A full factorial design study used to improve the extraction of cannabinoids from CBD-dominant cannabis flower using subcritical-CO_2 extraction

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B.S., West Virginia Wesleyan College, 2018

A REPORT

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

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KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

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Abstract

Subcritical carbon dioxide (sub-CO_2) extraction of cannabinoids can be optimized by understanding the parameters that significantly affect the extraction process. Extraction parameters, such as temperature, pressure, and extraction time, have been shown in multiple studies to affect the extraction process greatly. However, the effects of variabilities in the starting biomass are not often considered. To study the effect these variables have on the extraction process, a design of experiments approach was used to analyze prerecorded data from a cannabis oil production plant. A two-level, full factorial design with two variable components over eight batches was analyzed for process improvement. The factors considered include the initial total cannabidiol (CBD) content and initial moisture content, which was varied with a decarboxylating drying process prior to extraction. The effects of extraction time were also considered by individually analyzing the mass percent yield separated after 2, 4, and 6 hours for each trial. The statistical analysis determined that all variables considered, extraction time, the total CBD content, and a decarboxylating drying pretreatment (moisture content), had statistically significant effects on the mass percent yield of sub-CO_2 cannabis extract produced. These findings suggest that a decarboxylating drying process implemented before extraction would significantly increase the extraction efficiency by increasing the mass percent yield of CBD-dominant cannabis extract separated when using sub-CO_2 extraction.

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Abbreviations

ANOVA	analysis of variance
CBD	cannabidiol
CBDA	cannabidiolic acid
CO_2	carbon dioxide
DOE	design of experiments
SFE	supercritical fluid extraction
Sub-CO_2	subcritical carbon dioxide
THC	tetrahydrocannabinol
THCA	tetrahydrocannabinolic acid
%CBD	total CBD content
%Cont	percent contribution
%M	moisture content
%yield	mass percent yield of extract

1. Introduction

Cannabinoids extracted from cannabis flowers are becoming ever more valuable due to their potential medical advantages and therapeutic effects.[1,2] Due to its efficiency in isolating plant components, supercritical fluid extraction (SFE) has become an important extraction method that can separate valuable compounds on an industrial scale without the use of hazardous organic solvents. Carbon dioxide (CO_2) is a nontoxic solvent frequently used in SFE not only because its nonhazardous, but because its solubility and selectivity can be manipulated by making changes to the temperature and pressure. Additionally, CO_2 is chemically inactive, affordable, and accessible, making it an ideal solvent.[3–5]

When processing cannabis, it is difficult to achieve a balance between the quality and quantity of extracts produced. Cannabis processors can isolate desired compounds upon fine-tuning the selectivity by adjusting the extraction parameters; this leads to a more potent product the requires less post-processing. In other words, by changing variables such as the extraction temperature and pressure, the potency of extract products can be increased by minimizing the presence of unwanted coextracts, in turn, concentrating the cannabinoid of interest in the overall mass of extract produced. Due to a lack of understanding of how SFE is affected by the extraction parameters, variations in the starting materials, and the fluid dynamics of the extraction process, it is difficult to determine which parameters have a significant impact on the selectivity of the solvent and the extraction efficiency. To consider many potentially significant variables simultaneously, the implementation of experimental design techniques has proven useful.

A factorial design of experiments (DOE) is commonly used to assess the main and interaction effects between variables and on measured response variables. It is regarded as an efficient and information-rich approach for analyzing analytical data that reduces the resources, time, and effort required to determine the parameters that have a significant effect on a process.[6–9] By determining contributing factors in SFE, the process can be adjusted to optimize the extraction efficiency and selectivity for different compounds from cannabis. The purpose of this study is to use a full factorial DOE approach to determine how variabilities in the starting material affect the percent yield of material extracted from CBD-dominant cannabis. The variables considered include the total CBD composition, moisture content, and extraction time.

In terms of quality, subcritical CO_2 (sub-CO_2) has experimentally proven to be superior to supercritical CO_2 for the extraction of CBD because it minimizes the presence of co-extracts, thereby maximizing potency and reducing post-processing requirements. However, there is a lack of in-depth research into sub-CO_2 extraction of cannabis compounds, meaning the effects of extraction parameters and variations in the starting material when using sub-CO_2 are even less understood.

2. Background

Cannabis is an annual flowering herb of the *Cannabis* genus that has been used for thousands of years for its medicinal properties and fiber.[10] Because of the possible therapeutic effects and medical advantages associated with the phytochemicals found in the cannabis plant, the research and development into cannabis have grown exponentially. This has led to an increase in the acceptance and legalization of cannabis products.[11] In June 2018, the first cannabis-based medication hit the market for the treatment of epilepsy becoming the first legal medical cannabis product to be released.[12]

Cannabis contains more than 125 cannabinoids and more than 500 non-cannabinoids, such as flavonoids, alkaloids, phenols, and terpenes.[13–15] Although many cannabinoid phytochemicals have been shown to have biological activity, research has mostly focused on cannabidiol (CBD), a non-psychoactive cannabinoid, and tetrahydrocannabinol (THC), a psychoactive cannabinoid.[14,15] The former (CBD) has been shown to have antipsychotic, anxiolytic, and antiepileptic effects that can potentially help those with ailments, including Alzheimer's[16] and schizophrenia.[17] The latter (THC) has been shown to help in the treatment of illnesses such as fibromyalgia,[18] Parkinson's disease,[19] and glaucoma.[20] Naturally, these compounds are found in the plant in their acidic states, cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA) and are known for having therapeutic effects of their own.

The concentration of cannabinoids within the plant is dependent on a variety of factors including the plant’s age, strain, and growing conditions (nutrition, humidity, light level) as well as the type of plant tissue (leave, stem, root).[21] As seen in Figure 1, the cannabis plant's stem serves as the primary support for the entire plant and the origin of its many parts.

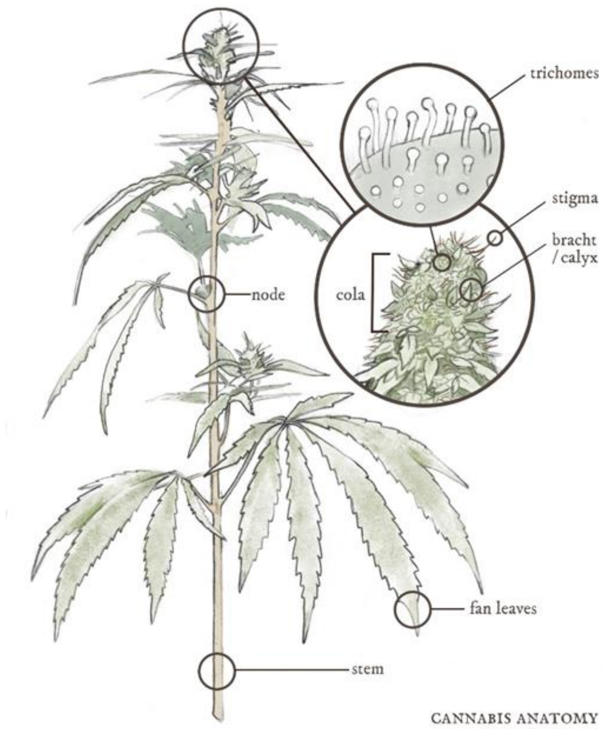

Figure 1: Anatomy of cannabis plant
(Image source: Rebekah Brewer, "Understanding the Cannabis Plant," Accessed December 06, 2022 via https://nationalholistic.com/understanding-the-cannabis-plant-physiology)

