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Synergistic Interactions and Emulsion Preparation of Antioxidant Potential of Cannabidiol and Cold Brew Coffee

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Abstract

Antioxidants play an important role in mitigating the damage caused by oxidative stress in the human body. Over time, the oxidative stress can lead to diseases such cardiovascular disease, neurodegenerative disorders and even cancer. Notably, both cannabidiol and coffee antioxidants. **Purpose:** To investigate the interaction between the antioxidant potential observed from the combination of CBD and cold brew coffee and compare it to their individual effects. Additionally, develop a methodology for emulsion preparation for analysis of antioxidant potential of a beverage made of cold brew coffee infused with CBD. **Materials and Methods:** Two cold brew coffee and two CBD oil brands were used. A high-shear mixer was used for emulsion preparation. In this step, six emulsions were created using different parameters (emulsifier presence, rotation speed of high-shear mixer, and time). A qualitative analysis was performed prior to freezing to find the stable emulsion. Samples with stable emulsion were then freeze-dried for 24 hours before antioxidant potential assessment. Lyophilized samples went through a series of extraction steps, and the resulting extracts were evaluated using Trolox Equivalent Antioxidant Capacity (TEAC) assay for antioxidant potential. A Chi-square test assessed agreement between expected and observed antioxidant potentials in mixtures. A one-way ANOVA with Tukey's test determined significant differences among Trolox levels for cold brew coffee and CBD blends. **Results:** No visible separation was found for emulsion with parameters 10,000 rpm, without emulsifier and time of 5 minutes. A statistically significant higher antioxidant potential for blends C1CBD1 (0.510 mmol TE/100 mL wet weight, $p=0.009$, $p<0.05$) and C1CBD2 (0.571 mmol TE/100 mL wet weight, $p=0.03$, $p<0.05$) in their observed TEAC results compared to their expected values based on their individual tests was found. Similarly, a higher antioxidant potential was found for blends C2CBD2 and C2CBD1 compared to their expected values. However, neither were

statistically significant. **Conclusions:** Blending cold brew coffee with CBD in a beverage can enhance antioxidant activity and potentially offer potential health benefits. However, additional investigation is required to elucidate how different proportions of these components influence antioxidant potential.

Keywords: antioxidant, synergistic effects, cold brew, cannabidiol

MONTCLAIR STATE UNIVERSITY

Synergistic Interactions and Emulsion Preparation of Antioxidant Potential of Cannabidiol and Cold Brew Coffee

By

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SYNERGISTIC INTERACTIONS AND EMULSION PREPARATION OF ANTIOXIDANT POTENTIAL OF CANNABIDIOL AND COLD BREW COFFEE

A THESIS

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Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1 The coffee plant

Coffee holds a prominent position as one of the world's most popular beverages, enjoyed by millions worldwide. (Gökcen and Şanlier, 2019). It is usually made from mature seeds of *Coffea arabica* Linn, which belongs to the *Rubiaceae* family, growing at altitudes between 1000-2000 meters. These seeds are found in the red fruits of the plant and are processed by removing it from the fruit and sometimes the outer layer of the seed, known as the spermoderm. The seeds of botanical genus *Coffea* may be raw, roasted, whole, or ground. The beverage produced from these seeds is called coffee (Sharma, 2020). Out of the 70 known species of coffee, only three are commonly cultivated. *Coffea arabica* is the most cultivated, accounting for 75% of the world's coffee production, followed by *Coffea canephora* which provides almost 25% of total production (widely known as coffee *Robusta*), and *Coffea liberica* and other species which contribute less than 1% of the total (Sharma, 2020).

The fruits of the *Coffea* plant, often referred to as cherries, must undergo immediate processing upon harvesting to prevent spoilage. There are two primary processing techniques: the dry method, which involves sun-drying the coffee beans, and the wet method, which is done by removing the outer layers of the coffee cherry and subsequent fermentation of the beans for a period ranging from 18 to 24 hours. During this fermentation process, the mucilage, a sugary, viscous substance surrounding the bean, begins to break down. It's worth noting that the chosen processing method can significantly influence the coffee's flavor profile. The wet method typically results in a cleaner, more vibrant taste than the dry method (Illy, 2002). Next, the beans are roasted and prepared for their intended use, often being ground for sale to the final consumer. After the coffee beans have been processed, they need to be stored properly to maintain their quality. Coffee

should be stored in a cool, dry place, away from light and moisture. Whole beans should be ground just before brewing to ensure maximum freshness (Illy, 2002).

1.2 Historical evolution of coffee and cold brew coffee

The origin of coffee can be traced back to Ethiopia, where it was consumed as a brewed beverage made from the roasted seeds of the *Coffea* plant. The cultivation and trade of coffee began in the Arabian Peninsula. Coffee was primarily grown in the Yemeni district of Arabia in the 15th century, and by the 16th century, its consumption had spread to Persia, Egypt, Syria, and Turkey. Coffee consumption was not only limited to private homes but was also prevalent in public coffee houses, known as *Qahveh Khaneh*, which emerged in cities throughout the Near East. These establishments quickly gained popularity as social hubs for various activities, such as coffee consumption, socializing, music, entertainment, chess playing, and news sharing. The coffee houses became crucial centers for the exchange of information and became commonly referred to as "Schools of the Wise." In Mecca, a holy city attracting thousands of pilgrims from all over the world each year, the knowledge of the "wine of Araby" (i.e., coffee) began to spread (Teketay, 1999; National Coffee Association USA, n.d.)

The global spread of coffee began to accelerate when European travelers brought it back to Europe. Coffee became popular throughout Europe in the 17th century, particularly in major cities where coffee houses thrived as social hubs. In England, these were known as "penny universities," offering coffee and stimulating conversation for a penny. London alone had over 300 coffee houses by the mid-17th century, giving rise to various businesses (Pendergrast, 2019). In the United States, despite coffee being introduced in the mid-1600s, popularity grew after the Boston Tea Party in 1773 (National Coffee Association USA, n.d.). In the early 18th century, the plant was first introduced to the Caribbean area, then to Brazil, which has held the title of the

planet's primary coffee producer since the mid-1800s. Brazil is responsible for a significant portion of the global supply of standard-grade arabica coffee. Finally, the plant was introduced to the Cordilleras, which is regarded to produce some of the highest quality coffee in the world (McCook, 2017).

While cold brew has gained popularity in recent years, its history traces back to the 1600s when Japan began using cold brewing techniques with tea leaves soaked in cool river water. Around 40 years later, this method was applied to coffee, marking the inception of the first recorded instance of cold brew coffee. The period between 1920 and 1950 witnessed the use of finely ground coffee in Cuba. Later, a significant milestone in cold brew coffee occurred with the invention of the Toddy cold brew coffee system by American Todd Simpson, inspired during a trip to Peru. This method was an innovative way of cold brew coffee, and at the same time convenient and versatile, providing a cold brew coffee with a smooth flavor profile. Later, in 1969, Japan introduced its first vending machines selling canned chilled coffee. Fast forward to the 2010s, cold brew is now globally available in various flavors, infused with CBD, and packaged in different forms (Kasperowicz, 2019).

1.3 Cold Brew Coffee Background

Cold brew coffee's unique characteristics are primarily attributed to its unconventional brewing process. Cold brew coffee is a method of coffee preparation in which ground coffee beans are steeped in cold water, between a temperature of 4 °C to 25 °C, for an extended period, typically 12 to 24 hours. It has been gaining popularity due to its smoother taste and lower acidity when compared to traditional methods of hot water extraction, such as pour over, French press, drip brewing, and others. (Rao and Fuller, 2018).

Exploring the nuances of cold brew coffee further, Morresi et al. (2020) investigated how variations in the grind size and brewing time affected antioxidant capacity, sensory properties, and consumer acceptance of cold brew coffee. The study demonstrated that variations in grind size and brew time can significantly affect the final product's characteristics, including its antioxidant content and sensory attributes. The authors found that the grind size of 0.65 mm had a higher antioxidant content compared to 1.15 mm and 1.65 mm, but these results did not affect the likability among panelists in sensory traits. Understanding these variables is crucial for optimizing cold brew coffee's potential health benefits, production, and tailoring it to consumer preferences.

In another relevant study, Kyroglou at al. (2022) employed vacuum cycles to produce cold brew coffee and compared them to traditional methods. The research aimed to determine the ideal parameters for creating a cold brew coffee that would be accepted in sensorial analysis. The authors assessed the impact of vacuum cycles and pressure on physicochemical attributes (TDS%, pH, acidity, phenols, caffeine, and color) and conducted sensory evaluation. The optimal conditions were found to be two vacuum cycles at 205 mbar, resulting in lower physiochemical properties and higher sensory scores.

When considering the broader context of the coffee industry, it's important to note that coffee roasting and the manufacture of coffee products in the United States are considered "foods of no nutritional significance" and exempt of nutrition labeling according to the Food and Drug Administration (FDA) through the Federal Food, Drug, and Cosmetic Act (FDA, 2018). Additional exempt foods are tea leaves, instant coffee and tea, dehydrated vegetables for condiment purposes, flavor extracts, and food coloring. Even though coffee products are considered exempt under this regulation, the FDA mandates proper labeling of all food products, including coffee, with accurate information regarding product identification, allergen information

(if necessary), ingredients, net weight or volume, any health claims or nutritional content statements, and contact information. While specific regulations for cold brew coffee production and labeling do not currently exist due to its recent emergence in popularity, many coffee companies voluntarily adhere to existing FDA regulations and provide detailed information on their cold brew product labels.

