THESIS

CHARACTERIZING SMOKE TAINT IN HOPS (*HUMULUS LUPULUS*) AND INVESTIGATING THE IMPACT OF DEFOLIATION STRESS ON PHYTOCANNABINOID CONTENT IN INDUSTRIAL HEMP (*CANNABIS SATIVA*)

Submitted by

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ABSTRACT

CHARACTERIZING SMOKE TAINT IN HOPS (HUMULUS LUPULUS) AND INVESTIGATING THE IMPACT OF DEFOLIATION STRESS ON PHYTOCANNABINOID CONTENT IN INDUSTRIAL HEMP (CANNABIS SATIVA)

The family *Cannabaceae* contains at least 10 genera, with *Cannabis* (hemp) and *Humulus* (hop) being two of the most economically important. Both genera have long been valued by humans for their chemical constituents and are used today for both medicinal and recreational purposes. However, adverse environmental factors may impact the chemical profile of these important crops, leading them away from a true-to-type quality. This thesis will explore the effects of an abiotic stress on the chemical profile of each crop: smoke-taint in hops and defoliative hail damage of hemp.

The Pacific Northwest contains 97.5% of U.S. commercial hop acreage and has also seen an increase in the number and severity of wildfire events in recent years. While there is extensive research from the wine industry on the impact of smoke taint in grapes, our knowledge of smoke taint in hops is limited. Here, we aimed to characterize smoke taint in hops using laboratory simulated wildfires with distinct fuel types and non-targeted gas chromatography-mass spectrometry. Our results reveal an overall variation in the chemical profiles between smoked and control hops and across fuel types and the detection of known and novel smoke taint markers including guaiacol, 4-methylguaiacol, and xylopyranose. This research provides evidence to support the use of established smoke taint markers for hop analysis and lays the groundwork for future studies to investigate various fuel types and their impact on hop quality.

The United States has seen an abrupt increase in commercial industrial hemp production since the Agricultural Improvement Act of 2018. However, the historical prohibition of this crop has resulted in a lack of basic physiological research to guide management practices. For example, abiotic stress can stimulate plants to increase production of secondary metabolites such as phytocannabinoids and this is of high importance to farmers as they as they must balance optimization of CBD yield (crop value) with regulatory requirements (THC < 0.3% by mass) that could lead to crop loss (mandated destruction). In this study we evaluated the impact of defoliation stress (to simulate hail damage) at three different growth stages. Our results indicate that defoliation stress during late flowering yielded no significant change in phytocannabinoid production. However, defoliation stress during vegetative and early flowering yielded a significant increase in phytocannabinoids, including total CBD and THC, at harvest.

ACKNOWLEDGEMENTS

Where do I even begin? It seems that countless people have helped me along the way, so I'll do my best to not leave anyone out. The first, and probably the most obvious to anyone that has ever set foot on the third floor of Nutrien is Jackie. As busy as she always is, one can always come to her whether it be for guidance, training, etc. The significance of her contribution to the success of the students she mentors cannot be overstated. Several present and past students in our lab group have also played an integral role in my journey. Rachel has been such a great colleague with whom to bounce around ideas, whether it be related to our studies or our work in the HLA Graduate Student Council. The same goes for Eva; she has been a great second set of eyes. Both Katies have done more for my professional life than anyone could ask. Their belief in me has really helped along the way. Janina and Tyler for their work on the hemp project and just being great people with whom to work and hang out. Val, Maggie, Beth, and the rest of the Prenni group for the consistently great feedback on posters and presentations. One thing that I have always valued about our lab group is the culture of growth and support, and that, of course, starts at the top. Jessica is incredibly skilled at recognizing her students and setting for them their own individual bar. Her many admirable qualities are no secret, and I definitely realize how fortunate I have been to work under her mentorship.

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that can really boost anyone's mood. Mackenzie, also for her work on the hemp project, and anyone else that I may have missed by name.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 - INTRODUCTION

1.1 Cannabaceae

The *Cannabaceae* family currently consists of at least 10 genera, with the most economically important being *Celtis*, *Cannabis*, and *Humulus*.[1, 2] Phylogenetic analysis suggests that *Cannabis* and *Humulus* share a close evolutionary relationship, forming their own distinct clade within the *Cannabaceae* family sister groups belonging to their own clade within their family, diverging from their most common ancestor around 27.8 million years ago.[3] Throughout human history, species belonging to *Cannabis* and *Humulus* have been used both as medicine and for recreation, with *Humulus* also being desired for its preservative qualities. Their cultivation and utilization have been intertwined with various cultures throughout time and remain so to this day. It is, therefore, important to investigate chemical compounds, whether they be metabolites or contaminates, that may impact the quality of these important crops. This thesis will explore those aspects, while highlighting the impact of defoliation in hemp and smoke-derived contamination in hops.

1.1.1 Hops (Humulus lupulus)

The *Humulus* genus currently has 3 recognized species, those being *H. japonicus* (*H. scadens*), *H. lupulus*, and *H. yunnanensis*, with *H. lupulus* being widely cultivated around the world.[4] The earliest written record of hop cultivation dates back to Germany in 736 CE, although some archaeological evidence suggests its use in the household centuries, or perhaps even millennia, earlier.[5, 6] In his 1597 book, *The Herball or Generall Historie of Plantes*, English herbalist John Gerard described various uses for hops which included benefits to the liver and spleen, the cleansing of one's blood, removing skin boils, a bread additive, and of course, "…[to]

manifest… the wholesomenesse of beere above ale; for the hops rather make it a physicall drinke to keepe the body in health."[7]

Hops are dioecious perennial bines that wrap around a support by thigmotropism in a clockwise rotation and climb by phototropism up to 20 feet high with the aid of hooked trichomes.[8] Female hop plants produce inflorescences known as strobiles, commonly referred to as "cones." A central strig supports several bracts which each support two bracteoles which enclose the lupulin glands (glandular trichomes).[5] These glands produce the soft resins and essential oils for which hops are valued. The soft resins, which are responsible for the bitter quality of hops, include the alpha (humulone, adhumulone, and cohumulone) and beta acids (lupulone, adlupulone, and colupulone), the former of which are largely isomerized during wort boiling into more soluble isoforms.[5] The aroma and flavor qualities of hops are largely attributed to the essential oils produced in the lupulin glands. The essential oils in hops are classified as either hydrocarbons, oxygenated derivatives, or sulfur containing compounds.[5] Perhaps the most important hydrocarbons are the monoterpene myrcene and the sesquiterpenes humulene and β-caryophyllene, with ratios of the latter two often being used to distinguish cultivars from one another.[5]

1.1.2 Hemp (Cannabis sativa L.)

The *Cannabis* genus has historically been split into three species: *C. sativa, C. indica, and C. ruderalis*. However, it has been suggested for some time that they may all be classified as *C. sativa*. A recent genomic review by Lapierre et al. "strongly supports the hypothesis of a highly diverse monotypic species."[9] Perhaps a more useful way of classifying cannabis is through chemotaxy. Currently, there are 5 recognized chemotaxis of cannabis: Type I is the "drug-type" which contain high concentrations of Δ^9-tetrahydrocannabinol (Δ^9-THC); Type II which is the "medical—type" and contains a 1:1 ratio of Δ^9-THC and cannabidiol (CBD); Type III is considered "industrial hemp" that contains higher concentrations of CBD (being the predominant constituent of this type) and very low to non-existent levels of Δ^9-THC; Type IV fiber-type with cannabigerol (CBG) as the main constituent; and Type V chemotype being a fiber-type hemp with very little to no cannabinoids.[10] Going forward, I will use the blanket term "cannabis," unless referring to a specific chemotype.

Cannabis is a dioecious plant that is desired for its fiber as well as its unfertilized female flowers. The female flowers are covered in glandular trichomes which produce several terpenes, including cannabinoids. There are three main trichome morphologies present on the cannabis flower: bulbous, sessile, and stalked, with only the latter two producing significant levels of cannabinoids.[11] The "core intermediate" from which all other cannabinoids are derived is cannabigerolic acid, which serves as a substrate for other cannabinoid synthases to produce compounds such as Δ^9-tetrahydrocannabinolic acid (THCA), cannabidolic acid (CBDA), and cannabichromenic acid (CBCA).[11]

1.2 Environmental Stress

Plants, unlike animals, cannot seek shelter or move to a more suitable location when environmental conditions become extreme. Therefore, they have developed metabolic pathways that enable them to produce chemicals for protection. These are collectively known as 'secondary metabolites' (sometimes referred to as 'specialized plant metabolites'). Secondary metabolites are any metabolite produced by the plant that does not contribute to its everyday function or structure and can be used to protect against a range of conditions including intense ultraviolet radiation and herbivory. Secondary metabolites are often desirable and economically valuable, such as cannabinoids. However, plants may respond to a specific stress at varying degrees of intensity at different growth stages. By understanding how a plant responds to a specific type of stress at a particular growth stage, horticulturalists are able to induce production of secondary metabolites by introducing stress to the plant.

1.3 Methods of Characterization

1.3.1 Analytical Techniques

The development of analytical instrumentation throughout the 20th century has allowed the field of metabolomics to flourish in recent years. The ability to describe the metabolome of important crops allows us to obtain important data that can aid in optimization of agricultural practices for the production of high-value crops. The various analytical instruments detect different types of molecules (e.g., volatile vs. nonvolatile compounds), so the choice of analytical method imparts a degree of bias in the analysis.

A common first step in any chemical analyses of organic material is chromatography. Chromatography is an analytical technique that separates compounds based on properties such as molecular size or polarity. Gas chromatography (GC) achieves this partition using a temperature gradient which impacts the equilibrium of compounds between the volatile gas phase and a solid stationary phase. Compounds can be introduced into the GC as either a gas (headspace) or liquid injection. A benefit to headspace sampling is the ability to employ the use of solid phase microextraction (SPME) which requires minimal sample preparation and can serve to concentrate volatile compounds prior to chromatographic separation. Conversely, liquid injection allows for the option of adding a derivatization step which increases the volatility of semi-volatile compounds enabling their separation by GC. Another common method of separation is liquid chromatography (LC). Liquid chromatography can separate compounds based on various properties such as charge, polarity, and/or size. In the case of reverse phase LC, separation is based on polarity and is achieved using a gradient of increasingly non-polar solvent and a hydrophobic stationary phase, with the most non-polar compounds being the last to elute.

Following separation, compounds are detected by mass spectrometry (MS). The mass spectrometer will first ionize compounds which enables separation of ions based on their mass-to-charge ratio (m/z). Chromatography coupled with mass spectrometry (GC-MS or LC-MS) can either be performed as a targeted or non-targeted analysis. A targeted analysis utilizes the mass analyzer to filter out ions that with an m/z outside of the target analyte, increasing the instruments sensitivity to the targeted analytes. This type of approach requires a-priori knowledge of the compound targets and can generate absolute quantitation. Non-targeted analysis sacrifices selectivity and sensitivity but enables the detection of a much broader range of compounds. Further, non-targeted analysis will generate relative quantitation and does not require a target list of compounds, thus facilitating novel discovery.

1.3.2 Sensory

In addition to analysis though instrumentation, sensory analyses offer valuable insights into consumer perception. While analytical techniques enable characterization at the molecular level, they do not reflect the human sensory experience – an important factor for consumer acceptance of high value crops like hops and hemp. Sensory analyses are utilized across various industries and play a critical role in assessing the quality of food and beverage products. Panels may either be ‘trained’ or untrained, the latter being commonly referred to as a ‘consumer’ sensory panel. Trained sensory panels undergo rigorous training to ensure consistency and precision in their evaluations. Important training objectives include standardizing their sensory lexicon to ensure a common association between aromas and descriptors, as well as reference testing to confirm proper identification of various aromas/flavors. On the other hand, a consumer sensory panel typically consists of target consumers of the product and receive little to no training. A benefit to consumer sensory panels is that, in the end, it is their opinion that matters. However, untrained consumers will have varying levels of sensory acuity, resulting in less objective results.

Panelists typically assess qualities such as aroma, flavor, appearance, etc. and play an essential role in maintaining a product that is true-to-type. Characterization is performed on the finished product (beer) and as a critical component of raw material selection and quality control. Sensory analysis adds a human component to the characterization of product quality and is an indispensable component in ensuring product excellence and maintaining consumer expectations.

