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Determination of primary active constituents in *Cannabis* preparations by high-resolution gas chromatography/flame ionization detection and high-performance liquid chromatography/UV detection☆

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Abstract

For a complete quantitative analysis of primary active constituents in *Cannabis* preparations Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN), we have compared two different chromatographic techniques, high-resolution gas chromatography (HRGC)/flame ionization detection (FID) and high-performance liquid chromatography (HPLC)/UV. The two different methods have been validated using crude drug (hashish) with methyloleate and tetraphenylethylene as internal standard for HRGC/FID and HPLC/UV, respectively.

Keywords: Validation; Liquid chromatography; Gas chromatography; Δ^9-THC; CBD; CBN

1. Introduction

The active substances content of *Cannabis* is very complex and still to be defined. From *Cannabis*, to date, more than 500 chemical compounds have been identified, of which more than 60 belong to the class of cannabinoids; of these, the most important are: cannabinol (CBN), cannabidiol (CBD), and some isomers of tetrahydrocannabinol (THC), Δ^9-THC and Δ^8-THC (Fig. 1).

The main pharmacologically active cannabinoid is Δ^9-THC and it is used as a reference substance in evaluating the drug intensity of various preparations based on *Cannabis* (hashish, marijuana, hash oil). In addition, Δ^9-tetrahydrocannabinolic acid is also found in the resin, this compound is not itself active, but is readily converted to Δ^9-THC by the heat produced when *Cannabis* resin is smoked [1,2].

From the botanical point of view there is only one species of canapa plant, "*Cannabis sativa* L.", but there are two types of *Cannabis sativa*: one has low content of Δ^9-THC and high content of cannabidiol;

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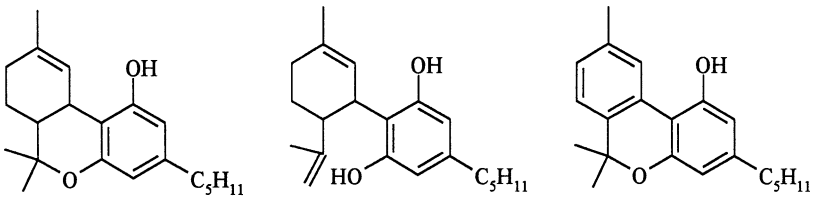

Fig. 1. Chemical structures of cannabinoids: 1, Δ^9-THC; 2, CBD; 3, CBN.

the other, a low content of cannabidiol and a high content of Δ^9-THC. The first is used in the production of fiber (fiber type); the second for its euphoric effects (drug type) [3]. In regard to cultivation, when one has to evaluate whether the plant is "drug type" or "fiber type," it is important and useful to determine both the active principle content and the phenotypic index, and as a result, the CBD and CBN content too. It is then possible, applying the formula for the percentage contents of the three fundamental cannabinoids, to obtain what is defined as the "phenotypic ratio":

$$\text{phenotypic ratio} = \frac{\%\Delta^9\text{-THC} + \%\text{CBN}}{\%\text{CBD}}$$

A value greater than one can indicate "drug type" [4–6].

On the other hand, for forensic application, prevailing scientific opinion considers that to define a *Cannabis* plant as "drug type" it should have in its leaves and inflorescences a Δ^9-THC content equal to at least 0.5% [5].

In forensic toxicology, the analytic investigations on preparations of *Cannabis* are carried out almost exclusively using high-resolution gas chromatography (HRGC) [3]. This technique makes it possible to adequately analyze the material, but the high temperature required in this method tends to lead to an overestimation of the THC content, mainly due to the decarboxylation of the tetrahydrocannabinolic acid [7].

Gas chromatographic technique is, therefore, useful in forensic toxicology to verify the psychoactivity of the vegetal material, but it is difficult to defend it in analytic toxicology where it would be more useful to examine the content of cannabinoids with a technique like that in high-performance liquid chromatography (HPLC) [8–12] which does not alter the contents of the active principles.

