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Quantitative analysis of Δ^9-tetrahydrocannabinol (Δ^9-THC) in cannabis plants using the Fast Blue BB (FBBB) and 4-aminophenol (4-AP) colorimetric tests

Nicole B. Valdes, Roberta Gorziza, José R. Almirall *

Department of Chemistry and Biochemistry, and Center for Advanced Research in Forensic Science (CARFS), Florida International University, 11200 SW 8th Street, Miami, FL 33199, USA

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ABSTRACT

The Fast Blue BB (FBBB) and 4-aminophenol (4-AP) colorimetric tests have been reportedly used for the qualitative determination of Δ^9-THC in plants and for the differentiation between marijuana and hemp-type cannabis. We report the miniaturization of the FBBB colorimetric reaction on a silicone treated filter paper substrate and the analytical figures of merit for a quantitative determination of Δ^9-THC for the first time. The reaction between Δ^9-THC and FBBB forms a red chromophore that fluoresces when irradiated with visible (480 nm) or UV (365 nm) light, providing a 3-fold increase in sensitivity. Portable instruments are introduced for the objective color determination for both tests and for the fluorescence reading of the THC + FBBB complex. We report a fluorescence signal with Δ^9-THC, Δ^8-THC, and CBN. The limit of detection (LOD) was determined to be 1.6 ng/μL with precision ~12 % RSD for standard Δ^9-THC solutions ranging between 5 and 20 ng/μL. The linear dynamic range for this test is reported between 1.6 ng/μL and 20 ng/μL for the portable fluorescence detector. The miniaturization of both colorimetric tests and the increased sensitivity of the FBBB test using fluorescence analysis, coupled to portable instruments allows for limited quantitative analysis of cannabis plants in the field.

Introduction

According to the 2022 United Nations Office on Drugs and Crime (UNDOC) World Drug Report, there are approximately 209 million cannabis users worldwide with an increasing trend [1]. The Controlled Substances Act of 1970 classified cannabis as illegal at the federal level in the United States and the 2018 Farm Bill further distinguishes between legal hemp-type cannabis and marijuana-type cannabis by Δ^9-tetrahydrocannabinol (Δ^9-THC) content in the plant. Cannabis plants containing 0.3 % (w/w) or more Δ^9-THC are classified as controlled marijuana at the federal level while cannabis plants containing less than 0.3 % Δ^9-THC are legal to cultivate and distribute as hemp [2]. As of August 2023, 23 states and the District of Columbia have legalized marijuana for both recreational and medicinal use, and 16 additional states have legalized marijuana for medicinal use or decriminalized the drug [3]. With these recent changes to federal and state laws, it is now critical to be able to determine if cannabis plant material is hemp (legal) or marijuana with a simple method that can be used in the laboratory or in the field.

It is possible to conduct an array of analytical tests on plant material to confirm a classification between hemp or marijuana in the laboratory. Typically, presumptive tests are first performed on the plant sample followed by confirmatory tests using instrumentation such as gas chromatography coupled to mass spectrometry. Colorimetric tests are presumptive tests used to determine the presence of an analyte by reacting a small amount of sample that contains the analyte of interest with a chemical reagent that results in a color change depending on the functional groups present in the compound [4]. These tests are widely used due to their fast and simple protocols that consume a small amount of sample, but these tests often lack selectivity. The Duquenois–Levine test is commonly used for presumptive testing of marijuana, but has been shown to produce false positive results when used with other materials, like patchouli, that contain a phenol group [5,6]. In addition, the Duquenois–Levine test does not distinguish between hemp-type cannabis and marijuana-type cannabis plants. Law enforcement agencies in the US and around the world have started to use the 4-aminophenol (4-AP) test as part of a qualitative cannabis typification scheme. The 4-AP test, which originated in the Swiss Forensic Institute in Zurich

* Corresponding author.
E-mail address: almirall@fiu.edu (J.R. Almirall).

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differentiates between hemp-type cannabis, which contains low THC concentrations and high cannabidiol (CBD) concentrations, and marijuana-type cannabis, containing low concentration of CBD and high concentration of THC [7]. Cannabis with high CBD concentrations reacted with 4-AP produces a pink color, while a high concentration of THC in cannabis results in a blue color. If the THC:CBD ratio is close to 1, the resulting color is purple and considered to be an inconclusive 4-AP test. The Virginia Department of Forensic Science recommends using the Duquenois–Levine test to determine whether the plant material is cannabis, followed by the 4-AP test as a cannabis typification test [8]. The 4-AP test is used as a qualitative test and there has not been any reporting to date of a colorimetric test to quantify THC in a plant sample to the best of our knowledge.

Our group recently reported the miniaturization and optimization of the 4-AP test, focusing on decreasing volumes of the reagents and increasing the stability of the color formed [9]. These reported improvements overcome color degradation previously observed only a few minutes after the reaction is initiated resulting in a brown/black color, hypothesized as oxidation of the unreacted 4-AP reagent [9]. By reducing the volumes of the reagents and adjusting the stoichiometry, the blue or pink color formed was stable for more than 40 min compared to the ~8 min using the original volumes for the test [9].

Our group has also reported the combination of the 4-AP test with another colorimetric test that allows for the differentiation between marijuana and hemp-type cannabis, the Fast Blue BB (FBBB) colorimetric test [10–12]. When reacted with individual cannabinoids, the FBBB molecule forms a complex with Δ^9-THC that produces a red color, with CBD forming an orange color, and with cannabinol (CBN) forming a purple color [11]. The resulting chromophore has been confirmed by mass spectrometry and ^{1}H NMR, and due to its stability can remain unchanged for more than 7 days [11]. The THC + FBBB complex also fluoresces when irradiated with blue visible light (480 nm) or ultraviolet (UV) light (365 nm) [12]. This fluorescence is a result of the extended conjugation of the pi bond system afforded by the added rigidity of the complex (Fig. 1d). The complex formed by the reaction of FBBB with CBD, on the other hand, does not fluoresce since it lacks the structural rigidity (Fig. 1e). The formation of a chromophore/fluorophore complex involving THC and FBBB allows for a dual confirmation using this test—the formation of the red color as well as the fluorescence signal. A recent publication by Gorziza et al. validated the use of the FBBB test to detect Δ^9-THC from oral fluid samples, showing its applicability to field use [13].

As the legal status of marijuana continues to change in the United States, it is important to implement simple yet sensitive fieldable methods to differentiate between marijuana and hemp cannabis plant material. Current color tests used serve as qualitative presumptive tests, where the color indicates the presence (or absence) of Δ^9-THC, but not the concentration in the sample. This study focuses on the ability to quantitate Δ^9-THC in a sample using the fluorescence of the THC + FBBB complex observed with a portable fluorescence reader to gather objective data that can be related to Δ^9-THC concentration within a limited dynamic concentration range. Figures of merit for the test such as precision and bias, linear dynamic range, and limits of detection for the FBBB test are reported here, for the first time. This work also introduces a portable colorimeter that can improve both the 4-AP and the FBBB color test evaluation by reducing subjectivity. Both the portable fluorescence reader and the portable colorimeter were compared to digital microscopes previously used for the FBBB method validation in cannabis plant extracts [12,14].