As the popularity of cold brew coffee continues to rise, it is possible that more studies will be conducted to further refine methods of production and possibly lead to the development of specific regulations tailored to this coffee beverage in the future.

1.4 Cannabis plant

Cannabis stands as one of the most ancient plants cultivated for human purposes. Archaeological findings indicate its cultivation for fiber and rope use dating as far back as 12,000 BCE in central Asia (Friedman and Sirven, 2017). In the United States its history spans centuries, from its early use in colonial times for industrial and medicinal purposes to its federal prohibition with the Marijuana Tax Act of 1937. The Controlled Substances Act of 1970 classified Cannabis as a Schedule I controlled substance, leading to an extended period of federal prohibition (U.S. Customs and Border Protection, 2019). However, changing attitudes and the emergence of the medical and recreational cannabis movements in the late 20th century paved the way for a shift in cannabis policy.

According to Duggan (2021) the absence of essential understanding regarding certain facets of cannabis research is a consequence of the global prohibition in the past, which has significantly impeded scientific inquiries in this domain. While cannabis is still classified as a Schedule I controlled substance, over time, numerous states in the United States began legalizing medical and recreational cannabis stemming from a growing acceptance of cannabis use among

the public. This changing situation has led to different cannabis regulations across different states, a resurgence of interest in hemp-based products, and ongoing debates about federal cannabis policy reform (Bridgeman and Abazia, 2017).

Cannabis is an annual flowering plant from the *Cannabaceae* family, the same botanical family as hops (a common ingredient used to flavor beer). This genus comprises three species: *Cannabis sativa, Cannabis ruderalis,* and *Cannabis indica*. Nevertheless, these three species can crossbreed, leading to a certain fluidity in defining their species boundaries (Schilling et al., 2020).

Cannabis sativa strains have tall, slender plants with narrow leaves. They are often associated with higher levels of tetrahydrocannabinol (THC), the psychoactive compound accountable for the euphoric effects related with Cannabis use. The *Cannabis indica* strains are characterized by shorter, bushier plants with broader leaves. These strains typically have higher levels of cannabidiol (CBD), a compound that does not cause euphoric effects and is known for its medicinal properties. *Indica* strains are often associated with relaxation and sedation. Lastly, *Cannabis ruderalis* is less common and tends to have lower THC levels. It is primarily used for breeding and hybridization due to its autoflowering traits, which can be advantageous for cultivation (Gloss, 2015).

1.5 Chemistry of Cannabis

The primary constituents of cannabis are cannabinoids, terpenoids/terpenes, and flavonoids (responsible for the plant's pigmentation). Cannabinoids are the most well-known group of compounds in cannabis, with over 100 different cannabinoids identified to date. The two most abundant and studied cannabinoids are Δ9-tetrahydrocannabinol (THC) and CBD. While THC is most well-known for the psychoactive effects of cannabis, CBD is known for its various potential therapeutic properties (Duggan, 2021).

THC interacts primarily with the endocannabinoid system in the human body, binding primarily to cannabinoid receptors 1 and 2 in the central nervous system. This interaction leads to various psychoactive effects, including changes in mood, perception, and cognitive function (Zou and Kumar, 2018). Beyond its recreational use, THC has shown therapeutic promise. It is used medically to alleviate symptoms such as pain, nausea, and muscle spasms, particularly in conditions like chronic pain, chemotherapy-induced nausea, and multiple sclerosis (Bridgeman and Abazia, 2017).

CBD, in contrast to THC, does not induce a high or altered state of consciousness. It is non-psychoactive and well-tolerated even at high doses. CBD's mechanisms of action are diverse. Unlike THC, it has a minimal affinity for CB1 and CB2 receptors, instead interacting with other receptor systems and influencing the endocannabinoid system indirectly (Zou and Kumar, 2018). Among these receptors CBD shows agonist activity in the receptors TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and PPAR-γ (Raup-Konsavage and Vrana, 2023).

Cannabis contains chemicals known as terpenes and terpenoids. They are aromatic compounds responsible for the plant's unique flavors and aromas. Common terpenoids found in cannabis include myrcene, limonene, and pinene, each with its distinct aroma and potential therapeutic effects. For example, myrcene is associated with sedative effects, while limonene may have anxiolytic and mood-enhancing properties. Terpenoids are thought to work synergistically with cannabinoids, influencing the overall effects of cannabis through what has been coined as the entourage effect (Russo, 2011), further research is needed to understand these effects.

1.6 Cannabis use as a food ingredient

Cannabis has been used for its therapeutic effects as well as a food ingredient since ancient times (Farinon et al., 2020). Cannabis-infused edibles refer to food items that incorporate extracts

from the cannabis plant, additionally cannabis products including those that use hemp seeds. These infusions are present in a diverse range of food and beverages such as coffee, dairy products, baked goods such as muffins, brownies, chocolates and even films for vegetables and fruits (Iftikhar et al., 2021).

In a review, Farinon et al. (2020) found that incorporating hemp seeds into animal feed can impact products such as eggs, milk and meat, leading to improvements in the fatty acid profiles of these items. However, incorporating hemp seeds into enriched or fortified foods meant for human consumption can alter their rheological and sensorial properties, posing challenges to consumer acceptability. Additionally, the authors emphasized the need for further research to better understand the functional compounds in hemp seeds, their molecular mechanisms, optimal concentrations, and suitable matrices to preserve functionality in the gastrointestinal tract.

While cannabis has been used in food applications such as baked goods, beverages and other food items intended for human consumption, its acceptance is still not widely studied. In a study conducted by Charlebois et al. (2018), the authors explored the acceptance of cannabis as a food ingredient by Canadian citizens before it became a legal drug in Canada in 2018. The authors conducted an online survey across Canada. As a result, they found that almost half of the respondents (45.8%) would be willing to try products with cannabis, where baked goods ranked among the top choices (46.1%). Regarding the concerns about cannabis as a food ingredient, more than half of the respondents (58.5%) concerns were related to children eating cannabis food products by accident. Charlebois et al. (2020) in their most recent work, did a second assessment to understand if consumer’s perceptions had changed after cannabis legalization in Canada. The authors described that while cannabis itself was legal, products like edibles, i.e., cannabis applied in a food or beverage were not yet fully legalized, at the time of the study. The results for the

second assessment were analogous to the one in 2018, with an increase regarding safety concerning children and pets (60%). The findings underscore the immediate need for regulation of edibles containing CBD and/or THC.

1.7 Antioxidants background

Antioxidants have been shown to play an essential role in mitigating oxidative stress, which is associated with health conditions, such as cancer, cardiovascular disease, and neurodegenerative disorders. (Pisoschi et al., 2021). Oxidative stress happens when there is an imbalance between the production of free radicals and the body's antioxidant defense mechanism. These reactive oxygen species (ROS) are naturally produced in the body but some environmental exposure such as pollution, can increase its levels (Bjørklund and Chirumbolo, 2017). Antioxidants are readily available from dietary sources, including fruits, vegetables, nuts, and seeds, containing vitamins C and E, beta-carotene, and flavonoids, among others. Within the body, endogenous antioxidants such as glutathione and superoxide dismutase are produced to combat oxidative stress, with several other species (Pisoschi et al., 2021).

Antioxidant substances function via various chemical mechanisms, including the transfer of hydrogen atoms (HAT), the transfer of single electrons (SET), and the capability to bind transition metals. In HAT, antioxidants donate a hydrogen atom (H) to free radicals. Free radicals exhibit high reactivity due to their unpaired electrons, and by donating a hydrogen atom, antioxidants can stabilize them. This process transforms the free radical into a stable molecule and prevents it from causing further damage in the body such as cellular or protein damage. They can also neutralize free radicals by transferring a single electron to them through SET mechanism. This electron transfer helps stabilize the free radical by pairing its unpaired electron. Some antioxidants can bind to transition metals like iron and copper. Transition metals can catalyze the production of

harmful free radicals in a process called the Fenton reaction. By binding to these metals, antioxidants can prevent them from participating in reactions that generate free radicals (Francenia Santos-Sánchez et al., 2019).

Antioxidants can be found in a wide variety of foods. During food preparation, which includes cooking, mixing, or any other cooking processes, different substances can become mixed. Consequently, various antioxidants may mix in the process. These antioxidants have the potential to interact with each other, which could possibly result in additive, synergistic or antagonistic effects.

In the context of antioxidants, additive effects occur when multiple antioxidants are used or consumed together, leading to combined or cumulative benefits. These effects are expected when two different compounds are combined, with the anticipation that their potential will be added together. Skroza et al. (2015) investigated the possible combined or synergistic antioxidant effects when resveratrol was combined with other phenolic compounds in two-component mixtures, they concluded that as these compounds can exhibit unpredictable interactions when combined, which becomes challenging to determine the cumulative antioxidant activity of mixtures by solely examining the data pertaining to individual components.

