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Detection and quantification of selected cannabinoids in oral fluid samples by protein precipitation and LC-MS/MS

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ABSTRACT

Cannabis is the most widely consumed illicit drug worldwide. As consumption rates increase, partially due to the decriminalization of its use for medicinal and recreational purposes, analytical methods for monitoring different cannabinoids in several biological matrices have been developed. Herein, a simple and fast extraction procedure to extract natural cannabinoids from oral fluid (OF) samples was developed and fully validated according to the ANSI/ASB 2019 Standard Practices for Method Validation in Forensic Toxicology. Using only 0.2 mL of neat OF, the analytes [Δ^9-tetrahidrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), cannabinol (CBN) and cannabidiol (CBD)] were extracted by protein precipitation with a mixture of methanol:acetonitrile (80:20, v/v); the extracts were centrifuged, evaporated to dryness and reconstituted in 100 μL of methanol. Analysis was performed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The developed methodology produced linear results for all compounds, with working ranges of 0.1–50 ng/mL for THC, 0.5–50 ng/mL for THC-OH, CBN and CBD, and 0.05–1 ng/mL for THC-COOH. Ion suppression was observed for THC, CBN and CBD, which did not impair sensitivity considering the low limits of quantification (LOQs) and limits of detection (LODs) obtained (which varied between 0.05 and 0.5 ng/mL). The extraction procedure produced great recoveries, and the compounds were stable. No interferences were found, and the method proved to be extremely fast, selective, precise, and accurate for use in routine analysis. The method was successfully applied to authentic samples.

1. Introduction

Cannabinoids remain the most widely consumed drugs of abuse on a global scale. The growing legalization of their use for both medicinal and recreational purposes and, therefore, the rise in accessibility, type of products and consumption methods, makes it crucial that laboratories world-wide develop new and enhanced methods for accurately determining these compounds in several biological matrices [1].

Oral fluid (OF) has become increasingly popular in the fields of clinical and forensic toxicology for assessing recent use of drugs of abuse [2], being analyzed in cases of drug treatment, workplace drug testing, pain management, and the assessment of driving under the influence [1]. Some advantages of this matrix comprise its non-invasive collection procedure, that can be performed by non-medical personnel, its reduced biohazard risks, the possibility for on-site collection and screening, the minimal risk for adulteration of the samples, and the presence of non-metabolized drugs, which may indicate recent drug use. Also, considering the window of detection for OF samples, this matrix exhibits a strong correlation with blood concentrations in cases of recent use [3]. These advantages over blood and urine lead to OF being one of the most important alternative matrices studied in the clinical and forensic fields. However, the potential unavailability of enough sample amount for

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analysis is one of the main drawbacks of this specimen [1].

Cannabis smoking leads to high levels of Δ^9-tetrahidrocannabinol (THC) in the oral mucosa, hence why this compound is usually the target analyte for detecting cannabis use in OF. According to the Substance Abuse and Mental Health Services Administration (SAMHSA) [4], the cut-off value for THC in OF screening should be 4 ng/mL, while for confirmation a level of 2 ng/mL is recommended. However, because of its short detection windows, lower values can be necessary to evaluate THC consumption since although THC can be detected in OF samples immediately after consumption, after dissipation of the initial contamination its concentration in OF decreases rapidly [5,6]. THC concentrations in OF are generally higher than in blood within the first few hours post-consumption, primarily because of oral contamination rather than systemic absorption. The concentration of THC in this matrix drops quickly, typically falling below 5–10 ng/mL within two to four hours after smoking. For chronic users, THC levels may persist at 1–5 ng/mL for more than 48 hours. Recent cannabis use can be confirmed if THC concentrations in OF exceed 10 ng/mL. Even in occasional users, this threshold can be reached up to 26 hours after exposure, while in frequent users, it can be detectable for over 72 hours. Thus, similar to blood and urine analyses, OF testing can identify low THC concentrations for several days post-consumption [1]. While in theory THC can be detected in non-users due to second hand smoke, the presence of its metabolite THC-COOH helps distinguishing these situations and prevents issuing false positive results.

Several methods have been published on the determination of cannabinoids in OF samples, applying different extraction approaches and chromatographic techniques. However, for accomplishing limits of detection (LODs) as low as 0.05 ng/mL [7], 0.10 ng/mL [3], 0.13 ng/mL [8], 0.78 ng/mL [9], 1 ng/mL [2,10,11], or 2 ng/mL [5], sample cleanup methods such as solid-phase extraction (SPE) [2,3,8] and liquid-liquid extraction (LLE) [11] have to be employed. These procedures are in general time consuming and may require large amounts of solvents. Protein precipitation seems therefore appealing, and some researchers [5,9,10] have already used this approach for cannabis analysis.

This work describes a fast and sensitive method for the determination of THC, 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), cannabinol (CBN) and cannabidiol (CBD) – represented in Fig. 1 – in OF samples by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) using protein precipitation as sample clean-up. The method was fully validated according to the guiding principles of ANSI/ASB Standard 036 [12].

2. Materials and methods

2.1. Chemicals and reagents

LC–MS grade (≥99.9 %) methanol, water and acetonitrile were purchased from Honeywell Riedel-de-Haën™ (Seelze, Germany). Formic acid (98–100 %) was obtained from Supelco Merck (Darmstadt, Germany) and ammonium formate 1 M (p.a.) was purchased from Fluka/Sigma-Aldrich (Steinheim, Germany). Certified standards of THC, THC-OH, THC-COOH, CBN, and CBD at 1 mg/mL were purchased from Cerilliant Corporation (Round Rock, Texas, USA), as well as the internal standards (ISs), THC-d3 at 100 μg/mL, THC-OH-d3 at 100 μg/mL and THC-COOH-d3 at 1 mg/mL.

