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Quantitative determination of five cannabinoids in blood and urine by gas chromatography tandem mass spectrometry applying automated on-line solid phase extraction

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Abstract

Cannabis is the most frequently consumed illegal substance worldwide. More recently, an increasing number of legal cannabis products low in psychoactive Δ^9-tetrahydrocannabinol (THC) but high in non-intoxicating cannabidiol (CBD) are being more widely consumed. While the detection and quantification of THC and its metabolites in biological matrices is an important forensic-toxicological task, additional detection of CBD is also important, for example, when examining the plausibility of consumer's statements. This report describes the method validation for the quantitative determination of THC and its two major metabolites, 11-hydroxy-THC (OH-THC) and 11-nor-9-carboxy-THC (THC-COOH), as well as CBD and cannabinol (CBN) in whole blood and urine. The method employs automated on-line solid phase extraction coupled to gas chromatography tandem mass spectrometry (GC–MS/MS). The method was fully validated according to guidelines of the Swiss Society of Legal Medicine (SGRM) and the Society of Toxicological and Forensic Chemistry (GTFCh). The method fulfilled the validation criteria regarding analytical limits, accuracy and precision, extraction efficacy, and sample stability. The limits of detection (LODs) in whole blood and urine were 0.15 ng/mL for THC, OH-THC and CBD, 0.1 ng/mL for CBN, and 1.0 ng/mL for THC-COOH. The limits of quantification (LOQ) in whole blood and urine were 0.3 ng/mL for THC, OH-THC and CBD, 0.2 ng/mL for CBN, and 3.0 ng/mL for THC-COOH. The fully validated and automated method allows sensitive and robust measurement of cannabinoids in whole blood and urine. Detection of CBD provides additional information regarding consumed products.

KEYWORDS
cannabinoids, driving under the influence of drugs, GC–MS/MS, on-line solid phase extraction

1 | INTRODUCTION

The phytocannabinoid Δ^9-tetrahydrocannabinol (THC) is predominantly responsible for the experienced mental high felt by consumers of cannabis products,[1,2] whereas cannabidiol (CBD), a further well-known cannabinoid, is regarded as non-intoxicating,[3] while thought to exert other effects including anticonvulsive, anti-inflammatory, mildly sedative, and anxiolytic properties.[4–6] Cannabinol (CBN), a non-enzymatically generated degradation product from THC,[7,8] can also be found in trace amounts in cannabis products.

Cannabis rich in THC is the most frequently consumed illicit substance worldwide[9]; however, legalization efforts for medical and recreational use have been on the increase, particularly in recent years.[10–13] Often products low in THC can be legally purchased.[14,15] For instance, under current Swiss law, cannabis flowers containing less than 1% THC are not banned under the narcotics law.[16] Dried flower buds of these cannabis varieties are advertised as having high concentrations of CBD.[17]

With regard to driving, Switzerland, among other countries,[18] pursues a zero tolerance for THC, meaning that detection of THC proves driving inability regardless of impairment.[19] A limit concentration of 1.5 ng/mL THC in whole blood is applied, above which THC is considered as detected.[20] In practice, the Swiss-wide measurement uncertainty of ±30% has to be applied, which means that the legal limit is only exceeded with sufficient certainty at a measured concentration of ≥2.2 ng/mL. However, not only consumption of drug-type cannabis high in THC but also legal CBD-cannabis (which contains less than 1% THC) can lead to blood concentrations exceeding this limit.[21,22] According to Swiss guidelines,[23] detection of THC and its two major metabolites 11-hydroxy-THC (OH-THC) and 11-nor-9-carboxy-THC (THC-COOH) by accredited forensic-toxicological laboratories is mandatory for concluding driving inability. Nonetheless, the detection of further plant ingredients is of major interest, as it might allow one to distinguish the uptake of CBD-rich cannabis from drug-type, THC-rich products. This is of particular interest if drug abstinence has to be verified in countries where CBD-rich/THC-low cannabis products are legally available and consumed. Additionally, minor cannabinoids including CBN are discussed as potential markers to identify recent cannabis uptake,[24–27] to distinguish the use of different types of cannabis,[28] and play a role in anti-doing testing.[29]

The major THC-metabolite THC-COOH is glucuronidated by several UDP-glucuronyltransferases.[30] The ratio of glucuronidated to free THC-COOH in blood is highly variable and ranges from approximately 0.5 to 5 and is dependent on consumption frequency and time difference between intake and blood sampling.[31–33] Cut-off concentrations of free THC-COOH and/or its glucuronide in blood have been proposed as a marker for frequent THC-consumption.[33,34] Based on the work by Fabritius et al.,[34] the Swiss Society of Legal Medicine (SGRM) has implemented a cut-off concentration of 40 ng/mL for free THC-COOH in whole blood, above which a frequent consumption (i.e., twice or more per week) is presumed. In cases involving driving under the influence of drugs (DUID), a concentration of ≥40 ng/mL of THC-COOH can result in an evaluation of general driving fitness.

