FORENSIC DETECTION AND DISCRIMINATION OF *CANNABIS* TYPES

SOMSARUAY WATANASIRI

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Thesis

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Somsaruay Watanasiri

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FORENSIC DETECTION AND DISCRIMINATION OF *CANNABIS* TYPES

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ABSTRACT

This study demonstrated the use of three methods to identify and discriminate the drug-type and fiber-type of *Cannabis* plants. *Cannabis* samples collected from seven different geographical areas in Thailand were subjected to chemical and biological tests. The presumptive Fast Blue B salt test clearly distinguished the drug-type samples from the intermediate drug-type and fiber-type samples. The drug-type gave a red to purple-red color. The other two gave orange to red-orange color. The Thin Layer Chromatography (TLC) fingerprints developed in N-hexane: Dioxane: Methanol system at the ratio of 70:20:10 gave the best resolution in separating the cannabinoids. TLC fingerprint of the drug-type samples clearly showed the major Tetrahydrocannabinol (THC) and/or Cannabinol (CBN) bands, but not the Cannabidiol (CBD) band. In contrast, TLC fingerprint of the fibre-type samples clearly showed the CBD band; however, the THC band shown was very light. In addition, an orange band with Rf value of 0.20, suspected to be Cannabidiolic acid (CBDA), was present in all fibre-type samples. It was noted that THC and CBD bands were present in comparable concentrations in intermediate drug-type samples. For DNA analysis, the Tetrahydrocannabinolic acid (THCA) synthase marker (Kojoma, *et. al.*) clearly distinguished the fiber-type and drug-type samples, but not for the *Cannabis sativa*- *trn*L-F chloroplast DNA marker.

This study suggests the use of Fast Blue B salt test in field application as a presumptive test. The method is easy and inexpensive. The test not only indicates the presence of cannabinoids, but also the type of *Cannabis*. The TLC fingerprints and DNA analysis can be used as confirmatory test for detecting and discriminating *Cannabis* types. The chemical tests discriminate the cannabis types according to the difference of the major cannabiniods contents and their quantities; whereas, the biological test discriminates the types by analysis of the DNA polymorphisms.

KEY WORDS: FORENSIC/ *CANNABIS* TYPES/TLC/ DNA/FAST BLUE B SALT

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บทคัดย่อ

ตัวอย่างพืชสกุล *Cannabis* จาก 7 แหล่งพื้นที่เพาะปลูกได้ผ่านการวิธีทางเคมี คือ การเกิดปฏิกิริยาให้สีกับ Fast Blue B salt และ Thin Layer Chromatography และวิธีการทางการตรวจสารพันธุกรรมโดยอาศัยเครื่องหมายทางโมเลกุล จำนวน 2 ตำแหน่ง จากผลการตรวจพบว่า การเกิดปฏิกิริยาให้สี สามารถแยกตัวอย่างพืชประเภทเสพติดซึ่งให้สีแดงถึงม่วงแดง ออกจากพืชประเภทกึ่งเสพติดและเส้นใยซึ่งให้สีส้มถึงแดงส้มได้ เนื่องจากสีเหล่านี้เกิดจากองค์ประกอบทางเคมีที่แตกต่างกันของตัวอย่างพืชแต่ละประเภท การวิเคราะห์โดย Thin Layer Chromatography ในระบบตัวทำละลายที่ประกอบด้วย เฮกเซน-ไดออกเซน-เมทานอล ในอัตราส่วน 70:20:10 ให้ผลในการแยกสารสำคัญได้ชัดเจน ซึ่งในตัวอย่างพืชประเภทเสพติดจะพบสาร THC และ/หรือ CBN ในปริมาณมาก โดยไม่พบสาร CBD ในทางตรงกันข้าม ตัวอย่างพืชประเภทเส้นใยจะพบสาร CBD ในปริมาณมาก และพบ THC ในปริมาณที่น้อยมาก รวมทั้งพบแถบสีส้มที่มีค่า Rf ประมาณ 0.20 ซึ่งคาดว่าน่าจะเกิดจากสาร CBDA ซึ่งเป็นสารตั้งต้นของ CBD ในตัวอย่างพืชประเภทเส้นใย ส่วนในประเภทกึ่งเสพติดนั้นจะพบทั้งสาร THC และ CBD ในปริมาณที่เท่ากัน จากผลการตรวจโดยอาศัยเครื่องหมายโมเลกุลจำนวน 2 ตำแหน่งนั้น Drug-type marker ซึ่งจำเพาะกับพืชสกุล *Cannabis* ประเภทเสพติด ให้ผลในการแยกประเภทได้ชัดเจน ส่วนอีกตำแหน่งนั้น คือ *Cannabis sativa*-specific marker ไม่สามารถใช้ในการแยกประเภทได้ แต่สามารถใช้ในการตรวจหาพืชสกุล *Cannabis* ได้

การเกิดปฏิกิริยาให้สีกับ Fast Blue B salt นั้น นอกจากจะเป็นวิธีการตรวจสอบเบื้องต้น เพื่อบ่งบอกว่าเป็นพืชสกุล *Cannabis* แล้ว ยังสามารถใช้ในการตรวจสอบตัวอย่างภาคสนามเพื่อแยกชนิดของพืชโดยคร่าวก่อนส่งไปตรวจสอบยืนยันโดยวิธี Thin Layer Chromatography หรือ วิธีการตรวจสารพันธุกรรมก็ได้

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
A	Adenine
bp	Base pair
C	Cytosine
CBD	Cannabidiol
CBN	Cannabinol
cm	Centimeter
˚C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
g	Gram
G	Guanine
HPLC	High Performance Liquid Chromatography
hr	Hour
kb	Kilo base pairs
<	Less than
$MgCl_2$	Magnesium Chloride
µl	Microliter
µM	Micromolar
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
min	Minute
>	More than
ng	Nanogram
ONCB	Office of Narcotics Control Board

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LIST OF ABBREVIATIONS (CONT.)

pmole	Picomole
QSBG	Queen Sirikit Botanic Garden
rpm	Rounds per minute
s	Second
TLC	Thin Layer Chromatography
T	Thymine
T_m	Melting temperature
TBE	Tris boric acid-ethylenediaminetetra acetic acid
V	Voltage
v/v	Volume per volume
w/v	Weight per volume

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CHAPTER I
INTRODUCTION

1.1 Introduction

Cannabis had been wildly cultivated over thousands years ago as a source of fiber, food, oil, medicine and inebriant. According to their primary psychoactive Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), *Cannabis* can be generally classified into three chemotypes, drug, intermediate drug and fiber-type. *Cannabis* plant which has high content of THC with no or low content of CBD is considered to be drug-type; whereas, which of those has low content of THC with high content of CBD is considered to be fiber-type and the plant which has a comparable content of THC and CBD is considered to be an intermediate drug-type. Drug-type cannabis usually contains more than 2% THC; whereas, fiber-type contains as little as 0.15% THC (1). Therefore, in order to control the narcotic drug-type *Cannabis* a maximum limit of 0.3% THC in *Cannabis* plants were agreed in the international regulation (2), (3).

It is believed that cross-pollination of *Cannabis* types in the field may lead to the presence of many local varieties in which chemical compositions varies and may cause the THC content to exceed the permitted limit. Therefore, all types of *Cannabis* possession and cultivation is still disputed and prohibited in Thailand and other countries. In 2005, the Royal Project Foundation of Thailand has realized the enormous usage of fiber-type *Cannabis* and subsequently started the research as to develop fiber-type *Cannabis* (hemp) for legal commercial cultivation (4), (5). In a situation that *Cannabis* cultivation for fiber is becoming legally widespread, it challenges the Office of the Narcotics Control Board (ONCB) which has a duty to prevent and suppress drug abuse in Thailand to provide a strategy in preventing and suppressing the distribution of drug arise from hemp cultivation. It is a question that how we can correctly identify the cultivated plant in a suspected cultivate plantation.

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Currently, the ONCB laboratory follows the methods recommended by the Division of Narcotic Drugs, United Nations for cannabis detection and the analysis of the chemical compounds. Both physical examinations and chemical analysis were recommended in the manual. The physical examinations are macroscopic and microscopic analysis to observe the nature and physical characters of the material; alleged. The analytical techniques for cannabinoids described are colour test, Thin layer chromatography (TLC), High performance liquid chromatography (HPLC) and Gas chromatography (GC). The color test is considered as a presumptive test. Therefore, a confirmatory test for the presence of THC, CBD and CBN would then be subsequently done (Vienna., 1987 #10). It is also suggested that a positive physical examination and a positive TLC result are sufficient to confirm the identification of cannabis (6). There is no report on using TLC for the discrimination of drug-type from fiber-type *Cannabis*.

The earlier forensic approaches especially at the DNA level are mainly used to distinguish *Cannabis* from other plants. In 1998, Limacre and Thorpe reported the application of DNA technology to distinquish *Cannabis* from other plants. They used the "*Cannabis sativa*-specific primer" which amplified the intergenic sequence between *trn*L and *trn*F gene in the chloroplast (7). Nowadays, the more important task in forensic application is to discriminate illegal drug-type from legal fiber-type plant. Recently, Kojoma *et al*. (2005) reported the "drug-type marker" which is the molecular marker for discriminating drug-type from fiber-type cannabis (8).

In this study, we demonstrate the use of three tests; color test, TLC which are recommended and DNA analysis to discriminate and identify the cannabis sample collected from various cultivated sources in Thailand. Theses provide useful information not only about the chemical composition of cannabis types cultivated in various sources of Thailand and also the information on approach that can be taken practically for cannabis detection in field and laboratory.

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1.2 Objective

1.2.1 To demonstrate the ability of the *Cannabis sativa*-specific primers and the Drug-type marker for discriminating drug-type and fiber-type *Cannabis* cultivated in Thailand.

1.2.2 To compare the ability of DNA-based method with others recommended chemical-based methods; colour test and TLC for discriminating drug-type and fiber-type *Cannabis* strain cultivated in Thailand.

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CHAPTER II
LITERATURE REVIEWS

2.1 The genus *Cannabis*

2.1.1 Introduction

Cannabis (Cannabaceae) is a dioecious, annual, herbaceous, wind-pollinated plant. It has been cultivated thousands years ago throughout the temperate and tropical zones of the world for fiber in its stem, the oil in its seeds, and the psychoactive substances in female flowering tops (9), . *Cannabis* cultivated for fiber and seeds is called “Hemp” and which of those cultivated for narcotic purpose is called “Marijuana” (also spelled “Marihuana”). *Cannabis* is the only plant containing cannabinoids, a group of compounds which contain no nitrogen (10). Therefore, testing of cannabinoids content is one way to indicate the presence of *Cannabis* in Forensics Laboratories.

Cannabinoids consists of many constituents including psychoactive and non-psychoactive substances. The three principal cannabinoids constituents are psychoactive delta-9-tetrahydrocannabinol (Δ^9-THC), non-psychoactive cannabidiol (CBD) and cannabinol (CBN) which are usually the most abundant. CBN, a by-product of THC, is formed during improper storage of the flowering tops or resin, or during a chemical manipulation (9). In fresh material, most, if not all of the cannabinoids are present as one or both of two delta-9-tetrahydrocannabinol acid (Δ^9-THCA) derivatives, A and B acids (see figure 2-1). These compounds are unstable and undergo slow spontaneous decarboxylation to release the neutral cannabinoids, a process that is considerably accelerated by the temperatures involved in smoking (see figure 2-8) (10).

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Figure 2-1. Structure of delta-9-tetrahydrocannabinolic acid (Δ^9-THCA) derivatives (10)

R^1 = COOH, R^2 = H, Δ^9-tetrahydrocannabinolic acid A

R^1 = H, R^2 = COOH, Δ^9-tetrahydrocannabinolic acid B

2.1.2 Taxonomy

An attempt to delimit *Cannabis* taxa has been made since 1780s by using various characteristics such as morphology, inebriant ability, chemical component and genetic variation (11-13). Hillig (2005) has divided *Cannabis* based on genetic evidence by studied allozyme variation of *Cannabis* accessions of diverse geographic origins and found two major gene pools, the *C. sativa* and *C. indica* gene pool. This study supported the formal recognition of *C. sativa*, *C. indica*, and possibly *C. ruderalis* as separate species and leave the ranks of subspecies and variety available for further classification which rejected the single species concepts of Linnaeus, and Small and Cronquist but represents a synthesis of the species concepts of Lamarck, Delile, Janischevsky, Vavilov, Schultes et al. and Anderson. Furthermore, the study showed that none of the previous taxonomic concepts adequately circumscribe the sativa and indica gene pools. As a result, a polytypic concept of *Cannabis* is proposed which recognizes three species, *C. sativa*, *C. indica* and *C. ruderalis* and seven putative taxa (11). After all, the taxonomic treatment of members in the *Cannabis* genus is not yet clearly identified and required more study to further resolve the problem.

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Kingdom: *Viridiplantae*
Phylum: *Streptophyta*
Subclass: *rosids*
Order: *Rosales*
Family: *Cannabaceae*
Genus: *Cannabis*

Figure 2-2. The taxonomy classification of Genus *Cannabis* (14).

2.1.3 Morphology

Paris and Nahas (1984) had described the morphological characteristics of *Cannabis* as followed;

The morphological characteristics of this plant are distinct enough to make it easily identifiable (see figure 2-3, 2-4). Its palmated leaves are supported by a slender stalk 5 to 7 cm long, with a narrow groove along the upper side. Each leaf presents 5 to 11 leaflets radiating from the top of the stalk. The soft-textured leaflets, which may be as long as 25 cm, are most often narrow and lanceolate shape finely sharpened to a narrow point; the edges of the leaflets are regularly dentated like the blade of saw, with deep ridges running diagonally from the center to the periphery. The upper side of the leaf is dark green, contrasting with the lighter color of its underside.

The male flower clusters, with their pollen-laden stamina standing out from the leaves on individual flowering branches, are covered with bristly hair. The flowers consist of five whitish or greenish hairy petals, 3 mm long and five pendulous stamens which anther opens lengthwise.

