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Key Odorants of *Pycnanthemum incanum* and Stereochemistry of Chiral Compounds

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Key Odorants of *Pycnanthemum incanum* and Stereochemistry of Chiral Compounds

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Melissa Marie Dein

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ABSTRACT

Pycnanthemum, a genus in the Lamiaceae family, is comprised of a diverse group of aromatic plants commonly known as wild mountain mint. Due in part to their high terpene content, *Pycnanthemum* species have broad potential for health-promoting, cosmetic, culinary, and food flavoring applications. However, to fully understand the scope of applications and benefits of the different members of the *Pycnanthemum* genus a deeper understanding of the chemical composition of the plants is needed. To gain insight into the odorants driving the characteristic aroma of *Pycnanthemum spp.*, a species with a pungent mintlike odor, *Pycnanthemum incanum*, was selected for comprehensive odorant characterization. Odorants present in *P. incanum* were identified by coupling solvent assisted flavor evaporation (SAFE) and aroma extract dilution analysis (AEDA), which afforded 24 odorants including 14 odorants with flavor-dilution (FD) factors ≥ 4 . Selected odorants were quantitated by stable isotope dilution assays (SIDAs), and odor-activity values (OAVs) were calculated. The odorants with the highest OAVs included β -ionone (floral, violet; OAV 300), myrcene (terpeny, OAV 120), linalool (floral, citrus; OAV 79), and pulegone (mint, medicinal; OAV 58). In addition, an odor recombination model was developed based on the quantitative data and sensorially compared to the original plant odor. Descriptive analysis confirmed that the sensory attributes of the odor recombination model showed no differences from those of the original *P. incanum* plant material. Lastly, enantiomeric proportions of chiral odorants in *P. incanum* were determined by chiral

chromatography and the results revealed that the enantiomeric ratios were unique; however, comparable to those reported in other plant species. The characterization of key odorants contributing to the odor of *Pycnanthemum incanum* provided valuable insight into the chemistry of this underutilized plant and provides a foundation for future studies aimed at the characterization of other species in the *Pycnanthemum* genus.

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1 INTRODUCTION

Pycnanthemum incanum (L) Michx. (Figure 1) is a species of wild mountain mint endemic to North America commonly known as hoary mountain mint. *Pycnanthemum incanum* plants have been identified in wild populations throughout the Eastern United States in diverse habitats ranging from lush mountain tops and river beds to rocky embankments and roadsides. The tolerance of the species to varying environments indicates promising cultivation potential. Likewise, *P. incanum* is a highly aromatic plant and its unique odor suggests vast herbal, culinary, or essential oil applications. Regardless of its potential, minimal research has been focused on this underutilized plant and therefore an understating of the chemistry driving the plant odor is currently lacking.



Figure 1: Flowering *Pycnanthemum* sp.

The first objective of this study was to comprehensively identify the key odorants present in the vegetative aerial parts of *P. incanum*. This was achieved by employing solvent assisted flavor evaporation (SAFE) followed by aroma extract dilution analysis (AEDA). By using SAFE, a high vacuum distillation technique frequently used for flavor analysis, odorants were gently isolated from organic extracts of plant material and separated from non-volatile components. AEDA was utilized to distinguish the volatile compounds in the isolates that were odor-active and the relative importance of each odorant to the overall odor of the *P. incanum* plant. This analysis involved preparing serial dilutions of the volatile isolate and analyzing each dilution by gas chromatography-olfactometry (GC-O) until odors were no longer perceivable in the diluted volatile isolates.

The second objective was to employ stable isotope dilution assays (SIDAs) to quantitate the most impactful odorants as determined by AEDA and calculate odor activity values (OAV) for the odorants. To accurately quantitate the odorants, deuterium or carbon 13 labeled isotopes analogous to each odorant were used as internal standards for the SIDAs. A known concentration of isotope was incorporated with the analyte prior to extraction, distillation, and analysis. After analysis by gas chromatography-mass spectrometry (GC-MS) the concentration of each analyte in the original sample was calculated using ion ratios between the isotopic internal standard and the analyte.

The third objective was to construct an odor recombination model based on the quantitative data and sensorially compare the model to the original plant material. Free choice profiling was used to determine odor descriptors that were relevant to consider for sensory analysis of the *P. incanum* odor. Quantitative olfactory profile analysis was used to compare the intensity of key

odor descriptors between the odor recombination model and the original plant odor, thus validating the quantitative results.

The final objective was to determine enantiomeric proportions of key chiral odorants in *P. incanum* by means of chiral gas chromatography-mass spectrometry. Enantiomeric ratios were compared to that of other plant species to provide insight about biosynthetic pathways that play a role in generating the odorants in the *P. incanum* plant.

This work provided the first comprehensive evaluation of odor-active compounds present in *Pycnanthemum incanum*, as well as the genus as a whole. Thus, this study laid the groundwork for future studies of other *Pycnanthemum* species and deepened the understanding of biosynthesis of odorants in the genus. As a result, this new knowledge has opened the door for future investigations aimed at characterizing the odorant variability in wild *P. incanum* populations as well as efforts for genetic improvement of the plant for commercial applications.

2 LITERATURE REVIEW

2.1. Botany

Pycnanthemum, commonly referred to as mountain mint, is a genus of approximately twenty different plant species in the mint family, Lamiaceae (Table 1). Early explorers began classifying mountain mints, however, the taxonomic classifications of the *Pycnanthemum* genus know today was set forth by Boomhour¹ (1941), Grant & Epling² (1943) and Chambers (1961).³ The *Pycnanthemum* genus is divided into two primary groups, the Incanum Group and the Virginianum Group, with some additional subgroups. The group of interest for this study, the Incanum Group, is comprised of seven species including *P. albescens*, *P. clinopodioides*, *P. curvipes*, *P. floridanum*, *P. incanum*, *P. pycnanthemoides* (Figure 2) and *P. loomisii* (Figure 3).

The species in the incanum group are distinguished by similar morphological characteristics including broad leaves, small flower clusters and a bilabiate calyx with three teeth in the upper lip and two teeth in the lower lip.⁵ However, it is difficult to decipher between species in the Incanum Group strictly based on morphological characteristics and the morphological evaluation done by Boomhour indicated the existence of interspecific hybrids may contribute to this difficulty.⁶

The presence of natural hybrids of *Pycnanthemum* in sympatric populations where different species occur in the same geographic area was confirmed by Chambers and Chambers, 1971. Four of the six populations observed were comprised of species from the Virginianum Group and the other two populations had natural hybrids of *P. albescens* and *P. loomisii*; and *P. clinopodioides* and *P. incanum*.⁷ Species of the Incanum Group are out-crossing with increased seed-set upon out crossing. This is due in part to these sexual species being protandrous so the anther of a flower

Table 1. Species of the *Pycnanthemum* genus.⁴

Pycnanthemum Species	Common name
<i>Pycnanthemum albescens</i> Torr. & A. Gray	whiteleaf mountain mint
<i>Pycnanthemum beadle</i> (Small) Fernald	Beadle's mountain mint
<i>Pycnanthemum californicum</i> Torr. ex Durand	Sierra mint
<i>Pycnanthemum clinopodioides</i> Torr. & A. Gray	basil mountain mint
<i>Pycnanthemum curvipes</i> (Greene) E. Grant & Epling	stone mountain mint
<i>Pycnanthemum flexuosum</i> (Walter) Britton, Sterns & Poggenb.	Appalachian mountain mint
<i>Pycnanthemum floridanum</i> E. Grant & Epling	Florida mountain mint
<i>Pycnanthemum incanum</i> (L.) Michx.	hoary mountain mint
<i>Pycnanthemum loomisii</i> Nutt.	Loomis' mountain mint
<i>Pycnanthemum monotrichum</i> Fernald	onehair mountain mint
<i>Pycnanthemum montanum</i> Michx.	thinleaf mountain mint
<i>Pycnanthemum muticum</i> (Michx.) Pers.	clustered mountain mint
<i>Pycnanthemum nudum</i> Nutt.	coastal plain mountain mint
<i>Pycnanthemum pycnanthemoides</i> (Leavenworth) Fernald	southern mountain mint
<i>Pycnanthemum setosum</i> Nutt.	awned mountain mint
<i>Pycnanthemum tenuifolium</i> Schrad.	narrowleaf mountain mint
<i>Pycnanthemum torrei</i> Benth.	Torrey's mountain mint
<i>Pycnanthemum verticillatum</i> (Michx.) Pers.	whorled mountain mint
<i>Pycnanthemum pilosum</i> Nutt.	whorled mountain mint
<i>Pycnanthemum virginianum</i> (L.) T. Dur. & B.D. Jacks. ex B.L. Rob. & Fernald	Virginia mountain mint



Figure 2: *Pycnanthemum pycnanthemoides* N. Car. (PI 619301) (CPYC 55.000 SD), specimen acquired from the USDA.



Figure 3: *Pycnanthemum loomisii* 1662-3 (PI 619280) (CPYC 28.001 PL), specimen acquired from the USDA.

releases its pollen before the stigma of the same flower has reached maturity. However, these species are also considered self-compatible due to the limited self-pollination observed.⁶

It was determined experimentally that interspecific hybrids between multiple species of the Incanum Group were possible but F₁ hybrids were only viable between crosses of *P. loomisii* and *P. albescens*; and *P. pycnanthemoides* and *P. incanum*. Different chromosome numbers between species in the Incanum Group limit the possibility for viable hybrids but this does not negate the possibility for natural hybrids since crosses of diploids and tetraploid species have been shown to produce sterile triploid species that could survive in nature and spread vegetatively.⁷

Cytological evaluation can aid in deciphering between species that are morphologically similar or intermediate between two species. However, species in the same primary group of the genus do not necessarily have the same base number; both diploid and tetraploid species exist in the Virginianum and Incanum Groups. For example, in the Incanum group *P. albescens*, *P. curvipes* and *P. loomisii*, are diploid whereas *P. albescens*, *P. clinopodioides*, *P. floridanum*, *P. incanum*, and *P. pycnanthemoides* are tetraploid. Artificial hybridization studies indicate that polyploid *Pycnanthemum* species evolved from the hybridization of different groups.⁸ Furthermore, cytology is influential in distinguishing species. For example, *P. loomisii* and *P. pycnanthemoides* are very difficult to separate morphologically but can be distinguished cytologically due to *P. loomisii* being a diploid species as compared to *P. pycnanthemoides* which is a tetraploid species.⁵ Another technique has shown some promise to aid in species determination, which involved determining the flavonoid content of *Pycnanthemum* spp. Using paper chromatography, Carr and Hunter were able to detect twenty-five flavonoids, primarily flavones and flavonols, present in methanolic leaf extracts of multiple species of *Pycnanthemum*. Though individual *Pycnanthemum* species and intraspecific hybrids could be characterized by their

flavonoid patterns, the technique was not adequate to determine parent species of intraspecific hybrids.⁹

2.2. Geographic Distribution and Habitat

Native species of *Pycnanthemum* have been identified throughout eastern North America, with some species reported as far west as California (Figure 4).⁶ At least sixteen different *Pycnanthemum* species have been identified in the southeastern portion of the United States, ranging from Tennessee and Virginia, south to Florida, and west through Mississippi, hence the classification of this Southeastern region of the U.S. as the center of diversity for the *Pycnanthemum* genus.⁸

Pycnanthemum species thrive in a variety of habitats ranging from highly disrupted areas such as road sides, rocky embankments, and forest edges to more remote undisturbed areas including mountain tops, open balds, and riparian zones. In natural habitats, *Pycnanthemum spp.* can grow to a height of 3 to 4 feet and are characterized by long woody square stems with minimal to moderate pubescence and leaf shapes ranging from ovate to lanceolate with toothed margins. Flowering between July and September, *Pycnanthemum spp.* can be easily identified by the whitened appearance of the leaf surfaces adjacent to terminal flower clusters with small purple spotted flowers (Figure 5).³

2.3. History and Medicinal Applications

Historically, Native American communities used *Pycnanthemum* plants for medicinal applications. A tea prepared from the leaves was taken as a stomach tonic to relieve colic, induce perspiration, and reduce fevers. The leaves were also prepared as poultices and applied topically to reduce pain from headaches and inflammation.^{11,12} However, beyond traditional Native

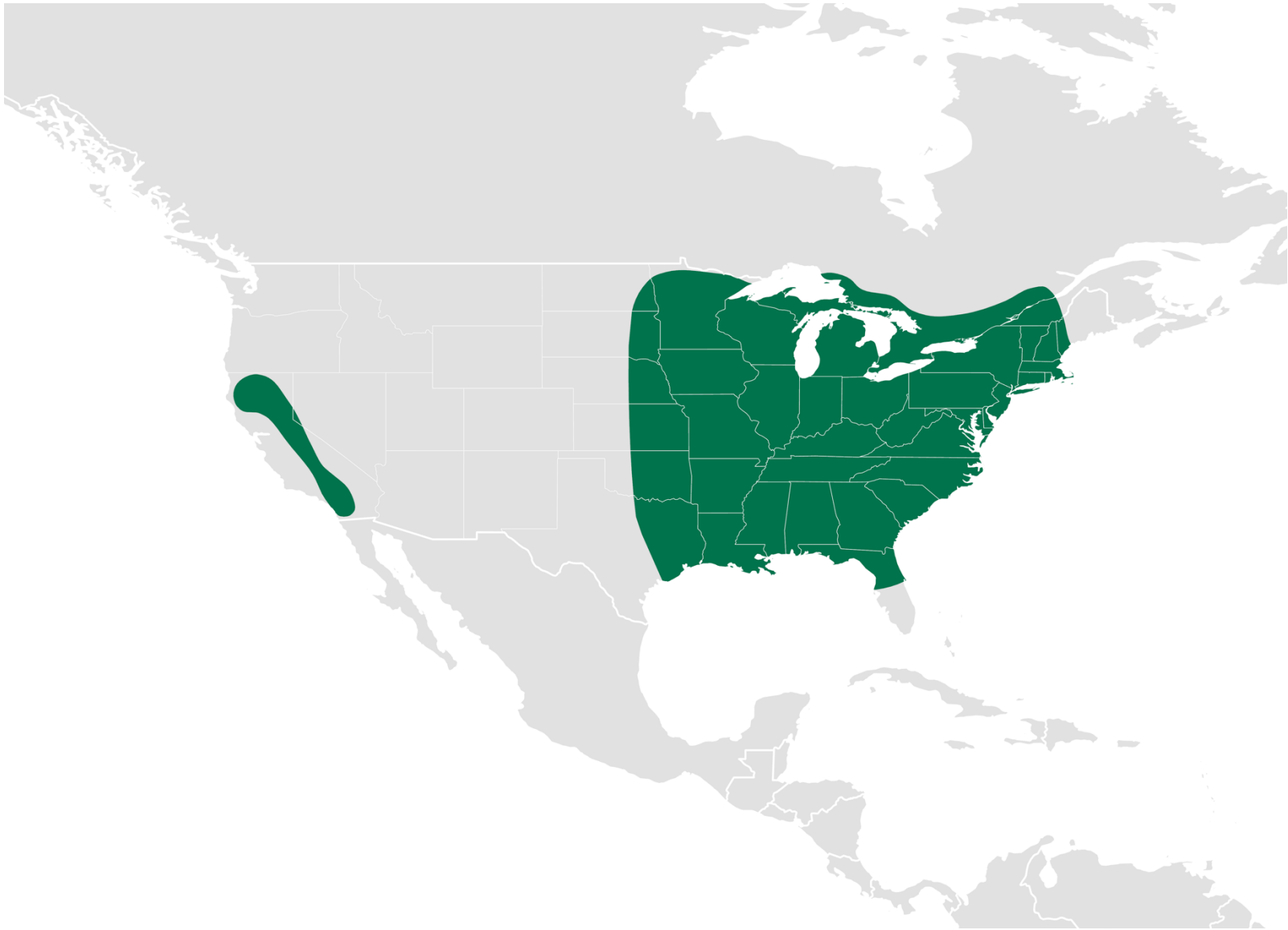


Figure 4: Geographic distribution of *Pycnanthemum* species in the United States.¹⁰



Figure 5. Flowering *Pycnanthemum* sp. displaying whitened leaves surrounding flower clusters.

American communities, the broad potential for medicinal, herbal (essential oil), and culinary applications of these plant species have not yet been explored, likely in part due to the lack of research surrounding the *Pycnanthemum* species.

2.1. Chemistry and Bioactivity

While extensive studies have established the taxonomy of plants in the *Pycnanthemum* genus, comparatively little research has explored the odor and chemical composition of *Pycnanthemum* species. One of the first evaluations of oils generated from *Pycnanthemum pilosum* was described as indicating the presence of pulegone, based on odor quality.¹³ Upon further evaluation, the presence of other volatiles, namely menthone and limonene, in *P. pilosum* oils were also identified.¹⁴ To date the most extensive evaluation of *Pycnanthemum* volatile oils was carried out by Shu and Lawrence in which Likens-Nickersons distillation was employed to isolate 40 volatiles from 3 varieties of *P. floridanum*.¹⁵ Although preliminary work elucidated some components in *Pycnanthemum* oils, to our knowledge there has been neither research assessing which volatiles are odor-active nor any comprehensive quantitation of odorants in any *Pycnanthemum* species.

The pungent odor of *Pycnanthemum spp.* is a distinguishable feature of plants in the genus, as is common for other genera in the Lamiaceae family. The unmistakable odor has aided in identification of *Pycnanthemum* species in the field, however, there has been some indication that diverse odor quality has been observed within the *Pycnanthemum* genus. For example, Sorensen and Matekaitis proposed an interspecific taxa, *Pycnanthemum virginianum* f. *citriodora*, due to a population of *P. virginianum* with a distinct lemon, citrus odor comparable to that of *Melissa officinalis* and *Monarda citriodora*.¹⁶ Regardless of odor diversity, a void in understanding

surrounding the chemistry driving the plant odor exists due to the lack of chemical characterization of the odorants produced by the plants.

In addition to the minimal understanding of *Pycnanthemum* chemistry, few studies have focused on the bioactivity of *Pycnanthemus spp.* There has been indication of antimicrobial activity of *Pycnanthemum tenuifolium* (narrow leaf mountain mint). In this study, ethanolic extracts of *P. tenuifolium* produced an 8mm zone of inhibition against *Staphylococcus aureus* in a disc diffusion assay.¹⁷ Likewise, extracts of roots and aerial plant parts of *P. flexuosum* have also been shown to inhibit hyaluronidase activity.¹⁸ Though little bioactivity has been reported for *Pycnanthmeum spp.*, the bioactivity of species in the *Mentha* genus, another genus of the Lamiaceae family, has been well documented. Specifically, peppermint tea and oil (*Mentha piperita* L.) have been reported to exhibit antioxidant, antitumor, antiviral, antifungal and antibacterial activity.¹⁹ Therefore, the broad spectrum of bioactivities in related genera suggests potential for *Pycnanthemum spp.* to exhibit similar properties if evaluated.

A concern surrounding food and cosmetic application of *Pycnanthemum spp.* and species with similar chemical profiles is toxicity. A single study on the toxicity of *P. albescens* oils on male Wistar rats determined an LD₅₀ of 3147 ± 362 mg/Kg after 48 hours as compared to peppermint oil with an LD₅₀ of 2426 ± 329 mg/Kg.²⁰ More recent studies have also focused on the acute toxicity of individual chemical compounds commonly found in mint species. In a study where rats were fed 200 to 800 mg/ Kg of menthone for 28 days, increased weight in the spleen and liver, as well as visual changes to the white mater of the rat's brains were observed.²¹ Likewise, a study by Thorup et al. 1983 evaluated the effects of administering menthol and pulegone to rats by gavage for 28 days. At doses of 200 to 800 mg/ Kg of menthol, again an increase in liver weight was observed.²² For the rats administered pulegone (80 to 160 mg/Kg), overall body weight

decreased, the level of creatine in the blood decreased and atonia was observed. Though toxicity warrants consideration when postulating food and cosmetic applications of *Pycnanthemum spp.*, dose is a critical factor to consider, as low levels of the aforementioned volatiles are present in many generally regarded as safe (GRAS) foods and flavorings. Also, at the present, the chemical composition of different species of *Pycnanthemum* is not well understood.

3 MATERIAL AND METHODS

The primary goal of this work was to characterize the key odorants contributing to the odor of *Pycnanthemum incanum* (CPYC 100.000, USDA accession PI 619340) and gain insight about the chemical composition of the species. In order to achieve this goal a combination of analytical and sensory techniques were employed (Figure 6). Prepared plant parts were extracted with organic solvent and an established distillation technique, SAFE, was used to isolate volatile compounds from non-volatile compounds. The volatile isolate was fractionated with solid phase extraction (SPE) to separate classes of compounds to aid in identification of odorants and to simplify chiral chromatography. AEDA of the SAFE isolate was employed to identify which volatile compounds were odor active and SIDA was used to quantitate key odor-active compounds. In parallel, free choice profiling resulted in a lexicon of descriptors to describe the plant odor. In addition, quantitative olfactory profile analysis was employed to verify that an odor recombination model could be prepared using the quantitative data that accurately represented the plant odor.

3.1. Plant Material

Pycnanthemum incanum seeds (CPYC 100.000, accession PI 619340) were acquired from the USDA National Germplasm Repository (Corvallis, OR) and grown on site in a climate-controlled Venlo style greenhouse (945 ft²). Supplemental lighting ensured 12 hours of light per day and the air temperature was maintained at 25 °C ± 3 °C. The seeds were started in professional grade seed starting mix in seed trays with clear moisture retaining covers (Figure 7). After germination (7 to 14 days) the seedlings were transplanted into 3-inch pots in Pro-Mix (Premier Horticulture Inc., Quakertown, PA) and placed on propagation trays (18 pots/tray). When fully established, the seedlings were again transplanted into 1-gallon nursery pots (Figure 8). During vegetative growth the plants were irrigated daily for 15 minutes at a rate of 10 mL/minute and

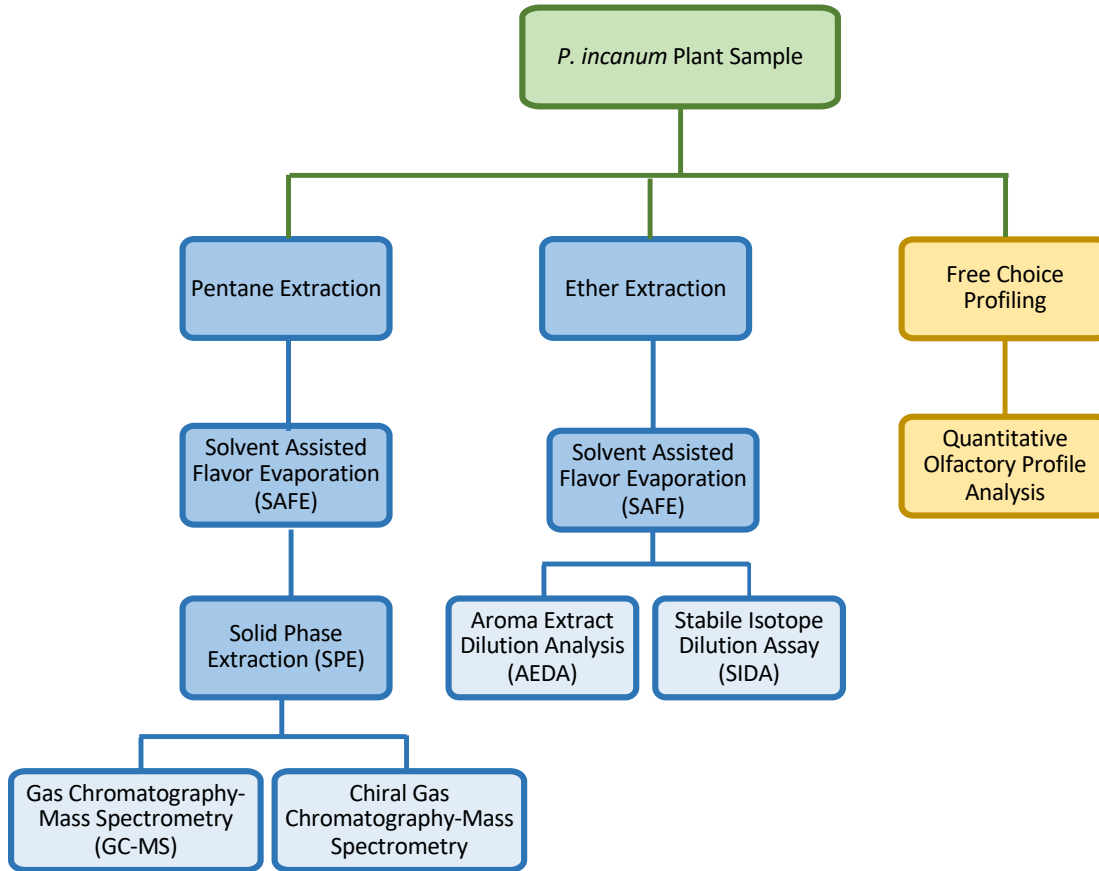


Figure 6. Flavor Analysis Flow Chart.



