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Miniaturized one-step extraction for the analysis of tetrahydrocannabinol Δ^9- and Δ^8- isomers, phytocannabinoids, and hexahydrocannabinol in whole blood using liquid chromatography tandem mass spectrometry

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ABSTRACT

Δ^9-tetrahydrocannabinol (Δ^9-THC) plays a major role in driving under the influence of drugs investigations and workplace drug testing. Additionally, cannabidiol (CBD), as well as semi-synthetic cannabinoids, like hexahydrocannabinol (HHC) and Δ^8-tetrahydrocannabinol (Δ^8-THC), are increasingly found on regulated and unregulated drug markets. A straightforward bioanalytical method was developed, covering 14 analytes, including Δ^9-THC, CBD, Δ^8-THC, and their respective metabolites, as well as HHC and a subset of minor phytocannabinoids.

Sample cleanup consisted of one precipitation step conducted in a 96-well plate format and requiring only 50 µL of whole blood. For analysis an ultra high-performance liquid chromatography system coupled to a triple quadrupole mass spectrometer was used. The method was validated in accordance with ICH M10 and guidelines in forensic toxicology.

The method proved to be precise and accurate with a maximal inter assay imprecision of 9.4 % and a maximal accuracy bias of 12.3 %. The validation revealed good recoveries (>86 %), highly consistent matrix effects (relative standard deviation; RSD ≤7 %) and suitable limits of quantification (LOQs), i.e. 0.5 µg/L for Δ^8- and Δ^9-THC. In addition, baseline separation of Δ^8-THC-COOH and Δ^8-THC from the corresponding Δ^9-THC isomers were achieved. Method validation demonstrated a fit for purpose analytical method covering legal thresholds for Δ^9-THC for driving. Samples from 4 individuals after inhalation of CBD-rich cannabis were analyzed, whereby 8 out the 14 analytes could be quantified, demonstrating the utility and suitability of the method.

The method reduces sample volume, solvent use, and workload, making it well-suited for automation and clinical or forensic applications.

1. Introduction

With nearly 228 million estimated users in 2022, cannabis ranks amongst the most popular recreational drugs worldwide [1]. Despite cannabis still being controlled in most regions in the world, recent years were marked by an increasing number of countries legalizing its recreational use [2–5]. Moreover, medical cannabis and cannabis-based products are becoming increasingly accessible, with 64 countries implementing such provisions as of 2021 [6]. Δ^9-tetrahydrocannabinol (Δ^9-THC) is the main psychoactive cannabinoid of *Cannabis sativa* and plays a major role in workplace drug testing and forensic toxicological investigations [7–11]. The latter includes driving under the influence of drugs (DUID) investigations, where alcohol and cannabis are the most prevalently detected drugs in impaired drivers [5,9]. Regarding driving, legal thresholds are commonly applied for Δ^9-THC [8], whilst its major metabolites hydroxy Δ^9-THC (Δ^9-THC-OH) and carboxy Δ^9-THC (Δ^9-THC-COOH) are typically also considered [5,12,13].

Δ^9-THC is only one of almost 150 phytocannabinoids found in *Cannabis Sativa*. Due to the natural origin, cannabis and cannabis based products can have considerable variance in the contents of these phytocannabinoids [14–16]. Cannabis plant varieties are commonly classified into different chemical phenotypes (also referred to as chemotypes or chemovars) based on the ratio of the two major cannabinoids Δ^9-THC and cannabidiol (CBD). *Phenotype I*, also known as “drug type” cannabis,

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is rich in Δ^9-THC. *Phenotype II* has equal amounts of Δ^9-THC and CBD and *phenotype III* describes what is often referred to as "industrial hemp" and is rich in CBD and low in Δ^9-THC [15,17]. Industrial hemp, which is often referring to cannabis with Δ^9-THC contents below 0.3 % (*w/w*), is generally considered to have no intoxicating effects. In recent years, hemp type (*phenotype III*) cannabis products, also referred to as CBD products, have gained popularity on the lifestyle and recreational drug markets [18,19]. The often remaining traces of Δ^9-THC in these CBD-rich products evoked studies investigating if legal Δ^9-THC thresholds for driving can still be breached when using these products [20,21].

The term minor cannabinoids is usually applied to describe further phytocannabinoids besides the major cannabinoids Δ^9-THC and CBD [22–24]. These lower abundant phytocannabinoids have gained interest, for instance in the quality control of cannabis containing pharmaceutical products. Notably, cannabis comprises not a single main constituent but a complex mixture of various phytocannabinoids. Adding to the complexity, phytocannabinoids are biosynthesized and thus present in the cannabis plant as carboxylic acid precursors. Extensive heating (e.g. vaporization) or smoking, leads to decarboxylation and the release of the free phytocannabinoids. Consequently, Δ^9-THC is predominantly present as Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) in the untreated cannabis plant (e.g. pre-smoking) [15,16,22]. Despite it being highly abundant in unprocessed cannabis products, Δ^9-THCA is generally considered a minor cannabinoid, particularly in the context of bioanalytical analyses [7,24].

Adding to the everchanging landscape surrounding cannabis' legal, medical and recreational use, a further current development encompasses the emergence of cannabinoids that are structurally related to Δ^9-THC that has been linked to have been the consequence of the initial popularity and widespread production of CBD [19]. These THC-like compounds are commonly referred to as "semi-synthetic" cannabinoids, despite some being of pure synthetic origin [25–27]. An example is Δ^8-THC that is also naturally present in *Cannabis sativa*, however in considerably lower amounts compared to Δ^9-THC, as it is formed from Δ^9-THC via oxidative degradation [19,28]. Δ^8-THC can be synthesized from CBD, which, together with its initial often legal status, resulted in the widespread production and selling of Δ^8-THC products [19]. Since Δ^8-THC is a positional isomer of Δ^9-THC, it shares the same chemical skeleton, only differing by the position of one carbon-carbon double bond [25]. Hexahydrocannabinol (HHC) is another semi-synthetic cannabinoid that first emerged in 2021 and can be synthesized using CBD. It has a broad accessibility and has been and still is sold via vape shops and internet vendors [29]. Although many of these novel cannabinoids are little researched regarding their pharmacological activity, they are commonly sold as alternatives to cannabis [19,30,31]. Literature suggests cannabimimetic effects of various of these cannabinoids, including Δ^8-THC [32–34] and HHC [34–37], which renders their detection relevant in the medico-legal field. Due to its close structural similarity with Δ^9-THC, Δ^8-THC poses various analytical challenges [30, 38–40]. The same applies for the corresponding hydroxy-and carboxy-metabolites. To prevent misinterpretation of analytical results, the potential presence and analytical interference of Δ^8-THC should be assessed in the context of cannabinoid bioanalysis [31,39,40].

With an increasingly complex cannabis landscape leading to diverse challenges touching on clinical and forensic applications, there is a growing need for the implementation of easy and comprehensive bioanalytical methods covering not only Δ^9-THC and CBD but also minor cannabinoids and the recently emerged semi-synthetic cannabinoids [40]. Previously, the inclusion of minor cannabinoids, such as Δ^9-THCA, cannabigerol (CBG), cannabinol (CBN; an oxidative degradation product of Δ^9-THC) [14], and tetrahydrocannabivarin (THCV) into bioanalytical methods have been investigated for their potential to help distinguish the type of cannabis product used (e.g. medical or recreational) [41–43]. Furthermore, Rague et al. [44], gave evidence of CBG as an indicator of recent cannabis use.

In line with recent efforts, this study aimed to develop and validate an easy and robust bioanalytical method for the quantification of 14 cannabinoids in whole blood, including Δ^9-THC and CBD, and their major metabolites (hydroxyl and carboxy), the semi-synthetic cannabinoids Δ^8-THC and HHC, as well as the minor cannabinoids Δ^9-THCA, CBG, CBN, and THCV. An overview of the chemical structures and the metabolic pathways of Δ^8-THC, Δ^9-THC, and CBD [19] is given in Fig. 1. The study focused on optimizing the sample cleanup procedure to reduce the workload in forensic and clinical applications. Additionally, the applicability of the method was evaluated by analyzing study samples from four participants who inhaled CBD-rich cannabis as part of a controlled clinical study.

2. Materials and methods

2.1. Chemicals and reagents

All solvents and solvent additives were from LC-MS quality. Methanol (MeOH), acetonitrile (ACN), water, and formic acid by Biosolve were obtained from Chemie Brunschwig (Basel, Switzerland). Isopropanol (IPA) by Chemsolute was purchased from Th. Geyer (Zug, Switzerland) and dimethyl sulfoxide (DMSO) from Merck (Buchs, Switzerland).

2.2. Certified reference standards

All calibrated reference standards were purchased as a 1 mg/mL or 0.1 mg/mL solution in MeOH, ethanol or isopropanol and are classified as certified reference material. Solutions of Δ^9-tetrahydrocannabinol (Δ^9-THC), hydroxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-OH), Δ^9-tetrahydrocannabinol carboxylic acid (Δ^9-THC-COOH), Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), tetrahydrocannabivarin (THCV) and Δ^9-THC acid (Δ^9-THCA) and the deuterated (D) internal standards (ISTDs) Δ^9-tetrahydrocannabinol-D_3 (Δ^9-THC-D_3), hydroxy-Δ^9-tetrahydrocannabinol-D_3 (THC-OH-D_3), Δ^9-tetrahydrocannabinol carboxylic acid-D_9 (Δ9-THC-COOH-D_9), cannabidiol-D_3 (CBD-D_3), and cannabinol-D_3 (CBN-D_3) were obtained from Lipomed (Arlesheim, Switzerland). Hydroxy-Δ^8-tetrahydrocannabinol (Δ^8-THC-OH), Δ^8-Tetrahydrocannabinol carboxylic acid (Δ^8-THC-COOH), 7-hydroxy-cannabidiol (7-OH-CBD), 7-carboxy-cannabidiol (CBD-COOH), and the ISTDs Δ^8-tetrahydrocannabinol-D_3 (Δ^8-THC-D_3) and cannabigerol-D_3 (CBG-D_3) were purchased from Cerilliant (Round Rock TX, USA). (±)-HHC (mixture of 9S-HHC and 9R-HHC) used for method development and validation was obtained from Chiron (Trondheim, Norway). Pure reference standards of 9S-HHC and 9R-HHC manufactured by Cayman Chemicals (Ann Arbor, MI; USA) were kindly provided by the Institute of Forensic Medicine in Berne (Switzerland). 6α-hydroxy-cannabidiol (6-OH-CBD) was purchased from Cayman Chemicals (Ann Arbor, MI; USA). Stock solutions of all analytes were prepared by diluting the reference standards in DMSO. An ISTD working solution was prepared by dilution of ISTDs in a mixture of ACN:MeOH (1:1, *v/v*). These solutions were stored at −20 °C.

