

Method of confirmation by LC-MS/MS

INTRODUCTION

In the early 80 's many studies have been carried out on the determination of substances of abuse in keratinous matrix and then use of hair analysis as a means of previous use of these substances. Once incorporate into the hair, by the systemic circulation and by the contribution of sweat and sebum, substances of abuse remain impounded in the keratinous matrix in a stable way over time as a function of their lipophilicity, molecular weight, the pKa and the steric encumbrance. The analysis of substances of abuse in keratinous matrix is an ideal addendum to the analysis of blood or urine as it provides information regarding an earlier time than that covered by other biological matrices and refers to a longer time period (one or more months) as a function of the length of the hair.

Considering an average growth of 1 cm per month, you can learn the history of the consumption by analyzing segmented hair per cm or fractions of cm. It is also possible to highlight external contamination by examination of washing solvents by comparing them with the results obtained by digested hair. The search for substances of abuse in hair can be used to test use, abuse and misuse over time and to determine intensity and its history and then provide analytical data with medico-legal value. Determination in hair may be required in the following cases: deaths related to substances of abuse, assessment of ineligibility for driving, criminal liability, reliance custody of infants, prenatal exposure to substances of abuse and finally as confirmation of sporadic consumption or a dependency in workers.

Immunochemical tests can be used as first screening methods to analyze in a short time a large number of samples in a standardized, efficient and economical way to determine negative samples, i.e. samples that not contain Δ^9 -Tetrahydrocannabinol and its metabolite whose concentration is below a threshold value (cut-off).

Confirmation methods are used to verify false positive due to the non-specificity of screening tests. In the case of keratinous matrix, also no proper first screening tests can be performed. The "Society of Hair Testing" (SOHT) recommends the use of chromatographic separative methodologies coupled to chromatographic separative disclosure in mass spectrometry for the determination of substances of abuse in keratinous matrix for medical-legal purposes and/or clinical trials.

According to the guidelines of SOHT, this method allows to determine delta-9-THC and its metabolite delta-9-THC-COOH upon extraction from keratinous matrix and subsequent liquid-liquid extraction to eliminate the presence of complex matrix interferences.

Finally, the determination in Liquid chromatography/mass spectrometry (LC/MS/MS) takes places.



N° 009	Delta-9-THC and Delta 9 THC-COOH in hair by LC/MS	11/2025
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TECHNICAL FEATURES

Principle of the Method :

This method determines delta-9-THC and its metabolite delta-9-THC-COOH after hydrolysis of the keratin matrix, SPE extraction and LC-MS/MS in reading.

The method was validated according to the criteria established at the international level by the FDA and by the ICH.

ANALYTE	(Correlation Coefficient R ²) ± DS	LOD (ng/mg)	LOQ (ng/mg)
Delta-9-THC	0,9993 ± 0,0007	0,01	0,02
Delta-9-THC-COOH	0,9998 ± 0,0002	0,001	0,002

ANALYTE	Intra-Day Accuracy			Intra-Day-Precision			Inter-Day-CV%		
	(Error %)			(CV%)			(Error %)		
	Calibrator			Calibrator			Calibrator		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Delta-9-THC	8,11	5,76	6,43	6,49	6,67	6,97	7,91	5,76	6,1
Delta-9-THC-COOH	11,1	8,99	9,33	9,26	8,67	10,42	10,62	8,99	9,35

ANALYTE	Recovery (%)		
	Calibrator		
	Low	Medium	High
Delta-9-THC	91,1	62,9	75,1
Delta-9-THC-COOH	61,9	74,9	66,8

The table below shows for each analyte concentrations corresponding to the calibrators whose values Low, Medium and High cited above refer.

