Validation Review

Procedure Validation of 4-AP Color Test

Check the appropriate box: New Validation ☑ Modification ☐ Performance Check ☐

Sign and Date to indicate your acceptance of the procedure/instrument:

Examiner (if applicable) Brett Trotter Date 8-8-19

Unit Supervisor Glenn B. Everett Date 8-8-19

Technical Leader (if different than Supervisor) Glenn B. Everett Date 8-8-19

Crime Laboratory Regional Supervisor [signature] Date 8.8.19

Quality Assurance Manager [signature] Date 08-08-19

*All information must be submitted to the Quality Assurance Manager prior to the implementation of the procedure/instrument. If all technical staff training has not been completed, please include a proposed training plan and completion schedule.

Validation of 4-Aminophenol Color Test for Screening Industrial Hemp and Marihuana

Table of Contents

I. Purpose:

Plant material containing less than 0.3 percent Δ^9-tetrahydrocannabinol (THC) by dry weight is now legal in the state of Tennessee as well as federally. Testing methodology and in particular a screening method that can distinguish hemp (less than 0.3 % THC) from marihuana (greater than 0.3% THC) is needed. The Forensisches Institut Zürich developed a color test (4-aminophenol or 4-AP) that gives different color results based on the ratio of cannabidiol versus Δ^9-tetrahydrocannabinol. While this color test cannot determine the percentage of THC present in the sample by dry weight, it can be indicative of whether or not a sample might be marihuana or hemp. This color test's distinction between the ratios of cannabidiol (CBD) to THC would be useful as a screening or presumptive test for forensic scientist and law enforcement officers in the field.

This validation study will examine the results of the 4-Aminophenol (4-AP) for known hemp samples (obtained for the Tennessee Department of Agriculture), marihuana samples (submitted to the Tennessee Bureau of Investigation and used with the permission of submitting agencies), negative controls (oregano and cilantro), and positive controls (Cerilliant standards for multiple cannabinoids). These results will be compared to the expected results of blue (THC rich samples) and red/purple (CBD rich samples). Negative controls will be compared to blank results. The results for the various cannabinoid standards will also be documented to ensure that other cannabinoids would not interfere with interpretation of results from casework. A controlled study of known ratios of CBD to THC will be compared and monitored over time to give our laboratory and idea of what ratios give blue results versus red results. This controlled study will be prepared by pipetting known amounts of Cerilliant CBD and THC standards. A side by side comparison of purchased test kit results to in-house prepared reagents will be conducted to ensure that our prepared reagents give similar results to purchased test kits. This study will also give our laboratory confidence to recommend these test kits to other law enforcement agencies.

The 4-AP color test validation will be successful and fit for use when it has been demonstrated that the observed colors for known hemp samples are distinct and differ enough from observed colors for known marihuana samples. It will also be a criterion for successful completion of this study that positive and negative controls are identified so that each batch of prepared reagents can be independently tested for fitness for use in casework. Each analyst must complete a competency test which will be documented in this validation study before the method can be used in casework.

II. Methodology:

This color test consists of two reagents described below.

Reagent 1:
995 mL Ethanol or Reagent Alcohol
5 mL 2 N Hydrochloric Acid
300 mg 4 – Aminophenol
Reagent 2:
700 mL Water
300 mL Ethanol or Reagent Alcohol
30 g Sodium Hydroxide pellets

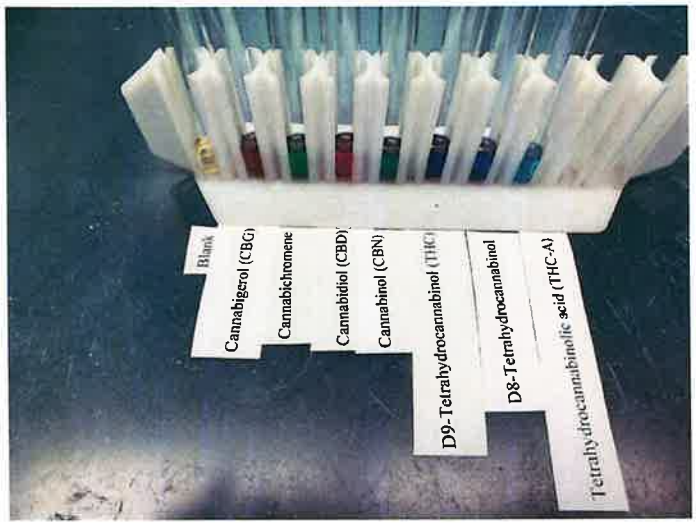
Reagent 1 is added to a small amount of plant material immediately followed by an equal portion of Reagent 2. Amber dropper bottles, disposable pipettes, and test tubes should be used when conducting this color test. The resulting color is observed after a brief period of vortexing the mixture. The resulting color should be documented after a brief period of time (typically less than a couple of minutes) as the mixture continues to darken over time. Both reagents should be stored in amber glass bottles in a refrigerator. The reagents should remain fit for use for ninety days. The reagent preparation, storage, and use were all recommended by another forensic testing laboratory that is also in the process of validating this color test. The test was developed by the Forensisches Institut Zürich as a field test kit. The recipe above can be adjusted as long as all the ratios of ingredients are all changed proportionally. The following chemicals were used to prepare Reagents 1 and 2 for this validation study.

Reagent Alcohol – Fisher – Lot # 179329
2 N Hydrochloric Acid – Fisher – Lot # 191098
4-Aminophenol – Aldrich – Lot # BCCB0380
Water – In-house – ELGA purification system 18.2 MΩ
Sodium Hydroxide pellets - Fisher - Lot # 117238

III. Cannabinoid Study:

Multiple cannabinoid standards were purchased from Cerilliant. The 4-AP color tests were run on these standards. These standards are listed in the table below. Some miscellaneous items were run as well. Patchouli oil, coffee, tea, and a factory cigarette butt were chosen either because of know interferences with the modified Duquenois-Levine color test or

Cannabinoid	Resulting color
Cannabigerol CBG	Reddish purple
Cannabichromene	Green
Cannabidiol CBD	Reddish purple
Cannabinol CBN	Bluish green
Δ^9 Tetrahydrocannabinol	Bluish purple
Δ^8 Tetrahydrocannabinol	Blue
Tetrahydrocannobinolic acid	Blue
Patchouli Oil	Yellow (similar to blank)
Coffee Grounds	Gray with blue overtones
Orange Black Spice Tea	Light Yellow
Found Cigarette Butt	Light Yellow

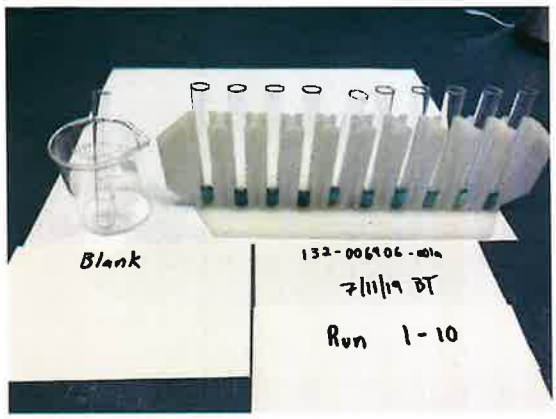


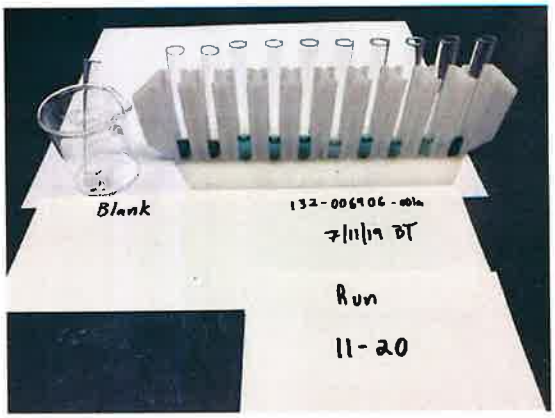
The expected results were a blue color for marihuana and a red color for hemp. The CBD standard did result in a red color at first then darkened to reddish purple over time. All of the standards darkened over time and when performing the test the best results were observed between twenty seconds and a minute. A lighter background made observing the results easier.

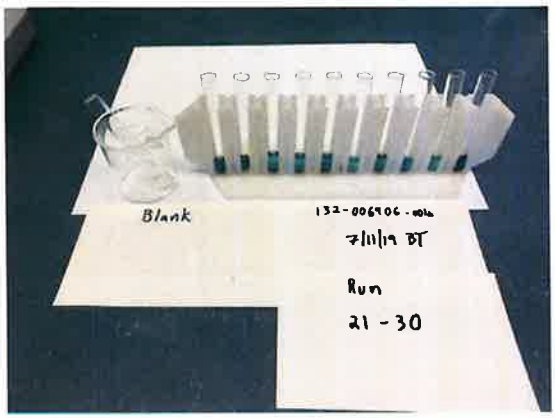
IV. Repeatability:

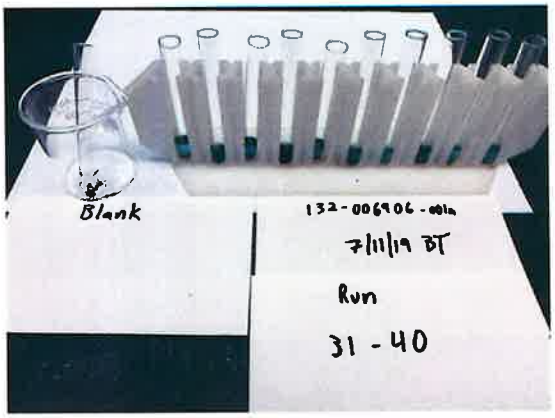
Plant material from T. B. I. case 132-006906 was approved for use in method development and was used to perform experiments in the 4-AP color test validation. A sample of this plant material was made homogeneous by grinding to a fine powder in a coffee grinder. This powder was used to perform 100 4-AP color tests. These samples were run in batches of ten with a blank run with each batch. The amount of plant material powder, 4-AP reagent # 1, and 4-AP reagent #2 were all approximately the same for each test. The resulting colors for all the plant material tests were blue and consistent. There was some variation to the intensity of the blue; however, this could be explained by the fact that some variations existed in the amounts of plant material analyzed or the amounts of reagents added. A stem was selected from the plant material and the leaves and buds were completely picked off the stem. This stem was then analyzed with the 4-AP color test. The stem resulted in a blue color that was similar to the color results obtained by analyzing the buds and leaves that were used to make the homogeneous sample. A seed was also selected from this plant material and analyzed with the color test. The seed resulted in a light yellow color that was consistent with the blank.

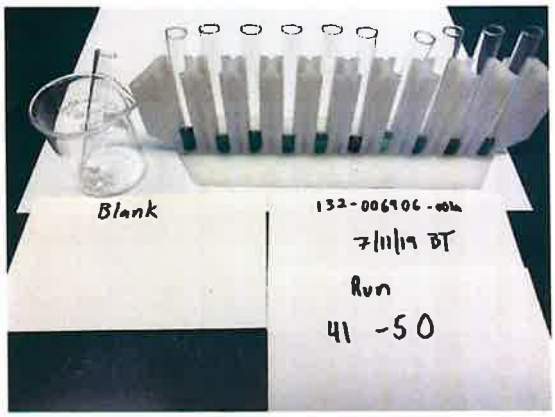
The pictures below were all taken within one minute of adding the reagents to the plant material. The samples were all vortexed for a couple of seconds to mix reagent # 1 and # 2 with the plant material.







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7/11/19 BT
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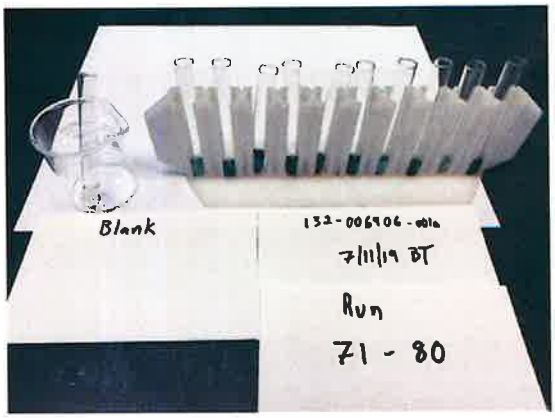
41 -50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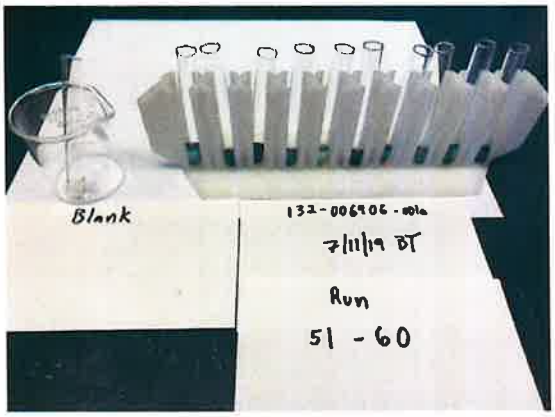
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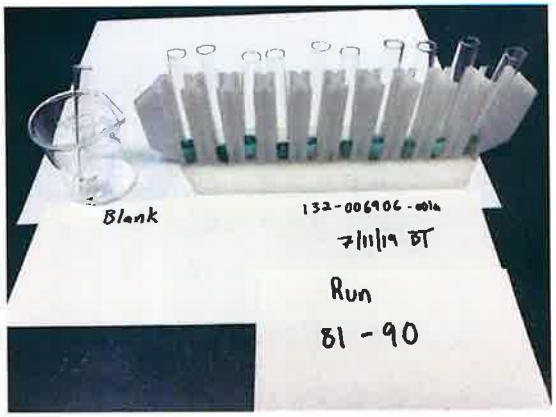
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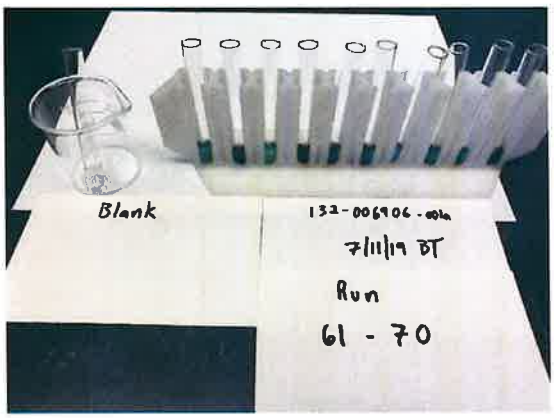
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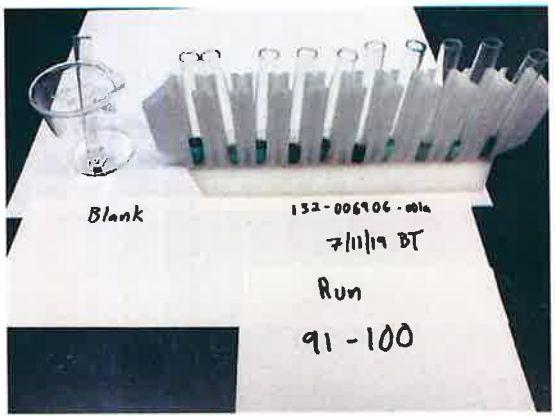
7/11/19 BT
Run
61 - 70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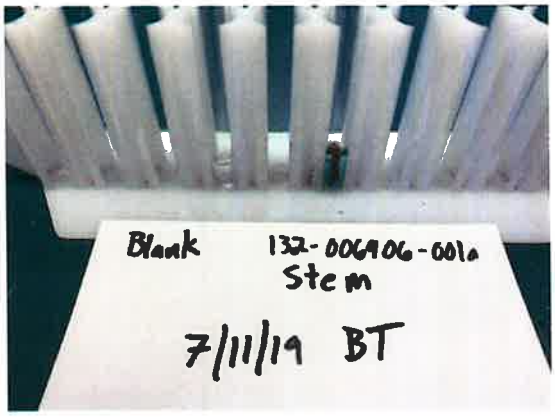
Run
91 - 100

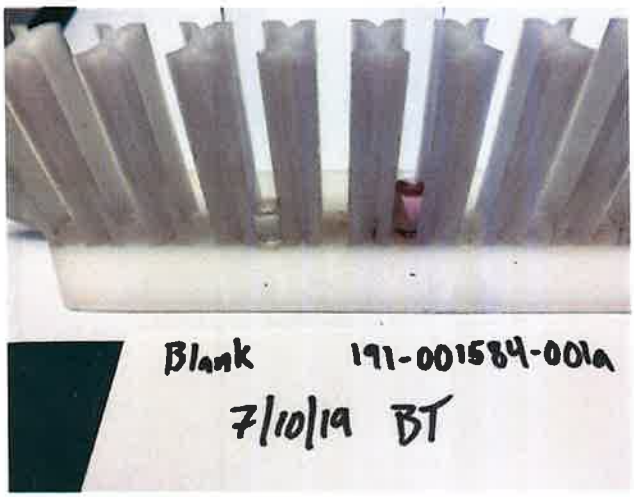

Blank 132-006906-001a
Stem
7/11/19 BT


Blank 132-006906-001a
Seed
7/11/19 BT

V. Plant material samples:

Our laboratory requested and received permission from some law enforcement agencies to use a portion of plant material exhibits for the purposes of method development and training. I analyzed these samples and the results are listed below. These samples had previously been quantitated for THC by our laboratory.

Laboratory number	Resulting Color	Quantitation Result
191-001912-002a	Reddish purple	0.38 % THC
191-001737-001a	Dark reddish purple	0.44 % THC
191-001584-001a	Light purple	0.33 % THC
191-001518-001a	Reddish purple	0.45 % THC
191-011450-001a	Dark blue	Greater than 1 % THC
191-002060-001a	Light reddish purple	Greater than 1 % THC
191-001496-001a	Reddish purple	0.38 % THC
181-025151-001a	Reddish purple	0.45 % THC
191-002292-001b	Blue	Not quantitated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7/10/19 BT


Blank 191-002060-001a
7/10/19 BT


Blank 191-001518-001a
7/10/19 BT


Blank 191-001496-001a
7/10/19 BT


Blank 191-011450-001a
7/10/19 BT

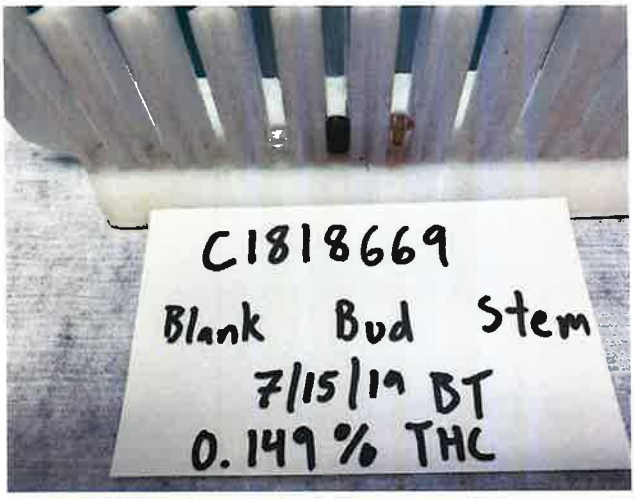

Blank 181-025151-001a
7/10/19 BT

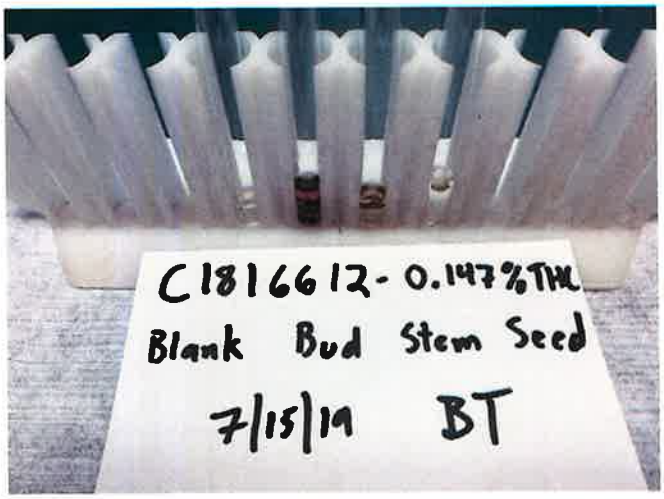
The plant material sample from 191-002060-001a had a quantitated THC value greater than one percent indicating that it was marihuana and therefore a controlled substance. However, the 4-AP color test result was reddish purple which is consistent with hemp samples. This result might be considered a false negative, however upon reviewing the gas chromatograph mass spectrometry data the amount of CBD in the sample is much greater than the amount of THC even though the THC percentage by dry weight exceeds the legal limit. The analyst must understand that the results of the color test are not quantitative in nature, but are limited to the ratio of CBD to THC. A blue result occurs when the amount of THC present in the sample exceeds the amount of CBD. A red or purple result occurs when the amount of CBD exceeds the amount of THC present in the sample. The resulting color cannot be used to accurately determine or make conclusions about the dry weight percentage of THC in the sample. This color test is a presumptive or screening test and must be performed in conjunction with other test in order to make conclusions about the legality of a plant material sample.

