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Differentiation between hemp-type and marijuana-type cannabis using the Fast Blue BB colorimetric test

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A B S T R A C T

The Fast Blue BB (FBBB) colorimetric test has been shown to differentiate between the different cannabinoids found in cannabis addressing the urgent need to distinguish between hemp-type cannabis, which is legal in every state of the US, from marijuana-type cannabis (≥0.3% w/w THC). FBBB forms a red chromophore in the presence of THC, an orange chromophore in the presence of CBD, a detectable fluorophore with THC but no fluorescence with CBD. We report, for the first time, a miniaturized reaction directly on a 3.5 mm diameter solid substrate of a material previously developed in our laboratory using a methanol extract from ~10 mg of plant sample. Different cannabinoids, various herbs and spices, and authentic cannabis samples were tested with the optimized color test, and the FBBB reaction was found selective for THC relative to other cannabinoids and herbs and spices. RGB (Red, Green, Blue) numerical codes were obtained for each color (and fluorescence) image produced by the reaction. Linear Discriminant Analysis (LDA) results correctly classify a set of 25 known plant samples (marijuana, hemp and herbs and spices) as either containing THC levels below 0.3% (w/w) (hemp) or as containing high THC levels (≥0.3% w/w) and low CBD levels (marijuana). Cannabis samples containing low THC (but just above 0.3%) and high CBD (THC:CBD ratios <2) were incorrectly classified, however. None of the herbs or spices tested were incorrectly classified as either hemp or marijuana.

Introduction

The Agricultural Improvement Act of 2018 permits the cultivation and legal trade of industrial hemp in the United States. This act defines hemp as *Cannabis sativa* and any part or derivative of the plant including seeds, extracts, cannabinoids, isomers, acids, salts, and salts of isomers with a total delta-9 tetrahydrocannabinol (THC) concentration below 0.3 % (w/w) on a dry weight basis [1]. This statute removed hemp-cannabis from its schedule I classification by using this definition to separate it from marijuana-type cannabis. Currently, there are no standardized methods to distinguish hemp from marijuana. Most forensic laboratories use chromatographic methods such as Gas Chromatography (GC) or High-Performance Liquid Chromatography (HPLC) methods coupled to mass spectrometry to quantitate the THC in suspicious plant materials. Furthermore, colorimetric tests that were once used to presumptively identify cannabis are not able to differentiate between hemp and marijuana, creating the need for an effective field test that can differentiate between hemp-type cannabis and marijuana-type cannabis.

Hemp and marijuana are two different strains of the Cannabis plant with the main difference between the two being the concentration of cannabinoids contained in them. The two most important cannabinoids in these plants are THC and cannabidiol (CBD). THC is the cannabinoid that causes a psychoactive response in the body giving the person a "high". It also has anti-inflammatory and analgesic properties, which make it desirable for medical use [2]. CBD is also known for these beneficial properties but is non-psychoactive, so it does not give a person a "high" when used [2]. Typically, hemp is CBD-rich containing low concentrations of THC causing its THC:CBD ratio to be below 1. Cannabis is considered marijuana when it has a concertation of total Δ^9-THC ≥ 0.3% (w/w), but usually has a THC:CBD ratio above 1 [3]. Elsohly et. al. reported that from 2009 to 2019 marijuana in the U.S. increased in THC potency across the decade from an average of 10% THC in 2009 to 14% THC in 2019. In 2019, the average CBD concentration in marijuana was found to be 0.6% (w/w), and that the THC:CBD ratio was above 20 across the decade [4]. This difference in THC:CBD ratios can be used in the design of an effective field test for the identification of marijuana-type cannabis.

The most common field tests performed for the presumptive identification of unknown drugs are colorimetric assays. These tests are considered presumptive as they only indicate the possibility of the analyte being present in the substance [5]. Until the Agricultural Improvement Act of 2018, the modified Duquenois-Levine (D-L) test was the color test used to presumptively identify a suspicious plant material as cannabis. Although used for many years, the D-L test is known to produce false positives with reaction of molecules containing a resorcinol backbone and an aliphatic chain [6,7]. Therefore, the D-L test is

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known to produce false positive results from plants such as patchouli, spearmint, and eucalyptus. Furthermore, THC, CBD, and many other cannabinoids contain both a resorcinol group and an aliphatic chain, resulting in a D-L test that is not selective enough to differentiate between the cannabinoids. This shortcoming is the reason that the D-L test is no longer a suitable field test for the identification of marijuana-type cannabis. There is now an urgent need for color tests that can differentiate between hemp (CBD-rich cannabis) and marijuana (THC-rich cannabis).

One colorimetric test that is currently being used to differentiate between hemp and marijuana is the 4-aminophenol (4-AP) test developed by the Swiss Forensic Institute in Zurich [8]. A recent validation study has shown that a pink color forms when the THC:CBD ratio is below 0.3 (CBD rich) and a blue color forms when the plant has a THC: CBD ratio above 3 (THC rich) [9]. A confirmatory chemical test such as GC-FID or GC–MS is still required after a positive 4-AP test. The test requires the use of at least 1 mL of one of its reagents, 4-aminophenol, to produce a color result. Although the 4-AP test has demonstrated capability as a presumptive test for cannabis, it has also been reported that it may not be selective for THC. False positive results have been obtained with sage, oregano, and several cannabinoids, such as cannabinol (CBN) [9]. A more selective and smaller-scale alternative presumptive test could improve the presumptive confirmation for marijuana in the field.

A colorimetric reagent that has been used for many years as a visualization reagent for cannabinoids when analyzing cannabis extracts through thin layer chromatography (TLC) is the Fast Blue BB (FBBB) reagent [10,11]. The FBBB test is selective among major cannabinoids, providing a red color for THC, an orange color for CBD, and a purple color for CBN. Ultraviolet-Visible Spectroscopy has shown that the FBBB + THC chromophore has an absorption band at 471 nm, which is responsible for its red color[12]. The Almirall lab previously reported the structure of the FBBB + THC chromophore using results from high-resolution mass spectrometry (Direct Analysis in Real Time Mass Spectrometry) and Hydrogen Nuclear Magnetic Resonance (H^1NMR). It was determined that, in basic conditions THC becomes a phenolate anion and that this anion attacks the diazo group in FBBB at the para position to form the chromophore (Fig. 1) [13]. A bathochromic (or "red") shift results from the extended conjugation in the chromophore and the $n \rightarrow \pi^*$ transition caused by the electrons in the diazo group of FBBB [10,14].

