Mountain pine beetle infestation: GCxGC-TOFMS and GC-MS of lodgepole pine (pinus contorta) acetone extractives.

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ABSTRACT

The Rocky Mountains and western U.S. forests are impacted by the infestation of mountain pine beetles (MPB). MPB outbreak is killing pine and spruce trees at an alarming rate. These trees present a fuel build-up in the forest, which can result in catastrophic wildland fires. MPB carry blue-stain fungi from the genus *Ophiostoma* and transmit infection by burrowing into trees to lay eggs. Once attacked by a pine beetle, the host tree produces resinous compounds around the burrow in an effort to kill the invading insect. Mortality rate of the trees is less than one year. Lodgepole pine acetone extracts were separated and fractionated. These extractives were class of chemicals were characterized and identified using gas chromatography-mass spectrometry (GC-MS) and 2 dimensional GC (GCxGC). Solid phase extraction was used to fractionate the infected and uninfected extractive-samples into various chemical classes. The extractives from the sapwood (SW) and heartwood (HW) were separately fractionated. They were separated into polar (P) and nonpolar (NP) components. The NP component was further fractionated into acid (A) components, neutral (N) components and into individual chemical classes.

The percentage of the infected SW and HW NP and P components were 36.4% SW-NP, 63.6 SW-P and 56.6% HW-NP, 43.4% HW-P. We anticipated an increase in the sapwood polar component. There was a decrease in the percentage on the nonpolar component. The percentage of uninfected sap and heart wood nonpolar and polar are 44.6% SW-NP, 55.4% SW-P and 46.0% HW-NP, 54.0% HW-P. The percentages in the uninfected extractives were similar in the SW and HW.

The chemical classes investigated were resin acids, fatty acids, fatty alcohols, triglycerides, sterols, lignans, phenols and low molecular weight carbohydrates. The extractives chemical classes showed differences in the concentration for the sapwood and heart wood. The resin acids decreased and the fatty acids were in trace amounts in the sapwood. The identifications of formed chemicals are still under investigation. The decreased concentrations in fatty acids are the normal response factor for trees in distress over a period of time.

KEYWORDS

Characterization of chemical classes, identification, fractionation, GC-MS, GCxGC, extractives, mountain pine beetle, infestation

1. INTRODUCTION

The mountain pine beetle (*dendroctonus ponderosae)* (MPB) presents a serious challenge to the health of the Lodgepole pine (*pinus contorta)* populations of North America. Outbreaks of the MPB have increased in both frequency and severity in various parts of North America, with devastating effects on the health of the forests.[1][2] The burrowing of the MPB poses two challenges to tree, it feeds on the phloem and lays eggs.[2] This, however, is not the main cause of death according to Hubbard et al. 2013.[3] They found that the blue staining-fungi of the genus *Ophiostoma* cause the cessation of water uptake by an infected tree, leading to its death. Other studies have found that the fungi is capable of killing the tree in less than a year after infecting it.[4]

Once attacked by a pine beetle, the host produces resinous compounds around the burrow in an effort to kill the invading insect.[4][5] A further response of the tree is a change in composition of extractives within the infected sapwood. Sugars and fatty acids decrease while monoterpenes and other extractives increase.[4] As a result of these changes, the infected sapwood has essentially the same extractives as heartwood except for the high level of monoterpenes.[4]

The aim of this study is to investigate why the blue stain will penetrate the sapwood but not the heartwood, and the differences in extractives among the heartwood and sapwood of infected and uninfected wood. The extractives were analyzed by gas chromatography mass spectrometry (GC-MS) and GCxGC. Due to complexity of the extractives, GC-MS does not by itself have the capacity to separate or resolve the peaks in wood extractives.[6] The unresolved peaks in wood extractives require the employment of multidimensional chromatographic methods. Comprehensive 2 dimensional GC (GCxGC) is a powerful analytical technique known for expanding the peak capacity of the chromatographic domain and as a result enhancing resolution and characterizing complex samples. GCxGC also has the potential of classifying known and unknown compounds through chromatographic retention patterns.[6] Coupled with time-of-flight mass spectrometry (TOFMS), GCxGC allows constituents of complex mixtures to be resolved using full range non-skewed mass spectral information and fast data acquisition rates.

This study will be utilizing a technique mentioned in a 2002 paper by Andre J. S. Hanneman et al. The method used for separation of the acetone extracts into polar and nonpolar fractions followed by further separation of the nonpolar fraction into acid and neutral fractions using solid phase extraction (SPE). The separated fractions will then be analyzed using gas chromatography.

