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REVISTA ifes ciência

USO DE MICRODISPOSITIVO DE PAPEL NA ANÁLISE DE AMOSTRAS DE MACONHA PARA CLASSIFICAÇÃO DO TEMPO DE APREENSÃO

USE OF A PAPER MICRODEVICE IN TO ANALYSES OF MARIJUANA SAMPLES FOR SHELF TIME CLASSIFICATION

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Resumo: A microfluídica é uma tecnologia de sistemas que manipula e estuda pequenas quantidades de fluidos, por meio de estruturas com dimensões de dezenas a centenas de micrômetros (microdispositivos), tendo como principais vantagens seu custo mínimo, rapidez e capacidade de fornecer resultados pontuais. Neste trabalho, a versão microfluídica em papel, considerada a mais barata, foi desenvolvida para realizar o teste do sal *Fast blue b* para calcular o teor de canabinol (CBN) em amostras de maconha apreendidas (n = 66). Além de usar o conhecimento das reações de conversão de outros canabinóides em CBN para descobrir a rastreabilidade temporal de amostras expostas ao meio ambiente. Uma câmera de smartphone combinada com o aplicativo Photometrix® foi utilizada para avaliar a colorimetria dos testes e construir as regressões de estudo, sendo a confirmação de valores feita por técnica tradicional de cromatografia líquida. A curva analítica da determinação de CBN apresentou coeficiente de determinação (R^2) = 0,9964 e *Root Mean Square Calibration Error*, RMSEC, = 0,576 para quantificação por microfluídica, e R^2 = 0,9947 para quantificação por cromatografia líquida. Para as amostras de maconha apreendidas, as concentrações de CBN variaram de 8,97 a 21,48 mg mL^{-1} com valor médio de 14,11 mg mL^{-1}, sendo quantificadas por colorimetria associada a ferramentas quimiométricas. Com todas as informações obtidas também foi possível avaliar o tempo de exposição ambiental, o que pode ser discutido tendo em vista essas concentrações e o conhecimento das amostras.

Palavras-chave: canabinoides; drogas de abuso; microfluídica; Photometrix®.

Abstract: Microfluidics is a systems technology that manipulates and studies small amounts of fluids, through structures with dimensions from tens to hundreds of micrometers (microdevices), having as main advantages its minimum cost, speed and ability to provide punctual results. In this work, the microfluidic paper version, considered the cheapest, was developed to perform the *Fast blue b* salt test to calculate the cannabinol (CBN) content in seized marijuana samples (n = 66). In addition to using knowledge of the conversion reactions of other cannabinoids to CBN to discover the temporal traceability of samples exposed to the environment. A smartphone camera combined with the Photometrix® application was used to evaluate the colorimetry of the tests and build the study

regressions, with confirmation of values being performed using the traditional technique of liquid chromatography. The analytical curve for CBN determination showed a coefficient of determination (R^2) = 0.9964 and Root Mean Square Calibration Error, RMSEC, = 0.576 for quantification by microfluidics, and R^2 = 0.9947 for quantification by liquid chromatography. For seized marijuana samples, CBN concentrations ranged from 8.97 to 21.48 mg mL^{-1} with a mean value of 14.11 mg mL^{-1}, being quantified by colorimetry associated with chemometric tools. With all the information obtained, it was also possible to evaluate the time of environmental exposure, which can be discussed in view of these concentrations and knowledge of the samples.

Keywords: cannabinoid; drug of abuse; microfluidics; Photometrix®.

1 INTRODUCTION

The technological exponential grows up of this Era promoted great advances in several areas, worth mentioning: medicine, chemistry, robotics, among others. With the advent of technological advances come the consequences, among them the increase in the number of illicit drugs. With the increase of its consumption, the ways of transportation and hiding become more and more efficient. Therefore, the technologies for analyzing samples need to evolve and become more efficient, it is worth noting that, if possible, as cheaply as possible.

The term drug is recurrent in history, because it started as a synonym for medicine, the word drug originates from the expression “droog” (old Dutch) which means “dry leaf”, because, in the past, most medicines were based on vegetables/ herbs/leaves and thus generated the ability to eliminate pain and ward off problems[1]. The updated term drug mentions how every substance that is introduced into a living organism modifies one or more of its functions[2]. This was necessary due to the high dissemination of drugs classified as illicit, given their use to escape psychological crises and even reality itself[3].

In Medicine and Pharmacology, the expression drug refers to any substance that prevents or cures diseases by causing physiological changes in organisms. Also called narcotics or narcotics, illicit drugs can be: (I) Natural: those extracted in natura, for example, from the *Cannabis* plant, marijuana is extracted, from the poppy flower, opium is obtained. (II) Semi-synthetic: made using plants, but undergo chemical processes in laboratories, such as crack, cocaine, heroin. (III) Synthetic: they are entirely produced in laboratories following specific techniques, such as ecstasy and amphetamine[4].

In Brazil, one of the drugs commonly used recreationally and whose consumption has been growing is marijuana. Specialists in the health area do not advise the use of marijuana, all of them disapprove due to the physiological evidence generated by the effect of the indiscriminate use of the drug. There are enough indications that it can be considered dangerous. The potential dangers for our minds are serious[5]. As a result, there is overwhelming consensus that this drug should not be used haphazardly, and no responsible medical body supports such action[6]. However, with the evolution of science, there are already plant-based products that help in the treatment of diseases, such as multiple sclerosis and autism[7].

Cannabis sativa L. (Linnaeus) is an herb that belongs to the Cannabaceae family and was botanically cataloged in 1753 by Carl Von Linné[8]. It is seen, therefore, that the advancement of science has made the cultivation of *Cannabis* cover a diversified market, from medicines to weaving, however, among these, the fact that this plant is used as a recreational drug has become a nuisance for consumers. From it, illicit preparations are obtained

such as marijuana, known worldwide also as hashish and hashish oil, which contain chemical compounds called cannabinoids, which are attributed the psychotropic properties that classify these derivatives as narcotics[8].

Due to its mechanism of action and its effects, cannabinoids is classified as a CNS (Central Nervous System) disturber. The responsible for the effect is the raw drug, since it is derived from the *Cannabis* plant as it is the dried flowers and leaves of this plant. It is found on the illegal market unaltered, raw, pressed into tablets and is commonly smoked. In Brazil, the *Cannabis* plant is also referred to as marijuana, a word that in Portuguese is believed to be an anagram of the word hemp, that is, in portuguese "MACONHA" is an anagram of "CANHAMO". Marijuana is considered an illicit drug in practically the whole world and the history related to its prohibition is long[9].

