DoE-assisted development and validation of a thin layer chromatography method for optimized separation of major cannabinoids in Cannabis sativa L. samples

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\textbf{ABSTRACT}

As interest in the medical use of cannabis and cannabinoids rises, the need to establish appropriate quality specifications for them also becomes evident. Consequently, various methods applied to the analyses of cannabinoids in \textit{C. sativa} have been reported. Among the chromatographic techniques used for this purpose, TLC stands out as a suitable alternative for fingerprint assessment. Nevertheless, structural similarity between cannabinoids imposes some analytical challenges. In this context, DoE-assisted optimization may favor achieving an adequate resolution between those analytes. Accordingly, this study aimed at developing and validating a TLC method for cannabinoid profile monitoring in \textit{C. sativa} inflorescences by employing a DoE-driven strategy. A mixture design was applied to estimate the ideal mobile phase composition. Data analyses focused on maximizing resolutions between eight major cannabinoids and improving band distribution throughout the chromatogram. The overall quality of the chromatographic profiles after method optimization was satisfactory. Method validation covered selectivity, precision, and ruggedness assessment. The Youden design was applied for simultaneous analysis of five experimental factors. The proposed method was shown to be fit-for-purpose, allowing chemotype differentiation, in addition to being stability-indicating. Moreover, the results can support the definition of control strategies to ensure consistent achievement of the intended method’s performance.

\textbf{KEYWORDS}

Cannabinoids; Cannabis sativa; design of experiments; quality control; thin-layer chromatography

\textbf{Introduction}

Scientific evidence on the efficacy of \textit{Cannabis sativa} L. and some of its main cannabinoids for the treatment of certain pathological conditions, such as refractory epilepsy, chronic neuropathic pain, and multiple sclerosis, has been consolidated in recent decades,\textsuperscript{[1–4]} which has been contributing to the increase in demand for cannabis-based medicinal products around the world.\textsuperscript{[4]} Given this scenario, there is a clear need for defining relevant quality specifications applicable to those products to reduce the risks associated with their use and favor the appropriate characterization of the medicines to be evaluated in future clinical studies.\textsuperscript{[5]}

Adequate monitoring of the chemical profile of articles of botanical origin plays a critical role in their quality control, complementing the information obtained from botanical tests. In that regard, chromatographic techniques are often applied to obtain fingerprints that reflect the qualitative profile of characteristic constituents of the plant species, being useful not only for identification/authentication purposes but also to assist in the assessment of the stability and batch-to-batch consistency of the material of interest.\textsuperscript{[6–9]} To that end, the chromatographic profile should ideally be
related to the pharmacological properties of the herbal active pharmaceutical ingredient (API). \[6\] Moreover, it should be able to indicate relevant similarities and dissimilarities between samples to allow to discern between species, as well as to distinguish intraspecific groups, such as cultivars, chemotypes, and varieties, when applicable. \[9\]

With regard to pharmaceutical-grade inflorescences of *C. sativa*, fingerprint analyses are often focused on the cannabinoid profile, since these secondary metabolites have a restricted distribution in the plant kingdom and are considered to be important identity markers for this species. \[5,10,11\] Moreover, the semi-quantitative assessment of cannabidiol (CBD), $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), and their acidic counterparts; cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA); is required to achieve chemotype differentiation. This, in turn, is relevant for ensuring the desired efficacy since the main chemotypes of this species are known to possess distinct pharmacological profiles. \[5,12\]

Among the chromatographic techniques applicable to this purpose, thin-layer chromatography (TLC) stands out in terms of its versatility, low cost, and relatively shorter analysis time, with the possibility of analyzing several samples and reference materials/standards in parallel. \[9,13,14\] Nonetheless, TLC is also sensitive to a series of environmental and experimental factors, such as chamber saturation, temperature, and relative humidity, which often leads to poor reproducibility. \[6,15\] Therefore, the effects of those factors on the method’s performance should ideally be evaluated so that the necessary controls can be established. \[13\] Furthermore, resolution between some structurally similar analytes may be challenging to achieve due to elution mode limitations and stationary phase characteristics. Some of these limitations may be surmounted with the application of high-performance thin-layer chromatography (HPTLC) plates, whose reduced thickness (<150 μm) and particle size (<10 μm) may improve analytes’ resolution. In addition, the advent of instruments that allow the automation of analytical steps, such as sample application, elution, and detection, may contribute to overcoming reproducibility issues. \[14\]