2.3. Whole blood matrices

For calibration, whole blood samples stabilized with 5 mg/mL potassium fluoride in S-Monovette® tubes (Sarstedt, Seleven, Switzerland) were pooled by mixing equal volumes of five blood donations obtained from four healthy volunteers. Quality control (QC) samples were diluted using a pool of equal volumes of three citrate-phosphate-dextrose stabilized whole blood samples from three healthy volunteers obtained from the local blood donation center (Interregionale Blutspende SRK AG, Berne, Switzerland). Blood samples were tested for the absence of cannabinoids prior to use in the preparation of calibration and QC samples.

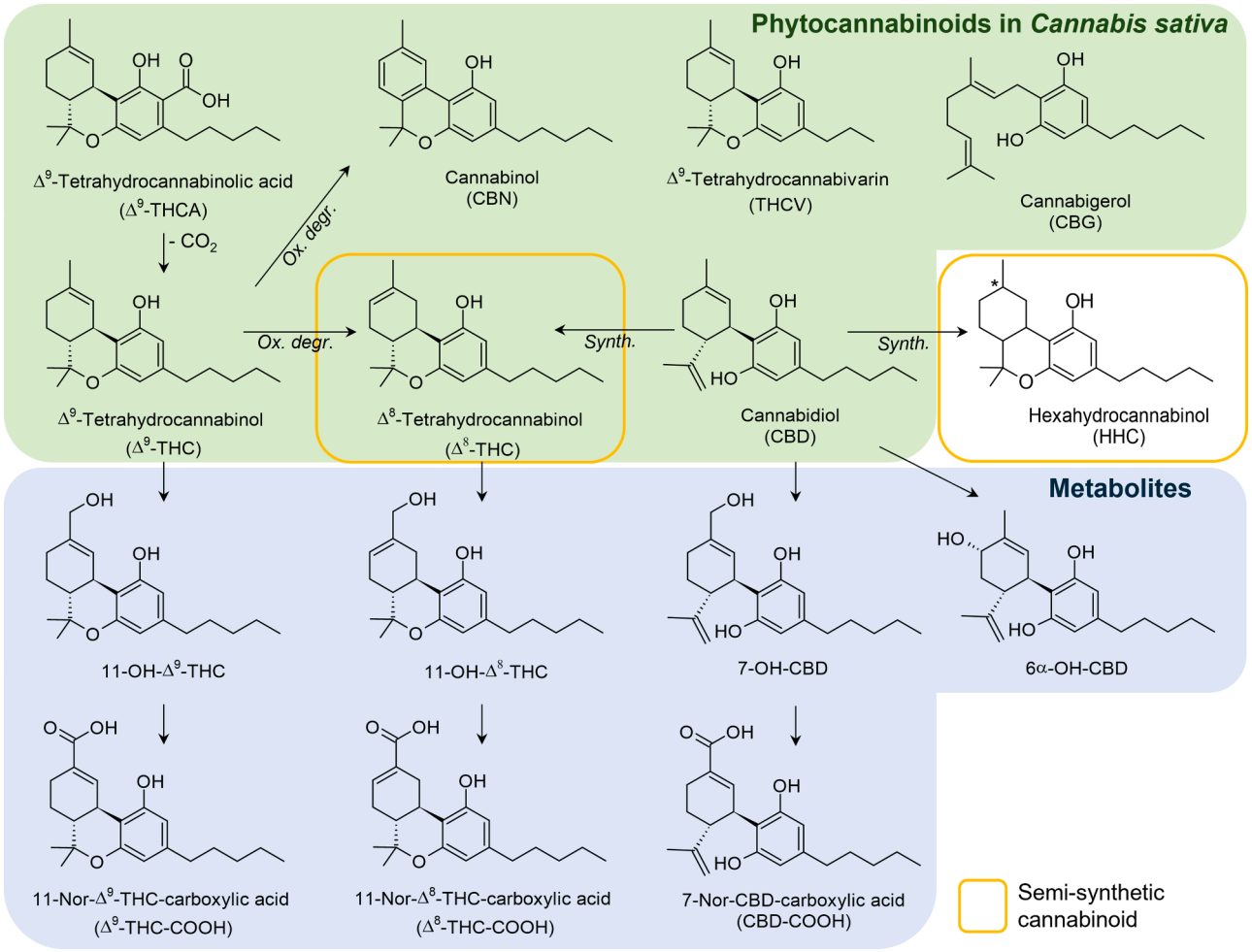

Fig. 1. Chemical structures and metabolic pathway of the analytes covered with the presented analytical method. "*" indicates HHC's stereocenter, "Ox. degr": oxidative degradation, "synth.": synthesis.

2.4. Sample preparation

An aliquot of 50 µL whole blood sample was pipetted into 0.75 mL push cap tubes that were arranged in a 96-well plate format using the 96-rack high cover all by Micronic (Lelystad, The Netherlands). The samples were precipitated using 150 µL of a ACN:MeOH mixture (1:1, *v/v*) containing ISTDs at a concentration of 5 µg/L (Δ^9-THC-D_3, Δ^8-THC-D_3, THC-OH-D_3, CBG-D_3, CBN-D_3), 10 µg/L (Δ^9-THC-COOH-D_9), or 2.5 µg/L (CBD-D_3). Samples were capped (Split TPE Capcluster by Micronic) and shaken for 10 min at 1800 rounds per minute (rpm) using an iShak BL UNO VT shaker by Neuation (Haslab, Biel-Benken, Switzerland). The samples were centrifuged for 30 min at 10 °C at 2397 g using a Rotina 380 R centrifuge by Hettich (Bäch, Switzerland). After centrifugation, the samples were ready for injection and the sample plate is placed into the cooled autosampler at 10 °C until analysis.

2.5. Ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis

For analysis, a Nexera Series ultra high-performance liquid chromatography (UHPLC) system from Shimadzu (Kyoto, Japan) coupled to an API 6500+ QTrap triple quadrupole tandem mass spectrometer (QQQ-MS) from AB Sciex (Baden, Switzerland) was used. Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B consisted of 0.1 % formic acid in MeOH. The UHPLC gradient began at 5 % mobile phase B, held for 0.5 min, mobile phase B was then increased to 50 %, after which it was ramped to 65 % (reached at 2.5 min), 80 % (reached at 7.5 min), and 98 % (reached at 8 min and held for 1 min). The system was then reequilibrated to the starting conditions (5 % mobile phase B) for one minute, resulting in a total runtime of 10 min per sample. An injection volume of 10 µL and a flow rate of 0.7 mL/min were used. A Kinetex XB-C18 analytical column (50×2.1 mm, 1.7 µm; Phenomenex, Basel, Switzerland) was used for the chromatography. The oven temperature was kept at 45 °C. The mass spectrometer was equipped with a heated electron spray ionization (ESI) source, operated at 400 °C, 35 psi curtain gas, 50 psi nebulizer gas, 50 psi desolvation gas and the collision associated dissociation (CAD) gas was set to 9 aru. All analytes except Δ^9-THCA were measured in positive ionization mode with a spray voltage set to +5500 V. Δ^9-THCA was measured in negative ionization mode with spray voltage set to −4500 V. Scheduled reaction monitoring (sMRM) was applied. The corresponding analyte-specific settings, retention times, and mass transitions are listed in Table 1.To assure correct identification of analytes, in addition to retention time match, a flagging threshold of an ion-ratio deviation of <40 % was applied directly within the SCIEX OS software. The target MRM ion-ratio is based on the average ratio within the calibration samples. A constant tolerance was applied.

2.6. Calibration and quality control (QC) samples

Working solutions for all calibrators and QC samples were prepared in DMSO by serial dilution of stock solution mixtures. The solutions were spiked into a pool of drug-free blood matrix, originating from a minimum of three subjects, to obtain the respective calibration standards and

Table 1
Mass spectrometric parameters of all analytes: mass transitions ("-1": quantifier, "-2" qualifier), retention times (RT), declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP).

Mass transition	Precursorion [m/z]	Fragmention [m/z]	RT [min]	DP [Volt]	EP [Volt]	CE [Volt]	CXP [Volt]
6-OH-CBD-1	331.1	201.1	2.47	51	10	33	14
6-OH-CBD-2	331.1	271.1	2.47	71	10	25	16
CBD-COOH-1	345.1	299.1	2.70	51	10	27	18
CBD-COOH-2	345.1	193.2	2.70	51	10	35	14
7-OH-CBD-1	331.1	201.1	2.76	51	10	33	14
7-OH-CBD-2	331.1	271.1	2.76	71	10	25	16
Δ^9-THC-OH-1	331.1	193.1	4.00	51	10	35	12
Δ^9-THC-OH-2	331.1	201.1	4.00	56	10	35	14
Δ^9-THC-OH-D_3-1	334.2	196.1	4.00	56	10	35	14
Δ^9-THC-OH-D_3-2	334.2	201.1	4.00	56	10	35	12
Δ^9-THC-COOH-1[a]	345.1	299.1	4.30	71	10	29	20
Δ^9-THC-COOH-2[a]	345.1	193.1	4.30	71	10	39	16
Δ^9-THC-COOH-D_9-1[a]	354.2	308.1	4.30	76	10	29	20
Δ^9-THC-COOH-D_9-2[a]	354.2	196.2	4.30	76	10	39	12
THCV-1	287.1	231.1	4.35	66	10	27	14
THCV-2	287.1	123.1	4.35	66	10	47	8
CBD-1	315.1	193.1	4.65	51	10	31	12
CBD-2	315.1	135.1	4.65	51	10	27	10
CBD-D_3-1	318.2	196.2	4.65	51	10	31	12
CBD-D_3-2	318.2	135.1	4.65	51	10	29	10
CBG-1	317.1	193.1	4.78	36	10	27	12
CBG-2	317.1	123.0	4.78	36	10	47	6
CBG-D_3-1	320.2	196.2	4.78	36	10	25	12
CBG-D_3-2	320.2	123.0	4.78	36	10	45	12
CBN-1	311.1	223.1	5.65	71	10	31	14
CBN-2	311.1	178.0	5.65	71	10	85	12
CBN-D_3-1	314.1	223.1	5.65	86	10	31	14
CBN-D_3-2	314.1	178.1	5.65	86	10	87	12
Δ^9-THC-1[a]	315.1	193.1	6.26	81	10	33	14
Δ^9-THC-2[a]	315.1	259.2	6.26	66	10	29	16
Δ^9-THC-D_3-1[a]	318.2	196.2	6.26	66	10	33	12
Δ^9-THC-D_3-2[a]	318.2	262.1	6.26	66	10	29	18
HHC-1	317.1	193.1	6.60	61	10	35	14
HHC-2	317.1	137.2	6.60	61	10	31	10
Δ^9-THCA-1	357.1	245.1	7.99	−95	−10	−42	−15
Δ^9-THCA-2	357.1	191.1	7.99	−95	−10	−44	−17

