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Determination of phytocannabinoids in cannabis samples by ultrasound-assisted solid-liquid extraction and high-performance liquid chromatography with diode array detector analysis

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ABSTRACT

The characterisation of cannabis plants, especially the determination of specific phytocannabinoids, has gained enormous importance in the last decade, mainly due to the recent changes in cannabis control in several countries or states. This is particularly relevant for the forensic, medical or recreative industry to have a rapid, inexpensive, and reliable methodology to identify and quantify phytocannabinoids. Furthermore, spiking cannabis products with Δ^8-tetrahydrocannabinol (THC) is a contemporary trend that demands improving or replacing current methods to include this cannabinoid. The current study presents an ultrasound-assisted solid-liquid extraction followed by high-performance liquid chromatography with diode array detection (HPLC-DAD) methodology to identify and quantify Δ^9-THC, Δ^8-THC, cannabidiol, cannabinol, Δ^9-tetrahydrocannabinolic acid and cannabidiolic acid in cannabis products. The herbal samples were extracted with ethanol:acetonitrile (50:50, v:v) by ultrasonication using only 50 mg of sample. The plant oils were diluted in ethanol. The optimised procedure allowed ≈100% extraction efficiency of the target cannabinoids. The validation assays showed that the method is linear ($R^2 > 0.997$), selective, sensitive, precise and accurate, with suitable limits of detection (0.125–0.250 μg mL^{-1}) and quantification (0.500 μg mL^{-1}). The method was successfully applied to cannabis samples, demonstrating its suitability for routine analyses. This contribution follows the current demand for fast and straightforward analysis services of this plant and its derivatives, using small amounts of sample. The present study compares very favourably against other works, particularly in regards to the extraction efficiency, speed of the overall procedure, method sensitivity, and ability to monitor Δ^8-THC spiked samples using a novel solvent mixture.

1. Introduction

The cannabis plant has been used by humankind for several millennia. Its use by ancient civilisations to produce textile fibres and therapeutic purposes dates back more than 5000 years [1,2]. Cannabis has many chemical compounds, with over 120 classified as phytocannabinoids, primarily responsible for its pharmacological properties [3,4]. Within this broad set, Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are present in higher amounts, primarily concentrated in the female inflorescences in their acidic forms, Δ^9-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) [5–7]. While Δ^9-THC is primarily responsible for the psychoactive activity, which may lead to euphoria and relaxation in humans [8–10], CBD is associated with anti-inflammatory, anticonvulsant, anxiolytic and analgesic properties, attenuating the psychoactive effects caused by Δ^9-THC consumption [11,12]. However, cannabis use can lead to undesirable effects such as cognitive impairment, paranoia, anxiety, chronic psychosis, or drug addiction [8,13,14].

The acid forms can be readily decarboxylated into their neutral forms, namely Δ^9-THC and CBD. Over time, Δ^9-THC can be converted to CBN or Δ^8-tetrahydrocannabinol (Δ^8-THC) [15,16]. CBN content in cannabis mainly depends on time and storage conditions [17]. CBN is highly stable against oxidative degradation and has been used as a marker to identify cannabis in archaeological findings [18]. Interestingly, Δ^8-THC has already been synthetically converted from CBD [19] and can be used to spike hemp plants [20]. This is particularly important, since it has psychoactive poten-

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tial and is not controlled in several countries. Moreover, there is a lack of public awareness that this compound is spiked in samples to circumvent the law. Nevertheless, in some countries, Δ^8-THC is becoming a controlled molecule.

In the forensic context, in 2018, a report from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), attributes 71% of registered seizures to cannabis products [21]. In 2020, cannabis remained the most commonly seized illicit substance (66% of seizures). To this end, there was a substantial contribution from an increased liberalisation of cannabis (or hemp) products available at "Cannabis shops" [22].

Regulatory legislation for substance abuse in the European Union is essentially based on the United Nations Conventions of 1961, 1971 and 1988 [23]. However, the legal penalties for the illicit use and supply of cannabis vary substantially between Member States. In Portugal, as well as in other European countries, *Cannabis sativa L.*, as well as a set of THC isomers, including Δ^9 or Δ^8, are under strict control [24]. According to most EU laws, cannabis is allowed for industrial purposes as long as THC levels do not exceed 0.2% (w/w) [25], and according to the new Common Agricultural Policy, since January 1st 2023, this value must increase to 0.3% [26]. For this reason, there is a need for analytical strategies that enable fast and reliable determination of cannabinoid content in the cannabis plant.

