mg ferrous ammonium sulfate (used to bind any remaining oxygen in the solution)
2) Add a known amount of sediment, representing 3-5 mg of organic carbon, to each vessel.
3) Using a glass pipet and pipet bulb, add 20 mL of the bubbled 2N NaOH solution to each of the sample vessels. When transferring the NaOH solution from the flask to the sample vessel, run the stream of NaOH down the sides of the vessel, taking care to minimize the amount of splashing that occurs so that the amount of oxygen introduced during this step is negligible. Try to rinse any particles off of the vessel sides as you do this.
4) Cap the vessels with their respective o-rings and caps, ensuring that the o-ring is oriented correctly (if you look at the o-ring, you will notice that the groove on one side is deeper than the groove on the other. It is very important that the deeper groove is facing up). Again, avoid splashing the solution around in the vessel from this point on.
5) Once the caps are hand-tight, use the capping station to completely seal the vessels. To do so, place the vessel in the capping station and rotate it until it sinks into a locked position. Then, push and hold the toggle switch in the "tighten" position until the tension meter reads a value in the "blue" range. Rotate the vessel in the capping station and repeat the tightening three more times.
6) Evenly place the sealed vessels into the turntable (Diagram Below). Connect the vessels using the 3 mm O.D. Teflon tubing pieces, leaving one port open on the first and last vessels. Make sure that the ferrules are tight.



7) Place the turntable onto the drive lug inside the microwave system cavity. Rotate it until it locks into place.
8) With the valve panel switch in the "nitrogen" position, open the Nitrogen gas supply on the manifold above the microwave and allow the tubing to flush with Nitrogen at a pressure of ~60 psi to blow out any water that has accumulated in the line. Once the tubing is dry, reduce the pressure to ~20 psi and connect the pressure sensing line to the open port in the first vessel and tighten the ferrule nut to secure the pressure sensing line. Allow the vessels to flush with Nitrogen for a minute or so.
9) Carefully insert the Teflon-coated Pyrex thermowell (rinse with dichloromethane "DCM" prior to placing it in the vessel) into the open port of

the last vessel until it nearly touches the bottom of the vessel – make sure it is not touching the bottom of the vessel. The thermowell position can be observed by holding the vessel up to a light source. Gently tighten the ferrule, but do not overtighten it, as doing so will crack the thermowell.

10) Carefully insert the temperature probe into the thermowell until it bottoms out. The temperature probes operate using fiber optics, so they are very fragile. DO NOT sharply bend or kink the probe as it will break the fibers inside and the probe will no longer work – probes are very expensive!
11) Plug the temperature probe into the receptacle located in the cavity ceiling. Use care during this step as the connection pieces are made of porcelain and can break easily.
12) Secure the pressure sensing line into the standoff located in the center of the turntable so the lines don't tangle during the run.
13) With the instrument door open, press the turntable key to rotate the turntable 2 or 3 times to ensure the sensing lines do not become entangled. Situate the vessel rack so that the control vessel (the one with the temperature probe in it) is located at the back center of the cavity when the turntable switches rotation directions. If necessary, reposition pressure line tubing and/or temperature probe line. Recheck turntable rotation and close the instrument door.
14) With the valve panel switch in the "nitrogen" position, increase the Nitrogen gas supply on the manifold above the microwave to 60 psi. Allow the vessels to sit at 60 psi until system has equilibrated. Open the door and carefully remove one vessel at a time and submerge in a beaker full of water and check for bubbles, which would indicate a leaky vessel or ferrule. If a vessel is leaking, make note of which vessel it is and continue leak-checking the rest of the vessels. Dry the vessel and place it back in the rack. If the ferrules are leaky, first hand tighten them and recheck the vessel for leaks. Once finished, release the pressure in the system by turning the valve panel switch to the "vent" position. Allow the vessels to sit for a couple of minutes to ensure that they are completely vented. Remove any leaky vessels from the system and uncap and check the o-ring and cap for dirt. If there is any debris, wipe the part with a small amount of methanol on a kimwipe. Recap the vessel(s) and reconnect to the system. Repeat the leak check and make certain that the vessels are not leaking. If a leak is still present, it may be necessary to transfer the contents to a clean vessel. If there are no leaks, continue to the next step.
15) Adjust the Nitrogen pressure regulator to ~12 psi. Turn the valve panel switch to the "nitrogen" position. Allow the vessels to sit for a couple of minutes to equilibrate. Turn the vacuum pump on and turn the valve panel switch to the "vacuum" position for about 90 seconds, or until the vacuum gauge on the valve panel reads -27 in Hg. Turn the valve panel switch back to the "nitrogen" position and allow it to remain there for 90 seconds, and until it reaches approximately 12 psi. To check the pressure during this step, follow the instructions on the machine for checking pressures below 50 psi. Turn the valve panel switch between "nitrogen" and "vacuum" at least five times,

