Molecularly imprinted polymer for selective determination of Δ9-tetrahydrocannabinol and 11-nor-Δ9-tetrahydrocannabinol carboxylic acid using LC-MS/MS in urine and oral fluid

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Abstract

The use of molecularly imprinted polymers (MIPs) for solid phase extraction (MISPE) allows a rapid and selective extraction compared with traditional methods. Determination of Δ9-tetrahydrocannabinol (THC) and 11-nor-Δ9-tetrahydrocannabinol carboxylic acid (THC-COOH) in oral fluid (OF) and urine was performed using homemade MISPEs for sample clean-up and liquid chromatography tan- dem mass spectrometry (LC-MS/MS). Cylindrical MISPE shaped pills were synthesized using catechin as a mimic template. MISPEs were added to 0.5 mL OF or urine sample and sonicated 30 min for adsorption of analytes. For desorption, the MISPE was transfered to a clean tube, and sonicated for 15 min with 2 mL acetone:acetonitrile (3:1, v/v). The elution solvent was evaporated and reconstituted in mobile phase. Chromatographic separation was performed using a SunFire C18 (2.5 µm; 2.1×20 mm) column, and formic acid 0.1 % and acetonitrile as mobile phase, with a total run time of 5 min. The method was fully validated including selectivity (no endogenous or exogenous interferences), linearity (1– 500 ng/mL in OF, and 2.5–500 ng/mL in urine), limit of detection (0.75 and 1 ng/mL in OF and urine, respectively), imprecision (%CV <12.3 %), accuracy (98.2–107.0 % of target), extraction recovery (15.9–53.5 %), process efficiency (10.1–46.2 %), and matrix effect (<−55 %). Analytes were stable for 72 h in the autosampler. Dilution 1:10 was assured in OF, and Quantisal™ matrix effect showed ion suppression (<−80.4 %). The method was applied to the analysis of 20 OF and 11 urine specimens. This is the first method for determination of THC and THC-COOH in OF using MISPE technology.

Keywords

Molecularly imprinted polymer

Oral fluid

Urine

THC

THC-COOH

LC-MS/MS

Introduction

Molecularly imprinted technology is a powerful tool in the development of highly selective analytical methods. Molecularly imprinted polymers (MIPs) are materials prepared in the presence of a target molecule (template) or closely related species (mimic) that serve as a mould for the formation of template-complementary binding sites. The formation of a complex between the target analyte and functional monomer is carried out in a porogenic solution prior to radical copolymerization in the presence of a large excess of cross-linking monomer(s). After polymerization, the template is removed and the resulting rigid three-dimensional cavities are complementary to the target analyte. Thus, MIPs can be created to recognize a large variety of target molecules often with antibody-like affinity and selectivity. MIPs are especially good candidates as adsorbents for sample preparation, for example in solid phase extraction (MISPE), due to their high selectivity and their ability to pre-concentrate the analytes or remove potential interferences from a complex sample matrix while providing a robust and reproducible analytical method regardless of sample matrix variability [1–3]. Generally, the MISPE procedures were carried out in the off-line mode for extracting analytes from different matrices, such as biological fluids, tissue samples, and plant samples [4, 5]. In addition, their use as scavengers to remove undesirable compounds from foods or biological fluids [6], and as screening tools in drug discovery [7] have also been demonstrated [8].

Cannabis is the most commonly used illicit drug through-out the world. The 2012 Substance Abuse and Mental Health Services Administration (SAMHSA) report showed a canna- bis prevalence of 7.3 % in current users older than 12 years old [9]. The 2012 European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) report showed a 3.6 % of cannabis consumption in the last month for the population be- tween 15 and 64 years old [10]. Specifically, Spain has the highest prevalence of cannabis use in Europe, with a prevalence of 7.6 % in the last month [10]. Thus, there is a great demand for reliable and sensitive methods for the determination of cannabis presence in biological samples. Its use is detected by determining the presence of the main active compound Δ9-tetrahydrocannabinol (THC) as well as its main metabolite 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). To our knowledge, only one paper describes the use of MISPE for the determination THC, THC-COOH, and 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH) in urine [11].

