Physical Analysis and Chemical Profiling of Illicit Herbal Cannabis using Multivariate Analysis

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ABSTRACT: An important aspect in forensic analysis is drug profiling. Information regarding the chemical properties of seized drug samples can be accumulated, which provides intelligence information to assist law enforcement agencies. Such information can be used to combat drug trafficking and abuse. In this study, twenty three illicit cannabis samples seized from Selangor and suburbs of Kuala Lumpur which were submitted to the Department of Chemistry Malaysia were analyzed. All cannabis samples were extracted using methanol-chloroform mixture in a ratio of 9:1. High performance liquid chromatographic (HPLC) technique was used to separate cannabinoids in illicit herbal cannabis samples using Onyx Monolithic column. Mobile phase consisting of methanol-water (75:25) was used as the eluent at a flow rate of 0.8 mL/min and analytes detected at 220 nm. Analysis of reproducibility of retention time and peak area has validated the robustness of silica based monolithic column for HPLC analysis of cannabis. Peak areas of the cannabis extracts were used to profile illicit cannabis samples. Profiling of cannabis samples were established using cluster analysis and principal component analysis (PCA). Results from cluster analysis suggest that the illicit cannabis samples could have originated from five different geographical origins. Although PCA produced almost similar groupings like cluster analysis, but is not a suitable tool for analysing small set of data. PCA is more suited to decompose large data set with more variables. Classification model from this work suggests that plant material from one geographical origin can be trafficked by different means of route.

Keywords: Cannabinoids, illicit herbal cannabis, high performance liquid chromatography (HPLC), principal component analysis, cluster analysis

Introduction

Drug profiling is an important aspect of forensic drug analysis. Drug profiling is the extraction of drug sample's chemical and/ or physical profile based on properties that they portray [1]. It is an intelligence-gathering exercise that includes evaluation of synthetic pathway or extraction method, identification of diluents, adulterants or impurities and identification of drugs geographic origin for plant derived substances [2]. Physical and chemical properties of a seized drug samples can be accumulated, and provides intelligence information to combat drug trafficking and abuse. Profiling of drugs is not a new phenomenon in western countries like United States, Germany, Netherlands, United Kingdom and Australia. For the past five years, illicit cannabis has significant number of drug related arrests in Malaysia [3] (Table1). This shows that cannabis is one of the most abused and highly trafficked illicit drugs in Malaysia. Unfortunately, information on seized cannabis samples is limited because profiling was not a routine analysis in Malaysia. The alarming number of seized cannabis samples submitted to Department of Chemistry Malaysia warrants a need for drug profiling in Malaysia.

Table 1: Drug-related arrests in Malaysia by drug type, 2006-2010

Drug type	2006	2007	2008	2009	2010
ATS	2865	1490	1787	1298	8551
Cannabis	5275	3385	1726	5207	3011
Codeine	180	91	70	50	71
Heroin	7963	4752	4974	5047	6483
Morphine	5889	4312	3640	3386	5181
Opium	7	14	9	5	31

Source: Drug Abuse Information Network for Asia and the Pacific (DAINAP)

Cannabis is a type of hallucinogen; the plant is annually propagated from seeds and grows vigorously with well drained soil, ample nutrients, and water [4]. All known cannabis

