Optimization of the quantification of Cannabidiol and Tetrahydrocannabinol methodology by High Performance Liquid Chromatography

Optimización de la metodología para la cuantificación de Cannabidiol y Tetrahidrocannabinol mediante Cromatografía Líquida de Alta Resolución

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Abstract. Cannabinoids are terpenophenolic compounds obtained from the species *Cannabis sativa* (Cannabaceae), which is worldwide distributed across multiple varieties. The concentration of each cannabinoid in a cannabis plant is closely related to the variety. There are specimens in which higher yields of one specific cannabinoid are found than the remaining cannabinoids. International organizations such as the United Nations Office on Drugs and Crime, recommend the application of High-Performance Liquid Chromatography as an analytical method for the quantification of cannabinoids. In the present work, the different variables that influence a chromatographic analysis such as temperature, sample pH, elution flow and wavelength have been evaluated and the chromatographic conditions for the analytical quantification of the metabolites cannabidiol and tetrahydrocannabinol have been standardized.

Keywords: Cannabis sativa; industrial hemp; HPLC; Medical Cannabis.

Resumen. Los cannabinoides son compuestos terpenfenólicos obtenidos de la especie *Cannabis sativa* (Cannabaceae), que se distribuye por todo el mundo en múltiples variedades. La concentración de cada cannabinoide en una planta de cannabis está estrechamente relacionada con la variedad. Hay ejemplares en los que se encuentran mayores rendimientos de un cannabinoide concreto que del resto de cannabinoides. Organismos internacionales como la Oficina de las Naciones Unidas contra la Droga y el Delito recomiendan la aplicación de la Cromatografía Líquida de Alta Resolución como método analítico para la cuantificación de cannabinoides. En el presente trabajo se han evaluado las diferentes variables que influyen en un análisis cromatográfico como la temperatura, el pH de la muestra, el flujo de elución y la longitud de onda y se han estandarizado las condiciones cromatográficas para la cuantificación analítica de los metabolitos de cannabidiol y tetrahidrocannabinol.

Palabras clave: Cannabis sativa, cáñamo industrial, HPLC, Cannabis medicinal.

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Introduction

Cannabis sativa is an ancient plant with quite global distribution and multiple uses (El Sohly et al., 2017). The plant is native to East Asia (De Backer et al., 2009). Cannabis phenotypes are highly variable and it is accepted that the plant has two subspecies: *C. sativa* subsp. *sativa* and subsp. *indica* (Hillig y Mahlberg 2004; Knight et al., 2010). Increased production of cannabis for medical and recreational purposes in recent years has led to a corresponding increase in laboratories performing cannabinoid analysis of cannabis and hemp (McRae y Melanson 2020).

Natural cannabinoids exhibit a terpenophenolic base structure (C_{21}), and are constituted by a group of approximately 120 compounds discovered to date (Radwan et al., 2021). Among them, the metabolites cannabidiol and tetrahydrocannabinol are of particular importance due to the growing interest in developing consumer products containing these compounds (Persia et al., 2023). The concentration of each of these cannabinoids is intrinsically related to the variety (Aizpurua-Olaizola et al., 2014; Deidda et al., 2019). In industry, those varieties with lower THC content are used for the production of hemp fibers, cannabis oil (Glivar et al., 2020). For veterinary supplements, low tetrahydrocannabinol content varieties of *C. sativa* are employed (Pinto y Requicha, 2024). To date, there are more products in which cannabinoids are present, such as cosmetics, nutritional supplements (Kanabus et al., 2021). In certain cases, THC and CBD are active ingredients (Wakshlag et al., 2020; Vasantha Rupasinghe et al., 2020).

In Paraguay, in 2020, the National Program for the Promotion, Development, Cultivation, Development of Production, Marketing and Research of Hemp and Industrial Hemp Cultivation was created. Paraguay exported, for the first time in its history, 20 tons of food derived from industrial hemp to the European Union in July of 2021, thus becoming the first country in Latin America to export food derived from *Cannabis* spp (Ferrere Legal Services, 2021). Currently, more than 700 peasant family farmers work and plant hemp throughout the country, which could be an emerging income category for small producers (La Nación, 2021).

According to Paraguayan legislation, industrial hemp or non-psychoactive Cannabis is that which "plants flowering tops with or without fruit, of the hemp plant, whatever its name, whose THC content is less than 0.5%. dry weight of THC" Decree No. 2,725/19 Presidency of the Republic of Paraguay. Although, within the changes in Paraguayan legislation (Decreto 2725/19, 2020), certain regulations have been established that include both the production and research of products based on *Cannabis sativa* and its derivatives, the current regulations do not contemplate or establish any standardized regulatory protocol for the quantification of cannabinoids within the national territory, which is crucial given the psychoactive principle (THC) that can be found in greater or lesser concentration depending on the variety of *Cannabis sativa* with which one works. To our knowledge, there are no studies or

bibliographies that support cannabinoid quantification issues at a national level. Based on these points, it is important to have a validated quantification method to apply to *C. sativa* products. The objective of this work was to develop a methodology for the quantification of cannabidiol and tetrahydrocannabinol using high-performance liquid chromatography with emphasis on optimal chromatographic conditions for quantitative analysis.

Materials and methods

Place of experimentation

The present research was carried out at the Multidisciplinary Center for Technological Research (CEMIT), located at the University Campus of the National University of Asuncion in the city of San Lorenzo.

Standardization of the method

As a starting point for HPLC standardization, the experimental conditions proposed by a Doehlert design generated by the Modde®10 software were used, with standard reagents for the CBD and THC metabolites (Deidda et al., 2019).

Variables studied

For the analysis of the influence of each of the variables in the chromatographic separation process, runs were performed modifying only the variable under study such as Wavelength; Temperature; pH; Flow. The concentration of the CBD and THC compounds was kept constant. The magnitude of the variable for which the detector quantified the largest area was determined.