The female flower inflorescence is larger, densely clusters, and do not project beyond the leaves. Each flower presents a green bract, a tubular sheath which surrounds the ovary and out of which project two long stigmas. The bract is covered with hairs and stalkless circular glands and glandular hair which secrete drops of resin (see figure 2-6). The female flower is pollinated by wind, and not by flying or crawling insects.

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After pollination the stigmas fall off, but the bract increases in size and produces a fruit, which is an achene: a hard shell containing a single seed. This achene is ellipsoid, flat, smooth, and of grayish or brown color. It is small with size in the axis 3 x 5 mm. The seed yields significant amount of oil (9).

Cannabis is normally dioecious (carrying separate sex) and dimorphic. Monoecious plants with both male and female flowers also occur mostly in several modern European fiber cultivars. *Cannabis sativa* also has the ability, rarely observed in nature, to reverse its sex after being transplanted. This reversal may be induced by changing daylight exposure of the plant and soil nitrogen content (15).

The male plant, which is 10-15% taller, is short-lived, dying after the pollen is shed and the reproduction cycle has been initiated. The female plant survives until killed by frost or until the seeds have matured. *Cannabis* plants vary enormously in height depending on genetic constitution and environment, but are typically 1 to 5 m (9, 15).

Cannabis is a very resistant plant that tolerates weather changes well, except for heavy frost. It is attacked by only a very few pests or insects. Its seed grow in many different soils and climates and require very little care once it has sunk its root in the ground. *Cannabis* has a distinctive odor. The flowering tops of the female plant secrete a clear, shiny, sticky resin. The resin is formed from the time the flowers first appear until the seeds reach maturity. The resin production might be an adaptive reaction of the plant to shield its maturing seed against heat and low moisture content in the air. Under this protective coating, the fertilized ovules may mature and develop into seeds (9). The resin of *Cannabis* is the part to contain the most concentrated psychoactive ingredients. It was shown that the Δ^9-THC content decreases in the various parts of *Cannabis* plant in the following order: bracts, flowers, leaves, smaller stems, larger stems, roots and seeds (16).

The morphological differences between drug-type and fiber-type *Cannabis* plant had been observed and described in the research of the botanical garden organization, ministry of natural resources and environment, Thailand. These differences are the height of the plant and the size of the leave. Fiber-type plant is mostly taller than 2 meters; whereas, drug-type is shorter than 2 meters. The size of fiber-type leave is larger than those of drug-type as shown in figure 2-5 (17).

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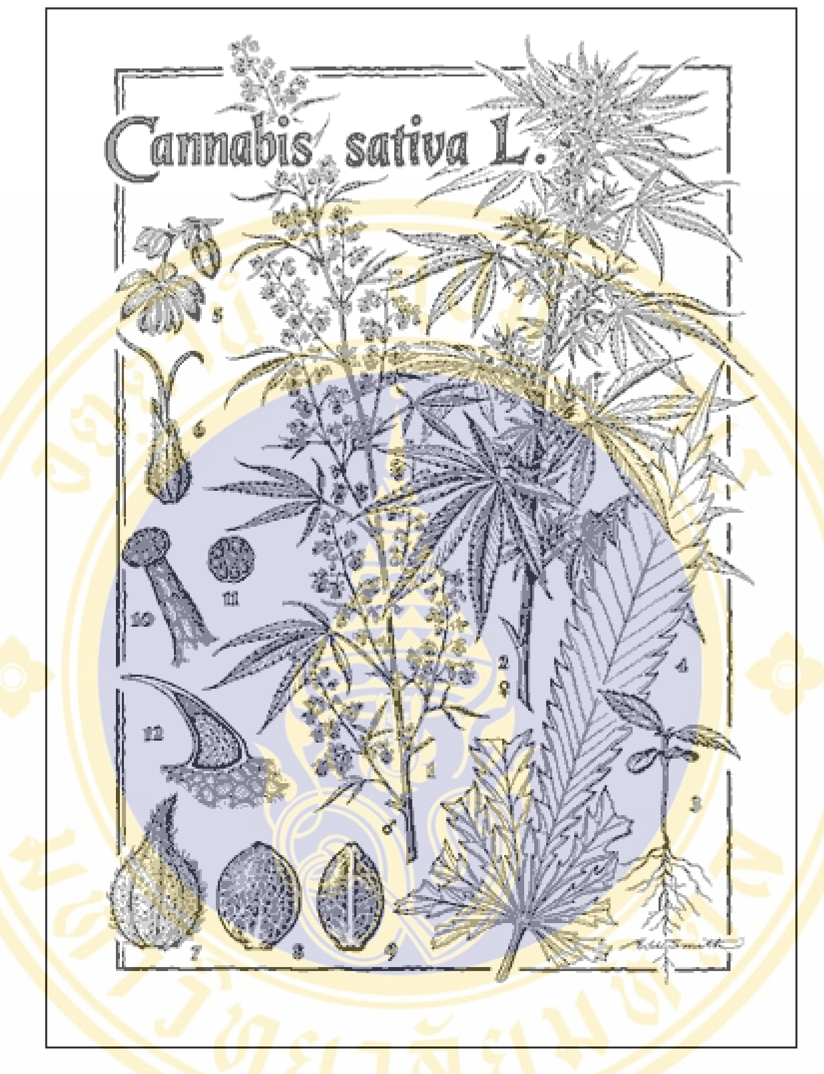

Figure 2-3. Composite plate of *Cannabis sativa* by Elmer Smith. 1. Flowering branch of male plant. 2. Flowering branch of female plant. 3. Seedling. 4. Leaflet. 5. Cluster of male flowers. 6. Female flower, enclosed by perigonal bract. 7. Mature fruit enclosed in perigonal bract. 8. Fruit (achene), showing wide face. 9. Fruit, showing narrow face. 10. Stalked secretory gland. 11. Top of sessile secretory gland. 12. Long section of cystolith hair (note calcium carbonate concretion at base). (44)

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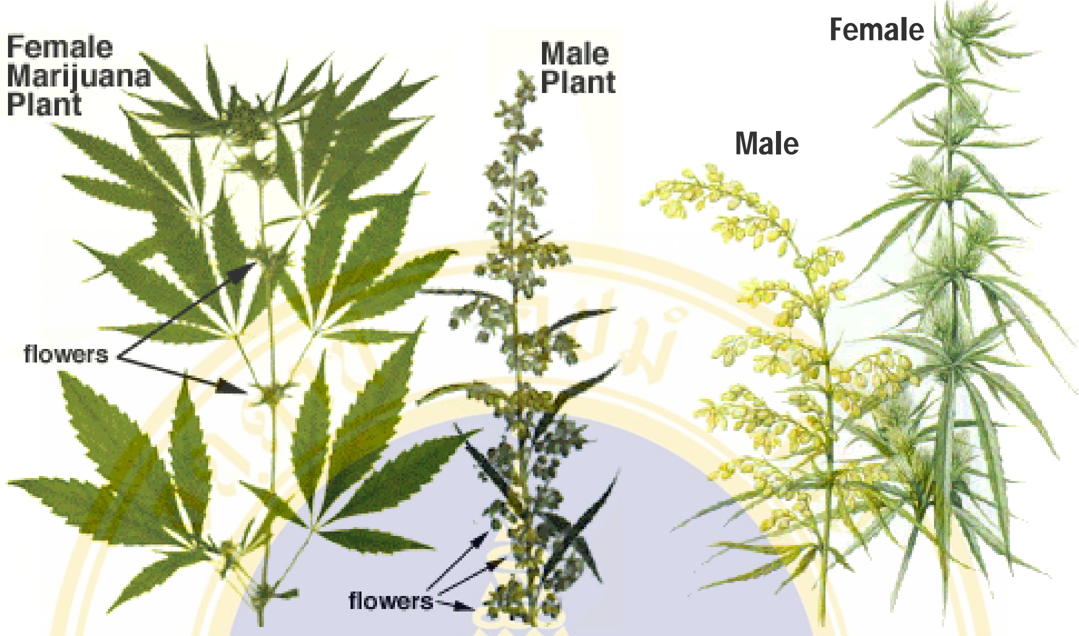

Figure 2-4. The drawing shows the morphological characteristics difference of male and female *Cannabis* plant.

Figure 2-5. The picture of fiber-type leave (upper) and drug-type leave which the size of fiber-type leave is larger than drug-type leave (17) .

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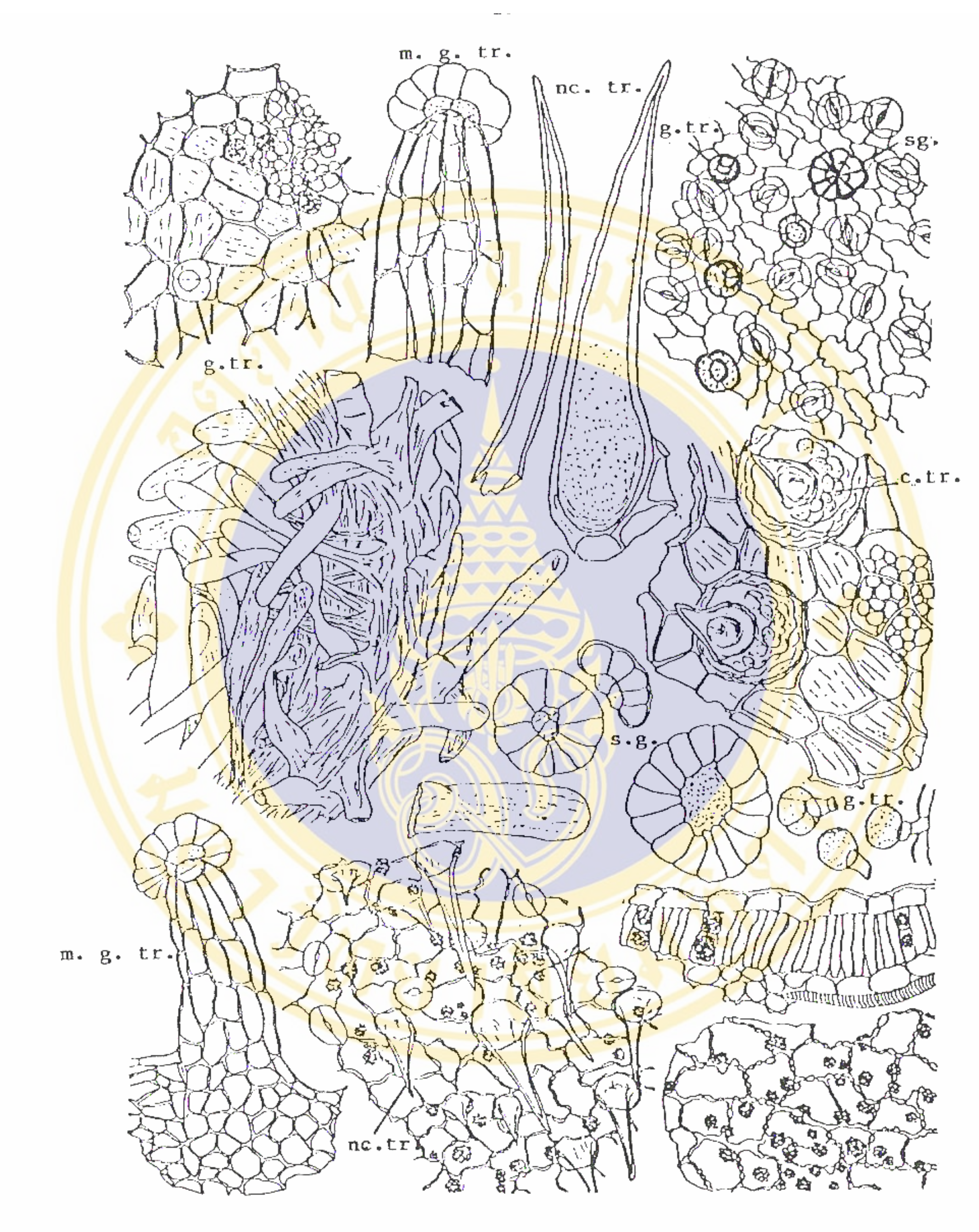

Figure 2-6. The microscopic Characteristics of *Cannabis sativa* L. (40)

G. TR.	:	Small glandular trichomes
M. G. TR.	:	Multicellular multiseriate glandular trichomes
S. G.	:	sessile trichomes
NC. TR.	:	Non-cystilistic trichomes

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2.1.4 Structure and biosynthesis of cannabinoids

Two naming conventions can be used for the cannabiniods and its derivatives. The dibenzofuran method is more common in the forensic context and will be used throughout this present study. All cannabinoids are oily and insoluble in water but soluble in solvents such as chloroform and petroleum ether. None of cannabinoids contain nitrogen thus none are alkaloids (18). The name and structure of principal cannabinoids of *Cannabis* are summarized in table 2.1.

Table 2-1. The principle cannabinoids of *Cannabis*.

Name	Structure
Δ^9-Tetrahydrocannabinol (Δ^9-THC or THC), equivalent to Δ^1-THC in the monoterpenoid system	
Δ^9-Tetrahydrocannabinolic acid (Δ^9-THCA)	

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Name	Structure
Cannabinol (CBN)	
Cannabidiol (CBD)	

Sources of the structures for this table: The National Library of Medicine (National Institutes of Health) chemical information database, http://sis.nlm.nih.gov/Chem/ChemMain.html.

The biosynthesis of cannabinoids has been studied since 1970s. Until 2001, Fellermeier *et al.* had shown clear evidence and confirm earlier hypotheses that cannabigerolic acid (CBGA) is a common precursor for the synthesis of both THCA and CBDA (see figure 2-7) (19), (20). The enzyme which catalyzes oxidative cyclization of CBGA to form THCA is Δ^1-tetrahydrocannabinolic acid synthase. The reaction is FAD –dependent and can be summarized as CBGA + $O_2 \rightarrow$ THCA + H_2O_2. THC, the neutral form of THCA, is derived from THCA by non-enzymatic decarxylation (see figure 2-8) (21).

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Figure 2-7. Biosynthesis of cannabichromenic acid (CBCA, 6), tetrahydrocannabinolic acid (THCA, 7), and cannabidiolic acid (CBDA, 8) in *Cannabis* plants. Isopentenyl diphosphate (IPP, 1); dimethylallyl diphosphate (DMAPP, 2); geranyl diphosphate (GPP, 3); olivetolic acid (OA, 4); cannabigerolic acid (CBGA, 5) are also shown in the scheme (19).

