The Pennsylvania State University

The J. Jeffery and Ann Marie Fox Graduate School

THE EFFECTS OF CULTIVATION AND EXTRACTION CONDITIONS ON THE PHYTOCHEMISTRY AND BIOACTIVITY OF *CANNABIS SATIVA* L.

A Dissertation in

Plant Biology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2024

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ABSTRACT

Cannabis sativa L. or hemp has become an in-demand crop due to its multiple uses and recent changes in the legality of its cultivation and consumption. However, due to decades of illegalization, our full comprehension of the plant's biology and its full effects on the human body are limited. This lack of understanding places many growers and consumers at risk of unwanted products or unknown effects. This dissertation investigates these unknowns, covering *Cannabis sativa'*s cultivation, extraction, and biological activity with special attention to its cannabinoid and terpenoid chemical profile. The chemical profile of the cannabis plant is a trove of bioactive metabolites featuring a myraid chemical compounds that vary in concentrations due to an overwhelming number of cultivars. Throughout this dissertation, we address the complexity of hemp's cannabinoid and terpenoid profile and answer questions that will aid researchers and commercial vendors in harnessing the chemical composition of this perplexing plant. A key finding in this dissertation is the effects soil health has on cannabinoid and terpenoid content in supercritical CO_2 extracts of hemp from outdoor grown inflorescence. Revealing that soil health can effect the cannabinoid composition and total terpenoid content based on cultivar. Moreover, this dissertation compared the cannabinoid and terpenoid profiles from three common hemp extraction techniques: hydrodistillation, supercritical CO_2, and solvent-based (ethanol) extraction. The results demonstrate how extraction protocol is a key regulator of cannabinoid/terpenoid abundance and diversity of any given hemp cultivar. Lastly, a novel *in vitro* bioactivity was revealed for the neutral cannabinoids cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), and cannabinol (CBN); shedding light on the importance of minor chemical compounds while postulating a possible mechanism of action responsible for the changes in blood pressure after consumption of a hemp extract. All together these studies add to the developing body of information surrounding hemp's cannabinoid and terpenoid compounds. However, follow-up

studies are required to corroborate the implications made by this dissertation and are essential to unraveling the unknowns surrounding this chemically diverse plant.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Joshua Kellogg, for the guidance, feedback, and support he provided throughout my graduate career. His support in both academic and personal matters was instrumental in the completion of this dissertation. I am also extremely thankful to Dr. Kent Vrana and Dr. Wesely Raup-Konsavage for the opportunities they offered and their insightful suggestions and encouragement in the work presented in this dissertation.

In addition, I would like to thank to all the members of the Kellogg lab with profound appreciation to Teal Jordan, Xiaoling Chen, and Ellie Abraham; your kindness and support will never be forgotten. A special thanks to Joshua Rogers, who played a pivotal role in executing several experiments reported in chapter 4. A warm appreciation goes out to my family and friends who continued to encourage me throughout my educational endeavors. The most significant acknowledgement goes out to my fianceé Avry, whose constant love and support provided me with the confidence and motivation to complete my educational career. I would also like to dedicate this dissertation to my dad who passed away in Nov 2021. I wouldn't be who I am today it wasn't for him.

Lastly, I wish to acknowledge the generous financial support from the PA Options for Wellness Fellowship Program. Without their funding, this research project would not have been feasible. I would also like to acknowledge Steve Groff of Cedar Meadow Farm (Holtwood, PA, USA) for his contributions of hemp material playing a major role in the completion of this dissertation. The authors would like to thank the Huck Institute's Metabolomics Cores Facility at the Pennsylvania State University for supporting mass spectrometry instrumentation and data collection.

Introduction

Cannabis sativa L. (e.g., cannabis or hemp) is well known for its ability to produce the non-psychoactive compound, cannabidiol (CBD)). CBD has been recognized for the treatment of chronic pain, inflammation, epilepsy, depression, and anxiety[1]. Aside from CBD and THC, *Cannabis indica* and *Cannabis sativa* are chemical troves of bioactive metabolites, possessing a diversity of compounds including several types of cannabinoids, terpenoids, and flavonoids[2]. Given this wide diversity of bioactive chemical compounds, growth and processing methods such as cultivation and extraction can play a large role in developing and harnessing the full spectrum of compounds that the cannabis plant can produce.

Cannabis use is growing in popularity as more states are legalizing it for its recreational use[3]. However, a complete understanding of the full effects of cannabis compounds beyond THC is still unknown, leaving many at risk for adverse effects or potential drug-drug interactions. Understanding mechanisms of action, biological activity of minor constituents, and synergy between cannabis compounds are of significant importance for public health. The effects of cultivation and processing procedures on the resulting chemical profile of cannabis extracts, and thus downstream biological effects, are also important, as they give producers and researchers refined tools to become more selective in the chemical profiles of any given cannabis or hemp products. The goal of this dissertation is to evaluate the effects of essential processing procedures on the bioactive chemical profile of hemp while exploring novel bioactivity that contributes to our current understanding of the effects of hemp compounds on the human body.

The first chapter includes a published review on secondary terpenes of hemp titled "Secondary Terpenes in *Cannabis sativa* L.: Synthesis and Synergy". This review was first published in *Biomedicines* (DOI: 10.3390/biomedicines10123142), and has been updated and renamed for the dissertation to include additional information on the cannabinoids of hemp. In this

chapter, I provide an overview of the biosynthesis of terpenes and cannabinoids, while covering the biological activities of secondary terpenes found in hemp. Secondary terpenes are terpenes that are found in lower concentrations in the cannabis plant that may contribute to the overall effects of hemp products. The literature surrounding secondary terpenes is limited as many reviews cover primary terpenes and cannabinoids that are present in larger concentrations within the inflorescence of the cannabis plant. Thus, this chapter emphasizes the importance of secondary terpenes as well as provides a perspective on the diversity and abundance of the different compounds that hemp can produce. This review also provides readers with a form of classification that categorizes cannabis based on the ratio of CBD and THC and a detailed list of the terpenes that can be found in each chemotype of cannabis. Furthermore, this chapter explores the concept of the 'entourage effect' a term describing the potential for synergy between compounds such as secondary terpenes and cannabinoids to achieve the effects experienced after using hemp products.

The second chapter titled "Impact of Soil Quality on Cannabinoid and Terpene Content of *Cannabis sativa* L." explores how the conditions of outdoor cultivation can affect the bioactive chemical profile of a hemp extract. While several cultivation methods for hemp exist, outdoor cultivation is especially effective in providing high yields of cannabis inflorescence with limited costs. A key component for the outdoor cultivation of hemp is soil composition. Several types of regenerative farming practices have been adopted by hemp growers to improve soil conditions and nutrients, including the use of cover crops, plants that are primarily grown for seasonal cover and other conservation purposes. This chapter is an original research article that compares the cannabinoid and terpenoid content of two hemp cultivars grown in two fields differing in soil preparation including the use of cover crops (with no-till) and conventional field consisting of tilled soil. A soil health assessment was conducted for each field providing physical, biological, and chemical characteristics. Overall, differences in organic matter, macronutrients, and micronutrients were observed between the two types of soil. This information was then paired with an analysis of

total cannabinoid and terpenoid profiles of supercritical CO_2 extracts of the two hemp cultivars from both fields. The outcome showed differences in individual cannabinoid content and total terpene concentration based on cultivar type for each field. This study provides outdoor growers with information on the effects soil health can have on cannabinoid and terpene content in hemp. However, studies with isolated treatments of the characteristics that contribute to the health of the soil are required to delineate the main driver between soil health and hemp cannabinoid and terpene content.

Chapter 3 is a study that was published in 2024 in the journal *Plants*, entitled "Effect of Hemp Extraction Procedures on Cannabinoid and Terpenoid Composition" (DOI: 10.3390/plants13162222). This study compared the cannabinoid and terpene profiles of three primary commercial procedures, using hemp inflorescence from a single hemp cultivar. The extraction techniques of hydrodistillation, supercritical CO_2, and solvent-based extraction (ethanol) were employed on the same harvest of a CBD/CBG dominant hemp cultivar and then evaluated for the resulting terpene and cannabinoid content using GC-MS and LC-MS/MS, respectively. An analysis of the resulting terpene and cannabinoid profiles revealed that hydrodistillation provided extracts with highly concentrated and diverse terpene profiles but with minimal amounts of cannabinoid content, supercritical CO_2 extracts were excellent at capturing neutral cannabinoids with minimal variety of terpenes, and ethanol extracts consisting of higher levels of acidic cannabinoids with minor terpene levels. The outcome of this study demonstrated how the process used to extract hemp can play a key role in the product composition altering the potential biological effects of any given hemp cultivar.

The fourth chapter is an original research paper exploring a novel biological effect of hemp compounds on an enzyme that helps control blood pressure titled "Angiotensin-Converting Enzyme (ACE) Inhibitory Activity of Cannabinoids from *Cannabis sativa* L.". Research surrounding the mechanism of action of hemp compounds on lowering blood pressure is limited .

The angiotensin converting enzyme or ACE, belongs to the renin-angiotensin system (RAS), a multi-hormonal system that helps constrict blood vessels in response to low blood pressure, and forms the vasoconstrictor angiotensin II from the biological peptide angiotensin I. Thus, ACE is an attractive target of high blood pressure medication. In this study, hemp extracts were tested for their ACE inhibitory characteristics using an *in vitro* enzymatic assay. Hemp extracts were then chemically fractionated to identify any cannabinoids or terpenes that may be responsible for the observed ACE inhibitory activity. Several resulting fractions demonstrated considerable amounts of neutral cannabinoids limited ACE activity (< 20% of control). This was paired with a molecular docking study of various cannabinoids and ACE, providing binding affinity scores that were compared to a known ACE inhibitor, lisinopril. A confirmation of the cannabinoid's ability to inhibit ACE activity was then carried out by testing purified standards of CBD, cannabichromene (CBC), cannabinol (CBN), and cannabigerol (CBG) against ACE activity. The subsequent tests revealed CBC and CBN to be the most potent of the four neutral cannabinoids tested. The outcome of this study demonstrates the ACE-inhibiting activity of hemp extracts, while also suggesting a possible mechanism by which bioactive compounds from hemp may act modulate blood pressure.

Together these chapters emphasize the complexity and plasticity of *Cannabis sativa*'s cannabinoid and terpenoid profile, and how its composition can alter its biological activity. Ultimately adding to the body of literature surrounding the processing of hemp and the full effects of its chemical constituents.

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Chapter 1

The Phytochemistry of *Cannabis sativa* L.

Abstract

Cannabis is a complex biosynthetic plant, with a long history of medicinal use. While the cannabinoids have received the majority of attention for their psychoactive and pharmacological activities, cannabis produces a diverse array of phytochemicals, including terpenes and flavonoids. These compounds are known to play a role in the aroma and flavor of cannabis but are also potent biologically active molecules that exert effects on infectious as well as chronic diseases. Furthermore, terpenes have the potential to play important roles as synergistic (e.g., "entourage") compounds that modulate the activity of the cannabinoids. This review highlights the diversity and bioactivities of terpenes in cannabis, especially minor or secondary terpenes that are less concentrated in cannabis on a by-mass basis. We will also explore the question of the entourage effect in cannabis, what studies to date have found supporting or refuting the concept of synergy in cannabis, and where synergy experimentation is headed to better understand the interplay between phytochemicals within Cannabis sativa L.

Introduction

Cannabis sativa L. is a dioecious plant of the Cannabaceae family and is perhaps most famous for its production of the psychedelic metabolite delta-9 tetrahydrocannabinol (D9-THC). Cannabis has been used in traditional medicine for millennia across several continents; cannabis has been used in traditional Chinese medicine therapies for the treatment of gout, pain, convulsions, insomnia, cough, headache, itching, and anemia[1], while in traditional Aryuvedic

practices cannabis has been recorded to stimulate digestion, function as an analgesic and sedative, as well as it's aphrodisiac, anti-parasitic, and anti-viral properties[2]. In addition to THC, cannabis produces a number of other cannabinoid compounds with potent activities. Cannabidiol (CBD) is one non-psychedelic cannabinoid that has emerged as a popular botanical supplement ingredient[3]: a majority of Americans are aware of CBD, and ca. 18% have tried or are regular users of CBD products[4]. The US hemp-derived market in cannabidiol (CBD) topped $4.7 billion in 2021 and is expected to reach $12.0 billion by 2026[5]. However, while many bioactivities can be ascribed to the presence of cannabinoids, cannabis is a prolific biosynthetic organism, producing over 750 known phytochemicals, including flavonoids and terpenoids, many of which possess putative medicinal properties[6] , yet the majority of these phytochemical constituents and their mechanisms of action have not been fully explored.

Terpenes (also termed isoprenoids) are the most diverse class of natural products and are the most abundant by mass[12]; in cannabis, terpenes account for 3–5% of the dry mass of the inflorescence[13]. Terpenes have incredible potential for bioactivity against both infectious and chronic health conditions[14–16] and have been employed for thou-sands of years for therapeutic purposes, including in anti-inflammatory, anti-microbial, antioxidant, antitumor, and antidiabetic capacities[17]. In addition, terpenes often pro-vide the foundation for the flavor and aroma of numerous plants and food products[18–20], including cannabis[21], granting the plant earthy or herbal aromas that combine with hints of sweet, citrusy, or piney scents. The terpene profile and content of cannabis has been reviewed previously[13,22,23]; however, analytical profiling studies, as well as cannabis phytochemistry reviews, traditionally focus on the more prevalent, terpenes such as myrcene, α-pinene, limonene, β-caryophyllene, linalool, humulene, ocimene, bisabolol, and terpinolene. The presence of a vast array of terpenes highlights the additional complexity of cannabis, as well as the further potential for bioactivity within this complex plant.

In botanical samples, mixtures of phytochemicals are often more effective than their individual constituents in isolation due to additive or synergistic interactions among compounds. Indeed, many chronic and infectious diseases are not regulated by a single cellular target, but often have multiple regulating pathways[24,25]. As organisms in a complex and dynamic ecological environment, plants have evolved to address this multi-factorial disease etiology through the synthesis of structurally and functionally diverse phytochemicals. Thus, cannabis may also exert its bioactive effects via a combination of multiple constituents. Originally hypothesized in the late 20th century and termed the "entourage effect"[26], synergy between different cannabinoids has been documented in several studies. However, the potential for synergy between cannabinoids and other chemical classes, especially terpenes, has remained underreported.

This chapter aims to synthesize recent studies and information regarding the compositional diversity of terpenes, especially 'minor' terpenoid structures (compounds that are less prevalent in the plant on a by-mass basis) that have not been the focus of other reviews yet are found in diverse cultivars of cannabis and have unique and varied bioactivities as well. This is a unique feature of this review. In addition, we will build on the body of knowledge regarding how terpenes can potentially work in concert with cannabinoids to enhance bioactivity, as this is a timely topic given the upswing in interest in cannabis and potential synergy/entourage effects.

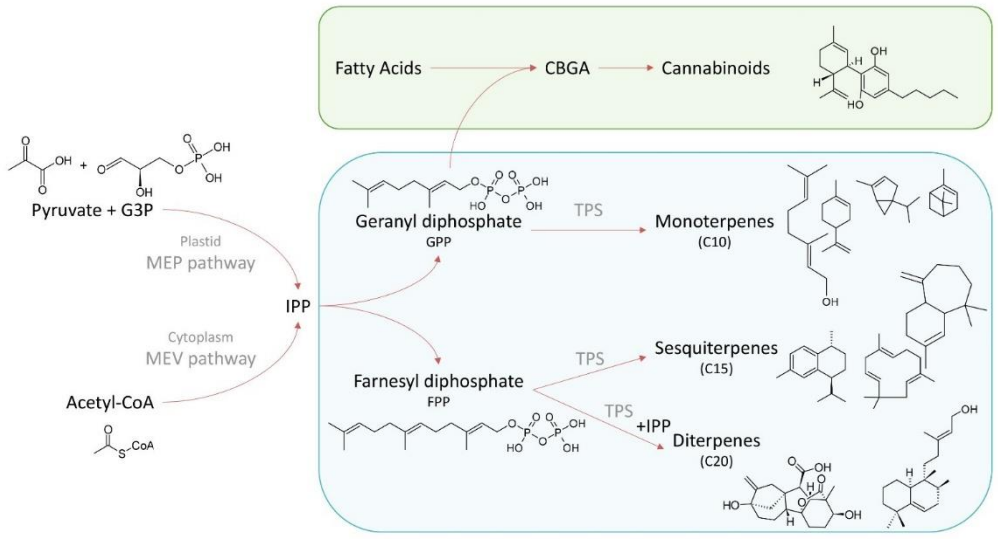
Cannabinoid Biosynthesis, Diversity, and Bioactivity

Cannabinoids are a group of compounds that contain a C32 terpenophenolic backbone[219]. The initial pathway for cannabinoid biosynthesis utilizes tetraketide synthase (TKS), catalyzing the condensation of hexanoyl-CoA and three malonyl-CoA forming 3,5,7-trioxododecaneoyl-CoA[220,221,222]. This molecule then undergoes cyclization and aromatization forming olivetolic acid which is then prenylated by an aromatic prenyltransferase forming the acidic precursor

cannabiergolic acid (CBGA)[223,224,225]. At this point in the biosynthetic process, the type of cannabinoid is determined, as CBGA is catalyzed by a specific cannabinoid acid synthase, e.g., cannabidiolic acid (CBDA) synthase catalyzes CBGA to form the acidic precursor CBDA that can be non-enzymatically decarboxylated to form neutral cannabinoid cannabidiol (CBD)[222.226].

Terpene Biosynthesis

Terpenes originate from the 5-carbon precursor isopentenyl diphosphate (IPP), which is biosynthesized from either pyruvate and glyceraldehyde (via the methylerythritol phosphate (MEP) pathway in plastids)[27] or from acetyl-coA (via the mevalonic acid (MEV) pathway in the cytoplasm)[28] (Figure **1-1**). One or more IPPs condense with di-methylallyl diphosphate (DMAPP) in a 1′–4 fashion to form geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), or geranylgeranyl diphosphate (GGPP, C20). GPP and FPP serve as substrates for a multitude of synthetic reactions, condensing together to form the precursors of carotenoids and steroids, or cyclizing to form a myriad of terpene natural products (e.g., monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20))[12,29]. GPP also condenses with a diphenol with an alkyl chain (e.g.,



olivetolic acid) to form the cannabinoids[30]. In cannabis, over 200 terpenes have been published to date[31]

Figure **1-1**: General scheme of terpene synthesis pathway in Cannabis sativa L.

Terpenoid biosynthesis is governed by a family of homologous enzymes, the terpene synthases (TPS)[29,32], which catalyze the formation of different types of terpenes, including monoterpenes, diterpenes, hemiterpenes, and sesquiterpenes. These essential enzymes are encoded in large gene families that have been broken down into seven subfamilies based on phylogenetic analyses rendering, TPS-a, -b, -c, -d, -e/-f, -g, and -h, each based on amino acid length and location of emergence, such as angiosperms or gymnosperms [29,33]. In angiosperms, the TPS-a subfamily contains sesquiterpene synthases (ses-qui-TPSs); the TPS-b subfamily contains monoterpenes synthases (mono-TPSs) and hemi-terpene synthases[34].

Booth et al. analyzed the genome and transcriptome of Purple Kush cannabis to identify more than 30 cannabis terpene synthases (CsTPS genes)[35], which has been expanded to over 14 cultivars, representing chemotypes I, II, and III[34,36,37]. The characterized TPS genes of cannabis are documented as being a part of the TPS-a and TPS-b subfamilies[29]. Only nine of the 30 CsTPS genes have been fully characterized with respect to their catalytic functions, eight of which are multi-product enzymes that can generate different terpene structures from either GPP or FPP substrates[35,38]. Interestingly, genetic variation in these CsTPS has been associated with differences in the Sativa-Indica scale of cannabis labeling. Genotyping 100 cannabis samples for > 100,000 single nucleotide polymorphisms revealed that Sativa- and Indica-labelled samples were indistinguishable from a genome perspective; however, variation in CsTPS genes translated to shifts in the terpene profile and was correlated with the current dichotomous label system, suggesting terpenes (and genetic markers associated with terpene biosynthesis) could have a large

role in governing the strain classification[39]. This biosynthetic plasticity could be one explanation for the diversity of terpenes found in cannabis; however, it is important to keep in mind that the CsTPS responsible for many cannabis terpenes remain unexplored. When considering the incredible diversity of cannabis terpenes, it is un-known how the expression levels of different CsTPS could vary with plant development stage, plant organ and cell-type, and environmental factors. In addition, non-enzymatic modifications of terpenes, such as cyclization and oxidation, can increase structural diversity independent of enzymatic biochemical reactions. Even post-harvest considerations can change the terpene profile, especially the smaller, more volatile hemiterpenes and monoterpenes[40]. More qualitative and quantitative studies are needed to comprehensively profile the terpenes found in cannabis and how those concentrations relate to ex-pression levels and functionality of the CsTPS.

Terpene Diversity in Cannabis

Over 20,000 terpenes have been identified in the Plantae kingdom, making these highly volatile compounds one of the most structurally and functionally diverse groups of natural products[41]. Cannabis is widely known for its assorted terpene profiles. To date, 200 terpenes/terpenoids have been detected in cannabis[42]. However, the complete identification and quantification of the vast majority of terpenes/terpenoids remains undetermined, blunting our knowledge of the impact of cannabis terpenes on plant and human health[43]. Thus, the complete identification of terpenes in cannabis may suggest a substantial assortment of cannabis terpenes unknown to current breeders and researchers.

With the tremendous diversity of compounds in cannabis, researchers seek to categorize the main chemical constituents of cannabis cultivars or ‘strains’ by establishing five classes of chemotypes based on cannabinoid ratios. These are classified as Chemotypes (I): high

THCA:CBDA ratio; (II) intermediate ratios of THCA:CBDA; (III) low THCA:CBDA ratio; (IV) high CBGA content/low ratio of THCA:CBDA; and (V) containing almost no cannabinoids[44]. This classification has drawn researchers to further categorize canna-bis chemical profiles by associating cannabinoid content with bioactive metabolites such as terpenes. Table **1-1** illustrates the concentration range (mg/g) of terpenes and terpene derivatives reported in published research articles investigating the terpene content of specific cannabis chemotypes. Chemical profiles of common cannabis cultivars continue to show that myrcene, β-caryophyllene, limonene, α-terpinene, and α-pinene are the most prominent terpenes that can be found in the first three chemotype varieties[34,45,46,47,48]. Terpene profiles of the remaining chemotypes are limited or have yet to be investigated. Conversely, the classification of secondary terpenes (terpenes found in lower concentrations) in cannabis chemotypes is limited, as they are often disregarded or unreported due to a lack of reference material. More studies on cannabis terpene chemotypes are required to identify the relationships between specific terpenes and cannabinoid content.

Birenboim et al., 2022, were the first to demonstrate a highly accurate classification of medicinal cannabis chemovars based on their cannabinoid and terpene profiles. Using a partial least-square discriminant analysis multivariate (PLS-DA) technique, Birenboim et al. were able to differentiate terpene content between the inflorescences of three major chemovars (high-THCA, high-CBGA, and a hybrid). They concluded that the terpenes of the three major classes were significantly different in their concentrations of different terpenes[49], providing evidence of the high-THCA class having a higher abundance of limonene, β-caryophyllene, β-pinene, α-humulene, γ-elemene, and seychellene. Within the hybrid class, α-pinene and β-myrcene are more pronounced, followed by a high abundance of γ-eudesmol, α-bisabolol, and guaiol in the high-CBGA class. However, these results represent 14 different cannabis chemovars, including seven high THC chemovars, five hybrid chemovars, and only two high-CBG chemovars. The plant material used was from commercial breeding lines that could not be affiliated to a specific

subspecies because of crossings between different cultivars over many generations. Moreover, several factors have been shown to influence terpene diversity, such as plant genetics, pest presence, overall plant health, soil composition, proper drying, curing, and microbiology[34,50,51,52,53,54,55].

Variations in terpene expression can also be dependent upon the stage of growth. In 2016, Aizpurua-Olaizola et al. analyzed the terpene and cannabinoid content of the leaves and flowers of cannabis chemotypes I, II, and III. For 23 weeks, a chemical profile was generated on a weekly basis, providing the researchers with a total content of canna-binoids and terpenes at different stages of growth. Researchers found that chemotypes II and III required more time to reach their peak production of monoterpenes compared to chemotype I. Major terpene differences were also observed between chemotypes I and III. The distinct terpenes of chemotype I included γ-selinene, β-selinene, α-gurjunene, γ-elemene, Selina-3.7 (11) diene, and β-curcumene, while chemotype III displayed β-eudesmol, γ-eudesmol, guaiol, α-bisabolol, or eucalyptol. This suggests a chemo-type-dependent terpene distribution, as the investigators describe the more prominent terpenes in chemotype III as having a higher correlation coefficient with CBDA and chemotype I terpenes having a higher correlation coefficient with THCA[53]. Despite the differences in terpene content at different stages of growth, limitations of terpenes and cannabinoid expression may be observed based on light exposure and select spectra.

A high abundance of terpenes and cannabinoids can be found on the surface of cannabis inflorescence and leaves in the glandular appendages known as trichomes[56,57]. Trichomes are believed to be a defense mechanism against several different stresses, including light stress[58,59]. This has led to the proposed ecological function of cannabinoids and terpenes aiding in protection against high light exposure[58]. Additionally, research has shown the altering effects LED light can have on THC and terpene concentrations, but not CBD[57,58]. One study provided evidence of supplemental green light increasing THC and terpene content in comparison to controls.

However, quantification of IPP and DMAPP were not conducted, leaving the mechanistic implications undetermined[52]. With the increasing application of LED lighting for indoor cultivation, the chemical profiles of the desired chemotype may be susceptible to changes based on light application. Nonetheless, with the information surrounding the factors that influence terpene concentrations, terpene biosynthesis, and genetic expression, new cultivars with desired cannabinoid and terpene profiles may become attainable as the research surrounding terpenes in cannabis continues.

Table **1-1**: Concentrations of terpenes found in cannabis. Concentration range is given by chemotype where available; Tr—trace (< level of quantitation).

Compound	Chemotypes	Rage of Average Concentrations Reported per Chemotype (mg/g Dry Weight)	Reference
Agrospirol	I	I: Tr–0.50	[45]
Alloaromandrene	I, II, III	I: 0.004–0.08 II: 0.08–0.10 III: 0.05–0.10	[53,60]
Aromadendrene	I	I: 0.02–0.13	[61]
α-Bisabolol	I, II, III	I: Tr–1.10 II: 0.57–1.22 III: 0.07–2.31	[34,45,46,53,60,62–64]
α-Bisabolene	I, II, III	I: 0.13–0.50 II: 0.11–0.29 III: 0.03–0.50	[53,61]
β-Bisabolene	I, II, III	I: 0.05–0.17 II: 0.18–0.51 III: 0.12–0.71	[53]
Borneol	I, II, III	I: 0.01–0.03 II: 0.05 III: 0.009–0.02	[34,61,63,64]
α-bergamotene	I, II, III	I: 0.024–1.18 II: 0.45–0.81 III: 0.018–0.68	[34,53]

Cis-bergamotene	I, III	I: 0.07–0.11 III: 0.21	[61]
Trans-bergamotene	I, III	I: 0.12–0.28 III: 0.04	[61]
Bulnesol	I, II, III	I: 0.10–0.50 II: 0.090–0.19 III: 0.070–0.49	[34,45,53]
γ-cadinene	I, III	I: 0.41–0.60 III: 0.02	[61]
Camphene	I, III	I: 0.002–0.09 III: 0.001–0.48	[34,60,63,64]
Camphor	I	I: 0.001–0.01	[61,64]
P-Cimene	I, III	I: 0.016 III: 0.01	[64]
β-Caryophyllene	I, II, III	I: 0.24–8.20 II: 0.86–3.90 III: 0.16–3.17	[34,45,46,60–65]
β-Caryophyllene oxide	I, II, III	I: 0.005–0.06 II: 0.02 III: 0.09	[60,61,63]
Trans-β-caryophyllene	I, III	I: 0.02–0.06 III: 0.06	[53,61]
δ-3-carene	I, II, III	I: Tr–0.60 II: Tr III: 0.065–0.070	[45,46,61,64,65]
α-Cedrene	I, III	I: 0.038 III: 0.023	[64]
β-Citronellol	I, III	I: 0.002 III: 0.001–0.003	[60,64]
α-curcumene	I, III	I: 0.008 III: 0.017	[60]
β -Curcumene	I, II, III	I: 0.014–0.61 II: 0.061–0.16 III: 0.016–0.09	[53,60]
Cyclounatriene	I, III	I: 0.02–0.13 III: 0.086	[34]
Elemene	I, II	I: Tr–2.70 II: Tr	[45,65]
γ-elemene	I, III	I: 0.104–1.89 III: 0.04–0.068	[34,53,61]
δ-elemene	I, III	I: Tr–0.392 III: 0.005	[34]
Eucalyptol	II, III	II: 0.010–0.07 III: 0.052–0.14	[53,60,63]
Eudesma-3,7(11)-diene	I, III	I: Tr–0.80 III: 0.05	[34,61,65]

Eudesmane	I, III	I: 0.33–0.55 III: 0.04	[34]
A-eudesmol	I, II	I: 0.02 II: 0.26	[63]
β-Eudesmol	I, II, III	I: Tr–0.92 II: 0.23–0.65 III: 0.085–1.01	[45,53,61,63,64]
γ-Eudesmol	I, III	I: Tr–0.80 II: 0.30–0.78 III: 0.010–1.03	[34,45,53,61]
α-farnesene	I, II, III	I: 0.02–0.06 II: 0.24 III: 0.002	[34,63]
β-farnesene	I, II, III	I: 0.019–1.96 II: 0.73–1.6 III: 0.008–1.4	[34,53,65]
Trans-β-farnesene	I, III	I: 0.31–1.06 II: 0.35 III: 0.05	[61,63]
Fenchone	I, II, III	I: 0.005–0.03 II: 0.02 III: 0.007–0.008	[60,63,64]
Fenchol	I, II, III	I: 0.047–1.09 II: 0.09–0.31 III: 0.028–0.138	[34,46,60–64]
Germacrene B	I, III	I: 0.25–1.27 III: 0.34	[34]
Geraniol	I, III	I: 0.01 III: 0.004	[63,64]
Geranyl Acetate	I	I: Tr–0.70	[46]
Guaiol	I, II, III	I: Tr–1.09 II: 0.27–0.87 III: 0.010–1.21	[34,45,53,61,63,65]
α-guaiene	I, III	I: Tr–0.50 II: Tr III: Tr	[45,65]
δ-guaiene	I, II	I: Tr–0.80 II: 0.80	[45,61,65]
α-gurjunene	I	I: 0.1–0.46	[53]
Humulene	I, II, III	I: Tr–4.00 II: 0.64–1.11 III: 0.26–0.93	[45,46,53,64]
α-Humulene	I, II, III	I: 0.09–1.93 II: 0.32–0.36 III: 0.14–0.27	[34,60,62,63,65]
Isopulegol	I, II	I: 0.02–0.04	[63]

		II: 0.02	
Ledene	I, II	I: 0.11–0.13 II: 0.05	[63]
Limonene	I, II, III	I: Tr–9.1 II: 0.079–1.14 III: 0.022–1.44	[34,45,46,53,60–64]
Linalool	I, II, III	I: Tr–3.10 II: 0.27–0.35 III: Tr–0.36	[34,45,46,53,60–64]
Cis-linalool oxide	I, III	I: 0.002 III: 0.005	[60]
Trans-linalool oxide	I, III	I: 0.002 III: 0.002	[60]
Menthol	I, III	I: 0.001 III: 0.001	[60]
β-Myrcene	I, II, III	I: 0.12–14.8 II: 0.20–3.02 III: 0.18–7.60	[34,45,46,53,60–65]
Nerolidol	I, II, III	I: 0.02 III: 0.01	[61]
Trans-nerolidol	I, III	I: 0.019–1.66 II: 0.09 III: 0.005–0.07	[60,63,64]
β-Ocimene	I, III	I: 0.21–1.38 II: 0.02 III: 0.19	[34,53,63]
Cis-Ocimene	I, II, III	I: 0.006–3.9 II: 1.00 III: 1.00	[45,60,61,64,65]
Trans-Ocimene	I, III	I: Tr–3.8 III: 0.007–0.01	[46,60,64]
α-phellandrene	I, II, III	I: Tr–0.60 II: Tr III: Tr	[65]
β-phellandrene	I, III	I: Tr–2.1 II: 0.70 III: 0.097–0.50	[34,65]
α-pinene	I, II, III	I: Tr–6.70 II: 0.068–4.63 III: 0.004–1.40	[34,45,46,53,60–65]
β-pinene	I, II, III	I: Tr–2.00 II: 0.054–0.80 III: 0.001–0.50	[34,45,46,53,60–65]
α-phellandrene	I, II, III	I: 0.003–0.7 II: Tr III: 0.001	[46,60,61]

2-pinanol	I, III	I: 0.036–0.16 III: 0.047	[34]
Sabinene	I, III	I: 0.005 III: 0.001	[60]
Cis-sabinene hydrate	I, II	I: 0.015–0.08 II: 0.003–0.03	[60,61,63]
α-selinene	I, II, III	I: 0.04–1.36 II: 0.26–0.65 III: 0.094–0.79	[34,53,63]
β-selinene	I, II, III	I: 0.093–0.61 II: 0.09–0.34 III: 0.10–0.22	[53,63]
γ-selinene	I, II, III	I: 0.09–0.63 II: 0.06–0.09 III: 0.03–0.14	[53,61,65]
δ-selinene	I, III	I: 0.10–0.36 III: 0.09	[34]
Selina-3.7 (11) diene	I, II, III	I: 0.03–1.89 II: 0.05–0.07 III: 0.06–0.092	[53]
β-Sesquiphellanderene	I, II, III	I: 0.09–0.48 II: 0.14–0.23 III: 0.074–0.19	[53]
α-Terpinene	I, II, III	I: Tr–0.10 II: Tr III: Tr–0.068	[45,60,64]
γ-Terpinene	I, III	I: 0.02–0.06 III: 0.01–0.06	[46,60,61,64]
Terpineol	I, II, III	I: Tr–0.70 II: 0.60 III: Tr	[45]
Terpinen-4-ol	I, III	I: 0.02 III: 0.01	[60]
α-Terpineol	I, III	I: 0.04–0.9 II: 0.29 III: 0.11–0.22	[34,46,60,62,64,65]
Terpinolene	I, II, III	I: Tr–13.9 II: 0.010–3.70 III: 0.019–2.90	[34,45,46,53,60,63–65]
Valencene	I, II	I: 0.001–0.06 II: 0.01 III: 0.16	[34,60,63]

Potential Roles of Secondary Terpenes

The biological activity of cannabis terpenes is a growing topic that been extensively covered in multiple reviews[13,23,66,67,68,69,70,71]. These reviews on the therapeutic properties of cannabis terpenes primarily cover the commonly encountered mono- and sesquiterpenes (e.g., β-caryophyllene, β-myrcene, α- and β-pinene, α-humulene, limonene, terpenoline, and linalool). For this reason, this review aims to further investigate nine secondary terpenes of cannabis, based on their abundance in the plant and their therapeutic potential (Figure **1-2**). Regardless of their minor presence, the significant therapeutic value of secondary terpenes could point towards stronger or novel synergistic effects. The following is a summary of the more uncommon but notable secondary terpenes/terpenoids in cannabis and their potential therapeutic value; it is impossible to describe all the pharmacological effects of terpenes/terpenoids in this paper, but we shall give some examples of how these compounds possess multi-functional bioactivity. It is worth noting that the compounds often have multiple potential activities, and there is overlap of activities between terpene compounds.

