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RESEARCH ARTICLE

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Preliminary data on the potential for unintentional antidoping rule violations by permitted cannabidiol (CBD) use

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Abstract

According to the World Anti-Doping Agency (WADA) regulations, cannabinoids use is prohibited in competition except for cannabidiol (CBD) use. For an adverse analytical finding (AAF) in doping control, cannabinoid misuse is based on identification of the pharmacologically inactive metabolite 11-nor- Δ^9 -carboxy-tetrahydrocannabinol-9-carboxylic acid (carboxy-THC) in urine at a concentration greater than 180 ng/ml. All other (minor) cannabinoids are reported as AAF when identified, except for CBD that has been explicitly excluded from the class of cannabinoids on WADA's Prohibited List since 2018. However, due to the fact that CBD isolated from cannabis plants may contain additional minor cannabinoids, the permissible use of CBD can lead to unintentional violations of antidoping regulations. An assay for the detection of 16 cannabinoids in human urine was established. The sample preparation consisted of enzymatic hydrolysis of glucuronide conjugates, liquid–liquid extraction, trimethylsilylation, and analysis by gas chromatography/tandem mass spectrometry (GC–MS/MS). Spot urine samples from CBD users, as well as specimens obtained from CBD administration studies conducted with 15 commercially available CBD products, were analyzed, and assay characteristics such as selectivity, reproducibility of detection at the minimum required performance level, limit of detection, and limit of identification were determined. An ethical committee approved controlled single dose commercially available CBD products administration study was conducted to identify 16 cannabinoids in urine samples collected after ingestion or application of the CBD products as well as their presence in spot urine samples of habitual CBD users. Variable patterns of cannabinoids or their metabolites were observed in the urine samples, especially when full spectrum CBD products were consumed. The presence of minor cannabinoids or their metabolites in an athlete's in-competition urine sample represents a substantial risk of an antidoping rule violation.

KEYWORDS

cannabinoids, doping, gas chromatography mass spectrometry, sport, urine

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1 | INTRODUCTION

The use of cannabinoids is prohibited in sports competition.¹ 11-Nor-delta-9-carboxy-tetrahydrocannabinol-9-carboxylic acid (carboxy-THC) is presently the main target analyte for the detection of cannabis misuse in doping control urine samples,² with a urinary threshold of 150 ng/ml.³ According to the World Anti-Doping Agency (WADA) Technical Document TD2019DL, a sample shall be reported as an adverse analytical finding (AAF) when the value exceeds the decision limit of 180 ng/ml.³ For other (minor) cannabinoids, no thresholds exist. Consequently, when identified in in-competition doping control urine samples according to WADA regulations,⁴ an AAF is reported by the antidoping laboratory.

Cannabidiol (CBD) does not produce the euphoria and tachycardia of Δ^9 -tetrahydro-cannabinol⁵ and was excluded from WADA's Prohibited List¹ on January 1, 2018. Yet it was clarified by WADA and others^{6,7} that CBD products, manufactured from cannabis plant extracts, may also contain THC,⁸ or varying concentrations of (banned) cannabinoids⁹ that could lead to an AAF when an athlete is subjected to routine doping controls.

Globally, there is a substantial increase in the use of over-the-counter CBD products, despite the lack of scientific data for the drug's efficacy for different indications.^{10–12} One pharmaceutical CBD product (Epidiolex®) is currently approved in Europe for the therapy of a serious form of epilepsy in children (Dravet syndrome).¹³ However, CBD products are frequently sold as nutritional supplements, novel foods, or cosmetics and can be purchased in pharmacies, organic shops, drugstores, supermarkets, and via the Internet, and a growing receptivity amongst elite athletes is conceivable.^{14–16}

Multiple publications are available for the analysis of cannabis intake,^{17–19} with analytical methods being largely based on gas

chromatography–mass spectrometry (GC–MS)^{20,21} and/or liquid chromatography–mass spectrometry (LC–MS).^{18,19,22} In order to clarify whether the permissible use of different CBD products can lead to unintentional violations of the antidoping regulations, similar analytical strategies were pursued in this study to assess the risks associated with CBD consumption by athletes during routine doping controls. Therefore, a GC–MS-based detection method for various cannabinoids in human urine was developed and validated, and 15 - different commercially available CBD products (oils, pastes, capsules, crystals) were purchased (Table 1). Elimination studies were performed in healthy volunteers who consumed the CBD products following the manufacturers' dosage recommendations and provided postadministration urine samples. The excretion study was performed with approval (number 060/2020) of the ethical committee of the German Sport University Cologne (Germany) and written informed consent was obtained from all participants. Urine samples were analyzed for the presence of doping-relevant cannabinoids by means of GC–MS. In addition, spot urine samples obtained from habitual CBD users were tested for cannabinoids using the established approach. These proof-of-concept investigations were performed to determine the risk of an inadvertent AAF resulting from the intake or application of permitted CBD products and to initiate a discussion about future reporting levels for minor cannabinoids in sports drug testing.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

All solvents and reagents were of analytical grade purity. *Tert*-Butyl methyl ether (TBME) was purchased from AppliChem

TABLE 1 CBD products for administration studies

| Product | Formulation | Application route | Manufacturer information | CBD dose (mg) |
|---------|-------------|-------------------|---|---------------|
| 1 | Oil | Oral | 63% Hemp seed oil, 37% hemp extract | 12 |
| 2 | Powder | Oral | 99.6% CBD crystals | 50 |
| 3 | Oil | Oral | 2400 mg CBD/10 ml hemp seed oil—Full spectrum (CBG, CBN) | 44 |
| 4 | Oil | Oral | 2000 mg CBD/10 ml hemp oil, hemp extract, isolate—full spectrum | 36 |
| 5 | Capsule | Oral | Hemp oil/300 mg hemp extract per capsule | Unknown |
| 6 | Oil | Oral | 1000 mg CBD/10 ml hemp seed oil | 50 |
| 7 | Oil | Oral | 3% CBD in hemp oil—full spectrum | 50 |
| 8 | Oil | Oral | 1000 mg CBD/10 ml hemp seed oil—decarboxylated | 50 |
| 9 | Oil | Oral | 500 mg CBD/10 ml hemp seed oil | 25 |
| 10 | Oil | Oral | 500 mg CBD/10 ml hemp oil—full spectrum (CBN, CBV, CBG, CBC) | 25 |
| 11 | Oil | Oral | 1000 mg CBD/10 ml hemp oil—full spectrum (CBN, CBV, CBG, CBC) | 50 |
| 12 | Aq sol | Oral | 250 mg CBD/10 ml water (aq sol: Aqueous solution) | 11.25 |
| 13 | Capsule | Oral | 25 mg CBD/hemp oil per capsule | 50 |
| 14 | Oil | Oral | 1000 mg CBD/10 ml hemp oil—full spectrum | 50 |
| 15 | Gel | Transdermal | 10% CBD | 20 |

