Driving Under the Influence of Cannabis: Pitfalls, Validation, and Quality Control of a UPLC-MS/MS Method for the Quantification of Tetrahydrocannabinol in Oral Fluid Collected With StatSure, Quantisal, or Certus Collector

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Background: "Driving under the influence of drugs" (DUID) has a large impact on the worldwide mortality risk. Therefore, DUID legislations based on impairment or analytical limits are adopted. Drug detection in oral fluid is of interest due to the ease of sampling during roadside controls. The prevalence of Δ^9-tetrahydrocannabinol (THC) in seriously injured drivers ranges from 0.5% to 7.6% in Europe. For these reasons, the quantification of THC in oral fluid collected with 3 alternative on-site collectors is presented and discussed in this publication.

Methods: An ultra-performance liquid chromatography–mass spectrometric quantification method for THC in oral fluid samples collected with the StatSure (Diagnostic Systems), Quantisal (Immunalysis), and Certus (Concateno) devices was validated according to the international guidelines. Small sample volumes of 100–200 μL were extracted using hexane. Special attention was paid to factors such as matrix effects, THC adsorption onto the collector, and stability in the collection fluid.

Results: A relatively high-throughput analysis was developed and validated according to ISO 17025 requirements. Although the effects of the matrix on the quantification could be minimized using a deuterated internal standard, and stability was acceptable according the validation data, adsorption of THC onto the collectors was a problem. For the StatSure device, THC was totally recovered from the collector pad after storage for 24 hours at room temperature or 7 days at 4°C. A loss of 15%–25% was observed for the Quantisal collector, whereas the recovery from the Certus device was irreproducible (relative standard deviation, 44%–85%) and low (29%–80%). During the roadside setting, a practical problem arose: small volumes of oral fluid (eg, 300 μL) were collected. However, THC was easily detected and concentrations ranged from 8 to 922 ng/mL in neat oral fluid.

Conclusion: A relatively high-throughput analysis (40 samples in 4 hours) adapted for routine DUID analysis was developed and validated for THC quantification in oral fluid samples collected from drivers under the influence of cannabis.

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INTRODUCTION

Due to the impact of "driving under the influence of drugs" (DUID) on the worldwide mortality risk,[1] several countries have adopted DUID legislations based on impairment or on analytical limits.[2–6] Although both the laws and the legal procedures relating to DUID vary around the world, most commence with an observation of external signs of impairment or suspected recent drug use by a police officer. Several jurisdictions also provide roadside testing by using rapid immunological tests to screen possible DUID suspects; typically, this on-site testing is performed on oral fluid or urine. However, commonly final judicial measures are only taken after subsequent confirmation of these preliminary screening results by analysis of a secondary specimen from the suspect. This additional sample is analyzed by a more specific laboratory-based technique; typically, gas or liquid chromatography is used in combination with mass spectrometry. In most countries, a blood sample is drawn for the purpose of confirmation. However, some variations exist, for example, Australia, which replaced the blood confirmatory protocol with one based on an oral fluid specimen.[6] Belgium has also followed this example and has recently passed a law to also permit oral fluid confirmation.[2] According to the current Belgian law, a driver will be sanctioned if Δ^9-tetrahydrocannabinol (THC), cocaine, or its metabolite benzoylecgonine (BE), 6-acetylmorphine (6-MAM) or morphine, amphetamine, or 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) is detected in oral fluid at concentrations higher than specified cutoff values. These values range from 5 ng/mL for 6-MAM and morphine; 10 ng/mL for THC and cocaine (or its metabolite BE) and up to 25 ng/mL for amphetamine and MDMA.[2] Thus, for Belgian drivers, the following procedure currently applies: once the initial suspicion of recent drug use has been established by a police officer (based on a checklist of external signs), the driver is apprehended at the roadside and an initial oral fluid screening test (Drugwipe 5^+, Securetec, Germany) is performed. If the screen is positive, immediate administrative measures (disqualification from driving for minimum 12 hours till 15 days) are taken and the confirmatory specimen is collected. To date, this specimen remains as blood;

however, it is to be replaced by oral fluid at the end of 2012. Although oral fluid is now a permitted confirmatory specimen in Belgium, it is still not officially being used, as the Belgian legislator is yet to specify an official oral fluid collection device. The latter is required, instead of collection via expectoration, due to practical considerations in a roadside setting. Furthermore, for a legal procedure, one single type of collector should be used for all persons tested as this collector will have an impact on the final drug concentration owing to device-specific parameters such as drug stability and adsorption issues.

Several articles and reviews concerning bioanalytical procedures for oral fluid have recently been published.[7–9] Classical analytical techniques such as gas chromatography–mass spectrometry or liquid chromatography–mass spectrometry in combination with sample pretreatment such as liquid–liquid (LLE) or solid-phase extraction sample treatment are still very popular for oral fluid analysis. However, the analyst should be aware that both endogenous compounds and any preservatives/buffers included in the particular oral fluid collection devices can have a significant influence on assay performance and reproducibility. For example, additives such as stabilizing salts, nonionic surfactants, and antibacterial agents have been shown to hamper sensitivity, precision, and accuracy of subsequent liquid chromatography–mass spectrometry measurements through matrix effects (ion suppression or ion enhancement).[10,11] For gas chromatography analysis, practical issues such as increased maintenance for various instrumental components, for example, liner, column, and ion source can also occur owing to the collector's buffers.[12]

