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Simultaneous Quantification of Δ^9 -Tetrahydrocannabinol, 11-nor-9-carboxy-Tetrahydrocannabinol, Cannabidiol and Cannabinol in Oral Fluid by MicroFlow-Liquid Chromatography-High Resolution Mass Spectrometry

Marta Concheiro^a, Dayong Lee^a, Elena Lendoiro^b, and Marilyn A. Huestis^{a,*}

^aChemistry and Drug Metabolism, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, 251 Bayview Boulevard, Suite 200, Room 05A721, Baltimore, MD 21224, USA

^bServicio de Toxicología Forense, Dpto. Anatomía Patológica y Ciencias Forenses, Facultad de Medicina, Universidad de Santiago de Compostela C/San Francisco s/n, Santiago de Compostela (A Coruña) 15782, Spain

Abstract

Δ^9 -tetrahydrocannabinol (THC) is the primary target in oral fluid (OF) for detecting cannabis intake. However, additional biomarkers are needed to solve interpretation issues, such as the possibility of passive inhalation by identifying 11-nor-9-carboxy-THC (THCCOOH), and determining recent cannabis smoking by identifying cannabidiol (CBD) and/or cannabinol (CBN). We developed and comprehensively validated a microflow liquid chromatography (LC)–high resolution mass spectrometry method for simultaneous quantification of THC, THCCOOH, CBD and CBN in OF collected with the Oral-Eze® and Quantisal™ devices. One mL OF-buffer solution (0.25mL OF and 0.5mL of Oral-Eze buffer, 1:3 dilution, or 0.75mL Quantisal buffer, 1:4 dilution) had proteins precipitated, and the supernatant subjected to CEREX™ Polycrom™ THC solid-phase extraction (SPE). Microflow LC reverse-phase separation was achieved with a gradient mobile phase of 10mM ammonium acetate pH 6 and acetonitrile over 10 min. We employed a Q Exactive high resolution mass spectrometer, with compounds identified and quantified by targeted-MSMS experiments. The assay was linear 0.5–50 ng/mL for THC, CBD and CBN, and 15–500 pg/mL for THCCOOH. Intra- and inter-day and total imprecision were <10.8% CV and bias 86.5–104.9%. Extraction efficiency was 52.4–109.2%, process efficiency 12.2–88.9% and matrix effect ranged from –86 to –6.9%. All analytes were stable for 24h at 5°C on the autosampler. The method was applied to authentic OF specimens collected with Quantisal and Oral-Eze devices. This method provides a rapid simultaneous quantification of THCCOOH and THC, CBD, CBN, with good selectivity and sensitivity, providing the opportunity to improve interpretation of cannabinoid OF results by eliminating the possibility of passive inhalation and providing markers of recent cannabis smoking.

Corresponding author, Professor Dr. Dr. Marilyn A. Huestis, Chief, Chemistry and Drug Metabolism, Biomedical Research Center (BRC), National Institute on Drug Abuse, NIH, 251 Bayview Blvd., Suite 200 Room 05A-721, mhuestis@intra.nida.nih.gov, Phone: 443-740-2524, Fax: 443-740-2823.

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Keywords

Oral fluid; THC; THCCOOH; CBN; CBD; LC-MS

1. Introduction

Cannabis is the most widely used illicit drug. According to the United Nations 2012 World Drug Report, 170 million people (3.8% of world's adult population) consumed cannabis at least once a year in the recent period [1]. More than 100 cannabinoids were identified in the cannabis plant [2], with Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) the most extensively studied. THC is the primary psychoactive constituent, metabolized to equipotent 11-hydroxy-THC (11-OH-THC) and non-psychoactive 11-nor-9-carboxy-THC (THCCOOH) [3–4]. Further phase 2 metabolic processes include conjugation of all three analytes with glucuronic acid and possibly sulfate, facilitating elimination [5]

Oral fluid (OF) monitoring to document drug exposure in workplace, drug treatment, pain management, and driving under the influence of drugs (DUID) programs has greatly increased. The main advantages of monitoring drug intake with this alternative matrix are an easy, non-invasive and gender-neutral collection under direct observation, reducing the opportunity for adulteration, and, depending upon cutoff concentration, a short drug detection window better reflecting recent consumption. Disadvantages of OF testing include small sample volume, reduced salivation after intake of drugs with parasympathomimetic properties, and low concentrations requiring high sensitivity.

The Substance Abuse and Mental Health Services Administration (SAMHSA) and the Driving Under the Influence of Drugs, Alcohol and Medicines (DRUID) European Union project list THC as the only target analyte for detection of cannabis use in OF at 2 and 1 ng/mL confirmation cut-off concentrations, respectively. However, only requiring THC in OF may yield interpretation issues. THC was detected in OF from non-smokers passively exposed for 3 h to cannabis smoke from multiple smokers in an Amsterdam cannabis café [6]. Detection of THCCOOH in OF provides clues of active cannabis smoking, as it is not present in cannabis smoke [7–8] and passively diffuses from blood into OF [9]. However, THCCOOH quantification requires highly sensitive analytical methods as it is present in low pg/mL concentrations. Thus, the detection of THCCOOH in OF offers a means of ruling out passive cannabis exposure, but requires highly sensitive detection.

