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Simultaneous Determination of Cannabidiol, Cannabinol, and Δ^9-Tetrahydrocannabinol in Human Hair by Gas Chromatography-Mass Spectrometry

Jin Young Kim[1,2], Sung Ill Suh[1], Moon Kyo In[1], Ki-Jung Paeng[2], and Bong Chul Chung[3]

[1]Drug Analysis Laboratory, Forensic Science Division, Supreme Prosecutors' Office, Seocho-dong, Seocho-gu, Seoul 137-730, Korea, [2]Department of Chemistry, Graduate School, Yonsei University, Seoul 120-749, Korea, and [3]Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, Seoul 130-650, Korea

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An analytical method was developed for evaluating the cannabidiol (CBD), cannabinol (CBN), and Δ^9-tetrahydrocannabinol (Δ^9-THC) level in human hair using gas chromatography-mass spectrometry (GC-MS). Hair samples (50 mg) were washed with isopropyl alcohol and cut into small fragments (< 1 mm). After adding a deuterated internal standard, the hair samples were incubated in 1.0 M NaOH for 10 min at 95°C. The analytes from the resulting hydrolyzed samples were extracted using a mixture of *n*-hexane-ethyl acetate (75:25, *v/v*). The extracts were then evaporated, derivatized, and injected into the GC-MS. The recovery ranges of CBD, CBN, and Δ^9-THC at three concentration levels were 37.9-94.5% with good correlation coefficients (r^2 >0.9989). The intra-day precision and accuracy ranged from -9.4% to 17.7%, and the inter-day precision and accuracy ranged from -15.5% to 14.5%, respectively. The limits of detection (LOD) for CBD, CBN, and Δ^9-THC were 0.005, 0.002, and 0.006 ng/mg, respectively. The applicability of this method of analyzing the hair samples from cannabis abusers was demonstrated.

Key words: GC-MS, Hair analysis, Cannabidiol, Cannabinol, Tetrahydrocannabinol

INTRODUCTION

The hemp plant *Cannabis sativa* is one of the most widely abused illicit drugs in Korea (Supreme Prosecutors' Office, 2004). A dry, pulverized green and/or brown mix of the flowers and leaves of *Cannabis sativa* is usually smoked either as a cigarette or in a pipe. Cannabis contains more than 420 chemicals including at least 61 cannabinoids (Turner *et al.*, 1980). Although cannabis contains many cannabinoid components, Δ^9-tetrahydrocannabinol (Δ^9-THC) is the most prominent psychoactive cannabinoid constituent, and the potency of marijuana is determined by its concentration . Cannabidiol (CBD) and cannabinol (CBN), like Δ^9-THC, are constituents that can be isolated from both *Cannabis sativa* and cannabis smoke (Staub *et al.*, 1999).

Δ^9-THC undergoes extensive metabolism in humans into 11-hydroxy- and 8-hydroxy-Δ^9-tetrahydrocannabinol and finally to 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH), which is excreted in the form of glucuronide conjugates with several different conjugated species (Wall *et al.*, 1970; Agurell *et al.*, 1976; Hawks *et al.*, 1982).

Identifying CBD, CBN, and Δ^9-THC in decontaminated hair indicates exposure to cannabis (Cirimele *et al.*, 1996; Baptista *et al.*, 2002; Musshoff *et al.*, 2002; Uhl and Sachs, 2004). Besides the parent drug, Δ^9-THC, determining the level of the main metabolite, THCCOOH, is recommended for distinguishing passive exposure from active, intentional ingestion. In contrast, determining the THCCOOH level can only be performed in a separate examination using gas chromatography-tandem mass spectrometry (GC-MS-MS), GC-MS-negative ion chemical ionization (NCI) with a high-volume injection or an additional clean up before GC-MS-NCI (Mieczkowski, 1995; Uhl, 1997; Sachs

Correspondence to: Jin Young Kim, Drug Analysis Laboratory, Forensic Science Division, Supreme Prosecutors' Office, Seocho-dong, Seocho-gu, Seoul 137-730, Korea
Tel: 82-2-535-4173, Fax: 82-2-535-4175
E-mail: paxus@spo.go.kr

and Dressler, 2000; Moore *et al.*, 2001; Uhl and Sachs, 2004). However, these latter analytical methods are time consuming and technically expensive. Therefore, an analysis of hair to identify cannabis use is often restricted to the identification of CBD, CBN, and Δ^9-THC, which can be detected by routine gas chromatography-mass spectrometry (GC-MS).

