2020

Validation of the 4-Aminophenol Chemical Test and Analytical Testing Scheme to Distinguish Marijuana and Hemp

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Validation of the 4-Aminophenol Chemical Test and Analytical Testing Scheme to Distinguish Marijuana and Hemp

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Forensic Science at Virginia Commonwealth University.

by
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Virginia Commonwealth University
Richmond, Virginia
March 2020

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Acknowledgement

The author wishes to thank several people. I would first like to show my sincere gratitude to my research mentor, Becky Wagner, for her guidance and encouragement throughout every step of this project. The door to Becky's office was always open whenever I had questions about my research or writing and without her help, this project would truly not have been possible. Besides my advisor, I would like to pay special regards to my research committee members, Scott Maye and Michelle Peace, for their insightful comments and assistance on this project and its subsequent writings.

My sincere thanks goes to the staff at Virginia Department of Forensic Science Central Laboratory, who provided me the opportunity to join their team as an intern and who gave me access to the laboratory and research materials. Their support is truly appreciated and this project would have been impossible without it. I would like to acknowledge the entire Controlled Substances Section at the Department of Forensic Science, particularly Deborah Brimijoin, Erin Przybylski, and John Payne. Without their passionate participation, input, and teaching, my project could not have been successfully completed. Additionally, I would like to thank Carl Wolf at Virginia Commonwealth University's Forensic Toxicology and Specialty Testing Lab for providing me access to his food product materials. Without his contribution, that portion of my project would not have been possible. I thank Sandra Rodriguez-Cruz and her team at the Drug Enforcement Agency Special Testing and Research Laboratory for their contributions and insight, which made this collaborative project possible.

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List of Abbreviations

Abbreviation	
4-AP	4-Aminophenol
CBC	Cannabichromene
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDV	Cannabidivarin
CBDVA	Cannabidivaric acid
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBL	Cannabicyclol
CBLA	Cannabicyclolic acid
CBN	Cannabinol
CBNA	Cannabinolic acid
C. sativa	Cannabis sativa
CBV	Cannabivarin
DCLS	Division of Consolidated Laboratory Services
DEA	Drug Enforcement Administration
DFS	Department of Forensic Science
D-L	Duquénois-Levine
exo-THC	Exo-tetrahydrocannabinolic
GC-FID-MS	Gas chromatography-flame ionization detection-mass spectrometer
HPLC-DAD	High performance liquid chromatograph-diode array detector
NIDA	National Institute on Drug Abuse
PCP	Phencyclidine
Δ^8-THC	Δ8-tetrahydrocannabinol

Abbreviation

THC	Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin
THCVA	Tetrahydrocannabivaric acid
TLC	Thin layer chromatography
UV	Ultraviolet

Abstract

VALIDATION OF THE 4-AMINOPHENOL CHEMICAL TEST AND ANALYTICAL SCHEME TO DISTINGUISH MARIJUANA AND HEMP

By Kenna Lewis, BS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Forensic Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2020

Research Mentor: Rebecca Wagner, PhD, Research Section Supervisor, Virginia Department of Forensic Science

With the passing of the 2018 Farm Bill, which legalized hemp, law enforcement agencies and forensic laboratories needed a more efficient testing scheme to differentiate marijuana and hemp Cannabis plants. The validation of the 4-aminophenol chemical test and the subsequent analytical scheme allows for this differentiation to be performed. The evaluation of eighteen different cannabinoids, including acids, demonstrated that compounds with structures similar to tetrahydrocannabinol (THC) produced a blue result and compounds with structures similar to cannabidiol (CBD) produced a pink result. Several titration curves with varying concentrations of cannabinoids indicated a pink result when the THC concentration was less than the CBD concentration and a blue result when the THC concentration was greater than the CBD concentration. When the concentrations on THC and CBD were nearly equal, inconclusive results were obtained. The impact of other cannabinoids was also evaluated with a titration evaluation. Although not all cannabinoids produced the expected color result when analyzed, the cannabinoids are minor components of Cannabis plant material and would not be expected to be in high enough concentrations to skew the color results. Preprocessed, processed, and casework plant material samples were used to validate the 4-aminophenol chemical test. The remainder of the newly proposed analytical scheme, including thin-layer chromatography and gas-chromatography-flame ionization detection-mass spectrometry was used to corroborate this validation. The validation of a high performance liquid chromatograph-diode array detector method to fully quantitate samples is still in progress. The 4-aminophenol chemical test also has potential as a viable screening method for oil and food samples as well, but further validation is required.

Introduction

Cannabis sativa (*C. sativa*), of the Cannabaceae family, is one of the oldest plants used for food, fiber, medicine, and as an illicit drug. Evidence suggests extensive Cannabis use over 5,000 years ago (3000 BC) in what is now Romania (1). Around 2800 BC, the first medical use of *C. sativa* was documented by Emperor Shen Neng in China as treatment of illnesses such as rheumatism, malaria, and gout (2). The earliest documented cultivation of Cannabis is in China during the sixteenth century (1600 BC to 1501 BC) and subsequently extended into the Middle East, Europe, and the Americas (3, 4). Marijuana also has been used to aid alleviating problems in childbirth, fatigue, absentmindedness, venereal diseases, snakebites, and more in countries such as Africa, China, and India (4). Today it is used by consumers for possible treatment for neuropathic pain, spasticity in Multiple Sclerosis patients, and nausea and vomiting in patients undergoing cancer treatment (3).

Federal restriction of the importation, cultivation, possession, and distribution of cannabis began in 1937 with the passing of the Marijuana Tax Act and prohibition under federal law occurred with the Controlled Substances Act of 1970 (1). The Controlled Substances Act was passed by Congress as Title II of the Comprehensive Drug Abuse Prevention and Control Act of 1970 and replaced the previous legislation, including the Marijuana Tax Act (5). It created a framework for how substances could be regulated based on abuse potential, safety, and medical utility. Today, marijuana remains federally illegal and a schedule I substance, meaning there is a high potential for abuse and dependence with no currently accepted medical usage.

As of June 2019, eleven states and the District of Columbia have adopted laws legalizing marijuana for recreational use (6). California was the first state to permit cannabis use for medical purposes under physician supervision with the enactment of the Compassionate Use Act in 1996 (4). In 2018, Vermont was the first state to legalize recreational marijuana through a legislative process, initiating a shift in the marijuana reform movement (7).

Cannabis is a dioecious plant, meaning male and female parts develop on separate plants if grown from a seed (3). In 1971, the plant was characterized into drug and fiber types based on the presence of the

most abundant cannabinoids in its leaves and buds (8). Plants with a THC/CBD ratio greater than one were classified as drug phenotype and plants with a THC/CBD ratio less than one were classified as fiber phenotype (9). Cannabinoids are a group of terpenophenolic compounds with a ring structure derived from geranyl pyrophosphate that act on cannabinoid receptors in brain cells and suppress neurotransmitter release (3).

The Agricultural Improvement Act of 2018 became effective on December 20, 2018, establishing a framework for the legal production of hemp in the United States. The act removed hemp from the definition of marijuana in the federal Controlled Substances Act and excluded tetrahydrocannabinols in hemp from the definition of tetrahydrocannabinols in schedule I (10). The act defines hemp as "the plant Cannabis sativa L. and any part of that plant, including the seeds thereof and all derivatives, extracts, cannabinoids, isomers, acids, salts, and salts of isomers, whether growing or not, with a delta-9-tetrahydrocannabinol concentration of not more than 0.3 percent on a dry weight basis" (10). Individual states can apply to the Department of Agriculture for regulatory authority of hemp production by submitting a regulatory plan to the Secretary of Agriculture (10). Individual states can also further restrict or prevent hemp cultivation, but cannot prohibit transportation or shipment of hemp and hemp products lawfully produced throughout the state.

Marijuana and industrial hemp are different strains of the Cannabis plant and the only way to distinguish them is to conduct quantitative testing to determine the concentration of THC in the plant material. THC is the active chemical component responsible for the psychoactive effects and is the main terpenophenolic compound and cannabinoid (3). THC can induce euphoria, alter sensory perception and relaxation, and cause the "high" that recreational Cannabis users seek (11). CBD is another important cannabinoid, which is a non-euphoriant and has shown therapeutic activity such as anti-inflammatory, anticonvulsive, anxiolytic, analgesic, neuroprotective, anticancer, and antioxidant effects (11). Cannabis plants with a THC concentration greater than 0.3% are considered marijuana type and Cannabis plants with a THC concentration less than 0.3% are considered hemp or fiber type. The quantity and ratios of the cannabinoids present are a function of botanical and cultivation factors, mode of preparation of the drug,

and of the conditions of storage including light, heat, and the duration of growth in soil (12). Currently, law enforcement has no presumptive field test that can readily differentiate illegally possessed marijuana from legally possessed hemp.

While the passing of the 2018 Farm Bill allows for the expansion of the industrial hemp industry and stimulates revenue, Cannabis with a THC content greater than 0.3% is considered marijuana and is still illegal to possess under federal law (10). The Commonwealth of Virginia legislation has established an affirmative defense for correctly registered individuals enabling the individual to be charged with possession of marijuana unless a laboratory test determines that the THC concentration is below the legal threshold (13). The burden of proof now falls on the individual in possession of Cannabis to raise the affirmative defense by claiming to be a registered grower, dealer, or processor of industrial hemp and proving a license. Current field tests implemented for the presumptive identification of marijuana cannot differentiate between marijuana and hemp. A rapid chemical field test for law enforcement to support or dismiss probable cause for possession of marijuana would provide a cost effective and efficient protocol for both law enforcement and laboratories.

Color tests are rapid and presumptive screening tools used to narrow down the possible identification of an unknown drug sample. Color tests are not specific on their own but can be advantageous because they promptly can indicate the presence or absence of a controlled substance. The Duquénois-Levine (D-L) field test, commonly used by law enforcement officers, can identify a substance as Cannabis plant material and can be used to establish probable cause to charge a suspect. The D-L test has been the most well-known color test used for identifying Cannabis since 1941 and is based on color developments, typically in a test tube, with a series of reagents (14). The Duquénois reagent, developed in 1937, consists of vanillin and acetaldehyde dissolved in ethanol (14). In an acidic environment, usually hydrochloric acid, the Duquénois reagent will react with the free position *para*- to the phenol group in the THC compound, producing an intense purple color (15). However, false positives are possible since many compounds contain a phenol group with such free *para*-position, including many other cannabinoids. The Levine modification (addition of chloroform) minimizes some of these interferences by only allowing molecules

with long aliphatic chains to cross into the chloroform layer, causing the purple color to extract into the chloroform layer, thus increasing the selectivity of the test (15).

It is important to know that the D-L test can also yield false positive results with other botanical materials, including patchouli, cypress, and eucalyptus, and inconclusive results with lavender, spearmint, oregano, and thyme (16). Therefore, it should not be used as the sole means of conviction or be overly relied upon by law enforcement. Common analytical testing schemes within forensic laboratories include gross morphological and microscopic examinations, the D-L test, thin layer chromatography (TLC), and mass spectrometry confirmation to identify the presence of cannabinoids in suspected plant material (17, 18). Morphological characteristics common in Cannabis include the palmate arrangement of the leaflets, the pinnate appearance of the leaflets, the serrated edges of the leaflet, the buds, and fluted stems and stalks (19). Microscopic examination of Cannabis plant material will allow for the observation of cystolithic hairs, nonglandular hairs, “bear claw” shaped hairs with a cystolith of calcium carbonate at the base, that are typically found on the upper side of the leaf (17, 18, 19). Microscopic examination of a Cannabis sample will also reveal the presence of coconut shaped seeds with a ridge around their circumference and veined with lacy markings (19). TLC is a method of separation that allows for an indication of the presence of cannabinoids. TLC can assist in distinguishing marijuana and hemp by allowing for the comparison of relative cannabinoid concentrations (19). This analytical testing scheme becomes more challenging when differentiating between marijuana and hemp, creating the need for a more complex analytical testing scheme.