As for the identification of such a class of substances, cannabinoids, the main tests in case of flagrante delicto are colorimetric tests, highlighting the presumptive Duquenois-Levine test and *Fast blue b* (double salt of *o*-Dianisidinabis chloride (diazotitizate) of zinc, ($C_{14}H_{12}Cl_2N_4O_2ZnCl_2$)). The proposed reaction for cannabinoids with *Fast blue b* occurs through coupling, which promotes the appearance of chromophores with the active part of the substance of forensic interest. In the colorimetric test with *Fast blue b*, the coloration initially yellowish, it can assume three different colors due to the presence of the main response components, the orange color for the presence of Cannabidiol (CBD), lilac/purple for the presence of Cannabinol (CBN) and reddish for the presence of Δ^9-tetrahydrocannabinol (Δ^9-THC)[10].

A noteworthy point when studying this drug is the conversion of Δ^9-tetrahydrocannabinol (Δ^9-THC) into CBN. This occurs because the oxidation of Δ^9-THC into cannabinol (CBN) is a known and well-understood chemical process, such as secondary decarboxylation, and it is important to highlight that Δ^9-THC is a psychoactive compound while CBN is a less psychoactive compound. This secondary decarboxylation process occurs when Δ^9-THC is exposed to a source of oxygen and prolonged heat, such as light and air, a process that is usually common when used as a narcotic due to storage and transport[9]. During this process, a part of the methyl group (-CH_3) is oxidized to form aldehyde, which is further hydrolyzed to form cannabinol[11]. While CBD is – arguably – currently the most well-known cannabinoid, CBD conversion could also potentially lead to other products that can cause psychotropic effects, which have not been examined by most studies thus far. Another point discussed here is the transformation of CBD into Δ^9-THC under in vivo conditions, here the probable transformation occurs by the interaction of the hydroxyl with the pi bond present by proximity, the probable mechanism follows the rupture of the pi bond for the formation of a heterocyclic structure, as shown by Δ^9–THC[11].

These conversions allow temporally classifying samples exposed to the environment, which helps to identify the distance from production as a function of transport time, and whether a sample has been on the streets for a long time or not. It is seen, however, that more precise techniques, if compared to traditional colorimetry, are necessary in the quantification of such substances[12]. Chemical investigation has its own branch, the forensic sciences that are based on the concept that there can be no crime without some evidence, that is, there is no transgression without the appearance of a trace that, after due analysis, has been verified, technically and scientifically, its relationship with the investigated fact.

One of the most used techniques in forensic sciences is High Performance Liquid Chromatography (HPLC), one of the most resolving tools in analytical chemistry due to its ability to

separate/isolate substances present in complex matrices. The technique is capable of separating compounds that are present in virtually any sample that can be dissolved in a liquid. The method uses the principle that a sample solution is injected into a column with the aid of a pressure pump, this column is made of a porous material, called the stationary phase, and a liquid, called the mobile phase, which is pumped at high pressure. Substances are separated on the basis of different polarity relationships/intermolecular interactions between mobile phase, analyte and stationary phase. Depending on the partition behavior of different components, elution occurs at different times[13].

Within the sample, analytes with higher affinity for the stationary phase will travel more slowly and will displace a shorter distance inside the column compared to analytes with lower affinity for the inner wall of the column, so these reach the detector first to the detriment of those with higher affinity. to the substance impregnated in the column. HPLC is versatile because: it is not limited to volatile and thermally stable samples, and the choice of mobile and stationary phases is wider. It is worth noting that the method has numerous analytical advantages, such as: simultaneous analysis of more than one substance, high resolution, high sensitivity, good repeatability, low sample quantity, moderate analysis condition, easy to fractionate the sample and purify[14].

However, there are chromatographic parameters that must be evaluated and controlled to maintain the efficiency of the technique, which makes the method quite expensive for a routine practice. The appropriate mobile phase, column, column temperature, pressure, and gradient must be found to provide adequate analyte compatibility and stability, as well as degradants and impurities. It is seen, therefore, that it is a valuable technique for exploratory analyzes and can be used as a counterproof of cheaper and faster analyzes[15].

A growing trend is colorimetric analysis, given the greater reliability since the development of digital images through the RGB (Red/Green/Blue) system. This is based on the organization of a matrix built by lines and columns, where the indices determine a point in the image, these points are defined as “image elements” or simply pixels. In this way, each pixel is built by an intensity of colors in the RGB system, that is, there is a response value for each of the three RGB colors that, when associated, provide different colors. Thus, the relationship between RGB color systems and colorimetric tests allow obtaining qualitative data and quantitative analytical measurements[16]. However, the generation of this image and its conversion to an analysis method is a little more complicated, given the high number of data generated.

The emergence of a pocket computer allowed solving one of the problems associated with colorimetry, with the advent of the smartphone both software and hardware enabled its integration into everyday life. The low cost combined with the immediate response makes the obtaining of data by analytical practices based on colorimetry gain prominence in several areas, such as food, beverages, fuels and more recently in drugs[17]. In airports, to assess the presence of narcotics, for example, tests are performed visually, that is, with the human eye, where the use of digital images could minimize the recurrent subjectivity in these types of analyses. In order to make these practices more efficient and available on a daily basis, laboratory miniaturization technologies are being developed.

One of the most recent technologies refers to Micro Paper Devices (µPADs), or paper microdevices. This technology aims at printing shapes on paper using a wax printer. The wax prevents the propagation of the solution by limiting the sample region in the case of solutions with polar

solvents, which makes the technique very selective for the analytes under study. The main tests for drugs such as cocaine, crack and marijuana can be carried out using solvents thus enabling the use of µPADs in their practices[18].

It is seen, then, that an association of this technique with drug analysis has a great chance of making illicit drug identification tests faster, more sensitive and cheaper. It is also worth noting that the high selectivity reported for the analysis opens the way for other possibilities how quality control of medicines based on this substances and tests with human body fluids in cases of people who used one of the aforementioned drugs.

2 THEORETICAL REFERENCE

The use of such an application in paper-based colorimetric techniques is not yet widespread, even though it is a cheap and fast practice, since colorimetry is dependent on the specificity of each substance, making it difficult to use for more complex matrices. However, a technique, microfluidics, has allowed the use of colorimetry even in more complex matrices, mainly due to its small analysis region.

