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Chemotypic Variation of Biogenic Volatile Organic Compounds in Midwest Forest and Atmospheric Role of Peroxyacyl Nitrates in Southeastern U.S.

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CHEMOTYPIC VARIATION OF BIOGENIC VOLATILE ORGANIC COMPOUNDS IN MIDWEST FOREST AND ATMOSPHERIC ROLE OF PEROXYACYL NITRATES IN SOUTHEASTERN U.S.

by

Shino Toma

A dissertation submitted to the Graduate College in partial fulfillment of the requirements for the degree of Doctor of Philosophy Chemistry Western Michigan University June 2017

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Biogenic volatile organic compounds (VOC), including isoprene and terpenes, impact atmospheric processes such as production of tropospheric ozone, formation and growth of secondary organic aerosol (SOA), global climate change, and human health. Although estimated emission rates of total biogenic VOC exceed anthropogenic emissions, there are many challenges to direct measurements because they are very reactive with atmospheric oxidants and some are still difficult to detect by current analytical techniques. Therefore, high uncertainties lead to discrepancies between individual and total biogenic VOC budget with atmospheric oxidants and an underestimation of SOA formation.

White pine (Pinus strobus) was once a dominant species in many parts of the Great Lakes region and is currently growing back. To estimate future air quality in light of Midwest forest succession, the composition of terpenes in white pine needles was analyzed over five growing seasons at the University of Michigan Biological Station and other forests in Michigan using gas chromatography with mass spectrometric detection. Early successional trees were selectively girdled in one forest in 2008, and the successional influence on total terpene concentration in this forest was observed by 2011. Terpene composition in pine needles displays chemotypic variation
within forests at the molecular biology level and has the potential to affect atmospheric chemistry. α-Pinene was the dominant terpene in white pine and accounted for 30-50% of all terpenes on a mole basis. However, 14% of the trees showed high levels of a single enantiomer of limonene in the same specific trees every year. The limonene contribution to hydroxyl radical reactivity was estimated to be approximately 4% higher than without consideration of chemotypic differences, which may be derived from genetic differences.

Peroxyacetyl nitrate (PAN), peroxypropionyl nitrate (PPN), and peroxymethacryloyl nitrate (MPAN), photooxidation products of VOC in the presence of nitrogen oxides (NOx), were measured during the Southern Oxidation and Aerosol Study (SOAS) field campaign in Alabama in summer 2013 using GC-ECD. The measured levels were lower than in past ground measurements in the southeast, with daytime means of PAN: 169, PPN: 5, and MPAN: 9 parts per trillion volume. Higher levels were seen in air influenced by urban sources. A multiple regression analysis indicates that biogenic VOC can account for 66% of PAN formation over the campaign. MPAN, which is derived solely from isoprene, is more strongly correlated to isoprene nitrates than to its precursor methacrolein, which indicates the possibility of an unreported atmospheric mechanism for formation of MPAN. This work reports the first relationship between gas-phase MPAN and organic particles from field measurements. The highest levels of MPAN correlate well with high levels of total organic mass in particles ($R^2=0.455$). In addition, chamber experiments of isoprene photooxidation in the presence of NOx showed a significant amount of a PAN-type compound identified as peroxyacryloyl nitrate (APAN), which is typically described as an anthropogenic product. APAN may assist in particle growth like MPAN.
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Shino Toma
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CHAPTER I

INTRODUCTION

1.1. Overview of Atmospheric Role of Biogenic Volatile Organic Compounds

Volatile organic compounds (VOCs) play a critical role in the troposphere as can be seen in the schematic diagram of VOC fate shown in Figure 1-1. VOC compounds lead to ozone (O₃) formation in the presence of nitrogen oxides (NOx), as well as to secondary organic aerosol (SOA) formation and growth. VOCs and SOA have the potential to change the climate on a regional and global scale (e.g. Laothawornkitkul et al., 2009; Peñuelas and Staudt, 2010; Williams and Koppmann, 2007).

![Figure 1-1. Schematic Diagram of the Fate of Volatile Organic Compounds During the Daytime in the Troposphere. HC means hydrocarbon (Based on Laothawornkitkul et al., 2009; Guenther et al., 1995; Müller, 1992)]
The total global emission rate of biogenic volatile organic compounds (BVOC) is estimated to be approximately 1200 teragrams of carbon (Tg C) per year (Guenther et al., 1995), while global anthropogenic VOC, including fossil fuel and biomass burning, is approximately 150 Tg C per year (Müller, 1992). Foliage is the largest source of total global BVOC emissions, accounting for over 90% (Guenther et al., 1995). The estimated BVOC (excluding methane, which has slow reaction rate with atmospheric oxidants) breakdown based on hydroxyl radical (OH) reactivity in North America is composed of mostly three categories: isoprene (51%), terpenes (31%), and oxygenated VOC (16%) (Guenther et al., 2000). Therefore, this work focused on influences of isoprene and terpenes on atmospheric chemistry.

1.2. Role of BVOC in Plants

The composition and amount of BVOC emissions are influenced by plant physiology. Plants emit BVOC for protection against environmental stress such as thermotolerance, oxidative stress, photoprotection, defense against herbivores or pathogens, and for reproduction such as attracting insects to accomplish pollination and seed dispersal and interactions such as communication or allelopathy with another plant (Peñuelas and Staudt, 2010). The volatilities of BVOC and their internal (genetic and biochemical) and external (abiotic and biotic) factors control the emission rate by altering BVOC synthesis, BVOC vapor pressure, or resistance to BVOC diffusion to the atmosphere in the short or long term (Peñuelas and Llusia, 2001). These factors are complex and it can be difficult to evaluate their relative importance on BVOC emission due to the large variability. To wholly elucidate BVOC emissions, it is important to study them with a multidisciplinary approach including genetic, physiological, ecological, and physico-chemical measurements (Peñuelas and Llusia, 2001).
Isoprene

Not all plants make isoprene and it is hard to predict which plants will produce isoprene (Fuentes et al., 2000). It has been suggested that isoprene plays a role in thermotolerance (Singsaas et al., 1997). Trees with large leaves, such as oaks, emit isoprene. Less advanced plants also commonly emit isoprene, while shade-tolerant plants cannot (Fuentes et al., 2000). Photobiosynthesis enhances isoprene emission, especially photosynthesis with red light (623-685 nm) more so than with blue light (420-450 nm) (Lerdau, 1991). Hence, isoprene emission is light-dependent. However, the foliage emission rate of isoprene is estimated using not only photosynthetic photon flux density but also leaf temperature due to dependency of enzymatic activity, which is responsible for isoprene emissions (Guenther et al., 1993).

Terpenes (Monoterpene and Sesquiterpene)

Not all plants make monoterpenes (MT) (Lerdau, 1991), although MT products have been reported from 46 families of flowering plants and all conifers (Banthorpe and Charlwood, 1980; Darnley, 1974). Many plants emit MT as a defense against herbivores and, for these plants, MT levels are inversely proportional to the number of insects present (Brooks et al., 1987; Farentinos et al., 1981). Mechanical stress, such as wounding or breaking leaves, leads to increased terpene emissions.

The scheme of terpenoid synthesis is shown in Figure 1-2. The 5-C precursors of isoprene, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are formed via two pathways, 2-C-methylerithritol-4-phosphate (MEP) and mevalonate (MVA) pathways (e.g. Laothawornkitkul et al., 2009; Martin, 2002; Zulak and Bohlmann, 2010). Isoprene and monoterpenes (MT) are produced from the MEP pathway in plastids, while sesquiterpenes (SQT)
are formed from the MVA pathway in the cytosol. Unlike isoprene, most plants store MT in specialized structures, which vary with plant taxon (Lerdau et al., 1997). For instance, species in the genus *Pinus* are known to store MT in the resin ducts of their needles (Fahn, 1979). Hence, environmental temperature and humidity directly control MT emissions from leaves (Tyson et al., 1974). The SQT are present in many plants as well. While the synthesis of MT is strongly activated by light, SQT are synthesized in the whole leaf without light activation (Gleizes et al., 1980). It is difficult to detect SQT directly in the gas-phase due to the low vapor pressures and high reactivity with ozone (Fuentes et al., 2000). The ozonolysis of β-caryophyllene, which is one of the best known SQTs, has been studied and it is known that the further oxidation products contribute to aerosol formation more than MT (see Table 1-1) because of its potential for causing atmospheric nucleation (Boris Bonn, 2003; Lee et al., 2006a).

Table 1-1. The Lifetime and SOA Yield of Selected Anthropogenic and Biogenic VOC with the Atmospheric Oxidants, OH Radical, O₃, and NO₃ Radical.

<table>
<thead>
<tr>
<th>VOC</th>
<th>OH (d)</th>
<th>O₃ (a)</th>
<th>NO₃ (a)</th>
<th>SOA mass yield by OH, %&lt;sup&gt;bd&lt;/sup&gt;</th>
<th>SOA mass yield by O₃, %&lt;sup&gt;c,d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropogenic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Butane</td>
<td>4.7</td>
<td>&gt; 4500</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-2-Butene</td>
<td>2.2</td>
<td>2.1</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>1.9</td>
<td>&gt; 4.5</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Biogenic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoprene</td>
<td>1.4</td>
<td>1.3</td>
<td>1.6</td>
<td>2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>α-Pinene (MT)</td>
<td>2.6</td>
<td>4.6</td>
<td>11 min</td>
<td>32 ± 0.1</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>β-Pinene (MT)</td>
<td>1.8</td>
<td>1.1</td>
<td>27 min</td>
<td>31 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>Terpinolene (MT)</td>
<td>37 min</td>
<td>13 min</td>
<td>0.7 min</td>
<td>31 ± 2</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>β-Caryophyllene (SQT)</td>
<td>42 min</td>
<td>2 min</td>
<td>3 min</td>
<td>68 ± 7</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>α-Humulene (SQT)</td>
<td>28 min</td>
<td>2 min</td>
<td>2 min</td>
<td>65 ± 1</td>
<td>45 ± 3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data from Atkinson (2000), Atkinson et al. (2003). Using concentration (molecule cm<sup>-3</sup>) OH radical, 12-h daytime average of 2.0×10<sup>6</sup>; O₃, 24-h average of 7×10<sup>11</sup>; NO₃, 12-h nighttime average of 2.5×10<sup>8</sup>.<br><sup>b</sup>Data from Lee et al., (2006a)<br><sup>c</sup>Data from Lee et al., (2006b)<br><sup>d</sup>SOA molar yields are expressed on a percent mass basis using the ration (µg aerosol m<sup>-3</sup> / µg terpene m<sup>-3</sup>) assuming an aerosol density of 1.25 g cm<sup>-3</sup>.
Figure 1-2. Terpenoid Biosynthesis via MEP and MVK Pathways. The number in circle means the number of molecule unit used in the synthesis. MEP: methyl erythritol 4-phosphate, MT: monoterpene, SQT: sesquiterpene, and DiT: diterpene. (Based on Martin et al., 2002; Zulak and Bohlmann, 2010; Laothawornkitkul et al., 2009)
1.3. Chemistry of BVOC in the Atmosphere

1.3.1. VOC Chemistry with NOx

Tropospheric composition in the absence of VOC depends on the concentration of NO and NO$_2$ according to the Leighton mechanism in Scheme 1 (Finlayson-Pitts and Pitts, 2000).

Scheme 1

\[
\text{NO}_2 + h\nu (\lambda \leq 420 \text{ nm}) \rightarrow \text{NO} + O(^3\text{P})
\]

\[
O(^3\text{P}) + O_2 + M \rightarrow O_3 + M
\]

\[
\text{NO} + O_3 \rightarrow \text{NO}_2 + O_2
\]

(O($^3\text{P}$): ground level triplet state, M: N$_2$ or O$_2$)

Since NO and NO$_2$ are interconverted very rapidly, the system is said to be in a “photostationary state” where there is no net reaction and the ratio of concentrations of NO, NO$_2$, and O$_3$ is a constant based on the rate constants for photolysis of NO$_2$ and for the reaction of NO with O$_3$.

However, VOC reacts with hydroxyl (OH) radical, most of which is produced by the reaction between photolysis of O$_3$ and water vapor (this reaction is shown in Section 4.3.1.3.), and forms alkyl radical (R·) (by abstraction of H for alkanes or OH addition for alkenes) and then alkylperoxy radical (RO$_2$·) in subsequent reactions as described in Scheme 2 for an abstraction reaction.

Scheme 2

\[
\text{RH} + \text{OH·} \rightarrow \text{R·} + \text{H}_2\text{O}
\]

\[
\text{R·} + \text{O}_2 + M \rightarrow \text{RO}_2\cdot + M
\]

\[
\text{RO}_2\cdot + \text{NO} \rightarrow \text{RO·} + \text{NO}_2
\]

\[
\text{RO·} + \text{O}_2 \rightarrow \text{R’CHO} + \text{HO}_2\cdot
\]

\[
\text{HO}_2\cdot + \text{NO} \rightarrow \text{OH·} + \text{NO}_2
\]
\[ 2\{\text{NO}_2 + \text{hv} + \text{O}_2 \rightarrow \text{NO} + \text{O}_3\} \]

\((\text{M: N}_2 \text{ or O}_2)\)

Since NOx reacts with the alkyperoxy radical instead of O3, as in Scheme 1, the overall reaction of Scheme 2 is:

\[ \text{R} \cdot + 4\text{O}_2 + 2\text{hv} \rightarrow \text{R’CHO} + \text{OH} \cdot + 2\text{O}_3 \]

These processes result in net formation of O3. Both biogenic and anthropogenic VOC lead to the formation of atmospheric O3 in the presence of NOx (Atkinson, 2000; Atkinson and Arey, 2003).

Further oxidation reactions of organics in the presence of NOx produce highly polar compounds, R’-C(O)OONO2 called peroxyacyl nitrates (PANs), as seen in Scheme 3 (Finlayson-Pitts and Pitts, 2000; Roberts, 2007).

\[
\text{Scheme 3} \\
\text{R’CHO} + \text{OH} \cdot \rightarrow \text{R’CO} + \text{H}_2\text{O} \\
\text{R’CO} \cdot + \text{O}_2 + \text{M} \rightarrow \text{R’C(O)OO} \cdot + \text{M} \\
\text{R’C(O)OO} \cdot + \text{NO}_2 \rightleftharpoons \text{R’C(O)OONO}_2
\]

PANs formation is a reversible reaction, as they can thermally decompose back to acylperoxy radicals with release of NOx.

1.3.2. Lifetime of BVOC with Atmospheric Oxidations

BVOC emitted into the air reacts with atmospheric oxidants such as the hydroxyl radical (OH) and ozone (O3) during the daytime, and nitrate radical (NO3) at night (Finlayson-Pitts and Pitts, 2000). Most BVOC compounds are unsaturated, hence, the alkene-OH reaction is initiated by OH addition to the double bond and results in a hydroxylalkyl radical via an intermediate (abstraction of H atom from either end of the double bond also occurs but it has a small probability).
The alken-O₃ reaction is initiated by O₃ addition across the double bond to form a primary ozonide compound. The primary ozonide is unstable, one of the peroxy O-O bonds and the C-C bond (formerly the double bond) cleave simultaneously and form an aldehyde or a ketone and a Criegee intermediate. The initial step of the alken-O₃ reaction is well known. However, the mechanism of further reactions in the gas phase is less well understood (Finlayson-Pitts and Pitts, 2000). Atkinson and co-workers have estimated the chemical lifetime (the time for decay of the VOC to be 1/e of its initial concentration) for both anthropogenic and biogenic VOC compounds with each oxidant in the troposphere (Atkinson, 2000; Atkinson and Arey, 2003). Table 1-1 shows that biogenic VOC compounds, especially MT and SQT, have shorter lifetimes than anthropogenic VOC compounds. This indicates that MT and SQT are highly reactive with oxidants compared to anthropogenic VOCs and can play a key role in secondary organic aerosol (SOA) formation, in spite of their smaller emission mass compared to isoprene.

1.4. Atmospheric Importance of Biogenic Secondary Organic Aerosol (SOA)

1.4.1. Biogenic Aerosol in the Northern Hemisphere

While primary aerosols are directly emitted into the atmosphere as particles, such as from fossil fuel burning and via mechanical processes (Roberts, 2007), Secondary organic aerosols (SOA) are formed by gas-to-particle conversion of VOC precursors to low- or non-volatile compounds. Polar VOC compounds have lower vapor pressure and higher water solubility than their precursors. This material can either condense to form tiny new particles or coagulate onto existing particles to form larger SOA (Kroll and Seinfeld, 2008). The organic aerosol (OA) is the largest single fraction of aerosol in the lower troposphere, with a range of 18 – 70%; with an
average of 45% at various locations in the Northern Hemisphere (Zhang et al., 2007). Recent research suggests that SOA accounts for a large fraction of the OA mass (Jimenez et al., 2009).

1.4.2 Influence of SOA on Climate and Human Health

Aerosols in the atmosphere interacts on the Earth’s radiation budget, and hence climate, both directly and indirectly. The “direct effect” is aerosol scattering of sunlight (cooling the surface) and absorbing of sunlight (net warming the surface), while the “indirect effect” is associated with cloud adjustments due to aerosols (Boucher et al., 2013; IPCC, 2013; Roberts, 2007; Seinfeld et al., 2016). The Fifth Assessment Report (AR5) from the Intergovernmental Panel on Climate Change (IPCC) estimated the atmosphere radiative forcing due to aerosol-radiation interactions (RFari) from different anthropogenic aerosol types for the 1750 – 2010 period using models, AeroCom II. The median of net RFari from total aerosol is -0.34 W m\(^{-2}\) and the negative number indicates that the aerosol contributes to cooling the climate system. The fraction of SOA was estimated as -0.03 W m\(^{-2}\). (Note: AR5 mentioned a large uncertainty for the total global BVOC emissions, which is expected to cause most of SOA.)

In addition, aerosols provide sites for heterogeneous chemistry and can cause significant respiratory health problems (Roberts, 2007).

1.4.3 Uncertainty of SOA Formation and Missing Chemistry of BVOC Oxidation

Although organic compounds are a substantial fraction of the atmospheric fine particulate matter, the formation of SOA from organic compounds by chemical transformation is highly uncertain (Kroll and Seinfeld, 2008). BVOC is expected to be the dominant contributor to global SOA formation due to their large global emissions and high reactivity with major atmospheric
oxidants (Kanakidou et al., 2005; Ng et al., 2006). SOA yields from selected BVOC by photooxidation and ozonolysis are compared in Table 1-1. Although the emission mass of terpenes is less than that of isoprene, terpenes, especially SQT, contribute a significant amount of SOA formation (Lee et al., 2006a; 2006b). SOA formation and growth depend on the structure of BVOC, especially the number and the location of double bonds. Ng and co-workers (2006) classified BVOC into two groups (ozonolysis or photooxidation) based on their laboratory experiments. For BVOC with only one double bond, the first oxidation step is rate-limiting and the first-generation oxidation products mainly contribute to SOA formation. For BVOC with more than one double bond, since continuous aerosol growth is observed even after all initial BVOC is consumed, the second oxidation step is expected to be rate-limiting and SOA is formed from the further oxidation of first-generation products. Ng et al. suggest that intermediates such as unsaturated oxidation products from BVOC are key to improving atmospheric models of SOA formation and growth. Hao and co-workers (2011) directly measured SOA yields from the oxidation of BVOC emitted from living trees using chamber experiments and suggested that SOA outcomes from real plant emissions depend on both the specific chemical profile of BVOC emissions and the oxidant species. Therefore, investigation into the mass and composition of BVOC emissions from real plants and their atmospheric activities is important to understand SOA formation.

1.5. Architecture of Dissertation Projects

This dissertation consists of two sections: Section 1 (Chapter 2) focuses on ecological and plant physiological effects on BVOC variation from needles of white pine (Pinus strobus), which is becoming a large part of Midwest US forests, and Section 2 (Chapters 3 and 4) focuses on the influence of BVOC emission on atmospheric chemistry of peroxyacyl nitrates (PANs), which are
formed from the oxidation of VOC in the presence of NOx (i.e., products from both natural and anthropogenic sources). In Chapter 2, large-scale screening of BVOC composition from a whole forest in Northern Michigan was conducted for five years as ecosystems transition during forest succession. Air-quality in future Michigan forests was evaluated using the atmospheric activity of each BVOC component. The effect of chemotypic variation of BVOC within one species in a population is discussed. In Chapter 3, the biogenic influence on PANs in the gas-phase and particle-phase is assessed under conditions of biogenic-anthropogenic interactions in southeastern U.S. Finally, in Chapter 4, a PAN-type compound from isoprene oxidation is reported and the evidence for identification and hypothetical formation pathway is described.

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CHAPTER II

SECTION 1: BIOGENIC VOLATILE ORGANIC COMPOUNDS IN PINE NEEDLES

2.1. Introduction

2.1.1. New Approach to Estimate BVOC Emission from Leaves in Midwest Forests

BVOC emission rate estimates mostly have been derived from leaf or branch enclosure measurements using a special bag and sampling setup (Ortega and Helmig, 2008; Ortega et al., 2008) with temperature and light corrections based on the Guenther model (Guenther et al., 1995). The setup and equilibration time for these enclosure measurements make it logistically difficult to evaluate a whole forest ecosystem. While isoprene is emitted as soon as it is produced in the plastid, terpenes are stored within foliage. Therefore, terpenes emission is related to vapor pressure within plant tissues and greatly controlled by temperature (Lerdau et al., 1994; 1997; 1995). If there is a relationship between terpenes emission rate and their concentrations this would follow Rault’s law, which expresses the partial vapor pressure of a mixture with the mole fraction of solvent. Therefore, measuring BVOC concentration in leaves could be an alternative to the branch enclosure technique.

Several field studies have suggested that some atmospheric reactivity is not accounted for by current emission estimates in temperate forests of North America inferred from high reactivity in the near-surface atmosphere. Goldstein and co-workers (2004) showed that forest thinning dramatically enhanced MT emission and ozone uptake in a California ponderosa pine plantation. They suggested that ozone uptake was due to reactions with unmeasured BVOC. Ozone reaction rates were fast enough to allow oxidation within the forest canopy with unmeasured BVOC at
approximately 10 times greater than the measured MT flux. In a northern Michigan forest, Di Carlo et al. (2004) directly measured that the total OH reactivity was significantly greater than expected based on measurement of individual compounds (missing OH reactivity). Assessment of BVOC distribution and concentration in leaves could help to understand unmeasured BVOC in the gas-phase.

Lerdau and co-workers (1994; 1995) studied the relationship of three MT species (α-pinene, β-pinene, and Δ-carene) in ponderosa pine (Pinus ponderosa) (1994) and Douglas-fir (Pseudotsuga menziesii) (1995). In ponderosa pine, concentrations of α-pinene and β-pinene in needles were highly correlated with the emission rates, but this was not true for Δ-carene. In Douglas-fir, all three MT showed a positive relationship. The authors suggested that terpene concentrations in leaves could be used as an important parameter to predict the emission rates in models, but the relationship depends on tree species and individual components (Lerdau et al., 1995). While the present work does not explicitly explore the relationship of BVOC between gas-phase emissions and needle concentrations, the relationship is plausible since the profile of compounds within a needle likely is related to gas-phase emissions scaled by volatility. Measuring BVOC concentrations in leaves could provide a benchmark for potential BVOC emissions in a whole forest and allows easy measurement of a large number of samples from a wide area in a short time and more accurate estimation of the BVOC pool in the biomass of a forest.

Atmospheric composition of BVOC species varies depending on forest structure and the type of ecosystems such as plant species, health of vegetation, weather, and location (Peñuelas and Llusia, 2001), and may be influenced by air quality. The forest composition in the Great Lake region of Northern America has been changing over time and an outline of forest history with dominant BVOC emission (e.g. Guenther, 2013) is shown in Figure 2-1. The upper Midwest
Forests are recovering from widespread logging and wild fires during the early 20th century (Frelich, 1995; Frelich and Reich, 1995; Schmidt et al., 1996). Isoprene is the dominant BVOC emission from the primary successional forest.

Eastern white pine (a strong terpene emitter) was once a dominant species in many parts of the Great Lakes region and is currently growing back to replace aspens (Bergen and Dronova, 2007; Whitney, 1986; 1987; Ziegler, 2010). Succession from aspen forests to a higher representation of pines will cause “chemical succession” whereby isoprene will give way to terpenes in the atmosphere. This will lead to a number of atmospheric implications and to issues of forest health. For instance, Lee et al. (2006) report that terpenes, especially SQT, produce SOA in higher yields than isoprene from photoxidation. Analysis of white pine needles will provide baseline information about the pools of BVOC available in the growing forest and will help predict future atmospheric BVOC levels in the Midwest forests. This research will report the identified BVOC levels in needles of understory white pine (n = 71) in Northern Michigan measured over a
large area during five growing seasons since 2008 in order to statistically survey the change of BVOC composition as changing with the forest succession and by year, and will attempt to estimate the atmospheric influence of chemotypic diversity within this population of white pine.

2.1.2. Summary of Master’s Thesis with Results in the Summers of 2008 and 2009

2.1.2.1. Explanation of a Biological Station

The investigation of large-scale screening of BVOC composition in needles of eastern white pine (*Pinus strobus*) was launched at the University of Michigan Biological Station (UMBS) in the summers of 2008 as my Master’s thesis project (Toma, 2010). The UMBS is located in Pellston in Northern Michigan (45°30’N, 84°42’W) and has two types of experimental forests. One is an intact early successional forest (Ameriflux) as control. The dominant present-day cover types of the UMBS forest are aspen (60.9%), northern hardwoods such as maple and American beech (16.6%), and upland conifer with white and red pines (13.3%) (Bergen and Dronova, 2007). In the Forest Accelerated Succession Experiment (FASET) forest, the stems of early successional tree species (aspen and birch) were selectively girdled in 2008, which expedited the growth of later successional trees such as white pine. The two forests are located within 1 km of each other and have similar ecosystems, including structure of forest, soil type and climate. The details of stem-girdling treatment and environmental condition in UMBS is described by Nave et al. (2011).

2.1.2.2. General BVOC Composition at UMBS

Understory white pine trees were randomly selected at Ameriflux (n = 35) and FASET (n= 36) in the summer of 2008 for the follow-up survey until 2012. Eight monoterpenes (MT, 10 carbons) were identified from extracts of white pine needles using GC-MS (Toma, 2010) as α-
pinene, camphene, β-pinene, myrcene, α-phellandrene, limonene, β-phellandrene, and terpinolene and 5 sesquiterpenes (SQT, 15 carbons): β-caryophyllene, germacrene D, germacrene B, γ and δ-cadinene. A sesquiterpene alcohol was also identified. These compounds accounted for 90% of total area in the chromatograms. α-pinene was the most dominant terpene in all samples and accounted for 30 - 50% of all terpenes on a molar basis. After that, germacrene D and a sesquiterpene alcohol (SQT-ol) were consistently seen in field samples. Since pure standards are not currently commercially available, the unknown SQT-ol was tentatively identified as germacrene D-4-ol by referring to the NIST 98 MS library and confirming the presence of the hydroxyl group with a TMS derivative.

2.1.2.3. Understory Trees

As a result of forest disturbance at FASET, the forest structures of FASET and Ameriflux may be different in the future. However, BVOC concentrations in needles from the same understory trees (n = 71) did not show statistically significant differences between the two forests in the two growing-seasons of 2008 and 2009. Although the concentration of total BVOC, which is the sum of MT and SQT, did not change significantly from 2008 to 2009, total MT increased by 37.0 %, while total SQT decreased by 69.4 %. The variation was hypothesized to be due to different environmental factors such as light and temperature on the bioproduction of these different BVOC.
2.1.2.4. Canopy Trees

A small number of overstory white pine trees were sampled from FASET (n = 4) in 2008 and Ameriflux (n = 8) in 2009 in order to compare the BVOC concentration based on height. The needles were collected from the top (6 – 17 m) and the bottom (2 – 4 m) of the trees, to test different light environments on the same individual. In 2008, the average of total MT from the top of trees was larger than at the bottom, while the trend of SQT showed the opposite. In 2009, all MT at the top of the trees were significantly higher than at the bottom by 30 – 50%, but SQT levels did not statistically change. Total BVOC concentration from the top of overstory trees was higher than from understory trees, which suggests that the magnitude of terpene compounds at UMBS will increase as understory white pine trees grow into the canopy.

2.1.2.5. High Limonene Trees (Chemotypic Variation)

In 2009, a different GC-MS system was used to analyze terpene compounds than in 2008. A HP 6890/MSD 5973 with HP-5MS column (30 m × 0.25 mm × 0.25 µm) was used in 2008, while SHIMADZU GC-2010/GCMS-QP2010 with SHR5XLB column (30 m × 0.25 mm × 0.25 µm) in 2009. The GCMS system in 2009 was able to separate D-limonene (4-isopropenyl-1-methylcyclohexene) from β-phellandrene (3-isopropyl-6-methylene cyclohexene), which the system in 2008 did not permit. The GCMS conditions in 2009 demonstrated that some understory trees (8 out of 71 trees) in the UMBS have a tremendously high D-limonene concentration that comprises up to 36% of the total BVOC, although the average of limonene composition was less than 5%. When the limonene concentration was plotted against the α-pinene concentration, which was the most dominant terpene composition in white pine needles from the UMBS, the results clearly showed two distinct trends (Toma and Bertman, 2012). The sites of high limonene trees
were not close to each other and included both Ameriflux and FASET forests. There was no common factor such as light environment, tree age, and tree diameter at breast height (DBH).

2.1.2.6. BVOC Composition in Gas-phase vs. Needles from Literature

Hunt et al. (1990) reported the same BVOC components in white pine needles from Ontario and Wisconsin and their results also showed the large amount of germacrene D and its alcohol (note: they could not separate \( \gamma \)-muurolene and germacrene D). Ortega et al. (2008) measured basal emission rates from seven white pine trees over the 2003-2005 growing seasons at the UMBS and other places using a blanch enclosure technique and reported the terpene composition in the gas-phase as 76.0 – 98.7% MT and 1.3 – 24.0% SQT. Their gas-phase MT composition was similar to the needle extractions from my work and \( \alpha \)-pinene was also the most dominant BVOC in gas-phase. However, the amount of SQT in gas-phase was lower than in needle extract and the composition was different. In contrast to the composition in needles, \( \beta \)-caryophyllene was higher than germacrene-D in gas-phase. \( \alpha \)-Farnesene, which was not seen in needle extracts, was the most dominant SQT in gas-phase. The difference of reported BVOC composition in gas-phase and measured in needle extracts is likely related to differences in volatility.

2.1.3. Objectives

1) Seasonal change of BVOC composition in needles was investigated over the life of pine needles.

2) Analysis of large-scale screening of BVOC composition in understory white pine needles was conducted at UMBS using two forests in order to predict future atmospheric BVOC
composition in Midwest forests as the forest succession changes from primary to secondary.

3) The investigation of BVOC composition of white pine needles was conducted in other forests in lower peninsula of Michigan.

4) Further study of chemotypic variation was conducted to assess the impact on atmospheric chemistry.

5) The enantiomeric composition of pinenes was investigated using a GCMS equipped with a chiral column.

2.2. Experimental

2.2.1. Sampling Location

2.2.1.1. WMU

Needles of white pines generally have a lifetime of two years. The changing BVOC composition in white pine needles was investigated based on the age of the needles and the season from three selected trees in the campus of Western Michigan University (WMU). The location of each tree on the campus is shown in Figure 2-2. The fresh young (newborn) needles (a tip needles of the branch with light green color) and one-year-old needles (the next layer away from the tip needles with dark green color, see Figure 2-3) were collected monthly from April in 2010 to July 2011 and from April 2010 to December 2010 respectively. The needles were constantly collected from the same branch, which were located on the south side of the tree.
Figure 2-2. Locations of Selected White Pine Trees in the WMU Campus. The capital letter as the symbol in a circle comes from the building name, which is near each sample tree. H: Haenicke Hall, W: Wesley Foundation, and O: Oaklands Res.

Figure 2-3. One-year-old White Pine Needles. This photo was taken in May, 2017 near Haenicke Hall, WMU.
The investigation of large-scale screening of BVOC in the needles of white pine understory trees was continuously conducted during summers of 2010 and 2011 at the University of Michigan Biological Station (UMBS). The one-year-old needles were collected from the same trees, which were selected in 2008, at Ameriflux (n = 35, S1-S35) and FASET (n=36, S36-S71). The distribution of the understory trees in both forests is shown in Figure 2-4. Some trees were unable to be sampled after 2008. The additional sampling was conducted in 2010 and 2011 and the sampling information of additional trees is described in Appendix A.
In 2010

- Annual sampling was conducted during July 20th through July 24th.
- 21 understory trees were added in Ameriflux (n = 11) and FASET (n = 10) in order to confirm existence of high limonene tree consistency in the population.
- In order to compare the BVOC composition at normal sampling period (which is around the end of July), three additional collections were performed on June 8th (sample tree: S1, S2, S3, S4, S7, and S16), July 1st – 4th (sample tree: S1-S71), and August 20th – 23rd (sample tree: S1-S71).
- Needles were collected from the top (6 -17 m) and the bottom (2 – 4 m) of overstory trees (n = 8) in Ameriflux.
- The needles of another species, red pine trees (*Pinus resinosa Ait.*), were collected (n = 5) in the UMBS for the comparison of BVOC composition to white pine needles.

In 2011

- Continuous sampling from understory trees was conducted in FASET on July 30th and Ameriflux on July 31st.

2.2.1.3. Other Sampling Sites in MI in 2011

In 2011, white pine needles were collected from four additional places in Michigan from North to South in order to compare the BVOC composition in the different environments. 20 trees were randomly selected at each place (understory trees were selected if possible). The additional sampling places are shown in Figure 2-5 and the sampling information is added in Appendix A.

1. Chaboiganing Nature Preserve is 13 km south from UMBS. Needles were collected on 8/1/2011 and 8/3/2011.
2. Hartwick Pines State Park is 114 km south from UMBS. This forest is more than 100 years old and has secondary growth hardwood forests in northern Michigan, and many of old grown white pine trees. Associations of understory trees were located aside from the old-growth trails. Needles were collected on 8/1/2011 with permission from DNR (Department of Natural Resources) in advance of sampling.

3. A forest in Crawford County, Grayling is near Hartwick Pines State Park. The location was guided by the DNR. There were many understory white pine trees, aspen, red pine, oak, and maple. Some trees were damaged from lightning; tree samples G4, G5, G6, G7, and G8 had burned trunks due to lightning damage. Sampling was conducted on 8/2/2011.

4. Fort Custer State Park is 25 km east of WMU. This forest is in a typical southern Michigan farm county constituting second growth forests and the remains of prairie. There are many oak and birch trees and few understory white pine trees. Samples were collected on 9/1/2011.

Sampling method and analysis of BVOC in needles were the same as at UMBS.
2.2.2. Sampling Method

This sampling method is described in Toma (2010; 2012). Briefly: nitrile examination gloves were worn for collecting needles to avoid contamination and heat from contact with skin. About 0.5 g (around 10 bundles) of one-year-old needles (which is the second layer away from the tip of the needles, see Figure 2-3) were collected from the southern branches at around 1.3 m using a stainless-steel tweezers and put into a plastic Ziploc bag. The bag was kept on dry ice in a Styrofoam box during the sampling. The tweezers were rinsed with hexane, which was used for BVOC extraction, before collecting each sample. Diameter of the trunk at breast height (DBH,
cm) at around 1.3 m AGL and temperature of needles were measured and tree age, tree height, surrounding vegetation, and light environment were estimated on each tree. In order to control contamination from the sampling process, blank samples (a plastic Ziploc bag was opened in the air and closed without needle samples) were taken for each sampling day or forest. After transporting to the laboratory, the collected needles were stored in a freezer at -80 °C until extraction, which was conducted within one month.

2.2.3. Extraction Method

Extraction was performed as described in Toma (2010). Approximately 0.2 g of frozen needles were cut into ~5 mm uniformly long pieces with stainless scissors, which were rinsed with hexane between samples. The needle pieces were soaked in 4 or 5 mL hexane (Sigma-Aldrich, n-Hexane CHROMASOLV) in a sealed glass vial at 23.0 °C for 24 h. Since hexane easily vaporizes, the solution contained 100 µmol L\(^{-1}\) of tridecane (Aldrich, 99+%) as an internal standard. In order to standardize the BVOC concentration from needles, the dry mass of needles in grams (dwg) was obtained by heating the residue at 55 °C in an oven for 24 h after transferring all of the extracted solvent into a GC vial.

2.2.4. GCMS Analysis

The BVOC in the extracted solutions was analyzed by gas chromatography-mass spectrometry (GCMS). As mentioned in the introduction of this chapter, the instrumentation in 2009 separated limonene and β-phellandrene, while that in 2008 could not. However, the peaks of limonene and β-phellandrene were still overlapping under the conditions in the 2009 instrumentation that resulted in some error in quantification.
In 2010, the optimization of GCMS conditions were performed again in an effort completely separate limonene and β-phellandrene. Currently, a standard of β-phellandrene is not commercially available. Consequently, angelica seed oil that contains β-phellandrene 60 % (by peak area) was used for optimization of the GCMS conditions. The essential oil was provided from Shiono Koryo Kaisha, Ltd. (Japan) through personal communication with Dr. Hideo Naoki. The same volumes of angelica seed oil and limonene (Fluka, ≥ 99.0 %) were added in hexane solvent and the solution was run on the GCMS under various split ratio and temperature programs, increasing sample time resolution, and setting Selected Ion Monitoring (SIM) mode. However, these conditions did not affect the separation. Manninen et al. (2002) reports terpenes from needle and wood of scots pine using a GCMS (HP 6890/MSD5973) with HP-5MS (30 m × 0.25 mm × 0.25 µm) that is exactly the same as our condition in 2008 and their approach also could not separate limonene and β-phellandrene. Polarity of the stationary phase is key to the separation of the compounds, and both the HP-5MS (Agilent) and SHR5XLB (SHIMADZU) are non-polar columns (containing 5% phenol). A polar column, Stabilwax (RESTEK) was used for the mixture solution and that resulted in separation of limonene and β-phellandrene, though γ-cadinene and δ-cadinene were not separated. The order of retention time of some compounds (e.g. myrcene and α-phellandrene) also changed relative to the polar phase. In this work, limonene is more important than cadinene due to its known reactivity in the atmosphere. Therefore, a polar column was employed to analyze terpene compounds after 2010. The GCMS conditions for 2008 – 2011 are summarized in Table 2-1.
Table 2-1. Parameters and Conditions of GCMS Analytical System Used in 2008-2011.

<table>
<thead>
<tr>
<th></th>
<th>2008a)</th>
<th>2009</th>
<th>2010/2011b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC/Detector</td>
<td>HP 6890/MSD 5973</td>
<td>SHIMADZU GC-2010, GCMS-QP2010</td>
<td>HP 6890/MSD 5973</td>
</tr>
<tr>
<td>Capillary column</td>
<td>HP-5MS (30 m × 0.25 mm × 0.25 µm)</td>
<td>SHR5XLB (30 m × 0.25 mm × 0.25 µm)</td>
<td>Stabilwax (30 m × 0.32 mm × 0.25 µm)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>1 - 0.3 µL (splitless)</td>
<td>1 µL (split 1:2)</td>
<td>1 µL (splitless)</td>
</tr>
<tr>
<td>Temperature program</td>
<td>1) 60 °C (2 min hold) to 180 °C at 7 °C min⁻¹</td>
<td>60 °C (2 min hold) to 200 °C at 4 °C min⁻¹</td>
<td>40 °C (2 min hold) to 200 °C at 4 °C min⁻¹</td>
</tr>
<tr>
<td></td>
<td>2) 60 °C (2 min hold) to 110 °C at 7 °C min⁻¹,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) The system in 2008 was not able to separate limonene and β-phellandrene.
b) The system in 2010 was not able to separate γ-cadinene and δ-cadinene.

A representative GC chromatogram of terpenes from white pine needle extracts is shown Figure 2-6 with retention time (RT) of identified terpene in Table 2-2 (Note: Stabilwax was used for this GC chromatogram). Major terpenes in white pine needles were identified using retention times and mass spectra of commercial standards and essential oils. Purified standards were used to identify α-pinene (Aldrich, 98%), camphene (Supelco, 98.5 %), β-pinene (Aldrich, 99 %), myrcene (Fluka, ≥95.0 %), α-phellandrene (SAFC), limonene (fluka, ≥99.0 %) terpinolene (Fluka, ≥95.0 %), β-caryophyllene (SAFC, ≥80.0 %), and germacrene-D (Treatt USA Inc., 40 %). β-Phellandrene was identified using Angelica seed oil (Shiono Koryo Kaisha, Ltd. Japan, 60 %) and germacrene B, γ-cadinene and δ-cadinene were identified using Hinoki essential oil (Shiono Koryo Kaisha, Ltd.). Germacrene D-4-ol was inferred from the NIST 98 MS library.

