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Abstract:	Cannabidiol prevalent (CBD-rich) cannabis derivatives are increasingly popular and widely available on the market as replacement of THC, tobacco substitutes or therapeutics for various health conditions. In this paper, we evaluate the impact of a repeated CBD-rich cannabis intake on levels of cannabinoids in biological samples. Urine, oral fluid and hair (pubic and head) samples were obtained from a naive user during a 26-day smoking period of one 250-mg CBD-rich cannabis joint/day containing 6.0% cannabidiol (CBD; 15 mg) and 0.2% delta-9-tetrahydrocannabinol (THC; 0.5 mg). In total, 35 urine, 8 oral fluid and 4 hair sample were collected. Cannabinoids concentrations were quantified by a UHPLC/MSn technique. The results suggested that the repeated exposure to CBD-rich cannabis (containing small amounts of THC) can generate positive results in biological samples. Urinary concentrations of 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) were quantitatively detected after 8 days from the smoking start and exceeded the 15 ng/mL cut-off limit on day-15 even in the urine sample collected 12 hours after the last intake. In the oral fluid collected on day-26, no cannabinoids were found before the cannabis intake, thus excluding accumulation, while THC was detectable up to 3 h after the cannabis intake, at concentrations progressively decreasing from about 18 to 6 ng/mL. Hair samples collected one week after the end of the study turned out negative for THC and THC-COOH, suggesting that this matrix is suitable to discriminate the chronic consumption of CBD-rich cannabis from THC-prevalent products. The obtained findings are relevant for the interpretations of cannabinoids levels in biological fluids, also in light of the legal implications of a positive result.
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Title Page (with authors and addresses)

Determination of cannabinoids in urine, oral fluid and hair samples after repeated intake of CBD-rich cannabis by smoking

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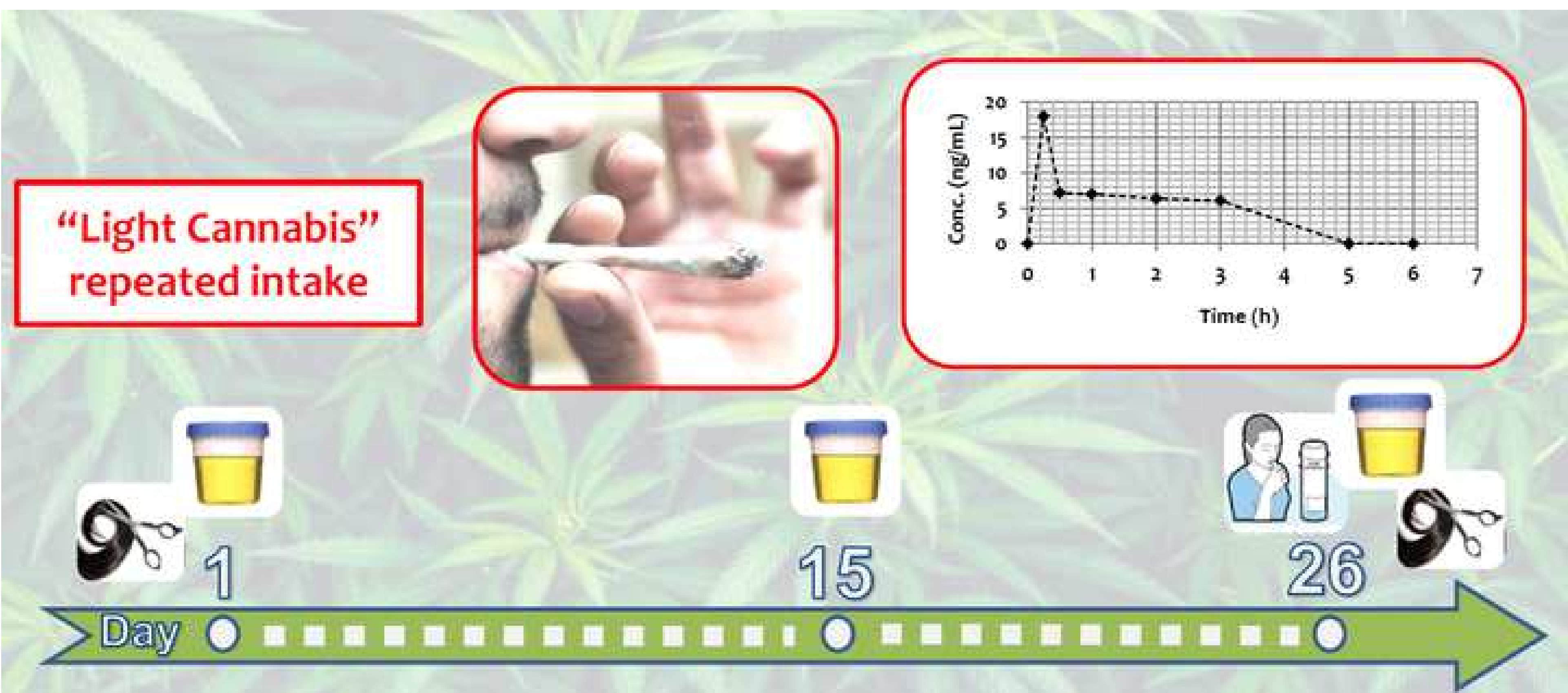
Graphical Abstract (for review)



