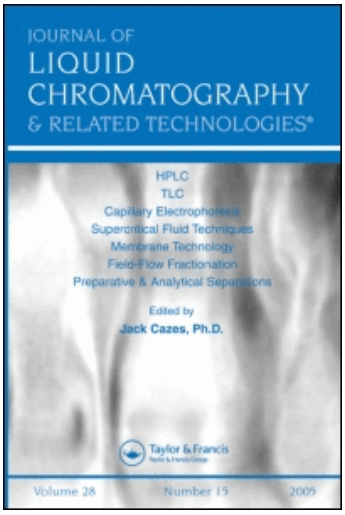

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THE OPTIMIZATION OF THE SEPARATION CONDITIONS FOR CANNABINOIDS FROM *CANNABIS SATIVA* L. *VAR INDICA* AND APPLICATION OF THE METHOD TO DETERMINE THE CONTENT OF Δ^9-TETRAHYDROCANNABINOL IN PLANT MATERIAL

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ABSTRACT

The method for determination of isomer Δ^9 of tetrahydrocannabinol in *Cannabis sativa* L. *var indica* has been developed. It is based on HPLC separation of isomers, Δ^9 and Δ^8, with resolution factor $Rs = 1.00$. The method validation involved specificity and precision. Based on analysis of the raw material – *marihuana*, the variation coefficient RSD was found to be 2.09%.

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INTRODUCTION

Cannabis sativa L. *var indica* is one of the oldest narcotic plants. Its cultivation originates from central Asia, and has been spread across all countries from tropical to temperate regions. They are widely spread and often illegally grown. The plant material reveals broad variation depending on the region of cultivation. In the last 15 years remarkable progress have been made in understanding the mechanism of narcotic activity of *Cannabis indica*. The most active cannabinoids is Δ^9-tetrahydrocannabinol (Δ^9-THC) (1). Besides that basic psychotropic substance, the plant material of *Cannabis indica* also contains Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD), and cannabinol (CBN). The problem of cannabinols analysis is mostly connected with determination of 9-carboxy-THC (the main metabolite of THC) in human urine (2). The analytical methods which have been used are: gas chromatography (GC) (3,4) and high pressure liquid chromatography (HPLC) with UV detection – Spherisorb C-8 column, acetonitrile: 50 mM phosphoric acid (65 : 35) (12) or electrochemical detection – Zorbax C-8 column, acetonitrile, methanol, 0.02 N sulfuric acid (13). According to the literature (7–10), the cannabinoids have been determined qualitatively in materials originating from different geographic regions.

The HPLC method applied RP-18 (Partisil) column and methanol/0.02 N sulfuric acid (80 : 20 v/v) as a mobile phase. The sample was extracted for 4 h at 80°C. However, the quantitative analysis has not been made.

Due to the strong psychotropic activity of the isomer Δ^9-THC, the object of this work was to develop a specific and precise HPLC method for its determination. For comparative studies, several standards of other cannabinoids such as: cannabinol and cannabidiol were used (Figure 1). The developed analytical method is based on HPLC. Additionally, the method was applied to determine Δ^9-THC in plant material (*marihuana*) after optimization of extraction conditions to recover cannabinoids.

EXPERIMENTAL

Apparatus

The HPLC chromatograph Shimadzu (Kyoto, Japan) was equipped with spectrophotometric SPD10AV *vp* and diode array SPDM10A *vp* detectors, two pumps LC-10AT *vp*, and autosampler SIL-10AD *vp*. To control the process and acquire data, the Computer Pentium III 733 MHz with Class VP version 5.0 software was used.

