Received: 3 February 2023 | Revised: 26 April 2023 | Accepted: 23 May 2023

DOI: 10.1111/1556-4029.15309

JOURNAL OF FORENSIC SCIENCES AAFS

ORIGINAL PAPER

Criminalistics

Chemical identification and optimization of the 4-aminophenol colorimetric test for the differentiation between hemp-type and marijuana-type cannabis plant samples

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Funding information
National Science Foundation, Grant/Award Number: #1739805

Abstract

The 4- Aminophenol (4-AP) colorimetric test is a fast, easy-to-use, and cost-effective presumptive assay of cannabis plant material producing different chromophores with THC-rich cannabis (blue color) and with CBD-rich cannabis (pink color). The main drawback of the 4-AP test is a brief observation window where the color rapidly changes to black, limiting the utility of the test. We now report for the first time, the identification of the product chromophores between 4-AP and CBD/THC as well as propose an explanation and a solution for the color degradation of the chromophores. The identification of the chromophores is provided by spectroscopic (UV–Vis), chromatography, and mass spectrometry (TLC and LC-QToF-MS). Oxidation of excess 4-AP (Reagent A) in the presence of NaOH (Reagent B) produces the black color observed for the previously reported 4-AP tests and reported in the literature. The adjustment of reactants concentrations and volumes of 4-AP:THC/CBD to a 1:1 ratio significantly reduces the black oxidation by-product and increases the observation window up to 2 h instead of the previously reported 5–10 min. For the first time, mass spectrometry and chromatography confirmed that the reaction of THC and CBD with 4-AP produced chromophores with m/z (M + H) = 420, consistent with proposed indophenol structures. The TLC method developed confirmed the separation between CBD and THC chromophores. The specificity of the test is also reported, showing false positive results for the presence of THC (blue color) for samples of thyme and oregano. LDA and SIMCA models showed that the optimized 4-AP procedure performs better than the previously reported 4-AP color test.

KEYWORDS
4-AP test, cannabis, CBD, colorimetric test, hemp, marijuana, THC, THC chromophore

Highlights

- The 4-AP color test was optimized to improve color stability.
- Oxidation of excess 4-AP in the presence of NaOH produces the black by-product.
- Adjustment of the stoichiometry of the 4-AP:THC/CBD to 1:1 increases the stability to 2 h.
- THC and CBD chromophores were identified by UV–Vis, TLC, LC-QToF-MS.

- LDA and SIMCA models showed better classification results when compared to the previous 4-AP test.

1 | INTRODUCTION

The recent changes in cannabis regulation (2018) have increased the demand for the differentiation between hemp-type and marijuana-type cannabis plant material [1]. In countries such as the United States and Switzerland, the change in the legislation allows for the legal cultivation and distribution of cannabis as hemp based on a threshold concentration of Δ9-tetrahydrocannabinol (THC) in the plant [2, 3]. According to the United States legislation [1], when the THC content is below 0.3% (w/w) on a dry weight basis, the cannabis material is classified as hemp (and may include CBD-rich cannabis plants). When the THC in cannabis plants is equal to or above 0.3% (w/w), the cannabis material is classified as marijuana, or THC-rich cannabis [2] and is considered a schedule I controlled substance in the USA. In Switzerland, the cannabis plant material is controlled when the THC is equal to or greater than 1% THC (w/w) in the plant [3]. In other countries such as Brazil, the restrictions are even more pronounced. The National Health Surveillance Agency from Brazil (ANVISA) allows the commercialization of CBD-rich products containing <0.2% THC. Products containing 0.2% THC or greater require special authorization from ANVISA and, once approved, may be used for palliative care or the treatment of specific diseases, such as multiple sclerosis [4, 5].

Such changes in cannabis regulation and the use of cannabidiol (CBD) for therapeutic and recreational purposes have increased interest in cannabis plant materials in the last few years and have resulted in both legal and illicit distribution of cannabis plants. Different cannabis strains emerged on the market, especially CBD-rich cannabis, which may vary from 0.3 to 25% of CBD [3, 6, 7]. However, THC-rich cannabis is still present in the market as can be seen in a study performed by Smith et al. [8], which described a chemotaxonomic analysis of commercial cannabis from six different states in the US. The analyses were performed by considering the distribution of each cannabinoid across the country. The results showed that the content of THC was much higher when compared to the content of other cannabinoids [8].

The legal and market changes necessitate the differentiation between hemp and marijuana cannabis plants using presumptive methods, considering that the current method (Duquénois–Levine test) employed for the characterization of cannabis plants does not distinguish between hemp and marijuana and therefore laboratory-based analytical methods that determine the total content of Δ9-tetrahydrocannabinol by liquid or gas chromatography are needed [2, 9–11]. Colorimetric tests are presumptive tests used to indicate the presence of a target compound by a simple color reaction, but these tests are not 100% selective for this class of compounds. In a colorimetric test, a reaction between a target compound and a reactant produces a color allowing its simple and fast indication without the need for extensive sample preparation. Different colorimetric tests have been used for presumptive cannabis identification over the years, such as the Duquénois–Levine test (D-L), the Fast Blue B test (FBB), Fast Blue BB test (FBBB), and more recently, the 4-aminophenol Test (4-AP) [6, 12–14]. The Duquénois–Levine test has been employed for more than 80 years to presumptively identify cannabis plant material but does not differentiate between hemp and marijuana. The D-L test produces a violet color for the cannabinoids and has as a main drawback the lack of specificity for cannabis plant material, giving false-positive results for other plants that contain a phenol group [15]. The Fast Blue B and the Fast Blue BB are more specific when compared to the D-L. Both produce an orange and red color for CBD and THC, respectively. However, the Fast Blue BB is even more specific than the Fast Blue B and does not produce false-positive results when tested for some plant species from Brazil according to Santos et al. and collaborators [13]. Recently, our research group has reported the optimization and the miniaturization of the FBBB test by using a solid substrate to perform the analyses and employing the measure of fluorescence through a portable microscope to differentiate between hemp and marijuana [6]. The work developed by Acosta and Almirall [6], demonstrates that CBD-rich cannabis does not fluoresce while THC-rich cannabis fluoresces, increasing the specificity and sensitivity of the test.

Another colorimetric test that has been used to test cannabis plant material is the 4-aminophenol color test (4-AP), first reported by the Zurich Police [3]. The 4-AP test has produced excellent results for the differentiation between hemp and marijuana as a "typification" test [2, 3, 16]. In the 4-AP test, the cannabis plant material is mixed with Reagent A (4-AP) and Reagent B (NaOH), producing either a pink color for the CBD-rich cannabis or a blue color for the THC-rich cannabis within a few minutes after initiation of the reaction. A commercial kit was developed by the Zurich Police by mixing the seized plant material with 4-AP and then NaOH (Reagent B) in a plastic container containing the reactants, showing the color change by the production of the chromophore, after only 2 min [3]. Sequentially, validation and optimization of the test were made by Lewis et al. [2] by removing the isopropanol from the Reagent A preparation.

The group also decreased the volume of reactants, by performing the reaction in a spot plate, adding 1 mL of Reagent A and a few drops of Reagent B, instead of the containers with high volumes of reagents used by the Swiss group [3]. Recently, our research group developed an optimization and miniaturization of the 4-AP test [16], using a low-volume extract of the cannabis plant material to perform the test, instead of the solid seized plant material, and using a silicone filter paper as a solid substrate to perform the reaction.

The solid substrate permits the use of even lower volumes of the reactants, when compared to the optimization performed by Lewis et al. [2], producing good results for the differentiation between hemp and marijuana. A validation study including 192 cannabis samples of known THC/CBD composition was reported

[16] using the miniaturized reaction, and however, also reported a color degradation of the chromophores after an 8 min observation window, as had been previously reported by others. The test originally developed by the Zurich Police has the main drawback of the formation of a black/brown color within a few minutes, limiting its application for the differentiation of hemp and marijuana in a field situation; in addition to the potential for inconclusive results produced when similar ratios of THC/CBD are present in the cannabis plant material.

The characterization of the chromophores produced by colorimetric reactions between THC/CBD for the FBBB test and the Duquenóis–Levine test using mass spectrometry and nuclear magnetic resonance (NMR) methods have been previously reported [13, 14, 17, 18] but the complete (structure) characterization of the 4-AP colorimetric test with THC/CBD have not. Some studies have been reported to use liquid chromatography coupled to diode array detector (LC-DAD), liquid chromatography coupled to mass spectrometry (LC–MS), gas chromatography coupled to flame ionization detector (GC-FID) and thin layer chromatography (TLC), to confirm the presence or the ratio of CDB/THC in the seized samples, without identifying the chromophore structures [2, 3, 12, 19]. The reason for the chemical reaction that leads to rapid color degradation has also not been previously reported. The purpose of this work is to report, for the first time, the optimization and subsequent improvement of the color stability of the chromophores produced by the 4-AP test, significantly reducing the oxidation of the 4-AP reagent in the presence of base and, therefore, reducing the formation of the black color by adjusting the stoichiometry of the reactants. We also report, for the first time, the identification of the chromophores using TLC, UV–Vis, and LC-QToF-MS, along with stability and selectivity studies in addition to the timed-resolved experiments, to confirm that along with the increase in color intensity, there is an increase in chromophore production.