Another practical consideration, when selecting a particular device, is the amount of specimen collected and whether the oral fluid is collected neat or is diluted by the collector buffers, as low volume (or diluted) collections may result in sensitivity issues or be too limited volume for repeated analysis or testing by a counter-expert. To minimize these issues, a common approach has been to develop multicompound analytical methods for oral fluid.[13–17] However, when doing so, one of the key challenges in method development is to achieve a suitable and reproducible recovery and sensitivity for all compounds of interest. This can be particularly problematic, for example, basic drugs and THC, the active constituent of cannabis. Cannabis is however the most commonly detected drug in the impaired driver population.[18–20] According to the European study "Driving Under the influence of Drugs, Alcohol and Medicines" published recently,[21] the prevalence of THC in seriously injured drivers ranged from 0.5% to 7.6% in Europe. In our own laboratory, 70% of blood samples analyzed from DUID-positive cases from 2010 to 2011 were positive for THC. For this reason, our objective was to develop a robust quantitative method for THC in oral fluid, based on ultra-performance liquid chromatography in combination with tandem mass spectrometry. Oral fluid samples were collected on-site using 3 alternative collection devices, which were to be evaluated. Although a simple sample preparation was used, a robust and relatively high-throughput analysis using a small sample volume was developed that satisfied ISO 17025 requirements. During development and validation, special attention was paid to factors such as matrix effects, THC adsorption onto the collector, and stability in the collection fluid. Some problems were identified and are discussed. In addition, some possible solutions for quality control (QC), according to ISO 17025, are proposed. Finally, the method was applied to a limited number of oral fluid samples collected during roadside controls, and these data are presented.

MATERIALS AND METHODS

For clarity of the article, a summary of the paragraph "Materials and Methods" is described in Figure 1.

Reagents and Materials

THC and its deuterated analogue THC-d_3 were obtained from Promochem (Molsheim, France), and these were supplied in methanol at 1 mg/mL and 100 μg/mL, respectively. Hexane [high-performance liquid chromatography (HPLC) grade] was purchased from Merck (Darmstadt, Germany). Methanol (ultra-performance liquid chromatographic mass spectrometric grade) and water (HPLC grade) were purchased from Biosolve (Valkenswaard, the Netherlands). Ammonium formate >99.995% was purchased from Sigma-Aldrich (Steinheim, Germany). Drug-free oral fluid was obtained from healthy volunteers. The StatSure device (StatSure Diagnostic Systems, Inc, Brooklyn, NY) consisted of a collector pad with an indicator that turns blue on collection of ±1 mL of oral fluid. The collector is thereafter transferred to a tube (supplied) containing 1 mL of stabilizing buffer. The Quantisal (Immunalysis, Pomona, CA) and Certus (Concateno, Abingdon, United Kingdom) devices consist of a collector pad with a blue indicator and a collection tube containing 3 mL of buffer. One milliliter of oral fluid is collected in both cases.

Preparation of Standard Solutions, Calibrators, and QC Samples

A primary stock solution of THC was prepared in methanol at a concentration of 2.0 μg/mL and stored at 4°C. This stock solution was further diluted with methanol to give working solutions at the following concentrations: 0.2, 0.04, and 0.01 μg/mL.

Oral fluid calibrators were prepared by adding an aliquot of the individual working solutions (25–100 μL) to 50 μL of neat oral fluid that was diluted with 50 μL (StatSure) or 150 μL (Quantisal, Certus) of stabilizing buffer to give a final concentration range from 5 to 320 ng/mL of THC in undiluted neat oral fluid.

The primary stock solution of the internal standard (IS) THC-d_3 was prepared in methanol at a concentration of 0.04 μg/mL and was stored protected from light at 4°C.

A batch of QC samples at low (15 ng THC/mL neat oral fluid) and high (240 ng THC/mL neat oral fluid) concentrations were prepared independently by a different laboratory technician, and aliquots of 1 mL were stored at −20°C (for a maximum of 2 months) before analysis. A methanolic mix stock solution (S) comprising 4.0 μg/mL amphetamine and MDMA, 1.5 μg/mL cocaine and BE, 1.0 μg/mL morphine, and 6-MAM and 2.5 μg/mL THC was prepared. For the batch of StatSure QC samples, 60 μL (low) or 960 μL of S was added to 10 mL of blank oral fluid. StatSure buffer was then added to give a final volume of 20 mL. For the Quantisal or

SAMPLE PREPARATION

	Standard	QC	PT	Sample
Stock solutions	Stock 2 µg/mL THC → W1: 0.2 µg/mL; W2: 0.04 µg/mL; W3: 0.01 µg/mL	Stock 2.5 µg/mL THC	/	/

Preparation

50 µL blank neat oral fluid +

StatSure	Quantisal	Certus
50 µL buffer	150 µL buffer	150 µL buffer

+

Cal	V (µL)	W	Conc (ng/mL)
1	25	3	5
2	50	3	10
3	25	2	20
4	50	2	40
5	100	2	80
6	40	1	160
7	80	1	320

StatSure	Quantisal	Certus
10 mL blank neat oral fluid	5 mL blank neat oral fluid	5 mL blank neat oral fluid

+

StatSure		
QC	V (µl)	Conc (ng/mL)
Low	60	15
High	960	240
Quantisal / Certus		
QC	V (µL)	Conc (ng/mL)
Low	30	15
High	480	240

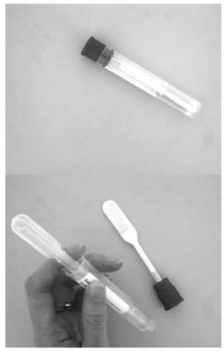
+

StatSure	Quantisal	Certus
Buffer until 20 mL	Buffer until 20 mL	Buffer until 20 mL

StatSure	Quantisal	Certus
100 µL sample	200 µL sample	200 µL sample

50 µL neat oral fluid provided by PT +

StatSure	Quantisal	Certus
50 µL buffer	150 µL buffer	150 µL buffer



StatSure	Quantisal	Certus
100 µL sample	200 µL sample	200 µL sample

Extraction

StatSure	Quantisal	Certus
Standard + 100 µL water + 50 µL IS	Standard + 50 µL IS	Standard + 50 µL IS
QC +100 µL water + 50 µL IS	QC + 50 µL IS	QC + 50 µL IS
PT + 100 µL water + 50 µL IS	PT + 50 µL IS	PT + 50 µL IS
Sample +100 µL water + 50 µL IS	Sample + 50 µL IS	Sample + 50 µL IS