[a] Same parameters were used for the corresponding Δ^8-THC isomers (sMRM window of 45 s).

quality control samples. The volume of stock solution spiked into the whole blood matrices was kept constant for all samples at a final percentage of 1 % (*v/v*) DMSO. The calibration ranges established for the analysis were as follows: 1.0 μg/L – 25 μg/L for 6- and 7-OH-CBD, 2.5 μg/L – 250 μg/L for Δ^8- and Δ^9-THC-COOH, and 0.5 μg/L – 25 μg/L for all other analytes. The QC levels were prepared at the following concentrations for all analytes except 6- and 7-OH-CBD and Δ^8- and Δ^9-THC-COOH: 0.5 μg/L (LOQ: lower limit of quantification), 1.0 μg/L (low QC; LQC), 8.0 μg/L (mid-range QC; MQC), and 20 μg/L (high QC; HQC). For the analysis of 6/7-OH-CBD, the following concentrations were prepared: 1 μg/L (LOQ), 2.0 μg/L (LQC), 8.0 μg/L (MQC), and 20 μg/L (HQC). For analysis of Δ^8- and Δ^9-THC-COOH, the following concentrations were prepared: 2.5 μg/L (LOQ), 5.0 μg/L (LQC), 80 μg/L (MQC), and 200 μg/L (HQC). Calibration solutions were prepared by a different person than the QC samples. During method validation, for the accuracy and precision measurements that took place over several days, calibrators and QC samples were aliquoted at 50 μL in 0.75 mL push-cap tubes (Micronic) and stored at −20 °C (storage no longer than 4 weeks). On the day of analysis, the frozen samples were thawed at room temperature and processed according to the protocol outlined previously.

2.7. Method validation

The method validation was conducted in accordance with the guidelines and frameworks in forensic toxicology from the Society of Toxicological and Forensic Chemistry (*Gesellschaft für Toxikologische und Forensische Chemie*; GTFCh) [45] and Swiss Society of Forensic Medicine (*Schweizerische Gesellschaft für Rechtsmedizin*; SGRM) [46]. In addition, experiments in accordance with the "ICH guideline M10 on bioanalytical method development and study sample analysis" from the European Medicines Agency (EMA) were incorporated into the method validation process [47].

2.7.1. Calibration curve and range

Calibration samples were prepared and measured encompassing a minimum of 5 different levels, spanning the anticipated working range. Each calibration level was spiked in whole blood, extracted in triplicate and measured in duplicate, resulting in 6 consecutive measurements per concentration level. The resulting calibration regressions were evaluated using the Valistat 1.0 software from Arvecon (Walldorf, Germany) regarding:

i) outliers (Grubbs-test, 95 % significance level) and
ii) homogeneity of variances (F-test and Cochran-test, 99 % significance level).

For each individual analyte, the maximum number of outliers that may be detected was two, and these outliers must not occur at the same concentration level. Outliers were excluded from the calibration equation. Weighing of calibration was considered for all calibrations and especially when homogeneity of variance was not given between first and last calibration point. For the calibration curve the following criteria had to be met

i) coefficients of determination r^2 of $\geq$0.99 and
ii) 75 % of the calibration points within 85–115 % (at LOQ: 80–120 %) margins of the nominal concentration (accuracy).

2.7.2. Accuracy and precision

Accuracy and precision of the analytical method were determined by measuring QC samples at LOQ, LQC, MQC, and HQC. The QC samples were prepared as described in Section 2.6. These samples were analyzed on eight different days in duplicate extraction. The following parameters were assessed according to GTFCH method validation guideline [48]:

- Inter-assay precision, which refers to the coefficient of variance (%CV) determined over all 16 measurements ($n = 2$ QCs, $n = 8$ days).
- Inter-assay accuracy bias, which was calculated as the bias between the nominal and calculated concentration over all 16 measurements ($n = 2$ QCs, $n = 8$ days).
- Repeatability refers to the precision of independent measurements obtained using the same method, laboratory, and operator on identical sample material within a short time period.
- Laboratory precision, which is defined as the precision of a given method for analyzing samples over different days.
- The 95 % β-tolerance interval integrates the accuracy and precision of the method, describing the range within which the actual values of a certain number of measurements are likely to fall with a given probability.

All equations used are provided in the supplementary material. In accordance with the ICH M10, GTFCh, and SGRM guidelines, the repeatability and laboratory precision are considered acceptable if the variation is ≤15 % (≤20 % at LOQ), the inter-assay accuracy falls within 85–115 % (LOQ 80–120 %), and the 95 % β-tolerance is ±30 % (±40 % at LOQ).

2.7.3. Carry-over

Carry-over was assessed by running two extracts of drug-free whole blood without ISTD (double blank) after the highest calibrator. Carry-over was considered insignificant if peak areas in these samples were ≤20 % of the peak areas at LOQ.

2.7.4. Selectivity

Selectivity testing comprised the analysis of seven different drug-free blood samples. First the samples were extracted with ISTD (blank) and without ISTD (double blank), to also assess any signals resulting from the ISTD analytes. Signal intensities in the drug-free matrix at the respective retention times of the different analytes had to be ≤20 % of the signals obtained at LOQ. If the interference exceeds this threshold, the LOQ must be increased. In an additional selectivity experiment, the same seven blood samples were spiked with all analytes at the LOQ. Acceptance criteria were accuracy between 80–120 % in four out of seven of the samples.

2.7.5. Recovery

Extraction efficiency was determined by extracting the different QC samples (LQC, MQC, and HQC) in triplicate. The signal intensities of these samples were then compared to signal intensities from fortified blood extracts, for which the analytes were spiked after protein precipitation. The resulting peak areas were compared with the spiked extracts corresponding to a 100 % extraction recovery. For the recovery to be considered acceptable, it had to be consistent and concentration independent, with RSDs of ≤20 % across all concentration levels.

2.7.6. Matrix effects

Seven different whole-blood sample extracts were fortified at the concentration levels of LQC and HQC. The same concentration levels were prepared in a mixture of water:MeOH:ACN (2:3:3; *v/v/v*), corresponding to the final extract composition lacking blood matrix constituents. These experiments were conducted in triplicate. The matrix effects were expressed as ratios of the signal peak areas with and without matrix. In line with the GTFCh guideline for method validation of forensic toxicological methods [48], matrix effects resulting in RSD of ≤25 % between different blood matrices were regarded acceptable for analytes with deuterated ISTDs, whilst a stricter limit of RSD ≤15 % was defined for analytes without deuterated ISTD (e.g. Δ^9-THCA that is referred to THC-COOH-D_9 ISTD and THCV that uses no ISTD). The more restrictive limits for analytes without deuterated ISTD are intended to ensure that matrix effects are less likely to affect the reliability of quantitative results.

2.7.7. Analyte stability

The stability of the analytes in the extracts following sample preparation was assessed for 24 h at 10 °C (autosampler stability) and for seven days at −20 °C. The QC samples of the accuracy and precision assays were used for this purpose (LOQ, MQC and HQC, $n = 2$). The freeze-thaw, bench-top, and long-term stability at −80 °C of the analytes in untreated whole blood was evaluated using LQC (LOQ: 6- and 7-OH-CBD), MQC, and HQC samples ($n = 3$). To assess the freeze-thaw-stability the samples were subjected to a freezing period for at least 20 h and subsequently thawed at room temperature on three occasions. To assess the bench-top stability of the analytes, the QC samples were stored at ambient temperature for a period of 8 h. Finally, QC samples were stored for a period of seven weeks at −80 °C, with the objective of evaluating the long-term stability of the analytes. The stability of the analytes at the different storage conditions was evaluated using freshly prepared calibrations, meaning that on each day of analysis blank blood was spiked with a previously prepared solution of standards in DMSO. A decrease or increase in concentration (accuracy) of ≤20 % indicated that the analytes were stable under the respective storage condition.

2.7.8. Additional experiment Δ8-THC-OH and Δ9-THC-OH

The final chromatography did not result in baseline separation of the hydroxy metabolites Δ^9-THC-OH and Δ^8-THC-OH. Therefore, full validation was conducted for Δ^9-THC-OH only. Further experiments were conducted to evaluate the performance of the method in the presence of Δ^8-THC-OH alone and in combination with Δ^9-THC-OH. Therefore, blank blood was spiked with either 10 µg/L Δ^8-THC-OH or Δ^9-THC-OH. Additionally, a mixture of 5 µg/L of each Δ^8-THC-OH and Δ^9-THC-OH was spiked into blank blood. Samples were extracted and analyzed in triplicate. Quantitation was performed with a calibration of Δ^9-THC-OH according to Section 2.6. Additionally, ion ratios of qualifier and quantifier ions for the spiked samples were further investigated. The ion ratio was calculated by dividing the peak area of the quantifier transition (331.1 → 193.1 *m/z*) by the peak area of the qualifier transition (331.1 → 201.1 *m/z*).

2.8. Method application

To examine the application of the presented method, whole blood samples of four study participants of the clinical study on CBD-rich cannabis and driving by Egloff et al. [21] were analyzed. Study participants vaporized and inhaled 300 mg cannabis, containing 14.6 % CBD (*w/w*) and 0.64 % Δ^9-THC (*w/w*) [21]. The study was approved by the Ethical Committee of Northwestern and Central Switzerland (EKNZ, BASEC ID: 2019-00639) and conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice (ICH-GCP). All study participants provided written informed consent prior to study participation and agreed upon further use of the samples beyond the initial study. Whole blood samples were collected prior to inhalation, directly after the last inhalation and 10, 20, 180, 240, and 300 min after inhalation using BD Vacutainer® blood collection tubes stabilized with lithium heparin (Medisave, Amsterdam, The Netherlands). For one of the study participants (№ 4), samples at 180 and 240 min were not available, as blood sampling failed at those timepoints. After study completion, the samples were stored at −80 °C until further analysis, resulting in a storage period of 4–5 years regarding the samples presented herein. Upon analysis, all samples were thawed at room temperature and samples were extracted

in duplicate. For the analysis, the study samples and QC samples (LQC, MQC, and HQC) were framed by calibration samples at the beginning and end of the sequence. Samples with concentrations above the upper limit of quantification were diluted with drugfree whole blood into the calibration range. The concentration-time curves were plotted using the GraphPad Prism 10 Software (San Diego, CA, USA), and the descriptive pharmacokinetic parameters maximal blood concentration (C_{max}) and time to reach C_{max} (t_{max}) were derived from the plots.