2.2. Preparation of working solutions

Working solutions were prepared in methanol by dilution of the stock solutions. The final concentrations were 1, 50, 500 and 5000 ng/mL for THC; 5, 50, 500 and 5000 ng/mL for THC-OH, CBN and CBD; and 1, 2, 4, 10 and 100 ng/mL for THC-COOH. An IS working solution at 1 μg/mL was also prepared in methanol. All solutions were stored at −20 °C in amber borosilicate glass vials, protected from light, until use.

2.3. Oral fluid samples

OF samples were collected by free spillage from the oral cavity to disposable polypropylene test tubes. Blank samples used for the method's optimization and validation were provided by laboratory personnel. Authentic OF samples were obtained from university students. The study adhered to the principles of the Declaration of Helsinki and received approval from the Ethics Committee of Universidade da Beira Interior (CE-UBI-Pj-2021–022-ID:801).

2.4. Sample preparation

After collection by passive drool and centrifugation (4500 rpm; 5 min), OF samples (200 μL) were transferred to plastic Eppendorf vials and spiked with IS solution (THC-d3, THC-OH-d3 and THC-COOH-d3) at 50 ng/mL, and 500 μL of an ice-cold mixture of methanol/acetonitrile (80:20, v/v) was added. After vortexing for 20 s, the samples were centrifuged at 14000 rpm for 10 min at 5 °C. The supernatant was transferred into a new glass tube and evaporated to dryness at 35 °C under a gentle stream of nitrogen. Extracts were reconstituted in 100 μL of LC–MS grade methanol, transferred to polypropylene autosampler vials, and a 5 μL-aliquot was injected into the instrument.

2.5. LC–MS parameters

Analysis was conducted using an ExionLC™ AC liquid chromatograph (SCIEX, Darmstadt, Germany) coupled to a Sciex QTrap® 6500+ mass spectrometer (SCIEX, Darmstadt, Germany). Extracts were injected onto a Waters™ Acquity UPLC® HSS T3, 1.8 μm (2.1 mm×100 mm) column (Waters, France), with the column oven maintained at 45 °C. Mobile phases consisted of LC–MS grade water with 2 mM ammonium formate and 0.1 % formic acid (A) and LC–MS grade methanol with 2 mM ammonium formate and 0.1 % formic acid (B). The flow rate was set at 0.4 mL/min. The gradient started at 90 % A:10 % B for the first 30 seconds, transitioning to 95 % B by 8 minutes and held for 3 minutes before reverting to the initial conditions after 11 minutes. This ratio was maintained for an additional 3 minutes, resulting in a total run time of 14 minutes. Positive multiple reaction monitoring (MRM) mode was employed for analysis, monitoring two transitions for each analyte and one for each IS. Key parameter settings included ion spray voltage of 5.5 kV, temperature of 250 °C, ion source gas 1 and 2 at 60, and curtain gas at 35. Nitrogen was used as the collision gas. Analyst® Instrumental Control and Data Processing Software version 1.7 and SCIEX OS version 2.1 (SCIEX, Darmstadt, Germany) were used for data acquisition and analysis, respectively. For THC, THC-OH and THC-COOH, the

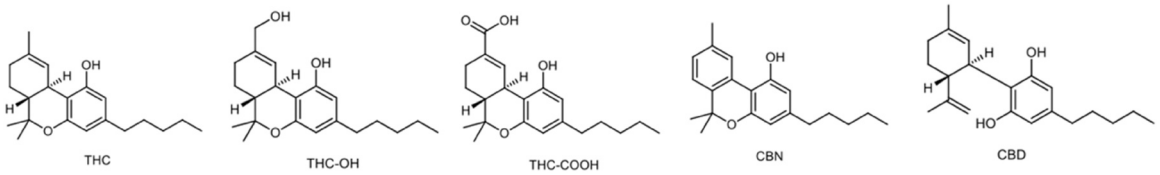

Fig. 1. : Chemical structure of the selected cannabinoids.

corresponding isotopically labeled IS were used. CBN and CBD were monitored using THC-d3, since this IS offered better linearity results for CBN, and is structurally similar to CBD (as observed in Fig. 1). MRM transitions, retention times (RTs) and specific LC–MS/MS parameters for each compound are presented in Table 1.

2.6. Method validation

The method was validated according to the guidelines of the ANSI/ASB Standard 036 [12], and the following parameters were studied: ionization suppression/enhancement, selectivity, linearity, precision and accuracy, carryover, limits of detection (LODs) and quantification (LOQs), recovery, and stability (autosampler, room temperature, and freeze-thaw stabilities). All calibrators and quality controls (QCs) were prepared in blank OF samples. OF samples from different origins were used to study some of the parameters, namely LODs, LOQs and matrix effects.

Identification criteria for positivity were established according to the guidelines of the World Anti-Doping Agency (WADA) [13], and included a signal-to-noise ratio equal to or greater than 3:1. Additionally, the relative retention time (RRT) of the chromatographic signal of the analyte had to be less than or equal to 1 % of that of the same substance present in the QC sample, if a deuterated analog was not used as IS – otherwise, the variation in the RRT of the compound had to be less than 0.5 %. The identification of two ions in the mass spectrum and the monitoring of their relative intensities were also part of the acceptance criteria.

3. Results and discussion

3.1. Matrix effects (ionization suppression/enhancement)

To study ion suppression or enhancement, two sets of samples were prepared. Set #1 consisted of unextracted standards at the study concentrations, that were injected six times; set #2 was prepared with OF samples from ten different origins (n=10), that were fortified with the same concentrations as set #1 after extraction and evaporation of the extracts. The extent of suppression or enhancement was assessed by the ratio between peak areas of both sets. All samples were prepared in duplicate, to study ion suppression/enhancement at both high and low concentrations. This parameter was also evaluated for the ISs under study. Average values should not exceed ±25 % [12].

As seen in Table 2, THC, CBN and CBD suffer from ion suppression at high concentrations, ranging from −29.97 to −32.13 %. To ensure that ionization suppression did not impact the method's LODs and LOQs, at least three times the number of samples from different origins were used to study these parameters, and matrix effects were found to be not significant at low concentrations and did not affect positivity assessment.