Materials and methods

Materials

Fast Blue BB Salt Hemi (zinc chloride) was obtained from Sigma Aldrich (St. Lous, MO, USA). 4-aminophenol, methanol, ethanol, and polystyrene spot plates were obtained from Fisher Scientific (Hampton, NH, USA). Methanolic standard solutions (1 mg/mL) of Δ^9-THC were obtained from Restek Corporation (Bellefonte, PA, USA). Methanolic standard solutions (1 mg/mL) of Δ^8-THC, CBD, CBN, CBG, and CBDA were purchased from Cerilliant Corporation (Round Rock, TX, USA). A concentrated Δ^9-THC standard (5 mg) was purchased with DEA authorization from Cerelliant. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Macron Fine Chemicals (Radnor Township, PA, USA). Whatman 1 PS Silicone Treated Filter Paper was obtained from Cytiva (Malborough, MA, USA). Sixty-nine marijuana samples were obtained from the Drug Enforcement Administration (DEA) and 24 marijuana samples were obtained from the National Institute of Standards and Technology (NIST). All of these authentic marijuana samples were previously analyzed by a GC–MS method to determine the concentration of Δ^9-THC in the samples [14]. A total of 122 hemp samples were used for these experiments. Seven hemp samples were obtained from NIST, 17 hemp samples were purchased from Blue Ridge Hemp Co (Asheville, NC, USA), 8 hemp samples were purchased from Tweedle Farms (Clatsop County, OR, USA), 2 hemp samples were purchased from Bammer (Santa Rosa, CA, USA), 15 hemp samples were purchased from Black Tie (Yuba City, CA, USA), 2 hemp samples were purchased from Lovewell Farms (Hope Valley, RI, USA), 3 hemp samples were purchased from Oasis Farms (North Las Vegas, NV, USA), 1 sample was purchased from Green Unicorn Farms (San Francisco, CA, USA), and 1 sample was purchased from One Sky Farms through The Green Nursery (Bloomington, IN, USA). All of the hemp samples were previously analyzed to determine the THC and CBD concentrations and reported previously [12,14]. From the samples purchased, an additional 66 hemp samples were created by mixing some of the hemp samples to create a variety of concentrations of THC and CBD. Cigars, Apollo Hop Pellets, Azacca Hops, Citra Whole Leaf Hops, and a variety of spices and teas were obtained from commercial retailers. These spices and teas

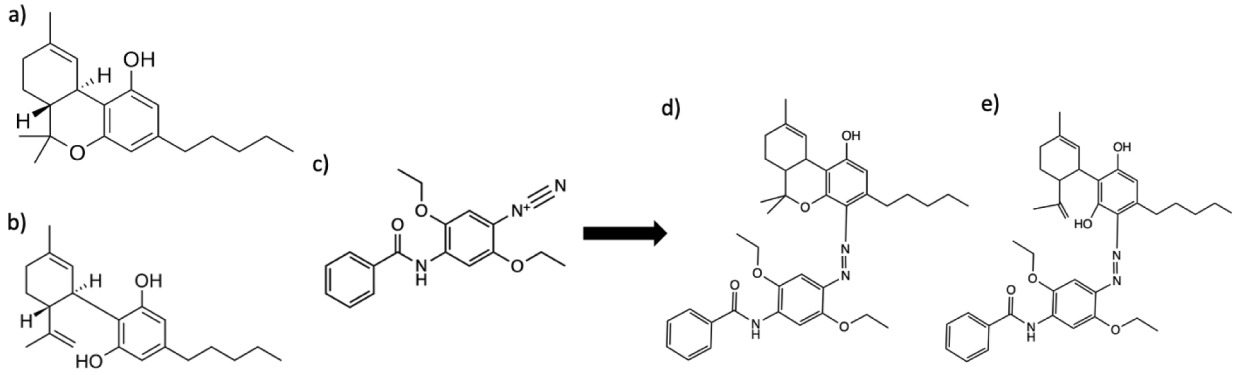


Fig. 1. Complexes formed when (a) Δ9-THC is reacted with (c) FBBB to form the (d) THC + FBBB product or when (b) CBD is reacted with (c) FBBB to form the (e) CBD + FBBB product [11].

include thyme, oregano, sage, black pepper, eucalyptus tea leaves, lavender flowers, parsley flakes, red pepper flakes, spearmint leaves, carobinha, and guarana.

Instrumentation

For color imaging purposes, a Dino-Lite AM4115ZT(R9) Digital Microscope was used from Dunwell Tech, Torrence, CA. To keep consistency between imaging the many samples, a 15.24 cm × 15.24 cm × 15.24 cm light box was made from a cardboard box and the inside was lined with white paper. The Dino-Lite microscope was inserted into a hole cut out at the top of the box for imaging. A 40 mm Calibrite ColorChecker Classic Nano was obtained from X-Rite (Grand Rapids, MI) and placed in the light box for imaging with the Dino-Lite to serve as a standard. To capture the fluorescence images, a Dino-Lite AM4115T-GRFBY Digital Microscope was used. The microscope used for fluorescence has a 480 nm excitation light source and emission filters at 510 and 610 nm. Fluorescence images were taken inside a cardboard box to keep extraneous light out of the sample images.

Although the Dino-Lite Digital Microscope allows for imaging of the samples, the images must be processed using ImageJ software to determine RGB values and other information. A colorimeter and fluorescence reader were used to collect objective values from the color and fluorescence, respectively. The Lishang LS172B 45/0 Colorimeter (Shanghai Lishang Technology Co. Ltd., Qingpu District, Shanghai, China) was used to obtain color values from both FBBB and 4-AP samples. This hand-held device is placed above the substrate to record a measurement. The colorimeter device allows a standard to be recorded and compares values of the standard to a sample and uses a designated threshold value to determine if the sample is similar or different from the standard color. This device is further described in the colorimeter section.

To obtain objective data from the fluorescence samples, a Dialunox® ESEQuant LR3 was used. This instrument is a lateral flow fluorescence measurement tool. Different fluorescence excitation and emission wavelengths are commercially available. The instrument used was equipped with a 365 nm excitation and 520 nm emission wavelength. Since the FBBB test results in a chromophore and fluorophore and the test is performed on a solid substrate, the substrate can be placed into the Dialunox® reader using a sample introduction drawer, and the scan results an intensity (mV) value for the sample. The Dialunox® was set to take three (3) 1 mm width measurements across the sample placed in the drawer and the intensities values were averaged.

Plant extract methodology

Cannabis plant extractions were performed for each marijuana and hemp sample without further drying the samples. For this method, approximately 10 mg of plant material was weighed and placed in a 1.8 mL amber vial with 1 mL of methanol added to the vial. The plant sample remained in the methanol for 10 min and vortexed twice for 20 s during the extraction. After the 10 min elapsed, a disposable glass Pasteur pipette was used to remove the supernatant and transferred to a new, clean amber vial. The plant extracts were kept in a freezer at −20 °C until use.

Fast blue BB and 4-AP reagent preparation

The 1 % FBBB solution was prepared by dissolving 10 mg of FBBB salt in 10 mL of methanol using a volumetric flask. Once prepared, the solution was kept in an amber vial and stored in the freezer, wrapped in aluminum foil until use. The 0.1 N NaOH solution was prepared by dissolving 0.4 g of NaOH pellets in 100 mL of methanol using a volumetric flask. The NaOH solution was then stored in a clear vial in the refrigerator [11].

The 4-AP reagent A was prepared using 8.67 mg of 4-aminophenol dissolved in 99.5 mL of ethanol and 0.5 mL of 2 M HCl. Reagent B was prepared using 3 g of NaOH in 70 mL of ethanol and 30 mL of deionized water. Both solutions were stored in clear vials in the refrigerator [15].

Fast blue BB test procedure

A 6.3 mm × 6.3 mm square of silicone treated filter paper was used as the solid substrate for the FBBB test. This substrate square was placed into a well tray lined with aluminum foil for efficient clean-up. These tests were performed by first adding 10 μL of the plant extract in methanol at room temperature to the substrate square followed by 10 μL of the FBBB reagent and 10 μL of 0.1 % NaOH in methanol. Once all reagents are added to the solid substrate, the color formation occurs immediately. The formation of a red color indicates a positive result for marijuana-type cannabis due to the presence of Δ^9-THC, and an orange color formation indicates a negative result. The tests were performed in triplicate.

Once the samples were dry (approximately 15 min), the substrate squares were placed in a 15.25 cm × 15.24 cm light box for imaging. A hole was cut at the top of the box for the insertion of the Dino-Lite AM4115ZT(R9) digital microscope for imaging. A Calibrite ColorChecker Classic Nano Target was also inserted into the imaging area for calibration purposes. All three replicates were placed in the light box on a piece of clear tape and imaged with the microscope. Since the chromophore complex of THC with FBBB fluoresces, a Dino-Lite AM4115T-GRFBY digital microscope with a 480 nm light source and 510 nm and 610 nm emission filters was used for fluorescence imaging. The fluorescence images were performed in a cardboard box to block out excess interfering light. RGB scores from the color images were obtained using the ImageJ software with the RGB plugin. Fluorescence intensity was measured using the Dialunox® ESEQuant LR3 instrument.