THC also was detected in chronic daily cannabis smokers' OF for up to 48 hours, and in some cases for as long as 28 days during monitored abstinence [10]. THCCOOH also was detected in low concentrations in some of these smokers' OF for an extended period (up to 4 weeks of abstinence) [10]. Recent studies by our group suggested the inclusion of CBD and CBN as markers of recent consumption (< 6h) [11–12]. Therefore, there are advantages to interpreting cannabinoid OF results when multiple cannabinoid concentrations are available, substantiating the importance of including these cannabinoids in the analytical method.

Methods are available for the quantification of THCCOOH at low pg/mL concentrations in OF. These methods were developed by gas chromatography tandem mass spectrometry (GC-MSMS) [13], two-dimensional gas chromatography-mass spectrometry (2D GC-MS) [14–15], and liquid chromatography tandem mass spectrometry (LC-MSMS) [16–18][19].

These previously published methods included THCCOOH only [13,20], or THCCOOH and THC [16–17]. Milman et al's [15] 2D GC-MS method quantified THC, CBD, CBN and THCCOOH. In that method, two different elutions from one OF sample and two different

injections on two instruments, one with negative chemical ionization for THCCOOH and the other with electron impact for THC, CBD and CBN, were required. Fabritius et al [19] developed a method by LC-MSMS after liquid-liquid extraction for the determination of THC, 11-OH-THC, CBN, CBD, THC-A and THCCOOH in OF. In this method, THCCOOH LOQ was 80 pg/mL.

LC-MSMS is an analytical technique of increasing interest in forensic and clinical toxicology laboratories, as simultaneous analysis of analytes with different polarities can be achieved with good sensitivity and without derivatization of analytes with low vaporization, saving time and cost. However, Lee et al [18] and Coulter et al [16] required THCCOOH derivatization to increase method sensitivity and to achieve a 10 pg/mL limit of quantification (LOQ) by LC-MSMS.

We developed and extensively validated a microflow LC-high resolution MS method for simultaneous determination of THC, CBN, CBD and THCCOOH in OF collected with two commonly utilized devices in the United States, the Quantisal™ and Oral-Eze® devices. All analytes were eluted by microflow LC-high resolution MS in the same analytical run with one injection. THCCOOH was detected with high sensitivity (LOQ 15 pg/mL) and no derivatization procedure was required. As proof of the method, we analyzed 30 authentic OF specimens collected with Quantisal (n=15) and Oral-Eze (n=15) devices from controlled administration studies.

2. Materials and methods

2.1. Chemicals and materials

THC, THCCOOH, CBD, CBN (1 mg/mL) and internal standards (IStd) THC-d₃, THCCOOH-d₃, CBD-d₃ and CBN-d₃ (100 µg/mL) were obtained from Cerilliant (Round Rock, TX, USA). Solid phase extraction (SPE) was performed with CEREX® Polycrom™ THC (3cc/35 mg) columns from SPEware Corporation (Baldwin Park, CA, USA). Glacial acetic acid, formic acid, ammonium acetate, methanol and acetonitrile were acquired from Fisher Scientific (Fair Lawn, NJ, USA). Hexane and ethyl acetate were purchased from Sigma (Milwaukee, WI, USA), and ammonium hydroxide 28–30% from JT Baker (Phillipsburg, NJ, USA). All solvents employed in the extraction were high-performance liquid chromatography (HPLC) grade and in the chromatographic system, LC-MS grade. Water was purified-in-house by an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA, USA). Quantisal devices for the collection of oral fluid and Quantisal transport buffer for diluting calibrator standards were obtained from Immunalysis Corporation (Pomona, CA, USA). Oral-Eze devices and Oral-Eze transport buffer were from Capitol Vial, Inc. (Auburn, AL, USA). Low-retention 1.5 mL microcentrifuge tubes were acquired from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation

Microflow LC-high resolution MS was performed on a Thermo Scientific Ultimate 3000 RSLCnano system operated in microflow mode coupled to a Thermo Scientific Q Exactive Mass spectrometer (Thermo Scientific, Fremont, CA). The Ultimate 3000 RSLCnano system consisted of a degasser, a tertiary loading pump, a binary eluting pump, a column oven and an RS Autosampler. SPE was performed using a positive pressure manifold CEREX Positive Pressure SPE Processor from SPEware Corporation (Baldwin Park, CA, USA). Evaporation under nitrogen was completed using a TurboVap LV® evaporator from Zymark (Hopkinton, MA, USA).

