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Final Summary Overview

Development of a THC Breath Analyzer using Chitosan Film with Colorimetric Dye

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Purpose

The development of a breathalyzer for the early detection of marijuana's recent use is an important matter considering the current legal status of marijuana-based products around the country. To achieve this goal, our initial approach was to develop a portable cartridge that would be able to react with cannabinoids selectively to detect THC use, but not CBD. Most THC breathalyzers in the market are merely collection devices that will need further laboratory analysis. Considering the working system of an alcohol breathalyzer, using a redox reaction would be risky for the THC approach as it would not be selective for the specific cannabinoids and it would give a positive result to any molecule capable of oxidizing the reagent. In this project we have worked with two hypotheses to explain it: (1) the limitation of the current breathalyzer systems for the detection of Δ9-THC is due to their lack of capacity to detect the analyte in situ due to the limitation of their composition, and (2) using unspecific reactions could lead to false positive results with cannabinoids that are not targeted. The goal of the project was to develop the reaction support for the development of the THC breathalyzer prototype.

Our initial idea was to create a sorptive matrix using chitosan that could hold the reaction between the Δ9-THC and the specific dye, generating a color. Modifying this sorptive barrier with a dye selectively reactive to Δ9-THC would allow the detection in situ. Sorptive solid barriers allow the expelled air to pass through but will retain the solid microparticles, allowing the reaction between the Δ9-THC and the dye to take place on the surface of this barrier, giving a response that can be either colorimetric (appearance or color change) or luminescent (light emission against UV irradiation). Our specific aims included (1) the development of a cartridge to support the reaction system; (2) the development of a reaction system to detect Δ9-THC using color assay; (3) the evaluation of the cartridge + reaction system for validation parameters, including sensitivity and linearity and; (4) the evaluation of the system stability to be commercially available. The aims and scope of this project specifically address the requirement identified by the 2022 Forensic Technology Working Group in Forensic Toxicology - "Proof-of concept to support portable, reliable, and robust roadside devices to test for marijuana use and/or measure impairment, including scientific foundation for new or existing devices."

Project Design and Methods

Revelation System – Dyes Choice

The Fast Blue Dyes Family is well-known as a colorimetric reagent for the analysis of cannabinoids. These tests are considered presumptive as they only indicate the possibility of the analyte being present in the substance. The Fast Blue dyes consist of three different compounds namely Fast Blue B (FBB), Fast Blue BB (FBBB), and Fast Blue RR (FBRR). Their structures are shown in Figure 1. Among them, FBB and FBBB are strong candidates as revealing reagents.

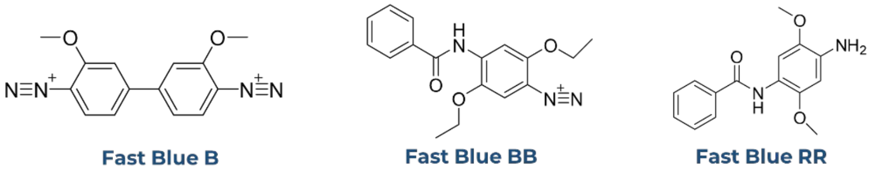


Figure 1 – Fast Blue B, Fast Blue BB, and Fast Blue RR structures.

The FBBB test is selective among major cannabinoids, providing a red color for THC, an orange color for CBD, and a purple color for CBN. Ultraviolet-Visible Spectroscopy has shown that the FBBB + THC chromophore has an absorption band at 471 nm, which is responsible for its red color. Previous studies showed that in basic conditions THC becomes a phenolate anion and that this anion attacks the diazo group in FBBB at the para position to form the chromophore. A bathochromic (or "red") shift results from the extended conjugation in the chromophore and the transition caused by the electrons in the diazo group of FBBB. A similar mechanism is showed for FBB, giving the positive result when in contact with Δ9-THC, a red color. FBRR was not evaluated as it reacts with cannabinoids giving the same color, and because of that it would give positive results for legal cannabinoids such as CBD. For example, using FBRR, CBD and CBG (cannabigerol) result in the same color formation.

The initial part of the studies focused on the FBB and FBBB as revealing agents. However, the final product uses FBBB as the colorimetric reagent. The reason relies on the fact that, in addition to different colors formed for Δ9-THC and CBD when using both dyes, FBBB fluoresces in the presence of Δ9-THC and not CBD, giving an extra detection layer.

Matrix Support Development

Production of the Sorptive Matrix based on Chitosan Gel

Our initial goal was based on the development of the chitosan film to serve as the colorimetric reaction support. This film would be modified with Fast Blue dyes. For that, a chitosan film was produced and the film was impregnated with Fast Blue dyes in different concentrations. To evaluate the reaction, THC was added to the film, and the color was measured spectrophotometrically. Chitosan is a naturally abundant poly-amino saccharide, which is a synthetic byproduct of chitin deacetylation as shown in Figure 2. It consists of straight chains of β-(1→4)-2-acetamido-2-deoxy-D-glucose and deacetylated units. The advantage of chitosan application relies on the capacity of these properties to be easily tunable.

Figure 2 – Conversion of chitin in chitosan

The high potential of chitosan for the adsorption of dye molecules is attributed to the abundance of hydroxyl and amine groups in its glucose units. Due to the presence of numerous functional groups having high reactivity and flexibility of the polymer chain, chitosan possesses wide applications in the preparation of various adsorbent materials such as films, membranes, aerogels, hydrogels, and composites. In addition, the use of chitosan gel as the base for the cartridge reaction would produce an environmentally friendly cartridge.