Quantification of each terpene was conducted using multi-point calibration curve with the purified standard at 10, 50, 100, 500, and 1000 µM (detection limit was around 5 µM under the condition in 2010 and 2011). 100 µM of tridecane (Aldrich, 99+ %) was added to each standard
solution and the peak ratio of the standard to tridecane was used for making the calibration curve. The standard was injected into the GCMS three times at each concentration and a linear fit, $R^2 \geq 0.99$ was obtained for each standard. β-Phellandrene was quantified using the average slope from all MT standards. Purification of germacrene-D standard (from the natural 40% source) was attempted using flash chromatography with hexane or 95% hexane + 5% ethyl acetate. However, separation of germacrene D was difficult from other SQT isomers. Therefore, germacrene D and other SQT except β-caryophyllene were quantified using the average slope from β-caryophyllene and α-humulene (Fluka, ≥98.0%) standards.
Figure 2-6. Representative GC Chromatogram of Terpenes from White Pine Needles Extracts. Needles were collected from near Haenicke Hall (file name: H10FB11A.D). Stabilwax was used for analysis (GCMS conditions were set for 2011 in Table 2-1).

Table 2-2. Identified Terpenes in Figure 2-6 with GC Retention Time.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>RT, min</th>
<th>Terpene</th>
<th>Peak No.</th>
<th>RT, min</th>
<th>Terpene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.03</td>
<td>α-Pinene</td>
<td>8</td>
<td>6.95</td>
<td>β-Phellandrene</td>
</tr>
<tr>
<td>2</td>
<td>3.71</td>
<td>Camphene</td>
<td>9</td>
<td>9.11</td>
<td>Terpinolene</td>
</tr>
<tr>
<td>3</td>
<td>4.49</td>
<td>β-Pinene</td>
<td>10</td>
<td>18.16</td>
<td>β-Caryophyllene</td>
</tr>
<tr>
<td>4</td>
<td>5.47</td>
<td>3-Carene</td>
<td>11</td>
<td>21.32</td>
<td>Germacrene D</td>
</tr>
<tr>
<td>5</td>
<td>5.87</td>
<td>α-Phellandrene</td>
<td>12</td>
<td>22.00</td>
<td>Germacrene B</td>
</tr>
<tr>
<td>6</td>
<td>6.00</td>
<td>β-Myrcene</td>
<td>13</td>
<td>22.75</td>
<td>γ&amp;δ-Cadinene</td>
</tr>
<tr>
<td>7</td>
<td>6.74</td>
<td>D-Limonene</td>
<td>14</td>
<td>30.26</td>
<td>Germacrene D-4-ol</td>
</tr>
</tbody>
</table>

RT of 100 µM tridecane (internal standard) was 9.69 min. RT means GC retention time.
2.2.5. SPME Measurement

Pine needle extracts made it easier to sample many trees but still does not give direct gas-phase emission data. The ideal comparison would be to measure the BVOC composition in gas-phase and in needles at the same location and time, since BVOC composition varies on a yearly basis. Several attempts were made to compare extracts to gas-phase. First, we attempted branch enclosure experiments in the UMBS, but we did not obtain decent results. Then, we tried a laboratory experiment in collaboration with Quinton Colwell who was a high school research student from Kalamazoo Area Mathematics and Science Center in 2012. In this experiment, headspace of needles harvested from local trees were analyzed as a proxy for emission rates using a Solid Phase Micro Extraction (SPME) technique. White pine needles were collected at near Haenicke Hall in Figure 2-2 and were divided for gas-phase analysis and leaf extract analysis. BVOC in gas-phase was absorbed on to a fiber (23-Gauge 85 µm Carboxen/PDMS or 23-Gauge 100 µm PDMS, SIGMA-ALDRICH) and was measured using a GC-FID (HP6890 with HTS PAL headspace autosampler, Agilent Technologies) installed with a Stabilwax (30 m × 0.32 mm × 0.25 µm, Restek) column. BVOC in the needle extract was analyzed using the same method as described earlier.

2.3. Results

2.3.1. Seasonal and Age Effects on BVOC Concentration

Figure 2-7 shows the impact of seasonal variation and needle age of needle BVOC (MT+SQT) concentration from three white pine trees found on the WMU campus. Mean temperature, which is plotted in Figure 2-7, was recorded from the Kalamazoo/Battle Creek
International Airport via https://www.wunderground.com. Most of the one-year-old needles were not available after December due to falling after they withered. I observed new needles appear around April (Figure 2-3). Therefore, the needles of white pine trees in Michigan have a two-year lifetime in which leaf buds start growing in the spring and cannot pass the second winter. In Figure 2-7 (a), the mean of the total BVOC from one-year-old needles in 2010 (born in 2009) varied by month. The level was relatively high during the summer and was lowest in winter. The ratio of SQT/MT was slightly changed by season and the range was from 0.47 – 0.35 as April to December with 0.42±0.05 (mean ± standard). Figure 2-7 (b) tracks BVOC concentration for more than one year since leaf bud stage (born in 2010). The concentrations of BVOC and SQT especially from new needles was low for the first three months and the ratio of SQT/MT was around 0.1 and very low at the beginning (10% or less). New needles also showed relatively high BVOC concentration during the summer and low level during the winter. After the first two months, the range of ratio SQT/MT became 0.37 – 0.47 through the leaf’s life with the mean being 0.41±0.03. During summer 2010 (July – September), the range of ratio from new needles was similar to the ratio from one-year-old needles in (a). Therefore, BVOC concentration from white pine needles varied by season and was higher in summer and the ratio of SQT/MT was strongly affected at the first two months as sprouts.
(a). Mean of BVOC concentration from one-year-old needles in 2010 (born in 2009) with the standard deviation (n=3).

(b). Mean of BVOC concentration from new needles born in 2010 with the standard deviation (n=3).

Figure 2-7. Monthly and Age Changes of Mean of BVOC (MT+SQT) Concentration with the Standard Deviation.
2.3.2. BVOC Composition of Understory Trees in UMBS since 2008  
(The Long-term Effects of the Girdling Disturbance in the FASET)

2.3.2.1. General Description

Table 2-3 lists the mean concentration and standard deviation of 13 major compounds (8 MT and 5 SQT) from selected understory trees in Ameriflux and FASET forests since 2008. Identical experiments were conducted in 2012 by Katie Bergh, an undergraduate from Kalamazoo College who worked on this as part of her Senior Individualized Project under my direction (Bergh, 2013). These 13 compounds accounted for more than 90% of total area in the chromatograms. Some trees showed 4-carene, which is a MT, but the level was near the detection limit or less than that (it was not detectable from most of trees). Hence, 4-carene is excluded from the list. Identical compounds were observed in all five years from both forests. Overall, the dominant terpene was α-pinene, which accounts for 30-50% of all BVOC on a mole basis over five years. Germacrene D-4-ol, which is an alcohol of SQT, was the second most abundant and accounted for approximately 20% of total BVOC except in 2009. Similar terpene components were observed in a previous report of pine needles in Ontario and Wisconsin (Hunt et al., 1990).

Table 2-4 shows the mean composition and the standard deviation of each terpene in total MT or SQT from 2008 to 2011. Most of MT components had similar composition for all four years in both forests. SQT components also showed analogous concentrations except in 2009.
Table 2-3. The Mean of each BVOC Concentration (µmol dwg⁻¹) with the Standard Deviation in Needles of Understory White Pine Trees at Ameriflux (Control Forest) and FASET since 2008.

<table>
<thead>
<tr>
<th>Year</th>
<th>Ameriflux</th>
<th>FASET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2008*</td>
<td>2009*</td>
</tr>
<tr>
<td></td>
<td>n=35</td>
<td>n=34</td>
</tr>
<tr>
<td></td>
<td>n=36</td>
<td>n=36</td>
</tr>
</tbody>
</table>

**MT**

- **α-Pinene**
  - Ameriflux: 13.201±5.104
  - FASET: 15.822±4.808

- **Camphene**
  - Ameriflux: 2.028±1.160
  - FASET: 2.363±1.018

- **β-Pinene**
  - Ameriflux: 3.994±1.424
  - FASET: 4.700±1.974

- **Myrcene**
  - Ameriflux: 2.218±1.234
  - FASET: 2.611±1.730

- **α-Phellandrene**
  - Ameriflux: 1.006±0.421
  - FASET: 0.937±0.450

- **Limonene**
  - Ameriflux: 1.943±1.255**
  - FASET: 2.374±1.953**

- **β-Phellandrene**
  - Ameriflux: 1.370±0.852
  - FASET: 1.540±1.071

- **Terpinolene**
  - Ameriflux: 0.174±0.123
  - FASET: 0.199±0.104

**SQT**

- **β-Caryophyllene**
  - Ameriflux: 0.962±0.328
  - FASET: 1.093±0.351

- **Germacrene D**
  - Ameriflux: 4.230±1.751
  - FASET: 5.116±1.863

- **Germacrene B**
  - Ameriflux: 1.126±0.428
  - FASET: 1.260±0.465

- **Y,β-Cadinene**
  - Ameriflux: 1.833±0.801
  - FASET: 2.073±1.134

- **Germacrene D-4-ol**
  - Ameriflux: 8.543±4.889
  - FASET: 8.586±6.118

37
<table>
<thead>
<tr>
<th>Year</th>
<th>2008*</th>
<th>2009*</th>
<th>2010</th>
<th>2011</th>
<th>2012***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ameriflux</td>
<td>n=35</td>
<td>n=34</td>
<td>n=33</td>
<td>n=33</td>
<td>n=31</td>
</tr>
<tr>
<td>FASET</td>
<td>n=36</td>
<td>n=36</td>
<td>n=36</td>
<td>n=36</td>
<td>n=36</td>
</tr>
<tr>
<td>Median</td>
<td>26.835</td>
<td>38.262</td>
<td>29.887</td>
<td>35.645</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>20.051</td>
<td>5.233</td>
<td>16.269</td>
<td>17.091</td>
<td>21.294</td>
</tr>
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<td>FASET</td>
<td>n=36</td>
<td>n=36</td>
<td>n=36</td>
<td>n=36</td>
<td>n=36</td>
</tr>
<tr>
<td>Total BVOC</td>
<td>43.294±13.823</td>
<td>42.025±19.933</td>
<td>47.626±19.882</td>
<td>50.303±18.416</td>
<td>87.11±35.39</td>
</tr>
<tr>
<td>Median</td>
<td>46.878</td>
<td>39.549</td>
<td>44.579</td>
<td>49.264</td>
<td></td>
</tr>
<tr>
<td>FASET</td>
<td>49.534±15.703*</td>
<td>49.319±19.018</td>
<td>50.158±21.315</td>
<td>58.904±21.833</td>
<td>111.4±40.10</td>
</tr>
<tr>
<td>Median</td>
<td>44.411</td>
<td>44.594</td>
<td>45.982</td>
<td>56.272</td>
<td></td>
</tr>
<tr>
<td>DBH (cm)</td>
<td>4.1±4.1</td>
<td>4.5±4.1</td>
<td>5.0±4.4</td>
<td>5.4±4.5</td>
<td></td>
</tr>
<tr>
<td>Ameriflux</td>
<td>3.3±2.8</td>
<td>3.6±2.7</td>
<td>3.9±2.8</td>
<td>4.1±2.8</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>20.3</td>
<td>17.8</td>
<td>21.9</td>
<td>22.5</td>
<td>22.5</td>
</tr>
</tbody>
</table>

* Data was obtained from M.S. thesis (Toma, 2010).
**Limonene was not completely separated from β-phellandrene in 2008.
***Data in 2012 was obtained by Katie Bergh (2013).
Mean temperature in July was obtained from UMBS data sets
http://umbs.lsa.umich.edu/research/datafile/july-weather-records.htm-0.
Table 2-4. The Mean Composition of Each Terpenes Against Total MT or Total SQT with the Standard Deviation in Needles of Understory White Pine Trees in Both Forests 2008 – 2011. (% mole based)

<table>
<thead>
<tr>
<th>Year</th>
<th>Site</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% per total MT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Pinene</td>
<td>Ameriflux</td>
<td>52.9±8.9</td>
<td>58.9±10.2</td>
<td>57.9±10.5</td>
<td>59.1±9.7</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>54.7±7.6</td>
<td>59.6±8.9</td>
<td>58.8±9.2</td>
<td>61.6±8.5</td>
</tr>
<tr>
<td>Camphene</td>
<td>Ameriflux</td>
<td>8.0±2.8</td>
<td>6.2±3.3</td>
<td>7.3±3.1</td>
<td>6.8±3.2</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>8.1±2.3</td>
<td>7.0±2.7</td>
<td>7.6±2.6</td>
<td>7.1±2.8</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>Ameriflux</td>
<td>17.0±5.7</td>
<td>15.4±6.7</td>
<td>15.1±5.3</td>
<td>15.1±5.8</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>16.0±4.0</td>
<td>13.6±4.6</td>
<td>13.6±4.7</td>
<td>13.4±3.9</td>
</tr>
<tr>
<td>Myrcene</td>
<td>Ameriflux</td>
<td>9.1±4.1</td>
<td>10.6±6.1</td>
<td>6.7±3.8</td>
<td>7.6±4.2</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>8.8±4.4</td>
<td>10.0±6.1</td>
<td>6.7±4.3</td>
<td>6.7±4.3</td>
</tr>
<tr>
<td>α-Phellandrene</td>
<td>Ameriflux</td>
<td>4.2±1.3</td>
<td>2.0±1.9</td>
<td>3.0±1.1</td>
<td>3.0±1.3</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>3.2±1.1</td>
<td>2.1±1.7</td>
<td>2.9±0.9</td>
<td>2.5±0.8</td>
</tr>
<tr>
<td>Limonene</td>
<td>Ameriflux</td>
<td>8.2±5.0*</td>
<td>2.7±5.6</td>
<td>4.8±6.7</td>
<td>3.7±5.9</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>8.5±7.4*</td>
<td>4.1±8.6</td>
<td>5.1±8.4</td>
<td>4.3±7.5</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>Ameriflux</td>
<td>4.2±4.5</td>
<td>3.9±1.4</td>
<td>3.9±1.4</td>
<td>3.9±1.4</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>3.5±1.8</td>
<td>4.1±1.1</td>
<td>3.6±1.0</td>
<td>3.6±1.0</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>Ameriflux</td>
<td>0.7±0.4</td>
<td>N/A</td>
<td>1.3±0.6</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>0.7±0.3</td>
<td>N/A</td>
<td>1.3±0.3</td>
<td>0.6±0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>% per total SQT</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Caryophyllene</td>
<td>Ameriflux</td>
<td>5.6±2.0</td>
<td>1.1±2.8</td>
<td>7.3±2.6</td>
<td>6.3±2.1</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>5.9±1.9</td>
<td>0.7±1.8</td>
<td>8.5±2.5</td>
<td>7.0±1.9</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>Ameriflux</td>
<td>23.9±8.5</td>
<td>54.5±18.4</td>
<td>25.3±8.4</td>
<td>28.7±10.0</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>27.2±8.0</td>
<td>62.6±19.6</td>
<td>31.5±8.8</td>
<td>35.5±11.0</td>
</tr>
<tr>
<td>Germacrene B</td>
<td>Ameriflux</td>
<td>6.4±2.1</td>
<td>3.5±4.0</td>
<td>9.4±3.2</td>
<td>8.0±2.8</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>6.7±2.1</td>
<td>6.2±5.6</td>
<td>10.5±3.6</td>
<td>9.5±3.7</td>
</tr>
<tr>
<td>Y,δ-Cadinene</td>
<td>Ameriflux</td>
<td>10.1±2.5</td>
<td>0.7±1.8</td>
<td>13.3±4.6</td>
<td>9.1±2.2</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>9.8±2.6</td>
<td>0.8±2.3</td>
<td>9.4±1.8</td>
<td>8.3±2.2</td>
</tr>
<tr>
<td>Germacrene D-4-ol</td>
<td>Ameriflux</td>
<td>42.9±12.1</td>
<td>40.2±18.2</td>
<td>44.6±12.1</td>
<td>48.0±13.3</td>
</tr>
<tr>
<td></td>
<td>FASET</td>
<td>38.4±11.3</td>
<td>29.7±19.1</td>
<td>40.1±13.4</td>
<td>39.6±14.8</td>
</tr>
</tbody>
</table>

*Limonene was not completely separated from β-phellandrene in 2008.

2.3.2.2. Ameriflux vs. FASET

The mean DBH in Table 2-3 indicates that the understory white pine trees in both forests have been growing by approximately 0.4 cm/yr in Ameriflux and 0.3 cm/yr in FASET.

All terpene components were combined as MT and SQT in order to compare the concentration between Ameriflux (control) and FASET (disturbed) forests statistically, because they had similar composition in total MT or total SQT, as shown in Table 2-3. First, the normality
of the data distributions of MT, SQT, and total BVOC (MT+SQT) was investigated using a Kolmogorov-Smirnov test. The results of P-values are shown in Table 2-5. The bold number, which is less than 0.05, indicates that the data does not have normal distribution. Non-normal distribution was frequently observed from data in FASET in 2008.

Table 2-5. P-Value for Normality Distribution of MT, SQT, and Total BVOCs in Ameriflux and FASET Using Kolmogorov-Smirnov Test.

<table>
<thead>
<tr>
<th></th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ameriflux</td>
<td>0.168</td>
<td>0.200*</td>
<td>0.200*</td>
<td>0.200*</td>
</tr>
<tr>
<td>FASET</td>
<td><strong>0.044</strong></td>
<td><strong>0.040</strong></td>
<td>0.200*</td>
<td>0.200*</td>
</tr>
<tr>
<td>SQT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ameriflux</td>
<td>0.200*</td>
<td>0.200*</td>
<td>0.034</td>
<td>0.200*</td>
</tr>
<tr>
<td>FASET</td>
<td><strong>0.002</strong></td>
<td>0.099</td>
<td>0.061</td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>BVOC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ameriflux</td>
<td>0.123</td>
<td>0.200*</td>
<td>0.200*</td>
<td>0.200*</td>
</tr>
<tr>
<td>FASET</td>
<td><strong>0.036</strong></td>
<td>0.050</td>
<td>0.051</td>
<td>0.127</td>
</tr>
</tbody>
</table>

* This is a lower bound of the true significance by SPSS.
Bold number indicates that data distribution does not have normality.

Since distribution is often non-normal, MT, SQT, and total BVOC concentrations were compared in Ameriflux and FASET forests using both a parametric test (independent t-test) and a non-parametric test (Mann-Whitney U test). If the P-value of the test is less than 0.05, the hypothesis that there is no difference between the two populations, is rejected, i.e. there is a difference between two forests. Results of the P-value are shown in Table 2-6 from 2008 to 2011. Independent t-test indicated MT in FASET was statistically higher than MT in Ameriflux in 2008 and 2011. The Mann-Whitney U test also indicated MT in FASET was statistically higher than MT in Ameriflux in 2011. Considering non-normal distribution of MT in FASET in 2008, the concentration did not show significant differences between two forests until 2011. Bergh (2013) reported total BVOC in FASET was significantly higher than that in the Ameriflux in 2012 ($p = 0.014$). The concentration of 6 of 13 terpenes, camphene, β-pinene, terpinolene, β-caryophyllene,
germacrene D, and germacrene D-4-ol, in FASET were significantly higher than those in Ameriflux in 2012 (Bergh, 2013). Therefore, the effect of girdling early successional trees began appearing in the forest structure three years after the disturbance.

Table 2-6. P-Value (2-tailed) from Independent t Test and Mann-Whitney U Test to Compare Terpenes in Ameriflux vs. FASET.

<table>
<thead>
<tr>
<th></th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Independent t-test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>0.022</td>
<td>0.138</td>
<td>0.338</td>
<td>0.010</td>
</tr>
<tr>
<td>SQT</td>
<td>0.389</td>
<td>0.201</td>
<td>0.785</td>
<td>0.880</td>
</tr>
<tr>
<td>BVOC</td>
<td>0.080</td>
<td>0.122</td>
<td>0.612</td>
<td>0.081</td>
</tr>
<tr>
<td>Mann-Whitney U test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>0.107</td>
<td>0.100</td>
<td>0.548</td>
<td>0.024</td>
</tr>
<tr>
<td>SQT</td>
<td>0.550</td>
<td>0.296</td>
<td>0.848</td>
<td>0.943</td>
</tr>
<tr>
<td>BVOC</td>
<td>0.275</td>
<td>0.130</td>
<td>0.665</td>
<td>0.136</td>
</tr>
</tbody>
</table>

The presence of difference due to p <0.05 (2 tailed)

Distributions of concentration of total MT, total SQT, and BVOC in Ameriflux and FASET 2008 – 2012 are in Figure 2-8 using a box-and-whisker plot. (Note: the original data in 2012 does not include limonene. This means MT and BVOC in 2012 is likely underestimated by 3-5 %.) In 2012, absolute concentrations of all MT, SQT, and BVOC were dramatically higher than over four past years. The magnitudes of all three categories in the year increased by a factor of two, more than the other four years in Ameriflux and FASET.
(a) Distribution of total MT in Ameriflux and FASET

(b) Distribution of total SQT in Ameriflux and FASET
2.3.2.3. MT vs. SQT

The concentration of total BVOC did not significantly change from 2008 to 2010. As reported in my master’s thesis (Toma, 2010), partitioning between total MT and total SQT varied by year. Since there is no statistically significant difference between the two forests over the three years, the data from both forests were combined for comparing total MT and total SQT. Both concentrations in 2009 were significantly different than 2008 and 2010. Total MT in 2009 had higher concentration with a wider range of standard deviation than 2008 and 2010. On the contrary, total SQT was lower in 2009 than in 2008 and 2010. MT and SQT in conifers are produced by
different biosynthetic pathways at different places (e.g. Martin, 2002). MTs are formed by
monoterpene synthase using geranyl diphosphate in plastids, while SQT formed from
sesquiterpene synthases and farnesyl diphosphate in the cytoplasm. The different responses of MT
and SQT might be related to enzyme activity and environmental factors such as light and
temperature. The mean temperature of UMBS in July 2009 was 17.8 °C, which was about 2-3 °C
cooler than in 2008 and 2010 (mean temperature was included in Table 2-3 and Figure 2-8 (c)).
Although the impact of ambient temperature on terpenes in white pines has not been studied
specifically, it is known that terpene synthase activity is affected by temperature (e.g. Grote and
Niinemets, 2008). Also, there is evidence that temperature can affect terpene concentration in
needles of at least one species of pines (Blanch et al., 2008). This one environmental factor is not
solely responsible for differences but is representative of different overall growing conditions
(including such as precipitation) that might have influenced the absolute levels of compounds
measured.

2.3.3. Canopy (Overstory) Trees in UMBS

Table 2-7 shows the mean concentrations of BVOC in needles from the top and the bottom
of overstory trees. Data from 2008 and 2009 (Toma, 2010) are included. A comparison of the mean
concentrations between the top and the bottom of the tree needles were conducted using the non-
parametric Mann-Whitney Test. The presence of a significance difference was indicated by a star
(p < 0.05). The mean concentrations of MT at the top of trees were significantly higher (30–50 %)
than at the bottom over three years, while SQT levels did not have as consistent a trend. SQT were
largely the same at the top and at the bottom in 2009 and 2010. Hence, total BVOC in needles at
the top of the canopy trees was also larger than understory trees by about 50% in 2009 and 2010.
The bottom needles of canopy trees essentially had the same composition as understory trees. This suggests that MT concentrations will increase as understory white pine trees grow into the canopy as a result of forest succession.

Differences in both light and temperature properties at each location in the canopy could affect these concentrations. Needles on large canopy trees exist in different light environments at the top and the bottom and a temperature gradient of several degrees can exist between the top and bottom of a forested environment (Gu et al., 1999). Terpene emissions into the atmosphere tend to be more sensitive to temperature than light because terpenes are stored within the leaf (Lerdau et al., 1994). Still, light dependent synthesis has been seen in expanding conifer needles (Lerdau and Gray, 2003). Results in Table 2-7 support the suggestion by Gleizes et al. (1980), who reported that synthesis of MT is more sensitive to photo-dependent activation than SQT. Consequently, light exposure may impact the production of MT more than SQT. The researchers showed that there is no MT formation without light, but there is, SQT formation in both light and dark. Determining the drivers of the variation of terpene levels at different place in the canopy will require further study.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total MT</th>
<th>Total SQT</th>
<th>Total BVOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008 (n=4)</td>
<td>Top *33.7</td>
<td>12.3</td>
<td>46.0</td>
</tr>
<tr>
<td>FASET</td>
<td>Bottom 25.1</td>
<td>*21.0</td>
<td>46.1</td>
</tr>
<tr>
<td>2009 (n=8)</td>
<td>Top *57.1</td>
<td>5.5</td>
<td>*62.6</td>
</tr>
<tr>
<td>Ameriflux</td>
<td>Bottom 29.2</td>
<td>5.2</td>
<td>34.4</td>
</tr>
<tr>
<td>2010 (n=10)</td>
<td>Top *46.3</td>
<td>12.8</td>
<td>*59.1</td>
</tr>
<tr>
<td>Ameriflux</td>
<td>Bottom 24.9</td>
<td>12.3</td>
<td>37.3</td>
</tr>
</tbody>
</table>

* indicates significant difference between top and bottom of needles based on a Wilcoxon signed test ($P = 0.05$). 2008 measurements made in FASET; 2009 and 2010 in Ameriflux.
2.3.4. BVOC Composition from Other Pine Trees

The same terpene components were observed in red pine (*Pinus resinosa*) needles as in white pine (*Pinus strobus*) needles, but the relative composition was quite different (Figure 2-9). In red pine needle extracts, the dominant component was β-pinene (48.9%), which accounts for a half of total BVOC, while α-pinene (27.7%) and myrcene (10.2%) were the next major components. The amount of SQT was very small in red pine needles. White pine at UMBS in 2010 showed mean MT and mean SQT accounted for 65% and 35% of total BVOC in needle extracts, respectively, while red pine showed mean MT and mean SQT accounted for 92% and 8%, respectively.

![Relative BVOC Composition from Red Pine and White Pine Needles Extracts in UMBS 2010](image)

Figure 2-9. Relative BVOC Composition from Red Pine and White Pine Needles Extracts in UMBS 2010. Total BVOC concentration (mean±standard deviation, μmol dwg⁻¹) for red pine (n = 5): 58.76±10.11, for white pine (n = 69): 48.95±20.53.
2.3.5. BVOC Composition from Other Forests in the Lower Peninsula of Michigan

Table 2-8 shows the mean DBH of 20 sampled white pine trees from four different forests and the mean total BVOC. In the forests of Chaboiganing Nature Preserve and Fort Custer Recreation Area, white pine needles were collected from large trees because understory trees were not found in these areas. Hence, the mean DBH of trees from these two forests were 1.5-3 times higher than that from forests of Hartwick Pines State Park and Crawford County, Grayling. The mean DBH from Hartwick Pines State Park and Crawford County, Grayling is twice as large as that from UMBS. However, the mean DBH is not directly proportional to the mean of total BVOC concentration. One-way ANOVA test, (a parametric test since all sampled data showed normal distribution), was performed to compare the total BVOC concentration among four forests, and resulted in no statistical difference (p=0.275). The level was similar to the mean of total BVOC concentration from Ameriflux in UMBS (p=0.095). Tukey’s post hoc test found the presence of significant differences of mean total BVOC concentration between FASET and Crawford County, Grayling (p=0.001) and between FASET and Fort Custer Recreation Area (p=0.006).

<table>
<thead>
<tr>
<th></th>
<th>Chaboiganing</th>
<th>Hartwick</th>
<th>Grayling</th>
<th>Fort Custer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBH, cm</td>
<td>14.0 (±14.6)</td>
<td>9.3 (±10.9)</td>
<td>8.2 (±11.9)</td>
<td>30.2 (±12.9)</td>
</tr>
<tr>
<td>Total BVOC, µmol dwg⁻¹</td>
<td>47.1 (±16.7)</td>
<td>45.8 (±19.8)</td>
<td>37.9 (±11.4)</td>
<td>40.2 (±19.5)</td>
</tr>
</tbody>
</table>

n=20 for each location. The parentheses indicate the standard deviation.

The same terpene components found at UMBS were observed in four additional forests in 2011. The relative BVOC composition (%) from the forests was plotted in Figure 2-10. The dominant MT as well as BVOC was α-pinene and the dominant SQT was germacrene D-4-ol.
2.3.6. Effects of Chemotypic Variation within Population of White Pine on BVOC Composition

2.3.6.1. UMBS

The same eight trees (S7, S16, S27, S34, S41, S49, S67, and S71) that showed high limonene concentration in 2009 – 2011 also showed this in 2012. Twenty one (21) trees (11 at Ameriflux and 10 at FASET) were added in 2010, and high limonene concentrations were found from five new sampled trees. This means that 14% of the trees sampled at UMBS (13 out of 92 trees) had high levels of limonene. Bergh (2013) also additionally sampled 26 trees in 2012 and three trees (approximately 12 %) showed high levels of limonene. Hence, this chemotypic
Variation is a common observation from the white pine population in the UMBS. Additionally, the same two distinct trends between limonene and the sum of other monoterpenes (OTM) seen in 2009 – 2011 (designated Mode I and Mode II) were observed every year and are included in each regression line in Figure 2-11.

Figure 2-11. The Correlation Between Limonene and Total Other Monoterpenes Concentration at UMBS in 2009 – 2011. Data from 2008 are not included because limonene was not adequately resolved by the GC.
Environmental Influence at UMBS

Trees in Mode II were randomly distributed in the two forests and were not necessarily close to each other in similar environmental parameters. Several environmental factors, such as light environment, tree age, and DBH were recorded for each sample, but there were no significant correlations between Mode I/Mode II ratios and any of these environmental factors. In 2012, Bergh (2013) investigated the influence of mycorrhizal colonization (comparison between percent mycorrhizal colonization and BVOC concentration) and root disturbance (comparison of BVOC concentration pre- and post- disturbance root). The study of mycorrhizae showed no significant relationship with BVOC except myrcene, which had statistically negative correlation (p = 0.011). There was no statistical effect of root disturbance on BVOC concentration (p ≥ 0.14). Therefore, the bimodal behavior is not related to measured environmental factors in the UMBS.

Methyl Jasmonate Influence

It is known that induction via enzyme activity can result in a change of composition of volatile compounds in trees. Martin et al. (2002; 2003) studied resin composition of Norway spruce in bark and wood before and after treatment with methyl jasmonate, which mimics insect damage. Limonene concentration was tremendously enhanced after the induction, but the activity of MT and SQT synthases declined 15 days after the treatment. This means that the induced change is a temporary influence on BVOC composition in trees. However, the bimodal behavior in limonene/OMT ratio in white pine in the UMBS seems to persist for a long time (over several seasons). Figure 2-12 shows the tracked limonene/OMT ratios from representative trees at Ameriflux of Mode I and Mode II trees during the growing season, from June to August in 2010. Even though absolute concentrations of OMT and limonene changed as the growing season
progressed, the limonene/OMT ratio for each tree stayed in the same mode. Figure 2-12 includes the tracked limonene/OMT ratio from white pine trees at the WMU campus (n=1), which is located near Wesley Foundation and is used in Figure 2-7, for a more expansive span (April–December in 2010). This study also showed that the ratio of limonene/OMT did not change throughout the growing season and reflected one of the same modes seen at UMBS (Mode I in Figure 2-11).

Hence, the bimodal behavior in the ratio of these two compounds from white pine trees may be not related to environmental factors and damage from insects, but is more likely due to a genetic difference. (However, the reported experiment with methyl jasmonate treatment observed for a single attack influence. In forests, prolonged attack is possible. Hence, damage by insects cannot be excluded completely.)
Figure 2-12. Consistency in the Relationship Between Limonene and Total Other MT in Specific Trees in UMBS Throughout a Growing Season in 2010. S7, S16, S27, S34, S41, S49, S67 and S71 belong to Mode II. Tracked data on the WMU Campus from April to December in 2010 is included.

2.3.6.2. At Other Forests in the Lower Peninsula of Michigan

Chemotypic variation of BVOC composition in white pine extracts within the same population was observed from other forests. The mean limonene concentration from four additional forests represented 1-3% of total BVOC concentration in Figure 2-10. The relationship between limonene and the total of OMT in all forests are shown in Figure 2-13 with linear regression lines of Mode I and Mode II obtained from the data in Figure 2-11. Three forests,
Hartwick, Grayling, and Fort Custer, resulted in low limonene concentration in which the relationship with OMT exactly fitted the linear regression line of Mode I in UMBS. In Chaboiganing Nature Preserve, two out of 20 trees showed high limonene concentration that accounted for 14 and 16 % of total BVOC. In Figure 2-13, these two trees’ data tended to be near the linear regression line of Mode II from the UMBS. The Chaboiganing Nature Preserve is located close to UMBS. This may indicate that the white pine tree populations of the two forests have similar expressed genes. High myrcene concentrations were frequently observed in all four forests. However, the relationship between myrcene and OMT (total MT – myrcene) in Figure 2-14 did not show distinct bimodal behavior like limonene did.

Figure 2-13. The Relationship Between Limonene and Total Other Monoterpenes Concentration in Four Additional Forests in Lower Peninsula Michigan in 2011. The two equations of linear regression lines were obtained from Figure 2-8.
2.3.7. Chiral GCMS Experiment for High $\alpha$-Pinene vs. High Limonene Trees

The Section 2.3.6. showed that while most of the trees held mainly $\alpha$-pinene in the needles, 14% of the understory white pine trees at UMBS contained relatively high levels of limonene. Understory trees were then categorized into four groups based on their $\alpha$-pinene and limonene concentration in needle extract, as shown in in Figure 2-15. The unit of concentration is micro mole per dry weight gram (of needle mass), $\mu$ mol dwg$^{-1}$. Representative trees were selected from each group for an experiment to test the chiral composition of each group. As shown in Figure 2-16, $\alpha$-pinene is directly proportional to total BVOC. Hence, each group is categorized as

- Group A: high $\alpha$-pinene (total BVOC) and high limonene – S16, S41, S7
- Group B: low $\alpha$-pinene (total BVOC) and high limonene – S7, S34
- Group C: high $\alpha$-pinene (total BVOC) and low limonene – S38, S40, S42
- Group D: low $\alpha$-pinene (total BVOC) and low limonene – S12, S3, S61

Group A and B belong to Mode II and Group C and D belong to Mode I, as described in Section 2.3.6.1. this work.

Figure 2-15. Relationship Between $\alpha$-Pinene and Limonene in the UMBS 2010 Summer.
Chiral GC experiments were conducted at Kalsec Inc. (Kalamazoo, MI) with gracious cooperation from Dr. Doug Williams and Dr. David Bolliet on March 25th and 28th, 2011. A chiral column, HYDEODEX-β-3P (25 m × 0.25 mm ID, Machery Nagel) was installed in a TRACEGC/POLARIS Q detector (Thermo Finnigan, Italy). Standards and essential oil were diluted by hexane to \( \sim 167 \mu \text{mol} \text{ L}^{-1} \). The injection volume was 1 µL with split mode (30:1). The injector and detector temperatures were held at 220 °C. The oven temperature program, based on the one developed by Wang et al. (1997), began at 60 °C and was held there for 5 min, then the temperature increased at 4 °C min\(^{-1}\) up to 140 °C. Each retention time was confirmed using authentic standards: (1S)-(−)-α-pinene (SIGMA), (1R)-(−)-α-pinene (Aldrich, 98 %), (S)-(−)-limonene (Alfa Aesar, UK, 97 %), and (R)-(−)-limonene (Fluka, ≥ 99.0 %). Angelica seed essential oil (Shiono Koryo Kaisha, Ltd., Japan) was used to confirm the separation of β-phellandrene from limonene.
Representative GC spectra using the chiral column for Mode I and II are shown in Figure 2-17. The order of retention time was (-)-α-pinene, (+)-α-pinene, (-)-limonene, and (+)-limonene. The column was able to separate β-phellandrene from limonene.

![Image](image-url)

**Figure 2-17.** Representative GC Spectra of Mode I and Mode II Using a Chiral Column.

The outstanding difference between samples in Mode I and samples in Mode II is the relative amount of (-)-limonene. The fraction of each (-) and (+) of α-pinene and limonene is summarized in Table 2-9 based on the area of GC peaks. In both Group A and Group B (Mode II), the average fraction with standard deviation of (-) and (+)-α-pinene from five trees are 60±5% and 40±5% respectively, while that of (-) and (+)-limonene were 88±2% and 12±2% respectively. In both Group C and Group D (Mode I), the average fraction with standard deviation of (-)-α-pinene from
six trees was 60±13%, this amount is higher than (+)-α-pinene, 40±13% and shows a similar behavior to Mode II. However, the average fractions of (-) and (+)-limonene in Mode I were 26±14% and 74±14% respectively and this observation was the opposite from that observed in Mode I. The ratio of total limonene/total α-pinene, where the total means sum of both (-) and (+) enantiomers, is plotted versus (-)-limonene in Figure 2-18. The results clearly show that trees in Mode II are consistently enriched in only the single enantiomer (-)-limonene. This is one piece of evidence in support of a genetic basis on the chemotype difference.

Table 2-9. The Mole Basis Fraction, % and the Concentration, (Conc., µmol dwg⁻¹) of (-) and (+) of α-Pinene and Limonene by GCMS with Chiral Column.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>(-)-α-pinene %</th>
<th>Conc.</th>
<th>(+)-α-pinene %</th>
<th>Conc.</th>
<th>(-)-limonene %</th>
<th>Conc.</th>
<th>(+)-limonene %</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>S16</td>
<td>56.0</td>
<td>9.2</td>
<td>44.0</td>
<td>7.2</td>
<td>86.9</td>
<td>9.2</td>
<td>13.1</td>
<td>1.4</td>
</tr>
<tr>
<td>A</td>
<td>S41</td>
<td>64.9</td>
<td>13.0</td>
<td>35.1</td>
<td>7.0</td>
<td>89.9</td>
<td>14.1</td>
<td>10.1</td>
<td>1.6</td>
</tr>
<tr>
<td>A</td>
<td>S71</td>
<td>64.2</td>
<td>6.3</td>
<td>35.8</td>
<td>3.5</td>
<td>90.9</td>
<td>9.7</td>
<td>9.1</td>
<td>1.0</td>
</tr>
<tr>
<td>B</td>
<td>S7</td>
<td>54.6</td>
<td>4.3</td>
<td>45.4</td>
<td>3.5</td>
<td>86.6</td>
<td>3.2</td>
<td>13.4</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td>S34</td>
<td>62.5</td>
<td>1.8</td>
<td>37.5</td>
<td>1.1</td>
<td>87.6</td>
<td>2.1</td>
<td>12.4</td>
<td>0.3</td>
</tr>
<tr>
<td>C</td>
<td>S38</td>
<td>51.5</td>
<td>16.4</td>
<td>48.5</td>
<td>15.5</td>
<td>33.4</td>
<td>0.5</td>
<td>66.6</td>
<td>1.0</td>
</tr>
<tr>
<td>C</td>
<td>S40</td>
<td>74.8</td>
<td>34.8</td>
<td>25.2</td>
<td>11.7</td>
<td>21.1</td>
<td>0.4</td>
<td>78.9</td>
<td>1.7</td>
</tr>
<tr>
<td>C</td>
<td>S42</td>
<td>63.2</td>
<td>22.9</td>
<td>36.8</td>
<td>13.3</td>
<td>34.5</td>
<td>0.4</td>
<td>65.5</td>
<td>0.8</td>
</tr>
<tr>
<td>D</td>
<td>S3</td>
<td>66.2</td>
<td>4.7</td>
<td>33.8</td>
<td>2.4</td>
<td>37.6</td>
<td>0.1</td>
<td>62.4</td>
<td>0.1</td>
</tr>
<tr>
<td>D</td>
<td>S12</td>
<td>67.2</td>
<td>7.6</td>
<td>32.8</td>
<td>3.7</td>
<td>28.1</td>
<td>0.1</td>
<td>71.9</td>
<td>0.3</td>
</tr>
<tr>
<td>D</td>
<td>S61</td>
<td>39.3</td>
<td>3.3</td>
<td>60.7</td>
<td>5.1</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Ref.</td>
<td>#1 (MO)</td>
<td>29</td>
<td>71</td>
<td>38</td>
<td>62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref.</td>
<td>#2 (MO)</td>
<td>23</td>
<td>77</td>
<td>28</td>
<td>72</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref.</td>
<td>#3 (MO)</td>
<td>35</td>
<td>65</td>
<td>30</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref.</td>
<td>#4 (MO)</td>
<td>31</td>
<td>69</td>
<td>19</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ref. indicates data comes from Wang et al., (1997). The data was resin sample from the trunk of white pine located in Rolla, MO.