2.3. Preparation of standard solutions

Calibrators' working solutions at 2.5, 5, 10, 25, 50, 100 and 250 ng/mL for THC, CBD and CBN, and 0.075, 0.1, 0.25, 0.5, 1 and 2.5 ng/mL for THCCOOH were prepared by appropriate dilution in methanol. The combined IStd solution (THC-d₃, CBD-d₃ and CBN-d₃ at 50 ng/mL and THCCOOH-d₃ at 0.5 ng/mL) was prepared in methanol. The low QC working solution at 7.5 ng/mL for THC, CBD and CBN, and at 225 pg/mL for THCCOOH; medium QC working solution at 37.5 ng/mL for THC, CBD and CBN, and at 500 pg/mL for THCCOOH; and high QC working solution at 150 ng/mL for THC, CBD and CBN, and at 1,500 pg/mL for THCCOOH were prepared in methanol from different lots than those employed for calibrators.

2.4. Preparation of calibrators and controls

The calibrators and controls were prepared spiking the calibrators' and controls's working solutions in drug-free OF-buffer mixtures (0.25 mL OF + 0.5 mL Oral-Eze buffer; 0.25 mL OF + 0.75 mL Quantisal buffer). The drug-free OF samples were obtained from healthy volunteers.

2.5. Specimen procedure

One mL OF-Quantisal buffer mixture (0.25 mL oral fluid + 0.75 mL buffer) or 0.75 mL OF-Oral-Eze buffer mixture (0.25 mL oral fluid + 0.5 mL buffer) was combined with 25 μ L IStd solution and gently vortexed. Proteins were precipitated by addition of 1 mL ice-cold acetonitrile, followed by vortexing. Tubes were centrifuged at $1,855 \times g$ for 7 min to pellet protein, and supernatants were decanted onto CEREX Polycrom THC extraction columns preconditioned with methanol (1 mL). Columns were washed with 3 mL deionized water/acetonitrile/ammonium hydroxide (85:15:1, v/v/v) and dried via positive pressure at maximum flow 28 psi for 20 min. The elution was performed with 3 mL hexane/ethyl acetate/acetic acid (75:25:1, v/v/v). Eluates were evaporated to dryness under a stream of nitrogen at 35°C in a Zymark TurbovapLV evaporator. Samples were reconstituted in 200 μ L of mobile phase 70 A: 30 B, v/v, vortexed briefly, transferred to a low-retention microcentrifuge tube, and centrifuged at $17,000 \times g$ at 4°C for 5 min. Finally, supernatants were transferred into screw top autosampler vials containing 0.2 mL glass inserts.

2.6. Microflow Liquid Chromatography

Chromatographic separation was achieved with a Hypersil Gold aQ 50 $\times$ 0.5 mm 3 μ m column (Thermo Scientific, Fremont, CA) with gradient elution with mobile phase A (10 mM ammonium acetate adjusted to pH 6 with 1% formic acid) and B (15% methanol in acetonitrile) at a 30 μ L/min flow rate at 35°C. The initial composition (30% B) was maintained for 0.5 min, B was increased from 30% to 90% over 5.5 min, held at 90% for 1.5 min, and returned to initial conditions over 0.5 min. A 2 min equilibration followed, yielding a total run time of 10 min.

2.7. Mass Spectrometry

The Q Exactive mass spectrometer was equipped with heated electrospray ionization source (HESI-II) and operated in the negative ionization mode. The spray voltage was 3 kV, capillary temperature 270°C, heater temperature 120°C, S-lense RF level 60, sheath gas flow rate 20 and auxiliary gas flow rate 2 (arbitrary units). Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the higher energy collision dissociation (HCD) cell, and as the damping gas in the C-trap. The instrument was calibrated in the positive and negative mode every 48 h. The mass spectrometer acquired a targeted-MSMS scan at a resolution of 17,500 (fwhm at m/z 200). Using this scan mode, the precursor ions are selected in the quadrupole (343.2 for THCCOOH, 346.2 for THCCOOH-

d₃, 313.2 for THC and CBD, 316.2 for THC-d₃ and CBD-d₃, 309.2 for CBN, 312.2 for CBN-d₃) using a 3 *m/z* window and subsequently fragmented in the HCD cell. A full scan of all fragmented ions originating from the precursor ion was performed, and specific product ions were utilized for data analysis (Table 1). The targeted-MSMS experiments were segmented in 2 windows, one for THCCOOH and the other for THC, CBD and CBN, to allow at least 12 points per peak.

2.8. Identification criteria

Identification criteria included RT within ± 0.2 min of average calibrator RT, presence of 2 product ions, and ion ratio between the quantifying ion and the qualifier ion within $\pm 20\%$ of that established by the calibrators.