The chitosan film was produced by dissolving 3.33 g of chitosan powder in 1 L of an aqueous acetic acid 2% (v/v) solution at room temperature by magnetic stirring for six h and vacuum filtering of the resulting solution. About 150 mL of the obtained solution was poured into 15 cm diameter Petri dishes, and acetic acid was evaporated in an oven at 65°C for 48 h. The film was then removed from the plates, cut into 1 x 1 cm squares, and stored in desiccators at 25°C for use. To modify the chitosan film for the impregnation with the Fast Blue (FB), an aliquot of 30 mL of a previously prepared FB solution was transferred to a 50 mL polyethylene bottle, followed by 30 mL of 0.1 mol/L sodium phosphate/sodium citrate buffer, and a 1x1 cm piece of the film. The tube was closed, and the solution was stirred under a shaker table for 2 hours. Then, the modified film was taken, left to dry for 10 minutes in a watch glass, and then this material was transferred to a Petri dish, where the addition of 10 µL of Δ9-THC 1000 µg/mL solution could be added. The colorimetric response was recorded using a smartphone. The aspect of the modified chitosan film after the sorption of the dye and the colors of the film after and before the addition of Δ9-THC are shown in Figures 3a and 3b, respectively.

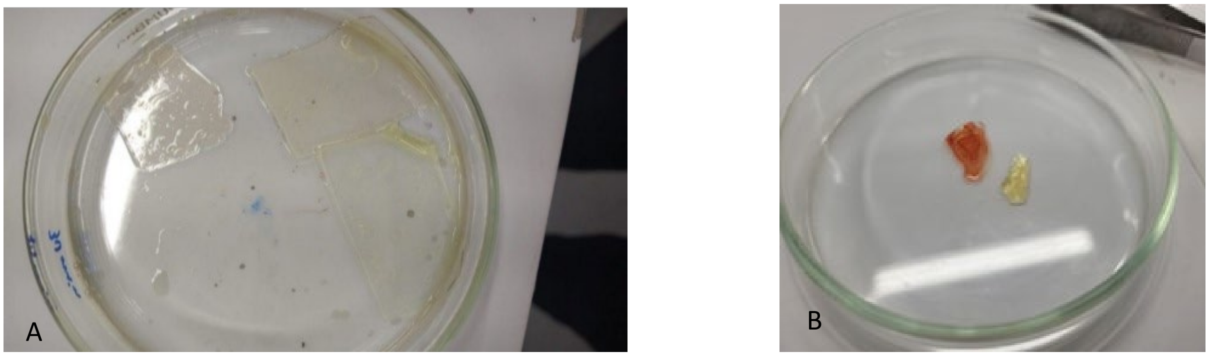


Figure 2 – (a) Modified Chitosan film with Fast Blue; (b) Modified chitosan film after (red) and before (yellow) Δ9-THC addition.

This preliminary result showed that in the presence of 9-THC, the chitosan gel impregnated with FBB changed from a light yellow to a dark orange-reddish color. After this result, the primary effort was to optimize the conditions that produce a better analytical response, which includes the study of the parameters inherent to the chitosan film production (such as chitosan mass, acetic acid concentration), the modification of the film with the fast blue dye (FBB concentration, pH of the solution, modification time, time to dry the film after the modification), and the better way to record the image.

Several studies were performed after these initial results to achieve the best colorimetric response. The first one was based on the influence of the concentration of the FBB concentration in the modification step. However, when this study was performed, inconsistent results were obtained. It was not observed correlation of the analytical response with the FBB concentration. In addition, stability results showed that the chitosan gel was not stable over time and would create a color change in the absence of Δ9-THC. In addition, the chitosan gel needs to be in an acidic solution due to its capacity to dehydrate easily when exposed to air. Due to future storage incompatibility, the chitosan gel was discarded.

Super Adsorbent Polymer Assays

The second option to obtain a solid sorptive matrix capable of reacting with Δ9-THC was to use superabsorbent polymer hydrogels (SPH). SPH is a class of polymeric hydrogels that are gently cross-linked systems that have the unique ability to imbibe water a hundred times its dried weight. Due to their extraordinary water retention ability, such as excellent hydrophilic properties, high swelling ratio, biocompatibility, and abundance in availability, they have been widely used in many fields, like agriculture and horticulture, drug delivery systems, sanitary, tissue engineering, immobilization of protein and cells, and for wastewater treatment. The ability of SPH to absorb water arises from hydrophilic functional groups attached to the polymeric backbone, while their resistance to dissolution arises from cross-links between network chains. Many materials, both naturally occurring and synthetic, fit the definition of hydrogels.

SPH may be synthesized in many "classical" chemical ways. These include one-step procedures like polymerization and parallel cross-linking of multifunctional monomers, as well as multiple-step procedures involving the synthesis of polymer molecules having reactive groups and their subsequent cross-linking, possibly also by reacting polymers with suitable cross-linking agents.

In the one-step procedure, it could be synthesized in a relatively simple approach in which a monomer, a radical initiator, and a cross-linking reagent are mixed with an appropriate solvent in an O_2-free flask. The polymerization will start after a proper stimulus (it could be hot or light, depending on the radical initiator). The prepared hydrogels need to be washed with distilled water to remove the monomers, oligomers, cross-linking agents, the initiator, the soluble and extractable polymer, and other impurities. Phase separation occurs and the heterogeneous hydrogel is formed when the amount of water during polymerization is more than the water content corresponding to the equilibrium swelling.

Typical solvents used for solution polymerization of hydrogels include water, ethanol, water–ethanol mixtures, and benzyl alcohol. The synthesis solvent may then be removed after formation of the gel by swelling the hydrogels in water. A variety of monomers, mostly acrylics, are employed to prepare hydrogels. With different chemical composition, the polymer shows different sorption capacity, charge, and mechanical strength.

