Comparison of Extraction Methods for Sample Preparation in the Quantification of Cannabinoids in Industrial Hemp

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ABSTRACT

Industrial hemp is legally defined in the United States by the Agriculture Improvement Act of 2018 (2018 Farm Bill) as Cannabis containing <0.3% total tetrahydrocannabinol (THC). The 2018 Farm Bill does not, however, specify standard methods for sample preparation or quantification of cannabinoids (including THC) in Cannabis. Extraction efficiency of phytochemicals is well-known to depend on the solvent and extraction method used. In this project, we evaluated the effect of sample preparation extraction methods on the quantitative analysis of five cannabinoids found in industrial hemp with regulatory or commercial significance: cannabidiol (CBD), cannabidiolic acid (CBDA), delta-9-tetrahydrocannabinol (THC), tetrahydrocannabinolic acid (THCA), and cannabinol (CBN). Extraction methods evaluated include: QuEChERS, diethyl ether, ethanol, and methanol. Extracts obtained via these methods were subject to quantitative cannabinoid analysis by UPLC/PDA. Standard curves for quantification of each cannabinoid were constructed using authentic standards for quantification. The concentrations of each cannabinoid in the plant material determined via each of the extraction methods were compared using one-way ANOVA followed by Tukey's HSD (significant difference defined as $p < 0.05$). All extraction methods evaluated returned different concentrations of total THC in the plant material. The QuEChERS extraction resulted in the highest calculated concentrations of THC, THCA and CBDA, reporting three to four times greater than obtained via other extractions evaluated. Classification of the starting plant material as hemp or marijuana depended on the extraction method used. These findings clearly and quantitatively demonstrate the need for standardization of extraction methods for hemp analysis and regulation.

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GENERAL AUDIENCE ABSTRACT

Hemp is a type of Cannabis plant that produces an insignificant amount of the psychoactive cannabinoid tetrahydrocannabinol (THC). Hemp was federally illegal in the United States until the 2018 Farm Bill differentiated hemp from Marijuana, defining hemp as containing less than 0.3% total THC. Standard methods for cannabinoid testing in hemp have not been defined. In this project, four extraction methods with potential use for sample preparation in hemp analysis were evaluated and compared. The extraction methods evaluated included: ethanol, methanol, QuEChERS, and diethyl ether. The concentrations of cannabinoids in each of the plant extracts were then determined using an appropriate analytical method and authentic standards for Cannabidiol (CBD), Cannabidiolic Acid (CBDA), THC, Tetrahydrocannabinolic Acid (THCA), and Cannabinol (CBN). Total concentrations of each cannabinoid in the plant material were then calculated using each extraction method. All extraction methods evaluated resulted in different concentrations of total THC in the plant material, with QuEChERS resulting in the highest calculated concentrations of THC, THCA, and CBDA. The identify of this plant material as hemp or marijuana depended on the extraction method used. This result is not surprising, as extraction efficiency of phytochemicals is well known to depend on the solvent and extraction method used. Nonetheless, our findings clearly demonstrate the need for standardization of extraction methods for hemp analysis and regulation.

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Chapter One: Introduction

Importance of Hemp and Outline of Project

"Industrial hemp" is a term used in regulation in the United States to define specific strains of plants of *Cannabis sativa.* US regulations define marijuana as the term to describe *Cannabis sativa* plants that contain a psychoactive chemical delta-9-tetrahydrocannabinol (THC or delta-9-THC) (NIDA 2020). In the US, the distinction between industrial hemp, or hemp, and Marijuana is legally defined by the amount of total THC present (115th Session of Congress 2018). Hemp plants must contain no more than 0.3% post-decarboxylated THC, otherwise the plant is determined to be marijuana and is a federally illegal substance in the United States although marijuana is not illegal by state law in an increasing number of states. Regulations on hemp vary from state by state in the United States, creating a complex regulatory environment surrounding the legal status of *Cannabis sativa* plant material. The potency of marijuana refers to the amount of THC that it contains.

Naturally occurring *Cannabis sativa* plant metabolites include a range of chemicals called cannabinoids, including THC, Cannabidiol (CBD), Cannabidiolic Acid (CBDA), Cannabinol (CBN), and Tetrahydrocannabinoic acid (THCA). These 5 cannabinoids are the target of focus for this project as we are primarily interested in compounds relevant to regulatory guidelines like THC, and compounds with high potential to influence the commercial value of the project, such as CBD, and the degradation products of these important cannabinoids. Figures 1 and 2 shows these target cannabinoids and how they

relate to one another. THCA is a precursor to delta-9-THC or CBN, as THCA is heated it decarboxylates into THC (Di Marco Pisciottano 2018). CBDA undergoes the same breakdown into CBD as shown in Figure 2 (Di Marco Pisciottano 2018). CBN is a degradation product of THC over time (Di Marco Pisciottano 2018). Methods that require dry products might show a difference in cannabinoid content as degradation is more likely due to the drying process. Dried products are expected to show a more decarboxylated content of THC and CBD, and a lower content of THCA and CBDA because of decarboxylation during drying. CBN will be less prevalent in methods where wet samples are used since they will not be heated.

Figure 1. Relationships and Molecular Structures of THCA, THC, and CBN

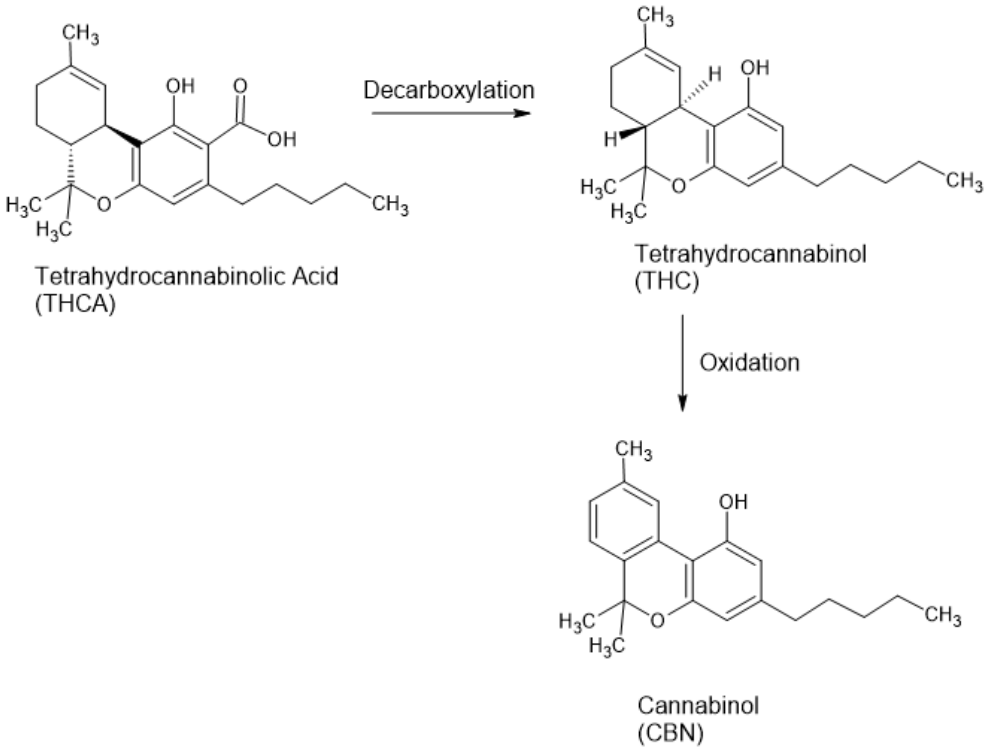
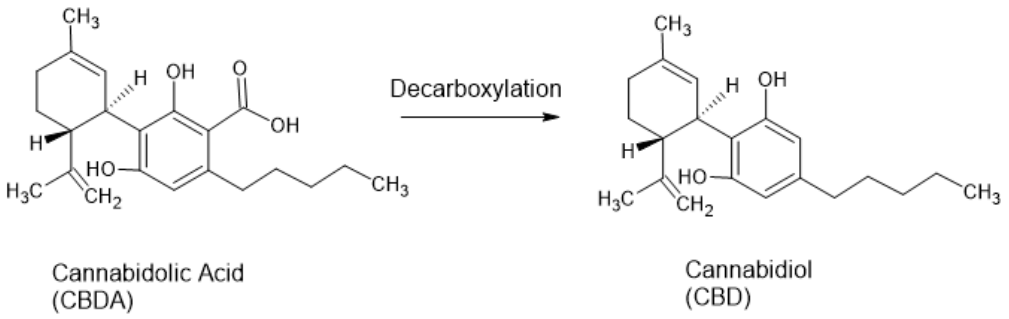


Figure 2. The Relationship and Molecular Structures of CBDA and CBD



Cannabidolic Acid (CBDA)

Cannabidiol (CBD)

The concentration of THC in hemp material is a regulatory concern, and there is no standard guideline for testing THC (Elkins 2019). Analytical methods for testing cannabinoids such as gas chromatography and liquid chromatography require that a liquid sample be injected into an analytical instrument, which necessarily involves obtaining an appropriate liquid for analysis from the solid plant material. The process for testing hemp plants generally involves obtaining a representative sample, drying the plant, grinding the plant into a powder, using solvents to extract cannabinoids from the powdered plant into a liquid, and testing the liquid extract using various chromatography methods to determine the concentration of cannabinoids.

There is a specification in the US regarding how the cannabinoids are measured as the Agriculture Improvement Act of 2018 states that the "procedure for testing using post-decarboxylation or other similarly reliable analytical methods, delta-9-tetrahydrocannabinol concentration levels of hemp produced" (115th Session of Congress 2018). This states that the legal THC requirement is referring to the total THC % which is delta-9-THC and decarboxylated THCA (115th Session of Congress 2018).