The 4-aminophenol (4-AP) chemical test is a presumptive test for the qualitative analysis of Cannabis plant material. The color test reagents can be prepared in a laboratory or purchased in commercially available Cannabis Typification field test kits (11). Marketing descriptions of the test indicate that it was designed to differentiate Cannabis plant material samples at a THC concentration of 1%. The 4-AP chemical test was originally developed by the Forensic Institute of Zurich (Zurich Police) in Switzerland, where it is used as a qualitative test prior to quantitative analysis of individual cannabinoids. A recent study by Hädener *et al*. showed that this colorimetric on-site test was successfully applied to

distinguish between CBD-rich type Cannabis samples with CBD concentrations higher than THC, and THC-rich Cannabis samples with CBD concentrations lower than THC (11). To conduct the differentiation, a small sample of dried or fresh Cannabis plant material is transferred into a vial, reagents are added, and the reaction is visually observed within two minutes. If the concentration of THC in plant material is greater than the concentration of CBD, the color test result will be blue, and is considered THC-rich/CBD-poor. If the concentration of CBD in plant material is greater than the concentration of THC, the color test result will be pink, and is considered THC-poor/CBD-rich. However, the study by Hädener *et al*. showed that plants with THC/CBD concentrations nearly equal or a THC/CBD ratio between 0.33 and 3 had a higher probability for false negative results. A false negative sample will produce a color change different from the expected pink or blue. Because the test is based on THC/CBD ratios, quantitative instrumental analysis is therefore required to determine the concentration of cannabinoids in the plant material.

The new analytical testing scheme for the identification and confirmation of marijuana includes first obtaining both a positive result with the D-L field test kit and a blue result from the 4-AP test by law enforcement at the scene. If the plant material indicates that it is THC-rich/CBD-poor with a blue result, the material will be submitted to the laboratory by the appropriate law enforcement agency for gross morphological and microscopic examination, TLC with optional 4-AP, and a semiquantitative screen on a gas chromatography-flame ionization detection-mass spectrometer (GC-FID-MS). GC-FID-MS is an efficient screening method with the ability to establish if the THC concentration in plant material is greater than or less than an administrative threshold, which is 2% (19). The GC-FID-MS method has been validated and consists of determining the ratio of total THC to internal standard in a sample using peak area from the FID (19). Total THC includes THC and its precursor tetrahydrocannabinolic acid (THCA), because the latter decarboxylates to produce THC during exposure to heat, such as smoking or in the injection port of the instrument. If the ratio of THC to internal standard in the case sample is less than the ratio of the 2% standard, the result is considered inconclusive. If the ratio of THC to internal standard in the case sample is greater than the ratio of the 2% standard, the sample can be identified as marijuana. The concentration of CBD can be identified if the method is being used to clarify the 4-AP color result. The results obtained

from the analytical scheme will produce an inconclusive result as to the Cannabis type or identify the plant material as marijuana. If inconclusive results are obtained, a full quantitation on a high performance liquid chromatograph-diode array detector (HPLC-DAD) will be completed if the samples are resubmitted by the law enforcement agency with a request for additional laboratory analysis. If the results of the analytical testing scheme are inconclusive for the type of Cannabis, the evidence can be resubmitted to the laboratory for quantitative testing. The HPLC-DAD method is currently in validation stages and will be used to further quantify the concentrations of CBD, THC, and THCA in plant material to enable the differentiate between marijuana and hemp.

Problems driven by the rapid growth of hemp-derived products have caused a rush to pass legislation clarifying the legal standards and required testing procedures. The new federal definition of hemp implies that a quantitative determination of the THC concentration is required to enable differentiation between marijuana and hemp. However, this process involves a significant increase in the amount of time required for laboratories to complete analytical testing of plant materials. Furthermore, since the D-L test only provides presumptive positive results for Cannabis material, and not the actual presence of THC, a positive result could be interpreted as a (false) positive for THC when it is not. Therefore, validation of a more selective field chemical test, such as the 4-AP test, provides laboratories and law enforcement personnel with an additional tool to presumptively differentiate marijuana from hemp without submissions to the laboratory, in an efficient and reliable manner. This color test allows for rapid presumptive screening of samples, is more selective than the Duquénois-Levine test, and allows for the classification of Cannabis plant material based on THC-rich or CBD-rich types.

In this manuscript, the validation of the 4-AP chemical test is presented and its use to distinguish between marijuana and hemp is investigated. This project was a collaboration between the Drug Enforcement Administration (DEA) and the Virginia Department of Forensic Science (DFS). The collaboration between DEA and DFS enabled a multi-agency validation of both the new 4-AP chemical test and the analytical scheme as a whole for differentiating marijuana and hemp. This validation included a comparison of results from over 30 agricultural hemp samples, assessment of numerous cannabinoid

reference materials, testing of household spices to investigate potential false positive responses, and evaluation of casework samples.

Materials and Methods

Reagents and Sample Preparation

Chemicals

4-Aminophenol was purchased from Tokyo Chemical Industry Company, LTD. (Tokyo, Japan). Ethanol, hydrochloric acid, and sodium hydroxide were purchased from Pharmco by Greenfield Global (Brookfield, CT, USA), Fisher Chemical (Waltham, MA, USA), and EmScience Lab Chemicals (Gardena, CA, USA), respectively. Deionized water was provided in-house. Cannabis Typification field test kits were purchased from Elixir Health Products Ltd. (London, England). Additional Cannabis Typification field test kits were purchased from Syndicate Chemistry (Orlando, FL, USA). Reference materials for eighteen cannabinoids were purchased from Cayman Chemical (Ann Arbor, Michigan, USA) and Cerilliant Corporation (Round Rock, Texas, USA). These cannabinoids included THC, THCA, Δ^8-tetrahydrocannabinol (Δ^8-THC), exo-tetrahydrocannabinolic acid (exo-THC), tetrahydrocannabivarin (THCV), tetrahydrocannabivaric acid (THCVA), CBD, cannabidiolic acid (CBDA), cannabidivarin (CBDV), cannabidivaric acid (CBDVA), cannabicyclol (CBL), cannabicyclolic acid (CBLA), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabinol (CBN), cannabinolic acid (CBNA), cannabivarin (CBV) and cannabichromene (CBC).

Reagent Preparation

The 4-AP chemical test utilizes 4-aminophenol in acidic ethanol and sodium hydroxide in ethanol/water as a presumptive test for the qualitative analysis of Cannabis plant material. Reagent A was prepared by dissolving 300 mg of 4-aminophenol in 995 mL of ethanol and 5 mL of 2 N hydrochloric acid. Reagent B was prepared by dissolving 30 g of sodium hydroxide in 300 mL of deionized water and 700 mL of ethanol. Both reagents were stored in the refrigerator in amber containers.

Sample Preparation

To perform the 4-AP chemical test in the laboratory, approximately 5 mg of plant material sample, which is about the size of a grain of rice, was added to a spot plate well or test tube. The sample was covered with Reagent A. Approximately two to four drops of Reagent B were then added to the well or test tube. The color change was observed and noted within the first one to two minutes after the addition of Reagent B. A negative control was performed with each analysis by adding Reagent A and Reagent B to an empty spot plate well or test tube. The negative control did not produce a color change. All plant material samples were analyzed in triplicate without grinding, drying, or processing.

To perform the test using the Cannabis Typification field test kit, approximately 5 mg of plant material was used for analysis. The field test kit consists of a pouch containing Reagent A and Reagent B in individual sealed glass vials, tweezers, and a clip to seal each pouch. To use the field test kit, the clip was removed, plant material was placed into the pouch using the provided tweezers, and the kit was resealed using the clip. Prior to breaking the glass vials containing Reagent A and Reagent B, the pouch was tapped gently on a hard surface to ensure the sample was at the bottom of the pouch. The vials were then broken followed by gentle shaking of the pouch. The color change was observed within one to two minutes.

Unless otherwise noted, validation experiments were performed using in-house reagents and materials. Commercial Cannabis Typification field test kits were only used for experiments where they are specifically described.

1. *Analysis of Reference Materials*

    a. *Individual Cannabinoid Analysis*

For the evaluation of individual cannabinoids using reference materials, eighteen cannabinoids were prepared at a 1% concentration in methanol to determine their color test result. The cannabinoids evaluated included THC, THCA, Δ^8-THC, exo-THC, THCV, THCVA, CBD, CBDA, CBDV, CBDVA, CBL, CBLA, CBG, CBGA, CBN, CBNA, CBV and CBC. Approximately 5 µL of the 1% solution was added to a spot plate well. Approximately 1 mL of Reagent A was added to the well followed by two to

four drops of Reagent B. The color change was observed and noted within the first one to two minutes after the addition of Reagent B.

b. Comparison of THC and CBD Concentrations

A titration curve was developed to evaluate the color change of varying concentrations of THC in the presence of 1% CBD. The THC concentration was evaluated at 0.1%, 0.3%, 1%, 2%, 3%, 4% and 5% while holding the CBD concentration constant at 1%. Triplicate evaluations were completed for each concentration. A titration curve was developed to evaluate the color change of varying concentrations of CBD in the presence of 0.3% THC to identify the impact of the result with varying concentrations of CBD. The resulting color change was evaluated at 0.1%, 0.3%, 0.5%, 0.8%, 1%, 2%, and 5% CBD. Triplicate evaluations were completed for each concentration.

c. Analysis of THC with Other Cannabinoids

To evaluate the impact of other cannabinoids present in the plant material upon analysis, THC in combination with the cannabinoids was assessed. The concentration of THC was held constant at 0.3% while a titration of another cannabinoid was completed. The titration included all cannabinoids previously listed in the Reagents and Sample Preparation section of Materials and Methods. The concentrations of the cannabinoids evaluated included 0.1%, 0.3%, 1%, 2%, and 5%. The 4-AP chemical test was performed in triplicate with the color noted approximately one to two minutes after the addition of Reagent B.

d. Analysis of CBD with Other Cannabinoids

To evaluate the impact on the result of other cannabinoids present in the plant material, CBD in combination with other cannabinoids was assessed. The concentration of CBD was held constant at 1% while a titration of another cannabinoid was completed. The titration included all cannabinoids previously listed in the Reagents and Sample Preparation section of Materials and Methods. The concentrations of the cannabinoids evaluated included 0.1%, 0.3%, 1%, 2%, and 5%. The color test was performed in triplicate with the color noted approximately one to two minutes after the addition of Reagent B.

e. Limitations and Interference Evaluation from Individual Cannabinoids

i. False Positive and False Negative Evaluations

Limitations and interferences from the results obtained from both individual cannabinoid analysis and cannabinoid titration experiments were evaluated. Any combination of cannabinoids which produces a blue color when no THC is present will be considered a potential false positive and any combination of cannabinoids which produces a pink color when THC is present will be considered a false negative.

ii. Acidic vs. Non-Acidic Cannabinoids

To investigate the impact of acidic cannabinoids on the overall color result, the presence of acidic and non-acidic cannabinoids was investigated.

iii. Reagent Addition Order

In order to determine if the reaction is reversible and to better understand the impact of the carboxylic acid, a 5% neat standard of THCA was evaluated, then a 1% solution of CBD was added to the mixture. If the reaction is reversible the color change should turn from blue to pink with the addition of the 1% CBD.

