










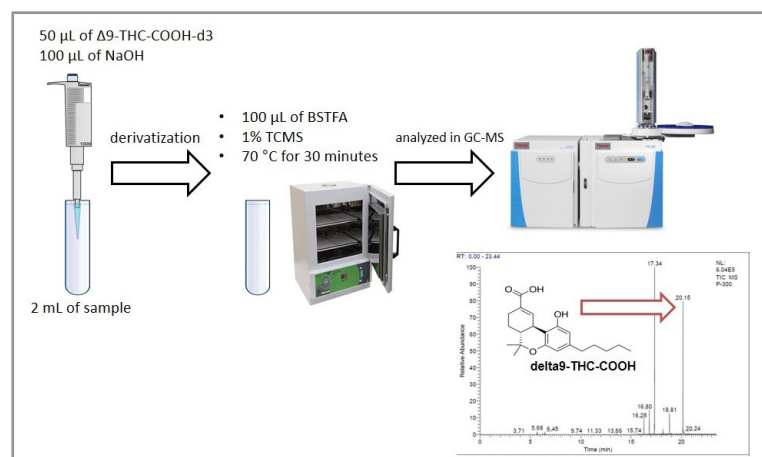
TECHNICAL NOTE

Quantitative analysis of Δ^9 -THC-COOH in Human Urine by the Liquid-Liquid Extraction technique and Gas Chromatography-Mass Spectrometry: Adaptation, Optimization and Validation

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The main active compound of *Cannabis sativa* is Δ^9 -tetrahydrocannabinol, which is quickly transformed into 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (Δ^9 -THC-COOH) in the human body. This research aimed to validate an efficient, fast and low-cost technique for Δ^9 -THC-COOH analysis in urine with adaptations of existing analytical methods. The validation process was carried out in accordance with guidelines published by ANVISA and with international guidelines. The analyte was extracted by liquid-liquid extraction and identified/quantified by gas chromatography

coupled to mass spectrometry. Linear curves ranges were from 5 to 300 ng mL⁻¹ ($r = 0.9993$; $y = 0.0269x - 0.0364$). Intra and inter-day precision varied from 3.38 to 9.04% and accuracy was between 83 to 112.9%. The Δ^9 -THC-COOH remained stable after 15-30 days of storage at -20 °C (long-term test), after 5 freeze-thaw cycles and post-processing for up to 72 hours. The method is fast, low-cost, with detection limits and quantification below the cut-off (15 ng mL⁻¹), which makes it useful and efficient for routine use at toxicology laboratories, for drug addiction and doping control, for forensic purposes and also for controlling the use of drugs of abuse by vehicle drivers.

Keywords: Δ^9 -THC-COOH; human urine; liquid-liquid extraction; gas chromatography-mass spectrometry.

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INTRODUCTION

Cannabis is the most consumed drug in the world. In Brazil, even though it is illegal, the scenario is not different. Data from reports published by the Alcohol and Drug Research Unit (UNIAD) state that *Cannabis* is the most consumed illegal substance in Brazil. 5.8% of the adult population report having used *Cannabis* at least once in life and 2.5% report having used it at least once in 2012 [1]. The main psychoactive compound of *Cannabis sativa* is Δ^9 -tetrahydrocannabinol (Δ^9 -THC), which can be quickly bio-transformed by liver enzymes into several by-products. One of them is 11-hydroxy- Δ^9 -THC. The oxidation of 11-hydroxy- Δ^9 -THC originates the inactive product 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (Δ^9 -THC-COOH). It can be conjugated with glucuronic acid and excreted in urine. Therefore, the identification of this analyte in urine is the best analysis procedure to check for an individual's exposure to the drug [2]. Δ^9 -THC-COOH is a major biotransformation product that can be identified in urine, blood and hair analysis by chromatography techniques. Most of the methods described for extraction of this analyte include mainly Solid Phase Extraction (SPE) and Liquid-Liquid Extraction (LLE), with separation and detection by GC-MS and LC-MS or LC-MS/MS techniques [3-10]. LLE is a method for isolating many drugs from biological matrices based on drugs separation between the aqueous phase (biological) and an organic extraction solvent. LLE has advantages over other techniques, once it is a cheap and simple operation. It is also a fast technique that provides good repeatability and high recovery of most cannabinoids [9].