The points in which smaller, thin stems develop are called nodes and produce fan leaves. Fan leaves typically have five leaves but have been shown to have as many as nine. The cola is the dense growth at the plant's top where individual blooms, or 'buds', join. The reproductive parts of the female flower are enclosed within the bract. Sugar leaves, the little leaves of the cola, hide the bract, which consists of tear-shaped nodules. The calyx, which is located at the base of the flower, is a layer of transparent tissue that is sometimes mistaken for the bracts. Stigmas, which resemble bright orange hair, grow from the female plant's pistils to gather pollen from the male plants. The stigmas of a maturing plant begin life white and gradually change to shades of amber and brown with age, allowing a farmer to determine a plant's maturity. Trichomes are the clear, bulbous structures that cover the plant's bracts, leaves, and stems. Occurring naturally as a defense method to protect against predators and harsh conditions, the mushroom-shaped glands produce a transparent resin that covers the cannabis flower and are the source of cannabinoids.[22,23] As trichomes produce cannabinoids, the highest concentrations of cannabinoids can be found in the trichomes (up to 60%). This is followed by unpollinated female flowers (up to 30%), pollinated flowers (up to 13%), leaves (0.05%), and stems (0.02%).[24] Cannabinoids have also been found in trace amounts in the plant's seeds,[25] roots,[26] and pollen.[27] The bracts have the greatest cannabinoid content (in percent by dry weight of the plant) as they are loaded with trichomes.[28]

Because they yield more cannabinoids as they age, mature female cannabis plants are standard for cannabinoid extraction.[21] The largest yield of cannabinoids is produced when female plants are cultivated in isolation from males to avoid pollination and the development of seeds.[29] As it has been shown that pollination decreases cannabinoid accumulation,[30] cannabinoid farmers carefully remove male plants before pollination. Of the hundreds of varieties of strains grown today, the most common varieties, C*annabis sativa* and C*annabis indica*, are most often mixed to produce hybrid varieties with varying cannabinoid compositions and effects.[31] This study utilizes the female plants from a hybrid variety of cannabis with high concentrations of the cannabinoid CBD.

Separating bioactive molecules from the plant matrix through phytochemical extraction is a key step in expanding the range of uses for these compounds in the pharmaceutical field. Numerous techniques for the extraction of cannabis have been published. These techniques include direct maceration, soxhlet extraction, ultrasound-assisted extraction, supercritical fluid extraction, and microwave-assisted extraction.[32] Of these, there are two extraction methods that have been more commonly investigated in literature and most frequently used in industrial applications. The first involves macerating plant material in an organic solvent (direct maceration) and then removing the solvent to concentrate the extract. The second is supercritical fluid extraction (SFE), which employs solvents under high pressure.[13,32–35]

A supercritical fluid is one that has been subjected to pressure and temperature beyond its critical point as seen in Figure 2. Many of the fluid's characteristics are intermediate between those of a gas and a liquid under these conditions. Table 1 shows that while the density of a supercritical fluid is comparable to that of a liquid, its viscosity is like that of a gas, and its diffusivity is in between the two states.[36]

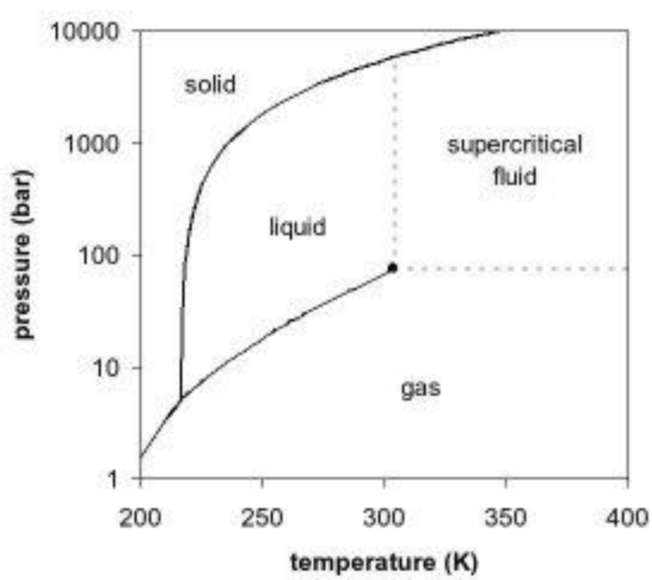

Figure 2: Phase diagram for a pure compound
(Image source: MarcJacobs, Public domain, via Wikimedia Commons)

Due to its unique physicochemical features, SFE offers various operational benefits over other extraction techniques. As a result of their low viscosity and high diffusivity, supercritical fluids have superior transport capabilities compared to liquids, may diffuse quickly through solid materials, and, as a result, can provide quicker extraction yields. The density of a

Table 1: Properties of gases, liquids, and supercritical fluids (data is from ref 36)

Fluid State	Density (g/cm^3)	Diffusivity (cm^2/s)	Viscosity (g s/cm)
Liquid	1	$<10^{-5}$	10^{-2}
Supercritical	0.3-0.8	10^{-3}-10^{-4}	10^{-4}-10^{-3}
Gas	10^{-3}	10^{-1}	10^{-4}

supercritical fluid may be adjusted by altering its pressure and/or temperature, which is one of the fluid's defining features. Since density is connected to solubility, the solvent strength of a fluid can be altered by adjusting the extraction temperature and pressure.[36]

SFE consists of two major stages: (1) extraction of the soluble compounds from the matrix when the solid sample absorbs the supercritical solvent; and (2) separation of solutes from the supercritical solvent. The process of selecting an appropriate solvent is vital. In reality, any solvent may be raised to the supercritical state, although certain criteria should be considered including the toxicity of the solvent, its physicochemical features that influence the supercritical state, the practicability, and the cost. A large variety of compounds have been employed for SFE including ethene, water, methanol, and carbon dioxide to name a few.[36] Since cannabinoids are non-polar molecules, they are most often extracted with nonpolar organic solvents such as hydrocarbons (e.g., butane), but have been shown to be soluble in polar solvents such as alcohols (e.g., ethanol).[13,32] Extractions using these solvents are effective; but, depending on the final product, often require extensive post-processing that necessitates extra testing to verify the removal of residual solvents. Additionally, these solvents can be expensive, and their toxicity, environmental danger, and flammability make them nonpreferred for large-scale extraction. Carbon dioxide (CO_2), on the other hand, stands out for cannabinoid extraction as it is a nonpolar solvent that's affordable, non-toxic, non-flammable, has a low critical temperature and pressure, and can extract heat-sensitive compounds.[13,35,37] Additionally, unlike organic solvents, CO_2 does not require additional postprocessing to remove residual solvents as it leaves biomass readily after cycles with minor adjustments to the temperature and pressure.[13,37]

In addition to cannabinoids, terpenes, volatiles, moisture, and heavy residues exit the plant matrix as co-extracts, with the latter made up of pigments, phospholipids, fatty acids, heavy metals, and other substances that resemble wax.[31] To increase purity and CBD concentrations, the plant waxes can be removed using a winterization post-process. This is a common process to further refine cannabinoid extracts but does require the use of additional solvents.[3] Winterization involves mixing the cannabinoid extract with an alcohol, followed by deep freezing the mixture at -112°F for at least 24 hours to separate plant waxes, which can then be removed by filtering. This step is often repeated multiple times as left-over waxes have been shown to interfere with the separation thermodynamics during solvent removal. The solvent is removed using a rotary evaporator and recycled back into the process for continued use. At the same time, the separated wax is set aside and can be used in various applications, including electronics, candles, and lubricants for equipment.[11]

The critical point of CO_2 occurs at a temperature of 87.8 °F and a pressure of 1071 psi. By using temperatures and pressures above this point, CO_2 is pushed into its supercritical state. Until recently, supercritical extraction was the most common method of CO_2 extraction as it produces high yields in a short amount of time. However, as the medicinal cannabis industry grows, quality is being prioritized over quantity. Because of this, subcritical CO_2 (sub-CO_2) is growing in popularity. Sub-CO_2 is subjected to lower temperatures and pressures, below the critical point, to create a subcooled liquid. In this state, sub-CO_2 has less solvent power than supercritical CO_2, making it capable of removing lighter oils while leaving behind heavier plant waxes. This produces a higher quality product that needs less if any, post-processing. This was verified experimentally via an additional study outside the scoop of this paper, in which extraction with subcritical parameters produced a product with minimal plant waxes, an increased CBD content, and decreased THC content when compared to supercritical extraction. Pictures comparing the subcritical and supercritical cannabis extracts collected from the same cannabis biomass can be found in Appendix A.