allowing the samples to equilibrate for at least 90 seconds between switches. After completing five vacuum evacuation/nitrogen purge cycles, turn the valve to the 'run' position, sealing the samples under a 12-psig nitrogen atmosphere. Turn the vacuum pump off.

16) Close the microwave door and start the microwave by selecting the method titled "800W-LIGNIN."
    - **a.** This program runs in the 800 Watt power mode and consists of a 5 minute ramp to 150 °C with a hold at 150 °C for 90 minutes. Under normal conditions, the samples will ramp up to temperature in the 5 minute window and remain between 148-153 °C, with a pressure between 60-70 psi for the duration of the run. **If pressure reaches above 100 psi stop the run immediately.**
17) At the conclusion of the oxidation a cooling cycle (automatic 10-15 minute post-run cycle) will begin. At the conclusion of the cooling cycle, the samples will still be too warm to remove them from remove them from the microwave. Let them sit in the microwave until they have reached a temperature below 70º C.
18) Turn the valve to the 'vent' position to release the remaining atmosphere of nitrogen gas. Disconnect the temperature probe from the roof of the microwave cavity. Disconnect the pressure sensing line from the vessel assembly and slide the pressure sensing line out of the stand off.
19) Remove the turntable from the cavity, loosen the ferrule nut and remove the tubes from each sample vessel.
20) Carefully lower each sample vessel into the socket of the Capping Station. Briefly push the toggle switch to the position marked "Loosen". The vessel cap will loosen by turning in a counterclockwise direction.

    CAUTION : Do not completely unthread the vessel cap in the Capping Station. Removing an uncapped vessel from the Capping Station may permit spillage and/or contamination of the samples.

21) Lift the vessel from the Capping Station and remove the cap.

1.3 Post-Reaction Extraction

22) To each vessel, add 50 uL of the 1 mM trans-Cinnamic Acid and Ethyl Vanillin recovery standard (suspended in pyridine) using a plastic pipettor. This solution is our recovery standards, so it is very important that the amount added to each vessel is precise. When evacuating the pipet, touch the tip to the side of the vessel just above the level of the sample liquid to ensure that the entire amount of standard makes it into the sample. Use a new pipet tip for each sample.

23) Swirl the sample in the vessel to suspend the particulates and carefully pour all of the material from the vessel into a clean 40 mL polypropylene centrifuge tube and close with a push-on cap.
24) Arrange the centrifuge tubes in centrifuge rotor, ensuring that the configuration is balanced.
25) Close the centrifuge lid and centrifuge at 4000 rpm at 4 °C for 10 minutes.
26) Once the program is complete, remove samples from the centrifuge and place in a test tube rack.
27) In a fume hood, open the centrifuge tubes and carefully decant the supernatant into clean 50 mL glass centrifuge tubes.
28) To the remaining solid in the plastic centrifuge tube, add 5 mL of the 2N NaOH solution and cap the tube. Use a vortex mixer to thoroughly mix the sample.
29) Perform a second centrifuging on the samples to act as a rinse of the solids (same as step 25). Following centrifuging, decant the liquid into the same 50 mL glass test tubes as the first round of liquid was put into and dispose of waste properly (see attached document "Cleaning Procedures- Waste").
30) Add 5 mL of 37% (12M) Hydrochloric acid to each sample using a plastic pipettor. Then add 8 mL of ethyl acetate to each sample using a volumetric pipette. Cap the tubes with Teflon-lined caps and shake for 1 min. Place tubes in a rack and allow the phases to separate, which usually only takes a few minutes. If there are bubbles where the ethyl acetate is 'stuck' in the aqueous layer, carefully swirl or use a pipette tip to release them to the proper layer. If you notice cloudiness or particulates in the bottom layer, check the pH of the bottom layer. It should be around 1. If it is too high, add small amounts (500 uL at a time) of 37% HCL until you see a change. Both layers should be translucent and not cloudy.
31) Once the phases have fully separated, carefully transfer the top (ethyl acetate) layer to a 20 mL scintillation vial using a Pasteur pipette. Use a new pipette for each sample, and make sure that the vial you are putting the sample into will accept a cap (some of our vials do not have caps that fit them).
32) Add another 8 mL of ethyl acetate to the test tube and cap and shake the samples again. This is a "rinse" of the sample.
33) Again, pipette the top layer off and add it to the same 20 mL scintillation vial as before. Dispose of the Pasteur pipets in the glass disposal box.
34) Cap the scintillation vials with the same Teflon-lined caps that were used with the 50 mL glass test tubes. Keep the caps with their original samples! If you are unsure if you mixed the caps, use a new cap so you don't risk cross-contaminating your samples.
35) At this point, the samples can be refrigerated until you are ready to continue. Sometimes, it works out well to get the samples to this point and refrigerate them overnight and finish processing them the next morning.

