

Thin Layer Chromatographic Analysis of Psychoactive Plant Cannabis Sativa L

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ABSTRACT:

Cannabis is one of the oldest known medicinal plant and is described in almost every ancient hand book on plant medicine but its use has banned in many parts of the world due to its psychoactive properties. Often different forms of cannabis drugs are referred to laboratories for forensic and legal purposes, Generally analysis report are based on preliminary identification of seized material and confirmation by tlc analysis and/or morphological examination .In the field also tests are performed using field testing kit by investigating officers. Due to non-specificity of colour tests the additional confirmation is needed. There are a number of TLC methods recommended for the qualitative and semi-quantitative analysis of cannabis, using a variety of different stationary phases (TLC plates) and solvent systems, and slightly different sample preparation and spot visualization techniques. Present review highlights thin layer chromatographic approaches made for the identification of cannabinoids in alleged cannabis drugs and cannabis products, Charred cannabis, herbal formulations and in biological fluids by various scientists as well as recommended procedures available in different official monographs. Review will prove its utility as a ready reference document to the forensic scientists.

Key words - Cannabis plant, Cannabinoid,, charred cannabis Thin layer chromatography

INTRODUCTION

The detection of drugs has an important role to play in many areas of society, such as sport, suspicious deaths, violent crime, and travel and work safety. Forensic laboratories assist law enforcement through the analysis of drugs seized by police. There are primarily two reasons for doing so. It is often difficult for police to determine exactly what has been seized. Analysis of the substances can tell police what is actually seized compounds, including adulterants, have been used to produce the drug or about their overall level of purity. Cannabis is one of the oldest known medicinal plant and is described in almost every ancient hand book on plant medicine but its use has banned in many parts of the world due to its psychoactive properties The history of the use and identification of Cannabis sativa L. is long and complex. It is one of the oldest cultivated plants, used for the production of oil from the seeds, and fibre from the stems for rope and fabrics, and has long been used as a psychoactive drug due to the presence of cannabinoids in the resins produced by the plant. Different forms of cannabis drug are referred to laboratories for forensic and legal purposes. Michael D. Cole (1) described that the methods used to identify cannabis products depend upon the nature of the products themselves. Herbal material can be identified on the basis of its morphological

characteristics alone, provided that certain of these are present. Where they are not (e.g. cannabis resin and hash oil), the identification is made on the basis of phyto-chemical identification and the proof of the presence of Δ^9 -THC, its precursor, cannabidiol (CBD), its breakdown product, CBN, precludes the use of the sample for comparative purposes.

Cannabinoids Contents of Cannabis Plant

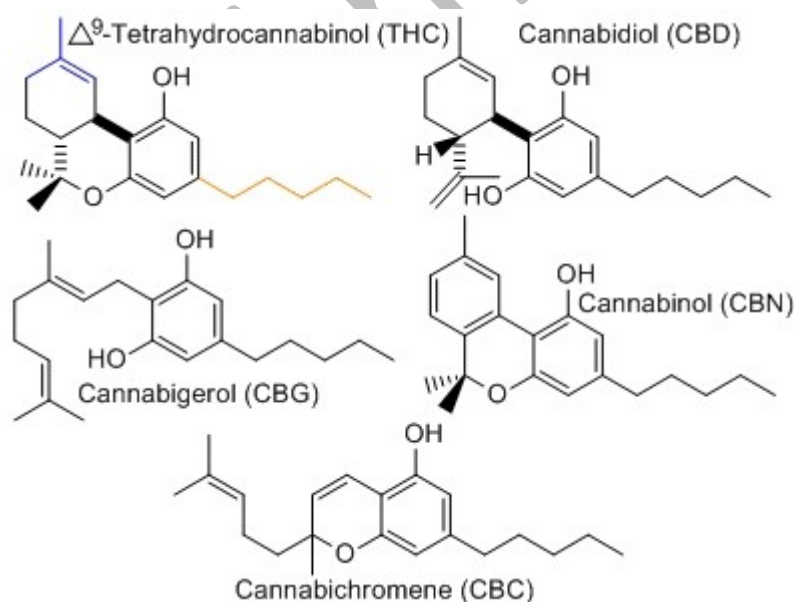
There are over 480 natural components found within the Cannabis sativa plant, of which 66 have been classified as "cannabinoids;" chemicals unique to the plant. The most well known and researched of these, delta-9-tetrahydrocannabinol (Δ^9 -THC), is the substance primarily responsible for the psychoactive effects of cannabis. The effects of THC are believed to be moderated by the influence of the other components of the plant, most particularly the cannabinoids.(2)

