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Optimization and validation of the fast blue BB (FBBB) screening test for the detection of delta-9-tetrahydrocannabinol (Δ9-THC) in oral fluid

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ABSTRACT

The fast blue BB test (FBBB) colorimetric test has been previously validated for the differentiation between marijuana-type and hemp-type cannabis plants. Individuals under the influence of delta-9-tetrahydrocannabinol (Δ9-THC) may be impaired to perform activities such as driving and the rapid, on-site detection of recent use of Δ9-THC could help to prevent traffic accidents. The current study describes, for the first time, the optimization and validation of the FBBB color test from oral fluid (OF) for field use. The FBBB reaction with Δ9-THC forms a red chromophore and a bright red fluorophore that is visualized with a portable fluorescence reader under UV light. The test was optimized for a miniaturized reaction on a 6.3 mm diameter glass fiber filter spot. The limit of detection (LOD) was established as 0.5 μg/mL or 500 ng/mL of Δ9-THC in OF (5 ng of Δ9-THC on the spot) by fluorescence detection. Other figures of merit include linearity in the 0.5–10 μg/mL range, acceptable precision (9.6–28% RSD) and an accuracy of ± 23.2–56.2%. Results from interference studies using different OF collection devices and substances are reported. Interferences might also occur when minor cannabinoids (CBD, CBN, CBG, and Δ8-THC) and Δ9-THC metabolites (THC-COOH and THC-OH) are also present in the matrix. Pre-loaded FBBB reagent is stable on the substrate when stored below 4 °C for 15 days and the color and fluorescence persist on the spot for at least 30 days post reaction. Future studies include development of an OF extraction procedure coupled to a prototype device for field application.

Introduction

According to the most recent World Drug Report [1] in 2019 the global number of cannabis users was estimated at >200 million. While there has been the legalization or decriminalization of the recreational use of marijuana in different countries such as Canada, Uruguay, and many states in the United States [1], marijuana remains illegal in most countries in the world and is considered a schedule I drug at the federal level in the USA. Cannabis plants are classified as marijuana-type, containing ≥ 0.3% w/w of delta-9-tetrahydrocannabinol (Δ9-THC) - the main psychoactive compound in the plant, or as hemp-type, containing mainly cannabidiol (CBD), and < 0.3% w/w of Δ9-THC [2]. Thus, there is an increase of commercial hemp-type cannabis consumables [3,4] that undergo regulation and require a quality control process in terms of the Δ9-THC content in the plants.

Individuals under the influence of Δ9-THC may be impaired for driving [5], working [6], or participating in sports competitions [7]. The screening of individuals under possible drug influence in those situations requires a reliable and low-cost field test that allows for the detection of recent intoxication. Oral fluid (OF) is a biological matrix that can be sampled for this purpose as it can reflect very recent (minutes to hours) drug use [8] unlike urine or blood. In addition, OF collection can be easily performed in the field in a non-intrusive manner, dispensing with the need for a trained technician required for blood collection, for example [9]. Immunoassay-based field detection tests are used in some countries for the identification of Δ9-THC in OF [10–14]. However, drawbacks have been reported with these tests including variability between different brands on the market for detection cut-offs, sensitivity, specificity, and accuracy, interferents and device usability including testing time and reading failures [15]. This study aims to demonstrate the utility of an alternative fluorometric-based reaction previously reported by our research group for the rapid, sensitive, and cost-effective detection of Δ9-THC in OF at concentrations that are indicative of recent drug use.

The fast blue BB (FBBB) test is a presumptive test that has been recently validated for the differentiation of marijuana-type and hemp-type cannabis in plant extracts on a miniaturized substrate [16]. Due to the extended conjugation of π-bonds in the reaction between FBBB

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and Δ9-THC in marijuana-type cannabis samples, a visible red chromophore can be observed [16,17]. Additionally, the reaction also produces a fluorophore [16] that enhances the selectivity and sensitivity of the test. The reaction between FBBB and CBD from hemp-type cannabis samples produces a visible orange color but no fluorescence [16].

The goal of this research was to optimize and to validate the FBBB test for the analysis of OF samples using a miniaturized substrate for Δ9-THC detection. The OF matrix presents some challenges when compared to cannabis plant extracts using organic solvents. OF composition (e.g., water, proteins, electrolytes, cells, and oral cavity bacteria) makes it a viscous and complex matrix [18] that could interfere with the chemical reaction between Δ9-THC and FBBB. OF collection can be performed either by passive drooling or using a commercial collection device [19]. Those devices include a swab-based pad that it is placed in the mouth, and then the pad is placed into a tube containing a buffer that helps with OF viscosity and preservation [18,19]. Many different OF collection devices are available in the market for drugs of abuse detection [20,21] and different buffers composition should be studied as possible interferants to the FBBB reaction. Another challenge to the translation of the FBBB test to the OF matrix is the Δ9-THC concentration. Cannabis plant extracts contain high concentrations of Δ9-THC, while in OF Δ9-THC concentrations can be as high as 1000 ng/mL after recent smoking of a marijuana cigarette and it can be as low as 1 ng/mL after a short period of time [22,23]. These scenarios were considered when the analytical parameters are optimized for the FBBB test application in OF.

The FBBB test optimization in OF included the choice of an adequate substrate for the reaction, determination of a limit of detection (LOD), evaluation of potential interferences, and stability studies. The semi-quantitative analysis of Δ9-THC in OF using the fluorescence generated in the reaction with FBBB was explored using a portable fluorescence reader. A calibration model, linearity, precision, and accuracy were evaluated as figure of merit for this instrument.

Materials and methods

Materials

Methanol (MeOH), Fast Blue BB (FBBB) Salt hemi (zinc chloride), and caffeine standard were purchased from Sigma Aldrich (St. Louis, Missouri, United States). Sodium hydroxide (NaOH) was purchased from Macron Fine Chemicals (Radnor Township, Pennsylvania, United States). Glass fiber filter G6 circles, Cytiva Whatman™ 1PS Disposable Phase Separating Paper (silicon treated filter paper), and Cytiva Whatman™ Grade 3 Qualitative Filter Paper Standard Grade were purchased from Fisher Scientific (Hampton, New Hampshire, United States). Methanolic solutions (1 mg/mL) of Δ9-THC, CBD, cannabinol (CBN), cannabigerol (CBG), delta-8-tetrahydrocannabinol (Δ8-THC), 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH), and 11-hydroxy-delta-9-tetrahydrocannabinol (THC-OH) were purchased from Cerilliant Corporation (Round Rock, Texas, USA). Synthetic OF (OraFlx®) was purchased from Dyna-Tech Industries (Lenexa, Kansas, USA). Quantisal™ and Intercept® OF collection devices were purchased from Immunalysis Corporation (Pomona, CA, USA) and OraSure Technologies (Bethlehem, PA, USA), respectively. Pure liquid nicotine (99.99%) was purchased from Freedom Smoke USA (Tucson, AZ, USA). Sodas, juices, teas, and beer were purchased in local stores in Miami, FL, USA. Guarana powder was purchased in Sao Paulo, Brazil.

Reagent preparation

The FBBB solution (1 mg/mL) was prepared by dissolving 10 mg of FBBB salt into 10 mL of MeOH. Since the solution of FBBB is sensitive to light [16] it was kept in the freezer (-20 °C) in an amber vial covered in aluminum foil for no longer than 40 days. The NaOH 0.1 N solution was prepared by dissolving 0.4 g of NaOH into a 100 mL of MeOH. The solution was kept in the refrigerator (4 °C) for no longer than 40 days.

Oral fluid collection and sample preparation

A commercial synthetic OF (OraFlx®) was tested as a potential matrix for this research. However, the results from Δ9-THC diluted in OraFlx® and FBBB reaction were not satisfactory. Instead, an Institutional Review Board (IRB) was obtained from Florida International University (IRB-22–0189) for human OF collection. All participants signed a consent form to donate OF as a blank matrix for this study, but no other personal information was collected. All participants were requested to not eat or drink anything for at least one hour prior to the collection.

The OF collection was performed by simply spitting (1–2 mL) into unidentified sterilized containers, involving at least eight different donors (males and females individuals above 18 years old) as needed. All samples were mixed, and the OF mix was kept frozen (-20 °C) in 2 mL aliquots until use. Prior to analysis, the OF was thawed and centrifugated at 3200 rpm for 5 min (Clay Adams® Brand Compact II Centrifuge, model 420225).

OF collection was also performed using Quantisal™ and Intercept® commercial collection devices. The Quantisal™ device provides a pad to be placed inside the mouth with a blue indicator when the pad absorbs 1 mL of OF. After the collection, the pad containing the OF was placed inside a tube containing 3 mL of buffer. The Intercept® device also provides a pad, with the recommendation of placing it on the cheek for 5 min. After collection, the pad is placed inside a tube containing 2 mL of buffer. For each device, samples from 9 different donors were mixed and kept frozen (-20 °C) in 2 mL aliquots until use. Since the collection devices buffers help with OF viscosity and debris, no centrifugation was performed in these samples.

The OF mix or the OF in buffer mix were submitted for the reaction with FBBB and NaOH on the miniaturized substrate. After confirming that no color or fluorescence was observed in the mix, standard working solutions of Δ9-THC and other cannabinoids/metabolites were prepared from 1 mg/mL stock solutions at different concentrations for the experiments.

Fast blue BB testing procedure

The FBBB testing procedure was optimized from the procedure previously described for cannabis plant extracts [16].

A 6.3 mm square spot of glass fiber filter was cut with a paper hole puncher and placed inside aluminum covered wells in a plastic tray. This procedure allowed for each sample to accommodate the reagents in a spot and to dry without cross contamination. The glass fiber filter spots were pre-loaded with 10 μL of FBBB 1 mg/mL and they were allowed to dry for 10 min. Then, 10 μL of blank or spiked OF sample was pipetted on the substrate, followed by 10 μL of the NaOH 0.1 N solution. It is possible to observe color from the reaction between FBBB and the cannabinoids/metabolites within 1–5 min, even before adding the NaOH. However, adding the NaOH was necessary to observe the fluorescence from the FBBB and Δ9-THC reaction. The spots were allowed to dry overnight, covered from light exposure. All the FBBB tests were performed on five replicates of OF and the experiments were performed at least twice over two different days.