+ 1 mL Hexane → Shaken 15' Centrifugation Evaporation +

200 µL ammonium formate/methanol (10/90 vv)

Analysis

Column	ACQUITY UPLC® BEH C18 2.1 mm x 100 mm x1.7 µm
Gradient	10% ammonium formate buffer (1mM) (A) and 90% methanol (B)
Run time	5 minutes
Flow	0.350 mL/min
Mass	Xevo-Triple Quadrupole mass spectrometer (Waters, Manchester, UK)
Ionization mode	Electrospray positive
Cone gas	Nitrogen, flow: 50 L/h
Desolvation gas	Nitrogen, flow: 1000 L/h, Temperature: 650°C
Collision gas	Argon, $5.8e^{-3}$ mbar

FIGURE 1. Schematic resume of sample preparation of standards, quality control samples (QC), proficiency test samples (PT), and samples collected during roadside police controls.

Certus QC samples, respectively, 30 or 480 µL, respectively, was added to 5 mL of blank oral fluid and buffer was added until a total volume of 20 mL.

In addition, proficiency test samples (Drugs in Oral Fluid Scheme, Lancashire, United Kingdom) were obtained from LGC Standards. For these samples, drugs have been

spiked into neat oral fluid. Before analysis, these oral fluid samples were thawed and then a 50 μL aliquot of the sample was added to either 50 μL of StatSure buffer or 150 μL of Quantisal or Certus buffer.

On-Site Biological Sample Collection

During the course of police controls, an on-site oral fluid screening test using the Drugwipe 5+ (Securetec)was performed as part of the Belgian legal procedure for DUID. If this test was positive, a blood sample was collected from suspected drugged drivers. In addition to this procedure, on a voluntary basis and with an informed consent, several drivers agreed to the collection of an oral fluid sample. Samples were collected with 1 of the 3 previously described collection devices and immediately placed in a refrigerator at 2°C–8°C. Samples were later weighed and then the solutions (a mixture oral fluid/buffer) were transferred to Greiner Bio-One tubes (Frickenhausen, Germany) and stored at −20°C until analysis.

During collection of the oral fluid sample, a dilution of the neat oral fluid will occur depending on the type of collector. In addition, the amount of neat oral fluid collected in realistic situations (eg, roadside) will not always be exactly 1 mL. Therefore, the actual THC concentration in neat oral fluid is calculated using the following Equation 1,[22] with the presumption that 1 mL of neat oral fluid weighs 1 g:

$$C_{THC} = \left[C_{UPLC/MS}\left(V_{buffer} + \left(W - WE\right)\right)\right] / \left[\text{Dilution factor}\left(W - WE\right)\right]$$

$C_{UPLC/MS}$: THC concentration calculated using the calibration curve

C_{THC}: THC concentration in neat oral fluid

V_{buffer}: buffer volume in collector device

W : weight collector after oral fluid collection

WE : mean weight of empty collector
(8.1284 g : StatSure collector;
10.0715 g : Quantisal collector;
10.3273 g : Concateno collector)

Dilution factor : volume of buffer + theoretical collected neat oral fluid volume. (1)

THC Extraction

Fifty microliters of the IS solution (0.04 μg THC-d_3/mL) was added to the samples. THC was extracted from the oral fluid/buffer mixtures via an LLE using hexane in a glass tube (5 mL; Chromacol, Zellik, Belgium). When using the StatSure device, 100 μL of water (HPLC grade) was added to 100 μL of the sample (StatSure dilutes oral fluid with a factor of ½). When using the Quantisal or Certus device, 200 μL of the sample was used (dilution factor of ¼) and no water was added before the extraction. One milliliter of hexane was added, and the "mixture" was shaken during 15 minutes on a Rock-n-Roller from Euroscientific (Lint, Belgium). After centrifugation at 1968*g* for 5 minutes, the organic layer was directly transferred into a high recovery vial (186000384c; Waters, Zellik, Belgium). In this vial, the extract was evaporated until dry using a Christ RVC 2-33IR vacuum centrifuge (QLab, Vilvoorde, Belgium). The extract was redissolved in 200 μL of a 1 mmol/L ammonium formate buffer/methanol (10/90 vol/vol) mixture.

Instrumentation and Chromatographic Conditions: Ultra-Performance Liquid Chromatography–Mass Spectrometric Method

A Xevo-Triple Quadrupole mass spectrometer (Waters, Manchester, United Kingdom) and a MassLynx V 4.1 data processing unit (Waters, Zellik, Belgium) were used. THC was separated from possible interferences on an ACQUITY UPLC BEH C18 2.1 mm × 100 mm × 1.7 μm column (Waters, Milford, MA). A gradient consisting of 10% ammonium formate buffer (1 mmol/L) (A) and 90% methanol (B) at starting point was applied. After 2 minutes, the gradient was set at 100% methanol to clean the column after the elution of THC. After 3 minutes, the initial conditions were set to prepare the column for the next run. Total runtime was 5 minutes, and a flow of 0.350 mL/min was set. The column temperature was 40°C. A full-loop injection system was used, and 5 μL of the extract was injected onto the column. This injection system was rinsed with a "weak" and "strong" wash consisting of, respectively, water (HPLC grade)/methanol (10/90 vol/vol) and pure methanol.

Ionization of THC and its deuterated analogue was achieved using electrospray in positive ionization mode. Nitrogen was applied as desolvation gas and cone gas at a flow rate of 1000 and 50 L/h, respectively. Argon was used as collision gas with a final pressure of 5.8 e^{-3} mbar. The desolvation gas temperature was 650°C, whereas the capillary voltage was 1 kV. To establish the "multiple reaction monitoring" (MRM) reactions, the program IntelliStart was used to optimize the cone voltage and collision energy for all of the transitions shown in Table 1.

