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The Italian panorama of cannabis light preparation: Determination of cannabinoids by LC-UV

Michele Dei Cas[a], Eleonora Casagni[b], Anna Saccardo[b], Sebastiano Arnoldi[b], Craig Young[c], Stefano Scotti[d], Edgardo Vieira de Manicor[e], Veniero Gambaro[b], Gabriella Roda[b,*]

[a] Department of Health Sciences, Università degli Studi di Milano, Via A. di Rudinì 8, 20142, Milan, Italy
[b] Department of Pharmaceutical Sciences, Università degli Studi di Milano, Via L. Mangiagalli 25, 20133, Milan, Italy
[c] Shimadzu Scientific Instruments, 7102 Riverwood Drive, Columbia, MD, 21046, USA
[d] Shimadzu Italia, Via Giovanni Battista Cassinis, 7, 20139, Milan, Italy
[e] Ecocanapa, Via Macchi 44, 20124, Milano, Italy

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ABSTRACT

Cannabis light preparations are products derived or containing dried female inflorescences of *Cannabis sativa* belonging to Chemotype III (THC/CBD ratio <<1); the total THC (THC + THCA) content in the crop must not exceed 0.2 % in accordance with the EU regulation.

In Italy the most recent law for industrial hemp (242/2016) states that only for farmers this limit is extended to 0.6 %. On the other hand, the Ministry of the Interior published a note stating that the sale or the presence in the markets of products (inflorescences, concentrates, essences and resins) or plants with concentrations higher than 0.5 % constitutes a crime. In this confusing legislation framework, it is very important to assess the legality of hemp, determining the total amount of THC. To this end a reliable LC-UV analytical method was developed and validated taking into account parameters such as precision, accuracy, linearity, repeatability of peak area and retention time, limit of detection (LOD = 0.002 % for all cannabinoids) and limit of quantification (LOQ = 0.005 % for all cannabinoids). Accuracy was expressed as the relative error (Er%), while precision was measured as the coefficient of variation (CV%). A CV% below 3 % and Er% between ± 6 % were obtained. The linearity was proven in the concentration range 0.005–1 % for THC, THCA and CBN and 0.005 %–50 % for CBD and CBDA.

The analytical method was applied to more than nine hundred cannabis light samples.

Based on the law 242/2016, only 18 % of the crops are to be considered legal for the market (total THC < 0.2 %). If the circular of the Ministry of the Interior should be converted as a proper law, a substantial amount of cannabis light preparations (24 %) would be considered illegal (total THC > 0.5 %). On the other hand, the most of the inflorescences (58 %) have a total THC content comprised between 0.2 % and 0.5 %, and it is not clear whether these products could be sold or not. Moreover, Cannabis light products are not authorized for human consumption, even if everybody knows that this is their primary use. In conclusion, the cannabis light panorama in Italy is quite confused and more specific and clear legislation should be proposed.

1. Introduction

Cannabis sativa is an herbaceous plant rich in cannabinoids, probably more than 90, with various pharmacological activities [1]. The maximum concentration in cannabinoids can be found in the female inflorescences (about 10 % of the composition of the flowers), but they are also present in the cellular and glandular hairs on the surface of the leaves. Only a negligible amount is contained in the stem and the seeds [2]. The production of cannabinoids is greatly influenced by the climate, the cultivation conditions, the exposure to sunlight and pollination [3]. In the past the genus cannabis was classified in three main species named

Abbreviations: THC, delta-9-tetrahydrocannabinol; Δ-8-THC, delta-8-tetrahydrocannabinol; THCA, tetrahydrocannabinolic acid; CBD, cannabidiol; CBDA, cannabidiolic acid; CBN, cannabinol; CBG, cannabigerol; CBGA, cannabigerol acid; CBC, cannabichromene; CBDV, cannabidivarin; THCV, tetrahydrocannabivarin; LC-UV, liquid chromatography coupled to ultraviolet spectroscopy detection; LOQ, limit of quantification; LOD, limit of detection; Er, relative error; CV, coefficient of variation; S/N, signal to noise ratio.

* Corresponding author.
E-mail address: gabriella.roda@unimi.it (G. Roda).

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Cannabis sativa L., a fiber-type one, *Cannabis indica Lam.*, a drug-type, and *Cannabis ruderalis Janish*, with intermediate characteristics. Nowadays, due to the intense breeding process, botanists define just one species of *Cannabis sativa L.* subdivided into different chemotypes [4], based on the dry-weight ratio of THC/CBD in the plant. Three are the main chemotypes: Chemotype I: THC/CBD ratio >>1.0, Chemotype II: THC/CBD ratio close to 1.0, Chemotype III: THC/CBD ratio <<1 plants with high CBD content, also known as hemp [5].