Solid-liquid extraction (SLE) [27–29], followed by reverse-phase high-performance liquid chromatography with ultraviolet (HPLC-UV) or diode array detection (HPLC-DAD) are the most commonly used approaches to simultaneously quantify neutral and acidic cannabinoids in cannabis plant materials [30,31]. The main advantage of HPLC is the ability to separate and quantify cannabinoids in neutral and acidic forms without the need for a derivatisation step [10,32,33] and with a much lower risk of sample decomposition. Sonication uses sound energy above 20 kHz (ultrasonic) to cause air bubbles in a liquid to implode in a process called cavitation. Moreover, US-SLE is considered a green method due to increased extraction efficiency, resulting in low solvent use [29,34]. Finally, US-SLE reduces the time required for extraction, decreases the cost of analysis and lowers potential cannabinoid loss due to degradation, which markedly benefits forensic science laboratories.

In the present study, we propose the development, optimisation and validation of an analytical methodology to monitor the six most significant cannabinoids in cannabis plant material, including Δ^9-THC and CBD, their acid forms, CBN and Δ^8-THC. To this end, SLE supported by sonication was used, with subsequent detection and quantification by HPLC-DAD.

2. Materials and methods

2.1. Reagents, standards and solutions

HPLC-grade absolute anhydrous ethanol (EtOH, 99.9%) and formic acid (COOH, 99.0%) were supplied by Carlo Erba (France). HPLC-grade methanol (MeOH, 99.9%), acetonitrile (ACN, 99.9%), and propan-2-ol (2-PrOH, 99.9%) were obtained from Honeywell (France). The ultrapure water type I used was obtained from a Sinergy-UV water purification system (Merck Millipore, Portugal). All certified reference materials (CRM), *i.e.* cannabinoids in solution, Δ^9-THC, CBD, Δ^8-THC and CBN, in methanol and Δ^9-THCA, CBDA in acetonitrile, were obtained from Dr Ehrenstorfer (UK) at a concentration of 1 mg mL^{-1}. Phemprocoumon (97.1%), used as an internal standard (IS), was obtained by Dr Ehrenstorfer (UK). Each calibration standard (0.5; 2.5; 5.0; 10.0; 15.0, and 25.0 µg mL^{-1}) was prepared individually from standard mixtures, thus avoiding error propagation. All solutions were prepared in methanol and IS to obtain a final IS concentration of 50.0 µg mL^{-1} and then stored and preserved in amber glass vials at recommended temperatures (−80 °C for mixtures containing Δ^9-THCA).

2.2. Cannabis samples

Cannabis herbal samples (7 dried flower tops) were provided by Kosmicare (Lisbon, Portugal), a non-profit organisation dedicated to Drug Checking services for healthy consumption, from the recreational use samples submitted for analysis. The other herbal samples (8 dried flower tops) and two "CBD oils" were purchased from "Cannabis shops" available to the public from different local stores in Almada (Portugal). The dried hop flowers were purchased from Novadiet (Spain).

2.3. Extraction optimisation

To optimise the extraction process, several parameters were evaluated using different design of experiment (DoE) approaches and assays to facilitate sample handling and decrease the volume of solvents and time needed for cannabinoid extraction. After the selection of the best solvent mixture to recover the target compounds, using a single centroid mixture design, another experimental design was performed to establish other relevant extraction conditions. In this case, we used a full factorial design with two continuous (2 levels each) and one categorical (with three levels) factors. The considered parameters were the solvent type (ACN, methanol and ethanol) and extraction volume (0.5 - 15 mL), the number of steps (1–3 extraction cycles) and the required sonication time (1 and 20 min). Full factorial design was used to determine the volume of extractant, ultrasonication time and the number of extraction steps.

The experiments were performed in duplicate (24 trials), testing 3 mL and 10 mL of acetonitrile: ethanol (50:50, v:v). For the extraction time, 1 min was compared with 20 min. The number of extraction cycles (1, 2 or 3) was also analysed. The optimisation procedures were performed using street samples previously analysed in preliminary studies to consider possible interferences of the matrix components. DoE analysis was performed using Minitab 17 Statistical Software (version 17.1.0, 2013).

2.4. Sample preparation

Sample preparation was carried out according to the United Nations Office on Drugs and Crime (UNODC) guidelines and other scientific literature [3,35,36], with several adaptations and after careful optimisation. All herbal samples were obtained already dried, and therefore it was only necessary to ground them manually using mortar and pliers, followed by IS spiking, to get a concentration of 50 µg mL^{-1}. 50 mg of sample was used since, in preliminary studies, it was determined that this small amount was suitable for analysis [20,37]. The resulting powder was subjected to 1 min of ultrasound-assisted solid-liquid extraction (US-SLE) (TPC UC-1000 Ultrasonic Cleaner, 40 kHz, 180 W, USA) using 10 mL of acetonitrile:ethanol (50:50, v:v). The final solution was filtered through a 0.2 µm PTFE filter, and 1 mL was placed in a vial for subsequent instrumental analysis. For cannabis oils, a simple dilution of the sample with ethanol containing the IS (50 µg mL^{-1}) was adequate [38–40].