1. Wavelength

In order to evaluate the impact of the wavelength variation in the detection process, runs were performed at different wavelengths keeping constant the conditions of temperature, elution flow, pH and concentration of the cannabinoids. As a starting point, the wavelength proposed by Deidda et al. (2019) of 222 nm was used and then modifications were made by varying the wavelengths between 200 and 222 nm for both the CBD and THC standards.

2. Temperature

Temperature adjustments were made keeping constant the values of flux, wavelength and pH of the mobile phase. The starting temperature was 53 ºC proposed by Deidda et al. (2019) and in successive injections, the temperature values were modified around the one proposed by the literature for subsequent analysis evaluating its influence on both the peak area and retention time for the compounds analyzed in the work.

3. pH evaluation

The pH of the mobile phase (acetonitrile and dipotassium phosphate) was the variable that was modified during the test, keeping the other chromatographic variables (temperature, elution flow rate and wavelength) constant. For this, we started from a pH equal to 3.45 proposed by Deidda et al (2019) and then with injections of the mobile phase brought to a pH of 3.15 and subsequent injections of the mixture with a pH corrected equal to 3.60.

4. Flow rate

The flow rate was evaluated by modifying it in different injections, keeping constant the values of temperature, pH of the mobile phase and wavelength in each of the runs for the study of this parameter. We worked under isocratic elution conditions with flow rates from 0.4 mL of mobile phase per minute (mL/m) to 1.3 mL/m.

Data processing and statistical analysis

To obtain the data and integrate the peaks, the Lab Solutions software of the SHIMADZU corporation was used in its version for LC chromatographs and the data were subsequently processed and analyzed with the Microsoft Excel program of the Office 365 package version 2021 (SHIMADZU Corporación, 2021; Microsoft Office 365, 2021). A Regression and Linear Correlation statistical treatment was performed on the data collected from all the chromatographic runs. Once the chromatographic conditions were standardized, the 5-point calibration curve was constructed. Five dilutions of decreasing concentration were injected for both the CBD standard and the THC standard. Using the Microsoft Excel program, a table of area as a function of concentration was constructed for the metabolites studied, the concentration being the independent variable and the area of the peak the dependent variable directly proportional to the concentration of the analyte.

Procedural part

a. Preparation of the standards

From certified standard solutions (SIGMA-ALDRICH®) six dilutions of decreasing concentration of the working standards for the CBD and THC metabolites of concentrations 1.03 mg/mL and 1.02 mg/mL, respectively, were prepared. Methanol grade HPLC was used as a solvent to prepare dilutions.

b. Preparation of the mobile phase 75:25

The mobile phase was made for a mixture of HPLC grade acetonitrile (J.T. Baker®) and a dipotassium phosphate solution (MERCK pro analysis). For the preparation of the phosphate solution, the necessary grams were weighed on an analytical balance to obtain a concentration of 5 mM of the salt in the final volume of the mobile phase mixture, which were dissolved in deionized water with the help of a glass rod until the total dissolution of the solute was visualized. The acetonitrile was then mixed with the

phosphate solution at a volume ratio of 75:25 (ACN:phosphate). The obtained mixture was brought to pH 3.45 with appropriate volumes of 12 M HCl and once the pH was reached, the obtained mixture was filtered with a vacuum filtration equipment using a membrane with a pore size of 0.45 µm (MF-Millipore®MembraneFilter) and then sonicated for 15 minutes.

c. Chromatographic conditions

The equipment and reagents provided by the Multidisciplinary Center for Technological Research were used. The High-Performance Liquid Chromatography (HPLC) equipment used throughout the process was composed of a SHIMAZDU brand chromatograph and a 250 mm long x 4.6 mm RESTEK brand C18 column. Both the CBD and THC standards and their respective dilutions were injected into the chromatography equipment of the CEMIT Medicines Quality Control Laboratory with the specifications already described in section 3.3 for evaluation. A diode array detector with an initial wavelength of 222 nm was used as the detection method. CBD and THC, whose areas obtained and integrated by the software used. The identification and corresponding quantification of the metabolites CBD and THC were carried out, whose areas were obtained and integrated by the software used.

Results and Discussion

1. Wavelength of equipment lamp (λ)

The following wavelengths chosen were: 200 nm, 210 nm, 215 nm and finally, 222 nm. We studied their influence on the peak areas given in Figure1, and record the retention times (see Figure 2).

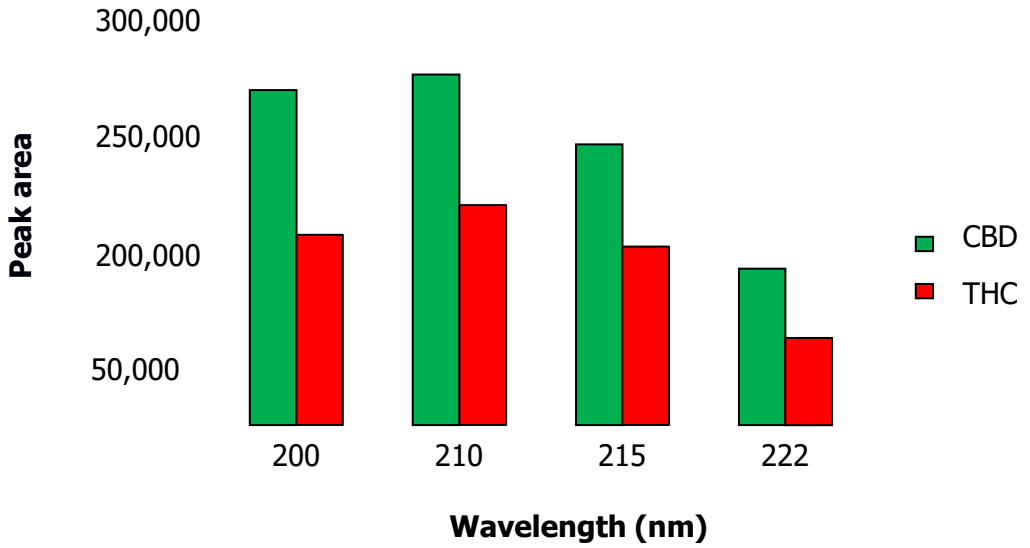

Figure 1. Peak area as a function of wavelength for CBD and THC. The highest absorbance of the peak areas of both Cannabidiol and Tetrahydrocannabinol is observed at a wavelength of 210 nm.