Highlights

- Repeated exposure to CBD-rich cannabis can generate positive results in drug testing
- THC oral fluid levels >2 ng/mL were observed few hours after CBD-rich cannabis use
- Urine THC-COOH concentration can exceed the threshold of 15 ng/mL
- No accumulation of THC in hair and pubic hair was observed

1 **Abstract**

2 Cannabidiol prevalent (CBD-rich) cannabis derivatives are increasingly popular and widely
3 available on the market as replacement of THC, tobacco substitutes or therapeutics for various
4 health conditions. In this paper, we evaluate the impact of a repeated CBD-rich cannabis intake on
5 levels of cannabinoids in biological samples. Urine, oral fluid and hair (pubic and head) samples
6 were obtained from a naive user during a 26-day smoking period of one 250-mg CBD-rich cannabis
7 joint/day containing 6.0% cannabidiol (CBD; 15 mg) and 0.2% delta-9-tetrahydrocannabinol (THC;
8 0.5 mg). In total, 35 urine, 8 oral fluid and 4 hair sample were collected. Cannabinoids
9 concentrations were quantified by a UHPLC/MS^n technique. The results suggested that the repeated
10 exposure to CBD-rich cannabis (containing small amounts of THC) can generate positive results in
11 biological samples. Urinary concentrations of 11-nor-9-carboxy-delta-9-tetrahydrocannabinol
12 (THC-COOH) were quantitatively detected after 8 days from the smoking start and exceeded the 15
13 ng/mL cut-off limit on day-15 even in the urine sample collected 12 hours after the last intake. In
14 the oral fluid collected on day-26, no cannabinoids were found before the cannabis intake, thus
15 excluding accumulation, while THC was detectable up to 3 h after the cannabis intake, at
16 concentrations progressively decreasing from about 18 to 6 ng/mL. Hair samples collected one
17 week after the end of the study turned out negative for THC and THC-COOH, suggesting that this
18 matrix is suitable to discriminate the chronic consumption of CBD-rich cannabis from THC-
19 prevalent products. The obtained findings are relevant for the interpretations of cannabinoids levels
20 in biological fluids, also in light of the legal implications of a positive result.

21

22 **Keywords:** cannabis; CBD; THC; urine; hair; oral fluid; drug testing, workplace.
23

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25

1. Introduction

In the past two decades, a growing interest has been observed in the therapeutic potential of cannabis and single cannabinoids, mainly cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC). More recently, CBD has gained prevalent attention for its potential therapeutic use as anti-inflammatory, anticonvulsive, anxiolytic, analgesic, neuroprotective, anticancer, and antioxidant agent [1–3]. In this context, a cannabis derived CBD product (Epidiolex®) was approved by the US Food and Drug Administration (FDA) for treatment of pediatric seizure disorders [4]. Likewise, the availability of non-medical CBD-rich products largely increased in recent years [5]. Cannabis products rich in CBD and low in THC can be legally sold and purchased in several countries [5,6]. In the European Union, the free cultivation of cannabis varieties containing up to 0.2% (0.6% in some specific cases, e.g. Italy) is allowed [7]. In Switzerland, hemp plants containing less than 1 % THC and 3-20% CBD are being sold for smoking as a tobacco replacement, and combined with other ingredients in daily products or with no need of specific declared use [8]. In the US and Canada, the commercialization of cannabis plants with THC concentration ≤0.3% is also allowed [9,10].