3.2. Selectivity and specificity

Ten blank matrix samples from different sources were extracted with the method described herein, and no significant matrix interferences were observed at the RT and monitored transitions. Thus, the method was considered selective.

To assess specificity, three blank samples from different origins were spiked with potentially interfering compounds, containing a mixture of 191 different drugs at 200 ng/mL (the full list of the substances present in this mixture is described in the supporting information). Again, no significant interferences were observed at the RTs and monitored transitions of the compounds under study, as seen in Fig. 2.

3.3. Linearity

To study the method's linearity, the working range was determined for each analyte. Spiked OF samples were analyzed and linearity was evaluated using six calibrators, evenly distributed within the working range, in five different runs (n=5). The concentrations of the calibrators were selected based on typical concentrations encountered in routine cases and as per concentrations reported in the literature. The method was found linear in the ranges of 0.1–50 ng/mL for THC, 0.5–50 ng/mL for THC-OH, CBN and CBD and 0.05–1 ng/mL for THC-COOH. The calibration curves showed mean determination coefficients (R^2) higher than 0.99. The analysis of residuals (sum of the deviations from the calculated concentration to the nominal concentration) revealed that the weighted linear regression ($1/x^2$) was the best model for the data obtained. Calibration data are summarized in Table 3.

3.4. Precision and accuracy

The study of these parameters was performed simultaneously with the evaluation of linearity, over 5 different runs.

Precision was expressed in terms of coefficients of variation (CV), and values lower than or equal to ±20 % were considered acceptable for all concentrations [12]. Inter-day precision was evaluated by analyzing the calibrators over five different runs (n=5). Intra-day precision was assessed for each analyte by analyzing five replicate samples (n=5) of one of the concentrations under study each day to study the repeatability of the method. To study intermediate precision (combined intra- and inter-day precision), QC samples (n=3) at four concentration levels were analyzed over each of the five days (n=15). QC concentrations were 0.1, 5, 20 and 40 ng/mL for THC; 0.5, 5, 20 and 40 ng/mL for THC-OH, CBN and CBD; and 0.05, 0.3, 0.5 and 1 ng/mL for THC-COOH.

Accuracy was expressed as relative errors (RE), or bias, indicating the disparity between the concentrations measured using the calibration equation and the nominal levels. Calculated concentrations within a ±20 % interval from the nominal value were considered acceptable for all studied levels [12].

Table 1
Retention time, MRM transitions and optimized LC–MS/MS parameters for the analysis of selected cannabinoids in oral fluid samples.

Analyte	Internal standard	Retention time (min)	Parent ion (*m/z*)	Product ions (*m/z*)	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)
THC	THC-d3	9.20	315	193	91	10	29	22
				123	91	10	39	14
THC-OH	THC-OH-d3	8.48	331	193	66	10	33	22
				201	66	10	33	24
THC-COOH	THC-COOH-d3	8.59	345	299	101	10	27	16
				193	101	10	35	22
CBN	THC-d3	9.02	311	223	100	10	35	10
				241	100	10	35	10
CBD	THC-d3	8.50	315	193	51	10	29	22
				123	51	10	41	18
THC-d3	-	9.20	318	196	91	10	29	22
THC-OH-d3	-	8.48	334	196	66	10	33	22
THC-COOH-d3	-	8.59	348	302	101	10	27	16

Table 2
Ion suppression/enhancement (%) for selected cannabinoids in oral fluid samples.

Analyte	Low concentration (n=10)		High concentration (n=10)	
	Concentration (ng/mL)	Ion suppression/enhancement (%)	Concentration (ng/mL)	Ion suppression/enhancement (%)
THC	2	−23.20	50	−32.13
THC-OH	2	11.86	50	−16.47
THC-COOH	0.1	8.07	1	−6.82
CBN	2	−19.52	50	−29.97
CBD	2	−23.01	50	−31.51
THC-d3	N/A	N/A	50	−25.06
THC-OH-d3	N/A	N/A	50	−1.07
THC-COOH-d3	N/A	N/A	50	10.22