The order of reagent addition was also evaluated. The same experiment above was performed by adding Reagent B prior to the addition of Reagent A. Reagents A and B were also added simultaneously. This experiment simulates the Cannabis Typification field test kit where the two ampules containing the reagents are broken simultaneously or in no specific order.

The reagent order for the 4-AP chemical test was also evaluated with plant material samples. A sample that was previously tested and determined to be inconclusive with pink to blue color result change and a validated marijuana sample were tested. A single analysis was performed with Reagent A followed by Reagent B, Reagent B followed by Reagent A, and both reagents added simultaneously.

iv. Test Tube vs. Spot Plate Well

Whether the 4-AP chemical test is performed in a test tube or a well-plate was also evaluated as a potential limiting factor of the test. A titration curve of varying concentrations of THCA (0.1%, 0.3%, 1%,

3%, and 5%) with a constant 1% concentration of CBD was created using both test tubes and a spot plate well and results were compared.

2. Analysis of Plant Material

a. Inter-Laboratory Comparison

A total of thirty-six agricultural plant material samples were prepared at the Division of Consolidated Laboratory Services (DCLS) in Richmond, Virginia and transferred to DEA and DFS for the 4-AP chemical test validation. DFS also received six additional samples for testing. All DCLS samples originated from Virginia hemp crops harvested during the fall of 2018. Samples obtained from DCLS were prepared by heating at 90°C until a constant weight was reached and subsequently ground and sieved prior to quantitative analysis for CBD, THC, and CBN concentrations using GC-FID-MS (20).

b. Freshly Harvested Agricultural Plant Material

Additional agricultural plant material samples from DCLS were obtained that were recently harvested and transferred to the laboratory. Three to four fresh stem clippings were obtained from a total of ten plants and put in the refrigerator. The samples were then placed into the drying cabinet at room temperature for several days to prevent molding. Once dry, the samples were placed back into the refrigerator. The plant material samples had very small buds if any and therefore the leaves were utilized for analysis. The 4-AP chemical test was performed in triplicate in conjunction with TLC and a 2% GC-FID-MS screen for THC.

c. National Institute on Drug Abuse (NIDA) Samples

A total of seven samples obtained from NIDA were analyzed with the 4-AP chemical test. Certificates of analysis were provided by NIDA and contained concentrations for THC and CBD. These concentrations were used to calculate THC/CBD ratios.

d. Suspected Marijuana Casework

Greater than twenty suspected marijuana plant material was analyzed with the 4-AP chemical test and verified using the GC-FID-MS 2% THC screen method. This experiment was performed to evaluate casework applications.

e. Immature Plant Material

Immature marijuana plant material has demonstrated higher concentrations of THCA than THC. The plants have demonstrated the greatest concentrations of THCA in parts of the plant that are undergoing prosperous growth (21). Cannabinoids are present almost exclusively in their acidic form when they are undergoing outdoor cultivation as a raw botanical material (22). As the plants begin to flower, the THCA concentrations drop in the mature leaves, and the THCA decarboxylates to THC (21). Therefore, to further understand the impact of acidic cannabinoids on the 4-AP chemical test, immature plant material samples were evaluated. Immature marijuana plant material samples were obtained from in-house Marijuana plants, grown by two DFS laboratories and were tested using the 4-AP color test. After approximately three to four-month gestation, the stems were harvested and refrigerated prior to analysis. The first plant sample was evaluated with no heating or drying. Additional samples of the same plant were evaluated after air drying for 24-hours, after air drying for 24-hours with subsequent heating for one hour at approximately 90°C, and after heating for one hour at approximately 90°C. A second immature plant sample was split into two groups and evaluated after being dried in a desiccator for two and five days.

f. Limitations and Interferences Evaluation from Household Spices

Potential interference from sixteen household spices was evaluated in a single analysis. The spices evaluated included sage, oregano, summer savory, marjoram, mint, crushed red pepper, black pepper, chili powder, parsley flakes, tobacco, hops, burr marigold, coffee, ditchweed, dragon's blood, and parsley spiked with phencyclidine (PCP), a hallucinogenic drug. The evaluation of spices was completed with a single analysis using the 4-AP chemical test.

3. Evaluation of Oil Products

Five oil products and four oil syringes marketed as hemp or CBD oils and one CBD crystal sample were evaluated using the 4-AP reagents. The following descriptions are directly taken from the manufactured bottles they were purchased in. Oil 1 was a Spectrum Essentials savory blend flax and hemp oil with natural garlic flavor, rosemary and oregano. Oil 2 was vanilla flavored Pharma CBD drops containing 500 mg of CBD. Oil 3 was Plus CBD oil spray, total plant complex, a peppermint dietary

supplement. Oil 4 was a Plus CBD oil spray containing peppermint. Oil 5 was Charlotte's Web hemp extract in MCT, a mint chocolate flavored dietary supplement. Syringe oils 1, 2, and 3 were Pharma CBD pure CBD extract gold oils. Syringe oil 1 was 15.9% gold oil, syringe oil 2 was 17% gold oil, and syringe oil 3 was 25% gold oil. Syringe oil 4 was a Real Scientific Hemp Oil [RSHO] containing 17% CBD-RSHO blue. The Endoca CBD crystals were THC free and contained 99% CBD and terpenes. Some of these products were previously quantitated on the HPLC and THC, THCA, and CBD concentrations were known. Additionally, eighteen oil samples from casework were evaluated using the 4-AP reagents. For all oil samples, approximately one drop of each oil was added to a spot plate well. The sample was covered with Reagent A. Approximately two to four drops of Reagent B were then added to the well. The color change was observed and noted within the first one to two minutes after the addition of Reagent B. A negative control was performed with each analysis by adding Reagent A and Reagent B to an empty spot plate well. The negative control did not produce a color change. All oil samples were analyzed in triplicate, unless the sample size did not allow for triplicate analysis.

4. Evaluation of Food Products

A variety of food products marketed as containing THC or CBD, and several food and beverage products produced at Virginia Commonwealth University's Forensic Toxicology and Specialty Testing Laboratory were evaluated using the 4-AP chemical test. The food products included a honey edible obtained from casework, Marmas blue raspberry soft candy, Magic Kitchen pebbles, Spot mixed fruit chews (Indica and Sativa) indicating 10 mg of THC, a Spot CBD dark chocolate bar, Baked Botanicals Don't Be Square peanut butter cups, and Spot classic brownies (Indica and Sativa) indicating 10 mg of THC. Two peanut butter cups with 5 mg and 10 mg THC were baked in the Forensic Toxicology and Specialty Testing Laboratory. The beverage products tested included Ocean Spray cranberry juice, Orangina soda, Richmond blend tea, black tea, Sprite, Snapple lemonade juice, Coca-Cola, black coffee, Bold Rock black raspberry hard cider, green tea, orange mango peach juice, Pepsi, apple juice, black cherry cream soda, tea with sugar, tea with ethanol, Synergy kombucha, and a CBD beer.

The Spot brand mixed fruit chews and Spot brand classic brownies were quantitated by the Forensic Toxicology and Specialty Testing Laboratory to obtain the THC and CBD concentrations. The peanut butter cups made in the laboratory were baked with 5mg and 10 mg of THC and no CBD. The remainder of the food products were not quantified. All beverage samples were spiked with equivalent concentrations of THC, CBD and CBN.

Approximately 5 mg of each food product and 1 mL of each beverage sample were added to a test tube. The sample was covered with Reagent A. Approximately two to four drops of Reagent B were then added to the test tube. The color change was observed and noted within the first one to two minutes after the addition of Reagent B. A negative control was performed with each analysis by adding Reagent A and Reagent B to an empty test tube. The negative control did not produce a color change. All food and beverage samples were analyzed in triplicate.

5. *Cannabis Typification Field Test Kit Stability Testing*

    a. *Temperature Stability*

The stability of the Cannabis Typification field test kits purchased from Syndicate Alliance in different environment conditions was evaluated by analyzing marijuana and hemp samples in duplicate at various temperatures and time points. The marijuana sample was confirmed marijuana plant material from casework and the hemp sample was a ground hemp certified reference material from Absolute Standards purchased from Emerald Scientific (San Luis Obispo, CA, USA). Four kits were initially evaluated and evaluated after 24-hours for the duplicate testing of a confirmed marijuana and confirmed hemp sample. Twelve test kits were then placed in a 40°C oven and twelve kits were placed in a -20°C freezer for one week. After one week, four kits from each group were removed and evaluated in duplicate with the same marijuana and hemp samples used at T = 0. The remaining kits were placed on the laboratory benchtop for one week to evaluate the kits at room temperature. After one week, four kits from each group were removed and evaluated in duplicate with the same marijuana and hemp samples previously evaluated. The remaining kits were then placed in the oven and freezer for one week. Test kits that were initially in the oven were placed in the freezer and test kits that were initially in the freezer were placed in the oven. After one week,

the remaining kits from each group were removed and evaluated in duplicate with the same marijuana and hemp samples previously evaluated.

b. Ultraviolet (UV) Light Stability

The stability of the Syndicate Alliance Cannabis Typification field test kits after exposure to UV light was evaluated by duplicate analysis of the same marijuana and hemp samples used for the temperature stability test. A total of sixteen field test kits were placed near a window exposed to sunlight at laboratory room temperature and humidity conditions. In one week time intervals, for four weeks, four field test kits were removed and evaluated using the marijuana and hemp samples in duplicate.

6. TLC and GC-FID-MS Analysis

All plant material samples that were used to validate the 4-AP chemical test were also used in conjunction with TLC and the newly validated GC-FID-MS method for the differentiation of marijuana and hemp. The following TLC and GC-FID-MS methods used were obtained from the DFS Controlled Substances Procedures Manual (19).

For TLC, 50 mg of each plant material sample was extracted using either hexane or methanol. Both hexane and methanol are acceptable extraction solvents for TLC, according to the DFS Controlled Substances Procedures Manual, and half of the samples were extracted using hexane and half were extracted using methanol. Approximately 0.8 mL of extraction solvent was added to each sample and vortexed for five seconds. The TLC mobile phase was 4% diethylamine in toluene with Fast Blue B salt (tetrazotized o-dianisidine zinc chloride salt) for a visualization spray. Fast Blue B selectively stains cannabinoid-containing plant tissues (17).

For the evaluation of the plant material samples using the GC-FID-MS method, a ratio of THC to internal standard is evaluated at minimum day of use using a 2% standard. The ratio is utilized to establish the ratio for the threshold. For example, if one day the ratio is 1.899 and a sample gives a ratio of 1.999 the sample is considered marijuana. If the ratio that day is 2.100 and the ratio for the sample is 1.999, the sample is considered inconclusive and further testing would be needed to differentiate between marijuana and hemp.

