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Journal of Pharmaceutical and Biomedical Analysis 36 (2005) 939–946
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A rapid and simple procedure for the determination of cannabinoids in hemp food products by gas chromatography-mass spectrometry

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Received 28 May 2004; received in revised form 30 July 2004; accepted 31 July 2004
Available online 22 October 2004

Abstract

A rapid and simple procedure using liquid–liquid extraction and subsequent gas chromatographic mass-spectrometric detection has been developed for determination of Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) in different hemp foods. After addition of Δ^8-tetrahydrocannabinol as internal standard, both solid and liquid specimens were extracted with two volumes of 2 ml of hexane/isopropanol (9:1): Chromatography was performed on a fused silica capillary column and analytes were determined in the selected-ion-monitoring (SIM) mode. The method was validated in the range 1–50 ng/ml liquid samples or 1–50 ng/g solid samples for THC and CBN, and 2–50 ng/ml or ng/g for CBD. Mean recoveries ranged between 78.8 and 90.2% for the different analytes in solid and liquid samples. The quantification limits were 1 ng/ml or ng/g for THC and CBN and 2 ng/ml or ng/g CBD. The method was applied to analysis of various hemp foods. THC content in different products varied 50-fold, whereas CBN and CBD were absent in some samples and achieved hundreds of ng/ml or ng/g in others. The concentration ratio (THC + CBN)/CBD was used to differentiate between the phenotypes of cannabis plants in different specimens. Products possibly originating from drug-type cannabis plants were found in the majority of analyzed specimens.

Keywords: Cannabinoids; Hemp food; Liquid–liquid extraction; Gas-chromatography-mass spectrometry

1. Introduction

Cannabinoids are a group of terpenophenolic compounds found in the hemp plant *Cannabis Sativa*. The highest cannabinoid concentration are found in the resin secreted by the plants' flowering buds [1]. Δ^9-Tetrahydrocannabinol (THC) is the psychoactive component of the hemp plant; other major nonpsychoactive costituents include cannabidiol (CBD) and cannabinol (CBN) [2]. Administered most commonly by smoking or ingesting, THC predominantly acts on the central nervous (CNS) and cardiovascular systems. Common CNS effects include euphoria, a sense of well-being, relaxation, tachycardia and alteration in blood pressure; hallucinations may appear at high doses [3].

On the basis of THC content, cannabis sativa plants are divided into fiber-type and drug-type [4]. Indeed, whereas the cultivation of drug-type hemp is prohibited in several countries, since 2001 the European Union allows cultivation of fiber hemp varieties with THC content of less than 0.2% [5]. After the legalization of fiber-hemp cultivation, the demand for hemp food products, mostly sold in esoteric stores, is significantly increasing because of supposed psychoactive properties associated with the potential THC content [6]. A wide variety of hemp-based products are available, including hemp leaves, hemp seed derivates, oil, flour, beverages (beer, lemonade and liqueur) and cosmetic products.

Some countries, such as Switzerland, assessed the health risks of THC in foods, especially when ingested by unsuspected consumers [6] and in cases of intoxication from hempseed oil or hemp infusion [3]. For this reason, Switzerland and Germany established maximum legal limits for the THC concentration in different foods [6]. Furthermore, it is

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doi:10.1016/j.jpba.2004.07.035

worth noting that a positive result to drug tests for marijuana use have been observed after ingestion of hempseed oil and other hemp foods, with the risk of producing a positive result in workplace drug testing [7–8]. Over the last 2 years, different types of hemp food gradually spread into the Italian natural foods stores and have been sold for "supposed" nutritional and health benefits. In this context, a check on cannabinoids content was decided by the governmental legal authorities [9–11]. Development of an easier to use, sensitive and specific method for determination of THC and other cannabinoids in hemp food products was needed. A few analytical methods available for routine determination of THC, CBN and CBD in hemp food products have been developed in recent years [4,6]. Generally speaking, gas chromatography-mass spectrometry was the favorite choice for cannabinoids analysis in both biological matrices and hemp products due to its versatility and feasibility [2,12–14]. Nonetheless, sample preparation appeared to be time consuming and required large amounts of extraction solvents. This method presents gas chromatography-mass spectrometry coupled with simplified sample preparation, rendering the assay suitable for high throughput laboratories. Furthermore, the assay has been validated to meet the acceptance criteria for bioanalytical method validation.