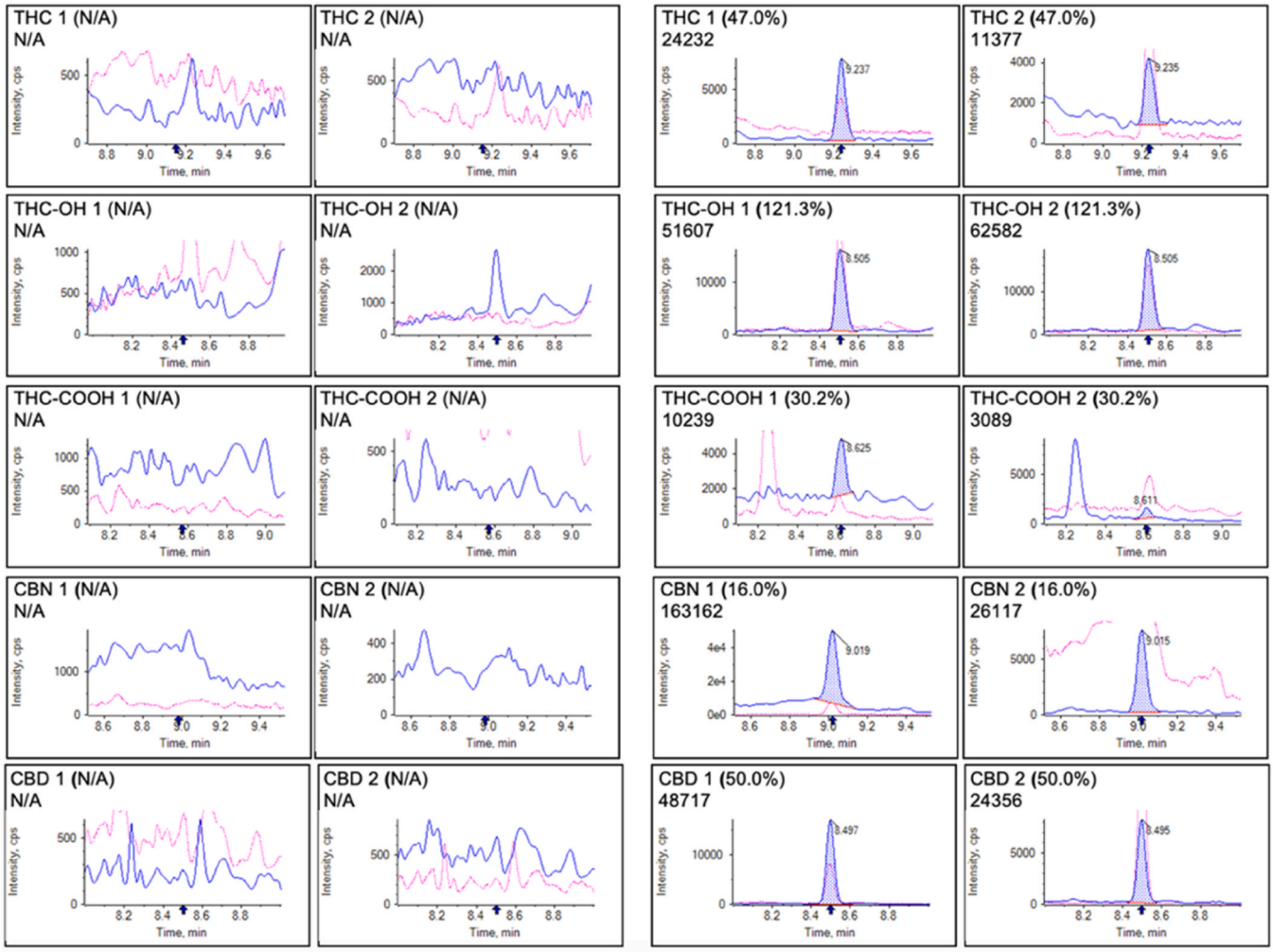

Fig. 2. : Extracted chromatograms obtained from OF samples spiked with 200 ng/mL of potentially interfering compounds (left) and from spiked OF samples at LOQ concentrations (right). LOQ of THC is 0.10 ng/mL; LOQ of THC-OH, CBN and CBD is 0.50 ng/mL; LOQ of THC-COOH is 0.05 ng/mL. The ion ratio for each analyte is given in the figure as well (%).

Inter-day precision showed satisfactory results, ranging from 0.58 % to 14.73 %. Bias for inter-day precision studies ranged between 0.08 % and 11.01 %.

Intra-day precision also presented good results, from 1.98 % to 12.55 %. In general, bias results for intra-day studies were satisfactory, with only three values outside the ±20 % acceptance criterion: 23.19 % for THC, 21.04 % for THC-OH, and 27.75 % for CBN, all at 20 ng/mL.

For intermediate precision studies, precision values varied between 4.16 % and 19.68 % and bias ranged between 0.18 % and 14.53 %.

Precision and accuracy data are summarized in Table 4.

3.5. Carryover

Instrument carryover was assessed by analyzing extracted blank OF samples immediately after analysis of the highest calibrator. It was considered that carryover was present if the peak area of the blank sample exceeded 20 % of the peak area of the LOQ for each analyte [14].

The blank samples presented 30 % of the peak area of the LOQ for THC and CBN. However, since results below the LOQ would not be reported, the adequacy of the method was not affected.

Table 3
Linear range, linearity, and correlation coefficients for selected cannabinoids in oral fluid samples (n=5).

Analyte	Weight	Linear range (ng/mL)	Linearity		R^2[a]
			Slope[a]	Intercept[a]	
THC	$1/x^2$	0.1 – 50	0.016 ± 0.001	0.000 ± 0.000	0.992 ± 0.007
THC-OH	$1/x^2$	0.5 – 50	0.048 ± 0.003	−0.003 ± 0.002	0.991 ± 0.006
THC-COOH	$1/x^2$	0.05 – 1	0.018 ± 0.001	0.000 ± 0.000	0.990 ± 0.005
CBN	$1/x^2$	0.5 – 50	0.019 ± 0.002	0.000 ± 0.000	0.990 ± 0.007
CBD	$1/x^2$	0.5 – 50	0.017 ± 0.002	−0.007 ± 0.001	0.991 ± 0.003

[a] Mean values ± standard deviation.

3.6. LOQ and LOD

Following the guidelines of the ANSI/ASB Standard 036 [12], LOQ values were determined as the lowest non-zero calibrator. Thus, nine different blank matrix sources were fortified with the analyte at the concentration of the lowest calibrator and analyzed alongside linearity studies, over 5 different runs (n=22). The method's acceptability parameters were met, with both CV and bias falling within the range of ±20 %.

To study LODs, samples spiked with decreasing concentrations of the analytes, starting from the LOQ values, were studied (n=9). The LODs were determined as the minimum concentration in which the peak could be clearly distinguished from a blank sample (as shown in Fig. 3), and the acceptance criteria (described in Section 2.6) were met. LOD was 0.05 ng/mL for THC and THC-COOH, 0.25 ng/mL for CBN, 0.35 for THC-OH and 0.50 ng/mL for CBD. Whereas for THC, THC-OH and CBN it was possible to obtain lower concentrations for the LODs, the same did not happen for THC-COOH and CBD. For these two compounds, concentrations below the LOQs did not provide peaks clearly distinguished from blank samples, and S/N ratios were lower than 3.0. Thus, the LOQs and LODs were the same for THC-COOH and CBD.

LODs and LOQs are summarized in Table 5.

Limits were deemed satisfactory considering the values found in literature. Pichini et al. [7] developed a methodology to quantify THC, THC-OH, THC-COOH, CBD and other cannabinoids by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) after extraction of the analytes from 100 μL of OF with acetone:acetonitrile (80:20, v/v). The team achieved an LOD of 0.05 ng/mL for THC and 0.08 ng/mL for THC-COOH; however, LOD values of 0.07 and 0.04 ng/mL were attained for THC-OH and CBD, respectively. Likewise, LOQ values were higher than the ones we obtained concerning THC and THC-COOH, but values for THC-OH and CBD were lower. Lin et al. [3] quantified the same analytes as the ones

Table 4
Intra-day, inter-day, and intermediate precision (CV) and accuracy (bias) of the proposed method for selected cannabinoids in oral fluid samples (QC: quality control samples; C: calibrators).