This paper describes an analytical method for the determination of THC and its main metabolite THC-COOH in oral fluid and urine, using a homemade MISPE for samples clean-up, and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Materials and methods

Chemicals and reagents

Reference standards THC and THC-COOH at 1 mg/mL in methanol, and THC-d_3 and THC-COOH-d_3 at 0.1 mg/mL in methanol were purchased from Cerilliant™ (Round Rock, TX, USA). Methanol LC-MS from Chromasolv® (St. Louis, USA), acetone reagent grade from Scharlau® (Sentmenat, Spain), and acetonitrile LC-MS from Panreac (Castellar del Vallès, Spain). Potassium hydroxide (KOH) in pellets, hydrochloric acid (HCl) 37 % reagent grade, and formic acid (98– 100 %) reagent grade were from Scharlau (Sentmenat, Spain). Water was purified with a Milli-Q water system (Milli-pore, Le-Mont-sur-Lausanne, Switzerland). Salivette® and Quantisal™ oral fluid collection devices were from Sarstedt (Nümbrecht, Germany) and Immunalysis (Pomona, CA, USA), respectively.

The molecularly imprinted polymer was synthesized using triethylene glycol dimethyl ether (TRIGLYME), poly(vinyl acetate) (PVAc), 4-vinylpyridine (4-Vpy), and (+)-Catechin hydrate (C, >98 %) from Sigma-Aldrich (Steinheim, Germany). Ethylene glycol dimethacrylate (EGDMA) and 2,2-azobis(2-methylpropionitrile) (AIBN) were obtain from Fluka (Buchs, Switzerland).

Synthesis of THC-MIPs

A mixture of TRIGLYME as low volatile solvent, and PVAc (7.5 %, wt % relative to pure solvent), as non-reactive linear polymer, was used. Catechin was selected as mimic template to avoid false positives due to bleending problems [3, 4]. At first this template was mixed with the functional monomer (M) 4-Vpy, the cross-linker (C) EGDMA and the porogen TRIGLYME at a ratio of 1:6:6 (T:M:C). AIBN was applied as initiator of the radical polymerization solution. A custom-made silicone device was used to contain the pre- polymerization solution and was placed in a suitable airtight container to keep nitrogen atmosphere after purging with nitrogen gas for 5 min to completely evacuate the air, thus avoiding the polymerization inhibition. Finally, the polymerization was carried out at 60 °C for 24 h. Small polymer cylinders were obtained, with the size of a pinhead and the shape of a small pill [12].

Several THC-MISPE-pills were synthesized following this procedure, and then subsequently washed with methanol as elution solvent to remove the template from the cavities. Polymers were washed until (+)-Catechin could no longer be detected at 277 nm in the elution solvent.

The pills are cylindrical in shape and the corresponding size and weight after synthesis and washing step with methanol to eliminate unreacted monomer and crosslinker fractions, are shown in Table 1. For the measure of the size of the pills, a 0.25–0.001-mm Electronic Digital Micrometer was used.

Oral fluid and urine samples

Fresh oral fluid (collected with the Salivette® device) and urine samples for the preparation of the calibrators and quality controls (QC) were donated by the staff personnel. Real oral fluid samples (collected with the Quantisal™ device) were obtained by the Spanish Traffic Police during 2012, and sent to our laboratory to confirm a positive on-site result for THC (with or without a positive result for opiates, cocaine, amphetamine and methamphetamine) using the Dräger DrugTest 5000 (Dräger, Lübeck, Germany). Real urine samples were obtained from a previous research project and were collected during 2013 from chronic cannabis users. Oral fluid and urine specimens were stored at −20 °C.

Preparation of calibration and quality control (QC) solutions

Different working solutions were prepared for calibrators and for QCs. A working solution containing THC and THC-COOH at 10 µg/mL was prepared by dilution of the individual ampoules (1 mg/mL) in methanol. Further dilutions with methanol were prepared to obtain working solutions at 1, 0.1, and 0.01 µg/mL. For the QC samples, working solutions at 2, 0.2, and 0.02 µg/mL were prepared. An internal standard (IStd) mixture containing THC-d3 and THC-COOH-d3 at 1 µg/mL was prepared in methanol.

Calibrators at 1, 2.5, 5, 10, 50, 100, and 500 ng/mL were generated by addition of 12.5, 25, or 50 µL of the appropriate working solutions to blank oral fluid or urine samples. QC samples at low (3 ng/mL for oral fluid and 6 ng/mL for urine), medium (30 ng/mL), and high (300 ng/mL) concentrations were prepared by addition of 15 or 75 µL of the corresponding QC working solutions.

Urine hydrolysis

Alkaline hydrolysis was performed to breakdown THC and THC-COOH glucuronide conjugates. KOH 12 M (25 µL) and IStd mixture at 1 µg/mL (25 µL) was added to 0.5 mL of urine. The mixture was incubated at 60 °C for 30 min. After hydro- lysis, the neutralization was performed with 300 µL HCl 1 M.

