Analysis of Cannabinoids in Fiber Hemp Plant Varieties (*Cannabis Sativa* L.) by High-Performance Liquid Chromatography[a]

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Key Words

Column liquid chromatography
Neutral and acidic cannabinoids
Fiber hemp plants
Cannabis accessions and environmental conditions

Summary

An analytical procedure was developed for the detection of neutral and acidic cannabinoids in herbal cannabis without the need of any preliminary derivatization.

The method was used to assay cannabinoid content of over one hundred fiber hemp samples grown in different Italian localities and harvested at different maturation level degrees during the summer. No interferences were observed due to the vegetal matrix.

The influence of genetic factors and environmental conditions on cannabinoid content is discussed; the results may be of interest to enhance potential of fiber hemp in compliance with law enforcement purposes.

Introduction

The fiber type of *Cannabis Sativa* L. has increased its importance in Europe as a raw material for paper and textile production. It is an interesting alternative source for the production of natural fibers.

Different hemp plants have considerable variations in the pattern of cannabinoid composition depending on source, geographical origin and environmental conditions [1, 2]. Hence, several laboratories used the measurement of the relative concentration of Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD) and cannabinol (CBN), the main cannabinoids, to characterize plant phenotype. According to Fetterman et al. [3] fresh plants with [(% Δ^9-THC) + (% CBN)] / [% CBD] ratio exceeding one are considered "drug phenotype", otherwise as "fiber phenotype". Small and Beckstead [4] distinguished three phenotypes depending on the combination of the Δ^9-THC and CBD content; in particular plants with less than 0.3 % Δ^9-THC were considered to have no psychoactive potency. Fournier and Paris [5] reported a tolerated maximum content of 0.5 % Δ^9-THC for fiber hemp; de Meijer et al. [2] characterized hemp accessions by the analyzed Δ^9-THC and CBD content and the correspondent ratio [% Δ^9-THC] / [% CBD].

The Italian law (D.P.R. 309/90) does not allow the cultivation of drug type cannabis, while the fiber type plants are legally cultivated in some regions of the Mediterranean area. During the past there was some uncertainty in the Italian law concerning the content of psychoactive substances in cannabis plants, which led to a number of interventions of the legal authorities against the cultivators of the fiber type cannabis plants. Therefore it is of relevant importance to determine which growing conditions produce the lowest levels of psychoactive constituents, particularly Δ^9-THC, the major active component.

The complexity and variability of cannabis samples rise several problems for the analyst and the adopted analytical solution depends on the type of information sought. Chemo-botanical investigations, i.e., require methods are which can assure accurate identification and quantification of the cannabinoid profile in fresh plant tissues. Analytical procedures, on the other hand, require the developments of new technique in order to improve the detection and separation of all cannabinoids present in plant tissues.

Since acidic cannabinoids, the predominant form present in living plants [6], are converted by high temperature to neutral cannabinoids when injected into gas-chromatograph [7], the direct GC analysis of cannabis extracts can determine only the total cannabinoid content [8]. Therefore a derivatization step is required prior

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to GC analysis to assess both acidic and neutral cannabinoids [9].

High-performance liquid chromatography (HPLC) detects cannabinoids in both the acidic and neutral forms and can provide an effective tool for more accurate assessment. Therefore numerous HPLC methods have been developed to determine the cannabinoid pattern in herbal cannabis and cannabis resin [6, 10–15]. Nevertheless some experimental conditions require long analysis times or can damage the analytical column in time owing to the acidic mobile phase.

The present work is part of an ongoing comprehensive research program to monitor the genetic, physical and chemical characteristics of hemp plants grown in Italy in order to define the cannabis accessions and the growing conditions producing the lowest cannabinoid content. The combined results may provide the bases to enhance agronomic potential of hemp in compliance with law enforcement purposes. In particular this paper describes a rapid HPLC procedure to analyze the main cannabinoids in a considerable number of cannabis samples from different origin and age. Given its simplicity our proposed method can be adopted for routine control on hemp crops.

Experimental

Materials

Methanolic solutions (1.0 mg mL^{-1}) of Cannabinol, Cannabidiol, Δ^9-Tetrahydrocannabinol and Δ^8-Tetrahydrocannabinol were purchased from S.A.L.A.R.S. (Como, Italy-Aut. Min. San. n. 800/UCS/SP.6364) and stored at –20 °C.

Samples of various hemp cultivars were kindly supplied by the "Istituto Sperimentale per le Colture Industriali" (Bologna, Italy).

Petroleum benzine low boiling (boiling range 40° ÷ 60 °C) was analytical grade (Merck).