Analyte	Concentration (ng/mL)	Sample type	Inter-day (n=5)			Intra-day (n=5)			Intermediate (n=15)		
			Measured[a]	CV (%)	Bias (%)	Measured[a]	CV (%)	Bias (%)	Measured[a]	CV (%)	Bias (%)
THC	0.1	C; QC	0.10 ± 0.00	0.58	0.08	0.10 ± 0.01	4.85	−3.75	0.11 ± 0.01	9.85	6.85
	2	C	1.98 ± 0.25	12.71	−0.97	1.98 ± 0.15	7.64	−1.06	N/A	N/A	N/A
	5	QC	N/A	N/A	N/A	4.64 ± 0.53	11.47	−7.16	4.55 ± 0.43	9.49	−8.94
	10	C	9.35 ± 0.37	3.96	−6.55	N/A	N/A	N/A	N/A	N/A	N/A
	20	QC	N/A	N/A	N/A	24.64 ± 1.17	4.73	23.19	22.33 ± 1.27	5.66	11.62
	25	C	27.56 ± 2.82	10.24	10.23	N/A	N/A	N/A	N/A	N/A	N/A
	40	C; QC	40.15 ± 1.44	3.58	0.38	39.14 ± 1.52	3.89	−2.16	39.11 ± 2.11	5.40	−2.23
	50	C	48.42 ± 1.26	2.60	−3.16	N/A	N/A	N/A	N/A	N/A	N/A
THC-OH	0.5	C; QC	0.50 ± 0.02	3.06	0.22	0.49 ± 0.03	5.97	−1.67	0.50 ± 0.04	7.35	0.42
	2	C	1.99 ± 0.26	12.92	−0.42	2.04 ± 0.15	7.22	2.11	N/A	N/A	N/A
	5	QC	N/A	N/A	N/A	4.81 ± 0.44	9.14	−3.89	4.56 ± 0.31	6.80	−8.77
	10	C	9.47 ± 0.53	5.59	−5.33	N/A	N/A	N/A	N/A	N/A	N/A
	20	QC	N/A	N/A	N/A	24.21 ± 0.72	2.99	21.04	22.57 ± 1.16	5.12	12.83
	25	C	27.47 ± 2.23	8.12	9.86	N/A	N/A	N/A	N/A	N/A	N/A
	40	C; QC	39.32 ± 2.40	6.11	−1.71	39.60 ± 2.59	6.55	−1.00	39.83 ± 2.57	6.46	−0.42
	50	C	48.69 ± 1.48	3.04	−2.63	N/A	N/A	N/A	N/A	N/A	N/A
THC-COOH	0.05	C; QC	0.05 ± 0.00	6.53	1.67	0.05 ± 0.00	9.36	−5.89	0.05 ± 0.01	19.68	6.56
	0.1	C	0.10 ± 0.01	14.73	−3.07	0.12 ± 0.01	9.69	16.04	N/A	N/A	N/A
	0.3	C; QC	0.29 ± 0.02	5.76	−2.81	0.30 ± 0.04	12.21	−1.10	0.29 ± 0.04	13.28	−2.82
	0.5	C; QC	0.51 ± 0.04	6.95	1.73	0.59 ± 0.02	4.18	18.03	0.52 ± 0.05	8.66	4.03
	0.7	C	0.71 ± 0.03	3.98	1.60	N/A	N/A	N/A	N/A	N/A	N/A
	1	C; QC	1.01 ± 0.05	4.94	0.88	1.02 ± 0.08	7.48	2.09	1.02 ± 0.06	6.25	1.97
CBN	0.5	C; QC	0.50 ± 0.01	2.88	0.39	0.52 ± 0.04	6.93	3.67	0.49 ± 0.05	9.94	−1.26
	2	C	1.98 ± 0.25	12.50	−1.24	2.02 ± 0.15	7.39	0.85	N/A	N/A	N/A
	5	QC	N/A	N/A	N/A	4.40 ± 0.55	12.55	−12.03	4.79 ± 0.52	10.85	−4.24
	10	C	9.50 ± 0.47	4.98	−5.00	N/A	N/A	N/A	N/A	N/A	N/A
	20	QC	N/A	N/A	N/A	25.55 ± 2.05	8.03	27.75	22.91 ± 1.41	6.17	14.53
	25	C	27.75 ± 2.60	9.35	11.01	N/A	N/A	N/A	N/A	N/A	N/A
	40	C; QC	40.30 ± 1.04	2.58	0.75	38.92 ± 0.77	1.98	−2.69	38.69 ± 3.56	9.21	−3.28
	50	C	47.05 ± 0.77	1.64	−5.91	N/A	N/A	N/A	N/A	N/A	N/A
CBD	0.5	C; QC	0.49 ± 0.01	1.68	−1.40	0.49 ± 0.01	1.17	−2.92	0.49 ± 0.02	4.16	−2.48
	2	C	2.13 ± 0.16	7.40	6.30	2.33 ± 0.28	11.88	16.45	N/A	N/A	N/A
	5	QC	N/A	N/A	N/A	4.89 ± 0.24	4.97	−2.12	4.31 ± 0.60	13.81	−13.75
	10	C	9.49 ± 0.68	7.22	−5.52	N/A	N/A	N/A	N/A	N/A	N/A
	20	QC	N/A	N/A	N/A	22.05 ± 1.46	6.62	10.25	20.04 ± 2.21	11.03	0.18
	25	C	27.61 ± 2.33	8.45	10.42	N/A	N/A	N/A	N/A	N/A	N/A
	40	C; QC	38.93 ± 2.63	6.74	−2.67	38.26 ± 3.15	8.22	−4.36	35.89 ± 3.47	9.68	−10.28
	50	C	46.44 ± 0.61	1.32	−7.13	N/A	N/A	N/A	N/A	N/A	N/A

[a] Mean values ± standard deviation.