Color and fluorescence analysis

The spots color was photographed using a Dino-Lite® AM4115ZT (R9) digital microscope (Dunwell Tech, Torrence, CA). The spots fluorescence intensity was captured and photographed using a Dino-Lite® AM4115T-GRFBY Digital Microscope (Dunwell Tech, Torrence, CA), which uses a 480 nm excitation light source, and emission filters of 510 nm and 610 nm. The software ImageJ [24] was used to collect the red, green, and blue (RGB) scores from fluorescence images.

A portable fluorescence reader device (Dianulox®, Stockach, Germany) was used to capture fluorescence from the spots, using a 365 nm

excitation filter with detection at 625 nm. The instrument was optimized to perform four readings (of 1 mm each) in different positions of the spot, with a LED current of 45/65. An intensity peak is generated from the average of the four readings for each sample (Fig. 1). The highest absolute height peak was considered as the fluorescence intensity for each sample.

A Visual Spectral Comparator 2000 (VSC2000) (Foster-Freeman, England) was used to confirm the LOD obtained by the fluorescence analysis with the Dianulox® portable fluorescence reader device. A spot filter was used on the sample spots to irradiate light with excitation filters between 400 nm and 540 nm. The long pass filter was set at 590 nm. The samples were scanned from 590 nm to 1000 nm for 0.2 s for fluorescence emission.

Method optimization and validation

Initially, the substrate optimization was performed for the OF matrix by testing different materials as a support for the FBBB reaction, evaluating color and fluorescence visualization.

With reference to the Standard Practices for Method Validation in Forensic Toxicology [25], the limit of detection (LOD), interference, and stability studies were performed as figures of merit to validate the FBBB test as a screening test for the detection of Δ9-THC in OF. These parameters were validated for the fluorescence captured from the reaction between Δ9-THC and FBBB using the Dianulox® portable fluorescence reader. The results were confirmed by the Dino-Lite® digital microscope with imaging and RGB scores. Additionally, a calibration model, linearity, precision, and accuracy were also evaluated for the fluorescence captured with the Dianulox® portable fluorescence reader for a semi-quantitative method validation using this instrument.

Different concentrations of Δ9-THC (100 μg/mL, 50 μg/mL, 20 μg/mL, 15 μg/mL, 10 μg/mL, 5 μg/mL, 3 μg/mL, 1 μg/mL, and 0.5 μg/mL) were diluted in OF and then tested using the FBBB reaction. Considering that 10 μL of the OF solution containing Δ9-THC were applied on the spot, these concentrations represent in total mass on the spot 1000 ng, 500 ng, 200 ng, 150 ng, 100 ng, 50 ng, 30 ng, 10 ng, and 5 ng of Δ9-THC, respectively. The LOD was defined as the lowest concentration of Δ9-THC in OF that would show fluorescence distinguishable from the blank. Using this experiment, a calibration curve model was built using the average of absolute height peaks for fluorescence captured with the Dianulox® portable fluorescence reader with ten replicates of each Δ9-THC concentration. The instrument's accuracy was calculated using the linear regression equation from the calibration curve, and the results were considered adequate within values ≤ 20%.

Intra and inter-day precision were evaluated by testing low, medium, and high concentrations of Δ9-THC (3 μg/mL, 20 μg/mL, and 50 μg/mL) in OF, subjected to the FBBB reaction. Five replicates of each concentration were used to calculate intra-day precision. This experiment was conducted over five different days to allow inter-day precision estimation. The results were considered adequate within a relative standard deviation (%RSD) of ≤ 20% at each level.

Interferences studies were performed by testing different cannabinoids (CBD, CBN, CBG, Δ8-THC), metabolites (THC-COOH and THC-OH), and different substances that could be possibly present in the oral cavity of individuals subjected to the FBBB test, such as caffeine, nicotine, sodas (Coca-Cola®, Guarana Antarctica®), teas (green tea and black tea), orange juice, and alcoholic drinks (Corona® beer, Absolut® Vodka, and Jack Daniels® Whiskey). Previously, guarana powder, a Brazilian fruit extract, was described as an interferent to the FBBB test in cannabis plant extracts, for producing a red color [26]. For this reason, both guarana powder and guarana Antarctica® soda were tested as possible interferents to Δ9-THC and FBBB reaction in OF.

All minor cannabinoids and metabolites standards were prepared individually in high concentrations (25 μg/mL solution in OF) and in a mix containing a high concentration (25 μg/mL) of each cannabinoid/metabolite and a high concentration (25 μg/mL) of Δ9-THC. All commercial beverages were diluted in OF (50:50, beverage: OF). The teas were prepared as recommended in the package, brewing the tea bag into a 200 mL cup of hot water. After cooling, the teas were also diluted in OF (50:50, tea: OF). Caffeine, nicotine, and guarana powder were prepared as a 25 μg/mL solution and then diluted in OF (50/50). Additionally, a high concentration (25 μg/mL) of Δ9-THC was also prepared by diluting it into solutions containing each substance previously diluted in OF (50/50). Using this strategy, we could identify if the substances would interfere by providing red color and/or fluorescence in the reaction with FBBB and if they could prevent the Δ9-THC reaction with FBBB. The interference studies were evaluated with the Dianulox® portable fluorescence reader and by analyzing the visual color and the fluorescence intensity captured with the Dino-Lite® digital microscopes.

Stability studies were conducted to evaluate for how long the color and the fluorescence could be observed on the spots containing the Δ9-THC and FBBB chromophore. Three different concentrations of Δ9-THC (3 μg/mL, 20 μg/mL, and 50 μg/mL) in OF were prepared for the FBBB reaction and dried overnight. Then, the fluorescence intensity was measured with the Dianulox® portable fluorescence reader and images were taken with the Dino-Lite® digital microscope. The samples were then kept in a drawer at room temperature covered in aluminum foil to be protected from light. The fluorescence intensity was measured again with the portable instrument and new images were taken at 15 and at 30 days after sample preparation.

Stability was also evaluated for the pre-loading of FBBB on the substrate, prior to the reaction. For this experiment, spots were pre-loaded with FBBB and kept under three different conditions: at room temperature (25 °C), in a refrigerator (4 °C), and in a freezer (-20 °C). After 15 days, three different concentrations of Δ9-THC (3 μg/mL, 20 μg/mL, and 50 μg/mL) in OF were prepared for the FBBB reaction and

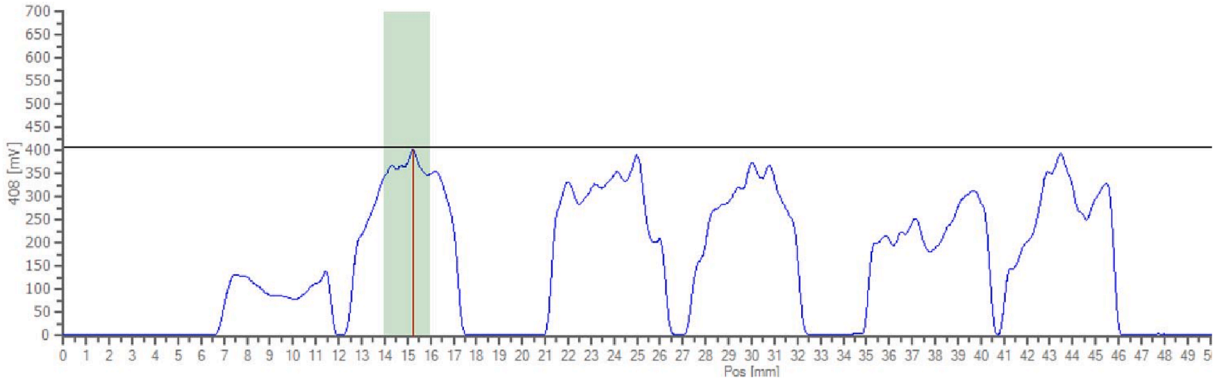

Fig. 1. Fluorescence intensity peaks captured with the Dianulox® portable fluorescence reader device. Samples: from left to right, one spot containing a blank sample of OF and five spots containing 500 ng of Δ9-THC in OF, submitted to the FBBB reaction. The red bar shows the absolute height from one of the peaks.

dried overnight. The fluorescence intensity was measured with the Dianulox® portable fluorescence reader and images were taken with the Dino-Lite® digital microscope.

Results

Substrate optimization

Planar Solid Phase Microextraction (PSPME), where the FBBB test was previously described in plant extracts [16], Cytiva Whatman™ 1PS Disposable Phase Separating Paper (silicon treated filter paper), Cytiva Whatman™ Grade 3 Qualitative Filter Paper Standard Grade, and glass fiber filter were evaluated as potential substrates for the FBBB reaction in OF. Silicon treated filter paper is a hydrophobic filter paper, Whatman filter paper grade 3 is a thick filter paper, and glass fiber filter paper has been described as an inert material that is adequate for fluorescence assays [27].

Table S1 (see supplementary material) shows the substrates performances as per visual color and fluorescence intensity for OF blank and for 15 μg/mL (150 ng) of Δ9-THC in OF on the spot, after the reaction with FBBB, captured and photographed with the Dino-Lite® digital microscopes. All the reactions were placed onto 3.5 mm circles of each substrate as previously described [16]. The only material that provided with a non-reactive blank on both color and fluorescence was the glass fiber filter (Table S1), while it provided a red color and a bright red color for fluorescence when Δ9-THC reacted with FBBB. Although the other materials also demonstrated functionality as substrates for the FBBB with Δ9-THC reaction in OF, a green fluorescence signal was observed in the OF blanks. Thus, glass fiber filter was chosen as the substrate for the FBBB reaction in OF. Afterwards, the size of the substrate was optimized to 6.3 mm squares, that could better accommodate the reaction for OF and it suits the Dianulox® portable fluorescence reader.

