# SURVEY OF *Cannabis sativa* L. FIBER TYPE CULTIVATED IN THAILAND.

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**A THESIS SUBMITTED IN PARTIAL FULFILLMENT**
**OF THE REQUIREMENTS FOR THE DEGREE**
**OF MASTER OF SCIENCE (FORENSIC SCIENCE)**
**FACULTY OF GRADUATE STUDIES**
**MAHIDOL UNIVERSITY**
**2007**

Thesis

Entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University

for the degree of Master of Science (Forensic Science)

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## ACKNOWLEDGEMENTS

I would like to gratefully thank Prof. Dr. Nuntavan Bunyapraphatsara and Dr. Nathinee Panvisavas, my major advisor and co-advisor, for their suggestion, patience, kindness and all support to help me complete the thesis.

I would like to express my appreciation to Assoc. Prof. Dr. Prapin Wilairat from National Doping Control Center, Mahidol University for his kind advice, suggestion and support about GC-MS analysis and for his the precious time to chair the thesis defending.

I would like to thanks Mr. Pipop Chamnivikaipong, the Director of Narcotic Crops Survey and Monitoring Institute and all ONCB staff for collecting the samples and taking care of me during the time in Chiangmai and Tak provinces.

I would like to gratefully thank Assoc. Prof. Dr. Ekarin Saifah, from Faculty of Pharmaceutical Science for his precious time to be the external examiner in my thesis defending committee.

I would like to thanks the scientists from CIF, Department of Chemistry and Faculty of Pharmacy for their help in analysis, advice and laboratory training.

I also wish to thank all forensic staffs, forensic friends for their friendship, help and support me, also listen to my problem and understanding.

Finally, I would like to thank my beloved parents, family and all my love to give me the heart, stand beside me and make me be happy. This love will help me to be strong and stay alive.

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SURVEY OF *CANNABIS SATIVA* L. FIBER TYPE CULTIVATED IN THAILAND

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## ABSTRACT

*Cannabis sativa* L. fiber type was collected from various cultivation areas in Tak and Chiangmai provinces in order to survey the cultivation situation in the campaign of commercial cultivation. The identification using color test, GC-MS and DNA analysis indicated only 3 of 58 samples were fiber type. These samples gave a negative result for drug-type marker which agreed with $\Delta^9$-THC <0.3%, phenotypic index $\frac{\%\Delta^9\text{-THC} + \%\,\text{CBN}}{\%\,\text{CBD}}$ and index ratio $\frac{\text{THC}}{\text{CBD}}$ less than 1. Tests on other samples tended to show that they were drug type. Furthermore, TLC chromatograms of these 3 samples showed the orange band (Rf.0.2) which may indicate CBDA, the precursor of CBD, intense CBD band and light band of THC while other samples gave the intense band of THC and CBD and/or CBN and also the extra orange band which may be classified as intermediate type. On the other hand, in field tests Fast blue B salt showed that the 3 samples of fiber type gave orange to orange–red color while a combination of colors from orange-red to purple were observed in other samples.

This survey indicates that *Cannabis* plants which are cultivated for fiber in Thailand tends to be drug type. Thus new pure fiber type seeds should be carefully identified before being distributed to the farmers and the cultivation areas should be controlled.

KEY WORDS: FORENSIC / *CANNABIS SATIVA* L. /CANNABINOID

76 pp.

การสำรวจการปลูกกัญชงในประเทศไทย (SURVEY OF *CANNABIS SATIVA* L. FIBER TYPE CULTIVATED IN THAILAND)

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บทคัดย่อ

การศึกษานี้ได้สำรวจสถานการณ์การปลูกกัญชงเพื่อเป็นแนวทางในการส่งเสริมการปลูกเป็นพืชเศรษฐกิจ โดยการสุ่มเก็บตัวอย่างจากแหล่งปลูกใหญ่ในจังหวัดตากและเชียงใหม่ และใช้วิธีการ color test, TLC, GC-MS และ DNA ในการตรวจสอบนั้น พบว่า จาก 58 ตัวอย่าง มีเพียง 3 ตัวอย่างเท่านั้น ที่ให้ผลการตรวจทุกวิธีว่าเป็นกัญชง โดยทั้ง 3 ตัวอย่าง ให้ผลลบจากการติดเครื่องหมายโมเลกุลพืชเสพติดซึ่งสอดคล้องกับกับปริมาณสารออกฤทธิ์เมื่อตรวจโดย GC-MS โดยพบว่าทั้ง 3 ตัวอย่าง นี้มี $\%\Delta^9$ –THC <0.3% และ Phenotypic index $\frac{\%\Delta^9\text{-THC} + \%\,\text{CBN}}{\%\,\text{CBD}}$ และ index ratio $\frac{\text{THC}}{\text{CBD}}$ น้อยกว่า1 ซึ่งระบุว่าเป็นกัญชง ในขณะที่ตัวอย่างอื่นมีแนวโน้มเป็นพืชเสพติด นอกจากนี้ยังพบอีกว่ากัญชงทั้ง 3 ตัวอย่าง ให้แถบสีส้มที่คาดว่าน่าจะเป็นสาร CBDA ที่เป็นสารตั้งต้นของสาร CBD ที่ Rf.0.2 เมื่อตรวจด้วย TLC และยังพบแถบเข้มของสาร CBD และแถบจางของ THC ด้วย ในขณะที่ตัวอย่างอื่นจะพบแถบเข้มของ THC และ CBD และบางตัวอย่างยังพบแถบของ CBN แถบส้มดังกล่าวยังพบในบางตัวอย่าง ซึ่งอาจจัดอยู่ในกลุ่มของพืชกึ่งเสพติด และเมื่อตรวจโดยการทำปฏิกิริยาการเกิดสีกับ Fast blue B salt ยังพบว่าทั้ง 3 ตัวอย่างยังให้สีส้มถึงส้มแดง ซึ่งเป็นสีของ CBD และ CBDA ในขณะที่ตัวอย่างอื่นให้สีผสมตั้งแต่ส้มแดง ถึง ม่วง

จากการสำรวจทำให้ทราบว่ากัญชงที่ปลูกในประเทศไทยมีแนวโน้มเป็นพืชเสพติด หากจะมีการส่งเสริม ควรมีการคัดเลือกและควบคุมสายพันธุ์ ตลอดจนพื้นที่การปลูก เพื่อป้องกันการแพร่กระจายของพืชเสพติดในประเทศไทย

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# LIST OF ABBREVIATIONS

| Abbreviation | Term |
|---|---|
| bp | Base pair |
| CBD | Cannabidiol |
| CBN | Cannabinol |
| cm | Centimeter |
| $^{\circ}C$ | Degree Celsius |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| g | Gram |
| GC-MS | Gas chromatography-mass spectrometry |
| HPLC | High Performance Liquid Chromatography |
| kb | Kilo base pairs |
| $MgCl_2$ | Magnesium chloride |
| µl | Microliter |
| mg | Milligram |
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimolar |
| min | Minute |
| m/z | Mass per charge |
| ng | Nanogram |
| ONCB | Office of Narcotics Control Board |
| THC | $\Delta^9$-tetrahydrocannabinol |
| TLC | Thin layer chromatography |
| pmole | Picomole |
| QSBG | Queen sirikit Botanic Garden |

# CHAPTER I
# INTRODUCTION

## 1.1 Introduction

*Cannabis sativa* L. (Cannabaceae) is a plant widely distributed throughout the temperate and tropical zones of the world and its cultivation is prohibited in most countries (1) including "the golden triangle" area where Thailand, Burma and Laos join constitutes. Although marijuana flourishes in the surrounding areas, high quality Thai marijuana does not necessarily come from Thailand. *Thai* or *Thai sticks* has become synonymous with marijuana originated from Southeast Asia but may have very well grown in Cambodia or Vietnam (2).

*Cannabis* has been a source of fiber, food, oil, medicine, and inebriant since prehistoric time (3). *Cannabis sativa* L. is classified as drug and fiber types based on morphological and chemical characteristics.

The drug type is cultivated illicitly and known as marijuana which is referred to the leaves and flowering tops of cannabis plants. It has been widely used among the young people for hallucination effect.

Marijuana contains carcinogens that are stored in fat cells for several months. Marijuana users experience the same health problems as tobacco smokers, such as bronchitis, emphysema, and bronchial asthma, increase heart rate, dryness of mouth, reddening of the eyes, impaired motor skill and concentration, and frequent hunger and an increased desire for sweet, as well as suppression the immune system are also included. Occasionally, hallucinations, fantasies, and paranoia are reported (2).

Another type of *Cannabis sativa* L. is commercially grown for fiber and used in the production of cloth and rope, edible seed oil, essential oil, cosmetic, lubricant and food. This type consists predominantly of stalks with only small amount of foliage present compared with the drug type (4).

In Thailand, The Royal Project Foundation of Thailand has realized the enormous (5-7) usage of fiber type and subsequently started to promote the commercial cultivation. The Office of the Narcotics Control Board (ONCB) which is the authority in drug control concerns that it may lead to expansion of drug type. Therefore it is needed to develop the appropriate method to identify the samples collected from the cultivated areas in order to control the cultivation of fiber type *Cannabis*.

Preliminary survey (5) found that the claimed fiber type contained high THC content which may be due to the cross pollination between two types. It is the purpose of this study to survey the cultivation situation of fiber type in order to set up the controlled plan before large scale production will be promoted.

## 1.2 Objective

To survey, qualitative and quantitative analysis of fiber type *Cannabis sativa* L. cultivated in Thailand by using chromatographic and DNA techniques.

# CHAPTER II
# LITERATURE REVIEW

## 2.1 The Genus Cannabis

### 2.1.1 Introduction

*Cannabis* (Cannabaceae) has been a source of fiber, food, oil, medicine, and inebriant since prehistoric times (3). *Cannabis* is an annual of flowering herb, usually dioecious. The leaves are palmately compound, with serrate leaflets. The first pair of leaves usually has a single leaflet, the number gradually increasing up to a maximum of about thirteen leaflets per leaf (usually seven or nine), depending on variety and growing conditions. *Cannabis* usually has imperfect flowers with staminate "male" and pistillate "female" flowers occurring on separate plants, although hermaphroditic flowers sometimes occur (8).

The plant is believed to have the habitat in the mountainous regions just north west of the Himalayas. Nowadays, this plant widely distributed throughout the temperate and tropical zones of the world and its cultivation is prohibited in most countries (1). *Cannabis sativa* has many common names: hemp, marijuana, bhang, ganja, hashish, etc (2). Hemp is usually referred to the varieties of Cannabis cultivated for non-drug use. *Cannabis* can produce a group of chemicals called cannabinoids which produce mental and physical effects when consumed. As a drug it usually comes in the form of dried leaves (marijuana), resin (hashish), or various extracts collectively known as hash oil. In the early $20^{th}$ century, it became illegal in most of the world to cultivate or possess *Cannabis* for drug purposes (8).

### 2.1.2 Taxonomy

Many taxonomists tried to delimit *Cannabis* taxa by using various characteristics such as morphology, inebriant ability, chemical component and genetic variation (3). Linnaeus, in 1753 recognized only *C. sativa* L. In 1785, the famous French biologist Lamarck described a putatively distinctive second species of *Cannabis, C. indica*(9). From chemotaxonomy and morphology, Small and Cronquist put *C. indica* to the rank of subspecies; *C. sativa* subsp. *sativa* and *C. sativa* subsp. *indica* (Lam.)(9). Moreover, many researches have proposed several species of the genus, of these, only *C. ruderalis* Janisch. is commonly accepted (3).

Small (1979) considered the amount of THC produced by *Cannabis* to be an ‘‘extremely important’’ taxonomic character and used gas chromatography (GC) to differentiate *indica* subspecies from *sativa* subspecies on the basis of their THC content(3).


**(a)** **(b)**

**Figure 2-1** a, b Morphological characteristic of *Cannabis* plant.

### 2.1.3 Morphology

*Cannabis sativa* is an annual wind-pollinated plant, normally dioecious and dimorphic, although sometimes monoecious (mostly in several modern European fiber cultivars). Some special hybrids, obtained by pollinating females of dioecious lines with pollen from monoecious plants, are predominantly female (so-called “all-female,” these generally also produce some hermaphrodites and occasional males). All-female lines are productive for some purposes (e.g. they are very uniform, and

with very few males to take up space they can produce considerable grain), but the hybrid seeds are expensive to produce. Staminate or "male" plants tend to be 10%–15% taller and less robust than the pistillate or "female". An isolation distance of about 5 km. is usually recommended for generating pure-bred foundation seed. A "perigonal bract" subtends each female flower, and grows to envelop the fruit. While small, secretory, resin-producing glands occur on the epidermis of most of the above-ground parts of the plant, the glands are very dense and productive on the perigonal bracts, which are accordingly of central interest in marijuana varieties. The root is a laterally branched taproot, generally 30–60 cm deep, up to 2.5 m in loose soils, very near the surface and more branched in wet soils. Extensive root systems are key to the ability of hemp crops to exploit deep supplies of nutrients and water. The stems are erect, furrowed, and usually branched, with a woody interior, and may be hollow in the internodes. Although the stem is often woody, the species is frequently referred to an herb or forb.

Plants vary enormously in height depending on genetic constitution and environment, but are typically 1–5 m. (heights of 12 m. or more in cultivation have been claimed)(10, 11).

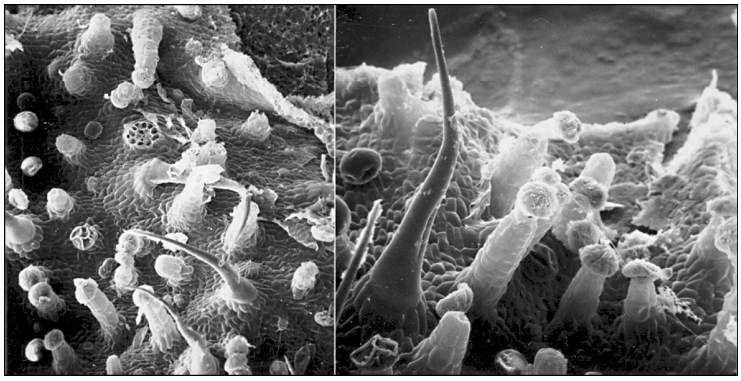


**Figure 2-2** Scanning electron micrographs of the abaxial surface of a perigonal bract (intoxicating part of the plant), and may contain 20% THC dry weight.

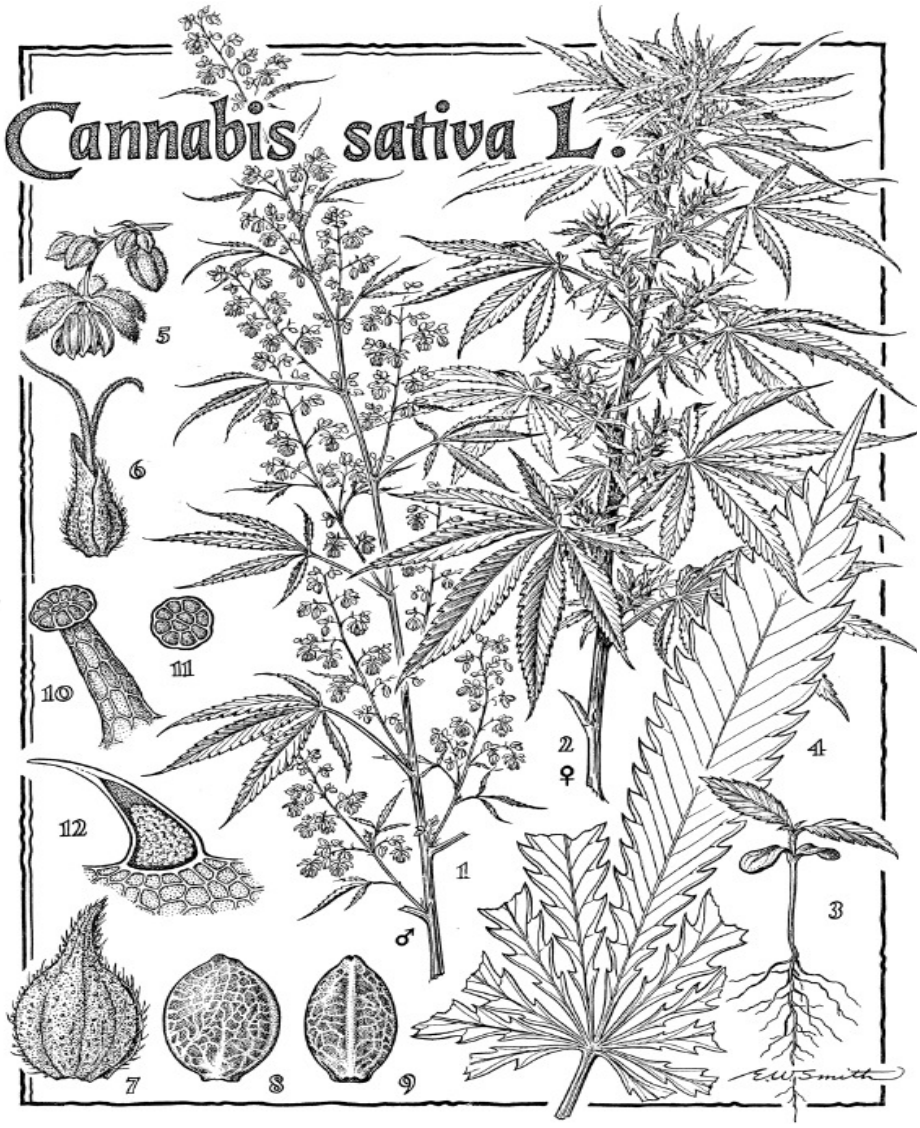



**Figure 2-3** *Cannabis sativa* L. This superb composite plate by artist Elmer Smith, often reproduced at a very small scale and without explanation in marijuana books, is the best scientific illustration of the hemp plant ever prepared. 1. Flowering branch of male plant. 2. Flowering branch of female plant. 3. Seedling. 4. Leaflet. 5. Cluster of male flowers. 6. Female flower, enclosed by perigonal bract. 7. Mature fruit enclosed in perigonalbract. 8. Seed (achene), showing wide face. 9. Seed, 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). Reproduced with the permission of Harvard University, Cambridge, MA.

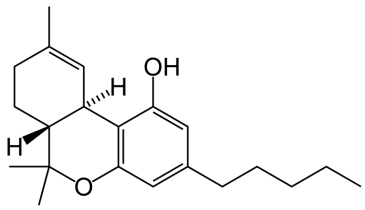
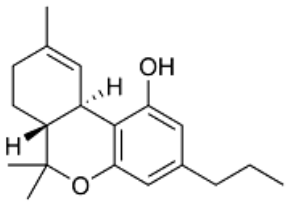
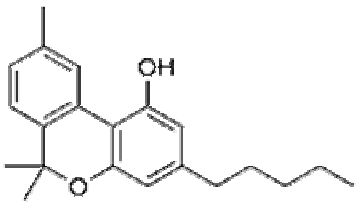
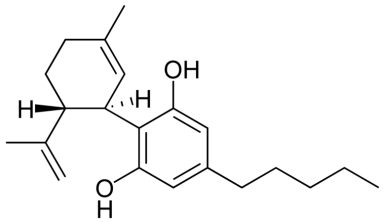
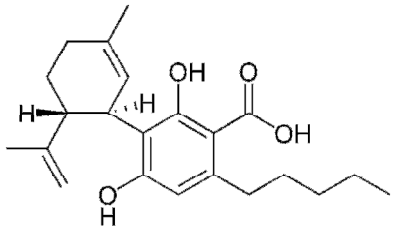
### 2.1.4 Chemical components

*Cannabis* plant contains a unique family of terpeno-phenolic compounds called cannabinoids which produce the "high" experience from smoking. They are found in the glandular trichomes that occur on most aerial surfaces of the plant. Approximately 61 cannabinoids (from > 420 of chemical compound in Cannabis) are known to exist, although some of these are breakdown products or artifacts. ш cannabinoid content of different strains of cannabis is associated with their geographic origins (3, 8).