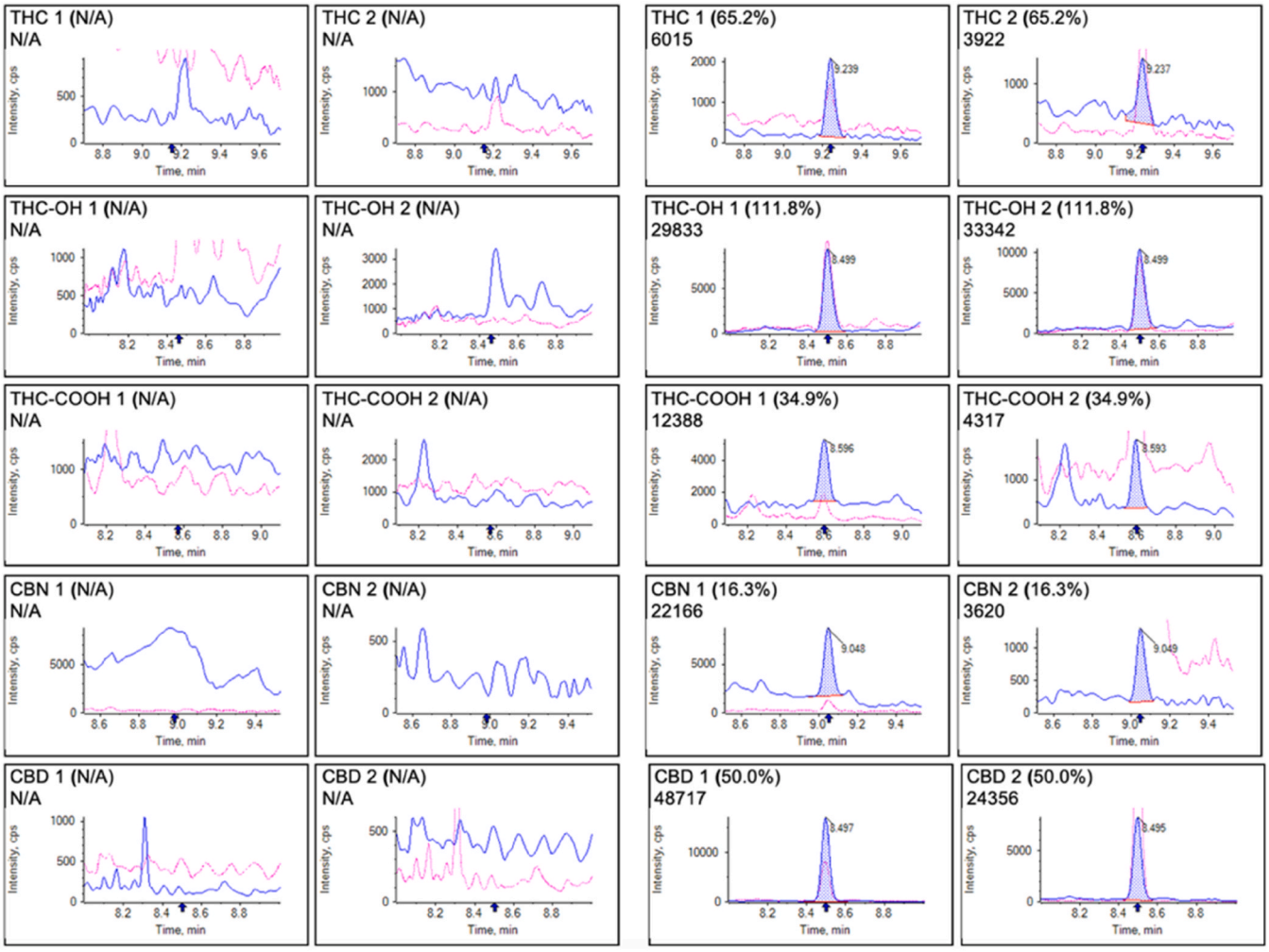

Fig. 3. : Extracted chromatograms obtained from blank OF samples (left) and from spiked OF samples at LOD concentrations (right). LOD of THC and THC-COOH is 0.05 ng/mL; LOD of THC-OH is 0.35; LOD of CBN is 0.25 ng/mL; LOD of CBD is 0.50 ng/mL. The ion ratio for each analyte is given in the figure as well (%).

Table 5
LOD (n=9) and LOQ (n=22) for selected cannabinoids in oral fluid samples.

Analyte	LOD (n=9)		LOQ (n=22)			
	Concentration (ng/mL)	CV (%)	Concentration (ng/mL)	Measured (ng/mL)[a]	CV (%)	Bias (%)
THC	0.05	10.83	0.10	0.10 ± 0.01	9.36	4.06
THC-OH	0.35	3.77	0.50	0.50 ± 0.03	6.57	0.31
THC-COOH	0.05	10.66	0.05	0.05 ± 0.01	17.68	3.66
CBN	0.25	8.35	0.50	0.50 ± 0.04	8.82	0.19
CBD	0.50	2.32	0.50	0.49 ± 0.02	3.54	−2.35

[a] Mean values ± standard deviation.

described herein and reached lower LOQ values for THC-OH, CBN and CBD. However, the team used 400 μL of sample, that was collected with a collection device, extracted the analytes by SPE and analyzed the samples with HPLC-MS/MS. Gerace et al. [10] used a similar extraction procedure to analyze THC and CBD, and reached LOD values of 0.50 ng/mL for both analytes. Reinstadler et al. [15] also obtained LOQ values of 0.50 ng/mL for THC, THC-OH and THC-COOH after analyzing 500 μL of OF collected from collection devices by extracting the samples twice with acetonitrile prior to analysis by LC-MS/MS. Sobolesky et al. [16] developed an SPE–LC-MS/MS method to quantify ten cannabinoids, including the five studied herein, and obtained LOQ values of 0.40 ng/mL for THC, THC-OH, CBN and CBD and 1.0 ng/mL for THC-COOH. The teams of Coulter [8], Borden [9] Mercier [2], and da Cunha [11] developed a method for monitoring THC in OF samples, and reached LOD values of 4, 0.78, 1 and 1 ng/mL, respectively. Lastly, Gorziza [5] studied THC and CBD in dried oral fluid spots (DOFS) by LC-MS/MS after extraction with methanol:acetonitrile (50:50, v/v) and reached LOD values of 2 and 4 ng/mL, respectively. Nevertheless, the herein described procedure is simpler and quicker and is therefore able to increase laboratory throughput, while allowing detecting low amounts of the analytes.