(Darmstadt, Germany) and distilled before use. β -Glucuronidase from *Escherichia coli* was supplied by Roche Diagnostics GmbH (Mannheim, Germany) and Red Abalone β -glucuronidase from Ango Science (Santiago, Chile). *N*-Methyl-*N*-trimethylsilyl-trifluoroetamide (MSTFA) was obtained from Machery & Nagel (Düren, Germany). All solutions and buffers were prepared using deionized water (Water Lab System, Millipore, Eschborn, Germany).

The following certified standards were purchased from LGC Promochem (Wesel, Germany): Δ^8 -tetrahydrocannabinol (Δ^8 -THC), Δ^9 -tetrahydrocannabinol (THC), 11-OH- Δ^9 -tetrahydro-cannabinol (11-OH-THC), CBD, cannabigerol (CBG), cannabinol (CBN), cannabidivarin (CBDV), cannabichromene (CBC), Δ^9 -tetrahydrocannabidivarin (THCV), Δ^9 -tetrahydrocannabinol acid (THCA), cannabidiol acid (CBDA), cannabigerol acid (CBGA), cannabinol acid (CBNA), cannabidivarinic acid (CBDVA), cannabichromene acid (CBCA), Δ^9 -tetrahydrocannabidivarinic acid (THCVA), d_3 - Δ^9 -tetrahydrocannabinol (d_3 -THC), d_3 -11-OH- Δ^9 -tetrahydrocannabinol (d_3 -11-OH-THC), d_3 -cannabidiol (d_3 -CBD), d_3 -cannabinol (d_3 -CBN). 17 α -Methyltestosterone was obtained from Serva, Heidelberg, Germany.

2.2 | CBD products

A total of 15 CBD products (14 products for oral administration, one gel for transdermal application) were obtained from Internet shops, pharmacies and CBD manufacturers (Table 1).

2.3 | Sample preparation

Cannabinoids are excreted unconjugated and conjugated (primarily as glucuronidated phase-II metabolites).^{18,20,23,24} In order to detect both, free and conjugated urinary cannabinoid metabolites, as well as to increase the analytical sensitivity, a hydrolysis step during the sample preparation is frequently recommended and employed. Hence, 2 ml urine was fortified with 50 ng of the internal standards d_3 -THC, d_3 -11-OH-THC, d_3 -CBD, d_3 -CBN, and 17 α -methyltestosterone. The samples were buffered to pH 7.0 with 0.75 ml 0.8 M phosphate buffer ($\text{Na}_2\text{HPO}_4\text{:NaH}_2\text{PO}_4$, 1:2, w:w). Twenty-five microliters β -glucuronidase from *E. coli* were added. The mixture was incubated at 50°C for 1 h. After cooling to ambient temperature, the pH was adjusted to 9.6 by the addition of 0.5 ml aqueous potassium carbonate and potassium hydrogen carbonate (20%, 1:1, w:w). Five milliliter TBME were added, and the mixture was shaken for 5 min and subsequently centrifuged at 598 g for 5 min. The organic layer was transferred to a fresh glass tube, evaporated to dryness at 50°C using a rotary evaporator under reduced pressure, and the dry residue derivatized with 100 μ l MSTFA/ NH_4I /ethanethiol 1000:2:3 (v:v:v) for 20 min at 60°C.

An alternative hydrolysis protocol in accordance with earlier published approaches was employed to verify the analytical method for the detection of cannabinoids. Here, 40 μ l β -glucuronidase from Red Abalone (5200 units) were utilized, and the hydrolysis was performed at pH 5 (adjusted using sodium acetate buffer) applying an incubation period of 4 h at 37°C.²⁴ The comparison of conjugate hydrolysis efficacy was done using five urine specimens obtained from administration studies performed with full spectrum CBD products. The samples were prepared twice in triplicate and hydrolyzed with *E. coli* or Red Abalone, respectively.

2.4 | Gas chromatography/tandem mass spectrometry

All analyses were performed using a Thermo Scientific TSQ 8000 tandem mass spectrometer coupled to a Thermo Scientific Trace 1310 gas chromatograph (Thermo Fisher Scientific, Dreieich, Germany). A J&W Scientific Ultra I (OV-1) column (length 17 m, I.D. 0.2 mm, film thickness 0.11 μ m) was employed, and helium was used as carrier gas at a head pressure of 16 psi.

An 1.8 μ l aliquot of the sample was injected into the GC system, which was operated in split (1:10) mode. The GC temperature was ramped as follows: initial temperature 185°C, program rate 3°C min⁻¹ to 234°C, program rate 40°C min⁻¹ to 310°C, constant temperature at 310°C for 2 min. The injection port and transfer line were heated to 300°C.

The trimethylsilylated analytes were measured using selected reaction monitoring (SRM) with electron ionization (EI), and the corresponding diagnostic ion transitions for each compound are presented in Table 2.

2.5 | Urine SG

Cannabinoid concentrations were adjusted to a urine specific gravity (SG) of 1.020 based on the following equation: $\text{Conc}_{\text{corr}} = \text{Conc}_{\text{measured}} * (1.020 - 1)/(SG - 1)$.

The SG measurements were performed on a PAAR Refractometer Abbemat 350 (Osterfildern, Germany) with automatic sampling.

