

**Phytochemical Analysis of Boreal Forest Flora of Northwestern  
Ontario, Canada**

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Declaration:

I hereby declare that this submission is original and my own research and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgment has been made in the text.

Jazmin Romaniuk

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## Abstract

The following research document has been written in the format of a thesis by publication. As such, there is an introductory and concluding chapter to provide additional information and context to the scope of the study, though the major research is contained in the three chapters (chapters 2-4). Each chapter focusses on the phytochemical analysis of one of three flora species selected from the Boreal forest habitat of Northwestern Ontario, Canada. Each of the chapters are prepared as manuscripts and contain an introduction, method, results, discussion, and conclusion, also including references that pertain to each individual chapter. The original research is in the process of being submitted for publication in peer reviewed journals. The three species that were chosen for this study were the Sweet flag (*Acorus calamus* var. *americanus* (Raf.) H. Wulff), Sweet gale (*Myrica gale* L.) and Wild ginger (*Asarum canadense* L.). These are aromatic plants and each of these plants contain and release volatile aromatic compounds that are detectable by human olfactory senses. Many of the aromatic compounds found in these plants have known bioactive properties. Testing using Gas Chromatography coupled Mass Spectrometry (GCMS) was conducted to identify and report on the phytochemical constituents present in the selected flora species. Phytochemical constituents can aid in the chemotaxonomic placement of flora species within its taxonomic placement, such as the determination of species sub-variety (ie. the *Acorus* variation, *americanus*). As well, phytochemical analysis can also identify compounds of bioactive interest, which includes medicinal properties, such as with the aromatic compound limonene and its sought-after anxiolytic-like properties (Lima et al., 2013). The introduction establishes the background geographical and botanical characteristics of the plants analysed. The analysis of *Acorus calamus* var. *americanus* (Raf.) H. Wulff by GCMS, in this research, characterises much of the phytochemicals and confirms the absence of  $\beta$ -asarone, a bioactive compound found in the European species, *Acorus calamus* L. Sweet gale, *Myrica gale* L., has previously been reported to have different chemical composition relative to its geographical location, making chemotaxonomic identification of species challenging. However, it does provide support for the chemotaxonomic classification of Sweet gale into various geographically defined

varieties, enabling determination of the geographic origins of plants identified. The phytochemical analysis has never been performed for Sweet gale found in Northwestern, Ontario for this chemotaxonomic geographical characterisation. The analysis of the *Asarum canadense* L., Wild ginger, has confirmed the absence of compounds (like  $\beta$ -asarone) that have been found in other related species and identified compounds pertinent for its medicinal use in Northwestern Ontario, Canada. The phytochemical analysis has also provided a geographically relevant chemotaxonomic characterisation for the region. The results of these analyses provide the opportunity for chemotaxonomic geographic identification of three of the Northwestern Ontario plant species that have research interest elsewhere in the world.

# Chapter One

## 1 Introduction

The Boreal Forest encompasses 11% of the entire earth's terrestrial surface (Bonan & Shugart, 1989). In Canada, it covers approximately 5.8 million hectares, that being more than 58.5% of the entire country (Anielski et al., 2003). In addition, Anielski states that approximately 35% of the world's wetlands exist in Canada. These wetlands are critical to the survival of many species in this forest region (Anielski et al., 2003). Animals, both herbivores and carnivores rely on forest resources for food, shelter and survival. In the past, humans have exploited these forests through food procurement, and have developed and employed techniques and technologies to ensure their long-term survival. The Boreal biome also contains numerous coexisting flora and fungal species that have developed survival strategies suitable for this taxing environment. This includes development of defences against Boreal Forest herbivores, predation and competition (Gong & Zhang 2014, Unsicker et al., 2009). These defence mechanisms can be physiological, such as leaf thickness, trichomes and herbivore mimicry (Gong & Zhang, 2014, Svoboda, 1998), but also include chemical defences. Some of the chemical defences, as noted by Heil and Gong & Zhang, are compounds such as saponins, terpenes, anthocyanins, among others, and are explored further within the following examination of Boreal flora.

The volatile compounds that serve as chemical defences in flora species are also utilized by humans for their medicinal bioactive properties. For example, terpenes and terpenoids are known to have anticancer, anti-inflammatory and anticarcinogenic properties which are used as treatments for cancer, chronic diseases and other illnesses (Sultana & Saify, 2003). To best identify these medicinal constituents among flora, the examination of the volatile compounds must include delineating the exact genus, species and geographical location for proper flora identification. The following review addresses why it is important for plants develop volatile compounds, the characteristics and use of bioactive compounds, and the chemical identification of flora. This section concludes with the botanical description of the flora under investigation as a prelude to introducing the methodological approach and anticipated results of the examination of selected plants. Such exploration of plant constituents could identify novel compounds that are useful in medicine. Exploring the potential of a "library" of knowledge, which is not frequently explored, is valuable for the growing research toward the development and future of medicine.

## 1.1 Flora develop chemical defences

Genetics, habitat, and environmental pressures aid in the production of phytoconstituent components that are defence mechanisms for flora survival. As defence from environmental pressures, plants are known to release volatile chemicals when damaged by herbivores or affected by fungal or microbial infections (Heil, 2014, Gong & Zhang, 2014). Heil (2014) describes these volatile chemicals as herbivore induced volatile organic compounds (HI-VOCs). The release of these HI-VOC compounds can be best illustrated as a plant warning, which Heil describes as a plant ‘communication’ system (Heil, 2014). The release of these HI-VOCs by the plant occurs directly after herbivore contact which then signals other nearby plants of their distress (Heil, 2014). Unsicker et al, states it is possible that volatiles can act as markers of a species identity and repel herbivore attack. They also state that the volatiles may also act to signal herbivores of the presence of competition as well as enemies (Unsicker et al., 2009). Many flora species develop bitter defence compounds such as, terpenes or terpenoids, found in flora such as pine. These compounds are known to have bioactive properties such as antibacterial, antiparasitic, antifungal and many other medicinal properties for humans (Sultana & Saify, 2003).

## 1.2 Bioactive plant compounds

There are many phytochemical compounds within flora that have bioactive properties that are medicinally useful for humans. Plants develop HI-VOCs and other phytochemical compounds for protection against bacteria and fungi (Heil, 2014), and humans can also extract these bioactive compounds to use as medicine to fight the same organisms. For example, (*E*)  $\beta$ -caryophyllene is emitted from maize roots, and it is thought that it enables the plant to battle herbivores (Rasmann et al., 2005). This compound has also been tested to be an effective anticonvulsant in rats, with minimal toxicity (Oliveira et al., 2016). Both plants and animals can utilize the compound for medicine and defence.

Many of these phytochemical compounds, detectable by olfactory senses, are developed in highly aromatic plants of the genus *Artemisia* spp. The foliage from this plant genus has a strong scent of eucalyptus when harvested as well as tasting bitter. C’avar et al. have found that constituents of *Artemisia annua* L. are antimicrobial (C’avar et al, 2012), which would effectively deter some forms of bacteria. As well, when one tastes a plant with active constituents, such as the sesquiterpene lactones in chicory, the compounds have a bitter quality



(Chadwick et al, 2013). Upon examination of the history of European pharmacology, people used wild bitter plants to prepare herbal elixirs to combat a variety of ailments (Egea et al, 2015). The aromatic or bitter flora that have been used to create elixirs containing medicinal bioactive constituents can treat the nervous system, respiratory problems, mental disorders, infectious and parasitic diseases (Egea et al, 2015).

Some bioactive compounds are also well known for use in traditional medicine practices, such as the monoterpene limonene. Limonene is a highly volatile compound that is found in the rinds of citrus fruit and in many other aromatic plants such as lavender albeit in smaller quantities (Lima et al, 2013). Limonene, amongst many other bioactive properties, is considered to have antimicrobial properties. A common practice across cultures involves inhaling limonene in plant essential oils for its anxiolytic effect (Lima et al, 2013). Also, the well-known compound nicotine of the tobacco plants of the *Nicotiana* genus, has been used for millennia (Ross, 2005). These plants have been grown, exploited and traded around the world throughout human history (Ross, 2005). Most of the members of *Nicotiana* genus, contain the compound nicotine in varying concentrations. The plant *Nicotiana alata* Link & Otto, which has a very low concentration of nicotine (0.003%), and may not be as desirable specifically for the nicotine compound as compared to *Nicotiana rustica* L., with 18% nicotine concentration (Eich, 2008).

Due to these variations in the presence and concentration of bioactive compounds, as noted with *Nicotiana* spp., the importance of proper plant identification is key to analysing a species with the desired concentration of compounds. A closer examination of the presence and variation of phytochemical constituents between species also allows one to understand pre-historic and historic plant use and practices. Flora containing bioactive compounds that affect humans are used for non-nutritional consumption in practices such as smouldering or smoking as well as infusions used as traditional medicines. These practices are very hard to detect and almost impossible to confirm in the archaeological record using traditional archaeological methods. Chemical analysis of archaeological material using a reference library of the phytochemical components of flora can be performed, allowing paleoethnobotanists to identify these previously invisible human activities.