Figure 7. *P. incanum* seedlings grown on seed trays.



Figure 8. Full grown *P. incanum* seedling in 1-gallon pot.

fertilized biweekly with 20-20-20 water soluble fertilizer (Southern Ag, Rubonia, FL). Leaves and stems of mature plants (~18 inches tall) were harvested prior to flowering and air dried for a minimum of 7 days at 25 °C. The dried plant material was stored in air tight glass containers with Drierite™ (Fisher Scientific, Waltham, MA) until further analysis.

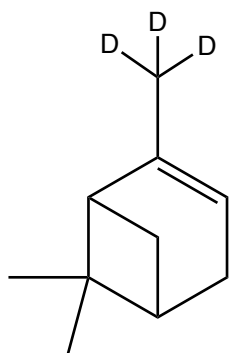
3.2. Reference Odorants and Other Chemicals

The following odorants were sourced from commercial suppliers as indicated: **1 – 4, 6 – 17, 19, 20, 23, 24** (MilliporeSigma, St. Louis, MO); **5** (Santa Cruz Biotechnology, inc., Dallas, TX); and **18** (Enamine LLC, Monmouth Jct., NJ). In addition, odorants used for quantitative olfactory profile analysis, including dihydrocarvone and (Z)-hex-3-en-1-ol, were also purchased from MilliporeSigma (St. Louis, MO).

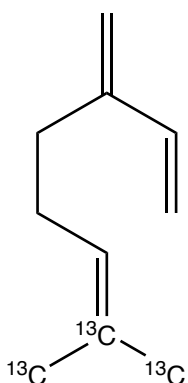
Unstabilized diethyl ether was acquired from Honeywell Burdick & Jackson, and freshly distilled prior to use. Anhydrous sodium sulfate was purchased from Fisher Scientific (Waltham, MA). A mixture of n-alkanes (C9 – C18) was obtained from Phenomenex (Torrance, CA) and n-alkanes (C19 – C26) were individually obtained from MilliporeSigma (St. Louis, MO).

3.3. Isotopically Labelled Odorants

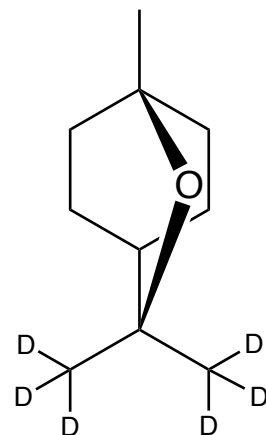
Labelled isotopes including (²H₃)-**1**, (¹³C₃)-**2**, (²H₆)-**3**, (²H₃)-**6**, (²H₈)-**7**, (²H₈)-**13**, (²H₃)-**14**, (²H₂)-**16**, (²H₂)-**17**, (²H₃)-**20** and (²H₃)-**23** were purchased from aromaLAB (Planegg, Germany). Additionally, (²H₃)-**10** was acquired from C/D/N Isotopes Inc. (Quebec, Canada). All isotopically labelled odorants were individually prepared in freshly distilled pentane at known concentrations or quantitated by GC-FID using external calibration curves generated from isotopically unmodified analytical standards. The structures of deuterium and ¹³C labelled isotopes are provided in Figure 9.



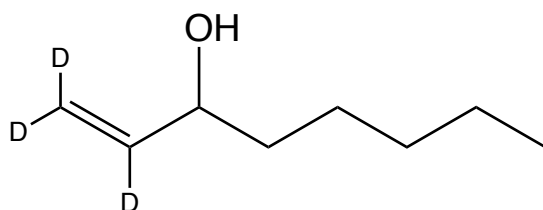
α-pinene-d3



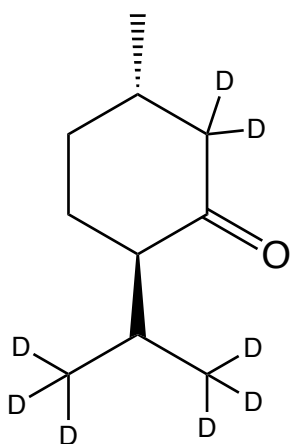
myrcene-¹³C3



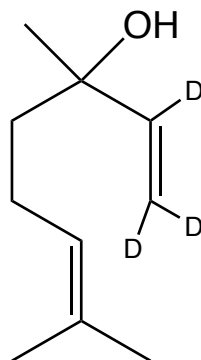
1,8-cineole-d6



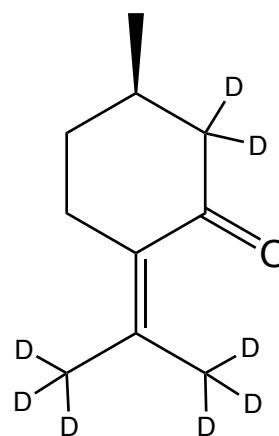
1-octen-3-ol-d3



menthone-d8

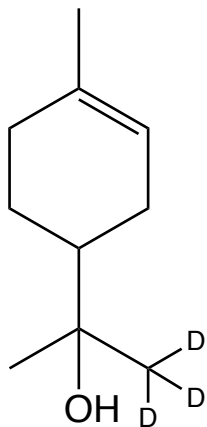


linalool-d3

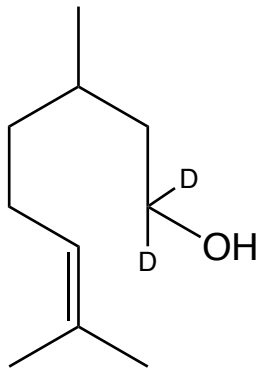


pulegone-d8

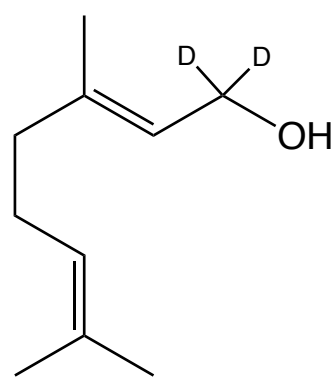
Figure 9a: Isotopically labelled compounds.



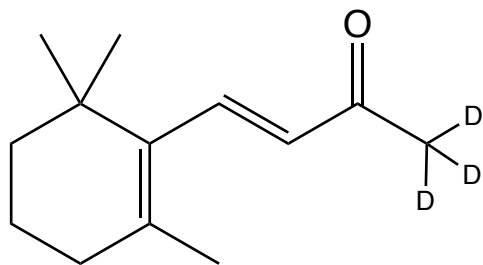
α -terpineol-d3



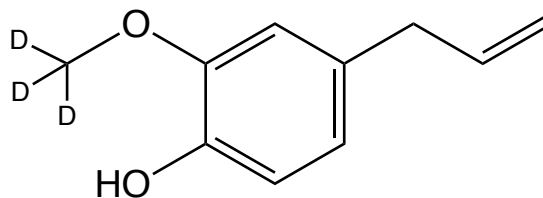
β -citronellol-d2



geraniol-d2



β -ionone-d3



eugenol-d3

Figure 9b: Isotopically labelled compounds continued.

3.4. Identification of Key Odorants of *Pycnanthemum incanum*

3.4.1. Extraction of Volatile Compounds

Dried leaves and stems of the *P. incanum* plants were ground to a fine powder using a mortar and pestle and the plant material (200 mg) was measured into a 250 mL centrifuge tube. Freshly distilled diethyl ether (25 mL) was added to the centrifuge tube and manually shaken for 5 minutes to extract the volatiles. The extract was centrifuged at 4500 rpm for 10 minutes and the organic phase decanted. A second extraction of the residual plant material was carried out in the same manner. The organic extracts were combined and submitted to solvent assisted flavor evaporation (SAFE) (Figure 10).

3.4.2. Solvent Assisted Flavor Evaporation (SAFE)

The gentle distillation of volatiles under a high vacuum distillation system thermostated at 41 °C resulted in the volatile fraction in diethyl ether. The volatile isolate was dried over anhydrous sodium sulfate, and excess solvent removed at 43 °C using a Vigreux column. The isolate was first concentrated to approximately 2 mL on the Vigreux column and then concentrated under a gentle stream of nitrogen to a final volume of ~200 µL.

3.4.3. Aroma Extract Dilution Analysis (AEDA)

Determining the odorants that contribute to the plant odor was achieved through aroma extract dilution analysis (AEDA). Serial dilutions of the SAFE isolate were prepared, resulting in 11 samples. These samples ranged from flavor dilution (FD) 1 (the concentrated SAFE isolate), to FD 1024 (the most dilute sample). Each of the samples (FD 1 to FD 1024) were analyzed in succession by gas chromatography-olfactometry. For each odor, the odor quality and lowest dilution at which the odor was perceivable, were determined.



Figure 10: Solvent Assisted Flavor Evaporation Apparatus.

3.4.4. Gas Chromatography – Olfactometry (GC-O)

An Agilent 7820A GC system (Agilent Technologies, Santa Clara, CA) was affixed with a Zebron™ ZB-FFAP GC capillary column (30 m x 0.32 mm OD x 0.25 µm film thickness) acquired from Phenomenex (Torrance, CA). Helium was used as a carrier gas with a flow rate of 1.5 mL/minute and volatile isolates (1 µL) were injected on column at 35 °C. This temperature was held for 1 minute before increasing the oven to 60 °C at a rate of 60 °C/minute. Then a second ramp to 240 °C at a rate of 6 °C/minute was followed by a final hold time of 10 minutes at 240 °C. After chromatographic separation by the capillary column, the effluent was divided by a glass Y splitter. Equal portions of the effluent were channeled through two deactivated fused silica columns (2.5 m x 0.20 mm OD). One channel delivered the effluent to a sniffing port equipped with a nose cone heated to 250°C, while the remaining effluent was channeled to a flame ionization detector (FID) connected to a chart recorder. The FID was maintained at 250 °C with air, hydrogen and makeup flow rates of 450 mL/minute, 40 mL/minute, and 45 mL/minute, respectively. As an odor was perceived by the panelist at the sniffing port, the corresponding retention time and odor character were simultaneously noted on the chart recorder. A schematic of the GC-O instrument is provided in Figure 11.

3.4.5. Gas Chromatography – Mass Spectrometry (GC-MS)

Volatile isolates were analyzed by an Agilent Technologies 78204 GC system (Santa Clara, CA) coupled to an Agilent Technologies 5977B mass spectrometry detector (Santa Clara, CA). The GC column employed was a Zebron™ ZB-FFAP GC capillary column (30 m x 0.25 mm OD x 0.25 µm film thickness) from Phenomenex (Torrance, CA) with helium as a carrier gas (1 mL/minute). Volatile isolates (1 µL) were injected cold on column and the initial temperature of 35 °C was held for 1 minute. The oven temperature was then increased to 60 °C at a rate of

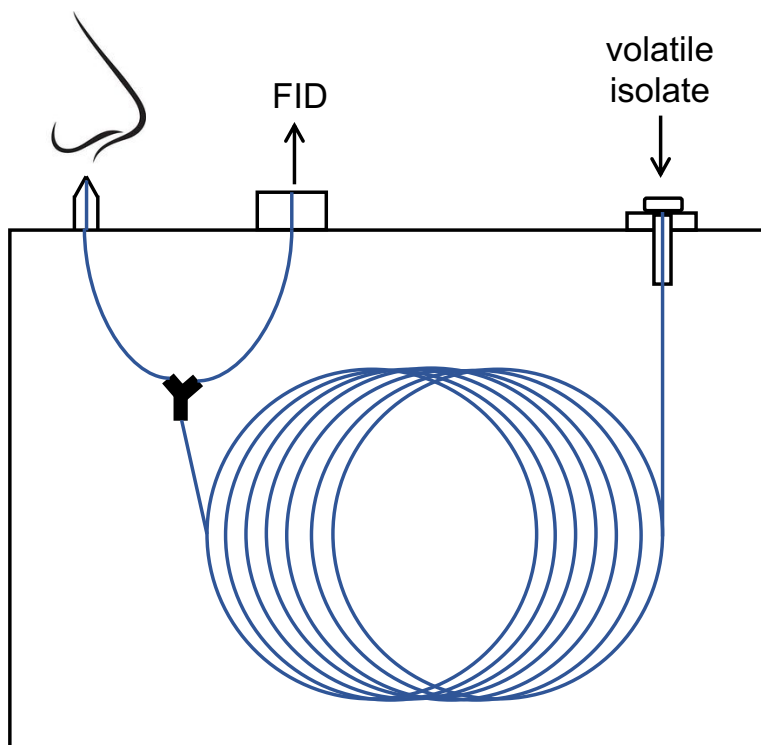


Figure 11. Diagram of Gas Chromatography-Olfactometry Instrument.

60 °C/minute followed by a second ramp at 6 °C/minute to reach 250 °C. The oven was then held at 250 °C for 5 minutes. The capillary column was connected via a heated transfer line to the mass spectrometer which operated in electron ionization mode at 70eV. Additionally, the MS source was maintained at 230 °C and the MS quadrupole at 150 °C.

To assist in identification of each odor-active compound a linear retention index (RI) was calculated for each of the peaks. This was achieved by analyzing a mixture of *n*-alkanes with increasing chain length (C9 to C26) by GC-FID and GC-MS and the retention time of each hydrocarbon was used to calculate an RI for each peak of interest. The equation used to calculate the retention indices was the following:

$$RI = 100 \times \left[n + (N - n) \frac{t_{r,a} - t_{r,n}}{t_{r,N} - t_{r,n}} \right]$$

Where *n* is the number of carbons in the alkane with the earlier retention time, *N* is the number of carbons in the alkane with the later retention time, *t_{r,a}* is the retention time of the analyte, *t_{r,n}* is the retention time of the earlier eluting hydrocarbon, and *t_{r,N}* is the retention time of the later eluting hydrocarbon.

3.5. Quantification of Odor-active Compounds from *Pycnanthemum incanum*

3.5.1. Stable Isotope Dilution Assays (SIDA)

To quantitate the levels of each of the odorants identified by AEDA, stable isotope dilution assays (SIDA) were employed. Deuterated and ¹³C labelled analogues for each of the analytes were acquired and individually prepared in pentane at known concentrations as internal standards. The dried plant material (50 mg – 500 mg) was ground to a fine powder with a mortar and pestle. A sample was measured into a glass culture tube, and freshly distilled ether (4 mL) was added.

Each of the labelled odorants (50 μL – 200 μL) were added volumetrically before manually shaking (5 minutes) to extract the plant material. After 2 hours of extraction at room temperature, the supernatant was filtered through anhydrous sodium sulfate (5 g) and analyzed by GC-MS. Using previously determined response factors (RFs) for ions of the analyte and analogous isotope, as well as integration of peaks from extracted ion chromatograms, concentrations for each analyte were calculated relative to the quantity of plant material analyzed. The ions and response factor used for each analyte are provided in Table 2.

3.5.2. Quantitation of Isomenthone

Neither a ^{13}C nor deuterium labelled standard was commercially available for isomenthone, so this odorant was quantitated using ($^2\text{H}_8$)-menthone. Menthone, a diastereomer of isomenthone, was selected as the internal standard for quantitation of isomenthone because of its structural similarity. A response factor was calculated for isomenthone and ($^2\text{H}_8$)-menthone (m/z 112/115, RF 0.99). Isomenthone was then quantitated by SIDA according to the sample preparation detailed above.

3.5.1. Odor Threshold Determination

For each of the odorants quantitated by SIDA, an odor threshold was determined to allow for calculation of odor activity values (OAVs). Odor thresholds for odorants **1 – 3**, **6**, **10**, **16**, **17**, **20**, and **23** were provided by the Sensory Systems Chemistry Group at Leibniz-LSB (Technical University of Munich, Freising, Germany). Odor thresholds for odorants **7**, **8**, **13**, and **14** were quantitated in water according to a method set forth by the American Society of Testing and Materials (ASTM). The ASTM method E-679 involved presenting participants with a sequence of triangle tests with two blanks (water) and one test sample (water with odorant), with the concentration of odorants increasing throughout the series.²³ For each triangle test, the three

Table 2. Labelled standard ions and response factors.

no.	odorant	labelled standard	Ion (<i>m/z</i>)		RF
			analyte	standard	
1	<i>α</i> -pinene	<i>α</i> -pinene-d3	93	96	0.57
2	myrcene	myrcene- ¹³ C3	69	72	0.96
3	1,8-cineole	1,8-cineole-d6	154	160	1.05
6	1-octen-3-ol	1-octen-3-ol-d3	57	60	0.81
7	menthone	menthone-d8	154	162	0.68
10	linalool	linalool-d3	93	96	0.85
13	pulegone	pulegone-d8	152	160	0.61
14	<i>α</i> -terpineol	<i>α</i> -terpineol-d3	136	139	1.27
16	<i>β</i> -citronellol	<i>β</i> -citronellol-d2	95	97	0.44
17	geraniol	geraniol-d2	93	95	1.00
20	<i>β</i> -ionone	<i>β</i> -ionone-d3	177	180	0.77
23	eugenol	eugenol-d3	164	167	0.84

samples, enclosed in sealable 20mL glass vials labelled with a letter (A, B or C), were presented to the participants in a random order. The series of triangle tests included six levels with a threefold difference in concentration of odorant between each level. All sensory tests were administered to participants (n=10) who had no self-reported sensory impairment.

3.6. Separation and Identification of Odor-active Stereoisomers from *Pycnanthemum incanum*

3.6.1. Solid Phase Extraction (SPE)

A volatile isolate was prepared in pentane by SAFE and concentrated as detailed above. A silica SPE cartridge (Strata® SI-1 Silica (55 μm , 70 \AA), 2 g/12 mL) from Phenomenex (Torrance, CA) was affixed to a SPE manifold and fractionation was executed under vacuum. The cartridge was conditioned with aliquots of pure pentane and ether: pentane (10mL), ether (5mL), and pentane (5mL). The volatile isolate (1mL) was loaded on the SPE cartridge and eluted with the following solvents: Fraction 1 (F1), pentane (100%); Fraction 2 (F2), pentane/ether (98/2, v/v), Fraction 3 (F3), pentane/ether (95/5, v/v); Fraction 4 (F4), pentane/ether (90/10, v/v); Fraction 5 (F5), pentane/ether (50/50, v/v); and Fraction 6 (F6), ether (100 %). Each fraction was individually concentrated to 200 μL under a gentle stream of nitrogen and analyzed by chiral GC-MS.

3.6.2. Chiral Gas Chromatography – Mass Spectrometry

An Agilent Technologies 78204 GC system coupled to a 5977B mass spectrometry detector (Santa Clara, CA) was used for chiral chromatography. Two cyclodextrin columns were used for chiral GC-MS, one with a based phase and another with a γ -cyclodextrin based phase (Figure 12). Both the Rt- γ DEXsa capillary column (2,3-di-acetoxy-6-O-tert-butyl dimethylsilyl gamma cyclodextrin) and Rt- β DEXsm capillary column (2,3-di-O-methyl-6-O-tert-butyl

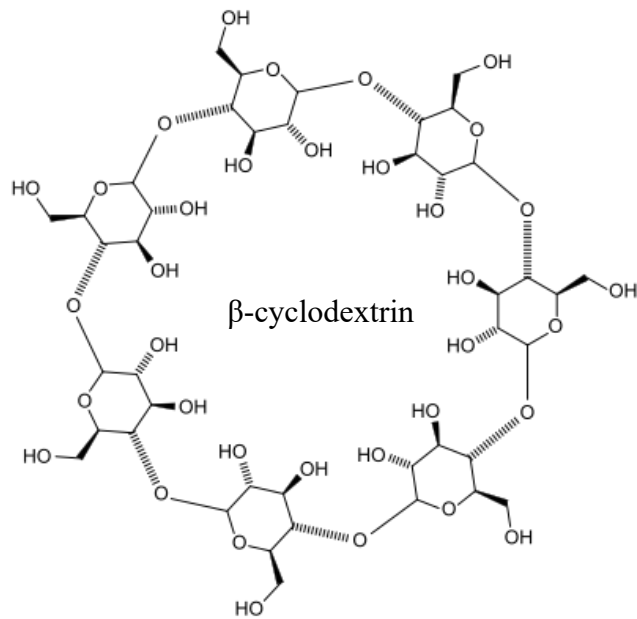
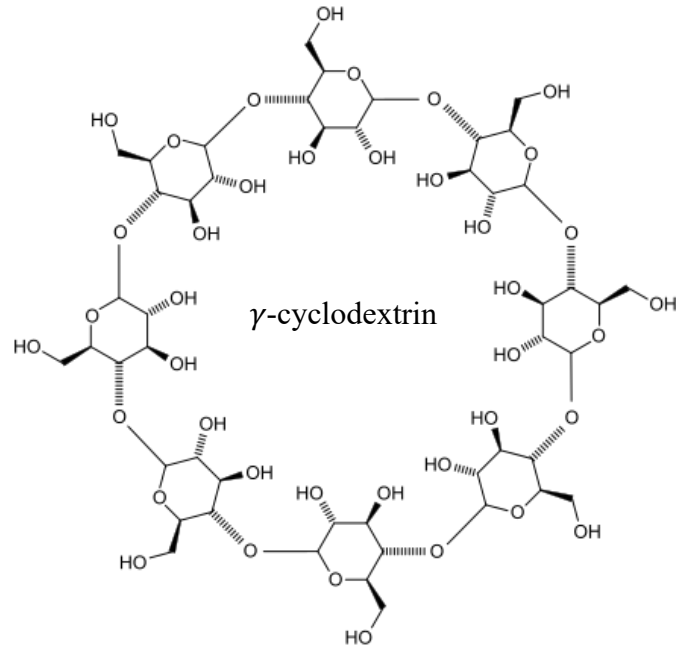


Figure 12: Structure of cyclodextrin stationary phases.

dimethylsilyl beta cyclodextrin) were from Restek (Bellefonte, PA) and both had a length of 30 m, 0.25 mm internal diameter, and 0.25 μm film thickness.

