Standard Operating Procedure

Lignin phenol calibration of methoxy groups in lignin substrates

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If you use this protocol, please cite:
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A. PURPOSE

Methoxy ($-OCH_3$) groups found on lignin molecules may be targets for quantification, e.g. to know reaction yields, structural and thus source composition for mixed substrates and to understand diagenetic alteration in buried wood, peat, lignite and bituminous coal. Here, we describe a calibration procedure for the reaction and analytical step that uses lignin monomers, of known stoichiometry, to quantify iodomethane incorporating both instrument and analytical yields into the calibration. Quantification of peak area response to evolved iodomethane from a lignin phenol of known stoichiometry, is needed in order to determine methyl wt% in methoxy groups on lignin bearing substrates (e.g., wood, peat, lignite and coal) of unknown stoichiometry, using gas chromatography (GC) flame ionization detection (FID).

B. NECESSARY MATERIALS

1. Select one or more lignin phenol monomers with methoxy groups, for example:

Common name	IUPAC Name	Chemical formula	Molar mass (g/mol)	# methoxy groups	Methyl (wt. %)
Vanillin	4-Hydroxy-3- methoxybenzaldehyde	$C_8H_8O_3$	152.149	1	9.9
Vanillic acid	**4-Hydroxy-3- methoxybenzoic acid**	**$C_8H_8O_4$**	**168.148**	**1**	**8.9**
Syringealdehyde	4-Hydroxy-3,5- dimethoxybenzaldehyde	$C_9H_{10}O_4$	182.17	2	16.5
Syringic acid	**4-Hydroxy-3,5- dimethoxybenzoic acid**	**$C_9H_{10}O_5$**	**198.174**	**2**	**15.2**

We found all 4 compounds yielded consistent calibrations. The aqueous and organic layers settle faster for acids compared to aldehydes. A combination of V and S phenols is recommended for relevance to both angiosperms and gymnosperms. Other considerations or preferences may be cost or relevance to specific applications. For example, vanillin, acetovanillone, syringealdehyde, and acetosyringone may be more abundant in nature.

C. PROCEDURE

1. WEIGH. Use a microbalance to weigh one or more monomeric lignin phenols, to yield a 4-point calibration curve, we suggest 2 vials each of vanillic acid and syringic acid.

2. REACTION AND EXTRACTION. Follow the **SOP "Lignin methoxy cleavage via the Zeisel method with liquid-liquid extraction of iodomethane."** NB. Recovery of isooctane volume added for the liquid-liquid extraction will not be complete, therefore be sure to record the

volumetric recovery of each step (increasingly dilute), and the total, as this critically denotes the fraction recovered, and is needed for the calculation step.

3. ANALYSIS. Analyze iodomethane in isooctane, evolved from the suite of phenol calibration standards, as well as samples of unknown stoichiometry, by GC-FID or GC-MS. You may wish to use FID for quantification and MS for confirmation of target analyte, or you may use GC-MS for both identification and quantification.

   NB: The analyte (iodomethane) elutes BEFORE the solvent (isooctane). If using GC-MS be sure to turn off the detector before the solvent elutes! For FID you may see both the analyte and the solvent peak.

   Instrumentation:
   We used gas chromatography (GC, Agilent 6890) coupled with a single quadrupole mass – selective detector (MS, Agilent 5973, using electron ionization with an ionization energy of 69.9 eV) for identification and flame ionization detection (FID) for quantification. The GC was equipped with a 7683 programmable injector.