2.5. HPLC-DAD analysis

The HPLC-DAD analysis was carried out on a benchtop Agilent 1100 series LC chromatographic system (Agilent Technologies, Germany) equipped with an autosampler (G1313A), thermostated column compartment (G1316A), quaternary pump (G1311A) and a diode array detector (G1315B). The data acquisition and system

control were performed using the Clarity Chromatography Station software (versão 7.4.1.88; DataApex). Multiples mobile phases compositions were tested, using different solvent (ACN, MeOH) at distinct ratios, with organic phases ranging from 65- 90%. Isocratic separation was performed for 30 min using a flow rate of 0.5 mL min^{-1}, composed of 75% acetonitrile and 25% ultrapure water with 0.1% formic acid (pH ≈ 2.8), always degassed before use through ultrasonic treatment (TPC UC-1000 Ultrasonic Cleaner, 40 kHz, 180 W, USA). A Kinetex 2.6 µm C18 (octadecyl) 150 mm x 2.1 mm reverse phase column equipped with a guard column C18, 2.1 mm (SecurityGuard ULTRA Cartridge, AJ0–8782, Phenomenex, USA) was used to separate the target compounds. The thermostated column compartment and the injection volume were set at 20 °C and 10 µL, respectively. The DAD was set at wavelengths of 230, 280, 254 and 320 nm, previously selected according to the literature review and by confirmation of the maximum absorbance of the compounds' spectra [20,41]. A wavelength of 230 nm was selected for quantification purposes since it maximised the instrument signal-to-noise ratio (S/N).

2.6. Method validation

The proposed methodology was validated in accordance with the guiding principles of Eurachem and UNODC [42,43]. This included sensitivity, selectivity, working range, linearity, precision, trueness, sample repeatability, and analytical standards stability. Blank assays were also performed using neat methanol (with and without IS). Except when specified, all validation assays were performed in triplicate.

The validation assays were performed using neat standard solutions since the proposed extraction process showed that all target cannabinoids were fully recovered from spiked hops (*Humulus lupulus*) at two concentration levels. This was used as a surrogate matrix for the cannabis plant since both are members of the *Cannabaceae* family [44,45].

Next, calibration standards prepared in methanol at different concentrations were analysed in triplicate to establish linearity. The acceptance criteria included a determination coefficient (R^2) greater than 0.99, residual plots showing model adequacy and homoscedasticity assays. Goodness-of-fit (GoF) tests were performed with a 95% confidence interval. Selectivity was assessed by determining the resolution of the analyse peaks in standard mix solutions and cannabis extracts with different cannabinoid contents. Furthermore, selectivity was evaluated by analysing hops, which had been processed as described for cannabis flowers.

The sensitivity of the methodology was ascertained through the limits of detection (LODs) and quantification (LOQs) for a concentration having a S/N of 3/1 and 10/1, respectively. The lower limit of quantification (LLOQ) was also evaluated and defined as the lowest concentration value within acceptable levels of trueness and precision, corresponding to the lowest concentration standard of the calibration plot (0.5 µg mL^{-1}). The LODs were estimated by visual examination of chromatograms from standard solutions containing decreasing concentrations of the target phytocannabinoids.

Intra and inter-day precision and trueness were evaluated for three levels of quality controls (1.0, 12.5 and 20.0 µg mL^{-1}) in pentaplicate. Precision was calculated as the relative standard deviation (RSD) for each quality control of individual cannabinoid on a single day (intraday precision) or on three consecutive days (interday precision). Interday precision was calculated as RSD_{pooled}. Intra and interday trueness was calculated as the relative error percentage (or bias). The acceptance criteria for precision and trueness were that bias and RSD should both be less than 15.0%.

In order to ensure the repeatability of sample results, two distinct samples of previously analysed cannabis plant material with different phytocannabinoids content were selected. For each, analyses were performed in triplicate, on the same day (intraday) or on three different days (interday). For intraday repeatability, RSD was determined, and for interday repeatability, RSD_{pooled} was calculated as described for precision. The acceptance criteria were that both RSD values should be less than 15.0% [46].

To assess the standard solutions stability, a standard mixture of the six target cannabinoids at 10.0 µg mL^{-1} was analysed by the described instrumental methodology several times for three months.

An assay was carried out with hops as a surrogate matrix to evaluate extraction efficiency. Spiking assays were performed with the six target cannabinoids as well as IS. The spiked extracts were compared with a standard mixture of the same cannabinoids in methanol at the same concentration and chromatographic conditions.

2.7. Application of the study in real samples

The developed methodology was applied to the analysis of 16 cannabis samples for recreational use, including eight herbal samples bought from "Cannabis shops", which claimed to be "legal" (<0.2% THC), commercially available CBD-rich cannabis flowers, and 2 "CBD oils". Every sample was analysed in duplicate.