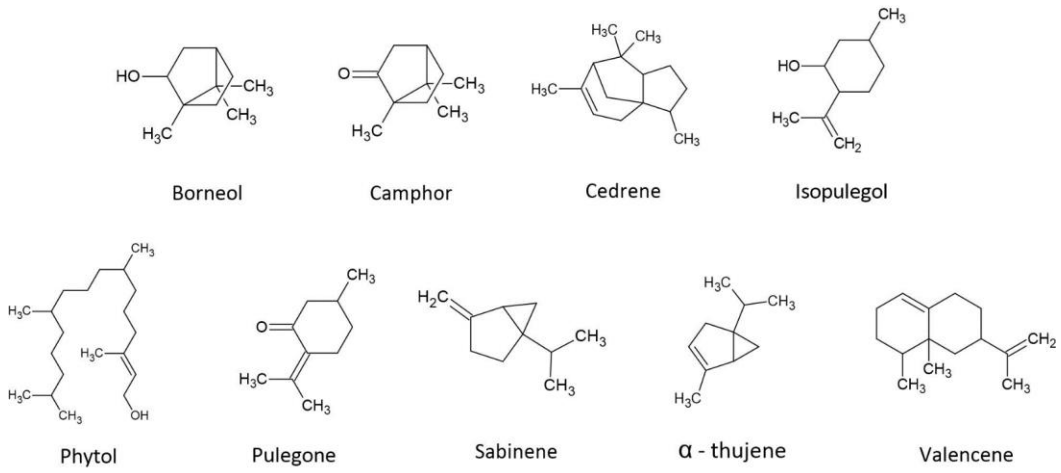


Figure **1-2**: Structures of secondary terpenes present in *Cannabis sativa* L.

Borneol

Identified as a monoterpene, borneol is a terpene derivative that can be found in several plant species, including *Cannabis sativa* L.[34,61,64]. The scent of this aromatic compound has been equated to a woody balsam aroma. Traditional Chinese medicine has employed the therapeutic properties of borneol for thousands of years as a resuscitation drug due to its active orifice-opening effects[72,73]. These effects are hypothesized to enhance blood–brain barrier (BBB) permeability[72,73,74], allowing for improved drug delivery to the central nervous system[73]. On top of its enhancement of BBB permeability, borneol also possesses anti-microbial, anti-inflammatory, anti-nociceptive, antithrombotic, neuroprotective, and genoprotective effects[75,76,77,78,79,80,81].

Camphor

This cyclic monoterpene ketone has been described as producing a strong mothball-like scent[82]. Like borneol, camphor has a long history of being used for its repellent and biological effects[83,84]. The medicinal properties of camphor oil include antibacterial, antiviral, antitussive, antimutagenic, anti-cancer, anti-inflammatory, antioxidant, and antidiabetic activity[85,86,87,88,89,90,91]. Studies evaluating camphor's biological effects typically involve wood extracts of *Cinnamomum camphora*, the camphor laurel tree that primarily consists of high levels of camphor and its derivatives[92].

Cedrene

Cedrene is a sesquiterpenoid that is classified as a secondary terpene of cannabis as only small amounts of the terpenoid have been identified in select cultivars. This sesquiterpenoid is commonly found in cedar and juniper trees[93,94]. The aroma produced by cedrene has been described as a woody, crisp scent. Like many aromatic compounds, the biological activities of cedrene have been explored primarily through extracts of cedarwood oil, which has been characterized as having copious amounts of the sesquiterpenoid[95]. Although many of the studies report the effects of full cedarwood oil, cedrene is a primary constituent of this oil and thus leads

to speculation that the biological activity of cedarwood oil is due to cedrene, with activities including antifungal, anti-microbial, and anti-cancer[96,97,98]. A few studies have been performed on isolated α-cedrene, suggesting potential anti-obesity properties[99,100].

Isopulegol

Identified as a monoterpene alcohol, isopulegol can be found at different concentrations in a variety of plants, including lemongrass, mint, eucalyptus, and several others[101,102,103]. The scent of isopulegol has been described as a minty fragrance[104]. Because of its presence in a diversity of plants, researchers have described its potential bioactivity, including antidepressant [105], antianxiety[105], anticonvulsant[106], gastroprotective[107], and anti-inflammatory activity[108]. Though isopulegol is said to contain several diverse bioactive properties, more research is required to characterize the mechanism in play.

Phytol

Phytol is a diterpenoid that has been described as having a grassy-fresh aroma[104]. Phytol is a common terpenoid of highly aromatic plants such as green tea, mint, tarragon, basil, and cannabis cultivars[109,110]. This terpenoid has been speculated to hold antioxidant[111,112], anti-inflammatory[113], analgesic[112], anti-cancer[111,114] anti-anxiety[115], anti-convulsant[116], and sedative[117] properties. Phytol and its derivatives have also been explored for toxicity in immune-compromised mice, suggesting non-toxic effects[118,119,120].

Pulegone

As a monoterpene, pulegone can be found in various aromatic herbs, but is commonly associated with the mint family, such as catnip and peppermint[121,122]. Known for its minty fragrance, pulegone has been identified at low concentrations in cannabis[22,123]. Researchers have suggested that pulegone contains anti-microbial[124], anti-anxiety[125], antipyretic[126], sedative[126,127], and anti-inflammatory[128] properties.

Sabinene

This bicyclic monoterpene can be found in a variety of different plant species and is often associated with a spicy flavor and aroma. Cannabis cultivars typically contain small concentrations of sabinene; however, some cultivars have been characterized as having more sabinene than others, such as Super Silver Haze and Arjan's Ultra Haze[104]. The medical benefits of this monoterpene suggest anti-inflammatory[129], antioxidant[130], and anti-microbial[131] properties. The known benefits of this terpene are limited, requiring more research and exploration of the effects of sabinene in cannabis.

Thujene

Like many monoterpenes, thujene can be found in a variety of plant-derived essential oils such as eucalyptus[132], frankincense[133], and dill[134]. Similar to humulene, α-thujene produces a woody, spicy aroma[135]. Researchers have tested the bioactivity of essential oils that consist of high levels of thujene, such as the essential oil of *Boswellia serrata*, which has been reported to consist of 61.36% α-thujene[136]. Investigations exploring the bioactivity of essential oils containing α-thujene suggest antioxidant[137], anti-inflammatory[138], anti-microbial[139], and analgesic[140] properties. Although these studies provide insight into the bioactivity of essential oils containing this compound, more research is required to delineate the therapeutic properties of isolated α-thujene.

Valencene

The sesquiterpene valencene produces an aroma that is often associated with citrus fruits such as Valencia oranges[141]. In cannabis, valencene has been reported in several different cultivars, but only at low concentrations[70]. Aside from its appealing scent, valencene's bioactivity has been explored through various essential oil profiles, speculating anti-inflammatory[142], neuroprotective[143], anti-allergic[144], and anti-microbial[145] properties.

While minor terpenes may not be the most abundant in cannabis, they have the potential to aid in the biological activities of cannabis. These terpenes demonstrate overlapping activity

with each other, often targeting the same biological function (even if mechanism of action remains unknown) (Figure **1-3**). Likewise, an overlap of therapeutic benefit between cannabinoids and these secondary terpenes may be inferred based on current cannabinoid research[60], suggesting a potential to increase the efficacy of these cannabinoids in an additive or synergistic manner.

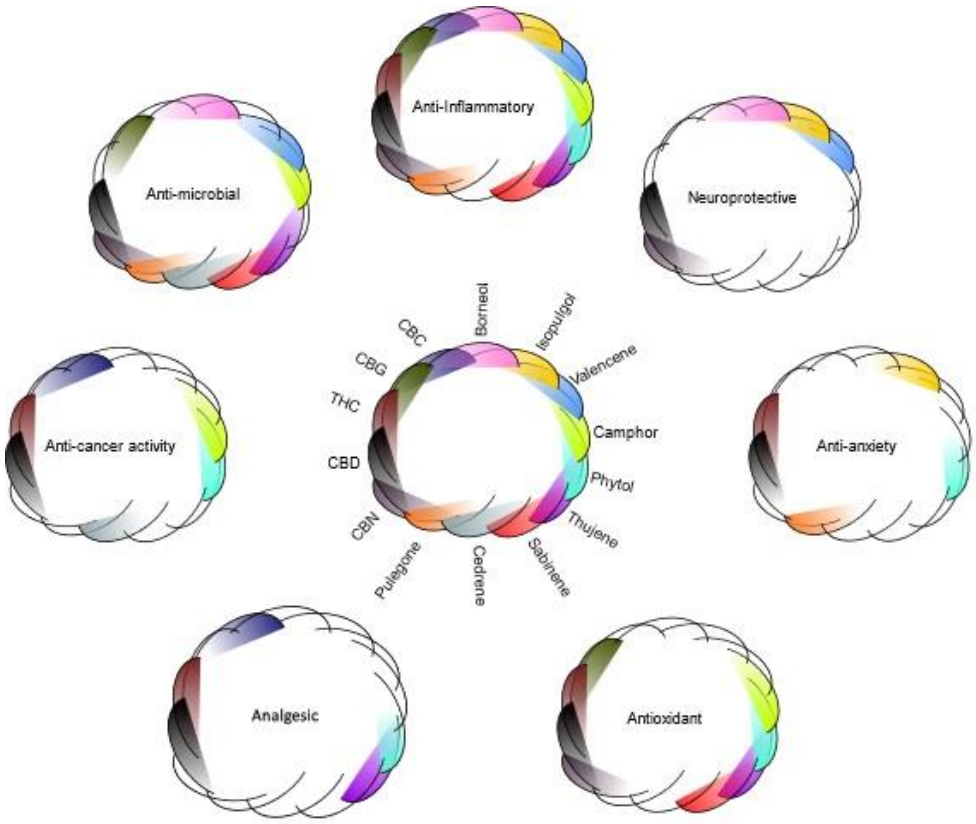


Figure **1-3**: Bioactivities of the nine secondary terpenes covered in this review, along with cannabis's principal cannabinoids (CBD, cannabidiol; THC, tetrahydrocannabinol; CBG, cannabigerol; CBN, cannabinol; CBC, cannabichromene). Bioactivity circles are color-coded to match the legend in the middle; the presence of a particular shading in the circle is indicative that the terpene or cannabinoid has been reported to possess that bioactivity. The figure demonstrates that not only do terpenes have multiple potential bioactivities, but different compounds possess overlapping activities, suggesting their potential to exert combination effects

Mechanism of Action for Terpenes—Pharmacologic Receptor Targets (TRPs)

Several studies have investigated the pharmacodynamics of the receptors for the major terpenoids found in cannabis (e.g., β-caryophyllene, β-myrcene, β-pinene, α-humulene, linalool). For instance, β-caryophyllene has been found to be an agonist at the cannabinoid receptor 2 (CB2), peroxisome proliferator-activated receptor gamma (PPARγ), and the toll-like receptor 4 (TLR4)/CD14/MD2 complex, while β-myrcene is an agonist at α2-adrenergic receptors and transient receptor potential cation channel subfamily V member 1 (TRPV1)[13,146,147,148,149]. The information on the receptors modulated by the minor terpenes found in cannabis is much more variable and will be the focus of the discussion below.

Borneol is an agonist of TRPM8. This activation of TRPM8 by borneol has been found to be temperature sensitive and dose-dependent across a range of concentrations, from 10 μM to 2 mM; however, no EC_{50} was reported because the study failed to reach a maximal response[150,151]. The activation of TRPM8 receptors by borneol has been found to activate glutamatergic and GABAergic transmission in the spinal cord, leading to anti-nociceptive activity[152,153]. The activation of TRPM8 by borneol has also been shown to enhance the chemosensitivity of lung cancer cell lines to doxycycline[151]. Borneol is also an agonist of TRPV3 (EC_{50} = 3.45 mM) channels[154]. Furthermore, borneol is an antagonist of the TRPA1 channel, with an IC_{50} of 0.2–0.3 mM in cell-based assays[155,156]. The activation of TRPV3 and inhibition of TRPA1 also likely contribute to the antinociceptive properties of borneol. Of note, the antagonist/agonist profile of borneol at these receptors matches that of several cannabinoids, including CBD, CBG, and THC; however, these effects occur at relatively high levels.

Camphor is a major terpenoid constituent of cannabis but is best known as an isolate from the camphor laurel (*Cinnamomum camphora*). This compound is FDA-approved as an additive to soothing creams and ointments and as a component of over-the-counter respiratory

treatments. Camphor has been found to act as an agonist at TRPM8 and TRPV3 and an antagonist at TRPA1, which is perhaps not surprising considering the structural similarity of camphor to borneol. Despite this structural similarity, camphor is less potent at both TRPV3 (EC_{50} = 6.03 mM) and TRPA1 (IC_{50} = 1.26 mM) compared to borneol[154,156]. At TRPM8, camphor has an EC_{50} of approximately 4.5 mM[157]. Additionally, camphor has been shown to be a partial agonist at TRPV1, with similar potency as at TRPV3 and TRPA1 (EC_{50} > 3 mM)[158,159]. The action of camphor at these receptors likely accounts for its analgesic activities. In addition, activation of the TRPV family of receptors has been linked to the ability of camphor to relax the trachea in rats, which may help explain its anti-congestive activities[160].

Cedrene has been identified as a potent agonist of the olfactory receptor 10J5 (OR10J5), a GPCR that is also found in liver and muscle tissue[161]. In human hepatocytes, cedrane has been shown to lower lipid levels through OR10J5. Furthermore, cedrane has been shown to reduce muscle atrophy induced by a high fat diet in mice, this action is mediated through the mouse ortholog of OR10J5, MOR23[162]. This study also found that cedrane increased muscle mass and strength, possibly through increasing expression of IGF1.

Isopulegol has been identified as an agonist of the most abundant $GABA_AR$ in the brain, α1β2γ2, with an EC_{50} of approximately 3.25 μM. Activation of the GABAR produces sedative effects, and these receptors are targets for both analgesics and anticonvulsant medications[163]. Isopulegol is also an agonist at TRPM8 and may also antagonize the TRPV1 receptor[164,165]. Either of these actions may account for the anti-nociceptive properties of isopulegol that have been described in mice[165].

Phytol and its metabolites can act as natural ligands for a variety of transcription factor receptors. This list includes the peroxisome proliferator-activated receptor (PPAR) α and γ; however, an EC_{50} was not reported because the assay did not reach a plateau at 100 μM, the highest concentration tested[166,167]. Additionally, phytol has been shown to be an agonist of

retinoid X receptors (RXR), with EC_{50} estimates ranging from 41.9 to 67.2 μM, depending upon the isotype[168]. Through activation of these receptors, phytol has been shown to reduce cancer cell viability in a number of cancer cell lines. Indeed, it has been found to have a lower IC_{50} in the lung adenocarcinoma cell line, A549, than the chemotherapeutic agent methotrexate[169]. Phytol induced apoptosis in this system through the activation of the TNF receptor, TRAIL, and FAS. Additionally, the authors used molecular docking to suggest that phytol may bind to glucose-6-phosphate dehydrogenase to inhibit tumor progression. In vitro, phytol has also been shown to increase the release of CA^{2+} reserves via activation of GPR40, a G-protein-coupled receptor that normally binds to free fatty acids, with an EC_{50} of 34.5 μM[170]. The activation of PPARs, RXRs, and GPR40 by phytol may also be of potential therapeutic benefit for the treatment of diabetes, and because of the ability to activate RXR receptors, phytol is also being pursued by the cosmeceutical industry as an anti-aging treatment in lieu of retinol (which is not well tolerated by all individuals due to its activation of TRPV1)[171,172].

Using a recently developed in vitro receptor binding assay, pulegone was shown to be the component in *Ziziphora clinopodioides* that binds and potentially activates β_1-adrenoceptors[173]. Pulegone has been found to be an agonist of avian TRPM8 at low concentrations; however, it antagonizes this receptor at higher concentrations[174]. This study also found that pulegone is an antagonist of TRPA1 at both low and high concentrations. Taken together, these data suggest that pulegone may have anti-nociceptive and analgesic utility.

Computer-based molecular docking research predicted that sabinene may be a potent interactor with L-asparginase from the bacterial pathogen, *Salmonella typhimurium*[175]. This study found that sabinene had a higher docking score than the antibiotic ciprofloxacin, suggesting that sabinene may have antibacterial properties and may be a good candidate for antibiotic development. Additionally, sabinene has been found to reduce levels of the inflammatory marker nitric oxide in cells exposed to lipopolysaccharide[129]. Another molecular docking study suggested

that sabinene may interact with the spike protein on the SARS-CoV2 (COVID-19) virus along with three cell membrane proteins (transmembrane serine protease 2, cathepsin B, and cathepsin L) that play a role in mediating viral entry into cells[176]. Additional studies will be needed to determine if any of these interactions occur in vivo, as well as which receptors might mediate the decrease in nitric oxide production caused by sabinene. An in silico study suggested that thujene may have a modest binding affinity for the SARS-CoV2 main protease and papain-like protease, but further work will be needed to confirm these findings[177]. Additionally, no studies could be located that identified potential human receptors for this terpene.

Valencene has been reported to be cardioprotective following myocardial infarction in rats, and this protection is mediated through the inhibition of the NF-κB pathway, oxidative stress, and cardiac hypertrophy; however, the receptors that mediate this inhibition were not examined[178]. Valencene has been found to be an antagonist of the calcium ion channel TRPV1 and the slow release calcium release-activated calcium channel protein 1 (ORAI1), which inhibited the melanin content in UVB exposed melanoma cells, and may therefore be useful for treating photo-aging of the skin[179]. This inhibition, may also mediate the ability of valencene to potentially treat atopic dermatitis[180].

Synergy and the Entourage Effect: Beyond Cannabinoids

Natural product discovery efforts are traditionally reductionist in nature, devoted to condensing a complex botanical extract down to a single bioactive agent for drug development purposes. This is true for cannabis research and development, where the single molecule approach remains the dominant approach[181]. However, botanical medicines, including cannabis, are in fact complex diverse concoctions of phytochemicals that have the potential of exerting differing and potentially complementary biological effects. In-deed, it is often observed that these

mixtures work in concert to achieve a specific physio-logical effect[182]. Compounds can work in a synergistic manner, in which each active compound potentiates the other to achieve a greater than expected benefit when combined (i.e., 1 + 1 > 2). If one compound, having no activity of its own, impacts the efficacy of an active molecule to increase activity (i.e., 1 + 0 > 1), this is known as an entourage effect[26]. As cannabis research has evolved, there has been a growing body of evidence that cannabinoids beyond THC demonstrate efficacy in humans [183,184] and that synergy/entourage could potentially play a large role in the bioactivity of cannabis extracts and products[22,185].

Botanical synergy and entourage have been demonstrated in cannabis, first in the combination of THC with other, "minor", cannabinoids. Johnson et al. (2010) tested a cannabis-based extract for patients with intractable pain and found that, while the THC dominant extract did not improve patient outcome versus the placebo (the mean pain Numerical Rating Scale (NRS) was a nonsignificant change of −1.01 vs. −0.69), a whole plant extract (the only difference being the presence of CBD) demonstrated a significant improvement in pain outcome (mean NRS of −1.37 vs. −0.69) compared to the placebo[186]. Animal studies focusing on analgesia also evidenced greater response from a full-spectrum cannabis extract as compared to pure CBD dosing[187]. Recently, experiments with a seizure mouse model looked at the effects of different strains of cannabis that all contained an equivalent CBD concentration. While all were effective, there were noticeable differences between the strains, and profiling 94 phytocannabinoids across 36 of the most commonly used Cannabis plants prescribed to patients in Israel led to the conclusion that these other cannabinoids have an impact on the overall efficacy of cannabis plant extracts[188]. In one in vitro study, one study of breast cancer cell lines revealed that the extract of the whole cannabis was more effective than a preparation featuring THC by itself; the boost in activity was attributed to the presence of "minor" cannabinoids cannabigerol (CBG) and tetrahydrocannabinolic acid (THCA)[189]. Complex fractions from cannabis extracts demonstrated synergistic interactions on colorectal cancer cell lines[190].

Cannabinoids have been widely studied for the treatment of epilepsy[191,192]; com-plex extracts containing multiple cannabinoids were found to treat severe epilepsy, such as Dravet and Lennox-Gastaut syndromes, at lower doses than trials using purer preparations (e.g., Epidiolex, which contains 97% CBD)[66,193]. A 2018 meta-analysis by Pamplona et al. of 11 studies demonstrated that the response rate at 50% improvement of seizure frequency was similar between the two groups, but the average daily doses were significantly different: 27.1 mg/kg/d for purified CBD as opposed to 6.1 mg/kg/d. for cannabis extracts[194]. Moreover, the incidence of adverse events was discernably higher in the CBD versus complex extract treatments ($p < 0.0001$), a result that the authors attributed to the lower dose utilized, which was achieved in their opinion by the synergistic contributions of other entourage compounds.

Most synergy studies have focused primarily on the interactions between cannabinoid structures, despite the fact that the original definition of the entourage effect arose from the interaction of 2-acyl-glycerol esters with cannabinoids[26]. As terpenes are a large and diverse family of phytochemicals found in cannabis, they have the potential to serve as potentiating agents working in concert with cannabinoids. Terpenes, broadly speaking, have been found to be broadly synergistic, helping modulate the activity of a number of other botanicals, pharmaceuticals, and compounds. The terpenes highlighted in this review also have a strong history of synergistic activity with other compounds. Borneol was shown to synergize with curcumin to induce apoptosis in human melanoma cells[195], potentiate the activity of berberine and baicalein in inhibiting in vitro and in vivo fungal growth[196], and function as a potentiating agent to sensitize cancer cells to doxorubicin treatment[151]. Sabinene[197] and pulegone[198] each indicated the potential for synergistic interactions with prescription antibiotics in treating bacterial infections, and β-caryophyllene and phytol demonstrated combination effects inducing apoptosis in skin epidermoid cancer cells[199]. Terpenes have also demonstrated efficacy in the treatment of mood and anxiety disorders, suggesting the possibility of combination effects with cannabinoids

for more effective treatments[200]. Thus, while the potential of canna-bis terpenes to possess additive or synergistic properties was originally posited as hypo-thetical based upon similar bioactivities[23], more recent studies have explored this possibility in earnest.

A 2021 study by LaVigne et al. found that α-humulene, geraniol, linalool, and β-pinene were cannabimimetic at the CB1 receptor and produced cannabinoid-like behaviors in a mouse model. Furthermore, the terpenes potentiated the effects of a cannabinoid agonist, suggesting synergistic activity[201]. Di Giacomo et al. treated triple negative breast cancer cells (MDA-MB-468) with hemp inflorescences and pure compounds of CBD, caryophyllene and cannabichromene. The presence of these other compounds induced the potentiating effects of CBD, likely mediated through CB2 activation[202]. However, separate studies observed that none of the terpenes α-pinene, β-pinene, β-caryophyllene, linalool, limonene, and β-myrcene were found to alter potassium channel signaling in AtT20 cells expressing CB1 and CB2 receptors, and did not interact with THC at the receptor[203], nor did they affect changes in intracellular calcium at the hu-man transient receptor potential ankyrin 1 (hTRPA1) or human transient receptor potential vanilloid 1 (hTRPV1) channels[204]. Using a radioligand ([3H]-CP55,940) to measure binding at the CB1 and CB2 receptors, none of the tested terpenes (myrcene, α-pinene, β-pinene, β-caryophyllene, and limonene) had interactions with receptors, nor did they modulate the binding of THC or CBD[205]. Similarly, no synergy was detected between myrcene and CBD in modulating inflammation and analgesic properties in a rat adjuvant monoarthritis model[206]. Research on colorectal cancer cells did not detect any enhancement of activity when terpenes were included as part of a complex CBD oil com-pared to the effect of CBD alone[207].

The divergence of results involving potential synergy or entourage effects has led to doubt surrounding the entourage effect in cannabis and whether it really holds pharmaceutical potential. Cogan (2020) references several studies where individual cannabinoids did not improve the clinical performance of THC or CBD[208]. However, the intellectual leap to label cannabis

potential synergistic interactions as “questionable” is perhaps premature. Botanicals or combinations exhibiting synergy or entourage does not necessitate that the effects take place at the same target to elicit a heightened response; com-pounds can exhibit “pharmacodynamic synergism” by acting at multiple cellular targets (seen in both antibiotic and cancer synergistic therapies)[209,210] and “pharmacokinetic synergism” by increasing the solubility or disposition (absorption, distribution, metabolism) of active constituents [211,212], and can limit side effects of the active constituent[213,214] or disrupt resistance mechanisms[215,216]. Indeed, the study Santiago et al. that purported the “absence of entourage” nevertheless suggested that synergy could still be taking place at a different molecular target than the CB receptors[203]. Therefore, as further studies are developed, it would be prudent to also employ phenotypic assays that encompass more than a single receptor/enzyme/target and can better deduce the combi-nation effects at complementary sites and pathways to deliver heightened results. Thus, the elevated skepticism or dismissal of synergism in cannabis is perhaps unwarranted at this time, as there exists a growing body of evidence suggestive that not only do multiple cannabinoids work in concert to produce heightened effects (or potentially lower deleterious side effects), as seen in the prescription drugs that utilize multi-component canna-bis extracts (Sativex® and Epidolex®) [186], but that terpenes/terpenoids can also potentially function as synergists with cannabinoids to deliver amplified results. Furthermore, studies from our own group, using an animal model of chemotherapeutic induced peripheral neuropathy, demonstrated an enhanced effect at reducing mechanical hypersensitivity by an extract containing equal parts CBG and CBD, in addition to other cannabinoids and terpenes, compared to pure CBG[217]. Additionally, in the same animal model we found that pure CBD was without an effect; however, when animals were treated with a complex hemp extract at the same CBD concentration, a reduction in mechanical sensitivity was observed[218]. These studies suggest that there is a potential interaction between cannabinoids and terpenes that can enhance the effect of pure cannabinoids alone. Moreover, the lack of

synergy in vitro may not hold true for results in intact organisms, and the entourage effect does not have to be present or absent in all systems or biomedical indications. The search for synergy and entourage effects within the diverse phytochemical landscape of cannabis remains in its infancy; to better understand these combination effects, further research on the potential combination effects of cannabis's polypharmacy is essential to establish mechanisms of interaction, cellular targets of interest, and adverse events.

Conclusions and Future Directions

While commonly thought of as a psychoactive plant producing one of the most famous mind-altering chemicals discovered by humans, cannabis is a biosynthetic engine, producing hundreds of diverse phytochemicals that have the potential to impact a wide variety of human health conditions. In particular, cannabis produces 200 terpene structures that are of interest, both as independently bioactive molecules as well as by modulating or potentiating the effects of cannabinoids or other phytochemicals from cannabis. Terpenes are already widely implemented in traditional medicines and pharmaceuticals, as well as in industrial processes, perfumery, cosmetics, and food additives. They demonstrate generally low toxic profiles and high bioavailability and are highly selective to TRP channels, among other targets. There are known cannabimimetic activities of some terpenes, and they already have shown synergy amongst each other in other in vitro and in vivo studies. Thus, there is a firm foundation for cannabis synergy and the involvement of terpenes in the flavor, aroma, and bioactivity of cannabis. Investigations into potential combination effects in cannabis is a growing field, one which requires rigorous experimental design and execution but has the possibility to evolve our understanding of cannabis's diverse pharmaceutical effects.References

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Chapter 2

Impact of Soil Quality on Cannabinoid and Terpene Content of *Cannabis sativa* L.

This chapter is based on joint work with Dr. Wesely Raup-Konsavage from Penn State Hershey Medical School and Steve Groff from Cedar Meadow Farm.

Abstract

Background

Cannabis sativa L or 'hemp' is an expanding commodity that relies heavily on its chemical constituents for its therapeutic effects and legal status. Growing conditions are critical for obtaining an anticipated chemical profile for any given hemp cultivar. A vital component of outdoor hemp cultivation is soil composition. This study evaluates the effects soil health has on cannabinoid and terpenoid content by comparing the extracts of two hemp cultivars grown identically in two fields differing in soil preparation including cover crop usage and conventional tillage.

Methods

Two hemp cultivars, Tangerine (CBD dominant) and Stem Cell (CBG dominant) were grown identically with differing soil composition from a cover crop field (CCs) and a conventional field that was tilled (CF). Hemp inflorescence from 200 hemp plants were extracted using supercritical fluid extraction techniques totaling 5 extracts of each cultivar per field. Each

extract was evaluated for its total terpene and cannabinoid content, as well as its terpenoid and cannabinoid profile. These results were then paired with a soil assessment of each field describing chemical, physical, and biological soil characteristics.

Results

A soil assessment of each field revealed significant discrepancies in the percentage of organic carbon, predicted soil protein, soil aggregate stability, magnesium concentration, and extractable potassium and phosphorous. Significant differences in CBD, CBDA, and THC concentrations were observed between field type depending on the cultivar type. Higher concentrations of d9-THC were observed in tangerine extracts from CF with an average of 29.63 mg/g while CCs tangerine extracts consisted of 3.29 mg/g. Stem cell extracts from CCs displayed roughly 39 mg/g more CBG than CF. Total terpene concentration correlated with cultivar type based on field. Tangerine extracts from CF contained an average of 33.29 mg/g of total terpenes compared to tangerine extract from CCs with 17.36 mg/g. Stem cell extracts from the CCs field contained an average of 20.33 mg/g of total terpenes while Stem cell extracts from the CF contained 6.11 mg/g of total terpenes. No differences in terpene composition were observed between either cultivar or field.