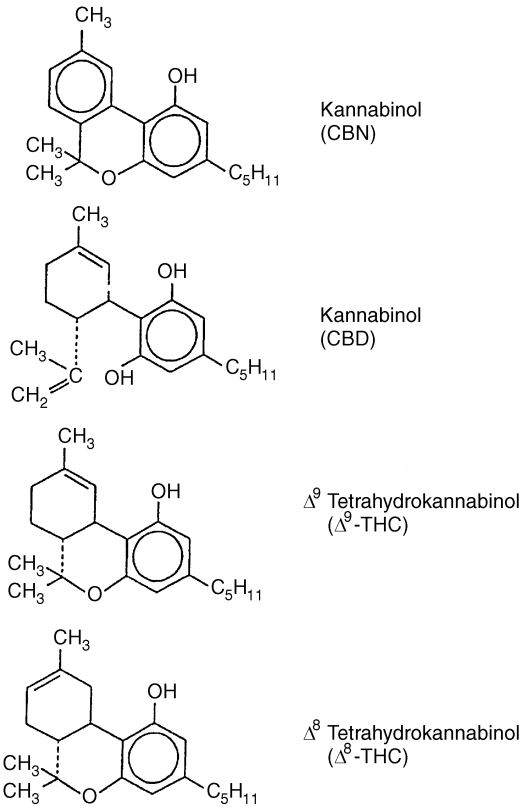


Figure 1. Chemical formula of main cannabinols present in *Cannabis sativa* L.

Chemicals and Standards

Methanol, water, ethanol, and acetonitrile were of HPLC isocratic grade, and petroleum ether, tetrabutylammonium dihydrogenphosphate were A.R. grade (Fluka), sodium hydroxide solution (0.1 mol/L) and standards – Δ^9-tetrahydrocannabinol – 100 mg/mL approx. 95%, Δ^8-tetrahydrocannabinol – 100 mg/mL approx. 95%, cannabinol (CBN), cannabidiol (CBD), were from Sigma.

Plant Material

Cigarettes containing top parts of *Cannabis sativa* – marihuana.

Conditions for HPLC Determination (Table 1)

Preparation of Standard Solution

10 μL of Δ^8-THC (100 mg/mL), 10 μL Δ^9-THC (100 mg/mL), and 1 mL CBD and CBN solutions (1 mg/mL), and about 10 mL methanol were placed in a 20 mL volumetric flask. The solution was immersed for 15 min. in an ultrasonic bath, filled up to volume with methanol, and thoroughly shaken.

Preparation of Sample Solution

About 0.1000 g of finely powdered and sieved (0.315 mm sieves) plant material was mixed with ca. 4 mL of a methanol. Next, it was extracted for 15 min. in an ultrasonic bath, placed for 40 min. in an automatic shaker, and filled up to 5 mL with methanol. The solution was filtered with a 0.45 μm filter.

RESULTS AND DISCUSSION

With the use of 4 typical standards for cannabinols, i.e. Δ^8-THC, Δ^9-THC, CBN, CBD, the method of separation and determination of Δ^9-tetrahydrocannabinol, responsible for psychotropic activity of *Cannabis sativa*, was developed. A mixture of acetonitrile and water was selected as a mobile phase. Acetonitrile gives lower UV absorption than methanol and due to smaller density gives lower back pressure. In the first stage of our study, the Supelcosil™ LC-ABZ column

Table 1. The Conditions of HPLC Measurements

	Method 1	Method 2
Column	Supelcosil™ LC-ABZ, 15 cm × 4.6 mm ID; 5 μm	Bakerbond™ PAH-16 Plus, 25 cm × 3.0 mm ID; 5 μm
Wavelength	220 nm	220 nm
Mobile phase	Acetonitrile/water (60 : 40 v/v) oraz 0.17 g tetrabutylammonium dihydrogenphosphate	Acetonitrile/water (60 : 40 v/v)
Flow speed of mobile phase	2 mL/min	1 mL/min
Column temperature	20°C	20°C
Sample loop	40 μl	20 μl

was used. However, there was a problem of specifity due to incomplete separation of isomers Δ^9-THC i Δ^8-THC. The other two cannabinols, CBN and CBD, gave peaks of shorter relative retention times. According to the chemical formula (Figure 1), the isomers Δ^9-THC and Δ^8-THC, differ only in the position of the double bond. With the use of Supelcosil^{TM} LC-ABZ column and mixture of acetonitrile and water, the peaks were broad and tailing with improper separation (the asymmetry coefficient equal to $f_{AS} = 2.45$). This effect can be explained by the surface structure of stationary phase (*n*-acylammine group) and the consequence of hydrogen bonding interactions connecting the polar analyte and stationary and mobile phases (9,10). The addition of 0.17 g/L tetrabutylammonium dihydrophosphate to mobile phase improved the separation coefficient for tetrahydro-cannabinol isomers to $Rs = 0.90$, with simultaneous improvement of peak shape for the same analyte ($f_{AS} = 1.2$) (Figure 2A).

