SEPARATION AND IDENTIFICATION OF CANNABIS COMPONENTS BY DIFFERENT PLANAR CHROMATOGRAPHY TECHNIQUES (TLC, AMD, OPLC)

N. GALAND*, D. ERNOUF, F. MONTIGNY***, J. DOLLET* and J. POTHIER***

*UFR des Sciences Pharmaceutiques, Laboratoire de Pharmacognosie, 31 Avenue Monge F-3720 – TOURS
** UFR des Sciences Pharmaceutiques, Laboratoire de Toxicologie, 31 Avenue Monge, F 37200 - TOURS
***UFR des Sciences Pharmaceutiques, Plateau d'Analyses Chimiques et Biochimiques, 31 Avenue Monge,
F-37200 – TOURS

Abstract

The use of Cannabis is illicit in numerous countries and the increasing consumption has led to a multiplication of scientific studies. New methods of planar chromatography such as Automated Multiple Development (AMD) and Optimum Performance Laminar Chromatography (OPLC) techniques can be used as a substitute for the traditional TLC for the identification and quantification of the Indian hemp components. Each method offers its own advantage: high resolution with neither diffusion nor spot stretching for AMD, speed, efficiency and the possibility of working in semi-preparative mode for OPLC.

1- Introduction

Because of the ever increasing use of Cannabis, it has become necessary to dispose of a whole range of efficient methods for the identification of its components and particularly for the characterization of the "narcotic compound": Δ^9-tetrahydrocannabinol (Δ^9-THC). Analyses can be carried out from plants or biological

fluids. In urine, blood and saliva samples Δ^9-THC and major metabolites, such as 11-nor-Δ^9-THC-9-carboxylic acid can be _____

authors to whom correspondence should be addressed

e-mail pothier@univ-tours.fr
e-mail nicole.galand@univ-tours.fr

often required (1). Cannabinoids can be detected by numerous and various analytical methods, including immunoassays (EMIT®, Elisa, fluorescence polarization, radioimmunoassay) (2,3,4,5,6), planar chromatography techniques: the classical Thin Layer Chromatography (TLC), the Optimum Performance Laminar Chromatography (OPLC), and the Automated Multiple Development (AMD), Gas Chromatography-Mass Spectrometry (GC-MS) (7,8,9), high performance liquid chromatography - Mass Spectrometry (HPLC- MS) (10). Generally, there is a good quantitative correlation between these methods and few discrepancies even in the borderline region, especially if the cut-offs through immunoassay techniques are low in spite of the different metabolites cross-reactivities.

Moreover a wide variety of methodologies has been recommended for the determination of marijuana samples or Cannabis plants: TLC (11), OPLC (12), HPLC (13), GC and GC-MS (14,15), capillary electrochromatography (16), time-resolved fluoroimmunological method (17), immunoassay (18). Most of these techniques require heavy and costly instruments and a lot of time

Planar chromatography is a suitable method to screen simultaneously numerous samples directly from plants. It has become a modern technique with the commercialization of numerous adsorbents and new appliances with automated development chambers such as OPLC and AMD which are interesting alternatives to classical TLC.

. OPLC opens up the possibility to analyze an important number of samples in a very short time. Moreover, this method can be used in semi-preparative mode to purify products by direct elution thanks to the fact that migration is linear in correlation with time. *h*Rf values are reproducible and each compound is eluted at a defined time.

AMD presents the best resolution without any spot diffusion and without oxidation since the micro-chamber is saturated with for instance methanol under a nitrogen atmosphere.

The aim of this study was to compare the performances of the TLC, AMD and OPLC techniques for the identification and quantification of the Cannabis components.

2- Experimental

The standard solutions of Δ^8-THC, Δ^9-THC and Cannabinol (CBN) were purchased from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France). Since Cannabidiol (CBD) is not available, it was isolated from Cannabis resin by OPLC in semi-preparative mode (see Results and discussion: *Isolation of standard Cannabidiol by OPLC semi-preparative*)

All the standard solutions were prepared in 0.5mg/1mL in methanol.

Cannabis resin (0.1g) was extracted by shaking at room temperature for 20 min with 10 mL of hexane. The filtrate was evaporated to dryness and the residue dissolved in 1 mL of toluene.

Hemp sample (0.5g) was extracted for 10 min with 20 mL of hexane. After filtration, the extract was evaporated in vacuum and the residue dissolved in toluene.

All solvents were purchased from Carlo Erba Réactifs (Val de Reuil - France) and then distilled.

A Linomat IV (Camag, Muttenz, Switzerland) was used for samples application.

