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Chemical Characterization of Pseudognaphalium obtusifolium by Gas Chromatography - Mass Spectrometry (GC-MS) to Assess Potential Therapeutic Phytochemicals and Toxicological Concerns Using Simulated Use Conditions

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry at Virginia Commonwealth University

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CHEMICAL CHARACTERIZATION OF PSEUDOGNAPHALIUM OBTUSIFOLIUM BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC-MS) TO ASSESS POTENTIAL THERAPEUTIC PHYTOCHEMICALS AND TOXICOLOGICAL CONCERNS USING SIMULATED USE CONDITIONS

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Currently, there is an increasing demand for natural therapies and herbal products to treat various ailments. It is generally believed that natural therapies have fewer side-effects than traditional western medicine; however, they are often used in different strengths and formulations without consistency of the levels of target compounds or knowledge about toxicity. Due to this growing trend, a comprehensive chemical evaluation of plants used for medicinal purposes is necessary.

Pseudognaphalium obtusifolium is a plant that has been used historically by Native Americans as an herbal medicine. It is a flowering plant belonging to the Asteraceae family indigenous to the Eastern United States. There are documented accounts of the Native Americans using the herb therapeutically. Reportedly, they used the plant to prepare tea and as filler for bedding. Additionally, they smoked the plant material.

To date, there has been little research published on the chemical composition of this plant. Thus, the objective of this work was to conduct a chemical survey of *P. obtusifolium* using methodologies that would simulate the three historical routes of administration (tea, bedding material, and smoke inhalation).

To determine the types of compounds that may be found in the plant, initial experiments using pressurized solvent extraction (PSE) with an ethanolic solvent were performed followed by analysis using gas chromatography – mass spectrometry (GC-MS) in scan mode. This extraction technique enabled a broad range of compounds to be identified.

For the analysis of the tea, the leaves and the flowers were ground and analyzed separately. The “tea” simulation was then performed using a water extraction which was then back extracted into dichloromethane for GC-MS analysis in Selected Ion Monitoring (SIM) mode. Seventeen target compounds (terpenes, terpinoids, flavanoids, etc.) were quantified using this method.

A bedding material simulation was performed using headspace solid phase micro-extraction (HS-SPME) to collect the volatile and/or semi-volatile components of the headspace. The compounds collected on the SPME fiber were then analyzed by GC-MS in scan and SIM modes to qualitatively and quantitatively determine the types of chemical compounds (most of which were terpenes) that may be off-gassed from bedding material. This analysis compared levels of compounds in two different crop years and four terpene compounds were quantified.

To simulate smoking of the plant material, the leaves and flowers were fashioned into smoking articles. Sample collection was performed by a smoking machine and smoke condensate was collected. The smoke condensate was then analyzed by GC-MS in scan mode. As combustion and pyrolysis of plant material are known to produce toxic products, specific potentially harmful compounds were investigated and quantified.

This chemical analysis of *P*. *obtusifolium* identified target compounds that can be found in the three simulated usage forms. Identification of these compounds gives insight on why the Native Americans may have used *P. obtusifolium* as an herbal medicine. Among the detected compounds, there were many unknowns. Elucidating these unknown compounds will be important in the effort to understand the full chemical profile of this plant.

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List of Abbreviations

Automated Mass Spectral Deconvolution and Identification System – AMDIS
Center for Disease Control and Prevention – CDC
Chemical Abstracts Registry Service – CAS
Dichloromethane – DCM
2,4-Dinitrophenylhydrazine – DNPH
Electron Ionization – EI
Flavor and Extract Manufacturers Association of the United States – FEMA
Gas Chromatography – Mass Spectrometry – GC–MS
Growth inhibitory dose at half maximal - GI_{50}
Headspace Solid Phase Microextraction – HS–SPME
Inhibitory concentration at half maximal - IC_{50}
International Conference on Harmonization -ICH
Internal Standard – ISTD
International Agency for Research on Cancer – IARC
International Standard Organization – ISO
Limit of Detection – LOD
Limit of Quantitation – LOQ
Mass to Charge Ratio – m/z
Methicillin Resistant *S. Aureus* – MSRA
National Institute of Standards and Technology – NIST
Polydimethylsiloxane – PDMS
Practical quantitation limit - PQL
Pressurized Solvent Extraction – PSE
Pseudognaphalium obtusifolium – *P*. *obtusifolium*
Quick-Easy-Cheap-Effective-Rugged-Safe (extraction method) – QuEChERS
Retention Time – RT
Selective Ion Monitoring – SIM
The Good Scents Company – TGSC
Total Particulate Matter –TPM
tris-(hydroxymethyl)-aminomethane – Trizma
U.S. Food and Drug Administration – FDA
Ultra-Performance Liquid Chromatography with Photodiode Array detection – UPLC-PDA

1 Introduction

Identification and chemical characterization of our native flora is an important area of scientific research. Structurally diverse natural flora can provide a valuable source of therapeutic agents that can be useful in the treatment of disease. There are multiple documented historical accounts that show that people used the plants found in their environment for medicinal purposes.

1.1 Historic use of plants as medicine

A classic example of people historically using plants for medicine is the treatment for malaria. The Center for Disease Control and Prevention (CDC) describes malaria as a mosquito-borne disease transmitted by the Anopheles mosquito [1]. The mosquito infected with the parasite *Plasmodium falciparum* or *Plasmodium malariae* transmits the parasite to humans following a bite [2]. When people are infected with the parasite they usually present with high fevers, followed by a host of other symptoms such as flu-like conditions, nausea, vomiting and, in extreme conditions, death [1]. In the 1630's Spanish conquistadors introduced the bark of the cinchona tree (*Cinchona officianalis*) to Spain. This bark was used by native Peruvians to cure fevers and treat other symptoms of malaria [3]. It has commonly been referred to as Jesuit's bark, cardinal's bark, or sacred bark, and the cure quickly spread across all of Europe [4]. For approximately 300 years, the bark of the cinchona tree was the only known effective remedy for malaria [3,4] .

For the treatment of malaria, the bark of the cinchona tree was first dried, ground to a fine powder, and then mixed into a liquid (commonly wine) before being consumed [3]. While this method was effective in the treatment of the disease, the cure could be

compromised if the bark that was not from the cinchona tree or the cinchona tree bark was not well preserved. To standardize treatment, in the 1820's two professors, Pierre Joseph Pelletier and Joseph Caventou, at the School of Pharmacy in Paris, isolated the active compound, quinine, from the tree bark enabling the physicians to prescribe the drug more accurately [4]. The professors also found that not only quinine was effective against malaria, but they were also able to isolate other cinchona alkaloids, including quinidine, cinchonine, and cinchonidine that were also effective anti-malarials [4]. In 1944, due to wartime pressure, American chemists Woodward and Doering accomplished a formal synthesis of quinine without the aid of natural sources [5]. Today malaria is still relevant; the CDC estimates that in 2016 there were 216 million cases of malaria worldwide with 445,000 cases ending in death [6]. Quinine is one of the drugs still used as a treatment for malaria [7]. For a short time, it was replaced by chloroquine but due the increasing chloroquine resistance by the organism *Plasmodium falciparum* it is again a key drug of choice for malaria treatment [3,7].

Aspirin is another common medicine whose inspiration for synthesis came directly from historical use of a plant. In the 18th century Reverend Edward Stone reasoned that since cinchona bark was effective in the treatment of fevers, due to its same bitter taste, willow bark could also be used in the treatment of fevers [8]. While his scientific method does not meet today's standard, he successfully treated people suffering from fevers with the willow bark. Later, researchers learned that salicin and other similar natural compounds isolated from willow bark, collectively called salicylates, effectively reduced fever, pain, and swelling [8]. The success of these willow tree natural product isolates led the German company, Bayer, to design a compound modeled after salicin and other

active metabolites that could compete with the natural salicylates available at that time [4]. Bayer designed O-acetylsalicyclic acid which is now recognized by the common drug name of aspirin [4]. Aspirin is still found in our medicine cabinets today where it is still used as an analgesic to treat fever, pain, and inflammation.

Currently another concern is increasing antibiotic resistance to β-lactam antibiotics. The Centers for Disease Control (CDC) has implicated methicillin – resistant *S. aureus* (MSRA), a bacterium, as being responsible for doubling the amount of hospitalizations from 127,036 to 278,203 from 1999-2005 in the United States [9]. This bacterium is commonly found on skin of healthy individuals and often becomes difficult to treat once it becomes resistant to multiple antibiotics [10]. Antibiotic resistance could lead to mortality and already some drugs of last resort such as vancomycin, which is a nephrotoxin, are becoming increasing ineffective in treating the disease [10,11].

In 2015, Harrison et al. were able to recreate a 1000-year-old antimicrobial remedy for MRSA using plants found in a 10^{th} century Anglo-Saxon medical text (Bald's Leechbook) [12]. The cure was listed in the medical text under Bald's eyesalve which was designed to treat eye infection. Reproducing the cure which consisted of garlic, leek, oxgall, wine, and brass was not effortless; the curative was written in Old English and first needed to be translated [12]. Next, there were no detailed amounts of each component. The research group found, upon reconstruction of this cure, reproducible results in the eradication of established Staphylococcus aureus biofilms in an in vitro model of soft tissue infection [12]. This cure known as an "ancientbiotic" outperformed vancomycin and was also able to eradicate MSRA in chronic mouse wounds that were infected with MSRA [12]. The Harrison group made three specific conclusions from this study: 1) plants and

plant models can continue to help in the development of new drug therapies, also 2) remedies of the past should be explored to see if they are capable of yielding new treatments for disease. 3) A more combinatorial approach capitalizing on the synergy of bioactive compounds may often be more therapeutic than the isolation of single target molecules [12]. This point is even further reinforced by the Harrison group which found that the combination of ingredients listed in the medieval text were crucial for full activity against MSRA [12].

1.2 Demand for natural therapies vs. understanding of safety and efficacy

Currently there is an increasing demand for natural therapies and the use of herbal products to treat various ailments. While scientific evidence exists regarding some complementary and alternative medicine (CAM) therapies, for most herbal medicines, there are key questions that are yet to be answered through well-designed scientific studies such as whether these therapies are safe and whether they work for the purposes for which they are used [13]. It is generally believed that natural therapies have fewer undesirable side-effects, but this is not true in all cases of natural product use. Furthermore, since there is limited regulation, they are often used in different strengths and formulations without consistency in content or knowledge about toxicity [14]. For this reason, The World Health Organization (WHO) published guidelines in 1998 that establish quality, safety, and stability protocols for plant materials [15]. These guidelines are tailored to industries that may use natural products commercially and have little effect on the home user. Plant products used as therapeutics have many challenging factors that could possibly affect the quality of the natural remedy [16].

A factor that is difficult to control from one plant to the next is the level of bioactive compounds. Bioactive compounds may be affected by many genetic and environmental factors. Some environmental factors that affect plant growth and development are temperature, carbon dioxide, light, water, nutrients, and breeding [17]. Variability can also come from the modes in which the plant is prepared for consumption [14].

Another factor that may lead to variation in the levels of bioactive species in plant material when testing is the multiple ways in which herbal solutions are stored and prepared after harvesting. Tseng et al. studied the effects of different drying methods and storage times in two grape cultivars to determine which condition yielded the greatest retention of bioactive compounds. Even though they determined the drying methods that retained the highest levels of bioactive compounds, these yields were reduced significantly after 16 weeks of storage at 15 ± 2 ºC [18].

1.3 Historical usage of *Pseudognaphalium obtusifolium* as a medicinal plant

P. obtusifolium is of interest because there are multiple documented accounts where parts of the plant have been used historically for medicinal purposes [19–24]. It also has an aromatic smell characteristic of terpenes which can also suggest bioactivity [25,26]. Historically the leaves and flowers of this plant have been used by Native American tribes as a medicinal herb in a variety of ways. The *Peterson Field Guide to Medicinal Plants and Herbs of Eastern and Central North American*, documents ways Indigenous Americans used the herb to cure a variety of ailments such as sore throats, pneumonia, cold, fever, upset stomach, abdominal cramps, rheumatism, vaginal

discharge disorder, bowel disorders, asthma, coughs, mouth ulcers, tumors, and as a diuretic [22]. The field guide lists the plant as also being a mild nerve sedative and antispasmodic [22].

It has been reported that the Creek Native American tribe referred to the herb as Ahålobaktsi or Halobåktci [24]. The annual report also records that the Creek used the herb as a cure for mumps, bad colds, upset stomachs, and as a rest aid. The Creek also used this herb to add a “perfume” to other medicines [24].

A 1983 dissertation by Croom has provided a more detailed summary than other accounts of the use of this plant by the Lumbee Native American tribe. In the Croom account, *P. obtusifolium* was one of the more popular plants of the Lumbee and was used in many herbal remedies to treat colds, flu, neuritis, asthma, coughs, and pneumonia [23]. The Lumbee also stuffed pillows (e.g., bedding) with the herb to prevent asthma attacks [23]. The herb is typically combined with other plants, but it is also used alone [19,23]. Smoking the herb is not mentioned in the Lumbee Native American historical account; however, other sources indicate that the plant was also smoked to treat asthma [20,21].

In a study of Lumbee traditional medicine in 2017, Rochet et al. tested water extracts of *P. obtusifolium* in addition to eight other plants. The extracts were prepared with 10 grams of the whole plant in 50-100 mL of water and were found to have a total polyphenolic content of 2.8 % using the Folin-Ciocalteu assay [27]. According to the study design, they found that *P. obtusifolium* extracts did not alleviate neurotoxicity caused by Parkinson’s disease related systems which was one of the focal points of their research [27]. There have been other groups that have done exploratory work to

investigate the herb's anticancer properties [28]; however, aside from the study by the Rochet et al. research group, there has been no published research on the compounds found in this plant.

1.4 *Pseudognaphalium obtusifolium* identification

P. obtusifolium has been identified as being indigenous to the eastern United States. *P. obtusifolium* is an annual flowering plant belonging to the Asteraceae family. It is found in dry clearings, fields, and woodland edges. The plant can grow to heights of approximately 1-2 feet tall with densely woolly stems (Figure1A and D) [22]. The leaves are green on top and silvery or white underneath (Figure1B). The numerous alternate leaves are narrow and attached directly to the base of the plant without a stalk (Figure1B) [29,30]. The small, off-white flowers are numerous consisting of many flower heads (Figure1C and D) [29,30]. The flowers become an off-white to tan color once dried.

Some observations made during the growing period is that the plant can grow in full sun but seems to thrive better in partial shade and dry sandy soil. The leaves and flowers have a complex fragrant aroma that can be described as "piney/lemony" with additional "caramel or maple syrup" notes. The aroma is more pronounced in the dried plant.

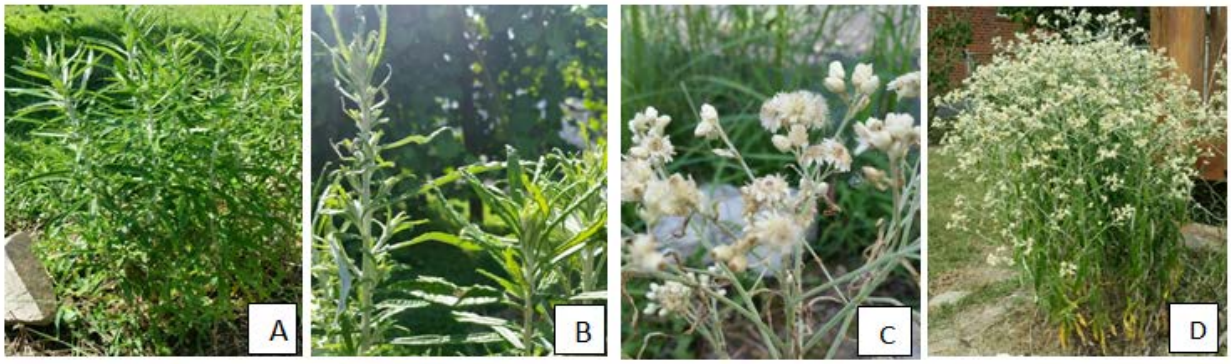

Figure 1. Stages of *Pseudognaphalium obtusifolium* development: (Left to right) A) June- July young plants; B) August - September flowers forming; C) and D) September – October plant in bloom.

1.5 Overview of sample preparation and extraction techniques

The chemical profiling of *P. obtusifolium* was tailored for the three common usage forms (tea, bedding material, and smoke inhalation) [21,23]. Pressurized solvent extraction and liquid/liquid solvent extraction were used to characterize compounds found in the tea while headspace extraction was used to identify compounds found in the bedding material. Compounds in the smoke were identified using smoke collection and extraction techniques.

1.5.1 Pressurized Solvent Extraction (PSE)

Solid-liquid extraction techniques are used for preparative purposes when analytes require separation from a solid sample matrix. The most widely used solid-liquid extraction technique for extracting bioactive compounds is Soxlet extraction [31]. Soxhlet extraction is not ideal for characterization of plant material because during Soxhlet extraction, heated solvent continuously flows over the solid material. This constant heated

flux coupled with lengthy extraction times could cause analyte degradation for heat labile compounds. Due this possibility alternative extraction techniques were explored.

Pressurized solvent extraction (PSE) also known as, pressurized fluid extraction (PFE), and accelerated fluid extraction (ASE) or enhanced solvent extraction (ESE), was chosen as an extraction method because it uses elevated temperature and pressure to increase both the rate and efficiency of the extraction thus allowing for shorter extraction times [32]. The elevated pressure allows the plant cell structures to be ruptured and the analytes more easily released, which leads to low solvent expenditures and faster extraction times [32]. PSE is usually automated; therefore, it also provides some standardization to the extraction process. For plant matrices, this standardization is necessary because there are many other factors that are difficult to control.

PSE yielded an aqueous extract but most of the less polar bioactive compounds, which were the target for this study, are more soluble in an organic phase. For this reason, the second step of the extraction process used liquid/liquid extraction to partition or separate the compounds based on their relative solubilities in two immiscible liquids. In this way the liquid/liquid extraction step also provides some measure of sample clean-up because many of the aqueous components may not be as soluble in an organic solvent extraction. Liquid/liquid extraction is described by equation 1 [33]:

Equation 1:

$$K_d = \frac{C_o}{C_a}$$

where K_d is the distribution ratio, C_o is the concentration of the target analyte in organic phase and C_a is the concentration of the target analyte in aqueous phase [33].

The K_d may be influenced by many factors such as temperature and pH of the solution.

1.5.2 Solid Phase Extraction (SPE)

Another extraction technique that was used in this study was solid phase extraction (SPE). This preparative extraction technique used for liquid samples is beneficial for reducing undesirable components of the plant matrix that can interfere with isolation of the desired analytes. In SPE desirable components of the plant extract can be retained on the solid phase (also referred to as the sorbent phase) and eluted with a compatible solvent yielding a cleaner sample for analysis [34,35]. Conversely, the undesirable elements of the plant matrix can be retained by the sorbent phase which also yield a cleaner sample.

There are a range of phases that are commercially available; they include silica, activated carbon, C_{18}, C_8, C_5, C_4, phenyl, diol, amino bonded silica, ion exchange phases and polymer phases [34]. There are also salting out extraction techniques such as Quick, Easy, Cheap, Effective, Rugged, and Safe (QueChERS) which combine salts and SPE materials to enhance extractions [35,36].

