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A STUDY OF THE ANALYSIS OF HEMP-DERIVED OIL PRODUCTS: DEVELOPMENT OF A METHOD FOR THE ANALYSIS OF PHYTOCANNABINOIDS IN MEDICINAL PRODUCTS

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A STUDY OF THE ANALYSIS OF HEMP-DERIVED OIL PRODUCTS: DEVELOPMENT OF A METHOD FOR THE ANALYSIS OF PHYTOCANNABINOIDS IN MEDICINAL PRODUCTS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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2022

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ABSTRACT OF DISSERTATION

A STUDY OF THE ANALYSIS OF HEMP-DERIVED OIL PRODUCTS: DEVELOPMENT OF A METHOD FOR THE ANALYSIS OF PHYTOCANNABINOIDS IN MEDICINAL PRODUCTS

Human interactions with cannabis have a history spanning millennia as a source of fiber, food, and medicine. The plant came across Asia, Europe, and Africa with the movements of people and travelers eventually coming to the Americas with European explorers. In colonizing North America, cannabis fiber was key to the production of canvas and ropes for ships. Although fiber was the driver for cannabis production in the United States, medicinal uses were prevalent and propelled the eventual regulations. The Pure Food and Drug Act of 1906 mandated accurate labeling on drugs such as cannabis followed by the Marihuana Tax Act of 1937 that essentially made cannabis illegal. The legal status of cannabis was not changed on the federal level until the Agricultural Act of 2014 which opened the door to varieties of cannabis, labeled as hemp, with Δ^9-tetrahydrocannabinol (Δ^9-THC) content less than 0.3 % per dry weight of plant material. The Agricultural Improvement Act of 2018 removed hemp from scheduling originally implemented in the Controlled Substance Act of 1970. As the doors opened to hemp, the market was flooded with hemp-derived products minimally regulated as the language of the Agricultural Improvement Act of 2018 was primarily directed at the agricultural commodity leaving individual states to develop and manage the rules for the agricultural commodity and finished products. As such, the industry has outpaced the regulations leaving consumers to rely on the product manufactures and anecdotal stories from family, friends, and the internet to guide their usage. Today, hemp-derived products can be found on seemingly every corner from the gas stations to grocery stores to pharmacies to boutique CBD stores. Consumers are turning to these products in addition to, and as an alternative to, standard medications. With little to no regulations regarding the finished products, and the quality concerns shown by other studies demonstrate the need for methodology to better understand what these hemp-derived products contain.

For these studies, 80 unregulated hemp-derived oil products representing 51 brands were purchased from 9 local (brick and mortar) and 21 online retailers between April 2 to May 9, 2021. The samples were selected to represent a cross section of local and national brands produced inside and outside of Kentucky. Additionally, Epidiolex® (the FDA-approved CBD product) was obtained (UK Investigational Drug Service Pharmacy) to

serve as regulated control. These samples were extracted and quantified by liquid-chromatography tandem mass-spectrometry (LC-MS/MS).

In these studies, the hemp-derived oil products were evaluated quantitatively for 17 cannabinoids comprising a range of metabolic products in the plant as well as potential degradants. The cannabidiol (CBD) content detected was compared to the label claim amount. Of the products tested, 31% contained more than 110% of the label claimed content and 15% contained less than 90% of the label claimed content. With regards to Δ^9-THC, 52 products contained Δ^9-THC ranging from 0.008 mg/mL to 2.071 mg/mL including 5 of 21 products labeled as "THC Free". Δ^8-THC was not detected in any products tested. Excluding CBD and Δ^9-THC, the minor cannabinoids most frequently detected in the samples were cannabidivarin (100% of samples tested), cannabigerol (77%), cannabichromene (72%), cannabinol (67%), cannabicyclol (67%), and cannabidiolic acid (51%). The concentrations of these minor cannabinoids varied widely from trace levels to several milligrams per milliliter (e.g., CBDA: 0.006 – 12.258 mg/mL).

Consumers are taking these products, often to treat a medical condition, without knowing the true contents. The inaccuracy of the label claim content with regards to CBD, the marketed cannabinoid in these products, could lead to unknown dosing. Additionally, consumers are taking these products without understanding the risks of unintentional consumption of Δ^9-THC. This accidental use of Δ^9-THC could have adverse effects on health and safety as well as potentially legal consequences in child custody and impaired driving or could impact employment, military, and sport eligibility status. Furthermore, many of the other 15 phytocannabinoids quantitated in this study have been shown to have pharmacological activity in pre-clinical studies. Overall, these studies clearly demonstrate that consumers are not being provided with an accurate and comprehensive unawareness of the contents of the products they are consuming.

KEYWORDS: Cannabidiol (CBD), Hemp-Derived Products, 2018 Farm Bill, Δ^9-Tetrahydrocannabinol (Δ^9-THC), Liquid-Chromatography Tandem Mass Spectrometry (LC-MS/MS), Phytocannabinoids

Erin Dager Johnson

04/06/2022

Date

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04/06/2022
Date

DEDICATION

To Brandon, you are the most patient and supportive partner that I could have asked for. This would not have been possible without you

Caitlin and Logan, thank you for your love, support, and encouragement. Just know that you decide your own future and the path you start on can change if you want it to.

To Dr. Clive Pearce, thank you for encouraging me to take a leap and supporting me throughout this journey. I am forever grateful for you believing in me.

.

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I would not be where I am today without Dr. Clive Pearce. Thank you for asking me to consider going to graduate school. Your faith in me and constant encouragement have helped me to complete this journey, and I am better for it. I would like to thank my friends and colleagues, Dr. Deborah Mawson, Dr. Mallory Morris, Ms. Traci Rust, and Ms. Holly Carter for encouraging me and cheering me on throughout this journey. I want to thank Mr. Jeff Worley for his editorial help.

Finally, I would like to acknowledge the support of my family. I was committed to this path before you joined our family, but Brandon, I could not have made it through graduate school without your love and support. You jumped into parenting two teenagers without hesitation picking up numerous responsibilities to give me the time and space to accomplish my goals. Caitlin and Logan, we made the decision as a family for me to go

to graduate school even though you didn't fully understand what the commitment meant. Your love and encouragement have helped carry me through the challenges. I can only hope that you see and understand that you are the architect of your own path, and that growth and learning are lifetime pursuits. I appreciate the encouragement and support of my parents, Chrisman and Kay Dager, and bonus parents, Kenneth and Ruth Ann Johnson.

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CHAPTER 1. INTRODUCTION

1.1 History

The history of Cannabis spans millennia with a speculated origin in Central Asia. [1] The breadth of Cannabis history may be attributed to its triple-purpose nature as a source of fiber, food, and medicine. [2] The use of cannabis for medicinal purposes can be traced back to Chinese oral traditions dating 2600 – 2700 BCE. [1-3] The cannabis plant spread throughout Europe, Arab countries, and Africa with the movements of people and travelers from Central Asia. [1] The documented medicinal uses of cannabis are numerous, but some are consistent with current clinically demonstrated uses such as emesis and convulsions. In addition to the medicinal uses of cannabis, the plant was important for use of the fiber. The use of cannabis fiber, commonly called hemp when fiber was the primary purpose for cultivation, became prominent as exploration expanded overseas since the fiber was key in the manufacture of ropes and sails. For this purpose, cannabis was brought to the Americas with European explorers. [4, 5] In the colonization of North America, cannabis (hemp) cultivation became widespread and a key agricultural commodity along with tobacco for export to England. [5] The use of cannabis to treat a variety of illnesses continued for millennia throughout many cultures, but in the 19th century, the modern history of medical cannabis began with the physician William O'Shaughnessy in India [1]. He applied the scientific method to the study of the pharmacological and toxicological properties of cannabis. [1, 3] It wasn't until 1964 , however, that active compounds in the cannabis plant were isolated and structure elucidated, including Δ^9-tetrahydrocannabinol (Δ^9-THC). [6]. Despite the long history indicating the therapeutic potential for *Cannabis*

sativa, the research has been slow in coming due to a complex legal history and societal obstacles.

1.2 Legal and Societal Hurdles

In the United States, the use of cannabis for medicinal purposes led to the rocky road in the plant's legal status journey. Although the primary purpose for cannabis cultivation in the United States from colonization was for the fiber, the history of medicinal uses of cannabis led to treatment of various conditions. In the latter part of the 19th century, individual states started passing legislation to control the adulteration of food and drugs, [7] and the Pure Food and Drug Act was passed by Congress for consumer protection in 1906. [8] A key point of this legislation was accuracy of labeling for drugs such as cannabis. [8] With the creation of the Federal Bureau of Narcotics in 1930 and appointment of Harry Anslinger as commissioner, the efforts to make cannabis illegal began. [9] As part of the fight to get rid of cannabis, a media campaign including print and film, such as *Reefer Madness*, sought to demonize cannabis. [9, 10] This work culminated with the passage of the Marihuana Tax Act in 1937, which only allowed the sale of cannabis products that were labeled with a tax stamp, which was substantial in cost and difficult to obtain, from the United States government. [11] Through the Marihuana Tax Act, legal cannabis was essentially eliminated from the United States with only a brief reprieve during World War II when cannabis (hemp) fiber was needed for the war effort. [12] A consequence of this legislation that has continued to hinder the scientific research was the lack of availability of cannabis to conduct research studies since only a single plot of cannabis started by the University of Mississippi in 1968 was considered legal. [13] The Marihuana Tax Act was deemed to be unconstitutional in 1969 and thus repealed. [14] The

following year, 1970, the Controlled Substance Act was passed making cannabis, specifically *Cannabis sativa*, and any parts thereof a Schedule 1 controlled substance. [15] By definition, a Schedule 1 controlled substance has no accepted medical use which significantly hindered access to *Cannabis sativa* and subsequently the cannabinoid constituents for the purposes of research into the therapeutic potentials. [15] Despite the legal hurdles, the discoveries of the components of the endocannabinoid system spurred growing interest in the potential medicinal benefits of the cannabis plant components.

The primary psychoactive component of the cannabis plant is Δ^9-THC and is the main contributor to the legal prohibition of cannabis, but interest in the non-psychoactive component cannabidiol (CBD) and the alternate uses of the plant for fiber and food resulted in rethinking of the restrictions. The Agricultural Act of 2014 (2014 Farm Bill) provided a legal definition to differentiate between marijuana and hemp, both of which are *Cannabis sativa*, and created an opening for the cultivation and research of hemp in a pilot program. Hemp was defined as *Cannabis sativa* containing not more than 0.3 % Δ^9-THC per dry weight of plant material. [16] This legislation came with the caveat that regulation of hemp cultivation was delegated to the individual states which has led to a patchwork of rules. [16] Since the passage of the 2014 Farm Bill, the cultivation of low Δ^9-THC *Cannabis sativa*, hemp, and market for subsequent products, has grown substantially. [17] With the interest and growth in industrial hemp, language was included in the Agricultural Improvement Act of 2018 (2018 Farm Bill) which removed hemp from the controlled substance list. [18]

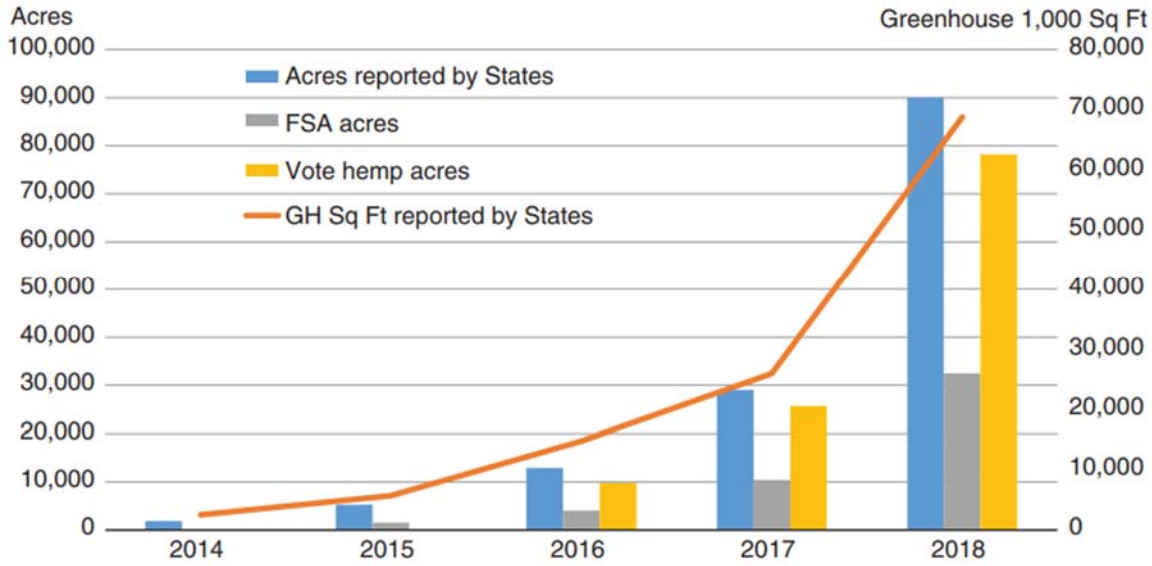


Figure 1-1 Hemp acreage from 2014 to 2018 in each year by data source [17]
Note: FSA = Farm Service Agency. GH Sq Ft = Greenhouse square feet. Not all States reported data on the same basis. Reported acreage may include planted, harvested, and/or licensed or approved acreage. Not all States reported greenhouse data. Farm Service Agency data include only data reported by Agency customers and are a simple total of reported acres. Source: USDA, Economic Research Service calculations based on data reported by State pilot program, USDA, Farm Service Agency, and Vote Hemp.

1.3 Endocannabinoid system

It would take more than 20 years after the discovery of Δ^9-THC to begin understanding the system to which cannabis constituents were thought to bind. In the search for the biological system that interacts with Δ^9-THC, researchers discovered the endocannabinoid system. Scientists have isolated and characterized two cannabinoid receptors, cannabinoid receptor type-1 (CB1) and cannabinoid receptor type-2 (CB2). [19, 20] CB1 and CB2 are G protein-coupled receptor with Δ^9-THC binding activity. [21] CB1 is primarily expressed in the brain while CB2 is more widely expressed peripherally. [19, 20] Once the receptors were identified, researchers sought to find the endogenous ligands for these receptors. *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) were discovered in 1992 and 1995, respectively. [22-24] Anandamide and 2-AG are synthesized from plasma membrane and hydrolyzed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. [25] As shown in Figure 1-2, the downstream signaling of the cannabinoid receptors has been demonstrated to affect multiple pathways.

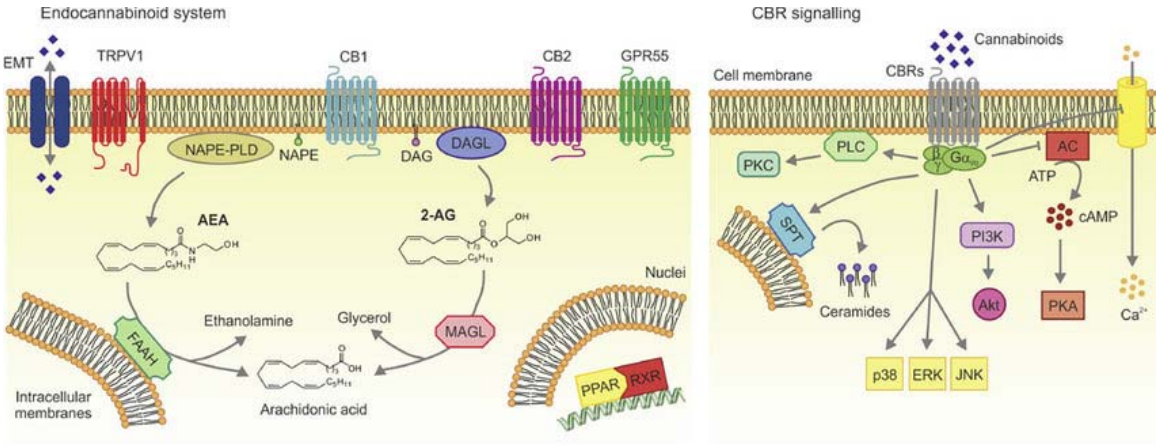


Figure 1-2 Endocannabinoid system and cannabinoid receptor signaling [25]
Left panel: The endogenous ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), of the endocannabinoid system (ECS) are synthesized on demand from membrane lipids by N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) for AEA and diacylglycerol lipase (DAGL) for 2-AG. AEA and 2-AG cross the cell membrane through a purported endocannabinoid membrane transporter (EMT). The main receptor targets of AEA and 2-AG are cannabinoid receptor 1 (CB1), cannabinoid receptor 2 (CB2), transient receptor potential vanilloid 1 (TRPV1), G-protein-coupled receptor 55 (GPR55), and peroxisome proliferator-activated receptors (PPARs). Arachidonic acid is released by the hydrolysis of AEA and 2-AG by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively.
Right panel: Cannabinoid binding to the cannabinoid receptor induces signaling of several pathways including inhibition of protein kinase A (PKA) pathway, activation of mitogen-activated protein kinase cascades (p38, JNK, and ERK), activation of protein kinase B (Akt) pathway, inhibition of calcium channels, activation of protein kinase C (PKC), and generation of ceramides.

1.4 Phytocannabinoids

Phytocannabinoids are a family of metabolites and breakdown products formed in various concentrations in the *Cannabis sativa* plant. Cannabinoid biosynthesis primarily takes place in glandular trichomes that develop on female flowers and, to a lesser extent, on the leaves. [26] The biosynthetic pathway for the major cannabinoids starts with hexanoyl-CoA reacting through enzymatic reactions with malonyl-CoA to form olivetolic acid, as shown in Figure 1-3. [26] The caromatic prenyltransferase, geranyltransferase, enzyme catalyzes the reaction of olivetolic acid and geranyldiphosphate to form cannabigerolic acid (CBGA). [26, 27] As shown in Figure 1-4, CBGA reacts with the respective oxidocyclase enzymes to form Δ^9-tetrahydrocannabinolic acid-A (THCA-A), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA). [27-31] The carboxylic acid moiety is labile, readily lost, resulting in the corresponding neutral forms cannabigerol (CBG), Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), and cannabichromene (CBC). [28] Cannabinolic acid (CBNA) and cannabinol (CBN) are a degradation product of the THCA-A pathway. [28, 30-32] Δ^8-tetrahydrocannabinol (Δ^8-THC) is a transformation artifact from Δ^9-THC. [28] Cannabicyclol acid (CBLA) and cannabicyclol (CBL) have a similar core skeleton to CBCA and CBC, respectively, and are considered artifacts resulting from 2+2 cyclization of the double bonds when exposed to light. [28] This group of phytocannabinoids is described as the olivetol series of phytocannabinoids because the biological precursor in the plant is olivetolic acid. [27, 32] A second series of phytocannabinoids is the varinol series derived from the enzymatic reactions with butanoyl-Co-A to form varinolic acid. Varinolic acid reacts with geranyldiphosphate, catalyzed by geranyltransferase, to form cannabigerovarinic acid (CBGVA). CBGVA

undergoes enzymatic modification to form Δ^9-tetrahydrocannabivarinic acid (THCVA) and cannabidivarinic acid (CBDVA), similar to the olivetol series. The carboxylic acid moiety is labile, and thus readily lost, resulting in the corresponding neutral forms, Δ^9-tetrahydrocannabivarin (THCV) and cannabidivarin (CBDV). [27, 32] A hemp-derived product could potentially contain any or all of these compounds.

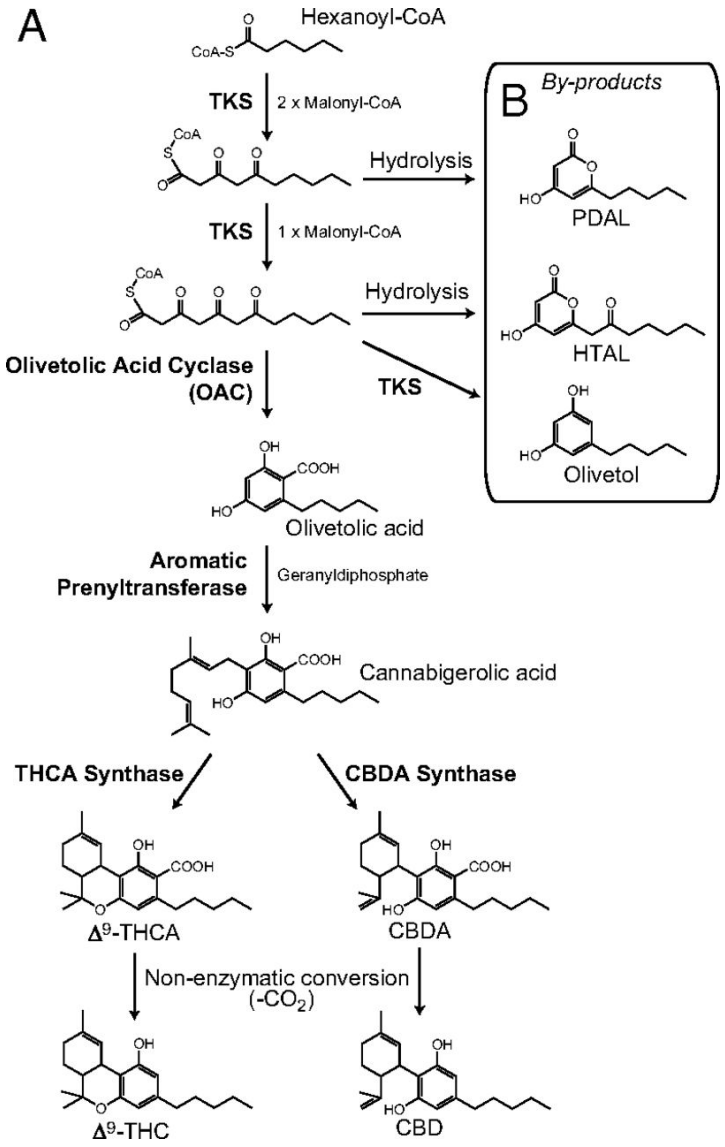


Figure 1-3. Biosynthetic pathway of CBGA [26]

The proposed cannabinoid biosynthetic pathway for cannabigerolic acid (CBGA). (A) The pathway leading to the major cannabinoids Δ^9-tetrahydrocannabinolic acid (THCA-A) and cannabidiolic acid (CBDA), which decarboxylate to yield Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), respectively. (B) Recombinant TKS enzyme produces triketide (PDAL) and tetraketide (HTAL and olivetol) by-products in vitro.