CHAPTER 2 – CHARACTERIZING SMOKE-TAINT IN HOPS (*HUMULUS LUPULUS*)

2.1 Introduction

The Pacific Northwest (PNW) region, which includes Washington, Oregon, and Idaho, contains 97.5% of U.S. commercial hop acreage accounting for 37.2% of worldwide hops acreage.[12] In 2023, the PNW harvested 54,318 acres of hops with a total crop value upwards of $562 million.[13] The high demand for U.S. hops sparked a steady increase in hop acreage and production in the region between 2014 and 2021; however, 2022 saw the first decrease in total acreage from the previous year, falling by nearly 11% between 2021 and 2023.[12, 13]

In recent years, wildfire events have been increasing in both number and severity within the PNW region. According to the Environmental Protection Agency, Idaho, Oregon, and Washington are all "top five" with respect to average annual burned acreage per state, ranking in at first, third, and fifth place, respectively.[14] Wildfire smoke is a complex mixture that includes water vapor, fine and coarse particulate matter, chemical pollutants (CO, NOx, etc.), and volatile organic compounds.[15] However, studies have shown that the composition of wildfire smoke can vary depending on the fuel source.[16, 17] Diverse landscapes coupled with periods of prolonged drought has resulted in expansion of potential wildfire fuel sources across the western United States. Of note, in 2021, drought conditions in Colorado led to an unprecedented urban wildfire (originating as a grass fire) resulting in the loss of over 1000 homes. Events such as these expose a gap in smoke-taint research of economically important crops with respect to different fuel sources which have been shown to yield smoke with variable volatile profiles.[18]

There is a wealth of research from the wine industry on the effects of smoke-exposed grapes (pre-and post-harvest) which has demonstrated detectible levels of volatile phenols (VPs) characteristic of smoke-taint in both berries and wine.[19-22] Several studies have explored treating grapes with simulated wildfire events, both pre- and post-harvest . These experiments involve some type of smoking enclosure where either several bunches or whole vines are enclosed with ductwork connecting it to a smoking device. Grapes and/or whole grapevines are then subjected to smoke that is derived from a specific type of fuel for a specified amount of time with few investigating different fuel sources.[17] However, the morphology and chemical composition of grapes differ significantly from that of hops which may influence the mechanisms of smoke-taint and impacts on downstream product quality. For example, grapes are a juicy berry filled with sugars that can conjugate to smoke-derived contaminates, whereas hops are primarily comprised of lignocellulosic material, lacking the sugar content of grapes.

Gas chromatography coupled with mass spectrometry (GC-MS) is commonly employed for the chemical analysis of grapes to detect smoke-taint.[19, 22-24] These analyses are usually targeted for specific compounds that have been described as indicative of smoke-taint, such as guaiacol, 4-methylguaiacol, and isomers of cresol.[23, 25] Solid-phase microextraction (SPME) is frequently used as a means to sample volatile compounds that are indicative of smoke-taint in grapes.[25] Recently, SPME GC-MS was effectively used for targeted detection and quantitation of known smoke-taint markers of smoke exposed hops.[26] Such targeted methods, however, do not enable detection of hop specific compounds or enable insight of variation in smoke-taint from different fuel sources. Here, we employed a nontargeted analytical approach to enable broad detection of the impact of smoke taint on the chemical composition of hops utilizing both SPME and derivatized liquid extraction sampling methods. Additionally, the use of laboratory simulated wildfire events allowed for systematic evaluation of multiple fuel types and amplification of smoke-taint impact to enable detection of low abundance compounds.

2.2 Materials and Methods

2.2.1 Hops

Two public hop cultivars, Cascade and Centennial, grown during the 2021 growing season were used for our laboratory simulated wildfire treatments. These hops were chosen due to their popularity as well as their public availability. Centennial hops were sourced from a commercial producer in Washington state and were dried prior to receipt. Cascade hops were privately grown in Colorado, harvested, and air dried to room temperature for 3 days. All hops were stored at -20 °C prior to treatment. No smoke exposure was reported for these hops during production.

2.2.2 Fuel Types

Laboratory simulated wildfire smoke treatments were conducted to amplify the impact of smoke exposure in hops and to enable control of fuel type. Fuel selection was adapted from Noestheden, Dennis, & Zandberg (2018). Briefly, four fuel types were used and included pine forest, deciduous forest, grassland, and urban materials (Figure A1). The pine (P. ponderosa) and deciduous (A. saccharinum) fuels consisted of equal amounts of both green and brown leaf material (20%), bark (30%), and soil organic matter (50%). The grassland fuel contained both green (30%) and brown (50%) leaf material, and soil organic matter (20%). Finally, the urban fuel contained drywall (30%), construction lumber (P. menziesii) (50%), rubber (10%), and plastic (10%).

2.2.3 Smoke Treatments

For each smoke treatment, approximately 4 g of whole hop cones were placed onto a stainless-steel baking pan lined with fresh aluminum foil. Then, 2 g of fuel were loaded into a Mitbak Portable Smoking Gun Machine. Fuel was allowed to burn with the blower motor on “low” until fuel was spent, and smoke was no longer filling the dome, at which point the blower was switched off. The total time of smoke exposure was 15 minutes for each treatment. Cascade and Centennial hops were smoked individually, and the smoker was cleaned between each treatment with 1% Alconox and 70% ethanol. Smoked and control hops were homogenized for 30 seconds using a Mueller Electric 550K Coffee Grinder and stored at -20 ºC until analysis.

2.2.4 SPME GC-MS Analysis

Approximately 0.5 g (± 0.4 g) of homogenized hop material were added to 20 mL Restek headspace vials which were then filled with 5 mL of 35% NaCl solution. An Agilent GC, model 8890 (Agilent Technologies), coupled with an Agilent mass spectrometer detector (MS), model 5977B XTR EI 250 (Agilent Technologies) was used to analyze SPME samples. The 20 mL headspace vials were incubated for 5 min at 50 °C using a CTC Analytics PAL System RSI 85 autosampler (Agilent Technologies). After preincubation, a SPME arrow (CTC analytics 1.1 mm DVB/PDMS) fiber was inserted into the headspace of the vial and extracted for 30 min. After extraction, the SPME arrow fiber was injected into a splitless injector (280 °C; purge flow = 15 mL/min) and desorbed for 2 min. A DB-WAXetr (60 m x 0.32 mm i.d., 0.25 µm film thickness) capillary column (Agilent Technologies) was used for chromatographic separation. The oven temperature program was as follows: 50 °C hold for 2 min, ramp at 20 °C/min to 150 °C and hold for 0 min, ramp at 5 °C/min to 260 °C and hold for 3 min. The total run time was 32 min. The carrier gas was hydrogen which was held at constant flow rate at 2 mL/min with a column head pressure of 11.45 psi. The MS detector functioned in the EI mode (70 eV) and was connected to the GC by a transfer line heated to 250 °C. The source temperature was set at 280 °C and the quad temperature at 150 °C. Masses were scanned between 35-550 m/z at 1.5 scans/sec.

2.2.5 Derivatization and Liquid Extraction

Vials were filled with 20 mg (±0.5 mg) of dried hop homogenate and derivatized with 25 mg/mL methoxyamine HCl in pyridine solution and MSTFA + 1% TMCS. 50 µL of methoxyamine HCl was transferred to sample vials containing dried supernatant and incubated for 40 min at 60°C, sonicated for 10 min, then incubated for a second time for 45 min at 60 °C. 50 µL MSTFA +1% TCMS was then added to each sample and incubated for 30 min at 60 °C. Samples were analyzed using a Clarus 690 gas chromatography system (PerkinElmer Waltham, MA, USA) coupled to a PerkinElmer Clarus SQ 8T mass detector. Separation was performed with a TG-5MS column (Thermo Scientific, 30m x 0.25mm x 0.25mm). One microliter of derivatized sample was injected at a 1:12 split ratio and 1.0 mL/min helium gas flow. The oven profile consisted of an 80 °C hold for 30 s, ramping 15°C/min to 330°C, with an 8-min hold at the end of the run. Masses between 50-620 m/z were scanned at 4 scans/sec after EI ionization operating at 70 eV. The injector temperature held at 285 °C, the transfer line held at 280 °C, and the source held at 260 °C.

2.2.6 Data Processing

Data files were converted to .cdf and preprocessed (peak picking and alignment) using XCMS as previously described in Yao et al. (2019).[27] The *RAMClustR* package operating within the R Programming (version 4.0.3) environment was used to deconvolute the compound spectra.[28] Metabolite annotations were performed by spectral searching against the NIST, GOLM and an in-house proprietary database in the in-house program RAMSearch (Colorado State University, CO, USA).[29] Annotations were also evaluated based on the expected linear relationship between retention time and retention index of the assigned structure.

2.2.7 Statistical Analysis

Data from both the SPME and liquid extraction sampling methods were z-score transformed and combined for multivariate statistical analysis. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed with SIMCA Software v. 16 (Sartorius Stedim Biotech, Umea, Sweden). Data were unit variance scaled prior to model building. The quality of the models is described by two metrics: R2 (indicating the overall model fit, PCA and OPLS-DA) and Q2 (indicating the predictive power determined after cross-validation, OPLS-DA). A Shapiro-Wilk test was performed to evaluate the normality of the sample distribution (Table A1). Subsequently, compounds that did not conform to the normality assumption underwent analysis using Mann-Whitney U tests (Table A2), while those meeting the assumption were subjected to Welch's t-test (Table A3). Analysis of different fuel types was performed using ANOVA with post-hoc Tukey HSD. Statistical analysis was done in R (version 4.3.1) with RStudio (version 2023.09.1) with Pearson correlation coefficients calculated in Excel (version 2401) and graphs made in GraphPad Prism (version 9.3.1).

2.3 Results and Discussion

Nontargeted GC-MS analysis enabled the detection and annotation of 74 compounds between both SPME and liquid extraction methods (Table A4). Principle component analysis (PCA; Figure 1A-B) of smoked and control hops of both cultivars found that 51.1% (PC1) of the variation was explained by cultivar and 12.1% (PC2) was due to smoke exposure (R^2=0.76). Orthogonal partial least squares discriminate analysis (OPLS-DA; Figure 1C-D) shows strong separation between control and smoked hops (R^2=0.78, Q^2=0.87). Compounds with Variable Importance in Projection (VIP) scores over 0.95 (22 compounds; Table A5) were chosen to represent compounds most reflective of smoke exposure in hops. All previously reported smoke derived compounds were also selected regardless of VIP score. Given that most of the known smoke compounds were correlated across cultivar (Table A6), the discussion below will highlight the Cascade cultivar for simplicity of interpretation.

