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A PERÍCIA CRIMINAL COMO INSTRUMENTO DE
PROMOÇÃO DOS DIREITOS HUMANOS

APPLICATION OF MULTIVARIATE ANALYSIS ON DIGITAL IMAGES OF *CANNABIS SATIVA* L. EXTRACTS

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ABSTRACT

Cannabis sativa L is one of the most used drugs in the world. Information about the plant's age and storage can help forensic scientists to identify and to track samples. The ratio between the cannabinoids tetrahydrocannabinol (THC) and cannabiniol (CBN) has been related to the degradation of cannabis with time. Thus, this study aimed to test Multivariate Image Analysis (MIA) to evaluate cannabis extracts according to their colors. Initially, 52 samples of *Cannabis sativa* L. extracts were analyzed by Gas Chromatography coupled to Flame Ionization Detector (GC-FID) to quantify THC and CBN. Afterwards, the extract samples were photographed and analyzed by two

different multivariate analysis tools: ChemoStat[®], a free chemometrics software, and PhotoMetrix PRO[®], an app for mobile devices. Using unsupervised methods of Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) it was found that the more intense the color of an extract, the greater the concentration of THC and in turn, the lighter colored extracts correspond to samples with CBN, without THC. The results suggest a simple method for previous clustering of samples that may precede chromatographic analyzes, assist in chemical profile studies or simply aggregate samples of similar profiles to be analyzed together.

KEYWORDS: *Cannabis sativa* L. Marijuana. Chemometrics. PCA. HCA. GC-FID.

1. INTRODUCTION

Cannabis sativa L. (Cannabaceae) is among the most consumed drugs in the world: only in 2018, there were about 192 million active users (UNODC, 2020). Usually, the aerial plant parts – such as leaves and inflorescences – are dried, compressed and used to make a cigarette for smoking. Cannabis life cycle includes germination and a vegetative state, followed by flowering and seed formation and, in the end, the senescence. During these phases, the ambient conditions and leaf storage, its developmental stage and the seed genetics are the main factors for the cannabis chemistry composition and cannabinoid variability (BONINI *et al.*, 2018; GONZÁLEZ, 2018; BORILLE *et al.*, 2017). More than 750 compounds have been already identified in the plant, including flavonoids, steroids, nitrogen compounds, terpenoids (which are responsible for cannabis fragrance) as well as the main group of compounds, the cannabinoids (BONINI *et al.*, 2018; GONZÁLEZ, 2018; BORILLE *et al.*, 2017). Amongst them, there is tetrahydrocannabinol (THC), its main psychoactive component, and cannabinal (CBN), a cannabinoid whose concentration is related to the plant's age and its storage conditions (BONINI *et al.*, 2018; GONZÁLEZ, 2018; BORILLE *et al.*, 2017). The THC decomposition to CBN is the main route to cannabis degradation (BORILLE *et al.*, 2017; CARBONE *et al.*, 2010; TURNER & ELSOHL, 1979). A previous study shows that THC and CBN concentration ratio could be related to the sample storage time, contributing to the evaluation of the approximate age of a given cannabis sample (ROSS & ELSOHL, 1999). Besides that,

chlorophyll degradation, and the consequent color loss of the leaves, is also associated to the plant's senescence (HORTENSTEINER, 2006). In this context, color has already been associated to plant degradation and age, for the Latin American plant *Ilex paraguariensis*, for example, in terms of food quality and storage (LEWINSKI *et al.*, 2015; NABECHIMA *et al.*, 2014).

In general, Gas Chromatography (GC) techniques are widely used in cannabinoid analyzes in routine toxicology laboratories, allowing for the unambiguous identification and quantification of cannabinoids (RAMIREZ *et al.*, 2019; BRIGHENTIA *et al.*, 2017; BRUCI *et al.*, 2012). Gas Chromatography-Mass Spectrometry (GC-MS) is a gold standard method for qualitative analysis of cannabinoids and the GC coupled to a Flame Ionization Detector (GC-FID), which is the recommended method for quantitative analysis (UNODC, 2009).