Andrenyak et al. [11] have achieved improved performance characteristics using LLE and MTSFA derivatization for the determination of cannabinoids in human plasma when compared with assays that used SPE as their main procedure. In another study, González-Mariño et al. [12] developed a LLE technique for the determination of cannabinoid and synthetic cannabinoid metabolites in wastewater as a simple and fast alternative to SPE protocols.

LLE procedures can be optimized in forensic analysis. Purshcke et al. [13] optimized a classical LLE technique for analysis of cannabinoids in human blood serum with a fully automated workflow, achieving fast and reliable results.

Most of these techniques used blood or hair as biological samples for drug analysis. Urine has several advantages over other matrices, such as the fact that its collection is less invasive. Besides, it can be obtained in large quantities and presents good conservation and stability of the analytes, which allows freezing [14].

When investigating a sample for psychoactive substances, the *National Institute on Drug Abuse (NIDA)* recommends associating screening methods with confirmatory techniques, such as gas chromatography coupled to mass spectrometry (GC-MS) [15]. Although most current methods use high performance liquid chromatography (HPLC), GC-MS is a standard Gold technique for the analysis of drugs. Its consumables are cheaper than in other techniques, and its specificity, sensitivity and limit of detection are good [9]. Moreover, GC-MS is available at many toxicology laboratories.

To provide a reliable determination of a certain analyte, the validation of methods is indispensable. It serves the purpose of demonstrating that a specific analytical technique has the performance characteristics required. In Brazil, the National Health Surveillance Agency (ANVISA) published the RDC N° 27, May 17th, 2012, and the RCD N°166, Jun 27th, 2017, which are guidelines for the validation of analytical and bioanalytical techniques to be carried out properly [16,17]. The parameters assessed through validation are also in accordance with the United Nations Office on Drugs and Crime (UNODC) [18].

Thus, this work aimed to adapt, optimize and validate a method for the detection and quantification of Δ^9 -THC-COOH in human urine by GC-MS. The compound Δ^9 -THC-COOH was chosen for analysis because it is considered a biomarker of exposure to *Cannabis* products and the most prevalent metabolite in urine samples [19]. The LLE-GC-MS technique proposed in this work combines the simplicity, speed and low cost of LLE together with the availability, low cost and effectiveness of GC-MS.

MATERIALS AND METHODS

Materials

Standard solutions of Δ^9 -THC-COOH (1.0 mg mL⁻¹ in methanol), along with their deuterated internal standards Δ^9 -THC-COOH-d₃ (1.0 mg mL⁻¹ in methanol) were purchased from Cerilliant Corporation® (Round Rock, TX, USA). The sodium hydroxide solution was provided by Biotec- LabMaster Ltda® (Paraná, Brazil). Acetic acid was supplied by Labsynth® (Diadema, Brazil). Methanol, n-hexane, ethyl acetate were purchased from Merck® (Darmstadt, Germany). BSTFA (bis-trimethylsilyl-trifluoroacetamida) and TCMS (trimethylchlorossilano) were purchased from Sigma Aldrich®, Round Rock, Texas, USA. Deionized water was provided by Milli-Q system, Millipore® (Barueri, Brazil). Working solutions were prepared through adequate dilution of the stock solutions with methanol for final concentrations of 10 µg mL⁻¹. All solutions were stored in a freezer at -20°C.