It is worth noting that microfluidic paper-based analytical devices (µPADs) are used as colorimetric sensors in low-cost biological analyses. Paper is mainly composed of a cellulose polymer, which makes it porous, favoring the microfluidity of liquids between the fibers due to the capillarity process[19]. There are advantages of using paper, which can be listed: the great abundance, low cost compared to other platforms for sensing, easy to obtain and handle, compatibility with large-scale production of microfluidic devices, possibility of long-term storage, easy physical modification and chemistry of its surface for bio-assays, easy disposal through incineration making it more environmentally correct, possibility of using reduced volumes of samples (micro to nanoliters depending on the resolution of the barriers created in the paper), white coloring (adequate for colorimetric tests), among others. However, some factors may also hinder the use of such a matrix, such as sensitivity to humidity and the homogeneity of its structure[20].

When using µPADs for colorimetric analysis there are listed gains such as portability, accessibility and simplicity of the devices. However, its applicability cannot always be carried out directly, requiring a previous treatment of the sample to avoid interferences in the color of the solution, inconsistencies in lighting, lack of uniformity, or the presence of particulate contaminants that can confuse the interpretation of the colorimetric result. It is possible to combine these pre-treatment processes in a single paper disposable device[21]. So much so that the number of publications on the subject is growing gradually over the years, publications arise from various fields of research.

Thus, this work seeks to compare optimized µPADs systems for the detection of the three main cannabinoids, CBN, CBD, and Δ^9-THC, in marijuana samples seized by the Civil Police of the state of Espírito Santo made available to the laboratory by a partnership. For this, a µPAD was developed to compare colorimetry against a standard. With the aid of a HPLC equipment, it was possible to validate with the traditional analysis technique.

As a response, the light intensity recorded by the Photometrix® application was compared with a calibration curve with seven points of known concentrations of CBN standard and mixtures of CBN, CBD, and Δ^9-THC in order to evaluate the influence of the matrix. Moreover, chemometric tools such as Principal Components Analysis (PCA) and Partial Least Squares (PLS), which are part of the Photometrix® application, were used to search for temporal and areal clustering of the samples as well as to predict the

content of Cannabinol (CBN) present in seized samples, respectively. Thus, the construction of the colorimetric profile of the tests for the samples can help in the identification of the substances, in addition to allowing the traceability of future seizures *in loco*.

3 MATERIALS AND METHODS

3.1 µPADS

A Samsung® J8 Smartphone with a software Photometrix® was used for colorimetric analysis by µPADs; the PLS technique to search for rapid quantification, and the PCA technique to search for clusters that can be correlated to physical properties of the 66 marijuana samples seized by the Civil Police of Espírito Santo and provided by a partnership with the laboratory (cooperation agreement No. 1007/2012).

The µPADs were designed in software Office® and printed on A4 papers with the ColorQube 8880 wax printer and the wax provided by the printer manufacturer. The cannabinoids were extracted from the samples by exposure to methanol. The colorimetric response confirming the presence of cannabinoids occurs when the solution 0.25 % m/v of *Fast blue b* salt presents marsala coloration, resulting from the mixture of three main colors, purple (positive response for CBN), orange (positive response to CBD), and red (positive response for Δ^9-THC).

The construction of the µPADs followed the development of circles of 8 mm in diameter, and the printing was done in a wax printer as already reported in previous paragraph. For full impregnation of the wax to the paper, the µPADs were heated in an oven at 120 °C for 5 minutes, which allows the wax to melt and impregnate the paper as a whole, leaving only the circular area still hydrophilic.

After the preparation of the µPADs, the same procedure was performed for all samples: 100 mg of the seized sample was added to a 4 mL vial and to this was added 2 mL of methanol; the vial was shaken and closed, and the extraction process took place for 24 h at room temperature in the laboratory. After extraction, 3 µL of 0.25% w/v *Fast blue b* solution was pipetted with a micropipette, and a 5-minute wait was allowed for the total and uniform impregnation of the µPAD by the solution. Then, 3 µL of the extract of each seized sample was pipetted onto the µPAD impregnated with the *Fast blue b* solution[21]. After 30 minutes, the Photometrix® application was used to build the PLS model and predict the CBN contents based on a calibration curve constructed with known solutions ranging from 1, 2, 3, 5, 10, 15 and 30 mg · mL^{-1} that were constructed from a standard solution of concentration 1 g · mL^{-1}.

Moreover, a PCA was performed to determine similarity among the samples of seized marijuana. Note that the PCA looked for similarity by area and time, that is, whether the samples would cluster by area of seizure or by time of production. The samples were divided into three municipalities (Serra, Vila Velha, and Vitória) and in 22 months ranging from August 2015 to May 2017; thus, each municipality has one sample per month, totaling 66 samples, as shown in Table 1S, it is noteworthy that these samples will be identified with date of seizure, municipality of seizure, and corresponding number in the analysis. For example, the sample 08/15 S (1) corresponds to a sample seized in August 2015 in the municipality of Serra, and will be identified as 1.

For data analysis, the application Photometrix® was used, which is available for free on the Android® and IOS® app stores (http://www.photometrix.com.br). The application contains multivariate analysis tools and allows performing PCA, PLS, and hierarchical cluster analysis (HCA).

The PCA analysis method performed by the application has as its

main characteristic speed and ease, counting on few steps to the final data, without the need for an image treatment to interpret the results and construct the graph.

To perform this test, a Samsung® J8 smartphone was used (16-megapixel camera, F 1.7 aperture, digital stabilization, 4032 × 3024-pixel resolution, and Android® 8.1 operating system). The samples were deposited in µPADs, 3 µL, with the aid of an automatic micropipette, then 3 µL of *Fast blue b* solution (0.25%, m/v) were deposited. After the pre-established time, that is, 30 minutes of total impregnation, photographs were taken with the µPADs in a white mini photographic studio (Photo Studio Led USB Portable), dimensions 22 cm × 25 cm × 25 cm, to standardize the images and avoid the influence of other interferences. Then the data were processed.

3.2 HPLC

The confirmation of the values of CBN, CBD, and Δ^9-THC content was confirmed by an injection of 5 µL of *Cannabis* extract in the liquid chromatograph Agilent Infitnity 1260 (Palo Alto, CA, USA) in Phenomenex® Gemini C18 column (250 × 4.6 mm, 5 µm) with oven at 35 °C in an isocratic flow of 1.5 mL · min^{-1} of 35:65 solution of Water: Acetonitrile for 25 minutes.

The instrument was controlled by the Agilent Open LAB Chromatography Data System® program. The detection occurred by the diode array detector (DAD) of the equipment monitoring the wavelength of 228 nm, since the literature states that this is the area of best study[22]. It is noteworthy that the calibration curves used in both the µPAD and HPLC analyses were constructed using CBN, CBD, and Δ^9-THC standards of concentration 1 g · mL^{-1} each produced by Cerilliant Corporation in the United States of America, in the municipality of Round Rock, Texas. The extracts were diluted 1000 times in order to fit the conditions of the equipment, since it has high sensitivity.