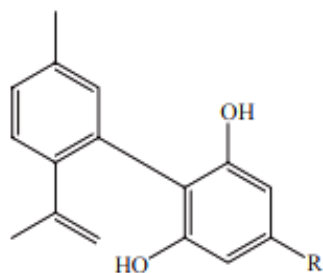
The cannabinoids are separated into subclasses. These are as follows:

Cannabigerols (CBG); Cannabichromenes (CBC); Cannabidiols (CBD); Tetrahydrocannabinols (THC); Cannabinol (CBN) and cannabinodiol (CBDL).

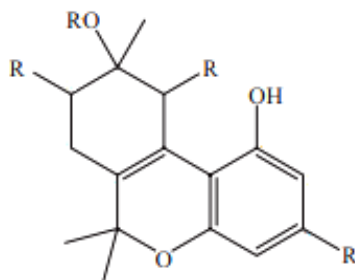
Other cannabinoids (such as cannabicyclol (CBL), cannabielsoin (CBE), cannabitrinol (CBT) and other miscellaneous types).

Structural formula of some of these are

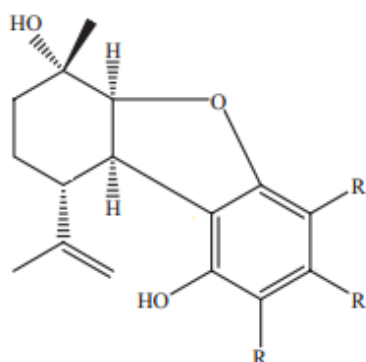




Cannabicyclol (CBL)



Cannabitrinol (CBT)



Cannabielsoin (CBE)

ANALYSIS OF CANNABIS:

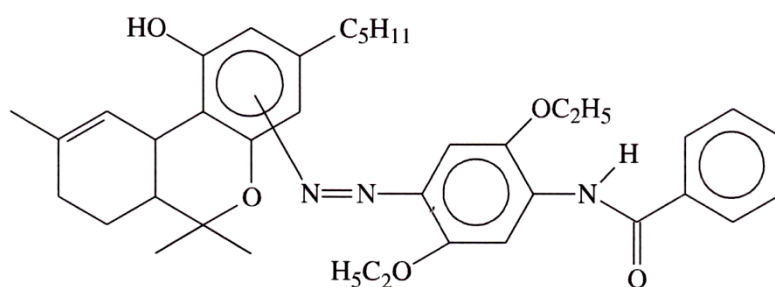
Colour tests

It is always suggested that positive colour test only provides an indication of the possible presence of cannabis-containing material and not a definitive identification of cannabis. It is therefore mandatory for the analyst to confirm such results by the use of additional, typically more discriminative techniques thin-layer chromatography and microscopy for cannabis plant material for positive identification, provided that at least three cannabinoids are identified by TLC (3).

Many colour tests have been suggested e.g. Fast Corinth V salt test, Fast Blue B salt test Duquenois-Levine test, Rapid Duquenois test, Beams alkaline and acid test however Duquenois-Levine test is commonly used color test. Modified Duquenois-Levine test, is an established screening test for the presence of cannabis. The test was initially developed in the 1930s by Pierre Duquenois, and was adopted in the 1950s by the United Nations as the preferred test for cannabis, and originally claimed to be specific to cannabis. After several modifications, it became known as the Duquenois-Levine test. However various studies showed that the test was not specific to cannabis, the study most cited in favour of the specificity of the D-L test is Thornton and Nakamura (4) however the authors themselves reported that the D-L test gave false positives, but declared the D-L test is confirmatory when combined with the presence of cystolithic hairs, which cannabis plants possess. However, many plant species have such hairs, and the study only confirmed that 82 of them did not give D-L test false positives.

