Research Article

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TLC Densitometry Analysis of Cannabis Flower Growing in Thailand

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Abstract: The inflorescence cannabis of Hang Kra Rog Phu Phan (*Cannabis sativa* L.) has attracted interests as a raw plant material due to its high tetrahydrocannabinol (THC) content. This study aimed to investigate the THC and cannabidiol (CBD) contents of inflorescence cannabis samples from different regions in Thailand. TLC densitometry determined the contents of THC and CBD on silica gel G_{60} F_{254} and hexane-dichloromethane (1:1) as a mobile phase. This method was validated for THC analysis and TLC method resulted in linear regression with the calibration curve of 300-900 ng for THC. The THC and CBD contents were in the range of 7.29 – 12.70 %w/w and 0.11 – 0.73 %w/w, respectively. Prior to the analysis, the decarboxylation reaction by heating in a hot air oven was a favorable sample preparation to maximize the THC and CBD contents. According to the Notification of the Department of Agriculture the Plant Varieties Act reported equal amounts of THC and CBD of this breed. However, all samples showed relatively high THC contents comparing to CBD contents. The highest THC content (12.70 %w/w) was from Nakhon Phanom 2.

Keywords: Inflorescence cannabis, Cannabinoids, TLC densitometry, Tetrahydrocannabinol

INTRODUCTION

Cannabis sativa L. (or Kancha or Ganja) was used in traditional medicine for the treatment of some symptoms i.e., insomnia, stomachache, and headache. Cannabinoids and volatile terpenes were the major components found in the flowers and leaves of cannabis. Delta-9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), are the main substances converted from tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) with heat or some others capable of triggering decarboxylation. These acidic precursor compounds were derived from cannabigerolic acid (CBGA) with THCA synthase and CBDA synthase, respectively. THC exhibited psychoactive properties while CBD did not possess this property. In addition, THC was rich in marijuana (drug type) and CBD was commonly found in hemp (fiber type). There are several varieties of cannabis breeds in Thailand, and Hang Kra Rog Phu Phan ST1 has been registered and characterized (1). This subspecies was categorized as *Cannabis sativa* L. This breed was studied and reported THC and CBD content in approximately equal amounts. In addition, they were claimed to have a high content of THC, which was a controlled substance. Skunk was a common name called inflorescence of cannabis. This study determined

the THC and CBD contents of some cannabis inflorescence cultivated in Thailand.

Thailand has announced cannabis as a restricted medicinal plant for medical uses. Several parts of Kancha were sold on the market, mostly including dried flower tops, leaves, and resin. Most of them were claimed to be the Hang Kra Rog Phu Phan breed due to high THC content. These plant materials may not have a high THC content, as claims. With narcotics regulation, THC, which was higher than 0.2 %w/w in the extract, was prohibited for recreational uses. Therefore, the control of THC and CBD content was crucial. Thai Herbal Pharmacopoeia (THP) launched monographs of Cannabis sativa Inforescentia Femina, Cannabis leaf, and Cannabis root. The criteria for THC content were that the THC content was not less than 0.2 %w/w and not less than 1.0 %w/w for Kancha leaves and flowers, respectively (2). Medical products containing Kancha were available in the markets for sleep aids and appetite enhancers. The products may be misused for recreation purposes, which were prohibited by law. This study focused on the identification and analysis of THC and CBD in Kancha Inforescentia Femina using thin layer chromatography (TLC).

Chromatographic techniques are recommended for the identification and assay of cannabinoids. Gas chromatography (GC), high-performance chromatography (HPLC) with a diode array detector and TLC are standard methods for analytical laboratories (3). However, TLC is a reliable method and low-cost method compared to HPLC. It can be applied for examining samples without sophisticated instruments. This study determined the THC content after the decarboxylation process. Decarboxylation can increase THC and CBD contents because this activation transforms THCA and CBDA to THC and CBD, respectively (4). TLC was used for the analysis of cannabinoid components in plants, cannabis products, and forensic samples also (5). Since cannabis especially flowers and leaves, is used as a raw material in many products, regulation of THC and CBD content is needed by the Thai Food and Drug Administration (FDA). Determining the contents of THC and CBD in plant materials is important for the characterization of marijuana or hemp (6). The THC and CBD contents of these cannabis samples from various areas of Thailand were compared. The developed TLC method was also studied and validated.

MATERIALS AND METHODS

Materials

Cannabis skunks were bought from the markets in Thailand. There were 14 samples from several cultivation areas in Thailand (Table 1). The samples were identified by a botanist (Mr.Nirun Vipunngeon), College of Pharmacy, Rangsit University. The powdered samples were examined microscopically and unicellular trichomes were showed in Figure 1. Acetone, dichloromethane, ethyl acetate, diethyl ether, methanol, and ethanol were ACS/AR grade and purchased from Fisher Scientific, UK and acetonitrile (HPLC grade) was bought from Dunkans Pure Chemicals, Korea. Anisaldehyde was purchased from Acros Organics, US.

Standard THC (Purity 98%) was isolated and purified from the Medicinal Cannabis Research Institute, College of Pharmacy, Rangsit University. Standard CBD (Purity 98%) was purchased from Department of Medical Sciences, Ministry of Public Health, Thailand.

Standard solution

Standard THC was prepared at a concentration of 3,100 µg/mL and diluted to a concentration of 93 µg/mL in dichloromethane. Standard CBD was prepared at a concentration of 1,000 µg/mL and diluted to 100 µg/mL in dichloromethane. Then, silica G_{60} F_{254} was applied for 2 to 7 µL. The calibration curves were obtained from the THC concentration of 186 – 651 ng and CBD concentration of 200 – 700 ng.