2 | MATERIALS AND METHODS

2.1 | Reagents and standards

4-aminophenol, ethanol, and diethylamine were purchased from Fisher Scientific. Methanol, chloroform, and ethyl acetate were purchased from Sigma Aldrich. NaOH and hydrochloric acid were purchased from Macron Fine Chemicals. Thymol and carvacrol were acquired from TCI America Inc. Δ-9-tetrahydrocannabinol (Δ-9-THC) and cannabidiol (CBD), at 1000 μg/mL in methanol were purchased from Restek Corporation (Restek France, Glastron, Inc.) and Cerilliant Corporation.

2.2 | Materials

Whatman™ 1PS Silicone Treated filter paper (125 mm diameter), TLC Silica Gel 60 F254 in aluminum sheets, and Hydrion (93) S/R pH Paper 0.0-13.0, were purchased from Fisher Scientific.

2.3 | Cannabis plant material and household herbs and spices

CBD-rich hemp strains that included certificates of analysis of cannabinoid content were purchased from Blue Ridge Hemp Co. and Tweedle Farms. Cigars, apollo hop pellets, whole leaf hops, oregano, sage, parsley, red pepper flakes, black pepper, lavender, and eucalyptus leaves were acquired in local markets of Miami, Florida. Two Brazilian species that previously resulted in false-positives for the FBB test [20] known as Carobinha (*Jacaranda decurrens* Cham) and Guaraná (*Paullinia cupana* Kunth), were also tested with the 4-AP color reaction. The cannabis research program managed by the National Institute of Standards and Technology (NIST) provided 31 cannabis samples that had been previously characterized for cannabinoid content using liquid chromatography-photodiode array (LC-PDA). Twenty-four of these cannabis samples were determined to contain >0.3% (w/w) THC and seven of these samples were determined to contain <0.3% (w/w) THC. The Drug Enforcement Administration (DEA) provided 69 marijuana samples with known THC and CBD composition for this study, according to described in the previous method [16]. The DEA samples were also characterized by liquid chromatography-mass spectrometry (LC–MS).

2.4 | Methods

2.4.1 | 4-AP reagent preparation

The reagents A and B employed for the 4-AP test were prepared following two different methods. The first preparation was in accordance with the method developed by Lewis et al. [2] and according to Acosta et al. [16], employing an excess of 4-AP for the reaction. The second method was the newly developed preparation adjusting the stoichiometry of the reaction to avoid excess 4-AP reagent. To prepare reagent A following the first method, 30 mg of 4-aminophenol was weighed and dissolved in 99.5 mL of ethanol and 0.5 mL of 2 M hydrochloric acid (HCl) [16]. For the second method, 8.67 mg of 4-aminophenol was weighed and dissolved in 99.5 mL of ethanol and 0.5 mL of 2 M hydrochloric acid (HCl). The Reagent B was prepared by dissolving 3 g of sodium hydroxide (NaOH) in 70 mL of ethanol and 30 mL of deionized water. Both reagents A and B were stored in the refrigerator at 4–8°C when not in use.

2.4.2 | Reaction in the presence and absence of oxygen

The influence of oxygen in the color degradation of the chromophores was tested by flowing air through plastic tubing into the samples. To introduce the nitrogen into the falcon containing the reaction, a hole was made in the screw top. The nitrogen was

pumped into the reaction over 60 min. The reactions were prepared in falcon tubes and scaled up to 8 mL, using an excess of 4-AP, as previously described [16] with a proportion of 20.7:1 (4-AP:THC/CBD).

2.4.3 | 4-AP test procedure optimization

The 4-AP test procedure was optimized using a silicone filter paper that was cut into 6 mm diameter round cut-outs by using a standard hole punch and then placed inside a disposable well plate to perform the reaction. The reaction was initiated by adding the THC/CBD extract or standard, followed by Reagent A (4-AP) and then by Reagent B (NaOH). The reaction was tested using different concentrations and volumes of the Reagent the A (4-AP) and testing blank samples along with the CBD and THC standards. The initial tests followed the same method developed by Acosta and Almirall [17], by adding 5 µL of THC/CBD standard, 30 µL of 4-AP, and 6 µL of NaOH. After the adjustments, the final method employed was performed by adding 10 µL of THC/CBD standard or extract, 10 µL of 4-AP (86.7 ppm), and 12 µL of NaOH.

2.4.4 | Stoichiometry of the reaction

After performing the tests with and without oxygen, the stoichiometry of the 4-AP and THC/CBD reaction was adjusted to remove the excess of 4-AP from the reaction and to adjust the amount of NaOH. All the tests were made with a 250 ppm solution of THC and CBD standards. After the first adjustments in the stoichiometry, the reactions to test the pink chromophore (CBD + 4-AP) and the blue chromophore (THC + 4-AP) were monitored over 60 min. Different amounts of NaOH were also tested to determine its influence on color stability. The optimization of the 4-AP procedure was also employed to test the ability of the test in detecting high amounts of THC when compared to the 4-AP. Thus, the proportions of THC/CBD:4-AP tested were (a) 6:1, (b) 4:1, (c) 3:1 and (d) 2:1.

2.4.5 | pH test

Three different solutions of Reagent B were prepared for the test. Solution 1 was made with 283.2 mg of NaOH in 30 mL of DI water and 70 mL of EtOH (pH 13). The solution was made with 34.2 mg of NaOH in 30 mL of DI water and 70 mL (pH 11). Solution 3 was made with 35.5 mg of NaOH in 300 mL of DI water and 700 mL of EtOH (pH 9). The liquid reaction was prepared by adding 50 µL of 250 ppm THC, 60 µL of reagent A, and 12 µL of prepared NaOH solution being tested. After adding the Reagent B, the reaction had the pH monitored for over 30 min. The minimum pH necessary for the reaction to occur was then determined using a pH paper (0.0–13).

2.4.6 | 4-aminophenol and chromophore identification

2.4.6.1 | *Quadrupole time-of-flight (QToF) mass spectrometry by flow injection analysis (FIA)*

The identification of the chromophores produced by the reaction between 4-AP + THC and 4-AP + CBD was first determined by Q-ToF-MS (6530 QToF-MS, Agilent Technologies) using FIA (Flow Injection Analysis) in positive (+) ion mode of an electrospray ionization source (ESI). The Q-ToF mass spectrometer was maintained at a gas (nitrogen) temperature of 200°C, drying gas flow at 6 L/min, the nebulizer was 35 psi, sheath gas temperature at 200°C and sheath gas flow at 12 mL/min, the capillary voltage was 4000 V, nozzle voltage at 500, fragmentor voltage of 135 V, skimmer voltage 65 V, octopole RF at 750 V. The acquisition range in the full scan mode was 100–1000 m/z and the MS/MS spectra were obtained at a Collision Energy (CE) of 20 V considering as parent ion (M + H) $m/z = 420.2533$. Data acquisition was achieved using MassHunter Data Acquisition Software B.05.00 and data analysis was performed using MassHunter Software Qualitative Analysis (B.07.00). For the first tests, a liquid reaction was prepared with a final concentration of 62.5 µg/mL for THC/CBD and diluted to 1 ppm in methanol and placed in the autosampler.

After the confirmation of the chromophore in the reaction by the full scan analysis, a time-resolved experiment was performed by monitoring the color change over 30 min at 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, and 30 min, for the THC + 4-AP chromophore (blue color) and the CBD + 4-AP chromophore (pink color). The color change was monitored by digital photography using the system developed by Acosta et al. [16], followed by analysis using QToF-MS at the specific times described above to confirm the increase in the production of the chromophores by monitoring the molecular ion $m/z = 420.2533$, along with the increase in the color intensity of the chromophore over time. The QTOF system uses as reference ions m/z = 121.050873 and $m/z = 922.009798$, corresponding to Purine and HP-0921, respectively.

2.4.6.2 | *Liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry (LC-QToF-MS)*

A LC-QToF-MS method was developed to confirm the presence of the chromophores in the reaction. For this test, THC, CBD, and the 4-AP standards were analyzed, followed by the same liquid reaction analyzed by FIA described above. Finally, the reaction was prepared using the solid substrate by two systems: one considering the excess of 4-AP over THC/CBD, 20:7, and the other using the same proportion of 4-AP and THC/CBD (1:1), after the stoichiometry adjustment. The reaction products were extracted after 8 min using 100 µL of methanol, followed by a 1:50 dilution in methanol. The stability of CBD + 4-AP and THC + 4-AP chromophores in the solid substrate was also tested over 8 h, by monitoring the presence of the chromophore molecular ion $m/z = 420.2533$, through LC-QToF-MS. The analyses were performed using a LC system with a 1290 Infinity autosampler, and

binary pump coupled to a 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer with JetStream electrospray ionization (ESI) source (Agilent Technologies). A Zorbax Eclipse Plus C18 column (3.0 × 100 mm × 1.8 μm) was used for the chromatographic separation and was kept under 15°C. Isocratic elution was performed using water +5 mM ammonium formate (solvent A) and MeOH +0.1% formic acid (solvent B), 10:90 (A:B). The flow rate was 0.4 mL/min and the injection volume was 1 μL. The total analysis run time was 10 min.