In urine samples, cleavage of phase-II-metabolites prior to analysis is standard practice. The ratio of free to glucuronidated THC-COOH in urine ranges from 1.3 and 4.5.[35] To measure total THC-COOH, cleavage of THC-COOH-glucuronide is often necessary. Cleavage of the glucuronide ester bond can be achieved chemically, for instance by basic hydrolysis, or enzymatically, usually by addition of β-glucuronidase.[36,37]

This paper describes an automated method for the detection and quantification of THC, OH-THC, THC-COOH, CBD, and CBN (Figure 1) in whole blood and urine via on-line solid phase extraction (SPE), followed by derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamid (MSTFA), coupled to gas chromatography tandem mass spectrometry (GC–MS/MS). The advantages of automated sample preparation lie mainly in a less labor-intensive sample preparation, increased sample throughput, and improved reproducibility. The method is based on a previously reported sample preparation;[22] however, a reduction of the sample volume from 0.5 to 0.25 mL and optimization of the extraction efficacy were key goals for this current method. The detection of CBD alongside THC and THC-metabolites provides additional information, especially with regard to potential differentiation between the consumption of drug-type cannabis from (legally available) products rich in CBD but low in THC. Compared with previously reported automated methods for the detection of THC, OH-THC, and THC-COOH,[38,39] this
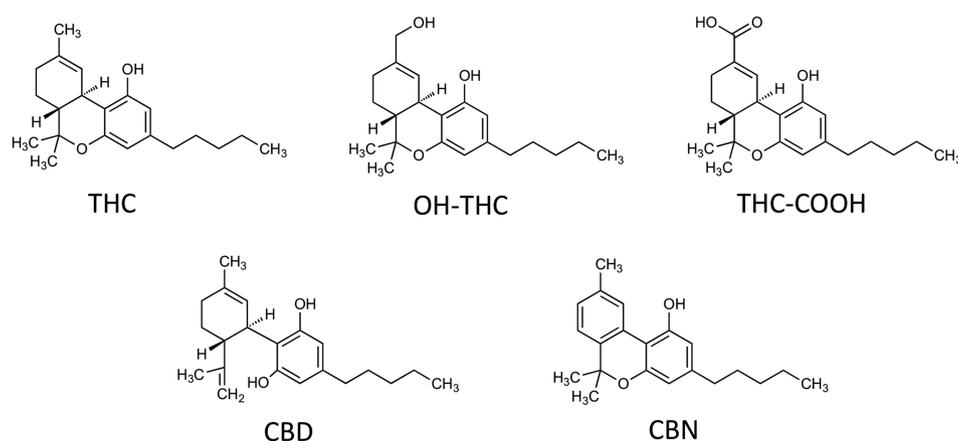
FIGURE 1 Chemical structure overview of the target analytes incorporated into the method: Δ^9tetrahydrocannabinol (THC), 11-hydroxy-THC (OH-THC), 11-nor-9-carboxy-THC (THC-COOH), cannabidiol (CBD), and cannabinol (CBN)

method represents an extension, by two analytes, for the analysis of two different matrices (urine and whole blood). Furthermore, special attention was paid to THC-COOH-glucuronide stability during whole blood sample preparation, to ensure accuracy in the determination of the free THC-COOH fraction.

2 | MATERIALS AND METHODS

2.1 | Materials

2.1.1 | Instrumentation

Automated sample preparation was conducted with a Multi Purpose Sampler II (MPS) by Gerstel (Mühlheim, Germany), which was equipped with several modules: one module for SPE (SPE-station), one for evaporation of solvents under reduced pressure and controlled temperature (mVap), one for shaking under controlled temperature (MPS Agitator), and a Solvent Filling Station 2 (SFS 2) for the supply of large volumes of solvents. The system contained two syringes: a 2.5 mL syringe for SPE steps, which included a gas supply for drying of the SPE cartridges, and a 10 μL syringe for addition of derivatization reagent and sample injection into the adjacent GC–MS/MS. Both syringe holders, called movers, were able to transport vials using magnetic caps. The mover, equipped with the larger syringe, could additionally grab and transport the specially fabricated SPE cartridges. Different solvent reservoirs as well as three trays for sample vials, eluate vials, and SPE cartridges were also mounted. The MPS was controlled by the supplied Maestro software (version 1.4.49.3). GC–MS/MS analysis was conducted with a Trace GC Ultra (Thermo Fisher Scientific, Reinach, Switzerland) equipped with an Optima™ 5 MS GC-capillary (length 30 m; ID 0.25 mm; Macherey Nagel, Oensingen, Switzerland) and coupled to a TSQ Quantum XLS MS-system (Thermo Fisher Scientific, Reinach, Switzerland). The GC–MS/MS was controlled by the Xcalibur software (version 4.1; Thermo Fisher Scientific, Reinach, Switzerland).

2.1.2 | Reagents and consumables

Flat-bottom (2 mL) and high-recovery (1.5 mL) glass vials, non-magnetic vial caps, SPE cartridges (Chromabond C18ec, 1 mL/100 mg) equipped with an adaptor for transportation, and LC–MS-grade water, acetonitrile (ACN), and methanol (MeOH) were purchased from Macherey Nagel (Oensingen, Switzerland). Magnetic vial caps were purchased from Gerstel. LC–MS-grade n-hexane, ethyl acetate (EtOAc), dichloromethane (DCM), MSTFA, acetic acid (AcOH), and β-glucuronidase (140 U/mL, from *Escherichia coli* K12; manufactured by Roche Reference) were purchased from Merck (Buchs, Switzerland). Certified reference standards (1 mg/mL) of THC, OH-THC, THC-COOH, CBD, and CBN as well as deuterated analogues (0.1 mg/mL) of THC-D3, OH-THC-D3, THC-COOH-D9, CBD-D3, and CBN-D3 were purchased from Lipomed (Arlesheim, Switzerland).