1.4 Drying

36) Add cleaned (muffled) sodium sulfate to each vial and shake for about one minute. The amount that you add will depend on how much water is in your sample. You want most of the sodium sulfate to form one big clump, but there should also be a few smaller clumps floating around. The clumps should move freely around the vial and not stick to the bottom or the walls. If you have added too little, the sodium sulfate will not clump and it will stick to the bottom of the vial with an oozy consistency.
37) Tap the vial on the counter to concentrate the sodium sulfate crystals on one side of the vial. Then, using a Pasteur pipette, transfer the liquid to a clean 20 mL vial. Try to avoid splashing the liquid on the rim of the new vial. These vials do not need to have caps.
38) Place the vials in a heated Turbovap (45 deg. C) and dry the samples down under a low N_2 flow. When the samples are nearly dry, rinse the walls of the vials with 1-2 mL of ethyl acetate and allow the samples to completely dry. The amount of time that it takes for the samples to dry will depend on how high the flow of nitrogen is, but it typically takes about 45 minutes. Check your samples frequently. Do not let the samples overdry in the Turbovap, as you run the risk of losing some of the more volatile compounds in your sample.
39) Once the samples are dry, immediately resuspend them in 800 uL of pyridine. If your samples are of lower organic content, the resuspension volume should be changed to 400 uL. Record the resuspension volume you use in the lab notebook. Do each sample individually by pipetting the pyridine in to the vial and swirling it around briefly to make sure that everything in the vial goes into solution. Use the same pipette tip that you used to pipette the pyridine into the vial to transfer this liquid into a 2mL amber vial with a telfon-lined cap. Use a new pipette tip for each sample.
40) The samples can now be stored in the freezer until you are ready to run them on the GC.

1.5 Gas Chromatography Analysis

See attached document “Sample prep for GC-FID analysis”

Cleaning Procedures (also see detailed cleaning protocols – attached)

Microwave Vessels and caps: Wash with Micro-90 soap and sponge. Thoroughly DI rinse and place in a 10% acid bath for at least a few hours, preferably overnight. Rinse vessels with DI water and hang on rack to dry. When washing the caps, be sure the keep the correct o-rings with their respective caps.

Tubing connection pieces: Rinse with DI water and place in acid bath.

Thermowell: DI-rinse after a microwave run, and wipe with dichloromethane immediately before a run.

All glassware: Wash with Micro-90 soap and scrub with a test tube brush. Thoroughly rinse with DI water and let air dry. Muffle.

Plastic caps: Wash with Micro-90 soap and sponge. Rinse with DI water and place in acid bath overnight (or for at least a few hours). Thoroughly rinse with DI water and allow to dry.

Waste

CuO sludge: There is a container for the sludge that is left over after centrifuging. Use a DI water wash bottle to rinse the residue from the plastic test tubes into this waste container.

90%Water, 10% ethyl acetate: There is a jug for the liquid that you are left with following the phase separation. Pour the remaining liquid from the glass test tubes into this jug.