The areas quantified by the detector lamp indicate that the wavelength of highest absorption for the cannabinoids used in this work was 210 nm; the peak area starts to decline both above and below thatwavelength.

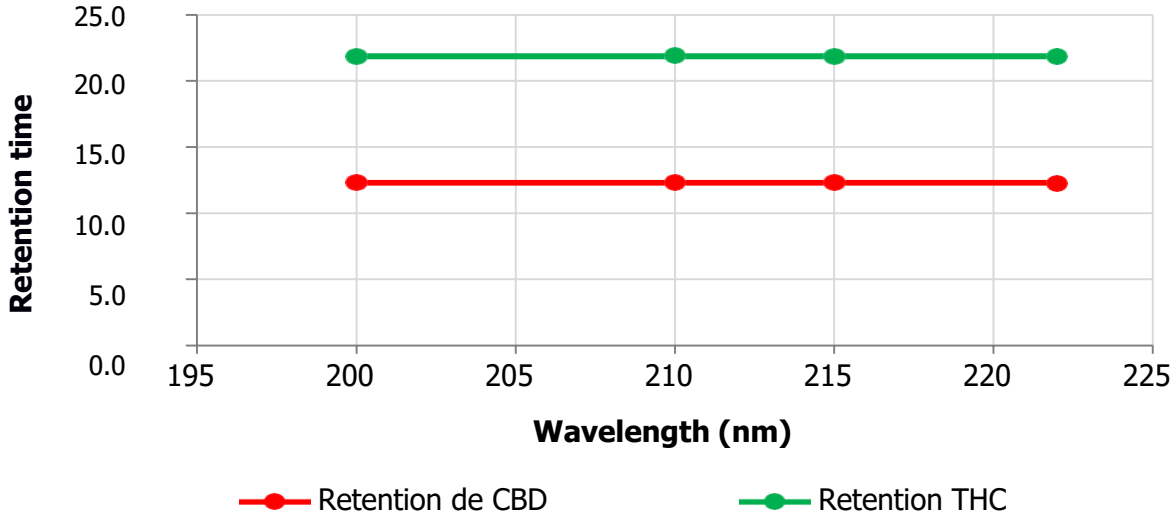

Figure 2. Retention time as a function of wavelength for CBD and THC. The retention times with the variation of the wavelength of the equipment (P>0.05), this phenomenon is observed for both CBD and THC.

2. Operation temperature

The following temperatures in degrees Celsius were considered: 51 ºC, 53ºC proposed by (Deidda et al., 2019), 54 ºC, 58 ºC and 60 ºC. All injections were performed at a flow rate of 1 mL/min of the mobile phase with a pH of 3.45; the wavelength used was 210 nm.

The observed peak areas corresponding to CBD were quite constant with values around 200,000 for the chosen temperature variations (Figure 3). Regarding the THC metabolite, peak areas were between 120,000 and 128,000 under the same conditions. The retention times of both metabolites decreased with increasing temperature (Figure 4).

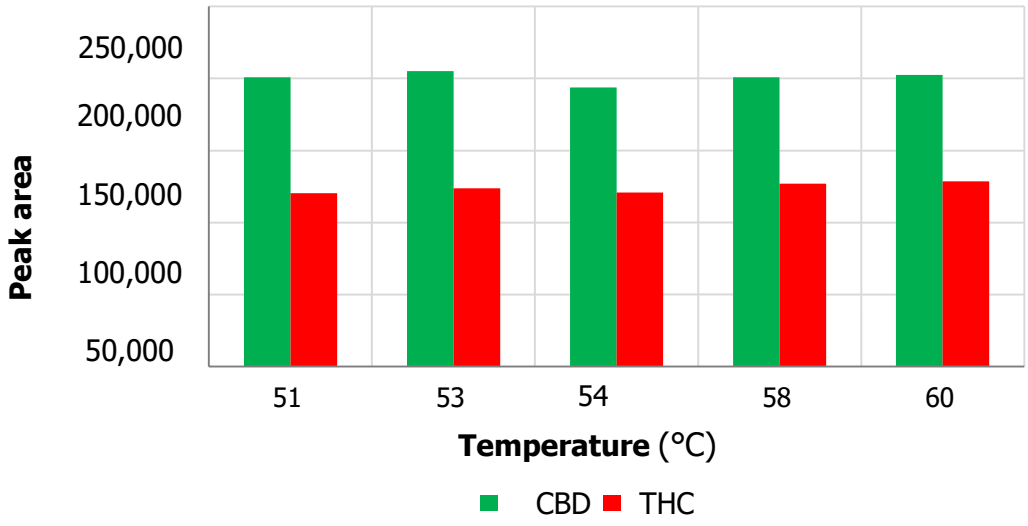

Figure 3. Peak area as a function of temperature variation for CBD and THC metabolites (P>0.05).

The longest retention time recorded corresponded to THC with a time of approximately 24.5 minutes at 51 ºC and 15.8 minutes for a temperature of 60 ºC. Likewise, the longest retention time recorded for CBD was 13.2 minutes at an injection temperature equal to 51 ºC and about 9.2 minutes at 60 ºC.

