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Validation of a combined Fast blue BB and 4-Aminophenol colorimetric test for indication of Hemp-type and Marijuana-type cannabis

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ABSTRACT

Two effective presumptive tests used to indicate hemp-type and marijuana-type cannabis are the Fast Blue BB (FBBB) and 4-Aminophenol (4-AP) colorimetric tests. We report the miniaturization of a 4-AP colorimetric reaction on a substrate to be used in combination with the previously reported FBBB test. Both tests use <50 μL of reagents to effectively indicate hemp and marijuana. The FBBB and 4-AP tests analyzed 99 authentic marijuana samples and 93 authentic hemp samples and the performance of both tests, individually and in combination, are presented here. Red, Green, and Blue (RGB) scores were obtained for the chromophores (and fluorophore in the case of FBBB) from magnified images of the reaction products on the substrate and Linear Discriminant Analysis (LDA) and Data Driven-Soft Independent Modeling of Class Analogies (DD-SIMCA) models were constructed using the RGB scores. The LDA results showed that FBBB and 4-AP are effective (>90 % correct classification) at classifying THC-rich marijuana (THC:CBD > 2) from hemp individually but have a slightly higher specificity when both tests are used in combination (greater than or equal to 95 % correct classification). Marijuana samples with a THC:CBD below two were considered outliers for the SIMCA models. However, sensitivity and specificity above 95 % were achieved with the SIMCA models when these samples were removed. These observations and statistical results suggest that FBBB and 4-AP may be used either individually or in combination to reliably indicate hemp and marijuana when the THC:CBD is above two.

Introduction

When the Agriculture Improvement Act of 2018 was enacted in 2019, it classified cannabis plants into two categories: hemp-type and marijuana-type. Hemp-type cannabis is defined as *Cannabis sativa* with a total delta-9 tetrahydrocannabinol (THC) concentration below 0.3 % (w/w) and is legal for cultivation and trade in the United States [1]. Marijuana-type cannabis is any *Cannabis sativa* that contains equal to or above 0.3 % (w/w) THC and is classified as a federally illegal Schedule I drug in the US. Although the legal threshold for cannabis to be considered marijuana is only 0.3 % (w/w), typical marijuana plants contain much higher concentrations of THC. Hemp-type cannabis typically contains a high concentration of CBD and low concentrations of THC. Recent reports of confiscated cannabis in the US and Switzerland show that THC-rich cannabis usually contains between 10 and 14 % THC and little to no CBD [2,3]. The study from Switzerland also found that CBD-rich cannabis contained concentrations of CBD up to 25 % and found that the level of total THC in these plants were below 1 % [3]. Even though hemp and marijuana are chemically different, these two cannabis strains have a similar physical appearance and smell that may confuse law enforcement officers attempting to distinguish between the two. The Duquenois-Levine (D-L) color test, a common presumptive test for cannabis, is unable to differentiate between hemp and marijuana. This is due to both THC and CBD containing a resorcinol backbone and an aliphatic chain triggering the same colorimetric result for both strains [4,5]. There is a great need for a fast and reliable test that can differentiate between hemp and marijuana in the field.

The main difference between hemp and marijuana are the concentrations of THC and CBD, therefore presumptive field tests should focus on the THC:CBD ratios of the cannabis plants. One such test that does this is the Fast Blue BB (FBBB) color test. FBBB has been used as a visualization reagent for Thin Layer Chromatography (TLC) analysis of cannabis extracts [6]. Ultraviolet–Visible Spectroscopy, High Resolution Mass Spectrometry, and Proton Nuclear Magnetic Resonance (1H NMR)

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[7,8] have characterized the reaction and product of FBBB and THC. From these studies it was determined that, under basic conditions THC becomes a phenolate anion. The diazo group in FBBB is then attacked by the phenolate anion and bonds at the para position to form a red chromophore. CBD reacts with FBBB using the same mechanism, forming an orange chromophore. Recently, it was discovered that the product formed with THC and FBBB fluoresces brightly under 480 nm light, while the product of CBD and FBBB does not [9]. The difference in color and fluorescence allows for FBBB to be a selective and sensitive test to indicate marijuana (THC-rich/CBD-poor) and hemp (CBD-rich/THC-poor). In this study, a miniaturized version of the FBBB reaction was performed on a 3.5 mm Planar Solid-Phase Microextraction (PSPME) [10] to analyze extracts from 25 different authentic marijuana and hemp samples. Images were acquired of the chromophores/fluorophores formed using a hand-held portable microscope at low (20X) magnification. Red, Green, and Blue (RGB) numerical codes were obtained for these images using ImageJ software and these numerical codes were then used to perform Linear Discriminant Analysis (LDA) to objectively classify the hemp and marijuana samples. The results of the analyses showed that FBBB is sensitive and specific when differentiating hemp from THC-rich marijuana (THC:CBD > 2). FBBB observation experiences difficulty in interpreting results for cannabis with THC:CBD between 0.3 and 2. This study also demonstrated that FBBB was selective for THC among other cannabinoids and plant materials [9].

Another color test that has demonstrated its capabilities as a presumptive test to indicate between hemp-type and marijuana-type cannabis is the 4-Aminophenol (4-AP) test. This test forms a blue color in the presence of THC-rich cannabis and a pink color with CBD-rich cannabis [3,11]. The 4-AP test has been found to be a fast (2-minute product formation) and effective test to indicate hemp and marijuana. However, one limitation of 4-AP is that after the 2-minute observation window, the color becomes too dark to properly interpret. There is also difficulty in interpreting results for cannabis with THC:CBD between 0.3 and 3. It has been reported that for samples containing THC:CBD between 0.3 and 3, a purple color forms instead of a distinct pink or blue [11]. In its current form, this test requires 1 mL of 4-Aminophenol to be reacted with 5 mg of a solid plant matter sample. The large amount of liquid and plant matter required for the reaction make it difficult to capture RGB scores that produce numerical and objective color interpretation results. Miniaturizing of the 4-AP color test on a solid substrate with plant extracts allows for an improved practical and objective field test.

FBBB and 4-AP have demonstrated capability as field tests to indicate hemp or marijuana. When used in combination, the tests provide improved specific classifications. To demonstrate this, the 4-AP reaction was miniaturized and placed onto a 6.35 mm silicone treated filter paper substrate in order to obtain a clear color image for the reactions. The modified 4-AP test and the previously developed FBBB miniaturized test [9] were used to analyze 192 authentic cannabis samples obtained from the National Institute of Standards and Technology (NIST), the Drug Enforcement Administration (DEA), and from commercial retailers. The RGB codes for the chromophore/fluorophore products were acquired and used to build chemometric models to classify the hemp and marijuana samples. These models show that, individually and when used in combination, FBBB and 4-AP perform well in classifying hemp and marijuana. These models struggle when the ratio of THC:CBD range from 0.3 to 2 for FBBB and from 0.3 to 3 for 4-AP.

Materials and methods

Materials

Methanol and chloroform were purchased from Sigma Aldrich (St. Louis, Missouri, United States). 4-Aminophenol, ethanol, and Cytiva Whatman™ 1PS Disposable Phase Separating Paper were purchased from Fisher Scientific (Hampton, New Hampshire, United States). 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).

Sixteen CBD-rich hemp strains were purchased from Blue Ridge Hemp Co (Asheville, North Carolina, United States) 7 CBD-rich hemp strains were purchased from Tweedle Farms (Clatsop County, Oregon). 0.5 g of Painted Lady hemp, Elektra hemp, and Spec 7 hemp were used to make hemp mixes with the other hemp samples available from commercial retailers. In this way, 63 hemp mixes were made for analysis bringing the total number of commercial hemp samples to 86. Cigars, apollo hop pellets, citra whole leaf hops, oregano, thyme, spearmint, sage, parsley, red pepper flakes, black pepper, lavender, and eucalyptus leaves were all purchased through various commercial retailers to represent plant materials that were not cannabis but could be mistaken for cannabis. The cannabis research program at NIST provided 31 previously characterized by Liquid Chromatography-Diode-Array Detector (LC-DAD) samples. Twenty-four of these cannabis samples were determined to be marijuana and seven of these samples were determined to be hemp. DEA provided 75 marijuana samples for this study. These samples were characterized by High Performance Liquid Chromatography (HPLC) using a Shimadzu LC-2030C Plus Cannabis Analyzer equipped with a Nexleaf CBX for Potency column (150 mm × 4.6 mm × 2.7 μm) using the manufacturer's "high sensitivity" method. Plant material samples analyzed via HPLC were first ground using an IKA grinder mill and passed through a 425 μm sieve to ensure homogeneity. Two approximately 100 mg portions were each extracted in 5 mL of 80:20 acetonitrile:methanol via brief vortexing and sonication for 15 min. Each sample was then centrifuged, and the supernatant was transferred to a 10 mL volumetric flask and diluted to volume. Samples were filtered through a 0.45 μm filter prior to injection.

Reagent preparation

The FBBB solution was made by dissolving 10 mg of FBBB salt in 10 mL of methanol. This solution was stored in the freezer in an amber vial wrapped in tinfoil. A methanolic solution of 0.1 N NaOH was made by dissolving 0.4 g NaOH in 100 mL of methanol and was stored in the refrigerator in a clear container.

The 4-AP reagent was made by dissolving 30 mg of 4-aminophenol in 99.5 mL of ethanol and 500 μL of 2 M HCl. A NaOH solution was prepared by dissolving 3 g of NaOH in 70 mL of ethanol and 30 mL of deionized water. Both reagents were stored in clear vials in the refrigerator.