7. HPLC-DAD Analysis

Before analyzing a sample on the instrument, an extraction procedure must be performed to separate the cannabinoids from the matrix. First, the plant material was dried in an oven at 40°C for 24-hours to remove excess moisture, ground twice for one minute at 5000 rpm, and weighed into two separate 100 mg replicates. The samples were extracted with a total of 5.0 mL of 80:20 acetonitrile:methanol with 0.5 mg/mL androstenedione. If the concentration of THC, THCA, or CBD is expected to be higher than the calibration range, additional dilutions can be performed. The sample will then be vortexed for 30 seconds, sonicated for 15 minutes, and centrifuged for two minutes at 1000 rpm. Once the extraction is complete, the liquid will be transferred to an appropriate autosampler vial and injected on the instrument. The HPLC-DAD instrument contains an Agilent Zorbax Eclipse XDB-C18, 3.0 x 150 mm column with 3.5 µm particle size. The column thermostat is set at 28°C. Mobile Phase A is 0.1% formic acid in water and Mobile Phase B is 0.1% formic acid in acetonitrile. The flow rate is 1 mL/minute and the injection volume is 5 µL. The gradient for Mobile Phase B is started and held at 60% for one minute, ramped to 70% in 1 minute, then to 77% in 4 minutes, held at 77% for 5 minutes, then to 95% in one minute, and held for one minute with 2.5 minutes of post time. The DAD signal is 220 nm with a reference wavelength at 360 nm. The spectrum ranges from 190 nm to 400 nm and the elution order is androstenedione, CBD, THC, then THCA. The concentrations of THC, THCA, and CBD can be determined using the peak area ratios obtained from the chromatograms of the standard curves for each cannabinoid and each replicate.

Results and Discussion

1. Analysis of Reference Materials

a. Individual Cannabinoid Analysis

Although the chemistry of the color change is still unclear, the color change of various cannabinoids appears to be correlated to structure. Cannabinoids similar in structure to THC, produced a blue color result, including Δ^8-THC, THCA, exo-THC, THCV, THCVA, CBL, CBLA, CBN, CBNA, and CBC. These cannabinoids contain an ether functional group, consisting of an oxygen atom forming single bonds with two carbon atoms on either side, two carbon atoms away from the aliphatic chain on the aromatic ring

structure. Cannabinoids that produced a pink color result include CBDV, CBG, CBGA, and CBV, CBDA, and CBDVA. These cannabinoids contain a hydroxyl group in the place of the ether, two carbon atoms away from the aliphatic chain on the aromatic ring structure, and a double-bonded carbon atom off of an ethyl group on the second ring structure of the molecule. CBDA and CBDVA have structures similar to CBD and produced purple results. A photograph of the results is shown in Figure 1. A summary of all cannabinoid structures can be found in Appendix A.

b. Comparison of THC and CBD Concentrations

The results for the titration experiment evaluating the color change of varying concentrations of THC in the presence of 1% CBD are outlined in Table 1. When the THC concentration was less than the CBD concentration, the color result was pink; and when the THC concentration was greater than the CBD concentration, the result was blue. When the CBD concentration was 1% and the THC concentration was 1% and 2%, inconsistent results were obtained, suggesting that when the THC and CBD concentrations are similar the test may produce inconclusive results. These results also suggest that the test does not differentiate based on the percentage of THC, but based on the ratio between THC and CBD.

The results for the titration experiment evaluating the color change of varying concentrations of CBD in the presence of 0.3% THC are outlined in Table 2. When the THC concentration was less than the CBD concentration, the result was pink; and when the THC concentration was greater than the CBD concentration, the result was blue. When the THC and CBD concentrations were nearly equal, the result color was purple, a combination of pink and blue, indicating an inconclusive result. This further indicates that the chemical test is not a 1% THC threshold test, but is based on the ratio of THC to CBD.

c. Analysis of THC with Other Cannabinoids

Results for the assessment of THC with other cannabinoids are summarized in Table 3. When the concentration of THC was at 0.3%, Δ^8-THC, THCA, exo-THC, THCV, THCVA, CBN, CBNA, CBL, CBLA, CBC, and CBV produced a blue result at all concentrations of the cannabinoid. CBDA, CBDV, CBDVA, CBG, and CBGA produced concentration dependent results. CBDA produced a blue result at low concentrations (0.1%, 0.3%, and 1%) and a purple result at higher concentrations (2% and 5%). CBDV

produced a blue result at low concentrations (0.1%), purple at 0.3% and 1%, and pink results at higher concentrations (2% and 5%). The associated acid CBDVA produced a blue result at low concentrations (0.1%, 0.3%, and 1%) and purple and pink results at 2% and 5%, respectively. CBG produced a blue color result at low concentration (0.1%), followed by a purple result at 0.3% and pink results at 1%, 2%, and 5%. The associated acid, CBGA, produced a blue result at the lowest concentrations (0.1% and 0.3%), a purple result at 0.3% and a pink result at higher concentrations (1%, 2%, and 5%). When holding the THC concentration constant, the color result could be predicted by the result of the individual evaluation.

d. Analysis of CBD with Other Cannabinoids

Results for the assessment of CBD with other cannabinoids are summarized in Table 4. When the concentration of CBD was held constant at 1%, THCVA, THCA, CBDA, CBDV, CBDVA, CBN, CBNA, CBLA, CBG, CBGA, CBC, and CBV produced a pink result at all concentrations of the cannabinoid. However, Δ^8-THC, exo-THC, THCV, and CBL produced concentration dependent results. Specifically, Δ^8-THC, exo-THC, and THCV produced a pink result at low concentrations (0.1% and 0.3%). Both Δ^8-THC and exo-THC produced blue/purple results at 1%, purple results at 2%, and blue results at 5%. THCV produced blue/purple results at 1% and 2% and blue results at 5%. CBL produced pink results at low concentrations (0.1% and 0.3%) and blue results at higher concentrations (1%, 2%, and 5%).

When holding the CBD concentration constant at 1%, all acids produced a pink result regardless of the individual compound color observation. The THC and CBD present must both be in their acidic or non-acidic form in order for the test to perform as expected, based on the cannabinoids ratios. When one of the cannabinoids is in the acidic form and the other is not, the results will be the color of the non-acidic cannabinoid on its own, as if the acid isn't present at all. Although not all compounds produced the expected color result when analyzed in the presence of CBD, the cannabinoids are all minor components of the plant material and would not be expected to be in high enough concentrations to skew the color test result when comparing the ratio of THC to CBD.

e. Limitations and Interference Evaluation from Individual Cannabinoids

i. False Positive and False Negative Evaluations

Based on the results noted above, it is recognized that not only THC, but also the presence of Δ^8-THC, exo-THC, THCV, and CBL could result in the production of a blue color result if these cannabinoids are present at concentrations greater than CBD. This color change could be interpreted as a presumptive positive marijuana result, even though there is no THC present. False negative results (pink) could also occur in the presence of high concentrations of CBDA, CBDVA, CBDV, CBG and CBGA, even though high concentrations of THC were present. No acids, including THCA, produced a color change in the presence of 1% CBD and therefore do not have an impact on the color test result. The titration experiments of CBD and THC with other cannabinoids produced several unexpected results when compared to the titration analysis of the cannabinoid in the presence of a constant 0.3% concentration of THC and a constant 1% CBD concentration. In general, titration experiments indicated that the color result of the individual analysis could not be used to predict the color result when either THC or CBD was also present in the sample.

ii. Acidic vs. Non-Acidic Cannabinoids

The experiments indicated that, when present, the neutral forms of THC or CBD dictate the final color observed. For example, no acids, including THCA, produced a color change in the presence of 1% CBD and therefore do not have an impact on the final pink color test result.

When CBD and THC were both in their acidic forms, CBDA and THCA, respectively, the expected color results were obtained with a pink result when the concentration of THCA was less than CBDA, and a blue result when the concentration of THCA was greater than CBDA. Similarly, when THC was combined with CBDA, the color result was blue, regardless if the concentration of THC was lower than the CBDA concentration. In conclusion, the CBD and THC must both be in their acidic or non-acidic form in order for the test to produce the expected results. When one of the cannabinoids is in the acidic form and the other is not, the results will be determined by the non-acidic cannabinoid, as if the acid isn't present at all.

iii. Reagent Addition Order

In order to determine if the reaction is reversible and to better understand the impact of the carboxylic acid, a 5% neat standard of THCA was evaluated and the expected result of blue was obtained. Next, a 1% solution of CBD was added to the mixture, and the mixture turned from blue to pink. Since the color test appears to be reversible, the chromophore that is formed must not be thermodynamically stable.

When adding Reagent B prior to the addition of Reagent A, the color results were the same as the addition of Reagent A followed by Reagent B. Reagents A and B were also added simultaneously and the color results remained consistent. This experiment indicates that reagent order does not impact the color result.

When the reagent order was evaluated with plant material samples, the results were comparable regardless of the order reagents were added. The inconclusive sample produced a pink/grey result and the marijuana sample produced a blue result.

iv. Test Tube vs. Spot Plate Well

Whether the 4-AP chemical test is performed in a test tube or a well-plate is also not a limiting factor of the test. The same results were obtained in a test tube and a well-plate for a titration curve of varying concentrations of THCA (0.1%, 0.3%, 1%, 3%, and 5%) with a constant 1% concentration of CBD. This indicates that the 4-AP chemical test can be performed using either test tubes or a spot plate well without any effects on the color results.

2. Analysis of Plant Material

a. Inter-Laboratory Comparison

A comparison of results from the DEA and DFS, with their associated CBD and THC concentrations, are in Table 5. Additionally, photographs for these samples are shown in Figure 2. The results confirmed that the 4-AP chemical test color result is based on the ratio of THC concentration to CBD concentration in the plant material. Plant material with a THC concentration greater than the CBD concentration produced a blue result and plant material with a THC concentration less than the CBD concentration produced a pink result. Samples with a nearly equal concentration of THC to CBD produced

an inconclusive result. A result was classified as inconclusive when the sample initially produced a pink result and later turned blue or when a color other than pink or blue developed within the two-minute observation window. After the two-minute observation window, all of the color results became significantly darker than the initial color produced. Given the ratio nature of the test, a plant material sample with a THC concentration greater than 1% may produce a pink result if the CBD concentration is greater than THC. This is shown in sample 82. Furthermore, a THC concentration of less than 1% may produce a blue result if the CBD concentration is less than the THC concentration. This is shown in several samples in Table 1, including sample 9, 14, 17, 19, 60, 61, and 83. The overwhelming agreement between results observed by DFS and DEA demonstrates the robustness of the typification test, while also confirming that the 4-AP chemical test is not based on the actual concentration of THC, but rather the ratio of THC to CBD.

b. Freshly Harvested Agricultural Plant Material

Two samples gave inconclusive results producing a grey color with a hint of blue or purple. All other samples produced a pink result or a dark pink nearly purple result. As indicated by the THC to internal standard ratio for GC-FID-MS analysis, all samples produced a ratio less than one indicating a concentration of THC to be less than 2%. These samples had significantly higher concentrations of CBD when comparing the CBD and THC response using GC-FID-MS.

