Forensic Science International 378 (2026) 112685

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Detection and quantification of selected cannabinoids in hair samples by liquid-liquid extraction and LC-MS/MS

Mónica Antunes [a,b], Susana Simões [b], Suzana Fonseca [b], João M. Franco [b], Mário Barroso [b,d,*], Eugenia Gallardo [a,c,e]

[a] RISE-Health, Departamento de Ciências Médicas, Faculdade de Ciências da Saúde, Universidade da Beira Interior, Covilhã 6200-506, Portugal
[b] Instituto Nacional de Medicina Legal e Ciências Forenses, Delegação do Sul, Serviço de Química e Toxicologia Forenses, Rua Manuel Bento de Sousa 3, Lisboa 1169-201, Portugal
[c] Laboratório de Fármaco-Toxicologia-UBIMedical, Universidade da Beira Interior, Estrada Municipal 506, Covilhã 6200-284, Portugal
[d] AlphaBiolabs, 14 Webster Court, Carina Park, Warrington WA5 8WD, UK
[e] Centro Académico Clínico das Beiras (CACB) – Grupo de Problemas Relacionados com Toxicofilias, Avenida Infante D. Henrique, Covilhã 6200-506, Portugal

ARTICLE INFO

Keywords:
Cannabinoids
Hair analysis
LC–MS/MS
MS^3

ABSTRACT

Cannabis remains the most used illicit drug worldwide, with rising use linked to medical and recreational decriminalization. This has driven the development of analytical techniques to detect cannabinoids in biological matrices. Hair offers key advantages due to its non-invasive collection, extended detection window, stability, and easy storage. This study presents the development and validation of a robust method for extracting natural cannabinoids from hair, following ANSI/ASB 2019, FDA, and Society of Hair Testing guidelines. Hair samples were washed with methanol and cut into small pieces. Approximately 20 mg of hair was incubated with 1 M NaOH and methanol (30 min, 50 °C). The mixture was acidified with acetic acid and underwent liquid-liquid extraction using hexane/ethyl acetate (90/10, v/v). The organic phase was evaporated and reconstituted in 1-pentanol/methanol (50/50, v/v). Analysis was conducted by LC-MS/MS using multiple reaction monitoring (MRM) and triple-stage mass spectrometry (MS^3). The method was selective, specific, precise, and linear, with working ranges of 5–2000 pg/mg for THC, CBN, and CBD; 50–2000 pg/mg for THC-OH; and 0.2–20 pg/mg for THC-COOH. Ion suppression was observed but did not affect sensitivity, with LLOQs and LODs from 0.2 to 50 pg/mg. Over 25 hair samples from university students tested positive for cannabis. THC ranged from 5.9 to 2430.7 pg/mg; one sample had THC-OH above LLOQ (61.4 pg/mg); THC-COOH ranged from 0.3 and 36.4 pg/mg; CBN from 5.7 to 461.0 pg/mg; and CBD from 5.7 to 850.2 pg/mg. Results aligned with self-reported use, confirming the method's forensic suitability.

1. Introduction

Cannabis remains the most widely used illicit drug in the world, with recent estimates indicating that 8 % of European adults have used cannabis in the last year. Although patterns of consumption vary across countries, the increasing decriminalization of cannabis for both medical and recreational purposes has contributed to expanding markets and a growing diversity of available products, including high-potency extracts, edibles, and cannabidiol (CBD)-rich preparations [1]. According to the United Nations Office on Drugs and Crime (UNODC), cannabis is consumed in quantities comparable to those of legal substances such as tobacco, alcohol, and caffeine [2,3]. While the plant has a long history of medicinal use—particularly in the treatment of chronic pain, neurological disorders, and chemotherapy-induced symptoms—its psychoactive properties, especially those of Δ⁹-tetrahydrocannabinol (THC), continue to raise significant public health and legal concerns [2,4]. Adverse effects on cognition, memory, motor function, and psychomotor performance have been linked to impaired driving, workplace accidents, or drug-facilitated crimes [2,5]. Moreover, the presence of synthetic cannabinoids in illicit products further complicates the risk profile [1]. As such, monitoring cannabis use has become critical in forensic toxicology. The need for accurate and sensitive analytical methods to detect

* Corresponding author at: Instituto Nacional de Medicina Legal e Ciências Forenses, Delegação do Sul, Serviço de Química e Toxicologia Forenses, Rua Manuel Bento de Sousa 3, Lisboa 1169-201, Portugal.
E-mail addresses: antunes.ss.monica@gmail.com (M. Antunes), susana.m.simoes@inmlcf.mj.pt (S. Simões), suzana.m.fonseca@inmlcf.mj.pt (S. Fonseca), j.miguel.franco@inmlcf.mj.pt (J.M. Franco), mario.j.barroso@inmlcf.mj.pt (M. Barroso), egallardo@fcsaude.ubi.pt (E. Gallardo).

https://doi.org/10.1016/j.forsciint.2025.112685
Received 28 July 2025; Received in revised form 4 October 2025; Accepted 5 October 2025
Available online 6 October 2025

a wide range of cannabinoids and their metabolites in various biological matrices is increasingly important. The choice of matrix—blood, urine, oral fluid, or hair, to name a few—can provide different windows of detection and insights into recent or long-term use [2].

In recent years, hair has emerged as a valuable biological matrix in forensic toxicology due to its ability to provide long-term information on drug consumption [2]. Most drugs, including cannabinoids, can be incorporated into hair during its growth phase via passive diffusion from the bloodstream into the hair follicle. Once deposited in the keratin matrix, these substances remain stable without undergoing further metabolism, offering a detection window that can span to several months or even years, depending on hair length [3,4,6]. Compared to conventional biological samples such as blood or urine, hair presents numerous advantages: it is stable over time, resistant to adulteration, easy to collect and store, and less influenced by short-term abstinence or external tampering [2,4,7,8]. The non-invasive nature of sample collection and the ability to reflect chronic or past exposure make hair particularly suitable for applications such as drug-facilitated crime investigations, child protection cases, workplace testing, and monitoring drug abstinence or relapse [7]. However, hair analysis is not without challenges. Factors such as cosmetic treatment, hair pigmentation, and environmental contamination can influence drug concentrations and complicate interpretation [4,7,8].

To rule out external contamination, samples must be thoroughly washed, being afterwards subjected to digestion, typically through alkaline hydrolysis [7,9], for drug detection. Among cannabinoids, THC, its acidic metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), cannabidiol (CBD), and cannabinol (CBN) are commonly targeted in hair testing [10]. Since THC and its neutral metabolites can be present due to external contamination, confirming active cannabis use requires the detection of specific metabolites like THC-COOH, which is only formed endogenously [6,7,11]. According to the Society of Hair Testing (SoHT), the cut-off values to confirm cannabinoid ingestion are 50 pg/mg for THC and 0.2 pg/mg for THC-COOH, with a screening threshold for THC set at 100 pg/mg [7]. However, the acidic nature of THC-COOH results in critically low incorporation levels in hair, as the matrix preferentially binds basic compounds [12]. As a result, highly sensitive techniques such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) are required to detect THC-COOH, since the SoHT recommended cut-off value is generally not achievable using a single mass spectrometer [10]. Also, recent research suggests that simultaneous detection of THC-COOH and the hydroxylated metabolite 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH) can strengthen the confirmation of active cannabis use and reduce false positives due to environmental exposure [12].

This study presents a robust and sensitive method for the determination of selected cannabinoids in hair samples by LC–MS/MS using liquid-liquid extraction (LLE) as sample clean-up. The method was fully validated according to the guiding principles of ANSI/ASB Standard 036 [13] and the Food and Drug Administration (FDA) Guidance for Industry [14]. The targeted analytes, whose chemical structures are shown in Fig. 1, serve as key indicators of cannabis consumption. THC is the primary psychoactive compound in Cannabis plants, while THC-OH is its main metabolite and THC-COOH is the primary secondary metabolite. Additionally, monitoring CBN and CBD – the principal components of medical cannabis – helps distinguish different consumption patterns.