2. Experimental section

2.1. Instrumentation

GC-MS analyses were carried out on a 6890 Series Plus gas chromatograph equipped with an Agilent 7683 autosampler and coupled to a 5973 N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation, Palo Alto, CA, USA).

2.2. Chemicals and materials

Cannabidiol (CBD), cannabinol (CBN), Δ^9-tetrahydrocannabinol (THC), Δ^8- tetrahydrocannabinol (used as internal standard, I.S.) were supplied by Salars (Como, Italy). *N*-methyl-(trimethylsilyl) trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) was obtained from Sigma-Aldrich (Milano, Italy). Ultrapure water and all other reagents of analytical grade were obtained from Carlo Erba (Milano, Italy). A diverse range of commercially available hemp food products were purchased in autumn 2003 from esoteric and nature stores in Italy. The products analyzed within this study were: beer, pastilles, liqueur, seeds, scented grass and oil. The blank products used in the validation studies (products similar in the composition to those previously mentioned but without any presence of cannabinoids, reported as "drug-free food products") were purchased from the same nature stores or at local supermarkets and analyzed to assess the absence of any hemp component before spiking them with cannabinoids standard solutions.

2.3. Preparation of standard solutions

Stock standard solutions containing THC, CBD and CBN at 1 mg/mL concentration were prepared in methanol. Working solutions at concentrations 1 μg/mL were immediately prepared by dilution of the stock standards with methanol and stored at −20 °C until analysis. The internal standard (I.S.) working solution was used at a concentration of 1 μg/mL.

Calibration standards containing 10 μl of I.S. working solution and THC, CBD and CBN at concentrations: 50, 20, 10, 5, 1 ng/ml for liquid samples and 50, 20, 10, 5, 1 ng/g for solid samples were prepared daily for each analytical batch by preparing tubes with suitable amounts of methanol working solutions, which were evaporated under nitrogen before adding 1 ml or 1 g of pre-checked drug-free food products. Several aliquots of two quality control samples containing 10 μl of I.S. working solution and cannabinoids at 15 and 25 ng/ml or ng/g were prepared for the different food products to be used for calculation of validation parameters. Calibration and quality control samples were treated and processed as unknown samples.

2.4. Samples and sample preparation

All the solid samples (pastilles, seeds and scented grass) were blended and homogenized in a standard mixer. Liquid samples (beer, liqueur and oil) were homogenized by shaking. An amount of 1 ml (liquid samples) or 1 g (solid samples) hemp product, added to 10 μl of I.S. working solution, underwent liquid–liquid extraction with 2 ml of hexane/isopropanol (9:1). The mixture was homogenized by vortex for 2 min and centrifuged at 1076 g/min for 5 min. The organic layer was separated and transferred to another tube and the sample was re-extracted with 2 ml organic mixture. The combined organic layers were evaporated to dryness at 40 °C under a nitrogen stream. The dried samples were derivatized in capped test tubes with 100 μl of MSTFA-2%TMCS at 70 °C for 30 min. For GC/MS analysis, a 1 μl amount was injected.

2.5. GC-MS conditions

Analyte separation was achieved on a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d, film thickness 0.25 μm) (Agilent Technologies, Palo Alto, CA, USA). The oven temperature was programmed at 120 °C for 2 min, increased to 290 °C at 20 °C/min and held for 10 min. Split injection mode (15:1) was used. Helium (purity 99%), with a flow rate of 1 ml/min was used as carrier gas.

The injection port, ion source, quadrupole, and interface temperatures were: 260, 230, 150 and 280 °C, respectively.

The electron-impact (EI) mass spectra of the analytes were recorded by total ion monitoring mode (scan range 40–550

m/z) to determine retention times and characteristic mass fragments. For quantitative analysis, the chosen characteristic mass fragments were monitored in the selected-ion-monitoring (SIM) mode: *m/z* 386, 371, and 303 for THC-TMS, *m/z* 458, 390 and 337 for CBD-TMS, *m/z* 382, 367 and 310, for CBN-TMS, *m/z* 382, 330 and 303 for I.S.-TMS.

2.6. Validation procedures

Prior to application to real samples, the method was tested in a 3-day validation protocol [16,17]. Selectivity, recovery, matrix effect, linearity, precision, accuracy, and limits of detection and quantification, were assayed.