Wang et al. (1997) reported the chirality behavior of α-pinene and limonene from the resins of white pine (Pinus strobus) trees (n=4) located in Rolla, MO, USA (Table 2-9). The sample size is small and sap could have been different from needles, but the reported range of the ratio of limonene/α-pinene, 0.07 – 0.09, indicates that all white pine trees from MO seem to belong Mode
I (high α-pinene and low limonene concentration). The fraction on (-) and (+)-limonene from reported trees was similar to the fraction in the Mode I, while the fraction of α-pinene was opposite of the results in the UMBS (except S61) and showed dominant (+)-α-pinene. This suggests that the enantiomeric composition varies based on locations (or populations). High limonene trees are enriched primarily in (-)-limonene at UMBS. (As a further research, it would be interested in a test if whether methyl jasmonate treatment causes a large amount of a single enantiomer of limonene or not.)

The concentration of each chiral component (µmol dwg⁻¹) is calculated using the chiral fraction in Table 2-9. The relationship of enantiomeric concentration is displayed in Figure 2-19. The (-) and (+) α-pinene were positively correlated in all groups with slopes that are very similar. The average with standard deviation of ratio (-)/(+) of α-pinene is 1.6±0.3 for Group A and B (Mode II) and
1.7±0.8 for Group C and D (Mode I). The slope of the correlation between (-) and (+) limonene is much greater in Groups A and B than in Groups C and D. The average with standard deviation of ratio (-)/(+) of limonene is 7.8±1.6 for the former and 0.4±0.2 for the latter.

Figure 2-19. Relationship of Concentrations of Enantiomeric Forms on a) α-Pinene and b) Limonene.

Figure 2-20 shows the relationship between limonene and α-pinene concentrations based on the same (+ or -) form where a direct proportionality was observed in Group A and B, but not in Group C and D in (-) forms. On the other hand, in (+) forms, a direct proportion was obtained from all four groups, but the sensitivity was different for Mode I and Mode II.
It has been reported that a single terpene synthase enzyme may produce multiple terpene products (e.g. Martin et al., 2004; Zulak et al., 2009). This means that α-pinene synthase and limonene synthase might produce both α-pinene and limonene in different amounts. Enzymes present in the white pine tree could control the folding of the substrate molecule and influence the enantiomeric compound produced (Dewick, 2002). Figure 2-21 shows a traditional proposed biosynthesis pathway for enantiomeric limonene and α-pinene (Dewick, 2001; Hyatt et al., 2007). Geranyl diphosphate (GPP), the monoterpane precursor is folded in two different ways via ionization to an allylic cation and results in forming enantiomeric linalyl diphosphate (LPP), which is an isomer of GPP. The cyclization of LPP generates achiral α-terpinyl cation, which is the precursor of both α-pinene and limonene.

If two enzymes, α-pinene synthase and limonene synthase, control the production of the α-terpinyl cation that can produce either/or both α-pinene and limonene, the enantiomeric products that are proposed for white pine trees in the UMBS, such as those in Table 2-10, are based on the results from Figure 2-19 and 2-20. It may be hypothesized that in the UMBS, α-pinene synthase
produces the same fractions of (+) and (-)-α-pinene for all groups, but the quantity of the terpene is relatively higher in Groups A and C, and produces relatively high (+)-limonene for all groups. While activity of limonene synthase is observed in Group A and B, i.e. only Mode II, the fraction of (-) is tremendously higher than (+).

<table>
<thead>
<tr>
<th>Group</th>
<th>α-pinene synthase</th>
<th>Limonene synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-pinene</td>
<td>limonene</td>
</tr>
<tr>
<td>A</td>
<td>(+) = (-)</td>
<td>(+) &gt; (-)</td>
</tr>
<tr>
<td>B</td>
<td>(+) = (-)</td>
<td>(+) &gt; (-)</td>
</tr>
<tr>
<td>C</td>
<td>(+) = (-)</td>
<td>(+) &gt; (-)</td>
</tr>
<tr>
<td>D</td>
<td>(+) = (-)</td>
<td>(+) &gt; (-)</td>
</tr>
</tbody>
</table>

— indicates no production.
Figure 2-21. The Biosynthesis Pathway for Enantiomeric α-Pinene and Limonene. (Based on Dewick 2002, Hyatt et al., 2007)
Establish a genetic basis for chemotypic variation can be challenging (Shelton et al., 2002). Analysis of the chemotypic difference in vegetation is a relatively new field in the study of atmospheric air quality. In order to find evidence of a genetic basis from the bimodal phenomenon as illustrated in Table 2-10, an attempt was made to sequence both limonene and α-pinene synthases from representative trees selected from each group A-D from the UMBS. However, the DNA sequencing of terpene synthases of white pine tree (Pinus strobus) was not available in the GenBank database, because the synthases had not yet been sequenced. As a stem of this project, the optimization on a DNA sequencing method for α-pinene and limonene synthases from white pine needles was conducted with the assistance of Noah Sorrelle, who was working with Dr. Todd Barkman’s group at WMU. Although the ultimate goal to show the evidence of genetic influence on chemotypic variation would be obtaining the protein expression from the DNA sequencing, the optimization is suspended at finding the appropriate DNA sequences to produce the protein. The approaches and results were shown Appendix C. Although efforts to observe a genetic basis were incomplete (Appendix C), results of chiral analysis suggest that genetics are a strong possibility.

2.3.8. Comparison Between Head Space BVOC by SPME vs. Extract of Needles

The results of BVOC concentration in extracts and a very simple experiment measuring gas-phase BVOC using SPME from same tree were normalized to α-pinene concentration (as α-pinene = 1) in Figure 2-22. α-Pinene was the dominant component in both, while germacrene D-4-ol, which is the most dominant SQT in the extract, was less present in gas-phase. A hydroxyl group of germacrene D-4-ol could lead a low vapor pressure (see Table 2-11 in Section 2.4.1.) and result in the compound remaining in the needles. In addition, it may be due to lower affinity for SPME material, because each SPME fiber has selective chemical affinity (this means the relative
composition of BVOC by SPME measurement is likely different from that by branch enclosure method). Therefore, optimization (including calibration of SPME for individual compounds and using several SPME fibers, which have different materials) would be required for assessing how well BVOC emissions can be estimated using SPME.

Ortega and co-workers (2008) measured BVOC emissions from white pine trees (n = 7) in UMBS in summer 2003 and 2005 using a branch enclosure technique. The normalized compositions of MT emissions are plotted with the normalized composition of MT in extract from Ameriflux (2008-2011) in Figure 2-23. Branch enclosure experiments also showed different MT composition from extract and each MT in gas-phase similar to SPME results. Although absolute concentrations may be different between the gas-phase and needle extracts (since the unit of emission rate is not to convert for the unit of extract in this work, we can’t directly compare the absolute concentration at this statement), the fraction of camphene, β-pinene, β-myrcene, limonene, β-phellandrene in gas-phase were more than twice as large as that in needle extracts.

Figure 2-22. The Normalized BVOC Compositions of White Pine Needles in Gas-phase (by SPME) and Extract. Each component was normalized to α-pinene concentration. White pine needles were obtained at WMU campus.
Figure 2-23. The Normalized BVOC Compositions of White Pine Needles in Gas-phase (by Branch Enclosure) and Extract in the UMBS. The data of BVOC emissions are obtained from Ortega et al., (2008).

2.4. Discussion

2.4.1. Estimated Atmospheric Impact of High Limonene Concentration

Currently, the effect of chemotypic variation is rarely considered when estimating emission rates of BVOC from a forest using branch enclosure measurements since it is hard to measure enough individuals using this technique. If the biosynthesis of terpenes and emissions into the atmosphere vary within a population as a result of genotypic variation and lead to different pools of BVOC, the emission rates based on limited sampling could result in systematic error in emission estimates. In the UMBS, 14% of trees show high concentration of limonene (Mode II in Figure 2-11). Limonene reacts with hydroxyl radical and ozone faster than α-pinene (Atkinson and Arey, 2003) (see Table 2-11). Hence, limonene emission over the forests in northern Michigan could be underestimated in current models. The atmospheric impact of Mode II level of limonene was
assessed by estimation of the OH reactivity. OH reactivity with limonene can be expressed relative to with other MT (OMT)

\[
\frac{d[OH]}{dt} = k_{OMT}[OMT] + k_{Limonene}[Limonene]
\]

\[
= \left(1 + \frac{k_{Limonene}[Limonene]}{k_{OMT}[OMT]}\right)k_{OMT}[OMT]
\]

\[
= \gamma k_{OMT}[OMT] \quad \text{(Eq. 2-1)}
\]

where \( \gamma \) is the contribution of limonene to OH reactivity relative to the rest of the MT. (NOTE: SQT was not included due to physical and kinetic parameters which are too uncertain and the dominant SQT, germacrene-D-4-ol may be less abundant in the gas-phase.) If BVOC emissions from white pine needles scales with extract concentration and temperature based on Raoult’s law, which is discussed in Section 2.4.2, the concentration ratio in gas-phase in \( \gamma \) is derived from the measured ratio in extract,

\[
\frac{[Limonene]}{[OMT]} = \frac{P_{Lim.}}{P_{OMT}} = \frac{X_{Lim.}}{X_{OMT}} \cdot \frac{P_{Lim.}}{P_{OMT}} \quad \text{(Eq. 2-2)}
\]

where \( P \) is the partial vapor pressure of a component, \( X \) is the mole fraction of the component in a solvent, and \( P^o \) is the vapor pressure of the pure component. Parameters for \( P^o \) and \( k \) at 298 K are shown in Table 2-11. The \( k_{OMT} \) and \( P_{OMT}^o \) were estimated by taking the sum of individual rate constant and vapor pressure scaled by the individual relative composition of concentration. The ratio \( X_{Lim}/X_{OMT} \) is obtained from the slope of regression lines in Figure 2-11. The relative contribution to OH reactivity, \( \gamma \) was calculated for Mode I (\( \gamma_I = 1.02 \)), Mode II (\( \gamma_{II} = 1.31 \)), and a mixture of Mode I and II (\( \gamma_{I\&II} = 1.07 \)) based on the distribution observed at UMBS (under 86 % Mode I and 14 % Mode II). This means the effect of limonene in Mode I is small, contributing about 2 % on OMT contribution to OH reactivity, while that in Mode II contributes 31 %. The
mixture of trees in both Mode I and Mode II that is currently found at UMBS is about 4% higher than when considering only pure Mode I, the most abundant chemotype. Identical method was applied to assess the relative contribution of each mode to O₃ reactivity, γ’ and resulted in Mode I ($\gamma_1' = 1.01$), Mode II ($\gamma_{II}' = 1.17$), and a mixture of Mode I and II ($\gamma_{I\&II}' = 1.03$).

In Figure 2-23 suggests the fraction of limonene in gas-phase is four times larger than that in needle extracts. The contribution of limonene due to chemotypic variation may be underestimated.

As these young forests at UMBS and the upper Michigan continue to transition to the next successional stage, terpenes will begin to rival or replace isoprene as the dominant BVOC in the near-canopy atmosphere. Since limonene reacts faster with ozone as well as with hydroxyl radical and yields more SOA than α-pinene, chemotypic variation within the forest population of white pine could be a significant factor in the BVOC impact on atmospheric chemistry.
Table 2-11. BVOC Parameters in Gas-phase Reactions at 298 K.

<table>
<thead>
<tr>
<th>BVOC Parameter</th>
<th>Rate constant with OH (10^{12} \times k) (cm(^3) molecule(^{-1})s(^{-1}))</th>
<th>Rate constant with O(_3) (10^{17} \times k) (cm(^3) molecule(^{-1})s(^{-1}))</th>
<th>Vapor pressure (Pa)</th>
<th>Mean concentration in UMBS 2011 (µ mol dwg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pinene</td>
<td>52.3 (^a)</td>
<td>8.4 (^a)</td>
<td>560.9 (^b)</td>
<td>21.59</td>
</tr>
<tr>
<td>camphene</td>
<td>53 (^a)</td>
<td>0.09 (^a)</td>
<td>438.7 (^b)</td>
<td>2.59</td>
</tr>
<tr>
<td>β-pinene</td>
<td>74.3 (^a)</td>
<td>1.5 (^a)</td>
<td>391.1 (^b)</td>
<td>4.81</td>
</tr>
<tr>
<td>3-carene</td>
<td>88 (^a)</td>
<td>3.7 (^a)</td>
<td>230.8 (^b)</td>
<td>0.04</td>
</tr>
<tr>
<td>α-phellandrene</td>
<td>313 (^a)</td>
<td>300 (^a)</td>
<td>195.2 (^b)</td>
<td>0.97</td>
</tr>
<tr>
<td>β-myrcene</td>
<td>215 (^a)</td>
<td>47 (^a)</td>
<td>320 (^c)</td>
<td>2.49</td>
</tr>
<tr>
<td>limonene</td>
<td>164 (^a)</td>
<td>21 (^a)</td>
<td>201.8 (^b)</td>
<td>1.41</td>
</tr>
<tr>
<td>β-phellandrene</td>
<td>168 (^a)</td>
<td>4.7 (^a)</td>
<td>260 (^c)</td>
<td>1.35</td>
</tr>
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</tr>
<tr>
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<tr>
<td>germacrene D</td>
<td>239.5513 (^c)</td>
<td>61 (^c)</td>
<td>3.04 (^c)</td>
<td>5.73</td>
</tr>
<tr>
<td>germacrene B</td>
<td>289.8733 (^c)</td>
<td>206 (^c)</td>
<td>1.38 (^c)</td>
<td>1.53</td>
</tr>
<tr>
<td>γ,δ-cadinene*</td>
<td>183.2546 (^c)</td>
<td>103.6600 (^c)</td>
<td>3.665 (^c)</td>
<td>1.66</td>
</tr>
<tr>
<td>germacrene D-4-ol</td>
<td>157.8248 (^c)</td>
<td>44.1375 (^c)</td>
<td>0.00824 (^c)</td>
<td>9.22</td>
</tr>
</tbody>
</table>

\(^*\) means the values are average of γ-cadinene and δ-cadinene.

The sources are from a) Atkinson and Arey (2003), b) Hoskovec et al. (2005), and c) calculated using EPI system (EPI SuiteTM v4.0 program). The results from EPI program are able to obtain via http://www.thegoodscentscompany.com (select a compound via the tab of “aromatic ingredients”, click “safety in use” at section of physical properties, and click the link of “EPI system” to view.) The source d) is Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2012 ACD/Labs).

2.4.2. Estimation of BVOC Emission Using Raoult’s Law

Raoult’s law is a relational expression for a component in between gas phase and liquid phase. Raoult’s law describes the partitioning between the organic liquid phase and the vapor phase of a homogenous solution (note: this is one of the limitations for Raoult’s law). The partial vapor pressure of a component \(P_i\) is given as

\[
P_i = X_i P_i^\circ\quad (Eq. 2-3)
\]
where $X_i$ is the mole fraction of the component and $P_i^{°}$ is the vapor pressure of the pure component at a particular temperature (298 K was used for this study). BVOC components in gas-phase from white pine needles in UMBS 2011 were estimated using Raoult’s law. The vapor pressure of each component is listed in Table 2-11. The mole fraction, $X_i$ was obtained using the mean concentration of each component in UMBS (combined the data from Ameriflux and Faset) in 2011. The concentration of each component in gas-phase, $[\text{BVOC}_i]_{gas}$ is estimated as

$$[\text{BVOC}_i]_{gas} = \frac{P_i}{P_{\text{total}}} \times n \quad (Eq. 2-4)$$

where $P_{\text{total}}$ is total pressure of air that is 99695.282 Pa (= 1atm), and $n$ is the number density of air that is $2.5 \times 10^{19}$ molecules cm$^{-3}$ at 25 °C (=298 K) and 1 atm. The concentrations of BVOC components in gas-phase using Raoult’s law and extract are normalized to $\alpha$-pinene, as shown in Figure 2-17. The results indicate that all SQT components, especially germacrene D-4-ol, were less present in the gas-phase due to the low vapor pressure of these components (see Table 2-11).
Gas-phase BVOC is consumed by oxidants such as the hydroxyl radical and ozone. When BVOC reacts with hydroxyl radical, the loss rate is described as

\[
\frac{d[BVOC_i]_{gas}}{dt} = -k[BVOC_i][OH] \quad (Eq. 2-5)
\]

where \(k\) is the rate constant. Since the hydroxyl radical has very short lifetime, the [OH] is assumed to be constant and the loss rate is treated as pseudo first order,

\[
\frac{d[BVOC_i]_{gas}}{dt} = -k'[BVOC_i] \quad (Eq. 2-6)
\]

where \(k' = k[OH]\). The loss rate by ozone is also obtained via pseudo first order. The rate constants of reactions with hydroxyl radical and ozone at room temperature (298 K) are placed on Table 2-11. The calculated loss rates via reaction with hydroxyl radical and ozone are shown in Figure 2-25, with mean BVOC concentration of the extracts from white pine needles in UMBS 2011. SQT
has relatively higher rate constant than MT in reactions with hydroxyl radical and ozone. However, the small presence in gas-phase that is derived from Raoult’s law results in low loss rate, even though gaermacrene D is one of major components in the extract. This was observed in certain amounts in the gas-phase using SPME techniques, as shown in Figure 2-22. However, the most of factors of SQT are still uncertain because these were predicted by a computational program, not by experimental results (see the explanation on source c) in Table 2-11).

![Figure 2-25. Estimation of Loss Rates of BVOC Components from White Pine Needles in UMBS 2011 with OH or O₃ Reaction.](image)

Lerdau et al. (1995) suggested that not all BVOC shows a proportional relationship in extractions and gas-phase. The predicted BVOC composition using Raoult’s law in Figure 2-24 is different from measured BVOC compositions in Figure 2-22 (by SPME) and 2-23 (by branch enclosure). In Figure 2-24, the estimated fraction of camphene, β-pinene, β-myrcene, limonene, β-phellandrene in gas-phase were less than the fraction of them in needle extracts. In reality, terpene compounds in needles are stored in a resin matrix so interact strongly with leaf material.
and each component has a different activity. Therefore, Raoult’s law may not be appropriate to predict BVOC emission from needle extracts.

Flores and Doskey (2015) suggest a different approach and introduced “activity coefficient”, which is calculated with Hansen solubility parameters including dispersive, polar, and H-bonding interactions of oleoresin solute. To predict emission rates of terpenes from storage terpenes in leaves using the algorithm, they used our data (Toma and Bertman, 2012) for storage terpene concentrations and Ortega’s (2008) data for emission rates of terpenes. They showed that the predicted emissions from our data using their algorithm also correlated to measured emissions by Ortega (R=0.95).

2.5. Summary

White pine (Pinus strobus) was once a dominant species across the US Midwest and is currently growing back from aspens. The forest succession results in “chemical succession on BVOC” whereby isoprene gives way to terpenes in the atmosphere. Although branch enclosure technique mostly has been employed to measure BVOC emission rate, there is still challenges for evaluating BVOC emissions in a large forest using the technique. Therefore, this study measured BVOC concentrations in extract of leaves, that could provide a benchmark of BVOC emissions from a large number of samples in a wide area with easy measurement in a short time. This allows more precise estimation of BVOC pool in the biomass population of a forest.

The large-scale screening of BVOC in white pine needle extract was conducted in the University of Michigan Biological Station (UMBS) in Northern Michigan for five years since 2008 Summer, in which BVOC level in needle extracts is relatively high. Understory trees of white pine were sampled from two types of forests; one forest is undisturbed forest (Ameriflux) as control
and the other forest is an experimentally disturbed forest (FASET) where early successional tree species were girdled in spring 2008 to make way for late successional species (including white pine). The common BVOC components (13 MT and 5 SQT) were observed in two forests. α-Pinene was the most dominant BVOC and germacrene D-4-ol was the most major SQT in white pine needles. The absolute concentrations of MT and SQT varied by year, but their fluctuating behavior was not same by year. The different fluctuating behavior in MT and SQT was observed by height difference of canopy trees. This may be due to their different response to environmental factors such as light and temperature. Although absolute concentration of each terpene component changes, the composition of each terpene by same number of carbon was less change by year. Statistical test assessed the BVOC structure from white pine needles in two forests had been same until 2010. The effect of expediting the growth of later successional trees may appear from 2011 after primary successional trees died and the BVOC concentration in FASET was dramatically higher than that in the control forest, Ameriflux in 2012 summer. Comparison of BVOC concentration of white pine needles based on the height within the canopy trees showed MT in needles at the top of the canopy trees consistently higher (30-50%) than at the bottom. The BVOC in needles at the top of the trees also higher than at the bottom. The BVOC in needles at the bottom of canopy trees have the same amount as understory trees. These observations suggest the terpene concentrations will increase as understory white pine trees grow into canopy in the process of forest succession. Hence, the changes in both area coverage and canopy structure will influence atmospheric composition in the Midwest forests.

White pine needles were collected four additional places in lower peninsula of Michigan in 2011. The same BVOC components were obtained in needles from all four forests. There was
no statistical difference on BVOC concentration in all four forests and the BVOC level was same to that in Ameriflux.

The large sample size of trees allows to show chemotypic variation among individuals within a population. 14% of white pine trees at the UMBS contains relatively high levels of a single enantiomer of limonene (up to 36% of total BVOC) in the same specific trees every year. The ratio limonene to other MT (OMT) concentrations in needles did not change as changing seasons. Assuming MT concentrations in needle extract scale with emission rate, the presence of high level of limonene trees enhances the relative contribution to OH reactivity due to reaction with MT by approximately 4% comparing to no presence of that at the current UMBS. This could be an indicator of the potential error in estimation of BVOC emission in a forest. As these young forests in the Midwest continue to transition and terpenes is replacing isoprene, individual trees variation could be a significant factor in the atmospheric chemistry.

2.6. References


Ortega, J., Helmig, D., Daly, R. W., Tanner, D. M., Guenther, A. B. and Herrick, J. D.:


CHAPTER III

SECTION 2-1: PANS IN THE SOUTHEASTERN U.S.

3.1. Introduction

3.1.1. Importance of Peroxyacetyl Nitrates on Biosphere-Atmosphere Interaction

Peroxyacetyl nitrates (PANs, RC(O)OONO\textsubscript{2}), oxidation products of VOC in the presence of nitrogen oxides (NO\textsubscript{x}, note: NO\textsubscript{x}=NO+NO\textsubscript{2}), play an important role in gas and particle atmospheric chemistry. PANs were discovered in polluted air such as the Los Angeles smog in the late 1950s (Stephens, 1987). Stephens’s group found that PANs act as the cause of “oxidant plant damage” as well as eye irritation. The pathway of PANs formation in the atmosphere is shown in Figure 3-1. PANs form from the reaction of NO\textsubscript{2} and peroxyacetyl radicals, which can be derived from OH-initiated oxidation of aldehydes. PANs are less stable than most other organic compounds in the atmosphere and thermally decompose back to peroxyacetyl radical and NO\textsubscript{2} by a first-order reaction (e.g. Bertman and Roberts, 1991; Nguyen et al., 2015). The decomposition rate depends on the concentration ratio of NO/NO\textsubscript{2} (Roberts and Bertman, 1992). Hence, PANs act as a NO\textsubscript{x} reservoir and affect the lifetime of NO\textsubscript{x} in the troposphere (Finlayson-Pitts and Pitts, 2000). PANs account for a significant amount of total reactive odd nitrogen (NO\textsubscript{y}), which is the sum of NO\textsubscript{x}, HNO\textsubscript{3}, N\textsubscript{2}O\textsubscript{5}, NO\textsubscript{3}, organic nitrates, and particle nitrate, in rural and forested areas in the Eastern United States (Trainer et al., 1993). Note: NO\textsubscript{y}-NO\textsubscript{x} is called NOz.
Figure 3-1. The Formation of PANs in the Atmosphere from OH-initiated Oxidation of Aldehyde in the Presence of NOx.

The frequently observed PAN species in ambient air are shown in Figure 3-2. Peroxyacetyl nitrate (PAN) is the simplest and most abundant of the PANs, while peroxypropionyl nitrate (PPN) and peroxymethacryloyl nitrate (MPAN) are also often observed in the field, although typically at an order of magnitude lower concentration than PAN (e.g. Nouaime et al., 1998; Roberts, 2002). PAN is formed from both anthropogenic precursors such as acetaldehyde and acetone as well as biogenic precursors like isoprene. Isoprene oxidation products, such as methylglyoxal and acetaldehyde, are the largest contributor of PAN formation and account for 37% of the global PAN budget (Fischer et al., 2014). PPN is formed from anthropogenic VOC (AVOC) such as propanal, propane, MEK, 1-butene, and larger alkanes, while MPAN is derived from a single BVOC, methacrolein (MACR), a product of isoprene oxidation(Carter and Atkinson, 1996).
Recent laboratory experiments have suggested that further oxidation reactions of MPAN with OH could be involved in the formation of SOA via epoxide intermediates (Chan et al., 2010; Lin et al., 2013; Nguyen et al., 2015; Surratt et al., 2010; Worton et al., 2013). Surratt et al. (2010) suggest that OH reaction with the double bond of MPAN leads to the formation of a hydroxynitrate that could be involved in the formation of secondary organic aerosol (SOA) under high-NOx conditions in chamber experiments. This reaction might be one of the important missing reactions for SOA formation and is currently excluded from atmospheric models. Nguyen et al. (2015) assessed three proposed mechanistic pathways to form 2-methylglyceric acid (2-MGA) from MPAN using laboratory photooxidation experiments under systematic variation of conditions. One of the pathways involves forming a 3-member lactone. They suggest 2-MGA also exists in gas phase and whether the lactone leads to gaseous 2-MGA or SOA depends on the ambient particle characteristic such as liquid water and pH, as well as atmospheric conditions (Section 3.3.8. shows the summary of these pathways). These pathways are also currently excluded from atmospheric models that include isoprene, the most abundant biogenic VOC in the world (Guenther et al., 2006).
Therefore, the contribution of MPAN photooxidation to aerosol radiative forcing may be underestimated. In addition, these hypotheses have not been examined with MPAN and particles in the field yet.

3.1.2. PANs Chemistry in the Southeastern U.S.

Much atmospheric research has been conducted in the Southeastern (SE) U.S. The region has a unique air quality as a result of geographical conditions such as warm temperature, high humidity, intense solar insolation, high natural emission of biogenic hydrocarbons, and a variety of anthropogenic pollution sources (Cowling et al., 1998). Portmann et al. (2009) found a significant inverse relationship between trends in daily temperature and average daily precipitation in the SE U.S. in summer. The temperature has not increased like other regions at the same latitude (30°-40°N). One hypothesis is that the cooler summer temperatures with high precipitation are related to the effect of a large amount of atmospheric aerosols in the SE. U.S. Goldstein and co-workers (2009) studied patterns of aerosol optical thickness (AOT) and suggested that the dominant AOT signal in the SE U.S. in summer is explained by secondary organic aerosol (SOA) formed from biogenic volatile organic compounds (BVOC) oxidation, resulting in a regional cooling during the summer. The Southern Oxidant and Aerosol Study (SOAS), part of the Southeast Atmosphere Study (SAS) in 2013, focused on biosphere-atmosphere interactions and the subsequent influences of BVOC on atmospheric oxidant chemistry and aerosol formation (https://www.eol.ucar.edu/field_projects/sas; http://soas2013.rutgers.edu).
3.1.3. Objectives

PANs were measured during the SOAS 2013 campaign to characterize the systematic behavior and levels of individual PAN species at an urban-impacted forest. Ground-based PAN compounds have been measured in the SE U.S. since the early 1990s to study tropospheric O$_3$ formation (e.g. Roberts et al., 1998). In this work, 2013 measurements were compared to measurements from a range of different sites in the SE U.S. In addition, statistical methods were developed to quantitatively assess the relative magnitude of biogenic and anthropogenic precursors to PANs formation. Finally, MPAN was compared with other nitrogen compounds in the gas phase, MACR and total isoprene hydroxynitrates (IN) and with organic nitrogen or total organic compounds in the particle phase to look for any relationship that might give insight into their influence on SOA formation. This is the first report to compare the relationship between gas-phase MPAN and organic particles from field measurements.

3.2. Experimental

3.2.1. Sampling Location

Ground-based measurements during the Southern Oxidant and Aerosol Study (SOAS) were conducted from 1 June to 15 July, 2013 at the Southeastern Aerosol Research and Characterization (SEARCH) Centreville (CTR) site, which was located in a forested area in the Talladega National Forest near Brent, Alabama, (lat: +32°54′11.81″, long: -87°14′59.79″). The major anthropogenic influence at this site comes from the cities of Tuscaloosa and Birmingham, which are located 50 km northwest and 80 km northeast, respectively.
3.2.2. Instrumentation

PANs were measured using a custom gas chromatograph (GC) equipped with a Shimadzu Mini-2 $^{63}$Ni electron capture detector (ECD) maintained at 55 °C (described by Nouaime et al., 1998). A polar column (RESTEK, Rtx-200, 15 m x 0.53 mm ID x 1 µm) was kept at 15 °C to minimize thermal decomposition of PAN compounds. UHP Helium at 8 cm$^3$ min$^{-1}$ was used as carrier gas and the detector was flushed with 3 cm$^3$ min$^{-1}$ UHP N$_2$ make-up gas. Ambient samples were drawn through a $\frac{1}{4}$” OD PFA Teflon tube from 8.2 m height above the ground at 1 SLPM (standard liters per minute) and a sub sample of this air was pulled through a 2 cm$^3$ sample loop at 50 sccm (standard cubic centimeters per minute). The sample loop was injected into the GC at 20 min intervals via a 6-port PTFE Teflon valve (Hamilton).

Figure 3-3 shows a schematic flow diagram used for sampling. In the “sample” mode, all three of the 3-way control valves (PFA Teflon solenoid valves, Fluoroware) in the box of sampling system are in the normally open position (NO – C). The intake air bypasses the charcoal trap and passes through the 6-port Teflon rotary valve to inject into the GC-ECD (injection volume 1cc) at atmospheric pressure. The normally closed position (NC – C) is used to calibrate the GC-ECD with gas samples generated from separate dilute solutions of synthetic PAN, PPN, and MPAN in dodecane or tridecane maintained at ice water temperature in diffusion cells (Williams et al., 2000). In this mode, the intake air passes through a charcoal trap to scrub all NOy compounds from the air. Calibration standards are added to the inlet in a matrix of ambient air to avoid any artifact that might arise from differences in background gas concentration between ambient samples and calibration samples. During the “calibration” mode, synthetic PAN in zero air (ZA) is mixed with the scrubbed air, which is then injected onto the GC-ECD. The quantitative amount of PAN in each synthetic compound was determined with a chemiluminescence NOx analyzer (Themo...
Environmental Instruments, Inc., Model 42S) equipped with a NO$_2$ converter with molybdenum, Mo held at 325°C. Calibration of the NOx analyzer was done against a NIST-traceable cylinder of 2 ppmv (parts per million by volume) NO in N$_2$ (SCOTT-MARRIN, INC). The theory with calculations of the NOx analyzer will be explained later. The baseline and sensitivity on the GC-ECD system were checked every day during the field campaign. Based on sensitivity and background measurements, the detection limits (S/N=2) for PAN, PPN and MPAN were estimated as 2.5 pptv, 3.6 pptv and 3.9 pptv, respectively. Uncertainty was estimated to be 20% RSD, most of which came from the chemiluminescence NOx analyzer (ECD was less than 2.5% RSD).
Figure 3-3. The Flow Diagram of PAN Injection into GC-ECD. Bold line indicates ¼” OD PFA Teflon tube and thin line does 1/8” OD PFA Teflon tube.
3.2.3. PANs Synthesis

The main procedures of PANs synthesis are described in Nielsen et al. (1982), Gaffney et al. (1984), and Bertman and Roberts (1991). In general, PANs, such as PPN and MPAN, in the liquid phase is synthesized from acyl anhydride in the presence of proton as catalyst.

\[
(RCO)_2O + 2H_2O_2 \xrightarrow{H^+} H_2O + 2RC(O)OOH
\]

\[
RC(O)OOH + HNO_3 \xrightarrow{H^+} H_2O + RC(O)OONO_2
\]

3.2.3.1. Liquid PAN (Peroxyacetyl Nitrate)

PAN is synthesized from peroxycetic acid using the following procedure:

1) First, all reagents and glassware were cooled before beginning the experiment. Note: reagents were put on ice and glassware was kept in freezer.

2) A 100 mL of round-bottom flask (RBF) with a magnetic stir rod was affixed to a stand and was put into ice water bath until half of RBF was immersed.

3) 20 mL of cold pure dodecane was added in the RBF while it was stirring for 5 minutes.

4) 3 mL of cold peroxycetic acid solution (30%, Aldrich) was added and stirred for 5 minutes.

5) 2 mL of concentrated H_2SO_4 was slowly added dropwise and allowed to flow down the side of the RBF. The solution was stirred for 5 minutes.

6) Then, 1.5 mL of concentrated HNO_3 (Aldrich) was slowly added dropwise and the solution was stirred for 15 minutes to ensure the reaction was complete before proceeding to the next step.

7) A 125 mL of separatory funnel was fixed a ring stand and held roughly 50 mL of ice water.

8) The contents of step 6 were carefully poured into the funnel.
9) The aqueous layer (bottom) is drained without shaking the funnel.

10) The organic layer was washed at least 3 times with approximately 25 mL of cold water.

11) After the aqueous layer was discarded, the organic layer was transferred from the funnel using a pipette into a small cold beaker kept on ice.

12) Anhydrous MgSO₄ powder (Fisher Scientific) was added to the organic solution to dry it.

13) The dried solution was then filtered through a plug of cotton and aliquoted into 1 mL vials.

14) The vials were kept in freezer (−20°C) until required for calibration of GC-ECD.

3.2.3.2. Liquid PPN (Peroxypropinoyl Nitrate)

PPN synthesis is initiated from propionic anhydride in two steps: a) formation of the peroxy acid from the propionic anhydride, and b) nitration of the peroxy acid.

1) First, all reagents and glassware were cooled before beginning the experiment.

2) A 100 mL of round-bottom flask (RBF) with a magnetic stir rod was affixed to a stand and was put into ice water bath until half of RBF was immersed.

3) 7.5 mL of propionic anhydride (Aldrich) was poured into the RBF and stirred for 5 minutes.

4) 0.1 mL of concentrated H₂SO₄ was slowly added dropwise and allowed to flow down on the surface of RBF inside.

5) Over the course of 5 minutes, with constant stirring, 0.75 mL of 50% H₂O₂ (Aldrich) was added dropwise and allowed to flow down the side of RBF as slowly as possible to avoid producing heat.

6) The mixture was stirred for 2 to 3 hours to dissociate the propionic anhydride. This step was finished when the ice in the bath melted.
The melted ice in the bath was replaced with fresh ice and 20 mL of cold tridecane (Aldrich) was added as organic solvent and was stirred for 5 minutes.

5 mL of concentrated H$_2$SO$_4$ was slowly added dropwise and allowed to flow down the side of RBF. The solution was stirred for 5 minutes.

2 mL of concentrated HNO$_3$ was slowly dropped down the side of RBF.

The solution was stirred for 15 minutes.

A 125 mL separatory funnel containing roughly 50 mL of ice water was affixed to a ring stand.

The contents of RBF were poured carefully into the funnel.

Steps 9) ~ 14) of the PAN synthesis were performed to purify the liquid PPN.

3.2.3.3. Liquid MPAN (Peroxymethacryloyl Nitrate)

The carbon double bond in MPAN makes it a more reactive compound than PAN or PPN. Hence, there are greater variation in product yield and purity of MPAN synthesis. In addition, since PAN is a decomposed product from MPAN, purification is another problem. This study used a method by Surratt et al. (2010) in which synthesized MPAN is consecutively washed with deionized water for purification instead of using a column.

1) All reagents and glassware were cooled before beginning the experiment.

2) A 100 mL of round-bottom flask (RBF) with a magnetic stir rod was affixed to a stand and was put into ice water bath until half of RBF was immersed. A magnetic stir bar shaped like a cross was used to powerfully mix the organic and aqueous solvents.

3) 5 mL of methacrylic anhydride (94 %, Aldrich) was added in the RBF and was stirred for 10 minutes.
4) A total of 50 µL of MeSO₃H (Aldrich) was slowly dropped to flow down on the side of RBF at six times in aliquots of 7 µL and 8 µL. MeSO₃H was used because it easily dissolved into the organic solvent and improved the reaction.

5) 0.6 mL of 50 % H₂O₂ was dropped down the side of RBF as slowly as possible to avoid producing heat. The solution became a milky color.

6) The contents were stirred for 5 hours. During that time, the ice in the bath was gradually melted to room temperature. At every one-hour interval, the RBF was taken from ice water bath and was soaked into a sonicator bath for less than 1 minute in order to ensure the solution was well mixed. Cold water was used in the sonicator bath to keep the RBF cold.

7) Ice in the bath was replaced. The solution was stirred for 10 minutes.

8) 10 mL of tridecane was added into the RBF and was stirred for 10 minutes.

9) 0.8 mL of MeSO₃H was dropped to fall down along the wall of the flask.

10) Over the course of 2 minutes, 0.8 mL of concentrated HNO₃ was slowly added dropwise.

11) The contents were stirred for 10 minutes and then soaked in the sonicator bath with cold water for less than 1 minute.

12) A 125 mL separated funnel was set up as in step 7) at PAN synthesis.

13) The solution of the RBF was carefully poured into the funnel.

14) The aqueous layer (bottom) was drained without shaking the funnel.

15) The organic layer was washed at least 16 times with approximately 25 mL cold water. MPAN synthesis accompanies producing PAN, a decomposed compound of MPAN. In order to remove PAN, it is important to sufficiently wash with water, because PAN is more water soluble than MPAN.

16) Steps 11) ~ 14) at PAN synthesis were performed to clean MPAN.
3.2.4. PANs Calibration with NOx Analyzer

3.2.4.1. Theory of Chemiluminescence NO-NO₂-NOx Analyzer

The level of gas-phase synthetic PAN standard from the diffusion cell is determined using a chemiluminescence NO-NO₂-NOx analyzer hereafter referred to as “NOx analyzer” (Model 42S, Thermo Environment). The NOx analyzer measures emitted light from the gas-phase reaction of nitric oxide (NO) and ozone (O₃). Initially, the reaction produces the excited state of nitrogen dioxide (NO₂*), which quickly decays to the stable energy state (NO₂) emitting light in the process. The light is detected with a photomultiplier tube (PMT). This process is represented by the following equation.

\[
\text{NO} + \text{O}_3 \rightarrow \text{NO}_2 + \text{O}_2 + h\nu
\]

In order to measure NO₂ or other reactive oxidized nitrogen (NOz, such as PANs) in the sample gas using a NOx analyzer, oxidized nitrogen must first be converted into NO. The Model 42S uses a molybdenum converter, which is heated to approximately 325 °C to convert oxidized nitrogen to NO. The reaction is summarized as follows:

\[
3 \text{ NO}_2 + \text{Mo} \rightarrow 3 \text{ NO} + \text{MoO}_3
\]

In this way, the resulting signal represents the sum of all reactive oxidized nitrogen (NOy). If a gas contains only NO and NO₂, then the total oxidized nitrogen is a measurement of NOx (= NO + NO₂), and NO₂ can be obtained by subtraction of NO from NOx.

Figure 3-4 is the schematic flow diagram of the way the NOx analyzer was used to calibrate the PAN GC. Zero air (ZA) is used as background gas, since room air might contain unknown concentration of NOx and NOz. The NOx analyzer draws about 1 LPM of air and since ZA must be supplied at the sample intake as overflow, the flow rate of ZA must be greater than 1 LPM. A
regulated amount of sample gas is introduced to the NOx analyzer through the Sample Intake and led to a series of mode control valves. When the valve position is in the “normally open” mode, the sample gas bypasses the NO$_2$ converter and moves to the prereactor solenoid. Then the sample is sent to the reaction chamber where it is mixed with O$_3$. Thus, the instrument reads the NO level in the sample gas. When the valve is in the “normally closed” mode, the gas sample flows through the NO$_2$ converter and all reactive oxidized nitrogen in the sample is converted to NO$_2$. 
Figure 3-4. Schematic Diagram of Chemiluminescence NO-NO₂-NOx Analyzer, Model 42S and the Related Devices.
Calibration of the NOx analyzer was performed with a NIST-traceable NO standard cylinder. Multiple standard cylinders were used for the data reported here. The information of used NO standard cylinders are summarized in Table 3-1.