2. Materials and methods

2.1. Chemicals and reagents

LC–MS grade (≥99.9 %) methanol and water were purchased from Honeywell Riedel-de-Haën™ (Seelze, Germany), along with hexane. Sodium hydroxide 1 M (volumetric solution) was obtained from Fisher Scientific (Loughborough, England). Formic acid (98–100 %), glacial acetic acid (100 % anhydrous, analytical grade), and ethyl acetate were supplied by Supelco Merck (Darmstadt, Germany), while ammonium formate 1 M (p.a.) and 1-pentanol were purchased from Fluka/Sigma-Aldrich (Steinheim, Germany). Certified standards of THC, THC-OH, THC-COOH, CBN, and CBD at 1 mg/mL were acquired from Cerilliant Corporation (Round Rock, Texas, USA), along with 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 0.4, 1, 2, 4 and 10 ng/mL for THC-COOH; 10, 50 and 500 ng/mL for THC, CBN and CBD; and 100, 500 and 1000 ng/mL for THC-OH. An IS working solution was also prepared in methanol, containing THC-d3 and THC-OH-d3 at 100 ng/mL and THC-COOH at 10 ng/mL. All solutions were stored at −20 °C in amber borosilicate glass vials and protected from light until use.

2.3. Hair samples

Hair samples were collected from the vertex posterior region of the head, near the scalp, using scissors, and stored in envelopes until analysis. Blank samples for method optimization and validation were provided by laboratory personnel, while authentic hair samples were obtained from university students. The study complied with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Universidade da Beira Interior (CE-UBI-Pj-2021–022-ID:801).

2.4. Sample preparation

Hair samples were washed twice with LC–MS grade methanol to remove surface contaminants such as salts, lipids, and drugs. After air-drying at room temperature, they were cut into pieces smaller than 1 mm. The final methanol wash was kept for testing if deemed necessary. Approximately 20 mg of hair was hydrolyzed in a 10 mL screw-top glass tube with 500 µL of 1 M NaOH and 500 µL of LC–MS grade methanol for 30 min at 50 °C. After cooling to room temperature, samples were centrifuged at 4500 rpm for 15 min, transferred to Eppendorf tubes, and spiked with an IS solution containing THC-d3 and THC-OH-d3 at 250 pg/mg and THC-COOH-d3 at 25 pg/mg. Following vortex mixing for 20 s, 50 µL of acetic acid was added, and the samples were centrifuged at 14000 rpm for 10 min at 5 °C. The contents of the Eppendorf tubes were then transferred to 10 mL screw-top glass tubes. After adding 4 mL of a hexane/ethyl acetate (90/10, v/v) mixture, samples were centrifuged at 4500 rpm for 15 min. The organic layer was collected and evaporated to dryness at 35 °C under a gentle nitrogen

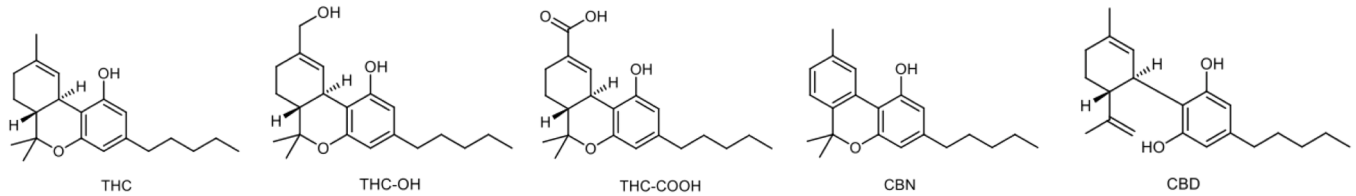

Fig. 1. Chemical structure of the selected cannabinoids.

stream. The dried residue was reconstituted in 100 μL of a 1-pentanol/LC–MS grade methanol (50/50, v/v) mixture, and 10 μL was injected into the LC–MS/MS system.

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, transitioning to 95 % B by 3 min and held for 3 min before reverting to the initial conditions after 6 min. This ratio was maintained for an additional 3 min, resulting in a total run time of 9 min. MS detection was performed in two modes: multiple reaction monitoring (MRM) and triple-stage MS analysis (MS^3). Positive MRM was used for the analysis of THC, THC-OH, CBN and CBD, monitoring two transitions for each analyte and one for each IS. Key parameter settings included an ion spray voltage (IS) of 5.5 kV, a source temperature of 250 °C, ion source gases 1 (GS1) and 2 (GS2) at 60 psi, curtain gas (CUR) at 35 psi, and collision gas (CAD) set to medium. Negative MS^3 was employed for the analysis of THC-COOH and its correspondent IS. In the MS^3 mode, first-generation product ions were further fragmented in the linear trap to produce second-generation product ions, which then underwent MS^3 transitions, as shown in Fig. 2 for THC-COOH. Key parameter settings for MS^3 included an IS of −4.5 kV, source temperature of 600 °C, GS1 at 50 psi, GS2 at 70 psi, CUR at 25 psi, and CAD set to high. Nitrogen was used as the nebulizer, heater, curtain gas, and collision-activated dissociation gas. Data acquisition and analysis were performed using Analyst® Instrumental Control and Data Processing Software (version 1.7) and SCIEX OS (version 2.1) (SCIEX, Darmstadt, Germany), respectively. For THC, THC-OH and THC-COOH, the corresponding isotopically labeled IS were used. CBN and CBD were monitored using THC-d3, since this IS provided better linearity results for CBN, and is structurally similar to CBD (as shown in Fig. 1). MRM and MS^3 transitions, retention times (RTs) and specific parameters for methods are presented in Table 1.

2.6. Method validation

The method was validated in compliance with the ANSI/ASB Standard 036 guidelines [13] and the FDA Guidance for Industry [14], assessing the following parameters: selectivity and specificity, linearity, limits of detection (LODs), lower limits of quantification (LLOQs), precision and accuracy, matrix effects (ionization suppression or enhancement), recovery, processed sample stability (in the autosampler), and carryover. All calibrators and quality controls (QCs) were prepared using blank hair samples. To evaluate specific parameters, namely LODs, LLOQs, and matrix effects, hair samples from various sources were used. Identification criteria for positive results were established following the World Anti-Doping Agency (WADA) guidelines [15], requiring a signal-to-noise ratio of at least 3:1. Furthermore, if a deuterated analog was not used as an IS, the relative retention time (RRT) of the analyte's chromatographic signal could not deviate by more than 1 % from that of the same substance in the QC sample; otherwise, the RRT variation had to remain below 0.5 %. Additionally, the identification process included detecting two ions in the mass spectrum and monitoring their relative intensities as part of the acceptance criteria.