A TLC-MAT Desaga (Bionisis, Le Plessis-Robinson, France), OPLC 50 (Bionisis, Le Plessis-Robinson, France) and AMD (Camag, Muttenz, Switzerland) were used for the chromatographic studies.

TLC and AMD were performed on 10x20 cm plates precoated silicagel HPTLC F_{254} (Merck Art. 11764).

OPLC was performed on HTSorb BSLA 011 and HT Sorb BSLA 003 (Bionisis Le Plessis-Robinson, France).

The chromatograms were derivated by spraying with Fast Blue Salt B reagent (19).

For Classical TLC and TLC-MAT, the eluent used was hexane-diethyl ether 80:20 v/v. For AMD, the elution gradient was acetone 100 (bottle 1); diisopropylether 100 (bottle 2); hexane 100 (bottles 3, 4, 5, 6); migration during 20 steps. For OPLC, the eluent was isooctane-diethyl ether 90:10 v/v. The external pressure was 50 bars, the flow rate 100 µL/min, the flash volume 75 µL, the eluent volume 1000 µL and the migration time 607 sec.

The GC-MS instrumentation used consisted in a Hewlett Packard system (HP 5890 series II gas chromatograph with a HP5989A quadrupole mass spectrometer). HP-5MS 15m X 0.25mm X 0.25 □m capillary column and helium (99.99%) carrier gas at a flow rate of 1.3 mL. min^{-1} were used. The injector temperature was maintained at 250°C and all injections were made in the splitless mode. The GC oven temperature was held at 50°C for 1 min and then programmed to 250°C at

10°C. min^{-1} and maintained for 10 min. The GC-MS transfer line was maintained at 280°C, electron ionisation at 70 eV and the mass spectrum scanned from m/z 35-450. Chromatographic data were acquired using HP Chemstation software.

3- Results and discussion

TLC

Comparison of various eluents used in TLC for the separation of Cannabinoids

TLC is a suitable method for screening different samples.

In the literature, the eluents which are mostly used are: eluent A: isooctane-ethyl acetate-acetic acid 30:10:1 v/v (20); eluent B: petroleum ether-diethyl ether 90:10 (21); eluent C: acetone-methylene chloride-diisopropyl ether-hexane 1:1:3:20 v/v (22); eluent D: toluene-chloroform-methanol 100:10:1 v/v (23); eluent E: hexane-dioxane 90:10 v/v double migration (24); eluent F: hexane-diethyl ether 80:20 v/v (19).

The eluents A and B result in a clean separation between Δ^9-THC and CBN, but not between Δ^9-THC and CBD.

With eluent C, the main Cannabinoids are separated but the spots are stretched.

The best results were obtained with eluent F, hexane-diethyl ether 80:20 v/v (table I) which allowed a clean separation of Δ^8-THC, Δ^9-THC, CBN and CBD (Figure 1).

The separation of Cannabinoids: Δ^9-THC, CBN and CBD by classical TLC is not easy because these derivatives possess chemical structures with very close

substitutes. Besides, the molecular weights of Δ^9-THC and Cannabidiol are the same (314.47) and the molecular weight of Cannabinol is very close (310.44).

The analysis of the chromatograms reveals two different groups of Cannabinoids: a first group, the least polar, composed of CBD, CBN, and Δ^9-THC (upper *h*Rf values) and a second one which consists in many compounds with lower *h*Rf .

The detection limit with the Fast Blue salt reagent is 0.25 µg for Δ^9-THC, CBD and CBN.

Different eluents were tested: isooctane, heptane, hexane, pentane with diethyl ether with a ratio of 90-10 v/v. The comparison between these four alkanes showed that the separation capability decreases when the carbon-bearing chain lengthens.

After this traditional TLC study, these compounds were studied with modern planar chromatographic methods such as AMD and OPLC with the aim of optimizing their separation and identification.

AMD

The “universal gradient” n°1 with methanol, methylene chloride, hexane is far too polar. Therefore, it was necessary to decrease polarity by replacing methanol with methylene chloride.

First, two gradients were tested:

Elution gradient 1A: methylene chloride 100; methylene chloride 100; methylene chloride-hexane 50:50; hexane 100; hexane 100; hexane 100; during 25 steps

Elution gradient 1B: diethyl ether 100; hexane-hexane 50:50; hexane 100; hexane 100; hexane 100.

These two eluents are interesting for revealing a most of the constituents of Cannabis but do not separate Δ^9-THC with CBN and CBD very clearly.

In AMD, the best separation of the three interesting compounds was realized in HPTLC with the elution gradient 1C: acetone 100; diisopropylether 100, hexane 100, hexane 100, hexane 100, during 20 steps (table I), (Figure 2).