GC-MS analysis

The analyses were carried out by using a TRACE 1300 GC System Gas Chromatograph coupled to a Thermo Scientific® ISQ Series quadrupole mass-selective detector (MSD) (Thermo Fisher Scientific, Milan, Italy), with the coupling of an AI 1310 automated analyzer. Separation of the analytes was done by using a capillary column with 5% of Phenyl Polysilphenylene-Siloxane (TR-5MS) (30 m x 0.25 mm x 0.25 µm), supplied by Thermo Scientific® (Milan, Italy). The temperature of the injector port was 280 °C, and the temperature of the interface was 250 °C. The oven ramp was set to initialize at 90 °C for 2 min, and then increase in 10 °C/min until reaching 220 °C, kept for 4 min and, then, increase again in 30 °C/min, reaching up to 290 °C, kept for 6 min. The whole process lasted approximately 23 min. The carrier gas (Helium) was adjusted to a constant flow rate of 1.0 mL/min, and 1 µL of the samples was injected in splitless mode. The mass spectrometer was operated in electron ionization mode (EI). Qualification and quantification of ions were performed in the selected ion monitoring (SIM) mode, and they were chosen based on selectivity and abundance, in order to maximize the signal-to-noise ratio in the extracts prepared. Three ions were monitored (the quantification ion is underlined): Δ^9 -THC-COOH *m/z* 371, 473 and 488.

Sample preparation

Urine samples free from the drugs (such as *Cannabis*) were provided by 10 volunteers who were nonusers. Five samples from drug abuse users were obtained by convenience sampling, as subjects were chosen according to sentinel events reports obtained via epidemiological monitoring programs at the University Hospital of Maringá (HUM) [20]. The study was approved by the Ethics Committee for Research on Human Beings from the State University of Maringá, under registration number 458.185.

Analytes were extracted by LLE. For that purpose, 2 mL of the sample, 50 ng mL⁻¹ of internal standard Δ^9 -THC-COOH-d₃ and 100 µL of NaOH 10% (v/v) were placed in 15 mL propylene tubes and taken to an incubator for hydrolysis at 60 °C for 20 minutes. The tubes were taken out of the incubator until reaching room temperature. Then, 2 mL of deionized water, 2 mL of acetic acid 10% (v/v) and 8 mL of extractor solvent were added to the tubes (n-hexane: ethyl acetate, 9:1, v/v), submitted to mechanical agitation for 30 minutes and centrifuged at 700 x g for 5 minutes. The supernatants were transferred to glass conical tubes and led to evaporate at 50 °C in water bath. For derivatization, 100 µL of BSTFA (bis-trimethylsilyl-trifluoroacetamide) and 1% TCMS (trimethylchlorossilane) were added to the dried residues, which were kept in an incubator at 70 °C for 30 minutes. The tubes were centrifuged at 448 x g for 3 minutes, the volumes were transferred to 2 mL vials, and 1 µL were injected and analyzed on GC-MS (Figure 1).