The experimental work we have carried out is based on the convalidation of two instrumental methods, HRGC and HPLC, applied to a single extract of vegetal material. The HRGC method we have previously published in an earlier study already experimented in gas chromatography [13], but we have reduced the quantity of vegetal material analyzed from 100 to 50 mg so as to render the sample appropriate for the analysis in HPLC without further treatment.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol and HPLC grade acetonitrile were purchased from J.T. Baker (Daventer, Holland). KH_2PO_4 and methyloleate were obtained from Merck (Darmstadt, Germany).

Δ^9-THC standard (10 mg ml^{-1}, vial of 1 ml) was obtained from S.A.L.A.R.S. (Como, Italy); CBD and CBN standards were purchased from Sigma; 1,1,2,2-tetraphenylethylene was purchased from Fluka and samples of hashish were obtained from illecit import seizures.

Ultrapure water (18.2 MΩ) was obtained by means of a Milli-Q apparatus by Millipore Corporation (Bedford, MA) and was used for mobile phase preparation. The mobile phase was vacuum filtered through a 0.45 μm pore size filter (Agilent Technologies).

2.2. Apparatus

2.2.1. HRGC

The HRGC analysis were performed using an Agilent Technologies (Palo Alto, CA) model HP 6890 series equipped with a split–splitless injector,

electronic pressure control, HP 6890 autosampler and flame ionization detector (FID).

The column used was an HP5 (5% phenyl methyl silicone) fused-silica capillary column (15 m × 0.32 mm i.d., 0.25 μm film thickness), which was also obtained from Agilent Technologies. Hydrogen and helium were used as the carrier and make-up gas, respectively. Hydrogen was obtained by Whatman gas generators (model 75-32-220-V452) and the flow rate of hydrogen was 1.3 ml min^{-1}; air helium and hydrogen were of high-purity grade.

Temperature programming was used for the successful elution of all the peaks of interest. The column temperature was programmed from an initial 180 to 220 °C at 40 °C min^{-1}, followed by a gradient of 5 °C min^{-1} to 240 °C and a gradient of 25 °C min^{-1}