Synergism refers to the cooperative or interconnected operation of two or more components, agents, or physiological functions in a way that their combined effect surpasses the cumulative impact of each operating independently. Finally, antagonism refers to a situation in which the combined action of two or more substances, when used together, results in an effect that is weaker or smaller than the sum of the effects produced by each substance individually (Olszowy-Tomczyk, 2020).

Previous studies have investigated additive, synergistic and antagonistic effects. Durak et al. (2015) reported an observed synergistic interaction between coffee and willow extract when assessing their ability to inhibit lipoxygenase activity. Similarly, Panya et al. (2017) found that rosmarinic acid and its esters, when combined with α-tocopherol and through the formation of additional antioxidants like caffeic acid, can work together synergistically to effectively inhibit lipid oxidation in oil and water emulsions, thus improving the oxidative stability of food products. In another study, Muhammad et al. (2017) conducted experiments involving the combination of epicatechin and catechin (found in chocolate) with gallic acid, tannic acid, quercetin, sinapic acid, cinnamic acid, eugenol, and cinnamaldehyde (found in cinnamon) in various proportions. Their results demonstrated a significant increase in antioxidant activity when cinnamon extract was added to the cocoa extract. This interaction exhibited a spectrum ranging from synergy to antagonism, with less synergy observed as higher proportions of cinnamon extract were used.

Previous investigations have studied antioxidant activity in binary systems of diverse substances (Durak et al., 2015; Panya et al., 2017; Muhammad et al., 2017) as well as the individual antioxidant properties of cannabidiol (Tura et al., 2019) and cold brew coffee (Rao and Fuller, 2018). However, to this date, no studies have examined their antioxidant effects when combined.

1.8 Antioxidant activity and nutrition in coffee and cold brewed coffee

Green coffee beans contain a variety of bioactive components known for their antioxidant properties. These components, including caffeine, chlorogenic acid, trigonelline, cafestol, and kahweol, are found in varying proportions depending on the bean's origin. When coffee beans undergo the roasting process, their chemical composition changes, resulting in the development of distinct flavors, smells, and colors. In roasted beans, melanoidins exhibit antioxidant capabilities and are form through non-enzymatic browning. As a result, the antioxidant capacity of brewed

coffee doesn't solely stem from the components present in green beans but also from those created during the roasting process (Liang and Kitts, 2014).

These antioxidants play a pivotal role in safeguarding cells from oxidative stress, a contributing factor to several chronic diseases. Coffee consumption has been linked to potential health advantages such as lowering the likelihood of some types of cancer, cardiovascular disease, and type 2 diabetes. However, the amount and type of coffee consumed can also have negative effects, such as increasing the risk of high blood pressure and heart disease in individuals with underlying medical conditions (Mejia and Ramirez-Mares, 2014).

In addition to its potential role in preventing disease, coffee consumption may also have cognitive benefits. For example, Johnson-Kozlow et al., (2002) conducted a study following a population from the Rancho Bernardo Study for four years (between 1988 to 1992) of 890 females and 638 males with average age of 72.6 and 73.3 years old, respectively. They found that coffee consumption was associated with improved cognitive performance in older women. They had a better performance in 50% of the tests and self-reported having a higher intake of coffee throughout their lives (caffeinated). Conversely, no relationship was observed between coffee consumption and cognitive process for men nor was there any association detected between decaffeinated coffee intake and cognitive processes for either males or females. In addition, Shukitt-Hale et al. (2013) studied rats to understand the effects of coffee in aged animals and cognitive behavior. They found out that animals in a diet including 0.55% of coffee supplements had better performance in cognitive tests compared to rats that had a standard diet. It is important to note that it is reported that the advantageous effects of coffee in protecting the nervous system are not solely attributable to caffeine but are instead attributed to other bioactive compounds found

in coffee. As a result, consuming moderate amounts of coffee may decrease physical and mental decline during aging.

Numerous studies have confirmed the antioxidant potential of coffee. Del Castillo et al. (2002) concluded that the greatest antioxidant capacity is found in coffee beans roasted to a medium degree, primarily because of the equilibrium achieved between the breakdown of phenolic compounds and the formation of Maillard reaction byproducts known as melanoidins during the roasting process. Rao and Fuller (2018) investigated the acidity and antioxidant activity of cold brew coffee and compared that to hot brew coffee. They found that cold brew coffee has lower acidity compared to traditional hot brewed coffee and suggested that the hot brewing method tends to draw out a greater amount of non-deprotonated acids compared to the cold brewing method. These acids might be accountable for the elevated levels of antioxidant properties observed in the hot-brewed coffee samples. In a 2020 study by Grim et al., coffee samples were made from Colombian beans roasted at various temperatures, both for hot and cold brews. Hot brew coffee displayed higher caffeine and total CQA levels and better antioxidant activity than cold brew. Coffee pH rose with roast temperature, and hot brews had more titratable acids and TDS than their cold counterparts. The relationship between roast temperature and coffee attributes was linear for hot brew and followed an inverted U-shaped trend for cold brew, highlighting the significant impact of roasting temperature on coffee chemistry, especially in crafting cold brew coffee beverages.

While coffee is most well-known for its antioxidant properties, it also serves as a valuable source of chromium and magnesium. These two essential minerals aid in regulating blood sugar levels by supporting the effective utilization of insulin (Sharma, 2020).

It is important to note that while coffee's antioxidant capacity is a promising area of research, individual reactions to coffee may differ from person to person. Therefore, further investigations are needed to not only understand the specific health effects of antioxidants in coffee comprehensively but also expand knowledge in cold brew research.

1.9 Antioxidant activity of cannabidiol

CBD, one of the major phytocannabinoids found in the cannabis plant, has gained significant attention in recent years due to its diverse range of potential therapeutic properties. An essential determinant of a CBD's antioxidant capacity lies in the presence and arrangement of hydroxyl groups or electron-donating groups, which can facilitate electron and hydrogen abstraction reactions. CBD also distinguishes itself from other cannabinoids by containing two phenolic groups, which contribute to its notable antioxidant potential. CBD's molecular formula (figure 1) comprises three distinct chemical components: limonene (green), phenol (blue), and an aliphatic group (red) (Borges & da Silva, 2017).

Figure 1- Chemical structure of CBD

(Borges & da Silva, 2017)

CBD has been shown to express antioxidant effects through several mechanisms. One of these mechanisms involves the enhancement of superoxide dismutase activity, an endogenous antioxidant enzyme responsible for dismuting superoxide radicals into oxygen and hydrogen peroxide. This action effectively reduces the levels of superoxide radicals, which are a primary

type of ROS responsible for cellular damage. Furthermore, CBD is believed to increase intracellular levels of glutathione, a potent antioxidant found in cells. Glutathione plays a pivotal role in cellular health by neutralizing ROS and safeguarding biomolecules from oxidative damage (Atalay et al., 2019).

Studies also have shown that CBD can reduce lipid peroxidation, protein oxidation, and DNA damage, suggesting its potential in mitigating oxidative stress-related diseases (Atalay et al., 2019; Sun et al., 2017).

1.10 Effects of food processing in antioxidants

Numerous studies have investigated how both thermal and non-thermal processes affect antioxidants, showing a range of positive and negative outcomes. Thermal processes, which encompass traditional methods crucial in cooking, baking, and food preservation, play a pivotal role in ensuring food safety (Phanumong et al., 2022). While heat can enhance the release of certain antioxidants by breaking down cell walls and making them more bioavailable, it may also lead to the degradation of sensitive antioxidants, such as vitamin C and polyphenols (Poljsak et al., 2021).

In a study, Al-juhaimi et al. (2018) reviewed several articles to compare conventional thermal processes with non-thermal processes such as gamma and UV radiation, UV light, pulsed electric fields, and hydrostatic pressure in the processing of fruits and vegetables. While findings are similar to Poljsak et al. (2021), the authors found that heat damage is particularly pronounced in colored foods that contain antioxidant pigments like anthocyanins. Nevertheless, they also found that certain studies have contradicted the notion that thermal damage can diminish the overall antioxidant properties of food products. Nonthermal methods, on the other hand, seem to have a less detrimental effect on essential health-promoting phytochemicals and may even improve their

bioavailability. Opting for these processes might be a strategic choice in enhancing the nutritional quality of food items.

Coffee roasting is a heat processing technique used to turn green coffee beans into darker variations to enhance sensorial characteristics (National Coffee Association USA, n.d). Previous studies have investigated the relationship between coffee roasting and its effect in antioxidant activity (Liang and Kitts, 2014; Rao and Fuller, 2018). All their findings were consistent with previous study from Del Castillo et al. (2002), where it was found that an increase in coffee roast degree is associated with reduced antioxidant activity. This is due to the loss of chlorogenic acid during the roasting process. Although other antioxidant components are produced as byproducts of the Maillard reaction that happens during roasting such as melanoidins, for example.