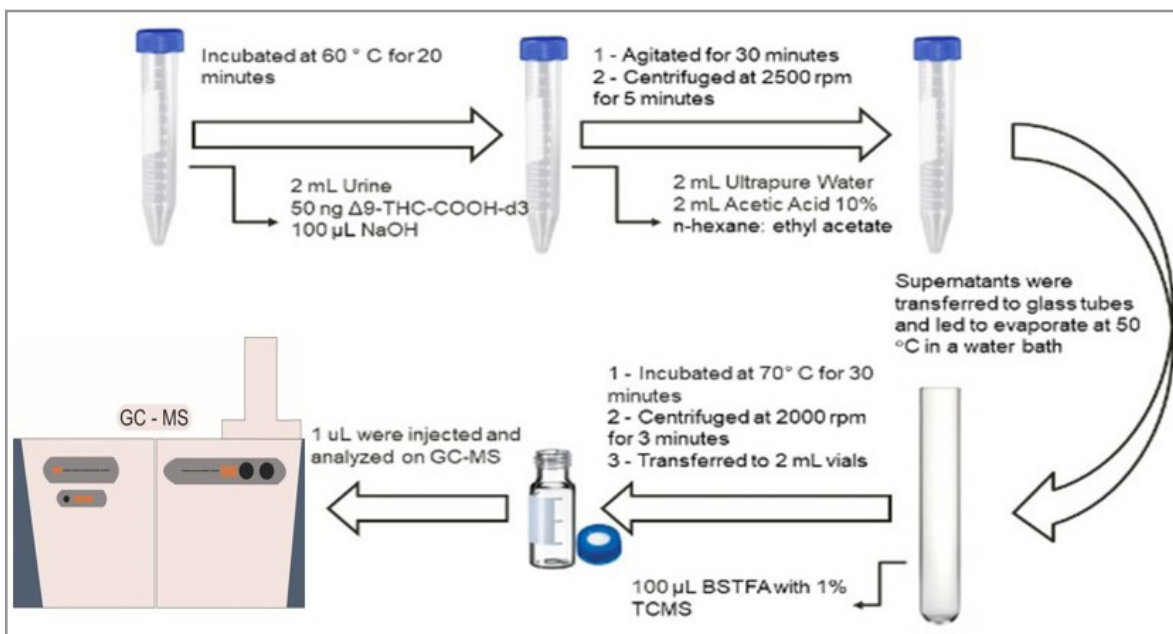


Figure 1. Sample treatment and LLE procedure for Δ^9 -THC-COOH analysis in urine.

Validation procedure

After the adaptations and optimization described, the method was validated in accordance with ANVISA and UNODC [16,18], comprising parameters such as selectivity, linearity, limit of detection, limit of quantification, precision, accuracy, matrix effect, carryover and stability.

Specificity / Selectivity

Specificity was assessed by evaluating the retention times (RT) of the peaks corresponding to the analytes evaluated. Two blank samples (with the addition of internal standards: 10 ng mL⁻¹ of Δ^9 -THC-COOH-d₃) were analyzed, in addition to 10 urine samples from different sources without the addition of internal standards to verify the presence of possible interferences. The presence or absence of any interfering peaks (endogenous substances), at a significant level, close to the analyte retention time and the internal standard, were assessed. The responses of interfering peaks close to the internal standard retention time must be less than 5% of the internal standard response [16].

Linearity

Linearity was accomplished with blank urine (pool sample) spiked with Δ^9 -THC-COOH standard solutions at different concentrations (5 ng mL⁻¹, 10 ng mL⁻¹, 15 ng mL⁻¹, 50 ng mL⁻¹, 150 ng mL⁻¹ and 300 ng mL⁻¹). The analyses of different concentrations, within the range established, were carried out in six repetitions. Calibration curves of the analytes were obtained by correlation between the signal response (area ratio of the analyte peak and the internal standard) and analyte concentration in the sample. Acceptance criteria included the correlation coefficient (r) above 0.99. A linear calibration model was used with 1/x weighting (inverse of the concentration), generally recommended for bioanalytical methods. When the error variance is not constant across the quantification range of the analytical method, it is necessary to use the weighting that has the lowest value to sum the relative errors of the nominal values of the calibration standards versus their values obtained by the curve equation. Application of the 1/x factor adequately compensates for the occurrence of heteroscedasticity [16].

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Determinations of the LOD and LOQ were done by fortifying different blank (urine) samples, analyzed in quadruplicates, using signal-to-noise ratio and visual evaluation methods, respectively [16]. The LOD was determined by verifying what was the lowest concentration assessed to have resulted in qualitatively chromatographic peaks (their magnitudes must be at least thrice higher than the noise peaks, signal/noise ratio $\geq 3:1$) [16]. Samples were fortified with decreasing concentrations of the analyte and assessed until no qualitatively chromatographic peaks were obtained. The LOQ estimated was considered to be the lowest concentration capable of obtaining detection (signal/noise ratio $\geq 10:1$), identification, accuracy and precision criteria in all fortified samples.