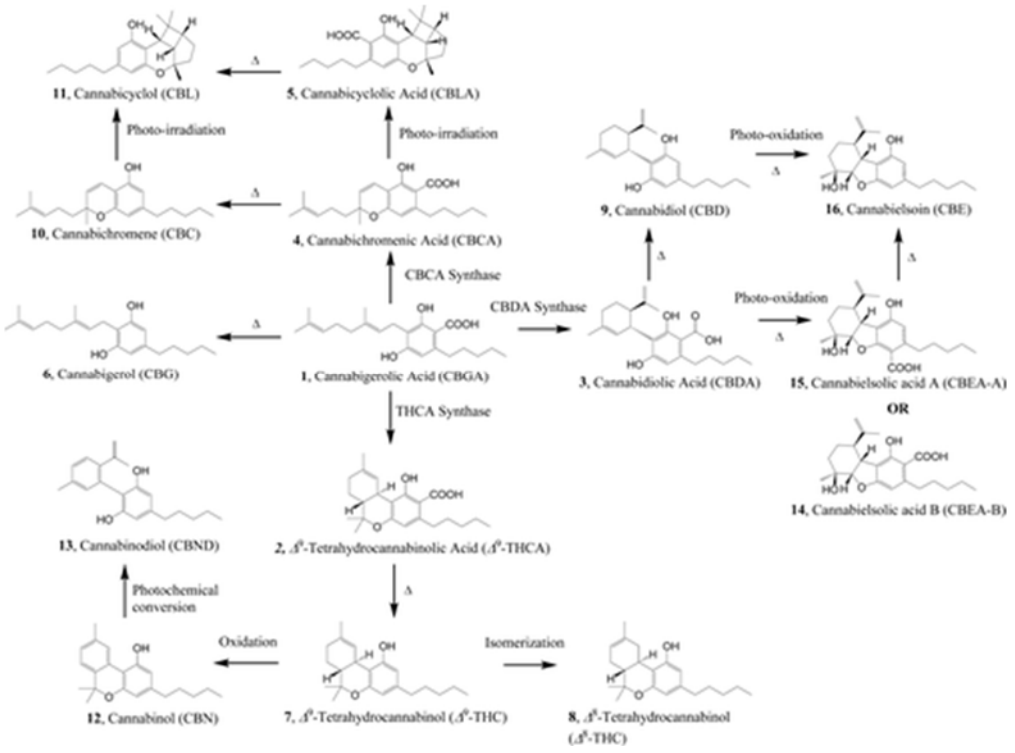


Figure 1-4 Biosynthesis of major cannabinoids [31]

Cannabinoids are formed through a combination of enzymatic and non-enzymatic processes. From CBGA (1), a series of enzymatic reactions produce THCA-A (2), CBDA (3), and CBCA (4). Non-enzymatic decarboxylation of CGBA (1) from heating (e.g., direct sunlight, smoking, or hot oven) produces CBG (6). In the THCA synthase arm, THCA-A (2) can convert to Δ9-THC (7) through heat induced decarboxylation followed by isomerization to Δ8-THC (8) or oxidation to form CBN (12). CBN (12) may convert to CBND (13) through a photochemical conversion. In the CBDA synthase arm, CBD (9) is formed by decarboxylation of CBDA (3) by heating while CBEA-A (15) and CBEA-B (14) are formed by heat and photo-oxidation. CBD (9) forms CBE (16) in a reaction to heat and photo-oxidation. In the CBCA synthase arm, CBCA (4) converts to CBC (10) by decarboxylation because of heating which can then convert to CBL (11) in response to photo-irradiation. Photo-irradiation causes CBCA (4) to convert to CBLA (5) which can undergo decarboxylation to form CBL (11).

1.5 Rationale

Cannabis sativa produces numerous chemicals such as phytocannabinoids through secondary metabolism. [27] More than 90 phytocannabinoids have been reported in the literature, but cannabidiol (CBD) and Δ^9-THC have been the main subjects of research into the potential therapeutic benefits. [27] CBD is a non-psychoactive component of *Cannabis sativa* while Δ^9-THC is the primary psychoactive component. In recent years, researchers have explored the therapeutic potential of other phytocannabinoids such as cannabidivarin (CBDV), Δ^9-tetrahydrocannabinolic acid (THCA-A), Δ^9-tetrahydrocannabivarin (THCV), cannabigerol (CBG), cannabichromene (CBC), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). [33, 34] In 1985, the Food and Drug Administration (FDA) approved a synthetic form of Δ^9-THC, Marinol®, for the treatment of anorexia associated with weight loss in patients with AIDS and as an antiemetic associated with cancer chemotherapy. [35] In the same year, the synthetic analog Nabilone, Cesamet®, received approval as an antiemetic. [36] In June 2018, the FDA approved Epidiolex®, cannabidiol derived from *Cannabis sativa*, for the treatment of two forms of epilepsy, Dravey syndrome and Lennox-Gastaut syndrome, expanding to include seizures associated with tuberous sclerosis complex in 2020. [37-39]

With the legalization of hemp, *Cannabis sativa* containing not more than 0.3% Δ^9-THC per dry weight of the harvested plant, hemp-derived products have become readily available to consumers. The purported therapeutic benefits of CBD have become the driver in the growth of the hemp industry. As these products are not FDA-approved drugs nor are they considered dietary supplements nor can they be added to human or animal food, the policies to ensure consumer safety are not clear or consistent. [40] Similar to the era that

led to the passage of the Pure Food and Drug Act of 1906, the lack of regulations in the manufacture of hemp-derived products leaves the consumer to rely on the claims of the manufacturer, which may be inaccurate and often incomplete. [41-43], In one study of 84 CBD products, only 31% of the products tested were accurately labeled (i.e., within 10% of the advertised amount) as to the CBD content, and 21% of the products tested contained Δ^9-THC, which was not declared on the label. [41] Given that many consumers are taking CBD products to treat multiple medical conditions without consulting a medical provider, the consumer may be unable to determine an appropriate dose of CBD as well as the risk for unintended exposure to a contaminant. [44] All indicators point to the continued growth of the hemp-derived product industry, commonly called the CBD industry, underscoring the importance of understanding these quality issues.

A first step in addressing the issues surrounding CBD industry requires a better understanding of what is in these products. Because the phytocannabinoids have been shown to have potential biological activity, there is a need to isolate and identify which cannabinoids are present and at what absolute and relative concentrations. As with any biological material, botanical products such as those derived from *Cannabis sativa* are a complex mixture of the botanical components and the additives to make it a consumer product. Examples are carrier oil, flavorings, sweeteners, and preservatives in this case. To isolate the phytocannabinoids of interest, a selective extraction procedure is necessary to separate the compounds of interest from the rest of the product components. Once the phytocannabinoids are isolated from the product mixture, a selective and quantitative analytical technique is required to identify the phytocannabinoids present and determine the concentrations in the products. Since matrix and matrix components can impact

extraction efficiency and quantitative accuracy, the studies presented here focus on oil products as the class of matrix. Development of methodology for the isolation and analysis of phytocannabinoids is a first step in understanding what consumers are taking and in better understanding the risks of unintentional consumption of contaminates.

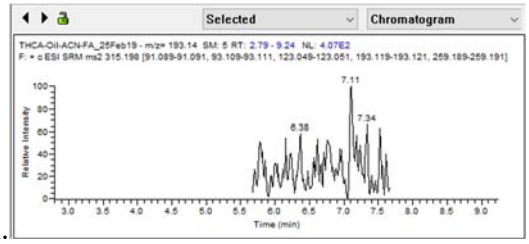
CHAPTER 2. METHODOLOGY

2.1 Development of the Extraction Procedure

To determine the phytocannabinoid content of a hemp-derived product, the phytocannabinoids needed to be extracted from the product, carrier oil for oil products. Liquid extraction techniques are the most commonly reported method for the isolation of phytocannabinoids from *Cannabis sativa* plant material. [28-30, 45-47] A thorough review of the literature examining the methods of hemp processors revealed three approaches to extraction of the desired materials from the hemp plant. Solvent extraction with organic solvent such as hexane was common in the past but less used currently. Now, most hemp processing is done with cold ethanol or supercritical CO_2 extraction. Supercritical CO_2 extraction results in a greater volume of oil extract recovery than cold ethanol, but the product contains more lipophilic substances from the plant biomass, requiring more processing to obtain purer cannabinoid products.

Liquid, or solvent, extraction has also been widely applied to isolate phytocannabinoids from hemp-derived products. [48] The analytical analysis tool can influence the choice of solvent as well as whether the scope of the analysis includes the carboxylic acid forms or only the neutral phytocannabinoids. Based on the literature, solvent extraction with methanol and acetonitrile were the most commonly reported techniques. The primary difference between these two solvents is that methanol is protic, and acetonitrile is aprotic. Both solvents were tested and compared based on extraction efficiency and risk of decarboxylation. To assess the extraction efficiency, samples of MCT (medium chain triglyceride) oil were spiked with CBD and Δ^9-THC, then extracted with each solvent system. The extraction efficiency was similar using either methanol or

acetonitrile. To assess the risk of decarboxylation from the extraction, a sample of MCT oil was supplemented with THCA-A and extracted with each solvent system. The samples were analyzed for Δ^9-THC, the decarboxylated form of THCA-A. As shown in Figure 2-1, Δ^9-THC was not detected in the acetonitrile extraction (A) sample but was detected in the methanol extraction (B) sample. It is important to note that reference material manufacturers prepare and ship certified reference standards of the carboxylic acid forms of phytocannabinoids in acetonitrile.

A. 

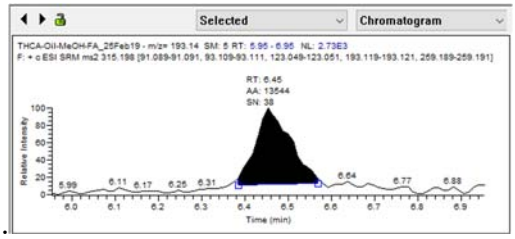
B. 

Figure 2-1 Δ^9-THC analysis in THCA-A samples a) acetonitrile extraction and b) methanol extraction

Negative matrix samples were spiked with THCA-A and extracted with acetonitrile (a) and methanol (b). The samples were analyzed for Δ^9-THC, the decarboxylated byproduct of THCA-A to determine the risk of conversion during the extraction procedure. Δ^9-THC was not detected in the sample extracted with acetonitrile (a) while Δ^9-THC was detected in the sample extracted with methanol (b).

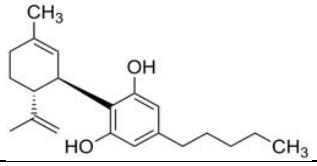
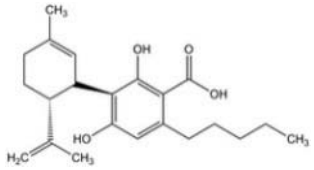
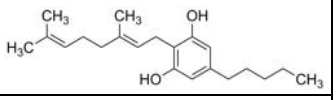
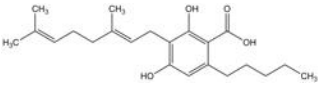
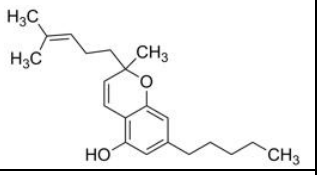
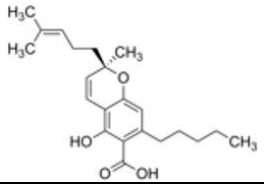
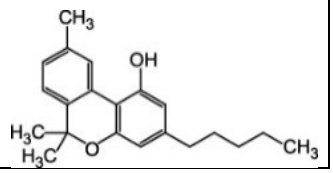
2.1.1 Surrogate matrix

For quantitative analysis, calibrator and quality control samples are prepared in matrix, and the test samples are compared to these samples of known quantities. The most logical matrix choice for the preparation of calibration and quality control samples would be hemp seed oil, but hemp seed oil contains trace amounts of Δ^9-THC and CBD. [49] The presence of trace levels of phytocannabinoids in the matrix has the potential to impact the accuracy of the analysis. As such, it was necessary to identify a surrogate negative matrix for the preparation of calibrator and quality control samples. After a thorough review of product labels, several oils used as carrier oils and additives in the manufacture of hemp-derived oil products as well as oils readily available at the local market were evaluated. Extra virgin olive oil, MCT oil, sesame oil, grapeseed oil, safflower oil, sunflower oil, liquid coconut oil, canola oil and vegetable oil were tested in addition to hemp seed oil. The primary criteria for comparison were recovery of analytes and behavior in assay compared to hemp seed oil. The results were similar across the oils tested. Extra virgin olive oil (EVOO) was selected as the surrogate negative matrix for ease of availability and matrix match to a commercially available proficiency test. [50]

2.2 Development of Instrumental Method

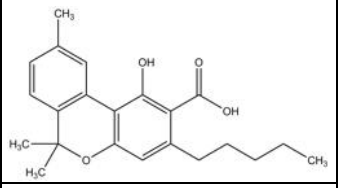
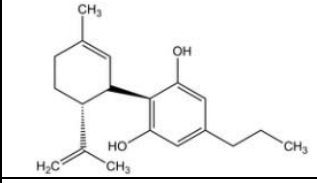
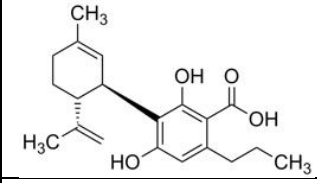
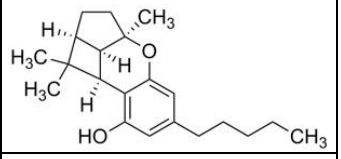
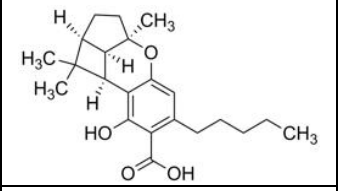
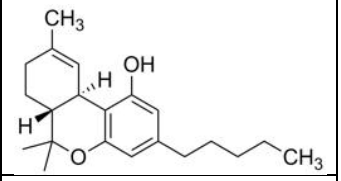
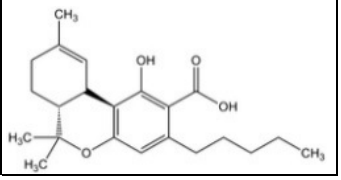
A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the analysis of hemp-derived oil products. The method was developed to separate the phytocannabinoid compounds in Table 2-1 through a combination of chromatography and mass to charge ratio.

Table 2-1 Phytocannabinoids included as target analytes in the analysis of hemp-derived oil products

CAS#	Phytocannabinoid Compounds	Abbrev.	Structure
13956-29-1	cannabidiol	CBD	
1244-58-2	cannabidiolic acid	CBDA	
25654-31-3	cannabigerol	CBG	
25555-57-1	cannabigerol acid	CBGA	
20675-51-8	cannabichromene	CBC	
185505-15-1	cannabichromenic acid	CBCA	
521-35-7	cannabinol	CBN	

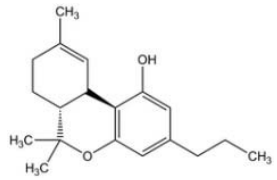
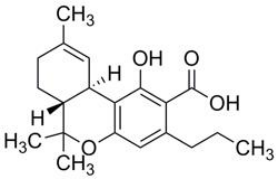
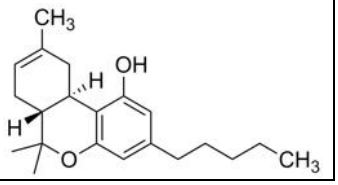
CAS#: unique, unambiguous identifier for each chemical substance; *Phytocannabinoid Compounds*: phytocannabinoid name; *Abbrev.*: common abbreviation; *Structure*: chemical structure of compound

Table 2.1 (continued) Phytocannabinoids included as target analytes in the analysis of hemp-derived oil products

CAS#	Phytocannabinoid Compounds	Abbrev.	Structure
2808-39-1	cannabinolic acid	CBNA	
24274-48-4	cannabidivarin	CBDV	
31932-13-5	cannabidivarinic acid	CBDVA	
21633-63-2	cannabicyclol	CBL	
40524-99-0	cannabicyclol acid	CBLA	
1972-08-3	Δ^9-tetrahydrocannabinol	Δ^9-THC	
23978-85-0	Δ^9-tetrahydrocannabinolic acid	THCA-A	

CAS#: unique, unambiguous identifier for each chemical substance; *Phytocannabinoid Compounds*: phytocannabinoid name; *Abbrev.*: common abbreviation; *Structure*: chemical structure of compound

Table 2-1 (continued) Phytocannabinoids included as target analytes in the analysis of hemp-derived oil products

CAS#	Phytocannabinoid Compounds	Abbrev.	Structure
31262-37-0	tetrahydrocannabivarin	THCV	
39986-26-0	tetrahydrocannabivarin acid	THCVA	
5957-75-5	Δ^8-tetrahydrocannabinol	Δ^8-THC	

CAS#: unique, unambiguous identifier for each chemical substance; *Phytocannabinoid Compounds*: phytocannabinoid name; *Abbrev.*: common abbreviation; *Structure*: chemical structure of compound

2.2.1 Liquid Chromatography (LC)

High-performance liquid chromatography is a chromatographic technique in which a pressurized liquid solvent mixture serves as the mobile phase and contains the sample. This mixture is passed through a column filled with a solid adsorbent material stationary phase. The presented method is a reverse-phase chromatography approach. The mobile phase has 2 components consisting of an aqueous solution and organic solution. The sample is loaded onto the column with a lower organic ratio. The analytes in the sample interact with the C8 ligands attached to the Kinetex® core-shell particles. As the organic ratio of the mobile phase increases, the analytes release from the C8 ligands in preference for the organic solvent. The differences in the preference for the C8 ligand to the organic solvent result in the chromatographic separation in the elution of the analytes off the column as shown by the example in Figure 2-2.

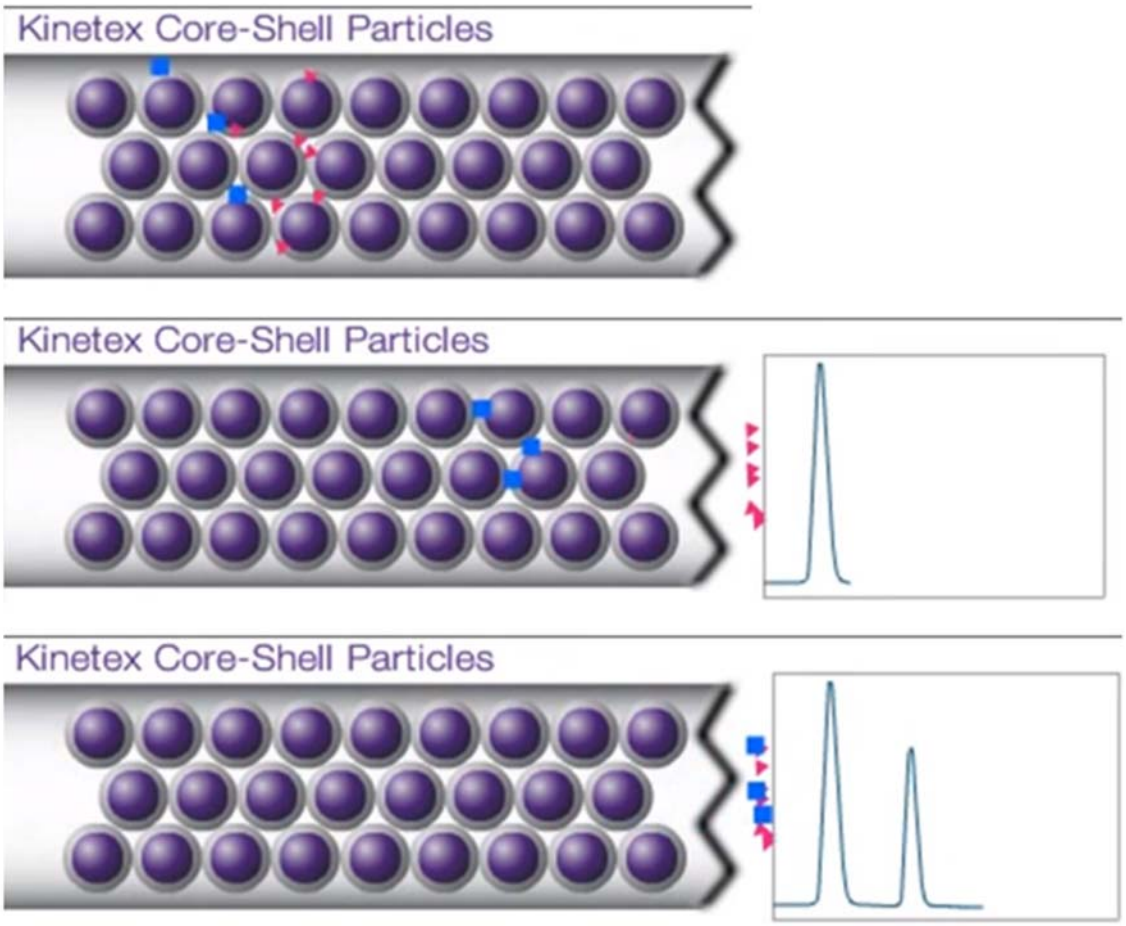


Figure 2-2 Chromatographic separation with Kinetex® Core-Shell Particles [51]
Diagram demonstrating the separation of analytes as they move through the pathways of the Kinetex core-shell particles and interact with the C8 ligands. Through a combination of how the compound interacts with the C8 ligand and the mobile phase, each compound elutes from the column at a unique retention time as demonstrated by the chromatographic peaks.

2.2.2 Tandem Mass Spectrometry (MS/MS)

The power of mass spectrometry as an analytical tool is greatly enhanced by the combination with a chromatographic technique such as liquid chromatography. Mass spectrometry is based on the principle of detecting a charged species in a mass-to-charge ratio (m/z). When liquid chromatography is combined with mass spectrometry, the mobile phase typically contains a modifier such as an acid that will impart a charge to the eluted analytes. As shown in Figure 2-3, the mobile phase carries the eluted analyte through the electrospray ionization (ESI) needle contained in the ionization probe. A voltage is applied to the needle imparting an excess of charges to the surface of the droplets emerging from the needle in a spray. Additionally, heated gases are used to evaporate the solvent droplets. As the size of the droplet decreases, the electrical density on the surface of the droplet increases. Once the critical point -Rayleigh stability limit- is reached, the droplets explode. This happens because the electrostatic repulsion is greater than the surface tension of the droplet. The ion species form of the analyte in the gaseous phase enters the ion transfer tube. The transfer tube applies heat to dry off any remaining solvent as well as a voltage to move the charged ion into the system.

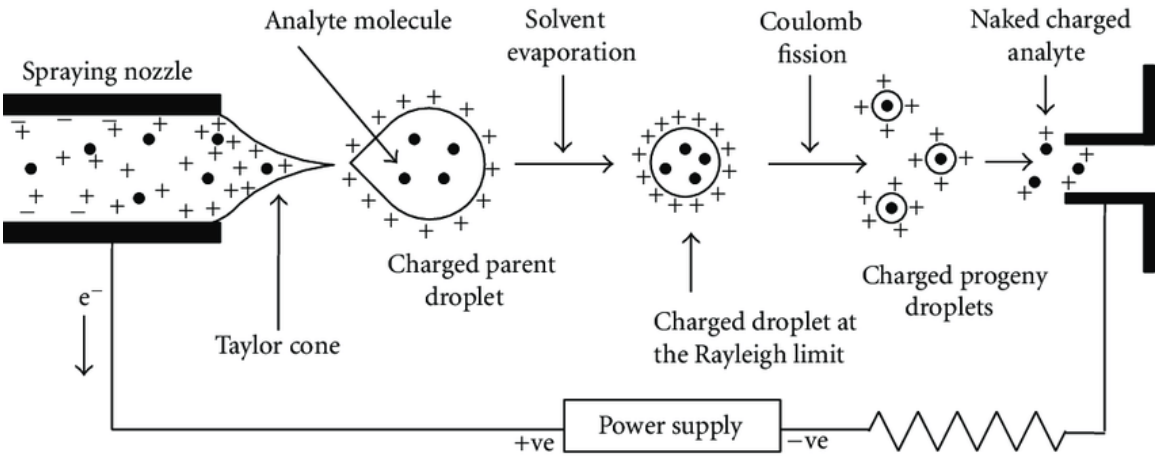


Figure 2-3 Schematic diagram of the electrospray ionization process in positive ionization mode [52]

The mobile phase containing a modifier (source of charge) carriers the analytes through the spray needle. The electrical field created from the positive charge on the spray needle and the positive charge of the liquid flowing through the spray needle leads to the formation of the Taylor cone. Small, charged droplets break off from the cone with the charge located on the surface of the droplet. Through heat and application of gases, the solvent is evaporated increasing the surface. At the same time, Coulombic repulsion moves the droplets away from each other and towards the heated capillary. The size of the droplet continues to decrease until it reaches the point (Rayleigh limit) where the surface tension can no longer sustain the Coulombic force of repulsion amongst the charges on the surface of the droplet. At this point, Coulombic fission leads to progeny droplets. This process occurs repeatedly until the point where nanodroplets from which the gas-phase charged analyte molecules (naked charged analyte) are formed. The charge (opposite of the analyte) on the heated capillary draws the naked charged analyte into the heated capillary.