The use of synthetic oral fluid and the fast blue BB test

The commercial synthetic OF OraFlx® was tested as a potential matrix for the FBBB method validation. For this purpose, a comparison experiment was conducted involving a blank sample of human OF, a blank sample of synthetic OF, a sample of Δ9-THC in a high concentration (25 μg/mL) diluted in human OF, and a sample of Δ9-THC in a high concentration (25 μg/mL) diluted in synthetic OF, all reacted with FBBB in the presence of NaOH.

The synthetic OF sample containing Δ9-THC did not show a red color as it shows for the human OF sample when reacting with FBBB (Table S2, see supplementary material). Additionally, it only displayed a faded red color for the fluorescence intensity captured with the Dino-Lite® digital microscope, compared to the bright red color shown for the human OF sample containing Δ9-THC (Table S2). Analyzing the Dianulox® portable fluorescence reader data (Table S2), the average fluorescence measured from the reaction involving the human OF blank, the synthetic OF blank, and the synthetic OF containing Δ9-THC present similar results (around 150), which is a much lower number compared to the human OF sample containing Δ9-THC (around 430). There is indication that the synthetic OF OraFlx® prevented the reaction between Δ9-THC and FBBB and therefore was it not selected as a matrix for validation in this research.

Pre-processing of oral fluid

Initially, non-processed human OF was tested for the FBBB reaction. However, high signals of fluorescence were observed with the reaction between OF blank samples and FBBB using the Dianulox® portable fluorescence reader (Fig. 2). When analyzing the fluorescence images of these spots acquired with the Dino-Lite® digital microscope, it was observed that the signals were most likely coming from the debris (e.g., proteins, cells, bacteria) present in the OF (Fig. 2a and 2b). To reduce these debris, the OF was submitted to centrifugation. Fig. 2d displays the reaction of centrifugated OF (Fig. 2c) with FBBB on the spot with no observable signal from the debris. Centrifugated OF samples also generated significant decreases in the fluorescence signal acquired with the Dianulox® portable fluorescence reader. Therefore, centrifugated OF was chosen as a matrix for the FBBB method validation.

Limit of detection (LOD) definition and calibration curve of Δ9-THC in oral fluid

Nine different concentrations of Δ9-THC (from 0.5 μg/mL to a 100 μg/mL, or 5 to 100 ng) were prepared in OF and then subjected to the FBBB reaction. Table 1 shows the visual color and the fluorescence for each concentration captured and photographed with Dino-Lite® digital microscopes for one replicate of each concentration. Table 2 shows the average fluorescence captured with the Dianulox® portable fluorescence reader for five replicates of each concentration. When analyzing the images taken with the Dino-Lite® digital microscopes, the red color from the Δ9-THC reaction with FBBB becomes visually distinguishable above 5 μg/mL of Δ9-THC (50 ng on the spot) (Table 1). The fluorescence intensity, however, can be observed starting at 0.5 μg/mL (5 ng of Δ9-THC on the spot) (Table 1). With increasing concentrations of Δ9-THC, both color and fluorescence intensity from the reaction with FBBB increase as well (Table 1).

When analyzing the fluorescence captured with the Dianulox® portable fluorescence reader, the average signal for the reaction between 0.5 μg/mL of Δ9-THC and FBBB was also higher than the average signal generated by OF blank samples (Table 2). Comparing increasing concentrations of Δ9-THC, it is possible to observe that the signal also increases up to 10 μg/mL of Δ9-THC. Higher concentrations (15, 20, and 50 μg/mL) of Δ9-THC show similar average fluorescence signals, but 100 μg/mL of Δ9-THC show the highest signal. Comparing the average fluorescence signal between replicates prepared in two different days, the %RSD is below 20% for all concentrations of Δ9-THC but for 0.5 μg/

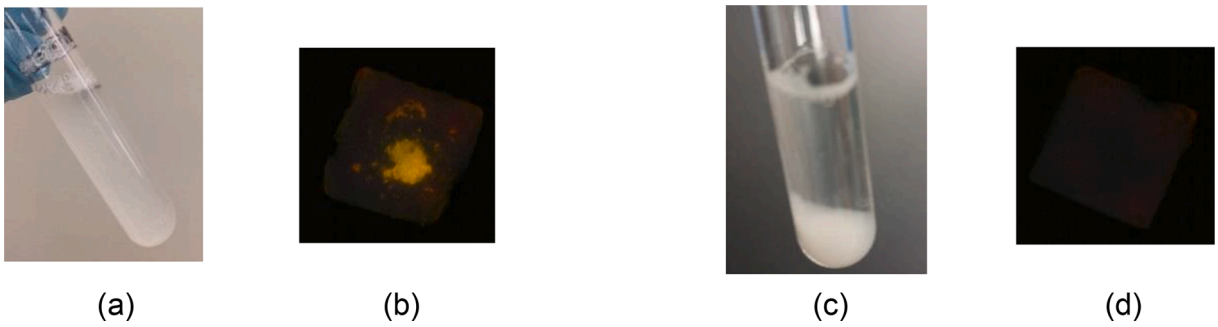

Fig. 2. Non-processed OF (a, b) versus centrifugated OF (c, d). Visual aspect (a, c) and fluorescence (b, d) captured and photographed with the Dinolite® digital microscope.

Table 1
Visual color and fluorescence captured and photographed with Dino-Lite® digital microscopes with increasing concentrations of Δ9-THC in OF on the spots, after reaction with FBBB.

Δ9-THC Concentration in OF	Color	Fluorescence
0 (blank)		
0.5 µg/mL (5 ng)		
1 µg/mL (10 ng)		
3 µg/mL (30 ng)		
5 µg/mL (50 ng)		
10 µg/mL (100 ng)		
15 µg/mL (150 ng)		
20 µg/mL (200 ng)		
50 µg/mL (500 ng)		
100 µg/mL (1000 ng)		

Table 2
Average fluorescence captured with Dianulox® portable fluorescence reader, with increasing concentrations of Δ9-THC in OF on the spots, after reaction with FBBB.

Δ9-THC Concentration in OF	**Dianulox® Day 1***	Dianulox® Day 2*	Average	**SD**[1]	% RSD
0 (blank)	118.6	139.0	128.8	14.4	11.1
0.5 µg/mL (5 ng)	130.8	201.4	166.1	49.4	30.0
1 µg/mL (10 ng)	178.8	219.6	199.2	28.8	14.4
3 µg/mL (30 ng)	237.8	301.8	269.8	45.2	16.7
5 µg/mL (50 ng)	234.0	334.6	284.3	71.1	25.0
10 µg/mL (100 ng)	418.9	444.0	431.4	17.7	4.1
15 µg/mL (150 ng)	365.6	437.4	401.5	50.7	12.6
20 µg/mL (200 ng)	347.4	369.0	358.2	15.2	4.2
50 µg/mL (500 ng)	374.8	374.4	374.6	0.2	0.07
100 µg/mL (1000 ng)	462.0	469.8	465.9	5.5	1.1

*Average of five replicates each day.
[1] SD: standard deviation.

mL and 5 µg/mL, indicating overall precision for the instrument measurements.

Fig. 3 shows the fluorescence analysis for different concentrations of Δ9-THC performed with a VSC2000 instrument to confirm the fluorescence previously captured with the Dianulox® portable fluorescence. The analysis was made using one replicate of each Δ9-THC concentration. Following the same pattern of the portable instrument, the reaction between low concentrations (0.5, 1, 3, 5, and 10 µg/mL) of Δ9-THC and FBBB showed increasing intensity peaks of fluorescence above the blank sample peak. Concentrations above 10 µg/mL of Δ9-THC (15, 20, 50, and 100 µg/mL) achieved the maximum intensity peak threshold for the VSC2000 instrument.

The fluorescence analysis using the Dino-Lite® digital microscope, the Dianulox® portable fluorescence reader, and the VSC2000 instrument confirm that 0.5 µg/mL (5 ng) of Δ9-THC in OF is the minimum mass amount necessary for the reaction with FBBB on a miniaturized spot of glass fiber filter. Therefore, it was established as the test LOD.

A calibration curve was also plotted for the fluorescence signals acquired with the Dianulox® portable fluorescence reader for the reaction

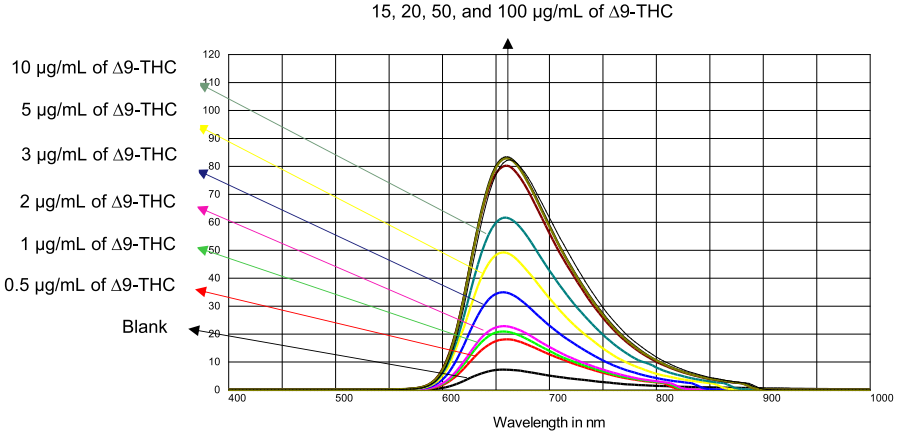

Fig. 3. VSC2000 analysis for different concentrations of Δ9-THC in OF on the spots, after reaction with FBBB. The graph shows the peak intensity (y) and the wavelength in nm (x) for the fluorescence emission.

between FBBB and different concentrations of Δ9-THC in OF, using the average from ten replicates of each concentration over two different days (Table 2). The average of the fluorescence for ten replicates of a blank (OF without Δ9-THC) reaction with FBBB was subtracted from the average of the fluorescence for ten replicates of each concentration. Fig. 4 displays the linear range that was observed for the reactions involving low concentrations of Δ9-THC (0.5, 1, 3, 5, and 10 μg/mL, or 5, 10, 30, 50, and 100 ng). The correlation coefficient was 0.9654.