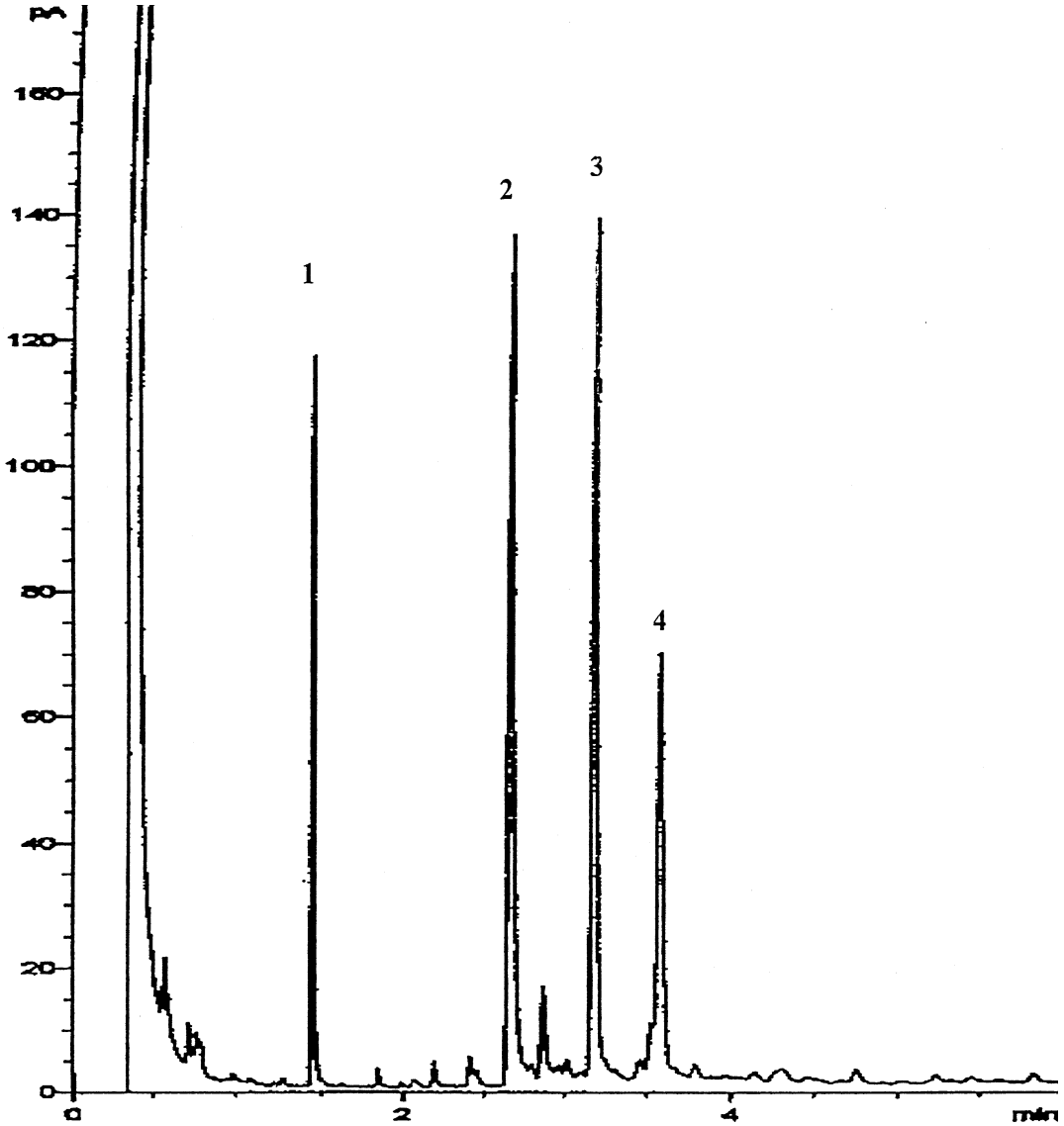

Fig. 2. Typical HRGC chromatogram of a hashish sample after extraction. Peaks: 1, IS; 2, CBD 0.39 mg ml^{-1}; 3, Δ^9-THC 0.40 mg ml^{-1}; 4, CBN 0.20 mg ml^{-1}.

to a final temperature of 280 °C. The total analysis time was 8 min. The injector and detector temperatures were 280 and 300 °C, respectively. The injector was operated in split mode with a split rate of 30:1.

Δ^9-THC, CBD and CBN were identified by comparing their retention time of the authentic compounds (S.A.L.A.R.S., Sigma). Under these conditions, the retention times of Δ^9-THC, CBD, CBN and IS were 2.99, 2.50, 3.36 and 1.37 min, respectively (Fig. 2). All data were acquired with HP Chemstation version A.06.03.

2.2.2. HPLC

The HPLC analysis were performed using a Hewlett-Packard model 1050 chromatograph with autosampler and UV detector (MWD 1050).

Separations were obtained on a reversed phase column (Lichrocart, C18 125-4 mm, 5 μm, Merck) connected with a guard column (Lichrosphere 100 RP18, 5 μm, Merck). The injection was effected through a 10 μl loop. The mobile phase, a mixture of acetonitrile—phosphate buffer (pH 5.0) (65:35 (v/v)), was filtered through a membrane filter and degassed by an ultrasonic apparatus. The flow rate was maintained at 1.5 ml min^{-1} (90 bar), the column was maintained at room temperature and detection was effected at 220 nm. Run time was 22 min.

Under these conditions, the retention times of Δ^9-THC, CBD, CBN and IS were 12.68, 6.13, 9.85 and 17.05 min, respectively (Fig. 3). All data were acquired with HP Chemstation A.01.03.

2.3. Standard solutions

Δ^9-THC standard solutions were prepared from standard solution 10.00 mg ml^{-1} in methanol up to final concentration of 0.50–0.20–0.10–0.05 mg ml^{-1}.