2.6 | Excretion study urine samples

Following written consent, study participants (three males, five females, 42–66 years old) were administered one CBD product each per trial, according to the recommended dosage of the manufacturer (Table 1), and urine samples were collected before and 8, 16, and 32 h after product administration. For five CBD products (numbers 2, 3, 7, 11, 14, Table 1), two volunteers were available for administration studies. The urine specimens were stored frozen until analysis for up to 8 weeks.

TABLE 2 Summary of compound properties

| Compound | Abbreviation | RT (min) | IT (m/z) | CE (V) | LOD (ng/ml) | LOI (ng/ml) |
|---|----------------------------------|----------|-------------|--------|-------------|-------------|
| Cannabidivarin | CBDV | 4.16 | 362.3/273.2 | 7 | 0.18 | 0.18 |
| | | | 362.3/319.3 | 7 | | |
| | | | 362.3/231.2 | 17 | | |
| Δ^9 -Tetrahydrocannabivarin | THCV | 4.93 | 358.3/315.3 | 9 | 0.35 | 0.18 |
| | | | 358.3/275.2 | 19 | | |
| | | | 358.3/261.2 | 23 | | |
| d ₃ -Cannabidiol | d ₃ -CBD | 6.14 | 393.3/304.3 | 7 | | |
| Cannabidiol | CBD | 6.21 | 390.3/301.3 | 7 | 0.15 | 0.80 |
| | | | 390.3/319.3 | 7 | | |
| | | | 390.3/244.2 | 11 | | |
| Cannabichromene | CBC | 7.11 | 303.3/246.2 | 24 | 0.10 | 0.10 |
| | | | 303.3/174.1 | 35 | | |
| | | | 303.3/231.2 | 27 | | |
| Δ^8 -Tetrahydrocannabinol | Δ^8 -THC | 7.21 | 303.3/246.2 | 24 | 0.45 | 0.30 |
| | | | 303.3/174.1 | 35 | | |
| | | | 303.3/231.2 | 27 | | |
| d ₃ - Δ^9 -Tetrahydrocannabinol | d ₃ - Δ^9 -THC | 7.45 | 374.3/292.2 | 9 | | |
| Δ^9 -Tetrahydrocannabinol | THC | 7.50 | 371.3/289.2 | 9 | 0.18 | 0.40 |
| | | | 371.3/305.3 | 7 | | |
| | | | 371.3/265.2 | 7 | | |
| Cannabidivarinic acid | CBDVA | 8.18 | 463.3/373.3 | 9 | 0.45 | 0.80 |
| | | | 463.3/147.1 | 33 | | |
| | | | 463.3/133.1 | 31 | | |
| d ₃ -Cannabinol | d ₃ -CBN | 8.79 | 370.3/310.3 | 25 | | |
| Cannabinol | CBN | 8.86 | 367.3/310.3 | 25 | 0.10 | 0.16 |
| | | | 367.3/295.2 | 30 | | |
| | | | 367.3/238.2 | 35 | | |
| | | | 367.3/323.3 | 31 | | |
| Cannabigerol | CBG | 8.95 | 337.3/321.3 | 9 | 0.95 | 0.46 |
| | | | 337.3/263.2 | 9 | | |
| | | | 337.3/249.2 | 13 | | |
| Δ^9 -Tetrahydrocannabivarinic acid | THCVA | 10.09 | 459.3/147.1 | 33 | 0.15 | 0.60 |
| | | | 459.3/337.3 | 9 | | |
| | | | 459.3/379.3 | 9 | | |
| Cannabidiolic acid | CBDA | 10.67 | 491.3/401.3 | 9 | 0.45 | 0.45 |
| | | | 491.3/133.1 | 33 | | |
| | | | 491.3/345.3 | 20 | | |
| | | | 491.3/311.3 | 26 | | |
| Cannabichromenic acid | CBCA | 10.90 | 419.3/257.2 | 19 | 0.40 | 0.30 |
| | | | 419.3/200.2 | 25 | | |
| | | | 419.3/271.2 | 30 | | |
| | | | 419.3/305.3 | 30 | | |
| d ₃ -11-Hydroxy-tetrahydrocannabinol | d ₃ -11-OH-THC | 12.39 | 374.3/292.2 | 9 | | |
| 11-Hydroxy-tetrahydrocannabinol | 11-OH-THC | 12.49 | 371.3/289.2 | 9 | 0.20 | 0.80 |
| | | | 371.3/305.3 | 7 | | |
| | | | 371.3/265.2 | 7 | | |
| | | | 371.3/329.3 | 9 | | |

(Continues)

TABLE 2 (Continued)

| Compound | Abbreviation | RT (min) | IT (m/z) | CE (V) | LOD (ng/ml) | LOI (ng/ml) |
|---------------------------------------|--------------|----------|-------------|--------|-------------|-------------|
| Δ^9 -Tetrahydrocannabinol acid | THCA | 13.05 | 487.3/147.1 | 35 | 0.45 | 0.90 |
| | | | 487.3/365.3 | 10 | | |
| | | | 487.3/407.3 | 9 | | |
| | | | 487.3/419.3 | 17 | | |
| Cannabinolic acid | CBNA | 14.64 | 483.3/147.1 | 35 | 0.40 | 0.80 |
| | | | 483.3/321.3 | 12 | | |
| | | | 483.3/335.3 | 15 | | |
| | | | 483.3/393.3 | 9 | | |
| Cannabigerolic acid | CBGA | 14.70 | 561.3/421.3 | 21 | 0.70 | 0.95 |
| | | | 561.3/403.3 | 19 | | |
| | | | 561.3/147.1 | 35 | | |
| | | | 561.3/477.1 | 21 | | |
| Methyltestosterone | MT | 14.94 | 446.3/301.3 | 20 | | |
| | | | 301.3/169.1 | 15 | | |
| Carboxy-THC | THC-COOH | 15.20 | 371.3/289.2 | 18 | | |
| | | | 371.3/305.3 | 12 | | |
| | | | 371.3/265.2 | 15 | | |

Note: For each compound examined, the observed retention time (RT), ion transitions (IT), and collision energy (CE) are presented. For target analytes (internal standards excluded), limit of detection (LOD) and limit of identification (LOI) are also shown.