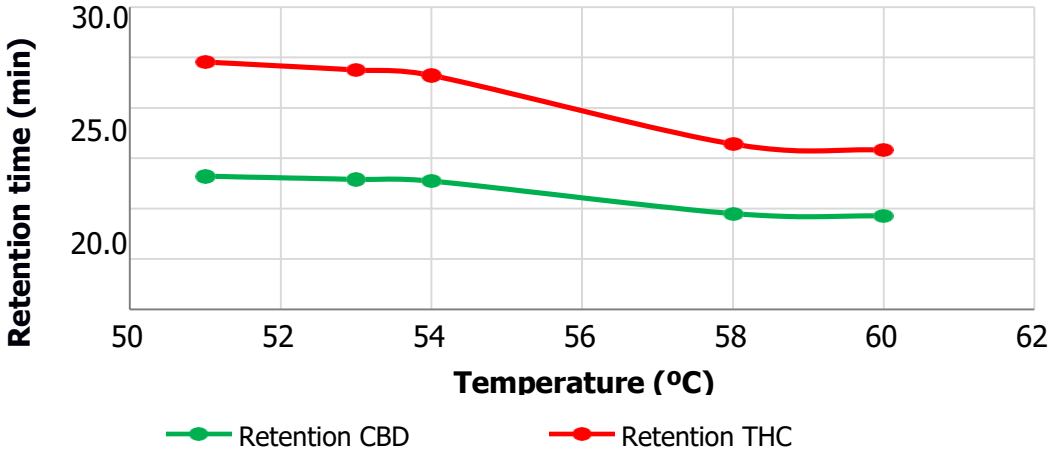

Figure 4. Retention time as a function of temperature variation for CBD and THC metabolites. This graph shows the influence of temperature on the retention times of the CBD and THC metabolites. As the temperature increases, the retention time for both metabolites decrease.

3. Sample pH

For the pH evaluation, other chromatographic variables such as temperature (about 53 ºC), elution flow and wavelength of 210 nm were kept constant. All injections were performed with the same concentration of CBD and THC, as used in the previous experiments. For CBD the peak areas were maintained at a value ofapproximately 260,000 within the pH range 3.15-3.60 arbitrarily chosen (Figure 5). The shortest retention time for CBD was achieved at pH 3.45 which was about 12.3 minutes approximately (Figure 6). Proceeding in the same way, for THC the shortest retention time was obtained at pH 3.45, and the areas were around 163,000 within the employed pH range.

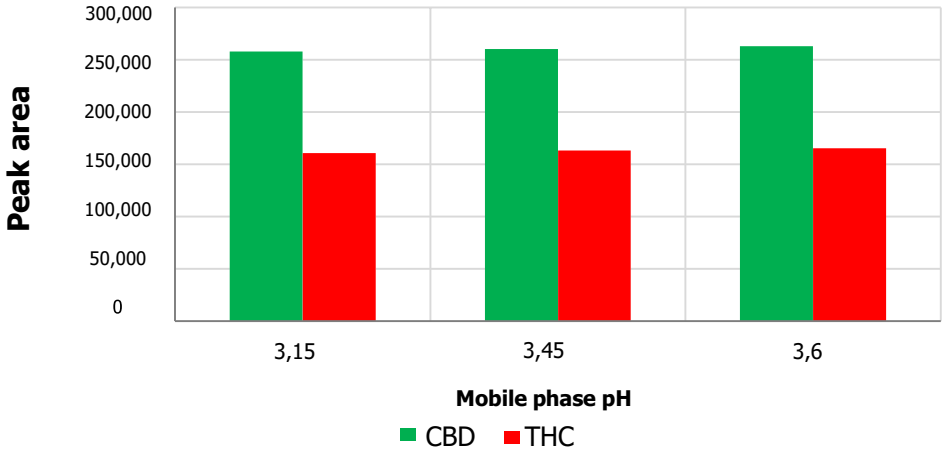

Figure. 5. Peak area as a function of mobile phase pH for CBD and THC metabolites. The influence of the pH on the peak area recorded for the CBD and THC metabolites is negligible.