Our laboratory requested and received multiple hemp samples from the Tennessee Department of Agriculture. Some of these samples had been quantitated and issued a report. Several of those samples were analyzed with the 4-AP color test and the results are found in the table below. When available a small amount of the bud (flowering portion) was sampled. If no bud was available then a portion of the leaf was sampled; this is indicated in the table.

TN Department of Agriculture Number	Resulting Color	Quantitation Result
C1818814	Reddish purple	0.150
C1818813 (leaf)	Light purple (leaf)	0.256
C1818813 (bud)	Reddish purple (bud)	0.256
C1818442	Reddish purple	0.304
C1818128	Reddish purple	0.306
C1818815 (leaf)	Light purple (leaf)	0.069
C1816402	Reddish purple	0.064
C1816608 (leaf)	Light purple (leaf)	0.203

C1816527	Reddish purple	0.054
C1816032	Reddish purple	0.169
C1816383	Reddish purple	0.051
C1816656	Reddish purple	0.126
C1816056	Reddish purple	0.221
C1816076	Reddish purple	0.128
C1816404	Reddish brown purple	0.103
C1816528	Reddish purple	0.089
C1816381	Dark Reddish purple	0.050
C1816447	Reddish purple	0.103
C1816033	Reddish purple	0.068
C1816075	Light reddish purple	0.117
C1816085	Reddish purple	0.133
C1816807	Reddish purple	0.121
C1818438	Reddish purple	0.069
C1818266 (bud)	Reddish purple (bud)	0.234
C1818266 (seed)	Light yellow (seed)	0.234
C1818669 (bud)-See picture	Dark Reddish purple (bud)	0.149
C1818669 (stem)–See picture	Light reddish purple (stem)	0.149
C1816802	Reddish purple	0.105
C1816068	Reddish purple	0.109
C1816612 (bud)-See picture	Reddish purple (bud)	0.147
C1816612 (stem)-See picture	Light reddish purple (stem)	0.147
C1816612 (seed)-See picture	Light yellow (seed)	0.147
C1816524	Light reddish purple	0.049





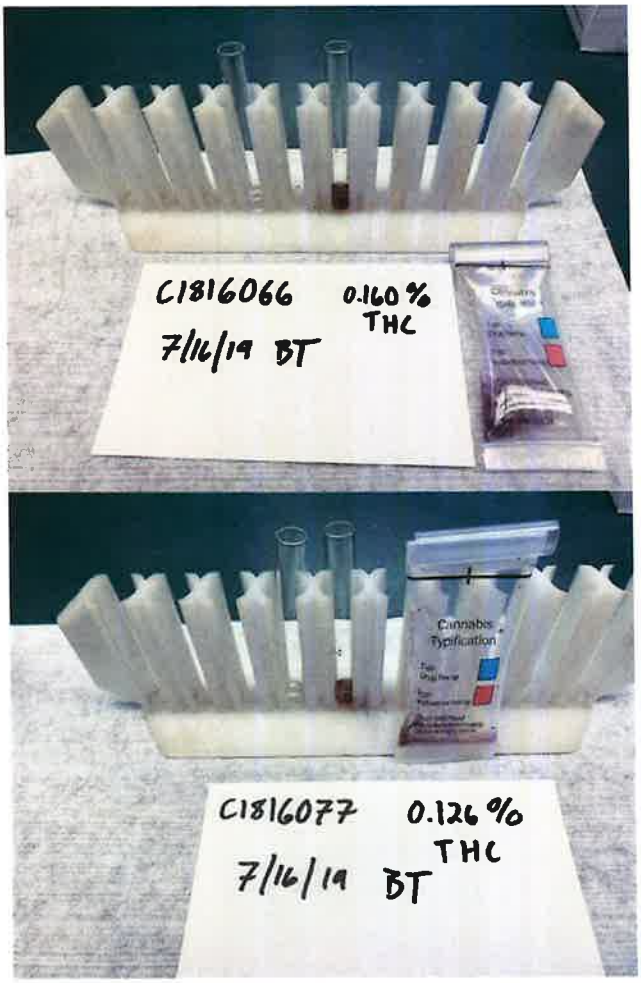
Throughout May of 2019 one analyst worked exclusively plant material and obtained GC/MS data for each exhibit. In total there were 167 exhibits of plant material analyzed. Assuming a 9:1 or greater ratio of THC to CBD would give a blue result and any ratio less than that would give a reddish purple result, 149 exhibits would have resulted in a blue 4-AP color

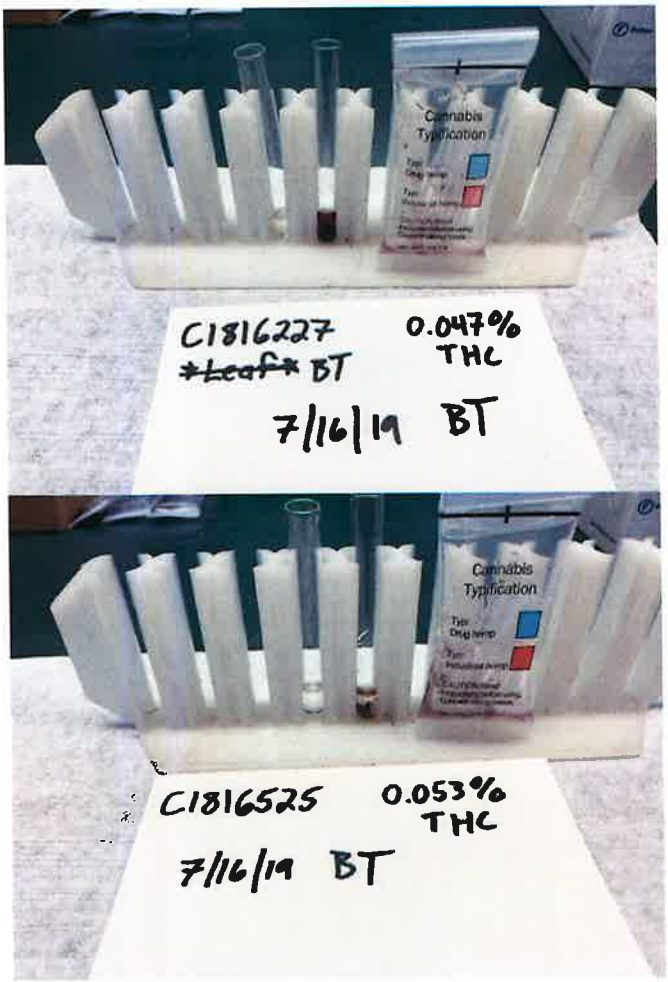
test result and 18 exhibits a reddish purple result. If these exhibits are representative of all the plant material exhibits our laboratory receives, then approximately 10.8 % of our cases would require further analysis (THC quantitation analysis or GC/MS analysis) in order to further ascertain their legality. The 89.2% of cases would have enough data to confidently identify marihuana as long as it was made clear no quantitative analysis or instrumental analysis was performed.

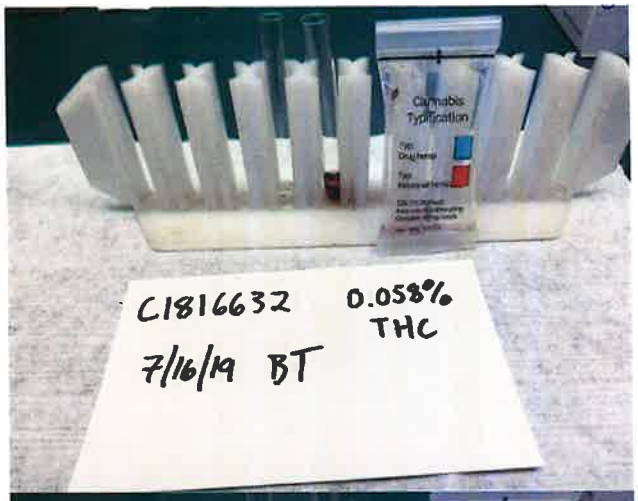
VI. Test Kit Evaluations:

On 7/16/2019 our laboratory received the field test kits. Hemp samples were run with our in-house prepared 4-AP reagent in conjunction with the purchased field test kits.

Agriculture ID #	TBI 4-AP color	Field Test color	TDA Quant Result
C1816066	Reddish purple	Reddish purple	0.160
C1816632	Reddish purple	Light reddish purple	0.058
C1816077	Light reddish purple	Light reddish purple	0.126
C1816525	Reddish purple	Reddish purple	0.053
C1816227	Dark reddish purple	Reddish purple	0.047
C1818446	Reddish purple	Reddish purple	0.253
C1818123 (Leaf)	Light reddish purple	Light reddish purple	0.138
C1818078	Reddish purple	Reddish purple	0.205
C1818129	Reddish purple	Reddish purple	0.093
C1818443	Reddish purple	Reddish purple	0.189



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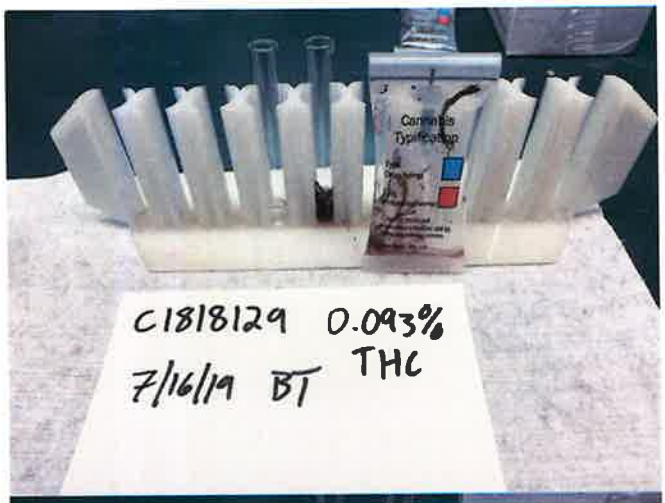
7/16/19 BT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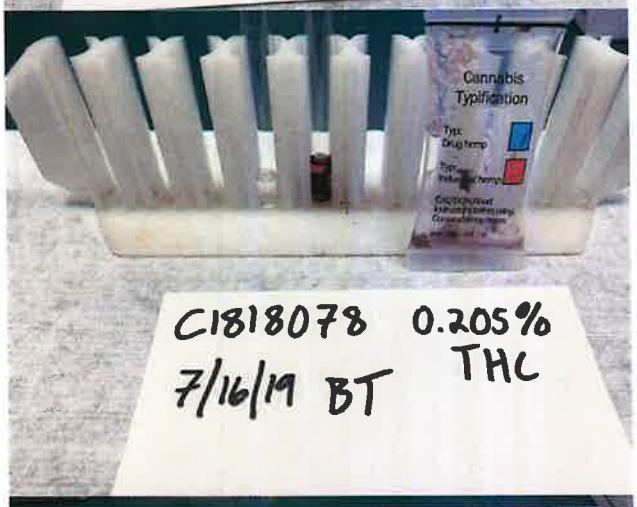
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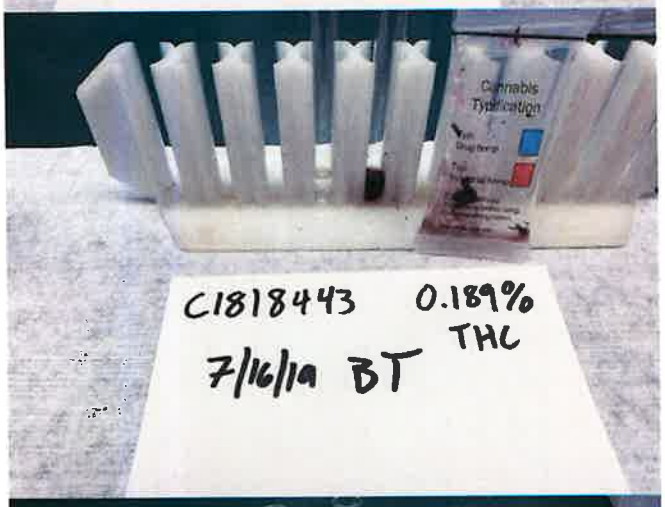
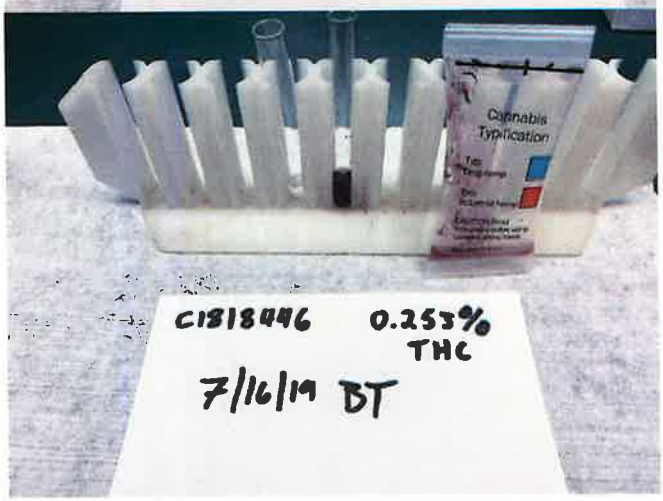

Cannabis Typification
C1818443 0.189% THC
7/16/19 BT

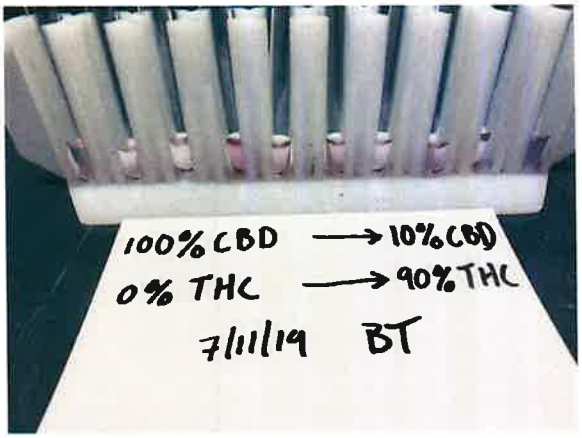

Cannabis Typification
C1818123 0.138% THC
7/16/19 BT


Cannabis Typification
C1818446 0.253% THC
7/16/19 BT

VII. THC to CBD Ratio Study:

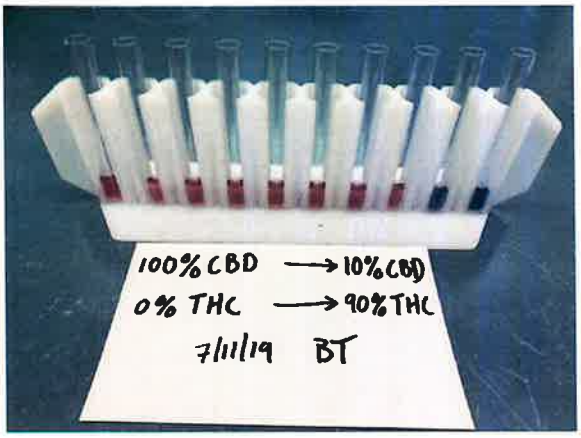
Δ^9-Tetrahydrocannabinol (Cerilliant lot # FE08221804) and Cannabidol (Cerilliant lot # FE08071702) standards were used to prepare a series of ten solutions with different ratios of THC to CBD. The solutions were prepared by pipetting these standards into test tubes in different ratios. The standards were in a concentration of 1 mg/mL. These mixtures were analyzed using the 4-AP color test. This experiment was conducted in an attempt to determine at what ratio of CBD to THC the resulting color changes from reddish purple to blue.

Ratio of THC to CBD	Resulting Color 1 Minute	Resulting Color 10 Minutes	Resulting Color 20 Minutes	Resulting Color 50 Minutes
0 : 10	Light purple	Reddish purple	Reddish purple	Reddish purple
1 : 9	Light purple	Reddish purple	Reddish purple	Reddish purple
2 : 8	Light purple	Reddish purple	Reddish purple	Reddish purple
3 : 7	Purple	Reddish purple	Reddish purple	Dark purple
4 : 6	Purple	Reddish purple	Reddish purple	Dark purple
5 : 5	Purple	Reddish purple	Reddish purple	Dark blue purple
6 : 4	Purple	Reddish purple	Reddish purple	Blue
7 : 3	Purple	Reddish purple	Bluish purple	Blue
8 : 2	Purple	Bluish purple	Blue	Blue
9 : 1	Blue	Blue	Blue	Blue



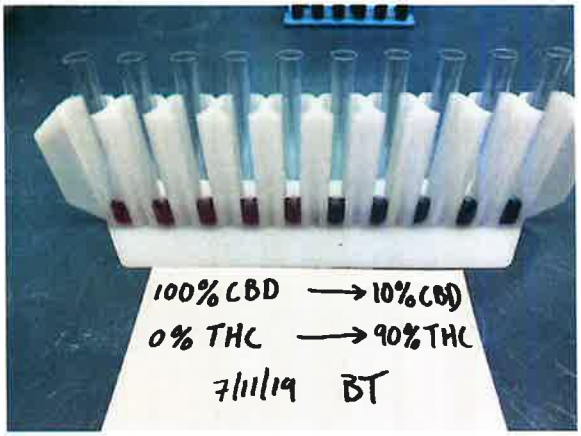
After 1 minute

After 10 minutes



After 20 minutes

After 40 minutes



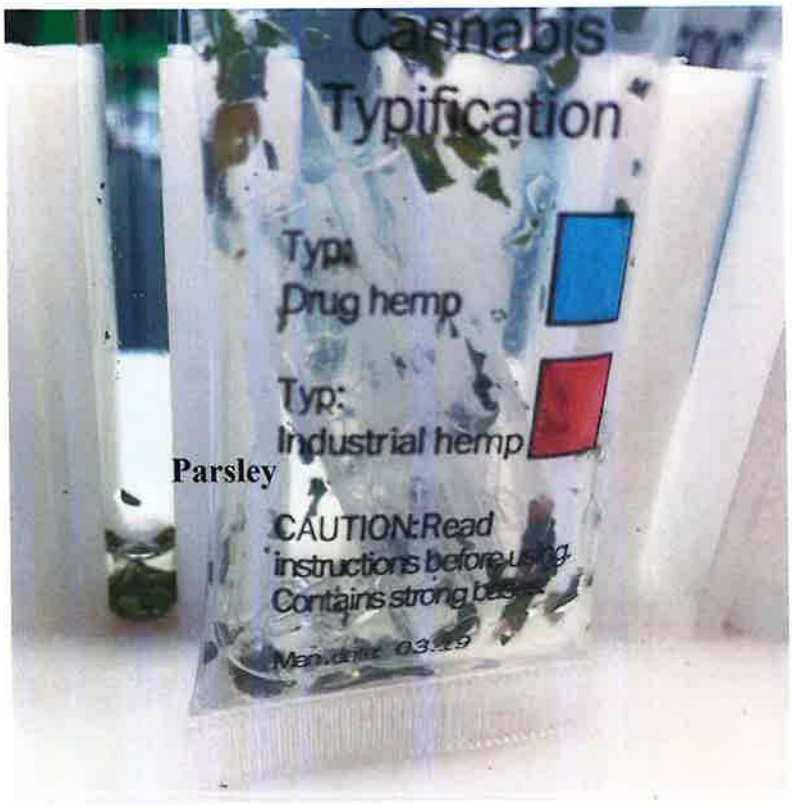
After 50 minutes

As the color test was allowed to sit the colors definitely darkened and the mixtures with lower concentrations of THC began to develop a bluer color. The results for the 3:7 THC:CBD never turned to a predominately blue color, but the results for the 4:6 THC:CBD and higher did change to a blue color. Once again, this study demonstrated that results of the color test should be interpreted within a minute or two of the addition of both reagents to the plant material.