Sample preparation

Cannabis samples (ground and sieved No. 60 mesh) were decarboxylated using a hot air oven at 130 °C for 20 minutes (4). Then 100 mg was accurately weighed and extracted with 5 mL of dichloromethane using an ultrasonic bath for 20 minutes. After removing solvent, the extracts were reconstituted in acetonitrile – methanol (8 – 2) for 2 mL.

TLC conditions

TLC densitometry was performed using a Linomat 5 sample application, visualizer and scanner 3 [Camag, Switzerland]. The mobile phase was hexane and dichloromethane (1:1) and monitored at wavelengths of 254 and 366 nm and under white light after spraying with anisaldehyde TS. For quantitative analysis, the standard solution was applied for 2 to 7 µL and the sample solutions were applied for 5 µL. The band length was 6 mm, the scanning speed was 20 mm/s, and the scanning wavelength was 210 nm. The retardation factor (R_f was then calculated using Wincats software version 1.4.5.

TLC method validation

The developed TLC analysis of cannabinoids was validated for linearity, accuracy, and precision according to ICH guideline Q2(R1) (7). The limit of detection and limit of quantification were calculated from (3.3 x slope)/SD and (10 x slope)/SD of the standard curve. Standard solution was applied to 4, 5, and 6 µL in triplicate. The analysis was performed in three different plates (n=3). The linear equation and coefficient of determination (R^2) were calculated from the standard curve. Percent recovery was determined for the accuracy and percent relative standard deviation (%RSD) was calculated for precision. The performance requirements were accepted according to the standard criteria (8). THC contents were compared using IBM SPSS statistic software version 22.

RESULTS AND DISCUSSION

Cannabis samples (dried plant materials) were obtained from 14 sources and 11 regions of Thailand including the northeastern, the middle area and the upper southern area of the country (Table 1). Samples were ground and subjected to microscopic testing to identify the flower top part. Figure 1 showed unicellular trichomes with and without cystolith and fragments of epidermis of the samples. These trichomes can be seen in abundance and were unique for flower parts (9). However, to confirm Hang Kra Rog Phu Phan breed as the seller claimed, it required molecular assays together with quantitative analysis of the chemical components.

The TLC method was developed for identification and quantitative analysis of THC and CBD. TLC method validation of THC analysis showed a linear calibration curve of THC in the range of 300 – 900 ng with a linear regression (Table 2). The method was accurate with %recovery of 95.54 – 102.79. The R_f value of THC was reproducible with %RSD less than 5.3% and 7.3% for intraday and inter-day, respectively. Figure 2 showed overlay spectrum of the THC and CBD standard bands to those of the samples with the same R_f value resulting the specificity of the method. The spectrum of cannabis sample showed the maximum absorption wavelengths of THC were 209 and 279 nm and corresponded to those of standard THC. However, the maximum absorption wavelength of CBD were 210 – 220 nm and 270 – 280 nm. There was slightly shifted to 250 nm. Figure 3 showed TLC densitometric chromatogram of standard THC and one of the cannabis samples. It showed clearly resolution between THC and CBD substances. Although HPTLC with HPTLC plate and advance instrument were more selective method than traditional TLC (6, 10), the validated TLC method in this study was simple and practical for qualitative and quantitative analysis of THC and CBD in plant materials.

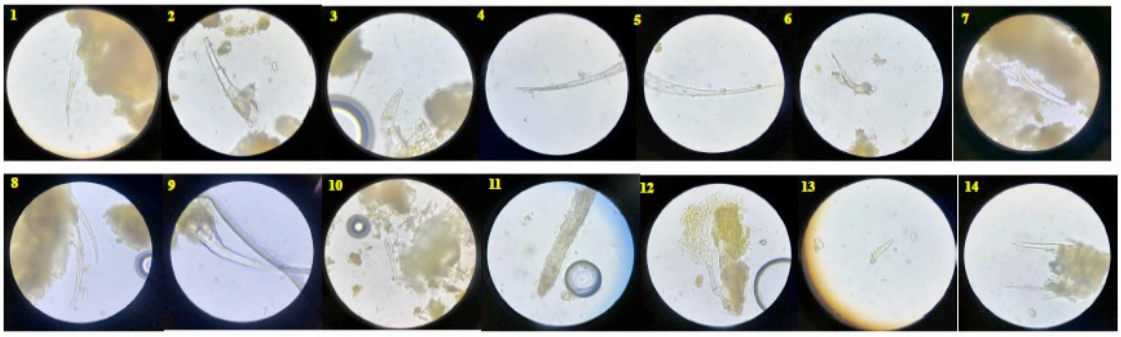

Figure 1. Trichome and epidermis found in cannabis samples

Table 1. Cannabis samples, cultivated areas and THC and CBD contents

Samples	Sources	%w/w	
		THC content	CBD content
1	Nakhon Phanom 1	10.42 ± 1.87	0.68 ±0.10
2	Nakhon Phanom 2	12.70 ± 3.13	0.11 ± 0.04
3	Buriram	8.66 ± 0.42	ND*
4	Pathum Thani	8.16 ± 0.22	0.73 ± 0.26
5	Phetchaburi	8.49 ± 0.07	ND*
6	Roi Et	7.67 ± 1.04	ND*
7	Ratchaburi	7.29 ± 0.29	ND*
8	Sakon Nakhon	7.43 ± 0.06	ND*
9	Sing Buri	10.28 ± 0.21	ND*
10	Nong Khai 1	8.04 ± 0.10	0.13 ± 0.07
11	Nong Khai 2	8.93 ± 0.10	ND*
12	Udon Thani 1	7.79 ± 0.02	ND*
13	Udon Thani 2	8.88 ± 0.43	0.66 ± 0.13
14	Ubon Ratchathani	8.55 ± 0.09	ND*