2.4.6.3 | *UV–Vis spectroscopy*

The UV–Vis analyses were performed in a Varian Cary 100 UV–Visible Spectrophotometer, with a UV–Vis scan rate of 600 nm/min in dual beam mode, in the region from 400 to 800 nm. Initially, the UV–Vis spectra were acquired for the blank sample (Methanol + Reagent A + Reagent B). The reactions of both chromophores (4-AP + CBD and 4-AP + THC) were prepared and scaled up to 4 mL, using the same proportions employed at the QToF-MS experiment. The color change and the spectra were monitored over 30 min at the same times monitored at QToF-MS. The analyses were performed using 4 mL quartz cuvettes.

2.4.6.4 | *Thin layer chromatography (TLC)*

A TLC method was employed for the evaluation of the reaction using different elution systems. The first system consisted of hexane: ethyl acetate: methanol (70:20:10), a second system consisted of chloroform: methanol (89:11), and a third system consisted of ethyl acetate with different proportions of diethylamine (3, 6, 8, and 10%). A TLC Silica Gel 60 F254 was used and cut into 6 cm × 9 cm plates. The samples were applied with an interval of 1.0 cm between them, and 0.5 cm from the borders. Six samples were analyzed: 1 (THC standard), 2 (CBD standard), 3 (Methanol), 4 (Blank), 5 (THC chromophore), and 6 (CBD chromophore). For the experiment with Thyme and Oregano were analyzed the following samples: 1 (THC standard), 2 (CBD standard), 3 (Blank), 4 (THC chromophore), 5 (Oregano chromophore), and 6 (Thyme chromophore). The solvent chamber was prepared 30 min prior to the analysis. 2 μL of each sample was applied three times using a pipette. After drying, the silica plate was placed into the chamber for about 10 min until the elution front reached the solvent mark. After drying, a UV light source (254 nm) was used to visualize the results immediately after the separation and then again 24 h after the TLC experiment.

2.5 | Selectivity study

2.5.1 | Use of potential interferents (herbs, spices, and tobacco)

To test the possible interferences using the 4-AP test after the optimization, the reaction was performed with 14 different samples including the following teas, spices, and tobacco samples: (1) Carobinha (*Jacaranda decurrens* Cham), (2) Guaraná (*Paullinia cupana* Kunth), (3) Sage (*Salvia officinalis*), (4) Lavender (*Lavandula* L.), (5) *Eucalyptus* L., (6) Apollo hop, (7) Spearmint leaf (*Mentha crispata*), (8) Citra Whole Leaf hops, (9) Cigar, (10) Red pepper, (11) Oregano, (12) Thyme (*Thymus vulgaris*), (13) Black pepper (Piper nigrum) and (14) Parsley (*Petroselinum crispum*). The extraction was employed using the same method developed by Acosta et al. [16], using methanol: chloroform (9:1) as solvents. The procedure was performed using the same conditions as the test employed for the cannabis plant material, according to the optimized version of the test: 10 μL of the extract, 10 μL of 4-AP (86.7 ppm), and 12 μL of NaOH. The samples were also extracted using just methanol to test the efficiency of the extraction without the necessity of adding chloroform. All the samples were acquired in a local market of Miami, Florida, USA, with the exception of the Brazilian species of Carobinha and Guaraná, which were acquired online from the Brazilian companies Flora Medicinal Pharmacy and Eurofito, respectively.

2.6 | 4-Aminophenol test procedure with authentic cannabis samples

A total of 186 cannabis samples composed of hemp ($n = 93$) and marijuana ($n = 93$) were tested by the optimized 4-AP procedure. The extraction of the cannabis samples was performed following the same protocol developed by Acosta et al. [16] using 1 mL of methanol: chloroform (9:1). In addition, an extraction using 100% methanol was also employed. The images of the resulting chromophore products were captured using a Samsung Galaxy S8 smartphone 15 min after the addition of the Reagent B (NaOH). The RGB codes were obtained from the images using Image J Software (ImageJ bundled with Java 1.8.0_172). Once the regions were selected a region of interest (ROI) RGB plugin was used to acquire the RGB codes.

2.7 | Chemometric models

RGB codes of the 4-AP chromophores were used to build Linear Discriminant Analysis (LDA) models using JMP software. Red (R), Green (G), and Blue (B) were used as the variables for analysis. The predefined classes for this model were hemp and marijuana. A total of 93 hemp samples and 93 marijuana samples were used to build the models. 48 hemp samples and 48 marijuana samples were used as the training sets to build the model, while the other 45 hemp samples and 45 marijuana samples were used to test the models. The 4-AP test was performed in triplicate for each sample. Each replicate was added as an individual object within the models.

Data-Driven Soft Independent Modeling of Class Analogies (SIMCA) models were also constructed for the marijuana class in this study. To perform SIMCA, a Chemometrics 2.0 add-in to

Microsoft Excel was used as previously described by our group [16]. R, G, and B scores of the images of the 4-AP chromophores were used as the variables. Alpha was set to 0.05 and gamma, which indicates outlier significance, was set to 0.01. All replicates were added to the dataset as individual objects. The training set was observed at each principal component (PC) to determine which samples were classified into the extreme category. Samples marked as extreme for each PC were selected to be added to the training set. The test set was then selected by using every third sample from the remaining samples so that 20%–35% of the full dataset was represented in the test set. Outliers were then observed and removed using a robust version of the models for the test and training sets at each PC. Although each replicate of a sample was added as an individual object whenever one replicate of a sample was marked as extreme or an outlier, all other replicates of the sample were treated similarly. Specificity is determined by testing hemp samples against the marijuana models made at each principal component. The models were then observed at each principal component (PC).

3 | RESULTS AND DISCUSSION

3.1 | 4-AP test procedure optimization and miniaturization

The miniaturization of the 4-AP test was performed to decrease the volume of reactants used so far, while the major purpose of the 4-AP test optimization was to improve the color stability and understand why the color was degrading to black/brown after only a few minutes. The first tests indicated that the use of excess of 4-AP could lead to its oxidation at basic pH, which then resulted in black color. Thus, more tests were conducted with the 4-AP + CBD and 4-AP + THC reactions and with blank samples using the method described by Acosta et al. [16], that was employing 20 times more 4-AP than THC/CBD, considering their molarity. The tests with the blank samples indicated the same black color produced for the 4-AP + CBD chromophore and 4-AP + THC chromophore. Thus, to confirm this hypothesis, different tests were made to prove the role of oxidation on color change and if the chromophore was suffering degradation or not.

The tests described in Section 2.4.2 were performed for (1) Reagent A (4-AP), (2) Methanol + Reagent A + Reagent B (blank sample employed during the tests), (3) 4-AP + CBD + Reagent B, and (4) 4-AP+ THC + Reagent B. For the Oxygen test, the reaction was prepared in falcon tubes following the same proportions used previously (17), with an excess of 4-AP. After connecting the tubes, the air was pumped into the liquid reaction over 60 min for the four samples. The results presented in Figure 1 show that oxidation occurs when there is an excess of 4-AP under the presence of a NaOH (Reagent B). As can be seen in Figure 1A–D, the same black/brown color is produced for the blank sample and both 4-AP + CBD and 4-AP + THC reactions, proving that the black color cannot be attributed to the chromophore degradation, but to the oxidation of the excess of 4-AP in basic solution.

To confirm the role of oxidation in the color change (production of the black/brown color), another test was made by pumping nitrogen into the reaction over 60 min, with the purpose of replacing the oxygen of the environment with nitrogen, to confirm that reducing the amount of oxygen would avoid the production of the black color. The test confirmed the hypothesis of the oxidation of the excess of 4-AP, since without oxygen, the black color was not produced for the blank or the CBD and THC reactions (Figure S1). The concentration of the blue and pink chromophores of THC and CBD, respectively, were noticeably reduced under nitrogen. This suggests that oxidation is required for both the formation of the chromophore and the black by-product. Reducing the amount of oxygen reduced the rates of both reactions but the rate of formation of the chromophore is faster.

After running the experiment to test the influence of the oxygen on the formation of the black color and proving that the oxidation is responsible for the color change, we wanted to confirm that by removing the excess of 4-AP from the reaction, the formation of the black color could be avoided since would not be an excess to be oxidized. The reaction was performed on the silicone solid substrate. In the previous study [16] a 20.7:1 molar excess of 4-AP to THC/CBD was used (4-AP = 8.24×10^{-8} mol: THC/CBD = 3.97×10^{-9} mol). In the current study, the conditions were modified and minimized to form an equimolar ratio of 4-AP to CBD/THC. The final volumes used were 10 µL of THC/CBD 250 ppm and 10 µL of 86.7 ppm 4-AP 1:1 (7.95×10^{-9} mol: 7.95×10^{-9} mol). After running the tests, the hypothesis of the oxidation of the excess of 4-AP under basic conditions was confirmed, since the black color was not produced anymore, by removing the excess of 4-AP, leading to a big improvement in the color stability for the pink chromophore (CBD + 4-AP) and for the blue chromophore (THC + 4-AP). Therefore, after adjusting the concentration and volume of 4-AP according to the stoichiometry of 4-AP:THC/CBD (1:1), the color lasted for about 40 min instead of 8 min, when compared to the method developed previously [16] (Figure 2). To show how color production varied with the different proportions of 4-AP:THC/CBD, an experiment was performed using the silicon filter paper as a solid substrate and 1:1, 10:1, and 20:1 ratios of the 4-AP:THC/CBD mixtures. However, it is important to highlight that the reaction with the 1:1 ratio of 4-AP:THC/CBD took more time to occur, giving a good blue and pink color at 15 min with a more intense color at 30 min. So, the reaction speed was different when compared to the first methods reported, that were employing an excess of 4-AP [2, 3].