The typical range of temperatures and pressures used in CO_2 extraction of CBD changes depending on various factors including the equipment being used for the extraction process, the type of plant material being extracted, and the desired purity of the CBD dominant extract produced. The typical range of temperatures and pressures used in supercritical CO_2 extraction of CBD are between 95°F to 140°F and 1000 psi to 5000 psi. The typical range used in subcritical CO_2 extraction is temperatures of 68°F to 95°F and pressures of 500 psi to 1500 psi. [31,38,39]

Aside from solvent type, temperature, and pressure, several additional factors are known to affect the extraction efficiency of phytochemicals from biomass, including particle size of the biomass, moisture content, extraction time, material-to-solvent ratio, solvent flow rate, agitation, and solvent pH.[13] It is estimated that 30–60 percent of the total cannabinoids in cannabis flowers are lost somewhere during the extraction and purifying process.[11] Because of this, it is important to determine what factors have the greatest effect on extraction efficiency; however, since many parameters impact the process and different researchers have different interests and perspectives, there is a noticeable shortage of research available.

A 2022 review by Hebah Ubeed, et al., explains the current knowledge gaps in the field.[13] This includes a lack of information about variabilities in the plant, the preparations of biomass, the influence of different extraction conditions, and internal effects on the body. This study focuses on providing information about how variabilities in the plant material affect the extraction process.

Dealing with plant biomass introduces several discrepancies as the chemical composition in the flowers is affected by the plant's general health and environmental factors.[11] To obtain a high-quality starting material and maximize cannabinoid content, factors including latitude, the season of harvest, and harvest timing should be considered. As these factors directly affect the cannabinoid content of the plant, this study uses variations in cannabinoid content, specifically total CBD content (%CBD), to study how these changes affect the extraction process. Furthermore, the retention of cannabinoids from cannabis after harvest is impacted by transportation, storage, and drying procedures and conditions, which further affect the cannabinoid content. These phytochemicals are fragile; thus, careful handling is essential during shipment, storage, and preprocessing. Further research on different drying settings and procedures is required to determine the optimal drying methods and conditions of cannabis before the extraction process as drying can change the phytochemicals in the plant.

Cannabis, like many plants, is made up of 80% water and requires drying before the plant can be used. Drying the plant inhibits microorganisms from rotting plant tissue and allows long-term preservation while retaining potency, flavor, therapeutic characteristics, and efficacy.[38] Hang-drying or air-drying cannabis plants after harvest is the oldest method that requires no special equipment. This method involves hanging whole plants in a cool dark room with a temperature between 65 and 80 °F and humidity between 45 and 55%, either on a string or on drying screens.[38] Oven drying is a faster, more direct way of drying. It can be used to decrease drying times and facilitate a decarboxylation process, a method that is often used when processing cannabis material dependent on the cannabinoid of interest.

In plants, CBD occurs naturally in its acid form, cannabidiolic acid (CBDA). When heated, CBDA converts to CBD through a decarboxylation process that removes a carboxyl group released in the form of carbon dioxide.[7] A visual of this conversion can be seen in Figure 3.

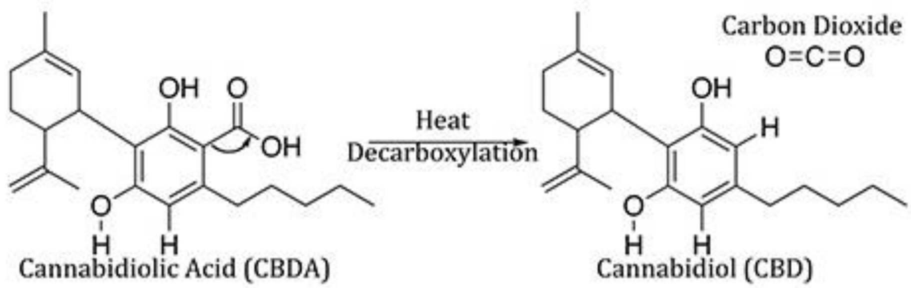

Figure 3: CBDA decarboxylation reaction
(Image source: Cedarstone Industry, "Decarboxylation Equipment for Hemp&CBD Products," Accessed February 15, 2023 via https://www.cedarstoneindustry.com/product-category/extraction-equipment/cbd-decarboxylation/)

During industrial processing, an extract high in CBDA is often collected and then converted to CBD after extraction during post-processing as there is less material to process. This study considers if a decarboxylating drying process implemented before extraction has a statistically significant effect on the mass percent of sub-CO_2 extract collected. As the vapor temperature of water is 212°F at atmospheric pressure and the CBDA to CBD conversion occurs around 230°F at atmospheric pressure, water is also removed during decarboxylation. As water is also removed, this study considers the combined effect decarboxylation and moisture content (%M) might have on the extraction efficiency by considering the extraction of CBDA at 10 %M and the extraction of CBD at less than 2 %M.

The extent of any SFE study depends on the resources available, the time it takes, how much it costs, and the investigators' aim. Over a year, information was gathered from an industrial process in which parameters and preprocessing conditions were altered to create an extract with a targeted chemical profile and consistency to meet a company's goals. In doing so, an extensive data set of more than 400 data points was formed, including information on the extraction of cannabinoids at various temperatures, pressures, extraction times, extractor volumes, extraction mass/volume ratios, cannabis strains, initial cannabinoid contents, and with varieties in pre-extraction processing adding varieties in moisture content and cannabinoid structure. A DOE approach was utilized in addition to the data set to analyze previously unstudied variables.

DOE is a type of controlled experimentation in which the variables of a process are varied to determine the impact on a dependent variable, in this case, the mass percent yield (mass of extract per mass of biomass) of CBD extract produced. DOE studies can be used to improve the efficiency of processes by determining which parameters that have the greatest influence. Importantly, this method considers several factors simultaneously and can determine if variables within the process interact with one another. The most common DOE used are a full factorial design, fractional factorial design, and Plackett-Burman design.[8,9,40]

This study utilizes a full factorial design to determine if variations in the cannabis biomass moisture content (%M) and total CBD content (%CBD) significantly affect the total mass percent yield (%yield) of sub-CO_2 extract collected throughout the extraction process.

3. Methodology

3.1 Overview of Processing Method

The procedures followed to obtain CBD from cannabis are shown in Figure 4. Once CBD dominant cannabis plants were grown and harvested, they were hung upside down to dry (in avoidance of sunshine to prevent photochemical change) before separating the flowers from the stems with deflowering equipment. The flowers were then dried further and processed to minimize particle size. Following grinding, the biomass underwent two different processes to compare. Process A uses sub-CO_2 extraction to extract biomass with large concentrations of CBDA to produce a CBDA dominant extract. Process B implements a decarboxylating drying process before extraction to convert CBDA to CBD.

