Chemical Variability in *Clinopodium nepeta*: A New Source of Nepetalactones

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ABSTRACT

Nepetalactoids are bioactive iridoid terpenes commonly found in the extracts and essential oil of *Nepeta cataria*. Nepetalactoids which include bioactive compounds such as the isomers of nepetalactone, nepetalic acid, dihydronepetalactone, and nepetalactam have been demonstrated to exhibit antioxidant and anti-inflammatory properties as well as and as repellents to disease-vectoring arthropods like ticks, mosquitos, and bedbugs. This paper describes the first report of nepetalactoids in *Clinopodium nepeta*. Three commercial cultivars of *Clinopodium nepeta* (designated CA018, CA021, and CA023 in the study) were greenhouse-grown, harvested at two time points, and extracted with methanol. The methanol extracts were analyzed using GC/MS to determine the aromatic volatile compounds while UHPLC-QTOF/MS was used to quantify the nepetalactoids. The resulting chemical profiles were evaluated statistically to identify potential chemotypes. CA018 was chemotypically distinct from the other two cultivars across both harvest times. Additionally, the chemical profiles of CA021 and CA023 were more similar to each other and yet also varied more between harvests than CA018. Significant differences in nepetalactoid profile between the three cultivars were observed between harvest times. 4aα,7α,7aβ-nepetalactone was present across both harvests and was the second most abundant compound by percent peak area in all three cultivars in harvest 2 (CA018 14.0%; CA021 11.9%; CA023 14.8%). In harvest 1 CA018 was significantly higher in 4aα,7α,7aα-nepetalactone than the other cultivars (harvest 1 and harvest 2 respectively CA018 20-0.7%; CA021 7.8-1.4%; CA023 6.8-2.4%). 4aβ,7α,7aβ-Nepetalactone was present across all three cultivars (Harvest 1 CA018 1.4%; CA021 0.9%; CA023 1.4%). CA018 and the other two cultivars also differed in piperitenone oxide and pulegone content with CA018 having relatively more piperitenone oxide and less pulegone compared to the other two cultivars. Mean piperitenone oxide content in harvest 1 and 2 were: CA018 23%-52.8% total peak area. CA021: 4.5%-6.0%, CA023 2.7%-3.6%. Pulegone in harvest 1 and 2 respectively: CA018 8.3%-10.5% total peak area. CA021: 19.1%-38.4%, CA023 16.9%-39.1%. The two chemotypes also consistently differed in having cis-sabinene hydrate and megastigmatrienone (present in CA018) or isomintlactone, mintlactone, and peperinic acid (CA021 & CA023). The results of this study indicate that *Clinopodium nepeta* could also serve as a source of nepetalactoids and given a diverse germplasm would be promising to explore for natural product development.

INTRODUCTION

Clinopodium nepeta (L.) Kuntze (syn. *Calamitha nepeta*), commonly known as the "Lessor Catmint," is a low-growing perennial herb that commonly

grows in dry meadows across the Mediterranean, Central Asia, and North Africa (Alan et al., 2011; Thorogood, 2019; 2021). The plant is noted for its fragrant grey-green oregano-like leaves with a minty aroma and has historically been used as a culinary herb and in folk medicines, and has shown potential in phytoremediation and as a ground cover plant (Vlachou et al., 2019). Additionally, extracts have also been demonstrated to have antioxidant, anti-tumor, and anticancer activity (Vlachou et al., 2023). More recently, some research has shown the efficacy of *C. nepeta* as an insecticide and insect repellent against two species of beetle, *Tribolium confusum* and *Sitophilus zeamais* (Debbabi et al., 2020; Pacifico et al., 2015).

Clinopodium nepeta is not a true catmint nor in the same genus as catmints (*Nepeta* spp.). However, *C.* and *Nepeta* genera are both members of the Lamiaceae family, and many *Nepeta ssp.* grow in similar regions and ecologies as *C. nepeta* (Thorogood, 2019; 2021). *Clinopodium nepeta* has also been demonstrated to produce many of the same non-iridoids aroma volatiles like β-pinene, β-caryophyllene oxide, and germacrene D which are also found in catmints like *Nepeta nepetella* (Chizzola, 2006). Despite these similarities we have not been able to find any studies that identified the presence of nepetalactone, the compound that the *Nepeta* genus (true catmint) is renowned for producing in *C. nepeta* (Gomes et al., 2021).

Nepeta cataria is the most recognized *Nepeta* species due to its eliciting a euphoric effect in cats, as a result of the plant producing nepetalactone (Lichman et al., 2020). In addition to its effect on cats, nepetalactone has demonstrated arthropod repellency against disease-vectoring organisms like ticks and mosquitoes (Birkett et al., 2011; Reichert et al., 2019; Shi et al., 2020). Nepetalactone is part of a related group of molecules, collectively as 'nepetalactoids.' These include the isomers of nepetalactone, dihydronepetalactone, the isomers of nepetalic acid, and nepetalactam. Given the importance of controlling disease-vectoring arthropods, significant effort and focus have been made in searching for plants and accessions within the *Nepeta* genus with high nepetalactoid content (Gomes et al., 2020; 2021).

In our goal of searching for additional plant sources of nepetalactoids, this study reports the grow-out and validation and finding for the first time such compounds in *C. nepeta*. Methanol extracts made from each cultivar were quantitatively evaluated for nepetalactoids using Ultra-High-Performance Liquid Chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS). Methanol extracts were also analyzed using Gas Chromatography/Mass Spectrometry (GC/MS) to cross-validate the UHPLC results and identify aromatic compounds in the *C. nepeta* extracts. Two harvests from all three cultivars were evaluated, and the results of the chemical analyses were subjected to statistical cluster analysis to identify potential chemotypes and chemotype stability across harvests.

MATERIALS AND METHODS

Cultivation, Harvest, and Extract Preparation. Seeds for the three commercial lines of *C. nepeta* were purchased from Jelitto Perennial Seed (Schwarmstedt, Germany). The three Jelitto lines identified by their catalog numbers CA018 ("White Cloud Strain"), CA021, and CA023 ("Blue Cloud Strain"). Accessions were seeded into 288 cell trays using Promix BX soil on 3/13/2020. Plants in each cell were thinned down to one plant per cell one week after germination. Plantlets were transferred to 1-gallon pots on 04/10/2020 when they had produced their second set of true leaves. This generated the study population of 6 plants per line.

