Volatile compounds in the foliage of balsam fir (*Abies balsamea*) analyzed by static headspace gas chromatography (HS-GC)

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Abstract

Many studies have focused on the influence of needle defense compounds that are produced when trees are attacked. Spruce budworm is the most important defoliator of conifers in eastern North America causing tree mortality. Volatile components such as terpenes are of importance as they are known to be agents of defense in plants and trees against many aggressors like spruce budworm. In this study, the Static Headspace Gas Chromatography (HS-GC) method was used to evaluate volatile compounds in the foliage of balsam fir (*Abies balsamea*) in order to compare the results obtained with the traditional GC-MS method. An advantage of analyzing plant volatile compounds with the HS-GC was the simplicity of execution, allowing a large number of samples to be treated. The most abondant volatile molecules were identified on the HS-GC chromatogram, except for some compounds such as α-thujene, fenchone, terpin-1-en-4-ol and α-terpineol. In addition to the qualitative analysis of terpene, a quantitative analysis of β-phellandrene was done to compare the variation of this compound between a control and a defoliated site. This study suggests that β-phellandrene was released as a response to injuries when the site was heavily defoliated by spruce budworm.

Keywords: Static Headspace, Balsam fir, *Abies balsamea*, β-phellandrene, monoterpene.

1. Introduction

In eastern North America, the spruce budworm (*Archips fumifurana Clem*.) is the best known defoliating insect of balsam fir [*Abies balsamea* L. (Mill.)] and black spruce [*Picea mariana B.P.S.* (Mill.)]. Since 2005, the area defoliated in Quebec (Canada) has doubled every year, exceeding 2 million ha in 2012 [1]. It is currently the insect that causes the most damage in the boreal forests of eastern North America and more specifically affects balsam fir [2]. This problem needs extensive study to understand the relationship between insect and tree, including how trees defend themselves. Many studies have focused on the influence of needle defense components, such as terpenes, which are produced when trees are attacked by defoliator insects [3, 4]. These molecules are of importance as they are known to be agents of defense in plants and trees against many aggressors like insects [5-9]. Conifers also produce secondary metabolites (such as β-phellandrene, β-pinene, δ-3-carene, β-caryophyllene, etc.) in their needles to defend themselves and ensure/increase their survival [10, 11]. To gather extensive qualitative and quantitative information about the defense components, many samples must be collected and analysed in order to understand their variations during the growing season (from May to October).

Static Headspace Gas Chromatography (HS-GC) has been used since the early days of gas chromatography and is a primary tool for the analysis of volatile organic compounds in the pharmaceutical, clinical and biological fields [12].

The possibility of obtaining a specific separation of volatile compounds in a sample offers several advantages over the traditional method by GC-MS or GC-FID. Unlike other GC methods, the HS-GC technique can prevent the non-volatile compounds in a sample from entering the GC inlet by holding the entire sample matrix (liquid or solid) in a vial while transferring only volatile components into the GC inlet and column [13]. Because of this, non-volatile compounds remain in the vial and not accumulate in the column which, in turn, will increase the lifetime of a GC column. In the literature, the main techniques used to determine the volatile compounds found in plants, such as terpenes, are the GC-MS or GC-FID methods [14]. However, the sample preparation step for GC-MS or GC-FID analyses can be time-consuming as samples generally require treatments before they can be analyzed. An initial extraction procedure by a solvent, or another type of extraction, is usually necessary to prepare the sample before it can be analyzed by GC methods. The most commonly used extraction method is steam distillation to obtain the essential oil of a plant. This requires a relatively long extraction time (about 2 hours) and a large quantity of plant material (about 200-500g) to obtain enough essential oil for analysis [15]. When there are many samples to be analyzed, this extraction method requires much more time than the HS-GC method.

The advantage of analyzing plant volatile molecules with HS-GC is the simplicity of execution: ground tissues are placed in a headspace vial that is sealed in a gas-tight enclosure before the sample is ready to be transferred to the gas chromatography system for analysis [13, 16]. In addition to the simplicity of sample preparation, the HS-GC method offers a good sensitivity and high speed of execution [17]. These advantages clearly show that headspace sampling is an interesting option to consider for the analyses

of samples in analytical chemistry thus helping in the understanding of a biological issue such as plant defense.

In this study, the Static Headspace Gas Chromatography (HS-GC) technique was used to (1) qualitatively evaluate volatile compounds like monoterpenes and sesquiterpenes from *Abies balsamea* foliage; (2) compare the results obtained with the traditional GC-MS method; (3) provide an example of a quantitative analysis of a selected monoterpene (β-phellandrene) from foliage of balsam fir. A comparison of results obtained by HS-GC and GC-MS will determine if the HS-GC method is comparable to the GC method. The quantitative analysis will demonstrate if a variation can be detected during the growing season between trees differently defoliated by spruce budworm.