Solid substrate preparation

The preparation of the PSPME substrate has been previously described in detail by Guerra et al. [12]. To make PSPME, glass fiber filters are washed, activated, and spin-coated with a sol–gel polydimethylsiloxane (PDMS). To complete the process, the filters were then cured in a high-temperature oven. The resulting PSPME substrate material was then cut into 3.5 mm diameter reaction disks using a Rapid Core Sampling Tool from Electron Microscopy Sciences.

PSPME was not used as the solid substrate for the 4-AP test, as the substrate did not absorb the reagents well. Instead, Cytiva Whatman™ 1PS Disposable Phase Separating Paper was used. These separating papers were made into 6.35 mm diameter reaction disks using a standard hole punch.

Sample preparation and extraction

The cannabis samples from NIST and DEA were previously homogenized to a fine powder. For the commercial hemp samples and other plant samples, 0.5 g of the plant material was homogenized using a tobacco spice grinder. For extraction, a 10–15 mg subsample was taken for each plant, placed into an autosampler vial, and were extracted with 1

mL of MeOH:$CHCl_3$ (9:1). The vials were then vortexed for 20 s twice during a 10 min extraction period. Following the extraction period, the supernatant was removed via pipette and placed into a clean autosampler vial. These extracts were then stored in the freezer until use.

Fast blue BB testing procedure

To prepare the PSPME substrate for analysis, the substrate is placed in the center of a 12 mm diameter carbon tape. This tape keeps the reagents from spilling around the substrate and concentrates them onto the substrate, enhancing color and fluorescence. To begin, 10 μL of the plant extract was pipetted onto the PSPME substrate. Next, 10 μL of 0.1 % FBBB solution immediately followed by 10 μL of 0.1 N NaOH are pipetted onto the substrate. The color change was observed immediately following the addition of the NaOH solution; red indicating a positive result for marijuana and orange a negative. The FBBB test was performed in triplicate per extract. Each substrate was photographed with a Dino-Lite AM4115ZT(R9) digital microscope (Dunwell Tech, Torrance, CA) and Dino-Lite AM4115T-GRFBY Digital Microscope. The Dino-Lite AM4115T-GRFBY is equipped with a 480 nm excitation light source and emission filters for 510 nm and 610 nm. To remove any possible interference from outside sources of light, fluorescence images were taken inside of a box. RGB codes were obtained for both visible and fluorescent images by using the ImageJ RGB measure plugin across each substrate.

4-aminophenol test procedure

The reagent volume for the 4-AP procedure was miniaturized in this study to reduce glare when photographing the chromophores. A solid substrate was also included in this procedure for this same reason. First, the 6.35 mm separator paper was placed inside a disposable spot plate. Next, 5 μL of plant extract was pipetted into the substrate. Once the paper had absorbed the extract, 30 μL of 4-AP was pipetted onto the substrate followed by 6 μL of the NaOH solution. A faint color developed in 2 min, distinct pink (CBD) or blue color (THC) could be observed at 5 min, and at 10 min the color began to degrade. The 4-AP test was run for each sample in triplicate. Photographs of the chromophores formed by 4-AP were taken 5 min after the reagents were mixed. A Samsung Galaxy S8 smartphone was used to take the photos.

Since the color of the product would degrade before the solvent evaporated, photos of the substrates were taken while they were still in liquid. Because of this, lighting became important to obtain precise RGB codes per sample. To reduce glare and shadow, a photobox was constructed. This photobox was a 15.24 cm × 15.24 cm × 15.24 cm cardboard box lined with white computer paper to allow for light to disperse evenly throughout the area. Two holes were cut at the top, one for the LED flashlight that was used as the light source and one for the Galaxy S8 camera to take the photos with (Fig. 1.). Once the reaction began, the spot plate was put into the photobox. The autoexposure on the camera was fixed to be standard for all photos. After 5 min, a photo was taken capturing all three replicates of the sample. RGB codes were then obtained from the images taken using Image J. A color threshold tool was used to adjust the hue, saturation, and brightness of the image to only select regions with color in them so that any glare would be ignored. Once these regions were selected an ROI (region of interest) RGB plugin was used to obtain the RGB codes for the regions.

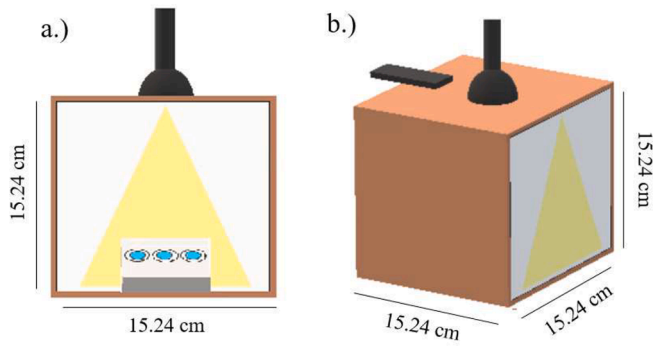

Fig. 1. Diagram of the photobox used to capture images of the 4-AP reaction, a.) Front view and b.) Side view.

Analysis of the chemometric models

To objectively determine how well the FBBB and 4-AP classified the cannabis samples as hemp or marijuana, two different supervised modeling methods were used. The first model method used was Linear Discriminant Analysis (LDA). LDA is known as a "hard" model method in which samples are classified between groups which have previously been defined. For these models, the predefined groups were marijuana, hemp, and other. The "other" group was constructed using plant material that was not cannabis and therefore contained no THC or CBD. RGB of the visible images of the FBBB chromophore (R, G, and B), RGB of the fluorescence image of the FBBB fluorophores (R-F, G-F, B-F), and RGB of the 4-AP chromophores (R-AP, B-AP, and G-AP) were used as variables in the LDA. The LDA analysis was performed using the JMP software. For this analysis, different combinations of variables were used to determine how specific FBBB and 4-AP were individually and when used together. All 31 NIST samples, the 41 commercial hemp samples, and DEA Samples 1–30 were used to build the training sets, while DEA Samples 31–75 and 45 hemp samples were used as a test set meant to validate the model. In the models, each replicate for each sample was added in as an individual object.

Data Driven-Soft Independent Modeling of Class Analogies (DD-SIMCA) was also used to determine the sensitivity and specificity of the FBBB and 4-AP tests. DD-SIMCA uses a one-class modeling method as opposed to a multiclass method, such as LDA. Here, only one class is predefined and samples either fall into the predefined class or out of it. There is also the option for the sample to fall into the extreme category, which means the sample is part of the class but is close to being an outlier. DD-SIMCA is also useful because one can adjust the acceptance threshold for both Type-I error and the outlier significance by adjusting the alpha and gamma values of the model, respectively. The Type-II error is then later calculated when comparing an alternate class to the class trained for the model. For this modeling method, samples in the data set would categorize as in the training set, test set, or in the alternative set. Ideally, all samples for the predefined class will fall in the training and test sets and the samples put into the alternative set will be marked as outliers.

To perform the DD-SIMCA, the Chemometrics 2.0 Excel add-in was used. All nine variables used in the LDA are used in the DD-SIMCA models. Since DD-SIMCA is a one class method, separate models were used to classify hemp and marijuana. Preprocessing in the form of centering and scaling was available in the SIMCA excel software, however no improvements to the models were found by performing them. Therefore, no preprocessing was done to the data for analysis. For all models, alpha was set to 0.05 and gamma was set to 0.01. All replicates of all cannabis samples were used in the data set. The training set was then observed at each PC (principal component) to determine which samples were falling into the extreme category. Samples that were marked as extreme for each PC were selected to be added into the training set. The test set was then selected by using every third sample from the remaining samples in such a way that 20–35 % of the full dataset was represented in the test set. A robust version of the models created was used to detect any outliers in the models. Outliers were then removed from the training and test sets for each PC. Samples that did not belong to the class that was being modeled were used to test the specificity of the models made. These samples were known as the alternative set. Once the training and test set were fully selected, the figures of merit

for each principal component were calculated in the SIMCA software. The PC with the best sensitivity and specificity was chosen for modeling. Once the best PC was selected, it was then assured that the data did not contain irregularities by referring to the extreme plots of the models. These extreme plots examine if the observed number of extremes are equal to the expected number of extremes. If datapoints are found outside of the acceptable range, this signals an irregularity with the data or that the wrong PC is being used.

Results

Analysis of cannabis samples

In total, 192 cannabis samples were analyzed with the FBBB and 4-AP test methods (Supplementary Table S1). Out of these samples, 93 samples were known to be hemp and 99 samples were known to be marijuana. Using FBBB and 4-AP in combination allows for a 3-pronged approach to classify cannabis as marijuana or hemp by using the color of the FBBB chromophore, fluorescence of the FBBB fluorophore, and the color of the 4-AP chromophore. THC-rich samples form a red color and fluoresce under 480 nm light for FBBB and a blue color with 4-AP (Fig. 2**a**.). Most CBD-rich samples form an orange color and not fluoresce with FBBB and a pink color with 4-AP (Fig. 2**b**.). There were some samples that were found to be inconclusive based on observations, such as DEA Sample 39 (Fig. 2**c**) and NIST Sample 20. For these samples, one or two indicators in these tests would be in opposition with the other. In the case of DEA Sample 39, its extract formed a light red chromophore that fluoresced under 480 nm light, but it formed a green color with 4-AP. This is not in agreement with the FBBB results. Similarly, for NIST Sample 20, it formed an orange color with FBBB, however it fluoresced slightly under 480 nm and formed a grayish blue color with 4-AP. DEA Sample 39 was found to have THC and CBD concentrations below 1 % and NIST Sample 20 has a THC:CBD of 1.4, indicating that at low concentrations or when the concentrations of THC:CBD are roughly the same, FBBB and 4-AP may provide inconclusive results.