Thin layer chromatographic analysis

TLC can be the method of selection if the object is to examine a large number of samples rapidly and simultaneously and it is a low-cost method also. There are a number of TLC methods recommended for the qualitative and semi-quantitative analysis of cannabis, using a variety of different stationary phases (TLC plates) and solvent systems, and slightly different sample preparation and spot visualization techniques. Many of those methods also produce acceptable results but each method that is newly introduced are subjected to validation and/or verification prior to routine use.(5). According to Michael D. Cole (6) the most frequently used mobile phase for TLC is toluene, although other systems can be used. The chromatogram is developed, removed from the solvent and the mobile phase allowed to evaporate at room temperature. If toluene (or another dangerous solvent) is used, drying of the chromatographic plate should be carried out in a fume cupboard. Once the plate is dry, the compounds present should be examined. The most commonly used reagent is 1% Fast Blue BB in water, which has replaced the previous use of the carcinogenic Fast Blue B. A variety of red, orange, brown and yellow compounds form as a result of the reaction of the Fast Blue BB with the cannabinoids to form diazonium salts



Diazonium salts

Some of the selected thin layer chromatographic methods suggested by different investigative agencies, forensic scientists and scholar of pharmaceutical sciences have been already reviewed by Goutam and Goutam(7) In the present paper efforts have been made to highlights the thin layer chromatographic approaches made for the identification of herbal cannabis, herbal formulations, cannabis products, charred cannabis and in biological fluids by various scientists as well as recommended in different official monographs of repute, for ready reference to the forensic scientists. (Table 1 and 2)

Table -1 Thin layer Chromatography- Rf value of the Cannabis constituents

Cannabinoids	Solvent system											
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
9-THC	11	31	30	29	00	01	31	50	35	36	32	39
CBN	94	95	52	20	90	77	97	45	25	63	27	28
CBD	94	95	05	36	88	76	97	60	45	16	36	44

Table-2-Solvent systems and adsorbents

Solvent systems	Adsorbents (tlc plates)	Mobile phase
T ⁷ 1	Silica gel G, 250 µm thick dipped in, or sprayed with, 0.1% potassium hydroxide in methanol and dried	Methanol: Strong Ammonia solution
T ⁹ 2	Silica gel G, 250µm	Ethyl acetate: Methanol: Strong Ammonia solution (80:85:10)
T ¹⁰ 3	Silica gel G, 250 µm thick, dipped in, or sprayed with, a 10% solution of silver nitrate, and dried.	Toluene, using unsaturated (open tank) condition
T ¹¹ 4	gel G, 250 um thick, sprayed with diethylamine immediately before use	Xylene: Hexane: Diethylamine (25:10:1).
T ¹² 5	Silica gel G, 250µm	Chloroform : Ethanol (90:10).
T ¹³ 6	Silica gel G, 250µm	Chloroform : Cyclohexane : Acetic acid (40:40:20)
T ¹⁴ 7	Silica gel G, 250µm	Chloroform : Methanol: Propionic acid (72:18:10)
T ¹⁵ 8	Silica gel G, 250µm	Hexane: Diethyl ether (80:20)
T ¹⁶ 9	Silica gel G, 250µm	Benzene: n- hexane: Diethylamine (25:10:1)
T ¹⁷ 10	*Silica gel G, Silver nitrate(300µ)	Benzene
T ¹⁸ 11	Silica gel G, 250µm	Pet ether: Diethyl ether (80:20).
T ¹⁹ 12	Silica gel G, 250µm	Cyclohexane : Di- isopropyl ether : diethylamine(52:40:8)

**In system T10 silica gel G- silver nitrate(300µm)- plate coating is prepared as a slurry by dissolving 6gm of silver nitrate in 38 ml of water and suspending 18 gm of silica gel G in the solution. The slurry is applied to the plate with a spreader to a thickness of 300µ. the coated plates are allowed to set at room temperature for 10 min and then activated in a dark oven controlled to 85-90 °C for 35 minutes. Store plates in a dark desiccators containing anhydrous calcium sulphate.*

Visualization – fast blue B salt- dissolved in 15 mg of fast blue B salt in 20 ml of 0.1 N sodium hydroxide

Location reagents

Fast blue B solution – Cannabidiol gives an orange colour, cannabinol gives a violet colour, and delta 9 tetrahydrocannabinol gives a red colour. The colours may be intensified by over spraying with 1 M hydroxide or by exposing the plate to ammonia fumes.

Duquenois reagent- after spraying with the reagent, blue to violet colours are given by cannabinoids when over sprayed the plate with hydrochloric acid.