Certified reference standard for the β-glucuronide of THC-COOH manufactured by Cerilliant was purchased from Merck (Buchs, Switzerland). Quality control (QC) blood samples with pre-quantified high and low cannabinoid concentrations as well as other narcotics were purchased from ACQScience (Rottenburg am Neckar, Germany). Validation data were statistically evaluated by the Valistat software 2.0 provided by Arvecon (Walldorf, Germany) and Excel from Microsoft Professional Plus 2010 (version 14.7237.5000). Negative blood and urine samples were obtained in anonymized form from voluntary employees from the institute. Blood samples were collected in either 4 mL vacutainer® (Becton Dickinson GmbH, Heidelberg, Germany) containing 1.5 mg/mL sodium fluoride and 3 mg/mL Na_2EDTA or 9 mL Monovette® by Sarstedt fortified with potassium fluoride (4.5 mg/mL; custom-made by Hôpital du Valais, Sion, Switzerland). Urine was collected in 100 mL sample cups made from high density (HD) polyethylene (Sarstedt, Sevelen, Switzerland) and poured into 30 mL tubes made from polypropylene (also from Sarstedt) for storage. Blood and urine specimens were stored at $-20°C$ until analysis.

2.2 | Methods

2.2.1 | Sample pretreatment

As shown in Figure 2, 0.25 mL of whole blood (fluoride stabilized) or urine was spiked with 10 μL of internal standard (ISTD) solution, resulting in final concentrations of 3 ng/mL for THC-D3, OH-THC-D3, CBD-D3, and CBN-D3 and 30 ng/mL for THC-COOH-D9. Whole blood samples were then precipitated by drop-wise addition of 1 mL of 75% ACN in water (method A) while vortexing. For urine, after addition of the ISTD solution, each sample was first enzymatically de-glucuronidated by addition of 10 μL of β-glucuronidase followed by incubation for 60 min at $40°C$ and subsequently precipitated by addition of 0.75 mL ACN (method B). All samples, regardless of whether they were prepared using method A or method B, were then centrifuged for 10 min at *2500 g*. After centrifugation, the supernatant—hereafter referred to as the primary extract—was transferred into a flat bottom sample vial and placed on the sample tray of the MPS.

2.2.2 | Automated SPE

Cartridges were subsequently conditioned with 2 mL methanol (MeOH), 2 mL water, and 1 mL of a 0.1 M aqueous AcOH solution (each at a flow rate of 150 μL/s). Afterwards, 1 mL of the primary extract (method A) was mixed with 1.5 mL of 0.1 M AcOH in the 2.5 mL syringe of the MPS. Alternatively, 0.75 mL of the primary extract (method B) was mixed with 1.75 mL of 0.1 M AcOH. The diluted sample was then transferred onto the cartridge (15 μL/s). Next, the cartridge was subsequently washed first with 1 mL of 0.1 M AcOH and then with 1 mL of 30% ACN (both at 15 μL/s). Finally, the cartridge was dried in a stream of nitrogen for 5 min after which the analytes were eluted by addition of 0.9 mL ACN. Due to the dead
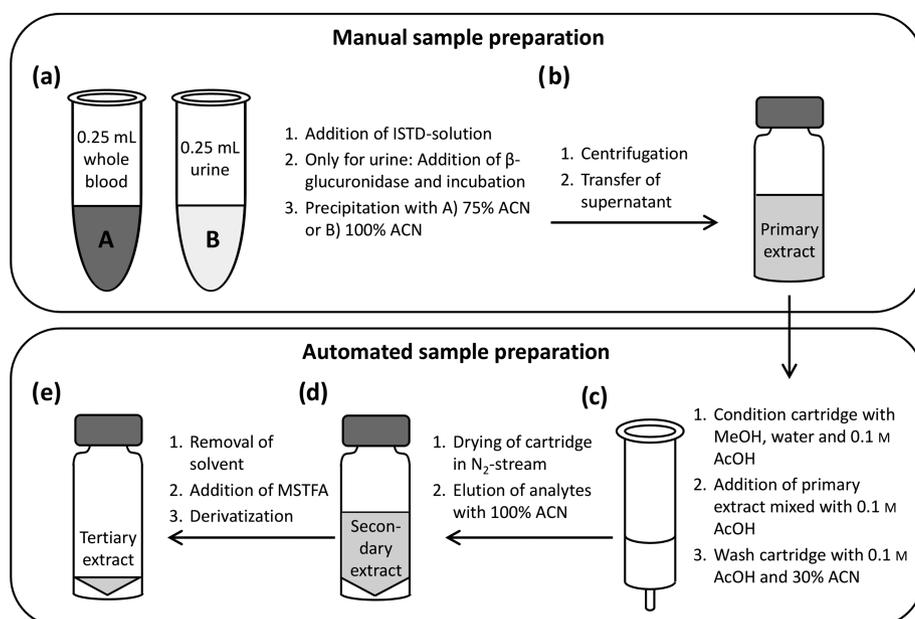
FIGURE 2 Schematic overview of the optimized sample preparation protocol (method A and B): (a,b) pretreatment of samples to generate primary extract; (c,d) solid phase extraction (SPE) parameters to generate secondary extract; (e) derivatization to generate tertiary extract

volume of the cartridge, this resulted in 0.75 mL elution solution, from here on referred to as the secondary extract. The secondary extract was collected in a high-recovery vial equipped with a magnetic cap. After transfer of the vial to the mVap module, the solvent was evaporated to dryness (8 min at 70°C and 80 mbar). Afterwards, 30 μL of MSTFA were added to the dried sample and the mixture derivatized for 20 min at 90°C in the Agitator module. The final, derivatized extract containing the trimethylsilyl-(TMS-)derivatives of the analytes, from here on referred to as the tertiary extract, was then analyzed by GC–MS/MS. A schematic overview of the process as described above is shown in Figure 2.