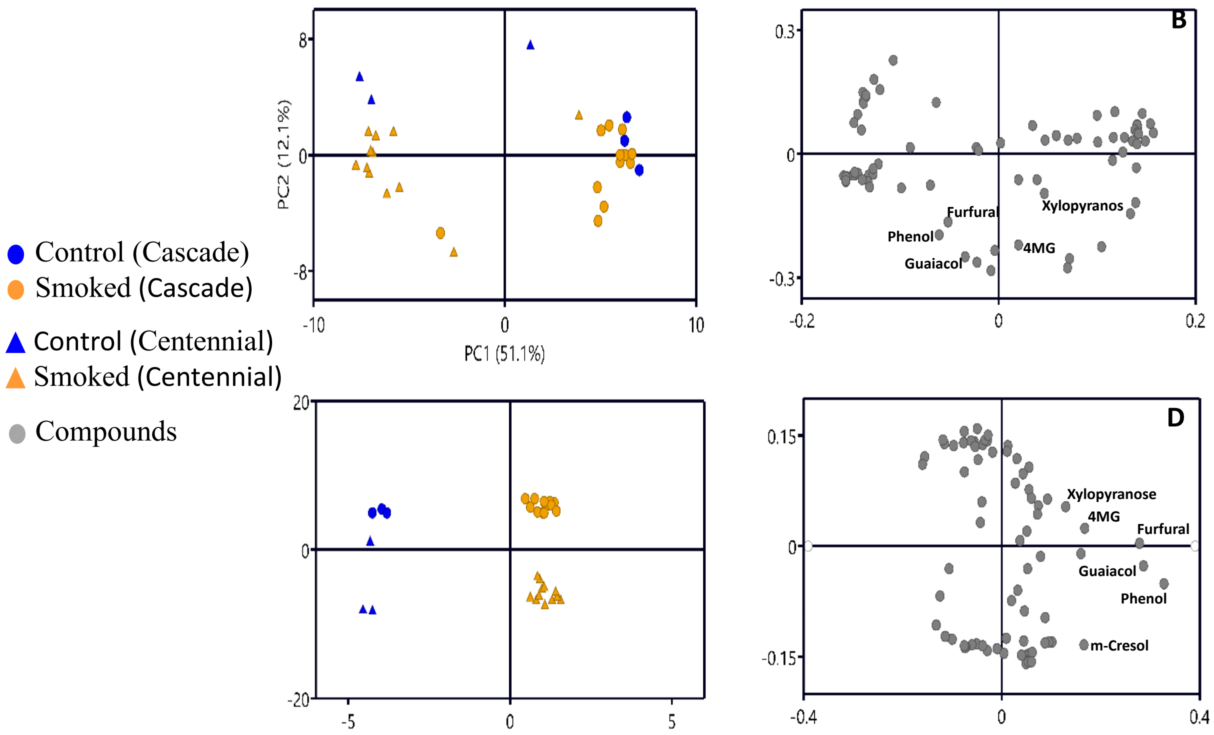

Figure 1. (A) Principal component analysis (PCA) scores plot. PC1 indicates that 51.1% of variation is due to cultivar, while 12.1% of variation is explained by smoke exposure (R^2=0.76) with triangles representing the cultivar Centennial and circles representing the cultivar Cascade. **(B)** PCA loadings plot showing smoke-derived compounds are most responsible for variation. **(C)** Orthogonal partial least squares discriminate analysis (OPLS-DA) scores plot. Triangles represent the cultivar Centennial and circles represent the cultivar Cascade (R^2=0.78, Q^2=0.87). **(D)** OPLS-DA loading plot highlighting the smoke-derived compounds are most responsible for separation between control and smoke exposed hops.

2.3.1 Variation between control and smoked hops.

Nontargeted GC-MS analysis resulted in the detection of several known smoke taint markers that are commonly employed in the targeted analyses of smoke taint in wine. These compounds include guaiacol (p=<0.0001), 4-methylguaiacol (p=0.0044), and m-cresol (p=<0.0001) (Figure 2); all of which were found to be elevated in the smoked hops compared to controls across both cultivars. Two additional compounds, furfural (p=<0.0001) and phenol (0.0044) displayed a similar quantitative pattern. These compounds are less frequently reported in smoke tainted grapes and have not been previously reported in smoke tainted hops. Furfural is an aldehyde derived from xylose which can be released when the hemicellulose fraction of wood undergoes pyrolysis.[30, 31]

Finally, xylopyranose (p=0.0308) was also found to be significantly elevated (Cascade only) in smoked hops versus controls. Xylopyranose, the 5-carbon ring form of xylose, is the

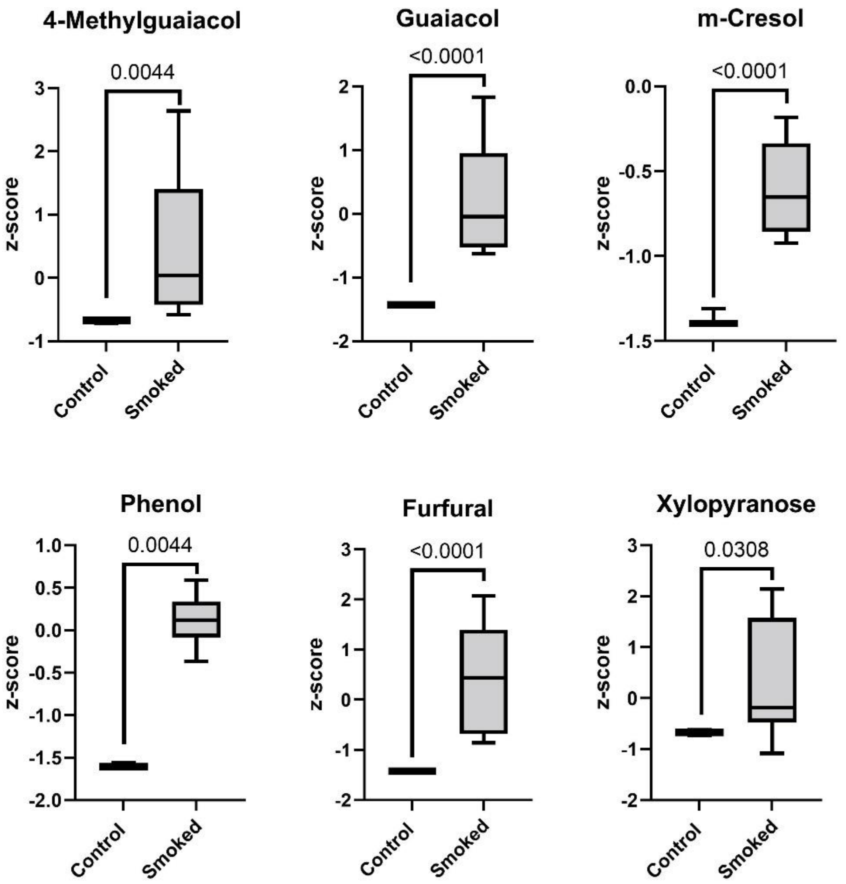

Figure 2. Compounds (VIP > 0.95) found in significantly ($p < 0.05$) higher levels in smoked hops with respect to controls (Cascade cultivar). Guaiacol, m-cresol, and furfural were analyzed with Welch's t-test, whereas phenol, 4-methylguaiacol, phenol, and xylopyranose were analyzed with Mann-Whitney U tests.

monosaccharide backbone of xylan in the hemicellulose fraction of wood and plant material. The lack of previous detection in smoke tainted grapes is likely attributed to the reduction in the hemicellulose fraction of grape berries as ripening occurs.[32] To our knowledge this represents the first reported evidence of this compound in smoke taint research.

2.3.2 Variation across fuel types.

Exploring the impact of different fuel types revealed discernable variations in volatile/semi-volatile profiles (Figure 3). Significant differences were observed in guaiacol abundance, with the urban treatment exhibiting elevated guaiacol abundance compared to all

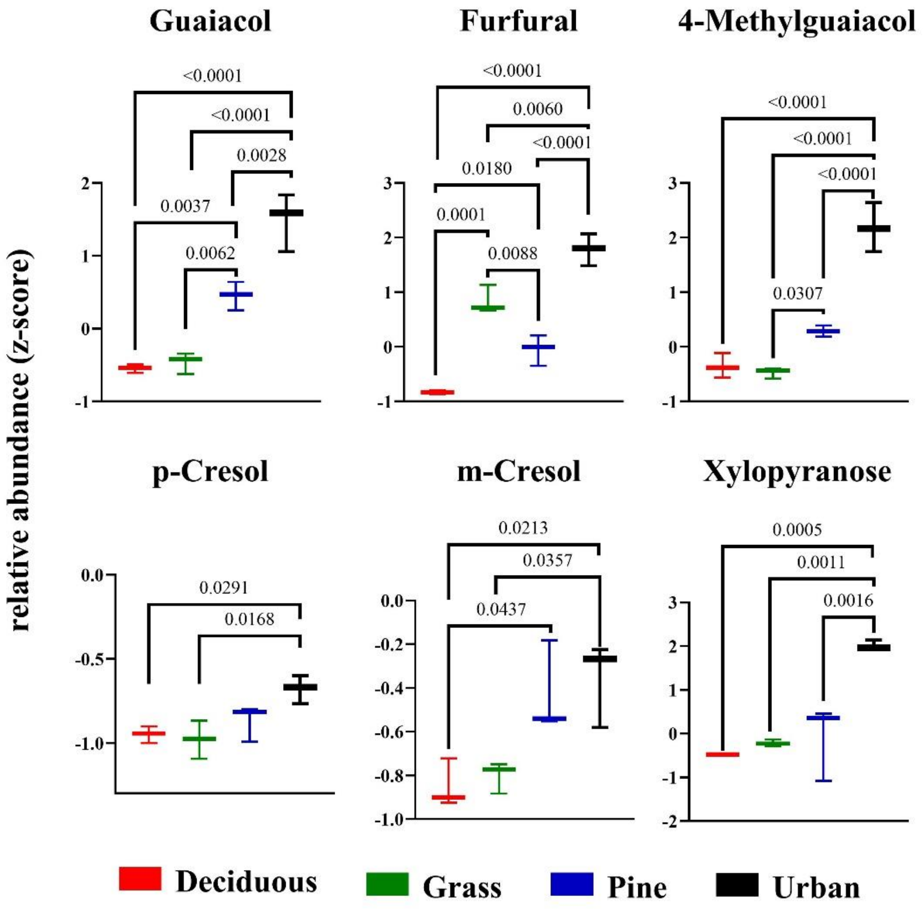

Figure 3. Compounds (VIP > 0.95) found to significantly ($p < 0.05$) vary between fuel types through ANOVA and post-hoc Tukey HSD. VIP score for p-Cresol was 0.76, but included as it is a common smoke taint compound.

other treatments. Moreover, the pine fuel type demonstrated higher guaiacol abundance than both deciduous and grass treatments. Similarly, 4-methylguaiacol was most abundant in the urban treatment, followed by the pine treatment which was statistically more abundant than the grass treatment. Abundance of both m- and p-cresol were also higher in the urban treatment when compared to deciduous and grass treatments, while m-cresol exhibited higher abundance in the pine treatment compared to the deciduous treatment. Interestingly, furfural was found in divergent abundance across all treatments, with the highest abundance found in the urban treatment, followed by grass, pine, and deciduous, respectively.

Xylopyranose was significantly more abundant in the urban fuel treatments compared to all other treatments. Polymers of wood (cellulose, hemicellulose, and lignin) can break down into their monomeric units under pyrolysis. A previous study investigated the hemicellulose fraction of different tissues in various types of plants, including conifers, broad-leaved trees, and grasses, with results suggesting that coniferous sapwood has a greater fraction of hemicellulose than broad-leaved/coniferous bark and grasses.[33] This suggests that the significantly higher abundance of xylopyranose in the urban treatment could be result of the use of *P. menziesii* sapwood in the urban fuel compared to the *P. ponderosa* and *A. saccharinum* bark in the pine and deciduous smoke treatments, respectively, as well as the grass smoke treatment.

Taken together, the results from this study revealed significant changes in the profile of volatile/semi-volatile compounds in smoked hops compared to controls. The use of a nontargeted analytical approach enabled the detection of a unique compound previously unreported in the smoke taint literature (xylopyranose), with potential implications for understanding the influence of different fuel sources. Additionally, commonly reported smoke taint markers such as guaiacol and m-cresol were detected at significantly higher abundance in smoked hops. To expand upon these results, future studies to investigate various fuel types may seek to monitor the temperature of the smoke chamber throughout treatments. This study supports the use of established wine smoke taint markers for characterization of hop smoke taint and suggests the importance of considering diverse fuel types and their varying impact on hop quality, providing a foundation for further exploration into hop-specific smoke taint markers.

CHAPTER 3 – EVALUATING METHODS FOR DETECTING SMOKE IN HOPS THROUGH SENSORY ANALYSIS

3.1 Introduction

Descriptive analyses are an important part of the hop selection and breeding process. Hop distributors and brewery raw materials specialists work closely with hop farmers to ensure a consistent and high-quality supply of hops for brewers. Sensory analysis plays a pivotal role in the process of selecting the ideal lots of a particular cultivar with the desired sensory characteristics. Sensory panels also contribute to quality control by ensuring that hop lots are consistent from harvest to harvest and during storage. Consistency is crucial for breweries aiming to produce beers with reliable and predictable sensory attributes. Alternatively, novel sensory characteristics may be desirable to the brewer.