The Rt- γ DEXsa column was used to separate enantiomers of α -pinene (**1**), menthone (**7**), isomenthone (**8**), and α -terpineol (**14**). The Rt- β DEXsm column was used to separate enantiomers of 1-octen-3-ol (**6**), linalool (**10**), pulegone (**13**), and β -citronellol (**16**). Samples were injected on column with helium as a carrier gas (1 mL/minute). Initially the GC oven was held at 40 °C for 1 minute followed by three consecutive temperature ramps. The first temperature increase was at a rate of 5 °C/minute to reach 120 °C, the second at 3 °C/minute to 135 °C and the third at 5 °C/minute to 230 °C with an additional hold time of 5 minutes at 230 °C. The mass spectrometer was operated in EI mode with the same parameters as outlined above. A second method was developed to achieve separation of 1-octen-3-ol enantiomers. For this method, the helium flow rate was 5 mL/minute and the sample were injected on column at 40 °C. The initial oven temperature (40 °C) was maintained for 1 minute and then increased at a rate of 0.5 °C/minute to reach 100 °C, followed by a second ramp at 5 °C/minute to reach 150 °C and then increased at 10 °C/minute to reach 230 °C, with a final hold time of 5 minutes at 230 °C.

3.7. Sensory Evaluation of *P. incanum* Aroma Recombination Model

3.7.1. Free Choice Profiling

Free choice profiling was implemented to generate common odor descriptors of the *P. incanum* plant material. The plant material was ground to a fine powder with a coffee grinder and 100 mg measured into a 20 mL glass scintillation vial. Participants (n=10) were administered the sample for orthonasal evaluation and asked to provide three odor descriptors using their own lexicon. All of the odor descriptors provided by the participants were combined and based on the

most frequently provided descriptors it was come to a group consensus which descriptors were most suitable to evaluate by quantitative olfactory profile analysis. Likewise, a reference odorant was also agreed upon that effectively represented the odor of each of the selected odor descriptors. The selected odorant and correlating attributes used for quantitative olfactory profile analysis were the following: menthone (*mint, fresh*), linalool (*citrus*), (*Z*)-hex-3-en-1-ol (*green*), geraniol (*floral*), pulegone (*mint, medicinal*), eugenol (*spice*), 1-octen-3-one (*mushroom*), α -pinene (*pine*), 1,8-cineole (*eucalyptus*), and dihydrocarvone (*solvent*).

3.7.2. Odor Recombination Model

An odor recombination model was designed to verify that the quantitative data generated by stable isotope dilution analysis could be used to effectively recreate the odor of the original *P. incanum* plant. The odor model was compounded by combining the quantified odorants in pure form at a concentration 500 times higher than quantified in the plant material in a carrier such as water or oil. This stock solution of the odor model was then incorporated into an appropriate matrix at the odorant concentrations determined in the plant material. Multiple iterations of odor model prototyping was completed by incrementally increasing the number of compounds included in the odor model to include those with lower OAVs while also determining the most suitable carrier and matrix to assess the odor recombination model by quantitative olfactory profile analysis.

Based on the initial odor model prototyping an aqueous odor model was determined to be the best suited model to assess sensory attributes of the *P. incanum* plant odor. Therefore, both an aqueous extract of *P. incanum* plant material and the *P. incanum* odor recombination model incorporated in water were subjected to quantitative olfactory profile analysis. The aqueous extract was prepared by grinding dried *P. incanum* plant material in a coffee grinder and combining the plant powder (750 mg) with DI water (250 mL). The mixture was extracted for 3 min (25 °C) and

then filtered prior to sensory evaluation. The *P. incanum* odor recombination model was prepared by combining all quantitated odorants with OAVs ≥ 1 in water at the concentration determined by SIDAs. The odor model was then incorporated in water at an equivalent concentration of 3 g plant material per kg water.

3.7.3. Quantitative Olfactory Profile Analysis

For quantitative olfactory profile analysis, each reference compound was prepared in water at a concentration 100 times its odor detection threshold, and administered in a 20 mL glass scintillation vial. The participants were first asked to smell a reference solution to become familiar with the odor attribute of interest. They were then provided two 150mL beakers wrapped in aluminum foil and covered with a watch glass, each containing 20 mL of a sample (aqueous plant extract and aqueous odor model). The participants were then asked to rate the intensity of the given attribute in each sample on a seven-point scale with 0.5 increments, where 0 = not observable, 1 = weakly observable, 2 = moderately observable, and 3 = strongly observable. This procedure was repeated for each of the ten reference solutions, and scores were averaged across participants (n=10) using Microsoft® Excel Version16.21 for Office 360 (Microsoft Corporation, Redman, WA). In addition, the quantitative olfactory profile analysis data was evaluated by a one-way analysis of variance (ANOVA) comparing the attribute means between the odor model and plant extract. The analysis was done using JMP Pro 14.0.0 software (SAS Institute, Cary, NC) with a significance level of 0.05.

4 RESULTS AND DISCUSSION

4.1. Odor Quality of *Pycnanthemum incanum* Plant

An initial evaluation of the odor characteristics of *P. incanum* plant material was carried out by free choice profiling. Participants were asked to smell an aliquot of the dried and ground plant material and asked to provide descriptors from their own vocabulary that were most suitable for the odor qualities they perceived. They were asked to base their judgement strictly on the olfactory experiences, not visual observations. The most common descriptors provided by participants related to the mint-like character of the plant such as *fresh mint*, *medicinal mint*, and *eucalyptus*. However, beyond the mint-like character, other notes included *citrus*, *green*, *floral*, *spicy*, *mushroom*, *pine* and *solvent* (Table 3). A reference odorant was then selected that exhibited the representative odor quality of each of the key attributes to be used for quantitative olfactory profile analysis. The odor descriptors, and the corresponding odorant, were as follows: *mint, fresh* (menthone), *citrus* (linalool), *green* ((*Z*)-hex-3-en-1-ol), *floral* (geraniol), *mint, medicinal* (pulegone), *spice* (eugenol), *mushroom* (1-octen-3-one), *pine* (α -pinene), *eucalyptus* (1,8-cineole), and *solvent* (dihydrocarvone) (Table 3).

4.1. Identification of Key Odorants

Volatiles present in the *P. incanum* plant were isolated by organic extraction of dried and ground plant material using diethyl ether. The volatiles were further isolated from the non-volatiles by SAFE and concentrated to a small volume. A sensorial evaluation of the volatile isolate demonstrated that the odorants had been retained during the distillation process because of the pungent mint-like odor, reminiscent of the starting material. The concentrated isolate was then subjected to AEDA to determine an FD factor for each of the odorants and to determine which odorants contribute to the odor character of *P. incanum*.

Table 3. Odor Descriptors of *P. incanum* Provided by Free Choice Profiling and Odorants Selected for Quantitative Olfactory Profile Analysis.

Odor descriptor examples	Count	Selected odor descriptor	Selected odorant
mint, peppermint, wintergreen	13	mint, fresh	menthone
citrus, fruity	2	citrus	linalool
green, leafy, woody	4	green	(<i>Z</i>)-hex-3-en-1-ol
fresh, sweet mint, leafy	4	floral	geraniol
medicinal, mothball, fecal	4	mint, medicinal	pulegone
anise, cinnamon, spicy	3	spicy	eugenol
woody	1	mushroom	1-octen-3-one
hay, woody	2	pine	α -pinene
cooling, menthol, ephemeral	4	eucalyptus	1,8-cineole
citrus, medicinal	2	solvent	dihydrocarvone

To aid in the identification of key odorants perceived during AEDA, the SAFE isolate was analyzed by GC-MS to acquire a mass spectrum for each of the odorants. The total ion chromatogram of the SAFE isolate is provided in Figure 13. In addition, a hydrocarbons standard was used to accurately determine the retention index of each of the compounds of interest. However, some compounds coeluted with others preventing an accurate mass spectrum from being acquired. Therefore, SPE was used to separate classes of odorants and reduce coelution when analyzed by GC-MS. The total ion chromatograms of the SAFE isolate compared to each SPE fraction are provided in Figure 14. Through a combination of odor quality perceived by GC-O, peak retention index, and the retention index of authentic standards 24 odor-active compounds were identified. A flavor dilution chromatogram depicting the 24 odor-active compounds and their FD factors is provided in Figure 15. The IUPAC name for all compounds referred to by common names can be found in the appendix.

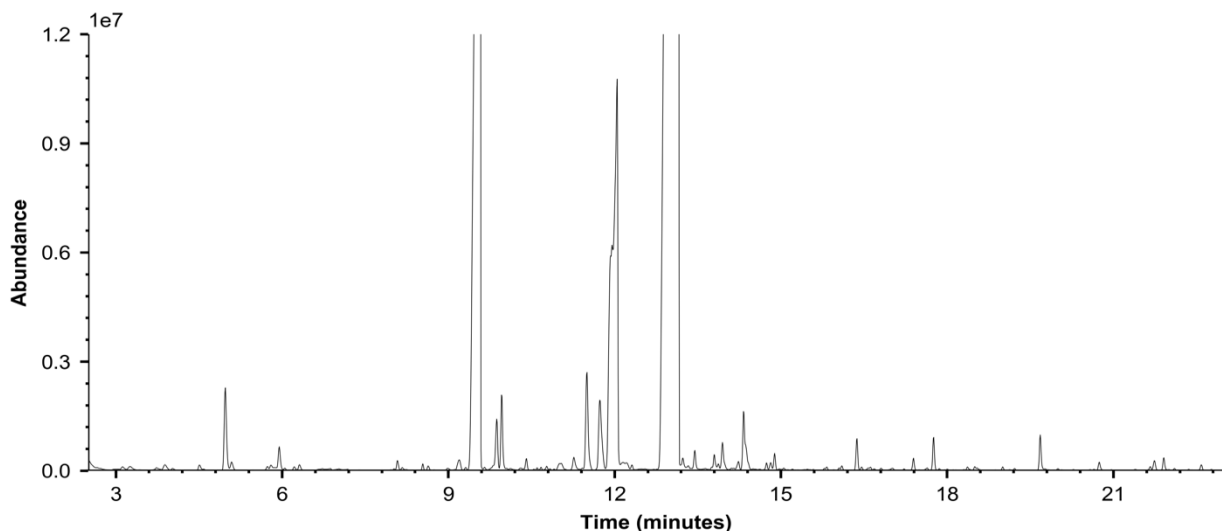


Figure 13. Total Ion Chromatogram of *P. incanum* SAFE isolate analyzed by GC-MS (FFAP column).

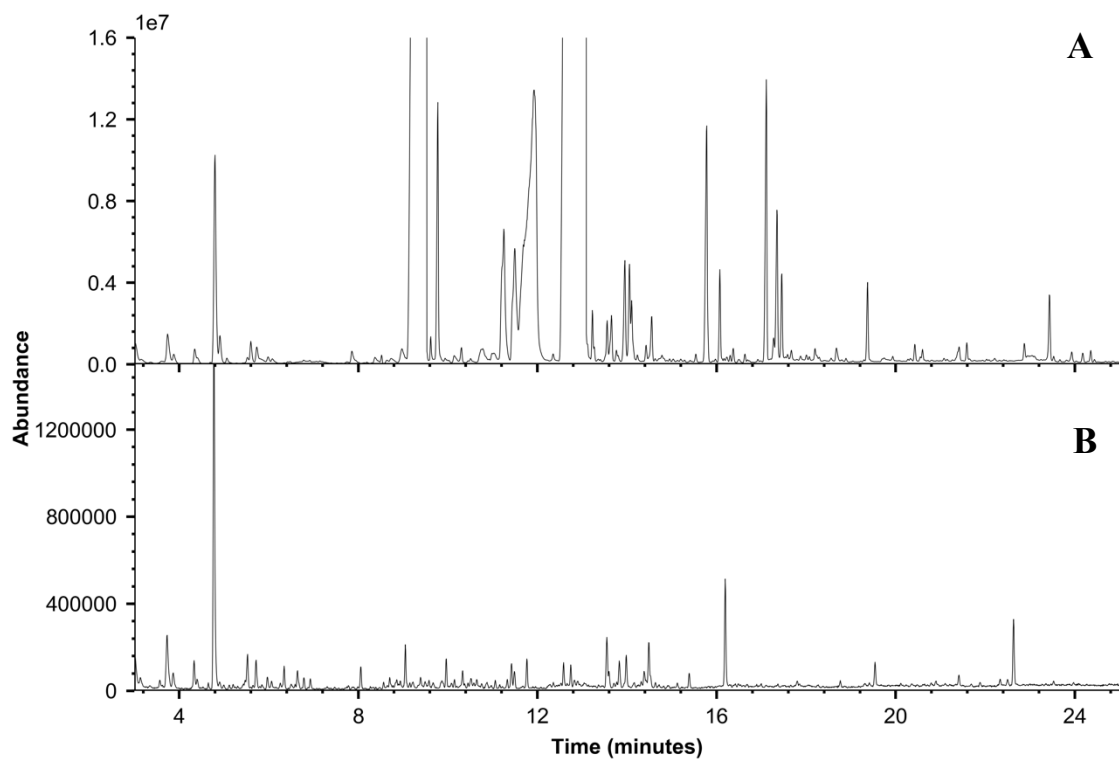


Figure 14a. Total Ion Chromatograms of A) SAFE isolate prior to fractionation and B) SPE Fraction 1 (GC column; FFAP phase).

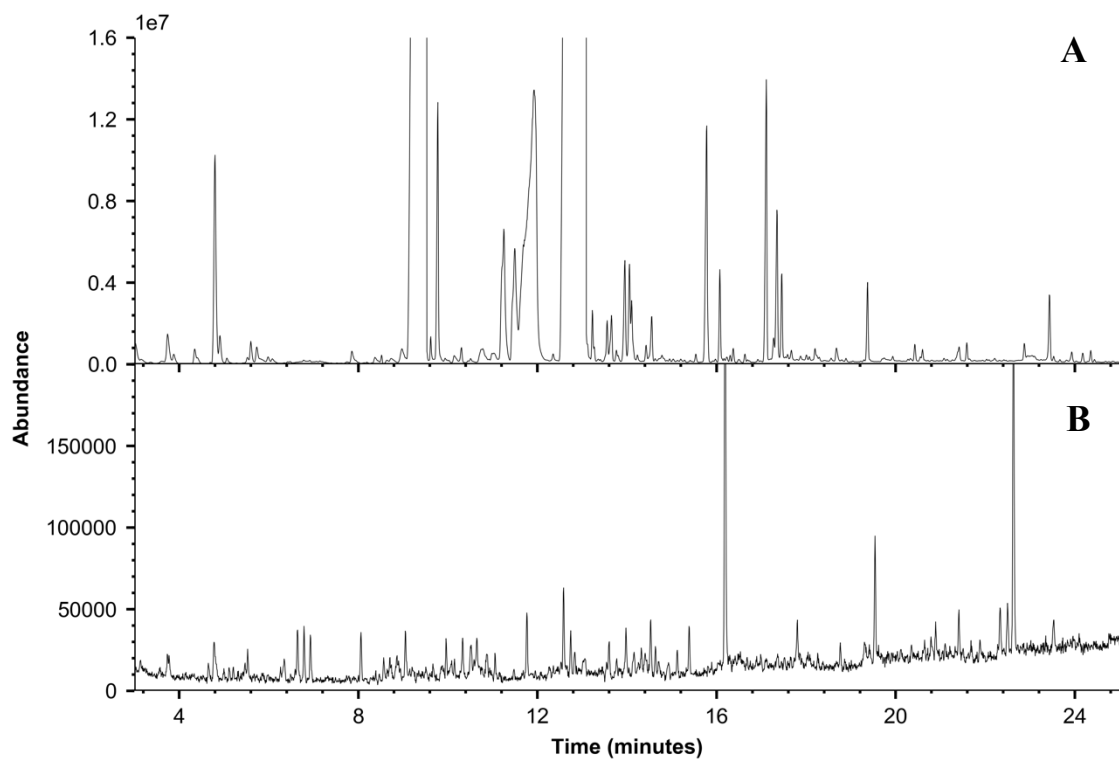


Figure 14b. Total Ion Chromatograms of A) SAFE isolate prior to fractionation and B) SPE Fraction 2 (GC column; FFAP phase).

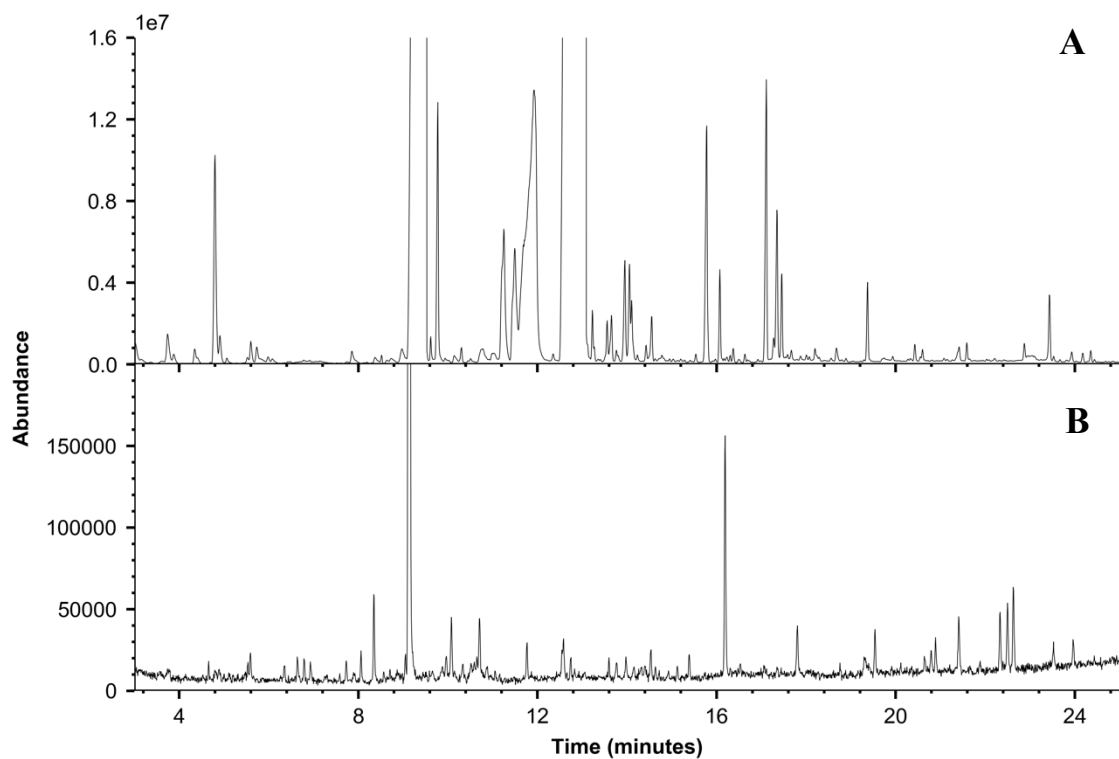


Figure 14c. Total Ion Chromatograms of A) SAFE isolate prior to fractionation and B) SPE Fraction 3 (GC column; FFAP phase).

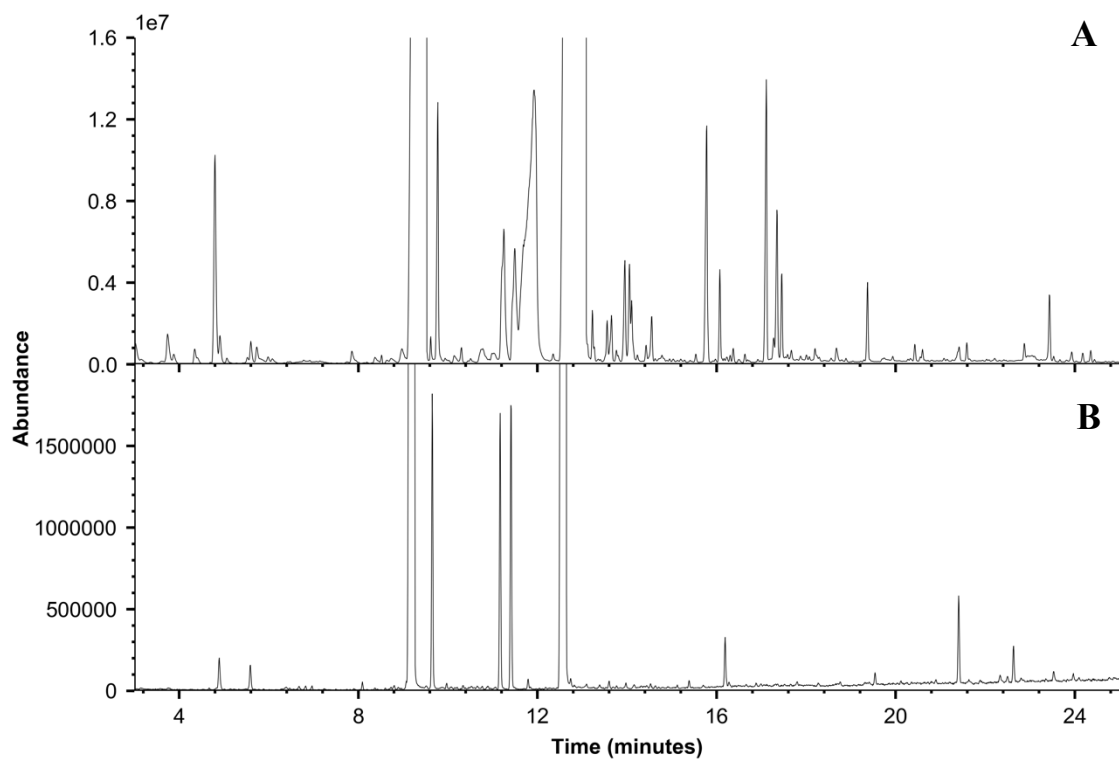


Figure 14d. Total Ion Chromatograms of A) SAFE isolate prior to fractionation and B) SPE Fraction 4 (GC column; FFAP phase).

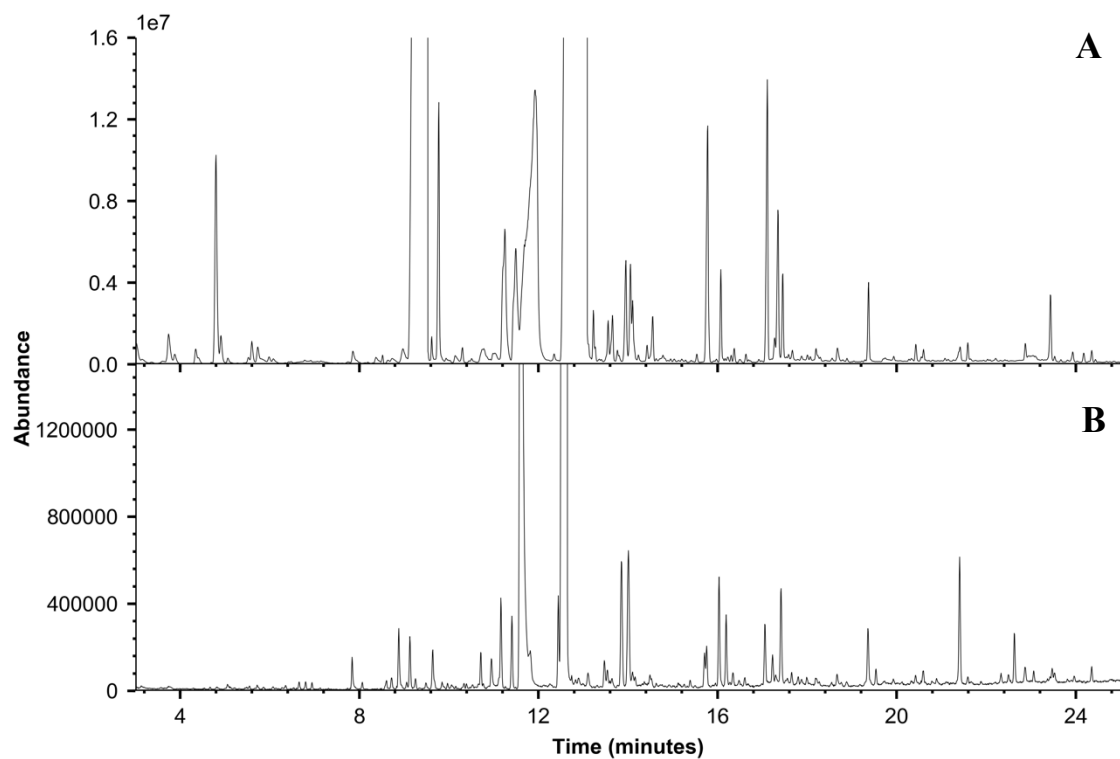


Figure 14e. Total Ion Chromatograms of A) SAFE isolate prior to fractionation and B) SPE Fraction 5 (GC column; FFAP phase).