The three main constituents of *Cannabis sativa* are $\Delta^9$ -tetrahydrocannabinol ($\Delta^9$-THC), cannabidiol (CBD) and cannabinol (CBN). $\Delta^9$ -THC is the main active constituent, which is found in the same amount in male and female cannabis grown under the same conditions. It was shown that the $\Delta^9$-THC content in the various parts of cannabis plant decreases in the following order: bracts, flowers, leaves, smaller stems, larger stems, roots and seeds (1, 12). A second intoxicating isomer, $\Delta^8$ -THC, is much less abundant in *C. sativa*, occurring only in trace amounts, if at all, and is somewhat less potent than $\Delta^9$-THC. Still another homologue, occasionally found in large amount, is tetrahydrocanabiverol (THCV), which has also been reported to be less psychoactive than THC (13).

THC is the psychoactive ingredient of *Cannabis,* and CBD, is an antipsychoactive ingredient (14). And cannabinol (CBN) is a degeneration or transformation product produced when THC ages, has been said to have some limited euphoriant activity, although most literature states that this cannabinoid is not euphoriant (13).

**Table 2-1 Structures of Cannabinoid compounds in *Cannabis***

| Name | Structure |
|---|---|
| $\Delta^9$-Tetrahydrocannabinol (or THC) or $\Delta^1$-Tetrahydrocannabinol in monoterpenoid system |  $C_{21}H_{30}O_2$ , MW = 314.46 g/mol |
| Tetrahydrocannabivarin (THCV, THV) |  $C_{19}H_{26}O_2$ , MW = 286.41 g/mol |
| Cannabinol (CBN) |  $C_{21}H_{26}O_2$ , MW = 310.43 g/mol |
| Cannabidiol (CBD) |  $C_{21}H_{30}O_2$ , MW = 314.46 g/mol |
| Cannabidiolic acid (CBDA) |  $C_{22}H_{30}O_4$ , MW = 358.48 g/mol |

### 2.1.5 Biosynthesis

The biosynthesis pathway was shown in figure 2-4. The gene encoding THCA synthase has been identified by Sirikantaramas *et al.*(35). The result supported the work of Fellermeier *et al.*(36) and shown clear evidence to confirm earlier hypotheses that cannabigerolic acid (CBGA) is a common precursor for the synthesis of both THCA and CBDA.

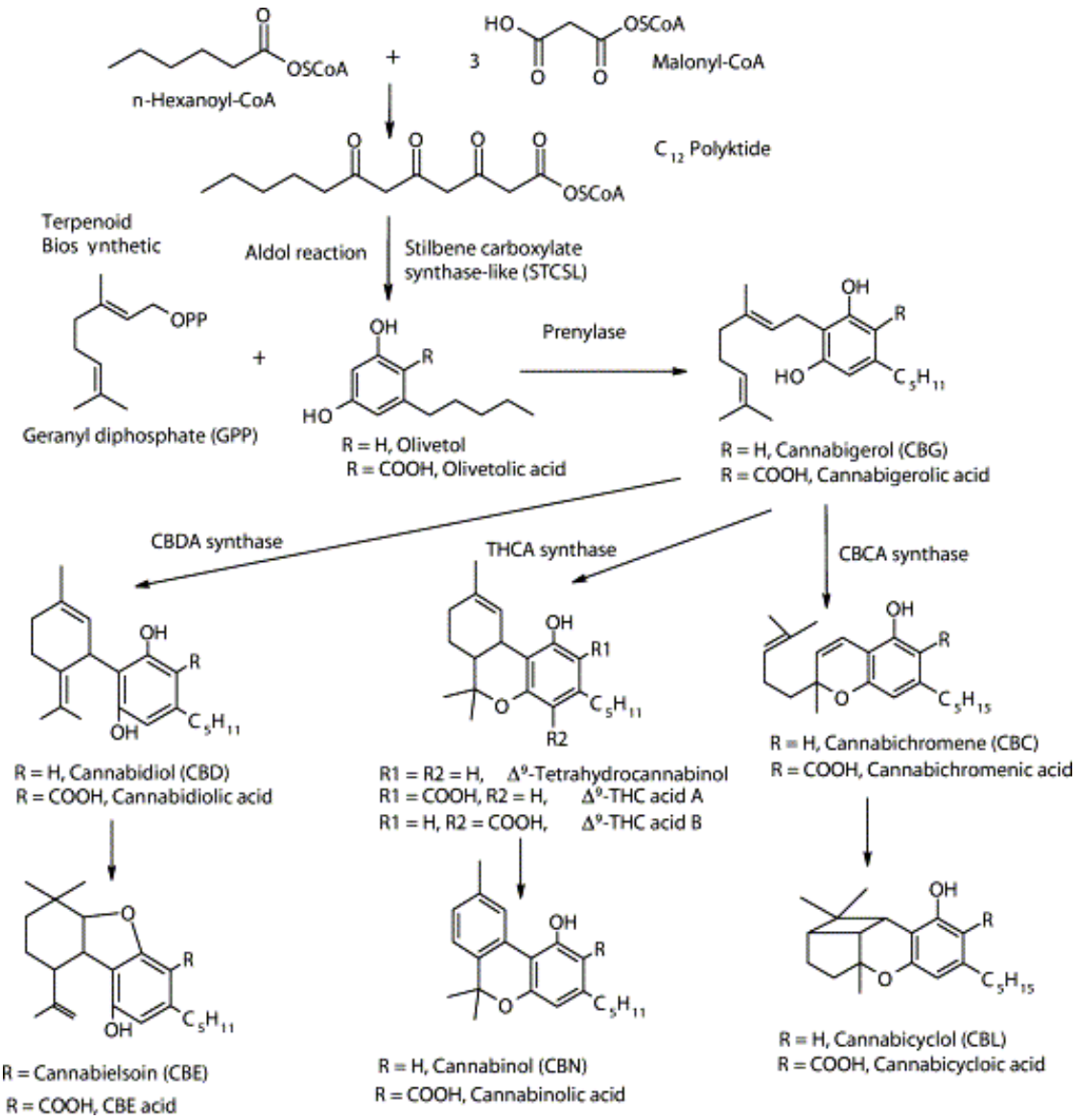



**Figure 2-4** Biosynthesis pathways of cannabinoids in *Cannabis* plant.

## 2.2 The usage of *Cannabis* plant.

### 2.2.1 Drug type *Cannabis*

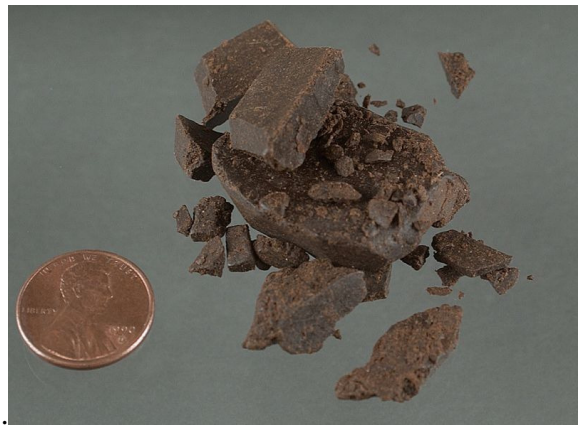
*Cannabis sativa* L. drug type (also known as marijuana) contains a group of chemical compounds which can cause of hallucination and produce the “high” from smoking (2, 3, 8, 13-16). It is widely used for illicit drug throughout the world including Thailand. *Cannabis* is illegally cultivated throughout Thailand especially in the northeastern and northern parts of Thailand where the geography and climate is suitable (2, 5, 17).

Drug type *Cannabis* has a few forms of products. First is herbal product, this type is prepared from the dried flowering tops and leaves which contains 0.5 -5% of THC while the "sinsemilla" variety may contain 7-14% of THC. Most users smoke hand-rolled cigarettes.

Second is Resin (or Hashish), made from THC-rich glandular hairs known as trichomes, as well as varying amounts of *Cannabis* flower and leaf fragments. The flowers of a mature female plant contain more trichomes than other parts of the plant. The product remaining is resembled in a fine powder and compressed into slabs. Most resin products contain 2-10% THC although it can be as high as 10-20% of THC.

The last form of drug type product is Liquid *Cannabis* (or Hashish oil). It is a highly potent and viscous substance obtained by using an organic solvent to extract THC from hashish (or marijuana), concentrating the filtered extract and in some cases subjecting it to further purification. It contains 15-50% THC, however THC as high as 70% have been detected in some samples (2, 16, 18).

a). 


b). 

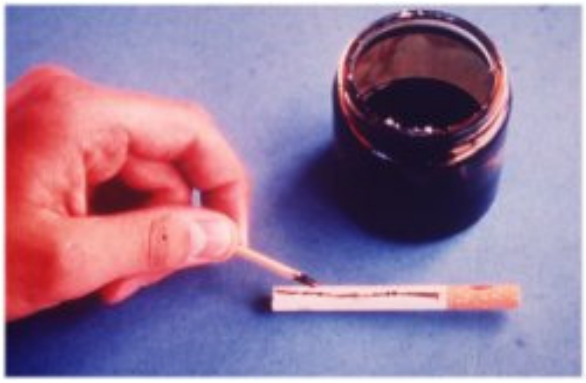
c). 

**Figure 2-5** a). Dried marijuana, b). Resin or Hashish, c). Liquid Cannabis or Hashish oil

### 2.2.2 Fiber type *Cannabis*

*Cannabis sativa* L. fiber type (also known as hemp or industrial hemp) is another type of *Cannabis* plant which cultivated for fiber and/or seed production, commonly differentiated from strains cultivated for medicinal or recreational use(3).

*Cannabis sativa* L. was most valued as a fiber source, considerably less as an intoxicant, and only to a limited extent as an oilseed crop. Hemp is one of the oldest sources of textile fiber, with extant remains of hempen cloth trailing back 6 millennia. Hemp grown for fiber was introduced to western Asia and Egypt, and subsequently to Europe somewhere between 1000 and 2000 BCE (10).

*Cannabis sativa* L. is extremely unusual in the diversity of products for which it is or can be cultivated. 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"(10).

**Table 2-2** World import and export hemp fiber in 2004.

| **Import Value 20 million US dollars.** | | **Export value 16.5 million US dollars.** | |
|---|---|---|---|
| **Country** | **Percentage of Import (%)** | **Country** | **Percentage of Export (%)** |
| Belgium | 20 | China | 27 |
| Spain | 19 | France | 18 |
| Italy | 10 | UK | 11 |
| Germany | 5 | Belgium | 7 |
| USA | 5 | Romania | 7 |
| Japan | 5 | Italy | 6 |
| Others | 36 | Others | 24 |

Source: Global Trade Atlas

In Thailand, hemp was widely cultivated among the Hill Tribe people in Northern part of Thailand. Because the area has a suitable climate, soil and water. They grown hemp for fiber for weaving the hemp clothes (11). But in Thailand, growing hemp still illegal under the Narcotics Act because of the drug situation and from the close characteristics between hemp and marijuana. So The Office of the Narcotics Control Board (ONCB) which is the authority in drug control concerns that it may lead to expansion of drug type. The growers have to ask permission from the Food and Drug Administration (FDA) in the Ministry of Public Health and have to cultivate under the control of the Office of The Narcotics Control Board (ONCB) (5-7, 11).

The information from the ONCB officer has shown a four big cultivation areas, Chiang mai, Chiang rai, Pa-Yao and Tak, where the Hmong Hill Tribe people live.

**Table 2-3** Cost value of hemp fiber strain Por Sor 1 (พันธุ์ พ.ศ.1) compared with other plant fibre in Thailand. From the study of the Office of Agricultural research and Development region 1 by Dr.Permsak Supapornheminth 2005(11).

| Type of Product | Product kg / rai (1600 $m^2$) | Sale Bath / kg. | Sale Bath / rai( 1600 $m^2$) |
|---|---|---|---|
| Fresh plants of Por Sor 1. | 4,600 | 0.5-2.0 | 2,300-9,200 |
| Dried fiber - pine (for paper)<br>- jute (for handed craft paper) | 193 | 22-30<br>70-100 | 4,246-5,790<br>13,510-19,300 |
| Whole dried eucalyptus fiber | 645 | 18-20 | 11,610-12,900 |
| Hemp fiber for weaving in Chiangmai market. | 160-193 | 180-350 | 28,800-67,550 |

**Table2-4** The Comparative investment cost and incoming between hemp and other economic plants (11).

| Plants | Jute fiber for paper[a] | Eucalyptus Cut every 5 years[b] | Field-corn for animal feeding[c] | Cotton[c] | Fresh Hemp[d] |
|---|---|---|---|---|---|
| Product / Rai | 1,200 kg. | 12 tons | 535 kg. | 225 kg. | 4,600 |
| Cost | 2 Bt/kg. | 780 Bt/ton | 3.69 Bt/kg. | 14.49 Bt/kg. | 0.5-2 Bt/kg. |
| Incoming (Bt / Rai) | 2,400 | 9,360 | 1,974.15 | 3,260.25 | 2,300-9,200 |
| Invest (Bt / Rai) | 1,778.65 | 6,215 | 1,896.71 | 3,299 | 500 |
| Profit (Bt / Rai) | 477.35 | 3,145 | 77.44 | -38.75 | 1800 |

Source of information;

a. Department of Agricultural Extension

b. Office of Agricultural Economics

c. Agricultural Statistics 1998/1999, Office of Agricultural Economics

d. Dr.Permsak Supapornheminth, 2005

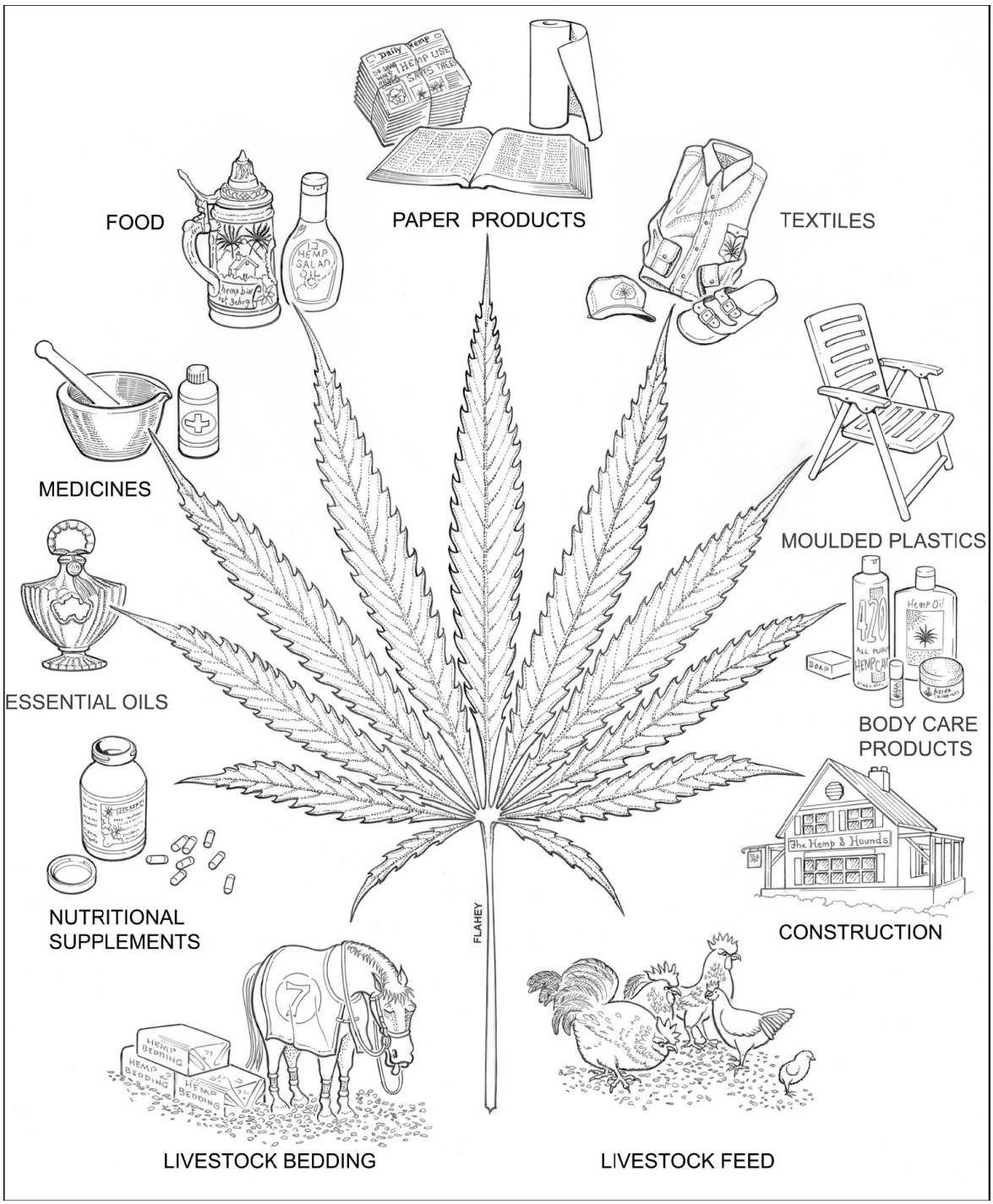



**Figure 2-6** Major uses of Industrial Hemp (10).

## 2.3 *Cannabis* Detection

### 2.3.1 Physical Examination

#### 2.3.1.1 Macroscopic Examination

Macroscopic examination or visual identification includes 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(19). Many of the morphological characteristics of individual *Cannabis* plants are greatly influenced by environmental factors. There is an enormous variation in sizes and shape.

Further more if it is being grown for fiber, there are over 200 plants per square metre. This forces the plants to compete to for sunlight and grow straight up, often up to 3 or 4 metres high in 4 months. This will produce the desired long, strong fibers in the stalk. But if it is being grown for its psychoactive value, the plants are well spaced out and generally kept to a shorter shrub shape, with many flowers. All males would be eradicated from the field to prevent seeds, as THC production slows down once the flowers have been pollinated, and smokers do not want seeds in their cannabis.

The differences between drug and fiber types by macroscopic are as follows (10, 11, 16, 20).