THC-MISPE extraction

MISPE extraction consisted of two steps: adsorption and desorption of the analytes. For the adsorption of THC and THC-COOH into the MIP structure, 0.5 mL of oral fluid or urine was added to a clean tube with a THC-MISPE-pill, and sonicated for 30 min. The MISPE-pill was subsequently transferred to a clean tube, and analytes desorption was per- formed with 2 mL of acetone/acetonitrile (3:1, v/v) sonicating for 15 min. This solvent was evaporated and reconstituted in 50 µL of methanol and 50 µL of formic acid 0.1 %. After centrifugation at 14,500 rpm (10 min), the supernatant was collected and injected into the LC-MS/MS.

THC-MISPE wash

Two washing steps were performed using 10 mL of acetone:acetonitrile (3:1, v/v) and sonication during 15 min for MISPE-pills reuse. Absence of MISPE carry-over was tested analyzing the second wash solvent after the extraction of 500 ng/mL of THC and THC-COOH (n=5) in oral fluid and urine samples.

LC-MS/MS analysis

The HPLC system was an Alliance 2795 Separation Module with an Alliance series column heater/cooler coupled to a Quattro MicroTM API triple quadrupole (Waters Corp., Mildford, MA, USA). Chromatographic separation was performed with a SunFire™ I.S. C18 (2.1 mm×20 mm, 2.5 µm) analytical column (Waters Corp., Mildford, MA, USA), maintained at 26 °C. Formic acid 0.1 % (A) and acetonitrile (B) were used as mobile phase at a flow rate of 0.5 mL/min working applying the following gradient: 40 % B at 0 min, increased to 65 % over 0.8 min, increased to 100 % B over 2 min, and return to initial conditions at 2.1 min, and equilibrate until 5 min.

The MS was operated in electrospray in positive mode (ESI+). The following parameters were applied: capillary voltage, +3 kV; source block temperature, 130 °C; desolvation gas (nitrogen) temperature, 400 °C; desolvation and cone gas (nitrogen) flow rate 800 and 80 L/h, respectively. Argon was employed to promote analyte fragmentation in the collision cell. Optimal cone voltage, precursor-to-product ion transitions and collision energies were selected by performing a direct infusion of each individual analyte into the MS connected with a “T” valco to the LC effluent using formic acid 0.1 % and acetonitrile (50:50, v/v) as mobile phase at 0.1 mL/ min. Two precursor-product transitions were monitored for each compound, and only one for IStds. The selected quantifier transitions were: 315.2>193.2 (cone voltage, 30 V; colli- sion energy, 25 eV) for THC, 345.1>299.2 (35 V, 22 eV) for THC-COOH, 318.2>196.2 (35 V, 25 eV) for THC-d3, and 348.1>330.3 (35 V, 18 eV) for THC-COOH-d3. The qualifier transitions were: 315.2>135.1 (30 V, 25 eV) for THC and 345.1>327.2 (35 V, 18 eV) for THC-COOH. Data acquisition was controlled with Masslynx 4.0 software and processed with Quanlynx software (Waters Corp., Mildford, MA, USA).

Identification criteria

Identification criteria included retention time (RT) within ±0.2 min of mean calibrator RT, presence of 2 product ions, and ion ratio between the quantifying ion and the qualifier ion within ±20 % of that established by the calibrators [13].

Validation

The method was validated in oral fluid and urine for the following parameters: selectivity, linearity, limit of detection (LOD) and quantification (LOQ), accuracy and imprecision (within-day, between-day, and total), extraction recovery, matrix effect, process efficiency, and stability after 72 h in the autosampler. Matrix effect due to Quantisal™ buffer and dilution integrity were also determined in oral fluid.

Selectivity of the method was evaluated for endogenous and exogenous interferences. Interferences from endogenous matrix components were evaluated by analyzing 10 oral fluid samples and 10 urine samples from different sources after fortification with the IStd solution. Endogenous interferences were considered insignificant if THC and THC-COOH were in a concentration lower than the LOD. Exogenous interferences were evaluated by the analysis of oral fluid or urine samples fortified with THC and THC-COOH at the LOQ and with potentially interfering common drugs of abuse and medicines (morphine, 6-monoacetylmorphine, codeine, methadone, 2- ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylendioxy ethylamphetamine, cocaine, benzoylecgonine, cocaethylene, ecgoninemethylester, lysergic acid diethylamide, ketamine, norketamine, gamma-hydroxybutyric acid, nicotine, cotinine, fentanyl, amitrityline, paroxetine, zolpidem, zopiclone, ibuprofen, omeprazol, acetaminophen, diclofenac, naproxen, temazepam, alprazolam, 7-aminoflunitrazepam, clonazepam, diazepam, flunitrazepam, lormetazepam, lorazepam, nordiazepam, oxazepam, triazolam, nitrazepam, and bromazepam) at 500 µg/mL. Sufficient specificity was achieved if the analytes of interest quantified within ±20 % of LOQ.

