

# Fast Confirmation for Marijuana Metabolite: THC-COOH, Ultra-Fast LC-MS/MS Run Time, and Application to Routine Samples

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**Summary.** A fast, reliable, inexpensive, and practical method with a low determination limit and high recovery has been developed for the determination of the marijuana metabolite in routine analysis. THC-COOH in urine was validated using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Before an easy single-step extraction with Toxi-Tubes, basic hydrolysis was performed at 60 °C for 30 min. LC-MS/MS analysis takes 2.5 min for each sample, and the retention time of the analyte is 1.75 min. Specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, repeatability, and intermediate precision (inter-day) system suitability parameters were determined in the validation study. The recovery of the extraction method was 88.67 (±5.91). LOD and LOQ values were 1.41 and 5.00 ng mL<sup>-1</sup>, respectively. The method showed linear response between the values 5.00 and 500.00 ng mL<sup>-1</sup>. The repeatability was 9.64% (relative standard deviation, RSD%), and the intermediate precisions (RSD<sub>R</sub>%) were 10.73%, 13.74%, and 8.11% at 10.00, 100.00, and 200.00 ng mL<sup>-1</sup> concentration levels, respectively. No statistically significant difference was found in ANOVA analysis, between three consecutive days in intermediate precision study, for 90% confidence level. HorRat values were between 0.34 and 0.61. The method was applied to CEDIA positive samples, obtained from the Trabzon Group Presidency of Turkish Council of Forensic Medicine, successfully.

**Key Words:** LC-MS/MS analysis of THC-COOH, cannabinoids, urine, validation, HorRat

## Introduction

Cannabis is the most widely used drug in the world because of its effects of relieving and altering state of consciousness and its price [1]. Cannabinoids are the natural components of marijuana (Cannabis) [2]. The most impor-

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tant cannabinoids are  $\Delta^9$ -THC and  $\Delta^8$ -THC [3]. The effect of Cannabis depends on the amount of  $\Delta^9$ -THC which exists in 20 times more in amount than  $\Delta^8$ -THC. Despite of the very low concentration of  $\Delta^9$ -THC in urine, the maximum psychologic effect lasts for 4–6 h.  $\Delta^9$ -THC metabolizes very quickly. Firstly, it is converted to its active metabolite 11-nor- $\Delta^9$ -hydroxy-THC (11-OH-THC) through hydroxylation, and then, it is oxidized to form inactive 11-nor- $\Delta^9$ -carboxy-THC (THC-COOH) which is conjugated with glucuronide, existing in both the free and glucuronide form and mainly excreted in urine [4].  $\Delta^9$ -THC metabolites may be detected in urine for up to 12 days following a single oral dose. THC-COOH is the main metabolite and the only one that is essential to perform cannabis urine test [5]. THC-COOH concentrations greater than 1.50 ng mL<sup>-1</sup> in urine are compatible with the marijuana taken in past 8 h [6]. THC-COOH concentrations in abusers are observed in high concentrations like 2705.0 ng mL<sup>-1</sup>. U.S. Ministry of Defence has evaluated the values  $\geq 15.0$  ng mL<sup>-1</sup> as positive [7].

High-performance liquid chromatography (HPLC) [8, 9], thin-layer chromatography (TLC) [10], and immunoassay techniques [11] are used in the screening of THC-COOH metabolite. Many analytical techniques such as gas chromatography–mass spectrometry (GC-MS) [12–14] and liquid chromatography–tandem mass spectrometry (LC-MS/MS) [5, 15, 16] were used in the confirmation. Since there is a risk of cross-reactions and false-positives in immunoassay methods [11], alternative methods are recommended for confirmation. GC-MS was the most widespread analytical confirmation method. However, concentration losses for THC-COOH may occur during derivatization procedure in GC-MS assays. Since there is no need for the analytes to be transformed into volatile form in LC-MS/MS analysis, analyte loss due to derivatization is not in question.

There are methods developed with sample preparation techniques like liquid–liquid extraction [17], solid-phase extraction [5, 18, 19] used for THC-COOH determination in the literature, with low detection and quantification limits. Most of them have relatively long sample preparation or chromatographic analysis times. There is a fast determination method with classical LC-MS/MS instrument and column (3.5 min run time) and low detection limit; however, phosphoric acid was used in hydrolysis procedure at 80 °C, and the matrix was directly given to LC-MS/MS after centrifugation without any cleanup procedure [20]. Introduction of a new rapid method without phosphate use will prevent the risk of clogging the MS inlet capillary in routine analyses [21].