This wording allows different detection measures to be used, and liquid chromatography can be compared to the total THC measures from gas chromatography methods, which uses heated columns for separation where there is potential for the heat applied during the analytical method to affect the outcome and the reliability of this comparison.

Cannabis first began to be regulated in the United States with the passing of the 1906 Pure Food and Drug Act which states that packages must declare the quantity of *Cannabis indica* included in products (Pure Food and Drug Act 1906). The Federal Controlled Substances Act in 1971 declared marijuana as a Schedule I controlled substance, which classifies it as a drug that cannot be prescribed or administered due to the abuse potential (Gabay 2013). The passing of the 2018 Farm Bill allowed the production of industrial hemp federally across all 50 states. Because of the previous illegal status of marijuana, there are no established standard methods specifically for hemp for analytes such as cannabinoids or pesticides. State agencies released guidelines on how to harvest plant material for testing, but these procedures can vary by different laboratories (VDAC 2021). Both state-run facilities and third party "for profit" laboratories are offering regulatory testing of THC in hemp. Each laboratory develops its own methods for sample preparation and analysis, and performs the validation of methods in house (DCLS 2019). These facilities do not release their methods publicly, and there is no certification or method to corroborate what validation protocols the laboratory follows. The storage of hemp varies by facility, and some places keep samples at room temperature while others refrigerate or freeze the sample prior to testing (DCLS 2019 and IALR 2021). There is no standard method of drying samples as some

laboratories dry samples at room temperature for 48 hours, and there is no standard target moisture content to define when a sample is sufficiently dry (IALR 2021). Other methods specify oven drying at 90°C and weighing the sample after each heating process until the sample no longer loses weight when it is heated (DCLS 2019). Laboratories may specify which solvent is used during extraction but the specifics regarding amount of plant material to volume of solvent and contact time is not standardized nor documented publicly, even though these factors are expected to influence extraction efficiency (IALR 2021). Methanol is a commonly used solvent for hemp extraction due to the low cost. And the ratio between plant material and solvent is expected to affect the overall cannabinoid content measured (IALR 2021).

Currently the FDA does not allow THC or CBD added to food and there is no standard method for testing cannabinoids in food (FDA 2020). Notwithstanding, this is an area of tremendous interest to the food industry, due to high consumer demand for such products, and the uncertain regulatory horizon in the US and globally. A commonly used extraction method for testing pesticides in food is called the QuEChERS method (Ferracane 2021). With the increasing attention on hemp additives in food and beverages, the QuEChERS method is being evaluated as a possible testing method for edible (food) products that contain hemp additives (Lee 2020). Because there are different methods being used to determine cannabinoid content based on the type of material being tested, (e.g. hemp flowers, CBD oil preparation, food products containing hemp extracts, and others), it is important to consider how the results obtained through these different sample preparation and analytical methods compare to one another.

This project focuses on the different extraction methods, the solvents used, and if the extraction method required wet or dry sample material. Ethanol and methanol are solvents commonly used by laboratories because they are cost efficient, easy to find, and will interact with the fat soluble cannabinoids. Extraction methods with these alcohol solvents employ different ratios of solvent to plant material, differences in timing that range from 5 min contact to one hour of contact (DCLS 2019), and some processes use two extractions of the same sample as opposed to one extraction (AOAC 2018). Thus, it is useful and timely to compare extraction methods commonly used for sample preparation to determine THC percent in hemp plant material, and to compare methods that are used on plant material with methods that are used on food products.

Objective and Hypothesis

The objective of this project is to compare the effects of four different extraction methods used in sample preparation for hemp analysis on the quantitative results for cannabinoids in the extract. This will allow the determination of the effects of different solvents and extraction procedures on the recovery of the major cannabinoids of regulatory and/or commercial importance. Our hypothesis is that the different solvents used in the extraction process impacts the total concentration of cannabinoids reported and that methods designed for wet products will extract more cannabinoids compared to methods that use only alcohol solvents.

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Chapter Two: Literature Review

Introduction to Hemp

Hemp is a type of *Cannabis sativa* plant that contains low concentrations of delta-9-tetrahydrocannabinol (delta-9-THC), a psychoactive cannabinoid. Cannabis plants have been heavily regulated within the United States since 1906 due to concerns over the intoxicating effects of delta-9-THC (Pure Food and Drug Act 1906). In the US, a distinction between Marijuana and hemp is defined by the concentration of delta-9-THC either in the whole plant or in any parts of the plant as sampled (115th Session of Congress 2018). Marijuana is defined in the United States as containing higher than 0.3% total THC (115th Session of Congress 2018), and industrial hemp contains less than 0.3% total THC. Marijuana was classified as a Schedule 1 drug in the United States in 1971, and was outlawed by the Federal Controlled Substances Act (Gabay 2013). The

production of hemp plants for research purposes were only recently legalized with the passing of the 2014 Farm Bill, and the 2018 Farm Bill allowed for commercial production of industrial hemp (113th Session of Congress 2014, 115th Session of Congress 2018). Regulation in the 2018 Farm Bill defines that THC concentration must be determined by post-decarboxylated or similar methods, in order to quantify the Total THC concentration which includes both THCA and delta-9-THC. THCA is calculated to % delta-9-THC by converting THCA into delta-9-THC by using molecular weight differences between the molecules for a conversion factor of 0.88.

The classifications of different species of Cannabis is widely discussed as modern practices focus on the concentration of THC as a species defining phenotype. The recorded study of differences in cannabis plants dates back to 1785 where evidence showing that *Cannabis sativa* produced fiber and oil and *Cannabis indica* was grown for its intoxicating effects (Small 1976). Small and Cronquist identified that the primary difference between Northern and Southern grown plants in that area is the intoxicating effects. The concentration of 0.3% delta-9-THC was used to separate different classes of the Cannabis plant: those used historically for drug purposes, and those historically used primarily for fiber and oil (Small 1976).

Modern references on cannabis focuses on regulation as the defining factor for what is classified as hemp or marijuana. Hybrids of *indica* and *sativa* are bred, based on the desirable characters of either higher or lower THC production (Chen 2019). Hemp was widely grown in the United States from the 1800's, and until 1937, hemp provided a

source of rot resistant fiber for paper, textiles, and rope (USDA 2020). Health concerns related to the consumption of cannabis led to the passing of the Marihuana Tax Act of 1937, where all cannabis products were taxed and there were no distinctions provided between hemp or marijuana strains. The import of raw hemp fiber for use in textile manufacturing increased from 500 pounds to 1.5 million pounds in the 1990's. (USDA 2000), demonstrating consumer demand for specialty textiles in the United States.

Hemp seeds can serve as a source of dietary amino acids and hemp seed oil is used for food, fuel, or lubricants (USDA 2000). Oil products from hemp and hemp seed are used in health care products and food items as a nutritional supplement (USDA 2000). Recently, hemp oil containing products have been marketed as containing a "naturally ideal" ratio 3:1 of Omega 6:Omega 3 fatty acids (Nutiva 2021). However, a higher amount of Omega 3 (linolenic acid) than Omega 6 (linoleic acid) is generally associated with improved health outcomes, as excessive amounts of Omega 6 (e.g. a higher ratio of Omega 6:Omega 3 FA) are expected to contribute to inflammation (Simopoulos 2002). This is just one example of the type of confusing health claims routinely being made on food and dietary supplements containing hemp.

The profitability of hemp products is widely debated, due to the substantial capital costs required to produce hemp extracts that remain in demand in the dietary supplement and food ingredient sector, despite the current FDA regulations prohibiting such use. In 1995, estimated total costs of hemp acres were $286 for fiber and $196 for seed (USDA 2000). The approximate yield per acre for hemp fiber was 2.8-6.1 tons in 1993 (USDA 2000).

Regardless of the large cost associated with production, hemp is increasing in popularity with interest developing in CBD products. Hemp acres planted in 2019 averaged 120 compared to 77 in the previous year (Hemp & CBD Industry Factbook 2019). Estimated projections for hemp related CBD products are over $10 billion by 2024 in the United States (Hemp & CBD Industry Factbook 2019).

Regulations

The passing of the 2018 Farm Bill allowed for the commercial production of industrial hemp in the United States for the first time in 48 years (115th Session of Congress 2018). Industrial hemp production has seen a rise in 2019 due to federal United States regulations after changes made in the 2018 Farm Bill Legislation (115th Session of Congress 2018). Federal guidelines allow the production, processing, and sales of cannabis plants that contain less than 0.3% delta-9-tetrahydrocannabinol (delta-9-THC), as measured on a dry weight basis. The wording in the federal regulations states that the concentration of the whole plant or plant parts must be less than 0.3% post-decarboxylated THC. This leads to confusion as different parts of the plant are known to contain different levels of THC (Hemphill 1980).

The updates in the 2018 Farm Bill updated language to specify that the regulation on THC is for total THC content. Decarboxylation of THCA converts the acid component to THC, which occurs with heat or drying (De Backer 2009). The total THC content is delta-9-THC and tetrahydrocannabinolic acid (THCA) together. Using the molecular weight of 314.45g/mol for THC and 358.47g/mol THCA, the equation of 314.45g/mol /

358.47g/mol = 0.88. This shows a conversion factor of 0.88 is used to convert THCA to THC to calculate the total THC content: THCA * 0.88 + delta-9-THC = Total THC content.

There are safety concerns in the United States regarding the production and sale of hemp products, due to the intoxicating effects of THC, as well as the unknown effects of other hemp produced compounds when consumed by humans.

Economic Impact

The cannabis plant has been used for the fiber, oil, seeds, and resin produced for centuries (Small 1976). Currently, companies and consumers are exploring the potential for health benefits of cannabidiol (CBD) and other cannabinoids (Hemp & CBD Industry Factbook 2019). According to the group "Hemp & CBD Industry", in 2019 CBD retail sales in the United States were listed as 1.2 billion dollars. The industry is expected to see a growth of up to 10.3 billion dollars for annual CBD sales by 2024 (Hemp & CBD Industry Factbook 2019).