For comparison and confirmation, RBG codes were obtained from the fluorescence images captured with the Dino-Lite® digital microscope. The red scores produced the most variation of color between different concentrations of Δ9-THC (Table 1), and a calibration curve model was built using the average red scores for five replicates of each concentration (Fig. 5). The average of the fluorescence for five replicates of a blank (OF without Δ9-THC) reaction with FBBB was subtracted from the average of the fluorescence for five replicates of each concentration. Fig. 5 shows the same linear range observed for the reactions involving low concentrations of Δ9-THC (0.5, 1, 3, 5, and 10 μg/mL, or 5, 10, 30, 50, and 100 ng), with a correlation coefficient of 0.919, corroborating the findings of the calibration model using the Dianulox® portable fluorescence reader. This experiment confirmed that it is possible to estimate different concentrations of Δ9-THC in OF using the FBBB test using the Dianulox® portable fluorescence reader.

Accuracy and precision - Dianulox® portable fluorescence reader

Accuracy, intra, and inter-day precision were evaluated for a semi-quantitative analysis of the fluorescence from the Δ9-THC in OF and FBBB reaction captured with the Dianulox® portable fluorescence reader. To estimate the accuracy of Dianulox® portable fluorescence reader measurements, the average fluorescence of ten replicates of each Δ9-THC concentration and FBBB reaction was applied in the linear regression equation (y = 2.8087x + 26.424) estimated for the calibration curve (Fig. 4) using this instrument. Each of the expected concentrations (0.5, 1, 3, 5, and 10 μg/mL, or 5, 10, 30, 50, and 100 ng), corresponding to the real Δ9-THC concentrations in OF, was estimated. Table 3 shows the expected concentrations and the accuracy results from the calculations. Only higher concentrations of Δ9-THC (5 and 10 μg/mL) demonstrated excellent accuracy (<20%).

The precision of the instrument was evaluated at three different concentrations of Δ9-THC in OF (low, medium, and high – 3, 20, and 50 μg/mL, or 30, 200, and 500 ng), submitted to the FBBB reaction in five replicates, over five different days. Table 4 shows the results for five replicates prepared on day 1 (intra-day) and on five different days (inter-day). The averages, the standard deviation, and the %RSD between the

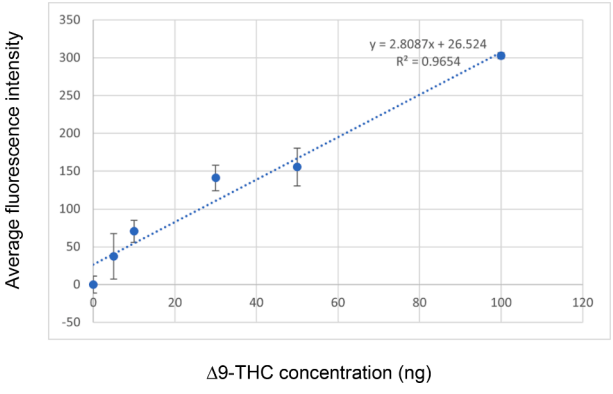

Fig. 4. Linear regression estimated for the fluorescence from the reaction between FBBB and different concentrations of Δ9-THC in OF, captured with the Dianulox® portable fluorescence reader. Each point in the curve represents the average of ten replicates for the reaction. Errors bars represent the %RSD between the ten measurements.

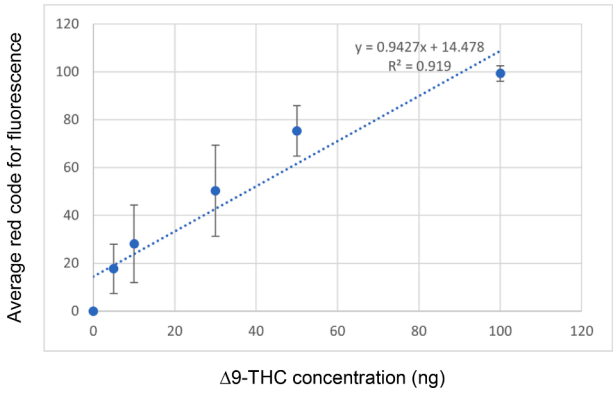

Fig. 5. Linear regression estimated for the fluorescence from the reaction between FBBB and different concentrations of Δ9-THC in OF, captured from images and RGB codes taken with a Dino-Lite® digital microscope. Each point in the curve represents the average of five replicates for the reaction. Errors bars represent the %RSD between the five measurements.

Table 3
Accuracy calculated from the linear regression equation (y = 2.8087x + 26.424) for the average fluorescence (ten replicates) from the reaction between FBBB and each of the concentrations inside the linear range observed between 5 and 10 μg/mL of Δ9-THC in OF, captured with Dianulox® portable fluorescence reader.

Δ9-THC concentration in OF	Expected concentration based on the equation	Accuracy
0.5 μg/mL (5 ng)	3.8	–23.2
1 μg/mL (10 ng)	15.6	56.2
3 μg/mL (30 ng)	40.7	35.8
5 μg/mL (50 ng)	45.9	–8.1
10 μg/mL (100 ng)	98.3	–1.6

five replicates were calculated. For intra-day precision, the %RSD below 20% indicate overall precision for all three concentrations (Table 4). For inter-day precision, however, the low concentration (3 μg/mL or 30 ng) showed an increased variation (28%) between inter-day replicates (Table 4).

Analysis of oral fluid collection devices buffers for the FBBB test

Quantisal™ and Intercept® OF collection devices were chosen to be tested for the detection of Δ9-THC using the FBBB test.

Three different concentrations of Δ9-THC (3, 20, and 50 μg/mL, or 30, 200, and 500 ng) were diluted in OF previously collected in Quantisal™ and Intercept® buffers, and then tested for the FBBB test. A blank sample containing only the device buffer and a blank sample containing human OF mix in the buffer were also tested as negative controls. Δ9-THC samples diluted in OF collected by spitting were used as positive controls.

Table S3 (see supplementary material) shows the images from the visual color and the fluorescence intensity acquired from the reaction between OF in Quantisal™ buffer samples and FBBB (one replicate each), compared to the reaction between OF samples and FBBB (one replicate each), captured with Dino-Lite® digital microscopes. As expected, blank samples did not show red color and as concentrations of Δ9-THC increased, the red color intensity increased for all samples. As for the fluorescence intensity, the blank sample of Quantisal™ buffer showed a faded red signal, while the blank sample of OF in Quantisal™ buffer did not show any color. Increasing concentrations of Δ9-THC increased the red color intensity for both OF in Quantisal™ buffer and OF samples without buffer. Interestingly, OF in Quantisal™ buffer samples showed a stronger color and fluorescence intensity for the lower

Table 4
Intra-day precision calculated from five replicates of three different Δ9-THC concentrations (low, medium, high) diluted in OF, after reaction with FBBB (day 1).

Δ9-THC concentration in OF	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Intra-Day Average	SD[1]	% RSD
3 μg/mL	161	279	269	237	243	237.8	46.4	19.5
20 μg/mL	321	337	322	441	316	347.4	52.9	15.2
50 μg/mL	402	391	374	313	394	374.8	36.0	9.6
Δ9-THC concentration in OF	**Average Intensity Day 1***	**Average Intensity Day 2***	**Average Intensity Day 3***	**Average Intensity Day 4***	**Average Intensity Day 5***	**Inter-Day Average**	**SD[1]**	**% RSD**
3 μg/mL	237.8	301.8	344.0	355.2	167.2	281.2	78.6	28.0
20 μg/mL	347.4	369.0	356.8	434.4	281.0	357.7	54.8	15.3
50 μg/mL	374.8	374.4	407.8	486.0	396.4	407.8	46.0	11.3

*Average of five replicates each day.
[1] SD: standard deviation.

concentration of Δ9-THC (3 μg/mL or 30 ng), when compared to OF samples.

Table S4 (see supplementary material) displays the fluorescence average intensity of five replicates of both OF in Quantisal™ buffer and OF samples without buffer for two experiments conducted in the Dianulox® portable fluorescence reader over two different days. The average, the standard deviation, and the %RSD between the two experiments were evaluated to estimate the instrument's precision. The %RSD was below 20% for all the reactions presented on Table 9. Overall, OF in Quantisal™ buffer samples generated lower fluorescence intensity signals for the blanks and for all three different concentrations of Δ9-THC, except for the concentration of 30 ng, compared to OF without buffer samples. Both the buffer blank sample and the OF in buffer sample showed similar average results with the Dianulox® portable fluorescence reader. Increasing concentrations of Δ9-THC in OF in Quantisal™ buffer provided increasing values for fluorescence intensity as well, corroborating the visual analysis with the Dino-Lite® digital microscopes. These results indicate that the Quantisal™ OF collection device can be used with the FBBB test for Δ9-THC detection.

Table S5 (see supplementary material) shows the images from the visual color and the fluorescence intensity acquired from the reaction between OF in Intercept® buffer samples and FBBB (one replicate each) compared to the reaction between OF without buffer samples and FBBB (one replicate each), captured with Dino-Lite® digital microscopes. As expected, blank samples did not show red visual color or fluorescence intensity and increasing concentrations of Δ9-THC increased the red color intensity for both OF in Intercept® buffer and OF samples. OF in Intercept® buffer samples showed visual color and fluorescence intensity quite similar to OF samples.

In Table S6 (see supplementary material), the fluorescence intensity average of five replicates of both OF in Intercept® buffer and OF samples without buffer for two experiments conducted in the Dianulox® portable fluorescence reader over two different days. The average, the standard deviation, and the %RSD between the two experiments were evaluated to estimate the instrument's precision. The %RSD was below 20% for all the reactions. The average fluorescence was similar between OF in Intercept® buffer and OF samples, when considering the blank and the low concentration of Δ9-THC (3 μg/mL or 30 ng). Higher concentrations of Δ9-THC (20 and 50 μg/mL, or 200 and 500 ng) showed lower fluorescence intensity signals in OF in Intercept® buffer samples compared to OF samples. However, like in OF samples without buffer, increasing concentrations of Δ9-THC in OF in Intercept® buffer samples provided increasing values for fluorescence intensity as well, corroborating the visual analysis with the Dino-Lite® digital microscopes. These results indicate that the Intercept® OF collection device can be used with the FBBB test for Δ9-THC detection.