Various TLC or HPTLC methods applicable to the identification and fingerprint monitoring of *C. sativa* samples are reported in scientific literature and official quality monographs. \[5,16-20\] Those methods are diverse in terms of the chromatographic conditions applied, involving either polar (e.g., silica gel) or non-polar (e.g., C-18) stationary phases, as well as a manifold of mobile phases. \[5,16\] Despite that, one may note that most of those methods were conceived for monitoring a limited set of cannabinoids only and that the ability to provide adequate resolution between the analytes of interest is often not appropriately demonstrated. In fact, the resolution between neutral cannabinoids $\Delta^8$-THC, cannabiol (CBN), and CBD in traditional normal-phase TLC systems has been considered especially challenging to achieve. \[21\] Accordingly, the use of reversed-phase plates, often of the HPTLC type, has been advocated for separating the aforementioned cannabinoids. \[16\] In this scenario, the German Pharmacopoeia indicates the use of a reversed-phase HPTLC method in both cannabis inflorescences and cannabis extracts \[22\] monographs. Similar methods have also been recommended by a Cannabis Expert Panel from the United States Pharmacopoeia (USP) \[5\] and by the American Herbal Pharmacopoeia in its “Cannabis Inflorescence and Leaf” monograph. \[20\] However, one should consider that HPTLC reversed-phase chromatographic plates are markedly more expensive, which can make such methods impractical for routine analysis in resource-limited situations, especially in developing countries.

The application of systematic approaches involving Analytical Quality by Design (AQbD) tools, such as the Design of Experiments (DoE) has proved advantageous to help overcome the analytical challenges that may arise during the development of chromatographic methods for cannabinoids and cannabinoid analyses, allowing for a better understanding of the influence of experimental factors on analytical performance. \[23,24\] Accordingly, in the case of TLC/HPTLC-based methods, the application of multivariate analysis based on mixture design for mobile phase optimization could be a promising strategy to achieve the maximum resolution of a set of cannabinoids of interest. Notwithstanding, as far as we know, there are no reports in the literature of such a development approach applied to TLC/HPTLC methods for cannabinoids analyses in *C. sativa* samples.

In view of the above, this study aimed to develop and validate a low-cost normal-phase TLC method focusing on the identification of eight major cannabinoids in *C. sativa* samples by employing a DoE-assisted approach. The authors also sought to evaluate the potential influence of selected experimental factors on the method’s performance by applying the Youden test.

**Materials and methods**

**Chemicals and reagents**

Single-component reference standards solutions (1.0 mg/mL) of cannabidiolic acid (CBDA), tetrahydrocannabinolic acid (THCA), cannabigerolic acid (CBGA), cannabidiol (CBD), cannabigerol (CBG), cannabiol (CBN), $\Delta^8$-tetrahydrocannabinol ($\Delta^8$-THC), and $\Delta^8$-tetrahydrocannabinol ($\Delta^8$-THC) were purchased from Cerilliant/Sigma-Aldrich (USA). Solvents used in sample preparation and mobile phase composition were of analytical grade. Vanillin-sulfuric acid derivatizing reagent was prepared as described elsewhere. \[25,26\]

**Plant material**

*C. sativa* samples of THC-dominant chemotype, consisting mainly of dried pistillate inflorescences, were obtained from collaborators participating in projects conducted under the validity of a Technical-Scientific Cooperation Agreement between our laboratory (Labtoxico/UFRGS) and the Regional Superintendence of the Federal Police in Rio Grande do Sul (SR/PF/RS). The herbal material was identified by Prof. Dr. Lilian Auler Mentz (Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil). Samples of
a commercial product (Real Scientific Hemp Oil®-Hemp Med), obtained through a technical collaboration with the Associação Nacional dos Usuários de Canabidiol-Anuc (Rio Grande do Sul, Brazil, CNPJ 28.644.035/0001-82), were also evaluated for comparison purposes, as representatives from the CBD-dominant chemotype, since inflorescences of chemotypes other than the THC-dominant were not available.

Reference solutions preparation

Mixed cannabinoid reference solution A (CS.A, system suitability solution) was prepared by diluting standard stock solutions of CBDA, THCA, CBD, CBN, and Δ⁹-THC in methanol to achieve final concentrations of 20 μg/mL. Mixed cannabinoid reference solution B (CS.B) was prepared by diluting standard stock solutions of CBGA, CBDA, THCA, CBG, CBD, CBN, Δ⁹-THC, and Δ⁸-THC in methanol to achieve final concentrations of 20 μg/mL.

Sample preparation

THC-dominant chemotype

Twenty milligrams of the powdered sample (granulometry range 180–250 μm) were accurately weighed and transferred to a glass tube with a screw cap, followed by the addition of 4.0 mL of methanol. Ultrasound-assisted extraction was carried out for 10 min, at room temperature (25 ± 5 °C). Test tubes were then centrifuged at 3000 rpm for 5 min. The supernatant was transferred to an amber glass flask, preserving the plant material contained in the tube. The extraction procedure was repeated with the reserved plant material, using the same solvent volume. Extracts obtained were pooled, filtered, and transferred to a 20 mL amber glass volumetric flask. Volume was made up of methanol (Final drug/extract ratio: 1 mg/mL).

