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

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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.

Abstract

The most recent law about Cannabis Light in the Italian legislation panorama is the law 242/2016 which states that the total THC content in the crop must not exceed 0.2 % and in any case, not exceed 0.6 % only for farmers. A further note was published from the Ministry of the Interior (20/07/2018 number of protocol 2018/43586) in which it was remarked 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. In this frame, the legality of hemp, according to current legislation, was achieved by a reliable LC-UV determination. Based on the law 242/2016, only 18% of the crops are to be considered legal for the market (THC tot < 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 (THC > 0.5%). On the other hand, the most of the inflorescences (58%) have a THC tot 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. The production of cannabinoids is greatly influenced by the climate, the cultivation conditions, the exposure to sunlight and pollination [2].

In the past the genus Cannabis was classified in three main species named *Cannabis sativa L.*, a fiber-type one, *Cannabis indica Lam.*, a drug-type, and *Cannabis ruderalis Janish*, with intermediate characteristics. Nowadays, it is more used a monotypic classification, based on a successful hybridization within the genus, where the Cannabis plants are all classified as Cannabis sativa, but subdivided into various chemotypes [3]. Cannabis chemotypes are determined as the dry-weight ratio of THC/CBD in the plant. Three are the main chemotypes: plants with a THC/CBD ratio >>1.0 belong to Chemotype I, plants which have a ratio close to 1.0 are included in Chemotype II, instead a THC/CBD ratio <<1 indicates plants with low THC content, and it is also known as hemp [4].

Cannabis light preparations are referred to dried inflorescences with a concentration, established by law, lower than 0.2% in THC [5]. 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 %. A further note was published from the Ministry of the Interior (20/07/2018 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 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 employment. 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 [5,6] 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 [6–14].

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 Italian growers 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 (Figure 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.

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. the extract concentration after sample preparation. The linearity was proven according to the regression line by the method of least squares and expressed by the coefficient of correlation (R^2). LOQ is the lowest concentration that encounters a S/N>10 whereas LOQ a S/N>3. Method validation results were listed in Table 1.

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

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 Figure 2. Here, only the concentration of CBDA, CBD, THC, and THCA has been monitored, since the concentration of the other cannabinoids resulted in generally not significant for legal purposes or under the LOQ. The total content of THC was calculated as follow: (THCA x 0.877) $+\Delta^9$-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 between the samples (CV%: 47-201), which may fluctuate according to genetic factors and environmental influences.

3.4 Legal consideration

In this legal context, only 18% of the inflorescences analysed can be liberally sold complying the limit of 0.2% of THC. However, the circular previously mentioned (20/07/2018 number of protocol 2018/43586) has not a legal status since it is not considered a law and thus it cannot bind either citizen or the judge. Referring to the most recent law in this field (n. 242/2016) the seizure or the destruction of hemp crops can be applied only in the case of products with a concentration of THC > 0.6%. A recent sentence (12/2018) of the Italian Court "*Terza Cassazione*" stated that to be considered a drug of abuse Cannabis Light besides overcoming 0.2% THC, has to show a significant potential of abuse, that is considered when the amount of THC is above 0.5%, confirming the circular of the Ministry of the Interior. Hence, according to what previously specified, 76% of the inflorescences analysed showed a THC content inferior to 0.5% and their commercialization by farmers do not constitute a crime. The remaining 24%, which is outside this concentration established by law, must be destroyed. Further legislation will be promulgated in order to clarify the products with a concentration between the 0.2-0.5% sold by retailers. The law 242/16 does not take into account the possibility that the farmer sells inflorescences to retailers, who can introduce them on the market as Cannabis Light products. This possibility is not mentioned, but on the other hand, it is not forbidden. In this legal uncertainty, many companies were opened, selling industrial hemp inflorescences. Our results can likely represent a view of Cannabis Light products on the Italian market. As the products having a THC content higher than 0.6% must be destroyed, most of the inflorescences might have a THC content between 0.2 and 0.5%, with significant consequences related to the detention of such products either for the seller or for the buyer. Cannabis Light products are not commercialized for human use but, exclusively, as *"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). Who buy these products, on the other hand, very likely decide to consume them as recreational tools, with possible harm to health. In this frame, we were interested in evaluating the chemotype of the analyzed samples. A histogram (Figure 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 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 Figure 4.

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 Figure 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 (TCH tot) and y (CBD tot). This relationship (Figure 4) 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.