Geladi *et al.* (1986) introduced the use of image multivariate data as analytical signals in chemistry, and from the joint application of multivariate data analysis tools and of digital image processing known as Multivariate Image Analysis (MIA) (GELADI *et al.*, 1986). MIA keeps up with computational advances and has been gaining space with the use of algorithms that allow the simultaneous manipulation of a large amount of data. Analytical methodologies that employ MIA have advantages such as speed, low cost, less waste generation and greater logistical facility when compared to conventional analytical methodologies (DAMASCENO *et al.*, 2015) and has been studied in several areas, according to color variability (BRERETON, 2009). MIA is comprised of a set of tools that can be applied to the characterization of many different samples, such as Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). PCA consists of data matrix transformation, which can represent the high number of variables within a small number of factors, reducing the experimental dimension. HCA is a useful analysis to determine objects' similarity and to identify anomalous samples (BRERETON, 2009). Free software like ChemoStat® and PhotoMetrix PRO® can be used for MIA applications. ChemoStat® software was designed for exploratory data analysis. The software works on spectral data, acquired from infrared or image chemometrics, to the decomposition of color layers by way of pixels (HELFER *et al.*, 2015). PhotoMetrix PRO®

(HELFER *et al.*, 2017) is a colorimetric analysis tool, developed for mobile devices, which employs PCA, HCA and PLS methods; this app uses the mobile camera to capture digital images, decomposing them into scores and loadings for multivariate analysis. This app has already been used in environmental analysis (LUMBAQUE *et al.*, 2019; GRASEL *et al.*, 2016), in food analysis (BOCK *et al.*, 2018; HELFER *et al.*, 2018) and in Forensic Documentoscopy (GORZIZA *et al.*, 2020; VITTORAZZI *et al.*, 2020).

The aim of this study is to differentiate cannabis extracts samples by its color intensities, using MIA techniques, with both ChemoStat[®] and PhotoMetrix PRO[®] software. These results were then compared to known quantitative values of THC and CBN, using GC-FID, in order to verify the possibility of a correlation between the extract's color and its cannabinoid content, for a possible sample age estimate.

2. MATERIALS AND METHODS

2.1 CANNABIS SATIVA L. SAMPLES

Fifty-two samples of *Cannabis sativa L.* were obtained through a joint project between the Department of the Federal Police of Rio Grande do Sul and the Federal University of Rio Grande do Sul (CAPES Pró-Forenses n° 23038.006845/2014-91). The samples were obtained from different seizures, as well as from outdoor cultivation and were later received in the laboratory in March 2019 without more precise information about the collection period. The sample preparation was adapted from the UNODC guideline (UNODC, 2009). The samples were dried and crushed with the aid of a ball mill until homogeneous, but without specific granulometry. Then, 50 mg of a sample was transferred to a 5 mL volumetric flask. 5 mL of HPLC grade methanol (Merck Milipore[®]) acted like an extractor solvent. The extraction was done with the aid of an ultrasonic bath (Eco-Sonics Ultronique Q3.0 / 25A) for 12 minutes, at temperature of 25 °C followed by centrifugation (Centrifugador Excelsa 2 - Fanem LTDA) at 1500 rpm for 3 minutes. Finally, the supernatant was transferred to an identified amber flask and kept refrigerated until analysis.

2.2 GAS CHROMATOGRAPHY COUPLED TO FLAME IONIZATION DETECTOR (GC-FID) ANALYSIS

The extracts were analyzed on the same day of the extraction in a gas chromatograph (GC) coupled to a flame ionization detector (FID) (Perkin Elmer, model XL GC Autosystem), with a manual injector and equipped with a column DB5-MS (30 m x 0.25 mm x 0.25 μ m). Nitrogen was used as the carrier gas (flow of 1.4 mL / min). The initial oven temperature was 230°C, for 2 minutes. Then, a temperature gradient was applied, with an increase of 15°C per minute, finally reaching 300°C. The final temperature was kept for 5 minutes. The injector was set for 20:1 split mode, at 280°C, and the detector was maintained at 300°C. The volume of sample injected was 1 μ L. These analyzes were carried out after the analytical validation of the method. The validation followed the relevant recommendations (BRASIL, 2017) and the data is reported in an article submitted for review.

2.3 MULTIVARIATE IMAGE ANALYSIS (MIA)

After the GC-FID analysis, 200 μ L of each cannabis extract was pipetted into a 96-well culture plate, for the image capturing. Figure 1 shows the sample disposition. All image collection was done with an android technology smartphone model G6 Play XT-1922-7 (Motorola®) with a 13 MP camera. The smartphone was placed on a 7 cm platform (Figure 2), in order to standardize all image collection and to focus the camera. The platform with the smartphone remained fixed, and the 96-well plate was positioned below the smartphone and moved spot by spot, manually, to capture each image. The ambient lighting conditions were all the same for all samples.