3.7. Recovery

To evaluate recovery, two sets of samples (n=3) were extracted for each concentration of the QC samples. The analytical method was

applied to these samples, with the first set fortified before extraction and the second set fortified immediately after extraction, just before the evaporation of the extracts. The obtained results are summarized in Table 6 and ranged from 78.54 % to 115.53 %, indicating that the method exhibits sufficient extraction efficiency for all the analytes.

3.8. Stability

Three different studies were performed to assess the stability of the analytes.

Autosampler stability was assessed by reanalyzing the extracts at the concentrations of the QCs (n=3) after being kept in the autosampler (at 15 °C) for 24 h.

Room temperature stability was studied by evaluating samples that were left at room temperature for 24 h before extraction.

Freeze/thaw stability was evaluated by analyzing samples that underwent three cycles of freeze (at −20 °C) and thaw (at room temperature) before extraction.

All three studies were performed by comparing these extracts to freshly prepared samples. Analytes would be considered stable if the CV and bias were within ±20 %. The results obtained from the stability study are summarized in Table 7.

We concluded that the compounds remain mostly stable in the autosampler for 24 hours, except for THC, THC-COOH, and CBN at the concentrations of their respective LOQs, with CV higher than 20 % for THC and THC-COOH, and bias over 20 % for CBN.

Regarding room temperature stability, only THC and THC-COOH are not stable at low concentrations. THC is not stable below 5 ng/mL, exhibiting a bias higher than 20 % for both concentrations. THC-COOH is also not stable at the LOQ, with a CV exceeding 20 %.

None of the studied cannabinoids exhibit stability at low concentrations after undergoing freeze-thaw cycles.

All compounds exhibit stability under the three studied conditions at the two highest concentrations of the QCs.

Based on the results obtained in the stability study, samples should be analyzed as soon as possible after collection to prevent false negatives. Furthermore, freezing should occur promptly after collection, and thawing should only take place at the time of analysis.

3.9. Application to authentic samples

The method was successfully applied to real samples collected from university students. Nine samples were analyzed until now, and seven showed positive results for at least three of the compounds under study. The concentrations of each cannabinoid in each sample are presented in Table 8.

THC concentrations varied between 0.04 and 898.23 ng/mL, THC-OH concentrations ranged from 0.09 to 1.18 ng/mL, and THC-COOH concentrations ranged from 0.17 to 4.62 ng/mL. CBN concentrations spanned from 1.26 to 44.57 ng/mL, while CBD concentrations ranged from 0.44 to 1007.86 ng/mL. Highly concentrated samples were diluted with blank oral fluid to fit the linear range and were reanalyzed.

The number of samples analyzed is yet too small to draw major conclusions about the levels found, since analysis was performed mainly to assess the method's applicability. However, when comparing to other works found in literature [2,3], we concluded that we were able to quantify the analytes in the same concentration ranges as other works, without the need of sample collection devices, which may interfere with analyte recovery.

4. Conclusions

A LC–MS/MS method was developed and comprehensively validated for the quantification of selected cannabinoids in OF samples. Protein precipitation proved to be highly efficient for the extraction of cannabinoids from OF, allowing obtaining good recoveries (78.54–115.53 %) and requiring minimal sample volume (200 μL). LOQs were 0.1 ng/mL for THC, 0.5 ng/mL for THC-OH, CBN and CBD, and 0.05 ng/mL for THC-COOH, and as such this method combines an extremely fast extraction procedure with extremely low LOQ values, well below the recommended cut-offs. Notably, the LOQ for THC was twenty times lower than the cut-off of 2 ng/mL for THC confirmation. All analytes proved stable, particularly at medium to high concentrations. The method showed no interferences, was selective and precise, inexpensive, easy, sensitive, and extremely fast to perform.

OF was collected by passive drool, which eliminates the need for collection devices and associated analytical problems (e.g. irreversible analyte adsorption, which may be an issue for some compounds). The use of small specimen volumes, along with the demonstrated high sensitivity, offers notable advantages, particularly when faced with limited specimen availability. This is especially crucial in the context of OF testing, where the capability to perform multiple examinations on a single sample is highly valuable.

Cannabinoids are known to adhere to certain materials, such as plastic. Thus, several tests were made prior to the validation of the analytical method to assess the suitability of the laboratory's equipment. We found that the plastic tubes used for collection, the plastic Eppendorf vials used for extraction, the glass tubes used for reconstitution of the samples and the plastic vials used for chromatographic analysis did not influence negatively the analytes.

Considering other studies found in literature, this method stands out for its speed, robustness, and effectiveness in obtaining extremely low LOD and LOQ values, especially for THC and THC-COOH. Compared to methods that achieved lower LODs and LOQs [3,16], our work stands out for its speed and low cost, compared to methods that use time-consuming and expensive techniques like SPE and require collection devices to collect the samples.