<table>
<thead>
<tr>
<th>Place, Term</th>
<th>NO supply (year)</th>
<th>NO conc, ppmv</th>
<th>NO₂ conc, ppmv</th>
<th>Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>WMU, ~March 2013</td>
<td>Scott-Marrin, Inc.</td>
<td>0.784</td>
<td>0.029</td>
<td>± 2%</td>
</tr>
<tr>
<td>Purdue University, May 2013</td>
<td>Praxair (2011)</td>
<td>5.17</td>
<td></td>
<td>± 5%</td>
</tr>
<tr>
<td>SOAS 2013</td>
<td>Scott-Marrin, Inc.</td>
<td>2.001</td>
<td>&lt; 0.010</td>
<td>± 2%</td>
</tr>
<tr>
<td>WMU, Oct 2013~</td>
<td>Scott-Marrin, Inc.</td>
<td>1.999</td>
<td>&lt; 0.010</td>
<td>± 2%</td>
</tr>
</tbody>
</table>

The NOx analyzer provides the level of reactive oxidized nitrogen as a linearized voltage, so the sensitivity of the NOx analyzer is obtained as a standardized unit, i.e. concentration of NO per 1 volt.

\[
\text{Sensitivity} = \frac{\text{Flow}_{\text{NO std.}}}{\text{Flow}_{\text{Intake}}} \times \frac{\left[\text{NO}\right]_{\text{std.}}^{\text{conc.}}}{\left[\text{NO}\right]_{\text{std.}}^{\text{vol.}} - \left[\text{NO}\right]_{\text{ZA}}^{\text{vol.}}} \times \frac{1}{\text{Volt}_{\text{std.}}} \quad (\text{Eq.3-1})
\]

- \(\text{Flow}_{\text{NO std.}}\): Flow amount from NO standard cylinder
- \(\text{Flow}_{\text{Intake}}\): Flow amount of Intake
- \(\left[\text{NO}\right]_{\text{std.}}^{\text{conc.}}\): Concentration of NO standard cylinder
- \(\left[\text{NO}\right]_{\text{std.}}^{\text{vol.}}\): Volt intensity of NO standard cylinder
- \(\left[\text{NO}\right]_{\text{ZA}}^{\text{vol.}}\): Volt intensity of NO from Zero air cylinder
3.2.4.2. Converter Efficiency in Ideal Conditions

The “converter efficiency” describes what fraction of an NO\textsubscript{2} sample is converted to NO by the Mo converter and is an important factor used to assess the accuracy of the NO\textsubscript{x} analyzer. A constant fraction of the NO standard is reacted to NO\textsubscript{2} using a titration ozone generator, and the resulting NO\textsubscript{2} is fed into the Mo converter. Figure 3-3 shows (a) a flow schematic for the titration system, and (b) a graph of idealized voltage signal at different conditions using NO standard cylinder with purity of 100%. Since the system is overflow by ZA, signals for NO (solid line) and NO\textsubscript{x} (dash line) at the condition A are due to zero air supply. If the purity of the NO standard cylinder is precisely 100%, NO\textsubscript{x} consists of NO only because NO\textsubscript{2} is not contained at the condition B. When the O\textsubscript{3} lamp is turned on while NO standard is still being provided at the condition C, the NO signal decreases because O\textsubscript{3} reacts the NO to NO\textsubscript{2}. The NO\textsubscript{2} is converted via process (2) and (3) in Figure 3-5 (a). It is necessary to leave some amount of NO to calculate converter efficiency.

Where x is the titrated NO with O\textsubscript{3} lamp and y is recovered NO in NO\textsubscript{2} converter. These are calculated as follow:

\[ x = [NO]_B - [NO]_C \]  
\[ y = [NOx]_C - [NO]_C \]

(\text{Eq.3-2})

(\text{Eq.3-3})

Where, brackets indicate voltage intensity of each oxidized nitrogen at the condition of subscript in Figure 3-5 (b). Then, converter efficiency with ideal condition is obtained by:

\[ \text{Converter Efficiency} = \frac{x}{y} \times 100\% \]  
\[ (\text{Eq.3-4}) \]

If all of the titrated NO is recovered in the NO\textsubscript{2} converter, x = y and NO\textsubscript{x} level at the condition C should to be same with that at the condition B.
3.2.4.3. Converter Efficiency in the Practical Conditions

In practice, the system does not behave in the idealized fashion described above. A description of real laboratory voltages under the different conditions is shown in Figure 3-6. Solid and dash lines are voltage of NO and NOy respectively. (In case of contamination by NOz in the actual condition, NOy is used instead of NOx in the ideal condition with NO standard cylinder.) In fact, if the NO standard cylinder contains a little NO2, then NOy at the condition B does not consist of NO only. In addition, since it is difficult to completely recover titrated NO in the NO2 converter with Mo, the NOy level at the condition C is not same with that at the condition B,

Figure 3-5. The Ideal Theory to Calibrate NOx Analyzer with 100 % Purified NO Standard Cylinder. (a) Schematic conversion process of NO and NO2 from NO standard cylinder. (b) Volts intensity at different conditions. Signal with solid line is NO and signal with dash line is NOx. Zero air is supplied with overflow.
$[\text{NO}_x]_B > [\text{NO}_x]_C$. The dropping voltage of NOy at the condition C (length of $z'$ in Figure 3-6) is related to incomplete conversion of NO$_2$ in the NO$_2$ converter. The NO$_2$ is the sum of original NO$_2$ in the cylinder and product of NO titration by O$_3$ lamp locating upstream of the converter. Since the signal of synthetic PAN is obtained as NOy as in condition D in Figure 3-6, it is important to consider the presence of extra NO$_2$. Therefore, the accurate converter efficiency at the actual conditions is calculated from the volt intensity of “net NOy with NO standard cylinder” (condition B) and “net NOy with the titration” (condition C). First, the percent amount of NO titrated is calculated by

$$\text{titrated } NO = \frac{[\text{NO}]_B - [\text{NO}]_C}{[\text{NO}]_B - [\text{NO}]_A} \times 100\% \quad (\text{Eq.3-5})$$

Brackets indicate voltage of each oxidized nitrogen at the condition of subscript in Figure 3-6. The voltage of net NOy with NO standard cylinder and net NOy with the titration are the results of subtracting the voltage of inactive NOy, that is not related to titration process. These are calculated as following,

$$Untitrated [\text{NOy}] = ([\text{NOy}]_B - [\text{NOy}]_A) \times (1 - \text{titrated } NO) \quad (\text{Eq.3-6})$$

$$\textit{net} [\text{NOy}]_{\text{NOstd.}} = ([\text{NOy}]_B - [\text{NOy}]_A) - \text{untitrated} [\text{NOy}] \quad (\text{Eq.3-7})$$

$$\textit{net} [\text{NOy}]_{\text{titrated}} = ([\text{NOy}]_C - [\text{NOy}]_A) - \text{untitrated} [\text{NOy}] \quad (\text{Eq.3-8})$$

$\text{titrated } NO$ : Percent amount of NO titrated

$\text{Untitrated } [\text{NOy}]$ : Volts of untitrated NOy

$\textit{net} [\text{NOy}]_{\text{NOstd.}}$ : Volts of net NOy with NO standard cylinder

$\textit{net} [\text{NOy}]_{\text{titrated}}$ : Volt of net NOy with the titration

Therefore, actual converter efficiency is obtained by

$$\text{Actual Converter Efficiency} = \frac{\text{net} [\text{NOy}]_{\text{titrated}}}{\text{net} [\text{NOy}]_{\text{NOstd.}}} \times 100\% \quad (\text{Eq.3-9})$$
When the tubing from a diffusion cell containing synthetic PAN is installed into the NOx analyzer under overflow of zero air, the signal of PAN is obtained as NOy (the length of (a) in Figure 3-6) and there is no signal of NO. In order to determine the concentration of synthesized PAN, $\text{Conc.} \text{PAN}$, the sensitivity of the analyzer needs to be modified with the actual converter efficiency, $\text{Actual Conv.Eff.}$, with the formula,

$$\text{Conc.} \text{PAN} = ([\text{NOy}]_D - [\text{NOy}]_A) \times \text{Sensitivity} \times (2 - \text{Actual Conv.Eff.}) \quad (\text{Eq.3-10})$$

The error in the synthetic PAN concentration was calculated with formula (Eq.3-1) ~ (Eq.3-10) with error propagation. Overall accuracy is conservatively estimated to be $\pm 20\%$ average.

![Diagram](image)

**Condition A**
- Zero air

**Condition B**
- Zero air
- + NO std.

**Condition C**
- Zero air
- + NO std.
- + PAN

**Condition D**
- Zero air

Figure 3-6. Practical Voltages of NOx Analyzer Under the Different Conditions. Solid line is NO and dash line is NOy.
3.2.5. Software

Software used to operate the GC-ECD, analyze data, and assess statistical relationships are listed in Table 3-2.

Table 3-2. Summary of Used Software and Purpose.

<table>
<thead>
<tr>
<th>Software</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAQFactory (Ver. 5.80, AzeoTech, Inc)</td>
<td>• ON/OFF for mode control valves</td>
</tr>
<tr>
<td></td>
<td>• Monitoring of MFC volts</td>
</tr>
<tr>
<td></td>
<td>• Controlling GC-ECD program for sampling</td>
</tr>
<tr>
<td></td>
<td>• Monitoring of NO and NOy using chemiluminescence NO-NO₂-NOₓ analyzer</td>
</tr>
<tr>
<td>PeakSimple (32 bit, Ver. 3.29, SRI Instruments)</td>
<td>• Integrating peaks at GC-ECD</td>
</tr>
<tr>
<td>Igor Pro (Ver. 6.3, WaveMetrics)</td>
<td>• Plotting data results</td>
</tr>
<tr>
<td>SPSS (Ver. 16, IBM)</td>
<td>• Analyzing data with statistical tests</td>
</tr>
<tr>
<td>R (Ver. 3.1.0 GUI 1.64, The R Foundation)</td>
<td>• Analyzing data with statistical tests</td>
</tr>
</tbody>
</table>

3.2.6. Reference PANs data in 1999

Measurements of PANs using the same methods to those described in this work were made in Dickson, TN from 15 June to 14 July, 1999 as part of the Southern Oxidants Study (SOS) (Cowling et al., 1998) and are referred to in the text. The site was located near Montgomery Bell State Park about 60 km west-southwest (upwind) of downtown Nashville (Chen, 2001). While Dickson is in a different part of the southeast, the distance from a major urban area makes this site a good comparison with the 2013 SOAS data.
3.2.7. Data from Other Research Groups Used in This Analysis

Several datasets from SOAS collaborators were needed for the analysis presented in this work. Measurements of NOy, NOx, and O3, wind direction and temperature, solar intensity, and boundary layer height were made by Atmospheric Research & Analysis, Inc. (ARA) as described by Hidy et al., (Hidy et al., 2014). Total isoprene hydroxynitrate (IN) concentrations were measured by Purdue University using a chemical ionization mass spectrometer (CIMS) with operating conditions described by Xiong et al. (2015). MACR was measured by de Gouw and Goldstein groups (NOAA/CIRES/UC Berkeley) using a GC-MS. Data on total organic alkyl nitrates in the particle phase (apANs) was available from UC-Berkeley using a thermal dissociation method described by Day (2002). Total organic mass in particles was quantified by University of Colorado (Jimenez group) with a High Resolution Time of Flight Aerosol Mass Spectrometer (HR-ToF-AMS). Hydroxyacetone (HAC) in the gas phase was obtained from California Institute of Technology (Wennberg group) using a Chemical Ionization Time of Flight Mass Spectrometer (ToF-CIMS).

3.3. Results and Discussion

3.3.1. Post-verification of Experiments in SOAS 2013

3.3.1.1. Sensitivity of GC-ECD with Flow Rates of He and N₂

The ECD was run under non-standard conditions to enhance sensitivity for the thermally labile PAN compounds. Typically, ECD detectors are run at elevated temperature (~250°C) with large makeup flow. In this work, the ECD was run at 55°C with minimum makeup flow. As a result, the sensitivity of the instrument responded to small changes in flow rates. Experiments to
field-test the PAN system and analyze products from smog chamber runs of BVOC chemistry were conducted at WMU and Purdue University before attending SOAS 2013 campaign in AL. The calibration slope for PAN in SOAS 2013 varied from what was measured at WMU in April 2013 and Purdue University in May 2013 (Table 3-3). The GC-ECD responds to gas flow rates and to the ratio of carrier: make-up. It is important to improve the sensitivity of ECD to detect PANs at levels near/below detection limit like PPN and MPAN in SOAS 2013. Hence, the change in sensitivity of the GC-ECD was investigated with various flow rates of gases to determine the optimum. (Note: the calibration curves were generated for each place. Therefore, the estimated concentrations of PANs at each place are reliable, although the sensitivities of PANs from each place were different on ECD.)

Table 3-3. Average RT and Calibration Fitting Slope of PAN at WMU, Purdue, and SOAS 2013. The equation of calibration to estimate the concentration of PAN is Area = Slope*Concentration (ppt).

<table>
<thead>
<tr>
<th>Place</th>
<th>Average of total ECD flow, mL min⁻¹</th>
<th>Average of RT of PAN, min</th>
<th>Calibration Fitting Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>WMU in 2013 April</td>
<td>11.1±0.3</td>
<td>5.05±0.03</td>
<td>1.75 ± 0.11 (R²=0.855)</td>
</tr>
<tr>
<td>Purdue</td>
<td>11.6±0.1</td>
<td>5.15±0.09</td>
<td>1.91 ± 0.04 (R²=0.975)</td>
</tr>
<tr>
<td>SOAS 2013 in AL</td>
<td>11.6±0.2*</td>
<td>5.16±0.12</td>
<td>0.70 ± 0.01 (R²=0.994)</td>
</tr>
</tbody>
</table>

Average of ECD flow and average of RT of PAN are shown with the standard deviation. Calibration fitting slope is done with the standard error. * indicates the record of flow rates was measured until June 17th due to damage on the flow meter.
Figure 3-7 shows the relationship of PAN area at GC-ECD (a) with make-up gas flow N₂ and (b) with flow rate ratio of He/N₂. As the flow rate of N₂ increased, PAN area decreased. However, increment of PAN area was strongly associated with increment of flow ratio of He/N₂. Their R² was 0.937. This suggested that the sensitivity of GC-ECD decreases with increment of flow rate of N₂ while it increases with increment of flow ratio of He/N₂.

![Figure 3-7](image_url)

Figure 3-7. The Relationship Between ECD Area of PAN and (a) Flow Rate of N₂, and (b) Flow Ratio of He/N₂. The bar indicates the standard deviation of ECD area of PAN by three times repeated measurements.
The slope of the regression line of GC-ECD peak area on concentration of standards indicates the sensitivity of the GC-ECD to the compound. Figure 3-7 suggests that the different sensitivities of PAN on GC-ECD from various places in Table 3-3 are related to the ratios of flow rates. This assumption was investigated from the slopes of calibration curves for PAN under various ratios of He/N₂ flow rates. The relationship between the slope of calibration curve and the ratio of flow rates are shown in Figure 3-8 and a linear regression line was obtained as

\[ \text{Slope} = 0.941 \times \text{He/N₂} - 0.689 \quad (Eq. \ 3-11). \]

High \( R² \), which was 0.877, indicates that the slope of calibration curve of PAN (sensitivity) should be predictable from the flow ratio of He/N₂ using the equation 3-11. This hypothesis was tested by comparing in an expected slope of calibration curve and the experimental slope at the flow ratio. If a required sensitivity (calibration slope of PAN) is “1.00”, the flow ratio should be 1.795 according to Equation 3-11 (this data is plotted in Figure 3-8). The average of actual total flow during the SOAS 2013 (11.6 mL min\(^{-1}\)) was used. During the campaign, actual total flow rate was measured occasionally while running GC-ECD with field PANs, but each flow rate, He and N₂, was not, because that needed to hold up measuring field sample. Hence, the flow rates of He and N₂ were obtained by the formula described below (first the flow rate of N₂ was obtained, and then the flow rate of He was calculated) and resulted in 7.45 mL min\(^{-1}\) and 4.15 mL min\(^{-1}\) respectively.

\[ \frac{He}{N₂} = \frac{11.6 - N₂}{N₂} = 1.795 \]

Synthetic PAN in a diffusion cell was injected onto the GC-ECD under calculated flow rates (actual flow rates of He and N₂ were 7.45 mL min\(^{-1}\), and 4.15 mL min\(^{-1}\) respectively). the experimental slope of calibration curve was 0.896 (Figure 3-8), that is 9.4% off from the regression line (Slope=1). A similar experiment was attempted with different synthetic PAN at different flow
ratios. The flow rates of He, and N<sub>2</sub> were adjusted to 9.00 mL min<sup>-1</sup> and 3.00 mL min<sup>-1</sup>, respectively (i.e., the ratio He/N<sub>2</sub> was 3.0). The expected slope using Equation 3-11 was 2.134, while the observed slope was 2.511, which is 15.1% off from the regression line (Figure 3-8). Therefore, the slope of the calibration curve is related to the flow rate ratio He/N<sub>2</sub> and it is predictable using the regression line between the slope and flow rate ratio with 10-15% error. The magnitude of error might be similar to that of uncertainty of the overall GC-ECD system including NOx analyzer, which was around 20%.

Although total flows were similar, the average of the calibration slope of PAN at SOAS was less than 1, while that in Purdue and WMU were two times larger than that in SOAS. This indicates the flow rate ratio He/N<sub>2</sub> might have been less than 1.795 mL min<sup>-1</sup>, or the flow rate of He might be smaller than 7.45 mL min<sup>-1</sup> and N<sub>2</sub> might be larger than 4.15 mL min<sup>-1</sup>. Other possible reasons could be caused by a shift of flow rate ratio after transferring to Alabama. In addition, it was necessary to adjust the flow rates of carrier and make-up gases up to at least two decimal places of mL min<sup>-1</sup> due to higher sensitivity of ECD.
3.3.1.2. GC Retention Time and Room Temperature

During the SOAS 2013 campaign, the retention time (RT) of PANs was non-constant on the chromatograms. Diurnal behavior of average RT of PAN is shown in Figure 3-9 with 1 hour intervals. The average RT of PAN was shortest and unstable during the daytime from 9 am to 8 pm, while it stayed approximately 5.2 min during the rest of the day. The instrumental system was housed in a trailer (our trailer was #9) in the SOAS 2013 campaign and other research groups sharing the trailer also observed noise on their instruments during the daytime, especially mass spectrometers. The air conditioning and vent system did not function well during the daytime in the heat of the Alabama summer. Diurnal behavior of average interior temperature, which was obtained from a thermometer equipped to ECD system, in the trailer #9 is plotted in Figure 3-10.
Temperature increased during the daytime and matched with the period in which RT of PAN also changed. The highest interior temperature was almost simultaneous with the shortest RT of PAN at around 5 pm. The highest interior temperature might affect the temperature of the GC column and shortened the RT of PAN during the daytime. The column is actively cooled with thermoelectrics that had trouble when the room got too hot. (Since the temperature of the column was not recorded by the data logger, this assumption cannot be confirmed using data in SOAS 2013.)

Figure 3-9. Diurnal Plot of Retention Time (RT) of PAN with All Data in SOAS 2013 in Alabama. Average of interval time is 1 hour.
3.3.1.3. Defining Daytime for Comparison

Hydroxyl radical (OH) plays a key role in fate of PANs. Tropospheric OH is produced by photolysis of various species in the atmosphere. The major pathway to create OH is photolysis of i) O$_3$ and vapor water, ii) gaseous nitrous acid (HONO) and iii) H$_2$O$_2$ (Finlayson-Pitts and Pitts, 2000).

i)\[ O_3 + h\nu (\lambda \leq 336 \text{ nm}) \rightarrow O(^1D) + O_2 \]
\[ O(^1D) + H_2O \rightarrow 2OH \]

ii)\[ HONO + h\nu (\lambda < 400 \text{ nm}) \rightarrow OH + NO \]

iii)\[ H_2O_2 + h\nu (\lambda < 370 \text{ nm}) \rightarrow 2OH \]
Therefore, photochemical reactions, like those that produce PANs, are most active during the daytime. PANs can also be produced at night, however the nitrate radical (NO$_3$) instead of OH is the dominant oxidant and the amount is smaller than during the daytime. In the SOAS 2013 campaign, solar radiation (W m$^{-2}$) was measured by the ARA group using a LI-200SA Pyranometer (LI-COR) and the diurnal behavior is shown in Figure 3-11. Light was detected from 6 am to 8 pm and the highest level was reached around noon.

The planetary boundary layer (PBL) is the lowest part of the atmosphere (~100 to 3000 m in altitude) and is directly influenced by the earth’s surface (Finlayson-Pitts and Pitts, 2000; Stull, 1988). PBL growth during the day is another important factor that affects PANs concentration. In general, air is mixed well and a convective layer is formed above the surface layer in midday. After sunset, a radiation inversion is caused by rapid cooling of the earth’s surface and results in formation of a stable nocturnal BL, in which air does not mix rapidly due to greater air density. Above this layer is called a residual and this traps compounds that are mixed well in daytime or are transported from different area. As the sun rises, heating of the ground results in mixing of the nocturnal BL and the residual layer. Since PANs are conserved in the residual layer during the night at cool temperatures, the process of mixing layers during the daytime has a significant impact on PANs concentration. The diurnal behavior of the BL height measured by ARA in Figure 3-12 shows that the BL in SOAS 2013 started growing quickly at approximately 9 am, reached ~1000 m around noon and then gradually increased till 5pm.

In this study, the daytime was defined as 10 am to 4 pm local time (CDT) as the most photochemically intense period with a well-developed boundary layer.
Figure 3-11. Diurnal Behavior of Solar Radiation During SOAS 2013. (This data is available from ARA.)

Figure 3-12. Diurnal Behavior of Boundary Layer Height in SOAS 2013. ARA measured using a ceilometer, photon counting of back-scattered pulse of near-IR light (1064 nm) via LIDAR.
3.3.2. General Behavior of PANs in 2013

3.3.2.1. Time Series of PANs During Entire Campaign

During the SOAS 2013 campaign, PAN, PPN, and MPAN were regularly observed on the GC-ECD. Their average retention times on the chromatograms were 5.16, 10.88, and 17.46 minutes respectively. Unknown peaks were occasionally detected at 8.74 and 15.67 minutes. The peak at 8.74 minutes was confirmed as peroxyacryloyl nitrate (APAN) and the approach for identification of this compound will be discussed in the next chapter.

Figure 3-13 shows a time series of PAN, PPN, and MPAN throughout the campaign. Data that were below detection limit (BDL) are plotted at half of the reported detection limit for that compound. This was done to distinguish BDL from missing data due to tests, calibrations, and the periodic existence of a noise interference that often appeared during this campaign and could not be eliminated. Relatively high levels of PAN were observed as periodic spikes during the campaign, but overall PAN levels were lower than most other measurements in the southeast made over the last 20 years. A local biomass burning event was observed on June 4\(^{th}\), which resulted in an unusually high level of PAN of around 1600 pptv.

General descriptive statistics for all daytime data are summarized in Table 3-4. (The data on June 4\(^{th}\) was removed from these statistical analyses.) PAN was consistently the most abundant peroxyacryl nitrate compound and the mean concentration during daytime was 34 and 19 times larger than that for PPN and MPAN, respectively. In Table 3-4. “PANs” describes the sum of individual PAN, PPN, and MPAN values. NO\(_y\) is the sum of reactive oxidized nitrate compounds in the troposphere including organic (like PANs) and inorganic compounds. Diurnal behavior of the ratio of [PANs]/[NO\(_y\)] is plotted in Figure 3-14, which shows the ratio in daytime is higher
than that at night. The median of the ratio of \([\text{PANs}] / [\text{NOy}]\) during daytime was 0.14, which suggests that PANs ordinarily accounted for about 14 % of NOy during the daytime at SOAS 2013. Parrish et al. (1993) reported the median range of \([\text{PANs}] / [\text{NOy}]\) from four different sites in eastern North America was 15-25%. Roberts et al. (2002) reported the average ratio in Nashville, TN in 1999 was 10 % with overall data, 15 % at midday, and 3 % at night. The average of absolute PAN concentration was 674 pptv in Nashville 1999. Although PANs concentration of SOAS 2013 was five times less than that of Nashville 1999, the proportion of PANs to NOy was similar.

Peroxyacryloyl nitrate (APAN) was also observed occasionally during the campaign (average 3.9±5.6 pptv during daytime). Although APAN has been reported to arise from other anthropogenic sources (Roberts et al., 2001; Tanimoto and Akimoto, 2001), it did not show a significant relationship with PPN in this work. The coefficient of determination, \(R^2\), in Spearman’s rank correlation test was 0.068 (\(p=0.904\)).

Note: during the SOAS 2013 campaign, two other research groups measured the sum of total PANs without identification of each species. The comparison of total PANs measurements from these two groups with the measurements from WMU are described in Appendix D.
Figure 3-13. Time Series of Detected Peroxyacyl Nitrates During SOAS 2013 Campaign Using GC-ECD.
Table 3-4. General Descriptive Statistics of PANs and Other Trace Gases During SOAS 2013 Campaign. (Data on June 4th is not included.)

<table>
<thead>
<tr>
<th></th>
<th>All day</th>
<th>Daytime (10am–4pm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN</td>
<td>Number of data</td>
<td>2813</td>
</tr>
<tr>
<td></td>
<td>Mean ± STD (ppt)</td>
<td>126 ± 110</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>99</td>
</tr>
<tr>
<td>PPN</td>
<td>Number of data</td>
<td>2402</td>
</tr>
<tr>
<td></td>
<td>Mean ± STD (ppt)</td>
<td>4 ± 5</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>2</td>
</tr>
<tr>
<td>MPAN</td>
<td>Number of data</td>
<td>2346</td>
</tr>
<tr>
<td></td>
<td>Mean ± STD (ppt)</td>
<td>5 ± 7</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>2</td>
</tr>
<tr>
<td>O3</td>
<td>Mean ± STD (ppb)</td>
<td>26 ± 13</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>25</td>
</tr>
<tr>
<td>NOx</td>
<td>Mean ± STD (ppb)</td>
<td>0.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.4</td>
</tr>
<tr>
<td>PANs/NOy</td>
<td>Mean ± STD</td>
<td>0.11 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.10</td>
</tr>
</tbody>
</table>

STD means standard deviation.
PPN and MPAN include data of below detection limit, 1.8 and 1.9 pptv respectively.
PANs = PAN + PPN + MPAN

![Figure 3-14. Diurnal Plot of the Ratio of [PANs]/[NOy] in SOAS 2013.](image-url)
3.3.2.2. Relationship with Wind Direction

Polar plots of PAN compounds are shown in Figure 3-15 as a function of wind direction in SOAS 2013. The bold trace line in each plot indicates concentration of a PAN compound averaged over 20 degrees of wind direction, and the solid lines from the center are the frequency of wind direction over the entire measurement period. Although surface air most frequently came from the south during the SOAS 2013 campaign, air from the north contained levels of PANs that were twice as large as from south. The averages of PAN, PPN, and MPAN with air from north were 181.9, 5.3, and 8.4 pptv respectively, while averages of air from south showed 94.6, 2.8, and 3.6 pptv. This northern distribution is also seen with NOx and O$_3$ reflecting the influence of anthropogenic pollution sources from Birmingham and Tuscaloosa. These observations indicate that PANs production during SOAS 2013 was NOx sensitive and controlled by NOx concentrations. (This topic will be discussed more in Section 3.3.4.) Other trace gases, NO, NO$_2$, NOy and O$_3$ are plotted with wind direction in Figure 3-16.
Figure 3-15. Polar Plots of PANs Concentrations as a Function of Wind Direction in SOAS 2013.

Figure 3-16. Polar Plots of NO, NO₂, NOy and O₃ Concentrations as a Function of Wind Direction in SOAS 2013. (Measurements were performed by ARA.)
3.3.2.3. Diurnal Behavior of PANs and Influence of Air Temperature

Diurnal plots of mean and median values of PANs as a function of time of day throughout the measurement period filtered by surface wind direction (Figure 3-17) indicate a pattern in PAN, PPN, and MPAN from the north and a much weaker pattern in southerly air. These observations also indicated the influence of NOx sensitivity in the air from the north like Section 3.3.2.2. All three PANs levels were highest with wide range of the standard deviations during the daytime on average, when OH chemistry was active and the air was mixed well in the boundary layer. The mean and median diurnal cycles of PAN and MPAN in air from the North showed a pattern even though the standard deviations were greater, while that for PPN was less clear because of its low concentration over the campaign. The levels of both PAN and MPAN from the North started increasing around 7-8 am and reached the highest levels around 10-11 am then decreased by about 30% until around 2 pm. Frequently during the campaign there was a higher level in the late afternoon/early evening.
Figure 3-18. Diurnal Behaviors of PAN, PPN, and MPAN During SOAS 2013 in AL with Wind from South and North.
This diurnal pattern of PAN was generally similar to those reported at Youth Inc. in Nashville in 1995 (Nouaime et al., 1998), at Cornelia Fort Air Park in Nashville 1999 (Nouaime et al., 1998; Roberts, 2002), and at TexAQS 2000 in Houston (Roberts et al., 2001), although the range of absolute concentrations was quite different. The observation that PAN levels dropped slightly in the afternoon was seen in these other sites also. PANs are very sensitive to temperature and decompose readily at higher temperatures. Accordingly, the influence of air temperature was investigated on the lifetime of PAN under the conditions of SOAS 2013. The following scheme shows the process of PAN loss.

PAN, formed by the reaction of peroxyacetyl (PA) radicals and NO\(_2\) at the rate \(k_{-1}\), is thermally decomposed to PA at the rate \(k_1\). On the other side, PA reacts with NO to be other products at the rate \(k_2\). Equations, 3-12 and 3-13, are applied for each process.

\[
\frac{d[PAN]}{dt} = -k_1 [PAN] + k_{-1} [PA] [NO_2] \quad (Eq. 3-12)
\]

\[
\frac{d[PA]}{dt} = k_1 [PAN] - k_{-1} [PA] [NO_2] - k_2 [PA] [NO] \quad (Eq. 3-13)
\]

The \(k_1\), \(k_{-1}\), and \(k_2\) were calculated using the Arrhenius equation, \(k(T) = A \cdot \exp(-Ea/RT)\). The values of \(A\), Arrhenius factor and \(Ea\), activation energy were obtained from Atkinson et al. (2006) as

\(k_1 = 5.4 \times 10^{16} \exp(-13830/T)\)
\[ k_1 = 1.2 \times 10^{11} \left( \frac{T}{300} \right)^{-0.9} \]

\[ k_2 = 7.5 \times 10^{12} \exp\left( \frac{290}{T} \right) \]

where, \( T \) is thermodynamic temperature (K).

When \([PA]\) is in steady state, \(d[PA]/dt = 0\). Then, the PAN loss rate is calculated as the equation 3-14 by substituting 3-13 into 3-12.

\[
- \frac{d[PAN]}{dt} = k_1[PAN]\left(1 - \frac{k_1}{k_1 - k_2[NO]/[NO_2]}\right) \quad (Eq. 3-14)
\]

For convenience, the contribution to PAN loss rate, \(\mu k_{PAN}\) is defined as

\[
\mu k_{PAN} = k_1(1 - \frac{k_1}{k_1 - k_2[NO]/[NO_2]}) \quad (Eq. 3-15)
\]

This means equation 3-14 is

\[
- \frac{d[PAN]}{dt} = \mu k_{PAN} [PAN] \quad (Eq. 3-16)
\]

The diurnal behavior of average of air temperature in SOAS 2013 is shown in Figure 3-18. The average air temperature reached the highest level at around 3 pm, at which time PAN levels dropped slightly. However, Equation 3-13 demonstrates the loss rate of PAN is influenced by the ratio of \([NO]/[NO_2]\) as well as air temperature. Since it was difficult to obtain true PAN concentrations in the field devoid of thermal decomposition, the contribution to PAN loss rate, \(\mu k_{PAN}\) in SOAS 2013 was evaluated in Figure 3-19. The \(\mu k_{PAN}\) increased from 5 am to 8 am and held relatively stable until 4 pm. Therefore, air temperature had little effect on the slight drop of PAN level in afternoon.
Figure 3-18. Diurnal Plot of Air Temperature in SOAS 2013.

Figure 3-19. Diurnal Pattern of Contribution to PAN Loss Rate in the Air from North in SOAS 2013.
Boundary layer (BL) volume is another factor that can change PAN concentration. As the BL develops and mixes with the residual layer in the early morning, the PAN level increases. On the other hand, expansion of the BL volume during the daytime as a result of surface warming results in dilution of PAN concentration. Figure 3-20 estimates the dilution as BL height increases during SOAS 2013. For this assessment, a constant PAN concentration of 100 pptv was used for the calculation. Based on a comparison of the air volumes between minimum and maximum height of BL, there is a possibility that PAN is diluted by at the most a factor of 5.

Therefore, despite typical temperature increases during the afternoon, because of variation of [NO]/[NO₂], there is little change in the PAN decomposition rate constant, which suggests that PAN levels fluctuate during early afternoon mostly due to boundary layer growth. Overall, the diurnal patterns of PAN and MPAN in SOAS 2013 could result from the influence by both photooxidation activity and by dynamics of the planetary boundary layer.

![Figure 3-20. Estimated Dilution Effect on Assumed PAN Concentration by Developing of Boundary Layer in the Daytime.](image-url)
3.3.2.4. Correlation of PANs with Ozone

Both PANs and O$_3$ are produced from the reactions between VOC and NOx. Therefore, it is expected that PANs have a strong correlation with O$_3$ in polluted areas (e.g. Roberts, 2002; Trainer et al., 1993). In SOAS 2013, the diurnal behavior of average O$_3$ (Figure 3-21) shows higher levels in the air from the north than that from the south, similar to the behavior of PAN (Figure 3-17). The O$_3$ concentration in the air from the north started increasing around 7am, reached a peak around 6pm, and slowly decreased overnight, while PAN showed maximum concentration around noon. Roberts (2007) suggests O$_3$ has longer lifetime than PANs in the warm summertime surface layer.

Figure 3-21. Diurnal Plot of O$_3$ with Wind from South and North in SOAS 2013.
A Spearman’s rank correlation test, which is a non-parametric test because data did not have a normal distribution, is shown for PAN and O₃ for 9am to 7pm in Table 3-5. The significant values were less than 0.01 for both north and south. The correlation between PAN and O₃ from the north was weaker than from the south. When PAN concentration was less than approximately 400 pptv, O₃ increased with increasing PAN concentration, while, when the PAN concentration was higher than 400 pptv, which was occasionally observed in the air from north, the O₃ plateaued with increasing PAN (Figure 3-22). Trainer et al. (1993) reported this systematic behavior for the relationship between O₃ and NOₓ at higher NOₓ levels from six sites in the eastern US and Canada and suggested that O₃ formation depends on the ratio hydrocarbon/NOₓ. Therefore, the relative low correlation in O₃ and PAN from the north air might result from this limiting O₃ formation at higher PAN.

Table 3-5. Spearman’s Rank Correlation Test Between O₃ and PAN for 9 am – 7 pm Based on the Wind Directions in SOAS 2013.

<table>
<thead>
<tr>
<th></th>
<th>North</th>
<th>South</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN</td>
<td>rho</td>
<td>Sig. (2-tailed)</td>
</tr>
<tr>
<td></td>
<td>0.443</td>
<td>8.413e⁻¹²</td>
</tr>
<tr>
<td></td>
<td>0.673</td>
<td>&lt; 2.2e⁻¹⁶</td>
</tr>
</tbody>
</table>

Rho means Spearman's rank correlation coefficient. Sig. (2-tailed)" means the two-tailed p value.
3.3.3. Historical PANs with Other Trace Gases in the Southeastern U.S.

PAN compounds have been measured at various rural and urban locations within the SE U.S. over the last 20 years. Seven sites are shown on the map in Figure 3-23 to compare with SOAS 2013. Statistical distributions of daytime PAN concentrations from SOAS 2013 and the additional seven sites are plotted in Figure 3-24. (Note: original data of PANs and other trace gases from Elberton, ROSE, Henderson, and Cornelia Fort Ground Site were obtained through a personal communication with Dr. James Roberts from National Oceanic and Atmospheric Administration.) Urban areas show relatively high levels and a wide distribution. Results from rural areas, where there is an expected dominance of biogenic influence, varied depending on the place and the year. Some distributions were similar to urban areas, while others showed a remarkably narrow range with low concentration. Different levels of PAN were observed in 1990 and 1992 at the same
sampling site, ROSE. PAN at SOAS 2013 was the lowest of all other previous observations in the SE U.S. Ozone was also lowest of these studies (Figure 3-25). Although PAN levels were relatively low in Elberton 1990 and ROSE 1992, ozone from the sites showed levels similar to those from urban areas. The distribution of NOx, a reactant of PAN, shown in Figure 3-26, may be reflective of PAN levels. The urban areas showed high levels of NOx, while the rural areas, except Dickson 1999, showed low levels of NOx.

Figure 3-23. Various Locations of Other Measurements in the Southeastern U.S. since 1990. Sampling term: Elberton (June 24th – July 13 in 1990), ROSE 1990 (June 10th – July 20th in 1990), ROSE 1992 (June 19th – July 2nd in 1992), Henderson (June 22nd – July 19th in 1994), Youth Inc. (June 29th – July 26th in 1995), Dickson (June 13th – July 15th in 1999), Cornelia Fort Ground Site (June 14th – July 14th in 1999), and SOAS 2013 (June 1st – July 15th in 2013). (Data map: Google map, 2016)
Figure 3-24. Distribution of Daytime PAN Concentration at Various Locations in the Southeastern U.S. (Nouaime et al., 1998; Roberts, 2002; Roberts et al., 1998). Whisker Bottom: 10th%, Box bottom: 25th%, Mid-line of Box: median, Box Top: 75th%, Whisker Top: 90th%. The red dot indicates the mean. Box with green filled indicates PAN from rural area and orange filled box is from urban area.

Figure 3-25. Distribution of Daytime O₃ Concentration at Various Locations in the Southeastern U.S.
While the sensitivity of PAN production from NOx and VOC reaction has been less reported, the relationship of O₃ production with the reactants has been well investigated and displays a highly nonlinear relationship under many conditions (Finlayson-Pitts and Pitts, 2000; Milford et al., 1994). In Figure 3-27, the relationship is expressed by a two-dimensional depiction in which primary emission components, VOC (ppbC) and NOx (ppb), are plotted in x and y axes, and midday rates of O₃ production (ppb h⁻¹) are demonstrated with isopleths. When the VOC/NOx ratio is high (i.e., high VOC and low NOx), O₃ concentration is effectively controlled by NOx concentration, and this regime is called “NOx-limited”. HO₂, which is derived from VOC, reacts with NO and produces OH radicals and NO₂. Hence, an increase in OH results in an increase of O₃ concentration. When the VOC/NOx ratio is low, (i.e., low VOC and high NOx), O₃ concentration is sensitive to VOC concentration but not to NOx concentration. This regime is
called “VOC-limited”. NO₂ competes with VOC for reaction with OH radicals and produces HNO₃ without forming O₃. Typically, rural areas are NOₓ-limited while urban and suburban areas are VOC-limited (Chameides et al., 1992; Finlayson-Pitts and Pitts, 2000).

Figure 3-27. Sensitivity of O₃ Production in the Reaction Between NOₓ and VOC. Isopleths indicate midday rates of O₃ production (ppb h⁻¹) calculated using a box model. OH-reactivity is normalized to the reactivity and concentration of propylene. Observed data from four different types of locations are included. (adapted from Finlayson-Pitts and Pitts, 2000)

Figure 3-28 shows the relationship between O₃ concentrations and NOₓ concentrations at various locations in the SE U.S. over 20 years. The trend line with a dashed line in the figure indicates that O₃ in several places had similar concentration ranges, approximately 50 – 70 ppbv, even though average NOₓ concentrations were varied. The VOC/NOₓ ratios might be located in same level of ozone isopleth. Only SOAS 2013 showed a different level of O₃, 30 ppbv, although
the low NOx level was similar to Elberton 1990 and ROSE 1992 (see Figure 3-26). This is expected since the concentrations of VOC in Elberton 1990 and ROSE 1992 were higher than in SOAS 2013, and high VOC/NOx ratios resulted in high sensitivity to NOx concentration and produced large amounts of O3.