3. Results

3.1. Method development

During method development different organic solvents and ratios for protein precipitation were evaluated, i.e., pure ACN, 1:1 MeOH:ACN (*v/v*), and pure MeOH. Best results regarding blood-pellet formation and sensitivity (investigated using signal to noise ratios of Δ^9-THC and Δ^8-THC at 0.5 µg/L) was observed for a 1:1 mixture of MeOH:ACN (*v/v*). Additionally, volumes and ratios of whole blood to organic solvent were varied to assess the lowest amount of whole blood required for sufficient sensitivity (0.5 µg/L) for Δ^9-THC (investigated using signal to noise ratios of Δ^9-THC and Δ^8-THC at 0.5 µg/L). A suitable ratio of whole blood to precipitation solvent was found at 50 µL of sample and three times the volume (150 µL) of organic solvent. The resulting pellet after centrifugation proved stable and remains compact during the period of analysis, thereby allowing for direct analysis, without additional transferring of the supernatant. Different analytical columns were tested, namely: Hypersil Gold Phenyl (100×2.1 mm, 1.9 µm), Accucore Biphenyl (100×2.1 mm, 2.6 µm), and Hypersil Gold C18 (100×2.1 mm, 1.9 µm) from Thermo Fisher Scientific (Reinach, Switzerland), as well as Acquity UPLC BEH C18 (150×2.1 mm, 1.7 µm) from Waters (Baden-Dättwil, Switzerland), and Kinetex XB-C18 (50×2.1 mm, 1.7 µm), Kinetex C8 (50×2.1 mm, 2.6 µm), and Kinetex C18 (50×2.1 mm, 2.6 µm) from Phenomenex (Basel, Switzerland). Best performance, regarding chromatographic resolution of Δ^9-THC and Δ^8-THC, was obtained using the Kinetex XB-C18 analytical column. Mobile phase constitutions were varied, i.e., different MeOH:ACN ratios for the mobile phase B, and the addition of modifiers (e.g. ammonium formate). Best results regarding peak shape and peak resolution were achieved using 0.1 % formic acid (*v/v*) in methanol for mobile phase B. The chromatographic method development aimed for baseline separation of the isomers Δ^9-THC and Δ^8-THC, as well as their metabolites. Baseline separation was achieved for Δ^9-THC and Δ^8-THC, partial (but satisfactory) separation was achieved for Δ^8-THC-COOH and Δ^9-THC-COOH. Insufficient chromatographic resolution was obtained for the Δ^8- and Δ^9-THC hydroxy-metabolites, which in the final method co-elute. Therefore, the method validation was performed for Δ^9-THC-OH. For HHC, a certified reference standard containing a mixture of the 9R- and 9S-enantiomers was used. The chromatography resulted in two partially separated peaks for these enantiomers. The method was therefore validated for mixtures of these enantiomers, using the integral over both peaks to calculate the total HHC peak area. A chromatogram showing the separation of all analytes using the final optimized method is shown in Fig. 2.

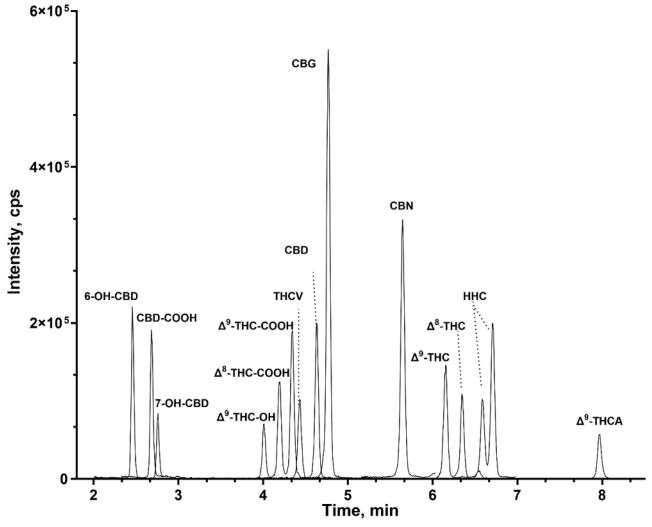

Fig. 2. Chromatogram obtained using the final method (Section 2.5) of a blood sample spiked with analytes at 5 µg/L, except for 6-OH-CBD and 7-OH-CBD which were spiked at 15 µg/L. Note HHC is present in two peaks resulting from the partial separation of its 9R- and 9S-diastereomers.

3.2. Method validation

3.2.1. Calibration range

The final calibration ranges, retention times, and respective ISTDs per analyte are shown in Table 2. The regression and corresponding coefficients of determination (r^2), outliers, and results of the homogeneity testing are given in *Supplementary Table S1*. The working ranges proved linear with $r^2 \geq 0.995$. Final calibration ranges of 0.5–25 µg/L were achieved with acceptable linearity and accuracy for Δ^8-THC, Δ^9-THC, and Δ^9-THC-OH, as well as most further cannabinoids (CBD, CBD-COOH CBN, HHC, CBG, THCV and Δ^9-THCA), whilst the applied higher calibration ranges from 2.5 - 250 µg/L proven suitable for the two carboxy metabolites (Δ^8-THC-COOH and Δ^9-THC-COOH). For 6-OH-CBD and 7-OH-CBD the calibration proved to be linear, accurate and precise between 1.0 –25 µg/L. Weighing of $1/x^2$ proved to be suitable for all

Table 2
Retention times (RT), final calibration ranges, and respective internal standard (ISTD) transitions used for normalization of the validated method.

Analyte	RT [min]	Calibration range [µg/L]	ISTD transition
Δ^9-THCA	7.99	0.5 - 25	Δ^9-THC-COOH-D_9–2 [10 µg/L]
Δ^9-THC	6.15	0.5 - 25	Δ^9-THC-D_3–1 [5 µg/L]
Δ^9-THC-OH	4.04	0.5 - 25	Δ^9-THC-OH-D_3–1 [5 µg/L]
Δ^9-THC-COOH	4.30	2.5 - 250	Δ^9-THC-COOH-D_9–1 [10 µg/L]
CBD	4.65	0.5 - 25	CBD-D_3–1 [2.5 µg/L]
6-OH-CBD	2.47	1.0 - 25	Δ^9-THC-OH-D_3–2 [5 µg/L]
7-OH-CBD	2.76	1.0 - 25	Δ^9-THC-OH-D_3–2 [5 µg/L]
CBD-COOH	2.70	0.5 - 25	Δ^9-THC-COOH-D_9–1 [10 µg/L]
CBN	5.65	0.5 - 25	CBN-D_3–1 [5 µg/L]
CBG	4.78	0.5 - 25	CBG-D_3–1 [5 µg/L]
THCV	4.35	0.5 - 25	no ISTD
Δ^8-THC	6.35	0.5 - 25	Δ^8-THC-D_3–1 [5 µg/L]
Δ^8-THC-COOH	4.15	2.5 - 250	Δ^9-THC-COOH-D_9–1 [10 µg/L]
HHC (mix of enantiomers)	6.60	0.5 - 25	Δ^9-THC-D_3–1 [5 µg/L]

analytes. *Supplementary Figure S1* shows the resulting calibration curves for all quantifier and qualifier ions. The mean observed accuracy bias ($n = 6$) per calibration level and over all analytes was ≤6.6 % and the mean observed precision was ≤11 %. *Supplementary Table S2* shows the detailed results for all concentration levels.

3.2.2. Accuracy and precision

Accuracy and precision were determined at four concentrations (LOQ, LQC, MQC, and HQC). *Supplementary Table S3* shows the tested QC levels and their respective results including inter-assay precision and accuracy as well as repeatability, laboratory precision, and the β-tolerance intervals for all analytes. The accuracy bias of all analytes is illustrated in Fig. 3. The inter-assay accuracy bias and precision was ≤12.3 % and ≤9.4 %, respectively, considering all analytes and QC levels. Repeatability bias was ≤9.0 % and the laboratory precision was ≤9.6 %. The 95 % β-tolerances were between −23.8 % and 30.0 %, and, thus, within the acceptance interval of ±30 %. Thus, the acceptance criteria for inter-assay precision and accuracy, repeatability, laboratory precision, and β-tolerance interval were met for all analytes. However, the method signal to noise was not adequate for the tested LOQ level at 1 µg/L for 7-OH-CBD, as further elaborated in Section 3.2.4. Since the tested LOQ level proved unsuitable for 7-OH-CBD the LOQ levels was omitted and, therefore, only three QC levels included (Fig. 3 and *Supplementary Table S3*).

3.2.3. Carry-over

Carry-over in blank extracts measured after the highest calibrators was, on average, 0.18 % of the area of the highest calibrator. Compared to the areas of the LOQs, the highest carry-over was 12.9 % for 6-OH-CBD. The carry-over was ≤20 % of the LOQ for all analytes. In practice, blank samples are measured between samples, thereby minimizing risk of carry-over to a large extent.

3.2.4. Selectivity

Most analytes showed selectivity over the different blood matrices with no relevant interfering signals thereby fulfilling the requirements. Interfering signals surpassing the defined 20 % of the signal at the LOQ was observed for 7-OH-CBD (1 µg/L) with interferences of about 64.8 %, as can be seen in Fig. 4, which shows overlays of the 7 blank blood matrices and the signal at LOQ. The extrapolated LOQ of 7-OH-CBD is