Cannabis light preparations are referred to dried inflorescences with a concentration, established by law, lower than 0.2 % in THC [6]. The most recent regulation in this field in the Italian legislation panorama is the law 242/2016 *"Dispositions for the promotion of cultivation and supply chain of agro-industrial cannabis"* which is focused on the disposition on the cultivation of *Cannabis sativa*. It states that the total THC content in the crop must not exceed 0.2 % and in any case, not exceed 0.6 %. On 20/07/2018, the Ministry of Interior published further legislation on the matter (protocol number 2018/43586) concerning *"the legal and operational aspects related to the commercialization of hemp with low THC content and relations with the drug law"*. This note states that the limit of 0.6 % of total THC content can be applied only to the farmer who *"due to natural causes and without having in any way contributed to his conscious intervention develops a culture with concentration limits of the active ingredient higher than those allowed (0.2 %)."*

Concerning the regulatory framework, according to the circular, the limit of 0.6 % of total THC content cannot be extended to the commercial operators who sell the inflorescences, the resin-based products and the textile hemp with a concentration of active principle between 0.2 % and 0.6 %. Moreover, it was remarked (note published from the Ministry of the Interior 20/07/2018 number of protocol 2018/43586) that the sale or the presence in the markets of products (inflorescences, concentrates, essences and resins) or plants with concentrations higher than 0.5 % fall in the definition of *illicit drugs or psychotropic substances subjected to the supervision and control of the Ministry of Health* and thus their detention and commercialization constitutes a crime (n. 309/90). The note also recalled that law 242/2016 does not provide for the sale of inflorescences for personal consumption through smoking or other similar methods of use. Given the above legislation framework, it is mandatory to have an analytical method for the determination of the total THC content in the commercialized hemp inflorescences to verify their legality. The cannabinoids are usually carboxylated in plant material, and high temperature in the GC apparatus causes the degradation of the acidic forms [6,7] irreversibly. Therefore, this study aimed to take an overview of the concentration of the principal cannabinoids in cannabis light preparations by using an HPLC/UV technique which does not require any derivatization or the use of high temperature [7–15].

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetonitrile, toluene, (-)-Δ9-THC methanol solution at 1 mg/mL, CBD methanol solution at 1 mg/mL, standard solutions at 1 mg/mL in acetonitrile of THCA, CBDA, CBN (all analytical grade > 99 %) were purchased from Sigma-Aldrich (St.Louis, USA). Water (18.2 Ω cm^{-1}) was prepared using a Milli-Q System (Millipore, Darmstadt, Germany).

2.2. Extraction from plant-based preparations

Female inflorescences of industrial hemp (n = 922) were obtained from growers and retailers of several Italian growing areas from January to June 2018. They were 1) stripped of the stem, leaves and seeds, 2) ground into a mortar to reduce the size of the particles and then 3) mixed thoroughly to ensure homogeneity. Plant residues (about 50 mg) were placed in a centrifuge tube with 5 mL of methanol and vortexed three-times for about 1 min/each. Samples were centrifuged for 5 min at 4000 RPM, and the clear supernatant was withdrawn. Each vial was prepared as follows: 100 μL of supernatant, 900 μL methanol.

2.3. HPLC/UV analysis

The analytical system consisted of an HPLC/UV Prominence-*i* LC-2030C-Cannabis Analyzer for Potency (Shimadzu Corporation, Kyoto, Japan). The separation was attained on a reversed-phase Shimadzu NexLeaf CBX for Potency, 2.7 μm (150 mm x 4.6 mm) analytical column, preceded by a security guard cartridge. The linear gradient was between eluent A (water) and eluent B (acetonitrile) both containing 0.085 % phosphoric acid. The flow rate was 1.6 ml/min and the column temperature was 35 °C. The elution gradient was set as below: 0–7 min (70–85 % B), 7.0–7.1 min (85–95 % B), 7.1–8.0 min (95 % B), 8.0–8.1 min (95-70 % B) and 8.1–10 min (70 % B). The UV detection was monitored at fixed 220 nm. Qualitative analysis were performed on the following 11 cannabinoids (Fig. 1): CBDV (Rt = 2.55), CBDA (Rt = 3.39), CBGA (Rt = 3.67), CBG (Rt = 3.87), CBD (Rt = 4.04), THCV (Rt = 4.21), CBN (Rt = 5.65), Δ9-THC (Rt = 6.53), Δ8-THC (Rt = 6.66), CBC (Rt = 7.35), and THCA (Rt = 7.61). Quantification was restricted to five cannabinoids: CBDA, CBD, CBN, Δ9 –THC, THCA.