The aims of this study were to establish and validate a GC-MS method for the simultaneous determination of CBD, CBN, and Δ^9-THC in human hair, and to investigate the chemical stability of these analytes in the alkaline medium used for extracting the drugs from the matrix. This GC-MS method was successfully applied to the analysis of CBD, CBN, and Δ^9-THC in the hair samples of cannabis abusers.

MATERIALS AND METHODS

Materials

The CBN and CBD were purchased from Sigma Israel Chemicals LTD. (Jerusalem, Israel). The Δ^9-THC and Δ^9-tetrahydrocannabinol-d_3 (Δ^9-THC-d_3) were purchased from Cerilliant (Austin, TX, U.S.A.) in vials at a concentration of 1000 and 100 μg/mL in methanol, respectively. The methanol, *n*-hexane, isopropyl alcohol, and ethyl acetate were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). All solvents were of high performance liquid chromatography (HPLC) grade. The *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), and *N*-trimethylsilylimidazole (TMSI) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.).

Preparation of solutions

The CBD and CBN (10 mg) were dissolved in methanol (10 mL) to prepare a stock solution. Subsequently, 1, 10, and 100 μg/mL CBD and CBN solutions were prepared by dilution. Working standards solutions of Δ^9-THC (1 μg/mL, 10 μg/mL) and Δ^9-THC-d_3 (1 μg/mL) were prepared using the appropriate dilution with methanol. All of these solutions were stored at -20°C in the absence of light until needed.

Stability

The stability of CBD, CBN, and Δ^9-THC under alkaline digestion conditions at 95°C for 10 min was determined by measuring the recovery of the analytes after digestion using 1 mL of a 1.0 M NaOH solution spiked with each analyte at three concentration levels (10, 75, and 200 ng/mL) in five replicate experiments.

Preparation of Materials

The drug-free human hair was obtained from a 36-year-old male volunteer, and was used as the control and blank matrix for preparing the matrix-matched calibration solutions for CBD, CBN, and Δ^9-THC. Hair samples (n = 22) from possible cannabis abusers were included in the analysis among the suspected abusers' samples received from the Narcotics Department at the Seoul District Prosecutors' Office between June and November 2004. These samples, weighing more than 30 mg, were generally pulled out or cut as close as possible to the skin from the posterior vertex. The total length of the hair samples was measured, and any special treatment features were noted. The samples were stored under dry conditions at room temperature.

Sample preparation

The hair samples were washed three times with 10 mL isopropyl alcohol, dried in a fume hood, cut with scissors into small fragments (<1 mm), and weighed. A hair sample (50 mg) was then transferred to a silanized glass test tube (16 × 100 mm) containing 50 ng of Δ^9-THC-d_3 as an internal standard. The analytes were isolated from the protein matrix *via* consecutive hydrolysis in a 1.0 M NaOH solution at 95°C for 10 min in. After cooling, 5 mL *n*-hexanė-ethyl acetate (75:25, *v/v*) was added to the incubation mixture and the analytes were extracted by mechanical shaking for 20 min at approximately 60 cycles per minute (cpm) and centrifuged for 5 min at 2800 × *g*. The organic phase was transferred to a silanized test tube (12 × 100 mm) and evaporated under a nitrogen stream using a TurboVap LV evaporator. The residue was dried in a vacuum desiccator over P_2O_5-KOH for at least 30 min. The trimethylsilyl derivatives were formed by a reaction with 40 μL of MSTFA-TMCS-TMSI (100:2:5, *v/v/v*) in a dry heating block at 60°C for 15 min. An aliquot (1 μL) of the sample solution was injected into the GC-MS.

Gas chromatography-mass spectrometry

GC-MS analysis was performed using an Agilent Technologies 6890N GC (Palo Alto, CA, U.S.A.) coupled to a 5973N Mass Selective Detector. The system was controlled by Drug Analysis Chemstation G1701CA software (C.00.00, Agilent Technologies). The gas chromatograph was equipped with a capillary column (Ultra-1, 25 m × 0.20 mm i.d., 0.33 μm, J&W Scientific, Folsom, CA, U.S.A.). The GC temperature program used is as follows: initial temperature was 180°C, held for 0.5 min, increased to 240°C at a rate of 5 °C/min, held for 2.5 min, and then increased to 300°C at a rate of 25 °C/min. The injector temperature was 260°C and a splitless injection was used with a split-valve off-time of 0.5 min. The flow rate of the carrier gas (helium) was 1.0 mL/min. The mass spectrometer was operated at 70 eV in the electron impact mode with selected ion monitoring (SIM) for quantification. The selected ion groups of the derivatized analytes monitored

are as follows: *m/z* 390 and 301 (CBD); *m/z* 367 and 368 (CBN); *m/z* 386 and 303 (Δ^9-THC); *m/z* 389 (Δ^9-THC-d_3).