2.7 | Spot urine samples of CBD users

In addition to the above-mentioned controlled administration studies, also spot urine samples of seven customary CBD users were collected. The volunteers provided additional information concerning the formulation, dosage, and frequency of the product usage. The urine specimens were stored frozen until analysis within 8 weeks. The collection of spot urine samples was performed in agreement with the ethical committee of the German Sport University Cologne (Germany).

2.8 | Determination of cannabinoids in human urine

The identification of the cannabinoids was performed by comparison with adequate reference standards using a minimum of three precursor-product ion transitions, meeting the requirements of the WADA Technical Document TD2015IDCR.⁴

The estimation of the analytes' concentration was performed by means of calibration curves (working range 1–11 ng/ml), utilizing the peak area ratios of the quantifier ion transitions of analyte and internal standard. For substances with concentrations outside the calibration curve, the urine volume was adjusted.

3 | ASSAY VALIDATION

The assay validation was performed according to the requirements of the WADA ISL.²⁵

3.1 | Selectivity

Selectivity is the ability to differentiate the analyte of interest from endogenous matrix interferences or from other substances present in the sample. Ten different blank urine specimens with known origin were prepared and analyzed as described above in order to probe for interfering peaks in the selected ion transitions at the expected retention time. Additionally, it was shown that the positive quality control sample produced the expected signal at the correct retention time for each target analyte.

3.2 | Reproducibility of detection at the MRPL

Ten different blank urine specimens with known origin, spiked with 1 ng/ml of each target analyte, were prepared and analyzed to demonstrate the reproducibility of detection at the minimum required performance level (MRPL).

3.3 | Limit of detection

Six different representative urine samples, fortified at five different percentage concentrations of the MRPL (5% = 0.05 ng/ml, 10% = 0.1 ng/ml, 20% = 0.2 ng/ml, 50% = 0.5 ng/ml, 100% = 1.0 ng/ml), were prepared and analyzed. The estimation of the limit of detection (LOD) was performed by using a detection response curve. The LOD is estimated as the

concentration at which the response curve shows a 95% analyte detection rate.

3.4 | Limit of identification

The limit of identification (LOI) is the lowest concentration of an analyte, which meets the WADA TD IDCR⁴ criteria in 95% of representative samples.

Six different representative urine samples, fortified at five different percentage concentrations of the MRPL (5% = 0.05 ng/ml, 10% = 0.1 ng/ml, 20% = 0.2 ng/ml, 50% = 0.5 ng/ml, 100% = 1.0 ng/ml), were prepared and analyzed. The estimation of the LOI was performed by using a detection response curve. The LOI is estimated as the concentration at which the response curve shows a 95% analyte identification rate.

4 | RESULTS AND DISCUSSION

The temporal indication of cannabis use is important in clinical evaluations, in workplace drug testing, in crash and incident investigations, in verifying or falsifying the accuracy of court testimonies, and for routine doping controls. Here, Δ^9 -tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (carboxy-THC) are the most frequent analytes for the situational assessment. Additional cannabinoids, for example, CBD, CBN, CBG, THCV, and THCVA were suggested for inclusion into such evaluations in order to improve clinical and forensic result interpretations in blood, plasma, and oral fluid.^{18,19,26} The detection of these analytes and other minor cannabinoids as well as their urinary metabolites in in-competition doping control urine samples would constitute an AAF. The herein presented pilot study focused on seven intact minor cannabinoids (i.e., CBG, CBN, CBC, CBDV, CBGA, THCA, and CBDA) without corresponding predicted or established metabolic products. Targeting urinary phase-I and/or phase-II metabolites might further extend the detection windows for these minor cannabinoids, and follow-up investigations would be warranted if prolonged retrospectivity is desired, considering the fact that cannabinoids are currently banned in-competition only.

4.1 | Assay validation

In order to assess the developed assay's analytical suitability, the parameters selectivity, reproducibility of detection at the MRPL, LOD, and LOI were determined according to the requirements of the WADA ISL.²⁵ Regarding selectivity, the investigation of 10 different blank urine samples generated no interfering signals at the expected retention times for the analytes. The detection of all cannabinoids was possible at the 1 ng/ml MRPL. LOD and LOI were determined by a detection response curve with five concentrations (Table 2). For several substances (CBCA, CBG, THCV, Δ^8 -THC), the determined LOI

was lower than the corresponding LOD. This is attributed to the use of a higher urine volume in the confirmation procedure. Figure 1 shows the extracted ion chromatograms of the investigated cannabinoids and internal standards.

The use of β -glucuronidase from Red Abalone as discussed in CBD-dedicated analytical approaches²⁴ resulted in an increase in urinary CBD (1.2-fold–twofold) and CBDV (twofold–threefold) concentrations compared to hydrolysis with *E. coli*, suggesting a more efficient hydrolysis of glucuronic acid conjugates. However, an unsatisfactory deconjugation of relevant steroid profile markers (androsterone, etiocholanolone, 5 α -androsterane-3 α ,17 β -diol, 5 β -androsterane-3 α ,17 β -diol, testosterone, and epitestosterone) was observed and, in the light of the fact that the use of β -glucuronidase from *E. coli* is obligatory in sports drug testing initial testing procedures for steroid profile-related analyses,²⁷ it was decided to use β -glucuronidase from Red Abalone in the confirmatory analysis targeting cannabinoid-related analytes.

The fact that the precursor cannabinoid acids can generate their decarboxylated counterparts under the influence of heat and light and by means of spontaneous decarboxylation²⁸ was taken into consideration, especially since the decarboxylation of cannabinoid acids can occur in the hot injector port of the GC or during the manufacturing process of CBD products.²⁹ As decarboxylation rates for the cannabinoid acids as determined in our study are 0.2% and 8.8%, the degradation was considered negligible.

