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SEPARATION, QUANTITATION AND ISOLATION OF CANNABINOIDS FROM *CANNABIS SATIVA* L. BY OVERPRESSURED LAYER CHROMATOGRAPHY

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SUMMARY

Two overpressured layer chromatography (OPLC) methods have been developed for the separation of neutral and acidic cannabinoids. The first is an adaptation of Korte's well known method to the OPLC system, which improves its reproducibility. The second one is a new technique based on the phenomenon of chromatographic solvent demixing. The eluent itself is also divided into zones. In the α-zone the neutral cannabinoids and in the β-zone the acidic ones are separated. As a result of the good and reproducible separation, there is a possibility to quantitate cannabinoids by densitometry. The on-line version of OPLC proved suitable for the isolation of hemp constituents.

INTRODUCTION

Thin-layer chromatography (TLC) has been applied to the detection, isolation and determination of cannabinoids, the particular constituents of hemp (*Cannabis sativa* L.), for examination of the composition of cannabis from various sources for phytochemical, forensic and toxicological purposes[1].

The TLC separation of cannabinoids is difficult because of their similar chemical structures. Most techniques yielding a good separation are relatively complicated and time-consuming for a simple and accurate routine analysis. Because some of them involve multicomponent solvent systems or preparation of special home-made plates or their troublesome impregnation, they have only limited use for quantitative analysis. Therefore, it is necessary to develop better techniques.

In this paper we report the application of overpressured layer chromatographic (OPLC) methods which yield better, quicker and simpler separation, quantitation and isolation of major neutral and acidic cannabinoids than the earlier techniques.

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EXPERIMENTAL

Chemicals

The solvents used for cannabinoid extraction and for chromatography were reagent grade (Reanal, Budapest, Hungary). Tetrazotized *o*-dianisidine chloride (Fast Blue B or FBB) for the detection of the spots of the cannabinoids was obtained from Fluka (Buchs, Switzerland). The synthetic cannabinoids, Δ^1-tetrahydrocannabinol (Δ^1-THC), $\Delta^{1(6)}$-tetrahydrocannabinol ($\Delta^{1(6)}$-THC), cannabidiol (CBD), cannabichromene (CBC, cannabigerol (CBG), cannabinol (CBN), and the plant material were supplied by the United Nations Organisation (UNO) Narcotics Laboratory, Wien, Austria and by the Research Institute of Agriculture, Kompolt, Hungary.

The concentration of the test samples in each case was 0.1%.

Sample extraction

A 1-g amount of each powdered and homogenized plant sample was extracted three times with 20 ml methanol–chloroform (9:1, v/v) by ultrasonic vibration. The pooled extract, were filtered and evaporated in vacuum at room temperature.

For OPLC analysis; the crude extract was dissolved in 2 ml of the extraction solvent.

For gas chromatographic (GC) analysis; it was dissolved in 2–10 ml of the extraction solvent containing 4-androstene-3, 17-dione as the internal standard at a concentration of 2 g/l.

GC analysis

The GC analysis was performed with a Chrom 5 chromatograph (Laboratorní, Přistroje, Prague, Czechoslovakia) equipped with an hydrogen flame ionization detector and an IT2 Type integrator. Cannabinoid standards were chromatographed and the column calibrated using the internal standard method. A special column (2.6 m × 2 mm I.D.) called "tandem" was used. The upper 9/10 was packed with 3% OV-1 and the lower 1/10 with 3% OV-17, both on Supelcoport 80–100 mesh. The column and detector were operated at 190 and 250°C respectively. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min.

OPLC analysis

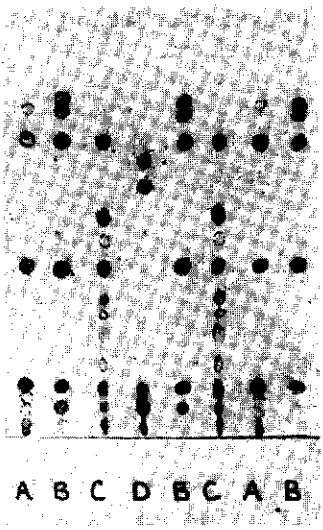
A Chrompres 10 and a Chrompres 25 system obtained from Labor MIM (Budapest, Hungary) were used. For the isolation of the cannabinoids, they were equipped with an UV detector (Liquodet 308, Labor MIM) using the on-line detection mode at 250 and 300 nm. Chromatography was carried out on precoated silica gel 60 F_{254} HPTLC sheets (Merck, Darmstadt, F.R.G.) with sealed edges. The pump was set to supply the mobile phase at rates of 0.3–0.7 ml/min according to the exact setting. The resulting linear velocities were between 0.8 and 1.5 cm/min. The sample application was carried out by a Limonat III (Camag, Muttenz, Switzerland) and by a 1-μl Hamilton syringe. The spots were visualized under UV light and with FBB. The neutral cannabinoids were quantitated by a Shimadzu CS-920 high-speed TLC scanner at 215 nm by comparison with the cannabinoid standard.