Test interferences

Cannabinoids interferences

Four cannabinoids (CBD, CBN, CBG, and Δ8-THC) and two metabolites (THC-COOH and THC-OH) were tested as possible interferants to the detection of Δ9-THC in OF using the FBBB test.

Table 5 shows the visual color and the fluorescence intensity captured with the Dino-Lite® digital microscopes for one replicate of the cannabinoid/metabolite reaction with FBBB and for one replicate of the cannabinoid/metabolite reaction with FBBB in the presence of Δ9-THC (25 μg/mL or 250 ng), compared to a blank sample of OF and to a positive control of Δ9-THC (25 μg/mL or 250 ng). As it can be observed, individually, CBD and CBG reaction with FBBB provided an orange color and absence of fluorescence. CBN provided a light purple color and a faded red color for fluorescence. As for the reaction between Δ8-THC or the metabolites (THC-COOH and THC-OH) and FBBB, a red color and a bright signal for fluorescence was observed, similar to the FBBB reaction with Δ9-THC. When a high concentration (25 μg/mL or 250 ng) of Δ9-THC was added to a high concentration of each cannabinoid/metabolite (25 μg/mL or 250 ng), the color for the reactions involving CBD, CBN, and CBG remained different than red, and the fluorescence intensity showed a much less bright signal than the positive control of Δ9-THC itself (25 μg/mL or 250 ng). Δ8-THC or the metabolites (THC-COOH and THC-OH) reaction with FBBB in the presence of Δ9-THC (25 μg/mL or 250 ng) remained generating a red color and a bright fluorescence signal, like the positive control.

Table 6 shows the fluorescence intensity average of five replicates of the cannabinoid/metabolite and for five replicates of the cannabinoid/metabolite in the presence of Δ9-THC (25 μg/mL or 250 ng) for two experiments conducted in the Dianulox® portable fluorescence reader over two different days. Table 6 also shows the average of five replicates for the negative control (OF blank sample) and for the positive control (Δ9-THC, 25 μg/mL or 250 ng) of the experiments. The average, the standard deviation, and the %RSD between the two experiments were evaluated to estimate the instrument's precision. The %RSD was below 20% for all the reactions presented on Table 6. Overall, the results with the Dianulox® portable fluorescence reader corroborate with the conclusions of the experiments with the Dino-Lite® digital microscopes (Table 5). Individually, CBD, CBN, and CBG reaction with FBBB showed an average fluorescence below the OF blank, while Δ8-THC and the metabolites (THC-COOH and THC-OH) reaction with FBBB show an average fluorescence similar to the reaction between Δ9-THC and FBBB. When mixed with a high concentration of Δ9-THC, CBD, CBN, and CBG reaction with FBBB quenched the fluorescence from the reaction between Δ9-THC and FBBB. Δ8-THC and the metabolites (THC-COOH and THC-OH) reaction with FBBB, in the presence of Δ9-THC generated an average fluorescence similar to the reaction between Δ9-THC and FBBB.

These results indicate that CBD, CBN, and CBG could potentially interfere with the Δ9-THC detection in OF by preventing both color and

Table 5
Color and fluorescence captured with Dino-Lite® digital microscopes for the reaction between Δ9-THC, minor cannabinoids (CBD, CBN, CBG, and Δ8-THC), and metabolites (THC-COOH and THC-OH). On the right side of the table, the images show the cannabinoids and the metabolites submitted for the reaction individually in a high concentration (25 μg/mL); on the left side of the table, the images show the cannabinoids and the metabolites in a high concentration (25 μg/mL) in the presence of a high concentration of Δ9-THC (25 μg/mL).

Cannabinoid	Color	Fluorescence Intensity	Cannabinoid + Δ9-THC (250ng)	Color	Fluorescence Intensity
Blank			-	-	-
Δ9-THC (25 μg/mL)			-	-	-
CBD (25 μg/mL)			CBD (25 μg/mL)		
CBN (25 μg/mL)			CBN (25 μg/mL)		
CBG (25 μg/mL)			CBG (25 μg/mL)		
Δ8-THC (25 μg/mL)			Δ8-THC (25 μg/mL)		
Metabolite	**Color**	**Fluorescence Intensity**	**Metabolite + Δ9-THC (250 ng)**	**Color**	**Fluorescence Intensity**
THC-COOH (25 μg/mL)			THC-COOH (25 μg/mL)		
THC-OH (25 μg/mL)			THC-OH (25 μg/mL)		

Table 6
Fluorescence captured with the Dianulox® portable fluorescence reader for the reaction between **Δ**9-THC, minor cannabinoids (CBD, CBN, CBG, and **Δ**8-THC), and metabolites (THC-COOH and THC-OH). The reaction was performed using a high concentration of the cannabinoid/metabolite (25 μg/mL), and then a high concentration of the cannabinoid/metabolite 25 μg/mL) in the presence of a high concentration of **Δ**9-THC (25 μg/mL).

Cannabinoid	Day 1*	Day 2*	Average	SD[1]	% RSD
Blank	142.0	188.6	165.3	32.9	19.9
Δ9-THC (25 μg/mL)	401.6	413.0	407.3	8.0	1.9
CBD (25 μg/mL)	95.2	96.6	95.9	0.9	1.0
CBD (25 μg/mL) + Δ9-THC (25 μg/mL)	288.0	286.6	287.3	0.9	0.3
CBN (25 μg/mL)	154.2	138.4	146.3	11.1	7.6
CBN (25 μg/mL) + Δ9-THC (25 μg/mL)	176.4	232.0	204.2	39.3	19.2
CBG (25 μg/mL)	94.6	94.6	94.6	0	0
CBG (25 μg/mL) + Δ9-THC (25 μg/mL)	149.2	156.0	152.6	4.8	3.1
Δ8 THC (25 μg/mL)	489.4	459.8	474.6	20.9	4.4
Δ8 THC (25 μg/mL) + Δ9-THC (25 μg/mL)	495.6	584.2	539.9	62.6	11.6
Metabolite	**Day 1**	**Day 2**	**Average**	**SD[1]**	**Day 2**
THC-COOH (25 μg/mL)	457.6	467.0	462.3	6.6	1.4
THC-COOH (25 μg/mL) + Δ9-THC (25 μg/mL)	422.4	328.4	375.4	66.4	17.7
THC-OH (25 μg/mL)	456.8	469.0	462.9	8.6	1.8
THC-OH (25 μg/mL) + Δ9-THC (25 μg/mL)	375.2	358.8	367.0	11.5	3.1

*Average of five replicates each day.
[1] SD: standard deviation.

fluorescence appearance from the Δ9-THC/FBBB reaction, producing false negative results. On the other hand, Δ8-THC and the metabolites (THC-COOH and THC-OH) could potentially interfere with the Δ9-THC detection in OF by producing false positive results with the FBBB test.

Previous studies [22,23] show that minor cannabinoids (such as CBD and CBN) are often detected in OF in much lower concentrations than Δ9-THC. For this reason, a new experiment was designed using a low concentration of CBD, CBN, and CBG (5 μg/mL or 50 ng) in the presence of a high concentration of Δ9-THC (25 μg/mL or 250 ng). Table 7 shows the visual color and the fluorescence intensity captured with the Dino-Lite® digital microscopes for one replicate of the cannabinoid mixed with Δ9-THC reaction with FBBB compared to a blank sample of OF and to a positive control of Δ9-THC (25 μg/mL or 250 ng). It also presents the fluorescence intensity average of five replicates of the cannabinoid and Δ9-THC reaction, for two experiments conducted in the Dianulox® portable fluorescence reader over two different days. The average, the standard deviation, and the %RSD between the two experiments were evaluated to estimate the instrument's precision. The %RSD was below 20% for all the reactions presented on Table 7. The results for color and fluorescence intensity using both the Dino-Lite® and the Dianulox® instruments show that Δ9-THC could be easily detected in the presence of lower concentrations of CBD, CBN, and CBG. The results observed with the Dino-Lite® digital microscope show the same red color and bright red color for fluorescence for the minor cannabinoids in the presence of Δ9-THC and for the positive control of Δ9-THC alone. Comparing the average fluorescence intensity captured with the Dianulox® portable instrument, the fluorescence for the minor cannabinoids in the presence of Δ9-THC is lower than the positive control, but it is still much higher than the OF blank.

Other substances interferences

A total of eleven substances that could potentially be present in the oral cavity of any individual, prior to OF collection, were tested for the FBBB test individually and in the presence of a high concentration of Δ9-THC (25 μg/mL or 250 ng). Although there are numerous other possibilities for drinks and substances that could be also found in OF, different types of common drinks, including sodas, juice, teas, and alcoholic beverages were tested as a representative group as possible interferants to the test (Tables S7 and S8, see supplementary material). Standard solutions of caffeine and nicotine were also tested. The interference studies were evaluated using both the Dino-Lite® digital microscopes and the Dianulox® portable fluorescence reader instruments,

Table 7
Testing of minor cannabinoids (CBD, CBN, and CBG) in a low concentration (5 μg/mL) in the presence of a high concentration of Δ9-THC (25 μg/mL). Images were collected with Dino-Lite® digital microscopes, and the fluorescence intensity was captured with the Dianulox® portable fluorescence reader.

Δ9-THC concentration	Visual Color	Fluorescence Intensity	Dianulox® Day 1*	Dianulox® Day 2*	Average	SD[1]	%RSD
OF blank			142.0	188.6	165.3	32.9	19.9
Δ9-THC (25 μg/mL)			401.6	413.0	407.3	8.0	1.9
CBD (5 μg/mL) + Δ9-THC (25 μg/mL)			335.6	289.6	312.6	32.5	10.4
CBN (5 μg/mL) + Δ9-THC (25 μg/mL)			366.6	407.0	386.8	28.5	7.3
CBG (5 μg/mL) + Δ9-THC (25 μg/mL)			278.6	303.0	290.8	17.2	5.9

*Average of five replicates each day.
[1]SD: standard deviation.

for imaging and fluorescence signals estimation.