The visualization of the chemical constituents was accomplished by spraying fast blue salt B reagent (19).

The different Cannabinoids were identified by their *h*Rf and the colour of the spots: purple for Δ^9-THC, orange red for CBD and violet for CBN.

OPLC

Analytical OPLC

Applying the classical TLC eluent hexane-diethyl ether 80:20 v/v, OPLC gave clear separation but the different stripes took a “zigzag” shape because the viscosity of the eluents was too low and the silicagel plates were not homogeneously permeated deep inside their structure. To solve this problem, viscosity was increased by replacing hexane with a higher homologous such as isooctane which doesn’t change the elution power of the eluent but increase inner pressure, leading to higher *h*Rf values, thus improving considerably the shape of the stripe - the best results being obtained with isooctane-diethyl ether 90:10 v/v as eluent (table I) (Figure 3). Moreover, hexane-diethylether 80:20 v/v used in semi-preparative offers the advantage of evaporating easily because of its low viscosity.

Semi-preparative OPLC applied to isolation of standard Cannabidiol

CBN and Δ^9-THC were obtained from Sigma-Aldrich. Because obtaining CBD was not possible, this compound was isolated from Cannabis resin by OPLC in semi-preparative mode. In the literature, few works have reported this technique. First of all, this method had been tested on Opium extract (25) and on xanthines from tea leaves extract (26). In the case of Cannabis extract, two series of compounds are shown: the first one has *h*Rf above 50 which is easily and quickly carried out and the second one has *h*Rf below 50. For the latter, it was necessary to increase the eluent polarity. The aim of this work was to obtain pure compound from resin of Cannabis by coupling the chromatograph to a fraction collector. The extract was applied in line with Linomat IV and eluted with hexane-diethyl ether 90:10 v/v. The migration of the eluent was performed during the time required to begin the elution process. Because OPLC allows for linear migration in correlation with time, it was possible to determine the instant when Cannabidiol was collected. Thanks to OPLC, it was possible to obtain pure Cannabidiol.

The advantage of OPLC compared to TLC in semi-preparative mode is that no scraping and eluting of bands are necessary. In OPLC, the components can be eluted from the plate and obtained pure by coupling to a fraction collector.

Every elution fraction was evaluated by analytical TLC with hexane-diethyl ether 80:20 v/v as eluent. After derivation by fast blue salt B reagent (19), four fractions were obtained giving only one spot in TLC. The control of structural study performed by GS-MS analysis allowed the identification of a compound present in the sample as being Cannabidiol (figure 4).

.

Structural analysys of Cannabidiol

From chromatographic data obtained by GC/MS, the organic compounds were identified by computer. Standard reference mass fragmentograms listed in the National Institute of Standards and Technology library (NIST) were compared to the specific results obtained here.

The total ion chromatogram obtained shows one major compound. A search in the database spectral library indicated that this substance might very likely be “2-[3-methyl-6-(1-methylethenyl)-cyclohex-2-en-1-yl]-5-pentyl benzene-1,3-diol (Figure 1). The identified compound is also known under the synonym Cannabidiol (CBD). This assumption is in full agreement with the mass spectrum of CBD investigated by Baptista (27). Furthermore, the chromatograms show other substances, with low concentrations, which can be assigned as aliphatic and ethylenic hydrocarbons. Unfortunately these compounds cannot be identified with certainty because of their poor mass spectra resolution.

Conclusion

The modern planar chromatography techniques are reliable because they are automated and inexpensive, they allow a better resolution than classical TLC and can potentially replace slower and more costly methods (GC-MS). Thus increasing the productivity of the laboratory thanks to their ability to analyse several samples at the same time (up to 20).

So, with traditional TLC , it was possible to separate Δ^9-THC, CBD and CBN from cannabis resin and Indian Hemp herb but, this method didn’t offer a clean

separation of the most polar compounds: four spots for cannabis extracts with classical TLC and eight spots for the resin could be obtained with AMD.

AMD offers high resolution without any stretching of spots, the focalisation of which gives the possibility of making dosage by scanner densitometry.

These two modern techniques, OPLC and AMD, are reproducible because they are completely automated. They can provide an interesting information about the composition of different samples of Indian Hemp and opens up the possibility to determine the geographical origin of different samples.

The benefits of OPLC compared to TLC are many. Namely: efficiency, reproducibility, small consumption of developing eluent and shorter analysis delay. Consequently, the spots have a more regular shape and diffusion and stretching is not as pronounced as in TLC. Another major advantage of OPLC compared to TLC is the possibility to extend this method to semi-preparative chromatography in which no scraping and eluting of bands are necessary because the components can be drained from the plate and obtained pure by coupling OPLC to a collector.