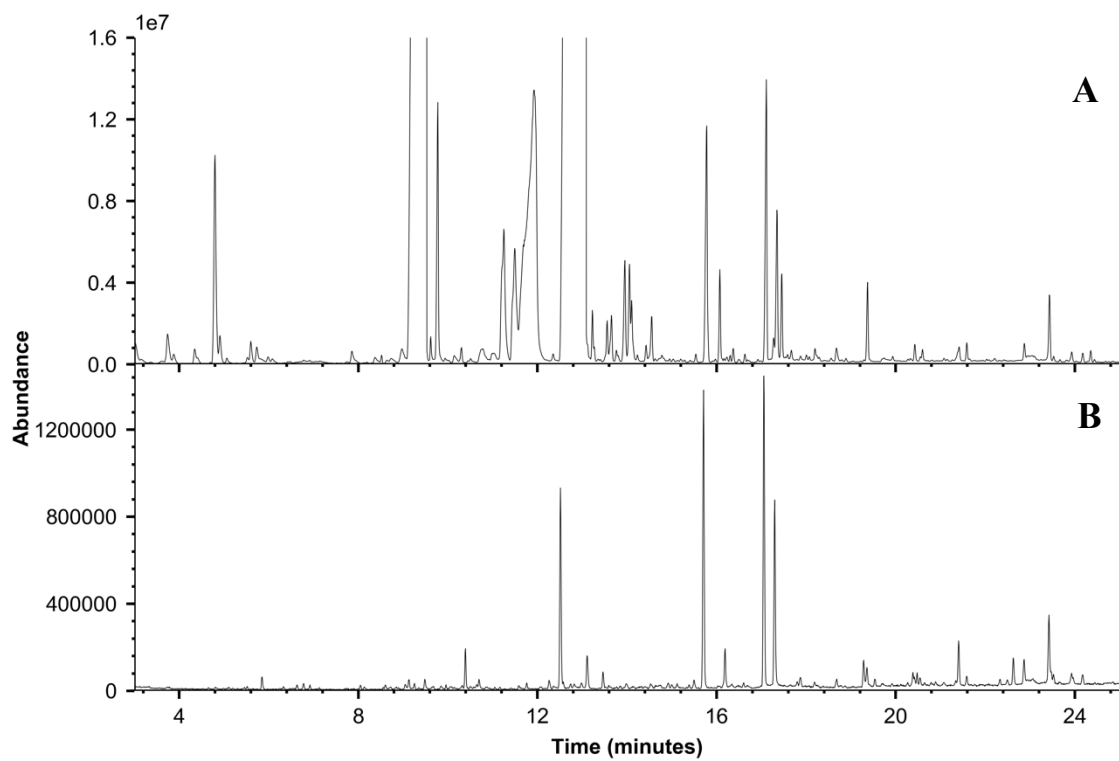


Figure 14f. Total Ion Chromatograms of A) SAFE isolate prior to fractionation and B) SPE Fraction 6 (GC column; FFAP phase).

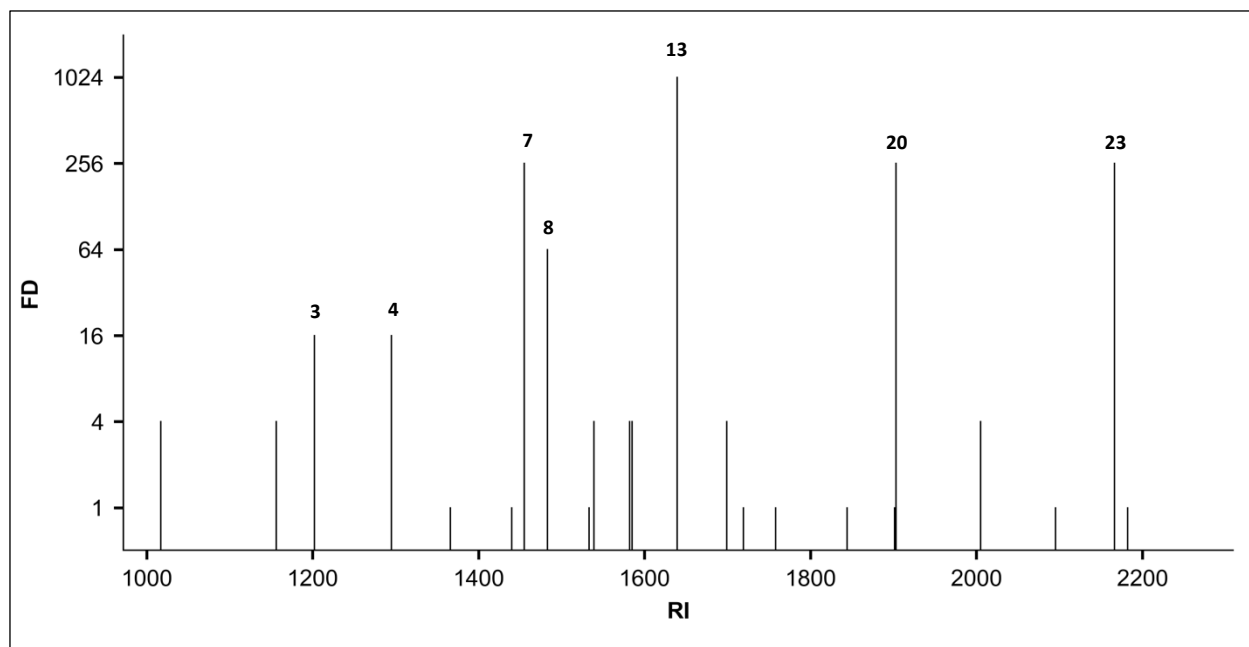


Figure 15. Flavor Dilution Chromatogram of Key Odorants of *P. incanum*. Numbers depict odorants with FD factors ≥ 16 .

(3) 1,8-cineole; **(4)** 1-octen-3-ol; **(7)** menthone; **(8)** isomenthone; **(13)** pulegone; **(20)** β -ionone; **(23)** eugenol.

As a result of AEDA, 24 compounds were identified as odor-active, including 5 odorants with FD factors ≥ 64 (Table 4, Figure 16). The odorant with the highest FD factor was pulegone (FD 1024), which has a mint, medicinal odor quality. Other odorants with high FD factors were menthone (FD 256), β -ionone (FD 256), and eugenol (FD 256), followed by isomenthone (FD 64). The menthone and isomenthone also exhibit mint-like odor quality whereas β -ionone and eugenol have floral and clove odor qualities, respectively. In addition, the mass spectrum of each of the compounds acquired by GC-MS, which aided in identification of the odorants, are provided in Figures 17 - 21.

Nine odorants were identified with FD factors of 16 and 4 (Table 5, Figure 22). The odorants with FD factors of 16 included 1,8-cineole, which has a eucalyptus odor quality, and 1-octen-3-one, which has a mushroom odor quality. Of the 7 odorants with FD factors of 4, only one had a mint-like odor quality, namely neomenthol. All other odorants in this group exhibited a variety of odor attributes including pine (α -pinene), terpeny (myrcene and α -terpineol), floral, citrus (linalool), cucumber ((2*E*,6*Z*)-nona-2,6-dienal), and metallic (*trans*-4,5-epoxy-(2*E*)-dec-2-enal). In addition, the mass spectrum of each of the compounds acquired by GC-MS, which aided in identification of the odorants, are provided in Figures 23- 30.

Ten odorants were only perceivable in the most concentrated SAFE isolate, therefore having an FD factors of 1 (Table 6, Figure 31). A number of floral compounds, including β -citronellol, geraniol, and *cis*-jasnone, as well as some mint-like compounds, including piperitone and piperitenone, were identified with an FD factors of 1. Other odorants in this group exhibited diverse odor attributes such as geranium ((5*Z*)-octa-1,5-dien-3-one), mushroom (1-octen-3-ol), fatty ((2*E*)-non-2-enal), metallic (*trans*-4,5-epoxy-(2*E*)-undec-2-enal), and phenolic (thymol).

Table 4. Odorants Identified in *P. incanum* Plant Material with Flavor Dilution Factors \geq 64.

no.	odorant	odor quality	RI on		FD factor
			FFAP	DB-5	
13	pulegone	mint, medicinal	1639	1241	1024
7	menthone	mint, fresh	1455	1155	256
20	β -ionone	violet	1903	1487	256
23	eugenol	clove	2166	1367	256
8	isomenthone	mint, vegetable	1483	1164	64

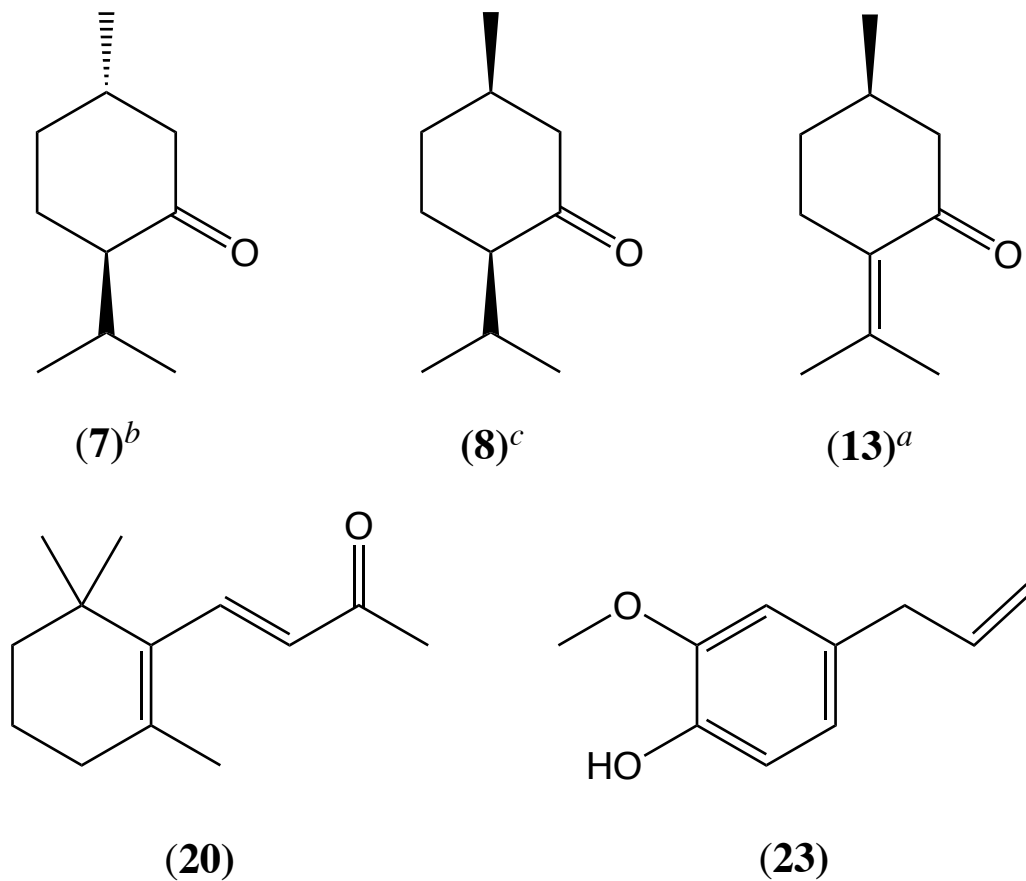


Figure 16. Structure of Odorants Identified in *P. incanum* Plant Material with Flavor Dilution Factors ≥ 64 .

^a(*R*)-(+)-pulegone. ^b(2*S*,5*R*)-(–)-menthone. ^c(2*R*,5*R*)-(+)-isomenthone.

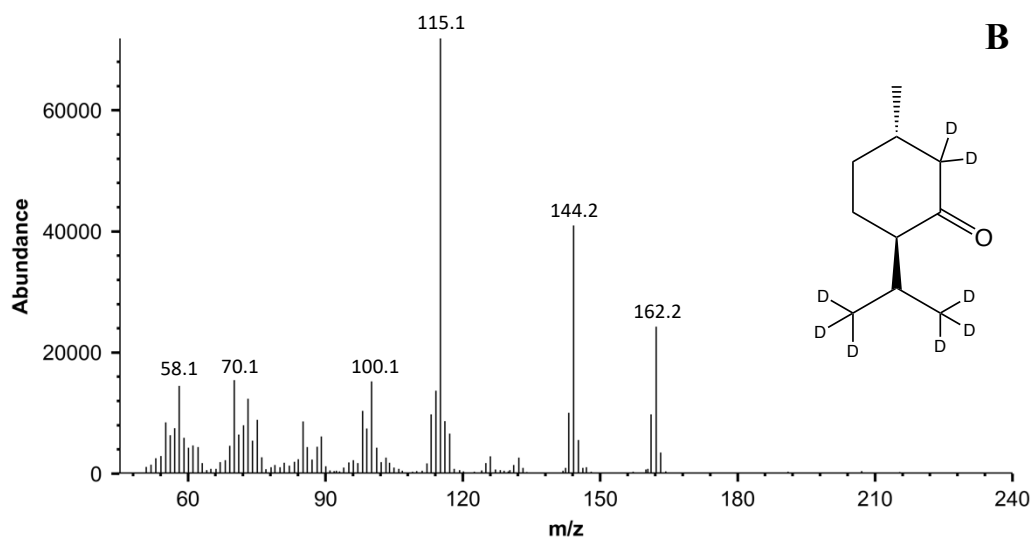
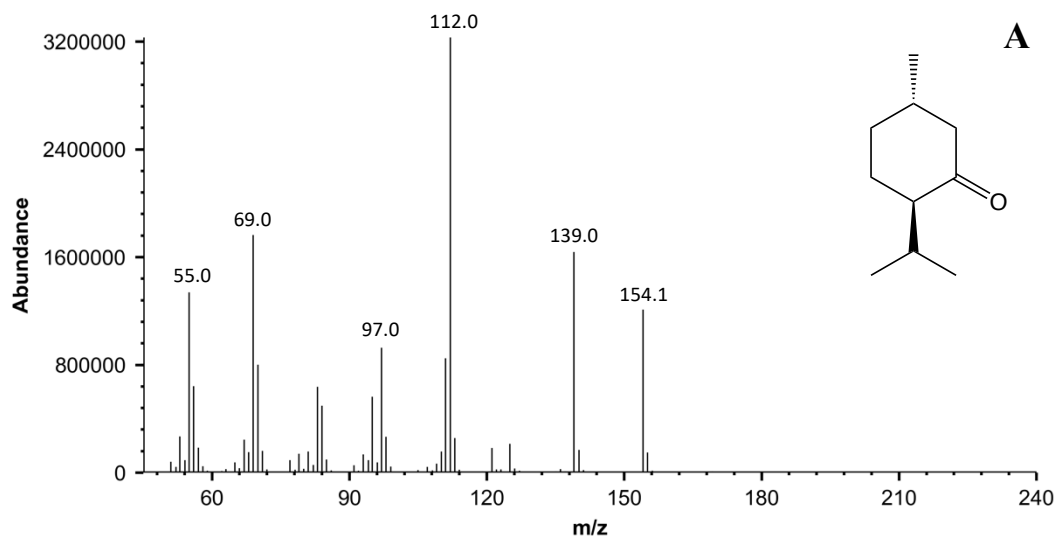


Figure 17. Mass Spectrum of A) menthone (7) and B) menthone-d8.

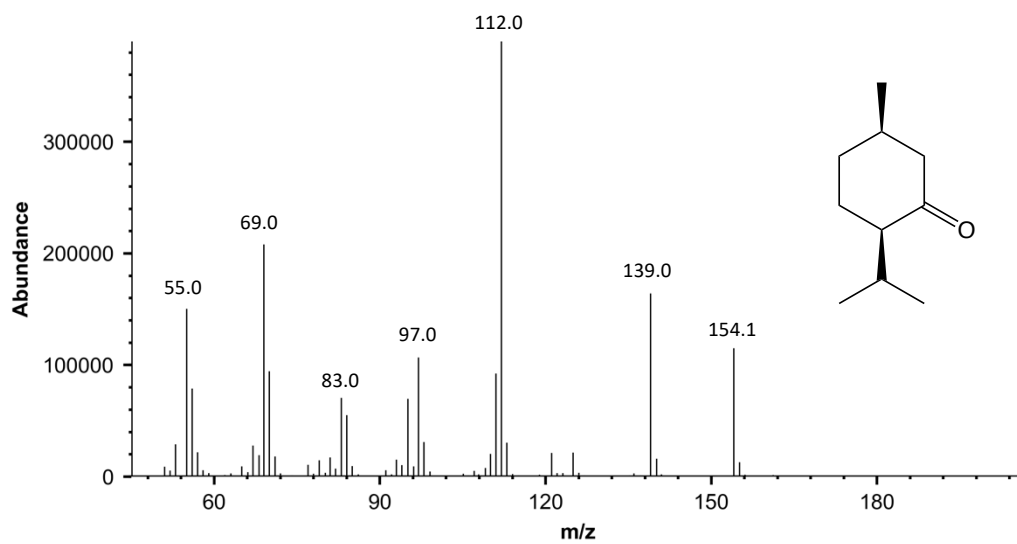


Figure 18. Mass Spectrum of isomenthone (8).

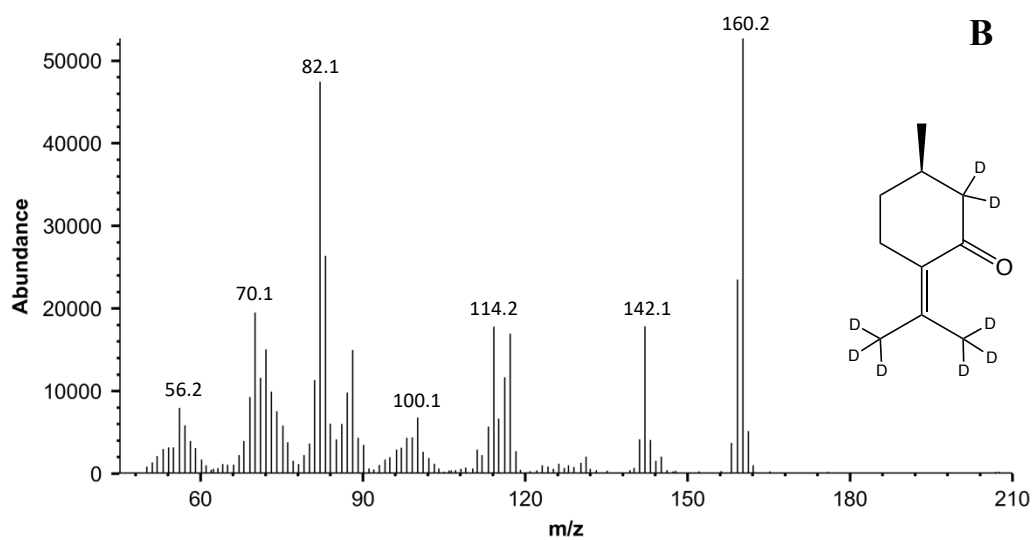
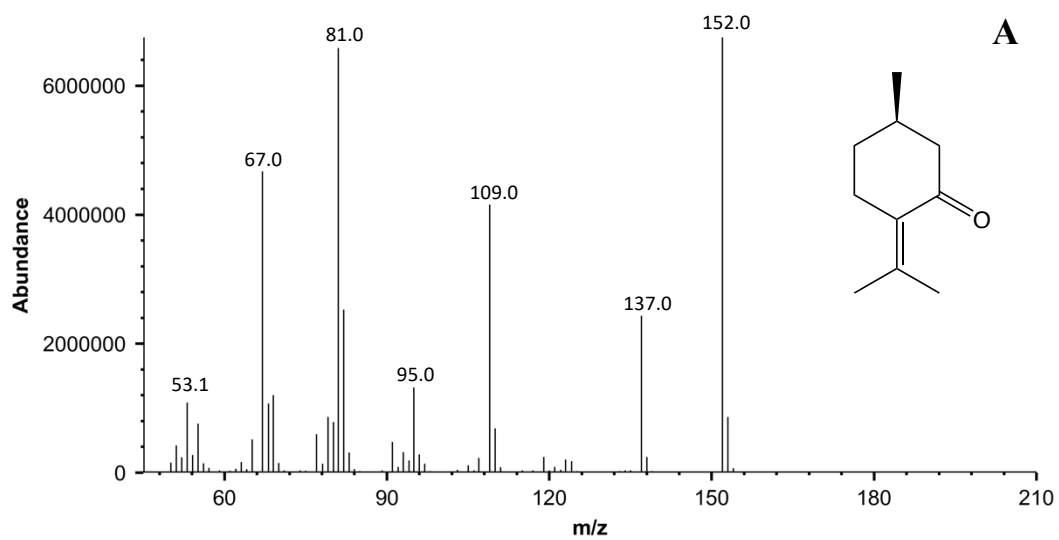


Figure 19. Mass Spectrum of A) pulegone (13) and B) pulegone-d6.

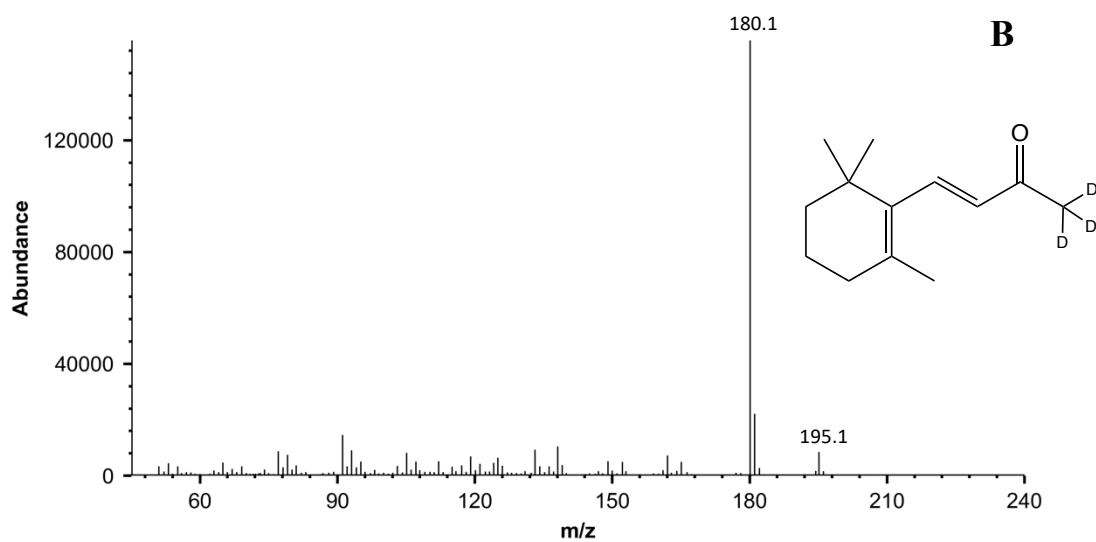
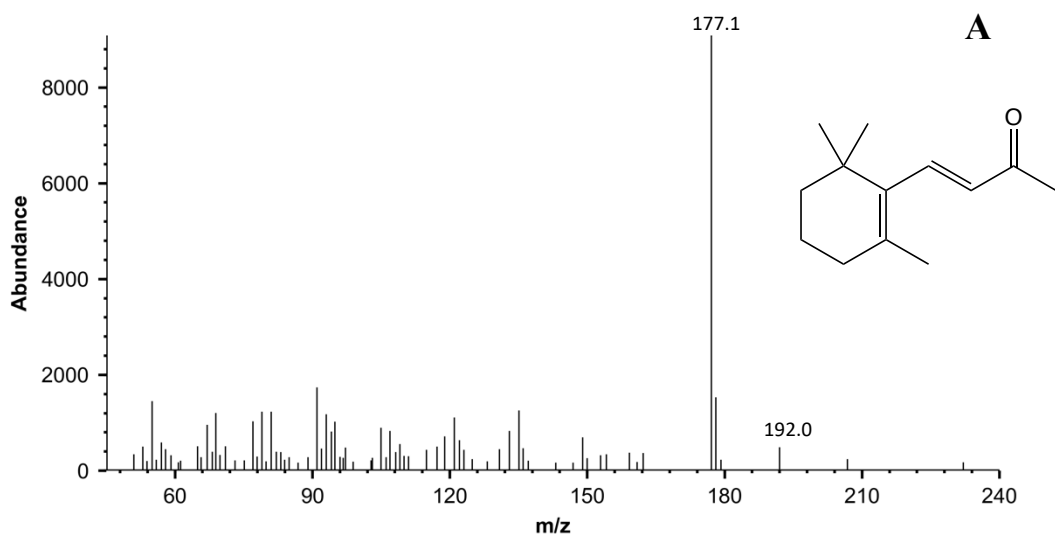


Figure 20. Mass Spectrum of A) β -ionone (20) and B) β -ionone-d₃.