3. Results

3.1. Selectivity and specificity

To evaluate selectivity, blank matrix samples from ten different sources were extracted using the described extraction method. No significant matrix interferences were detected at the RTs or monitored

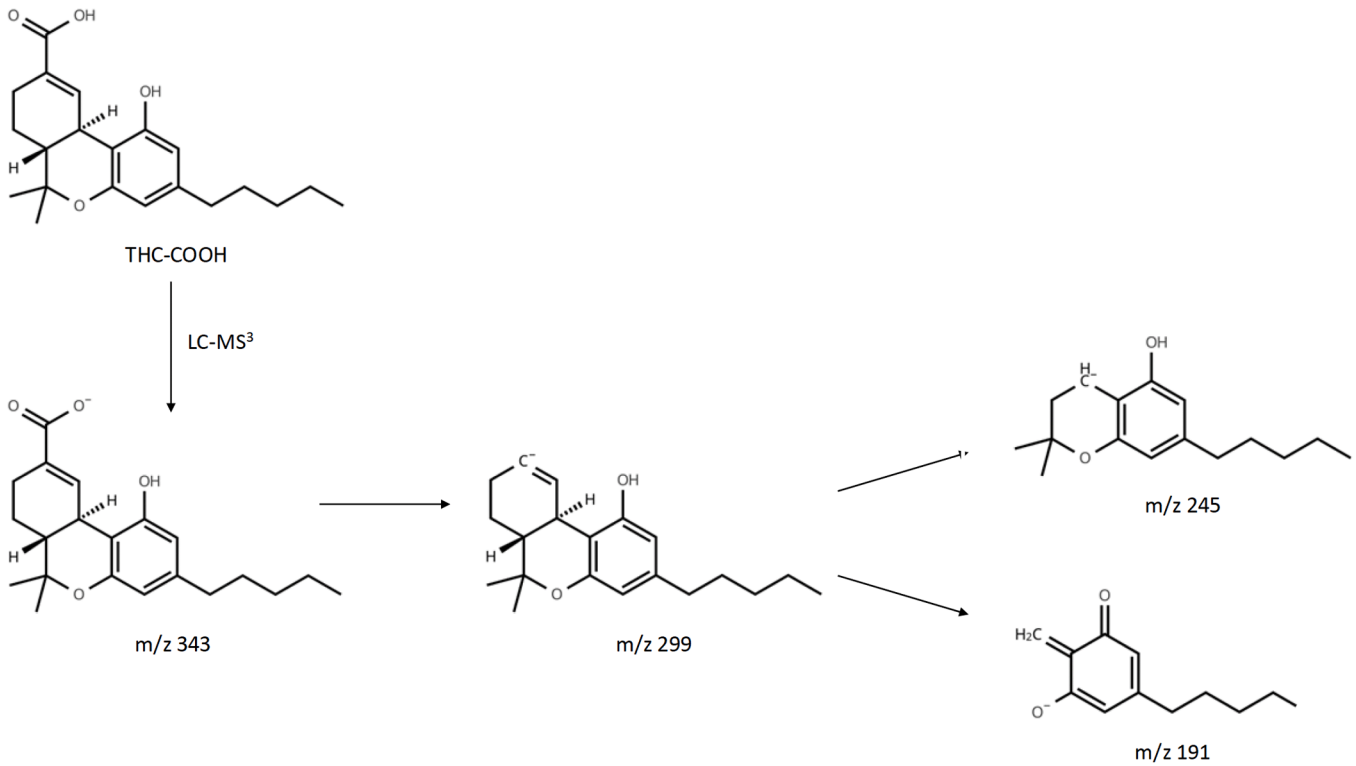

Fig. 2. Fragmentation pattern of THC-COOH analyzed by LC-MS^3.

Table 1
Retention time (RT), MRM and MS3 transitions and optimized LC–MS/MS parameters for the analysis of selected cannabinoids in hair samples (Q1: parent ion; Q3: product ion; DP: declustering potential; EP: entrance potential: CE: collision energy; CXP: collision cell exit potential).

Analyte	RT (min)	Q1 (*m/z*)	Q3 (*m/z*)	MS^3 range scanned (*m/z*)	MS^3 range for quantification (*m/z*)	DP (V)	EP (V)	CE (eV)	CXP (V)
THC	4.77	315	193	N/A	N/A	91	10	29	22
			123			91	10	39	14
THC-OH	4.15	331	193	N/A	N/A	66	10	33	22
			201			66	10	33	24
THC-COOH	4.19	343	245	175–250	240–250	−100	−10	−28	N/A
CBN	4.55	311	223	N/A	N/A	100	10	35	10
			241			100	10	35	10
CBD	4.20	315	193	N/A	N/A	51	10	29	22
			123			51	10	41	18
THC-d3	4.77	318	196	N/A	N/A	91	10	29	22
THC-OH-d3	4.15	334	196	N/A	N/A	66	10	33	22
THC-COOH-d3	4.19	346	302	N/A	N/A	−101	−10	−28	−16

transitions, confirming the method's selectivity.

For specificity assessment, three blank samples from different sources were spiked with a mixture of 191 potentially interfering compounds at 200 pg/mg (a complete list of these substances is provided in the supporting information). Additionally, the same samples were spiked with both the interference mixture and the target analytes to determine whether the presence of these compounds affected peak detection. As shown in Fig. 3, no significant interferences were observed at the RTs or monitored transitions of the analytes under study.

To determine whether the ISs interfered with their corresponding non-deuterated analytes, and vice versa, two blank samples were prepared. One sample was spiked with ISs at the study concentrations (THC-d3 and THC-OH-d3 at 250 pg/mg; THC-COOH at 25 pg/mg), while the other was spiked with the highest calibrator concentration, excluding

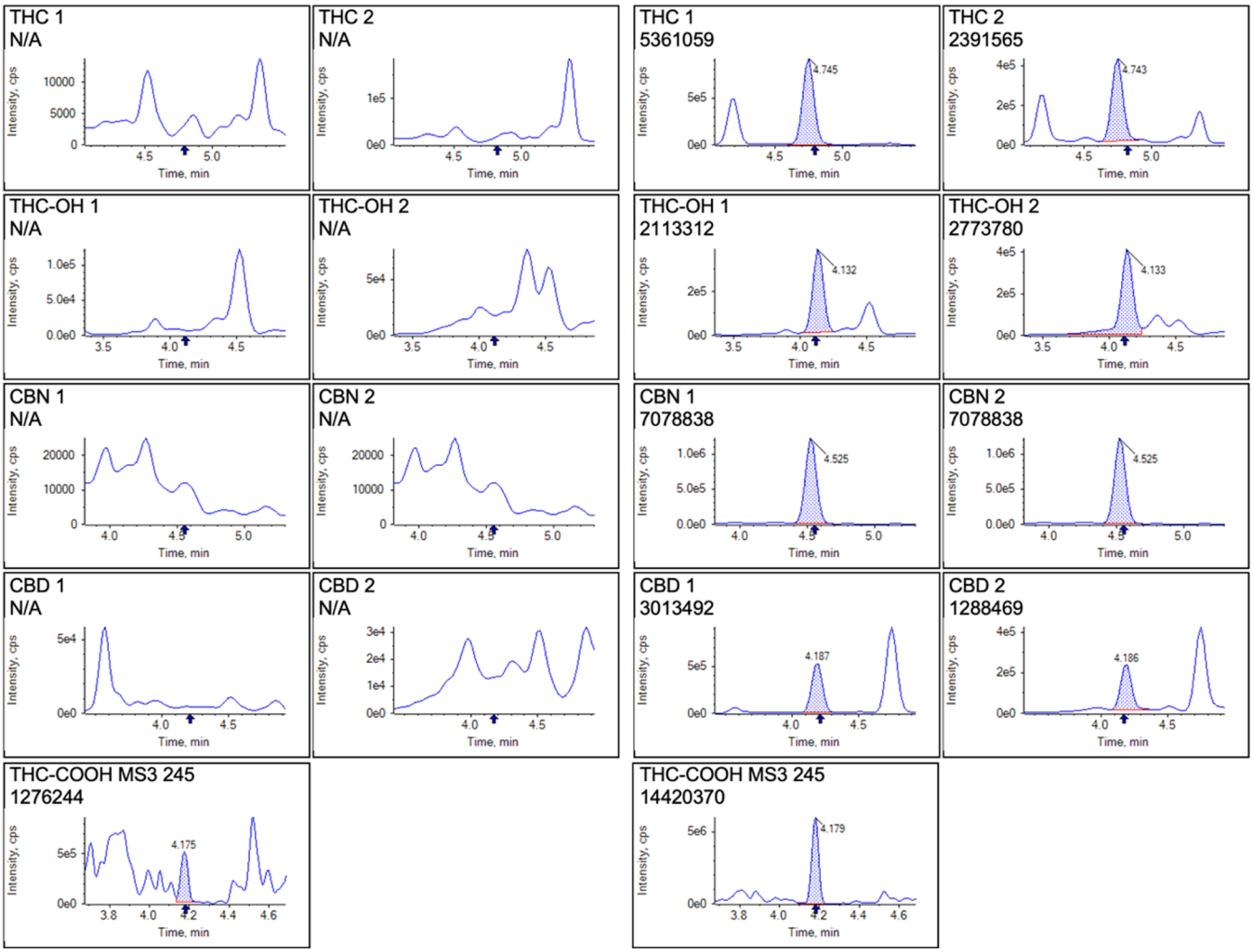

Fig. 3. Extracted chromatograms obtained from hair samples spiked with 200 pg/mg of potentially interfering compounds (left) and from hair samples spiked with 200 pg/mg of potentially interfering compounds and selected cannabinoids with the following concentrations: THC, CBN and CBD at 200 pg/mg, THC-OH at 500 pg/mg and THC-COOH at 5 pg/mg (right).