All 93 of the hemp samples in this study were CBD-rich, and expected to produce an orange color and no fluorescence with FBBB and a pink color with 4-AP. NIST Samples 11 and 12 produced no color with FBBB or 4-AP. NIST Sample 14 produced a light orange color with FBBB but no color with 4-AP. It is important to note that none of hemp samples produced a false positive result. All marijuana samples with a THC:CBD > 2 produced a red color and fluorescence with FBBB, and a blue color with 4-AP, with the exception of DEA Sample 39. Marijuana samples with THC:CBD < 2 produce either a result that would be considered a false negative result, such as NIST Sample 22 or DEA Sample 43, or inconclusive results, such as NIST Sample 20. Inconclusive results were found to be likely with samples containing a THC:CBD close to one.

Linear discriminant analysis

Linear Discriminant Analysis was performed using the RGB codes for the visible and fluorescence FBBB chromophore/fluorophore images and the visible 4-AP chromophore images. All 192 samples were analyzed in triplicate with the FBBB and 4-AP tests. Each replicate was added to the LDA dataset as an independent sample, therefore the full dataset contained 576 data points. In this dataset DEA Samples 31–75 and 45 of the commercial hemp samples were used as the test set to validate the model.

The first LDA model constructed used the RGB of the visible chromophores and RGB of the fluorophores formed by FBBB as variables (six variables total) to determine the specificity of the FBBB test alone. The training set model had an r^2 of 0.60 and correctly classified 92 % of the samples and the test set had a r^2 of 0.91 and correctly classified 99 % of samples within it. The canonical structure of the model showed R-F had the highest correlation with marijuana-type cannabis and that G-F and G had the highest correlation with hemp-type cannabis. The misclassified samples in the training set were NIST Samples 9, 10, 18, 19, 20, 21, 22, 23, and 29. All but one of these samples had a THC:CBD below two, meaning they were not THC-rich. NIST Sample 29 was the exception to this, containing a THC:CBD of 3.231. The one replicate of NIST Sample 29 that misclassified could be explained by having a higher G score and lower R score than most marijuana samples. The test set samples that misclassified were DEA Samples 43 and 73. The misclassification of DEA Sample 43 was unsurprising as it was found to be CBD-rich containing very little THC. DEA Sample 73 was found to be THC-rich, however the chromophore/fluorophore formed had high G scores and low R-F scores causing misclassification by the model.

Next, a model using RGB of the 4-AP test was constructed to see how well it compares the FBBB model. The training set for this model had an r^2 of 0.82, correctly classifying 97 % of the samples. The r^2 for the test set was 0.72 and correctly classified 96 % of the validation samples. In this model, G-AP is closely correlated with marijuana-type cannabis and R-AP is correlated with hemp-type cannabis. The marijuana samples misclassified in the training set were NIST Samples 6, 10, 19, and 23, all containing a THC:CBD below two. Two replicates of NIST Sample 14, a hemp sample, misclassified as marijuana in the training set. This sample did not form a color with 4-AP likely leading to its misclassification. In the test set, DEA Samples 43, 50, 52, and 75 misclassify as hemp. Once again DEA Sample 43 misclassified, as expected, due it being CBD-rich and forming a pink color. DEA Samples 50, 52, and 75 however are all THC-rich cannabis samples. Although they were THC-rich they formed very light colors with the 4-AP test. This caused them to have a higher R-AP score than other marijuana samples, leading to their misclassification.

One aspect of this study was to determine whether 4-AP and FBBB increase in specificity when used complimentary to each other. To determine this an LDA model was constructed using the RGB of the

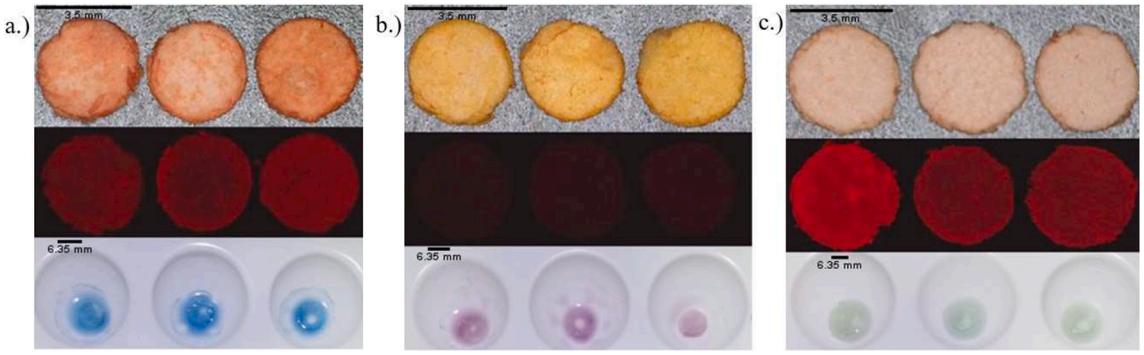

Fig. 2. FBBB chromophore, fluorophore, and 4-AP color results for a.) NIST Sample 7 (THC-rich), b.) NIST Sample 16 (CBD-rich), and c.) DEA Sample 39 (inconclusive result).

visible FBBB chromophore, the fluorescence FBBB fluorophore, and the RGB of the 4-AP chromophore (9 variables total). The training set for this model had r^2 of 0.82 with 95 % of the set correctly classified, while the test set had an r^2 of 0.81 with 98 % of this set correctly classified. In this model, R-F was found to be highly correlated with marijuana and R-AP was found to be closely correlated with hemp. The training set misclassifications were NIST Samples 6, 9, 10, 19, and 23, all of which contain THC:CBD below two. Out of the hemp samples, two replicates of NIST Sample 14 were misclassified as marijuana. NIST Sample 14 produced a light orange color with FBBB and no reaction with 4-AP. This produced RGB with high B and high R-AP score, likely leading to its misclassification. In the test set, DEA Samples 43, 52, and 75 were all misclassified. These misclassifications were likely due to their high R-AP scores, with the exception of DEA sample 43 which is CBD rich.

In all the models described above, the LDA models misclassified samples of marijuana with a THC:CBD below two. This decreased the specificity of the models. To better understand the specificity of these two tests individually and together, the marijuana samples with a THC:CBD below two were removed from the training and validation sets. With these 13 samples removed from the dataset the new number of data points in the set was 537. This improved the training set and most of the test sets for all three models.

The training set of the model using FBBB color and fluorescence obtained a r^2 of 0.97 and the test set had an r^2 of 0.98. In this version of the model, 99 % of the training set is correctly classified and 99 % of the test set is correctly classified. The same as the model with the full dataset, R-F is highly correlated with marijuana and G and G-F are highly correlated with hemp. The two training set misclassifications are of NIST Sample 29, which has a THC:CBD of approximately 3. These misclassifications as hemp are likely due to their higher G score and lower R-F scores when compared to other marijuana samples. The sample that misclassified in the test set was DEA Sample 73 for the same reasons as discussed above. A comparison of the LDA before and after marijuana samples with THC:CBD < 2 were removed from the dataset can be seen **in** Fig. 3.

The 4-AP model also showed improvements in the training model of the LDA when the low THC samples were removed (Fig. 4). The new model had an r^2 of 0.99 with a 99 % correct classification in the training set, however the performance in the test set decreased. The test set had an r^2 of 0.55 and only 96 % correctly classified of the test set. In this model, G-AP is correlated with marijuana and R-AP is correlated with hemp. One replicate of NIST Sample 3 was misclassified as hemp in the training set. The poor fit of the test set can be explained by the misclassified samples in the test set: DEA Samples 39, 50, 52, and 75. DEA 39 was misclassified due to the green color it formed with the 4-AP chromophore. This sample produced ambiguous results due to its low concentration of THC and CBD. NIST Sample 3 and DEA Samples 50, 52, and 75 were misclassified likely due to the light blue color they produced giving a high R-AP score which is highly correlated with the hemp samples. It is important to note that none of these misclassified samples produced a pink color indicative of hemp. This indicates that if results have low concentrations or a poor extraction, this could lead to an inconclusive result with 4-AP.

The LDA model that combines all nine variables from FBBB and 4-AP had a great improvement once the 13 marijuana samples with THC:CBD below two were removed (Fig. 5). In this model, the training set had a correct classification rate of 100 % and the test set had a correct classification rate of 99 %. The training set for this model was found to have an r^2 value of 1.00, meaning there is a clear separation of the hemp and marijuana classes, and the test set had an r^2 value of 0.98. Like the model before the sample removal, R-AP was found to be the variable most closely correlated with hemp and R-F was found to be closely correlated with marijuana. The sample that was misclassified in the test set was DEA 75. As explained previously, DEA Sample 75 produced a light blue color with 4-AP which increased its R-AP score leading to its misclassification.