RESULTS AND DISCUSSION

Partition technique

One of the best TLC systems for cannabinoids is Korte's method[2], which based on modifying the adsorbent properties of silica gel with dimethylformamide (DMF) in carbon tetrachloride and on two or three-fold elutions with non-polar solvents. Nevertheless this method is not reproducible because the R_F values of the components depend on the proportions of the stationary phase (DMF) and the mobile phase, which cannot be regulated precisely owing to the manifold drying and developing. In addition, because of the long separation time (*ca.* 4 h) the degradation of the components should be reckon into account.

Fig. 1 shows a chromatogram obtained with an OPLC system. An HPTLC sheet was impregnated with dimethylformamide–carbon tetrachloride (4:1) and after drying at ambient temperature (30 min) and sample application it was developed in cyclohexane. Because of the shorter developing time thee components do not degrade. All the major neutral cannabinoids can be separated by this method, but the cannabinoid acids (R_F = 0.0–0.1) interfere with CBD and CBG. Thus, in the case of the analyses of plant materials, similarly to the GC analysis when the total cannabinoid content can be detected analytically, the extract should be heated in order to convert the acids into neutral cannabinoids either before or after the extraction. On the basis of our experiments, the decarboxylation of the acids in a 1-g sample of plants takes place in 5 min. at 200°C.



	$\overline{rR_{F/CBN}}$ (n=8)	C.V.(%)
$\Delta^{1(6)}$THC	1.89	14
CBC	1.81	15
Δ^{1}THC	1.64	12
Fettrot 7 B	1.37	32
Δ^{1}THCV	1.29	6
CBN	1.00	
CBD	0.36	12
CBG	0.22	16
	Start	

Fig. 1. Separation of cannabinoids by the OPLC partition technique. The sheet (20 cm × 20 cm precoated silica gel 60 F_{254}) was impregnated with dimethylformamide–carbon tetrachloride (4:1) and dried for 30 min at room temperature (22 ± 2°C). Mobile phase: cyclohexane; linear velocity; 1 cm/min. Time: 40 min. A = Extract of fibre type cannabis: B = neutral cannabinoids; C = extract of hashish type cannabis; D = six-component dye test mixture. $\overline{rR_{F/CBN}}$ = Average relative R_F, where $R_{F/CBN}$ = 1.00; C.V. = coefficient of variation. Δ^1THCV = Δ^1-Tetrahydrocannabivarin.

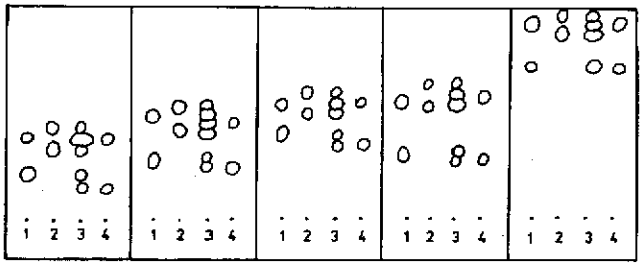


Fig. 2. Separation of neutral cannabinoids in an UM chamber by the adsorption technique. Sheet: 10 cm × 5 cm precoated silica gel to F_{254}. 1 = CBC + $\Delta^{1(6)}$-THC; 2 = CBD + Δ^{1}-THC; 3 = neutral cannabinoids; 4 = CBN + CBG. Mobile phases used, from left to right: xylene, toluene, benzene, chloroform and dichloromethane.

Adsorption technique

In OPLC separations, first we tested one-component eluents having polarities corresponding eluents which had been used in traditional methods 1,3–7 (Fig. 2).

Since all the neutral components can be separated well in toluene, we did not examine any two- or more-component solvent systems. In this eluent the cannabinoid acids have R_F values less than 0.10. The resolution can be improved by continuous development.

In the case of eluent systems comprising two or more components of different polarities, chromatographic solvent demixing takes place. This can be detected because some impurities in the sorbent migrate with the demixing front, and is more pronounced in systems without a vapour space. We used this effect to separate the neutral and acidic cannabinoids simultaneously with the same eluent.