Method Validation

The method was validated according to the protocol described in a recent publication by Wille et al.[23] This protocol was based on suggested experiments/parameters and decision criteria described by the US Food and Drug Administration, the Center for Drug Evaluation and Research, and the Center for Veterinary Medicine guidelines,[24] the guidelines provided by the European Medicines Agency (EMEA),[25] and several reviews published by Peters and Maurer and Peters et al.[26,27]

Selectivity was evaluated by analyzing blank oral fluid samples of 6 different individuals and 2 zero samples (IS spiked to blank oral fluid). The evaluation of extraction efficiency and matrix effects was performed according to the method published by Matuszewski et al[28] at low (15 ng/mL)

TABLE 1. MRM Parameters

Compound	Precursor Ion, Da	Product Ion, Da	Cone, V	Collision, eV
THC quantifier	315.23	193.01	32	44
THC qualifier	315.23	122.97	32	34
THC-d_3	318.29	196.06	32	34
CBN quantifier	311.22	222.97	36	30
CBN qualifier	311.22	293.16	36	22
CBD quantifier	315.29	193.06	28	32
CBD qualifier	315.29	122.91	28	42

and high (240 ng/mL) THC concentration (n = 6). Adsorption of THC onto the oral fluid collectors was evaluated at low and high concentrations (n = 6) by collecting spiked oral fluid with the collection pad until the indicator turned blue. Thereafter, the collection pad was put into the collection tube and the oral fluid/buffer mixture was analyzed directly, after 24 hours at room temperature, after 1 week at 4°C, and after 14 days at 4°C. The percentage of THC recovery was calculated comparing the obtained results after those storage conditions with the theoretical spiked value. For evaluation of linearity, 7-point curves (5, 10, 20, 40, 80, 160, and 320 ng/mL) were constructed using internal standardization (n = 8). A linear regression type with a 1/× weighting factor resulted in a calibration model with adequate "goodness of fit."[23] Precision and bias of the method was evaluated over the linear dynamic range at 4 different concentration levels, that is, the limit of quantification (LOQ) (5 ng/mL), 15 ng/mL (low), 60 ng/mL (medium), and 240 ng/mL (high). Each concentration was analyzed in 2 replicates on 8 separate days. Using an analysis of variance calculation, bias, repeatability, and intermediate precision were assessed.[23] Analyte stability determinations comprised freeze/thaw cycle stability (3 cycles) and long-term stability in the oral fluid/buffer mixtures (2 months, −20°C or 4°C) and processed sample stability of the obtained extracts (autosampler stability: 24 hours). All analyte stability determinations were determined at low (15 ng/mL THC) and high (240 ng/mL THC) concentrations with 6 repetitions (3 pools of oral fluid analyzed 2 times). Controls and stability samples were prepared at the same time and analyzed before and after stability treatment. Calculation of the stability parameters was done using the obtained peak areas for autosampler stability. For the freeze/thaw and long-term stability, IS was added just before analysis and the peak area ratios were used. An acceptance interval of 90%–110% was applied for the ratio of the mean stability sample concentration versus the mean control concentration. Moreover, an acceptance interval of 80%–120% of the control sample mean was applied for the 90% confidence interval of the stability samples.

RESULTS

Method Development and Validation

Chromatographic conditions were optimized to separate THC from cannabinol (CBN) and cannabidiol (CBD) as observed in Figure 2. A batch of 40 samples (30 samples, 7 calibrators, and 1 blank and 2 QC samples) could be prepared and analyzed within 4 hours ensuring relatively high throughput in the laboratory.

No chromatographic interferences were observed for THC during validation, as the area under the curve of the "interferences" was lower than 0.80% (StatSure), 0.40% (Quantisal), and 2.29% (Certus) of the area under the curve of a THC peak at the LOQ concentration. Cross fragmentation of THC-d_3 was lower than 0.10% for StatSure, 0.48% for Quantisal, and 1.68% for the Certus device compared with the area of THC at LOQ. Table 2 shows the results of the extraction efficiency experiment, the matrix effects, the mean calibration curve obtained, and the accuracy and stability data. In addition, information concerning THC adsorption onto the collector is described.

Quality Control

Reproducibility of the method was checked by participation in the "Drugs in Oral Fluid Scheme" proficiency test organized by LGC Standards. Only 3 samples were positive for THC. For sample 1, a mean concentration of 2.4 ng/mL in neat oral fluid with a standard deviation of 1.8 for all participating laboratories (n = 3) was obtained. This was beneath our LOQ of 5 ng/mL (semiquantitative results were 3.25 ng/mL using the StatSure buffer, 3.45 ng/mL using the Quantisal buffer, and 3.95 ng/mL using the Certus buffer). For sample 2, the theoretical spiked concentration was 6 ng/mL but both participating laboratories, including ours, had a negative result. For sample 3, the theoretical spiked concentration was 14.7 ng/mL, whereas the mean of the laboratories (n = 11) was 11.7 with a deviation of 6 ng/mL. According to our method, sample 3 contained 10.4 ng/mL.

Daily QC of the method was followed by in-house–prepared QC samples. During validation, the criteria for acceptance of the run were set; QCs should be within 15% of their nominal value. Other criteria were that 75% of the calibrator should fall within 15% of the nominal value (or 20% for the LOQ), and the coefficient of correlation (r^2) of the calibration curve should be higher than 0.990. Before samples could be accepted, the MRM ratio should also be within 20% and the retention time within 5% of the THC peak in the QC.