VIII. Controls:

It will be important to verify that each time the reagents (4-AP #1 and 4-AP # 2) are prepared that they give consistent results to the previous preparations. Therefore, when preparing these reagents they should be run against the same controls. The negative control will be a non-controlled spice such as oregano or cilantro. The positive control will be a Δ^9-tetrahydrocannabinol standard.

The McCormick's Parsley proved to be a good choice as a negative control. No color developed in either the 4-AP color test prepared in our laboratory or with the field test kit that was purchased.



Parsley run with 4-AP color test and field test kit

Conversely, the McCormick's Oregano did not prove to be a good choice as a negative control. Oregano has cystolithic hairs and gave both blue color results for the prepared 4-AP reagents and the purchased field test kit. After obtaining the unexpected blue result for oregano, the spice was run five more times with our prepared reagents and the results were consistently blue. Two different analysts ran the test to ensure there was not a technique issue causing contamination of the oregano. A modified Duquenois Levine test was completed on the oregano as well as a gas chromatograph mass spectroscopy test (GC/MS) to verify that the source of

oregano had not been contaminated with THC. The Duquenois Levine and GC/MS testing were negative for THC on the oregano.

Oregano run with 4-AP color test and field test kit

The Δ^9-tetrahydrocannabinol standard gave blue results to both the field test kit and the prepared 4-AP reagents. This result was expected and demonstrates that a THC standard is an appropriate positive control to verify fitness for use with prepared 4-AP reagents. The THC standard used in this study was purchased from Lipomed (Lot # 135.1B71.0L6).

Δ^9-Tetrahydrocannabinol Standard with 4-AP color test and field test kit

Purchased Cannabidiol standard will be used as a positive control for the result of the 4-AP color test associated to hemp. The 4-AP color test was conducted on a Cerilliant CBD standard and resulted in a reddish purple color. These three controls will be run at the time of preparation of the color test reagents.

While the false positive results for oregano are disappointing, they do not preclude the 4-AP color test from providing useful results. If run in conjunction with the modified Duquenois Levine or instrumental analysis, analyst could still distinguish marihuana from hemp and other plant materials. It is also reminds us that color test are and can only be used as presumptive test. It will be necessary to combine the 4-AP color test with other methodology in order to make conclusions about the legal status of the submitted plant material.

IX. Conclusions

Current testing procedures in our laboratory for plant material do not allow for a distinction between marihuana and hemp without a GC/MS THC quantitation. Our quantitation method is time consuming, challenging, and only performed when requested and approved. The addition of the 4-AP color test would allow for a cheap and fast test that would give the analyst confidence to distinguish illegal cannabis from legal cannabis.

Sample selection between different components of the plant was investigated on a limited basis. For a select number of samples stems, seeds, and or leaves were analyzed at the same time as portions of the flowering bud. The validation showed that analyzed seeds resulted in a color similar to the blank and could not be interpreted in a manner which distinguishes hemp and marihuana. Analysis of leaves and stems did result in interpretable colors, but were not as quick and vibrant as the results from the bud. When sampling the plant material portions from the bud should be taken, but if not available the analyst could sample leaves or as a last resort stems. Seeds should not be analyzed with the 4-AP color test.

Identifying oregano as a false positive indicated that this color test should be a supplement to our current testing and not a replacement for the Duquenois Levine test. The results encountered while testing 191-002060-001a also demonstrated the importance of understanding the capabilities of this color test. Because the test only investigates the ratio of CBD to THC in a sample, it is possible for a sample to contain greater than 0.3 % THC and still give a reddish purple hemp result. Plant material cases exist that will need quantitative analysis in order to determine their legal status.

Every experiment that involved a side by side comparison of the 4-AP reagents to the field test kit yielded similar colors. It can be concluded that the formulation used to prepare our reagents is in fact the same or very similar to the manufacturer's. Comparing the purchased field test kits in to the prepared 4-aminophenol reagents not only validated our capability to prepare the reagents, but also allows us to recommend these test kits with some confidence to other law enforcement agencies. The implementation of field test kits could reduce the number of submitted evidence lessening the burden of the forensic chemistry unit. Several agencies have already reached out to our laboratory seeking a field test for hemp and marihuana.

X. References:

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CANNABINOID LEVEL IN THE LEAVES AS A TOOL FOR THE EARLY DISCRIMINATION OF CANNABIS CHEMIOVARIANTS

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Summary

One hundred and seventy-six plants of 22 different lots of *Cannabis sativa* L., grown at the Botanical Garden of Siena (Italy) were chromatographically analysed in order to define the cannabinoid content in their leaves.

The content of the major cannabinoids, Δ^9-tetrahydrocannabinol, cannabidiol, cannabinol and cannabichromene, determined weekly in vegetative and floral leaves enabled the determination of the chemical types of the plants, according to Turner's classification.

The plants were easily distinguishable in drug, intermediate and fiber types. The cannabinoid characteristic of each type remains predominant, as compared with the other cannabinoids, throughout the whole period of growth, including the floral stage and after harvesting.

On this basis, the predominant concentration of a specific cannabinoid can be used reliably for forensic application concerning drug-suspected material in very young plants.

Key words: Narcotics; *Cannabis sativa*; Cannabinoids; Chemiovariants.

Introduction

At the present, *Cannabis* is generally regarded as a monotypic genus consisting of a single species, *Cannabis sativa* L., with different chemical types [1]. The chemical phenotypes are identified by their cannabinoid contents. Consequently, much work has been carried out on these compounds, e.g. Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN), cannabichromene (CBC), cannabidiol (CBD), which occur only in *Cannabis* plants [2]. In this regard, chemical research was also encouraged due to the legal implications concerning the misuse of marijuana in recent years.

Chromatographic analysis provides unequivocal identification of the cannabinoids. This analytical method is utilized in the chemical characterization of drug or fiber (non-drug) strains of *Cannabis* [3]. In a classical

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report, Small and Beckstead [4] proposed a chemical classification of *Cannabis* variants which distinguished four chemical phenotypes on the basis of cannabinoid contents. Later, different criteria have been suggested by other authors [5,6]; recently Turner [7] proposed a simpler classification restricted to three chemiovariants: (a) the fiber type, in which the major cannabinoid is CBD; (b) the intermediate type, in which there are equal amounts of CBD and Δ^9-THC; (c) the drug type, in which Δ^9-THC is the major compound.

The aim of this research is to examine the content of cannabinoids in the leaves during the life cycle of plants coming from different countries and grown in a similar environment. In this way, we seek to determine if the distinction of the three types described above occurs in any given developmental stage of the plant. In fact, since the possession of the drug type of *Cannabis sativa* L. is prohibited in most countries, the legal evaluation demands a rigorous and unequivocal means of identifying plant material, as drug or non-drug type, even when very immature specimens are seized. Unfortunately, as outlined in recent papers [6,8], the need cannot be easily met at present because of the lack of knowledge regarding the fluctuation of cannabinoid ratios during the life cycle of plants.

Experimental

Plant material

Twenty-two seed samples of *Cannabis sativa* L. were acquired from a variety of sources, including commercial firms, seed exchange institutes and police seizures.

Plants were grown outdoors in 22 different lots in the Botanical Garden of Siena (Italy) from April 30 to August 30, 1982. Eight specimens were grown in each lot, resulting in a total of 176 plants; all of them developed normal inflorescence. Male and female plants were equally represented.

After 16 weeks of growth, the plants (1.70—2.50 m long) were harvested and dried indoors at room temperature.

Vegetative leaves were collected after the 4th week of growth. Floral leaves — i.e. those neighbouring flowers — were gathered after the 8th week of growth. Samples of both leaf types were collected weekly until the 16th week and, then, bi-weekly until the 28th week of the drying process.

Chemicals

For the chemical analyses, CBD, Δ^9-THC, CBC and CBN standards were purchased from Supelco, Inc. (Crans, Switzerland); all solvents and other chemicals were for chromatography and furnished by Merck, Inc. (Darmstadt, Germany).

Chemical analyses

Separate chemical analyses were carried out on single vegetative and floral leaves of each
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Results and discussion

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floral leaves of each plant. Twenty-five milligrams of dried leaf powder were extracted for 4 h with petroleum ether. After centrifugation the ether extracts were dried under a nitrogen stream and the dry residue was dissolved in 1 ml of cyclohexane. After the addition of a known amount of α-colestane (internal standard), 1 μl of the extract was injected into a gas-liquid-chromatograph with packed column (GLC) for analysis. In some cases high-resolution gas-chromatography (HRGC) was used in order to obtain clearly separated peaks of CBC and CBD [9].

Instrumentation

GLC was performed with a Fractovap apparatus Mod. 4200 (C. Erba Inc., Milan, Italy) equipped with a flame ionization detector in conjunction with a Spectra-Physics SP 4000 central-processor, a SP 4020 data interface and a 4050 printer-plotter. A silanized glass column (2 m × 0.4 cm) was packed with 3% OV 17 (phenyl-methyl-silicone on 100—120 mesh Gas-Chrom) and operated on isothermal temperature of 230°C. Injector and detector temperatures were maintained at 300°C. Carrier gas was nitrogen (flow rate, 50 ml/min). HRGC was performed with a HRGC apparatus Mod. 4160 (C. Erba Inc., Milan, Italy). The following chromatographic conditions were used: glass capillary column, 15 m × 0.4—0.45 mm ID; stationary phase SE 52; injection "on column"; programmed temperature, multi-step system: 50°C/isothermal for 3 min, rate 5°C/min, 100°C isothermal for 2 min, rate 10°C/min, 170°C isothermal for 1 min, rate 2°C/min, 250°C isothermal for 30 min; hydrogen carrier gas at 3 ml/min; flame ionization detector.

Results and discussion

The CBC, Δ^9-THC, CBN and CBD contents were determined chromatographically in each plant in order to define the different *Cannabis sativa* types, according to Turner's classification [7]. The analytical data, collected from 176 plants, showed that 80 of them belonged to the drug type, 40 to the intermediate type and 56 to the fiber type.

A significant uniformity was observed in the chemical composition of the plants of each considered lot.

Figure 1 shows the development of the major cannabinoids present in the three described types during the life cycle of the plants, starting from the 4th week after planting until maturity and after harvesting. It demonstrates that, in the drug type (Figs. 1A,B), the content of the main cannabinoid (i.e., Δ^9-THC) remains nearly constant in the vegetative leaves (Fig. 1A), but it markedly increases in the floral leaves (Fig. 1B) during blooming.

In the intermediate type (Figs. 2A,B), Δ^9-THC and CBD increase in both vegetative (Fig. 2A) and floral (Fig. 2B) leaves during plant maturation. The levels of the two compounds are similar until the 13th—14th week.

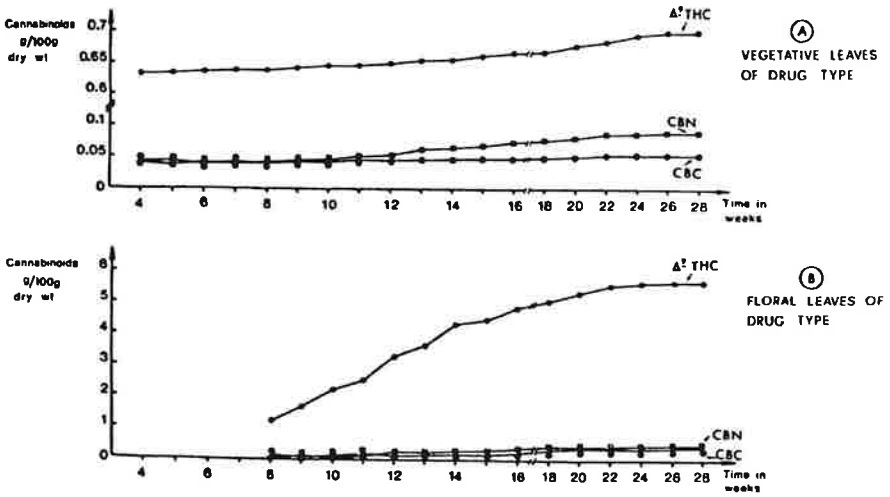


Fig. 1. Cannabinoid contents in vegetative (A) and floral (B) leaves of growing (up to 16 weeks) and harvested plants belonging to a drug type *Cannabis sativa* lot. All data points are an average of the analyses on 8 separately tested plants.

Afterwards CBD becomes slightly prevalent in the vegetative leaves (Fig. 2A), while Δ^9-THC becomes prevalent in the floral leaves (Fig. 2B).

In the fiber type (Figs. 3A,B), CBD is always predominant; however, while its values are constant in the vegetative leaves (Fig. 3A), in the floral ones (Fig. 3B) CBD increases continuously during the full developmental cycle of the plants.

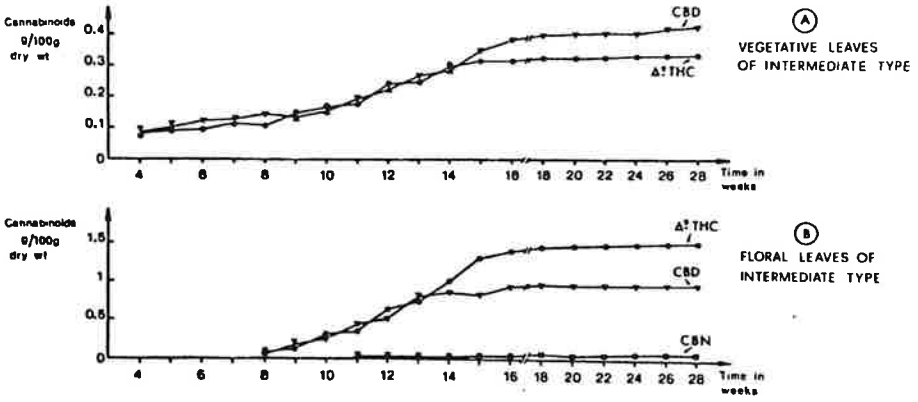
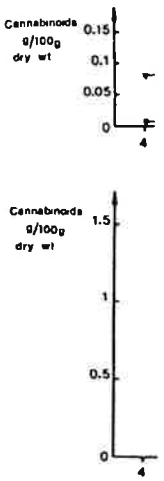


Fig. 2. Cannabinoid contents in vegetative (A) and floral (B) leaves of growing (up to 16 weeks) and harvested plants belonging to an intermediate type *Cannabis sativa* lot. All data points are an average of the analyses on 8 separately tested plants.



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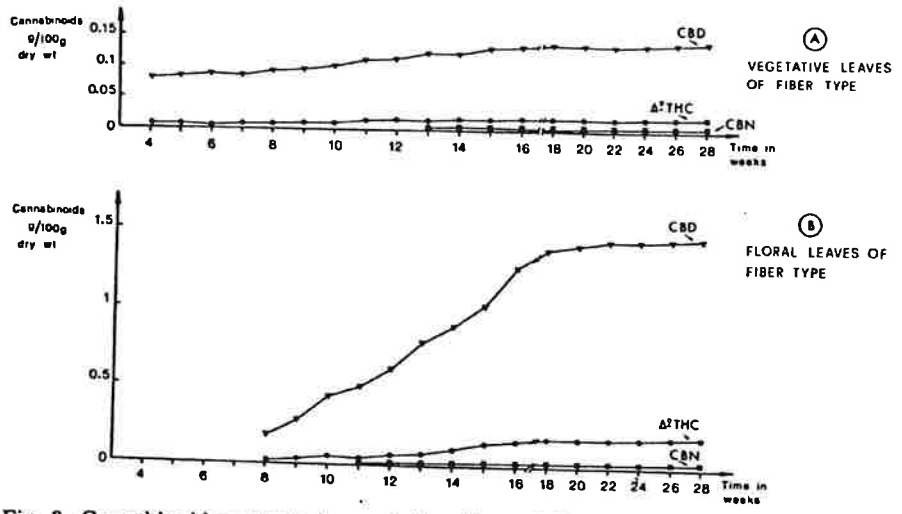

Fig. 3. Cannabinoid contents in vegetative (A) and floral (B) leaves of growing (up to 16 weeks) and harvested plants belonging to a fiber type *Cannabis sativa* lot. All data points are an average of the analyses on 8 separately tested plants.

CBC is constantly absent in any stage of the life cycle of the plants belonging to the intermediate and fiber types.

The results obtained by the analyses performed on vegetative and floral leaves of the three type plants grown under the same conditions leads to the following conclusions:

(1) The amount of the major cannabinoids in the vegetative leaves of the drug and fiber types undergo only minor changes during the whole period of growth, including the floral stage and after harvesting; in the intermediate type an increase in Δ^9-THC and CBD occurs during the growth.
(2) In the floral leaves, the content of cannabinoids characteristic of each type of *Cannabis sativa* progressively increases with aging and, to a lesser degree, after harvesting.
(3) Both in vegetative and in floral leaves, the cannabinoids characteristic of each type of *Cannabis sativa* remain predominant, as compared with the other cannabinoids, throughout the whole period of growth, including the floral stage and after harvesting.

Therefore, by measuring the levels of the principal cannabinoids, it is possible to ascertain *Cannabis sativa* chemovariants even when plants are in an early stage of development. In fact, from the graphs reported in Figs. 1, 2 and 3, it is evident that very young samples already have the chemical features of the type to which they belong.

In agreement with the results reported by Fournier [10], the present studies do not show a cyclic fluctuation in the amounts of the cannabinoids in the various types of *Cannabis sativa*; rather they show that each type has a constant feature. This is at variance with previously published results [6,8,11,12]. If this is linked to the fact that in these cited cases the analyzed materials included different, randomly gathered plant portions, it is a matter of speculation.

The fact that no cyclic variation has been found in the level of cannabinoids in the different types of *Cannabis sativa*, allows the evaluation of drug-suspected material even in very young plants.

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ORIGINAL ARTICLE

Cannabinoid concentrations in confiscated cannabis samples and in whole blood and urine after smoking CBD-rich cannabis as a "tobacco substitute"

Marianne Hädener[1] · Tim J. Gelmi[1] · Marie Martin-Fabritius[1] · Wolfgang Weinmann[1] · Matthias Pfäffli[2]

Received: 2 October 2018 / Accepted: 19 December 2018

Abstract
In Switzerland, only cannabis with a total Δ^9-tetrahydrocannabinol (THC) content higher than 1% is controlled by the narcotics legislation. Cannabis products rich in cannabidiol (CBD) and low in THC can be legally sold as tobacco substitutes. In this paper, we address analytical and forensic toxicological issues related to the increasing availability and consumption of these products. Based on the analysis of 531 confiscated cannabis samples, we could establish classification thresholds: plant material with a ratio of total THC/total CBD ≥ 3 is graded as THC-rich/CBD-poor, whereas samples with a ratio ≤ 0.33 are categorized as CBD-rich/THC-poor cannabis. We also evaluated an on-site test kit as a rapid alternative to the laborious liquid or gas chromatography (LC or GC)-based techniques normally used for the differentiation between THC- and CBD-cannabis. Furthermore, we determined whole blood and urine cannabinoid levels after smoking different doses of legal CBD-cannabis. A male volunteer smoked one cigarette within 15 min and four cigarettes within 1 h and within 30 min, respectively. Cigarettes contained on average 42.7 mg CBD and 2.2 mg THC. Blood samples were collected up to 1.1 h and urine samples up to 27.3 h after the beginning of smoking. All urine samples tested negative by three immunochemical assays for detection of cannabis use. This is an important finding for abstinence monitoring. However, we found that the trace amounts of THC present in CBD-cannabis can produce THC blood levels above the Swiss legal limit for driving, and thus render the consumer unable to drive from a legal point of view.