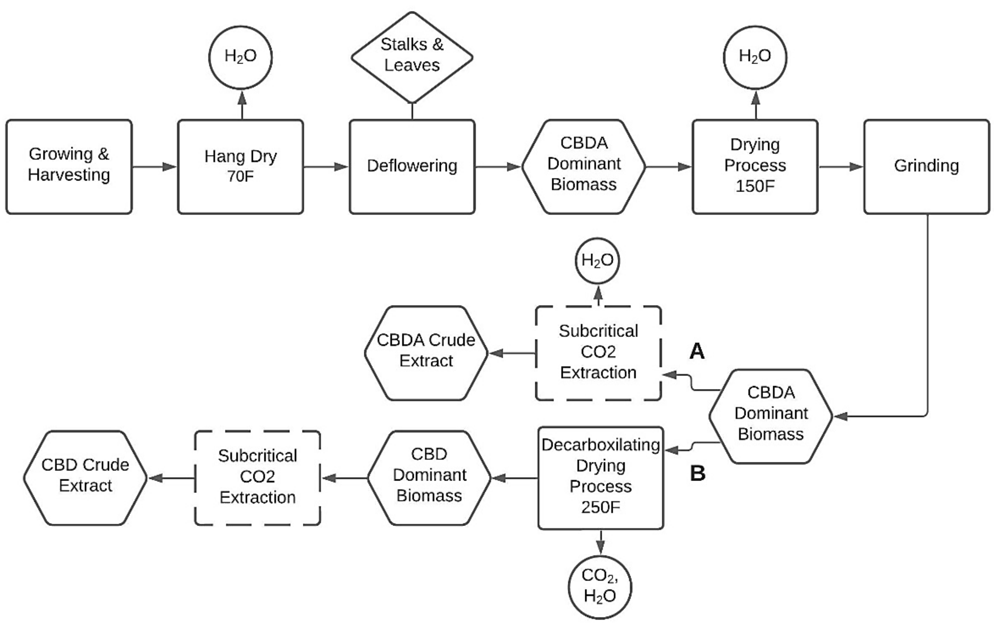

Figure 4: Process flow diagram followed to produce cannabis extracts

3.2 Plant Material

The CBD-dominant cannabis strain, "Cherry Wine," with a hybrid *sativa*/*indica* genotype, was planted by two farms located in Upshur County, West Virginia. Both lots were planted in May of 2019 in temperature-controlled greenhouses that remained between 65°F and 85°F. In June 2019, the plants were transplanted into the ground to continue their growth before harvesting in September 2019. The flowers of the cannabis plants were harvested from both farms and hung to dry for 7-10 days at 70°F in a controlled environment. The flowers were stripped from the plant's stalk and dried on a conveyer belt dryer at 115°F for 45-60 minutes to reduce the %M to below 10%. From there, the dried material was transported to an extraction facility where the material was ground (Trimleaf plant shredder machine, 3lb model) to obtain a particle size <2.0mm. Part of the plant material then went straight to extraction (process A) while the other part underwent a decarboxylating drying process (process B). The moisture content (%M) was determined with a moisture meter (General Tools, Model MMD7NP) before drying and extraction.

3.3 Decarboxylating Drying Process (Process B)

To decarboxylate the cannabis biomass, a one-inch layer of ground flower material was distributed on large aluminum sheets and placed in a 35 cubic foot oven at 250°F for 3 hours. To ensure even heating, the oven door was opened every hour to mix the material. Through testing, it was verified that this method produced a 90-95% conversion of CBDA to CBD repeatedly.

3.4 Cannabinoid Quantification

To determine the cannabinoid content of both the cannabis flowers and extracts, samples were sent to ACS Laboratory (Sun City Center, FL) for analysis using HPLC. According to the certificate of analysis provided, the dried, ground flower material from farm A had a total CBD content (%CBD) of 10.6% and while the flower from farm B had a 9.7 %CBD before decarboxylation.

Total CBD is defined as the total amount of CBD present in a sample, considering the potential for CBDA to convert to CBD. Total CBD is calculated as the sum of the amount of CBDA (% $W_{CBDA}/W_{biomass}$) multiplied by a correction factor of 0.877 plus the amount of CBD (% $W_{CBD}/W_{biomass}$) as shown in Equation 1. This formula accounts for the loss of mass due to the decarboxylation of CBDA.

Equation 1: $$\boldsymbol{Total\ CBD = CBD + 0.877CBDA}$$

3.5 Subcritical/Supercritical CO_2 Extraction Equipment

The subcritical carbon dioxide (sub-CO_2) extraction was performed using The Duplex SFE unit (Apeks Supercritical by Gibraltar, Johnstown, OH). The solvent, food-grade CO_2, was supplied by AirGas. To reach the required pressure, CO_2 was fed into the extraction vessel containing the plant material using a diaphragm compressor. The CO_2 and oil were then pumped out of the extraction vessels and decompressed via an orifice into a separator vessel, as seen in Figure 5. The reduction in pressure allows the CO_2 to convert back to a gas which naturally separates from the denser oils that fall to the bottom of the separator vessel and into a collecting cup. The CO_2 continues around the closed-loop system until the trial completes. The internal temperature is controlled through a heated jacket on the extraction vessel.

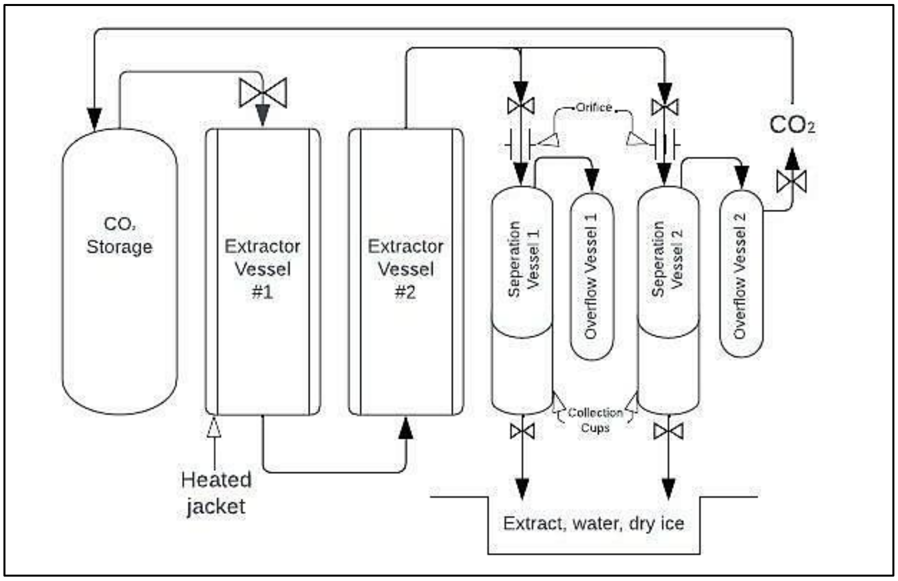

Figure 5: Subcritical/supercritical CO_2 extraction unit

3.6 Extraction Parameters

The values for extraction parameters were derived from the equipment manufacturer's (Apeks Supercritical by Gibraltar, Johnstown, OH) recommendations and previous studies. Control variables include extraction temperature, extraction pressure, extractor volume, extractor run time, solvent flow rate, amount of biomass per extraction vessel, particle size, and the strain of starting material. Values for each controlled variable are given in Table 2. The extraction temperature was set to 75°F and 1200psi, respectively, per the manufacturer's recommendation for sub-CO_2 extraction. The flow rate and extractor volume were determined by the manufacturer's design and could not be varied. The extraction run time was determined by previous experimental studies to reduce residual cannabinoid content in the spent biomass while producing a quality extract in a minimal time. Based on the results, the total extraction time was set to 6 hours.

Independent variables of the study include total CBD content (%CBD) and the combined effect of moisture content (%M) and cannabinoid type. The dependent variable studied is the total mass percent yield (%yield) of extract collected throughout the extraction process. As it is known that the material-to-solvent ratio affects the extraction efficiency of phytochemicals from plant materials, only data points with a similar initial biomass weight were considered (11.0 ± 0.34 pounds per extraction vessel). The maximum sample holding capacity of the reactor was 80 L across 4 extraction vessels. Due to data availability, this study only considered trials ran at half capacity, 40L across 2 extraction vessels. With different cannabis strains having different plant structures and cannabinoid contents that are known to affect the extraction process, "Cherry Wine" was the only CBD-dominant cannabis strain studied. As for particle size, all cannabis biomass underwent the same grinding conditions for the same amount of time, producing a particle size <2.0mm.