4.2 | Excretion study urine samples

In Europe, CBD is primarily obtained from hemp containing a maximum of 0.2% THC, with the declaration on most products of “THC free.” Nevertheless, a recent study of 67 food products on the German market (mostly CBD oils) showed detectable THC in 25% of the tested products,^{29,30} and the analysis of CBD oils in the Netherlands further corroborated this finding.³¹ The manufacturers of the products administered in this study evidently employed low-THC cannabis material for their CBD products as no significant signals for THC and related metabolites were detected in the investigated urine samples.

Urine samples collected 8, 16, and 32 h after CBD administration according to the manufacturer's recommended dose (Table 1) were analyzed for cannabinoids. In all urine samples collected 8 h after administration, CBD was detectable in concentrations higher than 5 ng/ml (Table 3). Despite similar amounts of CBD in the different products, urine concentrations were highly variable; for example, 50 mg CBD in products 2, 6, 7, 8, 11, 13, and 14 led to 28–746 ng/ml CBD in urine. Administration of 44 mg CBD in product 3 yielded maximum urinary CBD concentrations of 4485 ng/ml, and 36 mg CBD via product 4 resulted in 1424 ng/ml urinary CBD 8 h after application. Of note, both products were declared as full spectrum products. These results may be attributed to an incorrect CBD content declaration (which was not confirmed prior to the administration study) or to individual metabolism differences in the volunteers.

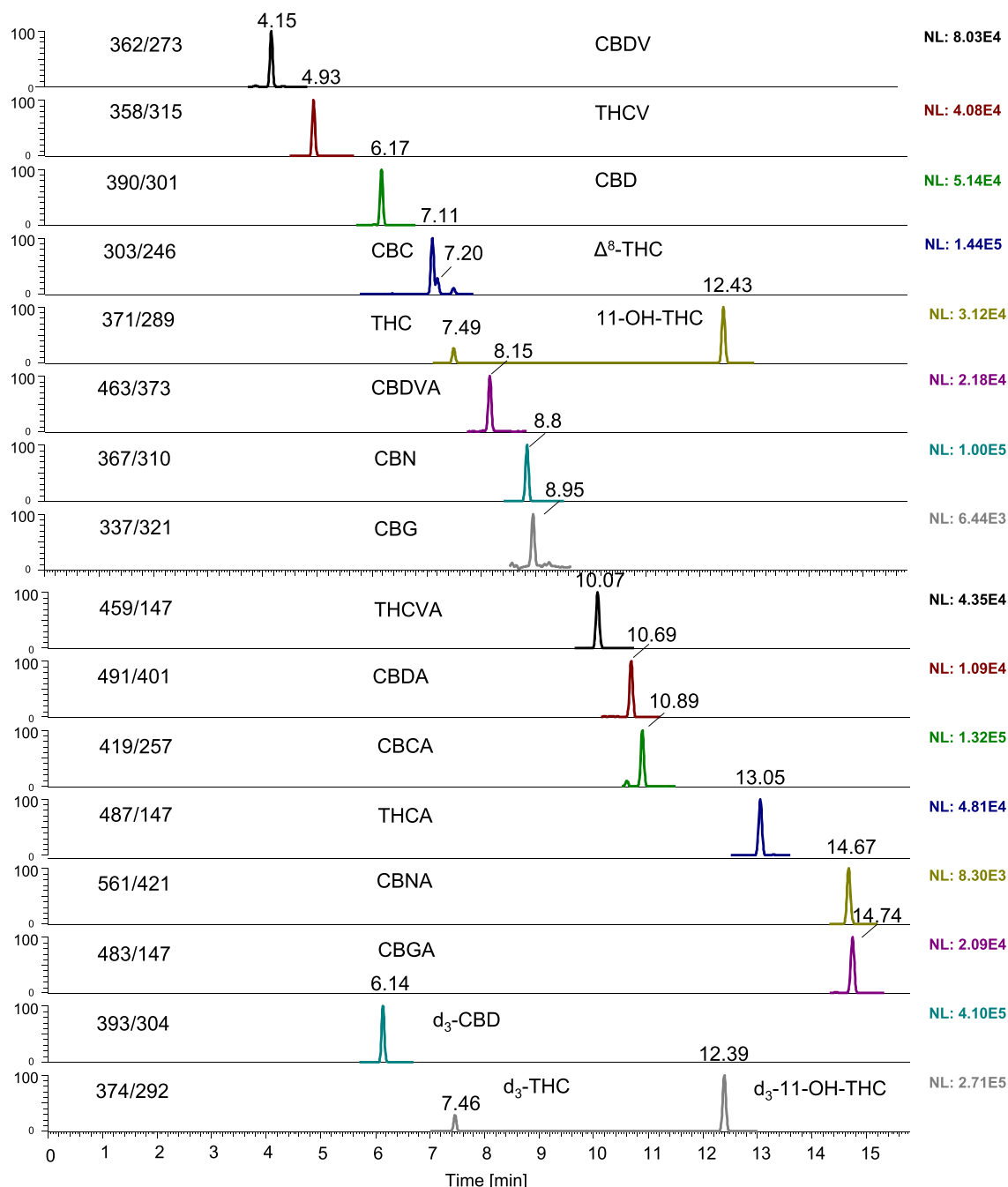


FIGURE 1 Extracted ion chromatograms of cannabinoids and respective internal standards (10 ng/ml) [Colour figure can be viewed at wileyonlinelibrary.com]

Intake of full spectrum cannabinoid CBD extracts and products without specific CBD concentration declarations resulted in the detection of various prohibited cannabinoids (Table 3) in the study participants' urine over more than 30 h. This suggests a considerable risk of antidoping rule violations for athletes (Table 3). The most abundant urinary cannabinoid besides CBD was CBG reaching urine concentrations up to 800 ng/ml, followed by CBDV (maximum urinary concentration ca. 95 ng/ml), CBN (maximum urinary concentration ca. 4.6 ng/ml), and CBC (maximum urinary concentration ca. 4.5 ng/ml). A full spectrum hemp seed oil (product 7) yielded

positive CBGA, THCA, and CBDA (Table 3) urine concentrations, while none of the cannabinoid acids CBDVA, CBCA, CBNA, THCVA, THCA nor THCV, Δ⁸-THC, THC or 11-OH-THC were detected in the elimination study urine samples. Alarming however is that in 8 of 15 individuals (53%) the 8 h urine samples contained CBG, and in 12 of 15 individuals (80%) CBDV was detected, all of which would constitute an AAF if the sample was collected from an athlete in-competition. In three instances, CBG, CBC, and CBDV were identified even 32 h postadministration (Table 3). The administration of five selected products (product numbers: 2, 3, 7, 11, 14) to an additional