2.2.3 | GC–MS/MS parameters

An injection volume of 1 μL was chosen. The programmed temperature vaporization (PTV) inlet was used in splitless mode. An initial temperature of 70°C was held for 0.5 min. The PTV inlet was heated with a ramp for 14.5°C/min up to 250°C, with a transfer time of 1 min. After the transfer step, the PTV was cleaned at a temperature of 300°C for 10 min.

Helium (≥99.9999%; Carbagas, Gümligen, Switzerland) was used as the carrier gas at a flow rate of 1.5 mL/min. The oven temperature gradient program started at 70°C, which was held for 0.5 min. The temperature was then increased by 80°C/min until 200°C was reached. In a second ramp, the temperature was increased for 10°C/min to a final temperature of 300°C, which was held for another 3 min. Including the cool-down period of 5 min, one GC-run required 18 min.

The transfer line and ion source were held at 250°C. MS was used in electron ionization mode (EI; emission current of 50 μA, electron energy of −70 eV). Argon (≥99.999%, Carbagas) was used as the collision gas at 1.2 mTorr. All analytes were measured in positive ion

mode. In Table 1, the MS/MS detection parameters via single reaction monitoring (SRM) are summarized.

Due to the small sample volumes of fully prepared samples, that is, the tertiary extract and potential decomposition of analytes, a continuous workflow was chosen. Hereby, samples are prepared one at a time, whereby preparation of a subsequent sample occurs while the previous one is being analyzed by GC–MS/MS. Overall, the method enables immediate injection of a fully prepared sample every 46 min, including blank measurements in-between samples.

2.2.4 | Method development

To evaluate the influence of pH, mixing of the primary extract with water and AcOH solutions (0.1 M, 0.5 M, and 1.0 M) prior to SPE was assessed. Washing of the SPE-cartridge with 30% versus 40% ACN was evaluated. For the elution of analytes from the cartridge, different volumes of ACN (0.5–1.5 mL) were evaluated. Required duration for drying different volumes of ACN (0.5, 0.75, 1.0, and 1.25 mL) in the mVap module at fixed temperature and pressure (5, 7.5, 10, and 12.5 min) were evaluated. Furthermore, different durations of derivatization at 90°C were evaluated (2.5, 5, 10, 15, 20, 25, and 30 min).

2.2.5 | Validation

The method was validated according to the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh) and the SGRM.[40,41] Validation parameters included selectivity and specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy with precision and trueness, extraction efficiency, testing for carry-over, stability of samples at various time points during sample preparation,

TABLE 1 Used parameters for single reaction monitoring mode for analytes and ISTD

Retention time (min)	Analyte	Transition	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
8.19	THC-D3	Quantifier	389.30	374.30	13
		Qualifier	374.30	292.30	20
8.21	THC	Quantifier	386.30	371.30	13
		Qualifier	371.20	289.30	20
10.01	OH-THC-D3	Quantifier	374.30	292.30	15
		Qualifier	374.30	308.10	15
10.03	OH-THC	Quantifier	371.30	289.30	15
		Qualifier	371.30	305.10	15
10.97	THC-COOH-D9	Quantifier	380.30	292.30	15
		Qualifier	380.30	314.10	15
11.02	THC-COOH	Quantifier	371.30	289.30	15
		Qualifier	371.30	305.10	15
7.36	CBD-D3	Quantifier	393.07	319.20	10
		Qualifier	393.07	304.50	10
7.38	CBD	Quantifier	390.20	319.20	10
		Qualifier	390.20	301.00	10
8.83	CBN-D3	Quantifier	370.50	310.30	20
		Qualifier	370.50	295.30	30
8.85	CBN	Quantifier	367.40	310.20	20
		Qualifier	367.40	295.20	30

Abbreviation: ISTD, internal standard.

stability of THC-COOH-glucuronide in whole blood samples, and hydrolysis of THC-COOH-glucuronide by enzymatic cleavage via β-glucuronidase in urine samples. In order to be used as a test sample, unless otherwise indicated, 0.25 mL drug-free whole blood or urine was spiked with ISTD and the required reference analyte(s) (which had first been diluted in MeOH as required).

Selectivity was tested by analysis of six blank samples (only MSTFA) and two drug free samples (containing only ISTD).

Specificity was tested by fortifying blood and urine with phytocannabinoids including target analyte precursors THC-acid (THCA), CBD-acid (CBDA), and CBN-acid (CBNA) found in cannabis plant material, synthetic cannabinoids, drugs of abuse, and relevant benzodiazepines. A detailed list of substances can be found in the supporting information.

LOD and LOQ were tested in whole blood and urine according to DIN 32645 by generation of 9-point calibration curves spanning the expected analytical limits. For THC, OH-THC, and CBD, the concentrations ranged from 0.1 to 1.0 ng/mL (interval of 0.1 ng/mL between levels), for CBN, the curve ranged from 0.06 ng/mL up to 0.6 ng/mL (interval of 0.06 ng/mL between levels), and for THC-COOH, the concentrations ranged from 1.0 ng/mL up to 10 ng/mL (interval of 1.0 ng/mL between each calibrator). Additionally, calculated LOD were verified by determination of signal-to-noise(S/N)-ratio using Xcalibur software, which needed to be greater than 3.

Linearity was tested in both, whole blood and urine, respectively, by measuring calibrations in six replicates, followed by Mandel *F* test for linearity, Grubb's test for outliers, and Cochran's *C* test for variance homogeneity (each at a 99% level of significance). Calibration levels for THC, OH-THC, and CBD were 0.3, 0.5, 1.0, 2.0, 5.0, 10, 15, and 20 ng/mL. For CBN, the levels were 0.18, 0.3, 0.6, 1.2, 3.0, 6.0, 9.0, and 12 ng/mL, and for THC-COOH, levels at 3.0, 5.0, 10, 20, 50, 100, 150, and 200 ng/mL were included. Additionally, linearity of CBD was tested at higher concentrations, employing levels at 20, 50, 100, 150, 200, 250, and 300 μg/L.