Sensory methods involve the use of panelists who assess the aroma, flavor, and overall sensory characteristics of hop samples. Breweries will often have in-house sensory panels that undergo consistent training to ensure accurate assessment. Their sensory evaluations help determine if a particular lot of hops aligns with the desired profile for a specific beer style. Panelists will work together to develop a common vocabulary to describe the aromatic profile of the hops. This descriptive vocabulary is important to ensure consistent and accurate ratings. Training materials for sensory panels can be commercially available, such as the Aroma Standards Kit by Barth Haas. These kits contain vials of solutions with distinct aromatic profiles, for example citrus, herbal, and floral. In recent years, the American Society of Brewing Chemists (ASBC) sensory subcommittee has been experimenting with different aroma standards to develop methods of detecting and characterizing smoke-taint. Some of these experimental standards include beef jerky, burned corn chips, etc.

When sampling hops during a sensory analysis, several methods can be employed. Perhaps the quickest method in terms of preparation is the hand-rub technique, where piles of hops are placed on a table for panelists to take into their hands and rub back and forth (to release volatile aroma compounds) prior to evaluation. Downsides to this method include (1) the amount of clean up afterwards and (2) the amount of handwashing that must be done between samples to minimize carry-over. Another method that is widely used is the 'grind' method, where hops are ground into fine particles and placed into some sort of vessel, such as a cardboard cup for the panelists to evaluate. A third method, which has been in development by the ASBC sensory subcommittee, is to create a hop slurry using 5% ethanol in water. While methanol extractions are common for chemical analysis of hops, a 5% ethanol solution is preferred for sensory as it mimics a beer matrix.

The prevalence of smoke-tainted hops has escalated in recent years and has raised alarm in the brewing and raw materials industries. At present, sensory assessments lack training and evaluation concerning smoke-taint attributes; however, industry demand highlights the urgent need for incorporation of such training and evaluation into sensory practices. The objective of our preliminary sensory analysis was to evaluate several experimental standards to characterize smoky qualities in hops and to compare the 5% ethanol slurry to the conventional hop grind method for detecting smoky attributes.

3.2 Experimental Design and Methods

For this experiment, 10 panelists evaluated 6 hops samples, 5 of which were grown in the Pacific Northwest region during the 2020 growing season. Among our 6 samples, 5 were dried cones, and the remaining one was received as T90 pellets. Panelists were trained on 4 aroma standards from the John I. Haas Aroma Standard Kit (citrus, floral sweet, fruit, and herbal), 4 experimental smoke standards (beef jerky, burned corn chips, charred sisal twine, and Lapsang Souchong tea), and one "extreme" example of smoky hops, which was previously smoked using applewood chips as fuel. The sensory panel took place over two consecutive days. On day one, participants underwent a training session lasting approximately 30-45 minutes, followed by a sensory evaluation employing the hop grind method. On the second day there were two sensory sessions, with the first utilizing the hop grind method, followed by the 5% slurry session.

3.2.1 Sample Preparation

All hop samples were homogenized using a coffee grinder for at least 15 seconds, then 3 g aliquots were placed into 250 mL glass jars. One jar was reserved as the hop grind sample. Ground hops in the second jar were suspended in either 75 mL or 30 mL of 5 % ethanol in water solution for cones or pellets, respectively. These slurry samples were then vortexed and allowed to sit for one hour at room temperature prior to the sensory session. The jars were wrapped with aluminum foil and an alpha-numeric code was fixed to the outside. Twine and corn chips were prepped the morning of session 1. The smoky standards were placed in 250mL jars.

3.2.2 Training

Panelists were introduced to the concept of a sensory panel and its role in the industry. It was emphasized that they were not to measure of good versus bad (favorable/unfavorable), but rather what can they detect. Training reference standards included: citrus (Haas), floral (Haas), herbal (Haas), sweet fruity (Haas), beef jerky, burned Frito-style corn ships, charred sisal twine, and lapsang souchong tea. Panelists were also provided an example of extreme smokiness in hops through a side-by-side comparison of smoke treated and smoke free Centennial hop pellets.

All aroma standards remained available for the panelists to reference throughout the sensory sessions.

Panelists were asked to not react in any way, whether that be verbal or physical, so as not to influence other panelists. Panelists were also asked to not look inside of the jars when sampling their aromatic profiles. The logic behind this request was that their response may be influenced by the color of the hop material (bright green, perhaps, being more favorable to a dull green).

The sensory ballot (Appendix A2) rating scale was set from 1 to 3. If any specific target aroma was not detected, it was rated as 1; if 'somewhat detected' a 2, and if 'definitely detected' it was rated a 3. The sum of each aromatic attribute, such as citrus, was summed for each sample to obtain a total score. For example, if 7 people rated 'beef jerky' at 1 and 3 people rated it a 2, then the final score for 'beef jerky' in that specific sample would be 13.

2.6 Results and Discussion

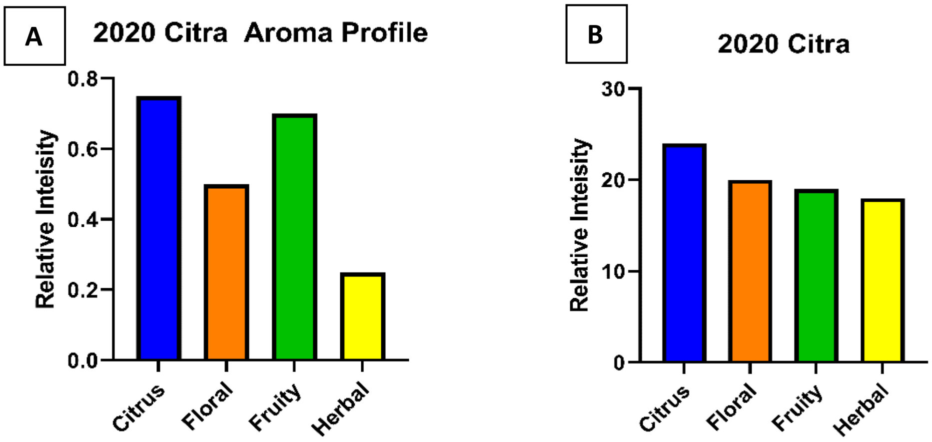

Figure 4. (A) Official 2020 aroma ratings for Citra hops alongside (B) aroma ratings from our sensory panel.

Sensory results were scored by the sum of all ratings for each descriptor. First, we evaluated how well our panel did at assessing desirable qualities in unsmoked hops. Figure 4 shows quality ratings for Citra hops, with the official 2020 ratings for citrus, floral, sweet fruit, and herbal (Figure 4A) alongside the scores from our panel (Figure 4B). These results indicate that our panel did well at detecting these desirable aromas in their relative intensities, although they detected slightly less “fruit” and more “herbal”.

Next, we evaluated how well these desirable aromas were identified in hops that were exposed to natural wildfire smoke during harvest. Figure 5 shows the official 2020 sensory ratings for Mosaic hops (Figure 5A) along the ratings from our panel (Figure 5B). Here, our panel appears to have inversely rated the relative aroma intensities with respect to the official ratings, suggesting that smoke may impact the ability to detect desirable aromas in hops leading to a flavor and/or aroma that is not true-to-type. However, these results may also indicate that our panel was simply unable to detect these attributes with the same accuracy as a trained sensory panel.

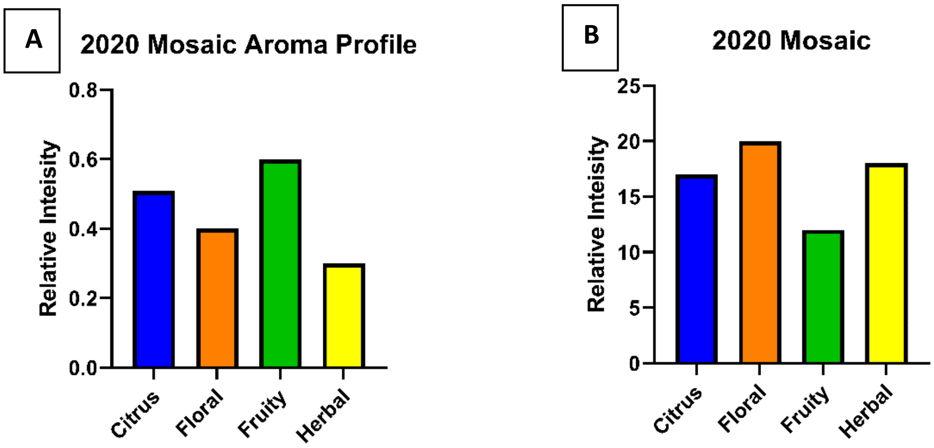

Figure 5. (A) Official 2020 aroma ratings for Mosaic hops alongside (B) aroma ratings from our sensory panel.

Finally, we evaluated how our panel rated the various smoke aromas between the grind and slurry preparation methods (Figure 6). Overall, the results suggest that the slurry method (Figure 6B) may have enabled improved panelist detection of smoke aromas. While the ‘smoky’ and ‘jerky’ aroma ratings were slightly less in the slurry method, the tradeoff was an increase in detection of the ‘burned corn chips,’ ‘charred twine,’ and ‘Lapsang Souchong tea’ attributes with an overall more balanced score between attributes.

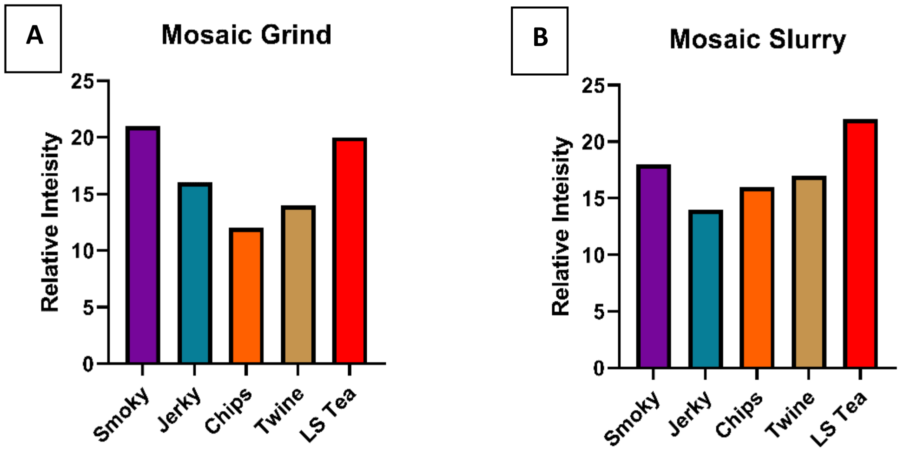

Figure 6. Sensory panel ratings for smoke aromas in Mosaic hops through the (A) grind and (B) slurry methods.

An important consideration when evaluating the performance of the two preparation methods is that the slurry method closely matches the method in which hops are enjoyed: a 5% ethanol solution. Ethanol has been used as a polar solvent in the extraction phenols, such as guaiacol, and thus may improve the ability to detect these compounds responsible for smoky aromas through olfactory detection.[34] However, additional research is required using a trained sensory panel to standardize smoke detection in hops. Taken together, these preliminary results suggest that our panel was able to successfully identify desirable sensory attributes as well as smoky qualities, when present, and the slurry method is worth further exploration.

CHAPTER 4 - INVESTIGATING THE IMPACT OF DEFOLIATION STRESS ON PHYTOCANNABINOID CONTENT IN INDUSTRIAL HEMP (*CANNABIS SATIVA*) [1]

4.1 Introduction

Commercial production of industrial hemp is relatively new in the United States (U.S.), a result of the Agricultural Improvement Act of 2018 which defined hemp as "the plant Cannabis sativa L. and any part of that plant… with a delta-9 tetrahydrocannabinol concentration of not more than 0.3 percent on a dry weight basis."[35] Hence, larger scale commercial industrial hemp cultivation in the U.S. is still in its infancy and the extended prohibition of *Cannabis sativa* L. has resulted in a gap in the critical physiological research needed to support commercial production.

In just one year, from 2021 to 2022, the value of U.S. hemp production decreased by over 60%.[36, 37] The state of Colorado experienced an even sharper decrease in hemp production during this time, likely due to overproduction of the crop. Specifically, in 2021, 4087 hectares acres were planted compared to only 648 hectares in 2022. Importantly, only approximately 30% of acres planted were harvested for product, a rate much lower than the national average. It has been reported that an average of 10.8% of hemp planted between 2018 and 2020 tested over the 0.3% THC limit.[38] While no data is available on the specific reasons for such low yields, it is likely that challenges faced by Colorado hemp growers to maintain total THC content under the 0.3% threshold was a contributing factor.