3. Results and discussion

3.1. Method development and instrumental optimisation

In order to obtain the best instrumental conditions using HPLC-DAD, the cannabinoids under study were analysed individually to check their retention times and UV/Vis spectra. Different parameters were studied to obtain the best resolution, selectivity and sensitivity. Based on the literature [36,47], several methods, using gradient and isocratic separation, were tested using different mobile phases with distinct solvent ratios. The choice of 75% ACN resulted in a more stable baseline and optimal separation of the target cannabinoids compared to the other compositions. Wavelengths of 230, 280, 254 and 320 nm were tested using the DAD, in accordance with the literature [20,41]. From our studies, 230 nm was selected for the target phytocannabinoids quantification since it provided the best S/N. The remaining wavelengths proved to be quite useful, permitting an increased specificity of the method and allowing the identification of interfering compounds.

3.2. Extraction optimisation

The UNODC has already established an US-SLE based methodology to extract the target cannabinoids from herbal matrices efficiently. However, this approach uses large amounts of sample (500 mg) and solvent, as well as several analytical cycles. In order to circumvent these limitations, we decided to develop an optimised US-SLE approach based on the UNODC guidelines using only 50 mg of herbal cannabis sample. This included selecting the best extraction solvent to extract the target compounds, and to minimise solvent consumption. Moreover, the number of extractions and sonication time was also optimised.

According to UNODC recommended methods for neutral and acidic cannabinoids analysis [35], the most suitable extraction solvents are methanol, ethanol and mixtures with chloroform or acetonitrile [48]. Chloroform was not included in this study since it is incompatible with the employed chromatographic conditions and would add unnecessary analytical steps. Thus, ethanol, methanol and acetonitrile were selected for a single centroid mixture design to maximise extraction yields.

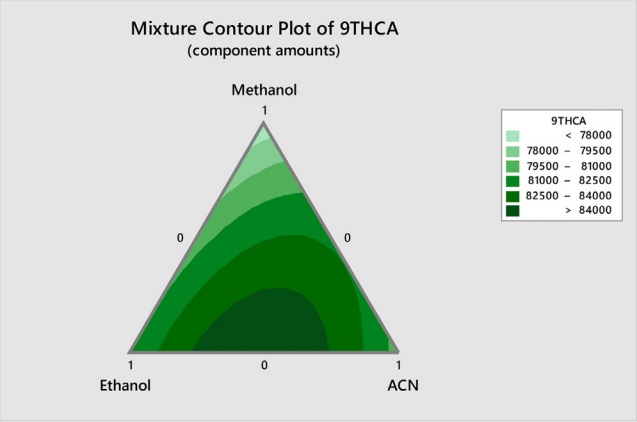

Fig. 1. Contour Plot of the solvent mixture for Δ^9 -THCA. The areas represented in darker colour reflect greater extraction yields, suggesting that the best results were obtained using an equivolume mixture of acetonitrile and methanol.

The choice of single centroid mixtures design resulted from the number of solvents used. This allowed to test each solvent individually, and also mixtures of two or more solvents (DoE in Table A1 of the supplements). Fig. 1 shows the contour plot for Δ^9-THCA, where the ideal mixture is ethanol:acetonitrile (50:50, v/v). Similar results were obtained for all compounds (see Figure A1 in supplemental files). This data suggests that the combined use of polar aprotic and protic solvents positively contributed to maximising the extraction yields of all target compounds.

This result was quite surprising since the use of acetonitrile for phytocannabinoid extraction is scarce in the literature [29]. Previously, just two studies used water: acetonitrile [37] and methanol: acetonitrile [49] mixtures. To the best of our knowledge, the current study is the first using the mixture of ACN and ethanol, ensuing a novel approach and more successful analysis of phytocannabinoids in plant material, particularly in herbaceous samples.

Fig. 2 shows the results of the performed factorial design. As it can be observed, and according to the significance values (P-value) of each factor and respective interactions for each of the cannabinoids in herbaceous sample, the only significant factor affecting the extraction performance was the volume used, for a 95% confidence interval (see Figure A2 as an example). Thus, an approach was established using only one cycle with just one minute in the sonication step, significantly accelerating the extraction procedure.

Finally, since the extraction volumes proved to be the most limiting factor in the proposed method, five different volumes were tested to maximise the extraction yields and, at the same time, minimise the usage of organic solvents. Five values, including 0.5 mL, 2 mL, 6 mL, 10 mL, and 15 mL of solvent were tested in triplicate. The obtained signal, up to 6.0 mL, was slightly lower than those for greater solvent volumes. There was no significant difference between 10.0 and 15.0 (Figure A3). Thus, the optimum volume of extracting solvent was set at 10.0 mL.