In this context, the commercialization of CBD-rich cannabis products with low THC content and variable CBD concentration, is proliferating. Moreover, cannabis farmers have been working to create new cannabis varieties rich in CBD. Varieties expressing up to 25% total CBD and less than 1% total THC (typically 0.3–0.7%) within the floral tissue have recently been farmed [11].

CBD-rich products can either be sold as dried plant material to be inhaled using conventional methods (e.g., joints, bowls and vaporizers) or as concentrated CBD extracts intended for oral (e.g., oils, tinctures), pulmonary (e.g., vaporizers or vape pens) or topical (e.g., cream) consumption [12–14].

Considering the diffusion of CBD-rich material, several studies were recently addressed to evaluate the consequences of the single or repeated intake of these products on drug testing in biological

52 fluids [8,11,12,15,16]. In particular, it is relevant to know whether or not the consumption of low
53 THC/high CBD products can result in positive testing at cannabinoids screening within, for
54 example, workplace, roadside, or driving relicensing procedures. In this paper, we investigated the
55 impact of repeated CBD-rich cannabis intake on the cannabinoids levels in urine, oral fluid, and
56 hair samples, after a prolonged smoking period of 26 days.
57
58 **2. Experimental**
59
60 *2.1. Chemicals and Materials*
61 Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabidiolic acid (CBDA), 11-nor-9-
62 carboxy-Δ9-tetrahydrocannabinol (THC-COOH) and deuterated cannabinoids used as internal
63 standard (THC-d3, CBD-d3 and THC-COOH-d3) were purchased from Merck KGaA (Milan,
64 Italy). Methanol, acetonitrile, ethylacetate, dichloromethane, n-exane and glacial acetic acid were
65 provided by Merck KGaA (Milan, Italy) while formic acid (LC-MS grade) was obtained by Fisher
66 Scientific (Geel, Belgium). Sodium hydroxide was purchased from Carlo Erba Reagents
67 (Cornaredo, Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus
68 (Millipore, Bedford, MA, USA). For the determination of THC-COOH in urine, the working
69 solution was prepared by dilution in methanol of THC-COOH at a final concentration of 1 μg/mL.
70 The internal standard working solution was prepared in methanol at THC-COOH-d3 final
71 concentration of 10 μg/mL. For the determination of cannabinoids in oral fluid, the working
72 solution containing THC and CBD at a concentration of 1 μg/mL was prepared in methanol. An
73 equivalent internal standard mixture containing THC-d3 and CBD-d3 at a concentration of 1 μg/mL
74 was prepared in methanol. For the determination of cannabinoids in hair samples, the same working
75 solutions used for oral fluid were used. In addition, two working solutions of THC-COOH and
76 THC-COOH-d3 were prepared by dilution in methanol at final concentration of 0.1 μg/mL. All
77 stock and working solutions were stored at -20°C until used.

78

79 *2.2. Study design and biological samples*

80 A healthy male subject (45 years, 72 kg, 169 cm, BMI 25.2; regular tobacco cigarette smoker), who
81 declared no consumption of cannabinoid-containing products prior to the study, gave written
82 informed consent and accepted to donate urine, oral fluid and hair samples prior, during and after a
83 daily smoking of a joint containing 250 mg of CBD-rich cannabis for 26 consecutive days. The
84 cannabinoid content of the CBD-rich cannabis (6.0% CBD and 0.2% THC) was preliminarily
85 verified by means of HPLC-UV and GC-MS methods routinely employed in our laboratory.
86 Consequently, the daily exposure to CBD and THC via inhalation was estimated to be 15 and 0.5
87 mg, respectively. Observation of side effects was excluded from the study because of the small
88 number of tested subjects. The study was conducted in accordance with ethical standards
89 established in the Helsinki Declaration.

90 Urine samples were collected every day, 12 hours after smoking, except days 1, 15, and 26 when
91 urine samples were collected both before smoking (U-t_0) and in the time interval 1-10 h after
92 smoking. No urine sampling was carried out on day 16. The oral fluid samples were collected in a
93 test tube on day 26 before smoking (OF-t_0), and 0.25, 0.5, 1, 2, 3, 5 and 6 hours after smoking. Prior
94 of collection, the participant washed his mouth twice with water.

95 Head and pubic hair samples were collected one day before the beginning of the study and one
96 week after the end of the study [17]. Urine and oral fluid samples were stored at -20°C until the
97 analysis, while hair samples were stored in dark and at room temperature before the analysis.