In addition to characterizing the chromophore, the previous study evaluated the selectivity of FBBB for THC detection. Eight different types of tea, 3 hop products, and 3 authentic hemp buds were extracted and tested using FBBB. This test was performed by adding 10 μL of the extract to a filter paper, followed by 10 μL of 0.1% FBBB and 0.1 N NaOH. Extracts that were made from methylene chloride produced only 1 false positive with one of the teas [13]. Of note, none of the hemp samples produced a false positive result, displaying an orange color indicative of CBD [13]. These results support the selective nature of the FBBB test for use as a presumptive field test to distinguish between hemp, marijuana, and other plant materials.

In the previous study, filter paper, a Capillary Microextraction of Volatiles (CMV) device, and CMV strips were used as possible substrates to perform the FBBB test. A CMV device is an open-ended 2 cm glass capillary tube that contains seven 2 cm by 2 mm glass filter strips have been coated with vinyl-terminated polydimethylsiloxane (vt-PDMS) that

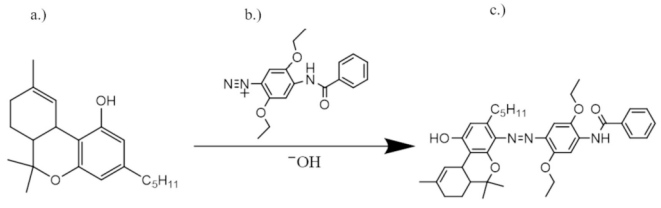

Fig. 1. Reaction between THC (a) and Fast Blue BB (b) forming the THC + FBBB (c) product chromophore/fluorophore [13].

was developed by the Almirall lab as an alternative to Solid Phase Microextraction (SPME) [15]. The modified glass filters that make up the CMV, known as Planar SPME (PSPME), have excellent absorption/adsorption capabilities and can withstand high temperatures. It was found that when the FBBB test is performed on one of the PSPME strips the LOD for THC was 100 ng, which is significantly lower than the known LOD for the D-L test, 5000 ng of THC [16]. Using PSPME as a substrate is advantageous over regular filter paper since it can withstand high temperatures allowing the chromophores formed to be detected using DART-MS with very little background [13].

In this current study, the capabilities of using FBBB as a presumptive field test to differentiate between hemp and marijuana are presented. We also report a fast and easy extraction method for plant material that can be used in the field. A previously reported substrate (PDMS-coated microfiberglass) known as PSPME support [13,17] was used for the FBBB reaction (Fig. 2). Six cannabinoids, 5 retail hemp samples, 20 authentic cannabis samples, tobacco, hops, herbs, and essential oils were tested with the FBBB reagent. RGB (Red, Green, Blue) numerical codes were obtained for each color result to confirm the color produced by the reaction in an objective manner. The fluorescence results of the FBBB + THC fluorophore is reported for the first time. The fluorescence spectra of the FBBB + THC product are distinguishable from the spectra of FBBB + CBD chromophores. The RGB score combined with the fluorescence of the FBBB + THC chromophore/fluorophore enhances the selectivity of the FBBB test for marijuana. Linear Discriminant Analysis (LDA) was performed to determine whether FBBB could be used to classify cannabis correctly as hemp-type and marijuana type.

Materials and methods

Materials

Methanol and chloroform were purchased from Sigma Aldrich (St. Louis, Missouri, United States). Methanolic solutions (1 mg/mL) of THC, CBD, cannabinol (CBN), cannabigerol (CBG), delta-9-tetrahydrocannabolic acid (THCA), and cannabidiolic acid (CBDA) were all purchased from Sigma-Aldrich. Standard working solutions of these cannabinoids were made from 1000 ppm stock solutions. A 1 mL mixture containing THC, CBD, CBG, CBN, THCA, and CBDA in acetonitrile at 500 μg/mL was purchased from Cayman Chemical (Ann Arbor, Michigan, United States). Terpene mixture 1 and terpene mixture 2, each containing 21 different terpenes (1 mg/mL) commonly found in the Cannabis plant, were also purchased from Cayman Chemical. Fast Blue BB Salt hemi (zinc chloride) was purchased from Sigma Aldrich and NaOH was purchased from Macron Fine Chemicals (Radnor Township, Pennsylvania, United States).

Spec 7 strain hemp, Purple emperor strain hemp, Eighty-Eight strain hemp, Painted Lady strain hemp, and Elektra Strain Hemp were all purchased from Blue Ridge Hemp Co. The certificates of analysis of each of the strains was reported by Blue Ridge Hemp Co.'s confirming that the cannabis purchased contained <0.3% total THC. Cigars, apollo hop pellets, citra whole leaf hops, oregano, sage, parsley, red pepper flakes, black pepper, lavender, and eucalyptus leaves were all purchased from commercial retailers. Two herb spice tobacco grinders were purchased from commercial retailers. The Cannabis research program at the National Institute of Standards and Technology (NIST) provided 20 cannabis samples, all of which had the % total THC and % total CBD previously determined through Liquid Chromatography-Photodiode Array (LC-PDA).

Reagent preparation

A 0.1% (w/v) solution of FBBB was made by dissolving 10 mg of FBBB salt in 10 mL of methanol. This solution was stored in the freezer (-20 °C) in an amber vial. A methanolic solution of 0.1 N NaOH was made by dissolving 0.4 g NaOH in 100 mL of methanol. This solution

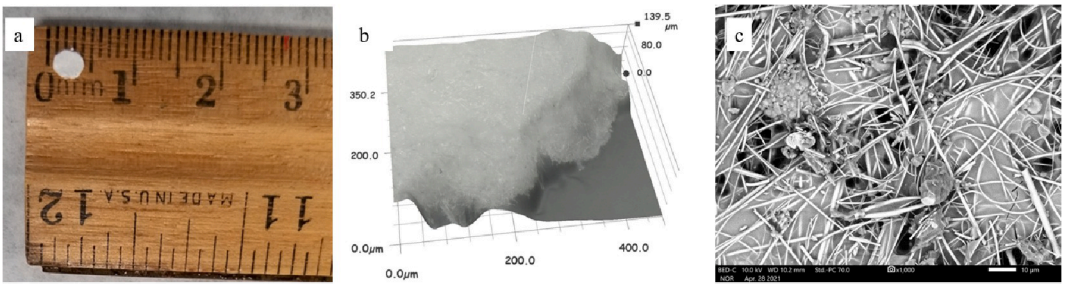

Fig. 2. Photograph of the 3.5 mm PSPME substrate (a), Depth image of the PSPME substrate at 700x (b), and a Scanning Electron Microscope image of the PSPME substate at 1000x (c).

was stored in the refrigerator (0 °C) in a clear container.