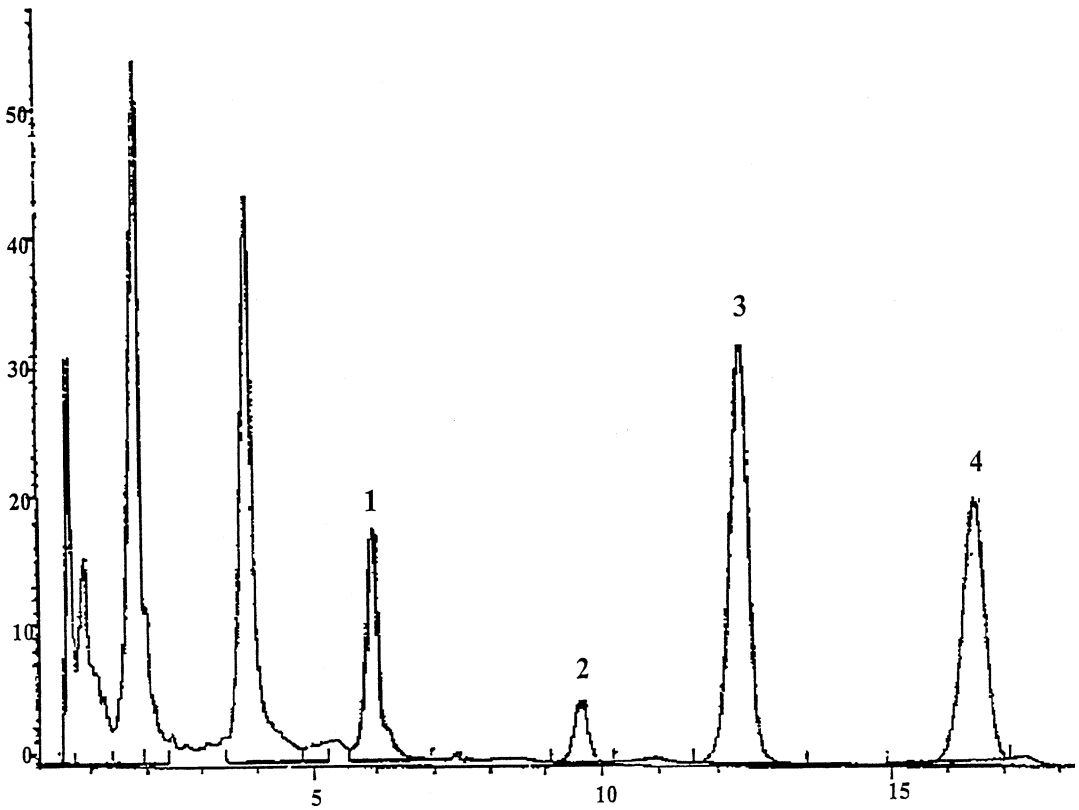

Fig. 3. Typical HPLC chromatogram of a hashish sample after extraction. Peaks: 1, CBD 0.15 mg ml^{-1}; 2, CBN 0.04 mg ml^{-1}; 3, Δ^9-THC 0.49 mg ml^{-1}; 4, IS.

CBD and CBN standard solutions were prepared from standard solution 1.00 mg ml^{-1} in methanol up to final concentration of 0.50–0.25–0.1–0.05 mg ml^{-1} for CBD and 0.2–0.1–0.04–0.02 mg ml^{-1} for CBN.

2.4. *Cannabis's* standard samples

Cannabis's standard samples (CSS) containing Δ^9-THC, CBD, CBN, were obtained weighing different amounts of hashish and quantifying them by using the calibration curve.

CSS were prepared to be used in the determination of precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). Preparations of *Cannabis* (hashish and marijuana) as such are, if not properly stored, subjected to degradation. Nonetheless, the concentration of active principles obtained following the extraction of *Cannabis* preparation remain unchanged if properly stored.

2.5. IS solution for HRGC

Internal standard solution (IS) was prepared dissolving 0.1 ml of methyloleate in a 500 ml volumetric flask. Methanol was used as the diluent.

2.6. IS solution for HPLC

Internal standard solution (IS) was prepared by accurately weighing tetraphenylethylene (10 mg) and transferring into a 200 ml volumetric flask. It was dissolved, made up to volume with methanol and mixed.

