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A Qualitative and Quantitative HPTLC Densitometry Method for the Analysis of Cannabinoids in *Cannabis sativa* L.

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Abstract:
Introduction – Cannabis and cannabinoid based medicines are currently under serious investigation for legitimate development as medicinal agents, necessitating new low-cost, high-throughput analytical methods for quality control.
Objective – The goal of this study was to develop and validate, according to ICH guidelines, a simple rapid HPTLC method for the quantification of Δ^9-tetrahydrocannabinol (Δ^9-THC) and qualitative analysis of other main neutral cannabinoids found in cannabis.
Methodology – The method was developed and validated with the use of pure cannabinoid reference standards and two medicinal cannabis cultivars. Accuracy was determined by comparing results obtained from the HTPLC method with those obtained from a validated HPLC method.
Results – Δ^9-THC gives linear calibration curves in the range of 50–500 ng at 206 nm with a linear regression of $y = 11.858x + 125.99$ and $r^2 = 0.9968$.
Conclusion – Results have shown that the HPTLC method is reproducible and accurate for the quantification of Δ^9-THC in cannabis. The method is also useful for the qualitative screening of the main neutral cannabinoids found in cannabis cultivars.

Keywords: HPTLC; *Cannabis sativa*; Δ^9-THC; HPLC; cannabinoids

Introduction

Cannabis (*Cannabis sativa* L.) is an important medicinal plant with a long history of human use. To date approximately 500 compounds have been identified in cannabis. Of particular importance are the cannabinoids, also known as phytocannabinoids, a group of C21 terpenophenolic compounds unique to cannabis (Turner *et al.*, 1980; Ross and ElSohly, 1995; ElSohly and Slade, 2005). Currently there is an increasing interest in the development of cannabinoids and cannabis preparations as legitimate medicines for a variety of medical applications. Some of these applications include, but are not limited too, multiple sclerosis, chronic pain, glaucoma, asthma and cardiovascular conditions, and as an antiemetic (Williamson and Evans, 2000). Δ^9-Tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive constituent in cannabis and has been the focus of a great deal of pharmacological and medicinal research (Costa, 2007). Consequently low-cost, high-throughput analytical methods for the qualitative and quantitative analysis of Δ^9-THC and other neutral cannabinoids are in high demand.

Cannabinoids are enzymatically biosynthesised in the plant as their corresponding carboxylic acid forms (Taura *et al.*, 2007). Neutral cannabinoids are formed via decarboxylation (loss of CO_2) of the acidic cannabinoids during exposure to light, heat (e.g. smoking), or as a result of prolonged storage (Thakur *et al.*, 2005). Cannabinol (CBN) is the most common oxidative degradation product of Δ^9-THC found in aged cannabis (Fig. 1) (McPartland and Russo, 2001). Δ^8-Tetrahydrocannabinol (Δ^8-THC), a regioisomer of Δ^9-THC, has also been found in cannabis samples at low concentrations but is commonly regarded as an extraction artefact. Numerous degradation products of Δ^9-THC and CBN are reported; however, they are usually present in very low concentrations or are only formed under very specific conditions (Turner *et al.*, 1980). Therefore, in order to analyse cannabis for its total Δ^9-THC potential, its precursor Δ^9-THC acid A (THCA) and degradation product CBN must be taken into consideration.

Studies have shown that Δ^9-THC, Δ^8-THC and CBN can be separated and potentially quantified using various TLC methods; however, these methods require the use of over-pressured layer chromatography (OPLC) instrumentation or automated multiple development (AMD) systems (Oroszlan *et al.*, 1987; Szabady *et al.*, 2002; Galand *et al.*, 2004). These studies do not take THCA into consideration and validation was not performed for decarboxylation, rendering these protocols unacceptable for total Δ^9-THC quantification. Numerous methods are available