98 All collected samples were screened for the presence of cannabinoids (CBD, THC and THC-
99 COOH) by means of validated UHPLC-MS^n methods. The main validation parameters are
100 described in the supplementary material (Table S1). In order to adjust the quantitative confirmation
101 results based on urine dilution, urinary creatinine was measured by the Jaffe method using an
102 Abbott Architect instrument (Abbott Laboratories, IL, USA). Therefore, every cannabinoid level
103 was normalized with respect to 100 mg/dL of excreted creatinine.

104 *2.3. Sample preparation*

105

106 *2.3.1. Urine*

107 Urine samples (500 µL) were spiked with 2.5 µL of internal standard solution and 20 µL of sodium
108 hydroxide solution (10 N). The mixture was incubated at 55°C for 15 minutes. Then the samples
109 were cooled at room temperature and added with 10 µL of glacial acetic acid for neutralization (pH
110 6.5-7.5). After vortexing, 50 µL of mixture was transferred into a new tube and diluted with 450 µL
111 of a solvent mixture methanol/acetonitrile (80:20 v/v). Finally, the tube was centrifuged at 14,000
112 rpm for 5 minutes and a 4 µL aliquot of the solution was injected into the UHPLC-MS^n system.

113

114 *2.3.2. Oral fluid*

115 Oral fluid samples (250 µL) were spiked with 10 µL of internal standard solution, then 500 µL of a
116 solvent mixture methanol/acetonitrile (80:20 v/v) was added. After vortexing for 10 seconds, the
117 samples were centrifuged at 14,000 rpm for 5 minutes. 50 µL of surnatant was transferred into a
118 new tube and a 2µL-aliquot of the solution was injected into the UHPLC-MS^n system.

119

120 *2.3.3. Hair*

121 The present sample preparation introduced minor modifications with respect to the standard
122 operating procedure [18]. Hair sample (50 mg) was washed twice with dichloromethane (2 mL,
123 vortex mixing for 3 min). After complete removal of the washing solvent, the hair aliquot was dried
124 at room temperature under a gentle nitrogen flow and then cut into 1-2 mm segments. The sample
125 was then fortified with 5 µL of internal standard before the addition of 2 mL NaOH 10N and
126 subsequent incubation at 75 °C for 60 min. After cooling at room temperature, 1 mL of glacial
127 acetic acid was added to adjust the pH value down to 3.5-4.0. Afterwards, the sample was extracted
128 with 10 mL of n-hexane/ethylacetate 90:10 (v/v). The organic phase was separated, dried under a

129 nitrogen flow at 45°C and reconstituted with 50 µL of acetonitrile/methanol 50.50 (v/v). Finally, an
130 aliquot of 5 µL methanol solution was directly injected into the UHPLC-MS^n system.
131
132 *2.4. UHPLC-MS^n methods*
133 Cannabinoids concentrations in urine and hair samples were determined using an Agilent Infinity
134 1290 UHPLC system (Agilent Technologies, Milan, Italy) coupled to a 4500 QTRAP hybrid triple
135 quadrupole/linear ion trap mass spectrometer with a Turbo V ion source (SCIEX, Darmstadt,
136 Germany). Analyst software version 1.6.1 (SCIEX, Darmstadt, Germany) was used for data
137 acquisition and analysis. A UHPLC Ace Excel Super C18 column 75 mm × 2.1 mm i.d., 2 µm
138 (Agilent Technologies, Scotland), protected by a C18 guard column, was used for the separation of
139 the target analytes. The column oven was maintained at 45°C. The elution solvents were
140 water/formic acid 5mM (solvent A) and acetonitrile (solvent B). After an initial isocratic condition
141 at 50% A for 0.3 min, the mobile phase composition was varied by a linear gradient (A:B; v/v) from
142 50% to 100% B in 3.0 min; then isocratic elution at 100% B was maintained for 0.5 min. The flow
143 rate was 0.6 mL/min and the total run time was 5 min, including re-equilibration at the initial
144 conditions before each injection. For urine analysis, mass spectrometric data were detected in
145 negative electrospray ionization (ESI) mode and MS/MS (MS^2) selected reaction monitoring (SRM)
146 acquisition, with an ion spray voltage of -4500 V and an ion source temperature of 600 °C. Nitrogen
147 was employed as the collision gas (5×10^{-3} Pa). For hair analysis, mass spectrometric data were
148 acquired following two different experiments. THC and CBD were detected in positive ESI mode
149 and MS^2 acquisition (ion spray voltage: 4000 V; ion source temperature: 550°C) while THC-COOH
150 was detected in negative ESI mode and MS^3 acquisition (ion spray voltage: -4500 V; ion source
151 temperature: 650°C). THC and CBD in oral fluid samples were determined using a Shimadzu LC-
152 20A Series system (Shimadzu, Duisburg, Germany) interfaced to an API 5500 triple quadrupole
153 mass spectrometer (SCIEX, Darmstadt, Germany) with an electrospray Turbo Ion source operating
154 in the positive ion mode (ion spray voltage: 4000 V; ion source temperature: 550°C). The