Hemp production has been pursued in the United States due to the variety of products that can be obtained from farming hemp. Different varieties of hemp are bred in order to produce less THC and more CBD, to provide better quality products to producers wanting to make CBD products and oils. After the passing of the 2018 Farm Bill, 47 states reported hemp growing programs with over 90,000 acres planted (USDA 2020).

Hemp Products

Cannabis or hemp is raised for the stalk, seeds, or flowers. The stalks can be used for textiles, rope, paper, and construction materials. The seeds are used for their oil and can be used as tinctures or food products. The flowers of the hemp plant can be processed for the oil, and is used in tinctures and oils in order to obtain the alleged health effects from the cannabinoids present (Hemp & CBD Industry Factbook 2019).

Cannabidiol (CBD) is a compound found in the hemp plant that can be extracted and/or purified, and sold as an oil diluted product. Hemp species are bred in order to increase the amount of CBD produced with a lower amount of THC. CBD is believed to offer various health benefits such as anti inflammatory and antipsychotic effects (Burstein 2015). The anti inflammatory effects of CBD have resulted in demand for these products by people suffering from various disease conditions such as arthritis, gastrointestinal disease, meningitis, and diabetes (Burstein 2015). Studies have compared the effects of CBD on cigarette and cocaine cravings as an effort to market CBD as a treatment for addictive behavior and withdrawal symptoms (Mongeau-Perusse 2021). CBD has been researched as a treatment for Huntington's disease, however results did not show significant differences compared to placebo results (Consroe 1991). Winiger et al explored the use of cannabis as a sleep aid, and found that increased consumption of cannabis led to an overall lower sleep quality, while differences in ages correlated to CBD consumption and better sleep results (Winiger 2021). As hemp products enter the US and global market under regulations that vary by country, adequate and accurate labeling presents a challenge to producers and regulators (Pepper 2020). Warning labels address that the

products may be habit forming, or increase psychosis or schizophrenic symptoms, and should not be used while operating heavy machinery (Pepper 2020).

The Food and Drug Administration (FDA) holds a position that CBD can only be used in pharmaceutical products, and cannot be added to food, beverages, or dietary supplements (FDA 2020). The FDA ruling is based on the lack of research on CBD, and unanswered questions on the long term use of the compound, such as what level of intake triggers risks, and how the different consumption methods affect the cannabinoid compounds (FDA 2020). Baby hemp leaves are sold to be consumed as a salad (Mi 2020). As more research is desired on cannabis in foods, the method for testing THC in different food matrices needs to be standardized. The effects of the human digestive system on cannabinoids needs to be researched in order to determine serving size and dosage recommendations. The effects of different matrices of food is a concern when measuring cannabinoids in foods. Lipids, proteins, fatty acids, and other substances in foods can contribute to the matrix effect, making extraction of cannabinoids in foods difficult and inconsistent (Rutkowska 2020). The QuEChERS method of extraction is targeted as the method of extraction for measuring cannabinoids that were added to food products (Christinat 2020).

Key Analytes

Tetrahydrocannabinol, or delta-9-THC, is a psychoactive compound found in cannabis (Baker 1981). THC-A is tetrahydrocannabinolic acid and, when heated, THC-A

decarboxylates and becomes delta-9-THC (Baker 1981). The acid forms of these compounds have been found to break down during the processing of the plant material during drying or extraction, and this affects the quantification and measurement of these different compounds.

The U.S. regulation for hemp requires the concentration of delta-9-tetrahydrocannabinol (delta-9-THC) to be less than 0.3% of dry weight basis after post-decarboxylation, or through a similar process. This means that the legal regulation of THC in hemp is based on the total of delta-9-THC and THC-A. Crops that test above 0.3% THC are required to be destroyed due to state and federal laws regulated under the Farm Bill.
Gas chromatography with flame ionization detector (GC-FID) has been used for cannabinoid analysis as this method heats the samples and decarboxylates the compounds, to report delta-9-THC as the total THC content. Regulations did not previously distinguish between delta-9-THC or total percentage of THC before the 2018 Farm Bill (115th Session of Congress 2018). As more testing is desired for cannabis products to determine product quality, liquid chromatography has been used in order to obtain values pre-decarboxylation, and the total THC is calculated by adding the delta-9-THC and THCA values (Baker 1981). Cannabinol (CBN) is a degradation product of delta-9-THC and is not expected in fresh plant samples (Messina 2015). Cannabinolic acid (CBDA) is the decarboxylated form of CBD (Martinenghi 2020).

Current Hemp Analytical Methods and Key Limitations

Testing for cannabinoids can be done on oils, tinctures, or raw hemp material. Raw plant material undergoes an extraction process prior to testing in order to separate the oils from

the plant material (AOAC 2018). Some methods require the plant material to be dried prior to extraction because the solvents used are mostly hydrophobic, and water would interfere with the solvent interactions and cannabinoids. After extraction, the liquid product and standards are diluted in solvents such as acetonitrile or methanol and prepared for testing by gas chromatography or liquid chromatography. The quantification is determined by the method and equipment used and can be gas chromatography-flame ionization detector which reports total THC, or liquid chromatography using a photodiode array detector (PDA or DAD) alone or in combination with mass spectrometers where THCA is measured separately from delta-9-THC but they are calculated together in order to report the total THC content to compare with decarboxylated methods. The methods vary by the particular laboratory, which may be a state regulatory facility, a third party lab, or an equipment supplier. Because methods are not standardized, the storage, drying, extraction, and detection methods vary between every laboratory and are dependent on what equipment and supplies are available. Labs may use similar extraction processes and different detection equipment, or other combinations of these elements which makes comparing results between labs unreliable.

Detection Methods

The type of equipment used to perform the test determines what compounds can be detected. Using a GC-FID means that the sample will be decarboxylated by heat, and therefore acid forms will not be found. THC-A will be decarboxylated into delta-9-THC. Any methods using GC analysis will only report Total THC, and will not be able to provide the breakdown of THC-A and delta-9-THC separately present in the sample. Because the federal legal requirement specifies that the regulation is

post-decarboxylation, the GC-FID method was historically used for regulatory analysis. However, liquid chromatographic methods must be used to report both THC-A and the naturally occuring delta-9-THC concentrations. While GC-FID methods are sufficient for regulation guidelines, production areas that want to quantify the full spectrum of cannabinoids would need to use liquid chromatography to avoid breakdown of compounds due to the high heat applied by gas chromatography.

Extraction methods

The extraction process of cannabinoids is dependent on the desired function. Extraction for analysis of cannabinoids for regulatory purposes is different from extracting cannabinoids in a processing facility for the intent to sell the final product either as an oil or additive. Solvent choices for laboratory analysis includes ethanol, methanol, acetonitrile, or diethyl ether. These chemicals are not desirable in commercial processing for hemp oils expected to be consumed because they are not intended for consumption. Industrial extraction processes may use food grade oils, supercritical carbon dioxide, ethanol, butane, and physical pressure extraction (Casiraghi 2018, Elkins 2019). Olive oil provides a food safe alternative to toxic solvents and can be used to extract cannabinoid oils from cannabis as well as being used to dilute hemp oils for a consumer product (Casiraghi 2018). Samples with high concentrations of THC require decarboxylation prior to mixing the plant with olive oil (Casiraghi 2018). Different extraction methods call for different ratios and contact time for the plant and solvent.

The Association of Official Analytical Collaboration (AOAC) International published a method for quantifying cannabinoids in cannabis that uses two sequential ethanol

extractions with 89-91% ethanol, in which the sample is shaken for 30 min and filtered (AOAC 2018). Methanol is used to dilute standards, and samples are diluted 10 and 100 fold to run against a five point standard curve (AOAC 2018).

Methanol is a commonly used solvent for laboratory analysis for hemp (IALR 2021). The following method was given by a regulatory laboratory: 0.2 grams of dried hemp mixes with 40mL of methanol for five min at 500rpm (DCLS 2019). The methanol and hemp mixture settles for one hour before collecting 1mL of the extract for LC analysis. Using smaller samples of hemp creates a concern that the analytical sample will not be representative of the whole plant material. Cannabinoid concentrations vary within the stem, leaves, flowers and seeds, and a small will not be able to represent an entire plant.

The QuEChERS method is a commonly used extraction method for testing pesticides in food products and uses an acetonitrile extraction along with $MgSO_4$ to separate out hydrophobic compounds from water soluble products (AOAC 2007).

Hops-14 is a diethyl ether and methanol extraction used to extract alpha and beta acids from hops. This method uses diethyl ether to extract cannabinoids while the methanol is used because it is inter miscible with water and helps to separate out the hydrophobic and hydrophilic compounds. However, there are drawbacks to using diethyl ether as it is explosive and evaporates quickly (Gallagher 1991).

Limitations of current hemp analysis protocols

The extraction methods that use ethanol or methanol require the plant material to be dried before testing to remove any water present. Typical drying processes heat the sample and can drive off volatile compounds and therefore lead to lower quantification of the cannabinoids. The type of drying method is not specified, which can lead to discrepancies in overall testing values. High heating temperatures will drive volatile compounds off, decreasing the total concentration quantified. Heating can cause degradation in the different cannabinoids present: CBN is formed from THC, and THCA and CBDA decarboxylate into THC and CBD, respectively, affecting the separate quantification of these separate compounds.

The QuEChERS method is intended for food products, and requires wet materials for the extraction process (AOAC 2007). This method was created for pesticide testing in food products, with the intent to be used for a variety of food matrices while being able to interact and provide a full comprehensive measurement of what compounds are present. The QuEChERS method has not been evaluated for THC and CBD specifically. The QuEChERS method is being reviewed as a method to use to detect THC in food products (Lee 2020), but is not typically used for determining THC in the raw plant material. The methods for foods and plants should be evaluated together in order to provide comparable results.