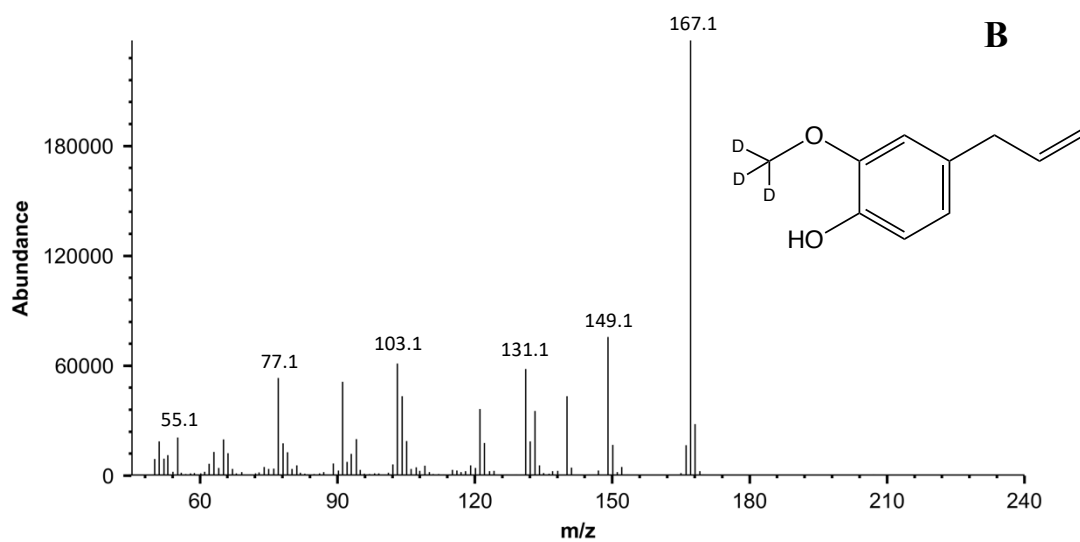
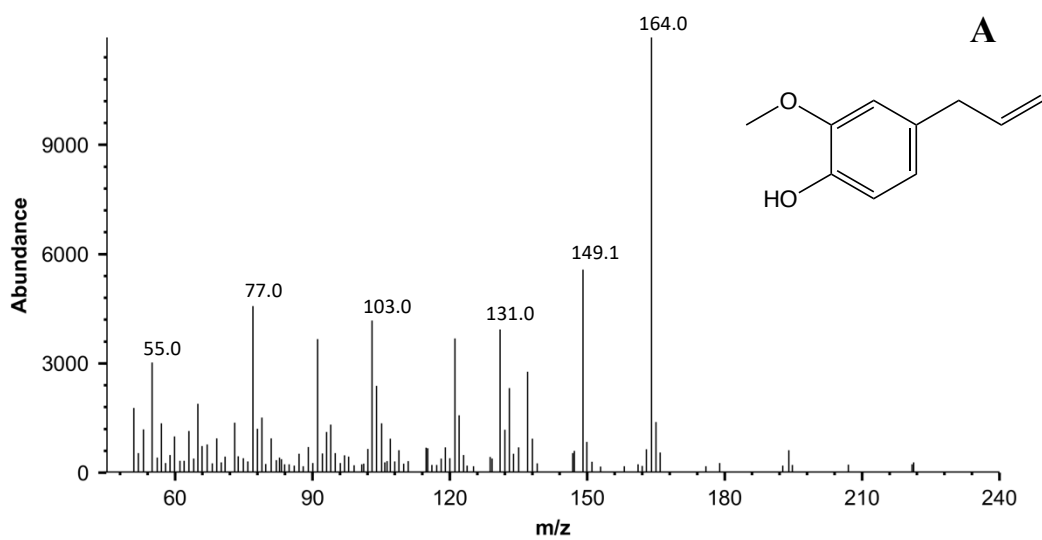


Figure 21. Mass Spectrum of A) eugenol (23) and B) eugenol-d3.

Table 5. Odorants Identified in *P. incanum* Plant Material with Flavor Dilution Factors ≥ 6 (excluding those previously mentioned, $FD \geq 64$).

no.	odorant	odor quality	RI on		FD factor
			FFAP	DB-5	
3	1,8-cineole	eucalyptus	1202	1029	16
4	1-octen-3-one	mushroom	1295	978	16
1	α -pinene	pine	1017	931	4
2	myrcene	terpeny	1156	991	4
10	linalool	floral, citrus	1539	1101	4
11	(2 <i>E</i> ,6 <i>Z</i>)-nona-2,6-dienal	cucumber	1582	1153	4
12	neomenthol	mint	1585	1166	4
14	α -terpineol	terpeny	1699	1192	4
21	<i>trans</i> -4,5-epoxy-(2 <i>E</i>)-dec-2-enal*	metallic	2005	1380	4

*Identified by odor quality and retention times.

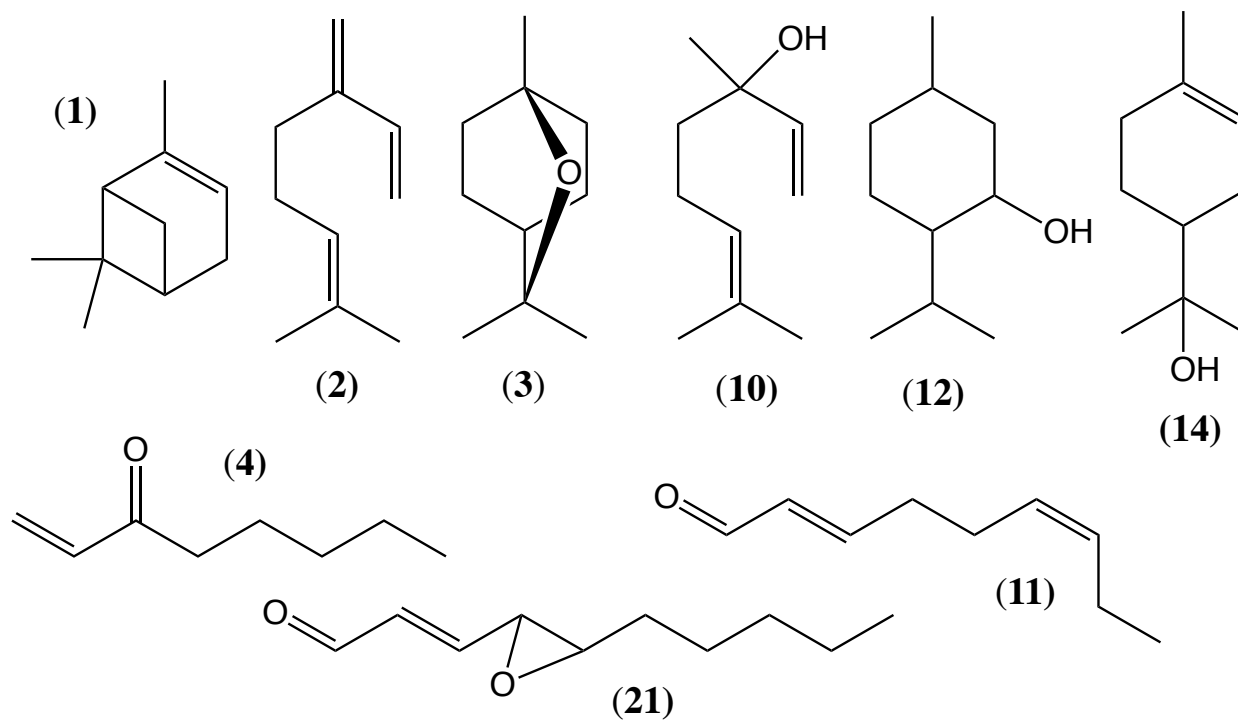


Figure 22. Structures of Odorants Identified in *P. incanum* Plant Material with Flavor Dilution Factors ≥ 6 (excluding those previously mentioned, $FD \geq 64$).

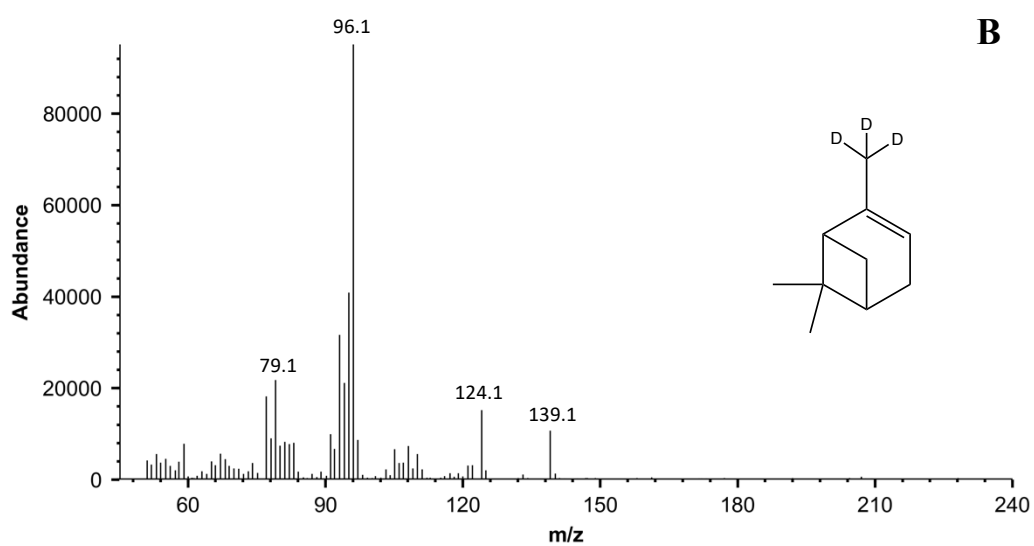
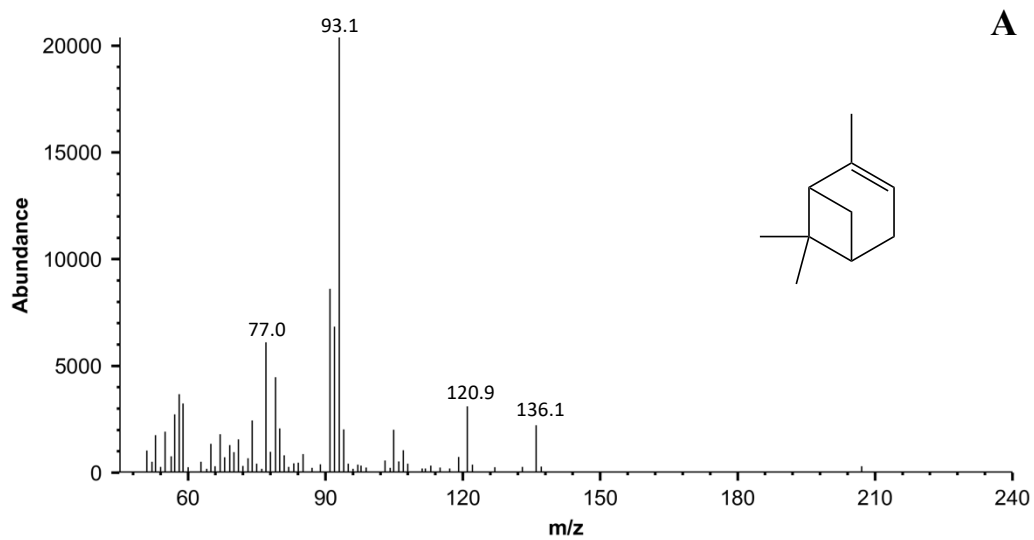


Figure 23. Mass Spectrum of A) α -pinene (1) and B) α -pinene-d₃.

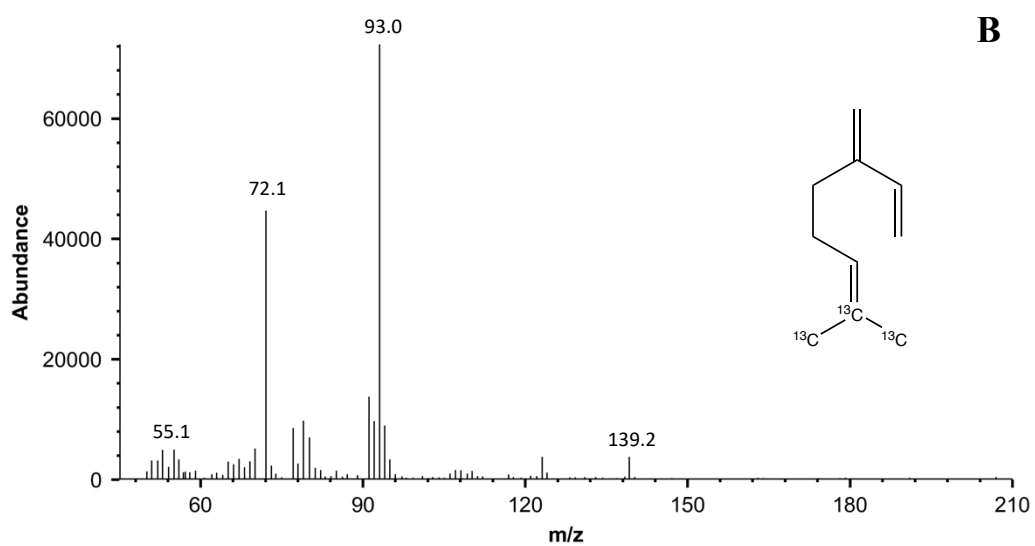
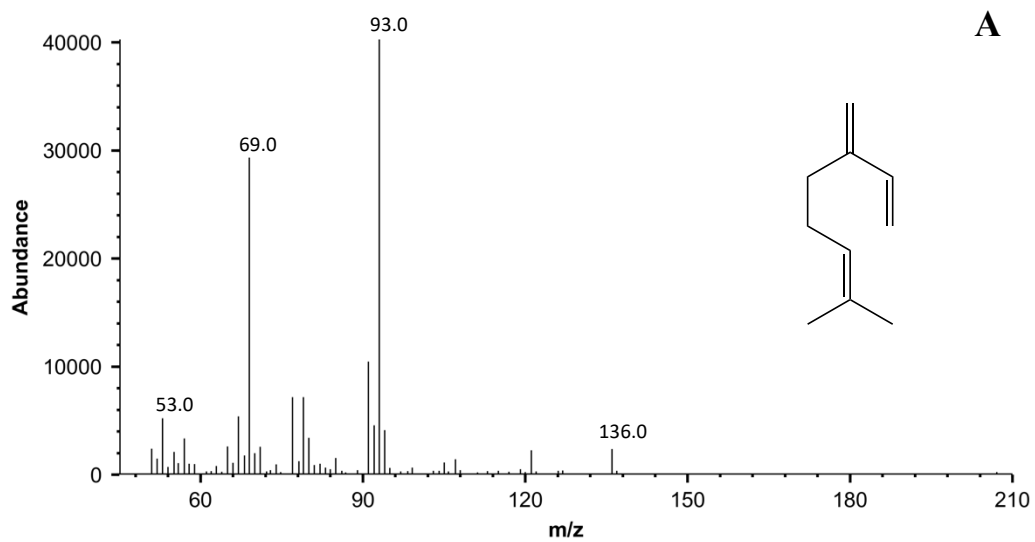


Figure 24. Mass Spectrum of A) myrcene (2) and B) myrcene-¹³C₃.

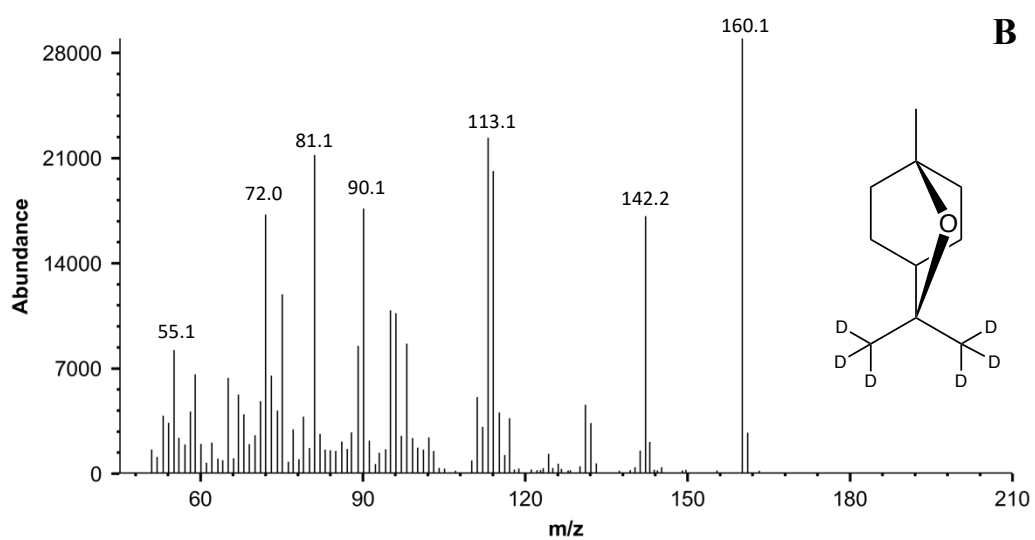
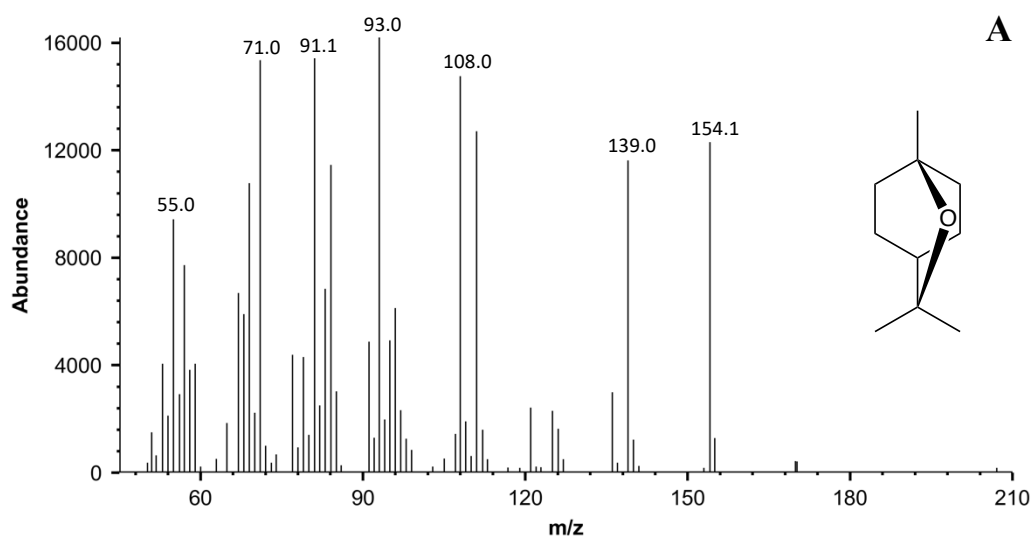


Figure 25. Mass Spectrum of A) 1,8-cineole (3) and B) 1,8-cineole- d_6 .

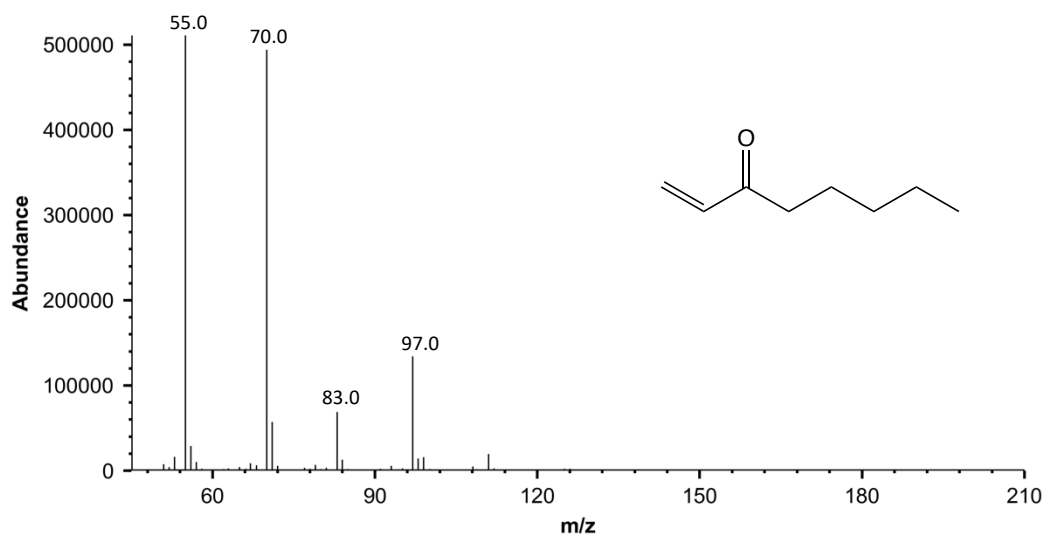


Figure 26. Mass Spectrum of 1-octen-3-one (4).