CBD-dominant chemotype (commercial extract)

Twenty micrograms of the sample was dissolved in methanol and subjected to an ultrasound bath for 20 min. The solution was transferred to a volumetric flask and the volume was made up to 20 mL with methanol.

Chromatography

TLC analyses were performed on precoated silica gel 60 F245 aluminum sheets (Merck or Macherey-Nagel, Germany). Samples were applied in the form of bands (6 mm) using an Automatic TLC Sampler 4 (ATS 4, Camag, Switzerland), with minimum distances of 15 mm from the plate edges, 10 mm from the base, and 3 mm between bands. Chromatographic development was carried out in a 10 × 10 cm twin trough glass chamber with stainless steel lid (Camag, Switzerland) previously saturated with the mobile phase. The minimum development distance was 8 cm. Densitometric scanning, when applicable, was carried out before derivatization using a TLC Scanner 4 (Camag, Switzerland) in the absorbance mode (220 nm). Chromatographic plates were derivatized by spraying a vanillin-sulfuric acid reagent solution, followed by heating (105 °C, 5 min). Results were then observed under visible light and recorded with a smartphone digital camera.

Software

Equipment control, data acquisition, and processing were performed by WINCATS® 1.4.8 (Camag, Switzerland). Minitab® 17 (Minitab LLC, USA) was used for DoE planning and data analyses during the method optimization. Data analyses from the Youden test were assisted by ActionStat® Pharma (Estacamp, Brazil).

Method optimization

Screening

Preliminary analyses involved the screening of different TLC methods previously reported in the literature for qualitative analyses of C. sativa samples[5,17,20,26–28] regarding the potential for providing an appropriate chromatographic separation of the main cannabinoids found in the assessed samples. Other eluting systems composed of binary mixtures of hexane-chloroform, hexane-dichloromethane, or hexane-acetone, in different proportions, were also evaluated for potential application in normal-phase TLC. From the results obtained with the aforementioned conditions, modified eluent systems were preliminarily evaluated to improve the quality of the obtained chromatographic profiles. The effect of the mobile phase acidification with small amounts of glacial acetic acid on the characteristics of the chromatographic profile was also evaluated at this stage.

DoE-assisted mobile phase optimization

An experimental Mixture Design (MD) was applied for the simultaneous evaluation of a series of mobile phases containing hexane, ethyl acetate, and methanol in different proportions, acidified with glacial acetic acid (0.2%). The experimental matrix was elaborated based on a cubic simplex lattice design, with triplicates of the central point, totaling 15 experiments. Upper and lower limits were defined for the proportion of each component to restrict the experimental region based on the results from the previous steps (Table S1 and Table S1 and Figure S1, Supplementary Material). Each experiment was carried out by applying 20 μL of the CS.B, in duplicate, on 5 × 10 TLC plates. The resulting chromatograms were evaluated regarding the cannabinoids’ retention factors (Rf) and the resolutions (Rs) between each nearest pair. The “ideal separation” response function (IS)[29] was also evaluated as an indicator of the overall analytes’ separation. Polynomial models correlating the response variables to the mobile phase composition were estimated by applying a “stepwise” adjustment method (α = 0.15). Contour plots relating the responses of interest to the proportions of the components of the mobile phase were also generated.
Profiles obtained after the parallel analyses of different cannabinoid pairs at the same time. Moreover, the suitability of the optimized mobile phase was then confirmed by applying it to the analyses of mixed cannabinoid standard solutions A and B (CS.A and CS.B) and sample solutions.

Method validation

Ruggedness

The effects of some experimental factors on the method’s performance were assessed by applying the Youden experimental design. This study was performed before the assessment of other merit figures to integrate development and validation steps. The experimental variables selected for evaluation at this stage were the stationary phase manufacturer; the previous drying of the TLC plates in an oven; the chromatographic chamber configuration, including the presence or absence of filter paper, and the saturation time; in addition to small changes in the composition of the mobile phase likely to occur in routine analyses (Table 1). The experimental matrix was designed using ActionStat®, providing for the simultaneous evaluation of the aforementioned variables at two levels in eight experiments (Table 1). Analyses were carried out in 5 × 10 cm TLC plates. Twenty-five microliters of CS.B were applied in duplicate. The cannabinoid’s Rf and resolutions (Rs) were evaluated as response variables. Data analysis was performed using a hypothesis test based on Lenth’s method with a significance level of 5%.