**Table2-5** Physical characteristics differentiation between hemp and marijuana

| **Characteristics** | **Hemp** | **Marijuana** |
|---|---|---|
| Height | Tall, can be 25 feet in high | Tend to be short and bushy |
| Leaves | Larger, non-sticky | Smaller, longer but sticky |
| Stems | More hollow at the internode, less wood | Less hollow, more wood |
| Flowers | ND. | ND. |
| Seed | ND. | ND. |

ND = Not difference

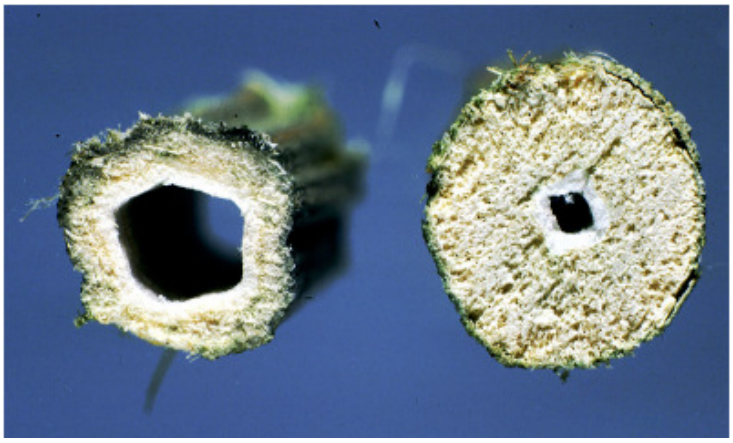


**Figure2-7** Cross sections of stems at internodes of a fiber plant (left) and of a narcotic plant (right). Fiber cultivars have stems that are more hollow at the internodes, i.e. less wood, since this allows more energy to be directed into the production of bark fiber.

**Figure 2-8** The pictures of fiber-type (upper) and drug-type leaf (lower) which the size of fiber-type leave is larger than drug-type leave.

**Figure 2-9** Marijuana plants (upper) tend to be short and bushy and hemp (lower) is taller and less space of growing.

Most of macroscopic characters of both drug and fibre types are the same. It's quite difficult to distinguish between two types and required taxonomist to identify.

#### 2.3.1.2 Microscopic Examination

The leaves of cannabis plants should be examined for the presence of glandular and bracts(19). Several studies of *Cannabis* have suggested that cannabinoids are accumulated primarily in the epidermal glandular trichomes. The abundant of trichomes on the surface of the fruiting and flowering tops of marijuana is the microscopic characteristic of marijuana products. Beside the glandular trichomes, non-glandular trichomes which lack of cannabinoids also are present on the plant epidermis in high numbers (3, 8, 21).

The drug type has a lot of glandular trichromes on the leaf surface. This glandular trichromes will make the sticky resin on the leaf surface, while the fiber type contains less number of glandular tricomes and non-sticky.

**Figure 2-10** Glandular trichome which is more abundant on drug type leaf surface.

### 2.3.2 Chemical Examination

#### 2.3.2.1 Color test

**Fast Blue B salt test**, this test is widely used in field test because it is easy to carry the reagents and perform the test. Fold two filter papers into quarters and open partly to form a funnel; place a small amount of pulverized marijuana or resin or a very small drop of liquid into the centre of the upper paper, add two drops of petroleum ether allowing the liquid added to penetrate to lower filter paper, and then separated the two filter papers, discarding the upper paper and allowing the lower filter paper to dry. Finally, a very small amount of the solid reagent, preparing by diluting Fast blue B salt with anhydrous sodium sulphate in the ratio of 1:100, was added to the lower paper and then added two drops of a 10% (w/w) aqueous solution of sodium bicarbonate. The result shows a purple-red colored stain at the centre of the filter paper which is indicative of a marijuana product; this color is a combination of the colors of the different cannabinoids which are the major components of marijuana: THC = red, CBN = purple, CBD= orange(5, 15-17).

**The rapid Duquenois test (Duquenois-Levine test)** Place a small amount of the suspect material in a test tube shake with 2 ml of solution 1 (Five drops of acetaldehyde and 0.4 g of vanillin are dissolved in 20 ml of 95% ethanol) for one minute; add 2 ml of concentrated hydrochloric acid, shake the mixture and then allow it to stand for ten minutes; if a color developed add 2 ml of chloroform. If the lower (chloroform) layer becomes violet color this indicates the presence of a marijuana product (The national narcotics laboratory, Vienna)(16).

#### 2.3.2.2 Chromatographic separation methods

##### 2.3.2.2.1 Thin-layer chromatography

Following the Division of Narcotic Drugs, United Nations recommendation, TLC using Silica gel 60 $F_{254}$ and three solvent systems is used to identify cannabis (16).

System A Petroleum ether : Diethyl ether = 8:2

System B Hexane : Dioxane = 9:1

System C N-hexane: Dioxane : Methanol= 7:2:1

Kitpipit T. (17) and Watanasiri S. (5) found that system C is the most suitable system to differentiate fiber and drug types.

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

##### 2.3.2.2.2 High performance liquid chromatography

High Performance Liquid Chromatography is the most widely used among the analytical techniques, due to its sensitivity, its suitability for separating nonvolatile species or thermally fragile ones, and above all, its wide applicability to substances that are of prime interest to industry, to many field of science including forensic science. The marijuana detection method using High Performance Liquid Chromatography is shown in figure 2-11a (17).

Many studies used this technique to analyze cannabis samples such as Marjorie Che`ze, *et al*. (2005) used liquid chromatographic separation to detect cannabinoid content in forensic hair samples. The mobile phase consisted of a mixture of acetonitrile and 2 mM formate buffer. The detection was performed on a triple stage quadrupole, fitted with electrospray ionization (ESI) orthogonal source, and allowed

the simultaneous detection of benzodiazepines in positive polarity and selected reaction monitoring mode (22). Kojoma *et al* (2006) used HPLC to determine cannabinoid content in fiber and drug types and found that the amount of THC content is lower than 0.2% in fiber type (23).

#### 2.3.2.2.3 Gas chromatography

In gas chromatography (GC), the sample is vaporized and injected onto the head of chromatographic column. Elution is brought about by the flow of an inert gaseous mobile phase. The marijuana detection method using gas chromatography is shown in figure 2-11b.

M. Stefanidou *et al*, (1998) used GC-MS capillary column to determine cannabinoid content in marijuana samples seized in forensic application and used the quantities and ratio of $\Delta^9$-THC/CBD content to classify the types of cannabis sample(1). Klemens M.*et al*, (2003) studied the variation of $\Delta^9$-THC content in single plant hemp varieties and used GC-MS capillary column EI-mode and SIM-mode to quantify $\Delta^9$-THC and calculate the ratio of THC/CBD.

In 2004, Karl W.H. *et al*, studied the chemotaxonomic of *Cannabis* collected from around the world. GC-MS was used for the analysis of cannabinoid, the cannabinoid content and the ratio of THC/CBD were determined in order to assign the sample to its chemotype (3).

| | |
|---|---|
| METHOD 1 | |
| Operating conditions | |
| Column | 250 mm by 4.6 mm i.d. |
| Packing material | Octadecyl-silica (medium load of $C_{18}$ on Partisil 5) |
| Mobile Phase | 0.02 N $H_2SO_4$ 20% v/v |
| | Methanol 80% v/v |
| Flow rate | 2.0 ml per minute |
| Detection | UV at 220 nm or |
| | UV at 254 nm |
| Injection volume | 10 µl by syringe or loop injector. |
| Quantitation | by peak areas, internal standard method. |
| Internal standard | di-n-octyl phthalate. |
| METHOD 2 | |
| Operating conditions | |
| Column | 150 mm by 4.6 mm I.D. |
| Packing material | Octadecyl-silica (Spherisorb S3 ODS 2 HPLC GRADE 3µm) |
| Mobile Phase | Methanol 85 |
| | Water 14.2 |
| | Acetic acid 0.8 |
| Flow rate | 1.5 ml. per minute |
| Operating temperature | ambient |
| Detection | UV at 230 µm. |
| Injection volume | 2-3 µl. |
| Quantitation | by peak areas, internal or external standard methods. |

**Figure 2-11a**: High-performance liquid chromatographic condition for the detection of marijuana. (The national narcotics laboratory, Vienna)

Packed column technique

| | |
|---|---|
| Detector | FID (Hydrogen 30 ml per minute, air 300-450 ml per minute). |
| Column | 6 ft (or 2 m), I.D. 2 to 4 mm. |
| Packing | 3% OV-17 or SE-30 or OV-1 |
| Carrier gas | Nitrogen at 30 ml per minute. |
| Operating Conditions: | Injector temperature: 270°C. |
| | Oven temperature: between 240-260°C isotherms, |
| | (Depending upon the actual packing) |
| Detector temperature: | 300°C |
| Internal standard | n-tetradecane or n-docosane or other suitable n-alkene; |
| Other standards | frequently used: androst-4-ene-3, 17-dion |
| | dibenzylphthalate or cholestane. |

2. Capillary column technique

| | | |
|---|---|---|
| Detector | FID. | |
| Column | OV-1-Chemically boned fused silica capillary 10 m. by | |
| | 0.52mm I.D. | |
| Film thickness | 1 µm | |
| Carrier gas | Helium | |
| Flow Rate | 2 ml per min. | |
| Injection technique | Split-Splitless | |
| Operating Temperatures: | Injector: | 290°C. |
| | Oven: | 240°C. |
| | Detector: | 290°C. |

**Figure 2-11b.** Gas liquid chromatographic condition for the detection of marijuana. (The national narcotics laboratory, Vienna)

### 2.3.3 DNA Technology

Historically, the identification of botanical trace evidence has relied on morphological and histological analyses. Then, the chemical identification was developed to identify the unique of chemical compound in each plant species.

Nowadays, the advance of the polymerase chain reaction (PCR) and the compilation of plant DNA sequence information in the database (e.g., Genbank) by molecular taxonomist, make it possible to use DNA-based technique for the identification of plant species.

In 1998, Linacre and Thorpe published the paper on DNA-specific primers. The nucleotide sequences between the *trn*L and *trn*F genes in the chloroplast of *Cannabis sativa* have been determined and *Cannabis sativa* specific nucleotide sequences within the intergenic spacer between the *trn*L 39 exon and *trn*F gene were identified. Primers, made to these sequences, have been tested on a range of different plant extracts but PCR product was obtained only in the presence of *Cannabis sativa* (24).

In 2006, Kojoma *et al*, studied DNA polymorphism in THCA gene of *Cannabis* fiber and drug types, and was able to identify a specific PCR marker for the THCA synthase gene for the ‘‘drug-type’’ strains. This PCR marker was not detected in the ‘‘fiber-type’’ strains (23).

## 2.4 *Cannabis* type discrimination and classification

From the unique of chemical compounds in *Cannabis* plant called cannabinoid, this characteristic made the chemotaxonomic important to discriminate the type of cannabis plant. Many publications tried to show that the chemical analysis was better than morphological method in the classification of *Cannabis*. Quantitative characterization involves determining a plant’s THC/CBD ratio (the inverse ratio is sometimes used) and assigning it to a discrete chemical phenotype (chemotype) (3).

There are several indexes which are have been suggested by different groups of scientists as follows.

a).The phenotypic index is defined by Fetterman *et al.* as $\frac{\%\Delta^9\text{-THC} + \%\ \text{CBN}}{\%\ \text{CBD}}$ (12).

If phenotypic index exceeds 1, the plant is classified as phenotype I, which represents the drug-*Cannabis*, while if phenotypic index is less than 1; the plant is classified as phenotype II, which represents the non-drug *Cannabis*.

b). Debruyne *et al.* (1981) (25) had combined the use of high-pressure liquid chromatography (HPLC) and high-resolution gas chromatography and established a criterion by calculating the peak area ratios of different sample varieties;

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

If at least one of these two ratios exceeds 1, the plant is classified as drug-*Cannabis*, while if both ratios are less than 1, the plant is classified as non-drug *Cannabis*. The result of these calculations is that in the resinous *Cannabis* samples at least two of the major cannabinoids ($\Delta^9$ -THC and CBN) contains high amounts (25). It must be emphasized that in some samples of *Cannabis* the low proportion of $\Delta^9$ -THC is not incompatible with their resinous character, as it is known that in these samples the CBN, a degradation product of $\Delta^9$-THC, is present in large proportions (26). This leads to the suggestion that $\Delta^9$ –THC+CBN would approximate the $\Delta^9$-THC content, irrespective of degradative changes.

c). Paris and Nahas (1984) (27) has described 3 chemical types by THC and CBD 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%)

d). Small and Beckstead (1973)(3) recognized the ratio $\frac{THC}{CBD}$ and classified *Cannabis* into 3 chemotype as follow:

Chemotype I = plants which has high THC/CBD ratio (>>1.0)

Chemotype II = plants which has intermediate ratio (close to 1.0)

Chemotype III= plants which has low THC/CBD ratio (<<1.0)

e). According to De. Meijer et *al.*(1992) (34). The criteria for classification is the cannabinoid content.

| Phenotype | %[THC] | %[CBD] | [THC]/[CBD] |
|---|---|---|---|
| Non-drug | <0.5 | ≥0.5 | <1 |
| Intermediate | ≥0.5 | ≥0.5 | |
| Drug | ≥0.5 | <0.5 | >1 |

f).European union (28).

According to the European Union, the maximum permitted content in fiber *Cannabis* varieties is 0.3% $\Delta^9$-THC. Plants contain $\Delta^9$ –THC less than 0.3% are considered to have no psychoactive potency and this value has been generally used to differentiate and classify the cannabis samples into drug-type and fiber-type (1, 16, 28).

After the year 2001, the limit of THC content will be lowered to 0.2% from the Council Regulation (EC) No.1420/98. By this regulation, plants sample were selected random at the end of their flowering period and with stalks and seeds removed (29, 30).

A sample size of 50 plants is fixed by EU regulations (Commission Regulation (EC) No.1177/2000) for routine analysis and bigger samples are collected where result are not clear (31).

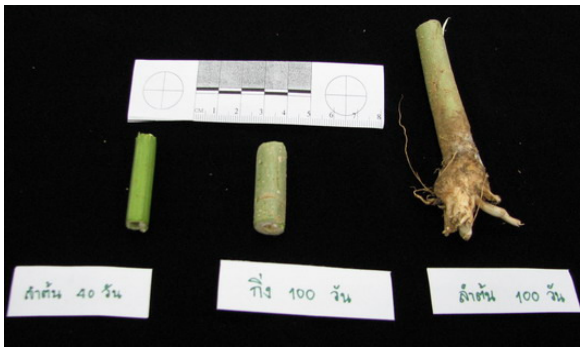
# CHAPTER III
# MATERIALS AND METHODS

## 3.1 Samples collection

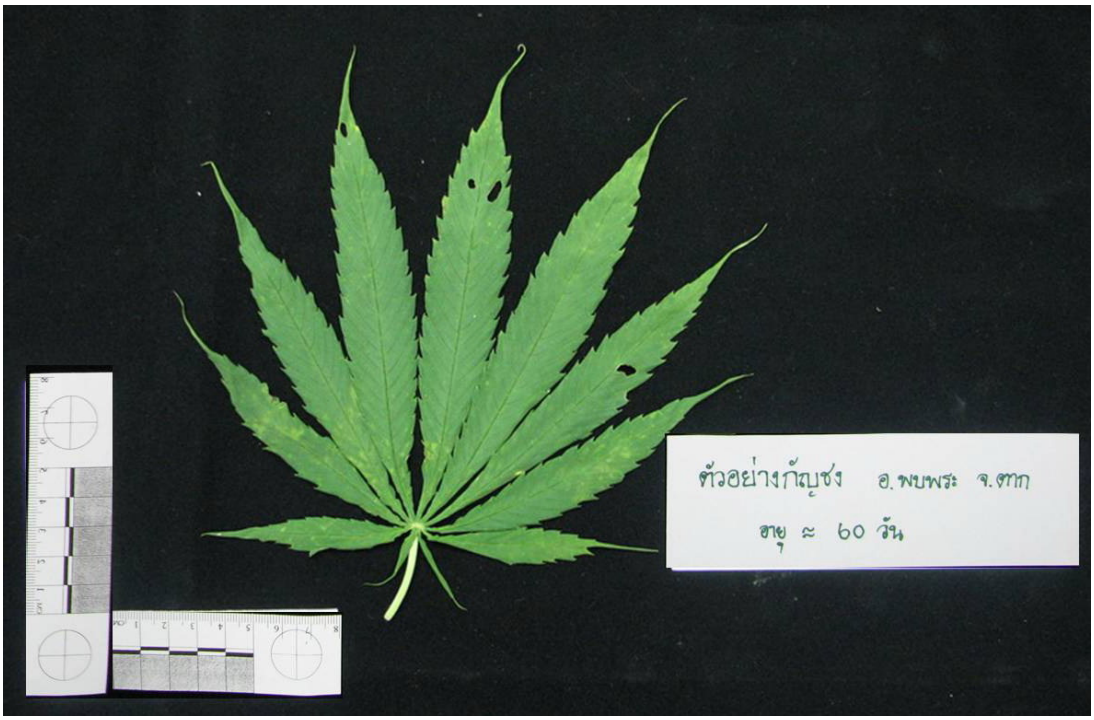
Fresh and dry leaves of hemp (*Cannabis sativa* L.) were collected from the commercial cultivation areas by the officer of the Narcotics Control Board (ONCB), Thailand. Approximately 100 mg of fresh leaves were cut into small pieces and put into an Eppendorf tube before quickly frozen in liquid nitrogen. The plant materials were stored at – $80^0$ 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.


**a).**

**b).**




**c).**




**d).**

**Figure 3-1 a), b), c) and d).** *Cannabis* plant samples

### 3.2 Chemical analysis

The plant fiber-type samples were subjected to three chemical testing and DNA-based methods. The chemical-based methods which are Fast Blue B salt test, TLC and GC-MS. Fast Blue B salt test and TLC were conducted as suggested in recommended methods for testing cannabis, in the manual adopted by national narcotic laboratories, United Nations (16). GC-MS method was modified the condition suggested by M.Stefanidou (1).

#### 3.2.1 Presumptive test

##### 3.2.1.1 Fast blue B salt test

The test was performed on a filter paper according to the recommended method in the manual of the Division of Narcotic Drugs, United Nations. Two filter papers were folded into quarters and opened partly to form a funnel. Then, a small amount of pulverized marijuana was placed into a centre of the upper paper. Then two drops of petroleum ether were added, allowing the liquid to penetrate to lower filter paper, and then the two filter papers were separated, the upper filter paper was discard and allowing the lower filter paper to dry. Finally, a very small amount of the solid reagent; Fast blue B salt: anhydrous sodium sulphate (1:100), was added to the lower paper. Then two drops of a 10% (w/w) sodium bicarbonate were added. The change of color on the filter paper was observed. The red-orange color on the lower filter paper suggested the presence of cannabinoids. The result was collected by photography.

##### 3.2.1.2 Thin layer chromatography

The test procedure was performed according to the recommended method in the manual of the Division of Narcotic Drugs, United Nations (5, 16, 17). 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 N-hexane: dioxane: methanol = 70: 20:10.

This solvent combination is suitable for not only the neutral cannabinoids but also the cannabinoid acid (16). 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 dissolving 50 mg of Fast blue B in the 1 ml of water then 20 ml of methanol was added. The plate was sprayed with the Fast blue B salt solution and left at room temperature for color development.