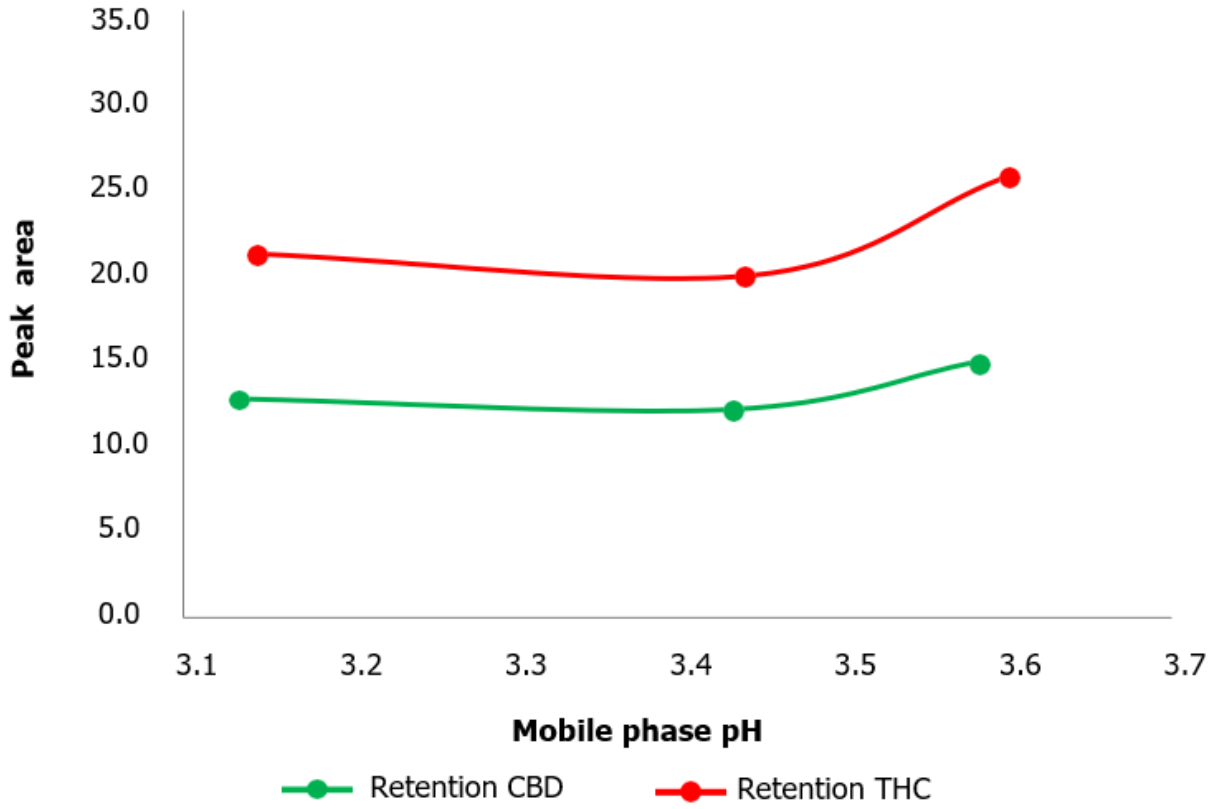

Figure 6. Retention time as a function of mobile phase pH for CBD and THC metabolites. The graph shows how retention times are affected by the variation of ambient pH. As the pH increases, the retention time increases.

Flow rate

To analyze the impact of the flow on the outcome, a constant temperature of 60 ºC,a wavelength of 210 nm and a pH of 3.45 were used. For each of the chromatographic runs, constant concentrations of CBD and THC standards were used. The standards were injected considering a range of mobile phase flow rates, from 0.4 mL/min to 1.3 mL/min. The largest areas recorded by the detector were ata flow rate of 0.4 mL/min giving areas of 501,854 and 299,083 for the CBD and THC compounds respectively (Figure 7). On the other hand, the smallest areas were obtained for both metabolites at a flow rate of 1.3 mL/min. Retention times were alsovisibly affected by the elution flow rate, giving in both cases the longest retention time at a flow rate of 0.4 mL/min, of approximately about 23.0 min for CBD and about 39.2 min for THC. The shortest retention time was achieved at a flow rate of 1.3 mL/min, reaching about 7.8 minutes for CBD and 13.9 minutes for THC (Figure 8).

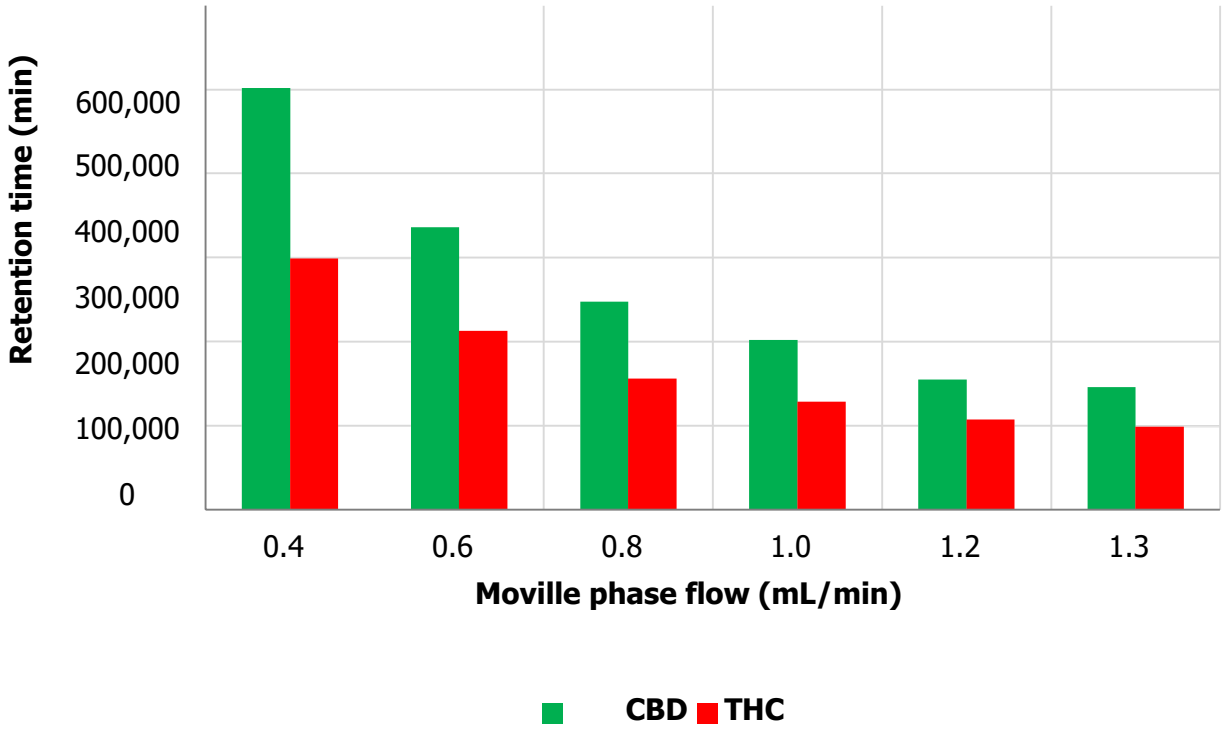

Figure 7. Peak area as a function of mobile phase flow for CBD and THC metabolites. The graph exposes the influence of mobile phase flow variation on the peak area of CBD and THC cannabinoids, the largest areas were recorded at a flow rate of 0.4 mL/min for both metabolites.