Figure 3-28. Ozone Levels for Each Site in the Southeastern U.S. as a Function of the Concentration of NOx. The bars indicate the average deviation. The dashed line is the curve fitting of exponential x offset using Igor Pro. The curve fitting line is as

\[ [O_3]_{ppbv} = 59.427 - 23.853 \exp\left(-\frac{[NO_x]_{ppbv} - 0.336}{0.022}\right) \]

The chi square is 1.869.

Since PAN is another product in addition to O3, it is expected to show similar behavior to O3 in its relationship to NOx. PAN levels at various locations are plotted as a function of the concentration of NOx in Figure 3-29. According to the figure, urban areas have high PAN concentration with high NOx, while rural areas do low PAN concentration with low NOx. The
different pattern of fitting line from that of O₃ vs. NOx indicates that PAN is more sensitive to NOx than O₃ and has a different pattern of isopleths than the two-dimensional depiction above. The equation in Figure 3-30 for PAN production seems to indicate that PAN production is related to (pseudo) 1ˢᵗ order kinetics of NOx loss rate. On the other hand, the equation in Figure 3-28 for O₃ production resulted in a constant level of O₃ production. PAN production over 20 years in the SE U.S. was sensitive to NOx concentration, but O₃ production was less so.

The fitting line in Figure 3-29 shows that PAN levels increase as NOx concentrations increase, but the rate of increase was lower at high levels of NOx conditions. This suggests that PAN production is VOC-limited at higher NOx levels, similar to the theory for O₃ sensitivity in Figure 3-27. Despite its high level of NOx, PAN in Henderson 1994 was at the same level as PAN in Youth Inc. 1995, which had one third the level of NOx. This indicates that the conditions in Henderson 1994 would be low VOC/NOₓ and result in low PAN production because of consumption of OH without PAN forming in the VOC-limited regime. In ROSE 1990, although the NOx level was similar to that in Elberton 1990, the PAN production in ROSE 1990 was twice as large as in Elberton 1990. In this case, the ratio VOC/NOₓ might be high and in the NOₓ-limited regime. Hence, plenty of OH enhanced the PAN concentration despite the low NOₓ level.
Figure 3-29. PAN Levels for Each Site in the Southeastern U.S. as a Function of the Concentration of NOx. The bars indicate the average deviation. The solid line is the curve fitting of exponential x offset using Igor Pro. The curve fitting line is as

\[
[\text{PAN}]_{\text{pptv}} = 1109.6 - 947.1 \exp \left(- \frac{[\text{NO}_x]_{\text{ppbv}} - 0.336}{2.902}\right)
\]

The chi square is 1.222.

3.3.4. Linear Combination Model to Quantify Contribution of Biogenic and Anthropogenic Hydrocarbon Precursors

3.3.4.1. Estimation of Ozone Production from AHC and BHC (Linear-Combination Model, Reported Method)

A linear-combination model was introduced by Williams et al. (1997) to quantify the relative importance of biogenic and anthropogenic hydrocarbons (BHC and AHC respectively) in \(O_3\) production based on measurements of PANs species. Because the thermal decomposition rates
of all three compounds are similar (Roberts and Bertman, 1992), [PAN] can be represented as a weighted linear combination of [PPN] and [MPAN],

\[ [PAN] = a[MPAN] + b[PPN] \]  \hspace{1cm} (Eq. 3-17)

In the literature (Roberts, 2002; Roberts et al., 1998; Williams et al., 1997), the coefficients \(a\) and \(b\) in Eq. 3-17 were determined with the filed data using least-squares calculations. Since \(O_3\) had some level of positive correlation with PAN, biogenic and anthropogenic contributions to \(O_3\) production were calculated using both coefficients as follows:

\[ BHC \ O_3 = (O_3\text{-background}) \times a_i[MPAN]/(a_i[MPAN] + b_i[PPN]) \]  \hspace{1cm} (Eq. 3-18)

\[ AHC \ O_3 = (O_3\text{-background}) \times b_i[PPN]/(a_i[MPAN] + b_i[PPN]) \]  \hspace{1cm} (Eq. 3-19)

The fraction of \(O_3\) production attributed to either AHC or BHC depended on the amount of measured MPAN and PPN and coefficients \(a_i\) and \(b_i\). The background is obtained from the intercept. This linear-combination model was applied to PAN, PPN, and MPAN in Youth Incorporated 1995, Dickson 1999, and SOAS 2013 from 9am – 7pm, and each coefficient in Eq. 3-17 is shown in Table 3-6. The appended \(R^2\) is the coefficient of determination of the least-squares fitting of calculated PAN from Eq. 3-17 versus measured PAN. Youth Inc. is located 32 km southeast of Nashville, an urban area in Figure 3-24. Dickson is a rural area and is expected to have similar environmental conditions to SOAS 2013, since both sites are located upwind of urban areas. The fraction of estimated \(O_3\) production from AHC and BHC are plotted with measured \(O_3\) in Figures 3-30, 3-31, and 3-32, respectively. Solid lines indicate a degree of influence (Roberts, 2002; Roberts et al., 1998; Williams et al., 1997). At Youth Inc. 1995, more than 50% of the predicted \(O_3\) production resulted from AHC (see Figure 3-30). A subset of high concentrations of \(O_3\) more than 75% (see red oval in the figure) can be attributed to AHC. Hence, the influence of AHC was dominant on \(O_3\) production in Youth Inc. 1995. The influence of BHC on \(O_3\) production
in Youth Inc. 1995 was already reported by Roberts et al. (1998) and was compared to that from New Henderson 1994. At Dickson 1999, the estimated O$_3$ production from both AHC and BHC is shown in Figure 3-31. When measured O$_3$ was more than 70 ppbv (above blue dash line in the figure), most of high levels of estimated O$_3$ production were derived from AHC while low levels resulted from BHC. Hence, the influence of AHC was relatively higher than that of BHC in Dickson 1999. Overall, in SOAS 2013 in Figure 3-32, more than 50% O$_3$ production is estimated to come from BHC. Hence, the influence of BHC was dominant in SOAS 2013.

<table>
<thead>
<tr>
<th></th>
<th>$a_i$</th>
<th>$b_i$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Youth Inc. 1995</td>
<td>4.511</td>
<td>6.695</td>
<td>0.808</td>
</tr>
<tr>
<td>Dickson 1999</td>
<td>5.259</td>
<td>5.798</td>
<td>0.758</td>
</tr>
<tr>
<td>SOAS 2013</td>
<td>8.434</td>
<td>7.255</td>
<td>0.629</td>
</tr>
</tbody>
</table>

Figure 3-30. The Estimated Fractions of O$_3$ Production from BHC versus AHC to Measured O$_3$ in Youth Inc. 1995 for 9am – 7pm. The oval in red indicates a subset of high concentrations of O$_3$. 
Figure 3-31. The Estimated Fractions of O$_3$ Production from BHC versus AHC to Measured O$_3$ in Dickson 1999 for 9am – 7pm. Dash lines indicate 70 ppbv of measured O$_3$.

Figure 3-32. The Estimated Fractions of O$_3$ Production from BHC versus AHC to Measured O$_3$ in SOAS 2013 for 9am – 7pm. The data on June 4$^\text{th}$ was excluded. The values of a half of the detection limit were used for below detection limits of PPN and MPAN.
3.3.4.2. Anthropogenic vs. Biogenic Contribution to PAN Production (Multiple Linear Regression, Proposing Method)

The approach using Eq. 3-17 through 3-19 does not directly include an evaluation of the strength of the relationship between PAN and each factor, MPAN or PPN using statistical hypothesis testing. In this work, the data was analyzed with a multiple linear regression (MLR) model as Eq. 3-20 with further statistical tests in order to compare the significance of participation of the measurement of MPAN and PPN for the prediction of [PAN]. This approach allows us to assess the relative importance of BHC and AHC as PAN precursors using the strength of correlation with PAN.

\[
[PAN] = A + B_1[MPAN] + B_2[PPN] \quad (Eq. \ 3-20)
\]

In Eq. 3-20, [PAN] is treated as a response variable and [MPAN] and [PPN] are used as independent predictor variables. \( B_1 \) and \( B_2 \) are partial regression coefficients on [MPAN] and [PPN]. Multiple regression analysis was conducted in two steps. First, the significance of the overall MLR model Eq. 3-20 was analyzed using an analysis of variance (ANOVA) \( F \) test, which explores whether at least one of the predictor variables significantly contributes to the predicted response variable in order to avoid increasing type I errors that reject a null hypothesis when it is true. The \( F \)-value, which is obtained from the ANOVA table, is used to examine whether there is at least one significant useful predictor variable. However, it is impossible to directly find out which predictor variable is significantly useful. Therefore, in the next step, the significant utility of each respective predictor variable was explored using a t-test (post hoc test). The respective \( t \)-value was calculated from each partial regression coefficient divided by the standard error. When results of a \( t \)-test indicate that there is significant importance of individual independent predictor variables, the standardized partial regression coefficient, \( \beta_i \), between each independent predictor
variable and the response variable allows us to compare the relative contribution of each independent predictor variable within the model. The strength of model fitting was given by the coefficient of determination, $R^2$. Tatsuoka (1971) proved the coefficient of determination in MLR, $R^2$, is equal to the sum of the product of the standardized partial regression coefficient, $\beta_i$, and the zero-order correlation, $r_{ic}$, which is a simple bivariate correlation with a response variable. That is, $R^2 = \sum \beta_ir_i$ (Eq. 3-21).

Therefore, we used the partial (or fraction of) $R^2$ in each predictor variable with the response variable, [MPAN]-[PAN] and [PPN]-[PAN] to describe the relative importance of BHC and AHC respectively to the observed [PAN]. Each partial $R^2$ is obtained as

$$R^2_{MPAN-PAN} = \beta_{MPAN-PAN} \times r_{MPAN-PAN}$$ (Eq. 3-22)

$$R^2_{PPN-PAN} = \beta_{PPN-PAN} \times r_{PPN-PAN}$$ (Eq. 3-23)

Where, $\beta_{MPAN-PAN}$ and $\beta_{PPN-PAN}$ are the standardized partial regression coefficient, and $r_{MPAN-PAN}$ and $r_{PPN-PAN}$ are the zero-order correlation in each pair.

High multi-collinearity causes effects on the results of a MLR analysis (Mendenhall et al., 2009). Although the assumption of the MLR analysis on [PAN] takes a stance that each predictor variable is derived from different hydrocarbon precursor independently, the values of “tolerance” or “variance inflation factor (VIF)” were helpful to assess the degree of multi-collinearity. The tolerance is calculated as $1 - R^2_{MPAN-PPN}$, where $R^2_{MPAN-PPN}$ is the coefficient of determination between MPAN and PPN and VIF is $1/$tolerance. Large VIF values indicate strong multi-collinearity of predictor variables. According to Stevens (2012), a value of VIF greater than 10 indicates effective multi-collinearity.

Results of these statistical tests on the MLR model for daytime SOAS 2013 data are summarized in Table 3-7. Similar PANs data collected from Dickson, TN during the SOS
experiment in 1999 and urban data from Youth Inc. in 1995 are used as comparable references.

The $p$-value, on $F$ from the ANOVA with less than 0.001 in Table 3-7 indicated that the MLR model in all the SOAS 2013, Dickson 1999, and Youth Inc. 1995 was able to reproduce measured PAN concentrations using MPAN and PPN as independent variables. Therefore, it appears that the model Eq. 3-20 is valid for expressing the relationship between PAN, MPAN, and PPN and at least one independent predictor variable is significantly useful.

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of data</th>
<th>$p$ of $F$-value</th>
<th>$R$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Youth Inc. 1995</td>
<td>568</td>
<td>&lt;0.001</td>
<td>0.917</td>
<td>0.840</td>
</tr>
<tr>
<td>Dickson 1999</td>
<td>486</td>
<td>&lt;0.001</td>
<td>0.876</td>
<td>0.766</td>
</tr>
<tr>
<td>SOAS 2013</td>
<td>498</td>
<td>&lt;0.001</td>
<td>0.775</td>
<td>0.601</td>
</tr>
</tbody>
</table>

Table 3-8. Summary of Coefficients on Each Independent Predictor Variable in Post-hoc Test.

<table>
<thead>
<tr>
<th></th>
<th>Youth Inc. 1995</th>
<th></th>
<th>Dickson 1999</th>
<th></th>
<th>SOAS 2013</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPAN</td>
<td>PPN</td>
<td>MPAN</td>
<td>PPN</td>
<td>MPAN</td>
<td>PPN</td>
</tr>
<tr>
<td>Coefficient in model</td>
<td>4.151</td>
<td>6.657</td>
<td>5.098</td>
<td>5.762</td>
<td>7.596</td>
<td>6.910</td>
</tr>
<tr>
<td>Std. error of coefficient</td>
<td>0.281</td>
<td>0.147</td>
<td>0.305</td>
<td>0.178</td>
<td>0.469</td>
<td>0.725</td>
</tr>
<tr>
<td>$p$ of t-test</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VIF</td>
<td>1.104</td>
<td>1.104</td>
<td>1.036</td>
<td>1.036</td>
<td>1.427</td>
<td>1.427</td>
</tr>
<tr>
<td>$\beta_i$</td>
<td>0.261</td>
<td>0.802</td>
<td>0.374</td>
<td>0.725</td>
<td>0.549</td>
<td>0.323</td>
</tr>
<tr>
<td>Zero-order correlation, $r_i$</td>
<td>0.508</td>
<td>0.882</td>
<td>0.509</td>
<td>0.795</td>
<td>0.726</td>
<td>0.624</td>
</tr>
<tr>
<td>Partial $R^2 = \beta_i r_i$</td>
<td>0.133</td>
<td>0.707</td>
<td>0.190</td>
<td>0.576</td>
<td>0.399</td>
<td>0.202</td>
</tr>
</tbody>
</table>

$p$ is significant value. $\beta$ is standardized partial regression coefficient. All dataset was during the daytime, 10 am – 4 pm.

A summary of coefficients of independent respective predictor variables is shown in Table 3-8. Since all VIF values were less than 10, there was no impact of multi-collinearity in the MLR analysis in both SOAS 2013, Dickson 1999, and Youth Inc. 1995. Significant levels of the t-test of all independent predictor variables showed less than 0.001. This indicates that both predictor variables, MPAN and PPN, were useful for predicting PAN. Therefore, respective coefficient values were available to estimate PAN. In SOAS 2013, $R^2$ of MLR was 0.601 and partial $R^2$ of
PAN-MPAN and PAN-PPN were 0.399 and 0.202 respectively. This means 60% of all data was explained by the MLR model with 40% contributed from BHC and 20% from AHC. At Dickson 1999, 77% of all data was explained by the MLR model with 19% from BHC and 58% from AHC. At urban area, Youth Inc. 1995, 84% of all data was explained by the MLR model with 13% from BHC and 71% from AHC. The $R^2$ of MLR in SOAS 2013 data was lower than that in Dickson 1999 and Youth Inc. 1995, which might result from the lower absolute PANs levels in the SOAS 2013; in particular, the SOAS MPAN and PPN data included a large number of BDL measurements, while the Dickson 1999 and Youth Inc. 1995 data did not. The medians of PAN, MPAN, and PPN in Dickson 1999 were 483.5, 25.4, and 24.7 pptv respectively. These were three times higher than that of SOAS 2013. The medians of PAN, MPAN, and PPN in Youth Inc.1995 were 706.5, 32.4, and 64.5 pptv respectively. Also, in Dickson 1999 and Youth Inc. 1995, NOx levels were seven times higher than at the SOAS 2013 campaign. Another reason for a low $R^2$ might come from the presence of different hydrocarbon that also produce PAN but is not included in the current MLR, or long-range transport of PAN. These factors would be included in the residual of $R^2$ (or $1 - R^2$).

In Figure 3-33, the relative importance of BHC and AHC was standardized to compare the SOAS 2013 data to the Dickson 1999 and Youth Inc. 1995 data. The standardized relative percentile was calculated as $R^2_{\text{MPAN-PAN}}/ R^2 \times 100$ for BHC and $R^2_{\text{PPN-PAN}}/ R^2 \times 100$ for AHC. Overall the SOAS 2013 data had a significant biogenic influence that accounted for 66% of PAN formation and was two times larger than the anthropogenic influence. On the other hand, the anthropogenic influence in the Dickson 1999 data accounted for 75% of PAN, which was three times larger than the biogenic influence. Although both sampling locations were located in rural areas in similar environmental forests, the results using MLR indicate that the background of HC
precursors was different. A predominance of AHC influence (84%) was observed in the Youth Inc. 1995 data, five times higher than the BHC influence.

![Comparison of Standardized Relative Contribution to PAN Formation from Biogenic and Anthropogenic Hydrocarbons During the Daytime in Youth Inc., TN in 1995, in Dickson, TN in 1999, and SOAS, in Centreville, AL in 2013.](image)

Figure 3-33. Comparison of Standardized Relative Contribution to PAN Formation from Biogenic and Anthropogenic Hydrocarbons During the Daytime in Youth Inc., TN in 1995, in Dickson, TN in 1999, and SOAS, in Centreville, AL in 2013

### 3.3.4.3. Comparison of Two Methods to Estimate Influence of BHC and AHC Using Linear-Combination Model and Multiple Linear Regression

In Section 3.3.4.1, the influence of BHC and AHC was obtained from estimated ozone production by running a linear-combination model among PAN, PPN, and MPAN. Then, each fraction was estimated from the fraction of coefficients in Eq. 3-17. In Section 3.3.4.2, the model was developed with further using a multiple linear regression (MLR) with statistical tests, which
included an evaluation of the strength of the relationship in dependent and independent variables.

Both results are summarized in Table 3-9.

Table 3-9. The Relative Importance Influence of BHC and AHC Using Two Linear-Combination Models.

<table>
<thead>
<tr>
<th></th>
<th>Youth Inc. 1995</th>
<th>Dickson 1999</th>
<th>SOAS 2013</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>By linear-combination model</strong>&lt;br&gt;(estimating ozone production)&lt;br&gt;(9am – 7pm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$ in $[\text{PAN}]<em>{\text{calcu}}$ vs. $[\text{PAN}]</em>{\text{ambient}}$</td>
<td>0.808</td>
<td>0.758</td>
<td>0.629</td>
</tr>
<tr>
<td>BHC-$O_3$ (Eq. D7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope in BHC-$O_3$ vs. $[O_3]_{\text{ambient}}$</td>
<td>0.095±0.014</td>
<td>0.221±0.026</td>
<td>0.645±0.020</td>
</tr>
<tr>
<td>$R^2_{O_3}$ in BHC-$O_3$ vs. $[O_3]_{\text{ambient}}$</td>
<td>0.047</td>
<td>0.090</td>
<td>0.610</td>
</tr>
<tr>
<td>AHC-$O_3$ (Eq. D8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope in AHC-$O_3$ vs. $[O_3]_{\text{ambient}}$</td>
<td>0.905±0.014</td>
<td>0.779±0.026</td>
<td>0.355±0.020</td>
</tr>
<tr>
<td>$R^2_{O_3}$ in AHC-$O_3$ vs. $[O_3]_{\text{ambient}}$</td>
<td>0.819</td>
<td>0.551</td>
<td>0.322</td>
</tr>
<tr>
<td><strong>BHC/AHC</strong></td>
<td><strong>0.10</strong></td>
<td><strong>0.28</strong></td>
<td><strong>1.82</strong></td>
</tr>
</tbody>
</table>

|                          |                 |              |           |
| **By MLR with statistical test**<br>(10am – 4pm) |                 |              |           |
| $R^2$ in MLR in Eq.D8    | 0.840           | 0.766        | 0.601     |
| $R^2_{MPAN-PAN}$ (standardized) | 0.133           | 0.190        | 0.399     |
| (standardized)           | (0.158)         | (0.248)      | (0.664)   |
| $R^2_{PPN-PAN}$ (standardized) | 0.707           | 0.576        | 0.202     |
| (standardized)           | (0.842)         | (0.752)      | (0.336)   |
| **BHC/AHC**              | **0.19**        | **0.33**     | **1.98**  |

In linear-combination model, the validity of coefficients on MPAN and PPN was confirmed using the coefficient of determination $R^2$ from the least-squares fit line by plotting calculated PAN concentration, $[\text{PAN}]_{\text{calcu}}$ versus PAN measured in ambient air, $[\text{PAN}]_{\text{ambient}}$. The fraction of BHC and AHC contributions is estimated from the Figure 3-30, 3-31, and 3-32 which estimated O$_3$ production (BHC-$O_3$ and AHC-$O_3$) with measured O$_3$. However, it was difficult to express quantitatively the influence fraction from looking at the data distribution in these figures,
especially data from Dickson was evenly scattered. Hence, the slopes of the fitted lines for BHC-O$_3$ and AHC-O$_3$ with measured O$_3$ from ambient air represent the average of relative O$_3$ productions from each source and are used for estimating BHC and AHC influences in this work.

In MLR, $R^2$ is statistically evaluated to measure validity of the model in Eq. 3-20. In this work, MLR model and each partial correlation coefficient in Youth Inc. 1995, Dickson, 1999, and SOAS 2013 were statistically valid due to their p-values (in Table 3-7 and 3-8). The fraction of BHC and AHC contributions were directly reflected $R^2$ as the parts, $R^2_{MPAN-PAN}$ and $R^2_{PPN-PAN}$.

The ratio BHC/AHC contributions in Table 3-9 shows that the BHC/AHC by MLR for Youth Inc. 1995 was two times larger than the ratio by linear-combination mode, while the ratios by MLR for Dickson 1999 and SOAS 2013 were similar to the ratios by linear-combination model.

As another comparison, Roberts and co-workers (2002) estimated that over 80 ppbv O$_3$ comprised contributions of 20% BHC and 80% AHC at Cornelia Fort Airpark in summer 1999 for daytime data from 8am – 8pm using the linear-combination model. They treated data which were above 80 ppbv O$_3$ because of major bias of data distribution. The ratio BHC/AHC is 0.25. I attempted to evaluate the influences using the MLR method in this work for same time range with their report. The standardized partial $R^2$ indicates that BHC influence was 10.9% and the AHC was 89.1% and BHC/AHC was 0.12. The MLR method helps treat whole data points even though the distribution of data is wide.

Although both approaches gave similar results, the MLR includes statistical strength of factors and directly treat $R^2$ in MLR for expressing BHC and AHC influences with the fractions, while estimating of the influences using linear-combination mode includes multiple errors and some $R^2_{O3}$ on the slope was week. Therefore, MLR is more accurate and simpler way to evaluate the BHC and AHC contribution.
3.3.5. Comparison of Contribution of Isoprene Oxidation with Computational Modeling

Groff (2015) used two approaches to assess the contribution of isoprene oxidation to PAN formation by 1) orthogonal distance regression (ODR) between field measurements of MPAN and PAN and 2) simulation of the production of peroxyacetyl (PA) radicals, precursors of PAN using an ambient 0-D photochemical model.

1) ODR

The contribution of isoprene oxidation to PAN formation was estimated by \( \frac{d[\text{PAN}]}{d[\text{PAN}]_{\text{isoprene}}/dt}/(d[\text{PAN}]_{\text{all sources}}/dt) \). MPAN is solely formed from isoprene and the relative yield of \( (d[\text{MPAN}]/dt)/(d[\text{PAN}]_{\text{isoprene}}/dt) \) was obtained from an isoprene oxidation chamber experiment. The reaction was initiated with 1.37 ppm isoprene, 268 ppb NO, and 206 ppb NO\(_2\) under 5% RH in a 5.5 m\(^3\) Teflon cylindrical bag. OH radical was produced by photolysis of HONO. The yield was estimated to be 0.15±0.03 RSD. This means,

\[
\frac{d[\text{MPAN}]/dt}{d[\text{PAN}]_{\text{isoprene}}/dt} = 0.15 \quad (\text{Eq. 3-24})
\]

Hence, the PAN from isoprene oxidation is estimated as

\[
d[\text{PAN}]_{\text{isoprene}}/dt = \frac{d[\text{MPAN}]/dt}{0.15} \quad (\text{Eq. 3-25})
\]

Therefore, the relative contribution of isoprene to total PAN formation was expressed using Eq. 3-25 according to the following equations.

\[
\frac{d[\text{PAN}]_{\text{isoprene}}/dt}{d[\text{PAN}]_{\text{all sources}}/dt} = \frac{d[\text{MPAN}]/dt}{0.15} \times \frac{1}{d[\text{PAN}]_{\text{all sources}}/dt} \quad (\text{Eq. 3-26})
\]

\[
\frac{d[\text{PAN}]_{\text{isoprene}}/dt}{d[\text{PAN}]_{\text{all sources}}/dt} = 6.6 \times \frac{d[\text{MPAN}]/dt}{d[\text{PAN}]_{\text{all sources}}/dt} \quad (\text{Eq. 3-27})
\]
The $d[MPAN]/d[PAN]$ was obtained from field measurements in SOAS 2013, which were done in this work. The value is the slope of linear least squares fit of [MPAN] vs [PAN].

2) Ambient 0-D photochemical model

A 0-D ambient kinetic model simulated the relative contribution to peroxyacetyl (PA) radicals from VOCs present at the field site based on the Master Chemical Mechanism (MCM) v3.3. The ambient model included not only isoprene and its oxidation products but also acetone, acetaldehyde, and terpenes as precursors.

These two methods were compared with the relative importance of BHC, $\beta_{MPAN,PAN}^{MPAN,PAN}$, from the MLR model in this work. Groff (2015) chose four days (6/3, 6/14, 6/26, and 7/12) of data from SOAS 2013 as appropriate to run the 0-D model because the PAN and NOx levels were relatively high. The time ranges were chosen so that the boundary layer height would be stable and any dilution effect would be minimal. Results of the comparison are plotted in Figure 3-34. Although the relative importance of BHC in the MLR model was less than 40% on June 3rd, it was significantly dominant on the other three days accounting for more than 68%. (Note: PPN on June 3rd did not have a significant level to predict PAN in MLR analysis, $p=0.600$.) By comparison, the estimated contribution of isoprene oxidation using ODR on June 3rd had the steepest slope, however, the range of the 95% confidence interval on this day was large. This means there was less accuracy to explain the relative contribution of isoprene oxidation. On the other three days, the relative contributions of isoprene oxidation using ODR were estimated 23 – 49 %, that number is the smallest among the estimations using three methods. Groff considered that might be due to the differences in MPAN/PAN between the chamber experiment and ambient conditions. Assuming a constant yield of MPAN/PAN in the chamber experiment has some
inherent uncertainty, as isoprene, the parent VOC, was added to the chamber to initiate the reaction, while precursors in ambient air conditions are in constant flux. Therefore, the ratio of secondary to primary oxidation products varies between chamber and ambient conditions, with likely relatively greater primary products under chamber conditions. In addition, photolysis rates also are significantly different between the chamber and the field conditions. In the ambient 0-D photochemical model, the correlation between MPAN and PAN was not related to estimating the relative contribution of isoprene oxidation to PAN production, since the contribution was obtained by simulating PA radical productions. The results of the 0-D model suggest that isoprene oxidation significantly contributed to PAN formation with a mean range of 55–73% over all selected days. The relative contribution of isoprene oxidation determined by PA radicals was typically 7–25% lower than by MLR model analysis on three days, except June 3rd. Hence, both methods, the MLR and the 0-D model, indicate that isoprene oxidation was the main source when high levels of PAN were observed during SOAS 2013.
Estimates of the Relative Contribution of Isoprene Oxidation to PANs Formation During 4 Specific Days of SOAS 2013 Using Three Different Approaches: Multiple Regression Analysis, ODR with Chamber Data, and Simulation of PA Radicals Using a 0-D Model. The * indicates the significant level of t-test. The C.I. means confidence interval.

**Figure 3-34.** Estimates of the Relative Contribution of Isoprene Oxidation to PANs Formation During 4 Specific Days of SOAS 2013 Using Three Different Approaches: Multiple Regression Analysis, ODR with Chamber Data, and Simulation of PA Radicals Using a 0-D Model. The * indicates the significant level of t-test. The C.I. means confidence interval.
3.3.6. MPAN vs. IN vs. MACR

Isoprene photooxidation mechanisms (Figure 3-35) consider MACR to be a first generation product with MPAN being a secondary product derived from MACR (Bertman and Roberts, 1991). The OH adduct of isoprene that is the intermediate leading to MACR in these mechanisms is also a precursor of isoprene nitrates (IN). Decomposed IN may form MACR (e.g. Fisher et al., 2016; Lockwood et al., 2010; Mao et al., 2013). Techniques able to measure IN are relatively new and there is no previous data for them in the SE U.S. According to the pathways in Figure 3-35, a strong relationship is expected between IN and MACR and between MACR and MPAN, while a weak relationship is expected between IN and MPAN.

The relationship between MPAN with MACR and IN at SOAS 2013 are plotted in Figure 3-36. The MACR-MPAN had larger spread than IN-MPAN. The results of zero-order (Pearson) correlation tests of MACR, MPAN, and IN relationships are shown in Table 3-10. Contrary to expectations, the correlation coefficient between MACR and MPAN for the entire campaign is
0.236 and there was no significant daytime relationship, when both MACR and MPAN normally showed high concentrations. However, MPAN was strongly correlated with IN over the whole campaign (r=0.69). Interestingly, IN was significantly correlated with MACR as well as MPAN. As a further assessment of these relationships, a “partial correlation test”, which enables comparison of the strength of correlation between two variables without the effect of multiple correlations with a third variable, was conducted for each pair, and the results in Table 3-10 shows that overall, IN has a significant positive correlation with both MACR and MPAN when the effect of the other variable is eliminated.

Figure 3-36. Relationship of MPAN with IN and MACR in SOAS 2013. All data are plotted. IN concentrations were measured by Purdue University using a chemical ionization mass spectrometer (CIMS). MACR was measured by de Gouw and Goldstein groups using a GC-MS.
Table 3-10. Coefficients of Zero-Order (Pearson) and Partial Correlation Among MACR, MPAN, and IN.

<table>
<thead>
<tr>
<th></th>
<th>MACR vs. IN</th>
<th>MACR vs. MPAN</th>
<th>MPAN vs. IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-order (none)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All data (N=303)</td>
<td>0.466**</td>
<td>0.236**</td>
<td>0.685**</td>
</tr>
<tr>
<td>10 am – 4pm (N=103)</td>
<td>0.389**</td>
<td>0.133</td>
<td>0.583**</td>
</tr>
<tr>
<td>9pm – 4am (N=86)</td>
<td>0.701**</td>
<td>0.369**</td>
<td>0.640**</td>
</tr>
<tr>
<td>Partial correlation (control)</td>
<td>(MPAN)</td>
<td>(IN)</td>
<td>(MACR)</td>
</tr>
<tr>
<td>All data (N=303)</td>
<td>0.430**</td>
<td>-0.130</td>
<td>0.669**</td>
</tr>
<tr>
<td>10 am – 4pm (N=103)</td>
<td>0.386**</td>
<td>-0.124</td>
<td>0.581**</td>
</tr>
<tr>
<td>9pm – 4am (N=86)</td>
<td>0.651**</td>
<td>-0.145</td>
<td>0.575**</td>
</tr>
</tbody>
</table>

** indicates P<0.01 level (2-tailed). Data on June 4th was not included.

Although daytime data over the whole campaign did not show significant correlation between MACR and MPAN, they were correlated on specific days. Since PAN chemistry in SOAS 2013 was NOx sensitive (see Section 3.3.2.2. and 3.3.3.), the comparison analysis was separated based on mean NOx level. The mean NOx levels during daytime (10am – 4pm) at SOAS were around 0.35 ppbv. Table 3-10 shows that MPAN was significantly correlated to both MACR and IN under relatively high NOx conditions (above 0.35 ppbv), while it was not under relatively low NOx conditions (below 0.35 ppbv). Coefficients of partial correlation for MACR-MPAN (controlling IN influence) and MPAN-IN (controlling MACR influence) were smaller than these values of zero-order correlation under relatively high NOx condition. This suggests that MACR, MPAN and IN correlated best during the daytime under relatively high NOx condition (Figure 3-
On the other hand, MPAN had a weak relationship to MACR but was significantly correlated to IN under relatively low NOx conditions. These comparisons suggest that there might be a common intermediate in isoprene oxidation that is shared between IN and MPAN (Figure 3-38). The formation pathway of MPAN via IN based on these field data could be as good as, if not superior to, the one via MACR under the atmospheric conditions experienced at SOAS. In this case, the distribution of isomers of IN would be crucial. However, the relative isomer yields are quite uncertain (Xiong et al., 2015). In addition, it is important to include the influences of loss processes of IN, MACR, and MPAN as further research.

Figure 3-37. Relationship of MPAN with IN and MACR Under Relatively High NOx Condition during 10am – 4pm.
3.3.7. Gas-phase MPAN vs. Organic Mass in Particles

The Figure 3-39 summarizes the currently proposed mechanism for SOA formation via isoprene oxidation (Crounse et al., 2012; Kjaergaard et al., 2012; Nguyen et al., 2015; Paulot et al., 2009; Surratt et al., 2010). To investigate the possibility that MPAN is involved in one of the pathways leading to SOA formation, gas-phase MPAN observations were compared with particle mass during SOAS. Since it is unclear whether, if MPAN was taken into the particle phase, nitrogen from MPAN would be retained in the particle, two types of components were investigated: a) total organic nitrogen in particles (in the form of organic nitrates) and b) total organic compounds in particle phase.
Figure 3-39. Proposing Processes to Form SOA via Isoprene Oxidation. Compounds in red means main isoprene oxidation compounds and compounds in black are proposed compounds (Crounse et al., 2012; Kjaergaard et al., 2012; Nguyen et al., 2015; Paulot et al., 2009; Surratt et al., 2010) Arrows in magenta indicates the reaction involves NOx, arrows in blue doesn’t.

The resulting relationships are shown in Figure 3-40 for all data and daytime data for June 28th – July 15th in SOAS 2013. The plots show that high MPAN was frequently observed during the daytime simultaneously with high mass of both particle measures. Direct comparison of the absolute slopes of the regressions is difficult due to the units for mass of particles (Note: these particle data were quantified by different groups with different methods. See Section 3.2.7. or the caption in Figure 3-40). However, the $R^2$ of total organic compounds in particles to MPAN during the daytime, 0.455, is larger than the $R^2$ of organic nitrates in particle with MPAN, 0.120. This may suggest that if an MPAN + OH adduct is involved in particle growth, nitrogen is lost before being taken into particle. This is the first report of the relationship between gas-phase MPAN and
particles from field data. In order to further inspect the mechanism in Figure 3-39, we attempted to compare gas-phase MPAN with other proposed compounds. Nguyen et al. (2015) proposed hydroxymethyl-methyl-α-lactone (HMML), an intermediate in a mechanism of SOA formation from isoprene oxidation (Figure 3-39). However, HMML was not available from SOAS 2013. These data are consistent with the mechanism that involves hydroxyacetone (HAC) as a further decomposition product from HMML. Although HAC was measured in the campaign, MACR oxidation produces HAC without MPAN formation (Crounse et al., 2012) and the levels of MACR are too high to distinguish with field data the production of HAC from MPAN (Table 3-11).

Figure 3-40. The Relationship Between MPAN in Gas Phase and the Mass of Particles. a) Organic nitrates by Cohen group (UC-Berkeley) with a thermal dissociation method, and b) total organic compounds by Jimenez group (U of Colorado) with a HR-ToF-AMS, for June 28th – July 15th in SOAS 2013 (the time period when data on organic nitrates in the particle phase was available). The $R^2$ and $\chi^2$ in a) for 10 am – 4pm are 0.120 and 0.337 respectively. The $R^2$ and $\chi^2$ in b) for 10 am – 4pm are 0.455 and 269.28 respectively.
Table 3-11. Coefficients of Zero-Order (Pearson) and Partial Correlation Among MACR, MPAN, and HAC.

<table>
<thead>
<tr>
<th></th>
<th>MACR vs. HAC</th>
<th>MACR vs. MPAN</th>
<th>MPAN vs. HAC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zero-order (none)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All data (N=236)</td>
<td>0.623</td>
<td>0.265</td>
<td>0.240</td>
</tr>
<tr>
<td>10 am – 4pm (N=70)</td>
<td>0.353</td>
<td>0.186</td>
<td>-0.367</td>
</tr>
<tr>
<td>9pm – 4am (N=73)</td>
<td>0.808</td>
<td>0.401</td>
<td>0.558</td>
</tr>
<tr>
<td><strong>Partial correlation (control)</strong></td>
<td>(MPAN)</td>
<td>(HAC)</td>
<td>(MACR)</td>
</tr>
<tr>
<td>All data (N=236)</td>
<td>0.598</td>
<td>0.153</td>
<td>0.099</td>
</tr>
<tr>
<td>10 am – 4pm (N=70)</td>
<td>0.461</td>
<td>0.363</td>
<td>-0.471</td>
</tr>
<tr>
<td>9pm – 4am (N=73)</td>
<td>0.769</td>
<td>-0.102</td>
<td>0.433</td>
</tr>
</tbody>
</table>

** indicates P<0.01 level (2-tailed).
3.4. Summary

Influence of Isoprene and PANs Chemistry in the Southeastern U.S.

During SOAS 2013, the sum of PAN, PPN and MPAN accounts for an average of 15% of NOy during the daytime. Urban-influenced air from the North showed enhanced PANs level and PAN and MPAN with air from North showed similar diurnal pattern, but not with PPN. Average PAN levels during the daytime was 169 ± 129 pptv. The levels of PPN and MPAN were often below the detection limit. In comparison with previously reported data, the level of total PANs is the lowest over the past few decades in the Southeastern U.S. The PAN concentration in SOAS 2013 was 4.3-6.1 times smaller than that from reported urban areas and was 1.5-3.3 times less than that in the reported rural areas. The relationships between PAN and NOx at various sites in SE U.S. showed an increase of PAN level with increasing NOx concentrations but the rate of increase was reduced at high levels of NOx conditions, possibly due to a switch from NOx-limited PAN formation to VOC-limited regimes, similar to the relationship between O3 and NOx.

A multiple linear regression (MLR) model was applied to compare the significance of participation of the measurement of MPAN and PPN for PAN prediction. The MLR method is more accurate and simple than a linear-combination method and allows us to statistically assess the relative importance of BHC and AHC using the strength of partial coefficients of determination. The results indicated that biogenic precursors account for 66% of PANs and was about twice as high as anthropogenic influence during the overall campaign. Three different methods to assess the influence of isoprene oxidation were compared for selected days, which observed high level of PAN. The MLR model and the ambient 0-D photochemical model indicated isoprene has the most dominant influence, accounting for 68-95% and 55-73% respectively, on PAN formation.
Over the whole campaign, MPAN more strongly correlated with IN than MACR, which is a direct precursor of MPAN. Interestingly, this was true even without the presence of MACR. When NO\textsubscript{x} concentration was relatively high, MACR, MPAN and IN all correlated well during the daytime, while under relatively low NO\textsubscript{x} conditions, MPAN significantly correlated to IN, but not to MACR. Further investigation is needed to assess whether MPAN is formed from IN as well as from MACR.

The relationship between gas-phase MPAN and organic particles from field measurements was first reported by this work. Relatively high levels of gas-phase MPAN were observed to correlate with daytime particle mass and was more closely related to total organic compound particles than to organic nitrogen in particles. This may suggest that the nitrogen of MPAN is removed by OH addition before being taken into the particle phase. These field data can suggest lab experiments to further investigate the mechanism of organic nitrates arising from isoprene oxidation.

3.5. References


Chen, W.: PAN, PPN and MPAN measurements and the quantitative relationship of PAN and MPAN in biogenic hydrocarbon conditions (Master's Thesis), Western Michigan University, Kalamazoo. 2001.


CHAPTER IV

SECTION 2-2: NEW PAN-LIKE PRODUCT FROM ISOPRENE OXIDATION

4-1. Introduction

4.1.1. Is APAN Formed from Isoprene?

A significant amount of an identified PAN-type compound has been observed after the PAN peak on GC chromatograms from chamber experiments of isoprene photooxidation in the presence of high NOx. A representative GC chromatogram from a chamber experiment is shown in Figure 4-1. According to the reported GC retention time order of PAN-type compounds from ambient air by Roberts and co-workers (2001), who used a GC-ECD with similar conditions to ours, the unidentified compound was tentatively proposed to be an APAN-like compound (Note: APAN is peroxycrolyl nitrate, CH$_2$=CHC(O)OONO$_2$). APAN is similar in structure to MPAN, but while a chemical mechanism to explain MPAN formation from isoprene-derived MACR, no such mechanism can be easily derived to explain APAN formation.