ISs (THC, THC-OH, CBN, and CBD at 2000 pg/mg; THC-COOH at 20 pg/mg). The analysis of the sample containing only the deuterated compounds did not result in any false positive identification of the analytes, confirming that the ISs did not cause interference. Similarly, the sample fortified with the target analytes, without ISs, showed no interference in IS detection.

The method was thus considered selective and specific.

3.2. Linearity, LLOQ and LOD

To study the method's linearity, the working range was determined for each analyte. Spiked hair samples were analyzed, and linearity was evaluated using eight calibrators, evenly distributed across the working range in five different runs (n = 5). The calibrator concentrations were selected based on typical values observed in routine cases and reported in the literature. The method demonstrated linearity within the following ranges: 5–2000 pg/mg for THC, CBN, and CBD; 50–2000 pg/mg for THC-OH; and 0.2–20 pg/mg for THC-COOH. The calibration curves showed mean determination coefficients (R^2) higher than 0.99. Residual analysis (sum of deviations from calculated to nominal concentrations) indicated that weighted linear regression (1/x) provided the best fit for the data. A summary of the calibration data is presented in Table 2.

Following the guidelines of the ANSI/ASB Standard 036 [13], LLOQ 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 acceptance parameters were met, with both coefficient of variation (CV) and bias falling within the range of ±20 %.

To study LODs, samples spiked with decreasing concentrations of the analytes, starting from the LLOQ values, were studied (n = 3). The LODs were determined as the minimum concentration in which the peak could be clearly distinguished from a blank sample (as shown in Fig. 4), and the acceptance criteria were met. LOD was 2 pg/mg for THC, CBN and CBD, 10 pg/mg for THC-OH and 0.2 pg/mg for THC-COOH. Whereas for THC, THC-OH, CBN and CBD it was possible to obtain lower concentrations for the LODs, the same did not happen for THC-COOH. For this compound, concentrations below the LLOQ did not provide peaks clearly distinguished from blank samples, and S/N ratios were lower than 3.0. Thus, the LLOQs and LODs were the same for THC-COOH. LODs and LLOQs are summarized in Table 3.

3.3. Precision and accuracy

The evaluation of these parameters was conducted alongside the linearity assessment over five separate runs. Precision was expressed as the CV, and values lower than or equal to ±20 % were considered acceptable at all concentration levels [13]. Inter-day precision was assessed by analyzing calibrators across five different runs (n = 5), while intra-day precision was determined by analyzing five replicate samples (n = 5) of a selected concentration each day to evaluate method repeatability. Intermediate precision (combined intra- and inter-day precision) was studied using QC samples (n = 3) at four concentration levels over five days (n = 15). QC concentrations were 5, 300, 1500, and 2000 pg/mg for THC, CBN, and CBD; 50, 300, 1500, and 2000 pg/mg for THC-OH; and 0.2, 7.5, 17.5, and 20 pg/mg for THC-COOH. Accuracy was expressed as relative error (RE), or bias, representing the difference between measured concentrations (calculated using the calibration equation) and nominal levels. A deviation within ±20 % of the nominal concentration was considered acceptable [13]. The method demonstrated satisfactory inter-day precision, with CV values ranging from 2.33 % to 13.13 %, and bias between 0.03 % and 8.56 %. Intra-day precision showed similarly strong results, with CV values from 1.32 % to 14.09 % and bias ranging from 0.78 % to 14.02 %. For intermediate precision, CV values varied between 5.07 % and 14.02 %, while bias ranged from 0.54 % to 6.14 %. A summary of precision and accuracy data is provided in Supplementary Table 1.

3.4. Matrix effects (ionization suppression/enhancement)

To evaluate ion suppression or enhancement, two sets of samples were prepared. Set #1 consisted of unextracted neat standards at the study concentrations, injected six times, while set #2 was prepared with hair samples from ten different sources (n = 10), fortified with the same concentrations as set #1 after extraction (before evaporation of the extracts). The extent of suppression or enhancement was determined by calculating the ratio of the average peak areas between the two sets. All samples were prepared in duplicate to evaluate ion suppression/enhancement at both high and low concentrations. This parameter was also assessed for the studied ISs. The acceptance criteria required that ionization suppression/enhancement values remain within ±25 % [13].

As shown in Table 4, all analytes exhibited ion suppression at both low and high concentrations, with values ranging from −49.76 % to −85.30 %. The exceptions were THC-COOH – which displayed ion suppression within ±25 % at low concentrations – and THC-COOH-D3. This can be attributed to the fact that THC-COOH was analyzed using negative ionization in an MS^3 run. For all other analytes at both concentration levels, and in accordance with the guidelines followed [1], additional measures were taken to ensure that ion suppression did not impact the method's LODs and LLOQs. Specifically, at least three times the number of samples from different origins were analyzed to evaluate these parameters, confirming that matrix effects were not significant at low concentrations and did not interfere with positivity assessment.

3.5. Recovery

To assess recovery, two sets of samples (n = 3) were extracted for each QC concentration. The analytical method was applied to both sets, with the first set fortified before extraction and the second set fortified immediately after extraction, just before evaporation of the extracts. The obtained results are summarized in Table 5 and ranged from 48.33 % to 104.71 %.

According to the guiding principles of the FDA [14], recoveries between 80 % and 120 % are considered acceptable. However, if the extent of the recovery of an analyte is consistent and reproducible, recoveries below these values can be accepted. Considering the results showed in Table 5, the CV of the recovery values for THC, THC-OH, CBN

Table 2
Linear range, linearity, and correlation coefficients (R^2) for selected cannabinoids in hair samples (n = 5).

Analyte	Weighting factor	Linear range (pg/mg)	Linearity		$R^{2\,(a)}$
			Slope[(a)]	Intercept[(a)]	
THC	1/x	5 – 2000	0.004 ± 0.000	0.000 ± 0.004	0.997 ± 0.002
THC-OH	1/x	50 – 2000	0.004 ± 0.000	0.090 ± 0.063	0.995 ± 0.003
THC-COOH	1/x	0.2 – 20	8.162 ± 2.006	6.691 ± 2.718	0.996 ± 0.003
CBN	1/x	5 – 2000	0.005 ± 0.001	0.007 ± 0.003	0.997 ± 0.002
CBD	1/x	5 – 2000	0.002 ± 0.000	0.000 ± 0.002	0.996 ± 0.003

(a) Mean values ± standard deviation.