Conclusion

This is the first study to show differences in total terpene concentrations of outdoor cultivated hemp grown in different soil conditions. Cover crop rotation exhibited effects of increased total terpene content and cannabinoid concentration based on hemp cultivar. Cover crop rotation can be an effective practice in retaining cannabinoid composition and increasing terpene concentrations, however, genetics of the plant may play a significant role in the response to the regenerative soil conditions.

Background

Cannabis sativa L., commonly referred to as 'hemp', has become a rising commodity in the United States with values of hemp production totaling $291 million in 2023[1]. Hemp is a versatile crop that has many uses depending on the plant part including its fibers, leaves, inflorescence, and seeds which have resulted in a variety of products such as industrial textiles, animal feeds, food supplements, therapeutic oils, tinctures, and topicals[2]. Cannabis sativa L. is a diecious plant meaning both female and male plants are produced[3]. Female plants contain copious amounts of the bioactive compound cannabidiol (CBD) which has demonstrated potential therapeutic properties including neuroprotective, antiepileptic, anxiolytic, analgesic, and anti-cancer properties[4]. Aside from CBD, hemp inflorescence also contains a plethora of bioactive compounds including other phytocannabinoids and therapeutic aromatic compounds such as terpenoids[5,6].

Cannabinoids and terpenoids are both groups of compounds that are classified as plant secondary metabolites that play critical roles in plant defense, communication, and competition[7,8]. When it comes to the biosynthesis of these compounds, studies have described shared precursors between cannabinoids and terpenes along with evidence of genetic variation for specific enzyme synthases of individual cannabinoids and terpenoids[8,9,10,11]. Additionally, researchers have explored the effects of environmental factors such as temperature, light quantity and quality, photoperiod, and soil nutrients suggesting these factors may also play a role in the total yield in the concentrations of cannabinoids and terpenoids in hemp extracts[12,13,14,15,16,17,18,19]. Although the role of soil nutrients in the synthesis of cannabinoids and terpenoids is unclear, changes in macronutrient levels have been observed to alter cannabinoid and terpene yield[12,18,19].

One of the most common practices among hemp growers is outdoor cultivation which has been shown to provide higher biomass at a lower cost when compared to indoor cultivation[20].

Efforts of outdoor cultivation have led farmers to pursue sustainable practices such as the incorporation of cover crops which have been shown to enhance different aspects of the soil's physical, biological, and chemical characteristics in comparison to traditional farming practices including soil tillage[21]. To date, there are no studies that have explored the effects of cover crop soil on cannabinoid and terpenoid content.

This study aims to assess the effects soil health has on cannabinoid and terpenoid content in extracts from outdoor-grown hemp inflorescence. Two cultivars of hemp were grown identically in two fields, a conventional field with tilled soil and a no-till field that has had long-term application of cover crops. Hemp inflorescence from each field was then harvested and extracted using supercritical fluid extraction techniques, and then evaluated for their total cannabinoid and terpenoid content and profile. It is hypothesized that cover crop application will increase soil health thus, providing elevated levels of these bioactive compounds in hemp extracts. The results of this study demonstrate that soil characteristics and genetics of hemp cultivars play a major role in specific cannabinoid and terpene yield, as well as total terpene content.

Methods

Hemp growth and harvest

Hemp cultivars Tangerine and Stem Cell (CBG dominant) were grown and harvested by Cedar Meadow Farms (Holtwood, PA, USA). A total of 200 plants were grown on two separate fields; a no-till cover crop field (CCs) and a conventional (tilled) field (CF) with no cover crop application. Each field consisted of 50 plants per cultivar in a randomized order to eliminate position bias. Plants were transplanted in June of 2023 and harvested in October 2023. All fields

were treated with identical amounts of sunlight and watered based on the occurrence of precipitation, with no additional fertilizer applied to either field. Temperature, humidity, pressure, and the total amount of precipitation during the growth period were gathered using online databases from local weather stations Fig. S1 & Table S5. Cover crops hairy vetch, triticale, winter oats, and crimson clover were grown in the cover crop field before transplanting hemp cultivars. No cover crops were grown in the CF; the soil was tilled before transplanting. A comprehensive assessment of soil health was conducted in March of 2021 by the Cornell Soil Health Laboratory (Cornell University, Ithica, NY, USA). All plants were harvested identically, placed within bead trays, and then transferred to designated reefer units based on cultivar to eliminate any cross-contamination after drying. A dehumidifier was added to each unit where hemp inflorescence was dried over a period of seven days. Dried inflorescence was then vacuumed sealed and stored at room temperature with limited light exposure until extraction.

Extraction and Characterization of Hemp Inflorescence

The extraction of dried hemp inflorescence was carried out as previously described[23,24]. In short, 250-325g, was ground and extracted using an SFT-SP1100 extraction system manufactured by Supercritical Fluid Technologies, Inc. (Newark, DE, USA). Extractions were performed at 55°C for 35 min at 413 bars, the extract was then collected from the collection vessel. Winterization was performed by dissolving the extract in 90% ethanol (10% extract by weight) for 24-48 h at -20°C and then filtered before ethanol evaporation. The final extract was dissolved in fractionated coconut oil (Pure Body Naturals, West Chester, OH, USA) at 300 mg/ml and decarboxylated at 95°C for 1 h.

Analysis of Extract Composition

Analysis was conducted by Keystone State Testing Laboratories (Lower Paxton Township, PA, USA) as previously described[24]. Briefly, cannabinoid content was determined by reverse phase HPLC using a Restek (State College, PA, USA) Raptor AERC C-18 column (2.7 μm particle size, 150 mm, 4.6 mm ID, and matching guard length 5 mm), mobile phase A: 1% phosphoric acid in LCMS grade water, and mobile phase B: 1% phosphoric acid in LCMS grade acetonitrile (ThermoFisher, Waltham, MA, USA). Data acquisition and integration were achieved with LabSolutions (Ver 5.87 SPI, Shimadzu). 11 cannabinoid reference standards (Shimadzu; Columbia, MD, USA) were calibrated with a 7-point curve from 0.5 μg/mL – 100 μg/mL. The detection of cannabis terpenes was carried out using a Shimadzu 8050 GCMS-MS (Shimadzu, Columbia, MD, USA) and a collection of 42 terpene compounds as calibration standards (CAN-TERP-MIX1H and CAN-TERP-MIX2H [Spex; Metuchen, NJ, USA]). A two-way ANOVA statistical test was employed to determine significant differences in compound concentrations using GraphPad Prism (GraphPad Software, Boston, MA, USA).

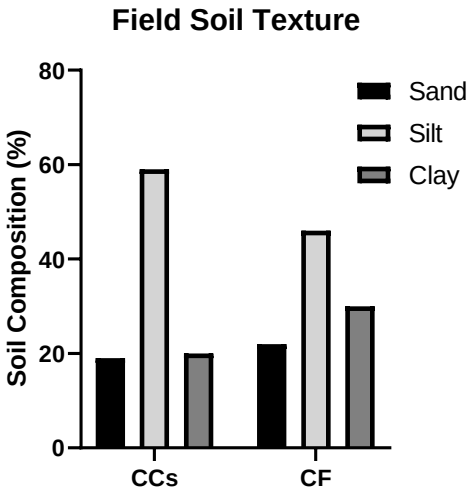
Results

Soil Quality Assessment

The assessment of soil quality provided an overall quality score for each field, including the physical, biological, and chemical characteristics. The CCs field was identified as silt loam soil textures while CF consisted of a clay loam soil texture (Figure **2-1A**). The physical characteristics provided indicators of predicted available water capacity and aggregate stability (Fig.1B). Both fields had similar predicted available water capacity but differed in aggregate stability with CCs measuring at 35.2% and CF at 5.5%. Biological characteristics revealed

similarities in organic matter (%) CCs and CF with values of 4.7% and 3.6 %, respectively. CCs contained the highest percentage of total carbon and nitrogen. As well as more soil respiration and active carbon (Figure **2-1C**). Chemical characteristics displayed a range of variation between fields. Soil pH for CF was reported at 6.9 while the CCs field displayed a pH of 7.1. High levels of extractable phosphorous were detected in CF with 47.8 ppm in comparison to the CCs field at 19.9 ppm. The amount of extractable potassium also differed between fields with CCs containing 437 ppm and CF with 216.2 ppm. Concentrations of minor elements such as iron, manganese, and zinc were similar across both fields. However, CCs field displayed the most amount of magnesium with 184 ppm and CF with 122.2 ppm (Figure **2-1D**).

A)



B)

Physical Soil Characteristics	*Cover Crop Field*	*Conventional Field*
Predicted Available Water Capacity (grams of water) / (grams of soil))	0.27	0.25
Aggregate Stability (%)	35.2	5.5

C)

Biological Characteristics	*Cover Crop Field*	*Conventional Field*
Organic matter (% total biomass)	4.7	3.6
Soil Organic Carbon (%)	2.93	1.8
Total Carbon (%)	2.95	1.82
Total Nitrogen (%)	0.26	0.15
Predicted Soil Protein (mg/g)	9.9	6.6
Soil Respiration (mg)	0.9	0.6
Active Carbon (ppm)	889	502

D)

Chemical Characteristics	*Cover Crop Field*	*Conventional Field*
Soil pH	7.1	6.9
Extractable Phosphorous (ppm)	19.9	47.8
Extractable Potassium (ppm)	437	216.2
Magnesium (ppm)	184	122.2
Iron (ppm)	0.7	0.4
Manganese (ppm)	7.4	7.5
Zinc (ppm)	1.9	2.3

Figure **2-1**: **(A)** soil composition (%) of no till and conventional soil application. **(B)** Physical soil characteristics **(C)** Biological soil characteristics **(D)** Chemical soil characteristics.

Extraction Yield

Total yield was calculated by dividing the post-extraction weight by the pre-extraction weight of the plant material. Total wax (%) was determined by the weight of the extract subtracted from the winterized extract weight. The two hemp cultivars, Stem Cell and Tangerine had similar extraction yields, with approximately 6% yield in the cover crop field and 5% from the conventional field (Figure **2-2A**). Total wax (%) followed a similar trend, with Stem Cell samples displaying 47% wax in the conventional field and 44% in the cover crop field (Figure **2-3C**). Tangerine samples displayed 46% wax in the conventional field and 45% wax in the cover crop field (Figure **2-3B**).

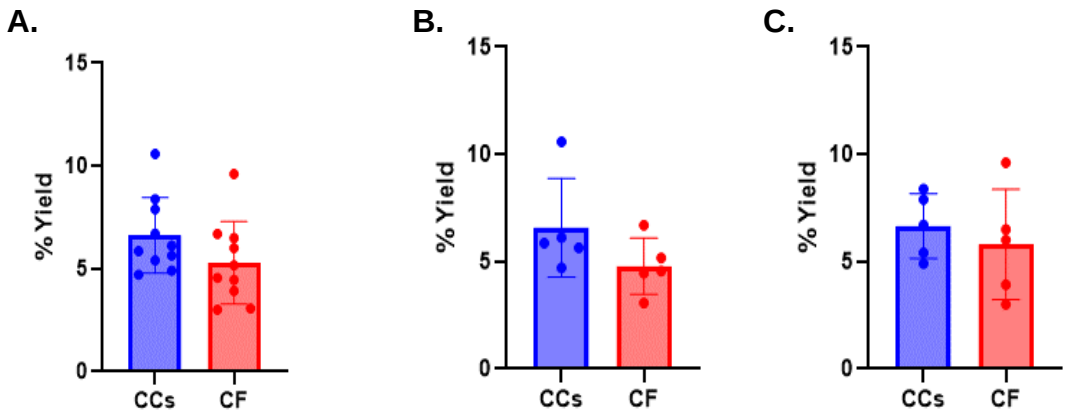


Figure **2-2**: (A) Combined extraction yield (%) mean ± SD; n=10 of two hemp cultivars grown in a field with cover crop application with no till versus a conventional field with tilled soil and no cover crop application, (B) tangerine extraction yield (%) mean ± SD; n=5 per cultivar. (C) Stem cell extraction yield (%) mean ± SD; n=5 per cultivar

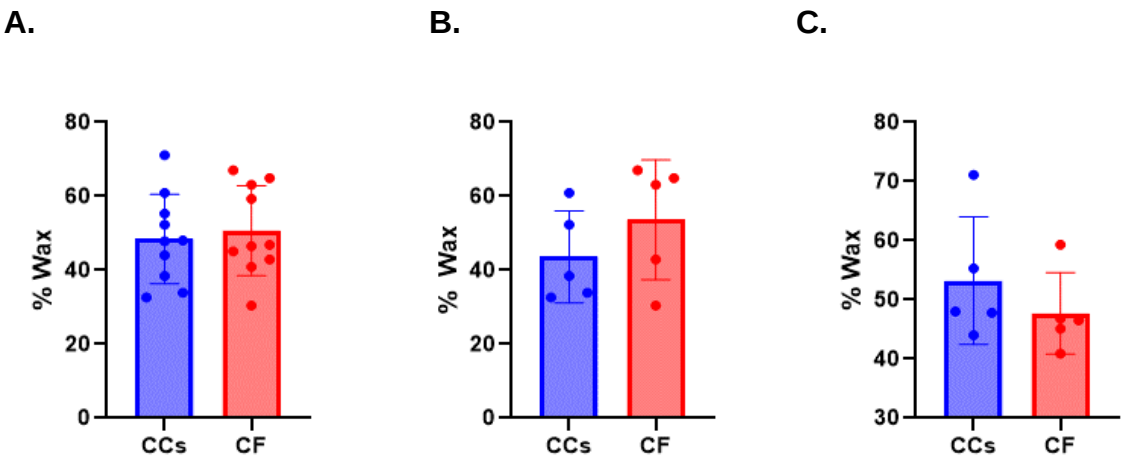


Figure **2-3**: (A) Combined wax (%) mean ± SD; n=10 of two hemp cultivars grown in a field with cover crop application with no till versus a conventional soil with no p application (B) Tangerine extract wax (%) mean ± SD; n=5 per cultivar. (C) Stem cell extract wax (%) mean ± SD; n=5 per cultivar.

Cannabinoid and Terpene Content

The total cannabinoid content was determined for both cultivars. There were no significant differences observed for either cultivar between each field (Figure **2-4A**). However, Tangerine in both displayed a similar range of cannabinoid concentration between 117 – 141 mg/mL (Figure **2-4D**). As for Stem Cell samples, the average total cannabinoid concentration for cover crop samples was 134 mg/mL, while the average total concentration for the conventional field was 111 mg/mL (Figure **2-4G** & **Appendix A**). Moreover, differences in total terpene concentrations were observed between each field based on the cultivar (Figure **2-4B**). The average concentration of total terpenes for Tangerine samples was significantly greater for the conventional field (Figure **2-4E**), while the cover crop field for Stem Cell samples generated

significantly more terpene concentrations compared to the conventional field (Figure **2-4H**). When combining both terpenoid and cannabinoid content, Stem Cell samples displayed a mean of 154 mg/mL in the cover crop field and 117 mg/mL (Figure **2-4I**).

When evaluating the cannabinoid profiles between each field, larger amounts of d9-THC were observed in the CF for the Tangerine samples and the CCs field resulted in extracts containing more CBG, CBGA, and CBDA with minuscule amounts of THC (Fig. 5A & Table. S1). The extracts from Stem cell samples showed little variation between the different fields, however, CBG content in extracts from CCs were much greater in comparison to extracts from CF. Stem Cell sample extracts from both fields produced similar amounts of CBD, CBC, CBDA, and d9 THC (Figure **2-6A** & **Appendix A**).

The terpenoid profiles for Stem Cell samples were similar across each field, however, Stem Cell samples from CCs displayed more minor terpenoids in comparison to samples from the CF (Fig. 6B). The terpenoids with large concentrations for Stem Cell samples were trans-caryophyllene, beta-farnesene, and alpha-bisabolol (**Appendix A**). Stem cell extracts from fields CF generated profiles with more beta-farnesene than trans-caryophyllene, which were contrasting results in comparison to CCs Stem Cell extracts. Terpenoid profiles of extracts from the Tangerine cultivar revealed similar terpenoid variation across all field types including minor terpenoid constituents. Tangerine extracts from CF contained the most amount of beta-farnesene followed by trans-caryophyllene. Terpenoid content of the remaining identified terpenoids were found in smaller concentrations in the CCs field in comparison to the CF (Figure **2-5B**).

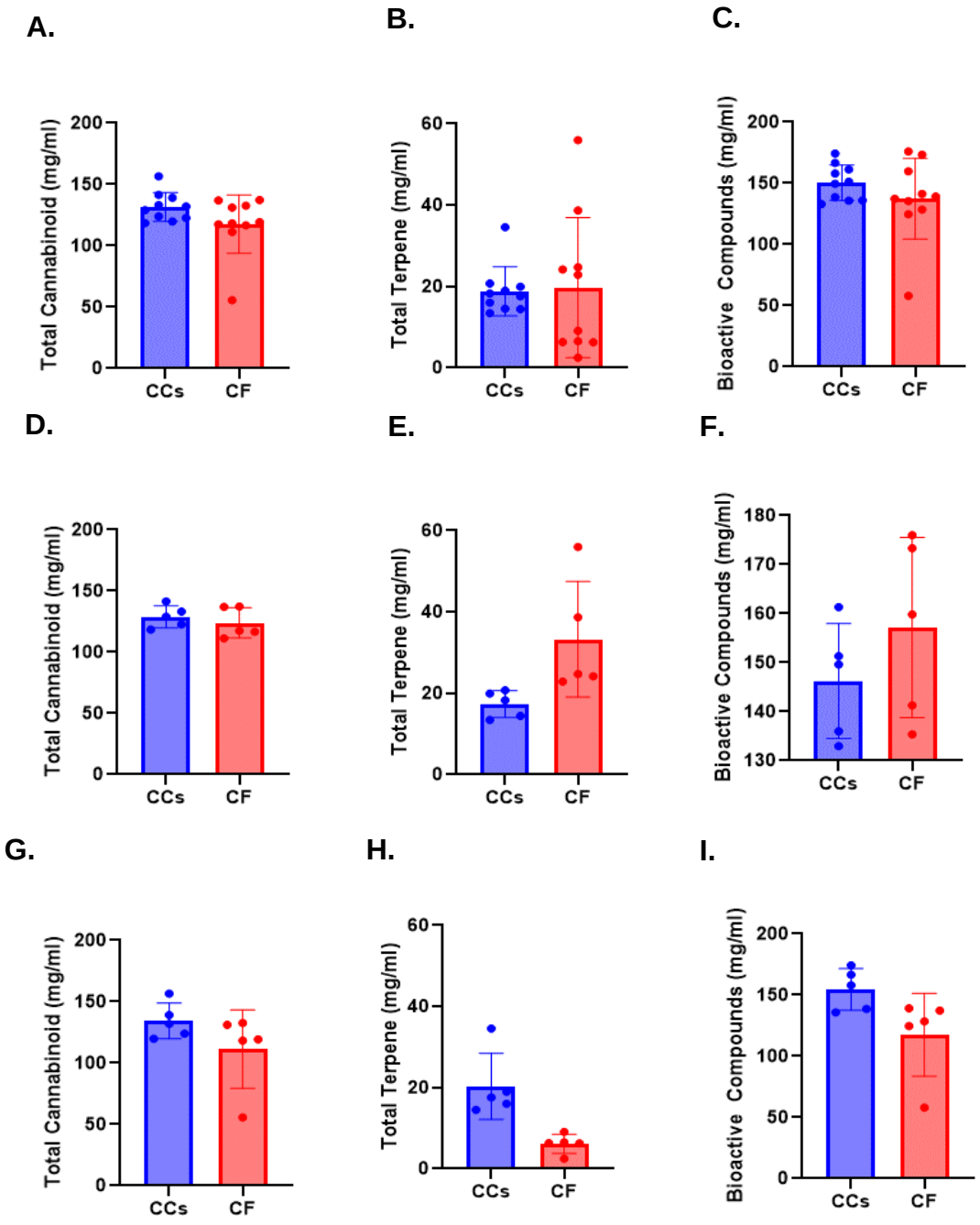


Figure **2-4**: (A) Combined cannabinoid content mean ± SD; n=10 per cultivar of both cultivar extracts (mg/mL) for no till and conventional soil. (B) Combined total terpene content (mg/mL). (C) Combined bioactive compounds (cannabinoids and terpenes (mg/mL)). (D - F)

Tangerine extract mean ± SD; n=5 per cultivar. (G - I) Stem Cell extracts mean ± SD; n=5 per cultivar.

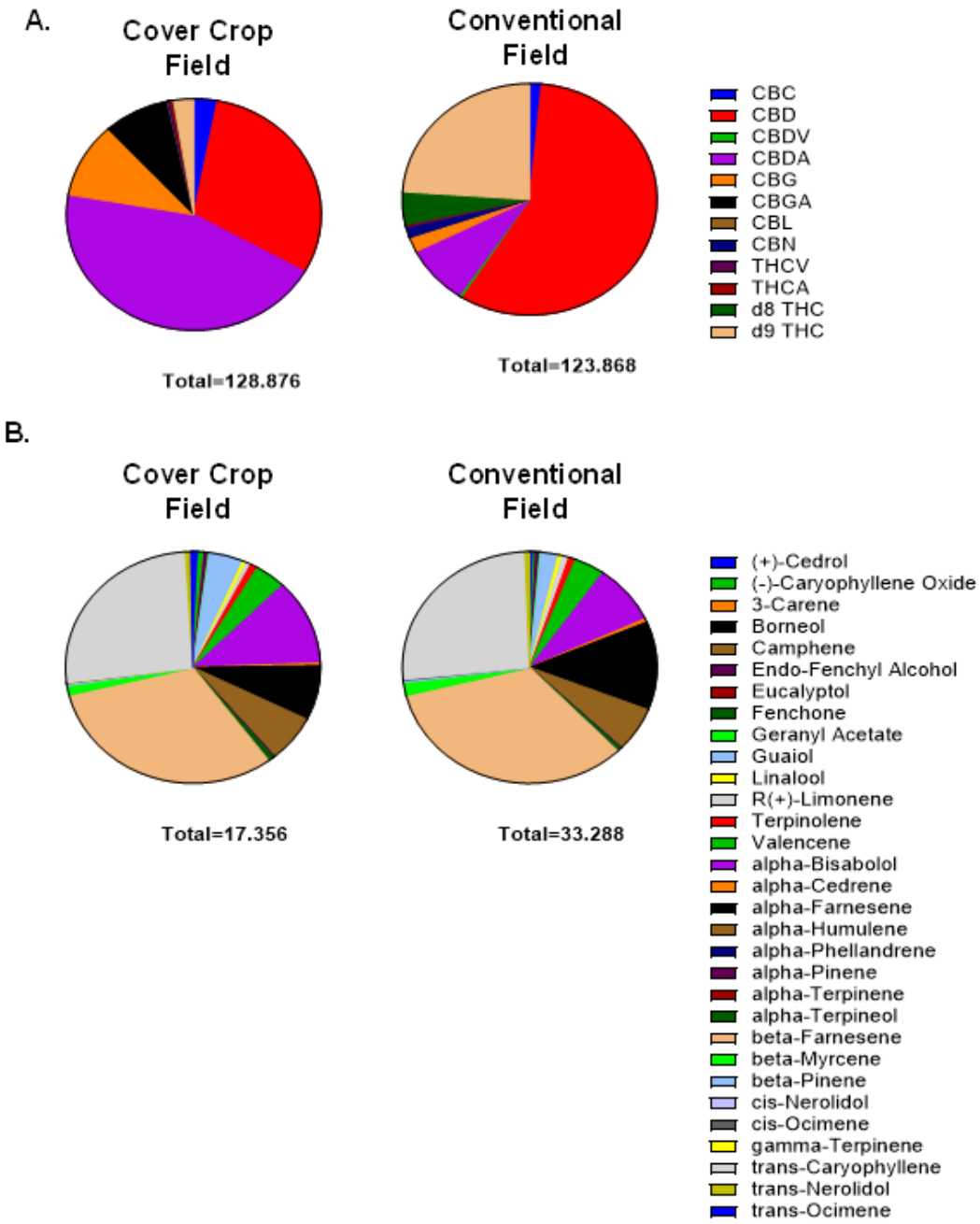


Figure **2-5**: Tangerine extract cannabinoid and terpene profiles (mg/mL) (A) cannabinoid of no till soil with cover crop application versus a conventional field with tilled soil (B) terpenes of no till vs conventional.

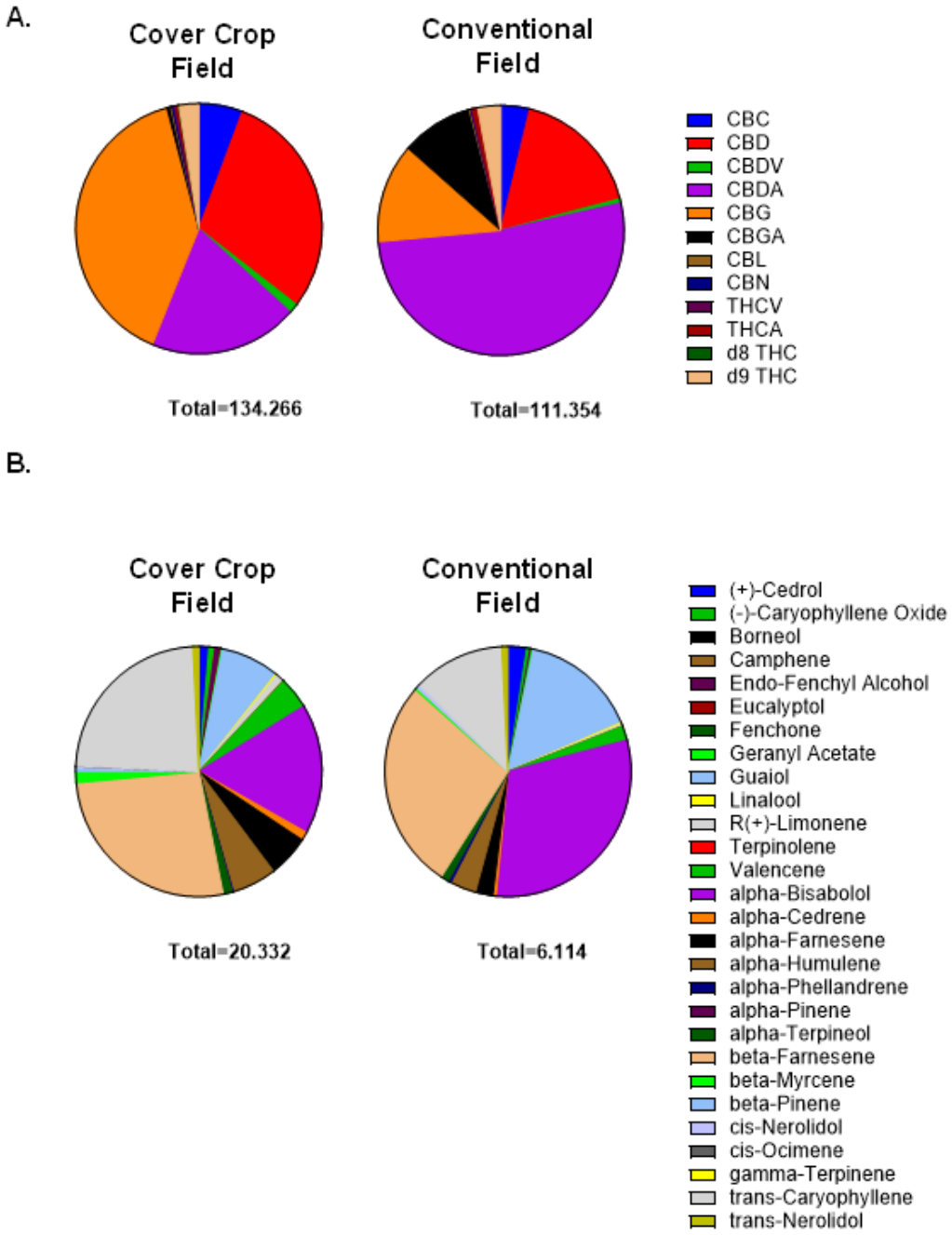


Figure **2-6**: Stem Cell extract cannabinoid and terpene profiles (mg/mL) (A) cannabinoid of no till soil with cover crop application versus a conventional field with tilled soil (B) terpenes of no till vs conventional.

Discussion

This study is the first to evaluate how cover crop usage can effect cannabinoid and terpene content. A comparison of hemp extracts of two different cultivars grown in both cover crop soil and tilled soil revealed significant differences in specific cannabinoid concentration and total terpene content. It was hypothesized that cover crop soil would provide elevated levels of cannabinoid and terpene content in hemp extracts.

An assessment of soil properties revealed differences in physical, biological, and chemical soil characteristics. Some of the most notable differences between cover crop field and non-cover crop field were the percentage of organic carbon, predicted soil protein, soil aggregate stability, magnesium concentration, and extractable potassium and phosphorous. Several studies have explored the effects nutrients such as nitrogen, phosphorous, and magnesium have on CBD content resulting in no effect on CBD yield[18,25,26]. Although this study provides differences between soil characteristics, the outcome of this study displayed no significant differences in total cannabinoid content. However, differences in THC and CBG concentration were observed between cultivars with field-type dependency. Furthermore, this is the first study to investigate the effects soil health has on terpene content in hemp, however, several physical, biological, and chemical soil characteristics differ between each field; making it difficult to conclude any interpretations of the outcome observed. Significant differences in total terpene content were observed between cultivars based on field type. This outcome adds to the growing body of evidence suggesting a relationship between the genetics of the hemp cultivar and its effect on soil nutrient uptake[25,26,27].

While this investigation provides differences in terpene content and cannabinoid profile when using cover crops during the out-of-season months, many limitations were present throughout the study. Results of the soil health assessment were collected in 2021, resulting in

potential differences compared to the actual health of the soil at the time of growth in 2023. However, these results may only be slightly different from the data that was reported as it is recommended to test soil every three years as significant differences may not occur[28]. Moreover, this study evaluates the content of CO_2 hemp extracts, leaving room for alterations in cannabinoid and terpene content depending on the extraction technique applied[29]. Conversely, this study does provide evidence of the cannabinoid and terpenoid content of a popular hemp product, making it applicable to real-world applications. Limitations in the complete soil characteristics exist as these methods do not provide information on the soil microbiome, which is reported to be stimulated by the use of cover crops[30].

Conclusion

Overall, this study provides evidence of alterations in cannabinoid profile and total terpene content when comparing hemp cultivars grown in cover crop soil and tilled soil. Two cultivars were grown identically in both tilled soil and cover crop soil, displaying genetic dependency on cannabinoid and terpene content based on soil type. The outcome of this study provides outdoor growers with information on the effects soil health can have on cannabinoid and terpene content in hemp. However, studies with isolated treatments of the characteristics that contribute to the health of the soil are required to delineate the main driver between soil health and hemp cannabinoid and terpene content.

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Chapter 3

Effect of Hemp Extraction Procedures on Cannabinoid and Terpene Composition

Abstract

A variety of techniques have been developed to extract hemp phytochemicals for research and consumption. Some of the most common processes in the industry include supercritical CO2 ex-traction, hydrodistillation, and solvent-based (ethanol) extractions. Each of these processes has the potential to differentially extract various phytochemicals, which would impact their efficacy, tol-erability, and safety. However, despite these differences, there has been no direct comparison of the methods and the resulting phytochemical composition. This work aimed to compare cannabinoid and terpene profiles using the three primary commercial procedures, using hemp inflorescence from a CBD/CBG dominant Cannabis sativa L. cultivar. Extracts were then evaluated for their terpene and cannabinoid content using GC-MS and LC-MS/MS, respectively. Hydrodistilled extracts contained the most variety and abundance of terpenes with β-caryophyllene to be the most concentrated terpene (25–42 mg/g). Supercritical CO2 extracts displayed a minimal variety of terpenes, but the most variety and abundance of cannabinoids with CBD ranging from 12.8–20.6 mg/g. Ethanol ex-tracts contained the most acidic cannabinoids with 3.2–4.1 mg/g of CBDA along with minor terpene levels. The resulting extracts demonstrated substantially different chemical profiles and highlight how the process used to extract hemp can play a large role in product composition and potential biological effects.