### **1.3 Chemotaxonomic Identification**

Physical botanical examination, entailing detailed descriptions, is normally sufficient to identify flora species but it may be difficult to identify subtle phenotypic variation. This can

become even more challenging when only some physical characteristics are present. Some features of a plant may not display consistent physical features required for the identification of a species. The most distinct and identifiable features used for species identification are seeds, flowers and leaves. Other parts of the plant may be more ubiquitous like roots and rhizomes.

Correct identification is challenging when these distinct features are absent and coupling physical botanical examination with phytochemical constituent analysis can provide a more reliable taxonomic identification. Likewise, when only the exudates (i.e. resins or gums) or extracts (i.e. essential oils) are all that is available, chemotaxonomic identification is all that is possible from their phytochemical composition. Chemotaxonomy provides reliable identification on the basis of characterization of species distinct chemical constituents. Each plant species may contain compounds and concentrations which are characteristic to that species. For example, Rajput et al. (2014) and Wagner specify that asarone compounds are absent in the diploid species *Acorus calamus* var. *americanus* (Raf.) H. Wulff (Wagner et al., 2011, Rajput et al., 2014) and this absence aids in the determination of this sub-variety. Chemotaxonomic identification can aid botanists in the identification of specimens when diagnostic features may not be present, as well as providing new reference material for paleoethnobotanists in the identification of pre-historic plant exploitation and practices.

#### **1.4 Phytochemical constituent investigation of flora**

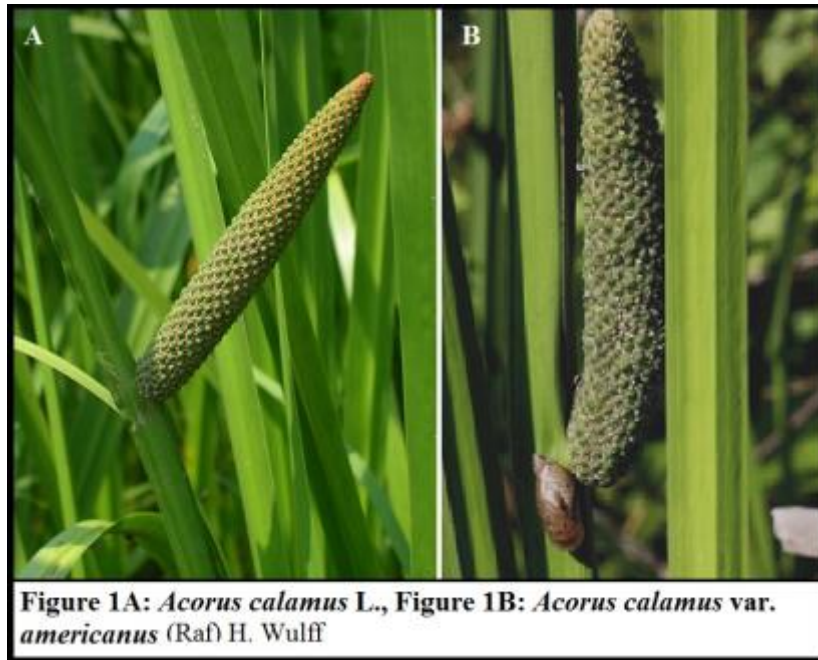
The following research investigates the phytochemical constituents of three aromatic flora species from the Boreal forest of Northwestern Ontario, Canada to delineate their phytoconstituents. It will identify the phytochemical constituents of local flora that could aid and enhance identification of species using the geographical location, physical description and chemical constituents for identification via Gas Chromatography Mass Spectrometry (GCMS).

##### **1.4.1 Plant specimens**

Plant collection took place over two growing seasons in 2014 and 2015. Based on the phytochemical literature and their observed volatility, flora samples were chosen by scent and bitter taste qualities, and through botanical examination. The plants chosen were the aromatic plants Sweet flag (*Acorus calamus* var. *americanus* (Raf.) H. Wulff) Wild ginger (*Asarum canadense* L.), and Sweet gale (*Myrica gale* L.). The botanical descriptions of the chosen specimens are offered below.

## Family: Acoraceae

*A. calamus* var. *americanus* (Raf.) H. Wulff, commonly known as Sweet flag, grow under similar conditions to its relative *Acorus calamus* L. found in Europe and elsewhere. It prefers living in a hydric habitat, swampy, marshy areas that have flowing water (Geiger & Banker, 2012). This North American species is a diploid variety of *Acorus*, unlike *A. calamus* which is a sterile triploid species located in Eurasia (Wagner et al 2011). The plant forms interlinking monotypic rhizome mats in the mud, growing leaf stalks ½ meter to 1 meter in height (Geiger & Banker, 2012). The leaf stalk's mid vein is equally raised which forms a smooth leaf surface. The flower grows club-like and irregularly shaped, refer to figure 1B below, at a jointed angle from a leaf stalk (Geiger & Banker, 2012).



1A Image: <http://www.anticaerboristeriaromana.it/oliessenziali.php>,

1B Image: NPIN Image

43192[http://www.wildflower.org/gallery/species.php?id\\_plant=ACAM](http://www.wildflower.org/gallery/species.php?id_plant=ACAM)

## Family: Aristolochiaceae

*Asarum canadense* L. is commonly called Wild ginger. It prefers living in the understory of deciduous forests. It grows near the surface of the humus layer in interwoven webs of rhizomes (Bicknell 1897, Damman and Cain 1998). The leaves are distinctly heart shaped, and

will grow a flower in its second year. The flowers are purple to maroon, and has three petals which arise in the early spring (Bicknell 1897, Damman and Cain 1998).

### **Family: Myricaceae**

*Myrica gale* L., known as Sweet gale, lives in a hydric habitat. The species inhabits most of the northern hemisphere. It is a deciduous dioecious shrub, lives in acidic soils, and can grow up to 2 meters tall (Svoboda et al 1998). The leaves are lanceolate shaped, rounded at the terminal end, with serrated edges. The leaves house glandular trichomes that release the volatiles in Sweet gale. Microscopic images of these glands could aid in taxonomic differentiation in species (Svoboda et al., 1998).

### **1.4.2 Methodological approach**

Analyses took place through solvent extraction and GCMS analysis. Plant samples are prepared by grinding the material into a powder or fine particle mixture, placed in a 2mL autosampler vial, and suspended in solvents consisting of ethanol, distilled water or acetonitrile. The extracts are then derivitised and analysed by GCMS. More detailed descriptions of methodology will be available in each of the following chapters.

### **1.4.3 Anticipated results**

Expected results are to identify chemical constituents from known, highly volatile flora species found in the Boreal forest. Compounds common to Flora species of Northwestern Ontario could include terpenes, phenols, and alkaloids. These are the same compounds that have neuroactive properties in humans, being used as medicines. The chemical constituents and compositions are unique to each species of plant, including their geographic locations and is an important factor for the delineation of taxonomic placement. The following chapters are the phytochemical analyses of these selected plants, each chapter focusing on an individual flora species local to Northwestern Ontario, Canada.

## Chapter Two

Phytoconstituents of *Acorus calamus* var. *americanus* (Raf.) H. Wulff Rhizomes collected in  
Northwestern Ontario, Canada

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**Introduction** – The phytochemical composition of Sweet flag, *Acorus calamus* L. from various geographical sources around the world, has been extensively studied and the bioactive compounds have been determined. The almost indistinguishable species of North American Sweet flag *A. calamus* var. *americanus* (Raf.) H. Wulff has not been fully analysed by GCMS. By performing phytochemical analysis of *Acorus calamus* var. *americanus* (Raf.) H. Wulff it is possible to provide chemotaxonomic identification of the North American variety of Sweet flag. The following study reports the GCMS analysis of rhizomes found in Northwestern Ontario, Canada.

**Objectives** – To provide a phytochemical characterisation of *A. calamus* var. *americanus* rhizome using Gas Chromatography Mass Spectrometry (GCMS).

**Material and Methods** – Samples were prepared by extraction of rhizome material with acetonitrile and ethanol. The solvents were removed and the GCMS analysis was performed on the extracts with BSTFA - TMS derivatisation in acetonitrile. The results were identified through a GCMS database.

**Results** – A range of sesquiterpenes and sesquiterpenoids were identified including  $\alpha$ -copaene,  $\alpha$ -cedrene,  $\beta$ -cedrene,  $\delta$ -guaiene, bergamotene,  $\alpha$ -longipinene, caryophyllene, thujopsene, isocaryophyllene,  $\beta$ -cubebene,  $\alpha$ -gurgujene,  $\alpha$ -muurolene, cedrenol,  $\gamma$ -muurolene,  $\delta$ -cadinene,  $\gamma$ -cadinene, acolamones, dehydroxy-isocalamendiol, calamendiol, trichoacorenol, acorenone and shyobunone.

**Conclusion** – The analysis showed that acetonitrile extracts contained the greater number of compounds extracted and that the phytochemical composition did not include any asarones commonly associated with *Acorus* species. This study has provided the phytochemical composition of *A. calamus* var. *americanus* that would allow for chemotaxonomic differentiation and further study into the bioactive components.