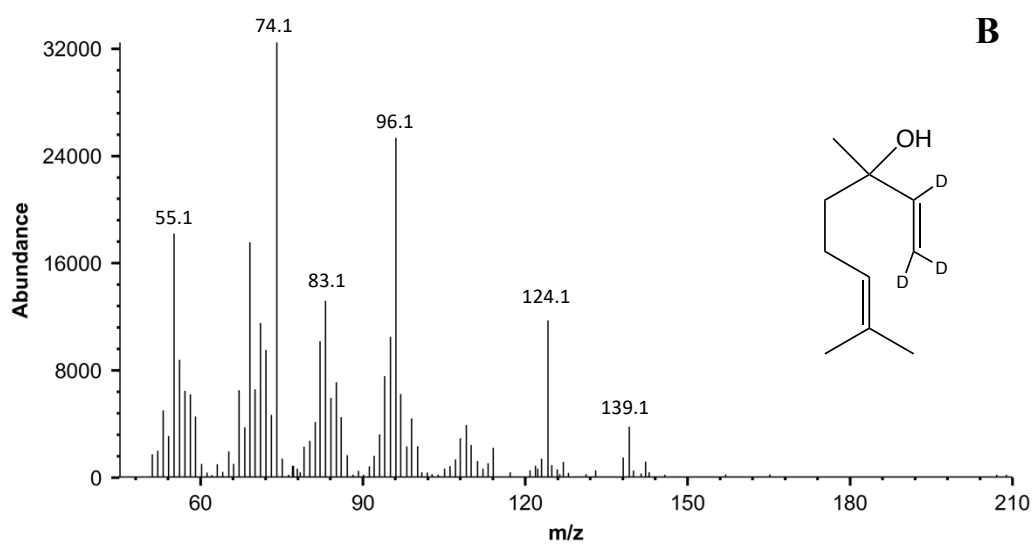
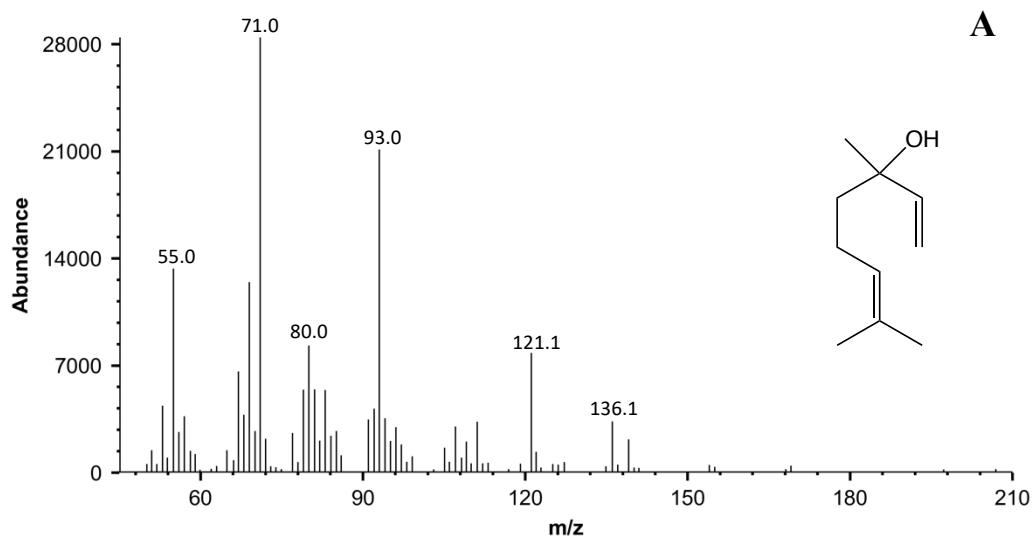


Figure 27. Mass Spectrum of A) linalool (10) and B) linalool-d3.

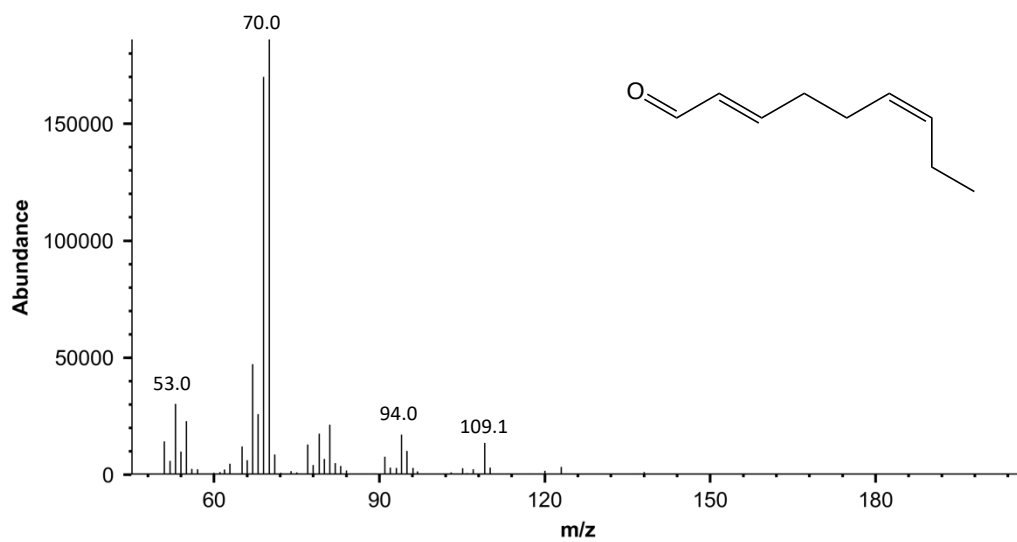


Figure 28. Mass Spectrum of (2E,6Z)-nona-2,6-dienal (11).

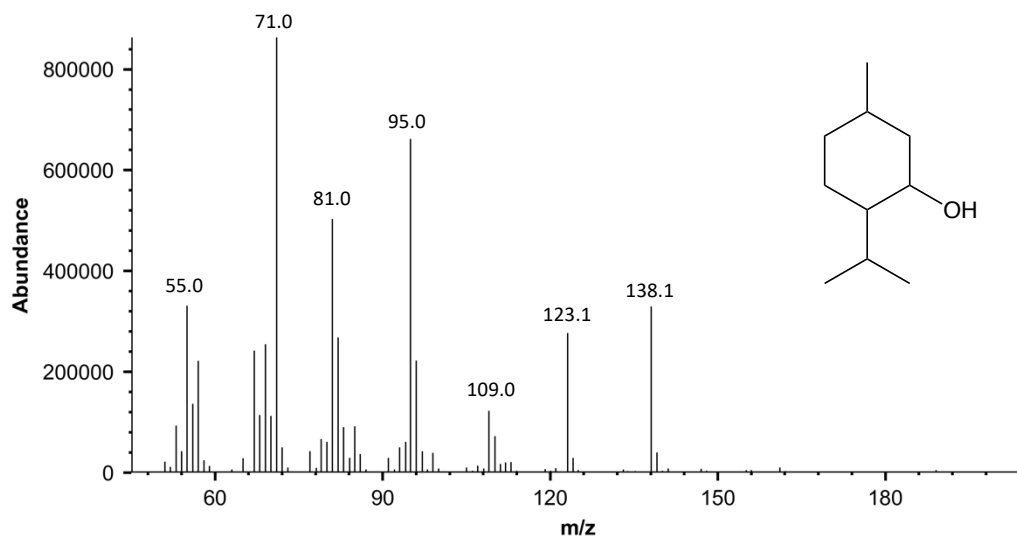


Figure 29. Mass Spectrum of neomenthol (12).

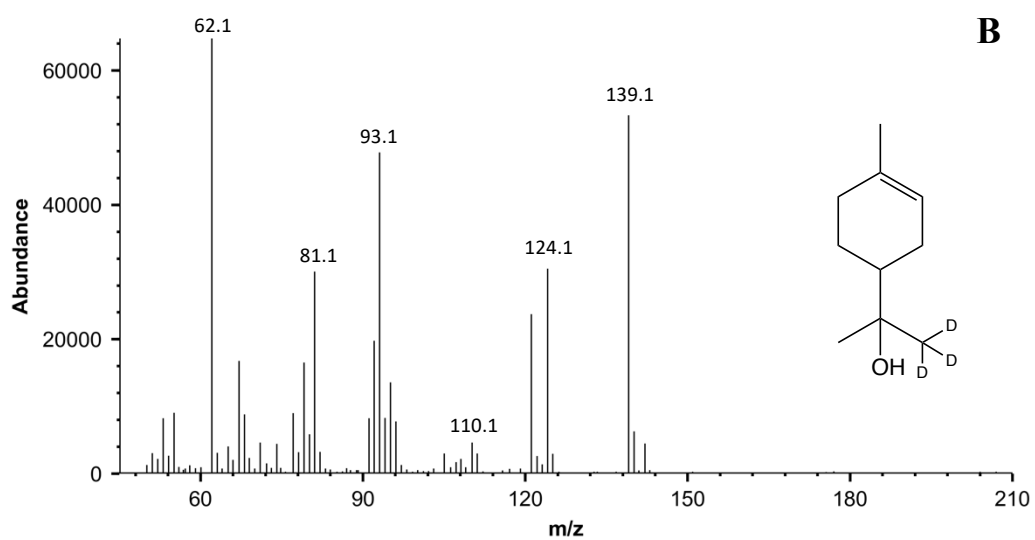
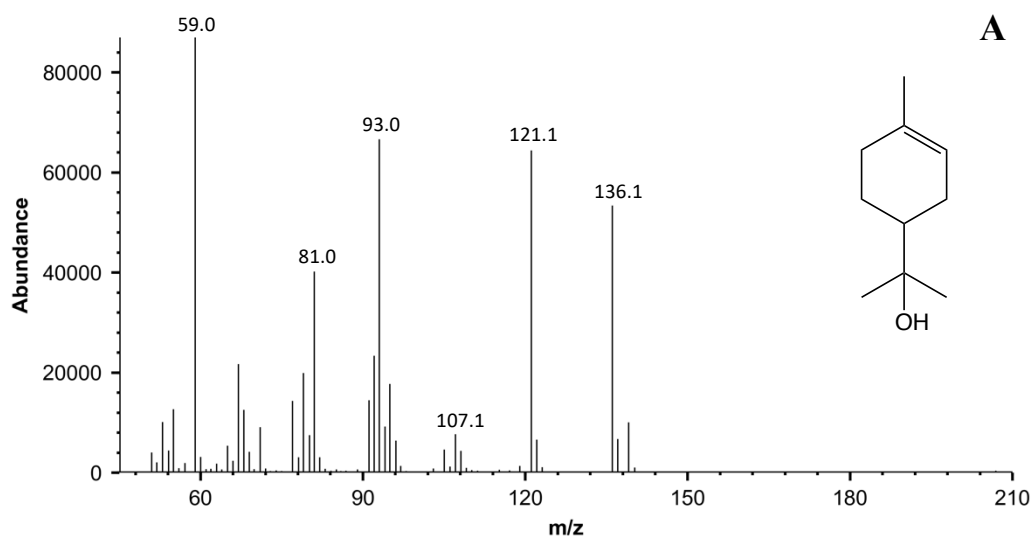


Figure 30. Mass Spectrum of A) α -terpineol (14) and B) α -terpineol- d_3 .

Table 6. Odorants Identified in *P. incanum* Plant Material with Flavor Dilution Factors of 1.

no.	odorant	odor quality	RI		FD factor
			FFAP	DB-5	
5	(5Z)-octa-1,5-dien-3-one	geranium	1366	982	1
6	1-octen-3-ol	mushroom	1440	979	1
9	(2E)-non-2-enal	fatty	1533	1161	1
15	piperitone	mint	1719	1254	1
16	β -citronellol	floral	1758	1230	1
17	geraniol	floral	1844	1257	1
18	piperitenone	mint	1901	1342	1
19	cis-jasmone	floral	1903	1400	1
22	<i>trans</i> -4,5-epoxy-(2E)-undec-2-enal*	metallic	2095	1485	1
24	thymol	phenolic	2182	1292	1

*Identified by odor quality and retention times.

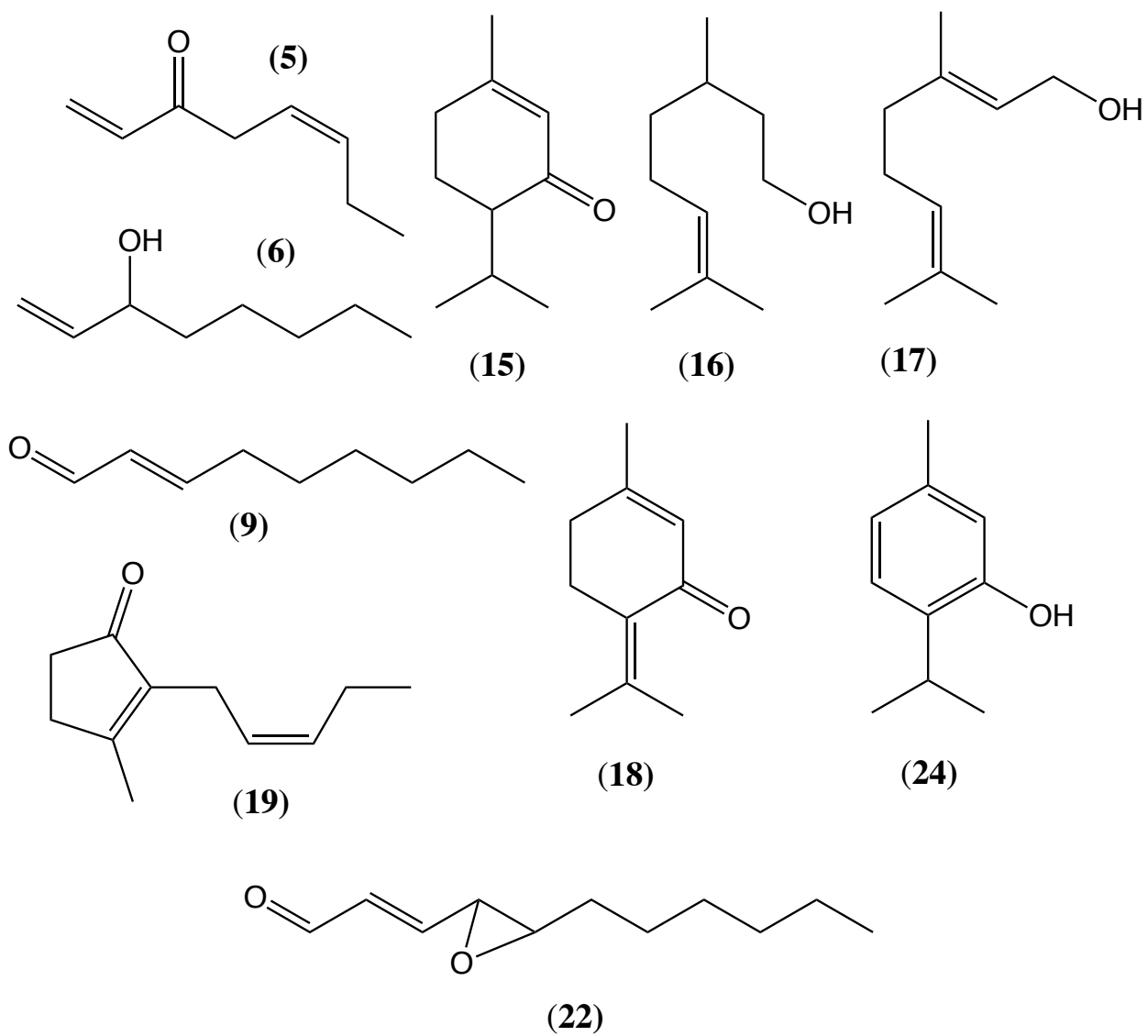


Figure 31. Structures of Odorants Identified in *P. incanum* Plant Material with Flavor Dilution Factors of 1.

Though some of the odorants with the highest FD factors exhibited a mint-like odor quality, namely pulegone, menthone, and isomenthone, it was observed that other odorants with high FD factors exhibited odor qualities such as floral, violet (β -ionone), clove (eugenol), eucalyptus (1,8-cineole), and mushroom (1-octen-3-one) which are not the predominant odor characters of *P. incanum*, but subtle odor nuances. Eleven of the odorants identified have been previously identified as volatiles in *P. floridanum*, including α -pinene, myrcene, 1,8-cineole, 1-octen-3-ol, menthone, isomenthone, neomenthol, pulegone, α -terpineol, piperitone, and piperitenone; however, they were not identified as odor-active.¹⁵ This study has been the first to identify these 24 volatiles as odor-active compounds in a member of the *Pycnanthemum* genus. Additionally, some odorants, namely β -ionone, eugenol, 1-octen-3-one, linalool and (2*E*,6*Z*)-nona-2,6-dienal, were identified as volatiles within the genus for the first time as a result of this study.

4.2. Quantitation of Key Odorants and Calculation of OAVs

To further verify the impact of odorants identified by AEDA, quantitation by SIDA was performed for odorants with FD factors ≥ 64 and a few other selected odorants. A small sample of dried plant material was combined with isotopically labelled standards prior to extraction with diethyl ether, and following filtration, analyzed by GC-MS. Each analyte was quantitated in at least triplicates and concentrations were averaged across replicates (Table 7).

The two odorants with the highest concentrations in the aerial plant tissue included pulegone and menthone, comprising 1.21% and 0.351% of the plant material, respectively. The presence of elevated levels of pulegone causes toxicity concern when considering pulegone containing selections of *P. incanum* for applications involving human consumption. The toxicity of pulegone, the primary component in the essential oil of *Mentha pulegium* L., commonly known as pennyroyal, has been well documented.²⁴ Both pulegone and its oxidative metabolite,

Table 7. Concentrations of Selected Key Odorants Identified in *P. incanum*.

no.	odorant	odor quality	concn ^a ($\mu\text{g}/\text{kg}$)
20	β -ionone	floral, violet	2110
2	myrcene	terpeny	48100
10	linalool	floral, citrus	15300
13	pulegone	mint, medicinal	12100000
17	geraniol	floral	10600
7	menthone	mint, fresh	3510000
3	1,8-cineole	eucalyptus	14900
23	eugenol	clove	5760
16	β -citronellol	floral	9520
1	α -pinene	pine	14100
6	1-octen-3-ol	mushroom	28900
8	isomenthone	mint, vegetable	104000
14	α -terpineol	terpeny	14200

^aMean of at least triplicate.

menthofuran, have been shown to have hepatotoxic properties by depletion of the antioxidant glutathione.²⁵ Even though *P. incanum* has been historically documented to be used by Native Americans as a tea, due to the high-pulegone content, plant selections with elevated levels of pulegone should not be used in preparations targeted for human consumption; however, the variability of pulegone in wild *P. incanum* populations is unknown.

To calculate OAVs and perform odor simulation experiments, an aqueous matrix was designed. Although previous studies have found that odorants can have different extraction rates when extracted in water,^{26,27} for the purposes of this study an aqueous infusion of the dried and ground aerial plant parts provided a suitable matrix for the sensory evaluations of the *P. incanum* odor recombination model. The aqueous plant extract was produced by extracting plant material (3 g) with 1 liter of water. Consequently, detection threshold values for each of the odorants of interest were determined in water and odor activity values were calculated (compound concentration/odor threshold) for the aqueous extract (Table 8). Of the 13 odorants quantitated, 11 had OAVs greater than 1, indicating they were influential in creating the odor of the *P. incanum* plant material in the aqueous matrix. The odorant with the highest OAV was β -ionone (OAV 300) which exhibits a floral, violet odor quality. Other odorants with high OAVs (OAV > 20) included myrcene (OAV 120), linalool (OAV 79), pulegone (OAV 58), geraniol (OAV 29) and menthone (OAV 22). Based on the results of the OAV calculation experiment, 11 of the 13 odorants had an OAV > 1, suggesting that these odorants potentially contribute to the odor of *P. incanum*; however, to determine definitively if all 11 odorants are necessary for the odor impression, omission experiments will need to be completed in the future.

Table 8. Aqueous Concentrations, Odor Thresholds and Odor Activity Values of key Odorants Identified in *P. incanum*.

no.	odorant	odor quality	Aqueous concn ^a ($\mu\text{g}/\text{kg}$)	odor threshold ^b ($\mu\text{g}/\text{kg}$)	ref. ^c	OAV ^d
20	<i>β</i> -ionone	floral, violet	6.33	0.021	28	300
2	myrcene	terpeny	144	1.2	29	120
10	linalool	floral, citrus	45.9	0.58	30	79
13	pulegone	mint, medicinal	36300	630		58
17	geraniol	floral	31.8	1.1	31	29
7	menthone	mint, fresh	10500	476		22
3	1,8-cineole	eucalyptus	44.7	4.0	29	11
23	eugenol	clove	17.3	1.8	30	9.6
16	<i>β</i> -citronellol	floral	28.6	4.9		5.8
1	<i>α</i> -pinene	pine	42.3	9.0	29	4.7
6	1-octen-3-ol	mushroom	86.7	45	32	1.9
8	isomenthone	mint, vegetable	312	630		< 1
14	<i>α</i> -terpineol	terpeny	42.6	59000		< 1

^aMean of at least triplicate. ^bOrthonasal odor threshold determined in water. ^cOdor threshold reference. ^dOdor activity value (concentration/odor threshold).

4.3. Sensory Evaluation of Odor Simulation Model

An odor simulation model was created by combining all 11 odorants with OAVs ≥ 1 in water at the concentration determined for the plant material. The odor model was incorporated into additional water to create the aqueous odor model (3 g odor model/1 Kg water). An aqueous extract was prepared in water at the same concentration as the odor model (3 g plant material/1 Kg water). The aqueous odor model was then compared to an aqueous extract of the original plant material by quantitative olfactory profile analysis (Figure 32). The results of the quantitative olfactory profile analysis indicated that the plant extract and aqueous odor model had comparable intensities for the sensory attributes considered with the p-values for each attribute as follows: *citrus*, $p=0.8409$; *green*, $p=0.1589$; *floral*, $p=0.1091$; *mint*, *medicinal*, $p=0.4376$; *spicy*, $p=1.0000$; *mushroom*, $p=0.3306$; *pine*, $p=0.5165$; *eucalyptus*, $p=0.8837$; *solvent*, $p=0.7288$; and *mint, fresh*, $p=0.4612$. Therefore, the statistical analysis suggested no differences existed between the aqueous odor model and odor simulation model at $p < 0.05$.

4.4. Enantiomeric Proportions of Chiral Odorants

A number of odorants identified in *P. incanum* naturally exist as optical isomers that cannot be separated without employing chiral chromatography. The relative composition of enantiomers is not frequently included for the purpose of odor analysis, but it is well known that for some chiral odorants, different enantiomers have different odor thresholds. For example, it was determined that the odor threshold of (*S*)-linalool is 80 times higher than that of (*R*)-linalool.³³ Thus, the enantiomeric proportions of certain odorants, including linalool, have the potential to impact the odor of a plant material. Likewise, odorants are often enzymatically derived in plant systems, suggesting that the generation of one enantiomer may be favored over another. Therefore, this study sought to gain further understanding of the enantiomeric composition of odorants present in

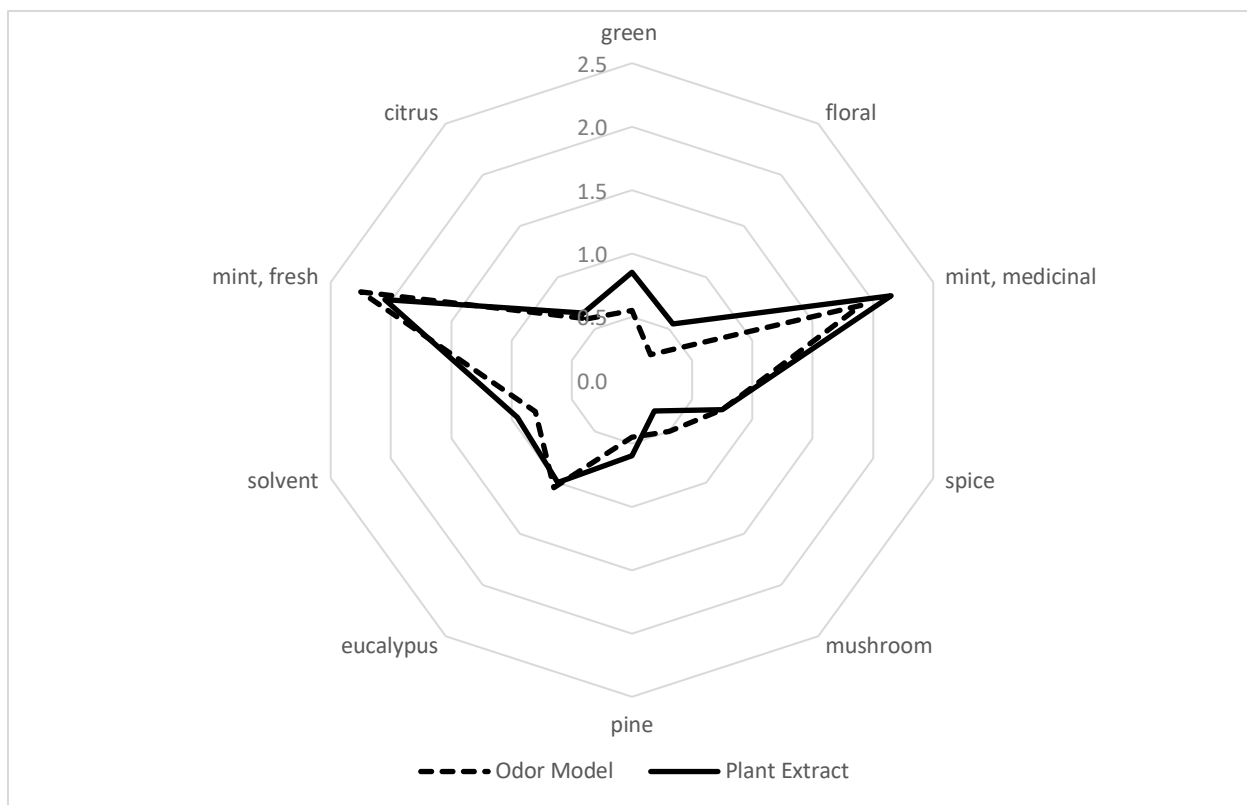


Figure 32. Olfactory Character of *P. incanum* Plant Material and *P. incanum* Odor Recombination Model.