Figure 2-8. The synthesis of THC (21).

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Later, it has been reported that this chemical characteristic is controlled by the B locus. By identifying several CBD- or THC-associated markers, a model of two alleles, B_D and B_T, being codominant had been proposed. The genotype of type I is B_T/B_T; whereas, type II is B_T/B_D and type III is B_D/B_D. Since there was a correlation between the types of *Cannabis* and genotypes of the B locus, this study suggested that allele B is probably responsible for coding the enzyme synthase for the conversion of common precursor cannabigerolic acid (CBGA) into CBDA or THCA (22). Plants with putative genotypes B_D/B_D, B_D/B_T, or B_T/B_T produce low (<0.20), intermediate (0.25-1.50), or high (>50) ratios of THC/CBD (13)

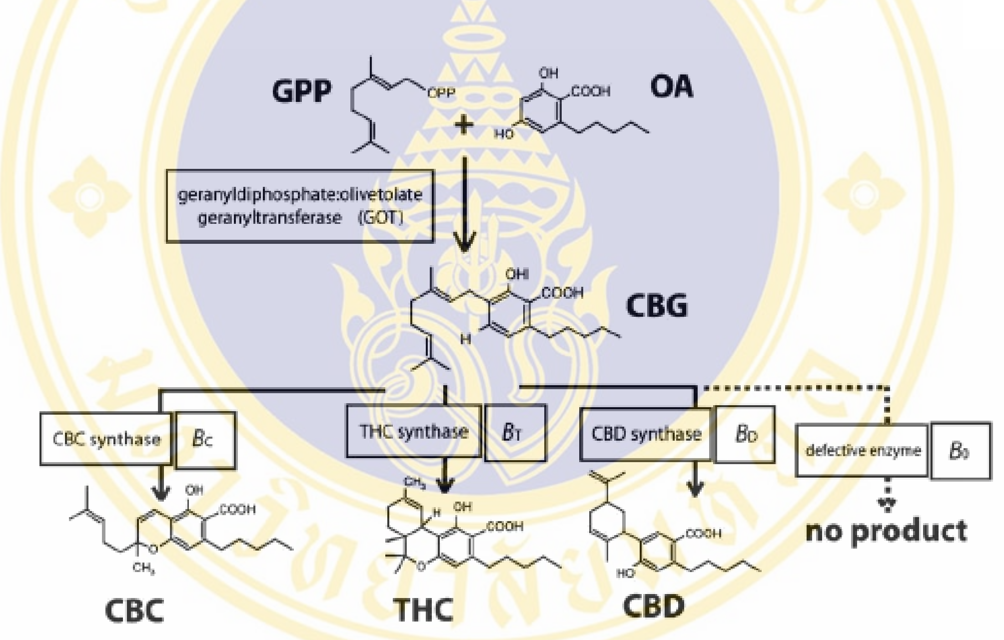

Figure 2-9. The biosynthetic pathway of the most common cannabinoids in *Cannabis* plants (23).

2.2 The *Cannabis* products

2.2.1 Illicit *Cannabis* products

According to the recommended methods manual for testing *Cannabis* of the Division of Narcotic Drugs, United Nations (40). Three types of illicit *Cannabis* products have been described which are herbal products (Marijuana), resin products (Hashish) and liquid *Cannabis* (Hashish oil). The type which is mostly found and causes a major narcotic problem in Thailand is the herbal products. *Cannabis* is

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illegally cultivated throughout Thailand especially in the northeastern and northern part of Thailand where the geography and climate is suitable.

Herbal product or marijuana is dry drug-containing parts consisting of only the fruiting, female flowering tops and leaves which had been stripped from the plant, allowed to air dry and followed by the process subsequently used to make various form of product. The high quality of herbal products is called "Sinsemilla" which mean "without seeds". Sinsemilla is produced by removal of male *Cannabis* plants from the environment of female *Cannabis* plants before the male has released its pollen. Therefore the female plants never become fertilized and produced no seeds. This technique has been practiced for many years in the Indian subcontinent to produce more yield of "ganja" (40).

Resin products or Hashish is produced from a variety of processes depending on the region of production. However, there is some similarity in the methods used to make *Cannabis* resin which sieving is an important part of the process. In Mediterranean countries, the herbal material is threshed, often against a wall, to separate the resin producing parts of the plant from those parts which do not produce resin. The material is then sieved to eliminate seeds and minor fibrous parts. The product remaining is resembled in a fine powder and compressed into slabs. *Cannabis* resin from the Indian subcontinent is produced by a rubbing or kneading process rather than a threshing process. The fruiting and flowering tops of the *Cannabis* plants may be rubbed between the palms of the hand or against rubber sheeting. The transferred resin is then scraped off with the edge of a metal instrument and collected into quantities suitable for pressed or rolled into slabs, rods, balls or whatever shape is favoured in the particularly locality (40).

Liquid *Cannabis* or Hashish oil is a liquid extract of either herbal *Cannabis* material or of *Cannabis* resin. It is made to concentrate the psychoactive ingredients prior to trafficking. Liquid *Cannabis* is prepared by the process of extracting chemicals from solid materials with continual refluxing of the extracting solvent, undertaken in chemical laboratories. Suitable organic solvents include ethanol, methanol, acetone and petroleum ethers. In general, liquid *Cannabis* is prepared to have the consistency of thick oil (40).

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Marijuana is usually defined as all parts of the plant *Cannabis sativa* L., excluding the stalk and sterilized seeds. Some jurisdictions defined as any plants of the genus *Cannabis*. This approach circumvents legal questions about species that have been raised in the past (18).

According to this manual, typical THC levels in the Herbal *Cannabis* is 0.5-5%, Resin *Cannabis* is 2-10% and Liquid *Cannabis* is 10-30%. These values are only a guide to levels likely to be encountered by the forensic analyst. Many samples of herbal, resin or liquid *Cannabis* may have THC content outside these limits (40).

Herbal Product (Marijuana) **Resin Product (Hashish)**

Liquid *Cannabis* (Hashish oil)

Figure 2-10. Three types of illicit *Cannabis* products; herbal product (marijuana), resin product (Hashish) and liquid *Cannabis* (Hashish oil)

2.2.2 Hemp products

"Hemp" is referred primarily to fiber-type *Cannabis sativa* plant. It is the one of the oldest sources of textile fiber. Hemp fiber is a high quality fiber with the property

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of flexibility, strength and durability. Hemp can be cultivated for the production of edible seed oil, essential oils, cosmetics, lubricants, fuels and moreover fibers for the paper and building industries (24). Popular Mechanics magazine (1938) touted hemp as “the new billion dollar crop,” stating that it “can be used to produce more than 25,000 products, ranging from dynamite to Cellophane.” (15). Table 2.2 shows the principle products of *Cannabis sativa* cultivated for fiber in Europe. Table 2.3 shows the products which hold the most promise for North America.

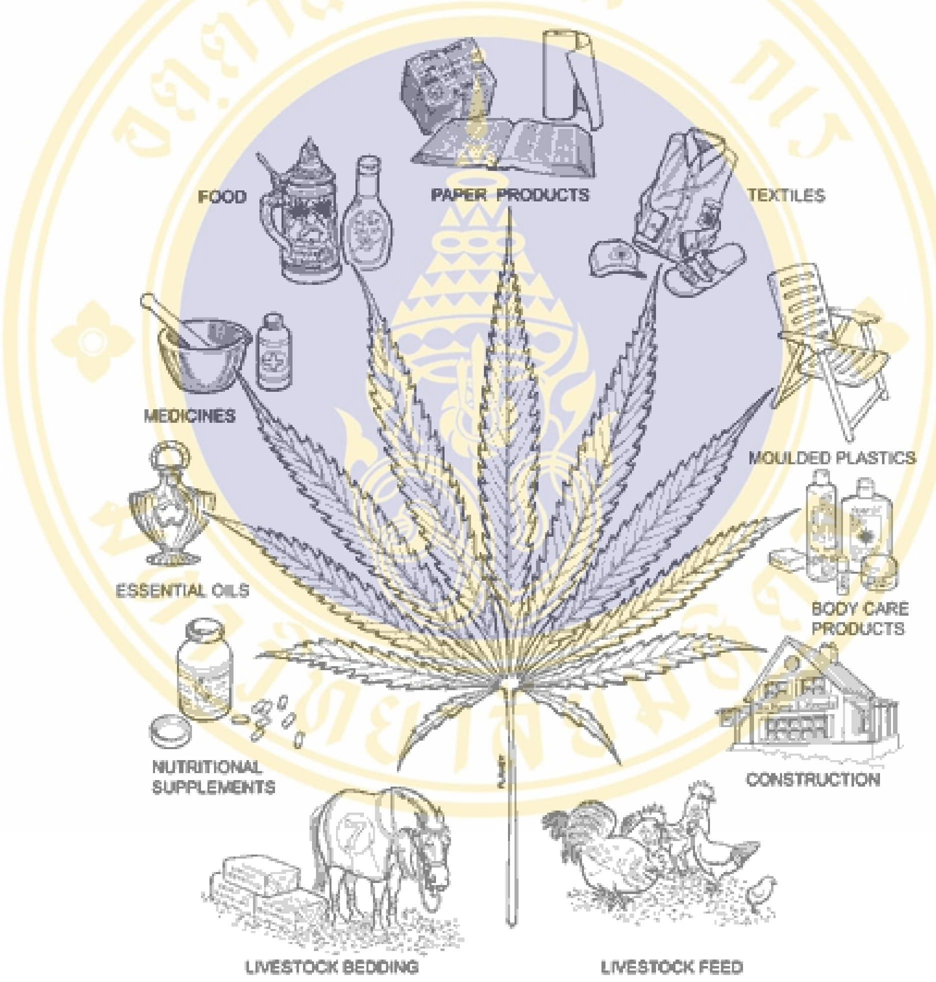

Figure 2-11. The diagram shows the major uses of industrial hemp (15).

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Table 2-2. Hemp fiber usage in the European Union in 1999 (25).

Class of product	Quantity consumed (tonnes)	Relative percentage
Specialty pulp (cigarette paper, bank notes, technical filters, and hygiene products)	24,882	87
Composites for autos	1,770	6
Construction & thermal insulation materials	1,095	4
Geotextiles	234	0.8
Other	650	2.2
Total	26,821	100

Table 2-3. Analysis of commercial *Cannabis* product potential in North America in order of decreasing value toward the right and toward the bottom (15).

Fruit (achenes)	**Long (bark) fiber**	**Woody stem core**	**Female floral (perigonal) bract**	**Whole plant**
Confectionary, baked goods	Plastic-molded products	Animal bedding	Medicinal cannabinoids	Alcohol
Salad oil	Specialty papers	Thermal insulation	Essential oil (for flavor & perfume)	Fuel
Body care "cosmetics	Construction fiberboard	Construction (fiberboard, plaster board, etc.)	Insect repellant	Silage
Animal food (whole seeds for birds, presscake for mammalian livestock)	Biodegradable landscape matting & plant culture products	-	-	-
Gamma-linolenic acid dietary supplements	Coarse textiles (carpets, upholstery)	-	-	-
Specialty industrial oils	Fine textiles	-	-	-

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In Thailand, hemp is primarily used by the Hmong Lai community for weaving clothes (26).

2.3 Testing of *Cannabis*

According to the manual for testing cannabis of the Division of Narcotic Drugs, United Nations (40), the methods for testing *Cannabis* products are mainly based on physical examination; macroscopic and microscopic examination and analytical chemical techniques; color test, TLC, HPLC and GLC. In order to establish the identity of any controlled drug, Division of Narcotic Drugs, United Nations suggest national narcotics laboratories to perform at least two independent analytical parameters and, when possible, three entirely different analytical techniques for the identification and analysis of *Cannabis* products should be used, for example: color test and any two of the available chromatography techniques (TLC, GLC or HPLC). Macroscopic and/or microscopic examination are also included as part of the testing protocol. These mandatory processes cause difficulties, complexities and retardment in the field of forensic work, presenting a challenge for forensic laboratories in dealing with an increase volume and a wide range of *Cannabis* products that encountered (40).

2.3.1 Physical Characteristics

Macroscopic examination or visual identification include a description of sample, morphological characteristics of the plants or herbal material, smell, number of pieces of resin, brand marks, whether the plant is flowering. Microscopic examination is used to examine the presence of hairs which are cystolithic, glandular, and unicellular trichomes (40), (18).

2.3.2 Color test

There are 2 color tests that have been used in *Cannabis* detection. First is Fast Blue B salt test and another is the rapid Duquenois test (Duquenois-Levine test). The Fast Blue B salt test can be performed either on filter paper or in test tube; whereas, the rapid Duquenois test is performed only in test tube. The positive result of Fast Blue B salt test for THC, CBD, and CBN is red, orange and purple, respectively. For the

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rapid Duquenois test, the positive result for *Cannabis* gives violet colored in the lower (chloroform) layer (40).

2.3.3 Chromatography

Thin Layer Chromatography (TLC) is one of the chromatography techniques used to separate individual components of a mixture on the basis of differences in their physical characteristics. The basic principles of chromatography involve the interaction of the substances with the stationary phase and mobile phase. The substances which interact strongly with the stationary phase will be retarded to the greatest extent; whereas, those which show little interaction will pass through with minimal delay, leading to differences in distances traveled or elution time. The essential components of TLC system are (6):

- Silica gel TLC plate; silica which acts as a stationary phase coated on aluminium sheet which acts as a chromatographic bed
- Solvent system; a solvent or mixture of solvents which suitable for the separation of the components of the samples. This solvent system acts as a mobile phase to carry the sample through the stationary phase.
- A development tank; a closed system which contains the solvent system and saturated the tank atmosphere with solvent vapour.
- A detection system to visualise the test substances.

In *Cannabis* detection, a positive TLC result, which identify a tested substances based on its Rf values compared to known reference standards, in conjunction with visual identification is sufficient to confirm to a court that the sample you examined was *Cannabis* (18).