**Keywords:** Phytochemistry, *Acorus calamus*, *Acorus calamus* var. *americanus*, Sweet flag, GCMS, chemotaxonomy

## **1 Introduction**

*Acorus calamus* L., Sweet flag, is well known for its bioactive properties (Adfa et al 2015, Kim et al 2009, Shi et al 2014, Vijayapandi et al 2012) including medicinal uses applicable to many ailments around the world (Imam et al 2013, Kumar et al 2015, Singh and Tripathi 2010). Many parts of the plant have been used traditionally, specifically the highly aromatic rhizome which has been used in Vedic as well as Chinese medicinal traditions for treating inflammation, congestion, as well as central nervous system (CNS) disorders (Mukherjee et al 2007, Rajput et al 2014). The North American variety of *Acorus*, is *A. calamus* var. *americanus* (Raf.) H. Wulff, a diploid species, differs from *A. calamus* L., a triploid variety of *Acorus* (Wagner et al 2011). The rhizome of *A. calamus* var. *americanus* (Raf.) H. Wulff is used similarly to other *Acorus* species and in some Indigenous North American medicine traditions (Mukherjee et al 2007), is known as *wike*, (*wee-ka*).

Each of the diploid and triploid varieties of Sweet flag grow in swampy and marshy waters (Geiger & Banker, 2012). The ideal habitat is in flowing marsh waters alongside bulrushes, arrowroot and wild rice (Geiger & Banker, 2012). The rhizomes grow into a woven mat in the mud, along with other roots and rhizomes of water plant species (such as the blue flag *Iris versicolor* L.) (Geiger & Banker, 2012). The flower appears club-like, variable in shape, and houses viable seeds (Geiger & Banker, 2012). This differs from the triploid variety that exhibits uniform conically shaped flowers (Geiger and Banker 2012). In 1979, Hoffman (Hoffman et al 2001) stated that the North American Cree peoples chewed the rhizome of Sweet flag to treat diverse ailments. Furthermore, they observed that in high doses, it is thought to cause modified states of consciousness (MSC), but the responsible constituent or mechanism of action remains unknown (Hoffman et al 2001).

The main bioactive constituent of the triploid *A. calamus*,  $\beta$ -asarone (cis-1,2,4-trimethoxy-1-propenylbenzene), is a known carcinogen at high concentration and possess a range of medicinal bioactive properties (Sundaramahalingam et al. 2013). Other phytoconstituents of *A. calamus* L. include sesquiterpenes and sesquiterpenoids such as caryophyllene,  $\beta$ -cedrene, and isoeugenol (Suman et al. 2014, Wagner et al. 2011, Avadhani et al 2013, Rana et al. 2013). Due to the similar use and habitat of Sweet flag varieties, it has been suggested that the North American species has a similar phytochemical composition to the other *Acorus* species dispersed around the world (Wagner, 2011). However, the phytochemical constituents of the Sweet flag may not be as synchronous as first suggested by researchers. Wagner (et al 2011) observed that

the diploid *A. calamus* var. *americanus* (Raf.) H. Wulff does not contain detectable amounts of  $\beta$ -asarone.

This research presents the GCMS analysis of the phytochemical constituents of *A. calamus* var. *americanus* (Raf.) H. Wulff collected from Northwestern Ontario, Canada. The resulting phytochemical constituents of *A. calamus* var. *americanus* (Raf.) H. Wulff allows for chemotaxonomic differentiation of Sweet flag varieties, also allowing the chemotaxonomic classification to distinguish between *Acorus* species.

## 2 Material and Methods

### 2.1 Flora Material

Rhizomes of *A. calamus* var. *americanus* (Raf.) H. Wulff were collected from Lake of the Woods, Ontario, Canada. The plant specimen verified as *A. calamus* var. *americanus* (Raf.) H. Wulff with the voucher specimen LKHD 104462 at the Claud Garton Herbarium, Lakehead University.

### 2.2 Flora extraction

The collected rhizomes were hung to dry for 5 days, washed in distilled water, and finely cut and crushed with a mortar and pestle. The crushed dried material appeared woody, fibrous with fine ground powder. The fine ground powder was separated into two samples (Aa1 and Aa2), weighed and placed into sterile 2ml glass autosampler vials. (Table 1).

**Table 1.** The samples subjected to analysis, coupled with the solvents used for their extraction.

<b>Samples</b>	<b>Weight (g)</b>	<b>Extract</b>
<b>Aa1</b>		
<b>JR2-V1</b>	0.0465	Ethanol
<b>JR2-V2</b>	0.0620	Ethanol
<b>JR2-V5</b>	0.0376	Acetonitrile
<b>JR2-V6</b>	0.0306	Acetonitrile
<b>Aa2</b>		
<b>JR2-V1</b>	0.0465g	Ethanol
<b>JR2-V3</b>	0.0306g	Acetonitrile

Two solvents were used for the extraction: ethanol (Sigma) and acetonitrile (Sigma). An aliquot of 1000 $\mu$ L of each solvent was added to individual 2mL autosampler vials. The



acetonitrile vials were capped as to hinder evaporation. The samples were placed on a heat block at 36°C for a minimum of 24 hours.

### **2.3 Derivatisation**

After incubation, the ethanol extracts were allowed to evaporate. An aliquot of 1000µL of acetonitrile was added to each of the evaporated samples to resuspend the extracts, 100µL of BSTFA with 0.1% TMS (Sigma) was added and the vials were purged with nitrogen gas and capped.

After incubation, an 800µL volume of the acetonitrile solution from the acetonitrile extracts was removed and added to new sterile 2mL autosampler vials. An aliquot of 100 of BSTFA with 0.1% TMS was added to the vials, purged with nitrogen gas and capped. The sealed vials of all the extracts were placed on the heat block at 121°C for 30 minutes for derivatisation immediately prior to loading in the GCMS.

### **2.4 GCMS analysis**

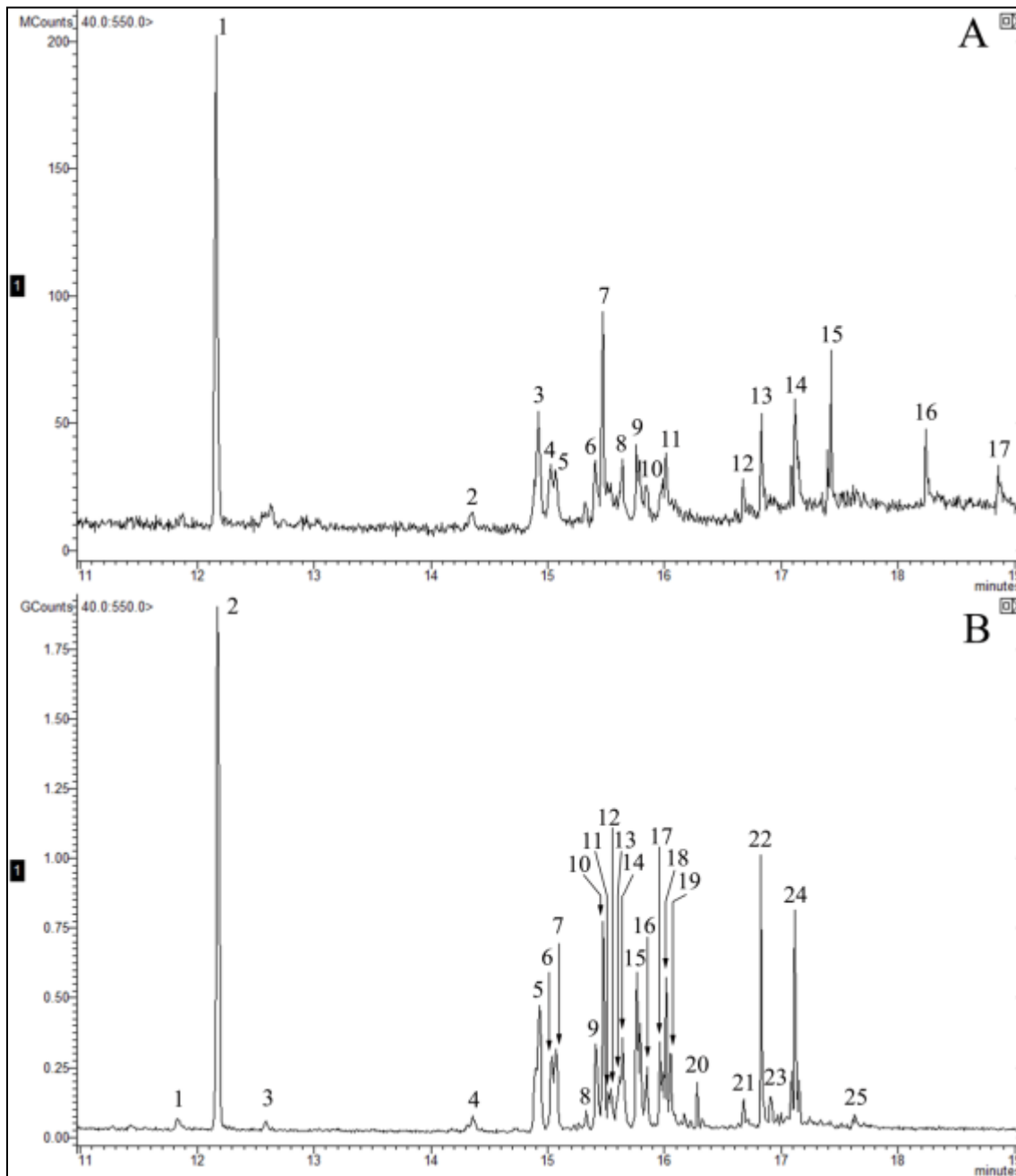
The analysis was performed on a quadrupole Varian model 450 gas chromatograph coupled with Varian model 300-MS mass spectrometer. The conditions for the analysis included helium carrier gas (1.0 mL/min), on a 30m Factor Four capillary column (ID = 0.25mm, VF-5 ms, DF=0.25 µm), with a splitless mode injection (port temperature 270°C), the column was set to 50°C for 2 minutes then increased to 155°C (rate 8°C/min) and then increased again to 275°C (rate 40°C/min), the final temperature was 275°C for 9 minutes and the interface temperature was set to 266°C. The ion source was set to 200°C and 70 eV under electron ionisation (EI) conditions. The scan range was 40 to 500 m/z and the output files were analysed using Varian MS workstation version 6.