Related to cold brew coffee, Phanumong et al. (2022) investigated two thermal processes, sterilization in a glass bottle and pasteurization in an aluminum foil pouch, to enhance shelf-life in a beverage made of cold brew coffee (*Robusta*) and coconut water. The authors found that sterilized coffee exhibited a significant decrease in caffeine content, total phenolic acid levels, and DPPH radical scavenging activity in contrast to pasteurization.

The extent of food processing into antioxidants also depends on factors like temperature, duration, and the specific antioxidant compounds present in the food.

1.11 Relevance of studying CBD and cold brew coffee combined

In recent years, cold brew coffee has become a popular alternative to the traditional hot brewed coffee due to its smoothness and lower caffeine content (Rao and Fuller, 2018). According to market research company The Brainy Insights (2023), this shift in consumer preference aligns with the substantial growth observed in the global cold brew coffee market. In 2022, the market was valued at $650.91 USD million dollars, but projections indicate it is set to reach $5.48 USD

billion by 2032. On the other hand, CBD sales declined about 10% from 2022 to 2023 in the United States, its main reason due to inflation post COVID-19 pandemic. However, even with these results, the market value of CBD infused drinks is expected to reach $6 billion USD by 2030, doubling its value of $3 billion USD since 2022 (Zion, Market Research, 2022).

Thus, the forthcoming results of this investigation carry significant ramifications for both health and industry. If an increase in antioxidant activity is found in the combination of cold brew coffee and CBD, it could mean potential health benefits for consumers. This could be particularly advantageous for individuals seeking ways to enhance their antioxidant intake, which is known to generally contribute to overall well-being. On the other hand, if an antagonist effect is found, this study could reduce the prevalence of products combining cold brew coffee and CBD.

If a synergistic effect is found, it could open new avenues for the development of therapeutic approaches using this unique beverage combination. Researchers and practitioners may explore the therapeutic applications of CBD-infused cold brew coffee for various health conditions, offering a novel and potentially effective treatment option.

For the food and beverage industry, there is an opportunity for product development. Companies can consider incorporating CBD and cold brew coffee into their product formulations to not only tap into the growing demand for health-conscious products but also to boost the antioxidant content of their offerings. This could help market these products as healthier options, potentially increasing sales and consumer satisfaction.

Additionally, the study's findings might encourage exploration of the use of combinations of these antioxidants for food preservation. This could have implications for extending the shelf life of certain products while also providing health benefits to consumers, a win-win scenario for both the industry and consumers.

Finally, the study's innovative approach in combining cold brew coffee and CBD may stimulate further research. Researchers may explore further the interactions between these substances and other compounds, leading to a better understanding of their potential synergistic or antagonistic effects in uncovering new applications in the fields of nutrition, health, and beyond. This suggests that the implications of this study extend not only to immediate practical applications but also to the exciting potential for future discoveries and advancements in the field.

Despite the availability of coffee beverages infused with cannabidiol (CBD), there is a lack of studies examining the combined antioxidant effects of these components. Thus, this study investigated the interaction between the antioxidant potential observed from the combination of CBD and cold brew coffee compared to their individual effects, as determined by the Trolox Equivalent Antioxidant Capacity (TEAC) assay.

Chapter 2: Manuscript I

TEAC Antioxidant Analysis of cold brew coffee and Cannabidiol

2.1 Abstract

Antioxidants are pivotal in alleviating oxidative stress, a contributing factor associated with diverse health issues like cancer, cardiovascular disease, and neurodegenerative disorders. During food preparation, the combination of antioxidants from different food sources can lead to synergistic interactions, enhancing their collective potential. Notably, both coffee and cannabis demonstrate antioxidant properties. **Purpose**: To investigate the interaction between the antioxidant potential observed from the combination of CBD and cold brew coffee and compare it to their individual effects. **Materials and Methods:** Two cold brew coffee brands and two CBD oil brands were used. A high shear mixer was used for emulsion preparation, which was then freeze-dried for 24 hours before antioxidant potential assessment. Lyophilized samples went through a series of extraction steps, and the resulting extracts were evaluated using Trolox Equivalent Antioxidant Capacity (TEAC) assay for antioxidant potential. The Chi-square of independence test was performed to assess the agreement between the expected values and the actual observed values from the TEAC assay. Additionally, a one-way ANOVA along with Tukey's test was employed to identify if there was significant difference among Trolox equivalents for blends of cold brew coffee and CBD. **Results:** A statistically significant higher antioxidant potential for blends C1CBD1 (0.510 mmol TE/100 mL wet weight, $p=0.009$, $p<0.05$) and C1CBD2 (0.571 mmol TE/100 mL wet weight, $p=0.03$, $p<0.05$) in their observed TEAC results compared to their expected values based on their individual tests was found. Similarly, a higher antioxidant potential was found for blends C2CBD2 (0.404 mmol TE/100 mL wet weight, $p=0.213$) and C2CBD1 (0.361 mmol TE/100 mL wet weight, $p=0.292$) compared to their expected values. However, neither were statistically significant. **Conclusions:** Combining cold brew coffee with CBD in a beverage can enhance antioxidant activity and potentially contribute to individuals'

health. However, further research is required to understand the impact of different substances ratios and types of cold brew coffee and CBD on observed synergistic effects.

2.2 Introduction

Antioxidants are known to play a crucial role in alleviating oxidative stress, a factor linked to various health issues such as cancer, cardiovascular disease, and neurodegenerative disorders (Pisoschi et al., 2021). Oxidative stress happens due to an imbalance between the production of free radicals and the body's antioxidant defense mechanism (Bjørklund and Chirumbolo, 2017). Antioxidants counteract the damaging effects of free radicals by donating electrons, stabilizing them, and preventing them from causing oxidative stress. These substances function via various chemical mechanisms, including the transfer of hydrogen atoms (HAT), the transfer of single electrons (SET), and the capability to bind transition metals (Francenia Santos-Sánchez et al., 2019).

At first, antioxidants were primarily studied due to their role in food preservation. Over time, it became evident that their significance extended beyond preventing oxidative processes in food, encompassing a crucial role in mitigating oxidative stress within the human body. Given its importance, the effects of antioxidants have been extensively studied in both the fields of nutrition and food science (Cömert and Gökmen, 2018).

Antioxidants come from endogenous and exogenous sources. Many antioxidants are synthesized within the body as parts of its normal metabolism, such as glutathione. Simultaneously, a significant portion of antioxidants come from dietary sources. Examples include fruits, vegetables, nuts, seeds, and other antioxidant-rich foods (Pisoschi et al., 2021).

Coffee is a source of antioxidants that has been vastly studied (Durak et al., 2015). Cold brew coffee has surged in popularity due to its smooth and less acidic flavor profile, making it an

appealing alternative to traditional hot brewed coffee (Rao and Fuller, 2018). Additionally, its convenience, ability to be prepared in large batches and stored, and versatility in terms of concentration and serving methods have also contributed to its widespread emerging preference.

Green coffee beans contain a variety of bioactive components known for their antioxidant properties. These components, including caffeine, chlorogenic acid, trigonelline, cafestol, and kahweol, are found in varying proportions depending on the bean's origin. When coffee beans undergo the roasting process, their chemical composition changes, resulting in the development of distinct flavors, aroma, and colors. In roasted beans, melanoidins, which form through non-enzymatic browning, also show antioxidant capabilities. As a result, the antioxidant capacity of brewed coffee doesn't solely stem from the components present in green beans but also from those created during the roasting process (Liang and Kitts, 2014).

A non-conventional source of antioxidants is *Cannabis sativa*. *Cannabis sativa* is a medicinal plant with a long history of use in various cultures. It contains several compounds, including cannabidiol (CBD), which has shown to possess antioxidant, anti-inflammatory, and neuroprotective properties, making it a promising therapeutic agent, despite the controversies involving Cannabis cultivation and uses (Bridgeman and Abazia, 2017) .

Cannabidiol and cold brew coffee antioxidant properties have been attributed to their ability to stabilize free radicals. While the antioxidant activity of cannabidiol is associated with the fact that it is a terpenophenolic, cold brew coffee, which pass through an extraction process and dilution, has been linked to its high levels of chlorogenic acid and other polyphenols (Atalay et al., 2019; Rao and Fuller, 2018). When food is mixed through cooking, beverage preparation or similar, antioxidants present in foods can get mixed. These antioxidants have the potential to interact with each other, which could possibly result in additive, synergistic or antagonistic effects.

In this context, the combination of CBD and cold brew coffee beverage could potentially lead to a synergistic effect. While an additive effect is what is usually expected, synergistic effects can also happen by boosting the antioxidant activity of products and affecting health benefits.

Previous investigations have studied antioxidant activity in binary systems of diverse substances (Durak et al., 2015; Panya et al., 2017; Muhammad et al., 2017) as well as the individual antioxidant properties of cannabidiol (Tura et al., 2019) and cold brew coffee (Rao and Fuller, 2018). However, to date, no studies have examined their antioxidant effects when combined.

2.3 Materials and Methods

2.3.1 Study design

All the experimental procedures for this project took place in the food research laboratory of the Department of Nutrition and Food Studies at Montclair State University. The research methodology for this thesis involved the utilization of an experimental factorial design to evaluate the antioxidant potential of both individual substances and mixture of CBD and cold brew coffee.