4 RESULTS AND DISCUSSION

4.1 µPDAS

After the methodology described in item 3.1, **Figure 1** was obtained, which corresponds to the µPDAs of the extracts of the seized marijuana samples (named 1 to 66) and the calibration curve of the CBN standard, with concentration ranging from 1 to 30 mg mL^{-1} (in the image named C1 to C30). The marsala staining shows the presence of the class of the investigated analytes, that is, cannabinoids, confirming that as a qualitative test, the analysis has notorious efficiency, with a 1.24 mg mL^{-1} detection limit. It is noteworthy that each spot – µPADs – consumes only 3 µL of methanolic solution, which makes the analysis non-destructive as it consumes a minimal amount of sample.

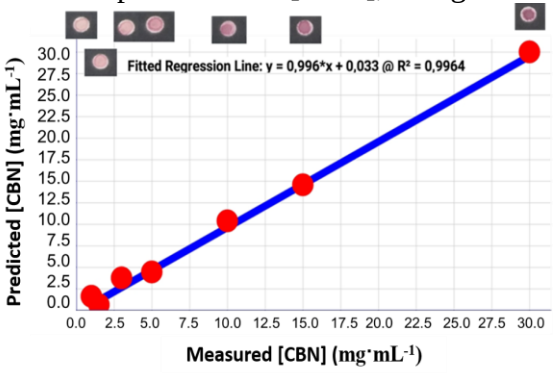
All samples analyzed (n = 66) showed a positive response for any of the cannabinoids Δ^9-THC, CBD, and CBN, since they give a colorimetric response to *Fast blue b*, even if differentiated[14], as Figure 1 shows. Δ^9-THC, CBD, and CBN present red, orange, and purple, respectively, in which the marsala color presented for the extracts of the seized samples should be a positive response to the presence of the cannabinoid group, resulting from the main combination of these substances[23].

Figure 1. Figure 1. Photograph of the finished test of seized marijuana samples—named from n = 1 to 66—and solutions of the CBN standard at concentrations from 1 to 30 mg mL^{-1}, being named C1, C2, C3, C5, C10, C15, and C30

Fonte: author

With the solutions obtained by diluting the standards, as already highlighted in item 3.1, it is possible to obtain the calibration curve and build a regression model for PLS (**Figure 2**). Note that the x-axis of the curve are the concentration values of the solutions prepared from the standard, while the y-axis represents their predicted values as a function of colorimetry with the aid of the Photometrix® application; for example, the sixth point of the curve has a known concentration value of 15 mg · mL^{-1} (x-axis) and a value of 14,7 mg · mL^{-1} when predicting by PLS with its image by the application. The curve showed R^2 = 0.996, besides an RMSEC equal to 0.5761, which informs the ratio of the mean square error of the values of the calibration curve, establishing that the constructed curve has good correlation with the prediction, that is, it is representative of reality. The analytic curve generated a regression equation: y = 0.996× + 0.033.

Figure 2. Calibration curve constructed by PLS for prediction of [CBN], in mg mL^{-1}



Fonte: author

Figure 2 presents the correlation equation between the actual value, the measured one (x axis), and the predicted value, the calculated one (y axis) as a function of the colorimetric intensity recorded by the smartphone's digital image. From the equation: y = 0.996x + 0.033 of the models built in **Figure 2**, it was possible to estimate the CBN concentrations in the *Cannabis* extract samples as a function of their colorimetric response, which are reported in **Table 1S**, where their values ranged from 8.64 to 21.48 mg mL^{-1} with mean percentage of 14.11 ± 2.47 mg mL^{-1} with Detection Limit of 1.24 mg mL^{-1} and Quantification Limit of 4.13 mg mL^{-1}. **Table 1S** presents the values obtained for samples, it is worth highlighting the identification already described in item 3.1.

As already discussed, the formation of the colorimetric spectrum of a *Cannabis* extract is constructed according to its composition. In this case, the three main substances that react colorimetrically to *Fast blue b* are CBN, CBD, and Δ^9-THC, being purple, orange, and red, respectively[23]. The result is unique for each extract since it comes from the composition of these three colors in different intensities, and since this is influenced by the proportion of each substance in the extract, it is impossible to distinguish these intensities with the naked eye.

However, an image structured in the RGB system uses the intensity of these colors for each of its points (pixels), being much more efficient in its distinction when building three-dimensional matrices of the colorimetric intensities obtained. With these matrices, it is possible to search for similarities of these intensities at multivariate level, which was applied in PCA, as shown in **Figure 3**.

After obtaining each CBN concentration value by PLS regression, the confirmation of these values or the discovery of a proportionality factor was performed by a traditional technique,

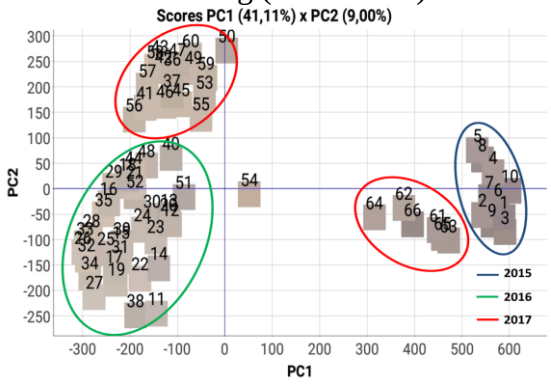
HPLC, which is, according to the United Nations Office on Drugs and Crime (UNODC)[24], the most recommended. But before that, a PCA was performed with the images generated by the positive response colorimetry (results in **Figure 1**) of each seized sample in order to seek correlations between the samples and to know if there were any clusters of similarity that could relate them by time of environmental exposure and/or region seized.

We believed that colorimetric differences would be influenced by region seized and/or time of exposure to the environment, and thus PCA was applied in the search for possible clusters. However, the component that most influenced the analysis was the CBN content, which allowed grouping by environmental exposure time in a trivial shelf time association. As previously stated, the samples are divided into three different municipalities over 22 months, from August 2015 to May 2017, totaling 66.