The drug-free food products (a common beer, some pastilles with composition in sugars and additives similar to that of the pastilles under examination, a base-liqueur used to prepare home-liqueurs, taraxacum seeds, olive oil, herbal infusion) were extracted and analyzed for assessment of potential interferences due to endogenous substances. The apparent responses at the retention times of the analytes under investigation and I.S. were compared to the response of analytes at the LOQ and I.S. at its lowest quantifiable concentration. Furthermore, potential interferences from principal drugs of abuse, opiates (6-monoacetylmorphine, morphine, codeine), cocaine and benzoylecgonine, amphetamine, methamphetamine and 3,4 methylenedioxymethamphetamine were also evaluated by spiking 1 ml or 1 mg of the above-mentioned products with 100 ng of each of the aforementioned substances and carrying out the entire procedure. The potential for carryover was investigated by injecting extracted drug-free products, with added I.S., immediately after analysis of the highest concentration point of the calibration curve on each of the 3 days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analytes under investigation.

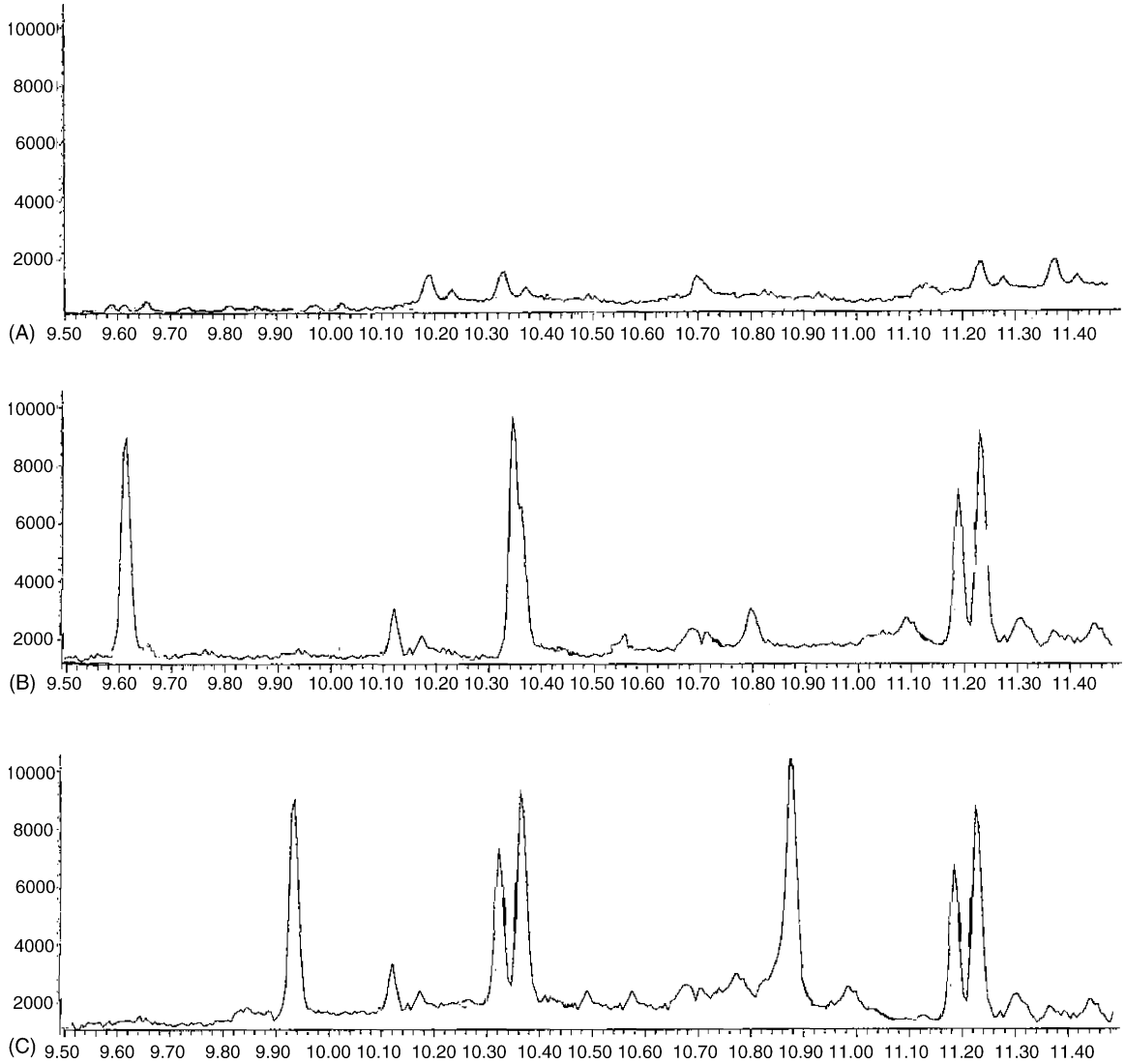

Fig. 1. Representative SIM chromatogram of an extract of pre-checked drug-free food products (A) beer; (B) scented grass; (C) seeds.

Analytical recoveries were calculated by comparing the peak areas obtained when quality control samples were analyzed by adding the analytical reference standards and the I.S. in the extract of drug-free food products prior to and after the extraction procedure. When the recoveries were assessed at three concentration levels (5, 15 and 25 ng/ml or ng/g), using four replicates at each level.

For an evaluation of matrix effects, the peak areas of extracted drug-free food products samples spiked with standards at a mean concentration level (10 ng/ml or ng/g) after the extraction procedure, were compared to the peak areas of pure diluted substances.

Calibration curves were tested over the quantification limit −50 ng/ml for liquid samples and 50 ng/g for all solid samples. Peak area ratios between compounds and I.S. were used for calculations. A weighted (1/concentration) least-squares regression analysis was used (SPSS, version 9.0.2 for Windows). Ten replicates of drug-free food products samples were used for calculating the limit of quantification. Standard deviation (S.D.) of the mean noise level over the retention time window of each analyte was used to determine the detection limit (LOD = 3 S.D.) and quantification limit (LOQ = 10 S.D.).

A total of five replicates at each of three quality control concentrations were added to drug-free food products, samples were extracted, as reported above and were analyzed for the determination of intra-assay precision and accuracy. The inter-assay precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Inter-assay precision was expressed as the relative S.D. (R.S.D.) of concentrations calculated for quality control samples. Inter-assay accuracy was expressed as the relative error of the calculated concentrations.

The effect of three freeze-thaw cycles (storage at −20 °C) on the cannabinoids stability in different food products was evaluated on quality control samples. The stability was ex-