For cannabis oils a simple dilution step using ethyl alcohol was performed prior to instrumental analysis. The addition of methanol resulted in an emulsion. The use of the other two alcohols (ethanol and isopropanol) resulted in transparent and translucent samples, with no need for extraction and centrifugation steps. Comparing the peak areas obtained by each solvent, results were quite similar, with ethanol giving a slightly higher signal, thus being defined as the solvent to be used for the dilution for the cannabis oil samples.

3.3. Extraction efficiency assays

The data shows that the target cannabinoids were fully extracted using the optimised approach, with efficiency values ranging from 98.8% to 105.6%. A comparison between the spiked and non-spiked hop matrix, and IS spiked, showed that no co-elutions occurred on the expected retention times of the target compounds.

The proposed fast US-SLE method (1 min) and the obtained high extraction efficiencies clearly show that the developed approach compares very favourably with previous works. *I.e.*, the average time of extraction times reported in the literature was above ten minutes [3,20,37,50]. Moreover, the present method uses only one extraction cycle, greatly increasing sample throughput.

3.4. Method validation

The working range was defined to take into account that cannabis samples must always be submitted to a dilution step to reduce possible interference from the matrix and avoid saturation of the chromatographic column. Thus, the linearity was established between 0.5 and 25.0 μg mL^{-1} for all target compounds. The R^2 values were between 0.9978 and 0.9988 for the six analysed cannabinoids, indicating optimum linearity. Homoscedasticity tests were also performed (as an example, CBD presented F_{calc} of 0.0044 in contrast with F_{tab} of 19) for each cannabinoid and residual plots, validating homoscedasticity for all compounds.

Precision and trueness were evaluated using quality control samples (QC) at three concentration levels, *i.e.*, 1.0, 12.5, and 20.0 μg mL^{-1} in pentaplicates. The RSD and the resulting bias values below (±)15% showed that the developed methodology meets the requirements of international guidelines (Table 1). The LODs and LLOQs were determined experimentally, obtaining similar results amongst the various cannabinoids. The LLOQ was 0.5 μg mL^{-1} for all compounds, and LOD was 0.250 μg mL^{-1} for CBDA and Δ^9-THCA, while for the rest of the cannabinoids was 0.125 μg mL^{-1}.

An independent standard mixture was quantified under the previously described conditions for three months. The variation between the first and last analysis was minimal (<1.8%), for all compounds under study. This result shows long-term stability of the standard mixtures and the instrumental system.

The low intra and inter-day RSD values obtained in the repeatability assays demonstrate that, despite the common variability associated with the complex nature of these matrices, the method, from sampling to the chromatographic detection of cannabinoids, showed suitable repeatability (intraday precision and intraday) and interday precision, as it was performed on similar aliquots. All RSD values were below 6%.

Overall, the validation parameters met the guidelines criteria, while presenting lower LOD and LLOQ values when compared to previous similar work [3,37,50].

3.5. Analysis of street samples

The developed methodology was applied to 17 herbal and oil cannabis samples. The results are displayed in Table 2.

Δ^8 -THC was only identified in sample A3, with a content of 0,06%. Therefore, this cannabinoid was not represented in Fig. 4. The amount of each cannabinoid in the samples (%, w/w for herbal, w/v for oil) was calculated using the applied dilution factor in the initial weighed herbal sample. Fig. 3 shows overlayed chromatograms from three different sources: a standard mixture of the six target cannabinoids, high Δ^9-THC herbal sample (A8) and a CBDA rich oil sample (B1), with a dilution factor of 250 and 1000, respectively.

Sample A8, shows a relatively high peak of Δ^9-THCA, but in contrast, low levels of CBDA and Δ^9-THC. CBD, CBN and Δ^8-THC were not detectable under these conditions. The "CBD" oil sam-