The synthesis's first step was adding 3 mL of methacrylic acid and 3 mL of acrylamide to the reaction flask under an inert atmosphere of nitrogen. Then, 0.20g of ammonium persulfate (APS) was added and stirred for 5 min with 0.25g of bis-methacrylate acid (MBA) cross-linking reagent. The mixture was stirred for 15 min, and then the solution was poured into 1 mL and 5 mL polypropylene syringes. The syringes were then placed in an oven at 65°C for 60 min. Then, they were removed, left at room temperature, and cut into 1 cm heights. The hydrogel cylinders were transferred to 1 L vials containing deionized water and left at room temperature to clean up the remaining unreacted monomers.

In this procedure, acrylamide and methacrylic acid act as monomer, MBA as cross-linking reagent and APS as radical initiator. Other polymers were synthetized in a similar procedure, but with different monomers, such as just methacrylic acid, just methacrylic acrylamide, just arylamide, the combination of 2, or all 3.

Not all the synthetized polymers show capacity to retain the dye, to be stable with time and to selectively react with THC after, but the polymer synthesized with 3 mL of methacrylic acid and 3 mL of acrylamide was stable.

The SPH has the chemical conditions to be used as solid support to pre-concentrate and produce a colorimetric response with Δ9-THC. However, as it needs to be maintained in water to keep its property, it does not have the mechanical characteristic to produce a stable and robust THC breathalyzer. Because of this, another solid phase needed to be studied.

3D Resin Assays using 3D Printer

3D Resin Development

As the solid support of chitosan and the super adsorbent polymer did not prove to be the most suitable solid surfaces to achieve our goal, a new study using additive manufacturing (3D printing) was initiated. Additive manufacturing can be defined as the "process of joining materials to make parts from 3D model data, usually layer upon layer, as opposed to subtractive manufacturing and formative manufacturing methodologies". Various technologies for 3D printing have been used consisting of fused filament fabrication (FFF), stereolithography (SLA), selective laser melting (SLS), inkjet and Polyjet printing, and laminated object manufacturing (LOM).

Due to its ease, versatility, and low cost, it was chosen to work with the technique of SLA. The main principle of this technique is that photo-curable resins are exposed to a laser and undergo a chemical reaction to become solid. The chemical reaction, called photo-polymerization, involves many chemical compounds such as photo-initiators, additives, and reactive monomers/oligomers. Usually, these materials are sold in a commercial product known as 3D printing resin.

A schematic representation of an SLS 3D printing is shown in Figure 3. A polymerizable resin (3) is located in the resin tank (9). The laser is located above (5) and with the help of an x-y scanning mirror (7) a laser beam (8) is formed and directed to the resin tank, where the material will be printed (1), layer by layer. In the end, the printed material is glued, upside-down, in the build platform (4). Usually, two other steps are needed, a wash step needed to remove the non-polymerizable resin attached to the material, and a photomechanical 3D cure step, to help finish the material.

Figure 3 – Schematic representation of an SLS 3D printer.

We hypothesized that mixing the Fast Blue dyes with a commercial 3D resin could be a reasonable reaction support for the colorimetric assay. The idea was to print the resin with the dye dissolved in it to create a physical reaction support. However, which dye, and which concentration would result in a better signal would need to be tested. To evaluate if the reaction with the dye in the resin would be possible, a preliminary test was performed. In our preliminary test, a solution was prepared by mixing 2 g of FBB in 100 mL of white 3D resin, adding 1 mL of NaOH 1 mol/L, and 1 mL of acetonitrile to facilitate the mix. This material was printed, and an addition of a drop of an ethanolic extract of Δ9-THC was added. The result is shown in Figure 4.

Figure 4 – Initial 3D printed support with FBB. The red color appears after the addition of one drop of a methanolic solution of Δ9-THC at 10 µg/mL.

This preliminary test showed that the use of a 3D resin is possible. However, it was necessary to obtain the best chemical composition ratio of this polymerizable mixture, especially the best concentration of the dye per mL of resin. In addition, it was necessary to evaluate the solubility of the dyes in the resins, trying to achieve the best relationship of solubility and color signal. To test the solubility, four resins were applied with different concentrations of Fast Blue dyes.

The Fast Blue dyes present low solubility in the resins, which are quite viscous, making mixing and homogenization difficult. Some strategies were applied to achieve better homogenization, such as hand stirring, vortex agitation, or ultrasound.

Influence of ultrasound *vs* vortex time

Ultrasound was applied to increase the homogenization of the dye in the resin before the printing process. To test the influence of the ultrasound, several solutions were prepared using the same procedure. The initial protocol for the preparation of the 3D resin consisted of weighing 2.8 g of dye in a 250 mL becker, followed by the addition of 150 mL of the Anycubic 3D white resin. After adding the resin, a steel spatula was used to stir the solution, in an attempt to achieve maximum homogenization. After stirring, the mixture was filtered, the remaining solid was discarded, and the solution was poured onto the 3D printer platform for printing the solid surfaces. For the ultrasound studies, the solution was prepared, however, instead of using spatula for handling homogenization, the flask containing the resin and the dye was transferred to a ultrasound water bath, where it was subjected to sonication for a predetermined period (15, 30, and 60 minutes). The best signal was obtained with 30 minutes of ultrasound homogenization. Despite the good signal, the homogeneity of the solution was visually not good enough, which could be the reason for the high standard deviation.