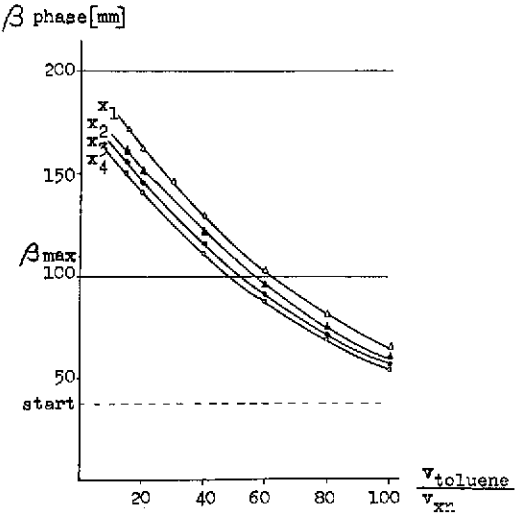


Fig. 3. The R_F values (on precoated silica gel 60 F_{254}) of the β-front in mixtures of toluene and some medium polar solvents. x_1 = Ethyl acetate; x_2 = dioxane; x_3 = acetone; x_4 = ethyl methyl ketone.

In addition to toluene, we attempted to apply solvents which, on the one hand, separate well the cannabinoid acids in the β-zone*, and on the other hand have a β-front** of lower R_F than that of the least neutral cannabinoid ($R_F\ \beta < R_F$ CBG).

First we measured the R_F values of the β-front in mixtures of toluene and some medium polar solvents (Fig. 3), then we examined the chromatographic behaviour of acidic cannabinoids in solvent systems of rR_F (β/Δ^1-THC) less than 0.65 in an ultramicro chamber (UM) (Fig. 4), The most selective system for cannabinoid acids is toluene–dioxane (60:1, v/v) which resulted in the most positive spots to Fast Blue B.

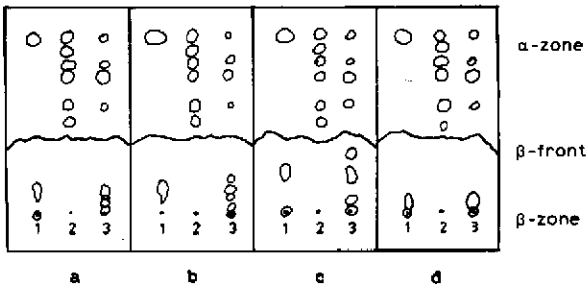


Fig. 4. Chromatographic behaviour of acidic cannabinoids in solvent systems of $rR_F\beta/\Delta^1$-THC = 0.65 in an UM chamber. Sheet: 10 cm × 5 cm precoated silica gel 60 F_{254}. Solvent systems: (a) toluene–ethyl methyl ketone (50:1); (b) toluene–acetone (55:1); (c) toluene–dioxane (60:1); (d) toluene–ethyl acetate (65:1). 1 = Extract of fibre type cannabis; 2 = neutral cannabinoids; 3 = extract of hashish type cannabis.

Fig. 5 shows the separation of the neutral cannabinoids in toluene–dioxane (60:1) by OPLC. In the α-zone*** the neutral and in the β-zone the acidic cannabinoids are present.

Since cannabinoids acid tests were not available, the extracts of two different types of plants (typical hashish, H) and fibre hemp (F) were examined. From the H sample 4 and from the F sample 1, FBB positive spots were detected in the β-zone. In all probability, the latter one is due to CBD acid as it gives an orange colour reaction and because it occurs in the extract of the fibre hemp where the main component is the CBD acid. The four FBB-positive spots, which with sample H gave red and violet reactions, were identified after OPLC isolation (see below).

In Fig. 5 the R_F values of the components are expressed as a percentage of that of Δ^1-THC. The position of the β-front relative to those of the neutral cannabinoids depends on the actual elution system, but at given chromatographic parameters (time, flow-rate, position of the eluent inlet and the samples on the chromatoplate, etc.) it can be characterized by its R_F value relative to that of Δ^1-THC.

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* β-zone = the part below the β-front of the plate. The mobile phase consists of toluene and another solvent component.