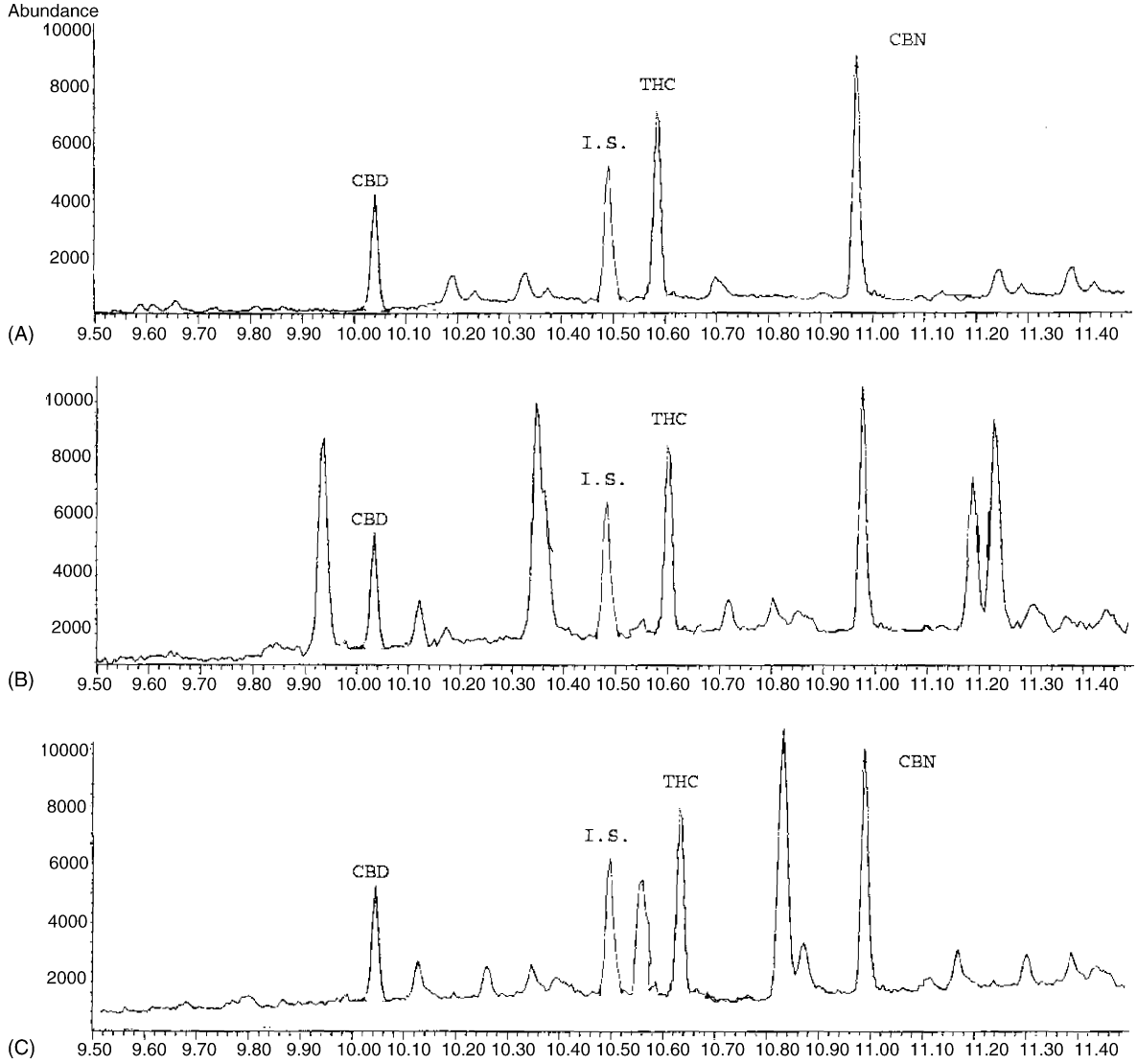

Fig. 2. Representative SIM chromatogram of an extract of pre-checked drug-free food products (A) beer; (B) scented grass; (C) seeds, spiked with 10 ng/ml or ng/g THC, CBD, CBN and I.S.

pressed as a percentage of the initial concentration of the analytes spiked in drug-free food products and quantified just after preparation.

3. Results and discussion

3.1. GC-MS

Representative chromatograms obtained following the extraction of f pre-checked drug-free food products (A: beer; B: scented grass; C: seeds) and 10 ng THC, CBN, CBD and I.S., spiked in 1 ml of drug free beer; 1 g scented grass and 1 g seeds are shown in Figs. 1 and 2, respectively. Chromatograms of extracts of hemp foods (liqueur, pastilles and seeds) containing different concentration of cannabinoids are presented in Fig. 3. When analyte concentrations in food products resulted in higher concentration than those of the calibration curve, a smaller amount of samples (usually 1/10 or 1/50 amount) was re-extracted and analyzed following standard procedure. Samples following the one exceeding the linear range in the chromatographic run were re-injected to check eventual contamination by carryover. Nonetheless, nor in this case any carryover was observed.