strains are wild pollinated and produce seeds that are called achenes [5]. There are three different species of cannabis plant: *C. sativa*, *C. indica* and *C. ruderalis*. Out of these three species, derivatives from *Cannabis sativa* are abused as illicit drug. Active component found in cannabis is cannabinoid, and so far sixty six cannabinoids are known to exist. Active component responsible for causing hallucinogenic properties are cannabidiol (CBD), cannabinol (CBN) and Δ^9-tetrahydrocannabinol (Δ^9-THC) [1]. Δ^9-tetrahydracannabinol compounds are found most abundant in the leaves and flowering tops of cannabis plants. THC content of cannabis varies at different parts of plant, generally decreasing in the following sequence: resin, flowers & leaves. Little THC is found on seeds, stem and roots. As a drug, cannabis exists in three distinctive forms, herbal cannabis (Marijuana), cannabis resin (hashish) or cannabis oil (hashish oil/ hemp oil). Chemical structure of three psychoactive cannabinoids is shown in (Figure 1).
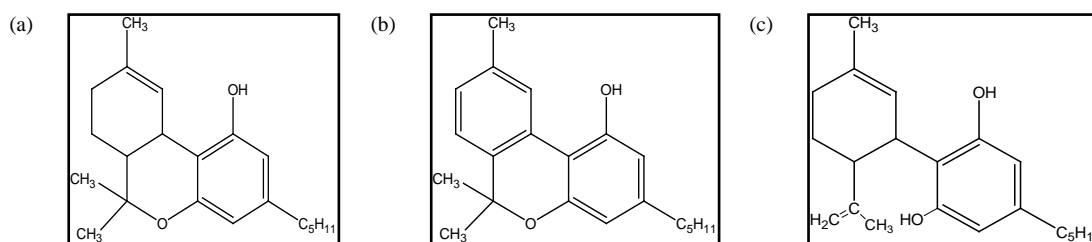

Figure 1: Chemical structure of three psycho active cannabinoids: (a) Δ^9THC (b) CBN and (c) CBD.

Forensic drug analysis encompasses identification of physical properties, qualitative analysis, and quantitative analysis. When a plant material is encountered, normally the sample is first examined using a stereomicroscope. The physical identification of marijuana by microscopic methods depends on observing short hairs on the upper side of the leaf known as cystolithic hairs. Qualitative analysis include color test and thin layer chromatography (TLC) analysis. Duquenous-Levine test (presumptive test), microscopic examination and TLC test maybe more than sufficient to rule out plants other than cannabis [6]. Microscopic and qualitative analysis are insufficient for profiling purposes. Cannabis samples are normally profiled based on their organic composition to trace the possible of source of any sample [7]. Results from instrumental analysis like gas chromatography (GC) and high performance liquid chromatography (HPLC) can be very useful in profiling illicit cannabis samples. The use of capillary columns has become very popular in analysis of cannabis using GC. Jenkins and Patterson analyzed 63 samples from various geographical areas using GC to observe relative proportions of CBD, CBN, and THC [8]. El Sohly *et al.* analyzed 157 samples from six different regions (Colombian, Jamaican, Mexican, Thai, Californian, and Hawaiian) using GC/MS technique. Statistical analysis of 175 peaks gave classification accuracies which ranged from 81% for Hawaiian samples to 100% for Jamaican samples. Despite their successful classifications, the authors found that they can only differentiate across relatively large regions, since the range of content of organic compounds is too small within a single country [9].

Unlike in GC where derivatization is necessary to analyze cannabis samples, HPLC does not require derivatization process. Most of the cannabis samples are analyzed using reversed-phase HPLC systems. In most studies, reversed-phase HPLC on a C18 column with an acidic acetonitrile-water as the eluent were used for separation of cannabinoids. Lehman and Brenneisen used water containing 8.64 g/L orthophosphoric acid:acetonitrile (85:15) as the acidic mobile phase in a gradient mode [10]. Hazakamp *et al.* analysed cannabinoids under acidic and basic conditions, with methanol-water containing 25mM formic acid (pH 3) as the acidic mobile phase and acetonitrile-phosphate buffer as the basic mobile phase [11]. The use of monolithic stationary phases for liquid chromatography has been reported by Zou *et al.* The silica skeletons of monolithic columns have macropores and mesopores which aid the efficient separation of analytes [12]. However, to date, no reports have been made that utilized monolithic reversed phase column for the separation of cannabis extracts.

Chromatographic data from GC or HPLC analysis can be profiled using multivariate statistical analysis. Principal component analysis is a type of multivariate analysis that linearly transforms an original set of variables

into a substantially smaller set of uncorrelated variables (principal components) that represent most of the information in the original set of variables. Using mathematical projections, PCA extracts and visualizes systematic patterns or trends in large data matrices [13] by considering variance of a data. It de-correlates the original data by finding the directions in which variance is maximized and then uses these directions to define a new basis. Score plot generated by PCA would allow us to find trends, patterns and outliers in the data more easily than would have been possible without the aid of this analysis. Remberger *et al.* employed PCA for the profiling of opium [14]. PCA was applied to evaluate data and correlate alkaloid concentrations to the origins of the opium samples. The origins of 27 opium samples were identified to originate from India, southern Europe, Middle East and Far East. PCA was also employed in profiling of 85 samples of various drugs and diluents using Raman spectra [15]. Profiling was done to distinguish and group the samples. Patterns among different drugs (cocaine, MDMA, and heroin) were revealed using PCA. Choi *et al.* used PCA to identify chemical profile of metabolites in cannabis tissues from different plantations [16].