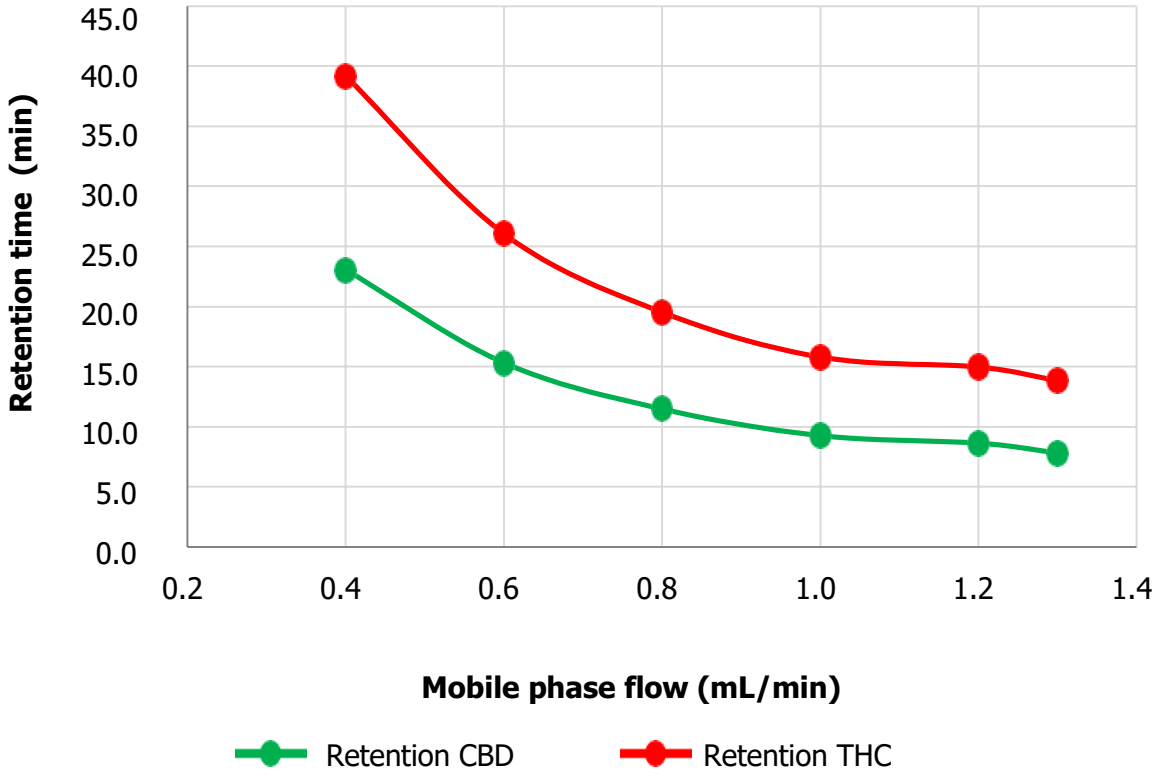

Figure 8. Peak area as a function of mobile phase flow for CBD and THC metabolites. Influence of flow variation on the retention times of CBD and THC metabolites. The lowest retention fluxes were observed at 0.4 mL/min.

Calibration curves

After analysis of the different variables, the following chromatographic conditions were chosen: Temperature: 53 ºC, A working flux rate: 0,8 mL/min (of mobile phase), Wavelength: 210 n, pH of the medium: 3,45. Once the described run conditions were programmed, the CBD and THC standards were diluted for the construction of the calibration curve.

a. CBD curve

The graph of CBD area obtained as a function of concentration (Figure 9) showed a linear correlation with an R^2 equal to 0.999 and an equation of the straight line equal to $y = 82273x + 21167$.

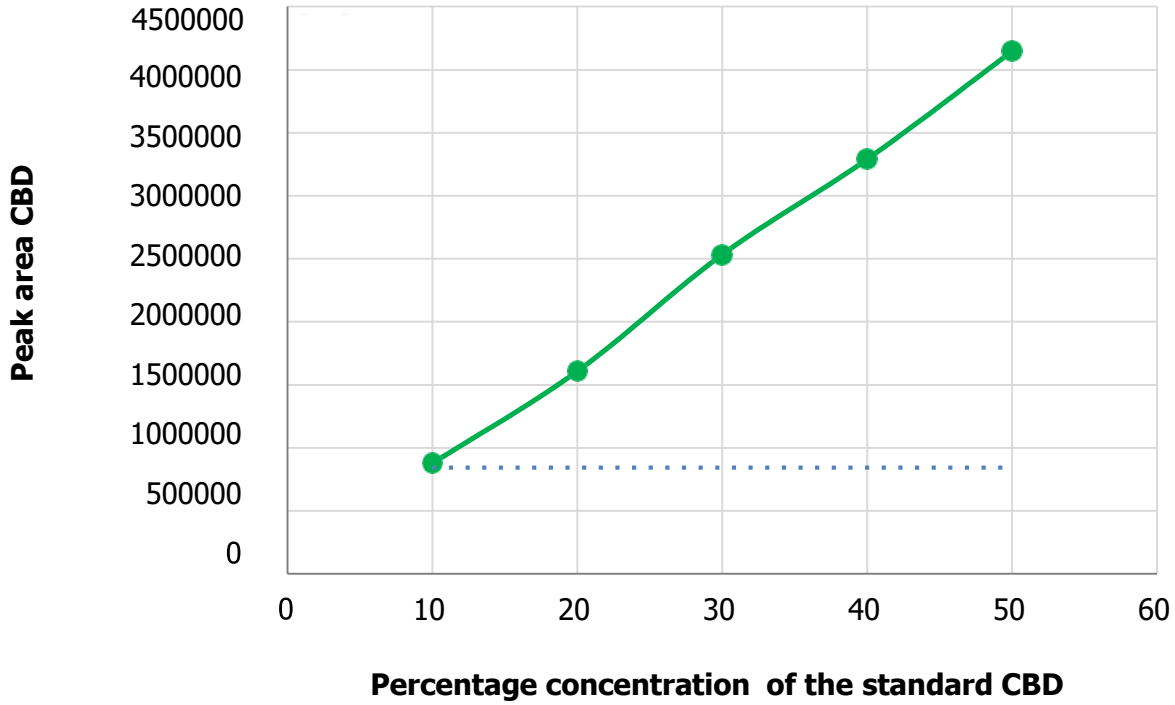

Figure 9. Calibration curve for CBD. CBD concentration is the independent variable and peak areas the dependent variable, presenting a positive slope.

b. THC curve

As with the CBD metabolite, the area of THC obtained was plotted as a function of concentration (Figure 10) and a linear correlation was obtained with an R^2 equal to 0.999 and an equation of the straight line equal to $y = 70985\ x - 39092$.