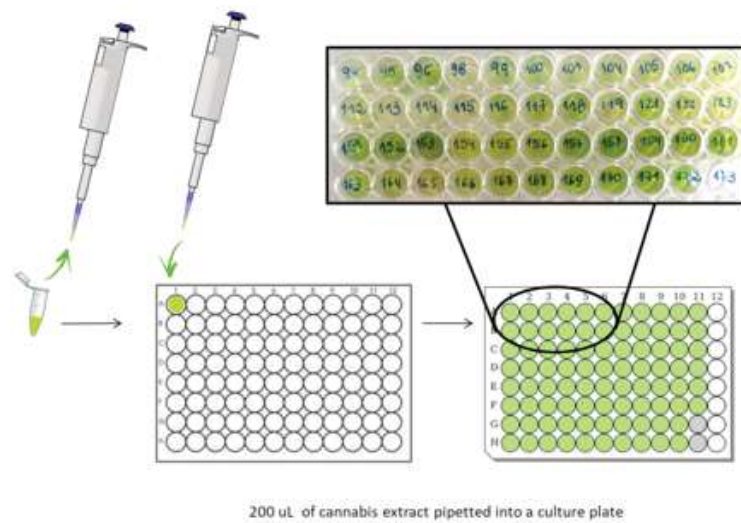


Figure 1: Cannabis extracts arranged on the plate for multivariate analysis.



Figure 2. Platform support for smartphone.

ChemoStat[®] software can be downloaded at <http://www.chemostat.com.br/>. For ChemoStat[®] analysis, after each image collection using the smartphone, each one was cut to a 32 x 32 pixel size, in three central vertical regions, using the software itself. As the results were reproducible, they were defined by the use of the central cut only.

In Multivariate Image Analysis (MIA), data from Red (R), Green (G) and Blue (B) histograms can be analyzed (or from each color's corresponding component relative R, relative B, relative G), and also Hue (H), Saturation (S), Value (V), Intensity (I) and Luminosity (L) channels.

These are imported components of an image file, either in the “bmp”, “jpg” or “png” formats. The chosen methods for image analysis were the exploratory multivariate PCA and HCA, by selecting the percentages of the R, G, B color channels and the H, S, V, I channels, in the mean-center mode. Excluding the L channel was better for the image’s analysis. The detection of anomalous samples (outliers) was evaluated by both the *Hotelling* T² method and the histogram visualization.

PhotoMetrix PRO[®] is a free app and it is available for Android, Windows Phone and iOS smartphones, and it can be downloaded at <http://www.photometrix.com.br>. The image collection is carried out with the app itself, which converts the data from the channels (R, G, B, H, S, V, I and L) of image acquisition, and processes it onto scores and loadings when it computes PCA. Figure 3 shows the PhotoMetrix PRO[®] layout and the set parameters for the analysis, performed the same way as the ChemoStat[®] analysis, using all the channels but the luminosity, and the pre-processing of mean centering the data on the average of each variable. The use of mean-center preprocessing was made to centralize the data in the middle of the graph, otherwise the generated results would occupy only one quadrant of the Cartesian plane. These parameters must be chosen before the acquired digital images by the app. The image collection was done using a ROI (region of interest) of 32 x 32 pixels, also the same image size used for ChemoStat[®] analysis. Euclidean distance with average linkage method was chosen to carry out the HCA.

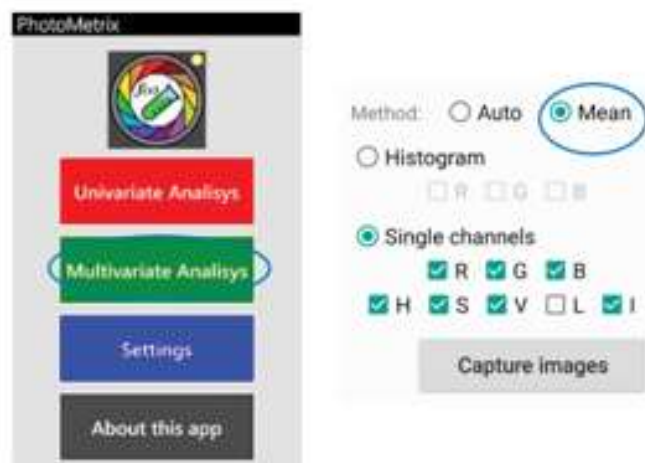









Figure 3: PhotoMetrix PRO[®] app settings.

















Initially, all tests were done in triplicate for each sample. However, due to the excessive number of images (156), the score graph results were difficult to interpret. Once each triplicate result was confirmed reproducible, only one digital image was collected for each sample, resulting in 52 analyzed images (as the ChemoStat® analysis).

















3. DISCUSSION AND RESULTS

3.1 GC-FID RESULTS

Table 1 shows the results of GC-FID analysis, for the quantification of the cannabinoids THC and CBN. Additionally, the digital images cut from each respective sample, used for the ChemoStat® software analysis, is also included to demonstrate its visual color. Based on the quantitative analyzes it is possible to verify that, of the set of 52 processed samples, 59.62% (31 samples) had both THC and CBN, and 25% (13 samples) had only CBN.