## Experimental

### Collection and Storage of Urine Samples (Cold Chain)

For the method development and validation, urine samples were collected from healthy volunteers, vortexed, and centrifuged. THC-COOH positive urine samples were obtained from the cases which are brought to Forensic Council of Turkey, Trabzon Group Headquarters. The samples were vortexed for 1 min, then centrifuged and decanted to a tube. Cut-off limit for THC-COOH was given as 50.0 ng mL<sup>-1</sup> by SEMPSHA, and the urine samples found in concentrations over this cut-off limit in CEDIA analysis were regarded as positive and collected for LC-MS/MS analysis. Real samples were kept in +4 °C approximately for 1 month, then brought to Istanbul University Institute of Forensic Sciences and kept at -20 °C, till the analysis time. The study was approved by the Local Ethics Committee of Istanbul University Cerrahpasa Medical Faculty.

### Instrumentation and Reagents

The LC-MS/MS system (Zivak Tandem Gold) consisted of an HPLC with a binary pump, a degasser, an autosampler, and a temperature-controlled column oven coupled to a triple quadrupole MS detector with an electrospray ionization (ESI) interface. Analyses were performed in ESI (-) mode. THC-COOH was analyzed at 45 °C on a reversed-phase column (Luna 3 $\mu$ m, C<sub>18</sub>, 50.0 × 3.00 mm, 100 Å, Phenomenex) with a guard column (4.0 mm × 2.0 mm, Phenomenex). A 2.5-minute pump program was developed using an isocratic elution applied with a flow of 0.30 mL min<sup>-1</sup>, where the mobile phase consisted of %20 A (aqueous 0.1 % formic acid) and %80 B (acetonitrile). Injection volumes were 10  $\mu$ L. The parameters were optimized for LC-MS/MS analysis as -5500 V for the needle voltage of the turbo ionspray source, -600 V for the shield voltage, 400 °C for drying gas temperature, 65 °C for API housing temperature, 55.0 psi for nebulizer gas pressure, 30.0 psi for drying gas pressure, 2.40 psi for CID gas pressure, 1600 V for detector, and 1.5 amu for SIM width. Analyses were performed by multireaction monitoring (MRM), using the precursor ion at *m/z* 345.0 and the fragment ion at *m/z* 299.0 (dwell-times: 0.100 s for each transition). Capillary voltage

was 70.0 V, and collision energy was 15.0 V. The retention time was 1.75 min. Shimadzu 902 Automatic analyzer (Roche-calibrated) was used for CEDIA analysis.

11-nor-9-Carboxy-tetrahydrocannabinol (THC-COOH) (Cerilliant Analytical Reference Standards, U.S.), sodium hydroxide, acetic acid, ethyl acetate, and hexane (Merck, Germany) were used for sample preparation. All reagents were of analytical grade. HPLC grade acetonitrile and HPLC grade methanol (Merck, Germany) were used for the mobile phase. Bidistilled water was obtained from Milli-Q equipment (Milli-Q, Germany).

### Sample Preparation

One milliliter of urine was spiked with 1.00 mL standard solution and vortexed, then 1.00 mL 1.0 N NaOH was added and revortexed. After 30 min hydrolysis at 60 °C, the samples were neutralized using 1.00 mL glacial acetic acid. Then, the samples were decanted to B type Toxi-Tubes and centrifuged for 10 min at 4000 rpm after 5 min vortex (extraction) procedure. The upper phases were separated and evaporated under N<sub>2</sub> atmosphere. The same hydrolysis and extraction procedure was applied for the blank and real samples. The extracts were dried under N<sub>2</sub> atmosphere and reconstituted in 1.00 mL methanol, and 10.0 µL was injected to LC-MS/MS.

During the method development, liquid-liquid extraction (LLE), solid-phase extraction (SPE), and Toxi-Tubes methods with 20-, 30-, and 60-minute hydrolysis procedure at 60 °C were investigated. The sample preparation method with Toxi-Tubes with the optimum hydrolysis time of 30 min which gave the best results was used.