The Hops-14 method uses wet plant material because methanol is combined with diethyl ether in order to separate out the water phase completely from the diethyl ether portion

(ASBC 2008). This method has not been evaluated for hemp products but could be applicable due to the similarities in cannabinoids and hops compounds.

Comparing LC and GC methods brings up questions regarding the use of heat during the GC-FID process. Sudol et al. (2020) address the auto-oxidation that occurs in diesel fuels when using GC methods, and brings forth the question that the high temperature of the injection port and column can degrade compounds before they reach the detector for GC methods, and decrease the overall content reported.

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Chapter Three: Experimental

Introduction

Cannabis sativa is a species of cannabis that has been bred specifically for medicinal use (Small 1976). Cannabis indica has historically been bred for psychoactive compounds. Delta-9-THC is the psychoactive compound naturally present in cannabis (Baker 1981). Cannabis has multiple different cannabinoids present, such as Cannabidiol (CBD), Cannabidiolic Acid (CBDA), Cannabinol (CBN), delta-9-tetrahydrocannabinol (THC), and Tetrahydrocannabinolic

acid (THCA). CBDA and THCA are the acidic forms of CBD and THC respectively. CBN is the degradation compound of THC. Regulations in the United States determine the total THC concentration as the distinguishing factor between hemp and marijuana (115th Session of Congress 2018).

Federal regulation in the United States classified cannabis as a restricted substance in 1971(Gabay 2013). The United States Agricultural Act of 2014 addressed defining cannabis that had less than 0.3% of delta-9-THC as industrial hemp, regardless of plant genotype, and allowed for research hemp production, but not commercial growing (113th Session of Congress 2014). However, this act in 2014 did not specify how THC was calculated and reported, and left speculation as to if the regulation was for only delta-9-THC and excluded THCA content. The passing of the 2018 United States Farm Bill allowed states to make their own regulations regarding industrial hemp, and specified the limit as 0.3% total THC (115th Session of Congress 2018).

When dealing with hydrophobic compounds, such as cannabinoid oils, ethanol is a desirable solvent as it is generally non-toxic and easy to find, as well as being able to interact with the target cannabinoids in hemp. Where ethanol is used, it can be argued that methanol could instead be used as it is a similar, cheaper compound, and methanol is commonly chosen in laboratories for in house validation testing for hemp extraction (IALR 2021).

The QuEChERS extraction was designed for pesticide testing in foods, as the acetonitrile solvent and salts used for the process can interact with multiple different matrices (AOAC 2007). Diethyl ether is used in order to extract alpha and beta acids from hops cones and pellets (ASBC 2008). This extraction was chosen because it was designed for wet and dry plant materials for hydrophobic compounds in hops. However, diethyl ether is hazardous and requires proper waste

disposal. For analytical testing purposes, the goal is to extract out a full comprehensive sampling of cannabinoids without allowing for degradation during processing. These four methods are tested and compared as to how they react with the target cannabinoids for regulatory and commercial purposes. The matrix effects on cannabinoids in different food products are being evaluated as to how it affects the analysis of the cannabinoids (Ciolino 2018).

Methods

Sample Prep

Two hemp plants (Cv. Hawaiian Haze) were obtained from the Virginia Tech Urban Horticulture Center in Blacksburg, VA on September 25, 2019, a week after commercial harvest maturity conditions as determined by visual observation of tricones on the plants. They were planted on July 5, 2019. These were field plants that had irrigation provided during their growth. The Hawaiian Haze hemp plant is a hybrid plant derived from Hawaiian and Haze parents as a high CBD and low THC producing strain (Gold CBD 2021).

The harvested aerial portions of the plant were frozen at -10ºC for approximately 48 hours, then the plant was destemmed and had the majority of the vegetative leaves removed, leaving primarily pruned flowers. The plant was always handled frozen in order to limit the loss of volatiles and oils. The pruned flowers from both plants were then mixed together to obtain one homogenous sample of sufficient volume for this study, vacuum sealed in gallon sized bags using a hand held vacuum sealer (FoodSaver, Atlanta, Georgia), and stored frozen at -80°C until the time of analysis.

The pruned flower material was removed from the freezer and again mixed thoroughly to obtain a homogenous sample which was then separated out into aliquots for the subsequent sample preparation treatments. Around 70g of this bulk sample was vacuum sealed and stored at -80°C for sample preparation using wet methods. Around 600g of the bulk sample was subsequently dried. This plant material was dried at 90ºC (85-95ºC) in a gravity convection lab oven (Quincy Lab Inc Model 10 oven) for 60 min. Fourteen containers were weighed, and wet plant material was placed in the containers and weights recorded, prior to placing the containers into the drying oven. The filled containers were cooled to room temperature in a desiccator and weighed. The heating, cooling, and weighing process was repeated until samples maintained a consistent weight. After the weight was consistent, indicating that the plant matter was completely dried, the samples were combined and frozen to -80ºC prior to grinding. The samples were frozen again at this stage to facilitate grinding as well as to limit the loss of any volatiles.

All of the dried plant material was combined and chopped with a Ninja express chopper (Model NJ100GR) for ten seconds at a time until the material was fine enough to shake through sieves. The ground material was shaken through a number 16 sieve with a 1.19mm mesh and then through a number 18 sieve with a 1mm mesh, and the sieved material was collected. Approximately 150 g of 1mm dried material was vacuum sealed and stored at -80ºC.

The ground samples were mixed thoroughly producing one homogenous sample of dried material. This dried material was then divided into four aliquots for the four extraction

methods, vacuum sealed and stored at -80ºC until extraction procedures were performed the next day.

Extraction Methods Evaluated

AOAC Extraction Method

The first extraction method evaluated was the AOAC 2018.11 method (AOAC 2018). The Association of Official Analytical Collaboration (AOAC) International is a 501(c)3 non-profit organization that validates scientific standards and methods. The 2018.11 method quantifies cannabinoids in dried plant materials or liquids, and does not address testing food products. This method was first written and published in 2018 in response to the newly federally legal hemp industry. A sample of the dried material was taken and processed according to the AOAC 2018.11 method. Triplicage 0.5 g portions from the sample were weighed and placed into 50mL centrifuge tubes with 20mL of ACS grade 91% ethanol (Fisher Scientific, High Point, North Carolina) added. The tubes were shaken for 30 min on a vertical shaker (Fisherbrand variable speed wrist motion) and at the end of 30 min, centrifuged in a Sorvall RC-5B Refrigerated Superspeed centrifuge (Dupont, Wilmington, Delaware) at 3000g for ten min. Each sample was filtered through Whatman grade 40 filter paper and collected into 50mL separately labeled volumetric flasks. The plant material was transferred back to the centrifuge tube to repeat the extraction, centrifugation, and filtration process. Each sample was diluted to 50mL final volume. A 3 mL aliquot of each of the triplicate extracts was filtered through a 0.22um PTFE filter (AQ Brand, Leland, North Carolina) into tubes. One mL of each extraction was transferred to LC tubes in triplicates where they were capped and queued at 4ºC for

UPLC analysis which started within 1hr of completing the extractions. The dilution factor of this extraction process is 100w/v with 0.5g of sample to 50mL ethanol. Samples were further diluted with 99.9% LC/MS grade methanol (Fisher Scientific, High Point, North Carolina) 1:1 in the UPLC vials for a final dilution of 200w/v. (AOAC 2018).

Methanol Extraction Method

For the method that is used by the Virginia State testing facility, a portion of dried material was removed from -80°C and triplicate samples (0.2g) were placed in separate centrifuge tubes with 40mL 99.9% LC/MS grade methanol (Fisher Scientific, High Point, North Carolina) added (DCLS 2019). Samples were mixed by a lab-line orbital environ-shaker (model 3527) shaker (Lab Line,Melrose Park, Illinois) for 5 min, and then allowed to settle for one hr at room temperature. A 3 mL aliquot of each extract was filtered through a 0.22um PTFE filter (AQ Brand, Leland, North Carolina) into tubes. A one mL aliquot of each filtered sample was transferred to LC glass screw neck vials with PTFE silicone septa (Waters, Milford, Massachusetts) where they were capped and held at 4ºC while queuing for UPLC analysis. The final dilution of this extraction process is 200w/v with 0.2 g of sample to 40mL methanol.

QuEChERS ExtractionMethod

The QuEChERS method was chosen because it is used on wet plant material to commonly extract out and test pesticides (AOAC 2007). The QuEChERS acronym stands for Quick, Easy, Cheap, Effective, Rugged, and SaFe (AOAC 2007). Because of the moisture content needed to react with the salts in this method, only wet material was used for this extraction method. Wet material was removed from a -80ºC freezer immediately

before extraction using the QuEChERS method. Triplicate 10g samples of wet material were placed in 50mL centrifuge tubes and mixed with 10mL of 1% acetic acid in Acetonitrile and 5g grams of Magnesium sulfate and Sodium Acetate (4/1, w/w). Tubes were shaken by hand and centrifuged at 3000 x g in a Sorvall RC-5B Refrigerated Superspeed centrifuge (Dupont, Wilmington, Delaware) for 5 minutes. 6 mL of solution was added to 1200mg MgSO4/PSA sorbent (3/1 w/w). Three mL of each extract was filtered through 0.22um PTFE filters (AQ Brand, Leland, North Carolina) into tubes, and 1mL of samples were transferred to LC tubes where they were capped for UPLC analysis and held at 4ºC until queuing for UPLC analysis. The dilution factor of this extraction process is 2 w/v with 10g of sample to 10mL solution. Samples were further diluted by 100v/v using 99.9% LC/MS grade methanol (Fisher Scientific, High Point, North Carolina) in the UPLC vials for a final dilution for measurement of 200 (AOAC 2007).