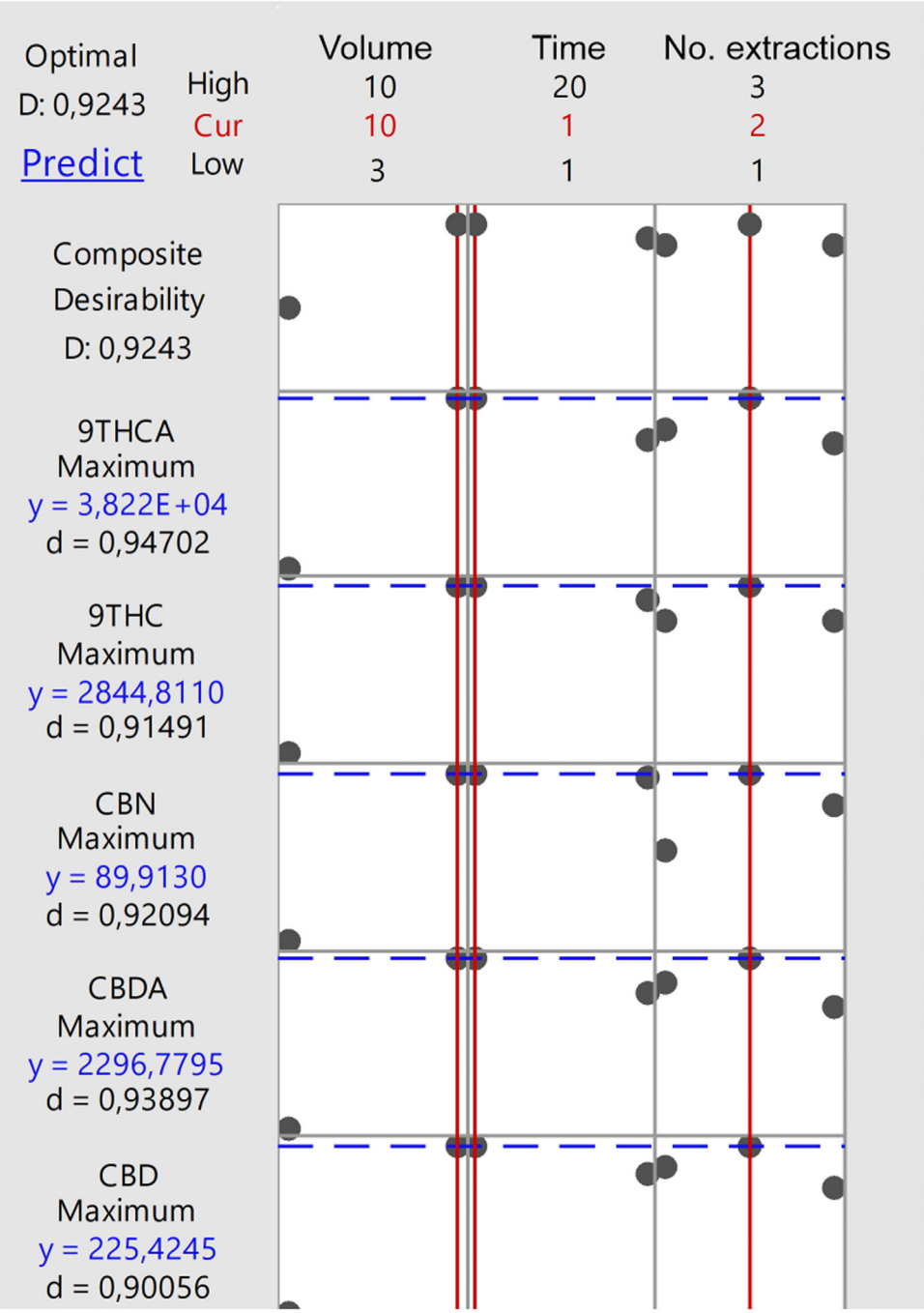

Fig. 2. DoE results for the determination of the necessary volume of extraction solvent (left), extraction time (middle) and number of extraction cycles (right). The 10 mL volume resulted in a higher signal for all cannabinoids. The number of extractions as well as the ultrasonic extraction time did not show significant variation.

ple, as labelled, was shown to have CBDA but residual levels of the compound in its neutral form.

Fig. 4 shows the target phytocannabinoids content in all analysed samples. There is one set of Δ^9-THC /Δ^9-THCA-rich samples, represented in dark and light blue, all from Kosmicare (A), and a second set of store-bought, CBD/CBDA-rich, dark and light green samples. Kosmicare street samples main compound, Δ^9-THCA, comprised contents from 0.45% (A1) to 17.43% (A3), with an average of 8.67%. Store-bought samples' main compound was CBD, with content ranging from 1.32% (A10) to 13.58% (A11), with an average of 6.17%. For the "CBD-rich" oils, labelled values were 5% (g mL^{-1}) for B1 and 10% for B2, of CBD, respectively. These values have not matched the quantified ones since CBD appears in minimal quantities and CBDA is the predominant compound in both samples (green%).

Given the 11% average THC content reported in the European Drug Report (EMCDDA, 2022) for herbal samples analysed in 2020, the amount of Δ^9-THC_T (acid and neutral forms) that appeared in the samples supplied by Kosmicare, with an average of 12.36%, turns out to be slightly higher. Noteworthy are samples A3 and A8, in which the quantified Δ^9-THC_T content was over 18%, illustrating the high phytocannabinoid content that the samples can have, and the heterogeneity of values that can exist in these substances of abuse available on the illegal market.

Regarding the contents indicated on their labels, both for analysed oils and herbaceous samples, only one of the quantifications

Table 1
Inter and intra-day precision, trueness, LOD and LOQ values for each cannabinoid at each Quality Control (QC) level.