Selectivity

Selectivity was assessed by comparing the chromatographic profiles obtained after the parallel analyses of different cannabinoid pairs. A mixed terpene standard solution containing β-caryophyllene, d-limonene, and β-myrcene (1 μg/mL in methanol), was analyzed in addition to the aforementioned solutions to investigate whether the Rf values of these secondary metabolites, also likely to be present in plant material, could coincide with those of the cannabinoids of interest. Minimum resolution of 1.5 between the critical cannabinoid pairs CBN/CBD and CBD/Δ9-THC in the CS.A chromatogram was established as a system suitability acceptance criterion.

A forced degradation study was also carried out as a complementary tool for selectivity assessment. To that end, a stock solution was prepared by spiking 200 μL of the sample solution obtained from the THC-dominant chemotype herbal material with a suitable amount of a mixed cannabinoid reference standard solution containing CBDA THCA, CBD, and Δ9-THC (500 μg/mL each), to give the final concentration of 100 μg/mL. Test solutions were then prepared by adding 100 μL of either a reagent or diluent and submitted to acidic, alkali, oxidative, photolytic, or thermal degrading conditions as described in Table 2. After the exposure time to the degradation conditions had ended, the volumes of the test solutions were made up with either a neutralizing reagent or diluent. The final solutions were then analyzed in parallel, following the proposed method (application volume: 10 μL). The obtained chromatographic profiles were compared to those obtained from a control solution to confirm the method’s ability to indicate the occurrence of degradations through notable changes in the observed cannabinoid profiles.

Table 1. Experimental variables included in ruggedness assessment, along with their respective levels, and experimental matrix created according to the Youden design.

| Experimental variable            | Levels | Experiment number |
|---------------------------------|--------|-------------------|
| TLC plate manufacturer (A)      | –1     | 1                 |
| Previous drying* (B)            | No     | Yes               |
| Mobile phase composition# (C)   | 85%    | 87%               |
| Chamber saturation time (D)     | 10 min | 20 min            |
| Chamber with filter paper (E)   | No     | Yes               |

*a10°C, 15 min.  
*bCorresponding to the hexane proportion.

table 2. Exposure conditions applied during the forced degradation study.

| Factor                      | Test solution preparation | Storage condition | Exposure time | Neutralization/dilution |
|-----------------------------|---------------------------|-------------------|---------------|-------------------------|
| Heating                     | 200 μL stock solution + 100 μL methanol | Amber glass vial; 60°C | 48 h          | 100 μL of methanol      |
| Light                       | 200 μL stock solution + 100 μL methanol | Transparent glass vial exposed to UV-vis light | 72 h          | 100 μL of methanol      |
| pH (acidic)                 | 200 μL stock solution + 100 μL HCl 0.05 M | Amber glass vial; 25°C | 48 h          | 100 μL of NaOH 0.05 M   |
| pH (alkali)                 | 200 μL stock solution + 100 μL NaOH 0.05 M | Amber glass vial; 25°C | 48 h          | 100 μL of HCl 0.05 M    |
| Oxidation                   | 200 μL stock solution + 100 μL H2O2 0.2% | Amber glass vial; 25°C | 72 h          | 100 μL of methanol      |
| Control                     | 200 μL stock solution + 100 μL methanol | Amber glass vial wrapped in aluminum foil; 25°C | 48 h          | 100 μL of methanol      |

Working point definition

Overlapping Resolution Mapping (ORM) was applied to assist in the identification of the experimental region most likely to provide a satisfactory resolution (Rs > 1.5) of all assessed cannabinoid pairs at the same time. Moreover, the optimal mobile phase composition was estimated by applying the Minitab tool for multiple response optimization. The suitability of the optimized mobile phase was then confirmed by applying it to the analyses of mixed cannabinoid standard solutions A and B (CS.A and CS.B) and sample solutions.

C. sativa samples from distinct chemotypes (see “plant material”), cannabinoids reference solutions (CS.A and CS.B), and a blank solution (methanol). A mixed terpene standard solution containing β-caryophyllene, d-limonene, and β-myrcene (1 μg/mL in methanol), was analyzed in addition to the aforementioned solutions to investigate whether the Rf values of these secondary metabolites, also likely to be present in plant material, could coincide with those of the cannabinoids of interest. Minimum resolution of 1.5 between the critical cannabinoid pairs CBN/CBD and CBD/Δ9-THC in the CS.A chromatogram was established as a system suitability acceptance criterion.