PSPME substrate preparation

The PSPME fabrication procedure has been previously described by Guerra et. al [17]. Briefly, glass fiber filters were washed, activated, and then spin-coated with a sol-gel polydimethylsiloxane. They were then cured in a high temperature oven. Once cured, the PSPME filters were cut into 3.5 mm diameter support using a Rapid Core Sampling Tool from Electron Microscopy Sciences.

Plant material extraction procedure

A 0.5 g subsample of hemp was homogenized in a spice grinder. A 10 mg sub-sample was then placed into an autosampler vial and extracted with 1 mL of MeOH:$CHCl_3$ (9:1). The vial was vortexed twice for 20 s over a 10 min extraction period. The supernatant was then pipetted from this vial into a clean amber autosampler vial. The extract was then stored in a freezer until use. This procedure was performed for all commercial hemp samples. For analysis of cigars, hops, and herbs, 10 mg was weighed out directly from the packaging for extraction. Homogenization using the spice grinder was performed on sage and eucalyptus since those plant materials were too large for this extraction directly out of the package.

Testing procedure

First, 10 µL of the plant extract or reference standards were pipetted onto the PSPME substrate. Next, 10 uL of 0.1% FBBB solution was then pipetted onto the substrate followed by 10 uL of 0.1 N NaOH. The color change was observed immediately following the addition of the NaOH solution. The solvents evaporate within 1–2 min as the color develops. A red color is indicative of THC and an orange color is indicative of CBD. The FBBB test was performed in 5 replicates per extract. Each substrate was photographed with a Dino-Lite AM4115ZT(R9) digital microscope (Dunwell Tech, Torrence, CA). A Dino-Lite AM4115T-GRFBY Digital Microscope was used to capture fluorescence images of the substrates. The Dino-Lite AM4115T-GRFBY uses a 480 nm excitation light source and contains emission filters for 510 nm and 610 nm. These images were taken in the absence of ambient light to remove interference from outside sources of light. The visible and fluorescence images are analyzed using the ImageJ software using the RGB measure plugin to obtain the average RGB numerical code across each substrate.

VSC2000 spectral analysis

A Visual Spectral Comparator 2000 (VSC2000) (Foster-Freeman) was used to obtain the fluorescence spectra of the chromophores formed by the FBBB reaction on the PSPME substrate. Magnification on the VSC2000 is automatically set at 5.5X. A spot filter was used to irradiate light from 400 nm to 540 nm onto the sample. The long pass filter was set at 590 nm. Camera integration was set at 0.2 s and the substrate was scanned from 590 nm to 1000 nm. Five spectra were collected for each substrate observed.

Results and discussion

CBD, THC, and hemp color and fluorescence results

A notable difference can be observed when FBBB is reacted with 1000 ng/µL of CBD and an extract of "Painted Lady" hemp containing 1263 ng/µL of CBD and no THC present compared to a reaction with 1000 ng/µL THC. When reacted with FBBB, the CBD solution and hemp extract both produce an orange color and THC produces a deep red color (Fig. 3a). The difference in color can be observed immediately after the NaOH is added to the reaction and is later confirmed through the chromophore's RGB code. A Dino-Lite digital microscope capable of fluorescence imaging shows that FBBB + THC fluoresces brightly under a 480 nm light source while FBBB + CBD and FBBB + Hemp do not fluoresce significantly (Fig. 3b). The fluorescence spectra obtained from the VSC2000 show a distinct difference in the fluorescence intensity and λ_{max} of FBBB + THC and FBBB + CBD and FBBB + Hemp (Fig. 3c). FBBB + THC has a fluorescence intensity near 70% and λ_{max}: 655 nm. FBBB + CBD has a fluorescence intensity below 13% and has a λ_{max}: 661 nm. Hemp + FBBB has a fluorescence intensity at 20% and a broader band with λ_{max}: 695 nm. This band at 695 nm interferes with the FBBB + CBD peak. A possible explanation for the band at 695 nm is that is chlorophyll from the plant is also extracted during the extraction and may be causing fluorescence at this wavelength. Chlorophyll is also known to absorb blue and red light and fluoresces in the red region of the visible spectrum [18]. Despite the wavelength difference between FBBB + CBD and FBBB + hemp, the intensity of the fluorescence bands 661 nm and 695 nm are

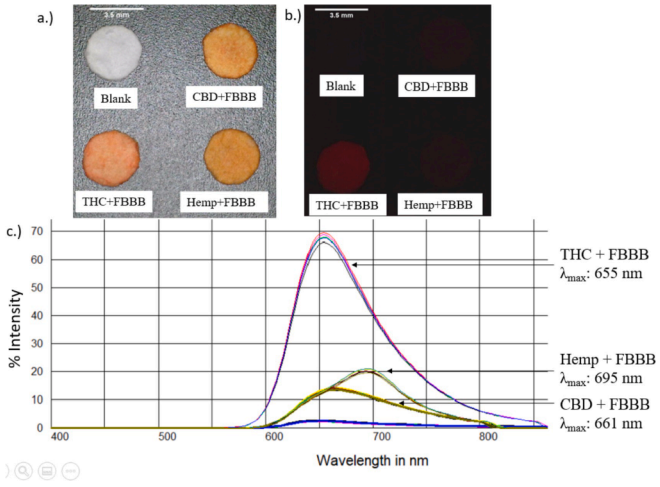

Fig. 3. (a), fluorescence image (b), and fluorescence spectra (c) for a blank, FBBB + 10,000 ng CBD, FBBB + Painted Lady hemp and FBBB + 10,000 ng THC.

significantly lower than that of FBBB + THC at 655 nm. The difference in visual color and fluorescence provides two ways to observe the chromophore/fluorophore formed and determine whether the plant material being observed is hemp or marijuana.

Temperature stability of the FBBB reagents

FBBB and NaOH were placed into amber vials and stored at room temperature (20 °C). A separate pair of vials containing the reagent were placed in a refrigerator (0 °C). Both sets of reagents were left at that temperature for one week and then evaluated using 1000 ppm THC and 1000 ppm CBD. After a week at room temperature, the FBBB reagent had gone from a yellow color to a clear color, indicating that it had become unstable at room temperature within the week. When used to test THC and CBD, it produced feint red and orange chromophores that could not be easily visualized. In addition to a decrease in color, the fluorescence of the THC + FBBB chromophore decreased as well. The refrigerated FBBB was evaluated and did not show a difference in color between tests done on day 1 and day 7. To test the long-term stability of FBBB in the refrigerator, THC and CBD was evaluated after 45 days of being in the refrigerator. There was no decrease in color or fluorescence after 45 days, demonstrating that although the FBBB reagent is unstable at room temperature, it is remains stable at refrigerated temperatures for at least 45 days.