The charged species of the analytes are directed by a series of lenses into the tandem quadrupole section of the mass spectrometer. The methodology employed for the presented studies is selective reaction monitoring (SRM), which works on the principle of a precursor ion being selected, then subjected to fragmentation, and the resulting fragment, product ion, being selected. To accomplish this, the charged species generated in ionization exit a series of directive lenses to enter quadrupole 1 (Q1). In Q1, the 4 rods of the quadrupole are grouped into 2 pairs with alternating current (AC) and direct current (DC) voltages applied in equal amplitude but opposite signs for each pair, as shown in the Figure 2-5 diagram. The frequency of the AC voltages is in the range of radio frequency and are thus referred to as RF voltages. The ratio of the DC voltage to the RF voltage needed to direct a charged species down the path of the quadrupole is specific to that precursor ion. Figure 2-4 provides a diagram of tandem mass spectrometry. The precursor ion of interest selected in Q1 is then sent to the collision cell (Q2) with argon gas, and a collision voltage is applied. The collision voltage excites the precursor ion which collides with the gas molecules and fragments into product ions. The product ions then enter quadrupole 3 (Q3) encountering the same selective RF and DC voltages described for Q1. The selected product ion from Q3 is directed to the electron multiplier detector where the counted ions are converted into an instrument signal.

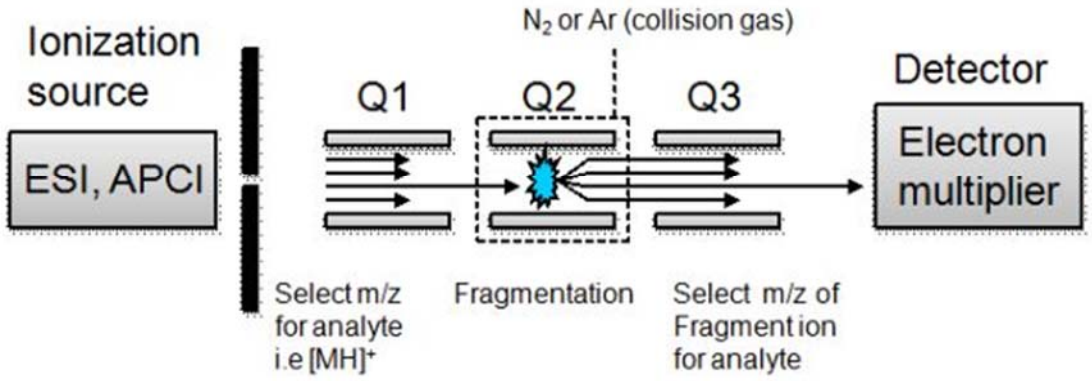


Figure 2-4 Diagram of selective reaction monitoring in tandem quadrupole mass spectrometry [53]

The charged molecules (ions) generated through electrospray ionization (ESI) are directed through a series of lenses (shown as solid black bars). The voltages applied to quadrupole 1 (Q1) are such to select out the precursor ion of interest from the group of ions coming through the lenses. The selected precursor ion is directed into the collision cell (Q2). In Q2, the collision energy determined in optimization and argon gas are applied in order to stimulate collision induced dissociation (CID). The product ions produced by CID enter quadrupole 3 (Q3) where voltages are applied to direct the product ions determined from optimization through Q3 then enter the electron multiplier (detector). The individual ions that make it through the pathway to the detector are counted and signal generated.

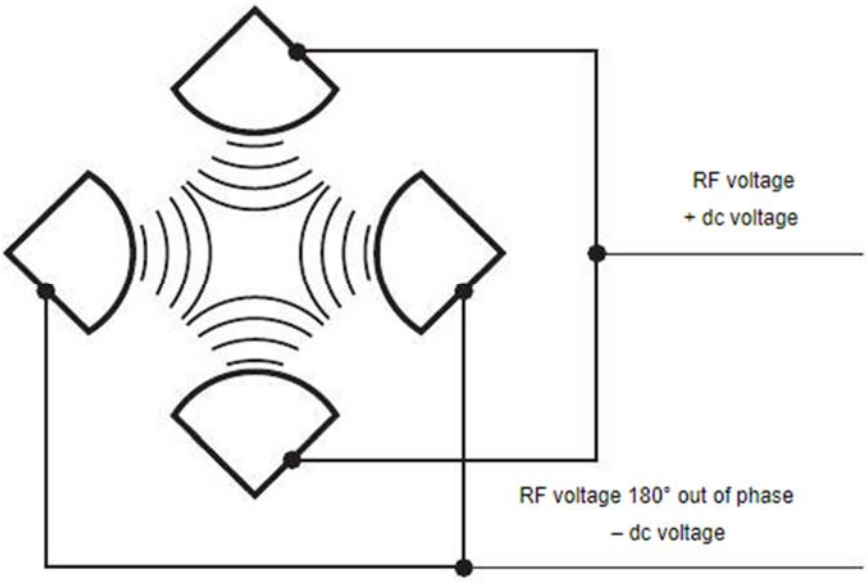


Figure 2-5 Diagram of a quadrupole with rod pairing with alternating and opposite voltage [54]

A quadrupole directs the path of the ion by alternating voltages 180° out of phase of each other moving the ion down the path of the pole.

Selective reaction monitoring (SRM) is a targeted technique that only looks for the specific precursor-product ion pairs programmed into the method. In order to determine the appropriate voltages and collision parameters for each precursor-product ion pair, the target analyte is optimized to the system by analyzing a reference material sample. The SRM analysis is a targeted approach looking for the specific list of compounds ignoring other components of the matrix. One way to think about this approach is that the blinders are on and only the desired compounds can be seen. To build in specificity, multiple precursor-product ion pairs can be programmed into the method for each analyte of interest. In the presented studies, 3 precursor-product ion pairs were programmed into the method for each analyte. The targeted approach to the SRM technique is an advantage and limitation as only the precursor-product ion pairs of interest are detected and other compounds in the sample will not be detected. The SRM technique is a good approach for quantitative analysis even at low levels because the electron multiplier counts each individual ion that hits the detector. For the presented studies, the quantitative capabilities of the SRM technique with a tandem mass spectrometry system were a suitable approach.

2.3 Sampling

The presented studies are focused on hemp-derived oil products. The goal in the selection of hemp-derived oil products was to acquire products from local and national brands readily available to consumers in central Kentucky. As such, 80 unregulated hemp-derived CBD oil products representing 51 different brands were purchased from 21 online and 9 local (brick and mortar) retailer sources between April 2 to May 9, 2021. The inclusion of online retailers allowed the researchers to ensure that the study included a representative selection of products produced in a variety of locations outside of Kentucky.

Epidiolex® (the FDA-approved CBD product) was also obtained (UK Investigational Drug Service Pharmacy) to serve as regulated control. Upon purchase, each product was randomly assigned a study identifier to blind researchers to product identification, but products 14 and 15 were lost in shipping and thus not included in the analyses. Products were stored according to packaging instructions or in a cool, dry space if no instructions were provided. All products were tested immediately after opening.

For the analyses, 3 samples were taken from each product and 3 replicates were prepared from each sample. The result for each analyte in each product tested is the mean of the 9 sample measurements.

Due to the challenges on the experimental analysis, these studies focused on hemp-derived oil products to the exclusion of other product types such as gummies, topicals, and vapes. At the time of purchase, oils were the most prevalent option available both online and in local stores.

2.4 Reagents and Standards

For all studies, reference materials were purchased from two different sources for the preparation of calibrator samples and quality control samples except for CBNA because only one source was available at the time of the study. The reference standard supplier used in the presented studies are ISO 17034 and ISO 17025 accredited, and all internal standards and reference standards, except CBD, were purchased as certified reference materials (CRM).

Both sources of CBD were purchased as neat powders, and working standards were prepared gravimetrically in acetonitrile. CBD was purchased from Cayman Chemical (Ann Arbor, MI, USA) for the preparation of calibrator samples and from Dr. Ehrenstorfer

(LGC Standards, Manchester, NH, USA) for the preparation of quality control samples. Cannabidiol-d_9 (CBD-d_9) was sourced from Cayman Chemical.

Δ^9-THC was purchased from Cayman Chemical for the preparation of calibrator samples and from Dr. Ehrenstorfer for the preparation of quality control samples. Δ^9-tetrahydrocannabinol-d9 (Δ^9-THC-d_9) was sourced from Cayman Chemical.

For the preparation of calibrator samples in the study of the minor cannabinoids, CBDV, THCV, CBG, CBN, Δ^8-THC, CBL, CBC, CBDVA, CBDA, THCVA, CBGA, THCA-A, and CBCA were purchased from Cayman Chemical while CBNA and CBLA were obtained from Cerilliant Corporation (Round Rock, TX, USA). For the preparation of quality control samples, CBDV, THCV, CBG, CBN, Δ^8-THC, CBL, CBC, CBDVA, CBDA, THCVA, CBGA, THCA-A, CBLA, and CBCA were purchased from Dr Ehrenstorfer while CBNA was obtained from Cerilliant Corporation. CBD-d_9, cannabigerol-d_9 (CBG-d_9), Δ^9-THC-d_9, and cannabichromene-d_9 (CBC-d_9) were sourced from Cayman Chemical. 11-nor-9-carboxy-tetrahydrocannabinol-d_3 (THC-COOH-d_3) and Δ^9-tetrahydrocannabinolic acid-d_3 (THCA-A-d_3) were purchased from Cerilliant Corporation.

For all studies, the reagents and solvents (LC/MS grade) used during the extraction and analysis were purchased from Fisher Scientific (Hampton, NH, USA). Extra virgin olive oil (EVOO), which was used as an analyte-free matrix was obtained from a local grocery retailer (Kroger, Cincinnati, OH, USA).

2.5 Sample Preparation

Prior to analysis, all sample containers were inverted multiple times to ensure contents were thoroughly mixed. Sub-aliquots of products were taken and transferred to

appropriately labeled containers where internal standard was added. After mixing, a fixed volume of acetonitrile was added and the samples were further mixed, then centrifuged (1811 x g, 20 mins). A 50 µl sub-portion of the supernatant was transferred to an autosampler vial and diluted with solvent and water to form a sample within an appropriate concentration range and composition (nominally 50:50 acetonitrile: water v:v) for analysis. The samples were capped and briefly vortex mixed prior to analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS). Samples with analyte concentrations above the calibration range were re-analyzed with dilution (10-fold) prior to internal standard addition.

2.6 Instrumentation – LC-MS/MS method

Analysis of samples was carried out via LC-MS/MS using a Thermo Accela 1250 quaternary LC system coupled with a TSQ Vantage mass spectrometer (Waltham, MA, USA). Separations were carried out using a reversed phase (C8) Kinetex® analytical column (2.1 x 100 mm, 2.6 µm) purchased from Phenomenex (Torrance, CA, USA). A gradient solvent program was employed using mobile phases of 0.1% formic acid in water (A) and in acetonitrile (B). Briefly, from a starting composition of 50% B, the percentage of organic mobile phase (i.e., B) was increased over 10 minutes, then an organic flush employed to remove residual matrix components before returning to the solvent starting composition. The solvent flow rate was 500 µL/min, and the total analytical run time was 14.25 min. The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in positive and negative ion modes using selective reaction monitoring (SRM). An example chromatogram of the compounds from Table 2-1 is shown in Figure

2-6. Figure 2-7 is an example chromatogram of the internal standards, stable-labeled cannabinoid analogs, included in the assay.

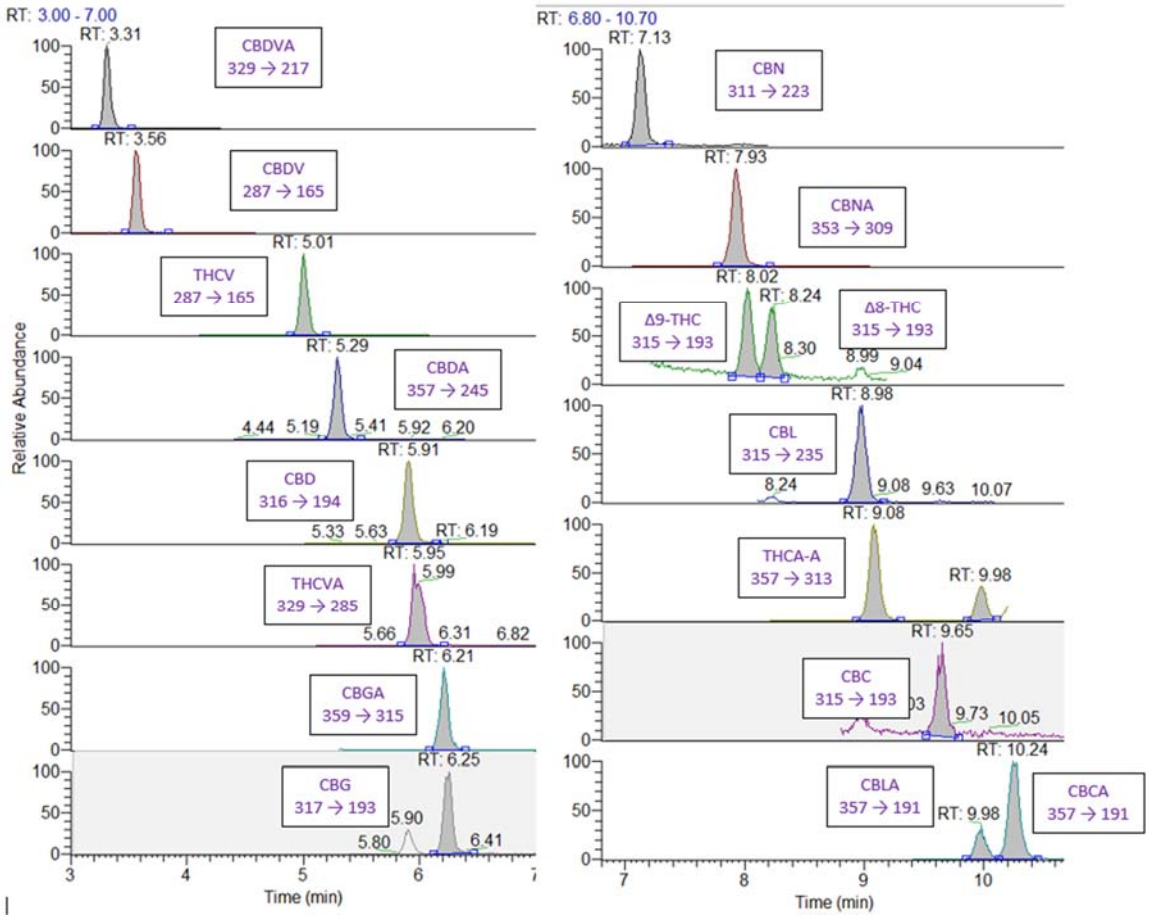


Figure 2-6 Chromatogram of the 17 phytocannabinoids included in the targeted analysis

Chromatogram of single selective reaction monitoring (SRM) transition [precursor ion → product ion] for each target cannabinoid demonstrating chromatographic resolution of each analyte.

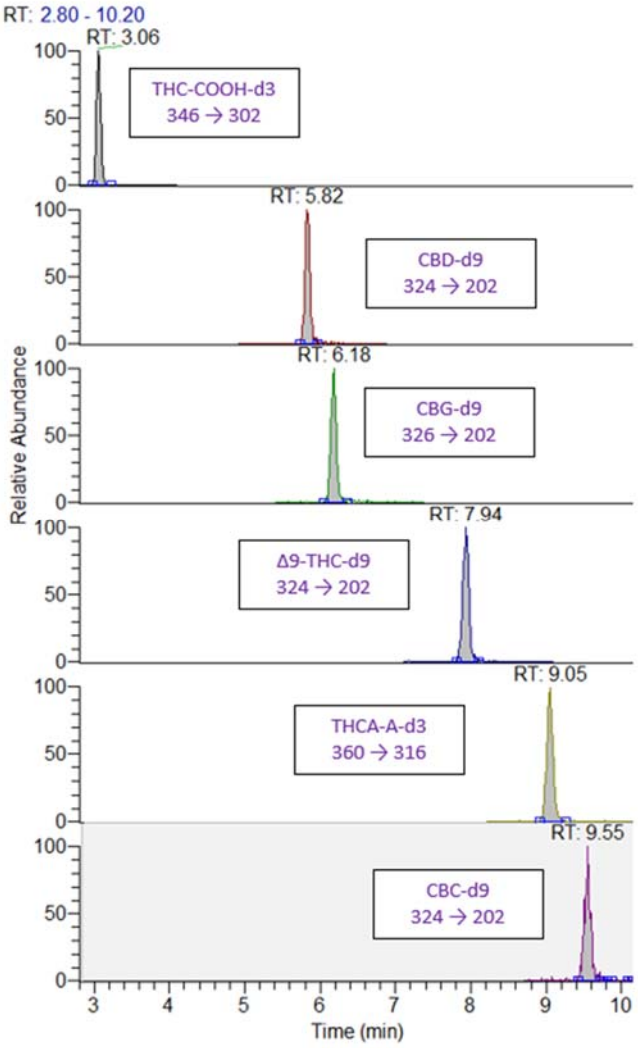


Figure 2-7 Chromatogram of the internal standards included in the analysis. Chromatogram of single selective reaction monitoring (SRM) transition [precursor ion → product ion] for each internal standard cannabinoid analog demonstrating chromatographic resolutions and used to quantitate of each analyte.

The SRM transitions monitored for the study of CBD and its internal standard are listed in Table 2-2. The SRM transitions monitored for the study of Δ^9-THC and its internal standard are listed in Table 2-3. The SRM transitions monitored for the study of minor cannabinoids and associated internal standards are presented in Tables 2-4 through 2-6. Table 2-4 shows the SRM transitions monitored in positive ionization mode, while Table 2-5 shows the SRM transitions monitored in negative ionization mode. The SRM transitions monitored for the internal standards are listed in Table 2-6.

Prior to each study batch, a system suitability sample containing the analytes and internal standards included in the study was analyzed to ensure that the instrument system was suitable for analysis. For each batch of samples, identification criteria were set for the detection of internal standards and target analytes based on the expected relative retention time (RRT) and ion ratio agreement (IR). The RRT was determined by taking the retention time of the target analyte and dividing by the retention time of the assigned internal standard. Across all batches, the RRT was within 1% except for THCVA which was within 2%. The IR was determined by taking the area response for the qualifier ion and dividing by the quantifier ion and converting to a percentage. A mid-range calibrator sample was used as the reference samples. The allowed tolerance for IR variance was within ± 10% absolute for IR of less than 25, within ± 25% relative for IR between 25 – 50, and within ± 15% absolute for IR greater than 50. These criteria were applied to the calibrator and quality control samples as well as to all test samples.

Table 2-2 Selective reaction monitoring (SRM) transitions for CBD and CBD-d_9

Analyte	Precursor Ion (m/z)	Product Ions (m/z)
Cannabidiol	316.2	194.1*
		260.2
		123.0
Cannabidiol-d_9	324.2	202.1*
		268.2
		123.0
*quantifier ion		

Analyte: compound monitored; *Precursor ion*: positively charged ion selected in Q1; *Product ion*: charged ion product resulting from fragmentation of the precursor ion; *m/z*: mass to charge ratio

Table 2-3 Selective reaction monitoring (SRM) transitions for Δ^9-THC and Δ^9-THC-d_9

Analyte	Precursor Ion (m/z)	Product Ions (m/z)
Δ^9-tetrahydrocannabinol (Δ^9-THC)	315.2	193.1*
		259.2
		123.1
Δ^9-tetrahydrocannabinol -d_9 (Δ^9-THC-d_9)	324.2	202.1*
		268.2
		122.9
*quantifier ion		

Analyte: compound monitored; *Precursor ion*: positively charged ion selected in Q1; *Product ion*: charged ion product resulting from fragmentation of the precursor ion; *m/z*: mass to charge ratio

Table 2-4 Selective reaction monitoring (SRM) transitions for minor cannabinoids monitored in positive ionization mode

Analyte	Abbreviation	Precursor Ion (m/z)	Product Ions (m/z)
Cannabidivarin	CBDV	287.2	165.1*
			231.1
			123.1
Δ^9-tetrahydrocannabivarin	THCV	287.1	165.1*
			123.1
			231.2
Cannabigerol	CBG	317.2	193.1*
			123.1
			137.0
Cannabinol	CBN	311.1	223.1*
			293.2
			208.1
Δ^8-tetrahydrocannabinol	Δ^8-THC	315.2	193.1*
			259.2
			123.1
Cannabicyclol	CBL	315.2	235.2*
			81.1
			123.1
Cannabichromene	CBC	315.2	193.1*
			259.2
			123.1
*quantifier ion			

Analyte: compound monitored; *Precursor ion*: positively charged ion selected in Q1; *Product ion*: charged ion product resulting from fragmentation of the precursor ion; *m/z*: mass to charge ratio

Table 2-5 Selective reaction monitoring (SRM) transitions for minor cannabinoids monitored in negative ionization mode

Analyte	Abbreviation	Precursor Ion (m/z)	Product Ions (m/z)
Cannabidivarinic acid	CBDVA	329.0	217.1*
			243.1
			283.2
Cannabidiolic acid	CBDA	357.1	245.1*
			271.1
			311.2
Δ^9-tetrahydrocannabivarinic acid	THCVA	329.0	285.2*
			217.1
			163.1
Cannabigerolic acid	CBGA	359.1	315.2*
			191.1
			297.2
Cannabinolic acid	CBNA	353.0	309.2*
			279.1
			222.0
Δ^9-tetrahydrocannabinolic acid-A	THCA-A	357.1	313.2*
			245.1
			191.1
Cannabicyclolic acid	CBLA	357.0	313.2*
			191.1
			217.1
Cannabichromenic acid	CBCA	357.0	191.1*
			313.2
			179.1
*quantifier ion			

Analyte: compound monitored; *Precursor ion*: positively charged ion selected in Q1; *Product ion*: charged ion product resulting from fragmentation of the precursor ion; *m/z*: mass to charge ratio

Table 2-6 Selective reaction monitoring (SRM) transitions for internal standards included in the analysis of the minor cannabinoids

Internal Standard	Abbreviation	Precursor Ion (m/z)	Product Ions (m/z)
11-nor-9-carboxy-tetrahydrocannabinol-d_3	THC-COOH-d_3	346.1	302.1*
			194.0
			248.1
Cannabidiol-d_9	CBD-d_9	324.2	202.1*
			268.2
			123.0
Cannabigerol-d_9	CBG-d_9	326.2	202.1*
			123.0
			138.0
Δ^9-tetrahydrocannabinol-d_9	Δ^9-THC-d_9	324.2	202.1*
			268.2
			122.9
Δ^9-tetrahydrocannabinolic acid-d_3	THCA-A-d_3	360.1	316.2*
			248.1
			194.1
Cannabichromene-d_9	CBC-d_9	324.2	202.2*
			123.1
			81.1
*quantifier ion			

Internal Standard: compound monitored; *Precursor ion*: positively charged ion selected in Q1; *Product ion*: charged ion product resulting from fragmentation of the precursor ion; *m/z*: mass to charge ratio

2.7 Method Performance

Prior to the analysis of samples, the method was evaluated for suitability through studies of selectivity, recovery, and matrix effects. To assess selectivity, three replicates of EVOO were prepared, extracted and analyzed for each of the following groups: 1) without spiking to assess the presence of interfering peaks resulting from the matrix, 2) spiked with only internal standards to assess proper identification of the internal standards and assess the presence of interference peaks for the target analytes stemming from the internal standards, and 3) spiked with only target analytes to assess proper identification of the target analytes and assess the presence of interference peaks for the internal standards stemming from the target analytes. Across all three groups no interferences were detected for any of the target analytes or internal standards. Additionally, the target analytes and internal standards were identified, correctly demonstrating that the developed method is selective for the target analytes and internal standards. Recovery was assessed by the comparison of pre-extraction spiked EVOO samples to post extraction spiked EVOO samples. Each group consisted of six replicates from which the mean peak area response was used to calculate the recovery of each target analyte, as shown in Table 2-7. To evaluate matrix effects, the post extraction spiked samples described for recovery were compared to six neat sample replicates, containing no matrix. The mean peak area response for each group was used to calculate the matrix effects shown in Table 2-7, ion suppression indicated by a negative result and ion enhancement indicated by a positive result. Recovery ranged from 87% to 100%, and matrix effects were minor.