Water was deionized and further purified by a Milli-Q Water Purification System (Millipore Corp., MA, USA). Methanol (Carlo Erba) and acetonitrile (Merck) were HPLC grade. The solvents were filtered through a 0.45 µm Millipore membrane filter and degassed by a constant flow of helium before and during use.

Instrumental

HPLC was performed on a Series 4 liquid chromatograph with a quaternary pump and a Model LC-85B variable wavelength UV detector (Perkin-Elmer, Norwalk, CT, USA). Data were acquired and processed by a Laboratory Computing Integrator (Perkin-Elmer LCI-100) and a 7500 Professional Computer with "Chromatographics 3" software (Perkin-Elmer). Separations were carried out on a LiChroCART-LiChrospher 100-RP-18 column (125 × 4 mm; 5 µm); a LiChrospher 100-RP-18 guard column (4 × 4 mm; 5 µm) preceded the analytical column (Merck). Sample solutions were injected using an automatic injector with a 20 µL loop (Perkin-Elmer ISS-101).

HPLC-MS analysis was performed on a single quadrupole SSQ 710A mass spectrometer with Particle Beam Interface (Finnigan MAT, Bremen, Germany) and equipped with a Jasco PU 980 pump (Tokyo, Japan). Solutions were injected manually into the LC-MS system through a Rheodyne (Cotati, CA, USA) model 7125-075 injection valve with a 20 µL loop. Data acquisition and mass spectrometric evaluation were on a Personal DEC Station 5000/120 data system (Digital Equipment, Unterföhring, Germany) with ICIS 7.0 software (Finnigan MAT, Bremen, Germany) and National Technical Information Services (NTIS; Springfield, VA, USA) mass spectral libraries.

Chromatographic Conditions

Chromatographic separations were performed at room temperature with a mobile phase consisting of methanol/water in the ratio 80:20 (*v/v*); the flow-rate was 1.0 mL min^{-1}. The same HPLC analytical column and guard column were used for both HPLC-UV and HPLC-MS procedures. The injection volume was 20.0 µL.

The detector wavelength in analytical HPLC-UV was set at 220 nm with 0.04 absorbance full scale (a.u.f.s.).

For the HPLC-MS analysis the operative parameters were essentially those we proposed previously for the simultaneous separation and identification of hashish constituents [15]. The mobile phase flow-rate was 1.0 mL min^{-1} in order to respect the maximum value allowed by this HPLC-MS system. In the particle beam interface the desolvation chamber temperature was 45 °C and the nebulizing gas was helium. Full mass spectra were acquired by scanning over the mass range *m/z* 45 to 700 once per second. The mass spectrometer was operated in the electron impact (EI) mode with the following parameters: ionization energy 70 eV; ion source temperature 250 °C, filament current 200 µA, conversion dynode power –15 kV and electron multiplier voltage 1500 V.

Standard Solutions and Calibration Graphs

Working reference solutions containing cannabinol, cannabidiol, and Δ^9-tetrahydrocannabinol were prepared by diluting with methanol the standard alcoholic solutions and processed in triplicate using the HPLC-UV conditions described above. Calibration range: 3.92 ÷ 44.44 µg mL^{-1} for CBD and Δ^9-THC; 0.65 ÷ 22.22 µg mL^{-1} for CBN.

Linear regression analysis was performed by the external standard method using Δ^8-tetrahydrocannabinol (29.00 µg mL^{-1}); the peak-area ratio of each cannabinoid to external standard was plotted versus the analyte concentration.

Table I. Total retention time (t_R), percent relative standard deviation of retention time ($RSD\ \%^{tR}$), retention factor (k) and separation factor (α) of the main standard cannabinoids.

Compound	t_R (min)	$RSD\ \%^{tR}$	k	α
Cannabidiol	5.37	0.35	5.17	
				1.95
Cannabinol	9.63	0.42	10.07	
				1.28
Δ^9-Tetrahydro-cannabinol	12.10	0.43	12.91	
				1.14
Δ^8-Tetrahydro-cannabinol	13.69	0.48	14.73	

Table II. Regression statistics for cannabinoid calibration curves.

Parameter [1]	CBD	CBN	Δ^9-THC
Slope	0.0327	0.0952	0.0466
CI slope	± 0.0004	± 0.0019	± 0.0011
Intercept	0.0070	0.0017	0.0212
CI intercept	± 0.0028	± 0.0021	± 0.0025
R^2	0.9992	0.9988	0.9984
N	18	18	18
Range	3.92÷44.44	0.65÷22.22	3.92÷44.44
LOD	0.119	0.032	0.077
LOQ	0.398	0.105	0.258

[1] CI: confidence interval; Range: calibration range ($\mu g\ mL^{-1}$); R^2: square correlation coefficient; LOD: limit of detection ($\mu g\ mL^{-1}$); N: number of points; LOQ: limit of quantitation ($\mu g\ mL^{-1}$).