Keywords Tetrahydrocannabinol · Cannabidiol · Classification · Drug of abuse testing · Driving while impaired

Introduction

For centuries, cannabis has been cultivated as a source of fiber, food, and oil, as a medicinal plant as well as for recreational purposes [1]. To date, well over 400 constituents have been identified in cannabis, of which at least 70 are phytocannabinoids, a group of 21 terpenophenolic compounds to which the majority of the plant's biological activities have been ascribed [2]. Δ^9-tetrahydrocannabinol (THC) is generally accepted to be the compound responsible for the psychoactive properties of cannabis inducing euphoria, altered sensory perception and relaxation, also known as the "high" that recreational cannabis users seek. Additionally, THC has been found to have a number of therapeutic attributes including anti-inflammatory, antiemetic, appetite stimulant, analgesic, and antispasmodic effects [3]. However, THC has also been associated with a number of adverse effects, including tachycardia, anxiety, cognitive deficits, paranoia, as well as with an increased risk of developing chronic psychosis and dependence [4–7]. Since several years, other cannabinoids have come into the focus of research and legislation, the most prominent being cannabidiol (CBD), which is non-euphoriant and is thought to modulate the psychoactivity of THC, thus alleviating the adverse effects of THC. Furthermore, CBD has shown promising therapeutic activity per se, such as anti-inflammatory, anticonvulsive, anxiolytic, analgesic, neuroprotective, anticancer, and antioxidant effects [8, 9].

Marianne Hädener and Tim J. Gelmi contributed equally to this work.

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The cannabinoid composition of the cannabis plant material, i.e., the ratio of the different cannabinoids, is primarily determined by genetic factors (strain type), but is also affected by environmental and harvesting conditions (e.g., growing and storage conditions, state of maturity at harvest) [10]. Over the last few decades, recreational users seeking the ultimate "high" have selectively bred cannabis plants to produce highest THC and lowest CBD levels and until recent years, little effort has been made to breed plants with high CBD levels as there was no demand among recreational cannabis users. However, due to the growing interest in the therapeutic effects of CBD, cannabis breeders have been working to create new strains 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 bred [11]. The total CBD and total THC content (in % of dry weight) corresponds to the sum of the free cannabinoid and its pharmacologically inactive carboxylic acid precursor (CBD-acid and THC-acid A, respectively), corrected for weight of the carboxylic acid groups. Decarboxylation of the acids into their active neutral forms occurs spontaneously as the plant ages and, to a much larger extent, upon heating (e.g., upon smoking, vaporizing, or baking the plant material) [12].

In the European Union, cannabis plants containing no more than 0.2% total THC and producing no psychoactive effects can be legally cultivated for fiber and seeds production. In Switzerland, only plant material with a total THC content of 1% or higher is controlled by the narcotics legislation [13]. CBD-rich/THC-poor cannabis products have been on the Swiss legal market as tobacco substitutes for more than a year and their consumption has recently become increasingly popular, for both therapeutic and recreational purposes.

Since 2017, our laboratory has analyzed over 800 cannabis samples confiscated by Swiss police forces by a fully validated high-performance liquid chromatography-diode array detector (HPLC-DAD) method for determination of the cannabinoid profile [14]. In this work, we will give a representative overview on the concentrations of CBD and THC in the confiscated material and we will propose a ratio of total THC/total CBD to discriminate between CBD- and THC-cannabis on an analytical level. Furthermore, we will evaluate the performance of a recently introduced on-site test kit [15] for the rapid distinction between CBD- and THC-cannabis.

As strategy to prevent cannabis-impaired driving, Switzerland, among several other European countries [16], has adopted a zero tolerance law that prohibits driving with any detectable amount of psychoactive THC in the blood. The detection of THC in blood is deemed proof of the driver's acute impairment and inability to drive. According to the directives of the Federal Roads Office an analytical cut-off of 1.5 μg/L is employed for the reliable detection of THC in whole blood. In forensic practice, a confidence interval of 30% is taken into consideration, resulting in an effective cut-off of 2.2 μg/L (2.2 μg/L minus 30% is 1.54 μg/L, which is just higher than the analytical cut-off of 1.5 μg/L). Since CBD-rich cannabis products contain trace amounts of THC, the question arises whether their consumption might cause THC blood levels ≥2.2 μg/L, and thus render the consumer unable to drive from a legal point of view. Another analytical question commonly raised is whether smoking legal CBD-rich cannabis can produce urinary cannabinoid levels high enough to result in positive findings in immunological urine drug tests. Urine drug screening by immunochemical assays is often performed in cases of suspected drug-impaired driving, for workplace drug testing, and to monitor abstinence from drug use [17, 18]. Antibodies used in urinary immunochemical tests for detection of cannabis use primarily target the THC metabolite 11-nor-9-carboxy-THC (THCCOOH) and have variable degrees of cross-reactivity with other metabolites and cannabinoids [19].

Herein, we attempt to answer the aforementioned questions by reporting whole blood and urine cannabinoid levels measured in a male subject after smoking different doses of CBD-rich/THC-poor cannabis flowers.

Material and methods

Chemicals and reagents

Dried CBD-rich/THC-poor cannabis flowers (*Cannabis sativa* L.) were obtained from Swiss Cannabis SA (Härkingen, Switzerland). Acetonitrile (HPLC gradient grade, 99.9%) was purchased from Acros Organics (Geel, Belgium) and methanol (absolute, HPLC grade) from Biosolve (Dieuze, France). Butyl acetate (HPLC grade, 99.7%), formic acid solution (puriss p.a., 50% in water), and *E. coli*-type IX-A β-glucuronidase were obtained from Sigma-Aldrich (Buchs, Switzerland). Potassium dihydrogen phosphate (puriss p.a., ACS), di-sodium hydrogen phosphate dihydrate (analytical grade), and *H. pomatia* β-glucuronidase/arylsulfatase were acquired from Merck (Darmstadt, Germany). Deionized water was prepared in-house with a Direct-Q water purification system from Millipore (Zug, Switzerland). THC, 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), and CBD reference standards as well as their trideuterated analogs were purchased from Cerilliant (Round Rock, TX, USA). THC-acid A, CBD-acid, and cannabinol (CBN) reference standards were obtained from Lipomed (Arlesheim, Switzerland). Blank human blood and urine samples were obtained from the blood donor center in Bern, Switzerland and donated by a volunteer, respectively, and were tested for the absence of cannabinoids prior to use.

Colorimetric on-site test kit for differentiation of CBD- versus THC-cannabis

For differentiation of CBD- versus THC-cannabis, a liquid colorimetric test can be used by police forces on the street and on indoor or outdoor cultivation sites, or as preliminary tests in the laboratories. A commercial test kit is already in use by several Swiss police forces [15]. The kit contains two solutions: solution 1, consisting of 0.2 mg/mL 4-aminophenol in ethanol/isopropanol (95:5; *v/v*), which was acidified with 0.5% hydrochloric acid (HCl 2 N); solution 2, consisting of 30 mg/mL sodium hydroxide in ethanol/water (70:30; *v/v*).

To conduct the differentiation, a small sample of dried or fresh cannabis plant material is transferred into a glass vial and 1–2 mL of solution 1 (depending on sample size) and four drops of solution 2 are added. The vial is closed, shaken vigorously and left to stand for 2 min to allow reaction. Subsequently, the color of the solution is visually evaluated: a blue coloration indicates THC-rich cannabis; a pink coloration indicates CBD-rich cannabis. For quantitative determination of cannabinoids, the HPLC-DAD method, which is described hereafter, is used.

HPLC-analysis of cannabis products for determination of THC, THC-acid A, CBD, CBD-acid, and CBN

The HPLC-DAD method for the analysis of cannabis products has already been published [14] and has recently been updated by adding CBD-acid to the calibrators and the control samples, in the same concentrations as THC-acid A.

Data treatment and evaluation of confiscated cannabis samples

Since January 2017, 808 confiscated samples have been analyzed for cannabinoids at our institute. For the evaluation of the CBD and THC content, only samples containing dried or fresh cannabis flowers were considered. Samples consisting of (burnt) joint material, plant material without flowers, hashish, or oil were excluded. Furthermore, 119 samples were analyzed before CBD-acid had been included in the method and were therefore also excluded as no re-analysis was possible. Finally, 531 confiscated samples have been evaluated with respect to CBD and THC concentrations. Data evaluation and visualization were conducted using R version 3.5.1 [20].

Study design for the smoking experiment

A healthy male subject (42 years, 75 kg, 183 cm; regular tobacco cigarette smoker) participated in three experimental sessions, separated by 3 weeks, in which he smoked either one cigarette ad libitum for up to 15 min (session A); four cigarettes within 1 h (session B); or four cigarettes within 30 min (session C) containing 0.5 g of a 1:1 mixture of CBD-rich cannabis flowers and tobacco. The smoked amounts of CBD and THC averaged about 42.7 and 2.2 mg, respectively per cigarette. Whole blood samples were collected between cigarettes and up to 1.1 h and urine samples up to 27.3 h after the beginning of smoking.

Qualitative analysis of urine samples

Qualitative screening by homogeneous enzyme immunoassay was performed with 300 μL of urine on an AU480 analyzer (Beckman Coulter, Nyon, Switzerland) using the Immunalysis HEIA Cannabinoids kit (Pomona, CA, USA) according to the manufacturer's instructions. Furthermore, all urine samples were qualitatively analyzed for cannabinoids using the THC lateral flow immunoassay test and automatic P.I.A.^{2} readout system from Protzek (Lörrach, Germany) as well as with the Drug-Screen-Multi 12Z dip test from Nal von Minden (Moers, Germany). All three immunochemical assays were calibrated with the THC metabolite THCCOOH (50 μg/L cut-off for THCCOOH).

Quantitative analysis of blood and urine samples

Sample preparation

Two hundred microliters of whole blood and urine samples were spiked with 20 μL of internal standard solution (2 ng THC-d_3 and 11-OH-THC-d_3, 10 ng THCCOOH-d_3, 2 ng CBD-d_3) and processed by protein precipitation with 600 μL of acetonitrile for determination of free cannabinoid concentrations or by β-glucuronidase treatment followed by liquid-liquid extraction with 1 mL of butyl acetate for determination of total cannabinoid concentrations (free and glucuronidated). For hydrolysis of glucuronide conjugates of THC, 11-OH-THC, and THCCOOH, 10 μL of *H. pomatia* β-glucuronidase/arylsulfatase and 200 μL of 66 mM phosphate buffer pH 6 were added, and the samples were incubated at 47 °C for 2 h. Hydrolysis of CBD-glucuronide was achieved by adding 200 μL of 75 mM phosphate buffer pH 6.8 containing *E. coli*-type IX-A β-glucuronidase at a concentration of 5000 U/mL and incubating at 37 °C for 2.5 h. After mixing and centrifugation, the organic phases (acetonitrile/butyl acetate) were evaporated to dryness at 50 °C under nitrogen and reconstituted in 200 μL of acetonitrile/water/formic acid, 60:40:0.1; *v/v/v*. Five microliters of the prepared samples were injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

Calibration samples were prepared by addition of reference standard solutions to blank blood and blank urine, respectively, and were processed by protein precipitation as described above. Linear ranges in blood and urine were 0.5–20 μg/L for THC and 11-OH-THC, 2.5–100 μg/L for THCCOOH, and 1–

250 µg/L for CBD. Lower limits of quantification (LLOQ) were 1 µg/L for THC and 11-OH-THC, 5 µg/L for THCCOOH, and 1 µg/L for CBD. Limits of detection (LOD) were 0.5 µg/L for THC and 11-OH-THC, 2 µg/L for THCCOOH, and 0.1 µg/L for CBD.

LC-MS/MS instrumentation

Cannabinoid concentrations were determined using our published column-switching LC-MS/MS method [21] with some modifications. The LC-MS/MS consisted of an UltiMate 3000 HPLC system (Dionex, Olten, Switzerland) coupled to a 4500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer with a Turbo V ion source (SCIEX, Brugg, Switzerland). Analyst software version 1.6.2 (SCIEX, Brugg, Switzerland) was used for data acquisition and analysis.

LC was performed with a Mercury Synergi 4 µm Polar RP trapping column (20 × 2.0 mm) and a Kinetex 2.6 µm C8 analytical column (50 × 2.1 mm), both obtained from Phenomenex (Torrance, CA, USA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. A 5-µL aliquot of the prepared sample was loaded onto the trapping column with a 500-µL/min flow of 50% B and by dilution via a T-union with a 300-µL/min flow of mobile phase A. After 1 min, the trapped analytes were eluted in backflush mode to the analytical column by running a gradient from 30 to 97.5% B over 6.5 min at a flow rate of 300 µL/min. After an isocratic hold for 2.5 min, the columns were re-equilibrated for 2 min, resulting in a total runtime of 12 min.

Mass spectrometric data were acquired in positive electrospray ionization and selected reaction monitoring mode, with an ion spray voltage of 3500 V and an ion source temperature of 650 °C. Measured transitions were THC/CBD, m/z 315.2 → 139.1*, 315.2 → 123.0; THC-d_3/CBD-d_3, m/z 318.2 → 196.3*, 318.2 → 123.0; 11-OH-THC, m/z 331.2 → 313.2*, 331.2 → 193.2; 11-OH-THC-d_3, m/z 334.2 → 316.2, 334.2 → 196.2; THCCOOH m/z 345.2 → 327.3*, 345.2 → 299.2; THCCOOH-d_3, m/z 348.2 → 330.2, 348.2 → 302.2. Transitions marked with an asterisk were used for quantification.

Normalization of cannabinoid concentrations in urine

In order to correct for dilution of the urine and thus to facilitate result interpretation, the analyte concentrations were normalized to the creatinine content of the urine by means of the following formula [22]:

$$\text{Conc.}_{\text{CR normalized}} = \text{Conc.}_{\text{Sample}} * \frac{\text{CR}_{\text{Ref.}}}{\text{CR}_{\text{Sample}}}$$

$\text{Conc.}_{\text{CR normalized}}$ being the creatinine-normalized concentration (µg/L); $\text{Conc.}_{\text{Sample}}$ the analyte concentration (µg/L); $\text{CR}_{\text{Ref.}}$ the creatinine reference concentration, defined as 100 mg/dL; and $\text{CR}_{\text{Sample}}$ the creatinine concentration in the sample (mg/dL), measured spectrophotometrically by Jaffé's reaction on the AU480 analyzer during qualitative screening of the urine samples. Details of the creatinine determination are reported elsewhere [23].

Results

Colorimetric on-site test kit

Several confiscated samples containing dried or fresh cannabis flowers have been tested with the colorimetric on-site kit. The typical coloration difference of the solution between THC-rich/CBD-poor and THC-poor/CBD-rich cannabis is seen in Fig. 1.

CBD and THC concentrations of confiscated cannabis samples

In a first step, the data was simply examined on a scatterplot showing the concentration pairs of total THC and total CBD. This allowed the observation of three quite distinctive groups, which could be classified by means of the ratio of total THC/total CBD. Samples with a ratio ≥ 3 were classified as THC-rich/CBD-poor cannabis, whereas samples with a ratio ≤ 0.33

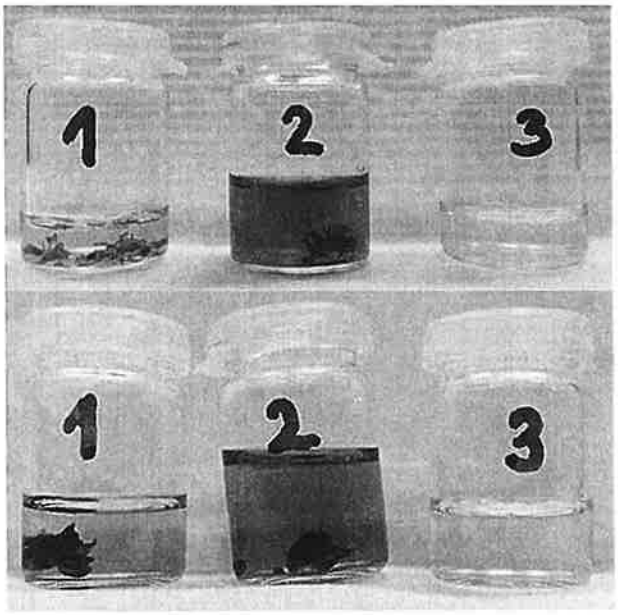

Fig. 1 Colorimetric on-site test (vial 1: cannabis sample with solution 1; vial 2: cannabis sample with solutions 1 + 2; vial 3: solution 1 as reference). Blue coloration (center top) indicates THC-rich cannabis; pink coloration (center bottom) indicates CBD-rich cannabis

were defined as THC-poor/CBD-rich cannabis. The majority of the confiscated samples ($n = 294$, 55%) could be classified as THC-rich/CBD-poor cannabis, whereas 39% of the samples ($n = 205$) were categorized as THC-poor/CBD-rich cannabis. A small number of samples ($n = 32$, 6%) showed a "mixed" cannabinoid profile with a total THC/total CBD ratio between 0.33 and 3 (Fig. 2).

When comparing the intragroup variability (Fig. 3), it can be noticed that the total THC concentrations of the confiscated THC-rich/CBD-poor cannabis samples ranged from 6.7 to 13.0% (interquartile range), with a median of 10.3%. Among the CBD-rich cannabis samples were flowers with up to 24.5% total CBD (25% CBD-acid, 2.5% free CBD, 0.66% total THC), the median concentration, however, is 8.5%. The total THC concentrations in this group are between 0 and 1.7%, with a median value of 0.3% and an interquartile range of 0.2 to 0.5%, concluding that the majority of the CBD-rich samples were below 1% in total THC, and thus legal according to the Swiss law (Fig. 3).

A short Internet research concerning the availability of CBD-cannabis on the Swiss market (Top 10 Google results with keywords "CBD hemp online") revealed that the median CBD concentration is 17%, with an interquartile range of 14.4 to 20.6%, and a maximum of 29.5%. The THC concentrations vary between 0.6 and 0.9% (interquartile range) and show a median of 0.7%.

Determination of cannabinoid concentrations in the CBD-rich cannabis flowers used in the smoking study

The CBD-rich cannabis flowers used in the smoking experiments were analyzed quantitatively for the major cannabinoids in duplicate by HPLC (Fig. 4, Table 1).

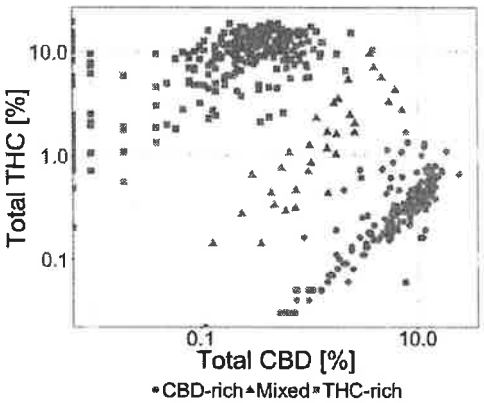

Fig. 2 Distribution of confiscated cannabis samples according to their total THC and CBD concentrations (logarithmic axis)

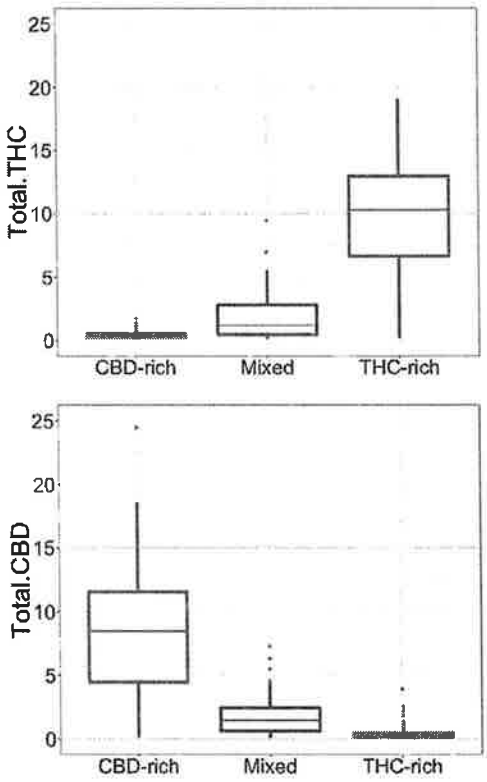

Fig. 3 Box plots showing the intragroup variability of the total THC and total CBD concentrations

Cannabinoid concentrations found in urine and whole blood after smoking CBD-rich cannabis

The applied immunochemical assays yielded negative results for all urine samples collected throughout the study. Quantitative LC-MS/MS analysis confirmed that urinary THCCOOH concentrations were below the LLOQ and thereby clearly below the 50 μg/L cut-off of the immunochemical assays (Table 2). Total CBD, THC, 11-OH-THC, and THCCOOH concentrations in urine were in the range of 7.87–148.79 μg/L, 0–1.34 μg/L, < 1–6.70 μg/L, and < 5–15.71 μg/L, respectively (Table 2). Whole blood cannabinoid concentrations are detailed in Table 3.