$$\text{LOD} = 3.3\sigma/S \qquad \text{LOQ} = 10\sigma/S$$

where σ = standard deviation and S = slope of the calibration curve

Intra-day and inter-day precision and accuracy

Intra-day precision was evaluated through the analysis, on the same day, of six replicates ($n=6$) of blank samples enriched with the analyte at three (3) control levels: low control (10 ng mL^{-1}), medium control (100 ng mL^{-1}) and high control (250 ng mL^{-1}). Inter-day precision was evaluated during three consecutive days, in five repetitions. Precision was calculated by using the coefficient of variation (CV). The acceptance criterion was $\leq \pm 15\%$ for all concentrations, except at the LOQ, in which $\leq \pm 20\%$ was accepted [16,18]. The values can be considered acceptable when varying $\leq \pm 20\%$ for the LOQ and $\leq \pm 15\%$ for all the other concentrations.

Accuracy was validated using 3 concentrations of the Δ^9 -THC-COOH standard (10 ng mL^{-1} , 100 ng mL^{-1} and 250 ng mL^{-1} , low, average and high concentrations, respectively), through the analysis during six consecutive days. It was calculated by using the percentage of the known concentration value (mean concentration measured/ theoretical concentration) $\times 100$. The values can be considered acceptable when varying from 80% to 120% for the lower limit of quantification and from 85% to 115% for all the other concentrations [16,18].

Matrix Effect

The matrix effect was determined by statistical evaluation of the slope coefficients of the calibration curves constructed with the analyte (standard) in solvent and with the sample (urine) fortified with the analyte (standard), with a level of significance of 5% (five percent) adopted in the hypothesis test [16]. The curve built in solvent was performed in deionized water, under the same conditions as the curve performed for linearity.

Stability

The stability parameter was evaluated in three situations: (1) long term, (2) freeze-thaw cycle and (3) post-processing [16]. In test (1), the time variation in which the matrix is stable when stored at $-20 \text{ }^\circ\text{C}$ was evaluated. For that, samples were analyzed at two concentrations: low (10 ng mL^{-1}) and high (250 ng mL^{-1}), after an interval of 15 and 30 days of storage. In test (2), resistance of the analyte was evaluated for its degradation under freezing-thawing cycles. Then, the low (10 ng mL^{-1}) and high (250 ng mL^{-1}) concentrations of the analyte were evaluated in samples after five cycles. Finally, test (2) evaluated the extracted samples, injected after 24, 48 and 72 hours at room temperature and without resuspension. Again, the analyte concentrations in the sample were low (10 ng mL^{-1}) and high (250 ng mL^{-1}).

Carryover

For the carryover evaluation, three injections of a single blank sample were made, one before and two after the injection of a sample at the highest point of the calibration curve (300 ng mL^{-1}). The results of the blank sample injections were compared with those obtained from the LOQ. The signal should not be detected at a concentration higher than the LOQ [16].

RESULTS AND DISCUSSION

The methodology of this study was based on a simple LLE technique with adaptations of existing methods [3,13]. The retention time obtained for Δ^9 -THC-COOH was 20.15 minutes, and three ions were monitored (the quantification ion is underlined): Δ^9 -THC-COOH m/z 371, 473 and 488 (Figure 2).