As mentioned before, LDA classifies samples into predefined classes. In the case of the model above there were only two classes predefined: hemp and marijuana. This only allows the LDA to indicate between two classes along only canonical 1. There are more plants than just hemp and marijuana, so for the final LDA model it was decided to add an “other” class. This class was constructed of 13 samples of plant materials that contained no cannabinoids tested with FBBB and 4-AP in triplicate. This class addition now allowed for the LDA to show differences in

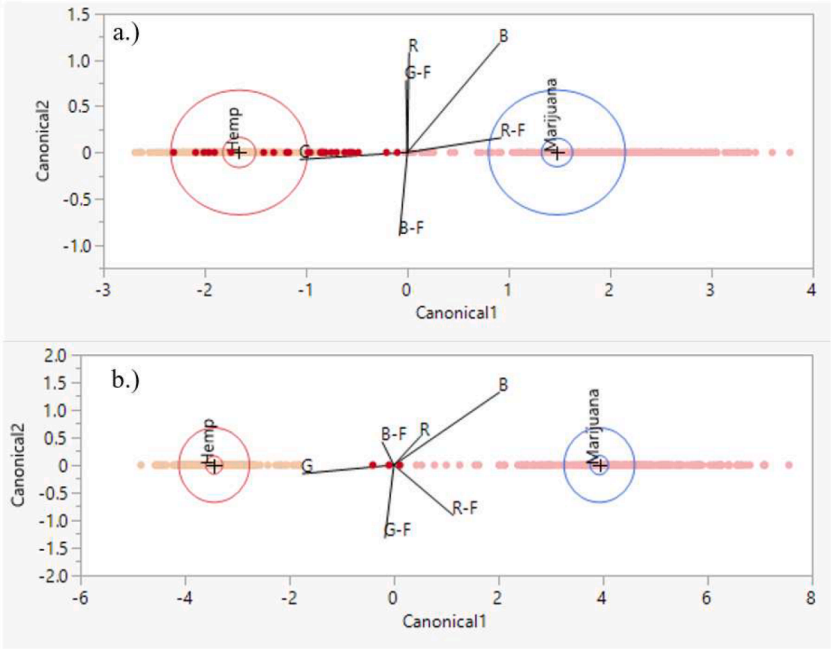

Fig. 3. Canonical plot of the LDA model using RGB of the FBBB chromophore/fluorophore before (a.) and after (b.) removing marijuana samples with THC:CBD below 2. Marijuana data points are shown in red and hemp data points are shown in orange. The highlighted points represent all misclassified samples in model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

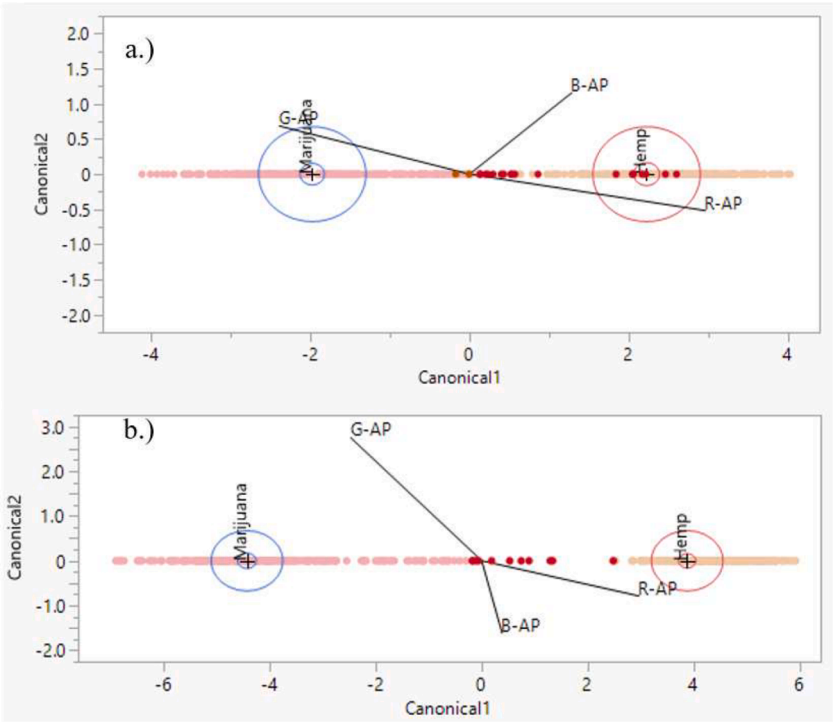

Fig. 4. Canonical plot of the LDA model using RGB of the 4-AP chromophore before (a.) and after (b.) removing marijuana samples with THC:CBD below 2. The highlighted points represent all misclassified samples in model. Marijuana data points are shown in red and hemp data points are shown in orange. The highlighted points represent all misclassified samples in model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

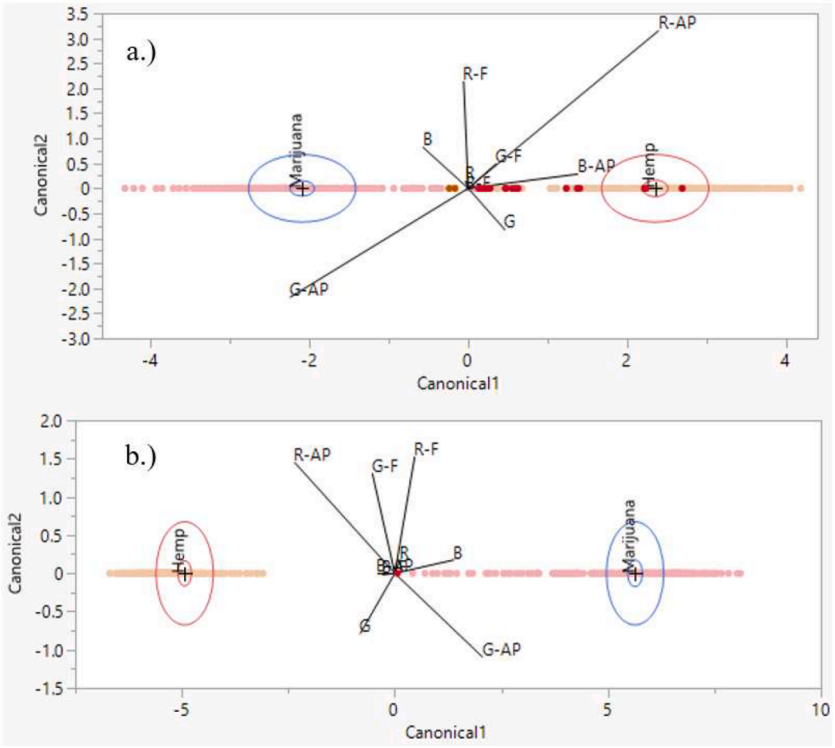

Fig. 5. Canonical plot of the LDA model using RGB of FBBB chromophore/fluorophore and RGB of the 4-AP chromophore before (a.) and after (b.) removing marijuana samples with THC:CBD below two. The highlighted points represent all misclassified samples in model. Marijuana data points are shown in red and hemp data points are shown in orange. The highlighted points represent all misclassified samples in model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

classification in canonical 2 as well as canonical 1 (Fig. 6). All nine variables were used in these models First, an LDA was made with all samples included in the dataset. The training set had an r^2 of 0.82 and correctly classified 95 % of the set and the test set had an r^2 of 0.85 correctly classifying 99 % of validation set. Hemp and marijuana were separated from the other class across canonical 1. Here, R, R-F, and B-AP were closely associated with the hemp and marijuana classes. Hemp and marijuana were separated from each other across canonical 2 with R-F highly correlated with marijuana and R-AP highly correlated to hemp. In the training set, NIST Samples 6, 9, 10, 18, 19, and 23 were misclassified as hemp and in the test set DEA Sample 43 was misclassified as hemp. All of these samples have a THC:CBD < 2 leading to their misclassification. None of the other class were misclassified in this model, showing the selectivity for these tests for cannabis among other plant material. This

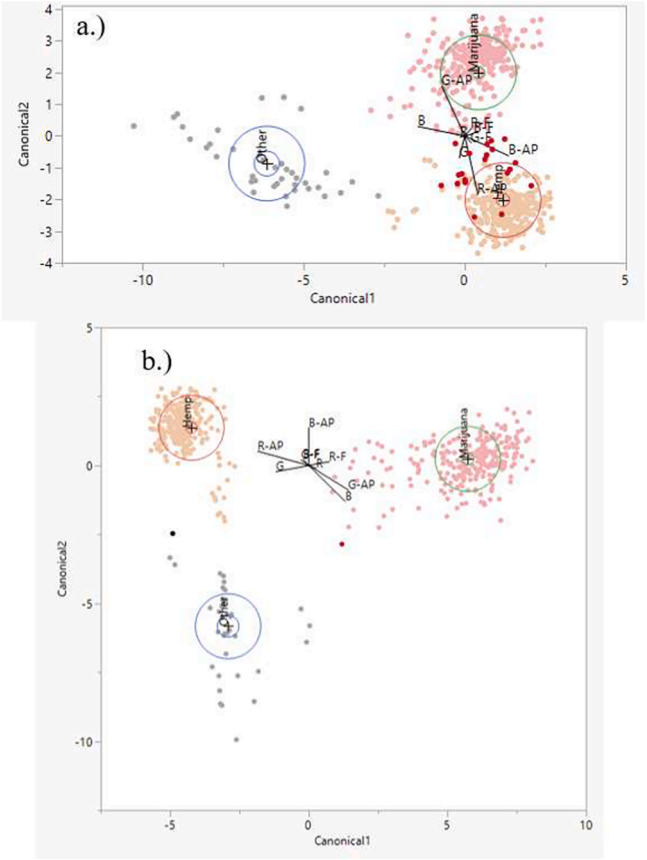

Fig. 6. Canonical plot of the LDA model using RGB of FBBB chromophore/fluorophore and RGB of the 4-AP with three classes: marijuana (red), hemp (orange), and other (black) before (a.) and after (b.) removing marijuana samples with THC:CBD below two. Misclassified points are highlighted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

model again indicates that 4-AP and FBBB are selective when used together to differentiate between hemp and THC-rich marijuana.