Cannabinoid	QC (μg/mL)	Trueness (%bias)			Precision (%RSD)			Inter-day	
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Trueness (%bias)	Precision (%RSD pooled)
CBD	1,00	11,64	14,49	14,49	8,02	1,83	3,51	−13,00	4,90
	12,50	2,32	−5,30	−5,30	0,36	4,43	0,94	4,00	2,90
	20,00	−2,75	−2,22	−2,22	1,39	0,59	0,32	2,60	1,00
CBDA	1,00	−0,53	−9,61	−9,61	6,96	6,48	4,60	5,80	6,90
	12,50	0,99	−3,54	−3,54	1,74	3,02	0,65	2,80	2,30
	20,00	−0,70	−0,41	−0,41	1,23	0,49	0,44	0,20	0,90
CBN	1,00	12,30	13,02	13,02	0,20	0,82	3,16	−13,30	4,40
	12,50	2,88	−4,81	−4,81	0,04	4,91	0,62	2,70	3,20
	20,00	−3,17	−2,91	−2,91	0,02	0,09	0,34	3,10	0,80
Δ9-THC	1,00	7,68	4,42	4,42	12,72	11,38	10,67	−6,40	13,00
	12,50	1,89	−1,15	−1,15	1,29	2,90	1,52	1,10	2,30
	20,00	−1,24	−0,06	−0,06	2,03	0,87	0,53	0,90	1,50
Δ8-THC	1,00	−1,95	2,21	2,21	0,30	11,10	12,84	1,10	3,60
	12,50	1,72	3,39	3,39	0,01	0,76	1,95	−3,30	1,60
	20,00	0,41	−0,55	−0,55	0,02	0,29	0,70	0,10	0,80
Δ9-THCA	1,00	14,50	9,03	9,03	0,05	10,45	8,78	−10,10	8,90
	12,50	0,66	−6,84	−6,84	0,01	4,20	0,60	5,90	2,70
	20,00	−4,61	−3,97	−3,97	0,03	0,32	0,32	4,50	0,90

Table 2
Herbaceous (A) and oil (B) samples analysed by HPLC-DAD and respective contents in each of the phytocannabinoids analysed by the method developed. The cases of non-quantifiable contents are indicated, as they are below the LOQ or not detectable when below the LOD.

Sample	CBD	CBDA	CBN	Δ^9-THC	Δ^9-THCA	CBD_T	Δ^9-THC_T
A1	0,03%	2,38%	1,48%	11,81%	0,45%	2,41%	12,26%
A2	0,05%	2,22%	0,69%	2,23%	8,61%	2,27%	10,83%
A3	0,04%	0,48%	0,02%	0,81%	17,43%	0,51%	18,24%
A4	0,02%	<LOD	0,86%	0,07%	0,45%	0,02%	0,52%
A5	7,87%	2,02%	0,02%	0,19%	0,25%	9,89%	0,45%
A6	0,08%	1,38%	0,04%	1,98%	13,63%	1,46%	15,61%
A7	0,04%	0,78%	0,09%	2,26%	7,94%	0,83%	10,20%
A8	0,04%	0,74%	0,02%	6,68%	12,18%	0,78%	18,86%
A9	7,36%	2,33%	<LOD	0,20%	0,15%	9,69%	0,35%
A10	1,32%	5,69%	<LOD	0,05%	0,05%	7,01%	0,09%
A11	13,58%	1,87%	<LOD	0,21%	0,60%	15,46%	0,81%
A12	9,93%	2,68%	<LOD	0,28%	0,26%	12,61%	0,54%
A13	2,68%	1,12%	<LOD	0,11%	0,06%	3,80%	0,17%
A14	2,72%	1,85%	<LOQ	0,12%	0,06%	4,57%	0,18%
A15	3,89%	7,38%	0,03%	0,30%	0,05%	11,27%	0,35%
B1	<LOQ	2,59%	0,01%	0,11%	<LOD	2,59%	0,11%
B2	0,03%	3,78%	0,08%	0,02%	<LOD	3,82%	0,02%

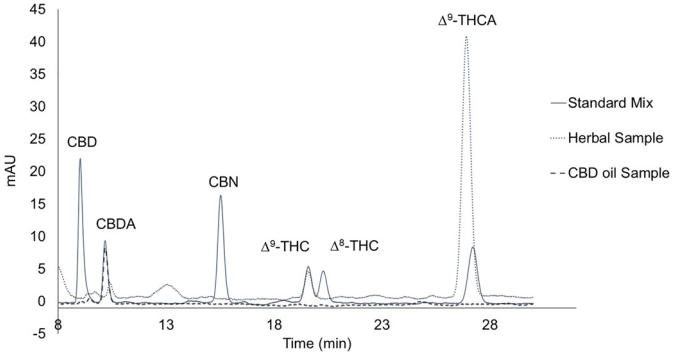

Fig. 3. Overlayed chromatograms of standard mix containing the 6 target cannabinoids at 5.0 μg mL^{-1} (solid line), as well as the analysis of a herbal cannabis sample (dotted) and a "CBD" oil sample (dashed).