2.3.2 Coffee Sample Preparation

Two different coffee brands were used in this study. One was donated by Law Coffee, located in Newark, New Jersey (C1). A second was acquired from a grocery store chain located in Holmdel, New Jersey (C2). Both brands use only ground coffee beans and water in the ingredients. Once received, the coffee samples were stored in the refrigerator at 4 ^{o}C. Prior to starting experiments, the cold brew coffee bottles were mixed into two single containers according to each brand and kept again in the refrigerator at 4 ^{o}C. This was done to avoid any possible difference between batches. The decision to use ready-to-drink beverages was made to be maximally comparable to products typically purchased by consumers.

Next, 325 mL of cold brew were homogenized in a high shear homogenizer (Silverson, Model L5M-A, East Longmeadow, MA, USA) for 5 minutes, followed by sonication in ultrasonic cleaner (Branson, Model 5800, Danbury, CT, USA) for 10 minutes. After this, samples of 16 g each were weighed and placed in deep freezer at –81 ^{o}C for 24 hours.

After 24 hours, the coffee samples underwent a 24-hour lyophilization in the freeze dryer before going through the extraction process. Subsequently, the resulting powdered coffee samples were transferred into hermetically sealed glass containers and preserved at a temperature of -81°C until extraction and phenolics analysis.

2.3.3 CBD Sample Preparation

Similarly, two different CBD brands were used in this study. Both brands used were THC free and had MCT oil and CBD extracted from hemp oil as ingredients. One was acquired from a company in Louisville, CO (CBD1) and the other one was purchased from a second company in Lafayette, CO (CBD2).

CBD samples were also mixed into two single containers according to brand and stored at room temperature according to manufacture instructions. Due to sample size limitations, CBD samples were only sonicated in ultrasonic cleaner for 10 minutes and did not go through homogenization. The samples were initially weighed and separated into 0.3 gram samples then subjected to 24 hours in a deep freezer at -81°C. Subsequently, the CBD samples underwent a 24-hour lyophilization in a freeze dryer before proceeding with the extraction process. While the CBD maintained its oily characteristics, the intent behind this process was to ensure process consistency with the other samples.

2.3.4 Cold brew coffee mixed with CBD

An emulsion of cold brew coffee infused with CBD was created by combining each type of cold brew coffee (C1 and C2) with their respective CBD components (CBD1 and CBD2). All samples went through triplicate assessments, and Table 1 presents the sample combinations used for measuring antioxidant potential (AntOX_Potential). Coffee brands are represented as C1 and C2, and the different brands of CBD are represented on the table as CBD1 and CBD2.

Table 1 - Possible combinations of sampling in triplicate

Sample	Brand_coffee	Brand_CBD	AntOX_Potential
1	C1	-	
2	C1	CBD1	
3	C1	CBD2	
4	C2	-	
5	C2	CBD1	
6	C2	CBD2	
7	-	CBD1	
8	-	CBD2	

The CBD dosage for the sample was determined by referencing existing RDT cold brew coffee beverages available in the market. Since most of these beverages contain CBD levels ranging from 15 to 25 mg, this study used 25 mg per 325 mL of cold brew coffee as the standard for the blend preparation.

For the preparation of the blend C1CBD1, 162.5 mL of cold brew coffee (C1) stored at 4°C was used. This volume was then combined with 250 µL of CBD1 in a beaker. The mixture

underwent homogenization in a high shear homogenizer for 5 minutes, followed by sonication in an ultrasonic cleaner for 10 minutes. Subsequently, 15 g samples were weighed and placed in a deep freezer at –81°C for 24 hours.

After 24 hours, the coffee samples underwent a 24-hour lyophilization in the freeze dryer before going through the extraction process. Subsequently, the resulting powdered C1CBD1 samples were transferred into hermetically sealed glass containers and preserved at a temperature of -81°C until extraction and phenolics analysis. The same procedure was conducted for samples C2CBD1 with respective samples of C2 and CBD1.

The procedure for samples C1CBD2 and C2CBD2 was identical, with the only variation being the volume of CBD used, this is because the brands had different amounts of CBD per serving. In the case of C1CBD2, 190 μL of CBD2 was mixed with 162.5 mL of cold brew coffee (C1). Similarly, for sample C2CBD2, 190 μL of CBD2 was used and mixed with 162.5 mL of cold brew coffee (C2).

2.3.5 Phenolics isolation

A series of extraction steps was employed to separate phenolics and water-based antioxidants from the lyophilized coffee, CBD samples and combined samples, adapted from previous work as described by Morresi (2019). For samples that contained cold brew coffee (only coffee and combined with CBD) the entire lyophilized sample was utilized and combined with a 4:1 acetone/water solution in a volume of 2.5 mL. For samples that contained only CBD oil, the sample was also mixed with 2.5 mL of a 4:1 acetone/water solution. The resulting mixtures were sonicated for 10 minutes, and then centrifuged at 1000g for another 10 minutes. After the centrifugation, the supernatant was separated and kept aside. Next, the samples containing coffee

were mixed with another 2.5 mL of the 4:1 acetone/water solution. If CBD oil only, the samples did not have a second step. Cold brew coffee samples were sonicated again for 10 minutes and centrifuged for 10 minutes. The second supernatant was removed and combined with the first one.

The samples then went through evaporation. The evaporation bath was set at 40°C, and the spider flasks rotated at 20 rpm. The evaporation process started at 307mBar and stayed at this pressure for about 30 minutes. Next, the pressure was reduced by 20mBar every 5 minutes until it reached a final pressure of 120mBar. The pressure was kept constant at 120mBar until there was no more solvents being present.

2.3.6 Antioxidant Potential

The antioxidant potential was evaluated through the Trolox Equivalent Antioxidant Capacity (TEAC) assay using a microplate reader (VersaMax with SoftMax Pro Software, Molecular Devices, San Jose, CA, USA). This approach enabled the evaluation of antioxidant efficacy by comparing how well each individual sample (CBD and cold brew) and mixed sample slowed oxidative reactions in comparison to Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a well-established powerful antioxidant. Consequently, the outcomes of this evaluation are characterized as "Trolox equivalents." The free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) served as the radical standard to gauge the sample's ability to counteract the radical in relation to a Trolox standard curve (protocol adapted from Brand-Williams et al., 1995).

Each sample from prior phenolic isolation was mixed with 0.3 mL of solvent containing 99.7/0.3 water/formic acid solution (15 µL of formic acid to approximately 4 mL of H_2O and then dilute with H_2O to 5 mL). Additionally, a 101.4405 µM DPPH solution was prepared mixing 1 mg

of DPPH in a 25 mL of a solution of methanol and water (20 mL methanol, 5 mL H_2O), the DPPH solution was then sonicated for 4 minutes.

To optimize readability in the microplate reader, various dilutions were applied to the samples. Specifically, samples C1, C2, and C2CBD1 underwent a dilution of 1/32. CBD1, being the exception, was utilized without any dilution. CBD2 required doubling its strength and volume. For C1CBD1, C2CBD2, and C1CBD2 samples, a dilution of 1/64 was performed. Variances in dilution were necessary due to observations made after several runs in the microplate reader, indicating that certain dilutions were stronger than others.

A 1.5 mM Trolox solution was also made by mixing 6 mg of Trolox in 16 mL of solution 1:1 made of acetone and water (17 mL of acetone and 17 mL of water).

In the microplate reader, the absorbance was configured to 517 nm for a kinetic measurement lasting 30 minutes at 27℃. Various combinations were prepared for evaluation: the sample blank involved mixing 10 µL of a 99.7/0.3 water/formic acid solution with 290µL of DPPH (101.4405µM); the 0mM Trolox sample was created by combining 10 µL of a 0mM Trolox Solution with 290µL of DPPH (101.4405µM); the 0.3 mM Trolox sample was formed by mixing 10 µL of a 0.3 mM Trolox Solution with 290µL of DPPH (101.4405µM); the 0.6 mM Trolox sample was prepared by mixing 10 µL of a 0.6 mM Trolox Solution with 290µL of DPPH (101.4405µM); the 1.2 mM Trolox sample was generated by combining 10 µL of a 1.2 mM Trolox Solution with 290µL of DPPH (101.4405µM); and the 1.5 mM Trolox sample was produced by mixing 10 µL of a 1.5 mM Trolox Solution with 290µL of DPPH (101.4405µM) in order to produce the curve. The final absorbance value was recorded after 30 minutes.

2.4 Statistical Analysis

For statistical analysis Jasp software (University of Amsterdam) was used. The tests performed were a chi-square of independence test to assess the agreement between the expected values, derived from the calculated proportional sum of the antioxidant potentials of the individual components (CBD and cold brew coffee), and the actual observed values for the antioxidant potential of the mixture.

Additionally, a one-way ANOVA (Analysis of Variance) along with Tukey's test was employed to identify if there was significant difference among Trolox for blends of cold brew coffee and CBD.