*ND = Not detected

Table 2. TLC analysis of THC method validation results

Parameters	Acceptance criteria	Results			
Linearity (300-900 ng)	$R^2 > 0.995$	$y = 2.0195x+286.06$, $R^2 = 0.999$			
Accuracy (%Recovery)	90 – 107				
620 ng		102.79 ± 3.11			
775 ng		95.54 ± 4.97			
930 ng		101.86 ± 2.08			
Precision (%RSD)	Intraday precision %RSD < 5.3	D1	D2	D3	(Inter-day)
620 ng	Inter-day precision %RSD < 7.3	1.57	1.40	1.49	5.37
775 ng		0.00	0.00	0.00	5.69
930 ng		0.00	0.00	0.00	5.69
LOD (ng)		99.37			
LOQ (ng)		301.14			

The R_f values of THC and CBD were 0.39 – 0.41 and 0.45 – 0.46, respectively, and were compared to reference standards for compound identification. It also clearly showed resolution between THC and CBD bands. The bands were not clearly observed at 254 and 366 nm (Supplement), but they were clearly observed after being sprayed with anisaldehyde TS. THC

and CBD bands of the samples were scanned using a scanner, and the areas were calculated for the contents according to their calibration curves. The THC and CBD contents are shown in Table 1. TLC analysis showed that THC contents were in the range of 7.29 – 12.70 %w/w and CBD contents were in the range of 0.11 – 0.73 %w/w, respectively. All samples showed that THC contents were higher than 1% w/w as mentioned in THP for Kancha inflorescence (2).

In addition, the average THC contents of these samples were 8.81 ± 1.46 %w/w which was slightly higher than the average THC content reported by Department of Agriculture. According to chemotype and genotype studies, the average Δ^9-THC content was 6.06 ± 0.43 %w/w and the average CBD content was 6.54 ± 0.28 %w/w (1). In contrast, CBD contents were much lower than reported for claim Hang Kra Rog Phu Phan ST1. Only 5 samples of total 14 samples that showed CBD contents in very small amounts.

The highest THC contents of cannabis samples were from Nakhon Panom 2, subsequently from Nakhon Panom 1 and Sing Buri, respectively. The highest CBD contents of cannabis samples were from Pathum Thani, subsequently from Nakhon Panom 1 and Udon Thani 2, respectively. The THC contents of these samples were statistically significant (p-value < 0.05) with one way ANONA. Among the samples from the same region (Nakhon Phanom 1, 2; Nong Khai 1, 2; Udon Thani 1, 2), THC contents were not significantly different. Overall, the average THC contents from the middle area

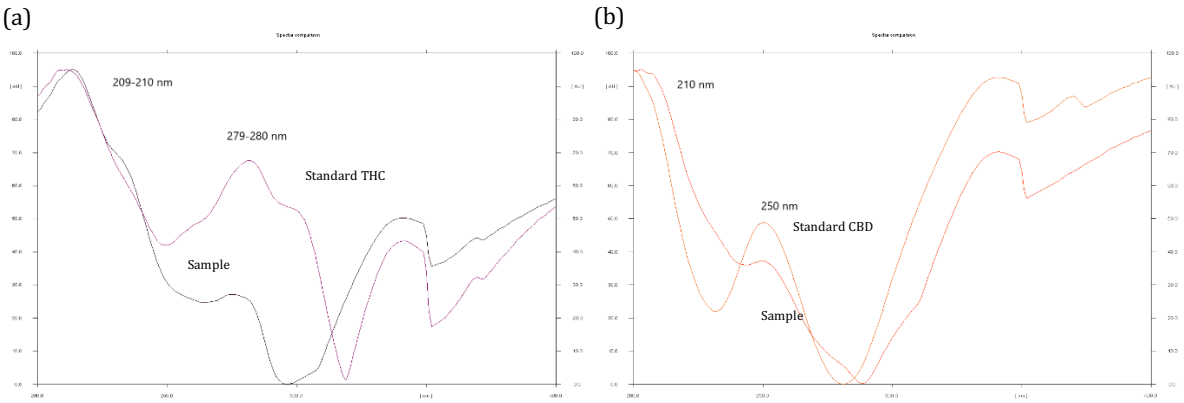

Figure 2. Overlay spectrum of (a) THC standard band and the corresponding band of sample (b) CBD standard band and the corresponding band of sample