Accuracy with precision and trueness was tested by duplicate measurements of low and high concentration QC-samples on 8 different days. For blood, commercially available QC-blood samples were used. Despite containing more than 20 drugs of abuse, the only relevant substances contained were THC, OH-THC, and THC-COOH; each QC therefore needed to be additionally fortified with CBD and CBN prior to sample preparation. The low concentrated QC contained 1.1 ng/mL THC and OH-THC, 5.5 ng/mL THC-COOH, and 1.0 ng/mL CBD and CBN. The high concentrated QC contained 20.7 ng/mL THC, 9.3 ng/mL OH-THC, 77.1 ng/mL THC-COOH, and 6.0 ng/mL CBD and CBN. Due to the importance of THC-COOH as marker for frequent consumption, the high concentrated QC was diluted with drug free whole blood to a final concentration of 40 ng/mL THC-COOH, resulting in a medium QC. For urine analysis, spiking urine with

analytes in larger batches to generate a QC pool resulted in measured values lower than allocated, likely due to unspecific binding of lipophilic cannabinoids to laboratory consumables or inhomogeneous interaction with matrices. These samples were therefore not suited for determination of accuracy. Finally, urine QC samples were generated by addition of all analytes to 0.25 mL of drug-free urine immediately before sample preparation. Different lot numbers of reference standard material were used in the QCs than for the calibration. Final concentrations in the low concentrated QC were 1.0 ng/mL THC, OH-THC, and CBD; 6.0 ng/mL THC-COOH; and 0.6 ng/mL CBN. The high concentrated QC contained 15 ng/mL THC, OH-THC, and CBD; 150 ng/mL THC-COOH; and 9.0 ng/mL CBN. Precision was assessed via repeatability (i.e., difference between duplicate measurements on the same day) and within-laboratory precision (i.e., difference between measurements on different days). Results are given as relative standard deviation (RSD), that is, RSD_r for intra-day and $RSD_{(T)}$ for inter-day precision. According to the guidelines,[40] both RSDs should not exceed 15% (or 20% when close to the LOQ). Trueness was assessed via the deviation from the target value (bias) over all measurements, whereby the bias should not exceed 15% (or 20% when close to the LOQ). As combined measure of $RSD_{(T)}$ and bias, the 95% β-tolerance interval (approximated: bias ± 2.508 $RSD_{(T)}$[40]) should not exceed 30% (40% when close to the LOD).[40]

Extraction efficiency (EE) was determined over the following calibration ranges: 0.5–15 ng/mL (THC, OH-THC, CBD), 5.0–150 ng/mL (THC-COOH), 0.3–9.0 ng/mL (CBN) via comparison of a control calibration to an extraction calibration. For the control calibration, target compounds and ISTD were added to the secondary extract after SPE of blank matrix, representing a 100% EE. For the extraction calibration, the target compounds were added to drug free samples prior to SPE, whereas ISTD was added to the secondary extract. With both data sets, a linear regression was performed. The EE was calculated by division of the slope of the extraction calibration by the slope of control calibration (reported as percentage).

Carry-over can occur via syringes and via the GC–MS/MS system. Carry-over via the 10 μL syringe, coming in contact with the tertiary extract, and the GC–MS/MS-system was preliminarily assessed by injection and analysis of pure MSTFA (blanks) directly after test samples containing 250 ng/mL THC-COOH derivatized in MSTFA (no matrix used). THC-COOH was chosen as test substance, as it is commonly the highest concentrated analyte after THC intake. Different wash conditions (*n*-hexane, EtOAc, and DCM) for the 10 μL syringe were evaluated. Carry-over via the 2.5 mL syringe, including all of the above sample preparation steps and GC–MS/MS, was assessed by sample preparation and analysis of test samples containing all analytes at the following concentrations: 150 ng/mL for THC, OH-THC, and THC-COOH; 300 ng/mL for CBD; and 20 ng/mL CBN. Analysis of test samples was followed by sample preparation and analysis of test samples containing only ISTD.

Sample stability was assessed at two time points during sample preparation. First, stability of the primary extract at room temperature

was assessed over a period of 3 days. Second, stability of the tertiary extract was evaluated by analyzing the same samples until complete evaporation of MSTFA after approximately 24 h. For both tests, six QC samples at low and high concentrations (see accuracy testing) were employed. Prior to automated sample preparation, the primary extracts of the QC samples were pooled and then redistributed to six vials.

Stability of THC-COOH-glucuronide in whole blood samples was tested by fortifying six blind samples with THC-COOH-glucuronide to a final concentration equivalent to 100 ng/mL free THC-COOH. The samples were pooled and split into 6 aliquots. These were periodically prepared and analyzed over 2.5 days (approximately every 12 h, last at 55 h). THC-COOH-glucuronide itself cannot be measured with the method described. However, by measuring the THC-COOH released by possible cleavage of the glycosidic bond during sample preparation, the stability of the glucuronide could be assessed. Additionally, stability of THC-COOH-glucuronide in the primary extract was investigated.

Cleavage of THC-COOH-glucuronide in urine samples was assessed in test samples fortified with THC-COOH-glucuronide (concentration equivalent to 100 ng/mL free THC-COOH), followed by addition of β-glucuronidase. Different durations of incubation (0, 1, 2, 4, 8, and 24 h) at 40°C were tested. To account for urine variability, the chosen duration was evaluated in five test samples from different donors.