Hops 14 Diethyl Ether Extraction Method

Fifty grams of dried and fifty grams of wet material were removed from -80ºC for the Hops14 extraction method (ASBC 2008). The Hops 14 method uses diethyl ether to extract alpha and beta acids from either fresh wet hop cones or dried hop pellets (ASBC 2008). This method was chosen for this project because it was written to be used for both wet and dry plant material, and would be a good source to compare differences between wet and dry testing. Three 10g samples of dry material, and three 10g samples of wet material were placed into separate 250mL flasks. Twenty mL of 99.9% LC/MS grade methanol (Fisher Scientific, High Point, North Carolina) and 100mL of BHT stabilized ACS diethyl ether (Fisher Scientific, Bengal, India) were added to the flasks, which were capped and placed on a vertical shaker (Fisherbrand variable speed wrist motion) for 30

min. At the end of 30 min, 40mL of 01.N HCL was added and mixture was placed back on the vertical shaker for 10 min. Flasks were allowed to settle for 10 min and 5mL of the diethyl ether phase was transferred to 50mL volumetric flask and brought to volume with 99.9% LC/MS grade methanol (Fisher Scientific, High Point, North Carolina). Samples were centrifuged at 3000x G in a Sorvall RC-5B Refrigerated Superspeed centrifuge (Dupont, Wilmington, Delaware) and filtered through 0.22um PTFE filter (AQ Brand, Leland, North Carolina) into tubes. One mL of each sample was transferred to LC vials and capped for analysis. The dilution factor for these samples is 100 with 10g sample to 100mL diethyl ether, and 5mL of that solution to 50mL total. The initial 20mL methanol does not factor into the dilution volume because it is used to separate the water and ether phases and is not included with the dilution in the final volume (ASBC 2008).

Cannabinoid Quantification by UPLC/PDA

Once extraction methods were performed, all of the liquid cannabinoid extracts samples were kept at -20^{o}C in the dark until preparing for cannabinoid analysis by UPLC. Once loaded onto the Waters ACQUITY UPLC H-Class 9 (Waters Corp, Milford, Massachusetts), samples were kept at 10^{o}C while awaiting injection. UPLC analysis was concluded for each set of extraction samples within 24 hrs of the extraction process as the Hops 14 method states that samples are stable for 24 hrs and that samples should be kept cool and in the dark (Hops 14). The ethanol and methanol extraction methods do not specify timing or stability of extractions after processing. One mg/mL certified standards in acetonitrile of CBD, CBDA, CBN, delta-9-THC, and THCA from Ceriluent were obtained from Sigma Aldrich and stored at -80^{o}C. One hundred uL of each standard was added to a 10mL volumetric flask and was diluted to volume with methanol for a stock

solution with a concentration of 10ug/L for each standard. Standards were created using instruction from the AOAC 2018.11 method as listed in Table 1. Two hundred fifty uL of 200ppm Ibuprofen internal standard was added to each vial that contained 1mL sample or standards. A 1% solution of LC/MS grade formic acid (Fisher Scientific, High Point, North Carolina) and ultra-pure HPLC grade water and a 1% solution of formic acid in LC/MS grade acetonitrile (Fisher Scientific, High Point, North Carolina) were prepared as solvents for UPLC analysis, and 10% methanol in water was prepared as a wash solution (Waters 2019).

Table 1: Preparation of Standard Concentrations of Cannabinoids for UPLC Analysis

Bought Standards [mg/mL]	V of std to stock solution (uL)	Total Volume of Std Stock Solution (mL)	Std Stock Solution [ug/mL]	V of std to add (mL)	Final [C] mg/mL	V Ibuprofen (mL)	V MeOH (mL)
1	125	10	12.5	0.25	2.5	0.25	0.75
1	125	10	12.5	0.5	5	0.25	0.5
1	125	10	12.5	0.7	7	0.25	0.3
1	125	10	12.5	1	10	0.25	0

Samples were run on a Waters UPLC according to the method “UPLC Separation for the Analysis of Cannabinoid Content in Cannabis Flower and Extracts by Waters” (Waters 2019). Analytical column CORTECS UPLC Shield RP18, 90A, 1.6um, 2.1 x 100mm was used on a LC System ACQUITY UPLC H-Class using a flow rate of 0.7mL/min. Mobile phase A consisted of ultra-pure HPLC grade water with 0.1% formic acid. Mobile phase B consisted of Acetonitrile acidified to 0.1% formic acid. The acid concentration remains consistent across buffers. This method used the PDA detector at 228nm wavelength at 4.8nm resolution. The injection volume was 20 uL for 1mg/mL

reference. Empower 3 CDS software (Waters, Milford, Massachusetts) was used to process and analyze data. For results that fell above the range of the standards, the sample was diluted 1:10 using 100uL of the extraction sample and 900uL of methanol. A 1:100 dilution was made using 100uL of the 1:10 dilution, and 900uL of methanol. Testing the different dilution factors were necessary as some of the cannabinoid concentrations required the higher dilutions. Figures 3 and 4 show chromatogram examples from the UPLC analysis.

Figure 3. Chromatogram Example of a Standard Sample

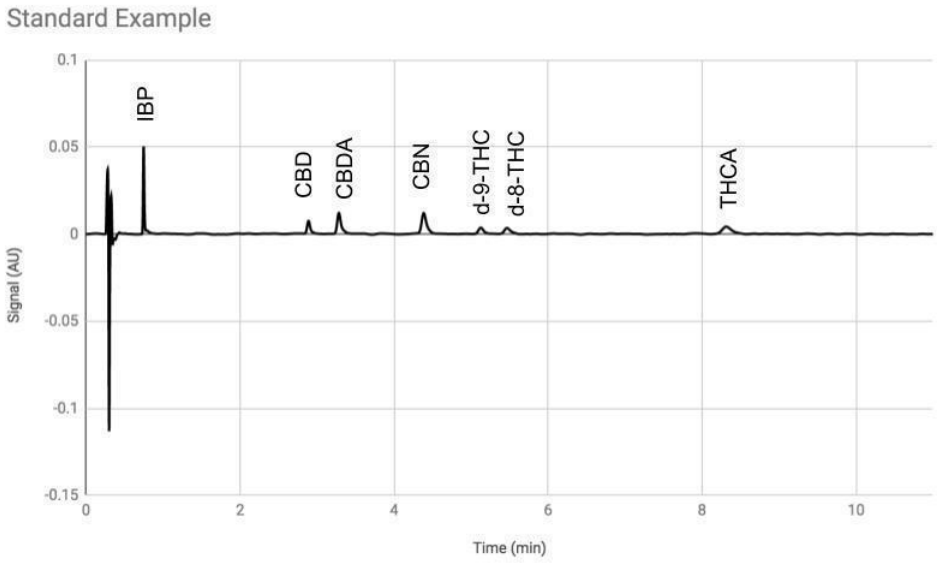
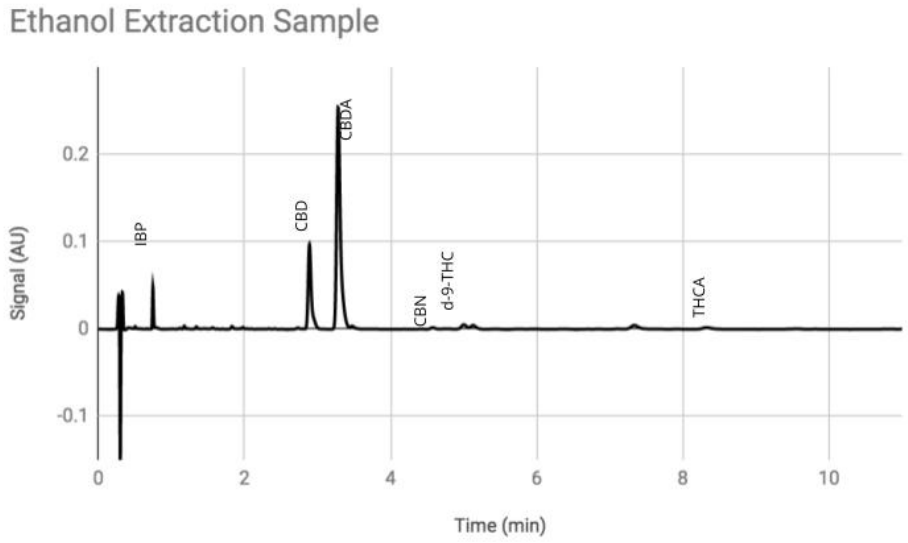


Figure 4. Chromatogram Example of an Ethanol Extraction Sample



Statistical Methods and Data Analysis

Data was analyzed using one-way ANOVA followed by comparison of treatment means using Tukey's HSD/ A significant difference was defined as $p<0.05$. Table 4 shows the p value differences between the methods.

Table 4: P values for Mean Comparison Between Methods

Method	Compared Method		CBD	CBDA	CBN	d-9-THC	THCA	Total THC
EtOH	MeOH	p-Value	0.9416	0.2934	0.6192	0.0322	0.9960	0.3773
EtOH	QuEChERS	p-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
EtOH	Hops Wet	p-Value	<0.0001	<0.0001	<0.0001	<0.0001	0.3372	0.0005
EtOH	Hops Dry	p-Value	<0.0001	<0.0001	<0.0001	<0.0001	0.0507	<0.0001
MeOH	QuEChERS	p-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MeOH	Hops Wet	p-Value	<0.0001	<0.0001	<0.0001	<0.0001	0.1791	<0.0001
MeOH	Hops Dry	p-Value	<0.0001	<0.0001	<0.0001	<0.0001	0.0204	<0.0001
QuEChERS	Hops Wet	p-Value	0.9985	<0.0001	1.000	<0.0001	<0.0001	<0.0001
QuEChERS	Hops Dry	p-Value	0.9416	<0.0001	0.2754	<0.0001	<0.0001	<0.0001

Hops Dry	Hops Wet	p-Value	0.8667	0.7809	0.2754	0.0491	0.8726	0.2105

Results and Discussion

Final values for % cannabinoids obtained using each of the extraction methods evaluated are shown in Table 3. Data collected from the UPLC was corrected for dilution factors, calculating in the mL of extract and grams of hemp used in the methods. Values were then calculated from ug/g to g/100g in order to determine percent values (w/w). Samples that used wet material were corrected for moisture totals from the data in table 7.1 to report values using dry weight basis.