Free CBD blood concentrations reached a maximum of 23.4 μg/L after smoking one cigarette, 47.6 μg/L after smoking four cigarettes within 1 h, and 105 μg/L after smoking four cigarettes within 30 min. THC blood concentrations reached a maximum of 1.4 μg/L, 2.1 μg/L, and 6.8 μg/L, respectively. 11-OH-THC was undetectable in all samples (LOD = 0.5 μg/L), and total THCCOOH was only detectable in one sample of session B, but below the LLOQ (< 5 μg/L). Total CBD concentrations were in the range of 16.8–148.0 μg/L (Table 3).

When evaluating the molar ratios of CBD-glucuronide to free CBD in whole blood, it can be noticed that the ratio changes over time. Immediately after smoking, the ratio is

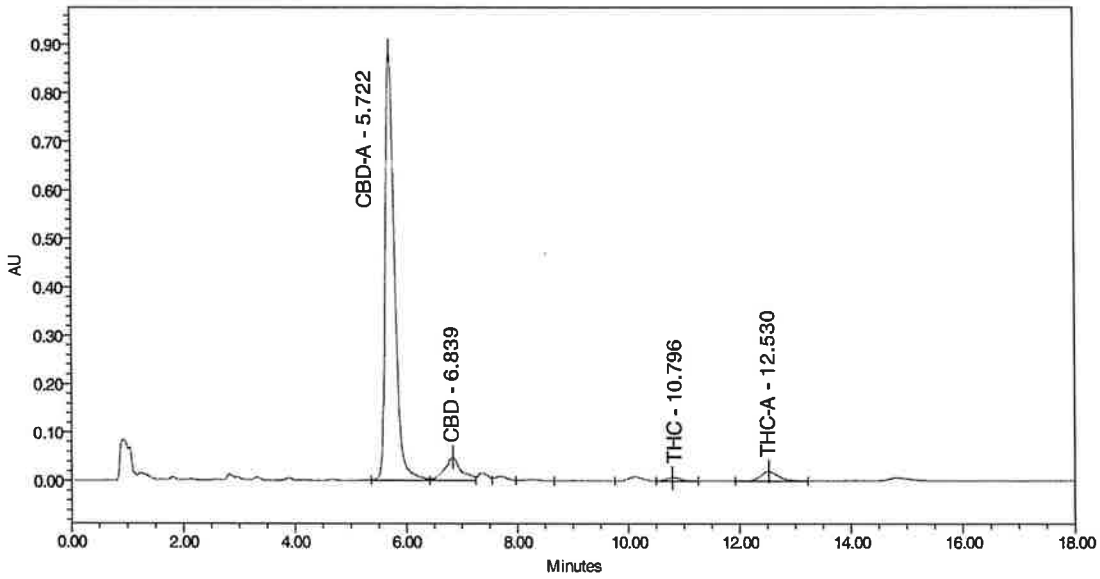

Fig. 4 HPLC-UV chromatogram of a CBD-rich cannabis sample. Detection at 210 nm (AU = absorption units). The compounds elute in the following order: CBD-acid (t_R: 5.72 min), CBD (t_R: 6.84 min), CBN (not detected, expected t_R: 9.02 min), THC (t_R: 10.80 min), THC-acid A (t_R: 12.53 min)

between 0.5 and 1.0, thus mostly in favor of free CBD and up to equality, which is reasonable for the absorption and early distribution phases. As time passes, CBD undergoes glucuronidation and thus the ratio increases (Table 4).

Discussion

Colorimetric on-site test kit and CBD and THC concentrations of confiscated cannabis samples

In Switzerland, the market for legal CBD-rich/THC-poor cannabis expanded rapidly over the past year. According to Swiss Federal Customs Administrations, only five companies were registered at the beginning of 2017, whereas now there are already more than 500 (personal communication). The availability and the consumption of these products are still a widely debated issue in the media and have posed a challenge for public health and especially to the judicial authorities, the police, and forensic experts/toxicologists, as CBD-rich/THC-poor cannabis cannot be differentiated morphologically and/or olfactorily from THC-rich/CBD-poor cannabis. Prior to the development of the new on-site test, the distinction was only possible by laborious analytical methods (mostly GC- or LC-based techniques), which resulted in high financial and administrative expenditures. This pre-test allows the police forces to directly conduct an analysis and take a decision on-site within a very short time and thus to greatly reduce these costs.

Table 1 Cannabinoid concentrations of CBD-rich flowers used in the smoking experiment

CBD	0.45%	THC	0.05%
CBD-acid	9.20%	THC-acid A	0.43%
CBD total	8.52%	THC total	0.43%

Such pre-tests have been successfully applied to distinguish between CBD-rich and THC-rich cannabis samples, whose CBD concentrations are significantly higher than the THC concentrations or vice versa. However, plants with similar concentrations of CBD and THC and therefore a total THC/total CBD ratio between 0.33 and 3 might pose problems when using the pre-test. Experiments with such material ($n = 15$) showed that there is a higher possibility of false negative results (26%). These false negative results indicated a pink coloration after 2 min but changed to a blue coloration after two more minutes, showing the correct result. It is supposed that the test needs more time to stabilize if the THC and CBD concentrations are similar. It is also possible that there is no color change at all, this happened with three samples containing very low concentrations (0.14–0.31% total THC and 0.36–0.75% total CBD). For future use of this pre-test, it is advised to wait at least 4 min prior to reading out the result. With regard to applicability, 1 mL of 4-aminophenol seems to be too little, depending on the sample size. Therefore, it was decided to add solution 1 until the sample was completely immerged.

Even if the coloration of the sample indicates CBD-rich cannabis, this does not automatically imply that it is legal cannabis according to the Swiss narcotics regulations. The total THC concentration needs to be less than 1%, which is verified through quantification of cannabinoids by an HPLC- or GC-based method. In most of the confiscated CBD-rich

Table 2 Urinary cannabinoid concentrations from sessions A–C (creatinine normalized). Total cannabinoid concentrations (free and glucuronidated) were obtained by hydrolysis with β-glucuronidase

	Time (hh:min)	THC total (μg/L)	11-OH-THC total (μg/L)	THCCOOH total (μg/L)	CBD free (μg/L)	CBD total (μg/L)	CBD gluc. (μg/L)
Session A				Cigarette 1			
	01:02	< LLOQ	< LLOQ	< LLOQ	n.d.	36.9	57.5
	12:55	n.d.	< LLOQ	< LLOQ	n.d.	15.4	24.0
Session B				Cigarettes 1–4			
	01:22	1.3	4.8	< LLOQ	1.3	148.8	230.2
	07:44	n.d.	6.7	15.7	< LLOQ	25.0	39.0
	20:00	n.d.	3.3	7.2	n.d.	14.6	22.8
	27:15	n.d.	1.8	5.6	n.d.	7.9	12.3
Session C				Cigarettes 1–4			
	22:22	n.d.	1.2	6.2	n.d.	8.6	13.3

cannabis samples—with a total THC/total CBD ratio < 0.33—the THC concentrations were below this limit. However, 4% of the samples ($n = 8$) showed THC concentrations between 1 and 1.7%, rendering them illegal. Nevertheless, these samples would probably have withstood the on-site test, since the CBD concentrations of all samples were at least 4.5 times higher than their corresponding THC concentrations. For future validation purposes, the minimal concentration ratio between CBD and THC should be determined, for which the test still gives a clear coloration.

Regarding the intragroup variability of the total THC and CBD concentrations in the confiscated cannabis samples, it can be noticed that the median and distribution of total THC concentrations in THC-rich/CBD-poor cannabis is in

Table 3 Whole blood cannabinoid concentrations from sessions A–C. Total cannabinoid concentrations (free and glucuronidated) were obtained by hydrolysis with β-glucuronidase

	Time (hh:min)	THC free (μg/L)	11-OH-THC free (μg/L)	THCCOOH total (μg/L)	CBD free (μg/L)	CBD total (μg/L)	CBD gluc. (μg/L)
Session A				Cigarette 1			
	00:16	1.4	n.d.	n.d.	23.4	35.2	18.4
	00:33	< LLOQ	n.d.	n.d.	18.0	29.7	18.3
	00:47	< LLOQ	n.d.	n.d.	9.8	20.3	16.3
	01:08	n.d.	n.d.	n.d.	6.9	16.8	15.5
Session B	00:00	n.d.	n.d.	n.d.	n.d.	n.d.	
	00:15	1.3	n.d.	Cigarette 1 n.d.	21.2	42.6	33.4
	00:31	2.0	n.d.	Cigarette 2 n.d.	34.1	85.4	80.0
	00:47	2.1	n.d.	Cigarette 3 n.d.	45.4	95.5	78.2
	01:01	2.0	n.d.	Cigarette 4 < LLOQ	47.6	122.0	116.1
Session C	00:00	n.d.	n.d.	n.d.	n.d.	n.d.	
	00:18	5.9	n.d.	Cigarette 1 Cigarette 2 n.d. Cigarette 3	64.7	90.2	39.8
	00:35	6.8	n.d.	Cigarette 4 n.d.	105.0	148.0	67.1
	00:51	2.2	n.d.	n.d.	49.4	120.0	110.1

Table 4 Molar ratios of CBD-glucuronide/free CBD in whole blood from sessions A–C

	Time (hh:min)	CBD free (nmol/L)	CBD gluc. (nmol/L)	CBD gluc./CBD free
Session A			Cigarette 1	
	00:16	74.4	37.5	0.5
	00:33	57.2	37.2	0.6
	00:47	31.3	33.3	1.1
	01:08	21.9	31.5	1.4
Session B	00:00	n.d.	n.d.	
			Cigarette 1	
	00:15	67.4	68.0	1.0
			Cigarette 2	
	00:31	108.4	163.1	1.5
			Cigarette 3	
	00:47	144.4	159.3	1.1
			Cigarette 4	
	01:01	151.4	236.6	1.6
Session C	00:00	n.d.	n.d.	
			Cigarette 1	
			Cigarette 2	
	00:18	205.7	81.1	0.4
			Cigarette 3	
			Cigarette 4	
	00:35	333.9	136.7	0.4
	00:51	157.1	224.5	1.4

accordance with data of the THC concentration values 2017 published in the narcotics statistics by the Swiss Society of Forensic Medicine [24].

When comparing the total CBD concentrations of the confiscated CBD-rich/THC-poor cannabis samples with the concentrations reported for CBD-cannabis available on the Swiss market, it becomes apparent that the median concentration offered (17%) is more than twice as high as the measured median concentrations in the seized material. The same applies to the THC concentrations. Even when including a measurement uncertainty, this still does not explain these differences. Some producers maintain a dedicated laboratory for the analysis of their samples, others use external laboratories. Differences in sample preparation (sampling, grinding, extraction method, etc.) certainly contribute to this discrepancy. Therefore, in our opinion, it is important to develop and introduce norms and standards for the analysis of cannabis samples.

Cannabinoid concentrations found in urine and whole blood after smoking CBD-rich cannabis

According to the producer, the cannabis flowers "Alessia," which were used in this study for the smoking experiments, contain between 7 and 17% total CBD. Our analysis showed clear separations of all major cannabinoids and a total CBD concentration of 8.52%, which lies within the producer's declaration, but at the lower end. The wide range of concentration given by the producer not only indicates uncertainty of measurement in the laboratory but probably also variance within the cultivation of the plants and the resultant products themselves. The main concerns, however, are the trace amounts of total THC (in this case 0.43%) present in the legal CBD-rich cannabis. The question arises whether the consumption might cause THC blood levels ≥ 2.2 µg/L (Swiss legal limit 1.5 µg/L, +30% confidence interval), and thus render the consumer unable to drive from a legal point of view.

Therefore, as a pilot experiment, one volunteer consumed one single and up to four cigarettes of legal CBD-cannabis in a rather short time (less than 1 h), simulating an acute use. In general, the estimation of the CBD and THC dose administered by smoking is very variable. Uncontrollable factors, such as differing efficiency of inhalation and losses by side-stream smoke and pyrolysis, have to be taken into account. It has been demonstrated that the bioavailability of THC ranges between 10 and 50% and that of CBD between 11 and 45%. Furthermore, pyrolytic destruction and side-stream smoke account for losses up to 30 and 50%, respectively [25]. In the present study, the cannabis cigarettes contained 42.7 mg CBD and 2.2 mg THC, which resulted in approximately 29.9 mg CBD and 1.5 mg THC released by smoking and an estimated inhaled dose of 14.9 mg CBD and 0.8 mg THC. Taking into

account the average bioavailability, 4.5 mg CBD and 0.2 mg were systemically available.

Urinary cannabinoid concentrations showed only trace amounts of THC (1.3 μg/L) 1 h after session B (four cigarettes in 1 h). It can be assumed that the urine in session C (four cigarettes in 30 min) would also have been positive if a sample had been taken within 1 h after the end of consumption. In both sessions, no THC was detected anymore after 20 h. Regarding THC metabolites, traces of 11-OH-THC and THCCOOH have been detected in all three sessions, but were only above the LLOQ (1 μg/L and 5 μg/L, respectively) in sessions B and C. However, as revealed by the highly sensitive LC-MS/MS method, all measured THCCOOH concentrations were well below the 50 μg/L cut-off for the applied immunochemical assays, which explains the negative results for all urine samples. The manufacturers do not provide any data on the cross-reactivity with THCOOH-glucuronide. The endogenous metabolite is (−)-*trans*-THCCOOH-glucuronide [26]. Commercially available THCCOOH-glucuronide reference standards are stereochemically different and are therefore not suitable for testing cross-reactivity. However, based on studies with other immunoassays, it can be assumed that cross-reactivity is extensive [27].CBD was predominantly detected as its glucuronide metabolite in all sessions, ranging from 12.3 to 230.2 μg/L. These concentrations were calculated from the difference between total and free CBD and corrected by a factor of 1.56, the quotient between the molar mass of CBD-glucuronide (490.59 g/mol) and CBD (314.46 g/mol). Regarding the free CBD and CBD-glucuronide concentrations, it can be noticed that only in two samples in session B free CBD was detected, of which one was over the LLOQ (1.3 μg/L after 1 h and 22 min). However, it can be assumed that a sample taken at a similar time in session C would also have been positive. Literature on the urinary excretion profile of CBD in humans after smoking is very scarce and has only been reported in one case. It showed that the two major compounds were nonmetabolized CBD (12.1%) and its O-glucuronide (13.3%) [28, 29]. These findings do not correspond to our results, but this may be related to the fact that the subject in this study was chronically treated with daily doses of 600 mg CBD over an unknown period of time. It is also conceivable that sample collection, storage, and processing conditions may be a reason for the differences. Furthermore, a small study has been conducted to investigate the impact of enzymatic and alkaline hydrolysis on CBD concentration in urine. Single intakes of 400 mg CBD showed traces (< LLOQ) of free CBD in urine, but mainly hydrolyzed CBD [30]. A comparison of concentrations, however, remains difficult as CBD was administered orally and the dose was around ten times higher. Other pharmacokinetic studies also showed that CBD, like THC, is subject to a significant first-pass effect; yet, unlike THC, is excreted to a large extent unchanged in the feces [31, 32]. Very recently, the Institute of Forensic Medicine Basel published a similar pilot study with CBD-cannabis [33]. However, in their study, the test person was a nonsmoker and consumed only one or two cigarettes over a 6-h time period. The CBD concentrations in urine after smoking one single CBD cigarette are in good agreement with the results of our pilot study. In addition, we detected THC in two urine samples and the THC metabolites 11-OH-THC and THCCOOH in all samples after smoking multiple CBD cigarettes within 1 h or 30 min.

Analyses of whole blood samples showed detectable THC concentrations in all three smoking sessions. In session C (smoking four cigarettes within 30 min), the THC concentrations exceeded the legal limit for driving under the influence of cannabis according to the Swiss Road traffic regulations. After consumption of two cigarettes within 15 min, the THC concentrations amounted to 5.9 μg/L, respectively to 6.8 μg/L after two additional cigarettes. Both values are clearly over the legal limit of 2.2 μg/L. The concentration dropped to 2.2 μg/L 20 min after smoking the last cigarette, and it can be assumed that it decreased below the legal limit within a few minutes after. It has to be considered that the cannabinoid concentration in the smoked CBD-cannabis flowers is consistent with the median concentration of the confiscated CBD-cannabis samples. However, the reported cannabinoid concentrations of CBD-cannabis available on the Swiss market are highly variable. Therefore, it could be possible to exceed the legal limit already after smoking one cigarette if the THC content of the CBD-rich cannabis and/or the quantities smoked are higher. In addition, other factors like the time taken to smoke a cigarette, puff duration, inhaled smoke volume, and breath-holding after inhalation have to be taken into account [34]. Furthermore, in session B, it can be observed that no considerable accumulation of THC occurs in whole blood. The THC concentrations after the second cigarette remain constant between 1.96 and 2.1 μg/L. This is consistent with observations in pharmacokinetic studies, showing that THC concentrations after smoking decrease by more than 50% within 15 min of reaching the maximum concentration and/or the last inhalation [25, 35–37]. However, accumulation can occur in the fat tissue, as THC is highly lipophilic [32]. It is possible to compare the THC concentrations obtained in this smoking study with concentrations measured in regular smoking studies with THC-rich cannabis, e.g., by Fabritius et al. [37], by comparing the consumed amount of cannabis and the resulting THC concentrations in whole blood. The smoked quantity of THC in the CBD-rich cigarettes was approximately 2.2 mg, compared to about 77 mg in the THC-rich cigarettes in the study by Fabritius et al. [37], which is roughly 3%. Taking into account the factors that can influence the whole blood cannabinoids concentrations (as discussed

above), the measured THC concentrations are fairly consistent and well comparable (1.4 μg/L versus ~40 μg/L after 15 min). Regarding the THC metabolites, 11-OH-THC was undetectable in all samples (LOD = 0.5 μg/L), and THCCOOH was only detectable in one sample of session B, but below the LLOQ.

CBD was detected in its free form and as its glucuronide metabolite in concentrations of 6.9 to 105.0 μg/L and 15.5 to 116.1 μg/L, respectively. Although the metabolism of CBD is well known, very few studies on the blood profile of CBD in humans after smoking are available. Ohlsson et al. conducted a study with deuterium-labeled CBD, which showed mean CBD concentrations in plasma of 32.6 μg/L 15 min after smoking [38]. This is equivalent to a whole blood concentration of 21.8 μg/L, taking into account a plasma/whole blood ratio of 1.5 [35, 39]. Since the quantity of CBD smoked in our study was about twice as high as in the study of Ohlsson et al. [38], our CBD concentration of 23.4 μg/L seems rather low. This discrepancy might be due to interindividual differences in the glucuronidation and elimination rate as well as in the smoking behavior. The recently published pilot study by the Institute of Forensic Medicine Basel also showed higher maximal concentrations of CBD and THC after smoking one cigarette [33]. This can be explained by the faster sampling time (10 min compared to 16 min) after the consumption. Otherwise, the results are in good agreement with our study.