Table 2-7 Recovery and Matrix effects results for each target analyte

Analyte	Recovery	Matrix Effects
CBDVA	100%	0%
CBDV	95%	+1%
THCV	94%	0%
CBDA	100%	0%
CBD	99%	-1%
THCVA	96%	+3%
CBGA	100%	-1%
CBG	95%	0%
CBN	95%	-1%
CBNA	99%	+1%
Δ^9-THC	96%	+1%
Δ^8-THC	87%	+1%
CBL	90%	-1%
THCA-A	96%	0%
CBC	93%	+1%
CBLA	90%	-1%
CBCA	97%	-1%

Analyte: Target analyte, *Recovery*: Percentage recovery was determined by comparison of pre-extraction spiked samples to post extraction spiked samples, *Matrix effects*: Matrix effects are a common concern in LC-MS. Matrix effects were determined by comparison of post extraction spiked samples to neat samples prepared without matrix. A positive (+) result indicates potential ion enhancement, and a negative (-) result indicates potential ion suppression.

Within the presented studies, each batch was self-validated using quality control samples spiked with each analyte in surrogate matrix, EVOO. For the CBD studies, each batch included a calibration curve consisting of 10 non-zero calibrator samples ranging from 0.5 to 100 mg/mL and 6 quality control sample replicates prepared at each of 4 different concentrations (2.0, 4.0, 40.0, and 80.0 mg/mL) for a total of 24 samples across the calibration range. For the study of Δ^9-THC and minor cannabinoids, each batch included a calibration curve consisting of 8 non-zero calibrators ranging from 0.005 to 1.500 mg/mL and 6 quality control sample replicates prepared at each of 4 different concentrations (0.010, 0.020, 0.600, and 1.200 mg/mL) for a total of 24 samples across the calibration range. The use of different sources for the preparation of calibrator and quality control samples helps to ensure quantitative accuracy by acting as a cross check of each other. Since CBNA was available from only a single supplier at the time of the study, two different preparations of working standards were made from the single source for spiking samples. To assess method performance, the accuracy and precision of the quality control samples were evaluated over four batches prepared on different days resulting in 24 measurements for each quality control sample concentration and a total of 96 samples. For the calibration curves, a linear or quadratic regression model was used with $1/x$ or $1/x^2$ weighting as appropriate. The coefficient of determination (R^2) was greater than 0.98 for all analytes in all batches.

The method performance of the quality control samples across all 17 phytocannabinoids targeted in the presented studies is summarized in Table 2-8. For CBD, the inter-batch relative error, a measurement of accuracy, was less than 9% across all concentrations of quality control samples, and the inter-batch coefficient of variation

(%CV), an indicator of precision, was less than 8%. The inter-batch relative error and inter-batch %CV of Δ^9-THC were within 9% across all quality control sample concentrations. For the minor cannabinoids, the relative error was within ± 15% across all target analytes at all quality control samples concentrations except for THCVA and CBCA which measured up to ± 32% and 27%, respectively. The inter-batch %CV was within 15% across all minor cannabinoid analytes across all quality control sample concentrations. The method performance showed precision across analytes.

Table 2-8 Method Performance of quality control samples

CBD	QC samples	2.0 mg/mL	4.0 mg/mL	40.0 mg/mL	80.0 mg/mL
	Mean conc.	2.2 mg/mL	4.3 mg/mL	39.7 mg/mL	79.4 mg/mL
	Std Dev	0.08 mg/mL	0.17 mg/mL	1.71 mg/mL	6.08 mg/mL
	Relative error	7.7%	8.5%	-0.7%	-0.8%
	Inter-batch CV	3.6%	4.0%	4.3%	7.7%

Δ^9-THC	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.009 mg/mL	0.019 mg/mL	0.573 mg/mL	1.095 mg/mL
	Std Dev	0.0008 mg/mL	0.0010 mg/mL	0.0278 mg/mL	0.0538 mg/mL
	Relative error	-6.7%	-5.3%	-4.5%	-8.7%
	Inter-batch CV	8.5%	5.2%	4.9%	4.9%

CBDVA	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.009 mg/mL	0.019 mg/mL	0.604 mg/mL	1.144 mg/mL
	Std Dev	0.0014 mg/mL	0.0007 mg/mL	0.0430 mg/mL	0.0998 mg/mL
	Relative error	-8.8%	-3.5%	0.6%	-4.7%
	Inter-batch CV	14.9%	3.8%	7.1%	8.7%

CBDV	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.009 mg/mL	0.018 mg/mL	0.519 mg/mL	1.061 mg/mL
	Std Dev	0.0004 mg/mL	0.0008 mg/mL	0.0231 mg/mL	0.0592 mg/mL
	Relative error	-9.9%	-12.3%	-13.6%	-11.6%
	Inter-batch CV	4.9%	4.8%	4.5%	5.6%

QC samples: theoretical concentrations of quality control samples; *Mean conc.*: mean observed concentration of the 6 quality control sample replicates; *Std Dev*: standard deviation; *Relative error*: percent relative error across all batches from theoretical concentration as a measurement of accuracy; *Inter-batch CV*: percent coefficient of variation across all batches as a measurement of precision

Table 2-8 (continued) Method Performance of quality control samples

THCV	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.011 mg/mL	0.020 mg/mL	0.604 mg/mL	1.231 mg/mL
	Std Dev	0.0004 mg/mL	0.0010 mg/mL	0.0257 mg/mL	0.0667 mg/mL
	Relative error	7.4%	2.0%	0.7%	2.5%
	Inter-batch CV	3.9%	5.0%	4.2%	5.4%

CBDA	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.010 mg/mL	0.019 mg/mL	0.573 mg/mL	1.082 mg/mL
	Std Dev	0.0011 mg/mL	0.0008 mg/mL	0.0267 mg/mL	0.0692 mg/mL
	Relative error	-4.2%	-4.4%	-4.5%	-9.8%
	Inter-batch CV	11.9%	4.4%	4.7%	6.4%

THCVA	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.008 mg/mL	0.019 mg/mL	0.573 mg/mL	1.082 mg/mL
	Std Dev	0.0009 mg/mL	0.0008 mg/mL	0.0267 mg/mL	0.0692 mg/mL
	Relative error	-22.6%	-32.4%	-6.0%	-6.8%
	Inter-batch CV	11.5%	4.5%	9.7%	10.2%

CBGA	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.009 mg/mL	0.018 mg/mL	0.569 mg/mL	1.132 mg/mL
	Std Dev	0.0010 mg/mL	0.0008 mg/mL	0.0308 mg/mL	0.1187 mg/mL
	Relative error	-6.9%	-9.1%	-5.2%	-5.7%
	Inter-batch CV	11.1%	4.6%	5.4%	10.5%

QC samples: theoretical concentrations of quality control samples; *Mean conc.*: mean observed concentration of the 6 quality control sample replicates; *Std Dev*: standard deviation; *Relative error*: percent relative error across all batches from theoretical concentration as a measurement of accuracy; *Inter-batch CV*: percent coefficient of variation across all batches as a measurement of precision

Table 2-8 (continued) Method Performance of quality control samples

CBG	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.010 mg/mL	0.019 mg/mL	0.572 mg/mL	1.151 mg/mL
	Std Dev	0.0004 mg/mL	0.0008 mg/mL	0.0232 mg/mL	0.0655 mg/mL
	Relative error	0.5%	-3.6%	-4.6%	-4.1%
	Inter-batch CV	4.0%	4.1%	4.1%	5.7%

CBN	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.010 mg/mL	0.019 mg/mL	0.572 mg/mL	1.151 mg/mL
	Std Dev	0.0005 mg/mL	0.0008 mg/mL	0.0232 mg/mL	0.0655 mg/mL
	Relative error	4.1%	-3.7%	-4.5%	-4.3%
	Inter-batch CV	5.2%	5.1%	5.0%	6.5%

CBNA	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.009 mg/mL	0.020 mg/mL	0.605 mg/mL	1.184 mg/mL
	Std Dev	0.0011 mg/mL	0.0010 mg/mL	0.0272 mg/mL	0.0701 mg/mL
	Relative error	-5.4%	-0.5%	0.9%	-1.3%
	Inter-batch CV	11.3%	4.8%	4.5%	5.9%

Δ^8-THC	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.010 mg/mL	0.021 mg/mL	0.617 mg/mL	1.197 mg/mL
	Std Dev	0.0005 mg/mL	0.0014 mg/mL	0.0315 mg/mL	0.0722 mg/mL
	Relative error	3.0%	2.8%	2.8%	-0.2%
	Inter-batch CV	4.9%	6.8%	5.1%	6.0%

QC samples: theoretical concentrations of quality control samples; *Mean conc.*: mean observed concentration of the 6 quality control sample replicates; *Std Dev*: standard deviation; *Relative error*: percent relative error across all batches from theoretical concentration as a measurement of accuracy; *Inter-batch CV*: percent coefficient of variation across all batches as a measurement of precision

Table 2-8 (continued) Method Performance of quality control samples

CBL	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.011 mg/mL	0.022 mg/mL	0.649 mg/mL	1.252 mg/mL
	Std Dev	0.0005 mg/mL	0.0009 mg/mL	0.0285 mg/mL	0.0646 mg/mL
	Relative error	12.2%	8.0%	8.1%	4.4%
	Inter-batch CV	4.2%	4.1%	4.4%	5.2%

THCA-A	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.009 mg/mL	0.019 mg/mL	0.583 mg/mL	1.147 mg/mL
	Std Dev	0.0010 mg/mL	0.0009 mg/mL	0.0253 mg/mL	0.0494 mg/mL
	Relative error	-6.6%	-6.3%	-2.9%	-4.4%
	Inter-batch CV	10.3%	4.5%	4.3%	4.3%

CBC	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.010 mg/mL	0.018 mg/mL	0.543 mg/mL	1.116 mg/mL
	Std Dev	0.0008 mg/mL	0.0014 mg/mL	0.0286 mg/mL	0.0744 mg/mL
	Relative error	-3.2%	-11.5%	-9.5%	-7.0%
	Inter-batch CV	8.4%	7.9%	5.3%	6.7%

CBLA	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.010 mg/mL	0.019 mg/mL	0.611 mg/mL	1.173 mg/mL
	Std Dev	0.0009 mg/mL	0.0010 mg/mL	0.0240 mg/mL	0.0537 mg/mL
	Relative error	-3.5%	-5.5%	1.8%	-2.3%
	Inter-batch CV	9.7%	5.2%	3.9%	4.6%

QC samples: theoretical concentrations of quality control samples; *Mean conc.*: mean observed concentration of the 6 quality control sample replicates; *Std Dev*: standard deviation; *Relative error*: percent relative error across all batches from theoretical concentration as a measurement of accuracy; *Inter-batch CV*: percent coefficient of variation across all batches as a measurement of precision

Table 2-8 (continued) Method Performance of quality control samples

CBCA	QC samples	0.010 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
	Mean conc.	0.008 mg/mL	0.015 mg/mL	0.468 mg/mL	0.878 mg/mL
	Std Dev	0.0010 mg/mL	0.0010 mg/mL	0.0226 mg/mL	0.0507 mg/mL
	Relative error	-24.4%	-24.5%	-22.0%	-26.9%
	Inter-batch CV	13.1%	6.3%	4.8%	5.8%

QC samples: theoretical concentrations of quality control samples; *Mean conc.*: mean observed concentration of the 6 quality control sample replicates; *Std Dev*: standard deviation; *Relative error*: percent relative error across all batches from theoretical concentration as a measurement of accuracy; *Inter-batch CV*: percent coefficient of variation across all batches as a measurement of precision

The inter-batch relative error results for THCVA and CBCA warranted investigation. Since CBD was purchased as a neat powder for the preparation of calibrator and quality control samples, the concentrations of the working standard solutions used for spiking samples was verified through the working standard preparation records. The remaining 16 phytocannabinoid target compounds were purchased as CRM solutions at a concentration of 1 mg/mL with certificates of analysis stating the accuracy of the solution. The stock solution was then diluted into a working standard solution used to spike samples. It is worth noting that the same working standard solution was used for the preparation of all four concentrations of quality control samples. To assess the matching of the working standard solutions, 10 vials were prepared of each working standard solution at a consistent concentration. The samples were analyzed, and the mean peak area response was calculated for each of the 16 analytes. In comparison of the working standard solution used to prepare calibrator samples to the working standard solution used to prepare quality control samples, the quality control working standard was 10% and 26% lower than the calibrator working standard for THCVA and CBCA, respectively. These differences explain the results observed for the method performance. The source of the discrepancy is likely differences in the concentrations of the CRM stock solutions or error in the preparation of the working standard solutions. As the CRM stock solutions were exhausted by the end of the study, the issue could not be explored further. The results for all 16 analytes are summarized in Table 2-9.

Table 2-9 Comparison of working standard solutions for the preparation of calibrator samples and quality control samples

Analyte	% Difference of QC working std compared to CAL working std
CBDVA	-6.3%
CBDV	-11.7%
THCV	0.3%
CBDA	-8.2%
THCVA	-9.5%
CBGA	-5.5%
CBG	-5.8%
CBN	-5.0%
CBNA	-2.8%
Δ^9-THC	-7.8%
Δ^8-THC	2.5%
CBL	6.3%
THCA-A	-7.5%
CBC	-6.8%
CBLA	-2.9%
CBCA	-25.7%

The mean peak area for the 10 replicates of the calibrator working standard solutions was compared to the mean peak area of the 10 replicates of the quality control sample working standard solutions according to the equation [(CAL/QC)/CAL] and converted to a percentage.

2.8 Conclusion

The method developed for the project reported here has been demonstrated as suitable for the quantitative analysis of 17 phytocannabinoids in hemp-derived oil products. The reproducibility of results shows the consistency and stability of the developed methodology. For CBDV, THCVA, and CBCA, the differences observed in the working standard solutions used to prepare calibrator and quality control samples explains the results observed for the method performance. The method was determined to be suitable and reproducible for the extraction and quantitative analysis of 17 phytocannabinoid compounds.

CHAPTER 3. LABEL ACCURACY OF UNREGULATED CANNABIDIOL (CBD) PRODUCTS: MEASURED CONCENTRATION VS. LABEL CLAIM

3.1 Abstract

Background: The legalization of hemp in the United States has led to tremendous growth in the availability of hemp-derived products, particularly cannabidiol (CBD) products. The lack of regulatory oversight in this industry has resulted in the marketing and sale of CBD products with questionable ingredients and quality. The aim of the current study was to examine the CBD content in 80 commercially available hemp-derived CBD products purchased from online and local retailers. Epidiolex® was also included in the study as a positive control.

Methods: The products were extracted by solvent extraction and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The analytical findings were compared to the label claims for CBD content.

Results: The label claims for CBD content ranged from 7.5 to 60 mg/mL, while LC-MS/MS analysis detected a range of 2.9 to 61.3 mg/mL. Of the 80 products evaluated, 37 contained CBD concentrations that were at least ± 10% different than the concentration listed on the label – 12 products contained < 90%, while 25 products contained >110%.

Conclusions: These data suggest that additional regulation is required to ensure label accuracy since nearly half of the products in this study were not properly labeled (i.e., not within a ± 10% margin of error). Consumers and practitioners should remain cautious of unregulated and often-mislabeled CBD products due to the risks of taking too much CBD (e.g., drug-drug interactions, liver enzymes elevations, increased side effects) and the consequences of taking too little (e.g., no clinical benefits due to underdosing).

The results of this study support the continued need for good manufacturing practices and testing standards for CBD products.

3.2 Introduction

Cannabidiol (CBD) is a non-psychoactive component of *Cannabis sativa* that has been the subject of increasing interest due to its purported therapeutic benefits. For decades, the close association of CBD to Δ^9-tetrahydrocannabinol (Δ^9-THC) (and previous Schedule I status of CBD) hampered the research of the potential medicinal benefits. The landscape has been changing since 1) the passage of the Agricultural Improvement Act of 2018 (2018 Farm Bill) which legalized products derived from hemp, defined as the plant/plant parts of *Cannabis sativa* with a Δ^9-THC concentration of no more than 0.3 percent of dry weight, [18] and 2) the removal of CBD from the US Drug Enforcement Administration (DEA) list of controlled substances. This legalization of hemp and hemp-derived products has led to rapid growth in the CBD industry. Epidiolex®, a purified oral solution of CBD, was approved by the US Food and Drug Administration (FDA) in June 2018 and is now approved for the treatment of three forms of epilepsy Lennox-Gastaut, Dravet syndrome, and epilepsy associated with tuberous sclerosis. [38, 55] With the exception of Epidiolex®, CBD products are largely unregulated and currently considered neither drugs nor legal dietary supplements nor can it be added to food. [40, 42] Although the FDA has utilized the authority under the Federal Food, Drug, and Cosmetic Act (FDCA) to enforce some regulation of CBD products (e.g., false marketing claims), there has been public pressure for the FDA to establish clear regulatory guidelines for CBD products. [56]

The FDA held a public hearing in June 2019 regarding CBD regulation and heard concerns from scientists regarding the chemical constituents of unregulated CBD products, including contamination from fungus, harmful by-products from the manufacturing

process, and the presence of dangerous drugs [JWH compounds, cathinones]. [57] The FDA and several research groups have also examined CBD concentrations in products and have reported generally consistent findings indicating concern with the label accuracy. Between 2015 to 2016, the FDA issued warning letters to 14 businesses with products containing less CBD than indicated on the label, including instances of CBD content being negligible or less than 1% of the label claimed content. [58] In one study of 84 CBD products, only 31% of the products tested were accurately labeled (i.e., within 10% of advertised CBD content). [41] Another study of CBD products in Mississippi showed that only 2 out of 20 products were within 10% of the advertised CBD content. [42] The issue of label accuracy is not unique to the United States. A study in the Netherlands showed that out of 16 CBD oil products tested only 5 contained CBD within 10% of the label claimed amount [59], and a study in Italy found that of 14 CBD oil products tested, only 5 contained CBD consistent within 10% of the labeled content. [60] In a study from the United Kingdom, the researchers reported that 11 of the 29 CBD oil products tested contained CBD within 10% of the advertised amount. [61]

For the current study, hemp-derived products (n=80) were purchased at various stores in central Kentucky and from online retailers from April 2 to May 9, 2021. The products were analyzed for CBD content, and the results were compared to the product label claims. Whereas previous studies have evaluated products available online [41] or local retailer in a specific state [42], this study investigated both online and local retailers. This study also included the FDA-approved product Epidiolex® as a regulated control. Additionally, this study focused solely on oil products since oils were the most prevalent option at time of purchase.

3.3 Methods and Materials

3.3.1 Sample Selection

CBD-containing products were acquired via online, and brick and mortar retail sources. Of the 80 samples, 44 CBD products were purchased from USA-based online retailers and the remaining 36 CBD products were purchased from local retailers within central Kentucky (e.g., CBD shops, head shops, health food markets, and health/wellness stores) that carried both local and national brands. The inclusion of online retailers ensured the representation of products produced in a variety of locations outside of Kentucky. Epidiolex® (the FDA-approved CBD product) was also obtained (University of Kentucky Investigational Drug Service Pharmacy) to serve as regulated control.

Upon purchase, each product was randomly assigned a study identifier to blind researchers to product identification. Products 14 and 15 were lost in shipping and thus not included in the analyses (81 total products including Epidiolex®). Upon receipt, product packaging and seals were inspected to ensure product integrity. The lot numbers were recorded, and the products were stored according to packaging instructions or in a cool, dry space if instructions were not provided. All products were tested immediately after opening.

3.3.2 Reagents and standards

Reference materials were purchased from two different sources for the preparation of calibrator samples and quality control samples. CBD was purchased from Cayman Chemical (Ann Arbor, MI, USA) for the preparation of calibrator samples and from Dr. Ehrenstorfer (LGC Standards, Manchester, NH, USA) for the preparation of quality control samples. Cannabidiol-d_9 (CBD-d_9) was sourced from Cayman Chemical. Reagents

and solvents (LC/MS grade) for use during the extraction and analysis were purchased from Fisher Scientific (Hampton, NH, USA). Extra virgin olive oil (EVOO), which was used as an analyte-free matrix was obtained from a local grocery retailer (Kroger, Cincinnati, OH, USA).

3.3.3 Sample Preparation

Prior to analysis, all sample containers were inverted multiple times to ensure contents were thoroughly mixed. Sub-aliquots of products were taken and transferred to appropriately labeled containers where internal standard was added. After mixing, a fixed volume of acetonitrile was added, and the samples were further mixed, then centrifuged (1811 x g, 20 mins). A 50 µl sub-portion of the supernatant was transferred to an autosampler vial and diluted with solvent and water to form a sample within an appropriate concentration range and composition (nominally 50:50 acetonitrile: water v:v) for analysis. The samples were capped and briefly vortex mixed prior to analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS). Samples with analyte concentrations above the calibration range were re-analyzed with dilution (10-fold) prior to internal standard addition.

3.3.4 Instrumentation

Analysis of samples was carried out via LC-MS/MS using a Thermo Accela 1250 quaternary LC system coupled with a TSQ Vantage mass spectrometer (Waltham, MA, USA). Separations were carried out using a reversed phase (C8) Kinetex® analytical column (2.1 x 100 mm, 2.6 µm) purchased from Phenomenex (Torrance, CA, USA). A gradient solvent program was employed using mobile phases of 0.1% formic acid in water (A) and in acetonitrile (B). Briefly, from a starting composition of 50% B, the percentage

of organic mobile phase (i.e., B) was increased over 10 minutes, then an organic flush employed to remove residual matrix components before returning to the solvent starting composition. The solvent flow rate was 500 µL/min, and the total analytical run time was 14.25 min. The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in positive ion mode using selective reaction monitoring (SRM). Monitored transitions for CBD and its internal standard were shown previously in Table 2-2.

3.4 Results

A total of 81 CBD products were tested (n=80 unregulated products + the FDA-approved product Epidiolex® [n=1]). For product labelling, an allowable variance of ± 10% was used, similar to other label accuracy studies [41, 60, 61], with detected CBD concentrations >110% of labeled value indicating the product was under-labeled (i.e., the product contained more CBD than the label indicated) and detection of <90% of labeled CBD concentration indicating the product was over-labeled (i.e., the product contained less CBD than label indicated). Products within ± 10% (i.e., 90% - 110% of labeled value) were categorized as accurately labeled. The observed concentration value was determined by taking the mean of 9 measurements for each sample.

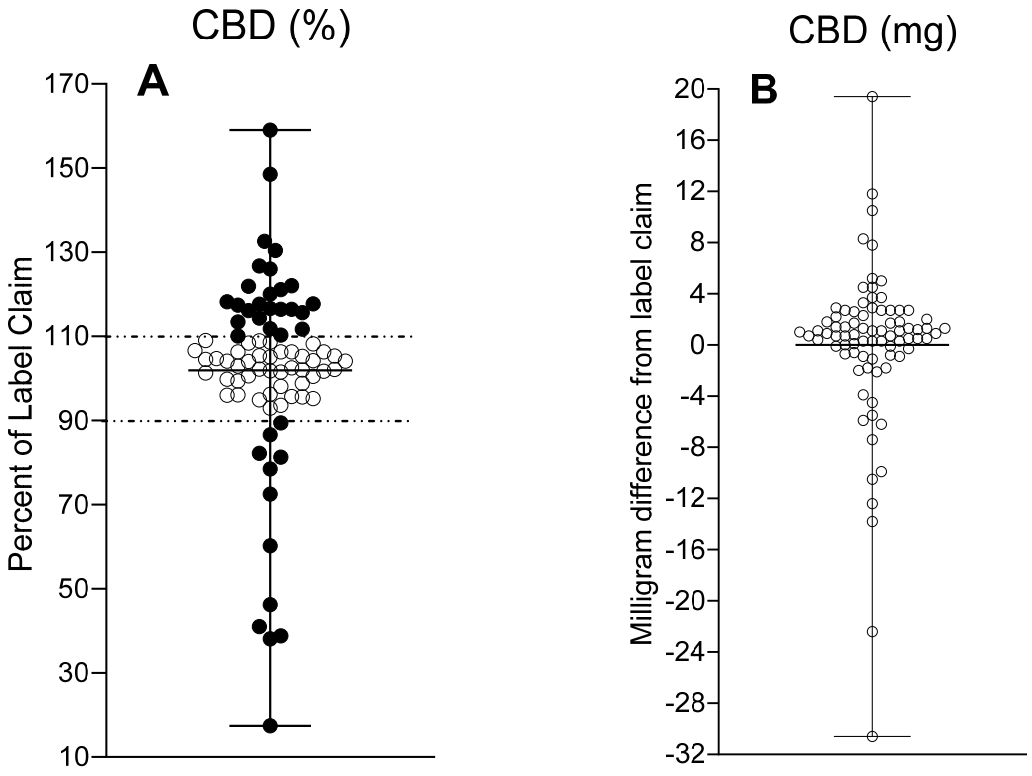


Figure 3-1 CBD measurements in 80 commercially available CBD oil products and Epidiolex®.