Validation of analytical method

In order to evaluate the specificity of the method, a blank hair sample was prepared, analyzed, and examined for its response in each of the analyte and internal standard (IS) chromatographic profiles. The linearity of the method for CBD, CBN, and Δ^9-THC was examined in the concentration range 0.05-5.0 ng/mg. A six-point calibration curve was established at each concentration using 50 mg samples of drug-free human hair spiked with methanolic solutions of analytes. The internal standard versus analyte concentrations was determined using linear regression analysis of the peak area ratios of the analyte. The percentage accuracy was determined by dividing the measured concentration by the theoretical concentration and multiplying this result by 100. The inter-day and intra-day validation was performed by calculating the precision and accuracy for four concentrations (0.05, 0.2, 1.5, and 4.0 ng/mg) corresponding to the quality control (QC) samples of the method. The limits of detection (LODs) for CBD, CBN, and Δ^9-THC were calculated based on the injected mass giving a signal that was three times the peak-to-peak noise of the background signal. The limit of quantification (LOQ) was defined as the lowest concentration that could be determined with a coefficient of variation (CV) < 20%. The extraction recoveries of each analyte were quantified at three concentrations (0.2, 1.5, and 4.0 ng/mg) of five replicates.

RESULTS AND DISCUSSION

Gas chromatography-mass spectrometry

The chromatographic separation of CBD, CBN, and Δ^9-THC was optimized for improving the peak shape and retention characteristics (Fig. 1). The analytes were identified by a retention time comparison with the reference standards. The SIM chromatograms for the analytes clearly indicated the order of elution (Table I).

Fig. 2 shows the EI mass spectra of the trimethylsilylated (a) CBD, (b) CBN, (c) Δ^9-THC, and (d) Δ^9-THC-d_3. The molecular and fragmented ions were formed in good abundance in all cases, and were used as the quantification ions. A derivatization reaction was performed to convert the polar -OH groups of the analytes into thermally stable, non-polar groups. The EI mass spectra of trimethylsilylated CBD, CBN, and Δ^9-THC in the human hair samples were consistent with those of the reference standards.

Decontamination

Passive drug absorption on hair samples must be considered as a possible source of any false-positive results. Therefore, an extra step was needed to remove external contaminants before beginning the analytical procedure. In order to confirm that CBD, CBN, and Δ^9-THC were adsorbed on the hair surface and eluted with washing, the positive control samples from the suspected