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Precision

The approach for precision assessment was adapted from Reich et al. and involved two levels, namely repeatability, and intermediate precision. Six independent replicates of the C. sativa sample solution were analyzed in parallel on the same TLC plate (application volume: 10 μL). The procedure...
was replicated on two additional TLC plates, on the same day. After the chromatographic development and derivatization, at least two bands corresponding to the cannabinoids of interest were selected in each chromatogram for the purpose of assessing Rf variability. For intermediate precision assessment, the aforementioned procedure was repeated on two additional days (1 plate per day), and the combined results from the 3 days were then compared. Acceptance criteria were defined so that the Rf difference should be not more than 0.02 within the same plate, not more than 0.05 between different plates on the same day, and not more than 0.1 between analyses carried out on different days. Moreover, the overall relative standard deviation (RSD%) of the Rf values recorded for each selected marker should be not more than 5% for repeatability and not more than 10% for intermediate precision.

**Results and discussion**

**Method optimization**

Among the screened mobile phases, the ternary system hexane-ethyl acetate-methanol (70:20:10, v/v/v) resulted in the most satisfactory chromatographic profile regarding the overall cannabinoids separation. Nevertheless, the neutral cannabinoids showed high Rf values (>0.8), with poor resolution between them. Mobile phases of lower polarity (e.g., petroleum ether-ethyl ether 80:20, v/v or hexane-ethyl ether 80:20, v/v) were unsuitable for the carboxylated cannabinoids. In its turn, chloroform or chloroform-methanol mixtures (e.g., 19:1, v/v) were excessively polar for the analytes of interest, resulting in low-quality chromatographic profiles. These results illustrate first of all the hardship of separating a diverse set of cannabinoids by TLC and, secondly, the difficulty of satisfactorily reproducing TLC methods already described in the literature. The latter can be attributed, at least in part, to the susceptibility of the technique to suffer interference from several experimental factors, as already mentioned in this paper. This highlights the need for appropriate validation of TLC-based methods,\[13,31\] as well as for establishing control strategies to ensure the consistent achievement of the desired method performance.

Also in the screening step, the effect of adding small amounts (up to 1%) of glacial acetic acid to hexane-ethyl acetate binary mixtures was evaluated to standardize the ionization state of acidic cannabinoids and avoid excessive retention of their ionized forms by the stationary phase. It was observed that the acidification of the mobile phase enhances the overall quality of the chromatographic profiles, reducing the broadening of bands and improving the distribution of acidic cannabinoids throughout the chromatogram.

The aforementioned results supported the planning of the experimental mixture design utilized in the optimization step. Accordingly, hexane, ethyl acetate, and methanol were selected as the three components of the simplex lattice. The experimental domain was restricted to maintain the mobile phase polarity within an adequate range for the analysis of the cannabinoids of interest, according to the results obtained in the screening phase (Table S1 and Supplementary Material). Glacial acetic acid was also added to the solvent mixtures in the final proportion of 0.2%, thus ensuring the maintenance of the acid cannabinoids in their protonated form. Polynomial models were estimated from the experimental results for each of the response variables to describe their behavior as a function of the mobile phase composition. In this study, the models were fitted using pseudocomponents. This approach is indicated to reduce the correlation between the model’s coefficients in cases where the experimental domain is restricted. Moreover, the “stepwise” adjustment method was applied so that only significant terms were included in the models ($\alpha = 0.15$).

With regard to the models estimated to describe the Rf variation, the S statistics were relatively low ($S < 0.06$), indicating a good agreement between the experimental data and the values adjusted by the model (Table 3). Furthermore, the adjusted determination coefficients ($r^2_{adj}$) for those models were higher than 89% (Table 3), thus indicating a good potential to describe the variation of these responses as a function of the mobile phase composition. The predictive determination coefficients ($r^2_{pred}$) also presented acceptable values (>83%), indicating good potential for predicting responses to new observations. The proximity between these