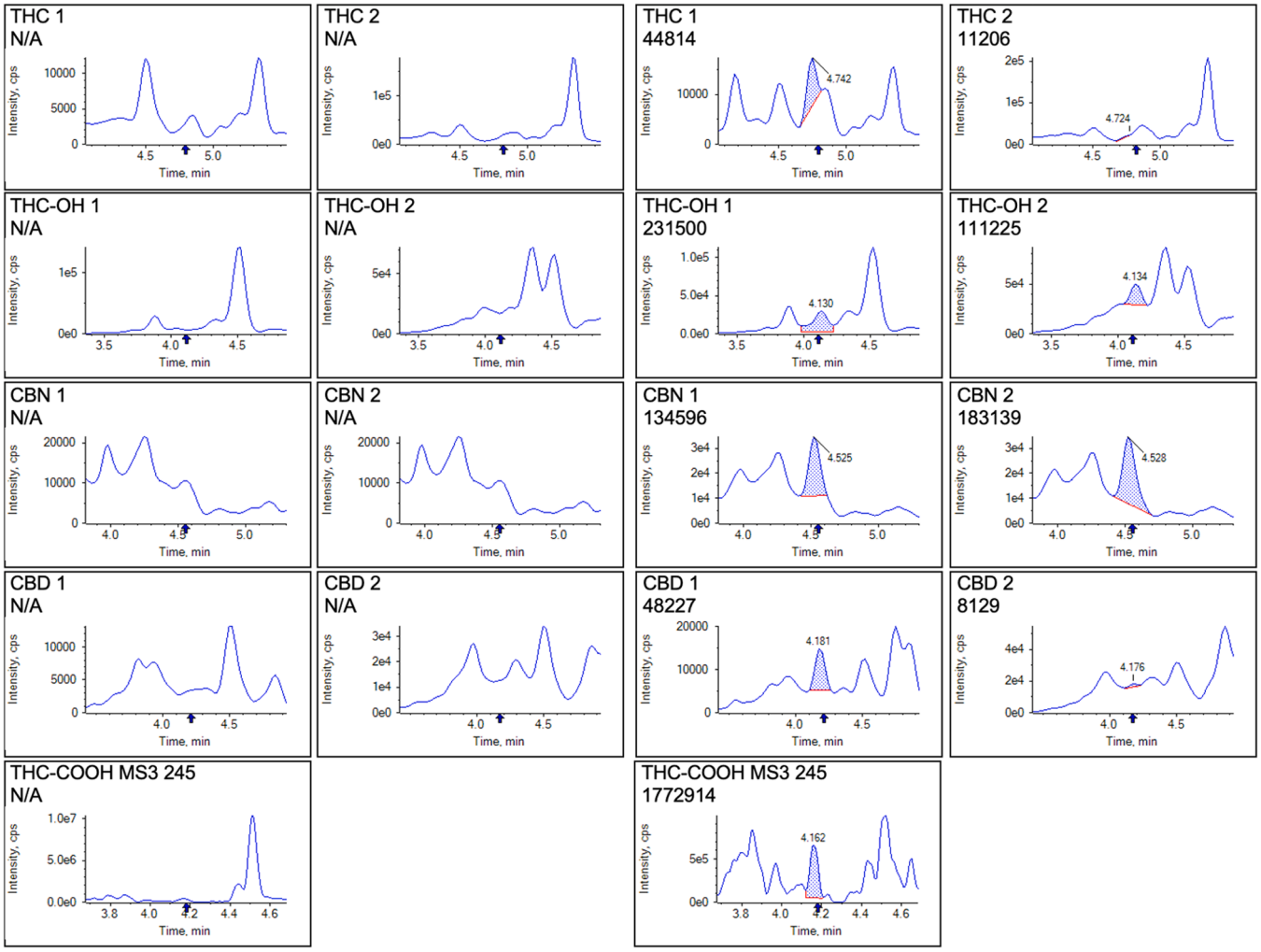

Fig. 4. Extracted chromatograms obtained from blank hair samples (left) and from spiked hair samples at LOD concentrations (right). LOD of THC, CBN and CBD is 2 pg/mg; LOD of THC-OH is 10 pg/mg; LOD of THC-COOH is 0.2 pg/mg.

Table 3
LOD (n = 3) and LLOQ (n = 22) for selected cannabinoids in hair samples.

Analyte	LOD (n = 3)				LLOQ (n = 22)			
	Concentration (pg/mg)	Measured (pg/mg)[a]	CV (%)	Bias (%)	Concentration (pg/mg)	Measured (pg/mg)[a]	CV (%)	Bias (%)
THC	2	1.96 ± 0.11	5.84	−1.79	5	4.83 ± 0.65	13.39	−3.30
THC-OH	10	11.02 ± 2.16	19.56	10.21	50	49.07 ± 6.08	12.40	−1.85
THC-COOH	0.2	0.19 ± 0.02	12.04	−2.80	0.2	0.19 ± 0.02	9.57	−5.14
CBN	2	2.33 ± 0.04	1.57	16.47	5	4.76 ± 0.60	12.68	−4.76
CBD	2	2.24 ± 0.34	14.97	11.99	5	4.82 ± 0.59	12.21	−3.70

[a] Mean values ± standard deviation.

Table 4
Ion suppression/enhancement (%) for selected cannabinoids in hair samples.

Analyte	Low concentration (n = 10)		High concentration (n = 10)	
	Concentration (pg/mg)	Ion suppression or enhancement (%)	Concentration (pg/mg)	Ion suppression or enhancement (%)
THC	20	−64.67	500	−65.76
THC-OH	200	−83.18	1000	−83.40
THC-COOH	1	−22.00	10	−49.76
CBN	20	−67.40	500	−68.34
CBD	20	−80.21	500	−79.85
THC-d3	N/A	N/A	250	−70.09
THC-OH-d3	N/A	N/A	250	−85.30
THC-COOH-d3	N/A	N/A	25	−18.75

Table 5
Extraction recovery (%) of selected cannabinoids in hair samples (n = 3).

Analyte	Concentration (pg/mg)									CV (%)
	0.2	5	7.5	17.5	20	50	300	1500	2000	
THC	N/A	77.60	N/A	N/A	N/A	N/A	53.27	56.43	54.20	19.15
THC-OH	N/A	N/A	N/A	N/A	N/A	67.50	54.34	50.21	49.75	14.96
THC-COOH	104.71	N/A	52.13	52.94	48.33	N/A	N/A	N/A	N/A	41.63
CBN	N/A	78.04	N/A	N/A	N/A	N/A	55.43	58.76	57.42	16.84
CBD	N/A	69.87	N/A	N/A	N/A	N/A	48.36	55.86	53.77	16.09

and CBD are within ± 20 %. CV for THC-COOH is higher but considers the only recovery value within the 80–120 % accepted interval.

The reported extraction recovery values can be attributed to the strong interactions of cannabinoids with the keratinous hair matrix, including hydrophobic and hydrogen bonding, which can reduce extraction efficiency [19]. Additionally, LLE may sometimes produce emulsions at the interface between aqueous and organic phases, potentially leading to analyte losses or variability in recovery [19]. However, these extraction results did not affect the method's LODs, LLOQs, precision, accuracy, or linearity.

3.6. Processed sample stability

Autosampler stability was assessed by reanalyzing the extracts after being kept in the autosampler (at 15 °C) for 24, 48 and 72 h. The initial response (0 h) was established based on injections performed on the day of sample extraction. Analytes were considered stable if the CV and bias remained within ±20 % of the results obtained for the sample prepared at time zero. Stability data are summarized in Supplementary Table2.

We concluded that THC, THC-OH, CBN and CBD remain stable in the autosampler for 72 h, since CV and bias remains within ± 20 % for all concentrations studied. On the other hand, a decrease in THC-COOH at the LLOQ is noticeable after 24 h in the autosampler, with CV and bias around 30 %. After 48 h, CV increases to 90.77 %, and 72 h after extraction of the analytes, no peaks can be found for THC-COOH in any concentration.