2. Materials and methods

2.1 Sample sites

Sampling was performed simultaneously on two research sites in the Laurentide Wildlife Reserve in the province of Quebec (Canada) from May to October 2011. The control site did not show any signs of defoliation by spruce budworm and is located near (about 1 km) the defoliated site. The defoliated site was classified as heavily defoliated in 2011 [18]. Six dominant trees per site were randomly selected (Table 1) and the foliage was sampled in the middle of the tree canopy.

2.2 Sampling

Needle samples from each tree were collected every two weeks starting in spring 2011 [Days of the Year (DOY) 139] except during the period of heaviest defoliation in June when sampling was performed weekly. The needles were placed in paper bags and stored at -20 °C. The age of the selected needles varied between 1 and 3 years [19]. The needles were immersed in liquid nitrogen at -196 °C to stop all enzymatic activities and 5 g was ground (Retsch MM200 Vibrant) for 5 minutes at 1 µm. The needles were then stored at -20 °C until analysis of chemical compounds.

2.3 Analytical Method

An amount of 50 mg of needle powder and 10µL geraniol standard (20.0 mg in 1mL of dichloromethane) were placed in a 10mL HS-vial. Geraniol standard was chosen as intern standard because it is not found naturally in balsam fir needles. Each sample was prepared and analyzed three times in order to ensure good repeatability. For quantitative

analysis, a calibration curve (R^2 = 0.995) was produced with five different concentrations of α-phellandrene:

$$y = 0.1075x + 0.0825$$

Where y is the peaks area obtained by HS-GC and x is the concentration of α-phellandrène.

The chemical standard of β-phellandrene was impossible to find from Sigma Aldrich or any other company. For this reason, α-phellandrene was chosen as a standard because it has the same response as β-phellandrene. The concentration of β-phellandrene in the needle was expressed as gram per gram of foliage weight used for the analysis and converted into a percentage. Each sample was analyzed in triplicate for the qualitative analysis.

An Agilent 7890C GC system with an Agilent PAL autosampler (headspace module) was used for analyses using helium as the carrier gas (flow 1mL/min). The column was a DB-5MS (30 m x 0.25 mm x 0.25 µm). Injector and needle temperature were maintained at 250 °C and 90 °C, respectively. Sample incubation time was 900 seconds at 80 °C and the injection volume was 500µL in splitless mode. The temperature programme was 40 °C for 2 min, then a rise of 2 °C/min and held at 135 °C for 15 min. For GC-MS analysis, an essential oil from the foliage of balsam fir was obtained by hydrodistillation as described by Simard et al. 1988 [20]. In summary, 200g of balsam fir foliage was placed in a 2 L flask and 1 L of distilled water was added. Steam distillation was carried out for 2 h at atmospheric pressure. The same conditions were reproduced for the GC-MS analysis but the injection volume was 1µL in split mode (200:1).

For this study, all chemical standards were purchased from Sigma Aldrich-Fluka (Oakville, ON). The compounds were identified by a comparison of the retention time and mass spectra with the literature [14, 15, 21], plus a database from LASEVE Laboratory of the University of Quebec at Chicoutimi (UQAC) and a commercial database.

3. Results and discussion

Most of the volatile molecules listed in the literature [20, 21] were found in the chromatogram of the balsam fir essential oil of the control site analyzed by GC-MS (figure 1). However, the following components were absent: limonene, linalol, para-cymene, fenchene, sabinene and α-terpinene. These elements are usually described in the literature, but are not essential to the certification of the essential oil of balsam fir unlike the presence of bornyl acetate [22, 23].

In comparison to GC-MS chromatogram, an example of the chromatogram obtained by HS-GC is illustrated in figure 2. After several tests under different conditions, the method demonstrated in this article was the most optimal. A good signal was observed, suggesting that the equilibration time was long enough to allow the compounds to evaporate properly. In most of the obtained chromatograms, the baseline was flat and noise free. The components that are displayed on the GC-MS chromatogram (figure 1) were very similar to those of the HS-GC (figure 2). The retention times of the compounds in the HS-GC chromatogram were offset by about two minutes.

Some differences can be observed between these two analysis methods. Some compounds in figure 1 are not present in figure 2. such as α-thujene (3), fenchone (10), terpin-1-en-4-ol (16) and α-terpineol (17). The absence of these compounds may be explained by insufficient volatility or concentration of these compounds for HS-GC detection. On the other hand, certain compounds are visible in figure 2 (Maltol, α-longipinene) and not in figure 1. However, the typical components of balsam fir foliage (β-pinene, Δ-3-carene, borneol, bornyl acetate, etc.) were successfully identified by HS-GC (figure 2). This method therefore offers a sufficient performance in qualitative

analyses and demonstrates that the chromatogram obtained by the HS-GC analysis of balsam fir needle samples is comparable to the GC-MS chromatogram. Moreover, the HS-GC technique offers the opportunity to perform a qualitative analysis without any extraction steps prior to analysis.