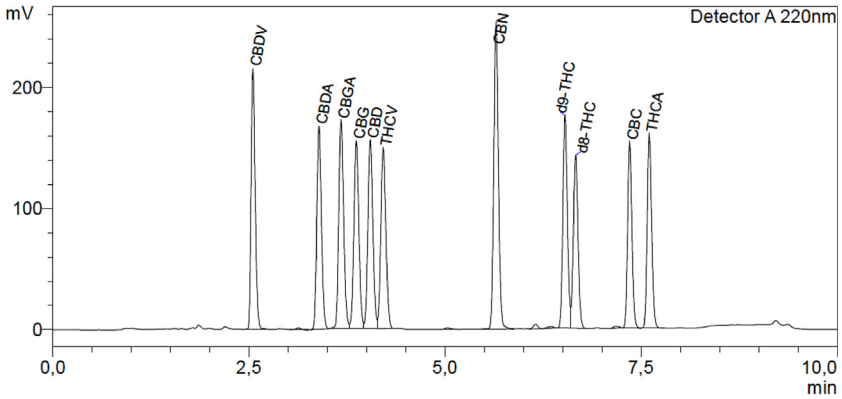

Fig. 1. Chromatographic separation of 11 cannabinoids in a standard sample at a concentration of 0.5 % (50 ppm, 50 μg/mL) of each cannabinoid.

3. Results and discussion

3.1. Method validation

The testing protocol was completed by Shimadzu Corporation, including parameters such as precision, accuracy, linearity, repeatability of peak area and retention time, limit of detection (LOD) and limit of quantification (LOQ). In the Shimadzu testing protocol, precision and accuracy were calculated using different replicates of samples in different working days. Accuracy was expressed as the relative error (Er%), while precision was measured as the coefficient of variation (CV%). A CV% below 15 % and Er% between ± 15 % were considered suitable. Six-point calibration curves were calculated by plotting peak area of each cannabinoid *vs*. its concentration. The linearity was proven according to the regression line by the method of least squares and expressed by the coefficient of determination (R^2). LOQ is the lowest concentration that encounters a S/N > 10 whereas LOD a S/N > 3. Method validation results were listed in Table 1.

This analytical method saves time and it is very easy to apply, allowing the determination of cannabinoids without derivatization as required by GC methods [6,7]. Respect to other HPLC methods [4,7] the advantage is that the separation of eleven cannabinoids is achieved in less than 8 min. Quantitative analyses are performed avoiding the use of the internal standard, thanks to the calibration system optimized by Shimadzu Corporation.

3.2. Application on cannabis light preparation

Female inflorescences of Italian industrial hemp (n = 922) were analysed by HPLC/UV Shimadzu Prominence-i LC-2030C-Cannabis Analyzer for Potency in order to determine the presence and the levels of 11 cannabinoids. Single results were listed in table S1, whereas an overview is shown in Table 2 and Fig. 2.

In this paper we report only the concentration of the principal cannabinoids (CBDA, CBD, THC, and THCA), since these are the most important for legal purposes.

The total content of THC was calculated as follow: (THCA x 0.877) +Δ^9-THC; in the same way the total content of CBD: (CBDA x 0.877) + CBD in which 0.877 correspond to the ratio between the molecular mass of decarboxylated form/carboxylated form. As expected, the concentrations of the cannabinoids are very heterogeneous and not-gaussian distributed (Figure S1) between the samples (CV%: 47-201), which may fluctuate according to genetic factors and environmental influences.

To the best of our knowledge, there is no study involving a so high number of hemp products in the Italian market.

3.3. Legal consideration

Only 18 % of the analysed inflorescences can be liberally sold in accordance with the EU regulation, complying with the limit of 0.2 % of THC. On the other hand, referring to the most recent Italian law (n. 242/2016) the 10 % of the samples should be destroyed, because they have a concentration of THC > 0.6 %.

The 58 % of the samples containing a THC level between 0.2 and 0.5 % are legal in accordance to the recent sentence (12/2018) of the Italian Court "*Terza Cassazione*" and the note of the Ministry of the Interior, fixing the cut-off of THC at 0.5 %.

The remaining 14 % of the samples with a THC content between 0.5 and 0.6 % is still unclear whether they are legal or not.