Introduction

Cannabinoids and terpenoids are widely recognized as the primary therapeutic agents of hemp (*Cannabis sativa* L.) inflorescence and extracts[1,2]. These phytochemical families can be found in copious amounts in the trichrome structures of female hemp inflorescence, and in smaller quantities within the leaves[3]. Cannabinoids are a group of compounds that contain a C32 terpenophenolic backbone, derived from the condensation of hexanoyl-CoA and three malonyl-CoA molecules and undergoing multiple cyclization and aromatization to produce myriad structures (>100 cannabinoids are currently known)[4,5,6,7,8,9,10]. Similarly, cannabis contains a large diversity of terpenoid structures, due to the wide biosynthetic capacity of the plant; there are >30 cannabis terpene synthases (CsTPS) genes identified (yet only 9 are fully characterized)[11,12,13]. Originating from multiple condensation reactions of the 5-carbon precursor isopentenyl diphosphate, terpenoids are classified according to the number of isoprene units (e.g., monoterpenes (two isoprene units), sesquiterpenes (three isoprene units), and diterpenes (four isoprene units)[1,11,14,15,16,17,18,19].

Hemp products, consisting of various cannabinoids and terpenoid profiles, have grown exponentially as the plant has been more widely accepted and legalized and the benefits have become more apparent[20,21]. A commercial hemp product's unique biological activity or flavor is determined by its levels of cannabinoids/terpenoids, which in turn is influenced by a number of variables, including strain differences, growing conditions, post-harvest practices, or the extraction processes used[22,23,24,25,26,27]. However, the selection of extraction process can ultimately lead to more diversity and abundance of specific chemical constituents based on compound properties, providing industries and researchers a direct approach to harnessing the biological effects of cannabis compounds.

There are a number of extraction techniques that have been developed for the extraction of phytochemicals from hemp inflorescence; the most prominent in the industry include supercritical fluid CO_2 extraction (SFE), solvent-based extractions, hydrodistillation, ultrasonication-assisted, and microwave-assisted extraction[28,29,30,31,32]. Supercritical fluid extraction is among the most popular extraction techniques due to its high specificity, lack of organic solvents, yield capacity, and extraction time[32,33]. SFE technique often utilizes CO2 in a supercritical fluid state by creating high pressure and high temperature conditions that permits the separation of non-polar compounds[34]. Organic solvent extraction methods (including ethanol, butanol, and isopropyl extractions) vary as the phytochemicals they extract depend on the solvent polarity and hydrogen bonding characteristics[35]. Solvent extractions are a primary method of producing botanical supplements and have proven to be a cost-effective approach for the extraction of cannabinoids; however, they may result in a fuller-spectrum extract, an extraction of all chemical constituents within the plant matrix depending on temperature, lack of winterization, or decarboxylation[31,36]. Hydrodistillation is a third technique that allows for the collection of aromatic compounds from plant material, often used when producing "essential oils"[37]. This utilizes a distillation apparatus that separates the essential oil from condensed vapor obtained from a boiling mixture of water and plant material. Other extraction approaches have been investigated, such as eutectic solvents, pulsed electric fields, and hydrodynamic extractions[38,39,40].

Given the popularity of SFE, hydrodistillation, and solvent-based extractions in the cannabis industry; there have been several studies that have examined the production of cannabinoids and terpenoids when employing these extraction techniques.. Including the investigation by Mazzara et al., 2022 analyzed terpene, polyphenol, and cannabinoid of hydrodistilled extracts from nine commercial varieties of Cannabis sativa L.[41]. Providing evidence of how any hemp cultivar when subjected to hydrodistillation will result in copious amounts of terpenes as much as 30.2% of total concentration. Likewise, in 2021 Bowen et al.,

reported the impact of solvent-based extraction (ethanol or isopropyl alcohol) and super critical CO_2 on the chemical profile of flower from single cannabis cultivar[36]. Interestingly, more neutral cannabinoids were detected in solvent-based extracts and more acidic cannabinoids in CO_2 extracts, an outcome believed to be influenced by the timing of decarboxylation.

However, thus far no study has directly compared the terpene and cannabinoid content of the three dominant extraction techniques from the same botanical material. The present investigation compared three hemp extraction techniques frequently seen in commercial applications: aqueous ethanol maceration, hydrodistillation, and supercritical fluid extraction. One hemp cultivar, grown in three different geographical locations, was harvested and processed identically with the resulting extracts analyzed for their targeted chemical profiles and quantification of known bioactive compounds. This study provides direct evidence that the extraction method yields distinct phytochemical profiles and could be employed to obtain more selective cannabinoid or terpene chemical profiles.

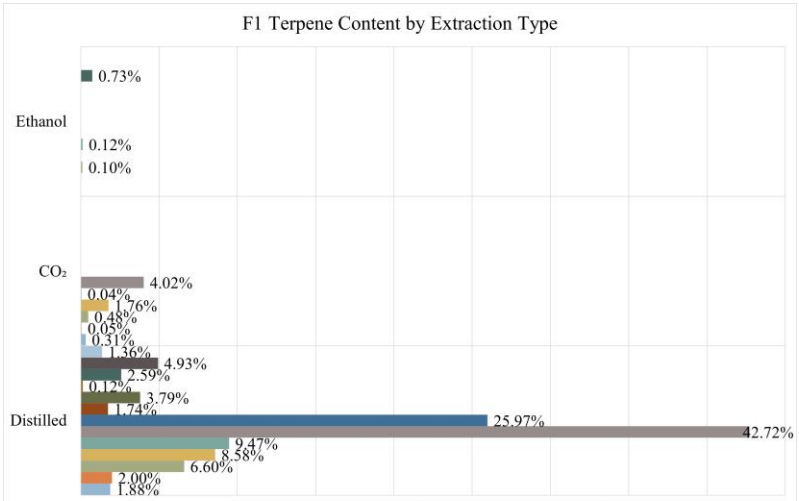
Results

Quantification of Terpenes, Cannabinoids, and Extraction Yield

Terpene concentration varied widely depending upon the hemp sample and extraction type. Hydrodistilled samples, displayed the most variety and abundance of terpenes, with β-caryophyllene the most concentrated terpene, ranging from 25–42 mg/g (Figure **3-1** and **Appendix B**). CO_2 extracts displayed contents of primary terpenes such as β-caryophyllene and humulene but at lower concentrations compared to hydrodistilled samples. Ethanol extracts showed the least diversity in terpene composition, but contained amounts of geranyl acetate that were comparable to distilled extracts at a concentration range of 0.73–1.1 mg/g. No geranyl acetate was identified in CO_2 extracts. Cannabinoid diversity was more prevalent within the CO_2

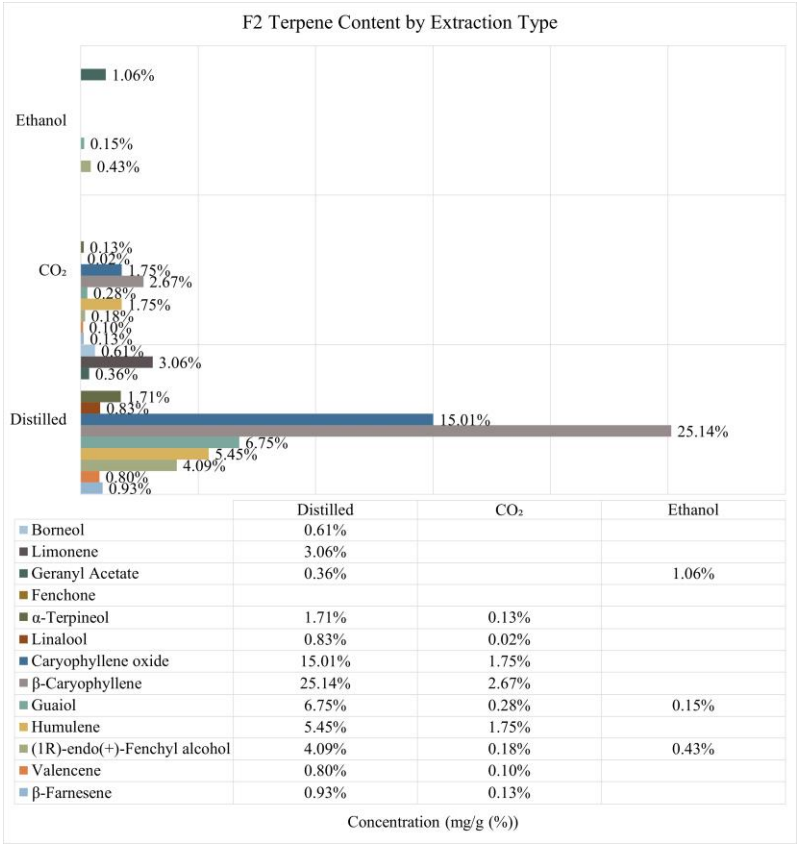
extracts, and displayed all six targeted cannabinoids (Figure **3-2** and **Appendix B**). CO_2 extracts also contained the most CBD and CBG ranging from 12.8–20.6 mg/g and 9.5–16.9 mg/g, respectively. Ethanol extracts contained the most CBDA ranging from 3.2–4.1 mg/g and CBGA around 0.1 mg/g across all extraction types. CBD and CBC were the only identifiable cannabinoid within distilled extracts at concentrations less than 1 mg/g. Furthermore, each technique generated contrasting yields of extract mass with ethanol providing the greatest yield between 22–24%, CO_2 ;1.3–1.8%, and distilled, 0.08–1.1% (Table **3-1**).

(A)

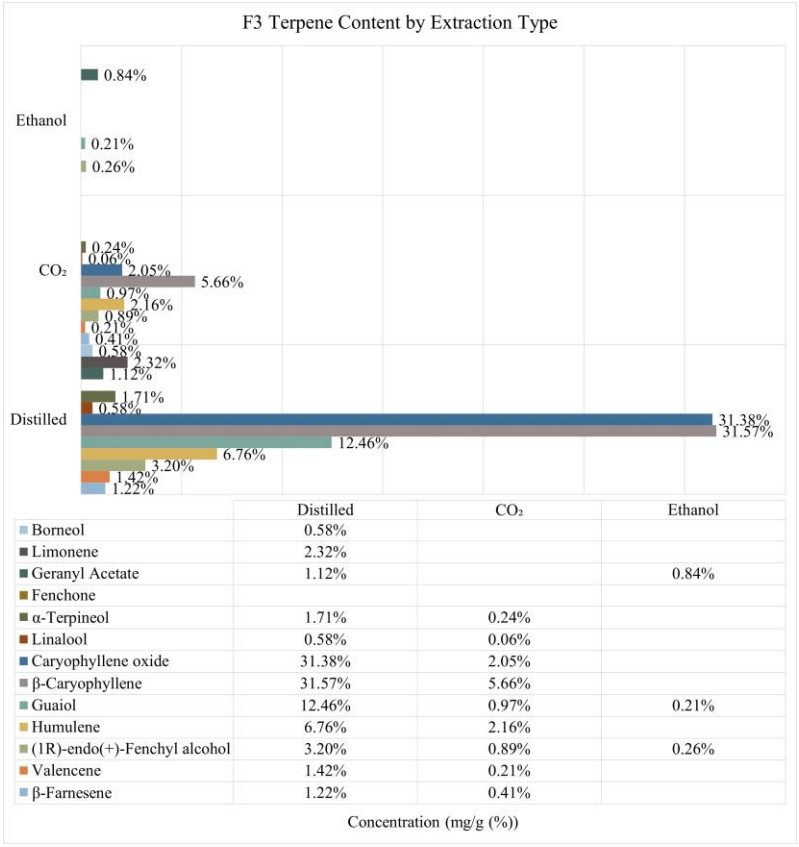


	Distilled	CO_2	Ethanol
Borneol	1.36%		
Limonene	4.93%		
Geranyl Acetate	2.59%		0.73%
Fenchone	0.12%		
α-Terpineol	3.79%		
Linalool	1.74%		
Caryophyllene oxide	25.97%		
β-Caryophyllene	42.72%	4.02%	
Guaiol	9.47%	0.04%	0.12%
Humulene	8.58%	1.76%	
(1R)-endo(+)-Fenchyl alcohol	6.60%	0.48%	0.10%
Valencene	2.00%	0.05%	
β-Farnesene	1.88%	0.31%	

(B)



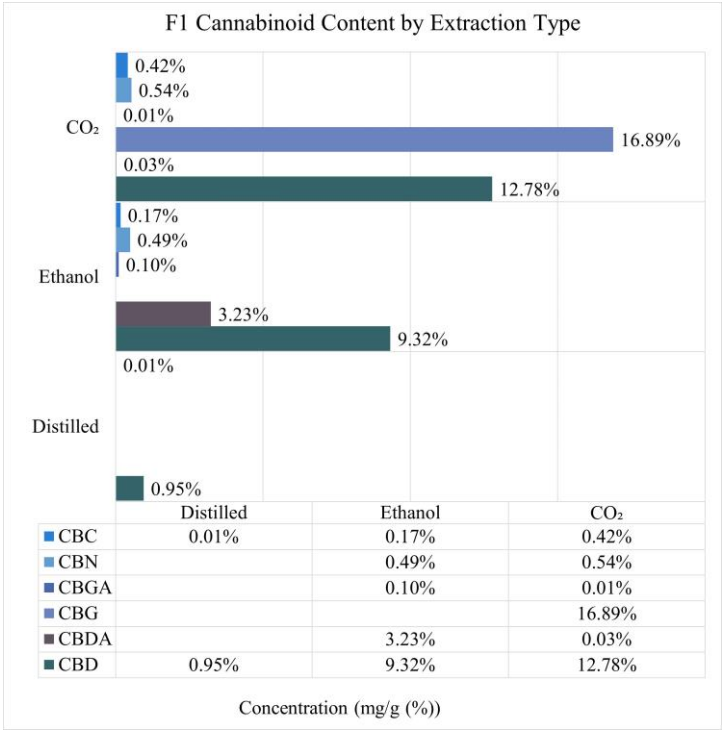
	Distilled	CO_2	Ethanol
Borneol	0.61%		
Limonene	3.06%		
Geranyl Acetate	0.36%		1.06%
Fenchone			
α-Terpineol	1.71%	0.13%	
Linalool	0.83%	0.02%	
Caryophyllene oxide	15.01%	1.75%	
β-Caryophyllene	25.14%	2.67%	
Guaiol	6.75%	0.28%	0.15%
Humulene	5.45%	1.75%	
(1R)-endo(+)-Fenchyl alcohol	4.09%	0.18%	0.43%
Valencene	0.80%	0.10%	
β-Farnesene	0.93%	0.13%	



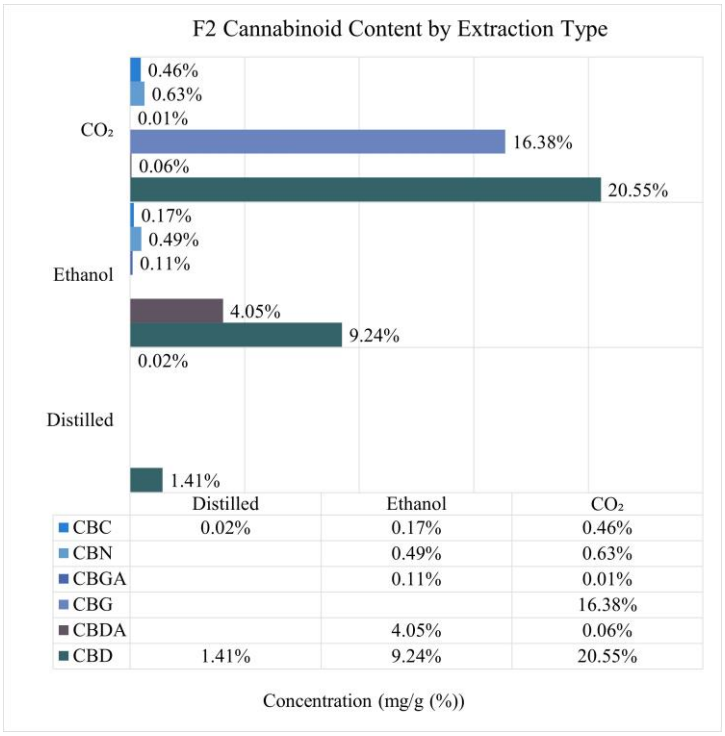
	Distilled	CO_2	Ethanol
Borneol	0.58%		
Limonene	2.32%		
Geranyl Acetate	1.12%		0.84%
Fenchone			
α-Terpineol	1.71%	0.24%	
Linalool	0.58%	0.06%	
Caryophyllene oxide	31.38%	2.05%	
β-Caryophyllene	31.57%	5.66%	
Guaiol	12.46%	0.97%	0.21%
Humulene	6.76%	2.16%	
(1R)-endo(+)-Fenchyl alcohol	3.20%	0.89%	0.26%
Valencene	1.42%	0.21%	
β-Farnesene	1.22%	0.41%	

(C)

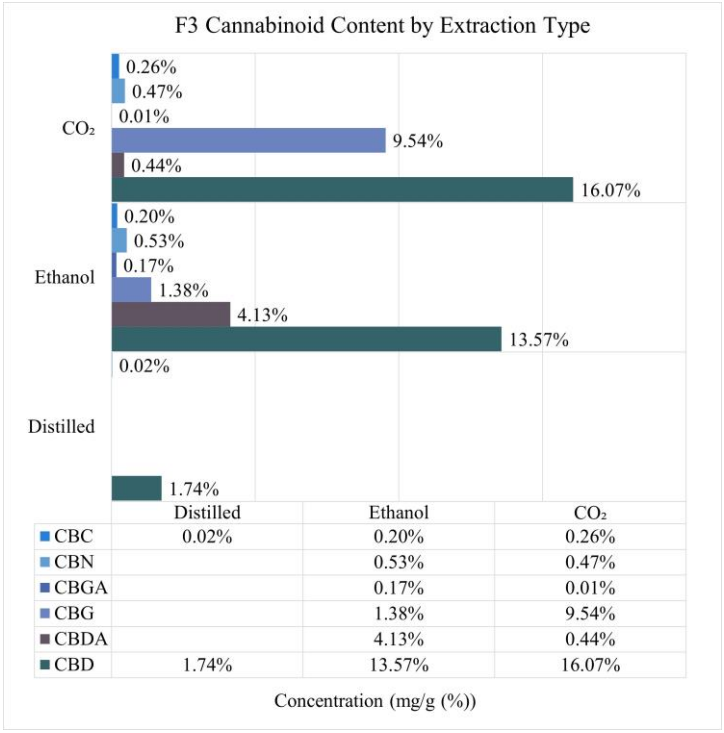
Figure **3-1**: Relative composition of identified terpene concentration from each extraction method. Data labels indicate absolute quantitation (mg/g (%)) of each compound in the corresponding extract. (A) F1 samples, (B) F2 samples, and (C) F3 samples.


F1 Cannabinoid Content by Extraction Type
CO₂
Ethanol
Distilled
0.42%
0.54%
0.01%
16.89%
0.03%
12.78%
0.17%
0.49%
0.10%
3.23%
9.32%
0.01%
0.95%
Distilled Ethanol CO₂
CBC 0.01% 0.17% 0.42%
CBN 0.49% 0.54%
CBGA 0.10% 0.01%
CBG 16.89%
CBDA 3.23% 0.03%
CBD 0.95% 9.32% 12.78%
Concentration (mg/g (%))

(A)


F2 Cannabinoid Content by Extraction Type
CO₂
Ethanol
Distilled
0.46%
0.63%
0.01%
16.38%
0.06%
20.55%
0.17%
0.49%
0.11%
4.05%
9.24%
0.02%
1.41%
Distilled Ethanol CO₂
CBC 0.02% 0.17% 0.46%
CBN 0.49% 0.63%
CBGA 0.11% 0.01%
CBG 16.38%
CBDA 4.05% 0.06%
CBD 1.41% 9.24% 20.55%
Concentration (mg/g (%))

(B)



(C)

Figure **3-2**: Relative composition of identified cannabinoid concentration from each extraction method. Data labels indicate absolute quantitation (mg/g (%)) of each compound in the corresponding extract. (A) F1 samples, (B) F2 samples, and (C) F3 samples.

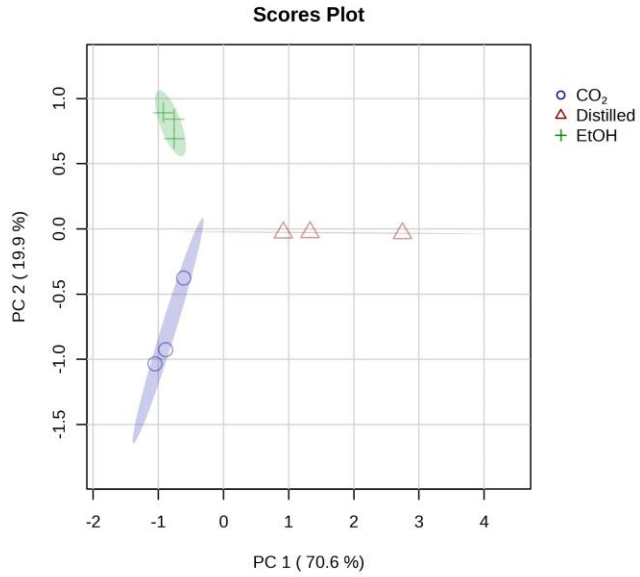
Table **3-1**. Extraction yield % ± SD (w/w) of different extraction techniques across three hemp samples.

	F1			F2			F3		
	Distilled	**Ethanol**	**CO_2**	**Distilled**	**Ethanol**	**CO_2**	**Distilled**	**Ethanol**	**CO_2**
Extraction yield *w/w* (%)	1.11	24.29 ± 0.02	1.84 ± 0.01	0.18	22.03± 0.01	1.56 ± 0.01	0.08	24.27 ± 0.01	1.30 ± 0.00

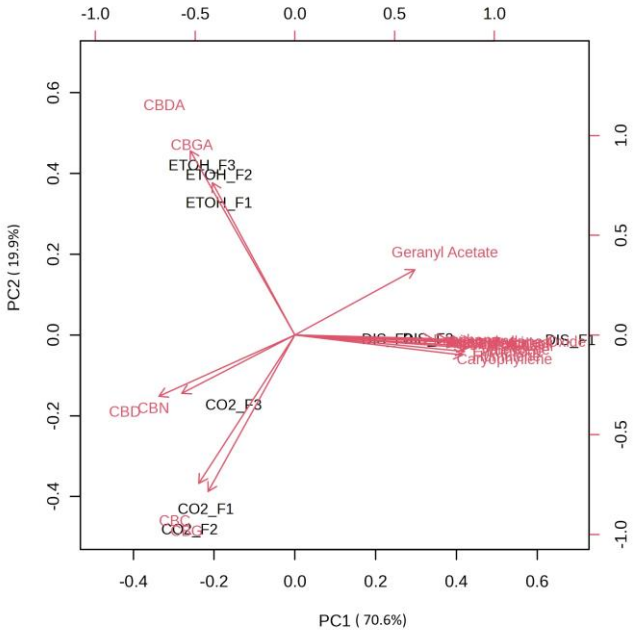
Statistical Analysis of Feature Relative Abundance

Principal component analysis (PCA) of the 19 identified compounds revealed a distinct clustering of the extraction techniques regardless of hemp sample (Figure **3-3A**). The use of PCA reduces the dimensionality of our dataset while preserving crucial variance in the data structure,

thus allowing for a better visualization and interpretation of the data. In an unsupervised setting, two principal components based on peak area displayed the clustering of samples with identical extraction techniques based on similar compounds. CO_2 and ethanol samples demonstrated a tight cluster between each technique while a larger variation in the distilled samples resulted in a slight overlap in confidence level with CO_2 extracts. The PCA biplot revealed that neutral cannabinoids had a higher correlation to CO_2 samples while cannabinoid acids CBDA and CBGA correlated with ethanol extractions (Figure **3-3B**). Terpenes showed a more variable correlation; however, most of the identified terpenes were present within the hydrodistillation samples with the exception of geranyl acetate which was also found in the ethanol extracts. These patterns were also observed in the heat maps displaying the relationship between specific compounds and samples. Heat map (Figure 4A) displayed the phytochemical landscape of compounds based on averages across extraction type.

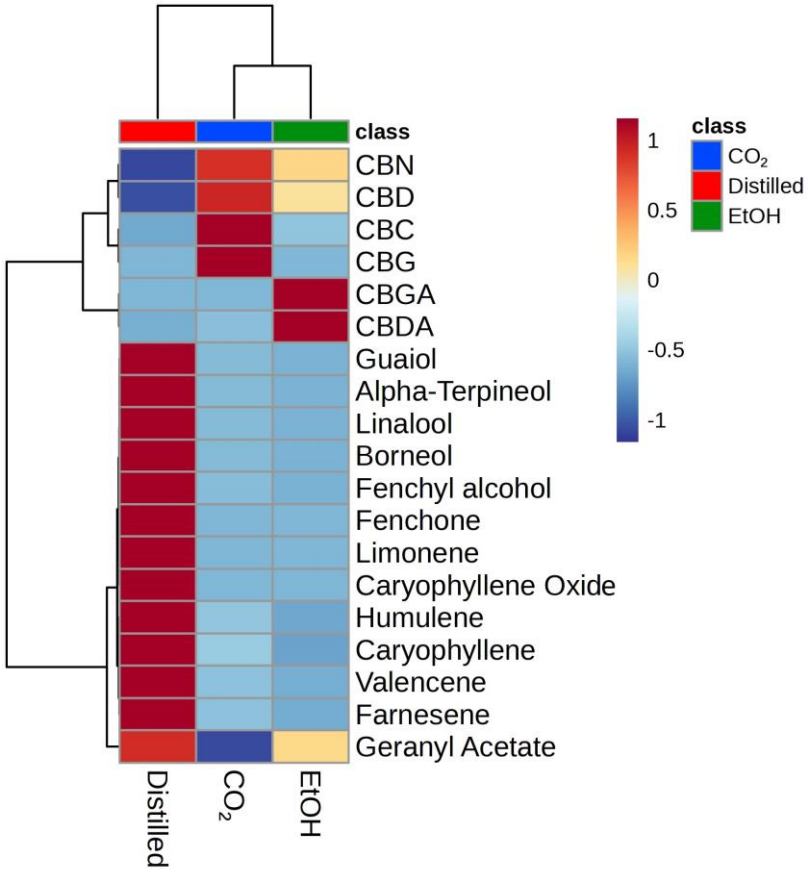


(A)

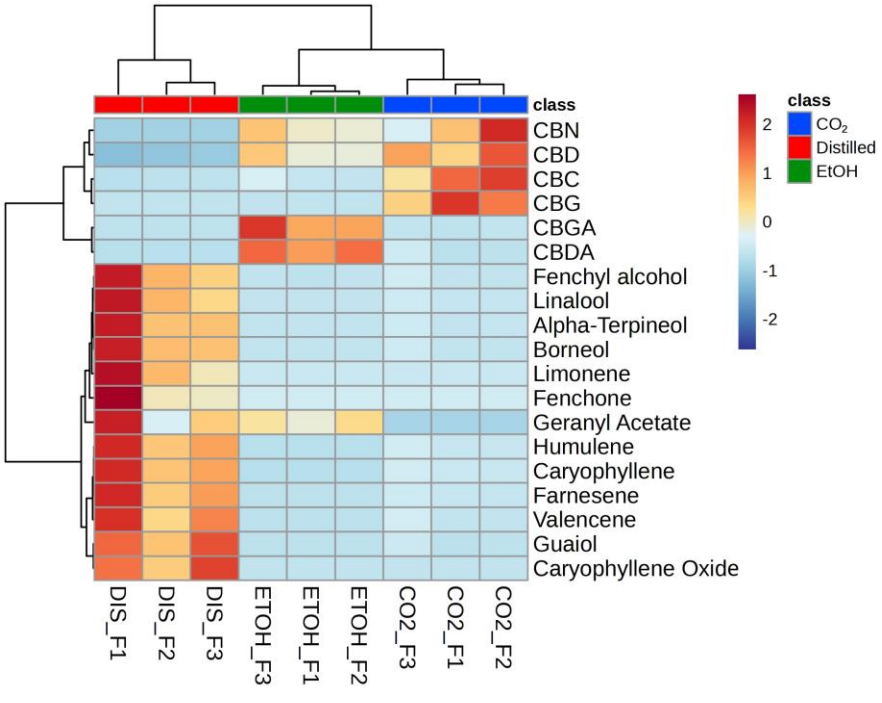


(B)

Figure **3-3:** (A) Principal component analysis (PCA) plot of samples based on peak area of identified metabolites. (B) Biplot of samples with identified metabolite peak area as variables.


class
CBN
CBD
CBC
CBG
CBGA
CBDA
Guaiol
Alpha-Terpineol
Linalool
Borneol
Fenchyl alcohol
Fenchone
Limonene
Caryophyllene Oxide
Humulene
Caryophyllene
Valencene
Farnesene
Geranyl Acetate
Distilled
CO2
EtOH
1
0.5
0
-0.5
-1
class
CO2
Distilled
EtOH

(A)


class
CBN
CBD
CBC
CBG
CBGA
CBDA
Fenchyl alcohol
Linalool
Alpha-Terpineol
Borneol
Limonene
Fenchone
Geranyl Acetate
Humulene
Caryophyllene
Farnesene
Valencene
Guaiol
Caryophyllene Oxide
DIS_F1
DIS_F2
DIS_F3
ETOH_F3
ETOH_F1
ETOH_F2
CO2_F3
CO2_F1
CO2_F2
2
1
0
-1
-2
class
CO2
Distilled
EtOH

(B)

Figure **3-4**: Heat map depicting the relative abundance of identified metabolites to samples. (A) average of triplicate strains per extraction technique. (B) individual samples across each extraction method. Samples are plotted as columns while identified metabolites are plotted as rows. Heatmaps created using MetaboAnalyst 6.0.

The acidic cannabinoids CBDA and CBGA were predominantly found within ethanol extracts, while the CO2 extract contained the neutral cannabinoids CBC, CBN, CBD, and CBG. At an individual level, slight variability was evident due to phytochemical differences between F1, F2, and F3 (Figure **3-4B**). Ethanol samples displayed similar compound distributions between F1, F2, and F3. Distilled extracts' terpene composition varied between samples, with F1 displaying a high concentration compared to F3 and F2, with the exception of guaiol and caryophyllene oxide being more concentrated in F3 distilled samples. CO2 extracts clustered together in general with greater relative abundance of neutral cannabinoids, though there were still differences between hemp source, with high significance between; CBC and CBG for F1, and all neutral cannabinoids for F2.

Discussion

The differences in cannabinoid and terpene composition between the extraction methods demonstrate the varying chemical profiles a single hemp variety can generate. As expected, the difference in polarity of the solvents often drives the variation we see in the resulting extract. Super critical CO_2 extraction proved to be the most efficient type of extraction for both neutral cannabinoids and primary terpenes of the hemp cultivar. Distilled extracts yielded primary and secondary hemp terpenes but were significantly lower in the diversity and concentration of cannabinoids. Ethanol extractions provided an approach capable of pulling out cannabinoid acids

with moderate terpene presence. Comparable amounts of geranyl acetate between distilled and ethanol extracts suggest that ethanol may be an optimal solvent for the extraction of terpenoids containing monocarboxylic acids. However, one investigation evaluating ethanol extracts differing in solvent temperature has shown evidence of a decrease in overall terpene content in extracts charged with room temperature ethanol compared to −40 °C ethanol[42]. This suggests that differences in terpene content evaluated in this study could be dependent upon the conditions that were applied. However, common across the three extraction techniques was their ability to extract CBD at various concentrations. One distinct difference between the processes of the super critical CO2 and solvent (ethanol) extractions is the added step in the CO_2 extraction of decarboxylating the extract. This typically converts the acidic cannabinoids (e.g., CBDA and CBGA) into their neutral forms (CBD and CBG, respectively)[7,10]. Thus, as expected, the ethanol extraction yielded a higher amount of acidic cannabinoids which would otherwise be lost during the decarboxylation step of the supercritical CO2 extraction. Due to the nature of the decarboxylation process (i.e., the high heat (ca. 95 °C) for a long duration (~60 min)), this would not be readily amenable to ethanol-based extracts. Thus, this highlights another difference between these two processes that may make one more suitable over another for different research or commercial purposes.