(A) The percentage of CBD label claim content with ± 10% tolerance denoting under-labelling (>110%) and over-labelling (<90%). (B) Deviation from CBD label claim in milligram.

The measured CBD content was compared to the advertised CBD content on the package label, and the percentage of label claim was determined for the CBD content. The CBD content percentage per label claim and milligram deviation from the label claim are shown in Figure 3-1. Of the 81 products tested, 31% [95% CI, 20% - 41%] were under-labeled (n=25), 15% [95% CI, 7% - 23%] were over-labeled (n=12) and 54% [95% CI, 43% - 65%] were accurately labeled (n=44). Epidiolex® was within 4% of its labeled concentration (label: 100 mg/mL; analyzed: 96.1 mg/mL) and is represented in the accurately labeled group. Across all samples, the observed CBD concentrations ranged from 2.9 mg/mL to 96.1 mg/mL, and the values for percent of label claim ranged from 17% (Product 13) to 159% (Product 79). For under-labeled products (shown in Table 3-2), the average amount of CBD was 121% of label claim with a range of 110.1% (Product 68) to 159% (Product 79). For over-labeled products (shown in Table 3-3), the average percent of label claim was 61% with a range of 17% (Product 13) to 89% (Product 76).

Table 3-1 List of samples containing at least 10% MORE CBD than label claim (i.e., under-labeled samples)

Sample Identifier	Source	Label Claim mg CBD / mL	Observed mg CBD / mL	Difference mg CBD / mL	Percent of Label Claim
79	Online	20.0	31.8	11.8	159
12	Online	40.0	59.4	19.4	148
40	Local	10.0	13.3	3.3	133
2	Local	17.0	22.2	5.2	130
59	Local	10.0	12.7	2.7	127
58	Local	30.0	37.8	7.8	126
3	Online	17.0	20.7	3.7	122
1	Local	17.0	20.7	3.7	122
77	Online	50.0	60.5	10.5	121
47	Local	25.0	30.0	5.0	120
43	Local	25.0	29.5	4.5	118
33	Local	10.3	12.2	1.8	118
39	Local	10.0	11.8	1.8	118
38	Local	10.0	11.7	1.7	117
4	Online	50.0	58.3	8.3	117
22	Online	16.7	19.4	2.7	116
26	Local	16.7	19.4	2.7	116
37	Local	8.3	9.7	1.3	116
81	Online	16.7	19.3	2.6	116
36	Local	8.3	9.5	1.2	114
66	Online	16.7	18.9	2.2	113
67	Online	16.7	18.6	2.0	112
42	Local	25.0	27.9	2.9	112
11	Online	8.4	9.3	0.9	110
68	Online	16.7	18.3	1.7	110

Sample Identifier: sample identification number; *Source*: purchase source; *Label Claim*: Concentration of CBD in the product according to the label in mg/mL; *Observed*: Mean observed concentration of CBD detected in each product in mg/mL; *Difference*: Absolute difference between the observed concentration; *Percentage of Label Claim*: Percentage of observed CBD concentration compared to concentration claimed on the label.

Table 3-2 List of samples containing at least 10% LESS CBD than label claim (i.e., over-labeled samples)

Sample Identifier	Source	Label Claim mg CBD / mL	Observed mg CBD / mL	Difference mg CBD / mL	Percent of Label Claim
76	Online	16.7	14.9	-1.8	89
30	Local	33.3	28.9	-4.5	87
5	Online	33.3	27.4	-5.9	82
7	Online	33.3	27.1	-6.2	81
29	Local	34.5	27.1	-7.4	79
80	Online	20.0	14.5	-5.5	73
31	Local	25.0	15.1	-9.9	60
28	Local	41.7	19.2	-22.4	46
48	Local	17.9	7.3	-10.5	41
24	Online	50.0	19.4	-30.6	39
45	Local	20.0	7.6	-12.4	38
13	Online	16.7	2.9	-13.8	17

Sample Identifier: sample identification number; *Source*: purchase source; *Label Claim*: Concentration of CBD in the product according to the label in mg/mL; *Observed*: Mean observed concentration of CBD detected in each product in mg/mL; *Difference*: Absolute difference between the observed concentration; *Percentage of Label Claim*: Percentage of observed CBD concentration compared to concentration claimed on the label.

The CBD content was determined for 80 non-regulated hemp-derived CBD oil products as well as Epidiolex®. Of the 44 unregulated products purchased from online retailers, 25% [95% CI, 12% - 38%] (n=11) were under-labeled, 14% [95% CI, 3% - 24%] (n=6) were over-labeled, and 61% [95% CI, 47% - 76%] (n=27) were accurately labeled for CBD content. For the 36 unregulated products purchased at local retailers, 39% [95% CI, 23% - 55%] (n=14) were under-labeled, 17% [95% CI, 5% - 29%] (n=6) were over-labeled and 44% [95% CI, 28% - 61%] (n=16) were accurately labeled.

As each observed concentration result was determined from the mean of 9 concentration measurements. Using these 9 measurements, the standard error of the mean (SEM) was calculated for each product. The observed mean concentration ± SEM for each sample is reported in Table 3-3.

Table 3-3 CBD concentrations ± standard error to the mean in mg/mL

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	20.7	0.2	30	28.9	0.2	57	15.9	0.2
2	22.2	0.3	31	15.1	0.2	58	37.8	0.7
3	20.7	0.2	32	47.9	1.0	59	12.7	0.2
4	58.3	1.1	33	12.2	0.1	60	35.7	0.4
5	27.4	0.2	34	27.2	0.4	61	35.9	0.5
6	16.0	0.1	35	23.4	0.2	62	33.8	0.6
7	27.1	0.3	36	9.5	0.1	63	54.5	0.9
8	16.7	0.1	37	9.7	0.1	64	33.3	0.4
9	52.7	0.7	38	11.7	0.1	65	16.9	0.3
10	52.3	1.1	39	11.8	0.1	66	18.9	0.3
11	9.3	0.1	40	13.3	0.2	67	18.6	0.3
12	59.4	0.8	41	17.8	0.2	68	18.3	0.2
13	2.9	0.0	42	27.9	0.4	69	16.9	0.4
16	17.3	0.2	43	29.5	0.4	70	16.9	0.3
17	17.7	0.1	44	49.1	0.5	71	17.5	0.3
18	21.4	0.2	45	7.6	0.1	72	17.3	0.2
19	16.0	0.2	46	16.9	0.2	73	48.0	0.7
20	17.4	0.3	47	30.0	0.4	74	10.5	0.1
21	24.7	0.3	48	7.3	0.1	75	7.5	0.2
22	19.4	0.4	49	8.9	0.1	76	14.9	0.2
23	17.3	0.3	50	26.1	0.3	77	60.5	1.2
24	19.4	0.4	51	15.8	0.2	78	18.1	0.3
25	51.1	1.3	52	16.6	0.2	79	31.8	0.5
26	19.4	0.3	53	18.1	0.2	80	14.5	0.7
27	33.8	0.4	54	17.1	0.2	81	19.3	0.3
28	19.2	0.2	55	15.6	0.2	82	61.3	0.9
29	27.1	0.4	56	17.1	0.3	83	96.1	1.5

Sample ID: sample identification; *Conc.*: mean observed sample concentration in mg/mL;
± *SEM*: standard error of the mean of the observed concentration measurements

3.5 Discussion

Recent studies of CBD products have led to quality concerns regarding the accuracy of product labelling, especially with regard to CBD content. [41, 42, 59-61] For this study, 80 unregulated CBD oil products were purchased from online retailers and local retailers in Central Kentucky. The products purchased for the study represented the range of CBD product manufacturers from local small businesses to companies with nationwide distribution, and Epidiolex® was included as a regulated control. Of the products tested, 54% were found to have CBD concentrations consistent with the advertised amount on the label while 31% were found to contain more than 110% of the label claim and 15% were found to contain less than 90% of the label claim amount of CBD. The results of this study are consistent with the findings of previous studies.

Since December 2018, hemp-derived CBD products have inundated the U.S. market in a variety of forms, including ingestible oils, gummies, beverages, topical creams, and inhalation liquids (i.e., vape pens), with sublingual oils being the most common. [44] During this time, the regulatory status of CBD oils has been vague and imprecise. The 2018 Farm Bill legalized the low Δ^9-THC *Cannabis sativa* plant from which these CBD oils are made, but the legality of these products is questionable because the FDA has stated that CBD oils cannot be considered drugs, dietary supplements, or added to foods, human or animal. [18, 40] Consumers have increasingly explored and used CBD oils for the purported benefits primarily as a specific therapy for medical conditions and secondarily for general health and well-being. [44] Corroon and Phillips reported that consumers are taking CBD products to treat multiple medical conditions, with an average of 2.67 medical conditions per consumer. [44] However, there is a disconnect between the products that

consumers are actually taking (i.e., the unregulated CBD products analyzed here) and the CBD products that are being tested in clinical trials (e.g., Epidiolex®; other pharmaceutical grade/regulated products). [42] Consumers often assume, wrongly, that the CBD products that they are taking are manufactured with the same level of control as an FDA-approved product. In addition, although Epidiolex® is currently FDA-approved for the treatment of three seizure conditions, CBD has not been FDA-approved to treat other conditions. Despite its popularity for the treatment of pain, anxiety, insomnia and other conditions, there is not substantial scientific evidence to support its use for these conditions; the controlled clinical trial data is limited or non-existent for these indications, and what data is available suggests little to no efficacy. [62, 63] Despite this lack of empirical evidence, consumers are searching for accurate information about CBD and often determining their own treatment plans from anecdotal evidence acquired from internet research, family members, or friends. [44] Since consumers are taking CBD products without medical guidance, it is imperative that, at a minimum, product labels convey clear and accurate information on CBD content to best allow consumers to be accurately informed about the doses being taken. The inaccuracy of labeling means that vulnerable consumers will not receive the expected dose of CBD, leading to concerns with respect to efficacy, side effects, and consumer safety. With the range of CBD concentrations available to consumers, 7.5 mg/mL to 60 mg/mL in this study, even small percentages of label inaccuracy could result in significant variation of CBD dosage from the intended dose, especially considering the potential for dosing multiple times per day.

The oral bioavailability of CBD has been estimated to be 6% due to extensive first pass metabolism. [62] CBD metabolism occurs in the liver through the actions of

cytochrome P450 isozymes. [64, 65] More specifically, the primary metabolites of CBD, 7-hydroxy-CBD and 6-hydroxy-CBD, have been shown to be mediated by CYP2C19 and CYP3A4. [64] *In vitro*, Bansal et al. reported time dependent inhibition of CYP1A2, CYP2C19 and CYP3A, demonstrated by a decrease in activity of 83%, 75%, and 85%, respectively. [66] Clinical studies of epilepsy using Epidiolex® have demonstrated potential inhibition or induction of CYP2C19, CYP3A4, CYP2C8, CYP2C9, CYP1A2, CYP2B6, UGT1A9 and UGT2B7. [39, 65] The risk of dose-dependent drug-drug interaction with CBD taken in combination with other medications and/or dietary supplements emphasizes the need for accuracy in labeling to better assist the consumer in determining appropriate dosing.

CBD effects on liver enzymes can lead to of hepatocellular injury. [39] In a study of acute and sub-acute toxicity, CBD dose-dependently increases both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) along with an increase in the liver-to-body weight ratios, resulting in an increased total bilirubin. [67] In some clinical trials, elevated liver aminotransferase enzyme levels were >3 times the upper limit of the normal range and led to patient withdrawal. [68, 69] Additionally, Ewing *et al.* showed differential regulation of more than 50 gene-markers related to hepatotoxicity after administration of CBD. [67] Elevation of markers of liver injury after administration of CBD have been shown to occur in a dose-dependent manner.

3.6 Conclusion

The results of this study add to the evidence from studies in several countries demonstrating that CBD content in over-the-counter CBD oil products is often inconsistent with the label claims. Inaccurate labeling has the potential to present safety risks to the

consumer. Since most consumers are using CBD products as therapeutic treatments for some types of real or perceived medical conditions, the dosing is important when considering the potential for CBD accumulation, elevation of liver enzymes, and drug-drug interactions. The findings reported here emphasize the continued need for clear and consistent regulation from federal and state agencies to ensure label accuracy of CBD products and subsequent enforcement. These results also indicate the need for continued development of good manufacturing practices and testing standards. Since consumers are taking CBD products for an ever-increasing range of conditions, independent of medical guidance, the accuracy of content labeling is important for the safety of the consumer.

CHAPTER 4. CANNABIDIOL (CBD) PRODUCT CONTAMINATION: QUANTITATIVE ANALYSIS OF Δ^9-TETRAHYDROCANNABINOL (Δ^9-THC) CONCENTRATIONS FOUND IN COMMERCIALLY AVAILABLE CBD PRODUCTS

4.1 Abstract

Background: Regulations and product labelling have not kept pace with the growth of the hemp-derived CBD market. We have evaluated the risk of Δ^9-tetrahydrocannabinol (Δ^9-THC) contamination in 80 unregulated products with comparison to a regulated control, Epidiolex®.

Methods: A cross section of local and national brands of hemp-derived oil products was purchased from local retailers in central Kentucky and online. These samples were extracted by solvent extraction and quantified by liquid-chromatography tandem mass-spectrometry (LC-MS/MS) using a validated method.

Results: Of the 80 unregulated products and Epidiolex®, Δ^9-THC was detected above the limit of quantification (LOQ = 0.005 mg/mL) of the assay in 52 samples, ranging from 0.008 mg/mL to 2.071 mg/mL. Twenty-one of the products tested were labeled as "THC-Free," and 5 of these products contained detectable levels of Δ^9-THC ranging from 0.015 mg/mL to 0.656 mg/mL.

Conclusions: Consumers are taking hemp-derived CBD products without understanding the risks of unintentional consumption of Δ^9-THC. This accidental use of Δ^9-THC could have adverse effects on health and safety as well as potentially legal consequences in child custody and impaired driving. Δ^9-THC drug test findings could impact employment, military, and sport eligibility status.

4.2 Introduction

Hemp derived products are increasingly available in retail stores and through online retailers throughout the United States due in part to the passage of the Agricultural Improvement Act of 2018 (2018 Farm Bill), which permitted sales of hemp products, including cannabidiol (CBD). CBD, a cannabinoid with limited to no abuse potential has been a driver in the market growth of the hemp-derived/CBD industry due to purported therapeutic benefits. [70-72] There is one FDA-approved CBD product currently on the market (Epidiolex®); this leaves the vast majority of the CBD products sold in the United States unregulated. These unregulated products are sold both online and in various marketplaces and are not currently considered drugs nor legal dietary supplements by the FDA. [40, 42] Although the FDA has utilized authority under the Federal Food, Drug and Cosmetic Act (FDCA) to enforce some regulation of hemp-derived CBD products, including false marketing claims, there is no regulation or oversight regarding the contents of these products. [56] This has allowed CBD products to be sold to consumers which contain 1) no measurable CBD, 2) various concentrations of synthetic cannabinoids as well as other drugs, and 3) other contaminants including residual solvents and heavy metals. [41-43, 61]

Recent reports indicate that many CBD products may contain appreciable concentrations of the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC). [41-43, 59-61] This oversight has likely occurred due to an imprecise definition of the federal legal limit of Δ^9-THC that is permitted in CBD products, along with little to no regulatory oversight of the manufacture, sale, and distribution. For example, the 2018 Farm Bill defined hemp as *Cannabis sativa* plant/plant parts containing concentrations of Δ^9-THC

of no more than 0.3 percent of dry weight. [18] This definition is based on the content of plant material from which the product is derived and offers no clear guidelines on how much Δ^9-THC is permitted in finished products such as oils, gummies and salves (as the plant biomass has been removed). Additionally, the 2018 Farm Bill leaves further regulation of hemp and hemp-derived products, beyond the general definition, up to the individual states. As such, an inconsistent patchwork of laws has emerged leading to a lack of clear guidance for consumers and producers. The FDA released guidance on how to calculate Δ^9-THC content in hemp-derived products for investigation of new drug (IND) applications and new drug applications (NDA) [73]; however, this guidance has not been adopted for unregulated hemp-derived CBD products that make up the vast majority of the current market.

Although previous studies reported on the range of Δ^9-THC found across the CBD products or the number of products testing above the limit of quantification (LOQ), there is little information quantifying the amount (mg/mL) of Δ^9-THC in each of the products and none were validated against a regulated control. [41-43, 74] The aim of the present study was to perform detailed quantitative analysis of the Δ^9-THC content in unregulated hemp-derived CBD products validated against a regulated control. The current study randomly sampled hemp-derived products available for purchase at various stores in central Kentucky and from online retailers. The Δ^9-THC content of each sample was determined by internally controlled LC-MS/MS analysis. None of the products tested had specific listings of Δ^9-THC content on the label. The current study details the Δ^9-THC concentrations for 80 unregulated products as well as Epidiolex®, a regulated CBD product derived from *Cannabis sativa*.

4.3 Materials and Methods

4.3.1 Sample Selection

Eighty unregulated hemp-derived CBD oil products representing 51 different brands were purchased between April 2 to May 9, 2021. The inclusion of online retailers ensured that these studies included a representative selection of products produced in a variety of locations outside of Kentucky. Epidiolex® (the FDA-approved CBD product) was also analyzed (UK Investigational Drug Service Pharmacy) to serve as regulated control.

Each product was randomly assigned a study identifier to blind researchers to product identification. Products were stored according to packaging instructions or in a cool, dry space if no instructions were provided. All products were tested immediately after opening.

Due to the challenges on the experimental analysis, the presented study focused on hemp-derived oil products to the exclusion of other product types such as gummies, topicals, and vapes. At the time of purchase, oils were the most prevalent option available.

4.3.2 Reagents and standards

Reference materials were purchased from two different suppliers with ISO17025 and ISO17034 accreditation for the preparation of calibrator samples and quality control samples. Δ^9-THC was purchased from Cayman Chemical (Ann Arbor, MI, USA) as a certified reference material for the preparation of calibrator samples and from Dr. Ehrenstorfer (LGC Standards, Manchester, NH, USA) as a certified reference material for the preparation of quality control samples. Δ^9-tetrahydrocannabinol-d_9 (Δ^9-THC-d_9) was sourced from Cayman Chemical. Reagents and solvents (LC/MS grade) for use during the

extraction and analysis were purchased from Fisher Scientific (Hampton, NH, USA). Extra virgin olive oil, which was used as an analyte-free matrix was obtained from a local grocery retailer (Kroger, Cincinnati, OH, USA).

4.3.3 Method Validation

The validation of the method for the analysis of Δ^9-THC included studies of linearity, accuracy, precision, recovery, and matrix effects. The accuracy and precision of the method was determined by the analysis of 3 calibrator and control sets with each set consisting of 8 calibrators, ranging from 0.005 to 1.500 mg/mL, and 6 control samples replicates prepared at each of 4 different quality control levels (0.005, 0.020, 0.600, and 1.200 mg/mL) across the calibration range. Recovery of Δ^9-THC was determined by comparison of pre-extraction supplemented samples and post-extraction supplemented samples. In the same experiment, matrix effect was determined by the comparison of post-extraction supplemented samples to neat samples, containing no matrix.

4.3.4 Sample Preparation

Prior to analysis, all sample containers were inverted multiple times to ensure contents were thoroughly mixed. Sub-aliquots, 3 replicates of 50 µL, of products were taken and transferred to appropriately labeled containers where internal standard was added. After mixing, a fixed volume of acetonitrile was added, and the samples were further mixed, then centrifuged (1811 x g, 20 mins). A 50 µl sub-portion of the supernatant was transferred to an autosampler vial and diluted with solvent and water to form a sample within an appropriate concentration range and composition (nominally 50:50 acetonitrile: water v:v) for analysis. The samples were capped and briefly vortex mixed prior to analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS). Samples with

analyte concentrations above the calibration range were re-analyzed with dilution in solvent (10-fold) prior to internal standard addition.

4.3.5 Instrumentation

Analysis of samples was carried out via LC-MS/MS using a Thermo Accela 1250 quaternary LC system coupled with a TSQ Vantage mass spectrometer (Waltham, MA, USA). Separations were carried out using a reversed phase (C8) Kinetex® analytical column (2.1 x 100 mm, 2.6 µm) purchased from Phenomenex (Torrance, CA, USA). A gradient solvent program was employed using mobile phases of 0.1% formic acid in water (A) and in acetonitrile (B). Briefly, from a starting composition of 50% B, the percentage of organic mobile phase (i.e., B) was increased to 65% B over 10 minutes, then an organic flush employed to remove residual matrix components before returning to the solvent starting composition. The solvent flow rate was 500 µL/min, and the total analytical run time was 14.25 min. Through the use of reference material, the method was demonstrated to chromatographically separate Δ^9-THC from common interferences such as CBD and Δ^8-tetrahydrocannabinol (Δ^8-THC).

The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in positive ion mode using selective reaction monitoring (SRM). Monitored transitions for Δ^9-THC and its internal standard were shown previously in Table 2-3.

4.4 Results

4.4.1 Method Validation

The purpose of the validation study was to determine the method performance for the quantification of Δ^9-THC in oil matrix. For the calibration range, 0.005 to 1.500 mg/mL, a linear calibration model was used with $1/x^2$ weighting and the coefficient of

determination (R^2) greater than 0.99 for all batches. The inter-batch accuracy and precision were calculated for each quality control sample level and the results are shown in Table 4-1. Recovery of Δ^9-THC was 96%, and a minor ion enhancement was observed of +1%. The method was demonstrated to be suitable for the quantitative analysis of Δ^9-THC.

Table 4-1 Summary of method validation studies characterizing Δ^9-THC quantification

Quality control samples	0.005 mg/mL	0.020 mg/mL	0.600 mg/mL	1.200 mg/mL
Mean concentration	0.0051 mg/mL	0.0213 mg/mL	0.6199 mg/mL	1.1583 mg/mL
Standard deviation	0.0005 mg/mL	0.0009 mg/mL	0.0232 mg/mL	0.0392 mg/mL
Inter-batch relative error	4.1 %	6.4 %	3.3 %	-3.5%
Inter-batch coefficient of variation	10.4 %	4.4 %	3.7 %	3.4 %

Quality control samples: theoretical concentrations of the quality control samples; *Mean concentration*: mean calculated from the concentrations of the 9 observed sample measurements; *Standard deviation*: standard deviation calculation of the 9 observed sample measurements; *Inter-batch relative error*: relative error of the mean of the 9 observed sample measurements compared to the theoretical concentrations reported as a percent, indication of method accuracy; *Inter-batch coefficient of variation*: calculation of the variation observed for the 9 sample measurement reported as a percent, indication of method precision

4.4.2 Δ^9-THC Determination in Products

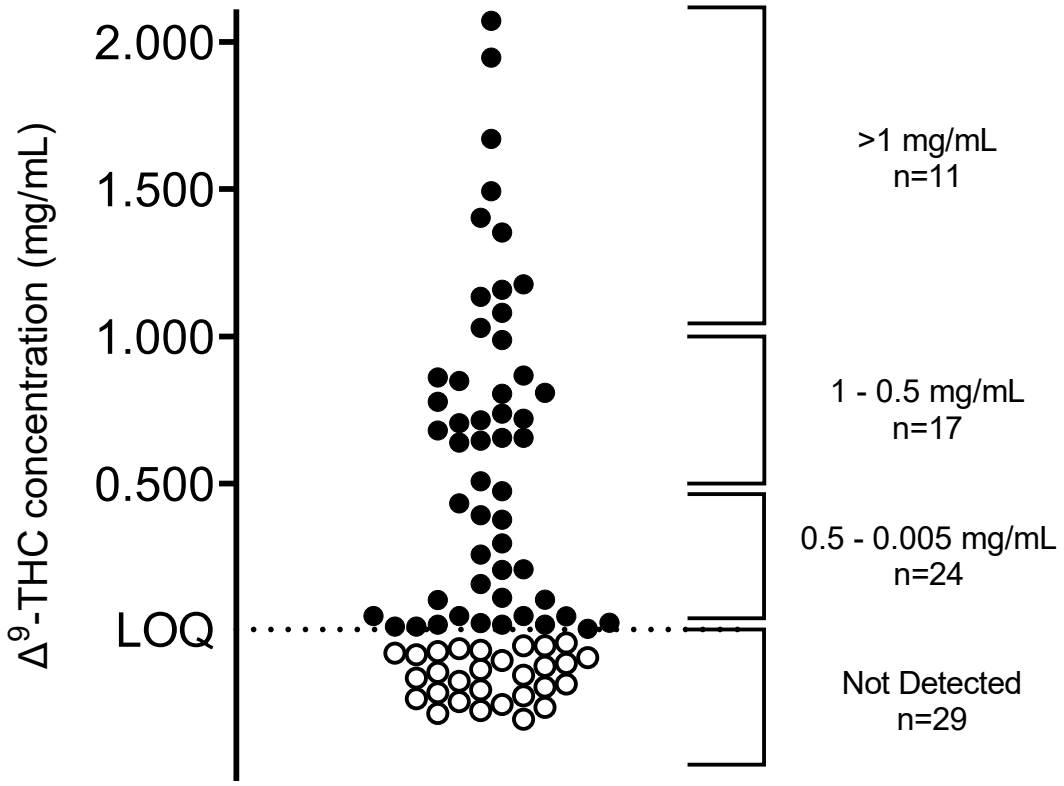


Figure 4-1 Δ^9-THC concentrations in 80 commercially available hemp-derived oil products and Epidiolex®
Visual representation of the of Δ^9-THC concentrations observed in the products tested grouped by concentration range.