### Validation

Validation was performed by means of specificity, linearity, LOD and LOQ, recovery, repeatability, and intermediate precision (inter-day and inter-analyst studies). The specificity is the ability of the method to measure the analyte response in the presence of all potential impurities [22], and it was assessed through comparing the chromatograms of blank methanol with a standard solution and blank urine sample with a spiked urine sample. To find out the linear range and mean recovery, 0.20, 0.30, 0.50, 1.00, 5.00, 10.00, 25.00, 50.00, 100.00, 200.00, and 500.00 ng mL<sup>-1</sup> THC-COOH standard solutions were analyzed directly three times and then they were spiked to 1.00 mL blank urine samples and analyzed using the developed method. A

matrix-matched calibration technique was used to overcome the matrix effects on the method. Three pre-extraction and three post-extraction spiked samples were prepared for each concentration level, and each sample was analyzed three times. The results were also used in LOQ determination according to Eurachem method [23]. Relative standard deviation (RSD%) values obtained from a minimum of six repeats for each spiked concentration level were plotted versus the corresponding concentrations. The concentration corresponding to 15.0% coefficient of variation (CV) was determined as the LOQ. The lower concentrations which had a higher CV than LOQ level were not included in the calibration graph. LOD was calculated using the standard deviation of nine analysis results of the samples with low analyte concentration ( $1.00 \text{ ng mL}^{-1}$ ). The slope of the calibration graph was used in LOD calculation:  $\text{LOD} = t \times s / \text{slope}$  ( $t_{95\%} = 3.3$ ) [24, 25]. LOD and LOQ values were calculated both for instrumental method and the whole method including the sample preparation in urine. The extraction recovery at seven concentration levels were determined through comparing the peak areas of the analyte in the samples that were spiked before sample preparation with those in the samples that were spiked after blank urine sample preparation. Repeatability and reproducibility (inter-day and inter-analyst) of the method were assessed using ANOVA and HorRat calculations at three concentration levels: 10.00, 100.00, and 200.00  $\text{ng mL}^{-1}$ . One-way ANOVA analysis was performed in three concentration levels using Excel.  $F_{\text{experimental}}$  values were compared to the  $F_{\text{critical}}$  values at 90% confidence level, to determine if significant variation existed between the days or analysts. Repeatability and inter-day precision ( $\text{RSD}_R$  %) values were calculated using ANOVA results. The HorRat values were calculated using  $\text{PRSD}_R = \text{predicted RSD\% (from the Horwitz equation)}$  and  $\text{RSD}_R = \text{experimentally measured RSD\% value for the selected concentrations}$  (AOAC Official Methods Of Analysis, 2012). Horwitz equation  $\text{PRSD}_R (\%) = 2^{(1-0.5\log C)}$  was used for the calculation of  $\text{PRSD}_R$ . In this equation,  $C$  is the concentration found or added, expressed as a mass fraction. All calculations were performed in Excel.

## Results and Discussion

In this study, an economical determination method with high recovery and low detection and quantification limits was developed and validated for Cannabis metabolite, THC-COOH, using a single Toxi-Tubes extraction and LC-MS/MS. The method was applied to 12 CEDIA-positive real samples which were among the cases under investigation in Turkish Council of Fo-

rensic Medicine Trabzon Group Headquarters. Hydrolysis was applied before the extraction. The peak area of 345 > 299 transition was observed in LC-MS/MS, for the analyte. A very fast (2.5 min) LC-MS/MS analysis method was developed. The MS cleavage of parent ion is given in Fig. 1.

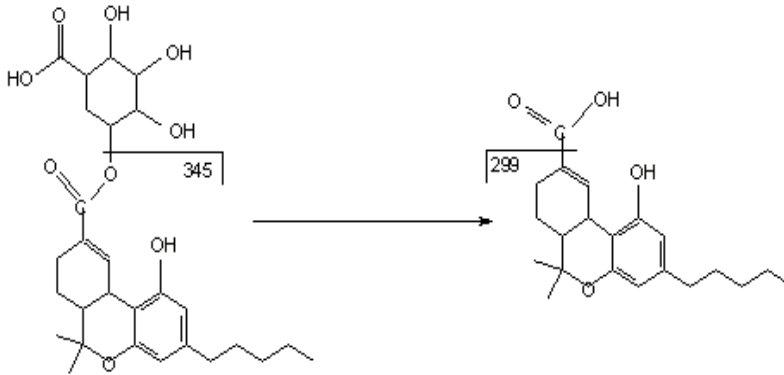


Fig. 1. The cleavage of parent ion to secondary ion in MRM transition of THC-COOH

Validation procedure of the method was performed by means of specificity/selectivity, linearity, accuracy (as recovery), LOD and LOQ, and precision (as repeatability and intermediate precision).

