Analysis of Dioicine Content in the Leaves of the Kentucky Coffeetree (*Gymnocladus dioicus*) Across the Growing Season

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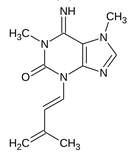
Background & Significance

The seeds of the Kentucky Coffeetree (*Gymnocladus dioicus*) have been used to brew a coffee-like beverage in historical accounts.[1-3] The tree contains Dioicine, an alkaloid that is related to caffeine, which we reported in 2009. Dioicine is mainly found in the leaves, though small amounts are found in the bark and roots.[4] We examined six specimens of *G. dioicus* on the ISU campus, collecting leaves on a weekly basis for analysis of dioicine levels with respect to light, height, compass direction and time. We are also consolidating extracts to isolate the alkaloid for bioactivity studies.

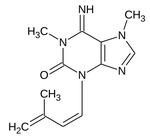
Kentucky Coffeetree
(*Gymnocladus dioicus*)

Dioicine

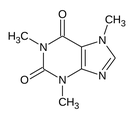
Dioicine, 3-(-3-methylbuta-1,3-dienyl)-1,7-dimethylisoguanine is a lipophilic, prenylated, purine alkaloid. It is structurally similar to caffeine. The hydrolysis of dioicine produces 1,7-dimethylisoguanine, a compound very similar to 1,7-dimethylxanthine, the primary metabolite of caffeine in man5[3] This may explain the utility of roasted coffeetree seeds as a coffee substitute. Dioicine is observed in the coffeetree as two mass spectrometrically identical isomers. The major isomer which elutes later has been isolated and determined to be *trans* (*E*).[4]



(*E*)-Dioicine



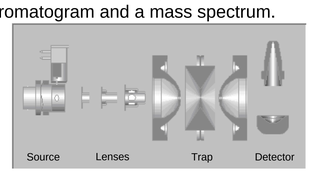
(*Z*)-Dioicine



Caffeine

Gas Chromatography-Mass Spectrometry

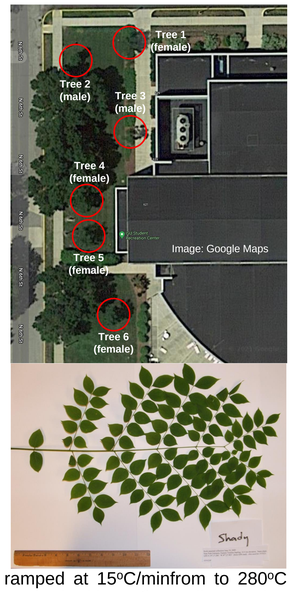
A Thermo Trace GC Ultra capillary gas chromatograph and iTQ 1100 ion trap mass spectrometer were used. The GC separates compounds in the gas phase. The compounds elute at characteristic retention times, then pass through a transfer line, and finally to the mass spectrometer (MS). The MS ionizes the molecules, then separates and detects the product ions. As seen below, the source produces ions at the beginning of the process and pushes them toward the lenses. The lenses focus the beam of particles using electric field gradients and progressively smaller paths so they can enter the ion trap. Once the trap is filled, the ions are scanned out using radio frequency voltage into the detector, where they are measured. This produces a chromatogram and a mass spectrum.



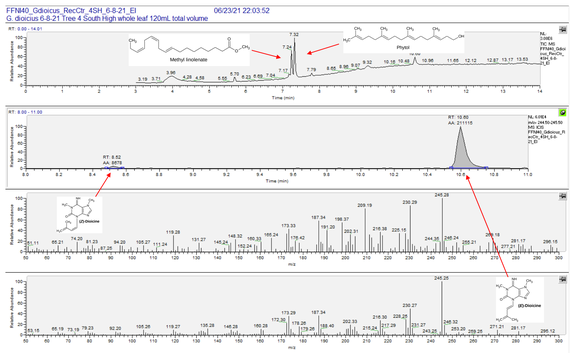
The most common ionization technique used in GC-MS is electron ionization, also called electron impact (EI).[4] A current passes through the filament in the source, which produces high energy (70 eV, ~1600 kcal/mol) electrons, which are then directed in a beam across the source using magnets. When analyte molecules pass through this beam, a valence electron is knocked out producing an unstable radical cation (molecular ion, M+). The molecular ion fragments into smaller ions and radicals. Only ions are seen on the mass spectrum because uncharged particles cannot be trapped and scanned.