2.9. Validation

Validation parameters included linearity, limits of detection (LOD) and quantification (LOQ), imprecision, accuracy, extraction efficiency, process efficiency, matrix effect, selectivity, carryover, dilution integrity and autosampler stability studies. A complete validation was performed for both the Quantisal and Oral-Eze collection device buffers. Linearity was determined by least-squares regression with $1/\times$ or $1/\times^2$ weighting. Acceptable linearity was achieved when the coefficient of determination was at least 0.99 and calibrators quantified within $\pm 15\%$ of target, except LOQ within $\pm 20\%$. LOD and LOQ were evaluated with decreasing analyte concentrations in drug-fortified OF-buffer mixtures. LOD was defined as the lowest concentration with acceptable chromatography, presence of all product ions with signal-to-noise ratios of at least 3, correct ion ratio and a retention time (RT) within ± 0.2 min of the average calibrator RT. LOD was evaluated fortifying oral fluid samples from 3 different sources. LOQ was the lowest concentration that met LOD criteria and a signal-to-noise ratio of at least 10, imprecision lower than 20%, and bias between 80–120%. LOQ was evaluated by 5 replicates on 3 different days with OF samples from different sources each day.

Imprecision and bias were determined at 3 concentrations (low, medium and high QCs) with 5 replicates on 4 different days ($n=20$). Imprecision, expressed as coefficient of variation (%CV) of the measured values, was expected to be less than 20%. We calculated pooled intra-day, inter-day, and total imprecision as defined by Chesher [21]. According to the author [21], within-run imprecision is subtracted from the standard deviation of the mean run averages to obtain a pure between-run component of imprecision. The estimates of imprecision standard deviation within-day (SW), between-day (SB), and total (ST), were:

$$SW = \sqrt{\text{MeanSquareWithinDay}}$$

$$SB = \sqrt{\text{MeanSquareBetweenDay} - \text{MeanSquareWithinDay}/n}$$

$$ST = \sqrt{SW^2 + SB^2}$$

In the SB equation, n is the number of replicates. It is possible for mean square between-day imprecision to be lower than mean square within day, with the quantity under the square-root sign being negative in this case. If this occurs, SB is set to 0, and inter-day imprecision

is then 0. Bias was evaluated as the percentage of target concentration ($n=20$), and was required to be 80–120%.

Extraction efficiency for each analyte was measured at each QC concentration. Blank samples were fortified with QC solution before and after sample extraction. Percent extraction efficiency was expressed as mean analyte area of samples ($n=5$) fortified with control solution before sample extraction divided by mean area of samples ($n=5$) with control solution added after sample extraction. Matrix effect was assessed by comparing analyte peak areas in 10 different blank extracted samples fortified with QC solutions after sample extraction, to peak areas of samples at the same nominal concentrations prepared in mobile phase (neat, $n=3$). Matrix suppression or enhancement was calculated as follows: $(100 \times \text{mean peak area of fortified samples after extraction} / \text{mean peak area of neat samples}) - 100$. Process efficiency examined the overall effect of extraction efficiency and matrix effect on quantification of analytes of interest. Mean analyte peak areas of 5 samples fortified before extraction were compared with mean peak areas of 3 neat samples prepared in mobile phase at the same concentration.

Interferences from endogenous matrix components were evaluated by analyzing OF samples from 10 healthy non-drug-consuming subjects. IStd was added and blank OF samples were analyzed as unknowns. Endogenous interferences were considered insignificant if analytes of interest were $< \text{LOD}$. Method specificity was demonstrated by fortifying low QC samples with high concentrations (1,000–2,000 ng/mL) of potentially interfering licit and illicit drugs. Drugs and metabolites examined included cocaine, opiates, amphetamines, synthetic cannabinoids, and medicines. A complete list of exogenous interferences evaluated is included in the supplementary table. Sufficient specificity was achieved if analytes of interest quantified within $\pm 20\%$ of low QC target concentrations.

Lack of carryover was demonstrated by injecting IStd-fortified blank samples immediately after samples spiked at 100, 500 and 1,000 ng/mL for THC, CBD and CBN and at 1, 5 and 10 ng/mL for THCCOOH. Carryover was considered negligible if the measured concentration was $< \text{LOD}$. Dilution integrity was evaluated by diluting samples containing 60, 1,500 and 6,000 ng/mL for THC, CBD and CBN, and 0.6, 15 and 60 ng/mL for THCCOOH, with blank OF-buffer mix to achieve 1:2, 1:50 and 1:200 dilutions. Samples at 60 ng/mL for THC, CBD and CBN, and 0.6 ng/mL for THCCOOH were diluted 1:2; samples at 1,500 ng/mL for THC, CBD and CBN, and 15 ng/mL for THCCOOH were diluted 1:50; and samples at 6,000 ng/mL for THC, CBD and CBN, and 60 ng/mL for THCCOOH were diluted 1:200. After IStd addition, samples were extracted as described previously. Dilution integrity was maintained if samples quantified within $\pm 20\%$ of 30 ng/mL for THC, CBD and CBN, and 0.3 ng/mL for THCCOOH after 1:2, 1:50 and 1:200 dilutions.

Autosampler stability was evaluated by injecting reconstituted low, medium and high QC samples stored 24 h at 5°C on the autosampler ($n=3$). Stability was considered acceptable if QC samples quantified within $\pm 20\%$ of freshly prepared QC samples ($n=3$).