chromatographic parameters (column, mobile phases and gradient) were the same used for the analysis of cannabinoids in urine and hair samples. The SRM transitions for analytes and internal standard detection are shown in Table 1.

3. Results

3.1. Urine

A total of 35 urine samples was collected during the study period. THC-COOH was detected in 27 samples (77% of the total) at concentrations in the interval <LOQ-23 ng/mL (LOQ: 6 ng/mL). In detail, 20 samples contained THC-COOH in trace (LOD<THC-COOH<LOQ), while in the remaining 7 samples the THC-COOH concentration was between 10 and 23 ng/mL. The results of all urine samples taken during the 26-day smoking period are shown in Table 2. All urine concentrations are reported as creatinine normalized concentrations.

3.2. Oral fluid

Figure 1 shows the concentrations of THC and CBD in oral fluid on day 26, before and after the daily smoking of the CBD-rich cannabis joint. After a 25-day period of regular intake of CBD-rich cannabis joints, THC and CBD concentration in oral fluid before smoking (day 26) resulted negative. The first collection 0.25 h after the start of CBD-rich cannabis smoking revealed the highest THC and CBD concentrations at 18 and 292 ng/mL, respectively. These concentrations rapidly decreased after 15 min at the levels of 7.2 and 131 ng/mL for THC and CBD, respectively. THC was detectable in oral fluid at concentration of 6.1 ng/mL up to 3 h after the cannabis intake. The sample collected 5 h after the cannabis intake resulted negative for the presence of THC. Similarly, CBD concentration declined after a 5 h period to 20 ng/mL, while 6 h after the cannabis intake the measured CBD concentration was 1.7 ng/mL.

181 *3.3. Hair*

182 Head and pubic hair tested before the study turned out negative for the presence of CBD, THC and
183 THC-COOH. Head and pubic hair collected one week after the end of the study revealed the
184 presence of CBD at concentrations of 312 and 178 pg/mg, respectively. The same samples resulted
185 negative for THC and THC-COOH.

186

187 **4. Discussion**

188

189 To the best of our knowledge, this is the first study simulating a repeated intake of CBD-rich
190 cannabis over a prolonged period of time (26 days). Meier and co-workers published a study
191 simulating a frequent use of cannabis (content: 34 mg CBD, 1.6 mg THC) following the intake of 2
192 joints per day by inhalation, for 10 consecutive days [8]. A maximum THC-COOH concentration of
193 42.5 ng/mL was observed in the urine sample collected on the day-8. In our study, the amount of
194 daily consumed THC was substantially lower (0.5 mg) and corresponded to the consumption of a
195 single legal joint per day. Nevertheless, the presence of THC-COOH in urine was ascertained at 13
196 ng/mL concentration since day-8. Moreover, on day-15 the urine sample collected before the daily
197 intake of cannabis turned out positive to THC-COOH at 15 ng/mL concentration, that is the
198 recognized international cut-off used to prove recent cannabis consumption in several prohibited
199 circumstances (e.g. workplace, driver license release/renewal, roadside controls, drug addiction,
200 firearm license, etc.) [19]. This finding is coherent with the known THC accumulation in the body
201 fat during the smoking period. On the other hand, after the initial 15 days of the observation period,
202 the THC-COOH urine concentrations showed a decrease, remaining below the LOQ in all samples.
203 Again, the observed decrease is in accordance with the influence of CBD on the excretion profile of
204 certain drugs (including THC), as already reported in the literature [20,21]. However, other factors
205 such as physical exercise, nutrition and other biological parameters may play a role in the individual
206 metabolism.