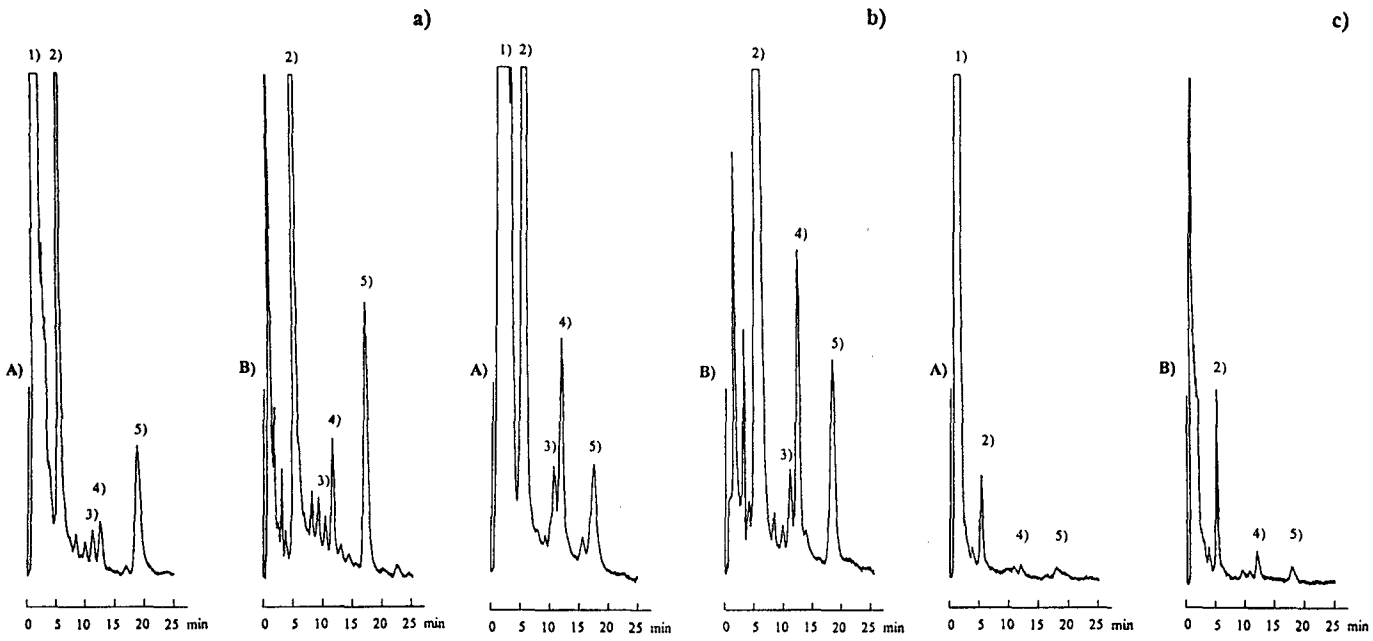

Figure 1

Representative HPLC-UV chromatograms of unheated (A) and heated (B) extracts of various hemp plants harvested in August: **a)** sample 1M, Carmagnola cultivar, Anzola (Bologna); **b)** sample 8F, Fibranova cultivar, Rovigo; **c)** sample 3M, Ungherese cultivar, Rovigo. Mobile phase: methanol/water, 80:20 (*v/v*). Flow-rate: 1.0 mL min^{-1}. Detection UV at 220 nm with 0.04 a.u.f.s. Peaks: **1** = acidic cannabinoids; **2** = cannabidiol; **3** = cannabinol, **4** = Δ^9-tetrahydrocannabinol; **5** = cannabichromene.

Preparation of the Samples

In this work we analyzed three cultivars of *Cannabis Sativa* L. (Carmagnola, Fibranova and Ungherese) grown in different Italian localities and harvested at different maturation level degrees during the summer (July, August, September). The examined samples included leaves from the upper third of the plant.

After harvesting and air drying to constant weight (40 °C, 24 h), leaves from each hemp plant were powdered in liquid nitrogen and mixed to give a homogeneous material. The samples were stored in the dark at 4 °C in order to avoid the spontaneous decomposition at room temperature of acidic cannabinoids into the correspondent neutral form [4].

Powdered samples were prepared for HPLC by weighing 150 mg portions into centrifuge glass tubes and adding 3.0 mL of petroleum benzine low boiling. The tubes were agitated in an ultrasonic bath for 10 min at room temperature and then centrifuged at 3000 r.p.m. for 5 min. The extraction procedure was repeated three times and the resulting clear supernatants were combined. Aliquots of the extracts (2.0 mL) were evaporated under a gentle stream of nitrogen, dissolved in 1.0 mL acetonitrile by sonication for 5 min at room temperature and the obtained solutions were injected in triplicate into the liquid chromatograph to assess neutral cannabinoids.