Concerning the molar ratios of CBD-glucuronide to free CBD and the hydrolysis of glucuronide conjugates in general, the degree of glucuronide cleavage should be discussed. In-source fragmentation of the glucuronide conjugates of THC, 11-OH-THC, THCCOOH, and CBD during electrospray ionization leads to the appearance of an additional, earlier eluting peak in the extracted ion chromatogram of the respective unconjugated analyte. Since these chromatographic peaks could not be detected in the extracted ion chromatograms of the hydrolyzed samples, enzymatic hydrolysis of all glucuronide conjugates was assumed to be complete.

Limitations

As interindividual variation in the smoking behavior and the cannabinoid metabolism is very high, the major limitation of this pilot study is that it involved just one participant. This makes the interpretation of results and the comparison with other studies challenging. To overcome these problems, future studies should include a higher number of participants and a longer sampling period. In addition, a test procedure to assess the influence of CBD on fitness to drive should be developed. The current legal situation of THC is based on a "zero tolerance" system using cut-off values. However, in our opinion, it is problematic to make statements about the level of impairment based only on substance concentrations.

Conclusions

Smoking experiments with legal CBD-rich/THC-poor cannabis with one volunteer showed that the legal limit of 2.2 μg/L can be exceeded when smoking multiple cigarettes within a certain time. The consumption of one cigarette only led to a THC concentration of 1.4 μg/L in whole blood. However, various factors, such as interindividual differences in smoking behavior and metabolism or cannabis potency, have to be taken into consideration as well. Therefore, and because possible impairing effects of CBD have not yet been investigated in detail, people should generally be advised to refrain from driving for at least 1 h after smoking CBD-cannabis.

Based on the analysis of a large collective of confiscated cannabis samples, we propose the following classification: plant material with a ratio of total THC/total CBD ≥ 3 is graded as THC-rich/CBD-poor, whereas samples with a ratio ≤ 0.33 are categorized as CBD-rich/THC-poor cannabis. Ninety-four percent of the analyzed samples could be assigned to one of these two categories.

Finally, there is a need for the introduction of norms and standards for the analysis of cannabis samples in the near future, as potency testing and quality control of legal tobacco substitute products will gain more importance. Our analyses showed fairly large discrepancies between the concentrations stated by the producers and those measured in the laboratories. These can be attributed, among other factors, to different sample preparation methods (sampling, grinding, extraction method, etc.). In addition, analyses have shown that the newly introduced on-site test kit for differentiation of CBD- and THC-cannabis is very promising, but needs further validation.

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Compliance with ethical standards

According to the ethics committee of the Canton of Bern, Switzerland, this study does not apply to Art. 3a of the Federal Act on Research involving Human Beings (Human Research Act) since it is a study only involving one participant—who was the principal investigator himself in a self-experiment. Therefore, they did not decide on the application listed with the project ID No. 2018-01037. However, written informed consent was obtained from the participant of the cannabis smoking study.

All procedures performed were in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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Cannabis Typification Test

The morphological and olfactory characteristics of so-called "industrial hemp" (containing predominantly CBD) and "drug hemp" (containing predominantly THC) are so similar as to make them indistinguishable in the field.

The Zurich Forensic Science Institute (Switzerland) has developed a chemical typification test allowing police in the field a quick and easy method of differentiation between the two types of cannabis.

From August to October 2017, a field test study with the Cannabis Typification Test was carried out by police forces in the Zurich area.

In a second phase, the study was extended to other law enforcement bodies in Switzerland.

Over 300 tests were performed on dried cannabis material during this period of time, enabling feedback on ease of handling and reliability of the testing kit and comprehensibility of the manual.

At the end of 2017 after some minor optimization to improve readability of the manual and durability of the testing kit, police forces in the Zurich area officially started to use the Cannabis Typification Test.

The test set consists of a clear transparent plastic bag (approx. 3 x 6 cm) containing a pair of disposable tweezers and two small glass ampules with the chemical reagents.

Small portions of cannabis samples can be placed inside the plastic bag by using the tweezers. After reclosing the bag, the two ampules are crushed by squeezing them with the fingertips, thus allowing the chemical reagents to react with the sample within approx. two minutes.

A reddish color of the test liquid indicates «industrial hemp» (containing predominantly CBD).

A bluish color indicates «drug hemp» (containing predominantly THC).

For any questions regarding the handling and reliability of the Cannabis Typification Test, please contact the Zurich Forensic Science Institute (info@for-zh.ch).

For purchasing the Cannabis Typification Test, please contact www.ca-re.ch.

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ORIGINAL ARTICLE

Cannabinoid concentrations in confiscated cannabis samples and in whole blood and urine after smoking CBD-rich cannabis as a "tobacco substitute"

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Abstract
In Switzerland, only cannabis with a total Δ^9-tetrahydrocannabinol (THC) content higher than 1% is controlled by the narcotics legislation. Cannabis products rich in cannabidiol (CBD) and low in THC can be legally sold as tobacco substitutes. In this paper, we address analytical and forensic toxicological issues related to the increasing availability and consumption of these products. Based on the analysis of 531 confiscated cannabis samples, we could establish classification thresholds: plant material with a ratio of total THC/total CBD ≥ 3 is graded as THC-rich/CBD-poor, whereas samples with a ratio ≤ 0.33 are categorized as CBD-rich/THC-poor cannabis. We also evaluated an on-site test kit as a rapid alternative to the laborious liquid or gas chromatography (LC or GC)-based techniques normally used for the differentiation between THC- and CBD-cannabis. Furthermore, we determined whole blood and urine cannabinoid levels after smoking different doses of legal CBD-cannabis. A male volunteer smoked one cigarette within 15 min and four cigarettes within 1 h and within 30 min, respectively. Cigarettes contained on average 42.7 mg CBD and 2.2 mg THC. Blood samples were collected up to 1.1 h and urine samples up to 27.3 h after the beginning of smoking. All urine samples tested negative by three immunochemical assays for detection of cannabis use. This is an important finding for abstinence monitoring. However, we found that the trace amounts of THC present in CBD-cannabis can produce THC blood levels above the Swiss legal limit for driving, and thus render the consumer unable to drive from a legal point of view.

Keywords Tetrahydrocannabinol · Cannabidiol · Classification · Drug of abuse testing · Driving while impaired

Introduction

For centuries, cannabis has been cultivated as a source of fiber, food, and oil, as a medicinal plant as well as for recreational purposes [1]. To date, well over 400 constituents have been identified in cannabis, of which at least 70 are phytocannabinoids, a group of 21 terpenophenolic compounds to which the majority of the plant's biological activities have been ascribed [2]. Δ^9-tetrahydrocannabinol (THC) is generally accepted to be the compound responsible for the psychoactive properties of cannabis inducing euphoria, altered sensory perception and relaxation, also known as the "high" that recreational cannabis users seek. Additionally, THC has been found to have a number of therapeutic attributes including anti-inflammatory, antiemetic, appetite stimulant, analgesic, and antispasmodic effects [3]. However, THC has also been associated with a number of adverse effects, including tachycardia, anxiety, cognitive deficits, paranoia, as well as with an increased risk of developing chronic psychosis and dependence [4–7]. Since several years, other cannabinoids have come into the focus of research and legislation, the most prominent being cannabidiol (CBD), which is non-euphoriant and is thought to modulate the psychoactivity of THC, thus alleviating the adverse effects of THC. Furthermore, CBD has shown promising therapeutic activity per se, such as anti-inflammatory, anticonvulsive, anxiolytic, analgesic, neuroprotective, anticancer, and antioxidant effects [8, 9].

Marianne Hädener and Tim J. Gelmi contributed equally to this work.

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The cannabinoid composition of the cannabis plant material, i.e., the ratio of the different cannabinoids, is primarily determined by genetic factors (strain type), but is also affected by environmental and harvesting conditions (e.g., growing and storage conditions, state of maturity at harvest) [10]. Over the last few decades, recreational users seeking the ultimate "high" have selectively bred cannabis plants to produce highest THC and lowest CBD levels and until recent years, little effort has been made to breed plants with high CBD levels as there was no demand among recreational cannabis users. However, due to the growing interest in the therapeutic effects of CBD, cannabis breeders have been working to create new strains 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 bred [11]. The total CBD and total THC content (in % of dry weight) corresponds to the sum of the free cannabinoid and its pharmacologically inactive carboxylic acid precursor (CBD-acid and THC-acid A, respectively), corrected for weight of the carboxylic acid groups. Decarboxylation of the acids into their active neutral forms occurs spontaneously as the plant ages and, to a much larger extent, upon heating (e.g., upon smoking, vaporizing, or baking the plant material) [12].

In the European Union, cannabis plants containing no more than 0.2% total THC and producing no psychoactive effects can be legally cultivated for fiber and seeds production. In Switzerland, only plant material with a total THC content of 1% or higher is controlled by the narcotics legislation [13]. CBD-rich/THC-poor cannabis products have been on the Swiss legal market as tobacco substitutes for more than a year and their consumption has recently become increasingly popular, for both therapeutic and recreational purposes.

Since 2017, our laboratory has analyzed over 800 cannabis samples confiscated by Swiss police forces by a fully validated high-performance liquid chromatography-diode array detector (HPLC-DAD) method for determination of the cannabinoid profile [14]. In this work, we will give a representative overview on the concentrations of CBD and THC in the confiscated material and we will propose a ratio of total THC/total CBD to discriminate between CBD- and THC-cannabis on an analytical level. Furthermore, we will evaluate the performance of a recently introduced on-site test kit [15] for the rapid distinction between CBD- and THC-cannabis.

As strategy to prevent cannabis-impaired driving, Switzerland, among several other European countries [16], has adopted a zero tolerance law that prohibits driving with any detectable amount of psychoactive THC in the blood. The detection of THC in blood is deemed proof of the driver's acute impairment and inability to drive. According to the directives of the Federal Roads Office an analytical cut-off of 1.5 μg/L is employed for the reliable detection of THC in whole blood. In forensic practice, a confidence interval of 30% is taken into consideration, resulting in an effective cut-off of 2.2 μg/L (2.2 μg/L minus 30% is 1.54 μg/L, which is just higher than the analytical cut-off of 1.5 μg/L). Since CBD-rich cannabis products contain trace amounts of THC, the question arises whether their consumption might cause THC blood levels ≥2.2 μg/L, and thus render the consumer unable to drive from a legal point of view. Another analytical question commonly raised is whether smoking legal CBD-rich cannabis can produce urinary cannabinoid levels high enough to result in positive findings in immunological urine drug tests. Urine drug screening by immunochemical assays is often performed in cases of suspected drug-impaired driving, for workplace drug testing, and to monitor abstinence from drug use [17, 18]. Antibodies used in urinary immunochemical tests for detection of cannabis use primarily target the THC metabolite 11-nor-9-carboxy-THC (THCCOOH) and have variable degrees of cross-reactivity with other metabolites and cannabinoids [19].

Herein, we attempt to answer the aforementioned questions by reporting whole blood and urine cannabinoid levels measured in a male subject after smoking different doses of CBD-rich/THC-poor cannabis flowers.

Material and methods

Chemicals and reagents

Dried CBD-rich/THC-poor cannabis flowers (*Cannabis sativa* L.) were obtained from Swiss Cannabis SA (Härkingen, Switzerland). Acetonitrile (HPLC gradient grade, 99.9%) was purchased from Acros Organics (Geel, Belgium) and methanol (absolute, HPLC grade) from Biosolve (Dieuze, France). Butyl acetate (HPLC grade, 99.7%), formic acid solution (puriss p.a., 50% in water), and *E. coli*-type IX-A β-glucuronidase were obtained from Sigma-Aldrich (Buchs, Switzerland). Potassium dihydrogen phosphate (puriss p.a., ACS), di-sodium hydrogen phosphate dihydrate (analytical grade), and *H. pomatia* β-glucuronidase/arylsulfatase were acquired from Merck (Darmstadt, Germany). Deionized water was prepared in-house with a Direct-Q water purification system from Millipore (Zug, Switzerland). THC, 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), and CBD reference standards as well as their trideuterated analogs were purchased from Cerilliant (Round Rock, TX, USA). THC-acid A, CBD-acid, and cannabinol (CBN) reference standards were obtained from Lipomed (Arlesheim, Switzerland). Blank human blood and urine samples were obtained from the blood donor center in Bern, Switzerland and donated by a volunteer, respectively, and were tested for the absence of cannabinoids prior to use.

Colorimetric on-site test kit for differentiation of CBD- versus THC-cannabis

For differentiation of CBD- versus THC-cannabis, a liquid colorimetric test can be used by police forces on the street and on indoor or outdoor cultivation sites, or as preliminary tests in the laboratories. A commercial test kit is already in use by several Swiss police forces [15]. The kit contains two solutions: solution 1, consisting of 0.2 mg/mL 4-aminophenol in ethanol/isopropanol (95:5; *v/v*), which was acidified with 0.5% hydrochloric acid (HCl 2 N); solution 2, consisting of 30 mg/mL sodium hydroxide in ethanol/water (70:30; *v/v*).

To conduct the differentiation, a small sample of dried or fresh cannabis plant material is transferred into a glass vial and 1–2 mL of solution 1 (depending on sample size) and four drops of solution 2 are added. The vial is closed, shaken vigorously and left to stand for 2 min to allow reaction. Subsequently, the color of the solution is visually evaluated: a blue coloration indicates THC-rich cannabis; a pink coloration indicates CBD-rich cannabis. For quantitative determination of cannabinoids, the HPLC-DAD method, which is described hereafter, is used.

HPLC-analysis of cannabis products for determination of THC, THC-acid A, CBD, CBD-acid, and CBN

The HPLC-DAD method for the analysis of cannabis products has already been published [14] and has recently been updated by adding CBD-acid to the calibrators and the control samples, in the same concentrations as THC-acid A.

Data treatment and evaluation of confiscated cannabis samples

Since January 2017, 808 confiscated samples have been analyzed for cannabinoids at our institute. For the evaluation of the CBD and THC content, only samples containing dried or fresh cannabis flowers were considered. Samples consisting of (burnt) joint material, plant material without flowers, hashish, or oil were excluded. Furthermore, 119 samples were analyzed before CBD-acid had been included in the method and were therefore also excluded as no re-analysis was possible. Finally, 531 confiscated samples have been evaluated with respect to CBD and THC concentrations. Data evaluation and visualization were conducted using R version 3.5.1 [20].

Study design for the smoking experiment

A healthy male subject (42 years, 75 kg, 183 cm; regular tobacco cigarette smoker) participated in three experimental sessions, separated by 3 weeks, in which he smoked either one cigarette ad libitum for up to 15 min (session A); four cigarettes within 1 h (session B); or four cigarettes within 30 min (session C) containing 0.5 g of a 1:1 mixture of CBD-rich cannabis flowers and tobacco. The smoked amounts of CBD and THC averaged about 42.7 and 2.2 mg, respectively per cigarette. Whole blood samples were collected between cigarettes and up to 1.1 h and urine samples up to 27.3 h after the beginning of smoking.

Qualitative analysis of urine samples

Qualitative screening by homogeneous enzyme immunoassay was performed with 300 μL of urine on an AU480 analyzer (Beckman Coulter, Nyon, Switzerland) using the Immunalysis HEIA Cannabinoids kit (Pomona, CA, USA) according to the manufacturer's instructions. Furthermore, all urine samples were qualitatively analyzed for cannabinoids using the THC lateral flow immunoassay test and automatic P.I.A.^{2} readout system from Protzek (Lörrach, Germany) as well as with the Drug-Screen-Multi 12Z dip test from Nal von Minden (Moers, Germany). All three immunochemical assays were calibrated with the THC metabolite THCCOOH (50 μg/L cut-off for THCCOOH).

Quantitative analysis of blood and urine samples

Sample preparation

Two hundred microliters of whole blood and urine samples were spiked with 20 μL of internal standard solution (2 ng THC-d_3 and 11-OH-THC-d_3, 10 ng THCCOOH-d_3, 2 ng CBD-d_3) and processed by protein precipitation with 600 μL of acetonitrile for determination of free cannabinoid concentrations or by β-glucuronidase treatment followed by liquid-liquid extraction with 1 mL of butyl acetate for determination of total cannabinoid concentrations (free and glucuronidated). For hydrolysis of glucuronide conjugates of THC, 11-OH-THC, and THCCOOH, 10 μL of *H. pomatia* β-glucuronidase/arylsulfatase and 200 μL of 66 mM phosphate buffer pH 6 were added, and the samples were incubated at 47 °C for 2 h. Hydrolysis of CBD-glucuronide was achieved by adding 200 μL of 75 mM phosphate buffer pH 6.8 containing *E. coli*-type IX-A β-glucuronidase at a concentration of 5000 U/mL and incubating at 37 °C for 2.5 h. After mixing and centrifugation, the organic phases (acetonitrile/butyl acetate) were evaporated to dryness at 50 °C under nitrogen and reconstituted in 200 μL of acetonitrile/water/formic acid, 60:40:0.1; *v/v/v*. Five microliters of the prepared samples were injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

Calibration samples were prepared by addition of reference standard solutions to blank blood and blank urine, respectively, and were processed by protein precipitation as described above. Linear ranges in blood and urine were 0.5–20 μg/L for THC and 11-OH-THC, 2.5–100 μg/L for THCCOOH, and 1–

250 µg/L for CBD. Lower limits of quantification (LLOQ) were 1 µg/L for THC and 11-OH-THC, 5 µg/L for THCCOOH, and 1 µg/L for CBD. Limits of detection (LOD) were 0.5 µg/L for THC and 11-OH-THC, 2 µg/L for THCCOOH, and 0.1 µg/L for CBD.

LC-MS/MS instrumentation

Cannabinoid concentrations were determined using our published column-switching LC-MS/MS method [21] with some modifications. The LC-MS/MS consisted of an UltiMate 3000 HPLC system (Dionex, Olten, Switzerland) coupled to a 4500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer with a Turbo V ion source (SCIEX, Brugg, Switzerland). Analyst software version 1.6.2 (SCIEX, Brugg, Switzerland) was used for data acquisition and analysis.

LC was performed with a Mercury Synergi 4 µm Polar RP trapping column (20 × 2.0 mm) and a Kinetex 2.6 µm C8 analytical column (50 × 2.1 mm), both obtained from Phenomenex (Torrance, CA, USA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. A 5-µL aliquot of the prepared sample was loaded onto the trapping column with a 500-µL/min flow of 50% B and by dilution via a T-union with a 300-µL/min flow of mobile phase A. After 1 min, the trapped analytes were eluted in backflush mode to the analytical column by running a gradient from 30 to 97.5% B over 6.5 min at a flow rate of 300 µL/min. After an isocratic hold for 2.5 min, the columns were re-equilibrated for 2 min, resulting in a total runtime of 12 min.

Mass spectrometric data were acquired in positive electrospray ionization and selected reaction monitoring mode, with an ion spray voltage of 3500 V and an ion source temperature of 650 °C. Measured transitions were THC/CBD, m/z 315.2 → 139.1*, 315.2 → 123.0; THC-d_3/CBD-d_3, m/z 318.2 → 196.3*, 318.2 → 123.0; 11-OH-THC, m/z 331.2 → 313.2*, 331.2 → 193.2; 11-OH-THC-d_3, m/z 334.2 → 316.2, 334.2 → 196.2; THCCOOH m/z 345.2 → 327.3*, 345.2 → 299.2; THCCOOH-d_3, m/z 348.2 → 330.2, 348.2 → 302.2. Transitions marked with an asterisk were used for quantification.