A second LDA was performed with all marijuana samples containing THC:CBD below two removed to better determine the specificity of 4-AP and FBBB to classify between hemp, marijuana, and the other class.. The resulting LDA model had a training set with an r^2 of 0.996, correctly classifying 99.7 % of the training set. The test set had an r^2 of 0.99, correctly classifying 99 % of the samples in the set. Across canonical 1, R-F and R were closely correlated with the marijuana class while R-AP, G-AP, and G were closely correlated with the hemp and other classes. Across canonical 2, B was more closely correlated with the other class while B-AP and R were more closely correlated with hemp and marijuana. The only sample to misclassify in the training set was one replicate of Citra hops. This was likely because it formed a yellow color with FBBB leading to a FBBB RGB score like that of hemp. One replicate of DEA Sample 39 misclassified as other. This is likely due to the light color it produced with the FBBB tests and the green color produced with 4-AP. It is important to note that none of the other classes misclassified as marijuana. This includes oregano which turned a blue color with 4-AP. This shows that when FBBB is used alongside 4-AP it can possibly prevent a false positive result from being obtained.

Data Driven-Soft Independent modeling of class analogies (DD-SIMCA) results

DD-SIMCA was performed using data in a similar fashion to the LDA analysis. RGB codes for the visible and fluorescence FBBB chromophore/fluorophore images and the visible 4-AP chromophore images were obtained for each replicate of each sample, totaling to 576 samples in the models. Although each replicate was put as an individual sample whenever one replicate of a sample was marked as extreme or an outlier, all other replicates of the sample were treated similarly. Since DD-SIMCA is a one class model, individual models were made for the hemp and marijuana samples.

First, the DD-SIMCA model utilized the RGB of the visible and fluorescence images of the FBBB reaction. The total number of variables utilized for this model was six. A total of six PCs were found to be usable for modeling, however PC_{max} (the maximum number of principal components used for modeling) was set to five. A total of 75 marijuana samples were placed into the training set and 24 samples were placed into the test set. No outliers were found in either the test or the training sets. Across all five principal components, the test set sensitivity was 100 %. The training set sensitivity gradually declined from 95 % to 93 % as the PCs were increased. Importantly, the specificity for these models were poor for all principal components except for PC 2 (Fig. 7**a**). PC 2 was selected to be used with 94 % sensitivity in the training set, 100 % sensitivity in the test set, and 100 % specificity when distinguishing from the alternative set (hemp). However, despite the excellent figures of merit, the extreme plots of the test set and the alternative set showed great irregularities in the dataset, (Fig. 8**a,b**). These irregularities were so apparent, it was determined that this would not be a reliable model to classify hemp and marijuana.

As with the LDA analysis the DD-SIMCA models were also observed when CBD-rich marijuana samples were removed from the dataset. Here, NIST marijuana samples containing THC:CBD < 2 were removed from the dataset prior to modeling. This represented 12 marijuana samples in total removed from the dataset. With these samples removed the model using the FBBB variables were reconstructed. In total, 64 samples were in the training set, 22 samples were in the test set, and one outlier was detected. The one outlier detected was DEA Sample 43, likely due to it having a very low THC:CBD. Across the five principal components the sensitivity of the training set was above 90 % and the test set sensitivity was 100 % across all five principal components. The main figure of merit that improved was specificity which was 100 % from PC 2–4 (Fig. 7**b**). Although, PC 2 was found to have a higher sensitivity than the other PCs, its extreme plots in the test and alternative set still showed great irregularities. PCs 4 and 5 showed the next best mix of sensitivity and specificity. The training models constructed with these PCs were found to have a sensitivity of 92 %. The models were found to 100 % specific not including any of the hemp samples in the marijuana set. The extreme plots of the models made from PCs 4 and 5 were similar to each other and showed improvements to the models constructed using the full dataset (Fig. 8**c,d**). It should be noted that, although improved, the test set extreme plot still showed an irregularity.

Models using the six FBBB variables were also constructed for hemp. A model was constructed using the full dataset and the dataset in which NIST marijuana samples with THC:CBD < 2 were removed. Similarly, six PCs were available to choose from, but the PC_{max} was set to five. For both models, 59 samples were in the training set, 23 samples were in the test set, and 11 samples were found as outliers and removed from the dataset. The 11 samples were NIST samples 11, 12, 13, 14, and 15, Cannatonic × Harlequin strain, Eighty-Eight strain, Pineapple Bleu Genius strain, Orange Peel Strain, Elektra Hawaiian Haze strain mix, and Elektra Pink Panther mix. NIST 11, 12, 13 and 14 were likely marked as outliers since they formed either no reaction with FBBB or formed a light chromophore when reacting with FBBB. NIST 15, Eighty-Eight, Elektra Hawaiian Haze and Elektra Pink Panther were misclassified due to having a high B score. Orange Peel and Pineapple Bleu Genius produced orange colors with FBBB, but their RGB had low G scores leading to misclassification. One replicate Cannatonic × Harlequin had a lower B score than the rest of the hemp samples leading it to be marked as an outlier. For the models using the full dataset, PC 4 showed the best sensitivity and specificity of the different models. This model had a

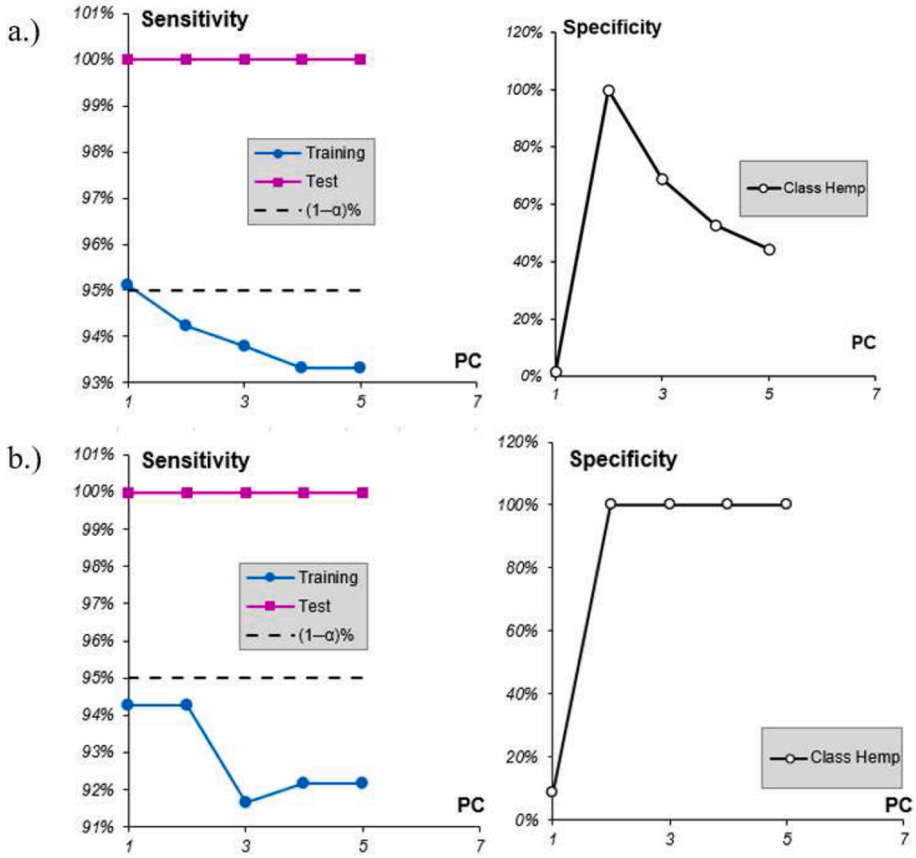

Fig. 7. Figures of merit for a.) Marijuana SIMCA models using the full dataset and b.) Marijuana SIMCA models using a dataset with low THC:CBD NIST samples removed.

training set sensitivity of 96 % and test set sensitivity of 100 %. The specificity for the PC 4 model was 96 %. For the PC 4 model, NIST Samples 9, 10,18, 19, and 23 and DEA Sample 43 were found to be included in the hemp models. These samples all have a THC:CBD < 2, once again showing that below THC:CBD of 2 the FBBB is likely to give a false positive result. This is confirmed when the hemp models constructed with a dataset in which the THC:CBD < 2 NIST marijuana samples are removed. In these models, PC 4 was used for modeling once more, with the test set and training set sensitivity the same as the model using the full dataset, however, the specificity increased from 96 % to 99 %. In this set, the only marijuana sample that is found to belong to the hemp set is DEA Sample 43.

Next, DD-SIMCA models were constructed for 4-AP using the RGB of the 4-AP images obtained. For these models, three principal components were available to use for modeling and PC_{max} was set at three. In total, 60 marijuana samples were in the training set, 25 marijuana samples were in the test set, and 14 samples were removed as outliers. The samples removed were NIST Samples 3,4, 6, 9, 10, 19, 21, 22, 23, and 24 and DEA Samples 39, 43, 50, and 75. NIST Samples 6, 9, 10, 19, 21, 22, 23, and 24 and DEA Sample 43 all have THC:CBD < 2 producing light pink to pink colors with the 4-AP test. NIST Samples 3 and 4 and DEA Samples 39, 50, and 75 are all THC-rich cannabis but produced light colors with the 4-AP reaction leading to the samples being marked as outliers. Once these outliers were removed, the only viable model option was to use PC 2. Models using PC 2 were found to have a training set sensitivity of 92 %, a test set sensitivity of 97 %, and a specificity of 100 %.