4-AP test procedure

The 6.3 mm × 6.3 mm silicone treated filter paper used for the FBBB tests was also used for the 4-AP tests. The substrate square was placed in a polystyrene spot plate (20 mm diameter and 10 mm deep) for the test procedure. For the reaction, 10 μL of the plant extract was added to the substrate, followed by 10 μL of reagent A, and 12 μL of reagent B. Tests were performed in triplicate. After approximately 15 min, the images were taken while the samples were still wet. The three replicate substrate squares were placed in the light box on a piece of clear tape for imaging using the Dino-Lite AM4115ZT(R9) digital microscope. The Calibrite ColorChecker Classic Nano was also inserted into the imaging area for calibration purposes.

Figures of merit for quantitative validation of FBBB using a portable fluorometer

The Dialunox® fluorescence reader was used to perform quantitative analyses of the samples using the FBBB reaction. Linear dynamic range, limit of detection (LOD), limit of quantitation (LOQ), precision, and bias were determined to evaluate the method. Standard Δ^9-THC solutions were prepared in methanol at the following concentrations by serial dilution: 0.5, 1, 5, 10, 15, 20, 25, 50, 100 ng/μL to determine the dynamic range of the FBBB colorimetric test as well as the LOD. The FBBB test procedure (see section 2.5) was followed, and the fluorescence intensity was measured with the Dialunox® instrument. The LOD was calculated using 3.3 times the standard deviation of the signal of the blank divided by the slope of the regression line. The LOQ was determined using 10 times the standard deviation of the signal of the blank divided by the slope of the regression line.

A calibration curve was prepared in a methanolic hemp extract to account for the matrix effects present in a cannabis plant extract during the FBBB test. The hemp used contains 0.18 % THC and 5.8 % CBD

naturally in the plant, according to the certificate of analysis for that sample. The plant was extracted as described in section 2.3 and the respective volumes of a Δ^9-THC standard solution were added to the extract to achieve 1, 2.5, 4, 5, and 7.5 % Δ^9-THC in the extract.

To test the precision of the FBBB test, a methanol blank and three concentrations of Δ^9-THC in methanol were used: 5, 10, and 20 ng/μL. Tests were performed in five replicates ($n = 5$) following the procedure described in section 2.5 over the course of 5 consecutive days. Once dry, the samples were measured using the Dialunox® instrument and the results were recorded. The standard deviation was calculated and the relative standard deviation (as% RSD) was reported as the precision of the inter and intra-day analyses.

The bias of the FBBB test was calculated using standard Δ^9-THC solutions and with spiked hemp samples to simulate marijuana-type cannabis with known concentrations of Δ^9-THC. Two standard Δ^9-THC solutions at 6 and 12 ng/μL Δ^9-THC in methanol were measured using the Dialunox® and evaluated using the calibration curve created using standard Δ^9-THC solutions. Ten samples were prepared between the 1 to 7.5 % Δ^9-THC range in the hemp extract, as well as 5 samples prepared above the range to determine the performance of more concentrated samples between the range of 12 to 18 % Δ^9-THC. The hemp samples were extracted as described in the *plant extraction* section. The equation for the linear regression from the calibration curves using standard Δ^9-THC solutions and hemp solutions were used to translate the fluorescence intensity to a corresponding concentration. The absolute bias was calculated by using the following equation:

$$\text{Bias } (\%) = \left|\frac{\text{Mean Concentration Calculated} - \text{Actual Concentration}}{\text{Actual Concentration}}\right| \times 100 \quad (1)$$

Eq. (1): Calculation of absolute bias.

The bias was then used to calculate the uncertainty in the concentration of THC reported by multiplying the bias by the actual concentration.

Introduction of a portable colorimeter for 4-AP and FBBB tests color evaluation

The Lishang colorimeter was used to measure the color values of the samples. The colorimeter has a lens aperture of 8 mm in diameter to perform the color readings. Because of this, measurements with the Lishang colorimeter were performed on a 10 mm silicone-coated substrate, and the volumes used for both the FBBB and 4-AP tests were doubled due to the increase in surface area. The colorimeter produces values that represent brightness (L-value), a red to green channel (*a*-value), and a blue to yellow channel (*b*-value). A larger (more positive) *a*-value indicates a more intense red color present in the sample measured and a smaller (more negative) *b*-value indicates a more intense blue color. The instrument uses the L, *a*, and *b*-values to calculate a color difference value (Eq. (2)). The color difference value (ΔE^*ab) is used to determine if a color passes or fails when compared to the standard when a threshold value is set.

$$\Delta E^*ab = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

Eq. (2): Color difference equation [16].

The color difference value was used to evaluate the colorimetric response of increasing concentrations of Δ^9-THC. Standard Δ^9-THC solutions were prepared in methanol (0.25, 0.5, 1, 5, 10, 15, 20, 25, 50, 100 ng/μL) and were reacted with the FBBB and 4-AP tests and compared to a standard methanol blank. The reactions were performed on the 10 mm silicone-coated substrate and each concentration was tested in triplicate and measured using the colorimeter.

Results

Dynamic range and limit of detection for fluorescence measurements

When standard solutions of Δ^9-THC were tested with FBBB and the resulting average fluorescence intensity for triplicate samples is plotted, the resulting response curve shows a logarithmic trend (Fig. 2a). Linearity is observed for a portion of the concentration range, and the error bars represent the standard deviation of the replicate trials. When concentrations above 20 ng/μL Δ^9-THC are removed, the remaining samples containing between 0.5 to 20 ng/μL Δ^9-THC can be fitted to a linear regression line with a coefficient of determination (R^2) value of 0.95. With this concentration range, the LOD was calculated to be 2.3 ng/μL Δ^9-THC and LOQ calculated to be 6.9 ng/μL Δ^9-THC. When the dynamic range is decreased to 0.5 to 10 ng/μL and plotted and fitted to a linear regression line (Fig. 2b), the resulting R^2 is 0.99 and lower LOD and LOQ are achieved at 1.6 and 4.8 ng/μL, respectively. Depending on the level of accuracy needed in the concentration of Δ^9-THC, either the 2.3 to 20 ng/μL or 1.6 to 10 ng/μL dynamic range can be used for quantitation.

A calibration curve was also prepared using a hemp extract as the matrix rather than methanol, as was done in the previous test. By using the hemp extract as the matrix, the other components present in a typical cannabis plant extract are taken into consideration for the reaction with FBBB. Fig. 3 shows the correlation between the concentration of Δ^9-THC present in the hemp extract and the average fluorescence intensity for 5 replicate trials for each concentration. The error bars represent the standard deviation of the replicate trials. The R^2 for this linear regression is 0.9947. The LOD was calculated to be 1 % Δ^9-THC. Samples more concentrated than 7.5 % Δ^9-THC were not prepared since at approximately 7.5 % Δ^9-THC is when the Δ^9-THC and FBBB exist at a 1:1 molar ratio stoichiometry. When Δ^9-THC is in excess, there was found to be a plateau in fluorescence signal past the 1:1 stoichiometry.

Dynamic range and limit of detection for color

Tests performed with both FBBB and 4-AP correlate the Δ^9-THC concentration in a sample with an increase in the color intensity. The colorimeter takes into consideration the L, a, and b-values to determine the color difference value. Fig. 4 indicates that the higher the concentration of Δ^9-THC, the more intense the color is from the blank sample that was used as a standard. The dashed red line in the graph set at 5 indicates the pass/fail threshold on the colorimeter. When the scatterplots were fitted with a linear regression, the concentration of Δ^9-THC that correlates with a "pass" value for both tests can be determined. With the FBBB test, it is found that a sample containing Δ^9-THC would be differentiated from the blank at a concentration of 6 ng/μL, indicating that the color of the sample is different enough from the blank to be distinguishable. The 4-AP test, on the other hand, requires a concentration of 25 ng/μL Δ^9-THC to identify a sample above the threshold value of 5, indicating a color difference from the blank. Results from this test suggest that the FBBB test is over 4 times more sensitive than the 4-AP test when using color as the only observation. When including the sensitivity advantages of fluorescence of the THC + FBBB complex, the test improves.