Based on the literature data (11) and taking into account the structure and properties of tested analytes, further attempts at improving peak separation were performed. As a result, a new chromatographic column, Bakerbond^{TM} PAH-16 Plus, recommended for separation of analytes of extended but planar structures was applied.

The experimentally determined separation coefficient R_s (resolution) with application of the new column and mixture of acetonitrile and water (60 : 40 v/v) as a mobile phase, was equal to $Rs = 1.00$, with simultaneous improvement of peak shape $f_{AS} = 1.00$ (Figure 2B).

On the basis of UV absorption spectra of standard solutions, the analytical wavelength was determined at 220 nm.

Further analytical studies were continued by two parallel methods. The method using column Supelcosil^{TM} LC-ABZ and mobile phase: acetonitrile and water (60 : 40 v/v) with addition of 0.17 g tetrabutylammonium dihydrophosphate, was called Method 1. Method 2 used the column Bakerbond^{TM} PAH-16 Plus and mobile phase: acetonitrile and water (60 : 40 v/v). The analysis was performed at 20°C (Table 1).

The linearity of both methods was determined based on 8 dilutions of standard substance, Δ^9-THC, in the range of 20 to 90 μg in 1 mL solution (Figure 3A, 3B). The correlation coefficients (R) for Methods 1 and 2 were equal to 0.99 and 1.00, respectively.

The limit of detection, defined as a sum of average noise plus 3 times standard deviation value was found for Method 1 to be equal to 0.50 μg /mL, and 0.125 μg/mL for Method 2. The limit of determination, defined as a sum of average noise plus 10 times standard deviation value was found for Method 1 to be equal to 2 μg/mL, and 1 μg/mL for Method 2.

The statistical evaluation of both analytical methods was made using the computer program Medisat. The variation coefficient, expressed as a relative standard deviation (RSD) determined on the basis of analysis of 9 independent