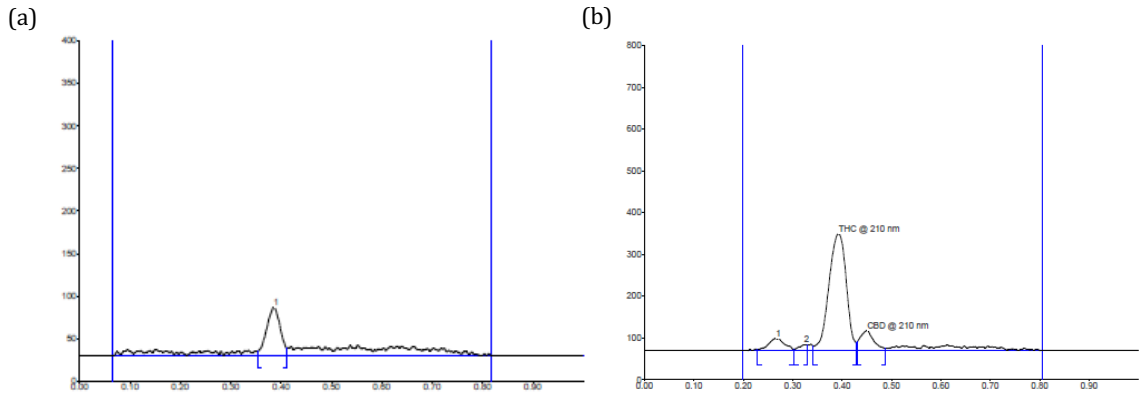

Figure 3. TLC densitometric chromatogram of (a) standard THC at the concentration of 651ng and (b) sample solution (10 mg/mL)

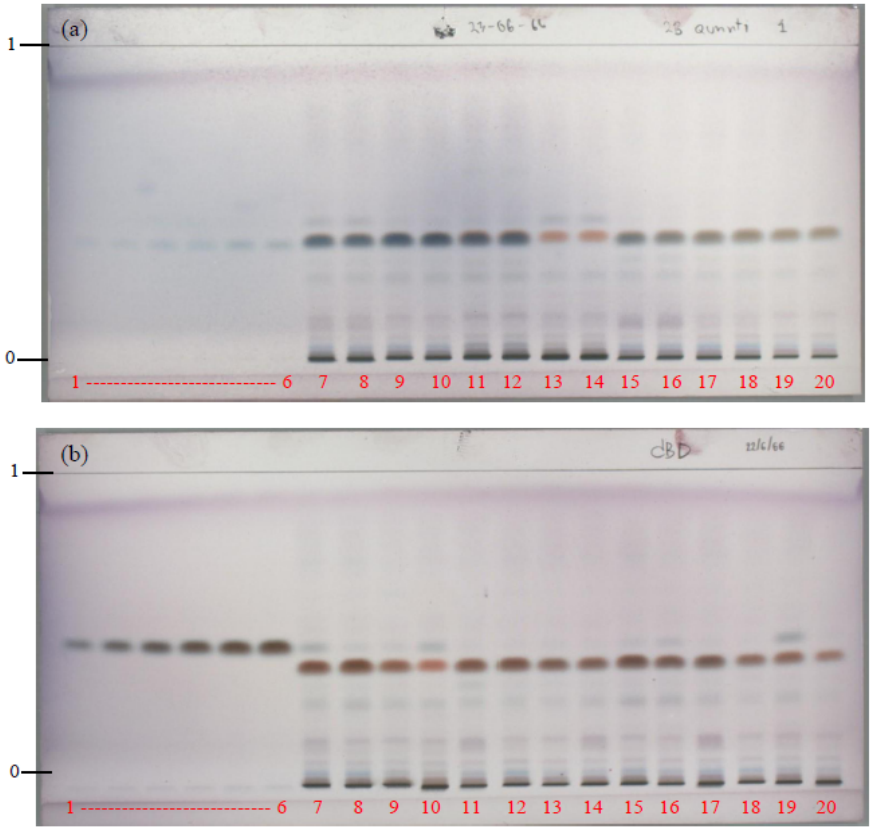

Figure 4. (a) TLC of standard THC (Tracks 1 – 6) and samples (Tracks 7 – 20) and (b) TLC of standard CBD (Tracks 1 – 6) and samples (Tracks 7 – 20) after derivatization with anisaldehyde TS and under white light.

(9.22 ± 1.24 %w/w) were slightly higher than those from the northeastern (8.91 ± 1.78 %w/w) and the upper south areas (7.89 ± 0.71 %w/w) of Thailand.

Variation of THC and CBD contents of these samples may be due to different cultivars, different cultivation areas and different harvesting time including drying processes. Although CBD was legalized in Thailand for medical uses, THC in cannabis was still a controlled substance. Cannabis plants were allowed for medicinal purpose with limited regulation. Chronic THC consumption has been relevant to serious side effects i.e., cognitive deficits, anxiety, paranoia, chronic psychosis, and dependence. Therefore, control of THC and CBD substances were important and required the validated laboratory method.

In addition, dried flowers were decarboxylated by heating in a hot air oven. The decarboxylation condition was applied from the study of Wang et al., 2016 because it was reported to increase THC and CBD contents (4). Another study by Beadle, 2020 reported that high temperature and short reaction time yield higher THC conversion, while lower temperature and longer reaction time maximized CBD content (11). The sample solutions were prepared by ultrasonic extraction with dichloromethane. Although in THP diethyl ether and petroleum ether (2:8 v/v) were suggested as the mobile phase, in this study, hexane and dichloromethane (1:1 v/v) were used, which was slightly modified from the study of Debruyne et al., 1994 (3).

Figures 4 showed TLC profiles of THC and CBD in the samples after derivation with anisaldehyde

TS. Anisaldehyde sulfuric spray reagent resulted in dark blue-violet bands after heating (12, 13), while aqueous Fast Blue B salt spray reagent gave bright purple to orange and red color immediately after spraying. Actually, Fast Blue B salt (0.1 – 0.5%) was recommended and more specific for cannabinoids (6, 9).