Another aliquot of each extract (2.0 mL) was evaporated to dryness by nitrogen and decarboxylated at 200 °C for 3 min according to the literature [16]. The samples were then allowed to cool to room temperature, dissolved in 1.0 mL acetonitrile by sonication for 5 min and analyzed in triplicate for total (acidic and neutral) cannabinoid content.

Results

The choice of the experimental conditions was guided by the need to obtain chromatograms of various complexity within short analysis time, as required when numerous samples are to be analyzed.

Under the HPLC conditions described above the main standard cannabinoids are completely separated in less than 25 min. Table I reports the correspondent values obtained for the total retention time (t_R), percent relative standard deviation of retention time (RSD % ^{tR}), retention factor (k) and separation factor (α).

Table II shows the regression statistics for cannabinoid calibration curves. The curves showed satisfactory values for square correlation coefficients (R^2) and 95 % confidence intervals (CI); limits of detection (LOD) and limits of quantitation (LOQ) were calculated according to the calibration curves [17].

Due to the lack of commercial standards with certified quality, we preferred performing indirect quantitation of acidic cannabinoids by measuring the amount of the corresponding neutral cannabinoids before and after decarboxylation at 200 °C, as described above.

Before analyzing hemp extracts, experiments were carried out to check the stability of neutral cannabinoids during heating. Two identical aliquots of the same standard solution were evaporated to dryness by nitrogen and only one was placed in an oven at 200 °C for 3 min. The dried samples were then dissolved with the same volume of acetonitrile and analyzed by HPLC. The correspondent chromatograms were substantially identical, confirming that the short heating step caused no decomposition of neutral cannabinoids.

Subsequently we analyzed the hemp plant extracts to determine their cannabinoid content. Satisfactory separations of the neutral cannabinoids from the complex plant matrix can be achieved under the chromatographic conditions described above. While the repetitive runs of identical samples were found to be highly reproducible, both qualitative and quantitative differences were found in different plants. Figure 1 (a, b, c) shows representative examples of unheated (A) and heated (B) extracts, giving some idea of the diversity of chromatograms obtained from different cannabis samples. Cannabidiol, cannabinol and Δ^9-tetrahydrocannabinol were located in the chromatograms by running pure standards; acidic cannabinoids (peak 1) were identified by peak enhancement of the correspondent neutral form in the heated extracts. Some unheated extracts were also subjected to the procedure described by Smith

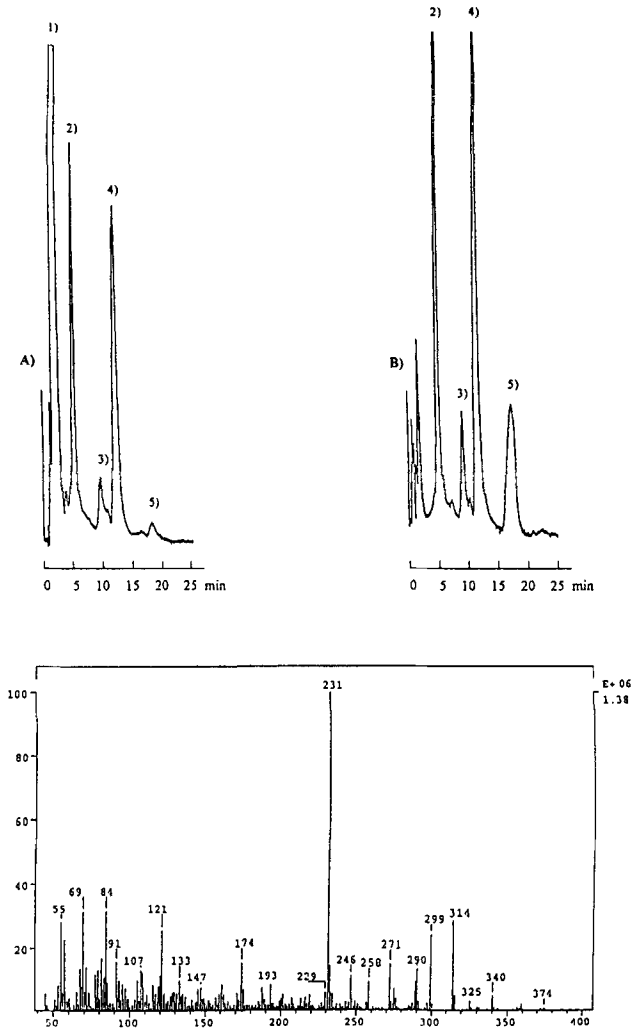

Figure 2

Above: HPLC-UV analysis of an unheated (A) and heated (B) extract. Sample: 1F, harvested in July, Ungherese cultivar, Budrio (Bologna). Chromatographic conditions and peaks: see Figure 1. Below: experimental background-subtracted mass spectrum of acidic fraction recorded during HPLC-MS analysis of the same unheated extract. Operative conditions: see text.