Figure 4-2 shows the timeseries of APAN with other measurements from a chamber experiment of isoprene photooxidation in presence of high NOx conducted as one of a series of chamber experiments at Purdue University in 2013 (experimental conditions are described in Appendix E). The concentration of the APAN-like compound in Figure 4-1 was estimated using the calibration curve for PAN. The concentration of NO$_2$ reached the maximum level (the maximum [NO$_2$]/[NO] ratio) an hour after initiating the experiment, and then the level started decreasing. At this time, the amount of the APAN-like compound started increasing along with
O₃, aerosol, and the other PAN-type compounds, PAN and MPAN. Clearly, the amount of this compound is significant and warrants further study.

Figure 4-1. Representative Chromatogram of APAN-like Compound from Chamber Experiment of Isoprene Photooxidation with High NOx. (in the Chamber Experiment II at Purdue University)
As described in Chapter 1, Isoprene is the most abundant non-methane hydrocarbon emitted into the atmosphere, with estimated global emissions of 440-660 Tg C yr\(^{-1}\) (Guenther et al., 2006). Based on chamber experiments conducted starting from isoprene, the amount of APAN formed from isoprene would make it a significant oxidized nitrogen compound in the troposphere. However, APAN is considered only an anthropogenic product, and there has not yet been a report that APAN comes from isoprene oxidation. The mechanism of APAN formation from isoprene is difficult to imagine because of the unsaturated three carbon fragment required. This chapter studies (i) the conditions required to generate an APAN-like compound using a series of chamber experiments at WMU; (ii) the compound is confirmed as a PAN-type species using thermal decomposition technique and identified by comparison to a synthesized APAN.
standard; (iii) the APAN-like compound from ambient air during the SOAS 2013 campaign will be discussed with other PANs in the gas phase and proposed products in the particle phase.

4.1.2. What is APAN?

APAN was first reported by Tanimoto (1999) as an unidentified compound from ambient air during a high-pollution episode in a suburban area of Tokyo, Japan. The compound was identified as APAN by comparing the negative ion chemical ionization mass spectrum of the compound with a synthesized standard (Tanimoto and Akimoto, 2001). Tanimoto and Akimoto (2001) proposed that APAN is formed from 1,3-butadiene, which is an anthropogenic hydrocarbon, via acrolein. The relevant APAN formation mechanism is shown in Figure 4-3.

![Figure 4-3. Proposal APAN Formation from 1,3-Butadiene via Acrolein.](image)

Recent laboratory experiments show that acrolein contributes to SOA formation, and the highest SOA yield were obtained with high [NO$_2$]/[NO] ratio (Chan et al., 2010). Further research was conducted by Jaoui and co-workers (2014). They generated SOA from 1,3-butadiene in the presence of NO$_x$ in a smog chamber and measured the photooxidation products.
Acrolein and APAN were detected in the gas phase and glyceric acid was observed in both gas and particle phases. The mechanism of SOA formation from 1,3-butadiene was proposed based on those proposed from isoprene (Lin et al., 2013; Nguyen et al., 2015; e.g. Surratt et al., 2010). Similar to isoprene photooxidation, the presence of the epoxide compound intermediate, acrylic acid epoxide, which is a product from further reaction of APAN with OH radical, would be key to formation of glyceric acid and its oligomers in the particle phase. Therefore, APAN is expected to play an important role in SOA formation as well as MPAN.

4.1.3. Objectives

Isoprene produces several PAN-type compounds, such as PAN, MPAN, and HPAN (peroxyhydroxyacetyl nitrate, HOCH$_2$C(O)OONO$_2$) (e.g. Zheng et al., 2011). Methylvinyl ketone (MVK) and methacrolein (MACR) are the largest first generation isoprene oxidation products (e.g. Biesenthal and Shepson, 1997). MPAN is produced via MACR only (as shown in Figure 4-4) and HPAN is produced from glycolaldehyde, which is a product of further reaction of MVK. PAN is obtained from MVK, MPAN or other pathways (e.g. Phillips et al., 2013). APAN is not easily derived from any of the major products of isoprene oxidation. Therefore, in this study, photooxidation is initiated with isoprene, MVK, or MACR under high NOx conditions, and the presence of an APAN-like compound is investigated to understand the pathway from isoprene. Since the molecular weights of APAN and HPAN are between PAN and PPN, the unidentified peak between the retention times of PAN and PPN is could be APAN or HPAN. Therefore, the retention time of HPAN is confirmed using glycolaldehyde oxidation.

PAN-type compounds are thermally labile, and this property can help identify PAN-type compounds. The theory is explained in Section 4.2.3. In addition, a standard material is required
to identify as APAN. The synthetic procedures for APAN needed to be optimized and to confirm the RT on GC and the mass spectra of standard compound before comparing the standard compound to an APAN-like compound from chamber experiments.

If isoprene also forms APAN, this means APAN is formed from both anthropogenic and biogenic precursors. In order to assess the anthropogenic and biogenic influences on the field APAN formation, the APAN is compared with PPN, which has anthropogenic precursor, and MPAN, which has biogenic (isoprene only) precursor. In addition, gas-phase APAN was compared to proposed APAN photooxidation products in particle to test the contribution of APAN to particle formation from the field samples.

Figure 4-4. Unknown Relationship of Isoprene Oxidation with APAN Formation.
4.2. Experimental

4.2.1. Smog Chamber Experiments of Isoprene Oxidation at WMU

The WMU experiments were performed in a ¾” plywood box (W. 121.92 cm × D. 87.95 cm × H. 58.10 cm) with the inside surface covered by an aluminum foil and four black lights (length 118.11 cm, 40 W) inside the box. Photochemical reactions occurred in a custom-made Teflon bag, which was placed in the box. A flange adapter with a tube fitting and a gasket were installed at the center of the Teflon bag. The size of the tubing, which was connected to the inlet of the bag, was varied based on the experimental process and is described later.

4.2.1.1. Smog Chamber Experimental Conditions

Table 4-1 lists the laboratory conditions for the series of chamber experiments in this work at WMU. These experiments were conducted under high-NOx conditions with 50% relative humidity (RH). First, photooxidation experiment started from an isoprene standard (Aldrich) as a precursor. Then, the first-generation products of isoprene oxidation, MACR (Aldrich) and MVK, were used as precursors to determine whether these compounds were involved in the pathway of APAN-like formation in Figure 4-4. Glycolaldehyde, which is a precursor of HPAN, was used to check the GC retention time of HPAN against APAN. Acrolein photooxidation was conducted to confirm the identity of synthetic APAN (this experiment will be mentioned in Section 4.2.4.3.).
Table 4-1. The Conditions of Chamber Experiments at WMU.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Date</th>
<th>Volume of bag (L)</th>
<th>Added HC</th>
<th>Added volume (µL)</th>
<th>Concentration (ppb)</th>
<th>Added volume of NOx (µL)</th>
<th>Concentration (ppb)</th>
<th>RH (%)</th>
<th>Sitting time to make HONO (hr)</th>
<th>Ratio HC/NOx by concentration</th>
<th>Ratio HC/NOx by injected volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/29/14</td>
<td>88</td>
<td>isoprene</td>
<td>0.2</td>
<td>546</td>
<td>10</td>
<td>114</td>
<td>50</td>
<td>2</td>
<td>4.79</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>2/5/14</td>
<td>88</td>
<td>isoprene</td>
<td>0.6</td>
<td>1639</td>
<td>30</td>
<td>341</td>
<td>50</td>
<td>2</td>
<td>4.81</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>2/12/14</td>
<td>88</td>
<td>isoprene</td>
<td>0.6</td>
<td>1639</td>
<td>30</td>
<td>341</td>
<td>50</td>
<td>1</td>
<td>4.81</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>2/19/14</td>
<td>88</td>
<td>glycolaldehyde 7E\textsuperscript{G}</td>
<td>3310</td>
<td>30</td>
<td>341</td>
<td>50</td>
<td>1</td>
<td>9.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2/26/14</td>
<td>88</td>
<td>MACR</td>
<td>0.4</td>
<td>1335</td>
<td>20</td>
<td>227</td>
<td>50</td>
<td>1</td>
<td>5.88</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>2/28/14</td>
<td>88</td>
<td>MVK</td>
<td>0.4</td>
<td>1325</td>
<td>20</td>
<td>227</td>
<td>50</td>
<td>1</td>
<td>5.84</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>3/7/14</td>
<td>88</td>
<td>isoprene</td>
<td>0.6</td>
<td>1639</td>
<td>20</td>
<td>227</td>
<td>50</td>
<td>1</td>
<td>7.22</td>
<td>0.03</td>
</tr>
<tr>
<td>8</td>
<td>5/23/14</td>
<td>88.1</td>
<td>isoprene</td>
<td>0.18</td>
<td>500</td>
<td>17.5</td>
<td>200</td>
<td>50</td>
<td>1</td>
<td>2.50</td>
<td>0.01</td>
</tr>
<tr>
<td>9</td>
<td>5/29/14</td>
<td>140.2</td>
<td>isoprene</td>
<td>0.29</td>
<td>500</td>
<td>14.5</td>
<td>103</td>
<td>50</td>
<td>overnight</td>
<td>4.85</td>
<td>0.02</td>
</tr>
<tr>
<td>10</td>
<td>6/4/14</td>
<td>140.2</td>
<td>MVK</td>
<td>0.24</td>
<td>500</td>
<td>12</td>
<td>86</td>
<td>50</td>
<td>overnight</td>
<td>5.81</td>
<td>0.02</td>
</tr>
<tr>
<td>11</td>
<td>6/11/14</td>
<td>72.7</td>
<td>acrolein</td>
<td>0.3</td>
<td>1502</td>
<td>15</td>
<td>206</td>
<td>50</td>
<td>1</td>
<td>7.29</td>
<td>0.02</td>
</tr>
<tr>
<td>12</td>
<td>6/13/14</td>
<td>72.7</td>
<td>MVK</td>
<td>0.24</td>
<td>963</td>
<td>12</td>
<td>165</td>
<td>50</td>
<td>1</td>
<td>5.84</td>
<td>0.02</td>
</tr>
<tr>
<td>13</td>
<td>6/24/14</td>
<td>72.7</td>
<td>MVK</td>
<td>0.24</td>
<td>963</td>
<td>12</td>
<td>165</td>
<td>50</td>
<td>1</td>
<td>5.84</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Experiment 7 was conducted for investigating thermal decomposition experiment. Experiment 9 was for confirmation of RT. The experiments No. 1 through 7 were conducted with Ben Lenning who was a high school research student from Kalamazoo Area Mathematics and Science Center in 2014. RH means relative humidity.

4.2.1.2. General Operation of Chamber Experiment

The main processes of the isoprene photooxidation experiments in a chamber are described below.

1) Fill a Teflon bag with zero air gas (ZA, Airgas) including H$_2$O and inject precursor hydrocarbon and NOx.

2) Settle the bag in the darkness to form HONO.

3) Turn on UV lights to initiate OH radical reactions.

4) Take GC samples from the bag for 3-4 hours.

Before Step 1, the time needed to fill the bag at 5 Standard Liters per Minute (SLPM)
using a calibrated mass flow controller (MFC) needed to be determined. Initial ZA filled in the bag was evacuated using a vacuum pump to purge the bag inside and was replaced with new zero air. This purge process was repeated at least twice. The bag contained no air prior to starting the experiment. In order to make 50% RH condition, first the ¼” OD PFA Teflon tube was connected to the inlet of the bag. A water bubbler was installed upstream of the inlet (Teflon bag) (Figure 4-5 (a) with pathway i), and zero air went through the water bubbler and filled half the volume of the Teflon bag at 5 SLPM, which was controlled using a MFC. After the bag was half-filled, the water bubbler was disconnected from the bag, and another ¼” OD PFA Teflon tube with a stainless steel tee (Swagelok) was connected to the inlet of the bag as shown in pathway ii) in Figure 4-5 (a). One port of the tee was sealed using a GC inlet septum. After resuming airflow to fill the remaining volume of the bag, starting materials such as isoprene and NOx were introduced to the bag under flow by injecting with a syringe through the septum. When liquid compounds like the isoprene standard were injected into the tee, the tee was heated with a heat gun before and during injecting the compound in order to vaporize it. NOx (NO\textsubscript{2} standard, Sigma-Aldrich) was injected using a gas-tight syringe (Hamilton). After the bag was filled with zero air and reactants, the tube was removed from the flange adapter of the bag and the adapter was closed with a screw cap. (Note: since glycolaldehyde (experiment No.4 in Table 4-1) is a solid, the required amount of solid was added into a stainless steel well, which was threaded on the top edge, and then the well was installed into the tee. The well was heated with the heat gun to carry the compound to the bag as gas.)

The bag was kept in the plywood box in the dark, at least one hour in order to generate HONO, which creates OH radicals with lights. HONO forms by a heterogeneous NO\textsubscript{2}-H\textsubscript{2}O reaction on the surface of the bag in the dark (Finlayson-Pitts and Pitts, 2000) as
\[ \text{NO}_2 + \text{surface reduced site} + \text{H}_2\text{O} \rightarrow \text{HONO} + \text{surface oxidized site} + \text{OH}^-, \]

or another reaction is a surface catalyzed reaction as

\[ \text{NO} + \text{NO}_2 + \text{H}_2\text{O} \rightleftharpoons 2\text{HONO}. \quad \text{(surface)} \]

At Step 3, the cap of the flange adapter was replaced with a tube fitting reducer from 1/4” to 1/8” and air from the bag was pulled into a GC-ECD through 1/8” OD PFA Teflon tube at 50 Standard Cubic Centimeters per Minute (sccm) sample loop as the pathway in the Figure 4-5 (b). A blank sample was taken from the bag before turning the lights on inside the BOX. When light was turned on, HONO was photolyzed rapidly and generated OH radical as

\[ \text{HONO} + \text{hv} \rightarrow \text{OH} + \text{NO}. \]

At Step 4, GC samples were taken every 20 min. The sample air from the bag was introduced to the GC-ECD system without dilution. During the experiments with light exposure, the temperature of the chamber (box) increased from room temperature up to around 30 °C.

Figure 4-5. Scheme of (a) Filling Zero Air into a Teflon Bag Adding i) Humid and ii) Hydrocarbon Source and NOx and (b) Injecting Sample into a GC-ECD.
4.2.3. Observation of Thermal Decomposition

PAN-type compounds thermally decompose into a peroxyacyl radical and NO₂ via a first order reaction. The rate of the thermal decomposition of PAN homologs is relatively similar since the energies of the R-C(O)OO-NO₂ bond is independent of the nature of R group (Roberts and Bertman, 1992). Therefore, if an unidentified peak decomposes by heating at a theoretical first order reaction rate, which is calculated using the kinetics for PAN (see below), this is evidence to support the proposal that the unidentified compound is a PAN-type compound.

To observe the thermal decomposition rate of a PAN-type compound, a 85 cm stainless coil (O.D. 1/8”, i.d. 1/16”) (blue line in Figure 4-6) was spliced into Teflon tube and the coil was placed in the boiling-water bath. The residence time of air in the metal coil was 2.018 sec at a flow rate of 50 sccm. The first order rate constant (k) was calculated using the Arrhenius’ equation as,

\[ k = A \exp^{\frac{-E_a}{RT}} \quad (Eq. 4-1). \]

Where, \( A \) is Arrhenius factor, \( E_a \) is activation energy, and \( R \) is gas constant. The parameters, \( A \) and \( E_a \) of PAN were \( 10^{17.4} \) and 28.5 respectively (Roberts and Bertman, 1992). At the boiling point of water, \( k \) was estimated as 5.09 sec\(^{-1}\). When the number of PAN molecules at 0 time is expressed as \( N_0 \), and that at time \( t \) is \( N_t \), there was relationship with \( k \) below,

\[ N_t = N_0 e^{-kt} \quad (Eq. 4-2). \]

The ratio of number of PAN molecules at t time to initial condition is

\[ \ln \frac{N}{N_0} = -kt \]

\[ \ln \frac{N_0}{N} = kt \quad (Eq. 4-3). \]

Hence, the ratio of the number of PAN molecules before and after passing through the coil was obtained by substituting 5.09 sec\(^{-1}\) and 2.018 sec for \( k \) and \( t \) in \( Eq. 4-3 \) as,
\[
\ln \frac{N_0}{N} = 10.265
\]

\[
\frac{N_0}{N} = 28724.086
\]

The percent of thermally decomposed PAN molecules was calculated by

\[
\{1 - (N/N_0)\} \times 100 = 99.997\% \quad (Eq. 4-4).
\]

Therefore, theoretically, 99.997\% of PAN-type compounds will be decomposed by passing through the 85 cm stainless coil (i.d. 1.5875mm) at 50 sccm flow rate while it is immersed in a boiling water bath. Since metal surfaces can scrub PAN type compounds, different lengths of the same type of coil, 33 cm or 230 cm, were used to determine how a peak of a PAN type compound was observed after passing through the coil at room temperature (Since the coil was not heated at room temperature, I could observe how much PAN was scrubbed by the surfaces of stainless coil without the effect of thermal decomposition). The percent of thermally decomposed PAN molecules (Eq. 4-4) was 98.14 \% for 33 cm coil and 100 \% for 230 cm coil at 100 °C at 50 sccm flow rate.

Figure 4-6. Diagram of Thermal Decomposition Experiment for Unidentified Peak as a PAN-type Compound from a Chamber Experiment.
4.2.4. Optimization of Synthesis of Liquid APAN Standard

4.2.4.1. APAN Synthesis Directly from Acryloyl Chloride

PPN and MPAN in the liquid phase are synthesized from their anhydride analogs with acid catalysis via two steps shown below:

\[
\text{H}^+ \quad \text{Step 1.} \quad (\text{RCO})_2\text{O} + 2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + 2\text{RC(O)OOH} \\
\text{H}^+ \quad \text{Step 2.} \quad \text{RC(O)OOH} + \text{HNO}_3 \rightarrow \text{H}_2\text{O} + \text{RC(O)OONO}_2
\]

However, acrylic anhydride, which is the anhydride compound of APAN, is currently not commercially available. Roberts et al. (2001) and Tokarek et al. (2014) synthesized APAN from a marketed acryloyl chloride (CH\(_2\)CHC(O)Cl). Tokarek et al. (2014) used 8.0 mL of acryloyl chloride and 5.0 mL of 50 % H\(_2\)O\(_2\) for making the peracid compound and 5.0 mL of concentrated H\(_2\)SO\(_4\) and 6.0 mL of HNO\(_3\) for being nitrated in Figure 4-7 (a). The stoichiometric ratio of HC (hydrocarbon source): 50 % H\(_2\)O\(_2\): H\(_2\)SO\(_4\): HNO\(_3\) was almost 1: 1: 1: 1 under the reported conditions.

Another synthesis was reported by Tanimoto and Akimoto (2001). First, acrylic anhydride was made from the reaction between acrylic acid and its corresponding acid chloride, acryloyl chloride using triethylamine (TEA) as a base catalyst. The remaining steps for the peracid and nitrated compound were the same as for the synthesis of PPN and MPAN that were described in Chapter 3. Their stoichiometric ratio, HC: (H\(_2\)SO\(_4\), acid catalyst for peracid): 50 %H\(_2\)O\(_2\): H\(_2\)SO\(_4\): HNO\(_3\) was almost 3.3: (0.1): 1: 1.1: 1.4. The ratio was similar to our procedures for PPN and MPAN synthesis.

Both methods were attempted to synthesize liquid APAN as the standard.
Figure 4-7. Reported Methods for APAN Synthesis. The (a) by Roberts et al. (2001) and Tokarek et al. (2014) and the (b) by Tanimoto and Akimoto (2001).

The amount of chemicals was scaled down to 1/5 of the PPN synthesis method described in Chapter 3.

1) First, all reagents and glassware were cooled before beginning the experiment.
2) A 50 mL of round-bottom flask (RBF) with a magnetic stir rod was affixed to a stand and was put into ice water bath until half of the RBF was immersed. The temperature was around 0~2 °C
3) 1 mL of acryloyl chloride (Sigma-Ardrich or Alfa) was added into the RBF and was stirred for 5 minutes. 20 µL of cooled MeSO₃H was slowly added dropwise down on the side of the RBF, and then 150 µL of cooled 50% H₂O₂ was added in the same way.
4) The mixture was stirred for 2-3 hours.
5) Ice in the bath was replaced as needed.
6) 1-4 mL of cold tridecane (Aldrich) or dodecane was added into the RBF and was
stirred for 5 minutes.

7) 1 mL of cooled MeSO₃H was slowly added dropwise down the wall of the flask and was stirred for 5 minutes.

8) 400 µL of cooled, concentrated HNO₃ was slowly added dropwise and the solution was stirred for 15 minutes.

9) 5 mL of cold water was added into a 15 mL of glass test tube.

10) The solution was transferred from the RBF to the test tube.

11) The solution was mixed with the water by pipetting sufficiently.

12) The aqueous layer (bottom layer) was removed from the tube by pipetting.

13) The organic layer was washed at least 5 times with cold water using step 11 and 12.

14) Anhydrous MgSO₄ powder (company) was added the organic solution to dry it.

15) The dried solution was filtered through a plug of cotton and was transferred to a diffusion cell to run with GC-ECD.

This method for APAN synthesis was difficult to optimize. First, the amount of chemicals was scaled down to half and ¼ of the reported amount by Tokarek et al. (2014). This optimization used acryloyl chloride supplied from two different companies, Sigma-Aldrich and Alfa Aesar with different stabilizers, < 200 ppm MEHQ and 400 ppm phenothiazine, respectively. When the stoichiometric ratio of acryloyl chloride to H₂O₂ was 1:1, both chemicals produced a small explosion at Step 3, although the 50% H₂O₂ was cooled and slowly dropped in order to prevent the mixture from heating by H₂O₂ addition. The solution turned from colorless to yellow and showed a small blast with vapor in the middle of H₂O₂ addition. In another attempt, an acetone-dry ice bath was used instead of an water ice bath to reduce heating during the addition of 50% H₂O₂. However, this approach did not resolve the problem because the
acryloyl chloride solution froze. Acryloyl chloride from Aldrich (with < 200 ppm MEHQ stabilizer) was less stable than the chemical from Alfa (with 400 ppm phenothiazine) for adding of more 50% H₂O₂, though both caused small explosions. Unfortunately, Tokarek et al. (2014) did not mention the brand name of supplied chemical.

In the third method, a different stoichiometric ratio was applied for APAN synthesis. The ratio was changed based on the PPN synthesis protocol that was described in the chapter 4, as acryloyl chloride: (H₂SO₄, acid catalyst for peracid): 50 %H₂O₂: H₂SO₄: HNO₃ = 4: (0.1): 1: 6: 3 with or without acid catalyst for peracid. In order to verify the stoichiometric ratio of the PAN-type compound synthesis, PAN was pretested to produce from acetyl chloride using the ratio and resulted in synthesized successfully. However, no APAN was formed from acryloyl chloride with this ratio. Occasionally, the solution became yellow-brown and an amount of precipitate was observed after adding 50 % H₂O₂. Therefore, this method still requires further optimization.

4.2.4.2. APAN Synthesis from Anhydride Compound

Tanimoto and Akimoto (2001) synthesized APAN via the anhydride compound.

Synthesis of Acrylic Anhydride

1) All reagents and glassware were cooled before beginning the experiment. A 500 mL round bottom flask (RBF) with a magnetic stir rod was placed into ice-water bath until half of the flask was immersed. NaCl was added to the ice-water bath to keep the temperature of ice water bath less than 0 °C. A magnetic stir bar shaped like a cross was used to powerfully mix the organic and aqueous solvents.
2) Acryloyl chloride was dispensed into a small vial and the vial was cooled on ice to prevent Acryloyl chloride from reacting with water vapor, as suggested by Tanimoto and Akimoto (2001).

3) 100 mL of dichloromethane (VWR) and 3.5 mL of acrylic acid (Aldrich) were poured in the RBF. 7.5 mL of triethyl amine was added to the mixture while it was stirring.

4) 4 mL of cooled acryloyl chloride was slowly added dropwise into the RBF. The color of the solution changed from colorless to milky yellow.

5) The mixture was stirred for 2 hours at 0 °C or below.

6) The solution was washed twice with cold distilled water using a 500 mL separatory funnel in order to remove HCl in the aqueous phase. Since dichloromethane is denser than water, the target compound would be in the bottom layer.

7) Instead of heating the solution at 50 °C as Tanimoto and Akimoto did (2001), a vacuum rotary evaporator was used to remove dichloromethane from the mix solution. As a result, the brown colored solution that remained contained acrylic anhydride.

8) The aliquots were refrigerated to prevent polymerization. The anhydride functional group was confirmed by IR spectra using a Fourier transform infrared spectroscopy (Nicolet is5, ThermoFisher). Figure 4-8 shows two bands at 1759 and 1796 cm⁻¹ due to anhydride structure.
Figure 4-8. Confirmation of Anhydride Group of Synthesized Acrylic Anhydride in IR Spectra.

**Synthesis of APAN**

9) New 50 mL RBF with a magnetic stir rod was soaked in ice water bath. 40 µL of H₂SO₄ and 300 µL of 50 % H₂O₂ were transferred into a 2 mL vial individually and the vials were cooled on ice.

10) 2 mL of the synthesized acrylic anhydride was poured into the RBF.

11) A total of 40 µL of H₂SO₄ was slowly dropped into acrylic anhydride while stirring.

12) A total of 300 µL of 50 % H₂O₂ was slowly added to the mixture. The solution became yellow-brown color and a certain amount of brown precipitate occasionally appeared at this step as a result of polymerization.

13) The solution was stirred for 2 hours until the ice water bath warmed to room temperature.
14) The melted ice water bath was replaced with fresh ice. 360 µL of H$_2$SO$_4$ and 360 µL of HNO$_3$ in separate 2 mL vial were cooled on ice.

15) 2 mL of cooled dodecane (campany) was added into the mixture.

16) A total of 360 µL of H$_2$SO$_4$ was slowly added dropwise to the side of RBF, and then total 360 µL of HNO$_3$ was dropped down in the same way at nearly 0 °C. The mixture was stirred for extra 15 minutes.

17) The synthesized APAN in the organic layer was washed with cold deionized water at least five times using a separatory funnel.

18) Anhydrous MgSO$_4$ powder was added the separated organic solution to remove any residual water and the solution was filtered through a cotton plug.

19) Around 1 mL of liquid synthesized APAN was transferred to a diffusion cell, which was kept in a liquid nitrogen container filled with ice, for GC-ECD measurement.

20) The property of PAN-type compound, thermal-decomposition was confirmed by passing through a heated stainless coil, which was downstream of the diffusion cell like Figure 4-9. Gas-phase APAN from the synthetic liquid standard was delivered from the diffusion cell with ZA at 50 sccm.
Since the surface of the 85 cm stainless coil scrubbed almost all of the synthetic liquid APAN, 33 cm length of the stainless coil was used for testing thermal decomposition. The red GC chromatogram trace line in Figure 4-10 was synthetic APAN flowed through the cooled coil on ice. In contrast, the blue trace line was synthetic APAN flowed through the heated coil at the boiling water temperature (100 °C). Comparison of the two traces show that heating treatment resulted in loss of the peak of synthetic compound. Therefore, the synthetic compound was confirmed as a PAN-type compound.
Figure 4-10. Property of Thermal Decomposition of Synthetic Liquid APAN. 33 cm of stainless coil was heated by boiling water.

4.2.4.3. GC Retention Time of Synthetic Liquid APAN vs. Gas-phase APAN from Acrolein Oxidation with NOx

The synthetic liquid APAN was confirmed by comparing the GC retention time (RT) of gas-phase APAN, which was produced from a photochemical reaction of acrolein with NOx in a smog chamber experiment (e.g. Jaoui et al., 2014). Both APAN sources needed to be loaded to GC-ECD at the same time to verify whether the RT were the same. To load both sources simultaneously, the synthetic liquid APAN in a diffusion cell was connected to the line from the chamber to GC-ECD by a tee fitting as shown in Figure 4-11. The liquid-synthesized compound was carried with ZA at 25 sccm to the bypass. Since the pump, which was installed downstream of the GC-ECD, pulled 50 sccm air, the balance 25 sccm gas was pulled from the chamber. For producing APAN in the chamber, 0.3 µL (1502 ppbv) acrolein (contains hydroquinone as stabilizer, 90 %, Sigma-Aldrich) and 15 µL (206 ppbv) NO₂ were injected into 72.7 L teflon bag.
with 50 % RH. Photochemical reaction was started after keeping the bag for 1 hour in darkness. The results in Figure 4-12 show that the RT of synthetic liquid APAN (red trace line in the chromatogram) matched the RT of gas-phase APAN from acrolein photooxidation.

Figure 4-11. Diagram of Experiment for Comparing GC Retention Time for Liquid APAN and Gas-phase APAN by Photooxidation. Samples were derived from diffusion cell and photochemical reaction chamber.
Figure 4-12. Comparison of the Retention Time of Synthetic Liquid APAN with Gas APAN from Acrolein Photooxidation.

4.2.4.4. EI Mass Spectra of Synthetic Liquid APAN

The synthetic liquid APAN from Section 4.2.3.3. was not purified enough for NMR analysis. The mass spectrum of APAN (molecular mass: 133) using electron impact (EI) has not been reported yet, while that using chemical ionization (CI) was reported by Tanimoto and Akimoto (2001) and CIMS was not available at WMU. The EI mass spectrum of the synthetic APAN was obtained by Tim Glidewell from our group. The experiment was conducted using a GC-MS (HP 6890/MSD 5973) and the conditions were optimized based on the established method by Bertman and Roberts (1991) for avoiding the loss of compound due to thermal decomposition by GC system. The m/z 30, 46, and 55 were obtained from the synthesized compound and these indicated characteristic major peaks from APAN fragmentations, as shown in Figure 4-13.
Consequently, the synthetic liquid compound was verified as APAN because it thermally decomposed, had the same GC retention time as gas-phase APAN from acrolein photooxidation, and had characteristic m/z fragments of APAN by EI.

Figure 4-13. GC Mass Spectra (EI) of Synthetic APAN with the Mass Fragmentations.

4.2.5. Comparison of GC Retention Time of an APAN-like Compound from Chamber Experiments with APAN Standard

Using the same system as in Figure 4-11, the GC retention time of an APAN-type product from isoprene photooxidation was compared with synthetic liquid APAN. The synthetic liquid APAN was added to a diffusion cell. In the chamber system, 0.3 µL (500 ppbv) isoprene and 14.5 µL (103 ppbv) NOx were injected into a 140.2 L Teflon bag under 50 % RH. The Photochemical reaction was begun after keeping the bag overnight in darkness. The retention
time of unknown PAN-type product was compared with that of synthetic liquid APAN (standard).

4.3. Results

4.3.1. Summary of Chamber Experiment with Various Hydrocarbons of Isoprene Oxidation

- Isoprene, MACR, and MVK

Since Teflon bags for chamber experiments were not completely sealed, the total volume of the chamber was different in each experiment. Therefore, to compare the results from different chamber experiments, the amount of APAN produced was standardized by the amount of PAN produced, which was observed from all experimental conditions. The peak area ratios of \([\text{APAN}]/[\text{PAN}]\) from Isoprene, MACR, and MVK with high concentration NOx under 50% RH are shown in Figure 4-14. The same amount of APAN and PAN were formed from isoprene photooxidation reactions. However, the amount of APAN was small when starting from MACR and MVK photooxidation reactions. Experiments No. 6 and 13 (in Table 4-1) with MVK did not show APAN formation (or below detection limit). Therefore, APAN was formed from isoprene without going through MACR or MVK.
Figure 4-14. The Timeseries of Ratio of APAN/PAN from Chamber Experiment with Isoprene, MACR, and MVK Under High NOx Condition with 50% RH. The exp. with number responds to the chamber experiment in Table 4-1.

- Glycolaldehyde

Since the molecular mass of HPAN (MW 137) is close to that of APAN (133) and HPAN is a product of isoprene photooxidation, the retention time of HPAN was confirmed with glycolaldehyde. Figure 4-15 shows the GC retention time of HPAN was just before the retention time of PPN and was completely separated from the retention time of APAN. Therefore, an APAN-type compound is solo component. (Note: since the detector of GC-ECD was replaced with another one after July 2013, the RT of PANs in the figure was different from that at Purdue University and SOAS 2013 campaign.)
4.3.2. Thermal Decomposition of an APAN-like Compound from Chamber Experiment of Isoprene Photooxidation

APAN observed from isoprene oxidation under the conditions of Experiment 7, shown in Table 4-1 was tested for decomposition. The red trace line in Figure 4-16 shows the chromatogram of PAN type compounds from the chamber 95 minutes after lights were turned on. The air went through the 85 cm stainless steel coil at room temperature before loading to the GC-ECD. The black-blue trace line in Figure 4-16 (a) shows the chromatogram of the compounds after passing through the heating 85 cm coil. The results show the diminishment of the APAN-like compound by heating. Therefore, the unidentified compound from isoprene oxidation was confirmed as a PAN type compound due to its thermal decomposition. (Note: Some amount of
PAN still remained by heating with the 85 cm coil. But essentially all PAN was thermally decomposed using a 230 cm coil, which provides long residence time. See Figure 4-16 (b).

Figure 4-16. Property of thermal decomposition of products from isoprene oxidation. (a) 85 cm or (b) 230 cm of stainless steel coil was heated by boiling water.
4.3.3. Comparison of RT of APAN-like Compound from Isoprene Oxidation vs. Liquid APAN Standard

APAN produced from isoprene oxidation in the chamber experiment No. 9 (in Figure 4-1) was analyzed by GC-ECD together with the liquid synthesized APAN standard into GC-ECD. The chromatogram in Figure 4-17 shows a single peak, which was larger than PAN, at the same retention time to liquid APAN standard by itself. According to Figure 4-14, the amount of APAN to PAN was close to a 1 to 1 ratio. Hence, the single peak was sum of the APAN-like compound from the chamber and liquid APAN standard. This is further evidence to indicate that the unidentified compound from isoprene oxidation is APAN.

Figure 4-17. Comparing the Retention Time APAN-like Compound from Isoprene with Liquid APAN Standard.
4.4. Discussion

4.4.1. Verification of APAN

The tentatively identified APAN peak showed thermal decomposition at the same rate as PAN (theoretically, more than 98% of PAN-type molecules are diminished within the residual (or transit) time in the heating stainless coil). The order of GC retention time (RT) of the APAN-like compound matched that of the RT reported from the field APAN studies during TexAQS 2000 (Roberts et al., 2001), which was measured using a similar instrument system and the order of elution was between PAN and PPN. When the RT ratio of APAN (or like)/MPAN was standardized by the RT of PAN as the origin in Eq. 4-5,

\[
\frac{RT_{APAN \; or \; like}}{RT_{MPAN}} = \frac{RT_{APAN \; or \; like} - RT_{PAN}}{RT_{MPAN} - RT_{PAN}} \quad (Eq. \; 4-5)
\]

it was 0.259 at the Purdue University and 0.263 at WMU. These RT ratios from both places also almost matched the ratio from TexAQS 2000, which was 0.258. In addition, the RT of the APAN-like compound from isoprene photooxidation matched the RT of synthetic liquid APAN. Consequently, this evidence supports the identification of the compound as APAN. However, because APAN production from isoprene photooxidation under high NOx conditions has not been discussed in the literature, it is important to confirm the mass spectrum of the product from the chamber experiment.

4.4.2. Field APAN in SOAS 2013

APAN was occasionally observed during the SOAS campaign (Chap 3), and the timeseries of APAN during SOAS 2013 is shown in Figure 3-14 with other PANs. Since an APAN standard was not available at the time, the concentration of APAN was estimated using
the calibration curve for PAN in the campaign. Unlike the laboratory experiments, the APAN level during SOAS 2013 was much lower than the PAN level and was very often below the detection limit (1.8 pptv).

In order to assess the influence on the field APAN from anthropogenic and biogenic sources, the relationship between APAN and PPN (from anthropogenic precursors) or MPAN (from biogenic precursor) during SOAS 2013 is plotted in Figure 4-18, without the data that was below the detection limit. Only APAN from June 4th was strongly correlated to PPN, which is derived from anthropogenic VOC. A local biomass burning was reported on the day (Washenfelder et al., 2015). Generally, APAN during SOAS 2013 did not show a positive correlation with PPN by Pearson test ($r=-0.221$, $p=0.072$) and Spearman’s rank correlation test ($\rho=-0.314$, $p=0.010$), although APAN was expected to arise from other anthropogenic sources (Note: data from June 4th was excluded). Similarly, MPAN from June 4th is positively correlated to APAN, but the ratio of MPAN to APAN was less than PPN to APAN. MPAN from the rest of the days did not show a significant statistical relationship with APAN, Pearson test ($r=-0.029$, $p=0.803$) and Spearman’s rank correlation test ($\rho=0.045$, $p=0.701$). Relationship with biogenic precursors, like the smog chamber experiment of isoprene photooxidation, was observed on June 12th during the campaign, shown in Figure 4-19. There might be other days that showed biogenic influence, however, the field instrument often experienced very bad baseline noise on the GC that totally obscured the peak. APAN was observed with a small amount of MPAN in the absence of PPN. The maximum isoprene level was 9 ppbv during the day and the level was not exceptional during the campaign. In addition, concentrations of NOx and CO were low. Hence, there is a question why APAN with biogenic chemistry was observed only on June 12th. Another issue is why the field APAN concentration was 10x lower than the PAN concentration when
smog chamber experiments showed a significant amount of APAN from isoprene photooxidation.

Figure 4-18. The Correlation of APAN with PPN or MPAN During the SOAS 2013. Data on June 4th, which had a local biomass burning, was separately plotted.

Figure 4-19. APAN Presence with Biogenic Chemistry During the SOAS 2013.
4.4.3. Comparison of Gas-phase APAN with Its Photooxidation Products in Particles

Through a personal communication with Dr. Jason Surratt from the University of North Carolina Chapel Hill, the field Study of APAN was compared with photooxidation products glyceric acid and its corresponding organosulfate derivative in PM$_{2.5}$ (fine particle matter less than 2.5 $\mu$m) during SOAS 2013. The proposed formation mechanism of APAN photooxidation products is shown in Figure 4-20. The mechanistic pathways suggested by Dr. Surratt, is a tentative proposed mechanism for SOA formation from acrolein (Jaoui et al., 2014), and a proposed mechanism for SOA formation from MPAN by Nguyen et al. (2015). Subsequent reaction of APAN with OH radical produces glyceric acid epoxide (GAE) and a lactone. The uptake chemistry of GAE in acidic particles produces glyceric acid and its organosulfate by nucleophilic addition. PM$_{2.5}$ was collected on 2 filters per day, 8 am to 7 pm and 8 pm to 7 am (for an intensive day, 4 filters were set per day: 8 am to 11 am, 12 pm to 3 pm, 4 pm to 7 pm and 8 pm to 7 am). Glyceric acid and its corresponding organosulfate compound were measured using a GC/MS and an UPLC/(-)ESI-HR-QTOFMS, respectively. Timeseries data from our APAN study that was conducted during the SOAS 2013 is shown in Figure 4-21. Each point for glyceric acid and its organosulfate represented the average concentration for each period. The standard deviation was estimated to be approximately 13.8%. Dr. Surratt noticed that these data still needed to be calibrated for quantification, but the timeseries behavior should be the same. Unlike APAN, glyceric acid and its corresponding organosulfate compound were always observed during the SOAS 2013. The magenta line circle in Figure 4-21 is the average APAN for the PM$_{2.5}$ sampling time (note: the APAN data that was lower than the detection limit was ignored). The relationship between the APAN average with glyceric acid or organosulfate was investigated from June 1$^{st}$ to June 14$^{th}$, when APAN levels were high; this data is shown in
Figure 4-22. Both combinations showed a weak relationship in this campaign; the coefficient of determination, $R^2$ in glyceric acid – APAN and in its organosulfate – APAN was 0.012 and 0.078, respectively. Since most of the APAN levels during SOAS 2013 was near or lower than the detection limit, the time resolution of filtration was much different from APAN sampling span. The loss rate of APAN was not considered here, and it was still difficult to evaluate the relationship between APAN in the gas-phase and its photooxidation products in the particle phase. Jaoui et al. (2014) reported field measurements of APAN in the gas phase and glyceric acid as 1,3-butadiene products in both gas and particle phases from several locations around the United States. However, the authors showed the presence of common products in the gas and particle phases, but they did not directly compare the compounds in the two phases. Additional research is required to investigate the proposed mechanism shown in Figure 4-20. Laboratory experiments that determine what reaction initiates APAN photooxidation are needed for the direct comparison between APAN in gas phase and glyceric acid and its organosulfate in particle phase.
Figure 4-20. Glyceric Acid and Its Organosulfate Formations from APAN.