Another important issue is that cannabis light products are not commercialized for human use, but exclusively for "*technical use.*" In this way, these products are not subjected to the standard controls planned for the products that are employed for human use (pesticides, fertilizers, microbiological tests and so on). Consumers who buy these products, on the other hand, very likely decide to consume them as recreational tools, with possible harm to health.

3.4. Determination of the chemotype

As the classification of cannabis is still a controversial issue [16] and due to the legal uncertainties the determination of the chemotype can be very important to help assessing the legality of a sample and the membership to a particular type of cannabis. A histogram (Fig. 3) of the THC/CBD ratios ($\log_{10}$) for the all 922 samples shows that the plants have to be assigned to Chemotype III and then are classified as industrial hemp. This result indicates that the chemotype based classification of hemp is necessary to assess

Table 1
Method validation results. Results are expressed in % dry weight.

	Linearity range	R^2	Slope	LOD	LOQ	CV%	Er%
CBDA	0.005-50	0.9981	1.33E+07	0.002	0.005	2.10	1.47
CBD	0.005-50	0.9999	1.37E+06	0.002	0.005	1.72	1.54
CBN	0.005-1	0.9998	2.08E+06	0.002	0.005	–	–
Δ^9 -THC	0.005-1	0.9994	1.41E+06	0.002	0.005	2.21	−4.35
THCA	0.005-1	0.9997	1.27E+06	0.002	0.005	2.50	−5.26

Table 2
Cannabinoids range of concentration in cannabis light preparations (n = 922).

	% CBDA	%CBD	% CBD tot	% Δ9 THC	% THCA	% THC tot
Min.[1]	0.11	0.04	0.21	0.03	0.03	0.05
Max.	23.83	18.3	21.36	0.6	1.04	1.02
Mean	9.502	0.7301	9.024	0.05794	0.36	0.3787
SD[2]	4.731	1.47	4.312	0.08695	0.2035	0.1964
SEM[3]	0.1558	0.04841	0.142	0.002864	0.006707	0.006467
25% Percentile	5.94	0.14	5.693	0	0.21	0.23
Median	9.14	0.31	8.77	0	0.33	0.37
75% Percentile	12.99	0.6925	12.41	0.09	0.5	0.52
CV	49.79 %	201.35 %	47.78 %	150.08 %	56.54 %	51.86 %

[1] Min: indicated the lowest concentration above LOQ.
[2] SD: standard deviation.
[3] SEM: standard error of mean.

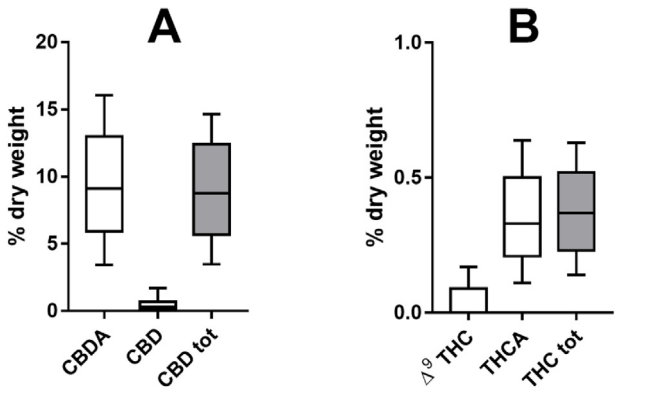

Fig. 2. Cannabinoids concentration (as % dry weight) from n = 922 samples of cannabis light preparations: (**A**) CBD, CBDA, and CBD tot and (**B**) delta-9 THC, THCA and THC tot. The boxes stretch from the 25 to the 75 percentile; the line across the boxes indicates the median values; the whiskers arising from the boxes indicate extreme values (10–90 percentile).

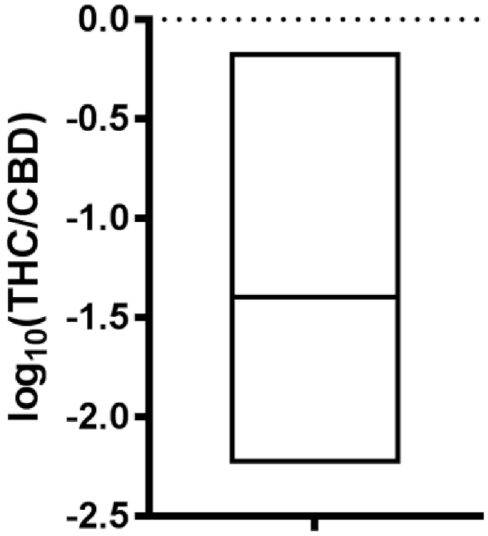

Fig. 3. Log_{10} of the ratio total THC/total CBD in all the analysed samples: a ratio THC/CBD <<1; (log_{10}(THC/CBD)<<0) designated Chemotype III plant or industrial hemp. The boxes stretch from min to the max and the line across the boxes indicates the median values.