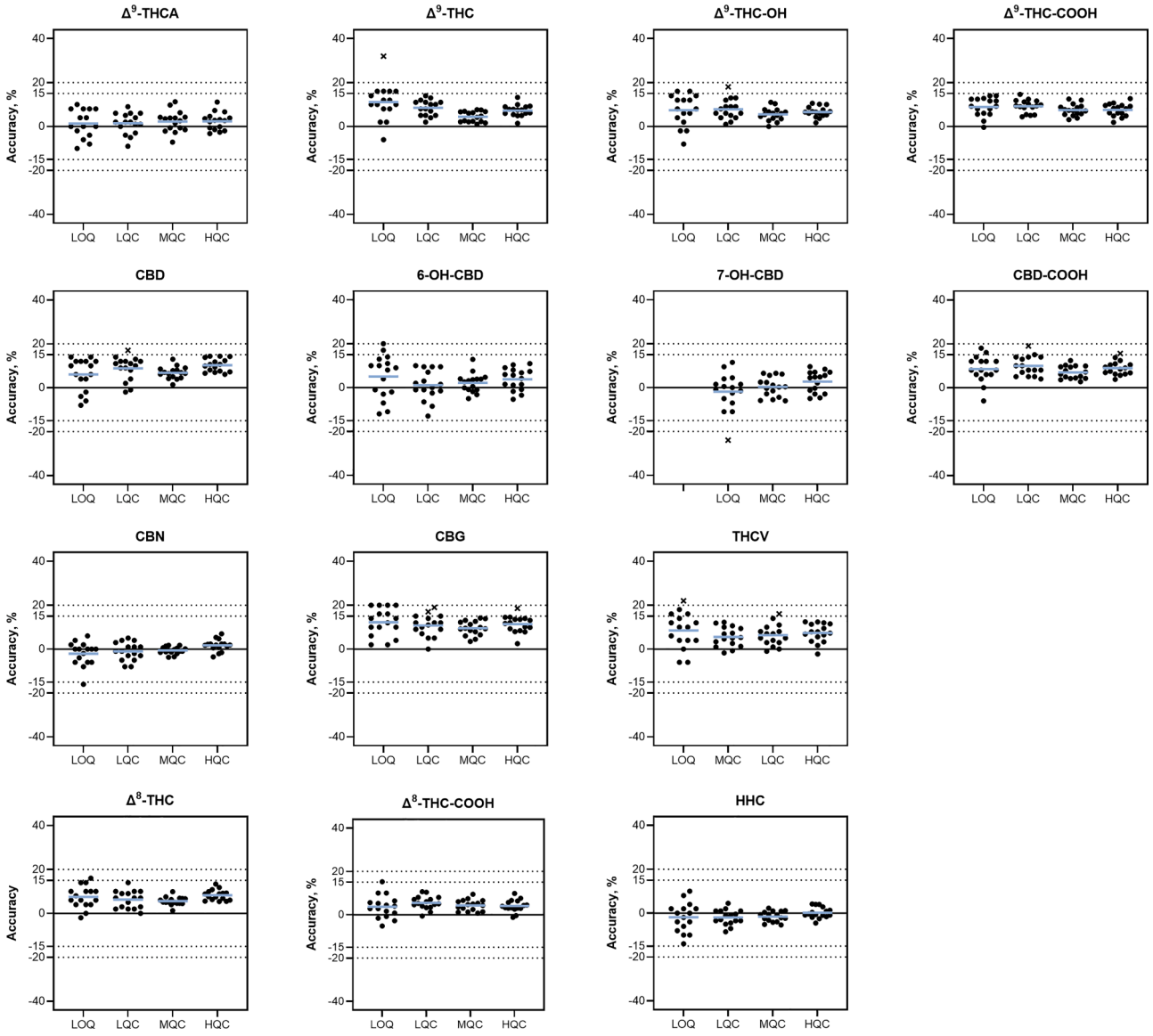

Fig. 3. Inter-assay accuracy bias data. Data points >15 % (LOQ: 20 %) are marked with the symbol x. The blue line represents the mean of all data points per QC level. Using Grubbs' test (α = 0.01, $G = 3.525$), one LOQ data point of Δ^9-THCA (0.85 µg/L ≙ 70 % bias) was excluded as an outlier.

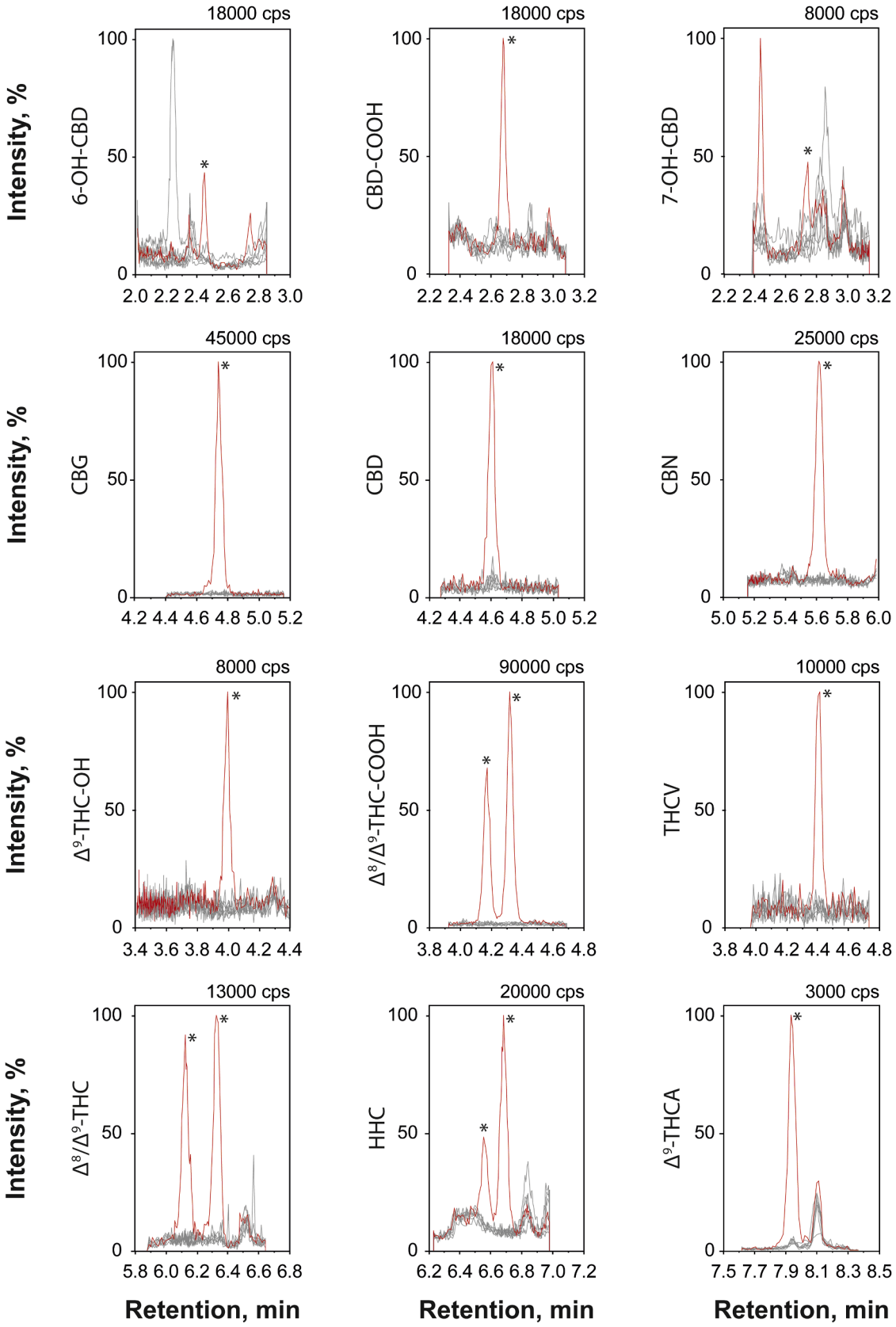

Fig. 4. Results from the selectivity experiment showing overlays of 7 blood samples (grey) and the analytes signal (quantifier; red) at LOQ (1 μg/L for 6-OH-CBD and 7-OH-CBD; 2.5 μg/L for Δ^8- and Δ^9-THC-COOH; and 0.5 μg/L for rest). "*" marks the analyte peak.

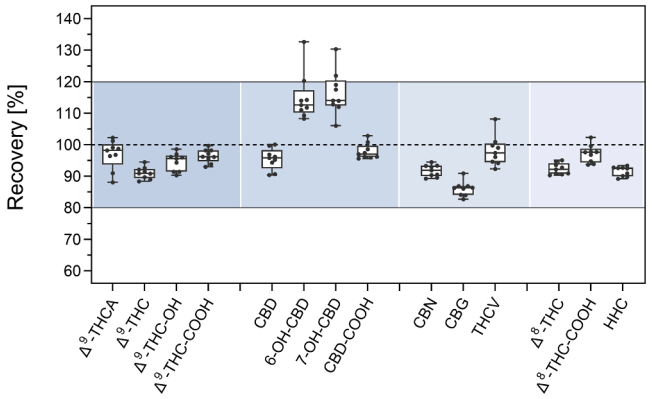

Fig. 5. Visualization of the recovery results.

expected at 2.5 µg/L. When investigating the spiked LOQ levels, all analytes passed the required acceptance criterium of not having more than two out of the seven samples with bias >20 %. The average bias and precision spanning all analytes were ≤9.4 % and ≤18.6 %, respectively. *Supplementary Table S4* summarizes all results from the selectivity experiments. It is noteworthy that 7-OH-CBD passed aforementioned selectivity assessment based on bias and precision, despite exhibiting an unsatisfactory signal intensity at the investigated LOQ level of 1 µg/L (Fig. 4 and *Table S4*). 7-OH-CBD requires further validation of the LOQ at higher concentrations, with an expected LOQ of 2.5 µg/L.

3.2.5. Recovery

Recoveries of >86 % were observed for all analytes, as visualized in Fig. 5. The recoveries were highly consistent and concentration independent, as demonstrated by RSDs of ≤5.5 % determined across different concentration levels. It is notable that 6-OH-CBD and 7-OH-CBD yielded mean recoveries of 114.9 % and 116.4 %, respectively. The data indicated a tendency towards higher recoveries at lower concentrations, which is likely attributable to the increased variability observed at low levels. The individual results for all analytes at the different QC levels are shown in *Supplementary Table S5*.

3.2.6. Matrix effects

Suppressing matrix effects were most pronounced for Δ^9-THC (−33 %), Δ^8-THC (−27 %), CBN (−28 %) and HHC (−35 %) as well as their corresponding internal standards (Δ^9-THC-D_3, Δ^8-THC-D_3 und CBN-D_3), whilst ion enhancement was detected for Δ^9-THCA (+27 %). Signal intensities of other analytes were not affected by the matrix. The observed matrix effects were found to be highly reproducible with RSDs of ≤7 %. Moreover, the matrix effects were concentration-independent, as indicated by a bias between lower and higher concentrations of on average 3.9 %. Fig. 6 visualizes the observed matrix effects and Table 3 shows the observed matrix effects (ME) at the two concentration levels (LQC and HQC) and the percentage difference between the two concentrations. In view of these findings, the impact of matrix effects was found to be negligible, and did not compromise the reliability of the method.

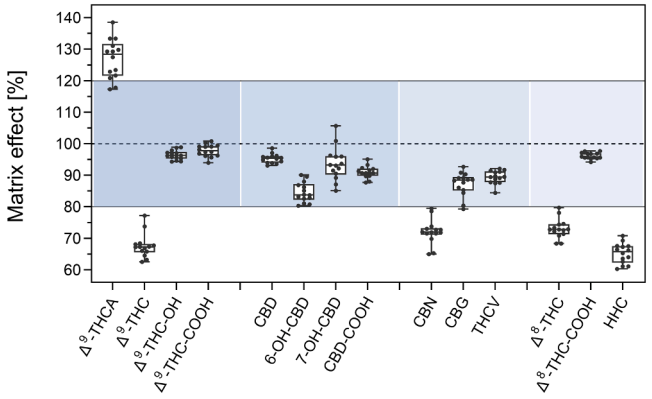

Fig. 6. Visualization of the matrix effects.