Normalization of cannabinoid concentrations in urine

In order to correct for dilution of the urine and thus to facilitate result interpretation, the analyte concentrations were normalized to the creatinine content of the urine by means of the following formula [22]:

$$\text{Conc.}_{\text{CR normalized}} = \text{Conc.}_{\text{Sample}} * \frac{\text{CR}_{\text{Ref.}}}{\text{CR}_{\text{Sample}}}$$

$\text{Conc.}_{\text{CR normalized}}$ being the creatinine-normalized concentration (µg/L); $\text{Conc.}_{\text{Sample}}$ the analyte concentration (µg/L); $\text{CR}_{\text{Ref.}}$ the creatinine reference concentration, defined as 100 mg/dL; and $\text{CR}_{\text{Sample}}$ the creatinine concentration in the sample (mg/dL), measured spectrophotometrically by Jaffé's reaction on the AU480 analyzer during qualitative screening of the urine samples. Details of the creatinine determination are reported elsewhere [23].

Results

Colorimetric on-site test kit

Several confiscated samples containing dried or fresh cannabis flowers have been tested with the colorimetric on-site kit. The typical coloration difference of the solution between THC-rich/CBD-poor and THC-poor/CBD-rich cannabis is seen in Fig. 1.

CBD and THC concentrations of confiscated cannabis samples

In a first step, the data was simply examined on a scatterplot showing the concentration pairs of total THC and total CBD. This allowed the observation of three quite distinctive groups, which could be classified by means of the ratio of total THC/total CBD. Samples with a ratio ≥ 3 were classified as THC-rich/CBD-poor cannabis, whereas samples with a ratio ≤ 0.33

Fig. 1 Colorimetric on-site test (vial 1: cannabis sample with solution 1; vial 2: cannabis sample with solutions 1 + 2; vial 3: solution 1 as reference). Blue coloration (center top) indicates THC-rich cannabis; pink coloration (center bottom) indicates CBD-rich cannabis

were defined as THC-poor/CBD-rich cannabis. The majority of the confiscated samples ($n = 294$, 55%) could be classified as THC-rich/CBD-poor cannabis, whereas 39% of the samples ($n = 205$) were categorized as THC-poor/CBD-rich cannabis. A small number of samples ($n = 32$, 6%) showed a "mixed" cannabinoid profile with a total THC/total CBD ratio between 0.33 and 3 (Fig. 2).

When comparing the intragroup variability (Fig. 3), it can be noticed that the total THC concentrations of the confiscated THC-rich/CBD-poor cannabis samples ranged from 6.7 to 13.0% (interquartile range), with a median of 10.3%. Among the CBD-rich cannabis samples were flowers with up to 24.5% total CBD (25% CBD-acid, 2.5% free CBD, 0.66% total THC), the median concentration, however, is 8.5%. The total THC concentrations in this group are between 0 and 1.7%, with a median value of 0.3% and an interquartile range of 0.2 to 0.5%, concluding that the majority of the CBD-rich samples were below 1% in total THC, and thus legal according to the Swiss law (Fig. 3).

A short Internet research concerning the availability of CBD-cannabis on the Swiss market (Top 10 Google results with keywords "CBD hemp online") revealed that the median CBD concentration is 17%, with an interquartile range of 14.4 to 20.6%, and a maximum of 29.5%. The THC concentrations vary between 0.6 and 0.9% (interquartile range) and show a median of 0.7%.

Determination of cannabinoid concentrations in the CBD-rich cannabis flowers used in the smoking study

The CBD-rich cannabis flowers used in the smoking experiments were analyzed quantitatively for the major cannabinoids in duplicate by HPLC (Fig. 4, Table 1).

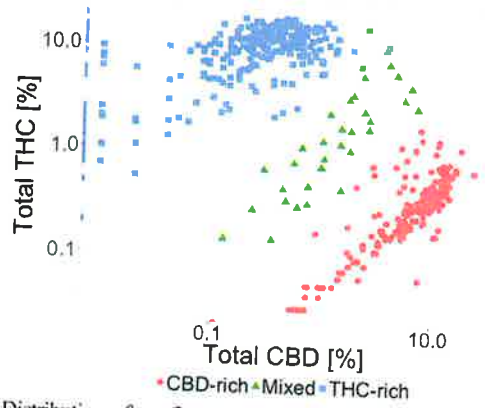

Fig. 2 Distribution of confiscated cannabis samples according to their total THC and CBD concentrations (logarithmic axis)

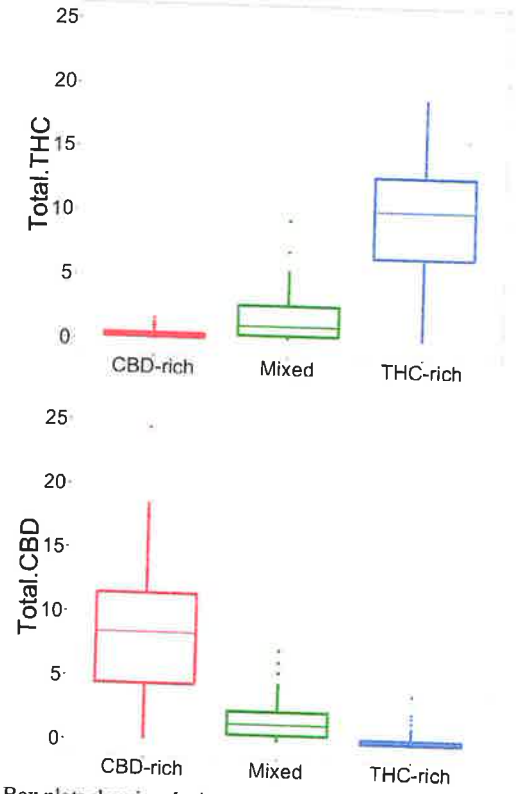

Fig. 3 Box plots showing the intragroup variability of the total THC and total CBD concentrations

Cannabinoid concentrations found in urine and whole blood after smoking CBD-rich cannabis

The applied immunochemical assays yielded negative results for all urine samples collected throughout the study. Quantitative LC-MS/MS analysis confirmed that urinary THCCOOH concentrations were below the LLOQ and thereby clearly below the 50 µg/L cut-off of the immunochemical assays (Table 2). Total CBD, THC, 11-OH-THC, and THCCOOH concentrations in urine were in the range of 7.87–148.79 µg/L, 0–1.34 µg/L, < 1–6.70 µg/L, and < 5–15.71 µg/L, respectively (Table 2). Whole blood cannabinoid concentrations are detailed in Table 3.

Free CBD blood concentrations reached a maximum of 23.4 µg/L after smoking one cigarette, 47.6 µg/L after smoking four cigarettes within 1 h, and 105 µg/L after smoking four cigarettes within 30 min. THC blood concentrations reached a maximum of 1.4 µg/L, 2.1 µg/L, and 6.8 µg/L, respectively. 11-OH-THC was undetectable in all samples (LOD = 0.5 µg/L), and total THCCOOH was only detectable in one sample of session B, but below the LLOQ (< 5 µg/L). Total CBD concentrations were in the range of 16.8–148.0 µg/L (Table 3).

When evaluating the molar ratios of CBD-glucuronide to free CBD in whole blood, it can be noticed that the ratio changes over time. Immediately after smoking, the ratio is

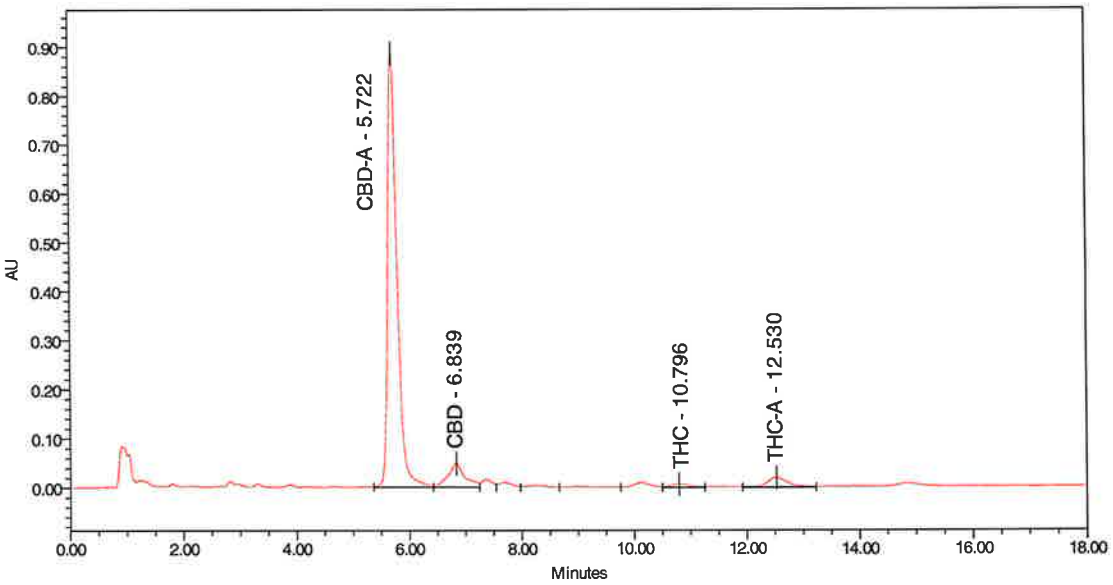

Fig. 4 HPLC-UV chromatogram of a CBD-rich cannabis sample. Detection at 210 nm (AU = absorption units). The compounds elute in the following order: CBD-acid (t_R: 5.72 min), CBD (t_R: 6.84 min), CBN (not detected, expected t_R: 9.02 min), THC (t_R: 10.80 min), THC-acid A (t_R: 12.53 min)

between 0.5 and 1.0, thus mostly in favor of free CBD and up to equality, which is reasonable for the absorption and early distribution phases. As time passes, CBD undergoes glucuronidation and thus the ratio increases (Table 4).

Discussion

Colorimetric on-site test kit and CBD and THC concentrations of confiscated cannabis samples

In Switzerland, the market for legal CBD-rich/THC-poor cannabis expanded rapidly over the past year. According to Swiss Federal Customs Administrations, only five companies were registered at the beginning of 2017, whereas now there are already more than 500 (personal communication). The availability and the consumption of these products are still a widely debated issue in the media and have posed a challenge for public health and especially to the judicial authorities, the police, and forensic experts/toxicologists, as CBD-rich/THC-poor cannabis cannot be differentiated morphologically and/or olfactorily from THC-rich/CBD-poor cannabis. Prior to the development of the new on-site test, the distinction was only possible by laborious analytical methods (mostly GC- or LC-based techniques), which resulted in high financial and administrative expenditures. This pre-test allows the police forces to directly conduct an analysis and take a decision on-site within a very short time and thus to greatly reduce these costs.

Table 1 Cannabinoid concentrations of CBD-rich flowers used in the smoking experiment

CBD	0.45%	THC	0.05%
CBD-acid	9.20%	THC-acid A	0.43%
CBD total	8.52%	THC total	0.43%

Such pre-tests have been successfully applied to distinguish between CBD-rich and THC-rich cannabis samples, whose CBD concentrations are significantly higher than the THC concentrations or vice versa. However, plants with similar concentrations of CBD and THC and therefore a total THC/total CBD ratio between 0.33 and 3 might pose problems when using the pre-test. Experiments with such material ($n = 15$) showed that there is a higher possibility of false negative results (26%). These false negative results indicated a pink coloration after 2 min but changed to a blue coloration after two more minutes, showing the correct result. It is supposed that the test needs more time to stabilize if the THC and CBD concentrations are similar. It is also possible that there is no color change at all, this happened with three samples containing very low concentrations (0.14–0.31% total THC and 0.36–0.75% total CBD). For future use of this pre-test, it is advised to wait at least 4 min prior to reading out the result. With regard to applicability, 1 mL of 4-aminophenol seems to be too little, depending on the sample size. Therefore, it was decided to add solution 1 until the sample was completely immerged.

Even if the coloration of the sample indicates CBD-rich cannabis, this does not automatically imply that it is legal cannabis according to the Swiss narcotics regulations. The total THC concentration needs to be less than 1%, which is verified through quantification of cannabinoids by an HPLC- or GC-based method. In most of the confiscated CBD-rich

Table 2 Urinary cannabinoid concentrations from sessions A–C (creatinine normalized). Total cannabinoid concentrations (free and glucuronidated) were obtained by hydrolysis with β-glucuronidase

	Time (hh:min)	THC total (μg/L)	11-OH-THC total (μg/L)	THCCOOH total (μg/L)	CBD free (μg/L)	CBD total (μg/L)	CBD gluc. (μg/L)
Session A				Cigarette 1			
	01:02	< LLOQ	< LLOQ	< LLOQ	n.d.	36.9	57.5
	12:55	n.d.	< LLOQ	< LLOQ	n.d.	15.4	24.0
Session B				Cigarettes 1–4			
	01:22	1.3	4.8	< LLOQ	1.3	148.8	230.2
	07:44	n.d.	6.7	15.7	< LLOQ	25.0	39.0
	20:00	n.d.	3.3	7.2	n.d.	14.6	22.8
	27:15	n.d.	1.8	5.6	n.d.	7.9	12.3
Session C				Cigarettes 1–4			
	22:22	n.d.	1.2	6.2	n.d.	8.6	13.3

cannabis samples—with a total THC/total CBD ratio < 0.33—the THC concentrations were below this limit. However, 4% of the samples ($n = 8$) showed THC concentrations between 1 and 1.7%, rendering them illegal. Nevertheless, these samples would probably have withstood the on-site test, since the CBD concentrations of all samples were at least 4.5 times higher than their corresponding THC concentrations. For future validation purposes, the minimal concentration ratio between CBD and THC should be determined, for which the test still gives a clear coloration.

Regarding the intragroup variability of the total THC and CBD concentrations in the confiscated cannabis samples, it can be noticed that the median and distribution of total THC concentrations in THC-rich/CBD-poor cannabis is in

Table 3 Whole blood cannabinoid concentrations from sessions A–C. Total cannabinoid concentrations (free and glucuronidated) were obtained by hydrolysis with β-glucuronidase

	Time (hh:min)	THC free (μg/L)	11-OH-THC free (μg/L)	THCCOOH total (μg/L)	CBD free (μg/L)	CBD total (μg/L)	CBD gluc. (μg/L)
Session A				Cigarette 1			
	00:16	1.4	n.d.	n.d.	23.4	35.2	18.4
	00:33	< LLOQ	n.d.	n.d.	18.0	29.7	18.3
	00:47	< LLOQ	n.d.	n.d.	9.8	20.3	16.3
	01:08	n.d.	n.d.	n.d.	6.9	16.8	15.5
Session B	00:00	n.d.	n.d.	n.d.	n.d.	n.d.	
				Cigarette 1			
	00:15	1.3	n.d.	n.d.	21.2	42.6	33.4
				Cigarette 2			
	00:31	2.0	n.d.	n.d.	34.1	85.4	80.0
				Cigarette 3			
	00:47	2.1	n.d.	n.d.	45.4	95.5	78.2
				Cigarette 4			
	01:01	2.0	n.d.	< LLOQ	47.6	122.0	116.1
Session C	00:00	n.d.	n.d.	n.d.	n.d.	n.d.	
				Cigarette 1			
				Cigarette 2			
	00:18	5.9	n.d.	n.d.	64.7	90.2	39.8
				Cigarette 3			
				Cigarette 4			
	00:35	6.8	n.d.	n.d.	105.0	148.0	67.1
	00:51	2.2	n.d.	n.d.	49.4	120.0	110.1

Table 4 Molar ratios of CBD-glucuronide/free CBD in whole blood from sessions A–C

	Time (hh:min)	CBD free (nmol/L)	CBD gluc. (nmol/L)	CBD gluc./CBD free
Session A			Cigarette 1	
	00:16	74.4	37.5	0.5
	00:33	57.2	37.2	0.6
	00:47	31.3	33.3	1.1
	01:08	21.9	31.5	1.4
Session B	00:00	n.d.	n.d.	
			Cigarette 1	
	00:15	67.4	68.0	1.0
			Cigarette 2	
	00:31	108.4	163.1	1.5
			Cigarette 3	
	00:47	144.4	159.3	1.1
			Cigarette 4	
	01:01	151.4	236.6	1.6
Session C	00:00	n.d.	n.d.	
			Cigarette 1	
			Cigarette 2	
	00:18	205.7	81.1	0.4
			Cigarette 3	
			Cigarette 4	
	00:35	333.9	136.7	0.4
	00:51	157.1	224.5	1.4

accordance with data of the THC concentration values 2017 published in the narcotics statistics by the Swiss Society of Forensic Medicine [24].

When comparing the total CBD concentrations of the confiscated CBD-rich/THC-poor cannabis samples with the concentrations reported for CBD-cannabis available on the Swiss market, it becomes apparent that the median concentration offered (17%) is more than twice as high as the measured median concentrations in the seized material. The same applies to the THC concentrations. Even when including a measurement uncertainty, this still does not explain these differences. Some producers maintain a dedicated laboratory for the analysis of their samples, others use external laboratories. Differences in sample preparation (sampling, grinding, extraction method, etc.) certainly contribute to this discrepancy. Therefore, in our opinion, it is important to develop and introduce norms and standards for the analysis of cannabis samples.

Cannabinoid concentrations found in urine and whole blood after smoking CBD-rich cannabis

According to the producer, the cannabis flowers "Alessia," which were used in this study for the smoking experiments, contain between 7 and 17% total CBD. Our analysis showed clear separations of all major cannabinoids and a total CBD concentration of 8.52%, which lies within the producer's declaration, but at the lower end. The wide range of concentration given by the producer not only indicates uncertainty of measurement in the laboratory but probably also variance within the cultivation of the plants and the resultant products themselves. The main concerns, however, are the trace amounts of total THC (in this case 0.43%) present in the legal CBD-rich cannabis. The question arises whether the consumption might cause THC blood levels ≥ 2.2 µg/L (Swiss legal limit 1.5 µg/L, +30% confidence interval), and thus render the consumer unable to drive from a legal point of view.

Therefore, as a pilot experiment, one volunteer consumed one single and up to four cigarettes of legal CBD-cannabis in a rather short time (less than 1 h), simulating an acute use. In general, the estimation of the CBD and THC dose administered by smoking is very variable. Uncontrollable factors, such as differing efficiency of inhalation and losses by side-stream smoke and pyrolysis, have to be taken into account. It has been demonstrated that the bioavailability of THC ranges between 10 and 50% and that of CBD between 11 and 45%. Furthermore, pyrolytic destruction and side-stream smoke account for losses up to 30 and 50%, respectively [25]. In the present study, the cannabis cigarettes contained 42.7 mg CBD and 2.2 mg THC, which resulted in approximately 29.9 mg CBD and 1.5 mg THC released by smoking and an estimated inhaled dose of 14.9 mg CBD and 0.8 mg THC. Taking into

account the average bioavailability, 4.5 mg CBD and 0.2 mg were systemically available.