2.7. Buffer

The buffer solution was prepared by dissolving 1.72 g potassium phosphate monobasic in water to 1 l adjusted to pH 5 with KOH 0.1N.

2.8. Samples solutions preparation for HRGC/HPLC

Fifty milligrams of hashish were placed in a 10-ml conical centrifuge tube. Five milligrams of methanol are added and mixing on the Vortex mixer for 10 s, resting for 5 min. Stirring and resting is repeated twice. Samples are centrifuged at 4000 × *g* for 5 min [13].

Aliquots of organic layer were pipetted into vials for autosamples and added to IS solution (0.5 ml organic layer + 0.5 ml of IS for HRGC and 0.1 ml + 0.9 ml of IS for HPLC).

3. Validation

Hashish as well as marijuana are complex vegetal matrices containing a variety of compounds not completely know; thus, it is not easy to determine "blank" samples of hashish to be added with know amount of standards (Δ^9-THC, CBD and CBN) in order to obtain ideal standard samples.

After a preliminary calibration made using absolute standards, we decided to validate the method employing the different weights of hashish (10–200 mg) in order to obtain samples containing different concentration of active principles without influencing the validity of the method, as shown by calculated statistical parameters.

The observation of an interference peak on CBN does not limit the validity of this method. Finally, it remains to be considered that in forensic toxicology samples having concentration of Δ^9-THC of 0.5% (0.05 mg ml^{-1} after solubilization—extraction through the proposed method) are cut-off values in order to consider a street material as a drug of abuse.

3.1. Linearity

The linearity of the method was verified using 12 samples of Δ^9-THC, CBD and CBN standard at the known concentration in the range 0.05–0.50 mg ml^{-1} for Δ^9-THC and CBD, and 0.02–0.20 mg ml^{-1} for CBN.

Linear regression lines were obtained by plotting the peak area (ratios of Δ^9-THC, CBD, CBN standard's peak area to IS peak area) versus the analyte concentration using the least squares method (Table 1).

3.2. Precision

The intra-day precision was evaluated by replicate analysis ($n = 6$) of two type of hashish containing the following concentration of Δ^9-THC, CBD and CBN: 0.703, 0.371 and 0.078 mg ml^{-1} for HRGC, and 0.220, 0.079 and 0.022 mg ml^{-1} for HPLC.

Table 1
Regression analysis of the determination of Δ^9-THC, CBD, CBN in HRGC and HPLC

Parameters	Δ^9-THC	CBD	CBN
GC			
Linear range (mg ml^{-1})	0.050–0.500	0.050–0.500	0.02–0.200
Number of solutions (n)	12	12	12
Calibration curve	$y = 0.643x - 0.0663$	$y = 0.638x - 0.0367$	$y = 0.032x - 0.0267$
Correlation coefficient (r)	0.9997	0.9999	0.9998
Intercept (a)	−0.0663	−0.0367	−0.0267
±S.D. of a	0.0900	0.0565	0.0319
Slope (b)	0.643	0.638	0.032
±S.D. of b	0.0330	0.0198	0.0279
S.D.% of b	2.20	1.28	1.80
HPLC			
Linear range(mg ml^{-1})	0.050–0.500	0.050–0.500	0.02–0.200
Number of solutions	12	12	12
Calibration curve	$y = 0.270x - 0.0200$	$y = 0.622x - 0.1056$	$y = 0.428x - 0.0048$
Correlation coefficient (r)	0.9989	0.9999	0.9995
Intercept (a)	−0.0200	−0.1056	−0.0048
±S.D. of a	0.0784	0.060	0.0242
Slope (b)	0.270	0.622	0.428
±S.D. of b	0.02852	0.022	0.0281
S.D.% of b	4.27	1.51	4.22

For inter-day precision, the samples were analyzed in triplicate on six different days over a 6-week period ($n = 18$) (Table 2).

The sample with concentration 0.7 mg ml^{-1} has been evaluated by our routine method and this determination has been carried out because this sample of street drug of abuse was the only available, and it was deemed necessary to evaluate the accuracy and precision of the method in the actual conditions. Moreover, it should be noticed that the statistical parameters should not be evaluated on the basis of absolute values, but rather on the basis of the variations determined by a sequence of analysis. Thus, we decided not to modify data obtained through our routine analysis.