The stability of FBBB as a preloaded salt on the PSPME substrate was also evaluated at different temperatures. Ten microliters of 0.1% (w/v) FBBB were pipetted onto 3.5 mm PSPME substrates, and the solvent was allowed to evaporate. These substrates were left at room temperature (20 °C), refrigerated temperatures (0 °C), and freezing temperatures (-20 °C). They were evaluated with using a "Painted Lady" hemp extract 15 min, 1 h, 2 h, 3 h, and 4 h after FBBB was loaded onto the substrate. For the substrates left at room temperature there was a loss of orange color between 15 min and an hour. After the first hour, barely any reaction could be visualized, once again showing that FBBB is not a stable reagent at room temperature. The preloaded substrates stored at low temperatures (0 °C), produced a consistent orange color from 15 min to 4 h. This shows that FBBB could be preloaded onto a PSPME substrate and kept stable at cold temperatures. However, it should be noted that the orange color produced when the FBBB is preloaded is duller and less intense than the color produced when FBBB is applied after the extract. A similar experiment was performed with THC, this time only evaluating the FBBB stability at refrigerated temperatures. The characteristic red color indicative of the presence of THC did form; however, like the hemp, the color was duller than when performing the usual procedure. Finally, FBBB was preloaded onto PSPME substrates and left in refrigerated temperatures for a week and were then used to evaluate 1000 ppm THC and 1000 ppm CBD solutions. Red and orange colors did form, respectively, but they were duller like all the other chromophores formed using FBBB as a preloaded salt. The fluorescence spectra of THC + FBBB were less intense than a typical FBBB + THC chromophore, but the λ_{max} was 655 nm, which is consistent with FBBB + THC. Even with the decreased intensity there was a stark difference between the FBBB + THC and FBBB + CBD fluorescence spectra (Fig. 4.)

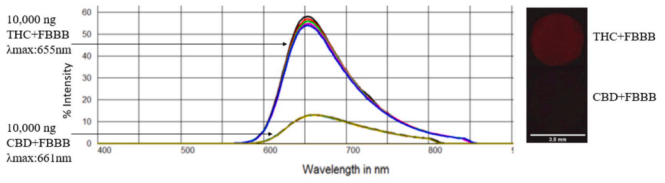

Fig. 4. Fluorescence spectra of FBBB + THC and FBBB + CBD formed from a PSPME substrate that was preloaded with FBBB and left in a refrigerator for 1 week prior to evaluation.

Colorimetric calibration of THC

An experiment was performed to determine how color and fluorescence changes as the concentration of THC increases when performing the FBBB test. The concentrations evaluated for THC were 0%, 0.25%, 0.50%, 1.0%, 2.5%, 5%, 7.5%, and 10% (w/w). The mass loadings for these experiments were calculated assuming that 10 mg of cannabis was being extracted in 1 mL of solvent. For these experiments, 10 μL was used for each solution and each concentration of THC was evaluated in triplicate. Following the reaction, images were obtained and the average RGB was obtained. The color results of this experiment could be seen in Table 1. As expected, as the concentration of THC increased, the color and fluorescence also increased. The FBBB + THC chromophore was visualized when 0.5% THC was present, meaning that the limit of detection for THC on the PSPME substrate was determined to be approximately 500 ng by color and by fluorescence using the Dino-Lite microscope. When the spectra of this sample were collected with the VSC2000 a band the fluorescence intensity at 655 nm at 30% was able to be observed.

Colorimetric calibration of THC in the presence of 2.5% CBD

Color changes with increasing concentration of THC while maintaining a fixed concentration of CBD at 2.5%(w/w) are also reported. The concentrations evaluated for THC were 0%, 0.25%, 0.50%, 1.0%, 2.5%, 5%, 7.5%, and 10% (w/w). This experiment was meant to mimic the ratios of THC and CBD that may be found in cannabis, so the mass loadings for these experiments were also calculated assuming that 10 mg of cannabis was extracted in 1 mL volume of solvent. These experiments were performed by pipetting 10 uL of 250 ng/μL CBD onto the PSPME substrate and the solvent was allowed to evaporate. The THC solutions were then added in increasing concentrations and the FBBB test was performed. As with the previous experiment, each concentration of THC was evaluated in triplicate and the average RGB was obtained. The results are shown in Table 2. When there was more CBD than THC present of the substrate, the FBBB produced an orange color even as the concentration of THC was increased. It was not until the THC:CBD ratio was above 1 that a red color could be visualized. The red color became more intense as the concentration of THC increased, as expected. There was a decrease in fluorescence intensity when comparing THC in the presence of CBD to THC alone, however. This is to be expected since FBBB reacts with both THC and CBD leading to a decrease in FBBB + THC being formed. When the THC:CBD ratio was 1 and above, the fluorescence intensity was between 30% and 50%. These experiments indicate that when the THC:CBD ratio is at or below 1, false negative or ambiguous results will be expected.

Colorimetric calibration of CBD

Similar to the experiment performed with THC, the intensity and fluorescence of the FBBB + CBD chromophore was evaluated as concentrations of CBD were increased. The concentrations evaluated for CBD were 0%, 0.25%, 0.50%, 1.0%, 2.5%, 5%, 7.5%, and 10% (w/w). As shown in Table 3, as the concentration of CBD increased, the intensity of the orange color also increases. The color can be visualized when 500 ng of CBD was reacted with FBBB, once again indicating that the LOD of cannabinoids on the PSPME substrate is ~500 ng. Even with increasing concentrations of CBD, the fluorescence of the FBBB + CBD chromophore remained low. This was confirmed in the fluorescence spectra collected from the VSC2000 where the fluorescence intensity for all concentrations were observed to remain below 20%.

Colorimetric calibration of CBD in the presence of 2.5 % THC

A similar experiment to the previous section was performed by evaluating increasing concentrations of CBD in the presence of 2.5% (w/

Table 1
Average RGB as the concentration of THC increased from 0.0% to 10% when evaluated with FBBB.

% THC (w/w)	THC mass loading (ng)	Average R	Average G	Average B
0.0%	0	210	210	217
0.1 %	100	210	210	217
0.25%	250	204	202	199
0.5%	500	208	200	184
1%	1000	228	188	150
2.5%	2500	236	166	119
5%	5000	245	152	107
7.5%	7500	244	151	112
10%	10000	244	153	116

Table 2
Average RBG code as %THC is increased in the presence of 2.5% CBD when evaluated with FBBB.