** β-front = solvent demixing front in the case of eluent systems comprising two components of different polarities.

*** α-zone = the part above the β-front of the plate. The mobile phase consists of toluene only.

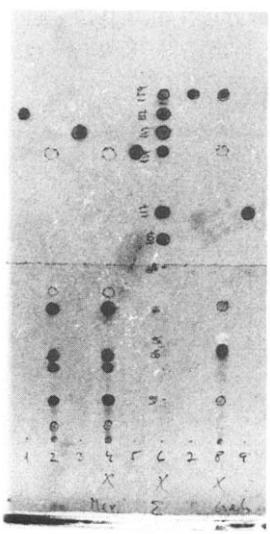

Fig. 5. Separation of cannabinoids by the OPLC adsorption technique. Sheet: 20 cm × 10 cm precoated silica gel 60 F_{254}. Solvent system: toluene–dioxane (60:1); linear velocity, 1 cm/min. Time: 30 min. 1 = $\Delta^{1(6)}$-THC; 3 = CBN; 2–4 = extract of hashish type cannabis; 5 = Δ^1-THC; 6 = neutral cannabinoids; 7 = CBD; 9 = CBC; 8 = extract of fibre type cannabis.

Quantitation

Due to the effective, reproducible OPLC separation there is a possibility for densitometric quantitation on the chromatoplates.

First the remission UV spectra of the neutral standards were measured on the chromatoplates. Each cannabinoid has an adsorption maximum in the range of 210–230 nm, so they were measured at 215 nm.

To prepare the calibration curve, 1.0–1.0 μl of the standards were applied to the chromatoplates. After development as in Fig. 5, the chromatograms were measured by densitometry. Fig. 6 shows the calibration plot for the neutral cannabinoids and the densitogram of the test mixture. The plot is linear in the range of 0.1–1.5 μg. The quantitation of cannabinoid acids is not possible by this method since they are not stable enough to make a standard mixture.

Isolation of cannabinoids by OPLC

To identify the FBB-positive components detected in samples 2–4 (Fig. 5) we isolated them from plant extract by on-line OPLC. A 200-μl volume of the extract was applied to an HPTLC sheet in a band. Since the aim was the separation in the β-zone, hexane–dioxane (4:1) was used as the eluent. The fractions corresponding to each peak were collected. The purity of the fractions was tested by TLC and GC.

Fig. 7 shows the on-line chromatogram and the TLC of the fractions. Two neutral (Δ^1-THC, CBN) and four acidic (CBDA, CBCA, CBNA, Δ^1-THCA) cannabinoids were isolated by on-line OPLC with UV detection. An other eleven components, some of which are FBB positive, were collected and their identification is in progress.