Table 3: % Dry Weight of Cannabinoids

Method	CBD	CBDA	Total CBD	CBN	D9THC	THCA	Total THC
	Dry Weight %	Dry Weight %	Dry Weight %	Dry Weight %	Dry Weight %	Dry Weight %	Dry Weight %
EtOH 1-1	3.086	3.774	6.481	0.026	0.179	0.094	0.262
EtOH 1-2	4.714	4.618	4.618	0.041	0.178	0.117	0.281
EtOH 1-3	3.074	4.112	4.112	0.023	0.168	0.086	0.244
EtOH 2-1	2.520	5.132	5.132	0.024	0.184	0.108	0.279
EtOH 2-2	2.690	3.832	3.832	0.040	0.156	0.115	0.257
EtOH 2-3	4.312	3.118	3.118	0.032	0.185	0.089	0.263
EtOH 3-1	4.860	3.594	3.594	0.041	0.177	0.096	0.262
EtOH 3-2	4.696	3.644	3.644	0.025	0.167	0.066	0.225
EtOH 3-3	2.182	5.206	5.206	0.037	0.166	0.110	0.262
MeOH 1-1	3.698	4.262	4.262	0.036	0.211	0.085	0.285
MeOH 1-2	5.920	4.778	4.778	0.030	0.216	0.107	0.310
MeOH 1-3	4.330	4.266	4.266	0.025	0.211	0.101	0.300
MeOH 2-1	4.648	4.252	4.252	0.045	0.230	0.118	0.334
MeOH 2-2	3.726	5.478	5.478	0.031	0.236	0.115	0.337
MeOH 2-3	2.676	4.824	4.824	0.037	0.214	0.120	0.320
MeOH 3-1	2.568	5.260	5.260	0.035	0.225	0.115	0.326
MeOH 3-2	3.162	4.878	4.878	0.038	0.246	0.106	0.339
MeOH 3-3	3.548	4.676	4.676	0.038	0.227	0.108	0.322
Q 1-1	0.085	10.848	10.848	0.000	0.783	0.483	1.206
Q 1-2	0.119	8.739	8.739	0.000	0.645	0.283	0.893

Q 1-3	0.119	7.594	7.594	0.000	0.561	0.316	0.838
Q 2-1	0.077	11.313	11.313	0.000	0.558	0.613	1.096
Q 2-2	0.100	9.800	9.800	0.000	0.635	0.619	1.177
Q 2-3	0.118	9.835	9.835	0.000	0.659	0.614	1.198
Q 3-1	0.107	9.174	9.174	0.000	0.628	0.531	1.094
Q 3-2	0.070	9.819	9.819	0.000	0.528	0.358	0.842
Q 3-3	0.103	11.368	11.368	0.000	0.576	0.441	0.963
HD 1-1	0.336	0.708	0.708	0.005	0.029	0.021	0.047
HD 1-2	0.313	0.634	0.634	0.004	0.029	0.015	0.043
HD 1-3	0.306	0.717	0.717	0.004	0.020	0.021	0.039
HD 2-1	0.336	0.615	0.615	0.004	0.031	0.017	0.045
HD 2-2	0.311	0.648	0.648	0.004	0.020	0.017	0.035
HD 2-3	0.324	0.544	0.544	0.004	0.025	0.022	0.044
HD 3-1	0.378	0.602	0.602	0.004	0.026	0.009	0.034
HD 3-2	0.244	0.614	0.614	0.004	0.021	0.020	0.039
HD 3-3	0.293	0.641	0.641	0.004	0.029	0.009	0.037
HW 1-1	0.008	0.814	0.814	0.000	0.073	0.047	0.114
HW 1-2	0.010	1.195	1.195	0.000	0.074	0.061	0.127
HW 1-3	0.013	1.001	1.001	0.000	0.054	0.038	0.087
HW 2-1	0.013	1.012	1.012	0.000	0.068	0.021	0.086
HW 2-2	0.009	0.914	0.914	0.000	0.079	0.086	0.155
HW 2-3	0.010	0.944	0.944	0.000	0.058	0.024	0.079
HW 3-1	0.008	1.036	1.036	0.000	0.095	0.039	0.129
HW 3-2	0.011	1.013	1.013	0.000	0.073	0.048	0.115
HW 3-3	0.008	1.060	1.060	0.000	0.086	0.032	0.114

Figure 5. Mean Values of the Concentration of Five Cannabinoids vs. Extraction Methods

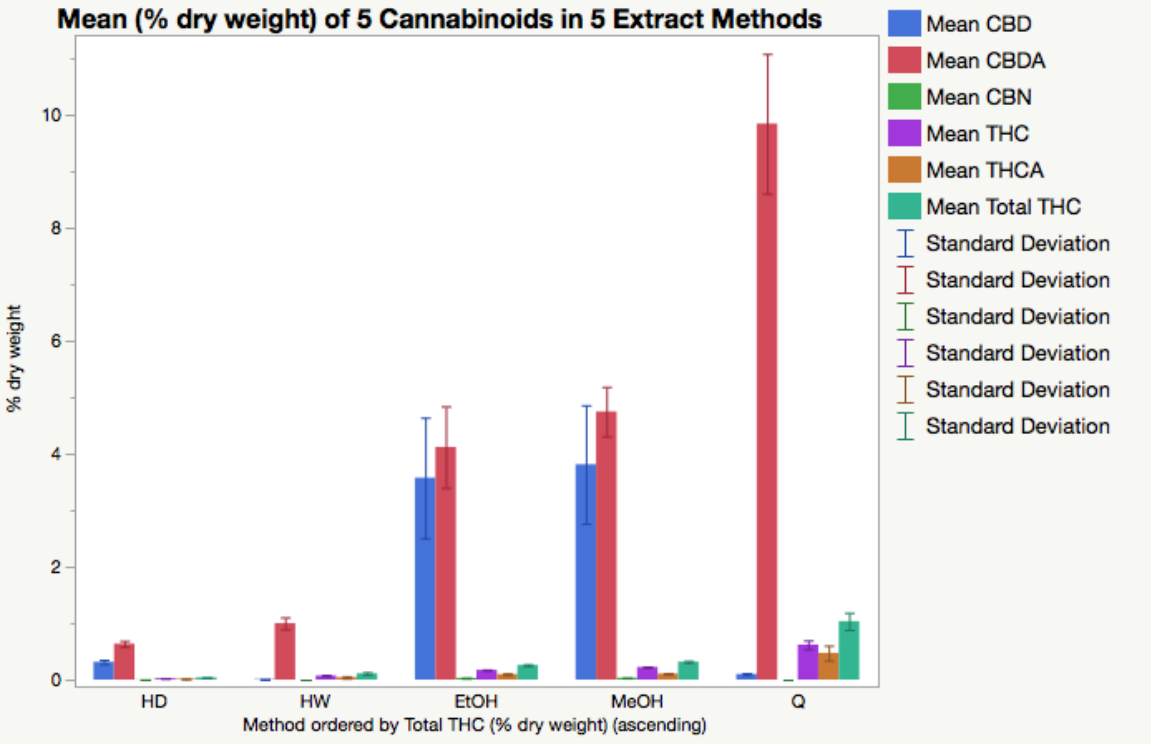
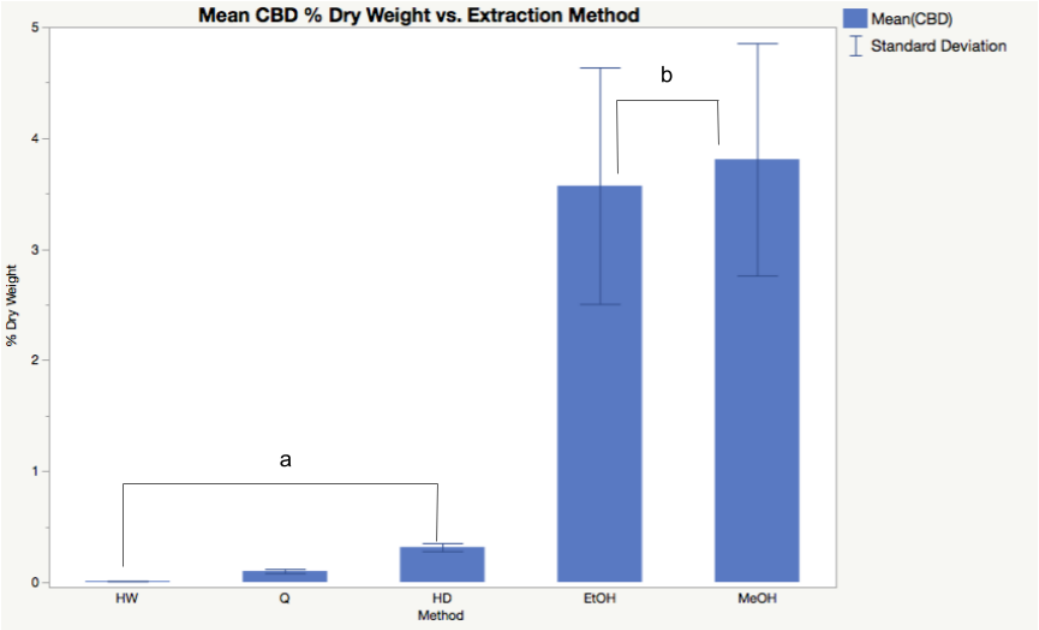


Figure 5 shows the mean of the results between each extraction method and the 5 cannabinoids tested.