Sample	Digital Image Cut	THC (µg/mL)*	CBN (µg/mL)*
94		-	-
95		-	30.70
96		-	26.23
98		< LLOQ**	50.87
99		-	-
100		-	-
101		-	28.29

Sample	Digital Image Cut	THC ($\mu\text{g/mL}$)*	CBN ($\mu\text{g/mL}$)*
104		< LLOQ	27.30
105		< LLOQ	58.71
106		30.09	37.19
107		26.16	39.71
110		< LLOQ	36.00
111		-	37.06
112		< LLOQ	33.03
113		-	36.18
114		-	46.92
115		-	35.88
116		-	32.55
117		-	30.29
118		-	30.86
119		-	83.09
121		< LLOQ	28.95
122		35.48	36.81

Sample	Digital Image Cut	THC ($\mu\text{g/mL}$)*	CBN ($\mu\text{g/mL}$)*
123		34.47	116.64
124		-	32.55
151		41.18	25.95
152		33.08	29.00
153		39.27	23.55
154		112.14	40.04
155		153.01	55.65
156		123.68	43.08
157		54.40	31.24
158		88.39	31.14
159		46.03	27.49
160		107.27	31.78
161		84.35	28.85
162		47.30	23.24
163		< LLOQ	29.30
164		57.04	43.95















Sample	Digital Image Cut	THC ($\mu\text{g/mL}$)*	CBN ($\mu\text{g/mL}$)*
165		27.52	29.82
166		91.09	35.42
167		60.56	23.44
168		58.11	28.78
169		63.47	40.14
170		46.61	27.55
171		63.09	28.07
172		-	40.51
173		-	-
174		-	-
175		-	-
176		-	-
177		-	-
178		-	-

Table 1: Quantification results of cannabis extracts by GC-FID with digital images of the samples.

*average of three determinations; ** LLOQ: Lower Limit of Quantification; - : not detected

3.2 PCA AND HCA PERFORMED ON CHEMOSTAT®

The analysis performed by the ChemoStat® software did not require any pre-treatment resources after transforming the digital images into a data matrix. Figure 4 shows PCA results for the 52 cannabis samples. It is possible to observe that PC1 presents 82.37% variance and PC2 presents 15.42% variance, showing a decreasing trend in the concentration on color (from right to left and from upper-lower).

The THC and CBN samples have more intense color and are in the upper right, as the color becomes weaker, diagonally to the left. The extracts have a THC concentration below the detection limit and/or have only CBN. Samples marked in grey display no quantification for cannabinoids, meaning no presence of THC or CBN.

Figure 5 shows the PCA loadings, indicating that for PC1, saturation is responsible for 83.29% of the variance and for PC2, the hue (predominance of color) justifies 14.57% of the variance.

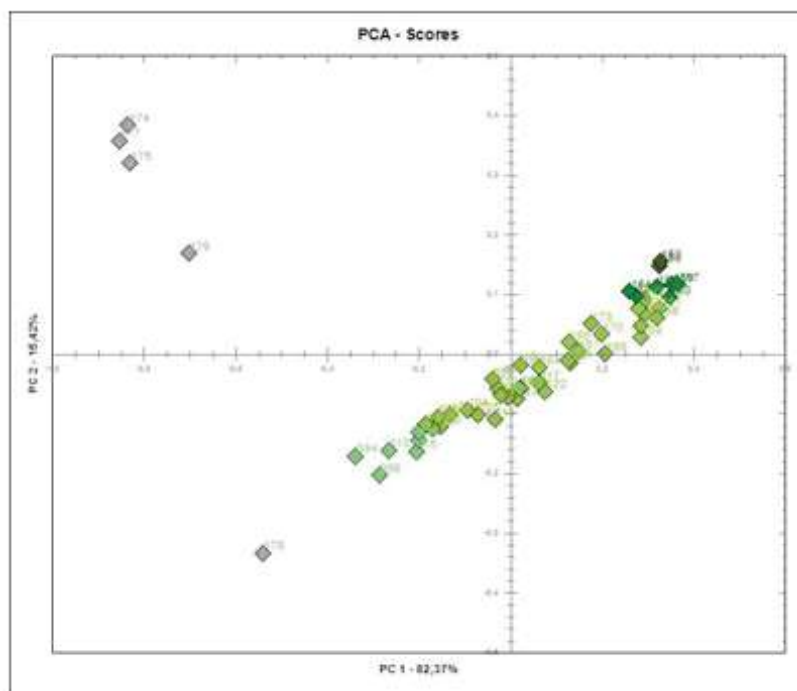


Figure 4: PCA results from the analysis of 52 samples using ChemoStat®.