Keil Lab Equipment Cleaning Protocols

Glassware:
All glassware should be thoroughly cleaned with Micro-90 Soap and DI Water. A sponge should be used to clean inside of beakers, and a test tube brush should be used to clean inside of test tubes and vials. Thoroughly rinse items (at least 4 times) with DI water to remove the soap residue. Allow items to air-dry flat on a large kimwipe on the countertop. Items should appear clean (no dark rings, spots, etc.) after these steps. If not, clean them again. When completely dry, wrap like items in aluminum foil packets so they may be combusted (i.e. separate wide-mouth vials from narrow-mouth vials). For beakers and other open-top items, place a piece of aluminum foil over the opening for combusting. Write a large letter "K" on the foil with a sharpie – this is so we can distinguish our items from other labs using the muffle furnace. The sharpie writing will disappear during combusting, but the imprint of the "K" will remain on the foil. Be sure to only take our lab's items from the muffle furnace when combusting is complete, as other labs will combust their glassware along with ours.

Plastic and Teflon:
All plastic and Teflon items (i.e. caps, liners, microwave vessels, etc.) should be thoroughly cleaned with Micro-90 Soap and DI Water. A sponge should be used to clean inside of beakers, and a test tube brush should always be used to clean inside of test tubes and vials. Thoroughly rinse items (at least 4 times) with DI water to remove the soap residue. Items should appear clean (no dark rings, spots, etc.) after these steps. If not, clean them again. If an item still appears dirty, set it aside and ask someone else in the lab for advice before continuing in the cleaning process. Place items in a 10% Hydrochloric Acid bath, making sure that they are fully submerged. Be sure to wear gloves and goggles to protect yourself from splashes when placing items in the acid bath. Let items remain in the acid bath overnight. Remove items from the acid bath (wear gloves and goggles) and thoroughly rinse with DI water (at least 4 times). Hang microwave vessels on the drying rack above the sink to dry. For caps and liners, lay a piece of foil on the countertop with a large kimwipe over it and place items on top of the kimwipe to dry. Cover items with an additional kimwipe.

Special instructions:

Microwave vessel bodies: These can be particularly tricky to get clean. The sponge must be squished down into the vessel and moved around the vessel to remove all residue. Also use the sponge to clean the rings on the outside of the vessel. IMPORTANT: **do not** use a test tube brush to clean the vessels, as it may scratch the Teflon.

Microwave vessel caps: Separate the o-ring from the inside of the cap when cleaning, but keep the o-ring with its respective cap – the vessels seal better if the same o-ring is always used.

Vial/Test tube caps: If you use a cap that requires inserting a Teflon liner, remove that liner when cleaning. Be sure to use a sponge to wipe all caps and liners.

New Items: All new glassware must be combusted prior to use. All new plastic ware (except for GC vial caps) must be acid-washed prior to use.

Sample prep for GC-FID analysis

Supplies:
2 mL screw cap GC Vials (agilent 5182-0715)
250 uL glass flat-bottom vial insert (agilent 5181-3377)
Blue screw caps, PTFE-lined (agilent 5182-0717)
0.25 mM Lignin External Standard
Pyridine
BSTFA + TMCS, 99:1

1. Label 2mL GC vial (fitted with a flat-bottom glass insert) with appropriate sample name. Use only cleaned and muffled vials and inserts.
2. For sample preparation, pipet 50 uL of sample (in pyridine) and 50 uL of BSTFA into the vial. Cap the vial, ensuring that it is tightly sealed. Roll the solution around in the vial, making sure to get any drops that may have accumulated on the upper end of the vial insert.
3. For standard preparation, pipet the appropriate amounts of 0.25 mM Lignin External Standard, Pyridine and BSTFA into the vial to yield the desired injection concentration. Again, cap the vial tightly and roll the solution around to thoroughly mix it. See attached External Standard Dilutions document for more details.
    a. If there is left-over BSTFA, you can save it for a short period of time (usually a couple of days) as long as it is in an amber vial fitted with a Teflon-lined cap and the headspace is purged with nitrogen. Store the vial in the refrigerator.
4. Derivitize the samples by heating them for exactly 10 minutes at 60º C in the dry-block heater. Use the stopwatch to time the heating.
5. While the samples are heating, prepare the GC for the sample sequence.
    a. Open the lignin (online) Chemstation program and ensure that the program is in “Method and Run Control” mode.
    b. Load method LIGNINCONSTFLOW.M. The GC will start heating up to operating temperatures and you should hear the “pop” of the FID igniting.
    c. From the “Sequence” menu, select Sequence Parameters and insert your name in the Operator Name field and change the subdirectory folder name to the current date. A pop-up window will appear asking if you’d like to create the new folder; hit yes. Press OK.
    d. Fom the “Sequence” menu, select Sequence Table. This is where you will insert all sample information and tell the GC the order in which you would like the samples to run. Make sure that the GC is set up to go into shutdown mode (method: “SHUTDOWN350DET”) at the end of the sequence. See example table on next page.
    e. From the “Sequence” menu, select Save Sequence As and rename the file name to the current date.
    f. From the “Sequence” menu, select Print Sequence and check the boxes “Sample information part” and “Method and Injection Info part.”