### Specificity and Selectivity of the Method

The THC-COOH and I.S. chromatograms of spiked urine were compared with blank methanol chromatogram and blank urine chromatogram which was extracted using the same procedure. There was no interference arising from the urine matrix and solvent, on the peaks of analyte and I.S. (Fig. 2). Also, the analyte and I.S. peaks were observed in their specific ion channels in different retention times. Thus, the method was proved to be specific and selective.

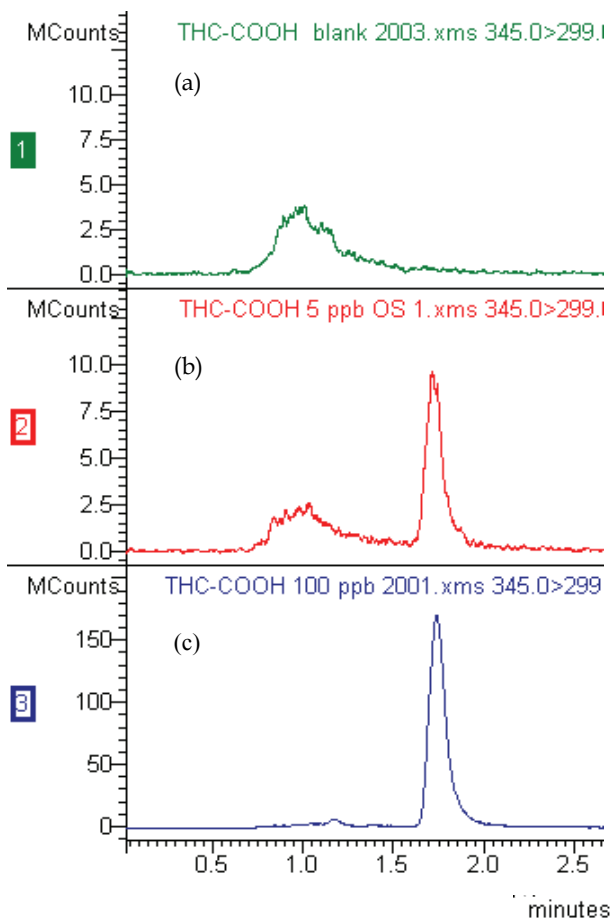


Fig. 2. Chromatograms of blank urine (a); urine samples including THC-COOH at LOQ level  $5.00 \text{ ng mL}^{-1}$  (b) and  $100.00 \text{ ng mL}^{-1}$  (c)

### Accuracy (Recovery)

Accuracy was tested by means of recovery. Pre- and post-extraction spiked samples in  $5.00$ ,  $10.00$ ,  $25.00$ ,  $50.00$ ,  $100.00$ ,  $200.00$ , and  $500.00 \text{ ng mL}^{-1}$  concentrations were analyzed, and a mean recovery of  $\%88.67 (\pm 5.91)$  was obtained (Table I).

Table I. Mean recoveries and standard deviations for 5.00, 10.00, 25.00, 50.00, 100.00, 200.00, and 500.00 ng mL<sup>-1</sup> concentration levels

THC-COOH spiked (ng mL <sup>-1</sup> )	THC-COOH found (ng mL <sup>-1</sup> )	Recovery %	Mean %
5.00	4.37 (±0.15)	87.32(±2.93)	88.67 (±5.91)
10.00	8.11 (±0.13)	81.09 (±1.26)	
25.00	21.21 (±0.38)	84.82 (±1.52)	
50.00	42.36 (±1.25)	84.72 (±2.51)	
100.00	90.07 (±2.33)	90.07 (±2.33)	
200.00	193.40 (±2.92)	96.70 (±1.46)	
500.00	479.90 (±7.24)	95.98 (±1.45)	

### Linearity and Linear Range

The standard solutions which were prepared at 11 concentrations between 0.20 and 500.00 ng mL<sup>-1</sup> were analyzed. The calibration graph and LOQ calculation using Eurachem graph have shown that the LC-MS/MS method was linear in the range of 0.25–500.00 ng mL<sup>-1</sup>. The equation of the calibration graph was  $y = 8.68 \times 10^6(\pm 1.00 \times 10^5)x - 1.30 \times 10^7(\pm 1.75 \times 10^7)$ , with an  $r^2$  value of 0.9989. After that, the calibration graph was constructed in urine matrix using these standard solutions and found to be linear in the range of 5.00–500.00 ng mL<sup>-1</sup>. The equation of the calibration graph was  $y = 4.39 \times 10^6(\pm 3.86 \times 10^4)x - 1.31 \times 10^7(\pm 8.04 \times 10^6)$ , with an  $r^2$  value of 0.9996.