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
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

4. Conclusions

The legality of hemp, according to current legislation, was hereby achieved by a reliable LC-UV determination. Cannabinoids quantification obtained with the HPLC/UV method proposed by Shimadzu Corporation. The LC system allows a time saver and direct determination of analytes since the acidic forms of the cannabinoids, due to thermal degradation, can be analysed in GC only prior

derivatization. Based on the law 242/2016 only 18% of the crops are to be considered legal for the market (THC tot < 0.2%). If the circular of the Ministry of the Interior 20/07/2018 number of protocol 2018/43586 was converted as a proper law a substantial amount of Cannabis light preparation (24%) would be considered illegal. On the other hand, the most of the inflorescences (58%) have a THC tot content comprised between 0.2% and 0.5%, and it is not clear whether these products could be sold or not. Another problem is that Cannabis Light product are not authorized for human consumption, even if everybody knows that is their primary use. Furthermore, the law. n. 242/2016 cannot be referred to as *lex specialis* in respect to the d.P.R n. 309/90 in the case of detection and commercialization of products, generically identified as technical collector's product, ornament or scent for the environment, with a total THC content greater than 0.5

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Figures.

Figure 1. Chromatographic separation of 11 cannabinoids in a standard sample at a concentration of 0.5% (50 ppm) of each cannabinoid.
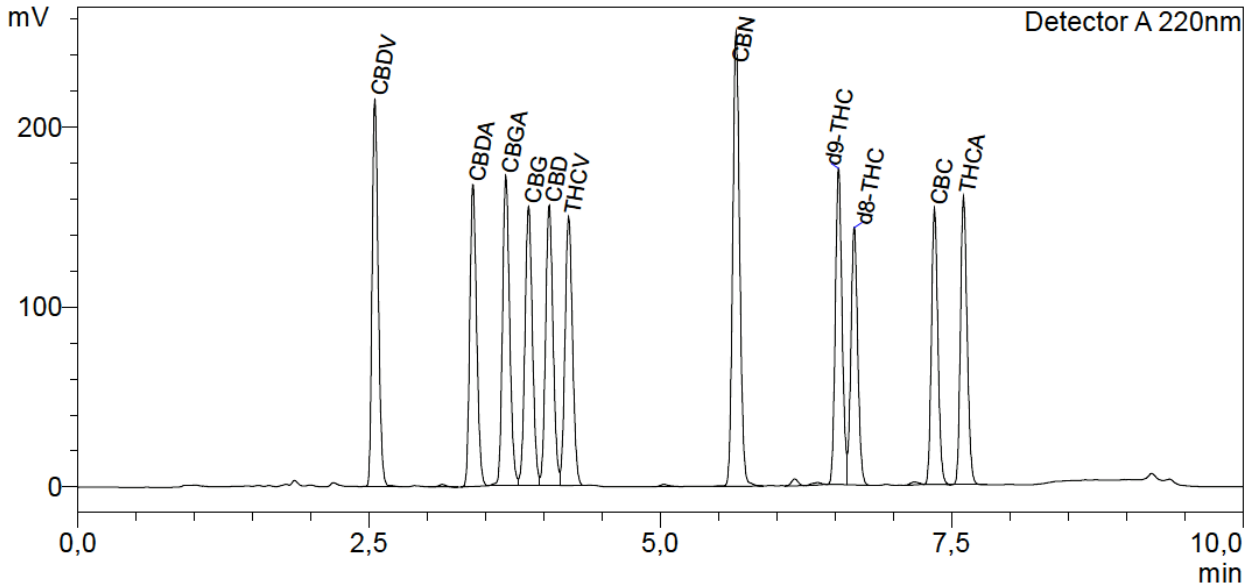