### **2.5 Identification of compounds**

The chromatogram and ion spectra were used for the identification of each compound. Each peak in the chromatogram was analysed by comparison to the database of the National Institute of Standards and Technology (NIST). The ion spectra generated from the sample was compared with the spectra of the best-matched compounds in the NIST database. Each match was then interrogated manually to ensure accurate identification by matching the relative ion peaks ( $M^+$ ,  $M+1$ ,  $M-15$  etc.).

### 3. Results

The results from the various extracts of the same solvent produced results that were consistent with each other. Only one example of each run is shown (Figure 1).



**Figure 1.** The chromatograms of the ethanol extract (A) and the acetonitrile extract (B).

**Table 2.** The chemical constituents of *A. calamus* var. *americanus* acetonitrile extract

Peak	Ret. (min)	MW	Chemical Compound
1	11.835	122.12	benzoic acid
2	12.174	92.09	glycerol
3	12.592	196.29	bornyl acetate
4	14.356	204.36	thujopsene
5	14.901	204.36	$\alpha$ -copaene
6	15.035	204.36	$\beta$ -cedrene
7	15.071	204.36	bergamotene
8	15.333	204.36	$\alpha$ -longipinene
9	15.411	204.36	$\delta$ -guaiene
10	15.474	204.36	isocaryophyllene
11	15.517	204.36	$\beta$ -cubebene
12	15.538	204.36	$\alpha$ -cedrene
13	15.616	204.36	$\alpha$ -gurgujene
14	15.645	204.36	caryophyllene
15	15.765	220.35	cedrenol
16	15.85	204.36	$\alpha$ -muurolene
17	15.956	220.35	shyobunone
18	15.984	204.36	$\gamma$ -muurolene
19	16.013	204.36	$\gamma$ -cadinene
20	16.282	204.36	unidentified
21	16.678	204.36	unidentified
22	16.827	220.35	dehydroxy-isocalamendiol
23	16.905	222.37	trichoacorenol
24	17.117	220.35	acorenone
25	17.627	238.37	calamendiol

\*The peak number corresponds to the number in figure 1, panel B.

The acetonitrile extraction produced many chemical compounds that include monoterpenoids, sesquiterpenes, sesquiterpenoids and benzoic acid and glycerol (Table 2). Out of the 25 main peaks in the chromatogram (figure 1, panel B), only 23 were identified, although the remaining two unidentified compounds were sesquiterpenes. The ethanol extraction produced only 16 main peaks in the chromatogram (figure 1 panel A). However, these compounds were identified as sesquiterpenes, sesquiterpenoids, fatty acids, xylofuranose and glycerol (Table 3). Out of the 16 main chromatographic peaks, only 14 were identified. One of the remaining unidentified peaks is likely a sesquiterpene, while the other compound does not reliably match any other compound in the database.

**Table 3** The chemical constituents of *A. calamus* var. *americanus* (Raf.) H. Wulff ethanol extract

Peak	Ret. (min)	MW	Chemical Compound
1	12.154	92.09	glycerol
2	14.342		unidentified
3	14.923	204.36	$\gamma$ -cadinene
4	15.044	204.36	$\beta$ -cedrene
5	15.065	204.36	aromadendrene
6	15.405	290.44	methyl octadec-6,9-dien-12-ynoate
7	15.469	204.36	$\gamma$ -himachalene
8	15.639	204.36	Copaene
9	15.752	204.36	$\gamma$ -muurolene
10	15.844	204.36	$\alpha$ -muurolene
11	16.014	204.36	$\gamma$ -cadinene
12	16.673	204.36	unidentified
13	16.828	220.35	dehydroxy-isocalamendiol
14	17.119	220.35	acorenone
15	17.423	150.05	xylofuranose
16	18.239	256.43	Palmitic acid

\*The peak number corresponds to the number in figure 1 panel A.

#### 4. Discussion

The comparison of the two solvents used for the extraction of phytochemicals showed that the acetonitrile extracts yielded the largest number and quantity of compounds (Table 2). While the acetonitrile extract recovered many sesquiterpenes, the ethanol extract recovered other compounds like xylofuranose and palmitic acid. The chemical constituents common to *Acorus* species have been found in the rhizomes of *A. calamus* var. *americanus* (Raf.) H. Wulff such as sesquiterpenes thujopsene,  $\beta$ -cedrene, and caryophyllene (Suman et al 2014, Wagner et al 2011, Avadhani et al 2013). Some of these compounds have been shown to be bioactive such as thujopsene and  $\beta$ -cedrene, which are known to be active in inhibiting human liver cytochrome enzymes (Jeong et al 2014). There were significant differences between the currently reported diploid variety and the triploid species, including the absence of asarones, the presence of cedrenes and other sesquiterpenes and sesquiterpenoids (Rajput et al 2014, Avadhani et al 2013). This study of *A. calamus* var. *americanus* (Raf.) H. Wulff also supports other research regarding *Acorus* species and varieties that have identified the major compounds in the rhizome, including acarenones and calamendiols, and various parts of the plant have yielded shyobunones, bornyl acetate, palmitic acid, caryophyllene (Rajput et al 2014, Avadhani et al 2013).

Most importantly, it has been confirmed that *A. calamus* var. *americanus* (Raf.) H. Wulff does not contain GCMS detectable amounts of  $\beta$ -asarone, which is the known bioactive compound in other *Acorus* species (Wagner et al 2011, Rajput et al 2014). The challenges involved in differentiating between North American species *A. calamus* var. *americanus* and Eurasian species of *A. calamus* L. has been noted by other researchers (Geiger and Banker 2012). Geiger and Banker (2012) clarified the differences between North American and Eurasian *Acorus* species and noted that there is a common misnaming of *A. calamus* var. *americanus* (Raf.) H. Wulff as *A. calamus* L. Geiger and Banker (2012) also explained the variation in the physical appearance of *A. calamus* L. and *A. calamus* var. *americanus* (Raf.) H. Wulff. Both survive in aquatic habitats, however, the most distinguishable, identifiable feature is the flower. *A. calamus* var. *americanus* (Raf.) H. Wulff flowers are club-like, variable and contain viable seeds, while *A. calamus* L. flowers are uniformly, conically shaped and sterile (Geiger and Banker 2012).

Detailed characterisation of the flower can aid in correct identification of a plant species. However, if the flower is unavailable, phytochemical analysis can be employed for accurate identification. Extensive research of the chemotaxonomic identification was provided by Wagner et al. (2011), and concluded that the variation of  $\beta$ -asarone content can be used to differentiate the species and varieties when this compound is minimal or absent (such as North American variety *A. calamus* var. *americanus* (Raf.) H. Wulff).  $\beta$ -asarone is reported in the concentrations of 3-13% in the triploid (n) European species (*A. calamus* L.) and up to 80% in the Asian tetraploid (n) variation (*A. calamus* var. *angustatus* (Raf.) Besser) (Wagner et al 2011). This current research provides the phytochemical composition of the rhizomes of the North American *A. calamus* var. *americanus* (Raf.) H. Wulff which supports Wagner et al (2011) reporting the absence of  $\beta$ -asarone. However, this research also indicates additional phytochemical differences in the terpene content that could also be exploited in the chemotaxonomic identification of the *Acorus* species and varieties. Combining plant physiological observations with the phytochemical data aids in accurate identification of the *Acorus* plants. Chemotaxonomic identification would be the only reliable means of identifying separated plant parts and specimens without flowers or flora collected outside the flowering season.

## **5. Conclusion**

This research confirms that the North American variety of Sweet flag, *A. calamus* var. *americanus* (Raf.) H. Wulff, exhibits an absence of the bioactive compound  $\beta$ -asarone. For the

first time, it has also provided a comprehensive list of the phytochemical composition of the *A. calamus* var. *americanus* (Raf.) H. Wulff rhizome from both ethanol and acetonitrile extracts. *A. calamus* var. *americanus* (Raf.) H. Wulff may be more advantageous for medical research and use and is worth further investigation unlike *A. calamus* constituting the unfavourable carcinogen,  $\beta$ -asarone. This comprehensive phytochemical characterisation provides the ability to identify *A. calamus* var. *americanus* (Raf.) H. Wulff from other species of *Acorus* flora found in North America. This allows for a very reliable chemotaxonomic identification of plant parts excluding flowers and plant extracts.