The homogenization of the mixture was also evaluated when performed using a vortex instead of an ultrasound. The goal of this experiment was to evaluate if the vortex could influence the solubilization and homogenization of the resin and dye. As this experiment needed to be performed in a polypropylene tube, the amount of resin and dye was decreased to adapt to the new volume. For this procedure, 500 mg of the dye was weighed inside a propylene tube, then 25 mL of the resin was transferred. The mixture was subjected to vortex homogenization during the studied time (5, 10, and 20 min). After vortexing, the mixture was filtered and used to print the pieces. After printing, an aliquot of 10 µL of Δ9-THC 100 µg/mL was added. The image was recorded with a smartphone, and the ImageJ software was used to transform the image into an analytical signal. The use of a vortex to homogenize the mixture was not successful, as a longer vortex time caused a decrease in the analytical signal.

Influence of Nature and Dye Concentration

As previously described, the Fast Blue family are common dyes applied in forensic laboratories for the analysis of seized drugs. The vast literature about these dye families and the chemical specificity of the reaction led to the application of this revelation system for the colorimetric device.

In this system, the dye needs to be sufficiently soluble in the resin, stable, to resist the printing process, to generate homogenous pieces, be able to react with THC, and to produce a reproducible colorimetric reaction that behaves according to the Beer principles. To achieve the best results, the initial study was based on the concentration of the dyes chosen and their effects on the color signal formed. As previously described, the dyes of choice were Fast Blue B (FBB), Fast Blue BB (FBBB), and Fast Blue RR (FBRR).

For the assay, FBB, FBBB, or FBRR were weighed (0.25g, 0.50g, 0.75g, 1g, and 1.5g) in a 50 mL polyethylene tube, followed by the addition of 25 mL of the white 3D Anycubic resin resulting in different resin/dye solution at 1%, 2%, 3%, 4%, and 6%, respectively. Mixtures were homogenized, filtered, and used to print a solid material in the format of strips, with the dimensions of 1 cm h x 2 cm w x 3 cm l. For each experiment, a set of seven strips was printed. Then, the strips were washed for 10 minutes with ethanol, dried with paper prior to use.

After the strips were dried, 10 µL of 1000 µg/mL solution of Δ9-THC was added to the surface of the strip. After a few seconds, the reaction was completed, and the change of color was recorded. A flat scan was applied to register the image. The software ImageJ was used to measure the color intensity before and after the THC addition. The obtained value was used to calculate the analytical signal, analogous to the absorbance signal (A= -log P/P0), where A is the analytical signal, P0 is the color intensity of the strip before the THC addition, and P is the color intensity of the strip surface after the THC addition. The results were plotted as a curve of Analytical Signal x Fast Blue concentration for each fast blue used (B, BB, and RR), and are shown in Figure 5.

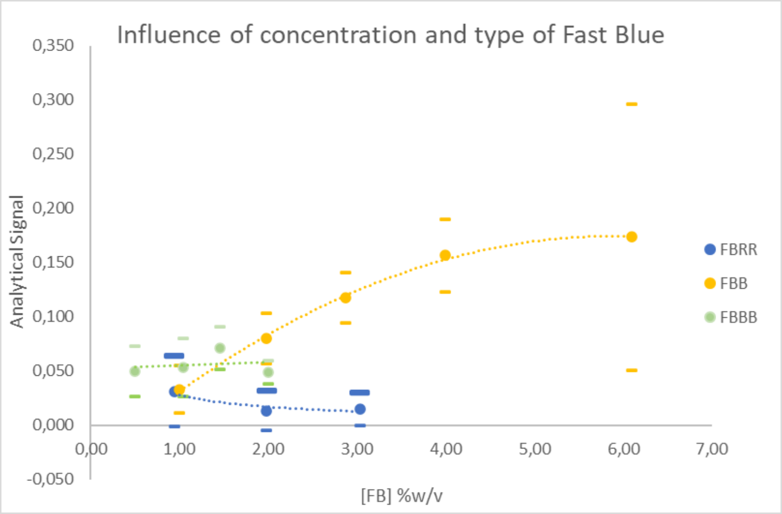


Figure 5 - Study of the influence of nature and concentration of the Fast Blue dyes on the analytical signal obtained for the same THC concentration.

The best analytical signal was obtained using the FBB dye at 4% w/w concentration. Above these concentrations, there was no significant increase in the analytical signal, with an increase in the standard deviation, showing higher variation. The dyes FBBB and FBRR did not show any improvement or better results than the ones obtained using FBB; thus, the dye of choice was FBB at 4%.

To increase the response and improve the solubility of the dyes in the resin, other commercially available resins were introduced as possible sorptive supports. Before, the resin applied was the 3D AnyCubic Resin, the one suggested by the printer manufacturer.

For the resins study, five different resins were evaluated. The information about the resin, brand, color, and composition is described in Table 1. The 3D Anycubic was added to this study for comparison reasons.

Table 1: 3D Resins chosen for the study.

Short name	Brand	color	composition

Creality	Creality Plant-Based 3D Printer Resin	Pearl White	PLA-Based Resin
Siraya	Siraya Tech Tenacious 3D Printer Resin Flexible 405nm UV-Curing Resin with High Impact Resistance Standard Photopolymer Resin for LCD DLP 3D Printing	Transparent	not informed
Anycubic	ANYCUBIC Water Washable Resin, 3D Printer Resin with Low Viscosity	Transparent	not informed
Elego	ELEGOO 3D Printer Resin 405nm LCD Fast UV Curing Standard Resin Photopolymer Resin High Precision Non Toxic Low Odor for DLP/LCD 3D Printing Translucent	Transparent	not informed
Sunlu	SUNLU 3D Printer Resin ABS Like Resin 1000g,	Transparent	ABS-Like

For this study, all resins were printed using the same methodology previously described for the 3D Anycubic. The FBB was added in different concentrations, and the solubility of the dye in the resins was evaluated. The washing process of each resin was respected. The advantage of using the 3D AnyCubic was the fact that it can be washed with water only, differently of the other resins, where ethanol must be added to the washing process. The resin Sunlu was discarded at the beginning of the process as the dye was not soluble in it at all. To each piece, 10 µL of a Δ9-THC solution at 50 µg/mL. Figure 6 shows the solubility of the FBB at different concentrations in different resins.