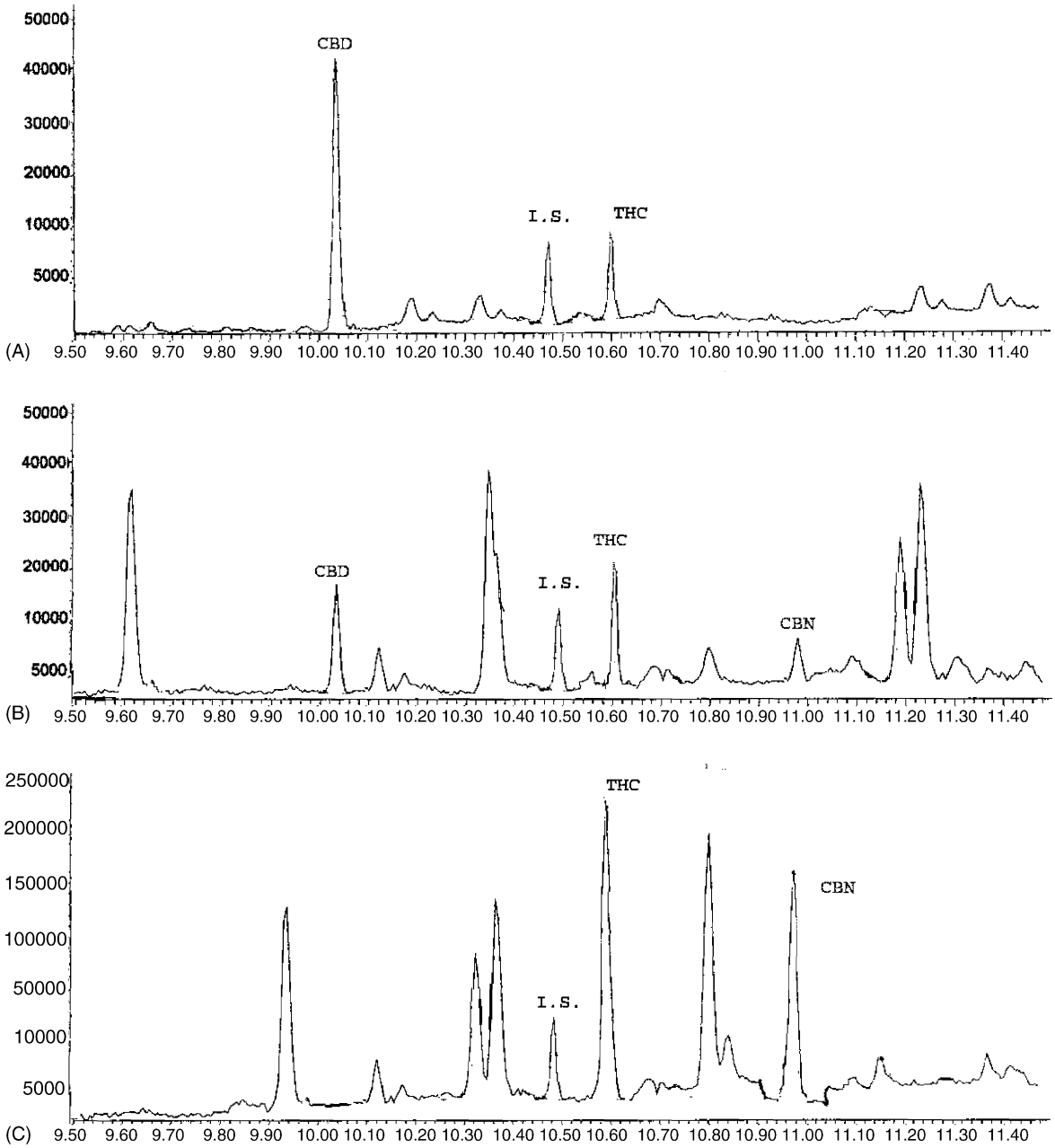

Fig. 3. Representative SIM chromatogram of an extract of: (A) 1 ml hemp liqueur containing 8.1 ng/ml THC and 91.3 ng/ml CBD; (B) 1 g hemp pastilles containing 22.9 ng/g THC, 8.3 ng/g CBD and 5.4 ng/g CBN; (C) 1 g hemp seeds containing 301 ng/g THC and 142.0 ng/g CBN.

A chromatographic run was completed in 21 min, and afterwards initial conditions were restored in 3 min. No additional peaks due to endogenous substances that could have interfered with the detection of compounds of interest were observed. None of the drugs of abuse other than the analytes under investigation interfered with the assay. Drug-free food product samples injected after the highest point of the calibration curve did not present any traces of carryover. With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted drug-free food products samples versus those for pure diluted standards showed less than 10% analytical signal suppression due to coeluting endogenous substances.

3.2. Validation results

Tables 1 and 2 summarize the method validation data. Linear calibration curves were obtained for the compounds of interest with a correlation coefficient (r^2) higher than 0.99 in all cases. The analytical recoveries (mean ± S.D.) obtained after liquid–liquid extraction at two concentration levels showed that there were no relevant differences between different food products. Limits of detection and quantification were considered adequate for the purposes of the present study. The results obtained for intra-assay and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria. [15,16]. With reference to the freeze/thaw stability assays for quality control samples, no relevant degradation was observed after any of the three freeze/thaw cycles, with differences from the initial concentration less that 10%. The stability of cannabinoids extracts at ambient and refrigerated temperature (+25 and +4 °C, respectively) was not assessed since as reported by international literature, these compounds present certain instability if simply refrigerated or maintained at room temperature [17,18]. For the same reason, once derivatized, samples were immediately injected.