Linearity was verified by preparing 4 calibration curves analyzed on 4 different days. Acceptable linearity was achieved if coefficient of determination (r2) was >0.99 and calibrators quantified within ±20 % of target at the LOQ, and within ±15 % at the other concentrations.

The LOD was defined as the lowest concentration with acceptable chromatography, presence of all product ions with signal-to-noise >3, retention time within ±0.2 min from all average calibrators, and acceptable ion ratio [13]. The LOD was determined by fortifying blank specimens (from 3 different sources) at decreasing concentrations. The LOQ was the lowest concentration that could be quantified with acceptable imprecision (≤20 %) and accuracy (80–120 % of target concentration), signal-to-noise ratio >10 [13]. LOQ was evaluated by the analysis of 5 replicates prepared using samples from different sources.

Imprecision and accuracy were assessed at 3 concentrations (low, medium and high QCs) with the analysis of 5 replicates on 4 different days (n= 20). Krouwer and Rabinowitz' recommendations [14] were followed for calculation of pooled within-day, between-day and total imprecision using one-way analysis of variance. Acceptable imprecision was achieved if %CV was ≤15 %. Accuracy was expressed as the percentage of the nominal concentration (n=20), and was required to be within 85–115 % of the target concentration.

Extraction recovery, matrix effect, and process efficiency were determined at two concentration levels (low and high QCs). Extraction recovery was calculated as the percentage after comparing mean peak areas of blank specimens fortified prior to extraction (n=6) with those obtained in specimens fortified after extraction (n=6). Matrix effect was determined by comparing mean peak areas in blank specimens (n=10, from different sources) fortified after extraction with mean peak areas of the analytes prepared in mobile phase (n=6). Matrix effect was calculated as follows: (100×mean peak area of fortified specimen after extraction/mean peak area of analytes in mobile phase)−100. Process efficiency examined the overall effect of the extraction recovery and the matrix effect. Process efficiency was determined by comparing mean peak areas of blank specimens fortified prior to extraction (n=6) with peak areas of samples at the same nominal concentrations prepared in mobile phase (n=6).

Matrix effect originated by the Quantisal™ buffer was evaluated at low and high QCs concentrations by comparing mean peak areas in blank oral fluid (n=10, from different sources) mixed with buffer (1:3, v/v) and fortified after extraction, with mean peak areas of the analytes prepared in mobile phase (n=6).

In order to prove analytes stability after in the autosampler (6 °C) samples at low, medium and high QC concentrations (n=5, each) were re-injected after 72 h storage. Analyte stability was considered acceptable if QC samples quantified within ±20 % of freshly prepared QC samples (n=5).

Dilution integrity was evaluated by diluting samples containing THC and THC-COOH at 3,000 ng/mL with blank oral fluid and Quantisal™ buffer (1:3) to achieved 1:10 dilution (n=5). After IStd addition, samples were extracted as de- scribed previously. Dilution integrity was maintained if diluted samples quantified within ±20 %.

Application to real specimens

The method was employed to determine cannabis consumption in 20 oral fluid and 11 urine specimens. Oral fluid specimens were previously analyzed using a solid phase ex- traction procedure with Strata X (3 cc, 60 mg; Phenomenex, Torrence, CA, USA) cartridges [15], and results were compared with those obtained after the application of the present method. Urine specimens were routinely analyzed using

0.5 mL of urine and hydrolyzing with 25 µL of KOH 12M (60 °C min, 30 min), following by neutralization with 200 µL of HCl 0.1M. A liquid–liquid extraction was performed with 1 mL acetic acid 20 % in water and 3 mL of hexane. After mechanical shaking (30 min) and centrifugation, the organic layer was evaporated, and the reconstitution solvent was analyzed by LC-MS/MS. Results were also compared with those achieved with the present method.

Results

Chromatographic conditions achieved sufficient resolution of all analytes within 3 min, with a total chromatographic run time of 5 min. The most abundant MRM transition was selected for quantification of all THC and THC-COOH. An additional MRM transition was monitored for identification purposes. Figure 1 shows a chromatogram of THC and THC- COOH in oral fluid (1A) and in urine (1B) at the LOQ.

THC-MISPE reuse

THC-MISPE can be reused without losing sensitivity after the described washing procedure. Each pill could be used at least 30 times without loss of sensitivity, as proved by the comparison of THC and THCCOOH areas and responses achieved in samples fortified at the LOQ, and analyzed at the beginning and the end of the complete method validation.