The Δ^9-THC content was determined from 81 products, including Epidiolex®. Data are reported as the mean of 9 sample measurements from 3 extractions from the source product, each divided into 3 separate samples for individual analysis, ± standard error of the mean (SEM). Of the 80 unregulated products analyzed in this study, Δ^9-THC was not detected in 29 products (36%). As shown in Figure 4-1, the Δ^9-THC concentration for the 51 products (64% of the unregulated products sampled) ranged from 0.008 mg/mL to 2.071 mg/mL. The mean concentration across these products was 0.620 mg/mL, and the median was 0.640 mg/mL. Epidiolex®, included as a regulated control for comparison, contained a Δ^9-THC concentration of 0.022 mg/mL (± 0.001). The mean Δ^9-THC concentration (± SEM) for each product is reported in Tables 4-2 – 4.4. Table 4-2 lists the Δ^9-THC concentrations for the 11 products with a result greater than 1 mg/mL, while the results for the 17 products with a Δ^9-THC concentration ranging from 1 mg/mL to 0.5 mg/mL are listed in Table 4-3. Table 4-4 details the results for the 24 products with a Δ^9-THC concentration ranging from 0.5 mg/mL to 0.005 mg/mL. Of the products tested, 21 were "THC Free" based on the product label, and of these, 5 (24%) contained detectable levels of Δ^9-THC (0.015 to 0.656 mg/mL) and are noted in Tables 4-3 and 4-4.

Table 4-2 List of samples with a Δ^9-THC concentration greater than 1 mg/mL.

Sample ID	Label Claim mg CBD / mL	Δ^9-THC concentration mg / mL (±SEM)
34	25.9	2.071 (± 0.041)
60	33.0	1.946 (± 0.029)
82	60.0	1.671 (± 0.032)
32	50.0	1.492 (± 0.015)
43	25.0	1.403 (± 0.007)
9	50.0	1.353 (± 0.011)
44	50.0	1.176 (± 0.010)
4	50.0	1.158 (± 0.007)
33	10.3	1.134 (± 0.007)
28	41.7	1.080 (± 0.008)
47	25.0	1.029 (± 0.009)

Sample ID: sample identification number; *Label Claim*: Concentration of CBD in the product according to the label in mg/mL; *Δ^9-THC concentration*: Mean observed concentration of Δ^9-THC detected in each product in mg/mL ± SEM

Table 4-3. List of samples with a Δ^9-THC concentration between 1 mg/mL and 0.5 mg/mL.

Sample ID	Label Claim mg CBD / mL	Δ^9-THC concentration mg / mL (±SEM)
29	34.5	0.987 (± 0.011)
35	25.0	0.867 (± 0.004)
2	17.0	0.861 (± 0.008)
22	16.7	0.849 (± 0.009)
24	50.0	0.809 (± 0.012)
58	30.0	0.806 (± 0.007)
3	17.0	0.778 (± 0.005)
56	16.1	0.738 (± 0.012)
53	16.7	0.720 (± 0.007)
21	25.0	0.715 (± 0.004)
16	16.6	0.706 (± 0.006)
1	17.0	0.680 (± 0.003)
51*	16.7	0.656 (± 0.006)
31	25.0	0.655 (± 0.007)
23	16.7	0.646 (± 0.010)
20	16.7	0.640 (± 0.007)
40	10.0	0.508 (± 0.005)

Sample ID: sample identification number; *Label Claim*: Concentration of CBD in the product according to the label in mg/mL; *Δ^9-THC concentration*: Mean observed concentration of Δ^9-THC detected in each product in mg/mL ± SEM

Table 4-4 List of samples with a Δ^9-THC concentration between 0.5 mg/mL and 0.005 mg/mL

Sample ID	Label Claim mg CBD / mL	Δ^9-THC concentration mg / mL (±SEM)
57	16.7	0.474 (± 0.003)
19	16.8	0.433 (± 0.004)
38	10.0	0.393 (± 0.004)
39	10.0	0.378 (± 0.004)
49	8.3	0.297 (± 0.003)
11	8.4	0.259 (± 0.003)
48	17.9	0.210 (± 0.002)
13	16.7	0.207 (± 0.002)
36	8.3	0.159 (± 0.001)
55	16.7	0.113 (± 0.003)
37	8.3	0.106 (± <0.001)
30	33.3	0.105 (± 0.003)
5	33.3	0.051 (± 0.001)
7	33.3	0.051 (± <0.001)
17*	16.6	0.051 (± <0.001)
64*	33.3	0.050 (± 0.002)
74	10.0	0.028 (± <0.001)
54*	16.7	0.027 (± <0.001)
83	100.0	0.022 (± 0.001)
27	33.3	0.022 (± <0.001)
67	16.7	0.022 (± 0.001)
6	16.7	0.016 (± <0.001)
12*	40.0	0.015 (± <0.001)
52	16.7	0.008 (± <0.001)

Sample ID: sample identification number; *Label Claim*: Concentration of CBD in the product according to the label in mg/mL; *Δ^9-THC concentration*: Mean observed concentration of Δ^9-THC detected in each product in mg/mL ± SEM

4.5 Discussion

The lack of clear regulations for hemp-derived products leaves consumers at risk for unintentional Δ^9-THC exposure. The present study of 80 hemp-derived CBD oil products represents a cross section of nationally distributed brands as well as brands reported to be local to Kentucky. Epidiolex® was included as a regulated control to allow for a comparison of quality standards between an FDA-approved drug to unregulated products. These data clearly demonstrate that with the lack of transparent and accurate label information stating a specific amount of Δ^9-THC in the product by volume consumers have no choice but to suspect the presence of Δ^9-THC in hemp-derived CBD products.

The results of the current study align with several other recent studies which have reported that many commercially available CBD products readily available and sold over the counter contain Δ^9-THC. [41-43, 60] Due to the possibility of intoxication or impairment, especially among children, it is important for the consumer to understand the possibility that CBD products contain Δ^9-THC. [41] Here we report that 52 products contained detectable concentrations of Δ^9-THC; 11 products had concentrations $\geq$ 1 mg/mL and one product contained $\geq$ 2 mg/mL. The regulated control, Epidiolex®, contained 0.022 mg/mL of Δ^9-THC. For comparison to the results reported here, the starting dose for dronabinol, synthetic Δ^9-THC, is 2.5 mg given orally twice daily, [35] and the National Institute on Drug Abuse (NIDA) has established a 5 mg dose of inhaled Δ^9-THC as the standard unit for research. [75] Although these doses of Δ^9-THC do not approximate those that would be expected after cannabis use (inhaling 0.25g of 20% Δ9-THC cannabis plant material $\approx$ total dose of 50 mg Δ^9-THC), there are still possible consequences of consuming these doses of Δ^9-THC, particularly since many consumers of

CBD products take CBD doses daily or multiple times per day. [44] For example, Herbst and Musgrave reported a case study of a 9-year-old child with refractory epilepsy presenting symptoms of an accidental overdose after consuming a CBD oil product that unknowingly contained Δ^9-THC. After evaluation in the emergency department, the child was admitted to the pediatric intensive care unit. The child's toxicology report indicated a urine 9-carboxy-11-nor- Δ^9-THC, the THC-COOH and inactive Δ^9-THC metabolite, concentration of 123 ng/mL. [76] By comparison, the THC-COOH urine threshold for a positive workplace drug test is 15 ng/mL. In general, Δ^9-THC can produce more serious consequences in the pediatric population than has been seen in adults, [77] suggesting that extreme caution is warranted when administering unregulated CBD products to children.

In addition to safety concerns, consumers of CBD products must also consider the potential impact of unintentional Δ^9-THC consumption on drug-testing outcomes such as workplace testing, limits for driving, criminal justice system testing, and sport doping. For workplace drug-testing programs in the United States, the urinary thresholds for THC-COOH (inactive metabolite) are 50 ng/mL for immunoassay tests and often 15 ng/mL for confirmatory by gas chromatography-mass spectrometry (GC-MS). [78] The World Anti-Doping Agency (WADA) and United States Anti-Doping Agency (USADA) have a threshold for THC-COOH of 150 ng/mL. [79] In addition, there is no widely accepted daily dose or total amount of Δ^9-THC that an individual can use to stay below these limits and prevent a positive drug test. However, some studies have suggested that positive drug tests can occur with doses of less than 0.4 mg of Δ^9-THC per day. [80-83] A joint report from Centre for Medical Cannabis (CMC), Association of the Cannabinoid Industry (ACI), and Conservative Drug Policy Reform Group (CDPRG) recommends a Δ^9-THC

safety limit of 0.021 milligrams per day. [84] In the current study, 30 products (37% of the samples tested) would exceed the 0.4 mg limit and 49 would exceed the 0.021 mg limit, including Epidiolex® with a 1 milliliter daily dose. Recent studies have shown positive findings for THC-COOH after multi-day administration of CBD oil products. [85, 86] As Δ^9-THC is highly lipophilic, its bioaccumulation in tissues such as brain, lung, heart, adipocytes, and liver results in its slow release from body stores. [87] A daily dose, and especially multiple doses per day, of a hemp-derived CBD product containing Δ^9-THC could lead to significant accumulation. Of the 81 hemp-derived CBD products included in this study, 52 contained a detectable amount of Δ^9-THC.

In an attempt to mitigate their risks, some consumers are seeking to attain the purported benefits of hemp-derived CBD products while avoiding the risks associated with Δ^9-THC consumption by looking for products labeled as "THC Free" and may assume that these products are safe. Here we report that 5 out of 21 (24%) of the products labeled as "THC Free" contained Δ^9-THC ranging from 0.015 mg/mL to 0.656 mg/mL. Contamination of Δ9-THC in products marketed as "THC Free" has resulted in workplace drug-testing positive findings. [88, 89] In one case, a truck driver of hazardous materials suffered a career-ending workplace drug testing violation for Δ^9-THC after consuming a CBD product listed as THC Free. [89] The FDA does allow "free from" claims for food products containing trace amount sodium, fat, and sugar, but a specified threshold of Δ^9-THC has not been set for hemp-derived CBD products. [90] The inadequacy of labelling information clearly poses a risk to the consumer of unintended or overconsumption of Δ^9-THC. [90]

Active-duty military and veterans are particularly vulnerable to consequences from contaminated products due to strict drug-testing rules. Due to the purported benefits of CBD for conditions including pain, anxiety, and post-traumatic stress disorder, hemp-derived CBD products have been heavily marketed to this group. However, the Department of Defense (DoD) has explicitly prohibited service members and civilian employees from using hemp-derived products other than a medical prescription for an FDA-approved drug based on the potential for Δ^9-THC contamination. [91] The DoD has issued guidance of a complete prohibition, regardless of stated or actual Δ9-THC content, to mitigate the risk of service members being charged for Δ^9-THC violations stemming from consumption of hemp-derived products. [92]

Similarly, athletes have increasingly turned to CBD products for their purported benefits, and in 2018, WADA removed CBD from its list of banned substances to permit its use. However, athletes are subject to anti-doping testing, and the presence of Δ^9-THC has led to suspensions and bans. [93, 94] Since 2018, there have been 60 CBD-related doping infringements reported, mostly involving Δ^9-THC. [95] This incudes Devin Logan, a US Ski and Snowboard athlete, who received a 3-month suspension after an adverse drug finding for Δ9-THC stemming from CBD product use. [96] Despite these risks, a study by Kasper et al. showed that male professional rugby players are turning to CBD products for pain management as an alternative to drugs such as opiates. [93] For similar reasons, the National Football League (NFL) has expressed interest in research on CBD and cannabinoids in pain management as an alternative to opiates. [97] Despite the potential Δ^9-THC contamination, athletes are using CBD products with the assumption that they

have pharmacological benefits for injury and recovery, and likely do not suspect Δ^9-THC contamination.

4.6 Conclusions

Hemp-derived products are increasingly available through online and local retailers. This study reports the quantification of Δ^9-THC in 80 of these unregulated hemp-derived products as well as Epidiolex®, the highly purified CBD product approved by the FDA. A wide range of consumers are taking unregulated hemp-derived products without a clear understanding of the risks of unintended consumption of Δ^9-THC. Here we report that many products labeled as being free of Δ^9-THC are not. Considering that the majority of hemp-derived CBD products contain some amount of Δ^9-THC, the unintended consumption of Δ^9-THC carries a range of risks, including adverse health effects, legal implications including child custody cases, driving while intoxicated laws, and risk to livelihood and military status. Carefully controlled research studies are needed on the specific Δ^9-THC dose threshold to prevent workplace and sports positive blood and urine drug tests. The results of these studies indicate an urgent need to require accurate quantification and labelling of these products and to clarify the limits of Δ^9-THC in marketed products to better ensure the safety of consumers.

CHAPTER 5. MINOR CANNABINOIDS

5.1 Abstract

Background: Though the majority of cannabinoid research has focused on Δ^9-THC and CBD, scientists have increasing expanded their exploration of the therapeutic potential of other phytocannabinoid compounds found in *Cannabis sativa*. Some of the research areas have included pain and inflammation, cancer, emesis, epilepsy, as well as antibacterial and anti-viral agents.

Methods: A cross section of local and national brands of 80 hemp-derived oil products was purchased from local retailers in central Kentucky and online. Epidiolex® was included as a regulated control. These samples were extracted by solvent extraction and quantified by liquid-chromatography tandem mass-spectrometry (LC-MS/MS). The targeted cannabinoids were cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabigerol (CBG), cannabichromenic acid (CBCA), cannabichromene (CBC), cannabinolic acid (CBNA), cannabinol (CBN), cannabidivarinic acid (CBDVA), cannabidivarin (CBDV), Δ^9-tetrahydrocannabivarinic acid (THCVA), Δ^9-tetrahydrocannabivarin (THCV), cannabicyclolic acid (CBLA), cannabicyclol (CBL), Δ^9-tetrahydrocannabinolic acid-A (THCA-A), and Δ^8-tetrahydrocannabinol (Δ^8-THC).

Results: Δ^8-THC was not detected in any of the products tested. Among the unregulated products included in this study, the most frequently detected cannabinoids, excluding CBD and Δ^9-THC, were CBDV (100% of samples tested), CBG (77%), CBC (72%), CBN (67%), CBL (67%), and CBDA (51%). Concentrations of these cannabinoids varied widely from trace concentrations to several mg/mL (e.g., CBDA: 0.006 – 12.258 mg/mL).

Conclusions:

Since several of these phytocannabinoid compounds have been shown to have biological activity, minor cannabinoids may be contributing to the purported therapeutic efficacy of hemp-derived products.

5.2 Introduction

The *Cannabis sativa* plant has been used for millennia for medicinal and recreational purposes. Of the over 100 compounds that have been identified in the plant, biological activity has been determined for only a small fraction of these compounds. The majority of research has focused on cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC). CBD, the main non-psychoactive component, has been approved by the FDA as an anticonvulsant. [37, 38] The presence of Δ^9-THC, the primary psychoactive component in Cannabis sativa, was a key reason for the Schedule I status for cannabis on the Drug Enforcement Administration (DEA) list of controlled substances, prohibiting research into any possible or potential medicinal benefits. [15] The Δ^9-THC concentration constitutes the legal distinction between hemp and marijuana though both are varieties of *Cannabis sativa*. The 2018 Farm Bill federally legalized products derived from hemp and removed these products from the DEA list of controlled substances. [18] Since the legalization of hemp and hemp-derived products, the industry has grown rapidly. The labeling of these products focuses on CBD and Δ^9-THC with little or no mention of the cannabinoid compounds that occur naturally in the plant, though at lower levels, and that are often referred to as "minor cannabinoids." Scientists are still identifying the large group of minor cannabinoids, because research has shown potential for these compounds to exhibit medicinal benefits.

5.2.1 Pain and Inflammation

Preclinical studies have indicated the potential for antinociceptive effects of CBD and Δ^9-THC which has led researchers to explore the potential of minor cannabinoids to control or mediate pain and inflammation in various models. One such study found that

cannabidiolic acid (CBDA) interferes with the development of inflammation. [98] Zagzoog *et al.* found an increase in tail flick latency after intraperitoneal injection with Δ^9-tetrahydrocannabinolic acid-A (THCA-A) as well as with Δ^9-tetrahydrocannabivarin (THCV). [99] THCV has been shown to reduce oedema and increase withdrawal latency to thermal hyperalgesia after carrageenan injection as well as to decrease pain behavior in a formalin-induced nociception model [100] In a model of corneal injury, Δ^8-tetrahydrocannabinol (Δ^8-THC) was shown to reduce pain scores and inflammation, determined by neutrophil infiltration, after topical administration. [101] Cannabinol (CBN) was reported to increase the reaction time to a thermal stimulus in a hot plate model demonstrating acute antinociception. [102] In models examining visceral pain, CBN produced significant antinociceptive effects. [103-106] Formukong *et al*. reported that cannabigerol (CBG) was an effective nociceptive in visceral pain and showed anti-inflammatory properties in a model of erythema. [105] CBG and cannabichromene (CBC) have been shown to increase tail flick latency in a model of thermal pain. [99, 107-109] In models of inflammatory bowel disease, CBG was reported to be effective as a preventative as well as a therapeutical treatment in the reduction of inflammation, while CBC was shown to reduce inflammation-induced hypermotility in the intestine. [110, 111] Research indicates that a variety of phytocannabinoids may provide potential treatment for numerous types of pain and inflammation, but more research is needed to better understand the mechanism of action for these compounds and to determine if human trials are consistent with the preclinical findings.

5.2.2 Cancer

The anti-inflammatory effects of cannabinoids have led scientists to explore the potential of these compounds as anti-cancer agents. Takeda *et al.* reported that CBDA is a selective inhibitor of COX2 as well as a suppressor of genes involved in metastasis of cancer cells. [112, 113] In a separate study, it was reported that THCA-A, CBDA, CBG, and cannabigerolic acid (CBGA) inhibited the COX-1 enzyme while THCA-A, CBG, and CBGA inhibited the COX-2 enzyme but not CBDA. [114] The source of the enzymes and detection methods differed among these studies, indicating that further research is warranted.

5.2.3 Emesis

Marinol® was approved by the U.S. Food and Drug Administration (FDA) for nausea associated with cancer chemotherapy but comes with the psychoactive side effects that would be expected with a synthetic form of Δ^9-THC. [35] Scientists have investigated the potential for CBDA and THCA-A, cannabinoids without psychoactive side effects, to inhibit vomiting and nausea in rodent models for anticipatory nausea, toxin-induced vomiting, and motion-induced vomiting. The results indicate that CBDA and THCA-A show promise as a treatment for these conditions. [115-118] Additionally, Rock *et al.* found that CBDV and THCV may produce anti-nausea effects in a toxin-induced nausea model. [119] The minor cannabinoids may provide non-psychoactive alternatives in the treatment of nausea associated with cancer chemotherapy.

5.2.4 Epilepsy

In 2018, Epidiolex®, a highly purified CBD oil from *Cannabis sativa*, was approved by the FDA for the treatment of two forms of refractory childhood epilepsy. [37] Research indicates the potential for anticonvulsant activity from other cannabinoids. Of similar structure to CBD, CBDV has been the subject of several pre-clinical studies for various models with mixed results, suggesting potential for therapeutic value. [120, 121] Anderson *et al.* reported that CBDV, CBDA, CBDVA, CBGA, CBC, and CBCA displayed anticonvulsant properties against hyperthermia-induced seizures. [122-124] Though not as potent as CBD and Δ^9-THC, CBN has been shown to possess anticonvulsant activity in an electroshock mouse model. [125] Hill *et al*. showed that THCV reduced seizure incidence in a pentylenetetrazole-induced seizure model [126] These studies show that the minor cannabinoids have potential therapeutic value in various models of epilepsy.

5.2.5 Antibacterial

With the emergence of microbial pathogens that are resistant to current drugs, researchers have looked to explore the antimicrobial properties of cannabinoids. Early studies found that Δ^9-THC and CBD showed activity against gram-positive bacteria though effectiveness was greatly reduced by the presence of serum, suggesting a reduction in the bioavailability of these compounds by unknown serum components. [127] These early studies opened the door for scientists to explore the antibacterial activity of other cannabinoids. Appendino *et al.* reported potent activity against six drug-resistant strains of Staphylococcus aureus, including a variety of Methicillin-resistant forms (MRSA), by CBDA, CBC, CBGA, CBG, THCA-A, and CBN. [128] Additionally, Farha *et al.* reported potent activity of CBG, CBN, cannabichromenic acid (CBCA), and Δ^8-THC against

MRSA USA300 and biofilm formation. [129] Another study reported that the bactericidal activity of CBCA was more rapid than vancomycin against Methicillin-resistant *Staphylococcus aureus* (MRSA). [130] These studies highlight the potential for the minor cannabinoids as a source of antibacterial drug leads for drug-resistant pathogens.

With the reported antibacterial activity of cannabinoids, scientists have conducted research into the potential implications of cannabinoids in oral hygiene. In one study, dental plaque was collected from adults and applied to a petri dish pretreated with CBC, CBN, CBG, and CBGA as well to 3 different brands of toothpaste. The average bacterial colony count for each group was lower for the cannabinoid treatments compared to the toothpaste treatments. [131] The same researchers also conducted a study comparing cannabinoid containing mouthwash to over-the-counter products with chlorhexidine digluconate 0.2% (CHX 0.2%) as a control. In this study, the mouthwash containing CBG inhibited bacterial growth from dental plaque similar to CHX 0.2%. [132] With the inactivation shown by van Kilgeren in the presence of serum, a topical application such as in oral hygiene may show promise for the antibacterial properties of cannabinoids.

5.2.6 SARS-CoV-2

Natural products, such as phytocannabinoids, have been shown to be a successful source of drugs and drug leads. In a recent study, CBDA and cannabigerolic acid (CBGA) were reported to bind to the spike protein of SARS-CoV-2 of two variants, thus blocking cell entry. [34] Though promising, much more work will need to be conducted to determine if these *in vitro* findings could potentially translate to *in vivo* antiviral therapies.

5.2.7 Current Study

Although some of the minor cannabinoids have been identified and shown to have therapeutic potential for many different indications, the quantification of the various compounds has not been reported, limiting any potential conclusion as to their relative contributions. With the rapid growth of the hemp industry, the preclinical studies are being cited to consumers as definitive proof of the therapeutic benefits of hemp-derived products are misleading and potentially dangerous. More research and controlled clinical trials are needed to understand the efficacy and mechanisms of action of these compounds in humans. The current study aims to provide a critical step in the scientific process by providing methods to quantify the phytocannabinoid content in a cross section of unregulated hemp-derived oil products including Epidiolex®. Quantifying the contents of hemp-derived products will help scientists begin to determine the relative contributions and interactions of individual components found in these products for further research. While previous studies reported on only a limited number of cannabinoids, the present study details the concentrations of 15 minor cannabinoids. [41-43, 59-61] The study includes tandem liquid chromatography mass spectrometry (LC-MS/MS) determinations of CBDVA, CBDV, THCV, CBDA, THCVA, CBGA, CBG, CBN, CBNA, Δ8-THC, CBL, THCA-A, CBLA, CBC, and CBCA.