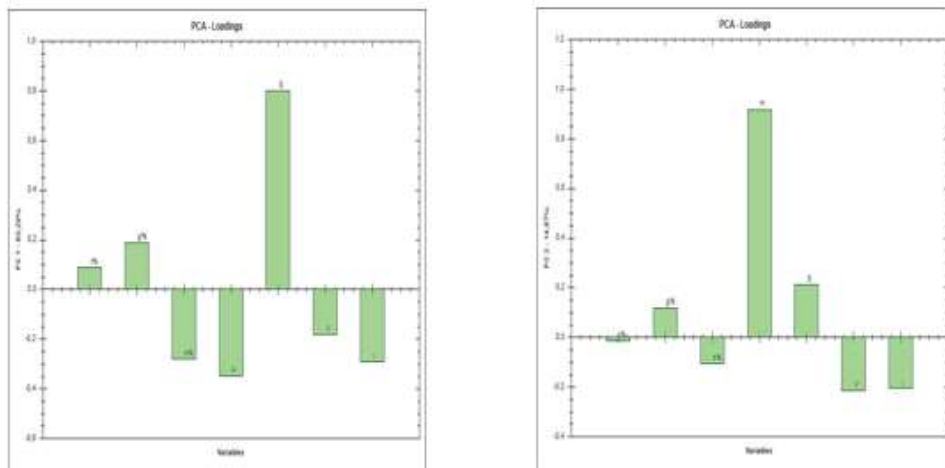


Figure 5: PC1 and PC2 loadings for PCA performed with ChemoStat®.

HCA results are shown in Figure 6. The first division, on the left, highlights samples in which neither THC nor CBN was quantified. Furthermore, there is a tendency of grouping between samples that have a more intense color compared to the others. Except for sample 178, which corresponds to a blank analysis (methanol). This is why the sample is by itself, separate from the others.

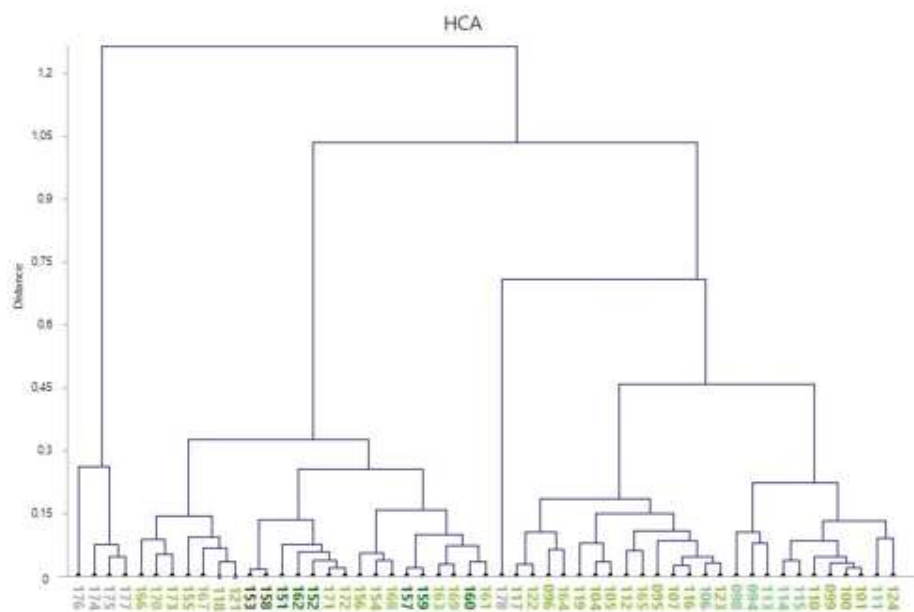


Figure 6: HCA results for 52 cannabis samples using ChemoStat® software.

3.3 PCA ANALYSIS ON PHOTOMETRIX PRO® APP FOR MOBILE DEVICE

Figure 7 illustrates PC1 versus PC2 graph score for PCA of the 52 cannabis samples analyzed by PhotoMetrix PRO®. Although it is not possible to clearly observe the identification of samples. It is possible to perceive a diminishing difference in color. The most intense green color samples are located in the upper left portion of the graph, corresponding to the samples in which the THC and CBN cannabinoids were quantified. It is noticeable that the images are becoming lighter in the lower left portion and these are the samples in which THC was detected below the limit of quantification and/or only the CBN was quantified. The set of samples identified from 173 to 176 (grey samples) forms an isolated group in the upper right region and the sample 177 is isolated below the others; these samples correspond to the extracts in which no cannabinoids were found.

The loadings of both PC1 and PC2 (Figure 8) indicate that the hue is the main factor responsible for the variance in samples.