Table S7 shows the visual color and the fluorescence intensity captured with the Dino-Lite® digital microscopes for one replicate of the substance and for one replicate of the substance in the presence of Δ9-THC (25 μg/mL or 250 ng) after reaction with FBBB. Control samples included blank OF as negative control and Δ9-THC (25 μg/mL or 250 ng) as a positive control. Of all substances listed on Table S7, only nicotine, guarana powder, and the teas (black tea and green tea) showed a red color in the reaction with FBBB, that could be mistaken by the red color that appears for the reaction between Δ9-THC and FBBB. However, none of these substances showed the red fluorescence intensity that we can observe in the reaction between Δ9-THC and FBBB. When the substances were mixed with a high concentration of Δ9-THC (25 μg/mL or 250 ng), a red color could be then observed for all the reactions, except for the orange juice. Orange juice showed a mixed color of green and red for the fluorescence intensity when mixed with Δ9-THC and FBBB. As for the fluorescence, guarana powder and the teas (black tea and green tea) quenched the fluorescence of Δ9-THC and FBBB reaction.

Table S8 shows the fluorescence intensity average of five replicates of the substance and for five replicates of the substance in the presence of Δ9-THC (25 μg/mL or 250 ng) for two experiments conducted in the Dianulox® portable fluorescence reader over two different days. Table S8 also shows the average of five replicates for the negative control (OF blank sample) and for the positive control (Δ9-THC 25 μg/mL or 250 ng) of the experiments. The average, the standard deviation, and the %RSD between the two experiments were evaluated to estimate the instrument's precision. Coca Cola® soda and black tea showed the highest variation between replicates, individually. In the presence of Δ9-THC; caffeine and vodka Absolut® showed the highest variation between replicates.

When analyzed individually, the substances caffeine, nicotine, guarana powder, teas (black tea and green tea), and vodka Absolut® showed fluorescence scores lower than the negative control and it would not be mistaken by the fluorescence of Δ9-THC and FBBB reaction. Guarana Antarctica® soda and whiskey Jack Daniels® show slightly higher signals than the negative control, but no fluorescence was observed with the Dino-Lite® digital microscope for these substances (Table S7). Coca Cola® soda, orange juice, and Corona® beer showed fluorescence signals even higher than the positive control. However, these three beverages show a green signal when the spot is analyzed with the Dino-Lite® digital microscope (Table S7), instead of the red signal that we observe for Δ9-THC and FBBB reaction. Therefore, these substances show a different fluorescence pattern when reacting with FBBB. Caffeine, nicotine, guarana powder, and the teas (black tea and green tea) seemed to quench the fluorescence from the Δ9-THC and FBBB reaction when analyzing it with the Dianulox® portable fluorescence reader. When comparing it to the Dino-Lite® digital microscope imaging analysis (Table S7), only guarana powder and the teas (black tea and green tea) did in fact quench the fluorescence of Δ9-THC and FBBB reaction.

Stability studies

Tables S9 and S10 (see supplementary material) show the results for the spots stability over time, kept in the dark under room temperature. For a medium concentration (20 μg/mL or 200 ng) and a high (50 μg/mL or 500 ng) concentration of Δ9-THC, both color and fluorescence can be still observed after 30 days. The fluorescence analysis using both the imaging with the Dino-Lite® digital microscope (Table S9) and the Dianulox® portable fluorescence reader (Table S10) indicates a little decrease in the signal over time, but those concentrations can still be easily detected. The low concentration of Δ9-THC (3 μg/mL or 30 ng), however, show a similar imaging pattern over time (Table S9), and an increasing signal for fluorescence captured with the Dianulox® portable fluorescence reader (Table S10). It seems that the chromophore/fluorophore formed by Δ9-THC and FBBB is stable, but in a low concentration of Δ9-THC, such as 3 μg/mL or 30 ng, the amount of product is not enough to be observed over time. In this case, the higher fluorescence signals that were observed with the Dianulox® instrument could be related to OF and/or FBBB degradation over time.

Table S11 (see supplementary material) shows the results for the stability of pre-loaded FBBB spots, which were kept at three different temperatures for 15 days: at room temperature (25 °C), in a refrigerator (4 °C) and in the freezer (-20 °C). At day 15, low, medium, and high concentrations (3, 20, and 50 μg/mL or 30, 200, and 500 ng) of Δ9-THC in OF were applied on the spots for reaction, and then compared to freshly FBBB pre-loaded spots. When kept at room temperature, all the pre-loaded FBBB spots turned into a light pink color before reaction and did not show significant fluorescence signals for the Dinolite® microscope imaging or for the Dianulox® instrument (Table S11). The pre-

loaded spots that were kept under lower temperatures (4 °C and −20 °C) performed similarly to freshly pre-loaded FBBB spots, providing both red color and fluorescence signals that could be observed with the Dinolite® microscope imaging and with the Dianulox® instrument (Table S11). However, the Dianulox® instrument analysis showed lower fluorescence signals for the blank samples and for the three concentrations of Δ9-THC applied on those spots.

Discussion and conclusions

The FBBB test was previously validated for the differentiation between marijuana-type and hemp-type cannabis [16]. The chemical reaction was optimized for 10 μL of cannabis plants extracts from organic solvents (1 mL of a mixture of MeOH and chloroform), 10 μL of FBBB, and 10 μL of NaOH, both prepared in methanolic solutions, on top of a substrate named PSPME [16]. The volatile small volume of sample and reagents (30 μL) is able to dry very quickly on the substrate allowing for fast results involving color (red in the presence of Δ9-THC and orange in the presence of CBD) and fluorescence (in the presence of Δ9-THC) that were captured with Dinolite® digital microscopes. Δ9-THC and CBD concentrations in cannabis plant extracts are relatively high and based on the red color from the chromophore formed by FBBB and Δ9-THC, or the orange color from the chromophore formed by FBBB and CBD, the test LOD was defined at 50 μg/mL or 500 ng [16]. The FBBB test in cannabis plants extract was subjected to interference studies for different cannabinoids (CBN, CBG, delta-9-tetrahydrocannabolic acid (THCA), and cannabidiolic acid (CBDA)), herbs, and spices [16]. Stability studies have shown that the FBBB reagent is not stable at room temperature and needs to be kept refrigerated while being protected from light [16].

This current work demonstrates the applicability of the presumptive FBBB test for Δ9-THC detection in OF, for the first time. We report the test's optimization on a different substrate (6.3 mm of glass fiber filter), using only 10 μL of OF, and the same small volume (20 μL) of reagents (FBBB and NaOH). Different from the cannabis plant extracts, Δ9-THC concentrations in OF can be significantly lower and in this matrix the fluorescence analysis provided more information about low concentrations of Δ9-THC than color. For this purpose, along with the Dinolite® digital microscopes spots analysis, we introduce a portable fluorescence reader (Dianulox®) for the analysis of the fluorescence between Δ9-THC and FBBB reaction. This instrument could demonstrate that different concentrations of Δ9-THC can be estimated in OF using the FBBB test. The OF matrix, however, is much more complex than cannabis plant extracts and issues such as OF collection and sample preparation need to be addressed accordingly.

While OF sample collection presents advantages for field drug testing, the matrix complexity is a challenge for method development. When OF is collected by simply drooling, the matrix viscosity and the presence of debris that are naturally in the oral cavity (e.g., proteins, cells, bacteria) are potential interferents to a test and this difficulty was observed in the fluorescence analysis from non-processed OF and FBBB reaction. Although it is possible to observe the fluorescence from the reaction between Δ9-THC and FBBB using non-processed OF, blank samples (non-spiked OF) provide a higher fluorescence signal than centrifugated OF. Therefore, non-processed OF interferes with the FBBB test by reducing its sensitivity. To overcome this issue, we performed OF centrifugation before spiking the matrix with Δ9-THC and submitted it to the FBBB test. This sample treatment was necessary to demonstrate the FBBB test applicability for the detection of Δ9-THC in OF. However, this procedure would not be adequate in a real sampling scenario due to the possibility that Δ9-THC might be lost along with the OF debris in a centrifugation process.

A different approach of handling OF viscosity and debris is to collect OF using a commercial device. However, the impact of devices preservation buffers should be evaluated in the method validation of a test for drug detection in OF [20,28]. We performed experiments with the synthetic OF OraFlx® and according to the manufacturer (Dyna-Tech Industries), the buffer solution contains sodium azide 0.1% as a preservative. Therefore, our results show that this buffer preservative can prevent the reaction between Δ9-THC and FBBB. In the reaction between Δ9-THC and FBBB, the electrons from the phenolate ion in the Δ9-THC attack the diazo group on the FBBB compound, forming the red chromophore complex [29]. It is suspected that the presence of the sodium azide preservative interferes with the formation of the chromophore complex since the THC interacts with the sodium azide rather than the FBBB, resulting in no color formation. Due to this finding, collection devices that contain a sodium azide buffer should not be used as to not interfere with the FBBB reaction.

Commercial devices buffers such as OralEze™ (ThermoFisher Scientific™) or SalivaSampler™ (StatSure™ Diagnostics Systems) contain sodium azide [21] and should be avoided for use with the FBBB test. The devices Quantisal™ (Immunalysis Corporation) and Intercept® (OraSure Technologies) contain non-azide buffer preservatives and they were chosen to be evaluated in this research with the FBBB test. The OF collection process involving the pad and the buffer, for both devices, was sufficient to handle the OF debris and no centrifugation was necessary prior to FBBB evaluation tests. Overall, the two devices demonstrated an adequate performance without interfering in the reaction between different concentrations of Δ9-THC (3, 20, and 50 μg/mL or 30, 200, and 500 ng) and the FBBB reaction. However, while these commercial OF collection devices help with providing a clean matrix for analysis, the buffer volume standardized for each device adds a critical dilution factor to Δ9-THC analysis in OF. Both Quantisal™ and Intercept® devices pads collect 1 mL of OF, and then they are diluted into 3 mL and 2 mL of buffer, respectively. Low concentrations of Δ9-THC in OF diluted in such volumes of buffer would be difficult to detect in a screening test.