### 3.2.2 Gas chromatography – Mass spectrometry

This method is widely used in many laboratories to identify and determine the cannnabinoid content. In this study, the analysis of sample was modified from the method suggested by M. Stefanidou *et al.* in 1998 (1). Each dried and grounded sample was extracted overnight with 1 ml. of n-hexane. After centrifugation, 500 µl of the supernatant was transferred to new vial and 10 µl of 1000 µg/ml diphenylamine in methanol was added as internal standard. Each sample was evaporated with nitrogen steam and the dry residue was reconstituted with 500 µl of ethyl acetate. One microliter of this sample was analyzed by GC-MS.

The condition of the analysis was as follows: GC-MS Agilent 5973: capillary column, Hewlett-Packard HP-5MS crosslinked 5% phenylmethylsilicone (30 m × 0.25 mm I.D.,film thickness 0.25 mm); oven temperature, initial temperature,100°C; initial time, rate15°C/min, final temperature 300°C, final time 8.00 min; injection port temperature, 220°C; interface temperature, 300°C. Spilt mode with ratio 20:1. The helium flow-rate was 1 ml /min. (Full condition see appendix).

One point calibration curve was prepared by 500 µl of standard solutions THC, CBD or CBN in methanol at concentration 500, 530 and 52 ppm. respectively. Added with 10µl of diphenylamine as internal standard at concentration 1000 µg/ml of methanol. One microliter of each standard solution was analyzed by GC-MS.

The data was analyzed by Chemstation Integrator program and the cannabinoid content was calculated by using the area of major ion and internal standard: $\Delta^9$-THC (m/z 299, 314, 231), CBD (m/z 231, 174, 314), CBN (m/z 295, 238, 310) and diphenylamine (m/z 169).

### 3.3 DNA analysis

#### 3.3.1 DNA preparation

DNA was extracted from all the plant samples using the Dneasy® Plant Mini kit (QIAGEN, Germany) (32). Following the manufacturer's protocol, about 100 mg wet weight or 20 mg dry weight of plant samples were used as starting material. Each sample 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 lyse 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 clear lysate and mixed by pipetting. Six hundreds 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 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.

### 3.3.2 DNA quantitation by yield gel method

The quantity of extracted DNA was estimated by comparing the band intensity of extracted genomic DNA samples with those of Lamda *Hin*dIII markers in 1% agarose gel. The quantity and size of genomic DNA samples was analyzed using GeneTools (SyneGene, UK) programme.

### 3.3.3 DNA amplification

All DNA samples were amplified by primer sequences as shown in table 3-2 (5, 17, 23, 24). Polymerase chain reactions were carried out using the following primers pairs;

#### 3.3.3.1 *Cannabis sativa* - specific primers (G and H)

All samples were amplified by primer pairs G and H of the *trn*L-*trn*F locus in chloroplast for checking DNA and the presence of *Cannabis sativa.* The simplex PCR amplifications using primers G and H were performed in a total volume of 25 µl. Each reaction contained 20 ng of extracted DNA, 200 µM of each deoxyribonucleotide triphosphate or dNTPs (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 197 bp (5, 17, 24).

#### 3.3.3.2 Drug - type marker (g and h)

After amplification by primers G and H, DNA samples were amplified by primer g and h which is the THCA synthase region. A portion of the THCA synthase gene in DNA samples were amplified 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 (5, 17, 23).

**Table 3-1** Sequences of primers used in the present study

| Primer name | Nucleotide sequences 5' - 3' | $T_a$ |
|---|---|---|
| *Cannabis sativa* –specific, G | GAG GGT TTC TAA TTT GTT ATG TT | 60° |
| *Cannabis sativa* –specific, H | ACTAGAGGACTTGGACTATGTC | 60° |
| Drug-type marker, g | AATAACTCCCATATCCAAGCA | 57° |
| Drug-type marker, h | AGG ACT CGC ATG ATTAGT TT | 57° |

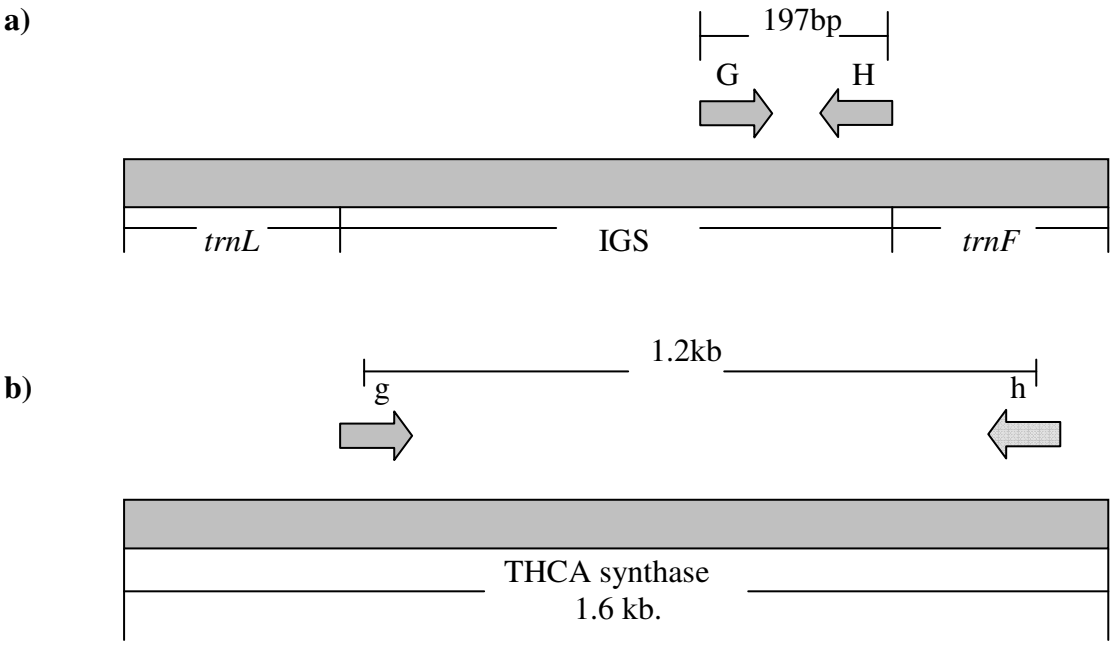



**Figure 3-2** a, b The diagram shows the position of primer pairs used to amplify the DNA samples.

### 3.3.4 PCR products detection

Gel electrophoresis was used for the detection of the PCR product fragments. A 2% (w/v) agarose gel in 1x TBE buffer was prepared. Then 2 µl of 10 mg/µl ethidium bromide was added to the warm gel and mixed well before pouring into the gel tray with well-casting comb. The PCR products were mixed with 10X loading dye before loaded into the gel. The mixtures were loaded to the gel which submerged in a TBE buffer tank. One hundred base pairs (100 bp ladder) was used as a size standard marker. Electrophoresis was carried out at 75 V for 90 min to separate the PCR products. The gel was photographed by using SyneGene Gel documentation under UV light (5, 17).

# CHAPTER IV
# RESULTS

## 4.1 Samples collection

Total samples were collected by ONCB officer from various sources. Sources of samples subjected to the present study were summarize in table 4-1.

**Table 4-1** Sources of samples subjected in the present study.

| Sources | No. of samples |
|---|---|
| From Queen Sirikit Botanic Garden, Chiangmai. | 11 |
| From Hmong Villagers Mae-rim, Chiangmai. | 4 |
| From Hmong villagers, Pop-pra, Tak. | 38 |
| From Hmong villagers, Muang, Tak. | 5 |
| **Total** | 58 |

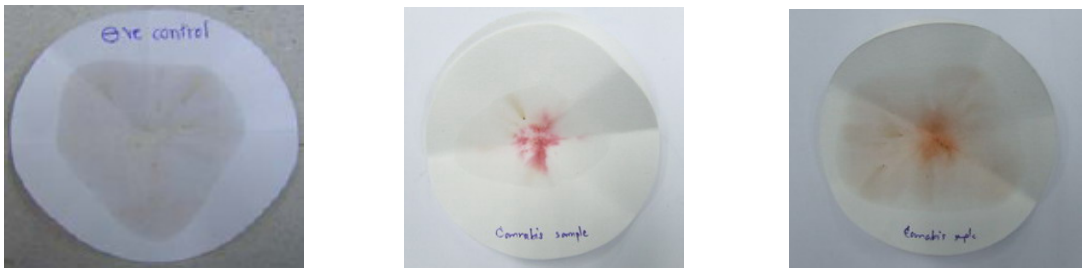
* Queen Sirikit Botanic Garden (QSBG), established in 1993 by the Botanic Gardens Organization of Thailand. QSBG is under the Ministry of Natural Resources and Environment.

## 4.2 Chemical analysis of *Cannabis* sample

### 4.2.1 Presumptive test

#### 4.2.1.1 Fast blue B salt test

The test showed the presence of cannabinoid content in the samples. Most of samples showed varying shade of Orange-Red and Red-Purple colors according to the major cannabinoids (see Figure 4-1). The results of color test were shown in table 4-2



Negative control Red-Purple Orange-Red

**Figure 4-1** The results of color test in *Cannabis* samples.

#### 4.2.1.2 Thin layer chromatography

Thin layer chromatography of all the samples showed the THC (red) some with CBD (orange) and CBN (purple) bands. Sample Hp59 showed only CBD band (see table 4-2 and figure 4-3)

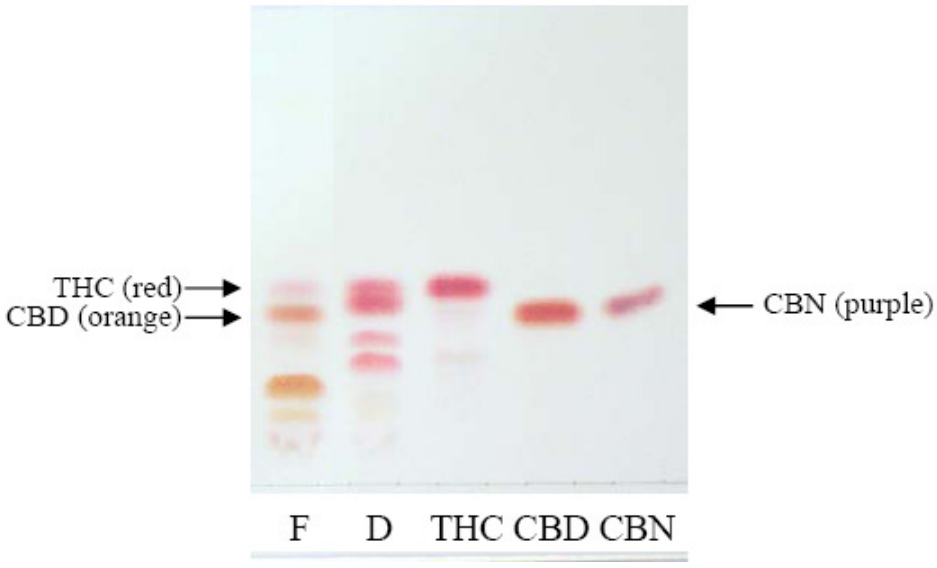
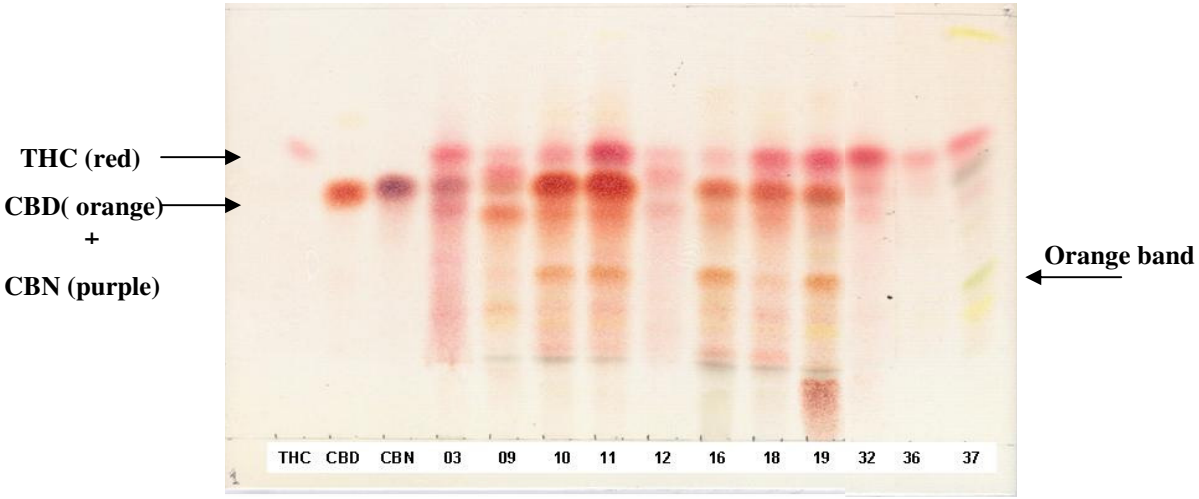
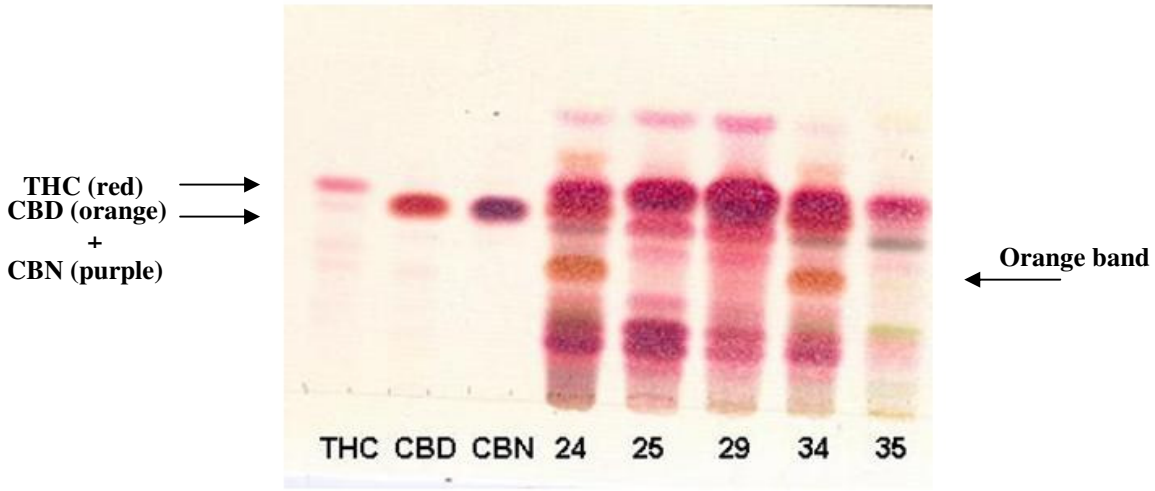


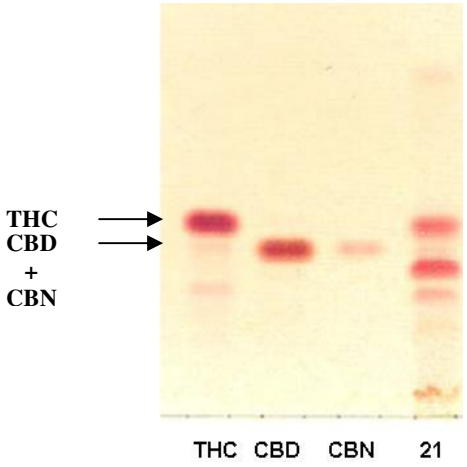

**Figure 4-2** TLC chromatogram of standard cannabinoids and samples of drug type (D) and fibre type (F).


THC (red)
CBD( orange)
+
CBN (purple)
Orange band
THC CBD CBN 03 09 10 11 12 16 18 19 32 36 37