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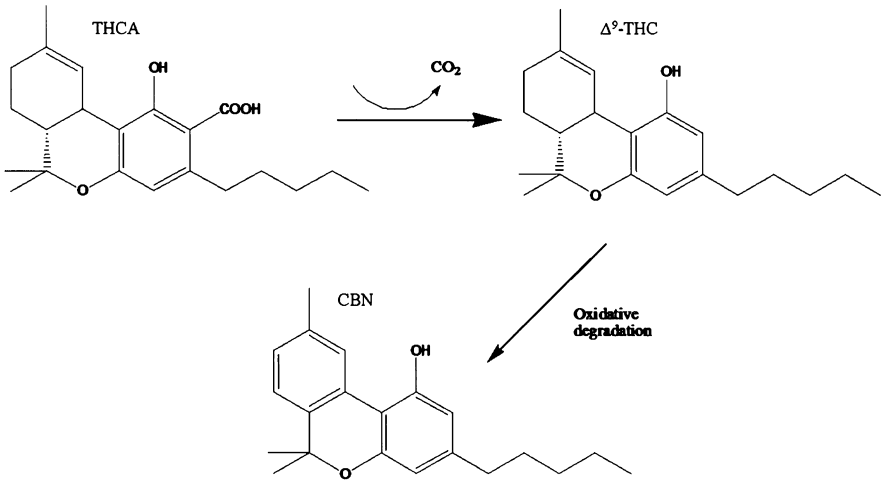

Figure 1. THCA decarboxylation into Δ9-THC and degradation into CBN.

to decarboxylate THCA into Δ^9-THC; however, their effectiveness was not tested with a THCA reference (Raharjo and Verpoorte, 2004).

Recent advances in TLC, specifically the development of high-performance thin-layer chromatography (HPTLC), have made TLC a more attractive method for the quantification of herbal drug constituents (Sherma and Fried, 2003). A reliable HPTLC method could offer advantages over both HPLC and GC methods for analysing cannabis, including lower running costs, more rapid analysis time and the ability to analyse multiple samples simultaneously. Methods for preparative isolation of the main acidic and neutral cannabinoid reference standards have now been developed (Hazekamp *et al.*, 2004a) and their spectroscopic and chromatographic data have been published (Hazekamp *et al.*, 2005). The lack of an adequate HPTLC method for the quantification of Δ^9-THC in cannabis and unavailability of both acidic and neutral cannabinoid reference standards directed the objective of this study: the development and validation of a rapid and accurate HPTLC method using ICH (1996/2005) guidelines for the quantification of Δ^9-THC and qualitative analysis of the major neutral cannabinoids in cannabis. Method validation was performed with pure cannabinoid standards and method performance was evaluated by analysing two different cannabis varieties and comparing results with those obtained with a validated HPLC system (OMC, 2008).

Experimental

Chemicals and reagents

Chloroform (≥99.8%, A.C.S. reagent stabilised with amylenes) and Fast Blue B salt were purchased from Sigma-Aldrich (Steinheim, Germany). Absolute ethanol and methanol analytical reagent-grade and HPLC-grade were purchased from Merck Biosolve Ltd (Valkenswaard, The Netherlands). Cannabinoid references for Δ^9-THC, THCA, Δ^8-THC, CBN, cannabidiol (CBD), Δ^9-tetrahydrocannabivarin (THCV), cannabigerol (CBG), and cannabichromene (CBC) were prepared from cannabis (Hazekamp *et al.*, 2004a, b). All cannabinoid reference standards were dissolved in reagent-grade ethanol.

Plant material

Cannabis was obtained from Bedrocan BV (Veendam, The Netherlands). Two different strains were used to represent both a relatively high total Δ^9-THC variety (Bedrocan: ca. 18%) and a relatively low total Δ^9-THC variety (Bediol: ca. 6%) (OMC, 2008). The plant material consisted of dried female flowertops, with 5–10% water content.

Decarboxylation and extraction procedure

Decarboxylation of the plant material was achieved by placing a sample (1 g) in a glass test tube (30 mL) and covering it with aluminium foil. The test tubes were placed in boiling water (100°C) for 2 h. The plant material was transferred to falcon tubes (50 mL) for extraction, followed by rinsing with ethanol (3 × 5 mL). The volume of ethanol was brought to 40 mL and the falcon tubes were placed on a shaker for 15 min at 300 rpm. The samples were centrifuged for 5 min and the supernatant collected in a 100 mL volumetric flask. Ethanol (25 mL) was added to the plant material and the extraction procedure repeated twice. The total volume of ethanol in the volumetric flask was brought to 100 mL. Samples were filtered through a 25 mm syringe filter with a 0.45 µm PTFE membrane. The first few millilitres of sample were discarded because of cannabinoids that might absorb to the filter and a new filter was used for each sample. The filtrate was finally diluted in ethanol 10 times for Bediol and 20 times for Bedrocan for analysis.