These results suggest a mechanism for the formation of the black by-product and a means to prevent this and extend the window of time to observe the color of the 4-AP color test. A potential mechanism may involve the oxidation of 4-AP to 4-benzoquinone imine followed by the reaction of another molecule of 4-AP [21–23]. Reducing the concentration of 4-AP will reduce the rates of both these reactions. The reaction of 4-benzoquinone imine with 4-AP is expected to be slower than the reaction with alky-substituted phenols with a free *para*-position (CBD, THC, carvacrol, and thymol) (see

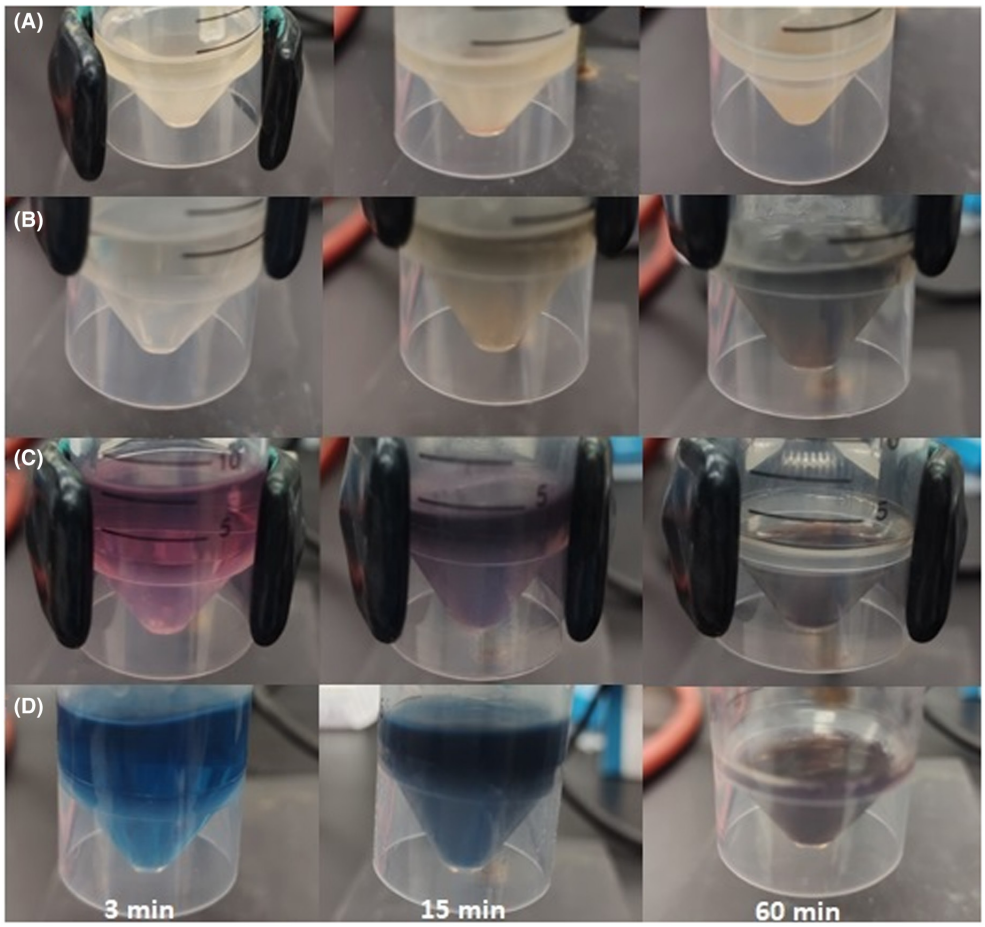


FIGURE 1 Experiment showing the influence of the oxygen on the formation of the black/brown color after pumping air into the reaction. (A) 4-aminophenol, (B) Blank, (C) CBD + 4-AP + Reagent B and (D) THC + 4-AP+ Reagent B.

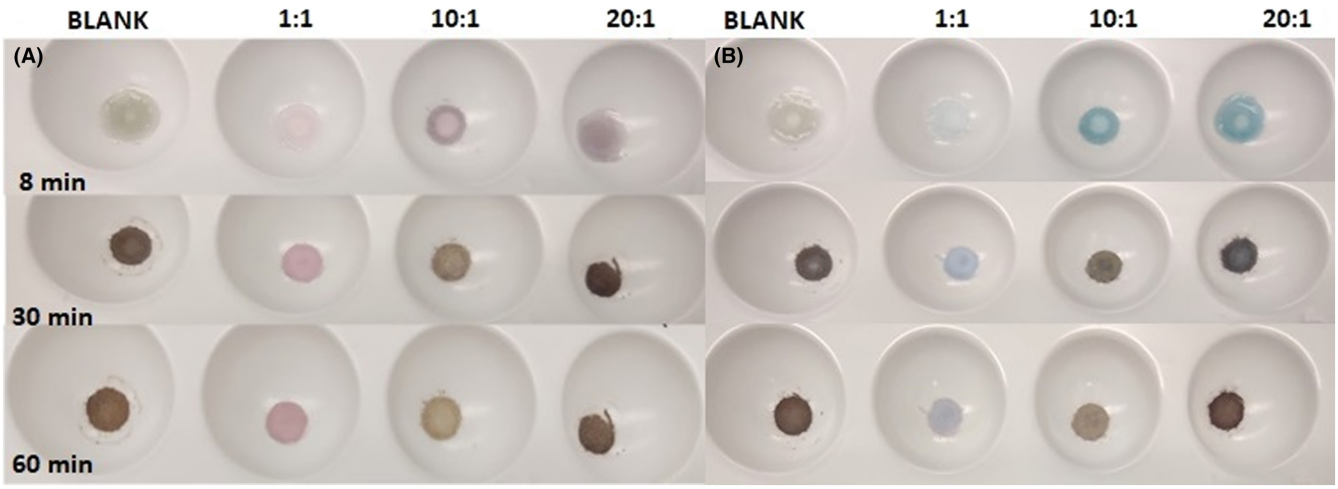


FIGURE 2 (A) 4-AP + CBD reaction and (B) 4-AP + THC over 60 min using different stoichiometric ratios of 4-AP:CBD and 4-AP:THC (1:1; 10:1 and 20:1).

Figure 10). This is consistent with our results that showed, that with a 1:1 molar ratio of 4-AP to the phenolic analyte (CBD, THC, thymol, or carvacrol) the reaction favored the chromophore over the black by-product.

To confirm the minimum pH required for the reaction, a test was performed using different pHs of Reagent B (pH 13, pH 11, and pH 9). Following the first reaction tested with the method developed by Acosta et al. [16], which employed 6 μL of NaOH at 300 ppm,

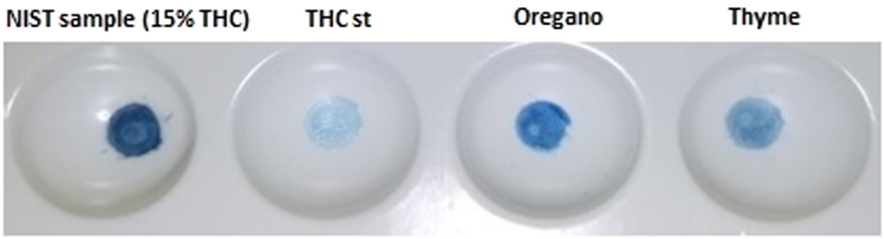


FIGURE 3 Picture showing the 4-AP reaction with a THC-rich cannabis sample containing 15% of THC (150,000 ng), the THC standard (15,000 ng), the oregano extract and the thyme extract. The picture was taken after 15 min.

the reaction did not occur when the pH 11 or 9 were tested. For the pH 14, the reaction occurred but there was a decrease to the pH 11. Thus, a series of tests were performed using different pHs of Reagent B (pH 13, pH 11, and pH 9). Based on these results together with the somewhat acidic nature of Reagent A (pKa 10.4), it was observed that it was necessary that the 4-AP (Reagent B) was converted into its anionic form for successful oxidation to the benzoquinone imine, a key intermediate in the formation both of the black by-product and also of the indophenol chromophore. High pH will also favor both the reaction of *p*-benzoquinone imine with 4-AP by deprotonation of 4-AP and also the reaction of *p*-benzoquinone imine with CBD/THC by deprotonation of these phenols. It is important to highlight that Reagent A has an initially acidic pH (2–4); thus, Reagent B must generate a basic solution (pH 13–14), to maintain the pH basic enough (higher than 10.4), for the reaction to occur. Different amounts of NaOH were also tested to check if the color stability could be improved (for more than 60 min) using the new optimized method (1:1) 4-AP:THC/CBD. The results obtained for 6, 12, 18, and 20 μL of NaOH showed that the color lasted for almost 2 h when 20 μL of NaOH were employed, 1.30 h when 18 μL were employed, 1 h when 12 μL were employed and 40 min when 6 μL were employed. The pH for the reactions that used 18 and 20 μL of NaOH was maintained at 12–13. When a volume higher than 20 μL was tested there was no change in the results when compared to those found with 20 μL. Based on the experiments obtained we can confirm that the stability of the color can last until 2 h if a higher amount of NaOH is used, which helps to keep the pH between 12 and 13. So, besides decreasing the concentration and volume of 4-AP, by adjusting the stoichiometry and avoiding the excess of 4-AP, the maintenance of the pH between 12–13 also contributed to ensure color stability. After all the optimization was performed, we chose to test using 12 μL of NaOH, which provides 1 h of color stability, since no additional stability was needed for the tests. Based on what was shown here, each laboratory could use its own method, maintaining the color stability for as much as is required. The reaction and the chromophore structure were first proposed by Lewis et al. [2]. It was also observed that by decreasing the concentration and volume of the 4-AP, and by increasing the volume of NaOH, we changed the reaction speed, which is now occurring more slowly when compared to the first reactions previously reported [2, 16].