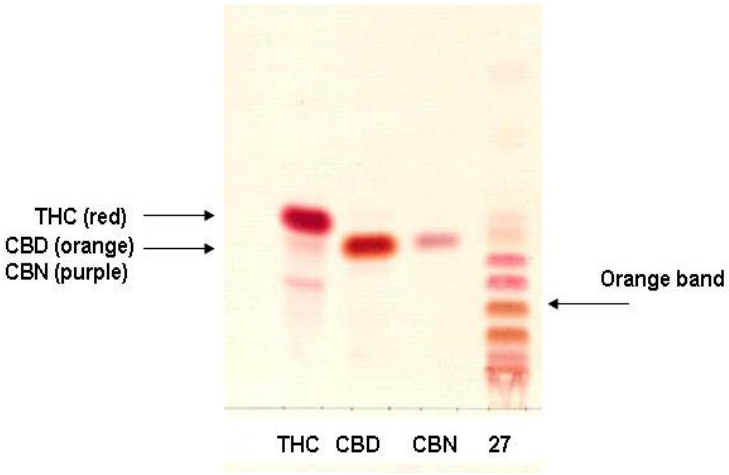
**a).**


THC (red)
CBD (orange)
+
CBN (purple)
Orange band
THC CBD CBN 24 25 29 34 35

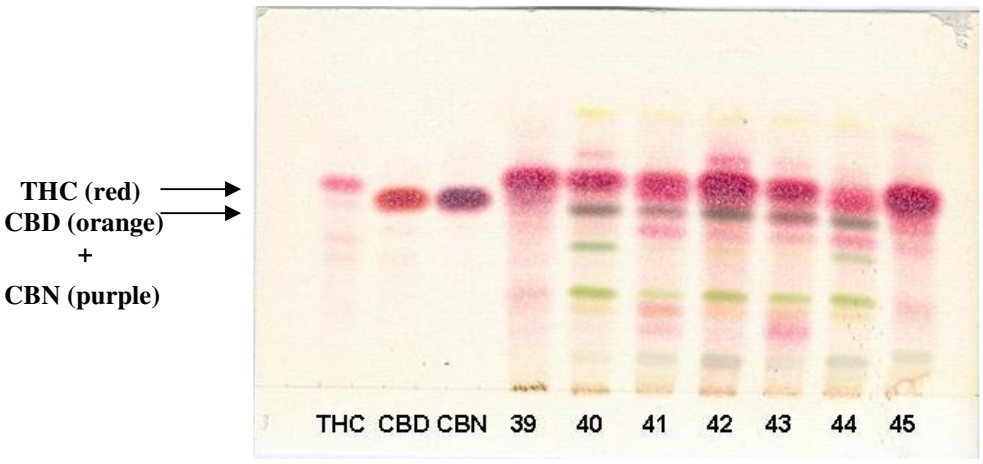
**b).**


THC
CBD
+
CBN
THC CBD CBN 21

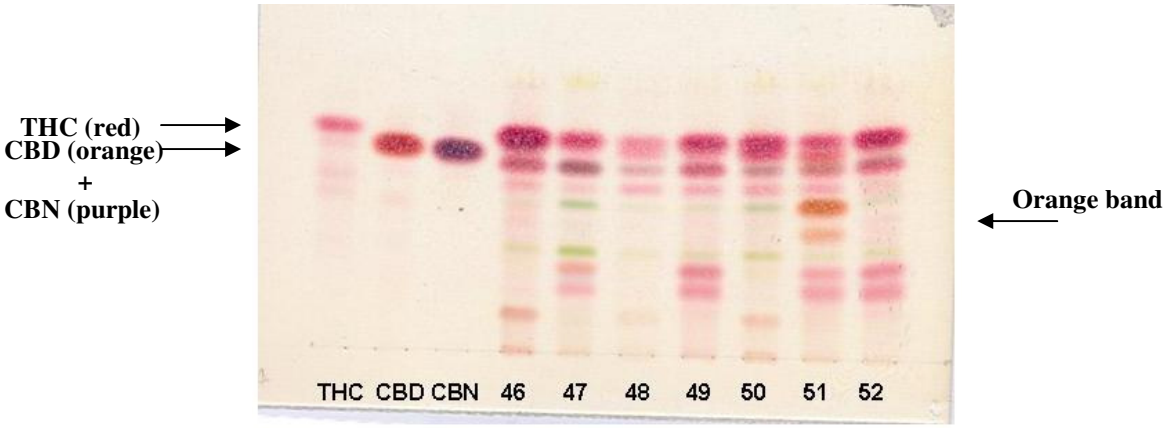
**c).**


THC (red)
CBD (orange)
CBN (purple)
Orange band
THC CBD CBN 27

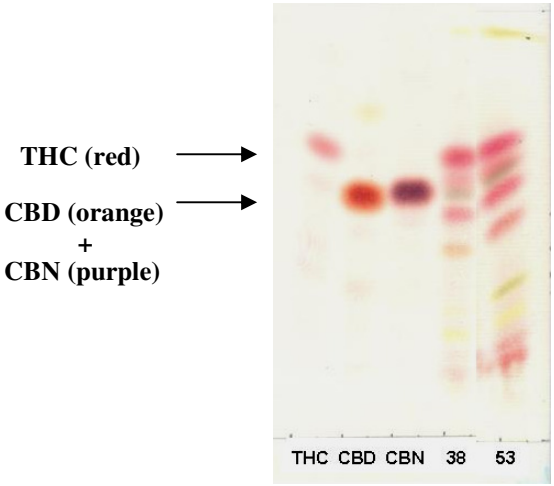
**d).**


THC (red)
CBD (orange)
+
CBN (purple)
THC CBD CBN 39 40 41 42 43 44 45

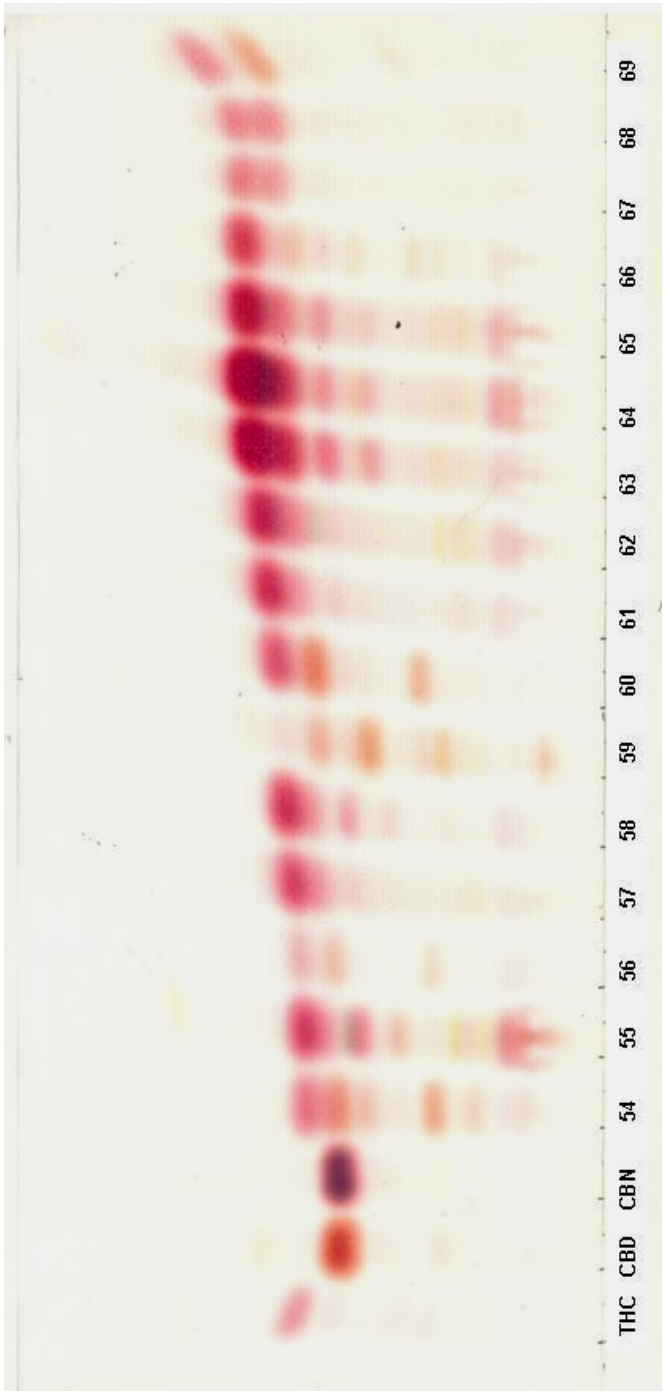
**e).**


THC (red)
CBD (orange)
+
CBN (purple)
Orange band
THC CBD CBN 46 47 48 49 50 51 52

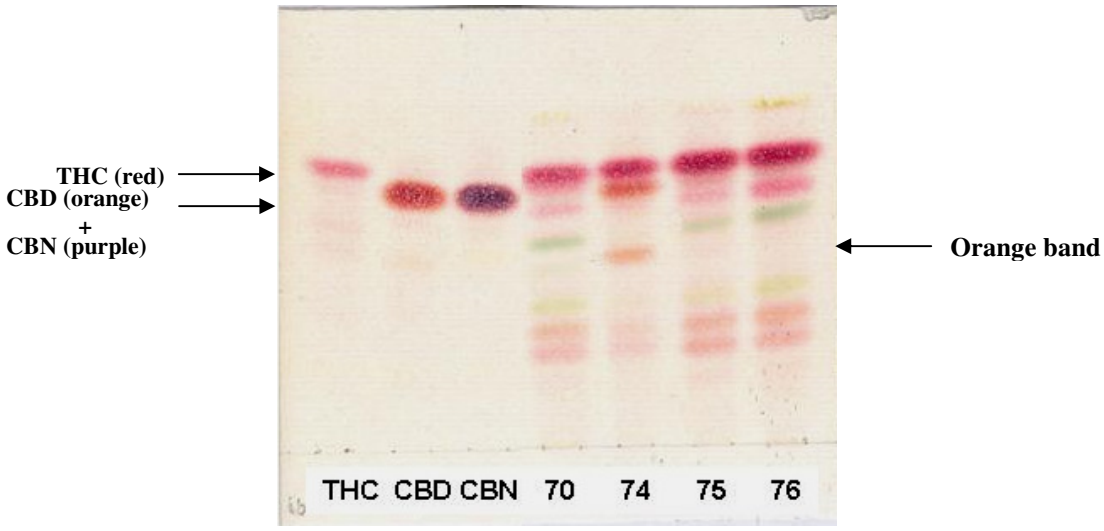
**f).**


THC (red)
CBD (orange)
+
CBN (purple)
THC CBD CBN 38 53

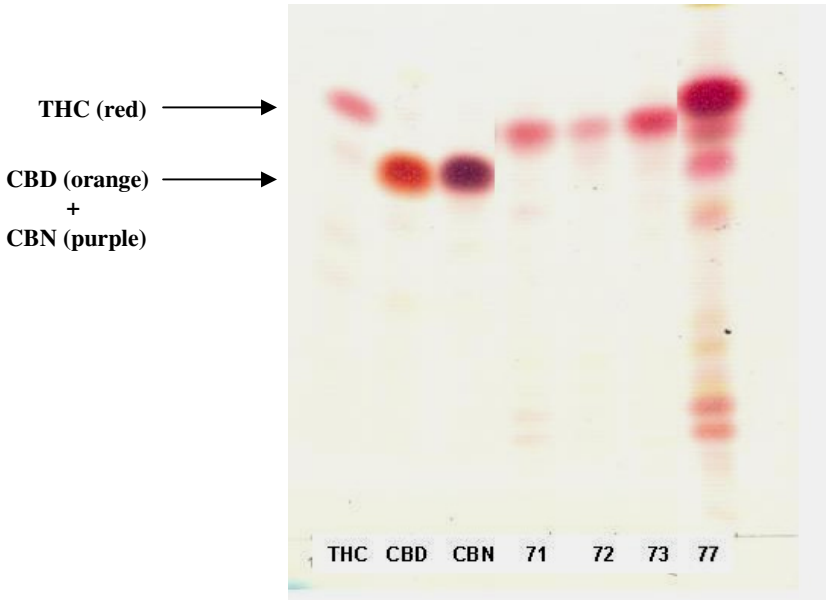
**g).**


THC
CBD
CBN
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
THC (red)
CBD (orange)
+
CBN (purple)
Orange band

**h).**




**i).**




**j).**

**Figure 4-3 a).- j).** TLC chromatograms of *Cannabis* samples.

**Table 4-2** The results obtained from presumptive color test and TLC of *Cannabis* samples

| **Samples** | **Results** | | | |
|---|---|---|---|---|
| | **Color test** | **Thin layer chromatography** | | |
| | | **THC** | **CBD** | **CBN** |
| Grown in QSBG | | | | |
| Hp03 | Red-purple | + | + | + |
| From Coordinate MA851857, 1013 m. from sea level, New Hmong Mae-Sa Village Moo.6,Pong-yang,Mae-rim,Chiangmai | | | | |
| Hp09 | Orange-red | + | + | + |
| Hp10 | Orange | + | + | + |
| Hp11 | Orange-red | + | + | + |
| Hp12 | Purple | + | + | + |
| Grown by seed from QSBG | | | | |
| Hp16 | Orange-red | + | + | + |
| Hp18 | ,, | + | + | + |
| Hp19 | ,, | + | + | + |
| Hp21 | Red-purple | + | + | + |
| Hp24 | ,, | + | + | + |
| Hp25 | ,, | + | + | + |
| Hp27 | Red-orange | + | + | + |
| Hp29 | Red | + | + | + |
| Hp 32 | Red-purple | + | + | + |
| Hp34 | Orange-red | + | + | + |
| From Hmong Kee-ree - ras Village, Pop-pra, Tak | | | | |
| Hp35 | Red-purple | + | + | + |
| Hp36 | ,, | + | + | + |
| Hp37 | ,, | + | + | - |
| Hp38 | ,, | + | + | + |
| Hp39 | ,, | + | + | + |
| Hp40 | ,, | + | + | + |
| Hp41 | ,, | + | + | - |
| Hp42 | ,, | + | + | + |
| Hp43 | ,, | + | + | + |
| Hp44 | ,, | + | + | + |

**Table 4-2** The results obtained from presumptive color test and TLC of *Cannabis* samples (cont.)

| Samples | Results | | | |
|---|---|---|---|---|
| | Color test | Thin layer chromatography | | |
| | | THC | CBD | CBN |
| From Hmong Kee-ree-ras Village, Pop-pra, Tak | | | | |
| Hp45 | Red-purple | + | + | + |
| From Coordinate MU810212, 853 m. from sea level, New Kee-ree-ras Village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | |
| Hp46 | Orange-red | + | + | + |
| Hp47 | Red-purple | + | + | - |
| Hp48 | ,, | + | + | - |
| Hp49 | ,, | + | - | - |
| Hp50 | ,, | + | + | - |
| Hp51 | Orange-red | + | - | - |
| Hp52 | Red-purple | + | + | - |
| Hp53 | Purple | + | + | - |
| Hp54 | Orange | + | + | - |
| Hp55 | Red-purple | + | - | - |
| From Coordinate MU 809201, 842 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | |
| Hp56 | Orange-red | + | + | - |
| Hp57 | Orange | + | + | + |
| From Coordinate MU 808151, 721m. from sea level, Ruam Thai pattana village 4,Ruam-Thai Pattana, Pop-pra, Tak | | | | |
| Hp58 | Orange-red | + | + | + |
| Hp59 | Orange | - | + | - |
| Hp60 | ,, | + | + | + |
| From Coordinate MU 798201, 830 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | |
| Hp61 | Red-orange | + | + | + |
| Hp62 | Red-purple | + | + | + |
| Hp63 | ,, | + | + | + |
| Hp64 | Orange-red | + | + | + |
| Hp65 | ,, | + | + | + |
| Hp66 | ,, | + | + | + |

**Table 4-2** The results obtained from presumptive color test and TLC of *Cannabis* samples(cont.)

| Samples | Results | | | |
|---|---|---|---|---|
| | Color test | Thin layer chromatography | | |
| | | THC | CBD | CBN |
| From Coordinate MU 798201, 830 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | |
| Hp67 | Red-purple | + | + | + |
| Hp68 | ,, | + | + | + |
| Hp69 | Orange-red | + | + | + |
| From Coordinate MU 814219, 864 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | |
| Hp70 | Red-purple | + | + | - |
| Hp71 | ,, | + | + | + |
| Hp72 | ,, | + | + | - |
| From Coordinate MU 990460, 824 m. from sea level, Ton Ma-muang village Moo.13, Mae-Tor, Muang, Tak | | | | |
| Hp73 | Red-purple | + | + | + |
| Hp74 | Orange | + | + | - |
| Hp75 | Red-purple | + | + | - |
| Hp76 | ,, | + | + | - |
| Hp77 | ,, | + | + | + |

Note: + = Positive result, - = Negative result

### 4.2.2 Gas chromatography- Mass spectrometry

The cannabinoid content in *Cannabis* samples were determined by the major ion of each cannabinoid. The major ions of $\Delta^9$-THC are m/z 299, 314, 231; CBD are m/z 231, 174, 314; CBN are m/z 295, 238, 310 and diphenylamine is m/z 169. The quantitation was calculated by using the area ratio of cannabinoid and internal standard.

The retention time of $\Delta^9$-THC, CBD, CBN and diphenylamine were 12.79, 12.29, 13.14 and 7.06 min, respectively. The total ion chromatogram of *Cannabis* sample was shown in Figure 4-4 and the results were shown in Table 4-3.

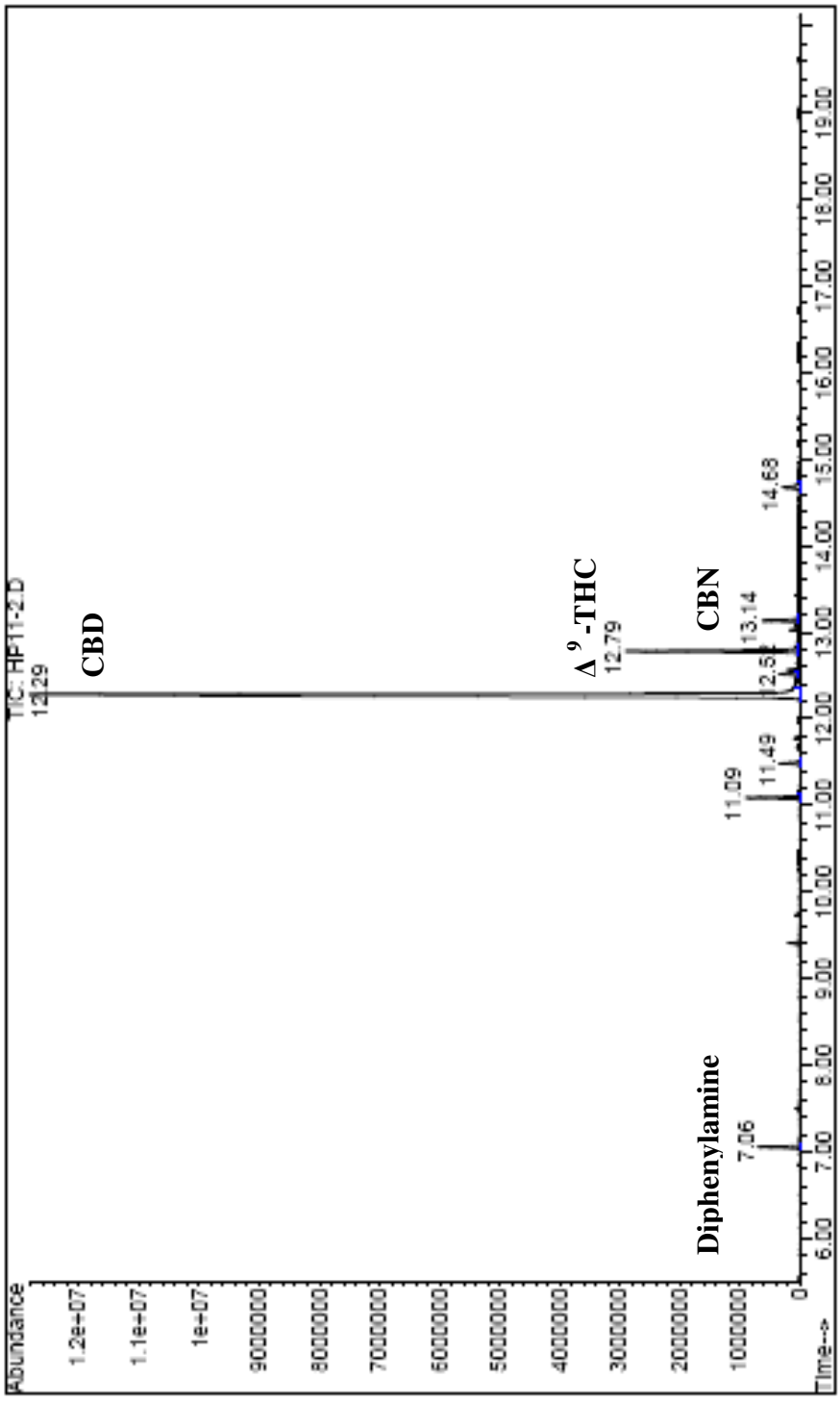



**Figure 4-4** Total ion chromatogram of *Cannabis* sample and diphenylamine as internal standard.

**Table 4-3** The cannabinoid content of *Cannabis* samples.

| Samples ID | Cannabinoid content | | | Chemical characterization | | |
|---|---|---|---|---|---|---|
| | %$\Delta^9$-THC | %CBD | %CBN | Phenotypic index $\frac{\%\Delta^9\text{-THC} + \%\text{ CBN}}{\%\text{ CBD}}$ | $\frac{\text{THC}}{\text{CBD}}$ ratio | |
| | | | | | Ratio | Chemotype |
| Grown in QSBG | | | | | | |
| Hp03 | 0.11 | 0.01 | 1.5E-04 | 11.02 >1 | 11.0 >1 | I |
| From Coordinate MA851857, 1013 m. from sea level, New Hmong Mae-Sa Village Moo.6, Pong-yang, Mae-rim, Chiangmai | | | | | | |
| Hp09 | 0.29 | 0.13 | 7.6E-03 | 2.29 >1 | 2.23 >1 | I |
| Hp10 | 0.18 | 0.85 | 2.9E-03 | 0.22 <1 | 0.21 <1 | III |
| Hp11 | 2.67 | 1.33 | 3.1E-02 | 2.03 >1 | 2.00 >1 | I |
| Hp12 | 1.10 | 0.03 | 5.3E-02 | 38.43 >1 | 36.66 >1 | I |
| Grown by seeds from QSBG | | | | | | |
| Hp16 | 0.14 | 0.30 | 1.3E-03 | 0.47<1 | 0.46 <1 | III |
| Hp18 | 1.17 | 0.28 | 1.8E-02 | 4.24 >1 | 4.17 >1 | I |
| Hp19 | 1.18 | 0.20 | 6.3E-03 | 5.93>1 | 5.90 >1 | I |
| Hp21 | 1.50 | 0.16 | 6.6E-04 | 9.38 >1 | 9.38 >1 | I |
| Hp24 | 2.60 | 0.39 | 2.9E-02 | 6.74 >1 | 6.66 >1 | I |
| Hp25 | 11.90 | 0.30 | 1.0E-02 | 39.70>1 | 39.66 >1 | I |
| Hp27 | 11.00 | 0.30 | 2.0E-02 | 36.73 >1 | 36.66 >1 | I |
| Hp29 | 7.32 | 0.12 | 6.4E-02 | 61.53 >1 | 61.00 >1 | I |
| Hp32 | 9.61 | 0.26 | 9.1E-02 | 37.31 >1 | 36.96 >1 | I |
| Hp34 | 2.98 | 0.51 | 1.5E-02 | 5.87 >1 | 5.84 >1 | I |
| From Hmong Kee-ree - ras Village, Pop-pra, Tak | | | | | | |
| Hp35 | 2.03 | 0.05 | 9.4E-03 | 40.79 >1 | 40.60 >1 | I |
| Hp36 | 1.46 | 0.03 | 7.1E-03 | 48.90 >1 | 48.66>1 | I |
| Hp37 | 0.61 | 0.01 | 1.5E-03 | 61.15 >1 | 61.00 >1 | I |
| Hp38 | 3.39 | 0.04 | 7.3E-03 | 84.93 >1 | 84.75 >1 | I |
| Hp39 | 4.70 | 0.13 | 3.4E-02 | 36.42 >1 | 36.15 >1 | I |
| Hp40 | 2.20 | 0.10 | 8.4E-03 | 22.08 >1 | 22.00 >1 | I |
| Hp41 | 2.00 | 0.03 | 5.9E-03 | 66.86 >1 | 66.66 >1 | I |
| Hp42 | 11.30 | 0.30 | 5.6E-02 | 37.85 >1 | 37.66 >1 | I |
| Hp43 | 3.10 | 0.14 | 1.4E-02 | 22.24 >1 | 22.14 >1 | I |
| Hp44 | 0.60 | 0.02 | 3.0E-03 | 30.15>1 | 30.0 >1 | I |
| Hp45 | 3.01 | 0.10 | 4.3E-02 | 30.53 >1 | 30.1 >1 | I |