Moreover, possibility of carryover was evaluated by the analysis of the first and second washing solvent after the extraction of oral fluid (n=5) and urine (n=5) samples fortified at 500 ng/mL. THC was not detected in the first washing solvent; however, THC-COOH was quantified close to the LOQ, and therefore, a second washing step was required for this analyte.

Validation

No interferences from any extractable endogenous compound were observed after the analysis of 10 different blank oral fluid or urine samples. Exogenous interferences were not detected after adding high concentrations of common drugs of abuse and medicines in samples spiked with THC and THC-COOH at the LOQ. Moreover, THC and THC-COOH quantified within ±20 % of target, indicating no interferences with the analytes of interest.

Linearity of analyte-to-IStd peak area ratio versus theoretical concentration was verified by least-square regression with 1/x weighting factor. Curvature tested on a set of 4 calibration curves yielded determination coefficients (r2) above 0.998, with residuals within ±15% for all calibrators. Dynamic range in oral fluid was 1–500 ng/mL and in urine samples 2.5– 500 ng/mL. LOD were 0.75 and 1 ng/mL, and LOQ 1 and 2.5 ng/mL for oral fluid and urine, respectively. Linearity results are summarized in Table 2.

Imprecision (within-day, between-day and total imprecision) and accuracy results were satisfactory at all tested concentrations in both matrices (Table 3). Within-day, between-day and total imprecision were <5.7 %, 7.0 %, and 8.2 % in oral fluid, and <10.8 %, 5.8 %, and 12.3 % in urine, respectively. Accuracy was also satisfactory, ranging from 98.7 % to 104.4 % and 98.2–107.0 % of nominal the concentrations in oral fluid and urine, respectively.

Extraction recovery, matrix effect, process efficiency and matrix effect originated by the Quantisal™ buffer data are shown in Table 4. Extraction recoveries ranged from 26.3 % to 53.5 % in oral fluid, and 15.9–34.5 % in urine, with the lowest values for THC at low QC in oral fluid, and for THC-COOH at high QC in urine. In oral fluid, THC and THC-COOH showed ion suppression at the low QC (50.4 % and 41.5 %, respectively), but no matrix effect was observed at the high QC. %CV within the 10 different specimens employed in this experiment was <20 % for both analytes. Higher matrix effect was observed in the presence of the Quantisal™ buffer, with ion suppression ranging from 50 % to 80.4 % depending on the analyte, and a %CV ≤20 % except for THC at low QC. Higher ion suppression was observed for both analytes at low and high QC in oral fluid, ranging from 36.7 % to 54.9 %. %CV within the 10 different specimens was ≤20 %, except for THC-COOH a high concentrations (%CV=28.1 %). Process efficiency ranged between 10.1 % and 46.2 % in both matrices.

THC and THC-COOH were stable after 72 h storage in the autosampler at 6 °C, with a percentage loss <3.1 % in oral fluid and <2.2 % in urine. Dilution integrity was studied by the analysis of in oral fluid samples at 3,000 ng/mL diluted tenfold (n= 5). Accuracy was 103.2 % (%CV= 3.5) and 105.7 % (%CV=3.9) for THC and THC-COOH, respectively.

Application to real specimens

Twenty oral fluid specimens with a previous positive result for THC were reanalyzed using the present method. Only THC was detected in these specimens using both methodologies, with concentrations ranging between 4.7 and 16,334.1 ng/mL using the traditional method and 14.7 to 16,621.6 ng/mL using the MISPE method. Imprecision between methods was evaluated, and expressed as %CV. For one specimen (case 13), THC concentration using MISPE extraction was lower than the LOQ and, therefore, the imprecision could not be evaluated. %CV was <20 % in all cases, except for cases 1 and 2 (%CV=48.9 % and 22.9 %, respectively; Table 5).

Eleven urine specimens obtained from chronic cannabis users were reanalyzed with and without hydrolysis using the present method, and results were compared with those obtain- ed with the liquid-liquid extraction protocol (Table 6). All the specimens were positive for THC-COOH; however, no positive results were found for THC. THC-COOH concentrations after hydrolysis ranged between 60.7 and

367.6 ng/mL using the traditional method and between 60.7 and 323.7 ng/mL using the MIP method. The imprecision between methods was <9.6 % in all cases.

Discussion

An analytical method for the determination of THC and THC- COOH was developed using MIP technology for sample clean-up and LC-MS/MS for detection.