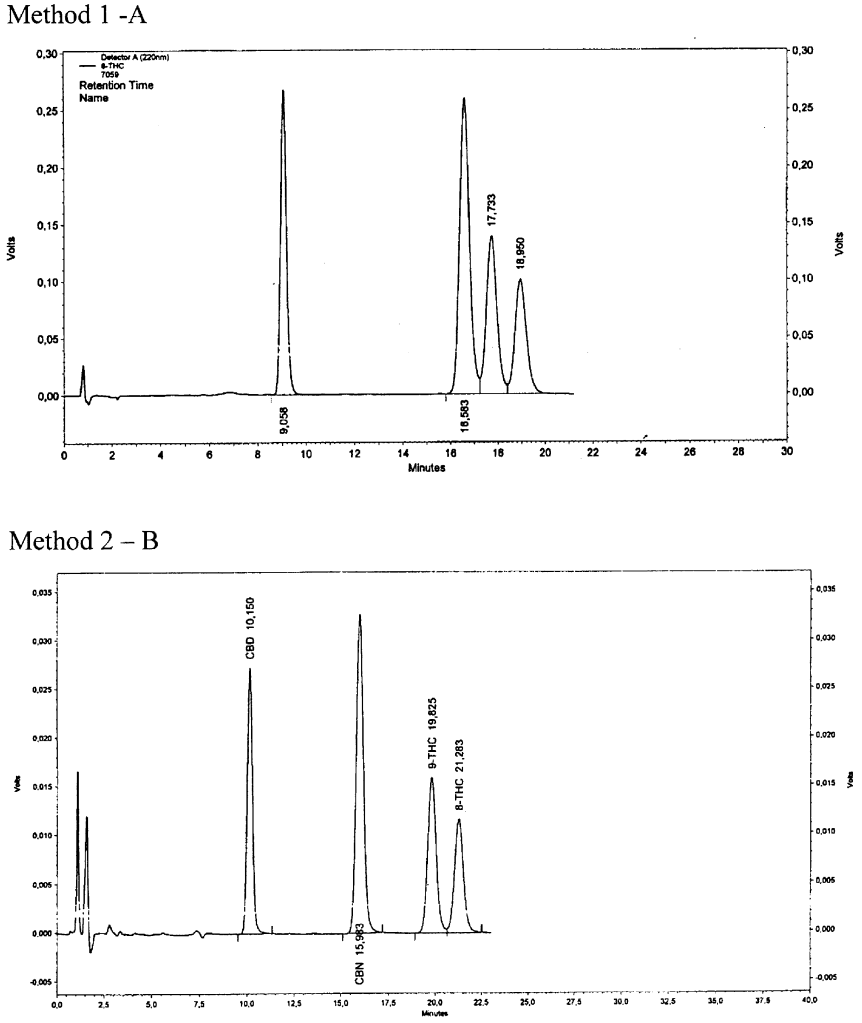
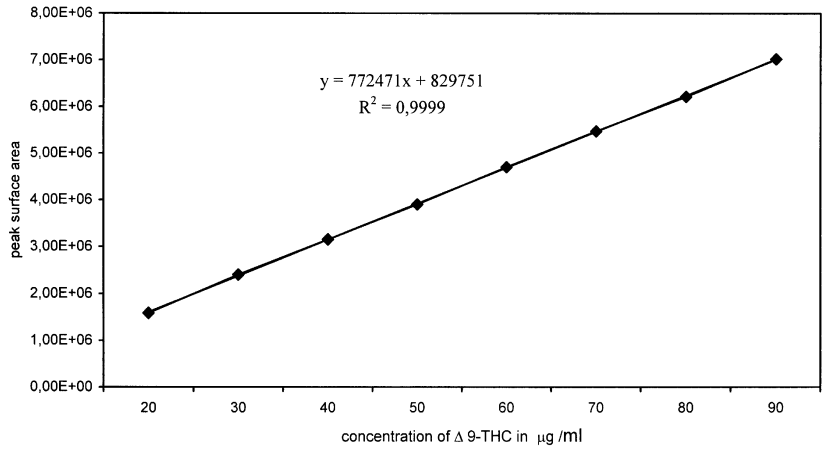


Figure 2. Chromatogram of standard solution of cannabinols.

samples of standard substances, was equal to 2.11%, and 1.80%, for Methods 1 and 2, respectively (Table 2). The use of a statistical comparative method based on estimation of significance of the differences between averages of two trials (T-Student test), gave empirical $T_{emp.} = 2.2525$ and theoretical $T_{teor} = 2.1200$ values, with the confidence level $\alpha = 0.95$. The value $T_{emp.} > T_{teor.}$, therefore, the