[18] in order to isolate the acidic cannabinoids, subsequently decarboxylated and analyzed as their correspondent neutral form. The obtained chromatograms confirmed our previously results.

Moreover combined HPLC-MS analyses were performed to aid in peak identification.

HPLC-MS data, m/z values of principal mass fragments (relative abundance as percentage of the base peak):

peak 2 (CBD) : 314 (10), 246 (20), 232 (15), 231 (100);

peak 3 (CBN) : 310 (12), 295 (100), 239 (6), 238 (17);

peak 4 (Δ^9-THC) : 314 (75), 299 (100), 271 (54), 258 (32), 243 (40), 231 (78);

peak 5 (CBCh, cannabichromene) : 314 (4), 299 (4), 232 (17), 231 (100), 174 (19).

Mass spectrometric data for acidic fraction (peak 1) are in good agreement with HPLC-UV analyses, as illustrated in Figure 2 for one of the analyzed samples. Here

Table III. Mean values of cannabinoids, expressed as per cent values of the total dry substance, found in Carmagnola, Fibranova and Ungherese cultivar plants.

Fiber hemp plant varieties *(Cannabis Sativa L.)*	CBD			Δ^9-THC		
	Total	Neutral	Acidic	Total	Neutral	Acidic
Carmagnola-Anzola (Bo)						
Female plants: July (10)	0.19	0.17	0.02	0.01	0.01	0.00
August (10)	0.73	0.34	0.39	0.03	0.02	0.01
September (9)	0.31	0.17	0.14	0.02	0.01	0.01
Male plants: July (9)	0.18	0.13	0.05	0.01	0.01	0.00
August (9)	0.30	0.16	0.14	0.02	0.01	0.01
Fibranova - Rovigo						
Female plants: July (8)	0.25	0.20	0.05	0.01	0.01	0.00
August (10)	0.55	0.44	0.11	0.02	0.02	0.00
September (3)	2.30	0.44	1.86	0.04	0.02	0.02
Male plants: July (10)	0.21	0.16	0.05	0.01	0.01	0.00
August (9)	0.19	0.19	0.00	0.01	0.01	0.00
Ungherese - Rovigo						
Female plants: July (3)	0.05	0.04	0.01	0.04	0.04	0.00
August (4)	0.28	0.11	0.17	0.35	0.17	0.18
Male plants: July (3)	0.22	0.10	0.12	0.01	0.00	0.01
August (3)	0.43	0.12	0.31	0.02	0.01	0.01
Ungherese - Budrio (Bo)						
Female plants: July (2)	0.15	0.10	0.05	0.04	0.03	0.01
August (3)	0.35	0.15	0.20	0.44	0.33	0.11
Male plants: July (2)	0.07	0.05	0.02	0.03	0.02	0.01
August (3)	0.12	0.08	0.04	0.01	0.01	0.00

* In brackets: number of harvested plants

are reported the HPLC-UV analyses of its unheated (A) and heated (B) extract together with the related mass spectrum of the acidic fraction recorded for the former by HPLC-MS. The acidic cannabinoids present here are cannabidiolic acid (CBDA), Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and cannabichromenic acid (CBChA), as clearly demonstrated by comparing the HPLC-UV peak areas and by the mass fragments with m/z = 174, 231, 246, 271, 299; 314. It was not possible to distinguish between the two known isomers of Δ^9-THCA (Δ^9-THCA-A; Δ^9-THCA-B), but it is likely that the isomer encountered was Δ^9-THC acid A since this is a major acidic constituents of cannabis.

The hemp samples analyzed by HPLC gave a variety of chromatograms differing in both qualitative and quantitative characteristics. The concentration of CBD, CBN and Δ^9-THC in the unheated (neutral compounds) and heated (sum of neutral and corresponding carboxylic acids) extracts was determined by the linear least-squares fits previously calculated. The obtained results are given in Figures 3–5 and are expressed as per cent values of leaf powder dry weight; for graphical reasons the values of CBD and Δ^9-THC are multiplied for 100 and 1000, respectively. Table III summarizes the mean values; CBN content was lower than 0.001 % in most of the samples, both unheated and heated, and therefore not shown.