An adaptation of precipitation polymerization was used to prepare a small pill of MIP, with the size of a pinhead, which does not need any further treatment to be employed. Instead of THC, catechin was selected as the template molecule to avoid bleeding problems in case of trace amounts analysis.

The method was fully validated in oral fluid and urine following the international recommendations of FDA and SWGTOX [16, 17]. All of the studied parameters (selectivity, linearity, within-day, between-day and total imprecision, ac- curacy, extraction recovery, matrix effect, process efficiency, Quantisal™ buffer matrix effect and stability) yielded satisfactory results.

Extraction of THC and its metabolite THC-COOH from biological matrices is usually performed by liquid-liquid (LLE) or solid phase extraction (SPE). Hexane or ethyl acetate are the organic solvents more frequently used in LLE procedures [18–20]. SPE is usually performed using silica based octadecyl (C18) or octyl (C8) sorbents, and silica based or copolymeric mixed mode (hydrophobic and anion exchange) sorbents [11]. The use of SPE with molecularly imprinted polymer as sorbent allows a more selective and simply extraction procedure compared with this traditional methods. Recently, a method was published for the detection of THC, and its metabolites THC-COOH and THC-OH in urine by SPE with molecularly imprinted polymer as sorbent [11]. In this method, a homemade MIP was synthesized using THC- OH as template, methacrylic acid as functional monomer, EGDMA as cross-linking and methanol as the porogenic solvent. A SPE cartridge was prepared with this homemade MIP and 2 mL of urine were extracted. Cartridges were conditioned with 2 mL of methanol and water. After loading the sample at pH=3, cartridges were washed with 2 mL water (twice) and 100 µL of methanol. Elution was performed with 2 mL of chloroform:ethyl acetate (60:40, v/v). Evaporated extracts were derivatized with BSTFA+1 % TMCS during 30 min and analyzed by gas chromatography–mass spectrom- etry. The sorbent can be reused after soaking the MISPE for 15 min with 3 mL of HCl:methanol (1:36, v/v).

Our MISPE method is less complex than that of Nestic et al. [11] as it is not necessary to condition the cartridges before loading the samples, and the elution of the analytes is performed directly after the adsorption, without a previous washing step of the THC-MISPE pills. Moreover, in our method only 0.5 mL are employed, achieving similar LOQs and LODs than those achieved by Nestic et al. (2 to 3 ng/mL

and 1 to 2.5 ng/mL, respectively). However, Nestic et al. obtained better extraction recoveries for THC and THC- COOH (>70.3 % and >74.0 %, respectively), probably due to a more specific template (THC-OH) used for the synthesis of their MISPE cartridges. The analysis of urine specimens from chronic cannabis users revealed the presence of the metabolite in all cases, but not the parent analyte due to THC extensive metabolism [21]. However, THC was the only analyte detected in oral fluid, as THCCOOH concentrations in this matrix are usually in the pg/mL range [22–25].

To the best of our knowledge, this is the first method for the determination of THC and THC-COOH in oral fluid using a MISPE extraction. THC is the primary target in oral fluid to detect cannabis intake. A cut-off of 0.02 ng/mL for THC- COOH has been recently suggested to discriminate between passive exposure and active consumption of cannabis [25], which was not achieved with the MISPE employed in the present method. Therefore, the main limitation of the present method is the high LOQ for THC-COOH which did not allow the detection of this analyte in the cases that have been analyzed in the present study. However, the THC cut-off recommended by the Guidelines for research on drugged driving [26] or in DRUID project [27] (2 or 1 ng/mL, respectively) to identify THC consumption in oral fluid was achieved with the present analytical method.

Our team is currently working on the use of a more specific MISPE template for THC or THC-COOH, which could allow to increase the specificity and sensitivity for THC and THC- COOH in urine and oral fluid samples.

Conclusions

A new MISPE-LC-MS/MS method for the analysis of THC and its main metabolite THC-COOH has been developed in oral fluid and urine. The method has been fully validated and applied to real samples. THC and THC-COOH concentrations found in these specimens were compared with those obtained with the routine methods used in our laboratory. MISPE demonstrated to be a useful tool for the detection and quantification of THC and THC-COOH in different biological matrices, allowing a simple, fast, and specific sample extraction.