The two inconclusive results noted with the 4-AP chemical test that did not corroborate with the GC-FID-MS results may be due to the fact that the ratio of THC to CBD on the GC-FID-MS screen is based off of total THC which concentration includes decarboxylated THCA. These plant material samples may have low enough THC concentrations to produce an inconclusive result with the 4-AP chemical test.

c. NIDA Samples

All seven samples produced inconclusive color results that were a grey/blue color indicating that the concentrations of THC and CBD were nearly equal. From the certificate of analysis provided with the samples, the ratio of THC to CBD concentration was calculated, producing values of 0.605, 0.596, 0.517, 0.418, 0.492, 0.377, and 0.294. These are consistent with the inconclusive color results observed, as the

THC to CBD ratios are within or near the uncertainty region of 0.3 to 3, previously reported by Hadener *et al* to produce inconclusive results. These results are summarized in Table 6.

d. Suspected Marijuana Casework

Suspected marijuana plant material was analyzed with the 4-AP chemical test and verified using the GC-FID-MS 2% THC screen method. All samples produced a blue result indicating that the THC concentration was greater than the CBD concentration, with the exception of sample 3, 7, 16, and 21. Sample 3, 7, and 21 produced a purple or inconclusive result, indicating that the concentrations of THC and CBD were nearly equal. Samples 3, 7, and 21 showed CBD peak areas higher than THC, but within the inconclusive range previously discussed. Sample 16 produced a pink result, indicating that the THC concentration was less than the CBD concentration. Comparison of the 4-AP chemical test color results with the GC-FID-MS analytical method confirmed the results. Sample 16 produced a pink result, indicating that the THC concentration was less than the CBD concentration. Comparison with the GC-FID-MS method confirmed the results because the ratio of THC to internal standard was less than one. These results are summarized in Table 7.

e. Immature Plant Material

After heating the immature plant samples on a heating block for one hour at approximately 90°C a faint blue result was observed well after the two minute observation period. No reactions were observed with all other conditions. For the second immature plant sample, after the two minute observation window, a blue/grey color was produced. This indicates that there may be less THCA present in the immature plants prior to flowering than anticipated. Therefore, immature plant material samples may not produce a reaction within the one to two-minute observation window of the 4-AP chemical test.

f. Limitations and Interferences Evaluation from Household Spices

No significant reaction was noted for any of the household spices tested, except for sage and oregano. Sage produced a light blue color change and oregano produced a blue result, showing potential for false positives.

3. Evaluation of Oil Products

A total of five hemp oil products, four hemp oil syringes, and one sample of CBD crystals were evaluated using the 4-AP reagents. All ten samples produced a pink color result, with the exception of oil 1 which produced a blue color result. Upon HPLC-DAD quantitation, oil 2 contained less than 1% CBD, less than 1% THCA, and no THC was detected. Oils 3 and 4 contained less than 1% CBD and no THC or THCA was detected. All four syringe oils contained less than 1% THC and THCA. Syringe oil 1 contained 14.4% CBD, syringe oil 2 contained 18.9% CBD, syringe oil 3 contained 26.1% CBD, and syringe oil 4 contained 16.9% CBD. The CBD crystals contained 99% CBD and no THC was detected. These results are summarized in Table 8.

Eighteen oil products from casework were evaluated using the 4-AP reagents. Casework oils 1, 2, 4, 5, 6, 7, and 8 were evaluated in triplicate and the remainder of the oils were evaluated in a single analysis due to a limited sample size. Oils 1, 4, 5, 6, 7, 8, and 10 produced blue/purple results and oils 2, 3, 9, 11, 12, 13, 14, 15, 16, 17, and 18 produced blue results. Based on these results, it is expected that all eighteen casework oil samples tested have a THC concentration greater than the CBD concentration. However, the samples should be quantified using HPLC-DAD to confirm the ratios.

These results show that the 4-AP chemical test may have the potential ability to evaluate oil products based on the ratio of THC and CBD, as it does with reference materials and plant material. It was expected for all of the oil products to produce a pink result, since they were advertised as hemp products and the quantitative results showed that the CBD concentrations were greater than the THC concentrations. The concentrations of THC, THCA, and CBD were not determined for Oil 1 using HPLC-DAD, but it is possible that the blue result obtained is a false positive due to the presence of oregano, as indicated on the bottle. When evaluated individually, oregano produced a blue result with the 4-AP test. Quantitation using HPLC-DAD should be performed to confirm the false positive. Quantitation should also be performed on the casework samples evaluated to further assess the ability of the 4-AP test to evaluate oils. Additional samples should be tested prior to extending the 4-AP chemical test to oil products.

4. Evaluation of Food Products

The honey edible and Spot fruit chews (Indica and Sativa) with 10 mg of THC produced blue results when analyzed with the 4-AP test. The Marmas blue raspberry soft candy and Magic Kitchen pebbles produced pink results. The Spot fruit chew (Indica) was quantitated and consisted of 10.90% THC and 0.08% CBD and the Spot fruit chew (Sativa) consisted of 9.90% THC and 0.09% CBD. The honey edible, Marmas soft candy, and Magic Kitchen pebbles were not quantitated. These results are summarized in Table 9. These results indicate that the 4-AP test is based on the ratio of THC and CBD in fruit/candy/gummy food materials that contain these cannabinoids as seen with plant material samples.

None of the food products that contained chocolate, including the purchased chocolate products and the products created in the Forensic Toxicology and Specialty Testing Laboratory had any significant color change with the addition of the 4-AP reagents. They did not produce blue or pink results. The Spot classic brownie (Indica) was quantitated and consisted of 9.20% THC and less than 0.5% CBD and the Sativa brownie consisted of 10.90% THC and less than 0.5% CBD. Based on these concentrations, it would be expected that the 4-AP test would result in a blue color with these products. Additionally, the peanut butter cups made in the Forensic Toxicology and Specialty Testing Laboratory contained 5 mg and 10 mg of THC and no CBD, so it would also be expected that the 4-AP test would result in a blue color. These results are also summarized in Table 9.

The mechanism behind why fruit/candy/gummy food products produce expected results with the 4-AP chemical test and chocolate food products do not must be further explored. However, chocolate contains a small amount of caffeine and larger amounts of theobromine, both stimulants that are almost identical except for one methyl group (23). It is possible the structure of these compounds may play a role in the lack of reaction obtained with the 4-AP chemical test in chocolate food products but this reaction should be further investigated.

None of the beverage products that were tested produced a significant reaction, with the exception of the black coffee, green tea, and tea with sugar. The black coffee produced a blue result and the green tea and tea with sugar produced faint pink results. This indicates that black coffee containing cannabinoids may

produce a false positive with the 4-AP chemical test. Since all of the beverage products consisted of 0.014% THC, CBD, and CBN, it would be expected that all of the results were inconclusive (purple or grey) because the concentrations of THC and CBD are equal. The mechanism behind why only these select three beverage products produced a reaction, especially different color reactions when the THC and CBD concentrations were the same, and the other fifteen beverages did not should be further explored. The 4-AP chemical test does not appear to be a viable screening method for evaluating beverage products for the presence of THC and CBD.

5. *Cannabis Typification Field Test Kit Stability Study*

    a. *Temperature Stability*

At a time period of zero, two replicates of the marijuana sample produced a blue reaction in the Cannabis Typification test kit and two replicates of the hemp sample produced a pink reaction within a two-minute time period. Test kits at all time points and conditions produced the expected color result of blue for marijuana and pink for hemp in duplicate within the two-minute time period.

Additionally, two marijuana and two hemp samples were tested at the 24-hour time point, immediately after removal from the refrigerator and freezer. Expected results were obtained. This portion of the stability study was performed to evaluate the effects of extreme temperatures that are possible in a realistic field setting. Changes in temperature from hot to cold and cold to hot do not seem to affect the stability of the 4-AP reagents inside of the kits. Law enforcement officers will likely be in a setting where the kits have been stored in their vehicles in warm or cold temperature and need to be used for immediate testing. The results obtained demonstrate the ability for the test kits to perform in these situations.

    b. *Ultraviolet Light Stability*

After one week of exposure to UV light in the laboratory window, expected results were obtained. After two, three, and four weeks of exposure to UV light, the ampules inside of the test kit began to discolor and appear a light pink instead of clear. As time went on the pink discoloration became darker. This was not observed in the test kits from the temperature stability study that were not exposed to UV light. Although

a color change was observed in the ampules, it did not have an impact on the color result and expected results were obtained.

6. TLC and GC-FID-MS Analysis

A summary of results obtained from the 4-AP chemical test, TLC, and GC-FID-MS screen for the plant material samples obtained from DCLS and used for the inter-laboratory comparison is described in Table 10. The analytical scheme requires the use of the 4-AP chemical test (optional), TLC, and GC-FID-MS method. To further understand the individual tests, a comparison of all three was done. The production of a blue result with the 4-AP chemical test and/or the indication of THC and/or CBD using TLC plus a THC to internal standard ratio greater than two using the GC-FID-MS screening method would result in a positive marijuana conclusion.

All samples in Table 10, with the exception of 8, 9, 14, 17, 19, 34, 46, 60, 61, 70, 73, and 83 produced a pink result with the 4-AP chemical test and were positive for both THC and CBD when TLC was performed. Samples 8, 9, 14, 17, 19, 60, 61, and 83 produced a blue color result and samples 34, 46, 70, and 73 produced inconclusive results. The TLC results for samples 8, 9, 14, 17, and 19 resulted in a positive THC spot and no CBD spot was indicated. Sample 61 was positive for THC and CBD with TLC, had a THC to internal standard ratio of 0.513, no CBD was detected using GC-FID-MS, and according to the DCLS quantitation had a THC concentration of 0.82% and a CBD concentration of 0.07%. Therefore, the blue result with the 4-AP chemical test is expected.

For all thirty-six samples evaluated using GC-FID-MS, the ratio of THC to internal standard was less than the administratively established ratio of two. Given the testing scheme criteria for establishing a positive marijuana result, none of the processed DCLS agricultural samples would be reported as marijuana and would not need to be fully quantified using HPLC-DAD to differentiate between marijuana and hemp.

In addition to the processed agricultural plant material samples, the freshly harvested agricultural plant material, suspected marijuana plant material, immature marijuana plant material, and NIDA reference plant material samples were all evaluated with the analytical scheme. Based on the results obtained from all sample types, correlation data was obtained and the proposed analytical scheme was verified.

Inconclusive results may vary with the 4-AP chemical test and presence of THC and CBD using TLC, but all inconclusive results were corroborated with the GC-FID-MS results within this verification.

7. HPLC-DAD Analysis

The HPLC-DAD method was used to evaluate the concentrations of THC, THCA, and CBD in the NIDA reference plant material samples previously discussed. Results obtained were not comparable to the results provided to DFS when the samples were initially tested and therefore, the method is still in the method development and validation process. Once the validation of this method is complete, all plant material samples validated using the 4-AP chemical test, TLC, and GC-FID-MS methods were expected to be analyzed using HPLC-DAD. This would complete the validation of the entire analytical scheme to differentiate marijuana and hemp.

However, since the completion of this project, there has been a legislative shift regarding cannabinoid quantitation. The language has changed from requiring quantitative values for both THC and THCA to allowing quantitation of total THC, which includes THC and post-decarboxylated THCA. Therefore, either HPLC or GC-FID can be utilized for quantitative analysis. The legislation regarding the legalization and decriminalization of marijuana is constantly changing.

Conclusions

Based on the correlation data presented, the proposed analytical testing scheme for the differentiation of marijuana and hemp plant material samples including TLC and a GC-FID-MS screen is validated. Evaluations were performed on processed agricultural samples, freshly harvested agricultural samples, suspected marijuana samples, immature plant material, and reference plant material to ensure the validity of the scheme. A blue result from the 4-AP test, a TLC result positive for THC and/or CBD, and a ratio greater than two with the GC-FID-MS method is indicative of marijuana. Inconclusive results may be observed with the 4-AP chemical test and when visualizing THC and CBD using TLC. The inconclusive results seen were corroborated with the THC to internal standard ratio and THC to CBD ratio obtained from the GC-FID-MS method.