When using the graph PC1 × PC2, clustering was already observed (**Figure 3**); based on the identifications of the samples, the groups represented the period of its apprehension. The explanation may be related to the conversion of Δ^9-THC into CBN, in which the 2015 and 2016 samples are expected to have a higher concentration of CBN (**Figure 4**). This temporal classification of drug exposure can help in identifying the distance of production as a function of transport time, and whether a sample has been on the streets for a long time or not. Note that by increasing the number of samples, which was possible by the incorporation of new samples to the chemometric model, the temporal method could be more selective. Moreover, the 2017 samples are in two groups, possibly due to the time required for the conversion of Δ^9-THC into CBN in a natural way, which may thus influence the results.

Unfortunately, there was no clustering by area in any of the analyzed graphs of the PCs; it is possible that the suppliers are not consistent with the seizure locations. Note that the 2017 samples are in 2 groups, possibly due to the fact that this is the time required for the conversion of Δ^9-tetrahydrocannabinol (Δ^9-THC) into CBN in a natural way and thus may have influenced the results.

Figure 3. PCA of seized samples of marijuana (n = 66) with temporal clustering (PC1 × PC2)



Fonte: author

Furthermore, the fact that the reaction of Δ^9-THC with *Fast blue b* generates a reddish coloration influences the identification of the purple coloration of CBN with the same reagent, generating the marsala obtained, which groups the samples in a similar way to the older ones. However, the Δ^9-THC converting to CBN decreases this reddish over time, which entails a decrease in color intensity, resembling the samples to the 2016 group. In other words, the difference in the colors of each substance, perceptible by imaging algorithm, allows us to observe the conversion of the substances over time, clustering the samples into temporally different portions.

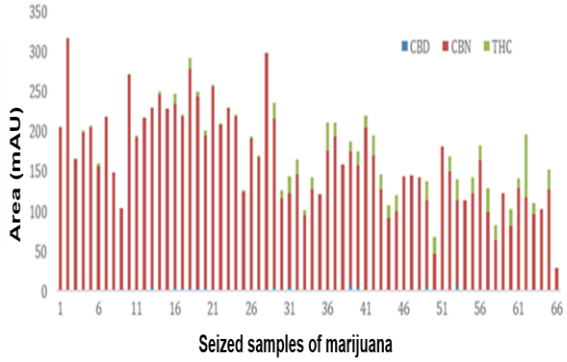
4.2 HPLC-DAD

Figure 4 shows areas results obtained by HPLC of three cannabinoids of *Cannabis* extracts from seized samples of marijuana (n = 66). It is containing the area values for cannabinoid compounds CBD, CBN, and Δ^9-THC (retention times = 9, 12, and 15 minutes, respectively). Over time (from t = 2015 to 2017), the Δ^9-

THC content, in green, starts to decrease and the CBN content, in red, gradually increases, as sample 1 (August 2015) is classified as the oldest compared with the sample 66 (May 2017), for example.

That is, the younger sample, the higher its Δ^9-THC content, which decreases over time due to its possible conversion into CBN, as already explained in the introduction. This reinforces the possibility of using the conversion of Δ^9-THC substances to CBN as a temporal identifier of environmental exposure, that is, shelf time, since the reaction time is known and thus can be used for this. Finally, the later conversion of other substances present, such as CBD, into CBN, starts to influence the response, increasing its value by promoting the emergence of another, higher intensity group, 2015. This stems from the temporal differences in the conversions of CBD into CBN and Δ^9-THC into CBN[25]. All these values were also investigated by the traditional HPLC technique.

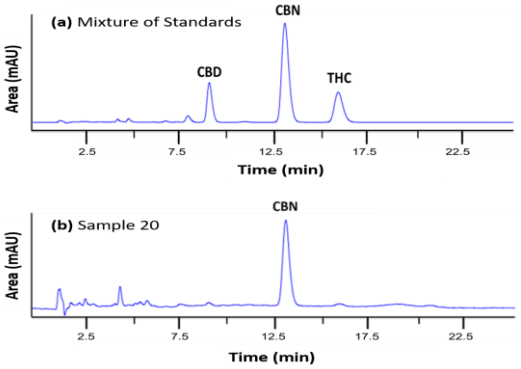
Figure 4. Number of seized samples of marijuana (n = 66) in function of detection of CBD, CBN, and Δ^9-THC (in mAU).



Fonte: author

The use of HPLC allowed to validate the possibility of quantifying CBN by colorimetry with μPADs since two more substances, Δ^9-THC and CBD, also react to *Fast blue b* by generating color, that is, a whole class of prohibited substances reacts to the same substance (**Figure 5**). The chromatography was performed by diluting the samples 1000 times in order to meet the study region of the equipment. However, each substance of these substances generates different colors in different spectral regions, which allows investigating the samples in a unique way for a particular substance, that is, CBN, since the equipment allows choosing the wavelength to be studied.

Figure 5. Chromatogram of *Cannabis* extract from seized marijuana sample, in which (a) represents a curve with the standards equimolar mixture and (b) a typical *Cannabis* extract from marijuana sample, sample 20.



Fonte: author

The intense signal observed in the seized samples (**Figure 5b**) corresponds to the substance CBN; this was observed in several other samples and in the standards used for the calibration curve, thus confirmed by the retention time of approximately 13 minutes, unlike CBD, which has a retention time of approximately 9 minutes. The chromatograms of other seized samples are presented in **Figure 1S** in the Supplementary Material. The longer a sample is exposed to the environment, the higher the amount of CBN, since the Δ^9-THC, with a retention time of 16 minutes, converts to CBN over time. Consequently, its concentration tends to decrease over time.

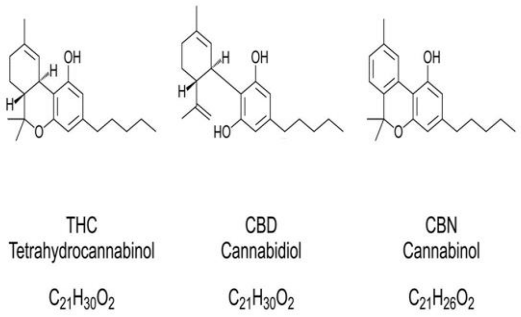
Given the chromatograms of the samples used in this work (**Figure 1S**), the difference reported can be observed when

comparing the set of chromatograms of samples from 7 to 12 and the set of samples from 61 to 66 that have a difference of 20 months. In the second set, the "younger," Δ^9-THC has a relevant area, whereas in the first set, the "older," Δ^9-THC signal practically does not appear[36], confirming what was proposed by the PCA analysis and also observed in **Figure 4**.

To construct the calibration curve by HPLC, the concentrations of 1, 3, 5, 10, 15, and 30 mg mL^{-1} of CBN were used, which generated the chromatograms of the Supplementary Material, as **Figure 2S** shows. From the results of the areas of the standards, it was possible to construct the calibration curve (**Figure 7**) for the calculations of the CBN contents in the seized samples.