Cluster analysis is another method to divide a group of objects into classes, where similar objects are in the same class [17]. Unlike PCA, which reduces dimensionality within the data set, cluster analysis searches for objects closer to each other in the data space. Hierarchical cluster analysis (HCA) is type of cluster analysis that identifies homogeneous groups of objects or variables based on selected characteristics using an algorithm that starts with each sample in a separate cluster and combines clusters until only one is left. Distance (dissimilarity) or similarity measures are generated comparing each pair of sample and each one of these objects will be very similar to the other ones in the same cluster. HCA classifies the data in a relatively simple and direct manner, with the results being presented as dendogram, a diagram that displays the distance or similarity between groups and provide a visual means of estimating relationships among multidimensional points [18]. Cluster analysis was used to produce 37 groups in 1000 drug samples based on chromatographic data [19]. Zhang *et al.* employed cluster analysis to evaluate similarity and/ or dissimilarity of 17 impurity profiles in seizures of metamphetamine hydrochloride drugs in China [20].

The main purpose of this study is to establish a profiling work for illicit cannabis samples in Malaysia. Forensic analysis for illicit cannabis samples is already a normal procedure in Malaysia whereby the forensic chemist performs basic analysis like identification of physical properties and qualitative analysis, ignoring totally other information that may be useful for the police. Profiling work can give substantial information on drug trafficking in Malaysia. Results from profiling works can be gathered and used to create database which can be helpful for intelligence purposes. This study was therefore undertaken to extract more intelligence information regarding the identity of illicit cannabis sample seized by employing a multivariate approach for profiling cannabis.

Experimental

Sample collection
Twenty three illicit herbal cannabis samples seized from nine different areas of Selangor and suburbs of Kuala Lumpur and submitted to Department of Chemistry Malaysia were analyzed. All samples were labeled according to their area of seizure and replicate extractions. Figure 2 depicts a map on districts of Selangor and suburbs of Kuala Lumpur showing clearly the area of seizures.

Chemicals and reagents
Analytical grade methanol (QRec Bright Chem Sdn. Bhd) and chloroform (Scharlau Chemie, Spain) were used to extract herbal cannabis samples. HPLC grade methanol was obtained from Fisher Scientific, UK and J.T. Baker, USA. Other reagents used were distilled water and ultra high quality (UHQ) water obtained from PURELAB® Option-R water purification system in Chemistry Department of Malaysia laboratory, Petaling Jaya. Cannabinoid standards [cannabidiol (CBD), cannabinol (CBN), and (-)-Δ^9 tetrahydrocannabinol (THC)] with concentration of 1000 ppm in methanol were obtained from LIPOMED, Switzerland.

Figure 2: A map depicting the area where illicit cannabis samples were seized by the police (1: Rawang, 2: Klang, 3: Subang Jaya, 4: Petaling Jaya, 5: Sentul, 6: Chereas, 7: Ampang, 8: Kajang, 9: Sepang). Sentul and Cheras are suburbs of Kuala Lumpur.

Apparatus
For the extraction of illicit cannabis samples, a vortex mixer from Glas-Col® Multi-Pulse Vortexer (USA), a digital ultrasonic bath model Bransonic® 5510 (USA) and a centrifuge from Thermofisher Scientific Heraeus® Multifuge® 3S+ (Germany) were employed. Microscopic analysis was performed using a Leica EZ4D stereomicroscope (United Kingdom).