Activated carbon, C_{18}, and QueChERS were experimented with in this work. Activated carbon removes nonpolar compounds such as compounds responsible for pigmentation as well as sterols. C_{18} phases are beneficial for removing nonpolar interferences such as fats and waxes. QueChERS is a technique that combines salting out with sorbent phases [35,36]. Salting out describes an event where the solubility of a nonelectrolyte substance in water decrease with increasing salt concentration [35,36]. The QueChERS technique in this study used NaCl combined with the sorbent $MgSO_4$.

The purpose of $MgSO_4$ was to remove excess water from the extraction matrix. Sample cleanup steps are important because they may prolong the life of the GC or UPLC column while also improving sample recoveries and accuracy [37].

1.5.3 Headspace Extraction

When identifying compounds found in the bedding material, another sample extraction technique referred to as headspace extraction was employed. Headspace extraction is a technique that is used to characterize gas phase components that are a part of a solid or liquid sample. The headspace sampling technique, referred to as static headspace is represented by a closed sampling system where vapor phase and the sample are in equilibrium [38]. In static headspace the sample may be heated for a set time so that the volatile components of the sample are in equilibrium between the gas-phase and the sample. The power of the headspace separation technique comes from the fact that the more volatile compounds move into the gas phase while the heavier, less volatile compounds remain in the bulk sample. When equilibrium is reached a portion of the gas phase is collected and analyzed by GC-MS. The headspace equilibrium is described by the equation 2 below [38].

Equation 2

$$A \propto C_g = \frac{C_o}{K + \frac{V_g}{V_s}}$$

The variable *A* represents the GC-MS peak area. C_g is the analyte concentration in gas-phase. C_o is the analyte concentration in the sample. *K* is the partition coefficient

of a given compound between the gas-phase and sample-phase. V_g is the volume of the gas-phase. V_s is the volume of the sample phase [38]. The variables in this equation can be optimized to yield increased peak areas.

1.5.3.1 Headspace using solid phase microextraction (SPME)

The headspace application used for this study was performed using solid phase microextraction (SPME) fibers (see Figure 2.A). In this method, a thin coat of polymer is added to a thin silica support [39]. Particles embedded in the polymer as well as the polymer itself have extraction and sample concentration capabilities. A needle protects the fiber (Figure 2.A) which is then fitted into a SPME fiber holder (Figure 2.B) for injection onto the GC column [39].

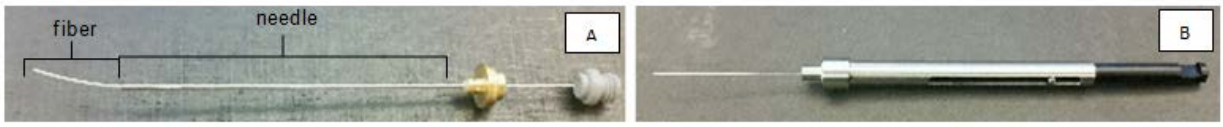

Figure 2. SPME fiber assembly: (A) the exposed fiber inside the SPME fiber assembly; (B) the holder assembly used for injection onto the GC.

Once gas-phase molecules are trapped by the fiber, they are introduced directly into the heated GC injector port where they are then thermally desorbed from the fiber and introduced to the GC column [36,37]. The SPME process should be optimized for the samples of interest so that reproducible data are acquired.

The sampling process starts when the gas-phase molecules in the headspace extracted by the porous coating comes to equilibrium with the molecules in the sample in the closed sampling system. Equation 2 above describes the partition coefficient of a

given analyte between gas-phase and sample-phase. Equation 3 describes the distribution constant between the fiber coating and sample [39]:

Equation 3

$$K_{es} = \frac{C_{fs}}{C_s}$$

The variable, $K_{es,}$ represents the analyte distribution constant between the fiber and coating. C_{fc} is the analyte concentration of the fiber coating, and C_s is the analyte concentration in the sample [30].

The distribution constant, $K_{es,}$ is affected by sample dilution, temperature, ionic strength, pH, and organic solvent. Optimization of the before-mentioned parameters improves sensitivity of the technique.

1.5.4 Smoke collection and extraction

Since the plant was smoked by the Native Americans, an extraction of the smoke was performed for chemical profiling of the smoke condensate. The plant material was stuffed into a cylindrical paper article resembling an unfiltered cigarette. Sample collection was performed using an automated smoking machine which simulates smoking of the smoking article. It should be noted that no smoking machine can replicate all the patterns of individual smokers [40]. Rather, machine testing seeks to characterize the analytes found in smoke and provides a level of standardization across smoke testing laboratories [40]. Smoke is usually divided into two phases, a particulate-phase and a vapor-phase [41]. To trap the particulate-phase, smoke from the smoking article is collected on a cellulose acetate pad [40]. The cellulose acetate pad is then extracted with the appropriate extraction solution for collection of target analytes. To

trap molecules in the vapor-phase, the smoke is pulled into a glass smoke collection trap called an impinger. The impinger is filled with the appropriate extraction solution that effectively traps target analytes of interest and can be cooled if necessary, to increase the trapping efficiency for target compounds. The cellulose acetate pad can be combined with the impinger trapping system when also exploring compounds that may be found in the particulate-phase [40]. After smoke condensate collection, the extraction solution can be analyzed by various analytical methods.

The simulation of smoking was a combination non-targeted and targeted analytical approach (Figure 14.A). Smoke collection techniques were used to obtain a liquid sample for a non-targeted gas chromatography-mass spectrometry (GC-MS) analysis while a targeted approach was used to access the levels of select carbonyls by Ultra-Performance Liquid Chromatography with Photodiode Array (UPLC-PDA) detection.

1.6 Sample separation and detection techniques

Once sample collection and extraction are complete, the extract needs to undergo some form of elucidation analysis so that the compounds in the extract are identified. In this work, gas chromatography coupled with mass spectrometry (GC-MS) is used almost exclusively for characterization of compounds. Smoke condensate chemical profiling was the one exception where Ultra-Performance Liquid Chromatography with Photodiode Array Detection (UPLC-PDA) was used in addition to the GC-MS technique.

1.6.1 Ultra-Performance Liquid Chromatography with Photodiode Array (UPLC-PDA) detection

UPLC relies on a series of pumps to pass pressurized solvent through a column coated with absorbent packing materials (stationary phase) [42]. These pumps can operate at high pressures (15,000 – 20,000 psi) which allows smaller (<2 µm) stationary phase particles to be used at increased linear velocities. Increased linear velocities improve analyte resolution while decreasing analysis runtimes. This stationary phase separates the target analytes from the extraction mixture based on the interactions of the analytes with the solvent mobile phase and the column materials. UPLC systems can separate analyte mixtures based on the polarity, molecular size, or electrical charge of the compound. For separation by polarities, there are two types of partition chromatography, normal phase and reversed phase. Normal phase employs a highly polar column stationary phase in tandem with a nonpolar mobile phase for separation. Reversed phase uses a nonpolar column stationary phase with a relatively polar mobile phase for analyte separation [42]. The work for this study was done using reversed phase techniques where nonpolar C_{18} ligands were attached to the silica column packing material.

The UPLC was coupled with a PDA detector for analyte detection. A PDA detector is capable of measuring sample extract components that have an absorption spectrum in the ultraviolet or visible region of light. The light from a deuterium lamp is directed into the flow cell containing the sample mixture. A diffraction grating splits the light into different wavelengths and diverts the light to multiple photodiode arrays fabricated with semiconductor material [43]. This material converts the light signal into

an electrical signal that corresponds to a wide range of wavelengths. The measurement is acquired by adjusting the deuterium lamp so that the wavelength of light from single or multiple predetermined wavelength(s) is directed into the flow cell. The flow cell is filled with sample and mobile phase solvent from the UPLC. Absorbance is measured by measuring the difference in light intensity between the front and the back of the flow cell. Continuous eluate flow from the UPLC allows spectra to be obtained by absorbance measurements measured in ≤ 1 second intervals. Retention times are used to identify components by spectral comparison [43]. Ideally the spectrum is compared with a standard analyte for compound identification and quantification.

1.6.2 Gas Chromatography Mass Spectrometry (GC-MS) detection

Gas chromatography mass spectrometry (GC-MS) in scan mode (non-targeted approach) and SIM (targeted approach) are powerful analysis techniques. The GC aids in the separation of a sample extract by separating volatile and semi-volatile compounds in a complex mixture into individual compounds and works best for small molecules (< 600 Daltons)[44–46]. Once a sample extract is injected onto the capillary column of the GC, it is propelled though the stationary phase of the column by an inert gas called a carrier gas (in all experiments, helium was used as the carrier gas). The stationary phase is the coating on the inside wall of the column that interacts with the sample extract while the components of the sample are transported down the column by the carrier gas [44–46]. The temperature affects how the sample interacts with the column; therefore, the column temperature can be adjusted to assist with analyte separation. The strength of these interactions, between the analytes in the sample and the column stationary phase

facilitate the compounds' separation. The stationary phase of the column is selected based on the types of compounds that will be targeted for analysis [44–46].

As compounds exit the GC column, they are detected using mass spectrometry [45]. In the most commonly used ionization mode, electron ionization (EI), the compounds exiting the column are bombarded with 70 eV electrons that fragments the compounds into positively charged ions. The most common MS is the single quadrupole where ions are separated based on their different mass to charge ratios (m/z) using alternating DC and RF current as the ions are propelled through them using a network of electrostatic lenses [45]. After separation by the mass analyzer the ions are detected with an electron multiplier. Computer software produces a mass spectrum of the ionized peak intensity as a function of the m/z ratios of the fragments. Full scan is a quadrupole setting that can monitor a range of compounds with fragments of typically 50-600 m/z in size. By starting with a lower m/z of 50, background ions which may be the result of residual air and carrier ions are excluded [46].

Due to the consistent fragmentation patterns resulting from 70 eV EI, computer spectral libraries can aid in the interpretation of these mass spectra and provide rudimentary compound identification [46]. The use of the full scan setting in conjunction with spectral mass libraries is ideal for identifying compounds and compound classes that may be found in an unknown sample [46]. Computerized AMDIS (automated mass spectral deconvolution and identification system) software compares the m/z fragmentation patterns of the experimental data to the patterns in the spectral libraries. Quality matches are assigned based upon the m/z found and the ratio of their intensities [46]. For common compound identifications using AMDIS, usually identifications above

80 are reliable, 70–79 are often correct, and 60–69 are uncertain [47]. For this body of work, the spectral match was set at ≥ 80 to decrease the chance of peak misidentification by the AMDIS software algorithms. Peaks were positively identified at a spectral match ≥ 80 if no certified standard was available. It is understood; however, that even the best match qualities do not always positively identify an unknown peak. Likewise, in many instances poor match qualities may often correctly identify a compound, thus it is important to provide confirmation with reference compounds when available.

Selective Ion Monitoring (SIM) is another capability of the quadrupole analyzer. In SIM mode, one or multiple target masses are monitored over the course of the chromatographic separation [46]. With this technique, the sensitivity can increase up to three orders of magnitude because the mass analyzer is not sampling a complete range of masses. A computerized system can select for different targeted masses over the course of the separation. This technique is helpful if the peak is only partially resolved because a unique ion can be selected which may not be a part of a co-eluting peak [46].

1.7 Sample analysis quantification techniques

This study used external calibration curves which are constructed using known concentrations of a standard compound for sample quantification. This work was also performed with and internal standard method to quantify data where a known amount of the internal standard is added both to standards and unknown samples to use the ratio of the response between the analyte and the internal standard to determine analyte concentrations. Standard addition was used to determine method accuracy with the determination of % recovery.

1.7.1 Calibration curves based on external and internal standards

External standards are used when injections are reproducible and there are not multiple sample preparation steps. An external standard response factor is calculated using the following equation [45].

Equation 4

$$RF = \frac{Area_{std}}{Conc_{std}}$$

The variable, RF, represents the response factor. $Area_{std}$ is the peak area of the standard analyte. $Conc_{std}$ is the concentration of the standard. This method is easily generalized with the use of multiple standards to create a calibration curve.

If the sample preparation procedure has multiple extraction steps the use of an internal standard calibration method will improve the reproducibility of the quantitation [46]. The internal standard is an analyte added to all samples (including calibration curves and blanks) in a known amount. Since the internal standard is added to all samples the signal from the unknown analyte can be compared to the signal from the calibration standards to quantify the unknown compound (see Figure 3). This is detailed in the calculation scheme below (see Equations 5-7).

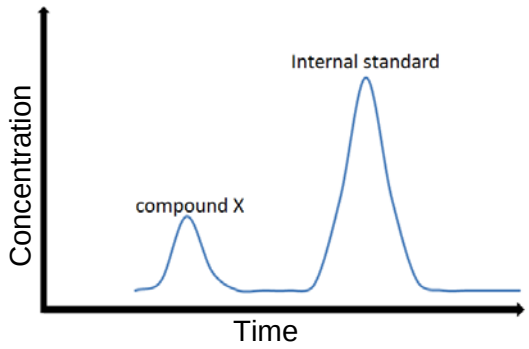

Figure 3. Example chromatogram of compound X and internal standard

Equation 5

$$RRF = \frac{Area_X}{Area_{ISTD}} \times Conc_{ISTD}$$

The Relative Response Factor (RRF) is represented by Equation 5, where $Area_X$ is the area of the unknown compound. $Area_{ISTD}$ is the area of the corresponding internal standard peak and $Conc_{ISTD}$ is the concentration of the internal standard for the calibration standard.

This method can also be generalized for the use of multiple standard samples using linear regression of a calibration curve. The concentration of the target analytes is then determined from the calculated RRF for the sample and the y-intercept as shown with the following equation [46]:

Equation 6

$$Conc_{unknown} = \frac{RRF - Intercept}{slope}$$

The variable, $Conc_{unknown}$, represents the calculated analyte concentration (µg/mL). The variables, intercept (which is the y-intercept) and slope are from the calibration curve equation. The concentration of the analyte can then be converted into µg/gram concentration units as shown in Equation 7.

Equation 7

$$Conc\ (\mu g/g) = Conc_{unkown} \times \frac{Vol}{wgt}$$

Conc (µg/g) is the amount of analyte in one gram of *P. obtusifolium*. $Conc_{unknown}$ (µg/mL) is the sample concentration of *P. obtusifolium* from the calibration. Vol is the volume (mL) of the extraction solution. Wtg is the *P. obtusifolium* sample mass weight (g).

The internal standard must be well resolved from the peaks of interest and not be found in the sample [45]. Preferably, the internal standard should also be available in high purity. Multiple extraction steps were associated with the complex matrix of *P. obtusifolium* plant material (see section 2.5.2); therefore, an internal standard method was needed.

1.7.2 Recovery experiments to evaluate method performance

Ideally a certified reference material with a known concentration would be evaluated by the established method. The method procedure would then be evaluated against how closely the quantitated concentration matched the established concentration of the certified material. If there is no certified reference material, method performance can be evaluated by recovery experiments. The purpose of assessing

recovery for this work was to demonstrate that the concentration of the target analyte reported is “true”. The “trueness” of a value is used to evaluate extraction efficiency.

Since the term “recovery” has been described and calculated in many ways both the term and the procedure for evaluating recovery must be described. This work uses the 1999 technical report, “Harmonised Guidelines for the use of Recovery Information in Analytical Measurement”, to define the type of recovery used for this work [48]. The 1999 technical report describes the type of recovery performed in this work as “surrogate” recovery where the recovery of a pure compound or element specifically added to the test material as a spike as depicted in Figure 4 and Equation 8 [48].

The report that the1999 technical report based its definitions on was published by the International Union of Pure and Applied Chemistry Analytical (IUPAC) in conjunction with the International Standard Organization (ISO) and The Association of Official Analytical Chemists (AOAC); these organizations standardize common terms and procedures on an international and global level [48].

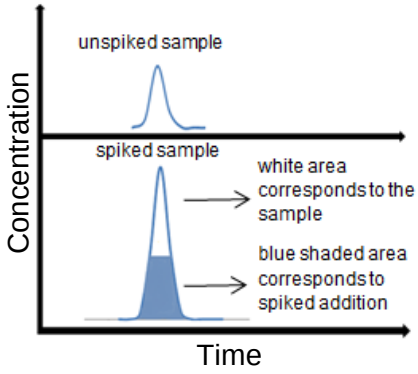

Figure 4. Illustration of surrogate recovery method

Equation 8

$$\% \text{ Surrogate Recovery} = \left(\frac{\text{Spike Sample Conc - Unspiked Sample Conc}}{\text{Spike Concentration}}\right) \times 100\%$$

The Spike Sample Concentration is the sample concentration determined experimentally in the spiked matrix. The Unspiked Sample Concentration is the sample concentration determined experimentally before the addition of a spike. Spike Concentration is the concentration that corresponds to the spike amount added.

Before surrogate recovery is performed, acceptance criteria should be established for the results. For instance, it may be reasonable to expect given the complex matrices and extraction steps that % recovery will have some uncertainty associated with the measurement. This uncertainty should be reported in the method so that the limitation of the analytical measurement is recognized [48].

2 Overview: Chemical characterization of *P. o*btusifolium based on historical use

As previously discussed, this study explores chemical characterization of *P. obtusifolium* based on the historical practices. Following historical usage forms capitalizes on the understanding of the plant that the native people developed through years of use. This work explored the chemical composition of simulated tea, bedding, and smoke exhaust. The leaves and flowers were analyzed separately to determine if they yielded the same chemical profiles.

The experimental approach used in this work is shown graphically in Figure 5. First, a chemical survey was conducted to determine which types of compounds may be found by using a hydroethanolic extract of the plant. This first step provided insight as to

what compounds could be found in a solvent extraction. Next, a chemical profile was performed on an aqueous extract. This extract was modeled after the way Native Americans used the plant to prepare a tea. The aqueous extract will be referred to as the tea simulation or tea. The headspace sample collection technique was used to elucidate compounds that may be found when the plant material was used as bedding material. Smoking the leaves of the plant was also documented; therefore, a smoking simulation of the plant was performed to determine compounds that may be found when smoked.