| Table 3. Fit indicators of the proposed polynomial models referring to the assessed response variables. |
|---------------------------------------------------------------|
| Response variable | $S$ | $r^2$ | $r^2_{adj}$ | $r^2_{pred}$ | Lack-of-fit (p-value) |
|--------------------|-----|-------|-------------|---------------|----------------------|
| Rf CBGA            | 0.0123 | 99.91% | 99.72% | 94.18% | 0.329 |
| Rf CBD             | 0.03797 | 98.31% | 97.10% | 94.44% | 0.566 |
| Rf THCA            | 0.04358 | 97.20% | 96.27% | 94.65% | 0.784 |
| Rf CBC             | 0.03963 | 97.59% | 96.39% | 93.38% | 0.661 |
| Rf CBN             | 0.05365 | 94.22% | 92.30% | 86.32% | 0.696 |
| Rf CBG             | 0.04791 | 95.42% | 93.13% | 87.07% | 0.791 |
| Rf Δ9-THC          | 0.05817 | 92.46% | 89.94% | 83.98% | 0.626 |
| Rf Δ9-THC          | 0.05513 | 92.18% | 89.57% | 83.17% | 0.584 |
| Rs (CBD/CBG)       | 0.15942 | 93.22% | 89.84% | 83.60% | 0.785 |
| Rs (THCA/CBD)      | 0.7037 | 93.08% | 86.16% | 77.05% | 0.819 |
| Rs (CB/GC)         | 0.3689 | 98.92% | 97.83% | 95.12% | 0.995 |
| Rs (CB/CBN)        | 0.4428 | 92.63% | 87.37% | 77.11% | 0.921 |
| Rs (Δ9-THC/CBD)    | 0.1848 | 99.03% | 97.67% | 92.60% | – |
| Rs (Δ9-THC)        | 0.4760 | 77.81% | 70.41% | 51.05% | 0.682 |
| Ideal separation (IS) | 0.0458 | 99.63% | 99.12% | 95.65% | 0.333 |
two coefficients also suggests the absence of overfitting for the evaluated models. As for the models referring to the resolutions between the critical pairs of cannabinoids, these indicators were more variable, with the $r^2_{pred}$ values of the models corresponding to the CBDA/CBGA and Δ⁸-THC/Δ⁹-THC pairs being relatively low (28.30 and 51.05%, respectively). The $r^2_{adj}$ and $r^2_{pred}$ of the other models were >70% (Table 3). Notwithstanding, as the purpose of the proposed models is only to indicate trends that allow the identification of the experimental region favorable to obtain the desired results, and not to be used as calibration models, the fact that the adjustment indicators assume relatively low values in some cases was not considered critical in the present study. The lack of fit was not significant for the assessed models ($\alpha = 0.05$).

The contour plots corresponding to the resolutions between the adjacent cannabinoid pairs are presented in Figure 1. It can be observed that each of those response variables is affected differently by the composition of the mobile phase, which indicates that obtaining a satisfactory resolution between all cannabinoids at the same time is a challenging task. Thereafter, an Overlapping Resolution Map (ORM) was constructed to assist in achieving the aforementioned objective (Figure 1h). For this purpose, a minimum resolution of 1.5 was set as an acceptance criterion as this can be considered sufficient to allow distinguishing adjacent bands after derivatization with the naked eye. The white area in the obtained overlaid contour plot represents the experimental region within which these criteria would be met.

In addition, the behavior of the "ideal separation" (IS) function within the experimental domain was also assessed, complementing the aforementioned approach. While the ORM focuses on the resolution of each adjacent analyte pair, the IS assessment seeks to maximize the global separation of the analytes, considering their distribution throughout the chromatogram, so the lower the value of the IS function, the better the band distribution in the chromatographic profile. Accordingly, the IS contour plot (Figure 2) indicates the regions of the experimental domain with the best potential to achieve this objective.

Subsequently, the Minitab® response optimizer tool, which is based on Derringer’s desirability function, was applied to assist in the estimation of the mobile phase composition that would provide satisfactory resolutions between all the assessed cannabinoids, in addition to maximizing the band distribution throughout the chromatogram. To that end, both the resolutions between adjacent cannabinoids and the IS function values were combined as response variables in the optimization analysis. The acceptance criteria were defined according to the aforementioned objectives (Table S2, Supplementary Material). The results obtained from the response optimization analysis indicate that the ideal composition of the mobile phase to achieve the desired chromatographic performance would be hexane-ethyl acetate-methanol (85:10:5, v/v/v), acidified with glacial acetic acid (0.2%v/v). The composite desirability index was 0.8915 (Figure S2, Supplementary Material). The working point that corresponds to the optimized condition is represented in Figure 1 and Figure 2 by a star-shaped symbol. One can notice that, in fact, this point is within the suitable experimental region indicated by the ORM analysis.

The suitability of the optimized chromatographic condition was experimentally demonstrated by applying it to the analysis of mixed cannabinoid standards solutions and sample solutions. The results from this analysis show that the resolutions between all analytes of interest were satisfactory, as was the overall quality of the chromatographic profiles.

**Method validation**

**Ruggedness**

The experimental design suggested by Youden and Steiner in 1975 has been widely applied to ruggedness analyses during validation studies for allowing the simultaneous evaluation of the effects of up to 8 experimental parameters with a reduced number of experiments. Despite that, examples of the application of this approach to the validation of qualitative TLC methods are scarce in the literature. Notwithstanding, the large number of experimental factors that can interfere with the performance of TLC methods makes this approach very promising to be applied in such situations. Accordingly, in the present study, the Youden method was applied to investigate the effects of changes in some experimental variables on the method’s performance.