207 The THC and CBD concentrations in the oral fluid sample collected after a 25-day period of regular
208 intake of CBD-rich cannabis joints but shortly before the last consumption tested negative, possibly
209 excluding the occurrence of a cannabinoids accumulation in this matrix during the smoking period.
210 Conversely, the presence of THC in oral fluid was ascertained up to 3 h after the cannabis intake at
211 concentrations ranging from 6 to 18 ng/mL, while the measured THC/CBD ratio was below 0.1 in
212 all samples. These findings are in accordance with previous studies published by Pacifici and co-
213 workers, who analyzed oral fluid after single and repeated intake of "light cannabis" (content: 58
214 mg CBD, 2.0 mg THC) [15,16]. In the single intake simulation study, THC was detected in oral
215 fluid in the concentration range 2.5-21.5 ng/mL 30 min after smoking, and then at a minimum value
216 of 1.0 ng/mL after 3 h. As noted in our study, CBD concentrations were usually one order of
217 magnitude higher than those of THC. Considering the repeated intake simulation study [16] (four
218 "light cannabis" cigarette smoked in a 4h period for a total intake of 6.4 mg THC and 232 mg
219 CBD), the mean concentration of THC passed from 17.6 ng/mL (30 min after the last cannabis
220 intake) down to 0.9 ng/mL (4 h after the last cannabis intake). Moreover, the THC/CBD ratio
221 resulted below 2 in all samples, suggesting a use of CBD-rich cannabis instead of THC-prevalent
222 products [16]. Nevertheless, all these findings revealed that positive THC testing in oral fluid may
223 occur for a 3-h period following a smoking intake of cannabis products containing less than 0.2% of
224 THC. This is particularly relevant in the context of roadside and workplace drug testing, in which
225 legal limits are provided [22–24]. Finally, regarding the keratin matrix, hair samples (head and
226 pubic) turned out negative for the presence of THC and THC-COOH, while CBD was detected at
227 high concentration in them. In this case, the hair matrix proved able to discriminate the chronic
228 consumption of CBD-rich cannabis from the THC-prevalent products.
229

230 **5. Conclusions**

231

In conclusion, this study based on a single subject suggests that the repeated exposure to CBD-rich cannabis (containing small amounts of THC) can generate positive results when biological samples are tested. In particular, positive urine results for THC-COOH, using the confirmatory cut-off of 15 ng/mL, were obtained after a 15-days period of consumption. On the other hand, it was demonstrated that there is no accumulation of cannabinoids in oral fluid after the repeated intake experimented in this study. However, THC concentrations above the confirmatory cut-off of 2 ng/mL were observed in the few hours after the CBD-rich cannabis products intake. Finally, the experimental results indicate that no accumulation of THC and THC-COOH in the hair matrix is likely to occur, thus suggesting the potential use of hair analysis to discriminate between the repeated consumption of CBD-rich cannabis and THC-prevalent products. The main limitation of this study is the small number of samples tested. Further studies, increasing the population involved, should be carried out to corroborate these preliminary findings, which are of ever-growing importance with the progressively increased availability of CBD-rich cannabis. Considering the legal implications of a positive result, robust criteria for the interpretation of cannabinoids levels in biological fluids are urgently needed.

CRediT authorship contribution statement: Conceptualization: E.G. and A.S.; Methodology: D.D., E.G. and A.S.; Validation: D.D. and S.P.B.; Formal analysis: D.D. and S.P.B.; Investigation: D.D. and E.G.; Data curation: D.D. and E.G.; Writing—original draft preparation: E.G., A.S. and M.V.; Writing—review and editing: E.G., A.S. and M.V.; Supervision, A.S and M.V.. All authors have read and agreed to the published version of the manuscript.

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Figure caption

Figure 1. Time course of THC and CBD concentrations in oral fluid on day 26 before (OF-t_0) and after the daily smoking of the CBD-rich cannabis joint.