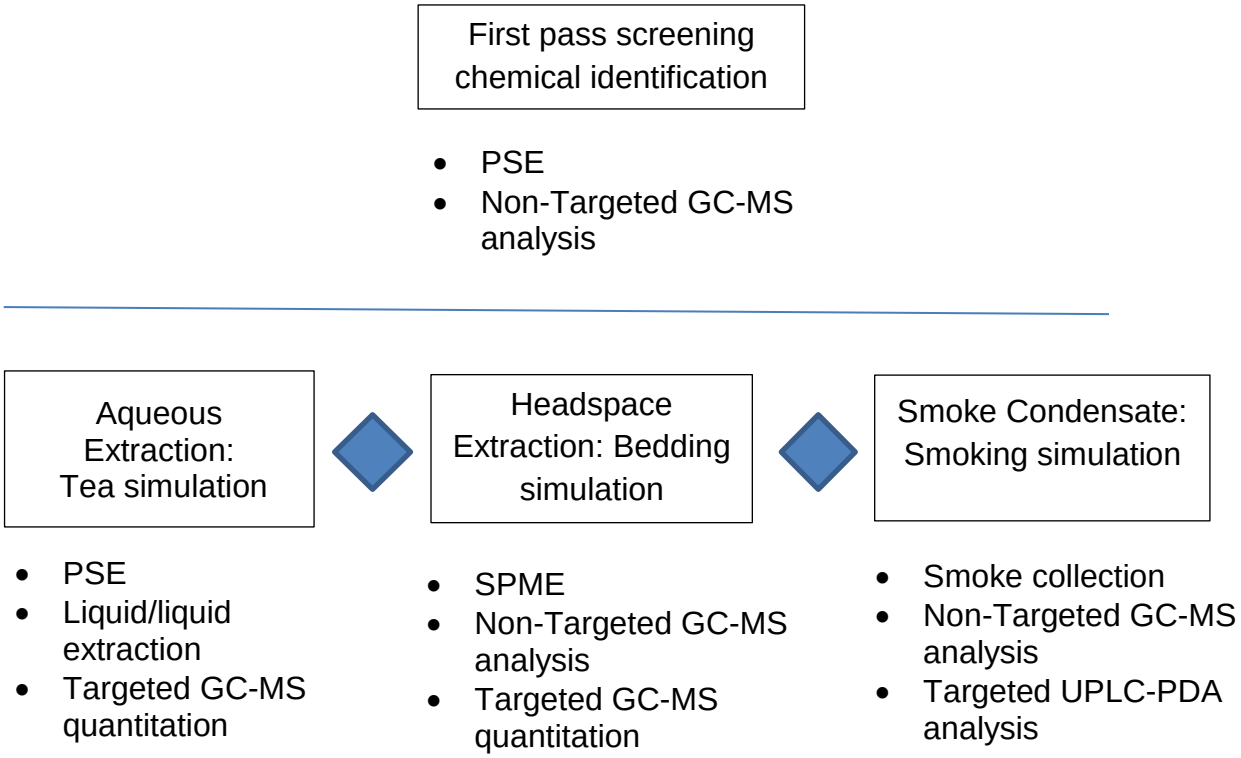

Figure 5. Schematic of chemical characterization plan for *P. obtusifolium* based on historical use

2.1 Plant material collection

Plants used for this study were obtained from Tellico, TN (Wild Pantry) and grown to maturity in Richmond, VA. The plants used in this study were formally identified by botanist John Hayden working in conjunction with Lewis Ginter Botanical Gardens of Richmond, VA. Formal identification was necessary because there are many references to common names. The Oklahoma Museum of Natural History records 11 common names for this plant: common everlasting, Indian posy, sweet everlasting, life everlasting, sweet-scented life everlasting, blunt-leaved everlasting, old field balsam, white balsam, catfoot, rabbit tobacco, and fragrant cudweed [20]. This herbal plant has also been reclassified from *Gnaphalium obtusifolium* to its current name *Pseudognaphalium obtusifolium* [29].

2.2 Preparation of plant material for study

Plant flowers and leaves were harvested in Richmond, VA during the fall seasons of 2017 and 2018. Croom documents that the plants were typically harvested after the fall frost or during the winter but also states that the Lumbee would also gather the leaves once they turned brown [23]. Once the mature flowers developed, they were allowed to dry on the plant. No further drying was performed in keeping with efforts to duplicate historical usage, and because further drying could possibly reduce the volatile components of the plant. The dried leaves and flowers were then separated from the whole plant. Next, a cryogenic grinding technique was used to preserve the volatile components of the sample and provide sample homogenization across the entire study. Liquid nitrogen (-196 ºC) was chosen to grind the samples because its low temperature

significantly reduces the vapor pressure of the sample components so that the volatile components are largely preserved. Liquid nitrogen also makes the sample brittle which allows it to be ground at shorter grinding times which also reduces sample exposure to high grinding temperatures. For this study, all samples were cryo-ground with liquid nitrogen using a Retch Grindomix GM200 at a grinding speed of 6000 rpm for 30 seconds. After grinding, the leaves and flowers both had a soft and light textured "fleecy" consistency (see Figure 6). The leaves and flowers were then stored separately in glass amber bottles or Mylar bags at room temperature until further analysis.

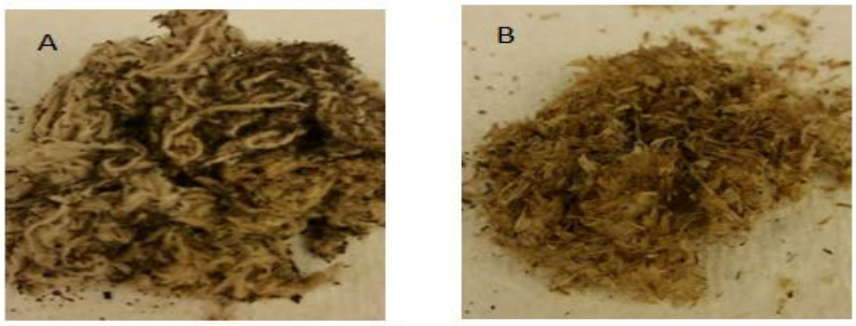

Figure 6. Ground leaves and flowers of *P. obtusifolium*. A) Shows the ground leaves and B) depicts the ground flowers of *P. obtusifolium*.

2.3 Initial analyte screening of plant material

Before starting the chemical characterization of analytes in the tea, a screening experiment was done using 70:30 ethanol: water extraction solution. This hydroethanolic extract was selected because in an evaluation of several medicinal plants ethanolic extractions of 50% and 70% ethanol: water were shown to have increased extraction efficiencies of bioactive compounds [49]. The results of the

screening experiment are used to target bioactive compounds that may be found in the tea or other usage forms.

2.3.1 Initial Pressurized Solvent Extraction (PSE) of plant material (70:30 ethanol: water extraction)

Extraction of bioactive ingredients was key to the chemical profiling investigation because *P. obtusifolium* was used by Native Americans to provide a therapeutic benefit. To maximize the extraction bioactive components, pressurized solvent extraction (PSE) was used to extract the leaves and flowers with a hydroethanolic extract as the solvent. In the current literature, PSE is often used to extract bioactive ingredients from natural sources [31]. The elevated pressure allows the plant cell structures to be ruptured at decreased temperatures, thus improving the rate of and the efficiency of the extraction [32]. In this study an automated Pressurized Solvent Extractor (SpeedExtractor E-914/E916 manufactured by Buchi) was used which adds uniformity to the extraction procedure. There is also normal variability that exists with plant material. To this end, all instrument parameters in the preparation of plant extracts should be as standardized as possible.

In this extraction approximately 4 grams of the leaves and flower were extracted separately. The plant material (leaf and flower) was weighed directly into stainless steel 120 mL extraction cells. The void space in the 120 mL cell after the plant material was added was maximized with steel ball bearings. The amount of steel ball bearings were weighed so the same mass could be used each time the extraction was performed. The extraction parameters for the SpeedExtractor E-914/E916 are detailed in Table 1 below.

Table 1**.** Pressurized Solvent Extraction Parameters

Parameter	Description
Temperature	100ºC
Pressure	150 bar
Solvent	70:30 ethanol: water
Extraction cell	120 mL
Time	60 minutes

2.3.2 Initial GC-MS identification of compounds present in the plant material

High analyte yields were important so that the filtrate could be injected directly on the column of the GC-MS in SCAN mode. Other GC-MS parameters for the hydroethanolic screening experiment are found in Table 2.

Table 2. GC-MS Parameters for the Hydroethanolic Screening Experiment

Parameter	Description
Column	DB-17MS (30 m X 0.25 mm X 0.25 µm)
Injection temperature	250 ˚C
Injection mode	Split (50:1)
Injection volume	1 µL
Temperature gradient	40 ˚C hold for 1.25 minutes ramp 15˚C/minute to 300 ˚C
Analysis time	77 minutes
Ion source	Electron ionization
Transfer line temperature	280 ˚C
Ionization energy	70 eV
Source temperature	230 ˚C
Acquisition type	SCAN mode

The compounds and classes of compounds found in the hydroethanolic extraction was used solely as a guide to understand the types of compounds potentially present in *P.*

obtusifolium. This also enabled optimization of extraction and GC conditions when exploring usage forms (tea, bedding, and smoke).

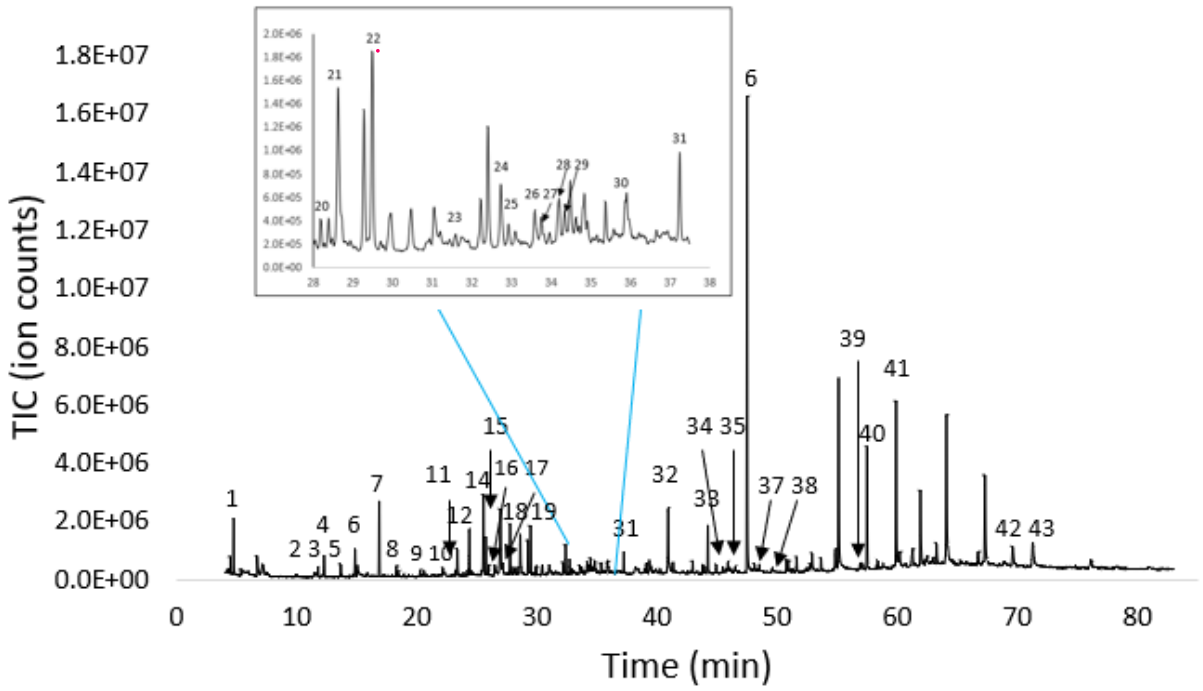

Figure 7. Chromatogram of 70:30 ethanolic extract in SCAN mode where some peaks were identified by spectral match. Unlabeled peaks could not be identified with a match factor ≥ 80. Peak details are given in Tables 3 and 4.

Figure 7 shows a total ion chromatogram (TIC) of the GC separation and the MS detection of the hydroethanolic extract in full SCAN mode. Approximately 36 % of the peaks were identified in the chromatogram and of the 43 compounds identified by spectral match, 11 of these compounds were confirmed using purchased reference compounds and these are noted in Tables 3 and 4. The identified peaks are numbered and correspond to Tables 3 and 4, where the compound class, retention time (RT), and Library Quality Matches are provided. Compound identification in analysis of GC-MS

data is achieved by matching the experimental mass spectra to the mass spectra in the NIST reference library [50].

As mentioned in section 1.6.2, only peaks with a match quality ≥ 80 were considered identified with the understanding that even the best match qualities do not always positively identify an unknown peak thus it is important to provide confirmation with reference compounds.

Table 3. Terpenes Found in the 70:30 Ethanol: Water Screening Experiment

Class	RT order	Compound	CAS No	Quality Match	Confirmation by reference compound
terpenes	11.55	(R)-(+)-limonene	5989-27-5	98	yes
	18.31	(−)-menthol	2216-51-5	91	yes
	25.44	β-ylangene	20479-06-5	95	no
	25.57	β-caryophyllene	87-44-5	99	yes
	25.76	β-copaene	18252-44-3	99	no
	27.17	alloaromadendrene	25246-27-9	95	no
	27.79	γ-muurolene	30021-74-0	96	no
	27.94	α-muurolene	31983-22-9	96	no
	28.20	(+)-β-selinene	17066-67-0	95	yes
	29.28	γ-cadinene	39029-41-9	98	no
	29.50	δ-cadinene	483-76-1	95	no
	31.60	α-calacorene	21391-99-1	98	no
	32.74	(-)-caryophyllene oxide	1139-30-6	99	no
	32.93	α-elemene	5951-67-7	90	no
	33.60	cadina-1(2),4-diene	16728-99-7	93	no
	33.76	humulene-1,2-epoxide	19888-34-7	91	yes
	34.21	2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene	150320-52-8	96	no
	34.35	(-)-α-cadinol	481-34-5	99	no
	44.25	kaur-16-ene	562-28-7	99	no
terpene alcohol	48.54	trans-sinapyl alcohol	20675-96-1	96	no

Table 4. Other Compounds Found in the 70:30 Ethanol: Water Screening Experiment

Class	RT order	Compound	CAS No	Quality Match	Confirmation by reference compound
acid	4.77	acetic acid	64-19-7	90	no
	35.91	tetradecanoic acid	544-63-8	98	no
	40.95	n-hexadecanoic acid	57-10-3	99	no
	45.91	linoleic acid	60-33-3	99	no
	46.52	linolenic acid	463-40-1	97	no
	20.31	benzoic acid	65-85-0	93	no
aldehyde	13.69	benzaldehyde	100-52-7	93	yes
alkane	56.90	nonacosane	630-03-5	90	no
ester	11.80	ethyl hexanoate	123-66-0	98	yes
	12.30	hexyl acetate	142-92-7	86	yes
	14.88	isoamyl isovalerate	659-70-1	91	yes
	16.89	ethyl 2-methyl-2-propionylacetate	759-66-0	91	no
	20.65	ethyl benzoate	93-89-0	93	yes
ketone	24.24	3-ethyl-4-methyl-1H-pyrrole-2,5-dione	20189-42-8	94	no
	24.39	4-phenyl-2-butanone	2550-26-7	98	no
	37.25	6,10,14-trimethyl-2-pentadecanone	502-69-2	99	no
	50.80	5-Methyl-5-(4,8,12-trimethyltridecyl)dihydrofuran-2(3H)-one	96168-15-9	99	no
	57.51	pinostrobin chalcone	18956-15-5	99	no
flavonoid	59.93	pinocembrin	480-39-7	99	yes
phenol	26.52	hydroquinone	123-31-9	81	no
sterols	47.52	trans-geranylgeraniol	24034-73-9	99	no
	69.63	stigmasterol	83-48-7	96	no
	71.30	gamma sitosterol	83-47-6	99	no

2.4 Characterization of compounds found in the tea simulation

A chemical survey was performed in an aqueous extract with the objective to more closely model the Native American preparation of an herbal tea. For this reason, the aqueous extract is referred to as the “tea simulation” or “tea” in this document. A tea extract was chosen because the leaves and flowers were chewed or prepared as a tea for coughs or colds according the Peterson Field Guide to Medicinal Plants and

Herbs [22]. There is no known recorded account of an exact recipe detailing how much of the herb is used for tea; however, most accounts boiled the plant material [19,23].

2.4.1 Aqueous Pressurized Solvent Extraction (PSE) of plant material

In keeping with the objective of staying close to the historical account, water was used for PSE extraction. With the exception of water as the solvent all other parameters were kept the same as the hydroethanolic extraction shown in Table 1. Plant material from the 2017 crop only was used for the tea simulation.

2.4.2 Liquid/liquid extraction procedures

A water extraction closely mimics a natural extraction; however, when GC-MS analysis is performed, it is not without problems. Ideally it would be advantageous to inject the aqueous extract directly on the GC-MS column; however, many bioactive analytes have more nonpolar character and, therefore, are poorly soluble in water which also leads to poor reproducibility. In addition to this, a water sample injected into the GC must first pass the injection port where vapor expansion (a 1 μL aliquot of water has a vapor expansion volume of approximately 1000 μL) occurs because of the high injection port temperature [51]. The high vapor expansion of water causes the sample to expand or “flash” to the top of the injection port. This phenomenon is called backflash. During this event, the entire sample is not introduced to the column where it can cause sample loss and poor reproducibility [51]. Loss of resolution, poor peak shape, and ghost peaks are also problems associated with backflash. This is not ideal if reproducible injections are desired. Backflash can be minimized by using split injections or a solvent with a low expansion coefficient [51]. Plants have complex plant matrices that often require clean

up steps that amplify the analyte of interest [52]. Complex matrices also may cause non-reproducible injections and thereby yield poor quantitative analysis results. To address this problem, a quick, single step, extraction clean-up step (Figure 8) was developed that considered the relatively small sample size (~50 mL). Using the cleanup scheme shown in Figure 8 reproducible GC-MS injections were achieved.

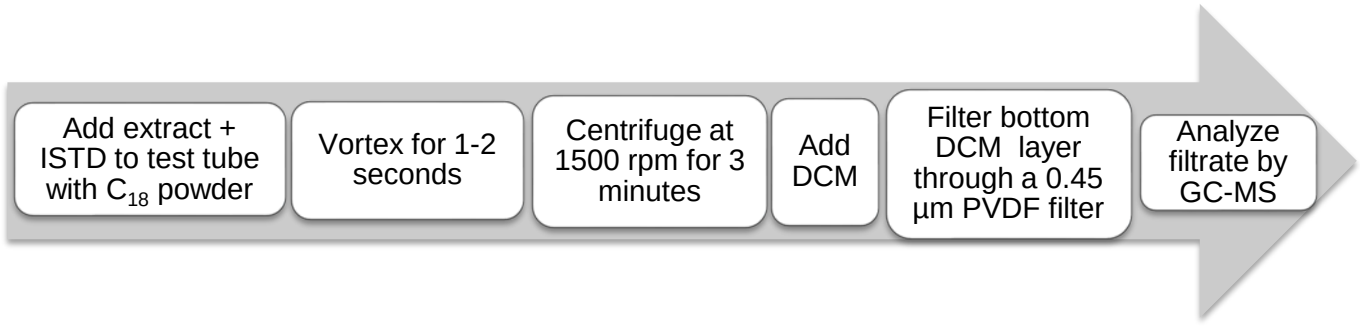

Figure 8. Leaf and flower sample cleanup procedure. The aqueous extract was simultaneously cleaned and back-extracted with dichloromethane (DCM).

In deciding what the best solvent would be for liquid/liquid extraction, hexane was first considered due to its lower density of approximately 0.65 g/cm^3 at 20 ˚C [53]; it is positioned above the water extract. This positioning made it easier to pipet off the top organic hexane layer for GC-MS analysis. It also makes the freeze pour method of extraction easier. In the freeze pour method, samples were frozen at -80 ˚C for 5 minutes. The hexane layer was then easily extracted when pipetting without having to be mindful of the frozen aqueous layer. However, it was observed that hexane was not able to successfully extract the majority of compounds that had been identified in the 70:30 ethanoic extraction.

Next, dichloromethane (DCM) was explored as a possible solvent choice for liquid/liquid extraction. DCM has a density of approximately 1.33 g/cm^3 at 20 ˚C [53]; because its density is higher than water, it formed the bottom layer in the liquid/liquid extraction making the separation process more cumbersome. DCM forms a separate layer from water but unlike hexane which was clear, a brown hydrosol is also formed in the DCM layer. This hydrosol makes reproducible injections unlikely. Due to the hydrosol formation, an additional cleanup step for the DCM extract was necessary. To make the DCM layer more homogenous, C_{18} particles, QuEChERS ($NaCl/MgSO_4$), and activated carbon were added to the aqueous sample matrix in an attempt to shuttle compounds that have a slightly higher affinity for the organic phase into the DCM layer. Activated carbon was used to try to remove the larger lipid molecules so that the smaller bioactive compounds could be separated from the matrix for more accurate GC-MS interpretation.