Table 3
Mean matrix effects (ME) at the low QC (LQC) and high QC (HQC) and the respective RSDs and %-difference of the ME of the different concentration levels.

Analyte	ME (LQC) [%]	RSD [%]	ME (HQC) [%]	RSD [%]	Difference LQC/ HQC [%]
Δ^9-THCA	126.3	5.2	127.3	4.3	−0.3
Δ^9-THC	67.4	6.9	67.7	4.8	−0.4
Δ^9-THC-OH	96.9	1.8	95.9	1.2	1.1
Δ^9-THC-COOH	96.3	1.2	99.4	0.9	−3.3
CBD	94.8	1.3	95.8	1.7	−1.1
6-OH-CBD	85.9	4.4	83.3	2.4	3.0
7-OH-CBD	94.6	7.3	92.6	3.5	2.2
CBD-COOH	92.0	1.7	90.0	2.1	2.2
CBG	87.1	4.5	87.3	4.5	−0.3
CBN	71.9	5.9	72.3	5.5	−0.6
THCV	88.8	2.9	90.0	1.6	−1.3
Δ^8-THC	72.9	4.9	73.1	4.0	−0.3
Δ^8-THC-COOH	96.2	1.3	96.1	0.8	0.1

3.2.7. Analyte stability

The stability results for all the tested conditions are visualized in Fig. 7. Autosampler stability of the final extracts after precipitation stored at 10 °C for 24 h was sufficient for most analytes, apart from Δ^9-THC-COOH. For Δ^9-THC-COOH a mean deviation of >15 % was observed, as the analyte signal showed a tendency to increase over time. Therefore, Δ^9-THC-COOH needs to be considered with care when samples are reinjected. Precipitated samples that were stored for seven days at −20 °C were found to be stable, with a mean deviation of ≤15 % for all analytes. Benchtop and freeze-thaw stability assessments of the analytes in untreated whole blood revealed limited stability of CBG and CBN, whilst satisfactory stability was observed for all other analytes. At low concentrations CBG and CBN showed signal declines of approximately 20 %. CBG and CBN therefore require special attention when faced with delays in sample preparation resulting in longer periods at room temperature and might not be accurate for samples undergoing repeated freezing and thawing. The storage of samples at −80 °C for a period of seven weeks revealed that the mean concentrations of Δ^9-THC, CBN and THCV differed by >15 % from the initial concentrations. CBD and HHC showed declines in concentration of about 15 %. The stability of 6-OH-CBD and 7-OH-CBD could not be assessed at the lowest concentration because it was below the LOQ for 7-OH-CBD and the sensitivity of the mass spectrometer for 6-OH-CBD decreased compared to previous validation assays. However, the MQC and HQC concentrations showed no relevant decrease or increase. Overall, storage of samples at −80 °C for seven weeks indicated that hydroxylated and carboxylated metabolites were more stable than the parent analytes without additional functionalization.

3.2.8. Additional experiment Δ^8-THC-OH and Δ^9-THC-OH

The accuracy bias and precision for the different spiked samples containing either Δ^8-THC-OH or Δ^9-THC-OH alone or an equimolar mixture of both isomers was ≤12.5 % and ≤2.1 % over all samples, respectively (Fig. 8). These data indicate that both isomers (Δ^8-THC-OH and Δ^9-THC-OH), as well as an equimolar mixture of both isomers, can be accurately and precisely quantified using a Δ^9-THC-OH calibration. Furthermore, it was found that the isomers show a small difference in retention time. As demonstrated in Fig. 9, the ion ratio for Δ^8-THC-OH

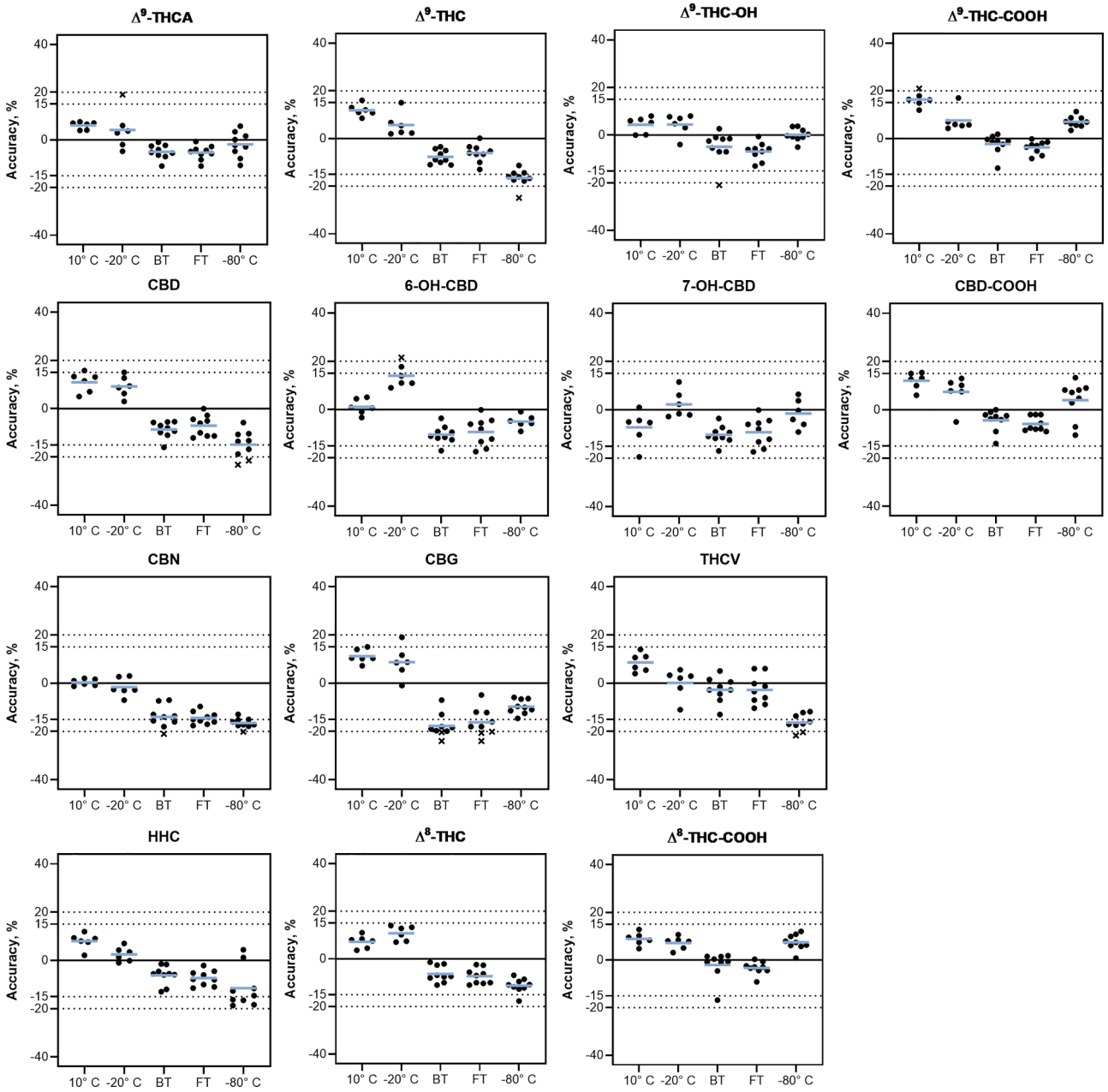

Fig. 7. Analyte stability in the sample extracts at 10 °C for 24 h (autosampler stability) and at −20 °C for 7 days and analyte stability in whole blood for the benchtop (BT) and freeze thaw (FT) experiments, and during storage at −80 °C for 7 weeks. Data points >20 % are marked with the symbol x. The blue line represents the mean of all data points per condition.

was found to be 0.3, while that for Δ^9-THC-OH was 1.0. Hence, a ratio of 0.3 for Δ^8-THC-OH means that the qualifier ion is around 3–4 times more abundant than the quantifier ion. Together with the small retention time shift of the isomers, the difference in the intensity of the qualifiers provide additional markers for the identification of the isomers during routine analysis. However, in practice, authentic samples containing Δ^8-THC-OH should preferably be quantified using a Δ^8-THC-OH calibration to circumvent potential matrix effects.

3.3. Method application: analysis of study samples of individuals after vaporizing CBD-rich cannabis (n = 4)

Applicability of the method was evaluated by analysis of blood samples from four study participants after vaporization of CBD-rich cannabis. As degradation was observed during storage at −80 °C for some analytes (see Section 3.2.7), the prolonged storage period of the study samples of 4–5 years could have had an impact on the absolute analyte concentrations reported herein. Despite potential analyte degradation, nine out of the fourteen analytes included in the method were quantifiable in at least one of the samples. Resulting concentration-time curves for all detected analytes despite CBN (since it was only detected in study participant 1 at <0.5 µg/L in the first time point after inhalation), are displayed in Fig. 10. Δ^8-THC, Δ^8-THC-COOH, and THCV which are naturally present in cannabis and cannabis derived products, were not detected in any of the investigated samples. Moreover, 6-OH-CBD, one of the cannabidiol (CBD) metabolites, was not quantifiable in any of the study samples. HHC could not be measured due to its absence in the vaporized cannabis product. The variability in analyte concentrations among the four study participants was high, yet the pattern of concentration-time profiles remained comparable. Study

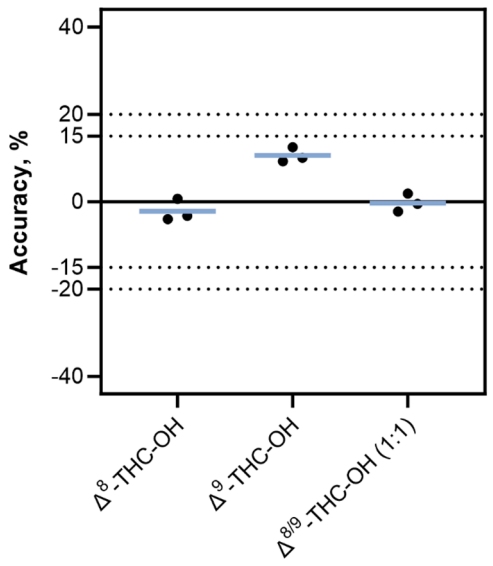

Fig. 8. The accuracy bias of Δ^8-THC-OH, Δ^9-THC-OH, and a mixture of Δ^8-THC-OH and Δ^9-THC-OH (1:1, equimolar) was determined using a calibration curve of Δ^9-THC-OH in $n = 3$ samples each.

participants 1 and 2 showed the highest concentrations for CBD, Δ^9-THC and CBG. Consequently, the presence of Δ^9-THCA, 7-OH-CBD, and Δ^9-THC-OH was only detected in these two participants. A difference in the extent of metabolization could be observed for the secondary metabolite Δ^9-THC-COOH. The maximal concentration of Δ^9-THC was found to be markedly higher in subject 2 than in subjects 3 and 4. However, the levels of Δ^9-THC-COOH were comparable in all three subjects. CBD, Δ^9-THC, CBG, Δ^9-THCA and the metabolites 7-OH-CBD and Δ^9-THC-OH exhibited maximal concentrations (C_{max}) directly after inhalation, while the secondary metabolites CBD-COOH and Δ^9-THC-COOH were present at their highest concentrations after 10 to 20 min after inhalation. Those metabolites also showed the longest detection periods with a plateau for up to 5 h (latest sampling point). The minor cannabinoids CBG and Δ^9-THCA were detected in the sampling point after inhalation with the latest detection at 20 min and could not be detected anymore at the next sampling point at 120 min. In summary, the developed method proved to be operational for the reasonable assessment of the pharmacokinetics of CBD, THC, and their major metabolites, as well as the measurement of minor cannabinoids such as CBG and Δ^9-THCA in the first minutes after cannabis inhalation.