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[1] This chapter was recently accepted for publication in Industrial Crops & Products, for which I was first author. Sandoval, B., Bowen, J. K., Richards, T., Chaparro, J. M., Lee, M., Uchanski, M., Cranshaw, W., and Prenni, J. E. (2024). Impact of Defoliation Stress on Phytocannabinoid Content in Industrial Hemp. *Industrial Crops & Products*. Article number: 118226.

The state of Colorado routinely experiences intense sunlight, extreme temperatures, and severe thunderstorms that include damaging hail. Such environmental stressors can trigger plants to produce organic compounds broadly known as secondary metabolites.[39] In hemp, these secondary metabolites include phytocannabinoids which are synthesized in glandular trichomes, primarily in female flowers but are also found in low concentrations in the leaves and stems.[40] Secondary metabolite production is typically localized and may or may not be dependent on growth stage. Previous research has shown that phytocannabinoid concentrations tend to increase for several weeks post anthesis.[41]

Hail events are common in Colorado, with 205 events reported in 2022[42], and frequently cause significant crop damage.[42] However, there is little data on how hail events might impact yield and quality of hemp crops. One recent study demonstrated that defoliation stress close to harvest may increase the concentration of cannabichromene (CBC), cannabidiol (CBD), cannabigerol (CBG), and tetrahydrocannabinol (THC),[43] though elevated phytocannabinoid concentrations were not statistically significant from control plants that did not experience defoliation. In Colorado, hailstorms often occur throughout the growing season and thus, in addition to better understanding the impact of defoliation stress, there remains a knowledge gap in our understanding of how the timing of this type of abiotic stress will impact the phytocannabinoid content at harvest.

Understanding the impact of environmental stress on the phytocannabinoid content of hemp is an important consideration of hemp growers as they as they must make management decisions to balance optimization of CBD yield (crop value) with regulatory requirements (THC < 0.3% by mass) that could lead to mandated destruction. The present study sought to expand on what is known about phytocannabinoid synthesis in response to abiotic stress by simulating defoliation from hail damage at different growth stages. We hypothesized that defoliation stress may impact the synthesis of phytocannabinoids, and that this impact is dependent on the growth stage of the plant when the stress occurs. To test this hypothesis*,* *C. sativa* L. 'Unicorn' plants were treated with simulated hail damage at three different growth stages (vegetative, early flowering, and late flowering) and phytocannabinoid content was evaluated using UPLC-MS/MS.

4.2 Materials/Methods

4.2.1 Plant Material & Cultivation

Cannabis sativa L. 'Unicorn 1' is a short-day, dioecious clonally produced high-CBD cultivar. It was approved as an industrial hemp seed variety by the Colorado Department of Agriculture in 2019. Transplants were acquired from the producer (Colorado Hemp Research Institute) and were planted into an outdoor conventional field on June 17, 2020, at the Agricultural Research, Development, and Education Center (ARDEC South) at Colorado State University in Fort Collins, Colorado (lat.40.611804 N; long. -104.997144 W; elevation 1525 meters). A total of 15.9 kg of fertilizer (1.75 kg N, 8.27 kg P2O5, 0 kg K2O) was applied over 0.31 ha. On a per hectare basis, this equates to 51.3 kg/ha product application rate (5.6 kg N/ha, 26/7 kg P2O5/ha, and 0 kg K2O/ha). Plants were spaced 0.9 m apart within rows, with 0.76 m between rows and a total of 24 plants/row. The field was flood irrigated to the point of saturation once per week throughout the season to keep hemp plants in a well-watered state with minimal drought stress. The approximate calculated water applied at each weekly irrigation was 0.022 hectare meters (2.1 acre inches). Treatment and control plants were randomly assigned ($n \geq 6$).

4.2.2 Defoliation and Sampling

Our experiment consisted of three defoliation events (DE) which occurred at different growth stages: DE1 (July 15, 2020, 28 days after planting) corresponds to vegetative, DE2 (August 17, 2020, 61 days after planting) corresponds to early flower, and DE3 (September 14, 2020, 89 days after planting) corresponds to late flower. For each defoliation event, plants were whipped with a flail that included multiple wire strings with metal bolts at the end of each (Figure A3). During each treatment, approximately 50% of the plant was defoliated (based on visual inspection; Figure A4). This 50% treatment threshold was chosen as a reasonable level that would ensure the plants experienced stress typical of hail damage but were not at risk of plant death. Control plants were sampled at the same time as treatment plants for each defoliation event. The treatment resulted in leaves that were stripped or tattered, and lateral branches that were bruised or bent.

Initially, the final harvest date for all three defoliation treatment groups was planned for September 28, 2020. However, due to a predicted frost event, the final harvest date for plants in the DE1 and DE2 treatments was moved up to September 7th to avoid any possible cold-temperature stress response. DE3 treatments took place after the frost event (which occurred on September 8th, 2020) and these plants were harvested on September 28th. Plants in the DE3 treatment group (and corresponding controls) were covered with frost blankets during the frost event.

The top two inches were sampled from three inflorescences (apical and two lateral) from each plant at harvest. Additionally, a subset of treated and control plants in the DE2 treatment group were sampled at both 1 and 14 days (D1 & D14) after defoliation to observe phytocannabinoid concentration at additional timepoints. Plants in the DE3 treatment group were also sampled 1 day after defoliation. Due to destructive sampling mimicking our defoliation treatments, each plant was sampled only a single time (except for DE2 D1 and D14). Flower material was separated from leaf material and placed in zip seal bags. Metabolism was quenched by placing the freshly harvested plant material on dry ice during transport followed by storage at -80°C until processing for analysis.

4.2.3 Phytocannabinoid Analysis

Plant material was lyophilized, sieved to remove seeds and stems, and homogenized for 5 min using a Next Advance Bullet Blender Storm 5 bead beater at speed setting 10. Homogenized inflorescence material (20 mg ± 0.5 mg) was weighed into 2 mL glass vials. Samples were extracted using 80% MeOH (80% LC-MS grade methanol and 20 % LC-MS grade water v/v) spiked with delta-9-THC-d3 and CBD-d3 at a final concentration of 25 ng/mL. A 12-point standard curve was prepared with the following concentrations (ng/mL): 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000, 2000, and 4000. To account for the higher concentration of CBDA in samples, a separate calibration curve for CBDA was used with the following concentrations (ng/mL): 24.41, 48.82, 97.65, 195.31, 390.63, 781.25, 1562.5, 3125, 6250, 12500, 25000, 50000, 75000, and 100000.

Extracted samples were analyzed using a quantitative ultra performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) method for 20 phytocannabinoids in hemp plant tissue as previously described[44]. Briefly, 5 µL of extract were injected onto an LX50 UPLC system equipped with a LX-50 solvent delivery pump (20-µL sample loop, partial loop injection mode; PerkinElmer, Shelton, CT, USA). An ACQUITY UPLC HSS T3 column (1 x 100 mm, 1.8 µm; Waters Corporation, Milford, MA, USA) was used for chromatographic separation. The column was maintained at 45°C, mobile phase A consisted of LC-MS grade water with 0.1% formic acid and mobile phase B was 100% acetonitrile. The elution gradient was held at 59% B for 11.5 min, increased to 99% B from 11.5 min to 16.5 min, then decreased to 59% B from 16.5 to 21.5 min. The column was re-equilibrated for 4 min for a total run time of 25.5 min. The flow rate was 200 µL/min. Detection was performed on a PerkinElmer QSight 220 triple quadrupole mass spectrometer (MS), with an electrospray ionization source, operated in selected reaction monitoring (SRM) with positive/negative polarity switching. SRM transitions for each compound were optimized through analysis of authentic standards (Table A7). Source conditions were: source temperature 325 °C, drying gas 120 (arbitrary units), hot-surface induced desolvation (HSID) temperature of 250°C, electrospray voltage of -4500 eV or 4500 eV, and nebulizer gas flow of 350 (arbitrary units). The MS acquisition was scheduled by retention time with 1.5 min windows.

4.2.4 Data analysis

Data processing was performed using Simplicity 3Q™ software (Version 1.5, PerkinElmer) as previously described [44]. Briefly, peak retention times corresponding to SRM transitions for each phytocannabinoid were validated against authentic standards. Quantitation was performed by generating a standard curve from authentic phytocannabinoid standards from 1.95-4000 ng/mL or 24.41-100000 ng/mL for CBDA. All signals were normalized to the appropriate internal standard and the concentration of compounds in experimental samples were determined based on linear regression of the calibration curve. Regression parameters and limits of detection (LOD) and quantification (LOQ) for detected phytocannabinoids can be found in Table A8. Peak areas were integrated and exported for statistical analysis (Microsoft Excel, version 2308) and graphed using GraphPad Prism (Version 8.2.1). Data was analyzed by unpaired two-tailed t-test, with all (treatment groups compared to their respective controls. For statistical analysis, phytocannabinoid quantities under the LOD were entered as 0.5 × LOD.

4.3 Results & Discussion

LC-MS/MS analysis resulted in the detection and quantitation of 12 phytocannabinoids including: cannabidiol (CBD), cannabidiolic acid (CBDA), cannabidivarin (CBDV), cannabidivaric acid (CBDVA), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabinol (CBN), cannabinolic acid (CBNA), delta-(9)-tetrahydrocannabinol (THC), delta-(9)-tetrahydrocannabinolic acid (THCA), tetrahydrocannabivarin (THCV), and tetrahydrocannabivaric acid (THCVA). The quantitative data for all samples is presented in Appendix Tables A9(a-c).

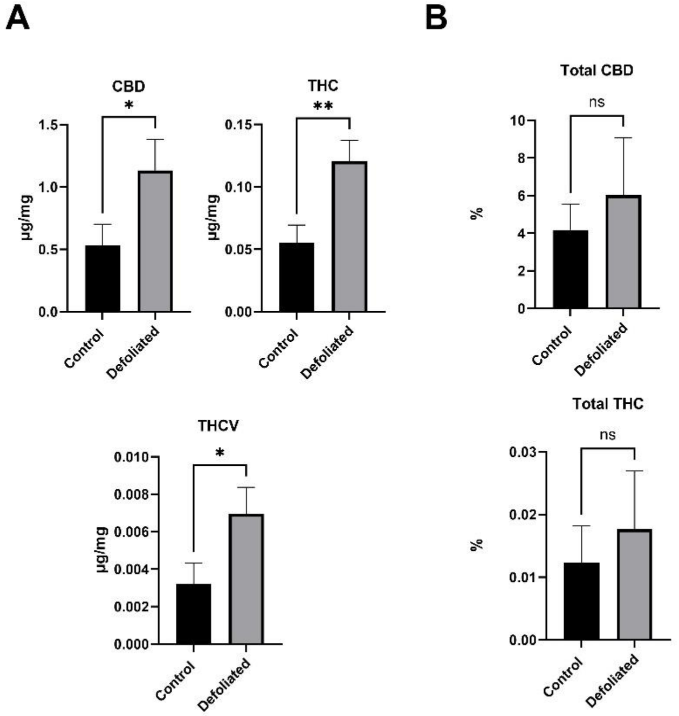

Figure 7. (A) Concentration (mg/mg of dried flower) and (B) % by dry weight of total potential CBD and THC after complete decarboxylation of phytocannabinoids measured at harvest following defoliation during vegetative growth and in corresponding controls (no defoliation treatment). Error bars represent the standard deviation of the mean. Significance was determined by t-tests, where * indicates $p < 0.05$ and ** indicates $p < 0.01$.

The first defoliation event (DE1) was carried out on July 15, 2020, and plants were sampled at harvest on September 7, 2020. Results demonstrated that defoliation during vegetative growth resulted in significantly higher ($p < 0.05$; Table A10) concentrations of CBD, THC, and THCV (μg/mg of dried flower) at harvest as compared to controls (Figure 7A). However, yield of these compounds is typically represented as % by dry weight of total potential after complete decarboxylation (e.g., total % CBD = %CBD + (0.877*%CBDA)). This value is also the basis for determining compliance with regulatory thresholds. No significant difference in total % of any phytocannabinoids was observed at harvest following defoliation during vegetative growth (Figure 7B, Tables A11 - A13).