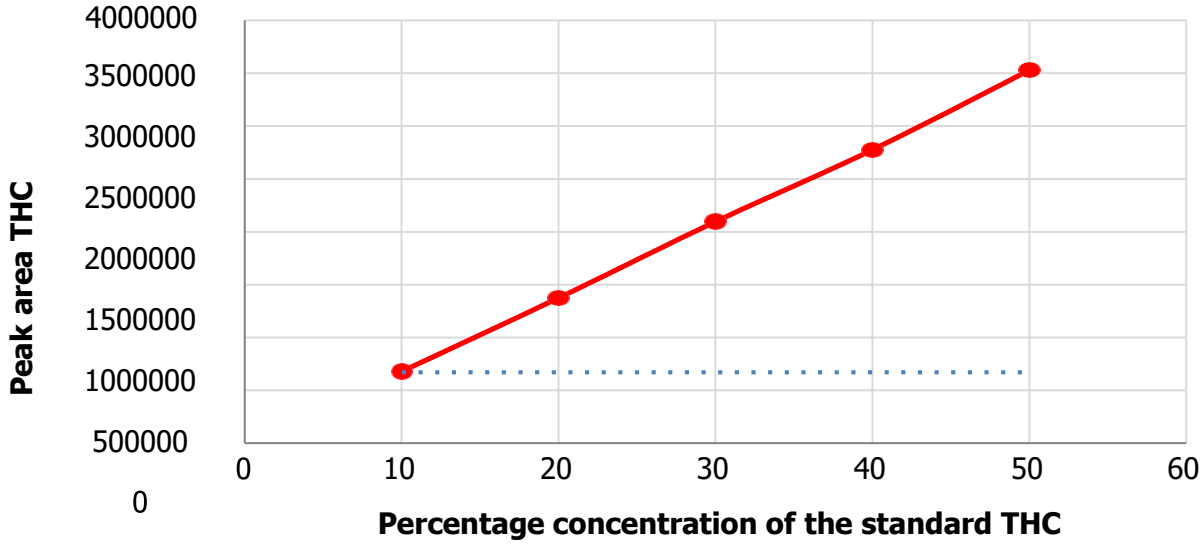

Figure 10. Calibration curve for THC. THC concentration is the independent variable and the peak areas the dependent variable, presenting a positive slope.

Elution flow

The influence of elution flow on the chromatographic run is clearly visible from Figure 7. This effect is detected for both metabolites equally; with increasing flow rate of the mobile phase, the recorded peak area decreases. Regarding CBD and THC compounds, the area is inversely proportional to the elution flow rate. The flow rate at which the standard is injected affects the speed at which the analyte elutes and this has a direct impact on the recorded area, because the higher the flow rate in which the metabolite is immersed, the less time the detector will have to perform a purified quantification of the area. On the other hand, the flux not only affects the magnitude of the area, but also the retention time. As can be seen in Figure 8, the retention time for the study compounds decreases with increasing flow rate, so behave the area values. The retention time also correlates inversely with the flow velocity, since increasing the velocity of the mobile phase increases the rate of carryover of CBD and THC in the column. This results in a shorter time required for their passage through the column.

Operation temperature

Analyzing Figure 3, it can be seen in detail that particularly for the studied metabolites, there are only slight changes in the quantification of the metabolite areas. All temperature variations were performed at the same elution flow rate. This confirms that no major changes in the peak areas should be observed for CBC and THC. However, observing Figure 4 elucidates the influence of temperature on the retention times of both compounds. Increasing the temperature slightly decreases the retention times of both CBD and THC. This phenomenon could be explained by the fact that a higher temperature accelerates the overall kinetics of all the eluting molecules, which would result in a contribution, albeit small, to a higher net elution rate. Therefore, slight variations in the retention times of the CBD and THC compounds with respect to experiments at different temperatures but maintaining constant flow conditions, pH and wavelength. Thus, it could be said that the influence of temperature would be

negligible for the quantification of the peak areas, but not so with the retention times in which perceptible variations are observed, although the latter are small (Yang 2008; Corradini et al., 2011).

Wavelength

The wavelength proposed by relevant references is about 222 nm (Deidda et al., 2019). Different wavelength modifications were carried out keeping the other chromatographic variables constant. As can be seen in Figure 1, the wavelength with the highest absorbance for the working species was 210 nm, obtaining areas with values more than twice as high as for the wavelength of 222 nm, suggested by the literature. As always, the result of such a measurement is a result of all parameters. In this study the working conditions were different from those of Deidda et al. (2019) , who used a 150 mm column while in the present work a 250 mm column was employed. Also, Deidda et al. (2019), worked with elution flows different from those suggested by the previous bibliography, so such changes would be expected when not working under the same conditions, regardless of the fact that the same compounds were studied. It should be noted that the absorbance of the species is not only closely related to the employed wavelength but also to the already elapsed lifetime of the chromatograph lamp.