For the 4-AP test, the colorimeter value of interest is the *b*-value, which measures color on a blue to yellow channel. A more positive b-value indicates a more intense yellow color, while a more negative value indicates a more intense blue color. Fig. 5b shows that as the concentration of Δ^9-THC increases, the b-value becomes increasingly more negative (a more intense blue). With the FBBB test, the relevant colorimeter value is the a-value, which measures the red to green channel. A more positive a-value indicates a more intense red color, while a more negative value indicates a color closer to green. With the FBBB samples, the a-value increases as the concentration of Δ^9-THC increases as seen in Fig. 5a. For both tests, as the Δ^9-THC concentration increases, so does the intensity of the resulting chromophore.

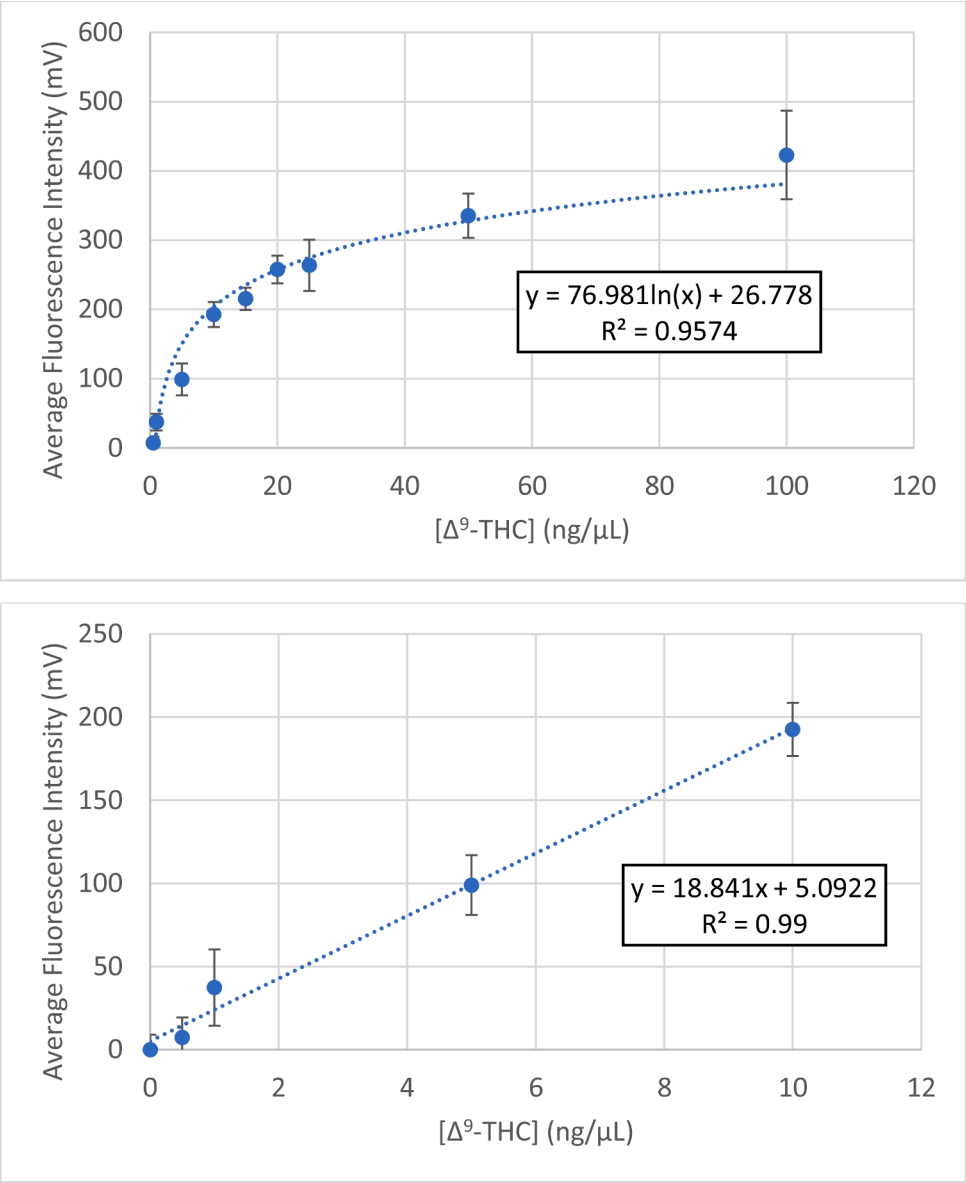


Fig. 2. a. Resulting logarithmic fluorescence of Δ^9-THC standard solutions (0.5 to 100 ng/μL) using the FBBB test ($n = 3$). b. Limited linear dynamic range of the FBBB test with Δ^9-THC (0.5 to 10 ng/μL).

Precision and bias of fluorescence for FBBB

Precision was evaluated for the FBBB test using the fluorescence intensity measured with the Dialunox® instrument. For this, a methanol blank and 5, 10, and 20 ng/μL Δ^9-THC concentrations were tested with the FBBB test in 5 replicates over 5 consecutive days. Inter-day and intra-day precision are reported in Table 1. The intra-day precision reported as percent relative standard deviation (RSD) is reported in Table 1 for 1 of the 5 days tested, resulting in a maximum RSD of 13 %. The inter-day precision reported as percent RSD was 12 % or below for the three concentrations tested.

Bias tests were performed using both the 1.6 to 10 ng/μL Δ^9-THC and 2.3 to 20 ng/μL Δ^9-THC calibration curves using Δ^9-THC standards. Two concentrations were chosen that fall within the linear dynamic range: 6 and 12 ng/μL Δ^9-THC. Solutions were prepared in methanol and 5 replicate samples were performed using the FBBB test and measured using the Dialunox® fluorescence reader. In the 1.3 to 10 ng/μL Δ^9-THC range, the absolute bias calculated for the 6 ng/μL Δ^9-THC sample was 33 %. The 6 and 12 ng/μL solutions were then used to test the bias using the curve with the larger dynamic range, 2.3 to 20 ng/μL Δ^9-THC. Five replicate samples were performed and the fluorescence was measured using the Dialunox®. The absolute bias for the 6 and 12 ng/μL concentrations were 17 % and 35 %, respectively.

Marijuana samples typically contain an average of 15 % Δ^9-THC as well as many other cannabinoids and terpene compounds. Because of these compounds, when the marijuana samples are extracted, many other compounds are extracted from the plant, resulting in a solution with some potential interferents. To test the bias of cannabis plant extracts with known amounts of Δ^9-THC, 15 hemp plant extracts were prepared with known amounts of Δ^9-THC added. These samples were prepared by one researcher and performed as blind samples by another researcher to avoid any potential bias from the researcher performing the experiments. Hemp extracts were used for this evaluation to control the amount of Δ^9-THC present. The equation for the regression line in Fig. 2 was used to determine the calculated concentration of Δ^9-THC from the fluorescence. Samples 1 to 10 were prepared with varying amounts of Δ^9-THC within the 1 to 7.5 % Δ^9-THC range in the Pinewalker hemp extract and the FBBB test protocol was performed by