Some samples were also analyzed without derivatization by GC on a Varian 3400 gas chromatograph equipped with flame ionization detector and a fused silica column (30 m × 0.25 mm I.D.) coated with DB5 MS J&W. The operative conditions were helium flow-rate: 0.8 mL min^{-1}, injector and detector temperature: 250 °C, temperature program: 1 min isothermal at 200 °C, increasing with 12 °C min^{-1} to 285 °C and then 5 min isothermal at 285 °C. The obtained data for total content were in good agreement with HPLC results.

All samples showed different content of cannabinoid depending on their maturity stage.

In Carmagnola cultivar (Figure 3, Table III) the female plants presented levels of neutral and total CBD increasing from July to August and then decreasing; in particular the mean content of neutral CBD in August was twice (0.34 %) the amount found in July and September (0.17 %). The level of total CBD, sum of neutral compound present as such and of that arising from de-

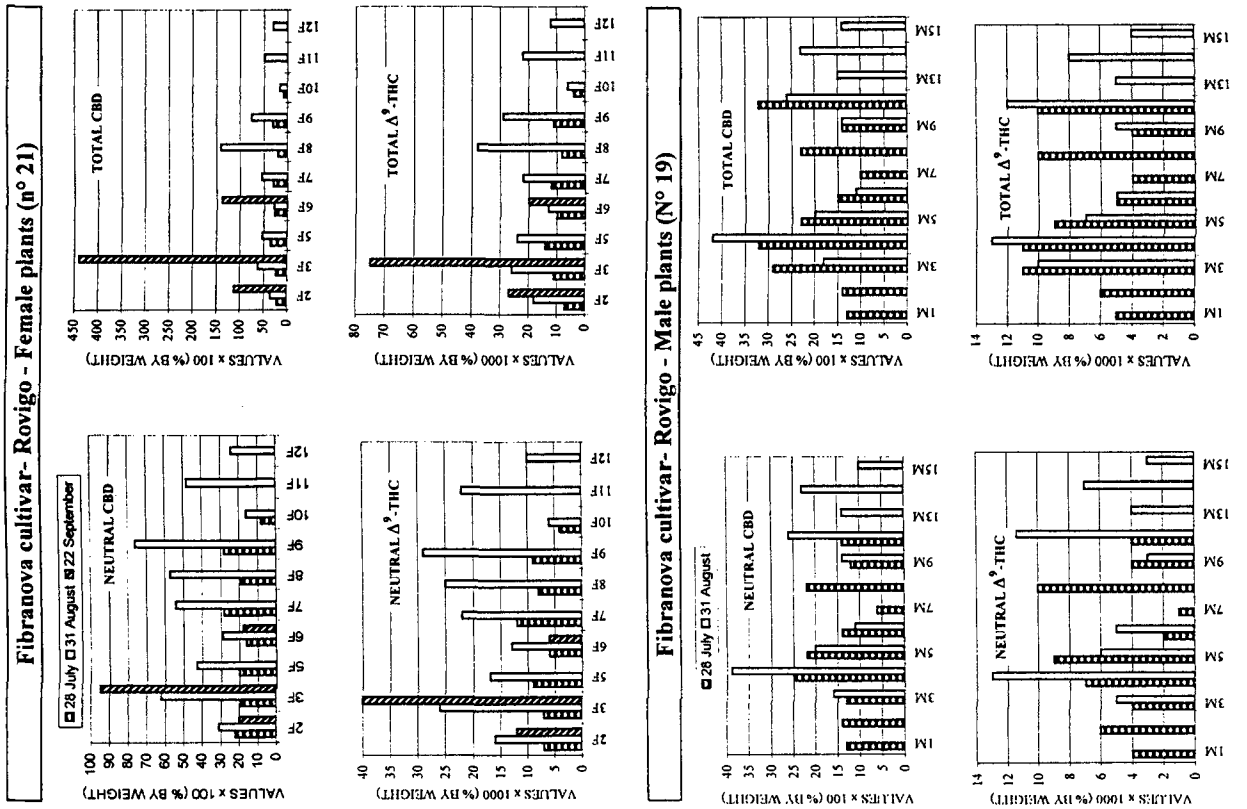

Figure 4
Average CBD and Δ^9-THC content of individual plants belonging to Fibranova cultivar and grown in Rovigo.