It was challenging to find clean-up steps that were optimal for all compounds identified in the 70:30 ethanoic extraction (see Tables 3 and 4). Three compounds from the hydroethanolic extraction were chosen to be indicators of extraction performance. These compounds, 4-phenyl 2-butanone, α-caryophyllene, and pinocembrin, were selected because they could be positively identified by a standard match as well as they were representative compounds that proved to be problematic in some extraction protocols. In exploring extraction aids, the performance of C_{18} particles were evaluated for sample clean-up. An addition of C_{18} particles to the DCM extraction step removed enough of the colloidal layer that the sample could be injected on the instrument and yield reproducible injections. A 50 mg amount of activated carbon was added to see if

the compound yields could be improved. It was found that activated carbon decreased the yields of pinocembrin. It was speculated that the reduction for pinocembrin could be associated with the high surface area of the activated carbon. Activated carbon also reduced the area counts of other compounds significantly than if C_{18} particles were used exclusively. QuEChERS ($MgSO_4$/NaCl) when evaluated alone could not sufficiently reduce the hydrosol formation in the DCM layer so that reproducible injections were achieved. When C_{18} particles were added in 50 mg increments to a 1 mL aliquot of extracted sample, area counts for 4-phenyl 2- butanone were increased; however, there was a decrease in other analyte areas. As shown in Table 5, there was no net benefit to peak areas when QuEChERS was used. When using C_{18} particles only, there remained peaks that were not optimized; however, the peak shape was improved over other extraction aids (activated carbon and QuEChERS). Table 5 shows that C_{18} particles alone performed better than the other extraction aids in removing the lipids, waxes, and colorizing agents to such an extent as to produce reproducible injections and suitable Gaussian peak shapes over a wide range of compounds.

Table 5. Peak Areas of Analytes used as Indicators of Extraction Performance

Target Compounds	QuEChERS + C_{18} particles	Activated carbon + C_{18} particles	C_{18} particles
4-phenyl 2-butanone	4990[1]	2010[1]	2917[1]
α-caryophyllene	152	155	599
pinocembrin	6310	not detected	8186

[1]Poor peak shape

Since the extraction procedure had multiples steps, it was necessary to ensure reproducible extractions. To achieve this, 1-octanol was used as an internal standard (ISTD) and a biphasic extraction (see extraction scheme in Figure 8), was performed where the 1 mL of aqueous plant extract was added to a test tube containing 300 mg of

C_{18} particles and vortexed for 1-2 seconds. Next, a 1 mL aliquot of DCM was added to the test tube. The mixture was then centrifuged for 5 minutes at 3000 rpm. After centrifugation, a 2 mL syringe was fitted with a PVDF 0.45 μm filter and the organic sample layer was introduced into the syringe. In the case that aqueous layer was also pipetted, it was easily identified in the narrow syringe by a color change in the sample. The aqueous organic layer remained brown-colored while the DCM layer was lightly colored and clear. The layers were easily identifiable, and the bottom DCM layer could now be quickly filtered directly into a GC autosampler vial. All standards were prepared in DCM because many of the compounds had greater solubility in DCM than water. After a mixed standard solution was prepared, aliquots of the standard solution mix were then added to water in equal ratios and liquid/liquid extraction was performed. Since most standard solutions were clear, extra care was taken during the extraction procedure; however, the two phases could still be easily identified in the 2 mL syringe. It was noted that if the liquid and C_{18} particle ratios were constant, the extraction volumes could be varied as needed.

2.4.3 GC-MS identification and quantification of compounds found in tea

The 70:30 ethanolic extraction spectral match information was helpful especially because the extract could be injected directly on the column “as is”. Peaks from this extract were large enough to be viewed in the GC-MS scan mode. However, the plant extract in water had reduced extraction efficiency and required multiple clean-up steps to achieve reproducible injection results. In contrast to the hydroethanolic extract, lower analyte concentrations were found in the aqueous extract, SIM mode became necessary to effectively detect many analytes and generate quantitative data. The

internal standard was added after PSE, at the beginning of the liquid/liquid extraction step to account for analyte loss during sample clean-up. The final DCM extracts were injected directly into the GC-MS.

The GC-MS system used for this study consisted of a 7890B GC coupled to a 5977A mass selective detector (Agilent Technologies, Inc.) with a PAL LSI 85 autosampler system. The GC-MS method parameters are detailed in Table 6.

Table 6. GC-MS Parameters

Parameter	Description
Column	DB-5MS (30 m X 0.25 mm X 0.25 µm)
Injection temperature	250 ˚C
Injection mode	Split (50:1)
Injection volume	1 µL
Temperature gradient	40 ˚C hold for 1.25 minutes ramp 15 ˚C/minute to 130 ˚C ramp 5˚C/minute to 169 ˚C ramp 15˚C/minute to 250 ˚C ramp 15˚C/minute to 300 ˚C
Run time	23.78 minutes
Ion source	Electron ionization
Transfer line temperature	280 ˚C
Ionization energy	70 eV
Source temperature	230 ˚C
Acquisition type	SIM mode

Data analysis was carried out using Agilent MassHunter Quantitative Analysis software (version B.07.01). Compound identification was done by comparing each mass spectrum with the spectrum in the National Institute of Standards and Technology (NIST) 2014 spectral library. There were many instances where the match quality of the peak was poor (< 80) therefore the peak could not be confidently identified with a spectrum in the NIST library. Compound identification was also done by comparing the retention time and mass spectra with that of a known reference standard.

For the aqueous extraction, a partial validation of the method was conducted to ensure that the method can give accurate sample concentrations for the analytes that had been identified. Table 7 has the results of the validation method parameters that were investigated including calibration, linearity, repeatability, accuracy.

Table 7. Method Optimization Parameters

Compound	(R^2)[1]	Std. Error[2]	PQL (µg/mL)[3]	Standard 1 Repeat-ability (%RSD)[4]	Accuracy					
					leaf extract % recovery (n=3)			flower extract % recovery (n=3)		
					low level	mid level	high level	low level	mid level	high level
benzaldehyde	0.996	0.0318	0.0295	4.2	109	109	100	110	77	93
4-phenyl-2-butanone	0.996	0.0304	0.0792	1.0	99	90	91	104	74	88
ethyl hexanoate	0.995	0.0328	0.0754	2.6	108	71	93	104	75	93
hexyl acetate	0.995	0.0352	0.0082	3.9	113	109	102	102	84	91
isoamyl isovalerate	0.996	0.0260	0.0060	6.6	93	98	97	90	98	73
ethyl benzoate	0.999	0.0097	0.0005	3.5	110	100	96	104	86	89
ethyl hydrocinnamate	0.999	0.0154	0.0003	3.3	112	106	112	116	105	105
dimethyl 3,3'-thiodipropanoate	0.995	0.0122	0.141	3.6	108	101	89	101	84	83
1-dodecanol	0.995	0.0351	0.198	18.3	106	104	110	93	92	103
pinostrobin	0.999	0.0229	0.0913	3.1	119	112	121	105	120	111
pinocembrin	0.995	0.0398	1.13	2.8	103	74	102	78	91	110
β-caryophyllene	0.998	0.0459	0.0003	3.4	95	101	104	92	82	99
α-caryophyllene	0.997	0.0280	0.0110	2.6	114	81	88	99	70	77
linalool	0.996	0.0327	0.0598	5.2	108	88	88	102	76	86
butylated hydroxytoluene (BHT)	0.994	0.0825	0.326	7.0	131	88	84	104	124	130
menthol	0.995	0.0328	0.0670	4.1	101	88	88	117	79	87
(-)-caryophyllene oxide	0.997	0.025	0.184	3.4	73	82	96	71	73	88

[1]Correlation coefficient
[2]Standard error
[3]PQL = Practical limit of quantitation
[4]Relative standard deviation of standard 1

The practical quantitation limit (PQL) is defined as the concentration of the lowest standard (standard 1) [54]. The %RSD for all replicate injections of standard 1 were under 10% with the exception of 1-dodecanol which indicates that for this compound

further optimization is needed. Calibration curves showed good linearity where all coefficients of determinations (R^2) were > 0.990. This demonstrates that the variability in the results is explained by the calibration curve 99% of the time and the remaining 1% may derive from other sources such as natural variability and measurement error or failure of the straight-line model to entirely correlate the relationship between the unknown analyte and the concentration. Weighting was used with each calibration curve since all the calibration curves in this study covered several orders of magnitude. Most calibration curves show increased error with increased concentration; therefore, weighting allows the data at the low end of the calibration curve to have a better fit when a least squares regression is used to fit the sample data to the calibration curve [55]. The simplest appropriate weighting model applied to all calibration curves was 1/x weighting. The results of the calibration curve demonstrate that the GC-MS method used was suitable for determining the concentration of these analytes in the sample extract.

Accuracy is defined as the difference between the measured value and the true value [56,57]. For this work, analyte % recovery was used as one of the indicators for accuracy. The ICH guidelines state that the chemist may need recovery information over all concentration ranges [48,57] . If complete coverage is not available, it may be suitable to estimate recovery at some critical level of analyte concentration [48]. In this work, the calibration curve was established to bracket the range of the sample. The sample extract was fortified with known quantities of the target compounds at the low, medium, and high levels in the range of the calibration curve. Percent recovery was performed in triplicate for each level (low, medium and high) and calculated according to

Equation 8 (section 1.7.2). Surrogate recoveries for all analytes were between 70 – 130%. The accuracy associated with the method is represented by the recoveries for each compound. The average (n=3) concentration (µg/g) for all analytes is given by Equation 9 using pinocembrin as the analyte for the example calculation.

Equation 9

$$\text{Conc}_{\text{leaf}} = (\text{Conc}\ \mu g/mL) \times \left(\frac{EV}{Wtg}\right) \times \left(\frac{TAV}{SAV}\right) = 128\ \ {}^{\mu g}/_{g}\ \text{of pinocembrin in leaf}$$

The concentration (µg/g) of the analyte in leaf (*Conc*$_{leaf}$) is determined by the concentration from the calibration curve, *Conc µg/mL* (pinocembrin = 10.1 µg/mL). *EV* (50 mL), is the extraction volume used to extract the plant material. The weight, *Wtg* (4 g), represents the sample mass. Total aliquot volume, *TAV* (1.01 mL), is calculated by adding the amount of internal standard (0.01 mL of 1-octanol) and the sample analyte volume, *SAV* (1 mL). The flower and leaf extracts were analyzed separately. The calibration curve was quantitated using 1/x weighting. The range of the calibration curve, the average of the samples (n=3), standard deviation, and %RSD are presented in Tables 8 and 9.

Table 8. Analysis of the Flower Extract

Class	Compound	CAS No	Calibration Range (µg/mL)	Average (µg/g) n=3	SD[1]	%RSD[2]
carbonyls	benzaldehyde	100-52-7	0.03-0.5	1.6	0.016	0.99
	4-phenyl-2-butanone	2550-26-7	0.07-1.4	3.5	0.043	1.21
esters	ethyl hexanoate	123-66-0	0.07-1.3	0.69	0.039	5.64
	hexyl acetate	142-92-7	0.007-0.14	0.63	0.025	3.99
	isoamyl isovalerate	659-70-1	0.006-0.12	0.093	0.01	9.99
	ethyl benzoate	93-89-0	0.0005-0.01	0.0045	0.00074	16.5
	ethyl hydrocinnamate	2021-28-5	0.0003-0.005	0.0037	0.00012	3.22
	dimethyl 3,3'-thiodipropanoate	4131-74-2	0.09-1.9	1.9	0.048	2.53
fatty alcohols	1-dodecanol	112-53-8	0.21-4.2	48	0.16	0.34
flavonoids	pinostrobin	480-37-5	0.09-5.8	19	1.9	10.2
	pinocembrin	480-39-7	1.01-12.1	66	3.3	5.1
terpenes	β-caryophyllene	87-44-5	0.003-0.006	0.0064	0.0013	20.1
	α-caryophyllene	6753-96-6	0.009-0.27	0.061	0.0039	6.42
terpene alcohol	linalool	78-70-6	0.05-1.0	13	0.07	0.546
phenols	butylated hydroxytoluene (BHT)	128-37-0	0.422-4.22	6.9	0.14	2.04
	menthol	89-78-1	0.06-1.1	7.6	0.15	1.91
terpenoid	(-)-caryophyllene oxide	1139-30-6	0.18-3.7	2.1	0.22	10.4

[1]Standard deviation
[2]Relative standard deviation

Table 9. Analysis of the Leaf Extract

Class	Compound	CAS No	Calibration Range (µg/mL)	Average (µg/g) n=3	SD[1]	%RSD[2]
carbonyls	benzaldehyde	100-52-7	0.03-0.5	1.97	0.07	3.33
	4-phenyl-2-butanone	2550-26-7	0.07-1.4	5.00	0.21	4.26
esters	ethyl hexanoate	123-66-0	0.07-1.3	0.77	0.013	1.64
	hexyl acetate	142-92-7	0.007-0.14	0.64	0.039	6.06
	isoamyl isovalerate	659-70-1	0.006-0.12	0.028	0.012	42.4
	ethyl benzoate	93-89-0	0.0005-0.01	0.011	0.001	9.12
	ethyl hydrocinnamate	2021-28-5	0.0003-0.005	0.0072	0.00026	3.52
	dimethyl 3,3'-thiodipropanoate	4131-74-2	0.09-1.9	1.87	0.031	1.66
fatty alcohol	1-dodecanol	112-53-8	0.21-4.2	40	1.7	4.15
flavonoids	pinostrobin	480-37-5	0.09-5.8	5.11	0.30	5.92
	pinocembrin	480-39-7	1.01-12.1	128	11	8.8
terpene	β-caryophyllene	87-44-5	0.0003-0.006	0.068	0.00037	5.42
	α- caryophyllene	6753-96-6	0.009-0.27	0.095	0.0063	6.66
terpene alcohol	linalool	78-70-6	0.05-1.0	1.5	0.079	5.12
phenols	butylated hydroxytoluene (BHT)	128-37-0	0.422-4.22	5.76	0.78	13.5
	menthol	89-78-1	0.06-1.1	6.91	0.13	1.93
terpenoid	(-)-caryophyllene oxide	1139-30-6	0.18-3.7	1.89	0.012	0.065

[1]Standard deviation
[2]Relative standard deviation

2.4.4 Discussion of bioactive compounds found in the tea simulation

Of the 17 compounds that were quantified, there are only three compounds (dimethyl 3,3'-thiodipropanoate, pinostrobin, and, pinocembrin) that have no flavor profile associated with them (see Table 10). Table 10 shows the chemical class, compound name, CAS number, the flavor profile described by the Flavor and Extract Manufacturer's Association (FEMA), and a FEMA number if it exists [58]. The diverse taste profile includes apple, chamomile, floral, and wood flavors. The taste profile may explain why Croom records *P. obtusifolium* as one of the most popular plants of the Lumbee tribe as it was a part of many herbal remedies [23].

Table 10. Flavor Profile of Seventeen Quantitated Compounds

Class	Compound	CAS #	FEMA flavor profile or reference flavor profile	FEMA # or reference
carbonyls	benzaldehyde	100-52-7	bitter almond, burnt sugar, cherry, malt, roasted pepper	2127
	4-phenyl-2-butanone	2550-26-7	floral, herbal, strawberry	TGSC[2]
esters	ethyl hexanoate	123-66-0	apple peel	2439
	hexyl acetate	142-92-7	apple, banana, grass, herb, pear	2565
	isoamyl isovalerate	659-70-1	green	2085
	ethyl benzoate	93-89-0	chamomile, celery, fat, flower, fruit	2422
	ethyl 3-phenylpropionate	2021-28-5	flower, honey	2455
	dimethyl 3,3'-thiodipropanoate	4131-74-2	NA[1]	NA[1]
fatty alcohol	1-dodecanol	112-53-8	soapy, waxy	TGSC[2]
flavonoids	pinostrobin	480-37-5	NA[1]	NA[1]
	pinocembrin	480-39-7	NA[1]	NA[1]
terpenes	β-caryophyllene	87-44-5	fried, spice, wood	2252
	α-caryophyllene	6753-98-6	woody	TGSC[2]
terpene alcohol	linalool	78-70-6	coriander, floral, lavender, lemon, rose	2635
phenols	butylated hydroxytoluene	128-37-0	toasted cereal	2184
	menthol	89-78-1	mint, cool	2665
terpenoid	(-)-caryophyllene oxide	1139-30-6	herb, must, spice, wood	4085

[1]NA indicates that a flavor profile was not found
[2]Information from The Good Scents Company (TGSC) website accessed May 2019

Table 11 shows that many of the compounds that have flavor profiles have also been identified as having potential therapeutic properties by literature sources.

Table 11. Potential Therapeutic Properties of Seventeen Quantitated Compounds

Class	Compound	CAS No	Potential properties	References
carbonyl	benzaldehyde	100-52-7	anti-cancer	[59]
	4-phenyl-2-butanone	2550-26-7	appetite enhancing	[60]
ester	ethyl hexanoate	123-66-0	antimicrobial	[61]
	hexyl acetate	142-92-7	[1]NA	[1]NA
	isoamyl isovalerate	659-70-1	[1]NA	[1]NA
	ethyl benzoate	93-89-0	[1]NA	[1]NA
	ethyl hydrocinnamate	2021-28-5	[1]NA	[1]NA
	dimethyl 3,3'-thiodipropanoate	4131-74-2	[1]NA	[1]NA
fatty alcohol	1-dodecanol	112-53-8	antibacterial	[62,63]
flavonoid	pinostrobin	480-37-5	cancer chemoprevention activity & anti proliferative, antioxidant	[64,65]
	pinocembrin	480-39-7	neuroprotective activity, cancer chemoprevention activity, antimicrobial	[66,67]
terpene	β-caryophyllene	87-44-5	anticancer and analgesic	[68]
	α-caryophyllene	6753-98-6	anti-inflammatory	[69]
terpene alcohol	linalool	78-70-6	anxiolytic	[70]
phenols	butylated hydroxytoluene	128-37-0	antioxidant, anti-hepatocarcinogen	[71,72]
	menthol	89-78-1	antimicrobial, antiviral, antioxidant, cooling agent	[73]
terpenoid	(-)-caryophyllene oxide	1139-30-6	anticancer and analgesic	[68]

1. NA indicates that no potential properties have been identified.

Interestingly, the presence of the fatty alcohol, 1-dodecanol, or more commonly referred to as lauryl alcohol, was not surprising because the tea simulation had a soapy consistency. The two flavonoids (both also found in honey) that were positively

identified were pinostrobin and pinocembrin [65]. This was of interest because these compounds have a wide range of biological activity. Pinostrobin is a potent inducer of mammalian phase 2 chemoprotective and antioxidant enzymes [65]. Other studies such as Junior et al. highlight the antiproliferative effects of pinostrobin against the breast cancer cell line (MCF-7; GI_{50} <0.25 µg/mL) and the leukemia cell line (K562; GI_{50} = 0.91 µg/mL) [64]. The GI_{50} is the concentration of the drug that inhibits 50 % of maximal cell proliferation. The concentration for pinostrobin determined by analysis of the flower and leaves in the tea simulation were approximately 1.5 µg/mL and 0.4 µg/mL respectively. Rasul et al. highlighted some biological effects of pinocembrin. This compound has shown cytotoxicity against the leukemia cell line (HL-60; IC_{50} < 100 ng/mL) [66]. Pinocembrin has also shown antimicrobial activity against *P. italicum* and *C. albicans* with a minimal inhibitory concentration of 100 µg/mL [66]. The concentration for pinocembrin determined by analysis of the flower and leaves in the tea simulation were approximately 5.2 µg/mL and 10.1 µg/mL respectively. The concentration for pinostrobin and pinocembrin reflect the relative concentration achieved when 4 grams of flower or leaf material is extracted with 50 mL of water by PSE. Additionally, pinocembrin may be used as a neuroprotective due to its anti-excitotoxic effects; it also has been shown to reduce brain edema by global cerebral ischemia-reperfusion (GCI/R) [66]. Brain edema is defined as an abnormal accumulation of fluid associated with volumetric enlargement of the brain. This is the type of brain edema a person may experience after cardiac arrest [74].