Plants were grown in the New Jersey Agricultural Experiment Station (NJAES) Research Greenhouse until harvested. Plants were grown in a completely randomized fashion within the same room of the greenhouse and re-randomized across the after harvest 1. Material from each plant was harvested when the plants were in full-flower by cutting and collecting the biomass from 5 cm above the soil line. Material from each plant was harvested separately, placed in its own designated bag, and dried at 45°C for 3 days in a Memmert plant dryer. The dried plant material from each plant was then

ground to a fine powder (<2mm particle size) using a Waring WSG 30 spice grinder. Plants were harvested twice, on 6/8/2020 and 7/15/2020.

Extracts were prepared by measuring out ~100 mg of dried plant material into 15 ml conical polypropylene centrifuge tubes with recorded sample masses. 5 ml of methanol was added to the tubes. samples were vortexed for 30 seconds and sonicated in a Bransonic® Ultrasonic bath for 10 minutes and left to extract for 24 hours. Tubes were then centrifuged for 10 minutes before 1.0 mL of supernatant was placed in a microfuge tube and centrifuged at 13k rpm for 10 minutes. After centrifuging, 950 µL of the supernatant was then transferred to an LC vial. This initial concentration was used for the GC/MS analysis. The UHPLC samples were prepared from the same initial extract but diluted by a factor of ten by taking 100µL of the initial extract and adding it to 900µL methanol. This mixture was also transferred to a microfuge tube and centrifuged at 13k rpm for 10 minutes before being transferred to an autosampler vial. These two concentration extracts were prepared from each plant for both harvests. Samples were stored at - 80 °C until analyzed.

GC/MS. Gas chromatography/mass spectrometry was performed using a Shimadzu 2010 Plus chromatograph with an injection volume of 1 µL from the initial concentration methanol extract. A SH-Rxi-5Sil MS column was used to separate constituents with chromatography-grade helium as the carrier gas. Carrier gas was set to a flow rate of 1ml per minute. An inlet temperature of 250 °C was used with an injection split ratio of 5. The column temperature was set to 35 °C for 4 minutes, followed by a 20 °C increase per minute until reaching 155°C. This was held for 1.25 minutes and was then followed by a temperature increase of 10°C per minute until reaching 250°C. Peaks were integrated using the GCMSsolution v4.3© (Shimadzu) software.

The mass spectrometry was carried out in a Shimadzu TQ8040 triple-Q Mass Spectrometer. The interface temperature was set to 250°C, and the ion source was set to 200°C. The solvent cut time was defined as 3.5 minutes, and the detector was set to 0.04 kV with a threshold of 1000. The obtained mass spectra for the individual constituents were used to identify them by comparing them to known mass spectra in the literature and mass spectra libraries (NIST05.lib, NIST05s.lib, W10N14.lib, and W10N14R.lib). N-alkane (C8-C20) standards were used to generate retention indices, which were calculated, using the Lucero et al. (2009) Retention indices (RI) calculating tool, to confirm compound identification by comparing them to retention indices described in the literature. Pure standards of α-pinene, β-pinene, β-caryophyllene, camphor, camphene, eucalyptol, farnesene, dihydronepetalactone, 4aα,7α,7aα-nepetalactone, and 4aα,7α,7aβ-nepetalactone were run and used to corroborate mass spectra and Kovat Index (KI) further. The results of the six plants per line were averaged and presented as the mean percentage peak area.

UHPLC-QTOF/MS. Analysis of the diluted methanol extract was performed on an Agilent 1290 Infinity II LC in tandem with an Agilent 5456 QTOF, using a Waters Acquity UHPLC BEH C18 1.7 µm (2.1 x 50 mm) column equipped with an Acquity BEH C18 1.7 µm (2.1 x 5 mm) pre-column. During analysis, the column and precolumn were heated to 30°C. The mobile phase chosen was a combination of water (solvent 'A') and acetonitrile (solvent 'B'), and made with 0.1% formic acid v/v. The flow rate was 0.4 ml/min with a solvent gradient of 0-0.2 mins 20-30% B, 0.2-3.5 mins 30-45% B, 3.5-4.5 mins isocratic 45% B, 4.5-4.6 mins 45-100% B, 4.6-7 mins isocratic 100% B, with a post-time of 1.2 mins. The first 0.3 min and last 0.1 min of each sample were sent to waste instead of MS. Positive mode was used during data acquisition, with the range set to 100-1200 m/z and an acquisition rate of 6 spectra/s. A gas temperature of 300°C (12 l/min), a sheath gas temperature of 325°C (12 l/min), and a nebulizer pressure of 25 PSI were produced by the AJS ESI. The capillary voltage was 4000 V compared to the nozzle voltage of 2000 V, with the Fragmentor set to 130 V. Reference masses of 121.050873 and 922.009798 m/z were used for the instrument. All data was collected in centroid form. Nepetalic acid (NA1, NA2), 4aα,7α,7aα nepetalactone (NL1),

4aα,7α,7aβ nepetalactone (NL2), dihydronepetalactone (DNL), and nepetalactam (Note: no nepetalactam was quantified by UHPLC-QTOF/MS in any samples from the study population though was detected by GC) were confirmed and quantified according to a standard curve and a combination of MassHunter Qualitative Analysis and MassHunter Quantitative Analysis. The standard curve was generated using a stock solution of reference standard where all target compounds had an initial concentration of 10 µg/mL. Seven 2-fold serial dilutions were made and analyzed to generate a standard curve of a known quantity (1x to 1/128x concentration). MassHunterQualitative Analysis was used to verify the existence of borderline peaks (M. Wang et al. 2007). 4aβ,7α,7aβ-Nepetalactone (NL3) was quantified according to the NL1 standard curve, distinguished from NL1 and NL2 by its different retention time, and confirmed by GC/MS (data not presented). The final quantitative profile of nepetalactoids for each *C. nepeta* line tested was determined by averaging the quantities from the six plants per cultivar per harvest and quantities presented in micrograms of compound per milligram of dried plant material.