resulted in information in agreement with the labelled one. For the oils, the levels of Δ^9-THC were found to be within the legal limits (<0.2% THC), but in the herbal samples, less than half complied. Regarding CBD/CBDA levels, most sample contents were far below the information provided on the product packaging, as reported by Hädener and Zivovinovic and collaborators on legally sold and recreational use cannabis samples [3,37]. These results demonstrate the need to control the products available in these shops and the importance of services providing analysis and information to the general public. These products are sold as "collector's items" and are not "fit for human consumption", although they guarantee “relaxing sensations” and being the “cure” for "pain, stress, PTSD and nausea". Nevertheless, following Regulation (EU) No. 1307/2013 if plant varieties do not have THC levels exceeding 0.2%, they are considered fit for industrial use. What is less clear is what the term "THC levels" indicates. If only the neutral form of Δ^9-THC is considered, the compound from which it precedes, Δ^9-THCA, is left aside. This compound is found in larger quantities in plants, making it extremely important to establish the total (Δ^9-) THC content. It should be emphasised that when subjected to heat, by smoking or being cooked, this compound decarboxylates and is converted to its neutral form [51,52], with the known psychoactive effects attributed. In practical terms, using samples A4, A5 and A9 as examples, taking or not taking the acid form into account, changes their disposition in legal terms (see appendices).

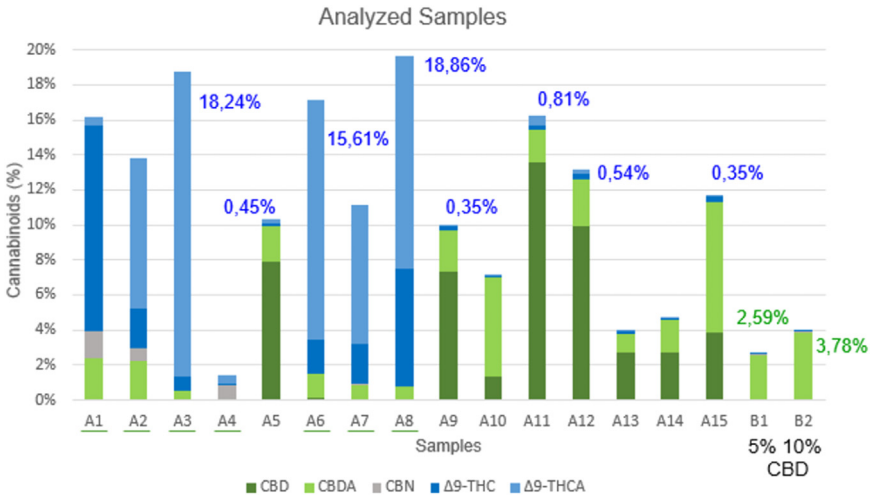

Fig. 4. Samples analysed and their quantified levels of cannabinoids. "A" represents herbal samples while "B" refers to oil samples. "A" refers to street samples. Blue values (%(w/w)) represent total Δ^9 -THC while green ones refer to CBDA only (w/v). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

In the present work, we propose a new US-SLE/HPLC-DAD method that meets the increasing demand for fast, cost-effective, and reliable cannabis monitoring. The developed method proved to be linear, sensitive, selective, true, and precise, with high extraction efficiencies, allowing effective discrimination of the six main phytocannabinoids in real herbal and oil cannabis samples. Overall, the developed method reduces the sample preparation time and the amount of solvents used, increasing the laboratorial efficiency in cannabis analysis. Additionally, the disparity of contents found, show the need for established analytical techniques that can monitor, not only the main compounds in these products (i.e., Δ^9-THC and CBD), but other substances that can be used to circumvent legal loopholes (ex. Δ^9-THCA and Δ^8-THC). It's necessary rigorous control of phytocannabinoids content in cannabis samples, namely those available to the community, due to consequences for public health while posing an economic and legal issue. Furthermore, the lack of clear and objective legal definitions, that allows for misinterpretation of analytical results, must be revised. In the future, it is intended to extend the study to a larger number and nature of samples.

Declaration of Competing Interest

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

CRediT authorship contribution statement

Beatriz Correia: Conceptualization, Methodology, Writing – original draft, Investigation. **Samir Marcos Ahmad:** Conceptualization, Methodology, Supervision, Validation. **Alexandre Quintas:** Writing – review & editing, Supervision.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464191.

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