Based on the results obtained in the stability study, samples should be analyzed as soon as possible after extraction to prevent false negatives, solemnly based on THC-COOH.

3.7. Carryover

Instrument carryover was evaluated simultaneously with linearity assays by analyzing extracted blank hair samples immediately after the highest calibrator. Carryover was considered present if the peak area of the blank sample exceeded 20 % of the peak area of the LLOQ for each analyte [13]. For THC-COOH, the blank samples showed 40 % of the LLOQ peak area. However, since results below the LLOQ would not be reported, the method's adequacy remained unaffected.

3.8. Application to authentic samples

The method was successfully applied to real samples collected from university students. 189 samples have tested positive for at least one of the targeted compounds. THC concentrations ranged from 5.17 to 609.74 pg/mg, with only six samples testing positive for THC-OH above the LLOQ (from 50.04 to 81.92 pg/mg). THC-COOH concentrations varied between 0.24 and 18.15 pg/mg. CBN levels were found between 5.22 and 460.90 pg/mg, while CBD concentrations ranged from 5.11 to 850.32 pg/mg.

Several samples tested positive for THC but negative for its metabolites. Thus, the second methanol wash was analyzed and tested positive for THC, suggesting potential external contamination.

Various samples showed cannabinoid concentrations near the LLOQ values. Additionally, three samples tested below the LOQ for THC but were positive for the secondary metabolite THC-COOH. Eleven samples tested positive for CBD only, suggesting medicinal rather than illicit cannabis use.

These results were compared with self-completion questionnaires filled out by the volunteers. In these surveys, several parameters were assessed, including age, gender, and cannabis consumption patterns. Fig. 5 presents the distribution of positive results according to age, gender, tobacco use, mode of consumption, and consumption pattern. We found that most of the consumption occurs among individuals aged 19–22 years, predominantly male, who also smoke tobacco (particularly between 3 and 10 cigarettes per day). The most frequently reported mode of consumption was cannabis weed, with a daily use pattern.

4. Discussion

Hair samples were washed twice with LC–MS grade methanol to remove potential external contaminants. Methanol is widely used in this context due to its ability to efficiently solubilize surface impurities without compromising the integrity of the hair matrix [6]. Importantly, the second methanol wash was retained and analyzed separately to identify false positives due to external contamination, allowing for a more accurate interpretation of cannabinoid active use. Only 20 mg of hair was used per sample, a relatively small quantity compared to other methods reported in the literature [2,9,20,21], making the procedure especially valuable when sample availability is limited. Digestion of the hair allows the release of the analytes from the matrix, and was achieved using alkaline hydrolysis, a well-established technique for effectively releasing analytes embedded within the matrix [9–11]. Digestion was performed for just 30 min (at 50 °C), minimizing degradation risks, and improving overall efficiency. Following hydrolysis, samples were acidified with acetic acid, a step necessary to neutralize the alkaline solution. LLE was conducted with 4 mL of a hexane/ethyl acetate mixture (90:10, v/v), a smaller solvent volume than typically reported in the literature [10,22], highlighting the method's efficiency. After evaporation to dryness, the residue was reconstituted in 100 µL of a 1-pentanol/LC–MS grade methanol mixture (50:50, v/v), as proposed by Dulaurent et al. [10]. This reconstitution strategy was critical because analytes were found to be trapped within oily residues that could not be solubilized by methanol alone. The inclusion of 1-pentanol, with its dual polar and apolar characteristics, ensured proper solubilization by interacting both with the lipophilic components of the hair and the injection solvent, thus improving injection efficiency and analyte recovery.

Detection of THC-COOH in hair remains analytically challenging due to its extremely low concentrations and the high background signal typical of the matrix. Our method successfully achieved an LLOQ of 0.2 pg/mg for this analyte, in accordance with the threshold proposed by the SoHT. Although LC-MS/MS techniques typically do not require derivatization, several published methods have incorporated chemical modification or complex hardware to achieve the necessary sensitivity for THC-COOH detection in hair. For example, Al-Zahrani et al. [9] employed methanolic HCl and FMPTS for derivatization following polymeric strong anion mixed-mode solid-phase extraction (SPE), enhancing the analyte's lipophilicity and improving detection sensitivity. Their method achieved an LOD of 0.1 pg/mg and an LOQ of 0.2 pg/mg for THC-COOH. On the other hand, Cho et al. [11] combined

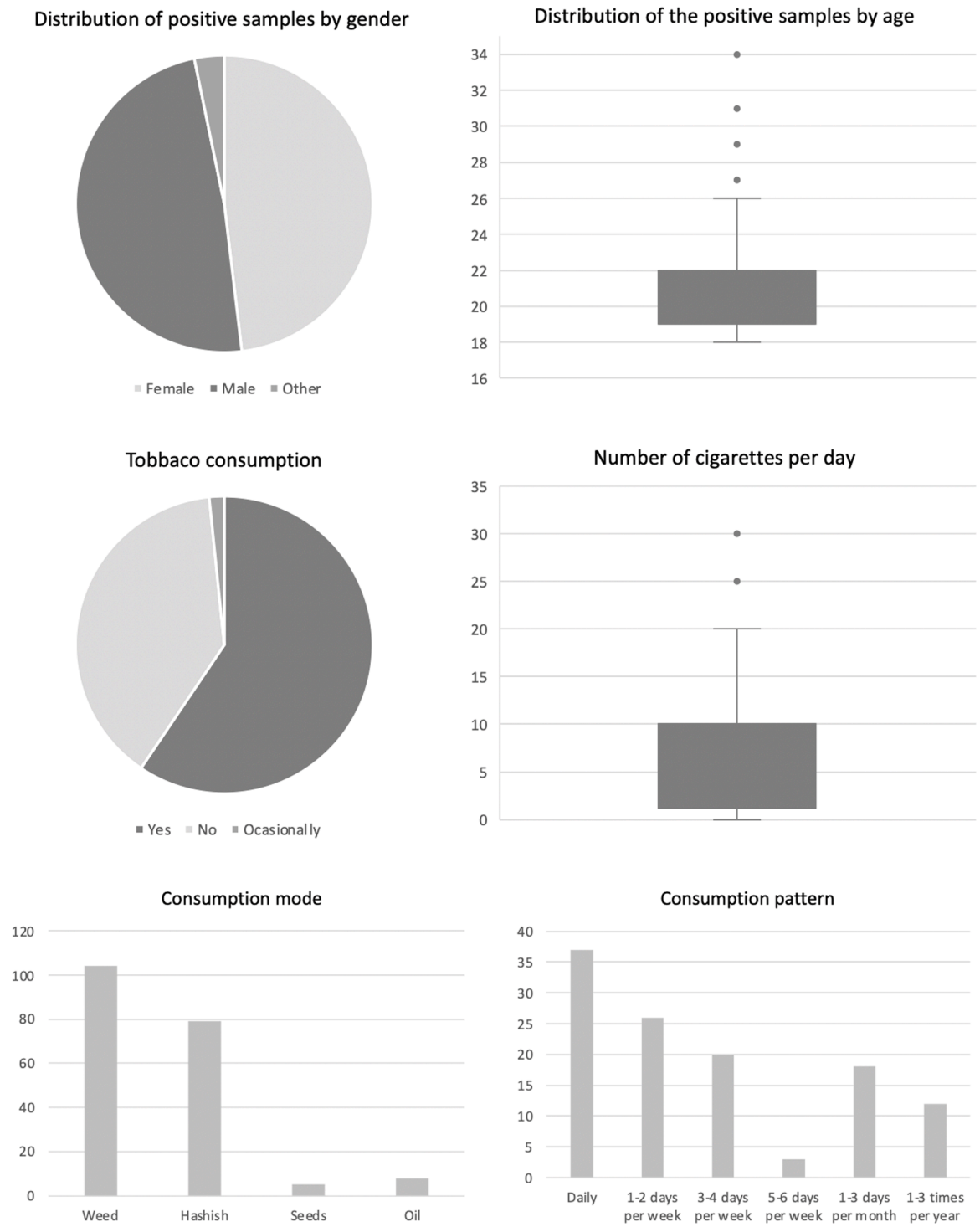

Fig. 5. Distribution of positive cases stratified by age, gender, tobacco use, mode of cannabis consumption, and consumption pattern.