Herbarium specimen preparation. A specimen from each cultivar was prepared and submitted to the Chrysler Herbarium (Herbarium, 2023). Plants with all four plant organs (flowers, roots, stems, and reproductive organs) were gently removed from the pot they were grown in after the second harvest. Plants were placed in an 18 in by 24 in plant press with cardboard, construction paper, and paper towels (in that order) separators, sandwiching each plant sample. The stack of cardboard paper and plants was pressed between a small wooden press with pressure applied by two tension straps. This was then placed in the same plant dryer mentioned above for three days. Species identification was confirmed by keying out plants according to several guides based on currently accepted names and the synonym *Calamitha nepeta* (Polunin, 1969; 1987; Polunin and Smythies, 1973; Thorogood, 2019; 2021). Pressed samples were then submitted and mounted by the Chrysler Herbarium and assigned the catalog numbers: CHRB0130378 (CA018), CHRB0130385 (CA021), CHRB0130383 (CA023).

Statistics. Analysis of variance (ANOVA) was performed on the dry mass yield of the harvests, comparing the relative yields of the three cultivars. The Tukey HSD was used for post hoc analysis. Additionally, the quantified results of the nepetalactoid analysis (UHPLC-QTOF/MS) were evaluated by Multivariate Analysis Of Variance, MANOVA using Roy's Largest Root Test to detect significant differences using an alpha value of 0.05 (Roy, 1953). This was performed using the Jasp statistical suite 0.18.2 (JASP Team (2023)., n.d.).

Clustering analysis was performed using R studio on the averaged and standardized ($x_{standardized} = (x - \bar{x}) / s$. where x: real x-value. $\bar{x}$: Sample mean s: Sample standard deviation) GC/MS peak area data to compare and visualize the differences between the three cultivars within and between harvests. Hierarchical clustering was performed using Pvclust with significant differences between extracts assessed using approximately unbiased p-value (au) with $\alpha = 0.95$ (Suzuki and Shimodaira, 2006). The distance method used was Euclidian, while the clustering algorithm was Ward.D2 (Murtagh and Legendre, 2014). An nboot of one million was used for pvclust.

The same scaled data used for pvclust was also used for principal component analysis (PCA). PCA was performed using the 'prcomp' r function and visualized using the Factoextra r package (Kassambara and Mundt, 2020). The significance of differences between extracts' chemical profiles was determined based on the "approximately unbiased p-value" (au) with $\alpha = 0.95$ (Suzuki and Shimodaira, 2006).

RESULTS

Yield and Phenotypic Observations. The three cultivars of *C. nepeta* showed significant differences in growth pattern, phenotype, and yield. There was a clear difference in flower color between CA018 and CA023, given their names reflect the expected floral color ('White Cloud' and 'Blue Cloud'). The flower color of CA021 was lilac and speckled purple color as the CA023 cultivar (Figure 1).

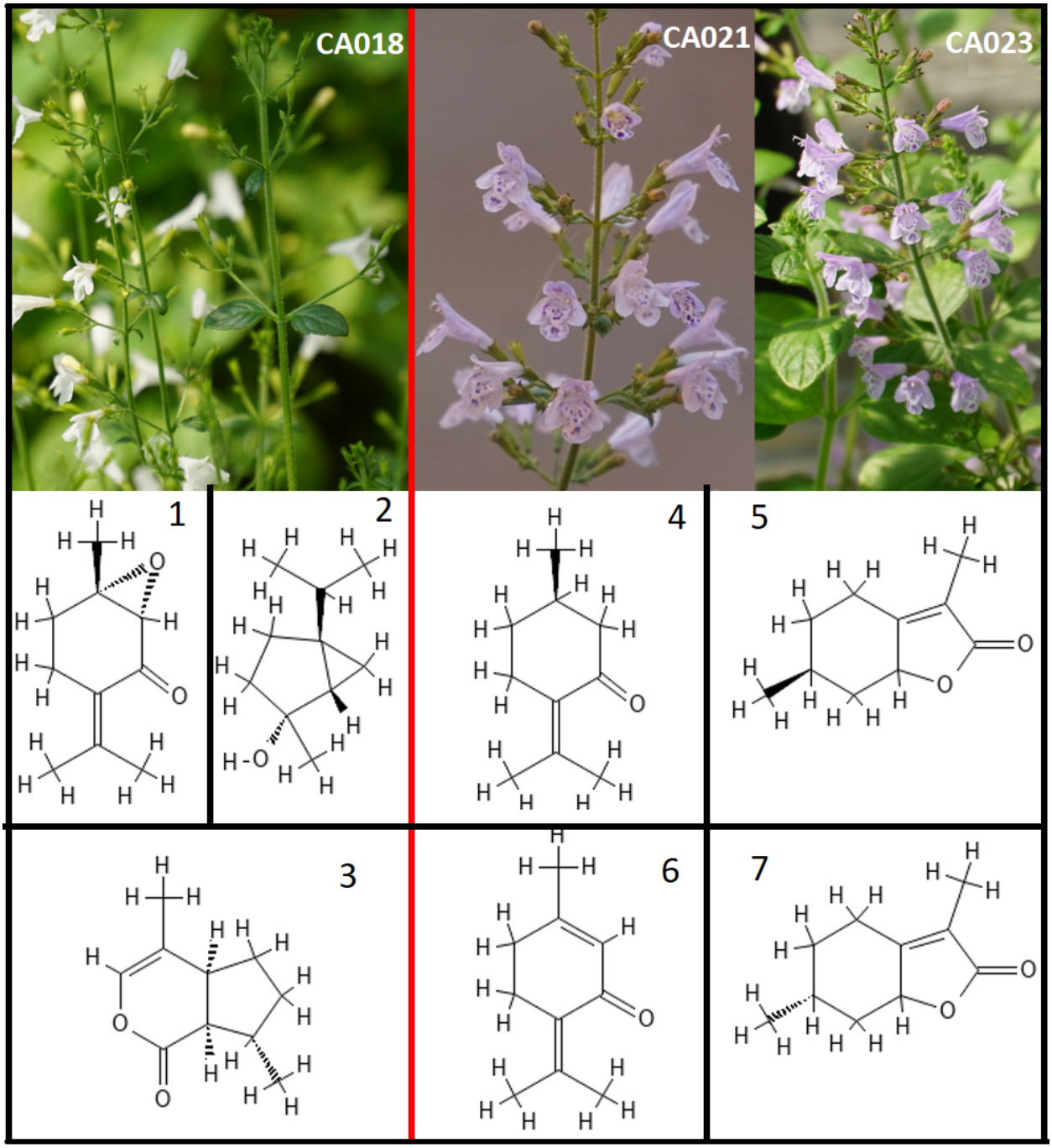

Figure 1: Close-up photographs of *Clinopodium nepeta* lines CA018, CA021, and CA023 inflorescences with compounds associated with the identified chemotypes. CA018 was found to accumulate relatively high amounts of in piperitenone oxide (1), cis-Sabinene hydrate (2), and at least in harvest 1 4aα,7α,7aα-nepetalactone (3). The CA021 and CA023 chemotypes accumulated relatively high amounts of pulegone (4), isomintlactone (5), piperitenone (6), and mintlactone (7).