An example of a quantitative analysis was performed by using the HS-GC technique on a specific monoterpene, β-phellandrene, in order to rapidly analyze the variation of this molecule in trees defoliated by spruce budworm during the growing season (figure 3). Some studies in the literature suggest that β-phellandrene is released by the tree as a response to injury [17] when the insect is feeding on the foliage. The quantitative analysis of β-phellandrene by HS-GC suggests that the two studied sites follow a similar pattern throughout the sampling with quantities ranging between 0 and 96%. The observed variations during spring (increase from 0% to 20% at DOY 161) and fall (increase of 5% to 35% at DOY 287) are consistent with the seasonal variations of monoterpenes described in the literature [24, 25] for a defoliated site and control site. At DOY 168 (mid-June), an important decrease (about 20% to 0% of β-phellandrene) was observed for both sites. A similar seasonal variation of β-phellandrene was observed between sites except for DOY 174 (figure 3). For the control site, a significant decrease appeared (0% of β-phellandrene at DOY 168 and 174) while for the defoliated site, an increase of the monoterpene from 0% to 15% was observed. This increase in the defoliated site occurred exactly during the period of strong defoliation (end of June) [26] when a rain of spruce budworm faeces was observed on this sampling date [27, 28]. This difference between the two sites may suggest that the β-phellandrene was released by trees on the defoliated site in 2011 as a response to injuries.

Accordingly, we hypothesized that β-phellandrene could contribute to the defense of trees against spruce budworm during that precise period.

4. Conclusion

Static Headspace Gas Chromatography was a useful technique for qualitative and quantitative studies of balsam fir foliage and this method was comparable to the traditional GC methods. In addition to the simplicity of sample preparation, the HS-GC method offers good sensitivity and a high speed of execution. It allows a rapid analysis of the composition of volatile compounds in a multi-sample sequence. Also, the example of a quantitative analysis of a selected monoterpene (β-phellandrene) has demonstrated a variation between trees differently defoliated by spruce budworm during the growing season. These results suggest that β-phellandrene was released by trees on the defoliated site in 2011 as a response to injuries and could contribute to the tree defense against spruce budworm in that precise period.

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Table

Tree	Diameter (cm)	Height (m)
C	18.97±2.31	16.40±1.55
D	15.42±2.60	13.57±2.12

Table 1 Diameter (DBH 1.3m) and average height of the trees in the control site (C) and defoliated site (D).

Figure list

Figure 1

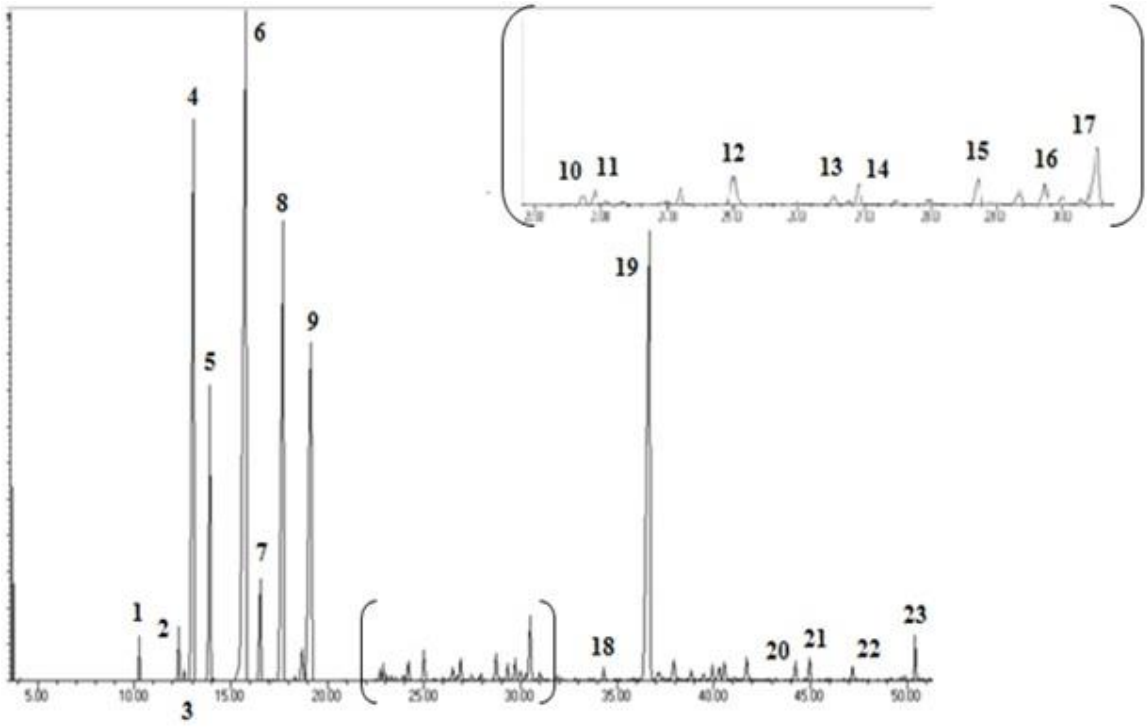
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Figure 2