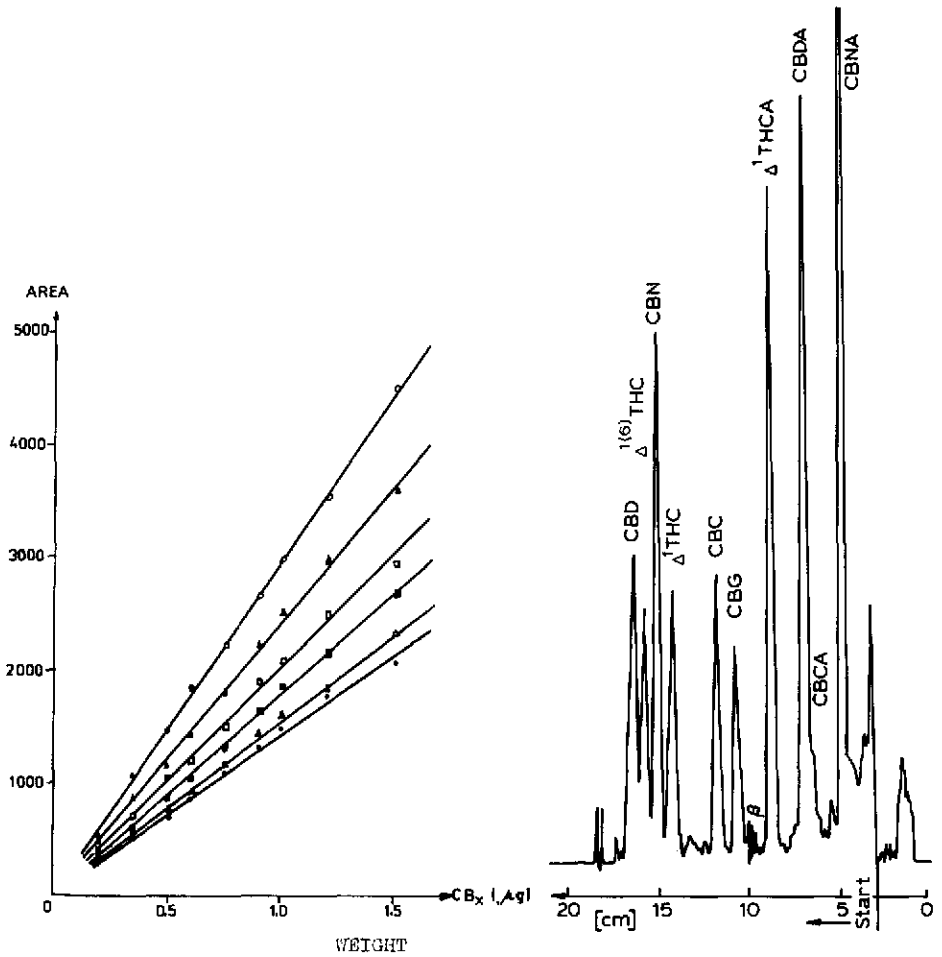

Fig. 6. Densitogram of the cannabinoid test mixture (neutral cannabinoids; extract of hashish type cannabis) and the densitometric responses of neutral cannabinoids after separation by OPLC. For chromatographic parameters see Fig. 5. Wavelength: 215 nm. ○ = CBN, $y = 3002.20\,x - 15.94$ ($r^2 = 0.9997$); ▲ = CBG, $y = 2448.04\,x - 10.23$ ($r^2 = 0.9971$); □ = Δ^1-THC, $y = 2030.39\,x + 11.23$ ($r^2 = 0.9970$); ■ = CBD, $y = 1817.52\,x - 18.96$ ($r^2 = 0.9998$); △ = $\Delta^{1(6)}$-THC, $y = 1562.87\,x - 2.61$ ($r^2 = 0.9999$); ● = CBC; $y = 1430.03\,x - 7.58$ ($r^2 = 0.9998$).

CONCLUSIONS

The described OPLC methods yield a better separation than the earlier TLC methods. The analysis time was also shortened.

By OPLC in combination with densitometry (on 200 mm × 200 mm chromatoplates), there is a possibility for rapid quantitative and simultaneous analysis of 13–18 samples (4–6 min per sample) for cannabinoids, which is specially important in series analyses (plant breeding, drug analysis). Our work resulted in the isolation

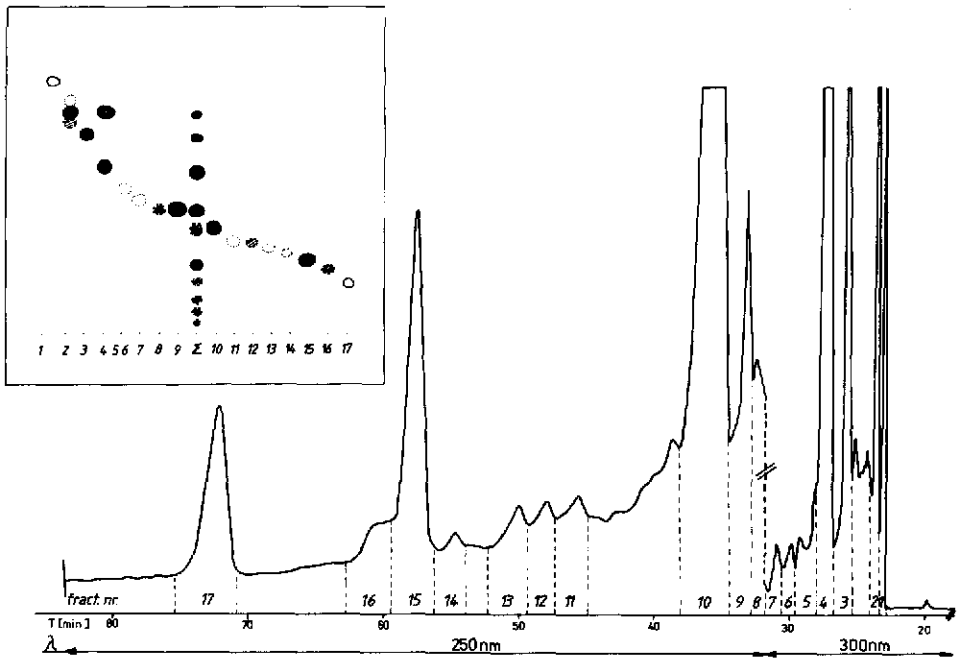

Fig. 7. The elution chromatogram and TLC of the fractions after isolation by on-line OPLC. Fractions: 1, 5, 7, 10–14, 16, 17 = unknown; 2 = Δ^1-THC; 3 = CBN; 4 = Δ^1-THCA; 8 = CBDA; 9 = CBCA; 15 = CBNA; Σ = extract of hashish type cannabis. Solvent system: hexane–dioxane (4:1).

of pure fractions of cannabinoid acids which can be analysed without any further purification by UV, TLC, GC and mass spectrometry.

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