The three cultivars were found to have significant differences in yield for the first harvest (Figure 2). The Tukey HSD revealed a significant difference between the dry mass yield of CA018 from that of CA021 but not CA023, while CA021 and CA023 dry mass yield were not significantly different from one another. There were no significant differences in yield at harvest 2. Notably, CA018 had less densely spaced flowers with fewer and smaller leaves on sections of the stem in full flower (Figure 1). The absence of yield differences between the three lines at harvest 2 could partly be due to plants having reached the maximum allowable size given the pot size and growing environment, as has been shown to occur with other Lamiaceae species. (Majkowska-Gadomska et al., 2018).

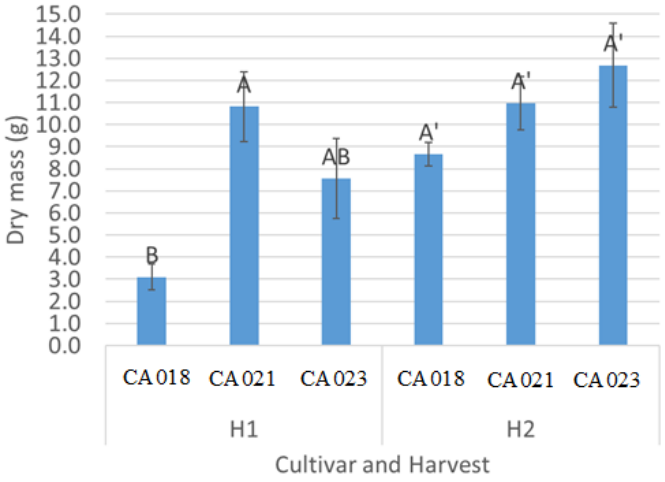

Figure 2: Average harvest yields of the three *Clinopodium nepeta* cultivars (CA018, CA021, CA023) in grams dry mass from harvest 1 (H1) and harvest 2 (H2). CLD based on Tukey HSD with a 95% confidence interval.

Natural Products Chemistry. Both UHPLC-QTOF/MS and GC/MS confirmed the presence of nepetalactone in all samples of *C. nepeta* at each of the two harvests. This is the first confirmed report of nepetalactone in *C. nepeta* or any member of the *Clinopodium* genus. While other studies have evaluated the biological activity, phenolics, and essential oil of *C. nepeta* (Beddiar et al., 2021; Božović and Ragno, 2017; Chizzola, 2006; Kirimer et al., 1992) no study has previously reported nepetalactone. Notably, the three isomers of nepetalactone detected and identified in these accessions are the three isomers most commonly found in species of *Nepeta* (Gomes et al., 2021).

The three isomers, 4aα,7α,7aα-nepetalactone, 4aα,7α,7aβ-nepetalactone, and 4aβ,7α,7aβ-nepetalactone were detected in addition to nepetalactam and nepetalic acid in all three cultivars of *C. nepeta* in the harvest 1 extracts. The extracts made from harvest 2 of all *C. nepeta* cultivars showed a higher concentration of 4aα,7α,7aβ-nepetalactone relative to the first harvest but little to none of the other isomers or other nepetalactoids. This was evident from both the higher UHPLC quantified 4aα,7α,7aβ-nepetalactone content seen in harvest 2 and the higher relative peak area of 4aα,7α,7aβ-nepetalactone in all the GC/MS profiles between harvest 1 and harvest 2 (Table 1 and Table 2). Notably, 4aα,7α,7aβ-nepetalactone was the second most abundant compound detected via GC/MS in all three *C. nepeta* cultivars and the only isomer detected by UHPLC in harvest 2. However, 4aα,7α,7aα-nepetalactone present across the extracts of harvest 2 but only by GC/MS though a much reduced peak areas compared to harvest 1.

In addition to the discovery and quantification of nepetalactone and related compounds with both analytical methods, the results of the GC/MS analysis isolated a total of 44 compounds across both harvests of all three cultivars. A total of 41 of the isolated compounds were identified (Table 2), and the content of nepetalactoids of the three cultivars in both harvests was quantified using the UHPLC/MS (Table 1). The second harvest of all three cultivars had fewer compounds than that seen from the corresponding extract in harvest 1.

Harvest 1 of CA018 was evaluated by GC/MS, where 99% of total peak area was explained by 24 isolated peaks. Of these, 21 were identified. The majority of CA018 harvest 1 average peak area was comprised of piperitenone oxide (23.05%), 4aα,7α,7aα-nepetalactone (20.00%), 4aα,7α,7aβ-nepetalactone (9.33%), pulegone (8.32%), neophytadiene 1(8.28%), nepetalactam (3.66%), and cis-sabinene hydrate (3.44%) which accounted for 76.07% of total peak area. When quantified by UHPLC harvest 1 of CA018 had the highest content of nepetalic acid 1, nepetalic acid 2, and 4aα,7α,7aα-

nepetalactone in the study with an average content of 1.572 µg/mg, 1.939 µg/mg, and 0.21 µg/mg of dry plant material respectively. CA018 Harvest 1 also had the highest 4aα,7α,7aα-nepetalactone relative peak area seen in the study. Harvest 1 of CA018 also saw a similar relationship with 4aα,7α,7aβ-nepetalactone and 4aβ,7α,7aβ-nepetalactone where CA018 had the lowest and the second highest quantified contents (0.367µg/mg and 0.013 µg/mg respectively), and relative peak area (9.3% and 1.36% respectively) in the study.