3 | RESULTS AND DISCUSSION

3.1 | Method optimization

Mixing of the primary extract with water or 0.1 M, 0.5 M, and 1.0 M AcOH resulted in equal signal intensity for THC, OH-THC, CBD, and CBN. Regarding THC-COOH, all acidic solutions yielded a roughly 10-fold higher peak intensity compared with pure water, likely due to improved retention of protonated, that is, neutral THC-COOH on the C18 SPE-cartridge. The use of 0.5 M and 1.0 M AcOH did not improve signal intensity compared with the 0.1 M solution, which is why the latter was chosen. Regarding washing of the cartridge, 40% ACN led to smaller signal intensities of all analytes compared with 30% ACN, which is why the latter was chosen. It was found that addition of 0.9 mL ACN to the cartridge resulted in approximately 0.75 mL of the secondary extract. The use of larger volumes did not lead to higher signals. The secondary extract could be efficiently dried in the mVap-module at 80 mbar and 70°C for 8 min. Finally, longer derivatization times led to higher peak intensity. Hence, 20 min was chosen for the final method as it provided an optimal combination of signal intensity and time consumption.

Stability of THC-COOH-glucuronide was assessed by measurement of test samples fortified with THC-COOH-glucuronide, whereby detection of free THC-COOH indicates cleavage of the glycosidic bond. Due to the 40 ng/mL cut-off value for free THC-COOH in whole blood,[34] above which a frequent consumption is assumed, it is

crucial that the THC-COOH-glucuronide remains stable during sample preparation and analysis. Initially, blood samples were precipitated by addition of 0.75 mL 100% ACN—a widely used precipitation technique for blood and serum.[42,43] Free THC-COOH was measured corresponding to a cleavage of approximately 25% of the glucuronide. The cleavage was similar, regardless of whether fluoride stabilized blood from Vacutainers® or Monovettes® was fortified. When six primary extracts were subsequently measured over a period of 2 days, no time-dependent increase in cleavage became evident. Furthermore, reanalysis of the tertiary extract after 24 h did not result in higher concentrations of free THC-COOH. These results suggest that cleavage did not occur during storage of the primary nor of the tertiary extract. Consequently, preparation steps at acidic pH or increased temperatures were suspected to cause glucuronide cleavage, for instance mixing the primary extract with 0.1 M AcOH, SPE in acidic conditions, evaporation of the secondary extract at 70°C in the mVap module, or derivatization of the tertiary extract at 90°C in the Agitator. However, neither the use of less acidic solvents, nor a reduced temperature in the mVap module, nor a reduced duration and/or temperature in the Agitator module led to a reduction of the cleavage. Finally, it was found that cleavage could be reduced to <3%, when blood samples were diluted with 0.25 mL of water prior to precipitation with 0.75 mL ACN. The same result was observed, when blood samples were directly precipitated with 1 mL of 75% aqueous ACN, ultimately leading to the successfully validated method A. Interestingly, when blood samples were diluted with 0.125 mL instead of 0.25 mL water prior to addition of 0.75 mL ACN, a cleavage of 12.5–15% could be observed. Potential reasons for the cleavage might be a change in pH or differences in matrix of the resulting primary extract.

Hydrolysis of THC-COOH-glucuronide in urine was assessed in test samples spiked with THC-COOH-glucuronide, whereby the extent of cleavage was assessed by measurement of free THC-COOH. Cleavage of more than 90% of THC-COOH-glucuronide was already achieved by mere addition of β-glucuronidase without any incubation, which most likely resulted from a combination of enzyme activity at room temperature during sample preparation and, potentially, some cleavage during the addition of ACN prior to automated sample preparation (as seen for whole blood samples when precipitated with 100% ACN). An increase in the durations of the incubation from 0.5 to 24 h did not result in improved cleavage and did not lead to a loss of signal intensity. Hence, 0.5 h was initially chosen. To account for variation in urine specimens with regard to dilution and pH value, urine samples from five different voluntary donors were fortified with THC-COOH-glucuronide, followed by addition of β-glucuronidase and incubation for 0.5 h. Mean cleavage in all samples after 0.5 h was 90%, whereby the smallest observed cleavage was 74%. Because longer incubation was not associated with any loss of signal, an incubation period of 1 h (0.5 h plus a safety margin of 0.5 h) was chosen for the final method. Also for CBD determination in urine, enzymatic hydrolysis of phase-II-metabolites seems crucial.[44] However, as CBD-glucuronide was not available as a reference standard, no evaluation of the cleavage step could be performed for this method. Furthermore, also phase-I-

metabolites of CBD and CBN were not commercially available at the start of the method validation and therefore not incorporated in the method.