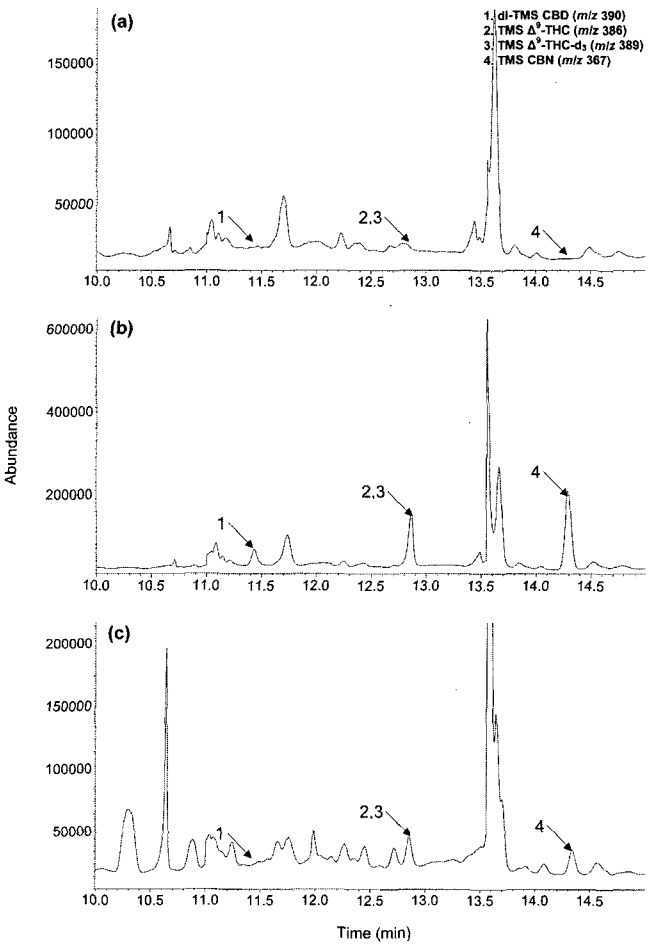

Fig. 1. SIM GC-MS chromatograms of CBD, CBN, and Δ^9-THC in human hair. (a) blank hair, (b) hair sample spiked at 0.5 ng/mg of each analyte, (c) positive control sample.

Table I. *Retention times and characteristic ions of trimethylsilylated* (TMS) CBD, CBN, and Δ^9-THC

	R_T (min)[a]	RR_T[b]	Molecular mass (g/mol)	Characteristic ions (*m/z*)[c]
di-TMS CBD	11.43	0.89	458	**390**, 301
TMS Δ^9-THC-d_3 (IS[d])	12.83	-	389	389
TMS Δ^9-THC	12.85	1.00	386	**386**, 303
TMS CBN	14.28	1.11	382	**367**, 368

[a]Retention time
[b]Retention time relative to that of the internal standard
[c]The ion used for quantification is given in bold
[d]Internal standard

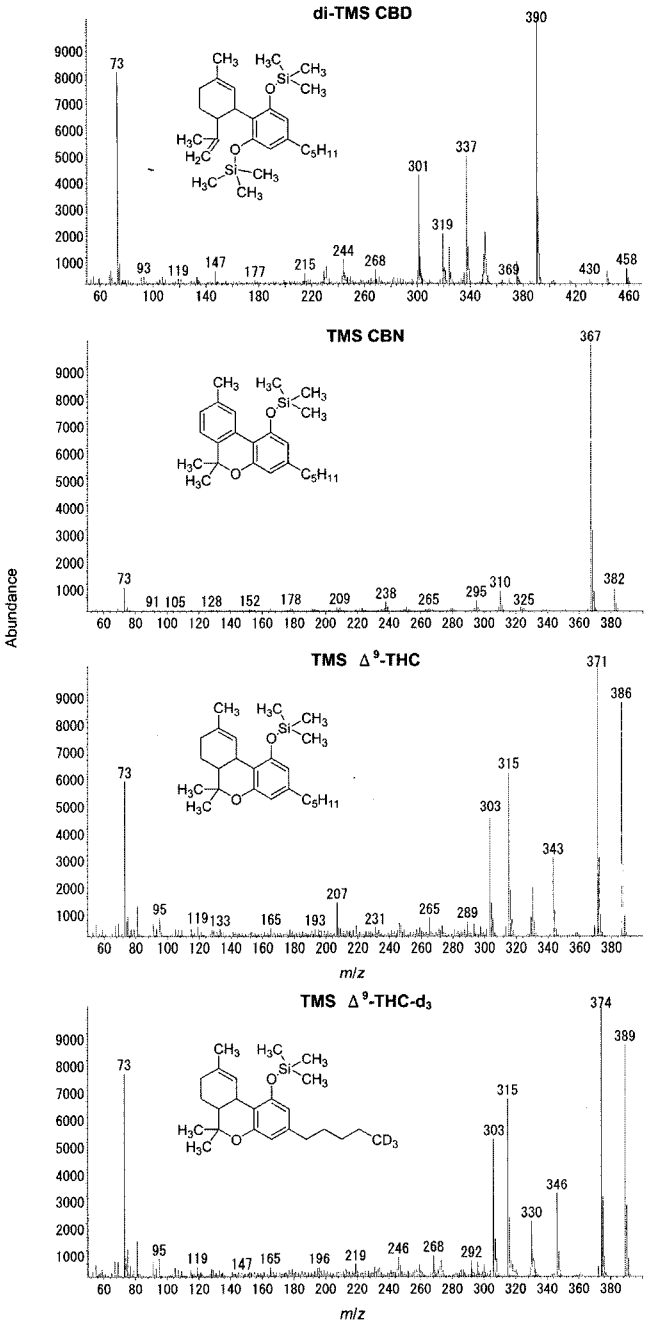

Fig. 2. EI mass spectra of trimethylsilylated CBD, CBN, Δ^9-THC, and Δ^9-THC-d_3 (internal standard).

abusers were washed using the aforementioned washing procedure and washed again with 10 mL isopropyl alcohol. The washing efficiency of the isopropyl alcohol wash after the normal wash of the hair samples was evaluated. None of the target analytes were detected in the isopropyl alchohol wash, demonstrating that the hair-washing procedure is sufficient for decontamination.