CA018 had the lowest content of 4aα,7α,7aβ-nepetalactone from harvest 2 with 0.434 µg/mg dry weight. A total of 17 compounds counted for 99% of the total identified peak area of the CA18 harvest 2. Primarily the peak area of CA018 harvest 2 was comprised of piperitenone oxide (52.77%), 4aα,7α,7aβ-nepetalactone (14.02%), pulegone (10.49%), neophytadiene 1(4.09%), cis-sabinene hydrate (3.35%), and piperitenone (2.85%) which accounted for 87.58% of total peak area.

Harvest 1 of CA021 identified 31 compounds were making this cultivar's extract most chemically diverse. Of these 28 were identified which accounted for 96.44% of total peak area. This harvest was primarily composed of pulegone (19.08%), neophytadiene 1 (13.02%), 4aα,7α,7aα-nepetalactone (7.77%), 4aα,7α,7aβ-nepetalactone (7.32%), piperitenone (6.13%), piperitenone oxide (4.55%), and neophytadiene 3 (4.38%) which together comprised 62.25% of total average peak area. The results of the UHPLC showed that CA021 had the lowest nepetalic acid contents compared to the other cultivars of harvest 1 (NA1= 0.576 µg/mg dry weight. NA2 = 0.719 µg/mg dry weight). The UHPLC found no 4aβ,7α,7aβ-nepetalactone though trace amounts were detected in the GC/MS (0.88% total peak area).

Harvest 2 of CA021 was comprised of 25 compound and 75.4% of total peak area was made up of pulegone (38.42%), 4aα,7α,7aβ-nepetalactone (11.94%), neophytadiene 1 (6.37%), piperitenone oxide (6.02%), piperitenone (4.85%), mintlactone (3.99%), and β-caryophyllene oxide (3.77%) total 75.35% of mean total peak area. While CA021 at harvest 2 had the lowest percentage area composed of 4aα,7α,7aβ-nepetalactone compared to the other extracts in harvest 2, CA018 had the second highest quantified amount in the study with 0.535 µg/mg dry weight.

The extract of harvest 1 CA023 isolated 29 compounds with 26 identified, accounting for 96.98% of total peak area. The major compounds isolated were pulegone (16.87%), neophytadiene 1 (14.99%), 4aα,7α,7aβ-nepetalactone (7.45%), 4aα,7α,7aα-nepetalactone (6.83%), 1,3,4,5-tetrahydroxy cyclohexanecarboxylic acid (5.77%), N-3-butenyl-n-methyl-cyclohexanamine (4.62%), neophytadiene 3 (4.55%), and piperitenone (3.93%) which altogether accounted for 65.01% of total peak area. Both LC/MS and GC/MS detected 4aβ,7α,7aβ-nepetalactone at the highest concentrations of this study in the first harvest of CA023 with an average 0.020 µg/mg dry weight making up 1.44% of peak area. CA023 harvest 1 also had the highest concentration of 4aα,7α,7aβ-nepetalactone of the cultivars with 0.434 µg/mg dry weight.

CA023 also had the highest concentration of 4aα,7α,7aβ-nepetalactone in harvest 2 with an average of 0.897 µg/mg dry weight, and the highest relative concentration by relative peak area in the harvest and the study with 14.84% of total peak area. Harvest 2 was also found to be composed primarily of pulegone (39.14%), neophytadiene 1 (7.12%), peperinic acid (3.70%), caryophyllene oxide (3.69%), and piperitenone oxide (3.55%) which combined with 4aα,7α,7aβ-nepetalactone made up 72.05% of total peak area.

DISCUSSION

Several differences between the chemical profiles of cultivars were observed both in the GC/MS and UHPLC-QTOF/MS analysis of the extracts. Cultivar played a prominent role in chemical profiles. The results of the MANOVA performed on the quantified nepetalactoids in Harvest 1 yielded a p-value of 0.019, thus leading to the rejection of the null hypothesis of no difference existing between the nepetalactiod content and cultivar. As Harvest 2 only found 4aα,7α,7aβ-nepetalactone when quantifying by UHPLC-QTOF/MS, MANOVA was determined to be

inappropriate for evaluating differences between cultivars in Harvest 2's nepetalactiod content. ANOVA of the UHPLC-QTOF/MS quantified 4aα,7α,7aβ-nepetalactone content did not reveal any significant differences based on cultivar. Differences in the chemistry of each cultivar were further evaluated through statistical clustering and principal component analysis of the GC/MS results.

When the scaled GC/MS profiles of the extracts were evaluated by pvclust it was found that CA018 extracts were not significantly different from each other between the extracts made from the two harvests (Figure 3). CA018 extracts were found to be significantly different from all other extracts but clustered significantly based on their au of 95 at leaf 3. The chemical profiles of CA021 and CA023 clustered strongly within the same harvest with both the harvest 1 and harvest 2 clusters having au of 100.

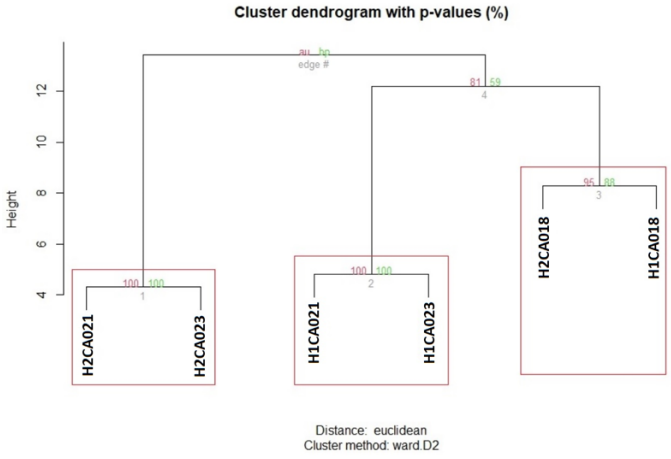

Figure 3: Dendrogram From pvclust. Rectangles denote significant clusters based on Approximately Unbiased p-value (au) with confidence ≥ 95%.