HPLC chromatography

HPLC profiles were acquired on a Waters (Milford, MA, USA) HPLC system consisting of a 626 pump, a 717plus autosampler and a 2996 diodearray detector (DAD; 228 nm), controlled by Waters Millennium 3.2 software. Full spectra were recorded in the range of 200–400 nm. A Vydac (Hesperia, CA, USA) C_{18} analytical column 218MS54 (4.6 × 250 mm, 5 µm), with a Waters Bondapak C_{18} (2 × 20 mm, 50 µm) guard column was used. The mobile phase con-

sisted of a mixture of methanol–water containing 25 mM of formic acid in gradient mode; methanol : water from 65:35 to 100:0 over 25 min, then isocratic to 28 min. The column was re-equilibrated under initial conditions for 4 min. Flow rate was 1.5 mL/min and total runtime was 32 min. All determinations were carried out at ambient temperature.

HPTLC chromatography

An automated sample applicator, CAMAG ATS-4, was used for spotting HPTLC plates and a densitometric and UV scanner, CAMAG Scanner type 3, was used for plate analysis (Camag, Muttenz, Switzerland). Both instruments were controlled by Wincats software version 1.4.3. The plates [20 × 20 cm, HPTLC silica gel 60 coated on aluminium foil (Merck, Steinheim, Germany)] were cut in half to make 20 × 10 cm plates, pre-washed by developing in methanol, follow by air drying (10 min) and a brief heating of 10 s with a hot air blower to remove any dust particles. The laboratory was kept at 23 ± 2°C with a relative humidity of 30–60%. Samples were applied 1 cm from the bottom and at least 2 cm from the sides of the plate as 6 mm bands in 5 µL aliquots using the applicator. Plates were developed in a 24 × 15 × 8 cm custom-made glass twin trough chamber saturated for 15–20 min with 20 mL chloroform. Plates were developed to a height of 6 cm, for 9 min ± 30 s, and were allowed to dry at ambient conditions in a ventilated fumehood for 10 min. Plates were then scanned at 206 nm using the UV scanner. Quantitative evaluation was done via peak areas. Spot identification was based on R_f value comparison with standards, which was confirmed by a UV scan from 200 to 400 nm, and visual evaluation by dipping the plates in a 0.1% aqueous Fast Blue B solution for 5 s, a selective stain for cannabinoids.

HPTLC method validation

Limit of quantitation, limit of detection, linear dynamic range and repeatability. The limit of detection (LOD) was determined empirically by testing dilutions of Δ^9-THC and CBN standard solutions until no spot could be observed at 206 nm. The linear range was initially found by spotting a wide range of Δ^9-THC and CBN standards (10–1000 ng). The determined linear dynamic range, LOQ, and repeatability of the method was confirmed by repeated analysis three times on three separate days with standards of Δ^9-THC and CBN at 50, 100, 250, 400 and 500 ng. The percentage relative standard deviation (RSD) was calculated at each standard level for each experiment.

Table 1. Cannabinoid R_f values

Cannabinoid	R_f
CBD	0.52
Δ^9-THC	0.47
Δ^8-THC	0.49
CBN	0.47
THCV	0.43
CBG	0.37
CBC	0.33

Mobile phase: chloroform.

Instrumental precision. In order to determine the precision of the applicator, 400 ng Δ^9-THC, 400 ng CBN, and a cannabis extract (Bediol) were spotted five times in 5 µL aliquots on a single plate. These samples were developed, scanned and RSD calculated. In order to determine scanner precision, single spots from a 5 µL aliquot of three samples containing 400 ng Δ^9-THC, 400 ng CBN, and a cannabis extract (Bediol) were developed and scanned five times and the RSD calculated.

Accuracy. To determine the accuracy of this method for the analysis of cannabis, the HPTLC method was compared with a validated HPLC method. Three samples each of Bediol and Bedrocan (no decarboxylation) were extracted and analysed by HPLC for their total Δ^9-THC content (OMC, 2008). Total Δ^9-THC content was calculated as follows: Δ^9-THC% = THCA% * (314.47/358.48) + Δ^9-THC%. Three samples each of Bediol and Bedrocan (decarboxylated) were extracted and analysed by HPTLC in triplicate runs and HPLC in a single run. Bediol and Bedrocan extracts were diluted 10× and 20× in ethanol, respectively, prior to HPTLC analysis.