The amount of plant material used for extraction can be increased to increase overall compound yields. It should be noted; however, that when the therapeutic profiles

of the 17 compounds identified in Table 11 from the tea simulation are viewed collectively, they seem to correlate with the Native American use for colds, flu, pneumonia, fevers, mouth ulcers, and tumors [22,23].

2.5 Characterization of key compounds found in bedding simulation

As outlined in Figure 5, a chemical survey was performed using headspace (HS) coupled with GC-MS. The focus was on headspace because there are two documented accounts of the leaves or flowers being used as a stuffing for pillows referred to as bedding. In one account, the dried flowers were used as a sedative filling for the pillows of those that may have had pulmonary tuberculosis [75]. In another account the leaves only were used by the Native Americans to prevent asthma attacks [23]. The goal in this study was to understand the rationale behind the Native American historical use of *P. obtusifolium* as a bedding material by using (HS)-GC-MS to explore the volatile composition of this herb.

2.5.1 Headspace-Solid Phase Microextraction (HS-SPME) with GC-MS to identify volatile compounds found in bedding

Sample preparation for the headspace analysis is described in section 2.2 Aliquots of the cryo-ground plant material were weighed into 20 mL headspace vials with septa screw tops and used for analysis.

As mentioned before, the headspace technique used for this work was performed using solid phase microextraction (SPME) fibers. Extraction and concentration simultaneously occur on the fiber when gas-phase molecules are adsorbed onto the surface of the polydimethylsiloxane (PDMS) film. The porous particles embedded in the

PDMS film that are responsible for adsorption were a combination of carboxen (CAR) and divinylbenzene (DVB). The particle size for the CAR particle is between 300 to 400 μm and has a pore size of approximately 0.5 - 0.8 nm [76]. The particle sizes for the DVB particle range from approximately 150 – 1000 μm with pore sizes of approximately 2 – 50 nm [76]. The small pore size of the CAR particles makes it more efficient for the adsorption of volatile, low molecular weight (40 -150 g/mol) species. Conversely, the larger pore size of the DVB particles makes it suitable for adsorption of higher molecular weight (50-300 g/mol) semi-volatile species from the headspace. For this work, a PDMS fiber embedded with CAR and DVB particles was chosen for its ability to extract both volatile and semi-volatile sample components.

Once gas-phase molecules are trapped by the fiber, they are introduced directly into the heated GC injector port where they are then thermally desorbed from the fiber and introduced to the GC column. The SPME process should be optimized for the samples of interest in order to acquire reproducible data. As mentioned in section 1.5.3.1, the distribution constant, $K_{es,}$ is affected by sample dilution, temperature, ionic strength, pH, and organic solvent. According to the design of this study, only sample dilution was considered as a factor to influence K_{es}. No additives were introduced into the extraction procedure such as solvents or salts in keeping with what analytes would normally be found in the headspace when used as a bedding material. For this same reason temperature was also not optimized. The plant material was sampled at two temperatures, ambient and 40 ˚C. The two temperature conditions represent the range of compounds may be inhaled from the bedding material from the moment of human contact until the bedding is heated a few degrees (˚C) beyond normothermia.

The only other parameter that needed to be adjusted was the sample dilution parameter. Sample dilution was varied by increasing or decreasing the sample size in a 20 mL headspace vial with a screw top septum cap. The final headspace parameters used for qualitative GC-MS profiling are shown in Table 12.

Table 12. HS-SPME and GC-MS Parameters

Sample (weighed in 20 mL headspace vial)	0.1 g of dried ground leaf/flower material
Sample incubation time	5 minutes
Sample incubation temperature	40 °C or ambient
Sample extraction time	1 minutes
Desorption	1 minute @ 250 °C
Post fiber conditioning time	5 minutes @ 270 °C
Fiber	DVB/CAR/PDMS
Temperature gradient	55 ˚C hold for 0.5 minutes ramp 13 ˚C/minute to 257 ˚C ramp 60˚C/minute to 300 ˚C
GC-MS analysis time	16.75 minutes
Column	Restek (1301sil MS) 30 m x 0.25mm ID x 1.00 µm
Oven	250 °C
Carrier gas	helium
Acquisition type	SCAN and SIM

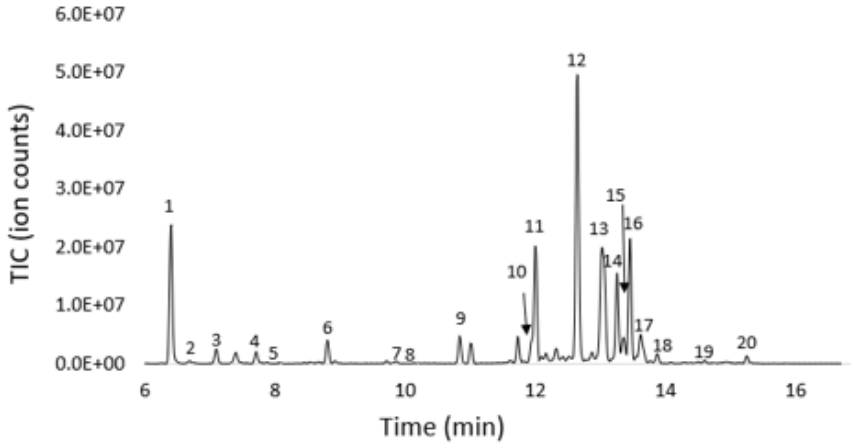

Figure 9. GC-MS chromatogram of the leaf headspace sample with a sample incubation temperature of 40°C. The legend for this figure is shown in Table 13.

After headspace optimization, analysis of the vapor phase sample was performed by GC-MS with a SCAN range of m/z 40-400.

The identification was done using the spectral searching against the National Institute of Standards and Technology (NIST) database. A certified reference standard match was also used for spectral identification where possible; however, some compounds identified by the NIST spectral match program were not available for purchase. Approximately 66 % of the peaks in the leaf and 40 % of the peaks in the flower were identified. Figure 9 shows a typical chromatogram for the chemical profile of the headspace for the leaf sample (a chromatogram of the flower headspace sample is included in the Appendix). The legend for Figure 9, detailed in Table 13, denotes compounds that were identified by both spectral match and/or a certified reference standard.

Table 13. Legend for Compounds Identified in the 2018 Leaf Headspace at 40 °C

Peak #	RT	Peak name	CAS
1	6.214	α-pinene	80-56-8
2	6.492	camphene	79-92-5
3	6.900	β-mycrene	123-35-3
4	7.521	R)-(+)-limonene	5989-27-5
5	7.881	γ-terpinene	99-85-4
6	8.730	linalool	78-70-6
7	9.660	ethyl octanoate	106-32-1
8	10.103	methyl salicylate	119-36-8
9	10.822	ethyl nonanoate	123-29-5
10	10.290	α-copaene	3856-25-5
11	11.900	ethyl decanoate	113-38-3
12	12.455	β-caryophyllene	87-44-5
13	12.831	α-caryophyllene	6753-98-6
14	13.060	α-muurolene	31983-22-9
15	13.158	α-selinene	473-13-2
16	13.256	δ-cadinene	483-76-1
17	13.419	calmamene	483-77-2
18	13.681	calacorene	293-02-3
19	14.743	γ-muurolene	30021-74-0
20	15.053	cadelene	483-78-3

2.5.2 Identification and comparison of volatile species in 2017 and 2018 crop years

The leaf and flower samples were analyzed separately. Two crop years (2017 and 2018) were analyzed and compared for the leaf and flower in triplicate aliquots for ambient and 40 ˚C. The ground leaf and flower analyzed with the sample incubation time of 40 ˚C showed increased sample yields for both the 2017 and 2018 crop years over the samples collected at ambient temperature; therefore, only the graphs of the comparisons at the 40 ˚C conditions are reported. The Croom account of the Lumbee Native Americans discussed that the bedding material was used for one year [23]. There are significant differences in the analyte concentration levels between the crop years (see Figure 10 and Figure 11).

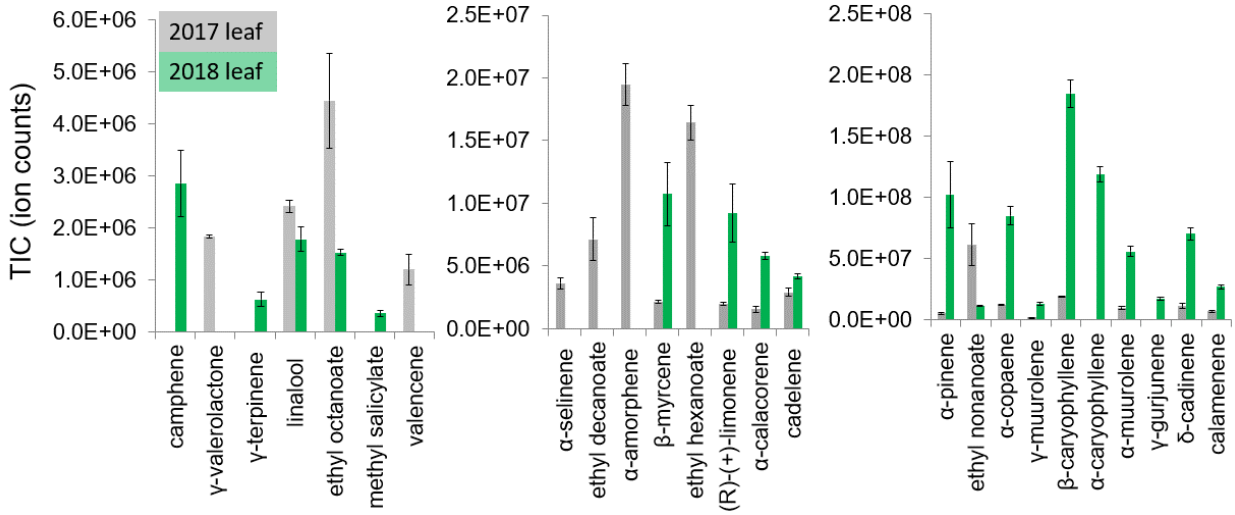

Figure 10. Qualitative headspace analysis of the 2017 vs. 2018 leaf at 40 °C.

Comparison of the headspace analysis extracted at 40 ˚C for the 2017 and 2018 crop years revealed that camphene, γ-terpinene, methyl salicylate, α-caryophyllene, and

γ-gurjunene were not found in detectable levels in scan mode for the 2017 crop. Conversely, γ-valerolactone, valencene, α-selinene, ethyl decanoate, α-amorphene, and ethyl hexanoate were not found in detectable levels in the headspace of the 2018 crop. Notably, β-caryophyllene followed by α-caryophyllene and α-pinene in the 2018 (40 ˚C) crop had higher total ion counts than all other compounds.

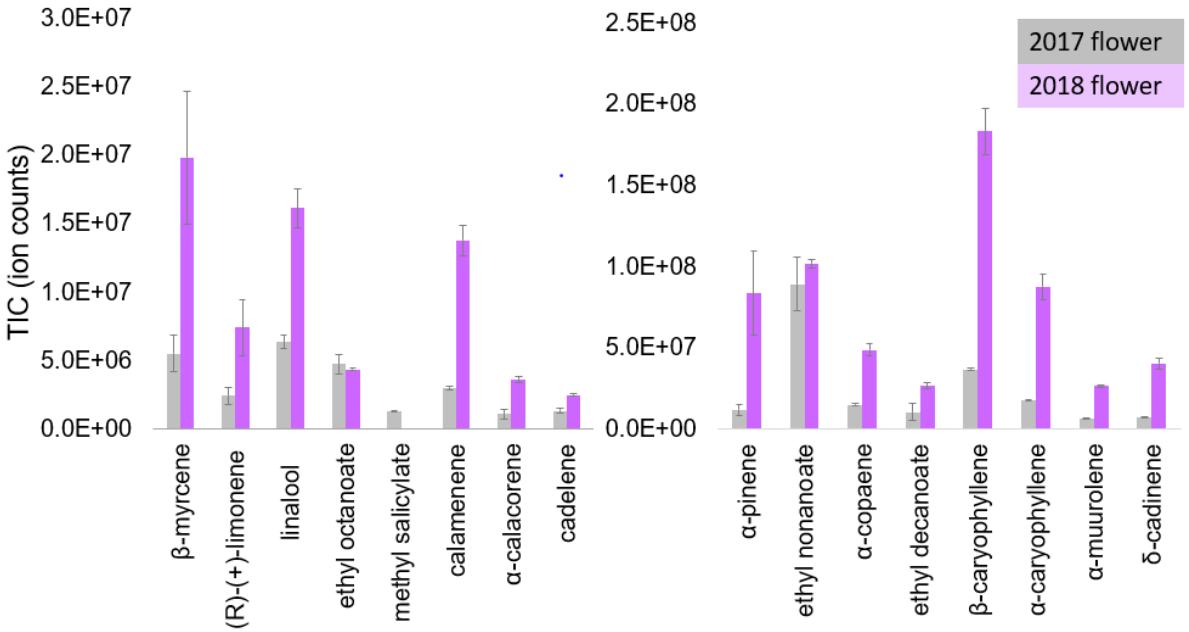

Figure 11. Qualitative headspace analysis of the 2017 vs. 2018 flower at 40º C.

A comparison of the 2017 and 2018 headspace flower (40 ˚C) samples revealed that for all identified compounds, methyl salicylate is only found in the 2017 (40 ˚C) flower sample. This was of note because it was found in detectable levels in the 2018 (40 ˚C) leaf sample but not the 2017 (40 ˚C) leaf sample. With the exception of methyl salicylate, ethyl octanoate, and ethyl nonanoate all identifiable compounds showed increased ion counts in the 2018 (40 ˚C) flower sample relative to the 2017 (40 ˚C) sample. Overall, β-caryophyllene had the highest ion counts in the 2018 crop followed

by ethyl nonanoate, α-pinene, and α-caryophyllene. Ethyl nonanoate, followed by β-caryophyllene, and α-caryophyllene had the highest ion counts in the headspace of the 2017 flower crop.

Many of the compounds identified by NIST spectral match or by standard confirmation in the headspace of the 2018 (40 ˚C) flower vs. 2018 (40 ˚C) leaf comparison were higher in the leaf. As shown in Figure 12, of the compounds identified γ-gurjunene was only found in the leaf; however, 5 compounds (camphene, γ-terpinene, methyl salicylate, γ-muurolene, and γ-gurjunene) were only found in the 2018 leaf.

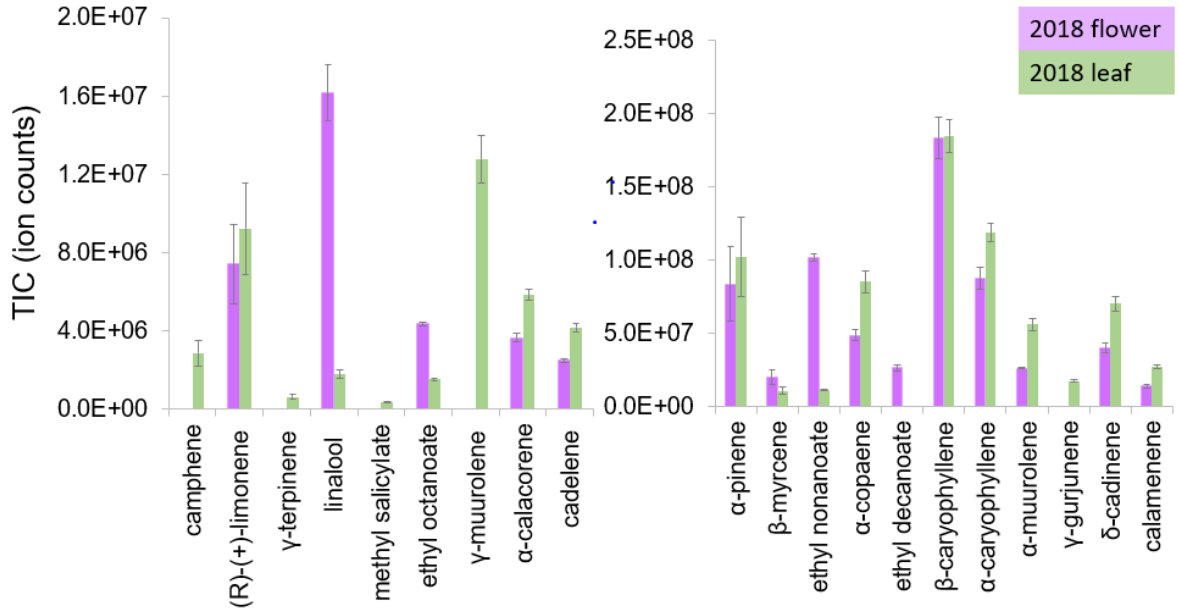

Figure 12. Qualitative headspace analysis of the 2018 leaf vs. flower sample at 40°C.

2.5.3 HS-SPME GC-MS quantification of four key compounds in the 2018 crop year

Headspace quantitation was performed with the leaf and flower to determine if the DVB/CAR/PDMS fiber could be used as a quick tool to quantify volatiles in the plant material. A SPME method of quantitation would be a quick assay used as a baseline

“freshness” test of the stored plant material. This test would ensure that the stored plant had a baseline level of volatile terpenes to evaluate the shelf life at different storage conditions. This is especially important since the 2017 and 2018 headspace comparison of the plant material showed a significant reduction in many of the volatile terpenes that could possibly have health benefits. The 2018 crop year had the highest yields; therefore, quantitation was performed with four selected terpenes using HS-SPME GC-MS.

Table 14. MS Selected Ion Monitoring Parameters for Quantitative Determination of Target Compounds

Compound name	Quantitation and Qualification ions
pinene	Quant ion 93, Qual ions 91,92
(R)-(+)-limonene	Quant ion 93, Qual ions 68,67,79
β-caryophyllene	Quant ion 93, Qual ions 91,105,133
α-caryophyllene	Quant ion 93, Qual ions 80,121

HS-SPME-GC-MS conditions were the same as shown in Table 12 (using the 40 °C temperature parameter only). The headspace quantitation was performed in SIM mode using both quantitation and qualifier ions (Table 14). Calibration curves were performed by exposing the SPME fiber in a 20 mL headspace vial to 20 µL of standard reference material at six concentration levels. The concentration of the standard had to be adjusted so that it would not saturate the fiber. Saturation of the fiber caused the calibration curve to be nonlinear. Saturation also extended fiber bake-out times before the fiber could again be used for analysis. For the DVB/CAR/PDMS fiber the standard concentrations had to be kept low to prevent fiber saturation and produce linear calibration curves. Therefore, the amount of plant material had to be sufficiently reduced in the headspace vial as well, so that the volatile components would fall in the range of the curve. The calibration curve ranges, coefficient of determination, and accuracy are

listed in the Table 15. To evaluate the accuracy of the HS-SPME method, a matrix fortification study was performed to determine if the compounds could be accurately recovered from the sample matrix. To prepare the plant material for fortification, the plant material was baked in an oven at 70 ˚C for 8 hours or until the target terpenes were less than the calibration standard 1. The baked sample matrix of the leaf and flower was then fortified with a known concentration and the recovery was calculated. Table 16 shows the average of 6 measurements, standard deviation (SD), percent relative deviation (%RSD) of the target volatile terpenes based on the calibration curves generated for both the leaf and flower.