3.3. Accuracy

Accuracy was established by comparing the peak area ratios for amounts of Δ^9-THC, CBD, CBN in CSS and the peak area ratios for the same analytes in

Table 2
Intra and inter assay precision

	Intra-day ($n = 6$)		Inter-day ($n = 18$)	
	Mean[a] ± S.D.	CV (%)	Mean[a] ± S.D.	CV (%)
GC				
Δ^9-THC	4.453 ± 0.057	1.28	4.285 ± 0.089	2.07
CBD	2.329 ± 0.030	1.30	2.240 ± 0.034	1.50
CBN	0.475 ± 0.007	1.40	0.461 ± 0.021	4.63
HPLC				
Δ^9-THC	0.578 ± 0.011	1.82	0.560 ± 0.025	4.50
CBD	0.221 ± 0.004	1.63	0.213 ± 0.008	3.84
CBN	0.088 ± 0.001	1.50	0.092 ± 0.004	4.50

[a] The mean was referred by the peak area ratios of the analyte to the internal standard.

Table 3
Recovery

	Nominal concentration (mg ml^{-1})	Nominal A_x/A_{IS} [a]	Found A_x/A_{IS} [a]	%Rec[a] ($n = 6$) (mean ± S.D.)	CV (%)
GC					
Δ^9-THC	0.701	4.439	4.390	98.885 ± 1.828	1.85
CBD	0.370	2.323	2.263	97.423 ± 1.655	1.70
CBN	0.078	0.475	0.438	92.211 ± 1.604	1.74
HPLC					
Δ^9-THC	0.220	0.570	0.541	94.801 ± 3.266	3.45
CBD	0.079	0.220	0.216	98.560 ± 3.330	3.38
CBN	0.022	0.087	0.093	106.997 ± 5.224	4.88

[a] The mean was referred by the peak area ratios of the analyte to the internal standard.

the *Cannabis* preparation. It is expressed as a recovery percentage (%Rec) and the results are shown in Table 3.

3.4. Limit of detection

The LOD, is estimated at three times the signal to noise ratio (S/N = 3).

Since the impossibility of obtaining a specific blank, attention was focused on a peak area, provided by a peak (PN) that was always present in the vegetal extracts with mobility comparable to that of interest to us. The ratio between the PN area was evaluated with that of the internal standard, with the ratios obtained from the analysis of samples containing concentrations of Δ^9-THC, CBD, CBN such that the ratios between the peak areas with the internal standard were at least three times that obtainable with the PN substance.

The results obtained are set out in Table 4, and refer to the following concentrations: 0.023, 0.018 and 0.015 mg ml^{-1} for Δ^9-THC, CBD and CBN, respectively, for HRGC and 0.015, 0.011 and 0.007 mg ml^{-1} for Δ^9-THC, CBD and CBN, respectively, for HPLC (Figs. 4 and 5).

3.5. Limit of quantification

The LOQ for this assay procedure was investigated by evaluating the accuracy and precision of analysis of CSS (Table 5).

Table 4
Limit of detection (LOD) of Δ^9-THC, CBD CBN in HRGC and HPLC

	PN mean[a] + S.D. ($n = 6$)	LOD	PN mean[b] − S.D. ($n = 6$)	LOD
GC				
Noise	0.012 + 0.0030	0.015		
Δ^9-THC			0.083 − 0.0022	0.081
CBD			0.080 − 0.0099	0.070
CBN			0.071 − 0.0049	0.066
HPLC				
Noise	0.002 + 0.0005	0.002		
Δ^9-THC			0.032 − 0.0010	0.031
CBD			0.027 − 0.0018	0.025
CBN			0.008 − 0.0010	0.007

[a] The mean was referred by the peak area ratios of the peak at $t_r = 1.97$ min for HRGC (Fig. 4) and $t_r = 5.55$ for HPLC (Fig. 5), to the internal standard.