Analysis of Oral Fluid Samples Collected During On-Site Police Controls

In Table 3, the results obtained during the roadside controls are shown. The collected amount of oral fluid, the THC concentration obtained in the oral fluid/buffer mixture, but also the calculated THC concentrations in the neat oral fluid, and the THC plasma concentration are described. The estimated expanded uncertainty of measurement U for the THC concentration was determined during validation and ranged from 6% for the StatSure to 7% for the Quantisal up to 11% for the Certus analysis using the formula $u = \sqrt{[u\ (\mathrm{RSD_{IP}})^2]}$ with $\mathrm{RSD_{IP}}$ defined as intermediate precision determined during validation.[23] Using the formula to calculate the THC concentration in neat oral fluid from the THC concentration

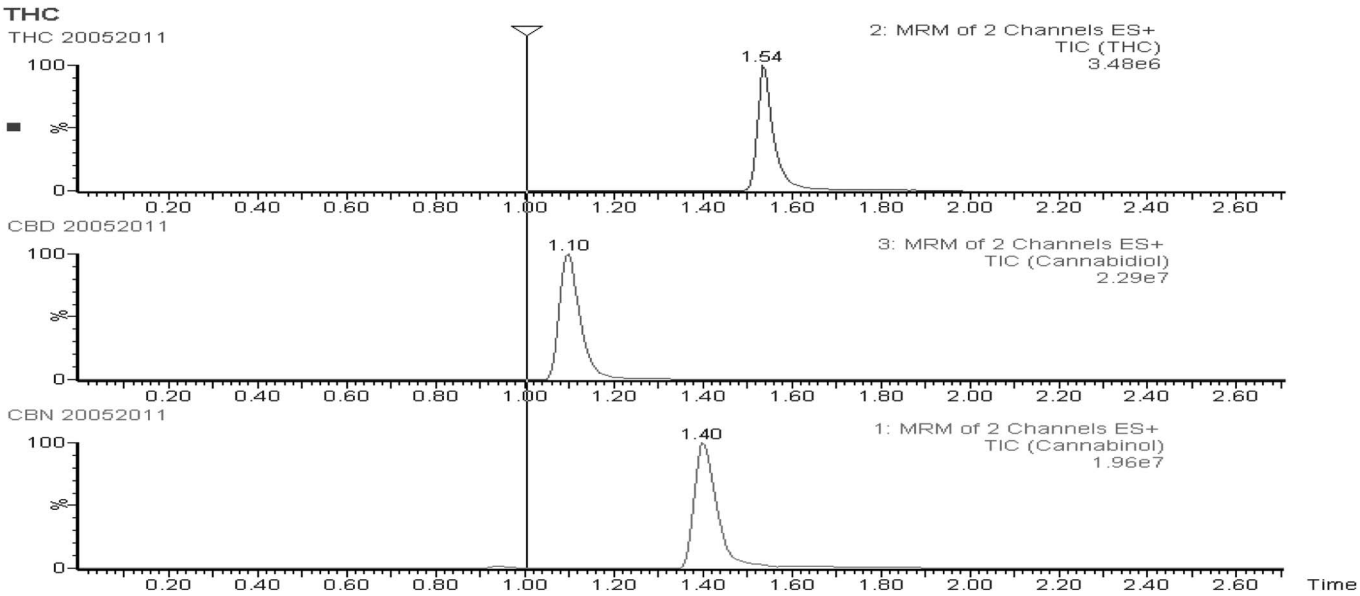


FIGURE 2. Chromatographic separation of THC from CBN and CBD.

determined in the collected mixture (neat oral fluid + stabilizing solution), a combined relative uncertainty of 0.06 ng/mL is calculated via

$$u = \sqrt{[u\ (\mathrm{RSD_{IP}})^2 + u(\mathrm{Vbuffer})^2 + u(\mathrm{weight\ collector})^2]},$$

with $\mathrm{RSD_{IP}} = 0.056$, the relative standard deviation (RSD) of buffer volume = 0.03 (n = 6),[12] the uncertainty of the balance = 0.1 mg, and the RSD of the weight of the empty collector = 0.19 (n = 30).[29]

DISCUSSION

Method Development and Validation

During method development, special attention was paid to the ease of implementation of the method within an ISO 17025 laboratory and to a relative high throughput. In addition, CBD was separated from THC to ensure proper identification and quantification of THC as CBD is also present in oral fluid after recent cannabis consumption and has the same MRM transitions as THC. 11-Hydroxy-Δ^9-THC (OH-THC) and 11-nor-Δ^9-THC-9-carboxylic acid (COOH-THC), the 2 major metabolites of THC, will not interfere in our developed method as the sample preparation and chromatography are not adapted to ensure sufficient sensitivity for both compounds; COOH-THC is only detectable in the picograms per milliliter concentration range in oral fluid.[9,30] It was, however, our aim to develop an easy and robust method to perform fast oral fluid analysis in the DUID context with "a per se" legislation with fixed cutoffs to detect recent drug use linked to the time frame in which impairment normally is observed. COOH-THC can give extra information for interpretation as it rules out passive contamination, and this is certainly necessary when cutoffs of 2 ng/mL are suggested as postulated by the Substance Abuse and Mental Health Services Administration for workplace drug testing or with a cutoff of 1 ng/mL proposed for research on drugged driving.[31] The Belgian legislator has, however, chosen a THC cutoff concentration of 25 ng/mL for screening and 10 ng/mL for confirmation in neat oral fluid to ensure detection of recent cannabis use and to diminish prosecution of a driver due to passive contamination from THC smoke[30,32] or residual THC in chronic smokers.[33] In addition, possible contamination due to cannabis smoke is diminished by taking the sample in a police van and not in the subject's car as THC can adsorb easily on the collector pad.[30] CBD and CBN are screened during the analysis but are not quantified in our method as it is not specified in the Belgian legislation. CBN and CBD could give extra information concerning recent cannabis use; however, CBN is not stable in neat oral fluid or samples collected with the Quantisal device,[34] and CBD content in cannabis plants can vary dramatically,[30] leading to difficulties in interpretation.