Figures 6-10 shows the differences of the individual cannabinoids between methods. Lower case letters (a, b, c, d, e) denote significant differences between the methods. The QuEChERS (Q), and wet (HW) and dry (HD) diethyl ether extractions reported the lowest values of CBD with no difference between these three methods. The ethanol and methanol extractions reported the highest CBD percentage with no significant difference between those two methods.

Figure 6. Mean Values of the Concentration of CBD vs. Extraction Methods



The QuEChERS method reports the highest CBDA values between methods, as depicted in Figure 7. The difference between CBDA and CBD and the extractions could be attributed to degradation occurring from CBDA to CBD in the ethanol and methanol extractions.

Figure 7. Mean Values of the Concentration of CBDA vs. Extraction Methods

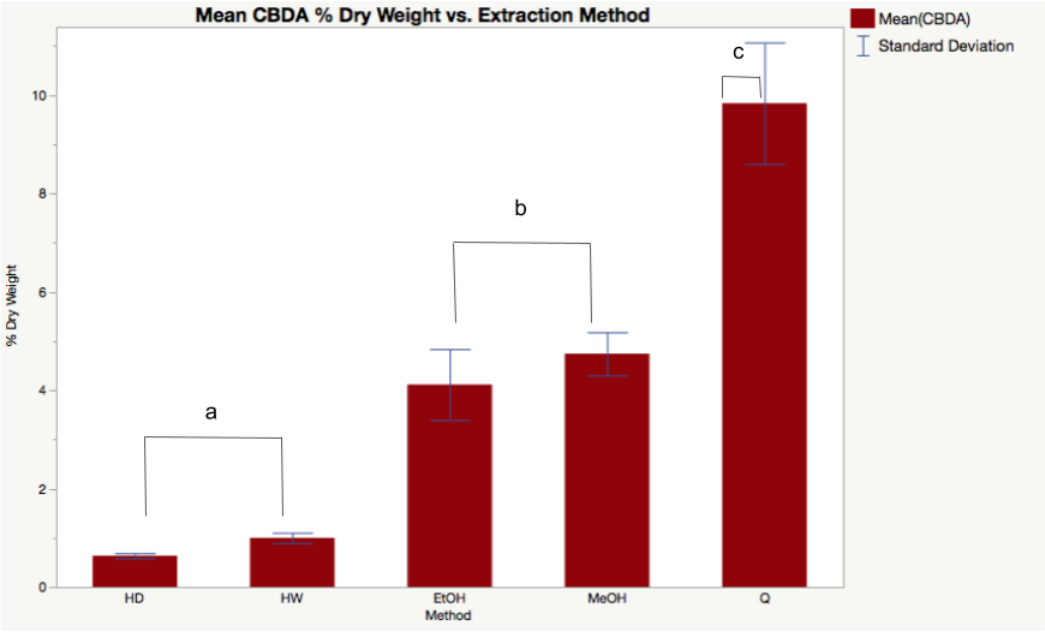


Figure 8 shows the CBN levels in each extraction. The two methods performed on wet plant material, HW and Q, had no detectable levels of CBN, and was not significantly different from the measurable CBN values when compared to the dry diethyl ether extraction. The ethanol and methanol extractions had higher levels of CBN measured, which could be contributed to the drying procedure used.

Figure 8. Mean Values of the Concentration of CBN vs. Extraction Methods

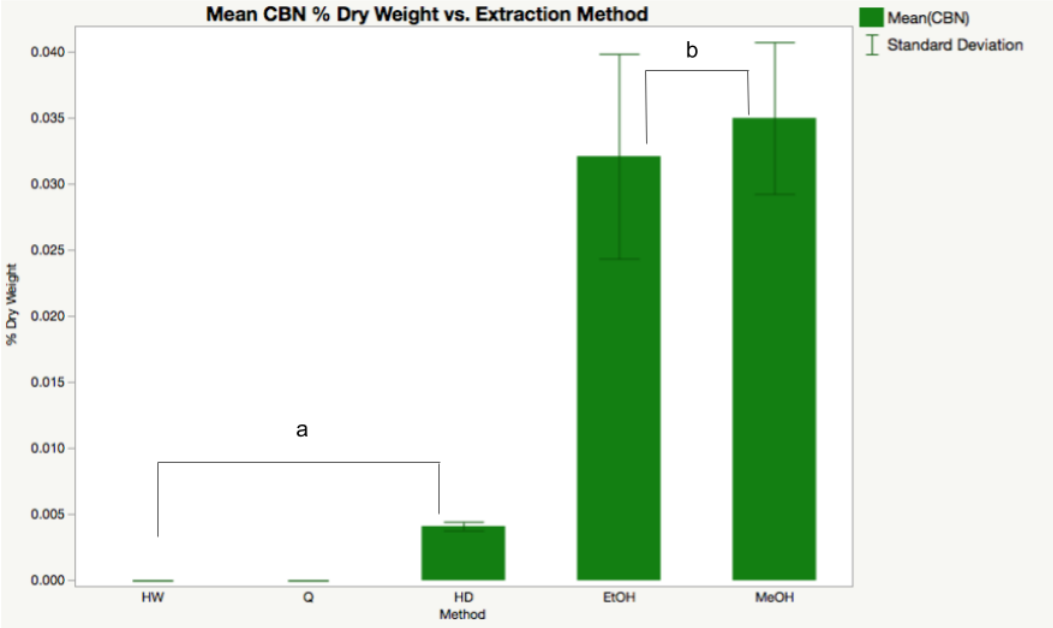
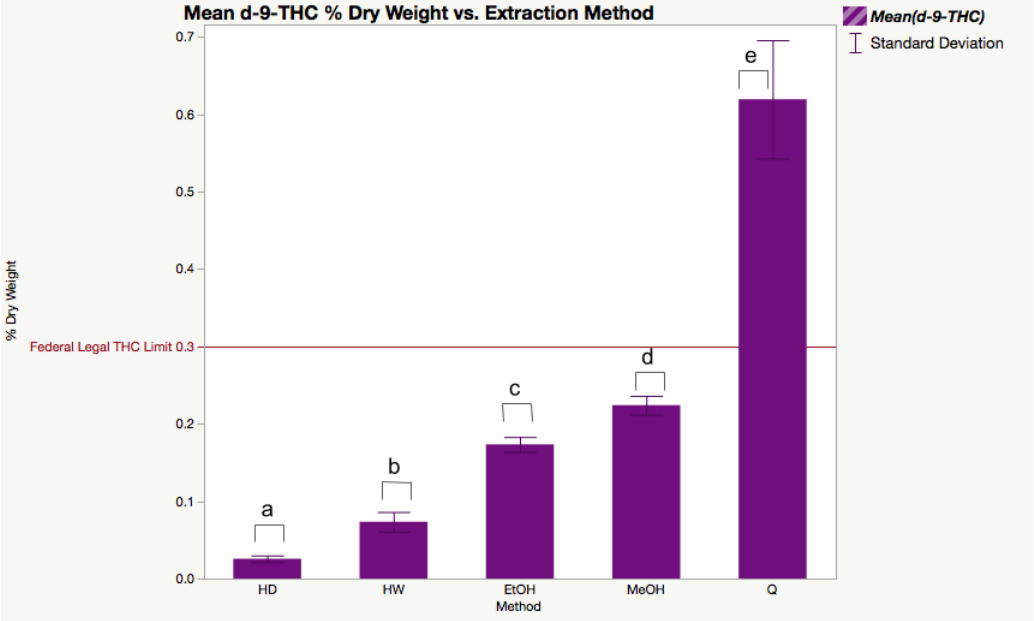


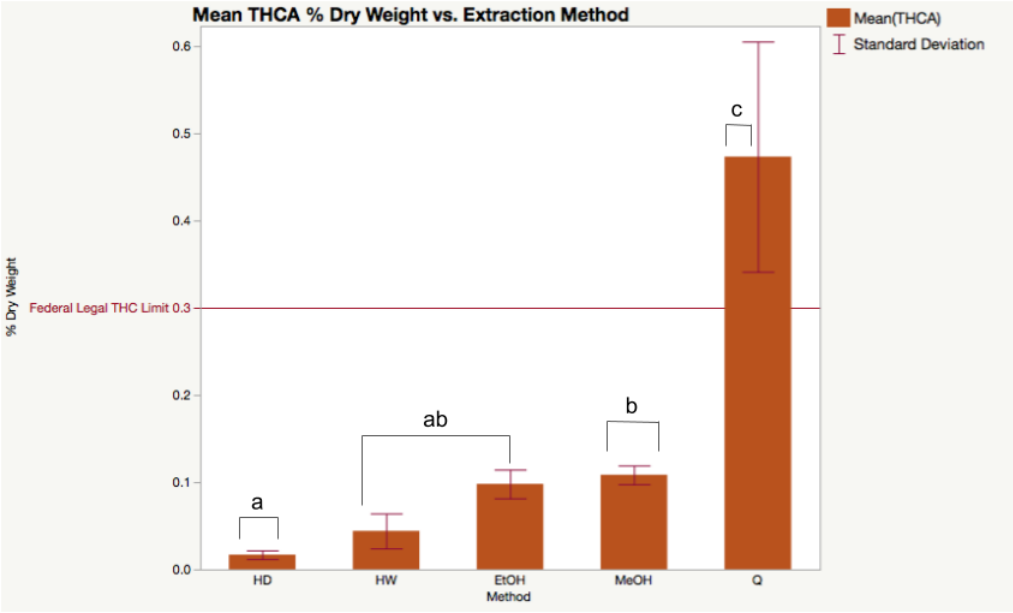
Figure 9 shows the delta-9-THC concentrations across the extraction methods. The QuEChERS extraction reported the highest levels of delta-9-THC, with all methods showing significant difference from each other.