% CBD (w/w)	CBD mass loading (ng)	Average R	Average G	Average B
0.0%	0	215	215	214
0.1 %	100	213	211	198
0.25%	250	217	212	188
0.5%	500	228	213	169
1%	1000	230	204	144
2.5%	2500	237	183	106
5%	5000	234	164	87
7.5%	7500	233	164	93
10%	10000	238	160	82

w) THC. The concentrations evaluated for CBD were 0%, 0.25%, 0.50%, 1.0%, 2.5%, 5%, 7.5%, and 10% (w/w). 10 uL of 250 ng/μL THC was pipetted onto the PSPME substrate and then followed by 10 μL of increasing concentrations of THC. The FBBB test was performed and color results were recorded by obtaining the average RGB(Table 4). The orange color indicative of CBD could not be properly visualized until the THC:CBD ratio was 1. As the THC:CBD ratio decreased, the orange color became more apparent. The presence of 2.5% THC increased the intensity of the CBD + FBBB flouresence. Even when 2.5% THC was present in the presence of 10% CBD, the fluorescence intensity at 661 nm was above 20%, while for 10% CBD alone, it was below 20%. These results once again demonstrate that when the THC:CBD ratio is well above 1, a red color is to be expected. However, as the ratio gets closer to and below 1, ambiguous results will be obtained.

Color results for reference materials

The color results obtained from performing the FBBB test on cannabinoids reference materials were also valuated. 1 mg/mL certified reference standards of THC, THCA, CBD, CBDA, CBG, and CBN were all evaluated following the standard FBBB testing procedure in 5 replicates. These cannabinoids were chosen due to their prevalence in the cannabis plant. THC produces a red color, THCA produces a light purple color, CBD, CBDA, and CBG produce an orange color, and CBN produced a pink/purple color. As shown in the scatterplot in Fig. 5, all chromophores formed are clearly separated from the color that is formed from FBBB + THC. The orange chromophore formed by CBD, CBDA, and CBG is separated from THC due to having a higher G value than the FBBB + THC chromophore. The fluorescence of FBBB + CBD, FBBB + CBDA, and CBG were well below that of FBBB + THC, having an intensity below 20% and a λ_{max} at 661 nm. CBN had a high fluorescence intensity of 60% but its λ_{max} was at 661 nm distinguishing it from FBBB + THC. THCA had a fluorescence intensity of 40% at λ_{max} 661 nm. A cannabinoid mix containing 0.5 mg/mL of these 6 cannabinoids was tested with the FBBB test and an orange color distinct from the red color of FBBB + THC was obtained. The fluorescence of the chromophore formed by this mixture resulted in an intensity of 25%. A mixture containing 0.5 mg/mL THC and THCA and a mixture containing 0.5 mg/mL CBD and CBDA were prepared and tested using FBBB. FBBB + THC/THCA mixture showed an enhancement of red color compared to the pure FBBB + THC color, however it also decreased the fluorescence intensity obtained from the chromophore. The CBD/CBDA mixture produced an orange color with weak fluorescence properties, as expected. Finally, two terpene mixtures containing 21 terpenes that are commonly found in cannabis were tested

Table 3
Average RGB as the concentration of CBD increased from 0.0% to 10% when evaluated with FBBB.

% THC (w/w)	THC Mass Loading (ng)	% CBD (w/w)	CBD Mass Loading (ng)	THC/CBD ratio	Average R	Average G	Average B
0.0%	0	2.5%	2500	0	229	180	119
0.1 %	100	2.5%	2500	0.04	231	176	113
0.25%	250	2.5%	2500	0.10	231	187	134
0.5%	500	2.5%	2500	0.20	236	179	124
1%	1000	2.5%	2500	0.40	230	181	131
2.5%	2500	2.5%	2500	1.00	242	164	123
5%	5000	2.5%	2500	2.00	240	153	112
7.5%	7500	2.5%	2500	3.00	243	146	105
10%	10000	2.5%	2500	4.00	244	144	107

Table 4
Average RGB code as %CBD increased in the presence of 2.5% THC when evaluated with FBBB.

% CBD (w/w)	CBD Mass Loading (ng)	% THC (w/w)	CBD THC Loading (ng)	THC/CBD ratio	Average R	Average G	Average B
0%	0	2.5%	2500	0	233	171	122
0.1 %	100	2.5%	2500	25	238	178	128
0.25%	250	2.5%	2500	10	234	177	128
0.5%	500	2.5%	2500	5	232	175	126
1.0%	1000	2.5%	2500	2.5	239	179	122
2.5%	2500	2.5%	2500	1	236	160	104
5.0%	5000	2.5%	2500	.5	230	140	82
7.5%	7500	2.5%	2500	.33	243	150	80
10%	10000	2.5%	2500	.25	234	154	91

with FBBB. No color or fluorescence was produced from these reactions. These color results show that FBBB is very selective among the cannabinoids and terpenes, particularly THC as none of the other cannabinoids tested produced a red color or high fluorescence intensity at 655 nm when evaluated.

Color results with hops, herb, spices, and tobacco

Elektra hemp, two cigars, apollo hop pellets, citra whole leaf hops, oregano, sage, parsley, red pepper flakes, black pepper, lavender, and eucalyptus leaves were all evaluated using FBBB. The cigars, red pepper flakes, thyme, sage, spearmint, lavender, and parsley did not have any color change as a result of the FBBB reaction. The apollo hops pellets resulted in a yellow color, the citra whole lead hops and ecualypus resulted in a light orange color, and oregano resulted in a light yellow color. An RGB scatterplot of THC, hemp, Apollo hop pellets, Citra whole lead hops, eucalyptus, and oregano shows that there is a clear separation between the FBBB + THC chromophore and the other chromophores, demonstrating the selectivity for FBBB as a test for THC-rich cannabis (Fig. 6). FBBB + hemp has a higher G score and lower R score than FBBB + THC, demonstrating a clear separation of color between the two. Fluorescence was visualized using the Dino-Lite for the citra whole leaf hops, apollo hop pellets, eucalyptus, oregano. spearmint, and parsley. Spectra from the Vsc2000 showed that the apollo hop pellets and citra whole leaf hops had fluorescence spectra distinct from FBBB + THC and FBBB + Elektra hemp. Eucalyptus, oregano. spearmint, and parsley all

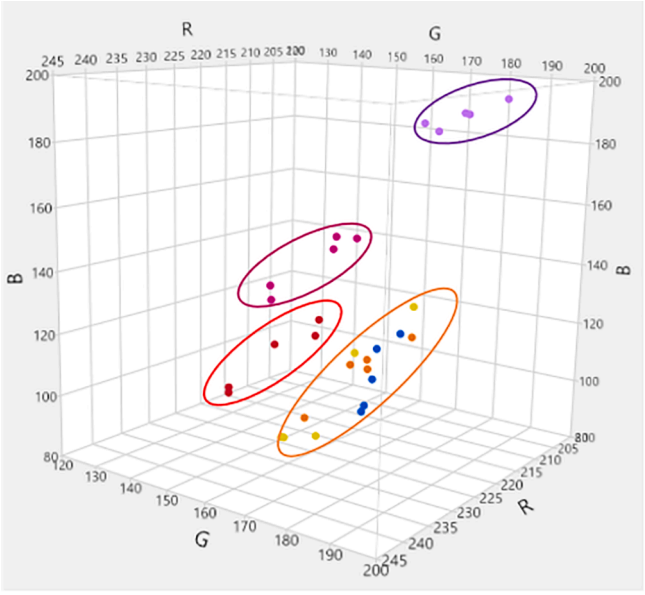

Fig. 5. RGB scatter plot of the THC (red), THCA (light purple), CBD (orange), CBDA (yellow), CBG (blue), and CBN (purple). CBD, CBDA, and CBG (orange circle), THC (red circle), CBN (purple circle), and THCA (light purple circle) show a clear separation through RGB score.