Selectivity, defined as the ability to differentiate and quantify the analyte in the presence of other components in the sample, was assured for the 3 evaluated collectors. As the applied method will not report THC concentrations lower than the LOQ, possible coeluting interferences with a response lower than 20% of the LOQ is still acceptable according to the EMEA guidelines.[25] Due to the zero samples, the usability of THC-d_3 as IS could be confirmed, as cross fragmentation was very low.

The extraction efficiency of the LLE using hexane was high, reproducible (RSD $<$ 15% as required by international guidelines), and independent of concentration and oral fluid collection type. This led to the conclusion that this easy and fast extraction technique will lead to robust results. Via calculation of the matrix influencing factor *f*, ion suppression was observed. This ion suppression ranged from 25% to 55% for the StatSure and Quantisal device, respectively. However, the required sensitivity of 5 ng/mL, even for the Quantisal device, is easily obtained. For the Certus device, no matrix effects were observed during the validation. According to our experiments, the matrix effects were for a great deal

TABLE 2. Validation Data

	LOQ 5 ng/mL	Low 15 ng/mL		Medium 60 ng/mL	High 240 ng/mL	
StatSure						
Extraction efficiency (RSD), %	—	99 (4)		—	117 (11)	
f	—	−0.23		—	−0.25	
ME_{IS} (RSD), %	—	99 (3)		—	99 (7)	
Mean calibration curve (r^2)		$Y = 0.925x + 0.163$ (1.000)				
Accuracy, %						
Bias	−0.1	−3.2		−2.2	−1.8	
Repeatability	2.7	1.7		2.6	2.2	
Intermediate precision	4.2	2.7		2.9	2.4	
Autosampler stability						
Mean recovery, %	—	99		—	90	
90% CI of area "stability" samples	—	7532–9891		—	138,145–144,187	
80%–120% of area "control" samples	—	7066–10,599		—	125,082–187,625	
Freeze/thaw stability						
Mean recovery %	—	101		—	95	
90% CI response stability samples	—	0.32–0.35		—	5.1–5.3	
80%–120% response control samples	—	0.26–0.40		—	4.3–6.4	
Long-term stability	—	−20°C	4°C	—	−20°C	4°C
Mean recovery, %	—	106	100	—	102	100
90% CI response stability samples	—	0.31–0.32	0.28–0.29	—	5.21–5.33	5.08–5.22
80%–120% area control samples	—	0.26–0.40		—	4.26–6.38	
Collector recovery (RSD), %						
T = 0	—	60 (7)		—	74 (13)	
T = 24 h, RT	—	100 (6)		—	106 (5)	
T = 7 d, 4°C	—	97 (16)		—	104 (5)	
T = 14 d, 4°C	—	62 (28)		—	69 (19)	
Quantisal						
Extraction efficiency (RSD), %	—	107 (5)		—	99 (7)	
f	—	−0.55		—	−0.44	
ME_{IS} (RSD), %	—	88 (3)		—	94 (9)	
Mean calibration curve (r^2)		$Y = 0.860x + 0.208$ (0.999)				
Accuracy, %						
Bias	−0.5	−2.9		−2.6	−2.1	
Repeatability	4.4	3.6		2.1	2.5	
Intermediate precision	4.5	3.4		2.3	2.8	
Autosampler stability						
Mean recovery, %	—	102		—	95	
90% CI area stability samples	—	4917–5251		—	83,733–86,375	
80%–120% area control samples	—	3983–5975		—	71,731–107,597	
Freeze/thaw stability						
Mean recovery, %	—	99		—	93	
90% CI response stability samples	—	0.26–0.27		—	3.49–4.01	
80%–120% response control samples	—	0.22–0.32		—	3.24–4.84	
Long-term stability	—	−20°C	4°C	—	−20°C	4°C
Mean recovery, %	—	89	101	—	95	110
90% CI response stability samples	—	0.25–0.26	0.27–0.29	—	3.80–3.86	4.32–4.58
80%–120% response control samples	—	0.22–0.32		—	3.24–4.86	
Collector recovery (RSD), %						
T = 0	—	40 (11)		—	43 (26)	
T = 24 h, RT	—	80 (16)		—	74 (12)	
T = 7 d, 4°C	—	85 (15)		—	73 (18)	
T = 14 d, 4°C	—	60 (10)		—	70 (8)	

(*continued on next page*)

TABLE 2. (*Continued*) Validation Data

	LOQ 5 ng/mL	Low 15 ng/mL		Medium 60 ng/mL	High 240 ng/mL	
Certus						
Extraction efficiency (RSD), %	—	120 (4)		—	112 (3)	
f	—	0.11		—	0.02	
ME_{IS} (RSD), %	—	100 (3)		—	102 (4)	
Mean calibration curve (r^2)		$Y = 0.825x + 0.070$ (0.999)				
Accuracy, %						
Bias	3.1	−0.8		−0.7	−1.2	
Repeatability	10.5	5.6		2.4	2.6	
Intermediate precision	12.2	5.6		3.2	3.5	
Autosampler stability						
Mean recovery, %	—	102		—	99	
90% CI response stability samples	—	1972–2082		—	31,337–33,353	
80%–120% response control samples	—	1585–2377		—	26,110–39,166	
Freeze/thaw stability						
Mean recovery %	—	100		—	90	
90% CI response stability samples	—	0.28–0.29		—	3.76–4.10	
80%–120% response control samples	—	0.22–0.32		—	3.24–4.86	
Long-term stability	—	−20°C	4°C	—	−20°C	4°C
Mean recovery, %	—	102	98	—	106	103
90% CI response stability samples	—	0.35–0.35	0.32–0.34	—	5.82–5.98	5.61–5.85
80%–120% response control samples	—	0.27–0.41		—	4.43–6.65	
Collector recovery (RSD), %						
T = 0	—	<LOQ		—	19 (35)	
T = 24 h, RT	—	44 (85)		—	37 (71)	
T = 7 d, 4°C	—	80 (44)		—	29 (31)	
T = 14 d, 4°C	—	<LOQ		—	10 (6)	