Influence of pH

According to Figure 5, the peak areas for CBD and THC remained relatively constant, only subtle changes in magnitude were observed due to pH variations of the mobile phase. The injections were performed at the same temperature and flow rate, and the wavelength was also kept constant throughout the process for the study of this variable. Following the same logic discussed above for the analysis of the elution flow and temperature, by keeping these variables constant, no major differences should be observed in the quantification of the areas by the detector. What can be seen in Figure 6 is the influence of the pH on the retention time for CBD and THC. It can be observed that the ideal pH for this methodology was 3.45. Values below or above this pH result in an increase in the retention times for both metabolites. This can be explained by the fact that varying the pH directly affects the degree of ionization of susceptible species in the mobile phase, which in turn directly affects the affinity of CBD and THC metabolites to the mobile and stationary phases. The affinity of these species to the stationary phase for pH values different from 3.45 is increased, consequently the elution speed is lower and the retention times increase (Yang, 2008; Ahuja y Dong 2005).

Calibration curve

Extremely high correlation coefficients were obtained in both calibration curves, indicating a very close relationship between the dependent and independent variables (area and concentration, respectively). Furthermore, a positive slope was observed for both, the curves elaborated for CBD and THC (Figure 9 and Figure 10). This consistently denotes that an increase in their concentration in the analyte it is accompanied by an increase in the peak area, which is intrinsically related to the concentration in the analyte in a directly proportional manner. (Figure 11, Figure 12).

Chromatograms

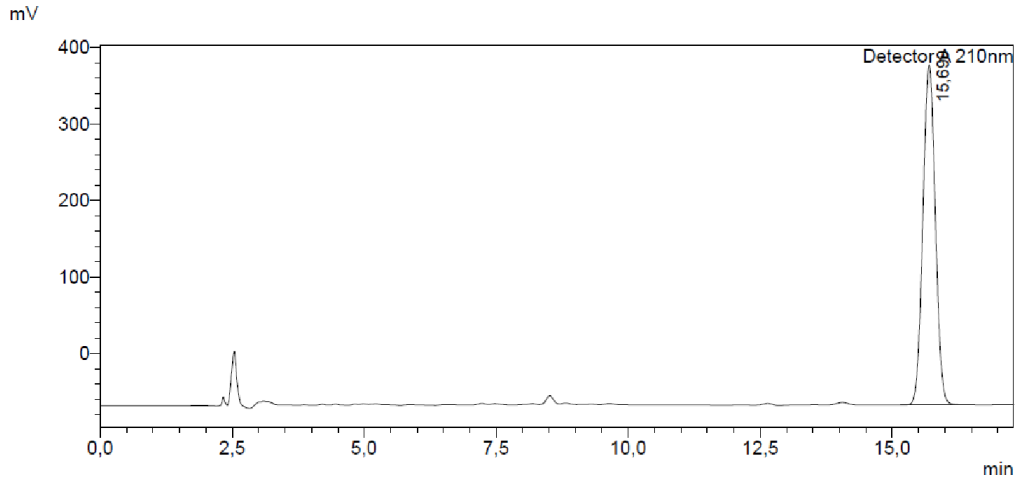

Figure 11. Chromatogram of the CBD standard. The peak of Cannabidiol is recorded at approximately 15 minutes for the previously given conditions.

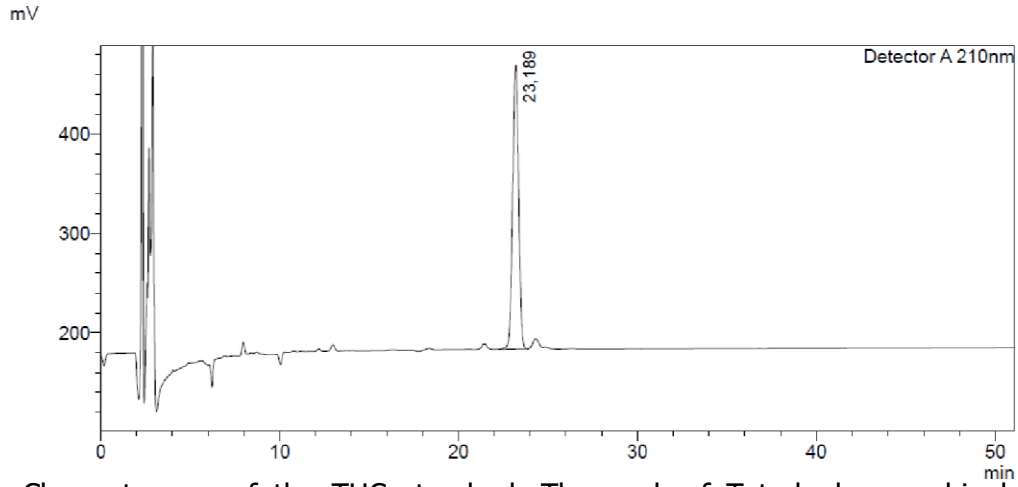

Figure 12. Chromatogram of the THC standard. The peak of Tetrahydrocannabinol is recorded approximately 23 minutes under the condition applied.

Conclusions

The chromatographic conditions for the standardization of a methodology for the quantification of cannabinoids CBD and THC were obtained, and the calibration curves for both analytes were successfully constructed. This work could be a starting point for the study of other cannabinoids and could serve as reference for their quantification, when making the proper adjustments according to the characteristics of the equipment to be used.

Authors' contributions: Conceptualization: J. F. A., A. R. **Data Curation** : J. F. A. **Formal Analysis :** J. F. A., E. F. B. **Funding Acquisition** : J. F. A., H. D. N., A.S.O. **Investigation :** J. F. A., H. D. N., A.S.O., E.F.B. .; **Methodology :** J. F. A., H. D. N., A.S.O. **Project Administration :** H. D. N., A.S.O. ; **Resources :** H. D. N., A.S.O. **Software :** A. R. **;** **Supervision** : H. D. N., A.S.O.

Validation : J. F. A., E.F.B.**.; Visualization :** E. F. B., A. R. ; **Writing – Original Draft :** J. F. A., H. D. N., A. S. O., E. F. B. , A. R. ; **Writing – Review & Editing** : J. F. A., H. D. N., A. S. O.

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