This validation indicates that the 4-AP chemical test is a viable screening option within the proposed analytical scheme for evaluating plant material samples for the differentiation of marijuana and hemp. The results obtained with the 4-AP test were corroborated with TLC and GC-FID-MS method results.

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Figure 1: Photograph of Individual Cannabinoid Reference Material Color Results

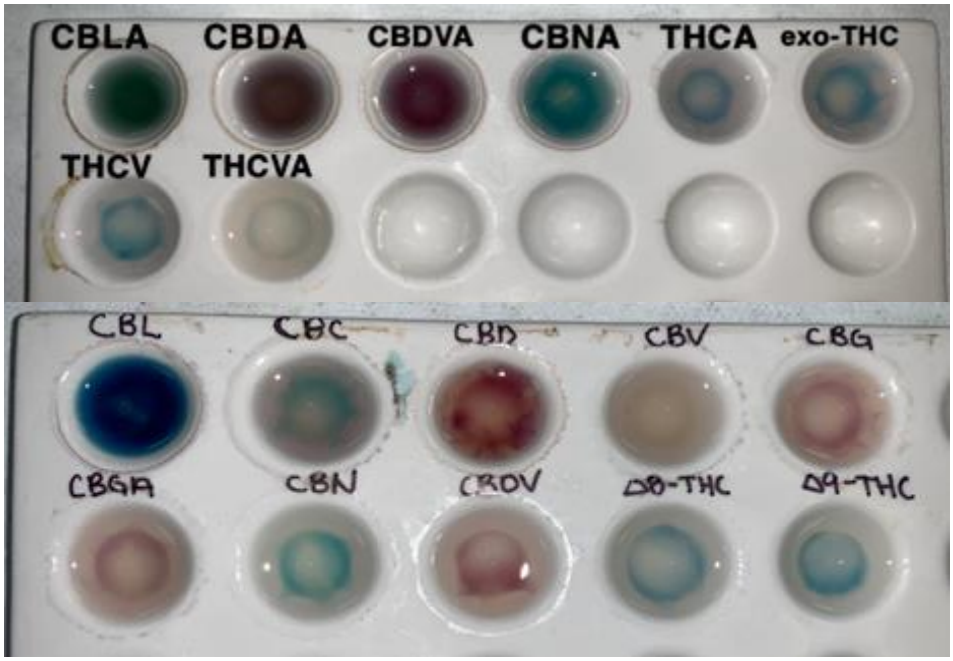


Table 1: 4-AP Chemical Test Results for Varying Concentrations of THC in the Presence of 1% CBD

	Color Results		
Sample	1	2	3
1% CBD, 0.1% THC	Pink	Pink	Pink
1% CBD, 0.3% THC	Pink	Pink	Pink
1% CBD, 1% THC	Pink	Pink	Pink then Blue
1% CBD, 2% THC	Pink	Pink	Purple
1% CBD, 3% THC	Blue	Blue	Blue
1% CBD, 4% THC	Blue	Blue	Blue
1% CBD, 5% THC	Blue/Purple	Blue	Blue

Table 2: 4-AP Chemical Test Results for Varying Concentrations of CBD in the Presence of 0.3% THC

	Color Results		
Sample	1	2	3
0.3% THC, 0.1% CBD	Blue	Blue	Blue
0.3% THC, 0.3% CBD	Purple	Purple	Purple
0.3% THC, 0.5% CBD	Purple	Purple	Purple
0.3% THC, 0.8% CBD	Purple	Purple	Purple
0.3% THC, 1% CBD	Pink	Pink	Pink
0.3% THC, 2% CBD	Pink	Pink	Pink
0.3% THC, 5% CBD	Pink	Pink	Pink

Table 3: 4-AP Chemical Test Results for Varying Concentrations of Cannabinoids with 0.3% THC

	0.3 % THC				
Cannabinoid	0.10%	0.30%	1%	2%	5%
Δ^8-THC	Blue	Blue	Blue	Blue	Blue
exo-THC	Blue	Blue	Blue	Blue	Blue
THCV	Blue	Blue	Blue	Blue	Blue
THCVA	Blue	Blue	Blue	Blue	Blue
THCA	Blue	Blue	Blue	Blue	Blue
CBDA	Blue	Blue	Blue	Purple	Purple
CBDV	Blue	Purple	Pink then Purple	Pink	Pink
CBDVA	Blue	Blue	Blue	Purple	Pink
CBN	Blue	Blue	Blue	Blue	Blue
CBNA	Blue	Blue	Blue	Blue	Blue
CBL	Blue	Blue	Blue	Blue	Blue
CBLA	Blue	Blue	Blue	Blue	Blue
CBG	Blue	Purple	Pink	Pink	Pink
CBGA	Blue	Blue	Purple	Purple	Purple
CBC	Blue	Blue	Blue	Blue	Blue
CBV	Blue	Blue	Blue	Blue	Blue

Table 4: 4-AP Chemical Test Results for Varying Concentrations of Cannabinoids with 1 % CBD

	1% CBD				
Cannabinoid	0.10%	0.30%	1%	2%	5%
Δ^8-THC	Pink	Pink	Blue/Purple	Purple	Blue
exo-THC	Pink	Pink	Blue/Purple	Purple	Blue
THCV	Pink	Pink	Blue/Purple	Blue/Purple	Blue
THCVA	Pink	Pink	Pink	Pink	Pink
THCA	Pink	Pink	Pink	Pink	Pink
CBDA	Pink	Pink	Pink	Pink	Pink
CBDV	Pink	Pink	Pink	Pink	Pink
CBDVA	Pink	Pink	Pink	Pink	Pink
CBN	Pink	Pink	Pink	Pink	Pink
CBNA	Pink	Pink	Pink	Pink	Pink
CBL	Pink	Pink	Blue	Blue	Blue
CBLA	Pink	Pink	Pink	Pink	N/A
CBG	Pink	Pink	Pink	Pink	Pink
CBGA	Pink	Pink	Pink	Pink	Pink
CBC	Pink	Pink	Pink	Pink	Pink
CBV	Pink	Pink	Pink	Pink	Pink

Table 5: Inter-Laboratory Comparison of 4-AP Chemical Test Results on DCLS Preprocessed Plant Material Samples, DEA vs. DFS

Sample	% THC	% CBD	DFS Results	DEA Results	DFS Typification	DEA Typification
1	0.14	2.81	Pink to purple	Pink	Pink	Pink
2	0.16	3.42	Pink to purple	Pink	Pink	Pink
6	0.31	4.96	Pink to purple	Pink	Pink	Pink
8	1.03	0.05	Blue	Blue	Blue	Blue
9	0.73	0.05	Blue	Blue	Blue	Blue
10	< 0.10	1.81	Pink to purple	Pink	Pink	Pink
14	0.69	0.14	Blue	Blue	Blue	Blue
17	0.81	0.14	Blue	Blue	Blue	Blue
19	0.43	0.14	Blue	Blue	Blue	Blue
21	0.46	10.60	Pink to purple	Pink	Pink	Pink
25	0.18	5.04	Pink to purple	Pink	Pink	Pink
27	0.24	6.64	Pink to purple	Pink	Pink	Pink
29	0.22	4.93	Pink to purple	Pink	Pink	Pink
31	0.11	3.61	Pink to purple	Pink	Pink	Pink
32	0.20	6.52	Pink to purple	Pink	Pink	Pink
34	1.29	2.00	Pink then blue/grey	Pink to dark blue	Inconclusive	Inconclusive
35	0.26	7.72	Pink to purple	Pink	Pink	Pink
37	0.21	6.19	Pink to purple	Pink	Pink	Pink
43	0.11	1.32	Pink to grey	Pink	Pink	Pink
44	< 0.10	1.05	Pink to grey	Pink	Pink	Pink
46	0.11	0.64	Pink to grey	Pink	Pink	Pink
52	0.46	10.05	Pink to purple	Pink	Pink	Pink
60	0.38	0.10	Blue	Blue	Blue	Blue
61	0.82	0.07	Blue	Blue	Blue	Blue
69	0.29	11.04	Pink to purple	Pink	Pink	Pink
70	2.27	3.99	Pink then blue/grey	Pink to dark blue	Inconclusive	Pink
71	0.97	6.37	Pink to purple	Pink	Pink	Pink
73	2.89	4.07	Pink then blue/grey	Pink to dark blue	Inconclusive	Pink
74	0.39	11.51	Pink to purple	Pink	Pink	Pink
75	0.56	14.18	Pink to purple	Pink	Pink	Pink
77	2.71	5.94	Pink then blue/grey	Pink to dark blue	Inconclusive	Pink
79	0.59	15.62	Pink to purple	Pink	Pink	Pink
81	0.43	13.64	Pink to purple	Pink	Pink	Pink
82	2.05	10.25	Pink to purple	Pink	Pink	Pink
83	0.90	0.05	Blue	Blue	Blue	Blue
85	0.36	8.15	Pink to purple	Pink	Pink	Pink
3	0.15	2.78	Pink to purple	N/A	Pink	N/A
12	0.15	1.25	Pink to purple	N/A	Pink	N/A
13	0.13	0.60	Pink to purple	N/A	Pink	N/A
22	0.47	11.85	Pink to purple	N/A	Pink	N/A
76	0.27	8.27	Pink to purple	N/A	Pink	N/A
84	0.20	5.32	Pink to purple	N/A	Pink	N/A

Figure 2: 4-AP Chemical Test Photograph for Preprocessed Plant Material

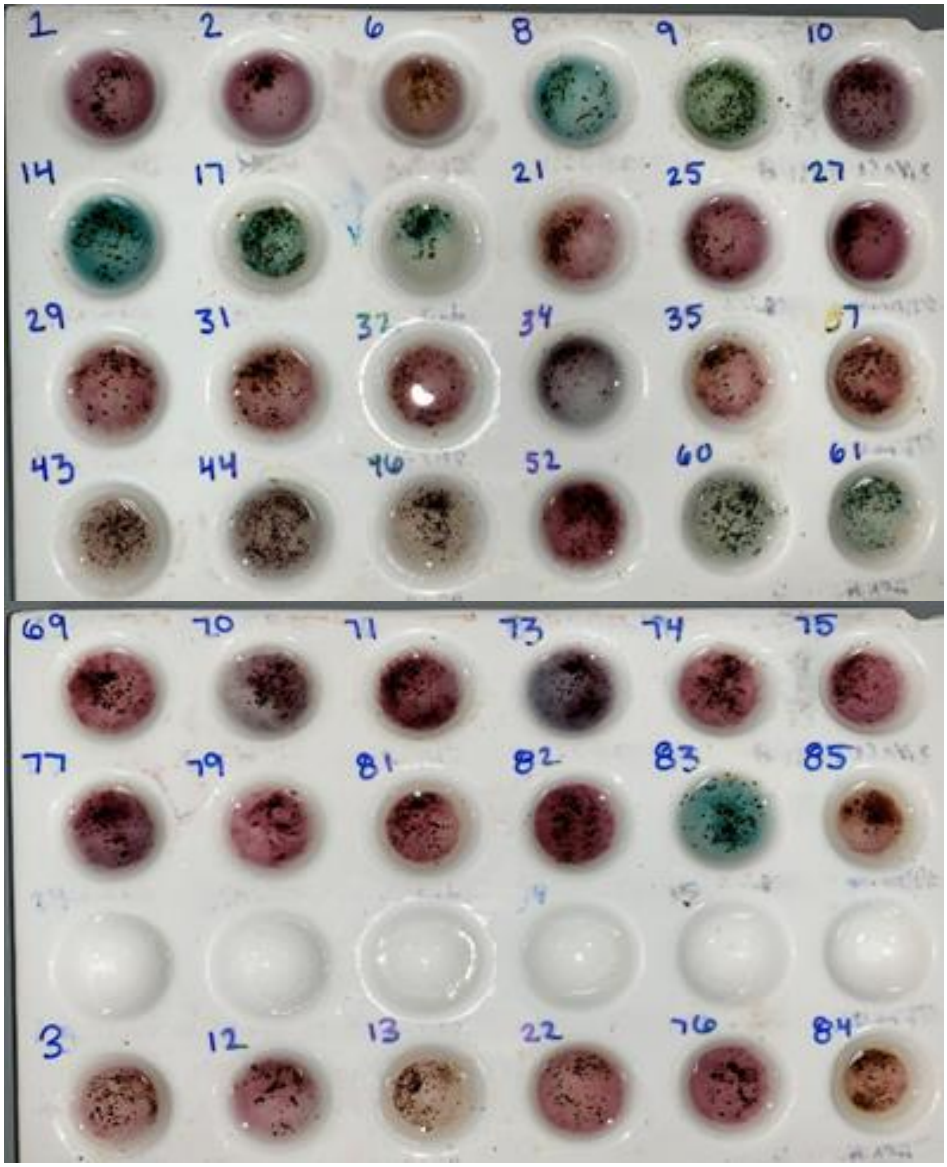


Table 6: 4-AP Chemical Test Results for NIDA Plant Material Samples with THC and CBD Concentrations