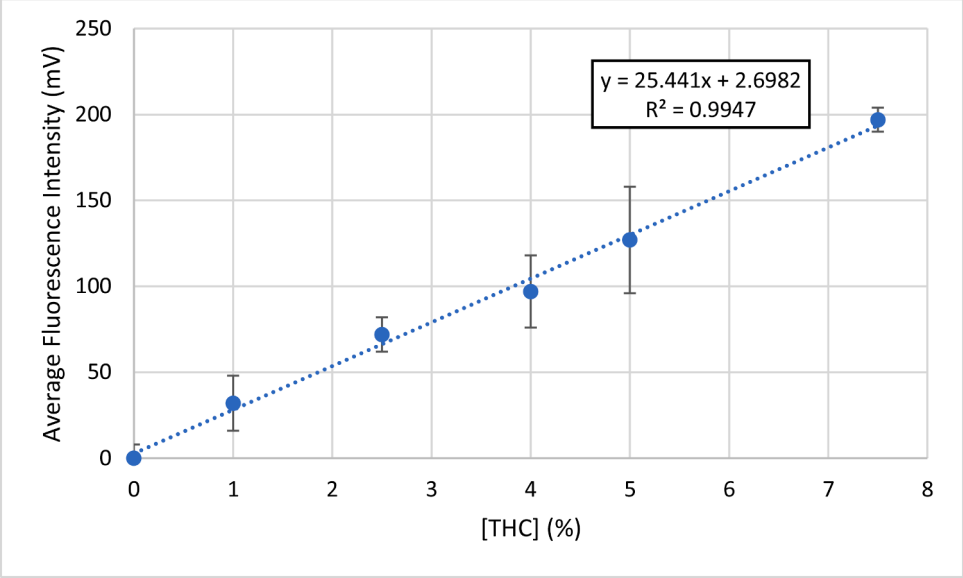


Fig. 3. Calibration curve of increasing Δ^9-THC concentrations prepared in Pinewalker hemp extract.

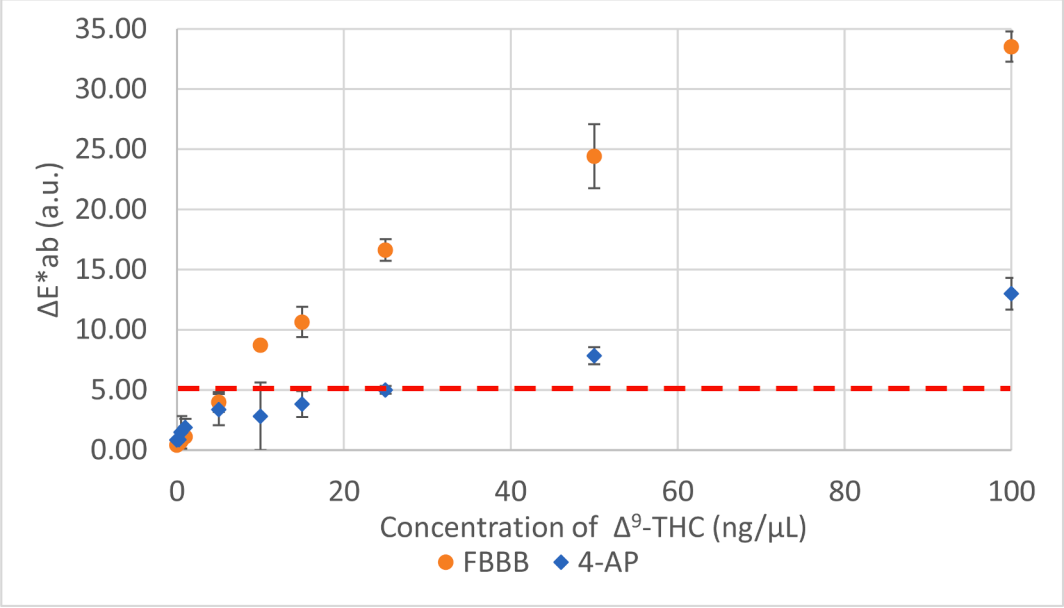


Fig. 4. Using the Lishang Colorimeter, a threshold value set at 5 differentiates a sample from the measured blank ($n = 3$).

another researcher. Samples 11 to 15 were prepared with concentrations above 7.5 % Δ^9-THC in the Pinewalker hemp extract. Each sample was tested in 5 replicate trials. The bias for these 15 samples are reported in Table 2. The absolute bias for samples 1–10 are all below 20 %. The samples that were prepared with higher concentrations above 7.5 % Δ^9-THC resulted in an absolute bias between 38 % and 56 %, increasing with the increase in concentration of Δ^9-THC. These increase in bias was expected since above the 7.5 % Δ^9-THC, the stoichiometry of the FBBB reaction is no longer 1:1. There is still fluorescence detected when the Dialunox® fluorescence analyses are performed, but since the reaction is no longer stoichiometrically balanced, the fluorescence signal does not increase with the increase in Δ^9-THC concentration.

Since the composition of cannabis plants vary between types of cannabis, types of hemp, and even within the plant, it was of interest to perform the bias experiments using other hemp plants to determine if there was a large discrepancy between the other hemps and the calibration curve prepared in Pinewalker hemp. Nine other hemp samples were extracted, and 4 % Δ^9-THC was added to each sample to ensure the Δ^9-THC concentration was within the 1–7.5 % Δ^9-THC range. The total concentration of Δ^9-THC column in Table 3 takes into consideration the existing concentration of Δ^9-THC in the hemp plant as well as the added 4 % Δ^9-THC. Each hemp sample was reacted with FBBB and measured with the Dialunox® fluorescence reader in 5 replicates. The hemps selected all contained lower concentrations of CBD (%) and were all kept in the freezer and thawed prior to use. CBD has been reported previously to quench the fluorescence when tested in a mixture with Δ^9-THC [13]. Although these samples were chosen intentionally due to their low CBD concentration, the samples still contained between 5.7 and 11 % CBD. Interestingly, even with higher concentrations of CBD, this did not seem to have a direct influence on the bias. Although the bias for these 9 other hemp samples is higher than the previous test where varying concentrations were prepared in the

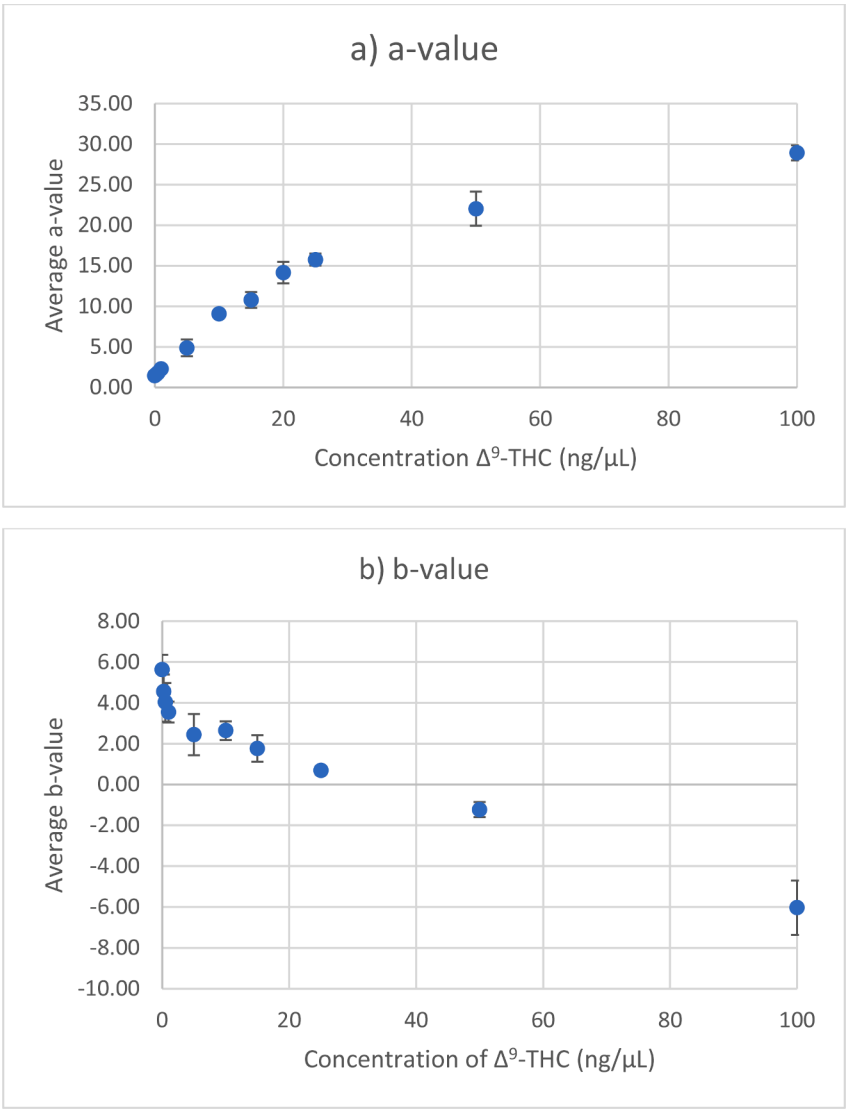


Fig. 5. a. Average a-value ($n = 3$) using colorimeter for the FBBB test. b. Average b-value ($n = 3$) for the 4-AP test.