ANALYTE	Concentration (ng/mg)		
	Calibrator		
	Low	Medium	High
Delta-9-THC	0,1	1	10
Delta-9-THC-COOH	0,005	0,05	0,5

Components of the kit (50 test) :

Reagent A – Diluting Solution, **1 x 10 ml**

Reagent B – Digestion Solution, **1 x 50 ml**

Reagent C – Deuterated Internal Standards, **1 x 0,2 ml**

Reagent D – Hair Wash Solution, **1 x 250 ml**

Reagent S – Blank Hair, **1 x 2000 mg**

Reagent F – Chemical Standard Sol., **1 x 500 µl**

Reagent G – Extraction Solution, **1 x 420 ml**

Reagent H – Stabilization Solution 1, **1 x 10 ml**

Reagent I – Stabilization Solution 2, **1 x 10 ml**

Reagent L – Acidificant Solution, **1 x 2 ml**

Reagent M1 – Mobile Phase M1, **1 x 500 ml**

Reagent M2 – Mobile Phase M2, **1 x 500 ml**

All the reagents are ready to use and stable for 3 years at 2-8 °C, except Reagents C and F stored at -20 °C.

See Warnings
Store at -20 °C

Code CC53016
(Packed separately – see data sheet)

Minimum Instrumental equipment required:

Instrument LC-MS/MS triple quadrupole (mode MRM)
Interface ESI (+)
Binary Pump
Solvent evaporation system (dryer)
Operational Computer

Hair collection procedure:

Recommended length: 5 cm, starting from the scalp. A strand is cut, not torn (the bulb has no purpose at the end of these investigations) in the region of the vertex back of the head, of at least 200 mg which is divided into 2 aliquots of similar weight (A and B) each of which is fixed to the proximal end. These aliquots are placed in separate non-transparent containers with airtight caps, sealed with irremovable tape and stored at room temperature. The hair must be cut with scissors that have been cleaned with a small amount of alcohol and well dried.

Hair collection procedure :

And it is necessary to cut 200 mg of hair from the pubic region; the hair thus collected is divided into two aliquots (A and B).

ANALYTICAL PROCEDURE :

A) PREPARATION of unknown samples

STEP 1 : Wash hair intact inside a glass tube of 10 ml with 2 ml of **Reagent D – Hair Wash Solution**

Seal the tube with the cap

Vortex for 1 minute

Centrifuge at 3.500 rpm for 5 minutes

Open glass tube

STEP 2 : Evaporate to dryness the solvent with the aid of a light stream of helium or nitrogen paying attention to remain in the hair / fur on the bottom of the tube. Alternatively remove with a clean forceps with Acetone (not supplied in the kit), the hair / fur from the tube and place on paper towels until completely dry. In this last case the blotter paper should be changed for each sample.

Repeat steps 1 and 2 for two consecutive

STEP 3 : Cut your hair, washed and dried, in segments of 1-2 mm with scissors cleaned with Acetone (not supplied in the kit)

STEP 4 : Weigh 50 mg of sample in a Pyrex glass tube with screw cap clean and previously named.

B) PREPARATION of CALIBRATORS ON 5 LEVELS

Preparation of the Standard Chemical:

Prepare 1 glass tube, called A and dilute the Chemical Standard (**Reagent F**) with **Reagent A – Diluting Solution** as reported in tab. Below:

Tubes	<u>Reagent F –</u> Chemical Standard Sol.	<u>Reagent A –</u> Diluting Sol.
A	20 µl	1.980 µl

Close the tubes – Vortex for 1 minute

Prepare 6 glass tubes of 10 ml called C0-C1-C2-C3-C4.

Add at each tube (C0-C1-C2-C3-C4-C5) the standard chemical just prepared referring to the table below:

Calibrator C0	Calibrator C1	Calibrator C2	Calibrator C3	Calibrator C4	Calibrator C5
20 µl Reagent A – Diluting Sol.	40 µl of A	100 µl of A	500 µl of A	20 µl of Chemical Standard (Reagent F)	40 µl of Chemical Standard (Reagent F)

Evaporate to dryness with the aid of a stream of helium or nitrogen (in this step the temperature must be < 40 °C).

Add to each tube of the calibrators 50 mg of **Reagent S – Blank Hair** (supplied in kit).

At this point Calibrators of 6 points are ready and the concentrations to be used for the calibration curve will be:

Analyte	Calibrator C0	Calibrator C1	Calibrator C2	Calibrator C3	Calibrator C4	Calibrator C5
Delta-9-THC	0 ng/mg	0.02 ng/mg	0.05 ng/mg	0.25 ng/mg	1 ng/mg	2 ng/mg
Delta-9-THC-COOH	0 pg/mg	0.2 pg/mg	0.5 pg/mg	2,5 pg/mg	10 pg/mg	20 pg/mg

C) PREPARATION OF INTERNAL STANDARDS

Reagent C – Internal standard Sol supplied in the kit should be diluted 1:10 with water before each use, in the necessary amount for the entire assay.