CI, confidence interval; *f*, matrix influence factor; ME_{IS}, matrix effect compensated by the IS calculated as percent recovery; r^2, correlation coefficient; T = 0, direct analysis; T = 24 h, RT, after 24 hours at room temperature; T = 7 d, 4°C, after 7 days at 4°C; T = 14 d, 4°C, after 14 days at 4°C.

attributed to the stabilizing buffers in the collectors and not to the differences in blank oral fluid matrices. However, when calculating the results using the deuterated IS, the matrix effect was completely compensated (RSD < 9%). Therefore, matrix-independent quantification could be guaranteed according to these validation data.[23]

Linear calibration curves using 1/× weighting were applied in daily routine. This calibration resulted in a goodness of fit with percentage of relative error lower than 15% for the calibrators except for the LOQ (<20%). The results demonstrated in Table 2 give the mean slope and intercept for 8 curves analyzed on 8 different days.

Accuracy of the method was proven as bias, repeatability, and intermediate precision were acceptable according to the international guidelines of 15%. LOQ could be fixed at 5 ng/mL as bias and imprecision were lower than 20%. From our validation data, it was clear that a lower LOQ could be obtained. However, as the Belgian legislator[2] has determined a cutoff of 10 ng/mL, an LOQ of 5 ng/mL was appropriate.

No instability of THC was observed in the extracts after 24 hours in the autosampler, meaning that samples could be reanalyzed the day after if a problem (eg, leakage of the column) occurred. In addition, THC was stable in the oral fluid sample collected with StatSure, Quantisal, or Certus after 3 freeze–thaw cycles and after storage at −20°C for 2 months. These results demonstrate that a reanalysis of the sample for contraexpertise purposes is no issue as long as it has been stored in the freezer in polypropylene tubes without the collection pad. However, although the stabilizing solution in the oral fluid collector will prevent THC degradation by stabilizing oral fluid pH and inhibiting enzymatic degradation, a recent publication by Lee et al[34] has demonstrated intersubject THC stability. In our study, only 3 pools of oral fluid were evaluated.

The choice of the collection device has a huge impact on the THC concentration in oral fluid due to adsorption onto the collector. Moreover, the storage/transportation conditions of the collected oral fluid sample have an influence on the THC recovery from the collector. During roadside oral fluid testing, police officers will take an oral fluid sample and will have to transfer this sample to the laboratory. In general, a collected sample will therefore not be analyzed directly after sampling (T = 0). The StatSure and Quantisal collection devices are acceptable for THC analysis as the recovery is high (>73%), concentration is independent and reproducible (RSD < 18%) under realistic transportation conditions, and a maximum 24 hours of room temperature during police controls and 7 days at 4°C for transportation. The StatSure has a 100% recovery, probably due to the specific design of the collector pad that can be easily and hygienically separated

TABLE 3. THC Concentrations in Oral Fluid and Plasma Obtained From Drivers Under the Influence of Cannabis

Sample	1	2	3	4	5	6	7
Collection device	StatSure	Certus	Quantisal	Quantisal	Quantisal	Quantisal	Quantisal
Oral fluid volume, mL	−0.27	1.13	0.38	0.58	0.33	0.43	0.36
Collection time							
Oral fluid	05:20 AM	07:30 AM	06:10 AM	00:45 AM	05:05 AM	—	—
Blood	—	—	06:30 AM	01:05 AM	05:45 AM	—	—
THC oral fluid concentration, ng/mL							
Analysis	691.9	8.6	36.5	594.2	18.5	141.9	237.6
Calculated	*x*	7.8	81.4	921.6	47.0	280.8	554.7
THC plasma concentration, ng/mL	28.3	7.7	3.2	6.5	9.5	—	—

from the collection device and squeezed to obtain all the collected oral fluid. The Certus collector is not ideal for THC detection in oral fluid samples due to the high degree of adsorption and the irreproducible results (Table 2). In addition, the blue indicator fluid can leach out the collector pad into the collection fluid depending on the storage time, leading to analytical problems such as increased matrix effect. It is clear that depending on the temperature and time of storage, the degree of THC recovery from the collector can vary. Although the experiment was based on realistic temperature and time frames of roadside collections, these results (Table 2) are still observations under "ideal" laboratory conditions. Therefore, it is important to minimize these variations with a protocol for roadside applications concerning sampling and storage.