Figure 9. Mean Values of the Concentration of delta-9-THC vs. Extraction Methods



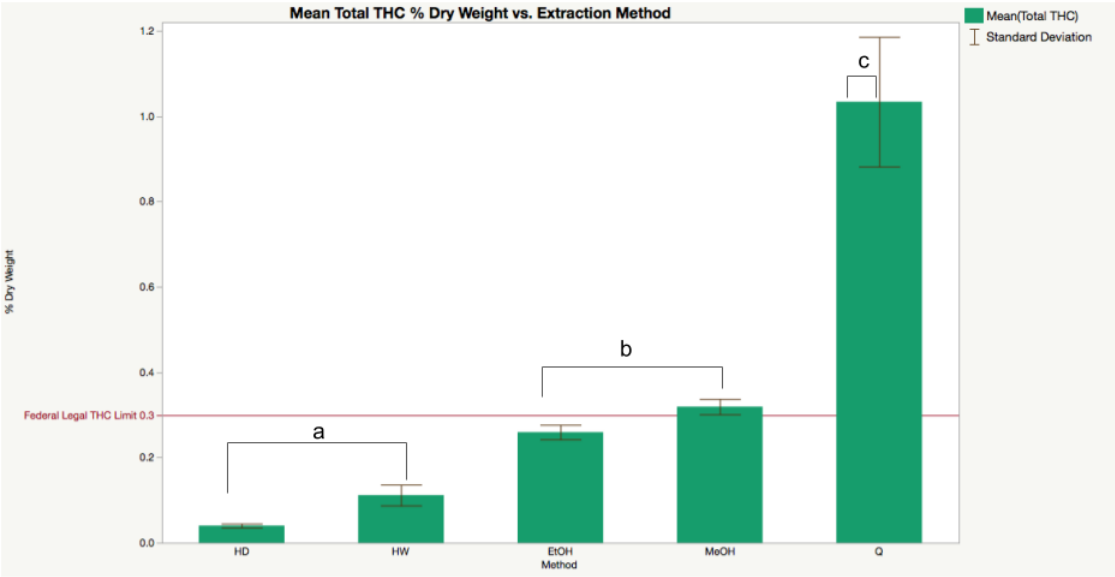
THCA concentrations among extraction methods are depicted in Figure 10. The QuEChERS method is once again showing the highest levels of THCA compared to all other extractions measured.

Figure 10. Mean Values of the Concentration of THCA vs. Extraction Methods



Calculated total THC values per method are illustrated in n Figure 11. The QuEChERS extraction reported the highest level of Total THC concentration in the sample. The ethanol and methanol extractions are not significantly different from each other, but are different from the other methods tested. It is important to note that the delta-9-THC levels of the methanol extraction were measured below the federal U.S. legal limit of 0.3%, but total THC calculated is listed above 0.3% when the THCA concentrations are factored in.

Figure 11. Mean Values of the Concentration of Total THC vs. Extraction Methods



Each analytical method was different between all the other methods for delta-9-THC when the means were compared. The ethanol and methanol methods were not different from each other for the other parameters of CBD, CBDA, CBN, THCA, and Total THC. The QuEChERS method was different from both the ethanol and methanol methods in every parameter. The QUECHERS method reported higher results for both delta-9-THC and THCA for a higher total THC reported compared to the other methods. The methods that used ethanol and methanol with dry material and included water interactions through the extraction process had higher values of CBN. The diethyl ether method that used ether on dry products showed lower levels of CBN, and the two methods (diethyl ether and QuEChERS) that used wet material did not have CBN detected. The ether methods, both wet and dry, showed lower amounts of all compounds detected while the QuEChERS method showed the highest values of CBDA, Delta-9-THC, and THCA.

Conclusions

Table 4 shows the confidence intervals and p values of each method compared to another method. Using a significant value of $p < 0.05$, the delta-9-THC value compared between the ethanol and methanol methods are significantly different.

The QuEChERS method has significant differences with p values of <0.0001 on all cannabinoids when compared to both the ethanol and methanol methods. The QuEChERS method is significantly different with p values <0.0001 for CBDA, d-9-THC, THCA and total THC when compared to both the wet and dry hops method. There is no significant difference for CBD and CBN between those methods. CBN was not detected in QuEChERS and the wet hops method, and the CBN reported in the other three

methods can be attributed to the drying process where CBN is formed by THC degrading due to heat.

The CBN values were affected by drying before testing. The ethanol and methanol extractions required drying, whereas the diethyl ether method was written for both dry and wet materials, and the QuEChERS method requires a certain amount of water present for the salts to interact with the material. Drying temperature and time could affect the production of CBN. No detectable CBN values were present in either of the methods that started with wet material and lower amounts of CBN were detected in the diethyl ether method with the dry material when compared to the ethanol and methanol methods that used dry product. Decarboxylation is a hydrolysis reaction, which requires the presence of water to occur. The nature of the diethyl ether and QuEChERS methods limit the degradation of THCA and CBDA because the compounds used separate water out of the material. This preserves the THCA and CBDA content and does not allow the compounds to degrade further after the extraction process. The methods using ethanol and methanol require dry products to extract the oils, but some water remains and is able to contribute to the hydrolysis of THCA and CBDA into THC and CBD respectively. The amount of samples and the amount of solvent (w/v) is not defined, and is a factor to look into regarding consistent extraction methods. The methanol extraction method used 0.2g of plant material, and the ethanol extraction used 0.5g of dried plant. Small plant samples do not allow a representative sample for an entire plant. Berthold et al discusses the quantification of 12 cannabinoids in a laboratory hemp sample with using methanol extraction, and compares concentrations between composite and flower samples

(Berthold 2020). A significant difference was found between the mean differences in the total CBD and total THC values between the composite and flower samples (Berthold 2020). Plant age can also determine amounts of cannabinoids in the different parts of the plants, as Hemphill et al determined that cannabinoids concentration decreased with leaf age (Hemphill 1980). Having a larger sample material used for the testing increases the representation of the entire plant.

The QUECHERS method showed the highest value in CBDA, delta-9-THC, and THCA. This illustrates that the other methods are not as comprehensive in extracting cannabinoids. Because the QuEChERS method uses salts and amine exchange materials to react with hydrophobic compounds, the method is not compound specific and reacts with cannabinoids as well as pesticides and other hydrophobic molecules (Atapattu 2020).

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Chapter Four: Future Work

Plant and sample storage

Harvest time will affect the composition of cannabinoids in the plant, and comparing THC and CBD content between plant maturity can help determine which species of hemp would provide more desirable cannabinoid content depending on the final product needed. Different cannabinoids are found at different parts of the plant and decrease in concentration over time (Hemphill 1980).

Hemp storage techniques currently vary in the United States as hemp production is newly regulated. Light and dark conditions should be considered as Mazzetti et al explore the effects of lights on CBD concentrations, suggesting that CBD oxidizes in the presence of light (Mazzetti 2020). Samples evaluated in a thirty day period showed degradation of CBD (Mazzetti 2020). Farmers work to determine how to keep their product from

molding or damaged by pests before being processed or sold, and the plant is often hung in barn areas with uncontrolled temperature and humidity to dry. Evaluating different humidity and temperatures for drying fresh plants would provide valuable information.

The samples obtained were frozen prior to pruning and extraction. This was done in order to prevent any degradation of cannabinoids over time and to prevent mold growth. The interactions between different temperatures and freezing and thawing impact on the raw plant material should be evaluated. AOAC methods recommend that samples are frozen prior to grinding in order to minimize loss of volatile compounds (AOAC 2018). Storage conditions such as an air tight container or in a plastic bag should be compared, as well as different temperatures of -4^{o}C, -20^{o}C, and -80^{o}C.

Drying Methods

Open air oven is often discussed as the procedure for drying hemp plants for extraction. Ninety ^{o}C was the recommended temperature used (DCLS 2019). Procedures specified for the product to be heated at 90^{o}C, cooled after an unspecified amount of time, and weighed to determine moisture lost and the process is repeated until the weight after drying is the same. This creates inconsistency on how long samples are heated, and contributes to differences in loss of analytes. Comparing heating temperatures of a lower temperature and longer time, or a specific time range for 90^{o}C heat can help determine a more consistent drying process for cannabis regulations. Drying with vacuum ovens should be explored, as drying can be achieved with lower temperatures to avoid the loss of volatile compounds or degrading analytes.

Water interaction and solubility

CBDA and THCA degrade into CBD and THC through decarboxylation, which involves the loss of CO_2 and requires water for the interactions to occur. Evaluating water activity in plant material could be beneficial for determining how to limit the degradation of these cannabinoids. The freezing process of plant samples and cooling process of keeping diluted samples at 4ºC while they await testing on the LC could affect water interaction. Amount of extraction solvent can be affected by the overall oil content. The solubility of the different cannabinoids and ratio of cannabinoids to each other can affect how much solvent will be needed for maximum extraction.

Time of degradation after extraction

The methods that had hydrolysis reactions occur showed a higher difference in CBD and CBDA measurements. It should be explored to determine when the hydrolysis reactions are happening within the samples and if the reactions are happening until an equilibrium is reached, until all of the CBDA content is decarboxylated to CBD, or if time is a factor. The reaction could be happening during the drying process, after extraction with ethanol and methanol, or after the sample is diluted and while the sample is waiting for analysis on the LC equipment.

Testing in Food Products

Foods that have CBD or THC as added ingredients are called “edibles”, which is an alternative way to consume *Cannabis sativa* products without smoking the plant material. Common food items that are sold with THC or CBD are baked goods, confections, or

gummy candies. There is concern over the dosage and effects of THC being added to foods as the chemicals go through the digestive system which decreases the dosage amount of THC into the bloodstream and delays the effect (NIDA 2020). The concern is that more product will be consumed due to the delay as compared to smoking the plant form.

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