ES. For an analytic session with 20 unknown samples and 5 calibrators are required 500 µl of each Standard Internal dilution (**diluted Reagent C**). Therefore you fetch 50 µl of **Reagent C – Internal standard Sol** and is diluted respectively with 450 µl of H₂O in a glass tube. Vortex for at least 10 sec. The solutions are now ready for use.

The **Reagent C – Internal standard Sol** undiluted and not used are closed again tightly and kept to the residual quantity in the freezer at -20°C.

D) DIGESTION OF KERATINIC MATRIX

STEP 5 : In each tube previously prepared (phase A and phase B) dispense:

	Calibrator C0 (prepared in STEP B)	Calibrator C1 (prepared in STEP B)	Calibrator C2 (prepared in STEP B)	Calibrator C3 (prepared in STEP B)	Calibrator C4 (prepared in STEP B)	Controls	Samples (prepared in STEP A)
Reagent C diluted – (Internal Std Sol. Diluted as shown in STEP C)	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl
Reagent E – Digestion Solution	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl

Seal the tubes with the cap – without invert gently mix the vial for 10 seconds

STEP 6 : Incubate at 90 °C for 1 hour

Cooling at Room Temperature

E) EXTRACTION OF DELTA-9-THC

- 1) At every tube called THC and prepared in STEP 6 add 4 ml of Reagent G – Extraction Solution

Seal the tubes with the cap – mix the vial for 10 seconds on stirrer

Vortex for 30 sec

Centrifuge at 3.500 rpm for 5 min

- 2) Transfer all the supernatant (organic phase) in CLEAN glass tubes called C0, C1, C2, C3, C4, C5, Sample 1, Sample 2, etc.
(N.B. Do not throw the water phase remained in the tube!)

F) EXTRACTION OF DELTA-9-THC-COOH

- 1) At the aqueous solution left after the extraction of delta-9-THC (STEP E, POINT 1) add 30 µl of Reagent L – Acidificant Solution

Vortex for 10 sec

- 2) Add to each tube 4 ml of Reagent G – Extraction Solution

Seal the tubes with the cap

Vortex for 1 min

Centrifuge at 3.500 rpm for 10 min

- 3) Transfer all the supernatant (organic phase) in glass tubes called C0, C1, C2, C3, C4, C5 Sample 1, Sample 2, etc. to which had been added the previous step in the organic phase (STEP E, POINT 2), resulting in a total volume of approximately 8 ml
- 4) Evaporate all the tubes to dryness with the aid of a stream of helium or nitrogen (in this step the temperature must be < 40 °C).

G) DILUTING AND INJECTION

- 1) Add to each tube (dry discharge) 200 µl of Reagent H – Stabilization Solution 1

Vortex for 10 sec

- 2) Add to each tube 200 µl of Reagent I – Stabilization Solution 2

Vortex for 30 sec

- 3) Inject 10-20 µl of solution in LC system

The solution is stable for 24 hours at 2-8 °C

GENERAL CONSIDERATIONS about QUANTITATIVE CALCULATION OF UNKNOWN SAMPLES

The calculation of the unknown samples is performed as a function of the equation obtained for the calibration curve where the abscissa axis shows the concentration in ng/mg and along the ordinate the ratio between the areas of the analyte/internal standard. To calculate the concentration in ng/mg hair in the case of unknown samples, please note that once you have the value of concentration on the line, it should be multiplied by the mg of hair that has built the calibration curve (50 mg) and divided by the actual weight of the sample under examination (eg. 10 mg if that is the weight of hair).