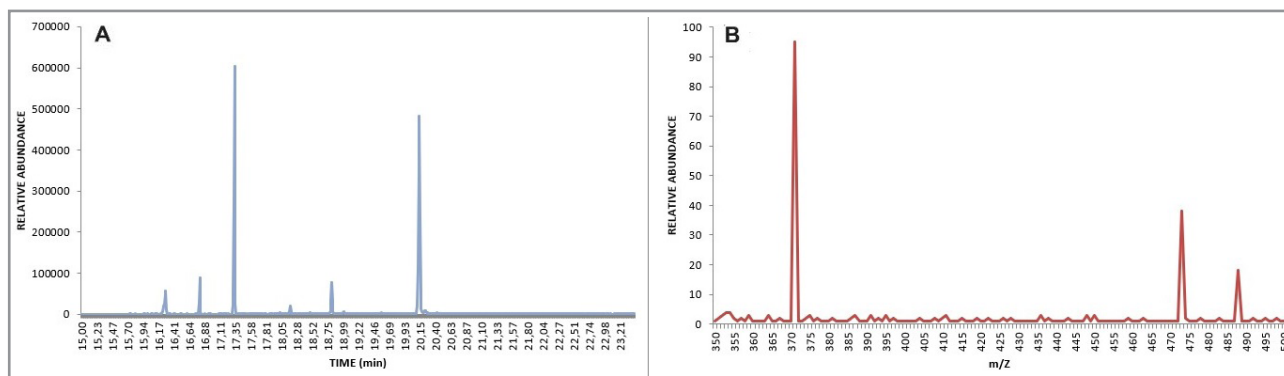


Figure 2. Chromatogram (A) and mass spectra (B) of Δ^9 -THC-COOH obtained by LLE/GC-MS. Where: (A) 20.15 min = retention time, (B) Quantifier and identification ion (m/z) of Δ^9 -THC-COOH (371, 473, 488).

The hydrolysis step was performed because over 80% of the THC-COOH excreted in the urine are conjugated with the glucuronic acid [19]. Therefore, hydrolysis prior to GC-MS analysis is required to better quantify the total of cannabinoids. Moreover, alkaline hydrolysis is the most effective one for the Δ^9 -THC-COOH glucuronide conjugate [2].

The chosen solvent mixture (n-hexane: ethyl acetate, 9:1, v/v) was the only one to have achieved quantifiable results in our extraction procedure. Other solvent mixtures tested, such as chloroform: ethyl acetate (80:20, v/v) did not present adequate sensitivity for chromatographic detection. With that mixture, it was not possible to quantify by GC-MS the analyte under study with precision and accuracy. Acetic acid was added to adjust the pH after the basic hydrolysis step. Derivatization using BSTFA and 1% TCMS proved to be more efficient than it is when using MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide), leading to better recovery rates for this technique. A good recovery rate was obtained using LLE. This technique is simple, cheap and fast to be performed at a laboratory of toxicological analyses.

The complete validation of LLE-GC-MS met a set of guidelines by national and international bodies [16-18,21]. Evaluation of samples with Δ^9 -THC-COOH standard addition and blank samples indicated no interference with the analysis of the analyte of interest, showing that there was adequate selectivity, with no interfering peak close to the retention time of all analytes and internal standards of interest. Figure 3 shows the chromatogram for an ultrapure water sample containing Δ^9 -THC-COOH standard at 300 ng mL⁻¹ (A), a urine sample without the analyte (blank sample, B), and a positive urine sample (C).

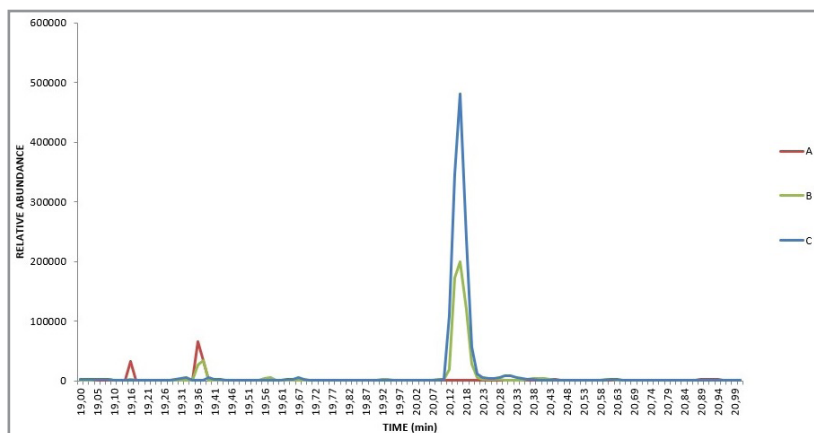


Figure 3. GC-MS Chromatogram obtained from LLE for samples containing Δ^9 -THC-COOH. Retention time of the analyte is 20.15. Where: (A) chromatogram of a urine sample not containing the analyte (blank sample), (B) concentration chromatogram of a positive urine sample at 121 ng mL^{-1} , and (C) chromatogram of an ultrapure water sample containing Δ^9 -THC-COOH standard at 300 ng mL^{-1}