The selection of the experimental factors to be evaluated was carried out to cover small changes likely to occur in routine analysis (e.g., the proportion of mobile phase components and the saturation time of the elution chamber); in addition to changes that may eventually occur while adapting the proposed method (e.g., the manufacturer of the TLC plate; the previous drying of the TLC plate in an oven and the use of filter paper in the elution chamber). The interpretation of the obtained results was focused on understanding the effects of these changes on the chromatographic behavior of the analytes of interest to allow establishing appropriate controls to favor the achievement of reproducible and good-quality chromatographic profiles.

The results obtained after the analysis of the CS.B applying the conditions described in the experimental matrix are presented in Table S3 (Supplementary Material). The overall variability of Rf values was relatively high, which highlights the need to identify the most influential parameters and define appropriate controls regarding them. Among the factors evaluated, the presence of filter paper in the elution chamber generated the most notable effects, having a statistically significant influence on the Rf of all assessed cannabinoids ($p < 0.05$), except THCA. Lenth’s plots indicate that the presence of filter paper tends to reduce the Rf values, with higher effects on neutral cannabinoids (Figure S3, Supplementary Material), resulting in a non-ideal distribution of analytes throughout the chromatograms. Therefore, it is recommended that the proposed method be applied without using filter paper. In turn, the use of TLC plates from different manufacturers and the change in mobile phase composition, with an increase in the proportion of...
the less polar component, had statistically significant effects ($p < 0.05$) on the migration of $\Delta^9$-THC and $\Delta^8$-THC only. However, the magnitude of the observed effects was notably smaller than that related to the presence of filter paper in the elution chamber (Figure S3, Supplementary Material). It is worth noting that the resolution between these analytes was not significantly affected (Figure S4, Supplementary Material), indicating that the method’s performance would
The cannabinoids of interest were well-distributed throughout the chromatographic profile. The densitograms obtained after parallel analysis of cannabinoids reference standards during routine analyses is indicated so that the method’s performance can be appropriately monitored.

**Selectivity**

The main goal of selectivity assessment for qualitative TLC methods is to demonstrate the ability to provide discriminative chromatographic profiles while allowing the identification of the identity markers of the plant species, if applicable. To that end, the proposed method must be challenged, using a set of structurally similar analytes, in addition to samples with different characteristics. Furthermore, the method’s stability-indicating properties should be demonstrated so that it can be reliably applied for fingerprint monitoring during stability studies. Accordingly, the conduction of forced degradation studies can complement the selectivity assessment, allowing to evaluate whether the method can indicate the occurrence of degradation reactions through notable changes in the pattern of bands observed in the obtained chromatographic profiles.

In the present study, the selectivity assessment focused on the demonstration of the method’s ability to allow distinguishing between samples from different chemotypes and different origins based on the qualitative evaluation of the cannabinoid profile. The densitograms obtained after parallel analysis of cannabinoids standard solutions, sample solutions, and blank solutions are shown in Figure S5 (Supplementary Material). An image of the obtained TLC plate recorded after derivatization with sulfuric vanillin SR is shown in Figure 3. One can notice that the analytical signals attributed to the cannabinoids of interest were well-distributed throughout the densitograms, and the resolutions between them were found to be sufficient for qualitative analyses. Likewise, the bands corresponding to the assessed cannabinoids observed after derivatization were easily differentiated from each other (Figure 3). The system suitability acceptance criteria were also met, with a minimum resolution of 1.33 between the critical pairs of cannabinoids present in the reference solution, determined after derivatization (Figure 3). Furthermore, the chromatogram corresponding to the blank solution did not show any visible signs in the region of interest (Figures S5 and Figure 3), thus indicating that the diluent would not present considerable interferences on the obtained results.

In addition to the aforementioned analyses, a mixed terpenoid standards solution containing d-limonene, β-myrcene and β-caryophyllene was also evaluated to evaluate the chromatographic behavior of these secondary metabolites under the proposed analytical conditions. One can observe that the chromatographic profile corresponding to this solution showed very intense bands, from pink to violet, in the region close to the plate front (Figure 3). The position and intensity of the band with Rf ≈ 1.0 indicate that most of the assessed terpenes were poorly retained by the stationary phase, resulting in coelutions in this region. This hypothesis was later confirmed by the parallel analysis of isolated standards of the selected terpenes, which also indicated the formation of a β-caryophyllene degradation product (data not shown). Thus, it was not possible to satisfactorily distinguish the
terpenes evaluated using the proposed method. Notwithstanding, the obtained results are valid to demonstrate that the terpenes most commonly found in the plant species are not likely to interfere with the identification of the cannabinoids of interest since their Rf values are markedly different.