In comparison, the THC and CBD contents in cannabis leaves were in the range of 1.36 – 5.44 %w/w and 0.88 – 1.20 %w/w, respectively (14). These reported contents were analyzed using HPLC. The cannabis flowers in this study were also analyzed with HPLC (Supplement). The contents of THC and CBD were in the range of 0.15 – 2.60 %w/w and 0.09 – 0.81 %w/w, respectively. The THC contents analyzed with HPLC were moderately lower than the content analysis with TLC because of the higher sensitivity and selectivity of HPLC. Additionally, sample preparations were prepared and extracted with acetonitrile in HPLC analysis. In HPLC profile showed three main cannabinoids THC, CBD, and cannabinol (CBN). CBN was the oxidative by-product of THC resulting from storage (15). However, CBN was barely seen in this TLC solvent system but it was eluted in HPLC chromatogram of cannabis sample (Supplement).

CONCLUSION

The average THC contents of fourteen inflorescence cannabis cultivated in Thailand were 8.81 ± 1.46 %w/w which was higher than THC content (6.06 ± 0.43 %w/w) reported for Hang Kra Rog Phu Phan ST1. However, CBD contents were not detected to very small amount (0.11 – 0.73 %w/w). This study reported validated TLC analysis of THC contents. The mobile phase system resulted in clearly resolution between THC and CBD. Sample preparation by decarboxylation prior to extraction increased THC content. The study suggested this TLC method was practical benefits potentially aiding in cost and time reduction for THC and CBD analysis in plant materials.

ACKNOWLEDGMENTS

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Supplement data

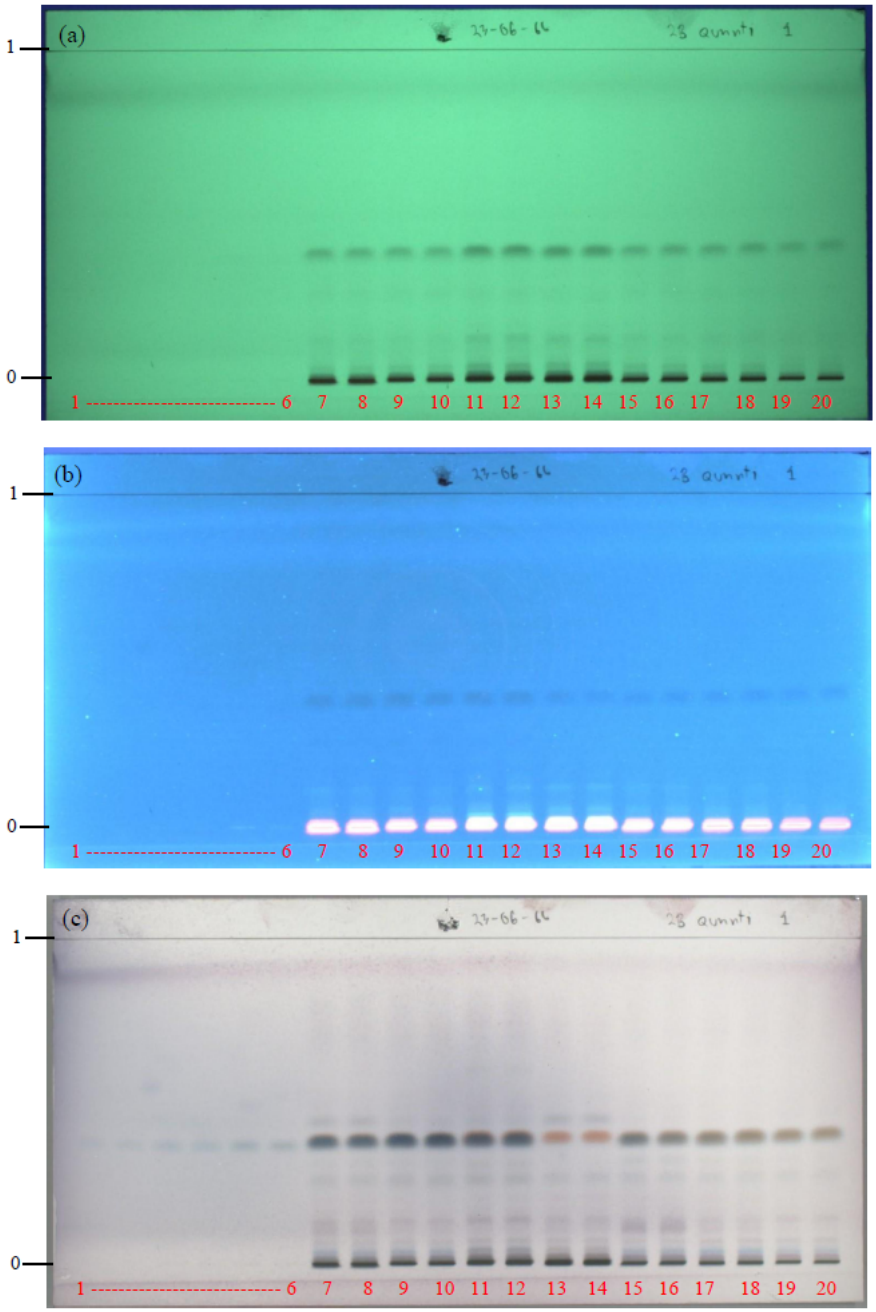

Figure S1 TLC profile of standard THC (Track1 1-6) and cannabis inflorescence (Tracks 7-20) (a) 254 nm (b) 366 nm (c) after being sprayed with anisaldehyde TS under white light