It is important to note that this study provides evidence of unique cannabinoid and terpene profiles obtained through the three different extraction techniques at discreet conditions, and thus it is reasonable to assume that altering various parameters of an extraction protocol may yield further modification of the phytochemical profile obtained. Several studies have evaluated the efficiency of SFE extraction under varying conditions of pressure, temperature, and solvent consumption; however, results are also dependent on the specific cultivar[43,44,45]. Increasing the total amount of plant material and minimizing the storage time may likely result in higher yields and possibly more diversity in terpenes and cannabinoids for the specific hemp cultivar. Additionally, modification of the ethanol extraction process may also lead to differences in

extract composition. In this study we make use of a simplified liquid solvent extraction approach that is easily reproducible and frequently employed at the industrial scale[46]. Previous publications involving ethanol extracts of cannabis species have observed that changes in solvent temperature and the minimizing pulverization can result in differences in compound abundance and extraction yield[42,47]. Although ethanol is excellent at drawing out bioactive compounds, and a primary method for other botanical supplement manufacturing processes, other compounds may also be present within the extract such as pigments from the plant material, limiting the selectivity. When considering hydrodistillation, one must consider the plant stage and handling process as both of these can influence the composition of terpenes at the time of extraction[48,49].

Furthermore, our approach of using two different analytical instrumentation methods (i.e., GC-MS and LC-MS/MS) revealed advantages for a potential multi-analytical approach to phytochemical analysis of cannabis but also revealed limitations of commonly accessible instrumentation. The GC-MS methodology employed in this investigation captured a range of different terpenes including a few that were observed after derivatization via trimethylsilyl addition. However, this analysis faced challenges as the GC-MS was equipped with only a single quad mass analyzer and only recorded low-resolution MS1 data; this was not sufficient to separate out some of the terpenes which have similar molecular composition and structure. Future work to compare chemical profiles of hemp extracts will utilize a GC analysis paired with tandem mass spectrometry allowing for better identification and capture of aromatic compounds[50]. On the other hand, LC-MS/MS analysis provided an advantage thanks to its additional fragmentation data, yet an LC approach made it difficult to capture all volatile compounds present within the extract. An untargeted approach may also be favorable for the identification of additional compounds that were not addressed in this investigation that may play a major role in the chemical and potential biological differences between extraction techniques.

Methods and Materials

Hemp Material and Solvents

A CBG/CBD dominant hemp cultivar “Tangerine” was grown, harvested, and donated by Cedar Meadow Farms, under three different field conditions and locations (hereafter referred to as F1, F2, and F3) (Holtwood, PA, USA). As a commercial hemp cultivar, the material is required to possess <0.3% THC by USDA regulation; therefore, THC and THCA standards were not included in this investigation. The inflorescence of each sampling that was used for extraction comparison were received 1–3 months prior to extraction and stored in vacuumed sealed bags at room temperature with limited light exposure. Once extracted, distilled samples were stored at 4 °C, ethanol extracts at room temperature, and super-critical CO_2 extracts were stored at room temperature, all extracts were stored in amber glass vials. All solvents were acquired through VWR (Radnor, PA, USA) and were of reagent or spectroscopic grade. Standards were provided by Cayman Chemical (Ann Arbor, MI, USA) and confirmed certified reference materials. The processing of hemp material was done in accordance with and permitted by the Pennsylvania Department of Agriculture (permit #42-001074).

Solvent Extraction

Methods for solvent extraction adapted and modified according to previously published protocols Kellogg et al., 2024 [51]; Szalata et al., 2022[52]. In brief, 1.0 ± 0.05 g of inflorescence material was ground to a fine powder using an electric spice grinder and transferred to a 50 mL conical tube, after which 20 mL of 96% aqueous ethanol was introduced to each tube. Samples were vortexed for 10 sec then sonicated at room temperature (RT) for 30 min. Samples were centrifuged at 4 °C for 5 min at 3000 rpm, and the supernatant transferred to a clean 25 mL volumetric flask. The extraction was repeated two additional times to reach final volume of 25 mL of supernatant. The combined supernatant was vacuum filtered on a Buchner funnel with No.

1 Watman paper. The collected supernatant was then placed under −20 °C conditions for 24 h, filtered, then transferred to a scintillation vial and allowed to dry at RT under N2. Each field sample was extracted in triplicate.

Hydrodistillation

Distillation was carried out using 30 g whole flower samples. The method was adapted using protocols from Abraham et al., 2023 [50]. Each sample was placed inside a 1000 mL round bottom flask, after which 600 mL of Milli-Q water was charged. The sample was then placed on a heating mantel and attached to a distilling apparatus (VWR, Radnor, PA, USA). Samples were brought to a boil then lowered to a medium temperature for a total of 60 min. Oil from the return tube of the distillation apparatus was collected from the discharge stopcock into a 20 mL scintillation vial. For complete removal of water remnants, each oil was filtered using a glass pipette containing a loosely packed layer of glass wool below 0.8 g of anhydrous sodium sulfate. The filtered oil was then captured in a tared 20 mL scintillation vial. All samples were extracted in duplicate except for a single sample with restricted mass (F3). There was insufficient material to allow for triplicate analyses of each sample; however, with three biological replicates, and the accuracy and repeatability of the instrumentation, we determined it was sufficient to allow for comparison across methodologies.

Supercritical CO_2 Extraction

Dried hemp flower, 55–105 g, was ground and extracted as previously described, with slight modifications [53], using an SFT-SP1100 extraction system manufactured by Supercritical Fluid Technologies, Inc. (Newark, DE, USA). Briefly, the extraction was performed at 55 °C for 35 min at 413 bars, the extract was then collected and dissolved in ethanol to a final concentration of 10% by weight. Winterization was carried out for 24–48 h at −20 °C and the extract was filtered before ethanol evaporation. The final extract was dissolved in fractionated coconut oil

(Pure Body Naturals, West Chester, OH, USA) at 300 mg/mL and decarboxylated at 95 °C for 1 h.

LC-MS/MS

All samples were reconstituted at 50 µg/mL in methanol containing 1 µM of chlorpropamide as an internal standard (Sigma Aldrich, St. Louis, MO, USA), except for the distilled extracts which were prepared at 1 mg/mL. Standards were prepared as a serial dilution from 30–0.003 µg/mL. The method was adapted from Anderson et al., 2024 [54]. Samples were injected onto an Acquity UPLC BEH Shield RP18 (1.7 µm, 2.1 mm × 100 mm) column (Waters Corporation, Milford, MA, USA) and eluted into a Thermo Orbitrap Exploris 120 (ThermoFisher Scientific, Waltham, MA, USA) with an H-ESI ion source in the negative polarity mode and set to a scan range of 300–400 m/z with a resolution of 120,000. Samples were injected at a volume of 5 µL with the following binary system of solvent A, 0.1% formic acid in water, solvent B, 100% acetonitrile, at the following gradient: 50% B for 9 min, 100% B for 2 min, then 50% B for 2 min. The mass spectrometer was operated with a spray voltage of 2500 V, vaporizing temperature of 350 °C, aux gas of 10 Arb, sheath gas of 50 Arb, sweep gas of 1 Arb, and a collision gas pressure of 1 mTorr.

GC-MS

Samples were prepared at a concentration of 1 mg/mL in GC-MS grade hexane with 1 µM of chlorpropamide. After being transferred to GC-MS vials, they were allowed to dry under a fume hood with open covers to prevent contamination of samples. Once dried, samples were introduced to pyridine and BSTFA (1:1), capped and allowed to sit for 60 min at room temperature to allow for the addition of a trimethylsilyl group for increased compound separation. Samples were then stored at −20 °C until MS analysis. Terpenoid standards were prepped in a similar manner but at a concentration range of 500 µg/mL–0.3 µg/mL. Cannabinoid standards were prepared at a concentration of 50 µg/mL for identification. Analysis of the samples and

standards were carried out using methods adapted from Abraham et al., 2023 [50] on an Agilent 5975C series GC-MSD with an Electron Ionization (EI) source operated in the positive mode with a scan range of 50–600 m/z [50]. All samples were injected at 1 µL on a 30 m × 250 µm × 0.25 µm Restek 13423 column (Restek, Bellefonte, PA, USA), which was initially set to 50 °C and pressure of 7.6522 psi. The oven temperature was ramped at 4 °C/min rate until 280 °C with a hold time of 2 min (61.5 min total run time).

Data Analysis

Compounds of interest in the GC-MS samples were identified based on MS1 data of the involved standards visualized in MassHunter Qualitative Analysis Agilent software (Qualitative Analysis 10.0). LC samples were identified using the MS2 data in MZMine3 [34,55]. Peak area measurements were accomplished using MZMine3 for GC samples, while measurements for LC samples were determined by Thermo Xcalibur Quantitation Analysis (ThermoFisher Scientific). MZmine Workflow parameters and standard concentration curves can be found within the supplemental material. MetaboAnalyst 6.0 was used for principal component analysis (PCA), loading plot, and heat map construction (www.metaboanalyst.ca). Relative abundance of identified features were used independent variables for each statistical analysis with a range scaling normalization of the data for the PCA. Equation (1) was used to quantify individual compounds, where "concentration of compound" was the calculated amount injected based on the corresponding calibration curves, "extraction vol/sample aliquot" was the sample preparation dilution. Equation (2) determined total extraction yield (%), based upon the ration between the dried extract mass and the dried plant material (inflorescence) mass. Extraction yields for distilled samples were limited to one replicate due to weight limitations.

$$\text{Compound}\,\frac{\text{mg}}{\text{g}}(\%) = \left(\begin{array}{c}\text{Concentration}\\ \text{of compound, }(\frac{\mu\text{g}}{\text{mL}})\end{array}\right)\left(\frac{\text{extraction vol. (mL)}}{\text{sample aliquot, (mg)}}\right)\left(\begin{array}{c}\text{conversion}\\ \frac{\mu\text{g to mg}}{\text{mg to g}}\end{array}\right)\times 100$$

$$\text{Extracton Yield (\%)} = \left(\frac{\text{Extract Dry Mass (mg)}}{\text{Inflorescence dry Mass (mg)}}\right) \times 100$$

Conclusion

Overall, this study provides evidence of the differences in cannabinoid and terpene composition obtained from hemp inflorescence after three predominant extraction techniques: supercritical CO2, solvent extraction (ethanol), and hydrodistillation. While supercritical CO2 extraction can achieve broad extraction and has a relatively short extraction time of large quantities of plant material, a supercritical CO2 extractor is expensive. Challenges with solvent extractions involving ethanol will likely make isolation of neutral cannabinoids difficult and result in a more diverse phytochemical profile. Likewise, hydrodistillation can be extremely effective at capturing terpenes within hemp inflorescences; however, the process has dramatically lower yields and a distinct lack of cannabinoids in the resulting extract. The results from this head-to-head investigation with identical plant material demonstrate how extraction techniques can allow commercial processors or academic investigators to be more selective in the extraction process and fine-tune their processes to develop more specific hemp extracts.

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Chapter 4

Angiotensin-Converting Enzyme (ACE) Inhibitory Activity of Phytocannabinoids from Cannabis sativa L.

Abstract

Background

Cannabinoids of *cannabis sativa* L. (hemp) are reported to affect blood pressure. However, the mechanistic effects/interactions of these cannabinoids on blood pressure regulation have yet to be fully explored. This study aims to characterize the interactions between cannabinoids and a main regulatory hypertension enzyme, angiotensin-converting enzyme-I (ACE), part of the renin-angiotensin system (RAS).

Methods

Inflorescence from locally grown hemp was extracted in ethanol to generate a crude hemp extract that was tested for its ACE inhibitory activity using an *in vitro* enzymatic reaction assay. The crude extract was then further separated using flash chromatography techniques, generating 21 fraction extracts that were evaluated for their ACE inhibitory activity and analyzed for their content of six hemp cannabinoids: CBD, CBG, CBN, CBC, CBDA, and CBGA. Binding affinities between ACE and the cannabinoids were then determined in an *in silico* molecular docking study, and then assayed to determine their IC_{50} values.

Results

A crude extract from the inflorescence of a CBD dominant hemp cultivar lowered ACE activity by 20 – 50% at a concentration of 0.1 mg/mL. Subsequent fractions displayed varying ACE inhibitory activity, with those containing more diversity in cannabinoid composition lowering ACE activity by greater than 85% when evaluated at concentrations of 200 μg/mL. Molecular docking between cannabinoids and ACE revealed similar or greater binding affinity scores

between CBG, CBN, CBC, and CBGA when compared to the known ACE inhibitor, lisinopril. However, all cannabinoids displayed a higher binding affinity for the N-domain of ACE. Following enzyme inhibition assays, CBC and CBN provided the lowest IC50 values at concentrations of 1.7 and 1.6 µM, respectively.

Conclusion

Overall, this study demonstrated ACE inhibiting activity of cannabinoids from *cannabis sativa* L. under *in vitro* conditions, while providing predicted binding scores that can help elucidate the interactions occurring between cannabinoids and ACE. CBC and CBN displayed an IC50 of 1.7 and 1.6 µM, respectively. These finding suggest a possible mechanism by which bioactive compounds from hemp lead to changes in blood pressure.

Background

The angiotensin-converting enzyme (ACE) is a key component of the renin-angiotensin system (RAS), a multi-hormonal system that maintains cardiovascular function including blood pressure. ACE acts by catalyzing the conversion of angiotensin I to angiotensin II, subsequently modulates vasoconstriction and increasing blood pressure[1]. However, ACE activity also contributes to the development of a variety of cardiovascular diseases[2]. Two forms of ACE are present in mammals: testis ACE and somatic ACE. The latter enzyme is widespread in highly vascular organs such as the retina and lung, but can also be found in blood vessels, kidney, intestine, adrenal gland, liver, and uterus[3,4]. ACE inhibitors are a frontline therapeutic agent in the treatment of hypertension in adults per the American College of Cardiology/American Heart Association guidelines[5]; and are effective in lowering blood pressure and controlling heart failure by blocking the formation of the vasoconstrictor angiotensin II and preventing the breakdown of vasodilator bradykinin [6].

Although cannabis has a long history of being used for medicinal purposes, efforts are still being made towards unraveling its full effects including its impact on blood pressure and cardiovascular health[7,8,9]. The relationship between cannabis and blood pressure has attracted increasing attention as cannabis use grows in the United States, both medically and recreationally. Several studies have observed variable effects on blood pressure in clinical trials, promoting a need for further information on the mechanism of action between cannabis compounds and systems that regulate blood pressure[10,11,12,13,14]. Crosstalk between the endocannabinoid system (ECS) and the RAS has gained interest with some links to the function of ACE[15]. Cannabinoid receptor I (CB1R) activation has been suggested to reduce the vasoconstricting and hypertensive effects of angiotensin II based on evidence from investigation evaluating vascular tissue from rats under different CB1R modulators[16]. The cannabis plant is often associated with the ECS as the main psychoactive compound delta-9-tetrahydrocannabinol (THC) has shown a high affinity for the ECS receptor, CB¬1R acting as a partial agonist and full agonist of the CB2R[17]. The primary cannabinoid in cannabis sativa L. or hemp is cannbidiol (CBD), a non-psychoactive cannabinoid, reported to be an inverse agonist of both CB¬1R and CB2R with a low binding affinity[18,19]. While minor cannabinoids, such as cannabigerol (CBG), cannabichromene (CBC), and cannabinol (CBN), which are found in smaller quantities in the cannabis plants, have been reported to have low affinity for the CB1R and CB2R receptors[20]. Given the distinct differences between cannabinoids and receptors in the ECS, researchers continue to explore a variety of receptor interactions to help better delineate the effects of exogenous cannabinoids. Research involving cannabinoids and receptors of the RAS have primarily focused on ACE2, in efforts to determine potential antiviral activity[15,21]. To date, peptides from hemp seed meal protein hydrolysates have been the only hemp product tested that exhibited ACE inhibitory effects both *in vitro* and *in vivo*[22,23]. However, there are no investigations studying the direct effects of cannabinoids from Cannabis sativa L. on ACE activity.

The present study addressed the ACE inhibitory activity of ACE inhibiting cannabinoids from hemp. In this study locally grown hemp inflorescence was evaluated for its ACE inhibitory activity using an in vitro enzyme reaction assay. The crude hemp extract was then separated into 21 fractions that were also evaluated for their ACE inhibitory activity as well as analyzed for cannabinoid content. Compounds in fraction extracts exhibiting high ACE inhibitory activity were tested in their pure form to determine their individual effects on ACE activity. Predicted ligand-receptor interactions from a molecular docking study and enzymatic reaction data were combined to provide information on the direct interactions between cannabinoids and ACE to broaden the information surrounding the mechanism of action compounds from hemp act on to effect blood pressure in the human body.

Methods

Solvents, Standards, and Hemp Material

All solvents were acquired through VWR (Radnor, PA, USA) and were of reagent or spectroscopic grade as needed. Cannabinoid and terpene standards used for identification and assessment of ACE inhibitory activity were purchased from Cayman Chemical (Ann Arbor, MI, USA) and confirmed as certified reference materials. Lisinopril (83915-83-7) was acquired from Sigma-Aldrich (St. Louis, MO, USA). Hemp inflorescence material was provided by Cedar Meadow Farms (Holtwood, PA, USA).

Hemp extraction and Fractionation

Inflorescence from a CBD dominant hemp cultivar (50.0 ± 0.05 g) was ground and transferred to a 1000 mL Erlenmeyer flask to which was added 900 mL of 96% aqueous ethanol. The sample was placed in a shaker overnight at 190 rpm at room temperature. The extraction slurry was then filtered through a No. 1 Whatman paper. The hemp pomace was re-extracted with an

additional 900 mL of 96% ethanol and allowed to shake overnight. The second solution was filtered and the two extraction rounds combined and held at -20°C for 24 h to allow for precipitation of lipids and fats. The final extraction solution was transferred to a 1000 mL round bottom flask and dried to completion using a BUCHI Rotavapor R-300 (New Castle, DE, USA) for the evaporation of the remaining ethanol.

The resulting crude extract (8.0 ± 0.05 g) was weighed and reconstituted in 50 mL of 100% methanol, and mixed with ca. 22 g 40-60 µm silica (VWR (Radnor, PA, USA) , homogenized, and allowed to dry via evaporation. The resulting mixture was charged to a 25 g Biotage Sfär DLV column (Uppsala, Sweden) and attached to a 120 g Biotage Sfär C18 D (Duo 100 Å, 30 µm) on a Biotage Selekt System. The sample mixture was then fractionated using a binary solvent system comprised of ethanol (A) and water (B) in a stepwise gradient: 50% A for 3 column volumes (CV); 55% A for 3 CV; 60% A for 3 CV; repeated. until 100% A, then held for 16 CV. A total of 224 25-mL fractions were collected, and grouped into a final set 32 fractions. Each fraction was transferred to a 1000 mL round bottom flask, rotovapped until dry, and transferred to a 25 mL scintillation vial. Of the 32, only 21 fractions contained enough extract for enzyme and LC-MS/MS analysis.

To eliminate pigment interference in the colorimetric ACE activity assay, 100 mg of the hemp crude extract and the 21 fraction extracts were reconstituted in 1 mL of 100% ethanol and vortexed until completely dissolved. Extracts were then introduced to 30 mg of activated charcoal, sonicated for 30 min, then filtered using a 1 mL syringe equipped with a 0.45 µm PTFE syringe filter (13mm, VWR (Radnor, PA, USA)). Additional charcoal and filtration was applied up to five more times depending on the opacity of each extract then allowed to dry at RT under N_2. Fractions were analyzed for their cannabinoid content before and after charcoal filtration to determine differences in concentration.

LC-MS/MS analysis of extracts

For cannabinoid quantification, extracts were reconstituted at 50 µg/ml in methanol containing 1 µM chlorpropamide as an internal standard. Cannabinoid standards (CBD, CBG, CBC, CBN, CBDA, and CBGA) were prepared in the same solvent and diluted from 30 – 0.003 µg/ml. Samples were injected (5 µL) onto an Acquity UPLC BEH Shield RP18 (1.7 µm, 2.1 x 100 mm) column (Waters Corporation, Milford, MA, USA) and eluted into a Thermo Orbitrap Exploris 120 (ThermoFisher Scientific, Waltham, MA) with an HESI ion source in negative polarity mode with a scan range of 300-400 *m/z* and a resolution of 120,000. Samples were eluted with the following binary gradient of solvent A (0.1% formic acid in water) and solvent B (100% acetonitrile): 50% B for 9 min, 100% B for 2 min, then 50% B for 2 min. The mass spectrometer was operated with a spray voltage of 2500 V, vaporizing temperature of 350 °C, aux gas 10, sheath gas 50, sweep gas 1, and a collision gas pressure of 1 mTorr.

Quantification of Cannabinoids

Cannabinoids were identified from the fragmentation patterns in comparison with authenticated standards in MZMine3 (Schmid et al., 2023). Peak area was determined through Thermo Xcalibur Quantitation Analysis (ThermoFisher Scientific). The following equation was used for the quantification of compounds based on concentration curves.

$$Compound\,\frac{mg}{g}\,(\%) = \left(\begin{matrix} Concentration \\ of\ compound\left(\frac{\mu g}{mL}\right) \end{matrix}\right)\left(\frac{extraction\ vol.(mL)}{sample\ aliquot(mg)}\right)\left(\begin{matrix} conversion \\ \frac{\mu g\ to\ mg}{mg\ to\ g} \end{matrix}\right) \times 100\%$$

In vitro ACE inhibitory assay – Cushman colorimetric assay

Hemp extracts and six cannabinoids (CBD, CBG, CBC, CBN, CBDA, and CBGA) were examined for ACE inhibitory activity using a modified version of the spectrophotometric method developed by Cushman and Cheung (1971). The rate of hippuric acid (HA) production was measured from hippuryl-L-histidyl-L-leucine (HHL) incubated with angiotensin-converting enzyme from rabbit lung (MilliporeSigma A6778, St. Louis, MO, USA). Each ACE-catalyzed reaction was carried out in a 1.7 mL microcentrifuge tube consisting of 130 µL sodium borate

buffer (100 mM, pH 8.3, 300 mM NaCl), 10 µL ACE (0.1 U/mL), 50 µL HHL (5 mM), and 10 µL sample. The reaction mixtures were incubated in a water bath at 37 °C for 120 min, after which the reactions were quenched with 75 µL of 1 M HCl, inverted gently, and stained with 150 µL of pyridine and 75 µL of benzene sulfonyl chloride (BSC) on ice for 15 min. 200 µL of each sample was then transferred to a 96-well clear, flat bottom plate, and read at 410 nm using a TECAN Infinite 200 PRO microplate reader (Männedorf, Switzerland). The crude R5 extract was tested at a concentration of 0.1 mg/mL and fraction extracts of 0.2 mg/mL in 0.5% DMSO. Cannabinoid standards were reconstituted at various concentrations in ethanol. The final concentration of ethanol in the assay was <5% v/v. The negligible effects of ethanol and 0.5% DMSO on ACE activity are reported in the supplemental material. Each experiment incorporated triplicates of each sample and control including lisinopril (0.5 nM) as a positive control, a blank (no ACE inhibitor), and an empty well (containing no ACE or inhibitor). ACE inhibition was then determined by the following formula.

$$\text{ACE inhibition } (\%) = \frac{(B - A)}{(B - C)} x\ 100\%$$

A = Sample B = Control with no inhibitor C = No inhibitor or ACE

IC_{50} values were obtained using Prism GraphPad Software. Data points exceeding enzyme activity above 100% were normalized for graphical representation. Statistical differences between mean values were determined using a one-way ANOVA (analysis of variance) through GraphPad Prism (https://www.graphpad.com).

Docking study – N-domain and C-domain of ACE

A molecular docking study was performed on the cannabinoids CBG, CBD, CBC, CBN, CBDA, and CBGA; along with Lisinopril and HHL using the N-domain (4CA6) and C-domain (1o86) of somatic ACE. Affinity binding scores were calculated using AutoDock Vina version 1.2.5[27,28] using an AutoDock4Zn protocol for docking with zinc metalloproteins[29]. Each ligand and

both receptors were prepped in accordance with the protocol. PDB files of 4ca6 and 1o86 were sourced from the RCSB Protein data bank[30], while cannabinoid structures were obtained from PubChem[31]. An average binding score was determined after three individual runs. The number of h-bond and the interacting residues are representative of the first position of each trial, additional poses can be found in **Appendix C**.

Results

Cannabinoids of fraction extracts

An LC-MS/MS analysis of the fractions showed differences in cannabinoid content before and after charcoal filtration. The purpose of charcoal filtration was to eliminate background absorbance of chlorophyll and other plant pigments, as samples containing these compounds would often reach max absorbance preventing measurements of HA. Before filtration, the 21 fractions displayed an assortment of targeted cannabinoid profiles, with CBD being the most common and highly concentrated cannabinoid. The flash chromatography gradient used for the generation of fractions resulted in several cannabinoids (CBG, CBN, CBC, and CBGA) eluted between fraction F8 – F13. The fractions with the highest amount of CBC and CBG, were F12 and F9, respectively. Low concentrations of CBN, CBG, and CBC were observed in fractions F17-F19. CBDA was observed across all fractions except F1 and F2. CBGA was found in low concentrations from F8 – F19 (Appendix Figure **C-2**).

After filtration, a significant drop in cannabinoid concentration and diversity was observed. Charcoal filtration caused the complete removal of CBG content in fraction extracts and lowered other cannabinoid concentrations as much as 22% for CBD (Appendix Figure **C-2**). Fractions F2 and F20, which exhibited low concentrations of CBD and CBDA before filtration, were below the level of detection for cannabinoids after filtration. Though yields were significantly decreased,

fractions still contained a diverse profile containing CBD, CBDA, CBGA, CBN, and CBC (Figure **4-2)**.

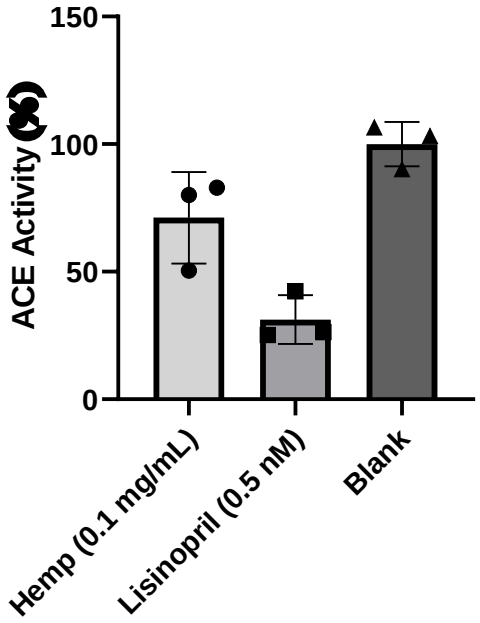


Figure **4-1**: ACE activity (%) of crude CBD dominant hemp extract (0.1 mg/mL) compared to Lisinopril (0.5 nM (positive control)) and blank samples (negative control) containing no ACE inhibitor. Each samples in triplicate; bar indicates mean residual ACE activity ± SEM.

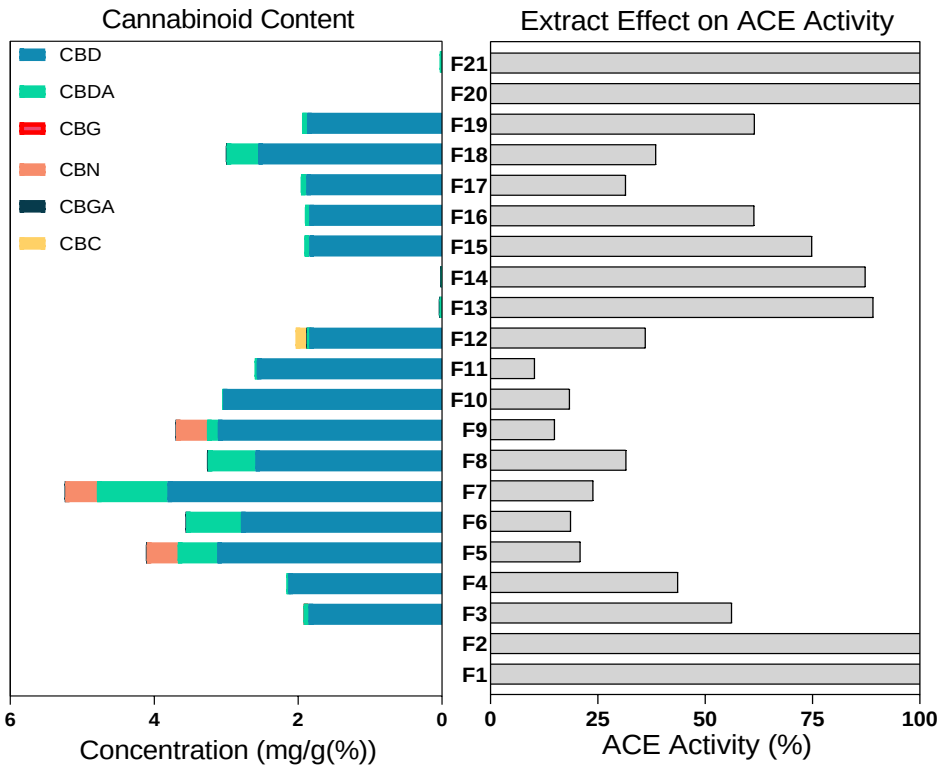


Figure **4-2**: (Left) Concentration (mg/g %) of cannabinoids in hemp fraction extracts after charcoal filtration. (Right) ACE activity (%) of filtered fraction extracts (0.2 mg/mL).

ACE inhibitory activity

Hemp extract and fractions were discovered to interfere with the colorimetric *in vitro* ACE inhibitory assay and were therefore clarified with activated charcoal as described above. The filtered extract was subsequently assayed at a concentration of 2.5 mg/mL and yielded a 30% decrease in ACE activity. Hemp extracts limited ACE activity from 50 – 80% (**Fig.1**). Hemp extract fractions were tested at a final concentration of 0.2 mg/mL (**Fig.2**). Overall, fractions F5 – F12 and F17 – F18, displayed >50% reduction in ACE activity, while F9 and F11 decreased ACE

activity by >85%. Notably, samples with low cannabinoid levels (e.g., F2, F20, and F21) did not affect ACE activity.

Individual hemp cannabinoids displayed significant ACE inhibitory activity; all were tested at a baseline concentration of 10µM. Neutral cannabinoids CBG, CBD, CBC, and CBN displayed higher inhibitory activity (≥75% inhibition of enzyme activity) in comparison to the acidic precursors CBDA and CBGA (50-60% inhibitory activity) (Figure **4-3**). CBN and CBC displayed the highest inhibition of ACE activity out of all the considered phytocannabinoids, while the positive control lisinopril displayed complete inhibition of ACE activity at 10µM.