Considering the experiments performed, it was shown that there is no necessity of employing excess 4-AP reagent to produce the chromophores. Consequently, the optimization and miniaturization call for a reduction in both the concentration and volume of 4-AP based on approximately 1:1 stoichiometric ratios of 4-AP reagent and the THC/CBD analytes reducing the oxidation products of 4-AP and improving the visualization of the respective chromophores. Another experiment was performed to determine the highest stoichiometric ratios of THC and CBD to 4-AP that can be detected in real samples of cannabis. As can be seen in Supplementary Material (Figure S2), high THC:4-AP and CBD:4-AP ratios (2:1, 3:1, 4:1, and 6:1), produced chromophores with a color stability of 1 h, proving that there is no necessity of using excess 4-AP for the reaction with the real samples. A cannabis sample provided by NIST and previously analyzed by HPLC containing ~15% of THC (w/w) was extracted (using the protocol described above) and tested to demonstrate the blue color when a low concentration and volume of 4-AP are utilized (4-AP:THC ratio equal 60:1; Figure 3). The results showed a dark blue after only a few minutes, confirming the presence of the THC-rich cannabis in the seized material and showing the color stability as expected, without the formation of the black color (Figure 3). The decrease in the volume and concentration of 4-AP allowed better results and color stability, achieving our purpose of performing the test optimization and miniaturization, employing less than 40 μL of reactants and avoiding the black color and the chromophore degradation.

The lower limit of detection (LOD) was also tested by performing the reaction with decreasing concentrations of the THC and CBD standards, giving a lower stoichiometric ratio for 4-AP:CBD and 4-AP:THC (2:1, 4:1, 5:1 and 10:1; Figure S3). The LOD was determined as the lowest concentration that allowed an easy identification of blue and pink colors, enough to indicate the presence of THC and CBD chromophores. 250 ng equivalent to 10:1 ratio of 4-AP:THC and 4-AP:CBD, was the lowest concentration tested, presenting a very light blue and pink color, not enough to give positive results for THC and CBD chromophores. So, the best results obtained for the identification of CBD and THC content was with a 500 ng (5:1) ratio. As expected, it was also observed that the blue and the pink colors were less intense for these ratios compared to the higher ratios tested at the optimization. The color stability for 250 ng (10:1) ratio of 4-AP:THC/CBD was also shorter than the others, since the difference between the 4-AP and THC/CBD ratio was higher, leading to the oxidation of the excess of the unreacted 4-AP (Figure S3).

3.2 | Selectivity—false-positive results

An experiment was performed with 14 herbs and spices to test the specificity of the optimized 4-AP test by using extracts prepared in chloroform: methanol (9:1) and just in methanol. Both extracts

showed the same results proving that there is no necessity of using chloroform for the extraction. The Brazilian samples of Carobinha (*Jacaranda decurrens* Cham), and Guaraná (*Paullinia cupana* Kunth), known to produce false-positive results for the FBB test [20], were also included in the test, and to date, were never tested before using the 4-AP test. The results showed a green/gray and orange/brown color for Carobinha and Guaraná, respectively, with no false-positive results as encountered in the FBB test [13]. However, two false positive results were observed after testing oregano and thyme, which produced a blue color similar to samples of THC-rich cannabis. Oregano was shown to produce a false positive result [2] and, however, to the best of our knowledge, thyme was never reported before as a false positive for the THC. Then, a comparison between the cannabis sample from NIST, which contains a high amount of THC (15% of THC), the THC standard, thyme extract, and oregano extract was performed, as can be seen in Figure 3. The NIST sample was also extracted using only methanol proving that there is no necessity of using chloroform, simplifying the method. The same samples were tested for the FBBB test and the 4-AP test except for the two Brazilian species [16] (Carobinha and Guaraná). However, according to the experiment previously performed [16] the only sample that developed a blue color was Oregano, also reported by Lewis et al. [2]. The hypothesis is that as the method performed before by Acosta et al. [16], was using a high concentration and volume of 4-AP (excess), there was a color change to black, preventing the proper identification of the blue color, like was performed here. All the color results obtained for the samples can be seen in Table 1 below.

Origanum vulgare (oregano) and *Thyme vulgaris* (thyme) are aromatic plants of Lamiaceae family with a large number of bioactive compounds [24]. The major compounds found in *Thyme vulgaris* are the diterpenoid thymol and its isomer carvacrol [25, 26]. Oregano also is known for presenting a high content of carvacrol and thymol [26, 27]. Both compounds can react with 4-aminophenol (Reagent A) at a basic pH producing the blue chromophore. To confirm the hypothesis, solutions of 1000 ppm of thymol and carvacrol were prepared in methanol and used to perform the 4-AP reaction with the optimized procedure, employing the solid silicone substrate. The results confirmed the hypothesis that thymol and carvacrol are the compounds responsible to produce the false-positive results for THC (blue color; Figure 4).

The protonated molecular ion m/z[(M + H) = 256.1332] was detected by QToF-MS for oregano, thyme, carvacrol, and thyme after preparing the 4-AP reaction. The structure was consistent with carvacrol indophenol, with a chromophore similar to that proposed by Lewis et al. [2] for THC and CBD. A TLC was also performed with a 4-AP reaction for the samples thymol, carvacrol, thyme, oregano, and THC. Thymol, carvacrol, thyme, and oregano showed the same retardation factor (RF), proving that thymol and carvacrol are the compounds responsible for the false-positive results presented by thyme and oregano. Therefore, when the TLC system was performed, both thymol and carvacrol chromophores showed a different (RF) when compared to the THC chromophore and could be used as a confirmation technique if there is a suspicion of a false positive result with oregano or thyme.

After the optimization and miniaturization of the 4-AP test, the identification was performed by confirming the presence of the chromophores using different analytical techniques such as UV–Vis, TLC, QToF-MS, and LC-QToF-MS.

3.3 | 4-AP CBD and THC chromophore's identification

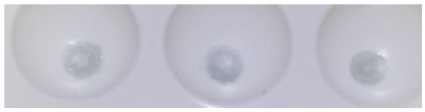
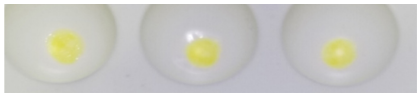
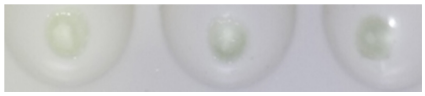
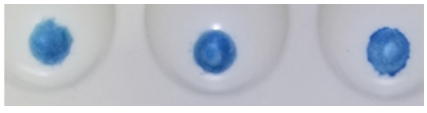
The first test performed to achieve the chromophore's identification was through the QToF-MS using flow injection analysis (FIA) as described in item 2.4.2. For this test, a liquid reaction was prepared using the same proportions employed by Acosta et al. [16] and diluted to 1 ppm in methanol. The injections were performed after 5 min of the reaction, confirming the presence of the molecular ion (MI + H) m/z = 420.2533 and proving the presence of the CBD + 4-AP and THC + 4-AP chromophores, along with the presence of the sodium adduct (MI + Na) m/z = 442, that was also detected in the MS spectrum (Figure 5).

After the identification of the chromophores by the FIA using the QToF-MS, a time-resolved experiment was performed to confirm that along with the increase in color intensity, there is an increase in chromophore production. For this experiment, a liquid reaction was prepared using a clear vial with an insert. After adding the NaOH (Reagent B), every time there was a color change pictures were taken and then a dilution to 1 ppm was prepared prior to the injection into the QToF-MS. The times monitored were 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, and 30 min. The results showed that the production of the chromophore increased along with the color intensity for 16 min for the THC chromophore and 12 min for the CBD chromophore (Figure 6).

The same time-resolved experiment was performed by UV–Vis by preparing the reaction using the same proportions employed by QToF-MS, confirming the increase in chromophore's production until 16 min for THC + 4-AP and until 12 min for the CBD + 4-AP (Figure 7). The λ max was 520 and 639 nm for the CBD + 4-AP and THC + 4-AP chromophores, respectively.