### **Conflict of interest statement**

The authors have no conflict of interest in this research.

### **Acknowledgements**

The authors would like to thank the staff of the Lakehead University Instrumentation Laboratory particularly Grzegorz Kepka and Michael Sorokopud for their help and support during this research. We would also like to thank Erika North from the Claud Garton herbarium at Lakehead University, for the botanical verification of *the A. calamus* var. *americanus* (Raf.) H. Wulff collected and used in this research.

## Chapter Three

### GCMS analysis of Phytoconstituents of *Myrica gale* L. leaves collected from Northwestern Ontario, Canada

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#### **Abstract**

**Introduction** – There have been numerous phytochemical analyses of Sweet gale, *Myrica gale* L. deriving from different geographical locations. Previous research has demonstrated that plants from different geographical locations have a different compliment of phytochemical constituents. This study addresses the phytochemical composition of the leaves from *M. gale* L. from Northwestern Ontario, Canada.

**Objectives** – To perform a phytochemical characterisation of *M. gale* L. (Sweet gale) leaves using Gas Chromatography Mass Spectrometry and determine if the flora material from Northwestern Ontario supports the same conclusion that plants from different locations contain different phytochemicals.

**Material and Methods** – Leaf samples were ground and solvent extracted with water, acetonitrile and ethanol. Gas Chromatography Mass Spectrometry (GCMS) analysis was conducted on the derivitised extracts. The phytochemical compounds were identified using the NIST database and manual comparison to reference spectra and samples.

**Results** – The results were consistent with other studies where a number of terpenes and terpenoids were identified including bergamotene, cadinene, caryophyllene, copaene,  $\alpha$ -elemene, limonene,  $\alpha$ -muurolene, myrcene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\alpha$ -phellandrene, cis-ocimene,  $\gamma$ -terpinene, selin-11-en-4-ol, selinenol along with other compounds of glycerol and docosadiynedioic acid.

**Conclusion** – The extractions using acetonitrile recovered a greater number of compounds. The phytochemistry of Northwestern Ontario Sweet gale has a different range of chemical compounds providing the characteristics for the possibility of geographical chemoidentification.

**Keywords:** Phytochemistry, *Myrica gale* L., Sweet gale, GCMS, terpenes

## 1 Introduction

Sweet gale (*Myrica gale* L.), found throughout the northern hemisphere, is a deciduous, dioecious shrub that favours growing in wetlands and on the banks of flowing water (Carlton et al., 1992). It can grow in damp, acidic soils, living abundantly in bogs and marshes (Svoboda, 1998), and favour forming in shrubby clumps (Skene, 2000). This plant is known to be highly aromatic, the leaves having secretory glands on both surfaces (Popovici, 2008). Throughout history, *M. gale* L. has been collected and used in making perfume, as an ingredient for beer prior to the use of hops, as well as their catkins being a source of candle wax (Svoboda, 1998). The fruit of *M. gale* L. have a long history of medicinal use, being used for cardiac disorders and stomach issues (Carlton, 1992, Popovici, 2008).

Phytochemical studies of *M. gale* L. grown in Ontario, Canada reported compounds such as myrcene, limonene,  $\alpha$  and  $\beta$ -Pinene, caryophyllene, terpinene, terpineol, copaene, linalool, calamene, camphene, and 1, 8-cineol (Svoboda, 1998). Svoboda *et al.* (1998) observed that the concentrations of these compounds vary between *M. gale* L. species growing in different countries. Carlton et al. (1992) reports that it is possible that *M. gale* L. found in Scotland can be distinguished from other European populations by phytochemical analysis. These authors state that *M. gale* L. synthesises the sesquiterpenoid germacrone as a chemical defence and claim that it is an adaptation to local pressures found in Scotland (Svoboda, 1998). It could be suggested that chemical constituents of *M. gale* L. can be dependent upon herbivore induced volatile organic chemical (HI-VOC) signalling (Heil, 2014). The production of compounds could vary in different geographic locations in the northern hemisphere.

This postulates that some plant species can vary their biochemical constituents to defend against herbivory and other attacks that are geographically specific. This raises questions regarding the mechanisms of biochemical synthesis of defensive compounds, and how environmental pressures affect the constituents composing an organism. Chadwick et al. (2013) proposes that the specific composition of sesquiterpenes is important for identification, as well as their response to allelopathic signalling. It is important to collect data from many locations to



have an understanding of the concentration and composition of *M. gale* L. for future comparison studies. This study is a phytochemical analysis of the chemical constituents of the leaves from *M. gale* L., collected from Northwestern Ontario, Canada, and analysed by GCMS.

## **2 Materials and Methods**

### **2.1 Plant Material**

Leaves of *M. gale* L. were collected from Rabbit lake, Kenora, Ontario, Canada. The plant specimen was verified as *M. gale* L. with voucher specimen LKHD 004149 at the Claud Garton Herbarium, Lakehead University, Ontario, Canada.

### **2.2 Plant extraction**

The leaves were collected and dried naturally. The material was crushed in a mortar and pestle. The resulting powder was added to sterile glass 2mL autosampler vials at the weight of 0.0072g. The extraction was performed using acetonitrile. A volume of 1000 $\mu$ L of the solvent was added to a sterile 2mL glass autosampler vial containing the ground leaf material. The vial was capped to prevent evaporation. The sample was placed on a heat block at 36°C for a minimum of 24 hours. The remaining solutions were separated by pipette and added to new autosampler vials.

### **2.3 Derivatisation**

100 $\mu$ L of BSTFA with 0.1% TMS (Sigma) was added and the vial was purged with nitrogen gas and capped. After incubation an 800 $\mu$ L volume of the acetonitrile solution from the acetonitrile extract was removed and added to new sterile 2mL glass autosampler vials. An aliquot of 100 $\mu$ L of BSTFA with 0.1% TMS (Sigma) was added to the vials, purged with nitrogen gas and capped.

The sealed vial of extract was placed on the heat block at 121°C for 30 minutes for derivatisation immediately prior to loading in the GCMS.

### **2.4 GCMS analysis**

The GCMS analysis was conducted using helium carrier gas (1.0 mL/min), on a 30m Factor Four capillary column (ID = 0.25mm, VF-5 ms, DF=0.25  $\mu$ m), on a quadrupole gas chromatograph (Varian 450) with mass spectrometer (Varian 300-MS). A splitless mode

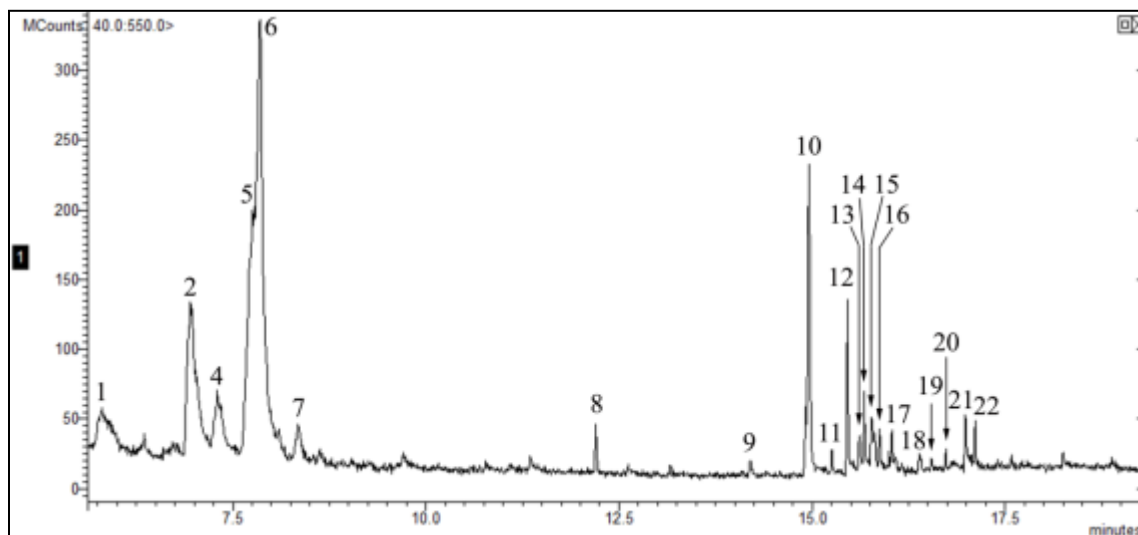
injection (port temperature 270°C) was used and the column temperature was set to 50°C for 2 min then increased to 155°C at a rate of 8°C/min and then increased again to 275°C at a rate 40°C/min. The final temperature was set to 275°C for 9 min and the interface temperature was set to 266°C. A 70 eV ion source was used under electron ionisation (EI) conditions and set to 200°C. The scan range was 40 to 500 m/z and the output files were analysed using Varian MS workstation version 6 (Romaniuk and Matheson *in submission*).

## 2.5 Identification of compounds

Each compound was identified using the chromatogram and ion spectra along with the database of the National Institute of Standards and Technology (NIST). The spectra for each compound identification was analysed manually and compared to reference spectra and samples.

## 3 Results

The most successful extract was acetonitrile (Figure 1). There were 22 chromatographic peaks that were analysed further however there were many smaller peaks that would make up minor constituents in the plant extracts.