Table 1
Intra-day precision for the three concentrations of Δ^9 -THC for one day of the precision tests and inter-day precision.

Δ^9-THC concentration (ng/µL)	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Intra-day average fluorescence intensity (mV)	SD	RSD (%)
5	272	218	238	237	222	237	21	9
10	280	295	266	244	209	259	34	13
20	313	351	321	365	398	350	34	10

Δ^9-THC concentration (ng/µL)	Average intensity day 1	Average intensity day 2	Average intensity day 3	Average intensity day 4	Average intensity day 5	Inter-day average fluorescence intensity (mV)	SD	RSD (%)
5	216	237	251	206	160	214	25	6
10	256	259	279	300	239	267	29	12
20	377	350	302	373	353	351	40	11

same hemp extract, the absolute bias is still reportedly below 30 %, which is a good indicator that this fluorometric test is beneficial to determine the Δ^9-THC content in a sample, even with varying concentrations of CBD.

Cannabinoid selectivity

Standard solutions of individual cannabinoids were prepared at 300 ng/µL in methanol to test the selectivity of the FBBB and 4-AP tests. The reactions were performed on the 10 mm substrate using the corresponding doubled volumes. Images and fluorescence readings were taken after 30 min. The color images are depicted in a table in Table 4. Δ^9-THC and Δ^8-THC resulted in a blue color with the 4-AP test and a red color with the FBBB test [11,17]. These results were expected since the two compounds are isomeric. CBD, CBG, and CBDA resulted in a pink color with the 4-AP test and an orange color with the FBBB test. CBN forms a blue color with the 4-AP test and a purple with the FBB test. Fluorescence intensities were measured for the FBBB samples using the Dialunox® instrument, the results are shown in Fig. 6. The methanol blank sample gave an average ($n = 3$) 173 mV intensity. Both Δ^9-THC

Table 2
Absolute bias (%) for samples of Δ^9-THC prepared in "Pinewalker" hemp.

Sample #	Actual concentration (% Δ^9-THC)	Calculated concentration (% Δ^9-THC)	Absolute bias (% bias)	Calculated concentration with uncertainty (% Δ^9-THC)
1	1.5	1.6	6.7	1.6 ± 0.1
2	2.8	2.3	18	2.3 ± 0.4
3	3.0	3.1	3.3	3.1 ± 0.1
4	4.6	4.5	2.2	4.5 ± 0.1
5	9.0	11	22	11 ± 2
6	8.0	9.3	16	9.3 ± 1.5
7	6.7	7.2	7.5	7.2 ± 0.5
8	5.0	5.6	12	5.6 ± 0.7
9	7.5	8.2	9.3	8.2 ± 0.8
10	7.5	8.7	16	8.7 ± 1.4
11	12	7.5	38	7.5 ± 2.8
12	14	8.6	39	8.6 ± 3.3
13	16	7.7	50	7.7 ± 3.9
14	17	7.9	54	7.9 ± 4.3
15	18	7.9	56	7.9 ± 4.4

and Δ^8-THC fluoresced significantly above the blank at an average of 689 mV and 779 mV, respectively. CBN gave a weaker fluorescence intensity at an average of 274 mV. CBD, CBG, and CBDA all resulted in a fluorescence intensity below that of the blank sample, indicating a quenching of the fluorescence.

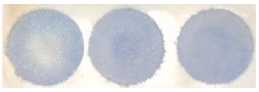
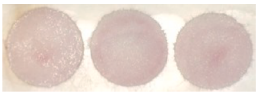
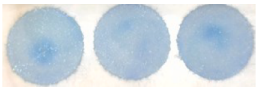
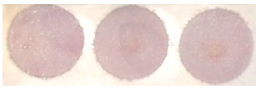
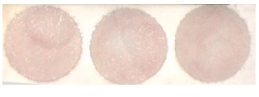
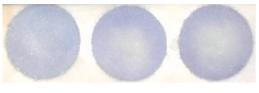
Chemometric analyses for cannabis plant samples tested with FBBB and 4-AP colorimetric tests

Previous chemometric analyses performed using the FBBB and 4-AP tests used only RGB codes obtained from color and fluorescence images taken using the DinoLite digital microscope. The interest of this test was to determine if there was an improvement in these models by using the objective reading for fluorescence using the Dialunox® instrument. The FBBB and 4-AP tests were used to evaluate a total of 202 hemp-type and marijuana-type cannabis samples. The cannabis plant samples used were the same as those used in the Acosta et al. [14]. Supplementary Table 1 describes the list of samples in the current study, which excludes 10 hemp and 8 marijuana samples from the past study, as well as it includes an additional 28 hemp [14]. Of the 202 cannabis plant samples, 111 samples were purchased or obtained as hemp-type cannabis and 91

Table 3
Absolute bias for 9 hemp samples at approximately 4 % Δ^9-THC.

Hemp sample	CBD concentration (% CBD)	Total concentration (% Δ^9-THC)	Calculated concentration (% Δ^9-THC)	Absolute bias of % Δ^9-THC (% bias)	Calculated concentration with uncertainty (% Δ^9-THC)
1	5.7	4.2	4.4	4.7	4.4 ± 0.2
2	6.3	4.2	3.1	26	3.1 ± 0.8
3	6.8	4.3	4.8	14	4.8 ± 0.7
4	7.0	4.3	4.2	<0.1	4.2 ± 0.1
5	7.2	4.3	3.0	29	3.0 ± 0.9
6	7.8	4.2	5.2	25	5.2 ± 1.3
7	8.2	4.2	3.9	7.3	3.9 ± 0.4
8	9.2	4.2	5.1	19	5.1 ± 1.0
9	11	4.3	4.1	5.6	4.1 ± 0.2

Table 4
Cannabinoid selectivity results for the 4-AP and FBBB tests with 6 cannabinoids. *Note*: The 4-AP color images were still wet at time of imaging.

Cannabinoid	4-AP color result	FBBB color result
Δ^9-THC		
CBD		
CBN		
CBG		
CBDA		
Δ^8-THC		

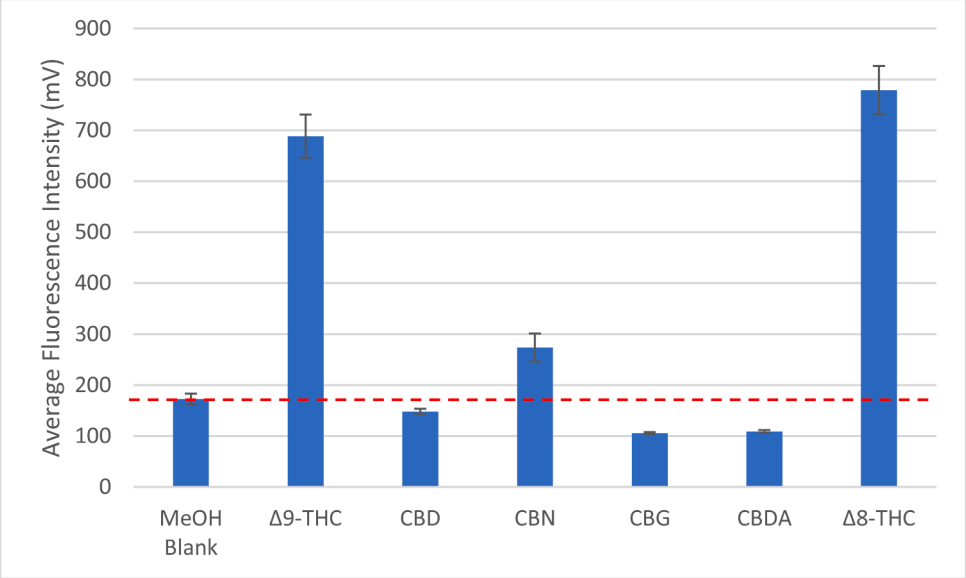


Fig. 6. Average fluorescence intensity ($n = 3$) for cannabinoids and FBBB reaction.

samples were obtained as marijuana-type cannabis. Notably, 12 of the samples that were purchased as hemp contained over 0.3 % THC according to the certificate of analyses provided by the vendors. The FBBB and 4-AP tests were performed in triplicate and allowed to dry for 15 min before images and measurements were taken. For the FBBB test, fluorescence intensity using the Dialunox® instrument, and for both tests, color images were taken and RGB codes were obtained using ImageJ.