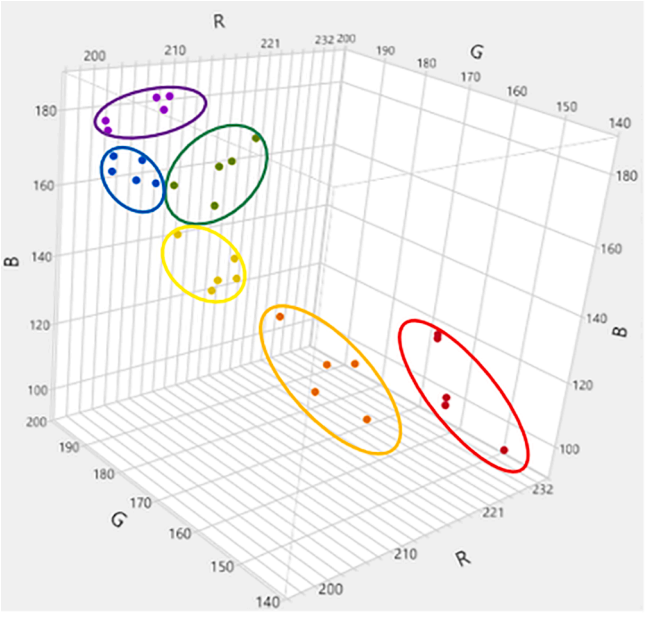

Fig. 6. RBG scatter plot of THC (red), Elektra hemp (orange), Apollo Hop pellets (green), Citra Whole Leaf Hops (yellow), Oregano (blue), and Eucalyptus (purple).

had λ_{max} at 695 nm, once again indicating that there may be fluorescence interference at 695 nm. The spectra for these plants produced a fluorescence intensity at 695 nm below 25%. In all cases, the chromophores formed did not show any color or fluorescence similar to FBBB + THC, once again demonstrating the selectivity of the FBBB test for THC-rich cannabis over other plant material.

Analysis of authentic hemp and marijuana samples

All five blue ridge hemp samples and 20 cannabis samples of known cannabinoid concentrations (obtained from NIST) were evaluated using the FBBB reagent with 5 replicates. For each replicate, a color image, a fluorescence image, and fluorescence spectra were obtained. The color results for all samples are summarized in Table 5. All of the hemp samples formed an orange color when reacted with FBBB, except for Sample 11 and Sample 12, which did not have any reaction. Of the 13 samples that are marijuana (THC >0.3% w/w), 6 of them produced an orange color instead of the red color indicative of THC. These six were Sample 6, Sample 9, Sample 10, Sample 18, Sample 19, and Sample 20. For samples 6, 9, 10, and 20 the total CBD was at a higher concentration than total THC, all containing a THC:CBD ratio below 1. Samples 18 and 19 had THC:CBD ratios of 1.0 and 1.4 respectively. The other marijuana type samples had a THC:CBD ratio much higher than 2 and formed a red color. Samples that had a THC:CBD ratio below 2 (samples 6, 9, 10, 18, 19, and 20) did not fluoresce brightly under the Dino-Lite microscope at 480 nm excitation. Importantly, the marijuana-type samples that either had no CBD or a high THC:CBD ratio did fluoresce brightly under the Dino-Lite at the same excitation. These results suggest that when there is more CBD than THC in the marijuana plant, or if the concentrations are similar, the FBBB will produce an orange color indicative of hemp rather than a red color indicative in marijuana. In addition, when the THC:CBD ratio is low, the fluorescence of the chromophore will also be low.

The fluorescence spectra from the VSC2000 for hemp-type samples showed a low % intensity at 655 nm, typically between 10% and 20%, and a higher intensity at 695 nm, between 15% and 40%. The exception to this were samples 11 and 12 whose extracts did not react with FBBB and had similar spectra to the blank. The marijuana-type samples with a low THC:CBD (below 2) showed similar spectra to the hemp samples, with fluorescence intensities at or below 20% at 655 nm for those with THC:CBD significantly lower than 1. Samples 18, 19, and 20, which have THC:CBD from 0.48 to 1.4, all showed slightly higher intensities at 655 nm than the hemp samples (between 19% and 31%). For marijuana-type samples with a THC:CBD above 2, the intensity of fluorescence increases between 40 and 70% at 655 nm and 695 nm. Low fluorescence intensity for hemp samples at 655 nm is expected since there is very little THC in these samples. For samples 6, 9, and 10 there was much more CBD than THC in the cannabis plant leading FBBB + CBD to form over FBBB + THC. Samples 18, 19, and 20 showed a slightly more intense band at 655 nm. This increase could be attributed to the fact that there is a similar concentration of CBD and THC in these samples and allowed for FBBB to react with both THC and CBD. In addition, all cannabis extracts contain a band at 695 nm. This interference is likely due to chlorophyll and other pigments from the plant material, however, even with this interference, the difference in fluorescence intensity between hemp and marijuana-type cannabis with a high THC:CBD is noticeable. When the THC:CBD ratio is below 2, the fluorescence intensity decreases. This is consistent with the results obtained from the color images and fluorescence images using the Dino-Lite microscopes. A comparison of a marijuana-type sample and a hemp-type sample through color images, fluorescence images, and the fluorescence spectra is shown in Fig. 7.

Linear discriminate analysis of cannabis samples

Linear Discriminant Analysis (LDA) was used as a supervised technique to determine whether FBBB can be used to correctly classify hemp-type cannabis and marijuana-type cannabis. Each sample described in Table 5 was evaluated in 5 replicates. For each replicate RGB of the color image, RGB of the fluorescence image, and the % intensity at 655 nm and 695 nm in the fluorescence spectra were recorded. The LDA analysis was performed using the JMP software.