Quality Control

A growing concern for analytical laboratories is the enormous evolution in QC assessment schemes. Although most method developers are now aware of the validation requirements according to international standards,[23,24,26,27,29] external quality assessment (EQA) requirements for analytical routine laboratories seem more complex to fulfill. Although EQA schemes for urine and blood/plasma are well established, this is not yet the case for oral fluid. A major problem is the fact that different oral fluid collectors can be used; a different collector can result in differences in recovery and stability for the collected drugs of abuse. At the moment, LGC Standards (Drugs in Oral Fluid Scheme) seems to be the only commercially available EQA scheme. During the past 2 years, 6 neat oral fluid samples obtained from volunteers possibly spiked with amphetamines or other stimulants; cannabinoids; cocaine and its metabolite benzoylecgonine, benzodiazepines, methadone, and buprenorphine; and several opiates were sent 3 times a year to participating laboratories. Three samples were positive for THC: one was a diluted oral fluid sample of a diazepam user, who occasionally smoked cannabis and thus THC was not spiked, and in the second sample, 6 ng/mL THC was spiked to neat oral fluid. In the third sample, the estimated spiked concentration was 14.7 ng/mL. Although our results (using the 3 buffers) for the first sample were within 1 SD of the mean value of all participating laboratories, we have to take into account that there were only 3 participants. This low number can result in high deviations of the results and thus difficulties in evaluation of the obtained data. In addition, the value was beneath our fixed LOQ of 5 ng/mL, so actually the proficiency test was more of a qualitative than quantitative test in our case. In the second sample, the 2 participating laboratories reported a negative result for the 6 ng/mL spiked THC concentration, leading to the impression that there might be problems with stability of THC in neat oral fluid or adsorption onto the transportation tube. A recent publication[34] indicates that THC in neat (expectorated) oral fluid is not stable even at storage temperatures of −20°C. Therefore, the organizers of proficiency tests should consider the use of a stabilizing buffer that increases the stability of the sample. In the third sample, an RSD of 51% was observed between the participating laboratories, suggesting a problem of interlaboratory reproducibility. Again, a problem with stability can be suggested as the estimated spiked concentration was 14.7 ng/mL. More reliable proficiency testing schemes with enough participants and better controlled samples (stability, adsorption, etc) are necessary in the near future to make conclusions concerning reproducibility between laboratories.

In addition to participation of proficiency tests, QC rules in daily routine were based on EMEA guidelines.[26] Because no QC samples were commercially available, in-house QCs were prepared. As batches of those in-house QC were not large, Westgard rules[35,36] were not followed. Instead, acceptance of a run was set at QC falling within 15% of their nominal value according to the accuracy rules as required by the US Food and Drug Administration and EMEA guidelines.[24,26] The applied QC samples were stable at −20°C for 2 months as evaluated by our long-term stability. However, when preparing QC samples, the laboratory has to be aware of possible intraindividual THC stability.[34] In addition, a blank sample and a calibration curve were analyzed and evaluated for each batch. Moreover, matrix effects during analysis of each sample were followed using a full scan simultaneously run with the MRM method.

Analysis of Oral Fluid Samples Collected During On-Site Police Controls

The first observation is the smaller amount of oral fluid collected in THC-positive drivers in comparison to drivers negative for all the drugs mentioned in the Belgian legislation (THC, amphetamine, MDMA, cocaine, BE, 6-MAM, and morphine). Although the collection of oral fluid was easily achieved for negatively screened drivers as indicated by the

blue coloration of the oral fluid collectors, in subjects under the influence of cannabis, smaller amounts of sample, that is, 300–500 μL were collected. One subject under the influence of THC even chewed on the collection device, leading to a negative value. This problem is likely to be observed more frequently with the StatSure device compared with the other collectors, as its collection pad is designed so it can easily be torn loose from the collector to ensure full extraction of the soaked up oral fluid. This design increases the recovery of THC from the collector but can be dangerous in real situations as the tested subject (under influence) can chew/swallow the collector pad.

In the cannabis smoking population, it was clear that 5 to 10 minutes of collection time was already felt to be enormously long by the drivers and some even got aggressive. Therefore, the collection was not performed until the indicator turned blue but stopped after a period of 5 minutes. This observation emphasizes the necessity for the laboratory to weigh the sample to determine the collected amount.

Even though small amounts of oral fluid were collected and also subsequently diluted with the stabilizing buffer, THC concentrations in the samples could easily be detected and quantified.[37]

Using the formula mentioned earlier in the "Materials and Methods/On-Site Biological Sample Collection" section, the concentration of THC per milliliter of neat oral fluid could be calculated by taking the dilution factor of the collector devices into account. This calculation was necessary as the Belgian legislator demands a comparison with a legal cutoff of 10 ng THC per mL of neat oral fluid and thus not per milliliter of collected sample. When using the formula above to determine the THC concentrations in neat oral fluid, one has to be aware that the formula includes some assumptions that will lead to an increased measurement uncertainty. The buffer volume in the collector will not exactly be 1 mL (StatSure) or 3 mL (Quantisal and Certus) as theoretically assumed.[16] In addition, the mean weight of the empty collector is determined by calculating the mean weight of 30 empty devices. The error of the balance was 0.1 mg and will have an implication on the determination of the weight of the "full" collector device. In addition, the THC concentration obtained via the validated method will also have a measurement uncertainty due to the sample analysis.

High oral fluid concentrations are observed, probably due to oral fluid contamination due to cannabis smoking.[9] In addition, the ratios of THC oral fluid to plasma range from 1 to 142, leading to the conclusion that for on-site collected samples, oral fluid concentrations cannot be used to calculate THC plasma concentrations and thus relate to an effect.[38] The ratio will be influenced by the utilization method (smoked or oral), the used dose, the time between consumption and detection, the time delay between oral fluid and blood collection, but also by the type of collector as the adsorption of THC onto the different collectors ranged from 30% to 90% loss of THC particles.

CONCLUSIONS

An accurate and fast ultra-performance liquid chromatography–mass spectrometric method for the quantification of THC in oral fluid samples collected with the StatSure, Quantisal, and Certus device was developed and validated. A daily QC program was developed, and problems of current proficiency schemes were discussed. During the method development, it was clear that the Certus device was not ideal due to adsorption of THC onto the collector. When applying the collection in a real case scenario, the type of collecting device must be chosen and the same type has to be used by all police officers to avoid judicial inequality. Moreover, in our study, a practical problem was observed: only small volumes of oral fluid were collected during a reasonable collection time of 5 minutes. However, THC concentrations could be easily detected, probably due to the contamination of oral fluid when cannabis is smoked. In general, this method is practical, easy, and fast and would be of interest for laboratories dealing with a per se legislation in oral fluid, with a large sample load. However, toxicologists should be aware that oral fluid THC concentrations cannot be used to predict degree of impairment and thus only gives an indication of recent use.

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