**Figure 1.** The chromatogram of the acetonitrile extracts from the leaves of *M. gale* L.

**Table 1.** The Phytochemical compounds identified in the acetonitrile extracts from the leaves of *M. gale* L.

<b>Peak</b>	<b>Ret. time</b>	<b>MW</b>	<b>Chemical compound</b>
<b>1</b>	5.801	136.23	$\alpha$ -pinene
<b>2</b>	6.963	136.23	$\beta$ -pinene
<b>3</b>	7.296	136.23	$\alpha$ -phellandrene
<b>4</b>	7.756	136.23	cis-ocimene
<b>5</b>	7.855	136.23	$\gamma$ -terpinene
<b>6</b>	8.337	136.23	myrcene
<b>7</b>	12.204	92.09	glycerol
<b>8</b>	14.207	204.36	$\alpha$ -muurolene
<b>9</b>	14.922	204.36	caryophyllene
<b>10</b>	14.958	204.36	limonene
<b>11</b>	15.255	204.36	copaene
<b>12</b>	15.453	204.36	unidentified
<b>13</b>	15.616	204.36	unidentified
<b>14</b>	15.673	204.36	bergamotene
<b>15</b>	15.786	204.36	cadinene
<b>16</b>	15.872	204.36	Selin-11-en-4-ol
<b>17</b>	16.034	204.36	$\alpha$ -elemene
<b>18</b>	16.382	204.36	unidentified
<b>19</b>	16.544	362.50	docosadiynedioic acid
<b>20</b>	16.728		unidentified
<b>21</b>	16.983	204.36	selinenol
<b>22</b>	17.111	204.36	unidentified

\*The peak numbers correspond to the peaks in Figure 1.

#### 4 Discussion

Phytochemical analysis of Sweet gale from different locations has been reviewed by Carlton et al (1992) and Svoboda et al (1998). The phytochemical composition of each of these geographically divergent plants varies both in terms of chemical compounds and concentrations. Carlton et al (1992) also demonstrates the change in concentration of these phytochemicals at different sampling times in the year. This study compares *M. gale* L. from Northwestern Ontario with previously published phytochemical constituents found in *M. gale* L. flora elsewhere in the northern hemisphere. The phytochemical analysis of *M. gale* L. from Northwestern Ontario is

consistent with the Ontario, Canada sample reported by Svoboda et al (1998). For example, the absence of germacrone in both the current study, as well as in Svoboda's analysis (Svoboda et al., 1998).

The monoterpene  $\alpha$ -thujene has been reported to vary in different geographical samples and was found in Canadian samples summarised by Carlton et al (1992). However, this current study with specimens from Northwestern, Ontario, along with Svoboda et al (1998), does not show  $\alpha$ -thujene in the Canada samples. As well, the monoterpene, phellandrene is not found in all *M. gale* L. plants and its presence also differs with  $\alpha$ - and  $\beta$ - forms of phellandrene. In the current samples, only  $\alpha$ -phellandrene was identified which is consistent with the review by Carlton et al (1992) that reports only  $\alpha$ -phellandrene present and  $\beta$ -phellandrene absent in other Canadian samples (Svoboda et al., 1998, Carleton et al., 1992). Elemene is reported in very few Sweet gale plants and is absent in other studies on Canadian plants. Interestingly, it was identified in the current Northwestern Ontario samples. Caryophyllene is reported at low concentrations in Canadian samples (Carlton et al 1992, Svoboda et al 1998), but the Northwestern, Ontario samples yielded it in higher concentration.

Many terpenes and terpenoids previously identified in *M. gale* L. from other locations in the northern hemisphere, were not found in the Canadian samples presented in the literature by Carlton et al (1992). As well, these terpenes and terpenoids were not found in the sample analysed from the current Northwestern, Ontario sample which include  $\beta$ -phellandrene, borneol, cis-3-hexanol, citronellol, citronellal, bornyl acetate, guaia-3, 7-diene, eremophilene, humulene, longifolene, aromadendrene,  $\gamma$ -muurolene,  $\epsilon$ -muurolene,  $\epsilon$ -cadinene, nerol, geraniol, calacorene, eudesmol, cadinol, farsenol, valencene,  $\beta$ -elemenone and germacrone. The absence of these compounds can aid chemotaxonomic identification for location specific species of *M. gale* L.

The lack of identification may be due to detection limits and thus may be present in concentrations too small to be detected using the current GCMS methodological approach. There are many terpenes and terpenoids that have been found in all geographical samples reported by Carlton et al (1992) and Svoboda et al (1998) and the current study. These include the compounds limonene, myrcene,  $\alpha$ -pinene,  $\beta$ -pinene. However, there are some reported by Carlton et al (1992) and Svoboda et al (1998) to be found in samples from all geographical locations that were not identified in the current Northwestern, Ontario samples. These include camphene, 1,8-cineol,  $\alpha$ -terpinene,  $\alpha$ -terpineol and terpinen-4-ol. Of these compounds 1,8-cineol and  $\alpha$ -terpineol. Their absence in the current samples may reflect the different extraction

methods, or that the phytochemical variations in flora is possibly due to environmental differences.

There were some compounds identified by Carlton et al (1992) that were found in low (<0.10 percent) concentrations in their Canadian Sweet gale samples. This includes camphene, amyl acetate,  $\alpha$ -p-dimethylstyrene, octyl acetate, benzaldehyde,  $\alpha$ -cubebene, guaia-6,9-diene,  $\delta$ -selinene and benzyl isovalerate. These compounds were not identified in the extracts of *M. gale* L. from Northwestern, Ontario. The results are consistent with the continental trends described by Carlton et al (1992) whereby  $\alpha$ -pinene and 1,8-cineol are major components of the leaf oils in Europe and relatively minor constituents in North American sweet gale, while myrcene and limonene comprise the major constituents in the North American leaf oils (Carleton et al, 1992).

The phytochemical characterisation of the leaves from *M. gale* L. from Northwestern Ontario contributes to our understanding of the geographically driven variation in phytochemical composition of this species. As noted by Svoboda et al (1998) there are differences in concentration and chemical constituents of each *M. gale* L. plant collected from France, Scotland, Finland, Netherlands, Spain and Ontario (Svoboda, 1998). Highlighting Finnish and Scottish samples, the authors found differences of phytochemical constituents within the species. Svoboda et al. notes that it is advisable to study individual flora species because populations are variable, as they have been shown chemically, depending on the location they are found.

*M. gale* L. does not appear to be affected by many diseases (Carlton, 1992), perhaps because of its volatile oils. Svoboda et al (1998) noted that that *M. gale* L. has secretory glands (volatile oil glands) that are responsible for the synthesis and secretion of products (Svoboda, 1998) used for protection. Carlton et al (1992) found that the antifungal activity is due to both monoterpenes and sesquiterpenes, stating that it is unclear why *M. gale* L. focusses terpenes in its leaf glands (Carlton, 1992). The authors propose that one possibility is that it may be due to how the leaf glands rupture and readily secrete the terpene compounds, the monoterpenes evaporating and leaving the strong sesquiterpenes to aid the wounded leaf (Carlton, 1992). These biophysical reactions to herbivory predations and environmental pressures, allow the plant to create compounds that are vital to the survival of the species. It is possible that the different ecological pressures and the associated compounds that are manufactured for survival may be a factor in this differing range of constituents of *M. gale* L. Further research is needed to determine this possibility.

## 5 Conclusion

This phytochemical analysis of the leaves of Sweet gale generated results consistent with other studies of Sweet gale from around the world, and contributes to our understanding of the geographically-based phytochemical differences identified in this species. This analysis differs slightly from other analyses performed on other Canadian materials, suggesting that this variation is not just seasonal and geographically different, but may vary between flora in similar geographic locations. However, this research does not contribute to our understanding as to why *M. gale* L. generates such diversity in the terpenes and terpenoids that are excreted from the leaf glands, which may suggest a greater function than simply defence. As mentioned in the discussion, it is possible that this variance may be due to finely varying difference in environmental pressures.

## Chapter Four

### GCMS analysis of Phytoconstituents of *Asarum canadense* L. Rhizomes collected from Northwestern Ontario, Canada

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#### Abstract

**Introduction** – Many ginger species exist around the world, which are known to be highly aromatic and medicinal. In Canada, a wild variety of ginger (*Asarum canadense* L.) can be found throughout the understory of deciduous forests. Phytochemical analysis of this wild ginger has identified terpenes and terpenoids that have bioactive properties. Studies have also identified the absence of  $\beta$ -asarone a compound found in other flora species of the same genus around the world, and has been found to have carcinogenic activities and hallucinogenic affects in humans.

**Objectives** – To phytochemical characterise the rhizomes of *A. canadense* L. from Northwestern Ontario, Canada, using Gas Chromatography Mass Spectrometry (GCMS).

**Material and Methods** – Phytochemical analysis of the rhizome material was performed by solvent extraction. The phytochemical compounds were identified through GCMS analysis and comparison to a database of known spectra and reference samples.