The first LDA model was constructed using % intensity at 655 nm and % intensity at 695 nm values as the variables. The resulting model had an R^2 of 0.61 and misclassified samples 6, 9, 10, 14, 15, 18, 19, 20. Samples 6, 9, 10, and 18–20 are marijuana-type samples with THC:CBD below 2, showing similar fluorescence spectra to hemp samples leading to their misclassification. Samples 6, 9, 10, and 18–20 were removed from the data set and LDA was performed again using the data from the 7

Table 5
Cannabis type, THC:CBD ratios, and color results for Blue Ridge Hemp samples and NIST samples.

Samp1e	Cannabis Type	THC:CIID	Color Result	Samp1e	Cannabis type	THC:CBD	Color Result
Painted Lady Hemp	Hemp	<0.05	Orange	NIST Sample 9	Marijuana	0.10	Orange
Eighty-Eight Hemp	Hemp	<0.05	Orange	NIST Sample 10	Marijuana	0.17	Orange
Elektra Hemp	Hemp	<0.05	Orange	NIST Sample 11	Hemp	<0.05	No RXN
Spec 7 Hemp	Hemp	<0.05	Orange	NIST Sample 12	Hemp	No THC/CBD	No RXN
Purple Emperor Hemp	Hemp	<0.05	Orange	NIST Sample 13	Hemp	0.07	Orange
NIST Sample 1	Marijuana	No CBD	Red	MST Sample 14	Hemp	0.32	Orange
MST Sample 2	Marijuana	No CBD	Red	MST Sample 15	Hemp	<0.05	Orange
MST Sample 3	Marijuana	No CBD	Red	MST Sample 16	Hemp	<0.05	Orange
MST Sample 4	Marijuana	No CBD	Red	MST Sample 17	Hemp	<0.05	Orange
MST Sample 5	Marijuana	80	Red	MST Sample 18	Marijuana	1.0	Orange
MST Sample 6	Marijuana	0.29	Orange	MST Sample 19	Marijuana	1.4	Orange
MST Sample 7	Marijuana	501	Red	MST Sample 20	Marijuana	0.48	Orange
MST Sample 8	Marijuana	310	Red				

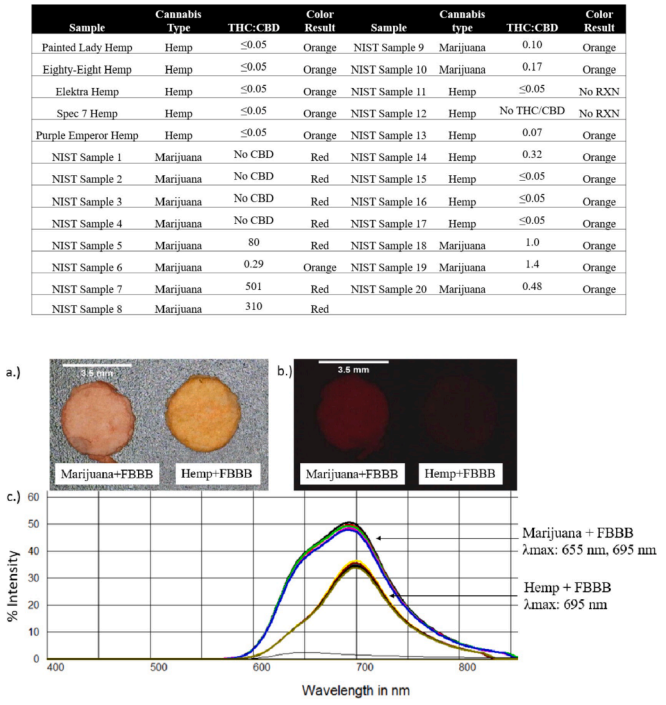

Fig. 7. (a), fluorescence image (b), and fluorescence spectra (c) for marijuana type cannabis (NIST Sample 5) and hemp-type cannabis (NIST Sample 16).

remaining marijuana-type samples (THC-rich cannabis) and the 12 hemp-type samples. This analysis resulted in an R^2 of 0.99_9 and no misclassifications.

LDA was also performed using the R, G, and B codes for each color image and fluorescence image. LDA of all the samples using RGB for the color images produced an R^2 of 0.51 and misclassified samples 3,4, 10, 18–20, and one replicate of 16 and 17 each. To improve the model, all marijuana type samples with THC:CBD below 2 were removed from the data set. Samples 11 and 12 were removed as well since they did not produce a color as they were likely the cause of the misclassification of samples 3 and 4, which produced a light red color. This did improve the model with the R^2 value of 0.95 and only misclassifying one replicate of sample 3. This indicates when using only RGB of the visible image, one should exclude samples that do not form a color as it may cause misclassification.

An LDA model of all samples using RGB of the fluorescence images taken for each replicate was also made. This LDA model misclassified multiple hemp-type and marijuana-type samples resulting in an R^2 of 0.46. When the marijuana type samples with THC:CBD below 2 were removed from the data set, there were no misclassifications and R^2 was 0.99_5. Finally, an LDA model was made to classify the marijuana-type samples with a high THC:CBD and all the hemp-type samples using the R,G and B (3 variables) from the color images and R-F,G-F and B-F (3 variables) from the fluorescence images for a total of 6 variables. This model resulted in a clear separation between hemp-type and marijuana-type cannabis resulting in an R^2 of 1.0 (Fig. 8a) with G (green in the visible) providing the highest correlation to hemp (low THC content) and R-F (red in fluorescence) providing the highest correlation to THC-rich cannabis (high THC:CBD). A Receiver Operating Characteristic (ROC) of the model showed that the area under the curve for both hemp and marijuana are 1, displaying excellent selectivity and sensitivity when combining color and fluorescence to discriminate from hemp-type cannabis (CBD-rich) and marijuana type cannabis (THC-rich) (Fig. 8b).

Conclusions

The FBBB test was used to evaluate 6 different cannabinoids, 5 commercial hemp strains, 20 cannabis samples, and various herbs and spices. It was determined that when FBBB reacts with THC, it forms a red chromophore that fluoresces under 480 nm light. Conversely, when reacted with CBD or CBD-rich products, such as hemp, an orange chromophore is formed, and this chromophore does not fluoresce. This is the first time, to the author's knowledge, that the fluorescence of the FBBB + THC chromophore/fluorophore is reported for a colorimetric test. This fluorescence is easily visualized using a portable Dino-Lite microscope and its spectra obtained with a VSC2000 spectrometer.