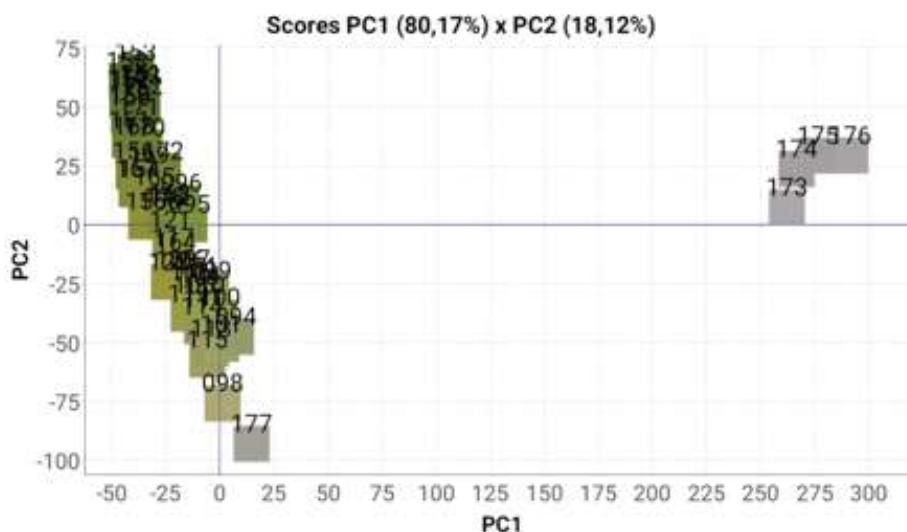


Figure 7: PCA results from the analysis of PC1 (80.1%) x PC2 (18.12%) using PhotoMetrix PRO®.

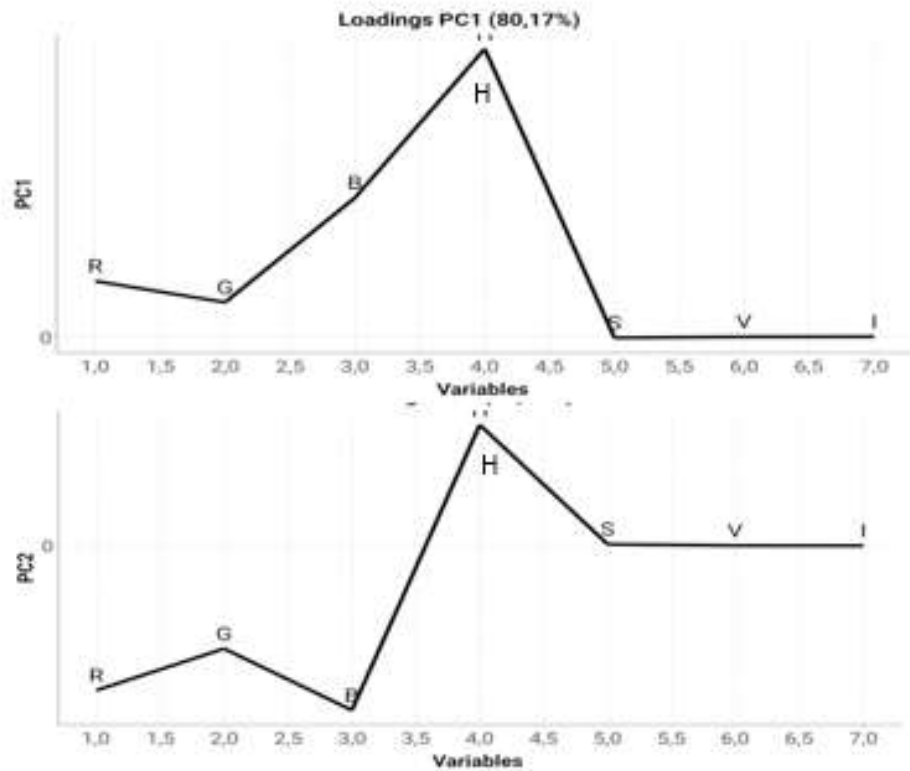


Figure 8: PC1 and PC2 loadings from PCA analysis with PhotoMetrix PRO®.

In order to verify whether the PhotoMetrix PRO® method was effective and reproducible, five samples were chosen at random and photographed five times in different modes. The first one was alternating the samples and the second was taking five photos in a row. Both tests were repeated three times, replicating the results, from that point only one image was taken from each sample, selecting the central image for MIA. Figure 9 shows the results for this experiment, demonstrating that the app is capable of differentiating the extracts.