The accuracy of the method in identifying Δ^9-THC and CBN in mixtures was also studied. Six solutions were prepared using Δ^9-THC and CBN standards, with three solutions prepared representing samples containing a relatively low amount of CBN and three of these solutions representing a relatively high amount of CBN. These solutions were analysed by HPTLC in triplicate runs and once by HPLC. HPTLC results were compared with the HPLC results and both data sets for the high and low CBN solutions were compared separately using a Student's *t*-test.

Results and Discussion

All cannabinoids studied were shown to have a >95% recovery with Δ^9-THC having >99.8 ± 3.7% (OMC, 2008). Decarboxylation was optimised for minimum degradation by studying the time required at 100°C to quantitatively convert THCA into Δ^9-THC. Time points tested ranged from 15 min to 4 h heating. According to HPLC analysis of decarboxylated cannabis samples the majority (97.66 ± 0.33%) of THCA was decarboxylated after 2 h at 100°C.

Chloroform was selected as a mobile phase since it gave adequate separation of Δ^9-THC from the other major neutral cannabinoids, namely CBG, CBC, CBD and THCV (Table 1). The colors of the compounds after staining with Fast Blue B were consistent with those reported (Hazekamp *et al.*, 2005). Chloroform stabilised with ethanol cannot be used as a mobile phase because ethanol increases the solvent strength of the mobile phase, causing spots to overlap in the HPTLC chromatogram. Figure 2 shows a typical HPTLC densitogram with clear separation of Δ^9-THC from other components. Because Δ^9-THC and CBN overlap in this chromatographic system we selected a scanning wavelength (206 nm) in which both compounds gave a similar extinction coefficient (see below) and could allow for the simultaneous quantification of both compounds.

HPTLC validation results

LOQ, LOD, linear dynamic range, repeatability. The linear dynamic range, LOQ and LOD at 206 nm for Δ^9-THC and CBN was determined to be 50–500, 50 and 10 ng respectively. The RSD values from intra and inter day repeatability experiments at each standard level were never greater then 5.0%, showing that the