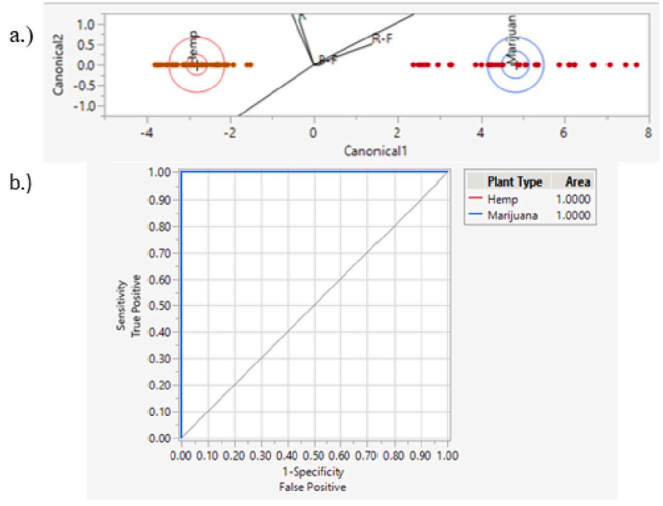

Fig. 8. (a) Canonical plot of the LDA model using RGB for color images and fluorescence images to classify 7 marijuana-type cannabis samples with THC:CBD >2 and 12 hemp-type cannabis samples and (b) ROC curve of the LDA model.

The intensity and wavelength of the fluorescence for the chromophore combined with the distinct red color it displays makes for a more selective and sensitive test to differentiate between marijuana and hemp. The structure for FBBB + THC has been previously determined by the Almirall lab, as shown in Fig. 1 [13]. The chromophore results from an extended conjugation of π-bonds decreasing the distance between energy transitions between the ground state and excited state. This extended conjugation causes a "red shift" of the FBBB chromophore, which is responsible for the red color and the fluorescence that is observed when THC reacts with FBBB. One theory for CBD + FBBB lacking fluorescence intensity is that CBD has a less rigid structure than THC. It is known that structure rigidity and a fused ring structure increases the quantum efficiency, and therefore fluorescence of a molecule. Since CBD is less rigid than THC and does not have a fused ring structure, it is prone to relaxation through internal conversion rather than through radiative means [19]. Therefore, FBBB + CBD likely relaxes through nonradiative mechanisms, which decreases overall fluorescence. The difference in both color and fluorescence that is observed for FBBB + THC and FBBB + CBD is an advantage that the FBBB test has compared to other tests for presumptive analysis of cannabis, which only use color.

The selectivity of the FBBB test was evaluated by analyzing 5 other cannabinoids, herbs, spices, essential oils, tobacco, and hops. None of these substances produced color like that of FBBB + THC nor fluorescence observed. For the colorimetric calibration experiments, it was shown that when the ratio of THC:CBD is above 1, a red color forms indicating that there is marijuana present. These experiments also found that the absolute LODs for THC on the PSPME substrates was as low as 500 ng, which is significantly lower than the LOD for the D-L test (~5000 ng). The THC LOD for the 4-AP test is not currently known but expected to be >500 ng. This study demonstrates that the FBBB test is very selective and sensitive for THC, forming a red color and an intense fluorescence that can be distinguished from other chromophores. In addition, this chromophore is long lasting, allowing the color and fluorescence to be observed long after the test is performed. This long-lasting color is attributed to the nature of the FBBB being a diazonium salt, which are known to be stable and even used to form dyes in textiles [20].

One limitation that was discovered for the FBBB test is that the reagent is not stable at room temperatures over more than a few days, losing its color and producing no reaction with THC or CBD. The FBBB reagent and the preloaded FBBB substrate were stable in the refrigerator/cooler (4 °C) for at least 45 days. The temperature instability is not ideal for field work since a kit using the Fast Blue BB test would likely be exposed to temperatures above 4 °C. For this reason, future work will focus on determining a method to maintain the FBBB stable at ambient temperatures.

The analysis of the Blue Ridge Hemp and NIST samples demonstrate that FBBB is very effective at discriminating between hemp-type samples with THC content <0.3% (w/w) and marijuana-type samples with a high THC content or THC:CBD ratios. Marijuana-type cannabis containing >0.3% (w/w) THC and high CBD (low THC:CBD ratio) could be misclassified as hemp but these types of samples are uncommon in seized drugs. The results of these LDA models using RGB inputs support the observed findings of the visual evaluation of the Blue Ridge and NIST samples with FBBB. The models show when FBBB is used to classify for marijuana, which has a low THC:CBD ratio, there is a decrease in specificity that causes these marijuana-type samples to be misclassified as hemp. When marijuana-type samples with THC:CBD <2 were removed from the LDA models, FBBB has high sensitivity and specificity for marijuana-type cannabis with a high THC:CBD ratio and shows a clear separation from hemp samples. In addition, the combination of RGB values from the fluorescence images and color images provided the most reliable model that correctly classified all 7 marijuana samples and 12 hemp samples.

This study has demonstrated the specificity and sensitivity of the FBBB reaction with THC compared with other cannabinoids. The combination of the red color and fluorescence of the FBBB + THC chromophore/fluorophore allows THC-rich cannabis to be distinguished from CBD rich cannabis. ElSohly et. al. analyzed confiscated cannabis in the US between 2009 and 2019 and found that the average THC:CBD ratio of the cannabis plants was found to be above 20 across the decade [4]. Although false negative results can be obtained for samples with a low THC:CBD ratio, FBBB is useful in discriminating between marijuana-type cannabis with a high THC:CBD ratio from hemp-type cannabis. Since most illicit cannabis in the US contains a high THC:CBD ratio, FBBB is applicable to field use as a presumptive test to distinguish between cannabis types. When compared to the other field tests on the market, FBBB is more selective as well, producing less false positive results among herbs, spices, and hops. This test uses a small volume of reagents and can be performed on a 3.5 mm PSPME substrate, which simplifies the analysis while allowing for portability. Finally, the observation time window for the FBBB + cannabinoids is longer than for other competing techniques such as the 4-AP reaction that has an observation window of a few minutes.

Future work will include validating the FBBB test by conducting an interlaboratory study with several operational laboratories and increasing the number of authentic cannabis samples of known cannabinoid concentrations. Future studies will also focus on better defining the analytical figures of merit for the reaction including LOD and the THC:CBD range in which ambiguous or false negative results are obtained using this test and the potential to conduct a concentration determination of the THC is some samples. FBBB will also be validated for field use, assessing operational parameters such as chemical stability of the reactants, storage limitations and the possibility of incorporating a portable spectrometer to determine the fluorescence spectra of the chromophore/fluorophore in the field. Additional studies will be conducted to determine how the FBBB test performs in comparison with, and in combination with, existing presumptive cannabis tests, such as the 4-AP test.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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