Figure 2. Cannabinoids concentration (as % dry weight) from n=922 samples of cannabis light preparations: (**A**) CBD, CBDA, and CBD tot and (**B**) delta-9 THC, THCA and THC tot.
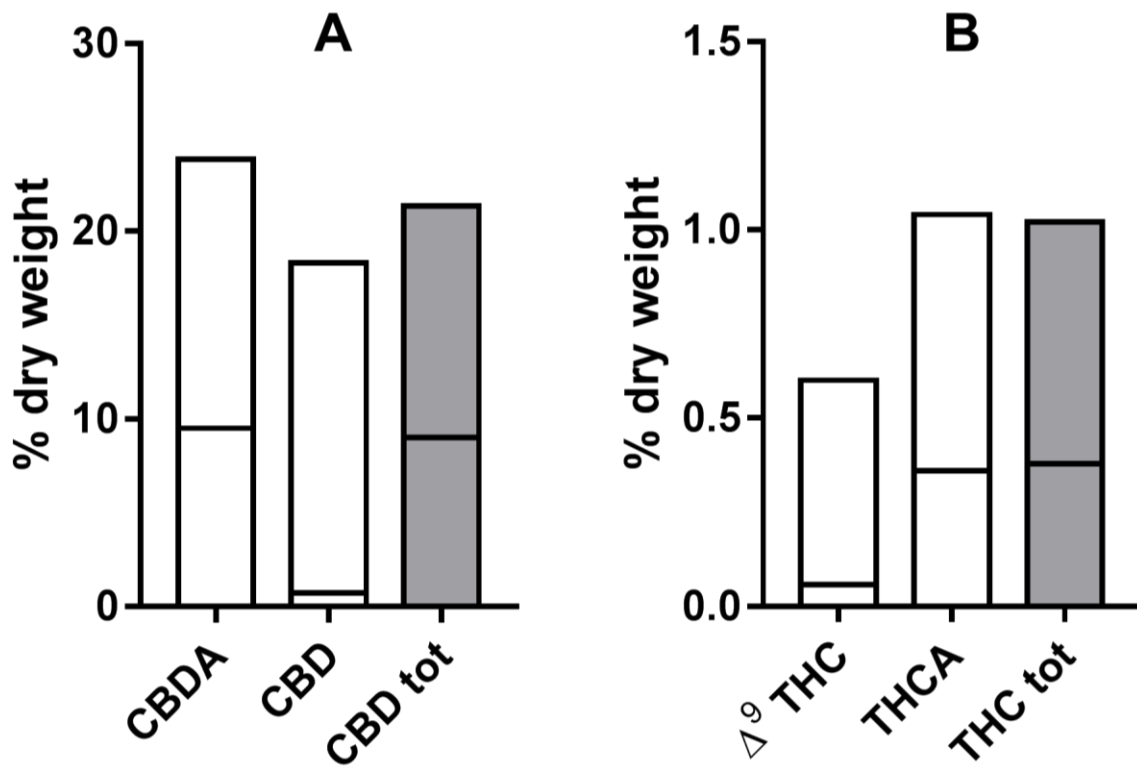
Figure 3. Log_{10} of the ratio total THC/total CBD in all the analysed samples: a ratio THC/CBD $<<1$; (log_{10}(THC/CBD)$<<0$) designated Chemotype III plant or industrial hemp.
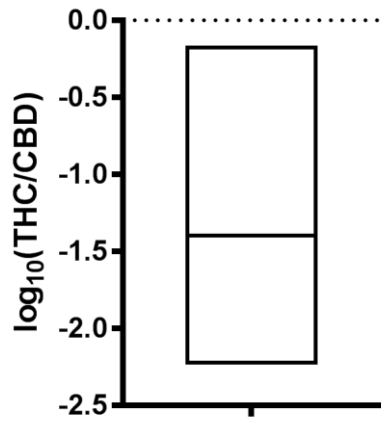

Figure 4. Cannabis light samples represented in a part of whole graph depending on THC tot (%) concentration.
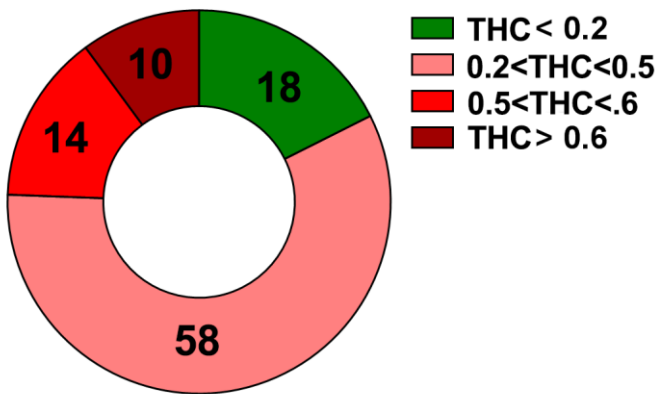
Figure 5. Linear correlation in light Cannabis samples (n=922) between CBD total concentration and THC total concentration; red dots indicated outliners (7%), which were automatically eliminated from the regression model.