NIDA Sample	Color Results	% THC	% CBD	Ratio of THC to CBD
2018-1445	Inconclusive	3.03	5.01	0.605
2018-1447	Inconclusive	2.46	4.13	0.596
2019-1464	Inconclusive	2.97	5.74	0.517
2019-1465	Inconclusive	2.02	4.83	0.418
2019-1466	Inconclusive	1.84	3.74	0.492
2019-1467	Inconclusive	1.20	3.18	0.377
2019-1468	Inconclusive	0.94	3.20	0.294

Table 7: 4-AP Chemical Test Results for Plant Material from Casework

	Color Results		
Sample	1	2	3
1	Blue	Blue	Blue
2	Blue	Blue	Blue
3	Purple	Purple	Purple
4	Blue	Blue	Blue
5	Blue	Blue	Blue
6	Blue	Blue	Blue
7	Blue/Purple	Blue/Purple	Blue/Purple
8	Blue	Blue	Blue
9	Blue	Blue	Blue
10	Blue	Blue	Blue
11	Blue	Blue	Blue
12	Blue	Blue	Blue
13	Blue	Blue	Blue
14	Blue	Blue	Blue
15	Blue	Blue	Blue
16	Pink	Pink	Pink
17	Blue	Blue	Blue
18	Blue	Blue	Blue
19	Blue	Blue	Blue
20	Blue	Blue	Blue
21	Inconclusive	Blue/Purple	Inconclusive
22	Blue	Blue	Blue
23	Blue	Blue	Blue

Table 8: 4-AP Chemical Test Results for Purchased Hemp Oils and Syringe Oils with Concentrations

	Color Results					
Sample	1	2	3	% THC	% CBD	% THCA
Oil #1	Blue	Blue	Blue			
Oil #2	Pink	Pink	Pink	ND	<1	<1
Oil #3	Pink	Pink	Pink	ND	<1	ND
Oil #4	Pink	Pink	Pink	ND	<1	ND
Oil #5	Pink	Pink	Pink			
Syringe Oil #1	Pink	Pink	Pink	<1	14.4	<1
Syringe Oil #2	Pink	Pink	Pink	<1	18.9	<1
Syringe Oil #3	Pink	Pink	Pink	<1	26.1	<1
Syringe Oil #4	Pink	Pink	Pink	<1	16.9	<1
Endoca CBD Crystals	Pink	Pink	Pink	ND	99	

Table 9: 4-AP Chemical Test Results for Food Products

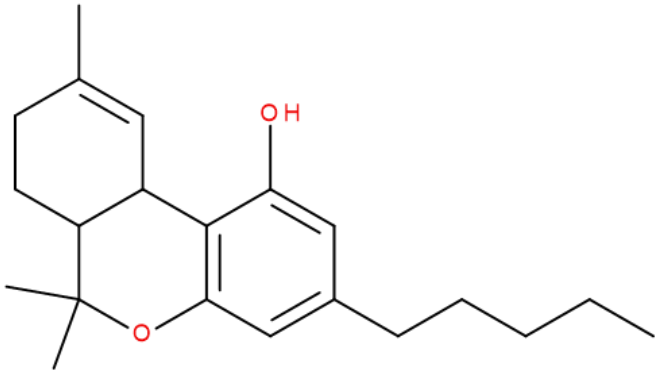
	Color Results				
Sample	1	2	3	% THC	% CBD
Honey Edible	Blue	Blue	Blue		
Marmas Blue Raspberry soft candy	Faint Pink	Faint Pink	Faint Pink		
Magic Kitchen Pebbles	Pink	Pink	Pink		
Spot mixed fruit chews, Indica, 10-mg THC	Blue	Blue	Blue	10.90%	0.08%
Spot mixed fruit chews, Sativa, 10-mg THC	Blue	Blue	Blue	9.90%	0.09%
Spot CBD dark chocolate bar	NSR	NSR	NSR		
Baked Botanicals Don't Be Square, Peanut Butter Cups	NSR	NSR	NSR		
Spot classic brownie, Indica, 10-mg THC	NSR	NSR	NSR	9.20%	<0.5%
Spot classic brownie, Sativa, 10-mg THC	NSR	NSR	NSR	10.90%	<0.5%
PB cups, 5mg	NSR	NSR	NSR		
PB cups, 10 mg	NSR	NSR	NSR		

Table 10: DCLS Samples 4-AP Chemical Test, TLC, and GC-FID-MS Screen Results Summary with Concentrations

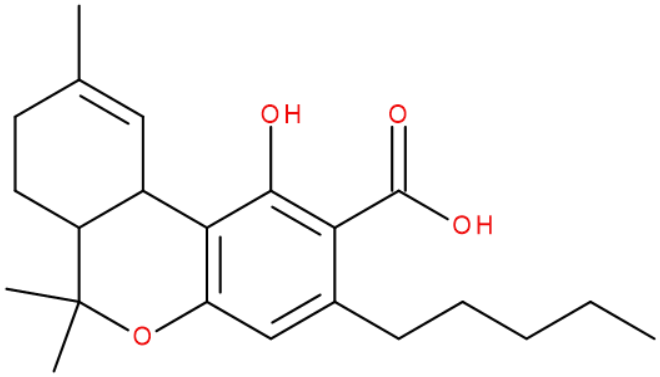
Sample	% THC	% CBD	4-AP Results	TLC Result	THC/CBD Ratio (GC-FID)
1	0.14	2.81	Pink	+CBD, +THC	0.141
2	0.16	3.42	Pink	+CBD, +THC	0.124
6	0.31	4.96	Pink	+CBD, +THC	0.195
8	1.03	0.05	Blue	+THC	0.738
9	0.73	0.05	Blue	+THC	0.382
10	< 0.10	1.81	Pink	+CBD, +THC	0.056
14	0.69	0.14	Blue	+THC	0.638
17	0.81	0.14	Blue	+THC	1.165
19	0.43	0.14	Blue	+THC	0.717
21	0.46	10.60	Pink	+CBD, +THC	0.366
25	0.18	5.04	Pink	+CBD, +THC	0.156
27	0.24	6.64	Pink	+CBD, +THC	0.166
29	0.22	4.93	Pink	+CBD, +THC	0.139
31	0.11	3.61	Pink	+CBD, +THC	0.103
32	0.20	6.52	Pink	+CBD, +THC	0.122
34	1.29	2.00	Inconclusive	+CBD, +THC	0.584
35	0.26	7.72	Pink	+CBD, +THC	0.149
37	0.21	6.19	Pink	+CBD, +THC	0.133
43	0.11	1.32	Pink	+CBD, +THC	0
44	< 0.10	1.05	Pink	+CBD, +THC	0
46	0.11	0.64	Pink	+CBD, +THC	0
52	0.46	10.05	Pink	+CBD, +THC	0.174
60	0.38	0.10	Blue	+CBD, +THC	0.292
61	0.82	0.07	Blue	+CBD, +THC	0.513
69	0.29	11.04	Pink	+CBD, +THC	0.211
70	2.27	3.99	Inconclusive	+CBD, +THC	1.181
71	0.97	6.37	Pink	+CBD, +THC	0.640
73	2.89	4.07	Inconclusive	+CBD, +THC	1.558
74	0.39	11.51	Pink	+CBD, +THC	0.348
75	0.56	14.18	Pink	+CBD, +THC	0.476
77	2.71	5.94	Inconclusive	+CBD, +THC	1.560
79	0.59	15.62	Pink	+CBD, +THC	0.519
81	0.43	13.64	Pink	+CBD, +THC	0.350
82	2.05	10.25	Pink	+CBD, +THC	1.424
83	0.90	0.05	Blue	+CBD, +THC	0.496
85	0.36	8.15	Pink	+CBD, +THC	0.308
3	0.15	2.78	Pink	+CBD, +THC	No Analysis
12	0.15	1.25	Pink	+CBD, +THC	No Analysis
13	0.13	0.60	Pink	+CBD, +THC	No Analysis
22	0.47	11.85	Pink	+CBD, +THC	No Analysis
76	0.27	8.27	Pink	+CBD, +THC	No Analysis
84	0.20	5.32	Pink	+CBD, +THC	No Analysis