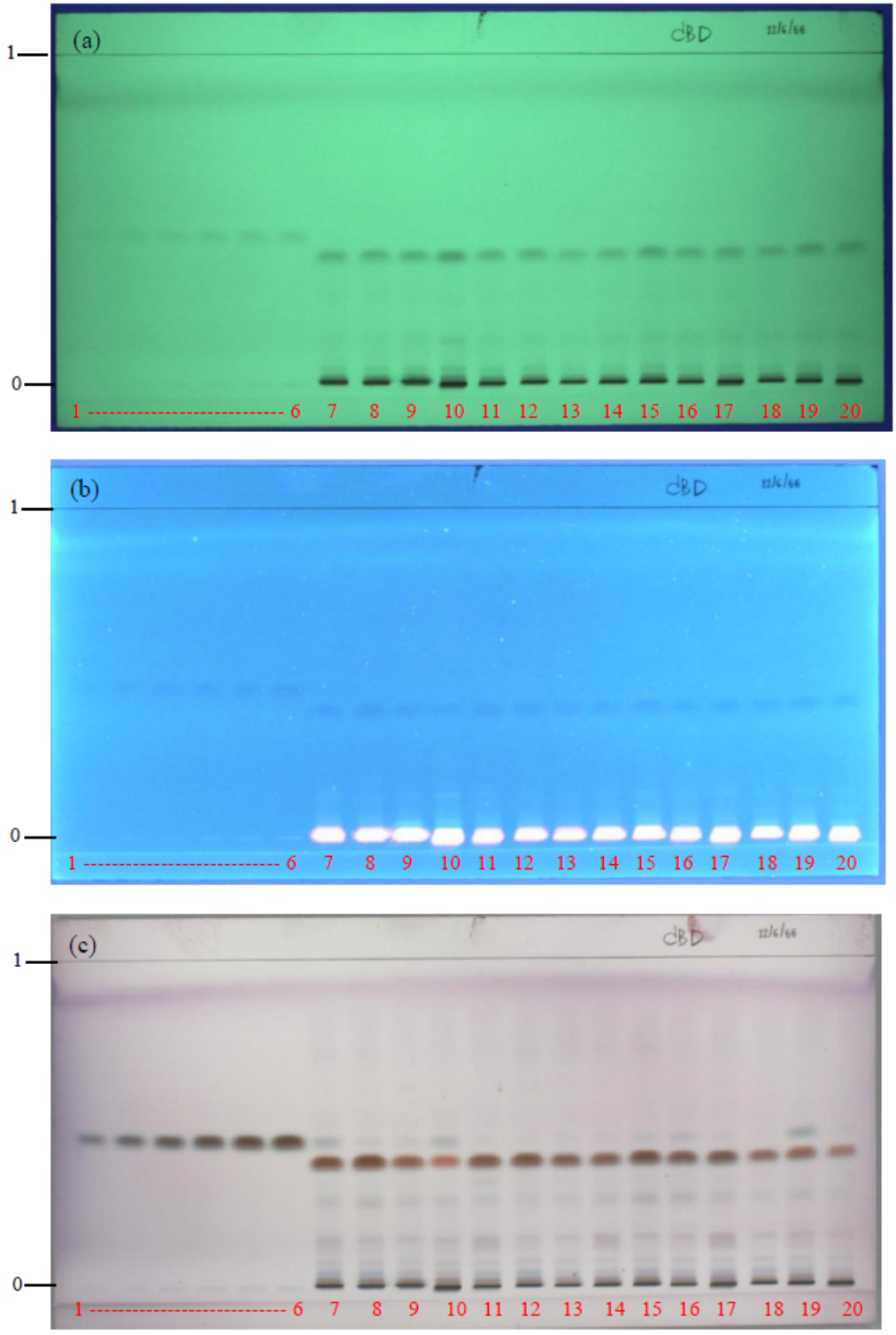

Figure S2 TLC profile of standard CBD (Track1 1-6) and cannabis inflorescence (Tracks 7-20) (a) 254 nm (b) 366 nm (c) after being sprayed with anisaldehyde TS under white light.