**Table 4-3** The cannabinoid content of *Cannabis* samples. (cont.).

| Samples ID | Cannabinoid content | | | Chemical characterization | | |
|---|---|---|---|---|---|---|
| | | | | Phenotypic index $\frac{\%\Delta^9\text{-THC} + \%\text{ CBN}}{\%\text{ CBD}}$ | $\frac{\text{THC}}{\text{CBD}}$ ratio | |
| | $\%\Delta^9$-THC | %CBD | %CBN | | Ratio | Chemotype |
| From Coordinate MU810212, 853 m. from sea level, New Kee-ree-ras Village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra, Tak | | | | | | |
| Hp46 | 6.36 | 0.10 | 2.7E-03 | 63.62 >1 | 63.6 >1 | I |
| Hp47 | 1.80 | 0.01 | 7.6E-04 | 180.08 >1 | 180.00 >1 | I |
| Hp48 | 1.20 | 0.01 | 6.0E-04 | 120.06 >1 | 120.00 >1 | I |
| Hp49 | 3.68 | 0.06 | 1.8E-03 | 61.36 >1 | 61.33 >1 | I |
| Hp50 | 2.50 | 0.04 | 1.4E-03 | 62.54 >1 | 62.50 >1 | I |
| Hp51 | 2.20 | 0.20 | 7.5E-04 | 11.00 >1 | 11.00 >1 | I |
| Hp52 | 4.80 | 0.06 | 2.0E-03 | 80.03 >1 | 80.00>1 | I |
| Hp53 | 1.40 | 0.03 | 5.0E-04 | 46.68 >1 | 46.66 >1 | I |
| Hp54 | 2.60 | 0.40 | 9.0E-04 | 6.50 >1 | 6.50 >1 | I |
| Hp55 | 2.10 | 0.05 | 8.0E-04 | 42.02 >1 | 42.00 >1 | I |
| From Coordinate MU 809201, 842 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | | | |
| Hp56 | 1.30 | 0.20 | 9.0E-04 | 6.51 >1 | 6.50 >1 | I |
| Hp57 | 1.70 | 0.03 | 9.0E-03 | 56.96 >1 | 56.66 >1 | I |
| From Coordinate MU 808151, 721m. from sea level, Ruam Thai pattana village 4,Ruam-Thai Pattana, Pop-pra, Tak | | | | | | |
| Hp58 | 9.14 | 0.16 | 2.5E-03 | 57.14 >1 | 57.13 >1 | I |
| Hp59 | 0.05 | 0.04 | 5.0E-05 | 1.25 >1 | 1.25 ≈ 1 | II |
| Hp60 | 3.94 | 0.51 | 3.1E-03 | 7.73 >1 | 7.72 >1 | I |
| From Coordinate MU 798201, 830 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | | | |
| Hp61 | 8.50 | 0.16 | 7.1E-03 | 53.17 >1 | 53.13 >1 | I |
| Hp62 | 2.58 | 0.06 | 2.8E-03 | 43.05 >1 | 43.00 >1 | I |
| Hp63 | 8.40 | 0.12 | 4.4E-03 | 70.04 >1 | 70.00 >1 | I |
| Hp64 | 9.70 | 0.30 | 6.0E-03 | 32.35 >1 | 32.33 >1 | I |
| Hp65 | 6.60 | 0.11 | 5.4E-03 | 60.05 >1 | 60.00 >1 | I |
| Hp66 | 10.17 | 0.21 | 5.9E-03 | 48.45 >1 | 48.42 >1 | I |
| Hp67 | 3.05 | 0.09 | 3.6E-03 | 33.92 >1 | 33.88 >1 | I |
| Hp68 | 2.47 | 0.10 | 3.0E-03 | 24.73 >1 | 24.70 >1 | I |
| Hp69 | 2.10 | 0.35 | 3.0E-03 | 6.01 >1 | 6.00 >1 | I |

**Table 4-2** The cannabinoid content of *Cannabis* samples.(cont.).

| Samples ID | Cannabinoid content | | | Chemical characterization | | |
|---|---|---|---|---|---|---|
| | | | | Phenotypic index $\frac{\%\Delta^9\text{-THC} + \%\text{ CBN}}{\%\text{ CBD}}$ | $\frac{\text{THC}}{\text{CBD}}$ ratio | |
| | $\%\Delta^9$-THC | %CBD | %CBN | | Ratio | Chemotype |
| From Coordinate MU 814219, 864 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | | | | | |
| Hp70 | 0.96 | 0.02 | 6.5E-04 | 48.03 >1 | 48.00 >1 | I |
| Hp71 | 4.10 | 0.10 | 2.3E-03 | 41.02 >1 | 41.00 >1 | I |
| Hp72 | 1.16 | 0.02 | 1.2E-03 | 58.06 >1 | 58.00 >1 | I |
| From Coordinate MU 990460, 824 m. from sea level, Ton Ma-muang village Moo.13, Mae-Tor, Muang, Tak | | | | | | |
| Hp73 | 5.02 | 0.06 | 7.3E-03 | 83.78 >1 | 83.66 >1 | I |
| Hp74 | 1.48 | 0.08 | 1.5E-03 | 18.52 >1 | 18.50 >1 | I |
| Hp75 | 2.33 | 0.02 | 3.4E-03 | 116.67 >1 | 116.50 >1 | I |
| Hp76 | 3.74 | 0.03 | 2.9E-03 | 124.76 >1 | 124.66 >1 | I |
| Hp77 | 9.70 | 0.20 | 1.0E-02 | 48.55 >1 | 48.50 >1 | I |

Note: NR= No result

The percentage of $\Delta^9$-THC content varied from 0.05% to 11.90%, the result showed that only 5 samples, Hp03, Hp09, Hp10, Hp16 and Hp59 which had $\%\Delta^9$-THC lower than 0.3%, indicating that they were fiber type. All the other samples had high % $\Delta^9$-THC which were classified as the drug type. But from the phenotypic index, $\frac{\%\Delta^9\text{-THC} + \%\text{ CBN}}{\%\text{ CBD}}$ (12), only 2 samples, Hp10 and Hp16, had ratio less than 1, which is fiber type. Using the ratio of $\frac{\text{THC}}{\text{CBD}}$ (3) only Hp10 and Hp16 had the ratio less than 1 and classified as chemotype III. Hp59 had the ratio close to 1 which was chemotype II. But all other samples had high ratio of $\frac{\text{THC}}{\text{CBD}}$, which was chemotype I.

## 4.3 DNA analysis

In this stage of analysis, DNA of *Cannabis* samples were amplified by using two primer pairs which were *Cannabis sativa* specific primers (G & H) and Drug type marker (g & h). The results were as follows.

### 4.3.1 *Cannabis sativa* specific primers (G and H)

All samples were successfully amplified and gave a positive results for *Cannabis sativa* specific primers (G and H) of the *trn*L-*trn*F locus in chloroplast to checking DNA and the presence of *Cannabis sativa* which reported by Linacre and Thorpe (1998)(24). The expected PCR product fragment is 197 bp. The positive control result and the sample results were shown in figure 4-5 and figure 4-6, respectively.

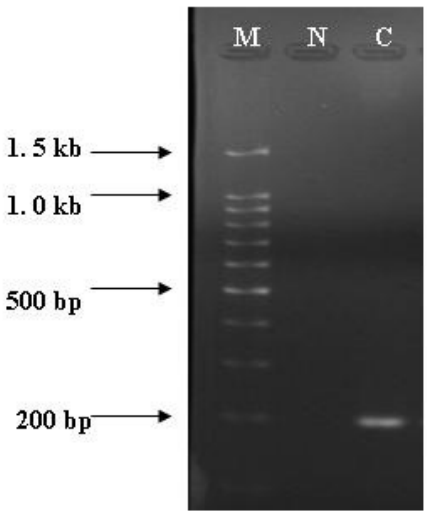
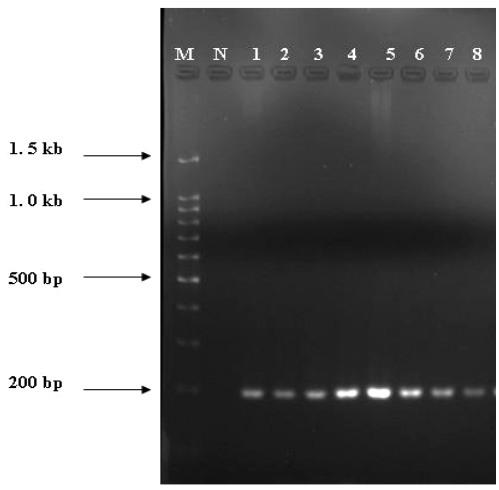
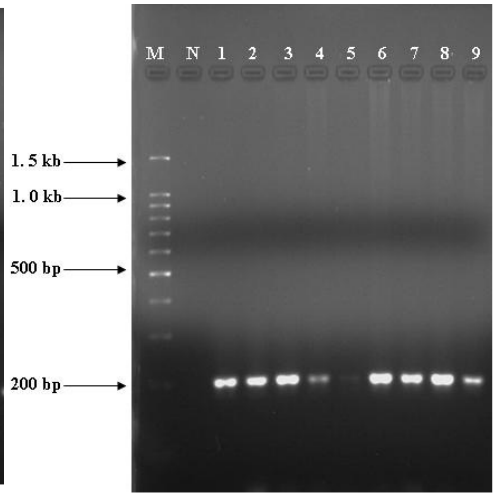


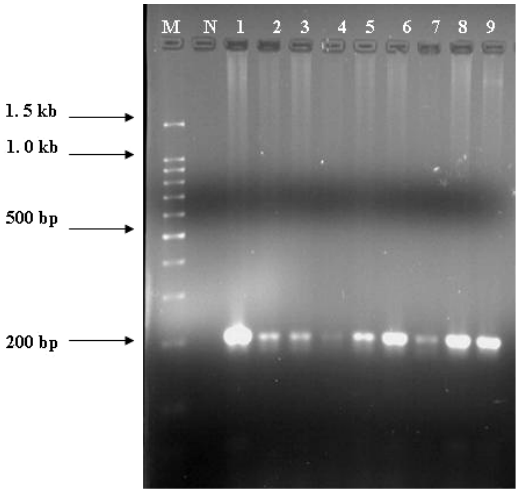

**Figure 4-5** Positive control (C) of PCR product (197 bp.) of *Cannabis sativa* specific primer compared with negative control (N) along with 100 bp ladder (M) as marker size.


M N 1 2 3 4 5 6 7 8
1. 5 kb
1. 0 kb
500 bp
200 bp

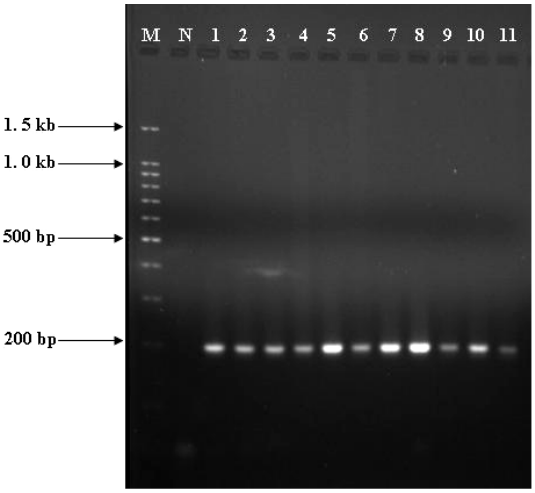
**a).**


M N 1 2 3 4 5 6 7 8 9
1. 5 kb
1. 0 kb
500 bp
200 bp

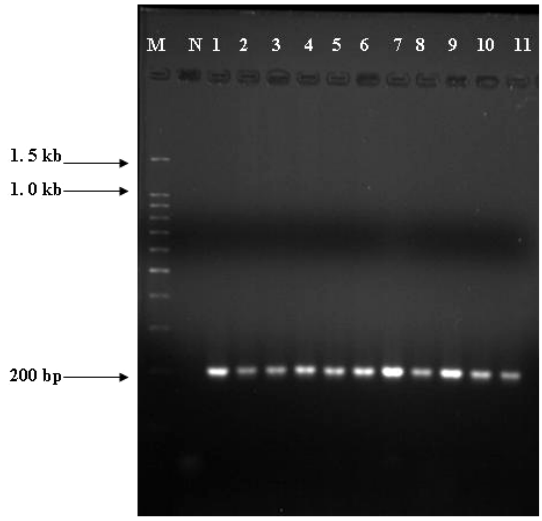
**b).**


M N 1 2 3 4 5 6 7 8 9
1. 5 kb
1. 0 kb
500 bp
200 bp

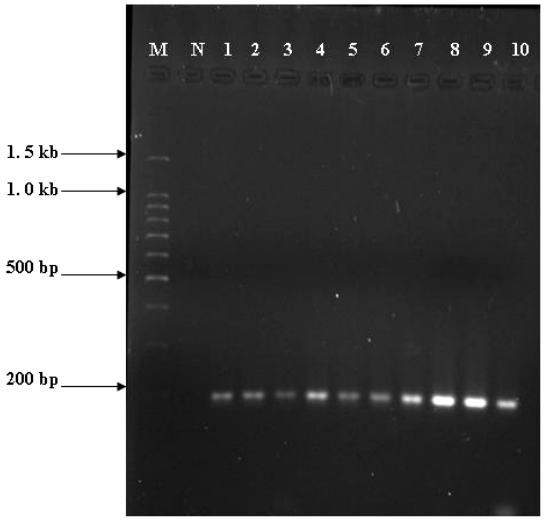
**c).**


M N 1 2 3 4 5 6 7 8 9 10 11
1. 5 kb
1. 0 kb
500 bp
200 bp

**d).**




**e).**




**f).**

| Lane | a). | b). | c). | d). | e). | f). |
|---|---|---|---|---|---|---|
| 1 | Hp03 | Hp21 | Hp37 | Hp46 | Hp57 | Hp68 |
| 2 | Hp09 | Hp24 | Hp38 | Hp47 | Hp58 | Hp69 |
| 3 | Hp10 | Hp25 | Hp39 | Hp48 | Hp59 | Hp70 |
| 4 | Hp11 | Hp27 | Hp40 | Hp49 | Hp60 | Hp71 |
| 5 | Hp12 | Hp29 | Hp41 | Hp50 | Hp61 | Hp72 |
| 6 | Hp16 | Hp32 | Hp42 | Hp51 | Hp62 | Hp73 |
| 7 | Hp18 | Hp34 | Hp43 | Hp52 | Hp63 | Hp74 |
| 8 | Hp19 | Hp35 | Hp44 | Hp53 | Hp64 | Hp75 |
| 9 | | Hp36 | Hp45 | Hp54 | Hp65 | Hp76 |
| 10 | | | | Hp55 | Hp66 | Hp77 |
| 11 | | | | Hp56 | Hp67 | |

M= 100 bp ladder, N= negative control

**Figure 4-6 a).-f).** The 197 bp. PCR products of *Cannabis sativa* specific primer of *Cannabis* samples on 2% agarose gel

### 4.3.2 Drug type - marker (g and h)

After amplified with *Cannabis sativa* specific primers, the DNA samples were amplified by drug type marker (g and h). The expected PCR product is 1.2kb. The detection of samples and positive control for drug and fiber types were shown in figure 4-7 and figure 4-8.