Method 1 -A



Method 2 -B

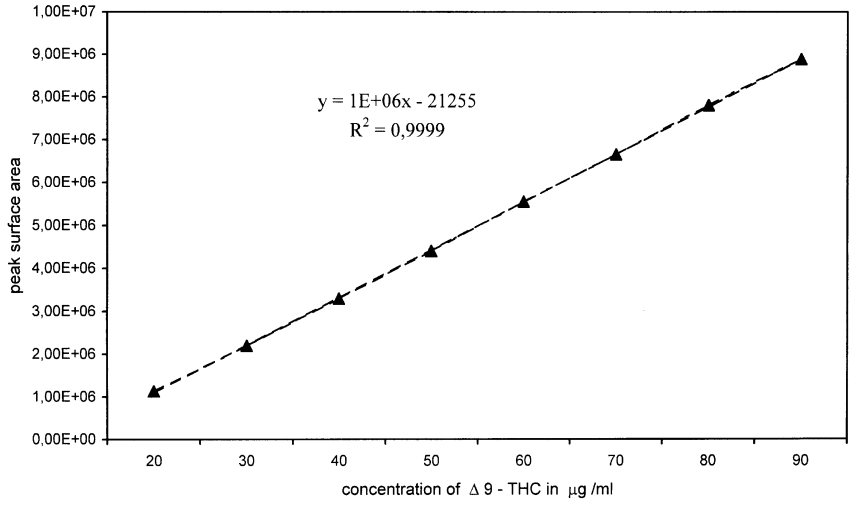


Figure 3. The relationship between peak surface area and content of Δ^9-tetrahydrocannabinol.

Table 2. Statistical Evaluation of the Method Reproducibility of Δ^9-Tetrahydrocannabinol Determination in Standard Solutions (Final Concentration Equal to Allowed Content of Δ^9-THC 5 mg/100 mL)

	Method 1	Method 2
Average for n = 5 mg/100 mL	5.021	5.055
Standard deviation, SD	0.1060	9.271×10^{-2}
Relative Standard Deviation, RSD	2.11%	1.83%
Standard error, SEM	3.749×10^{-2}	3.77×10^{-2}
Confidence limits of the average for $\alpha = 0.95$	$4.934 \leq m \leq 5.107$	$4.797 \leq m \leq 5.131$

difference between the averages is significant from a statistical point of view. Method 2 was selected for further analytical studies with the use of plant material.

To find optimal conditions for the extraction process, both highest recovery of tetrahydrocannabinol and its instability at elevated temperature were taken into account. The attempts of using ethyl ether as extraction medium, alkaline or acidic hydrolysis in methanol did not succeed. The results were irreproducible. On the other hand, the extraction of plant material using methanol at room temperature gave satisfactory results (Figure 4). The agreement of chromatograms

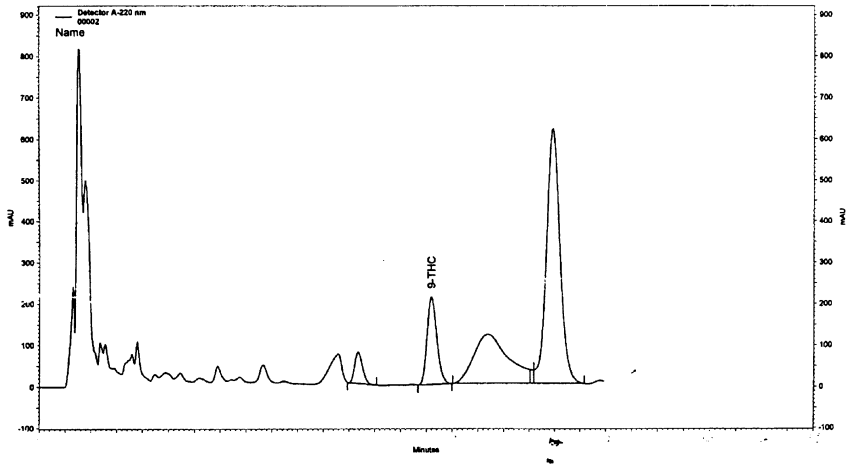


Figure 4. Chromatogram of methanolic extract of top parts of *Cannabis sativa – marihuana*.

with standard solution data was confirmed by UV spectra registered with the use of diode detector (Figure 5A, 5B).

On the basis of analysis of eight independent samples, the reproducibility of the method in relation to plant material was checked; the calculated variation coefficient (RSD) was equal to 2.09% (Table 3). The recovery of the method was