The other two chromatography techniques, Gas liquid chromatography and High performance liquid chromatography, are also used as confirmatory test for *Cannabis* detection. Either packed column techniques (with or without derivatisation) and capillary column technique for Gas liquid chromatography or two method of isocratic technique and Gradient technique are suggested in the manual (ST/NAR/8, 1987). For the details of these techniques in *Cannabis* detection, the reader is referred to the recommended methods for testing cannabis of the United Nations (40).

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2.4 Classification and Discrimination of *Cannabis* type

So far there are no botanical methods by which the two types of *Cannabis* plants, drug-type and fiber-type, may be distinguished. However, classification on the basis of cannabinoids content is widely accepted. There are several indexes, which are mostly concern about the amount of main cannabinoids content, suggested by different group of scientists as following reviews:

2.4.1. Fetterman *et al.* (1971) (16), (27) defined;

$$\text{The phenotype index} = \frac{\%\ \Delta^9\text{-THC} + \%\ \text{CBN}}{\%\ \text{CBD}}$$

The *Cannabis* plant which has the phenotype index more than 1 is classified to be "drug phenotypes" and which of those has the phenotype index less than 1 is classified to be "fiber phenotypes". Some scientists suggest to use the term "psychoactive" and "nonpsychoactive" or more precisely as the delta-9-THC phenotype and the cannabidiol phenotype instead of drug phenotypes and fiber phenotypes since no evidence concerning fiber content of the plant used in these experiments was given.

2.4.2. Small and Cronquist (1976) described 3 chemo-types (I, II and III) based on the THCA and CBDA percentage of the inflorescence dry weight.

- Chemo-types I has high percentage of THCA and low CBDA content
- Chemo-type II has comparable high percentage of THCA and CBDA
- Chemo-types III has low percentage of THCA

2.4.3. Debruyne *et al.* (1981) (28) had combined the use of high-pressure liquid chromatography (HPLC) and high-resolution gas-liquid chromatography and established a criterion by calculating the peak area ratios of different sample varieties;

$$\frac{\Delta^9\text{-THC}}{\text{CBD}} \quad \text{and} \quad \frac{\text{CBN}}{\text{CBD}}$$

These two ratios are low in the fiber-type; whereas, for the drug-type, at least one of these two ratios (28).

2.4.4. Paris and Nahas (1984) (9) has described 3 chemical types by percent of dry weight which are

- Drug-type (THC>1% and CBD=0)
- Intermediate drug-type (THC>0.50% and CBD>0.50%)
- Fiber-type (THC<0.25% and CBD>0.50%)

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2.4.5. According to the European Union, *Cannabis* plants with less than 0.3% Δ^9-THC concentration in the dry flowering tops are considered to have no psychoactive potency and this value has been generally used to delimit between drug-type and fiber-type *Cannabis* plants (2), (3). Textile *Cannabis* and resinous *Cannabis* can also be used to called drug-type and fiber-type plant, respectively.

Mandolino and Carboni had also described the other 2 chemotypes in his paper. Which are the prevalent CBG (CBG>0.3%) (29) and the zero cannabinoids with an undetectable amount of cannabonoids (23).

In addition, the Vienna convention also considers the plant material with psychoactive THC content higher than 0.3% of dry weight as illegal products (3). According to the classification mentioned above, type III obviously has an acceptable THC content to be legally and commercially cultivated. In fact, there are others factors apart from genetic characteristics causing variation in cannabinoids content which must be taken into account including environmental conditions (30), (31), (32), (33) such as light, moisture and oxygen (24), (34), geographic difference, the maturity, the sex, the part of the harvested plant, the treatment and storage of the plant, (35), (9). These factors can affect the THC content to vary and exceed the given limit (36), (37). Mechtler K. (2004) had studied the variations of delta-9-THC content in single plants of hemp varieties. In this study, 30-60 plants of each variety were sampled. The single plants with THC values far outside the bandwidth were found. This finding made a parametrical approach to estimate necessary sample size impossible. For a conclusion, a representative sample will be rather big and the data based on the analysis of a single plant will not necessarily give correct information on chemotype or provenance of the variety (38). As a result, hemp possession and cultivation is still disputed and prohibited in many countries, including Thailand. This prohibition is the major impediment for hemp to be commercially cultivated.

2.5 The study of forensic DNA Markers in *Cannabis*

Linacre and Thorpe (1998) had designed the *C.sativa*-specific primers (primer G, 5'-GAG GGT TTC TAA TTT GTT ATG TT-3'; primer H, 5'-ACT AGA GGA CTT GGA CTA TGT C-3'). These primers were tested on a range of different plant extracts which only gives a 197 bp PCR product in the presence of *Cannabis sativa* (7). These

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primers amplify a short intergenic spacer between trnL-trnF gene of chloroplast DNA. There is no report of testing these primers with the fiber type *Cannabis*. Since there was a report which discriminate drug-type and fiber-type *Cannabis* as different species, it is worthy to test these *Cannabis sativa*-specific primer with the fiber-type *Cannabis*.

Another approach of *Cannabis sativa* species identification exploited the properties of the Internal Transcibed Spacers I and II (ITS1 and ITS2) of the nuclar ribosomal genes. This DNA region was found to distinguish *Cannabis sativa* from any other plant species, including the closely related hop (23).

Recently, a difference between “drug-type” and “fiber-type” *Cannabis* strain has been identified using DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene, the gene encodes enzyme THCA synthase. Also, A specific PCR marker for the THCA synthase gene for the drug-type strains has been identified in this study (primer g, 5’- AAT AAC TCC CAT ATC CAA GCA-3’ ; primer h, 5’-AGG ACT CGC ATG ATT AGT TT-3’).This PCR marker can be detected with a PCR product of approximately 1.2 kb in the drug-type strains but not in fiber type strains (8).

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CHAPTER III
MATERIALS AND METHODS

3.1 Plant Samples

Plant samples were kindly supported by Office of the Narcotics Control Board (ONCB). Many types of samples were subjected to the present study as summarize in table 3-1. Samples were classified by the staff of ONCB and transported to the lab located in Bangkok. Fresh plants were frozen in liquid nitrogen and stored at -80 °C. Dry plants were dried in hot air oven at 40-50 °C for several hours before storing at room temperature. After the samples were collected and extracted, the remaining plant materials were destroyed immediately.

Figure 3-1 Photographs of samples; Drug-type (left) and Fiber-type (right).

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Table 3-1. Summary of plant samples subjected in the present study.

Sample Type	Source	No. of sample
Dried Drug-type plant	Illegally grown by Moo-sir hill tribe in the northern part of Thailand	10
Fresh Drug-type leave	Illegally grown in the southern part of Thailand	7
Fresh Fiber-type leave	Grown in the northern part of Thailand	4
Dried Fiber-type plant	Grown by the Maew villagers in the northern part of Thailand	1
Dried Fiber-type plant	Grown in the QSBG* located in the northern part of Thailand	3
Fresh fiber-type leave	Grown in the QSBG located in the northern part of Thailand	4
Fresh fiber-type plant	Grown by Hmong villagers in the northern part of Thailand	4
Fresh fiber-type plant	Grown from the seed colleted from the plant in northern part of Thailand	17
Seized marijuana	Illegal herbal product seized by legal officer	2

* QSBG is the Queen Sirikit Botanic Garden, established in 1993 by the Botanic Gardens Organization of Thailand. QSBG is under the Ministry of Natural Resources and Environment.

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The plant samples of authentic drug-type and fiber-type were tested by three testing methods which two are chemical-based method and another is DNA-based method. The chemical-based methods which are Fast Blue B salt test and TLC were conducted as suggested in recommended methods for testing cannabis, manual for use by national narcotics laboratories, United Nations (40); whereas, the DNA test was conducted according to Kojoma *et al.* (2006) (8) and Linacre and Thorpe (1998) (7).

3.2 Fast blue B salt test

The test was performed on a filter paper according to the recommended methods manual for testing cannabis of the Division of Narcotic Drugs, United Nations. The solid reagent is prepared by diluting Fast blue B salt with anhydrous sodium sulphate (1:100). Petroleum ether referred as solution 1 and 10% (w/w) aqueous solution of sodium bicarbonate referred as solution 2. Two filter papers were fold into quarters and partly opened to form a funnel. A small amount of dried pulverized cannabis plant was placed on the centre of the upper paper. Two drops of solution 1 were added allowing the liquid to penetrate to the lower filter paper. Then the two filter papers were separated; the upper was discarded and the lower filter paper was allowed to dry. After that, a very small amount of the solid reagent was added to the lower filter paper followed by two drops of solution 2. The produced color was then observed and recorded (40).

3.3 Thin Layer Chromatography

3.3.1 Preliminary test of TLC solvent system

One sample of male drug-type, female drug-type and female fiber-type were subjected to TLC using Silica gel 60 F_{254} and three solvent systems according to the recommended manual for testing cannabis of the Division of Narcotic Drugs, United Nations in order to obtain the suitable TLC condition.

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System A		*System B*		*System C*	
Solvents	Ratio	Solvents	Ratio	Solvents	Ratio
Petroleum ether	80	Hexane	90	N-hexane	70
Diethyl ether	20	Dioxane	10	Dioxane	20
				Methanol	10

3.3.2 TLC development of samples

The test procedure was performed according to the recommended methods manual for testing cannabis of the Division of Narcotic Drugs, United Nations. The amount of sample used in the extraction step was modified from the manual to a small amount. The herbal sample was prepared by pulverizing the plant materials into small aggregates to ensure rapid and complete extraction. Eighty milligrams of herbal sample was extracted with 1 ml of acetone for 15 minutes in an ultrasonic bath. The supernatant solution was transferred to a new vial. Four microliters of the sample solution was spot on the Silica gel 60 F_{254} coated on aluminium sheets (Merck) along with the THC, CBD and CBN standard solutions (Sigma). The developing solvent system consists of three solvents in a ratio as followed;

Solvents	Ratio
N-hexane	70
Dioxane	20
Methanol	10

This solvent combination is suitable for not only the neutral cannabinoids but also the cannabinoids acid (40). After developing, the TLC plate was dried prior to visualization with Fast blue B salt solution. The Fast blue B salt solution was prepared by dissolved 50 mg of Fast blue B in the 1 ml of water follow with 20 ml of methanol. The plate was sprayed with the Fast blue B salt solution and and left at room temperature for color development.

3.4 DNA Preparation

All the plant samples were extracted for DNA using the Dneasy® Plant Mini kit (QIAGEN, Germany). Following the manufacturer's protocol (39), about 100 mg wet weight or 20 mg dry weight of plant sample were used as starting material. The sample

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was grinded, within microcentrifuge tube using a glass pestle, under liquid nitrogen to a fine powder. Following the kit's protocol, 400 µl of buffer AP1 and 4 µl of Rnase A (100 mg/ml solution) were added to the grinded sample and followed by a vigorously vortexing. The sample was incubated for 10 min at 65 °C to lyses the cells. The sample was mixed 2-3 times during incubation by inverting tube. Then 130 µl of buffer AP2 was added to the lysate and mixed followed by 5 min incubation on ice to precipitates detergent, proteins, and polysaccharides. The lysate was applied to the QIAshredder Mini Spin Column (lilac) sitting in a 2 ml collection tube and centrifuged for 2 min at 14,000 rpm. The flow-through fraction was transferred to a new tube without disturbing the cell-debris pallet. One and a half volumes of buffer AP3/E was added to the cleared lysate and mixed by pipetting. Six hundred and fifty µl of the mixture was applied, including any precipitate which may have formed, to the DNeasy Mini Spin Column sitting in a 2 ml collection tube and then the spin column was centrifuged for 1 min at ≥8,000 rpm. The flow-through was discarded and this step was repeated with the remaining sample mixture. After the spin column was placed in a new 2 ml collection tube, 500 µl of buffer AW was added and the spin column was centrifuged for 1 min at ≥8,000 rpm. The flow-through was discarded. Another 500 µl of buffer AW was added and the spin column was centrifuged for 2 min at 14,000 rpm to dry the membrane. The spin column was transfered to a 1.5 ml microcentrifuge tube and 50 µl of buffer AE was added directly onto the DNeasy membrane. After a 5 min incubation at room temperature (25 °C), the spin column was centrifuged for 1 min at ≥8,000 rpm to elute the extracted DNA. The column was eluted twice with 50 µl of Buffer AE to increase DNA yield. Then the extracted DNA solution was stored at -20 °C for further analysis.

3.5 DNA Quantitation

3.5.1 UV-VIS Spectrophotometer

A quartz cuvette containing the DNA solution was placed in a UV-VIS spectrophotometer (Agilent Technologies, Thailand) to measured its UV absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) The DNA concentration was obtained by using the following formula ;

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DNA concentration = A_{260} value x diluting factor x 50

The purity of DNA is estimated by the calculation of A_{260}/A_{280} ratio which should have an A_{260}/A_{280} ratio of 1.8-2.0.

3.5.2 Yield Gel method

The quantity of extracted DNA was estimated by comparing the band intensity of extracted genomic DNA samples with those of 100 bp markers in 2% agarose gel. The quantity and size of genomic DNA samples was analyzed using GeneTools (SyneGene, UK) programme.

3.6 DNA Amplification

All of the primer sequences are shown in table 3-1. Polymerase Chain Reactions were carried out using the following primer pairs;

3.6.1 Drug-type Marker (g and h)

The strain of authentic drug-type and fiber-type were firstly confirmed by DNA test using Drug-type Marker. According to Kojoma *et al.*, a portion of the THCA synthase gene was amplified by primer g and h in a total volume of 10 µl. Each reaction contained 1 ng of extracted DNA, 200 µM of each deoxyribonucleotide triphosphate or dNTPs (Promega, USA), 1X PCR buffer, 2.5 mM $MgCl_2$, 0.5 units of *AmpliTag* Gold DNA polymerase (Applied Biosystem), 2 pmole of each primer and sterile distilled water. The reactions were carried out in GeneAmp PCR System 9700 (Applied Biosystem) using the following condition ; initial denaturation at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72°C for 2 min, followed by final extension at 72 °C for 5 min. The size of the amplified product was expected to be 1.2 kb (8).