Table 2: Extraction control variables

Extraction Temperature	75°F
Extraction Pressure	1200 psi
Extraction Vessel Volume	40L
CO2 Flow Rate	6.1 kg/min
Amount of Biomass per Extraction Vessel	11 ± 0.34 lbs
Cannabis Strain	Cherry Wine
Particle Size	<2.0mm

3.7 Total Mass Percent Yield (%yield)

Every 2 hours throughout the extraction process, the extract/CO_2 separation process was switched between separation vessels #1 and #2 (Figure 5). The extract was collected from the collection cup and the mass of the extract collected within the 2 hours was measured using a scale (Bonvoisin lab scale, 5000gx0.01g model). A record was kept of the yield collected after 2, 4, and 6 hours of extraction for each trial. The total percent yield (%yield) extracted was calculated by dividing the total mass of the extract produced up until that point by the initial mass of the cannabis biomass ($M_{extract}/M_{biomass}$).

3.8 Factorial Design Parameters

The values for the experimental factors considered in this full factorial study are shown in Table 3. Factors include moisture content (%M) and total CBD content (%CBD) at high and low levels. The %M was considered at 2% for CBD-dominant biomass that underwent a decarboxylating drying process and 10% for CBDA-dominant biomass that underwent standard drying processes but did not undergo an additional decarboxylating drying process. The %CBD was considered at 9.7% and 10.63% ($W_{CBD}/W_{biomass}$). The factorial design of 2^2 was utilized with 2 repetitions in each trial, giving an 8-run experimental design.

Table 3: 2^2 full factorial DOE factor values

			Low	High
Factor 1	Moisture Content	%M	2	10
Factor 2	Initial CBD Content	%CBD	9.7	10.6

In a typical experimental design, each experiment is carefully planned and performed in a randomized order. Randomization reduces the potential for bias in the selection process and levels the playing field between the impacts of known, unknown, and uncontrollable influencing factors. As this study uses data that was collected in advance, the run order was not randomized and therefore it is not possible to eliminate bias in the data point selection or equalize the impact of uncontrollable factors.

3.9 Statistical Analysis

The outputs of the experimental design were analyzed using statistical process control (SPC) software from BPI Consulting, LLC (*SPC for MS Excel)*.[41] Using the full factorial experimental design module offered by SPC for MS Excel, ANOVA (analysis of variance) was conducted on the experimental data. The confidence level of 95% ($p<0.05$) was considered statistically significant. Using the data, a linear regression model was also produced to allow for predictive modeling of the system. Even if the runs themselves have never been conducted, these models can be used to forecast the %yield expected for various combination of %M, %CBD within the system. The coefficients within the models are given in 'uncoded' units. Further explanation of ANOVA calculations and model coefficients can be found in Appendix B.

To specify the magnitude and direction of the relationship between a variable and the response variable, the coefficients from the model have also been given in 'coded' units. The coded coefficients allow for the importance of each variable to be quickly assessed by the sign and size of the coefficient for each factor. For example, a coefficient with a larger value equates to a large effect, while a negative coefficient equates to a negative effect.

4. Results and Discussion

The effect of %M and %CBD on the %yield obtained from the extraction process was determined using a full factorial design of experiments (DOE) with 8 experimental trials. To consider the effects of extraction time (T_{ext}) on the %yield obtained, the full factorial design was reconfigured to include T_{ext} as a third variable. Lastly, the 2^2 full factorial with two replicates was repeated for different values of T_{ext} to better understand how the effects of %M and %CBD change throughout the extraction process.

4.1 Full Factorial DOE with 2 Variables

A 2^2 full factorial design with 2 replicates, (8 experimental runs) was conducted to evaluate significant process parameters and develop a simple model. To determine the overall effects %M and %CBD had on the %yield of sub-CO_2 cannabis extract obtained during an extraction process, data collected following the extraction at 6 hours were considered. The %yield of extract collected from each extraction process can be seen in Table 4. The highest mass percent yield obtained was 14.65% and was collected from biomass with 2 %M and 9.7 %CBD. The lowest %yield was 7.87% and was obtained at a higher %CBD (10.6%) and higher %M (10%).

Table 4: 2^2 DOE collected after 6 hours of extraction

Run Order	%M	%CBD ($\% W_{CBD}/W_{biomass}$)	% Yield ($\% M_{extract}/M_{biomass}$)
1	10	9.7	11.00
2	10	9.7	9.54
3	2	9.7	14.65
4	2	9.7	13.72
5	10	10.6	7.87
6	10	10.6	8.11
7	2	10.6	12.59
8	2	10.6	12.57

Table 5 and 6 presents the significance of each input variable and their interactions as determined by ANOVA. The significance of each variable is indicated by the probability level (p-value). As this study was conducted with two replications for each factorial combination, the p-value of each component could be calculated. Appendix B provides further details about the calculations made for the statistical analysis. According to a 95% confidence interval, parameters with p-values less than 0.05 have a statistically significant effect on the experimental design's response. As determined by the p-values (Table 5), both %M ($p = 0.0006$) and %CBD ($p=0.0113$) had a significant effect on the %yield of the extract collected with %M having a greater effect overall. The interaction between %CBD and %M (%CBD&M) is deemed insignificant with a p-value of 0.4830. The full ANOVA table can be seen in Appendix C (Table 7A). From the regression model, the coded coefficients indicate that the effect %M and %CBD had on the %yield of sub-CO_2 extract collected was negative (Table 6), meaning an increase in %M and %CBD resulted in a decrease in mass %yield of extract. To estimate the %yield expected after 6 hours of extraction from the system for various values of %M and %CBD, the uncoded regression model can be used (Equation 2). Additional details on the regression model can be seen in Appendix B.

Table 5: Major results from 2^2 DOE ANOVA table

Variable	p-value	%Cont	
%M	0.0006	79.54%	**Significant factors ($p < 0.05$)**
%CBD	0.0113	16.60%	
%CBD&M	0.4830	0.50%	
Error		3.36%	
Total		100%	

Table 6: 2^2 DOE regression model coefficients

Variable	Coded Coeff	Uncoded Coeff
%M	-2.126	-0.5316
%CBD	-0.971	-2.158
Intercept	11.26	36.35
R2 (%)	96.64	

Equation 2: $$\%\boldsymbol{Yield} = \mathbf{-0.5316\ \%M - 2.1583\ \%CBD + 36.35}$$

4.2 Full Factorial DOE with 3 Variables

The previous results were determined after 6 hours of extraction. It would be expected that extraction time affects the %yield. To consider the effect extraction time had on the %yield, the full factorial method was repeated to include T_{ext} as a third variable. The full results for %yield collected after 2, 4, and 6 hours of extraction, including variable biomass conditions, can be seen in Table 7. These results can also be visualized in Figure 6. From general observations, trials at 10%M produced roughly half the amount of extract after 2 hours as trials with <2%M. Additionally, trials extracting CBD produced higher yields throughout the entire extraction process when compared to trials extracting CBDA. In other words, runs that use biomass that has undergone a decarboxylating drying process produced a higher yield of extract in less time.

Table 7: %yield collected from subcritical extraction of cannabis biomass after 2, 4, and 6 hours of extraction

Extraction Conditions				Results		
Run Order	%CBD	%M	Main Cannabinoid	Total percent yield (%yield) extracted after		
				2 Hours	4 Hours	6 Hours
1	9.7	10	CBDA	5.59	8.77	11.00
2	9.7	10	CBDA	4.96	7.72	9.54
3	9.7	<2	CBD	9.75	13.64	14.65
4	9.7	<2	CBD	9.32	12.97	13.72
5	10.6	10	CBDA	4.81	6.66	7.87
6	10.6	10	CBDA	4.31	6.42	8.11
7	10.6	<2	CBD	9.12	11.55	12.59
8	10.6	<2	CBD	8.52	11.69	12.57