Extraction of cannabis samples
Dried herbal cannabis materials (flowers and leaves) were randomly selected, pulverized (samples cut into small pieces using scissors) and further crushed using a mortar and pestle. 500 mg of dry homogenized herbal cannabis were extracted with 5 mL methanol-chloroform mixture (9:1 v/v) in a 15 mL extraction tube by vortex mixing for 10 seconds. The sample was subjected to sonication for 15 minutes and further vortexed on the 5th, 10th, and 15th minute. The sample was finally centrifuged at 2500 rpm for 25 minutes [6]. The above mentioned extraction method was modified from United Nations Office on Drugs and Crime, UNODC [15]. Table 2 describes labeling method used to distinguish illicit cannabis sample extracts. Twenty three illicit herbal cannabis samples generated fifty two extracts. All samples were subjected to replicate extractions, except for sample seized from Klang (KN1 and KN2) due to insufficient amount of sample.

Table 2: Labeling of illicit cannabis sample according to their area of seizure and replicate extraction.

Area of Cannabis seizure	Sample code	Replicate extraction	Extract labeling
Ampang	AM1	Duplicate	AM1 a, AM1b
	AM2	Triplicate	AM2 a, AM2 b, AM2 c
	AM3		AM3 a, AM3 b, AM3 c
Cheras	CH1		CH1 a, CH1 b
	CH2	Duplicate	CH2 a, CH2 b
	CH3		CH3 a, CH3 b
	CH4	Triplicate	CH4 a, CH4 b, CH4 c
Klang	KN1	Single	KN1 a
	KN2	Single	KN2 a
Kajang	KJ1	Duplicate	KJ1 a, KJ1 b
	KJ2		KJ2 a, KJ2 b
	KJ3	Triplicate	KJ3 a, KJ3 b, KJ3 c
	KJ4	Duplicate	KJ4 a, KJ4 b
Petaling Jaya	PJ1	Triplicate	PJ1 a, PJ1 b, PJ1 c
	PJ2		PJ2 a, PJ2 b
	PJ3	Duplicate	PJ3 a, PJ3 b
	PJ4		PJ4 a, PJ4 b
Rawang	RW1	Duplicate	RW1 a, RW 1b
Sepang	SP1	Duplicate	SP1 a, SP1 b
	SP2		SP2 a, SP2 b
Subang Jaya	SJ1	Triplicate	SJ1 a, SJ1b, SJ1 c
Sentul	SN1	Triplicate	SN1 a, SN1 b, SN1 c
	SN2		SN2 a, SN2 b, SN2 c

Visual inspection and microscopic analysis
Physical properties such as color and other botanical features for all illicit cannabis samples were recorded. Leaves, stems and seeds of herbal cannabis material were observed under stereomicroscope at magnifications of 16X and 35X. Microscopic analysis gives in depth information of physical properties of cannabis plant.

High Performance Liquid Chromatography

HPLC analysis of cannabis samples was performed on a Waters 2695 HPLC module equipped with a Waters 2996 photodiode array detector, an autosampler and data processing utilizing Empower software. Analyte separation was affected using a reversed phase Onyx Monolithic column (100 mm x 4.6 mm). Presence of cannabinoids in samples was confirmed by injecting individual cannabinoid standards prior to the analysis of cannabis samples. The column temperature was 30°C. The mobile phase consisted of methanol-water (75:25 v/v) in isocratic mode and the flow rate of the mobile phase was 0.8 mL/min. Run time for the analysis was set for 30 minutes and the analytes were detected at 220 nm. An aliquot of 10 µl of the samples was injected into the HPLC system. In between sample analyses, control solvent consisting of methanol-chloroform (9:1 v/v) was injected to prevent carry over [21].

Multivariate analysis

Data generated from chromatographic peak area of HPLC analyses were manipulated using Microsoft Excel and Unscrambler X 10.0. Prior to statistical analysis using the software, peak area of each cannabinoids was normalized using sum of the peak area. The normalized chromatographic data was initially stored in Microsoft Office Excel spreadsheet and later analyzed using Unscrambler X 10.0. The software incorporates functions to perform cluster analysis and PCA directly. Cluster Analysis is a valuable tool to understand the natural grouping of objects or samples. In this study, hierarchical cluster analysis (HCA) using single-linkage and squared Euclidean distance measures were employed. HCA single-linkage is also referred as nearest neighbor measures, which uses distance between closest samples to define a cluster.