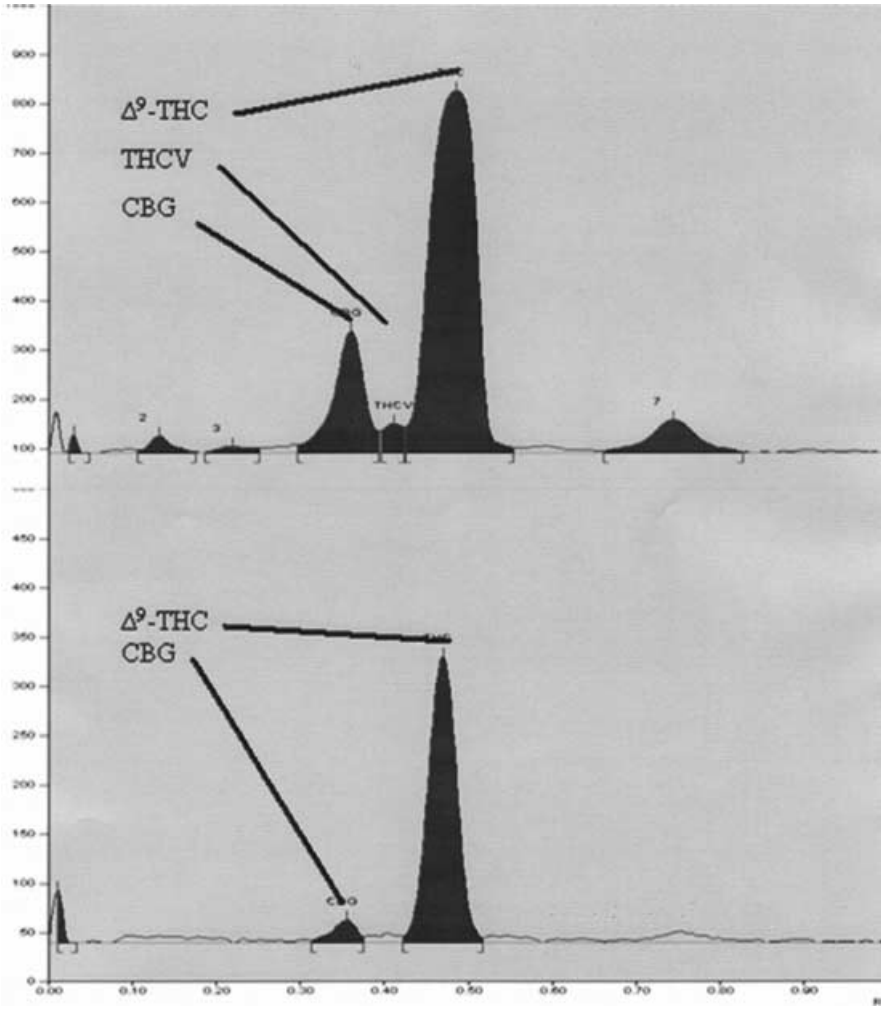

Figure 2. Upper densitogram shows an undiluted decarboxylated bedrocan sample with identified cannabinoids labeled. Lower densitogram shows a 20 x diluted bedrocan sample with clear baseline separation between identified cannabinoids.

Table 2. Repeatability results of the linear range[a]

Cannabinoid	Day 1 (n = 3)	Day 2 (n = 3)	Day 3 (n = 3)	Inter-day (n = 9)
Δ^9-THC	0.7–3.4	0.9–2.9	2.9–4.7	3.7–4.6
CBN	2.6–4.4	1.3–4.9	2.3–4.8	2.3–4.2

[a] Results show the range of RSD (%) values calculated from variation in the peak areas at each of the standard levels (50, 100, 250, 400, 500 ng) for each experiment (n = number of runs analysed).

Table 3. Precision of applicator needle[a] and scanner[b]

Sample	Needle (n = 5)	Scanner (n = 5)
Δ^9-THC 400 ng	0.4	0.4
CBN 400 ng	1.2	0.9
Bediol extract	1.6	0.6

[a] Results show RSD (%) values calculated from variation in peak area between spots (n = number of spots). [b] Results show RSD (%) values calculated from variation in peak area between scans (n = number of scans).

linear dynamic range is repeatable for Δ^9-THC and CBN (Table 2). The average linear regression equation was $y = 11.858x + 125.99$ with $r^2 = 0.9968$ for Δ^9-THC and CBN $y = 11.877x + 236.49$ with $r^2 = 0.9979$.

Instrumental Precision. The results from the needle precision and scanner precision experiments can be seen in Table 3. The low RSD values confirm that the needle is precise when spotting 5 µL of sample and that the scanner is precise when scanning at 206 nm.

Accuracy. Percentage recovery of Δ^9-THC in decarboxylated cannabis samples was determined by comparing total Δ^9-THC% from the non-decarboxylated Bedrocan and Bediol samples analysed by HPLC to the Δ^9-THC% determined from HPTLC and HPLC analysis of Bedrocan and Bediol samples subject to decarboxylation. Recovery of Δ^9-THC using our decarboxylation method was determined to be 85.8 ± 3.3% (RSD = 3.89). The lower recovery of decarboxylated cannabis samples could be a result of Δ^9-THC or THCA degradation; although no CBN or Δ^8-THC degradation was detected in any cannabis samples, this does not rule out the possibility of alternative or intermediate degradation. A correction

Table 4. Accuracy results

	HPTLC run 1	HPTLC run 2	HPTLC run 3	HPLC
Cannabis extracts, HPTLC results expressed as Δ^9-THC%, HPLC as total Δ^9-THC%, cannabis sample				
Bediol ($n = 3$)	6.4	6.5	6.4	6.2
Standard deviation	0.08	0.1	0.23	0.11
RSD (%)	1.21	1.48	3.54	1.92
Bedrocan ($n = 3$)	16.7	16.5	16.5	16.8
Std deviation	0.46	0.17	0.37	0.53
RSD (%)	2.76	1.01	2.21	3.1
Δ^9-THC/CBN mix solutions, results expressed as Δ^9-THC ng + CBN ng, sample solution				
Low CBN ($n = 3$)[a,b]	459	478	457	462
Standard deviation	3.4	9.6	2.2	5.6
RSD (%)	0.7	2	0.5	1.2
High CBN ($n = 3$)[a,c]	518	528	505	518
Standard deviation	6.5	8.5	5.3	3.9
RSD (%)	1.3	1.6	1	0.8

[a] Low CBN solutions contained Δ^9-THC 0.08 mg/mL, CBN 0.01 mg/mL. High CBN solutions contained Δ^9-THC 0.08 mg/mL, CBN 0.02 mg/mL. [b] *t*-Test *p*-value = 0.71. [c] *t*-Test *p*-value = 0.92. *n* = number of samples analysed, results show average.