2. MATERIALS AND EXPERIMENTAL METHODS

2.1. Wood samples

Lodgepole pine logs were delivered debarked from Colorado. The logs were cut and separated into heartwood and sapwood.

2.2. Preparation of wood samples

Wood chips from infected and uninfected lodgepole pine (*pinus contorta*) heartwood and sapwood were separately Wiley

milled and screened through a 40 mesh. Approximately 100 g of the sawdust was placed in an extraction thimble and extracted with 700 mL of 9:1 acetone:water for 8 hours using a Soxhlet extraction apparatus (2 cycles/h). The solvent was removed using a rotary evaporator.

2.3. ***Separation of polar and non-polar components***

Samples of acetone extracts were weighed into a pre-weighed glass vial. The internal standard tetracosane was added to each and dried with nitrogen. Extractives were washed with 30mL of 5:1 hexane:chloroform Each wash was mixed thoroughly using a vortex and sonicated. The liquid fraction (non-polar) was decanted into a pre-weighed vial and dried completely under nitrogen. Two trials were performed.

2.4. Solid phase extraction of non-polar components

The non-polar extractives (20-80 mgs) were dissolved in 1.0 mL of chloroform and fractionated using Isolute® aminopropyl cartridge. The columns were conditioned with hexane (2 x 1.5 mL) and loaded with the 1.0 mL non-polar extractives solution. The column was sequentially eluded into Fraction A, B and C. Fraction A was separated into Fraction D and E and Fraction B into F and G. The non-polar fraction summary is given in Table 1.

2.5. Solid phase extraction of neutrals and acids

The NP fraction was further fractionated into neutral and acid fractions. The NP extractives were dissolved in 1.0 mL of chloroform and fractionated as follows. The Isolute® aminopropyl cartridge column was conditioned with hexane (2 x 1.5 mL), and was loaded with the 1.0 mL of non-polar extractives solution. The neutral fractionation were eluted with 8.0 mL hexane:diethyl ether (8:1) followed by the second Acid fraction with 8.0 mL diethyl Ether:acetic acid (98:2) solvents. Samples were dried down in the hood and weighed. Samples were duplicated.

Table 1. Summary of fractionation used to separate the chemical classes. [8]

Fraction	Solvent	Ratio	Amount mL	Extracts
A	$CHCl_3$:Hexane	1:5	10.5	TG, SE, W
B	Ether: Hexane	8:1	9	S, DG, MG, Fal
C	Ether: AcOH	98:2	9	FA, RA
D	Hexane	—	18	SE, W
E	Ether	—	9	TG
F	Ether: Hexane	2:8	15	S, DG, Fal
G	Ether: MeOH	2:1	9	MG

2.6 Gas-Chromatography and Mass Spectrometer (GC-MS)

A Hewlett-Packard HP 5890 series II Plus gas chromatograph equipped with a HP 7673 auto injector and flame-ionization detector was used for analysis. The GC-MS was equipped with a DB-5 fused silica capillary column (ID, 0.25 mm, length, 30 m) and the proceeding temperature program: 160°C for 2 min, raising the temperature 7°C/min to 310°C and holding for 3.5 min.

2.7 Multi-dimensional GC

The Pegasus 4D GCXGC-TOF-MS system consisted of an Agilent 7890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph equipped with LECO dual stage quad jet thermal modulator and a split/split less inlet. Injections were made in both split less and split (25:1) modes at 250° C. The MS system was a LECO Pegasus (LECO, St. Joseph, MI, USA) working at -70 eV, transfer line 300° C, ion source 230° C and acquiring from 45 to 650 m/z at 150 spectra/second with a detector voltage of 1650 V. Columns used were Rxi-5silMS (Restek Corp), (30 m X 0.25 mmid, 0.25 μm film thickness) on the first dimension and the BPX-50 (SGE Analytical), (1.25mX0.10 mm I.D., 0.10 μm film thickness) on the second dimension. The experimental conditions for the columns were: primary oven temperature, 140° C (0.5 min), to 325° C at 7° C /min with a final hold of 7mins. The secondary oven temperature program mirrored that of the primary oven, only with a + 5° C temperature offset. The modulation period was 5 seconds. The carrier gas, helium, was used at a corrected constant flow of 1.5 mL/min. Compounds were identified by computer comparison of the mass spectra with the NIST library.