Table 15. Headspace Method Quantitation Parameters

Target Compound	Calibration range (mg/mL)	R^2	Leaf: Accuracy	Flower: Accuracy
			% recovery (SD)	% recovery (SD)
α-pinene	0.025 – 0.401	0.9982	113 (0.3)	103 (7)
(R)-(+)-limonene	0.005 – 0.080	0.9979	109 (0.4)	109 (3)
β-caryophyllene	0.015 - 0.242	0.9935	87 (18)	97 (6)
α-caryophyllene	0.005 – 0.082	0.9978	79 (13)	88 (6)

Table 16. Headspace Quantitation of Target Terpene Analytes

Target Compound	leaf average (mg/g) n=6	leaf SD	leaf RSD	flower average (mg/g) n=6	flower SD	Flower % RSD
α-pinene	10.6	2.3	21%	14.0	3.1	22%
(R)-(+)-limonene	1.3	0.2	13%	1.9	0.3	13%
β-caryophyllene	18.0	3.0	16%	90.5[1]	18.3[1]	20%[1]
α-caryophyllene	5.2	1.2	23%	6.2	1.3	21%

[1]β-caryophyllene calculations were performed with 0.02g of flower material and 0.1 g of leaf material
Note: Used 0.1 g of ground plant material for α-pinene, (R)-(+)-limonene, and α-caryophyllene

2.5.4 Discussion of bioactive compounds found in the bedding simulation

Most of compounds found in the bedding simulation had flavor profiles associated with them. The Native Americans may have found the collection of aromatic compounds pleasing. Table 17 shows the compound names for all the compounds found in the headspace along with the CAS number, FEMA (Flavor and Extract Manufacturers Association of the United States) flavor profile and FEMA number. If a FEMA number was not available, the flavor profile was acquired from another reference.

In addition to a pleasant odor, the bedding material also seems to have potentially therapeutic properties. Table 18 lists the chemical class, compound name, CAS number, potential therapeutic potential as well as a literature reference for all the compounds identified in the headspace.

The Native Americans used pillows stuffed with leaves to prevent asthma attacks, so it is not surprising that many of the compounds in the plant have been cited in the literature as possessing anti-inflammatory properties. There are also many compounds with anti-microbial capabilities. The collection of compounds in the bedding material could have also aided asthma sufferers if they were effective in the removal of pathogens that may complicate breathing. Linalool has a dual role of being an anxiolytic as well as an anti-inflammatory agent. The sedative properties of linalool would certainly be in keeping with the historical use of the plant as a sedative.

Table 17. The Flavor Profiles of Compounds in the Headspace

Compound	CAS No	FEMA flavor profile or reference flavor profile	FEMA # or other reference
methyl salicylate	119-36-8	almond, caramel, peppermint, sharp	2745
ethyl hexanoate	123-66-0	apple peel, brandy, overripe fruit, pineapple	2439
ethyl octanoate	106-32-1	apricot, brandy, fat, floral, pineapple	2449
ethyl nonanoate	123-29-5	floral	2447
ethyl decanoate	110-38-3	brandy, grape, pear	2432
α-pinene	7785-70-8	cedar wood, pine, sharp	2902
camphene	79-92-5	camphor, mothball	2229
β-myrcene	123-35-3	lemon, grapefruit, musty, spicy	[77]
(R)-(+)-limonene	5989-27-5	citrus, mint	2633
γ-valerolactone	108-29-2	herb	3103
γ-terpinene	99-85-4	bitter, citrus	3559
valencene	4630-07-3	citrus, green, wood	3443
α-selinene	473-13-2	NA[1]	NA[1]
calamenene	483-77-2	NA[1]	NA[1]
α-calacorene	21391-99-1	woody	TGSC[2]
cadelene	483-78-3	NA[1]	NA[1]
β- caryophyllene	87-44-5	fried, spice, wood	2252
α- caryophyllene	6753-98-6	woody	TGSC[2]
α-copaene	3856-25-5	woody, spicy, honey	NA[1]
γ-muurolene	30021-74-0	woody	TGSC[2]
α-muurolene	31983-22-9	NA[1]	NA[1]
γ-gurjunene	22567-17-5	NA[1]	NA[1]
δ-cadinene	483-76-1	thyme, herbal, woody, dry	TGSC[2]
α-amorphene	483-75-0	fruity	NA[1]
linalool	78-70-6	coriander, floral, lavender, lemon, rose	2635

[1]NA indicates that a flavor profile was not found.
[2]Information from The Good Scents Company (TGSC) website accessed May 2019

Table 18. Potential Therapeutic Properties of Compounds in the Headspace

Class	Compound	CAS No	Potential properties	References
ester	methyl salicylate	119-36-8	analgesic	[78]
fatty acid ester	ethyl hexanoate	123-66-0	antimicrobial	[61]
	ethyl octanoate	106-32-1	antimicrobial	[61]
	ethyl nonanoate	123-29-5	NA[1]	NA[1]
	ethyl decanoate	110-38-3	antimicrobial	[61]
terpenes	α-pinene	7785-70-8	chemotherapeutic, antibacterial	[79,80]
	camphene	79-92-5	antibacterial	[80]
	β-myrcene	123-35-3	anti-inflammatory	[81]
	(R)-(+)-limonene	5989-27-5	anti-inflammatory	[81]
	γ-valerolactone	108-29-2	antioxidant	[82]
	γ-terpinene	99-85-4	mild sedative	[83]
	valencene	4630-07-3	anti-mycobacterial	[69]
	α-selinene	473-13-2	NA[1]	NA[1]
	calamenene	483-77-2	anti-inflammatory, anti-plasmodial	[84]
	α-calacorene	21391-99-1	antibacterial, antifungal	[85]
	cadelene	483-78-3	anti-inflammatory	[86]
	β-caryophyllene	87-44-5	anticancer and analgesic, antibacterial, antifungal, anti-inflammatory	[68]
	α-caryophyllene	6753-98-6	anti-inflammatory, anti-inflammatory, antimycobacterial	[69]
	α-copaene	3856-25-5	antibacterial, antifungal,	[87] [82]
	γ-muurolene	30021-74-0	anti-inflammatory	[88]
	α-muurolene	31983-22-9	antifungal	[87]
	γ-gurjunene	22567-17-5	anticancer, anti-inflammatory	[89]
	δ-cadinene	483-76-1	anticancer, antibacterial, antifungal	[88,90]
	α-amorphene	483-75-0	Anti-inflammatory, antifungal	[84][91]
terpene alcohol	linalool	78-70-6	anxiolytic, anti-inflammatory	[92,93]

[1]NA indicates that no therapeutic benefit was found in current literature.

Table 15 shows that it may be possible to quantitate some of the headspace compounds using SPME. Quantitation of target headspace compounds may be used to

model the “freshness” of the bedding material. Freshness tests are indicative of how stable the compounds are over time, especially when used in bedding.

2.6 Characterization of identifiable toxic compounds in smoke condensate

The international Agency for Research on Cancer (IARC) has identified formaldehyde as a group 1 carcinogen [94]. This classification means that there is sufficient evidence to show that formaldehyde causes cancer in humans. IARC has also classified other carbonyl compounds, such as acetaldehyde as possibly carcinogenic [94]. It should be noted that the combination of compounds that are identified in the smoke output could possibly cause deleterious effects.

Additionally, the U.S. Food and Drug Administration (FDA) classifies 8 carbonyls found in tobacco cigarette smoke, formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, and 2-butanone, as harmful or potentially harmful [95]. There are established methods for determining levels of the 8 key carbonyl toxicants in tobacco mainstream smoke. One of these established methods was adapted for the analysis of carbonyls in *Pseudognaphalium obtusifolium* smoke extracts.

2.6.1 Sample preparation of plant material for UPLC-PDA analysis of carbonyls in smoke

The plant material must be in a form suitable for smoke collection before smoke analysis by UPLC-PDA can take place. To make a smoke extract, the plant material must be fashioned into a smoking article then smoked on a smoking machine. The following sections describe the smoke collection, extraction, and analysis.

2.6.1.1 Smoking article preparation and smoking machine setup

For this experiment, smoke condensate was collected using a method adapted from the International Organization for Standardization (ISO) method [40]. For comparison purposes the data obtained from the analysis of *P*. *obtusifolium* was compared to other published smoke data for marijuana and tobacco [96].

Acidified acetonitrile containing 2,4-dinitrophenylhydrazine (DNPH) was used to trap the smoke condensate in glass impingers for carbonyl analysis. A 10-port linear smoking machine (Cerulean) was used for smoke condensate collection. The smoking machine standardizes the puff volume, puff duration, and puff profile so that testing is reproducible. As mentioned in section 1.5.4, no smoking machine can replicate all the patterns of individual smokers. Rather, machine testing seeks to characterize the analytes found in smoke and provides a level of standardization across smoke testing laboratories.

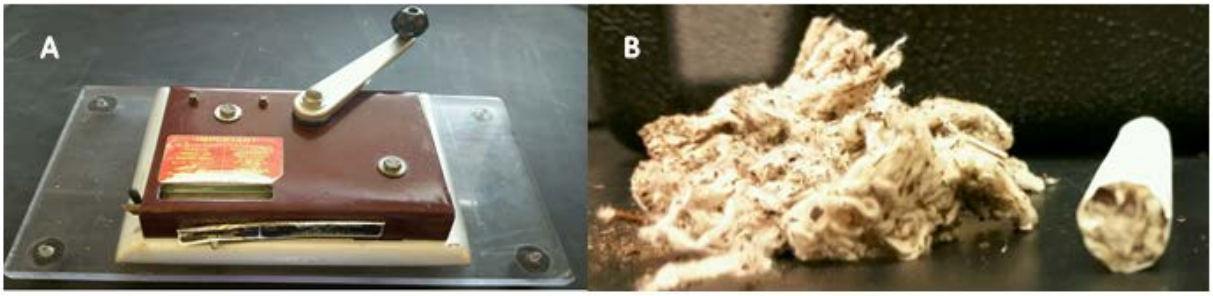

Figure 13. Smoking article fabrication. A) Depicts the Premier Supermatic Cigarette rolling machine used to make the smoking articles for the study. The leaf material and smoking article is shown in B.

A smoking article was fabricated with a Premier Supermatic Cigarette rolling machine, shown in Figure 13A, using cylindrical paper sleeves without a filter (Figure 13

B). These paper sleeves were packed with ground plant material from the leaves and flower separately. The weights for the first experiment are detailed in Table 19.

Table 19. Smoking Article Weights

Plant material	Target carbonyl experiment	
	Smoking article 1 Weight (g)	Smoking article 2 Weight (g)
Leaf rep 1	0.5813	0.5745
Leaf rep 2	0.6662	0.5659
Flower rep 1	0.6993	0.5936
Flower rep 2	0.6223	0.6613

2.6.1.2 Sample collection procedures for the smoke extract

To collect the smoke extract, the smoke from the ignited end of the smoking article is drawn through the length of the smoking article through a smoke adapter into two impingers (see Figure 14.A). Figure 14.B shows two (2) impingers connected to a smoking machine containing a solution of 2,4-dinitrophenylhydrazine (DNPH). As the aldehydes and ketones that are part of smoke stream reach the impinger they are trapped in the solvent.

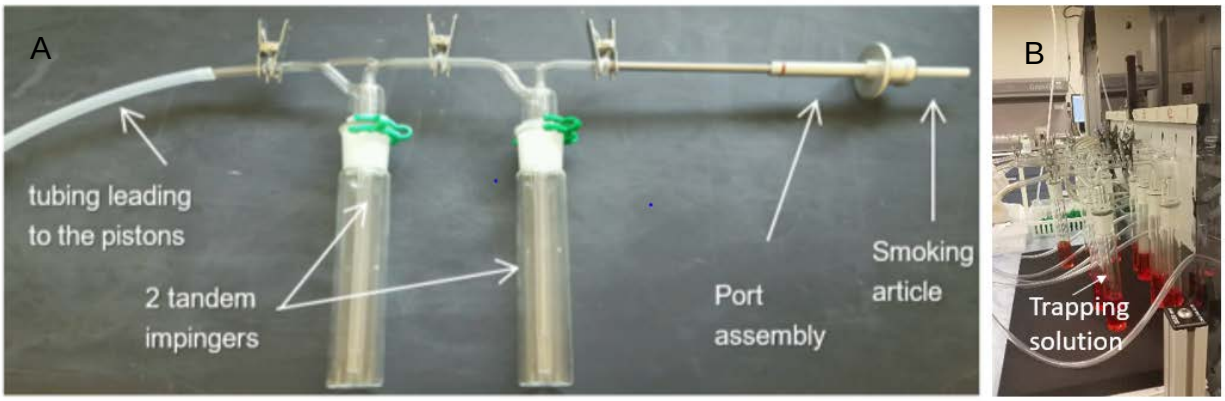

Figure 14. A) Depicts the impinger trapping system used to trap smoke condensate. B) Is a depiction of the impinger trapping system when attached to the smoking machine.

The carbonyls are stabilized by the DNPH derivatizing agent and become chromophores (see Figure 15) that are then able to be detected by the Photodiode Array (PDA) detector as a signal.

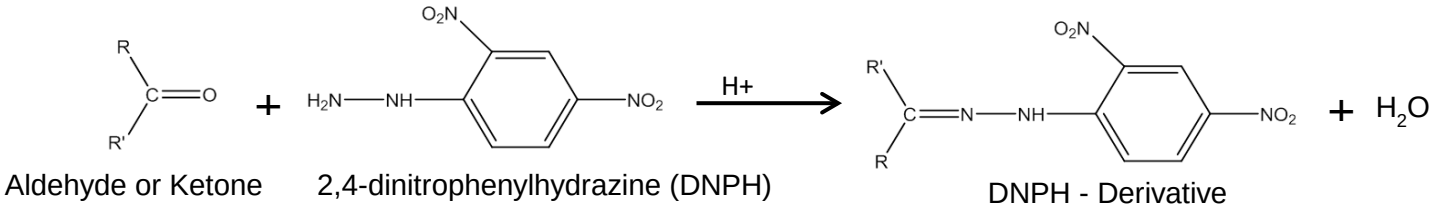

Figure 15. Reaction for 2,4-dinitrophenylhydrazine trapping system [101].

After the smoking article was fabricated, the smoking machine conditions were setup according to ISO 20778 [40]. Since carbonyls are found primarily in the vapor phase, impingers were used to funnel the vapor-phase into the DNPH trapping solution shown in Figure 14. A-B. Derivatization of the free carbonyls in smoke occurs and the DNPH-derivatives are formed (Figure 15). Smoking articles containing the flower material and leaf material were smoked and analysed separately. The smoking articles were smoked in duplicate to collect one sample. Therefore, four smoking articles for the leaf and the flower were prepared to produce two individual samples.

After smoke collection was complete, the impingers were disconnected from the smoking machine. The ISO 21160:2018 protocol was [40] followed with modifications; the smoke extract was not filtered, and the ratios were altered so that the sample was prepared directly into a 2 mL autosampler vial. The final autosampler vial contained 400 µL of smoke extract and 600 µL of tris-(hydroxymethyl)-aminomethane (Trizma)

solution [40]. The Trizma solution raises the pH of the reaction and thereby stops the formation of additional 2,4-dinitrophenylhydrazones which stabilizes the reaction.

2.6.2 Ultra Performance Liquid Chromatography (UPLC) with Photodiode Array detection (PDA) identification of target carbonyls

The 2,4 dinitrophenylhydrazone of the target carbonyls (formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, 2-butanone, and n-butyraldehyde) is what is measured by UPLC-PDA. The method used was adapted from *ISO 21160:2018* with the following differences [40] . This work differed from the ISO method because a UPLC was used instead of a High-Performance Liquid Chromatography (HPLC) system. This method also used different method conditions such as injection volume, column, mobile phase, and mobile phase gradient. The analysis time for this work was also reduced from approximately 44 minutes outlined in the ISO method to 10 minutes. Additionally, an Accustandard certified ISO guide 34 standard mix containing the eight target carbonyls was used. The elution order of the analytes remained the same and the UPLC parameters are listed in Table 20 and 21.

Table 20. UPLC Parameters

Parameter	Setting
column	InfinityLab Poroshell (2.1X100 mm, 2.7 micron)
Analysis time	10 minutes
Injection volume	2 µL
Column temperature	55 ºC
Auto-sampler temperature	5 ºC
PDA detection	365 nm
Weak wash solvent	10 % Acetonitrile in water
Strong wash solvent	acetonitrile

Table 21. UPLC Separation Conditions

Time (Min)	Flow Rate (mL/min)	Mobile Phase A (10 % Acetonitrile in Water) (%)	Mobile Phase B (75:25 Acetonitrile:Methanol) (%)
Initial	0.6	75	25
3.2	0.6	70	30
5.5	0.6	60	40
7.3	0.6	53	47
8.0	0.6	20	80
8.5	0.6	0	100
9.0	0.6	0	100
9.1	0.6	75	25
10.0	0.6	75	25

Waters TargetLynx software was used to generate a calibration curve to calculate response factors for each of the target carbonyl compounds. The calibration standards were made by using a certified standard to make serial dilutions of the standard solution containing formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, and 2-butanone in concentrations that bracketed the levels of carbonyls in the sample. The correlation coefficient for the eight calibration curves were > 0.999. There was no internal standard used for this evaluation. Target carbonyl concentrations were reported in µg/mL using TargetLynx software. The results from the calibration curve were adjusted to report the concentration of the free carbonyl (not the hydrazone). Table 22 and Equation 10 show that the concentrations of the

standards are based on the ratio of the molecular weight (MW) of the free carbonyl vs. the MW of the complex (carbonyl-DNPH derivative).

Table 22. Carbonyl Stock Standards

Target Carbonyl-DNPH complex	Cas #	MW of Target Carbonyl	MW of Target Carbonyl-DNPH complex	Purity of Target Carbonyl	DNPH-Carbonyl complex Final Conc. (µg/mL)	Free Carbonyl Final Conc. (µg/mL)
formaldehyde-DNPH	1081-15-8	30.03	210.15	100.0	354.4	50.6
acetaldehyde-DNPH	1019-57-4	44.05	224.18	98.0	4925	967.7
acetone-DNPH	1567-89-1	58.08	238.21	99.5	1004	244.8
acrolein-DNPH	888-54-0	56.06	237.15	99.0	419.4	99.1
propionaldehyde-DNPH	725-00-8	58.08	238.21	99.7	528.8	128.9
crotonaldehyde-DNPH	1527-96-4	70.09	250.22	98.6	179.5	50.3
2-butanone-DNPH	958-60-1	72.11	252.23	98.0	344.6	98.5
butyraldehyde-DNPH	1527-98-6	72.11	252.23	99.6	351.8	100.6

Equation 10. Concentration of the carbonyl standard

$$CC_{(\mu g/mL)=}\left(\frac{Carb_{MW}}{Complex_{MW}}\right)\times\left(\frac{Complex_{mg}}{TV_{mL}}\right)\times\left(\frac{p}{100}\right)\times\left(\frac{1000_{\mu g}}{1_{mg}}\right)$$

The concentration of the carbonyl (CC µg/mL) standard is determined by $Carb_{MW}$, the molecular weight of the free carbonyl (not complexed with DNPH), $Complex_{MW}$, the molecular weight of the carbonyl-DNPH complex (hydrazone), TV, the total volume of solution (mL), *p*, the purity of the neat carbonyl-DNPH complex and $Complex_{mg}$, the mass of the DNPH-complex (mg).