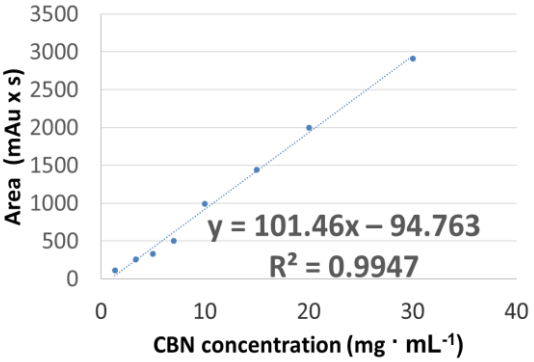
The standards were eluted individually and in triple equimolar mixture in order to observe if the presence of other standards had a significant influence on the results obtained, which was not observed, since these substances absorb better at other wavelengths. Thus, the calibration curve in the attempt of correlation was only CBN, since the presence of other analytes does not interfere in the HPLC technique. The chromatograms of the standard mixtures, equimolar mixture, confirmed the retention times of each substance, being CBD at 9 minutes, CBN at 13 minutes, and Δ^9-THC at 15 minutes. As the C18 column will better restrain apolar substances, the Δ^9-THC restrain only one hydroxyl group in a 21-carbon chain. CBN, on the other hand, even though having the same relation as Δ^9-THC, has two aromatic groups, increasing its polarity. And finally, CBD has the lowest retention time in the C18 column, having two hydroxyl groups in its composition, being the most polar among the three, as shown in **Figure 6**.

Figure 6. Molecular structure of compounds



Fonte: ChemDraw®

Figure 7. Calibration curve constructed by HPLC for [CBN], in mg mL^{-1}



Fonte: author

From the calibration curve equation, y = 101.46× – 94.763, CBN concentrations can be obtained in the seized samples by HPLC. **Table 2S** shows the values found for the samples and the correlation of the values obtained by HPLC.

Unfortunately, the results obtained by µPAD and HPLC showed no correlation. The linear correlation obtained was unsatisfactory, with R^2 = 0.0017 and correlation coefficient (R) = 0.041, which may be related to interfering factors that alter the color in the µPAD, making a linear correlation difficult, as there are other influences. Thus, the µPAD is a technology that is still not efficient for quantitative analysis, but is consolidated as a qualitative screening. Among these, can be highlighted the isomerization between the cannabinoids which occurs with the

passage of time of exposure to the environment, with excessive information for differentiation based on the image since each of these components generates a different color in the presence of *Fast blue b*. However, HPLC showed that µPAD is effective in identifying samples containing cannabinoids, in addition to identifying their temporal exposure to the environment.

5 CONCLUSIONS

The work proposal arose from the need for narcotic analyses to be increasingly faster, more versatile, cheaper, *in loco*, and minimally invasive in terms of samples. This is exactly what is observed with the results obtained. The analyses of *Cannabis* extracts from marijuana samples were positive in all cases and thus an effective tool in the identification of cannabinoids. The quantification showed no good correlation with the traditional analysis by HPLC; however, the chemometric resources allowed to group the samples by time of environmental exposure, which opens a range of possibilities in criminal investigation.

Among the possibilities emerged from this study the temporal tracking of seized samples, which, by a larger spectrum of samples, models of months or even days can be built to discover the *in loco* origin of drugs that are seized. It is thus possible to identify how long a drug has been exposed to the environment and to assess the origin of a shipment, helping in the quick and inexpensive identification of *Cannabis* extracts. Furthermore, low detection limit allows the practice of drug manipulation tests, proving whether a person manipulated marijuana or not.

AGRADECIMENTOS

To the Civil Police of Espírito Santo and the Federal University of Espírito Santo.

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REVISTA ifes ciência

SUPPLEMENTARY MATERIAL

USE OF A PAPER MICRODEVICE TO IDENTIFY CANNABINOIDS IN MARIJUANA SAMPLES SEIZED BY THE ESPÍRITO SANTO CIVIL POLICE - BRAZIL