Ultimately, a sample extraction procedure is commonly required in quantitative methods for Δ9-THC detection in OF [30] and it focuses on improving both the OF viscosity and the concentration for drug recovery aspects. Furthermore, an extraction procedure would help reduce the water content on the OF samples, allowing the spots to dry faster. The development of a sample extraction protocol is the next step for the research of Δ9-THC detection in OF using the FBBB test.

The method validation for the detection of Δ9-THC in OF using the FBBB test included the LOD definition, interferences, and stability studies. Additionally, a calibration model, linearity, accuracy, and precision were studied for the semi-quantitative evaluation of Δ9-THC in OF.

Sensitivity is the most challenging aspect of a Δ9-THC screening test in OF. After around 22 h after smoking [22,31], Δ9-THC concentrations in OF can be as low as 1 ng/mL. Because of this, different organizations published guidelines recommendations concerning Δ9-THC cut-off limits for OF field testing. In 2012, The European project *Driving Under the Influence of Drugs* (DRUID) [32] established the cut-off of 27 ng/mL for Δ9-THC detection in OF. In 2019, in the United States, the Substance Abuse and Mental Health Administration (SAMHSA) published mandatory guidelines for federal workplace drug testing programs [33], where the cut-off value for Δ9-THC detection in OF was established as 2 ng/mL for screening tests and as 1 ng/mL for confirmatory tests. Our experiments show that 500 ng/mL (0.5 μg/mL or 5 ng on the spot) of Δ9-THC can be detected in OF using the FBBB test on a miniaturized spot based on the fluorescence analysis. To obtain 5 ng of Δ9-THC mass on the spot, 10 μL a high concentrated solution of spiked Δ9-THC in OF (0.5 μg/mL) was applied. Therefore, a sample extraction protocol is still necessary for detecting low concentrations of Δ9-THC in OF (<500 ng/mL) so that it can be identified with the FBBB test.

Interference studies were evaluated for different cannabinoids (CBD, CBN, CBG, Δ8-THC), for Δ9-THC metabolites (THC-COOH and THC-OH), and for different substances that could be present in the oral cavity of individuals prior to the test. The cannabinoids CBD, CBN, and CBG had been previously tested, individually, for the FBBB in plant extracts [16], providing a different color and fluorescence patterns than Δ9-THC.

In this study, it was observed that these cannabinoids, when added in high concentrations in the presence of Δ9-THC, could prevent or decrease the color and fluorescence intensity from the reaction between Δ9-THC and FBBB. Nevertheless, these cannabinoids can only be found in OF in much lower concentrations than Δ9-THC [22,23,31]. When a new experiment was conducted using low concentrations of CBD, CBN, and CBG in the presence of a higher concentration of Δ9-THC, the red color and the fluorescence from the reaction between Δ9-THC and FBBB could be easily observed. Therefore, the presence of low concentrations of CBD, CBN, or CBG in OF should not interfere with higher concentrations of Δ9-THC and FBBB reaction.

Δ8-THC and the metabolites THC-COOH and THC-OH in reaction with FBBB demonstrated similar results than Δ9-THC for both color and fluorescence. However, the metabolites concentrations in OF are very low and should reflect the previous presence of Δ9-THC in the body [23]. As for Δ8-THC, this minor cannabinoid is also known for having psychoactive properties and it has been under investigation and discussion about its regulation [34–36]. In this scenario, the detection of Δ8-THC in OF samples, along with Δ9-THC, might add an extra value for the FBBB test in the field testing.

Of all substances tested as possible interferents to the FBBB in OF, Coca Cola® soda, orange juice, and Corona® beer, individually, have shown a fluorescence pattern that could be mistaken with the Δ9-THC pattern, using the Dianulox® portable fluorescence reader. Those could not be mistaken with Δ9-THC analyzing the images captured with the Dinolite® digital microscope. On the other hand, caffeine, nicotine, guarana powder, and the teas (black tea and green tea) seemed to quench the Δ9-THC reaction with FBBB using the Dianulox® portable fluorescence reader. When analyzed with the Dinolite® digital microscope only guarana powder and both black and green teas in fact quenched the reaction. It is important to highlight that these substances were all tested in relatively high concentrations (50/50 dilutions with OF). In a real OF sampling scenario for screening tests, there is a recommendation to wait at least 10 min prior to OF collection [37] that will reduce the possibility of interference contamination and this procedure should be also followed for OF sampling prior to the FBBB test. No medications or other drugs of abuse were tested for the FBBB test as possible interferents to Δ9-THC detection for this study. Future work will include additional interference studies, as it is recognized that many different substances could also be present in oral fluid.

Stability studies were performed to evaluate how long the reaction could still be observed, and if the FBBB reagent could be pre-loaded on the spots to facilitate field applicability. Both color and fluorescence of the Δ9-THC reaction with FBBB are stable for at least 30 days, allowing the test results to be kept as proof. Pre-loaded FBBB spots were only stable over time (15 days) when kept at lower temperatures, which could be a test limitation as it demands on-site refrigeration.

Calibration curves for different Δ9-THC concentrations in OF were plotted, based on the fluorescence analysis of Δ9-THC and FBBB reaction, captured with both the Dianulox® portable fluorescence reader and the Dinolite® digital microscope instruments. However, the fluorescence analysis using the RGB scores from images captured with the Dinolite® digital microscope does not allow for inter-day precision evaluation, as the imaging conditions (position, environment light), and consequently the RGB scores, may vary throughout different days. For this reason, the semi-quantitative analysis of Δ9-THC in OF samples was evaluated for the Dianulox® instrument. The figures of merit evaluated the quantitative analysis were linearity, precision, and accuracy. The linearity range observed for the fluorescence intensity from the Δ9-THC and FBBB reaction involved lower concentrations of Δ9-THC (from 0.5 μg/mL to 10 μg/mL, or 5 to 100 ng) with a correlation coefficient of 0.9654. This is an interesting finding as higher concentrations of Δ9-THC produce both red color and increased fluorescence intensity signals when reacting with FBBB and a positive test can be easily identified. The instrument‘s accuracy was tested for all the for all the Δ9-THC concentrations from the calibration curve (0.5, 1, 3, 5, and 10 μg/mL, or 5, 10, 30, 50, and 100 ng) using the linear regression equation. Higher accuracy values were found for the highest concentrations (5 and 10 μg/mL, or 50 and 100 ng) and similar results were obtained for intra and inter-day precision studies. The instrument demonstrated overall adequate precision results (<20%) for medium (20 μg/mL or 200 ng) and high (50 μg/mL or 500 ng) concentrations of Δ9-THC, while it was higher (>20%) for the low concentration of Δ9-THC (3 μg/mL or 30 ng). Even though the Dianulox® instrument did not fulfill all the requirements for a quantitative method validation recommended by the Standard Practices for Method Validation in Forensic Toxicology [25], these results show that the Dianulox® portable fluorescence reader demonstrates satisfactory performance to fit the purpose for a semi-quantitative analysis of Δ9-THC in OF using the FBBB test. Considering the simplicity of the Dianulox® instrument, it would not be expected that linearity, accuracy, and precision could reproduce the quantitative analysis performed by a mass spectrometry instrument. Therefore, a confirmatory test is still necessary after the initial identification of Δ9-THC in OF with the FBBB test.

Immunoassays are currently the screening method of choice for on-site drug detection in OF [37] but there are relatively high costs associated to this test. Most immunoassay devices available in the market require an OF collection device, often coupled to the test strip and a portable test reader [37]. Specifically for Δ9-THC detection, there are several issues reported with these devices [15]. Most studies involving immunoassays evaluate false positive and false negative tests rates by studying sensitivity, specificity, and accuracy [15,37]. Sensitivity, however, present values below expected (>90%) for some devices brands [15,37]. The cutoffs for Δ9-THC detection in OF vary between 4 and 100 ng/mL throughout different immunoassays in the market [15,37], and some of these values are considered high. Additionally, the overall testing time for immunoassays ranges from 2 to 30 min between brands [15]. Inadequate OF collection volume, failure tests, and instability to cold weather have been also reported for immunoassays involving Δ9-THC detection in OF [15].

The final goal of this research is to develop an OF collection and sample extraction system coupled to a prototype that can be used in the field and improve Δ9-THC detection in roadsides, workplace, or anti-doping programs. An ideal field test for the detection of Δ9-THC in OF would be low-cost, sensitive, and provide fast results.

The miniaturized substrate herein described, and the small volume of reagents required for the FBBB test make it a very affordable technique. The fluorescence measurements, however, would demand an initial investment to acquire a portable device and a computer for data analysis, which is not different than most of the immunoassays available in the market. The FBBB test sensitivity and the analysis time are expected to be improved with the development of an OF extraction method protocol. An OF sample extraction should allow higher Δ9-THC concentrations into smaller volumes of organic solvents, speeding up the chemical reaction with FBBB allowing for the spots to dry immediately for analysis. The FBBB test is a promising opportunity to improve Δ9-THC detection in OF in the field.

Future work will include further optimization of the FBBB test in accordance with the development of a prototype for field detection of Δ9-THC in OF. This device should be validated for field usage, including not only an OF collection and sample extraction protocols and new LOD definition, but the testing of real OF samples from marijuana users, and the assessment for the test‘s sensitivity, specificity, and accuracy, in comparison with an immunoassay. Additionally, different environmental situations (e.g., temperature, humidity) should be also studied for the prototype.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2023.100514.