An additional benefit of planar chromatographic techniques *versus* HPLC and GC lies in the fact that it is possible to detect in the Cannabis samples other addictive products belonging to different chemical classes (e. g. alkaloids like opiates and derivatives, cocaine, nicotine) mixed in a single Cannabis sample by using specific reagents: e.g. iodoplatinate or Dragendorff in the case of alkaloids.

TLC, OPLC and AMD can also supply interesting information as regards the composition of various samples of Hemp and offer the possibility of determine the origin of it.

Bibliography

1- J.E. Manno, B.R. Manno, P.M. Kemp, D.D. Alford, I.K. Abukhalaf, M.E. Mc Williams, F.N. Hagaman, M.J. Fitzgerald. Temporal indication of marijuana use can be estimated from plasma and urine concentrations of Δ^9-tetrahydrocannabinol, 11-hydroxy-Δ^9-tetrahydrocannabinol, and 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid. *J. Anal. Toxicol.*, **25**, 538-549 (2001).

2- D. Altunkaya, A.J. Clatworthy, R.N. Smith, I.J. Start. Urinary cannabinoid analysis: comparison of four immunoassays with gas chromatography-mass spectrometry. *Forensic Sci. Int.*, **50**, 15-22, (1991).

3- S. Kerrigan, W.H. Jr. Philips. Comparison of ELISAs for opiates methamphetamine-cocaine metabolite, benzodiazepines, phencyclidine, and cannabinoids in whole blood and urine. *Clin*. *Chem*., **47**, 540-547, (2001).

4- T. Korte, J. Pykalainen, P. Lillsunde, T. Seppala. Comparison of RapiTest with Emit d.a.u. and GC-MS for the analysis of drugs in urine. *J. Anal. Toxicol.*, **21**, 49-53, (1997).

5- A.D. Fraser, D. Worth. Monitoring urinary excretion of cannabinoids by fluorescence-polarization immunoassay: a cannabinoid-to-creatinine ratio study. *Ther. Drug. Monit.,* **24,** 746-750, (2002).

6- D. Perez-Bendito, A. Gomez-Hens, A. Gaikwad. Direct stopped-flow fluorescence polarization immunoassay of abused drugs and their metabolites in urine. *Clin. Chem.*, **40**, 1489-1493, (1994).

7- R.L. Foltz, I. Sunshine. Comparison of a TLC method with EMIT® and GC/MS for detection of cannabis in urine. *J. Anal. Toxicol.,* **14**, 375-378, (1990).

8- M.L. Weaver, B.K. Gan, E. Allen, L.D. Baugh, F.-Y. Liao, R.H. Liu, J.G. Langner, A.S. Walia, L.F. Cook. Correlations on radioimmunoassay, fluorescence polarization immunoassay, and enzyme immunoassay of Cannabis metabolites with gas chromatography/mass spectrometry analysis of 11-Nor-Δ^9-tetrahydrocannabinol-9- carboxylic acid in urine specimen. *Forensic Sci. Int.*, **49**, 43-56, (1991).

9- J. Teske, K. Putzbach, W. Engewald, R.K. Müller. Determination of cannabinoids by gas chromatography-mass spectrometry and large-volume programmed-temperature vaporiser injection using 25µl of biological fluid. *J. Chromatogr.* B, **772**, 299-306, (2002).

10- W. Weinmann, S. Vogt, R. Goerke, C. Muller, A. Bromberger. Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS. *Forensic Sci. Int.*, **113**, 381-387, (2000).

11- D. Debruyne, F. Albessard, M.C. Bigot, M. Moulin. Comparison of three advanced chromatographic techniques for Cannabis identification. *Forensic Sci. Int.*, **106**, 135-146, (1999).

12- P. Oroszlán, G. Verzár-Petri, E. Mincsovics and T. Székely. Separation, quantitation and isolation of Cannabinoids from *Cannabis sativa* L. by Overpressured Layer Chromatography. *J. of Chromatogr.*, **388**, 217-224, (1987).

13- O. Zoller, P. Rhyn, B. Zimmerli. High-performance liquid chromatographic determination of delta 9-tetrahydrocannabinol and the corresponding acid in

hemp containing foods with special regard to the fluorescence properties of delta 9-tetrahydrocannabinol.. *J. Chomatogr*. A, **872**, 101-110, (2000).

14-A.J. Poortman-Van Der Meer, H. Huizer. A contribution to the improvement of accuracy in the quantitation of THC. *Forensic Sci. Int*., **101**, 1-8, (1999).