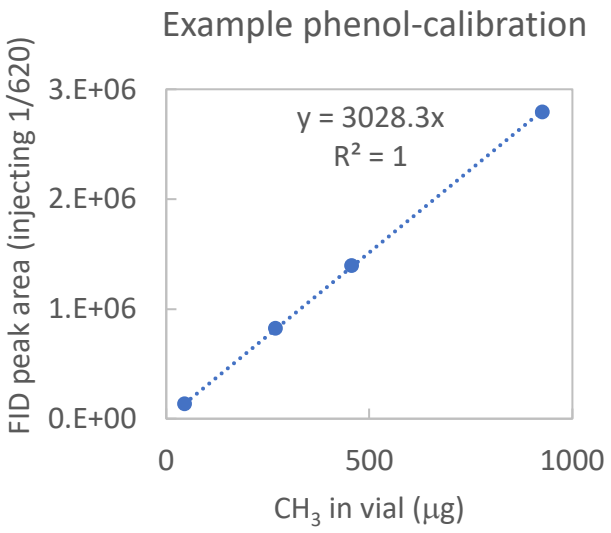
   GC conditions:
   - Liquid injections were performed by autosampler (PTFE/silicone/PTFE septa). NB, if the injector syringe has been recently used for other solvents such as hexane, switch out to another syringe as any hexane contamination will coelute with iodomethane.
   - Syringe rinse vials should be filled with isooctane.
   - Split/splitless (S/SL) inlet, at 150°C with a packed glass liner, in splitless mode.
   - We used a Rxi-5 ms column (30 m × 0.25 mm, film thickness 0.25 μm) with He carrier gas. *A different/shorter/packed column could be used provided the polarity is sufficient to separate iodomethane analyte from isooctane solvent.*
   - GC column constant flow rate 2 mL/min, passive split between FID and MS.
   - GC oven initial temperature of 32°C, held for 4 min, followed by a temperature ramp of 20°C/min up to 110°C for a total GC-FID run time of ~10 mins.
   - MS detection ended at 4.00 min before the solvent (isooctane) eluted, this was used to confirm analyte is iodomethane by m/z.

   Peak area integration
   - We used the FID trace for peak area integration (see D1 example chromatogram).
   - We routinely injected standards in triplicate and found repeat injection of the same vial yielded peak area reproducibility (instrument precision) of 2 %. If precision is similar, then a single analysis of a standard vial is likely sufficient.
   - Preparing multiple standard vials is more important given the larger uncertainty associated with the reaction and liquid-liquid extraction (estimated to be 6% relative to measurements of an iodomethane standard in isooctane). We therefore recommend 4 standard vials to assess experimental reproducibility.
   - Similar regression statistics (R^2 of 0.999) can be achieved using a single GC-FID measurement of 4 vials, as compared to a larger test of 18 vials and >triplicate measurements of each. 4 standard vials are sufficient to define the regression.
   - *OPTION: If you wish to monitor the yield of phenol reactions, then you may wish to run iodomethane calibration (see D2).*

4. CALIBRATION Regression of methyl abundance vs peak area for the phenolic standards can be used to generate a quantification calibration against which unknowns (e.g. lignin, wood, peat, lignite) can be quantified. Below is a suggested suite of standards and the resulting regression that we obtained. Be sure to set the intercept of the regression to 0. Use the slope of the line to convert peak areas of unknown samples to estimate methyl wt%.

   This is a clickable Excel worksheet:

Example solid phenol-based calibration for lignin methoxyl quantification				CH_3 molar mass (g/mol)	15.035
All of the calculations are embedded.				**slope of the line y = mx**	**3028**
Action: Fill in actual values in the green cells					
use consistent 150, 220, 250 extraction volumes for a total of 620 µL, in both standard and sample,					
or some other consistent volume, for sample and standard.					
Standard	**Vanillic acid**	**Vanillic acid**	**Syringic acid**	**Syringic acid**	
weigh standard, mass (mg)	0.501	3.010	3.005	6.100	
molecular weight (g/mol)	168.14	168.14	198.17	198.17	
#methyl groups	1	1	2	2	
methyl content (wt%)	8.9	8.9	15.2	15.2	
available CH_3 in vial (µg)	45	269	456	926	x
measure FID peak area (inject 1 µL/620µL)	137159	822954	1396494	2792987	y
Sample, unknown composition	low methyl	high methyl	low methyl e.g. coal, soil, suggest >3mg		
weigh sample, mass (mg)	3.000	3.000	high methyl e.g. gymnosperm lignite use <3mg		
measure FID peak area (inject 1 µL/620µL)	100000	30000000			
calculated original CH_3 in vial (µg)	33	9908			
methyl content (wt%)	**0.011**	**3.30**	accounts for extraction yield.		