TABLE 3 Cannabinoids (ng/ml) in excretion study urine samples, collected 8, 16, and 32 h after application of CBD products according to the recommended manufacturers' dosage

| Collection time (h) | CBD | | | CBG | | | CBN | | | CBC | | |
|-------------------------------|-----------|---------|---------|---------|---------|----------|----------|----------|----------|----------|----------|----------|
| | 8 | 16 | 32 | 8 | 16 | 32 | 8 | 16 | 32 | 8 | 16 | 32 |
| Product | ng/ml | | | ng/ml | | | ng/ml | | | ng/ml | | |
| 1 | 256 | 29 | 28 | | | | | | | | | |
| 2 ^a | 336-642 | 45-51 | 11-48 | | | | | | | | | |
| 3 ^a | 1096-4485 | 78-123 | 52-64 | 374-654 | 13-16 | <LOD-7.6 | <LOD-1.0 | | | | | |
| 4 | 1424 | 159 | 36 | 43 | | | 2.3 | | | 4.5 | 3.6 | |
| 5 | 248 | 21 | 10 | 797 | 27 | 8.6 | 1.1 | | | 1.6 | 0.9 | |
| 6 | 746 | 21 | 15 | | | | | | | 0.4 | | |
| 7 ^a | 28-96 | <LOD-13 | <LOD-12 | 24-84 | | | | <LOD-0.6 | | | | |
| 8 | 394 | 28 | 17 | | | | | | | | | |
| 9 | 318 | 53 | 18 | 49 | | | | | | | | |
| 10 | 224 | 48 | 13 | 129 | 5.5 | | 2.1 | | | 3.1 | 3.2 | 1.3 |
| 11 ^a | 469-625 | 32-41 | 9.4-29 | 73-129 | | | | 1.8-4.6 | <LOD-0.5 | 1.3-1.5 | 0.4-1.0 | 0.3-0.4 |
| 12 | 150 | 58 | 6.2 | | | | | | | | | |
| 13 | 318 | 53 | 18 | | | | | | | | | |
| 14 ^a | 194-210 | 26-28 | 6.1-10 | 1.7-240 | <LOD-15 | | 1-1-1.7 | | | <LOD-3.2 | <LOD-0.5 | <LOD-0.5 |
| 15 | 8 | 3.2 | 5.2 | | | | | | | | | |
| “Adverse analytical findings” | | | | 8/15 | 4/15 | 2/15 | 7/15 | 1/15 | 0/15 | 7/15 | 5/15 | 3/15 |
| | | | | 53% | 27% | 13% | 47% | 7% | 0% | 47% | 33% | 20% |

^aTwo volunteers.

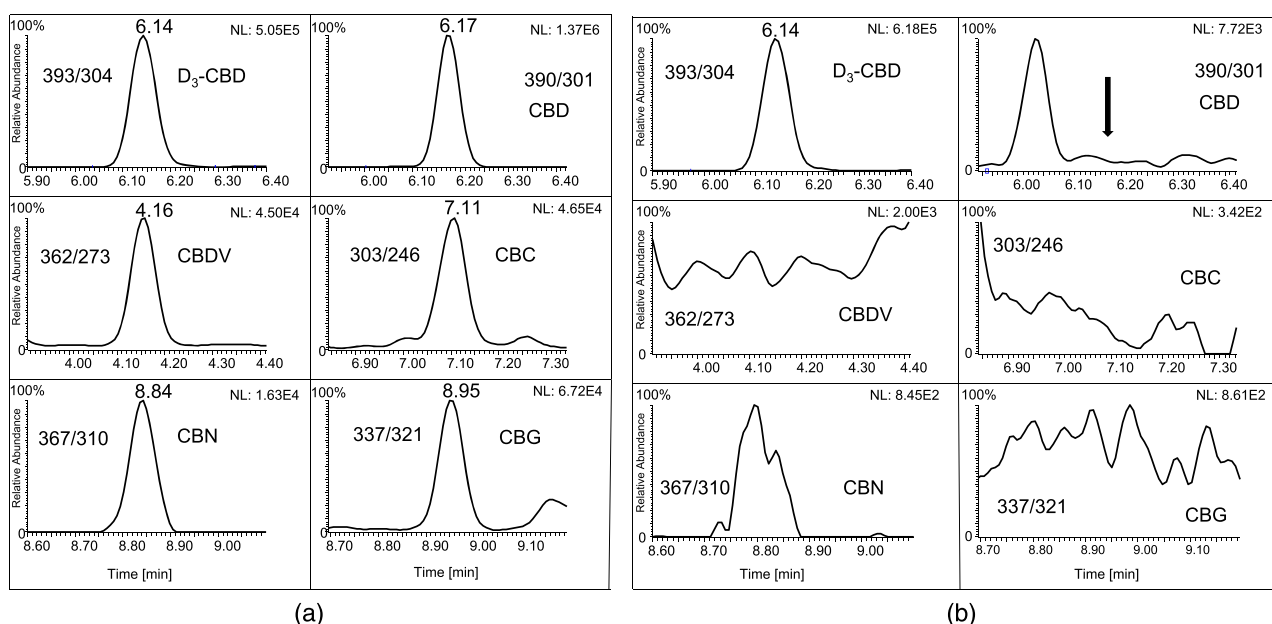
TABLE 3 (Continued)