Descending thin layer chromatography

Win, Pe (20) suggested a technique that uses descending thin layer chromatography (TLC) for identification of cannabinoids. Author used a partition system of two-dimensional descending TLC, in which toluene is used as the eluting solvent. The quantity of cannabinoids obtained by TLC had been confirmed by gas chromatography (GC). Cannabinol, cannabidiol, Δ^9 -tetrahydrocannabinol (THC) and Δ^8 -THC were used as reference substances in this analysis. A 20 x 60 cm TLC aluminum roll pre coated with silica gel F₂₅₄ (layer thickness 0.20 mm) was used.. The roll was impregnated with dimethyl formamide and dried with a hair dryer for 10 minutes; 10 x 20 cm impregnated plates were used for both one- and two-dimensional TLC. Toluene was used as the eluting solvent for descending and the spots were visualized by spraying with methanolic fast B salt solution. The geometrical form of the plates used in descending TLC. A plate in the form of an isosceles triangle with sides 21 cm, 21 cm and 10 cm in length was used for slow partition descending chromatography and claimed technique, useful for the analysis of cannabinoids.

Cannabis in herbal formulation

A. Mohammad et al (21) reported the use of surfactant in TLC analysis of an herbal formulation (Jatiphaladya) & developed a simple and reliable thin layer chromatographic (TLC) method using micellar solution of sodium dodecyl sulfate (SDS) as mobile phase for the identification of all the four herbal drugs with preliminary separation on silica gel 'G' TLC plate. The active components of drug were extracted in a mixture of ethanol and water (4:1), chromatographed on silica gel TLC plate using aqueous SDS (5%) as mobile phase and the resolved spots for Cannabis sativa (Rf-0.95), Myristica fragrans (Rf-0.64), Piper longum (Rf-0.41) and Embleia ribes (Rf-0.26) were identified using vanillin-sulfuric acid (2% solution of vanillin in 5% methanolic sulfuric acid). In order to realize most favorable mobile phase system in combination with silica gel 'G' as stationary phase, the effect of nature of surfactants (anionic, cationic or nonionic) and the level of concentration of each surfactant [sodium dodecyl sulfate (SDS), N-cetyl-N, N, N-trimethylammonium bromide (CTAB) or t-octyl phenoxydacaethoxy ethanol (TX-100)] on the mobility of all four active components was examined.

Determination of charred cannabis constituents

Kempe et al.(22) reported simple chromatography system for the determination of charred cannabis constituents. Eastman chromogram sheets, silica gel (6061), were used without activation. The mobile solvent used in all chromatograms was toluene;. Visualization of spots was accomplished by using a .15% aqueous-methanol (1 to 3) Fast Blue B spray. Known tetrahydrocannabinol (THC) cannabidiol (CBD) and cannabinol (CBN) 4 were chromatogramed with control hashish. The descending order of the separated components was CBD (orange), THC (scarlet),and CBN (violet) All the residue samples were charred and failed to show any marijuana characteristics. The samples were extracted with .3 ml or approximately 6 drops of petroleum ether for a few minutes. The chromatogram sheets were cut into 3 cm wide strips and spotted with approximately 3 μ l of the extract. The strips were then placed between glass plates, and the solvent was allowed to rise about 17 cm. Running

time was 105 minutes at room temperature (22°C). Authors chromatograms sprayed with Fast Blue B. and noted that several additional spots appeared on some chromatograms. The more predominate ones appeared below CBN. The R_f values were .19 and .40. It was noted that several additional spots appeared on some chromatograms. The more predominate ones appeared below CBN. It may be due to that CBD is not cyclized to THC during smoking. Authors reported that their results indicate the general lack of CBD in charred residue; however, THC and CBN are usually present. The question now arises as to why THC and CBN remain while CBD is absent in the smoked or charred marijuana. Even in the "roach" paper, CBD was absent while the THC spot was the most intense of all the samples.

Cannabis -Inflorescence and Leaf

TLC method suggested in American Herbal Pharmacopoeia is as follows (23)

Standards Preparations

Cannabinoid standards are dissolved in methanol at a concentration of 1 mg/ml.