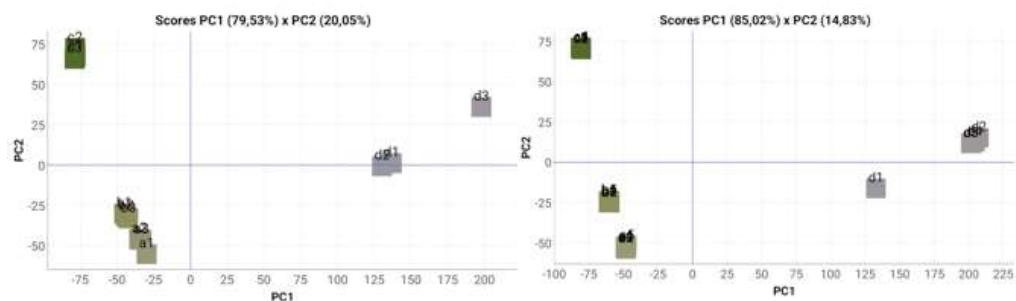


Figure 9: Differentiation test. On the left each sample was photographed randomly and the right sequentially.

The same test was carried out with the 5 randomized samples for HCA and the results corroborated with PCA (Figure 10), showing an appropriate differentiation between each group of samples.

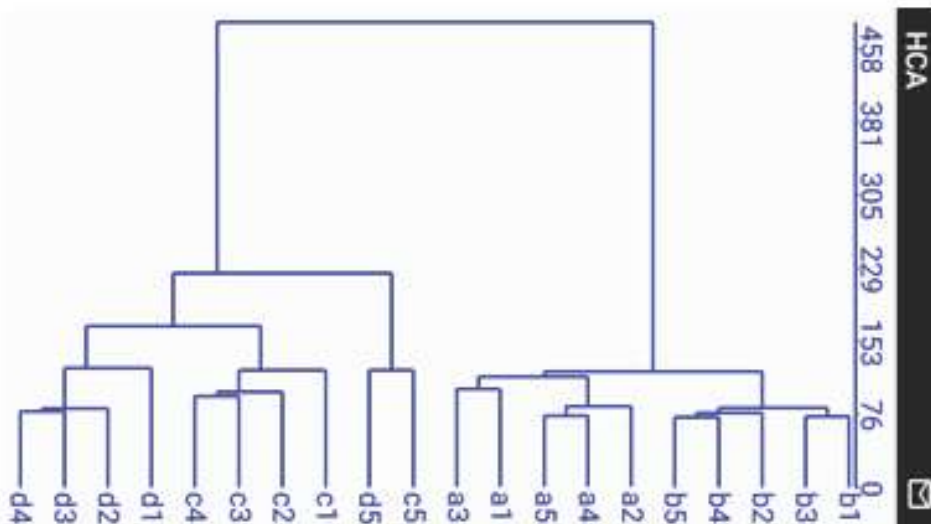


Figure 10: HCA results from tests with 5 random samples.

MIA tools can find correlations that have not been confirmed by the analytical methods result. Using two different software programs, PhotoMetrix PRO® and ChemoStat®, the results regarding THC and CBD contents in cannabis extracts are reproducible between themselves, making the use of the app possible for mobile devices, as a drug screening test on cannabis extracts spots or other colorful products. This is a simple and a low-cost tool that can be helpful for cannabis sample analysis in the forensic field. However, some precautions must be taken, such as verifying the reproducibility of MIA use, because the collection of digital images by different mobile devices and under non-standard conditions can interfere with the results. Gorziza *et al.*, carried out an experiment with pen inks using the same Photometrix PRO® software on two different smartphones, proving that there is a small variation of position between the samples in the PCA graph, but that did not change the final result (GORZIZA *et al.*, 2019). Vitorazzi *et al.* (2020), in a study of bank notes, determined the best region of interest for image acquisition, focal length and variations results in three different mobile devices. Even with variations, it was possible to differentiate authentic, counterfeit and reproduced banknotes for the study (VITORAZZI *et al.*, 2020). Therefore, the variations from one device to the other did not change the result of the clusters,

but rather the proximity between the samples in the groups. Hence, it is necessary to minimize the variations, standardizing the distance of image acquisition and performing repetitive analyzes to verify the reproducibility of the results.

Following the basic principles of forensic science, using different methods that have as a main objective to generate the same results, the proposal with this study is not to directly correlate the concentrations of cannabinoids with the images. The authors of this study propose a simple method for afore mentioned clustering of samples that may precede chromatographic analyzes, assist in chemical profile studies or simply aggregate samples of similar profiles to be analyzed together.