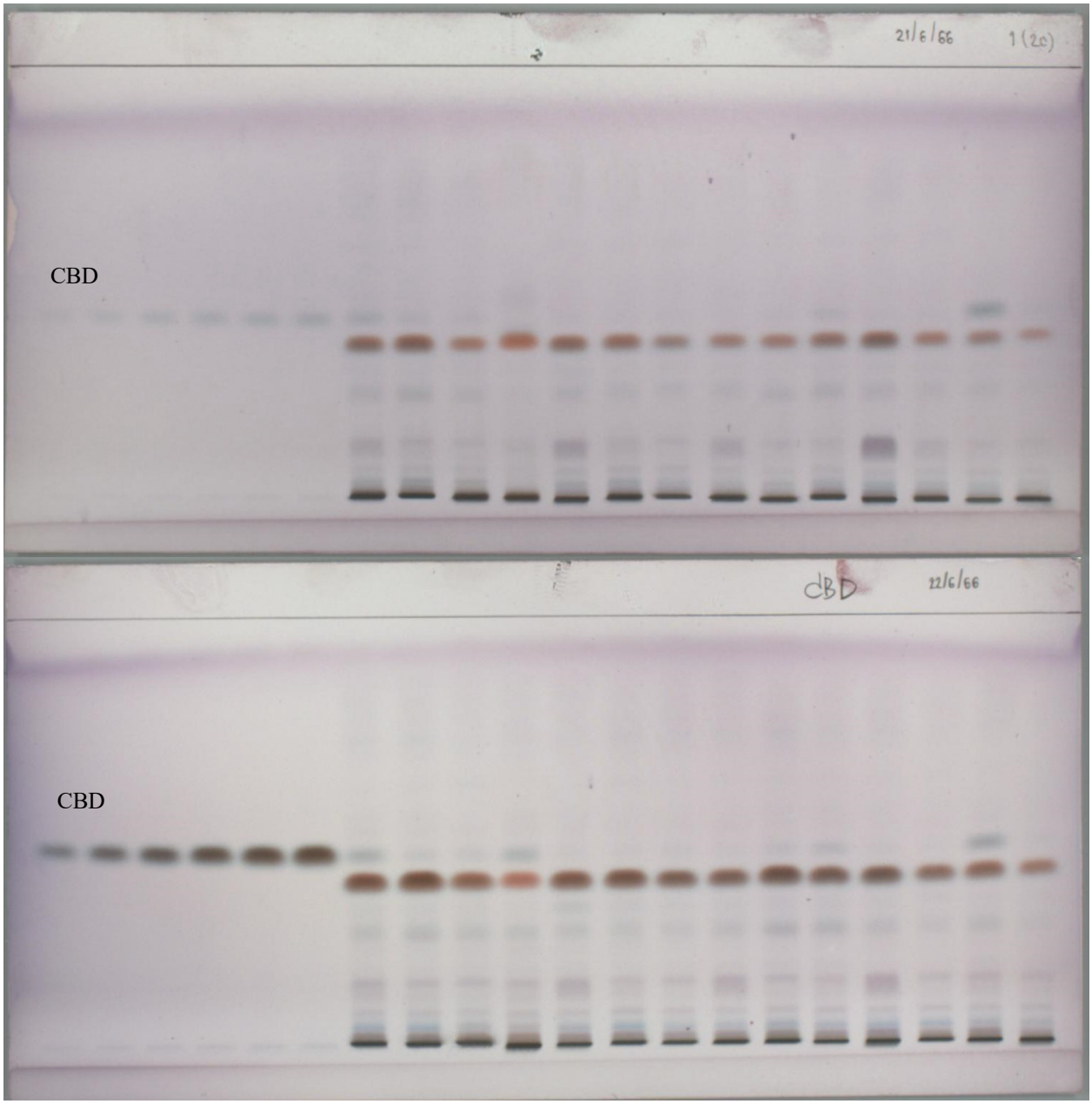

Figure S3 TLC profile of standard CBD (Track1 1-6) and cannabis inflorescence (Tracks 7-20) after being sprayed with anisaldehyde TS under white light.