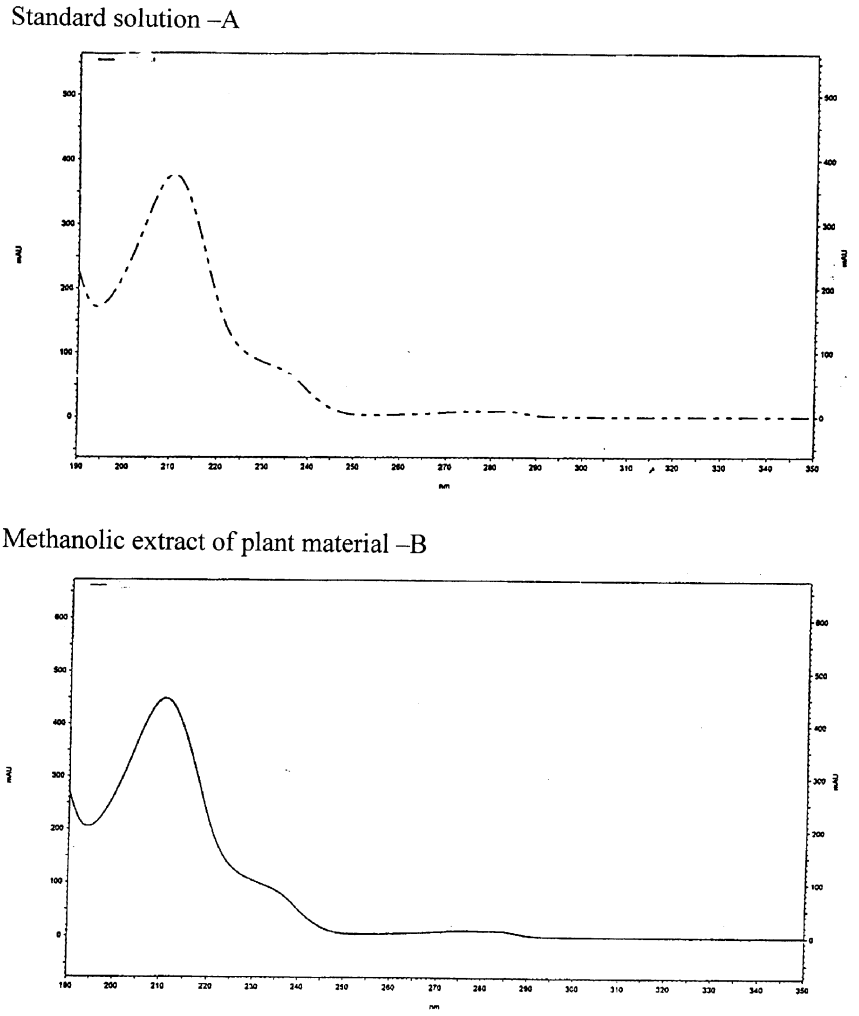


Figure 5. UV absorption spectra of Δ^9-THC peak in standard solution and methanolic extract (diode detector).

Table 3. Statistical Evaluation of the Method Reproducibility Based on Plant Material Samples

Average for $n = 8$ (w%)	0.355
Standard deviation	7.4150×10^{-3}
Relative Standard Deviation, RSD	2.09%
Standard deviation of the average	2.621×10^{-3}
Confidence limits for $\alpha = 0.95$	$0.349 \leq m \leq 0.361$

Table 4. Results of Recovery of Δ^9-THC in Samples of Plant Material

Weight of Plant Material, (mg)	Average Content of Δ^9-THC in Plant Material, (%)	Determined Content of Δ^9-THC (after addition of standard in amount of 0.36 mg per sample), (%)	Calculated Recovery Δ^9-THC, (%)
100.8	0.357	0.743	103.60
100.1	0.357	0.707	98.68
100.8	0.357	0.667	93.05
100.4	0.357	0.713	99.44
			$98.69 \pm 2.09\%$

checked by analysis of four samples of plant material fortified twice by addition of standard solution of Δ^9-THC. The difference in surface area of the appropriate peaks obtained from sample solution containing added standard and sample solution without standard, allowed the calculation of the recovery of standard substance (Table 4). It was equal to $98.69 \pm 2.09\%$.

The presented results confirm our supposition that the developed method of sample preparation and analyte determination fulfills the requirements for analytical methods and can be used to evaluate the amount of Δ^9-tetrahydrocannabinol in *Cannabis sativa*.

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