Stability study of analytes

The results after digestion with 1 mL of 1.0 M NaOH at 95°C for 10 min at the three concentration levels (10, 75, and 200 ng/mL) confirmed the expectation that the stability of the analytes might be affected during the digestion procedure. The severe digestion conditions appeared to affect the stability of CBD (Fig. 3). Therefore, the effect of the digestion procedure should be considered when interpreting the analysis of cannabinoids in hair.

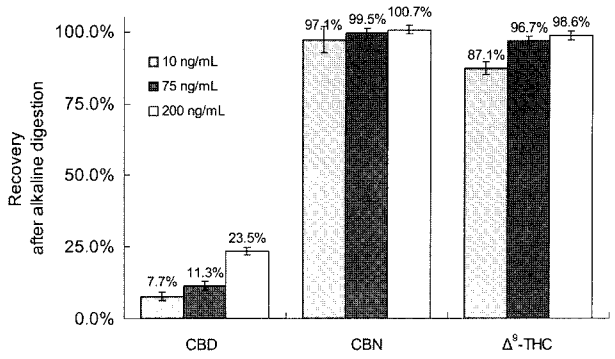

Fig. 3. The stability of CBD, CBN, and Δ^9-THC after digestion in 1 mL of 1.0 M NaOH solution at 95°C for 10 min.

Evaluation of validation data

The described method was validated by determining the selectivity, linearity, precision and accuracy, the limit of detection (LOD), the limit of quantification (LOQ), and recovery. After derivatization, CBD, CBN, and Δ^9-THC can be well defined chromatographically. A comparison of the SIM chromatograms for the blank hair sample and the hair sample fortified with the analytes and an internal standard confirmed there were no interfering peaks near the retention times of the analytes in the hair sample matrix (Fig. 1).

Table II. Validation data for the analysis of CBD, CBN, and Δ^9-THC in human hair

	CBD	CBN	Δ^9-THC
Concentration range (ng/mg)	0.05 – 5.0	0.05 – 5.0	0.05 – 5.0
Linearity[a] (r^2)	0.9989	0.9995	0.9992
LOD[b] (ng/mg)	0.005	0.002	0.006
LOQ[c] (ng/mg)	0.05	0.05	0.05
Recovery (% CV, n = 5)			
0.2 ng/mg	42.2 ± 5.8	93.4 ± 3.4	84.7 ± 2.4
1.5 ng/mg	37.9 ± 2.4	94.5 ± 1.5	85.9 ± 1.9
4.0 ng/mg	43.6 ± 0.4	94.0 ± 1.1	93.4 ± 1.8

[a]Linearity is determined by the linear correlation for the calibration curve.

[b]LOD : Limit of detection was defined as the concentration at which the characteristic ion was detectable on the corresponding mass chromatogram at S/N = 3 or greater.

[c]LOQ : Limit of quantification was defined as the lowest concentration on the calibration curve with a precision < 20% (% CV).

Table III. Precision[a] and accuracy[b] for the determination of CBD, CBN, and Δ^9-THC

Analyte	Nominal (ng/mg)	Intra-day (n=3)			Inter-day (n=5)		
		Mean (ng/mg)	Precision (% CV)	Accuracy (% bias)	Mean (ng/mg)	Precision (% CV)	Accuracy (% bias)
CBD	0.05	0.045	17.7	-9.4	0.047	6.2	-15.5
	0.2	0.23	5.2	14.8	0.22	4.9	12.1
	1.5	1.40	1.2	-6.6	1.39	1.8	-6.7
	4.0	4.04	5.3	0.9	4.08	3.6	2.1
CBN	0.05	0.047	11.9	-5.7	0.046	5.9	-7.3
	0.2	0.19	8.4	-4.2	0.20	9.0	-1.2
	1.5	1.49	1.6	-0.7	1.49	2.2	-0.9
	4.0	3.86	1.2	-3.5	3.85	3.3	-3.7
Δ^9-THC	0.05	0.057	12.9	14.1	0.057	12.3	14.5
	0.2	0.20	5.3	1.5	0.20	7.0	-1.7
	1.5	1.52	0.8	1.0	1.53	0.8	0.9
	4.0	3.89	3.3	-2.8	3.94	2.6	-1.4