2.5 Results

A total of 24 samples, in triplicate, were used in this study. Trolox Equivalent Antioxidant Capacity (TEAC) results are expressed in units of micromoles Trolox equivalents per 100 milliliters of wet weight (mmol TE/100 mL wet weight) and mean results are shown in Table 2. Trolox curves were plotted for each analysis and equations were used to calculate TEAC. The highest absolute value is attributed to the blend C1CBD2 (0.571 mmol TE/100 mL wet weight) and the lowest absolute value for TEAC results are shown for CBD1 (0.0834 mmol TE/100 mL wet weight).

Table 2 - Mean TEAC results for analyzed samples

Sample	TE/100 mL wet weight
Coffee 1	0.421
Coffee 2	0.355
CBD 1	0.0834
CBD 2	0.171
Blend (Coffee 1 CBD 1)	0.510
Blend (Coffee 2 CBD 2)	0.404
Blend (Coffee 1 CBD 2)	0.571
Blend (Coffee 2 CBD 1)	0.361

Additionally, the expected value for each coffee and CBD blend was determined, allowing an analysis of the specific effects of both components—the coffees and CBDs—individually. Table 3 shows the observed and expected value for each of the blended samples with related p-value. Expected values were computed by referencing Table 2, considering the results from individual samples along with the respective proportions. To illustrate, in the case of blend C1 CBD1, we utilized 162.5 mL of cold brew coffee and 250 µL of CBD1, resulting in 99.84% of the sample representing C1 and 0.06% representing CBD1.

A statistically significant higher antioxidant potential for blends C1CBD1 (0.510 mmol TE/100 mL wet weight, $p=0.009$, $p<0.05$) and C1CBD2 (0.571 mmol TE/100 mL wet weight, $p=0.03$, $p<0.05$) was found in their observed TEAC results compared to their expected values based on their individual tests shown in Figure 3 (expected TEAC for C1CBD1, 0421 mmol TE/100 mL wet weight and C1CBD2, 0.421 mmol TE/100 mL wet weight). Similarly, a higher antioxidant potential was found for blends C2CBD2 (0.404 mmol TE/100 mL wet weight, $p=0.213$) and C2CBD1 (0.361 mmol TE/100 mL wet weight, $p=0.292$) compared to their expected values (expected TEAC for C2CBD2, 0.355 mmol TE/100 mL wet weight and C2CBD1, 0.355 mmol TE/100 mL wet weight). However, the last two were not statistically significant.

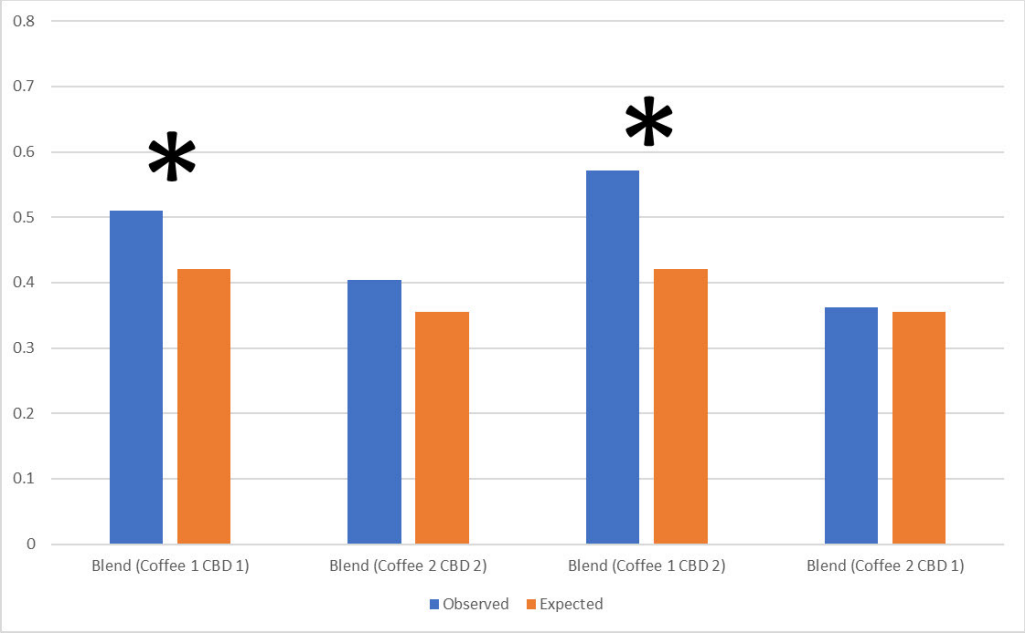
Table 3 - Observed and expect TEAC results for blends

Sample	Observed mmol TE/100 mL wet weight	Expected mmol TE/100 mL wet weight	p-value
Blend (Coffee 1 CBD 1)	0.510	0.421	0.009
Blend (Coffee 2 CBD 2)	0.404	0.355	0.213
Blend (Coffee 1 CBD 2)	0.571	0.421	0.03
Blend (Coffee 2 CBD 1)	0.361	0.355	0.292

p-values smaller than 0.05 indicate statistical significance.

Figure 2 shows graphically the differences between observed and expected TEAC for blends.

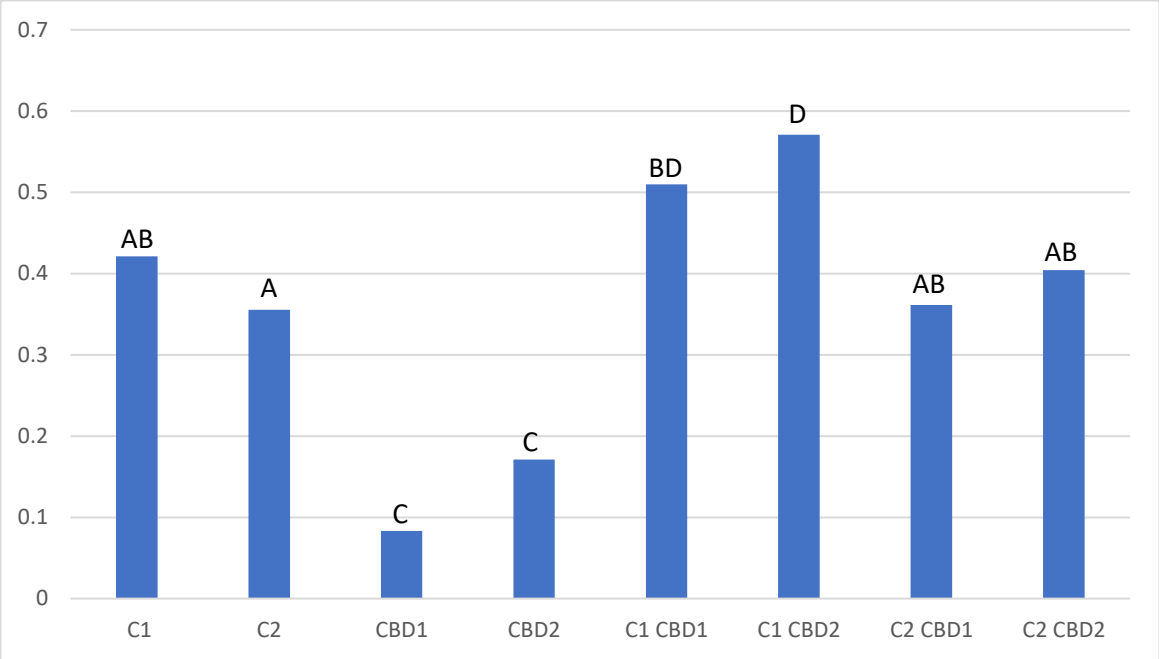
Figure 2 - Observed and expect TEAC results for blends



Asterisks indicate statistical significance, p-values smaller than 0.05

Additionally, one way ANOVA results are shown in Figure 3. Significant differences exist between samples placed in different columns that do not share the same letter ($\alpha = 0.05$).

Figure 3 - Comparison between samples



2.6 Discussion

The mean values found for TEAC for coffee samples C1 and C2 were 0.421 and 0.355 mmol TE/100 mL wet weight, respectively. These results align with Morresi et. al (2020), in which the authors investigated how variations in the grind size and brewing time affected antioxidant capacity of cold brew coffee. In Morresi's findings, Trolox means fell within the range of 1.52 to 2.17 mmol TE/100 mL wet weight, a magnitude consistent with the findings of this current study.

Similarly, in this study, mean values found for TEAC for cannabidiol samples CBD1 and CBD2 were 0.0834 and 0.171 mmol TE/100 mL wet weight, respectively. These findings align with a study conducted by Gruschow (2020), where the author explored the antioxidant potential, CBD content, and phenolic composition of various oils infused with cannabidiol. Gruschow reported values ranging from 0.0967 (for MCT oil infused with CBD) to 0.115 mmol TE/100 mL wet weight (for hemp oil infused with CBD). The resonance between these results and the present

study is noteworthy, as the CBD oils in our research incorporated a combination of hemp and MCT oils in their formulation.