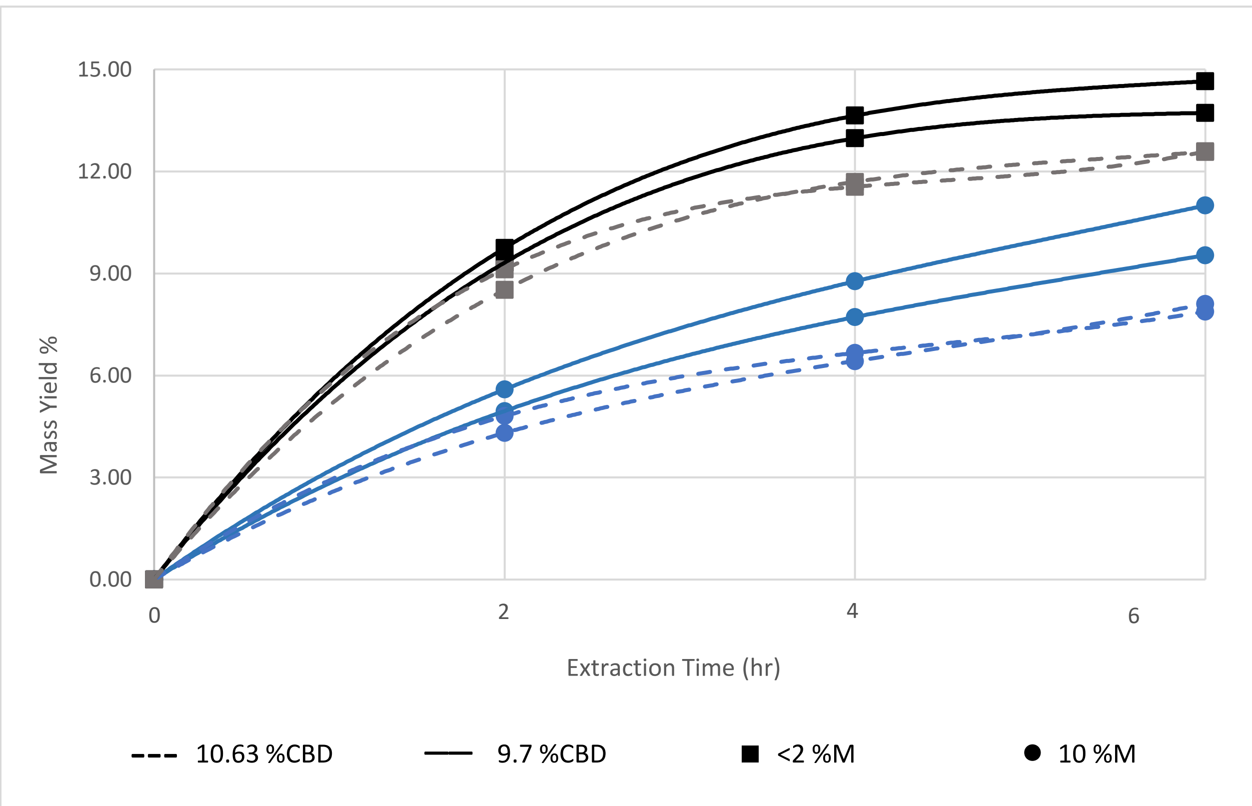

Figure 6: Effect of initial CBD content and moisture content on the product yield and extraction time

For the 8 experimental trials considered, the %yield at various extraction times were considered in a 2^3 factorial design to model the impact of %M, %CBD, and T_{ext} had on the %yield obtained. The design table can be seen in Table 8. The T_{ext} was considered at a low and high level of 2 and 6 hours. This design does not include repetitions for each factorial combination; therefore, the p-value could not be calculated to determine a level of statistical significance. Instead, the effects were assessed using the %contribution (Table 9).

Table 8: 2^3 DOE including T_{ext}

Run Order	%M	%CBD (% $W_{CBD}/W_{biomass}$)	T_{ext} (hours)	% Yield (% $M_{extract}/M_{biomass}$)
1	10	9.7	2	5.59
2	10	9.7	6	9.54
3	2	9.7	2	9.32
4	2	9.7	6	14.65
5	10	10.6	2	4.81
6	10	10.6	6	8.11
7	2	10.6	2	9.12
8	2	10.6	6	12.57

Table 9: %contribution for 2^3 DOE

Variable	% Contribution
%M	51.90%
%CBD	3.37%
T_{ext}	43.01%
%M & %CBD	0.00%
%M & T_{ext}	0.39%
%CBD & T_{ext}	1.07%
%M %CBD & T_{ext}	0.25%

The results shown in Table 9 indicate that %M has a greater effect than T_{ext} on the %yield, with %M contributing 51.9% and T_{ext} contributing 43.01% towards the variation in %yield extracted. For the interactions between variables, the calculated %contribution was minimal. Because of this, a new ANOVA was calculated excluding interactions. The results can be seen in Table 10. The elimination of interactions allowed the error term to have four degrees of freedom; and, therefore, p-values could be defined establishing a level of significance. When excluding interactions, %M, %CBD, and T_{ext} have a significant effect on the %yield as seen by the p-values in Table 10. The full ANOVA table can be seen in Appendix C (Table 9A). From the coded regression model coefficients (Table 11), the effect of T_{ext} on the %yield is positive. In other words, as T_{ext} increase, %yield increased. %M and %CBD had a negative effect as originally seen in section 4.1. Equation 3 can be used to estimate the %yield expected from the system at various values of %M, %CBD, and T_{ext}.

Table 10: Major results from 2^3 DOE ANOVA Table excluding interactions

Variable	p-value	
%M	0.0004	***Significant factors ($p < 0.05$)***
%CBD	0.0486	
T_{ext}	0.0006	

Table 11: 2^3 DOE regression model coefficients excluding interactions

Variable	Coded Coeff	Uncoded Coeff
%M	-2.201	-0.5503
%CBD	-0.5613	-1.247
T_{ext}	2.004	1.002
Intercept	9.214	21.17
R2 (%)	98.28	

Equation 3: $$\%Yield = -0.5503\ \%M - 1.247\ \%CBD + 1.002\ T_{ext} + 21.17$$

4.3 Full Factorial DOE with 2 Variables at Various Extraction Times

%M and %CBD were considered in a 2^2 factorial design with 2 replicates at T_{ext} values of 2, 4, and 6 hours individually to better understand how the effects of %M and %CBD change throughout the extraction process. The factorial design considering a 2-hour extraction time can be seen in Table 12 and the factorial design considered a 4-hour extraction time can be seen in Table 13.

Table 12: 2^2 DOE collected after 2 hours of extraction

Run Order	%M	%CBD (% $W_{CBD}/W_{biomass}$)	% Yield (% $W_{extract}/W_{biomass}$)
1	10	9.7	5.59
2	10	9.7	4.96
3	2	9.7	9.75
4	2	9.7	9.32
5	10	10.6	4.81
6	10	10.6	4.31
7	2	10.6	9.12
8	2	10.6	8.52

Table 13: 2^2 DOE collected after 4 hours of extraction

Run Order	%M	%CBD (% $W_{CBD}/W_{biomass}$)	% Yield (% $W_{extract}/W_{biomass}$)
1	10	9.7	8.77
2	10	9.7	7.72
3	2	9.7	13.64
4	2	9.7	12.97
5	10	10.6	6.66
6	10	10.6	6.42
7	2	10.6	11.55
8	2	10.6	11.69

The significance of each input variable after 2 and 4 hours, as determined by ANOVA, can be seen in the tables below. Table 14 presents the significance of %M and %CBD on %yield after 2 hours of extraction. Table 15 considers the %yield collected after 4 hours of extraction. The factorial design and analysis for the %yield collected after 6 hours of extraction can be seen in Section 4.1.

Table 14: Major results from 2^2 DOE ANOVA Table after 2 hours of extraction

Variable	p-value	*%Cont*
%M	0.0001	95.73%
%CBD	0.0588	2.70%

Table 15: Major results from 2^2 DOE ANOVA Table after 4 hours of extraction

Variable	p-value	*%Cont*
%M	0.0001	88.68%
%CBD	0.0060	9.91%

After 2 hours of extraction %M has a statistically significant effect while %CBD, on the other hand, provided little to no statistically significant effect with a p-value of 0.0588. After 4 hours of extraction, the calculated p-value for %CBD decreased to 0.0060; this %CBD becomes statistically significant after 4 hours. The full ANOVA tables can be found in Appendix C.