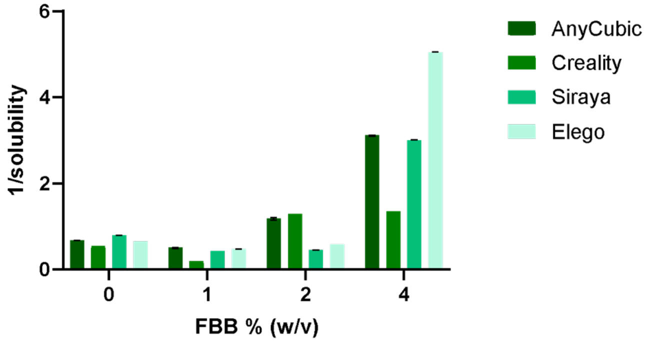


Figure 6 - Study of the solubility of FBB at different concentrations in four commercially available resins.

The Creality resin with FBB at 1% showed the best solubility and the lowest overall interference. However, when building the cartridges using this set, the cartridges developed color after hours at room temperature, even in the absence of Δ9-THC. In addition, due to the difficulty of homogenizing the dye in the resin (even considering the previous homogenization results), the presence of the dye in the composition of the cartridge was not uniform, showing parts where the cartridge possibly had higher concentrations of the dye and vice-versa, as shown in Figure 7.

Figure 7 – Cartridge printed using the Creality resin with FBB at 1%, showing the lack of homogenization of the presence of the dye. The color formed here is not a result of the reaction with Δ9-THC, showing the instability of this proposed set.

Considering the difficulty in developing a matrix where the dye would be soluble in the cartridge, the next option would be to consider the cartridge as the reaction support and not a piece of the reaction set.

Dye cover suitability

Dye directly deposited in the cartridge

For the evaluation of the reaction using the dye as a cover layer, the resin was printed as described in the manual. For these assays, the 3D AnyCubic returned as it requires just water in the washing process, making the production of the cartridge less toxic. For that, 20 μL of the colorimetric reagent was added after the cartridge was printed. This reagent was prepared using a variable amount of FBB salt with 1% (w/w) anhydrous sodium sulfate, which was diluted with 10% (w/w) sodium bicarbonate, dissolved in deionized water or methanol, and finally applied to the substrate and allowed to dry at room temperature. The sodium bicarbonate was used as the dye needs a basic environment to achieve a better color.

This system was not chosen due to the difficulty in spreading the dye homogenously on the top of the cartridge, making parts of the surface of the cartridge present different concentrations of the dye, which would make the reaction system inconsistent. To solve this problem, an addition of a reaction layer on the top of the cartridge was suggested.

Dye dissolved in agar

The addition of the agar layer was designed to create a top layer on the cartridge for best homogenization of the dye, allowing a more uniform reaction system. For that, 20 µL of the colorimetric reagent was added to a solution of agar 1.5%. The solution was added to the top of the printed cartridge and let rest for solidification. This reagent was prepared using a variable amount of FBB salt with 1% (w/w) anhydrous sodium sulfate, which was diluted with 10% (w/w) sodium bicarbonate, dissolved in deionized water or methanol, and finally applied to the agar solution. The sodium bicarbonate was used as the dye needs a basic environment to achieve a better color.

This system showed good homogeneity and acceptable spreading of the dye over the surface of the cartridge- However, due to the nature of the agar, mold was formed on the top of the cartridges stored at room temperature for one week, making the use of agar not a good option, considering the shelf-life of the final product.

Cartridge covered with ballistic gelatin

For this setting, the solution preparation was similar to the agar preparation, but adding the dye into the ballistic gelatin solution. The uniform layer using the ballistic gelatin was the best setting as it allowed an uniform distribution of the dye, and was able to be stored at room temperature for months without any alteration. Figure 8 shows the three systems after the addition of 10 µg of Δ9-THC.

3D Cartridge with FBB 1.5% cover layer

3D Cartridge with Agar layer + FBB 1.5%

3D Cartridge with Ballistic Gelatin layer + FBBB 2%

Figure 8 – Cartridge printed using the 3D AnyCubic resin with a direct cover layer of FBB at 1.5%, an agar layer with FBB at 1.5%, and the ballistic gelatin layer with FBBB at 2%. The choice of FBB 1.5% for the first settings was due to the pursuit of increasing the signal, as the solubility was not a problem anymore, and FBB at 1.5% showed a good signal. This set was also tested with ballistic gelatin, but the file with the picture was corrupted after a computer issue. The use of FBBB 2% in the ballistic gelatin was developed to improve the differentiation between CBD and Δ9-THC, as FBBB fluoresces in the presence of Δ9-THC but not CBD.

Due to stability, the good signal, linearity, and the possibility of improving the capacity to differentiate between Δ9-THC and CBD, the setting with ballistic gelatin with FBBB at 2% was chosen as the final cartridge design for the breathalyzer prototype.