The Harvest 1 CA021 and CA023 were more similar to the profiles of both CA018 based on the relative position of leaf four (Figure 3). When factoring in the results of the principal component analysis (Figure 4) with the pvclust the impact of harvest was determined to have a more significant impact on extract chemical composition than cultivar, and harvest had a more significant impact on CA021 and CA023 profiles than the profiles of CA018. This can be seen in the relative position of the respective data points along the primary principal component, which accounted for 47% of the total variance. All three cultivars are separated by harvest along the primary axis and are at opposite ends along PC1. In addition, within the same harvest, CA021 and CA023 are far more similar to each other than to CA018 (Figures 4 and 5). A notable difference between harvests was the fact that several compounds only appear in one harvest and not both, along with several compounds only existing in specific cultivars. This helps to explain the separation, given the sensitivity of PCA to binary relationships of the presence or absence of a variable (Abbott, 2014). The results of the PCA and pvclust lead to the conclusion that harvest had a more significant impact on the chemical profiles of CA021 and CA023 than on CA018. This also suggests that CA018 is a different chemotype compared to the other two cultivars.

CA018 extracts had relatively few compounds unique to the cultivar across both harvests but were consistently far more abundant than the other two cultivars in cis-sabinene hydrate, megastigmatrienone 2, and piperitenone oxide. Though the relative content of cis-sabinene hydrate in CA018 is below 5 % total peak area has been noted and used to identify different chemotypes in other Lamiaceae family members like thyme (Groendahl et al., 2008) and marjoram (Groendahl et al., 2008; Novak et al., 2002). Piperitenone oxide was consistently the most abundant compound in all CA018 extracts across both harvests and far exceeded the percentage peak areas and total average peak area (higher proportion and total area) of the other two cultivars. Piperitenone oxide has importantly been demonstrated to have repellent and insecticidal properties against the malarial vector *Anopheles stephensi* (Tripathi et al., 2004). The consistently higher relative content of piperitenone oxide, cis-sabinene hydrate, and megastigmatrienone 2 across harvests supports CA018 having a distinct chemotype from the other two cultivars (Patonay et al., 2021). This is further reflected where cis-sabinene hydrate, megastigmatrienone 2, and piperitenone oxide all vector toward the CA018 extracts and account for much of the variability of the second principal component (Figure 4). By contrast, the other two cultivars had many compounds in

common with each other across harvests that were far in excess and/or absent from CA018 extracts.

The compound that consistently had the highest concentration across harvests and cultivars (CA021 and CA023 chemotype) was pulegone. Pulegone was also observed to explain a large portion of the variability in both the primary and secondary principal components (Figure 4). Pulegone like piperinenone oxide has also demonstrated insect repellency and toxicity having demonstrated insecticidal effects against *Rhyzopertha dominica*, *Sitophilus oryzae*, and *Tribolium castaneum* (Ramadan et al., 2024) as well as being an effective fumigant against *Cryptolestes ferrugineus* (Fan et al., 2023).

Table 1: UHPLC-QTOF/MS quantified nepetalactoid content from both harvests of the three *Clinopodium nepeta* in µg/mg dry mass.

	NA1		**NA2**		**DNL**		**NL3**		**NL1**		**NL2**	
	Mean	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***
						Harvest 1						
CA018	1.572	0.503	1.939	0.631	0.001	0.001	0.013	0.013	0.210	0.034	0.367	0.121
CA021	0.576	0.056	0.719	0.040	0.000	0.000	0.000	0.000	0.115	0.011	0.426	0.068
CA023	0.790	0.201	0.762	0.242	0.000	0.000	0.020	0.013	0.092	0.015	0.434	0.046
						Harvest 2						
CA018	0.000		0.000		0.000		0.000		0.000		0.434	0.197
CA021	0.000		0.000		0.000		0.000		0.000		0.535	0.133
CA023	0.000		0.000		0.000		0.000		0.000		0.897	0.255

NA1= Nepetalic Acid 1, NA2= Nepetalic Acid 2, DNL= dihydronepetalactone , NL3= 4aβ,7α,7aβ-nepetalactone, NL1= 4aα,7α,7aα-nepetalactone NL2= 4aα,7α,7aβ-nepetalactone

This critical difference between the extracts of CA018 and CA021, CA023 of having pulegone over piperitenone oxide and vice versa implies a difference in the expression and or activity of (-)-isopiperitenone reductase as it is the enzyme at the branch point of the biosynthesis of pulegone vs. piperitenone oxide (Croteau and Venkatachalam, 1986; Mahmoud and Croteau, 2003; Ringer et al., 2003). In addition to the key difference of pulegone and piperitenone oxide content, the CA021 and CA023 group also contained piperitenone, caryophyllene oxide, and neophytadiene 1 at higher proportion across all harvests compared to CA018. The CA021 and CA023 chemotypes also contained peperinic acid, isomintlactone, and mintlactone in both harvests, which CA018 lacked altogether. This higher content of piperitenone in CA021 and CA023 compared to CA018 also supports the potential difference in the expression of terpenoid epoxidase which converts piperitenone to piperitenone oxide (Mahmoud and Croteau, 2003). The chemotypes identified based on differences in pulegone and piperitenone oxide content are supported by previous works that have identified similar patterns across *C. nepeta* accessions and cultivars (Alan et al., 2011; Marongiu et al., 2010).

The relative content of nepetalactone isomers is commonly used to distinguish chemotypes in populations of *Nepeta* spp. (Aćimović et al., 2022; Gomes et al., 2021; Mollova et al., 2023; Srivastava et al., 2021). The present study shows clear evidence for the presence of nepetalactoids in *C. nepeta* accessions, which can be used to distinguish chemotypes. Additionally, all three cultivars of *C. nepeta* at both harvests have relative amounts of nepetalactone in relative higher concentrations than that reported in the essential oil of many species of *Nepeta* spp., which either have no nepetalactone of relative continents of below 5% of total peak area (Gomes et al., 2021). Results of the MANOVA showed a significant difference between cultivars. CA018 exhibited significantly greater concentrations

of 4aα,7α,7aα-nepetalactone than the other two cultivars at only the first harvest. This is significant as 4aα,7α,7aα-nepetalactone and 4aα,7α,7aβ-nepetalactone are produced from biochemical pathways that require different enzymes and by extension, different genes (Hernández Lozada et al., 2022).