Urinary cannabinoid concentrations showed only trace amounts of THC (1.3 μg/L) 1 h after session B (four cigarettes in 1 h). It can be assumed that the urine in session C (four cigarettes in 30 min) would also have been positive if a sample had been taken within 1 h after the end of consumption. In both sessions, no THC was detected anymore after 20 h. Regarding THC metabolites, traces of 11-OH-THC and THCCOOH have been detected in all three sessions, but were only above the LLOQ (1 μg/L and 5 μg/L, respectively) in sessions B and C. However, as revealed by the highly sensitive LC-MS/MS method, all measured THCCOOH concentrations were well below the 50 μg/L cut-off for the applied immunochemical assays, which explains the negative results for all urine samples. The manufacturers do not provide any data on the cross-reactivity with THCOOH-glucuronide. The endogenous metabolite is (−)-*trans*-THCCOOH-glucuronide [26]. Commercially available THCCOOH-glucuronide reference standards are stereochemically different and are therefore not suitable for testing cross-reactivity. However, based on studies with other immunoassays, it can be assumed that cross-reactivity is extensive [27].CBD was predominantly detected as its glucuronide metabolite in all sessions, ranging from 12.3 to 230.2 μg/L. These concentrations were calculated from the difference between total and free CBD and corrected by a factor of 1.56, the quotient between the molar mass of CBD-glucuronide (490.59 g/mol) and CBD (314.46 g/mol). Regarding the free CBD and CBD-glucuronide concentrations, it can be noticed that only in two samples in session B free CBD was detected, of which one was over the LLOQ (1.3 μg/L after 1 h and 22 min). However, it can be assumed that a sample taken at a similar time in session C would also have been positive. Literature on the urinary excretion profile of CBD in humans after smoking is very scarce and has only been reported in one case. It showed that the two major compounds were nonmetabolized CBD (12.1%) and its O-glucuronide (13.3%) [28, 29]. These findings do not correspond to our results, but this may be related to the fact that the subject in this study was chronically treated with daily doses of 600 mg CBD over an unknown period of time. It is also conceivable that sample collection, storage, and processing conditions may be a reason for the differences. Furthermore, a small study has been conducted to investigate the impact of enzymatic and alkaline hydrolysis on CBD concentration in urine. Single intakes of 400 mg CBD showed traces (< LLOQ) of free CBD in urine, but mainly hydrolyzed CBD [30]. A comparison of concentrations, however, remains difficult as CBD was administered orally and the dose was around ten times higher. Other pharmacokinetic studies also showed that CBD, like THC, is subject to a significant first-pass effect; yet, unlike THC, is excreted to a large extent unchanged in the feces [31, 32]. Very recently, the Institute of Forensic Medicine Basel published a similar pilot study with CBD-cannabis [33]. However, in their study, the test person was a nonsmoker and consumed only one or two cigarettes over a 6-h time period. The CBD concentrations in urine after smoking one single CBD cigarette are in good agreement with the results of our pilot study. In addition, we detected THC in two urine samples and the THC metabolites 11-OH-THC and THCCOOH in all samples after smoking multiple CBD cigarettes within 1 h or 30 min.

Analyses of whole blood samples showed detectable THC concentrations in all three smoking sessions. In session C (smoking four cigarettes within 30 min), the THC concentrations exceeded the legal limit for driving under the influence of cannabis according to the Swiss Road traffic regulations. After consumption of two cigarettes within 15 min, the THC concentrations amounted to 5.9 μg/L, respectively to 6.8 μg/L after two additional cigarettes. Both values are clearly over the legal limit of 2.2 μg/L. The concentration dropped to 2.2 μg/L 20 min after smoking the last cigarette, and it can be assumed that it decreased below the legal limit within a few minutes after. It has to be considered that the cannabinoid concentration in the smoked CBD-cannabis flowers is consistent with the median concentration of the confiscated CBD-cannabis samples. However, the reported cannabinoid concentrations of CBD-cannabis available on the Swiss market are highly variable. Therefore, it could be possible to exceed the legal limit already after smoking one cigarette if the THC content of the CBD-rich cannabis and/or the quantities smoked are higher. In addition, other factors like the time taken to smoke a cigarette, puff duration, inhaled smoke volume, and breath-holding after inhalation have to be taken into account [34]. Furthermore, in session B, it can be observed that no considerable accumulation of THC occurs in whole blood. The THC concentrations after the second cigarette remain constant between 1.96 and 2.1 μg/L. This is consistent with observations in pharmacokinetic studies, showing that THC concentrations after smoking decrease by more than 50% within 15 min of reaching the maximum concentration and/or the last inhalation [25, 35–37]. However, accumulation can occur in the fat tissue, as THC is highly lipophilic [32]. It is possible to compare the THC concentrations obtained in this smoking study with concentrations measured in regular smoking studies with THC-rich cannabis, e.g., by Fabritius et al. [37], by comparing the consumed amount of cannabis and the resulting THC concentrations in whole blood. The smoked quantity of THC in the CBD-rich cigarettes was approximately 2.2 mg, compared to about 77 mg in the THC-rich cigarettes in the study by Fabritius et al. [37], which is roughly 3%. Taking into account the factors that can influence the whole blood cannabinoids concentrations (as discussed

above), the measured THC concentrations are fairly consistent and well comparable (1.4 μg/L versus ~40 μg/L after 15 min). Regarding the THC metabolites, 11-OH-THC was undetectable in all samples (LOD = 0.5 μg/L), and THCCOOH was only detectable in one sample of session B, but below the LLOQ.

CBD was detected in its free form and as its glucuronide metabolite in concentrations of 6.9 to 105.0 μg/L and 15.5 to 116.1 μg/L, respectively. Although the metabolism of CBD is well known, very few studies on the blood profile of CBD in humans after smoking are available. Ohlsson et al. conducted a study with deuterium-labeled CBD, which showed mean CBD concentrations in plasma of 32.6 μg/L 15 min after smoking [38]. This is equivalent to a whole blood concentration of 21.8 μg/L, taking into account a plasma/whole blood ratio of 1.5 [35, 39]. Since the quantity of CBD smoked in our study was about twice as high as in the study of Ohlsson et al. [38], our CBD concentration of 23.4 μg/L seems rather low. This discrepancy might be due to interindividual differences in the glucuronidation and elimination rate as well as in the smoking behavior. The recently published pilot study by the Institute of Forensic Medicine Basel also showed higher maximal concentrations of CBD and THC after smoking one cigarette [33]. This can be explained by the faster sampling time (10 min compared to 16 min) after the consumption. Otherwise, the results are in good agreement with our study.

Concerning the molar ratios of CBD-glucuronide to free CBD and the hydrolysis of glucuronide conjugates in general, the degree of glucuronide cleavage should be discussed. In-source fragmentation of the glucuronide conjugates of THC, 11-OH-THC, THCCOOH, and CBD during electrospray ionization leads to the appearance of an additional, earlier eluting peak in the extracted ion chromatogram of the respective unconjugated analyte. Since these chromatographic peaks could not be detected in the extracted ion chromatograms of the hydrolyzed samples, enzymatic hydrolysis of all glucuronide conjugates was assumed to be complete.

Limitations

As interindividual variation in the smoking behavior and the cannabinoid metabolism is very high, the major limitation of this pilot study is that it involved just one participant. This makes the interpretation of results and the comparison with other studies challenging. To overcome these problems, future studies should include a higher number of participants and a longer sampling period. In addition, a test procedure to assess the influence of CBD on fitness to drive should be developed. The current legal situation of THC is based on a "zero tolerance" system using cut-off values. However, in our opinion, it is problematic to make statements about the level of impairment based only on substance concentrations.

Conclusions

Smoking experiments with legal CBD-rich/THC-poor cannabis with one volunteer showed that the legal limit of 2.2 μg/L can be exceeded when smoking multiple cigarettes within a certain time. The consumption of one cigarette only led to a THC concentration of 1.4 μg/L in whole blood. However, various factors, such as interindividual differences in smoking behavior and metabolism or cannabis potency, have to be taken into consideration as well. Therefore, and because possible impairing effects of CBD have not yet been investigated in detail, people should generally be advised to refrain from driving for at least 1 h after smoking CBD-cannabis.

Based on the analysis of a large collective of confiscated cannabis samples, we propose the following classification: plant material with a ratio of total THC/total CBD ≥ 3 is graded as THC-rich/CBD-poor, whereas samples with a ratio ≤ 0.33 are categorized as CBD-rich/THC-poor cannabis. Ninety-four percent of the analyzed samples could be assigned to one of these two categories.

Finally, there is a need for the introduction of norms and standards for the analysis of cannabis samples in the near future, as potency testing and quality control of legal tobacco substitute products will gain more importance. Our analyses showed fairly large discrepancies between the concentrations stated by the producers and those measured in the laboratories. These can be attributed, among other factors, to different sample preparation methods (sampling, grinding, extraction method, etc.). In addition, analyses have shown that the newly introduced on-site test kit for differentiation of CBD- and THC-cannabis is very promising, but needs further validation.

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Compliance with ethical standards

According to the ethics committee of the Canton of Bern, Switzerland, this study does not apply to Art. 3a of the Federal Act on Research involving Human Beings (Human Research Act) since it is a study only involving one participant—who was the principal investigator himself in a self-experiment. Therefore, they did not decide on the application listed with the project ID No. 2018-01037. However, written informed consent was obtained from the participant of the cannabis smoking study.

All procedures performed were in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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XI. Competency Tests:

Performing the 4-AP color test does not differ from other color tests performed in the Forensic Chemistry unit. The amount sampled, the amount of reagents used, the time frame in which observations should be made, and vortexing the mixture are all similar to other color test. However, analyst should be trained in the subtleties and specifics of the 4-AP color test. It will also be prudent to document and demonstrate that the analyst correctly interpreted their observations for positive and negative samples. Correct identification of the color associated to hemp and marihuana is critical to the appropriate use of the color test reagents. Therefore, before use in casework the analyst must perform and pass a competency test as well as be trained in the specifics of the 4-aminophenol color test. The results of the competency test will remain in this validation study.

4-AP Color Test Competency Test Answer Key
Prepared by Brett Trotter on 7/26/2019

Item #	Identifier	Expected color
OO1	Parsley	Clear / Yellow
OO2	Marijuana - 132-006906	Blue / Green
OO3	Hemp - C1820094	Pink / Red

Date: 7-31-19
Trainer: Glenn Everett
Trainee: Laura Cole

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information			
Reagent 1	Reagent #1	4-AP	Expiration	10-8-19
Reagent 2	Reagent #2	4-AP	Expiration	10-8-19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	No Change	N/A	Pass ME
Unknown 1	No Change	001 Negative	Pass ME
Unknown 2	Blue	002 Marijuana	Pass ME
Unknown 3	Pink	003 Hemp	Pass ME

Laura Cole has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Laura Cole	7/31/19
Trainer	Glenn B. Everett	7-31-19
Supervisor	Glenn B. Everett	7-31-19
Technical Leader	Glenn B. Everett	7-31-19

Date: 7/26/19
Trainer: Brett Trotter
Trainee: Jennifer Sullivan

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	exp 10/8/19
Reagent 2	exp 10/8/19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	no change	N/A	Pass - BT
Unknown 1	no change	001	Pass - BT
Unknown 2	blue-marijuana (indicates)	002	Pass - BT
Unknown 3	pink-hemp (indicates)	003	Pass - BT

Jennifer Sullivan has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Jennifer Sullivan	7/26/19
Trainer	Brett Trotter	7/26/19
Supervisor	Kim B. Everett	7-26-19
Technical Leader	Kim B. Everett	7-26-19

Date: 7-31-19
Trainer: Glenn Everett
Trainee: Cassandra Franklin Beavers

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	4-AP Reagent #1 Expiration 10-8-19
Reagent 2	4-AP Reagent #2 Expiration 10-8-19

Color Test Results:

	Color produced	Sample ID	Pass/Fail	
Blank	NCD-(a)	N/A	Pass	MEC
Unknown 1	NCD -	001	NCSD Pass	MEC
Unknown 2	+ blue	002	Marijuana Pass	MEC
Unknown 3	+ rose	003	Hemp Pass	MEC

Cassandra Franklin Beavers has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Cassandra F. Beavers	7/31/19
Trainer	Glenn B. Everett	7-31-19
Supervisor	Glenn B. Everett	7-31-19
Technical Leader	Glenn B. Everett	7-31-19

Date: July 29, 2019
Trainer: SA/FS Brett Trotter
Trainee: SA/FS John Scott, Jr.

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	10/8/19
Reagent 2	10/8/19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	yellow - Neg.	N/A	Pass BT
Unknown 1	yellowish - Incons.	001	Pass BT
Unknown 2	Blue - THC	002	Pass BT
Unknown 3	Pink - CBD	003	Pass BT

John Scott has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	John Scott Jr.	9/8/29/19 7/29/19
Trainer	Brett Trotter	7/29/19
Supervisor	Glen B. Everett	7-30-19
Technical Leader	Glen B. Everett	7-30-19

Date: 7-31-19
Trainer: Glenn Everett
Trainee: Glen Glenn

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	4-AP Reagent #1 Expiration 10-8-19
Reagent 2	4-AP Reagent #2 Expiration 10-8-19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	NCO	N/A	Pass JDE
Unknown 1	NCO	001 Negative	Pass JDE
Unknown 2	Blue	002 Marijuana	Pass JDE
Unknown 3	Pink	003 Hemp	Pass JDE

Glen Jay Glenn has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee		7-31-19
Trainer	Glen B. Everett	7-31-19
Supervisor	Glen B. Everett	7-31-19
Technical Leader	Glen B. Everett	7-31-19

Date: 7-31-19
Trainer: Glenn Everett
Trainee: Ben Hubert

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	4-AP Reagent #1 expiration 10-8-19
Reagent 2	4-AP Reagent #2 expiration 10-8-19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	No color change	N/A	pass JDE
Unknown 1	No color change	001 Negative	pass JDE
Unknown 2	Blue	002 Marijuana	pass JDE
Unknown 3	Pink	003 Hemp	pass JDE

Ben Hubert has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee		7/31/19
Trainer	Glenn B. Everett	7-31-19
Supervisor	Glenn B. Everett	7-31-19
Technical Leader	Glenn B. Everett	7-31-19

Date: 7/29/19
Trainer: Brett Trotter
Trainee: Laura Newberry

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	4-AP Reagent #1 exp: 10/8/19
Reagent 2	4-AP Reagent #2 exp: 10/8/19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank		N/A	Pass BT
Unknown 1	no color - negative	001	Pass BT
Unknown 2	blue - marijuana	002	Pass BT
Unknown 3	red - hemp	003	Pass BT

Laura Newberry has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Laura Newberry	7/29/19
Trainer	Brett Trotter	7/29/19
Supervisor	Kim B. Everett	7-30-19
Technical Leader	Kim B. Everett	7-30-19

Date: 7-26-19
Trainer: Brett Trotter
Trainee: Glenn Everett

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	10/8/19 Expiration
Reagent 2	10/8/19 Expiration

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	No color change	N/A	Pass - BT
Unknown 1	No color change	Negative 001	Pass - BT
Unknown 2	Blue	Marijuana 002	Pass - BT
Unknown 3	Pink	Hemp 003	Pass - BT

Glenn Everett has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Glenn B. Everett	7-26-19
Trainer	Brett Trotter	7/26/19
Supervisor	Jennifer Hall	7.26.19
Technical Leader	Glenn B. Everett	7-26-19

Date: 7/26/19
Trainer: Brett Trotter
Trainee: Ella Carpenter

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	Exp 10/8/19 BT
Reagent 2	Exp 10/8/19 BT

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	NCO	N/A	Pass - BT
Unknown 1	NCO	01 - negative Cannabis	Pass - BT
Unknown 2	blue	02 - marijuana	Pass - BT
Unknown 3	pink	03 - hemp	~~Br~~ Pass - BT BT

Ella Carpenter has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Ella Carp	7/26/19
Trainer	Brett Trotter	7/26/19
Supervisor	John B. Emmett	7-26-19
Technical Leader	John B. Emmett	7-26-19

Date: 7/26/19
Trainer: Glenn Everett
Trainee: Brett Trotter

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	10/8/19 Expiration
Reagent 2	10/8/19 Expiration

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	No Color Change	N/A	Pass BDE
Unknown 1	No Color Change	001 - Negative	Pass BDE
Unknown 2	Blue	002 - Marijuana	Pass BDE
Unknown 3	Pink	003 - Hemp	Pass BDE

Brett Trotter has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Brett Trotter	7/26/19
Trainer	Glenn B. Everett	7-26-19
Supervisor	Glenn B. Everett	7-26-19
Technical Leader	Glenn B. Everett	7-26-19

Date: 7-26-19
Trainer: Brett Trotter
Trainee: Rebecca Hernandez

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	Exp. 10/8/19
Reagent 2	Exp. 10/8/19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	No color ⊖	N/A	Pass - BT
Unknown 1	No color ⊖	001	Pass - BT
Unknown 2	Blue; Indicates Marijuana	002	Pass - BT
Unknown 3	~~Reddish~~ purple Pinkish; Indicates Hemp	003	Pass - BT

Rebecca Hernandez has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Rebecca Hernandez	7-26-19
Trainer	Brett Trotter	7-26-19
Supervisor	Kim B. Everett	7-26-19
Technical Leader	Kim B. Everett	7-26-19

Date: 7/26/19
Trainer: Brett Trotter
Trainee: Andrea King

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	10/8/19
Reagent 2	10/8/19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	Clear ⊖	N/A	Pass - BT
Unknown 1	Clear ⊖	001	Pass - BT
Unknown 2	Blue ⊕ marijuana	002	Pass - BT
Unknown 3	Pink ⊕ Hemp	003	Pass - BT

Andrea King has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Andrea King	7/26/19
Trainer	Brett Trotter	7/26/19
Supervisor	Kim B. Everett	7-26-19
Technical Leader	Kim B. Everett	7-26-19

Date: 7/26/19
Trainer: Brett Trotter
Trainee: Laura Adams

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	10/8/19
Reagent 2	10/8/19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	Clear	N/A	Pass - BT
Unknown 1	Clear	901 NCSID	Pass - BT
Unknown 2	Blue	902 CBD THC MJ	Pass - BT
Unknown 3	Magenta pink	903 H emp	Pass - BT

Laura Adams has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Laura L Adams	7/26/19
Trainer	Brett Trotter	7/26/19
Supervisor	Ann B. Everett	7-26-19
Technical Leader	Ann B. Everett	7-26-19

Date: _____

Trainer: _____

Trainee: _____

Initial Competency Test for 4-Aminophenol Color Test for Supervised Casework

Reagents used:

	ID information
Reagent 1	
Reagent 2	

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank		N/A	
Unknown 1			
Unknown 2			
Unknown 3			

_____ Is authorized to performed supervised casework using the 4-aminophenol color test.

Date

Scott _____

_____

_____

_____

Date: 7/26/19
Trainer: Brett Trotter
Trainee: Lela Jackson

Initial Competency Test for 4-Aminophenol Color Test – Trained Scientists

Reagents used:

	ID information
Reagent 1	Exp 10/8/19
Reagent 2	Exp 10/8/19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	no color change	N/A	Pass - BT
Unknown 1	no color change	001	Pass - BT
Unknown 2	blue (marijuana)	002	Pass - BT
Unknown 3	purple (Hemp)	003	Pass - BT

Lela Jackson has successfully completed the competency test for the 4-aminophenol color test.

	Signature	Date
Trainee	Lela Jackson	7/26/19
Trainer	Brett Trotter	7/26/19
Supervisor	Helen B. Eiglt	7-26-19
Technical Leader	Helen B. Eiglt	7-26-19

Date: 8-7-19
Trainer: Glenn Everett
Trainee: Scott Jenkins

Initial Competency Test for 4-Aminophenol Color Test for Supervised Casework

Reagents used:

	ID information			
Reagent 1	4-AP	Reagent #1	Expiration	10-8-19
Reagent 2	4-AP	Reagent #2	Expiration	10-8-19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	No Color Change	N/A	pass MAE
Unknown 1	No Color Change	001 Negative	pass MAE
Unknown 2	Blue	002 Marijuana	pass MAE
Unknown 3	Pink	003 Hemp	pass MAE

Scott Jenkins Is authorized to performed supervised casework using the 4-aminophenol color test.

	Signature	Date
Trainee	Scott Jenkins	8-7-19
Trainer	Glenn B. Everett	8-7-19
Supervisor	Glenn B. Everett	8-7-19
Technical Leader	Glenn B. Everett	8-7-19

Date: 8-7-19
Trainer: Glenn Everett
Trainee: Becky Stout

Initial Competency Test for 4-Aminophenol Color Test for Supervised Casework

Reagents used:

	ID information		
Reagent 1	4-AP Reagent #1	Expiration	10-8-19
Reagent 2	4-AP Reagent #2	Expiration	10-8-19

Color Test Results:

	Color produced	Sample ID	Pass/Fail
Blank	C/C	N/A	pass JME
Unknown 1	C/C	001 Negative	pass JME
Unknown 2	Blue	002 Marijuana	pass JME
Unknown 3	Pink	003 Hemp	pass JME

Becky Stout Is authorized to performed supervised casework using the 4-aminophenol color test.

	Signature	Date
Trainee	Rebecca Stout	8-7-19
Trainer	Glenn B. Everett	8-7-19
Supervisor	Michelle Clark	08/07/19
Technical Leader	Glenn B. Everett	8-7-19