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Table 1. MIP "pill" dimensions (n=4)

Weight (g)		Size (mm)			
Weight before washing step	Weight after washing step	Height before washing step	Diameter before washing step (wide)	Height after washing step	Diameter after washing step (wide)
$1.93E^{-2}$	$1.28E^{-2}$	3.69	3.12	3.31	2.93

Table 2. Limits of detection (LOD) and quantification (LOQ), calibration ranges, and linearity results in oral fluid and urine

	Analyte	LOD (ng/mL)	LOQ (ng/mL)	Range (ng/mL)	Intercept±SD (*n*=4)	Slope±SD (*n*=4)	r^2±SD (*n*=4)
Oral fluid	THC	0.75	1	1–500	0.7309±0.4454	2.1987±0.2406	0.9997±0.0002
	THC-COOH	0.75	1	1–500	1.6892±0.2828	2.4344±0.1222	0.9996±0.0004
Urine	THC	1	2.5	2.5–500	0.7886±0.5863	1.9413±0.2735	0.9989±0.0010
	THC-COOH	1	2.5	2.5–500	2.0576±0.4989	1.6587±0.1131	0.9985±0.0013
THC: Δ9 -tetrahydrocannabinol, THC-COOH: 11-nor-9-carboxy-THC							

Table 3. Within-day, between-day and total imprecision, and accuracy at low (3 ng/mL in oral fluid and 6 ng/mL in urine), medium (30 ng/mL), and high (300 ng/mL) QC concentrations in oral fluid and urine

		Within-day imprecision (n=20, %CV)			Between-day imprecision (n=20, %CV)			Total imprecision (n=20, %CV)			Accuracy (n=20, % target)		
		Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
Oral fluid	THC	4.7	5.7	3.9	0.0	4.3	6.1	4.7	7.2	7.2	98.7	102.0	102.2
	THC-COOH	4.0	3.4	4.4	0.0	4.0	7.0	4.0	5.2	8.2	98.7	104.4	102.9
Urine	THC	6.6	2.4	3.6	1.2	2.0	3.3	6.7	3.2	4.9	98.2	107.0	104.3
	THC-COOH	4.3	4.1	10.8	5.6	0.0	5.8	7.1	4.1	12.3	101.5	106.7	105.7
THC: Δ9 -tetrahydrocannabinol, THC-COOH: 11-nor-9-carboxy-THC													

Table 4. Extraction recovery (ER; n=6), matrix effect (ME; n=10), and process efficiency (PE; n=6) at low (3 ng/mL in oral fluid and 6 ng/mL in urine) and high (300 ng/mL) QC concentrations in oral fluid and urine; and matrix effect originated by the Quantisal™ buffer (n=10) at low and high QC concentrations in oral fluid

	Analyte	% ER		% ME (%CV)		% PE		% ME Quantisal™ (%CV)	
		Low	High	Low	High	Low	High	Low High	
Oral fluid	THC	26.3	53.5	−50.4 (20.0)	-13.6 (10.9)	13.1	46.2	−80.4 (30.3)	-69.7 (17.5)
	THC-COOH	35.0	51.9	−41.5 (10.3)	-11.9 (11.8)	20.5	45.7	−59.8 (19.1)	-50.0 (16.9)
Urine	THC	26.6	34.5	−42.1 (17.3)	-54.9 (20.5)	15.4	15.6	–	-
	THC-COOH	32.4	15.9	−16.1 (12.2)	-36.7 (28.1)	27.2	10.1	–	-
THC: Δ9 -tetrahydrocannabinol, THC-COOH: 11-nor-9-carboxy-THC									

Table 5. THC concentration in 20 oral fluid specimens after applying two extraction procedures: solid phase extraction (SPE) or MIP-pill extraction, and imprecision between methods (%CV)

Case	THC (ng/mL) with SPE	THC (ng/mL) with MIP-pill extraction	Imprecision between methods (%CV)
1	292.1	142.0	48.9
2	104.2	75.1	22.9
3	308.3	244.6	16.3
4	54.9	50.0	6.6
5	1193.4	1,225.9	1.9
6	278.9	283.7	1.2
7	790.5	927.5	11.3
8	74.0	79.9	5.4
9	16,334.1	16,621.6	1.2
10	466.0	454.6	1.8
11	179.1	206.2	9.9
12	33.8	35.5	3.5
13	4.7	<LOQ	NA
14	18.9	20.0	4.0
15	116.3	111.6	2.9
16	80.3	83.1	2.4
17	216.2	204.0	4.1
18	210.5	213.6	1.0
19	26.4	22.2	12.2
20	17.7	14.7	13.1
THC: Δ9 -tetrahydrocannabinol			

Table 6. THC-COOH concentration found in 11 urine specimens after applying two extraction procedures (with and without hydrolysis): liquid–liquid extraction (LLE) or MIP-pill extraction, and imprecision between methods (%CV)