After performing the experiment using QToF-MS through FIA, a method was developed using liquid chromatography to perform the identification and separation of the chromophores, employing a reversed-phase column (C18). The method developed by LC-QToF-MS detected both chromophores at different retention times, 3.827 min for CBD + 4-AP chromophore and 4.485 min for THC + 4-AP chromophore (Figure 8). The chromophores were detected through MS and MS/MS analysis, by identifying the molecular ion m/z = 420.2533 and the main ions 377.1983, 364.1901, 300.1587, and 109.1008 with a mass error lower than 5 ppm. A mix of THC + 4AP and CBD + 4-AP was also tested to show the ability of the method to separate both chromophores. To the best of our knowledge, this is the first time that the identification of the THC and CBD chromophores produced by the 4-AP reaction is performed through chromatography and mass spectrometry. Until now, the confirmation of marijuana-type cannabis and hemp-type

TABLE 1 Color test results for the 4-AP test with extracts of teas, spices, and tobacco samples.

	Sample	Color	Result	Picture taken after 15 min
1	Carobinha	Green	Negative	
2	Guaraná	Gray-Blue	Negative	
3	Sage	Green/Light Blue	Negative	
4	Lavender	No color	Negative	
5	Eucalyptus	Green	Negative	
6	Apollo hop	Yellow	Negative	
7	Spearmint leaf	Green	Negative	
8	Citra Whole Leaf hops	Yellow	Negative	
9	Cigar	Light yellow	Negative	
10	Red pepper	Light yellow	Negative	
11	**Oregano**	**Blue**	**False-Positive**	
12	**Thyme**	**Blue**	**False-Positive**	
13	Black pepper	Light yellow	Negative	
14	Parsley	Light yellow	Negative	

The bold values significance added to distinguish the false-positives results.

cannabis primarily tested by the 4-AP color test was achieved by the identification/quantification of CBD or THC contents, and not by the identification of their chromophores [2, 3, 12]. The extraction of the chromophores with methanol directly from the solid substrate could also be used for the confirmation of the presence of marijuana-type cannabis or hemp-type cannabis, instead of using

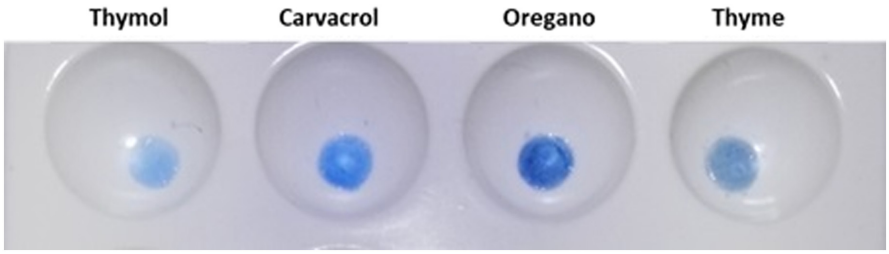


FIGURE 4 Picture showing the 4-AP reaction with thyme, oregano, thymol, and carvacrol using the solid silicone substrate.

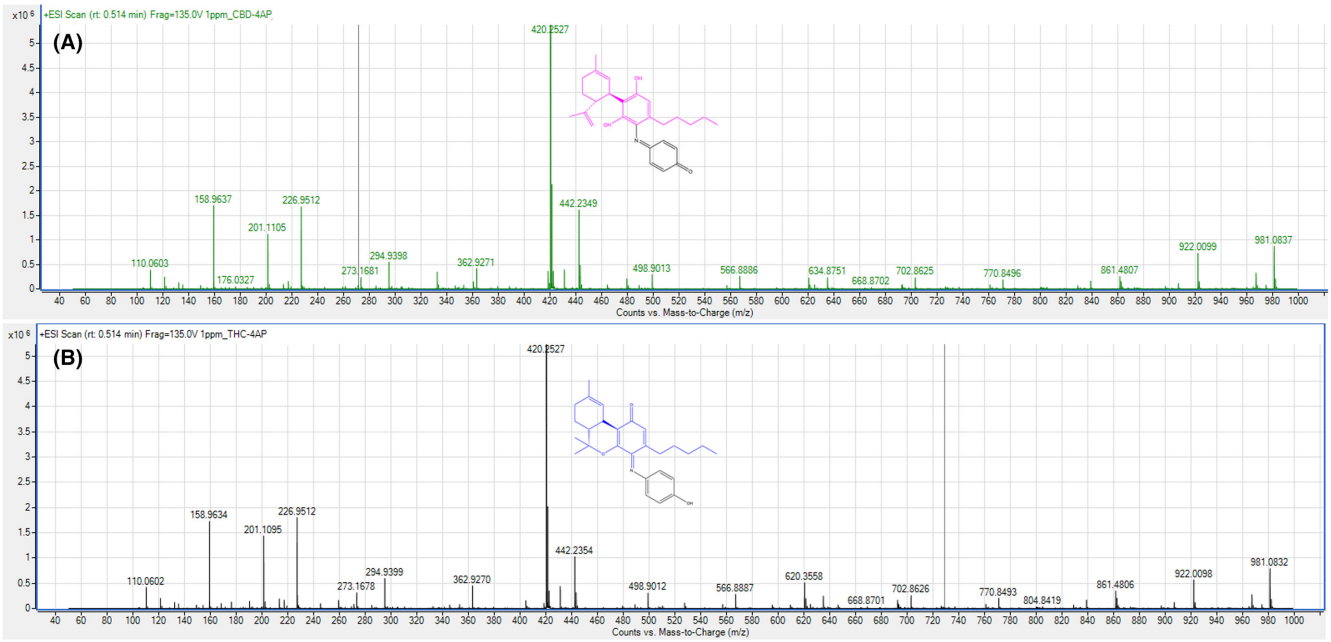


FIGURE 5 Mass spectra using full scan analysis for the CBD chromophore (A) and the THC chromophore (B).

the identification of CBD or THC by performing the analysis of the plant material. It is important to mention that the chromophores are thermolabile, so the use of methods that employ high temperatures as the direct analysis in real-time mass spectrometry (DART-MS) and the gas chromatography coupled to mass spectrometry (GC–MS) are not an option to identify the chromophores.

The long-term stability of the chromophores formed on the solid substrate was also tested using LC-QToF-MS, by preparing the reactions in triplicate and comparing the current method (4-AP:THC/CBD = 1:1) to the method developed by Acosta et al. [16] (4-AP:THC/CBD = 20:7). The average of the peak areas of the three replicates obtained from CBD + 4-AP chromophore and THC + 4-AP chromophore was monitored over time. The stability of both chromophores was monitored at 1, 2, 3, 4, and 8 h, by extracting the chromophore from the solid substrate with methanol. The results showed that the chromophores can still be detected after 8 h in both methods (1:1 and 20:7), giving another advantage by using the solid substrate, besides the reduced amount of reactants needed to perform the test. The CBD + 4-AP chromophore seems to be less stable when compared to the THC + 4-AP chromophore, presenting a higher decrease in the peak area after 4 h. The presence of unreacted THC and CBD was detected only for the first 2 h for the CBD and for 8 h for the THC chromophore, proving that the reaction speed is different for THC and CBD using the current method (Figure 9). With this experiment, we proved that even after several hours at room temperature the chromophores are still present on the substrate used for the reaction.

A TLC method was also developed to test the isolation of the product using three different elution systems (see item 2.4.6.4). All three methods showed the presence of chromophores, but the third method developed using ethyl acetate with 10% diethylamine was selected, giving a better separation between the THC and CBD chromophores and between both chromophores and the THC and CBD. The RF obtained for the THC, CBD standards, and CBD and THC chromophores were 0.83, 0.84, 0.49, and 0.67, respectively. The best chromophore isolation was achieved after 3 days of the reaction, showing no unreacted CBD, a small portion of unreacted THC, and no unreacted 4-AP. The chromophores of the 3rd day were also used as a "standard" to perform the TLC with the false-positives chromophores obtained for thyme, oregano, thymol, and carvacrol, which showed a different RF (0.54–0.56) when compared to the THC chromophore (0.67–0.70). By testing thymol and carvacrol we confirmed that both were reacting with 4-AP, producing the false-positive results. Thyme chromophore with a major percentage of