Results and discussion

Physical features of illicit cannabis samples

All twenty three illicit cannabis samples were in the form of compressed herbal slabs. Some prominent features of herbal material were noted such as the presence of leaves, stems and seeds. All samples existed as dark brown herbal material. Microscopic analysis of herbal material showed the presence of cystolithic hairs and grandular trichomes which are unique features and characteristics to the cannabis plant. Figure 3 shows cystolithic hairs on cannabis leaves at two different magnifications (16X and 35X). Head of grandular trichomes were also present in all cannabis samples (Figure 4). Although microscopic features were sufficient to confirm the identity of the plant material, but are insignificant for profiling purposes; hence chemometric analysis was employed for chemical profiling.
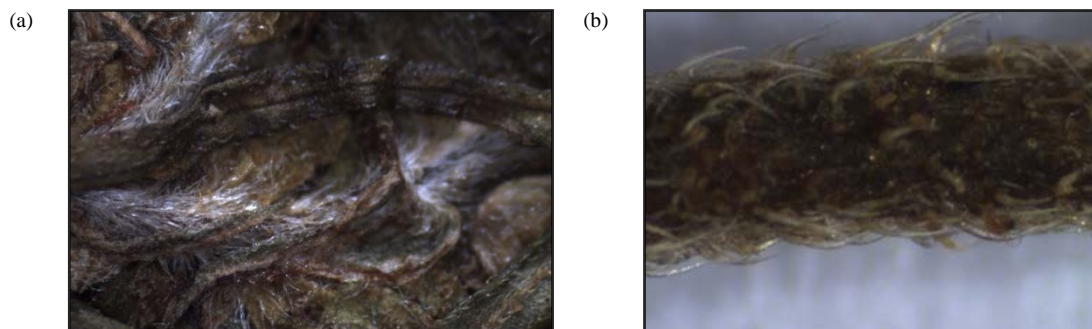

Figure 3: Cystolithic hairs observed on cannabis leaves at (a) 16× and (b) 35× magnifications

Figure 4: Head of grandular trichomes on cannabis leaves (35× magnification)

HPLC separation of cannabis

In order to analyze the chemical components in cannabis samples, all cannabis extracts and standards were subjected to high performance liquid chromatographic analysis. HPLC was the method of choice since it does not require the samples to be derivatized as required for GC analysis. Although acidic or basic mobile phase are often used for cannabis separation [9], methanol-water mixture (75:25 v/v) was investigated in this work for the separation of cannabis on a monolithic column. Reproducibility of HPLC separation in terms

of retention time and peak areas of three cannabinoids; namely cannabidiol (CBD), cannabinol (CBN), and (-)-Δ^9 tetrahydrocannabinol (Δ^9-THC) were determined for day to day (Table 3) and within day (Table 4) basis.

Table 3: Variation of retention time and peak area for day-to-day analysis of three cannabinoids (CBD, CBN and THC)

Analyte	Day-to-Day Reproducibility (n=6)					
	Retention Time				Peak Area	
	Mean	Standard Deviation	% RSD	Mean (x10^7)	Standard Deviation (x10^7)	% RSD
CBD	10.065	0.065	0.64	4.033	0.656	16.27
CBN	17.819	0.125	0.7	14.293	2.732	19.12
THC	22.584	0.225	0.99	5.449	4.927	9.04

Table 4: Variation of retention time and peak area for within-day analysis of three cannabinoids (CBD, CBN and THC)

Analyte	Within Day Reproducibility (n=3)					
	Retention Time				Peak Area	
	Mean	Standard Deviation	% RSD	Mean (x10^7)	Standard Deviation (x10^7)	% RSD
CBD	9.719	0.114	1.18	9.909	2.31	2.31
CBN	17.536	0.196	1.12	1.366	0.78	0.57
THC	22.332	0.331	1.48	14.455	1.17	1.17