As for the linearity of the method, the calibration curves were generated at the concentration range tested (5 to 300 ng mL^{-1}), and the coefficient of correlation was 0.9993 (linear regression equation: $y = 0.0269x - 0.0364$). Those were correctly adjusted by indicating the mathematical models at the level of 5% [21]. This linearity interval represents concentrations of Δ^9 -THC-COOH, usually found in urine samples of *Cannabis* users (5 to 300 ng mL^{-1}) [21,22].

The limits found in this study resulted in 5 and 10 ng mL^{-1} for LOD and LOQ, respectively. According to the Substance Abuse and Mental Health Services Administration (SAMHSA) [23], the suggested cut-off value in confirmatory assays for THC-COOH in urine is 15 ng mL^{-1} in routine analyses, usually performed by GC-MS. For LOD and LOQ values, the SAMHSA also preconizes that analytical methods for detection of Δ^9 -THC-COOH must be able to detect such analyte at values below the cut-off, which makes the method here tested in accordance with international guidelines and with other studies published in the literature [24,25].

The precision and accuracy parameters also presented satisfactory results. CV and RSD values were lower than 20% for LOQ concentration, and lower than 15% at other concentrations (Table I). Moreover, precision and accuracy were within acceptable ranges, as determined by the followed guidelines [16,18]. These parameters were also similar to those reported by other studies. Jamerson et al. (2005) obtained inter-assay precision ranging from 2.3 to 5.4% and intra-assay precision over 2% in their study with rapid quantification using GC-MS [26]. Nestić et al. (2013) obtained intra-assay precision ranging from 3.18 to 9.01% and inter-assay precision ranging from 0.99 to 8.80% [27].

Validation guidelines establish that linearity must be performed in the matrix [16,18]. This study was complemented in the evaluation of the matrix effect, once the linearity parameter in water was performed to verify the similarity between the curves in the presence and absence of the sample (urine). This evaluation consisted of comparing the values of the straight slope (0.0286 and 0.0269 for deionized water and urine, respectively). The parallelism of the straight lines is an indicator of the absence of interference from the constituents of the matrix, and its demonstration must be carried out by means of an adequate statistical evaluation, with a level of significance of 5% [16]. This matrix effect test proves that there is a parallelism of the lines, which indicates the absence of interference from the matrix. This does not mean that the lines are equal, since only the slope is considered (the linear coefficient is not). This fact proved the absence of interference from the components of the urinary matrix.

Table I. Analytical parameters of the method developed for detection and quantification of Δ^9 -THC-COOH, as assessed by LLE-GC-MS*

	Δ^9 -THC-COOH
Correlation coefficient (r)	0.993
LOD (ng mL ⁻¹)	5.00
LOQ (ng mL ⁻¹)	10.00
Intra-assay precision (CV%)	
Low concentration (10 ng mL ⁻¹)	3.66
Average concentration (100 ng mL ⁻¹)	4.46
High concentration (250 ng mL ⁻¹)	9.04
Inter-assay precision (CV%)	
Low concentration (10 ng mL ⁻¹)	3.38
Average concentration (100 ng mL ⁻¹)	4.46
High concentration (250 ng mL ⁻¹)	8.96
Accuracy (%)	
Low concentration (10 ng mL ⁻¹)	
Day 1	90.70
Day 2	112.90
Day 3	83.80
Day 4	93.80
Day 5	109.00
Day 6	83.00
Average concentration (100 ng mL⁻¹)	
Day 1	94.60
Day 2	107.60
Day 3	96.40
Day 4	102.00
Day 5	95.50
Day 6	92.70
High concentration (250 ng mL⁻¹)	
Day 1	100.70
Day 2	92.50
Day 3	91.80
Day 4	94.00
Day 5	95.90
Day 6	97.40

*LLE-GC-MS: liquid-liquid extraction-gas chromatography-mass spectrometry

The carryover and residual effect were not detected in the method we developed, even when we analyzed blank samples, injected after the last point of the calibration curve (300 ng mL⁻¹). Therefore, there was no need for comparison with the chromatograms obtained for blank samples fortified with concentrations corresponding to LOQ.