Collection and Sample Preparation

Leaves were collected from six trees on the ISU campus as indicated in the picture at right. Specimens were collected on north, east, south and west sides and at low (2-3m) and high (3-5 m) points in each tree using a telescoping lopper. Because of growth patterns directions and heights are approximate. Leaves are doubly compound and can reach nearly 1 m in overall length. Leaves were weighed and placed in 4 oz jars in a total volume of 120 mL methanol. Jars were agitated several times per day over 3-5 days to reach equilibrium. Samples were then taken directly without other preparation and injected directly into the GC-MS. Crude extracts (1µL) were injected into the GC and separated on an RTX-5MS (5% phenylsilicone/95%methyl-silicone) column at 1 mL/min flow at 100ºC (held 1 min) and ramped at 15ºC/minfrom to 280ºC (held 10 min). The MS was autotuned with perfluorotributylamine prior to analysis and used 70 eV energy, with automatic gain control (25 ms max ion time) and solvent delay of 3 min.



Coffeetree GC-MS



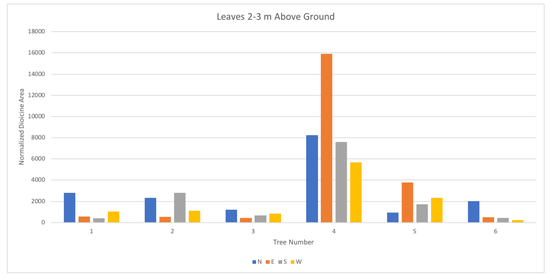
Data Analysis

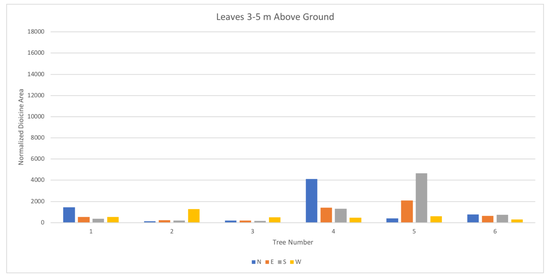
All compound peaks were integrated using extracted ion chromatograms to give maximum sensitivity and selectivity. Dioicine and its isomer were analyzed using their base peak of *m/z* 245. To account for instrument variability, we chose two constituent lipids as internal standards in our data analysis. We identified methyl linolenate, a fatty acid methyl ester, and phytol, a breakdown product of chlorophyll, by comparison of their spectra to those in the NIST library. To normalize our data, we calculated the ratio of the area of Z dioicine (8.5 min) and (E)-isomer (10.6 min) to area of methyl linolenate (7.2 min, *m/z* 79) Phytol (7.4 min, *m/z* 81) gave similar results. These areas were then multiplied by the total sample volume and divided by the mass of the leaf for each sample.

Results

We have collected seven weeks worth of samples, six of which are prepared for GC-MS analysis. Unfortunately, due to instrument breakdowns, we have not been able to run all of our samples in a single continuous batch and variability in instrument response has been problematic. To account for this, we have normalized areas to the one of two lipids. Until we are able to get one continuous run of samples, we have been analyzing each set of samples individually. Fortunately, the instrument is behaving well and we expect the full batch to run. However, we cannot yet assess how dioicine changes across the growing season.

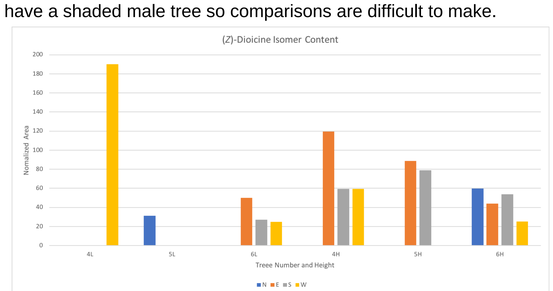
Looking at the normalized data below, it appears that overall the samples taken from lower in the tree had significantly more dioicine content, consistent with shading and possibly browser deterrence. and tree 4 overall had more dioicine than the other trees and was also quite shaded as is tree 5 which also has higher levels than the less shaded trees. Compass direction was not consistent in the data at hand nor was the sex of the tree. A more systematic evaluation should include male trees that are shaded.





Isomerism

The (*E*) isomer of dioicine is the major isomer present in all samples tested. However, the less abundant (*Z*) isomer is also observed. It elutes earlier than the trans isomer. We initially believed that dioicine underwent photoisomerization to produce the cis isomer. However, higher quantities of the cis are observed shaded leaves, arguing against this hypothesis. It is also the case that the trees that produce the cis isomer are uniformly female. However, we do not have a shaded male tree so comparisons are difficult to make.



Conclusions

In this study, we set out to explore differences in dioicine production and isomer composition in six trees based on light, leaf height, compass direction and season. Shaded leaves appear to consistently have larger amounts of dioicine than their sunny counterparts. Interestingly, the three trees that have little to no Dioicine present are the ones that are in full sun. Trees that are partly or mostly shaded produce more dioicine and have higher quantities of the cis-isomer, discounting photoisomerization as a reason for its presence. The major dioicine producers are female (though we have insufficient male specimens), suggesting a possible role for the alkaloid in protection of seed pods. Dioicine was also observed in the seeds and pods of female trees. A full analysis of samples across the season is currently underway and we will be able to determine more accurately the content over time.

Acknowledgements

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