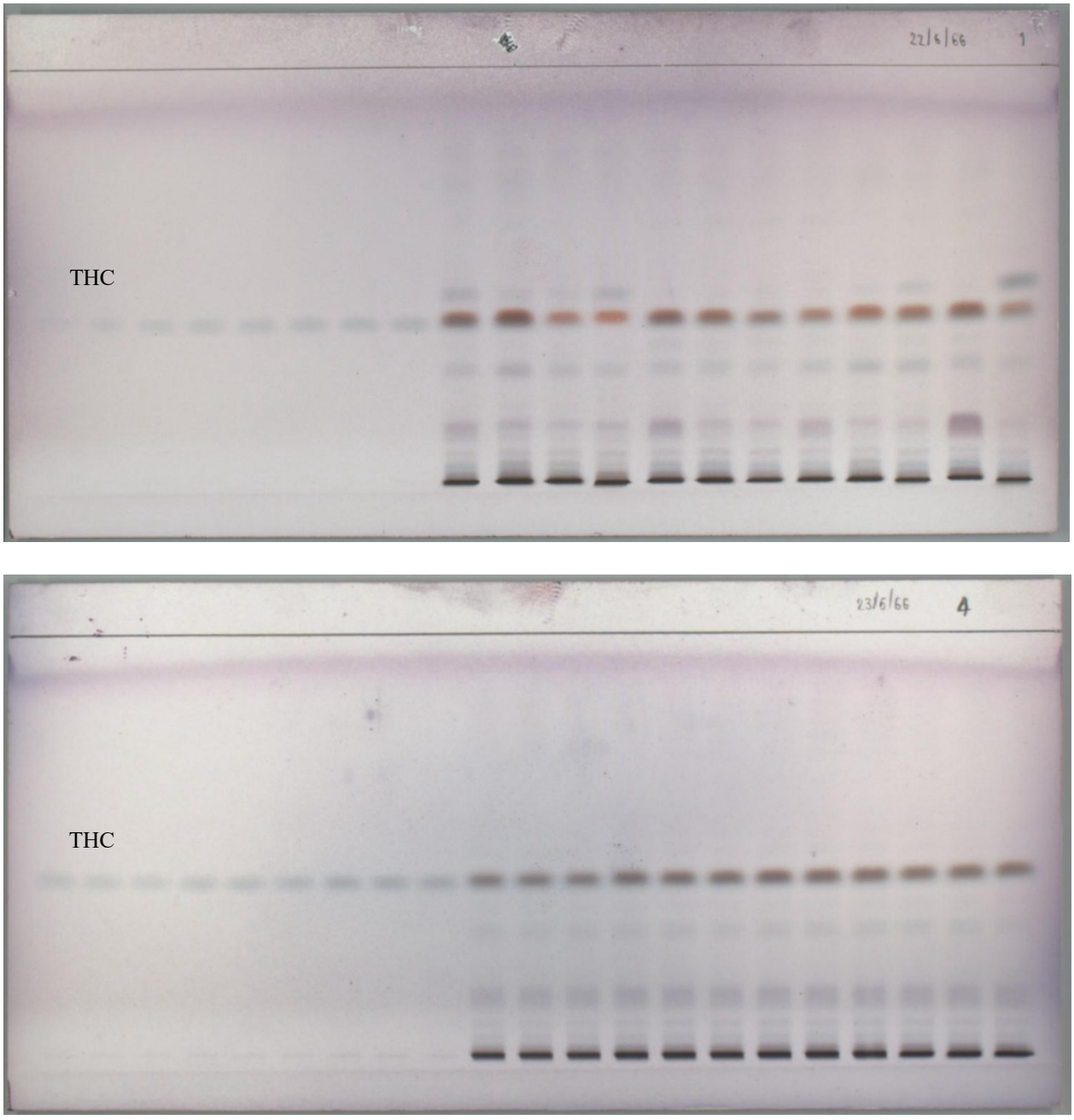

Figure S4 TLC profile of standard THC (Track1 1-8, 1-9) and cannabis inflorescence (Tracks 9-20, 10-21) after being sprayed with anisaldehyde TS under white light.

Table S1 HPLC method validation of THC analysis

Parameters	Acceptance criteria	Results			
Linearity (0.63-20 µg/mL)	$R^2 > 0.995$	y = 493532x + 417285 $R^2 = 0.9996$			
Range (5.2-15.6 µg/mL)	$R^2 > 0.995$	y = 305857x - 16848 $R^2 = 0.9996$			
Accuracy Conc. (µg/mL)	%Recovery = 80-110%	Avg ± SD			
5.2		100.71 ± 0.51			
10.4		99.29 ± 0.51			
15.6		100.24 ± 0.17			
Precision	%RSD < 5.3% for intraday precision < 7.3% for inter-day precision	Intraday D1	D2	D3	Inter-day
	Retention time				
5.2		0.08	0.04	0.04	0.11
10.4		0.04	0.00	0.00	0.04
15.6		0.04	0.00	0.00	0.04
	Peak area				
5.2		0.85	0.47	0.19	0.53
10.4		0.09	0.10	0.05	1.11
15.6		0.12	0.14	0.14	0.96
LOD (µg/mL)		0.36 ± 0.01			
LOQ (µg/mL)		0.93 ± 0.01			

Table S2 HPLC method validation of CBD analysis

Parameters	Acceptance criteria	Results			
Linearity (1.5-50 µg/mL)	$R^2 > 0.995$	y = 331643x + 125954 $R^2 = 0.9994$			
Range (5.1-15.3 µg/mL)	$R^2 > 0.995$	y = 312345x - 38288 $R^2 = 0.9981$			
Accuracy Conc. (µg/mL)	%Recovery = 80-110%	Avg ± SD			
5.1		101.54 ± 1.07			
10.2		98.46 ± 1.07			
15.3		100.66 ± 0.16			
Precision	%RSD < 5.3% for intraday precision < 7.3% for inter-day precision		Intraday		Inter-day
		D1	D2	D3	
	Retention time				
5.1		0.13	0.09	0.04	2.36
10.2		0.08	0.04	0.00	1.05
15.3		0.09	0.00	0.05	1.59
	Peak area				
5.2		0.75	0.44	0.32	0.53
10.4		0.15	0.28	0.78	1.11
15.6		0.11	0.55	0.17	0.96
LOD (µg/mL)		0.03			
LOQ (µg/mL)		0.10			

HPLC condition

Column: Poroshell EC-C_8 (3 x 150 mm, 4 µm) 35 °C

Mobile phase: (A) 0.1% Formic acid in water, pH 2.69 (B) Acetonitrile

Time (Min)	0	1	9	11	13	15
A	45	45	0	0	45	45
B	55	55	100	100	55	55

Flow rate: 0.5 mL/min

Detection: 210 nm

Table S3 THC and CBD contents analyzed with HPLC

Samples	Sources	%w/w	
		THC content	CBD content
1	Nakhon Panom 1	2.26 ± 0.00	0.47 ±0.03
2	Nakhon Panom 2	2.60 ± 0.03	0.44 ± 0.01
3	Buriram	1.48 ± 0.00	0.28 ± 0.00
4	Pathum Thani	0.15 ± 0.00	0.40 ± 0.02
5	Phetchaburi	1.98 ± 0.03	0.31 ± 0.00
6	Roi Et	2.17 ± 0.04	0.18 ± 0.00
7	Ratchaburi	1.87 ± 0.01	0.18 ± 0.00
8	Sakon Nakhon	1.73 ± 0.04	0.22 ± 0.01
9	Sing Buri	1.90 ± 0.24	0.34 ± 0.17
10	Nong Khai 1	1.93 ± 0.06	0.51 ± 0.02
11	Nong Khai 2	2.22 ± 0.04	0.24 ± 0.01
12	Udon Thani 1	1.27 ± 0.01	0.12 ± 0.00
13	Udon Thani 2	1.26 ± 0.04	0.81 ± 0.02
14	Ubon Ratchathani	0.68 ± 0.02	0.09 ± 0.01

Table S4 t-test statistical analysis

THC content	n	Mean	S.D.	t	df	sig
TLC	28	8.81	1.61	21.67	36	0.00*
HPLC	28	1.68	0.65			

* Statistical significance at *p*-value < .05

Table S5 One-way ANOVA Statistical analysis of 14 samples

Source of Variation	*SS*	*df*	*Mean Square*	*F*	*Sig*
Between Groups	55.347	13	4.257	3.967	0.008*
Within Groups	15.026	14	1.073		
Total	70.372	27			

* Statistical significance at *p*-value < .05