[a]Expressed as the coefficient of variance of the peak area ratios of the analyte/internal standard.
[b]*Calculated as [(mean calculated concentration – nominal concentration)/nominal concentration] × 100*

Tables II and III show the summarized quantitative validation parameters. The calibration standards at the six concentration levels for each analyte were used to construct the calibration curves. The correlation coefficients were 0.9989-0.9995 for all the analytes. The LOQ was defined as the lowest concentration that permitted precision within 20% (% CV). The extraction recoveries for CBD, CBN, and Δ^9-THC were determined at three concentration levels with five replicates. The peak-area ratios for each analyte in the samples that had been spiked with analytes prior to extraction were compared with the samples to which the same levels of analytes had been added after extraction. The recoveries of the analytes with the exception of CBD were 84.7-94.5%. The recovery range of CBD was 37.9-43.6%, demonstrating that the digestion procedure can influence the stability of the analyte during alkaline digestion.

The intra-day precision and accuracy were obtained by analyzing three replicates of four different spiked hair samples (0.05, 0.2, 1.5, and 4.0 ng/mg). The precision was determined by calculating the CV of the degree of agreement between the measured and nominal concentrations of the spiked samples. The inter-day precision and accuracy were determined by analyzing the same spiked, human hair samples on each of five consecutive days. For the intra-day (n = 3) assays, the precision ranged from 0.8 to 17.7% for CBD, CBN, and Δ^9-THC, while the accuracy ranged from -9.4 to 14.8%. For the inter-day (n = 5) assays, the precision ranged from 0.8 to 12.3% for these compounds, while the accuracy ranged from -15.5 to 14.5%. These results were considered satisfactory considering the complexity of the biological matrix.

Application method

The method described in this report was applied to the analysis of hair samples obtained from suspected cannabis abusers. Fig. 1(c) shows the SIM chromatogram for a positive control sample obtained from a confirmed cannabis abuser. Table IV shows the quantitative results of the analytes in hair samples. The measured CBN concentration in the hair samples was superior to those of either CBD or Δ^9-THC. The average concentration of CBD, CBN, and Δ^9-THC was 0.04, 0.36, and 0.14 ng/mg, respectively.

In 15 of the 22 hair samples, CBN was the cannabinoid detected most frequently both alone and in association with other cannabinoids. This is might be due to the pyrolitic degradation of Δ^9-THC to CBN when smoked (Strano-Rossi and Chiarotti, 1999). CBD was the second frequently detected compound. In one hair sample, only Δ^9-THC was detected and in six hair samples, only CBN was detected (Table V).

Table IV. Quantitative results of CBD, CBN, and Δ^9-THC in hair samples of suspected cannabis abusers

Analyte	Analyzed samples (n)	Positive samples[a] (n)	Range[b] (ng/mg)	Mean (ng/mg)	Median (ng/mg)
CBD	22	6	0.02-0.05	0.04	0.04
CBN	22	14	0.05-1.38	0.36	0.14
Δ^9-THC	22	4	0.06-0.27	0.14	0.09

[a]Qualified data below LOD and the lower end of the calibration range were included in the number of positive samples.
[b]The concentration value below the lower end of the calibration range *at three to four times higher than the normal amount of hair* was included in measured concentration range.

Table V. Qualitative results from CBD, CBN, and Δ^9-THC in the twenty-two hair samples

CBD	CBN	Δ^9-THC	Numbers of samples
+	+	+	1
+	+	-	5
-	+	+	2
-	+	-	6
-	-	+	1
-	-	-	7
Total			22

CONCLUSIONS

The GC-MS method showed a better sensitivity, good linearity, and lower LOD and LOQ than other methods. It also appears useful for simultaneously determining the presence and concentration of CBD, CBN, and Δ^9-THC in human hair. The data obtained from the hair samples cannabis of abusers indicated that CBN was the most frequently detected cannabinoid, followed in succession by CBD and Δ^9-THC. This study also showed that the digestion procedure can affect the stability of analytes. Therefore, it is essential to check the stability of the analytes under the digestion medium when interpreting the hair analysis results.

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