The reductions in the relative amount of 4aα,7α,7aβ-nepetalactone at the second harvest could potentially be attributed to seasonal variation, which has been previously described to impact nepetalactoid profiles in *Nepeta ssp.* (Schultz et al., 2004). Prior work reported significant impacts from seasonality and environmental conditions on the chemical profile of *C. nepeta* (Pacifico et al., 2015; Vlachou et al., 2023). The chemical composition of *C. nepeta* essential oils including pulegone and piperitenone oxide has been observed to be significantly impacted by the time of season and ambient temperature during cultivation (Pacifico et al., 2015; Vlachou et al., 2023). Yet, given the consistency of the high relative content across both harvests of compounds like pulegone and peperitenone oxide and the underlying biochemistry controlling their production (Croteau and Venkatachalam, 1986; Mahmoud and Croteau, 2003; Ringer et al., 2003) strongly suggests a genetic component controlling for these observed traits and supports the conclusion that CA018 and the other two cultivars are different chemotypes (Patonay et al., 2021). This difference in chemotype between CA018 and the other cultivars also corresponds with the difference in flower color with CA018 having white flowers and the others with speckled purple lilac flowers.

The results demonstrate the presence of nepetalactoids in each of the three cultivars of *C. nepeta*. Additionally, all three cultivars had contents of nepetalactoids that were similar to or higher than those reported in many *Nepeta* species (Gomes et al., 2021; Patel et al., 2022). The presence of insect-repellent compounds like nepetalactone in addition to having high contents in other arthropod-repellent compounds like piperitenone oxide (Tripathi et al., 2004) and pulegone (Fan et al., 2023; Ramadan et al., 2024) means *C. nepeta* could have significant potential for improvement and application against harmful arthropods. The consistency of which compounds were dominant in the extracts made from the different cultivars across harvest with CA018 being chemotypically distinct from CA021 and CA023 with the former being high in piperitenone oxide and having cis-sabinene hydrate, megastigmatrienone 2 while the other cultivars had little or none while the other group was higher in pulegone relative to peprinenone oxide.

Future studies should evaluate more accessions and cultivars of *C. nepeta* as well as potentially evaluate other understudied members of the *Clinopodium* genus for nepetalactoids and other bioactive compounds. Additionally, as many other papers have described there is significant variation within the *C. nepeta* species (Alan et al., 2011; Božović & Ragno, 2017; Chizzola, 2006; Debbabi et al., 2020; Vlachou et al., 2023).

Table 2: Aromatic volatiles of *Clinopodium nepeta* extracts from two harvests (H1 and H2) analyzed using gas chromatography coupled to mass spectrometry (GC/MS). RT = retention time (min). RI = Retention Indices experimentally calculated using a n-alkane (C8-C20) standard. The results are the mean of 6 replicates. 1 I= Identification method *= tentatively identified by MS. **=identified by MS and RI. MS and RI from literature presented in supplemental materials.

RT	RI	I[1]	Compound	CA018		CA021		CA023	
				H1	H2	H1	H2	H1	H2
7.69	925	**	1,2-cyclopentanedione	1.29	0.00	1.99	0.00	2.68	0.00
7.78	932	**	α -pinene	0.00	0.00	0.00	0.00	0.00	0.30
8.42	990	**	allyl formate	0.43	0.00	2.21	0.00	2.39	0.00
9.23	1069	*	cyclohexanamine, N-3-butenyl-n-methyl-	0.00	0.00	3.56	0.00	4.62	0.00
9.25	1071	**	cis-sabinene hydrate	3.44	3.35	0.00	0.00	0.00	0.20
9.86	1134	**	dihydroxy maltol	0.00	0.00	1.72	0.00	2.58	0.00
10.06	1156	**	menthone	0.00	0.00	0.00	0.00	1.04	0.00
10.10	1160	**	menthofuran	0.47	0.20	1.50	2.05	0.00	1.37
10.19	1170	**	isopulegone	0.00	0.00	0.00	0.20	0.00	0.43
10.38	1190	*	(cyclohexanone, 2-isopropyl-2,5-dimethyl-)a	0.00	0.00	0.00	0.11	0.00	0.40
10.43	1195	**	coumaran	0.00	0.00	1.10	0.00	1.35	0.00
10.58	1211	*	(cyclohexanone, 2-isopropyl-2,5-dimethyl-)b	0.00	0.00	0.00	1.95	0.00	1.64
10.76	1232	**	pulegone	8.32	10.49	19.08	38.42	16.87	39.14
10.95	1252	*	(3R,6R)-1,1,6-trimethyl-2-oxaspiro[2.5]octan-8-one	0.00	0.00	0.50	1.54	0.30	1.76
11.20	1279	*	1-Cyclohexanone, 2-methyl-2-(3-methyl-2-oxobutyl)	0.00	0.00	2.35	2.37	1.55	1.89
11.35	1295	*	(6R)-2,2,6-trimethyl-1-oxaspiro[2.5]octan-4-one	0.00	0.00	0.92	0.42	0.60	0.27
11.42	1302	**	guaiacol <ρ-vinyl->	0.15	0.00	1.00	0.00	0.45	0.00
11.61	1318	*	1,3-dioxolane, 2,2-dimethyl-4,5-di-1-propenyl-	0.00	0.96	0.00	0.00	0.00	0.00
11.78	1332	**	piperitenone	2.57	2.85	6.13	4.85	3.93	2.81
11.92	1344	**	menthofurolactone	0.00	0.00	0.00	3.65	0.00	3.10
12.03	1353	**	piperitenone oxide	23.05	52.77	4.55	6.02	2.70	3.55
12.11	1360	**	nepetalactone <4aα,7α,7aα->	20.00	0.73	7.77	1.36	6.83	2.37
12.44	1386	**	nepetalactone <4aα,7α,7aβ->	9.33	14.02	7.32	11.94	7.45	14.84
12.49	1391	**	nepetalactone <4aβ,7α,7aβ->	1.36	0.00	0.88	0.00	1.44	0.00
12.78	1420	**	β-caryophyllene	0.00	1.43	0.00	1.18	0.00	1.08
12.99	1444	**	menthofurolactone2	0.00	0.00	0.00	0.59	0.00	0.26
13.30	1479	*	3,4-dehydro-β-ionone	0.49	1.18	0.00	0.00	0.00	0.00
13.65	1512	**	mintlactone	0.00	0.00	1.91	3.99	1.17	3.48
13.92	1533	*	nepetalactam	3.66	0.00	1.11	0.00	1.38	0.00
14.03	1542	**	isomintlactone	0.00	0.00	0.54	0.58	0.49	1.02
14.30	1563	*	peperinic acid	0.00	0.00	2.36	3.62	2.08	3.70
14.50	1578	*	megastigmatrienone	0.00	0.89	0.00	0.00	0.00	0.00
14.59	1585	**	spathulenol	0.00	0.00	0.00	0.87	0.00	0.78
14.68	1591	**	β-caryophyllene oxide	0.64	1.59	1.90	3.77	1.75	3.69
14.83	1603	*	1 3 4 5-tetrahydroxy cyclohexanecarboxylic acid	1.82	0.00	1.36	0.00	5.77	0.00
15.05	1622	*	megastigmatrienone2	2.11	2.51	0.28	0.45	0.00	0.48
16.09	1707		unidentified 1	1.56	0.00	1.13	0.00	0.84	0.00
16.32	1726		unidentified 2	1.59	0.00	2.03	0.00	2.17	0.00
16.36	1729		unidentified 3	2.06	0.00	0.41	0.00	0.00	0.00
16.71	1757	*	loliolide	2.68	0.96	2.27	0.64	1.69	0.90
17.26	1800	*	neophytadiene 1	8.28	4.09	13.02	6.37	14.99	7.12
17.34	1807	*	(2E)-3,7,11,15-tetramethyl-2-hexadecene	0.74	0.00	2.03	0.00	2.92	0.00
17.52	1823	*	neophytadiene 2	0.97	0.71	2.70	1.16	3.42	1.36
17.72	1841	*	neophytadiene 3	3.00	1.27	4.38	1.90	4.54	2.04
			Total identified compounds	**94.79**	**99.9**	**96.44**	**99.9**	**96.98**	**99.9**