### LOD and LOQ Determination

Standard solutions were analyzed to determine LOD and LOQ values of the instrumental method which were found as 0.15 and 0.25 ng mL<sup>-1</sup>. To find the LOD and LOQ values for the whole method including sample preparation, the extracted urine samples were analyzed and the corresponding LOD and LOQ values were found as 1.41 and 5.0 ng mL<sup>-1</sup>.



## Repeatability and Intermediate Precision

Three concentration levels were used for precision calculations: 10.00, 100.00, and 200.00 ng mL<sup>-1</sup>. Four samples were used for each concentration in repeatability and intermediate precision studies. The mean of three analysis results was calculated for each sample. One-way ANOVA was used. The experimental *F* values obtained at each concentration level were compared with the critical *F* values from the table. Then, the mean sum of squares for intra- and inter-day results, grand mean, and the number of results were used in the calculation of repeatability and intermediate precision. All RSD% values were ≤12.22% for repeatability and ≤13.74% for intermediate precision. The results for each concentration were given in Table II.

Table II. Results of repeatability and intermediate precision among 3 days

Spiked <sup>a</sup> (ng mL <sup>-1</sup> )	Found <sup>a</sup> (grand mean) (ng mL <sup>-1</sup> )	Repeatability (RSD%)	Intermediate precision (RSD <sub>r</sub> %)	ANOVA <i>F</i> <sub>experimental</sub>	RSD <sub>R</sub> Horwitz	HorRat value <sup>c</sup>
		Within-day	Inter-day			
10.00	10.06	9.57	10.73	1.84 <sup>b</sup>	32.00	0.34
100.00	97.35	12.22	13.74	0.14 <sup>b</sup>	22.63	0.61
200.00	189.93	7.15	8.11	0.08 <sup>b</sup>	20.39	0.40

<sup>a</sup>Concentration.

<sup>b</sup><*F*<sub>critical, 90%</sub>.

<sup>c</sup>Acceptable limits: 0.25–1.33, *n*<sub>days</sub> = 4, sample per day: *n* ≥ 3.

Horwitz Ratio (HorRat) was used for assessment of acceptability of intermediate precision calculations. Horwitz equation originated from the empirical observation that reproducibility (interlaboratory) relative standard deviations tended to be around 4% when the analyte-mass fraction was *c* = 0.01 (that is, at a concentration of 1% by mass) [26]. Moreover, RSD<sub>R</sub>% tended to double approximately for every reduction in analyte concentration by a factor of 100. This relationship can be expressed mathematically as RSD<sub>R</sub>% = 2<sup>(1-0.5log<sub>10</sub>C)</sup>. HorRat is now one of the acceptability criteria for many of the recently adopted chemical methods of analysis of AOAC International, the European Union, and other European organizations (e.g., European Committee for Standardization and Nordic Analytical Committee). The original data developed from interlaboratory (among-laboratory) studies assigned a HorRat value of 1.0 with limits of acceptability of 0.5 to

2.0 [27]. The corresponding within laboratory relative standard deviations were found to be typically 1/2 to 2/3 of the among-laboratory relative standard deviations. HorRat values in this study were between 0.34 and 0.61, which were in acceptable limits (0.25–1.33) for intermediate precision (Table II). These values show that the experimental intermediate precision results in this study are in the acceptability range (Table II).

### Confirmation of the CEDIA (+) Results of the Real Samples with LC-MS/MS

After the validation of the method, 12 samples which were sent to Turkish Council of Forensic Medicine Trabzon Group Headquarters by the prosecution authorities and courts were analyzed using immunological analysis (CEDIA). The samples which were regarded as positive according to the cut-off value of the CEDIA instrument ( $50.00 \text{ ng mL}^{-1}$ ) were analyzed using the developed method. The LC-MS/MS results of these CEDIA positive samples are shown with their standard deviations and RSD% values in Table III.