P. incanum. A volatile isolate of dried *P. incanum* was prepared in pentane and fractionated by SPE to separate classes of odorants and reduce the amount of peak coelution during chiral GC-MS analysis. Each fraction was analyzed by chiral GC-MS, and using retention times of authentic reference standards as well as mass spectra, an enantiomeric ratio was determined (Table 9, Figure 33 - 41).

A single enantiomer was identified for five of the odorants including (*R*)-(-)-1-octen-3-ol, (*S*)-(-)- β -citronellol, (2*S*,5*R*)-(-)-menthone, (2*R*,5*R*)-(+)-isomenthone, and (*R*)-(+)-pulegone. In related genera including *Perilla* and *Mentha*, it has been reported that stereospecific conversion of geranyl diphosphate to (*S*)-(-)-limonene by limonene synthase is responsible for the presence of single enantiomers of limonene derivatives including (2*S*,5*R*)-(-)-menthone, (2*R*,5*R*)-(+)-isomenthone, and (*R*)-(+)-pulegone.^{34,35} Similar to *Perilla* and *Mentha*, the present study identified single enantiomers (>99%) of (2*S*,5*R*)-(-)-menthone, (2*R*,5*R*)-(+)-isomenthone and (*R*)-(+)-pulegone in *P. incanum*.

In contrast, scalemic mixtures of enantiomers were observed for α -pinene, linalool, and α -terpineol. Enantiomeric mixtures of these odorants have been reported in a variety of essential oils, with the relative percent of each enantiomer varying, depending on the plant material. Nonetheless, a 29.6:70.4 ratio of (+)- α -pinene to (-)- α -pinene has been reported in *M. spicata* 'Neerkalka' (29.6:70.4)³⁶ which is comparable to the composition determined in *P. incanum*: 29% (*R*)-(+)- α -pinene and 71% (*S*)-(-)- α -pinene. The composition of enantiomers determined for α -terpineol and linalool were also in line with previously reported values for other essential oils. The 85:15 ratio of (*S*)-(-)- α -terpineol to (*R*)-(+)- α -terpineol determined in this study is similar to that determined in *Laurus nobilis* L. oil (80:20).³⁷ Likewise, a ratio of 5:95 for (*R*)-(-)-linalool to (*S*)-(+)-linalool determined in this study is comparable to that of *Citrus sudachi* peel oil (7.03:92.97).³⁸

Table 9. Ratios of Enantiomeric Key Odorants Present in *P. incanum*.

no.	odorant	odor quality	FEE ^c	percent FEE ^d	SEE ^e	percent SEE ^d	Fraction ^f
1	<i>α</i> -pinene ^a	pine	(<i>R</i>)-(+)-	29%	(<i>S</i>)-(-)-	71%	F1
6	1-octen-3-ol ^b	mushroom	(<i>R</i>)-(-)-	> 99%	(<i>S</i>)-(+)-		F5
7	menthone ^a	mint, fresh	(2 <i>R</i> ,5 <i>S</i>)-(+)-		(2 <i>S</i> ,5 <i>R</i>)-(-)-	> 99%	F3
8	isomenthone ^a	mint, vegetable	(2 <i>R</i> ,5 <i>R</i>)-(+)-	> 99%	(2 <i>S</i> ,5 <i>S</i>)-(-)-		F5
10	linalool ^b	floral, citrus	(<i>R</i>)-(-)-	5%	(<i>S</i>)-(+)-	95%	F5
13	pulegone ^b	mint, medicinal	(<i>R</i>)-(+)-	> 99%	(<i>S</i>)-(-)-		F5
14	<i>α</i> -terpineol ^a	terpeny	(<i>S</i>)-(-)-	85%	(<i>R</i>)-(+)-	15%	F6
16	<i>β</i> -citronellol ^b	floral	(<i>R</i>)-(+)-		(<i>S</i>)-(-)-	> 99%	F6

^aSeparated by Rt- γ DEXsa capillary column. ^bSeparated by Rt- β DEXsm capillary column. ^cFirst eluting enantiomer. ^dRatio of peak areas from extracted ion chromatograms. ^eSecond eluting enantiomer. ^fSPE fraction that odorants were identified.

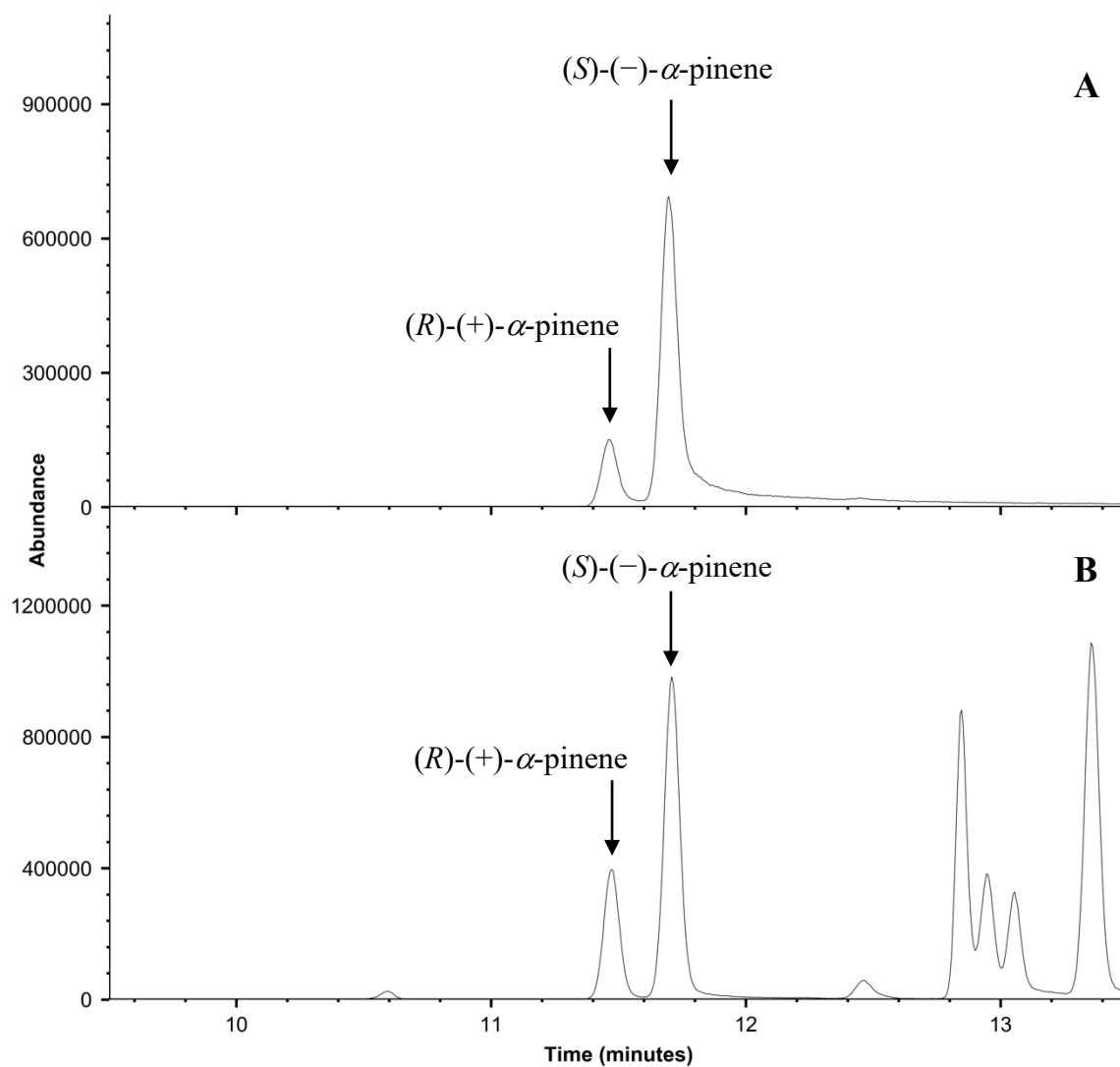


Figure 33. Separation of α -pinene enantiomers by Rt- γ DEXsa Capillary Chromatography.

A. Extracted ion chromatogram (m/z 93) of α -pinene reference standard (mixture of enantiomers); B. Extracted ion chromatogram (m/z 93) of SPE Fraction 1.

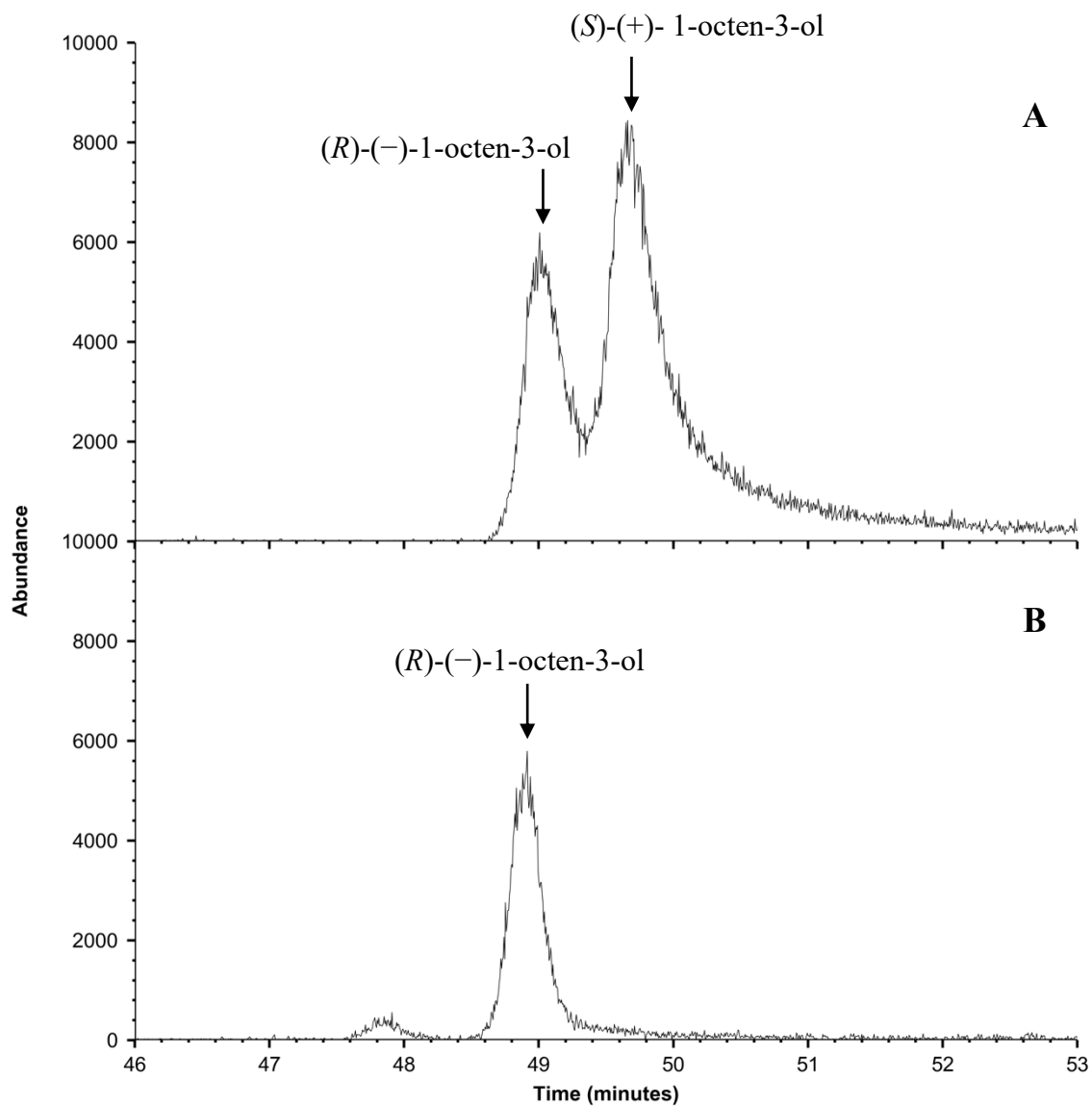


Figure 34. Separation of 1-octen-3-ol enantiomers by Rt- β DEXsm Capillary Chromatography.

A. Extracted ion chromatogram (m/z 57) of 1-octen-3-ol reference standard (mixture of enantiomers); B. Extracted ion chromatogram (m/z 57) of SPE Fraction 5.

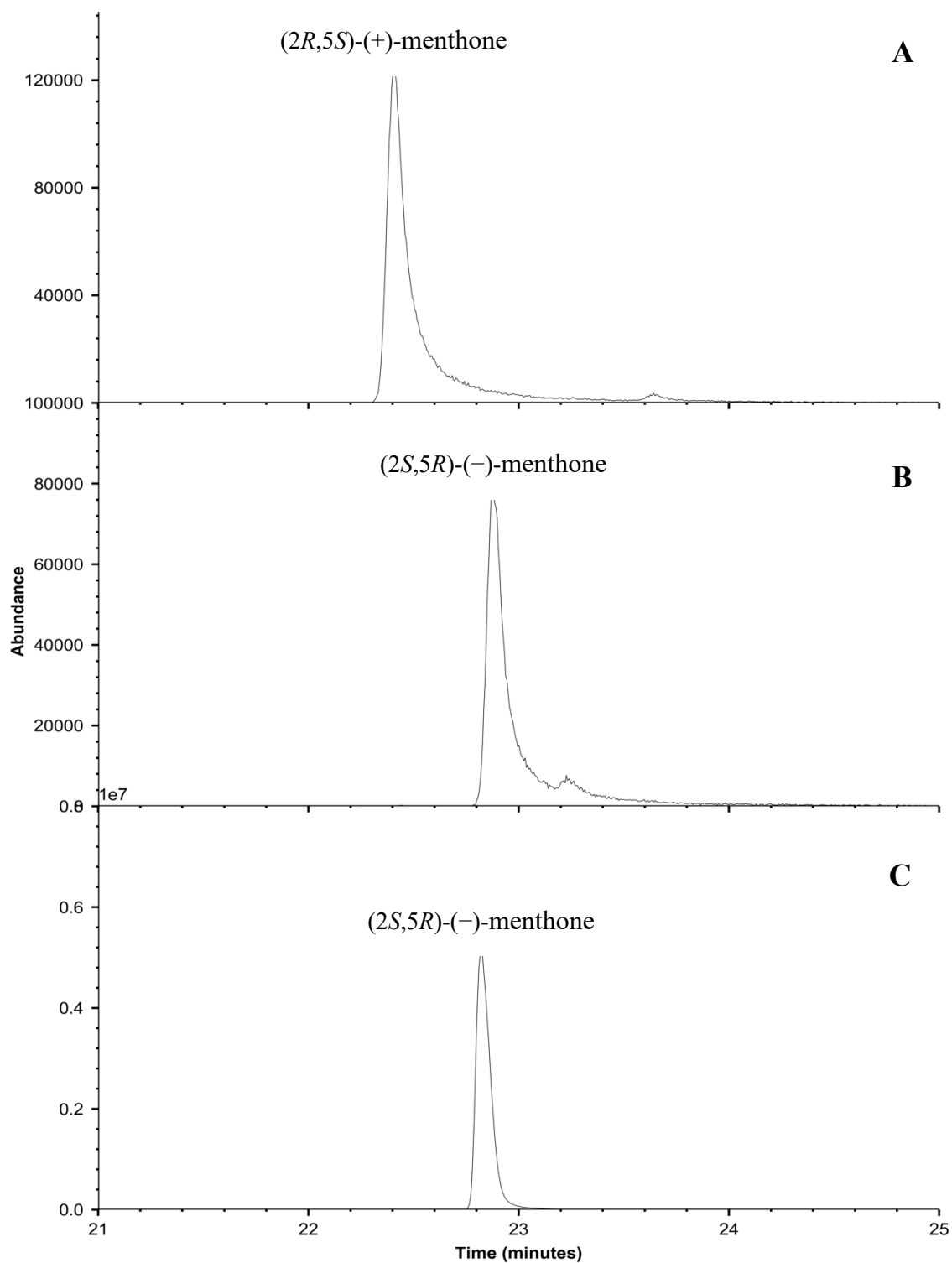


Figure 35. Separation of menthone enantiomers by Rt- γ DEXsa Capillary

Chromatography.

A. Extracted ion chromatogram (m/z 154) of (2R,5S)-(+)-menthone reference standard; B. Extracted ion chromatogram (m/z 154) of (2S,5R)-(-)-menthone reference standard; C. Extracted ion chromatogram (m/z 154) of SPE Fraction 3.

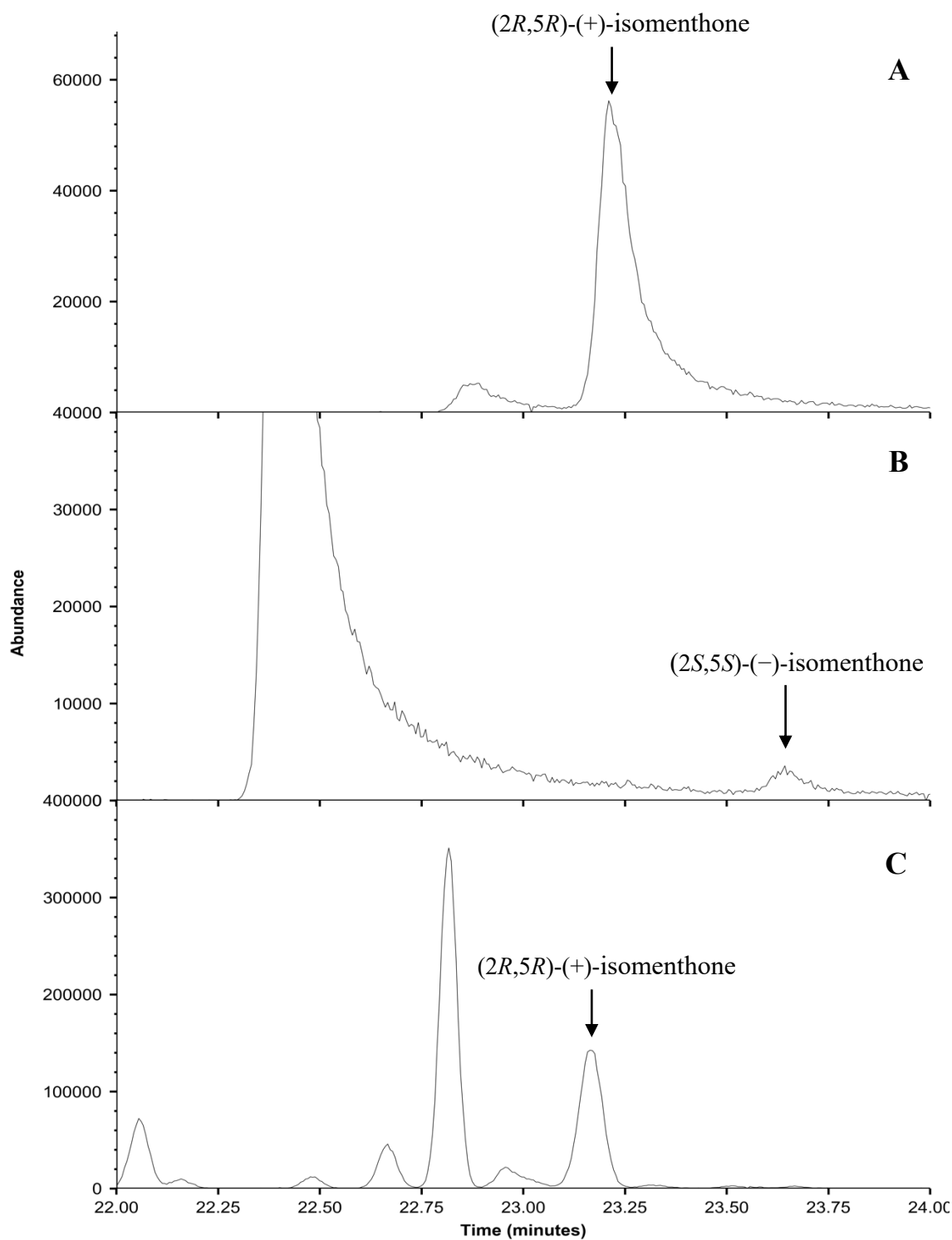


Figure 36. Separation of isomenthone enantiomers by Rt- γ DEXsa Capillary

Chromatography.

A. Extracted ion chromatogram (m/z 154) of (2R,5R)-(+)-isomenthone reference standard; B. Extracted ion chromatogram (m/z 154) of (2S,5S)-(-)-isomenthone reference standard; C. Extracted ion chromatogram (m/z 154) of SPE Fraction 5.