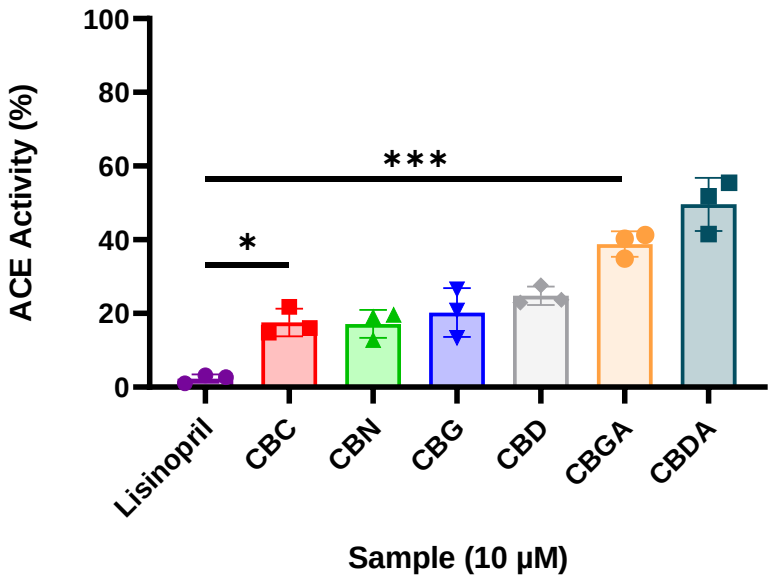
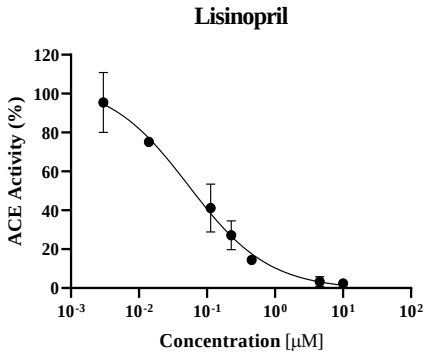
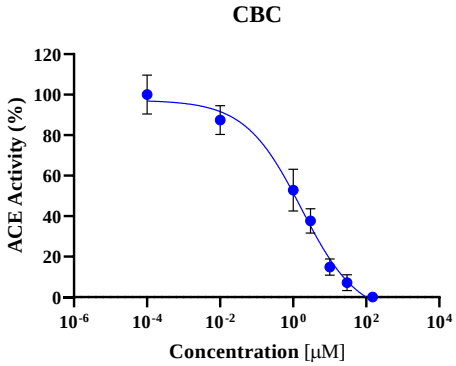


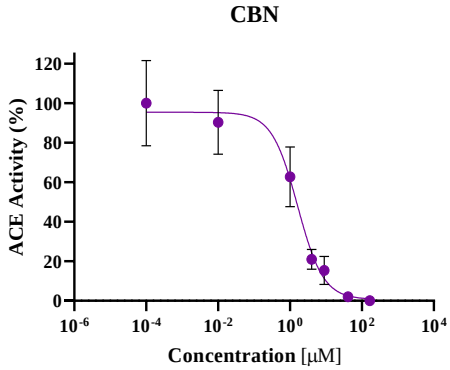
Figure **4-3**: ACE activity (%) of individual hemp cannabinoids compared to known ACE inhibitor, lisinopril. Samples were assayed at 10 µM in triplicate; bar indicates mean residual ACE activity ± SEM. Statistical differences (*) were determined using an analysis of variance (ANOVA) test through GraphPad Prism (www.graphpad.com).

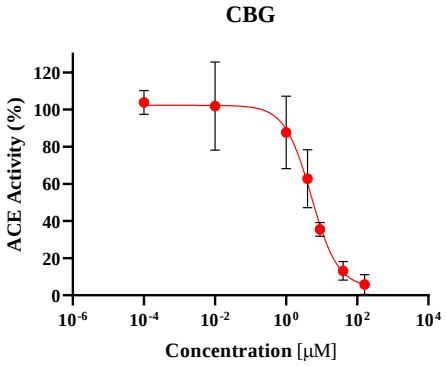
All neutral cannabinoids were further evaluated at a range of concentrations to determine IC_{50} values. At a range from 150 – 0.01µM, CBN and CBC had the lowest IC_{50} of the cannabinoids, with IC_{50}s 1.6 µM and 1.7 µM, respectively (Figure **4-4**). The more prevalent cannabinoids CBG and CBD, yielded IC_{50}s 5.1µM and 22.5µM, respectively. Given the high potency of Lisinopril, it was evaluated at a range from 10 µM – 0.003 µM, providing an IC50 of 0.058 µM.

Compound	*IC50 (µM)*
Lisinopril	0.057
CBC	1.7
CBN	1.6
CBG	5.1
CBD	22.5









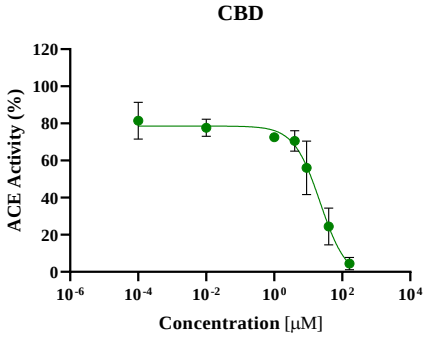


Figure **4-4:** Half-maximal inhibitory concentration (IC50) curves base on ACE activity of known ACE inhibitor (lisinopril) and phytocannabinoids from hemp.

Docking study

Molecular docking calculations predicting the binding affinity for each cannabinoid yielded nine potential conformations, from which a mean binding affinity score of the first conformation was obtained (Table **4-1**). No notable difference in the number of hydrogen bonds or interacting residues were observed between replicates. The binding affinity scores and RMSD l.b and u.b of all conformation for each ligand tested can be found in (**Appendix C**). All six cannabinoids including lisinopril displayed higher affinity for the N-domain than the C-domain. CBC displayed the strongest binding affinity score in both domains, with varying types of interactions including, conventional hydrogen bonds, pi-sigma, pi-pi stacked, alkyl/pi-alkyl, and metal-acceptor interactions (Table **4-1** and **Appendix C)**. The binding affinity score for CBDA was the weakest in both N- and C- domains. In comparison, three of the cannabinoids tested (CBG, CBGA, and CBC) resulted in a higher binding energy than lisinopril with varying numbers of hydrogen bonds. CBD was the only cannabinoid to display an interaction with the zinc cation in the C-domain of ACE. HHL had the highest binding affinity score for both the N and C-domains exhibiting scores of -11.56 and -11.65, respectively.

Table **4-1**: *In silico* average binding energy (kcal/mol) and number of predicted hydrogen bonds in the N- and C-domain of ACE for cannabinoids (CBD, CBG, CBN, CBC, CBDA, and CBGA), lisinopril, and hippuryl-His-Leu (HHL).

	N-Domain (4ca6)		*C-Domain (1o86)*	
Ligand	**Binding Energy (kcal/mol)**	**No. of H-bond interactions**	**Binding Energy (kcal/mol)**	**No. of H-bond interactions**
HHL	-11.56	7	-11.65	5
Lisinopril	-7.833	10	-7.642	4
CBD	-7.607	3	-6.518	1
CBG	-7.971	3	-7.656	4
CBN	-7.831	1	-7.115	1
CBC	-9.220	3	-8.631	3
CBDA	-7.021	3	-5.702	3
CBGA	-8.933	4	-7.764	7

Discussion

This study demonstrates the ACE inhibitory activity of cannabinoids under in vitro conditions. Predictions of receptor-ligand interactions were also presented to characterize the binding affinity that may be taking place between the select cannabinoids and ACE. When evaluated at 10μM, six cannabinoids found in cannabis sativa L. displayed inhibition of ACE activity with CBC and CBN lowering ACE activity to less than 15%. Likewise, in silico docking scores revealed binding affinities and interacting residues that are of similar value to the known ACE inhibitor, lisinopril. In this two-domain enzymatic structure, all compounds tested exhibit a preference to the N-Domian of ACE, with the exception for the synthetic substrate hippuryl-L-histidyl-L-leucine (HHL).

Additionally, hemp extracts containing various concentrations of the identified cannabinoids were also evaluated for their ACE inhibiting activity The results of this

studydemonstrated the ACE inhibitory effects a common hemp extract possesses. Though testing the hemp extracts proved to be successful, testing the authentic chemical profile of the plant extract proved to be difficult when using a colorimetric ACE assay. The implementation of active charcoal filtration proved useful in eliminating background absorbance from the extract color; however, major yield loss of compound concentrations was observed across all extracts tested. Considering this outcome, it is recommended that future investigations of hemp extracts on ACE activity should pursue measurements of HA at a wavelength 254 nm if using HHL as a substrate, a challenge that was faced in the study due to instrument limitation. Several other in vitro ACE assays can also be employed such as the Holmquist, Carmel and Yaron, Lam, and Elbl and Wagnar methods when exploring ACE inhibition of plant extracts: however, the safety and price of the materials involved in some of the methods should be considered along with instrumentation requirements[32,33,33,35,36].

This study also executed a targeted metabolite analysis of the hemp extracts evaluated, limiting the complete chemical profile to the cannabinoids of interest. A solvent extraction, using ethanol, is an excellent technique that provides a full spectrum extract containing a variety of metabolites that reside within a plant sample. The fraction extracts tested may have additional ACE inhibiting metabolites such as flavonoids and peptides which may have contributed to the effects observed, however these compounds were not identified in this study. Future work is recommended to employ an untargeted approach in the characterization of the chemical profile to better predict compounds that possess ACE inhibiting effects. Likewise, the method used for flash chromatography may prove to be more beneficial with the addition of increased column volumes per gradient elution, providing more time for cannabinoids to separate, as complete separation was not observed in this study.

Furthermore, when considering hemp products, one must consider the type of medium that is used for consumption. Many forms of hemp products have become widely

available such as hemp oils, edibles, vapor, smoke, and topicals. Although these media may contain concentrations of the cannabinoids addressed in this study, the metabolism of cannabinoids are distinct to the type of medium, limiting the possibility of cannabinoid and ACE interactions. For instance, ACE can be found in large concentrations within the pulmonary arteries of the lung, rendering smoke and vapor a probable medium that may ensue an ACE and cannabinoid interaction[37]. Moreover, the cannabinoids are less than 5% bioavailable when administered orally. Nonetheless, these results provide insight on the interactions between ACE and cannabinoids, a number of different interactions have been identified postulating potential pleiotropic effects of cannabinoids in cannabis sativa L.[38,39,40,41]. Similarly, the exploration of the 'entourage effect', where multiple compounds act to produce biological activity, may also be at play when testing complex extracts such as solvent extracts.

Conclusion

In total, this study suggests a possible mechanism for which cannabinoids consumption may influence blood pressure. This evidence is limited and provides the basis for how these compounds interact with ACE under fixed conditions. Cell and animal studies are required to verify these results as it provides a more complex environment with other molecular constituents that cannabinoids have shown preference to such as the ECS [20, 42]. Likewise, both the docking and in vitro study only reflect the interactions between a substitute for angiotensin I, providing no evidence for the prevention of the inactivation of the vasodilator peptide bradykinin, an additional function of current ACE inhibitors[43]. The cannabinoids preference for the N-domain of the ACE of is also something to consider when evaluating their overall effectiveness against ACE activity.

Finally, this study highlights the significance of minor cannabinoids. CBC and CBN displayed considerable ACE inhibitory characteristics in this study, insinuating their possible

contributions to other effects. Hinting at their importance when studying the effects cannabis products have in therapeutic research. Minor cannabinoid concentrations is also something to take into consideration as different chemovars or ‘strains’ are becoming more chemically diverse each year.

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Chapter 5

Conclusions and Future Directions

This dissertation focused on adding to the body of information that surrounds hemp's cannabinoid and terpenoid profile in terms of processing and biological activity. The investigations that make up this dissertation cover three main principal areas that specify the makings and use of a hemp product. Providing empirical evidence for questions pertaining to the growth, extraction, and biological activity of hemp (Figure **5-1**).

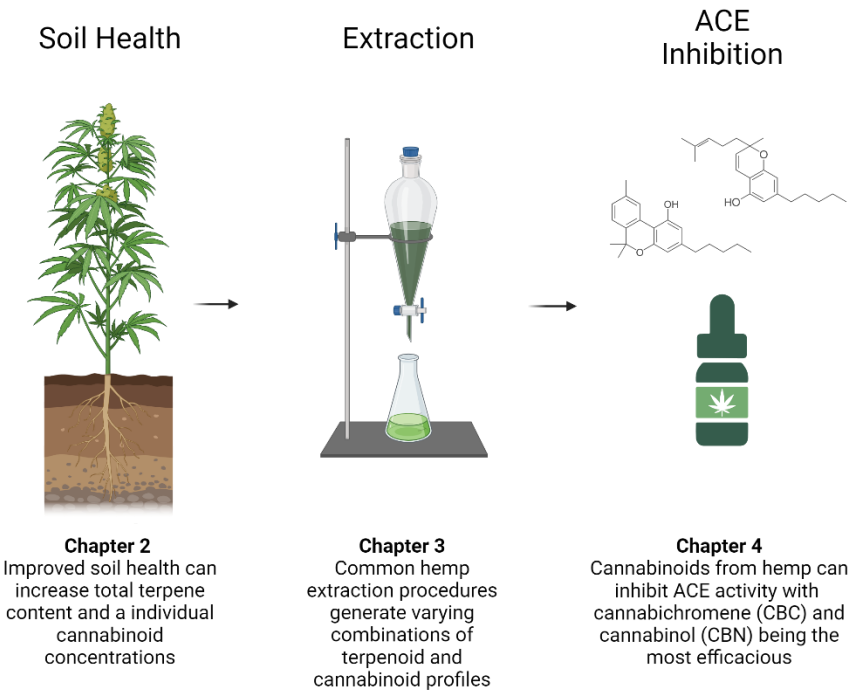

Figure **5-1**: Summary schematic describing the main conclusions of the research articles covered in chapters 2 – 4.

Chapter one is a review paper, published in Biomedicines, that provides an overview of the class of aromatic compounds that is limited in the literature, secondary terpenes in hemp. We discussed the biosynthesis of terpenes while highlighting the diversity and abundance of terpenes

that can be found across the many chemotypes of the cannabis plant. Moreover, we illustrate the biological activity of these minor terpenes and discuss the possibility of synergy between cannabis compounds. This article serves as a resource for researchers intrigued by the chemistry of cannabis to gain an overview of the chemical capacity of the cannabis plant. Hemp's ability to synthesize a multitude of terpenoid and cannabinoid flavors provides researchers and commercial manufacturers with a unique opportunity to capture various chemical profiles that could potentially target specific therapeutic applications. Several cannabinoids and terpenoids that have yet to be fully characterized, some of which may be responsible for the effects of hemp products, warranting future investigations that explore the unknown bioactive compounds in hemp.

As a medicinal plant, there are several cultivation practices that growers have implanted, with new practices being applied each year. Given the recent trend towards sustainable agriculture, little information is known about the effect regenerative farming such as the use of cover crops has on the therapeutic compounds produced by hemp. Chapter 2, we compare the cannabinoid and terpenoid profiles of two hemp cultivars grown in a traditional field consisting of tilled soil and a regenerative field that utilized cover crops in the off-season with soil that was left untilled. We found significant differences in individual cannabinoid content and total terpene concentrations between the fields, however; the type of cultivar showed to play a vital role in how well the plant performed in terms of cannabinoid and terpenoid yield. With these findings, we suggest that soil health can increase the total terpenoid content and individual cannabinoids of select hemp cultivars. However, future studies are required to determine the driving force in the changes observed for each cultivar type to surmise the effects of cover crop application on hemp cannabinoid and terpenoid content.

As a reference point for future studies, differences in soil biological characteristics were observed between the conventional field and the cover crop field, indicating a leading cause of the difference in total terpenoid concentration and individual cannabinoid content observed. The

current literature surrounding how biological soil characteristics impact cannabinoid and terpenoid production in *Cannabis sativa* L. includes studies covering nitrogen (N) supply and comparison studies between natural and artificial soil. An excess of N supply has shown a decrease in cannabinoid and terpenoid production while low N levels show an increase in terpenoid production[1]. While researchers comparing the hemp grown in outdoor soil and indoor synthetic soil, revealed significant variation in terpenes and cannabinoids profiles for the outdoor samples in comparison to indoor[2].

Chapter 2 adds to the body of evidence that biological soil characteristics play a role in terpenoid and cannabinoid production in hemp, which can be altered when using cover crops. To advance this area of research, it is recommended to conduct additional growth studies with the addition of exploring the microbial communities that make up each field type. An essential property of soil health is the composition of the soil's microbiome. Articles relevant to this topic emphasize the need for studies exploring the association between soil microbiota and its potential impact on terpenoid and cannabinoid production[3,4]. If executed, this work could be of immense value for researchers and commercial vendors as it may lead to enhanced organic production of terpenoids and cannabinoids in hemp.

After the cultivation of hemp, the next critical stage for many hemp products is the extraction phase. Chapter 3 investigated the effects hemp extraction procedures have on cannabinoid and terpene content. To achieve this, we used a single hemp cultivar and applied three common extraction techniques hydrodistillation, supercritical CO_2, and solvent-based extraction (ethanol). The resulting extracts were then evaluated for their cannabinoid and terpenoid content using methods of mass spectrometry (MS) including LC-MS/MS and GC-MS, respectively. These resulting profiles provided distinct cannabinoid and terpenoid profiles specific to the extraction procedure. Demonstrating the variety of chemical profiles a single hemp cultivar can generate while providing researchers and commercial vendors protocols that allow

them to be more selective in the chemical profile their hemp extracts possess. For example, if a diversity and high abundance of terpenes are desired one might consider the use of hydrodistillation. If extracts containing high concentrations of cannabinoids with moderate concentrations of terpenoids are desired, one will benefit from the use of supercritical CO_2. The use of ethanol extraction will yield small quantities of terpenes and moderate levels of cannabinoids.

Although successful at providing information on the contents of terpenoids and cannabinoids, each extraction procedure may yield several other compounds such as pigments or other aromatic compounds like flavonoids[5,6]. Underscoring a limitation of this study as several other compound groups and other cannabinoids and terpenoids that have not been fully characterized, were not identified during the MS analysis. This provides an opportunity for future work to include the use of an untargeted MS approach to identify all of the compounds that make up each extract.

Additionally, each extraction procedure performed in this study was subject to specific conditions that can be refined for further selectivity. For example, several factors such as extraction time, pH level, temperature, drying conditions, and material to solvent ratio, can influence the chemical composition of an extract[7]. Future studies may consider exploring one extraction technique with conditions to refine the chemical profiles for better cannabinoid or terpenoid yields. For example, when performing ethanol extraction differences in total terpene content, individual cannabinoid concentrations, and overall yield have been reported when using different temperatures of cold ethanol including - 20 ˚C, - 40 ˚C, and room temperature[8]. A similar study can be performed using hydrodistillation by adjusting the temperature of the heat source, as well as extraction time to determine differences in both cannabinoid and terpenoid levels.

Finally, in Chapter 4, we provide evidence of a potential hemp cannabinoid mechanism of action that relaxes veins, reducing blood pressure. This work adds to the current information surrounding the effects of hemp cannabinoids on the human body. Recent studies have reported that the cannabinoids cannabidiol (CBD) and cannabigerol (CBG) can reduce overall blood pressure in humans and mice, respectively[9,10]. However, the mechanism of action that is taking place for these effects to occur has yet to be described. Angiotensin converting enzyme (ACE) is a catalytic enzyme that forms vasoconstrictor peptide angiotensin II, as a result, ACE has become a common target for high blood pressure therapies leading to several discoveries of both synthetic and natural inhibitors[11]. The work in chapter 4, provides *in vitro* evidence of direct inhibition of ACE activity by the cannabinoids CBD, CBC, CBN, CBG, CBGA, and CBDA. All the select cannabinoids were tested against ACE activity at 10 µM using an *in vitro* enzyme activity assay. Each of the neutral cannabinoids were capable of limiting ACE activity to 20%. When testing each neutral cannabinoid for potency, it was revealed that cannabichromene (CBC) and cannabinol (CBN) had the lowest half-maximal inhibition concentration (IC50) of 1.7 and 1.6, respectively. These results suggest that neutral cannabinoids from hemp can inhibit ACE activity, thus presenting a mechanism of action of cannabinoids that leads to changes in blood pressure. Providing users with more information on the effects of hemp products containing these compounds if concerned with states of reduced blood pressure. However, more studies are needed to confirm the inferences implied including an exploration of inhibition characteristics and an examination under more complex conditions such as human cells.

Determining the inhibition characteristics would be of tremendous value as it provides more details as to how cannabinoids interact with ACE to lower its activity. To accomplish this, the enzymatic assay used in this study can be repeated using a minimum of seven concentrations of the substrate, HHL (e.g. 0 – 5 mM). Rather than determining ACE activity, total hippuric acid (the product of the reaction) will be determined after establishing a hippuric acid standard curve.

It is advised that lower concentrations of the cannabinoid be tested rather than higher levels as this will result in indistinguishable absorbances of all substrate concentrations. It is suggested that the cannabinoid concentration be tested at half of the IC50 concentration determined in Chapter 4, as it is low enough to reach the maximal rate of the reaction at the highest concentration of substrate. A blank containing no inhibitor must also be tested for comparison. Both the ACE and cannabinoid concentrations would remain the same for each concentration of substrate applied. The outcome of this study will provide more insight into the type of inhibition that is occurring based on V_{max} and K_m values. Based on the type of inhibition we can speculate that cannabinoids are binding to allosteric or orthosteric sites to achieve inhibition. Lastly, to confirm the working of the assay it urged that a known competitive ACE inhibitor such as lisinopril, be tested to confirm that the experiment was properly executed as this compound should result in as competitive inhibition type.

Additionally, the results that were reported in Chapter 4 were evaluated under fixed conditions requiring a more complex system such as mammalian cells to help corroborate the pronounced findings. It is suggested that a human cell line be used such as HK-2 (human kidney cells) which are reported to contain ACE[12]. It is advised to utilize a continuous cell line rather than primary cells as cultivation requires less upkeep and can result in more replicable experiments. To investigate the ACE inhibiting activity of cannabinoids in human cells it is suggested that an ACE activity kit be used to determine inhibition. This would require the treatment of cells using bovine serum albumin (BSA) at a concentration of 5mg/ml as research has shown that BSA treatment leads to overexpression of ACE protein[12]. After treatment, the ACE assay kit protocol details the remaining steps to allow for testing of ACE activity using fluorescence spectroscopy, including when to add the potential ACE inhibitor (i.e. CBD, CBG, CBC, or CBN). The concentration of cannabinoids to use for this assay must be between 0.01 to 5 µM for 24 h as concentrations above 5 µM have been shown to exhibit signs of cytotoxicity[13]. If

ACE inhibition is observed, testing at least seven concentrations of cannabinoid would allow for an identification of an IC50, which would be of value to many cannabinoid researchers. If no ACE inhibition is observed, we can rule out the possibility of cannabinoids from hemp possessing ACE inhibition activity.

In conclusion, this dissertation contributes to the growing body of information surrounding hemp's cultivation, extraction, and bioactivity. The inferences of each chapter provide novel perspectives that are valuable to growers, manufacturers, and consumers. For growers, increased soil biological characteristics can enhance cannabinoid concentrations and total terpene content, making it imperative to maintain the health of your soil. For manufacturers, extraction techniques are a powerful tool that can capture a specific cannabinoid and terpenoid profile of any given hemp cultivar. Finally, for consumers, the cannabinoids in hemp can inhibit ACE activity, meaning the consumption of hemp products containing these compounds can lead to a state of reduced blood pressure which may not be desirable for those suffering from metabolic disorders. Considering these statements, future studies are recommended to validate the inferences made in this dissertation. However, each chapter provides evidence inching us closer to the full understanding of hemp's chemical profile and the creation of tools necessary to harness its full therapeutic potential and safety.

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Appendix A: Supporting Information for Chapter 2

Table **A-1:** Tangerine cannabinoid concentrations (mg/mL) from hemp inflorescence grown in a conventional field (tilled soiled) and a cover crop field that applied cover crops in the off season with no tilled soil. Data means ± SD of n=5. Statistical significance was determined by a two-way ANOVA (***P < 0.001; **P < 0.01; *P < 0.05).

Cannabinoid	Cover Crop Field	Conventional Field	P-value	Summary
CBC	3.8±1.7	1.9±0.6	>0.9999	
CBD	39.0±11.2	71.3±14.4	<0.0001	***
CBDV	0	0.04±0.04	>0.9999	
CBDA	57.5±8.0	10.2±11.9	<0.0001	***
CBG	13.5±5.7	2.5±.8	0.0318	*
CBGA	10.8±5.6	0.03±.006	0.0350	*
CBL	0.1±0.02	0.05±0.04	>0.9999	
CBN	0.2±0.06	1.8±0.9	>0.9999	
THCV	0.2±0.04	0.4±0.04	>0.9999	
THCA	0.4±0.08	0.1±0.1	>0.9999	

Δ8-THC	0.1±0.1	5.7±3.6	0.7728	
Δ9-THC	3.3±1.0	29.6±11.6	<0.0001	***

Table **A-2:** Stem Cell cannabinoid concentrations (mg/mL) from hemp inflorescence grown in a conventional field (tilled soiled) and a cover crop field that applied cover crops in the off season with no tilled soil. Data means ± SD of n=5. Statistical significance was determined by a two-way ANOVA (***P < 0.001; **P < 0.01; *P < 0.05).

Cannabinoid	Cover Crop Field	Conventional Field	P-value	Summary
CBC	7.7±1.9	4.2±1.2	>0.9999	
CBD	39.8±26.8	19.1±12.6	<0.0001	*
CBDV	1.7±0.2	0.6±0.4	>0.9999	
CBDA	26.2±24.9	58±16.2	<0.0001	***
CBG	53.2±25	14.3±8	0.0318	***
CBGA	0.7±0.2	10.5±5.4	0.0350	
CBL	0.2±0.1	0.2±0.1	>0.9999	
CBN	0.4±0.3	0.1±0.1	>0.9999	
THCV	0.3±0.1	0.2±0.1	>0.9999	
THCA	0.5±0.06	0.7±0.3	>0.9999	

Δ8-THC	0.1±0.1	0	0.7728
Δ9-THC	3.5±1.1	3.4±1.1	<0.0001

Table **A-3:** Stem Cell terpenoid concentrations (mg/mL) from hemp inflorescence grown in a conventional field (tilled soiled) and a cover crop field that applied cover crops in the off season with no tilled soil. Data means ± SD of n=5. Statistical significance was determined by a two-way ANOVA (***P < 0.001; **P < 0.01; *P < 0.05).

Terpene	Cover Crop Field	Conventional Field	P-Value	Summary
(+)-Cedrol	0.124±0.019	0.172±0.097	>0.9999	
(-)-Caryophyllene Oxide	0.112±0.2	00.07±0.048	>0.9999	
3-Carene	0.004±0.008	0.002±0.004	>0.9999	
Borneol	0.002±0.004	0.004±0.005	>0.9999	
Camphene	0.008±0.004	0.004±0.005	>0.9999	
Endo-Fenchyl Alcohol	0.084±0.035	0.156±0.089	>0.9999	
Eucalyptol	0.006±0.005	0.006±0.005	>0.9999	
Fenchone	0.01±0.0	0.022±0.015	>0.9999	

Geranyl Acetate	0.008±0.004	0.024±0.009	>0.9999	
Guaiol	0.758±0.128	0.758±0.366	0.9174	
Linalool	0.084±0.028	0.166±0.107	>0.9999	
R(+)-Limonene	0.102±0.023	0.284±0.125	>0.9999	
Terpinolene	0.164±0.075	0.29±0.236	>0.9999	
Valencene	0.684±0.157	1.288±0.521	0.6620	
α-Bisabolol	2.082±0.476	2.778±1.373	0.0001	
α-Cedrene	0.066±0.015	0.166±0.063	>0.9999	
α-Farnesene	1.324±0.433	4.118±2.298	0.1348	***
α-Humulene	1.132±0.196	2.064±0.826	0.1189	
α-Phellandrene	0.014±0.019	0.012±0.004	>0.9999	
α-Pinene	0.01±0.007	0.018±0.013	>0.9999	
α-Terpinene	0.006±0.005	0.006±0.009	>0.9999	
α-Terpineol	0.134±0.046	0.18±0.083	>0.9999	
β-Farnesene	5.438±1.762	11.09±5.276	<0.0001	***
β-Myrcene	0.228±0.069	0.6±0.351	>0.9999	
β-Pinene	0.032±0.024	0.082±0.055	>0.9999	
Cis-Nerolidol	0.018±0.011	0.006±0.008	>0.9999	
Cis-Ocimene	0.026±0.011	0.056±0.047	>0.9999	

Γ-Terpinene	0.008±0.004	0.008±0.008	>0.9999	
Trans-Caryophyllene	4.526±0.818	8.666±3.105	<0.0001	***
Trans-Nerolidol	0.118±0.033	0.19±0.095	>0.9999	
Trans-Ocimene	0.044±0.064	0.002±0.004	>0.9999	

Table **A-4:** Tangerine terpenoid concentrations (mg/mL) from hemp inflorescence grown in a conventional field (tilled soiled) and a cover crop field that applied cover crops in the off season with no tilled soil. Data means ± SD of n=5. Statistical significance was determined by a two-way ANOVA (***P < 0.001; **P < 0.01; *P < 0.05).

Terpene	Cover Crop Field	Conventional Field	P-Value	Summary
(+)-Cedrol	0.266±0.144	0.15±0.052	>0.9999	
(-)-Caryophyllene Oxide	0.156±0.083	0.028±0.013	>0.9999	
Borneol	0.004±0.005	0	>0.9999	
Camphene	0.006±0.005	0	>0.9999	
Endo-Fenchyl Alcohol	0.14±0.136	0.016±0.005	>0.9999	
Eucalyptol	0.02±0.02	0	>0.9999	

Fenchone	0.016±0.011	0	>0.9999	
Geranyl Acetate	0.018±0.013	0.01±0.0	>0.9999	
Guaiol	1.558±1.027	0.934±0.355	>0.9999	
Linalool	0.046±0.021	0.012±0.004	>0.9999	
R(+)-Limonene	0.214±0.286	0.016±0.005	>0.9999	
Terpinolene	0.008±0.004	0.002±0.004	>0.9999	
Valencene	0.85±0.559	0.114±0.057	>0.9999	
α-Bisabolol	3.424±1.269	1.87±0.697	0.9993	***
α-Cedrene	0.226±0.189	0.026±0.011	>0.9999	
α-Farnesene	1.108±1.017	0.14±0.119	<0.0001	
α-Humulene	1.19±0.711	0.208±0.092	0.9236	
α-Phellandrene	0.02±0.044	0.024±0.009	>0.9999	
α-Pinene	0.01±0.017	0	>0.9999	
α-Terpineol	0.244±0.173	0.064±0.044	>0.9999	
β-Farnesene	5.436±1.692	1.67±0.957	<0.0001	***
β-Myrcene	0.306±0.379	0.018±0.008	>0.9999	
β-Pinene	0.08±0.115	0	>0.9999	
Cis-Nerolidol	0.04±0.007	0.03±0.007	>0.9999	
Cis-Ocimene	0.012±0.008	0	>0.9999	

γ-Terpinene	0.002±0.004	0	>0.9999	
Trans-Caryophyllene	4.776±3.581	0.734±0.328	<0.0001	***
Trans-Nerolidol	0.156±0.077	0.049±0.008	>0.9999	

JUNE	**TEMPERATURE**	**HUMIDITY**	**PRESSURE**
HIGH	95 °F (Jun 2, 1:53 pm)	93% (Jun 23, 3:59 pm)	30.21 "Hg (Jun 23, 3:59 pm)
LOW	50 °F (Jun 8, 5:53 am)	19% (Jun 4, 3:53 pm)	29.49 "Hg (Jun 14, 1:53 pm)
AVERAGE	70 °F	65%	29.85 "Hg

JULY	TEMPERATURE	HUMIDITY	PRESSURE
HIGH	96 °F (Jul 28, 4:53 pm)	94% (Jul 26, 7:05 am)	30.15 "Hg (Jul 26, 7:05 am)
LOW	62 °F (Jul 23, 4:53 am)	36% (Jul 31, 2:53 pm)	29.70 "Hg (Jul 29, 4:53 pm)
AVERAGE	78 °F	72%	29.92 "Hg

AUGUST	TEMPERATURE	HUMIDITY	PRESSURE
HIGH	91 °F (Aug 12, 1:53 pm)	94% (Aug 17, 4:18 am)	30.24 "Hg (Aug 17, 4:18 am)

LOW	57 °F (Aug 20, 6:53 am)	30% (Aug 31, 3:53 pm)	29.60 "Hg (Aug 10, 4:53 pm)
AVERAGE	75 °F	68%	29.92 "Hg

SEPTEMBER	TEMPERATURE	HUMIDITY	PRESSURE
HIGH	98 °F (Sep 4, 3:53 pm)	94% (Sep 12, 3:53 am)	30.36 "Hg (Sep 12, 3:53 am)
LOW	50 °F (Sep 17, 5:53 am)	31% (Sep 1, 4:53 pm)	29.73 "Hg (Sep 7, 3:53 pm)
AVERAGE	68 °F	74%	30.05 "Hg

OCTOBER	TEMPERATURE	HUMIDITY	PRESSURE
HIGH	83 °F (Oct 4, 3:53 pm)	97% (Oct 3, 7:53 am)	30.40 "Hg (Oct 3, 7:53 am)
LOW	36 °F (Oct 24, 6:53 am)	31% (Oct 24, 2:53 pm)	29.37 "Hg (Oct 21, 3:53 am)
AVERAGE	58 °F	73%	29.96 "Hg

Figure **A-1:** Weather data from Holtwood, PA (2.3 miles from fields) weather station provided by CustomeWeather (through Timeanddate;) descriptions of temperatures, humidity, and pressure for the months of June – October 2023.