MRM with MS^3 acquisition and a column-switching valve system to reduce matrix interferences, reaching comparable limits of detection and quantification. In 2024, SCIEX released a technical note outlining a THC-COOH detection protocol in hair based on SPE, also meeting the SoHT recommendation [16].

Vaiano et al. [17] developed and validated a sensitive LC-MS/MS method employing alkaline hydrolysis followed by LLE with a hexane/ethyl acetate mixture, and analysis via MRM on a C18 column. Their method achieved LOQs of 10 pg/mg for THC and 0.2 pg/mg for THC-COOH. While our approach is similar in terms of sample preparation, it extends the analytical scope to include additional cannabinoids—namely THC-OH, CBN, and CBD—providing a broader forensic profile. Furthermore, our method demonstrated enhanced sensitivity for THC, with an LOQ of 5 pg/mg, while maintaining the same LLOQ for THC-COOH (0.2 pg/mg).

Hehet et al. [18] presented a highly sensitive method for THC-COOH detection using $LC\text{-}MS^3$. Their method, which combined alkaline digestion with LLE, reached an LOQ of 0.1 pg/mg for THC-COOH and was validated on over 2000 hair samples. However, their analysis was limited to THC-COOH.

In contrast to THC-COOH, no cut-off currently exists for THC-OH. However, Casati et al. [12] proposed a value of 0.5 pg/mg in both scalp and body hair to distinguish between chronic use and external contamination, suggesting that the simultaneous detection of both THC-COOH and THC-OH may provide stronger evidence of active cannabis consumption. We managed to achieve a LLOQ of 50 pg/mg for THC-OH and a LOD of 10 pg/mg. In terms of incorporation, it is well established that neutral cannabinoids such as THC and THC-OH are deposited in hair more readily than acidic metabolites like THC-COOH. This is due to their greater lipophilicity, which correlates directly with their ability to bind to the lipid-rich structure of the hair shaft. Consequently, THC-OH is generally found at higher concentrations than THC-COOH in hair samples.

Our method delivers comparable sensitivity while enabling simultaneous quantification of THC, THC-OH, THC-COOH, CBN, and CBD, thereby offering a more comprehensive cannabinoid profile. Recently, there has been an increase in the development of LC-MS/MS methods for cannabinoid analysis in hair. However, studies including THC-OH remain scarce [6,11]. Our method addresses this gap by providing simultaneous detection of both neutral and acidic cannabinoids using LC-MS/MS and LC-MS^3. A key advantage of our method is its streamlined workflow. Earlier analytical methods for these compounds were based on gas chromatography (GC), which requires derivatization—especially for detecting THC-COOH due to its polarity and thermal lability. The method described herein does not require derivatization, valve-switching systems, or SPE, yet it achieves high sensitivity and specificity. Compared to SPE, LLE is faster, simpler, and less resource-intensive, requiring fewer preparation steps and no specialized cartridges. This enhances cost-effectiveness and operational efficiency, making the method well-suited for high-throughput applications. Despite the procedural simplicity, our method achieves robust analytical performance, including an LLOQ of 0.2 pg/mg for THC-COOH—equivalent to values reported in more complex methodologies. These results highlight the reliability, versatility, and practical advantages of our approach for routine forensic and toxicological analysis. Results of authentic samples were consistent with self-completion questionnaires filled out by the volunteers, which proves the method is reliable and robust.

5. Conclusions

In this study, we developed a robust and sensitive LC-MS/MS method for the simultaneous detection and quantification of multiple cannabinoids—THC, THC-OH, THC-COOH, CBN, and CBD—in human hair. The method combines alkaline hydrolysis, LLE, MRM and MS^3 spectrometry, achieving low LLOQ, particularly for THC-COOH, in line with the cut-off values proposed by SoHT.

Addressing the limitations of earlier GC-based methods that required derivatization – especially for THC-COOH – this approach enables direct, derivatization-free analysis of both neutral and acidic cannabinoids, including the underreported THC-OH.

In summary, this method offers a practical, sensitive, and comprehensive approach for the detection of cannabinoids in hair. It requires minimal sample and solvent volumes, uses a rapid alkaline hydrolysis step, benefits from the dual-solvent reconstitution to ensure analyte recovery, and achieves SoHT-compliant LOQs for THC-COOH. Furthermore, the inclusion of THC-OH, CBN, and CBD provides a broader interpretive framework for distinguishing between passive exposure and active cannabis use, and between medicinal and recreational use. Additionally, the washing step (and the consequent analysis of the second methanol wash) provides valuable insight into differentiating passive from active consumption. Hair samples may be segmented into 1-cm sections to establish a chronological profile of drug intake. However, in this study samples were analyzed as a whole, because most of the collected samples were low weight. These advantages position the method as a valuable tool for routine forensic and toxicological applications.

Ethics Declarations

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Faculdade de Ciências da Saúde da Universidade da Beira Interior (number CE-UBI-Pj-2021–022.). Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

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

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.

Acknowledgements

This work was developed within the scope of the CICS-UBI projects https://doi.org/10.54499/UIDB/00709/2020 and https://doi.org/10.54499/UIDP/00709/2020 and financed by national funds through the Portuguese Foundation for Science and Technology (FCT)/MCTES. Mónica Antunes acknowledges the PhD fellowship from FCT (2020.05765.BD).

Supporting information

The mixture used to evaluate the method's specificity contained the following compounds: acetazolamide, alprazolam, α-hydroxyalprazolam, amisulpride, amitriptyline, amlodipine, aripiprazole, atenolol, atorvastatin, atropine, bisoprolol, bromazepam, brotizolam, buflomedil, buprenorphine, bupropion, buspirone, captopril, carbamazepine, carvedilol, chlordiazepoxide, chloroquine, chlorpromazine, demethyl chlorpromazine, ciproheptadine, citalopram, desmethylcitalopram, clobazam, clomipramine, clomipramine-D3, desmethylclomipramine, clonazepam, 7-aminoclonazepam, clonidine, clozapine, desmethylclozapine, codeine, cyamemazine, cyclobenzaprine, demoxepam, diazepam, diclofenac, digoxin, metildigoxin-NH4 diltiazem, diphenhydramine, donepezil, dosulepin, desmethyldosulepin, doxylamine, duloxetine, enalapril, ergotamine, estazolam, felbamate, fentanyl, desmethyl fentanyl, flecainide, flunitrazepam, 7-aminoflunitrazepam, desmethylflunitrazepam, fluoxetine, desmethyl fluoxetine, fluphenazine, flurazepam, 2-hydroxyethylflurazepam, desalkylflurazepam, fluvoxamine, furosemide, gabapentin, halazepam, haloperidol, hydrochlorothiazide, hydroxyzine, ibuprofen, imipramine, indapamide, ketamine, N-desmethyl ketamine, ketoprofen, lacosamide, lamotrigine, laudanosine, lercanidipine, levetiracetam, levomepromazine, N-desmethyllevomepromazine, lidocaine, lisinopril, loprazolam, lorazepam< , lormetazepam, losartan, lovastatin, loxapine, desmethylmianserin, maprotiline, melperone, memantine, methadone, EDDP, metildigoxin, metoclopramide, metoprolol, mianserin, midazolam, α-hydroxymidazolam, mirtazapine, N-desmethyl mirtazapine, modafinil, morphine, naloxone, naltrexone, nifedipine, nimesulide, nitrazepam, 7-aminonitrazepam, nordiazepam, nortriptyline, olanzapine, oxazepam, oxcarbazepine, oxycodona, oxymorphone, paliperidone,