Given the window of detection of cannabis intake, OF emerges as a crucial matrix for examining recent cannabis use. However, it is important to take into consideration passive consumption when analyzing THC concentrations, as this compound may be present in second-hand cannabis smoke [17,18]. Thus, OF samples usually contain mainly THC rather than its metabolites, so quantification of THC-OH and THC-COOH requires a highly sensitive method [17], such as the one developed herein. The latter is rarely found in OF samples but can be detected in blood or produced from THC metabolism in the oral mucosa. In chronic cannabis users, THC-COOH may be present at very low levels, generally below 0.05 ng/mL. This metabolite's absence in cannabis smoke makes it a reliable marker for confirming active cannabis consumption. Therefore, the sensitive detection and monitoring of THC-COOH are essential for distinguishing between recent use and passive exposure [1].

Table 6
Extraction recovery (%) of selected cannabinoids in oral fluid samples (n=3).

Analyte	Concentration (ng/mL)							
	0.05	0.1	0.3	0.5	1	5	20	40
THC	N/A	109.00	N/A	N/A	N/A	93.29	89.98	95.10
THC-OH	N/A	N/A	N/A	95.27	N/A	91.14	92.45	96.02
THC-COOH	115.53	N/A	78.54	87.04	87.74	N/A	N/A	N/A
CBN	N/A	N/A	N/A	85.34	N/A	94.93	97.45	102.03

Table 7
Autosampler, room temperature and freeze/thaw stability (n=3) of selected cannabinoids in oral fluid samples.

Analyte	Concentration (ng/mL)	Autosampler stability (n=3)			Room temperature stability (n=3)			Freeze/thaw stability (n=3)		
		Measured[a]	CV (%)	Bias (%)	Measured[a]	CV (%)	Bias (%)	Measured[a]	CV (%)	Bias (%)
THC	0.1	0.11 ± 0.03	23.27	11.40	< LOQ	6.43	−24.03	< LOQ	18.61	−32.28
	5	4.30 ± 0.13	2.92	−14.08	3.93 ± 0.34	8.62	−21.46	3.34 ± 0.23	6.90	−33.27
	20	19.02 ± 1.35	7.11	−4.91	18.08 ± 0.77	4.25	−9.62	18.22 ± 1.67	9.15	−8.90
	40	41.71 ± 0.98	2.35	4.28	37.24 ± 5.24	14.06	−6.89	36.61 ± 4.70	12.84	−8.48
THC-OH	0.5	0.54 ± 0.03	5.34	8.54	0.50 ± 0.00	0.77	−0.80	0.53 ± 0.04	7.11	6.65
	5	4.47 ± 0.18	3.96	−10.51	4.45 ± 0.05	1.10	−11.03	3.60 ± 0.19	5.37	−28.06
	20	18.87 ± 1.24	6.56	−5.63	19.85 ± 0.39	1.94	−0.75	18.74 ± 1.79	9.57	−6.30
	40	41.14 ± 1.61	3.92	2.84	37.89 ± 4.15	10.95	−5.27	37.19 ± 6.91	18.57	−7.03
THC-COOH	0.05	0.06 ± 0.02	30.44	10.46	0.05 ± 0.03	72.32	−7.69	0.06 ± 0.01	22.05	25.11
	0.3	0.32 ± 0.03	8.20	6.35	0.32 ± 0.04	12.82	7.94	0.23 ± 0.02	9.91	−21.75
	0.5	0.52 ± 0.01	2.15	4.98	0.52 ± 0.01	1.98	4.55	0.45 ± 0.07	14.54	−9.86
	1	1.05 ± 0.10	9.82	5.27	0.90 ± 0.11	12.30	−9.88	0.94 ± 0.13	13.48	−5.61
CBN	0.5	0.69 ± 0.03	4.66	37.38	< LOQ	14.89	−7.29	< LOQ	9.70	−22.90
	5	4.86 ± 0.24	4.99	−2.74	4.53 ± 0.26	5.68	−9.36	3.27 ± 0.19	5.89	−34.65
	20	23.07 ± 0.10	0.43	15.36	20.14 ± 1.46	7.27	0.70	18.61 ± 3.05	16.41	−6.94
	40	45.17 ± 1.65	3.66	12.93	36.41 ± 3.50	9.60	−8.98	36.97 ± 6.91	18.68	−7.57
CBD	0.5	0.52 ± 0.00	0.84	3.04	0.51 ± 0.00	0.69	1.93	0.50 ± 0.00	0.75	−0.69
	5	4.38 ± 0.19	4.43	−12.40	4.34 ± 0.34	7.75	−13.26	3.37 ± 0.22	6.06	−25.95
	20	18.64 ± 1.06	5.68	−6.78	17.74 ± 0.60	3.37	−11.29	17.90 ± 1.80	10.04	−10.50
	40	38.28 ± 1.48	3.87	−4.31	36.07 ± 4.21	11.67	−9.82	35.14 ± 4.49	12.78	−12.15

[a] Mean values ± standard deviation.

Table 8
Analysis of authentic oral fluid samples.

Sample	Concentration (ng/mL)				
	THC	THC-OH	THC-COOH	CBN	CBD
1	Negative	Negative	Negative	Negative	Negative
2	Negative	Negative	Negative	Negative	Negative
3	898.23	1.18	4.09	44.57	1007.86
4	174.65	< LOQ	Negative	7.07	215.28
5	57.95	< LOQ	0.78	2.97	67.90
6	449.57	0.88	4.62	27.73	514.13
7	63.26	< LOQ	Negative	1.26	89.78
8	458.51	0.54	0.17	14.99	550.11
9	< LOQ	< LOQ	Negative	Negative	< LOQ

With the legalization of cannabis consumption for both medicinal and recreational purposes, it is extremely important to develop new methodologies for monitoring cannabinoids in different biological samples, as each sample is associated with different advantages for the study of these drugs. The study of THC and its metabolites alongside CBN and CBD – associated with medicinal cannabis intake – is crucial to discern recreational from medicinal consumption.

Ethics declarations

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee (number CE-UBI-Pj-2021-022.). Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Suzana Fonseca: Validation, Formal analysis. **João Franco:** Funding acquisition, Formal analysis. **Mónica Antunes:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Susana Simões:** Validation, Formal analysis. **Mário Barroso:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization, Validation, Formal analysis. **Eugenia Gallardo:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2024.112174.

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