6. When the vials are finished heating, remove them from the heating block and arrange them in the auto sampler tray in the correct position according to the sequence table.
7. Fill the wash vials with the appropriate solvents. Vial A11 gets dichloromethane and Vial B8 gets pyridine. Make sure that slots WA5, WA4, WB3 and WB2 have empty vials to collect the wash waste.
8. When you are ready to start the sequence, from the "RunControl" menu, select Run Sequence.

Line	Vial	Sample Name	Method Name	Inj/Vial	Sample Type
1	Vial 1	0.03 mM Lignin External Std	LIGNINCONSTFLOW	1	Sample
2	Vial 2	Sample 1	LIGNINCONSTFLOW	1	Sample
3	Vial 3	Sample 2	LIGNINCONSTFLOW	1	Sample
4	Vial 4	Sample 3	LIGNINCONSTFLOW	1	Sample
5	Vial 5	Sample 4	LIGNINCONSTFLOW	1	Sample
6	Vial 6	Sample 5	LIGNINCONSTFLOW	1	Sample
7	Vial 7	Sample 6	LIGNINCONSTFLOW	1	Sample
8	Vial 1	0.03 mM Lignin External Std	LIGNINCONSTFLOW	1	Sample
9		shutdown	SHUTDOWN350DET	1	Sample

External Standard Dilutions

A 0.25 mM external standard dilution is used to make the following dilutions. The 0.25 mM external standard dilution is made from a 10 mM external standard solution diluted with pyridine. If more 0.25 mM external standard solution is needed, dilute the 10 mM external standard solution with pyridine appropriately. For example, to make 5 mL of a 0.25 mM external standard, transfer 125 uL of the 10 mM solution into an amber scintillation vial and add 4875 uL of pyridine ($M_1V_1=M_2V_2$).

An external standard should be run in the GC during every sample run. Any of the following dilutions may be used, but be sure that the dilution used is noted.

Standards and Samples are diluted so that 50% of the volume is BSTFA. It is best to keep the total volume of samples and standards consistent. See tables below for standard dilutions for total volumes of 200 uL and 100 uL.

200 uL total volume

External Standard Dilution	0.01 mM	0.02 mM	0.03 mM	0.04 mM	0.05 mM
0.25 mM External Standard Dilution	8 uL	16 uL	24 uL	32 uL	40 uL
BSTFA + TMCS; 99:1	100 uL	100 uL	100 uL	100 uL	100 uL
Pyridine	92 uL	84 uL	76 uL	68 uL	60 uL

100 uL total volume

External Standard Dilution	0.01 mM	0.02 mM	0.03 mM	0.04 mM	0.05 mM
0.25 mM External Standard Dilution	4 uL	8 uL	12 uL	16 uL	20 uL
BSTFA + TMCS; 99:1	50 uL	50 uL	50 uL	50 uL	50 uL
Pyridine	46 uL	42 uL	38 uL	34 uL	30 uL

The Lignin External Standard is a mixture of 14 lignin compounds in a known concentration. Known masses of each compound are dissolved in pyridine to a given concentration and then combined to form a standard mixture. The compounds in the standard are:

4-Hydroxybenzaldehyde
4-Hydroxyacetophenone
4-Hydroxybenzoic Acid
3,4 dihydroxybenzoic Acid
Vanillin
Vanillic Acid
Acetovanillone
Ethyl Vanillin
Acetosyringone
Syringaldehyde
Syringic Acid
Trans-Cinnamic Acid
Ferulic Acid
p-Coumaric Acid