Percentage relative standard deviation (RSD) value for retention time on a day-to-day basis is below 1% for all three cannabinoids. The value of % RSD for peak area on a day-to-day basis is below 20%. In terms of within day analysis, % RSD for retention time was below 1.50%. Within day analysis of cannabinoids showed % RSD value of less than 2.50% for peak area. Reproducibility of retention time for both day-to-day and within day analysis is excellent with RSD of less than 2%. In terms of peak area, within-day analysis produced better reproducibility as compared to day-to-day basis. Therefore, all extracts were subjected to multiple injections in HPLC within a day when the samples were analyzed in order to obtain better reproducibility. Analysis of reproducibility in terms of retention time and peak area was also important in validating the robustness of silica based monolithic column. Since all three cannabinoids were well resolved, monolithic column employing methanol-water mixture was found to give good separation for HPLC analysis of cannabis samples and deemed suitable for routine use of cannabis analysis.

Cannabis profiling
Chemometric approach of cannabis profiling was carried out to establish links between different seizures of illicit cannabis. Multivariate analyses on normalized chromatographic data from HPLC analysis were based only on the three cannabinoids: CBD, CBN and Δ^9-THC. A dendrogram obtained from hierarchical cluster analysis of twenty three illicit cannabis samples showed five groups (Figure 5).

The result of the cluster analysis was in agreement with those established by visual inspection of the HPLC profiles that showed similar groupings. Comparison of HPLC profiles for each group is shown in Figure 6. All groups contained Δ^9-THC as the major peak except for group I where CBD was found to be the most intense peak. Moreover, comparison of HPLC profiles also revealed that cannabis extracts from group V has intense peak for both CBD and THC. From the cluster analysis, all seized illicit cannabis samples could have originated from five different geographical origins. HCA analysis provided an efficient means to recognize groups of samples based on the peak areas of chromatograms. Although HCA was successful in statistical discrimination, there is no objective means to distinguish boundaries between subgroups, nor does this type of analysis provide information on chemical composition of the samples.
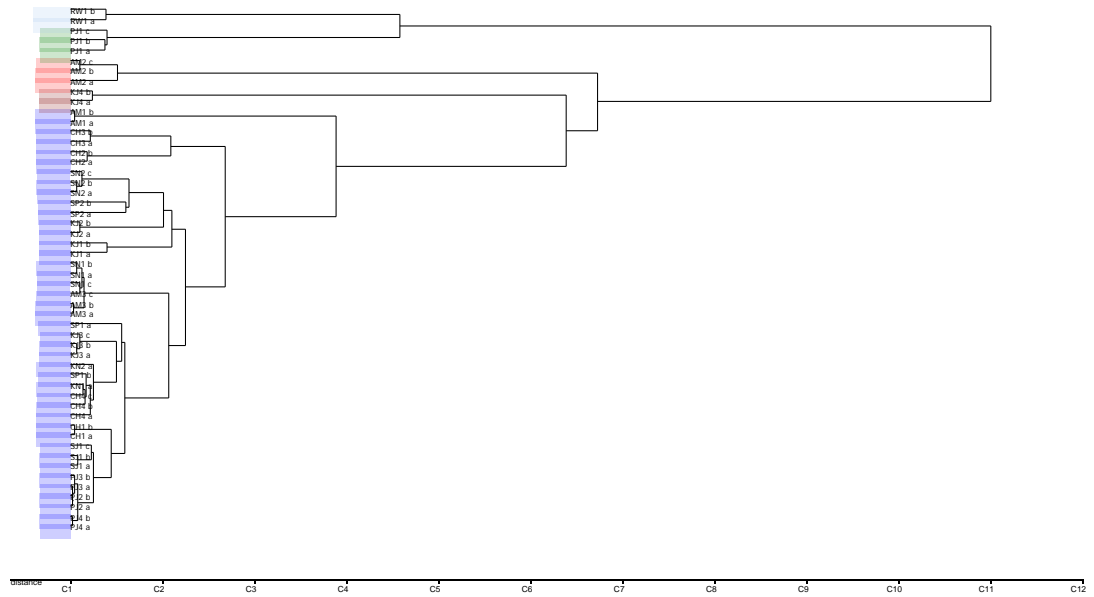
Figure 5: Dendrogram obtained from Cluster analysis of 23 illicit cannabis samples seized from nine different regions