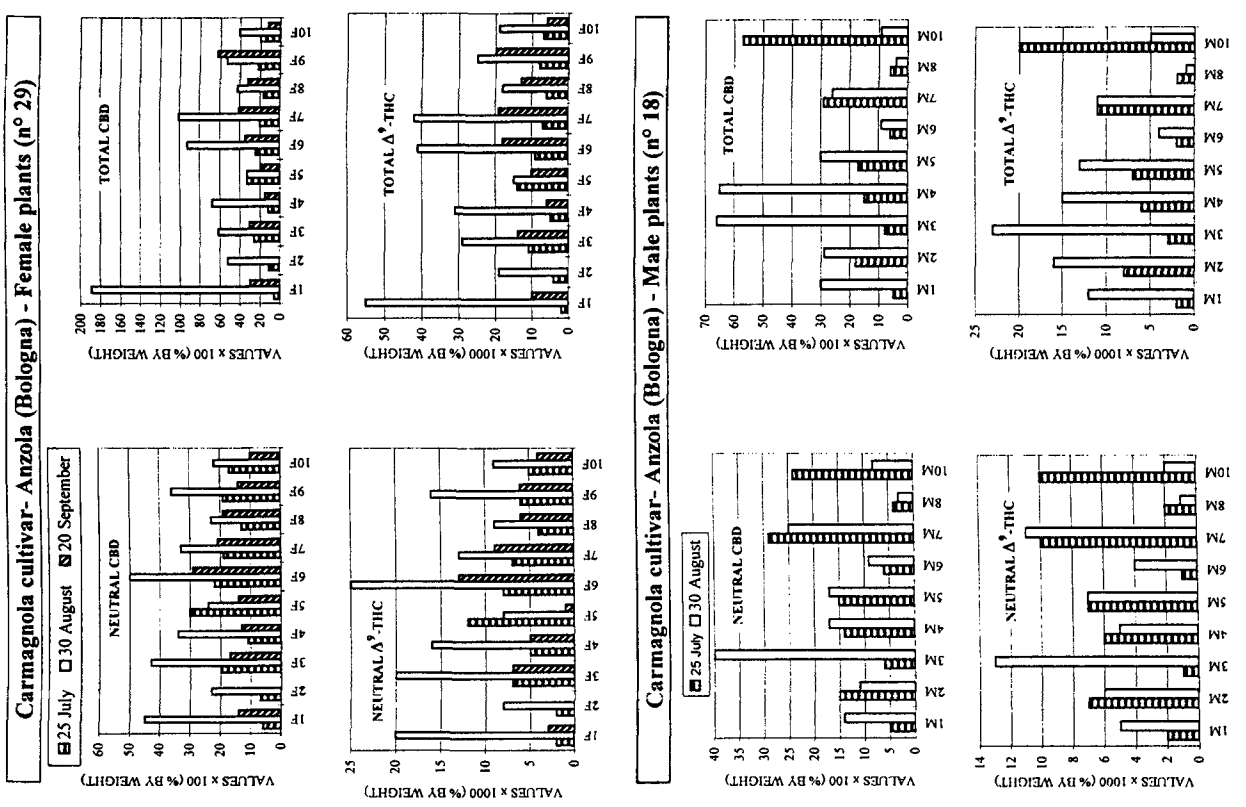

Figure 3
Average CBD and Δ^9-THC content of individual plants belonging to Carmagnola cultivar and grown in Anzola (Bologna).