4. Discussion

Herein, a simple one-step extraction procedure for the subsequent quantification of fourteen cannabinoids using UHPLC-MS/MS was developed and validated. The presented method proved suitable for application in forensic toxicological casework, as determined through method validation in line with forensic toxicological guidelines, regarding linearity, accuracy, precision, selectivity, carry-over, recoveries, and matrix effects.

The achieved LOQ of 0.5 µg/L for Δ^9-THC confidently covers the Swiss legal limit of 1.5 µg/L in whole blood, which in practice is as high as 2.2 µg/L, including the harmonized measurement uncertainty of ±30 % [49]. In the presented method, technical advances in mass spectrometry allowed for sufficient sensitivity, allowing for direct injection after protein precipitation, not requiring any concentration steps of the sample extract. This is remarkable, since cannabinoid bioanalysis in whole blood and plasma has often been associated with time-consuming and work-intensive sample cleanup procedures, often requiring evaporation and reconstitution of the extract after liquid-liquid extraction (LLE) or solid phase extraction (SPE) [10,20,41,44,50–58]. More straightforward extraction procedures employ disposable pipette extraction and phospholipid filtration [59,60]. Further approaches apply protein precipitation followed by online extraction, such as the methods published by Hädener et al. (covering Δ^9-THC-COOH and its glucuronide) [13] and Sempio et al. (covering 17 analytes) [61], requiring 100 µL and 200 µL of sample, respectively. Zancanaro et al. [62] presented an online extraction method based on the turbulent flow technology only requiring 50 µL of whole blood sample, however, validated only for Δ^9-THC and its hydroxy and carboxy metabolites, CBD, and CBN, whilst additionally presenting a higher LOQ for Δ^9-THC at 1 µg/L. In 2020, Pichini et al. [11] published a method for THC and CBD, as well as the carboxylic acid precursors Δ^9-THCA and cannabidiolic acid (CBDA) in serum, relying on a simple

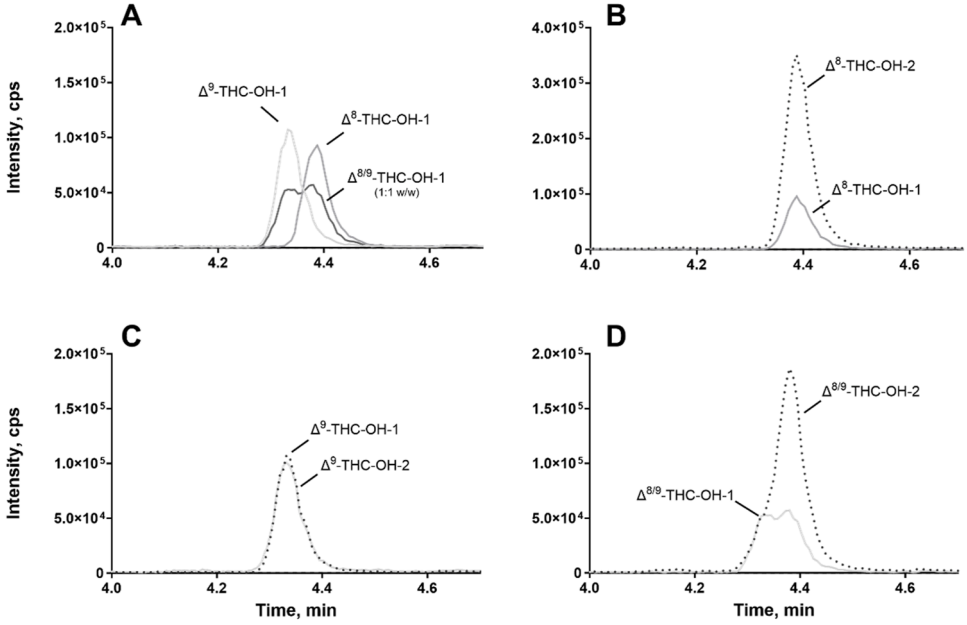

Fig. 9. A: Traces of quantifier ion for samples at same concentrations of Δ^8-THC-OH (10 µg/L), Δ^9-THC-OH (10 µg/L) and an equimolar mixture of both isomers (5 µg/L). B-D: Traces of quantifier ions (331.1→193.1 *m/z*) in solid light gray and qualifier ions (331.1→201.1 *m/z*) in dotted dark gray.

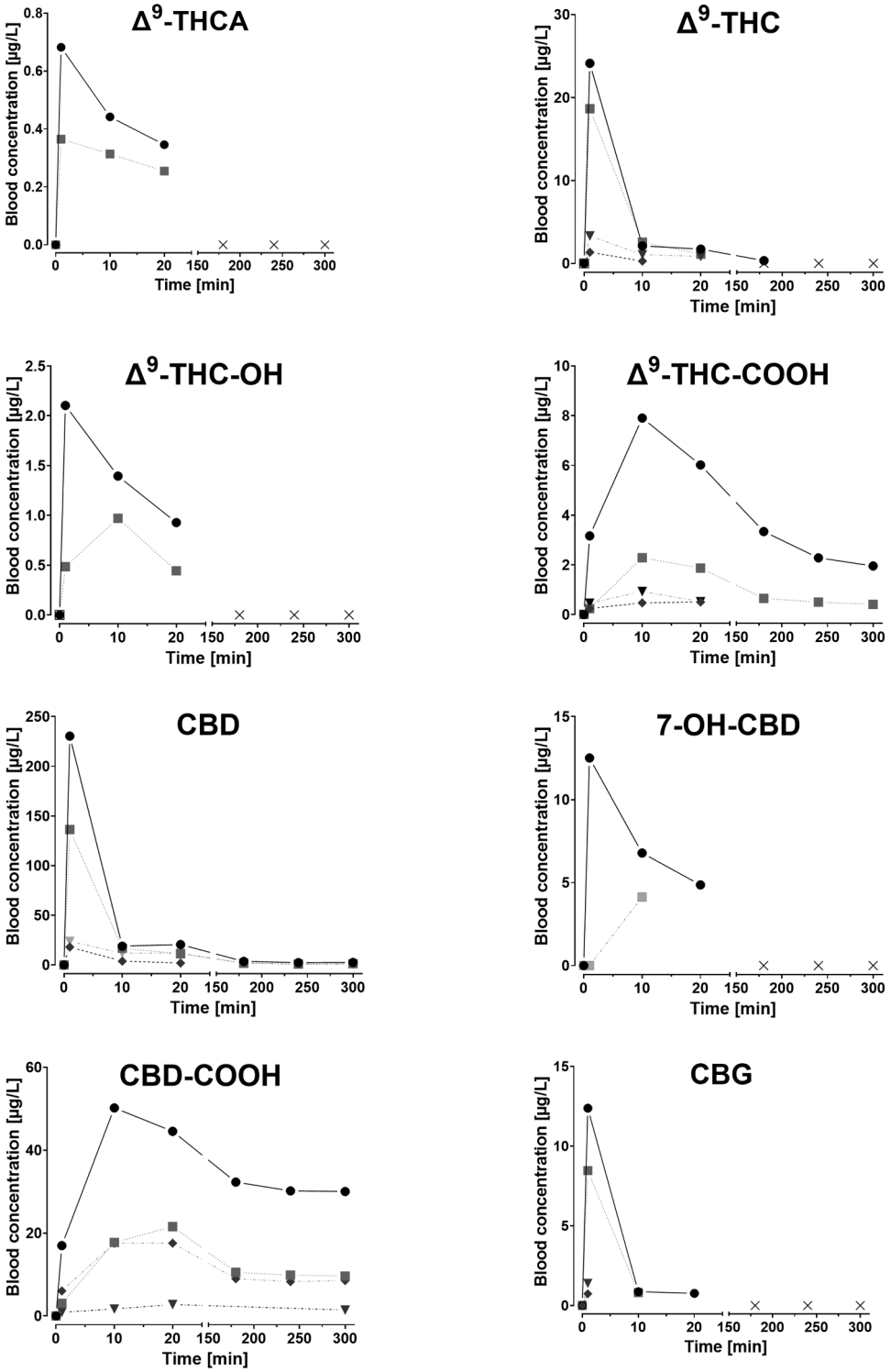

Fig. 10. Concentration time courses of Δ^9-THCA, Δ^9-THC, Δ^9-THC-OH, Δ^9-THC-COOH, CBD, 7-OH-CBD, CBD-COOH, and CBG in whole blood. 300 mg of CBD-rich cannabis inflorescences were administered via vaporization by four study participants. The study participants are shown individually: 1: black circle with continuous line, 2: gray rectangles with dotted line, 3: dark gray rhombus with dotted line and 4: light gray triangle with dashed line. Crosses indicate that the analyte was detected for none of the participants. Mean values of two injections of each sample are depicted.

precipitation step. This method, however, significantly differs from the presented work in the sample and extraction solvent volumes. For comparison, a summary of these methods is provided in Table 4.