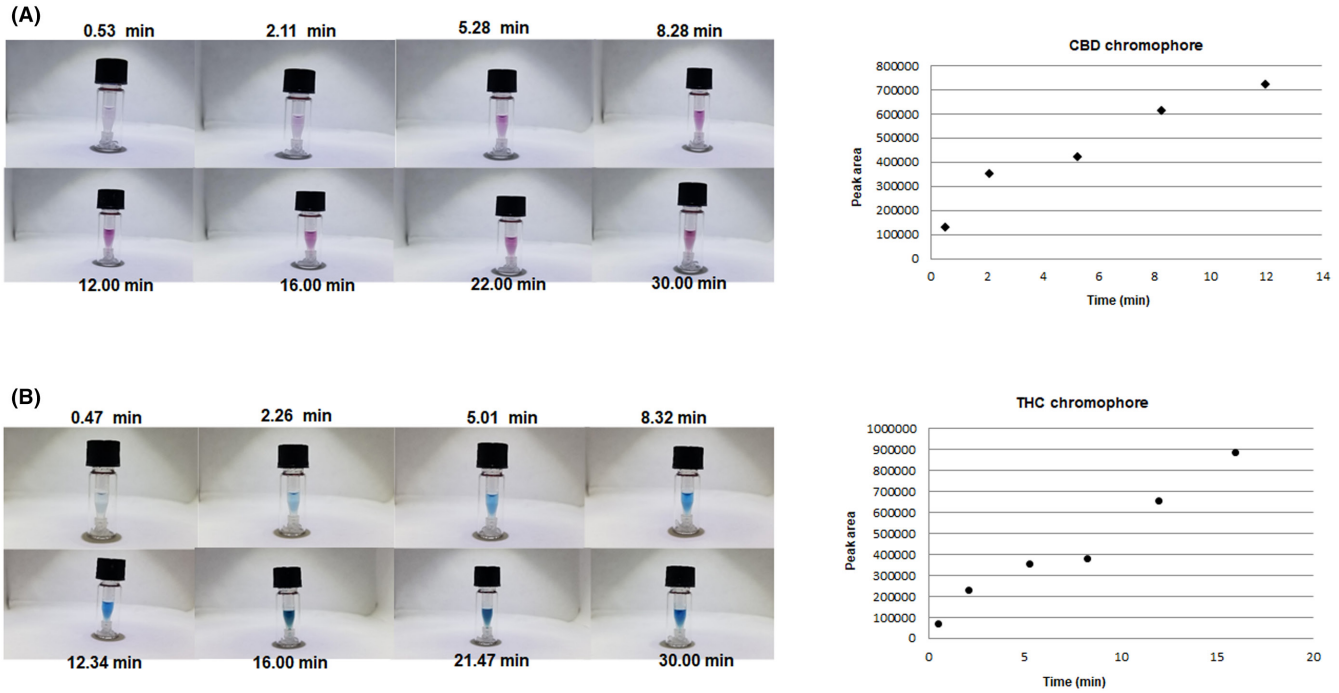


FIGURE 6 Time-resolved experiment showing the increase in the color intensity of the CBD chromophore (A) and THC chromophore (B) on the left; and the increase in the peak areas of the THC and CBD chromophores, (MI + H) m/z = 420.2533, obtained by FIA-QTOF, on the right.

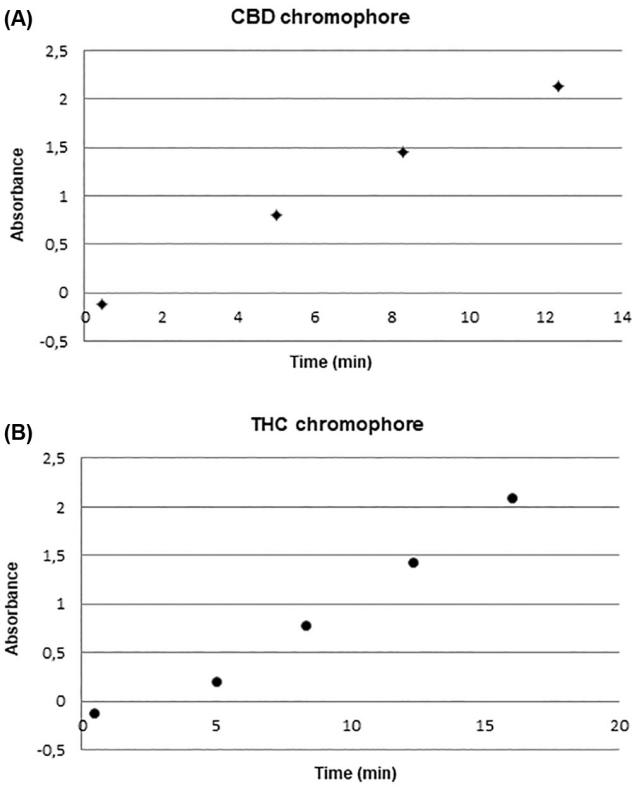


FIGURE 7 Graphs showing the increase of the absorbance × time obtained by UV–VIS for the CBD chromophore (A) and THC chromophores (B).

thymol showed to be less intense than the oregano chromophore, which presents a major percentage of carvacrol. Both chromophores showed the same RF, as mentioned above. Thus, the TLC method also showed to be a good tool for the confirmation of the false positive results obtained by the 4-AP color test, allowing the differentiation between the THC chromophore and the false-positives chromophores, by employing a simple and fast elution system developed here (Figure S5).

Combined, the results of these experiments suggest a possible mechanism for the reaction of 4-AP with CBD and THC that explains the different structures and colors of the chromophores. The chemical structure of indophenol [4-(4-hydroxyphenyl)iminocyclohexa-2,5-dien-1-one] and the intense blue color of the corresponding indophenolate sodium salt has a long history [28, 29]. Indophenol analogs have been prepared by the reaction of phenols with quinonechlorimides under alkaline conditions [30] and recently by the reaction of phenols with nitrosophenol in concentrated sulfuric acid [31]. Effects of the substituents on the phenolic ring on the color of the indophenol have been extensively studied [29]. Alkyl-substituted phenols containing only one hydroxyl group (THC, carvacrol, thymol) produce a blue chromophore, alkyl-substituted phenols containing two hydroxyl groups (CBD) produce a pink chromophore. As noted previously, the strongly alkaline conditions of the reaction will deprotonate 4-AP, favoring its oxidation to 4-benzoquinone imine. Under these conditions, the molecules of THC and CBD will be converted into phenolate anions leading to a reaction at the free *para*-position with *p*-benzoquinone imine. Initially, this produces a colorless leuco

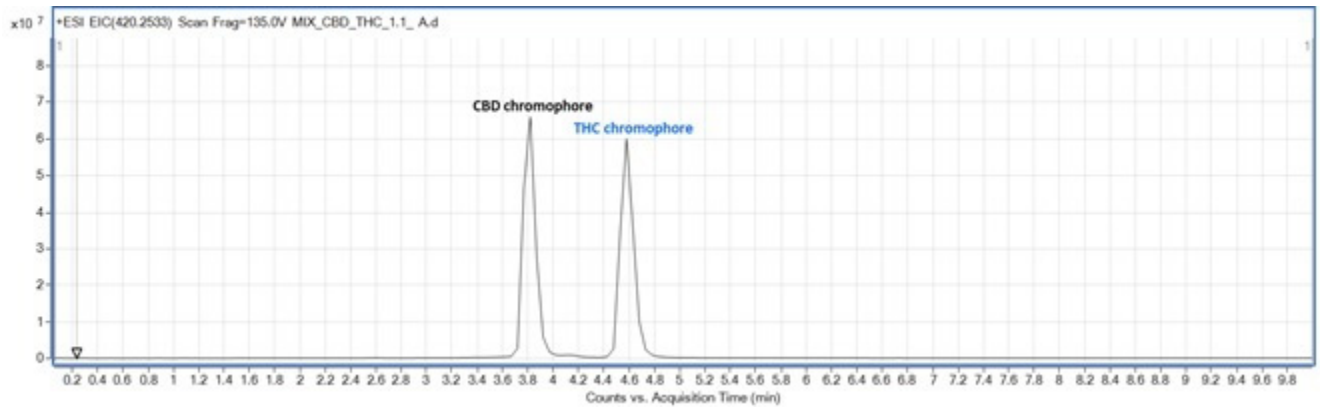


FIGURE 8 (A) Chromatogram showing the different retention times for the CBD chromophore (3.827 min) and for the THC chromophore (4.485 min) (B).

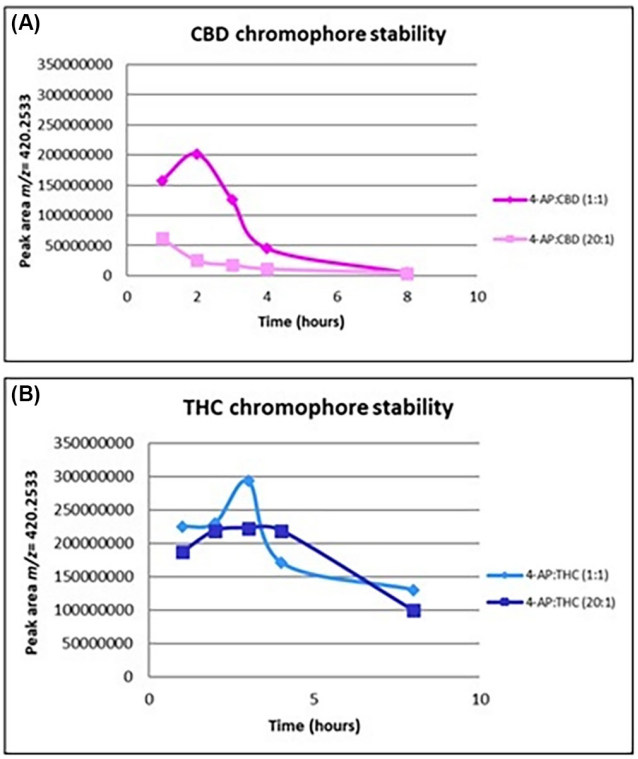


FIGURE 9 Stability plots for the CBD chromophore (A) and THC chromophore (B) on the silicone filter paper over time (8 hours) at room temperature, comparing different stoichiometric ratios of 4-AP:CBD and 4-AP:THC.

indophenol sodium salt, which, under the strongly alkaline reaction conditions, is oxidized by air to produce an indophenolate sodium salt (Figure 10). This mechanism is supported by (a) the need for strongly alkaline conditions, (b) the reduced rate of reaction caused by reducing the concentration of oxygen or 4-AP, (c) the competition between the reaction of either 4-AP or CBD/THC to react with 4-benzoquinone imine which produces the black by-product or the chromophore product, respectively, which can be made to favor the chromophore by reducing the concentration of 4-AP.