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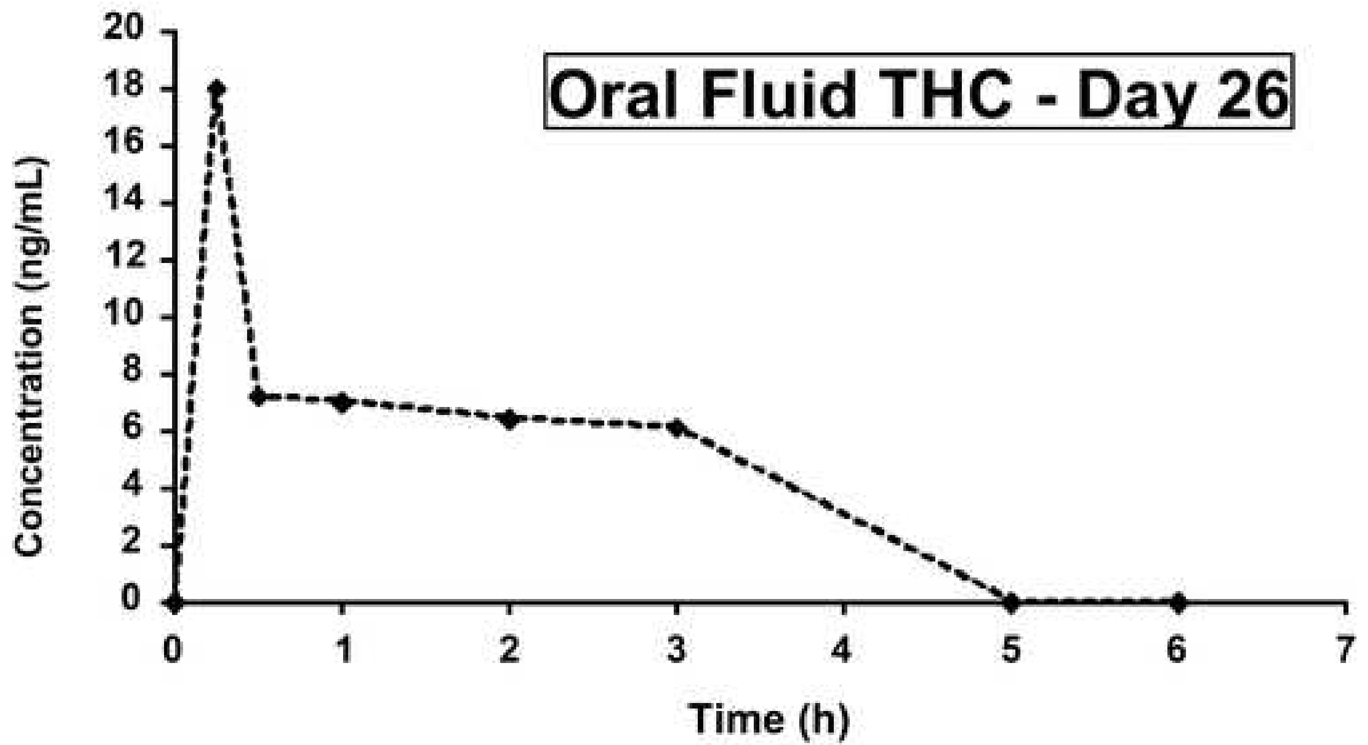