These results, paired with the results from section 4.1, can be visualized with the graph shown in Figure 7, showing the %contribution for each variable at various extraction times. As the process progresses, the effect of %M decreases while the effects of %CBD increases. As moisture is a coextract in the extraction process, %M naturally decreases with time explaining the decrease in %contribution throughout the process.

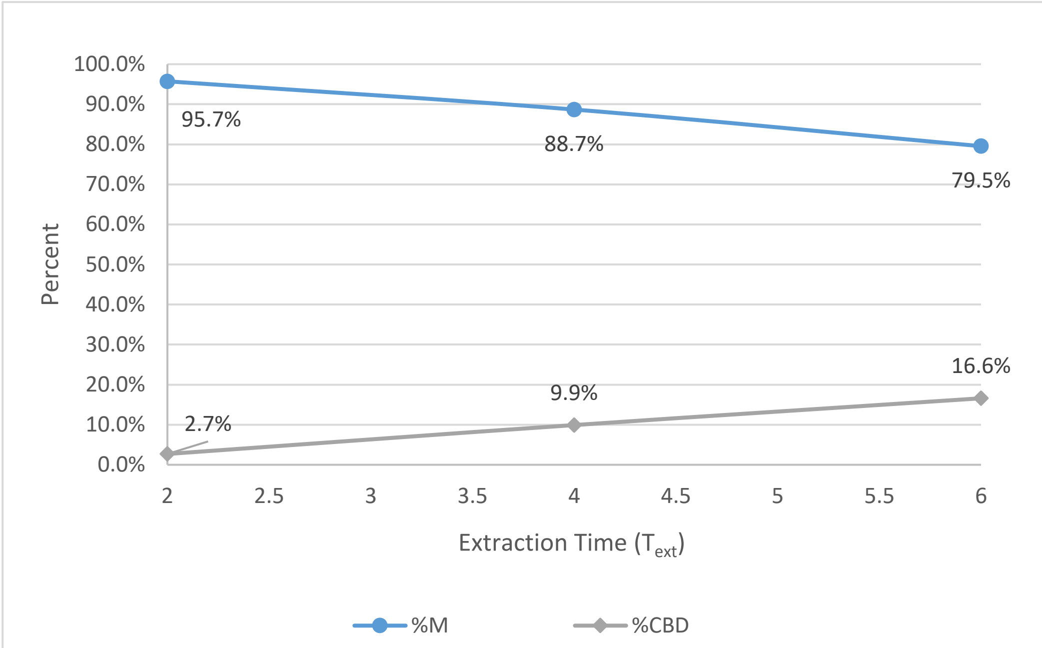

Figure 7: The effect, in terms of % contribution, %M and %CBD had on the variability in %yield of sub-CO_2 extract collected throughout the extraction process

5. Conclusions

Sub-CO_2 extraction is a recognized method in the industry for its abilities to separate phytochemicals from cannabis while minimizing the presence of heavy coextracts. This results in a high-quality product that requires less post-processing, if any, in comparison to supercritical-CO_2 extraction. There are, however, very few published works discussing the process or the effects of process variations. Without identifying the variables that have the greatest impact on the extraction process, the method cannot be optimized effectively.

A design of experiments approach allowed for efficient sampling of moisture content, CBD content, and extraction time to assess the influence these variables have on the total extraction percent yield. It was shown that the yield was significantly impacted by all variables considered, with moisture content having the largest effect overall. These results suggest that the extraction yield, from a subcritical CO_2 extraction process, would significantly increase by implementing a decarboxylating drying process prior to extraction. The effects caused by the chemical change of the main compound being extracted (CBDA to CBD) during the decarboxylation process were not considered.

6. Future Works

This study utilized a full factorial DOE approach to analyze pre-recorded data. A typical DOE is planned in advanced and ran in a randomized order to minimize bias from uncontrollable variables and in the data selection. This study could be improved upon by setting up the DOE before collecting the data so the order could be randomized. Additionally, a study planned in advanced would allow for additional details to be collected that were not available for this analysis. For example, the purpose of using subcritical CO_2 extraction over supercritical CO_2 extraction was to increase oil quality, which is most often determined by the cannabinoid content of the extract. As this data was taken from an industrial extraction process, cannabinoid data was not available for every trial due to cost. Therefore, the effect %M, %CBD, and T_{ext} had on the quality of extract could not be determined. Additionally, for the same reasons, the cannabinoid content remaining in the spent biomass was not measured for each trial to determine the effects of %M, %CBD, and T_{ext} on the amount of cannabinoids left in the biomass after processing which would give further insight into the extraction efficiency.

This study considered if implementing a decarboxylating drying process would significantly affect the extraction process. Since the drying process converts CBDA to CBD, it is unknown if moisture or the change in cannabinoid structure has a more significant effect on the extraction efficiency. To differentiate between the effects from moisture and the effects from structural differences, the extraction of CBDA at <2%M and the extraction of CBD at 10%M should be considered.

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Appendix A - Subcritical versus Supercritical Cannabis Extract

Supercritical Extract		
Subcritical Extract		

Figure 1A: Visual comparison of subcritical and supercritical CO_2 extracted cannabis extract (Image Source: Author Photos)

Appendix B - Statistical Analysis Additional Details

ANOVA Table

Using the full factorial experimental design module offered by *SPC for MS Excel*, ANOVA (analysis of variance) was conducted on the experimental data. Table 1A shows the basic ANOVA table calculations.

The first column contains the variation source. The causes of variation are split into two broad categories: between-trial variation and within-trial variation (error). The table's second column contains the sum of squares. The third column represents the number of degrees of freedom. *a* indicates the number of levels. *N* represents the number of total experimental runs. The fourth column represents the mean square. The mean square is calculated by dividing the sum of squares by the degrees of freedom. The F value appears in the fifth column. It is derived by dividing the mean square of the trials by the mean square error (*MSE = SSError (N-a))*. This number indicates whether there are statistically significant differences.

Table 1A: The basic form of an ANOVA table

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value
Between Trials	SS_{BTW}	a-1	SS_{BTW} / (a-1)	MS_{BTW} / MSE
Error (Within Trials)	SS_{Error}	N-a	SS_{Error} / (N-a)	
Total	SS_{Total}	N-1		

From the F-value, the p-value can be determined. This is the likelihood that the computed F values would be obtained if there were no differences between the trial values. According to the 95% confidence interval, parameters with p-values less than 0.05 have a statistically significant effect on the experimental design's response.

Regression Model

Based on the data, a regression model was created. As high and low values were used in this study, there was no midpoint data to determine curvature, therefore, a linear model following the form in Equation 1A was produced when excluding interactions.

Equation 1A: $$\boldsymbol{Y_{ijk} = M_i + C_j + ET_k + B}$$

Where:
Y_{ijk}= Response variable (%yield)
M_i = Effect of i^{th} % moisture content
C_j = Effect of j^{th} % CBD content
ET_k = Effect of k^{th} extraction time
B= y-Intercept

The regression model can utilize both uncoded and coded values for each factor dependent on the use. The uncoded model can be used to predict future outputs expected from the system for various input values of %M, %CBD, and T_{ext}. The coded equation, on the other hand, allows for the importance of each variable to be quickly assessed by the sign and magnitude of the coefficient. The following equation (Equation 2A) describes the link between a coded level of factor X (X_c) and the real level. For a factor X, X_L represents the low level and X_H represents the high level.