3. RESULTS AND DISCUSSION

Figure 1 shows the mass percentages of extractives separated from the lodgepole infected and uninfected sap and heart-wood. The HW and SW were separated into NP and P components. The percentages of the SW and HW separations have similar trends for the uninfected. There is a 10% difference between NP and P separation. It is noted in literature that extractives are similar for infected SW and HW.[4] However, the NP and P separation shows similar percentages for the uninfected wood (Fig. 1). The difference between the infected and uninfected NP SW component decrease by 9% percent (Fig. 1). The NP component includes fatty acids, resin acids and sterols. These classes of chemicals are used as a resource to respond for fuel and fighting foreign intruders on the tree. The uninfected P SW component is higher by 9%. The chemicals separated in this component are terpenoids, phytosterols and waxes. These changes in percentages could be directly related to NP uninfected SW component decrease.

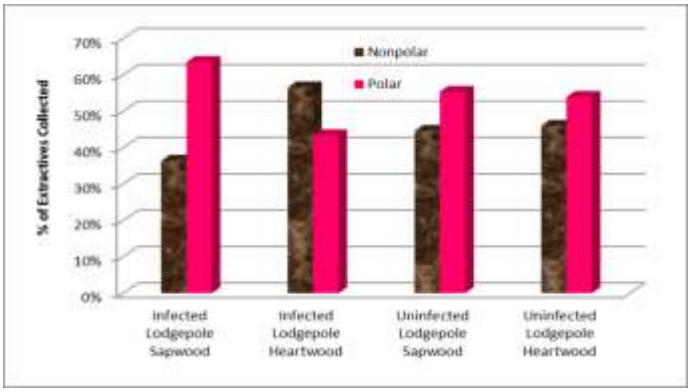

Figure 1. The uninfected and infected lodgepole heartwood and sapwood nonpolar and polar extractives collected.

The blue-stain fungi is not detected visually seen in the HW, however, the extractives chemical composition does show some changes, in particular, for P and NP components. The uninfected NP heartwood percentage increased by 12% (Fig. 1). The polar component deceased by 11%. The chemicals in the HW are noted to have biological activity. Therefore, chemicals in this area of tree may be metabolized to assist with the beetle attack. Further GCxGC based investigation can be used to determine what chemicals are contributing to the increase and decreases of these components.

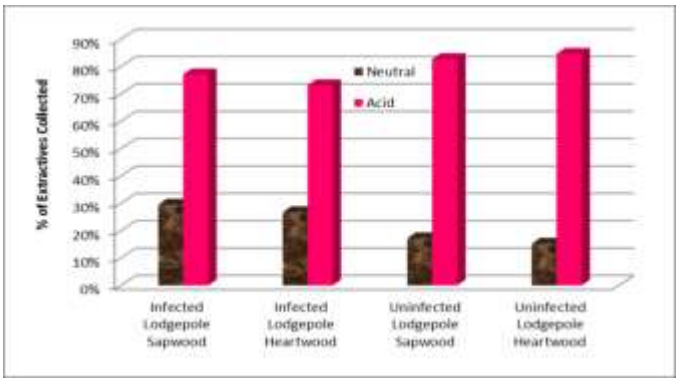

Figure 2. The uninfected and infected Lodgepole heartwood and sapwood neutral and acid extractives fraction collected.

To further understand the chemical composition of the nonpolar components they were fractionated to investigate the neutral and acid fractions. Figure 2 shows similar trends as the NP and P separation for the uninfected fractions. The uninfected SW and HW mass percentages are were similar. The uninfected neutral fractions for SW and HW were 17 and 15% respectively. The neutral fractions percentages increase by 13 and 12% percent for the infected fractionations (Fig. 2). The neutral composition does include terpenoids. Monoterpenes will increase due to the mountain beetle infestation in the tree. The acid fraction for SW and HW are the highest mass percentages in both fractions. There is a 6 and 12% decrease between uninfected to infected acid fractions. There was decrease for infected HW fractionation compared the infected SW fractionation. The percentage increase in the SW could be directly related to location of where the infestation takes place.

Other fractionations were done to check the mass percentages of various collections. These percentages are not shown. The nonpolar components were fractionated into different chemical classes of the extractives. The calculation showed trends that were similar those already discussed.