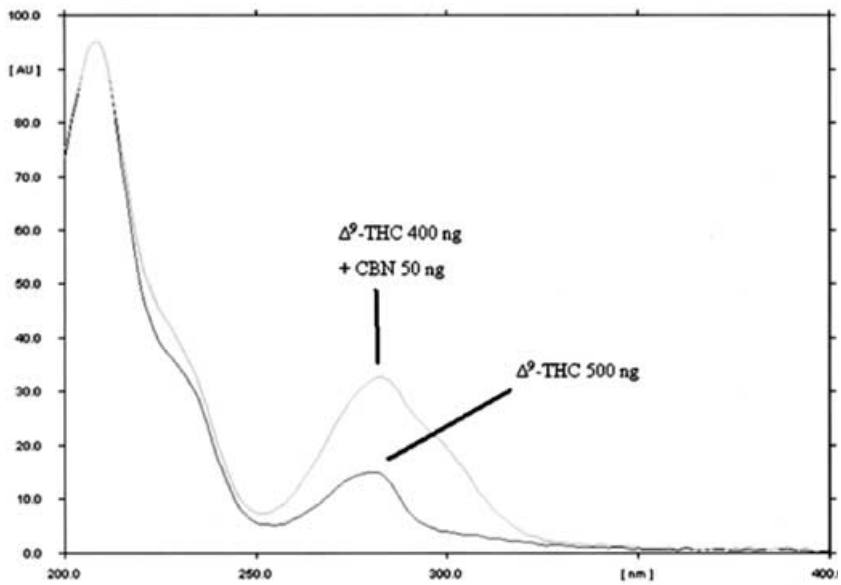

Figure 3. UV spectra (200 nm–400 nm) comparison of 09-THC versus 09-THC overlapped with CBN. The presence of CBN in a sample is visible by noticing the strong increase in absorbance at 280 nm.

factor of 1.17 was used to correct decarboxylated sample results obtained from our HPTLC method. Corrected results differ by no more then ± 0.5% total Δ^9-THC% in cannabis from HPLC results (Table 4). The low RSD values observed between the triplicate samples of Bedrocan and Bediol for each run indicate that both the HPTLC and HPLC analyses were accurate for Δ^9-THC quantification (Table 4).

CBN was not detected in HPLC analysis of cannabis samples before or after decarboxylation. However since CBN can be found in aged cannabis, standard solutions containing Δ^9-THC mixed with CBN were tested to determine if the presence of CBN would interfere with HPTLC results. UV spectra can be utilised to qualitatively determine the presence of CBN in a Δ^9-THC chromatographic spot (Fig. 3). The results of Δ^9-THC CBN mix experiments indicate that the HPTLC method allows for the simultaneous quantification of Δ^9-THC and CBN at 206 nm without a loss in accuracy and T-test values show that the HPTLC data set did not significantly differ from the HPLC data set (Table 4). Since it would be beneficial to determine CBN concentration in cannabis it was observed that when scanning at 320 nm Δ^9-THC up to 1000 ng is not detectable. CBN has a linear range of 50–750 ng at 320 nm with a linear regression of $y = 3.075x + 108.36$, $r^2 = 0.995$. By analysing Δ^9-THC and CBN mix solutions at 320 nm the amount of CBN in each solution could be determined; however the HPTLC method always slightly

overestimated the amount of CBN by an average of 17 ng (± 3.6) when compared with HPLC results (data not shown).

Conclusion

The method validated in this study offers advantages over previously developed TLC methods for cannabis analysis because it utilises a simple decarboxylation procedure to convert THCA into Δ^9-THC and requires only normal-phase HPTLC plates with an automated spotter and scanner. The method was shown to be comparable within a small degree of error (±0.5%) to results obtainable from a validated HPLC method. This chromatographic method can be utilised for qualitative fingerprinting of cannabis since good separation is achieved between the principle neutral cannabinoids (CBD, Δ^9-THC, THCV, CBG and CBC), forensic analysis of cannabis, quality control of hemp and quality control of medical cannabis.

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