Regarding the forced degradation study, the chromatographic profiles corresponding to the solutions submitted to degrading conditions were visibly different compared to the control condition (Figure S6, Supplementary Material). Among the conditions evaluated, the thermal degradation caused the most expressive change in the sample’s chromatographic profile, with a significant reduction in the intensity of the bands corresponding to the acid cannabinoids CBDA and THCA, accompanied by an increase in the intensity of the bands corresponding to their neutral counterparts, CBD and Δ⁹-THC. These changes are compatible with the occurrence of decarboxylation reactions. In turn, both acidic and alkaline conditions caused a decrease in the intensity of the bands corresponding to the monitored cannabinoids compared to the control condition, indicating a possible degradation of those compounds. However, the emergence of other bands of notable intensity was not observed in those cases, which hampers attempts to make conclusions regarding the eventual degradation products. The chromatographic profiles of the samples submitted to photodegradation or oxidative degradation were quite similar to the control profile, indicating that the degrading conditions applied in these cases were not sufficient to promote a more intense degradation of the monitored constituents. It is worth noting, however, that the main oxidation product of Δ⁹-THC, i.e., CBN, was shown to be identifiable by applying the proposed method, which indicates that degradation involving this pathway could be easily monitored. The aforementioned results indicate that the proposed method is capable of evidencing the occurrence of changes in the cannabinoid profile of *C. sativa* samples, therefore being likely to be used throughout stability studies.

**Precision**

The main goal of the precision assessment in the present study was to demonstrate the agreement between the chromatographic profiles obtained in different analyses by monitoring the variability of the Rf values of selected markers. The approach used was based on the protocol described by Reich et al., with modifications.

The chromatographic profiles obtained from the parallel analyses of sample solutions on the same TLC plate were quite similar, as demonstrated by the superposition of the corresponding densitograms (Figure 4). Two intense bands were observed in the obtained chromatographic profiles,
with $Rf \approx 0.36$ and 0.55, being attributed to the cannabinoids THCA and $\Delta^8$-THC, respectively (Figure S7, Supplementary Material). This pattern is compatible with a chromatographic profile characteristic of samples from the THC-dominant chemotype. Thereby, those analytical signals were selected for purposes of comparison between the different analyses. The chromatographic profiles obtained after repeating the analyses on two additional TLC plates on the same day were also quite similar, with little variability in the $Rf$ values of the selected markers (Table 4). The highest difference between the $Rf$ values was 0.02 for profiles from the same plate and 0.05 for those from different plates, with a maximum overall RSD of 3.26% (Table 4), thus fulfilling the previously established acceptance criteria.

In turn, the chromatographic profiles obtained after the analyses of the sample solutions on different days were similar to each other regarding the band pattern, although some differences in hue were observed (Figure S8, Supplementary Material). The overall variability of the $Rf$ values of the selected markers was slightly higher than that recorded on the first day of analysis (Table 5). Even so, the acceptance criteria were met. The aforementioned results suggest that the method is capable of providing chromatographic profiles with reproducible patterns. Nevertheless, parallel analysis of reference solutions of the analytes of interest is recommended to allow the correct assignment of the chromatographic signals since slight variations in hue and $Rf$ may occur.

### Conclusions

The proposed method proved to be adequate for the qualitative assessment of the cannabinoid profile of *C. sativa* samples, allowing the differentiation between relevant chemotypes of the plant species, in addition to being stability-indicating. However, since some cannabinoids of interest presented similar $Rf$ values, coelutions may occur under certain conditions, especially if the analytes’ concentrations in the sample are too high, causing band thickening. Therefore, it is recommended to apply one or more orthogonal methods based on other chromatographic techniques (e.g., HPLC-DAD) to complement the evaluation of the sample’s cannabinoid profile.

It is also worth mentioning that the DoE-assisted development approach allowed for a better understanding of the analytes’ chromatographic behavior as a function of the mobile phase composition, thus favoring the achievement of an optimal separation of the cannabinoids of interest. Moreover, the validation approach, which was adapted taking into account the peculiarities of the TLC technique and the method’s purposes, has satisfactorily demonstrated the achievement of relevant performance attributes. Notably, the Youden design applied to ruggedness assessment proved practical to support the definition of appropriate control strategies to ensure the method’s reproducibility.

Other analytical methods are being concomitantly developed in our laboratory, aiming at the drafting of pharmaceutical monographs for cannabis-based API and preparations.

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### Disclosure statement

Although one of the authors is a federal employee at the Brazilian National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária, ANVISA), the discussions included in this paper do not necessarily reflect the official position of the Agency nor imply regulatory obligations. That said, the authors report there are no competing interests to declare.

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### Data availability statement

The data that support the findings of this study are available from the corresponding author, MRS, upon reasonable request.

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