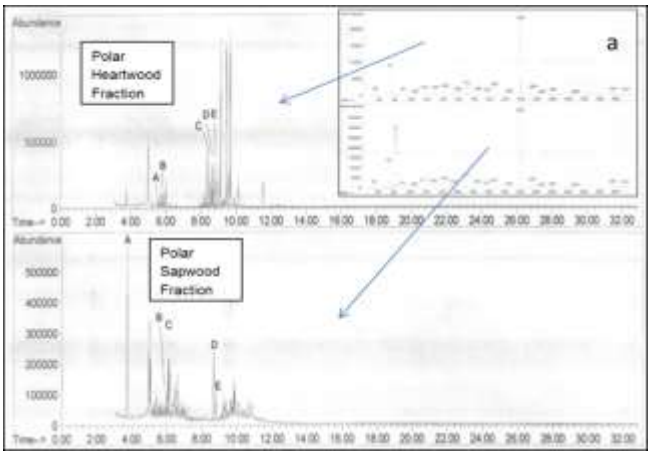

Figure 3. Silylated Polar Heartwood Extracts: (A) Dehydroabietic acid (B) Abietic acid (C) Pinocembrin; **Silylated Polar Sapwood Extracts:** (A) Dehydroabietic acid (B) Abietic Acid (C) Pinocembrin. (a) Mass spectra: Dehydroabietic acid

The polar fractions of the heartwood and sapwood produced nearly identical chromatograms (Figure 3). The separations were silylated before they were injected into the GC-MS. There were differences in concentrations with the sapwood having a slightly higher concentration of extractives. In both wood samples the resin acids dehydroabietic acid and abietic acid were found to be present as well as the flavonoid pinocembrin. The mass spectra are of dehydroabietic acid. When the pine beetle attacks a lodgepole pine tree, the tree usually responds by producing resinous compounds around the site of attack.[4] When the beetle burrows into the sapwood and not the heartwood, it is likely that the higher concentrations of resin acids in the sapwood fraction are due to the tree's resinous response. The higher concentration of pinocembrin may also be part of this response.

The identification of the chemicals in the various separations was difficult due to trace concentrations and co-eluting (similar retention time).

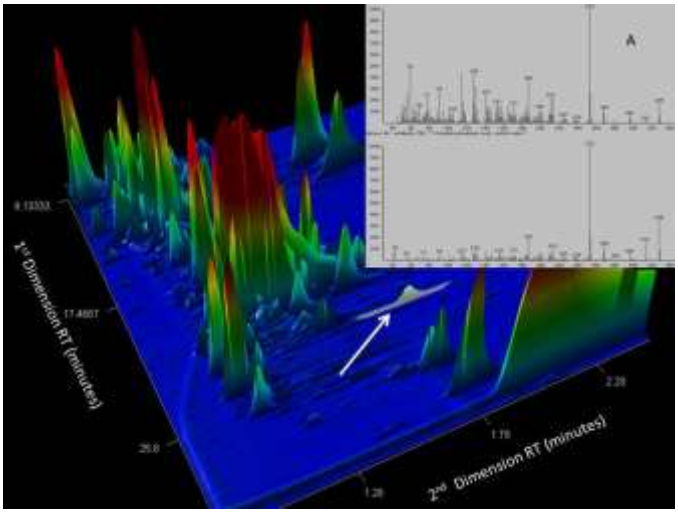

Figure 4. GCxGC chromatogram of infected neutral extractives sapwood. (A) Mass spectra of 7-oxodehydroabietic acid the highlighted peak.

GCxGC was able to resolve the co-elution of extractives with similar retention time. Even with various separations and fractionations co-eluting will always be an issue when

identifying wood extractives with GC-MS. GCxGC chromatogram in Figure 4 shows infected neutral sapwood wood extractives. The first dimension is the volatility and the second dimension is the polarity of the wood extractives. The highlighted peak is 7-oxodehydroabietic acid. The mass spectra from the resin acid had a 780 similarity match with NIST library. Several chemicals were identified along with unknown chemicals. The highlighted trace resin acid is identified even in the presence of highly concentrated extractives.

CONCLUSION

The polarity of the extractive chemicals and their increase for infected sap-wood and heart-wood is noted in literature. These changes are direct responses to the attack of the of the mountain beetle. GC-MS is not the best analytical tool for maximizing the identification of the wood extractives. GCxGC has more resolving power and the ability to deconvolute the complex mixture of the extractives. Future work will continue to maximize the separation process to identify trace chemicals and co-eluting compounds.

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