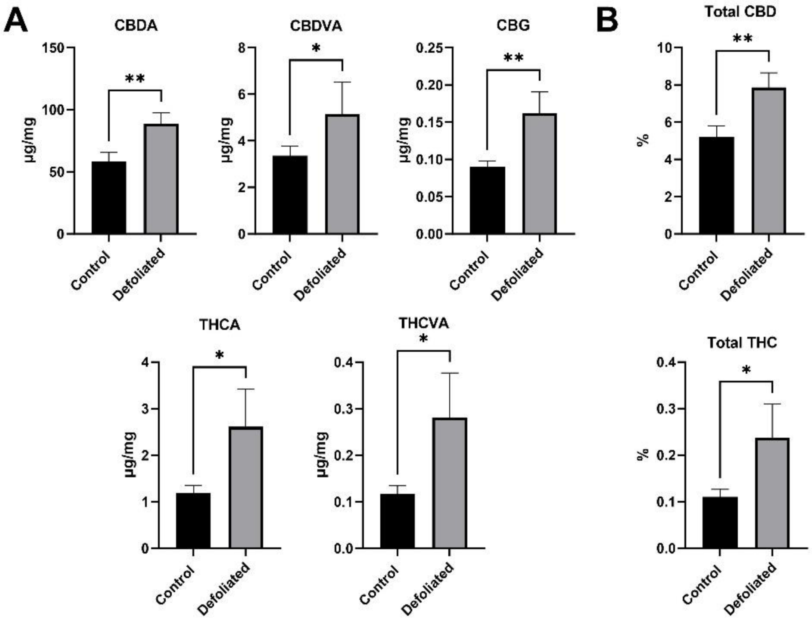

Figure 8. (A) Concentration (mg/mg of dried flower) and (B) % by dry weight of total potential (B) after complete decarboxylation of phytocannabinoids measured at harvest following defoliation during early flower and in corresponding controls (no defoliation treatment). Error bars represent the standard deviation of the mean. Significance was determined by t-tests, where * indicates $p < 0.05$ and ** indicates $p < 0.01$.

The second defoliation event (DE2) took place on August 17, 2020, and plants were sampled at harvest on September 7, 2020. Results demonstrate that defoliation during early flower resulted in significantly higher ($p > 0.05$) concentration (µg/mg of dried flower) of CBDA, CBDVA, CBG, THCA, and THCVA at harvest compared to control (Figure 8A; Tables S10). Importantly, this translated to a significant increase in both total % CBD and THC at harvest (Figure 8B; Tables A11 - A13).

Following defoliation during early flowering (DE2), a subset of control and treated plants ($n \geq 3$) were also sampled on August 18th and on August 31st corresponding with 1 and 14 days after defoliation, respectively. Focusing on the major phytocannabinoid synthesis pathway,[11] our results show a trend of increasing total % CBD and THC starting at 14 days after the defoliation event through harvest (Figure 9; Tables A11 - A13). This trend is not observed for total CBG, suggesting a metabolic shift favoring CBDA and THCA and potential stagnation of precursor synthesis (CBGA).

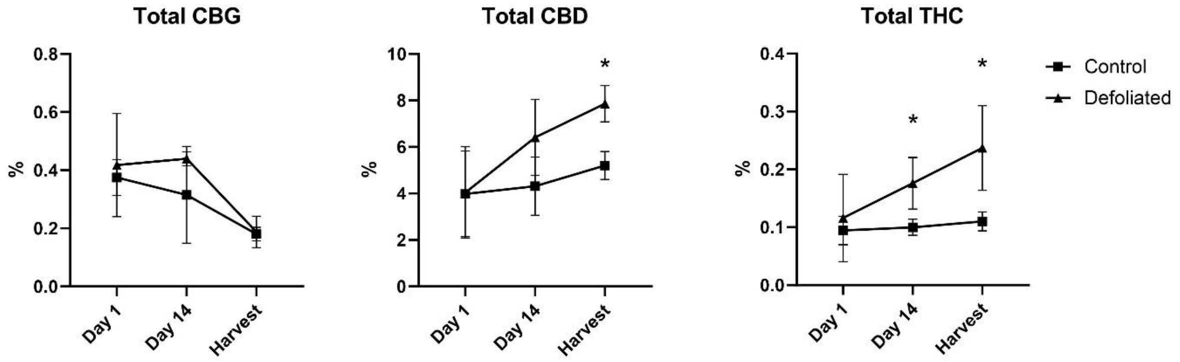

Figure 9. % by dry weight of total potential after complete decarboxylation of phytocannabinoids measured at day 1, day 14, and harvest following defoliation during early flower and in corresponding controls (no defoliation treatment). Error bars represent the standard deviation of the mean. Significance was determined by t-tests, where * indicates $p < 0.05$ and ** indicates $p < 0.01$.

The third defoliation event (DE3) was performed on September 14, 2020, and samples were collected September 15th and on September 28th, corresponding to 1 and 14 days after defoliation, respectively. No significant change in individual phytocannabinoid content (µg/mg of dried flower) or in total % CBD or THC was detected when compared to controls at harvest (day 14 after defoliation). These results support the recent work by Toth et al. (2021) that demonstrated that defoliation stress close to harvest (late flower) does not significantly impact phytocannabinoid concentrations [43]. It is important to note that DE3 was performed after the frost event that occurred on September 8th, 2020. However, the plants were protected, and no visible damage was observed. Furthermore, the results suggest that this event did not impact phytocannabinoid production based on the stable concentration of CBDA (not significant based on unpaired t-test, p= 0.6351) in control plants before and after (9/7, DE2 at harvest and 9/15, DE3 day 1; Table A9(a)).

4.4 Conclusion and Future Work

Our results demonstrate that defoliation during early flowering significantly impacted phytocannabinoid concentrations at harvest. This result highlights the challenge of managing product yield (total % CBD) with risk (total % THC). This is particularly relevant for hemp growers in Colorado where severe thunderstorms with hail occur often throughout the growing season. To our knowledge, this study represents the first report of the impact of defoliation at different developmental stages, providing critical information to aid farmers in developing data driven management strategies to optimize CBD yield while minimizing risk under current restrictions. Future studies are needed to evaluate this effect across growing seasons and for additional cultivars.

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APPENDIX

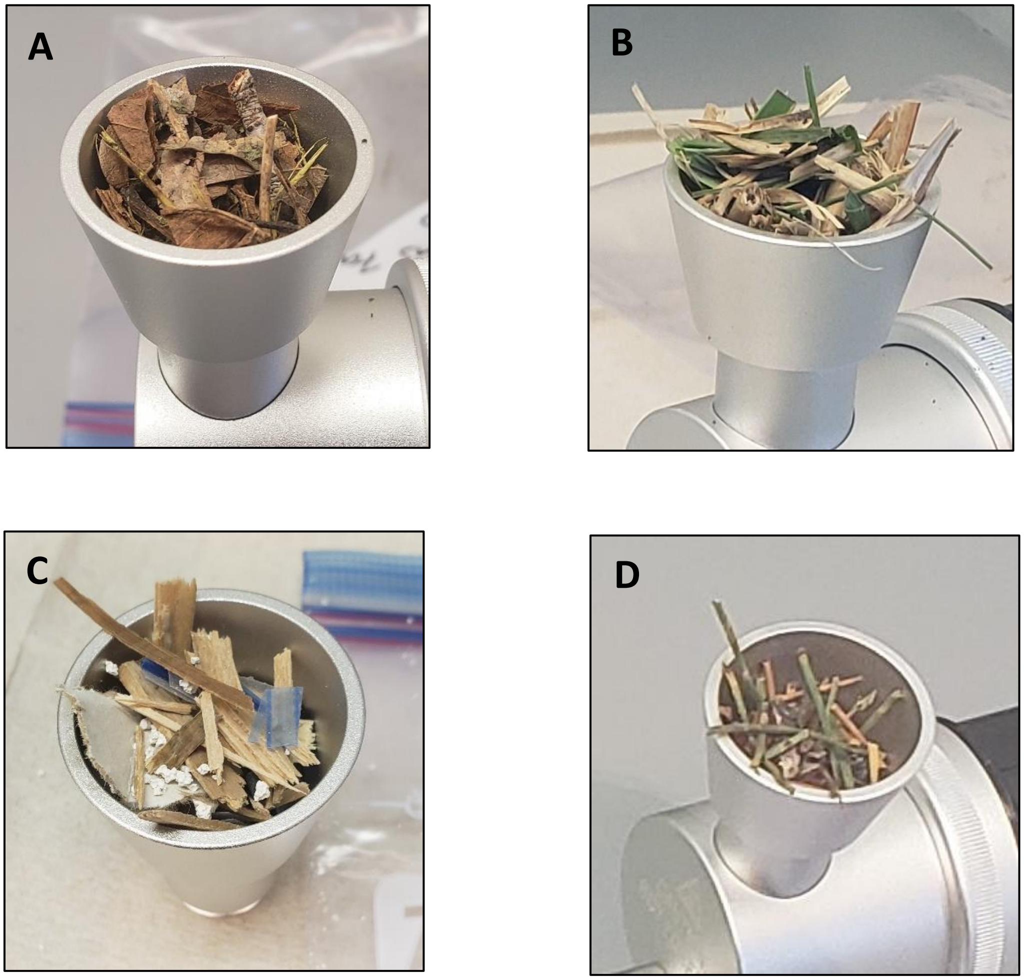


Figure A1: Fuel sources for simulated wildfires included (A) deciduous tree (*A. saccharinum*) material, (B) grass, (C) urban building material, and (D) pine tree (*P. ponderosa*) material.

Table A1. Shapiro-Wilk p-values (hops) for compounds with a VIP score greater than 0.95 and all known smoke compounds regardless of VIP score.

Table A1. Shapiro-Wilk p-values (hops) for compounds with a VIP score greater than 0.95 and all known smoke compounds regardless of VIP score.

Compound	**p-value**
Phenol	0.0033
Guaiacol	0.2320
Furfural	0.2432
m-Cresol	0.3109
4-Methylguaiacol	0.0018
Malic acid	0.7116

β-Ocimene	0.4770
β-Sitosterol	0.3349
β-Phellandrene	0.5512
Epicatechin	0.6190
Butenamide	0.4439
Xylopyranose	0.0041
Hexanoic acid	0.0532
Heptanoic acid	0.5137
β-Pinene	0.2989
Alanine	0.0087
α-Cubebene	0.2597
Ethanolamine	0.5902
Pinitol	0.7826
Shikimic acid	0.3814
Tocopherol	0.0051
p-Cresol	0.3369

Table A2. Mann-Whitney U p-values (hops) for compounds that failed the Shapiro-Wilk test between treatments and controls.

Compound	**p-value**
Alanine	0.0048
Tocopherol	0.3648
Xylopyranose	0.0308
Phenol	0.0044
4-Methylguaiacol	0.0044

Table A3. Welch's t-test p-values (hops) for compounds that passed the Shapiro-Wilk test between treatments and controls.

Compound	**p-value**
β-Sitosterol	0.0349
Butenamide	0.1692
Epicatechin	0.0076
Ethanolamine	0.3291
Malic acid	0.0820
Pinitol	0.6573
Shikimic acid	0.0834
m-Cresol	<0.0001
β-Pinene	0.8757
β-Phellandrene	0.7012
β-Ocimene	0.8596
α-Cubebene	0.9967
Heptanoic acid	0.5219
Furfural	<0.0001

Hexanoic acid	0.8261
p-Cresol	0.0571
Guaiacol	<0.0001

Table A4. All annotated compounds (hops) from both SPME and Liquid Injection sampling methods.