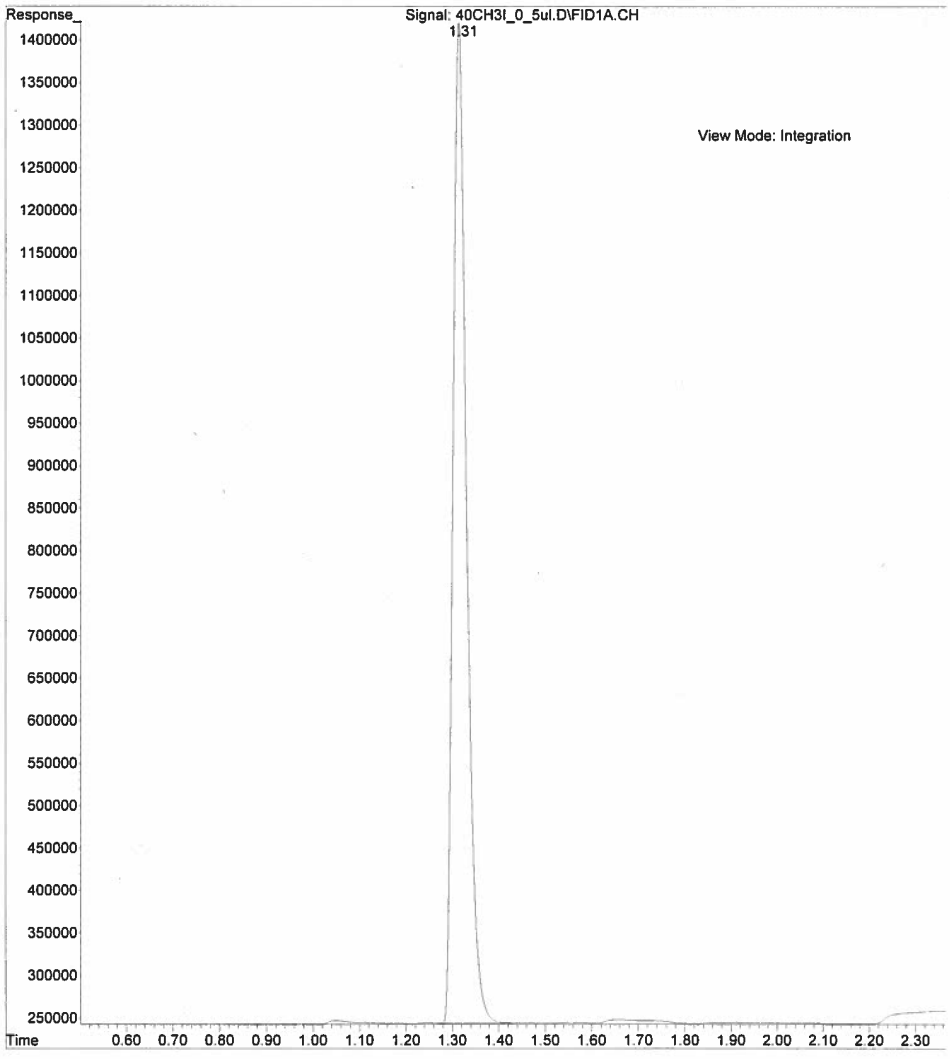


D. ADDITIONAL NOTES

1. Example GC chromatogram:

   The peak with retention time of 1.31 mins is iodomethane (the analyte), the isooctane (solvent) elutes later and must be excluded from the MS detector by shutting the MS off before the peak arrives.

```
File       :C:\MSDChem\1\DATA\2017\04\01\40CH3I_0_5ul.D
Operator   : HJL
Acquired   : 03 Apr 2017   4:09     using AcqMethod CH3I1SL_LIQ.M
Instrument :   Instrumen
Sample Name: 40CH3I_0_5ul
Misc Info  : 1/1000ul
Vial Number: 12
```



2. OPTIONAL Iodomethane calibration.

   If you wish to monitor the yield of phenol reactions, then you may wish to run iodomethane in isooctane, measuring 3 or more vials of different concentrations and comparing the slopes. However, this is not necessary as this phenol-based calibration provides like-with-like calibration for the quantification of methoxy evolved from solid substrates. As yields may vary between monomers, polymers or mixtures, the relative yield of wood and lignin standards relative to phenolic standards, may be monitored over time.

Clickable Excel Sheet:

Iodomethane standard	vial 1	vial 2	vial 3	vial 4	
iodomethane (µL)	0.5	1	3	5	
isooctane (µL)	1000	1000	1000	1000	
iodomethane injected (µg)	1.14	2.28	6.84	11.40	x
iodomethane in vial (mg)	1.14	2.28	6.84	11.40	
FID peak area (inject 1 µL/1000µL)	300507	559803	1687923	2747183	y
methyl mass injected (µg)	0.12	0.24	0.72	1.21