(a)
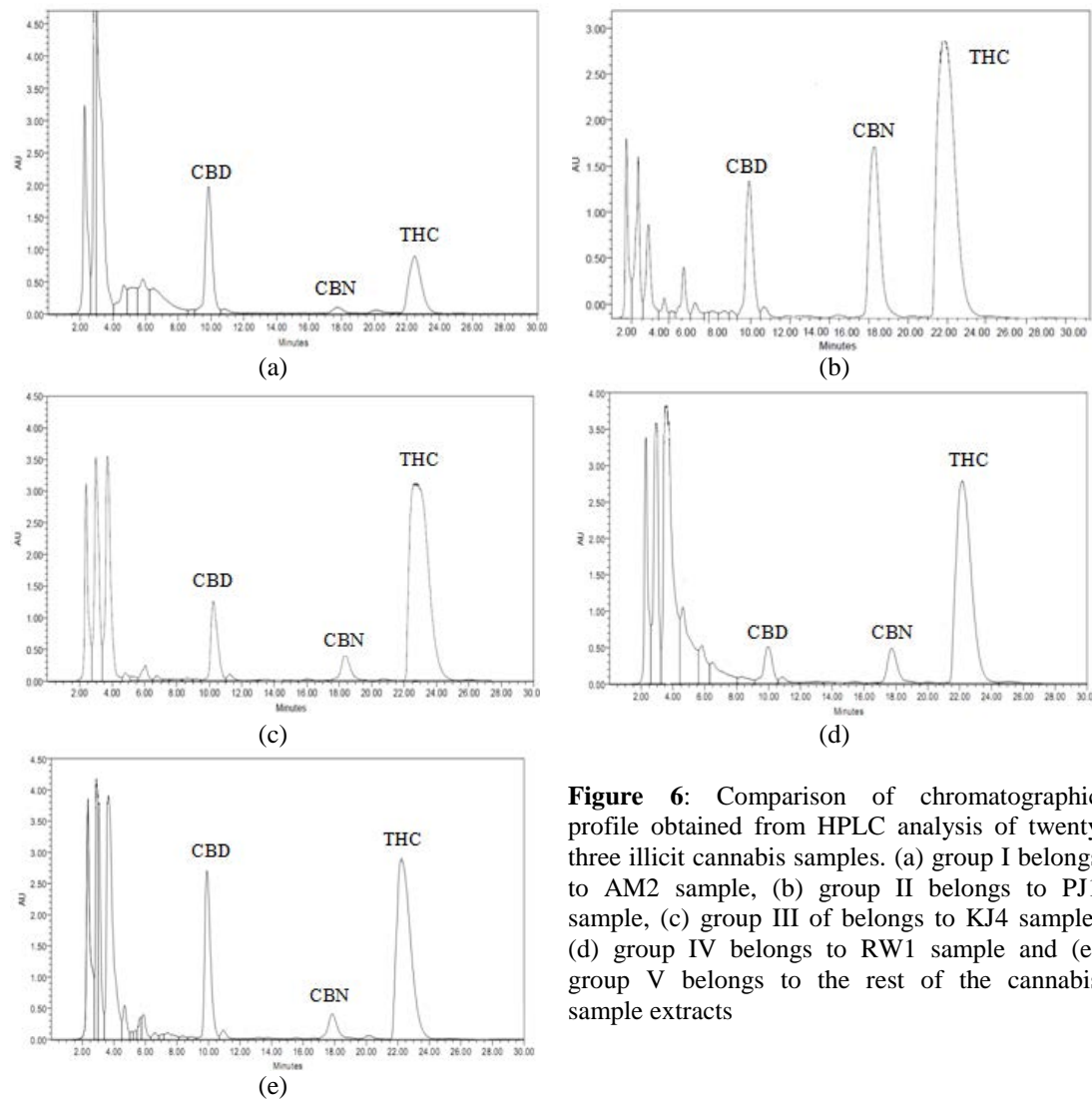

(b)

(c)

(d)

(e)

Figure 6: Comparison of chromatographic profile obtained from HPLC analysis of twenty three illicit cannabis samples. (a) group I belongs to AM2 sample, (b) group II belongs to PJ1 sample, (c) group III of belongs to KJ4 sample, (d) group IV belongs to RW1 sample and (e) group V belongs to the rest of the cannabis sample extracts