3.2 | Validation

3.2.1 | Whole blood samples

The method was successfully validated, in accordance with the guidelines of the GTFCh and SGRM.[40,41] No interfering signal was detected in any of the samples used to test selectivity and specificity. This indicates stability of analyte precursors THC-acid, CBD-acid, and CBN-acid, which upon decarboxylation would yield THC, CBD, and CBN. Decarboxylation is presumably prevented by protection of the carboxylic acid moiety via silylation. In Table 2, the results of the evaluation of LOD, LOQ, accuracy (given as RSD intra-day [RSD_r] and inter-day [$RSD_{(T)}$] and bias), EE, and linearity range are summarized. Overall, LOD and LOQ evaluated according to DIN 32645 lay clearly below the Swiss legal limit of 1.5 μg/L. S/N at LODs were satisfactory (≥175; see Supporting Information). Furthermore, maximum LODs of 1.0 μg/L for THC and 5 μg/L THC-COOH (in blood serum) as suggested by the German Society of Traffic Medicine were met.[45] Also, none of the RSDs or biases exceeded the minimally required 15%. EE of all analytes exceeded the required 50%, and linearity was shown for all analytes within appropriate concentration ranges. All 95% β-tolerance intervals of the low concentrated samples lay within the acceptable range of ±40%. For THC, OH-THC, and CBD, also higher concentrated samples lay within the acceptable ±30%. However, high levels of THC-COOH and CBN exceeded the acceptable ±30%. But none of the single measurements lay outside the range of ±30%. As THC-COOH at medium concentration (40 ng/mL) lay within the acceptable range, the method is nevertheless suited for routine use in Switzerland, where a cut-off of 40 ng/mL is used as a marker for frequent consumption. However, albeit not observed in any of the test samples, deviation of more than 30% from the true value cannot be excluded at higher concentrations. Regarding high concentrated CBN at 6 ng/mL, the 95% β-tolerance interval did not meet the required criteria of the GFTCh. CBN is a minor cannabinoid potentially occurring in trace amounts in authentic samples[24] but lacking legal implication. Therefore, this shortcoming is regarded as not relevant for forensic casework.

The preliminary test for *carry-over* in the 10 μL syringe revealed that washing of the syringe with *n*-hexane three times before and after injection was necessary to remove residual analytes (i.e., no signal in ensuing blank). Also, DCM and EtOAc were tested. DCM gave equal cleaning results but was dismissed due to its higher toxicity, whereas EtOAc did not fully remove residual analytes.

Additionally, washing of the 2.5 mL syringe and *carry-over* in the whole system from on-line sample preparation to GC–MS/MS analysis was investigated using test samples containing all analytes at high concentrations exceeding those observed in everyday routine work (150 ng/mL for THC, OH-THC, and THC-COOH; 300 ng/mL for

TABLE 2 Summary of validation of whole blood including limit of detection (LOD), limit of quantification (LOQ), relative standard deviation intra-day (RSD$_r$) and inter-day (RSD$_{(T)}$), bias, extraction efficiency (EE)

Analyte	LOD (ng/mL)	LOQ (ng/mL)	RSD$_r$ (%)	RSD$_{(T)}$ (%)	Bias (%)	EE (%)	Linearity range (ng/mL)	95% β-tolerance interval (%)
THC	0.15	0.3	5.3 (low)	10.9 (low)	−1.6 (low)	59	0.3–20	−28.9, 25.7 (low)
			3.5 (high)	5.0 (high)	−3.6 (high)			−16.1, 25.7 (high)
OH-THC	0.15	0.3	5.3 (low)	8.2 (low)	−2.4 (low)	65	0.3–15	−23.0, 18.2 (low)
			3.1 (high)	5.8 (high)	−0.4 (high)			−15.0, 14.1 (high)
THC-COOH	1.0	3.0	4.2 (low)	7.5 (low)	+2.0 (low)	55	3.0–150	−16.8, 20.81 (low)
			5.6 (medium)	5.6 (medium)	−13.7 (medium)			−27.8, 0.3 (medium)
			3.3 (high)	11.4 (high)	−7.5 (high)			−36.1, 21.1 (high)
CBD	0.10	0.3	6.3 (low)	11.5 (low)	+11.5% (low)	63	0.3–20	−12.2, 35.2 (low)
			3.6 (high)	6.9 (high)	+2.1 (high)		20–300	−12.1, 16.3 (high)
CBN	0.15	0.2	3.8 (low)	9.5 (low)	+3.1 (low)	63	0.2–12	−16.5, 22.7 (low)
			1.9 (high)	11.2 (high)	+11.2 (high)			−11.8, 34.2 (high)

Note: QC$_{low, blood}$ = 1.1 ng/mL (THC), 1.1 ng/mL (OH-THC), 1.0 ng/mL (CBD and CBN), 5.5 ng/mL (THC-COOH); QC$_{medium}$ = 40.0 ng/mL (THC-COOH); QC$_{high, blood}$ = 20.7 ng/mL (THC), 9.3 ng/mL (OH-THC), 77.1 ng/mL, 6.0 ng/mL (CBD and CBN).

TABLE 3 Summary of validation results of urine including limit of detection (LOD), limit of quantification (LOQ), relative standard deviation intra-day (RSD$_r$) and inter-day (RSD$_{(T)}$), extraction efficiency (EE), and 95% β-tolerance interval

Analyte	LOD (ng/mL)	LOQ (ng/mL)	RSD$_r$ (%)	RSD$_{(T)}$ (%)	Bias (%)	EE (%)	Linearity range (ng/mL)	95% β-tolerance interval (%)
THC	0.15	0.3	6.3 (low)	7.9 (low)	+10.1 (low)	82	0.3–20	−9.6, 30.0 (low)
			5.0 (high)	8.5 (high)	−2.5 (high)			−23.8, 18.8 (high)
OH-THC	0.15	0.3	7.3 (low)	7.3 (low)	+9.0 (low)	85	0.3–20	−9.3, 27.3 (low)
			10.2 (high)	10.3 (high)	−0.6 (high)			−26.4, 25.2 (high)
THC-COOH	1.0	3.0	6.7 (low)	7.9 (low)	+16.1 (low)	79	3.0–200	−3.7, 35.9 (low)
			3.5 (high)	6.6 (high)	−2.9 (high)			−19.5, 13.6 (high)
CBD	0.15	0.3	8.2 (low)	8.2 (low)	+7.7 (low)	85	0.3–20	−12.9, 28.3 (low)
			3.1 (high)	3.6 (low)	−1.9 (high)		20–300	−10.9, 7.1 (high)
CBN	0.1	0.2	3.3. (low)	6.5 (low)	−2.8 (low)	77	0.2–12	−19.1, 13.5 (low)
			2.6 (high)	9.1 (high)	+2.3 (high)			−14.0, 18.6 (high)