First, a principal component analysis (PCA) was performed using the RGB values obtained from the 4-AP and FBBB tests as well as the fluorescence intensity from the FBBB test was measured using the Dialunox® fluorescence reader (7 variables). Fig. 7 shows the results of the PCA. Using this chemometric method, two distinct groupings can be identified as the hemp-type and marijuana-type cannabis groups. The marijuana-type cannabis samples that fell outside of the circled grouping were noted to have THC:CBD ratios below 0.2 and gave pink results with the 4-AP test and orange results with the FBBB test. This limitation has been previously reported where samples that had a THC:CBD ratio below 2 gave inconclusive results [11,12].

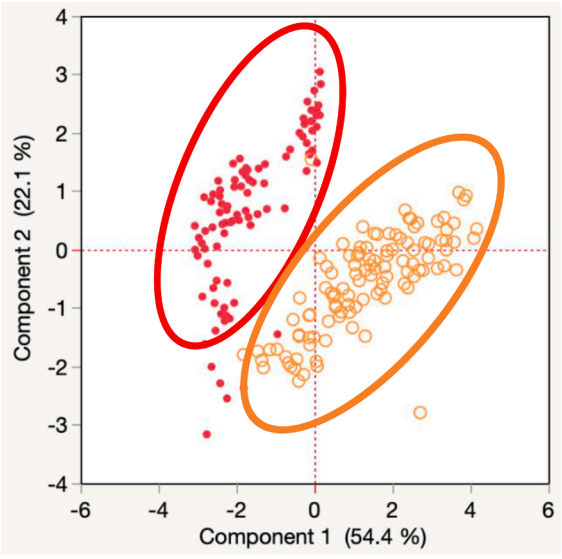


Fig. 7. PCA of all 202 hemp-type (hollow, orange circles) and marijuana-type (solid, red circles) cannabis. Note: Grouping circles are hand-drawn and do not represent statistical significance.

Next, linear discriminant analysis (LDA) was performed using the same data. LDA is a supervised method of dimensionality reduction of data. Discriminant analysis was performed on the cannabis samples in Acosta et al. [14] using both the FBBB and 4-AP tests and found that by using both tests to evaluate a sample, there is an increase in specificity [12]. The variables used for the discriminant analysis in Acosta et al. [14] were RGB codes of the color images and fluorescence images using the FBBB test, and the RGB codes for the color images using the 4-AP test [12]. A limitation for both tests was found to be when the THC:CBD ratio below 2, indicating that the cannabis sample is not THC rich. For all the discriminant analyses performed in Acosta et al., when the marijuana samples with THC:CBD below 2 were removed, there was a vast improvement in the models. When both the FBBB and 4-AP tests were used complimentarily, the LDA model had a reported 100 % correct classification rate for the training set and a 99 % correct classification rate for the test set.

Considering the previously reported results, an LDA model was constructed using JMP that involved 7 variables for each sample, RGB values for the color images of the FBBB and 4-AP tests and the fluorescence intensity of the FBBB samples using the Dialunox® instrument. Each cannabis sample was tested in triplicate and the values used for the LDA were the averaged values for each variable. The training set samples were separated into a "hemp" classification, which included cannabis plant samples that contained 0.3 % or less THC and a "marijuana" classification, which contained over 0.3 % THC. The training set used 67 marijuana samples and 39 hemp samples. The model predicted all the hemp and marijuana samples in their correct classes. A test set was also used to validate the model using 12 marijuana samples and 72 hemp samples. The model correctly classified 100 % of the hemp samples and 92 % of marijuana samples with only one misclassification. The marijuana sample that was misclassified had a THC:CBD of 3.2 and resulted in a purple color with the 4-AP test and orange color with FBBB. It should be noted that in the field, it is rare that marijuana samples have THC:CBD close to 3 or below. Typically, samples found in the field have concentrations of THC much higher than that of CBD.

Lastly, a k-fold cross-validation was performed using the 202 samples in the data set. Of the 202 cannabis samples, 15 marijuana samples had a THC:CBD at or below 3 which, as seen above, can result in misclassifications. K-fold cross-validation involves splitting the data set into K number of folds to evaluate a model given the data. For the purposes of

this data set, a k-value of 5 was chosen. In this case, the data set is split into 5 folds and in each iteration that the model is alternated, the test set is alternated. The average accuracy of the training sets was found to be 97.49 % and the average accuracy for the validation sets was 96.51 %.

Discussion

Colorimetric tests are widely used for field tests of drugs due to their portability and simplicity and require minimal training of field personnel prior to use. Qualitative field tests such as a liquid reaction of 4-AP with plant matter are currently used for the differentiation between hemp-type and marijuana-type cannabis samples. We now present, for the first time, a miniaturization of a field test using FBBB as a reactant deposited on a substrate surface and introduce a portable fluorescence reader and colorimeter to determine color and fluorescence more objectively for both the 4-AP and FBBB tests with THC. The results of this work demonstrate, for the first time, a linear correlation between the THC concentration and the fluorescence intensity for a limited range within a calibration curve created with known THC standards. We also now report the analytical figures of merit (linear range, precision, bias and limits of detection and selectivity) for quantitative analyses using the FBBB test for the determination of THC in cannabis plant samples within a limited range of concentrations.

The rigidity of the THC + FBBB complex along with the extended conjugation produces a bathochromic "red" shift that results in the fluorescence of the THC + FBBB complex [12]. An objective numerical fluorescence value can be obtained using the Dialunox® fluorescence reader to determine the concentration of Δ^9-THC in a sample based on a calibration with THC standards. Fluorescence analysis was used to determine the linear dynamic range of the FBBB test when evaluated with both standard Δ^9-THC solutions prepared in methanol, as well as Δ^9-THC solutions prepared from a hemp plant sample extract to account for the matrix effects. In addition to the linear dynamic range, the limits of detection (LOD) with color and fluorescence, precision, bias, and selectivity were investigated. When standard Δ^9-THC solutions were tested, a linear dynamic range between 1 and 10 ng/μL was found with an R^2 of 0.99, and a LOD and LOQ of 1.6 ng/μL and 4.6 ng/μL Δ^9-THC, respectively. The fluorescence makes it possible to detect Δ^9-THC even when there is no visible color difference from a methanol blank with the naked eye. For concentrations above 20 ng/μL Δ^9-THC, there is a logarithmic response in fluorescence intensity of the THC + FBBB complex (as seen in Fig. 2a). When the FBBB test was evaluated with Δ^9-THC solutions prepared in the hemp extracts, there was linearity between 1 and 7.5 % Δ^9-THC ($R^2 = 0.9947$) and a LOD of ~ 1 % Δ^9-THC. Even in the presence of a more complex matrix, the increase in Δ^9-THC concentration was evident with the increase in fluorescence signal.