Figure 7 shows a score plot for the fifty two cannabis extracts analyzed in this study using PCA. The first principle component (PC1) accounted for 54% of variance in the data, and second principal component (PC2) showed 28% of data variance, indicating total data variance of 82%. A large variance value corresponds to dynamics in the system and carries most of the information whereas small variances may well be noise and are always neglected. Hence the third principal component (PC3) carrying data variance of 18% was not included. Based on the score plot, it is indicative that there are five different groups among all cannabis sample extracts. Sample seized from Petaling Jaya (PJ1), Rawang (RW1), Kajang (KJ4) and Ampang

(AM2) are scattered apart from the dense area '1' which contains the rest of the samples. An overlap of groupings can be seen at dense area '1', proving that area of seizures cannot be used to profile the samples. Samples coming from different areas of seizure could have originated from same geographical origin. Although PCA and cluster analysis produced similar groupings, groupings from score plot are vague and not characteristic enough to define clustering among the cannabis samples. PCA is more advantageous in decomposing a large data set which must have at least four variables. However in this study, only three variables (three types of cannabinoids) were analyzed for profiling purposes.
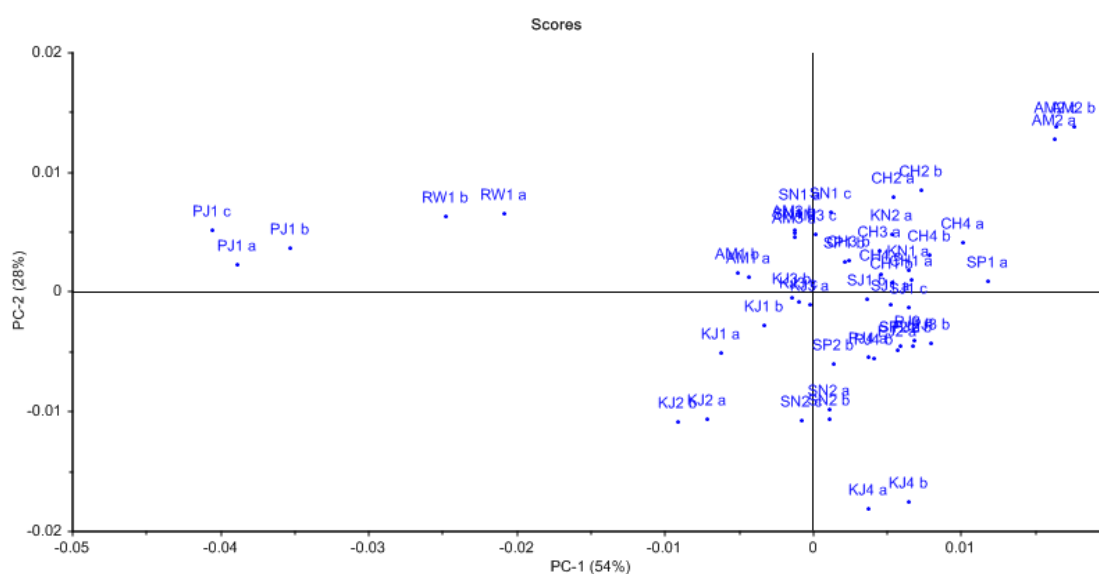

Figure 7: Score plot obtained from Principal component analysis of 23 illicit cannabis samples seized from nine different regions

Conclusion

In this study, a multivariate approach of twenty three illicit cannabis samples for chemical profiling was carried out. Cluster analysis indicated that the cannabis samples used in this study may have originated from five different geographical origins. Visual observation of the chromatographic profile also revealed five different patterns of chromatogram. Though PCA also generated a similar grouping, but this statistical tool is more appropriate if the analysis was based on higher number of variables. The area of seizure cannot be used to distinguish the samples, results from this study showed that samples seized from different regions can be grouped together. This indicated that a plant material from one geographical origin can be trafficked with different routes. Drug profiling work would be more conclusive if samples

from known geographical origin were compared against the seized samples from the police.

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