Figure 4-21. Timeseries Comparison of APAN with the Photooxidation Products, Glyceric Acid and Organosulfate of Glyceric Acid During the SOAS 2013. Average APAN is calculated based on the PM 2.5 sampling time.
4.4.4. Future Work for Finding the Mechanism of APAN Formation from Isoprene

4.4.4.1. Isoprene Photooxidation

The difference between isoprene (2-methyl-1,3-butadiene) and 1,3-butadiene is the presence of a methyl group. However, isoprene oxidation is initiated by the addition of an OH radical to one of the C=C bonds in isoprene. It is impossible to obtain 1,3-butadiene from isoprene. Acrolein is a photooxidation product from 1,3-butadiene and the subsequent reactions produce APAN (Tanimoto and Akimoto, 2001). Asatryan and co-workers (2010) mentioned that OH radical addition to acrolein has been relatively well-understood but further reaction with O₂ is still poorly understood. Paulot and co-workers (2009) summarized the current mechanistic pathways of isoprene photooxidation under high NOx. The pathway is branched based on the carbon positions by the combination of additional OH and O₂. The brief pathway of each channel is shown in Figure 4-23 with the branching %. The same author pointed out that β-
hydroxyperoxy channels via branching position (1,2 or 2,1) and (4,3 or 3,4), which account for 71% of pathway from isoprene photooxidation, have been well studied while δ-hydroxyperoxy channels via branching position (1,2 or 2,1) and (4,3 or 3,4), which account for 29% of that, have been less studied. The series of smog chamber experiments in this work demonstrated that APAN was formed from isoprene without going through MACR and MVK formation, which are products from subsequent reactions of β-hydroxyperoxy compounds. However, potential involvement of β-hydroxyperoxy channels to form APAN cannot be completely ruled out. The chemistry of δ-hydroxyperoxy channels contains rapid (E)/(Z) isomerization and results in more complicated pathways. The theoretical study of δ-hydroxyperoxy photoproducts was conducted by Dibble (2002) with computations and a 1,5 H-shift was proposed as dominant fate of (Z) configuration but the (E) analogue cannot undergo the shift. The mechanism with the branching ratio was investigated under low NO conditions by Peeters et al. (2014) and under high NO conditions by Nguyen and Peeters (2015). It is important to monitor the progress of investigations of δ-hydroxyperoxy channels to see if the products involve a 3-C fragment. If APAN is produced from the β-hydroxyperoxy (1,2 or 2,1) channel, the C3=C4 bond would be preserved in APAN and similarly, C1=C2 bond would be for (4,3 or 3,4) channel. If APAN comes from the δ-hydroxyperoxy channels, then the C2=C3 bond would remain. Isotopic labeling of isoprene would be helpful in understanding the hydroxyperoxy channels.
4.4.4.2. Potential of Isoprene Ozonolysis

The timeseries of the isoprene photooxidation experiment conducted at Purdue University in Figure 4-2 shows that high levels of $\text{O}_3$ (~180 ppbv) were produced before APAN was produced. Approximately 2.5 hours after initiating the experiment, $\text{O}_3$ level started slightly decreasing. The $\text{O}_3$ decrease might be related to wall loss on the surface of the chamber (isoprene nitrate also started diminishing around this time). However, the other possibility is that $\text{O}_3$ started being consumed by ozonolysis with alkenes, and the PAN-type compounds were still increasing after 2.5 hours. Hence, it is interesting to consider an ozonolysis reaction as a possible route to APAN. The mechanism of ozonolysis is described by e.g. Atkinson and Arey (2003) and Kroll and Seinfeld (2008). In the atmosphere, ozone attaches to a double bond of an alkene and creates a primary ozonide (POZ). The cleaved POZ produces a carbonyl compound and an energetically excited Criegee intermediate. This intermediate can react bimolecularly to form a stabilized
Criegee intermediate (“SCI channel”) or it can decompose to form an OH and an alkyl radical (“Hydroperoxide channel”).

The latest pathway of isoprene ozonolysis was proposed based on laboratory experiments by Nguyen and coworkers (2016). The experiments showed a very interesting compound, which might be related to APAN via a hydroperoxide channel of isoprene. Their chamber experiments were performed with ~100 ppb isoprene and 600 ppb of ozone under < 4-76%RH. The NO level was close to baseline and NO₂ was less than 5 ppbv during the experiments. Gas-phase organic products were measured using GC-FID and a triple-quadrupole chemical ionization mass spectrometer (CIMS) using CF₃O⁻. The proposed pathway of isoprene ozonolysis is shown in Figure 4-24. The products in red were observed during their chamber experiments. First, two POZ are formed from isoprene by the addition of O₃. Then, each POZ molecule decomposes into two pathways. One pathway forms MACR or MVK with CH₂OO (C₁ Criegee) while the other one forms formaldehyde and syn/anti configurations of MACR-OO* and MVK-OO* (C₄ Criegees). Subsequent bimolecular reactions were observed only from stabilized C₁ Criegee, while C₄ Criegees are unstable and further decompose into OH and alkyl radical products. Nguyen and coworkers proposed the presence of peroxyacryloyl radical, which is circled by red line in Figure 4-24, in the decomposition of syn MVK-OO*, and that compound is the direct acyl radical precursor of APAN. The authors assumed that the compound was based on a similar mechanism as RO₂ radical products with HO₂ in MVK+OH chemistry, which was studied by Praske et al. (2015). Although the peroxyacryloyl radical is still a prospective compound, observation of 3-carbon acryloyl products, which are expected products from further reaction of peroxyacryloyl radical with HO₂, indicates that it is not impossible to form APAN from isoprene. Nguyen’s group conducted these experiments in the absence of NOₓ. If isoprene ozonolysis takes
place under high levels of NO$_x$, then after the syn MVK-OO$^*$ decomposes, the acryloyl radical may react with NO$_x$ and produce biogenic APAN. This hypothetical route is shown in blue in Figure 4-24.

Figure 4-24. Published Proposal Ozonolysis Pathway of Isoprene. Nguyen and co-workers (2016) proposed ozonolysis pathway from isoprene based on their chamber experiments without NO$_x$ presence. Asterisks indicate activated Criegee intermediates. Their observed products are shown in red. My hypothesis pathway under high-NO$_x$ presence are indicated in blue.
This hypothesis could account for the results from our chamber experiments at WMU that showed APAN was formed from isoprene without the formation of MACR and MVK. In addition, this hypothesis explains why a large amount of APAN was obtained from the chamber experiments at Purdue University and WMU while the field levels of APAN was very small, (close to the detection limit) during the SOAS 2013 campaign. The large amount of APAN was produced under tremendously a high level of O₃ in the chamber at Purdue University. In contrast, the concentration of O₃ during SOAS 2013 was low, less than approximately 50 ppbv. The concentration of APAN with O₃ from SOAS is plotted in Figure 4-25 (a). Whenever APAN was observed during SOAS 2013, the PAN level was also relatively low for most days except June 4th when there was local biomass burning. To compare the condition (yield) of field experiments with laboratory experiments, the concentration of APAN and O₃ are standardized by PAN concentration in Figure 4-25 (b). The range of the [APAN]/[PAN] ratio during the SOAS 2013 had various distribution as well as the ratio from the chamber experiment at Purdue University. However, the [O₃]/[PAN] ratio from the chamber experiments was very small and the condition was not observed in SOAS field samples. The APAN from chamber experiments seemed to be sensitive to O₃ as a result of the wider range of [APAN]/[PAN] ratio at the narrow [O₃]/[PAN] ratio. Biogenic APAN from the field observed on June 12 are located at relatively low [APAN]/[PAN] and [O₃]/[PAN] ratios. The relationship between MPAN with O₃ is plotted in Figure 4-26 in the same way. The simple comparison of concentration of MPAN with O₃ in (a) has similar distribution to APAN. However, the [MPAN]/[PAN] ratio in 4-26 (b) shows a narrow range of distribution even though the ratio from the chamber experiment was less than 0.2. These results suggest that MPAN was less sensitive to O₃ than APAN. The line in red in both Figure 4-25(b) and 4-26(b) indicates the regression line during SOAS 2013. The coefficient of
determination from APAN and MPAN were 0.348 and 0.081 respectively. Consequently, O₃ has stronger correlation with APAN, but not with MPAN during SOAS 2013. Mysteriously, the MPAN yield was less than APAN (see Figure 4-2) in the chamber experiments, although MACR has a high branching ratio from isoprene in ozonolysis (see Figure 4-24). However, the chamber experiment at Purdue University was initiated as isoprene photooxidation and the yield of each component might be different from ozonolysis only or both due to a kinetic competition between photooxidation vs. ozonolysis. Hence, further chamber experiment of isoprene ozonolysis under high NOx presence is required to further investigate biogenic APAN formation.
Figure 4-25. The Relationship Between APAN and O$_3$ During SOAS 2013 and in the Chamber Experiment at Purdue University. (a) is a simple comparison of concentration of APAN with O$_3$. (b) is standardized the both concentration by concentration of PAN. The day with circle in blue showed a local biomass burning and in green indicated the presence of expected biogenic influence during SOAS 2013. The line in red indicates the linear regression with the coefficient of determination during SOAS 2013.
Figure 4-26. The Relationship Between MPAN and O₃ During SOAS 2013 and in the Chamber Experiment at Purdue University. (a) is a simple comparison of concentration of MPAN with O₃. (b) is standardized the both concentration by concentration of PAN. The day with circle in blue showed a local biomass burning and in green indicated the presence of expected biogenic influence during SOAS 2013. The line in red indicates the linear regression with the coefficient of determination during SOAS 2013.
4.5. Summary

A significant amount of an unidentified compound was observed from isoprene oxidation chamber experiments. The amount of this compound was almost the same as the amount of PAN, which was the most dominant PAN-type compound under these experimental conditions. According to the series of chamber experiments, the compound was formed without going through MVK and MACR intermediates, which are major first generation product from isoprene oxidation. The order of GC retention time of the compound was between PAN and PPN and the order matched the elution order of APAN from field samples reported by Roberts et al. (2001). The compound level was diminished by heating, a property that is characteristic of a PAN-type compound. Importantly, the RT of the compound matched the RT of the synthetic APAN standard. Therefore, this evidence supports that the unknown compound is APAN. However, APAN has been known to be derived from anthropogenic hydrocarbon. Hence, there is still a challenge to figure out the appropriate mechanism of APAN formation from isoprene.

APAN was occasionally observed during the SOAS 2013 field campaign in Alabama. Chapter 4 concluded that biogenic influence was dominant during the SOAS 2013, meaning that isoprene oxidation played an important role there. However, the level of APAN observed in the field was near or below the detection limit. APAN did not statistically correlate with PPN or MPAN. In addition, APAN did not statistically correlate to its proposed oxidation products, glyceric acid or organosulfate compounds in particle form. However, the low concentration of APAN during SOAS 2013 was not enough to evaluate these relationships statistically. Currently, the mechanism of SOA formation from APAN has been proposed based on the mechanism of that from MPAN. Hence, it is important to study the direct relationship between gas phase APAN and SOA formation with their intermediate compounds.
Since APAN has been known to be a product from anthropogenic hydrocarbon, it would be critical to demonstrate that isoprene also forms APAN. Hence, although the evidence from this work suggests that this occurs in the atmosphere, further research is required to find the appropriate mechanism and pathway of APAN formation from isoprene. Ozonolysis of isoprene might be key to obtain peroxyacryloyl radical from isoprene photooxidation.

4.6. References


APPENDIX A

Sampling Information for Extra Trees in 2010 and 2011

Note: the information for continuous research white pine trees since 2008 were described in my master’s thesis (2010).

UMBS in 2010

From Ameriflux (Sampling Day: 7/21/2010).

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<th>DBH, cm</th>
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<th>Vegetation</th>
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<td>A1</td>
<td>N45 33.640 W84 42.817</td>
<td>Med</td>
<td>21</td>
<td>5.3</td>
<td>&gt;4</td>
<td>Aspen canopy, White pine</td>
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<tr>
<td>A2</td>
<td>N45 33.773 W84 42.787</td>
<td>Low</td>
<td>13</td>
<td>2.3</td>
<td>&gt;2</td>
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<td>1.4</td>
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<tr>
<td>A4</td>
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<td>25</td>
<td>4</td>
<td>&gt;3</td>
<td>Aspen canopy</td>
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<tr>
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From FASET (Sampling Day: 7/22/2010).

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<th>DBH, cm</th>
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<td>3.5</td>
<td>&gt;3</td>
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<td>9</td>
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<td>1.6</td>
<td>Maple canopy, White pine</td>
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<td>3.5</td>
<td>2.5</td>
<td>Maple canopy, White pine</td>
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<td>Near S64</td>
<td>Low</td>
<td>24</td>
<td>3.8</td>
<td>2.5</td>
<td>Aspen, Maple, White pine</td>
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<td>1.5m NW from S71</td>
<td>Low</td>
<td>24</td>
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<tr>
<td>F10</td>
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<td>21</td>
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<td>1.7</td>
<td>Aspen, Maple, White pine</td>
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### Extra Canopy Trees from Ameriflux (Sampling Day: 7/24/2010).

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<td>N45 33.550 W84 42.852</td>
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### Red Pine Trees in UMBS (Sampling Day: 7/24/2010).

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<th>Vegetation</th>
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<td>4</td>
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<td>4.7</td>
<td>3</td>
<td>Red pine canopy, Maple</td>
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<tr>
<td>R5</td>
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<td>Med</td>
<td>31</td>
<td>2.5</td>
<td>2.6</td>
<td>Red pine canopy</td>
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</table>
Other Forest in MI in 2011

Hartwick Pines State Park (Sampling Day: 8/1/2011).

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<th>DBH, cm</th>
<th>Height, m</th>
<th>Vegetation</th>
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</thead>
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<td>H1</td>
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<td>1.7</td>
<td>white pine canopy, Maple, birch</td>
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<tr>
<td>H3</td>
<td>N44 44.574 W84 39.373</td>
<td>High</td>
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<td>2.3</td>
<td>White pine canopy, Oak, Maple, Birch</td>
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<td>3.5</td>
<td>White pine canopy, Oak, Maple, Birch</td>
</tr>
<tr>
<td>H5</td>
<td>N44 44.595 W84 39.373</td>
<td>High</td>
<td>9.7</td>
<td>&gt;8</td>
<td>White pine canopy, Oak, Maple, Birch</td>
</tr>
<tr>
<td>H6</td>
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<td>High</td>
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<td>&gt;13</td>
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</tr>
<tr>
<td>H7</td>
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<td>&gt;13</td>
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<tr>
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<tr>
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<td>White pine canopy, Scotch pine</td>
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<tr>
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<td>Med</td>
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<td>&gt;15</td>
<td>White pine canopy, Scotch pine, Maple</td>
</tr>
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</tr>
<tr>
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<td>&gt;10</td>
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A forest in Crawford County, Grayling (Sampling Day: 8/2/2011, heavy rain).

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<td>19.9</td>
<td>Burnt tree</td>
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Light environment for all trees was high. Vegetation: aspen, red pine, oak, maple, and lots of white pine trees.

<table>
<thead>
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<th>Sample</th>
<th>Position</th>
<th>Light</th>
<th>DBH cm</th>
<th>Vegetation</th>
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Fort Custer State Park (Sampling Day: 9/1/2011).

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</table>
APPENDIX B

Branch Enclosure Experiments in 2010

During August 20\textsuperscript{th} - 23\textsuperscript{rd} in 2010, a branch enclosure experiment was conducted in FASET for measuring BVOC emission from white pine needles of tree S71(high limonene), F9 (high germacrene D-4-ol), and F10 (high α-pinene) with cooperation from Dr. Detlev Helmig and his graduate student, Ryan Daly (University of Colorado). Ortega et al. (2008) described the system and methods in detail. A Teflon bag covered a branch with a couple of layers of needles from the tip and two Teflon tubes were connected to the bag. One tube was used to purge the bag inside with ambient air to remove water (moisture), then the air in the bag was sucked through another tube and passed to a cartridge, in which TENAX GR and carbotab were packed to adsorb terpenes or isoprene respectively. Several cartridges (up to 10) were installed in Autosampler, a customized instrument that was equipped with a timer, so that each cartridge continuously adsorbed the compounds every one or two hours during the daytime. Afterward, the adsorbed cartridges were sent to the laboratory of Helmig’s group while kept on dry ice to prevent the loss of compounds from thermal desorption. At the lab, BVOC emission from the white pine needles were analyzed using a GC-FID and a GC-MS with thermal desorption system. However, the results of chromatograph did not show either terpenes and isoprene. This may have been due to insufficient training of the technician and or by an unoptimized GC protocol. Additionally, a contact failure in the electric circuit could have caused no response of the Autosampler.

Reference
APPENDIX C

Optimization of Complementary DNA Cloning to Express Chemotypic Variation

This Section describes the strategy and optimized methods to investigate genetic variation within one species, white pine (*Pinus strobus*), to explain the chemotypic variation of BVOC in a population. From this section, RNA extraction from white pine needles was optimized starting from Azevedo’s method (2003). Four sets of primers were designed based on sugar pine sequences; two sets intended for $\alpha$-pinene synthase (AP1 and AP2) and another two sets for limonene synthase (LN1 and LN2). AP2 and LN2 worked to synthesize cDNA, but both had multiple products in electrophoretic analysis. The products, which roughly matched expected size, were isolated using agarose gel extraction and were put in a TOPO expression vector. The close sequencing of the plasmids was searched from GenBank database. The results suggested that the AP2 primers amplified some sort of chromosomal sequence of white pine (*Pinus strobus*) and did not match at all with any terpene synthases. More colonies from AP2 may need to be screened to obtain plasmid DNA, which produces terpene synthase. On the other hand, the LN2 primers resulted in some sort of terpene synthase, which was likely $\alpha$-pinene synthase or limonene synthase. Further optimization is needed to confirm whether $\alpha$-pinene synthase or limonene synthase is produced with enzyme assays in future work. Then, the hypothesis that chemotypic difference in Table 2-10 for white pine tree population in the UMBS would be derived from genetic expression will be tested using these established methods.
C.1. Strategy for Getting Protein Expression

The central dogma of molecular biology that is shown in Figure C-1 is the process of how genetic information transfers and manifests in biological systems (Crick, 1970; e.g. Farrell, 2010). Through transcription, genomic sequences of DNA are converted to messenger RNA (mRNA) by an RNA polymerase. In the ribosome, amino acids, which are transported by transfer RNA (tRNA), makes an assembly of polypeptide linkages (protein synthesis) based on the codon of the mRNA. This process is known as translation. Consequently, if the DNA sequences of α-pinene synthase and limonene synthase from the four groups at UMBS are determined and the resulting enzyme activity and/or gene expressions demonstrate the bimodal behavior, as suggested in the hypothesis in Table 2-10, this would be evidence to conclude that the chemotypic difference observed at UMBS arise from a genetic basis.

![Figure C-1. Central Dogma of Molecular Biology.](image)

The experimental approach was designed based on the method by Bohlmann et al. (1999), as illustrated in Figure C-2. Bohlman et al. (1999) worked on the expression of new monoterpene synthases from grand fir trees (Abies grandis) using cDNA cloning. Since there are no entries for
the DNA sequence of terpene synthase from the white pine in the GenBank database, extraction of pure RNA from white pine needles was needed to get DNA sequences. Complementary DNA (cDNA), which contains only exons (the portions of DNA that code for proteins), is created based on extracted RNA using reverse transcription and appropriate primers. cDNA can be confirmed through agarose gel electrophoresis. The desired DNA band is extracted from the gel by cutting, purified, ligated with a vector and then transformed into \textit{E. coli} to create plasmid DNA. The plasmid DNA is then sequenced. The synthases are assayed with GPP, a precursor of monoterpene, for terpene production. The products are identified using a chiral phase gas chromatography and mass spectroscopy (GC-MS). In this work, optimization studies were conducted up to the process of sequencing of plasmid DNA, but total expression was not achieved.
Figure C-2. Experimental Approach for Cloning and Expression Monoterpene Synthases from White Pine Needles Based on the Method by Bohlmann et al. (1999).
C.2. Experimental and Results

C.2.1. Sample Collection for the Optimization

The one-year-old needles were collected from white pine trees that were used for the experiments in Chapter 2 and located near the HAENICKE in Figure 2-1 on the campus of Western Michigan University. RNase (ribonucleases) degrades RNA. This enzyme is ubiquitous, including on skin cells (e.g. on hands) and is hard to deactivate even by autoclaving. Hence, all tools were wiped with RNase ZAP (Sigma-Aldrich) before sampling. Wiped Nitrile groves were worn during sampling. One bundle of needles was taken from the base of needles and was transferred into 1.7 or 2.0 mL RNase/DNase free micro-centrifuge tube (VWR). The tube was immediately frozen in liquid nitrogen. The tubes were kept in a -80 °C freezer until the RNA was extracted.

C.2.2. Optimization of RNA Extraction

C.2.2.1. Challenge of RNA Extraction from Pine Needles

RNA extraction using a commercial RNA extraction kit, RNeasy Mini Kit (QIAGEN), did not work on white pine needles. Rich phenolics, polysaccharides, and endogenous RNases in gymnosperm species make it hard to isolate intact and high-quality RNA (Azevedo et al., 2003). Phenolics easily oxidize and bind to nucleic acids (Loomis, 1974) and secondary metabolites hinder resuspension of precipitated RNA and enzymatic manipulation as well as interfere with absorbance-based quantification as contaminations (Wilkins and Smart, 1996). Azevedo et al. (2003), who worked on isolation of RNA from needles of adult maritime pine trees (Pinus pinaster), overcame this problem using proteinase K, an enzyme that cleaves proteins and
inactivates endogenous RNases. Hence, RNA extraction from white pine needles was optimized based on Azevedo’s method.

C.2.2.2. Preparation of Regents

All tools were wiped by RNase ZAP and were autoclaved except volumetric flasks, which were only wiped with RNase ZAP.

- 0.1% DEPC water
  Diethylpyrocarbonate (DEPC, Sigma-Aldrich) is an inhibitor of RNases. 1 mL of DEPC was added to a 1000 mL volumetric flask and diluted with milli-Q water. The solution was incubated at 37 °C overnight while shaking using a G24 Environmental Incubator Shaker (New Brunswick Scientific Co. INC, USA). Then, the solution was transferred to a bottle (Pyrex Media Bottle, VWR) and autoclaved to remove DEPC until the smell was no longer detected. Each bottle with the solution was autoclaved at least twice.

- 10 M LiCl
  42.40 g of lithium chloride (ACS reagent, Sigma) was placed in a 100 mL volumetric flask diluted with 0.1% DEPC water. The solution was transferred to another bottle (VWR) and autoclaved. This solution was kept in a refrigerator until RNA extraction was performed.

- 2 M LiCl
  10 M of LiCl solution was diluted to 1/5 with 0.1% of DEPC water. The solution was transferred to another bottle (VWR) and autoclaved. This solution was kept in a refrigerator until performing RNA extraction.
• 1 M Tris-HCl (for buffer solution)

12.12 g of tris (hydroxymethyl) aminomethane (Tris) (Ultra Pure™, Invitrogen) was placed in a 100 mL volumetric flask was diluted with 0.1% DEPC water. Hydrochloric acid (37%, EMPROVE, German) was added to solution until the pH reached 8.0. The solution was transferred to a glass bottle and autoclaved.

• 0.5 M EDTA (for buffer solution)

EDTA is not water soluble unless the pH reaches at least 8.0. Since the probe of a pH meter could not be placed into a 100 mL volumetric flask, 18.61 g EDTA (Disodium Salt Electrophoresis Grade, Fisher Scientific) was dissolved in approximately 90 mL of 0.1% DEPC water using a beaker. Roughly 2.3 g NaOH pellets (Mallinckrodt Specialty Chemical Co.) was gently added to the solution to adjust the pH to 8.0 while stirring. After EDTA was dissolved, the solution was transferred to a 100 mL volumetric flask and the flask was filled up to 100 mL with 0.1% DEPC water. The solution was transferred to a glass bottle and autoclaved.

• Extraction buffer

Extraction buffer was composed of 100 mM Tris-HCl, 2% (w/v) CTAB, 30 mM EDTA, 2 M NaCl, and 0.05% spermidine.

1) 60 mL of 0.1% DEPC water was poured in a small beaker on a hotplate stirrer. 11.69 g NaCl (minimum 99.5%, Sigma-Aldrich) was dissolved in the solution while stirring with heat.

2) 2g cetyltrimethyl ammonium bromide (CTAB) (Sigma-Aldrich) was gently added to the solution. CTAB was difficult to dissolve in water at room temperature. The solution was heated to more than 65 °C.
3) 10 mL of 1 M Tris-HCl was added to the solution.

4) 50 mg of spermidine (Sigma-Aldrich) was added to the solution.

5) The solution was transferred to a 100 mL volumetric flask and the flask was filled to 100 mL with 0.1% DEPC water.

C.2.2.3. RNA Extraction

The RNA extraction procedure was adjusted for 100 mg of pine needles, scaled down to 1/10 of Azevedo’s method.

1) 1.15 mL extraction buffer was poured into a 2 mL RNase/DNase free micro-centrifuge tube (VWR). 30 μL of 2-mercaptoethanol and 3 mg proteinase K (Sigma-Aldrich) were added to the buffer solution. Note: in Azevedo’s method, polyvinylpolypyrrolidone (PVPP) was added at this step. However, PVPP, a cross-linked form of polyvinylpyrrolidone (PVP), was not easy to handle because it was insoluble in water, even with heating, and easily formed bubbles due to its detergent nature. Hence, PVPP was directly added to the sample of needles at step 4.

2) The mixed buffer solution was incubated for 10 min at 42 °C.

3) Frozen white pine needles, which were stored in a 2 mL RNase/DNase free micro-centrifuge tube (VWR), was removed from a -80 °C freezer. A tiny amount of liquid nitrogen was poured into the tube after the cap was opened. The needles were ground to a fine powder using an autoclavable pellet pestle rod, the tip of which fits into the micro-centrifuge tube. Note: the particle size of the powder is very crucial to extract RNA effectively. When the particles were larger, the RNA yield was very poor or not detected.
4) 30 mg PVPP (Sigma-Aldrich) and the mixed buffer solution were added to the powdered needles. After the cap was closed, the tube was vigorously mixed using a vortex mixer to ensure that all ground needles were in solution, because the needles were stuck to the bottom of the tube by grounding with the rod.

5) The tube was incubated for 90 min at 42 °C.

6) The same volume (1.15 mL) of SEVAG, (24:1 v/v chloroform-isoamyl alcohol) was added to the tube containing the buffer and the needles. Note: because this addition caused the volume of solution to be greater than the 2 mL micro-centrifuge tube size, the mixture was separated into two tubes. The transfer of the needles and buffer required that the tip of a pipette tip was cut off to facilitate the transfer.

7) The tubes were vigorously mixed using the vortex mixer and were centrifuged at 15,000 g for 15 min at 4 °C in order to separate the different phases.

8) The top aqueous phase was transferred to new 2 mL micro-centrifuge tubes using a pipette.

9) Steps 6 – 8 were repeated. For this repeat, 0.575 mL SEVAG was used.

10) Around 160 µL of 10 M LiCl was added to the top phase and was vigorously mixed using the vortex mixer. Note: Azevedo’s method did not mention using vortex mixing at this step, however, doing so resulted in a higher RNA yield.

11) The solution in the tube was stored at 4 °C overnight to allow precipitation.

12) The tube was centrifuged at 15,000 g for 25 min at 4 °C. The supernatant was discarded.

13) The precipitated pellet was washed with 200 µL of 2 M LiCl and was centrifuged at 15,000 g for 25 min at 4 °C.

14) The supernatant was discarded and the pellet was dissolved in 50 µL of 0.1% DEPC water.
C.2.2.4. Confirmation of RNA concentration and Purity

RNA concentration and purity were determined using a UV-Vis Spectrophotometer and electrophoresis.

- **UV-Vis Spectrophotometer**

  1 μL of extracted solution was dropped on a UV-Vis Spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc.) and RNA concentration was measured using the RNA mode (260 nm). Contamination by polysaccharides was checked using absorbance ratios, $A_{260}/A_{230}$ and $A_{260}/A_{280}$ (Azevedo et al., 2003). The ideal range of the ratios of DNA and RNA by Farrell (2010) are listed in Table C-1. The out of the range of the ratio suggests the presence of contamination and requires the sample to be washed using the ethanol precipitation technique.

<table>
<thead>
<tr>
<th>absorbance ratios</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{260}/A_{280}$</td>
<td>1.8±0.1</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>$A_{260}/A_{230}$</td>
<td>2.0 – 2.4</td>
<td>2.0 – 1.8</td>
</tr>
</tbody>
</table>

The values were referred from Farrell, 2010.

- **Electrophoresis**

  1) 1×TAE (Tris-Acetate-EDTA mixture) buffer solution were poured into a gel electrophoresis box until the solution covers the gel. Note: when 50×TAE was diluted to 1×TAE, 0.1% DEPC water was used as the buffer solvent to prevent RNA degradation.

  2) 1.5% agarose gel tray, which was made by UltraPure™ Agarose (Invitrogen), was immersed into the buffer solution.
3) 3 µL of a loading dye was dropped on a hydrophobic sheet such as parafilm (Sigma-Aldrich). 5 µL of extracted solution was mixed with the dye drop using a pipette. 100 bp DNA ladder (BioLabs Inc., New England) was used as a molecular size standard and was mixed with the dye in the same fashion.

4) The mixtures were gently transferred into each well in the gel tray avoiding bubbles. The standard ladder was placed in the first lane.

5) Electrophoresis was run at approximately 94 V for 20 min.

6) The gel tray was taken out of the electrophoresis box and was soaked in EtBr solution (1 drop of EtBr/50 mL D.W.) for 10 min.

7) RNA fragments were observed using a gel imaging system, Gene Wizard Syngene (Synoptics Ltd., Made in UK).

Representative electrophoretic analysis for isolated RNA from white pine (Pinus strobus) needles is shown in Figure C-3. RNA was confirmed by two characteristic bands, 28S and 18S rRNA.
Figure C-3. Representative Electrophoretic Analysis of Isolated RNA from White Pine (*Pinus strobus*) Needles. The 73 lane was a cox sample from Dr. Barkman’s group and used as positive control.

C.2.3. Primer Design

Primers for creating cDNA were designed by Noah Sorrelle from Dr. Barkman’s group based on loblolly pine (*Pinus taeda*) and sugar pine (*Pinus tabuliformis*). The source of genes and the procedures using software are described later. All designed primer sequences are listed in Table C-2. Designed primers were ordered via http://www.idtdna.com. First of all, the mRNA sequences for the α-pinene synthase gene was designed based on loblolly pine. Only WP3 primers worked on cDNA production. Sugar pine (*Pinus tabuliformis*) is phylogenetically close to white pine
(Abramova, 2002). The primer sequences for the α-pinene and limonene synthase genes were redesigned using the sequences of sugar pine and AP2_SG and LN2_SG primers worked to create cDNA.

Note: the primers were supplied as fine salt in a tube. Water was added to create a 100 µM solution. (Note: the volume of water was needed to calculate based on moles of oligo, which is provided by a primer company, Integrated DNA Technologies, Inc.) Then, the tube was vigorously mixed using a vortex mixer and was briefly centrifuged. The solution was stored in a freezer at -20°C until RT-PCR. Primer was diluted to 10 µM, when it was used for the reaction.
Table C-2. Designed Primers for α-Pinene and Limonene Synthase of White Pine.

<table>
<thead>
<tr>
<th>Source</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Conc.</th>
<th>Tm</th>
<th>Test Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene (Pinus taeda)</td>
<td>GPS Forward</td>
<td>5'AGCTCTTCTGTTTCTGT GATCTCGTTGC3'</td>
<td>45.2</td>
<td>60.3</td>
<td>11/18/2011 N/P</td>
</tr>
<tr>
<td></td>
<td>GPS Reverse</td>
<td>5'AGCTTTAATAAGTGACA GGATCAATGACGTTCTC3'</td>
<td>32.5</td>
<td>60.8</td>
<td>(1194 bp)</td>
</tr>
<tr>
<td>α-Pinene (Pinus taeda)</td>
<td>WP2 Forward</td>
<td>5'AGCTCTTCTGTTTCTGT GATCTCG3'</td>
<td>33.4</td>
<td>56.4</td>
<td>1/20/2012 N/P</td>
</tr>
<tr>
<td></td>
<td>WP2 Reverse</td>
<td>5'TTATAAAAGTGACAGGAGA TCAATGACGTTCC3'</td>
<td>26.2</td>
<td>56.8</td>
<td></td>
</tr>
<tr>
<td>α-Pinene (Pinus taeda)</td>
<td>WP3 Forward</td>
<td>5'ACGTCCTCGAGTATGG TTGG3'</td>
<td>33.2</td>
<td>56.5</td>
<td>1/20/2012</td>
</tr>
<tr>
<td></td>
<td>WP3 Reverse</td>
<td>5'CGTATCCCTCGTAATCAA3'</td>
<td>32.8</td>
<td>53.7</td>
<td>✓</td>
</tr>
<tr>
<td>α-Pinene (Pinus lambertiana)</td>
<td>AP1_SG_F</td>
<td>5'ATGGACGAGGCTGAAA CTT3'</td>
<td>36.7</td>
<td>56.6</td>
<td>5/14/2012 N/P</td>
</tr>
<tr>
<td></td>
<td>AP1_SG_R</td>
<td>5'TTATAAAGTCACAGGAGA TCAACGAG3'</td>
<td>26.6</td>
<td>52.5</td>
<td>(1179 bp)</td>
</tr>
<tr>
<td>α-Pinene (Pinus lambertiana)</td>
<td>AP2_SG_F</td>
<td>5'ATGTCGGGGGGCGAGGGA AA3'</td>
<td>25.3</td>
<td>59.4</td>
<td>5/14/2012 ✓</td>
</tr>
<tr>
<td></td>
<td>AP2_SG_R</td>
<td>5'TCAGAGCATCTCTCCTC TGTG3'</td>
<td>29.3</td>
<td>54.3</td>
<td>(1524 bp)</td>
</tr>
<tr>
<td>Limonene (Pinus lambertiana)</td>
<td>LN1_SG_F</td>
<td>5'ATGCCGCAGGCAACGGA AAT3'</td>
<td>28</td>
<td>58.5</td>
<td>5/14/2012 N/P</td>
</tr>
<tr>
<td></td>
<td>LN1_SG_R</td>
<td>5'TCAGTGATCATGGCCGT TG3'</td>
<td>26.9</td>
<td>52.6</td>
<td>(1662 bp)</td>
</tr>
<tr>
<td>Limonene (Pinus lambertiana)</td>
<td>LN2_SG_F</td>
<td>5'ATGCCAGGAGGCTGAA ATCTT3'</td>
<td>35.1</td>
<td>53.6</td>
<td>5/14/2012 ✓</td>
</tr>
<tr>
<td></td>
<td>LN2_SG_R</td>
<td>5'TTACATAGCCACAGGT TGAAG3'</td>
<td>32.1</td>
<td>54.7</td>
<td>(1174 bp)</td>
</tr>
</tbody>
</table>

Conc. means concentration of the primer oligo (nMoles). Tm is the melting temperature (°C) in 50 mM NaCl solution. N/P indicates no production. The checked mark indicates the primer which produces cDNA. Parentheses indicates the expected length of cDNA base pairs (bp).
<Source and software for primer design>

- Design of primers from loblolly pine (WP1,2, and 3)
  - Go to the website “Primer 3”, http://frodo.wi.mit.edu/
  - Paste a sequence of *Pinus taeda* from a software, geneious 5.5.3 (Biomatters).
  - The website suggests appropriate primers. Check the size of primers. The size can be changed via “product size ranges”.

- Design of primers from sugar pine (WP1,2, and 3)
  - Go to the website “Dendrome”, http://dendrome.ucdavis.edu/
  - Select the link “tree genes” and then “summary by genes”.
  - Click “pinus”. Note: it is better to avoid species, which contains a lot of “EST” and “cDNA”, but prefer to select one that has a higher “TG Transcr Assembly”.
  - Select “Pinus lambertiana” and then “Resources”.
  - Download “454 Transcriptome Assembly” as FASTA format.

- Check the sequence in the GenBank database (NCBI)
  - Go to the website “Blast”, http://blast.ncbi.nlm.nih.gov/Blast.cgi
  - Select “Blast”, “nucleotide blast”, and then “Expressed Sequence Tags (est)” or “Transcriptom Shotgum Assembly (TSA)”.
  - Download software:
  - Confirm whether the sequence expresses like α-pinene synthase or limonene synthase.
• Comparing loblolly pine vs. sugar pine
  o Go to the website “ORF Finder” to convert nucleotide to amino acid sequences.
  o Compare two sequences by amino acid sequences using the software “Terminal”.

C.2.4. Creating Complementary DNA (cDNA) by Reverse Transcription PCR

C.2.4.1. Experimental Protocol

Reverse transcriptase PCR was performed using a hybrid protocol that combines the One Step RT-PCR Kit (QIAGEN) and SuperScript II Reverse Transcriptase (Invitrogen). This method used a two step reaction. A cox I sample from Dr. Barkman’s lab was used as a positive control throughout the experimental process.

• Preparation
  1) 2 µL of 100 µM primers were diluted to 18 µM with 9 µL RNase free water (QIAGEN) in a 0.2 mL PCR tube.
• 1st reaction
  2) 8 µL of autoclaved distilled water was pipetted into a PCR tube. 1 µL of 10 mM dNTP Mix (QIAGEN), 1 µL of 10 µM reverse primer, and 2 µL of mRNA sample were added to the tube and mixed using a pipette.
  3) The tube was heated at 65 °C for 5 min using a PTC-100™ Programmable Thermal Controller (MJ Research Inc.) or GeneApm PCR system 9700 (Applied Biosystems).
  4) The tube was quickly chilled on ice for approximately 2 min and then was briefly centrifuged.
5) 4 µL of 5X First-Strand Buffer (Invitrogen) and 2 µL of 0.1 M DTT (Invitrogen) were added to the tube.

6) The tube was incubated at 42 °C for 2 min.

7) 1 µL of SuperScript™ II RT was added.

8) The tube was incubated at 42 °C for 50 min, and then was heated at 70 °C for 15 min to inactivate the reaction.

- 2nd reaction

9) 38.1 µL of autoclaved distilled water was poured into a new PCR tube. (This volume varies to be 50 µL for total volume.) 5 µL of 10X PCR Buffer (Invitrogen), 1.5 µL of 50 mM MgCl₂ (Invitrogen), 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL of 10 mM dNTP Mix, 2 µL of cDNA from first-strand reaction (1st reaction), and 0.4 µL of Taq DNA polymerase (Invitrogen) were added to the tube in the order.

- PCR amplification

10) 30 cycles of PCR amplification were performed with the following conditions: denaturing at 94 °C for 30 sec, annealing at 50 °C for 45 sec, and extension at 72 °C for 2 min.

- Confirmation of cDNA synthesis

11) Synthesized cDNA was confirmed by a UV-Vis Spectrophotometer and electrophoresis. Representative results of electrophoretic analysis for synthesized DNA from white pine needles is shown in Figure C-4. In primers based on the sequence of α-pinene synthases from loblolly pine (Pinus taeda), WP3 created cDNA, while primers from sugar pine (Pinus tabuliformis) showed the bands with AP2_SG and
LN2_SG for α-pinene and limonene synthase respectively. The circled bands around 800 bp in Figure C-4 was closest to the expected size of target cDNA around 1000 bp (see Table C-2).

Figure C-4. Representative Results of Electrophoresis for Synthesized DNA from White Pine (*Pinus strobus*) Needle. Primers were designed from (a) loblolly pine (*Pinus taeda*) and (b) sugar pine (*Pinus tabuliformis*). The circled bands indicate the target size of cDNA.