3.4 | Chemometric models for the analysis of cannabis samples using the optimized 4-AP method

A recent study by our research group [16] tested 192 cannabis samples of known THC/CBD composition using a miniaturized 4-AP test and then used the RGB scores of images of the reactions with these samples. The RGB scores were used to construct Linear Discriminant Analysis (LDA) and Data-Driven Soft Independent Modeling of Class Analogies (SIMCA) models to evaluate the classification of hemp and marijuana using the 4-AP test [16]. A subset ($n = 186$) of these cannabis samples (93 hemp and 93 marijuana) were tested using the optimized 4-AP method presented here, using the same extracts prepared by the previous method.

The LDA models performed well in comparison to those from the previous study. When all 186 samples were used to build the LDA model, the training set was found to have correctly classified 99% of the training set with an r^2 of 0.88 and correctly classified 98% of the test set with an r^2 0.69. The samples that were misclassified were DEA Sample 43, NIST Sample 6, and Sour Suver Haze Hemp. DEA Sample 43 and NIST Sample 6 were found to be CBD-rich, therefore they formed a dark purple chromophore with 4-AP, leading to their misclassification when compared to the expected pink color. This is consistent with the results of the previous study [16]. Sour Suver Haze hemp formed a darker pink chromophore than other hemp samples, causing it to misclassify. However, there were marijuana samples that presented a THC:CBD <2 (CBD-rich) that formed ambiguous colors with the 4-AP test that were not misclassified but could affect how well the model classifies between hemp and marijuana. A LDA model that included the 13 marijuana samples with THC:CBD <2 removed from the dataset was constructed (Figure S4A). This model showed improvements, correctly classifying 100% of the training set and 99% of the test set. The models had better fits, with the training set having an r^2 of 1 and the test set having an r^2 of 0.99. In this model, the only misclassification was DEA sample 39, which formed a light green-blue chromophore with 4-AP leading to its misclassification. These models showed a great improvement over the models created by the previous method,

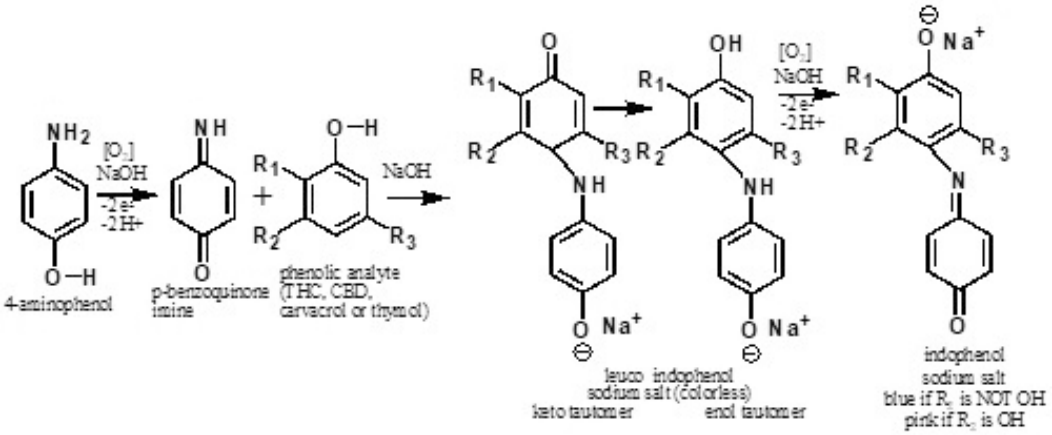


FIGURE 10 Proposed reaction mechanism between 4-AP and THC (R2 = ether), CBD (R2 = hydroxyl), thymol (R2 = hydrogen) and carvacrol (R2 = hydrogen). R1 and R3 are alkyl groups.

since the optimized 4-AP method allows for more intense colored chromophores to form over time, with no color degradation, as was observed with previously reported methods [2, 3, 16]. This reduces the number of samples that form light chromophores, which was an issue with the 4-AP test prior to the optimization [16] presented here.

SIMCA models were also constructed for the marijuana class with the full dataset and with the 13 THC:CBD < 2 marijuana samples removed. For both models, the maximum number of principal components available was three. Out of the three principal components, PC 2 was found to have the highest sensitivity and specificity for both models constructed (Figure S4B). The model constructed using the full marijuana dataset contained 53 samples in the training set, 24 samples in the test set, and 16 samples that were marked as outliers. The samples that were marked as outliers were found to have a THC:CBD < 2, such as NIST Sample 6 or DEA 43, or formed light or ambiguous colors with 4-AP, such as NIST Sample 3 and DEA Sample 39. This is consistent with the SIMCA results using the previous miniaturized 4-AP method [16]. However, the model in this study had higher sensitivity than previous models, with a training sensitivity of 95% and a test sensitivity of 100%. In addition, the specificity from hemp was found to be 100%. As with the LDA, a model was constructed with the 13 THC:CBD < 2 marijuana samples removed from the dataset prior to modeling. This model contained 51 samples in the training set, 25 samples in the test set, and 4 samples that were marked as outliers. The outliers in this model either formed a light chromophore with 4-AP, such as NIST Sample 3, or a color other than blue or pink, such as DEA Sample 39. This model also performed better than the 4-AP SIMCA models from the previous study, with a 96% training sensitivity, 99% test sensitivity, and 100% specificity.

4 | CONCLUSIONS

A "modified 4-AP" colorimetric test was miniaturized by using a solid silicone substrate to form the reaction in addition to reducing the volume of reactants to less than 40 µL and reducing the concentration of 4-AP from 300 to 86.7 ppm. Furthermore, we proved that there is no necessity of using chloroform to perform the extraction of the cannabis samples, simplifying the method. The optimization also allowed the color stability of the chromophores for up to 2 h using different volumes of NaOH, depending on the application of each laboratory. We also report, for the first time, that the reason for the color degradation of the 4-AP reaction/products to black is the oxidation of the excess of the 4-AP in the basic medium, and we now provide a simple solution to remove the excess 4-AP with an adjustment of the stoichiometry of 4-AP:THC/CBD to 1:1. The chromophores were identified for the first time using QToF, LC-QToF-MS, UV–Vis, and TLC. A simple extraction of the products with methanol from the silicone solid substrate followed by a LC-QToF-MS analysis confirmed the presence of both 4-AP-CBD and 4-AP-THC chromophores after 8 h, demonstrating that this extraction could be used as an alternative for the confirmation of CBD-rich or THC-rich cannabis, and proving that the silicone filter paper could be employed for further analysis, even several hours after the reaction was initiated and the color observed. The method developed by TLC was also able to confirm the presence and separation of the chromophores and can also be employed as an alternative for the confirmation of THC-rich cannabis and CBD-rich cannabis. The specificity of the test was also verified by testing different potential interferences (herbs, teas, and spices), including the Brazilian species of carobinha and guaraná, tested for the first time as interferences on the 4-AP reaction. The results did not show false-positives as was presented for the FBB test, showing a green color for guaraná (*Paullinia cupana* Kunth) and a purple/brown color for carobinha (*Jacaranda decurrens* Cham). However, the presence of false-positives for THC was observed for thyme and oregano, which also developed a blue color, same as THC. The false-positive results were attributed to thymol and carvacrol, diterpenes present in high concentration in thyme and oregano extracts. Even with the false-positives results, the modified 4-AP color test proved to be an excellent tool for

the preliminary differentiation of hemp and marijuana in the field, now without any color degradation, producing a pink color when the level of THC is about two times lower than the level of CBD, and a blue color when the level of THC is about two times higher than the level of CBD. Finally, after creating two different chemometrics models, both the LDA and SIMCA models showed that the optimized 4-AP method performs better than all previously reported 4-AP color test, supporting the results presented here. The classification rates and sensitivity of the models are improved over those constructed with the original miniaturized method. Both model types also showed that the 4-AP color test is likely to misclassify when marijuana samples have a THC:CBD < 2 which is consistent with the previous study. In addition, it was found that if the samples produce a light chromophore or form a color outside of blue or pink, it will also misclassify, such as NIST Sample 3 or DEA 39. However, this case was found to be less likely than with the previous method, as shown in the LDA models. This demonstrates that the optimized method can be expected to form deeper colors with samples in the field, with no color degradation, making it easier for users to distinguish between the pink and blue colors formed, and thus providing better results for the differentiation between hemp and marijuana. A detailed discussion and characterization of the structures of the chromophores presented here will be part of a further publication containing MS/MS and NMR data.

FUNDING INFORMATION

Funding provided by the National Science Foundation, Award #1739805.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

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How to cite this article: dos Santos MK, Acosta A, Capote R, Tabassam B, Ley J, Quirke M, et al. Chemical identification and optimization of the 4-aminophenol colorimetric test for the differentiation between hemp-type and marijuana-type cannabis plant samples. J Forensic Sci. 2023;00:1–16. https://doi.org/10.1111/1556-4029.15309