The analyte remained stable after 15 and 30 days of storage at $-20\text{ }^\circ\text{C}$ and after 5 freeze-thaw cycles. Post-processing stability was evaluated by re-injecting samples into the GC-MS apparatus after 24, 48 and 72 h. The results were compared with others obtained for freshly extracted samples that had a variation value lower than 15% (Table II). Stability results corroborate other results. Nestić et al (2013) used a spiked urine sample subjected to three freeze-thaw cycles and long-term stability. Those processed samples did not show significant change when analyzed [27].

Table II. Long-term, freeze-thaw cycle and post-processing stability of Δ^9 -THC-COOH in human urine assessed by LLE-GC-MS

Long-term stability (RSD%)		
	CB: 10 ng mL ⁻¹	CA: 250 ng mL ⁻¹
15 Days	3.21%	3.05%
30 Days	4.72%	4.61%
Freeze-thaw cycle stability (CV%)		
	CB: 10 ng mL ⁻¹	CA: 250 ng mL ⁻¹
After 5 Cycles	5.51%	8.32%
Post- Processing stability (CV %)		
	CB: 10 n g mL ⁻¹	CA: 250 ng mL ⁻¹
24h	3.54%	4.40%
48h	6.13%	9.93%
72h	5.40%	10.98%

These results obtained by validation are similar to those described in other studies that also used urine as a biological matrix. Abraham et al. [28] described the following results: $r = 0,999$, intra-assay precision over 2.40% and inter-assay precision ranging from 2.60 to 7.40%. Nestić et al. [27] obtained the following results: $r = 0,999$, intra-assay precision ranging from 3.18 to 9.01% and inter-assay precision ranging from 0.99 to 8.80%. However, these techniques aforementioned used extractions by SPE, which can increase the cost of the analyses.

According to the method validation parameters and given the simplicity of the validated method, together with the ease of obtaining the urine matrix, the developed method meets the needs of toxicological analyses and can be applied in forensic routine, assisting in solving cases of violent deaths, traffic accidents, doping and drug addiction control.

The method was applied to authentic urine samples from five individuals diagnosed with trauma in association with the use of drugs, who were taken care of at the emergency service. The following results were obtained: [1] positive (43.80 ng mL⁻¹), [2] positive (46.70 ng mL⁻¹), [3] positive (121.23 ng mL⁻¹), [4] positive (73.95 ng mL⁻¹) and [5] positive (95.02 ng mL⁻¹). These results show that the LLE-GC-MS method could be used in laboratory routine, with reliable results.

Most current techniques use SPE as an extraction method [5-10]. The technique presented in this study was developed using an optimized LLE-GC-MS. It has proven to be a cheap, simple and fast alternative in routine laboratories and forensic analysis, with reproducible and reliable results. Using urine as a biological sample has advantages, such as the fact that its collection is less invasive. Besides, it can be obtained in large quantities and presents good conservation and stability of the analytes.

CONCLUSION

The LLE-GC-MS validated method was efficient, since it met all the required parameters, in accordance with international and national guidelines established for analysis of Δ^9 -THC-COOH in urine. Therefore, we provide a result that is applicable to the reality of most laboratories with scarce financial support. The use of a technique of extraction and identification that is easy to be put into practice, with low costs and reliability, as it is the case of LLE-GC-MS, is of great value for lower expenses in laboratories with few resources. Other previously mentioned techniques use more expensive instruments, such as HPLC, and also extraction methods that increase the costs of an analysis, which is unfeasible in laboratory routine. The search for the development of more modern techniques is a desirable requirement, but they often do not apply to the reality of many routine laboratories. In addition, we use one of the most requested samples for drug research, that is, urine.

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