2.10. Method application

The method was employed to quantify THC, THCCOOH, CBD and CBN in 30 authentic OF specimens collected with Quantisal ($n=15$) and Oral-Eze ($n=15$) devices from 2 different protocols. Protocols were approved by the Johns Hopkins Institutional and by the National Institute on Drug Abuse Review Boards, respectively, and participants provided written informed consent. The Quantisal specimens were obtained from cannabis users enrolled in a study to examine oral THC (dronabinol®) administration on treatment of cannabis use disorders. In this study, participants resided on a closed research unit for 51 days, and received daily 0, 30, 60, and 120 mg oral THC in divided doses for 5 days. Each medication

session was separated by 9 days of *ad libitum* cannabis smoking [22]. We analyzed 15 OF specimens from 5 participants. These participants (n = 5) were chronic, daily cannabis smokers (all male and African American, aged 25–52 years) who reported cannabis smoking 29–30 days in the past month. Average frequency of cannabis smoking was 2.0–5.6 times per day; median (range) time since last smoking was 15.5 (1.1–109.4 h); and median (range) since last dronabinol dose was 2 (0–4) h. The 15 Oral-Eze specimens were obtained from 3 cannabis users who smoked a single cannabis cigarette (6.8% THC) *ad libitum*. These participants (n = 3) were self-reportedly occasional smokers (2 males, 1 female, African American/Mixed, aged 22–36 years) who reported cannabis smoking 0–2 days in the past 14 days. Average amount of cannabis cigarettes smoked was 4–6 joints per month, and median (range) time since smoking was 8 (3–21) h.

3. Results

All reported concentrations are neat OF concentrations prior to dilution with collection device preservation buffer. Linearity of analyte-to-IStd peak area ratio versus theoretical concentration was verified in OF samples with $1/\times$ or $1/\times^2$ weighted linear regression for THCCOOH and CBD, and for THC and CBN, respectively. Curvature tested on a set of 4 calibration curves yielded determination coefficients (r^2) above 0.99, with residuals within $\pm 15\%$ for calibrators, and within $\pm 20\%$ for LOQ. LOD and LOQ were 0.5 ng/mL for THC, CBD and CBN, and 0.015 ng/mL for THCCOOH. These results are summarized in Table 2.

Imprecision and bias were satisfactory at all three QC concentrations and with both the Quantisal and Oral-Eze buffers (Table 3). For Quantisal samples, extraction efficiencies ranged from 72.2 to 96.7%, process efficiencies from 12.2 to 49.1%, and matrix effect (ion suppression) from –86 to –38.3%. For Oral-Eze, extraction efficiencies ranged from 52.4 to 109.2%, process efficiencies from 28.7 to 88.9%, and matrix effect (ion suppression) from –48.4 to –6.9%. Matrix variation among 10 different oral fluid sources was <23.8%. Data are shown in Table 4.

Under the described conditions, no interferences from any extractable endogenous OF compound were observed (n=10). Method selectivity was demonstrated by adding high concentrations of potentially interfering licit and illicit drugs and metabolites to low QC samples. All analytes quantified within $\pm 20\%$ of target, indicating no interferences with analytes of interest. No carryover was detected for THCCOOH; however, 0.1% carryover was detected for THC, CBD and CBN.

The ability of the method to accurately quantify specimens containing high concentrations of analytes was evaluated by diluting 1:2, 1:50 and 1:200 with blank OF-buffer mix. Diluted samples accuracy was 85.1–97.8% (n=3 for each dilution factor) for Quantisal and 77.1–105.8% (n=3 for each dilution factor) for Oral-Eze samples. Analytes were stable in the autosampler at 5°C for 24 h, %difference was from –7.7 to 12.6%.

Thirty oral fluid specimens, 15 collected with Quantisal and 15 collected with Oral-Eze from controlled administration studies, were analyzed as proof of the method. Results obtained are shown in Fig.1 and 2, and Table 5.

4. Discussion

We developed a highly sensitive and specific microflow LC–high resolution MS method for the determination of THC, CBD, CBN and THCCOOH in OF collected with Quantisal and Oral-Eze collection devices. The method was extensively validated with each of the collection devices' buffers. It was necessary to validate all methods for each collection

buffer, as ratios of OF to buffer and buffer components vary by device. These changes may affect important validation parameters such as LOQ, matrix effect and extraction efficiency. In order to mimic collection conditions, drug-free OF-buffer mixtures (1:3, v/v, for Oral-Eze; 1:4, v/v, for Quantisal) were utilized as matrix for calibrators, controls and dilution integrity experiments.

OF can be collected by direct expectoration (spitting) or absorption onto a permeable pad or sponge, with or without an agent that stimulates OF production. Expectoration is less expensive and OF concentrations of xenobiotics can be directly measured. However, its collection can be difficult, of low volume and OF could be highly viscous, yielding problems during sample extraction. The chosen collection devices, Quantisal and Oral-Eze, have a blue indicator that becomes visible when 1 mL OF was collected. Both buffers avoid specimen degradation and bacterial growth.