Dye Concentration Optimization

Samples with varying Fast Blue B Dye concentrations at 1.5%, 2%, 2.5%, 3%, 4%, 5%, and 7% (w/v) were prepared and scanned using a flatbed photo scanner and Not Another PDF Scanner (NAPS2) scanning software. They were analyzed using the ImageJ software. In ImageJ, the images of the discs were processed using the "Histogram" function. An elliptical selection was made around the centroid of the substrate and recorded using the average of the green RGB channel. The discs were then treated with 50 ng THC (25 µL of 2 µg/mL THC).

At this moment, due to the different approaches concerning the colorimetric reaction, our team decided to also perform some tests using the Fast Blue BB dye. The use of FBBB relies on the fact that it fluoresces when in contact with THC, and it could improve the signal. The use of FBBB was discarded due to this lack of solubility in the resins. However, with this different approach to creating a cover layer, the solubility in the resin is irrelevant.

Samples with varying Fast Blue BB Dye concentrations at 1%, 2%, 3%, 5%, and 7% (w/v) were prepared & scanned under the same conditions. The discs were analyzed using the same ImageJ software techniques. The signal differences between the blank samples and those treated with 50 ng of THC were evaluated. Statistical analysis was performed using GraphPad.

The largest average signal difference observed with the Fast Blue B dye was 7.419 with the 5% FBB + Gel medium (Table 2). However, statistical differences were observed amongst all concentrations of Fast Blue B dye ($p < 0.05$).

Table 2. Summary of average signal differences between blank discs containing Fast Blue B dye at varying concentrations and discs treated with 50 ng THC.

CONCENTRATION FBB (%w/v)	SIGNAL DIFFERENCE	P-VALUE (n=3)
1.5	0.1607	<0.001
2	2.852	<0.001
2.5	2.580	<0.001
3	6.727	<0.001
4	6.716	<0.001
5	7.491	<0.001
7	5.926	<0.001

The largest average signal difference with the Fast Blue BB dye was 11.79 with the 5% Fast Blue BB + Gel medium (Table 3). However, there was a statistical difference between the two replicates. Statistical similarity was observed between the 2 replicate discs containing 2% Fast Blue BB solution, which output a mean signal difference of 9.06. Therefore, the subsequent variability test was conducted with 10 discs composed of a 2% Fast Blue BB + ballistics gel medium.

Table 3. Summary of average signal differences between blank discs containing Fast Blue BB dye at varying concentrations and discs treated with 50 ng THC.

CONCENTRATION FB BB (%w/v)	SIGNAL DIFFERENCE	P-VALUE (n=2)
1	5.995	<0.001
2	9.059	0.04
3	10.89	<0.001
5	11.79	<0.001
7	11.60	<0.001

Variability Study

Discs (10) containing 2% (w/v) Fast Blue BB & ballistics gelatin were scanned under the same conditions as above. The discs were treated with 20 ng of THC at room temperature and allowed to rest for 30 minutes. The treated discs were then scanned under the same conditions. These images were analyzed using ImageJ. The differences in signal intensities were calculated, and statistical analysis was performed using GraphPad to assess the variability between discs with identical treatment under identical conditions.

During the study of variability, the smaller difference between the signals means that the reaction is stable and is directly proportional to the amount of THC added to each disc. The nine discs evaluated were measured, and the results are shown in Figure 9.

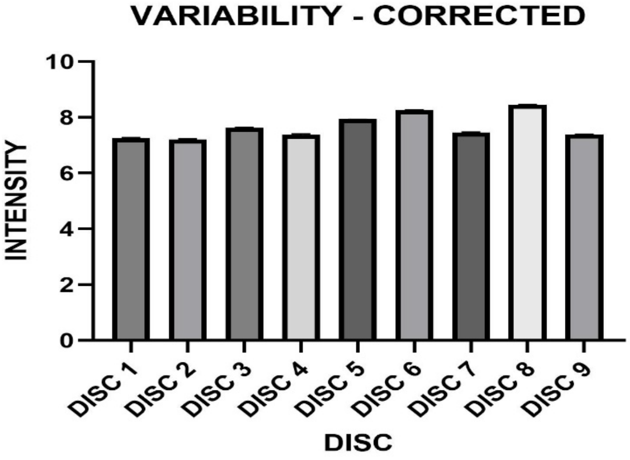


Figure 9 - Demonstration of the average signal intensity for 9 discs. Disc 10 was unable to be analyzed due to an error during the scanning process.

Linearity Study

Discs (5) containing 2% Fast Blue BB & ballistics gelatin were prepared. These discs were scanned and analyzed using ImageJ. The discs were treated with THC at an increasing concentration of 10 ng, 25 ng, 50 ng, 75 ng, and 100 ng.

A calibration curve was prepared using the 2% FBBB in ballistics gel to evaluate the relationship between THC concentration and signal, as, until the present report, the ballistic gel with FBBB 2% has been shown to be the most stable and reliable combination for the commercial cartridge. Figure 10 shows the calibration curve using THC ranging from 0 to 100 ng vs the signal obtained.

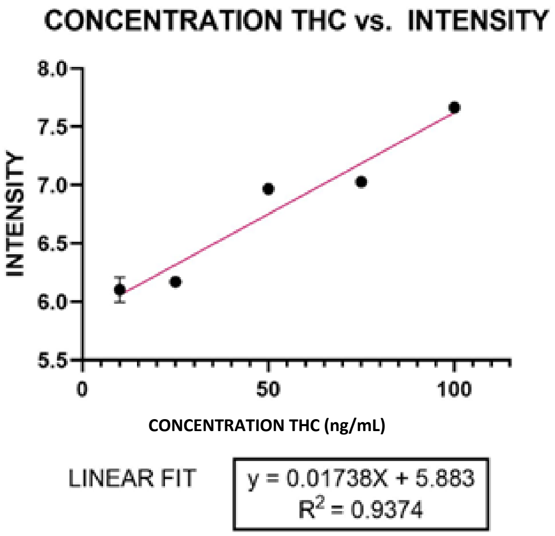


Figure 10 - Linear depiction of the relationship between the signal differences measured by the green RGB channel and the concentration of THC in the 2% Fast Blue BB + Gel medium.