| Collection time (h) | CBDV | | | CBGA | | | THCA | | | CBDA | | | THC-COOH | | |
|-------------------------------|---------|------|------|----------|------|------|----------|------|------|----------|------|------|----------|------|------|
| | 8 | 16 | 32 | 8 | 16 | 32 | 8 | 16 | 32 | 8 | 16 | 32 | 8 | 16 | 32 |
| Product | ng/ml | | | ng/ml | | | ng/ml | | | ng/ml | | | ng/ml | | |
| 1 | 3 | | | | | | | | | | | | | | |
| 2 ^a | 12-18 | | | <LOD-1.2 | | | | | | | | | | | |
| 3 ^a | 28-41 | | | <LOD-1.4 | | | | | | <LOD-1.0 | | | | | |
| 4 | 95 | | | 5.8 | | | 3.0 | | | | | | 5.2 | | |
| 5 | 9 | | | 1.1 | | | | | | | | | 2.3 | | |
| 6 | 19 | | | | | | | | | | | | | | |
| 7 ^a | 1.4-3.6 | | | 8.8-11 | | | <LOD-5.2 | | | 0.5-1.8 | | | 0.9-4.6 | | |
| 8 | 5 | | | | | | | | | 17-19 | | | 1.7-2.0 | | |
| 9 | | | | | | | | | | | | | | | |
| 10 | 10 | | | | | | | | | | | | | | |
| 11 ^a | 14-31 | | | <LOD-1.0 | | | | | | | | | | | |
| 12 | 1.0 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | | |
| 14 ^a | 23-25 | | | <LOD-3.3 | | | | | | 3.2-5.8 | | | 1.9-2.5 | | |
| 15 | | | | | | | | | | | | | | | |
| “Adverse analytical findings” | 12/15 | 6/15 | 2/15 | 1/15 | 1/15 | 0/15 | 0/15 | 1/15 | 0/15 | 0/15 | 0/15 | 0/15 | 0/15 | 0/15 | 0/15 |
| | 80% | 40% | 13% | 7% | 7% | 0% | 7% | 0% | 0% | 7% | 0% | 0% | 0% | 0% | 0% |

^aTwo volunteers.

TABLE 4 Cannabinoids (ng/ml) in CBD user spot urine samples

| Product | Product description | Route | Daily dosage | CBD ng/ml | CBG ng/ml | CBN ng/ml | CBC ng/ml | CBDV ng/ml | CBDVA ng/ml |
|---------|---------------------|-------------|--------------|-----------|-----------|-----------|-----------|------------|-------------|
| 1 | CBD gel | Transdermal | 10 mg | 5.2 | | | | | |
| 2 | CBD oil 10% | Oral | 3 drops | 85 | 16 | | | | |
| 3 | CBD oil 10% | Oral | 3 × 5 drops | 772 | 16 | 1.0 | 1.8 | 32 | 2.6 |
| 4 | CBD oil 5% | Oral | 3 × 4 drops | 116 | | | | 3.0 | |
| 5 | CBD oil 10% | Oral | 10 drops | 863 | 154 | 8.1 | 4.1 | 47 | |
| 6 | CBD oil 5% | Oral | 10 drops | 511 | 112 | 4.9 | 3.5 | 20 | |
| 7 | CBD oil 10% | Oral | 10 drops | 404 | | | | 5.9 | |

**FIGURE 2** (a) Cannabinoids in an authentic urine specimen of a cannabidiol (CBD) user (no 6). Extracted ion-chromatograms of CBD, cannabichromene (CBC), cannabigerol (CBG), cannabinol (CBN), cannabidivarin (CBDV) and internal standard d₃-CBD are shown. (b) Corresponding blank urine sample [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

set of volunteers resulted in urinary cannabinoid patterns and detection windows similar to those observed in the initial administration study (Table 3).

4.3 | Spot urine samples from CBD users

The analyzed spot urine samples showed high variability in cannabinoids presence (Table 4). Transdermal CBD gel application only achieved 5 ng/ml urinary CBD concentration, with no other cannabinoids detected.

Subjects taking CBD products for chronic pain therapy or to cure sleep disorders had CBD as well as CBG, CBN, CBC, CBDV, and CBDVA in their urine with maximum concentrations of 154 ng/ml for CBG and 47 ng/ml for CBDV (Table 4). A typical example is shown in Figure 2. These data contribute to the substantial risk of an antidoping rule violation for athletes when using CBD products.

5 | CONCLUSION

The use of permitted CBD products can lead to findings of prohibited cannabinoids in urine including e.g. CBG, CBN, CBC, and CBDV. While the herein presented pilot study focused merely on seven minor cannabinoids (i.e., CBG, CBN, CBC, CBDV, CBGA, THCA, and CBDA) besides CBD and the threshold substance THC-COOH, the risk of AAFs might be further aggravated by future consideration and potential inclusion of minor cannabinoid urinary metabolites into doping control analytical assays. Here, the unequivocal coherence between minor cannabinoid administration and corresponding urinary metabolite detection will require further investigations, and relevant reference material will be needed. In the light of the obtained data, comprehensive information and thorough education of athletes concerning the risks associated with the consumption of CBD products is of utmost importance in order to avoid the risk of an unintentional antidoping rule violation through the permitted use of CBD

products^{6,8} and, possibly, revisiting reporting levels for cannabinoids in the antidoping context are warranted.