THCCOOH determination in OF is becoming an important biomarker of active cannabis consumption. THCCOOH results from THC metabolism and is not present in the cannabis smoke [7]. Its concentrations in OF are in the pg/mL levels, therefore analytical methods have to be highly sensitive. A 20 pg/mL cutoff has been suggested in previous studies as a compromised to detect recent cannabis exposure [10–12]. Several published methods achieved this sensitivity with THCCOOH LOQ from 2 to 10 pg/mL [13,15–18,20], as well as the present method with THCCOOH LOQ at 15 pg/mL.

CBD and CBN also can be included in cannabis OF confirmation methods as markers of recent smoking. Recent studies showed that CBD and CBN windows of detection were 6 h after smoking cigarettes containing 2 mg CBD (0.25%) and 1.7 mg CBN (0.21%) [11–12]. However, absence of CBD and CBN in OF specimens does not rule out recent use. CBD and CBN content varies in cannabis depending on the cannabis strain, amount of sunlight, soil type and other factors [2]. Recent cannabis intake information is highly useful in DUID cases and accident investigations. However, only Milman et al [15] and Fabritius et al [19] determined CBD and CBN along with THCCOOH and THC. Although extracting all the analytes from the same specimen, Milman et al [15] had to perform 2 different elutions and 2 different injections in 2 instruments, one for THCCOOH and another one for THC, CBD and CBN. In the present method, we extracted the four analytes in the same elution step and we analyzed them in the same injection, saving time and costs. Fabritius et al [19] simultaneously determined a wide range of cannabinoids (THC, CBD, CBN, 11-OH-THC, THC-A and THCCOOH) in OF; however, THCCOOH LOQ (80 pg/mL) was much higher than the present method (THCCOOH LOQ 15 pg/mL), and higher than the suggested THCCOOH cut-off (20 pg/mL).

Lee et al [18] and Coulter et al [16] performed THCCOOH derivatization to achieve sufficient sensitivity with UHPLC-Triple quadrupole LC-MSMS systems. Coulter et al [16] monitored 2 characteristic fragments from THCCOOH molecule, but Lee et al [18] included a derivatization reagent (dansyl chloride) fragment as one of the 2 monitored transitions. This fragment monitoring did not contribute to the method specificity. He et al [17] developed a LC-MSMS method for the determination of THCCOOH and qualitative analysis of THC. These authors [17] also used a microflow LC and a Q Exactive instrument as in the present method. For THCCOOH determination, they evaluated the THCCOOH product ion m/z 299.2011. In our method, we monitored the product ions m/z 245.1546 (quantifier) and 191.1067 (qualifier). Although we found that also m/z 299.2011 was the most abundant ion in the neat solution injections, this product ion showed a very low signal to noise ratio and the ion ratio failed at low concentrations (LOQ) in OF samples. The identification of 2 product ions and the correct ion ratio is a mandatory identification requirement for LC-MSMS methods. High quality data are valuable in every forensic and

clinical setting. Inaccurate identification can lead to misinterpretation of data and to inaccurate drug monitoring.

The combination of microflow LC and Q Exactive high resolution mass spectrometer achieved good sensitivity for THCCOOH, with a LOQ in low pg/mL concentration. The lower flow of the microflow pump compared to HPLC/UPLC instruments yielded better signal in ESI. Also, it reduced the source contamination, and substantially decreased the solvent usage and waste generation. Compared to other low flow techniques such as nanoflow, microflow is more robust for biological specimen analysis with regard to small particles disrupting flow and increasing downtime. The mass spectrometer employed in this study, the Q Exactive offers high resolution and high mass accuracy, improving the selectivity and the sensitivity of the system.

In the present method, deuterated analogs for THC, CBN, CBD and THCCOOH were used as ISTDs. Coulter et al [16] employed THC-d₃ as internal standard for THC and also for THCCOOH because deuterated THCCOOH could be contaminated with unlabeled THCCOOH. We checked this possible contamination and we could not detect it. In every run, a negative sample only spiked with the ISTDs mix was analyzed, and THCCOOH was never detected at method's LOD (15 pg/mL). The use of the analyte deuterated analog improves the precision and accuracy of the method, and helps to compensate for the matrix effects.

5. Conclusion

We developed a sensitive and selective microflow LC–high resolution MS method for the simultaneous quantification of THC, CBD, CBN and THCCOOH in OF specimens collected with 2 different OF collection devices, Quantisal and Oral-Eze. Quantification of multiple cannabinoids provides the opportunity to improve interpretation of cannabinoid OF results by eliminating the possibility of passive inhalation and providing markers of recent cannabis smoking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- THCCOOH quantification in oral fluid could eliminate passive inhalation cases.
- CBD and CBN in oral fluid could be markers of recent consumption.
- Sensitive THC, THCCOOH, CBD and CBN quantification in oral fluid by LC-HRMS.
- THCCOOH LOQ was 15 pg/mL, and THC, CBD and CBN LOQ was 0.5 ng/mL.