**Results** – The results were consistent with other studies producing a range of terpenes and terpenoids including camphene, cymene,  $\alpha$ -pinene,  $\beta$ -pinene, sabinene, cyclohexene, 1-methyl-5-(1-methylethenyl)-,  $\beta$ -ocimene, terpinene,  $\alpha$ -terpinolene, limonene, allo-ocimene, linalool, borneol, linalyl acetate, 1-methylene-4-(1-methylethenyl)-cyclohexane, methyl eugenol ether,  $\beta$ -guaiene, selinene,  $\alpha$ -elemene along with several unidentified monoterpenes, sesquiterpenes and terpenoids.

**Conclusion** – The acetonitrile solvent under these extraction parameters was successful in extracting many chemical compounds. The phytochemical characterisation did not identify any asarones, contrary to previous phytochemical research of this genus.

**Keywords:** Phytochemistry, *Asarum canadense* L., Wild ginger, GCMS, terpenes

## 1 Introduction

Terpenes and terpenoids are aromatic hydrocarbons that are commonly found in the plant kingdom. One of the debated functions of these compounds is to provide chemical defences against local pathogens and herbivory (Sultana et al., 2013). Many of these aromatic compounds are bioactive in humans and people have used them for various medicinal applications. In the understory of the Boreal forest in Northwestern Ontario there are numerous flora that use chemical defense mechanisms, such as the strongly scented methyl salicylates of Wintergreen, or limonene of Wild ginger (Motto, 1985, Nikolić, 2013).

Commonly known as Wild ginger, *Asarum canadense* L. is an aromatic plant of which the rhizomes have the distinctive spicy scent of the plant. *A. canadense* L. survives in forest understories, inhabiting north-eastern North America from the Maritimes to the south-west in Missouri (Damman, 1998). *A. canadense* L. has distinct, heart-shaped leaves and purple to maroon flowers that sprout in the early spring (Damman, 1998, Bicknell, 1897). Many of the plants of the *Asarum* genus are considered to have bioactive medicinal properties, being used for treating hearing loss, skin conditions, and gastrointestinal disorders. Specifically, *A. canadense* L. is rendered to Wild ginger oil that is still used today in perfumery (Kopyt'ko, 2013).

Phytochemical studies have revealed that some of the compounds common to this species are aromatic hydrocarbons, phenols and monoterpenes (Kopyt'ko, 2013, Motto, 1985). Some of the compounds reported in phytochemical studies are linalyl acetate, methyl isoeugenol, terpineol,  $\alpha$  and  $\beta$ -pinene, limonene, among other aromatic constituents (Motto, 1985). Many of these compounds have been shown in the research to have bioactive properties such as antibacterial and antimicrobial effects (Rivas da Silva, 2012, Bacanli et al., 2015, Li et al., 2014, Hyldgaard et al., 2015). As well, some of the bioactive compounds identified in some of the species in the genus *Asarum* are carcinogenic, like  $\alpha$  and  $\beta$ -asarone, and are unsafe to use in herbal preparations (Kopyt'ko, 2013). Previous phytochemical analyses of the North American species *A. canadense* L. have been shown to not contain the asarone compounds (Kopyt'ko, 2013, Bicknell, 1897).

This study seeks to provide chemotaxonomic identification and to phytochemically characterise the rhizomes of *A. canadense* L. using GCMS to identify the terpenes and terpenoids which plants utilize for defense in the understory growth environment of deciduous forests.



## **2 Materials and Methods**

### **2.1 Flora Material**

Rhizomes of *A. canadense* L. were collected from Thunder Bay, Ontario, Canada, from within the understory of Sugar Maple (*Acer saccharum* Marshall) trees. The plant specimen was verified as *A. canadense* L. with voucher specimen LKHD 046201 at the Claud Garton Herbarium, Lakehead University, Ontario, Canada.

### **2.2 Flora extraction**

The rhizomes were laid out on sterile parchment paper to dry for 10 days, washed in distilled water, and finely cut. These pieces were crushed in a mortar and pestle in preparation for extraction. Water, ethanol and acetonitrile were used in a simple solvent extraction method employed for this analysis and 1000 $\mu$ L of each respective solvent was added to individual sterile 2mL glass autosampler vials. To ensure no evaporation, the vials were capped. The samples were placed on a heat block at 36°C for a minimum of 24 hours.

### **2.3 Derivatisation**

The samples in the ethanol solvent extractions were desiccated after their incubation. A volume of 1000 $\mu$ L of acetonitrile was added to each autosampler vial containing the desiccated samples. After these samples were re-suspended, 100 $\mu$ L of BSTFA with 0.1% TMS (Sigma) was added and the vials were purged using nitrogen gas and the vials were capped. After the incubation of the samples in the acetonitrile solvent, an 800 $\mu$ L volume of the supernatant was transferred to new sterile 2mL glass autosampler vial. An aliquot of 100 $\mu$ L of BSTFA with 0.1% TMS was added to the vials, they were purged with nitrogen gas and capped. The sealed vials of all of the extracts were placed on the heat block at 121°C for 30 minutes for derivatization immediately prior to loading in the GCMS.

### **2.4 GCMS analysis**

A Varian quadrupole gas chromatograph (model 450) couple mass spectrometer (model 300-MS) was used for this analysis. The samples were injected using a splitless injection mode with a port temperature of 270°C and run with a 30m Factor Four capillary column (ID = 0.25mm, VF-5ms, DF = 0.25 $\mu$ m) using helium as the carrier gas at a flow rate of 1.0mL/min. The column temperature was set to 50°C for the first 2 min then increased at a rate of 8°C/min to 155°C. The temperature was increased from 155°C with a rate of 40°C/min to 275°C and the

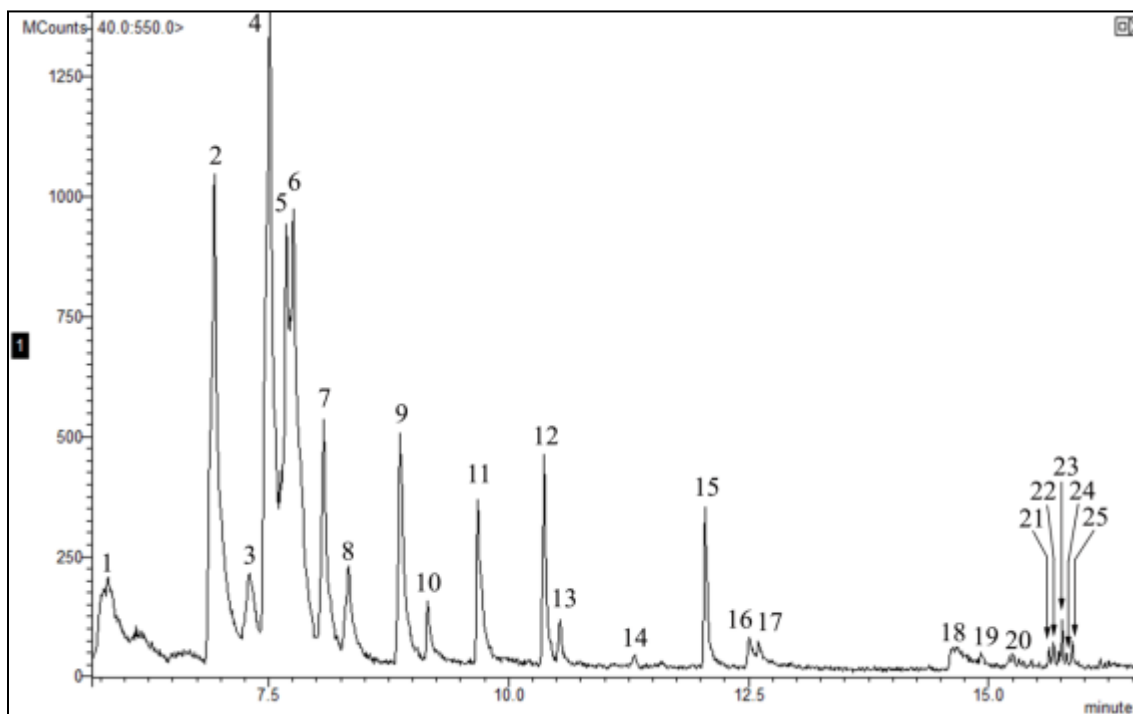
final temperature was 275°C for 9 min and 266°C was used as the interface temperature. The ion source was used under electron ionisation (EI) conditions with 70eV and the temperature was set to 200°C. The spectral scans were performed between 40 to 500 m/z.

## 2.5 Identification of compounds

The identification of the chemical compounds was made by using the output files and comparing them to the NIST98 mass spectral database using the MS workstation version 6 (Varian). The ion spectrum from each chromatographic peak was compared with the spectra from the NIST database and using the ion peaks ( $M^+$ ,  $M+1$ ,  $M-15$  etc.) each database match was then interrogated manually to ensure accurate identification.

## 3 Results

The acetonitrile solvent was successful in extracting many chemical compounds. There were 25 chromatographic peaks that were analysed further, however some of these compounds, specifically the terpenes and terpenoids could not be further identified. Further research will seek to identify them through Nuclear Magnetic Resonance spectroscopy (NMR). There were also several smaller peaks that would constitute the minor components of the flora extract.