paracetamol, paroxetine, pentobarbital, perampanel, perindopril, pethidine, desmethyl-pethidine, phenacetin, ethylphenidate, methylphenidate, phenobarbital, phenytoin, piracetam, pravastatin, prazepam, pregabalin, primidone, promethazine, propafenone, propranolol, desmethyl quetiapine, ramipril, reboxetine, reserpine, risperidone, rocuronium, salicylic acid, selegiline, sertraline, desmethylsertraline, sildenafil, simvastatin, strychnine, sulpiride, tadalafil, tapentadol, telmisartan, temazepam, tetramisole, thiopental, tiagabine, tianeptine, tiapride, ticlopidine, topiramate, tramadol, N-desmethyltramadol, O-desmethyltramadol, m-CPP, trazodone, triamterene, triazolam, α-hydroxytrazolam trimipramine, desmethyl trimipramine, valsarta, vardenafil, venlafaxine, O-desmethylvenlafaxine, verapamil, vigabatrin, vortioxetine, warfarin, ziprasidone, zolpidem and zonisamide.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2025.112685.

References

[1] European Monitoring Centre for Drugs and Drug Addiction, European Drug Report 2024, 2024, Trends and Developments.

[2] M. Antunes, M. Barroso, E. Gallardo, Analysis of cannabinoids in biological specimens: an update, Int. J. Environ. Res. Public Health 20 (2023) 1–36, https://doi.org/10.3390/ijerph20032312.

[3] J. Gonçalves, T. Rosado, S. Soares, A. Simão, D. Caramelo, Â. Luís, N. Fernández, M. Barroso, E. Gallardo, A. Duarte, Cannabis and its secondary metabolites: their use as therapeutic drugs, toxicological aspects, and analytical determination, Medicines 6 (2019) 31, https://doi.org/10.3390/medicines6010031.

[4] A.G. Nicolaou, M.C. Christodoulou, I.J. Stavrou, C.P. Kapnissi-Christodoulou, Analysis of cannabinoids in conventional and alternative biological matrices by liquid chromatography: applications and challenges, J. Chromatogr. A 1651 (2021) 462277, https://doi.org/10.1016/j.chroma.2021.462277.

[5] W. Hall, The health and psychological effects of cannabis use, Curr. Issues Crim. Justice 6 (2001) 208–220, https://doi.org/10.1080/10345329.1994.12036647.

[6] L. Mercolini, R. Mandrioli, M. Protti, M. Conti, G. Serpelloni, M.A. Raggi, Monitoring of chronic cannabis abuse: an LC-MS/MS method for hair analysis, J. Pharm. Biomed. Anal. 76 (2013) 119–125, https://doi.org/10.1016/j.jpba.2012.12.015.

[7] G.A.A. Cooper, R. Kronstrand, P. Kintz, Society of hair testing guidelines for drug testing in hair, Forensic Sci. Int. 218 (2012) 20–24, https://doi.org/10.1016/j.forsciint.2011.10.024.

[8] M. Barroso, E. Gallardo, D.N. Vieira, M. López-Rivadulla, J. Queiroz, Hair: a complementary source of bioanalytical information in forensic toxicology, Bioanalysis 3 (2011) 67–79, https://doi.org/10.4155/bio.10.171.

[9] M.A. Al-Zahrani, A.I. Al-Asmari, F.F. Al-Zahrani, H.J. Torrance, D.G. Watson, Quantification of cannabinoids in human hair using a modified derivatization procedure and liquid Chromatography–Tandem mass spectrometry, Drug Test. Anal. 13 (2021) 1095–1107, https://doi.org/10.1002/dta.3005.

[10] S. Dulaurent, J.M. Gaulier, L. Imbert, A. Morla, G. Lachâtre, Simultaneous determination of Δ9-Tetrahydrocannabinol, cannabidiol, cannabinol and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid in hair using liquid Chromatography-Tandem mass spectrometry, Forensic Sci. Int. 236 (2014) 151–156, https://doi.org/10.1016/j.forsciint.2014.01.004.

[11] H.S. Cho, B. Cho, J. Sim, S.K. Baeck, S. In, E. Kim, Detection of 11-nor-9-carboxy-tetrahydrocannabinol in the hair of drug abusers by LC–MS/MS analysis, Forensic Sci. Int. 295 (2019) 219–225, https://doi.org/10.1016/j.forsciint.2018.12.013.

[12] S. Casati, I. Angeli, A. Ravelli, M. Del Fabbro, M. Minoli, M. Orioli, 11-OH-THC in hair as marker of active cannabis consumption: estimating a reliable Cut-off by evaluation of 672 THC-Positive hair samples, Forensic Sci. Int. 304 (2019) 109951, https://doi.org/10.1016/j.forsciint.2019.109951.

[13] Scientific Working Group for Forensic Toxicology (SWGTOX) ASB Standard 036First Edition Standard Practices for Method Validation in Forensic Toxicology. J. Anal. Toxicol.2018, 1-40.

[14] U.S. Department of Health and Human Services; Food and Drug Administration; Center for Drug Evaluation and Research (CDER); Center for Biologics Evaluation and Research (CBER) *M10 Bioanalytical Method Validation and Study Sample Analysis - Guidance for Industry*; 2022;

[15] World Anti-Doping Agency *Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes*; 2021;

[16] X. He, O.G. Cabrices, A. Wang, M. Clabaugh, A.M. Taylor, Sensitive detection of the marijuana metabolite with the SCIEX triple Quad™ 4500 LC-MS/MS system, Sciex (2019) doi:RUO-MKT-02-6802-A.

[17] F. Vaiano, L. Scuffi, A. Lachi, C. Trignano, A. Argo, F. Mari, E. Bertol, THC and THC-COOH hair concentrations: influence of age, gender, consumption habits, cosmetics treatment, and hair features, J. Pharm. Biomed. Anal. 225 (2023) 115237, https://doi.org/10.1016/j.jpba.2023.115237.

[18] P. Hehet, T. Franz, N. Kunert, F. Musshoff, Fast and highly sensitive determination of tetrahydrocannabinol (THC) metabolites in hair using liquid chromatography-multistage mass spectrometry (LC–MS3), Drug Test. Anal. 14 (2022) 1614–1622, https://doi.org/10.1002/dta.3330.

[19] R. Jain, R. Singh, Microextraction techniques for analysis of cannabinoids, Trends Anal. Chem. 80 (2016), https://doi.org/10.1016/j.trac.2016.03.012.

[20] M. Cobo-Golpe, A. De-Castro-Ríos, A. Cruz, M. López-Rivadulla, E. Lendoiro, Determination and distribution of cannabinoids in nail and hair samples, J. Anal. Toxicol. 45 (2021) 969–975, https://doi.org/10.1093/jat/bkaa164.

[21] V.D. Schaefer, V.V. Müller, L. de L. Feltraco Lizot, R.Z. Hahn, A. Schneider, M. V. Antunes, R. Linden, Sensitive determination of 11-nor-9-Carboxy-Δ9-Tetrahydrocannabinol and complementary cannabinoids in hair using alkaline digestion and Mixed-Mode solid phase extraction followed by Liquid-Chromatography-Tandem mass spectrometry, Forensic Sci. Int. 328 (2021) 111047, https://doi.org/10.1016/j.forsciint.2021.111047.

[22] E. Gerace, S.P. Bakanova, D. Di Corcia, A. Salomone, M. Vincenti, Determination of cannabinoids in urine, oral fluid and hair samples after repeated intake of CBD-Rich cannabis by smoking, Forensic Sci. Int. 318 (2021) 110561, https://doi.org/10.1016/j.forsciint.2020.110561.