Standards Solution Stability- CBD, CBG, and CBN are stable in methanol, both at room temperature and with freezing. Δ^9 -THC, THCV, and CBC methanolic solutions are stable only when frozen and acid compounds are only stable in a freezer. Due to their instability, acid compounds should be prepared cool and stored and shipped frozen.

Reagent Preparation

Fast Blue reagent: Dissolve 0.5 g Fast Blue B salt (M P Bio chemicals, LLS) in 100 ml distilled water. **Vanillin/ H_2SO_4 :** Dissolve 6 g vanillin in 90 ml ethanol (95%). Add 10 ml of 98% H_2SO_4 . This reagent is relatively unstable and is best to use fresh each time.

Chromatographic Conditions- Stationary Phase: C18 (UV 254) TLC plates 150 μ m, 10 cm \times 10 cm (Sorbent Technologies).

Mobile Phase: 75:25 (V:V) methanol/water with 0.1% glacial acetic acid.

R_f values reported for cannabinoid standards

Phytocannabinoid	R _f
CBC	0.21
Δ^9 -THC	0.26
CBN	0.29
CBG	0.33
CBD	0.40
THCV	0.42
Δ^9 -THCA	0.61
CBDA	0.77

Wagner and Bladt (24) recommended the following thin layer Chromatographic techniques for the separation of cannabinoids as follows

Thin layer Chromatography

Reference solution

10 mg is dissolved in 10ml toluene

1mg synthetic THC is dissolved in 5ml $CHCl_3$,

5 μ l is used for tlc,

3 μ l is used for tlc

Adsorbent

Pre coated TLC plates with Silica gel F₂₅₄

Solvent system

n-hexane: diethyl ether 80:20

n-hexane: diethyl ether 90:10

Detection

UV-250 nm

Fast blue salt reagent(FBS No 15)
mine (vis)

Prominent quenching of cannabinoids

Cannabinoids appear orange red or Car

Standard thymol gives orange colour

Detection of THC-COOH

Kaistha and Tadras(25) advocated the use of Silica gel plates with Chloroform: methanol: con ammonia (85:15:2) as mobile phase. Fast blue RR(0.5%W/Vin equal volumes of methanol and water)was used for detection.

Kanter et al(26) developed a method for identifying total THC-COOH utilizing Silica gel G plates and sequentially

Developed them insolvent systems Acetone: chloroform: tri ethylamine (80:20:1)followed by Pet ether : ether : glacial acetic acid (50:50:1.5) . The procedure could detect a spot containing 0.5µg of THC-COOH.

Marijuana resin

Thornton and Nakamura(27) reported the Thin layer Chromatographic of marijuana resin and mention that a number of TLC systems were attempted with greater or lesser success Silica Gel G or Silica Gel H gave better separation than did absorbent layers made up with Alumina G or Kieselguhr G. Silica Gel G – AgNO₃ 5:1) gave good results not Substantially different from that of Silica Gel G alone, and was not utilized in further thin layer studied.

Thin layer Chromatographic mobility of marijuana constituents

Constituents	S1	S2	S3	S3	S4
Cannabidiolic acid	0.04	0.18	0.11	0.08	0.05
Cannabidiol	0.12	0.44	0.21	0.20	0.12
Cannabinol	0.38	0.66	0.29	0.54	0.26
Δ ¹ THC	0.48	0.70	0.36	0.60	0.30
Δ ⁶ THC	0.50	0.72	0.40	0.64	0.34
Δ ¹ THC acetate	0.71	0.84	0.80	0.85	0.60

Plates : 250 layer silica gel HF , activated at 100⁰ C for 1 hour

Solvent systems:

S1 : Benzene, S2 : Petroleum ether (60-80⁰ C b. p.): Ethanol (9:1)

S3: n-Hexane: p-Dioxane (9:1) ,S4: n-Hexane: Diethyl ether (4:1)

S5 Petroleum ether (60-80⁰ C b. p.): Diethyl ether (9:1)

CONCLUSION

There are a number of TLC methods for the qualitative and semi-quantitative analysis of cannabis, using a variety of different stationary phases (TLC plates) and solvent systems, and slightly different sample preparation and spot visualization techniques are suggested , many of those methods may produce acceptable results but each method that is newly introduced to

a laboratory must be validated and/or verified prior to routine use, as has rightly instructed in the Manual published by United Nations for use by national drug analysis laboratories (28)

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