DELTA-9-THC AND DELTA 9 THC-COOH IN HAIR BY LC/MS - Warnings

REAGENT C: DEUTERATED INTERNAL STANDARDS

Solution contains Delta-9-THC-D3, Delta-9-THC-COOH-D9	
Abuse Drugs:	Internal standard to be used for calibration:
DELTA-9-THC	Delta-9-THC-D3
DELTA-9-THC-COOH	Delta-9-THC-COOH-D9
Conservazione: store at – 20 °C	

COLUMN CONDITIONING

Install a new analytical column Accucore C18 (30 x 2.1 mm, 2.6 µm) or Zorbax RRHD C18 (50 x 2.1 mm, 1.8 µm), both thermostated at 50°C. Disconnect the detector and filter a solution of H₂O: ACN or MeOH (10 : 90 v/v), set flow at 300 ul / min for 15 min. **Don't recycle the washing solutions.** Condition the column with a solution of Mobile Phase M1: Mobile Phase M2 (95 : 5 v/v) set flow at 300 ul / min for 15 min.

It ISN'T possible to make analysis at recycling phase

If room temperature is > 20 °C store the Mobile Phase at 2-8 °C between an analytical session and another.

COLUMN CLEANING

Disconnect the detector. Flux a solution made of H₂O : ACN or MeOH (5 : 95 v/v) for 15 min at 300 ul/min and discharge. Store in a solution of H₂O : ACN or MeOH (5 : 95 v/v).

INJECTION NEEDLE WASH

Wash the syringe before each injection with Methanol.

PARAMETERS SET ON TRIPLE QUADRUPOLE SCIEX 4500

Cannabinoids Parameters ESI Positive

Curtain Gas (CUR): 40
Collision Gas (CAD): 10
Ion Spray Voltage: (IS): 4500 V
Temperature (TEM): 650°C
Ion Source Gas 1 (GS1): 45
Ion Source Gas 2 (GS2): 55

Cannabinoids Parameters ESI Negative

Curtain Gas (CUR): 40
Collision Gas (CAD): 10
Ion Spray Voltage: (IS): -4500 V
Temperature (TEM): 600°C
Ion Source Gas 1 (GS1): 45
Ion Source Gas 2 (GS2): 55

FRAGMENTATIONS

ANALYTE	PARENT ION
Delta 9-THC	315.2 (+)
delta 9-THC-COOH	343.1 (-)
DEUTERATED INTERNAL STANDARDS	
Delta 9-THC-D3	318.2 (+)
Delta-9-THC-COOH-D9	352.1 (-)

GRADIENT FOR HPLC SYSTEMS (ACCUCORE C18)

Time (min)	% M1 (PUMP A)	% M2 (PUMP B)	Flow (µl/min)
0	95	5	400
0.30	95	5	400
1.00	40	60	400
3.00	5	95	400
4.30	5	95	400
4.31	95	5	400
5.30	95	5	400

GRADIENT FOR UHPLC SYSTEMS (ZORBAX RRHD C18)

Time (min)	% M1 (PUMP A)	% M2 (PUMP B)	Flow (µl/min)
0.00	60	40	500
0.30	60	40	500
0.35	40	60	500
3.00	0	100	500
4.00	0	100	500
4.01	60	40	500
5.00	60	40	500

ACCESSORIES AND CONSUMABLES

CODE	DESCRIPTION	PACKAGING
CC53016	Calibrator in hair for delta-9-THC and delta-9-THC-COOH	1 x 2000 mg/ 1 x 0,5 ml / 1 x 10 ml
CC53019	Control in hair for delta-9-THC and delta-9-THC-COOH – Levels 1 and 2	1 x 1000 mg/ 1 x 0,5 ml / 1 x 12,5 ml
Z51843550	Glass vials of reduced volume	1 x 100 Pcs
Z51820717	Caps for glass vials of reduced volume	1 x 100 Pcs
Analytical Column for HPLC systems		
TF17126032130	Accucore C-18 Analytical Column (30 m x 2.1 mm – 2.6 µm)	1 Pc
Analytical Column for UHPLC systems		
Z959757902	RRHD C18 Analytical Column (50 x 2,1 mm. 1.8 µm)	1 Pc

Delta-9-THC IN HAIR

(Reference Chromatograms/Spectra)