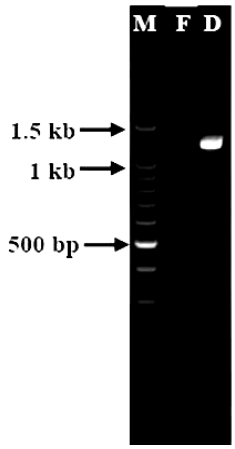
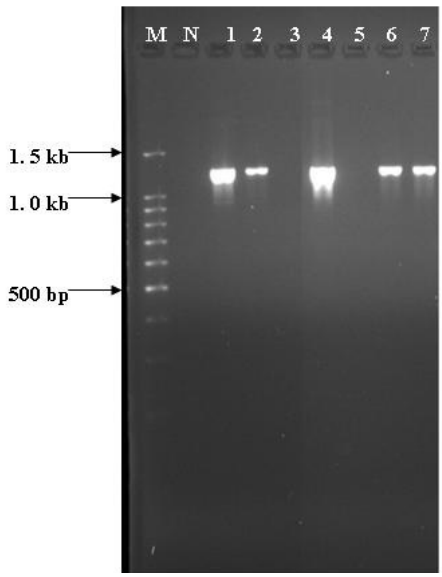
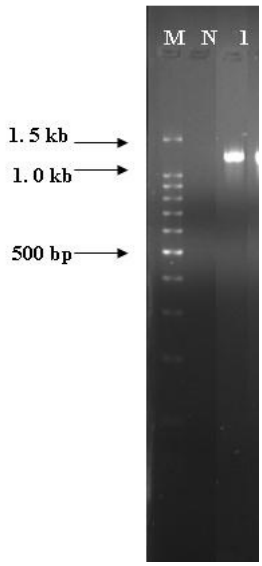


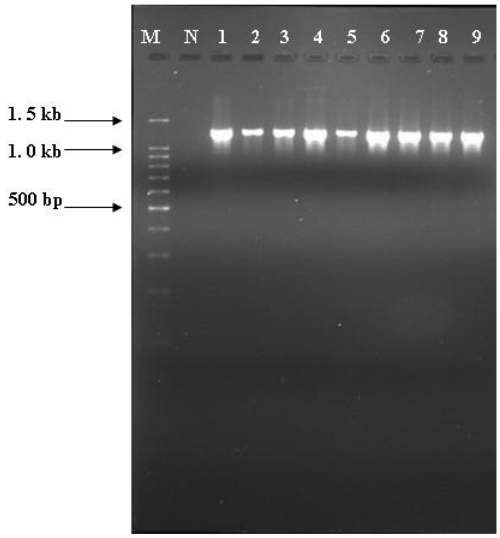

**Figure 4-7** Positive control of PCR product 1.2 kb of Drug type marker amplified with drug (D) and fiber (F) types with 100 bp ladder (M)



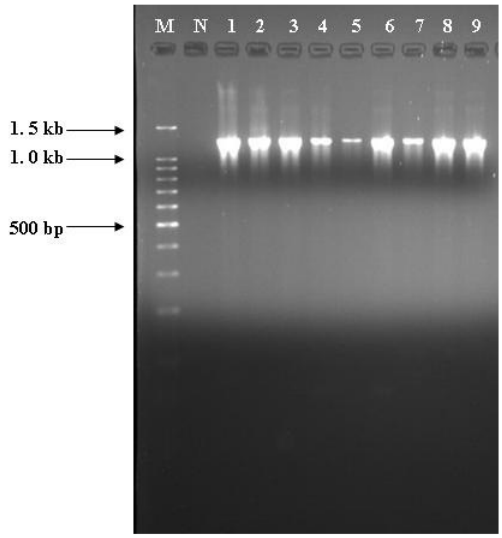

**a).**



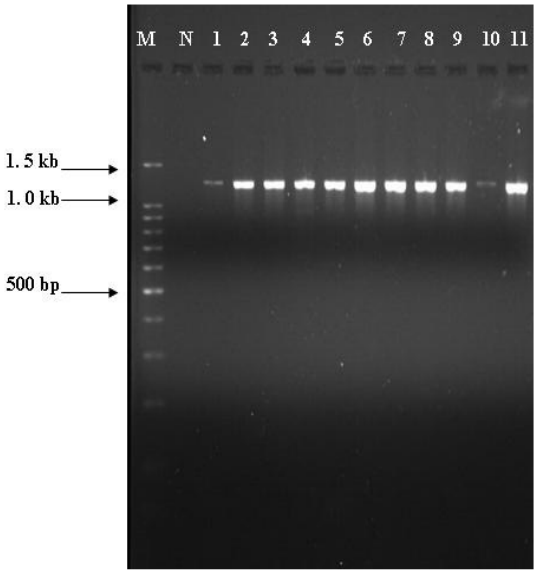

**b).**


M N 1 2 3 4 5 6 7 8 9
1. 5 kb
1. 0 kb
500 bp

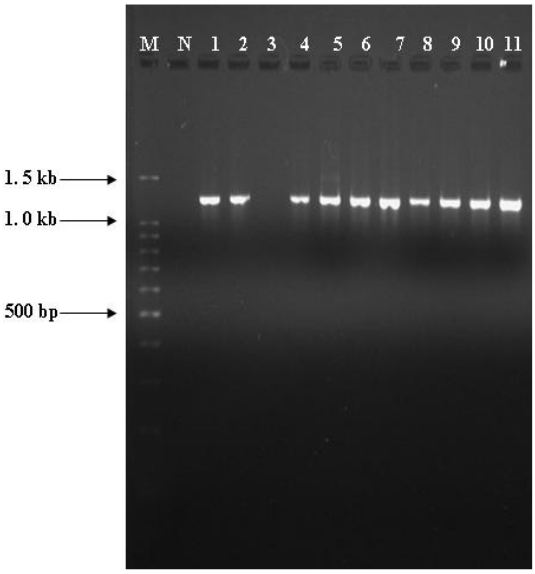
**c).**


M N 1 2 3 4 5 6 7 8 9
1. 5 kb
1. 0 kb
500 bp

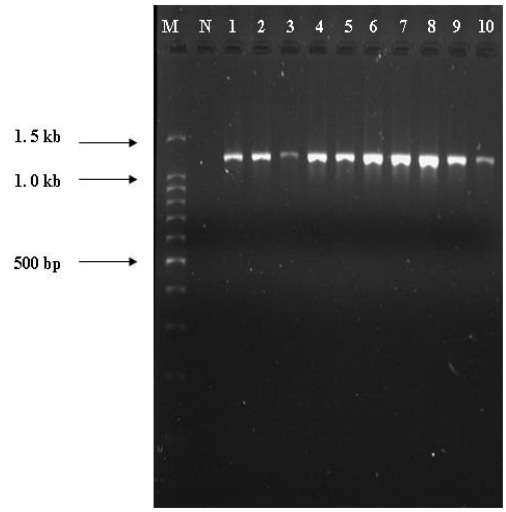
**d).**


M N 1 2 3 4 5 6 7 8 9 10 11
1. 5 kb
1. 0 kb
500 bp

**e).**


M N 1 2 3 4 5 6 7 8 9 10 11
1. 5 kb
1. 0 kb
500 bp

**f).**




**g).**

| Lane | a). | b). | c). | d). | e). | f). | g). |
|---|---|---|---|---|---|---|---|
| 1 | Hp03 | Hp11 | Hp21 | Hp37 | Hp46 | Hp57 | Hp68 |
| 2 | Hp09 | | Hp24 | Hp38 | Hp47 | Hp58 | Hp69 |
| 3 | Hp10 | | Hp25 | Hp39 | Hp48 | Hp59 | Hp70 |
| 4 | Hp12 | | Hp27 | Hp40 | Hp49 | Hp60 | Hp71 |
| 5 | Hp16 | | Hp29 | Hp41 | Hp50 | Hp61 | Hp72 |
| 6 | Hp18 | | Hp32 | Hp42 | Hp51 | Hp62 | Hp73 |
| 7 | Hp19 | | Hp34 | Hp43 | Hp52 | Hp63 | Hp74 |
| 8 | | | Hp35 | Hp44 | Hp53 | Hp64 | Hp75 |
| 9 | | | Hp36 | Hp45 | Hp54 | Hp65 | Hp76 |
| 10 | | | | | Hp55 | Hp66 | Hp77 |
| 11 | | | | | Hp56 | Hp67 | |

M=100 bp. ladder N=negative control

**Figure 4-8 a) – g).** The results of 1.2kb of PCR product of drug type marker of *Cannabis* samples on 2% agarose gel

Most samples showed positive results of drug type marker, except Hp10, Hp16 and Hp59 showed negative results indicating that these 3 samples were fiber type. The DNA results, *Cannabis sativa* specific primer and drug type marker, were summarized and shown in table 4-4.

**Table 4-4** The identification of *Cannabis sativa* L. samples using DNA techniques.

| **Sample ID** | **DNA results** | |
|---|---|---|
| | ***Cannabis sativa* specific primer (G & H)** | **Drug type-marker (g & h)** |
| Grown in QSBG | | |
| Hp03 | + | + |
| From Coordinate MA851857, 1013 m. from sea level, New Hmong Mae-Sa Village Moo.6,Pong-yang, Mae-rim, Chiangmai | | |
| Hp09 | + | + |
| Hp10 | + | - |
| Hp11 | + | + |
| Hp12 | + | + |
| Grown by seeds from QSBG | | |
| Hp16 | + | - |
| Hp18 | + | + |
| Hp19 | + | + |
| Hp21 | + | + |
| Hp24 | + | + |
| Hp25 | + | + |
| Hp27 | + | + |
| Hp29 | + | + |
| Hp32 | + | + |
| Hp34 | + | + |
| From Hmong Kee-ree - ras Village, Pop-pra, Tak | | |
| Hp35 | + | + |
| Hp36 | + | + |
| Hp37 | + | + |
| Hp38 | + | + |
| Hp39 | + | + |

**Table 4-4** The identification of *Cannabis sativa* L. samples using DNA techniques. (cont).

| **Samples ID** | ***Cannabis sativa* specific primer (G & H)** | **Drug type-marker (g & h)** |
|---|---|---|
| From Hmong Kee-ree - ras Village, Pop-pra, Tak | | |
| Hp40 | + | + |
| Hp41 | + | + |
| Hp42 | + | + |
| Hp43 | + | + |
| Hp44 | + | + |
| Hp45 | + | + |
| From Coordinate MU810212, 853 m. from sea level, New Kee-ree-ras Village (Romglao) Moo.9, Kee-ree-ras, Pop-pra, Tak | | |
| Hp46 | + | + |
| Hp47 | + | + |
| Hp48 | + | + |
| Hp49 | + | + |
| Hp50 | + | + |
| Hp51 | + | + |
| Hp52 | + | + |
| Hp53 | + | + |
| Hp54 | + | + |
| Hp55 | + | + |
| From Coordinate MU 809201, 842 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | |
| Hp56 | + | + |
| Hp57 | + | + |
| From Coordinate MU 808151, 721m. from sea level, Ruam Thai pattana village 4,Ruam-Thai Pattana, Pop-pra, Tak | | |
| Hp58 | + | + |
| Hp59 | + | - |
| Hp60 | + | + |
| From Coordinate MU 798201, 830 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | |
| Hp61 | + | + |
| Hp62 | + | + |

**Table 4-4** The identification of *Cannabis sativa* L. samples using DNA techniques. (cont).

| **Samples ID** | ***Cannabis sativa* specific primer (G & H)** | **Drug type-marker (g & h)** |
|---|---|---|
| From Coordinate MU 798201, 830 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | |
| Hp63 | + | + |
| Hp64 | + | + |
| Hp65 | + | + |
| Hp66 | + | + |
| Hp67 | + | + |
| Hp68 | + | + |
| Hp69 | + | + |
| From Coordinate MU 814219, 864 m. from sea level, New Kee-ree-ras village (Rom-glao) Moo.9, Kee-ree-ras, Pop-pra,Tak | | |
| Hp70 | + | + |
| Hp71 | + | + |
| Hp72 | + | + |
| From Coordinate MU 990460, 824 m. from sea level, Ton Ma-muang village Moo.13, Mae-Tor, Muang, Tak | | |
| Hp73 | + | + |
| Hp74 | + | + |
| Hp75 | + | + |
| Hp76 | + | + |
| Hp77 | + | + |

Note: + = Positive result, - = Negative result

# CHAPTER V
# DISCUSSION

Fifty-eight of *Cannabis sativa* L. fiber type samples were collected from various part of cultivation areas. The samples were claimed to be fiber type by the farmers and the ONCB officer. Samples were tested by 4 different methods which are widely used in forensic laboratories: color test, thin layer chromatography, gas chromatography-mass spectrometry and DNA.

Color test (Fast blue b salt test) is recommended method to use as field test to screen the high numbers of suspected material (5). The results showed the color of the major cannabinoid content due to the reaction with the Fast blue B salt. The color obtained varied from orange-red to red-purple. The test indicated the presence of *Cannabis sativa* L. and can distinguish the types of *Cannabis* in case of pure fiber type which has high concentration of CBD. But it is necessary to confirm using other test methods. If the amount of seized samples is not enough to perform the screening test, it is recommended to send the samples to the equip laboratory.

The samples were tested using TLC. Most of samples were in agreement with the results of Wattanasiri (2006). Fiber type will show the light band of THC and intense band of CBD with no CBN, but some samples gave intense bands of THC, CBD and CBN. Some samples showed a purple band of CBN which may be degraded product from THC during storage (13). To detect CBD band it is recommended to use both low and high concentration for TLC analysis. The orange band at Rf = 0.2, which was reported and proposed by Wattansiri (2006) to be CBDA band, did not appear in every sample. Sixteen samples which gave orange band, showed the intense CBD band. However, some samples with the orange band still showed intense THC band which should not be observed in the fiber type sample. According to Paris and Nahas in 1984 (27), samples which has both THC and CBD can be classified as intermediate type while fiber type should have the light band of THC and intense band of CBD. Thin layer chromatography is one of recommended

method to confirm the suspected sample which can be performed in the basic laboratory. But the skill of scientist and suitable TLC system are required.

The GC-MS condition was modified from M.Stefanidou *et al* (1). The three criteria to classify the drug and fiber types are shown in table 4-3. Most samples claimed by the farmers and the ONCB officer as fiber type, are more likely to be drug type based on the high concentration of $\Delta^9$-THC. Two samples, Hp03 and Hp09 which had $\Delta^9$-THC lower than 0.3%, should be classified as fiber type but the phenotypic index $\frac{\%\Delta^9\text{-THC} + \%\,\text{CBN}}{\%\,\text{CBD}}$ and $\frac{\text{THC}}{\text{CBD}}$ ratio indicated drug type. Therefore they should be classified as drug type (1).

For GC-MS analysis, the cannabinoid contents were calculated using only one calibration point, so the quantitation might not be accurate for samples which have values lower or higher than the calibration value. This error was observed in Hp59 which was chemotype II based on the ratio of THC/CBD, but based on % $\Delta^9$-THC this sample should be classified as chemotype III. Most samples showed high concentration of $\Delta^9$-THC and low concentration of CBD. This high ratio supported the classification of samples to be drug type (figure 5-1). However, the main purpose of this study is to survey the situation of fiber type cannabis growing in Thailand. These values are representation of the real situation of fiber type cannabis cultivated in Thailand and can be the guideline for cultivation control.

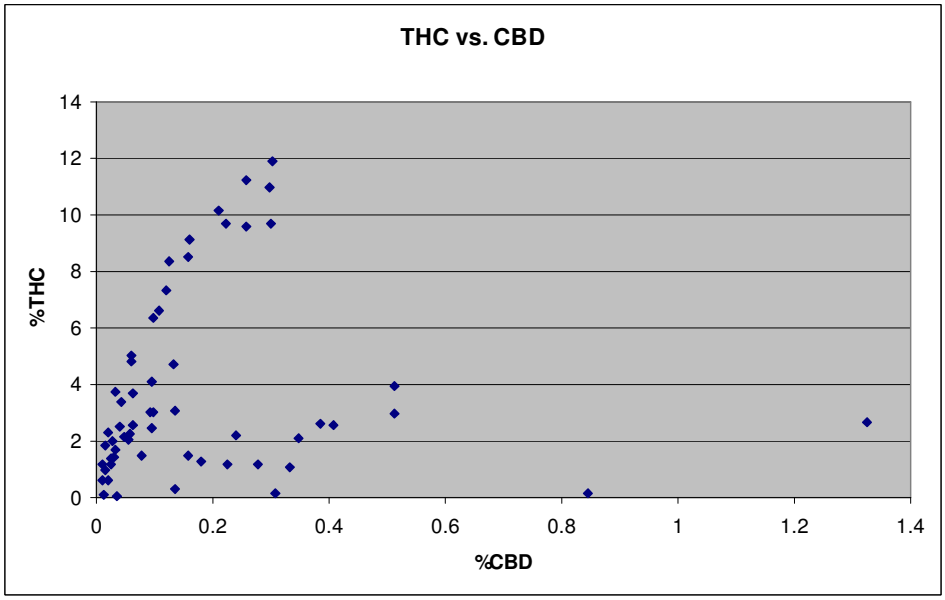



**Figure 5-1** The ratio of $\Delta^9$-THC and CBD

In this present study, all the samples were identified as *Cannabis sativa* L. using specific primer (G and H) (24). All samples showed positive result. DNA samples were amplified with a second primers pair (g and h), the drug type marker (23). Only 3 from 58 samples did not give PCR product using drug type marker indicating that these samples were fiber type. The result agreed with Kojoma *et al.* (2006) and showed the working of this primers pair in THCA synthase gene. But if the rarely silence mutation occur in the gene and the primers pair cannot match at the primers binding site, the drug type will give the negative result for drug type marker and make result is mistake of classification. This method can classify cannabis into 2 types, drug and fiber types, but for the intermediate type it will be included in drug type. DNA method is useful for analysis in early vegetative stage of plant. Old samples and degraded DNA sample may not be successfully amplified. The method requires the practical skill to perform. Mass routine analysis and extraction method can reduce the cost of test.

From the present study, *Cannabis sativa* L. samples were classified into *Cannabis* type according to chemical criteria as describe in section 2.4. Criteria which defined by Fetterman *et al.*(12), Debruyne *et al*. (25) and Small and Beckstead (3) were following in the same way of DNA analysis which can discriminate drug type from fiber type. But in criteria of Small and Beckstead (3) gave the number of intermediate type in Hp59 and gave the $\Delta^9$-THC as followed by EU.regulation (28)as previous described, therefore the suitable criteria which are useful to support the DNA analysis in this study is the concentration of $\Delta^9$-THC then use criteria Fetterman *et al.*(12), Debruyne *et al*. (25) and Small and Beckstead (3) to support, respectively. In Paris and Nahas (27) and De.Meijer *et al* (35) were not applicable to use for classification due to the low concentration of CBD in samples.

The classification result by chemical criteria was summarized in table 5-1.

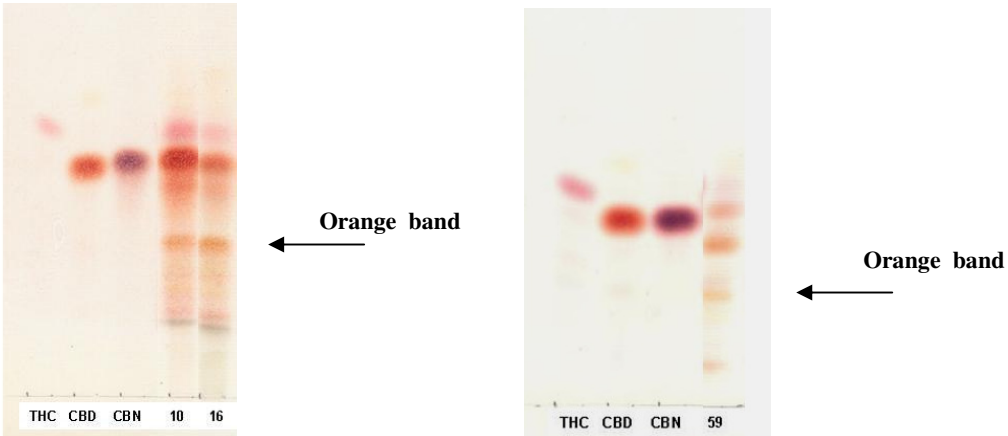
**Table 5-1** Classification of 58 *Cannabis* samples according to published criteria (section 2.4).

| Classification method(Ref.) | % Fiber type | % Intermediate type | % Drug type |
|---|---|---|---|
| a). Fetterman *et al.*(12) | 5.2 | N/A | 94.8 |
| b).Debruyne *et al*. (25) | 5.2 | N/A | 94.8 |
| c).Paris and Nahas (27) | N/A | N/A | N/A |
| d).Small and Beckstead (3) | 3.5 | 1.7 | 94.8 |
| e). De.Meijer *et al* (35) | N/A | N/A | 89.6 |
| f). EU.regulation (28) | 8.6 | N/A | 91.4 |
| g).DNA analysis(23) | 5.2 | N/A | 94.8 |

N/A= Not Applicable

Chemical analysis by GC-MS was used for a) to f), whereas the DNA analysis of the THCA synthase gene region was used for g).