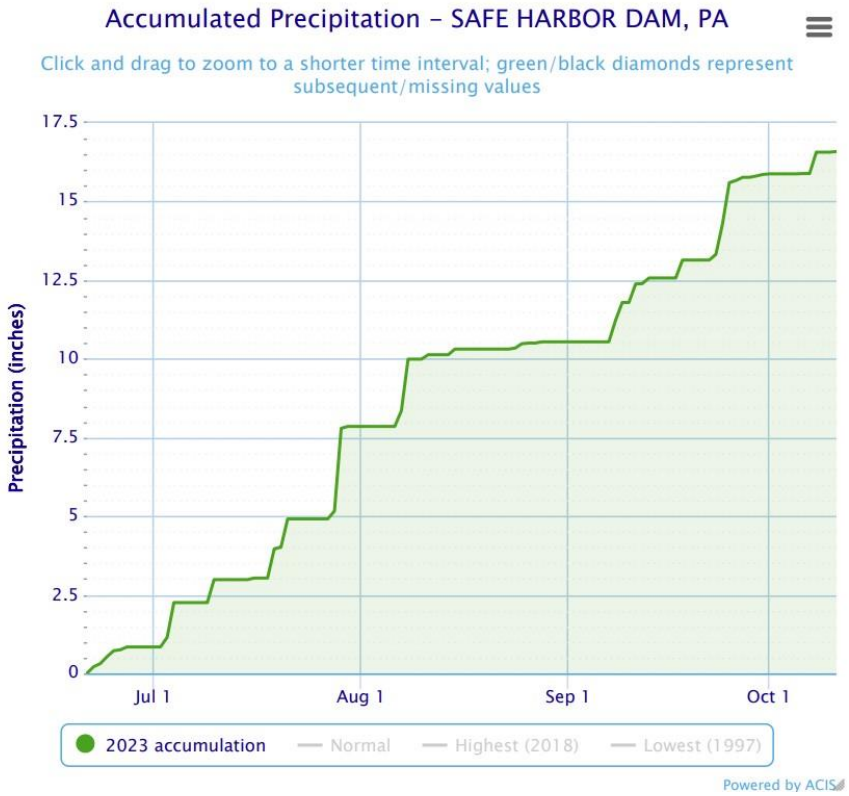

Figure **A-2:** Accumulated precipitation of the nearest weather station with precipitation data between the dates of Jun 21 – Oct 11 2023. Safe Harbor Dam, PA (9 miles from field).

Appendix B: Supporting Information for Chapter 3

Table **B-1**. Mean concentration of identified terpenes ± SD (mg/g) in sample extract. Limit of quantification (LOQ) and not detected (ND).

Terpene (mg/g)	F1			F2			F3		
	Distilled	Ethanol	CO_2	Distilled	Ethanol	CO_2	Distilled	Ethanol	CO_2
β-Farnesene	1.878 ± 0.014	ND	0.313 ± 0.000	0.933 ± 0.002	ND	0.131 ± 0.002	1.220	ND	0.409 ± 0.000
Valencene	1.995 ± 0.011	ND	0.048 ± 0.000	0.798 ± 0.002	ND	0.097 ± 0.001	1.423	ND	0.210 ± 0.001
(1R)-endo(+)-Fenchyl alcohol	6.600 ± 0.069	0.100 ± 0.002	0.475 ± 0.001	4.095 ± 0.001	0.431 ± 0.003	0.185 ± 0.003	3.205	0.261 ± 0.002	0.886 ± 0.001
Humulene	8.580 ± 0.056	ND	1.757 ± 0.001	5.447 ± 0.007	ND	1.751 ± 0.005	6.759	ND	2.158 ± 0.003
Guaiol	9.472 ± 0.046	0.117 ± 0.001	0.044 ± 0.001	6.753 ± 0.014	0.146 ± 0.001	0.278 ± 0.001	12.458	0.212 ± 0.000	0.971 ± 0.004
β-Caryophyllene	42.717 ± 0.323	ND	4.020 ± 0.008	25.138 ± 0.052	ND	2.673 ± 0.045	31.569	ND	5.664 ± 0.020
Caryophyllene oxide	25.973 ± 0.010	ND	ND	15.089 ± 0.013	ND	1.750 ± 0.010	31.380	ND	2.047 ± 0.002
Linalool	1.738 ± 0.016	ND	<LOQ	0.831 ± 0.000	ND	0.012 ± 0.000	0.581	ND	0.064 ± 0.000
α-Terpineol	3.793 ± 0.027	ND	<LOQ	1.713 ± 0.000	ND	0.393 ± 0.000	1.713	ND	0.245 ± 0.001
Fenchone	0.124 ± 0.002	ND	ND	<LOQ	ND	ND	<LOQ	ND	ND
Geranyl Acetate	2.587 ± 0.009	0.732 ± 0.014	ND	0.359 ± 0.001	1.064 ± 0.005	ND	1.119	0.844 ± 0.011	ND
Limonene	4.929 ± 0.036	ND	ND	3.064 ± 0.006	ND	ND	2.315	ND	ND
Borneol	1.358 ± 0.011	ND	ND	0.612 ± 0.000	ND	ND	0.582	ND	<LOQ

Table **B-2**. Mean concentration of identified cannabinoids ± SD (mg/g) in sample extract. Not detected (ND).

Cannabinoids (mg/g)	**F1**			**F2**			**F3**		
	Distilled	**Ethanol**	**CO_2**	**Distilled**	**Ethanol**	**CO_2**	**Distilled**	**Ethanol**	**CO_2**
CBD	0.95	9.32 ± 0.00	12.78 ± 0.01	1.41	9.24 ± 0.02	20.55 ± 0.07	1.74	13.57 ± 0.01	16.07 ± 0.05
CBDA	ND	3.23 ± 0.00	0.03 ± 0.00	ND	4.05 ± 0.01	0.06 ± 0.00	ND	4.13 ± 0.07	0.44 ± 0.01
CBG	ND	ND	16.89 ± 0.08	ND	ND	16.38 ± 0.05	ND	1.38 ± 0.01	9.54 ± 0.00
CBGA	ND	0.10 ± 0.00	0.01 ± 0.00	ND	0.11 ± 0.00	0.01 ± 0.00	ND	0.17 ± 0.00	0.01 ± 0.00
CBN	ND	0.49 ± 0.00	0.54 ± 0.00	ND	0.49 ± 0.00	0.63 ± 0.00	ND	0.53 ± 0.00	0.47 ± 0.00
CBC	0.01	0.17 ± 0.00	0.42 ± 0.00	0.02	0.17 ± 0.00	0.46 ± 0.00	0.02	0.20 ± 0.00	0.26 ± 0.00

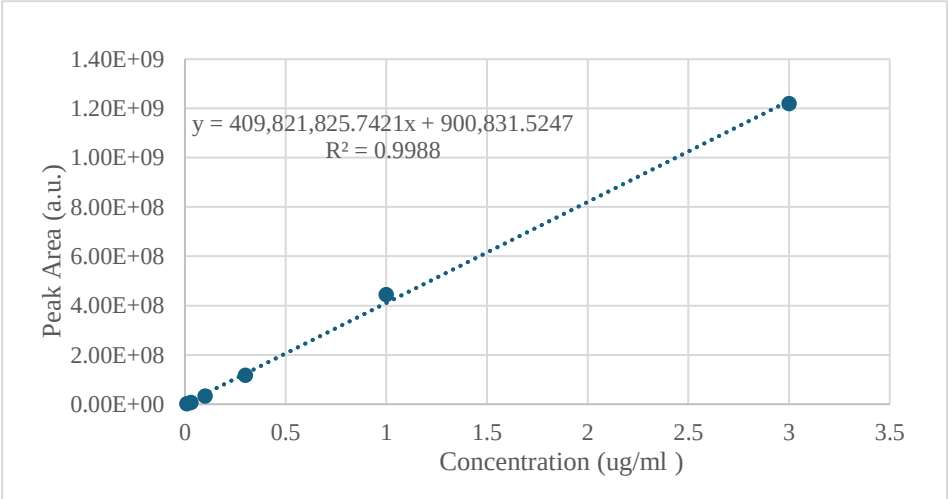

Figure **B-1.** Cannabidiolic acid (CBDA) calibration curve at a range of 30 – 0.003 µg/mL.

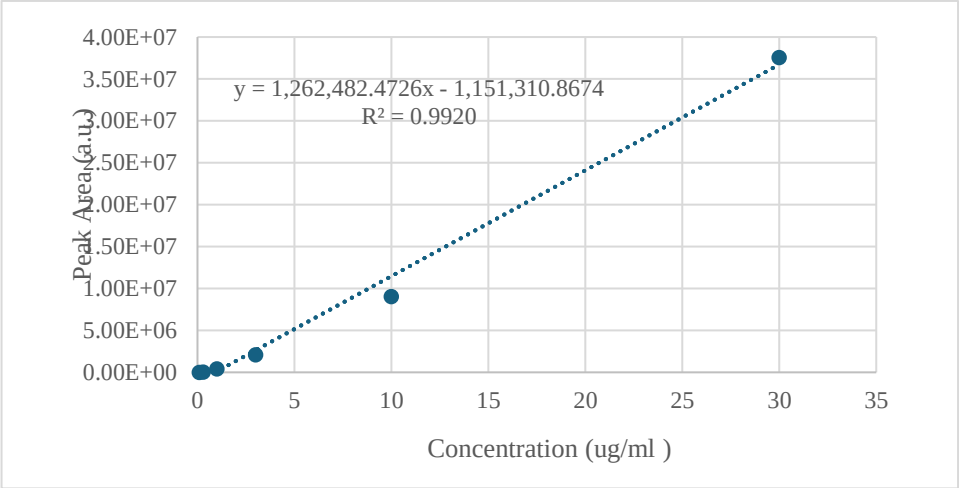

Figure **B-2.** Cannabidiol (CBD) calibration curve.

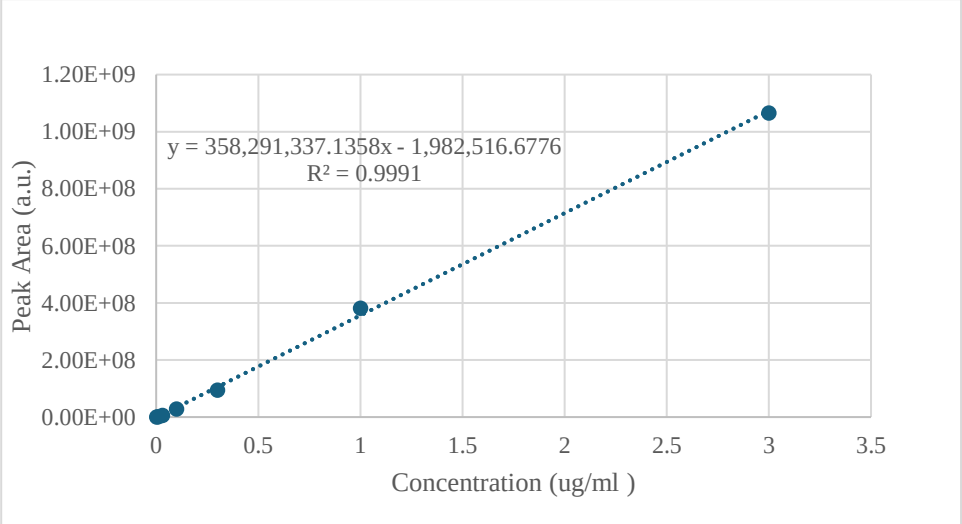

Figure **B-3.** Cannabigerolic acid (CBGA) calibration curve.

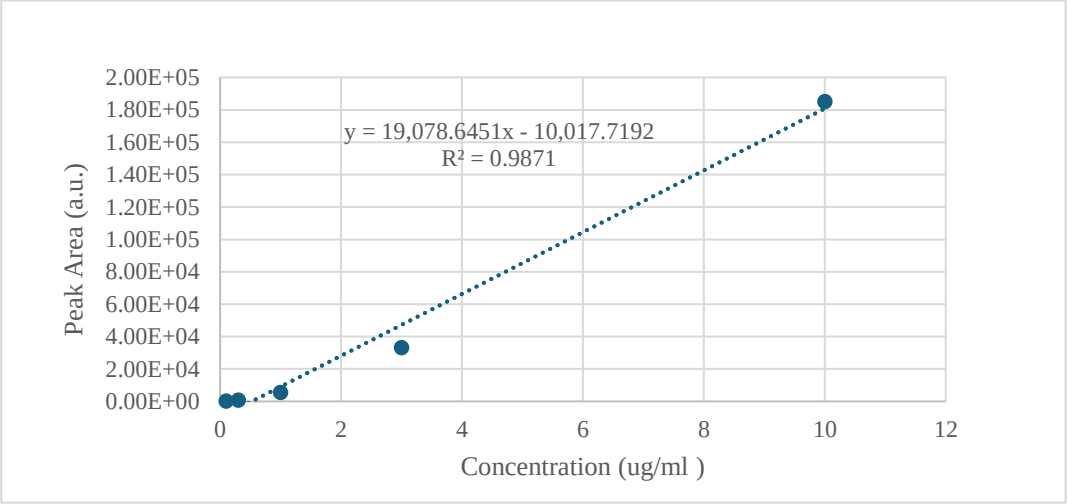

Figure **B-4.** Cannabigerol (CBG) calibration curve.

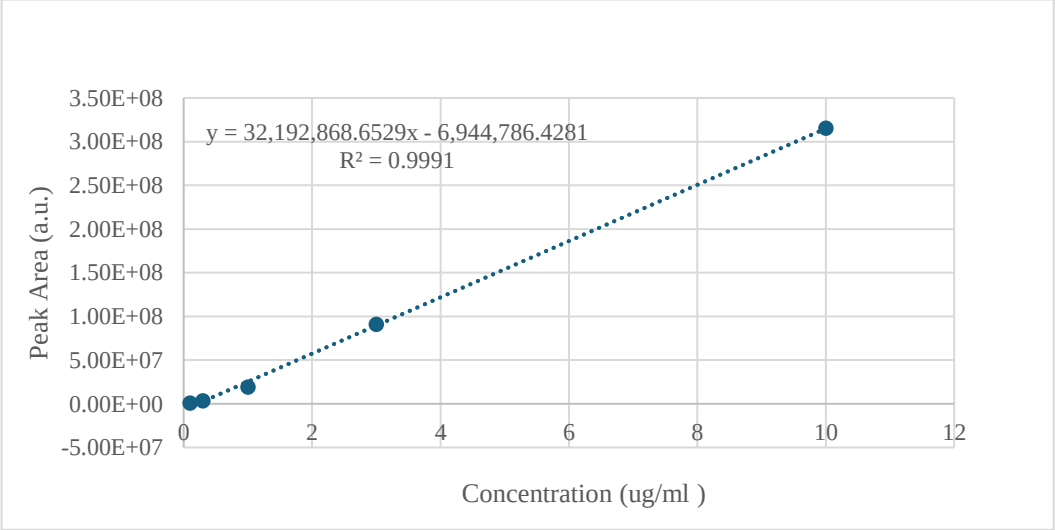

Figure **B-5.** Cannabinol (CBN) calibration curve.

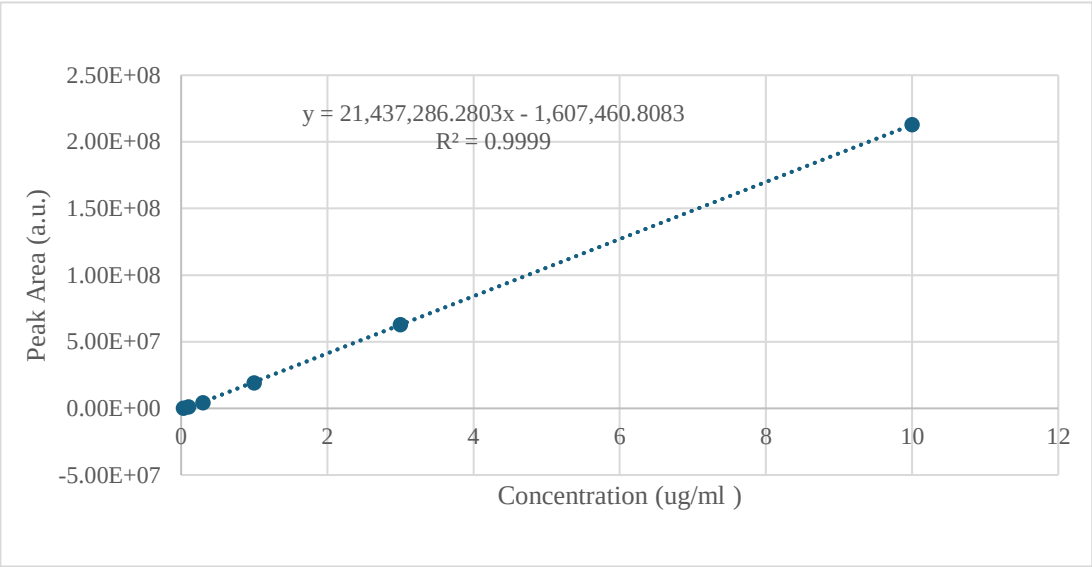

Figure **B-6.** Cannabichromene (CBC) calibration curve.

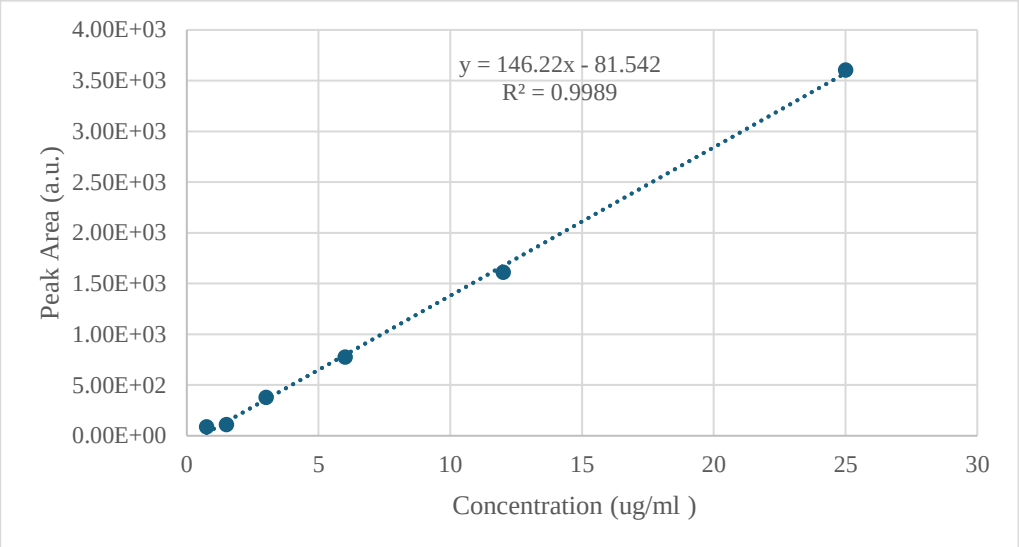

Figure **B-7.** Fenchone calibration curve a range of 50 ug/ml – 0.003 ug/ml.

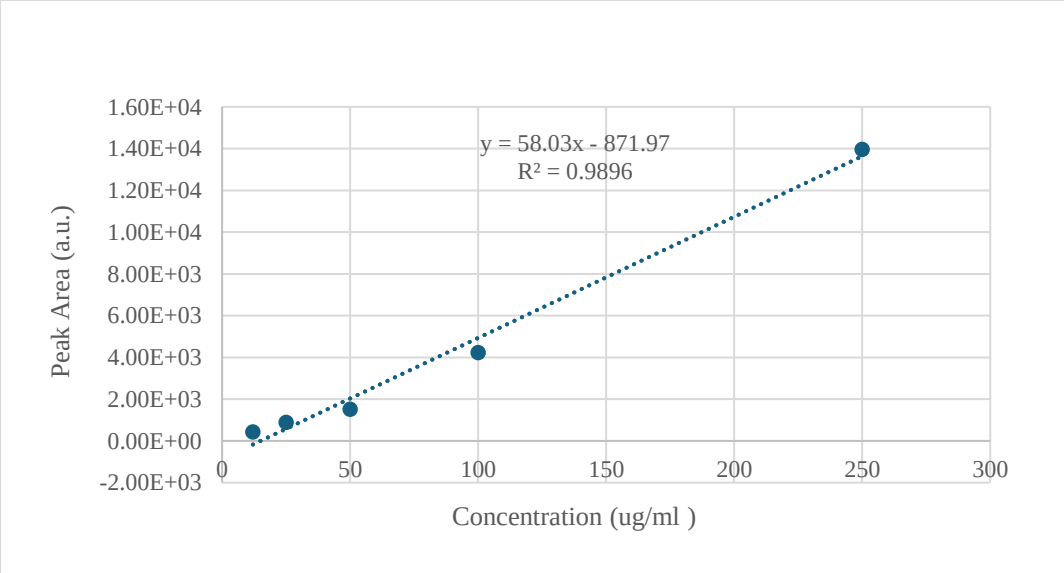

Figure **B-8.** Limonene calibration curve

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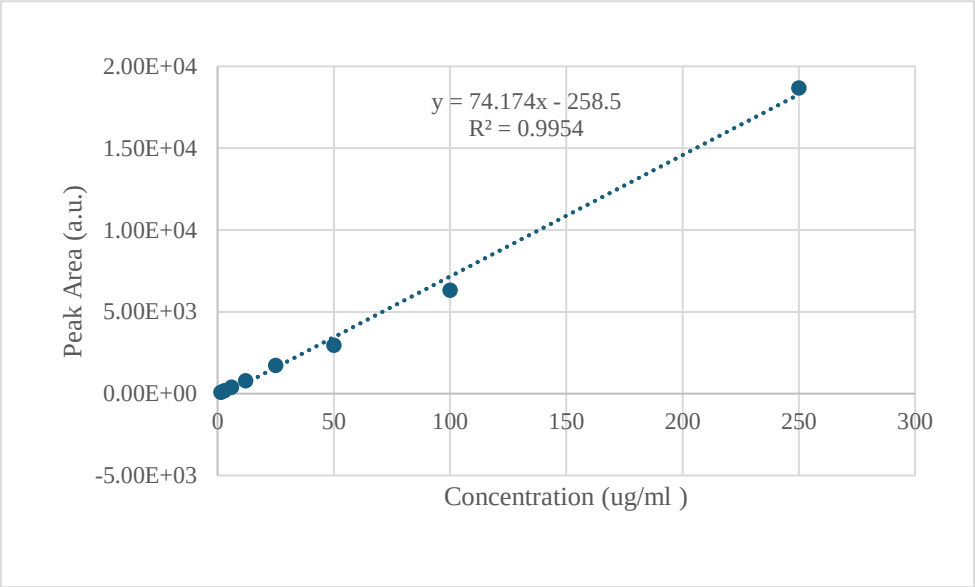


Figure **B-9.** endo(+)-Fenchyl alcohol calibration curve.

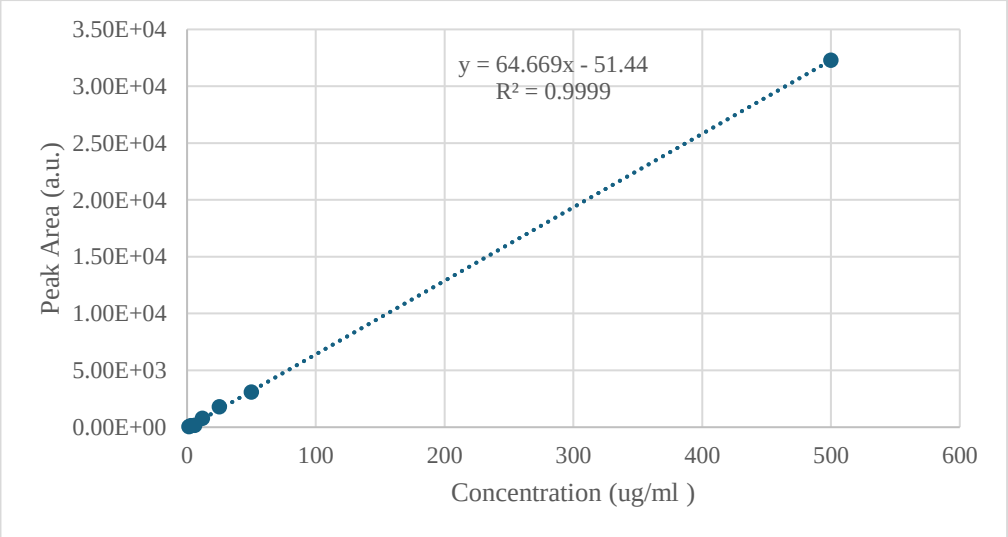


Figure **B-10.** β - caryophyllene calibration curve.

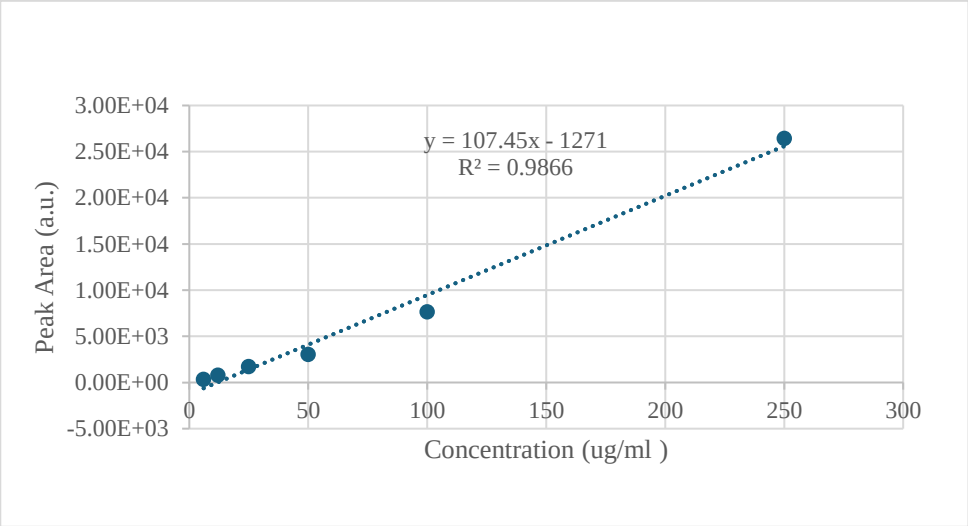


Figure **B-11.** Humulene calibration curve.

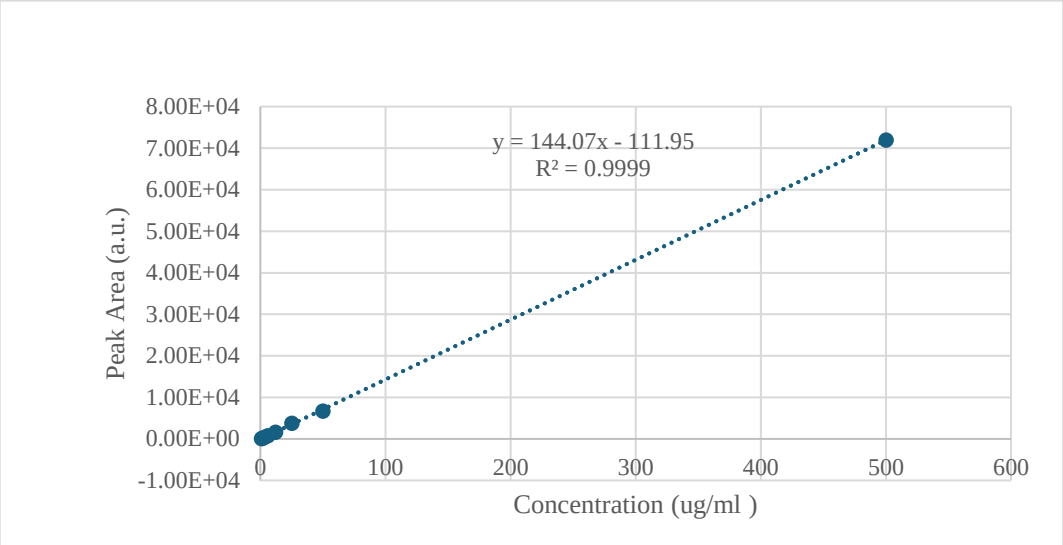


Figure **B-12.** Guaiol calibration curve.

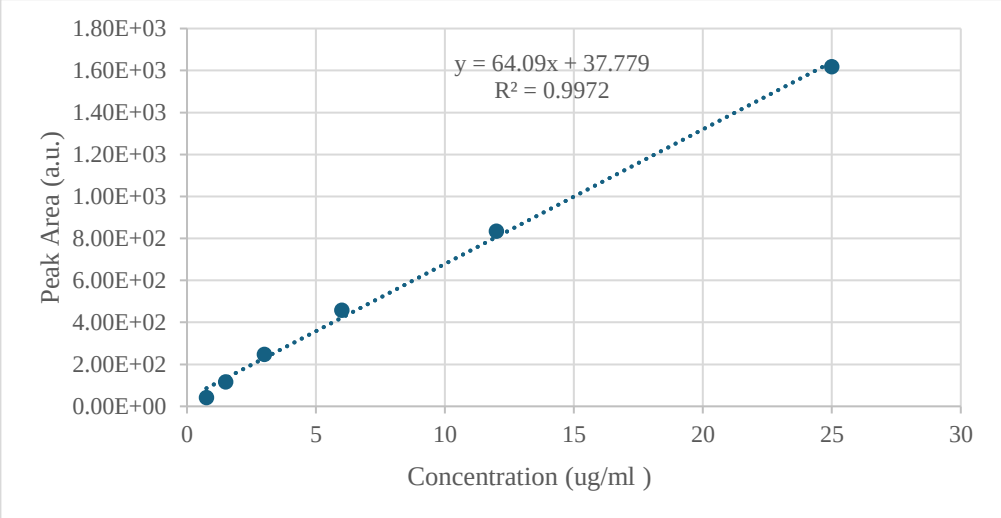


Figure **B-13.** Borneol calibration curve.

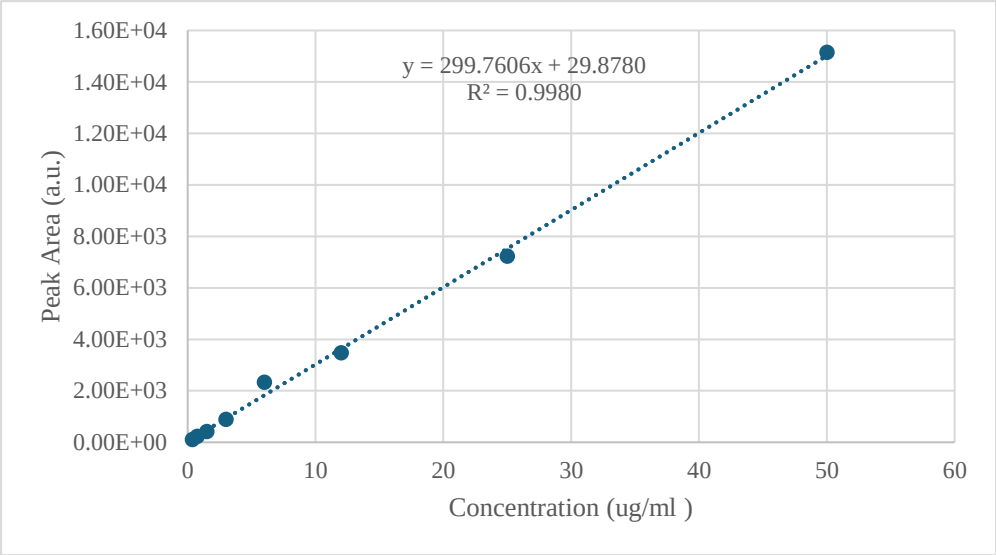


Figure **B-14.** Linalool calibration curve.