5.3 Materials and Methods

5.3.1 Sample Selection

Since hemp is the only recognized legal form of *Cannabis sativa* in Kentucky, the study described here contains only hemp-derived products. The goal in the selection of hemp-derived oil products was to acquire products from local and national brands readily

available to consumers in central Kentucky. A total of 80 unregulated hemp-derived oil products were purchased between April 2 and May 9, 2021. Epidiolex® (the FDA-approved CBD product) was also obtained (UK Investigational Drug Service Pharmacy) to serve as regulated control.

Upon purchase, each product was randomly assigned a sample identifier to anonymize product identification during the course of the study. Products were stored according to packaging instructions or in a cool, dry space if no instructions were provided. All products were tested immediately after opening.

To focus these studies and develop the methodology, the presented study is the analysis of hemp-derived oil products to the exclusion of other product types such as gummies, topicals, and vapes. At the time of purchase, oils were the most prevalent option available.

5.3.2 Reagents and Standards

Reference materials were purchased as certified reference materials from suppliers with ISO17025 and ISO17034 accreditation. Different sources were used for the preparation of calibrator samples and quality control samples. For the preparation of calibrator samples, CBDV, THCV, CBG, CBN, -Δ8-THC, CBL, CBC, CBDVA, CBDA, THCVA, CBGA, THCA-A, and CBCA were purchased from Cayman Chemical (Ann Arbor, MI, USA) while CBNA and CBLA were obtained from Cerilliant Corporation (Round Rock, TX, USA). For the preparation of quality control samples, CBDV, THCV, CBG, CBN, Δ8-THC, CBL, CBC, CBDVA, CBDA, THCVA, CBGA, THCA-A, CBLA, and CBCA were purchased from Dr Ehrenstorfer (LGC Standards, Manchester, NH, USA) while CBNA was obtained from Cerilliant Corporation. Cannabidiol-d_9 (CBD-d_9),

cannabigerol-d_9 (CBG-d_9), Δ^9-tetrahydrocannabinol-d_9 (Δ^9-THC-d_9), and cannabichromene-d_9 (CBC-d_9) were sourced from Cayman Chemical. 11-nor-9-carboxy-tetrahydrocannabinol-d_3 (THC-COOH-d_3) and Δ^9-tetrahydrocannabinolic acid-d_3 (THCA-A-d_3) were purchased from Cerilliant Corporation. Reagents and solvents (LC/MS grade) for use during the extraction and analysis were purchased from Fisher Scientific (Hampton, NH, USA). Extra virgin olive oil (EVOO), which was used as an analyte-free matrix, was obtained from a local grocery retailer (Kroger, Cincinnati, OH, USA).

5.3.3 Method Performance

For the present study, each batch was self-validated using quality control samples spiked with each analyte in surrogate matrix, EVOO. Prior to commencement of the study, the recovery of each analyte was determined by comparison of pre-extraction spiked samples and post-extraction spiked samples. In the same experiment, matrix effect was determined by comparison of post-extraction spiked samples to neat samples, containing no matrix. Each batch included a calibration curve consisting of 8 non-zero calibrators ranging from 0.005 to 1.500 mg/mL and 6 quality control sample replicates prepared at each of 4 different concentrations (0.010, 0.020, 0.600, and 1.200 mg/mL) for a total of 24 samples across the calibration range. The use of different sources for the preparation of calibrator and quality control samples helps to ensure quantitative accuracy by acting as a cross check of each other.

5.3.4 Sample Preparation

Prior to analysis, all sample containers were inverted multiple times to ensure contents were thoroughly mixed. Three sampling sub-aliquots of products were taken and transferred to appropriately labeled containers where internal standard was added at a

concentration of 0.020 mg/mL. After mixing, a fixed volume of acetonitrile was added and the samples were further mixed, then centrifuged (1811 x g, 20 mins). A 50 µl sub-portion, 3 replicates, of the supernatant was transferred to an autosampler vial and diluted with solvent and water to form a sample within an appropriate concentration range and composition (nominally 50:50 acetonitrile:water, v:v) for analysis. The samples were capped and briefly vortex mixed prior to analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS). Samples with analyte concentrations above the calibration range were re-analyzed with dilution in solvent (10-fold) prior to addition of internal standard.

5.3.5 Instrumentation

Analysis of samples was carried out via LC-MS/MS using a Thermo Accela 1250 quaternary LC system coupled with a TSQ Vantage mass spectrometer (Waltham, MA, USA). Separations were carried out using a reversed phase (C8) Kinetex® analytical column (2.1 x 100 mm, 2.6 µm) purchased from Phenomenex (Torrance, CA, USA). A gradient solvent program was employed using mobile phases of 0.1% formic acid in water (A) and in acetonitrile (B). Briefly, from a starting composition of 50% B, the percentage of organic mobile phase (i.e., B) was increased over 10 minutes, then an organic flush employed to remove residual matrix components before returning to the solvent starting composition. The solvent flow rate was 500 µL/min, and the total analytical run time was 14.25 min. Through the use of reference material, the method was demonstrated to separate all analytes through a combination of chromatography and mass.

The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in positive ionization and negative ionization modes using selective reaction

monitoring (SRM). Monitored transitions for analytes with positive ionization were previously shown in Table 2-4 and with negative ionization were previously shown in Table 2-5. The monitored transitions for the internal standards were previously listed in Table 2-6.

5.4 Results

5.4.1 Method Performance

The method performance was evaluated to determine the suitability of the method for the quantification of the minor cannabinoids. Recovery ranged from 87% to 100% for the target analytes while matrix effects ranged from minor ion suppression of -1% to minor ion enhancement of +3%. For the calibration range, 0.005 to 1.500 mg/mL, a linear or quadratic regression model was used with $1/x$ or $1/x^2$ weighting as appropriate. The coefficient of determination (R^2) was greater than 0.98 for all analytes in all batches. The details of the method performance are shown in Table 2-8.

5.4.2 Minor Cannabinoid Determination in Products

The content of 15 minor cannabinoids was determined in 81 hemp-derived oil products including Epidiolex®. Δ^8-THC was not detected in any of the products tested, and CBDV was detected in every product tested. Table 5-1 shows the range of cannabinoid content and frequency that products contained a specific cannabinoid for the products analyzed in the study. For each cannabinoid, the data is reported as the mean of 9 sample measurements from 3 extractions of the source product, each divided into 3 separate samples for individual analysis. The concentration data ± standard error of the mean (SEM) for each cannabinoid present is shown in Tables 5-2 through 5-15 by analyte.

Table 5-1 Range of concentrations observed for each minor cannabinoid and frequency of occurrence

Cannabinoid	Range (mg/mL)		% Products containing cannabinoid
CBDVA	0.007	0.129	7%
CBDV	0.019	0.809	100%
THCV	0.005	0.042	31%
CBDA	0.006	12.258	51%
THCVA	0.005	0.005	1%
CBGA	0.005	0.346	11%
CBG	0.006	2.233	77%
CBN	0.007	1.190	67%
CBNA	0.006	0.006	1%
CBL	0.006	0.322	67%
THCA-A	0.048	0.135	4%
CBC	0.006	3.330	72%
CBLA	0.019	0.019	1%
CBCA	0.005	0.531	16%

Cannabinoid: minor cannabinoid; *Range*: range of observed concentrations in mg/mL; *% Products containing cannabinoid*: frequency of cannabinoid occurrence across the study products

Table 5-2Observed CBDVA concentrations in mg/mL ± SEM

Sample ID	Concentration. (mg/mL)	± SEM (mg/mL)
13	0.019	<0.001
28	0.129	0.001
29	0.008	<0.001
33	0.007	<0.001
34	0.010	<0.001
48	0.028	<0.001

Sample ID: sample identification number; *Concentration*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-3 Observed CBDV concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.088	0.002	30	0.331	0.003	57	0.088	0.001
2	0.098	0.001	31	0.207	0.002	58	0.227	0.001
3	0.079	0.001	32	0.512	0.004	59	0.035	<0.001
4	0.234	0.001	33	0.152	0.001	60	0.218	0.003
5	0.120	0.002	34	0.195	0.002	61	0.196	0.003
6	0.074	0.001	35	0.149	0.001	62	0.106	0.001
7	0.119	0.001	36	0.091	0.001	63	0.216	0.004
8	0.050	<0.001	37	0.081	0.001	64	0.230	0.003
9	0.193	0.002	38	0.032	<0.001	65	0.126	0.002
10	0.184	0.002	39	0.032	<0.001	66	0.032	<0.001
11	0.030	<0.001	40	0.078	0.001	67	0.091	0.001
12	0.401	0.007	41	0.061	0.001	68	0.122	0.001
13	0.027	0.001	42	0.090	0.001	69	0.128	0.001
16	0.069	0.001	43	0.205	0.001	70	0.127	0.001
17	0.088	0.001	44	0.216	0.002	71	0.133	0.001
18	0.063	0.001	45	0.044	<0.001	72	0.128	0.001
19	0.136	0.001	46	0.079	0.001	73	0.112	0.002
20	0.100	0.001	47	0.153	0.002	74	0.026	<0.001
21	0.196	0.001	48	0.029	<0.001	75	0.019	<0.001
22	0.110	0.001	49	0.031	<0.001	76	0.102	0.001
23	0.098	0.002	50	0.072	<0.001	77	0.199	0.004
24	0.159	0.002	51	0.240	0.002	78	0.032	0.001
25	0.158	0.003	52	0.096	0.001	79	0.073	0.001
26	0.052	0.001	53	0.096	0.001	80	0.038	0.002
27	0.101	0.001	54	0.038	<0.001	81	0.058	0.001
28	0.207	0.002	55	0.192	0.004	82	0.809	0.006
29	0.206	0.002	56	0.094	0.001	83	0.354	0.003

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-4 Observed THCV concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
2	0.005	<0.001	33	0.023	<0.001
4	0.006	<0.001	34	0.029	<0.001
9	0.007	<0.001	35	0.005	<0.001
19	0.006	<0.001	43	0.011	<0.001
21	0.011	<0.001	44	0.007	<0.001
22	0.006	<0.001	47	0.009	<0.001
23	0.006	<0.001	51	0.011	<0.001
24	0.015	<0.001	53	0.008	<0.001
28	0.013	<0.001	56	0.009	<0.001
29	0.008	<0.001	58	0.006	<0.001
30	0.042	0.001	60	0.013	<0.001
31	0.010	<0.001	82	0.032	<0.001
32	0.032	<0.001			

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-5 Observed CBDA concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.535	0.007	36	0.038	<0.001
2	0.717	0.007	37	0.033	<0.001
3	0.277	0.003	38	0.017	<0.001
4	0.011	<0.001	39	0.017	<0.001
9	0.224	0.002	43	0.551	0.004
11	0.078	0.001	44	0.072	<0.001
13	1.879	0.020	48	5.469	0.068
16	0.030	<0.001	52	0.031	<0.001
17	0.006	<0.001	53	0.011	<0.001
20	0.133	0.002	56	0.006	<0.001
21	0.038	<0.001	58	0.420	0.002
22	0.009	<0.001	60	0.080	0.001
23	0.012	<0.001	65	0.048	<0.001
28	12.258	0.154	67	0.009	<0.001
29	0.886	0.008	69	0.050	<0.001
30	0.063	0.001	70	0.047	0.001
31	0.015	<0.001	71	0.051	<0.001
32	0.069	0.001	72	0.042	<0.001
33	0.467	0.006	73	0.016	<0.001
34	1.328	0.011	82	0.177	0.001
35	0.095	0.001			

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-6 Observed THCVA concentration in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
28	0.005	<0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-7 Observed CBGA concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.008	<0.001
2	0.008	<0.001
13	0.054	0.001
28	0.346	0.006
29	0.007	<0.001
33	0.013	<0.001
34	0.030	<0.001
48	0.026	<0.001
60	0.005	<0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-8 Observed CBG concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.379	0.004	38	0.120	0.001
2	0.236	0.001	39	0.118	0.001
3	0.251	0.002	40	0.089	0.001
4	1.366	0.011	43	0.358	0.002
5	0.006	<0.001	44	0.244	0.001
6	0.009	<0.001	46	0.044	<0.001
7	0.006	<0.001	47	0.581	0.005
9	0.499	0.005	48	0.078	0.001
11	0.085	0.001	49	0.183	0.002
12	0.019	<0.001	51	0.260	0.002
13	0.091	0.001	52	0.008	<0.001
16	0.468	0.003	53	0.438	0.003
17	0.290	0.003	55	0.210	0.001
18	0.029	0.000	56	0.176	0.002
19	0.241	0.002	57	0.331	0.003
20	0.459	0.006	58	1.239	0.009
21	0.574	0.003	59	0.418	0.004
22	0.605	0.006	60	0.652	0.003
23	0.238	0.004	61	1.309	0.010
24	0.464	0.007	64	2.233	0.034
27	1.291	0.009	65	1.180	0.008
28	0.568	0.008	67	0.067	0.001
29	0.861	0.005	69	1.193	0.010
30	0.461	0.005	70	1.195	0.010
31	0.393	0.004	71	1.230	0.013
32	0.812	0.006	72	1.196	0.015
33	0.274	0.004	74	0.096	0.001
34	0.582	0.007	76	0.156	0.002
35	0.279	0.002	77	0.151	0.001
36	0.050	0.001	82	1.201	0.010
37	0.036	<0.001	83	0.007	<0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-9 Observed CBN concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.141	0.001	38	0.031	<0.001
2	0.055	0.001	39	0.031	0.001
3	0.050	<0.001	40	0.052	0.001
4	0.237	0.002	43	0.042	0.000
9	0.108	0.001	44	0.133	0.002
11	0.019	<0.001	46	0.089	0.001
13	0.007	<0.001	47	0.029	<0.001
16	0.035	<0.001	48	0.108	0.002
17	0.380	0.004	49	0.016	<0.001
18	0.087	0.001	51	0.042	<0.001
19	0.083	0.001	53	0.060	0.001
20	0.008	<0.001	55	0.215	0.005
21	0.155	0.001	56	0.036	0.001
22	0.009	<0.001	57	0.019	<0.001
23	0.029	<0.001	58	0.045	0.001
24	0.042	0.001	60	0.089	0.001
27	1.190	0.010	64	0.915	0.011
28	0.024	<0.001	65	0.355	0.004
29	0.069	0.001	67	0.356	0.006
30	0.059	0.001	69	0.350	0.003
31	0.020	<0.001	70	0.351	0.003
32	0.191	0.002	71	0.371	0.004
33	0.053	<0.001	72	0.344	0.003
34	0.083	0.001	74	0.260	0.003
35	0.037	<0.001	76	0.497	0.007
36	0.021	<0.001	77	0.403	0.005
37	0.015	<0.001	82	0.242	0.002

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-10 Observed CBNA concentration in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
48	0.006	<0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-11 Observed CBL concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.023	<0.001	39	0.017	<0.001
2	0.032	<0.001	40	0.018	<0.001
3	0.031	<0.001	43	0.045	<0.001
4	0.177	0.002	44	0.122	0.002
5	0.037	0.001	46	0.008	<0.001
7	0.036	<0.001	47	0.083	0.001
9	0.322	0.002	48	0.026	<0.001
11	0.062	0.001	49	0.015	<0.001
16	0.024	<0.001	51	0.097	0.001
17	0.056	0.001	53	0.061	0.001
18	0.017	<0.001	55	0.029	0.001
19	0.031	<0.001	56	0.035	<0.001
20	0.029	<0.001	57	0.031	<0.001
21	0.155	0.001	58	0.062	0.001
22	0.006	<0.001	60	0.085	0.001
23	0.032	0.001	61	0.079	0.001
24	0.057	0.001	64	0.118	0.001
28	0.016	<0.001	65	0.059	0.001
29	0.080	0.001	67	0.049	0.001
31	0.037	0.001	69	0.057	<0.001
32	0.196	0.002	70	0.057	0.001
33	0.023	<0.001	71	0.059	0.001
34	0.073	0.001	72	0.056	<0.001
35	0.051	<0.001	74	0.037	<0.001
36	0.038	<0.001	76	0.060	<0.001
37	0.033	<0.001	77	0.072	0.001
38	0.017	<0.001	82	0.149	0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-12 Observed THCA-A concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
13	0.048	<0.001
28	0.135	0.001
48	0.052	<0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-13 Observed CBC concentrations in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)	Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.793	0.010	38	0.504	0.011
2	0.906	0.016	39	0.510	0.006
3	0.891	0.010	40	0.295	0.005
4	2.639	0.054	43	1.104	0.022
5	0.033	0.001	44	1.958	0.042
7	0.033	0.001	46	0.167	0.003
9	3.330	0.046	47	0.157	0.003
11	0.821	0.011	48	0.376	0.008
13	0.212	0.003	49	0.268	0.004
16	0.619	0.013	51	1.444	0.018
17	0.875	0.016	53	1.037	0.017
18	0.150	0.002	54	0.006	<0.001
19	0.128	0.002	55	0.203	0.002
20	0.884	0.011	56	0.684	0.010
21	0.868	0.011	57	0.647	0.016
22	0.970	0.011	58	0.204	0.005
23	0.454	0.007	60	1.458	0.031
24	0.654	0.007	61	0.117	0.003
27	1.070	0.016	64	1.253	0.015
28	1.008	0.012	65	0.628	0.009
29	1.421	0.027	67	0.702	0.012
30	0.021	<0.001	69	0.620	0.007
31	0.832	0.013	70	0.612	0.012
32	2.170	0.053	71	0.673	0.010
33	0.551	0.010	72	0.637	0.013
34	1.272	0.017	74	0.514	0.006
35	1.617	0.033	76	0.633	0.010
36	0.218	0.004	77	0.653	0.007
37	0.178	0.001	82	1.644	0.038
38	0.504	0.011			

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-14 Observed CBLA concentration in mg/mL ± SEM

Sample ID	Conc. (mg/mL)	± SEM (mg/mL)
48	0.019	0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

Table 5-15 Observed CBCA concentrations in mg/mL ± SEM

aSample ID	Conc. (mg/mL)	± SEM (mg/mL)
1	0.023	<0.001
2	0.029	<0.001
3	0.012	<0.001
9	0.014	<0.001
11	0.005	<0.001
13	0.098	0.001
28	0.531	0.010
29	0.014	<0.001
33	0.005	<0.001
34	0.021	<0.001
43	0.008	<0.001
48	0.178	0.003
57	0.007	<0.001

Sample ID: sample identification number; *Conc.*: mean observed sample concentration; ± *SEM*: standard error of the mean of the observed concentration measurements

5.5 Discussion

Hemp-derived products are increasingly marketed to consumers based on the presence of individual minor cannabinoids such as the emergence of Δ^8-THC products for the "legal high" or CBN-rich products marketed as sleep aids, or CBG-rich products marketed for alertness. Research has shown that these compounds have potential for therapeutic benefits across numerous indications, but more research is needed to understand the mechanisms of action and potential risks. Lacking a clearer understanding of the various cannabinoid compounds present in commercially available products limits our ability to ascribe the therapeutic or toxicological effects of individual components. Therefore, we developed methodology for the extraction and analysis of phytocannabinoids from hemp-derived oil products. The present study determined the cannabinoid content in 80 hemp-derived CBD oil products plus Epidiolex®. Of the 15 cannabinoids included in the study, only Δ^8-THC was not detected in any of the samples. For the other 14 cannabinoids, each compound was found in at least one product. At least one or more cannabinoid was detected in every product tested, including for Epidiolex®. The range of concentrations determined was broad as shown by CBDA found at 0.006 mg/mL to 12.258 mg/mL. Since product labels lack information on these cannabinoids, it is important to understand the content within hemp-derived products and the potential variability in cannabinoid content.

The results presented here indicate that the range of cannabinoids present in a product as well as the amounts of each cannabinoid present vary greatly. Though outside the scope of this project, it would be interesting to attempt to use statistical tools to determine if any correlations exist between the cannabinoids, suggesting common

biosynthetic pathways. Additionally, it would be interesting to determine if there are any correlations in the relative amounts of the cannabinoids present. Such information could be useful in the development of seeds and the determination of growing conditions to enhance development of certain cannabinoids while minimizing or eliminating others. With specific plant genetics and the increase in the utilization of greenhouses for the growth of hemp, the growing conditions associated with specific plant metabolic pathways could be controlled to enhance the production of the desired cannabinoid profiles. From the perspective of the manufacture of hemp-derived products, such statistical correlations could be used to refine the plant extraction process to isolate or remove specific cannabinoids. Through plant genetics, growing conditions, extraction processes, or any combination thereof, it is conceivable that products could be made with consistent cannabinoid profiles, and the methodology developed for the studies presented here could be used to verify the cannabinoid profiles of those products. This evaluation could help researchers understand the mechanisms and further explore the therapeutic potential of the phytocannabinoid compounds, their relative contributions, and their potential unacceptable side effects.

5.6 Conclusions

The results of this study demonstrate that the methods developed through this work is suitable for the identification and quantification of these 15 minor cannabinoids and could be expanded to others should research warrant. The fact that these compounds have been shown to have pharmacological activity demonstrates the need to better understand their presence and relative concentrations in various hemp-derived products that people are taking. These studies fill that need by providing scientists the ability to assess their

presence and their relative concentrations from various hemp-derived products. The presence of specific minor cannabinoids varied from product to product as well as the concentrations of those specific compounds. The methodology developed here will help consumers better understand the contents of the products they are taking. Additionally, determination of the cannabinoid profile in these hemp-derived products will enable researchers to evaluate the mechanisms of action and interactions of the components present in the product mixture. Optimization of seed genetics with growing conditions and manufacturing has the potential to generate products with specified phytocannabinoid profile to maximize therapeutic effect, while minimizing toxicological effect. The methodology presented in this study has the potential to help researchers better understand all aspects of cannabinoid research as the field grows exponentially.

CHAPTER 6. GENERAL DISCUSSION

6.1 Introduction

The purpose of this project was to develop methodology for the extraction and quantitative analysis of phytocannabinoid compounds from oil products derived from low Δ^9-tetrahydrocannabinol (Δ^9-THC) *Cannabis sativa*, commonly referred to as hemp. With the passage of the 2018 Farm Bill, the hemp-derived products industry has grown exponentially, but regulation has not kept pace. Consequently, quality issues have arisen such as products containing no measurable cannabidiol (CBD) despite label claim of CBD content, contamination with synthetic cannabinoids as well as other drugs, and presence of other contaminants including solvents and heavy metals. [41-43, 61]

The purpose of these studies was to develop methodologies to enable the evaluation of the phytocannabinoid contents in hemp-derived oil products. The study samples were readily available products purchased from local (brick and mortar) retailers as well as through online retailers. Local and national brands were represented, and the inclusion of online retailers ensured a representative selection of products produced in a variety of locations outside of Kentucky. In total, 80 hemp-derived oil products were purchased for these studies as well as Epidiolex®, an FDA-approved drug included as a regulated control.

6.1.1 Overview of Study 1: CBD

The first study evaluated the CBD concentrations in the test samples and compared the observed results to the CBD content claimed on the package label. For the products tested, 54% (n=44) were accurately labeled within ± 10% of the label concentration, 31% (n=25) contained CBD concentrations greater than 110% of the claim on the package label,

and 15% (n=12) contained less than 90% of the CBD claimed on the package label. These results expanded and are consistent with the findings of previous studies. [41, 42, 59-61] As consumers are typically taking these products to treat medical conditions and without consultation of a physician, people are subjected to safety risks such as CBD accumulation, elevated liver enzymes, drug-drug interactions, and lack of efficacy due to incorrect dosing. Clearly, regulations as well as good manufacturing practices and testing standards are needed to protect consumers who are increasingly turning to hemp-derived CBD products for a wide and growing range of perceived pharmacological uses.