**Figure 1.** The chromatogram of the acetonitrile extracts of the rhizomes of *A. canadense* L.

**Table 1.** Indicates the compounds found in the *A. canadense* L. rhizome acetonitrile extract.

Peak	Ret. time	MW	Chemical compound
1	5.836	136.24	$\alpha$ -pinene
2	6.934	136.24	$\beta$ -pinene
3	7.302	136.24	Sabinene
4	7.508	136.23	Camphene
5	7.685	134.21	Cymene
6	7.755	136.23	cyclohexene, 1-methyl-5-(1-methylethenyl)-
7	8.067	136.23	$\beta$ -ocimene
8	8.329	136.23	Terpinene
9	8.874	136.23	$\alpha$ -terpinolene
10	9.151	154.25	Unidentified
11	9.682	136.23	allo-ocimene
12	10.368	154.25	Linalool
13	10.531	154.25	Borneol
14	11.317		Unidentified
15	12.054	136.23	Limonene
16	12.507	196.29	linalyl acetate
17	12.599	136.23	1-methylene-4-(1-methylethenyl)-cyclohexane
18	14.624	178.23	methyl eugenol ether
19	14.922		Unidentified
20	15.248	204.36	$\beta$ -guaiene
21	15.630	204.36	Selinene
22	15.673	204.36	Unidentified sesquiterpene
23	15.765	204.36	$\alpha$ -elemene
24	15.814	204.36	Unidentified sesquiterpene
25	15.871	204.36	Unidentified sesquiterpene

The peak numbers refer to the labelled peaks in Figure 1.

#### 4 Discussion

The acetonitrile extracts were sufficient in extracting many chemical compounds from the *A. canadense* L. rhizomes using the simplest solvent removal method. The data has confirmed that the rhizome of *A. canadense* L. does not contain any asarone compounds, noted to be common to other species of the *Asarum* genus (Kopyt'ko, 2013). The chemical characterisation is consistent with other studies of *A. canadense* L., and include sabinene, camphene, cymene,  $\alpha$ -terpinolene, linalool, borneol, methyl eugenol, linalyl acetate, limonene, and  $\alpha$  and  $\beta$ -pinene (Motto, 1985). Several other compounds, not previously reported in the *A. canadense* L. rhizomes, were found. This includes cyclohexene, 1-methyl-5-(1-methylethenyl)-,  $\beta$ -ocimene, terpinene, allo-ocimene, 1-methylene-4-(1-methylethenyl)-cyclohexane,  $\beta$ -guaiene, selinene,  $\alpha$ -elemene, and several unidentifiable monoterpenes and sesquiterpenes. The current

analysis performed a simple solvent extraction from the rhizomes which would have reliably recovered the major constituents within the plant.

Many of the resulting compounds are known to be bioactive, with some of them having antibacterial and antimicrobial properties effective in protecting *A. canadense* L. living in the Boreal forest understory environment. Research has shown that the bioactive compounds  $\alpha$  and  $\beta$ -pinene have been effective in killing 100% of *Candida albicans* (C.P.Robin) Berkhout within 60 minutes, demonstrating that it is an effective fungicidal agent (Cristina da Rivas et al., 2012). Studies *in vitro* have shown that limonene has protective as well as antioxidant effects (Bacanli, 2015).

## **5 Conclusion**

This research has confirmed that the North American species of *Asarum*, *A. canadense* L., does not contain the carcinogenic compound asarone, as well as confirming the literature review of the phytochemical constituents of *A. canadense* L. This highly aromatic rhizome is composed of constituents that are volatile, and the distinct scent of the rhizome can be attributed to constituents such as limonene and methyl isoeugenol. Interestingly, this extraction and analysis revealed other compounds not previously reported. The compounds the plant manufactures also allow the species to defend itself biochemically by inhibiting bacterial and fungal growth. With further research, it may be possible that one could identify common constituents found in *A. canadense* L. and provide reliable chemotaxonomic identification for the species habitat and geographical location.



## Chapter Five

### 5.0 Conclusion

The analysis of these selected flora from Northwestern, Ontario has shown multiple terpene (monoterpene and sesquiterpene) and terpenoid compounds that can now be used to aid chemotaxonomic identification of flora species, and to report medicinal plant compounds indigenous to Canada. For these species, particularly *A. calamus* var. *americanus* (Raf.) H. Wulff, chemotaxonomic identification is important due to the current distribution of the plants and the physiological similarity of the species and varieties of *Acorus*. Current distribution of *Acorus* plants in North America makes it challenging to identify geographic variants that may have been introduced to the continent. Chemotaxonomic identification may resolve this problem.

The phytochemical composition of *A. calamus* var. *americanus* (Raf.) H. Wulff rhizomes, reported in Chapter 2, has been detected by GCMS analysis of this species variation. Unlike other species of *Acorus*, it does not contain the carcinogenic asarone compounds in the rhizomes. This bioactive chemical compound is characteristic of the triploid *A. calamus* L., and has been previously mistaken to be the main constituent of the Sweet flag species. The reported absence of this compound also aids in the accurate identification of plants of the *Acorus* genus, which has been shown to be a challenge in the literature with regular interchanging of the synonymous naming of *A. calamus* L. and *A. calamus* var. *americanus* (Raf.) H. Wulff (Geigar & Banker 2012). According to the ethnographic literature, *A. calamus* var. *americanus* (Raf.) H. Wulff is used in a similar way as Sweet flag is used in other parts of the world (Hoffman, et al., 2001).

Chapter 3 focusses on the phytochemical investigation of leaf extracts from *Myrica gale* L. found in Northwestern, Ontario, Canada. This is the first report of the phytochemical composition of the *M. gale* L. plants from Northwestern, Ontario. The results are consistent with previous phytochemical studies performed on samples from elsewhere in the Northern hemisphere. However, these compounds vary in composition, possibly due to differing geographical regions from which the specimens were collected. *M. gale* L. is noted to have secretory glands that release volatile compounds when the plant is affected by an herbivore predator or pathogen (Svoboda et al., 1998).

It is possible that the different ecological pressures (herbivory) in each habitat affect the associated compounds. The chemical concentration among the species of *M. gale* L. varies between countries, as stated by Svoboda et al (1998). The compounds that are manufactured and

expressed by the plant, due to the environmental pressures, may contribute to the differences in composition of *M. gale* L. from different geographic locations. This study yielded results consistent with the literature indicating that *M. gale* L. has many aromatic compounds like monoterpenes and sesquiterpenes and these volatile compounds have been shown to be used by humans in both traditional and modern medicine.

Chapter 4 describes the phytochemical compounds of the rhizomes of *A. canadense* L. While this genus is also known to have the carcinogen  $\beta$ -asarone, it is absent in this North American species found in the understory of deciduous forests. This is the first report of the phytochemical composition of the *A. canadense* L. plants from Northwestern Ontario and the results are consistent with previous phytochemical studies performed on samples from elsewhere. Reported aromatic compounds of *A. canadense* L., such as limonene and pinene, which are known to be bioactive constituents common to *Acorus* and *Asarum* species, were identified. These compounds are known to have antimicrobial, anxiolytic and antifungal bioactive properties (Lima et al, 2013). These compounds are thought to be a form of defence for the plants by emitting volatile compounds (HI-VOC's) in response to invasion by herbivores, microbials or invasive fungi (Heil, 2014).

Flora local to Northwestern Ontario that have bitter and aromatic qualities, also have GCMS detectable quantities of bioactive compounds. These bioactive compounds are manufactured by plants and it has been suggested that they are used as a defence mechanism against herbivory, and in comparison, the flora can be used by people as medicines of a similar function. Beneficially, the North American varieties of the medicinal plants of the genus *Acorus* and *Asarum* are absent in  $\beta$ -asarone. This raises the question of why the species of the *Acorus* and *Asarum* genus located in this region of the world do not have this compound.

As addressed in Chapter 3, *M. gale* L. found in geographic regions exhibit different chemical compositions. Further research regarding genetic and ecological investigations are needed to fully understand why these plants have unique compounds in the Canadian Boreal forest in comparison to the same genus' found across the world. After the analyses, we see that the compounds can vary within a genus, as with *Acorus* and *Asarum*, and vary in concentration between habitats and geographic regions. Therefore, it is important to investigate chemotaxonomic placement of plants coupled with botanical physiological observation so that one identifies flora correctly to take advantage of bioactive or medicinal compounds for future investigation and medicinal use.

## 5.1 Future Considerations

Further research in regards to the main active constituent of *Acorus calamus* var. *americanus* (Raf.) H. Wulff would be beneficial as this plant has a variety of uses that are highlighted in most ethnographic literature. The plant is used similarly around the world, though the constituent that is mostly attributed to many bioactive medicinal actions is the compound  $\beta$ -asarone. Further medical studies are needed to identify the main active constituent of *A. calamus americanus* (Raf.) H. Wulff. Also, further research regarding genetic and ecological investigations are needed to fully understand why flora have unique compounds in the Canadian Boreal forest in comparison to the same genus' found across the world. Finally, continued research is necessary to determine the different ecological pressures (herbivory) and the associated compounds that are manufactured for survival and expressed by the plant may be a factor in this differing range of constituents of flora. There may be other sets of compounds that were unable to be detected using the current technique. Further research will aim to identify compounds using other methods, such as Nuclear Magnetic Resonance spectroscopy (NMR).



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