[b] The mean was referred by the peak area ratios of the analyte to the internal standard.

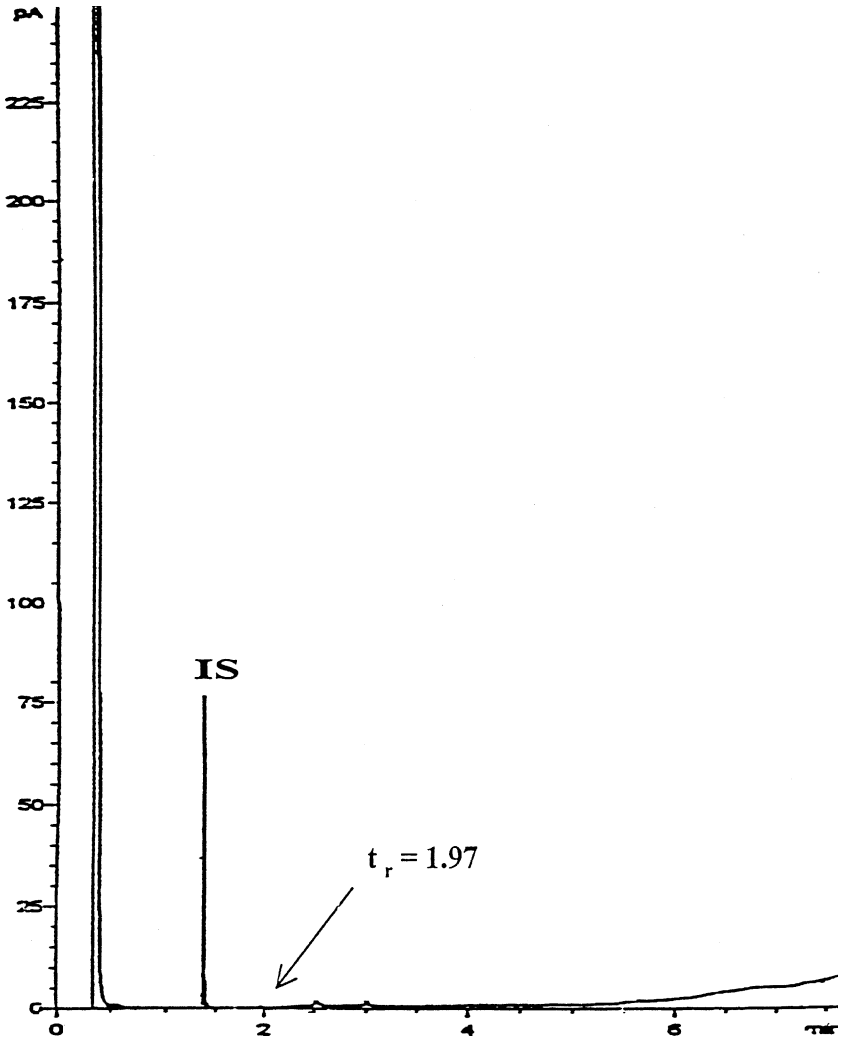

Fig. 4. LOD in HRGC.

Table 5
Limit of quantification (LOQ)

Compound	LOQ[a]	Mean[b] ± S.D. ($n = 6$)	CV (%)	%Rec[b] ± S.D. ($n = 6$)	CV (%)
GC					
Δ^9-THC	0.034	0.152 ± 0.0044	2.90	100.280 ± 1.410	1.41
CBD	0.041	0.222 ± 0.0094	4.22	100.868 ± 4.254	4.22
CBN	0.026	0.145 ± 0.0018	1.21	100.652 ± 1.219	1.21
HPLC					
Δ^9-THC	0.044	0.110 ± 0.0022	1.96	100.151 ± 1.4477	1.45
CBD	0.014	0.035 ± 0.0004	1.05	103.346 ± 2.5283	2.45
CBN	0.018	0.072 ± 0.0023	3.24	102.733 ± 3.9945	3.89

[a] Unit: mg ml^{-1}.
[b] The mean was referred by the peak area ratios of the analyte to the internal standard.