3.3. Analysis of samples

Results from the analysis of hemp food samples are listed in Table 3. Presented data are the mean and standard deviation of five different samplings of the same product batch. THC content in different food products varied 50-fold, while CBD and CBN were absent in some samples and achieved hundreds of ng/ml or ng/g in others. In the absence of a national specific legislation regarding maximum THC content in different hemp food products, the limit of 0.2% indicated by the European Union to allow hemp cultivation was considered the legal limit to allow hemp food distribution. Indeed, in all the analyzed samples THC concentration never exceeded the limit of 0.2% (Table 3).

In contrast to results obtained by other authors [4], which found CBD as the analyte at the highest concentration in the investigated hemp food, CBD and CBN concentrations in our studied products were always lower than that of THC.

Over the last two decades, the concentration ratio (THC + CBN)/CBD was proposed to differentiate between the phenotypes of cannabis plants [1]. Drug-type plants have a ratio greater than 1. Interestingly, even if the cannabinoids content in food products under investigation was extremely low, the

Table 1
Linearity, analytical recovery and limits of detection and quantification of the assay

Analyte	Correlation coefficient (r^2)	Analytical recovery % (5 ng/ml or ng/g)	Analytical recovery % (15 ng/ml or ng/g)	Analytical recovery % (25 ng/ml or ng/g)	LOQ (n = 10) (ng/ml or ng/g)	LOD (n = 10) (ng/ml or ng/g)
Beer						
THC	0.992 ± 0.007	83.8 ± 3.7	89.2 ± 7.8	88.2 ± 4.5	1	0.3
CBN	0.994 ± 0.003	81.2 ± 7.5	85.3 ± 8.0	87.2 ± 3.2	1	0.3
CBD	0.995 ± 0.005	83.2 ± 8.2	82.1 ± 2.2	85.2 ± 6.3	2	0.6
Pastilles						
THC	0.994 ± 0.004	83.2 ± 2.5	90.2 ± 8.3	88.5 ± 6.3	1	0.3
CBN	0.998 ± 0.001	80.2 ± 1.2	89.4 ± 6.7	80.2 ± 4.3	1	0.3
CBD	0.999 ± 0.001	81.5 ± 2.3	80.2 ± 1.8	82.4 ± 3.4	2	0.6
Liqueur						
THC	0.998 ± 0.001	83.0 ± 1.8	89.4 ± 8.8	85.3 ± 2.6	1	0.3
CBN	0.997 ± 0.005	84.7 ± 2.5	88.2 ± 4.5	89.2 ± 3.2	1	0.3
CBD	0.996 ± 0.007	83.5 ± 3.2	85.2 ± 3.2	82.1 ± 3.2	2	0.6
Seeds						
THC	0.995 ± 0.003	84.3 ± 1.7	80.1 ± 2.2	84.1 ± 2.5	1	0.3
CBN	0.996 ± 0.001	88.6 ± 2.3	82.2 ± 8.4	81.3 ± 3.2	1	0.3
CBD	0.995 ± 0.004	89.2 ± 3.2	84.2 ± 7.8	80.1 ± 6.3	2	0.6
Scented grass						
THC	0.994 ± 0.004	84.2 ± 3.2	85.8 ± 6.7	86.2 ± 1.5	1	0.3
CBN	0.998 ± 0.006	83.3 ± 2.4	80.2 ± 7.5	83.2 ± 3.2	1	0.3
CBD	0.998 ± 0.004	83.7 ± 3.2	83.2 ± 8.2	80.5 ± 2.3	2	0.6
Oil						
THC	0.995 ± 0.003	82.1 ± 1.9	84.1 ± 5.1	83.1 ± 2.3	1	0.3
CBN	0.996 ± 0.002	83.2 ± 2.1	85.1 ± 3.1	88.3 ± 6.2	1	0.3
CBD	0.991 ± 0.001	82.8 ± 2.2	80.4 ± 6.2	83.2 ± 2.1	2	0.6