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REFERENCES

- World Anti-Doping Agency, The 2020 Prohibited List. International Standard, **2020**, (04.05.2020), https://www.wada-ama.org/sites/default/files/wada_2020_english_prohibited_list_0.pdf
- Huestis MA, Mazzoni I, Rabin O. Cannabis in sport. *Sports Med*. 2011; 41(11):949-966.
- World Anti-Doping Agency, WADA Technical Document—TD2019DL v. 2.0.: Decision limits for the confirmatory quantification of threshold substances. , **2019**, (04.05.2020), https://www.wada-ama.org/sites/default/files/resources/files/td2019dl_v2_finalb.pdf
- World Anti-Doping Agency, Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes, 2015, (10.06.2020), <https://www.wada-ama.org/en/resources/science-medicine/td2015idcr>
- Lucas C, Galettis P, Schneider J. The pharmacokinetics and the pharmacodynamics of cannabinoids. *Br J Clin Pharmacol*. 2018;84(11): 2477-2482.
- Lachenmeier D, Diel P. A warning against the negligent use of cannabidiol in professional and amateur athletes. *Sports*. 2019;7(12):251.
- Habel S, Sproll C, Teipel J, Walch S, Lachenmeier D. Positive Cannabis-Urintests durch kommerzielle Cannabidiol-Produkte. *Toxichem Krimtech*. 2020;87:10.
- World Anti-Doping Agency, Summary of Major Modifications and Explanatory Notes 2018 Prohibited List, **2018**, (30.07.2020), https://www.wada-ama.org/sites/default/files/prohibited_list_2018_summary_of_modifications_en.pdf
- World Anti-Doping Agency, Questions and answers. Cannabinoids., **2019**, (19.06.2020), <https://www.wada-ama.org/en/questions-answers/cannabinoid#item-1584>
- Huestis M, Solimini R, Pichini S, Pacifici R, Carlier J, Busardo F. Cannabidiol adverse effects and toxicity. *Curr Neuropharmacol*. 2019; 17(10):974-989.
- Bergamaschi M, Queiroz R, Crippa J, Zuardi A. Safety and side effects of cannabidiol, a Cannabis sativa constituent. *Curr Drug Saf*. 2011;6 (4):237-249.
- Iffland K, Grotenhermen F. An update on safety and side effects of Cannabidiol: a review of clinical data and relevant animal studies. *Cannabis and Cannabinoid Research*. 2017;2(1):139-154.
- Devinsky O, Cross J, Laux L, et al. Trial of Cannabidiol for drug-resistant seizures in the dravet syndrome. *New Engl J Med*. 2017;376(21):2011-2020.
- Gamelin F-X, Cuvelier G, Mendes A, et al. Cannabidiol in sport: ergogenic or else? *Pharmacol Res*. 2020;156:104764.
- McCartney D, Benson M, Desbrow B, Irwin C, Suraev A, McGregor I. Cannabidiol and sports performance: a narrative review of relevant evidence and recommendations for future research. *Sports Medicine - Open*. 2020;6(1):27.
- Kasper A, Sparks S, Hooks M, et al. High prevalence of cannabidiol use within male professional Rugby union and league players: a quest for pain relief and enhanced recovery. *Int J Sport Nutr Exerc Metab*. 2020;30(5):315-322.
- Mareck U, Haenelt N, Geyer H, et al. Temporal indication of cannabis use by means of THC glucuronide determination. *Drug Test Anal*. 2009;1(11-12):505-510.
- Sempio C, Scheidweiler K, Barnes A, Huestis M. Optimization of recombinant β -glucuronidase hydrolysis and quantification of eight urinary cannabinoids and metabolites by liquid chromatography tandem mass spectrometry. *Drug Test Anal*. 2018;10(3):518-529.
- Scheidweiler K, Desrosiers N, Huestis M. Simultaneous quantification of free and glucuronidated cannabinoids in human urine by liquid chromatography tandem mass spectrometry. *Clin Chim Acta*. 2012; 413(23-24):1839-1847.
- Abraham T, Lowe R, Pirnay S, Darwin W, Huestis M. Simultaneous GC-El-MS determination of 9-tetrahydrocannabinol, 11-hydroxy-9-tetrahydrocannabinol, and 11-nor-9-carboxy-9-tetrahydrocannabinol in human urine following tandem enzyme-alkaline hydrolysis. *J Anal Toxicol*. 2007;31(8):477-485.
- Manno JE, Manno BR, Kemp PM, et al. Temporal indication of marijuana use can be estimated from plasma and urine concentrations of 9-tetrahydrocannabinol, 11-hydroxy-9-tetrahydrocannabinol, and 11-nor-9-tetrahydrocannabinol-9-carboxylic acid. *J Anal Toxicol*. 2001;25(7):538-549.
- Walton MJ, Anderson RA, Kicman AT, Elton RA, Ossowska K, Baird DT. A diurnal variation in testicular hormone production is maintained following gonadotrophin suppression in normal men. *Clin Chem*. 2007;66(1):123-129.
- Skopp G, Pötsch L, Ganßmann B, et al. Freie und glucuronidierte Cannabinoide im Urin - Untersuchungen zur Einschätzung des Konsumverhaltens. *Dent Rec*. 1999;10(1):21-28.
- Bergamaschi M, Barnes A, Queiroz R, Hurd Y, Huestis M. Impact of enzymatic and alkaline hydrolysis on CBD concentration in urine. *Anal Bioanal Chem*. 2013;405(14):4679-4689.
- World Anti-Doping Agency, World Anti-Doping Code. International Standard, **2019**, (16.06.2020), https://www.wada-ama.org/sites/default/files/resources/files/isl_nov2019.pdf
- Andersson M, Scheidweiler K, Sempio C, Barnes A, Huestis M. Simultaneous quantification of 11 cannabinoids and metabolites in human urine by liquid chromatography tandem mass spectrometry using WAX-S tips. *Anal Bioanal Chem*. 2016;408(23):6461-6471.
- World Anti-Doping Agency, Endogenous Anabolic Androgenic Steroids Measurement and Reporting, **2018**, (14.09.2020),
- Pellati F, Borgonetti V, Brighenti V, Biagi M, Benvenuti S, Corsi L. Cannabis sativa L. and nonpsychoactive cannabinoids: their chemistry and role against oxidative stress, inflammation, and cancer. *Biomed Res Int*. 2018;2018:1691428.
- Lachenmeier D, Bock V, Deych A, Sproll C, de Rezende T, Walch S. Hanfhaltige Lebensmittel - ein Update. *Deutsche Lebensmittel-Rundschau: Zeitschrift für Lebensmittelkunde und Lebensmittelrecht*; 2019;115(8):351-372.
- Lachenmeier D, Habel S, Fischer B, et al. Are side effects of cannabidiol (CBD) products caused by tetrahydrocannabinol (THC) contamination? *F1000Research*. 2020;8:1394.
- Hazekamp A. The trouble with CBD oil. *Med Cannabis Cannabinoids*. 2018;1(1):65-72.

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