A marijuana model was then constructed removing NIST samples with THC:CBD < 2 from the data set. In this model, 59 marijuana samples were in the training set, 21 samples were in the test set, and seven marijuana samples were removed as outliers. The outliers NIST Samples 3 and 4 and DEA Samples 39, 50, 52, and 75 which were likely outliers due to their light reactions with 4-AP giving an RGB that was distinct from that of the other marijuana samples. DEA sample 43 produced a pink color due to it being CBD-rich, leading it to be marked as an outlier. Once again, PC 2 was the only PC option to have an acceptable training and testing sensitivity and specificity. In this model, training sensitivity was 93 %, test sensitivity was 98 %, and specificity was 100 %. This model demonstrates the reality that sometimes 4-AP may produce a light reaction that makes it difficult to determine color. In addition, both marijuana models suggest that the color difference between CBD-rich and THC-rich cannabis may be more distinct than that of FBBB since the low THC:CBD samples were able to be marked as outliers in these models unlike the FBBB models.

For the 4-AP hemp models, the full dataset and the dataset with samples removed made no difference for the training and test models. The only model that had any significant change was that of the alternative set. For both models, 64 samples were in the training set, 26 samples were in the test set, and three samples were removed as outliers. The three samples that were removed as outliers were NIST Samples 11, 12, and 14. These samples all produced no color when reacted with 4-AP leading to their exclusion. For both models, the training set sensitivity was 95 % and the test sensitivity was 100 %. Specificity for the full dataset was 94 % with NIST Samples 6, 9, 10, 19, 21, 22, and 23 and DEA Sample 43 (all THC:CBD < 2) found to be included in the hemp model. When the NIST samples with THC:CBD < 2 were removed from the dataset, specificity increased to 99 % with only DEA Sample 43 being included in the hemp model.

Finally, SIMCA models using the RGB data from FBBB and 4-AP combined were used together representing nine variables in total. For these models, nine principal components were able to be selected from and PC_{max} was set to eight. For models examining the full dataset, 57

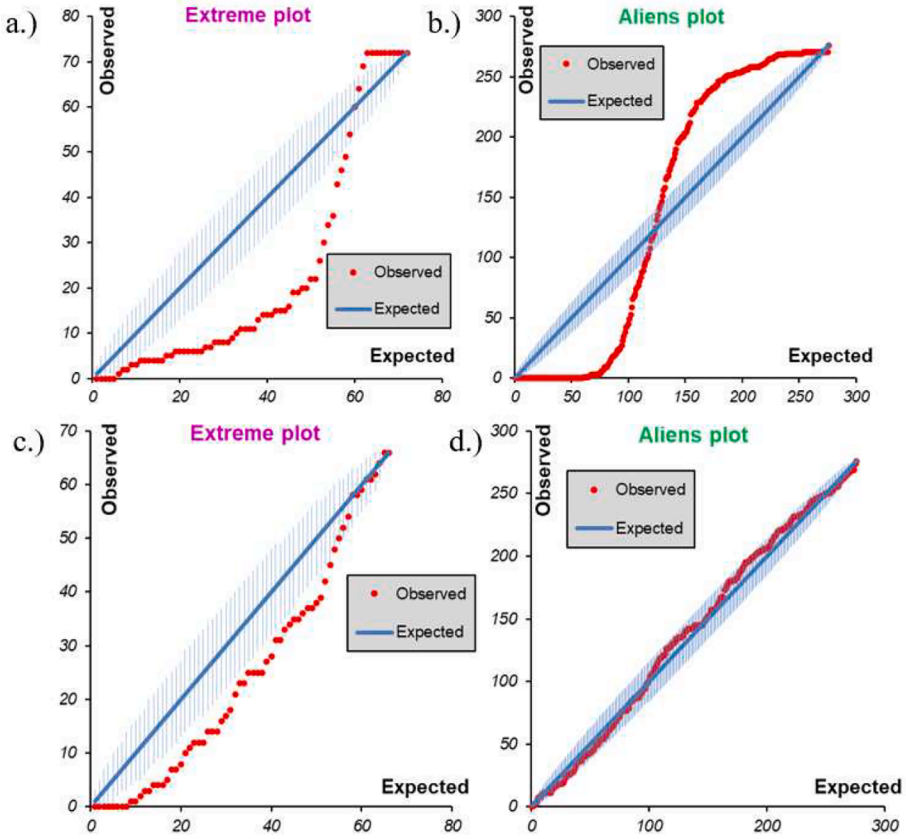

Fig. 8. Extreme plots for marijuana SIMCA full dataset test set (a.) and alternative set (b.) using PC 2 and the extreme plots for test set (c.) and alternative set (d.) for marijuana SIMCA model with THC:CBD < 2 NIST samples removed using PC 4.

marijuana samples were in the training set, 23 samples were in the test set, and 19 samples were marked as outliers. The outliers were NIST Samples 3, 4, 6, 9, 10, 18, 19, 20, 21, 22, 23, and 24 and DEA Samples 3, 15, 39, 43, 50, 73, and 75. NIST Samples 6, 9, 10, 18, 19, 20, 21, 22, 23 and 24 and DEA Sample 43 all have a THC:CBD < 2 producing chromophores expected for hemp leading them to be classified as outliers. NIST Samples 3 and 4 and DEA Samples 39, 50, 73, and 75 produced a light chromophore with either 4-AP, FBBB, or both. DEA Samples 3 and 15 produced a deep blue color with 4-AP and a red color with FBBB. Their product with FBBB also fluorescence brightly under 480 nm light. One reason they were marked as outliers was that their B score for FBBB RGB was found to be lower compared to most marijuana samples leading to these samples being marked as not belonging to the marijuana class. Across the eight PCs, PCs seven and eight performed the best. Models made with PCs seven and eight all had 95 % sensitivity in the training set, 93 % sensitivity in the test set, and 100 % specificity. The extreme plots for the models made with PC 7 had better fits and therefore were chosen as the best model in this set. The SIMCA models for the training, test and alternative sets can be seen in Fig. 9.

Next, SIMCA models for marijuana were constructed utilizing the dataset with the NIST samples with low THC:CBD removed. Here, 50 marijuana samples were used for the training set, 29 samples were included in the test set, and eight samples were removed as outliers. The outliers removed were NIST Samples 3 and 4 and DEA Samples 15, 39, 43, 50, 73 and 75. Most of the samples were marked as outliers due to the light colors they produced with either test. As explained previously, DEA Sample 15 likely misclassified due to the low B score from the FBBB chromophore. PCs five and six both showed promise as good SIMCA models to classify between hemp and marijuana. PC five had a training sensitivity of 98 %, a test sensitivity of 100 %, and specificity of 100 % and PC six had a training sensitivity of 96 %, a test sensitivity of 100 %, and specificity of 100 %. PC five had greater sensitivity than PC six, however, the extreme plot of the test model showed a great irregularity in PC five. For this reason, PC six was chosen as the preferred model instead (Fig. 10). Both the nine variable models using the full dataset and the dataset with low THC:CBD samples removed show that both FBBB and 4-AP will get false negative or inconclusive results when THC:CBD < 2. In addition, these models show inconclusive results may be obtained if a light reaction is had with either FBBB or 4-AP. It should be noted that both models had the highest training set sensitivity, 95 % and 96 % respectively, when compared to the other marijuana models made. The model made with the CBD-rich NIST samples removed showed a higher test set sensitivity than the full model, once again showing these models are strong when differentiating solely between THC-rich marijuana and hemp.

Finally, the hemp DD-SIMCA models using all nine variables were constructed. The model using the full dataset had 61 hemp samples in the training set, 25 samples in the test set, and seven samples that were removed as outliers. The samples that were removed as outliers were NIST Samples 11, 12, 13, 14, and 15 and Eighty-Eight strain and Pinewalker strain hemp. These were found to have formed a light chromophore with either FBBB or 4-AP. PC five was found to have the highest sensitivity and with a training sensitivity of 96 %, test sensitivity of 99 %, and a specificity of 99 %. For this model the only marijuana samples found to be included in the dataset were that of DEA Sample 43. When the THC-poor NIST samples were removed from the datasets, there was not much change in the models. PC five remained the best choice for modeling and the sensitivity and specificity are the same as the previous model (Fig. 11). Only DEA Sample 43 was included in with the hemp samples, which is expected due to its low THC:CBD. The hemp model results show the highest sensitivity when compared to the other hemp models. This shows that when used together the 4-AP and FBBB may increase the sensitivity of results which is beneficial to both tests.