The carbonyl concentration (CC) in the smoke was determined according to Equation 11. The concentration of the carbonyl (µg/smoking article) is calculated as the free carbonyl (see equation 10).

Equation 11

$$CC_{\mu g/\text{smoking article}} = Conc_{\mu \frac{g}{mL}} \times \left(\frac{TIV \times FV}{AV \times N} \right)$$

In this equation, CC represents the carbonyl concentration (µg/smoking article) of the free carbonyl in *P. obtusifolium* smoke and the $Conc_{\mu g/mL}$, is the calculated concentration from the calibration curve. TIV represents the total impinger volume which was 60 mL. The aliquot of extraction solution volume removed from impinger (0.4 mL) is denoted by AV. FV is the final volume of 1 mL (0.6 mL of Trizma+ 0.4 mL of extraction solution removed from impinger). Finally, N, is the number of smoking articles (SA) analyzed, which was two in this study.

2.6.3 Discussion of target carbonyl compounds found in the smoke simulation

Toxic compounds form through pyro-synthesis and thermal degradation during the burning of a smoking article. The heat of the combustion and the hot gases being produced causes pyrolysis of the plant material located immediately behind the combustion zone [41]. Hydrocarbons, aldehydes, alcohols, ketones, and acids along with carbon based nanoparticles are formed by pyro-synthesis and thermal decomposition and become part of what is inhaled in the smoke [41]. Additionally,

stable volatile materials such as alkaloids, water, and some waxy materials distill unchanged into the smoke stream.

Table 23. Carbonyl Target Yields in Leaf and Flower

Target compound	Leaf Concentration (µg/SA[2]):SD[3]	Flower Concentration (µg/SA[2]):SD[3]	IARC[1]
formaldehyde	28 (±13)	170 (±100)	1
acetaldehyde	145 (±78)	382 (±210)	2B
acetone	32 (±16)	70 (±36)	NA
acrolein	26 (±14)	81 (±43)	3
propionaldehyde	13.1 (±6.2)	30 (±15)	NA
crotonaldehyde	7.5 (±4.2)	33 (±17)	3
2-butanone	8.7 (±5.0)	20 (±13)	NA
butyraldehyde	7.8 (±3.8)	12.7(±6.1)	NA

[1]International Agency for Research on Cancer classifications
Group 1 – Carcinogenic to humans
Group 2A – Probably carcinogenic to humans
Group 2B - Possibly carcinogenic to humans
Group 3- Not classifiable as to its carcinogenicity to humans
[2]SA = smoking article
[3]SD = standard deviation

The data in Table 23 are from the analysis of 2 smoking articles as mentioned in section 2.6.1.2. The large standard deviations can be explained by the non-standardization of the smoking article process as well as the low number of replicate analyses. Even though the material was cryo-ground it still had a fluffy consistency which made fabrication of the smoking article challenging. This “fluffiness” made it difficult to pack the smoking article the same way each time. On several occasions the paper smoking sleeve split due to the pressure of the material. Table 23 also shows the IARC classification of each carbonyl where applicable.

An interesting observation in the target carbonyl yield data in Table 23 is that the flower has consistently higher concentrations of the target compounds than the leaf.

This could also be explained by the non-standardization of smoking article fabrication; there is approximately 8 % difference in the total combined weight between the leaf and the flower. When observing the differences in formaldehyde yields for the leaf and flower it seems unlikely that the difference is completely due to the difference in weight. One suggestion is that the leaf has fewer carbohydrates than the flower. Thermal degradation of cellulose is known to be associated with low molecular weight carbonyl formation [97].

To better comprehend the results, the analysis results for *P. obtusifolium* leaf and flower were compared to published values of carbonyl yields from smoking analyses of marijuana flower and tobacco leaf. When compared with yields from marijuana and tobacco, the yields from the flower and leaf are present in concentrations that are similar. This comparison was able to be made because the smoking protocol and smoke extraction process were similar to the method described by Moir et al. [96]. As shown in Figure 16, formaldehyde concentrations in the *P. obtusifolium* flower had levels that ranged higher than levels found in tobacco smoke. Acetaldehyde and propionaldehyde, had levels in the *P. obtusifolium* flower that ranged higher than those found for the marijuana smoking analysis. Acrolein and crotonaldehyde values were higher than marijuana while acrolein values approached levels found in tobacco smoke [96]. One important fact to note is that *P. obtusifolium* had a lower number of replicates (n=2) than both tobacco and marijuana which had 7 replicates each [96]. Another significant point is that all of these values can change based on how the smoking articles is fashioned as well as the growth conditions and how the plant material is prepared for testing.

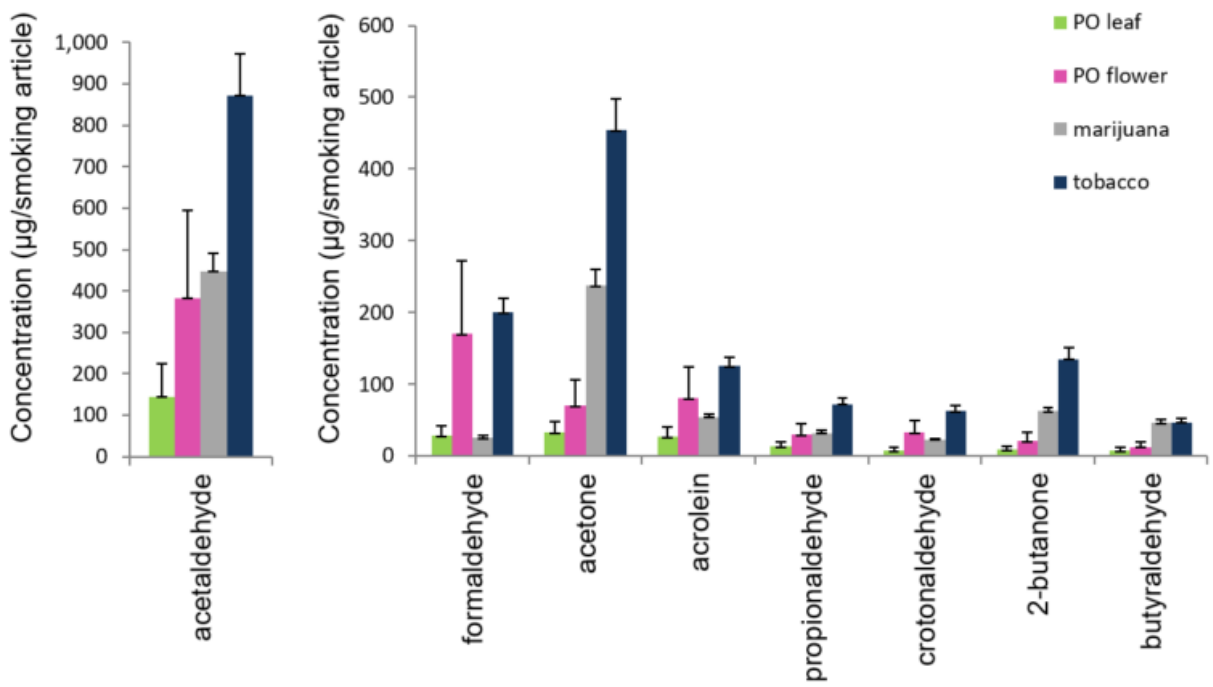

Figure 16. Comparison of *P. obtusifolium* (leaf and flower), marijuana, and tobacco smoke carbonyl yields. The comparison values for marijuana and tobacco smoke yields are from work done by Moir et al. [96].

As mentioned earlier, all eight target analytes found in the smoke of *P. obtusifolium*, have been identified by the FDA as being harmful or potentially harmful constituents in tobacco products and tobacco smoke. Additionally, the International Agency for Research on Cancer (IARC) has identified formaldehyde as a group 1 carcinogen (see Table 23) [94,95]. This classification means that there is sufficient evidence to show that formaldehyde causes cancer in humans. IARC has also classified other compounds on the list of target carbonyls such as acetaldehyde as possibly carcinogenic [95]. It should be noted that the combination of hazardous or potentially

hazardous chemicals that have been identified in the smoke output could possibly cause deleterious effects.

2.6.4 GC-MS survey of bioactive and toxic compounds in smoke condensate

In addition to analyzing the carbonyl content of the smoke condensates for *P. obtusifolium*, a smoke extract that captured both particulate-phase and gas-phase compounds was also collected and tested using GC-MS. The mass of total particulate matter collected on the pad and carbon monoxide were also measured.

For the GC-MS analysis, only one impinger was used in the impinger assembly instead of the two tandem impingers shown in Figure 14. Methanol was used as a trapping solution for the vapor-phase and a cellulose acetate pad inserted before the impinger was used to trap the particulate-phase. The impinger was filled with 10 mL of methanol fortified with 50 µL of a 1000 µg/mL stock solution of quinoline as the internal standard. The total concentration of quinoline in methanol was 5 µg/mL.

The methanol in the impingers was cooled to 0 ºC prior to the start of the smoke machine analysis to improve collection efficiency of volatile components. Three smoking articles for each the leaf and the flower were used for one smoke analysis run. Therefore, three smoking articles were used to obtain a single smoke analysis value.

The smoking machine was setup according to the ISO 20778 method [40]. After smoke collection was completed, an aliquot of the impinger contents for the leaf and flower were filtered through 0.45 µm filter directly into separate 2 mL autosampler vials for analysis by GC-MS.

After smoking the cellulose acetate pads were weighed for the leaf and flower before being placed in separate 40 mL amber bottles. The pads were then fortified with a 1000 µg/mL internal standard stock solution of quinoline resulting in an internal standard concentration of 5 µg/mL after adding 10 mL of methanol. The pads were then vortexed for 10 minutes to extract the particulate phase into the methanol. The pad for the flower and leaf sample was then filtered with a 0.45 µm filter directly into separate 2 mL autosampler vials for analysis by GC-MS. The estimated concentration (µg/g) was determined using the internal standard (ISTD) area and concentration for quinoline; see Equations 12 and 13 for an example calculation of styrene in the flower sample.

Equation 12: Example calculation for the estimated concentration (µg/g) of styrene in the flower

$$\text{Estimated concentration of target peak (styrene)} = \frac{\text{Conc}_{ISTD} \times \text{PA}_T}{\text{PA}_{ISTD}} = 10.6 \ \frac{\mu g}{mL}$$

The estimated concentration of the target peak (styrene) is calculated using the variables (Conc_{ISTD}, PA_T, and PA_{ISTD}). For this study, the concentration of the ISTD (Conc_{ISTD}) was 5 µg/mL of quinoline. The PA_T represents the peak area of the target peak (styrene peak area = 5.1) and the PA_{ISTD} is the peak area of the ISTD (quinoline peak area = 2.4). Once the peak estimated concentration (µg/mL) is calculated the estimated concentration can then be calculated on a per gram basis as in equation 13 below.

Equation 13

$$(\mu g/g)_{styrene} = \text{Estimated conc. } (\mu g/mL) \times \frac{\text{Ext Vol}}{\text{sample wgt.}} = 193 \ \mu g/g$$

The estimated concentration (µg/g) of the target peak (styrene) is calculated using the estimated concentration (µg/mL) of target peak (example: styrene = 10.6 µg/mL) an extraction volume (Ext Vol) of 10 mL. The sample weight (sample wgt. = 0.55 g) was the weight of smoking article prepared with plant material from the flower minus the weight of the paper wrapper. A Cerulean 10 port linear smoking machine used 5 main parameters (2 second puff duration, 30 second puff interval, a puff volume of 55 cc, and a sine wave) for smoke collection. Table 24 shows the average weight of the smoking article and number of smoking articles used for smoke collection. The average number of puffs, total particulate matter (TPM) and carbon monoxide (CO) are also shown.

Table 24. Smoking Article and Smoking Machine Data

Sample	Average smoking article weight (g)	smoking articles (#)	Puffs	TPM (mg/cig)	CO mg/cig
leaf	0.66 (n=3)	3	11.2	15.1	10.74
flower	0.55 (n=3)	3	13.9	2.2	9.81

The GC-MS was set up in scan mode. A DB-5MS (30 m x 0.25 mm x 0.25 µm) column was used for analysis. For the leaf smoke extract, approximately 22 % and 28% of the peaks in the particulate and vapor phase, respectively, were identified. For the flower smoke extract, approximately 20 % and 31% of the peaks in the particulate and vapor phase respectively were identified. The results in Tables 25 and 26 represent leaf and flower compounds trapped by the pad (particulate-phase) and impinger (vapor-phase). International Agency for Research on Cancer (IARC) classifications are also included where applicable.

Table 25. Smoke Analysis Survey of Leaf

Compound	CAS	Vapor/Particulate Phase	Estimated (µg/g)	IARC[1]
diethyl phthalate	84-66-2	particulate	4	NA[2]
vitamin E	59-02-9	particulate	279	NA[2]
stigmasterol	83-48-7	particulate	195	NA[2]
2-furanmethanol	98-00-0	vapor	29	2B
furfural	98-01-1	vapor	39	3
3-methyl-pyridine	108-99-6	vapor	29	3
(R)-(+)-limonene	5989-27-5	vapor	46	3
phenol	108-95-2	vapor	77	3
5-methyl-2-furaldehyde	620-02-0	vapor	27	NA[2]
3-methyl-2-cyclopentenone	2758-18-1	vapor	33	NA[2]
3-methylcyclopentane-1,2-dione	765-70-8	vapor	151	NA[2]
3-methyl-phenol	108-39-4	vapor	180	NA[2]
2-methoxy-phenol	90-05-1	vapor	58	NA[2]
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	28564-83-2	vapor	65	NA[2]
catechol	120-80-9	vapor	113	2B
(E)-5-isopropyl-8-methylnona-6,8-dien-2-one	54868-48-3	vapor	65	NA[2]
5-hydroxymethylfurfural	67-47-0	vapor	89	NA[2]
2-methoxy-4-vinylphenol	7786-61-0	vapor	33	NA[2]
indole	120-72-9	vapor	40	NA[2]
3-methyl-indole	83-34-1	vapor	59	NA[2]
3-(3,4-dihydro-2H-pyrrol-5-yl) - pyridine	532-12-7	vapor	22	NA[2]
4-methyl-3-phenyl-pyrazole	13808-62-3	vapor	56	NA[2]
neophytadiene	504-96-1	vapor	208	NA[2]
2,3'-dipyridyl	581-50-0	vapor	31	NA[2]
(E)-4-(3-hydroxyprop-1-en-1-yl)-2-methoxyphenol	32811-40-8	vapor	29	NA[2]
alloaromadendrene	25246-27-9	vapor	46	NA[2]
cotinine	486-56-6	vapor	37	NA[2]
(Z,Z,Z)-9,12,15-octadecatrienoic acid	463-40-1	vapor	57	NA[2]
scopoletin	92-61-5	vapor	48	NA[2]

[1]International Agency for Research on Cancer classifications: Group 1 - Carcinogenic to humans; Group 2A - Probably carcinogenic to humans; Group 2B - Possibly carcinogenic to humans; Group 3- Not classifiable as to its carcinogenicity to humans

[2]NA - No IARC classification

Table 26. Smoke Analysis Survey of the Flower

Compound	CAS	Vapor/Particulate Phase	Estimated (µg/g)	IARC[1]
toluene	108-88-3	particulate	645	3
2-methyl-2-butenenitrile	4403-61-6	particulate	81	NA[2]
o-xylene	95-47-6	particulate	260	NA[2]
5-(1-methylethylidene)- 1,3-cyclopentadiene	2175-91-9	particulate	91	NA[2]
styrene	100-42-5	particulate	193	2A
(R)-(+)-limonene	5989-27-5	particulate	410	3
1,2,4-trimethyl- benzene	95-63-6	particulate	23	NA[2]
benzonitrile	100-47-0	particulate	20	NA[2]
indene	95-13-6	particulate	19	NA[2]
toluene	108-88-3	vapor	376	3
o-xylene	95-47-6	vapor	231	NA[2]
furfural	98-01-1	vapor	29	3
bicyclo[4.2.0]octa-1,3,5-triene	694-87-1	vapor	151	NA[2]
1-ethyl-2-methyl-benzene	611-14-3	vapor	42	NA[2]
undecane	1120-21-4	vapor	13	NA[2]
(R)-(+)-limonene	5989-27-5	vapor	100	3
1,2,3-trimethyl- benzene,	526-73-8	vapor	9	NA[2]
benzaldehyde	100-52-7	vapor	11	NA[2]
benzonitrile	100-47-0	vapor	6	NA[2]
indene	95-13-6	vapor	12	NA[2]

[1]International Agency for Research on Cancer classifications: Group 1 - Carcinogenic to humans; Group 2A - Probably carcinogenic to humans; Group 2B - Possibly carcinogenic to humans; Group 3- Not classifiable as to its carcinogenicity to humans
[2]NA - No IARC classification

2.6.5 Discussion of compounds found in the smoke simulation

The results of the smoking survey (Tables 25 and 26) are approximates from single collections; however, they demonstrate the chemical composition collected from *P. obtusifolium* smoke condensate include many toxic compounds. The compounds in Tables 25 and 26 do not represent a comprehensive listing of all compounds found in the smoke of *Pseudognaphalium obtusifolium*. This list represents the compounds that were able to be identified by spectral match (match quality ≥ 80) or a standard match or both. As mentioned in section 2.6.4, approximately 22 % and 28% of the peaks in the

leaf particulate and vapor phase, respectively, were identified and approximately 20 % and 31% of the peaks in the flower particulate and vapor phase respectively were identified. Additionally, since this method was a general survey method, compounds that were not heat stable or compounds with high molecular weights, as well as non-volatile compounds may not be able to be identified by GC-MS. Further method optimization would be required for a comprehensive analysis and is beyond the scope of this research.

The International Agency for Research on Cancer (IARC) classifications are listed beside each compound identified in smoke. IARC cautions that Group 3 agents are not considered safe but rather this is the current classification based on the metrics and information they currently have [94]. New studies may support different classifications [94]. Styrene is in group 2A which suggests that it is probably carcinogenic to humans. This data further indicates that inhaling smoke from burning carbonaceous materials is known to be injurious to health. Styrene and toluene are volatile organic compounds that can induce inflammation of the lung that may lead to pathogenesis [98]. Xylene, (o-xylene, m-xylene, p-xylene) is an aromatic hydrocarbon that has been shown to shown to cause harm in many organ systems [99]. It can cause throat and nasal irritation in the respiratory system at exposure levels of 200 ppm in 3 -5 minutes. When exposed to the skin, xylene can also cause dermal erythema, vasodilatation, and dryness [99].

3 Conclusion and future work

The goal of this research was to provide a broad-based survey of the chemical composition of *P*. *obtusifolium* based on historical usage. As many of the compounds identified in this work have known bioactivity, the compounds found can provide insight into the potential therapeutic function of this native plant as it was used by indigenous populations. A chemical elucidation of this plant will also be useful for providing researchers an additional plant source for bioactive compounds.

In the tea simulation, using an aqueous extraction technique and GC-MS analysis, seventeen compounds were confirmed by spectral match and reference standard comparison. Compounds such as carbonyls, esters, fatty alcohols, flavonoids, phenols and terpenes were detected. These compounds are associated with many therapeutic properties (anti-inflammatory, antibacterial, anticancer properties, etc.). Quantitative analysis was also conducted. Concentrations of these compounds ranged from 0.006 µg/g to 128 µg/g.