Equation 2A: $$\boldsymbol{X_c = \frac{X - \frac{X_L + X_H}{2}}{\frac{X_H + X_L}{2}}}$$

Appendix C - Analysis of Data

Table 2A: 2 variables after 2 hours of extraction									
ANOVA Table							Model Information		
Factor	SS	df	MS	F	p value	%Cont	Factor	Coded Coeff	Uncoded Coeff
%M	36.30	1	36.30	243.6	0.0001	95.73%	%M	-2.130	-0.5325
%CBD	1.022	1	1.022	6.863	0.0588	2.70%	%CBD	-0.3575	-0.7944
%M*%CBD	0.000	1	0.000	0.000	1.000	0.00%	%M*%CBD	0.000	0.000
Error	0.5959	4	0.1490			1.57%	Intercept	7.048	18.31
Total	37.91	7				100.00%	R2 (%)	98.43%	

Table 3A: 2 variables after 2 hours of extraction excluding interactions									
ANOVA Table							Model Information		
Factor	SS	df	MS	F	p value	% Cont	Factor	Coded Coeff	Uncoded Coeff
%M	36.30	1	36.30	304.5	0.0000	95.73%	%M	-2.130	-0.5325
%CBD	1.022	1	1.022	8.579	0.0327	2.70%	%CBD	-0.3575	-0.7944
Error	0.5959	5	0.1192			1.57%	Intercept	7.048	
Lack of Fit	0.000	1	0.000	0.000	1.000	0.00%			
Total	37.91	7				100.00%	R2 (%)	98.43	

Table 4A: 2 variables after 4 hours of extraction									
ANOVA Table							Model Information		
Factor	SS	df	MS	F	p value	%Cont	Factor	Coded Coeff	Uncoded Coeff
%M	51.41	1	51.41	252.5	0.0001	88.68%	%M	-2.535	-0.6056
%CBD	5.746	1	5.746	28.23	0.0060	9.91%	%CBD	-0.8475	-1.867
%M*%CBD	0.0002	1	0.0002	0.001	0.9765	0.00%	%M*%CBD	-0.005	-0.0028
Error	0.8143	4	0.2036			1.40%	Intercept	9.928	32.68
Total	57.97	7				100.00%	R2 (%)	98.60%	

Table 5A: 2 variables after 4 hours of extraction excluding interactions									
ANOVA Table							Model Information		
Factor	SS	df	MS	F	p value	% Cont	Factor	Coded Coeff	Uncoded Coeff
%M	51.41	1	51.41	315.6	0.000	88.68%	%M	-2.535	-0.6337
%CBD	5.746	1	5.746	35.27	0.0019	9.91%	%CBD	-0.8475	-1.883
Error	0.8145	5	0.1629			1.40%	Intercept	9.928	32.85
Lack of Fit	0.000	1	0.0002	0.001	0.9765	0.00%			
Total	57.97	7				100.00%	R2 (%)	98.59%	

Table 6A: 2 variables after 6 hours of extraction									
ANOVA Table							Model Information		
Factor	SS	df	MS	F	p value	%Cont	Factor	Coded Coeff	Uncoded Coeff
%M	36.17	1	36.17	94.73	0.0006	79.54%	%M	-2.126	0.4200
%CBD	7.547	1	7.547	19.77	0.0113	16.60%	%CBD	-0.9713	-1.596
%M*%CBD	0.2278	1	0.2278	0.5967	0.4830	0.50%	%M*%CBD	-0.1688	-0.09375
Error	1.527	4	0.3818			3.36%	Intercept	11.26	30.64
Total	45.47	7				100.00%	R2 (%)	96.64%	

Table 7A: 2 variables after 6 hours of extraction excluding interactions									
ANOVA Table							Model Information		
Factor	SS	df	MS	F	p value	% Cont	Factor	Coded Coeff	Uncoded Coeff
%M	36.17	1	36.17	103.0	0.0002	79.54%	%M	-2.126	-0.5316
%CBD	7.547	1	7.547	21.50	0.0056	16.60%	%CBD	-0.9713	-2.158
Error	1.755	5	0.3510			3.36%	Intercept	11.26	36.35
Lack of Fit	0.2278	1	0.2278	0.5967	0.4830	0.50%			
Total	45.47	7				100.00%	R2 (%)	96.14%	

Table 8A: 3 variables

ANOVA Table						
Factor	SS	df	MS	F	p value	% Cont
%M	38.76	1	38.76	N/A	N/A	51.90%
%CBD	2.520	1	2.520	N/A	N/A	3.37%
Ext Time	32.12	1	32.12	N/A	N/A	43.01%
%M*%CBD	0.0006	1	0.0006	N/A	N/A	0.00%
%M*Ext Time	0.2926	1	0.2926	N/A	N/A	0.39%
%CBD*Ext Time	0.8001	1	0.8001	N/A	N/A	1.07%
%M*%CBD *Ext Time	0.1891	1	0.1891	N/A	N/A	0.25%
Total	74.69	7				

Model Information		
Factor	Coded Coeff	Uncoded Coeff
%M	-2.201	1.230
%CBD	-0.5613	1.154
Ext Time	2.004	7.313
%M*%CBD	0.00875	-0.1660
%M*Ext Time	-0.1913	-0.4574
%CBD*Ext Time	-0.3162	-0.6076
%M*%CBD *Ext Time	0.1538	0.04271
Intercept	9.214	-3.780

Table 9A: 3 variables excluding interactions

ANOVA Table						
Factor	SS	df	MS	F	p value	% Cont
%M	38.76	1	38.76	120.9	0.0004	51.90%
%CBD	2.520	1	2.520	7.860	0.0486	3.37%
Ext Time	32.12	1	32.12	100.2	0.0006	43.01%
Error	1.282	4	0.3206			
Total	74.69	7				

Model Information		
Factor	Coded Coeff	Uncoded Coeff
%M	-2.201	-0.5503
%CBD	-0.5613	-1.247
Ext Time	2.004	1.002
Intercept	9.214	21.17
R2 (%)	98.28%	

Appendix D - Fair Use Evaluations

Fair Use Evaluation Documentation

Compiled using the **Fair Use Evaluator** [cc] 2008 Michael Brewer & the Office for Information Technology Policy, http://librarycopyright.net/fairuse/

Name:	Cayla Collett
Job Title:	Student
Institution:	Kansas State University
Title of Work Used:	Full Factorial Design Study Used to Improve the Extraction of Cannabinoids from CBD-Dominant Cannabis Flower using Subcritical-CO2
Copyright Holder:	Rebekah Brewer
Publication Status:	Unknown
Publisher:	
Place of Publication:	
Publication Year:	
Description of Work:	Understanding the Cannabis Plant Infographic
Date of Evaluation:	December 6, 2022
Date of Intended Use:	December 6, 2022

Describe the Purpose and Character of Your Intended Use:

[+] Use is for research
[+] Use is socially beneficial (It promotes the creation of new knowledge by providing necessary background)
[+] Use is not-for-profit
[+] Use is clearly defined and is restricted in scope
[+] Use is one-time, or is only occasional or spontaneous

Fair ☑ ☐ ☐ ☐ ☐ ☐ ☐ Unfair

Describe the **Nature** of Your Intended Use of the Copyrighted Work:

[+] Work to be used is primarily of a factual nature

Fair Unfair

Describe the **Amount** of Your Intended Use in Relation to the Copyrighted Work as a Whole:

[+] Only the amount required to achieve the stated, socially-beneficial purpose or objective will be used (educational)
[+] The portion used is not the "heart" of the work
[+] Only limited and reasonable portions will be used

Fair Unfair

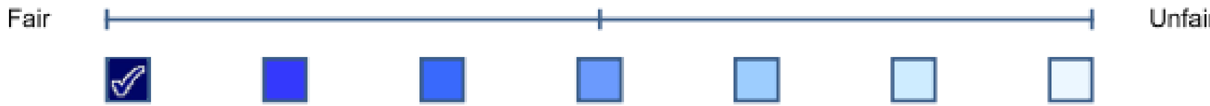
Describe the **Effect** of Your Intended Use on the Potential Market or Value of the Copyrighted Work:

[+] A market for the work as it will be used is absent or is negligible & use of the work will have little or no negative impact on its value or potential value

Fair Unfair

The Average **"Fairness Level,"** **Based on Your Rating of Each of the 4 Factors, Is:**

[see tool disclaimer for important clarifying information]:



Based on the information and justification I have provided above, I, Cayla Collett, am asserting this use is **FAIR** under Section 107 of the U.S. Copyright Code.

Signature: Cayla Collett

Date of Signature: 12/6/22

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