Based on the results, three samples, Hp10, Hp16 and Hp59 which gave the negative result for drug type marker, had the $\Delta^9$-THC lower than 0.3 % and the index ratio less than 1, can be classified as fiber type. TLC pattern gave light band of THC and intense of CBD band and no CBN, also the extra orange band on the low Rf.0.2 (as shown in figure 5-2a and b). Other samples should be classified as drug type due to high $\Delta^9$-THC content, although it was claimed to be fiber type by the farmer and the ONCB officer. Even though the high concentration of THC, the extracting THC from fiber type *Cannabis* is not easy to perform in terms of refining and CBD elimination, also the hazardous process (14), to promote the fiber type cultivation the government should checked the plant for real fiber type.




**a).** **b).**

**Figure 5-2 a). and b).** TLC chromatogram showing the extra orange band at Rf = 0.2

*Cannabis sativa* L. was cultivated throughout the world. Chemical component varies among the samples. Environmental conditions are one of the high impact factor to build up the variation such as warm climate, sunlight, the quality of soil in the cultivation field and high level from the sea can induce the high amount of psychoactive component(1). In the present study, the effect of the environment can not be concluded due to the difference of cultivation areas. Further more, the other important factor is the genetic variation which occurs in the genotype and express in phenotype. Morphology or chemical component of fiber type may change to be drug type. Cross pollination in critical area which cultivated fiber type close to drug type area may lead to get fiber type with high concentration of psychoactive component (14), drug type pollen can spread as far as in 12 kilometers, the cultivation area of Tak and Chiangmai provinces are open field, therefore it is possible to get the pollen of drug type cannabis from strong wind in the rainy season. It is recommended that to avoid contamination with drug type, the fiber type cultivation area should be 12 kilometers apart.

The advantages and limitation of each method used in this study was summarized in table 5-2.

**Table 5-2** The advantage, disadvantage and suitable methods use in the present study.

| Methods | Samples requirements | Advantages | Disadvantages | Suitable usage |
|---|---|---|---|---|
| Color test (Fast blue b salt) | -Fresh dried or old dried samples<br>-Gram of samples | -Easy and non complicate reagent to perform<br>-Low cost<br>-Practical and quick in field test<br>-Showed the presence of *Cannabis* plant | -Showed in combination color<br>-Can't distinguish between drug and fiber type<br>-False positive can be occurred<br>-required confirmatory test | -Pre screening in field test |
| Thin layer chromatography (TLC) | -Freshly, fresh dried or old dried samples<br>-Small amount in milligram | -Easier than another chromatographic methods without complication equipment<br>-Showed the unique pattern of samples which can be used in subtype discrimination<br>-Qualitative and semi-quantitative analysis<br>--Low cost and perform in basic lab | -Require standard solution<br>-Require suitable extraction and solvent system<br>-Require experience in the test performing | -Confirmatory test for suspect material |
| Gas chromatography-Mass Spectrometry (GC-MS) | -Freshly, fresh dried or old dried samples<br>-Small amount in milligram | -Qualitative and quantitative analysis<br>-Show pattern of chromatogram<br>-Low cost in mass routine lab analysis<br>-Good for volatile compound | -Require high technology equipment<br>-Only in fully equips lab<br>-High maintenances cost<br>-Not good in non-volatile compound | -Confirmatory and definitely test for suspect material |
| DNA technology | -Fresh samples<br>-Dried samples without DNA degraded<br>-Number of sample upon on extraction method | -Discriminate type of Cannabis plant<br>-Shows the presence of *Cannabis* plant<br>-Low cost (depend on extraction method) in mass routine lab analysis<br>-Useful for early vegetative stage plant | -Can't perform on degraded DNA<br>-Carcinogen expose | -Confirmatory test for suspect material |

# CHAPTER VI
# CONCLUSION AND SUGGESTIONS

1. *Cannabis sativa* L. fiber type cultivated in Thailand were collected and identified by 4 different methods, color test, TLC, GC-MS and DNA method. Samples were classified into types as follow:

**Table 6-1**. Classification of the surveyed *Cannabis* plant. (according to DNA method and chemical criteria by Fetterman *et al* (1971), Debruyne *et al* (1981) and Small and Beckstead (1973))

| Area | Number of plant analyzed | Fiber type | Drug type |
|---|---|---|---|
| QSBG, Chiangmai | 11 | 1 | 10 |
| New Hmong Mae-Sa Village, Mae rim, Chiangmai | 4 | 1 | 3 |
| Rom-glao village, Kee-ree-ras, Pop-pra, Tak | 35 | 0 | 35 |
| Ruam Thai pattana village 4,Ruam-Thai Pattana, Pop-pra, Tak | 3 | 1 | 2 |
| Ton Ma-muang village, Moo.13, Mae-Tor, Muang, Tak | 5 | 0 | 5 |
| Total | 58 (100%) | 3(5.2%) | 53(94.8%) |

2. In this present study, the best classification method to identify *Cannabis sativa* L. is DNA method because every sample which gave the negative result for drug type marker showed $\Delta^9$ –THC < 0.3%, this is also accepted in every chemical criteria. The suspect sample which had intermediate value of $\Delta^9$ –THC and index ratio will be excluded from non-drug type. The test can perform even in vegetative stage of plant and only small amount of material is required for the analysis. DNA testing can be further modified for high throughput screening of the suspect sample. This would also reduce the analysis cost. TLC can be used for screening to the

suspect sample, however, the sample have to be further analyzed by GC-MS as confirmatory testing and classification of cannabis type.

3. The orange band (Rf.0.2) which may be CBDA can be used to confirm fiber type in TLC method, however it can be presented in drug type or intermediate type which had high CBD.

4. Environmental condition and genetic variation are the important factors to induce the high concentration of cannabinoid content, before promoting commercial cultivation, the organization which is in charge of this programme should consider the cannabinoid content in the plants. The seeds should be screened for pure fiber seeds before distributing to the farmers to prevent the expansion of drug type in Thailand.

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# APPENDIX

```
          ============================================================
                                6890 GC METHOD
          ============================================================
OVEN
     Initial temp: 100 'C (On)                  Maximum temp: 325 'C
     Initial time: 0.00 min                     Equilibration time: 2.00 min
     Ramps:
              # Rate          Final temp   Final time
              1 15.00            300           8.00
              2 0.0(Off)
     Post temp:   300 'C
     Post time:   1.00 min
     Run time:   21.33 min
FRONT INLET (SPLIT/SPLITLESS)               BACK INLET (SPLIT/SPLITLESS)
   Mode: Split                                 Mode: Split
   Initial temp: 220 'C (On)                   Initial temp: 50 'C (Off)
   Pressure: 10.33 psi (On)                    Pressure: 0.00 psi (Off)
   Split ratio: 20:1                           Total flow: 45.0 mL/min
   Split flow: 19.7 mL/min                     Gas saver: Off
   Total flow: 23.4 mL/min                     Gas type: Helium
   Gas saver: Off
   Gas type: Helium

COLUMN 1                                    COLUMN 2
      Capillary Column                        (not installed)
      Model Number: Agilent 19091S-433
      HP-5MS, 0.25mm * 30m * 0.25um
      Max temperature: 350 'C
      Nominal length: 30.0 m
      Nominal diameter: 250.00 um
      Nominal film thickness: 0.25 um
      Mode: constant flow
      Initial flow: 1.0 mL/min
      Nominal init pressure: 10.33 psi
      Average velocity: 37 cm/sec
      Inlet: Front Inlet
      Outlet: MSD
      Outlet pressure: vacuum

FRONT DETECTOR (TCD)                        BACK DETECTOR (µECD)
      Temperature: 250 'C (Off)                  Temperature: 250 'C (Off)
      Reference flow: 20.0 mL/min (Off)          Mode: Constant makeup flow
      Mode: Constant makeup flow                 Makeup flow: 60.0 mL/min (On)
      Makeup flow: 7.0 mL/min (On)               Makeup Gas Type: Nitrogen
      Makeup Gas Type: Nitrogen                  Electrometer: Off
      Filament: Off
      Negative polarity: Off

SIGNAL 1                                    SIGNAL 2
      Data rate: 20 Hz                           Data rate: 20 Hz
      Type: test plot                            Type: test plot
      Save Data: Off                             Save Data: Off
      Zero: 0.0 (Off)                            Zero: 0.0 (Off)
      Range: 0                                   Range: 0
      Fast Peaks: Off                            Fast Peaks: Off
      Attenuation: 0                             Attenuation: 0

COLUMN COMP 1                               COLUMN COMP 2
     (No Detectors Installed)                    (No Detectors Installed)

THERMAL AUX 2
      Use: MSD Transfer Line Heater
      Description:
      Initial temp: 280 'C (On)
```

```
      Initial time: 0.00 min
           # Rate Final temp   Final time
           1 0.0(Off)
                                          POST RUN
                                             Post Time: 1.00 min
                                             Oven Temperature: 300 'C
                                             Column 1 Flow: 0.5 mL/min
TIME TABLE
    Time      Specifier                    Parameter & Setpoint
                        7673 Injector
               Front Injector:
                    Sample Washes              5
                    Sample Pumps               3
                    Injection Volume           1.0 microliters
                    Syringe Size               5.0 microliters
                    PostInj Solvent A Washes   3
                    PostInj Solvent B Washes   0
                    Viscosity Delay            0 seconds
                    Plunger Speed              Fast
                    PreInjection Dwell         0.00 minutes
                    PostInjection Dwell        0.00 minutes

               Back Injector:
          No parameters specified
                                  MS ACQUISITION PARAMETERS
General Information
------- -----------
           Tune File             :    atune.u
           Acquistion Mode       :    Scan
MS Information
-- -----------
           Solvent Delay         :    3.50 min
           EM Absolute           :    False
           EM Offset             :    0
           Resulting EM Voltage  :    1694.1

[Scan Parameters]

           Low Mass              :    40.0
           High Mass             :    400.0
           Threshold             :    100
           Sample #              :    2             A/D Samples 4

[MSZones]
           MS Quad               : 150 C maximum 200 C
           MS Source             : 230 C maximum 250 C


                       END OF MS ACQUISITION PARAMETERS

                     END OF INSTRUMENT CONTROL PARAMETERS
                     ------------------------------------
```

```
                          DATA ANALYSIS PARAMETERS
                          ------------------------
Method Name: C:\MSDCHEM\1\METHODS\CANNABIS.M

Percent Report Settings
-----------------------
    Sort By     : Signal

    Output Destination
       Screen       : Yes
       Printer      : No
       File         : No

     Integration Events               : AutoIntegrate

     Generate Report During Run Method: Yes

     Signal Correlation Window        : 0.020

Quantitative Report Settings
----------------------------
    Report Type: Summary

    Output Destination
       Screen       : Yes
       Printer      : No
       File         : No

    Generate Report During Run Method: No


                      END OF DATA ANALYSIS PARAMETERS
                      -------------------------------
```

## Total result to classification of *Cannabis* samples

| THC group | Sample ID | %THC | %CBD | %CBN | $\frac{\%\Delta^9\text{-THC} + \%\text{CBN}}{\%\text{CBD}}$ | $\frac{\text{THC}}{\text{CBD}}$ | Orange band at Rf.0.2 | Color test |
|---|---|---|---|---|---|---|---|---|
| Low THC (<0.3% THC and fiber type by DNA) | Hp59 | 0.05 | 0.04 | 5.0E-05 | 1.25 | 1.25 | + | Orange |
| | Hp16 | 0.14 | 0.30 | 1.3E-03 | 0.47 | 0.46 | + | Orange-red |
| | Hp10 | 0.18 | 0.85 | 2.9E-03 | 0.22 | 0.21 | + | Orange |
| Low THC < 1.0% but drug type by DNA | Hp03 | 0.11 | 0.01 | 1.5E-04 | 11.02 | 11.00 | - | Red-purple |
| | Hp09 | 0.29 | 0.13 | 7.6E-03 | 2.29 | 2.23 | - | Orange-red |
| | Hp44 | 0.60 | 0.02 | 3.0E-03 | 30.15 | 30.00 | - | Red-purple |
| | Hp37 | 0.61 | 0.01 | 1.5E-03 | 61.15 | 61.00 | - | ,, |
| | Hp70 | 0.96 | 0.02 | 6.5E-04 | 48.03 | 48.00 | - | ,, |
| 1%< THC < 5% | Hp12 | 1.10 | 0.03 | 5.3E-02 | 38.43 | 36.66 | - | Purple |
| | Hp72 | 1.16 | 0.02 | 1.2E-03 | 58.06 | 58.00 | - | Red-purple |
| | Hp18 | 1.17 | 0.28 | 1.8E-02 | 4.24 | 4.17 | + | Orange-red |
| | Hp19 | 1.18 | 0.20 | 6.3E-03 | 5.93 | 5.90 | + | ,, |
| | Hp48 | 1.20 | 0.01 | 6.0E-04 | 120.06 | 120.00 | - | Red-purple |
| | Hp56 | 1.30 | 0.20 | 9.0E-04 | 6.51 | 6.50 | + | Orange-red |
| | Hp53 | 1.40 | 0.03 | 5.0E-04 | 46.68 | 46.66 | - | Purple |
| | Hp36 | 1.46 | 0.03 | 7.1E-03 | 48.90 | 48.66 | - | Red-purple |
| | Hp74 | 1.48 | 0.08 | 1.5E-03 | 18.52 | 18.50 | + | Orange |
| | Hp21 | 1.50 | 0.16 | 6.6E-04 | 9.38 | 9.38 | - | Red-purple |
| | Hp57 | 1.70 | 0.03 | 9.0E-03 | 56.96 | 56.66 | - | Orange-red |
| | Hp47 | 1.80 | 0.01 | 7.6E-04 | 180.08 | 180.08 | - | Red-purple |
| | Hp41 | 2.00 | 0.03 | 5.9E-03 | 66.86 | 66.66 | - | ,, |
| | Hp35 | 2.03 | 0.05 | 9.4E-03 | 40.79 | 40.60 | - | ,, |
| | Hp55 | 2.10 | 0.05 | 8.0E-04 | 42.02 | 42.00 | - | ,, |
| | Hp69 | 2.10 | 0.35 | 3.0E-03 | 6.01 | 6.00 | + | Orange-red |
| | Hp40 | 2.20 | 0.10 | 8.4E-03 | 22.08 | 22.00 | - | Red-purple |
| | Hp51 | 2.20 | 0.20 | 7.5E-04 | 11.00 | 11.00 | + | Orange-red |
| | Hp75 | 2.33 | 0.02 | 3.4E-03 | 116.67 | 116.50 | - | Red-purple |
| | Hp68 | 2.47 | 0.10 | 3.0E-03 | 24.73 | 24.70 | - | ,, |
| | Hp50 | 2.50 | 0.04 | 1.4E-03 | 62.54 | 62.50 | - | ,, |
| | Hp62 | 2.58 | 0.06 | 2.8E-03 | 43.05 | 43.00 | - | ,, |
| | Hp24 | 2.60 | 0.39 | 2.9E-02 | 6.74 | 6.66 | + | ,, |
| | Hp54 | 2.60 | 0.40 | 9.0E-04 | 6.50 | 6.50 | + | Orange |
| | Hp11 | 2.67 | 1.33 | 3.1E-02 | 2.03 | 2.00 | + | Orange-red |
| | Hp34 | 2.98 | 0.51 | 1.5E-02 | 5.87 | 5.84 | + | ,, |
| | Hp45 | 3.01 | 0.10 | 4.3E-02 | 30.53 | 30.1 | - | Red-purple |
| | Hp67 | 3.05 | 0.09 | 3.6E-03 | 33.92 | 33.88 | - | ,, |
| | Hp43 | 3.10 | 0.14 | 1.4E-02 | 22.24 | 22.14 | - | ,, |
| | Hp38 | 3.39 | 0.04 | 7.3E-03 | 84.93 | 84.75 | - | ,, |
| | Hp49 | 3.68 | 0.06 | 1.8E-03 | 61.36 | 61.33 | - | ,, |
| | Hp76 | 3.74 | 0.03 | 2.9E-03 | 124.76 | 124.66 | - | ,, |
| | Hp60 | 3.94 | 0.51 | 3.1E-03 | 7.73 | 7.72 | + | Orange |
| | Hp71 | 4.10 | 0.10 | 2.3E-03 | 41.02 | 41.00 | - | Red-purple |
| | Hp39 | 4.70 | 0.13 | 3.4E-02 | 36.42 | 36.15 | - | ,, |
| | Hp52 | 4.80 | 0.06 | 2.0E-03 | 80.03 | 80.00 | - | ,, |

## Total result to classification (cont.)

| THC group | Sample ID | %THC | %CBD | %CBN | $\frac{\%\Delta^9\text{-THC} + \%\text{CBN}}{\%\text{CBD}}$ | $\frac{\text{THC}}{\text{CBD}}$ | Orange band at Rf.0.2 | Color test |
|---|---|---|---|---|---|---|---|---|
| THC > 5% | Hp73 | 5.02 | 0.06 | 7.3E-03 | 83.78 | 83.66 | - | Red-purple |
| | Hp46 | 6.36 | 0.10 | 2.7E-03 | 63.62 | 63.60 | - | Orange-red |
| | Hp65 | 6.60 | 0.11 | 5.4E-03 | 60.05 | 60.00 | - | ,, |
| | Hp29 | 7.32 | 0.12 | 6.4E-02 | 61.53 | 61.00 | - | Red |
| | Hp63 | 8.40 | 0.12 | 4.4E-03 | 70.04 | 70.00 | - | Red-purple |
| | Hp61 | 8.50 | 0.16 | 7.1E-03 | 53.17 | 53.13 | - | Red -orange |
| | Hp58 | 9.14 | 0.16 | 2.5E-03 | 57.14 | 57.13 | - | Orange-red |
| | Hp32 | 9.61 | 0.26 | 9.1E-02 | 37.31 | 36.96 | - | Red-purple |
| | Hp64 | 9.70 | 0.30 | 6.0E-03 | 32.35 | 32.33 | - | Orange-red |
| | Hp77 | 9.70 | 0.20 | 1.0E-02 | 48.55 | 48.50 | - | Red-purple |
| | Hp66 | 10.17 | 0.21 | 5.9E-03 | 48.45 | 48.42 | + | Orange-red |
| | Hp27 | 11.00 | 0.30 | 2.0E-02 | 36.73 | 36.66 | + | Red-orange |
| | Hp42 | 11.30 | 0.30 | 5.6E-02 | 37.85 | 37.66 | - | Red-purple |
| | Hp25 | 11.90 | 0.30 | 1.0E-02 | 39.70 | 39.66 | - | ,, |

**Color test by Fast blue B salt for *Cannabis* samples.**

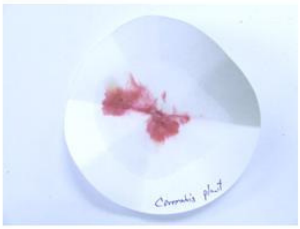

Negative control

Orange

Orange-red

Red-orange

Red



Red-purple

Purple

# BIOGRAPHY

| | |
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