15-S.A. Ross, Z. Mehmedic, T.P. Murphy, M.A. Elsohly. GC-MS analysis of the total delta 9-THC content of both drug and fiber-type cannabis seeds. *J. Anal. Toxicol.,* **4**, 715-717, (2000).

16-I.S. Lurie, R.P. Meyers, T.S. Conver. Capillary electrochromatography of cannabinoids, *Anal. Chem.,* **70**, 3255-3260, (1998).

17-M.A. Bacigalupo, A. Ius, G. Meroni, G. Grassi, A. Moschella. Time-resolved fluoroimmunoassay for delta(9)-tetrahydrocannabinol as applied to early discrimination of Cannabis sativa plant, *J. Agric. Food Chem.*, **47**, 2743-2745, (1999).

18-H. Tanaka, Y. Shoyama. Monoclonal antibody against tetrahydrocannabinolic acid distinguishes Cannabis sativa samples from different plant species. *Forensic Sci. Int.,* **106**, 135-146, (1999).

19-H. Wagner, S. Bladt. Plant Drug Analysis – A Thin Layer Chromatography Atlas 2nd ed., Spinger Verlag, Berlin, (1996).

20-G. Alemany, A. Gamundi, M.C. Nicolau, D. Serro. A simple method for plasma cannabinoid separation and quantification. *Biomed. Chromatograph.*, **7**, 273-274, (1993).

21-K D. Parker, J.A. Wright, A. F. Halpern, C.H. Hine. Preliminary report on the separation and quantitative determination of cannabis constituents present in plant material, and when added to urine, by thin-layer and gas chromatography. *Bull. Marc.*, **20**, 9, (1968).

22-B. Szabady, Z. Fater, S. Nyiredy. Comparative study of automated development chambers. *J. Planar Chromatogr.*, **12**, 82-85, (1999).

23-A. Schurr, and B.M. Rigor. Cannabis extract but not Δ^1-tetrahydrocannabinol, inhibits human brain and liver monoamine oxidase, *General Pharmacology: The Vascular System,* **15**, 171-174, (1984).

24-E. Stahl, Analyse Chromatographique et Microscopique des Drogues, Tec. et Doc., Paris, pp. 137-141, (1975).

25-J. Pothier, N. Galand, S. Fouchécourt, C. Viel. Isolation of alkaloids by Over Thin-Layer Chromatography, *Fitoterapia*, **68**, 42-44, (1997).

26-E. Mincsovics, M. Garami, L. Kecskès, B. Tapa. Personal Overpressured-Layer Chromatography (OPLC) Basic System Flexible Tool in Analytical and Semipreparative Work, *J. of AOAC Int.*, **82**, N° 3, 587-598, (1999).

27-M.J. Baptista, P. Venâncio Monsanto, E. Gouveia Pinho Marques, A. Bermejo, S. Ávila, A. Martelo Castanheira, C. Margalho, M. Barroso, D. Nuno Vieira. Hair analysis for Δ^9-THC-COOH, CBN and CBD, by GC/MS-EI. Comparison with GC/MS-NCI for Δ^9-THC-COOH. *Forensic Sci. Inter.*, **128**, 66-78, (2002).

Table I: *h*Rf and colours with Fast Blue Salt reagent of cannabinoïdes

Cannabinoids	*h*Rf			Colours with Fast Blue Salt B
	MAT/TLC	AMD	OPLC	
CBN	59	73	23	violet
Δ^9-THC	66	76	28	purple
CBD	73	79	34	orange red

Figure 1 - TLC-MAT of Cannabinoids

1 - Cannabidiol 1 µL, 2 - Cannabis extract 7 µL, 3 - Δ^8-THC 2 µL, 4 - Cannabinol 1 µL,
5 - Cannabis resin 2 µL, 6 - Δ^9-THC 3 µL, 7 - Cannabis extract 7 µL

Figure 2- AMD of Cannabinoids

1 - Cannabis extract 7 µL, 2 - Cannabidiol 1 µL, 3 – Cannabinol 1 µL,
4 - Cannabis resin 2 µL, 5 - Δ^9 THC 3 µL, 6 - Cannabis extract 5 µL,

Figure 3 - OPLC of Cannabinoids

1 - Cannabinol 1 µL, 2 - Cannabis resin 2 µL,
3 - Δ^9-THC 3 µL, 4 - Cannabidiol 1 µL

Figure 4 – *Top*: MS-GC of Cannabidiol obtained from Cannabis hemp by semi-preparative mode OPLC
Bottom: MS of Cannabidiol from NIST Library