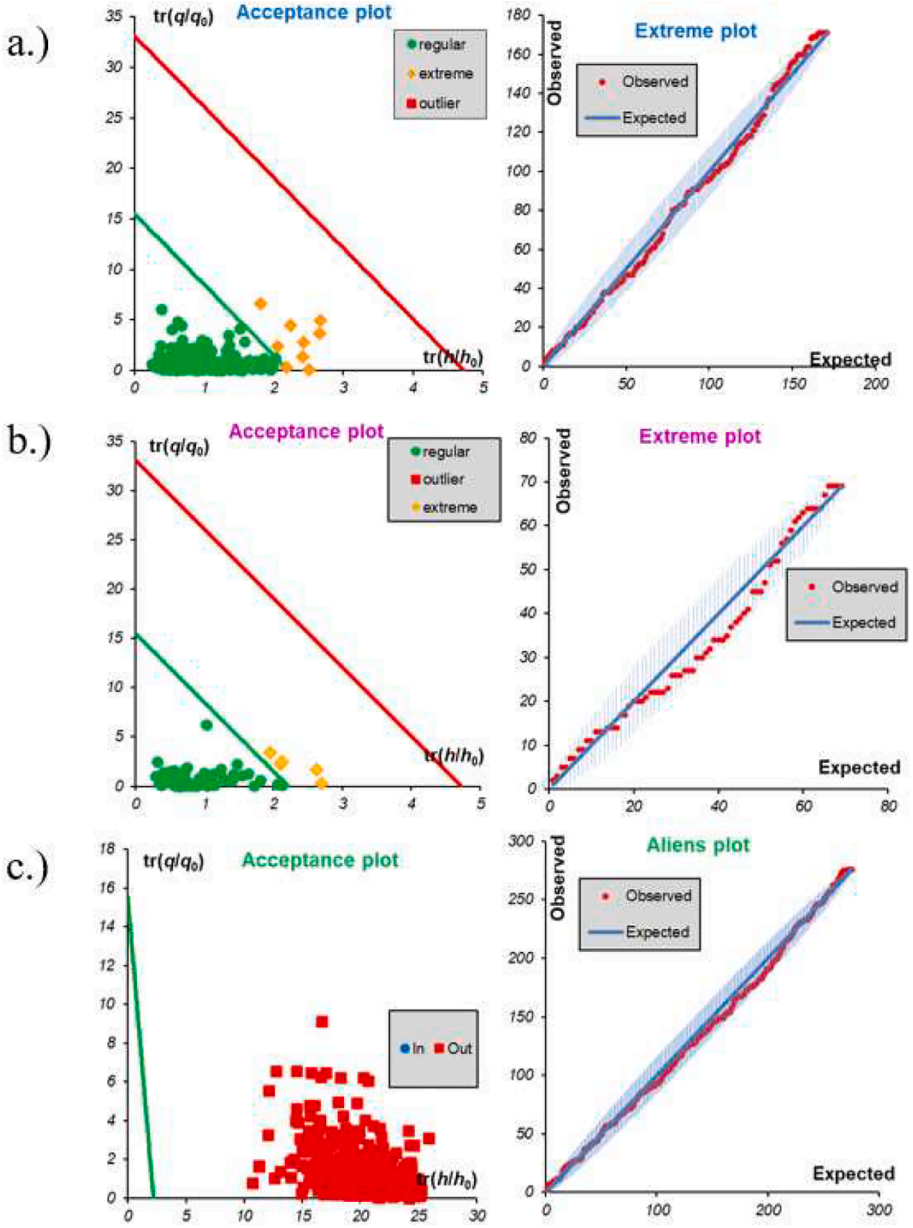

Fig. 9. The training SIMCA model (a.), test SIMCA model (b.) and alternative SIMCA model (c.) for the full marijuana dataset using PC 7.

Discussion and conclusions of results

FBBB and 4-AP have both demonstrated their ability to indicate hemp-type and marijuana-type cannabis, individually and together. This study has shown that when marijuana is THC-rich (THC:CBD $>$ 2), its extract will likely form a red chromophore,which fluoresces under 480 nm light, with FBBB and a deep blue color with 4-AP. Alternatively, hemp will form an orange chromophore, which does not fluoresce, with FBBB and a pink chromophore with 4-AP due to its high CBD content. Neither test produced a false positive result with any of the hemp samples. However, it was found that when THC:CBD is below two, false negative or inconclusive results for either test were likely. Inconclusive results were found mostly in samples that contained a THC:CBD close to one. Cannabis with THC:CBD close to one cause inconsistent results due to the fact that the color tests do not have enough resolution to indicate at this level. Similarly, marijuana samples with a THC:CBD $<$ 2 produce false negative results with these two tests. This is due to the high concentrations of CBD in these plants reacting with the reagents over the low levels of THC. With these tests there were some THC-rich cannabis samples that did not produce a false negative result but formed light colors with either 4-AP, FBBB, or both. For some samples, such as NIST Samples 3 and 4 and DEA Sample 39, the reason is likely that the concentration of cannabinoids in the cannabis is low and therefore the extract contains concentrations close to the limit of detection (LOD) of the tests. There are some samples, DEA Sample 75 for example, that contain a high concentration of THC, but produce a light reaction. This may be due to a systematic error during extraction, in which too little sample or too much solvent was added causing the concentration of the extract to be lower than the sample it represents. This is a consideration for implementing these methods with extracts in the field, however for most of the samples the extraction method was sufficient to achieve a robust color result for both tests.

Both 4-AP and FBBB have excellent sensitivity and specificity when differentiating between THC-rich and CBD-rich cannabis, however each test has limitations and advantages. 4-AP has the advantage of rapid color results, low detection limits, and stable reagents but has a limitation in that the time window in which one can observe the resulting color. It was previously reported that it takes 2 min for the 4-AP

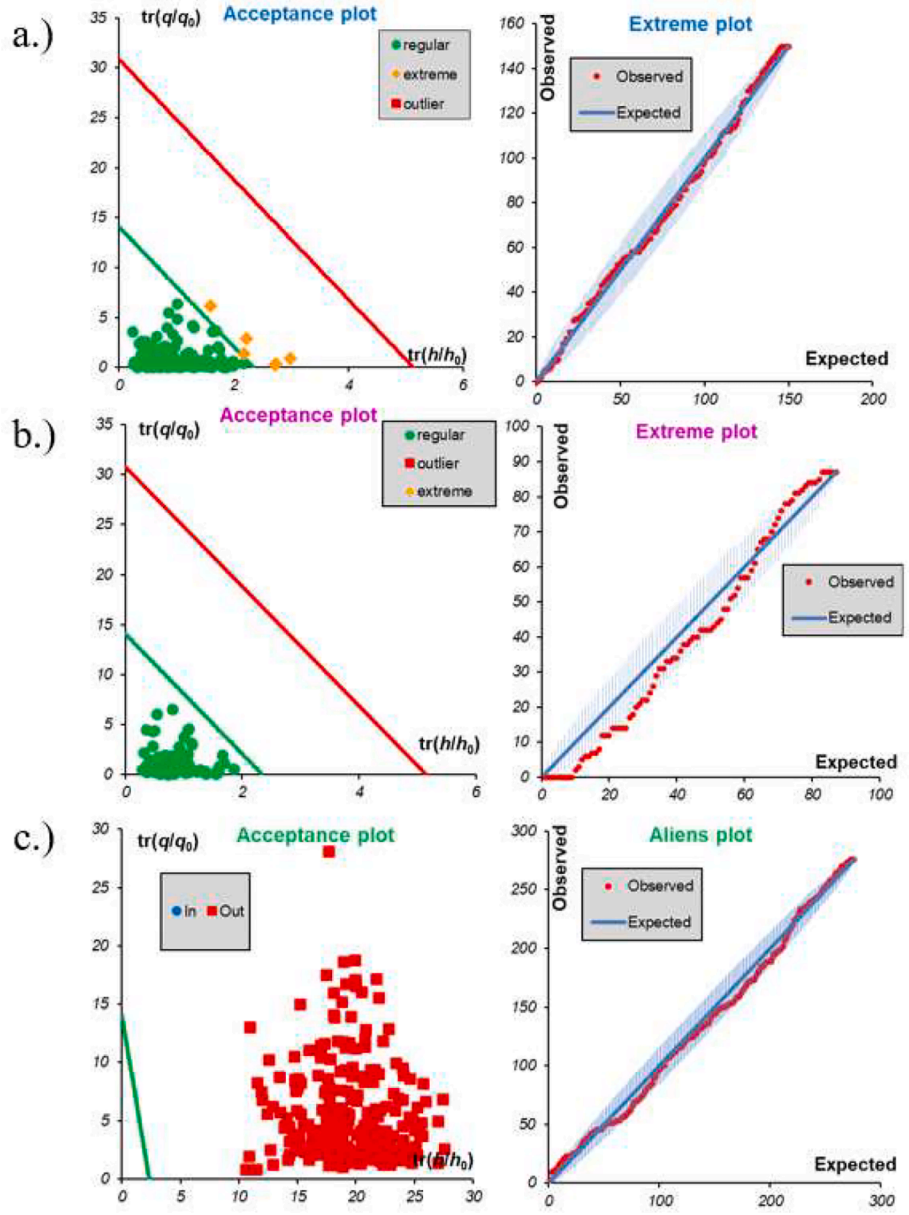

Fig. 10. The training SIMCA model (a.), test SIMCA model (b.) and alternative SIMCA model (c.) for the marijuana dataset with THC:CBD < 2 NIST samples removed using PC 6.

chromophores to develop [11]. This study found that for the miniaturized 4-AP method, it took 5 min for the color to fully develop. In addition, it was found that the color products for both THC + 4-AP and CBD + 4-AP began to degrade 10 min after reaction, eventually turning into a black and brown color, respectively. This only provides for an approximate 5 min observation window in which the color results can be acquired and interpreted. This degradation also occurred when the solvent of the reagents would evaporate, meaning all photos of the 4-AP chromophore had to be taken while the solvent was still present. This created glare from the liquid reflecting the light. The short time window and glare effect is not desirable for fieldwork as they may not allow for the chromophore to be captured properly. The FBBB test disadvantage is that the FBBB reagent is photosensitive and degrades when left in the light over prolonged periods. Previous studies have also shown that it may also be thermally sensitive as well [9]. This means that, for field use, the FBBB reagent should at the very least be concealed from light and perhaps kept in cool environments. Despite this limitation, FBBB does have many advantages over 4-AP. The reaction with FBBB and THC/CBD is immediate and once the solvents evaporate the chromophore can be observed on the PSPME substrate. The stability of the FBBB chromophore allows for color and fluorescence to be captured with ease. It also allows for the substrate to be collected as evidence and taken to the lab for further testing if necessary. FBBB also has the benefit of not just giving an intense color, but also intense fluorescence when reacted with THC. This provides two methods to presumptively confirm the results at the scene where 4-AP only has the color. The advantages and limitations of these tests are important to consider when deciding which one should be used in the field.