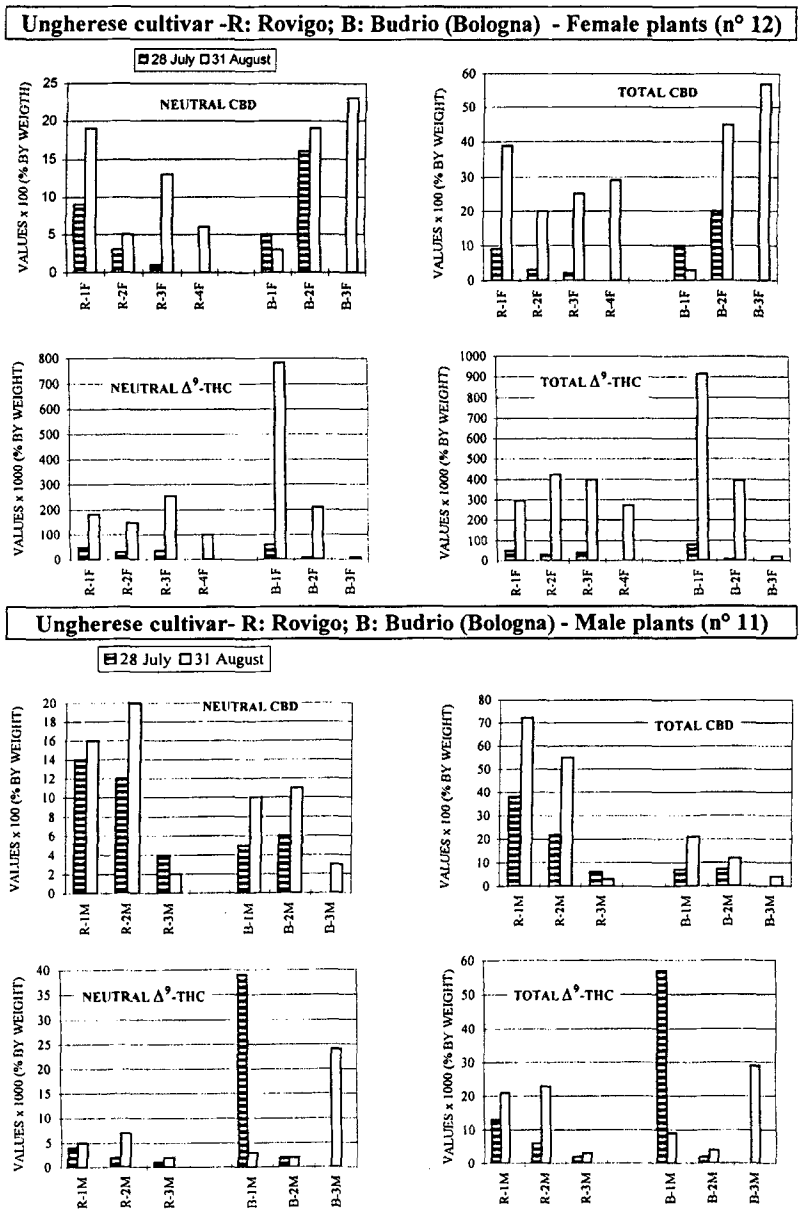

Figure 5

Average CBD and Δ^9-THC content of individual plants belonging to Ungherese cultivar and grown in Rovigo (series R) and in Budrio (series B, Bologna).

carboxylation of CBDA, was considerably higher in plants harvested in August (0.73 %) than in July or in September. Therefore the contribution of CBDA to total CBD content is 0.02 %, 0.39 %, 0.14 % in July, August and September, respectively. The mean values of both neutral Δ^9-THC and Δ^9-THCA approximated to 0.01 % in all female samples; the highest level (0.03 %) referred to total Δ^9-THC of samples harvested in August. Male plants presented neutral and total CBD content increasing from July to August; the enhancement for total CBD being mainly due to CBDA. The value of total Δ^9-THC averaged 0.01 % up to complete maturation level (August).

In Fibranova cultivar (Figure 4, Table III) the female plants presented level of neutral CBD and Δ^9-THC increasing from July to August and then unmodified, whereas the CBDA and Δ^9-THCA content was maximum in September. The values of total CBD and Δ^9-THC in September averaged 2.30 % and 0.04 %, respectively. In male plants the levels of total CBD and Δ^9-THC are mainly due to the correspondent neutral forms and are practically independent on the harvest time.

In Ungherese cultivar (Figure 5, Table III) the levels of neutral CBD and particularly of CBDA increased from July to August in all female samples; these enhancements were less marked in the male plants grown in Budrio than in those from Rovigo. The levels of neutral Δ^9-THC and Δ^9-THCA rose strongly in all female samples and were influenced by the time of harvesting, the

highest levels being reached in August. The mean level of total Δ^9-THC was 0.35 % and 0.44 % in female plants grown in Rovigo and Budrio, respectively, while it averaged 0.01 % in the male ones.

The levels of total Δ^9-THC are highest in female samples independently from their origin. It will be necessary, however, to process further samples for this cultivar to complete these results.

Conclusion

The method described here can accurately quantify the presence of neutral, acidic and total cannabinoids in plant tissue extracts and the direct coupling of HPLC and MS enables unequivocal identification of CBD, CBN, Δ^9-THC, CBCh and acidic cannabinoids. Moreover our method is fast and does not require any preliminary derivatization of the samples; dried plant leaves are extracted and analyzed efficiently without interferences due to the vegetal matrix.

The obtained analytical data showed that nearly all the examined samples, grown in different northern Italian areas, presented content of total Δ^9-THC very low (less than 0.04 %), except the few female samples of Ungherese cultivar harvested in August. Total CBD content was considerably higher than total Δ^9-THC in the majority of the plants and was influenced by the time of harvesting; it has to be stressed the important contribution of CBDA to the levels of total CBD. According to the graphs presented by de Meijer et al. [2] we could conclude that all the analyzed plants were to be classified as fiber, or non-drug, type.

On the basis of characteristic chromatographic profiles the chemical variation of several cannabis plants was examined; the obtained data are expected to be of interest to study the correlation between cannabinoid profiles, environmental conditions and geographical origin.

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