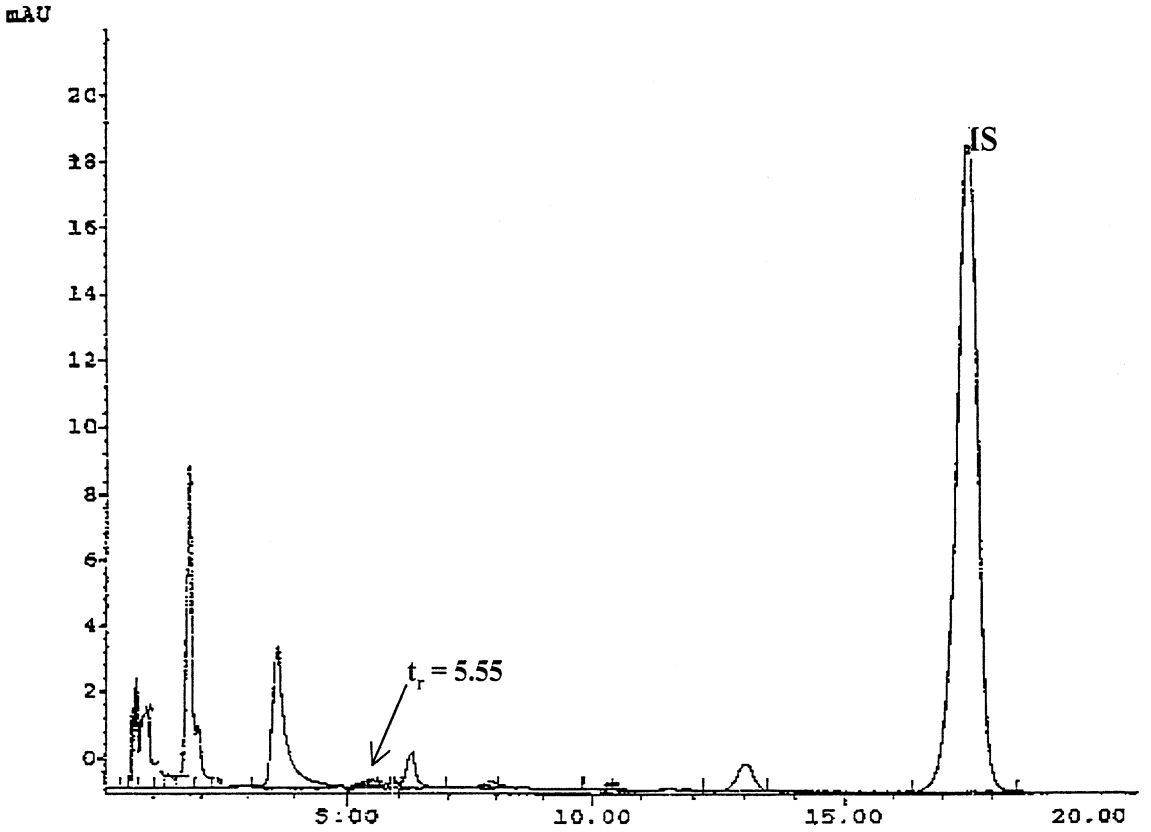

Fig. 5. LOD in HPLC.

4. Conclusions

The results showed that parameters of validation of both methods (specificity, linearity range, precision, accuracy, LOD and LOQ) proved to be entirely satisfactory and falling within the range of the standard operative procedure (SOP). By applying the two methods, on the same extract solution, for the characterization of the same material, hashish as well as marijuana, it was possible to obtain additional useful information in the toxicological investigation concerning these specific abuse drugs. HRGC/FID technique allows a best characterization of the material concerning its rate of psychoactivity. The employment of HPLC/UV data in forensic toxicology allows the identification of all easily decarboxilable acid compounds that could produce Δ^9-THC, the psychoactive component of *Cannabis* preparations.

This work shows that the HRGC/FID method is similar in terms of reliability, reproducibility and sensitivity to HPLC/UV. Both methods compare favourably with one another.

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