Table 1S. Result of CBN concentrations in *Cannabis* extract by μPADs

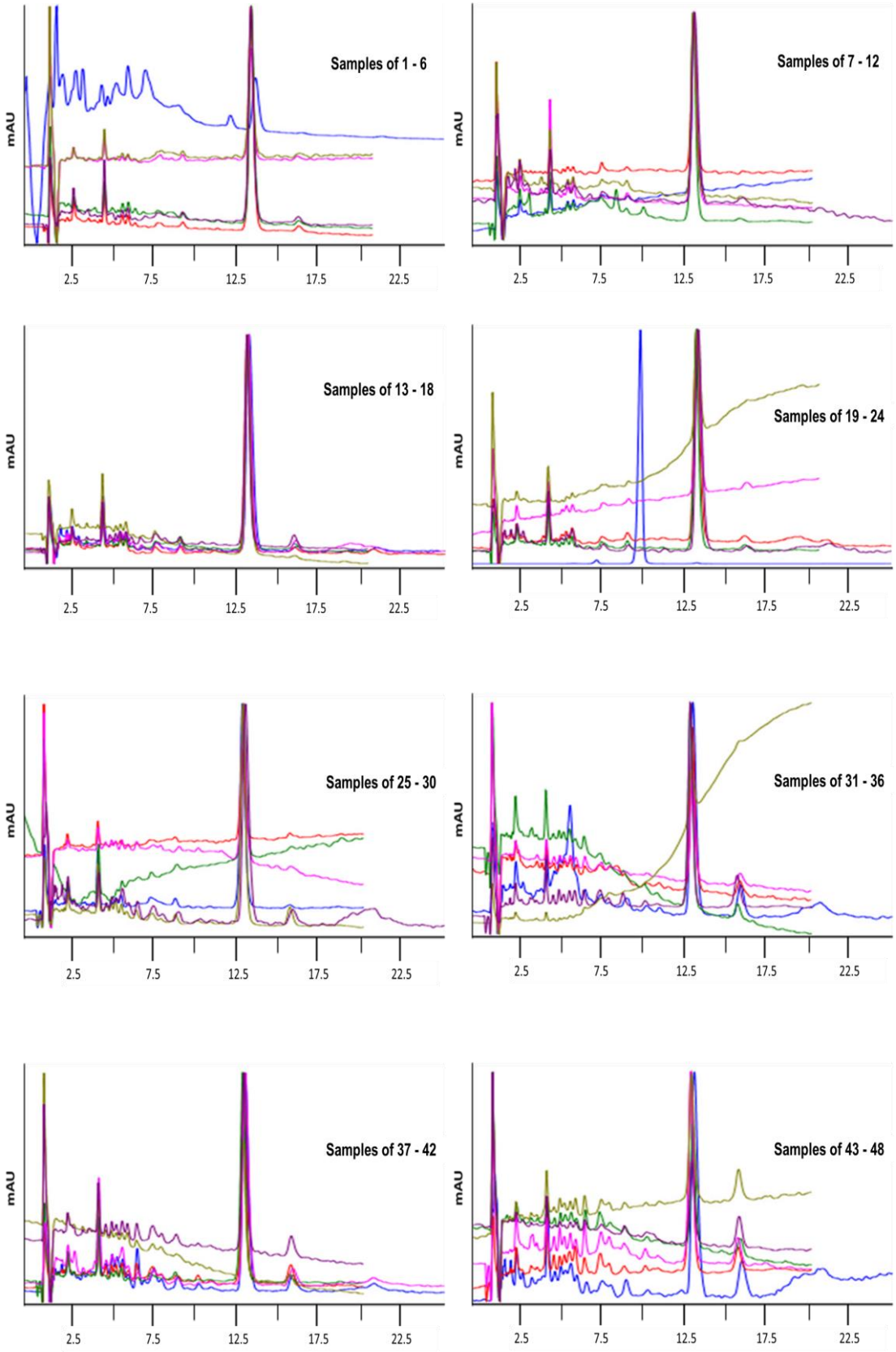
Sample	Concentration (mg mL^{-1})	Sample	Concentration (mg mL^{-1})
08/15 S (1)	21.48	04/16 S (27)	10.34
09/15 V (2)	19.87	05/16 V (28)	14.00
08/15 VV (3)	18.40	05/16 VV (29)	15.11
08/15 V (4)	17.20	05/16 S (30)	12.13
09/15 S (5)	20.51	06/16 V (31)	13.48
09/15 VV (6)	21.45	06/16 VV (32)	16.69
10/15 S (7)	18.75	06/16 S (33)	17.04
10/15 VV (8)	20.77	07/16 S (34)	14.28
10/15 V (9)	20.09	07/16 V (35)	15.35
11/15 S (10)	20.76	07/16 VV (36)	14.44
11/15 VV (11)	13.05	08/16 VV (37)	15.70
11/15 V (12)	9.31	08/16 S (38)	11.53
12/15 S (13)	9.97	08/16 V (39)	11.01
12/15 V (14)	8.64	09/16 V (40)	13.17
12/15 VV (15)	10.67	09/16 VV (41)	13.78
01/16 S (16)	10.89	09/16 S (42)	16.47
01/16 VV (17)	10.85	10/16 VV (43)	13.83
01/16 V (18)	13.42	10/16 S (44)	9.94
02/16 V (19)	13.27	10/16 V (45)	12.45
02/16 VV (20)	12.58	11/16 S (46)	11.64
02/16 S (21)	16.26	11/16 VV (47)	13.11
03/16 VV (22)	12.46	11/16 V (48)	12.08
03/16 S (23)	8.97	12/16 V (49)	13.76
03/16 V (24)	12.66	12/16 VV (50)	13.22
04/16 V (25)	11.99	12/16 S (51)	14.43
04/16 VV (26)	10.88	01/17 VV (52)	15.72

01/17 V (53)	12.50
01/17 S (54)	14.20
02/17 VV (55)	15.36
02/17 S (56)	12.53
02/17 V (57)	11.39
03/17 S (58)	16.42
03/17 V (59)	11.65
03/17 VV (60)	14.11
04/17 VV (61)	12.86
04/17 V (62)	16.91
04/17 S (63)	13.28
05/17 V (64)	14.59
05/17 S (65)	11.44
05/17 VV (66)	14.37
Average concentrations	**14.11**
Standard deviation	**2.47**

Table 2S. Result of CBN concentrations in seized marijuana extract by HPLC

Sample	Concentration (mg mL^{-1})	Sample	Concentration (mg mL^{-1})
08/15 S (1)	2.94	07/16 VV (36)	2.67
09/15 V (2)	2.88	08/16 VV (37)	2.85
08/15 VV (3)	4.06	08/16 S (38)	2.49
08/15 V (4)	2.54	08/16 V (39)	2.59
09/15 S (5)	2.95	09/16 V (40)	2.44
09/15 VV (6)	2.46	09/16 VV (41)	2.96
10/15 S (7)	3.08	09/16 S (42)	2.61
10/15 VV (8)	2.39	10/16 VV (43)	2.20
10/15 V (9)	1.96	10/16 S (44)	1.84
11/15 S (10)	3.59	10/16 V (45)	1.92
11/15 VV (11)	2.82	11/16 S (46)	2.35
11/15 V (12)	3.06	11/16 VV (47)	2.36
12/15 S (13)	3.15	11/16 V (48)	2.34
12/15 V (14)	3.35	12/16 V (49)	2.00
12/15 VV (15)	3.19	12/16 VV (50)	1.40
01/16 S (16)	3.19	12/16 S (51)	2.72
01/16 VV (17)	3.05	01/17 VV (52)	2.42
01/16 V (18)	3.64	01/17 V (53)	1.99
02/16 V (19)	3.30	01/17 S (54)	2.06
02/16 VV (20)	2.80	02/17 VV (55)	2.14
02/16 S (21)	3.44	02/17 S (56)	2.55
03/16 VV (22)	2.97	02/17 V (57)	1.91
03/16 S (23)	3.21	03/17 S (58)	1.56
03/16 V (24)	3.08	03/17 V (59)	2.15
04/16 V (25)	2.14	03/17 VV (60)	1.74
04/16 VV (26)	2.81	04/17 VV (61)	2.21
04/16 S (27)	2.58	04/17 V (62)	2.10
05/16 V (28)	3.88	04/17 S (63)	1.88
05/16 VV (29)	3.02	05/17 V (64)	1.94
05/16 S (30)	2.09	05/17 S (65)	2.19
06/16 V (31)	2.08	05/17 VV (66)	1.22
06/16 VV (32)	2.36	07/16 VV (36)	2.67
06/16 S (33)	1.85	08/16 VV (37)	2.85
07/16 S (34)	2.17	08/16 S (38)	2.49
07/16 V (35)	2.13	08/16 V (39)	2.59