The headspace analysis with SPME and GC-MS analysis, simulated the herb being stuffed into bedding such as a pillow. For this application, 25 compounds were identified by spectral match with 13 compounds also being confirmed by reference standard comparison. Most of the compounds found in the headspace were also of therapeutic benefit (see Table 18). Quantitative analysis was also conducted on four compounds, α-pinene, (R)-(+)-limonene, β-caryophyllene, and α-caryophyllene. It was also observed, that the concentrations of many compounds varied based on the harvest year and/or age of the plant material. Additionally, there were variations in

concentrations based on whether the plant material was harvested from the leaf or the flower.

The smoke extract was also evaluated in both leaf and flower, and approximately 57 compounds were identified. The smoke analysis was performed using a nontargeted (full scan GC-MS) and targeted approach (UPLC-PDA). As part of the targeted approach, the *P. obtusifolium* smoking article was smoked and analyzed for the presence of carbonyls, some of which are known carcinogens [94]. Using UPLC-PDA detection a derivatization method adapted from methods developed to characterize eight target carbonyls in tobacco was employed. This analysis showed that all eight target carbonyls were present in levels ranging from 0.1 µg/g to 5 µg/g. These levels were similar or greater than what has been reported in tobacco and marijuana smoke. In the non-targeted analysis, compound classes such as benzene, hydrocarbons, lipids, and steroids were also observed.

Table 27 shows the compounds detected across all analyses simulating historical usage. Overlap was observed for a few compounds in the tea, bedding, and smoke simulations.

Table 27. Compounds Identified in Usage Forms

Compound	CAS No	Tea	Bedding	Smoke
benzaldehyde	100-52-7	X		X
acrolein	107-02-8			X
propionaldehyde	123-38-6			X
butyraldehyde	123-72-8			X
crotonaldehyde	123-73-9			X
formaldehyde	50-00-0			X
5-methyl-2-furaldehyde	620-02-0			X
5-hydroxymethylfurfural	67-47-0			X
acetone	67-64-1			X
acetaldehyde	75-07-0			X

Table 28 (continued from Table 27) - Compounds Identified in Usage Forms

Compound	CAS No	Tea	Bedding	Smoke
2-butanone	78-93-3			X
furfural	98-01-1			X
cotinine	486-56-6			X
3-(3,4-dihydro-2H-pyrrol-5-yl) - pyridine	532-12-7			X
2,3'-dipyridyl	581-50-0			X
undecane	1120-21-4			X
styrene	100-42-5			X
5-(1-methylethylidene)- 1,3-cyclopentadiene	2175-91-9			X
neophytadiene	504-96-1			X
bicyclo[4.2.0]octa-1,3,5-triene	694-87-1			X
1,2,3-trimethyl- benzene,	526-73-8			X
1-ethyl-2-methyl-benzene	611-14-3			X
o-xylene	95-47-6			X
1,2,4-trimethyl- benzene	95-63-6			X
(Z,Z,Z)-9,12,15-octadecatrienoic acid	463-40-1			X
ethyl octanoate	106-32-1		X	
ethyl decanoate	113-38-3		X	
methyl salicylate	119-36-8		X	
ethyl nonanoate	123-29-5		X	
ethyl hexanoate	123-66-0	X	X	
hexyl acetate	142-92-7	X		
ethyl hydrocinnamate	2021-28-5	X		
dimethyl 3,3'-thiodipropanoate	4131-74-2	X		
isoamyl isovalerate	659-70-1	X		
ethyl benzoate	93-89-0	X		
pinostrobin	480-37-5	X		
pinocembrin	480-39-7	X		
indene	95-13-6			X
indole	120-72-9			X
3-methyl-indole	83-34-1			X
4-phenyl-2-butanone	2550-26-7	X		
3-methyl-2-cyclopentenone	2758-18-1			X
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	28564-83-2			X
(E)-5-isopropyl-8-methylnona-6,8-dien-2-one	54868-48-3			X
3-methylcyclopentane-1,2-dione	765-70-8			X
scopoletin	92-61-5			X
benzonitrile	100-47-0			X
2-methyl-2-butenenitrile	4403-61-6			X
3-methyl-phenol	108-39-4			X

Table 29 (continued from Table 27 and 28) - Compounds Identified in Usage Forms

Compound	CAS No	Tea	Bedding	Smoke
phenol	108-95-2			X
catechol	120-80-9			X
butylated hydroxytoluene (BHT)	128-37-0	X		
2-methoxy-4-vinylphenol	7786-61-0			X
menthol	89-78-1	X		
2-methoxy-phenol	90-05-1			X
(E)-4-(3-hydroxyprop-1-en-1-yl)-2-methoxyphenol	32811-40-8			X
1-dodecanol	112-53-8	X		
2-furanmethanol	98-00-0			X
diethyl phthalate	84-66-2			X
4-methyl-3-phenyl-pyrazole	13808-62-3			X
3-methyl-pyridine	108-99-6			X
stigmasterol	83-48-7			X
valencene	4630-07-3		X	
γ-valeraolactone	108-29-2		X	
β-myrcene	123-35-3		X	
γ-gurjunene	22567-17-5		X	
alloaromadendrene	25246-27-9			X
α-calacorene	21391-99-1		X	
γ-muurolene	30021-74-0		X	
α-muurolene	31983-22-9		X	
α-copaene	3856-25-5		X	
α-selinene	473-13-2		X	
α-amorphene	483-75-0		X	
δ-cadinene	483-76-1		X	
calamenene	483-77-2		X	
cadelene	483-78-3		X	
(R)-(+)-limonene	5989-27-5		X	X
α-caryophyllene	6753-96-6	X	X	
linalool	78-70-6	X	X	
camphene	79-92-5		X	
α-pinene	80-56-8		X	
β-caryophyllene	87-44-5	X	X	
γ-terpinene	99-85-4		X	
(-)-caryophyllene oxide	1139-30-6	X		
toluene	108-88-3			X
vitamin E	59-02-9			X

Four compounds from this group were identified as being in both the tea and the headspace (see Tables 27-29). Benzaldehyde and (R)-(+)-limonene were the only

compounds in smoke that overlapped with compounds found in the tea and headspace respectively. Many of the compounds identified in the smoke are toxic.

There were also many compounds that were not identified with the analytical tools used for this study. In some cases, peaks were detected but could not be identified with the spectral match criteria of ≥ 80. In addition, some compounds may not have been a part of the current MS spectral library. Also, peak overlap could result in poor library search results. Additionally, many compounds present in the *P. obtusifolium* may not have not been extracted by the techniques employed in this research. To identify more compounds in the plant matrix, sample preparation could be targeted to isolate the various chemical groups of interest. This targeting could include additional sample cleanup so that compounds may be differentiated from the larger sample matrix that currently hinders the identification of the compounds. Alternative extraction techniques could also be explored.

Another possible step in constituent identification especially suited for compounds in the tea simulation is preparative HPLC with tandem PDA and MS capabilities. In this technique the sample exits the detector(s) and goes to a fraction collector instead of going to waste. In this way isolation of and purification of target unknowns can be achieved. This technique is not without complications. Often suitable separation parameters take a considerable time to be optimized and often must be combined with other spectroscopy techniques such as Nuclear Magnetic Resonance (NMR).

NMR is not without limitations. NMR requires much larger sample concentrations and pure samples that are often constrained by complex plant matrices. Gas chromatography with high resolution (e.g., Time of Flight (TOF)-MS) is another technique that may provide principal component identification in complex matrices and used to supplement information provided by the GC-MS. High resolution MS can facilitate potential compound identification by determining the elemental compositions [46].

In addition to understanding what compounds are found in *P. obtusifolium,* studies should also be done to identify the optimal time of harvesting so that the levels of therapeutic compounds are maximized. The Croom research account documents that the Lumbee did not normally consume the plant until the fall or winter [23]. It was recorded by Croom that, “They [Lumbee] only collected the lower dried leaves on the plant if gathered in the summer. The green leaves were thought to make one sick, unless they had a strong stomach” [23]. This detail is of interest because there may be undesirable effects associated with using the herb collected outside of the harvest time. In future work, a chemical profile should be done on the green leaves of the plant before harvest to determine if there was any chemical basis for the warning in the Croom account [23]. There were additional accounts, though few, that either used the roots or the entire plant [19]. The roots and stems should also be analyzed to see if they offer the similar chemical profiles to that of the leaves and flowers or offer sources of other therapeutic potential [23].

When using natural sources such as plants as for therapeutic purposes there are many questions that need to be answered before we understand how plants influenced

medicine in historical native populations. When studying plants that potentially provide therapeutic benefits one main question should be answered - what is the role of this herbal remedy? Asking this question opens a myriad of other questions which allows the researcher to understand if the herb functioned as a curative, disease prevention, or other. Probing herbal plants for curative properties may have its difficulties but at least there is a target disease/cure paradigm in which to measure results. Exploring preventive cures is often less tangible and therefore more challenging but even more important. When we think of food as medicine we understand that a proper diet can prevent many ailments, this area of research has been highlighted in recent years particularly in the field of nutrigenomics which seeks to elucidate how diet can impact the human genome [100].

The Lumbee Native American tribe used *P*. *obtusifolium* as a curative for colds and flu symptoms, but it also could have acted as a preventive for asthma. As mentioned earlier, there are reports that the Creek also used this herb to add a "perfume" to other medicines [24]. In this case, at least part of the herb's function was to make the preparative medicine more palatable, but it also may have imparted synergistic effects. As many of the plant curatives were combined with other plants to provide therapeutic benefits, it is also helpful to recognize how each natural element supports efficacy. These questions require much experimentation and contributions from studies such as this work. Eventually, with enough experimental inputs, researchers may be able to model the function of each element found in herbal preparations to better understand the reason for use.

The future is promising; however, as more unknown compounds are identified, spectral libraries will be able to be updated which will enhance future searches and shorten the time for complex matrix identification. With the identification of more unknowns, ethno-pharmacologists will have an advanced chemical profile in which to evaluate the effectiveness of plant preparations. This broad range access to information may shorten the time it takes to discover new therapeutic plant compounds. One main caveat gleaned from the recreation of the Bald's Leechbook MSRA cure is that a combinatorial effect of multiple agents can be an effective curative [12]. It is also interesting to note that the researcher, Christina Lee, who initiated the Bald's Leechbook curative was an associate professor in Viking Studies. This highlights the power and importance of multiple disciplines working together to solve problems.

Furthermore, since we are presently disconnected from knowing exact historical formulations, the ethnobiologist must consider that plants may be misidentified due to the variety of common names that exist for an herb. The plant may also be reclassified (as in the case of *P*. *obtusifolium*). The ailments that the herb is supposed to treat can also be misinterpreted over time. To this end, we must also be willing to revisit and reevaluate past failed efforts.

Maybe one day in western society, we will seek to understand and embrace what nature has already provided. The ever-increasing frequency of drug resistant bacterial strains may necessitate or accelerate this need. Maybe one day plant preparations can legitimately be recognized by administrative bodies as suitable treatments for our most common ailments while also using novel therapeutic compounds as scaffolding to develop other useful remedies.

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5 Appendix

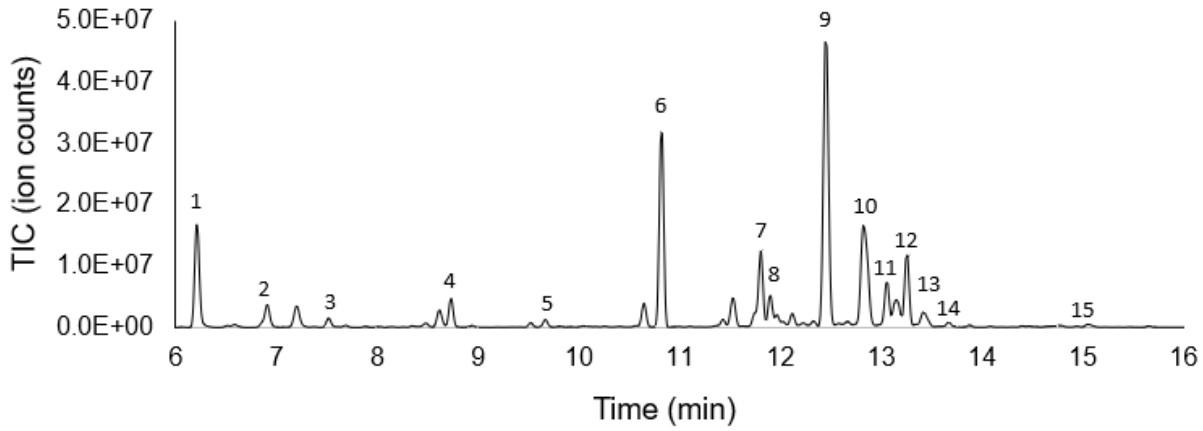

Figure A. GC-MS chromatogram of the headspace of the flower at 40°C. The legend for this figure is shown in Table A. Approximately 40 % of the peaks were identified.

Table A. Legend for Compounds Identified in the Flower Headspace at 40°C.

Peak #	Peak name	CAS
1	α-pinene	80-56-8
2	β-myrcene	123-35-3
3	(R)-(+)-limonene	5989-27-5
4	linalool	78-70-6
5	ethyl octanoate	106-32-1
6	ethyl nonanoate	123-29-5
7	α-copaene	3856-25-5
8	ethyl decanoate	113-38-3

Peak #	Peak name	CAS
9	β-caryophyllene	87-44-5
10	α-caryophyllene	6753-98-6
11	α-muurolene	31983-22-9
12	δ-cadinene	483-76-1
13	calamenene	483-77-2
14	α-calacorene	21391-99-1
15	cadelene	483-78-3

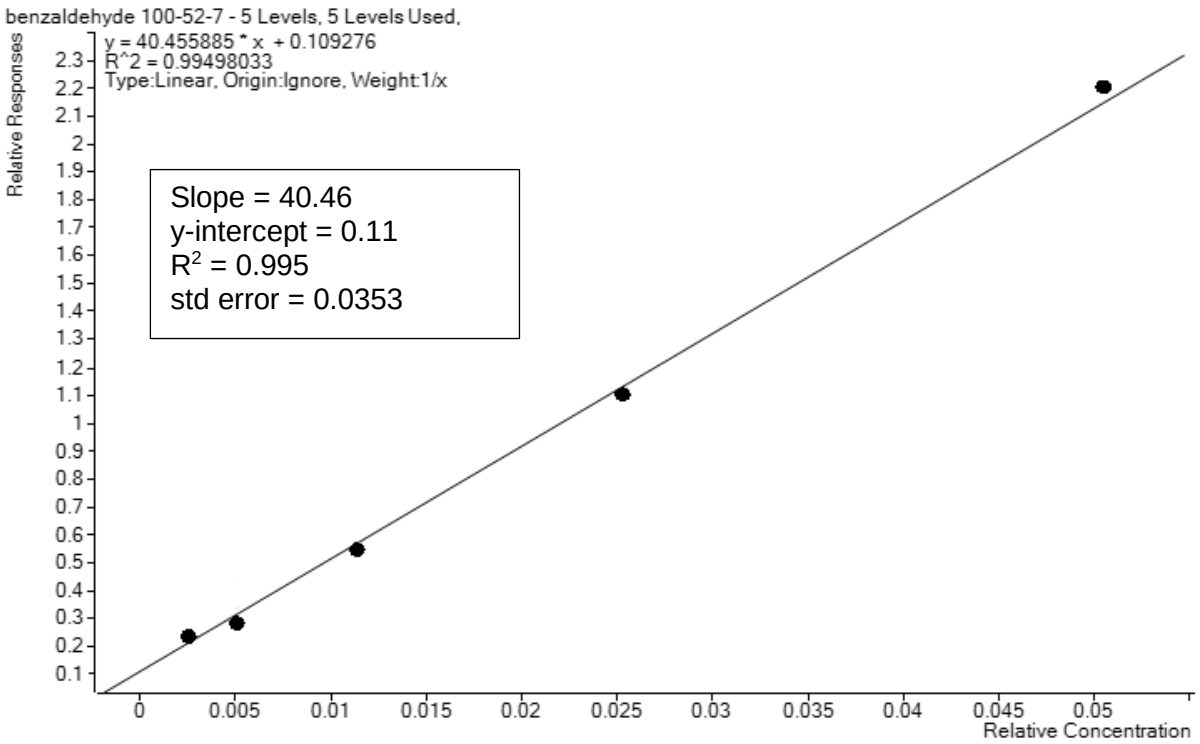

Figure B. Depicts the five point calibration curve for benzaldehyde where the relative response for benzaldehyde represents the peak area/internal standard area. The internal standard was 1-octanol.

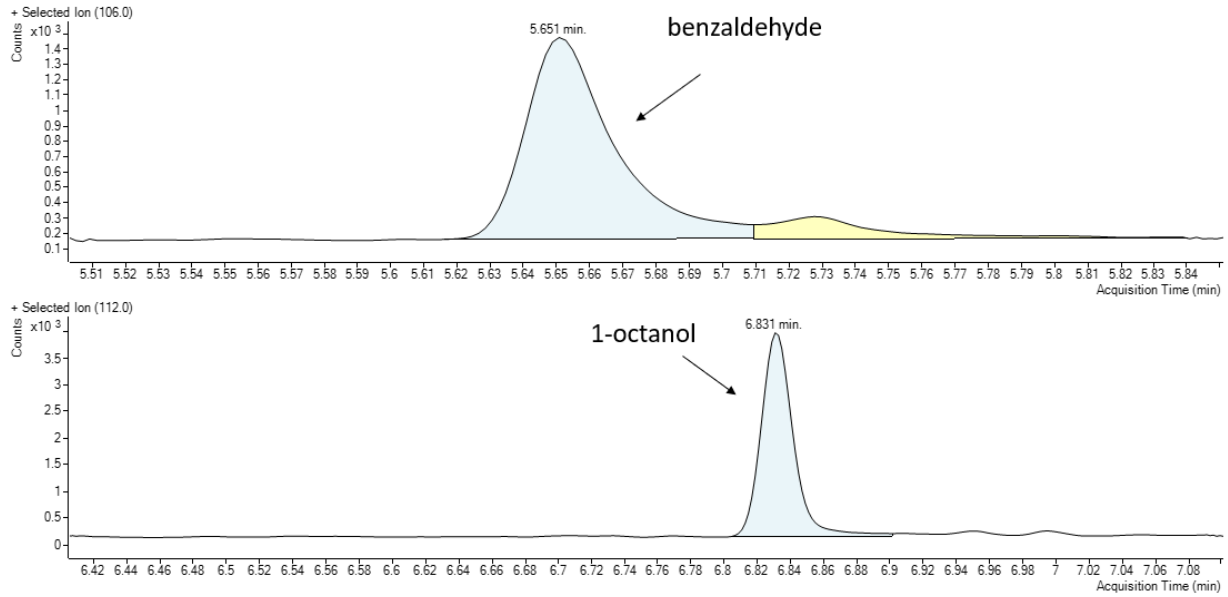

Figure C. A chromatogram of the benzaldehyde in the flower tea extract with 1-octanol as the internal standard.

6 Vita

Regina M. Ballentine is an American citizen. She graduated from Durham High School, Durham, North Carolina in 1987. She received her Bachelor of Arts in Chemistry from Virginia Commonwealth University, Richmond, Virginia in 2000 and has done analytical chemistry work for the last 20 years