Fig. 1. LC-MSMS chromatogram of an authentic oral fluid specimen collected with the Quantisal™ device (Specimen A-QT2). The specimen contained 5.4 ng/mL Δ^9 -tetrahydrocannabinol (THC), 72.7 pg/mL 11-nor-9-carboxy-THC (THCCOOH) and 0.7 ng/mL cannabinal (CBN).

Fig. 2. LC-MSMS chromatogram of an authentic oral fluid specimen collected with the Oral-Eze® device (Specimen G-OE4). The specimen contained 16.6 ng/mL Δ^9 -tetrahydrocannabinol (THC), 17.5 pg/mL 11-nor-9-carboxy-THC (THCCOOH), 0.6 ng/mL cannabidiol (CBD) and 1.6 ng/mL cannabinol (CBN).

Table 1

LC-MSMS parameters, retention times (RT) and internal standards (IStd).

Analyte	Precursor ion (m/z)	Product ions (m/z)	NCE (%)	RT (min)
THCCOOH	343.2	191.1067 <u>245.1546</u>	40	4.6
THCCOOH-d ₃	346.2	302.2206	40	4.6
CBD	313.2	179.1067 <u>245.1545</u>	40	6.2
CBD-d ₃	316.2	248.1734	40	6.2
CBN	309.2	171.0804 <u>279.1392</u>	55	6.6
CBN-d ₃	312.2	282.1581	55	6.6
THC	313.2	191.1068 <u>245.1544</u>	40	6.8
THC-d ₃	316.2	248.1735	40	6.8

NCE: Normalized collision energy; RT: retention time; THCCOOH: 11-nor-9-carboxy-THC; CBD: cannabidiol; CBN: cannabinol; THC: Δ^9 -tetrahydrocannabinol.

The quantification transition is underlined.

Table 2

Limits of quantification (LOQ) and linearity results in oral fluid-buffer mixtures. All concentrations are referred to neat oral fluid.

Oral fluid-Quantisal™ buffer					
Analyte	LOQ (ng/mL)	Linearity (ng/mL)	Intercept±SD (n=4)	Slope±SD (n=4)	r ² ±SD (n=4)
THC	0.5	0.5–50	0.0255±0.0068	0.2116±0.0191	0.9957±0.0033
THCCOOH	0.015	0.015–0.5	−0.0201±0.0022	0.0067±0.0004	0.9984±0.0007
CBD	0.5	0.5–50	0.0196±0.0111	0.2042±0.0157	0.9981±0.0020
CBN	0.5	0.5–50	0.0270±0.0130	0.1905±0.0176	0.9917±0.0062
Oral fluid-Oral-Eze® buffer					
Analyte	LOQ (ng/mL)	Linearity (ng/mL)	Intercept±SD (n=4)	Slope±SD (n=4)	r ² ±SD (n=4)
THC	0.5	0.5–50	0.0099±0.0090	0.2116±0.0114	0.9937±0.0033
THCCOOH	0.015	0.015–0.5	−0.0463±0.0169	0.0072±0.0001	0.9987±0.0004
CBD	0.5	0.5–50	0.0103±0.0036	0.2178±0.0092	0.9985±0.0014
CBN	0.5	0.5–50	0.0193±0.0066	0.1863±0.0089	0.9901±0.0065

THCCOOH: 11-nor-9-carboxy-THC; CBD: cannabidiol; CBN: cannabinol; THC: Δ^9 -tetrahydrocannabinol.

Table 3

Pooled intra-, inter- and total imprecision and bias in oral fluid-buffer mixtures.

Oral fluid-Quantisal™ buffer												
Analyte	Pooled intra-day imprecision (n=20, CV)			Inter-day imprecision (n=20, CV)			Total imprecision (n=20, CV)			Bias (n=20, % target)		
	Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
THC	7.4	6.5	5.7	6.2	3.5	4.7	9.6	7.4	7.4	95.1	98.4	98.5
THC COOH	4.9	2.7	2.8	4.1	3.2	3.3	6.4	4.2	4.4	86.5	88	92.2
CBD	6	6.2	4.1	3.9	2.7	3.7	7.1	6.8	5.5	93.3	100.2	98.3
CBN	6.5	5.6	7.5	8.6	6.4	5.1	10.8	8.5	9	97.4	101.4	99.9
Oral fluid-Oral-Eze® buffer												
Analyte	Pooled intra-day imprecision (n=20, CV)			Inter-day imprecision (n=20, CV)			Total imprecision (n=20, CV)			Bias (n=20, % target)		
	Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
THC	4.3	6.5	4	5.8	1.6	2.9	7.3	6.7	5	97.2	96.4	98.8
THC COOH	5.8	3	2.9	4.6	3.1	4.5	7.4	4.3	5.4	92.1	90	91.5
CBD	3.9	4.6	3	1.7	1.7	3.3	4.3	4.9	4.5	94.2	95.8	96.2
CBN	9	7.7	7.1	0	0	4.9	9	7.7	8.6	104.9	103.1	102.7

THCCOOH: 11-nor-9-carboxy-THC; CBD: cannabidiol; CBN: cannabinol; THC: Δ^9 -tetrahydrocannabinol.