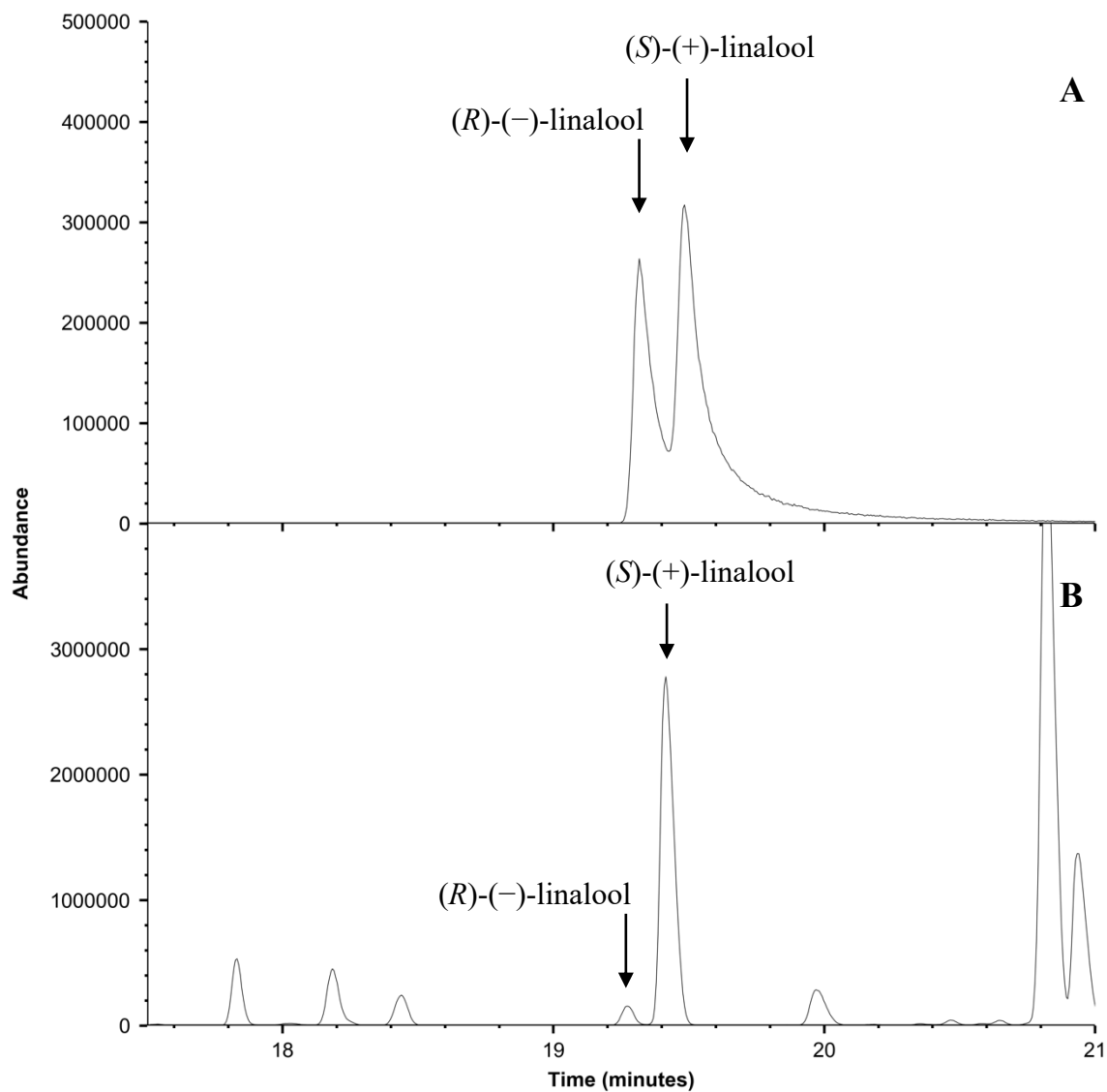


Figure 37. Separation of linalool enantiomers by Rt- β DEXsm Capillary Chromatography.

A. Extracted ion chromatogram (m/z 93) of linalool reference standard (mixture of enantiomers);

B. Extracted ion chromatogram (m/z 93) of SPE Fraction 5.

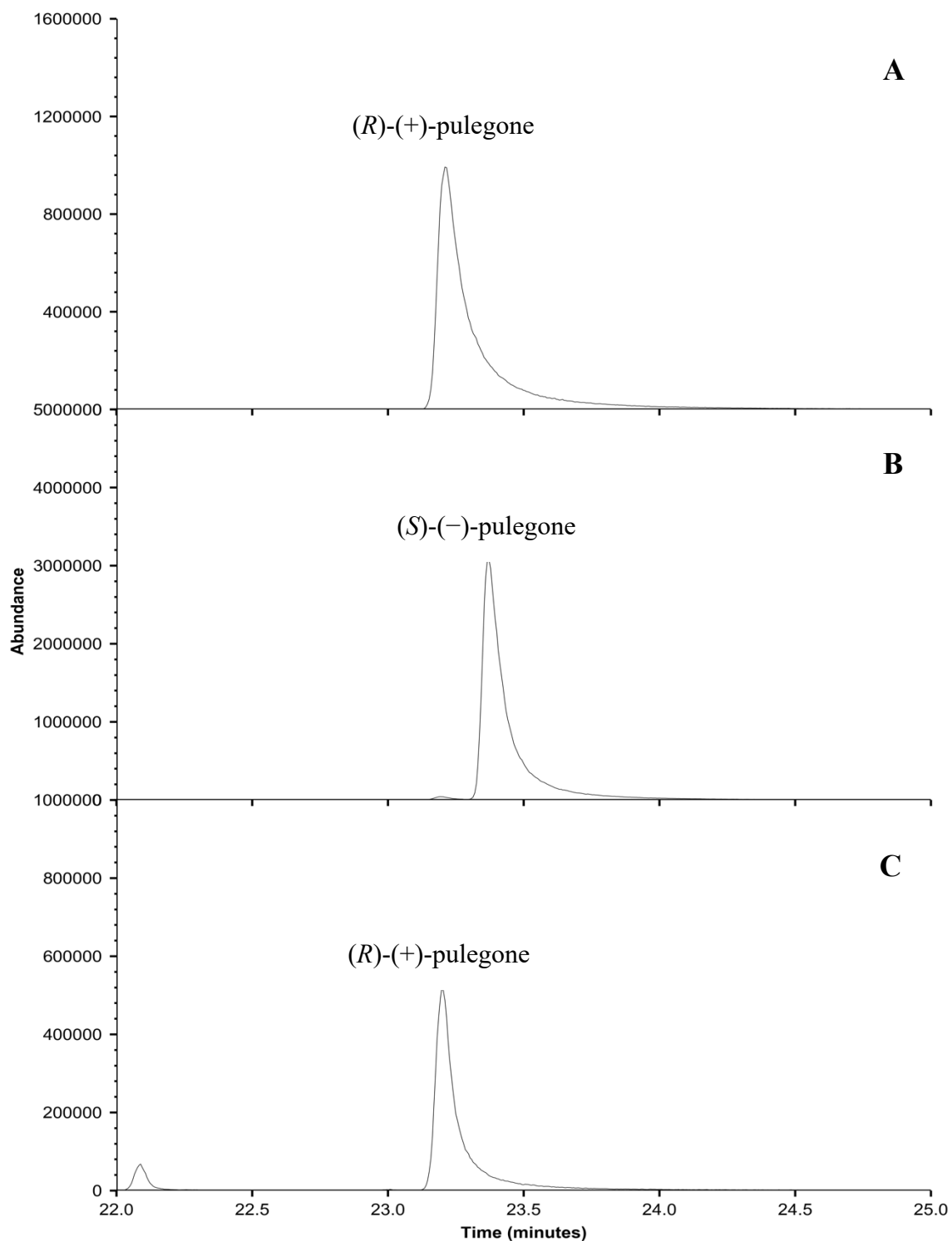


Figure 38. Separation of pulegone enantiomers by Rt- β DEXsm Capillary Chromatography.

A. Extracted ion chromatogram (m/z 81) of *(R)*-(+)-pulegone reference standard; B. Extracted ion chromatogram (m/z 81) of *(S)*-(-)-pulegone reference standard; C. Extracted ion chromatogram (m/z 81) of SPE Fraction 5.

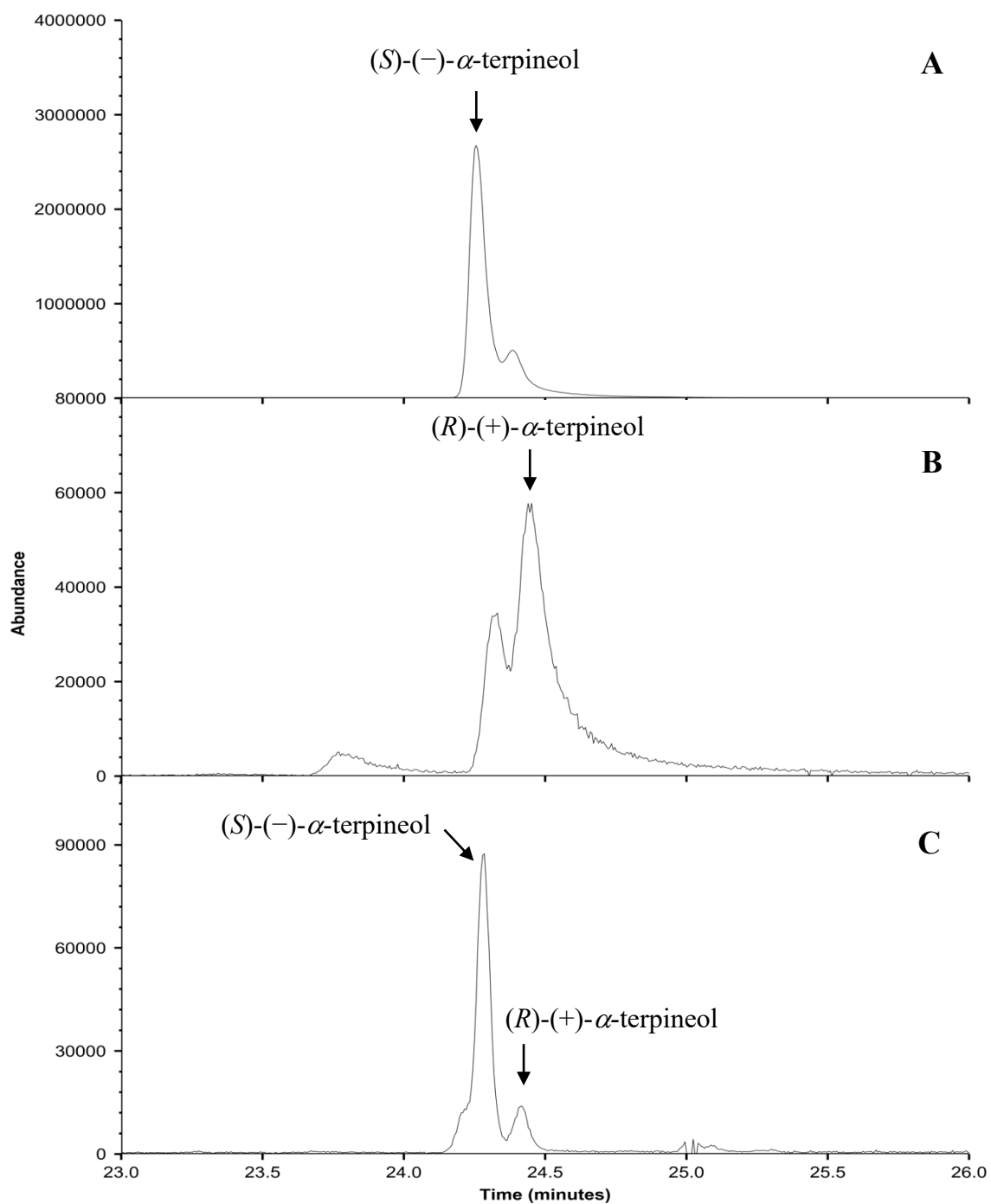


Figure 39. Separation of α -terpineol enantiomers by Rt- γ DEXsa Capillary

Chromatography.

A. Extracted ion chromatogram (m/z 136) of (S)-(-)- α -terpineol reference standard; B. Extracted ion chromatogram (m/z 136) of (R)-(+)- α -terpineol reference standard; C. Extracted ion chromatogram (m/z 136) of SPE Fraction 6.

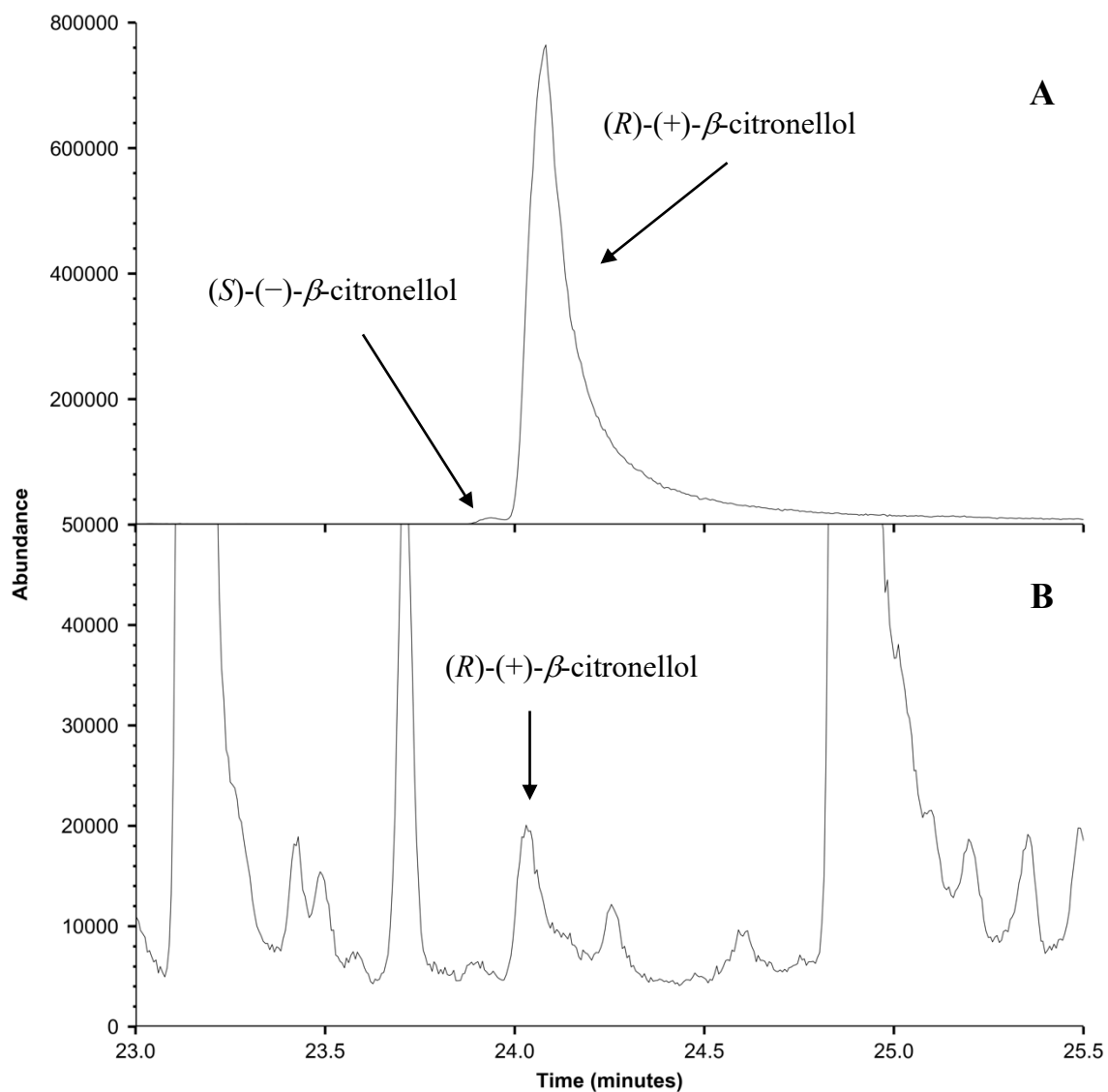


Figure 40. Separation of β -citronellol enantiomers by Rt- β DEXsm Capillary

Chromatography.

A. Extracted ion chromatogram (m/z 69) of β -citronellol reference standard; B. Extracted ion chromatogram (m/z 69) of SPE Fraction 5.

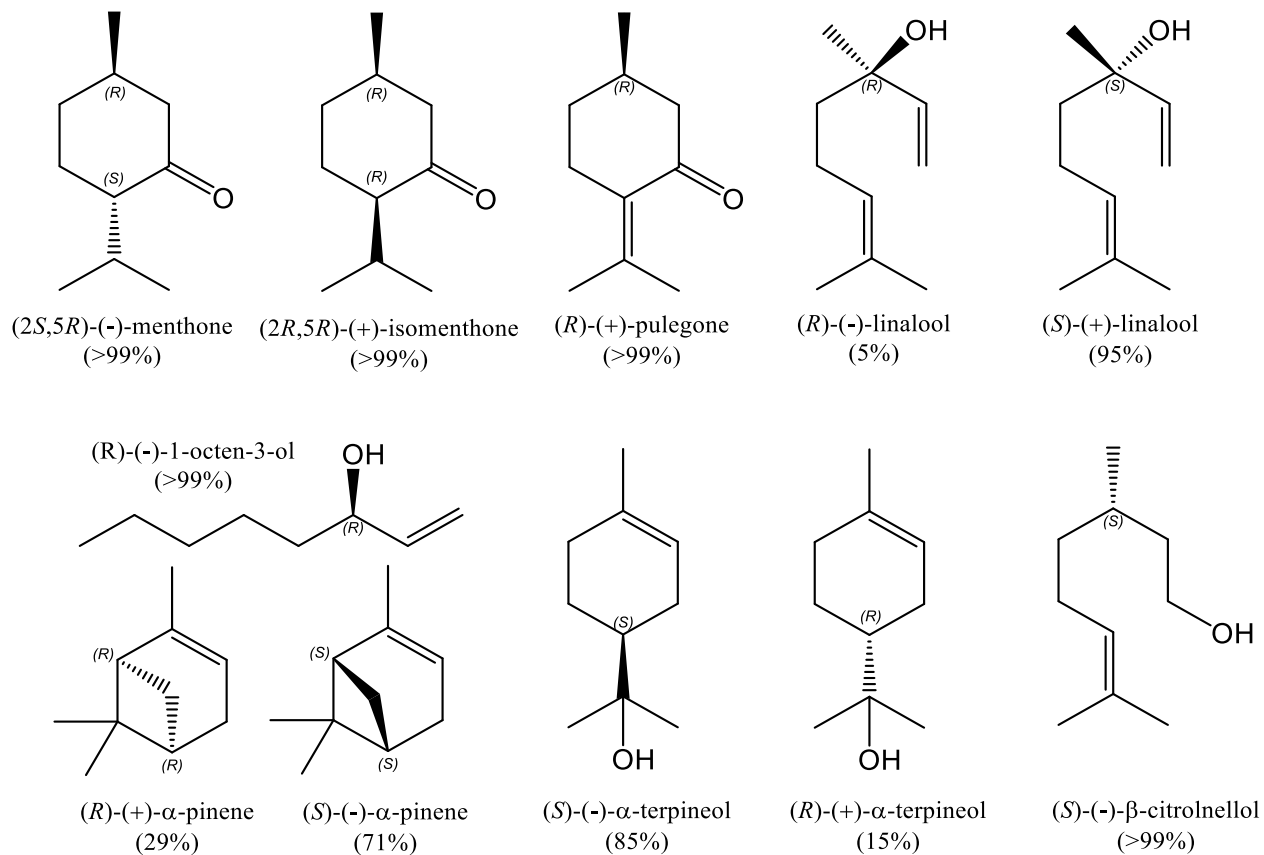


Figure 41. Chemical Structures of Key Chiral Odorants Characterized in *P. incanum*.

Furthermore, it has been proposed that linalool is a biosynthetic precursor to α -terpineol, resulting in the predominant enantiomers of these odorants having opposite signs.³⁷ For example, when (*S*)-(-)- α -terpineol is the predominant enantiomer then (*S*)-(+)-linalool will also predominate, as was observed for *P. incanum*. Overall, enantiomeric proportions of key odorants determined in this study are in agreement with previous work in other plant species and set a foundation for future studies on elucidation of the biosynthetic pathways that result in the volatile profile of *Pycnanthemum* species.

5 CONCLUSION

As a result of this study, 24 odorants were identified as contributing to the characteristic odor of *P. incanum*, including 13 that were identified for the first time as volatiles in the *Pycnanthemum* genus. Odorants with the highest flavor dilution (FD) factors included pulegone (FD 1024), menthone (FD 256), β -ionone (FD 256), eugenol (FD 256) and isomenthone (FD 64). Odor activity values were calculated for an aqueous extract of the plant material and 11 odorants were identified with OAVs >1. The odorants with the highest OAVs were β -ionone, myrcene, linalool, pulegone, geraniol and menthone. In addition, odor recombination experiments demonstrated that the key attributes of *P. incanum*, namely *mint, fresh*; *mint, medicinal*; and *eucalyptus*, could be closely matched by combining 11 odorants with OAVs >1 in an aqueous model. Though verification of the influence of each of these odorants should be further evaluated by omission experiments, this study afforded the first full characterization of the key odorants present in *P. incanum*.

This study also provided valuable insight about the chemical composition of *P. incanum* which may influence the applicability of *P. incanum* for food applications. The high levels of pulegone (1.2%; on a dry mass basis) in the aerial plant parts, raises toxicity concerns of using pulegone-containing *P. incanum* plants for human consumption. However, the diversity of odor quality that exists within wild population of *Pycnanthemum* suggests that the chemical composition in these populations may differ from that of the *P. incanum* accession studied herein (CPYC 100.000, accession PI 619340). Therefore, this work provides a solid foundation for future investigations into the unique odor of other species in the genus as well as wild populations of *Pycnanthemum*, while also opening opportunities for a variety of commercial applications.

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APPENDIX

IUPAC nomenclature for trivial compound names

trivial name	IUPAC nomenclature
1,8-cineole	1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane
(S)-(-)- β -citronellol	(S)-3,7-dimethyloct-6-en-1-ol
dihydrocarvone	2-methyl-5-prop-1-en-2-ylcyclohexan-1-one
<i>trans</i> -4,5-epoxy-(2 <i>E</i>)-dec-2-enal	(2 <i>E</i>)-3-[(2 <i>R</i> ,3 <i>R</i>)/(2 <i>S</i> ,3 <i>S</i>)-3-butyloxiran-2-yl]prop-2-enal
<i>trans</i> -4,5-epoxy-(2 <i>E</i>)-undec-2-enal	(2 <i>E</i>)-3-[(2 <i>R</i> ,3 <i>R</i>)/(2 <i>S</i> ,3 <i>S</i>)-3-pentyloxiran-2-yl]prop-2-enal
eugenol	2-methoxy-4-(prop-2-en-1-yl)phenol
geraniol	(2 <i>E</i>)-3,7-dimethylocta-2,6-dien-1-ol
β -ionone	(3 <i>E</i>)-4-(2,6,6-trimethylcyclohex-1-en-1-yl)but-3-en-2-one
(2 <i>R</i> ,5 <i>R</i>)-(+)-isomenthone	(2 <i>R</i> ,5 <i>R</i>)-5-methyl-2-propan-2-ylcyclohexan-1-one
cis-jasmone	(<i>Z</i>)-3-methyl-2-(pent-2-en-1-yl)cyclopent-2-en-1-one
linalool	3,7-dimethylocta-1,6-dien-3-ol
(2 <i>S</i> ,5 <i>R</i>)-(-)-menthone	(2 <i>S</i> ,5 <i>R</i>)-5-methyl-2-propan-2-ylcyclohexan-1-one
myrcene	7-methyl-3-methylideneocta-1,6-diene
neomenthol	(1 <i>S</i> ,2 <i>S</i> ,5 <i>R</i>)/(1 <i>R</i> ,2 <i>R</i> ,5 <i>S</i>)-5-methyl-2-propan-2-ylcyclohexan-1-ol
α -pinene	2,6,6-trimethylbicyclo[3.1.1]hept-2-ene
piperitenone	3-methyl-6-propan-2-ylidenecyclohex-2-en-1-one
piperitone	3-methyl-6-propan-2-ylcyclohex-2-en-1-one
(<i>R</i>)-(+)-pulegone	(5 <i>R</i>)-5-methyl-2-propan-2-ylidenecyclohexan-1-one
thymol	5-methyl-2-propan-2-ylphenol
α -terpineol	2-(4-methylcyclohex-3-en-1-yl)propan-2-ol

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Pycnanthemum incanum

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VITA

Melissa was born and raised in northeastern New Jersey. In high school she attended the Academy of St. Elizabeth in Convent Station, NJ and graduated in 2008. She completed her undergraduate education at Lafayette College in Easton, PA and graduated with a Bachelor of Arts in Chemistry in May 2012. After college, she spent approximately a year serving as a volunteer for AmeriCorps St. Louis. She was first introduced to the field of food science when she accepted a position as a lab technician for Mars Chocolate, NA in January 2014. Throughout her tenure at Mars Chocolate she held several research roles within the Flavor Chemistry group. In 2017 she moved to Knoxville, TN to pursue a full-time master's degree.