The statistically significant higher antioxidant potential found for blends C1CBD1 and C1CBD2 shows a synergistic antioxidant effect. This mirrors previous research that found synergistic effects when using a mixture of different compounds. Çelik, and Gökmen (2018) explored the interplay of antioxidants within a mixture through two distinct relationships. The first focused on the interactions of antioxidants in coffee, specifically its insoluble fraction with catechin and epicatechin, across various preparation methods. The second relationship, similarly, investigated the interactions between antioxidants in coffee (insoluble fraction) and dark chocolate. Antioxidant capacity was evaluated by assessing the percentage inhibition of the DPPH radical, same assay used in this study. The antioxidant capacity values for the infusions ranged from 0.953 to 1.184 mmol TE/100 grams, while the values for their insoluble fractions spanned from 0.045 to 1.05. The results revealed a synergistic effect in the combination of catechin and the insoluble fraction of espresso-prepared coffee. Other combinations did not exhibit a synergistic effect.

Additionally, in another study Acosta-Otálvaro et al. (2022) explored the antioxidant potential from a mixture of cocoa and coffee extracts. The phenolic compounds in cocoa and coffee individually demonstrated high bioavailability and valuable antioxidant capacity. When combined in different proportions, a synergistic effect was found for a mixture using a proportion of 3/1 (cocoa/coffee extracts). However, synergistic effects were not found for other mixture ratios. Thus, the study suggested that the overall benefit of using these combinations is not consistent across different proportions.

Durak et al. (2015) reported an observed synergistic interaction between coffee and willow extract when assessing their ability to inhibit lipoxygenase activity. Similarly, Panya et al. (2017) found that rosmarinic acid and its esters, when combined with α-tocopherol and through the formation of additional antioxidants like caffeic acid, can work together synergistically to effectively inhibit lipid oxidation in oil and water emulsions, thus improving the oxidative stability of food products.

The antioxidant content, as exemplified in cold brew coffee, may vary based on factors like coffee bean type, roasting process, and brewing method, as demonstrated in Morresi et. al (2020) and Rao and Fuller (2018). The lack of a significantly higher antioxidant potential in samples using coffee C2 could be attributed to its commercially available and pre-prepared nature, as well as the choice of bean. Thus, the distinct production processes for samples C1 and C2 may contribute to the observed differences in antioxidant results.

Regarding synergistic effects with CBD only one study was found to support this evidence. Dawidowicz et al. (2021) in their study, explored the synergistic and antagonistic effects of six different cannabinoids, including cannabigerol, CBD, THC, cannabigerolic acid, cannabidiolic acid (CBDA), and tetrahydrocannabinolic acid, both individually and in various combinations, using ABTS scavenging radical assay. According to findings from the study, both synergistic and antagonistic effects were found when cannabinoids are combined. Specifically, CBD demonstrated synergistic antioxidant effects when paired with CBG, resulting in enhanced antioxidant properties beyond what each cannabinoid could achieve alone. The authors also highlighted that the binary antioxidant mixtures can also display antagonistic effects on their overall antioxidant activity, depending on the proportions of their components. This is important to further research to understand how different ratios of components can affect antioxidant various in mixtures.

2.7 Conclusion

In this study, an investigation was conducted into the interaction between the antioxidant potential observed from the combination of CBD and cold brew coffee compared to their individual effects. A statistically significant higher antioxidant potential was found between the blends C1CBD1 and C1CBD2 when compared with their TEAC expected values based on their individual tests. In consequence, the combination of cold brew coffee and CBD can enhance antioxidant activity and potentially be beneficial to one's health. Further investigation is needed to understand the effects of the ratio for each component in antioxidant activity (CDB and cold brew coffee) as well as the use of different types of coffee and CBD to maximize results from this study.

2.8 Study limitations

This study has limitations. The exclusive use of DPPH as the sole method for assessing antioxidant potential may restrict the comprehensive evaluation of antioxidant levels in the samples, as it does not directly quantify the antioxidants present.

Furthermore, the absence of regulatory standards governing CBD oil production introduces a potential source of inconsistency, raising concerns about the accuracy of the labeled content in CBD-infused oils. The lack of standardized regulations may result in discrepancies between stated and actual CBD concentrations as well as truly being free of THC.

Additionally, in the case of CBD-only samples, using sonication alone without employing a high-shear mixer may impact the assessment of their antioxidant potential. This omission could introduce variations in the experimental conditions, potentially influencing the outcomes related to the antioxidant properties of the samples.

Finally, it's important to note that this study was conducted *in vitro*, meaning it was performed outside of a living organism, so the findings may not necessarily reflect what happens *in vivo*, within a living organism.

Chapter 3: Manuscript II

Emulsion preparation for Antioxidant Analysis of cold brew coffee and Cannabidiol

3.1 Abstract

Emulsions are colloidal systems where droplets of one liquid, the dispersed phase, are evenly distributed within a continuous phase of another liquid, with the two liquids being immiscible. They are vital in various industries including food, pharmaceuticals, cosmetics, and petroleum. **Purpose:** Create a method for emulsion preparation for antioxidant analysis of cold brew coffee and cannabidiol without the use of an added emulsifier. **Materials and methods:** Two cold brew coffee and two CBD oil brands were used. Samples were prepared and stored accordingly. A high shear mixer was used for emulsion preparation. Six different samples were prepared in triplicate possessing different characteristics and parameters (sample 1: soy lecithin added, 2000 rpm, 5 min; sample 2: soy lecithin added, 5000 rpm, 5 min; sample 3: no soy lecithin added, 5000 rpm, 5 min; sample 4: no soy lecithin added, 5000 rpm, 10 min; sample 5: no soy lecithin added, 10000 rpm, 5 min and sample 6: no soy lecithin added, 10000 rpm, 10 min). Emulsions were prepared accordingly and underwent a 10-minute sonication process in an ultrasonic cleaner. Qualitative observations were recorded. **Results:** sample 5 (no emulsifier added, 10,000 rpm for 5 minutes) and sample 2 (soy lecithin added, 5000 rpm for 5 minutes) had positive outcomes and a visible stability of emulsion was perceived, i.e., no separation between CBD and cold brew coffee. Other samples experienced unfavorable outcomes. **Conclusion:** sample 5 parameters can be used for antioxidant analysis of cold brew coffee and cannabidiol without the use of an added emulsifier. Further research is necessary to assess the stability of sample 5's emulsion and explore varying proportions of soy lecithin as an emulsifier. Additionally, studies could compare the antioxidant potential of emulsions with and without an emulsifier to better understand its results and impacts on health.

3.2 Introduction

Emulsions are colloidal systems composed of droplets of one liquid, a dispersed phase, is uniformly spread throughout a continuous phase, where the two liquids are not immiscible (Vaclavik et al., 2021). Emulsions play an important role not only in the food industry, but are also used in pharmaceutical, cosmetic and petroleum applications. Oil-in-water – oil is the dispersed phase and water is the continuous phase - are the most used emulsion in the food industry. Among the examples are milk, salad dressings, ice cream, bakery products and others (Vaclavik et al., 2021).

The different types of emulsions are associated with their droplets diameter and are: macroemulsions, nanoemulsion and microemulsion. The macroemulsions or conventional emulsions, are associated with a diameter size of 100 nm to 100 μm and are usually opaque and thermodynamically unstable. Nanoemulsion are similar to the first but diameter size falls within 20 nm and 100 nm and colorless. Lastly, microemulsions are also colorless, but their diameter ranges between 5–50 nm and it is the only one thermodynamically stable (McClements, 2010).

Emulsions can be facilitated by the addition of an emulsifying agent. The emulsifying agent can be a surfactant or a combination of surfactants, and it lowers the interfacial tension between the immiscible phases, preventing coalescence and promoting stability. Over time, emulsions may degrade due to various factors, including separation, flocculation, coalescence, partial coalescence, and Ostwald ripening. (McClements, 2010). The stability of emulsions is influenced by several factors including the droplets size distribution, type and concentration of emulsifiers, the ratio of the dispersed phase to the continuous phase, and the presence of external factors such as temperature, pH, and ionic strength (McClements, 2010).

While emulsifiers bring stability to an emulsion, they can affect antioxidant activity of the emulsion. Emulsifiers can interact with antioxidants in several ways, which may influence their stability and efficacy. Emulsifiers are typically present in the interface between oil and water phases in an emulsion, in this way, they might facilitate the interactions between antioxidants and this interface, potentially modulating availability and mobility of antioxidants (Mosca et al., 2013). In a study, Mosca et al. (2013) investigated the impact of emulsifier layer structure, presence of hydrophilic and lipophilic antioxidants, and radical initiators on lipid oxidation in emulsion systems using olive oil dispersed in a continuous phase of water with different emulsifiers. According to the authors, the combination of Tween 80 and Span 80 emulsifiers resulted in an interfacial layer more resistant to radical attack. Additionally, the study demonstrated a polar paradox concerning radical initiators and suggested that the most effective approach to protect emulsions involved is by using a combination of antioxidants in both phases to promote synergy and antioxidant regeneration mediated by the interfacial layer.