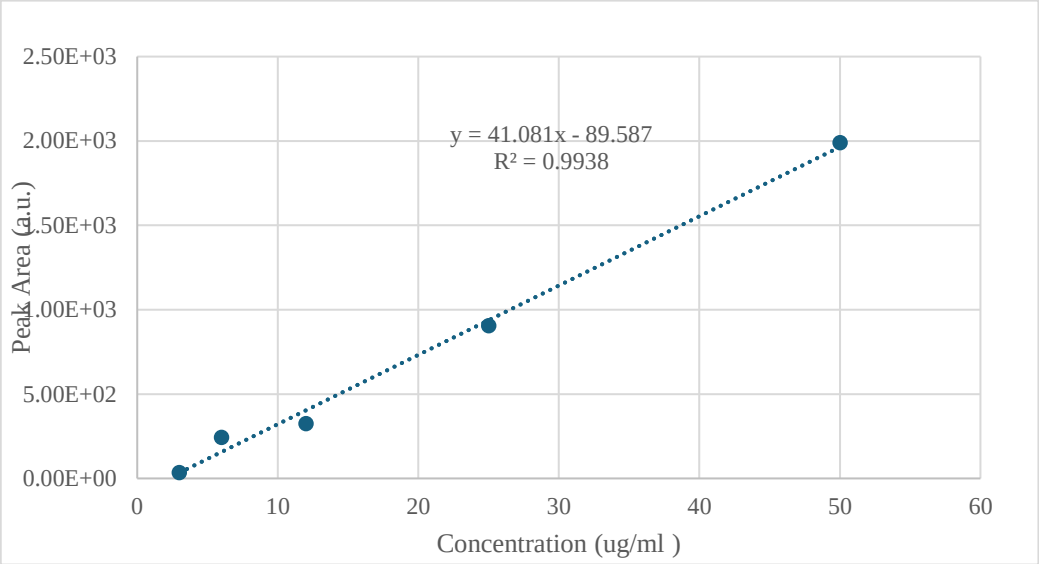


Figure **B-15.** Alpha-terpineol calibration curve.

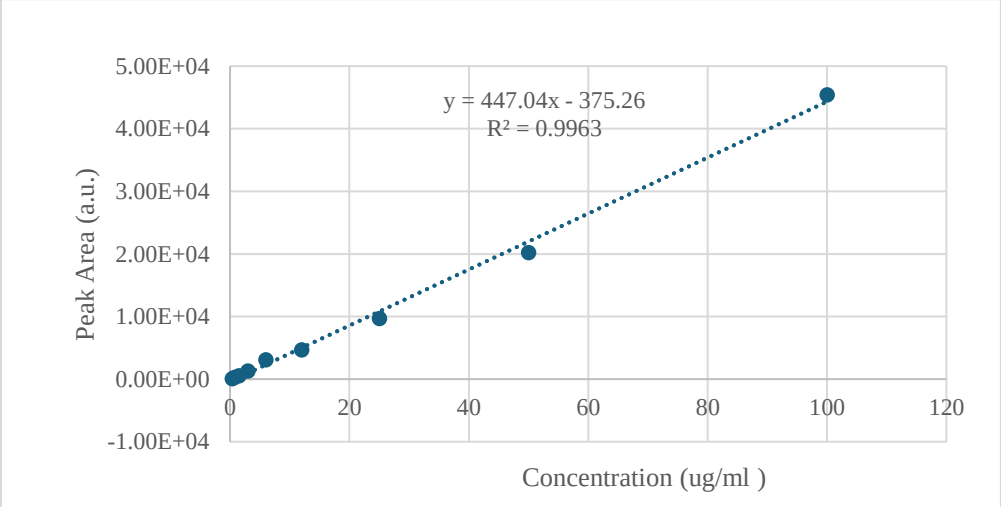


Figure **B-16.** Farnesene calibration curve.

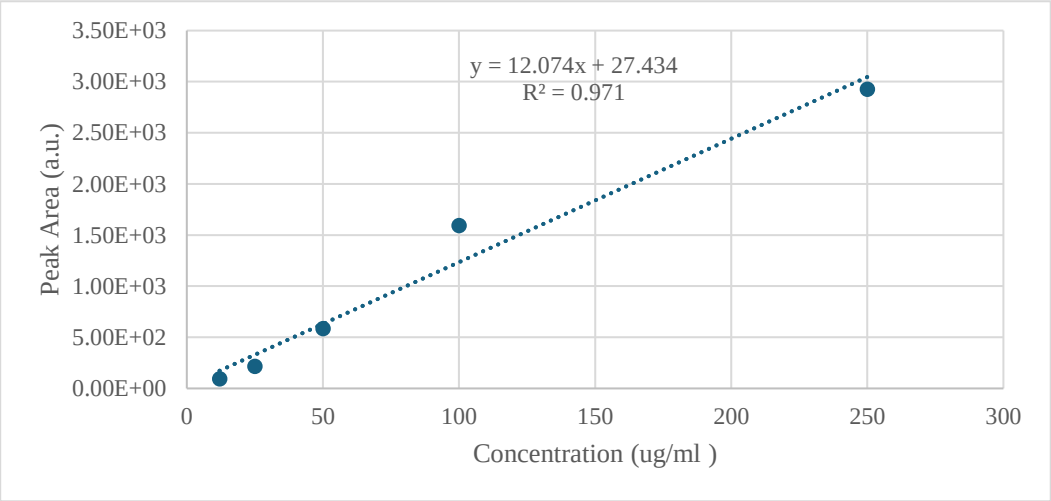


Figure **B-17.** Geranyl acetate calibration curve.

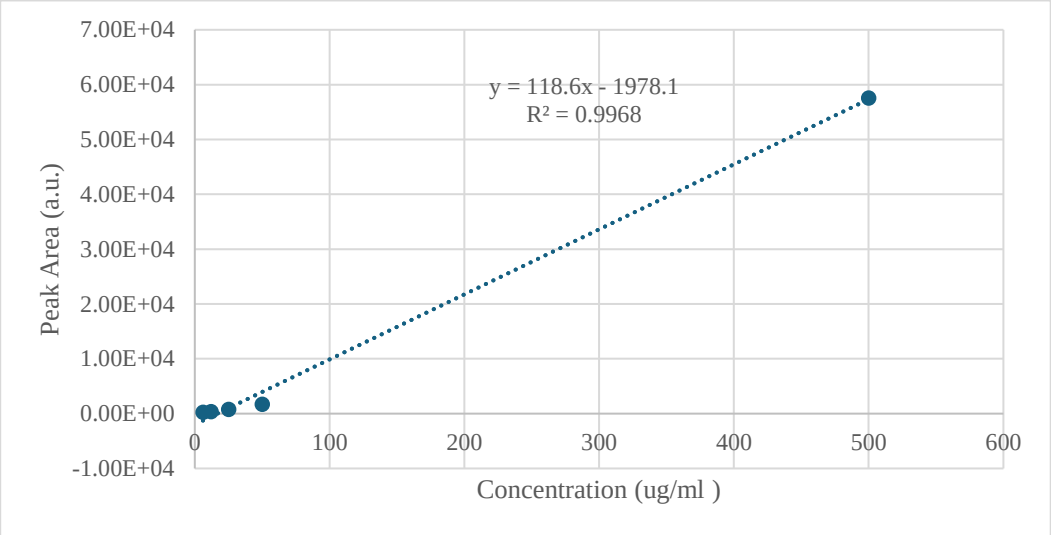


Figure **B-18.** Caryophyllene oxide calibration curve.

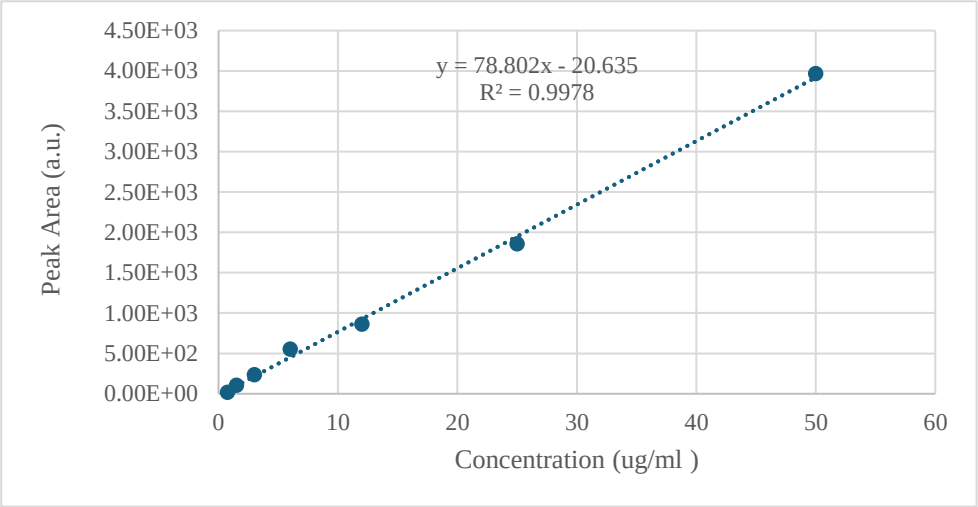


Figure **B-19.** Valencene calibration curve.

Appendix C: Supporting Information for Chapter 4

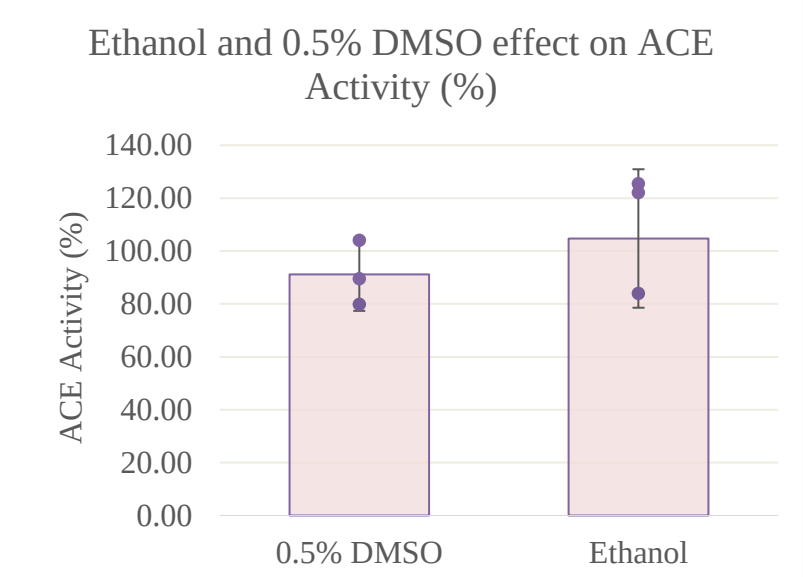
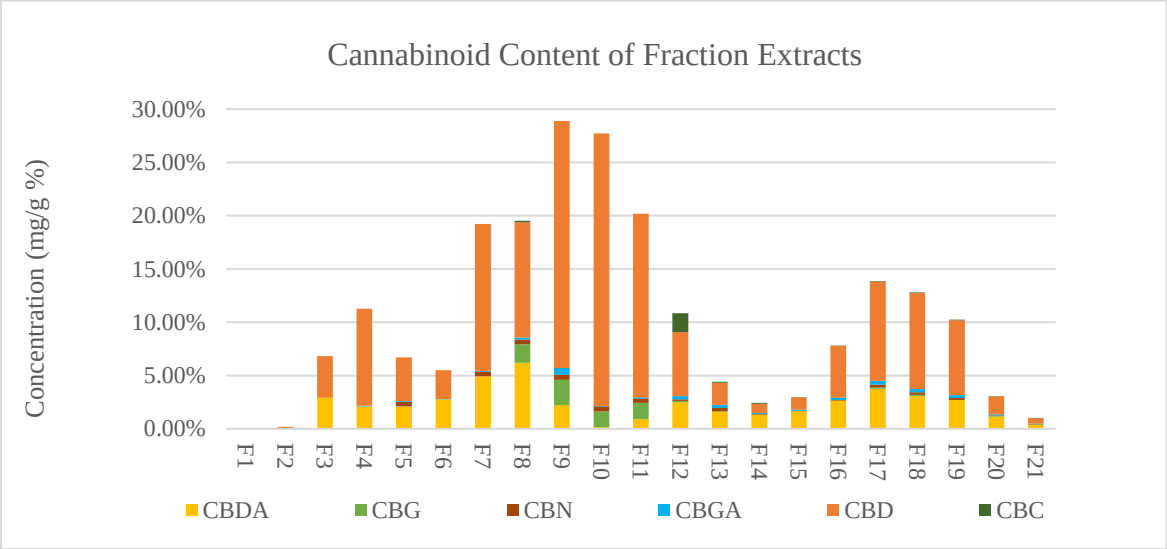


Figure **C-1:** ACE activity (%) of reconstitution solvents compare to lisinopril relative to ACE activity in blank (no inhibitor sample).



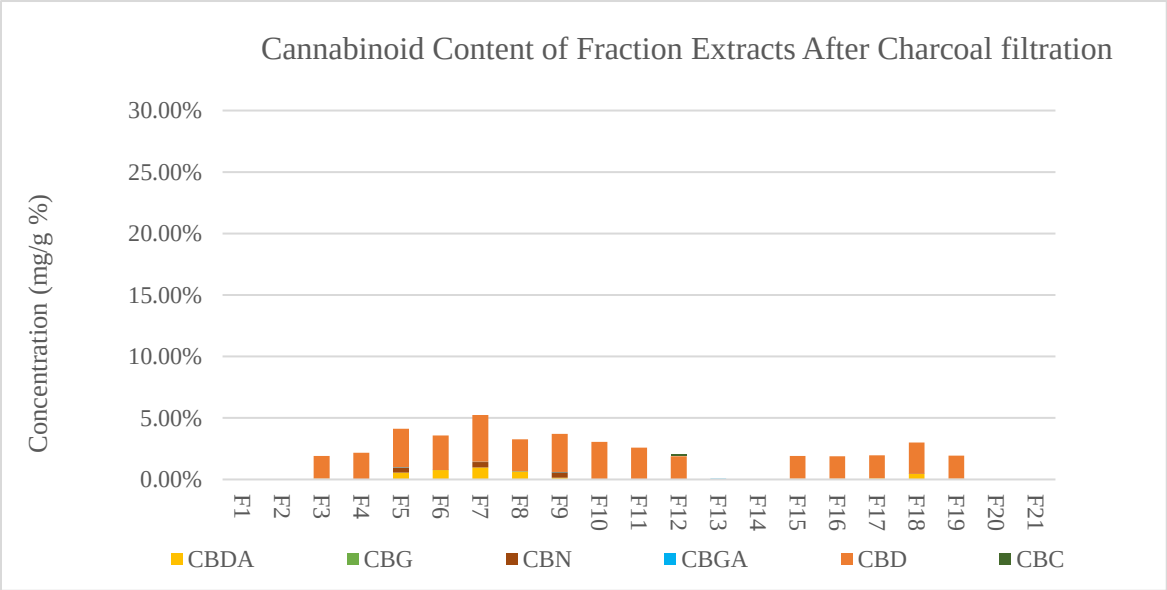
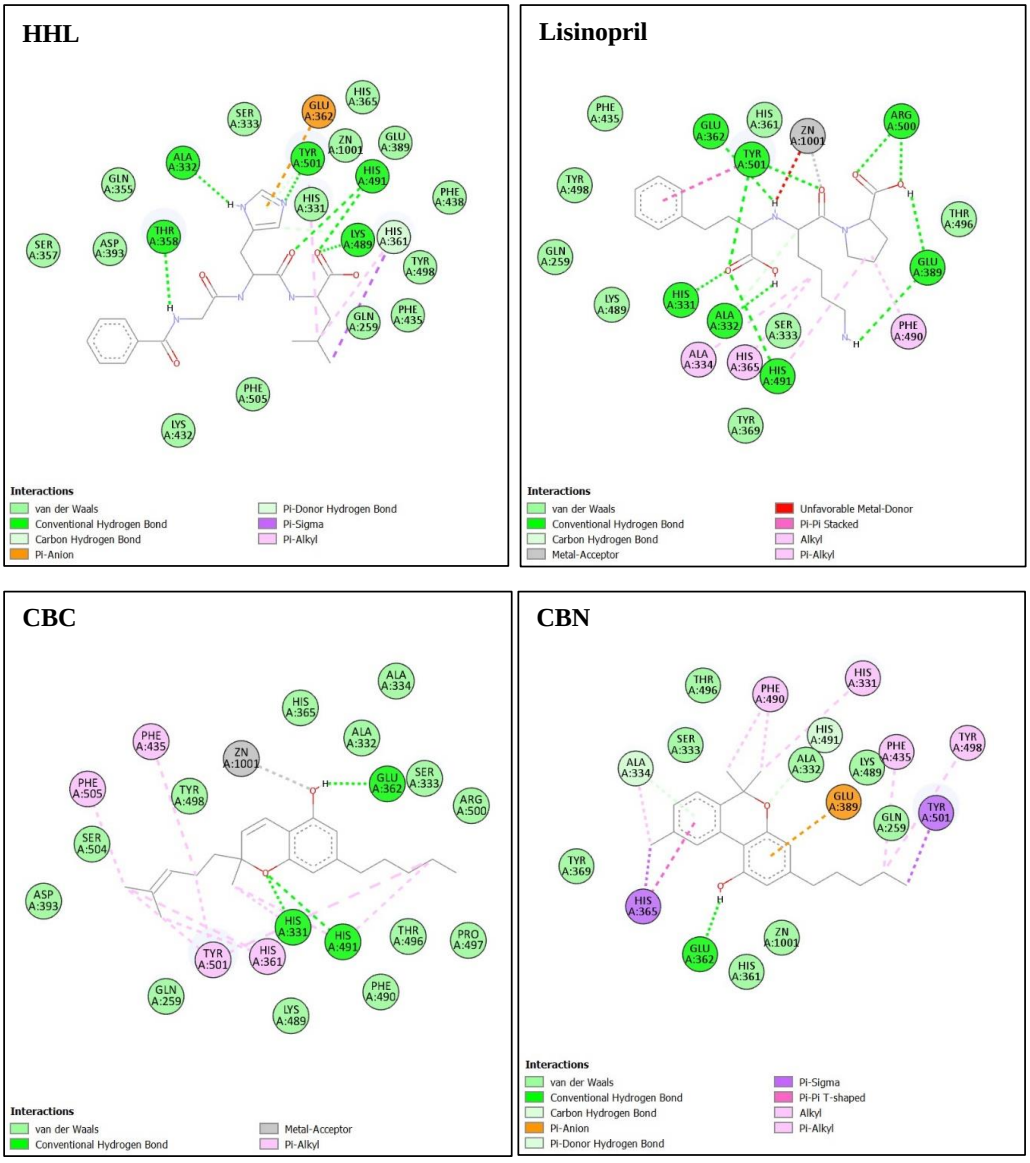


Figure **C-2.** Cannabinoid content (mg/g %) of fraction extracts before and after filtration using activated charcoal.

Residue interactions (N-Domain)



Residue interactions (N-Domain)

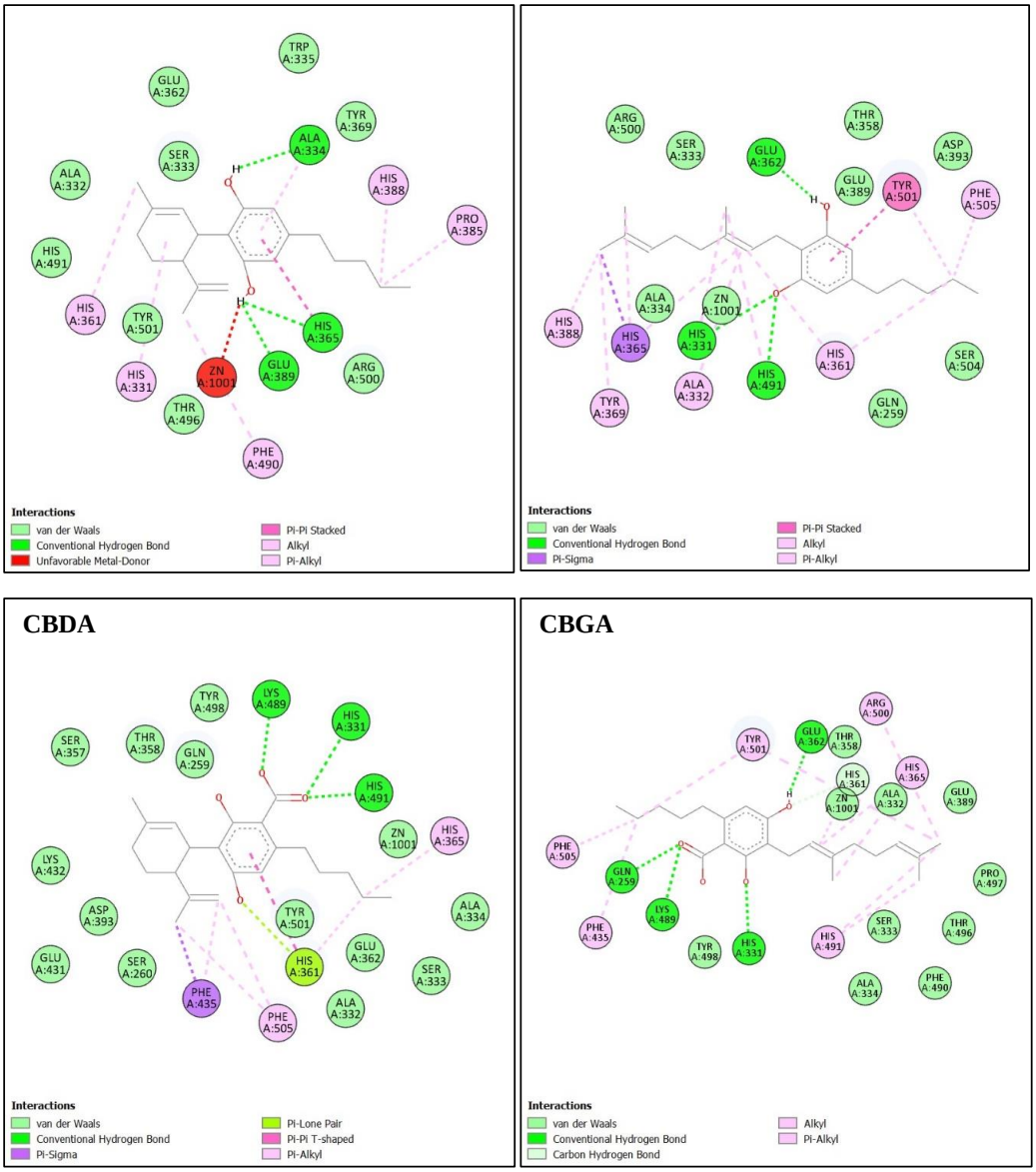
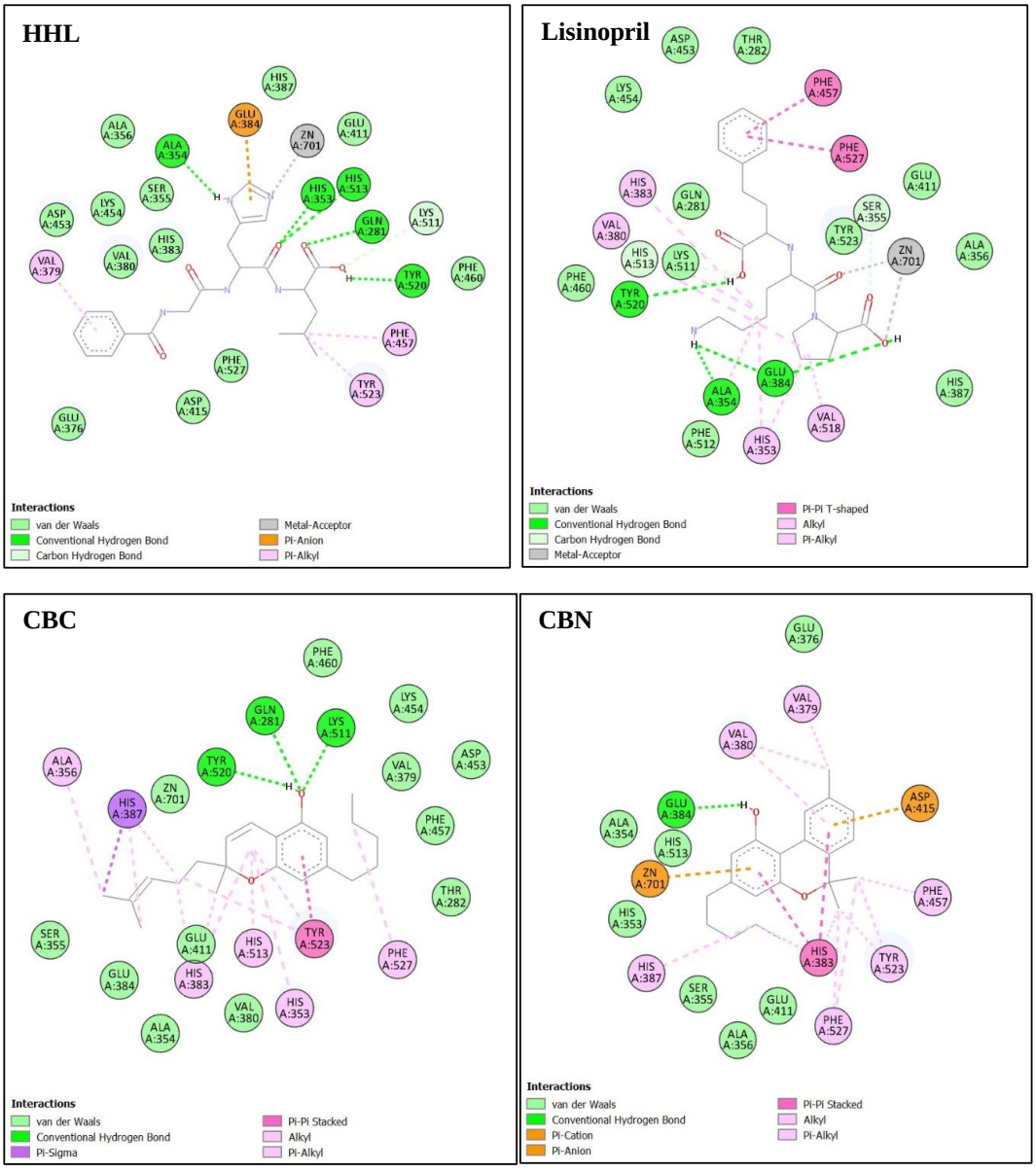


Figure **C-3:** Ligand and receptor residue interactions of the N-domain of ACE for substrate (HHL) , known ACE inhibitor (lisinopril), and six cannabinoids (CBD, CBG, CBN, CBC, CBDA, and CBGA).

Residue interactions (C-Domain)



Residue interactions (C-Domain)

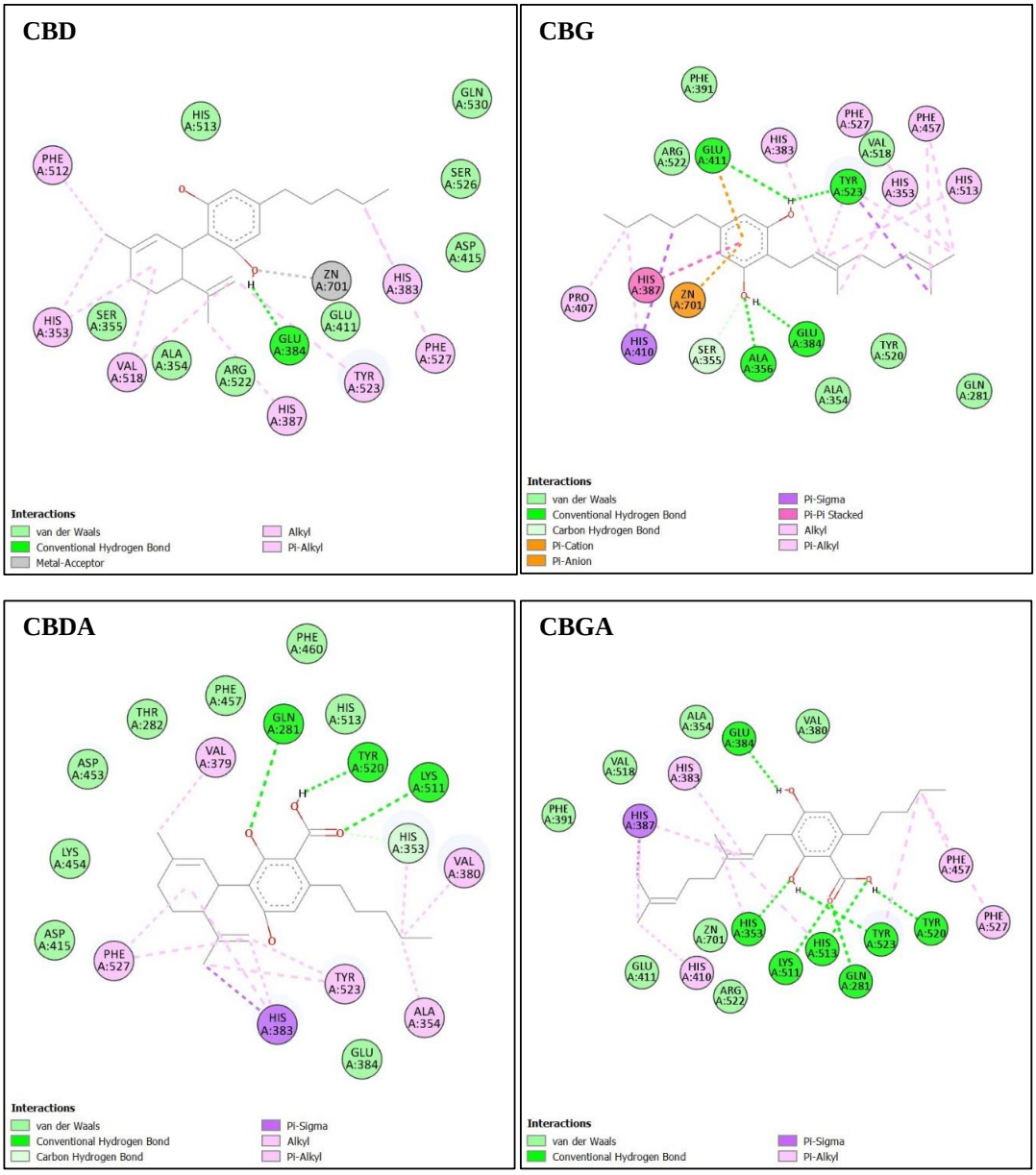


Figure **C-4:** Ligand and receptor residue interactions of the C-domain of ACE for substrate (HHL) , known ACE inhibitor (lisinopril), and six cannabinoids (CBD, CBG, CBN, CBC, CBDA, and CBGA).

VITA

Education

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- Graduate Program: Plant Biology
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Selected Publications

1) (In preparation) **Francisco T. Chacon,** Shawn A. Raup-Konsavage, Kelly Greenland, Robert Gearhart, Dhimant Desai, Joshua J. Kellogg, and Wesley M. Raup-Konsavage. Impact of Soil Quality on Cannabinoids and Terpenoid Content of *Cannabis sativa* L.. Journal of Cannabis Research.
2) (In preparation) **Francisco T. Chacon,** Joshua P. Rogers, Kent E. Vrana, Joshua J. Kellogg. Angiotensin-Converting Enzyme (ACE) Inhibitory Activity of Cannabinoids from *Cannabis sativa* L. Journal of Cannabis Research.
3) **Francisco T. Chacon,** Wesley M. Raup-Konsavage, Kent E. Vrana, Joshua J. Kellogg. Effect of Hemp Extraction Procedures on Cannabinoid and Terpenoid Composition. Plants 2024.
4) **Francisco T. Chacon,** Wesley M. Raup-Konsavage, Kent E. Vrana, Joshua J. Kellogg. Secondary Terpenes in *Cannabis saitva* L.: Synthesis and Synergy. Biomedicines 2022.
5) Maria A Ortega, Rhodesia M Celoy, **Francisco Chacon,** Yinan Yuan, Liang-Jiao Xue, Saurabh P Pandey, MaKenzie R Drowns, Brian H Kvitko, Chung-Jui Tsai. Altering cold-regulated gene expression decouples the salicylic acid-growth trade-off in Arabidopsis. The Plant Cell 2024.
6) Ivette Guzman, Krystal Vargas, **Francisco Chacon**, Calen McKenzie, Paul W. Bosland. Health Promoting Carotenoids and Phenolics in 31 *Capsicum* Accessions. HortScience 2020.

Selected Presentations

Poster Presentations:
1) *International Cannabis Research Conference (ICRS)* **2024**
2) *Cannabis Research Conferences (CRC)* **2023**
3) *The American Society of Pharmacognosy (ASP)* **2022**
4) *Society for Advancement of Chicanos/Hispanic & Native Americans in Science (SACNAS)* **2019**

Funding and Fellowships

1) PA Options for Wellness Fellowship **2023-2024**