3.6.2 Universal Primer (C and D) and *Cannabis sativa*-Specific Primers (G and H)

Seven samples of each drug-type and fiber-type plant were used for the amplification of these primers. The simplex PCR amplifications using primer C,D and G,H were performed separately in a total volume of 25 µl. Each reaction contained 20 ng of extracted DNA, 200 µM of each deoxyribonucleotide triphosphate or dNTPs

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(Promega, USA), 1X PCR buffer, 1.5 mM $MgCl_2$, 1.5 units of *Tag* polymerase (Applied Biosystem), 100 pmole of each primer and sterile distilled water. The reactions were carried out in GeneAmp PCR System 9700 (Applied Biosystem) using the following condition ; initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72°C for 30 s, followed by final extension at 72 °C for 7 min. The size of amplified products was reported to be 348 bp for CD and 197 bp for GH (7).

3.6.3 Universal Primer (C and F)

A pair of universal primer (C and F) obtained from Linacre and Thorpe was used to amplify the nucleotide sequence between the trnL and trnF genes in the chloroplast of both drug-type and fiber-type plants. The expected PCR products is approximately 818 bp (7). The amplifications were performed in a total volume of 50 µl. Each reaction contained 20 ng of extracted DNA, 200 µM of each deoxyribonucleotide triphosphate (dNTPs), 1X PCR buffer (Applied Biosystem), 1.5 mM $MgCl_2$, 1X Q solution, 2 units of *Tag* polymerase (Applied Biosystem), 100 pmole of each primer and sterile distilled water. The reaction were proceeded by GeneAmp PCR System 9700 (Applied Biosystem) at condition as followed ; initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72°C for 1 min, followed by final extension at 72 °C for 7 min.

3.7 Detection of PCR Products

Gel electrophoresis was used for the detection of the PCR product fragments. To prepared a 2% (w/v) agarose gel, 2 g of agarose powder was dissolved in 100 ml of TBE buffer. Then 2 µl of 10 mg/µl ethidium bromide was added to the warm gel and mixed well before pouring into the gel tray. The PCR products were mixed with 6X loading dye which helps the PCR products to gather at the bottom of well of the gel and act as an indicator for tracking. The mixtures were loaded to the gel submerged in a TBE buffer tank along with 100 bp ladder which act as a size standard. Electrophoresis was carried out at 75 V for 90 min to separate the PCR products. The gel was then photographed under UV light using SyneGene Gel documentation.

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3.8 Purification of PCR Products

A PCR product of drug-type and fiber-type sample amplified by each primer was selected for sequencing. The PCR products were purified from primers, nucleotides, polymerases, and salts using QIAquick PCR Purification Kit (QIAGEN, USA) before sending to Bio Service Unit (BSU), Bangkok, Thailand for sequencing. Following the kit's protocol, all centrifuge steps were carried out at 13,000 rpm. Five volumes of Buffer PB was added to one volume of the PCR mixture and mixed. To bind DNA, the mixture was applied to the QIAquick column sitting in a 2 ml collection tube and centrifuged for 30-60 s. The flow-through was discarded and the column was placed back into the same tube. To wash, 0.75 ml of Buffer PE was added to the column and centrifuged for 30-60 sec. The flow-through was discarded and the column was placed back into the same tube then the column was centrifuged for an additional 1 min. The column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of Buffer EB was added to the center of the QIAquick membrane and the column was centrifuged for 1 min. Ten µl of each primer at concentration of 10 µM were also sent along with the purified PCR products.

3.9 Analysis and comparison of sequences

The sequences of drug-type and fiber-type samples amplified by Universal primer (C, D and F) and *Cannabis sativa*-Specific Primers (G and H) were combined and compared between sample types for the base difference. These sequences were also compared with other sequences of this locus in the Entrez Nucleotide Database by blast programme (46), (47).

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Table 3-2. Sequences of primers used in present study.

Primer name	Sequence 5'-3'	T_a	Reported author
Universal primer, *C*	CGA AAT CGG TAG ACG CTA CG	54 °C	Linacre and Thorpe
Universal primer, *F*	ATT TGA ACT GGT GAC ACG AG	54 °C	Linacre and Thorpe
Universal primer, *D*	GGG ATA GAG GGA CTT GAA C	60 °C	Linacre and Thorpe
Cannabis sativa-Specific, *G*	GAG GGT TTC TAA TTT GTT ATG TT	60 °C	Linacre and Thorpe
Cannabis sativa-Specific, *H*	ACT AGA GGA CTT GGA CTA TGT C	60 °C	Linacre and Thorpe
Drug-type Marker, *g*	AAT AAC TCC CAT ATC CAA GCA	57 °C	Kojoma *et al.*
Drug-type Marker, *h*	AGG ACT CGC ATG ATT AGT TT	57 °C	Kojoma *et al.*

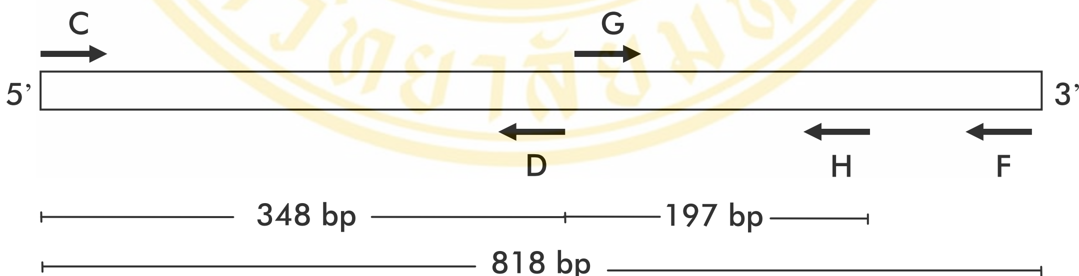

Figure 3-2. A diagram shows the positions of primers used in present study.

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CHAPTER IV
RESULTS

4.1 Identification of authentic drug-type and fiber-type sample

4.1.1 *Cannabis* presumptive testing by Fast Blue B salt

According to the recommended manual, the Fast Blue B salt was used to test for the presence of THC in suspected materials. The positive result of the test is only a presumptive indication of the possible presence of cannabis products or material containing cannabis products (40). The resulting color is a combination of the colors of the different cannabinoids which are the major components of cannabis. A positive color results for THC, CBD and CBN would show a red, orange and purple color, respectively. The results of drug-type and fiber-type samples in the present study showed a red-purple and orange-red color, respectively (see figure 4-1).

4.1.2 *Cannabis* confirmatory identification by TLC

There are three solvent systems suggested in the recommended manual (ST/R/8, 1987). System C which consists of N-hexane, Dioxane and Methanol at a ratio of 70:20:10 was used as developing system in present study because it can produces good separation of cannabinoids particularly the THCA and CBDA on the TLC plate which indicated by its highest number of bands separated and broad Rf range; whereas, other two systems cannot (see figure 4-2) (40). In system C, Δ^9-THC migrated in the highest Rf value among three constituents, followed by CBN and CBD. Although CBN migrates in the same Rf ranges as CBD, it could be differentiated by the color produced by Fast Blue B salt which were red for Δ^9-THC, orange for CBD, and purple for CBN. The standard compounds of THC, CBD and CBN were spotted along as references. The qualitative analysis of plant sample was performed by comparing Rf values with the Rf values of reference compounds. TLC pattern of authentic drug-type showed the presence of both THC and CBD but not CBN; whereas, authentic

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Figure 4-1. The resulting color of drug-type and fiber-type sample from the Fast Blue B salt test and the negative control.

Figure 4-2. The TLC pattern of male drug-type (lane 1), female drug-type (lane 2) and female fiber-type (lane 3) in three solvents system on Silica gel 60 F_{254} coated on aluminium sheets visualized by Fast blue B salt. System A is Petroleum ether : Diethyl ether (80:20), system B is Hexane : Dioxane (90:10) and system C is Hexane : Dioxane : Methanol (70:20:10). Arrow shows an extra band presence only in female fiber-type sample.

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fiber-type showed a slight amount of THC and a high concentration of CBD with no CBN. It should be noticed that no orange band was observed in the lower Rf region compare to the TLC pattern of fresh fiber-type. The TLC results of drug-type and fiber-type samples were different as shown in figure 4-3.

4.1.3 *Cannabis* confirmatory identification by DNA

According to Kojoma *et al.*, Drug-type marker (g and h) was used to confirm the sample type (8). Drug-type strain gives an approximately 1.2 kb-PCR product; whereas, no PCR product would be detected from the fiber-type strain. A universal primers C and D which amplified the intergenic spacer between *trn*L and *trn*F genes in the chloroplast (7) was used as positive control to demonstrate that the genomic DNA extracted were amplifiable. The DNA results of drug-type and fiber-type sample amplified by Drug-type marker are shown in figure 4-4

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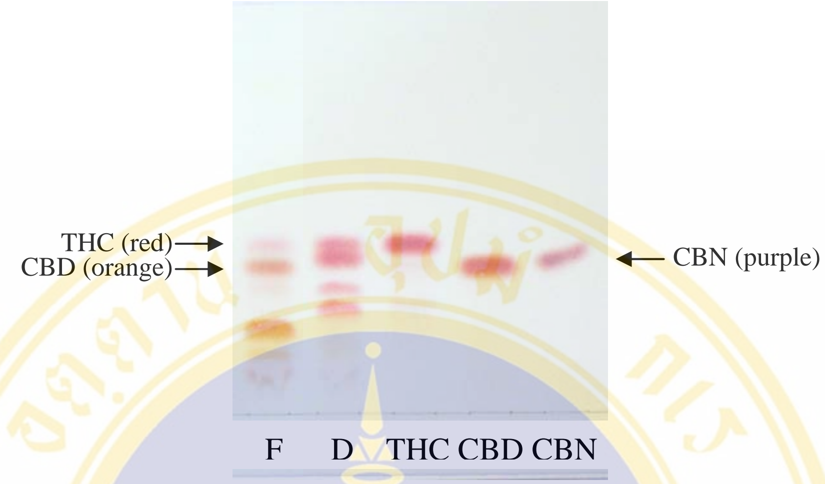

Figure 4-3. The TLC pattern of fiber-type (F) and drug-type (D) with three standards; THC, CBD and CBN.

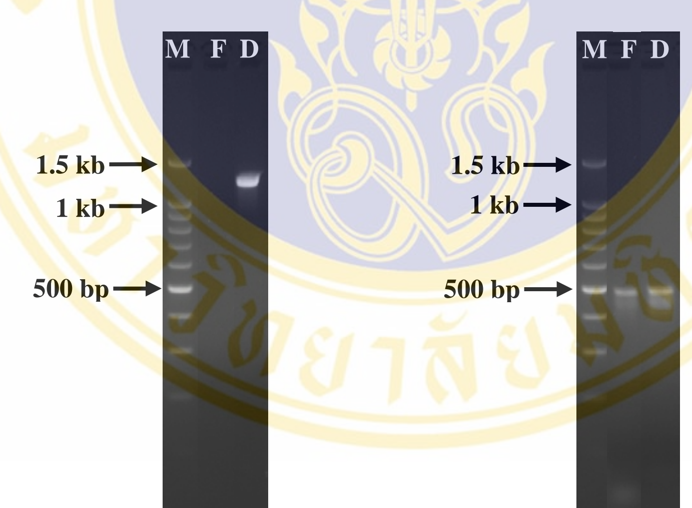

Figure 4-4. The PCR product on the 2% (w/v) agarose gel amplified by Drug type Marker g and h of drug-type (D) and fiber-type (F) samples (left). PCR product of IGS between trnL and trnF gene in the chloroplast amplified by universal primer C and D as positive control (right).

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4.2 Identification of *Cannabis* samples using a combination of TLC and DNA methods

A number of plant samples were subjected to TLC and DNA analysis and the results are summarized in the following table.

Table 4-1. The TLC and DNA result of selected sample from various sources in Thailand.

Sample	TLC band detected			Detected PCR product using	
	THC	CBD	CBN	Drug-type Marker (g and h)	Universal primers (C and D)
Fresh drug-type: Illegally grown in the southern part of Thailand					
Fresh drug-type #01	+	-	-	+	+
Fresh fiber-type: grown in the QSBG located in the northern part of Thailand					
Fresh fiber-type #02	+	+	-	-	-
Fresh fiber-type #14	+	+	-	+	+
Fresh fiber-type #15	+	-	+	-	-
Fresh fiber-type: grown from the seed colleted from the plant in northern part of Thailand					
Fresh fiber-type #03	+	+	-	+	+
Fresh fiber-type: grown by Hmong hill tribe in the northern part of Thailand					
Fresh fiber-type #35	+	+	+	+	+
Fresh fiber-type #37	+	+	-	+	+

TLC showed the presence of THC in all of these samples. Fresh drug-type sample showed only THC which fresh fiber- type, THC was presented with CBD or CBN. Only one sample from Hmong hill tribe gave THC, CBD and CBN.

Universal primers (C and D) were used as positive control. The size of amplified products using primer C and D is approximately 500 bp which is larger than reported by Linacre and Thorpe (1998) (7). From the results, two of these samples were unsuccessfully amplified because no PCR product was detected in the positive control.

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All these samples gave an approximately 1.2 kb-PCR product for Drug-type Marker indicating the drug-type strain of these samples (8).

4.3 Identification of *Cannabis sativa* sample from various sources in Thailand

4.3.1 Fast Blue B salt test

The Fast Blue B salt test was performed on 34 *Cannabis sativa* samples. The samples can be divided into 2 groups according to the color changes. Twenty one samples yielded red to purple-red color; whereas, 13 samples yielded orange to red-orange color. The Fast Blue B salt test of all tested samples were summarized in table 4.2-4.9.

4.3.2 Thin Layer Chromatography

Forty-one *Cannabis* samples were analyzed along with the THC, CBD and CBN standards. All of the fresh and dry plant samples showed the presence of THC with different concentrations. Two of the seized cannabis in a form of herbal product gave distinct patterns; one sample contained CBN and slight amount of THC were present; whereas, the other sample contained a dark band of both THC and CBN. In the group of fiber-type samples, the chromatograpic patterns and band intensities varied among samples. The TLC results are summarized in table 4.2-4.9 and shown in figure 4-5 to 4-12.