Note: QC$_{low, urine}$ = 1.0 ng/mL (THC, OH-THC, CBD), 6.0 ng/mL (THC-COOH), 0.6 ng/mL (CBN); QC$_{high, urine}$ = 15 ng/mL (THC, OH-THC, CBD), 150 ng/mL (THC-COOH), 9.0 ng/mL (CBN).

CBD; and 20 ng/mL CBN) followed by preparation of drug-free samples containing ISTD. After addition of the primary extract to the SPE cartridge, the 2.5 mL syringe was washed three times with MeOH and three times with water, whereas the 10 μL syringe was washed as described above. For none of the analytes was any carry-over observed in the blind samples prepared and analyzed immediately following the high concentrated test samples.

Regarding *sample stability* of the primary extract over the 3-day period, no trend towards a decrease of the absolute peak areas was observed. Stability testing of the analytes in the tertiary extract revealed an increase of the absolute peak area up to a factor of 3 within the observed time period, most likely due to evaporation of MSTFA. However, the analyte levels derived from the signal ratio of target compound to ISTD remained unchanged over the time span, thus indicating sufficient stability of all analytes in the tertiary extract to allow for re-injection up to 40 h after preparation.

Evaluation of THC-COOH-glucuronide stability in the primary extract during storage on the autosampler indicated a slight increase of cleavage from approximately 1.7% at 0 h to 2.6% after 55 h.

3.2.2 | Urine samples

The method was also validated for urine samples and fulfilled all requirements.[40,41] In Table 3, a summary of the evaluation of LOD, LOQ, accuracy (given as RSD intra-day [RSD$_r$] and inter-day [RSD$_{(T)}$] and bias), EE, and linearity range after sample preparation of urine samples can be seen. Overall, LOD and LOQ as determined according

to DIN 32645 lay significantly below the values of 10 ng/mL for both THC and THC-COOH as suggested by the German Society for Traffic Medicine.[45] S/N at LODs was satisfactory (≥359). Except for the low concentrated THC-COOH samples, for which a slightly higher bias of 16.1% was found, none of the RSD or biases exceeded 15%. As the level of the low THC-COOH sample was close to the lowest calibrator level (at 5.0 ng/mL), the bias is still in the acceptable range of <20%. EE of all analytes exceeded the required 50%, and linearity could be shown for all analytes within appropriate concentration ranges. The 95% β-tolerance interval lay within the acceptable range of ±30% (40% for low levels) for all analytes.

Carry-over was also evaluated for urine samples. Drug-free samples (prepared according to method B) subsequent to highly concentrated test samples (150 ng/mL for THC, OH-THC, and THC-COOH; 300 ng/mL for CBD; and 20 ng/mL CBN) did not indicate any carry-over using the syringe cleaning steps as previously described for whole blood samples.

Regarding *sample stability* of analytes in the primary and tertiary extract of urine samples, which were tested over 3 days and 40 h, respectively, no significant loss of signal could be observed for any analyte. For the tertiary extract, an increase in peak area was observed for all analytes, as described for the blood samples. Nonetheless, the derived analyte concentrations remained unchanged.

To elucidate if the method is fit for purpose, it was compared with the established method of analysis of cannabinoids as applied at the Institute of Forensic Medicine. Hereby, authentic blood and urine samples covering a range of concentrations were analyzed with both methods within a time frame of 1 week. No diverging results (i.e., difference >15%) were found (data not shown). Additionally, stored samples from previous ring trials (whole blood as matrix, because urine was not available) were re-measured and lay within acceptable ranges as provided by the ring trial provider. Chromatograms of negative and positive tested authentic whole blood and urine samples, especially at an analyte concentration at the analytical limits, are shown in Figures S13 and S14 in the supporting information.

4 | CONCLUSION

Employing automated sample preparation by on-line SPE, an efficient GC–MS/MS method for the detection of THC, OH-THC, THC-COOH, CBD, and CBN in whole blood and urine was developed and successfully validated. The validation results demonstrate that the analytical specification (particularly analytical limits) required by the Swiss legal limits[20] in blood for assessment of DUID cases[45] and for abstinence testing in urine, in accordance with the German Society for Traffic Medicine,[45] were completely fulfilled.

Challenges concerning THC-COOH-glucuronide stability occurred when fluoride stabilized whole blood samples were precipitated with pure ACN. Resulting overestimation of free THC-COOH would have led to undesirable consequences due to its use as a marker for frequent THC-consumption. Evaluation of THC-COOH-glucuronide stability during method development and validation is therefore crucial, especially as commercially available QC samples as well as ring trials often lack THC-COOH-glucuronide. This was sufficiently addressed by the optimization of the sample precipitation step, by which means the THC-COOH-glucuronide cleavage in blood was substantially reduced.

Albeit that slight underestimation at high THC-COOH levels and overestimation of high CBN levels in whole blood samples cannot be completely excluded, the method is fit-for-purpose for quantitative analysis in forensic toxicological assessment. The method requires only 0.25 mL of biological material. Due to a high degree of automation, the method enables a throughput of approximately 30 samples per 24 h with only minimal manual labor involved.

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