Table 2
Intra (n = 5) and inter assay (n = 15) precision and accuracy

Analyte	Intra-assay precision (R.S.D.)			Intra-assay accuracy (error %)			Inter-assay precision (R.S.D.)			Inter-assay accuracy (error %)		
	5 (ng/ml or ng/g)	15 (ng/ml or ng/g)	25 (ng/ml or ng/g)	5 (ng/ml or ng/g)	15 (ng/ml or ng/g)	25 (ng/ml or ng/g)	5 (ng/ml or ng/g)	15 (ng/ml or ng/g)	25 (ng/g or ng/ml)	5 (ng/ml or ng/g)	15 (ng/ml or ng/g)	25 (ng/g or ng/ml)
Beer												
THC	5.6	4.7	7.4	9.3	10.0	11.2	4.3	5.7	6.2	10.3	12.4	11.3
CBN	6.6	8.8	6.3	3.9	8.9	7.3	6.9	7.2	5.8	7.3	9.2	9.0
CBD	9.1	9.7	4.5	6.4	9.2	7.8	7.4	9.6	7.6	8.2	5.3	8.3
Pastilles												
THC	4.3	4.1	5.6	9.2	13.9	12.0	8.2	8.6	4.1	9.4	1.3	9.0
CBN	5.8	4.9	3.5	8.2	2.8	4.2	5.2	7.6	8.9	6.6	8.3	7.3
CBD	7.6	7.2	4.8	6.6	9.2	7.8	6.6	9.6	7.6	8.2	5.3	8.3
Liqueur												
THC	3.4	8.8	7.8	10.3	11.4	10.1	10.3	10.6	8.4	10.3	12.5	10.4
CBN	5.6	3.6	4.2	7.3	8.3	5.3	8.3	8.4	7.2	9.8	11.2	12.1
CBD	6.6	9.6	8.3	8.2	9.8	7.4	6.2	7.3	5.8	5.6	8.8	9.3
Seeds												
THC	4.8	5.7	5.3	9.9	12.4	9.9	9.9	8.5	6.6	9.9	10.4	9.9
CBN	5.6	4.7	3.9	8.5	11.7	1.8	4.5	1.8	5.4	7.3	9.2	8.8
CBD	7.4	7.4	6.4	9.4	11.3	8.7	3.4	2.5	6.7	2.5	8.8	12.2
Scented grass												
THC	5.9	2.7	3.7	8.4	9.9	10.1	4.4	2.7	4.3	5.2	5.5	11.3
CBN	6.6	2.8	4.2	7.4	8.5	7.8	6.4	4.7	8.2	6.3	8.4	9.9
CBD	4.2	6.0	5.3	9.0	10.3	6.8	9.0	8.6	7.3	10.1	9.9	8.8
Oil												
THC	5.2	4.3	5.2	8.9	10.1	9.9	7.9	10.2	9.9	5.4	12.4	9.9
CBN	6.8	2.5	3.2	6.7	8.7	7.3	4.7	9.1	8.7	5.2	11.3	10.3
CBD	5.3	6.6	5.8	8.8	9.2	8.1	6.8	7.4	5.8	9.6	8.8	8.6

Table 3
THC, CBD and CBN content (mean ± S.D., n = 5), % THC and phenotype ratio of the analyzed hemp food products

Product	THC	% THC	CBD	CBN	Phenotype ratio
Beer (ng/ml)	5.7 ± 1.1	0.57×10^{-6}%	4.8 ± 0.8	N.D.	1.1
Pastilles (ng/g)	23.2 ± 2.8	2.32×10^{-6}%	8.2 ± 1.2	5.0 ± 0.7	3.4
Liqueur (ng/ml)	8.0 ± 0.9	0.80×10^{-6}%	91.9 ± 3.5	N.D.	<0.1
Seeds (ng/g)	328.3 ± 36.3	32.83×10^{-6}%	N.D.	146.0 ± 4.5	>500
Scented grass (ng/g)	350.0 ± 45.4	35.0×10^{-6}%	21.9 ± 1.3	159.7 ± 2.3	23.2
Oil (ng/ml)	25.0 ± 0.5	2.5×10^{-6}%	3.7 ± 4.2	2.4 ± 0.3	7.4

phenotype ratio was higher than 1 in the majority of the products. Nonetheless, in our opinion total THC content should also be considered to support the possibility of products originating from drug-type cannabis plants [19].

4. Conclusion

This GC-MS method reported allows the determination of THC, CBN and CBD concentrations in different food products. The main characteristics of the assay are the rapid and simple extraction and sample preparation procedures and total analysis time. Owing to the minimum handling and time required, this procedure can be useful when large stocks of food samples from different origin have to be processed.

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