Table 4

Extraction efficiency, process efficiency and matrix effect in oral fluid-buffer mixtures.

Oral fluid-Quantisal™ buffer									
Analyte	Extraction efficiency (n=5)			Process efficiency (n=5)			Matrix effect (CV%) (n=10)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
THC	94.3	73.8	91.2	14.7	12.2	14.1	-84.8 (8.6)	-83.9 (12.2)	-84.6 (12.4)
THC COOH	72.6	72.2	79.3	39.2	41	49.1	-44.3 (7.4)	-43.3 (6)	-38.3 (7.4)
CBD	82.3	74.8	75.6	20.5	21.2	19.5	-75.4 (9.8)	-71.4 (9.1)	-75.1 (10.9)
CBN	96.7	77.5	93.4	13.8	12.5	12.7	-85.8 (9.2)	-84.3 (10.8)	-86 (12)
Oral fluid-Oral-Eze® buffer									
Analyte	Extraction efficiency (n=5)			Process efficiency (n=5)			Matrix effect (CV%) (n=10)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
THC	93.9	90.8	101.7	48.3	56.7	62.3	-48.4 (12.6)	-38.6 (6.4)	-39.9 (8.8)
THC COOH	52.8	83.1	92.9	43.4	73.5	88.9	-20.7 (23.8)	-14.2 (14.1)	-6.9 (11)
CBD	54.9	52.4	56.4	28.7	31.7	33.5	-47.6 (7.5)	-41.5 (6.4)	-42.1 (12.1)
CBN	92.1	85.5	109.2	50.5	53.5	66.1	-44.9 (11.1)	-38.2 (13.5)	-40.2 (13.4)

THCCOOH: 11-nor-9-carboxy-THC; CBD: cannabidiol; CBN: cannabinol; THC: Δ^9 -tetrahydrocannabinol.

Table 5

Drug concentrations in 30 authentic oral fluid specimens collected from different participants with Quantisal™ and Oral-Eze® devices. Quantisal specimens were collected from participants (A, B, C, D, and E) after oral THC dosing, and Oral-Eze specimens from participants (F, G and H) after smoking a 6.8% THC cigarette.

Specimen	T since smoking (h)	T since oral THC (h)	Oral THC dose(mg8)	THC (ng/mL)	THCCOOH (pg/mL)	CBD (ng/mL)	CBN (ng/mL)
A-QT1	7	0	40	ND	ND	ND	ND
A-QT2	107.4	0	10	5.4	72.7	ND	0.7
A-QT3	109.4	2	10	7.0	117.1	ND	0.9
B-QT4	108.2	2	40	11.7	365	ND	6.7
C-QT5	71.3	0	20	ND	80.0	ND	ND
A-QT6	1.1	4	40	6.4	152	ND	1.5
A-QT7	108.3	2	0	ND	24.8	ND	ND
D-QT8	11.5	1	20	5.7	ND	ND	4.6
D-QT9	108.5	2	40	ND	252	ND	ND
B-QT10	1.1	-	-	0.7	ND	ND	1.1
B-QT11	1.3	-	-	18.7	79.4	ND	2.0
C-QT12	14.5	0	40	34.8	125	ND	3.2
C-QT13	15.5	1	40	29.8	162	ND	2.9
E-QT14	23.4	3	0	20.6	170	ND	2.0
E-QT15	5.7	3	40	15.7	375	ND	1.9
F-OE1	4	-	-	24.0	ND	0.9	3.2
FOE2	8	-	-	15.5	ND	0.5	2.5
G-OE3	4	-	-	46.5	23.9	1.5	4.4
G-OE4	6	-	-	16.6	17.5	0.6	1.6
G-OE5	8	-	-	13.1	ND	ND	1.4
G-OE6	10.5	-	-	26.0	18.0	0.7	3.4
G-OE7	13.5	-	-	18.4	ND	ND	2.4
H-OE8	3	-	-	8.8	238	0.5	1.1
H-OE9	4	-	-	7.6	254	ND	0.9
H-OE10	5	-	-	13.5	626	0.5	2.4

Specimen	T since smoking (h)	T since oral THC (h)	Oral THC dose(mg8)	THC (ng/mL)	THCCOOH (pg/mL)	CBD (ng/mL)	CBN (ng/mL)
H-OE11	6	-	-	3.1	196	ND	0.8
H-OE12	8	-	-	4.5	291	ND	0.8
H-OE13	10.5	-	-	1.2	130	ND	ND
H-OE14	13.5	-	-	0.8	122	ND	ND
H-OE15	21	-	-	1.2	333	ND	ND

THCCOOH: 11-nor-9-carboxy-THC; CBD: cannabidiol; CBN: cannabinol; THC: Δ^9 -tetrahydrocannabinol; ND: Not detected; T:time; QT: Quantisal; OE: OralEze.