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Table 4.2. The Fast Blue B salt test and TLC results of ten samples of dried drug-type grown by Moo-sir hill tribe, one sample of fresh drug-type from the south of Thailand and two samples of seized cannabis.

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Dry drug-type: illegally grown by Moo-sir hill tribe in the northern part of Thailand No. of plant tested = 10					
Female dry drug-type #01	red -purple	+	-	+	4-5 : 1
Female dry drug-type #02	red-purple	+	-	+	4-5 : 2
Female dry drug-type #03	red -purple	+	-	+	4-5 : 3
Female dry drug-type #04	red -purple	+	-	+	4-5 : 4
Female dry drug-type #05	red -purple	+	-	+	4-5 : 5
Male dry drug-type #02	red -purple	+	-	+	4-5 : 6
Male dry drug-type #03	red -purple	+	-	+	4-5 : 7
Male dry drug-type #04	red -purple	+	-	+	4-5 : 8
Male dry drug-type #05	red purple	+	-	+	4-5 : 9
Male dry drug-type #06	red -purple	+	-	+	4-5 : 10
Fresh drug-type: illegally grown in the southern part of Thailand No. of plant tested = 1					
Fresh drug-type #01	N/A	+	-	-	4-5 : 11
Seized cannabis: illegal herbal product No. of sample tested = 2					
Seized Cannabis #01	purple	-	-	+	4-5 : 12
Seized Cannabis #02	red -purple	+	-	+	4-5 : 13

Note: + = Positive result, - = Negative result,
N/A = Not Applicable, U = Unable to determine

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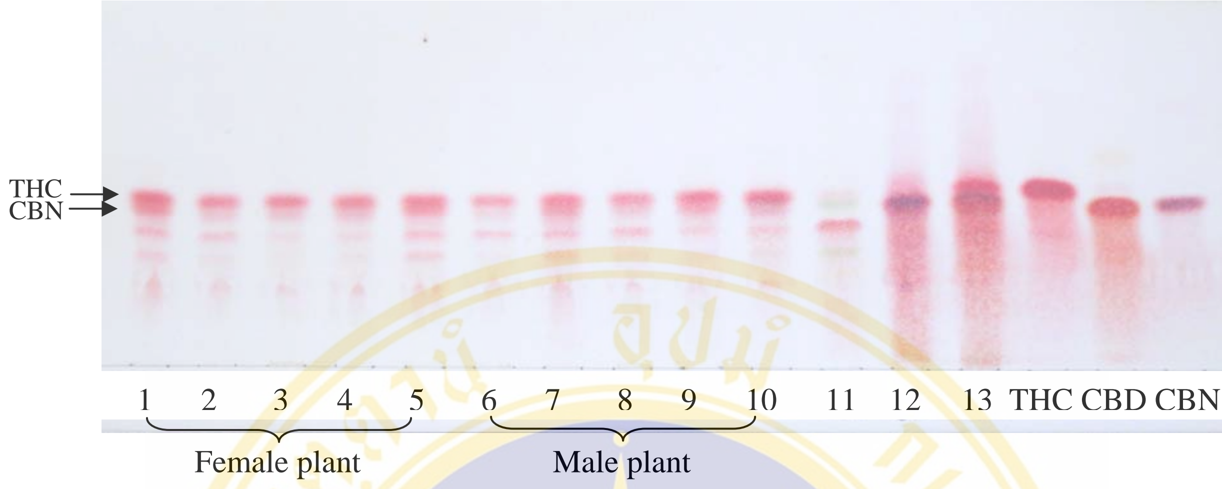

Figure 4-5. The TLC pattern of ten samples of dry drug-type; lane1-10, one sample of fresh drug-type; lane 11, two samples of seized cannabis; lane 12-13 as summarized in table 4.1 and three standards; THC, CBD and CBN.

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Table 4.3. The Fast Blue B salt test and TLC result of dry fiber-type grown by Maew hill tribe and dry fiber-type from QSBG located in the north of Thailand.

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Dry fiber-type: grown by the Maew hill tribe in the northern part of Thailand No. of plant tested = 1					
Female dry fiber-type #02	orange-red	+	+	-	4-6 : 2
Dry fiber-type: grown in the QSBG located in the northern part of Thailand No. of plant tested = 3					
Female dry fiber-type #01	red-purple	+	-	+	4-6 : 1
Female dry fiber-type #03	red-purple	+	-	+	4-6 : 3
Male dry fiber-type	red-purple	+	-	+	4-6 : 4

Note: + = Positive result, - = Negative result,
N/A = Not Applicable, U = Unable to determine

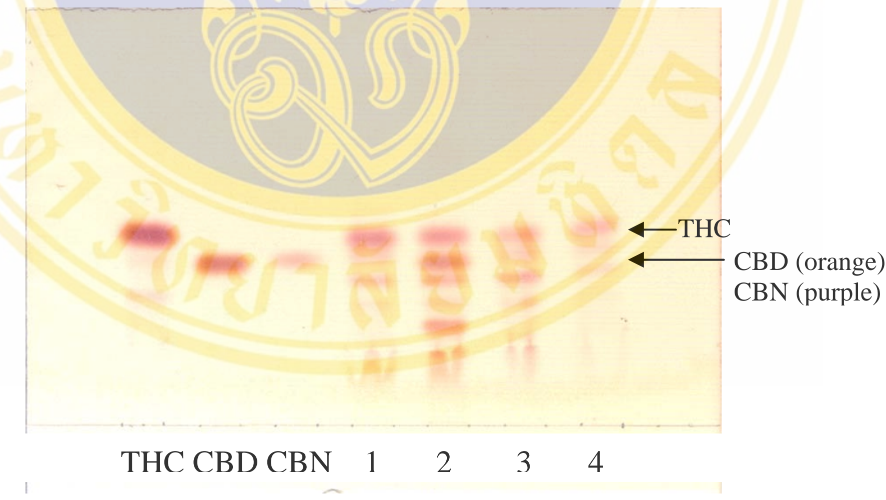

Figure 4-6. The TLC pattern of four dry fiber-type with three standards; THC, CBD and CBN. Lane 2 is a female plant sample grown by the Maew hill tribe. Lane 1 and 3 are the female plants grown in the QSBG. Lane 4 is a male plant grown in the QSBG

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Table 4.4. The Fast Blue B salt test and TLC results of fresh fiber-type from QSBG located in the north of Thailand and fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand.

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Fresh fiber-type: grown in the QSBG located in the northern part of Thailand No. of plant tested = 3					
Fresh fiber-type #02	N/A	+	+	-	4-7 : 1
Fresh fiber-type #14	N/A	+	+	-	4-7 : 2
Fresh fiber-type #15	N/A	+	-	+	4-7 : 3
Fresh fiber-type: grown from the seed colleted from the plant in northern part of Thailand No. of plant tested = 17					
Fresh fiber-type #03	orange-red	+	+	-	4-7 : 4

Note: + = Positive result, - = Negative result,
N/A = Not Applicable, U = Unable to determine

Figure 4-7. The TLC pattern of three samples of fresh fiber-type grown in the QSBG located in the northern part of Thailand; lane1-3, one sample of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand; lane 4 and three standards; THC, CBD and CBN.

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Table 4.5. The Fast Blue B salt test and TLC results of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand.

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Fresh fiber-type: grown from the seed colleted from the plant in northern part of Thailand No. of plant tested = 17					
Fresh fiber-type #16	orange-red	+	+	-	4-8 : 5
Fresh fiber-type #17	N/A	+	+	+	4-8 : 6
Fresh fiber-type #18	red-orange	+	+	-	4-8 : 7
Fresh fiber-type #19	red-orange	+	+	-	4-8 : 8
Fresh fiber-type #20	orange-red	+	+	-	4-8 : 9

Note: + = Positive result, - = Negative result,
N/A = Not Applicable, U = Unable to determine

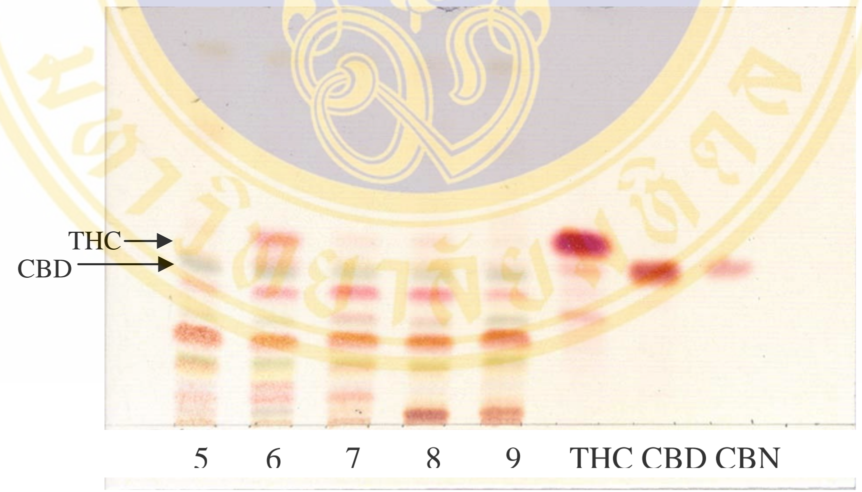

Figure 4-8. The TLC pattern of five samples of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand; lane 5-9 and three standards; THC, CBD and CBN.

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Table 4.6. The Fast Blue B salt test and TLC results of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand.

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Fresh fiber-type: grown from the seed colleted from the plant in northern part of Thailand No. of plant tested = 17					
Fresh fiber-type #21	red-purple	+	-	+	4-9 : 10
Fresh fiber-type #24	red-purple	+	-	+	4-9 : 11
Fresh fiber-type #25	red-purple	+	-	+	4-9 : 12
Fresh fiber-type #26	orange-red	+	+	-	4-9 : 13

Note: + = Positive result, - = Negative result,
N/A = Not Applicable, U = Unable to determine

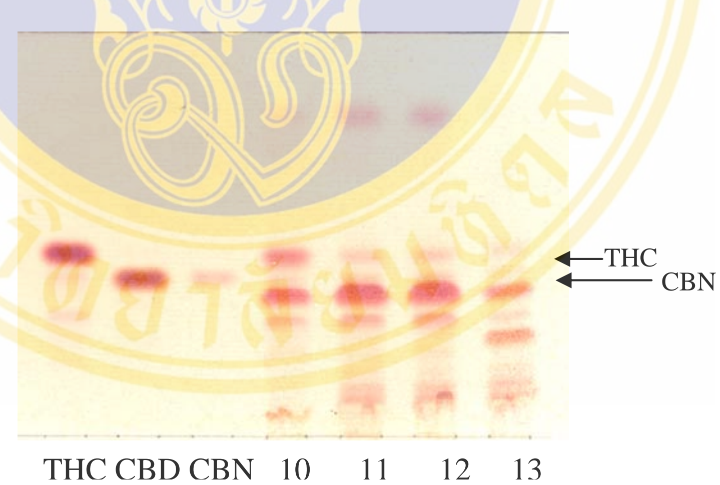

Figure 4-9. The TLC pattern of four samples of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand; lane 10-13 and three standards; THC, CBD and CBN.

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Table 4.7. The Fast Blue B salt test and TLC results of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand.

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Fresh fiber-type: grown from the seed colleted from the plant in northern part of Thailand No. of plant tested = 17					
Fresh fiber-type #27	red-orange	+	+	+	4-10 : 14
Fresh fiber-type #29	orange-red	+	+	-	4-10 : 15
Fresh fiber-type #30	red -purple	+	U	U	4-10 : 16
Fresh fiber-type #31	red-purple	+	-	-	4-10 : 17

Note: + = Positive result, - = Negative result,
N/A = Not Applicable, U = Unable to determine

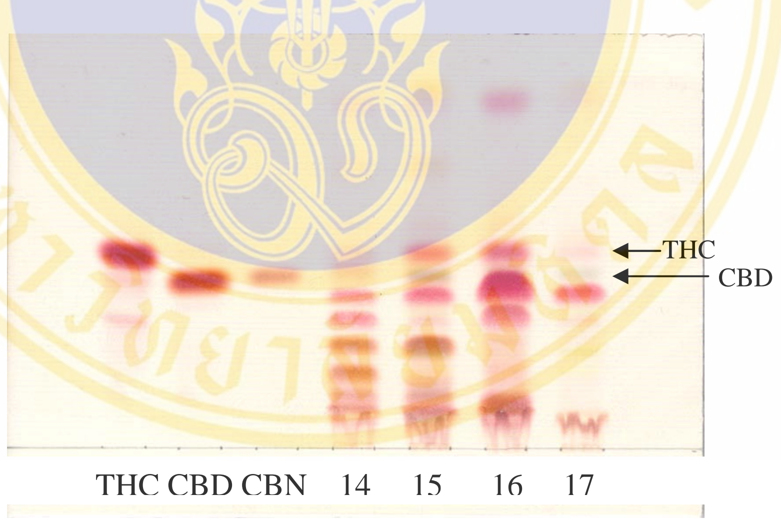

Figure 4-10. The TLC pattern of four samples of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand; lane 14-17 and three standards; THC, CBD and CBN.

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Table 4.8. The Fast Blue B salt test and TLC results of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand.

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Fresh fiber-type: grown from the seed colleted from the plant in northern part of Thailand No. of plant tested = 17					
Fresh fiber-type #32	red-purple	+	+	+	4-11 : 18
Fresh fiber-type #33	red -orange	+	+	-	4-11 : 19
Fresh fiber-type #34	orange- red	+	+	-	4-11 : 20

Note: + = Positive result, - = Negative result,
N/A = Not Applicable, U = Unable to determine

THC
CBD
THC CBD CBN 18 19 20

Figure 4-11. The TLC pattern of three samples of fresh fiber-type grown from the seed colleted from the plant in northern part of Thailand; lane 18-20 and three standards; THC, CBD and CBN.