the type of cannabis, e.g. drug type of fiber type, but it is not sufficient to assess if a specific sample is to be considered as a drug of abuse (THC > 0.5 %). The samples, according to their content in THC total, are grouped as shown in Fig. 4.

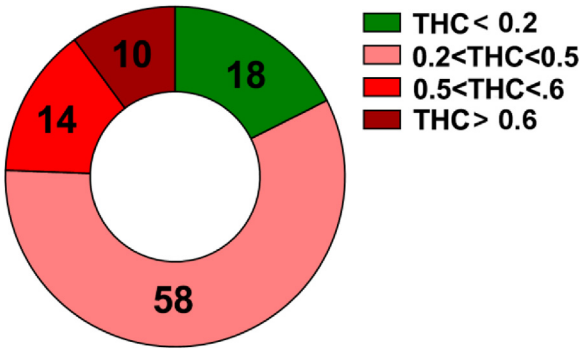

Fig. 4. Cannabis light samples represented in a part of whole graph depending on THC tot (%) concentration.

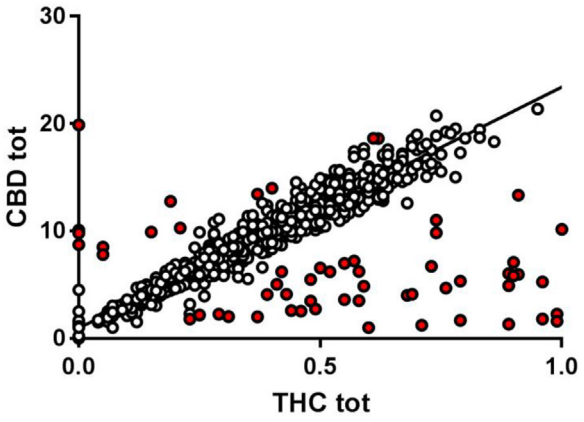

Fig. 5. Linear correlation in light cannabis samples (n = 922) between CBD total concentration and THC total concentration; red dots indicated outliners (7 %), which were automatically eliminated from the regression model (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.5. Linear correlation between CBD and THC levels

The determination of both CBD and THC levels in cannabis preparation allowed to confirm a linear correlation between the two analytes in the same sample in a population of n = 922, as shown in Fig. 5. The slope was 22,43 ± 0,2062 and y-intercept = 0,9625 ± 0,084 R^2 = 0.9326 indicates a strong positive correlation between the two variables x (THC tot) and y (CBD tot). This relationship (Fig. 4) that was studied in the early 2000s [16,17] is confirmed by our investigation with a much higher number of samples and it can be useful for both 1) predicting unreliable information declared on the labels of the products, allowing consumers to identify the most macroscopic frauds and 2) as a secondary confirmation after the analysis. Only a minor percentage (7 %) of the population was found to be an outlier (Q = 1 %), thus automatically removed from the linear regression model.

4. Conclusions

The legality of hemp, according to the current legislation, was hereby achieved determining the total THC amount by a reliable LC-UV determination. The LC method saves time and allows a direct determination of analytes since the acidic forms of the cannabinoids are not converted into the corresponding decarboxylated compounds.

According to EU regulation only 18 % of the crops are below the legal THC(total) content of <0.2 %. The 58 % of the samples containing a THC level between 0.2 and 0.5 % are legal in accordance to the recent sentence (12/2018) of the Italian Court, while it is still unclear whether the remaining 14 % of these hemp products with a THC content between 0.5 and 0.6 % would be legal or not.

Authorship statement

Michele Dei Cas: Conception and design of study; Drafting the manuscript
Eleonora Casagni: acquisition of data
Anna Saccardo: acquisition of data
Sebastiano Arnoldi: acquisition of data
Craig Young: analysis and/or interpretation of data
Stefano Scotti: analysis and/or interpretation of data
Edgardo Vieira de Manicor: analysis and/or interpretation of data

Veniero Gambaro: Conception and design of study

Gabriella Roda: Conception and design of study; Drafting the manuscript

All the authors above mentioned have approved the revised version of the paper

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