Figure 1S. Chromatograms of seized marijuana samples



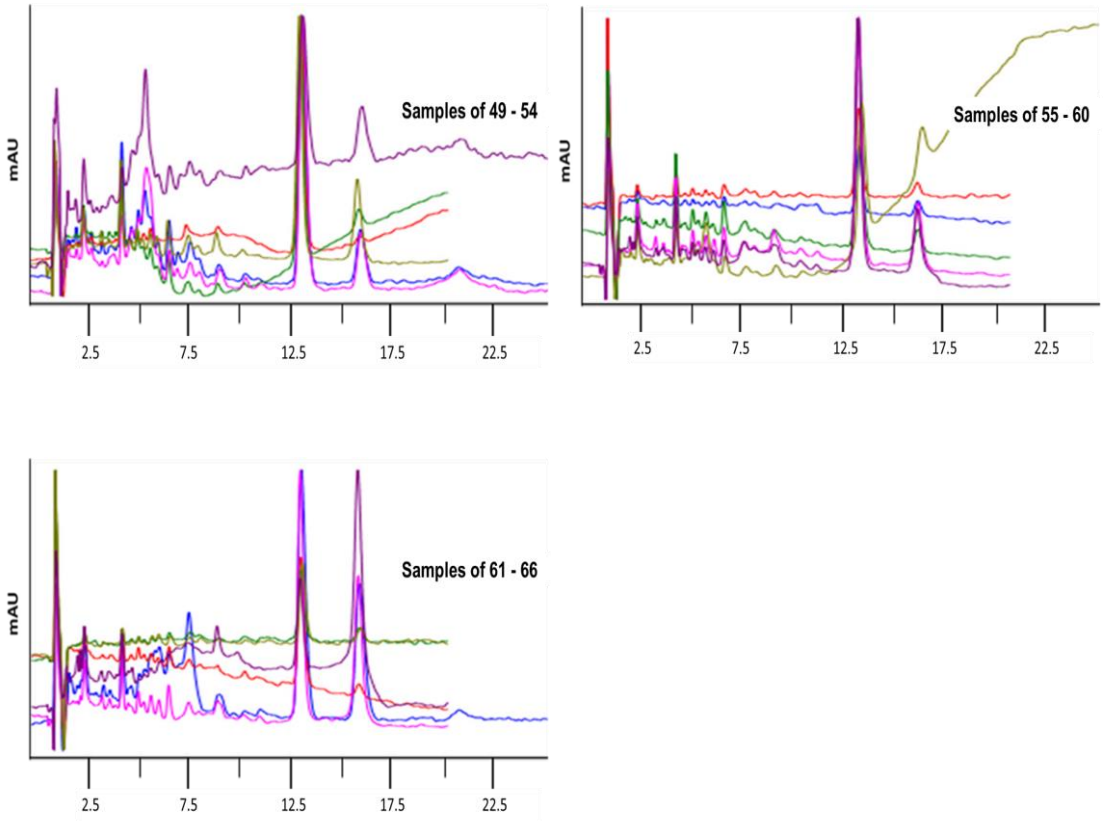
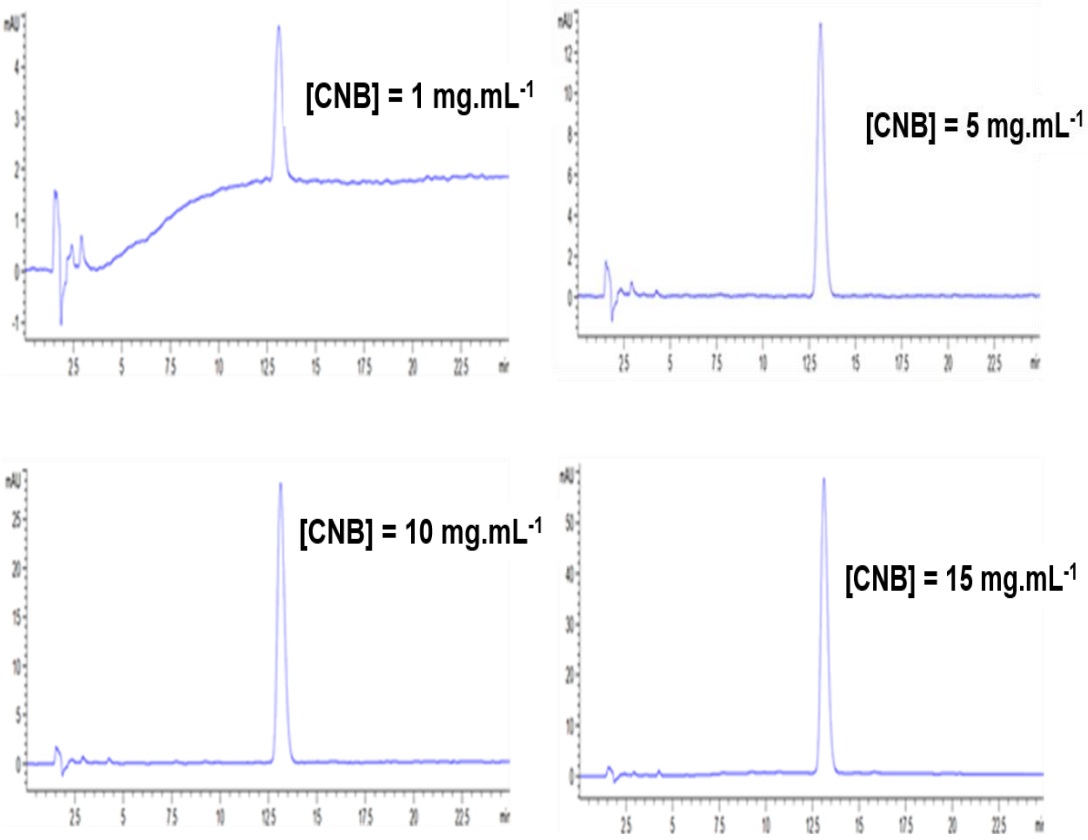
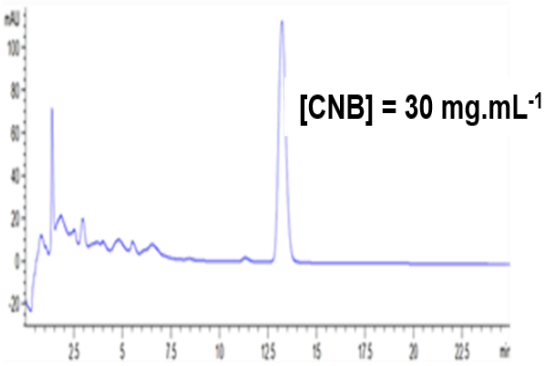


Figure 2S. Chromatograms of seized marijuana samples




[CNB] = 30 mg.mL-1