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Table 4.9. The Fast Blue B salt test and TLC results of fresh fiber-type grown by Hmong hill tribe in the northern part of Thailand

Samples Description	Fast Blue B salt test	TLC band detected			Figure : Lane
		THC	CBD	CBN	
Fresh fiber-type: grown by Hmong hill tribe in the northern part of Thailand No. of plant tested = 4					
Fresh fiber-type #35	orange-red	+	+	+	4-12 : 21
Fresh fiber-type #37	orange-red	+	+	-	4-12 : 22

Note: + = Positive result, - = Negative result,

N/A = Not Applicable, U = Unable to determine

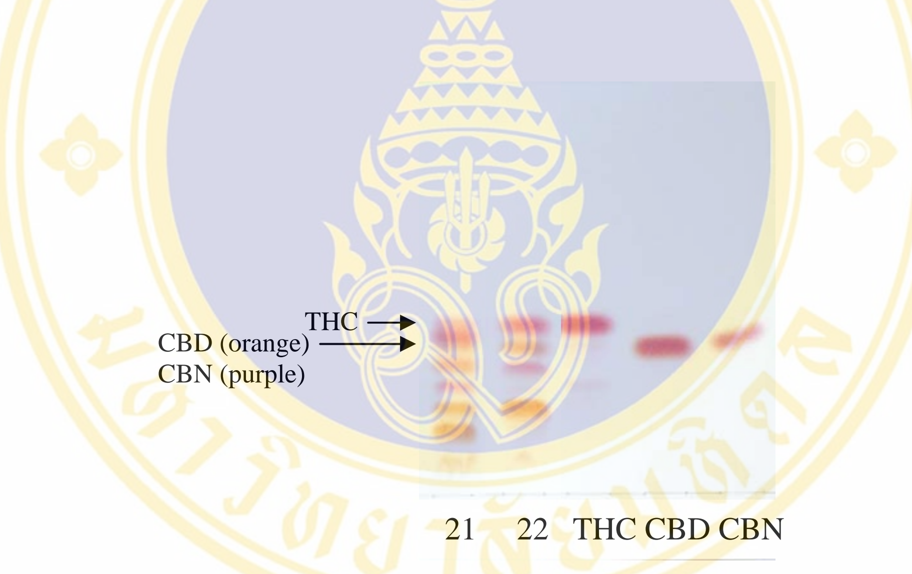

Figure 4-12. The TLC pattern of two samples of fresh fiber-type grown by Hmong hill tribe in the northern part of Thailand; lane 21 and 22 and three standards; THC, CBD and CBN.

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4.3.3 DNA detection using Drug-type Marker (g and h)

DNA detection using Drug-type Marker was performed on 16 samples of *Cannabis sativa* collected from various sources as summarized in table 4.10. The samples were identified using morphology by ONCB staff prior of the test as described in the sample description column. An approximately 1.2 kb-PCR product was detected in 14 samples which indicate the drug-type strain of these samples (8). The DNA results using Drug-type Marker and positive control are shown in figure 4-13 and 4-14, respectively.

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Table 4-10. The DNA results of sixteen samples amplified using Drug-type Marker (g and h) and universal primers (C and D) as positive control.

Sample Description	Detected PCR products using			Remarks
	Drug-type Marker (g and h)	Cannabis sativa-specific (G and H)	Universal primers (C and D)	
Fresh drug-type: Illegally grown in the southern part of Thailand No. of plant tested = 7				
Fresh drug-type CS_1	+	+	+	Lane 1
Fresh drug-type CS_2	+	+	+	Lane 2
Fresh drug-type #01	+	+	+	Lane 3
Fresh drug-type #02	+	+	+	Lane 4
Fresh drug-type #03	+	+	+	Lane 5
Fresh drug-type #04	+	+	+	Lane 6
Fresh drug-type #05	+	+	+	Lane 7
Fresh fiber-type: grown in the QSBG located in the northern part of Thailand No. of plant tested = 4				
Fresh fiber-type 1_2	+	+	+	Lane 8
Fresh fiber-type #02	-	-	-	Lane 9
Fresh fiber-type #14	+	+	+	Lane 14
Fresh fiber-type #15	-	-	-	Lane 13
Fresh fiber-type: grown from the seed colleted from the plant in northern part of Thailand No. of plant tested = 3				
Fresh fiber-type #03	+	+	+	Lane 10
Fresh fiber-type #04	+	+	+	Lane 11
Fresh fiber-type #05	+	+	+	Lane 12
Fresh fiber-type: grown by Hmong hill tribe in the northern part of Thailand No. of plant tested = 4				
Fresh fiber-type #35	+	+	+	Lane 15
Fresh fiber-type #37	+	+	+	Lane 16

Total sample tested = 16

Note: + = positive result, - = negative result

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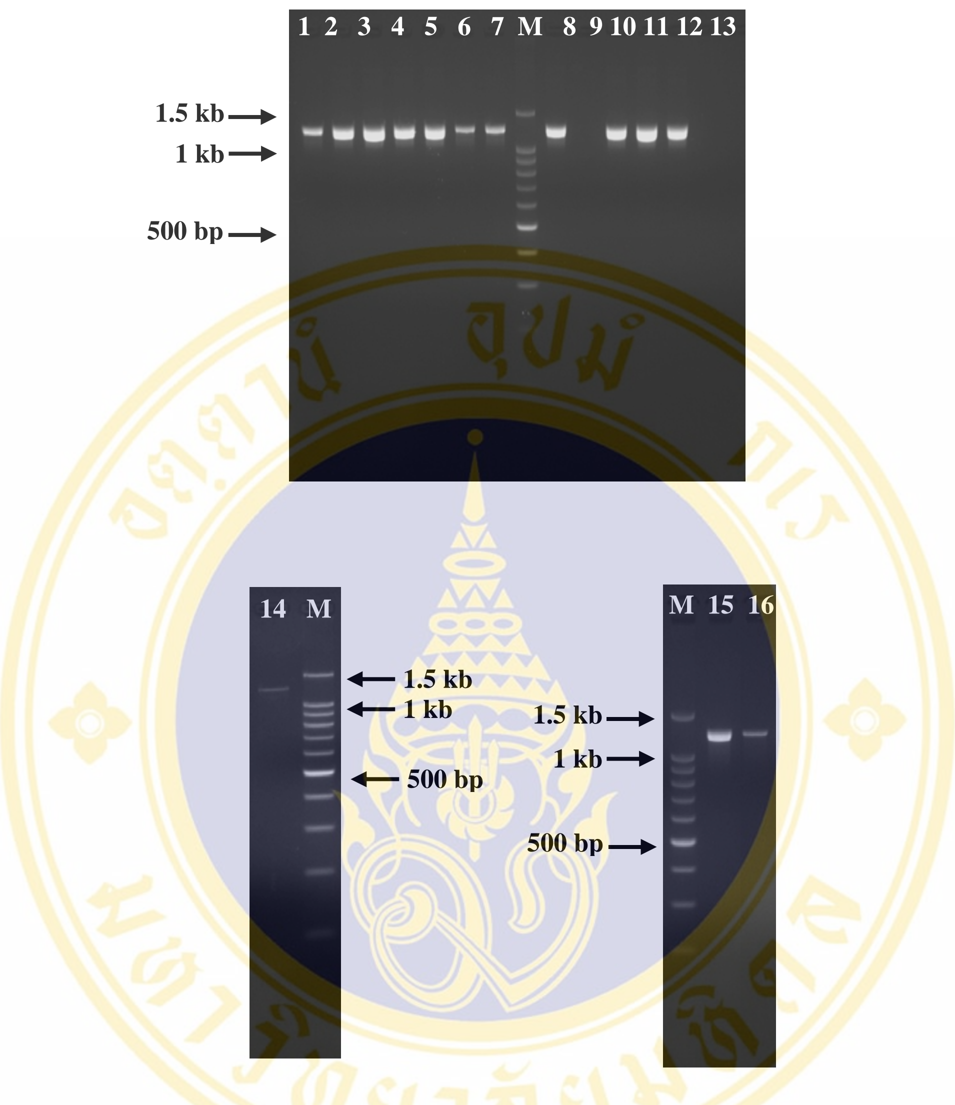

Figure 4-13. The PCR product on the 2% (w/v) agarose gel amplified by Drug-type Marker (g and h) of sixteen samples from various sources as summarized in table 4-10.

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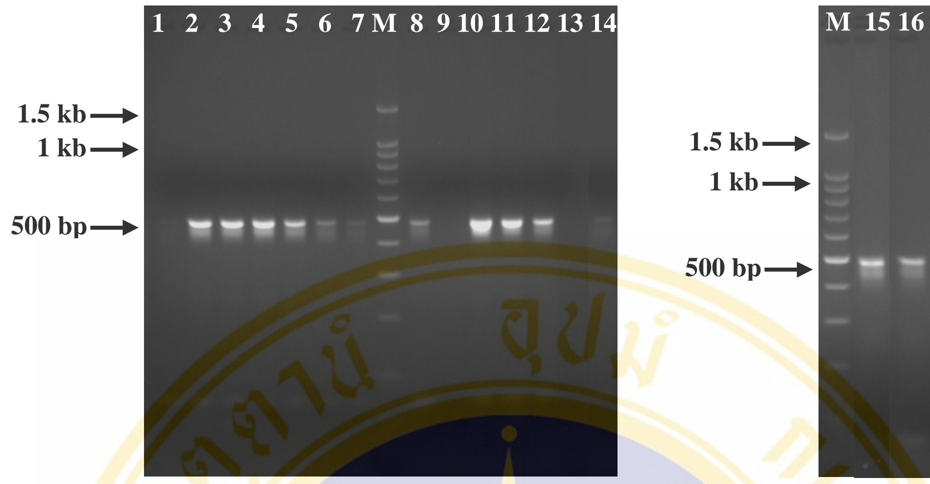

Figure 4-14. The PCR product on the 2% (w/v) agarose gel amplified by universal primers (C and D) as positive control of sixteen samples from various sources as summarized in table 4-10.

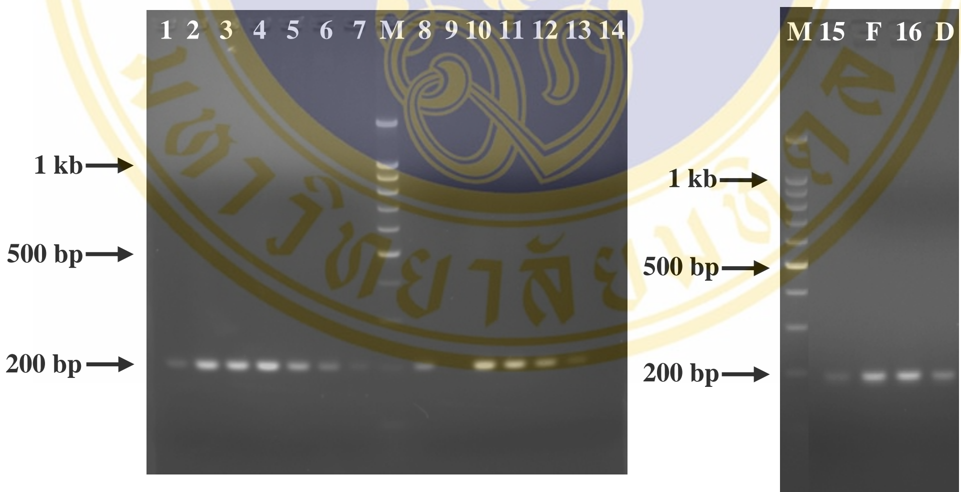

Figure 4-15. The PCR product on the 2% (w/v) agarose gel amplified by *Cannabis sativa*-specific primer (G and H) of drug-type (D), fiber-type (F) and sixteen samples from various sources of Thailand.

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4.4 Determination of intraspecific variation between drug-type and fiber-type strain

Noncoding sequences tend to evolve faster than coding sequences and the *trn*L-*trn*F intergenic spacer (IGS) is known to evolve faster than the other chloroplast loci and are often used to study relationships within species (41), (42). *Cannabis sativa*-specific primer (G and H) according to Linacre and Thorpe which amplified the IGS between *trn*L and *trn*F gene in the chloroplast (7) was used in the present study. Eighteen samples of *Cannabis sativa* including the authentic drug-type and fiber-type were analyzed. Both authentic drug-type and fiber-type samples showed an approximately 200 bp-PCR products. Sixteen samples collected from various sources also gave the same result as shown in figure 4-15.

The whole sequence of intergenic spacer between *trn*L and *trn*F gene was amplified using universal primers C and F. The PCR products were submitted for sequencing to investigate sequence variations between drug-type and fiber-type strain. The sequences of drug and fiber-types have 100% homology. The sequences are shown in the appendix.

In addition, according to Kohjyouma et al., the *trn*L-*trn*F IGS sequences of these drug-type and fiber-type samples were classified as “Type 2” and correlated to Kohjyouma’s study which found that the difference between the 2 chemotypes was not detected from the *trn*L-*trn*F IGS sequences (43).

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CHAPTER V
DISCUSSIONS

The strain of authentic drug-type or fiber-type was confirmed by the DNA method using Drug-type Marker according to Kojoma *et al.*(8). The TLC of these two authentic samples were different. Based on this study, the drug-type showed a dark band of both THC and CBD with no band of CBN; whereas, fiber-type showed a light band of THC and a dark band of CBD with no band of CBN. These patterns correlated to the concept described by Paris and Nahas (1984) which considered major cannabinoids contents of drug-type to contain THC (>1% of dry weight) and no CBD, on the other hand, fiber-type to contain higher content of CBD (>0.50% of dry weight) and lower content of THC (<0.25% of dry weight) (9). In addition, no orange band was presence in the lower Rf region (0.20-0.25) of drug-type compare to the chromatogram of fiber-type. This orange band is expected to be an indication of CBDA, a precursor of CBD in fiber-type. Therefore, this unique characteristic TLC chromatogram can be used to distinguish drug-type from fiber-type.

Fast Blue B salt test and TLC had been performed on a large number of samples. Twenty one samples yield red to purple color; whereas, 13 samples yields orange to red color. By comparing the results of both tests, it generally provides evidence of major cannabinoids content correlated to each other. Therefore, color test can be used not only as presumptive test to indicate the presence of *Cannabis* but also can be used as field test for roughly grouping the type of plant sample before subjecting the sample to be confirmed by other identification such as chromatography or DNA.