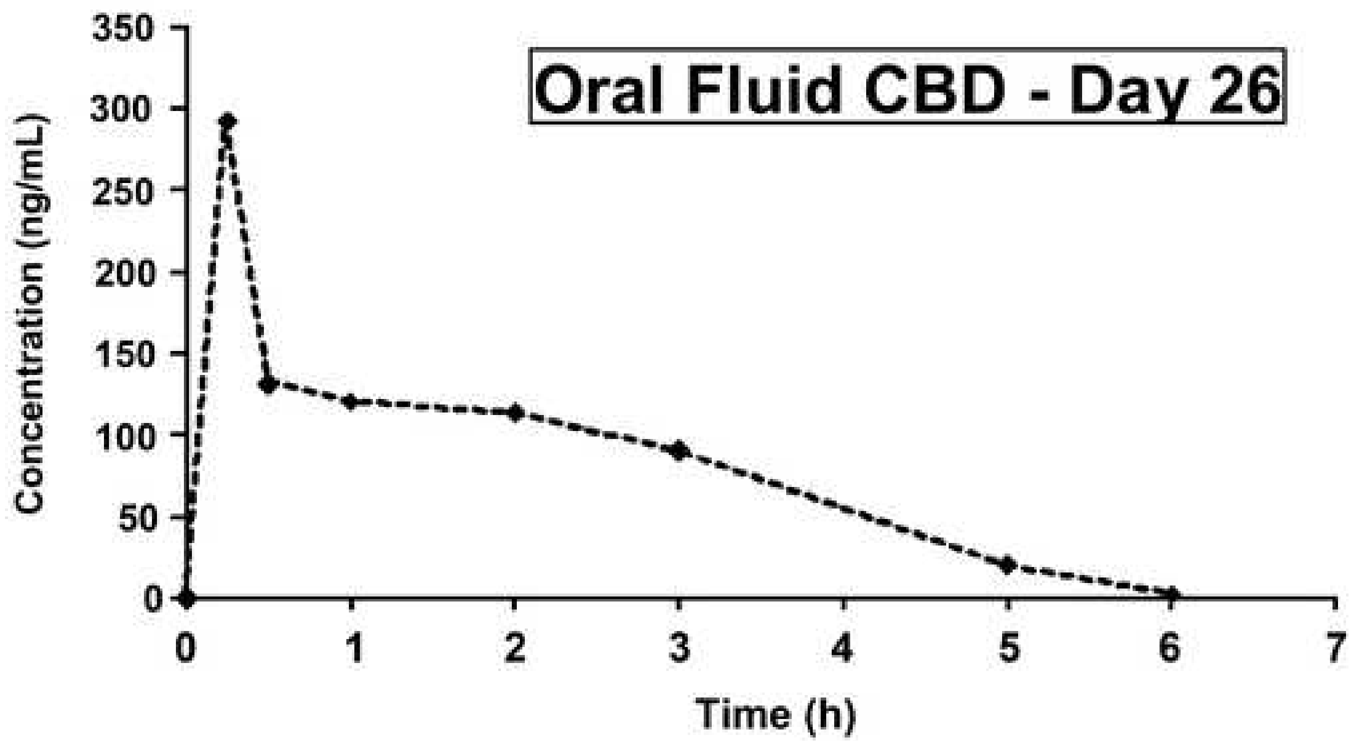


Table 1

Table 1. SRM transitions for the target compounds and the internal standards

Matrix	Compound	Ionization	SRM transitions (m/z)
Urine	THCCOOH	$[M-H]^-$	343.1 → 299.0 343.1 → 245.0 343.1 → 191.1
	THCCOOH-d3	$[M-H]^-$	346.1 → 302.0
Oral fluid **Hair**	CBD	$[M+H]^+$	313.2 → 244.8 313.2 → 106.6 313.2 → 311.0
	THC	$[M+H]^+$	315.2 → 193.2 315.2 → 123.1 315.2 → 259.2
	CBD-d3	$[M+H]^+$	316.1 → 248.0
	THC-d3	$[M+H]^+$	318.0 → 196.2
Hair	THCCOOH	$[M-H]^-$	343.2 → 299.0 (MS^2) 245.0 (MS^3)
	THCCOOH-d3	$[M-H]^-$	346.2 → 299.0 (MS^2) 245.0 (MS^3)

Table 2

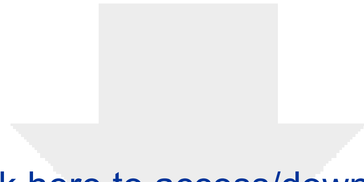
Table 2. THC-COOH concentration (creatinine normalized) in urine samples at different collection times during the 26-day study period.

Day	Collection time[1] (hh:mm)	Creatinine (mg/dL)	THCCOOH (ng/mL)
1	U-t_0	116	<LOD
	02:10	95	<LOQ
	04:30	138	<LOQ
2	13:30	100	<LOQ
3	12:00	126	<LOQ
4	16:00	109	<LOQ
5	16:00	92	<LOQ
6	12:00	169	<LOQ
7	12:00	207	<LOQ
8	12:30	61	13
9	12:30	90	<LOQ
10	12:30	58	12
11	12:00	171	<LOQ
12	12:30	87	<LOQ
13	12:00	120	<LOQ
14	12:00	84	10
15	12:00	113	15
	01:15	64	23
	02:55	85	19
	05:10	114	10
17	12:30	157	<LOQ
18	12:00	150	<LOQ
19	11:30	135	<LOD
20	13:30	124	<LOQ
21	12:20	163	<LOD
22	12:20	102	<LOQ
23	12:50	120	<LOQ
24	12:00	127	<LOD
25	10:30	114	<LOD
26	12:00	127	<LOD
	02:10	99	<LOQ
	03:45	153	<LOQ
	06:40	141	<LOQ
	08:10	179	<LOD
	10:10	166	<LOD

[1] Time elapsed between the last intake and the urine collection.

LOQ = 6 ng/mL

Table S1



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Optional e-only supplementary files

Table S1.docx