Colorimetric evaluation was performed using the FBBB and 4-AP tests using the Lishang colorimeter. When the 4-AP test was reacted with varying concentrations of Δ^9-THC, a color difference from the blank was detected at 25 ng/μL. The equivalent concentration that resulted in a color difference from the blank was 6 ng/μL using the Lishang colorimeter and the FBBB test. These results suggest that when Δ^9-THC is present in a sample, the FBBB test can distinguish the presence at approximately 4 times lower concentration using color alone when compared to the 4-AP test. The FBBB test proves to be even more sensitive when considering that fluorescence can be used to detect concentrations below the 6 ng/μL limit with color readings (as low as 1.6 ng/μL).

Precision and bias for the fluorescence measurements were also determined. When tested over 5 consecutive days, the precision of the test, using 3 different concentrations was 12 % RSD or lower. These relatively poor precision results are not unexpected for a colorimetric test, in comparison to more sensitive analytical instrumentation. Bias was determined using both standard solutions as well as using plant extracts. The methanolic Δ^9-THC solutions were validated in both the concentration ranges: 1–10 ng/μL and 1–20 ng/μL. A 6 ng/μL Δ^9-THC was prepared to test the bias in the 1–10 ng/μL range and resulted in an absolute bias of 33 %. When the larger dynamic range, 1–20 ng/μL, was used, 2 concentrations (6 and 12 ng/μL) were used to evaluate the bias. The bias for the larger dynamic range was found to be 17 % and 35 % for 6 and 12 ng/μL, respectively. To better understand how the FBBB test performs when used with a cannabis plant extract, hemp extracts were used and prepared with varying concentrations of Δ^9-THC. The samples prepared in the same hemp extract solution between 1 and 7.5 % Δ^9-THC resulted in a bias of 20 % or lower for all 10 samples. The 5 other samples prepared with concentrations above the 7.5 % Δ^9-THC resulted in a higher bias, as expected, most likely due to the stoichiometric ratio of Δ^9-THC and FBBB not being 1:1. Fluorescence intensity is not linear for samples above 7.5 % Δ^9-THC samples due to a lack of stoichiometric ratio between FBBB and the THC analyte but the test can report elevated [Δ^9-THC] (above 7.5 % Δ^9-THC), in its current form of the test. Nine other hemp extracts were prepared at approximately 4 % Δ^9-THC and once reacted with FBBB produced a bias of 28 % or lower, indicating that the FBBB test could be used with some confidence to determine the concentration of Δ^9-THC present in a sample.

The results in Fig. 6 indicate that when some cannabinoids (e.g. CBG and CBD) react with the FBBB test, there is quenching of the fluorescence observed. Both Δ^9-THC and Δ^8-THC resulted in similar fluorescence intensities when tested with FBBB, which was expected since the two compounds are isomers. CBN also resulted in fluorescence, but at only 39 % the intensity of Δ^9-THC. There are over 400 chemical components found in cannabis plants, 60 of those being cannabinoids [18]. The six (6) cannabinoids most likely to produce interferences were evaluated for this research, but we acknowledge that others could also be reacting with the FBBB test.

Chemometric methods were applied to the 202 cannabis plant samples to reveal groupings in the data and as a tool for classification and data visualization using both the FBBB and 4-AP tests. Previous chemometric analyses reported used RGB codes from color and fluorescence images for the FBBB test and RGB codes from color images from the 4-AP test. It was expected that adding instrumental measurement of fluorescence would improve the results of the models. The PCA performed used 7 variables and revealed two groupings: marijuana and hemp. The marijuana samples that fell outside of the marijuana grouping or into the hemp grouping all contained a THC:CBD of 2 or below. An LDA model was constructed using a training set that involved 67 marijuana samples and 39 hemp samples that were successfully classified into their respective classes. A validation of this model was performed using a test set that contained 72 hemp samples and 12 marijuana samples. The cross-validation correctly classified 100 % of the hemp samples and 92 % of the marijuana samples, with only one misclassification. The misclassified marijuana sample was composed of a THC:CBD of 3, which indicates that the marijuana was not THC-rich as is typically found to be in the field. A 5-fold cross-validation was performed to test the data using 5 different combinations of training and test sets. This validation method splits the full data set into 5 groups. The cross-validation used 4 of the groups of data points as a training set and then validated the model using 1 group as a test set. This was performed 5 times, each time alternating the group used as the validation set. The average accuracy for the training set was found to be 97.5 %, indicating that there is no over-fitting of the data. The average accuracy for the validation set was found to be 96.5 %.

Both the FBBB and 4-AP tests demonstrate the potential for quantitative analysis of Δ^9-THC within a limited concentration range. The colorimeter provides an objective value for a color reading and correlates the concentration of Δ^9-THC with the intensity of the chromophores that formed with the 4-AP and FBBB reactions. Moreover, the FBBB test has the advantage of using fluorescence detection of lower concentrations of Δ^9-THC even when the reaction with FBBB does not result in a very intense red color. Future work could include testing extracts from "fresh" marijuana plants for both colorimetric and fluorescence measurements. The FBBB test has been studied for potential

field applications and could also be applied to the analysis of THC in the oral fluid matrix [13]. Since this test involves small volumes and portable instrumentation, it can provide a cost-effective alternative to analyze cannabis plants in the field including the determination of THC concentrations in plants with good precision and accuracy.

CRediT authorship contribution statement

Nicole B. Valdes: Investigation, Methodology, Writing – original draft. **Roberta Gorziza:** Data curation, Formal analysis, Investigation, Methodology, Project administration. **José R. Almirall:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2024.100287.

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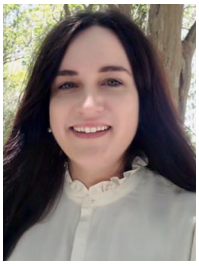
Nicole Valdes is a doctoral candidate in her final year of study in the Department of Chemistry and Biochemistry at Florida International University in Miami, FL. She received a BA and MS in Chemistry, both from FIU. Between finishing her undergraduate studies and beginning her PhD, Nicole earned a Certificate in Forensic Science. Nicole's research experience involves the study of fieldable techniques for forensic analysis including ignitable liquid residues in fire debris and the detection of the psychoactive component in marijuana, Δ^9-tetrahydrocannabinol, using the sensitive and selective Fast Blue BB test.



Roberta Gorziza is a Postdoctoral Associate at the Chemistry and Biochemistry Department of Florida International University in Miami, FL. She is a BS in Biomedical Science, with a MS in Molecular Biology and Genetics, and a PhD in Pharmaceutical Sciences, graduated from the Federal University of Rio Grande do Sul, in Brazil. Roberta started her career in forensic chemistry and toxicology in 2019, when she received a Brazilian scholarship to work as a visiting research scholar at West Virginia University in the United States, as part of her PhD program. After graduating in 2021, she has been working as a postdoctoral researcher in forensic science, acquiring experience in research, project management in the Center for Advanced Research in Forensic Science (CARFS), and teaching crime scene investigation and forensic chemistry courses.

José R. Almirall retired as a Distinguished University Professor and is now Emeritus Professor in Chemistry and Biochemistry at Florida International University (FIU) in Miami, FL. He received a B.S. in Chemistry from FIU, a M.S. in Chemistry from the University of Miami and a Ph.D. in Chemistry from the University of Strathclyde. He began his career at the Miami-Dade Police Department forensic laboratory as a practicing forensic chemist where he worked for 12 years prior to his academic appointment at FIU in 1998. He was the founding co-Director of the International Forensic Research Institute at FIU (1997) and the founding graduate program director of the MS in Forensic Science program at FIU (1998). He was also the founding Director of the National Science Foundation-funded Center for Advanced Research in Forensic Science (CARFS) at Florida International University. His research interests include the development of analytical methods for improving the forensic examination of trace evidence (materials) and developing statistical tools to improve the interpretation of chemical data. Prof. Almirall and his group have authored more than 165 peer-reviewed publications and over 750 presentations in analytical and forensic chemistry. Prof. Almirall currently serves as the co-Editor-in-Chief of *Forensic Chemistry,* an Elsevier journal.