This work aimed for the chromatographic separation of Δ^9-THC from its positional isomer Δ^8-THC. This is important because various laboratories reported interferences from Δ^8-THC, requiring adaptations to the analytical methods applied in forensic casework [30,39]. The separation of the Δ^8- and Δ^9- isomers of THC and THC-COOH was achieved within a short runtime of 10 min; however, this could be accomplished only very partially for the hydroxyl-metabolites Δ^8-THC-OH and Δ^9-THC-OH. This is in accordance with other studies presenting methods for the distinction of Δ^9-THC and Δ^8-THC. These studies also

Table 4
Overview of the presented and other bioanalytical methods for phytocannabinoids relying on protein precipitation. "CBC": cannabichromene, "CBDA": cannabidiolic acid, "CBDV": cannabidivarin, . "gluc": glucuronide.

Publication	Sample preparation	Runtime [min]	Analytes	Sample extraction [injection volume]	LOQ: Δ^9-THC, Δ^9-THC-OH, and Δ^9-THC-COOH
Presented method (Monti et al. [15])	Protein precipitation	10	Δ^9-THCA Δ^9-THC Δ^9-THC-OH Δ^9-THC-COOH CBD 6α -OH-CBD 7-OH-CBD CBD-COOH CBG CBN THCV Δ^8-THC Δ^8-THC-COOH	50 µL blood precipitated with 150 µL MeOH:ACN (1:1, *v/v*) [10 µL]	Δ^9-THC: 0.5 µg/L Δ^9-THC-OH: 0.5 µg/L Δ^9-THC-COOH: 2.5 µg/L
Hädener et al. [13]	Protein precipitation and online purification	4.5	Δ9-THC-COOH Δ9-THC-COOH-gluc	100 µL blood precipitated with 300 µL ACN [40 µL]	Δ^9-THC-COOH: 5.0 µg/L
Sempio et al. [61]	Protein precipitation and online purification	10	CBD 6α-OH-CBD 6β-OH-CBD 7-OH-CBD CBD-COOH CBD-gluc Δ^9-THC Δ^9-THC-gluc Δ^9-THC-OH Δ^9-THC-COOH Δ^9-THC-COOH-gluc CBC CBN CBG THCV CBDV THCV-COOH	200 µL plasma precipitated with 800 µL 0.2 M $ZnSO_4$/70 % MeOH [250 µL]	Δ^9-THC: 0.78 µg/L Δ^9-THC-OH: 1.56 µg/L Δ^9-THC-COOH: 0.78 µg/L
Zancanaro et al. [62]	Protein precipitation and online purification	10	Δ^9-THC Δ^9-THC-OH Δ^9-THC-COOH CBD CBN	50 µL blood precipitated with 150 µL ACN:MeOH (2:1, *v/v*) [50 µL]	Δ^9-THC: 1.0 µg/L Δ^9-THC-OH: 1.0 µg/L Δ^9-THC-COOH: 1.0 µg/L
Pichini et al. [11]	Protein precipitation	10	Δ^9-THCA Δ^9-THC Δ^9-THC-gluc Δ^9-THC-OH Δ^9-THC-COOH Δ^9-THC-COOH-gluc CBD CBDA	100 µL serum precipitated with 100 µL M3 reagent, 200 µL MeOH with internal standard, and 200 µL acetone:ACN (8:2, *v/v*) [10 µL]	Δ^9-THC: 0.12 µg/L Δ^9-THC-OH: 0.070 µg/L Δ^9-THC-COOH: 0.19 µg/L

focused on the separation of Δ^9-THC and Δ^8-THC and the corresponding carboxy metabolites, whereby the hydroxy metabolites have not been investigated [30,39,63]. Ballotari et al. [40] and Muir et al. [64] recently published quantitative HPLC-MS/MS methods for urine and blood, where they were able to distinguish the hydroxy metabolites. For the presented method, in instances where analytical data indicates the presence of either Δ^8-THC or Δ^8-THC-COOH, the hydroxy metabolites should be expressed as the sum of both isomers. Quantification of Δ^8-THC-OH alone or in combination with Δ^9-THC (as a sum) was shown to result in accurate and precise results when using the Δ^9-THC-OH calibration. This can be explained through the comparable intensities of the quantifier ion (331.1→196.1 *m/z*) for both isomers, whilst a significant difference in the ion ratios was observed for the qualifier ions. In combination with the detection of the Δ^8-THC or Δ^9-THC parent substances and/or the Δ^8-THC-COOH or Δ^9-THC-COOH secondary metabolites, this adds another level for identification of Δ^8-THC intake. A special focus was put on the chromatographic separation of the carboxy metabolites, as Δ^9-THC-COOH serves as an indicator of regular cannabis use, with a threshold of 40 µg/L generally used in Switzerland [65,66].

Besides Δ^8-THC and its metabolites, also HHC has been implemented into the method. With the presented method the 9S- and 9R- enantiomers are quantitated as a sum by using a reference standard for calibration containing a mixture of 9R- and 9S-HHC. The method is thus suitable for samples presenting a mixture of enantiomers. Additional measurements with pure enantiomers were conducted and are presented in *Supplementary Material ES1*. The metabolites of HHC have not been implemented and are therefore not detected. Recent studies have been published that investigated and reported the individual diastereomers and respective metabolites in biological specimens, such as oral fluid, blood, and urine [19,50,67,68]. Especially for screening applications in urine, the additional implementation of HHC metabolites to enhance the detection is advised [68].

Minor phytocannabinoids have been discussed before to help answer forensic questions about the drug product used [42,69] and to help data interpretation regarding use patterns, including time since last use [43,44]. There is still little known about the pharmacokinetics of many of these minor phytocannabinoids, for which analytical methods such as the presented could be used. With the presented method, CBG, THCV, and Δ^9-THCA can be further assessed for their potential role in refined data interpretation in forensic casework. Herein, the minor cannabinoid

THCV was not detected in the study samples, potentially attributable to the inhaled product being of the industrial hemp type (CBD-rich) [70]. Since THCV showed some degradation (decrease of ~15 %) during the stability assessments (section 3.2.7), the possibility that this analyte has initially been present at low levels and has degraded during storage cannot be ruled out. CBG could be detected in all four study participants, up to 20 min post-inhalation. These minor cannabinoids are typically present at trace levels (often <1 %, *w/w*) in dried cannabis plant material [15]. The presented method with LOQs of 0.5 µg/L for these minor cannabinoids will likely suffer from short detection windows. In return, as has been described before for CBG [44], the detection of minor cannabinoids as indicators of recent use remains worthwhile and should be subject of future research [69]. Further development work and the implementation of an on-line up-concentration step, or additional sample preparation steps as typically employed for the detection of minor cannabinoids in biological specimens, could improve the sensitivity for the assessment of their pharmacokinetic properties in humans [57].

For the analyzed study samples, despite the prolonged storage period of between 4–5 years various cannabinoids were still quantifiable. Stability investigations, however, showed that particularly Δ^9-THC, CBD, CBN, HHC and THCV showed a tendency to degrade (decrease of ~15 %) when stored at −80 °C for two months. In contrast, analytes with additional functionalization, such as the hydroxylated and carboxylated metabolites, were stable under the same storage conditions. Observations on the limited stability of Δ^9-THC, Δ^9-THC-OH, Δ^9-THC-COOH, CBD, and CBN in whole blood when stored at 4 °C and −20 °C were reported by Sørensen et al. [71], who propose the addition of antioxidants and/or storage at −80 °C to improve long term stability. Aforementioned authors also observed no significant decrease of various cannabinoids in whole blood when stored at −80 °C for up to the tested 5 months, however, using differing stabilizers than used herein. Long term stability data on cannabinoids and especially the herein included minor cannabinoids in whole blood and other human biological specimens are currently largely missing. Schweidweiler et al. [72] demonstrated acceptable stability of Δ^9-THC and its metabolites, THCV, CBD and CBG in oral fluid for 3 months at 4 °C. Regarding the final quantitated values in the presented study, a decrease in the analyte concentrations due to prolonged storage cannot be excluded. Based on our results and due to the current lack of stability assessments of cannabinoids in different human biological specimens, further assessments of stability, including long-term storage beyond 2 months and the impact of the storage vessel and the addition of antioxidants are recommended for samples expected to undergo prolonged storage.

Study samples from four individuals after vaporization and inhalation of CBD-rich cannabis were analyzed. The initial concentrations after inhalation showed a range of concentrations of the investigated cannabinoids. This is in accordance with findings from literature, particularly considering smoking and inhalation studies [21,73,74]. Δ^9-THC and CBD, as well as their metabolites Δ^9-THC-OH and 7-OH-CBD, presented at peak concentrations directly after inhalation. 6-OH-CBD could not be detected but has also shown to be present at lower concentrations compared to 7-OH-CBD in other studies [20,75]. The noise level of 6- and 7-OH-CBD was rather high for MS^2 analysis resulting in higher LOQs compared to the other cannabinoids. Therefore, the implementation of MS^3 experiments may have the potential to enhance the sensitivity of these CBD metabolites in whole blood. As expected, the secondary metabolites Δ^9-THC-COOH and CBD-COOH showed a delayed C_{max} compared to their primary metabolites. The minor phytocannabinoid CBG was detected in all study participants in the first sampling time-points after inhalation and showed a similar concentration time curve as the two major cannabinoids CBD and Δ^9-THC. Due to the prolonged storage of the study samples at −80 °C, some degree of analyte degradation cannot be excluded. Nevertheless, the developed method successfully detected both major and minor cannabinoids, demonstrating its suitability for the analysis of authentic samples, whilst offering an efficient protocol to extract and analyze larger sample loads. While the results demonstrate the method's applicability, absolute concentrations for the study samples should be interpreted with caution given potential uncertainties in long-term sample stability.

5. Conclusion

The presented method stands out through its simplicity in sample preparation and miniaturization that renders it suitable for automation. The simple workflow enables high-throughput applications encompassing larger sample loads, thereby making it suitable for use in larger scale clinical studies. By including a more comprehensive subset of phytocannabinoids, the role of these cannabinoids for refined data interpretation in the forensic and medical field can be further evaluated and should therefore be subjected to future research. Whilst previous methods for the bioanalysis of cannabinoids in whole blood matrices often relied on LLE and SPE, generally including various laborious and time-intensive sample preparation steps, the presented protocol demonstrated how also simple precipitation can achieve sufficient sensitivity for quantitative cannabinoid bioanalysis. However, the lower abundance of minor phytocannabinoids might require further enhancement of sensitivity. Stability issues of various analytes in whole blood have been observed and should be given special attention when storing blood samples for prolonged periods. The suitability of the method has been demonstrated by measuring clinical study samples of participants inhaling CBD-rich cannabis after vaporization.

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CRediT authorship contribution statement

Manuela Carla Monti: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Anna Stoll:** Writing – review & editing, Visualization, Investigation. **Götz Schlotterbeck:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Urs Duthaler:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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