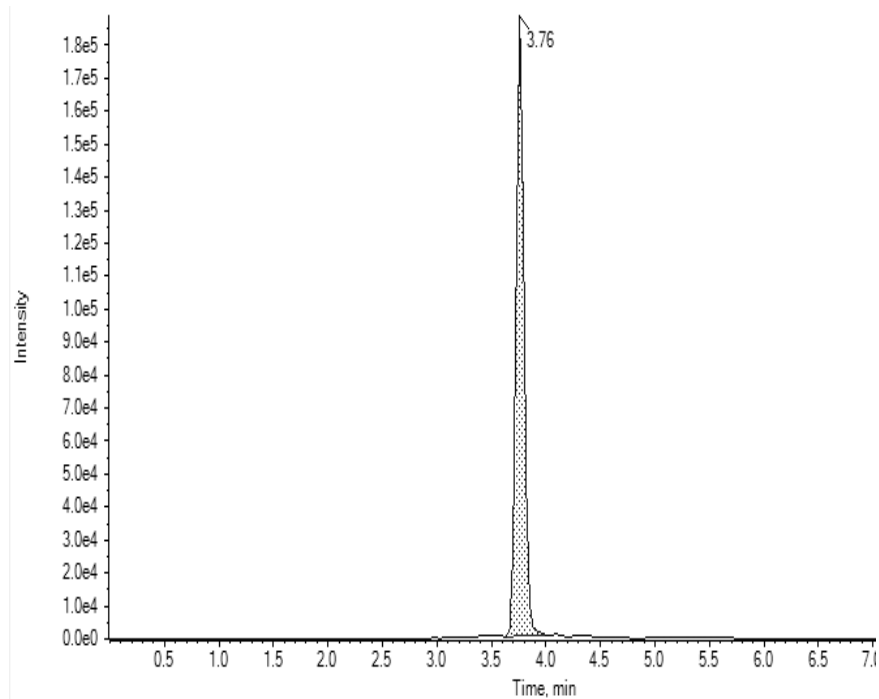


Fig. 1 :	Hair Calibrator
	R.T. 3.76 Delta-9-THC

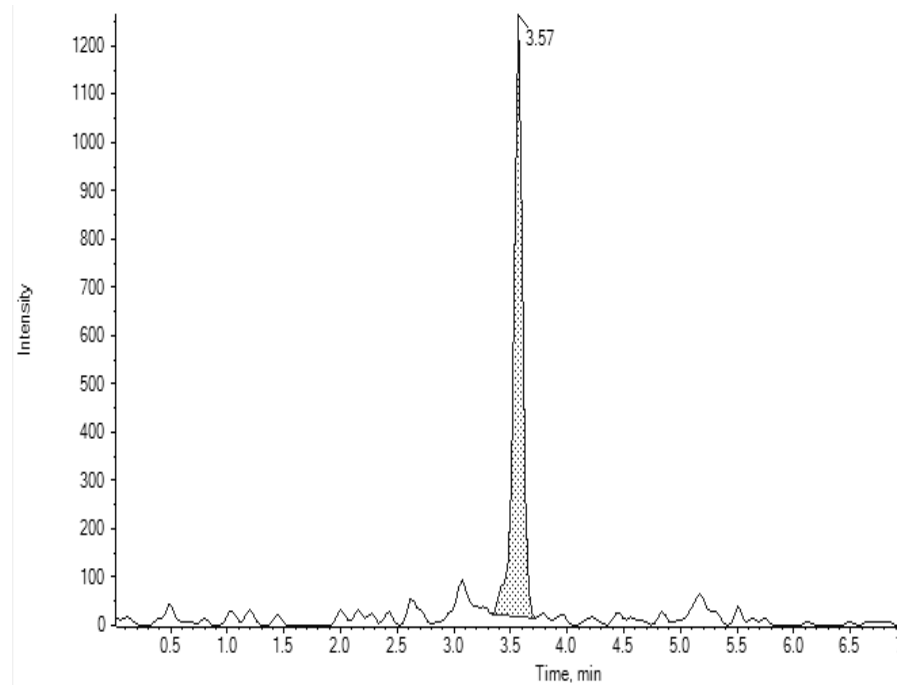


Fig. 2 :	Hair Calibrator
	R.T. 3.57 Delta-9-THC-COOH