Although emulsifiers can cause a synergistic effect on antioxidant activity, research is still not clear on how they can affect gut microbiome. For instance, in a review, De Siena et al. (2022) investigated different clinical trials, in vitro, and available research into the connection between emulsifiers and gut microbiota. Some studies have shown that certain emulsifiers can lead to inflammation and metabolic syndrome by altering the composition of the gut microbiome. On the other hand, some studies in the review have shown improved synergy between emulsifiers and the gut microbiome. While there are over 100 different types of emulsifiers used in the industry, food labels disclose the presence, but not the amount of emulsifiers. The authors emphasized the need for additional research for a clearer consensus.

As consumers increasingly seek out natural products, the food industry is experiencing a growing demand for natural food ingredients. This trend is evident in the domain of food additives, including emulsifiers and stabilizers, as highlighted in research by Feng et al. (2023). Previous studies have researched the use of emulsions in different beverages (Piorkowski and McClements, 2014). However, to date, no study has explored emulsions involving both CBD and cold brew coffee. Thus, the purpose of this study was to create a method for emulsion preparation for antioxidant analysis of cold brew coffee and cannabidiol without the use of an added emulsifier.

3.3 Materials and Methods

3.3.1 Study design

All the experimental procedures for this project took place in the food laboratory of the Department of Nutrition and Food Studies at Montclair State University.

Coffee brands are represented as C1 and C2, and the different brands of CBD are represented as CBD1 and CBD2. Samples consisting of CBD only did not go through homogenization due to limitation of sample size.

3.3.2 Cold brew coffee infused with CBD samples preparation

Two different coffee brands were used in this study. One was donated by Law Coffee, located in Newark, New Jersey (C1). A second was acquired from a grocery store chain located in Holmdel, New Jersey (C2. Both brands use only ground coffee beans and water in the ingredients. Once received, the coffee samples were stored in the refrigerator at 4 ^{o}C. Prior to starting experiments, the cold brew coffee bottles were mixed into two single containers according to each brand and kept again in the refrigerator at 4 ^{o}C. This was done to avoid any possible difference between batches.

Similarly, two different CBD brands were used in this study. Both brands used were THC free and had MCT oil and CBD extracted from hemp oil as ingredients. One was acquired from Louisville, CO (CBD1), and the other one was purchased from another company from Lafayette, CO (CBD2). CBD samples were also mixed into two single containers according to brand and stored at room temperature as manufacture instructions.

Cold brew coffee mixed with CBD was created by preparing an emulsion that combined each type of cold brew coffee (C1 and C2) with their respective CBD components (CBD1 and CBD2).

For the preparation of the blend C1CBD1, 162.5 mL of cold brew coffee (C1) stored at 4°C was used. This volume was then combined with 250 μL of CBD1 in a beaker.

The procedures for samples C1CBD2 and C2CBD2 were identical, with the only variation being the volume of CBD used. In the case of C1CBD2, 190 μL of CBD2 was mixed with 162.5 mL of cold brew coffee (C1). Similarly, for sample C2CBD2, 190 μL of CBD2 was used and mixed with 162.5 mL of cold brew coffee (C2).

3.3.5 Emulsion Preparation

Samples were prepared using a high shear homogenizer (Silverson, Model L5M-A, East Longmeadow, MA, USA) and an ultrasonic cleaner (Branson, Model 5800, Danbury, CT, USA). All samples were prepared in triplicate.

Samples comprising solely cold brew coffee and CBD were prepared using the same method employed for samples combining cold brew coffee with CBD.

A series of samples were prepared according to the different parameters such as rotation, time, and the use of an emulsifier (Table 4). Soy lecithin was only used as a comparative to the other parameters in the study. To comprehensively explore the antioxidant properties of cold brew

coffee and CBD independently, emulsifiers were intentionally omitted from this study to avoid potential interactions with antioxidants.

Table 4 - Parameters for emulsion preparation

Sample	Description	Rotation (rpm)	Time (minutes)
1	Sample with soy lecithin added*	2000	5
2	Sample with soy lecithin added*	5000	5
3	No emulsifier	5000	5
4	No emulsifier	5000	10
5	No emulsifier	10000	5
6	No emulsifier	10000	10

*Soy lecithin is one of the most used emulsifiers in the food industry and was chosen for this methodology; 0.5% of soy lecithin by weight of total liquid volume was used.

After homogenization, each sample underwent a 10-minute sonication process in an ultrasonic cleaner. Subsequently, samples were refrigerated for one hour to allow any existing foam to dissipate, if necessary.

3.4 Results and Discussion

The qualitative analysis of the samples prior to freezing and lyophilization processes revealed findings regarding the influence of emulsifier presence, rotation speed and time over the preparation of samples containing cold brew coffee and CBD. Sample 5, which lacked an emulsifier and went through rotation of 10,000 rpm for 5 minutes, exhibited superior outcomes compared to sample 1, where soy lecithin was added as an emulsifier to a lower rotation (2000 rpm) for the same duration. This observation suggests that the absence of an emulsifier in conjunction with higher agitation intensity might lead to favorable emulsion properties. This finding aligns with previous studies (McClements, 2010), indicating that increased shear forces

can enhance emulsion stability by promoting finer droplet size distribution and more efficient phase dispersion. Although this process generates an emulsion, the resulting emulsion may lack complete stability unless an emulsifier is added.

Furthermore, a similar trend was observed when comparing Sample 5 to Sample 2, where soy lecithin was utilized but at a higher rotation speed of 5000 rpm. While the results were similar – visible stability of emulsion was perceived, i.e., no separation between CBD and cold brew coffee – the goal of the study was to avoid the use of emulsifiers to not affect antioxidant potential, thus, the parameters found in sample 5 were the best for this study. In a previous research, Feng et al. (2023) investigated the use of natural emulsifiers, specifically melanoidins present in coffee as an emulsion stabilizer. In their methodology, they looked at properties and stability of oil-in-water emulsions containing 10% oil added with coffee melanoidins ranging from 0.25% to 4% and followed it for 28 days at room temperature. According to the results, melanoidins can create emulsions with a uniform droplet size distribution. Emulsions using 0.25% to 1% of melanoidins had phase separation. However, starting at 2% no separation was found. Thus, these components can serve as natural emulsifiers for foods. These results could explain why sample 5 with no emulsifier addition could have led to a visible stability for the mixture of cold brew coffee and CBD.

Contrarily, Sample 6, subjected to the same high rotation speed of 10,000 rpm as sample 5 but for an extended duration of 10 minutes, did not exhibit desirable outcomes. The prolonged mechanical agitation may have led to excessive heat generation due to friction, resulting in undesirable effects on the sample. Additionally, samples 3 (no emulsifier, 5000 rpm for 5 minutes) and 4 (no emulsifier, 5000 rpm for 10 minutes) did not show positive outcomes and in each qualitative analysis, it was possible to see the CBD and cold brew coffee phases separation.

3.5 Conclusion

In this study, an investigation was conducted to create a method for emulsion preparation for antioxidant analysis of cold brew coffee and cannabidiol without the use of an added emulsifier.

A qualitative analysis has shown that sample 5 (no emulsifier added, 10,000 rpm for 5 minutes) and sample 2 (soy lecithin added, 5000 rpm for 5 minutes) had positive outcomes and a visible stability of emulsion was perceived, i.e., no separation between CBD and cold brew coffee. In contrast, sample 6, under prolonged agitation at 10,000 rpm for 10 minutes, experienced unfavorable outcomes due to excessive heat generation from friction. Similarly, samples 3 and 4, subjected to lower rotation speeds and longer durations without an emulsifier, exhibited phase separation between CBD and cold brew coffee components.

Further investigation is needed to understand the stability of the emulsion for sample 5. Additional studies could compare antioxidant potential found in emulsion without emulsifier versus emulsion with addition of an emulsifier, with different proportions of soy lecithin, to understand its effects in antioxidant potential and health.

3.6 Study Limitations

An investigation of the diameter size of droplets of emulsion could have helped to understand the stability of emulsion.

Chapter 4: Conclusion

In conclusion, the development of cold brew coffee infused with CBD in a beverage presents an opportunity to increase its antioxidant activity, potentially promoting individuals' well-being. A statistically significant higher antioxidant potential was found between the blends C1CBD1 and C1CBD2 when compared with their TEAC expected values based on their individual tests. Similarly, a higher antioxidant potential was found for blends C2CBD2 and C2CBD1 compared to their expected values. However, for the latter, neither were statistically significant.

A qualitative analysis when creating a method for emulsion preparation for antioxidant analysis of cold brew coffee and cannabidiol without the use of an added emulsifier has shown that sample 5 (no emulsifier added, 10,000 rpm for 5 minutes) and sample 2 (no emulsifier added, 10,000 rpm for 5 minutes) had positive outcomes and a visible stability of emulsion was perceived, i.e., no separation between CBD and cold brew coffee. Other samples experienced unfavorable outcomes.

Further research is crucial to comprehensively understand the impact of varying ratios of CBD and cold brew coffee on antioxidant potential, as well as to explore various combinations of coffee types and CBD formulations to validate synergistic benefits. Additionally, investigation into the stability of the emulsion for sample 5 and the use of different proportions of soy lecithin as an emulsifier is needed. Comparative studies could also assess the antioxidant potential of emulsions with and without an emulsifier to discern its effects on both antioxidant potential and health outcomes.

Chapter 5: References

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