References

[1] UNITED NATIONS (2022) accessed July 26, 2022, https://www.unodc.org/res/wdr2021/field/WDR21_Booklet_3.pdf.
[2] Authenticated U.S. Government Information, 115th Congress 2d Session House of Representatives, Report 115-1072, Agriculture Improvement Act of 2018 - Conference Report H.R. 2, 2018. https://www.congress.gov/bill/115th-congress/house-bill/2 (accessed July 27, 2022).
[3] A. Manosroi, C. Chankhampan, C.J. Mai Sci, Pharmaceutical and Cosmeceutical Biological Activities of Hemp (Cannabis sativa L var. sativa), Leaf and Seed Extracts (2019). http://epg.science.cmu.ac.th/ejournal/.
[4] P. Cerino, C. Buonerba, G. Cannazza, J. D'Auria, E. Ottoni, A. Fulgione, A. di Stasio, B. Pierri, A. Gallo, A review of hemp as food and nutritional supplement, Cannabis Cannabinoid Res. 6 (2021) 19–27, https://doi.org/10.1089/can.2020.0001.
[5] D. McCartney, T.R. Arkell, C. Irwin, I.S. McGregor, Determining the magnitude and duration of acute Δ9-tetrahydrocannabinol (Δ9-THC)-induced driving and cognitive impairment: A systematic and meta-analytic review, Neurosci Biobehav Rev. 126 (2021) 175–193, https://doi.org/10.1016/j.neubiorev.2021.01.003.
[6] M.C. Hazle, K.P. Hill, L.M. Westreich, Workplace Cannabis Policies: A Moving Target, Cannabis Cannabinoid Res. 7 (2022) 16–23, https://doi.org/10.1089/can.2020.0095.
[7] S. Docter, M. Khan, C. Gohal, B. Ravi, M. Bhandari, R. Gandhi, T. Leroux, Cannabis Use and Sport: A Systematic Review, Sports, Health. 12 (2020) 189–199, https://doi.org/10.1177/1941738120901670.
[8] E.L. Karschner, M.J. Swortwood-Gates, M.A. Huestis, Identifying and Quantifying Cannabinoids in Biological Matrices in the Medical and Legal Cannabis Era, Clin Chem. 66 (2020) 888–914, https://doi.org/10.1093/clinchem/hvaa113.
[9] O.H. Drummer, Introduction and review of collection techniques and applications of drug testing of oral fluid, Ther Drug Monit. 30 (2008) 203–206, https://doi.org/10.1097/FTD.0b013e3181679015.
[10] A. Arroyo, M. Sanchez, E. Barberia, M. Barbal, M.T. Marrón, A. Mora, Comparison of the Cozart DDS 801 on-site drug test device and gas chromatography/mass spectrometry (GC/MS) confirmation results of cannabis and cocaine in oral fluid specimens, Australian Journal of Forensic Sciences. 46 (2014) 272–281, https://doi.org/10.1080/00450618.2013.832796.
[11] F. Musshoff, E.G. Hokamp, U. Bott, B. Madea, Performance evaluation of on-site oral fluid drug screening devices in normal police procedure in Germany, Forensic Sci Int. 238 (2014) 120–124, https://doi.org/10.1016/j.forsciint.2014.02.005.
[12] T. VanderLinden, S.M.R. Wille, M. Ramírez-Fernandez, A.G. Verstraete, N. Samyn, Roadside drug testing: Comparison of two legal approaches in Belgium, Forensic Sci Int. 249 (2015) 148–155, https://doi.org/10.1016/j.forsciint.2015.01.034.
[13] J. Liut, U. Bott, B. Madea, M. Krämer, A. Maas, Evaluation of RapidSTAT®, DrugWipe® 6S, DrugScreen® 5TK and DrugScreen® 7TR for on-site drug testing in German police roadside traffic patrol, Drug Test Anal. (2022), https://doi.org/10.1002/dta.3262.
[14] A.J. Krotulski, A.L.A. Mohr, M. Friscia, B.K. Logan, Field detection of drugs of abuse in oral fluid using the Alere™ DDS®2 mobile test system with confirmation by liquid chromatography tandem mass spectrometry (LC-MS/MS), J Anal Toxicol. 42 (2018) 170–176, https://doi.org/10.1093/jat/bkx105.
[15] S.C.D. Dobri, A.H. Moslehi, T.C. Davies, Are oral fluid testing devices effective for the roadside detection of recent cannabis use? A systematic review, Public Health. 171 (2019) 57–65, https://doi.org/10.1016/j.puhe.2019.03.006.
[16] A. Acosta, J. Almirall, Differentiation between hemp-type and marijuana-type cannabis using the Fast Blue BB colorimetric test, Forensic Chemistry. 26 (2021), https://doi.org/10.1016/j.forc.2021.100376.
[17] H.S. França, A. Acosta, A. Jamal, W. Romao, J. Mulloor, J.R. Almirall, Experimental and ab initio investigation of the products of reaction from Δ9-tetrahydrocannabinol (Δ9-THC) and the fast blue BB spot reagent in presumptive drug tests for cannabinoids, Forensic Chemistry. 17 (2020), https://doi.org/10.1016/j.forc.2019.100212.
[18] F.G. Bellagambi, T. Lomonaco, P. Salvo, F. Vivaldi, M. Hangouët, S. Ghimenti, D. Biagini, F. di Francesco, R. Fuoco, A. Errachid, Saliva sampling: Methods and devices. An overview, TrAC -, Trends in Analytical Chemistry. 124 (2020), https://doi.org/10.1016/j.trac.2019.115781.
[19] O.H. Drummer, Drug testing in oral fluid, accessed August 11, 2020, Clin Biochem Rev. 27 (2006) 147–159, http://www.ncbi.nlm.nih.gov/pubmed/17268583.
[20] K. Langel, C. Engblom, A. Pehrsson, T. Gunnar, K. Ariniemi, P. Lillsunde, Drug testing in oral fluid - Evaluation of sample collection devices, J Anal Toxicol. 32 (2008) 393–401, https://doi.org/10.1093/jat/32.6.393.
[21] Ł. Sobczak, K. Goryński, Evaluation of swabs from 15 commercially available oral fluid sample collection devices for the analysis of commonly abused substances: Doping agents and drugs of abuse, Analyst. 145 (2020) 7279–7288, https://doi.org/10.1039/d0an01379j.
[22] S. Anizan, G. Milman, N. Desrosiers, A.J. Barnes, D.A. Gorelick, M.A. Huestis, Oral fluid cannabinoid concentrations following controlled smoked cannabis in chronic frequent and occasional smokers, Anal Bioanal Chem. 405 (2013) 8451–8461, https://doi.org/10.1007/s00216-013-7291-5.
[23] D. Lee, M.A. Huestis, Current knowledge on cannabinoids in oral fluid, Drug Test Anal. 6 (2014) 88–111, https://doi.org/10.1002/dta.1514.
[24] W.S. Rasband, US National Institutes of Health, ImageJ Software, (1997). https://imagej.nih.gov/ij/ (accessed October 26, 2022).
[25] American Academy of Forensic Science (AAFS) Standard Board (ASB), Standard Practices for Method Validation in Forensic Toxicology., American Academy of Forensic Sciences. (2019).
[26] D. Cristiane Bordin, M. Messias, R. Lanaro, S. Oliveira, S. Cazenave, J. Luiz Costa, Análise Forense: Pesquisa de Drogas Vegetais Interferentes de Testes Colorimétricos Para Identificação dos Canabinoides da Maconha (Cannabis sativa L.), Quim Nova. 35 (2012).
[27] L. Zhang, K.S. Chen, H.Z. Yu, Superhydrophobic Glass Microfiber Filter as Background-Free Substrate for Quantitative Fluorometric Assays, ACS Appl Mater Interfaces. 12 (2020) 7665–7672, https://doi.org/10.1021/acsami.9b17432.
[28] D.J. Crouch, Oral fluid collection: The neglected variable in oral fluid testing, Forensic Sci Int. 150 (2005) 165–173, https://doi.org/10.1016/j.forsciint.2005.02.028.
[29] N.A. dos Santos, L.M. Souza, E. Domingos, H.S. França, V. Lacerda, A. Beatriz, B. G. Vaz, R.R.T. Rodrigues, V.V. Carvalho, B.B. Merlo, R.M. Kuster, W. Romão, Evaluating the selectivity of colorimetric test (Fast Blue BB salt) for the cannabinoids identification in marijuana street samples by UV–Vis, TLC, ESI(+)FT-ICR MS and ESI(+)MS/MS, Forensic, Chemistry. 1 (2016) 13–21.
[30] R.P. Gorziza, J.A. Duarte, M. González, L.E. Arroyo-Mora, R.P. Limberger, A systematic review of quantitative analysis of cannabinoids in oral fluid, J Forensic Sci. 66 (2021) 2104–2112, https://doi.org/10.1111/1556-4029.14862.
[31] G. Milman, D.M. Schwope, D.A. Gorelick, M.A. Huestis, Cannabinoids and metabolites in expectorated oral fluid following controlled smoked cannabis, Clinica Chimica Acta. 413 (2012) 765–770, https://doi.org/10.1016/j.cca.2012.01.011.
[32] H. Schulze, M. Schumacher, R. Urmeew, J. Alvarez, I.M. Bernhoft, H.G. de Gier, M. Hagenzieker, S. Houwing, A. Knoche, M. Pilgerstorfer, Driving Under the Influence of Drugs, Alcohol and Medicines in Europe—findings from the DRUID project, (2012) 1–58.
[33] SAMHSA, Mandatory Guidelines for Federal Workplace Drug Testing Programs, US Federal Register. (2019). https://www.samhsa.gov/sites/default/files/programs_campaigns/division_workplace_programs/final-mg-oral-fluid.pdf (accessed November 14, 2022).
[34] M.E. Bozman, S.V.R.R. Manoharan, T. Vasavada, Marijuana variant of concern: Delta 8-tetrahydrocannabinol (Delta-8-THC, Δ8-THC), Psychiatry Research Case Reports. 1 (2) (2022) 100028.
[35] G.T. Wurz, E. Montoya, M.W. DeGregorio, Examining impairment and kinetic patterns associated with recent use of hemp-derived Δ8-tetrahydrocannabinol: case studies, J Cannabis Res. 4 (2022), https://doi.org/10.1186/s42238-022-00146-9.
[36] A. Chan-Hosokawa, L. Nguyen, N. Lattanzio, W.R. Adams, Emergence of Delta-8 Tetrahydrocannabinol in DUID Investigation Casework: Method Development, Validation and Application, J Anal Toxicol. 46 (2022) 1–9, https://doi.org/10.1093/jat/bkab029.
[37] D. Buzby, A.L.A. Mohr, B.K. Logan, K.L. Lothridge, Evaluation of On-Site Oral Fluid Drug Screening Technology, 2021. www.ntis.gov.