The result shows good linearity and a direct relationship between the concentration of THC and the signal obtained.

Additional Studies with FBB

Due to its capacity to fluoresce and its linearity, FBB at 2% in ballistic gelatin was the support material of choice for the foundation system of the breathalyzer. However, further studies were conducted using similar systems to guarantee that the system of choice would be the combination of the qualities of all materials (the dyes and the reaction support).

Studies using 5% of FBB in ballistic gelatin were performed in parallel as our group insisted on trying to use FBB due to previous data. Data resulted from this additional experiment showed that the variability of the results in this system is low as shown in Figure 11, and it could be an important alternative to the final product.

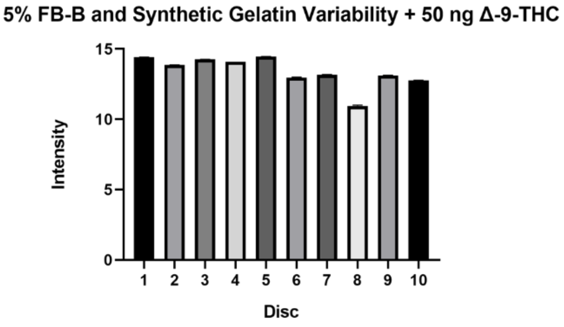
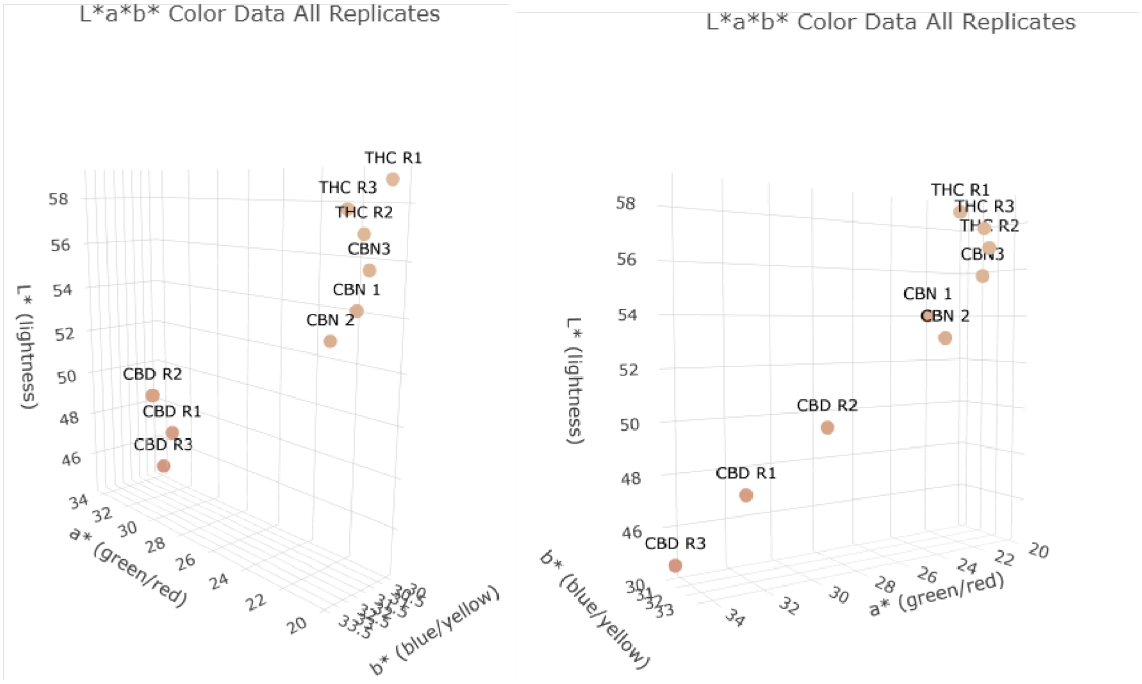


Figure 11 - 5% FB-B and Synthetic Gelatin Variability 50 ng Δ-9-THC.

Overall, the variability of this technique with 5% FB-B and 50 ng Δ-9-THC was much lower than that of previous techniques using a visual comparison method. A one-way ANOVA test was performed (P<0.0001, F=7368). As this is a colorimetric test, variability is expected. Further testing may be performed to see if the variability between replicates can be reduced. Overall, this method has provided strong intensity results with moderate variability based on a visual comparison.

In addition, using FBB at 5% in ballistic gelatin allowed to separate Δ-9-THC from CBD using the hue system, which is not dependent on the fluorescence as shown in Figure 12. This is an important option as optical systems based on fluorescence are usually more expensive to be developed when compared to systems based on visible light.


L*a*b* Color Data All Replicates

L*a*b* Color Data All Replicates

Figure 12 - 3D Plot of L*a*b* Values of 1 mg/mL THC, CBD, and CBN with Synthetic Gelatin and 5% FB-B.