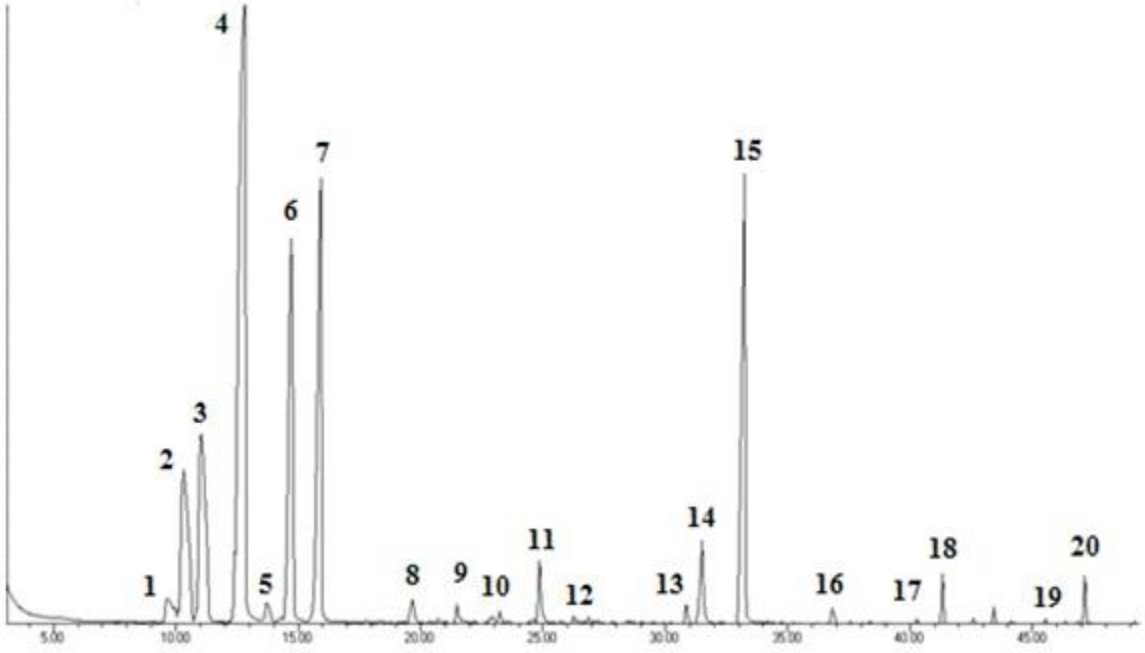
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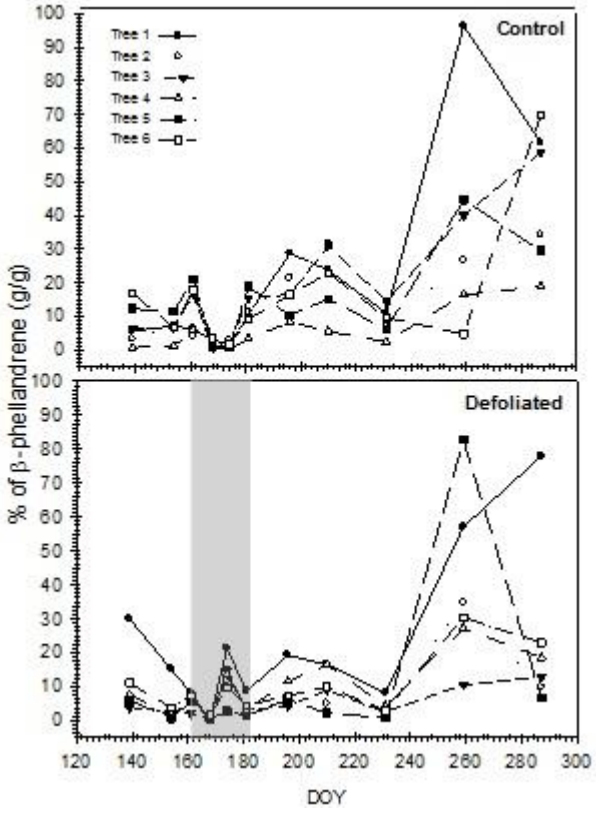
Figure 3


Control
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Tree 2
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Tree 4
Tree 5
Tree 6
% of β-phellandrene (g/g)
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80
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100
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