C.2.4.2. Touchdown PCR

Figure C-41 shows that primers produced multiple size of bands other than the target band. To improve the target band, the touch down PCR technique was performed at step 10 of the cDNA synthesis protocol described above by gradually reducing annealing temperature during PCR.
amplification. The initial annealing temperature was determined based on Tm of primers in Table C-2. Starting at 53.5 °C, the temperature was decreased by 0.5 °C to reach 48.5 °C (the final temperature) with 11 cycles.

C.2.5. DNA Extraction

cDNA products were effectively obtained with AP2_SG and LN2_SG2 primers, as shown in Figure C-5. However, both products still had multiple bands in electrophoresis. The target band was extracted using the QIAEX II Agarose Gel Extraction protocol described below.

Figure C-5. The Results of Electrophoretic Analysis for cDNA Synthesized with Touchdown PCR.
C.2.5.1. Agarose Gel Extraction

1) 1% agarose gel tray was made with double-size wells and was set in an electrophoresis system. Each cDNA product, mixed with 8 µL of a loading dye, was transferred into each well of the gel tray. Electrophoresis was run at approximately 82 V for 30 min.

2) The band with target size in the gel was sliced using a sharp blade. The band was visualized by exposing the gel to a portable UV light using a longer wavelength mode. Note: a gel imaging system, Gene Wizard Syngene, must not be used because the instrument has short wavelengths that can damage the DNA.

3) The sliced gel was weighed and was transferred to a RNase/DNase free micro-centrifuge tube. Three times volume of Buffer QX1 (QIAGEN) to the gel and 10 µL of QIAEX II (QIAGEN) were added to the tube and vortexed.

4) The tube was incubated at 50 °C for 10 min until dissolved and then was vortexed every 2 min until the color turned to yellow that indicates pH<7.5.

5) The mixture in the tube was centrifuged for 60 sec and the supernatant was removed with a pipette.

6) To wash the pellet, 500 µL of Buffer QX1 was added and the tube vortexed. The supernatant was removed after centrifuging the mixture for 30 sec.

7) The pellet was washed twice with 500 µL of Buffer PE (QIAGEN) like in Step 6.

8) The tube was spun to make sure the pellet in the tube down. After removing the remnant liquid with a pipette, the pellet was air-dried for 10-15 min until the pellet became white.

9) Buffer EB (QIAGEN), which is 10 mM Tris-Cl with pH 8.5, was warmed at 50 °C before using it. 30 µL of Buffer EB was added to the pellet to elute DNA and the mixture was incubated at 50 °C for 10 min.
10) The tube was centrifuged for 30 sec and the supernatant including DNA was transferred into new 2 mL RNase/DNase free micro-centrifuge tube by pipetting.

11) Step 9 and 10 was repeated with 20 µL of Buffer EB. Hence, the total volume of solution was 50 µL.

After the agarose gel extraction, the sample was continuously treated by the ethanol precipitation protocol in Section C.2.5.2. (This procedure could not be stopped and resumed later until Step 2.)

C.2.5.2. Ethanol Precipitation of DNA

1) 1/10 volumes (5 µL) of 3 M sodium acetate and 3 volumes (150+15 µL) of 70% ethanol were added into the 2 mL micro-centrifuge tube with isolated cDNA. Note: the solution was mixed by pipetting, because excess vortexing may break down DNA.

2) The tube was kept in a freezer overnight at -20 °C.

3) The tube was centrifuged for 25 min at 4 °C to sink a pellet and the supernatant was removed by pipetting.

4) The pellet was washed with 600 µL of 70% ethanol to remove any residual salt from the nucleic acid. After adding the ethanol, the tube was vortexed to make sure wet the inside of tube. The tube was centrifuged for 10 min at 4 °C to sink a pellet again and the supernatant was removed by pipetting.

5) The pellet with cleaned DNA was air-dried for 5 – 10 min and was suspended in 40 µL of Buffer EB (QIAGEN). The tube with the suspended pellet was stored at -20 °C in a freezer. Note: It is very important to suspend the DNA pellet in Buffer EB, which has pH 8.5, because DNA elution efficiency is dependent on pH and the maximum effect is obtained at pH 7.0 – 8.5. When autoclaved distilled water was added to the pellet, the yield was too
poor to observe by electrophoresis. The pH of water quickly lowered to around 5. This might be due to up-taking CO₂ from the air.

6) Isolated target DNA band was confirmed by electrophoretic analysis. Figure C-6 shows each extracted cDNA with AP2_SG and LN2_SG primers had a single band after conducting the gel extraction.

Figure 3-6. Results of Electrophoresis Analysis for Synthesized DNA with the Agarose Gel Extraction Treatment.
C.2.6. DNA Ligation with Plasmid Vector

After ethanol precipitation, the extracted DNA was sequenced using the Agencourt Clean SEQ protocol (Beckman Coulter). However, this approach resulted in no dye reaction and no sequencing. Therefore, plasmid DNA was synthesized using a TOPO TA Cloning Kit and its protocol (Invitrogen) before the sequencing analysis. Taq polymerase is able to attach a single deoxyadenosine (A) to the 3’ terminal of a PCR product (3’ A-overhangs). Topoisomerase I from *Vaccinia virus*, which recognizes the specific sequence 5’-CCCTT-3’, binds the phosphodiester of 3’-T(thymidine) in a vector with a covalent bond and cleaves one DNA strand. The covalent bond is subsequently attacked by the 5’OH group of the original cleaved strand. As a result, the DNA is unwound and Topoisomerase I is released from the DNA. TOPO vectors are already linearized attaching topoisomerase I at each 3’ phosphate. Therefore, the vector binds the overhang 3’-A of PCR product and forms plasmid DNA.

- **Preparation**
  
  10 capsules of LB-Agar Medium (MP Biomedicals, LLC) were added to 250 mL of distilled water and were autoclaved.

- **Pseudo PCR (3’ A-overhangs)**
  
  This process was performed without primers.
  
  1) 10 μL of 10X PCR Buffer, 0.75 μL of 50 mM MgCl₂, 1 μL of dNTP, 0.2 μL of *Taq* DNA polymerase, and 15.55 μL of autoclaved distilled water were added to a PCR tube.
  
  2) The tube was incubated for 1 min at 94 °C.
  
  3) 5 μL of cDNA was added to the tube.
4) The tube was incubated for 10 min at 72 °C and then placed on ice. Then, the TOPO cloning reaction was conducted immediately with the product. It is important to use fresh PCR product for the reaction.

- Topo cloning reaction

5) 4 µL of fresh DNA from Step 4, 1 µL of salt solution (Invitrogen), and 1 µL of TOPO vector were added to a new PCR tube and were mixed gently.

6) The tube was incubated for 5 min at room temperature and was placed on ice.

- Transformation by One Shot Top10 chemically competent E. coli

7) 2 µL of TOPO cloning reaction from Step 6 was added into a tube of One Shot Chemically Competent E. coli (Invitrogen) and was gently mixed without pipetting up and down.

8) The tube was incubated on ice for 30 min.

9) The tube was put into a water-bath at 42 °C for 30 sec without shaking and was then transferred to ice immediately (Heat-shock).

10) 250 µL of S.O.C. Medium (Invitrogen), which was at room temperature, was added to the tube.

11) The tube was capped tightly and was shaken horizontally at 37 °C for 1 hour by a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., Inc.). Note: the tube was fixed to the shaker with tape.

12) 100 µL of the transformation was spread on a pre-warmed LB plate (100×15mm) using a metal bar which had been sterilized using 70% ethanol and a burner.

13) The plate was covered with a lid and was left for ~15-20 min to fix the solution to the gel.
14) The dish was turned upside down and was incubated at 37 °C for 16 hour. Note: do not leave the dish more than 16 hours, because *E. coli* starts dying after that time.

15) The plate was stored in a refrigerator at 4 °C.

- Analysis of transformants

16) 5 mL of LB medium and 5 µL of ampicillin were added to an autoclaved 15 mL tube. The same amount of LB medium without ampicillin was added to a 2 mL tube as a control during the shaking in Step 19.

17) Well-developed colonies were selected from each petri dish. Two colonies were selected from the plate that contained the transformant with AP2_SG primers and the colonies were named AP2_1 and AP2_2. From the plate that contained transformant with LN2_SG primers, six colonies were picked out: LN2_1, LN2_2, LN2_3, LN2_4, LN2_5, and LN2_6. (Note: LN2_3, LN2_4, LN2_5, and LN2_6 were from reselected colonies, because the results from LN2_1, LN2_2 were not appropriate to sequence. This problem was mentioned in Section 3.2.7.3.)

18) A portion of each selected colony was stuck by a 200 µL pipette tip and the tip was directly put into the tube at Step 16, which already contained the medium.

19) The tube was horizontally shaken at 37 °C for 16 hours.

After incubation, plasmid DNA was isolated from the cells using a protocol in the next section.

C.2.7. Isolation of Plasmid DNA

C.2.7.1. Purification of Plasmid DNA

Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (QIAGEN) and its protocol.

1) The pipette tip was removed from the tube at Step 19 of section C.2.6.
2) Since the maximum capacity of the micro-centrifuge in the lab which was able to spin at cold temperatures, was 2.0 mL, two 2 mL micro-centrifuge tubes (tube-1 and tube-2) were prepared for each colony sample. 1.5 mL of mixture sample from Step 19 of Section C.2.6 was added into each tube (i.e., the total mixture sample was 3.0 mL).

3) The tubes with the sample were centrifuged for 15 min at 4°C. While centrifuging, Buffer P1 (QIAGEN) was kept on ice.

4) The supernatant was removed from the tube using a pipette, leaving the cell pellet.

5) 250 µL of Buffer P1 was added into tube-1 and the pellet was resuspended by pipetting up and down. The component in tube-1 was transferred into tube-2 and was mixed by pipetting up and down. (The pellets in two tubes were combined.)

6) 250 µL of Buffer P2 (QIAGEN) was added into the tube and the solution color was changed to blue. The tube was gently inverted to mix the solution without shaking. The tube was incubated for 3 min.

7) 350 µL of Buffer N3 (QIAGEN) was then added to the tube and was immediately and thoroughly mixed by inversion until the color of indicator in the tube disappeared. The solution became cloudy.

8) The tube was centrifuged for 15 min, resulting in the formation of a white pellet.

9) The supernatant was transfer to QIAquick Spin Columns. The spin columns were a combination of two tubes. The inner tube has a column that contains a silica membrane and traps DNA, and the outer tube is designed to hold the filtrate. The bottom part of inner tube was partially inserted into the outside tube. Therefore, the supernatant was loaded to the inner tube first.
10) The tubes were centrifuged for 1 min. Note: the capacity of inside tube was small. Therefore, the supernatant from Step 8 was divided into two aliquots and Steps 9 and 10 were repeated with the same tubes.

11) 750 µL of Buffer PE (QIAGEN) was added to the tube to wash the membrane. (Make sure to rinse the side of column also.) The tubes were incubated for 1 min.

12) The tubes were centrifuged for 1 min.

13) The filtrate was discarded from the outer tube by pipetting.

14) The empty outer tube covered the bottom part of inside tube again and the combined tubes were centrifuged again for 1 min to remove extra liquid from the column.

15) The cap of the inside tube was opened and was left in a fume hood for 4 min to evaporate the ethanol contained in Buffer PE.

16) The outer tube was replaced with new 2 mL micro-centrifuge tube and the inner tube was placed into it.

17) 60 µL of distilled water mixture (elute Buffer EB/dH2O = 1/9) was added into the inner tube to elute the DNA from the column, and tubes were left for 5 min. Note: normally, distilled water only was used for sequencing. However, the pH of Milli-Q was around 5.7. Hence, Buffer EB was mixed to prevent loss of DNA.

18) The cap of the outside tube was cut off to fit the centrifuge slot. The tubes were centrifuged for 1 min.

19) The isolated plasmid DNA was confirmed using electrophoresis and a UV-Vis spectrophotometer.
C.2.7.2. Protocol of PCR with Topo Genes

This process was continuously conducted from Step 18 in Section C.2.7.1. (The DNA from colony AP2-2 was eliminated, because the electrophoretic analysis at Step 19 in Section C.2.7.1. resulted in a band of the wrong size.)

1) 39.1 µL of EB buffer was poured into a new PCR tube. 5 µL of 10X PCR Buffer, 1.5 µL of 50 mM MgCl₂, 1 µL of 10 mM dNTP, and 0.4 µL of Taq DNA polymerase were added into a PCR tube.

2) 1 µL of 1/20 diluted plasmid DNA from Step 18 in Section C.2.7.1 with autoclaved distilled water, 1 µL of forward SG primer, and 1 µL of reverse SG primer were added into the tube. Total volume was 50 µL in the tube.

3) 30 cycles of PCR amplification were performed with conditions below:
   - Holding 2 min at 94 °C
   - 30 cycles of denaturing at 94 °C for 30 sec, annealing at 50 °C for 45 sec, and extension at 72 °C for 2 min
   - Holding 10 min at 72 °C
   - Reserving the tube at 4 °C

4) The DNA was confirmed using electrophoretic analysis with 1% agarose gel.

A single band of PCR product in electrophoresis was continued to PCR purification treatment in Section C.2.8.
C.2.7.3. Problem of PCR with Topo Genes

Figure C-7 is the PCR products of TOPO cloning in electrophoresis. AP2-1 showed a single band around 1200 bp (expected size), however both LN2-1 and LN2-2 had two bands around 1200 bp (expected size) and 600 bp (incorrect size), although PCR was conducted with SG primers. The presence of multiple bands would be an obstacle for sequencing. To confirm whether the multiple bands were derived from TOPO genes, PCR was conducted with TOPO primers (T7 primers), which were supplied by the kit, for DNA from LN2-1. The results in Figure C-8 show two bands that suggests the problem with LN2 was due to TOPO (T7) primers, not SG primers.

Figure C-7. Electrophoretic Analysis of PCR Product of TOPO Cloning for AP2 (α-Pinene synthase) and LN2 (Limonene synthase).
The strong presence of smaller size fragment bands is related to the presence of uncut plasmid vectors, particularly supercoiled DNA (e.g. Shakhmaeva et al., 2011) and these uncut vectors required treatment with specific enzymes that digest or cut each recognition site and produce a single band. Since our lab did not have the specific enzyme for TOPO genes, additional colonies were screened. (Note: colonies, LN2_3, LN2_4, LN2_5, and LN2_6, were selected at this process.) Reselected colonies from the petri dish at Step 15 in Section C.2.6. and were continuously treated with Step 18 and 19. The plasmid DNA from re-selected colonies were purified using the protocol in the Section C.2.7.1. The purified DNA from all colonies resulted in the supercoiled with linear bands in electrophoresis in Figure C-9. The purified DNA of LN2-3 and LN2-4 were selected to test for comparing PCR products using SG primers vs. TOPO (T7) primers in order to obtain solo size. The PCR conditions were same as the protocol in Section C.2.7.2. The PCR products were confirmed with electrophoretic analysis in Figure C-10. The products of LN2-3 and LN4 with SG primers showed a single band at between 900 – 1200 bp and the smaller sized band of supercoiled
disappeared, while the products with TOPO (T7) primers had another band at > 1500 bp. Hence, the PCR products with SG primers were applied for next treatment, PCR purification in Section C.2.8.

Figure C-9. Electrophoretic Analysis of Reselected Colonies for LN2 After Plasmid DNA Purification Treatment.
C.2.8. PCR Purification

C.2.8.1. PCR Purification

This purification was conducted on 50 µL DNA after the PCR amplification in Section C.2.7.2.

1) 3 volume of Buffer QG (150 µL) and 1 volume of isopropanol (≥99.5% A.C.S. reagent, Sigma-Aldrich) were added into the PCR tube with DNA from Step 3 in Section C.2.7.2 and were vortexed.

2) The mixture was transferred to a micro-centrifuge tube by pipetting and was centrifuged for 1 min.

3) The supernatant was removed by pipetting.
4) 500 µL of Buffer PE was added to the tube to wash and was left at room temperature for 2
   – 5 min.
5) The tube was centrifuged for 1 min.
6) The supernatant was removed by pipetting.
7) Steps 4 – 6 were repeated.
8) Additional centrifuging was conducted for 1 min to remove residual ethanol.
9) 50 µL of Buffer EB was added to elute the DNA for sequencing. The solution needed to
   be placed directly on the bottom of the tube without touching the side of the tube.
10) The tube was left at room temperature for 5 min.
11) The tube was centrifuged for 1 min.
12) The tube was stored in –20 °C.
13) The purified DNA was confirmed using electrophoretic analysis.

C.2.8.2. Ethanol Precipitation (Quick Version) Before Sequencing

1) 1/10 volume (5 µL) of 3M sodium acetate and 3 volumes (50 µL × 3) of 100% ethanol
   were added into the micro-centrifuge tube from Step 12 in Section 2.3.7.4.
2) The tube was placed on ice in a Styrofoam box and was covered by ice. The Styrofoam
   box was left in –20 °C for 2 hours or more.
3) The tube was centrifuged for 35 min at 4 °C.
4) The supernatant was discarded by pipetting.
5) 600 µL of 70% ethanol was added in the tube.
6) The tube was centrifuged for 15 min at 4 °C.
7) The supernatant was discarded by pipetting.
8) The pellet in the tube was air-dried until the ethanol evaporated.

9) 30 µL of autoclaved distilled water was added into the tube to re-suspend the DNA. The tube was left for 5 min to resettle. Note: for sequencing analysis, Buffer EB cannot be used to elute DNA.

10) DNA concentration was measured by a UV-Vis Spectrophotometer.

C.2.9. DNA Sequencing Analysis

C.2.9.1. DNA Sequencing Using Fluorescent Dye

DNA sequencing was conducted using GenomeLab Dye Terminator Cycles Sequencing with a Quick Start Kit (Beckman Coulter) and Agencourt CleanSEQ Dye-Terminator Removal (Beckman Coulter) and their protocols 608118AE December 2009 and 600v032. The size of AP2 and LN2 primers (including both forward and reverse) were expected to be approximately 2 kb each and the size of TOPO genes were also approximately 2 kb. Hence, the total size of primers, AP2+TOPO and LN2+TOPO, was 4 kb. According to the conversion table, which was provided from the protocol of Beckman Coulter, the double stranded DNA (dsDNA) concentration was estimated to be 260 ng/100 fmol based on the total size. The longer sequencing needed to have “template Pre-Heat treatment” before performing the DNA sequencing reaction.

1) “Template pre-heat treatment” was conducted with 2 (varied*) µL of DNA template from Step 9 in Section C.2.7.5. and 9 (varied*) µL of autoclaved distilled water. The components were added into a 0.2 mL PCR tube and were heated at 65 °C for 5 min using a thermal cycler. Note: the DNA template tube was warmed by hand and vortexed before transferring. Both forward and reverse primers could not be added in the same tube at Step 2, therefore, two test tubes needed to be prepared for each DNA template. The volume of
DNA template and distilled water (indicated with *) were varied based on the concentration of DNA at Step 10 in Section C.2.7.5. A calculation example is shown below.

<e.g.>

Since dsDNA of AP2 was estimated to be 260 ng/100 fmol, the DNA template needed to be 260 ng. When the concentration of PCR product from AP2-1 was 128.6 ng/µL using UV-Vis Spectrometry, the required volume to be 260 ng was approximately 2 µL. Total volume of the mixture, which was the sum of DNA template, TOPO primer (T7 primer), and Master mix, and distilled water, needed to be 20 µL. The volume of distilled water was adjusted to be this volume. In this case, required distilled water was 9 µL from 20 µL – (2 µL DNA + 1 µL primer + 8 µL master mix).

2) 1 µL of TOPO primer and 8 µL of Master mix (Beckman Coulter) were added to the tube (total volume 20 µL). Note: since the Master mix is sensitive to light, the bottle was covered by aluminum foil.

3) 30 cycles of PCR amplification were performed with the following conditions: denaturing at 96 °C for 20 sec, annealing at 48.5 °C for 20 sec, and extension at 60 °C for 4 min. Note: if Tm of a primer is < 55 °C, then annealing temperature would be (Tm – 5) °C.

4) Fresh stop solution, a mixture of 2µL of 3M sodium acetate, 2 µL of 100 mM EDTA, and 1 µL of 20 mg mL⁻¹ Glycogen (Beckman Coulter), was added to the tube.

5) 20 µL of CleanSEQ (Beckman Coulter) was added to the tube. Note: before use the bottle of CleanSEQ needed to be vortexed until the magnet beads were homogenously suspended in the solution.

6) 62 µL of 85% ethanol was added to the tube.
7) The tube was vortexed for 30 sec and was left to resettle for 10 min at room temperature in a polystyrene foam box with a lid to keep out light.

8) The tube was placed onto a well in a magnet plate and was left for at least 3 min to separate magnet beads with DNA from the solution. The magnet beads formed a ring on the side of the well and the solution became clear.

9) The supernatant containing residuals was removed by pipetting by placing the tip of a pipette on the bottom of the tube without contacting the beads on the sides.

10) 100 µL of 80% ethanol was added into the tube and was left for at least 30 sec to resettle.

11) As much ethanol was removed as possible by pipetting using 200 µL pipette tip and then 10 µL pipette tip.

12) The magnet plate with the tube was left in a fume hood to air-dry. However, the beads must not become completely dry, because dried beads result in lower signal intensities during sequencing.

13) 40 µL of formamide was added to the tube, which was taken then off the magnet plate to mix the solution by pipetting up and down to resuspend the beads, and was left for 5 min to resettle without centrifuging. Note: this process was still conducted in the fume hood.

14) The tube was placed back on the magnet plate to fix beads on the side of tube. The formamide solution with DNA was transferred into a well of a sample plate for sequencing analysis. The solution was slowly aspirated from the bottom of the tube by pipetting.

15) The surface of the solution was covered with 1 drop of mineral oil (Beckman Coulter). The empty wells in the sequencing plate were filled with formamide. (Do not make any empty well in the plate.)

16) Separation buffer (Genome Lab) was added into wells of buffer plate.
17) Plates were loaded to an DNA sequencing analyzer (CEQ Sequencing Analysis Beckman Coulter).

C.2.9.2. Nucleotide BLAST

Nucleotide sequences from white pine (*Pinus strobus*) needles were searched to find a similar biological sequence from GenBank, which is the database of National Center for Biotechnology Information (NCBI, U.S.A.), via https://blast.ncbi.nlm.nih.gov/Blast.cgi using a Basic Local Alignment Search Tool (BLAST), program BLASTN 2.6.1+. Selective significant alignment for each colony is listed in Table C-3. AP2-1, which from AP2_SG primers, amplified some sort of chromosomal sequence of *Pinus strobes*, but did not match at all with any terpene synthases. For LN_2 primers, which are supposed to produce limonene synthase, the sequences from LN2-4 and LN2-5 matched some sort of terpene synthase, which is likely α-pinene synthase or limonene synthase. The obtained sequences of LN2-4 and LN2-5 are shown after Table C-3.
Table 3-5. Results of Nucleotide BLAST for Each Colony.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Primer</th>
<th>Description</th>
<th>Sequence ID</th>
<th>Query coverage</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP2-1</td>
<td>Forward</td>
<td>Pinus strobus plastid, complete genome</td>
<td>KP099650.1</td>
<td>91%</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pinus strobus chloroplast, partial genome</td>
<td>FJ899560.1</td>
<td>91%</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Pinus strobus plastid, complete genome</td>
<td>KP099650.1</td>
<td>44%</td>
<td>95%</td>
</tr>
<tr>
<td>LN2-3</td>
<td>Forward</td>
<td>Pinus strobus plastid, complete genome</td>
<td>KP099650.1</td>
<td>88%</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>No significant similarity found</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN2-4</td>
<td>Forward</td>
<td>Pinus contorta (-)-alpha pinene synthase (TPS(-)apin1) mRNA, complete cds</td>
<td>JQ240303.1</td>
<td>81%</td>
<td>87%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pinus taeda (-)-alpha-pinene synthase mRNA, complete cds</td>
<td>AF543527.1</td>
<td>81%</td>
<td>87%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Picea abies (-)-limonene synthase (TPS-Lim) mRNA, complete cds</td>
<td>AY473624.1</td>
<td>70%</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Picea sitchensis (-)-limonene synthase mRNA, complete cds</td>
<td>DQ195275.1</td>
<td>70%</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Pinus taeda (-)-alpha-pinene synthase mRNA, complete cds</td>
<td>AF543527.1</td>
<td>83%</td>
<td>87%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Picea abies (-)-limonene synthase (TPS-Lim) mRNA, complete cds</td>
<td>AY473624.1</td>
<td>78%</td>
<td>79%</td>
</tr>
<tr>
<td>LN2-5</td>
<td>Forward</td>
<td>Pinus banksiana (-)-alpha/beta-pinene synthase (TPS(-)a/Bpin1) mRNA,</td>
<td>JQ240290.1</td>
<td>63%</td>
<td>84%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>complete cds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Pinus contorta (-)-alpha pinene synthase (TPS(-)apin1) mRNA, complete cds</td>
<td>JQ240303.1</td>
<td>93%</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pinus taeda (-)-alpha-pinene synthase mRNA, complete cds</td>
<td>AF543527.1</td>
<td>93%</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Picea abies (-)-limonene synthase (TPS-Lim) mRNA, complete cds</td>
<td>AY473624.1</td>
<td>90%</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Picea sitchensis (-)-limonene synthase mRNA, complete cds</td>
<td>DQ195275.1</td>
<td>90%</td>
<td>79%</td>
</tr>
</tbody>
</table>

Query coverage is the percent of the query length in the aligned segments over all segments.

Nucleotide Sequences from White Pine Needles

- LN2_4 with Forward primers

```
AAACANCCCGTTTGTGTNNGGACNNNNCAACTCTTTTGTAAATTNNNTATAC
CANANGGAGATACCCCTTTTACANAGCCCNAGTTGCAANNGTTGGTCGATCC
CCNAAGCTCTTTTGTCTCTGAGGCAAAAGCGTGATAGCTATCTCGATATTGTATC
CATAATGGAAAGCTCTGCTGATATCAAAAACATGTTTCTCGATATGCGTCGG
AACGTGGCTGTCAGGTTCAGTACGTTCTCCAGGTCAAGTATGCGATATCG
TATGCACTGTGGTGGGATAGCTATGAGGAGCTTCTCCCGTATCTCCTCGGATGG
TCTTTTCAATAAACAGATATAGACGAGCAGCTCTCTCTCCACGGGCCCTGTCAGC
TTTGATGCGATGCTGCTTACCCCATCACCCTCAGTATCGAAGTATGCGATATGC
TGAGGGCTTGGATGGAAGATGACACATTTGATCCTGAACTGACTATGCGATATG
```

252
TACCGTTCTCGTAGTACTCTTCAAAAGGTGGTAGCTACCAAGTGCTATCCAC
TTGCTTCTTGCATATACGAAATCAATACAATNCCCTCCCAGCATGCTCCAAACA
TANGTTAGCGTGTCTCGGCCTTGAGCCTTGTCTGCCCTCCCCGAGACATTTCGT
AGAGGGTGTGTAACATCATGATATATTCTCTCTCTATATATTTCGGAAGCCA
TTTGGGGNGGACNGATCCCCATCTCTNAATTGGCTTCTTGTGAAAANNNNTNN
NAAGCTCCTCTNACGGTTCCCCGANAA

- LN2_4 with Reverse primers

CNCNNANGNNTNNTCCNNNNCTCTTTGATCTCTGAGGCAAGTCGATGCTAT
CTCGATATTTGATCTCATAATGGAAAGCTCTGCTGATATCAAAAACATGTTTC
TTGNGCAGTTATGGGAACCTGGTCTACTAGGGTAACTTCCATATACCAATCT
CTTTAAACATACGTGCTCCAGTGCTGAGTGTGCTCTCGGCTGCTCTGGCTG

• LN2_5 with Forward primers

GNNNANNCTATTTCATAAAGNGANACTCTAGCTAAGATTCCTCGTGCTCGAGT
CTTTGACAGAGATAGGGGACCTGGTCTTTTGGAATATGTTGACACAGTATTTG
CACGAGATTAGAAGGAAATTATATGGAGCTTTTGGGACAGGATTAAG
AACAGAAATACAGAAGCTTATAGAACTTGGCAATTCAAAACACTGAGTTCAACATCT
TTCAATCTATAAAAAGAAGAAGTGAGTAGAGCTCTCTCAGATGTTGAAAGA
TTCCCCAGTCTCATACCTACCTTCACCTGCACGATCGAGGAAACTCTCA
CTCTGCTTCTTCTGCTCTCAGTTGGAGCCTCAACATTGCTAGAGACTCGGC
TTGCCAAAAGCTGTCATATACTGCTACTGTAATGATGATGACTGCGCGT

253
• LN2-5 with Reverse Primers

```
GNNTNNANNNTNNCCAGNCTTTTGAGTTTCTGAGGCAAGTCGGTACGAAT
GGTTGATATTTCTAGTATCCATAATGGAAAGCTCTGATATACAAAAACATGTTT
CTTGCAGTTAAGGCTTGTTCAGGTTTTGAAGATCCTCCAGTTTAAT
CCTTTAAATTACGTCAGGTATGATGAGCAGTTATGATGATGAGCAGCATCTCTC
CTGTTGATCTGGATTGCTTTTCATATAACCAAGATATAGACGAGAGTCTTATCT
CACGGGTCCTGTACGCTTTGAGACTGCTATCACCCTCTGAATCAGTAGATG
GCAGATGCAAAATCGTACGCTTTCAATGGAAGAGTCAACCTCTTTGAGGAT
GCTGAGGGAAGGAGATGTCGAGAATTGTTGCAATGCAGATATGCG
ATGAGCACTCCTACACTAACCTTTACGTTTCTCAGTAGATCACTCTTTTCAT
ATATTTGGTAAACCCATCTGTTGACGGTACGAGATCATCCACATCTAATTTGCT
TGAAGAGTCTCAGCTCTCAGCTTTACGGAAGAGTGACGTACATATCATNCAG
TACAGTGATGATGACGGCTTTGGCAAGCGAGTGTGG
```

References


APPENDIX D

Comparison of Total PANs Measurements from ARA, UC, and WMU

During the SOAS 2013 campaign, two other research groups measured the sum of total PANs without identification of each species. Atmospheric Research & Analysis, Inc (ARA) measured total PANs within 30 m of the WMU instrument and at the same height using thermal dissociation into NO$_2$ at 160 °C on top of ambient NO$_2$. The Cohen group from University of California, Berkeley (UC) measured total PANs using thermal dissociation from the tower approximately 100 m north of the WMU instrument and approximately 25 m above the ground.

General descriptive statistics of all three measurements are summarized in Table D-1. The sum of PAN, PPN, and MPAN in WMU data was treated as total PANs for comparison. WMU measurements below detection limits for PPN and MPAN were designated as half of detection limit for each compound. In comparison with PAN$_{WMU}$, PAN$_{ARA}$ had similar mean and median, while the measurement of PAN$_{UC}$ was 1.5 times higher than PAN$_{WMU}$, perhaps reflecting differences in sampling height/locations. The upper level of the tower was closer to and immediately above the surrounding canopy trees. The correlations with PAN$_{WMU}$ are plotted in Figure D-1. The dash line indicates least-squares regression line. The solid line is one-to-one correspondence with measurements of WMU. The slope indicates a magnitude of data in comparison to measurement of WMU. Measurements of UC and ARA differed by factors of 1.5 and 0.7 respectively.
Table D-1. Statistical Descriptions for Total PANs from WMU, ARA, and UC.

<table>
<thead>
<tr>
<th>Units (ppbv)</th>
<th>WMU</th>
<th>ARA</th>
<th>UC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.138</td>
<td>0.129</td>
<td>0.245</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.119</td>
<td>0.092</td>
<td>0.194</td>
</tr>
<tr>
<td>Std. error of mean</td>
<td>0.003</td>
<td>0.002</td>
<td>0.005</td>
</tr>
<tr>
<td>Median</td>
<td>0.103</td>
<td>0.111</td>
<td>0.204</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.013</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>Maximum</td>
<td>1.191</td>
<td>0.753</td>
<td>1.394</td>
</tr>
</tbody>
</table>

Figure D-1. Relationship of Total PANs with Other Research Groups during SOAS 2013 Campaign. The solid line is one-to-one correspondence with measurements of WMU.

In order to decide an appropriate statistical test for comparing PANs levels which were measured by three groups, two normal distribution tests were performed on total PANs from WMU, ARA, and UC using SPSS. The results are shown in Table D-2. According to Ghasemi and Zahediasl (2012), Kolmogorov-Simmov is the most popular test but Shapiro-Wilk is a more powerful test. All p-values on total PANs from the three groups were less than 0.05 in both Kolmogorov-Simmov and Shapiro-Wilk tests. This means, all data did not have normal
distribution. Therefore, nonparametric tests were applied to statistically assess the concentrations of total PANs from three groups. First, the correlations of measurements were investigated using Spearman’s rank correlation test and the results in Table D-3 indicate that the total PANs data from all three groups significantly correlated each other. The correlation coefficient, \( r_s \) of each pair (\( \text{PAN}_{\text{WMU}} - \text{PAN}_{\text{ARA}} \), \( \text{PAN}_{\text{WMU}} - \text{PAN}_{\text{UC}} \), and \( \text{PAN}_{\text{ARA}} - \text{PAN}_{\text{UC}} \)) was 0.754, 0.926, and 0.714 respectively \((p < 0.01)\).

| Table D-2. Normality Test for Total PANs Data from WMU, ARA, and UC. |
|-----------------|-----------------|-----------------|
|                 | Kolmogorov-Smirnov | Shapiro-Wilk     |
|                 | Statistic   | df    | p-value | Statistic   | df    | p-value |
| WMU             | 0.148       | 1507  | 0.000   | 0.776       | 1507  | 0.000   |
| ARA             | 0.108       | 1507  | 0.000   | 0.875       | 1507  | 0.000   |
| UC              | 0.117       | 1507  | 0.000   | 0.866       | 1507  | 0.000   |

\( \text{df} \) is degrees of freedom.

| Table D-3. Spearman’s Rank Correlation Test for Total PANs from WMU, ARA, and UC in SOAS 2013. |
|-----------------|-----------------|-----------------|
|                 | ARA             | UC             |
| WMU             | Corre. Coeff.   | 0.754**        | 0.926**        |
|                 | Sig. (2-tailed) | <0.000         | <0.000         |
| ARA             | Corre. Coeff    | 0.714**        |
|                 | Sig. (2-tailed) | <0.000         |

Corre. Coeff. is correlation coefficient. Sig. (2-tailed)** means the two-tailed \( p \) value.

Next, the Friedman rank sum test, a nonparametric test, was applied in order to compare the measurement levels of total PANs from three groups. This test is able to compare the ranks (or medians) among more than three dependent variables at once. The daytime (9am – 7pm) data were used for this assessment (the general statistical descriptions during the time were shown in Table D-4). The significance level, \( \alpha \), was set at 0.05. The results of the Friedman rank sum test and the following post hoc test are shown in Table D-5 (a) and (b), respectively. The \( p \)-values of the Friedman rank sum test were less than 0.05 and indicated that at least one of the rankings or median
was significantly different from the others. The following post hoc analysis was performed to assess the difference in significance of each pairwise using the Wilcoxon signed rank test. The results indicate that measurements of PANs_{UC} were significantly larger than those of both PANs_{WMU} and PANs_{ARA} due to p-value < 0.05 and that there was no statistical difference between PANs_{WMU} and PANs_{ARA} because of p-value > 0.05.

Table D-4. Statistical Description of Total PANs from Three Groups from 9am – 7pm.

<table>
<thead>
<tr>
<th>Units (ppbv)</th>
<th>WMU</th>
<th>ARA</th>
<th>UC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.186</td>
<td>0.176</td>
<td>0.295</td>
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<tr>
<td>Std. deviation</td>
<td>0.152</td>
<td>0.117</td>
<td>0.208</td>
</tr>
<tr>
<td>Std. error of mean</td>
<td>0.007</td>
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<tr>
<td>Minimum</td>
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<tr>
<td>Maximum</td>
<td>1.191</td>
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Table D-5. The Friedman Rank Sum and Post-hoc Tests for total PANs from Three Groups.

(a). Friedman rank sum test for total PANs from 9 am – 7 pm for three groups.

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<th>Friedman chi-squared</th>
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<td>445.3102</td>
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(b). The p-value on post-hoc tests using Wilcoxon signed rank test for comparing each pairwise.

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<td>UC</td>
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<td>WMU</td>
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Reference

APPENDIX E

Chamber Experiments of Isoprene Photooxidation at Purdue University in 2013

As a collaborative project from May 2\textsuperscript{nd} to May 8\textsuperscript{th} in 2013 at Purdue University, a series of chamber experiments were conducted to study the formation of organic nitrogen compounds via reactions of isoprene and other BVOC oxidation. All aerosols were expected to be generated via gas-to-particle conversion because no seed particles were introduced. Therefore, the project allowed us to evaluate a role of organic nitrogen on particle formation.

Photochemical reactions were performed in a cylindrical 5.5 m\textsuperscript{3} Teflon bag equipped with a mixing fan and UV lights. The details of the chamber experimental conditions including instrumentation were described in Groff (2015). Our objective was to measure PAN-type compounds using the custom GC-ECD used during SOAS, which was described in Chapter 3. Isoprene, ozone, NO, NO\textsubscript{2}, and total isoprene nitrates were measured by Purdue University. Isoprene was measured using a gas chromatograph equipped with a flame ionization detector (GC-FID) from Shimadzu on a Rtx-1, 30 m × 0.53 nm ID × 1.0 µm column (RESTEK). O\textsubscript{3} was monitored using a U.V. photometric O\textsubscript{3} analyzer (Model 49-003, Thermo Environmental Instruments). NO and NO\textsubscript{2} were detected via the chemiluminescence produced when NO reacts with ozone using a NO chemiluminescence analyzer (homebuilt). Total isoprene nitrates were measured using a chemical ionization mass spectrometer (CIMS) with Br\textsubscript{2} calibration (Xiong et al., 2015). The inside of the chamber was flushed with hydrocarbon free “zero” air (ZA), in order to remove any contamination prior to running each experiment. The conditions of experiments I, II, and III are listed in Table E-1. The chemicals were carried to the chamber with 6 L min\textsuperscript{−1} flow of zero air (ZA). Photooxidation was initiated by lights with H\textsubscript{2}O\textsubscript{2} or HONO.
A representative PAN chromatogram for smog chamber experiments I, II, and III are shown in Figure E-1. PANs formation was lower with lower amounts of NOx (experiment I and III), and higher with higher amounts of NOx (experiment II). PAN and MPAN are the primary PAN-type products from isoprene oxidation, and the GC retention time of each compound was confirmed using its liquid standard. Two unidentified peaks were observed; one appeared around 8.0 min, which was between PAN (RT ≈5.0 min) and PPN (RT ≈10.5 min), the other was around 15.4 min, which was just before MPAN (RT ≈16.6 min). Roberts and co-workers (2001) reported PAN-type compounds from ambient air during TexAQS 2000 in Houston using a GC-ECD with a RTX-200 (Restek), which was the same column as ours, and the order of the retention time was PAN, peroxycryloyl nitrate (APAN, CH$_2$=CHC(O)OONO$_2$), PPN, peroxy isobutyryl nitrate (PiBN, (CH$_3$)$_2$CHC(O)OONO$_2$), and MPAN. Therefore, the two unidentified peaks from our chamber experiments were tentatively proposed to be an APAN-like compound for the compound at RT 8.0 min and a PiBN-like compound for the one at RT 15.4 min. The APAN-like compound was studied in Chapter IV.
Figure E-1. Representative Chromatograms for Smog Chamber Experiment I, II, and III at Purdue University. Same scale range of intensity is applied for all panels.
In experiment II, the APAN-like compound produced under high NOx conditions was the second dominant PAN-type compound after PAN. Chamber experiment I also showed some amount of APAN, however the amount was not as much as was present in experiment II. Figure E-2 shows the ratio of \([\text{APAN}]/[\text{PAN}]\) and \([\text{MPAN}]/[\text{PAN}]\) from experiment I and II for the time series. In experiment II, the ratio of \([\text{APAN}]/[\text{PAN}]\) was larger than the ratio of \([\text{MPAN}]/[\text{PAN}]\) and increased up to 0.6, while \([\text{APAN}]/[\text{PAN}]\) from experiment I was less than 0.3 that was smaller than \([\text{MPAN}]/[\text{PAN}]\) from experiment I.

Figure E-2. The Ratio of APAN/PAN and MPAN/PAN from Experiments I and II at Purdue University.

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**APPENDIX F**

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