Within a L*a*b* color space, the L* values represent the luminance, or brightness, of the image. a* values represent the green and red color space, and b* the blue and yellow color space. The CBD points are clustered with higher values, a* values, and low L* values, which. The THC and CBN replicates have much larger L* values, and lower a* values. All three cannabinoids have relatively consistent b* values. CBN is a breakdown product of THC. This explains the colorimetric similarities between the two cannabinoids. THC and CBN being detectable together is acceptable, as both are psychoactive to some degree. The separation of CBD was the main goal, as CBD is not psychoactive. Preliminarily, the psychoactive cannabinoids were able to be separated in a 3D space from the non-psychoactive cannabinoids. This is a strong first step towards effective cannabinoid differentiation using a colorimetric system. Further analysis needs to be completed to determine at what concentration the cannabinoids can no longer be differentiated from one another.

In the end, cartridges could be based on FBBB at 5% or FBB at 2%, depending on the detection system developed. Current studies are being performed to evaluate if both systems could be conjugated under the same cartridge to maximize the analysis as FBB shows low limit of detection, decent variability, and hue differentiation, while FBBB allows highest linearity, decent variability, low detection limit, but lower selectivity.

Findings

This project resulted in the establishment of the foundational chemical profile needed for the development of a THC breathalyzer. Due to its excellent results, a patent application number PCT/US25/19696 was submitted to the United States Patent and Trademark Office. Figure 13 shows the draft of the future prototype to be developed.

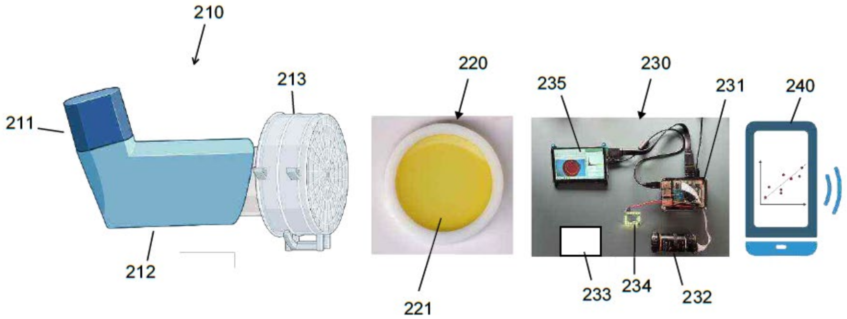


Figure 13 – Schematic of the developed prototype.

In addition to the chemical foundations developed during this project, our team also worked on the development of a detection system for the analysis of the colors obtained by the reaction system.

For the production of a successful prototype, three main pieces must be developed. The first part is the mouthpiece where the exhaled air will be collected. The idea is to create an air collector similar to a spacer, usually connected to inhalers for asthma treatment. In the prototype, the cartridge will be fixed at the end of the spacer where the Δ9-THC particles will collide and react. The second part is the cartridge itself, already discussed above. The third part is the detection system. For the evaluation of the Δ9-THC concentration, a colorimetric reaction occurs in the presence of the Δ9-THC molecule. The formation of a dark red color results in a positive for Δ9-THC. Like most of the color reactions, the reaction between Δ9-THC and Fast Blue dyes obey the Lambert-Beer Law, and the amount of color formed is directly proportional to the intensity of the color formed. To acquire the results, we are currently using optical scanners to obtain the image, which is subsequently treated and analyzed using the open software from NIH called ImageJ. Several approaches were tested for the acquisition of the image, such as using a ballistic microscope, photographic cameras (both portable and from cellphones), and table scanners. The image acquisition method that resulted in less shadow or color scheme variation was the table scanner. As the goal was to obtain a portable system, a table scanner coupled with a computer was not the best solution. Thus, our team developed a homemade portable image-acquiring system based on micro cameras and a Raspberry Pi where the Image J software is installed (Figure 14). In addition to the challenges of acquiring a high-quality image, as the ImageJ coding is open, our team was able to program some specificities in the software for the standardization of cartridge imaging analysis, consequently leading to better imaging processing.

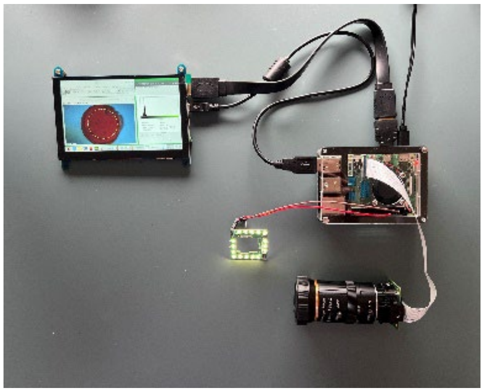


Figure 14 - Homemade detection system using Raspberry Pi and ImageJ coupled with a portable camera.

Implications for Criminal Justice and Practice in the United States

The consequences and legal penalties for driving under the influence (DUI) of alcohol are well publicized. However, what the general population ignores is the illegality of DUI of marijuana, in combination or not with other drugs. Laws defining what marijuana DUI means vary by state, as do applicable punishments. With the legalization of cannabis consumption in many parts of the USA, this lack of information from the general population has increased the risk of accidents and deaths caused by drivers intoxicated with marijuana. Due to the difficult and costly current methods available for the detection of marijuana in samples, it is vital to create and improve technologies for its detection in a fast, reliable, and in situ method for public safety and awareness. Recently, breath analysis has gained significant attention as a non-invasive method for the detection of chemicals or mixtures of chemicals in exhaled breath, but noncommercial dispositive for marijuana based in situ results, such as in alcohol breathalyzers, are not available. The outcome of this project is the fundamental chemical foundation and the initial prototype for the development of a Δ9-THC breathalyzer. The device developed in this project is a portable tool to increase safety on the roads and for Δ9-THC use monitoring.