Case	THC-COOH (ng/mL) with LLE		THC-COOH (ng/mL) with MIP-pill extraction		Imprecision between methods (%CV)	
	w/o hydrolysis	w/ hydrolysis	w/o hydrolysis	w/ hydrolysis	w/o hydrolysis	w/ hydrolysis
1	2.8	108.0	2.8	120.9	0.5	8.0
2	10.9	134.9	11.6	119.1	4.2	8.8
3	3.9	115.0	4.4	126.5	8.9	6.7
4	0.0	60.7	0.0	60.7	-	0.0
5	2.6	127.9	2.4	119.5	4.8	4.8
6	144.0	367.6	149.4	323.7	2.6	9.0
7	29.4	185.1	30.1	176.2	1.7	3.5
8	33.1	80.8	29.3	71.0	8.7	9.1
9	8.6	104.7	8.8	107.6	1.7	1.9
10	19.7	179.8	18.9	178.6	2.9	0.5
11	39.0	117.7	44.7	109.5	9.6	5.1
THC-COOH: 11-nor-9-carboxy- Δ9 -tetrahydrocannabinol						

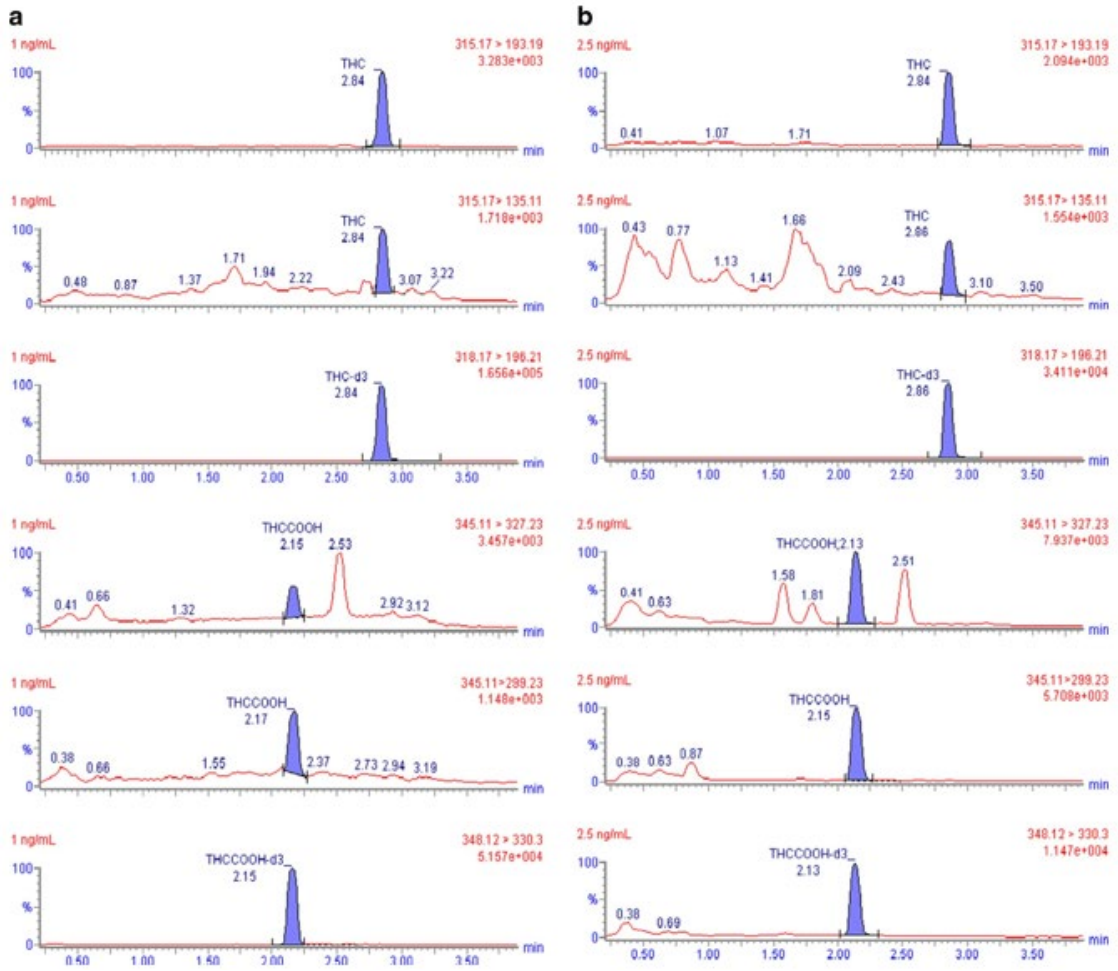

Figure 1. THC and THC-COOH in oral fluid (1A) (1 ng/mL) and urine (1B) (2.5 ng/mL) at the LOQ