Table III. LC-MS/MS results of these CEDIA positive samples which were obtained from the forensic cases and their RSD% values. Standard deviations are given in parentheses

Sample no.	CEDIA results <sup>a</sup> >50 ng mL <sup>-1</sup>	LC-MS/MS results <sup>b</sup> (ng mL <sup>-1</sup> )	RSD%
1	+	7.85 ( $\pm 0.82$ )	10.45
2	+	311.13 ( $\pm 19.90$ )	6.40
3	+	378.88 ( $\pm 36.35$ )	9.59
4	+	166.68 ( $\pm 9.16$ )	5.50
5	+	70.84 ( $\pm 10.12$ )	14.29
6	+	138.29 ( $\pm 3.02$ )	2.18
7	+	164.21 ( $\pm 16.50$ )	10.05
8	+	229.29 ( $\pm 28.73$ )	12.53
9	+	1006.78 ( $\pm 98.14$ )	9.75
10	+	46.43 ( $\pm 5.28$ )	11.37
11	+	241.54 ( $\pm 12.77$ )	5.29
12	+	11.58 ( $\pm 0.91$ )	7.86

<sup>a</sup> $n \geq 3$ .

<sup>b</sup> $n \geq 6$ .

While GC-MS seems to be the most used technique for the confirmation of the results of immunoassay analyses in the literature, because of its disadvantages as probable analyte losses during derivatization, health risk of derivatives, and higher limits of quantitation, LC-MS/MS is more advantageous in determination of THC-COOH.

Urine is a more preferable matrix for marijuana analysis, and its collection is easy and non-invasive. In the literature, its hydrolysis is performed through enzymatic [28] or basic hydrolysis [27] of the ester-glucuronide bond. Thirty minutes of basic hydrolysis was used in this study, which provides a fast sample preparation and very fast chromatographic elution time which is comparable with the elution times of analytes in ultra-performance liquid chromatography (UPLC)-MS/MS. There is an online SPE method for analysis of  $\Delta^9$ -THC and its metabolites in urine [29]. Chromatographic run time was 10 min. Since the availability of online SPE system, which is very expensive, is limited in routine laboratories, the present fast manual method with 2.5 min LC-MS/MS run time is still an attractive alternative for THC-COOH analysis. In a study with fast sample preparation [15], THC-COOH and THC-COOH-glucuronide analysis was performed in diluted urine without hydrolysis. However, the chromatographic elution time was 15.7. Montesano et al. [5] have determined THC-COOH, THC-OH, and two cannabinoids in urine using SPE after hydrolysis with  $\beta$ -glucuronidase and a total chromatographic run time of 5.8 min and the hydrolysis time was 2 hours. In another study [16], THC-COOH and THC-COOH-Gluc was determined in urine, using LLE. The hydrolysis time was 5 h and the chromatographic run time was 26.8 min for THC-COOH. There is a fast LC-MS/MS determination method in the literature with 3.5 min run time and low detection limit [20]; however, phosphoric acid was used in hydrolysis procedure at 80 °C, and the matrix was directly given to LC-MS/MS after centrifugation. Sulic et al. [30] used LLE and LC-MS in determination of THC-COOH in urine. The LOD and LOQ were 2.65 and 8.82 ng mL<sup>-1</sup>. The retention time was 21.54 min. A UPLC-MS/MS method with gradient elution was developed in another study [19]. The time was 1 h for enzymatic hydrolysis. SPE was applied. Recoveries, LOD, and LOQ values were not given for THC-COOH. Elution time was given as 6.34 min. Molecularly imprinted polymers were used in another study, where extraction time was more than 65 min [4]. In another study, Kwon et al. [31] have developed and validated a liquid chromatography-electrospray ionization-tandem mass spectrometric method (LC-ESI-MS/MS) for the direct determination of THC-COOH and its glucuronide in urine. The speed of extraction was the same; however, probably, since minimum solvent amounts were used,

the RSD% for intra- and inter-day precisions was >15%. The chromatographic run time is shorter, and linearity range is wider in our study. In a UPLC-MS/MS study where a UPLC column was used, a 1.55 retention time was achieved with a gradient elution [32]. Fast sample extraction columns were used after hydrolysis. However, LOD and LOQ values were higher in our study (5 and 10 ng mL<sup>-1</sup>). In the present study, the extraction time for THC-COOH was much shorter with Toxi-Tube B (which has not been encountered for THC-COOH determination, in the literature) and the chromatographic elution time is faster, almost as the elution time of the UPLC-MS/MS methods.

## Conclusion

No study with ≤2.5 min chromatographic run time and Toxi-Tube use has been encountered for THC-COOH, in the literature. The easiness of Toxi-Tube use, the ultra-fast chromatographic analysis time, very economical, fast, and a novel method with alkaline hydrolysis use, high recoveries, low quantitation limit, a comprehensive validation, and successful application in real CEDIA(+) samples have provided the method to be suitable for routine laboratories.

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