To objectively determine the sensitivity and specificity of the two colorimetric tests, RGB codes were obtained from images of the chromophores formed. This RGB represented the color of the chromophore as hard data that could be used to perform statistical analysis. This data was used in two types of multivariate models, LDA and DD-SIMCA, to see how well these tests classified marijuana and hemp. LDA is a supervised multiclass model in which only predefined groups are considered for selection. Therefore, datapoints are classified into the group they most

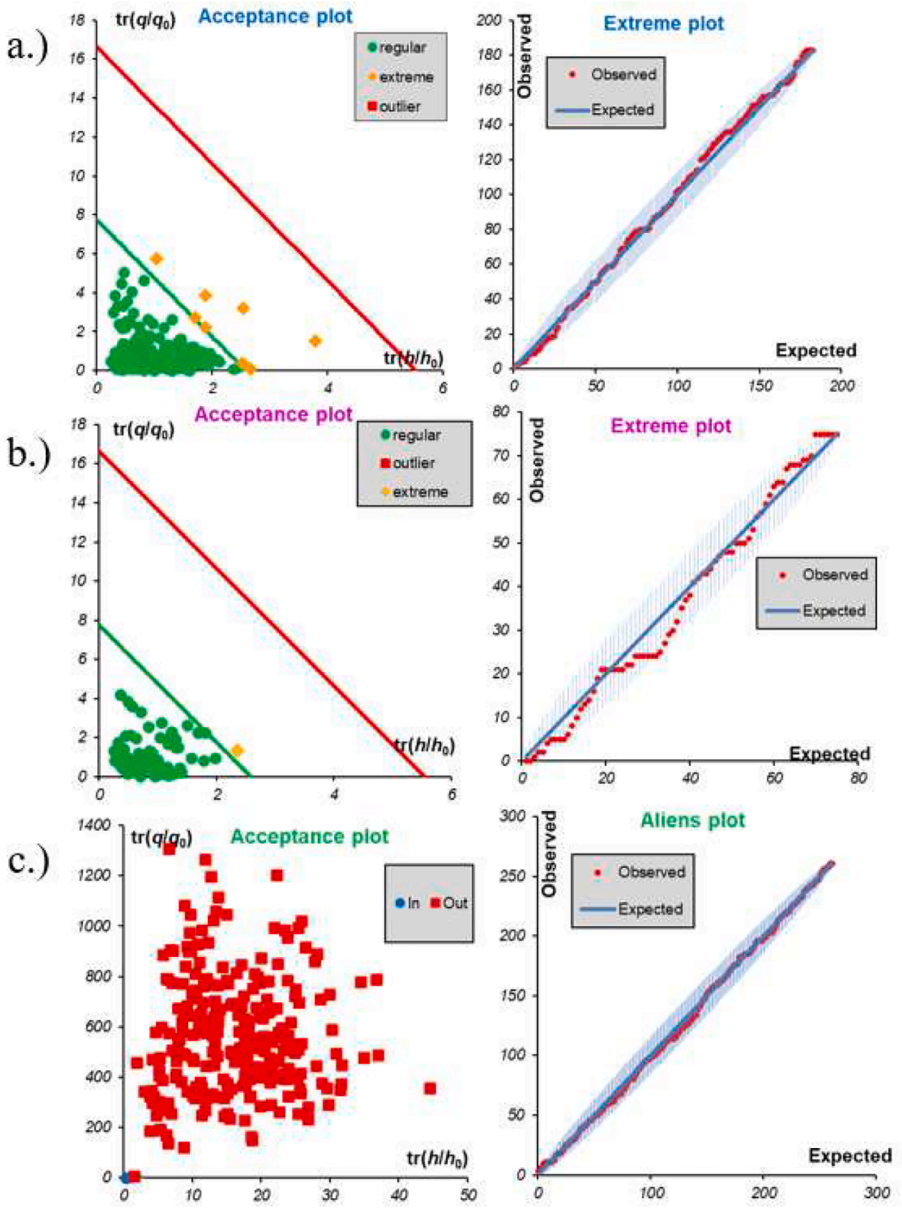

Fig. 11. The training SIMCA model (a.), test SIMCA model (b.) and alternative SIMCA model (c.) for the hemp dataset with THC:CBD < 2 NIST samples removed using PC 5.

closely resemble and could make an incorrect prediction if a group is not considered during modeling. None of the LDA models performed well when the CBD-rich marijuana samples were present in the dataset. Once these samples were removed from the dataset all LDA training models and most of the validation models were able to obtain $r^2 \geq 0.95$. FBBB had the weakest training model with an r^2 of 0.97. However, when compared to 4-AP, the validation model of FBBB had the better fit (r^2 0.98) than the validation model of 4-AP (r^2 0.55). The poor fit for the 4-AP score was attributed to samples which formed a light blue chromophore with 4-AP, leading to misclassification. When RGB from the FBBB test and 4-AP test were combined in a model, there was 100 % correct classification in the training model, 99 % correct classification for the test model, and r^2 values of 1 for the training set and 0.98 for the test set. This shows that when 4-AP and FBBB are used together there is a clear separation between the hemp and marijuana class. The samples that misclassified in the test set either produced a light reaction with the test reagents or were CBD-rich. To ensure that there were no incorrect predictions of hemp and marijuana it was important to consider an additional "other" class which contained the RGB codes for various non-cannabis plants. The additional class added another layer of depth to the model and more clearly showed the separation between classes. Once again the marijuana samples that were misclassified were CBD-rich or produced light reactions with either test. When the samples with THC:CBD < 2 were removed the model showed much improved results, with an r^2 of 0.996 in the training set and 0.99 in the test set. The LDA results show that FBBB and 4-AP work well as individual color tests to indicate hemp and marijuana and when used together provide slightly higher sensitivity and specificity.

While the LDA models work well, one drawback of the LDA models is if there is a plant that is not hemp or marijuana it could be misclassified as one of the two if there is not an additional class predefined beforehand. Realistically, a plant outside of the cannabis group may be tested in the field and would be considered as other. To better simulate a real life classification, DD-SIMCA was used. DD-SIMCA is a one class modeling method in which only one class is predefined. All new samples are then compared to the predefined class and are either fall into the class or out

of the class. The two classes which SIMCA models were made for in this study were hemp and marijuana.

All the hemp SIMCA models showed excellent sensitivity and specificity, only misclassifying marijuana samples which were CBD-rich as hemp. The hemp samples that were marked as outliers were ones that did not form a reaction or formed a light reaction with the colorimetric tests. The marijuana SIMCA models using the 4-AP variables and both 4-AP and FBBB variables both flagged marijuana samples with THC:CBD < 2 as outliers in their datasets. The FBBB model using the full dataset did not have outliers but did show a great irregularity in the dataset itself. Removing the CBD-rich NIST marijuana samples from the data set improved the sensitivity of the training and test sets for most models however, outliers were still detected in the 4-AP and combined models. These outliers were mostly samples which produced a light color with 4-AP, FBBB, or both tests. This reflects what is likely to be seen in the field. There may be cases in which the sample does not react fully with either test leading to an inconclusive result. It should be noted that all SIMCA models constructed with THC-rich marijuana samples all achieved sensitivity and specificity above 90 %. While the FBBB marijuana model did achieve high specificity, it was found to have irregularities within the dataset. This could possibly be due to not using enough variables for this type of analysis. The best performing marijuana model was found when 4-AP and FBBB variables were used for analysis. This was the only model to have a test set sensitivity above 95 % and a specificity of 100 %. The combined model for hemp also showed a sensitivity 96 % with a specificity at 99 %. The only marijuana samples that fit into the hemp datasets were those that contained a THC:CBD < 2. The SIMCA models show once again that FBBB and 4-AP work excellent individually but have the capability for higher sensitivity when used in combination.

Both model types were in agreement that the 4-AP and FBBB methods used in this study are able to indicate between hemp and marijuana. The models also demonstrated that these tests would give a false negative result for marijuana samples with a THC:CBD < 2, which is in agreement with previous studies [9,11]. The SIMCA models showed that there may be instances, whether due to low concentrations in plants or through systematic error, when the extracts will react poorly with the reagents causing a light reaction or inconclusive result. Despite this case, the models show a high sensitivity and specificity with 4-AP, FBBB, and both tests combined. The observational and statistical results demonstrate that FBBB and 4-AP are strong tests to use to distinguish between hemp and marijuana. However, both tests do give inconclusive or false results if the sample is CBD-rich or has a THC:CBD close to one. Both tests are applicable for field use separately or together, however FBBB has the advantage of forming a chromophore that lasts beyond the 4-AP time window, allowing for further analysis in the lab if needed. These results show that it would be beneficial to develop a test kit in which both the 4-AP and FBBB tests are used simultaneously. This would allow for a three pronged approach to confirming the presence of marijuana through the color of the FBBB, fluorescence of FBBB, and color of the 4-AP test. The utilization of both tests would allow for a rapid and accurate presumptive result. Future studies for these tests will look at developing a field test kit in which both tests can be used complimentary to each other.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2022.100448.

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