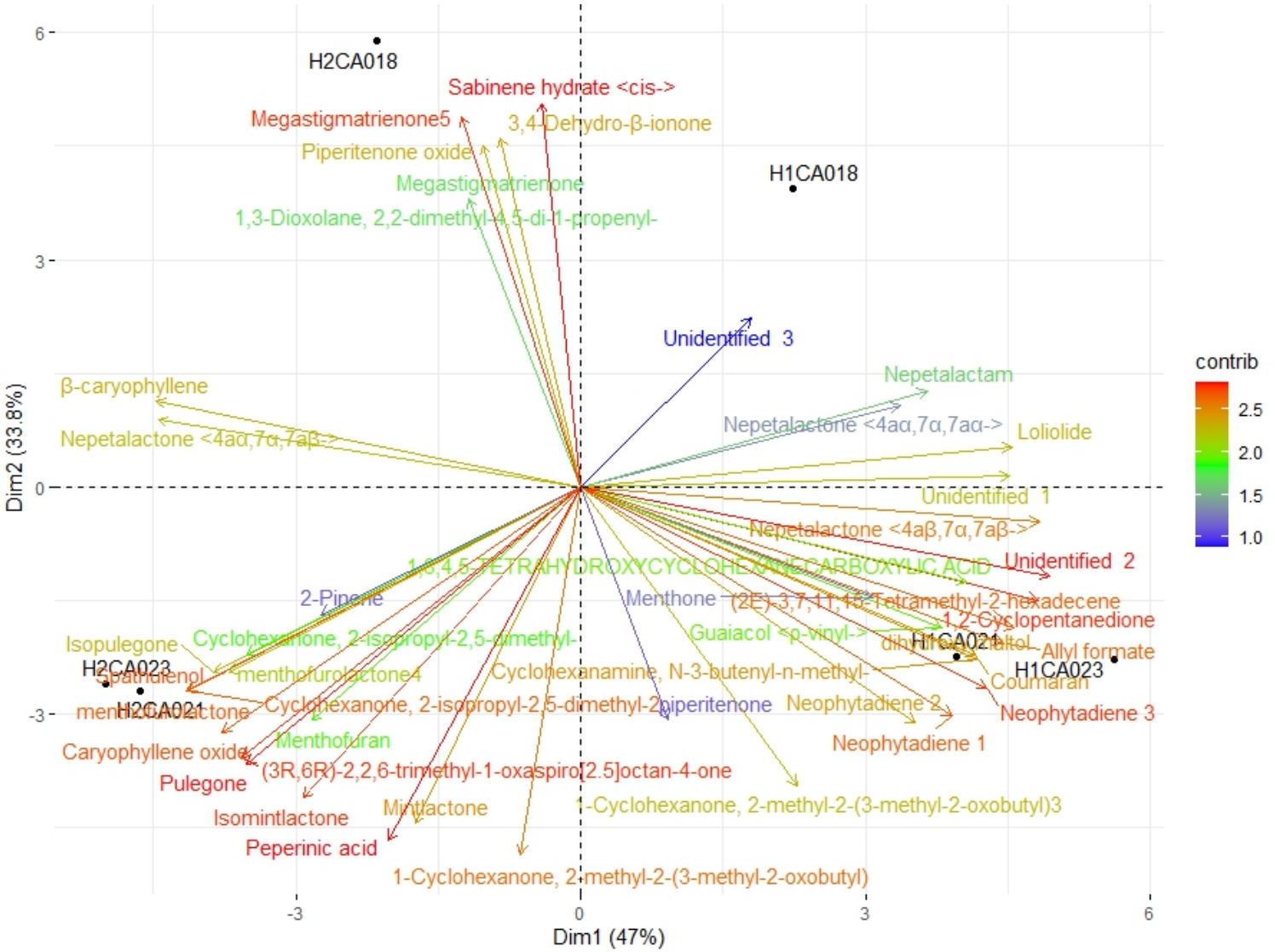

Figure 4: PCA biplot of scaled GC/MS peak area profiles of the three *Clinopodium nepeta* cultivars from both harvests. Eigenvectors are color-graded based on the relative contribution to PC1 and PC2.

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