SPME	**Liquid Injection**
2-Methyl-3,2-butenol	2,6-Dimethyl-5-heptenal
4-Methylguaiacol	2-Imidazolidone-4-carboxylic acid
α-Calacorene	4-Amino butanoic acid
α-Cubebene	Adipo-2,6-lactam
β-Caryophyllene	Alanine
β-Ocimene	Allantoin
β-Phellandrene	Alloinositol
β-Pinene	Asparagine
Δ-Amorphene	Aspartic acid
Furfural	beta-sitosterol
γ-Amorphene	Butanedioic acid
γ-Cadinene	Butenamide
Geraniol	Catechin
Geranyl butyrate	Citric acid
Germacrene D	Epicatechin
Guaiacol	Ethanolamine
Heptanioc acid	Fructose
Hexanoic acid	Galactose
Humulene epoxide	Glucose
Humulene	Glutamic acid
Lavandulyl butyrate	Glyceric acid
Linalool	Glycine
m-Cresol	Humulene
Methyl geranate	Lumichrome
p-Cresol	Malic acid
Perillene	Methionine
Phenol	Methylsuccinic acid
Styrene	Myoinositol
trans-Calamenene	N-Acetylglucosamine
	Ornithine
	Palmitic acid
	Phenylalanine
	Pinitol like
	Pinitol like 2
	Pyroglutamic acid
	Quinic acid
	Ribulose
	Serine
	Shikimic acid

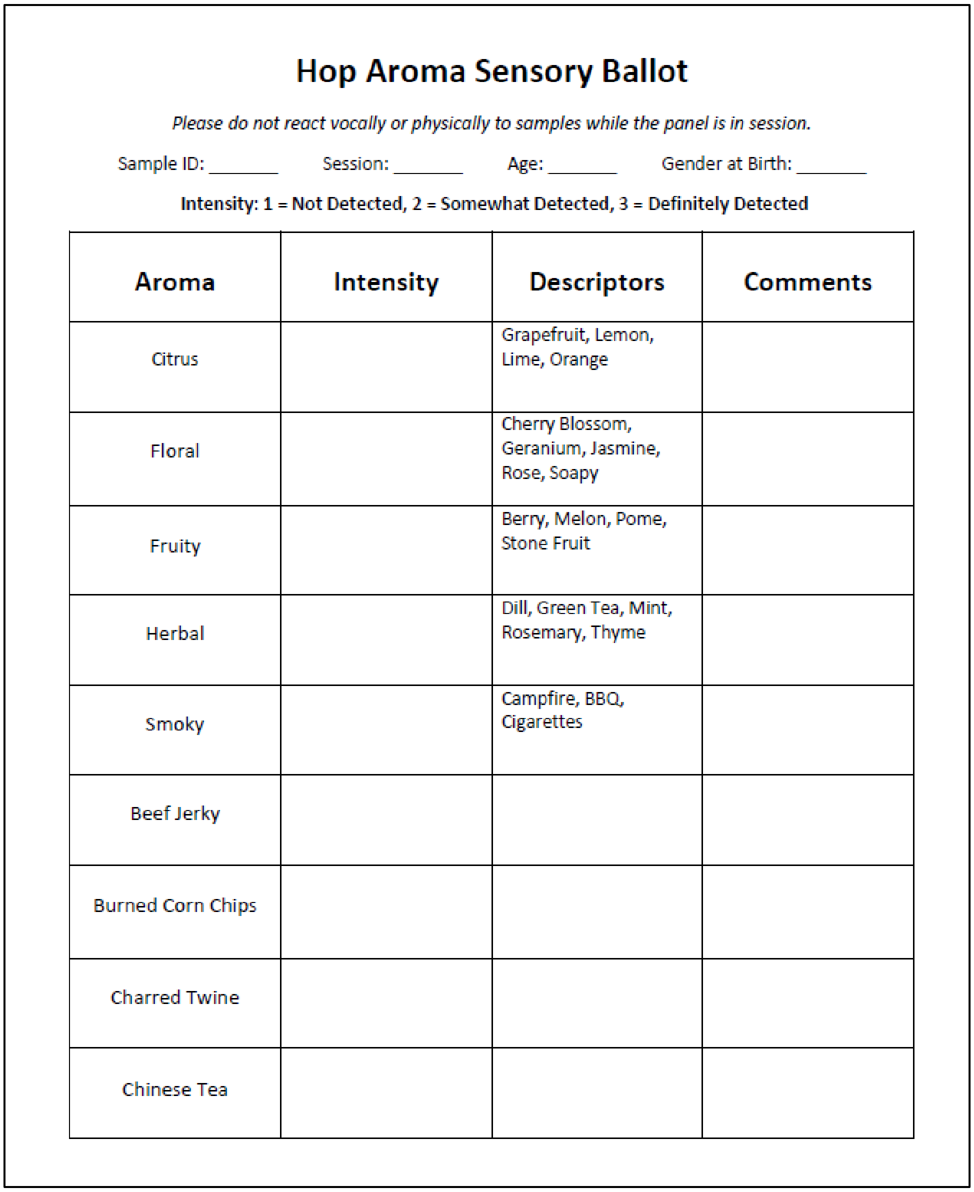
	Sorbose
	Sucrose
	Theronine
	Tocopherol
	Tyrosine
	Xylopyranose

Table A5. Compounds with variable importance in projection (VIP) scores greater than 0.95 and all known smoke compounds regardless of VIP score.

Compound	**VIP Score**
Phenol	2.51
Guaiacol	2.20
Furfural	2.15
m-Cresol	1.42
4-Methylguaiacol	1.41
Malic acid	1.39
β-Ocimene	1.36
β-Sitosterol	1.34
β-Phellandrene	1.23
Epicatechin	1.16
Butenamide	1.14
Xylopyranose	1.11
Hexanoic acid	1.10
Heptanoic acid	1.06
β-Pinene	1.05
Alanine	1.03
α-Cubebene	1.02
Ethanolamine	1.01
Pinitol	1.00
Shikimic acid	0.99
Tocopherol	0.96
p-Cresol	0.75

Table A6. Pearson correlation coefficient of reported smoke between cultivars. Critical value of 0.361 for Pearson's r with 28 degrees of freedom at p=0.05.

Compound	Furfural	Guaiacol	4-Methylguaiacol	m-Cresol	p-Cresol	Phenol	Xylopyranose
r	0.759	0.685	0.786	0.201	-.440	0.692	0.835



Hop Aroma Sensory Ballot

Please do not react vocally or physically to samples while the panel is in session.

Sample ID: _______ Session: _______ Age: _______ Gender at Birth: _______

Intensity: 1 = Not Detected, 2 = Somewhat Detected, 3 = Definitely Detected

Aroma	Intensity	Descriptors	Comments
Citrus		Grapefruit, Lemon, Lime, Orange	
Floral		Cherry Blossom, Geranium, Jasmine, Rose, Soapy	
Fruity		Berry, Melon, Pome, Stone Fruit	
Herbal		Dill, Green Tea, Mint, Rosemary, Thyme	
Smoky		Campfire, BBQ, Cigarettes	
Beef Jerky			
Burned Corn Chips			
Charred Twine			
Chinese Tea			

Figure A2: Sensory panel ballot.

Figure A3. Cat-of-nine-tails-style whip used in defoliation treatments,

Figure A4. *Cannabis sativa* L. ‘Unicorn’ plants before (a) and after (b) defoliation treatment.

Table A7. Selected Reaction Monitoring (SRM) transitions for targeted phytocannabinoid analysis.

	RT (min)	**Primary Transition**	**Confirmatory Transition**	**Polarity**	**CE (eV)**
CBT	14.65	315.1 -> 193.1	315.1 -> 122.9	+	-34
CBDA	3.7	357 -> 107	357 -> 245.1	-	56
CBDV	2.42	287.1 -> 164.9	287.1 -> 122.8	+	-46
CBDVA	2.11	329 -> 107	329 -> 217	-	37
CBD	4.66	315.1 -> 193	315.1 -> 122.9	+	-31
CBL	11.16	315.1 -> 81.1	315.1 -> 165.1	+	-37
CBLA	13.67	357 -> 191	357 -> 216.9	-	56
CBCO	2.98	257 -> 135	257 -> 146.9	-	34
CBC	12.55	315.1 ->193.1	315.1 ->122.8	+	-33
CBCA	13.87	357 -> 136	357 -> 147.9	-	42
CBG	4.45	317.1 -> 122.9	317.1 -> 193	+	-51
CBCV	5.72	287 -> 122.9	287 -> 164.9	+	-52
CBN	7.09	309 -> 279	309 -> 107.9	-	46
Δ9-THC	9.16	315.1 -> 192.9	315.1 -> 122.9	+	-36
Δ9-THCA	12.45	357 -> 245	357 -> 191	-	38
Δ8-THC	9.76	315.1 -> 193	315.1 -> 122.8	+	-62
CBGA	4.18	361.1 -> 343.1	343.1 -> 134.9	+	-20
CBNA	9.11	353 -> 279	353 -> 221.9	-	49
THCVA	5.69	329 -> 217.1	329 -> 163	-	41
THCV	4.23	287 -> 165.1	387 -> 122.9	+	-33

Table A8. Limits of detection (LOD), limit of quantification (LOQ), and regression slope for detected phytocannabinoids All regressions had $R^2 > 0.99$.

	LOD (µg/mg)	**LOQ** (µg/mg)	**Slope**
CBDA	0.304	1.014	0.026
CBDV	0.003	0.012	0.030
CBDVA	0.015	0.051	0.052
CBD	0.004	0.014	0.123
CBG	0.002	0.006	0.070
CBN	0.002	0.006	0.092
Δ9-THC	0.004	0.012	0.081
Δ9-THCA	0.003	0.011	0.100
CBGA	0.017	0.056	0.013
CBNA	0.001	0.002	0.360
THCVA	0.001	0.002	0.204
THCV	0.001	0.003	0.001

Table A9(a). CBD, CBDA, CBDV, and CBDVA concentrations (μg/mg). *0.5 × LOD.

Plant ID	Treatment	Defoliation Event	Sample Day	CBD	CBDA	CBDV	CBDVA
P009	CTRL	DE1	Harvest	0.36	64.50	0.03	4.46
P017	CTRL	DE1	Harvest	0.70	43.00	0.03	3.31
P031	CTRL	DE1	Harvest	0.54	33.80	0.03	1.84
P004	INJ	DE1	Harvest	1.42	96.50	0.11	4.75
P016	INJ	DE1	Harvest	0.98	76.30	0.07	6.25
P030	INJ	DE1	Harvest	1.00	29.40	0.04	1.83
P012	CTRL	DE2	1	0.05	29.60	0.00	1.11
P022	CTRL	DE2	1	0.06	37.10	0.00	1.27
P042	CTRL	DE2	1	0.16	69.10	0.01	2.88
P013	INJ	DE2	1	0.08	35.60	0.00	1.20
P021	INJ	DE2	1	0.16	71.70	1.74E-03*	2.75
P026	INJ	DE2	1	0.07	30.60	1.74E-03*	0.99
P022	CTRL	DE2	14	0.15	60.60	1.74E-03*	3.55
P028	CTRL	DE2	14	0.22	33.00	1.74E-03*	2.04
P036	CTRL	DE2	14	0.15	53.30	0.01	2.92
P013	INJ	DE2	14	0.13	66.70	1.74E-03*	2.98
P024	INJ	DE2	14	0.33	93.60	0.02	5.13
P026	INJ	DE2	14	0.14	58.30	0.00	3.11
P001	CTRL	DE2	Harvest	0.72	54.50	0.05	3.01
P003	CTRL	DE2	Harvest	0.81	50.90	0.04	3.05
P032	CTRL	DE2	Harvest	0.72	65.00	0.04	3.93
P039	CTRL	DE2	Harvest	0.51	63.90	0.03	3.44
P018	INJ	DE2	Harvest	1.04	98.90	0.09	7.16
P019	INJ	DE2	Harvest	0.93	79.90	0.06	4.16
P037	INJ	DE2	Harvest	0.50	93.20	0.04	4.87
P040	INJ	DE2	Harvest	0.74	82.90	0.04	4.36
P023	CTRL	DE3	1	0.36	74.50	0.02	4.78
P025	CTRL	DE3	1	0.39	59.90	0.03	3.65
P046	CTRL	DE3	1	0.44	51.80	0.02	2.71
P006	INJ	DE3	1	0.39	38.70	0.02	2.12
P038	INJ	DE3	1	0.35	76.60	0.02	4.79
P048	INJ	DE3	1	0.29	47.30	0.02	2.61
P029	CTRL	DE3	Harvest	1.16	66.80	0.07	4.42
P035	CTRL	DE3	Harvest	0.33	61.80	0.02	4.11
P007	INJ	DE3	Harvest	0.90	123.00	0.06	9.87
P014	INJ	DE3	Harvest	0.46	46.90	0.02	2.32
P034	INJ	DE3	Harvest	0.51	59.60	0.03	3.46
P043	INJ	DE3	Harvest	0.68	67.10	0.05	4.77

Table A9(b). CBG, CBGA, CBN, and CBNA concentrations (μg/mg). *0.5 × LOD.