6.1.2 Overview of Study 2: Δ^9-THC

The second study evaluated the Δ^9-THC concentrations in the test samples. Δ^9-THC was not detected in 36% (n=29) of the products tested. For the samples with detectable concentrations of Δ^9-THC, the levels ranged from 0.008 mg/mL to 2.071 mg/mL with a mean concentration of 0.620 mg/mL and median concentration of 0.640 mg/mL. Epidiolex® contained 0.022 mg/mL of Δ^9-THC. Of the products tested, 21 were labeled as "THC Free," but 24% (n=5) of those products contained detectable levels of Δ^9-THC (range: 0.015 to 0.656 mg/mL). Since the product labels do not directly state the presence of Δ^9-THC, consumers could unintentionally be exposed to an intoxicating drug. Such exposure carries a range of risks including adverse health effects, legal implications including, but not limited to, child custody cases, driving while intoxicated, and risk of failed drug test for employment or sports doping.

6.1.3 Overview of Study 3: Minor cannabinoids

The majority of research has focused on CBD and Δ^9-THC but more recently, a number of scientists have begun investigating the biological activity and therapeutic

potential of many of the other phytocannabinoids present in *Cannabis sativa*. The third study here evaluated the concentrations of 15 of these phytocannabinoid compounds in the test samples including Δ^8-THC, CBDVA, CBDV, THCVA, THCV, CBDA, CBGA, CBG, CBNA, CBN, CBLA, CBL, THCA-A, CBC, and CBCA. Fourteen of these cannabinoid compounds were detected in at least one of the samples tested. Δ^8-THC was the only compound not detected in any of the samples tested. The most frequently detected cannabinoids across the sample set were CBDV (100% of samples tested), CBG (77%), CBC (72%), CBN (67%), CBL (67%), and CBDA (51%). Concentrations of these cannabinoid compounds varied widely, from trace concentrations to several milligrams per milliliter, such as with CBDA concentrations 0.006 – 12.258 mg/mL. The impact of these compounds is yet to be fully understood, but several have been shown to have biological activity.

6.1.4 Limitations

These studies focused on hemp-derived oil products to the exclusion of other product types such as gummies, topicals, and vapes. Analytically, uniformity of matrix is necessary to ensure quantitative accuracy. Furthermore, at the time of purchase, oil products were the most prevalent option.

Based on the error observed in the performance of the quality control samples for THCVA and CBCA, the working standard solutions for the preparation of calibrator and quality control samples were compared to each other. The differences observed between the two solutions explains the error observed in the method performance. This error could have been prevented by comparison of the working standard solutions prior to the commencement of the study.

6.2 Regulatory Implications

The 2018 Farm Bill federally legalized the *Cannabis sativa* plant and subsequent products of the plant with the condition that the Δ^9-THC content is not more than 0.3% per dry weight of plant material from which the material is derived. [18] To calculate the Δ^9-THC in a plot, a small number of plant cuttings are taken, dried, and analyzed. Cuttings from as few as 5 plants for single plot, defined as a continuous field, are intended to determine a representative Δ^9-THC concentration and only Δ^9-THC. [133] The phytocannabinoids found in the *Cannabis sativa* plant, primarily the carboxylic acid form, are metabolic by-products, but the acid form can readily undergo decarboxylation to generate the neutral form. [134] With the same variety of plant grown in a single field, it is possible to have single plants with varying concentrations of phytocannabinoids. [45] Overall, the concentration of these metabolites can vary based on the age, growing conditions, variety, harvest conditions, storage conditions, and the extraction process. [134] The plant material, tested and deemed compliant with the 2018 Farm Bill, is processed removing the plant biomass. This step disconnects the extracted material from the legal framework of the 2018 Farm Bill. The crude extract from one or more crops, seed varieties, various growing conditions is processed and blended with a carrier substance, such as an oil, and various additives, such as flavorings, to create the product sold to consumers. As a result of the limitations of the scope of the 2018 Farm Bill, a product derived from plant material meeting the legality criteria could meet legal requirements regardless of the amount of Δ^9-THC in the final product achieved through concentration in the extraction and processing procedures. Essentially, the most accurate description of the *Cannabis sativa* grown on the farm is labeling it as a raw material.

As with any raw material, it undergoes numerous processes and changes before being available to a consumer. The methodology developed for the studies presented here quantifies the compounds made by the plant and that are present in the final product taken by the end user. To date, the Food and Drug Administration (FDA) has not established a clear path forward for *Cannabis sativa* products legalized by the 2018 Farm Bill despite pressures from various stakeholders. [57] that the issue is complex, in part because the 2018 Farm Bill focuses solely on Δ^9-THC in plant while the main intersection of the Food, Drug and Cosmetic Act (FDCA) with the industry is that CBD (Epidiolex®) is an FDA-approved drug and cannot be marketed as a medication in a non-FDA approved product. [18, 135] A downstream effect of the FDCA is that the FDA has declared that CBD, from *Cannabis sativa* or other sources, cannot be sold as dietary supplements nor can it be added to human or animal food. [40] By extension, the lack of categorization means that these products are not subject to any of the quality controls and regulations that would be applied under the applicable category. The only phytocannabinoids specifically mentioned by the FDA and 2018 Farm Bill are CBD and Δ^9-THC which begs the question as to whether the other cannabinoids shown to be present and to have potential biological activity should be a topic of consideration to regulators. With the explosive growth of this industry, the scientific data has not been available to assist the FDA in making scientifically based regulations. The studies presented here further expand the data available to the FDA, and the developed methodology provides a tool for further studies to grow the knowledge base.

6.3 Sport implications

With the removal of CBD from the WADA-prohibited substance list in 2018, athletes have increasingly sought CBD products for their purported anxiolytic, anti-

inflammatory, and neuroprotective effects. [136, 137] The research and controlled clinical trials are lacking, however, to demonstrate efficacious treatment for these conditions. [93, 136] The potential for cannabinoids to benefit athletes has enticed the National Football League (NFL) to award 1 million dollars to investigate the effects of cannabinoids on pain and recovery from sports-related injuries as well as pain management and neuroprotection from concussion. [138] A driver for athletes and sporting organizations is the search for an alternative to opioids, the current standard of care. [93] The use of cannabinoids to treat exercise- and sport-related injuries shows promise as alternatives to opiates, but more research is needed. In a study of chronic pain, a gel containing CBD, Δ^9-THC, CBDV, CBDA, and CBC was reported to reduce of opioid use in 53% of patients. [139] Preclinical studies of various models of pain and inflammation have shown the potential of cannabinoids such as CBD, CBDA, and THCA-A, which are non-psychoactive, to treat these conditions, but more research is needed to understand the mechanisms of action since cannabinoids have been reported to interact with numerous biological targets in the relevant pathways. [98, 99, 137, 139] The developed methodology presented in this study will be an important tool in assisting researchers to understand the cannabinoid profile of solutions for administration in the pursuit of better understanding the mechanisms of action of cannabinoids for the treatment of various conditions.

Even though WADA removed CBD from its prohibited substances list, Δ^9-THC is regulated through a urinary threshold of 150 ng/mL of the inactive metabolite (THC-COOH), and all other cannabinoids are prohibited at any concentration. Athletes who are subject to doping control are at risk of suspension for an anti-doping rule violation through inadvertent exposure to cannabinoids in hemp-derived products. [96] In one study where

participants consumed a single dose of one of 15 CBD products per label instructions, at least one WADA-prohibited cannabinoid was detected in the urine samples corresponding to 13 of the 15 products administered. [94] A key question for stakeholders in sports is whether cannabinoids are performance enhancing or performance diminishing or neither. With the current rules prohibiting cannabinoids, except CBD, methodology such as developed in the presented project is needed to evaluate the products that athletes are consuming, likely without understanding the risk. Manufacturers and athletes alike would benefit from such an analysis. Many athletes have a relatively short career compared to people in other professions. An anti-doping violation may prevent athletes from competing at their peak due to a suspension or may potentially impact a whole team for a violation determined in competition (e.g., loss of an Olympic medal for a team due to an individual member having a doping violation).

6.4 Labeling confusion

A key issue for concern in the hemp-derived product industry is the lack of clarity and industry standards with regards to labeling. The language used is not clear, and commonly used terms do not have commonly understood meanings. As an informational note, cannabis plant constituents could include cannabinoid compounds, flavonoids, and terpenoids. Product labels will often carry terms such as full spectrum, broad spectrum, or CBD isolate. Within the industry, the term full spectrum generally refers to extract from cannabis that has all the naturally occurring constituents. [140] Since the actual contents of the extract will vary based on the chemovar of the plant, growing conditions, storage conditions, and the extraction method itself, the term does not clearly inform the consumer as to what is in the product. There is less agreement on the meaning of the term broad

spectrum. This term is often applied to extracts where some but not all of the natural constituents of the plant are present with the most commonly removed compound being Δ^9-THC. Broad spectrum does not clearly describe what has been removed and what has been kept in the product. Another term commonly used on hemp-derived product labels is CBD isolate. This term refers to CBD isolated from any kind of cannabis whether high or low Δ^9-THC varieties. Consumers may assume that a product labeled as containing CBD isolate contains only CBD, but that assumption may be incorrect since the purity will depend on the extraction technique and quality controls of the manufacturing process. The terms full spectrum, broad spectrum, and CBD isolate lack a standardization of definition such as those applied by the FDA for terms such as fat free, sugar free, and salt free. [90] Without a standardization of definitions, these terms serve only to mislead and confuse consumers as to what is actually in the product being purchased.

Another aspect of label confusion is the CBD dosing information. Hemp-derived product labels typically lack information on cannabinoids in the product except for CBD. Often products will have a number on the front of the container referencing the total amount of CBD in the product. For example, a bottle labeled as containing 1000 mg of CBD does not inform the consumer as to the amount of CBD per dose. To further complicate the situation, dosing is done with an uncalibrated dropper lacking any volume markings, and dosing descriptions may be labeled as 5 drops. Such vague descriptions and dosing mechanisms do not enable the consumer to administer a controlled known dose of the product. As such descriptions are limited to CBD, no account is taken into consideration for other cannabinoid compounds that may be present in the product. Colorado has taken a step to try to better inform the consumer by requiring labeling of Δ^9-

THC content in total and per dose in hemp-derived products produced in the state. [141] It is a step in the right direction, but more work is needed to protect consumers, especially considering that hemp-derived products are being administered to children as well as adults for treatment of medical conditions.

6.5 Customized products

The history of cannabis is long, but the research of the phytocannabinoid compounds within the plant is in its relative infancy owing in great part to the regulations applied to *Cannabis sativa*. In the early stages of modern cannabis research, the focus was on the interactions of Δ^9-THC with the newly discovered endocannabinoid system. [19] As more phytocannabinoid compounds have been identified in *Cannabis sativa*, the scientific interest has grown in hopes of better understanding the medicinal uses of the plant throughout history and the modern anecdotal accounts of therapeutic benefits. The classical model of pharmaceutical drug development is based on a single active compound synthesized in a laboratory, whereas a natural product derived from a plant is a complex mixture of potentially active compounds interacting with a biological organism and each other. For example, CBD has been shown to behave as an CB1 negative allosteric modulator of Δ^9-THC reducing the psychoactive effects associated with Δ^9-THC. [142] The methodology developed by the studies presented here provides a starting point for researchers to identify and potentially create (through plant genetics, growing conditions, and extraction techniques) cannabis extract mixtures with specific phytocannabinoid profiles.

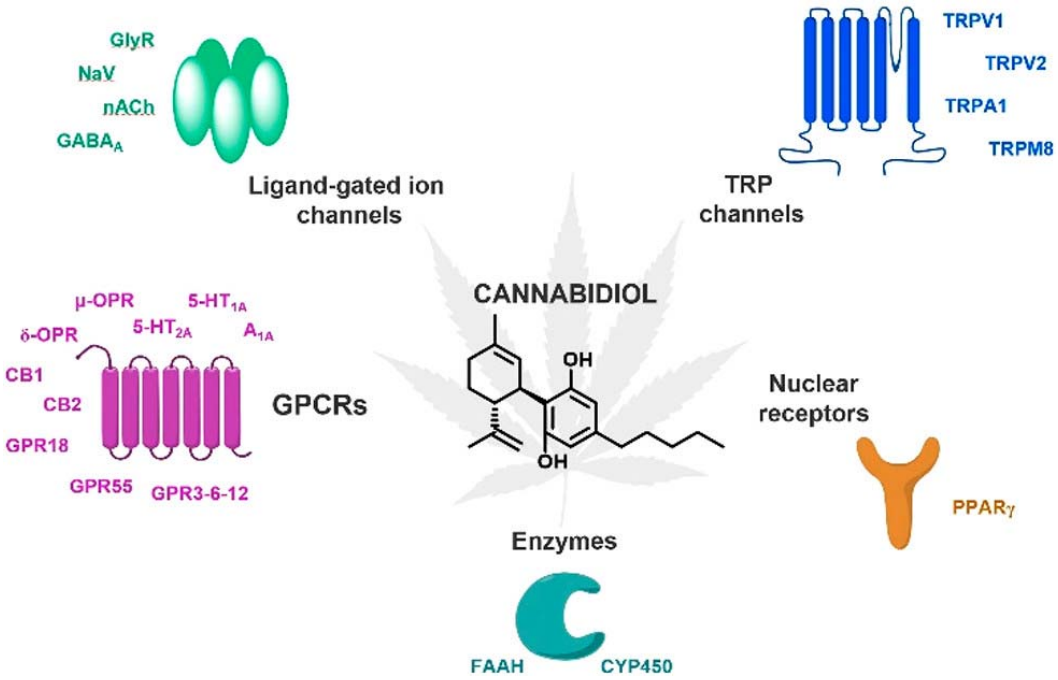


Figure 6-1 Molecular targets of CBD[142]
Visual summary of the receptors that have been reported thus far as targets of CBD.

After Δ^9-THC, CBD has been the most studied phytocannabinoid from *Cannabis sativa*. Numerous studies have indicated potential therapeutic benefit of CBD across an array of indications such as inflammation, analgesia, convulsion, anxiety, epilepsy, neuroprotection, and cancer leading to proposed interactions with several molecular targets shown in Figure 6-1. [142] Since CBD has poor binding to the orthosteric binding pocket of CB1 and CB2, the observed effects must be happening elsewhere, but the mechanism of action is not fully understood. [142] As binding studies indicate that phytocannabinoid compounds do not always bind to the cannabinoid receptors, the impact on the endocannabinoid system must be through a different interaction. [108] As Di Marzo *et al.* reported, phytocannabinoid action in the endocannabinoid system occurs well beyond the cannabinoid receptors. As shown in Figure 6-2, the actions of the phytocannabinoid compounds on the endocannabinoid system may in part be tied to the interactions these compounds have with the endogenous ligands, anandamide and 2-arachidonoylglycerol (2-AG). That fact that these ligands have been shown to bind to other receptors outside of the endocannabinoid system may help explain the broad range of therapeutic effects observed in the pre-clinical studies of cannabinoids. [21] The intricate interactions of phytocannabinoid compounds observed within the endocannabinoid system give indication as to the breadth of potential biological interactions on numerous biological systems associated with this group of compounds.

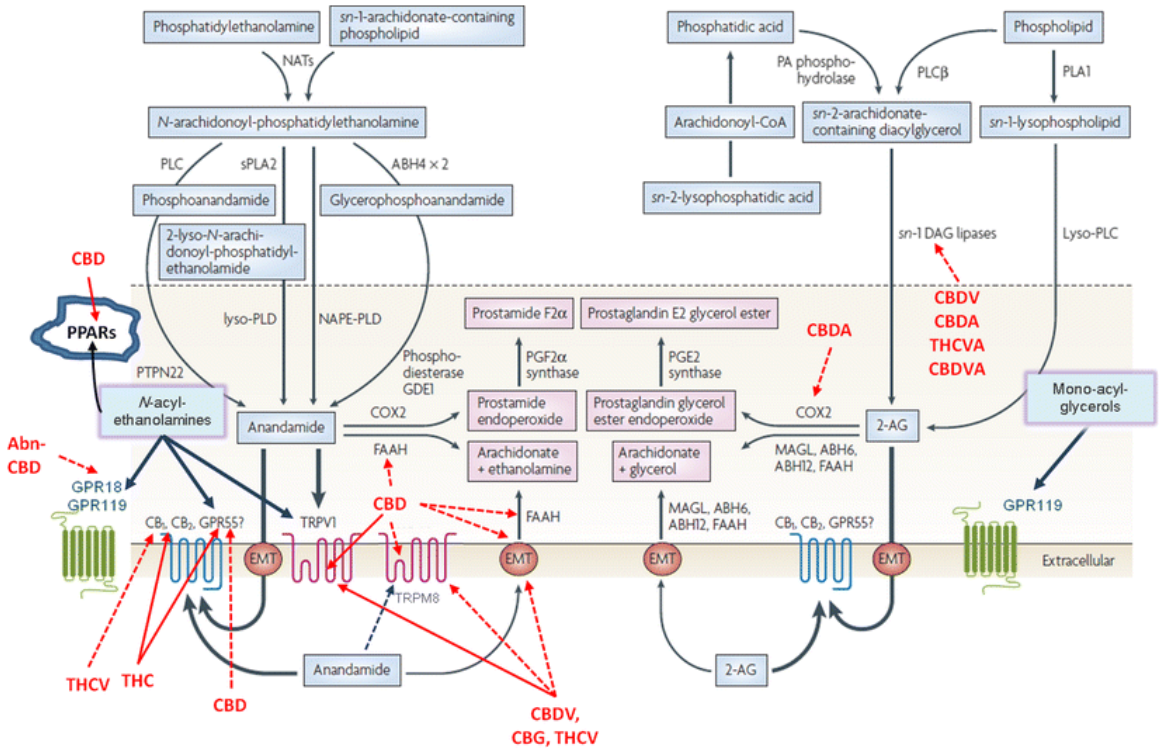


Figure 6-2 Phytocannabinoid interactions within the endocannabinoid system and connected biological processes [21]

The interactions of cannabinoid compounds extend well beyond binding with cannabinoid receptors, CB1 and CB2. The effect of several cannabinoid compounds has been linked with impact on the pathways involved with the biosynthesis and inactivation of the endogenous ligands, anandamide and 2-AG. The multitude of interactions highlights the complexity of the system and how administration of a mixture of cannabinoid compounds, as found in the presented studies, could complicate the observed results. The cannabinoid compounds have the ability to active (red solid arrows) or inhibit (red broken arrows) receptors and enzymes in the endocannabinoid system. The extend of the pharmacology of these interactions has not been fully assessed.

Consideration of the therapeutic potential for not just individual phytocannabinoid compounds but combinations of these compounds add another layer of complexity to our understanding of the pharmacological value of cannabis-derived products. Researchers have started investigating phytocannabinoids in combinations for various indications. [107, 108, 117, 118, 143-146], Taking a step beyond combining synthetic compounds, scientists are exploring the potential of botanical extract from *Cannabis sativa*. For example, De Petrocellis *et al*. reported differences in agonism of transient receptor potential (TRP) channels efficacy between pure cannabinoid and botanical drug substance (*Cannabis sativa* extract) rich in the cannabinoid being tested. [147] A limitation of this study was that the components of the botanical extract were not characterized, and thus influence on the observed differences could not be determined. [147]

The methodology developed here has the potential to drive cannabinoid research in multiple ways. First, the quantification of biologically active phytocannabinoids present at even low levels gives understanding to what components are in the botanical extract that could resulting in an observed effect. This is a starting point for researchers to identify what components may be responsible for a given effect, investigate the mechanism of action, and how the component may be impacting the action of other components in the extract. Second, methodology for the quantification of an array of phytocannabinoids can enable scientists to formulate blends of botanical extract with known and specific concentrations of a desired phytocannabinoid profile. Third, such methodology can help inform researchers in the development of plant varieties and optimization of growing conditions to produce desired phytocannabinoid profiles. Additionally, methodology for the quantification of phytocannabinoids can help inform the development of highly

specific extraction techniques and formulation approaches to include phytocannabinoids of interest and remove undesired phytocannabinoids.

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Education

University of Kentucky
Ph.D. in Pharmacology and Nutritional Science 2022

Marshall University
M.S. in Forensic Science 2012

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Professional Positions

University of Kentucky, Graduate Researcher	2018 – 2022
LGC Science Inc., *Principal Scientist*	2012 – present
Office of the Chief Medical Examiner, *Intern – Toxicology Lab*	2011
Marshall University Forensic Chemistry, *Graduate Research Assistant*	2010 – 2012
Lab Support (PerkinElmer), *Contract Research Scientist*	2010
Aerotek (Bristol-Myers Squibb), *Contract Research Scientist*	2009
Cara Therapeutics Inc., Senior Associate Scientist	2007 – 2008
Bayer Pharmaceutical Corporation	2002 – 2007
Associate Research Scientist II	2004 – 2007
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Peer Reviewed Publications

Erin Johnson, Michael Kilgore, and Shanna Babalonis. "Cannabidiol (CBD) product contamination: Quantitative analysis of Δ^9-tetrahydrocannabinol (Δ^9-THC) concentrations found in commercially available CBD products." (submitted November 2021) Under review

Shanna Babalonis, Erin Johnson, Michael Kilgore, and Michelle Lofwall." Cannabidiol (CBD): Unregulated Products May Contain the Psychoactive Cannabinoid THC" (submitted Nov 2021) Under review

Erin Johnson, Michael Kilgore, and Shanna Babalonis. "Label Accuracy of Unregulated Cannabidiol (CBD) Products: Measured Concentration vs. Label Claim." (submitted October 2021) Under review

Dave King, Paul Duffy, Saoirse Elizabeth O'Sullivan, Parveen Bhatarah, Erin Johnson, Andy Yates. "International heterogeneity in cannabidiol (CBD) consumer product regulations and guidelines; implications for the UK CBD food supplementation market" (submitted June 2021) Under review

Erin Johnson, Jasper van Heemst, Jeshurun Benavides, and Bob Gray. "Metabolism and excretion of the benzodiazepine analog etizolam in the horse." Drug Testing and Analysis. (2021) 13 (3) 583-594. doi: 10.1002/dta2967

Erin Johnson (Crum), Lorie Bishop, and Richard Sams. “Demonstration of a Step-Wise Approach to Addressing an Emerging Drug Threat Using Novel Fentanyl Analogues as a Case Study.” Oral presentation and Proceedings manuscript, International Conference of Racing Analysts and Veterinarians (ICRAV) 2018, Dubai, UAE.

Erin D. Crum, Kristen M. Bailey, Lauren L. Richards-Waugh, David J. Clay, Myron A. Gebhardt, and James C. Kraner. “Validation of blood and liver oxymorphone analysis using LC/MS/MS: concentrations in 30 fatal overdoses.” Journal of Analytical Toxicology (2013) 37 (8): 512-516. doi: 10.1093/jat/bkt077

Non-Peer Reviewed Publications

Erin Johnson, and Michael Kilgore, and Shanna Babalonis. “Phytocannabinoid Concentrations in Hempseed Protein Products.” Poster abstract. International Cannabinoid Research Society 2021 Symposium, Jerusalem, Israel. (June 2021) Virtual meeting

Erin Johnson, Michael Kilgore, and Shanna Babalonis. “LC/MS Analysis of 17 Phytocannabinoids in Hempseed Protein.” Poster abstract. Center for Clinical and Translational Science Research Days (April 2021) Lexington, Kentucky.

Erin Crum, Jeshurun Benavides, Nina Salazar, and Richard Sams. “LC-MSMS Analysis of Serum Samples for the Detection and Quantification of Corticosteroids in Equine Sport.” Poster presentation, American Society of Mass Spectrometry, Indianapolis, IN, 2017.

Erin D. Crum, BS, David J. Clay, BA, Kristen M. Bailey, MS, Myron A. Gebhardt, MS and James C. Kraner, PhD “Validation of liver and blood oxymorphone analysis using LC/MS/MS: Comparison with associated blood concentrations in fatal intoxications.” Poster presentation, American Academy of Forensic Science, Atlanta, GA, 2012. (www.marshall.edu/forensics/files/2012/09/Crum_Poster_rev4-2-3-12.pdf)