The senescence of the plant, which can occur due to the degradation of chlorophyll, and also by the oxidation of THC to CBN, can be observed through a color gradient that varies from intense green to lighter green, which identifies the quantified differences of cannabinoids (THC and CBD) among the studied cannabis extracts. It is worth mentioning that the evaluation carried out in this study is based only on the preliminary relationship between the analysis of THC and CBN by GC-FID and MIA. Many other plant metabolites contribute to the coloring of extracts, including other cannabinoids, precursors and/or derivatives of THC and CBN. As a qualitative screening method, the MIA will not always provide separation for all groups of samples. In this case, an analytical test such as GC-FID, CG-MS and/or LC-MS/MS should be used, as it should be used when confirmatory analysis is necessary. However, the MIA can indicate a tendency towards separation, helping forensic scientists to interpret and differentiate between marijuana samples.

4. CONCLUSION

Fifty-two samples of *Cannabis sativa L.* extracts were analyzed by GC-FID to quantify THC and CBN. Afterwards, the extracts were photographed and subjected to PCA and HCA analysis by ChemoStat® software and the PhotoMetrix PRO® app, showing a color gradient pattern that could differentiate samples and match quantified concentrations of THC and CBN or just CBN. The use

of chemometrics in forensic analysis can help in screening analytical methods, but it is not possible viable to replace the use of other standard analytical methods. The present study's recommendations require further research, but its use may reinforce the analytical findings in actual samples.

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APLICAÇÃO DA ANÁLISE MULTIVARIADA EM IMAGENS DIGITAIS DE EXTRATOS DE *CANNABIS SATIVA L.*

RESUMO

A *Cannabis sativa L* é uma das drogas mais usadas no mundo. Informações sobre a idade e armazenamento da planta podem ajudar os cientistas forenses a identificar e rastrear amostras. A proporção entre os canabinóides tetrahidrocannabinol (THC) e canabinol (CBN) tem sido relacionada à degradação da cannabis com o tempo. Assim, este estudo teve como objetivo testar a Multivariate Image Analysis (MIA) para avaliar os extratos de cannabis de acordo com suas cores. Inicialmente, 52 amostras de extratos de *Cannabis sativa L.* foram analisadas por Cromatografia Gasosa acoplada a Detector de Ionização de Chama (GC-FID) para quantificar THC e CBN. Em seguida, as amostras dos extratos foram fotografadas e analisadas por duas ferramentas diferentes de análise multivariada: ChemoStat, um software quimiométrico gratuito, e PhotoMetrix PRO, um aplicativo para dispositivos móveis. Usando métodos não supervisionados de Análise de Componentes Principais (PCA) e Análise Hierárquica de Cluster (HCA), verificou-se que quanto mais intensa a cor de um extrato, maior a concentração de THC e, por sua vez, os extratos de cor mais clara correspondem a amostras com CBN, sem THC. Os resultados sugerem um método simples de agrupamento prévio de amostras que podem preceder análises cromatográficas, auxiliar em estudos de perfis químicos ou simplesmente agregar amostras de perfis semelhantes para serem analisadas em conjunto.

PALAVRAS-CHAVE: *Cannabis sativa L.* Maconha. Quimiometria. PCA. HCA. GC-FID.

APLICACIÓN DE ANÁLISIS MULTIVARIADO SOBRE IMÁGENES DIGITALES DE EXTRACTOS DE *CANNABIS SATIVA L.*

RESUMEN

Cannabis sativa L es una de las drogas más consumidas del mundo. La información sobre la edad y el almacenamiento de la planta puede ayudar a los científicos forenses a identificar y rastrear muestras. La relación entre los cannabinoides tetrahidrocannabinol (THC) y canabinol (CBN) se ha relacionado con la degradación del cannabis a lo largo del tiempo. Por lo tanto, este estudio tuvo como objetivo probar el Análisis de Imagen Multivariante (MIA) para evaluar los extractos de cannabis según sus colores. Inicialmente, se analizaron 52 muestras de extractos de *Cannabis sativa L.* mediante cromatografía de gases acoplada al detector de ionización de llama (GC-FID) para cuantificar el THC y el CBN. Luego, las muestras de los extractos fueron fotografiadas y analizadas por dos herramientas de análisis multiva-

riante diferentes: ChemoStat[®], un software quimiométrico gratuito, y PhotoMetrix PRO[®], una aplicación para dispositivos móviles. Utilizando métodos no supervisados de Análisis de Componentes Principales (PCA) y Análisis de Conglomerados Jerárquicos (HCA), se encontró que cuanto más intenso es el color de un extracto, mayor es la concentración de THC y, a su vez, más extractos de color. corresponden a muestras con CBN, sin THC. Los resultados sugieren un método simple de agrupación previa de muestras que puede preceder a los análisis cromatográficos, ayudar en los estudios de perfiles químicos o simplemente agregar muestras de perfiles similares para analizarlas juntas.

PALABRAS CLAVE: *Cannabis sativa* L. Marihuana. Quimiometría. PCA. HCA. GC-FID.

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