The selection of solvent for the extraction is also important. Petroleum ether gives clean extract of only neutral cannabinoids; THC, CBD and CBN, it was used as extracting solvent in color test; whereas, acetone will extract neutral cannabinoids and cannabinoid acids; THCA and CBDA (40). To increase the polarity of TLC solvent system, 10 percent of methanol was added. This system had an advantage of giving a band of cannabinoids acids (THCA and CBDA) in the Rf range of 0.20-0.25; whereas,

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others two systems did not (40). Among the three solvent systems used in this study, the system consisting of N-hexane, Dioxane and Methanol at a ratio of 70:20:10 is suitable for discriminating drug-type and fiber-type *Cannabis*. However, multiple developments can be alternatively used for a better of cannabinoids.

According to the TLC results, dry drug-type samples (see figure.4-5; lane 1-10), which had been dried in hot air oven (40-50 °C), gave a clear evidence of THC as expected since THC is derived from a non-enzymatic decarboxylation of THCA which is considerably accelerated by the high temperatures or smoking. CBN is also present in this group of samples because it was a by-product of THC which formed during improper storage of the plant (9). On the other hand, fresh drug-type sample gave only a light band of THC with no band of CBD and CBN because it had been dried only a short period of time before extracting with acetone. Therefore, THC content was less than those in dry samples.

Two seized marijuana gave a different chromatographic patterns (see figure.4-5; lane 12-13). These herbal products derived from the flowering tops of female plant and had been dried and subsequently processed to make dried material as described in chapter II. Good quality marijuana should contain high content of THC which would depend on the plant materials and the production. In the present study, one of the seized marijuana contained high content of by-product CBN instead of psychoactive THC. In this case, an argument may arise since the regulation on illegal *Cannabis* products considers only the content of psychoactive THC as the criteria for illegal *Cannabis* detection not the by-product CBN. In forensic application, this issue should be concerned and clearly justified.

In dry fiber-type, one sample which grown by Maew hill tribe (see figure.4-6; lane 2) showed a comparable distinctive THC and CBD band and orange band in lower Rf (0.25) indicating the characteristics of intermediate drug-type. Unfortunately, DNA of this sample and all dry samples were too degraded due to the dry process and storage causing an unsuccessful DNA amplification. Others dry fiber-type samples which give a dark band of THC and CBN similar to the drug-type pattern indicates the

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characteristics of being drug-type rather than fiber-type. All dry fiber-type from QSBG gave band characteristics of drug-type.

In the group of fresh fiber-type, the samples grown from the seed showed various TLC patterns. Most of them showed band characteristics of fiber-type as indicate by the CBD band and the orange band in lower Rf region (0.20-0.25). Some of these samples showed characteristics of intermediate drug-type and the DNA analysis of the selected samples gave positive results to the Drug-type Marker which provides an evidence of the samples being drug-type or intermediate drug-type not fiber-type strain (see figure.4-6 to 4-11).

Another group of fresh fiber-type sample grown by Hmong hill tribe in the northern part of Thailand showed band characteristics of intermediate drug-type (see figure.4-12; lane 21, 22). These samples also gave positive results with Drug-type Marker.

In the present study, all the fiber-type samples that were tested by DNA gave positive result for Drug-type Marker (see figure.4-13). As for TLC, none of the fiber-type samples showed a distinct band of CBD similar to the authentic fiber-type. On the other hand, 8 of theses fiber-type samples gave a band pattern similar to drug-type; whereas, others remaining fiber-type samples gave an equal band intensity of both THC and CBD which can be considered as an intermediate drug-type characteristics. These provided the information that most of the samples which were previously classified as fiber-type were actually drug-type or intermediate drug-type rather than fiber-type. The probable cause of this situation of misclassification into fiber-type may due to variation of the *Cannabis* strain. *Cannabis sativa* is known to extensively spread worldwide and differentiated into many local variations which are difficult to distinguish by their morphology. These variations may spontaneously occur in nature by cross pollination between drug-type and fiber-type plant. Since drug-type *Cannabis sativa* has been widely cultivated in Thailand by the villagers for a long time before the encouragement of fiber-type cultivation to solve the narcotic problem, it can be cross pollinated with the near-by cultivated fiber-type, resulted in the intermediate drug-type strain progeny. Other factors which involved in the formation of plant

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constituents for instance; growth period of the plant, sex, condition during sample collection, storage before sample analysis, etc. may also play role in this chemical variation. However, more samples are needed for the accurate identification by DNA and would give more information about the strain type of these samples.

The results of DNA amplification by the *Cannabis sativa*-specific primer (C, D, G and H) showed that all the samples including the authentic drug-type and authentic fiber-type gave positive result for this marker (see figure.4-14,4-15). The results indicated that the *Cannabis sativa*-specific primer (C, D, G and H) which amplified the intergenic spacer between *trn*L and *trn*F gene in chloroplast does not have the ability of discriminating the drug-type from fiber-type plant because of their low intraspecific variation within the species of *Cannabis sativa*.

The three identification methods, color test, TLC and DNA has its own advantages and disadvantages and are suitable in different situation as summarized in Table 5-1.

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Table 5-1. Advantages, disadvantages and suitable usage of three test method; Fast Blue B salt test, TLC and DNA including the type of sample that can be tested by these methods.

Method	Type of sample	Advantages	Disadvantages	Suitable Usage
Fast Blue B salt test	-freshly dried sample -old dried sample -require sample not lager than a match head	-easy to perform -practical in the field -cheap -take only a few minutes to obtain result	-false positive can be obtained -result is subjective -require other test for confirmatory	-presumptive test which can be performed in the field for roughly indication of sample
TLC (chemical-based method)	-freshly dried sample -old dried sample -require sample in mg level	-cheaper than other chromatography techniques -does not require a fully equipped lab -can be used both qualitatively and semiquatitatively	-require standard sample -require experience in performing the test	-confirmatory test for suspected material
DNA (biological-based method)	-fresh sample -dried sample which is not DNA degraded -require 100 mg of fresh sample and 20 mg of dried sample	-suitable for cannabis detection and type discrimination since it detect at the molecular level -can be used to detect any type of sample that contain *Cannabis* DNA -the test can be further used for linkage analysis	-require fully equipped lab -unsuccessful to perform on DNA degraded sample	-confirmatory test for suspected material

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CHAPTER VI
CONCLUSIONS AND SUGGESTIONS

The three types of *Cannabis*; drug-type, intermediate-drug type, and fiber-type, can be distinguished by using both chemical and biological test.

In this study, we demonstrated that the drug-type DNA marker designed from the THCA synthase gene by Kojoma *et. al*., was suitable for discriminating the types of *Cannabis*. The 1.2-kb PCR product was amplified from only drug-type samples. Since the 200-bp PCR product was detected in all *Cannabis* samples PCR-amplified by the *Cannabis sativa*-specific primers (G and H), but not other kinds of plants, it is then suggested that this primer pair can be use to detect for the presence of *Cannabis*. Therefore, in order to make an accurate identification of the suspected *Cannabis* materials, we suggest the use of these two molecular markers together.

The Fast Blue B salt test can provide a presumptive indication of the *Cannabis* types. Due to the chemical composition of a sample, drug-type would yield a red to purple-red color whereas intermediate drug-type and fiber-type would yield an orange to red-orange color. Because this test is easy to perform and inexpensive, it can be used as presumptive test in the field to roughly indicate type of suspected plants or materials. In case that the result is positive for drug-type, it can provide an evidence for the officer to subsequently detain the samples before subjecting them to the laboratory for confirmation. However, the intermediate drug-type and fiber-type will give the same color shade for the result. Hence, a confirmatory test will be subsequently done to exclude the innocent fiber-type. To increase the accuracy of this test, a known combination of standard compounds or a color chart should be established as reference for help comparing the color result of each type.

TLC analysis of the *Cannabis* samples showed that the chromatogram patterns can be used as a fingerprint to identify the types of *Cannabis*. The TLC solvent system that was suitable for discriminating drug-type and fiber-type *Cannabis* consists of N-hexane, Dioxane and Methanol at a ratio of 70:20:10. In drug-type, a dark band of THC and/or CBN was detected. No CBD band was present. In fiber-type it was

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opposite, a dark CBD band and an orange band at Rf value approximately 0.20 , which is suspected to be CBDA, was clearly detected. The THC band presented was very light. For the intermediate drug-type, both THC and CBD bands were presented in comparable concentrations. An orange band and CBN band can be detected in the intermediate drug-type group as well.

We have also demonstrated that the DNA result using drug-type marker correlate to TLC results in 5 *Cannabis* samples. This also provided the evidence for misclassification of types, in which several drug-type and intermediate drug-type samples were categorized into fiber-type.

To detect and discriminate *Cannabis*, Fast Blue B salt can be preliminary used as field test followed by the confirmatory test, TLC or DNA analysis. TLC, which is chemical-based method, is a cheap and can be use as a qualitative and semiquantitative forensic tool. Although the DNA-based method is more expensive and requires a fully equipped laboratory, DNA based-method is more robust. DNA analysis can be use to detect, discriminate and identify the type of *Cannabis* in any sample that contained non-degraded *Cannabis* DNA. For the old *Cannabis* sample, which DNA may be degraded, TLC is more suitable to use for detection and discrimination. In addition, we suggest that DNA test can be further used as a pre-cultivation screening test to confirm the *Cannabis* types before cultivation. Moreover, the DNA samples remain can be further used in the linkage analysis for linking the plant to their parent which can provide the information of the plant strain and the source of cultivation.

The forensic application of the three methods, Fast Blue B salt test, TLC and DNA will serve as tools for authority to detect and discriminate types of *Cannabis* materials. Under circumstances that hemp cultivation will be promoted, we believe that these three analysis methods together not only would play an important role in the prevention of wrongful fiber cultivation, but also the misidentification of the plant type.

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APPENDIX

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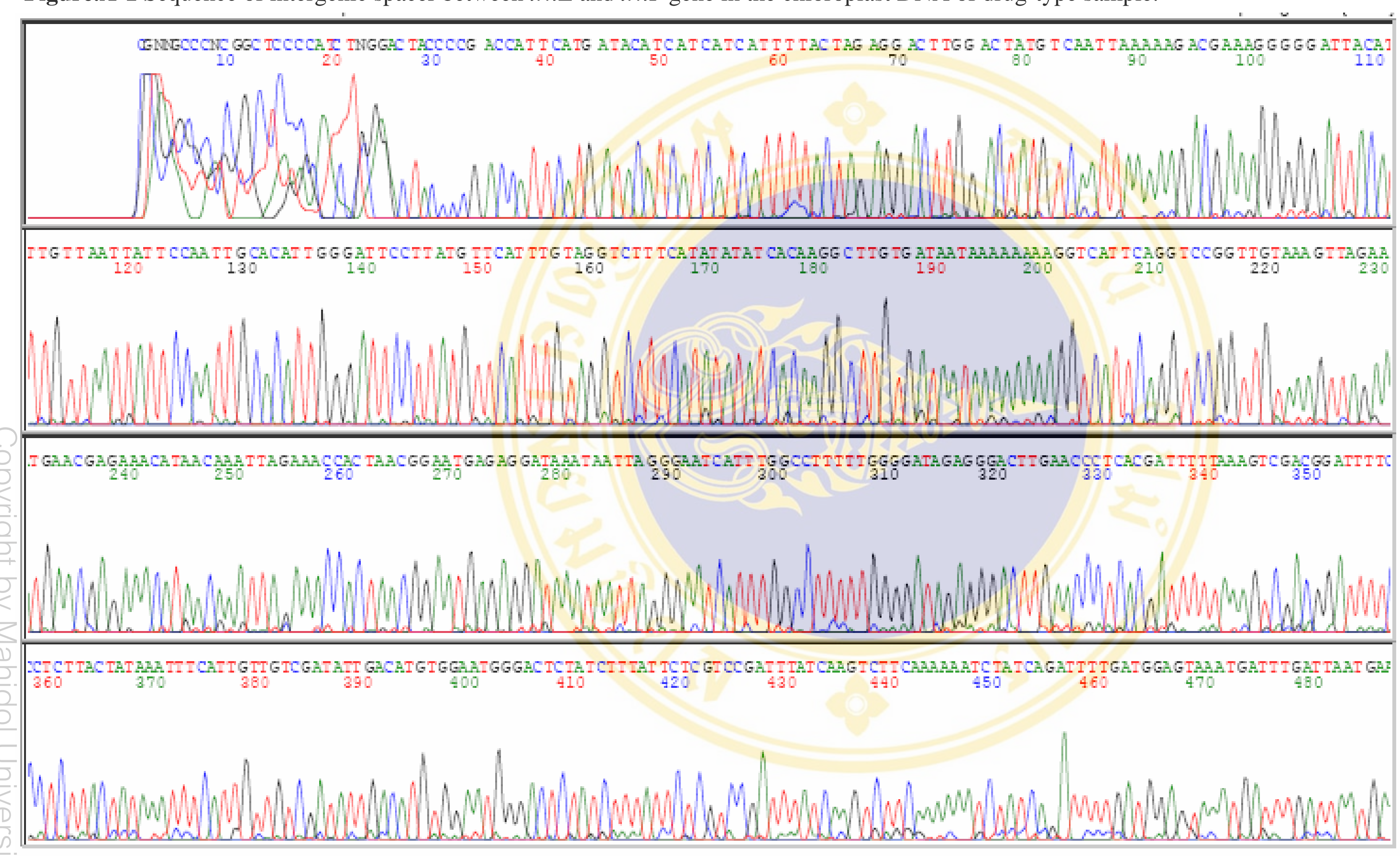
APPENDIX

Figure.A-1 Sequence of intergenic spacer between *trn*L and *trn*F gene in the chloroplast DNA of drug-type sample.



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APPENDIX

Figure.A-1 (Cont.) Sequence of intergenic spacer between *trn*L and *trn*F gene in the chloroplast DNA of drug-type sample.

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APPENDIX

Figure.A-2 Sequence of intergenic spacer between *trn*L and *trn*F gene in the chloroplast DNA of fiber-type sample.

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APPENDIX

Figure.A-2 (Cont.) Sequence of intergenic spacer between *trn*L and *trn*F gene in the chloroplast DNA of fiber-type sample.

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