Plant ID	Treatment	Defoliation Event	Sample Day	CBG	CBGA	CBN	CBNA
P009	CTRL	DE1	Harvest	0.133	1.670	8.20E-04*	0.007
P017	CTRL	DE1	Harvest	0.082	1.810	8.20E-04*	0.005
P031	CTRL	DE1	Harvest	0.042	1.100	0.001	0.008
P004	INJ	DE1	Harvest	0.145	2.020	0.003	0.020
P016	INJ	DE1	Harvest	0.149	2.230	0.001	0.012
P030	INJ	DE1	Harvest	0.053	1.110	8.20E-04*	0.008
P012	CTRL	DE2	1	0.044	3.540	8.20E-04*	0.001
P022	CTRL	DE2	1	0.062	4.910	0.000	0.003
P042	CTRL	DE2	1	0.086	4.150	0.001	0.005
P013	INJ	DE2	1	0.052	4.070	0.000	0.004
P021	INJ	DE2	1	0.118	6.900	0.001	0.006
P026	INJ	DE2	1	0.043	3.080	0.001	0.007
P022	CTRL	DE2	14	0.088	5.370	8.20E-04*	0.003
P028	CTRL	DE2	14	0.057	1.610	8.20E-04*	0.003
P036	CTRL	DE2	14	0.077	3.550	0.000	0.003
P013	INJ	DE2	14	0.084	5.200	0.001	0.004
P024	INJ	DE2	14	0.155	4.800	0.001	0.011
P026	INJ	DE2	14	0.080	4.660	8.20E-04*	0.004
P001	CTRL	DE2	Harvest	0.087	1.730	0.001	0.011
P003	CTRL	DE2	Harvest	0.083	2.080	0.002	0.014
P032	CTRL	DE2	Harvest	0.092	1.740	0.002	0.011
P039	CTRL	DE2	Harvest	0.100	2.250	0.001	0.009
P018	INJ	DE2	Harvest	0.197	2.810	0.002	0.025
P019	INJ	DE2	Harvest	0.137	1.520	0.001	0.016
P037	INJ	DE2	Harvest	0.173	1.780	0.002	0.015
P040	INJ	DE2	Harvest	0.140	1.700	0.001	0.011
P023	CTRL	DE3	1	0.125	3.500	0.001	0.010
P025	CTRL	DE3	1	0.060	1.700	0.001	0.012
P046	CTRL	DE3	1	0.062	2.280	0.001	0.012
P006	INJ	DE3	1	0.051	1.720	0.001	0.008
P038	INJ	DE3	1	0.127	2.490	8.20E-04*	0.007
P048	INJ	DE3	1	0.064	1.460	8.20E-04*	3.40E-04*
P029	CTRL	DE3	Harvest	0.110	2.530	0.002	0.018
P035	CTRL	DE3	Harvest	0.106	2.630	0.001	0.011
P007	INJ	DE3	Harvest	0.250	4.110	8.20E-04*	0.027
P014	INJ	DE3	Harvest	0.056	1.040	0.001	0.012
P034	INJ	DE3	Harvest	0.083	2.430	0.002	0.014
P043	INJ	DE3	Harvest	0.120	2.380	0.001	0.016

Table A9(c). Δ9THC, Δ 9THCA, THCV, and THCVA concentrations (µg/mg). *0.5 × LOD.

Plant ID	Treatment	Defoliation Event	Sample Day	Δ9THC	Δ 9THCA	THCV	THCVA
P009	CTRL	DE1	Harvest	0.040	1.630	0.002	0.211
P017	ctrl	DE1	Harvest	0.068	1.040	0.004	0.115
P031	CTRL	DE1	Harvest	0.057	0.926	0.003	0.083
P004	INJ	DE1	Harvest	0.139	2.760	0.008	0.264
P016	INJ	DE1	Harvest	0.106	2.470	0.006	0.251
P030	INJ	DE1	Harvest	0.116	0.684	0.007	0.070
P012	CTRL	DE2	1	0.008	0.860	5.17E-04*	0.055
P022	CTRL	DE2	1	0.008	0.958	0.001	0.056
P042	CTRL	DE2	1	0.015	1.370	5.17E-04*	0.145
P013	INJ	DE2	1	0.011	0.776	5.17E-04*	0.047
P021	INJ	DE2	1	0.026	2.280	5.17E-04*	0.157
P026	INJ	DE2	1	0.004	0.858	5.17E-04*	0.052
P022	CTRL	DE2	14	0.012	1.230	5.17E-04*	0.122
P028	CTRL	DE2	14	0.031	0.925	0.002	0.092
P036	CTRL	DE2	14	0.015	1.200	5.17E-04*	0.115
P013	INJ	DE2	14	0.012	2.120	5.17E-04*	0.150
P024	INJ	DE2	14	0.030	2.400	5.17E-04*	0.257
P026	INJ	DE2	14	0.014	1.440	5.17E-04*	0.120
P001	CTRL	DE2	Harvest	0.062	1.120	0.004	0.108
P003	CTRL	DE2	Harvest	0.086	1.370	0.004	0.138
P032	CTRL	DE2	Harvest	0.058	1.270	0.003	0.122
P039	CTRL	DE2	Harvest	0.041	0.990	0.003	0.097
P018	INJ	DE2	Harvest	0.121	3.750	0.004	0.410
P019	INJ	DE2	Harvest	0.093	2.190	0.003	0.216
P037	INJ	DE2	Harvest	0.044	2.590	0.001	0.297
P040	INJ	DE2	Harvest	0.060	1.920	0.003	0.202
P023	CTRL	DE3	1	0.037	1.850	0.002	0.197
P025	CTRL	DE3	1	0.059	2.430	0.005	0.213
P046	CTRL	DE3	1	0.049	1.160	0.002	0.108
P006	INJ	DE3	1	0.036	0.813	5.17E-04*	0.071
P038	INJ	DE3	1	0.033	1.920	5.17E-04*	0.172
P048	INJ	DE3	1	0.032	1.65E-03*	0.001	0.108
P029	CTRL	DE3	Harvest	0.125	1.640	0.007	0.175
P035	CTRL	DE3	Harvest	0.037	1.370	5.17E-04*	0.154
P007	INJ	DE3	Harvest	0.102	3.370	0.006	0.466
P014	INJ	DE3	Harvest	0.048	1.280	0.002	0.091
P034	INJ	DE3	Harvest	0.050	1.310	0.003	0.123
P043	INJ	DE3	Harvest	0.076	1.560	0.004	0.194

Table A10. Results from unpaired two-sample t-test of phytocannabinoid concentrations in defoliated plants versus controls. DE = Defoliation Event. Two-tail p-values are reported below; * indicates statistical significance ($p < 0.05$).

	DE1 Harvest	DE2 DAY1	DE2 DAY14	DE2 Harvest	DE3 DAY1	DE3 Harvest
CBD	0.0263*	0.8459	0.6985	0.4249	0.2612	0.7377
CBDA	0.4053	0.9718	0.1509	0.0018*	0.5849	0.7165
CBV	0.0811	0.3739	0.3857	0.1974	0.3662	0.9312
CBVA	0.5159	0.8971	0.3329	0.0492*	0.6237	0.7536
CBG	0.5108	0.8029	0.2764	0.0028*	0.9526	0.7808
CBGA	0.5545	0.7115	0.2782	0.9990	0.3799	0.9301
CBN	0.3490	0.9610	0.5103	0.9221	0.1539	0.6642
CBNA	0.1339	0.1505	0.2349	0.1157	0.0597	0.6629
THC	0.0068*	0.6613	0.9437	0.4033	0.0837	0.7370
THCA	0.3229	0.6634	0.0455*	0.0135*	0.2475	0.6455
THCV	0.0236*	0.3739	0.3739	0.5023	0.0720	0.9269
THCVA	0.4721	0.9997	0.1962	0.0147*	0.2773	0.6932

Table A11. Formulae used to calculate total phytocannabinoid percentage (w/w) with average percent and standard deviation in control plants.

% Total	**Formula**	DE1 Harvest	DE2 DAY1	DE2 DAY14	DE2 Harvest	DE3 DAY1	DE3 Harvest
CBD	%CBD + (0.877 × %CBDA)	4.183% ± 3.0	3.982% ± 2.0	4.312% ± 1.6	5.205% ± 0.78	5.483% ± 1.7	5.716% ± 3.0
CBDV	%CBDV + (0.867 × %CBDVA)	0.281% ± 0.20	0.152% ± 0.08	0.246% ± 0.11	0.295% ± 0.12	0.324% ± 0.12	0.374% ± 0.29
CBG	%CBG + (0.878 × %CBGA)	0.142% ± 0.04	0.375% ± 0.06	0.315% ± 0.17	0.180% ± 0.02	0.227% ± 0.08	0.237% ±0.01
CBN	%CBN + (0.876 × %CBNA)	0.001% ± <0.01	<0.001% ± <0.01	<0.001% ± <0.01	0.001% ± <0.01	0.001% ± <0.01	0.001% ± <0.01
THC	%THC + (0.877 × %THCA)	0.111% ± 0.03	0.094% ± 0.02	0.100% ± 0.01	0.110% ± 0.02	0.164% ± 0.06	0.140% ± 0.02
THCV	%THCV + (0.867 × %THCVA)	0.012% ± 0.01	0.007% ± <0.01	0.010% ± <0.01	0.010% ± <0.01	0.015% ± <0.01	0.015% ± <0.01

Table A12. Average percent and standard deviation in defoliated plants.

% Total	DE1 Harvest	DE2 DAY1	DE2 DAY14	DE2 Harvest	DE3 DAY1	DE3 Harvest
CBD	6.025% ± 3.0	4.041% ± 2.0	6.412% ± 1.6	7.863% ± 0.78	4.787% ± 1.7	6.571% ± 3.0
CBDV	0.378% ± 0.20	0.143% ± 0.08	0.325% ± 0.11	0.451% ± 0.12	0.277% ± 0.12	0.447% ± 0.29
CBG	0.168% ± 0.06	0.418% ± 0.18	0.439% ± 0.01	0.188% ± 0.05	0.174% ± 0.05	0.231% ± 0.12
CBN	0.001% ± <0.01	0.001% ± <0.01	0.001% ± <0.01	0.002% ± <0.01	0.001% ± <0.01	0.002% ± <0.01
THC	0.185% ± 0.10	0.116% ± 0.08	0.176% ± 0.04	0.237% ± 0.07	0.083% ± 0.08	0.172% ± 0.09
THCV	0.018% ± 0.01	0.007% ± 0.01	0.015% ± 0.01	0.025% ± 0.01	0.010% ± <0.01	0.019% ± 0.01

Table A13. Results from unpaired two sample t-tests assuming equal variance of Phytocannabinoid percentage in defoliated plants versus controls. DE = Defoliation Event. Two-tail p-values are reported below; * indicates statistical significance ($p < 0.05$).

% Total	DE1 Harvest	DE2 DAY1	DE2 DAY14	DE2 Harvest	DE3 DAY1	DE3 Harvest
CBD	0.3925	0.9714	0.1514	0.0017*	0.5819	0.7217
CBDV	0.5001	0.8952	0.3327	0.0494*	0.6217	0.7557
CBG	0.5457	0.7129	0.2709	0.8161	0.4005	0.9510
CBN	0.1587	0.1629	0.2081	0.1440	0.0493*	0.7072
THC	0.2852	0.6631	0.0465*	0.0148*	0.2413	0.6669
THCV	0.4413	0.9949	0.1990	0.0152*	0.2626	0.6956