Appendix A: Cannabinoid Structures

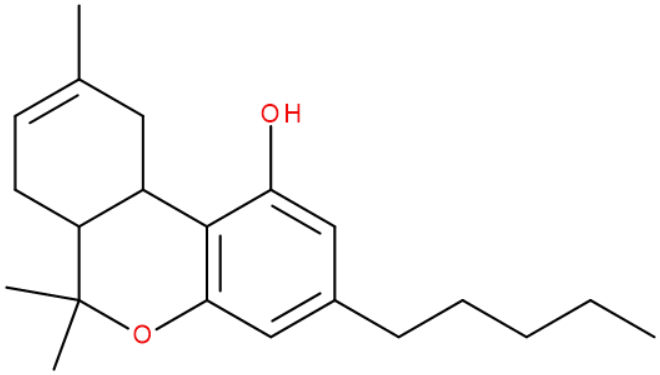
Δ^9-THC



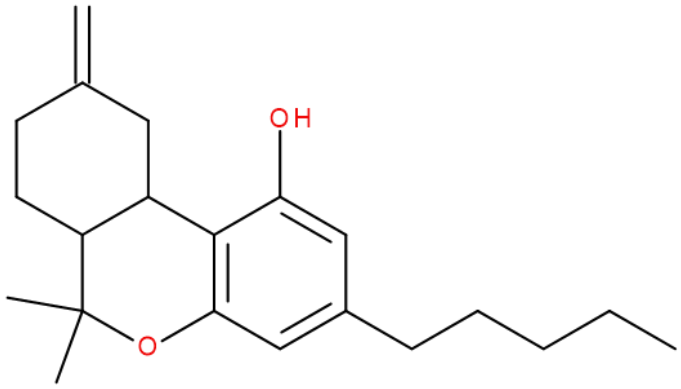
Δ^9-THCA



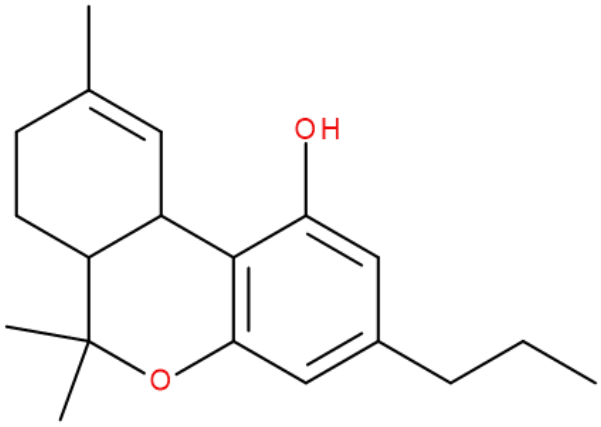
Δ^8-THC



exo-THC



THCV



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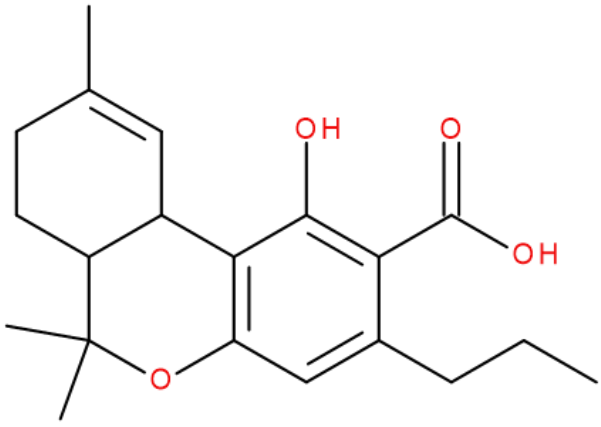

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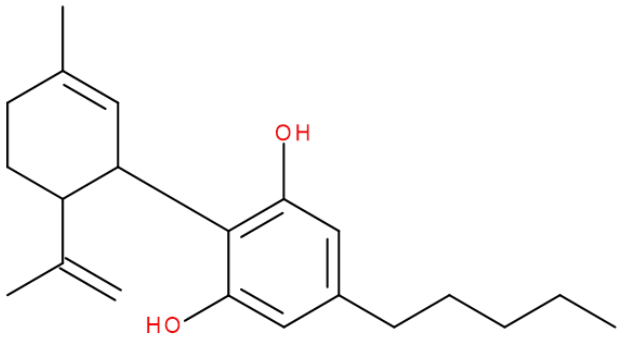

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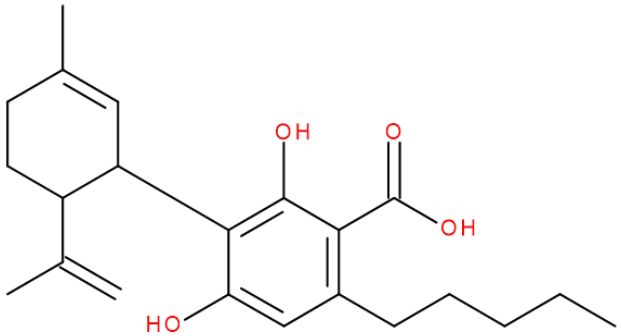
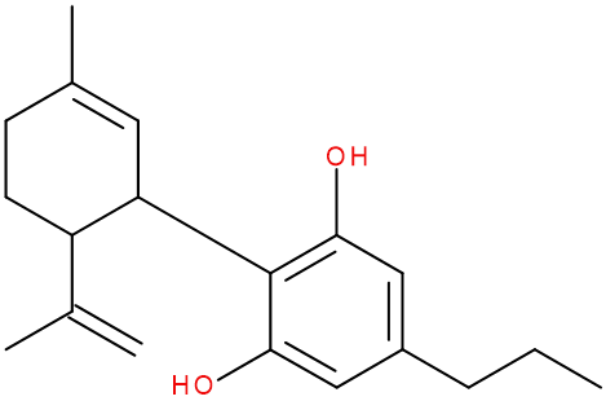


CBDV



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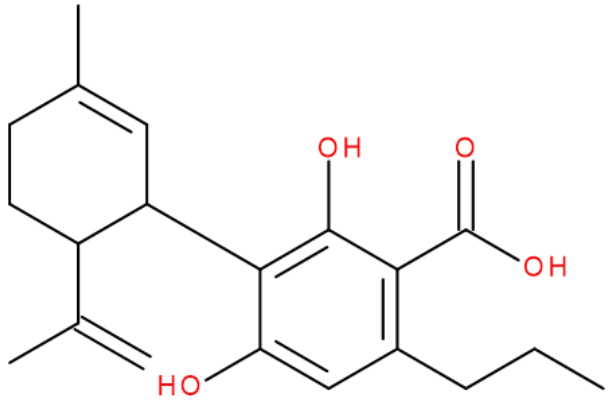

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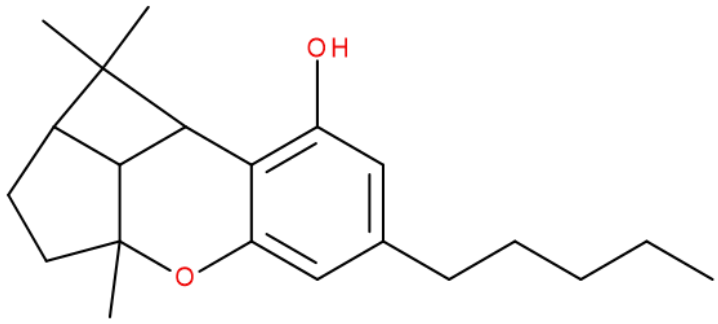
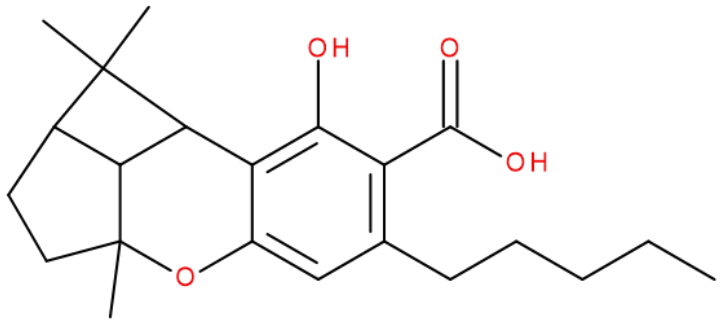
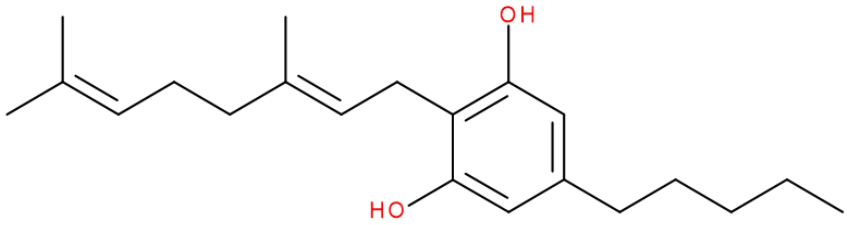


CBLA



CBG



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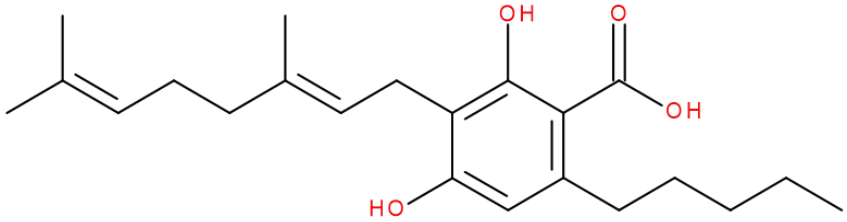

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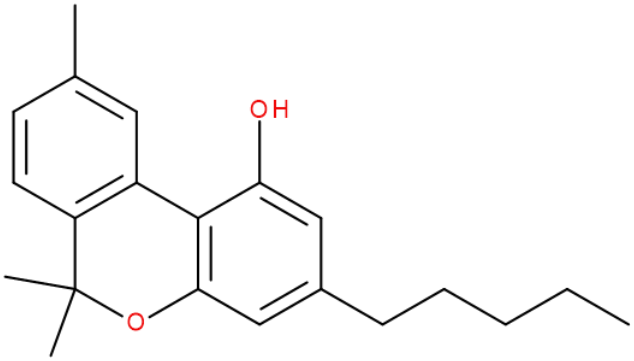

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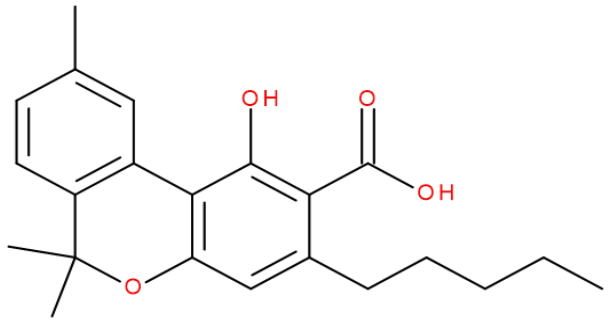

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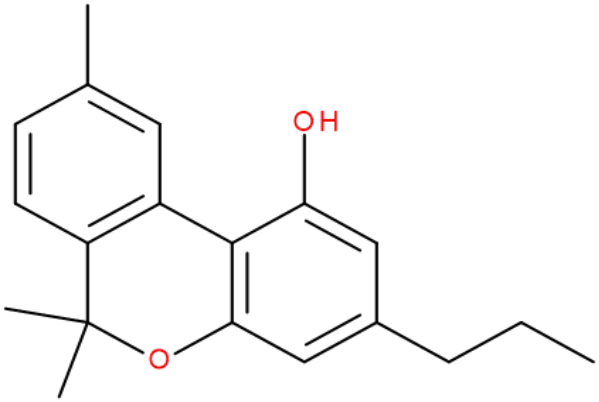

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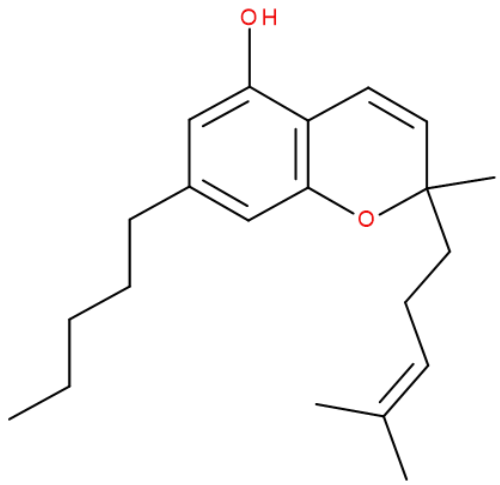

Vita

Kenna L. Lewis was born on November 27, 1996, in Buffalo, New York. She graduated from Sweet Home High School, Amherst, New York, in 2014. She received her Bachelors of Science in Forensic Science Technology, Bachelors of Science in Health Sciences, and Associates of Applied Science in Biological Sciences from Alfred State College, Alfred, New York in 2018. She will be graduating from Virginia Commonwealth University with her Masters in Forensic Science with a concentration in Drug Chemistry and Toxicology in May 2020. Kenna is employed at the Virginia Department of Forensic Science as a Forensic Administrative Assistant.